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Biodegradation And Biomodification Of Lignocellulose With A Main Focus On Lignin

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BIODEGRADATION AND BIOMODIFICATION OF LIGNOCELLULOSE WITH A MAIN FOCUS ON LIGNIN

by

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This dissertation, submitted by Ivana Brzonova in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done, and is hereby approved.

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Grant McGimpsey
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ABSTRACT

Lignin is the second most abundant biopolymer, and the first most abundant source of phenolic structures, on Earth. Recent research has focused on lignin monomers as replacements for phenolic monomers derived from nonrenewable resources. Biological decomposition and conversion may be one way to accomplish this objective. In this study, the effects of two basidiomycetous fungi (Coriolus versicolor and Trametes gallica) and two actinobacteria strains (Microbacterium sp. and Streptomyces sp.) and their combination on lignocellulose (kenaf) decomposition was evaluated.

The results showed that after 8 weeks of incubation up to 34 wt. % of the kenaf biomass was degraded, and the combination of fungi and bacteria was the most efficient. Lignin decomposition accounted for ~ 20 wt. % of the observed biomass reduction, regardless of the culture used. Most of this lignin was present as solubilized oligomers rather than monomers. Only after the monosaccharides were utilized by the microorganisms was the production of laccase, manganese-dependent peroxidase and lignin peroxidase enzymes induced, allowing lignin degradation to commence. The presence of carbohydrates was found to be detrimental for lignin degradation.

In a subsequent series of experiments, we targeted the degradation/modification of isolated industrial-kraft lignin while trying to reduce the process time by solubilizing lignin with DMSO to increase lignin availability for enzymes. The addition of 2 vol% DMSO to nutrient free aqueous media increased the lignin solubility up to 70% while the quasi-immobilized fungi (pre-grown on agar) maintained their ability to produce lignolytic enzymes. While biological treatment was done for 6 days, significant modification was already observed in less than 24 hours. The resulting product showed

the removal of phenolic monomers and/or their immediate precursors and a significant intramolecular cross-linking among the reaction products. Thus a new path for lignin biotreatment and further utilization was observed leading to the formation of polymers rather than monomers.

Our interest therefore shifted to lignin utilization as a biochemically modified macromolecule. The biologically modified lignin was isolated via two different paths: 1) precipitation by acidification followed by washing with water or alcohols, or 2) vacuum evaporation followed by drying. The results of novel detailed chemical analysis of the modified lignin polymers showed that each of the washing steps can be used as a modification process, since each of them produced a slightly different polymer, with varied thermal stability, swelling and buffer capacities.

The resulting lignin based polymers turned out to be insoluble in either organic solvents such as DMSO, DMF, NMP, dioxane etc., or in water. However, under alkaline conditions (1M NaOH) all of these new polymers were converted into pH sensitive anionic-hydrogels showing remarkable thermal stability and varied sulfur content, which, like other properties, could also be controlled by precursor polymer washing with solvents. One of these hydrogels was further converted into a nonporous membrane and tested as a filter for water desalination. The preliminary results of 78% salt rejection under 270 kPa obtained with 1.35 μm thick, ligninbased membranes are promising for further exploration.

DISSERTATION OUTLINE AND STATEMENT OF PURPOSE

This dissertation includes the following chapters:

- Chapter I provides an introduction and motivation for lignin utilization, summarizes the current state of art includes current strategies and the global demand for lignin-desired chemicals. Possibilities and challenges for biological method are also included.
- Chapter II is a review paper on the chemical and bio-modification of lignin macromolecules.
- Chapter III is a research paper on kenaf biomass biodecomposition by basidiomycetes and actinobacteria in submerged fermentation for production of carbohydrates and phenolic compounds.
- Chapter IV is a research paper on the fungal biotransformation of insoluble kraft lignin into a water soluble polymer.
- Chapter V is a research paper on lignin based insoluble polymers (anionic hydrogels) produced by basidiomycetes.
- Chapter VI presents addition work focused on biologically modified kraft lignin for water purification.

The General Purpose of the Specific Projects

Our goal was to first observe the behavior of selected microbial strains used under conditions which are similar to those described in the literature but with improved chemical characterization of the products. Many research papers reporting biodegradation of lignin are incomplete or even misleading since gravimetry in combination with UV-

Vis spectrophotometry were most commonly used methods. We decided to use fractionalization via TD-Pyr-GC-MS instead of regular GC-MS in order to characterize the products comprehensively including the products that are not GC-elutable. Further, kraft lignin biological treatment is usually focused on monomer production and uses very low substrate concentrations, high amount of nutrients and long decomposition times which yields in a mixture of chemicals with a low monomer yield.

After evaluation of the previous work done in this area we aimed at designing a biodegradation/biomodification system which could be suitable for industrial applications. After replacing fermentation with enzymation plus the addition of organic solvents in a nutrient free environment, we were able to biologically modify lignin in 6 days. This modification yielded a lignin-based polymer (rather than monomers) which was produced at high yields and possessed interesting properties with the potential for various applications, such as water treatment.

CHAPTER I

Lignin and its degradation/conversion

I.1 Introduction

The current socioeconomic and environmental situation calls for the replacement of fossil fuels and petrochemicals with alternatives from renewable and sustainable sources. The most abundant naturally occurring renewable source of carbon is lignocellulosic (LC) biomass comprised of cellulose, lignin and hemicellulose. However, these polymers have potential to be used as an inexpensive and renewable source of fine chemicals and biofuels only when decomposed, degraded or modified.(Smolarski, 2012)

Because the sun is a sustainable energy source, our current development in technology should be directed on the path to use this solar energy directly in an amount which can completely replace fossil fuels. In addition, our reliance on fossil fuels is not constrained to energy sources. Fossil fuels are the main source of chemicals, in the form of petrochemicals, used in many major industries including polymers, pharmaceuticals, foods, and textiles. Since we cannot convert solar energy directly into materials, we need a source of renewable carbon which would be converted into a broad spectrum of materials essential for everyday life. Lignocellulose is one of these candidates.

I.2 Motivation and background

Utilization and conversion of lignocellulosic biomass is done through physical, chemical, (Harmsen and Huijgen, 2010) or biological processes or their combination (Table 1). Biological processes are characterized with the longest processing time but

with only minimal requirements for both energy and chemicals, thus providing both economic and ecological competitiveness. If the processing time can be shortened using physical or chemical pre- or post-treatment methods, the advantages of biological processes could be realized in their full extent.

Table 1. Most common methods of lignocellulose pretreatment

Pretreatment	Process	Effects	Advantages and disadvantages
Physical	Milling, irradiation, hydrothermal, high pressure, expansion, extrusion, pyrolysis	Increase of surface and pore size Decrease of cellulose crystallinity Decrease in degrees of polymerization	Energy demanding Not able to remove lignin No chemicals required
Chem. and Phys-Chem.	Steam, Ammonia, CO_2, SO_2, alkali, acid, gas (dioxides), oxidation, solvent extraction of lignin (Ethanol-water, benzene-water, ethylene glycol, butanol-water, swelling agents),ionic liquids	Increase of surface and pore size Partial or complete delignification Decrease of cellulose crystallinity Partial hydrolysis of hemicellulose Polymerization decrease	Fast Require expensive chemicals Not environmentally friendly
Biological	Fungi, bacteria, enzymes	Partial or complete delignification Decrease in polymerization Partial hydrolysis of hemicellulose	Low energy requirement No chemical requirements Mild conditions High H_2O consumption Very slow Ecology friendly Very challenging for industry

Current research on bacterial degradation of cellulose and hemicellulose mainly focuses on conversion of these polymers to biofuels, particularly methanol and ethanol.(Alvira et al., 2010; Balat, 2011; Limayem and Ricke, 2012; Sarkar et al., 2012) However, ethanol is less energy dense (by ca. 30%) compared to diesel, gasoline or jet fuels. (Savage, 2011) Ethanol is also not suitable for heavy cars. Furthermore, it is corrosive both by itself and, particularly, upon its facile partial oxidation to acetic acid. Therefore it cannot be economically shipped, e.g., through existing pipelines. (Savage, 2011) Recent research focus was shifted to LC bioconversion to the less corrosive and more energy-dense *n*-butanol. An additional advantage of *n*-butanol as a fuel is that, in

cofntrast to ethanol, it does not have to be used in mixtures with diesel, gasoline or jet fuel for current engines. Furthermore, *n*-butanol separation from growth media is facilitated by its low solubility in water. Finally, *n*-butanol can be further converted into isobutene, which can serve as a feedstock for production of a wide spectrum of hydrocarbon fuel components. (Savage, 2011) These processes still need improvement, e.g., developing more efficient and more product-tolerant microorganisms.

The other alternative to ethanol production is the biomass conversion into gas phase hydrocarbons, methane, propane and butane. Selective production of longer-chain hydrocarbon fractions (petrol and diesel) from biomass by microorganisms is still not fully optimized but it could be done using synthetic biology protocols. (Savage, 2011) Another option is biodiesel production employing oleoaginous microorganisms,(Chakraborty et al., 2012; Galafassi et al., 2012; Hu et al., 2011; Huang et al., 2009; Jin et al., 2015; Miao et al., 2011; Ruan et al., 2014; Yu et al., 2011; Zeng et al., 2013) i.e., those that use the polysaccharide components of LC for massive production of fatty acid based oils (usually in the form of triglycerides), which can then be converted into biodiesel via transesterification.

To summarize the main applications of microorganisms to cellulose and hemicellulose conversion, microorganisms are used to produce small molecules, or to partially decompose (digest) lignocellulose to make subsequent processes less energy intensive. The same application of microorganisms can be done for lignin utilization. Lignin is the second main component of lignocellulose. (Alvira et al., 2010; Balat, 2011; Limayem and Ricke, 2012; Sarkar et al., 2012) It is a very recalcitrant phenolic polymer. Most of the lignin produced today is merely a by-product of carbohydrate production and

has low commercial value. Most ends up burned for heat and electric power generation. (Alvira et al., 2010; Balat, 2011; Limayem and Ricke, 2012; Sarkar et al., 2012) The pulp and paper industry estimated that 50M ton of lignin were extracted in 2010 but only 2% have been commercialized for production of dispersants, adhesives and surfactants or as antioxidants in plastics and rubbers. (Brebu et al., 2011; de la Torre et al., 2013)

Lignin decomposition into monomers has the potential to provide replacements for many petrochemicals currently used in the polymer, cosmetic, pharmaceutical, food and textile industries. (Varanasi et al., 2013) Industrial lignin is amorphous, highly polydisperse and behaves as a thermoplastic material, with its glass transition temperature (Tg) broadly varied. Tg can shift from 90 to 277 ^{o}C with an increase of the molecular mass, depending also on the process of lignin isolation, and thermal history.(Cateto, 2008) Recent applications of microorganisms for lignin utilization are very similar to those for the polysaccharide portion of lignocellulose. Biological methods are usually used for partial digestion/removal of lignin in the biomass to separate lignin from cellulose as lignin is undesirable during biofuel production. (Ji et al., 2012; Ravindran et al., 2012; Schilling et al., 2012; Tanaka et al., 2009; Y. Wang et al., 2013)

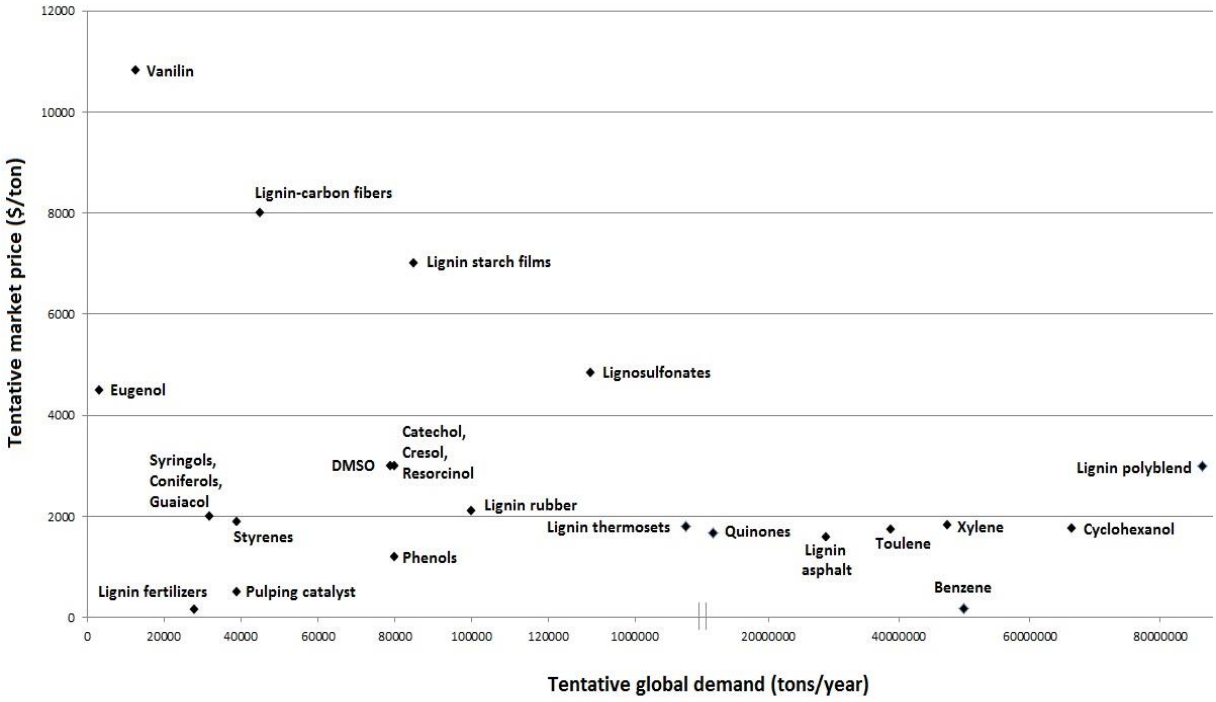


Figure 2. Chemicals produced from lignin, their tentative prices and global demand. (Holladay et al., 2007)

Various species of fungi and bacteria have bean explored for their ability to break down lignin into monomers, since chemical and physical methods of lignin conversion into monomers are currently providing low selectivity and small yields (up to 30%) while consuming large amounts of energy and catalysts.(Davis et al., 2016) More successful and selective is a chemical and physical conversion of lignin into small carboxylic acids.(Demesa et al., 2015) However they could also be easily produced from polysaccharides. (Gandini and Lacerda, 2015) In this area microorganisms offer much slower but more flexible and robust degradation systems in the form of enzymes, which could be possibly engineered to selectively disrupt specific lignin bonds. However this

process is hindered by spontaneous repolymerization of these monomers. (Evans and Palmer, 1983; Munk et al., 2015a)

Despite these challenges in lignin degradation, chemicals are industrially produced from lignin, their tentative prices and global demand are shown in Figure 2. A rough estimate of the global demand for these chemicals is 3.0×10^8 tons per year. (Holladay et al., 2007) Compared to this number, the estimated global production of crop residue is 4.0×10^9 tons per year of which 1.0×10^9 is lignin (based on the average 25% lignin abundance in biomass) as shown in Figure 1.(Eisentraut, 2010) These estimates show that a conversion of only 30% of the currently produced lignin would fulfill the global demand for these chemicals. This estimate does not include the current interest to use lignin monomers for production of highly functional polymers in industries such as medical, nutraceutical, pharmaceutical, electrical, etc. Such applications would increase the value of lignin monomers and thus make lignin decomposition methods more economically viable.

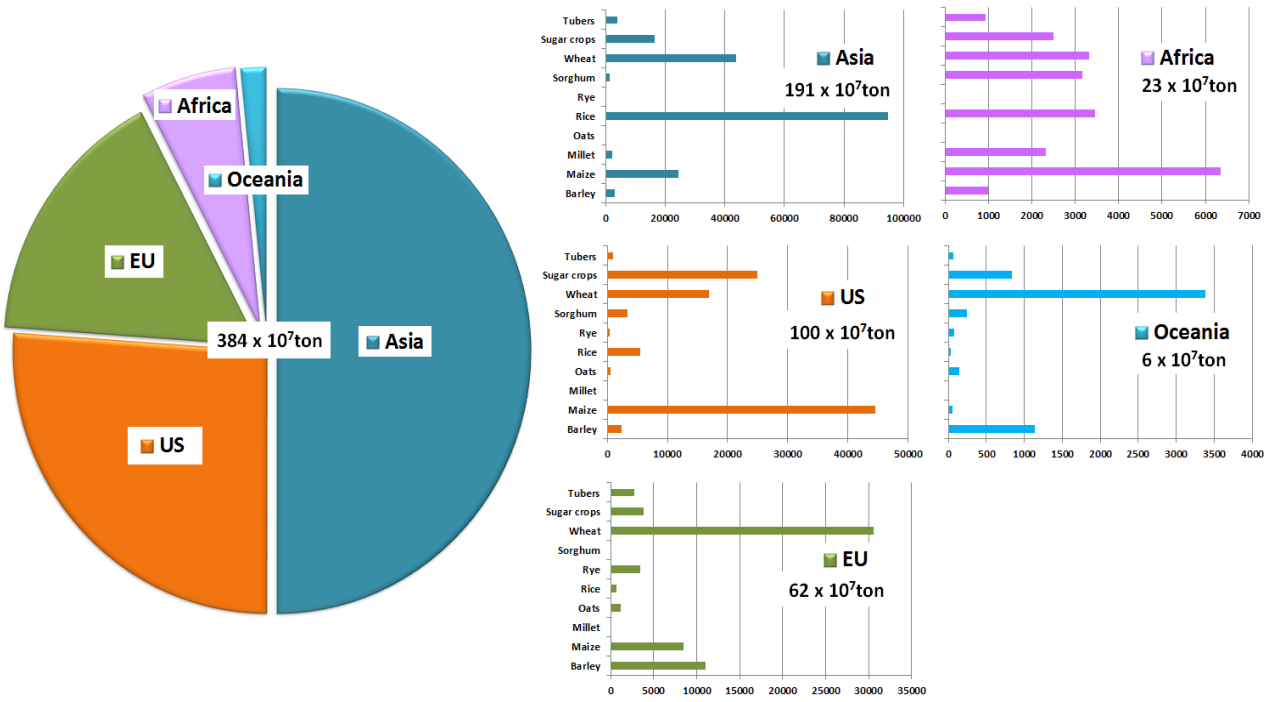


Figure 1. Tons of crop residue production in 2010 – based on *Renewable and Sustainable Energy Reviews 2015.*

Lignin decomposition into monomers followed by polymerization into highly functional polymers is not the only pathway that can be used. Industrial lignin can be utilized in its polymeric form without depolymerization, (Duval and Lawoko, 2014) but with purification and modification. Companies such as Domtar, MeadWestvaco, Lignol Innovations, La Rochette Venizel specialize on high purity lignin production. This is being done mainly through the removal of contaminants (hemicellulose) plus monomeric and oligomeric lignin species which can be followed by specific modifications that either add or remove certain functional groups. (Kai et al., 2016) After this treatment, polymeric lignin can be used for further conversion into functional materials and others. A

significatnt challenge in this area of lignin based polymers or oligomers is chemical characterization. (Asina et al., 2017; Kozliak et al., 2016)

Biological activities observed with different fractions suggest that lignin modified with various fungi might may offer solutions for treatment and prevention of many diseases. Applications are also promising antibiotics, anti-inflammatory ointments, treatments for cancer (polymer vaccines), scaffolds, drug delivery systems, wound healing, and also water purification systems. Table 3 provides a brief overview of the pharmaceutical applications of lignin (it should be noted, though, that the corresponding market prices and demands are hard to estimate).

Table 2. Lignin potential for the pharmaceutical industry

		Molecules	Effect	Ref.
Lignocellulose	Lignin	<0.5kDa lignin	Potential for HIV-1gene expression inhibition, broad antiviral spectrum	(Mitsuhashi et al., 2008; Sakagami et al., 2005a)
		~ 30kDa lignin	Inhibion of HIV protease	(Wang et al., 2015)
		Lignophenols (MW ~ 1500)	Lowering the weight of adipose tissues, plasma Tg levels, and hepatic expression of SREBP-1c mRNA	(Sato et al., 2012)
		Lignin	Antioxidant, anticancer	(Ugartondo et al., 2008)
		Bagasse, lignosulphonate, Curan 100, steam explosion	No harmful effect on skin or eyes	(Vinardell et al., 2008)
		Basidiomycetes metabolites in lgnolytic system	Chlorinated anisil and hydroqinone metabolites. - Antibiotic, pesticides	(Field et al., 1995)
	Lignin, polysaccharide complex	Lignin metabolite from Inonotus obliquus (MW 37561Da, 24945Da)	Antiproliferative, inhibited NF-?B activation	(Wang et al., 2015)
		758-5.3kDa	Antiherpes (HSV-1 and HSV-2) activity	(Zhang et al., 2007)
		Lignin with 20% carbohydrate complex	Immunopotentiating activity (antitumor, antimicrobial, antiparasite),	(Sakagami et al., 2005) (Sakagami et al., 2010)
		Biogenic silica particles from rice husk	Broad biomedical application	(Alshatwi et al., 2015; Athinarayanan et al., 2015)
	Poly-sacc haride	β – glucans (polysaccharide metabolite)	Immunomodulatory and anticancer activity	(El Enshasy and Hatti-Kaul, 2013; Gómez-caravaca et al., 2015; Grienke et al., 2014; Synytsya et al., 2009)
		Xylan derived oligosaccharides	Immunomodulatory, antioxidant, prebiotics	(Singh et al., 2015)
		Modified oligosaccharide	Broad medical applications	(Kuijk et al., 2014)

Any of these applications on industrial scale are challenging without a detailed chemical characterization. Therefore, in this work we are aiming at these applications by combining comprehensive chemical analysis of biologically modified lignin based macromolecules with a practical application of these products. We focus not only on monomers produced during biological treatment, but also on oligomers and polymers produced during a the biodegradation/modification of the biomass. Comprehensive analysis of these products is used as a tool to better understand the products and the process.

Table 3. Microbial degradation of lignocellulosic biomass

Microorganism used		**Substrate**	**Incubation time (days)**	**Lignin loss (%)**	**Biomass loss (%)**	**Conditions**	**Analysis**	**Ref.**
Fungi	**Bacteria**							
-	Mixed (from sludge) LDC (230 analyzed clones)	Reed straws Soaked in 3% NaOH/24 h Washed with distil water, pH7, 3-4cm, dried at 60C mineral rich medium	15	61	-	Submerged, statically/30 °C	Gravimetry	(Y. Wang et al., 2013)
T. hirsuta yj9	-	Corn stover (40-60 mesh) dried 72C for 2 days sterilized 121C/20min	42	71	73	30 °C/dark	Gravimetry	(Sun et al., 2011)
P. chrysosporium	Solid inoculant	Rice straw : vegetables : bran : soil (11:3:2:8) dried (40mesh), 55 and 45% mosture (2 steps)	49	30-40	40	37 °C/ solid state	Gravimetry	(Zeng et al., 2010)
G. geotrichum *M. verrucaria*	-	Rye straw (0.2-0.4mm) 7.4% moisture with mineral medium sterilized 121C/30min	30	53 60	-	Solid state /28 °C	Gravimetry	(Varnaitė et al., 2005)
T. versicolor *D. flavida* *D. squalens* *P. chrysosporium* *P. fascicularia* *P. floridensis* *P. radiata*	-	0.5% malt extract pH 5 sterilized 121C/15min (wheat straw)	32	12 18 16 17 25 23 19	-	25 °C / shaking	Gravimetry	(Arora et al., 2002)

-	*One step:* *Bacillus sp CS – 1* *Bacillus sp CS – 2* *S. thermoph. (NBRC13957)* *L. Bulgaricus (NBRC13953)* *T. fusca (NBRC 14071)* *Two steps:* *NBRC followed Bacillus 1* *Bacillus 1 followed NBRC*	Ballast salt medium (pH 8) Milled rice straw (40-80 mesh) Dried 60 °C/ 48h	3	One step: 20 - - - 18 Two steps 11 62	-	30 °C /37 °C shaking	UV at 280nm	(Chang et al., 2014)
D. squalens BEOFB 700 *F. pinicola BEOFB 600* *G. lucidum BEOFB 432* *L. betulinus BEOFB 500* *P. eringii HAI 501* *P. ostreatus HAI 592* *T. versicolor BEOFB 320*	-	Wheat straw	14	34 32 20 28 15 13 22	-	25 °C solid state	Gravimetry	(Knežević et al., 2013a)
T. pubescens: *BEOFB 330* *HAI 1200* *T. multicolor:* *HAI 426* *HAI 428*	-	Wheat straw/Oak sawdust (dried at 50C/ grounded to 0.5-1.0cm) Nitrogen 25mM, pH 6.5	14	58/2 56/3 43/6 28/3	-	25 °C / solid state	Gravimetry	(Knežević et al., 2013b)
mixed fungi	Mixed G+ bacteria	(Wheat straw, root veg. resid., bran, soil) Air dried, ground 2mm 65% water content COMPOSTING	50	26	-	35-65 °C	HPLC (monitoring quinone species)	(Huang et al., 2010)
P. chrysosporium CGMCC5.776	-	Cornstalk (40 mesh) dried 105C, sterilized 121C/20min, 70% moisture	15	34	10	29 °C / static	Gravimetry	(Zhao et al., 2012)
-	Thermopilic bacterial consortium (8 major microbes of aerobic/facultative anaerobic bacteria)	Bagasse rice straw corn stover eucalyptus pulp Sterilization 121C/15min	8	-	60 75 70	50 °C /static	Gravimetry	(Wongwilaiwalin et al., 2010)
P. flavido-alba ATCC 12679	-	grass, corn stover, wheat straw, wood fiber (1mm) 121C/ 20min sterilize 81% moisture Kirk mineral solution with glucose, proteins and ABTS	21	1 4 3 7	5 9 2 5	30 °C Static anaerobic	Gravimetry	(López et al., 2013)
P. chrysosporium BKMF-1767	-	Rice straw (60 mesh) Salt medium Sterilized/non sterilized 75% moisture	30	35-45	-	37 °C / Solid state	Gravimetry	(Yu et al., 2009)
P. chrysosporium	-	steam-exploded (3MPa/5min) rice straw, dried 105C/6h, sterilized 120C/30min (5 factors, each 4 levels)	15	9-65	10	29 °C / static	Gravimetry FTIR/SEM	(Zhang et al., 2012)

P. chrysosporium Hb *Fusarium sp. 82* *Fusarium sp.89* *F. moniliforme 821*	-	Solid state 70 % moisture (rice straw – milling 0.9mm mesh) 3 hours/ 105C to remove moisture Autoclaved 121C/20min	10	28 29 33 35	-	28 °C	Gravimetry	(Chang et al., 2012)
M. roridum LG7	-	Paddy straw Herbaceous weed Parthenium sp. (sterilized / non-sterile) (0.5-1cm) Sterilization 121C/15min	7	9/2 5/2	-	30 °C statically	UV-VIS, FTIR, SEM	(Tiwari et al., 2013)
P. chrysosporium ATCC 24725	-	Wheat straw ~6mm Sterilization 121C/1h	21	30	-	Solid state	Gravimetry, NMR, FTIR, Py-GC MS	(Singh et al., 2011)
P. chrysosporium	-	Wheat straw (Inorganic salts/tween 80)	7	25	27	37 °C	SEM, Gravimetry, FTIR, Pyr-GC, X ray diffraction	(Zeng et al., 2011)
-	Bacterial community XDC-2	rice straw wheat straw corn stalk (1mm) Submerged 5% (v/v)	12	45 9 16	40 25 20	35 °C/ static	Gravimetry GC-MS	(Hui et al., 2013)
P. chrysosporium ATCC MYA-4764	-	corn stover stored with ~15%moisture for 9 months. Ground to 5mm and dried, CO2 atmosphere	11	36		35 °C / solid state	Gravimetry	(Yao and Nokes, 2014)
P. tigrinus M109RQY *P. tigrinus M609RQY*	-	Casava peel Rice husk Rice straw milled 2mm, mineral solution, sterlized 121C/20min	15	41 11 68	31 5 26	30 °C / solid state	Gravimetry	(Ruqayyah et al., 2013)
P. chrysosporium *P. ostreatus*	-	oil palm empty fruit bunches 67% moisture salt medium	28	41 50	33 43	30 °C/ solid state	Gravimetry	(Piñeros et at., 2014)
P. chrysosporium PC2 *L. edode LE16* *P. ostreatus PO45*	-	1:10 Deionized water (sugarcane bagasse) 121C/ 1h sterilization	84	95 87 85	75 45 30	Solid state/ 25 °C / dark	Gravimetry, FTIR, NMR	(Dong et al., 2013)
T. pubescens BEOFB 330 *T. multicolor HAI 426*	-	wheat straw/oak saw dust Dried 50C/(0.5-1cm)	14	58/43 56/28	-	25 °C	Gravimetry	(Knežević et al., 2013b)

C. subvermispora	-	corn stover switchgrass wheat straw soybean straw hard- wood 5mm, 40C dried, 75% moisture (buffer and malt extract) Sterilization 121C/30min	18	29 27 4 0 17	13 12 5 3 10	Stationary/ 28 °C	Gravimetry / HPLC	(Wan and Li, 2011)
-	*Streptomyces UAH 23* *Streptomyces UAH 52* *S. viridosporus T7*	Wheat straw (40mesh) Dried 24h/50C Sterilization 1hour	28	APPL analysis	-	28-38 °C 200rpm	Pyr-GC-MS	(Rodriguez et al., 1997)
T. versicolor	-	oil palm biomass chips (2.5 cm x 1.5 cm x 0.2 cm) Sterilization 121C/20min 60-100% moisture	28	9	8	24 °C	SEM, Gravimetry	(Singh et al., 2013)
O. latemarginatus *R. vinctus* *P. chrysosporium* *C. versicolor*	-	Mineral solution	28	-	28 14 8 12	28 °C	Gravimetry	(Pereputan et al., 2013)
I. lacteus CD2 *E. taxodii 2538*	-	Bamboo (0.3-0.45mm) Sterilization 121C/45min	30	13 29	-	28 °C / 150 rpm	FTIR/ Gravimetry	(Zeng et al., 2012)
C. subvermispora	-	Cedar/Beech wood (2.0cm×2.0cm×0.5cm) acetone refluxing to remove extractives oven dried 60C	84	25/39	15/20	28 °C	Gravimetry	(Tanaka et al., 2009)
P. radiata *P. brevispora* *P. tremellosa TUW-L20* *D. squalens CBS 1000.73* *C. subvermispora:* *CBS 347.63* *FPL 90.031* *FPL 105.752* *H. fragiforme ZIM L108* *O. latemarginatus TUW-L10*	-	(spruce wood shavings 0.4-2.0 mm, oven dried) 121C/ 15min sterilization Moist static incubation.	14 30	3	6 6 6 4 5 6 7 4 2	30 °C	FTIR	(Fackler et al., 2006)
P. chrysosporium *T. reesei* *Coniochaeta sp. LF2*	-	Corn stower Mandel mineral salt medium	4.2	39 35 41	-	37 °C / 100 rpm	Gravimetry	(Ravindran et al., 2012)
G. trabeum/P. placenta	-	Aspen Spruce Corn stover (40 mesh) oven dried 100C for 48h, autoclaved 121C/1h	112	20/15 21/30 40/25	58/34 54/37 -	Solid state (cube jars)/ room temp.	Gravimetric, HPLC, (13C-TMAH) thermochemolysis, X-ray diffraction	(Schilling et al., 2012)

C. versicolor	-	Low nutrient solution sterilization (spruce sapwood)	84	75	-	22 °C / statical	UV VIS, HPLC, NMR, Gravimetry	(Norhasliida et al., 2014)
T. trogii MT	-	Wood blocks decay 2cm^3 121C/ 20min sterilization 90% humidity	120	75	-	28 °C	DEM, IR, LC-MS	(Ji et al., 2012)
P. sanguineus *O. latemarginatus* *C. versicolor* *R. vinctus*	-	Banana pseudostem 3x3 cm, 15% moisture	28	63 55 34 57	37 22 22 21	-	Gravimetry	(Norhasliida et al., 2014)

CHAPTER II

Chemical and bio-modification of lignin macromolecules: Review

II.1 Abstract

Lignin is the second most abundant biopolymer, and the first most abundant source of phenolic structures on Earth. Lignin is known for large variations in its complex structure, which depends on the source and on the isolation procedure applied. A lot of work has been done to utilize this rawmaterial in many ways. The main focus is on generating lignin monomers for replacement of phenolic monomers derived from nonrenewable resources.

Lately, economical reasons have increased the interest of utilizing lignin as a macromolecule. Purification, modification and lignin structure understanding plays the important role for lignin utilization. The chemical modification of lignin macromolecules is increasing the applications of lignin. Improved behavior and properties, together with added functionalities, are shown to be suitable for potential lignin applications in the polymer industry.

Less explored are the biological modifications of lignin macromolecules. However suggests great potential for lignin application in medicine. This review covers both the chemical modification of lignin macromolecules resulting in new functionalities and applications as well as the potential for biological modification of lignin macromolecules.

II.2 Introduction

The main focus is still towards lignin degradation into monomers or small molecules.(Asina et al., 2017) The main products of lignin decomposition are aromatic molecules, carboxylic acids and resins produced usually via thermal catalytic reactions.(Demesa et al., 2015; Ma et al., 2014) However, purification of these chemicals followed by further polymerization are expensive step essential to create desirable materials. Most of the research done in the area of lignin utilization addressees the prioritization of pathways to obtain monomers and polymers which are the are compatible with existing industrial infrastructure built for fossil resources.(Davis et al., 2016; Li et al., 2015; Upton and Kasko, 2015)

Lignin is a phenolic macromolecule with a highly complex structure. Lignin is comprised of monolignols, which are phenylpropanoid units where each of them has a different number of methoxy groups on the aromatic ring. (Laurichesse and Avérous, 2014a) These monolignols are connected through seven main linkages, where the β-O-4 ether bond is the most common the other ether bonds are α-O-4 and 4-O-5. Carbon-carbon bonds in lignin are usually β-β, β-5 and β-1, or branching bond of three units, dibenzodioxocin.(Duval and Lawoko, 2014; Thakur and Thakur, 2015) Combinations of these linkages and different monolignols results in different types of lignin varying in reactivity and branching. The functional groups in technical lignin are mainly phenolic and aliphatic hydroxyl groups, carboxyl and carbonyl groups. (Kai et al., 2016a)

The structure of lignin varies depending on the origin of the plant species and the isolation process.(Laurichesse and Avérous, 2014a) Heterogeneity and missing stereoregularity makes the chemical characterization and reaction prediction/simulations

very challenging. One of the best methods for lignin analysis used today is 31P NMR(Duval and Lawoko, 2014) complemented with GPC, LC-MS and GC-MS but even all these methods combined do not yield the exact structure and should be supplemented with fractionalization of the lignin or the lignin products prior to the analysis. (Asina et al., 2017; Kozliak et al., 2016) The most common type of lignin produced are listed in Table 1.

Table 1. Most common types of lignin (Duval and Lawoko, 2014; Kai et al., 2016)

Lignin Type	produced/available per year	Characteristic	Production
BENCH SCALE		**Closer to original lignin**	
Milled wood lignin (MWL)	NA	low molecular weight, low branching	low recovery yields (20%- 40%)
Mild acidolysis lignin	NA	Similar to MWL	> yield than MWL
Cellulolytic enzyme lignin	NA	Similar to MWL	> yield than MWL
Enzymatic mild acidolysis lignin	NA	high purity and higher yields (much higher molecular weight than MWL)	High, up to 90% yield
INDUSTRIAL SCALE		**Large structural changes compare to original lignin**	
Kraft lignin Tg 140-150 °C Td 340-370 °C	70 Mt / 90 kt	Contains sulphur (1-3%). Rich in phenolic hydroxyl groups and carbon-carbon bonds (result of cleavage of aryl ether bonds during the Kraft process and condensation reactions, respectively)	MeadWestvaco, Domtar
Lignosulfonates Tg 130 °C Td 250-360 °C	7 Mt / 1 Mt	Rich on sulfonate groups SO_3^-. 3.5-8% Sulphur, highly soluble in water (anionic polyelectrolytes). Surfactant properties, used as dispersants, water reducers in concrete, viscosity reducers	Borregaard LignoTech, Tembec, La Rochette Venizel, Nippon Paper Chemicals
Soda lignin Tg 140 °C Td 360-370 °C	~ 7 kt	Sulphur free, similar to kraft lignin, mostly from herbaceous plants.	Green value
Organosolv lignin Tg 90-110 °C Td 390-400 °C	~ 3 kt	Sulphur free, increased phenolic functionality due to acidolytic or alkaline cleavage of aryl ether linkages, no condensation reaction (organic solvents used), most similar to native lignin	CIMV, Lignol Innovations, DECHEMA

Most of the lignins, especially kraft lignin, are combusted to generate electricity for pulp and paper industries. There is high interest of lignin utilization in many areas of industry, especially in polymer production. Direct use of lignin in polymer applications are mainly as a filler, where lignin is blended with either synthetic or bio-polymers, in order to increase the fraction of a renewable material in these polymers and therefore produce "greener" polymers.(Laurichesse and Avérous, 2014a) Since lignin material is also a free radical scavenger, its presence in polymer blends can help decrease UV degradation or thermo-oxidation and therefore act as a stabilizer. (Duval and Lawoko, 2014) Lignin has better miscibility with polar polymers, since it is rich in OH groups. Typically 20-30% of lignin can be added in polymer blends, the only exception is polyvinylacetate where 85% of kraft lignin was incorporated. (Duval and Lawoko, 2014; Kai et al., 2016)

Direct application of lignin is also being evaluated for the production of carbon fibers but difficult melt processes make this production very challenging. In order to avoid these problems high purity of lignin is required for carbon fiber production. (S. Wang et al., 2016)

Lignin is also currently finding a place as a sorbent in the form of lignin based activated carbon. (Berrima, 2016; Dizhbite et al., 1999) The active functionalities of lignin - carboxyl and mainly the phenol groups - have shown a high affinity toward metal ions.(Verma et al., 1990) Therefore lignin derived activated carbon can be used for waste water treatment to remove toxic ions (heavy metals) such as chromium, copper, cadmium, lead, zinc, nickel, cobalt and mercury. The adsorption of these metals on lignin is highly pH dependent and depends on the oxidation state of the metal ion.(Yang and

Yan, 2012) Also the number of phenolic groups present in lignin is important. The difference can be large, for example cadmium (II) adsorption which can vary from 25 to 137 mg/g of lignin. (Duval and Lawoko, 2014)

Direct applications of unmodified lignin are very challenging and purification together with chemical modification are necessary for successful applications. The lignin functional group available for modifications are mainly OH groups, aliphatic (primary, secondary) or aromatic. Modifications can also be done on the free ortho-position of the phenolic ring. Lignin rich on guaiacyl moieties has reactive sites at the C5 position of the phenolic ring and the coumaryl type of lignin has reactive sites at C3 positions. (Duval and Lawoko, 2014; Kai et al., 2016)

In order to enhance blending with nonpolar polymers, the modification of OH groups is usually necessary and the most common method is alkylation (primarily methylation and/or ethylation) and acetylation. Most of these modifications are done while using rather toxic chemicals such as diazomethane or dimethyl sulfate for methylation. Therefore large scale industrial applications with these modifications are not recommended. Esterification of lignin is usually conducted with anhydrides (with 2-6 carbons chain length) and can result in increased solubility in styrene and miscibility with nonpolar polymers, such as polycaprolactone. (Duval and Lawoko, 2014; Kai et al., 2016)

Lignin is also being evaluated for different kinds of graft polymerization, such as 1) ring opening polymerization of monomers, which is initiated by lignin hydroxyl groups, 2) radical polymerization of vinylic monomers into lignin, 3) or more

complicated multistep methods. Another option is to use lignin as a macro-monomer where free phenolic positions and OH groups can be used for polymerization.(Duval and Lawoko, 2014; Kai et al., 2016; Laurichesse and Avérous, 2014) Crosslinking of these macro-monomers can be done to create thermosets or thermoplastics such as lignin phenyl formaldehyde resins, lignin based polyurethanes, or polyesters. (Duval and Lawoko, 2014)

Understanding the lignin structure and possible lignin modifications are essential in order to produce materials with controlled structures, highly functional materials, functional-lignin-based micro and nano structures, and nanoparticles.(Duval and Lawoko, 2014) The current focus is on nanostructured materials and nonporous materials mainly because of their broad applications as sorbents, insulating materials, catalysts, drug carriers, and more. (Marcelo et al., 2016; Yang et al., 2016)

Lignin's ability to self-organize under specific conditions is an attractive feature which can be largely utilized in nanotechnology and for pharmaceutical applications. Properties of lignin such as antimicrobial, antiviral, antioxidant, etc., also make lignin a great candidate for pharmaceutical applications.(Dong et al., 2011) Potential applications for drug encapsulation, ointments and wound dressing have been previously evaluated. (Figueiredo et al., 2017; Mahata et al., 2017)

In this review we will mainly focus on modification of lignin macromolecules in general and on application for energy storage and for medicine.

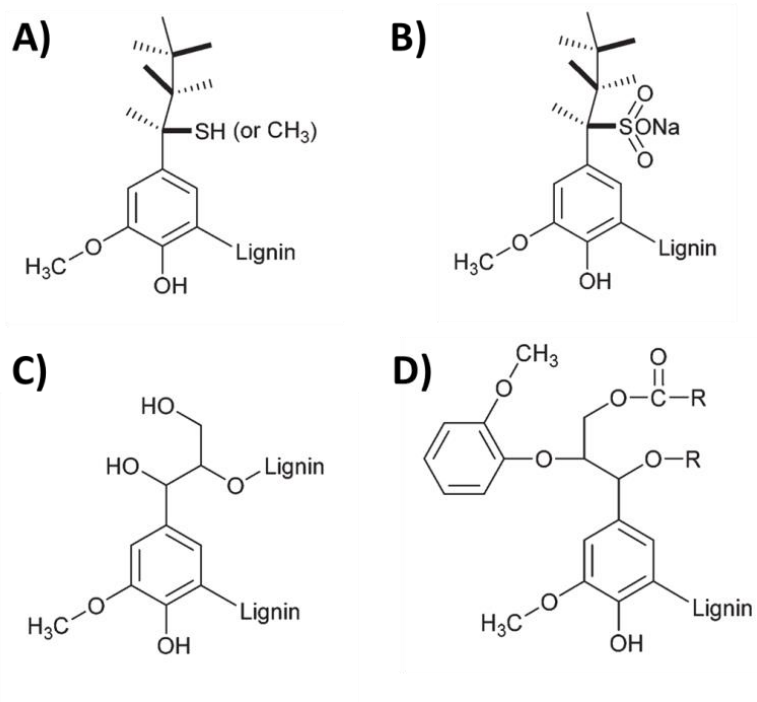


Figure 1. A) Kraft lignin, B) Lignosulfonate, C) Soda lignin, D) Organosolv lignin. (Kai et al., 2016)

II.3 Chemical modification of lignin macromolecules

Lignin, as a renewable resource and second most abundant polymer, has the potential to be used for large scale production of polymers, or be blended with synthetic polymers used today. Currently, the most explored area of lignin based polymers are lignin copolymers.

II.3.1 Lignin based polymers and copolymers

One of the largest groups of polymers are polyurethanes (PU) used as elastomers, foams, adhesives, coatings and fibers.

Phenol formaldehyde (PF) resin is commonly used as a thermoset as well as in adhesives, coatings, wood composites and advanced engineering plastics. PF has good mechanical properties and features high resistance against organic solvents and elevated temperatures. However, it has a fluctuating price since it depends on petroleum prices. Lignin based PF has a competitive price and properties.(Kai et al., 2016)

Phenol epoxy (PE) are polymers crosslinked using epoxy groups which react by ring-opening addition reaction with amine, carboxylic acids, thiols, isocyanates, etc. PE polymers feature strong adhesion to polar surfaces (e.g.: glass, ceramic) due to its hydroxyl groups. (Kai et al., 2016)

Polyethylene, polycarbonate, polybutyrate, poly(lactic acid), poly(caprolactone) polybutylene terephthalate are synthesized either via esterification. Diacids with diols or by self-condensation of carboxylic and hydroxyl acids. Lignin is rich in OH groups therefore it can react as a polyol in the polyester synthesis. Lignin OH groups can also be functionalized with carboxylic acids, acyl groups or epoxy groups.(Kai et al., 2016)

Table 2. Chemical modification of lignin

Modification	Chemical or/and method used	Effect	REF.
Functionalization of OH groups			
Alkylation (methylation, ethylation)	Diazomethane, dimethyl sulfate, methyl iodide	Enhanced compatibility with non-polar polymer matrices (aliphatic polyesters) Plastization, different mechanical properties	(Duval and Lawoko, 2014; Laurichesse and Avérous, 2014)
Esterification	Anhydrides (2-5 carbon chain length), calalyst: 1-methylimidazole	Increase solubility in nonpolar solvents & blending with nonpolar polymers	(Wang et al., 2011)
Phenolation	Phenol attached to benzylic carbons of lignin in acidic medium.	Additional reactive sites, side chain reactions cause fragmentation – MW decrease	(Alonso et al., 2005)
Addition of new active sides			
Oxypropylation	Reaction with cyclic ethers, propylene oxide reaction with lignin OH groups	Liquid lignin polyol-macromonomer for polyurethane synthesis only aliphatic hydroxyls since lignin phenolic OH have been replaced	(Duval and Lawoko, 2014)
lignin-g-polycaprolactone	Lignin hydroxyl groups can also initiate the ring opening polym. of caprolactone, to yield lignin-g-polycaprolactone copolymers	Ratios of chemicals can change between amorphous or crystallizestructure	(Kai et al., 2016)
Iron(III)-complex lignin nanoparticles	iron (III) isopropoxide [Fe(OiPr)3], THF	drug delivery applications, superparamagnetic behavior, cancer therapy and diagnosis (magnetic targeting, magnetic resonance imaging)	(Figueiredo et al., 2017)
Lignin-graft-Polyoxazoline (3-amino-1H-1,2,4 triazole)	A hydrophilic polyoxazoline chain is grafted through ring opening polymerization, possess homogeneous spherical nanoparticles of 10–15 nm. The copolymer was covalently modified with triazole moiety to fortify the antimicrobial and antibiofilm activities	The hydrogel was capable of down regulating the expression level of IL-1β in LPS induced macrophage cells, and to cause significant reduction of iNOS production. It supported cellular anti-inflammatory activity which was confirmed with luciferase assay, western blot, and NF-κB analysis. This novel lignin-based hydrogel tested in-vivo has shown the abilities to prevent infection of burn wound, aid healing, and an anti-inflammatory dressing material	(Mahata et al., 2017)
Copolymers graft (-g-)			
lignin-g-polylactic acid	Lignin reaction with lactide with an organocatalyst (ring opening polymerization) Phenolic OH react slower than aliphatic OH	further blending with PLA can result in homogenous polymer with UV blocking capability	(Duval and Lawoko, 2014)
lignin-g-poly(vinyl acetate)	Creation of a radical on the lignin macromolecule, which initiates polymerization of a monomer, lignin	good water solubility	(Duval and Lawoko, 2014)

lignin-g-polystyrene	radicals can be created by irradiation (or by peroxide). radical will react with lignin to form the lignin ‘‘macro’’-radical	hydrophobic, surface coating able to decrease of water sensitivity of wood	
lignin-g-polyacrylamide		good water solubility	(Duval and Lawoko, 2014; Feng et al., 2014)
lignin-g-poly(acrylicacid)			
lignin-g-poly(N-isopropylacrylamide)	controlled living radical polymerization-formation of long polymer chains	Low PDI 1.09 water soluble or stable suspensions if - solution hating above 32C results in precipitation of hydrophobic co-polymer fiber mats-electrospinning	(Kim and Kadla, 2010)
lignin-g-Polycaprolactone lignin-g-Polystyrene (polyethylene glycol)	Esterification enhance lignin miscibility with polycaprolactone. Oxyproplylation, OH groups termination with a diisocyanate followed by isocyanate copolymerimerization	High variability, depends on conditions, properties can be shifted by changing reaction conditions	(Duval and Lawoko, 2014; Murariu and Dubois, 2016)
lignin-based phenol formaldehyde resins	reaction with formaldehyde on *o or p* positions of phenol to obtain 3D network	often used as adhesive	(Guo et al., 2015; Zhao et al., 2016)
Lignin-based polyurethanes	is used as a source of OH groups, able to react with diisocyanates to yield polyurethanes. polyethylene glycol, polybutadiene glycol, polycaprolactone.	Usually highly crosslinked with high Tg	(Cateto, 2008)
Lignin-based polyesters	Lignin OH groups - polycondensation reactions with diacids or diacyl chlorides results in formation of lignin-based polyesters.	insoluble networks or thermoplastic polyester	(Kai et al., 2016)
Lignin - poly(arylene ether sulfone)	reaction of KL phenolic OH groups with 4,40-difluorodiphenyl sulfone	Very high thermal stability, Tg 150-170 C	(Duval and Lawoko, 2014)

II.3.2 Lignin based hydrogels

Another lignin modification – crosslinking – can result in a hydrogel formation. Hydrogels are attributed with broad functionalities, such as mucoadhesion, bioadhesion, possessing intrinsic properties and response to the environment (temperature, pH, chemicals, etc.). They have been used as scaffolds for growing tissues, in drug delivery systems and wound healing, and can be used for water purification or as sensors and valves in microsystems technology. Another common application is in food technology

(thickening agents, stabilizer).(Bag and Rao, 2006; Gupta et al., 2002; Hoare and Kohane, 2008; Kiick, 2009; Le Goff et al., 2015; Shen et al., 2011; Sun and Lee, 2014; Tobergte and Curtis, 2013; Wheeldon et al., 2008; Zanto et al., 2002; Zehner et al., 2015) Hydrogels are mainly produced from synthetic polymers, but lately there is increased interest in using renewable materials for hydrogel production. Ligninbased hydrogels can offer additional properties, including antimicrobial, antiviral, antioxidant, biocompatibility, biodegradability, low toxicity, eco-friendliness, and degradability. (Ciolacu, 2013; Nagam et al., 2016; Passauer, 2012a; Thakur and Thakur, 2015; Yesilyurt et al., 2016)

There are two basic types of hydrogels: physical and chemical. Physical hydrogels are usually crosslinked with different units by hydrogen or ionic bonding, hydrophobic association or molecular entanglement; these hydrogels can disintegrate over time. Chemical hydrogels contain covalent bonds between the macromolecules and tend to be permanent.(Bag and Rao, 2006) Types of these hydrogels include linear, block, graft copolymers, entangled networks of linear polymers, hydrophilic networks stabilized by hydrophobic domains, polyion–polyion complexes or H-bonded. (Nagam et al., 2016; Passauer, 2012a; Thakur and Thakur, 2015)

One of the hydrogel inherent features is their swelling. The swelling mechanism observed in hydrogels is mainly due to water adsorption by capillary pressure which includes physical and osmotic pressure in the capillary pores and caused by the concentration gradient from outside of the solvent absorbing polymer particles.(Passauer, 2012)

Polysaccharide based hydrogels are already commonly used compared to lignin hydrogels which are much less common mainly due the low polarity of lignin and its complex structure.(Thakur, 2015)

Some attempts to produce hydrogels were done with lignosulfonates because of their water solubility or with modified lignin such as hydroxypropyl lignin. Crosslinking is usually done using formaldehyde, glutaraldehyde, epichlorhydrin and diepoxy. The second most common method used is a copolymerization with acrylamide, vinyl alcohol, etc. Most of these gels have only a limited swelling capacity around 10 g(H_2O)/g(gel), with some exceptions such as 75 g(H_2O)/g(gel). Interestingly, some of the gels with low swelling capacity in water 8.5 g(H_2O)/g(gel) showed a larger swelling in 50% ethanol, resulting in swelling 14.5 g(H_2O)/g(gel). Modified kraft and organosolv lignin (by oxidation with hydrogen peroxide and Fenton reagent) was also used for hydrogel preparation through crosslinking with poly(ethylene glycol) and a swelling capacity around 70 g(H_2O)/g(gel). (Passauer, 2012)

Table 3. Lignin based hydrogels

Method used	Crosslinking agents used	Obtained properties	Ref.
Free radical polymerization	ceric ammonium sulfate with f N,N-methylenebisacrylamide for copolymerization of poly vinyl alcohol and polyacrylamide with lignin	Relatively high swelling capacity 60.0%	(Guo et al., 2013)
Chemical crosslinking	2-hydroxyethyl methacrylate poly(ethyl methacrylate) polyacrylamide poly (methacrylic acid) poly (acrylic acid) poly(glucosylethyl methacrylate) and poly(hydroxypropyl methacrylamide)	Active coating, sorbents, UV stabile	(Hoffman, 2012)
Physical crosslinking	Blending of xanthan and lignin	Novel superabsorbent thermo-oxidatively stable potential for pharmaceutical, cosmetic and food industries	(Raschip et al., 2007)
Graft copolymerization	acetic acid lignin copolymerization with N-isopropyl acrylamide (and N,N-methylenebisacrylamide cross-linker) with H_2O_2 initiator	Hydrogels pore size increased with acetic acid content	(Feng et al., 2011)
	Lignin-polyethylene glycol	Drug delivery	(Marcelo et al., 2016)
	lignin–phenol–formaldehyde hydrogels	Aerogels	(Amaral-Labat et al., 2013)
	Tannin-lignin and tanine-lignin-formaldehyde	Aerogels , thermal conductivity and mechanical resistance same as resorcinol–formaldehyde hydrogels	(Amaral-Labat et al., 2013)
	acetic acid lignin by chemical cross-linking with NCO-terminated polyurethane ionomers	slow-release fertilizer for different agricultural applications	(Peng and Chen, 2011)
	oligo(oxyethylene) lignin, oxidatively activated spruce organosolv lignin crosslinked with poly (ethylene) glycol diglycidyl ether	Swelling decreasing with increasing crosslinking. Application as a soil conditioner - agriculture, forestry.	(Passauer et al., 2012)
Lignin with other biopolymers	cellulose–lignin	Biological activity, fexible, energy storage	(Ciolacu et al., 2012; Xu et al., 2015)
	cellulose and lignin mixing in an alkaline solution cross-linked with Epichlorohydrin		
	lignin - starch starch, acryl amide, lignin/peat	Hydrogels for toxic metal ion removal	(Penaranda and Sabino, 2010)
	xanthan-lignin xanthan and lignin in alkaline medium with Epichlorohydrin crosslinker	Increase in thermo-oxidative stability Thermo-oxidative stability vanillin release as active aroma ingredient	(Raschip et al., 2011)
	alginate with lignin in aqueous alkali solution containing calcium carbonate - under carbon dioxide at 4.5 MPa	Scaffolds for tissue engineering, non-cytotoxic with good cell adhesion	(Quraishi et al., 2014)
	poly(ethylene glycol) methyl ether methacrylate grafted lignin (via atom transfer radical polymerization)	Tunable mechanical response excellent self-healing capability	(Berrima et al., n.d.)

II.4 Energy storage in Lignin based polymers

Lignin is being researched as a carbonaceous material for use in energy storage. However, lignin needs to be modified before such an application can be considered.

The most available and commonly used energy storage systems today are batteries. Such as flow, lead acid, Nickel-metal hydride (Ni-MH), nickel cadmium (Ni-Cad) and lithium ion batteries top the list. Flow batteries are based on reduction and oxidation reactions of two electrolyte solutions. During charging, one electrolyte is oxidized at the anode, while the other electrolyte is reduced at the cathode. The discharge cycle is the same process in reverse. (Díaz-González et al., 2012) Between the two electrolytes is an ion exchange membrane. (Alotto et al., 2014) Chemical energy is converted into electrical energy as electrons travel from anode to cathode as the electrolytes are pumped through the cell.(Alotto et al., 2014) Redox flow batteries have the advantage that power and energy capacity can be separated. The power is determined by the number of cells and size of the electrodes while energy capacity is determined by the volume and concentration of the electrolyte.(Xie et al., 2013)

The lead acid battery is made up of a lead dioxide positive electrode and a sponge lead negative electrode with sulfuric acid as an electrolyte. Both electrodes are converted into lead sulphate during discharge. During charging the electrodes return to the original state. The Ni-MH battery stores hydrogen in metal hyrdride alloys.(Díaz-González et al., 2012) The positive electrode is a nickel hydroxide while the negative electrode is a metal hydride. During discharge, the alkaline electrolyte reduces the metal hydride to metal while an electron leaves the negative electrode and a hydrogen atom goes from the electrolyte into the hydroxide matrix at the positive electrode. (Dunn-Rankin et al., 2005)

Nickel cadmium batteries consist of nickel species as the positive electrode and cadmium species as the negative electrode with an aqueous alkali solution as the electrolyte.(Díaz-González et al., 2012) During discharge, NiOOH reacts with water to

form Ni(OH)2 and OH- at the positive electrode, while at the negative electrode Cd reacts with OH- to form Cd(OH)2. Charging sees the same reactions going to completion in reverse.(Fan and White, 1991) A solution of KOH is the electrolyte. Lithium ion batteries operate based on the electrochemical reactions between positive lithium ions with analytic and catholytic active materials. The anolytic material is graphite and the catholytic material is often based on lithium metal oxide, such as lithium cobalate. The electrolyte is usually an organic liquid which contains dissolved lithium salts. During charging, the positive lithium ions flow from the positive electrode to the negative graphite electrode. (Díaz-González et al., 2012)

Other energy storage systems used today are pumped hydro storage, hydrogen-based energy storage, flywheel energy storage, superconducting magnetic energy storage and supercapacitor energy storage systems.(Díaz-González et al., 2012)

The problem with battery energy storage systems is that they are not really green. The recycling of the materials is very difficult and decomposition of these materials leads to release of toxic compounds in the environment. (Hsing et al., 2013; Nedjalkov et al., 2016)

As the number of intermittent renewable energy capturing devices increase, so does the need for efficient, cheap and clean energy storage systems. Lignin is an abundant biopolymer, cheap and readily available. Lignin is therefore being considered for use in a gel polymer electrolyte, (Díaz-González et al., 2012) as an anode material for lithium ion batteries (S. X. Wang et al., 2013; Zhang et al., 2015) and electrodes of a supercapacitor.(Milczarek and Nowicki, 2013; K. Wang et al., 2016; Xiong et al., 2016)

A supercapacitor is an energy storage device with high power density, fast charging/discharging process, reversibility and a long cycling life.(Xiong et al., 2016) Gong et al. explored a gel polymer electrolyte based on lignin for lithium ion batteries. The results showed excellent liquid electrolyte uptake, thermal stability, ionic conductivity, lithium ion transference number, electrochemical stability, compatibility with anode electrode and cell performance.(Gong et al., 2016)

An additional use of lignin for lithium ion batteries is as the anode material.(Zhang et al., 2015) Hierarchical porous carbon derived from lignin (LHPC) displayed a stable, high capacity, high cycling stability and rate capability. (Zhang et al., 2015)

Another material being used for anodes in lithium ion batteries is a fused carbon fibrous mat fabricated from a lignin-polyethylene oxide blend via electrospinning. Electrospun carbon fibers have the advantage in that they can form highly porous free-standing mats without the addition of any conductive agent or binder. (S. X. Wang et al., 2013)

Other area of research focusies on lignin-based carbon fibers (LCFs), which can function simultaneously as electrode active materials and current collectors for lithium ion batteries.(Tenhaeff et al., 2014) The conversion of the lignin fiber mat into a monolithic structure is a two-step process resulting in stable, free-standing, porous mats. (Tenhaeff et al., 2014)

Lignin is also being explored as a material for rod-shaped porous carbon for use as in electrical double layer capacitor electrode materials. The rod-shaped porous carbon

derived from aniline modified lignin had excellent characteristics, including high specific capacitance, small resistance and high stability in electrochemical performance. Aniline modified lignin would be an improvement from other carbon materials because the nitrogen content may increase capacitance performance. (K. Wang et al., 2016)

Another group did research involving a lignin composite. A kraft lignin carbon nanotube composite displayed persistent electrochemical activity and excellent charge storage properties when deposited on the surface of a gold electrode. The properties of the nanocomposite in addition to the abundance and low cost of kraft lignin make it an excellent prospect for applications such as supercapacitors. (Milczarek and Nowicki, 2013)

A lignin graphene hydrogel is also being explored for use as a possible material supercapacitor electrodes. When combined, lignosulfonate (lig) provides high pseudocapacitance as well as preventing graphene sheets from re-stacking. The lig/graphene hydrogel electrodes exhibited excellent rate capacity and cycling stability. (Xiong et al., 2016)

A bacterial cellulose-lignin-resorcinol-formaldehyde aerogel is another potential source of electrode material for supercapacitors. The blackberry-like structure and large mesopore concentration of this material aid ion transportation and adsorption, leading to high areal capacitance. (Xu et al., 2015)

Yet another material being considered for supercapacitor electrode material is highly porous activated carbon fibers generated from low sulfonated alkali lignin. The excellent electrochemical performance of the constructed supercapacitor affirms that low

sulfonated alkali lignin based activated carbon fibers make excellent carbon-based electrodes for supercapacitors in aqueous electrolytes. (Hu et al., 2014)

II.5 Biological modification of lignin – potential

Fungi, bacteria or their enzymes are used for lignin degradation or decomposition. Basidiomycetes are especially known to decompose or metabolize lignin in nature, therefore there is high interest to use them for the production of monomers. However, both the decomposition rates and the monomer production by these microorganisms are usually relatively low and depends on many variables.(Asina et al., 2017) The best results are usually obtained with engineered microorganisms or with specific enzymes. Less successful are experiments with industrial lignin where the yields of monomers are usually very low at <10%.

Many laccases and peroxidases are considered effective catalysts for lignin degradation/ decomposition of model compounds, however many of these enzymes were also found to cause polymerization.

Enzymes or living cells are not commonly used for lignin modifications even though biotransformations (enzymatic modifications) are known to enable reactions which would be difficult to obtain through organic synthesis. Therefore, enzymes being produced by various fungi and bacteria, or the whole organisms, might not only be used for decomposition of lignin into monomers, but also for the modification of the functional groups, purification, and probably also for spatial rearrangement.

Basidiomycetes are not only known to be natural lignin decomposers but also a large group of fungi including species which are known as medical mushrooms. Most of these fungi are tree fungi, their body is being, basically, metabolized wood.

Fungi need antibacterial and antifungal compounds in order to survive, therefore there is a great potential of fungi for production of bioactive metabolites which could be of benefit to humans (Table 4.). The largest known group of these molecules are immunomodulator which are mainly polysaccharides and β-glucans in complex with proteins, peptides or lignin. These compounds can play an important role in cancer treatment the biological activity depends on substituents, degree of branching and molecular weight, the high molecular weight fractions 1000-1200 kDa of branched β-glucans was found to have high activity.

Another group, less explored, are lignin macromolecules which were found to have antiviral properties, water-soluble lignins from *Inonotus obliquus* inhibited HIV protease with an IC 50 value of 2.5 mg/mL. The same effect was found for water soluble lignin from *L. edodes, C. versicolor.* Additionally, sulfated lentinan from the same fungi prevented HIV-induced cytopathic effect.

Precise analysis of these macromolecules is very challenging. However biological activities observed with different fractions suggest that the focus on the analysis of lignin modified with various fungi might offer a solution in the treatment and prevention of many diseases.

Table 4. Biological activity of biologically modified lignin macromolecules

	Molecules	Effect	Ref.
Lignin	<0.5kDa lignin	Potential for HIV-1gene expression inhibition, broad antiviral spectrum	(Mitsuhashi et al., 2008; Sakagami et al., 2005)
	~ 30kDa lignin	Inhibion of HIV protease	(Wang et al., 2015)
	Lignophenols (MW ~ 1500Da)	Lowering the weight of adipose tissues, plasma Tg levels, and hepatic expression of SREBP-1c mRNA	(Sato et al., 2012)
	Lignin	Antioxidant, anticancer	(Ugartondo et al., 2008)
	Bagasse, lignosulphonate, Curan 100, steam explosion	No harmful effect on skin or eyes	(Vinardell et al., 2008)
	Basidiomycetes metabolites in lgnolytic system	Chlorinated anisil and hydroqinone metabolites. - Antibiotic, pesticides	(Field et al., 1995)
Lignocellulose	Lignin metabolite from Inonotus obliquus (MW 37561Da, 24945Da)	Antiproliferative, inhibited NF-?B activation	(Wang et al., 2015)
	758-5.3kDa	Antiherpes (HSV-1 and HSV-2) activity	(Zhang et al., 2007)
	Lignin with 20% carbohydrate complex	Immunopotentiating activity (antitumor, antimicrobial, antiparasite),	(Sakagami et al., 2005) (Sakagami et al., 2010)

II.5.1 Biological/enzymatic modification

For the enzymatic modification of lignin, isolated enzymes or whole cells can be used, each of them has some advantages and disadvantages. Working with isolated enzymes is simpler, and the productivity is higher, owing to higher substrate concentration tolerance. In addition enzymes can also be recovered if immobilized, and it is possible to perform reactions in organic solvents, however cofactor recycling is enabled.

Comparing enzymes with whole cells systems, one can observe that whole cell systems do not require any cofactors, they have higher activities, produce fewer byproducts when resting cells are use, and immobilized cells can be reused. However the equipment for their application is more expensive, there are problems with scaling up experiments as well as requiring lower concentration tolerances for substrates.(Faber, 2011)

However, there exist almost any enzyme catalyzed reaction for almost any type of organic reaction, such as hydrolysis-synthesis of amides, epoxides, esters, lactones, ethers, nitriles, lactams; the oxidation-reduction of aromatics, aldehydes and ketons, alkanes, alcohols, sulfoxides and sulfides; alkylation and dealkylation; carboxylation, isomaration, Eve Michael-additon, and more. Even some reactions which were not done yet in organic chemistry, such as hydroxylation of aliphatic (selective functionalization of nonactivated positions in organic molecules).(Faber, 2011)

Table 5. Solvent compatibility with enzymes (Faber, 2011)

Solvent miscibility with water and effect	**Solvents (ordered from worst to the best)**
Miscible, concentration 10-50 v/v % can be used without deactivating the enzyme	DMSO, dioxane, formamide, DMF, methanol, acetonitrile, ethanol, acetone
Partially miscible, serious distortion of enzymes, application only with very stable enzymes (deactivation is common)	THF, ethyl acetate, pyridine, butanol, diethyl ether, propyl acetate
Low miscibility, enzyme distortion, can be used but activities are unpredictable.	Butyl acetate, dipropylether, chloroform, benzene
Immiscible, negligible enzyme distortion, high activity retention	Toluene, octanol, dibutyl ether, penthane, carbon tetrachloride, cyclohexane, hexane, octane, decane, dodecane.

A very dense lignin structure can be a problem for the biological/enzymatic modification of lignin. In the lignin modification system induced by microoranisms, there exist not only large enzymes but also small reactive molecules, which help with the degradation, decomposition and modification of lignin such as H_2O_2 or free radicals, such as OOH^-, OH^-, O_2^-, CO_2^-, either directly or through the Fenton reaction.(Jung et al., 2015) Enzymatic modification can be especially problematic when lignin enzymatic modification is being performed on not fully solubilized lignin substrate where enzymes are not able to access the polymer. In this case, utilization of organic solvents can be beneficial. At this moment, most biological treatment is being implemented towards lignin de-polymerization with the main focus being monomers. Usually isolated

lignolytic enzymes or fermentation with fungi and bacteria is being used. The yields of the monomers obtained with these methods are usually very low while the oligomeric or polymeric fractions are not being chemically characterized. Further, the separation of the monomeric products is complicated since many byproducts and nutrients exist in the media. To our best knowledge, the application of enzymation or the addition of organic solvents in order to increase lignin availability was not explored yet, or only minimally.(Brzonova et al., 2017; Milstein et al., 1994)

II.5.2 Modification *via* protein and peptide affinity

Lignin polymers can be cast into nanoparticles, thin films and coatings. Lignin negatively charged surface also makes lignin a great candidate for surface modification with peptides and proteins. Again, this lignin can be used in a broad application such as medical adhesives and products, cosmetic, food packaging and functional coatings.

Lignin is being recognized by some natural and synthetic molecules. Mainly, the interaction with proteins and peptides is currently an area of interest. The mechanism of interaction of these molecules with lignin is usually through hydrogen bonding, hydrophobic interaction and π—π stacking, CH—π interaction or coulombic interaction (Fig. 2). (Charlton et al., 2002; Österberg, 2016; Yamaguchi et al., 2016) Recently, the HFPSP (His-Phe-Pro-Ser-Pro) sequence was recognized in several peptides as lignin binding sequence. (Yamaguchi et al., 2016) When phenylalanine was substituted with Ile in the C416 (HFPSPIFQRHSH) peptide, the affinity for softwood lignin has decreased and affinity towards hardwood lignin stayed same. This indicates that structural differences can be recognized by the C416 peptide which is probably linked to recognition between the G and G/S lignin's. (Yamaguchi et al., 2016)

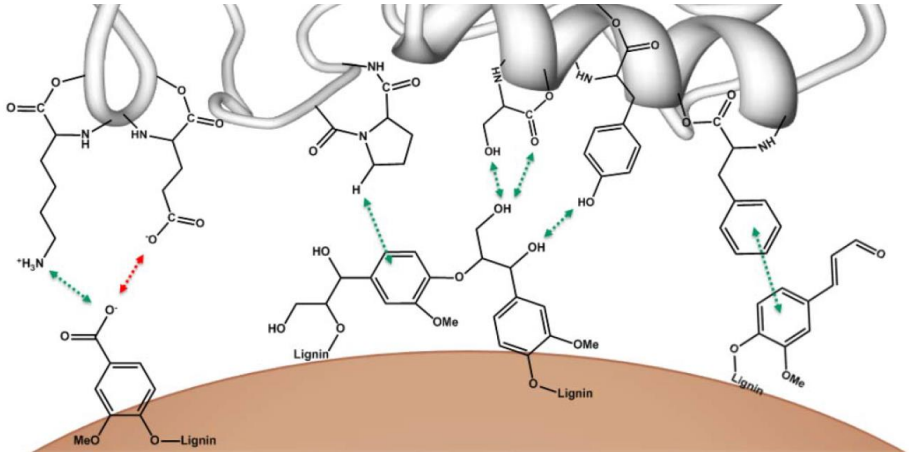


Figure 2. Peptide affinity towards lignin (Österberg, 2016)

Proteins as gelatin, casein, bovine serum proteins, conalbumin and albumin were tested for affinity towards lignin films. (Österberg, 2016) After protein adsorption, the mass of the lignin film was more than 800 ng/cm^2 for gelatin (the highest) and less than 100 ng/cm^2 for albumin (the lowest). A possible reason for this difference can be explained by changes in the protein orientation towards the lignin surface contact. The adsorption was also changing based on the media used: it decreased with the ionic strength (Salas et al., 2013) between water (highest), phosphate buffer, and PBS buffer (lowest). (Österberg, 2016) The correlation between ionic strength, protein adsorption and protein denaturation was also evaluated. (Salas et al., 2013) The correlation between adsorption and amino acids composition was also determined, the only positive correlation was found for Proline. (Österberg, 2016) Proline is known for its affinity towards polyphenols. (Baxter et al., 1997; Charlton et al., 2002) The differences in adsorption of glycine and β-conglycine on lignin surfaces were also evaluated. (Salas et al., 2013) This modification resulted in changes to the wettability of the surface which can improve adhesion to respective composites. (Salas et al., 2013)

These results suggest that peptides might be able to recognize specific areas of lignin. Some researchers reported sensitivity of lignin based biosensors similar to the results obtained with the ELISA method. (Cerrutti et al., 2015) Since specific peptides can self-assemble into specific structures while recognizing specific areas of the biopolymer, this could be used to not only help design functional nanomaterials but also as catalysts for nanoparticle preparation, immunosensing or as adsorbents for pattering. (Cerrutti et al., 2015; Date et al., 2008; Sawada et al., 2009) The affinity of lignin towards various proteins, the biocompatibility of lignin based matrices or sensors can be achieved. (Cerrutti et al., 2015)

II.6 Conclusion and perspectives

Modified lignin based macromolecules present large potential to be used in the polymer industry. The chemical modification of lignin is already being explored, showing us broad potential applications for the chemically modified lignin. Biological modifications of lignin macromolecules are in the very beginning stages in exploring what can be done. The preliminary data are showing very promising results which can prompt the exploration of new categories of antiviral medications, functional polymers, or other medical applications. Biologically modified lignin polymers have also potential to be used in water purification, energy storage systems and much more.

CHAPTER III

Kenaf biomass biodecomposition by basidiomycetes and actinobacteria in submerged fermentation for production of carbohydrates and phenolic compounds

III.1 Abstract

The efficiency and dynamics of simultaneous kenaf biomass decomposition by basidiomycetous fungi and actinobacteria were investigated. After 8 weeks of incubation up to 34% wt. of the kenaf biomass was degraded, the combination of fungi and bacteria was most efficient. Lignin decomposition accounted for ~ 20% of the observed biomass reduction, regardless of the culture used; the rest (80%) of biomass degradation was due to carbohydrate based polymers. Glucose, fructose, galactose and xylose were produced in tangible yields (26-38%) and then consumed by day 25 although some galactose persisted until day 45. Once monosaccharides were depleted, the production of laccase, manganese-dependent peroxidase and lignin peroxidase enzymes, essential for lignin decomposition, was induced. The products of lignin biodecomposition were shown to be water-soluble and characterized by thermal desorption-pyrolysis-gas chromatography.

III.2 Introduction

Lignocellulosic biomass (LCB) is one of the most abundant and economical sources of valuable chemicals and biofuels. LCB, whose production in the US is near 1.4 billion dry tons per year (Limayem and Ricke, 2012), is currently used for manufacturing of methanol and ethanol (Alvira et al., 2010; Balat, 2011; Limayem and Ricke, 2012; Sarkar et al., 2012) from its cellulose portion. By contrast, lignin is currently utilized mostly as a source of heat and electricity by its combustion, although several pioneering applications are envisioned for its use to produce replacement petrochemicals (Brebu et

al., 2011; de la Torre et al., 2013). This study focused on the use of kenaf (*Hibiscus cannabinus L.*) as a biomass source. Kenaf is a readily grown plant both in warm and colder climate zones, thus being a reasonable candidate for production of renewable chemicals (Öztürk et al., 2010).

The use of lignin-degrading fungi and, or cellulase-producing bacteria is essential for efficient biomass conversion. Based on the previous studies conducted with different biomass sources than kenaf, white rot fungi are considered to be the most efficient wood-degrading organisms (Evans and Palmer, 1983; Jo et al., 2010; Sun et al., 2004). Kenaf was also successfully degraded by white rot (Halis et al., 2012) and basidiomecetous (Pereputan et al., 2013) fungi. Table 1 lists the essential information on the previous work on microbial degradation of lignocellulosic biomass. More articles were published but those that are not included in Table 1 addressed only the degradation of carbohydrates rather than that of both carbohydrates and lignin. As can be seen from Table 1, most of the prior work focused on the degradation of agricultural wastes with lower lignin content. Most of the studies used fungi as lignin-degrading cultures; furthermore, most of the work was conducted using *P. chrysosporium* (Singh et al., 2011, Zeng et al., 2011, Zhao et al., 2012, Yao and Nokes, 2014). Neither bacteria nor indigenous microorganisms were thoroughly explored. Few studies involved the use of protocols assessing the conversion of both carbohydrates and lignin beyond simple gravimetric techniques (Crawford et al., 1983, Zeng et al., 2011, Singh et al., 2011).

As a step toward efficient and comprehensive LCB biochemical conversion, this study explores the application of white rot fungi and cellulase producing bacteria, as well

as that of naturally occurring microbial consortia. The white rot fungi used in this study, *Coriolus versicolor* (CV) and *Trametes gallica* (TG), are known to degrade all three major biomass components, i.e., lignin, cellulose and hemicellulose, at similar rates (Jo et al., 2010; Sun et al., 2004). *Microbacterium sp.* (MB) and *Streptomyces sp.* (SM) are cellulase producing bacteria (Hong et al., 2011; Maki et al., 2011) but may also degrade lignin (Seo et al., 2009) and phenolics (Zhang et al., 2013). A comprehensive suite of analytical techniques was applied, not only to assess the biomass conversion efficiency but also to analyze the main degradation products. The traditional gravimetrical analysis was supplemented with field emission scanning electron microscopy (SEM), high process liquid chromatography (HPLC) and thermodesorption – pyrolysis – gas chromatography – mass spectrometry (TD-Pyr-GC-MS), along with enzyme activity and pH monitoring. While using the TD-Pyr-GC-MS method, the innovative use of different temperature steps showed that the observed phenolics were the low-MW products of lignin degradation rather than non-decomposed lignin itself.

As for the novelty in the process logistics, a possibility of simultaneous efficient decomposition of both lignin and cellulose into smaller organic molecules was explored focusing on the dynamics of product evolution. To address this objective, the biomass treatment was conducted using several combinations of lignin converting basidiomycetous fungi and cellulase producing bacteria. The specific goal of this study was to apply *mixtures* of 1) lignin-degrading fungi (CV and TG) and 2) lignocellulose-degrading microorganisms (MB and SM), postulating that such a treatment would facilitate lignin utilization for generation of valuable chemicals due to potential synergy.

Table 1. Overview of lignocellulosic biomass microbial studies targeting lignin degradation.

	Microorganism used		Incubation time (day)	Lignin loss (%)	Biomass loss (%)	Analysis of degradation products	Reference
	Fungi	**Bacteria**					
Wood	*G. trabeum* *P. placenta*	-	112	21 15-30	54-58 34-37	HPLC, (13C-TMAH) thermochemolysis, X-ray diffraction	Schilling et al., 2012
	T. trogii MT	-	84	22	-	DEM, IR, LC-MS	Ji et al., 2012
	C. subvermispora	-	84	20	15-20	gravimetric	Tanaka et al., 2009
	P. ostreatus	-	180	9	-	gravimetry	Piškur et al., 2011
	C. versicolor	-	84	75	-	UV VIS, HPLC, NMR, gravimetry	Evans and Palmer, 1983
Kenaf	*P. sanguineus* *O. latemarginatus*	-	91	15 10		FTIR, SEM, gravimetry	Halis et al., 2012
	O. latemarginatus *R. vinctus* *P. chrysosporium* *C. versicolor*	-	28	-	28 14 8 12	gravimetry	Mohamed et al., 2013
Agricultural waste (straw, stalk)	-	*Bacterial community XDC-2*	12	45 9 16	25 38 20	GC-MS	Hui et al., 2013
	P. chrysosporium	-	21	30	-	Gravimetric, NMR, FTIR, Py-GC MS	Singh et al., 2011
			7	25	27	SEM, gravimetry, FTIR, Pyr-GC, X ray diffraction	Zeng et al., 2011
	-	*Streptomyces sp.*	28	-	-	Pyr-GC-MS	Rodriguez et al., 1997
	-	*S. viridosporuis*	56	APPL (Acid precitable polymer lignin) 20	36	Gravimetric, GPC, NMR, titration	Crawford et al., 1983
	P. chrisosporium	*S. viridosporus* *S. badius* *S. setonii*	21	APPL 19-27	-	HPLC, GPC	Pometto and Crawford, 1986
	P. chrysosporium	-	10	14-36	-	gravimetric	Yao and Nokes, 2014
	T. hirsuta	-	42	71	73	gravimetric	Sun et al., 2011
	P. chrysosporium	-	15	34	10	gravimetric	Zhao et al., 2012

III.3 Materials and Methods

III.3.1 Feedstock and microorganism

Air dried kenaf biomass was provided by the Department of Plant Sciences, North Dakota State University (Fargo, North Dakota, USA). Kenaf biomass was pulverized in a Thomas Wiley mill to yield the average particle size distribution between 2.0 - 1.0 mm. Cellulase producing bacteria (MB) and (SM) and basidiomycetous fungi, (CV) and (TG), were obtained from the Department of Biology of Lakehead University, Thunder Bay, Ontario, Canada. All other chemicals were purchased from Sigma Aldrich, St. Louis, MO, USA.

III.3.2 Biodecomposition setup

Erlenmeyer flasks (200 mL) used for biodecomposition experiments contained 2.0 g of kenaf and 75 mL of liquid medium. A medium for cultivation of both fungi and bacteria was prepared by mixing 2.7 g dextrose, 5.0 g sucrose, 1 g $MgSO_4$, 2.5 g peptone, 1.5 g yeast extract, 2.5 g NaCl and 1.5 g KH_2PO_4 in 1 L of deionized water (i.e., phosphate buffer, pH 5.5). Kenaf was sterilized along with the liquid medium for 20 min at 121 °C. Once cooled down, the microbial mixtures were inoculated. For inoculation with fungi, eight pieces of 4 × 4 mm of agar plates were applied. For bacterial inoculation, 1 mL of bacterial medium with an OD_{600} of 1.0 was added.

Experiments were conducted while varying three environmental parameters, stirring, light and temperature; namely, (1) both stirred and non-stirred media, (2) either under light or in the dark and (3) at room temperature vs. the incubator temperature, 29 ^{o}C. For experiments in the dark, the Erlenmeyer flasks were completely covered with

aluminum foil. The experiments with stirred media were performed in an incubator at 29 °C for 57 days, with a rotating speed of 60 rpm. The samples for analysis were taken on days 6, 11, 18, 25, 36, 46 and 57.

Both CV and TG were used in all binary and ternary combinatorial combinations among themselves and/or with MB and SM bacteria, plus the combination of all four strains and a mixture of two bacteria as specified in Table 2. Control samples included 1) sterile kenaf biomass with the corresponding dead (sterilized) microorganism(s) and 2) non-sterile kenaf biomass without any specific bacteria or fungi. Experiments were conducted in triplicate unless the variation in operational conditions resulted in no difference; then, the values obtained with and without illumination and/or shaking were combined.

Table 2. Experimental setup for kenaf biodegradation experiments. The microorganisms used are labeled with X while the applied operational conditions are labeled with Y.

Fungi		Bacteria		Operational conditions		
Coriolus versicolor	*Trametes gallica*	*Mycobacterium sp.*	*Streptomyces sp.*	Stationary /dark/ room temp	Stationary /light/[a] Room temp	Stirring /with less light/[b] at 29°C
X	-	-	-	Y	Y	Y
-	X	-	-	Y	Y	Y
X	X	-	-	Y	Y	Y
X	-	X	-	Y	Y	Y
X	-	-	X	Y	Y	Y
-	X	X	-	Y	Y	Y
-	X	-	X	Y	Y	Y
X	X	X	-	Y	Y	Y
X	X	-	X	Y	Y	Y
-	-	X	X	Y	Y	Y
X	X	X	X	Y	Y	Y
-	-	-	-	Y	Y	Y

[a] Sunlight passed through a window glass
[b] In an incubator which was placed in a room with sunblinds.

III.3.3 Chemical characterization

III.3.3.1 Gravimetric analysis

The overall analysis setup is shown in Fig. 1A, with the chromatographic analysis detailed in Fig. 1B. The biomass amount, its lignin and ash contents were measured gravimetrically using a standard method, NREL TP510- 42618 (Sluiter et al., 2011).

III.3.3.2 Monosaccharide analysis

Samples were filtered through a 0.2 µm filter and run through an Agilent 1200 HPLC (Waldbronn, Germany) with a refractive index detector, RID (Shanghai, China) and a 300 × 7.8 mm Transgenomic CHO-Pb column (Omaha, NE). The mobile phase was deionized water with a flow rate of 0.6 mL min^{-1}. Prior to the sample analysis, a set of external calibration standards were analyzed to calibrate the HPLC RID detector. The concentrations of standards ranged from 0.5 to 18 g L^{-1}. In addition, an internal sugar recovery standard with a concentration of 4.0 g L^{-1} was run every 8 injections to test for column parameter drift and validate the RID readings. The external standard and recovery standard solutions consisted of D-(+)glucose, D-(+)xylose, D-(+)galactose, L-(+)arabinose, and D-(+)mannose.

III.3.3.3 Scanning electron microscopy (SEM)

At the end of experiments (day 57), the liquid medium was filtered through a 0.02 µm filter; then, one droplet of this sample was placed on a glass cover plate and air-dried at room temperature (24±3 °C). The resulting dry residue was examined under a field emission scanning electron microscope, SEM (Hitachi, SU8010, Japan). The solid

fraction of the remaining kenaf biomass was washed three times with distilled water, centrifuged (13,000 rpm) and dried at 65 °C; then examined with the same microscope.

III.3.3.4 Analysis of biomass (lignin) decomposition products

The lignin decomposition products were analyzed by subjecting the filtered solid phase (Fig. 1B) to a thermal desorption-pyrolysis-gas chromatograph with mass spectrometric detection (TD-Pyr-GC-MS) Agilent 6890 (Santa Clara, CA) at three allotted times: 1) at the start of biodecomposition experiments, then 2) on day 25, after the depletion of the bulk of carbohydrates, and 3) at the end of experiments, on day 57. Liquid fractions of selected samples were centrifuged (10, 000 rpm, 5min) and filtered through a 0.2 µm filter. After the pH in the liquid fraction was adjusted to 6.0 with 0.1mM H_2SO_4, a brown precipitate appeared. This precipitate and liquid fractions were dried and analyzed separately on TD-Pyr-GC-MS using a CDS Analytical Oxford, PA 19363-0277, Inc., Pyroprobe 5200 Series with an Agilent 7890A GC and 5975C (Santa Clara, California) MS with electron ionization.

The pyroprobe program consisted of three temperature steps: TD at 350 °C for 3 min, 450 °C for 1 min (thermodesorption of adsorbed species and pyrolysis of oligomers) and 700 °C for 1 min (pyrolysis of high-molecular weight (MW) chemicals). Samples were collected after each temperature step on a TENAX trap and evolved at 300 °C through a heated transfer line to GC-MS. The analyses were performed using a 30 m long DB-5MS column with 0.25 mm I.D. and 0.25 mm film thickness. Ultra-pure helium (99.999%) was used as the carrier gas with a constant flow rate of 1.1 mL min^{-1} and split ratio 10:1. The GC oven temperature program started at 40 °C min^{-1}, followed by a gradient of 35 °C min^{-1} to 80°C then 15 °C min^{-1} gradient to 320 °C and hold for 7 min,

with a total analysis time of 25 min. The lignin decomposition was assessed as a percent response of the sum of three TD-Pyr (350, 450, 700 °C) steps.

III.3.3.5 Enzyme activity measurements

The enzyme activity measurements were conducted using a UV-vis Evolution ™ 600 Spectrophotometer (Thermo Fisher Scientific, Madison, WI) according to the procedures described by Hong et al., 2011. The laccase activity was determined based on the product absorption at 436 nm with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) as a substrate whereas that of manganese peroxidase (MnP) was measured at 469 nm using 2,6-dimethoxyphenol (DMP) as a substrate. Lignin peroxidase (LiP) was assayed at 651 nm with azure B as a substrate after a 1 h reaction at room temperature. For all of these assays, 250 µL of a filtered sample, 250 µL of a substrate (100 mM) and 500 µL of 0.01 M phosphate buffer were mixed. The reaction was initiated by the enzyme addition. The corresponding blanks contained no enzyme and consisted of 250 µL of the original sterile growth medium with 750 µL of 0.01 M phosphate buffer. One unit (U L^{-1}) of enzyme activity was defined as the amount of enzyme that transformed 1.0 µmol of a substrate per minute.

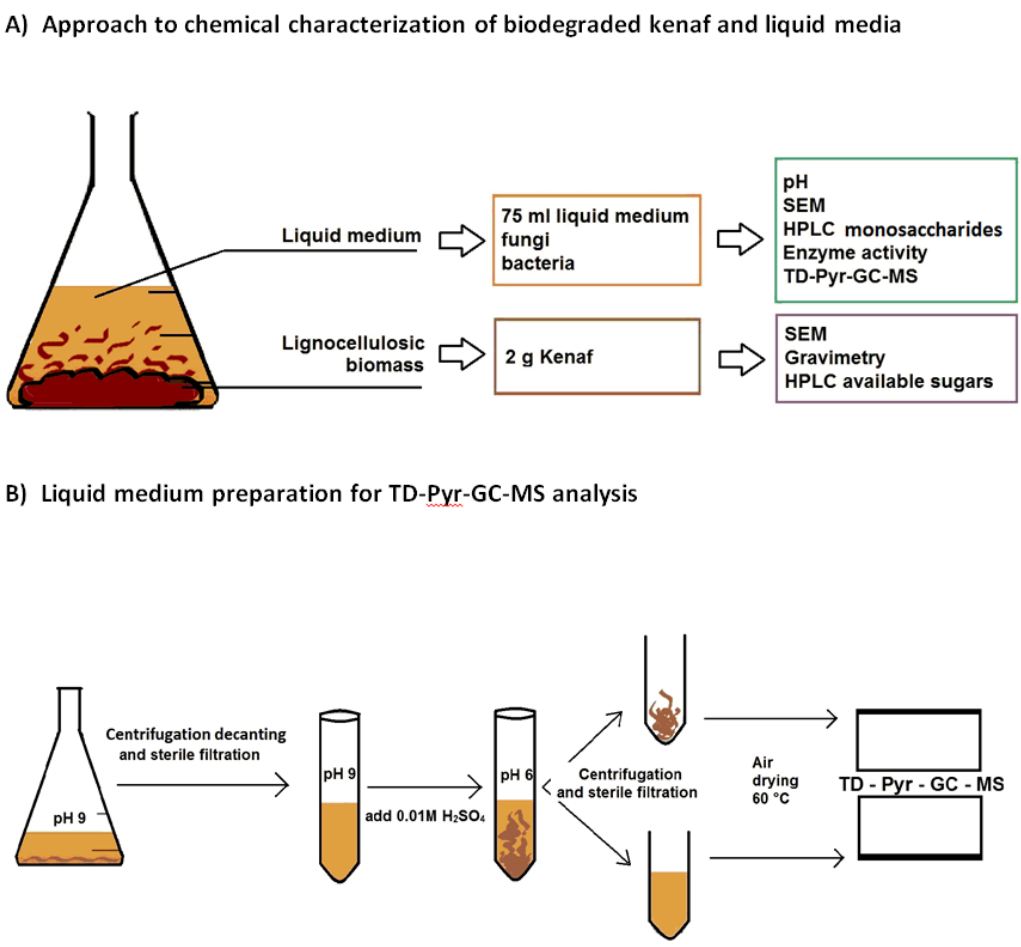


Fig. 1. The experimental setup. A) Decomposition experiment and analytical methods applied to biomass and medium. B) Detailed protocol for sample preparation for the TD-Pyr-GC-MS analysis of lignin decomposition products.

III.4 Results and Discussion

III.4.1 Kenaf biodecomposition

The gravimetric information obtained in kenaf biodecomposition experiments is provided in Table 3. The biodecomposition was statistically different from the blank in all of the runs. It was also statistically similar with the control containing only indigenous microorganisms (no specific microorganisms were added). The combination of all four microbial species turned out to be most efficient; 34 ± 2 % of the biomass was degraded in those runs in 57 days. Similar values were reported for bacterial (*Streptomyces*) biomass treatment, 36% of *Zea mays* decomposition in 56 days (Pometto and Crawford, 1986a; Crawford et al., 1983).

Table 3. Gravimetric characterization of kenaf biomass samples after 57 days of biodecomposition.

	Solid fraction [a]		**Soluble fraction** [b]
Biodecomposition organism	**Biomass** decomposition **(wt. %)**	**Lignin** decomposition **(wt.%)**	**(wt.%)**
CV	24 ± 1	19 ± 1	25 ± 1
TG	21 ± 1	20 ± 1	24 ± 1
CV, TG	29 ± 2	19 ± 1	26 ± 1
CV, TG, MB, SM	34 ± 2	20	31 ± 1
Biomass with only indigenous microorganisms (control)	22 ± 1	12 ± 2	17 ± 1
Biomass **sterilized** with no specific microbial pretreatment (blank)	3 ± 1	3 ± 1	3 ± 1

[a] Calculated using a sample preparation protocol described in Section 2.3.1 as compared to the original sample.
[b] Calculated as the mass of dissolved matter (after liquid evaporation) as compared to the mass of the original sample (Kenaf biomass plus solid medium components)
CV *Coriolus versicolor*
TG *Trametes Gallica*
MB *Microbacterium sp.*
SM *Streptomyces sp.*

The gravimetrically measured lignin biodecomposition percentages listed in Table 3 show similar results for both fungal species and their combination. Thus, a simultaneous application of lignin degrading fungi does not appear to result in a significant enhancement of biomass decomposition. However, the biomass decomposition was enhanced when both fungi and bacteria were applied. The numerical values were similar to those published in literature. Crawford et al. (1983) reported 19, 13, 13, 5 and 3% lignin decomposition using *Streptomyces viridosporus* for *Zea mays*, corn, quarckgrass, maple and spruce, respectively. i.e., the biomass source played the major role.

Lignin degradation may also depend on operational conditions. Evans and Palmer (1983) did not observe any lignin decomposition by *C. versicolor* in nitrogen-rich media (milled wood lignin), as well as in the absence of cellulose as a co-substrate. By contrast, 45% lignin decomposition in 14 days was reported under optimized conditions (nitrogen limitation) using solid-phase fermentation, with a lignin disc as a substrate. Nevertheless the yields obtained in this study are similar to those reported earlier.

SEM of the residual biomass showed a significant difference between the biomass incubated with specific fungal and bacterial strains and that incubated only with indigenous microorganisms (control). The SEM image of control was similar to the sterile blank. By contrast, the biomass particles were broken down into smaller pieces in the samples treated with specific microorganisms, perceivably as a result of biodecomposition. Particle size before the microbial decomposition, 1.0 ± 0.2mm, was similar to the particle size of kenaf incubated with indigenous microorganisms only (data

not shown). By contrast, the biomass particle size after the microbial decomposition became smaller, around 0.2mm (Figure 2).

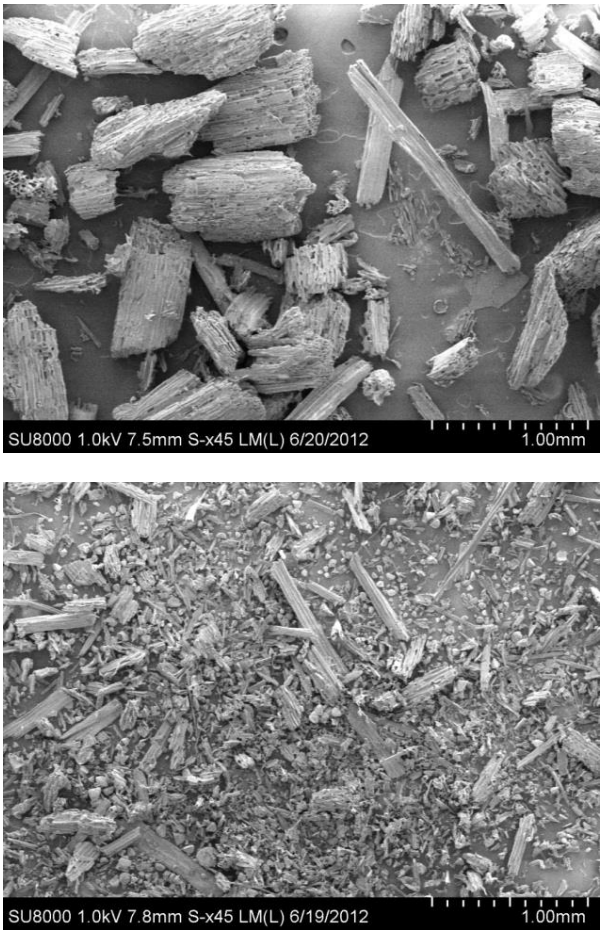


Figure 2. SEM of kenaf biomass following microbial degradation with CV, TB and MB (right) as compared to the control containing only indigenous microorganisms (left).

Structural changes were also observed; the original kenaf particles maintained their structure upon a shearing stress whereas the biomass particles after microbial decomposition readily fell apart into dust even under a small pressure applied. In the samples that underwent microbial decomposition, crystals were observed that may represent phenolic compounds, salts, or lipids. By contrast, these crystals were absent in

the samples without a specific microbial treatment. These changes indicate significant kenaf biomass degradation.

III.4.2 Cellulose and hemicellulose decomposition

Results from low-MW carbohydrate analysis (Fig. 3) showed the time-dependent release of glucose, xylose, galactose and fructose into the medium occurring at different times. At the beginning of incubation, only glucose and galactose (apparently produced during the sterilization) were present in concentrations of 8.0 and 0.4 g L^{-1}, respectively. During the microbial growth the glucose concentration increased by a value, which varied between 1.8 and 6.3 g L^{-1} for different cultures until day 6. The highest yield (38%) was obtained by a combination of all four strains. The glucose concentration then gradually declined approaching zero between days 11-18. The only minor exception was the incubation with the mixed culture of *Microbacterium* and *Streptomyces*, where the starting glucose concentration did not change until day 18 and then gradually declined to reach statistical zero on day 25. The galactose concentration increased until day 18 , to 1.6 and 2.1g $L^{-1}$and then gradually declined until day 46. The highest observed yield of galactose was 38% with *T. gallica* and *Microbacterium sp*.

Xylose was first detected on day 18 reaching the value between 0.2 and 1.7 g L^{-1} on day 18. The highest xylose yield, 26%, was observed when the biomass was incubated with both *Microbacterium sp. and T. gallica*. After day 18 the concentration of xylose gradually declined and the complete removal of xylose was recorded on day 36.

Fructose appeared on day 6, where the highest concentration was almost 6.3 g L^{-1} for all of the microbial combinations except for the fungus-free bacterial mixture, in

which the fructose concentration increased up to 2.2 g L^{-1}on day 11. The % yield of fructose could not be calculated as it was produced as a result of isomeration of other carbohydrates. Then the concentration was decreasing until day 25, when no fructose was observed in the medium. At the same time, near day 25, the medium virtually lost its fluidity in most of the samples becoming gel-like, apparently due to a massive mucus formation resulting from sugar conversion. By day 50, the mucus disintegrated and the medium regained its fluidity.

Similar maximum monosaccharide yields were reported in literature. Tanaka et al., 2009 and Taniguchi et al., 2005 obtained 50% and 33% glucose yields from rice straw using *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*, respectively. Wan and Li, 2007 reported a 66% glucose yield with *Ceriporiopsis subvermispora* from corn stover (Wan and Li, 2012). Even though the microorganisms used in the current work are not known to be the most efficient polysaccharide degraders, the maximum glucose yields obtained by us, e.g., 38 ± 6% for the combination of all four microorganisms, were similar to these values.

Thus, the bulk of monosaccharides were produced within the first 1-3 weeks of incubation. Different monosaccharides were produced sequentially rather than simultaneously, apparently reflecting the order of biodecomposition of several carbohydrate-based polymers. Thus, they can be harvested sequentially if sugars could be readily separated from the rest of the mixture. By day 28, most of the monosaccharides were removed from the medium, although the observation of abundant mucus formation indicated that some of them could be stored as readily digestible polymers.

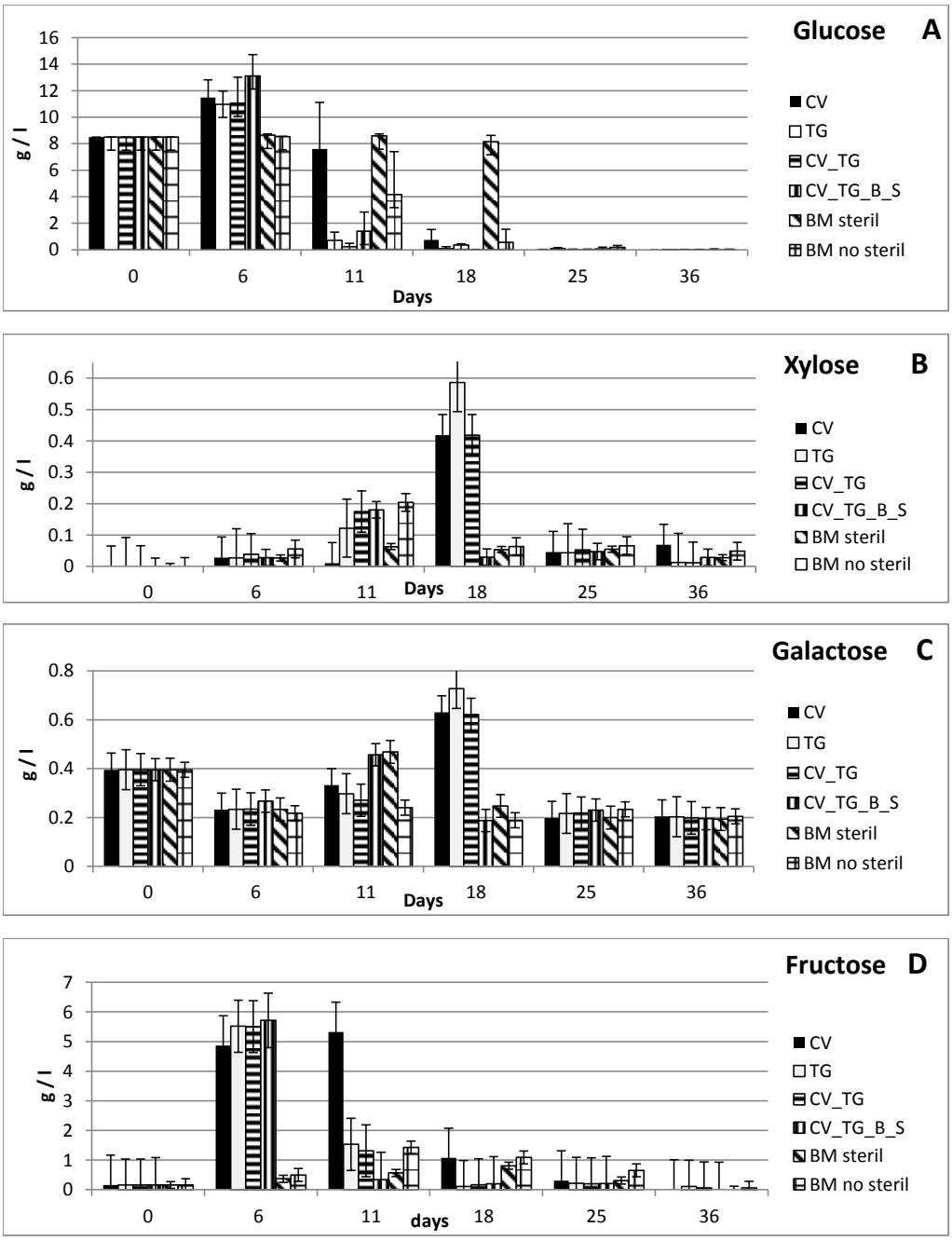


Fig. 3. Specific monosaccharide yields during the decomposition study: Glucose (A), Xylose (B), Galactose (C), Fructose (D). On day 46, no sugars were detected in the medium in detectable amounts.

III.4.3 Occurrence and analysis of lignin decomposition products

Chromatograms of day 57 dried filtered media were obtained using TD-Pyr-GC-MS at temperatures of 350, 450, and 700 °C. The reason for using different temperatures is the well-known paradigm that for most organic chemicals, thermodesorption of monomers occurs below or near 400 °C whereas chemicals evolving at much higher temperatures are the products of pyrolysis of oligomers and polymers (Beranek et al., 2013). Adsorption of monomers on both organic and inorganic residues could slightly shift the evolution temperatures higher. Thus, most of the monomers including phenolics were expected to appear at 350 °C; the rest of the monomers and either dimers or products of their decomposition should evolve at 450 °C whereas the decomposition products of polymers would be seen at 700 °C.

When comparing the TD-Pyr-GC-MS profile of the original kenaf biomass with those of the samples that underwent microbial decomposition, the occurrence of some of the observed phenolic compounds was similar. However as expected, some of them shifted from the high-temperature fractions in kenaf to lower-temperature fractions in the microbial treatment products suggesting decomposition of high - MW species to lower - MW species. Furthermore, several other phenolics (marked in Table 4) were observed only in the samples obtained after the microbial treatment. Thus, the TD-Pyr-GC-MS profiles of treated samples developed some specific signatures indicating lignin biodegradation.

Table 4. Distribution of lignin decomposition products in wt. % among the TD-Pyr-GC fractions in both the precipitate and supernatant obtained upon acid precipitation on day 57.

Temperature steps	Supernatant			Precipitate		
	CV, TG	CV, TG, B, S	Nonspecific MO	CV, TG	CV, TG, B, S	Nonspecific MO
350 °C	9 ± 2	9 ± 3	10 ± 3	2 ± 1	8 ± 2	8 ± 2
450 °C	52 ± 3	33 ± 5	12 ± 5	20 ± 4	15 ± 4	17 ± 5
700 °C	0 ± 2	1 ± 1	2 ± 2	1 ± 2	1 ± 3	4 ± 3
Sum of lignin derivatives	**61 ± 4**	**43 ± 4**	**24 ± 2**	**23 ± 4**	**24 ± 3**	**29 ± 3**

- On days 1 and 25, no quantifiable lignin products were observed

As expected, no lignin biodecomposition products were observed on day 1. No phenolics were observed in TD-Pyr-GC-MS fractions on day 25 either (data not shown). Only early-eluting nitrogen-containing compounds, perceived as protein decomposition products, were observed along with furans and furfurals, which are common products of sugar thermolysis. By contrast, phenolic compounds were observed on day 57 as abundant late-eluting peaks at 350 and 450 °C but not at 700 °C. Similar phenolic products of pyrolytic lignin decomposition were reported in literature (Jiang et al., 2010; Gosselink, 2011; Koncsag et al., 2012; Mansouri and Salvadó, 2006), thus confirming our data obtained by TD-Pyr-GC-MS.

The % of phenolic products among all eluted compounds (in all three temperature fractions) was estimated (Table 4, see the column labeled as "supernatant"). These semiquantitative results corroborate the qualitative information obtained with TD-Pyr-GC-MS. Namely, the majority of phenolics were observed in the 450 ^{o}C fraction; this relatively high elution temperature indicates the thermal decomposition of either lignin (which could dissolve in the alkaline medium toward the end of experiments) or its lower-MW oligomeric decomposition products. To ascertain whether the non-degraded

polymeric lignin remained in the solution as opposed to its partially degraded oligomeric fragments, an additional experiment was conducted.

In this additional experiment, sulfuric acid was added to the samples taken on day 57; the precipitates formed were subjected to TD-Pyr-GC-MS and the results were compared for the solution and precipitate. The pertinent classification of the peak sums is provided in Table 4. The precipitate turned out to contain a much smaller fraction of phenolics than the corresponding solutions, eluting mostly the products of lipid and protein thermal decomposition. For example, for the combination of CV and TV, phenolics in the liquid medium accounted for 61 ± 4% of the total GC-eluted peaks whereas their fraction in the precipitate was only 24 ± 2%. Gutiérrez et al., 2006 observed significant amounts of lipids (wax) present in lignin obtained from kenaf. This observation corroborates the recovery of large amounts of lipids precipitated with lignin in the current study.

Thus, the phenolic compounds observed in TD-Pyr-GC-MS experiments should be attributed to water-soluble oligomers produced as a result of lignin biodecomposition as opposed to non-degraded lignin. The lack of them observed on day 25 corroborates the data on carbohydrate biodecomposition and lignin-degrading enzyme activities (covered in the next section); the lignin started being degraded only after the bulk of readily digestible carbohydrates were depleted. It is of note that the non-specific control with indigenous microorganisms (i.e., with no specific fungi or bacteria added), showed less phenolics in the supernatant (Tables 4, 5 and Figure 4). By contrast, the fungal culture showed the greatest amount of phenolics in the solution, i.e., monomers and lower-MW oligomers, as expected for efficient lignin degrading cultures (Tables 4, 5). A relatively

smaller amount of low-MW phenolics found in the mixed culture containing both fungal and bacterial species compared to the strictly fungal culture may be explained by their further biodecomposition by bacterial species, through their catabolism.

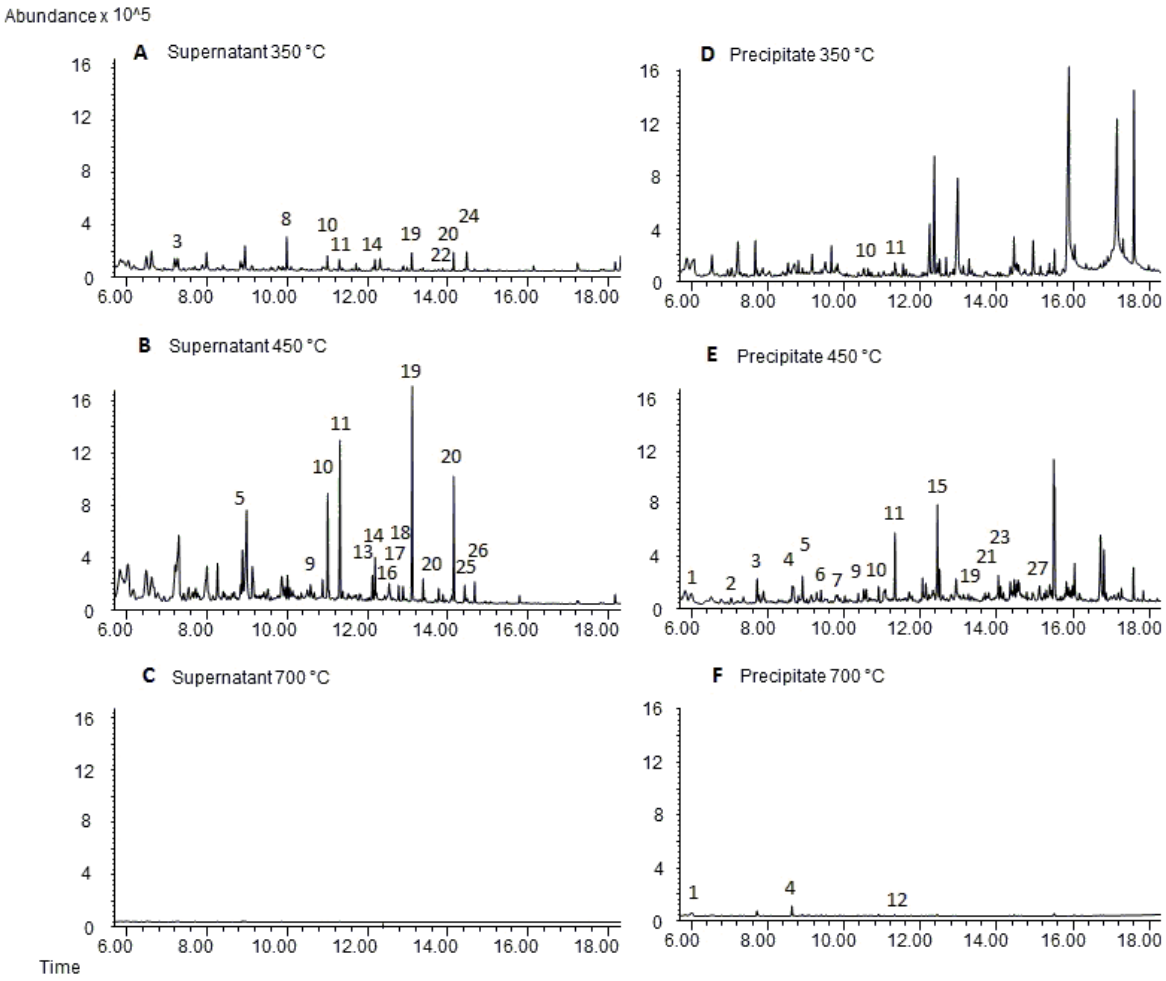


Figure 4. TD-Pyr-GC-MS chromatograms showing the distribution of methoxyphenols and aromatics (for labels of identified chemicals see Table 3) for biodecomposition with combination of C. versicolor and T. gallica after 57 days. Panels A (350 °C), B (450 °C), C (700 °C) represent the composition of the supernatant (decanted solution) after 57 days of incubation (as detailed in Section 3.6). Panels D (350 °C), E (450 °C), F (700 °C) reflect the composition of the precipitate in the same sample (57 days).

Table 5. Specific products of lignin decomposition evolving in TD-Pyr-GC-MS experiments detected in the representative runs using CV and TG. The highlighted chemicals were not present in the TD-Pyr-GC-MS of original kenaf biomass.

				Precipitate			Supernatant		
#	Lignin decomposition products	m/z [a]	tr (min) [b]	350 [c]	450 [c]	700 [c]	350 [c]	450 [c]	700 [c]
1	Toluene	39, 65, 91	6.00			++			
2	Styrene	44, 72, 104	7.04		++				
3	Phenol	39, 66, 94	7.72		+++	++	++		
4	Methylphenol	39, 77, 107	8.64		+++	++			
5	Guaiacol	81, 109, 124	8.90		+++	+ +		+++	+
6	Benzenediol	64, 81, 110	9.82		++				
7	Methylguaiacol	95, 123, 138	9.89		+++	++			+
8	Coumaran	65, 91, 120	9.99				+++		
9	Methoxybenzenediol	97, 125, 140	10.58		+++			+++	
10	Vinylguaiacol	107, 135, 150	11.03	+++	+++		+++	+++	+
11	Syringol	111, 139, 154	11.32	+++	+++	+ +	++	+++	
12	Dihydroxybenzoquinone	54, 69, 138	11.59			++			
13	Methoxysalicylic acid	53, 125, 153	12.12					+++	
14	Isoeugenol	55, 77, 103	12.18				+++	+++	
15	Isopulegol acetate	43, 81, 136	12.49		+++				
16	Acetovanillilone	43, 84, 151	12.54					+++	
17	Trimethoxy toluene	107, 139, 167	12.76					+++	
18	Guaiacyl acetone	43, 122, 137	12.88					+++	
19	Methyl syringaldehyde	77, 165, 180	13.13		++		+++	++++	
20	Propenylsyringol	77, 91, 119	13.37				+++	++++	
21	Homovanilic acid	94, 122, 137	13.79		++				
22	Dimethoxyhydroxybenzaldehyde	65, 111, 182	13.87				++		
23	Dimethoxymethylphenol	125, 153, 168	14.13		+++				
24	Coniferyl aldehyde	51, 77, 135	14.46				+++		
25	Coniferyl alcohol	77, 91, 137	14.48					+++	
26	Homosyringic acid	43, 167, 210	14.67					+++	
27	Hydroxymethoxybenzenemethanol	70, 125, 154	15.29		++				

[a] major ion (mass to charge ratio)
[b] retention time
[c] abundance as measured by the units of the MS peak using ion current:
> 1.000.000 ++++, > 100.000 +++, > 10.000 ++, > 1.000 +

III.4.4 Enzyme activity profiles

Lignin degrading enzyme activities peaked at different times (Table 6). However, all of them peaked after the bulk of monosaccharides had been degraded (25 day). This was expected because the lignin decomposition would become significant only when most of the readily biodegradable carbohydrates were depleted. After the peak of each enzyme activity was passed, the activity did not decline completely until the end of experiments, apparently reflecting the continued lignin decomposition.

Oxidative enzymes require oxygen through aeration; thus, as expected, the LiP activity was significantly greater for stirred samples in an incubator compared to the non-stirred samples run at room temperature. However, both MnP and laccase activities were statistically similar for stirred and non-stirred samples. Neither the other enzyme activities nor monosaccharide concentrations exhibited any dependence on this factor. Apparently, the oxygen demands were met even without stirring.

Table 6. Maximum enzyme activities and elapsed times at which they were observed.

	Laccase		Manganese Peroxidase		Lignin Peroxidase	
Biodecomposition with:	Day	U L^{-1} (n=3) [b]	Day	U L^{-1} (n=3) [b]	Day	U L^{-1} (n=2) [b]
CV	25	63 ± 11	25	7 ± 4	18	13 - 19
TG	18	25 ± 7	6-18	6 ± 1	18	7 - 11
CV, TG	6-18	25 ± 2	18	5 ± 2	18	6 - 16
CV, TG, MB, SM	18	33 ± 12	11-18	13 ± 4	18	5 - 21
Biomass with non-specific indigenous microorganisms (Control)	18	110 ± 25	18	22 ± 21	18	16 - 19
Biomass sterilized (Blank)	46	[a]	46	[a]	46	[a]

[a] Statistical zero

[b] U L^{-1} One unit of enzyme activity was defined as the amount of enzyme that transformed 1 µmol of substrate per minute.

n – Number of replicates

Akin et al. (1996) reported similar laccase, LiP and MnP activities for *T. gallica* degrading peat in low-nutrient mineral media. Hong et al. (2011) reported that *T. gallica* exhibited the highest laccase activity, 103 U L^{-1}, on day 25; the MnP activity by *C. versicolor*, 10 U L^{-1}, also peaked on day 25 whereas no significant LiP activity was observed. Our results obtained with the same fungi but with a different biomass source were qualitatively similar for laccase and MnP, although we also observed a significant LiP activity, between 13-19 U L^{-1}, on day 18. *T. gallica* consistently showed slightly lower values than *C. versicolor* for all enzyme activities. Pometto and Crawford (1983a) observed the peaks of lignin-degrading enzyme activities in *Streptomyces* much earlier than in the current study, on day 1 of cultivation. However, their entire measurement period was just 4 days.

Andreu and Vidal, (2011) determined that products of lignin decomposition served as mediators for laccase activity. We also observed significant although irregular increases of all three monitored enzyme activities after day 40 when products of lignin decomposition started to accumulate in the medium (see the previous section). These data are not shown in Table 6 because they could not be interpreted unambiguously since significant microbial contamination became noticeable at those incubation times.

Surprisingly, the levels of lignin-degrading enzyme activities did not fully correlate with lignin decomposition. In particular, the highest enzyme activity observed (Table 6) was that expressed by non-specific indigenous microorganisms (originally present in the kenaf biomass), which did not show any lignin and biomass decomposition beyond the limits of statistics (Table 3). On a similar token, *C. versicolor* exhibited the highest laccase and lignin peroxidase activities, yet showing no increase in lignin

decomposition (Table 3). Yet, the most efficient lignin degrading microbial combination including all four strains also showed relatively high levels of pertinent enzyme activities (Table 6).

III.4.5 pH profile

The pH decreased during the incubation for the first 11 days reaching the value of 3. The observed pH decrease can be explained by the accumulation of acidic products of sugar metabolism, e.g., pyruvic acid. However, after day 11, pH gradually increased to 9. The observed pH increase is unlikely to be the result of cell lysis, because cells continued to exhibit significant carbohydrate and lignin decomposition for most of the allotted time. Thus, it is more likely to reflect the formation of furfural and levoglucosan derivatives. The largest pH change was from 2.9 on day 6 to 8.6 on day 59 observed with *C. versicolor* in combination with *Microbacterium sp.*

The literature recommends the use of acidic media when the main expected products are carbohydrates, e.g., pH=4 (a lower pH might help with cellulose hydrolysis) for wheat straw biodecomposition by *Streptomyces* (Koncsag et al., 2012). However, whenever lignin biodecomposition was targeted, alkaline media were applied, e.g., pH=8.4 for *Streptomyces viridosporus;* a higher pH might be essential to neutralize phenols and make them more accessible (Giroux et al., 1988; Pometto and Crawford, 1986a). Thus, the pH profiles observed in the current work appear to be representative for both lignin and carbohydrate-based polymer biodecomposition.

III.5 Conclusion

The time profiles of carbohydrate and lignin biodecomposition by fungal and bacterial cultures show that the release and then removal of the bulk of monosaccharides occur prior to the onset of the lignin biodecomposition, even when using fungal cultures designed for fast lignin decomposition. This feature creates a potential of collecting the monosaccharide products separately from phenolics, for their subsequent use in production of carbohydrate-based biofuels and lignin-based valuable chemicals. The TD-Pyr-GC-MS data confirmed that lignin decomposition occurs during the microbial action. Fungi and bacteria yielded similar results although the combination of all four strains featured greater biomass decomposition

CHAPTER IV

Fungal biotransformation of insoluble Kraft lignin into a water soluble polymer

IV.1 Abstract

Low substrate solubility and slow decomposition/ biotransformation rate are among the main impediments for industrial scale lignin biotreatment. The outcome and dynamics of Kraft lignin biomodification by quasi-immobilized basidiomycetous fungi, *Coriolus versicolor*, were investigated in the presence of dimethyl sulfoxide (DMSO). The addition of 2 vol% DMSO to aqueous media increased the lignin solubility up to 70%, while the quasi-immobilized fungi maintained their ability to produce lignolytic enzymes. Basidiomycetous fungi were able to grow on solid media containing both 5-25 g/L lignin and up to 5 vol% DMSO, in contrast to no growth in liquid media as a free suspended culture. When a fungal culture pre-grown on agar was used for lignin treatment in an aqueous medium containing 2-5% DMSO with up to 25 g/L of lignin, significant lignin modification was observed in 1-6 days. The results suggest that lignin was biotransformed, rather than biodegraded, into an oxygenated and crosslinked phenolic polymer. The resulting product showed the removal of phenolic monomers and/or their immediate precursors based on gas chromatography and thermal desorption – pyrolysis – gas chromatography – mass spectrometry analyses. Significant intramolecular cross-linking among the reaction products was shown by thermal carbon analysis and ^{1}H NMR spectroscopy. An increase in polarity, presumably due to oxygenation, and a decrease in polydispersity of the lignin treatment product compared to untreated lignin were observed while using liquid chromatography.

IV.2 Introduction

Lignin is an abundant phenol-based biopolymer accounting for approximately 30% of renewable non-fossil organic carbon on Earth. (Laurichesse and Avérous, 2014b) Pulp and paper industry and biorefineries (utilizing cellulose for ethanol production) are the major sources of industrial lignin. (Laurichesse and Avérous, 2014b) However according to Smolarski, only 2% of lignin produced worldwide in 2010 was industrially utilized to yield organic products (Smolarski, 2012) whereas the bulk of lignin was simply burned as a source of heat and electricity. (Brebu et al., 2011; de la Torre et al., 2013) Yet based on the lignin structure, a number of valuable chemicals, e.g., vanillin, eugenol and other phenolics, could be produced from this feedstock. Thus, efficient decomposition and modification of lignin has potential to provide replacement of many petrochemicals currently used in polymer, cosmetic, pharmaceutical, food and textile industries. Not only monomers are desired products of lignin conversion. Lately, there has been an increasing interest in lignin based polymers. (Coronella, C.J.; Lynam, J.G.; Reza, M.T.; Uddin, 2014; Kai et al., 2016b)

Various methods of lignin processing have been evaluated, such as chemical or biological treatments and their combinations. (Dashtban et al., 2010; Pandey and Kim, 2011) Unfortunately, none of the currently explored protocols presents an efficient and economical way of lignin utilization. Chemical treatments are conducted at high temperatures to enable faster reactions but require significant energy consumption and expensive stoichiometric reagents or catalysts. By contrast, biological processes require minimum energy and only small amounts of inexpensive chemicals (medium

components) as they are conducted under ambient conditions. However, their significant disadvantage is an inherently slow rate. (Pandey and Kim, 2011)

Bacteria and enzymes are used more frequently than fungi for lignin biodegradation. Fungi, mainly basidiomycetes, are often utilized for lignocellulose breakdown as they do not need to be suspended in an aqueous medium and are able to decompose large solid blocks from within. However, fungi grow much slower than bacteria as the formation of mycelia takes several days. Once it happens, mycelia reduce the uniformity of thermal transfer hindering the bioreactor operation.(Thomas et al., 2013) These problems stem from a specific feature of basidiomycetes, as they naturally grow on solid state media. Yet, the use of basidiomycetous fungi would be advantageous, as they are able to efficiently mineralize lignin in nature.(Chang et al., 2012) Some basiomycetes preferentially digest lignin over carbohydrates.(van Kuijk et al., 2015) *Ceriporiopsis subvermispora* and *Pleurotus eryngii* growing on straw partially removed guaiacol-rich middle lamella lignin while *Phlebia* selectively removed lignin from secondary cell walls. (Hatakka, n.d.) Basidiomycetes were found not only to decompose lignin but also polymerize both lignin and its monomers, due to the production of various oxidoreductases. (Gouveia et al., 2012; Munk et al., 2015b)

The concentrations of Kraft lignin in water used in previous biological decomposition/ biomodification studies were usually as low as 0.5 g/L (Arun and Eyini, 2011; Chandra et al., 2007; Gibson et al., 2015; Lv et al., 2014; Morii et al., 1995; Zou et al., 2015a) or even lower, around 0.05 g/L. (Chang et al., 2014; Chen et al., 2012; Manangeeswaran et al., 2007; Yadav and Chandra, 2015) The highest concentration used was 5 g/L. (Zheng et al., 2013) This appears to be an inherent limitation, as Kraft lignin is

poorly soluble in water, only up to 0.05%. Kraft lignin is soluble in alkaline solutions at pH ≥ 9, but unfortunately most of the fungal lignolytic enzymes feature a peak of their activity at lower pH, 3-7. Due to poor solubility of lignin in water, the lignin particles cannot be immediately available to enzymes thus increasing significantly the reaction time. To overcome this problem, some biotransformation experiments employed a slightly elevated pH (7.5-9.0) for lignin dissolution. However, a representative laccase from *Trametes versicolor* starts to lose activity at pH as low as 7. (Margot et al., 2013) In addition, based on the Brenda-Enzyme database ("Der," n.d.), most of the enzymes responsible for lignin degradation/modification exhibit their optimum at pH <7, therefore lignin solubilization in aqueous media with NaOH is not the best choice for biological treatment of Kraft lignin.

While previous studies showed that dimethyl sulfoxide (DMSO) may be used in enzymatic biotransformations as a solubilization agent, (Faulds et al., 2011) to our best knowledge its use has not been reported in lignin fungal treatment. In this study, two hypotheses were tested: 1) lignin fungal bioconversion may occur in the presence of DMSO as an inert solvent and 2) fungi may exhibit a higher lignin degradation/modification rate in the presence of DMSO since solubilized lignin will allow easier access for the enzymes, i.e., a faster lignin transformation. We postulated that both the presence of DMSO and low amount of nutrients in the medium would inhibit excessive mycelial growth.

Lignin products, mainly oligomers and polymers, are known to be notoriously difficult to analyze because they are not amenable to GC due to their non-volatility.(Kozliak et al., 2016) In this work, we have adopted an approach for analysis of

such products, which comprises the second novelty of this study. The above-stated two hypotheses were evaluated using a suite of methods enabling comprehensive product characterization including gravimetric analysis, thermal carbon analysis (TCA), thermal desorption-pyrolysis-gas chromatography-mass spectrometry (TD-Pyr-GC-MS), liquid chromatography (LC) and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.

In particular, the combination of TCA and TD-Pyr-GC-MS allowed for thermal fractionation of lignin biotreatment products. TCA provided accurate quantification of these fractions while the application of TD-Pyr-GC-MS complemented these data with qualitative information on product speciation addressing both the monomeric phenolic species evolving at lower TD temperatures and pyrolytic fragments produced at high temperatures.

IV.3 Materials and Methods

IV.3.1 Feedstock, microorganisms and solvents

Two basidiomycetous fungal strains, *Coriolus versicolor* (CV) and *Trametes gallica* (TG), were obtained from the Department of Biology of Lakehead University, Thunder Bay, Ontario, Canada. The other fungal cultures used, *Ganoderma lucidum* (GL), *Pleurotus ostreatus* (PO) and *Pleurotus pulmonarius* (PP), were obtained from the Czech University of Life Sciences, Faculty of Agrobiology Food and Natural Resources, Prague, Czech Republic. Dried kenaf biomass was provided by the Department of Plant Sciences, North Dakota State University (Fargo, North Dakota, USA). Kenaf biomass was pulverized in a Thomas Wiley mill to yield particles with the average size distribution between 1.0 and 5.0 mm. Kraft lignin (alkali lignin) and all other chemicals

were purchased from Sigma Aldrich, St. Louis, MO, USA. The non-stabilized (i.e., without additional chemicals) solvents used, including DMSO, tetrahydrofuran (THF), dioxane and ethylene glycol of either HPLC or spectrophotometric grade, were obtained from VWR International, Radnor, PA, USA. Deuterated solvents for NMR experiments were purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, USA.

IV.3.2 Kraft lignin solubilization

The solubility of Kraft lignin (5 g/L) was evaluated in aqueous solutions containing 2, 5 and 10 % of organic solvents (DMSO, THF, dioxane and ethylene glycol), in NaOH aqueous solutions at pH 9.5 and also with distilled water at pH 6.5. Solubilization with organic solvents was performed in two steps. First, an organic solvent was added to Kraft lignin and the mixture was homogenized with stirring. Then increasing amounts of distilled water were added to the homogenized lignin to dilute the mixture down to the allotted percentage of the organic solvent. These samples were further homogenized for one hour in a shaker incubator at 30 °C. Then the samples were vacuum filtered through Whatman filter paper grade 5. The solid residues retained on the filter paper were considered to be insoluble lignin whereas the mass loss compared to the original lignin after passing through the filter paper was ascribed to solubilized lignin.

IV.3.3 Fungal growth and inoculum preparation

Fungi were pre-grown on a fungal agar medium, for which 3 g of kenaf, 3 g of lignin and 4.5 g of agar were mixed in 300 mL of distilled water. This medium was sterilized for 30 min at 121°C and poured on Petri dishes. Once cooled down, the plates were inoculated with ten 5×5 mm agar blocks containing fully grown cultures and incubated at room temperature under a dim light for 12 days to obtain full growth

covering the entire agar plate. These fully grown (on agar) cultures were further used as a source of quasi-immobilized fungi for liquid media inoculation. The agar blocks served as a source of nutrients for the fungi during the biotransformation experiments.

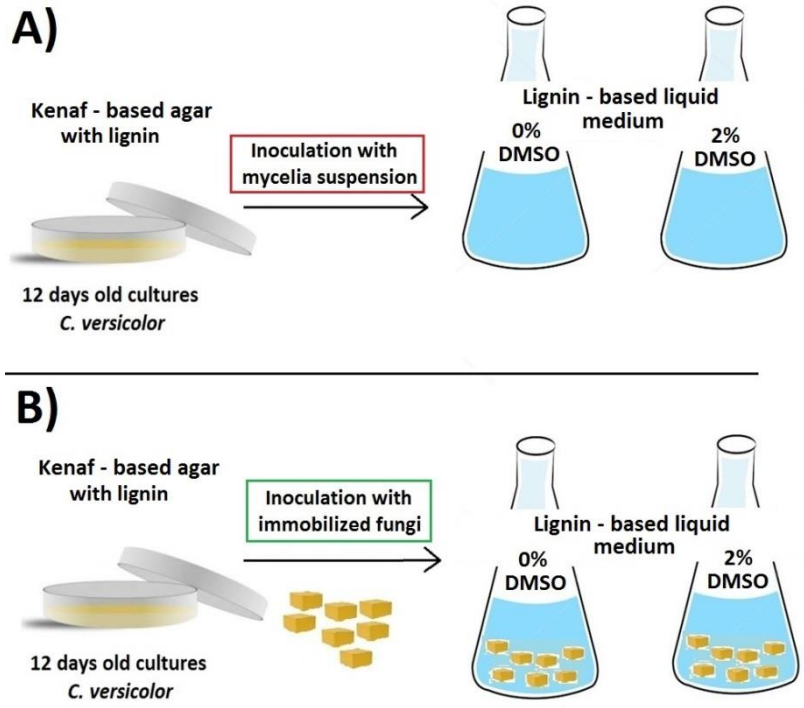


Figure 1. The experimental setup. (A) Biotransformation experiment using a common submerged cultivation inoculated by suspended mycelium (B) Inoculation with quasi-immobilized fungi where fully grown agar plates were cut into small blocks and placed into the lignin-based liquid media.

These quasi-immobilized fungi were used to inoculate liquid media, as shown in Figure 1. The liquid medium used for biotransformation was prepared with and without DMSO (≥2 vol% as specified). Erlenmeyer flasks (200 mL) used for biotransformation experiments contained 0.25 g of lignin and 50 mL of distilled water, yielding a lignin concentration of 5.0 g/L. For samples containing DMSO, 0.25 g of lignin were first dissolved in 1.0 mL of DMSO, and then distilled water (49 mL) was added to obtain a 2

vol% concentration of DMSO in the lignin containing medium. No additional nutrients or minerals were added. This lignin containing medium (pH 6.5) was not sterilized to prevent any solubilization or degradation caused by the sterilization process. High lignin content, absence of additional nutrients, DMSO presence and short duration of experiments were deemed sufficient to prevent microbial contamination. No contamination was observed.

For experiments, inoculation of lignin-containing liquid media was done in Erlenmeyer flasks as shown in Figure 1 with 5×5 mm agar blocks from 12 days old, fully grown cultures on agar plates. One quarter of such an agar plate was used for one Erlenmeyer flask. After the inoculation, the Erlenmeyer flasks were placed in an incubator at 29 °C with a shaking speed of 60 rpm. The samples for analysis were withdrawn every 24 hours for 6 days. Control samples contained agar blocks without fungi. Experiments were conducted in triplicate with and without the addition of DMSO (Fig. 1). Only the experiments with quasi-immobilized fungi (Fig. 1B) yielded detectable fungal growth (production of enzymes) whereas neither growth nor lignin transformation were observed in the flasks inoculated with suspended mycelia (Fig. 1A).
Even though Kraft lignin contains a small amount of impurities in the form of carbohydrates and minerals, the main source of the nutrients for this experiment is provided by the agar blocks, which contain nutrients in the form of Kenaf crop.

For determination of the activity of the extracellular enzymes (laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP)), the fungi were grown on agar plates under the same conditions as for the lignin degradation experiments as described above.

Then ¼ of an agar plate with a 12 days old fungal culture was cut into small pieces, ca. 5×5 mm in size. The resulting agar blocks were transferred into a 100 mL Erlenmeyer flask with an additional 5 mL of the liquid medium added and placed on a shaker operated under 90 rpm at 29 °C. After 24 hours of incubation, the liquid part containing a solution of extracellular fungal enzymes was centrifuged at 7,000 rpm for 1 min to remove mycelium and any pieces of agar.

The extracellular enzyme activities were measured for the liquid media inoculated with agar blocks (quasi-immobilized fungi). The extracellular enzymes isolated by centrifugation were used as a control, i.e., were also "incubated" for 6 days while monitoring their activities. For this control, 3 mL of inoculate wes added to 50 mL of a liquid media with 0.25 g of lignin, which was the same amount as in the medium for whole cells. The enzymatic activity was then measured every 24 hours for 6 days after incubation.To make these two data sets comparable, the same amount of fully grown agar plates was used, with either quasi-immobilized fungi or an extracellular enzyme mixture, with the agar plate washed and centrifuged.

IV.3.4 Chemical characterization

The overall product analysis protocol is shown in Fig. 2 with the essential details of each particular method described below. Upon completion of an experiment, the liquid and solid fractions were separated. The solid fraction represents non-solubilized and non-decomposed lignin. The liquid fraction represents solubilized, modified and/or decomposed lignin, which can also be called acid precipitable polymeric lignin (APPL) along with the supernatant, which would be present after APPL precipitation. APPL remained solubilized at pH >6. At lower pH, APPL precipitated (Fig. 2).

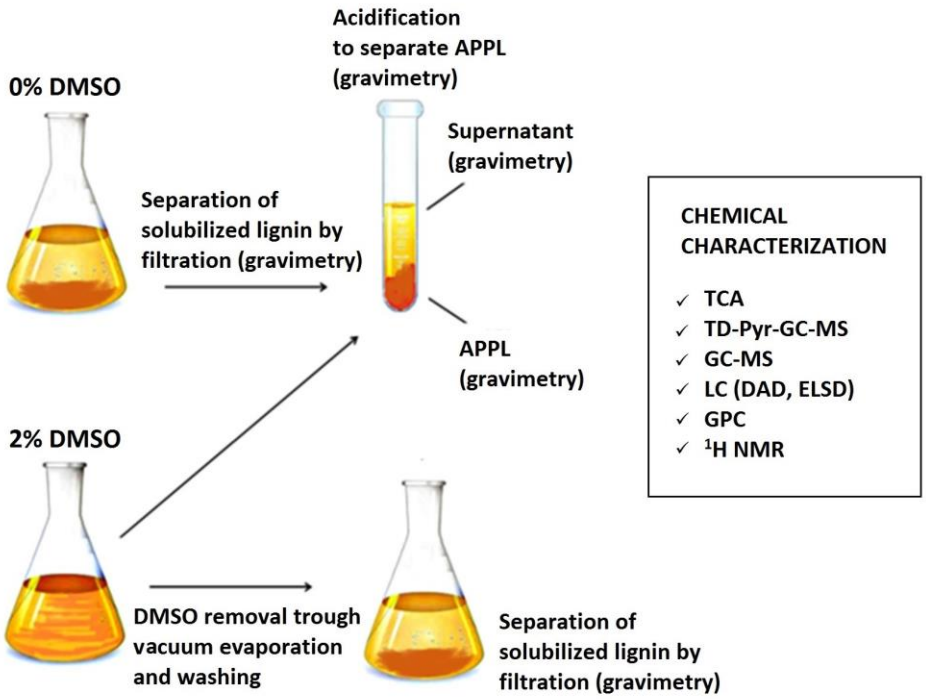


Figure 2. The overall sample preparation and analysis protocol. All the chemical characterization methods were used on the liquid part of the sample (supernatant) containing APPL in its fully solubilized form, whereas the gravimetric measurements were conducted on the solid fraction and on APPL in its precipitated form.

IV.3.5 Gravimetric analysis

The lignin decomposition and modification changes in APPL were assessed gravimetrically using a standard method, NREL TP510-42618.(Sluiter et al., 2011) To elucidate the role of DMSO in the treatment process and its impact on analysis, we characterized the samples containing 2% DMSO using two different approaches. First, they were evaluated just as those without DMSO by using gravimetric analysis. The liquid portion was acidified with hydrochloric acid to pH 3.5 and APPL was collected. However, the other half of the samples were subjected to DMSO removal prior to gravimetry, to separate the APPL fraction produced as a result of lignin solubilization

with DMSO. DMSO was removed using vacuum rotary evaporation of a 30-mL portion of the sample, where the water bath was heated up to 70 °C. 30 mL of sample was evaporated down to ~3 mL, and then 40 mL of distilled water was added to the sample, mixed and evaporated again. This "washing" step was repeated four times to remove DMSO from both the DMSO-containing control samples and those treated with *C. versicolor*. After the DMSO removal, samples were vacuum filtered through Whatman filter paper grade 5. The solid fraction collected on the filter paper was weighed after being dried to a constant weight in an oven at 75 °C.

Gas chromatography showed that this procedure was sufficient for the quantitative removal of this solvent. This procedure was verified by comparing the amounts of APPL in control samples (not treated with fungi) with and without DMSO. After the DMSO removal from the control samples, the same amount of APPL was recovered as in the samples without DMSO, in contrast to DMSO-containing *C. versicolor* treated samples.

IV.3.6 Enzymatic activity

Measurements of enzymatic activity were conducted as described by Hong et all. (Hong et al., 2011) using a UV-Vis Evolution™ 600 spectrophotometer (Thermo Fisher Scientific, Madison, WI). For analysis, the liquid portion of a sample was used. Filtered samples (250 µL) were mixed with 250 µL of a substrate solution (100 mM) in 500 µL of 100 mM sodium acetate buffer for laccase (pH 4.5), 50mM sodium tartrate buffer (pH 3) with the addition of 0.4 mM H_2O_2 for lignin peroxidase (LiP) and with 100mM sodium tartrate buffer (pH 4.5) with the addition of 1.0mM $MnSO_4$ for manganese peroxidase (MnP) activity measurement. The control samples contained no enzymes. One unit (U/L)

of enzyme activity was defined as the amount of enzyme that transformed 1.0 µmol of a substrate per minute.

The laccase activity was measured at 436 nm, with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) as a substrate. Azure B was used as a substrate for LiP activity measurements recording absorbance at 651 nm. MnP activity measurements were conducted with 2,6-dimethoxyphenol (DMP) as a substrate at 469 nm. All the measurements were performed after 30 min reaction at 23 °C. This measurement of enzyme activity is broadly used for a cursory determination of lignolytic enzymes produced by basidiomycetes. (Hong et al., 2011)

IV.3.7 Analysis of lignin biotransformation/decomposition products

IV.3.7.1 Thermal desorption and pyrolysis

A general fractionation of carbon in lignin and its biotransformation products was conducted by TCA on a thermal optical analyzer from Sunset Laboratory Inc. (WA, USA). This method was previously reported elsewhere. (Asina et al., 2016) The TCA thermal evolution profiles were obtained at 200, 300, 400, 500, and 850 °C under inert helium atmosphere followed by one additional step conducted under oxygen, with the temperature ranging between 550-850 °C, which allowed for quantification of the non-volatile char carbon portion in the samples. Prior to the analysis, the sample was dried at 145 °C for 4 min to remove DMSO. Samples without DMSO were dried using the same temperature to have comparable data. This high temperature drying step made the first, 200 °C, thermal desorption fraction inaccessible for an accurate TCA analysis. Therefore, additional complementary methods were used (LLE, GC-MS, TD-Pyr-GC-MS) to identify the low molecular weight fraction evolving at this low temperature.

TD-Pyr-GC-MS was conducted employing a thermal desorption–pyrolysis system (CDS 5000 Analytical) connected to an Agilent 7890 GC with an MS 5975C equipped with electron ionization (Santa Clara, CA). TD-Pyr temperature steps were set up to mirror the TCA analysis. The pyroprobe program consisted of 5 temperature steps: TD at 200 and 300 °C for 1 min (thermal desorption of adsorbed species and pyrolysis of oligomers) and 400, 500, and 850 °C for 1 min (pyrolysis of high-molecular weight (MW) chemicals). Samples were collected during each temperature step on a TENAX trap and then desorbed at 300 °C through a heated transfer line to GC–MS. The analyses were performed using a 30 m long DB-5MS column with 0.25 mm I.D. and 0.25 mm film thickness. Ultra-pure helium (99.999%) was used as the carrier gas with a constant flow rate of 1.1 mL min^{-1} and split ratio of 10:1 within a mass range of 33-1050 amu. The GC oven temperature program started at 40 °C min^{-1}, followed by a gradient of 35 °C min^{-1} to 80 °C then 15 °C min^{-1} gradient to 320 °C and hold for 7 min, with a total analysis time of 25 min.

IV.3.7.2 LLE-GC-MS

Prior to liquid-liquid extraction (LLE) of lignin biotransformation products, samples were adjusted to pH 6.5 with acetic acid. 4-Chloracetophenone (10 mg mL^{-1}) and *o*-terphenyl (10 mg mL^{-1}) in CH_2Cl_2 (DCM) were used as a recovery (RS) and internal standard (IS), respectively. An aliquot of the liquid sample portion (1 mL) with RS (50 µL) was extracted 3 times with 1 mL of DCM to obtain 3 mL of a DCM extract. The IS (75 µL) was added to this DCM extract, which was then analyzed by GC-MS using splitless injection with a splitless time of 0.2 min, on an Agilent DB-5MS column, 55 m long, 250 µm I.D, 0.25 µm film thickness with a carrier gas (He) flow rate of 1.5 mL min

$^{-1}$ and an injection temperature of 300 °C. The injection volume used was 0.2 µL. The temperature program was as follows: 50 °C hold for 1 min followed by 40 °C min^{-1} gradient up to 80 °C, followed by 20 °C min^{-1} gradient up to 320 °C hold for 7 min. The transfer line temperature was 280 °C and the solvent delay was 4 min. The mass spectrometer was operated within a mass range of 33-1050 amu. For derivatization, 1 mL of the liquid part of the sample was dried at 55 °C to remove water. Derivatization was conducted overnight at 70 °C by adding 100 µL of a commercial N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with trimethylsilyl chloride (TMCS) in a ratio of 99:1 to the dried sample. Then, 900 µL of DCM were added to the derivatized sample and the insoluble part of the sample was removed by centrifugation. Samples were run by GC-MS using the same method as for LLE samples, and the solvent delay was set at 6 min.

IV.3.7.3 Liquid chromatography

Reversed phase LC analysis was performed using an Agilent 1100 Series HPLC system (Santa Clara, CA, USA) with an Agilent G1315 B diode array detection (DAD) system and Varian 385-LC evaporative light scattering detector (ELSD) (Agilent, Santa Clara, CA, USA). A reversed-phase Zorbax Eclipse C18 column connected to a guard column (150 mm × 2.1 mm, particle size 3.5 μm and 10 mm × 2.1 mm, 5 um, Agilent Technologies, CA, USA) was used for separation. The column temperature was hold at 30 °C. The chromatographic data were acquired using Mass Hunter Workstation software (Agilent, Santa Clara, CA, USA).

2-Chloroacetophenone (Sigma-Aldrich, St. Louis, MO, USA) was used as an internal standard. The extracted wavelength was 290±2 nm. The samples were filtered

prior to the analysis. The injection volume was 5.0 μL. The mobile phase consisted of 0.50 mmol·L^{-1} ammonium acetate in water (solvent A), and 0.50 mmol·L^{-1} ammonium acetate in acetonitrile (solvent B).

The gradient program used for analysis started with an isocratic elution at 5% B for 10 min, followed by a linear gradient to 80% B from 10 to 20 min with 2 min hold and then a linear gradient to 95% B from 22 to 23 min and hold for 1 min. The last step was 24 to 27 minutes to 5% B followed by a 10 min hold. The flow rate was 300 μL min^{-1}. After the DAD, the flow was split equally and one half of it was delivered to ELSD at a flow rate of 150 μL min^{-1} and second half to the mass spec. The UV-Vis spectra were recorded from 190 to 700 nm with a step of 2 nm. The evaporative light scattering detector (ELSD) evaporation and nebulization temperatures were set to 70 °C.

The GPC analysis was conducted using a PL Gel Column (Agilent) with a particle size of 5 μm and pore size of 1000 Å and 1:1 THF-water as a mobile phase.

IV.3.7.4 ^{1}H NMR spectroscopic analysis

To prepare samples for NMR analysis, lignin biotransformation was performed in D_2O and in D_2O with 2% d_6-DMSO. The experiment was run for 6 days, then the biomass was removed and the sample was filtered through a 0.2 µm polytetrafluoroethylene (PTFA) filter. After the filtration, the solution was evaporated at 20 °C under a nitrogen gas stream to a volume of 0.5 mL. The concentrated sample was transferred to an NMR tube. ^{1}H NMR spectra were recorded for both untreated lignin and samples treated with fungi. ^{1}H NMR measurements were performed using a Bruker AVANCE 500 MHz NMR spectrometer. To obtain each spectrum, 128 scans were used.

Chemical shifts are reported in ppm relative to the signal of partially deuterated water (HOD) at 4.63 ppm.

IV.4 Results and Discussion

IV.4.1 Solvent and strain selection

DMSO, DMF, dioxane and ethylene glycol are known to solubilize Kraft lignin. We evaluated the impact of these solvents on lignin solubilization in aqueous media based on wt. % of solubilized lignin derived from the gravimetric determination of the solid residue. These gravimetric measurements are complemented in Table 1 by free energies of mixing (-ΔF) of these pure solvents which characterizes their lignin solubilization capacity (Brown, 1967). ("!!! LIGNIN SOLVENTS DMSO, DMF, ...pdf," n.d.) This parameter factors in the effective molecular weight of the dissolved lignin; the solvent suitability increases with negative ΔF.

Based on both parameters, DMSO was found to be the most suitable among the organic solvents tested being almost as efficient as an aqueous solution of sodium hydroxide at pH 9.5. One additional advantage of DMSO is its biochemical inertness: Fungi cannot use it as an alternate carbon and energy source, unlike most of the other organic solvents listed in Table 1. Combining these arguments with the relatively low toxicity of this solvent, DMSO was selected as a lignin solubilizing agent for the subsequent studies.

Table 1. Solubilization of lignin in aqueous media with various solvent additives and comparison to the reported free energy of mixing in pure solvents.

Solvent	Gravimetry (wt% of lignin solubilized)			Free energy of mixing, -ΔF (cal/mol) [1]		
	2% (solvent)	5% (solvent)	10% (solvent)	100 g/L (lignin)	200 g/L (lignin)	300 g/L (lignin)
DMSO	71	97	99	7.5	15.5	25
DMF	58	95	99	5	9	15.5
Dioxane	37	88	95	4.5	7.5	10.5
Ethylene glycol	20	66	91	NR	NR	NR
NaOH aqueous solution pH 9.5[2]		99		NR	NR	NR
pH 6.5[3]		7		NR	NR	NR

(1) Determined by Brown, 1967. Approximation of Kraft lignin solubility in various solvents where free energies of mixing are expressed as a function of concentration.
(2) Positive control – virtually all lignin dissolved.
(3) Negative control – minimum of lignin dissolved.
NR Denotes "Not Reported"

All five basidiomycetes used were capable of growing on lignin-containing agar media and on lignin/organic solvent-containing agar media. The growth data with and without DMSO are shown in Table 2 while the images of agar plates after 7 days are shown in Figure 3. The experiments conducted with 2% DMSO showed no significant inhibition of growth or any other differences with the DMSO-free medium (control). Even for 5% DMSO, the difference observed was merely qualitative rather than quantitative (Table 2). In this experiment, the growth was assessed as the radius of a circle with the color change induced by the fungi. Only *P. ostreatus* was able to grow on lignin solubilized at pH 9.

Table 2. Lignin biotransformation and fungal growth on solid agar media containing lignin and 5 vol % of organic solvents. The extent of lignin transformation was assessed by the color change and radius of the observed circle while the growth was observed by the development of visible mycelium on day 11. Several representative pictures of agar plates are shown in Fig. 3.

	Radius of fungal growth in (mm)					
	Water pH 7.0 (Control)	**Water pH 9.5**	**5% Ethylene Glycol**	**5% Dioxane**	**5% DMF**	**5% DMSO**
C. versicolor (CV)	**38 B Myc**	**0**	**16 P Myc**	**25 P Myc**	**7 P / 30 W**	**7 P / 30 W**
T. gallica (TG)	27 B Myc	0	16 P Myc	18 P Myc	10 P / 23 W	10 P / 23 W
P. ostreatus (PO)	30 B Myc	19 D Myc	11 P Myc	10 P Myc	9 P	10 P
P. pulmonarius (PP)	20 B Myc	0	13 P Myc	10 P Myc	10 P	10 P
G. lucidum (GL)	7 B	0	11 P Myc	8 P Myc	5 P	5 P

Myc – visible mycelium on day 11
D – dark, W – white, P – purple, B – brown

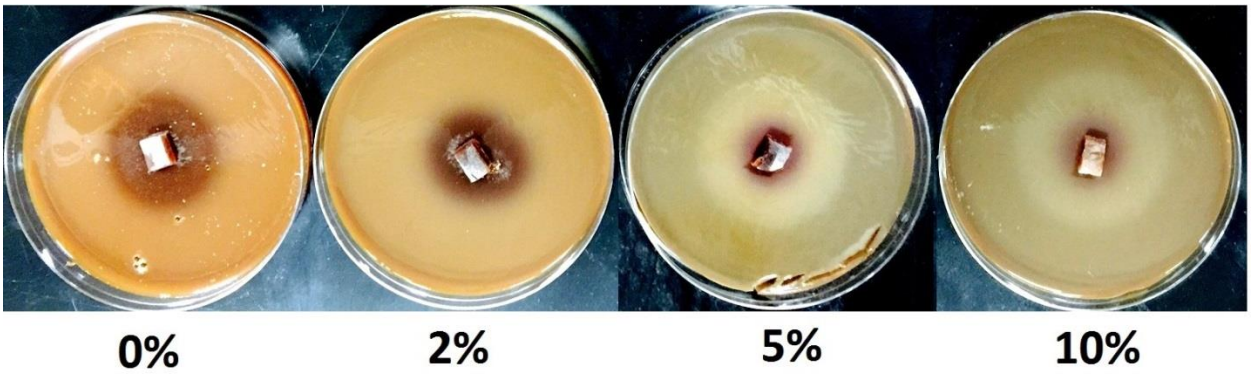


Figure 3. Kraft lignin based agar plates inoculated with *C.versicolor* on day 7, containing 0, 2, 5 and 10% of DMSO. Note that there is no visible mycelium (only color changes) at this moment on this agar plates. Visible mycelium starts to appear on or after day 11.

By contrast, all fungal strains used were able to grow on all media containing lignin and DMSO in 5% concentration. The recorded color change was from light brown to purple (when using organic solvents) to dark brown for the control having no solvent (Fig. 3). Lignin decoloration was observed only by CV and TG with DMSO and DMF. For low DMSO (≤2%) concentrations, the brown color formation was observed. For high

DMSO (≥5%) concentrations, the observed dark purple color was preceded by a white color formation, the radii of the circles of the corresponding colors are listed in Table 2.

However, these color changes were not accompanied by a visible mycelial growth. The mycelium started to appear at day 11 or later while the color changes of the lignin-containing agar showed up after ~24 hours. The fastest growth with the strongest mycelium buildup was observed for the control with no solvent, except for GL where the addition of ethylene glycol or dioxane improved the growth. Based on the fastest growth and perceived lignin transformation on DMSO-containing media, *C. versicolor* was selected for subsequent experiments.

As only minimal differences were observed in both color changes and growth between the media containing 0 and 2% DMSO (Fig. 3), the use of as little as 2% DMSO was deemed adequate for the subsequent experiments with liquid media containing 5 g/L of lignin, the maximum lignin concentration used in previous studies. (Zheng et al., 2013) This lowest concentration of DMSO was consistent with a potential application for industrial processes, where low amounts of organic solvents would be advantageous.

IV.4.2 Liquid media: Application of suspended and quasi-immobilized fungi

Quasi-immobilized fungi (pre-grown on agar) were used, since no growth in lignin containing liquid media was observed when innoculated traditionally with free suspended fungi (Fig. 1). Presumably, the biological modification of Kraft lignin has to be supported by the residual nutrients present in the inoculating agar blocks. None of the fungal strains evaluated was able to grow in lignin based liquid media without this nutritional supplement, which was thus deemed a limiting factor, just as for growth on

lignin-containing agar plates. Kraft lignin demethylation and/or phenolic ring opening, potentially occurring during the modification process, as well as carbohydrate impurities potentially present in commercial lignin, may provide additional nutrients to the fungi.

By contrast, significant lignin modification was observed in the liquid media with up to 10% DMSO when it was inoculated with fungi containing agar blocks, as opposed to free suspended cultures. The process was monitored by HPLC. Significant lignin biotransformation was observed in just 24 h despite the slow growth noted in the previous paragraph. Apparently, this high conversion rate was due to a large inoculum with quasi-immobilized fungi.

Given that the 2% DMSO addition also enabled the increase of lignin concentration to 10g/L (up to 25 g/L with ~5% DMSO), this development may enable an economical application of fungi for lignin treatment. This setup, using pre-grown quasi-immobilized fungal cultures, hosts a number of advantages as it significantly simplifies the product separation, enables the re-use of fungal pellets, excludes a potential excessive mycelial growth (Table 2) and makes contamination less likely.

IV.4.3 Effect of DMSO on enzyme activity

Enzyme activity was measured to obtain a reference for fungal growth under harsh conditions. Corroborating the HPLC data on fast lignin conversion during the first 24 h of incubation, the initial enzyme activity was significant. Quasi-immobilized fungi showed enzyme production over the entire duration of the experiment (Fig. 4A) compared to the isolated enzymes (Fig. 4B), for which the activity declined during the first 3 days. This comparison demonstrated that quasi-immobilized fungi replaced the

gradually inactivating extracellular enzymes, thus showing significant enzyme synthesis in the presence of DMSO. Moreover, the observed enzymatic activity of laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) increased in C. versicolor cultures with DMSO addition. This observation corroborates previous reports. (Busby et al., 1999; Shah et al., 2006; Watanabe and Ueji, 2001) It is of note that the DMSO concentration up to 2 % did not show any negative effect on the extracellular *in vitro* enzymatic activities of fungal laccase, LiP and MnP, the main lignin degradation enzymes.

The laccase production was found to be relatively low in our experiments, since the reported laccase activity of this strain is near 100U/mL under favorable conditions. (Revankar and Lele, 2006) The observed low laccase activity was presumably due to the nutrient limitation occurring under the specific conditions of this study, or perhaps even copper, being essential for this multicopper dependent oxidase.(Revankar and Lele, 2006) By contrast, the levels of LiP and MnP observed in our experiments were within the range reported in literature. (Sun et al., 2004; Zhao et al., 1996)

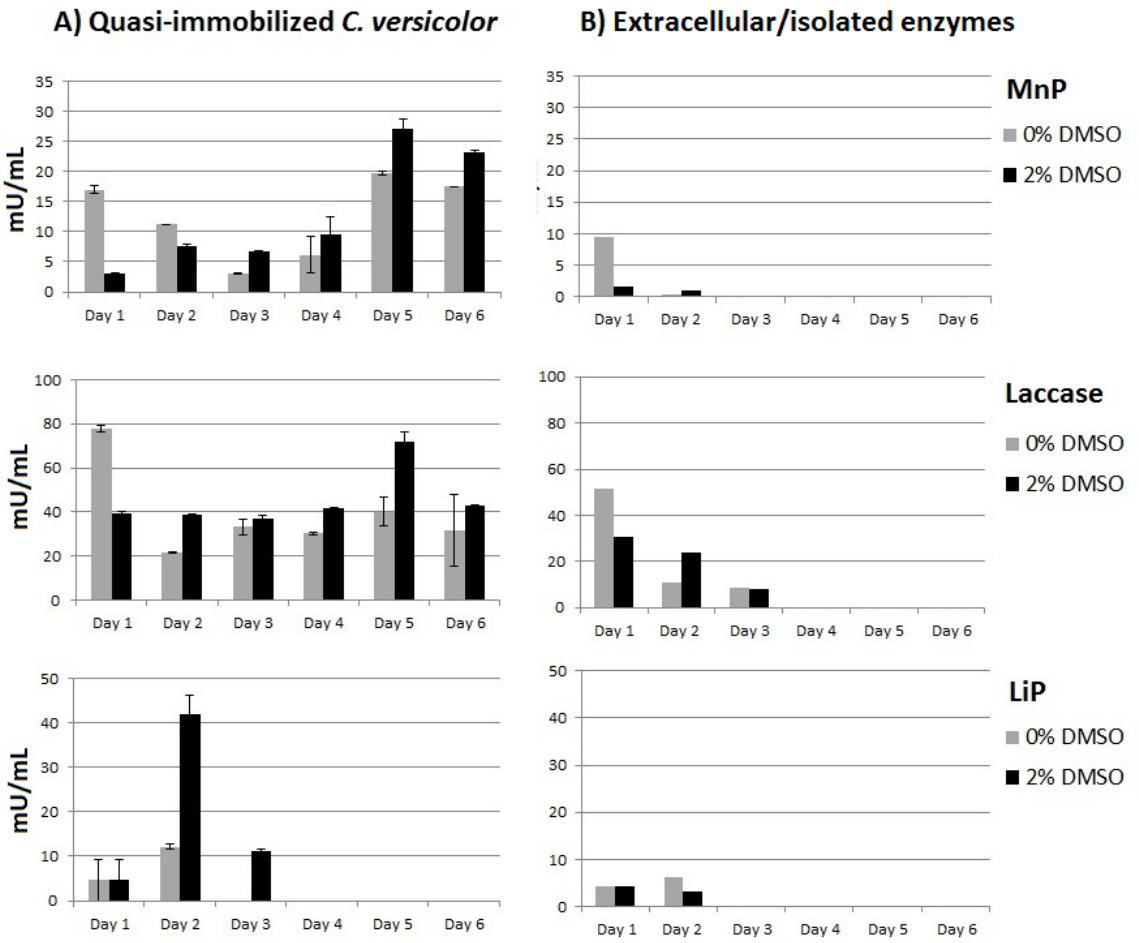


Figure 4. Effect of 2% DMSO on pertinent enzyme activity of *C. versicolor* (mU mL^{-1}). One unit of enzyme activity was defined as the amount of enzyme that transformed 1.0 µmol of substrate per minute for A) immobilized and B) extracellular enzymes separated from cells by centrifugation.

IV.4.4 Lignin solubilization and biotransformation based on gravimetry

Gravimetric analysis was the first essential step in the evaluation of lignin biotransformation by basidiomycetes. The recorded values for both the original lignin and its biodegradation product, with and without DMSO, are presented in Fig. 5. APPL was obtained both with the addition of 2% DMSO and with fungal treatments (Fig. 5A). To separate the effects of lignin solubilization and fungal treatment in the presence of

DMSO, samples containing DMSO were specifically processed to remove this solvent using vacuum evaporation, as detailed in section 2.3 (Fig. 5B).

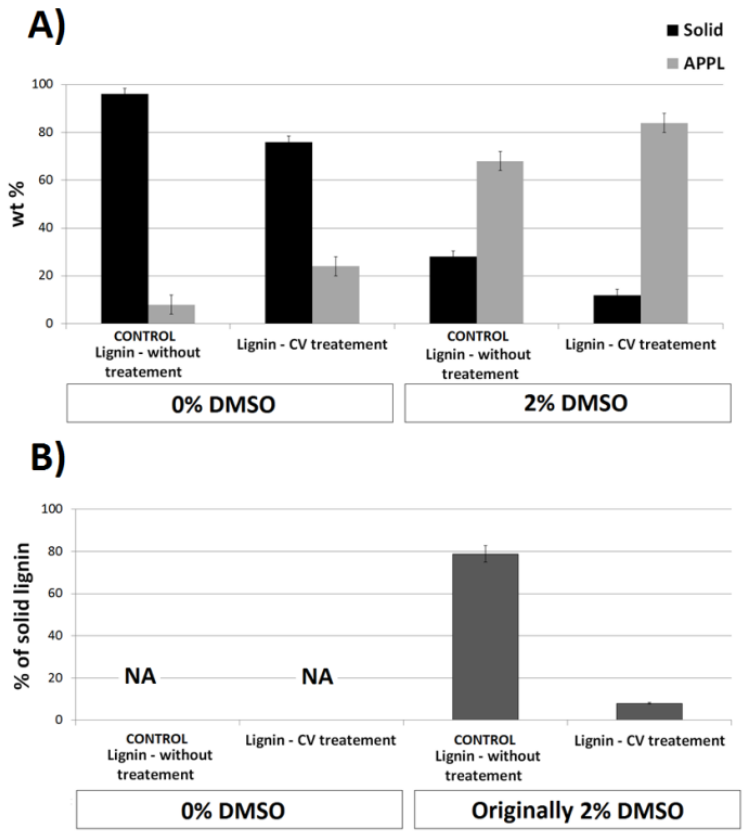


Figure 5. Effect on DMSO on lignin in both control and *C. versicolor* treated samples, after 6 days of incubation. A) Effect of DMSO on lignin solubility and APPL production. B) Percentage of solid lignin (water insoluble lignin) recovery after the DMSO removal by gradual vacuum evaporation.

As a result of DMSO removal, the original DMSO-solubilized lignin in the control sample without a fungal biotreatment was rendered insoluble in water. By contrast, most of the treated lignin remained in the solution even after the DMSO removal, as long as it remained in continuous contact with water as assured by the procedure used. Thus, the observed effects of biotreatment in the presence of DMSO could not be explained by solely lignin solubilization and should be ascribed to lignin biochemical conversion.

Addition of DMSO to aqueous media thus appears to improve not only the lignin solubility but also its availability to basidiomycetes. The result of the fungal treatment with 2% DMSO is a significant lignin bio-modification, faster than that without DMSO. Yet, the combined mass of the solid and APPL fractions did not change as a result of the fungal treatment, either with or without DMSO. The mass balance successfully closed at near 100% (Fig. 5A) thus evidencing lignin biotransformation rather than metabolic degradation.

Further insights into the nature of this process were obtained through product fractionation and chemical speciation analysis. The information presented in the remaining sections corroborates the observation of nearly perfect mass balance closure confirming that the observed lignin conversion is polymer modification rather than degradation into smaller fragments. The observed increased solubility of lignin polymer can be explained by its partial oxygenation. Demethylation described earlier as a common pathway for fungal lignin biomodification appears to be less likely, as it was shown to produce products insoluble in water. (Yang, 1981)

In literature APPL is often described as a product of lignin biodegradation.[13] However, this study shows that APPL generation may also be caused by simple lignin solubilization with either a fungal treatment or an appropriate solvent (Fig. 5). Thus it is essential to conduct appropriate control experiments with the same solvent system while assessing lignin biodegradation.

IV.4.5 Carbon fractionation and speciation

The thermal elution profiles of the biotreated lignin samples (liquid fractions only) are shown in Figure 6 for two different treatment times of 1 and 6 days in comparison to the control lignin without a fungal treatment. The observed increase of the carbon amount across-the-board, among all temperature fractions, (Fig. 6C and D) was due to the lignin solubilization by DMSO whereas the differences between the elution profiles of lignin and its biotreatment products (Fig. 6A and B) were due to the biochemical modification caused by fungi.

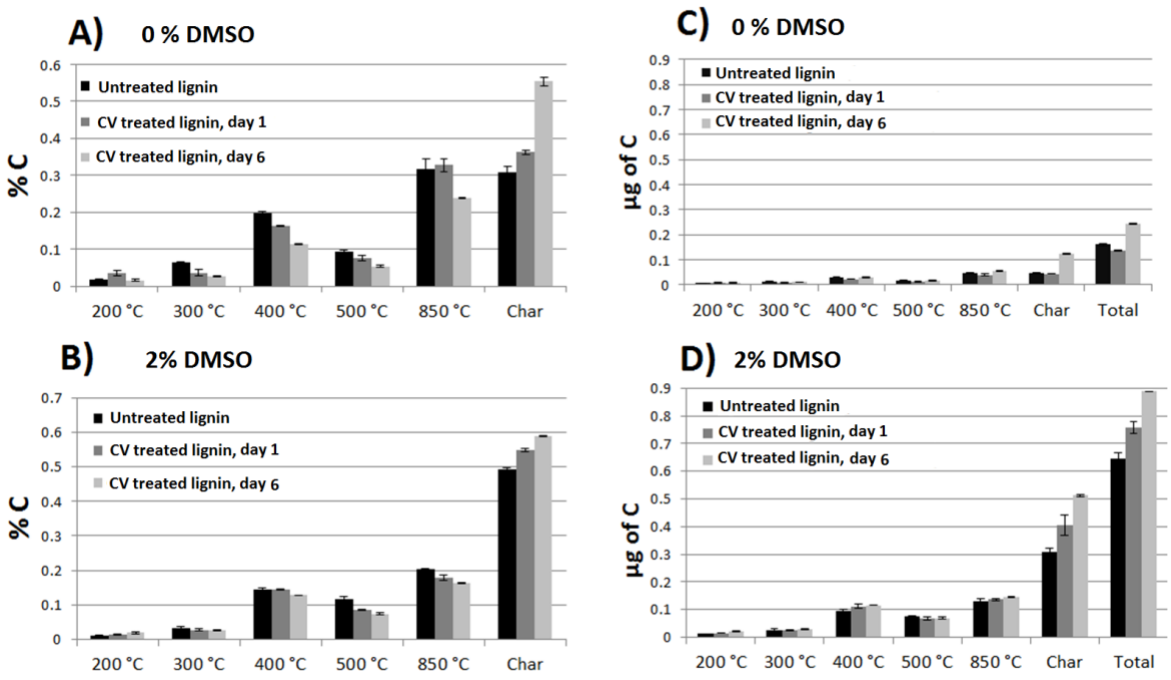


Figure 6. TCA temperature profiles showing thermal carbon elution. Panels A and B show the normalized % distribution of carbon fractions. Panels C and D show the absolute amounts of evolved carbon along with its total mass summed up among all the fractions (based on 64 % carbon in lignin determined by elemental analysis).

The most pronounced lignin solubilization, as expected, occurred as a result of DMSO addition: The solubilization is manifested as a significant difference between the

total carbon amounts depicted in panels C and D of Fig. 6, respectively. However, a more detailed inspection of the time profiles presented on these graphs showed that the solubilization gradually continued long after the DMSO addition (Fig. 6D). Further solubilization and modification were observed as a result of the subsequent fungal treatment. Even without DMSO, gradual lignin solubilization occurred as a result of the fungal treatment (Fig. 6C). Thus, lignin solubilization occurs due to two factors, fungal treatment and DMSO addition.

At first glance, the observed solubilization upon cultivation indicates lignin biodegradation, as suggested earlier. [13] It is often assumed, by default, that fungal lignin treatment leads to a stepwise decay yielding smaller fragments. This assumption is based on a greater product solubility in water, which is perceived to be a result of reduction in MW. However, based on TCA, the amounts of phenolic monomers (evolving at 200 and 300 °C) did not increase as a result of the fungal treatment, either with or without DMSO. Thus, corroborating the lack of carbon mass loss reported in the previous section, polymer lignin degradation into monomers or oligomers did not appear to be the preferred pathway.

Conversely, the observed changes in the TCA thermal elution profile suggest that polymerization is the main outcome of the short-term fungal treatment conducted. The observed volatile oligomer content was reduced and gradually converted into char precursors (carbon which does not evolve at 870 °C under helium, but only at 870 °C with oxygen, Fig. 6). Unexpectedly, these char precursors accumulated in the water-soluble fraction as the resulting solution had a dark purple color indicating the formation of a highly cross-linked yet water-soluble polymer.

The earlier studies on lignin degradation conducted in the presence of nutrients showed rather different products' patterns. For example, low-MW phenolics accumulated in the culture medium for up to 13 days in our experiments with *C. versicolor,* which were performed in the presence of glucose as a co-substrate (data not shown). However, many other studies indicated significant lignin polymerization as the main reaction pathway when treated with enzymes such as laccase. (Elegir et al., 2007; Munk et al., 2015b) The results obtained in this study indicate that the low abundance of a co-substrate acting as a readily available carbon and energy source under short-term fungal treatments appears to trigger the oxidative cross-linking of lignin and its smaller-MW fragments. In our previous study, while using the same fungal strain in its suspended form in a nutrient rich medium, we were able to obtain lower molecular weight species in a sizable yield, in contrast to the present study. (Asina et al., 2016)

TD-Pyr-GC-MS was used as a complementary method to TCA providing the chemical speciation of the eluting products (Fig. 7, Table 3), except for the non-volatile char precursors portion. The use of this method offered further insights into the process mechanism. Namely, the 200 °C fraction, which contained vanillin, guaiacol and acetovanillone, virtually disappeared after the treatment. The 300 °C fraction also showed a similar trend. This observation corroborates the TCA data obtained for similar temperature fractions.

However, the abundance of TCA fractions eluting at 200-300 °C could have been affected by the DMSO removal step at 145 °C. Therefore, the products were chromatographically analyzed prior to the thermal treatment to remove DMSO to ascertain whether the concentration of low-MW phenolics in lignin was affected by the

fungal treatments (detailed data are not shown). These analysis revealed the GC-elutable chemicals in non-treated lignin, i.e., phenolic monomers and dimers evolving from the GC column. By contrast and corroborating the data obtained by TCA and TD-Pyr-GC-MS, the samples obtained after the fungal treatments generated virtually no GC-elutable products yielding virtually "blank" chromatograms of the DCM extracts obtained after the fungal treatments, except for the added internal and recovery standards. Derivatization with N, O-bistrifluoroacetamide (BSTFA) to enhance the phenolic peaks neither revealed any new products nor showed a higher abundance of these compounds than those found in nonderivatized samples. Thus, the assumed quick and preferential removal of low-MW phenolics by fungi was confirmed.

The removal of phenolic monomers may be due to either their polymerization or catabolism by the fungi. Both of these effects may occur but the former appears to make a greater impact, as it correlates with the increase of the coked (polymeric) fractions observed by TCA after the fungal treatment (see the previous section). The higher-temperature pyrolytic fractions showed similar components for both the original lignin and its fungal treatment product thus corroborating the polymerization hypothesis. It is of note that the TD-Pyr-GC-MS did not show any products of protein thermal decomposition, e.g., indole, which is known as a distinct pyrolytic marker of protein presence.(Wang, n.d.) Thus, lignin solubilization after the fungal treatment, either with or without DMSO, is unlikely to be explained by its association with extracellular proteins. Glycosylation also seems to be an unlikely result of lignin modification because we were not able to detect any products of thermal decomposition of carbohydrates (e.g., levoglucosan or furfural).

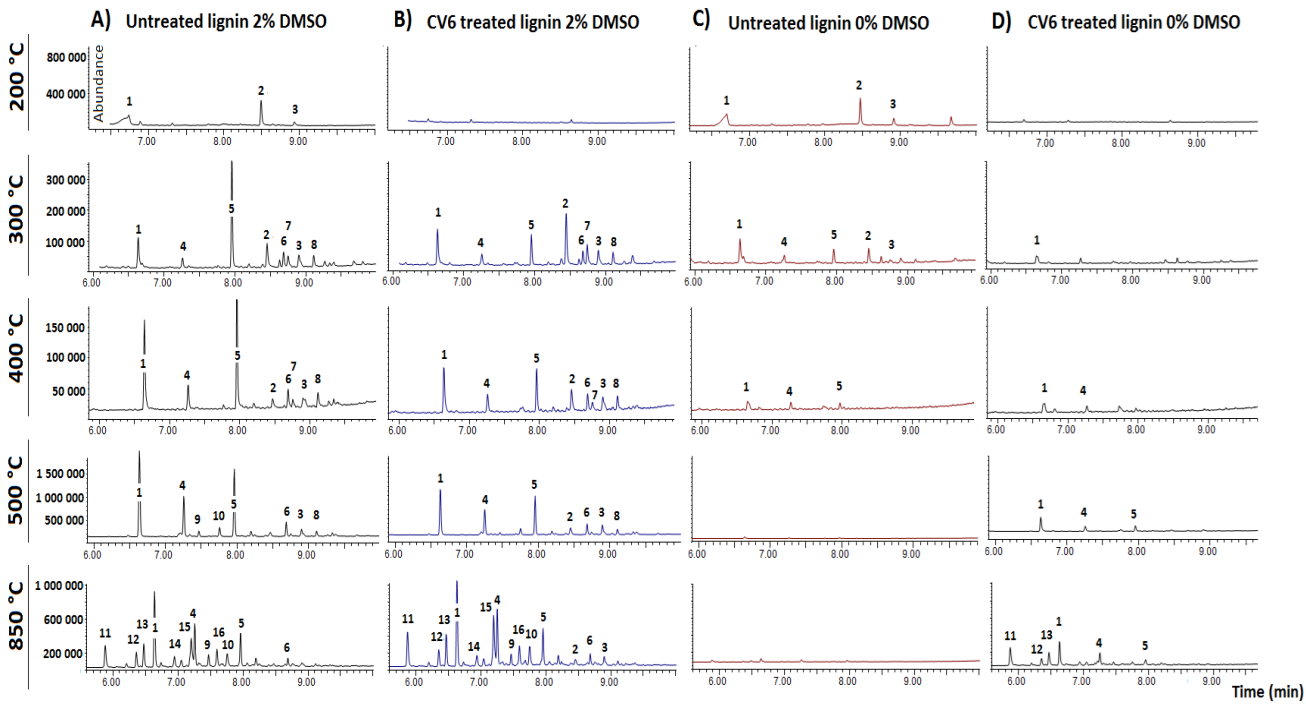


Figure 7. TD-Pyr-GC-MS analysis of individual TCA fractions obtained at 200, 300, 400, 500 and 850 °C. A and C are the chromatograms of control samples without treatment; B and D are those of the samples treated with *C. versicolor* for 6 days. The peak assignment with the corresponding *m/z* ratios is provided in Table 3.

Table 3. The peak identification with the corresponding ions of specific *m/z* ratios obtained in TD-Pyr-GC-MS based on library standards and literature.

#	Compound name	m/z	#	Compound name	m/z
1	Guaiacol	53, 81, 109, 124	9	Dimethoxytoluene	91, 109, 137, 152
2	Vanilin	81, 109, 123, 152	10	Ethylguaiacol	77, 122, 137, 152
3	Acetovanilone	108, 123, 151, 166	11	Phenol	39, 55, 66, 94
4	Methylguaiacol	67, 95, 123, 138	12	Methylphenol	51, 79, 90, 108
5	Vinylguiacol	77, 107, 135, 150	13	Methylphenol	51, 77, 90, 108
6	Isoeugenol	77, 103, 149, 164	14	Xylenol	77, 91, 107, 122
7	Propylguaiacol	94, 122, 137, 166	15	Benzenediol	64, 81, 92, 110
8	Acetonyleguiacol	94, 122, 137, 180	16	Pyrocatechol	78, 95, 106, 124

IV.4.6 Characterization of the fungi-treated lignin polymer with LC

To characterize the resulting polymer, two different liquid chromatography techniques, GPC and reverse phase LC, were employed. For GPC, peak elution profiles were not altered as a result of a 6-day fungal treatment with *C. versicolor* (not shown). The unaltered retention shows evidence that the lignin molecular weight was neither reduced nor increased significantly, given the low GPC sensitivity toward minor changes in MW. (Uliyanchenko et al., 2012) This finding is consistent with cross-linking being the main reaction path as opposed to biodegradation. However, as no change in MW was observed, most of the cross-linking transformations appear to occur intramolecularly rather than intermolecularly. Thus, the term 'intramolecular cross-linking' will be applied henceforth.

The results of the other reverse phase LC analysis, LC-DAD performed with a non-polar C18 column, are shown in Figure 8, show evidence for significant changes as a result of fungal treatment in the presence of DMSO. Both treated and untreated lignin exhibited the maximum UV-Vis absorbance at 290 nm. The difference between the treated and untreated lignin was the appearance of a new UV-Vis absorption maximum at 540 nm for the biotreated lignin product (Fig. 8B and D). This observation suggests that the product became chemically modified with diverse chromophores/functional groups, which are absent in the intact lignin.

The LC chromatograms obtained without DMSO were inconclusive, as the lignin samples without DMSO (Fig. 8A) represented only the water soluble portion of Kraft lignin compared to the samples with DMSO (Fig. 8B) where most of the lignin was dissolved.

Considering the samples obtained with DMSO, the HPLC-DAD peaks recorded at both 290 and 540 nm shifted towards shorter retention times with increasing fungal treatment time. Peaks could shift due to a loading effect (overloaded samples tend to elute earlier) or DMSO modification of the mobile phase. Yet, the IS peak retention did not change thus suggesting that the observed shift was not an artifact of the method. As this shift occurred on the reverse phase column, it may indicate an increase in product polarity, i.e., the extent of oxygenation, compared to the untreated lignin (Fig. 8B). This feature explains the increase in solubility in water observed for lignin after its treatment with fungi in this study.

The narrowing of the product's peak observed both with and without DMSO may indicate a decrease of the samples polydispersity (Fig. 8). The observed changes are consistent with the proposed intramolecular cross-linking of lignin oligomers, which would increase the sample uniformity (i.e., decrease polydispersity).

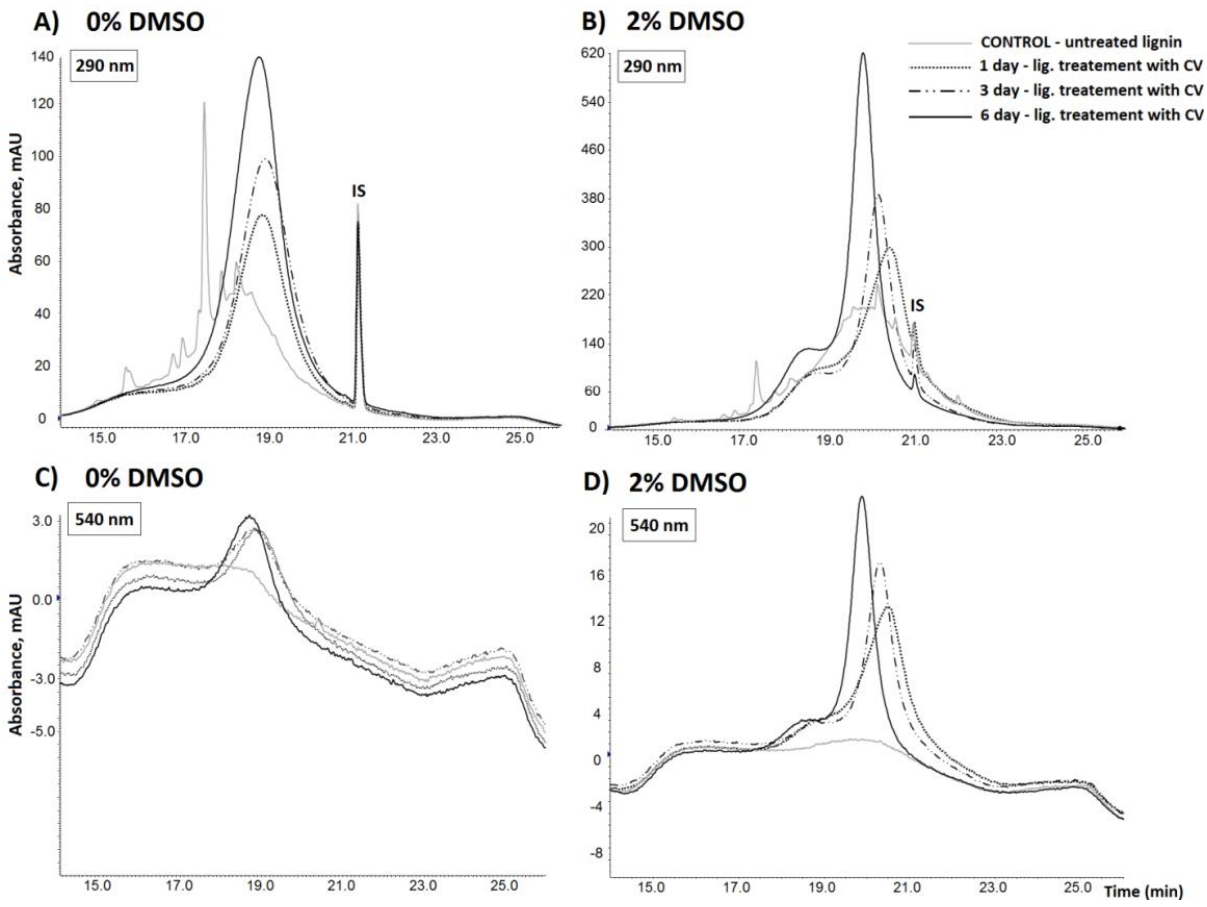


Figure 8. The extracted HPLC-DAD chromatograms of the lignin samples with the DAD detector wavelength set at 290 nm and 540 nm, respectively. Chromatograms were obtained for both the original lignin and its fungal biotransformation product. Panels A and B show the samples obtained in the experiments conducted without DMSO. Panels C and D show similar data obtained in the experiments with 2% DMSO in the cultivation medium.

IV.4.7 Confirmation of structural changes in treated lignin by ^{1}H NMR analysis

^{1}H NMR data corroborate the results obtained by TCA and TD-Pyr-GC-MS for the same liquid samples. The results of this analysis suggest significant structural changes after a *C. versicolor* treatment. A striking difference between the spectra of untreated and treated lignin is the disappearance of both phenolic 7.8-9.6 ppm (Joffres et al., 2014) and aromatic 6.5-7.9 ppm protons. In the spectra of untreated lignin, low-field signals are

clearly visible in spite of the expected deuterium exchange of phenolic hydrogens with D_2O.

The second feature specific for the spectra of biotreated lignin samples is the increase in the signal intensity in the 3.0-4.2 ppm region compared to that in the 6.5-7.9 ppm region. The integration ratio of the signals that appeared in the 3.0-4.2 and 6.5-7.9 ppm regions increased from 1.8:1 in the untreated lignin to 4.1:1 in the lignin treated for 6 days with *C. versicolor* without DMSO (Fig. 9A and B). Spectra of the samples with 2% DMSO showed a similar trend. For untreated lignin, the signal intensity ratio for the 3.0-4.2 and 6.5-7.9 ppm regions was 1.5:1, whereas after the treatment this ratio became as high as 5.9:1 (Fig. 9Cand D). Signals in the 6.5-7.9 ppm region are typically assigned to the hydrogens attached to aromatic rings whereas signals at 3.0-4.2 ppm are characteristic for protons of the (sp^3)CH_n-O fragment, benzylic hydrogens of the Ar$C\underline{H}_nCH_nO$ moiety as well as those at the lignin β-β linkage. (Kim et al., 2014) Therefore, the NMR data suggest that after treatment the number of aromatic hydrogens significantly decreases.

Since the ratio of aromatic and sp^3 hydrogens shifts toward the latter after fungal treatment, demethylation followed by cross-linking is a less likely pathway in this system even though it is common for earlier reported lignin modifications. (Filley et al., 2002; Ibrahim et al., 2011; Kandybovich et al., 2014; Zou et al., 2015b)

As of note, the altered ratios observed for the samples containing 0 and 2% of DMSO are likely due to the difference in solubility of lignin polymers in the two

solvents. Samples with 2% DMSO were transparent solutions whereas those without DMSO were non-transparent colloids.

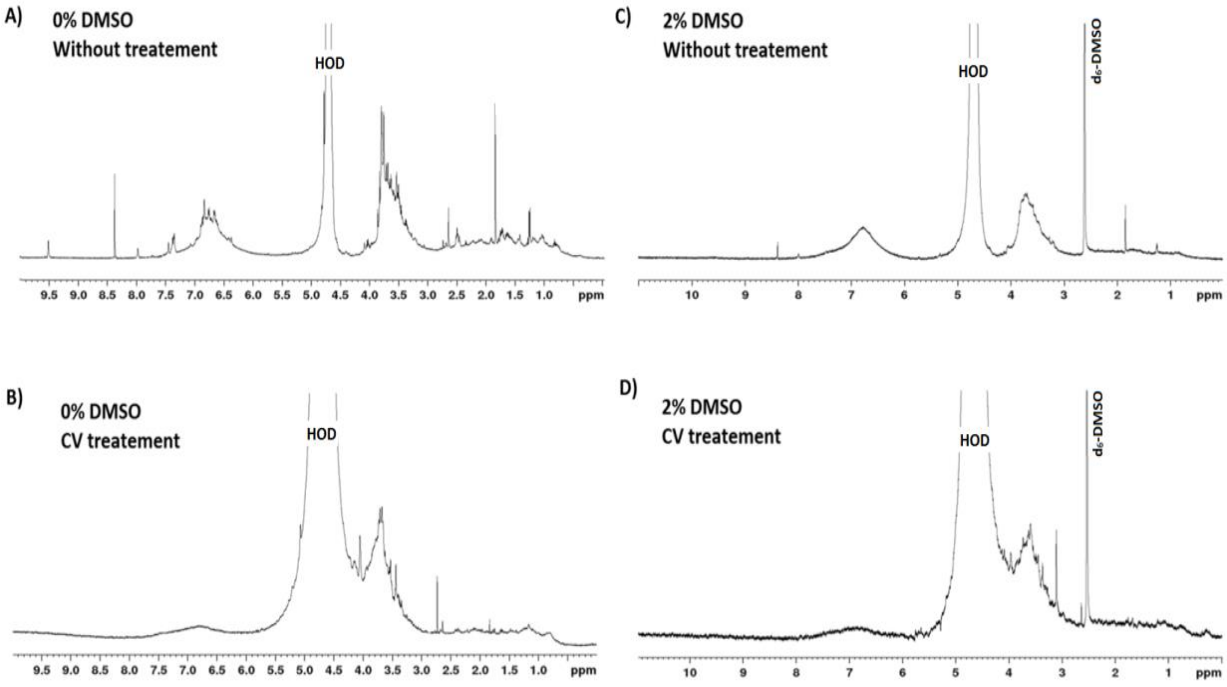


Figure 9. ^{1}H NMR spectra of treated (6 days) and untreated lignin in D_2O containing 0 and 2% DMSO.

Crestini et al. observed a similar decrease in aromatic hydrogens after a lignin treatment with white rot fungi. (Crestini et al., 1998) They interpreted this observation as that the lignin degradation by white rot fungi leads to the phenolic ring opening, which is ascribed to MnP and LiP. However, an aromatic ring opening would signify biodegradation whereas the results obtained in our study using other analysis methods indicated structural modifications involving aromatic rings rather than biodegradation, for example, oxygenation leading to quinone formation, intramolecular oxidative cross-

linking and cyclization. Based on literature, laccases are most likely involved in reactions shown in panels A, G and H of Figure 10 and they can also possibly be involved in cyclization. (Kalliola, 2015; Leutbecher et al., 2011; Ming et al., 2016) Most of these reactions form reactive intermediates whose further processing can lead to crosslinking.

The observed spectral features, such as the disappearance of the signals belonging to phenolic OH groups as well as a significant decrease in aromatic hydrogens, are consistent with oxidative transformations involving aromatic rings and phenolic OH moieties rather than aromatic ring opening. This observation corroborates the data obtained by TCA and TD-Pyr-GC-MS for the same samples. In particular, TD-Pyr-GC-MS yielded the same monomers evolving in high-temperature pyrolytic fractions for both untreated and treated lignin samples. This result is consistent with partial cross-linking of the existing phenolic fragments as the main path of lignin fungal modification but inconsistent with biodegradation, which would diversify the monomers' profile.

Examples of possible structural changes in lignin after the treatment are provided in Figure 10. Panels 10A and H show the most likely oxygenation paths. The rest of Figure 10 shows possible intramolecular cross linking reactions and an oxidative ring opening reaction (Fig. 10G). As the above-reported results showed, fungal treatment resulting in an increase in the polarity of lignin, therefore we assume that oxygenation (Fig. 10H) and oxidative ring opening (Fig. 10 G) are the most common structural changes in the treated lignin. Because there is no significant difference in the average molecular weight of lignin before and after treatment, structural changes are shown occurring due to intramolecular processes as opposed to cross-linking between two or more polymeric chains of lignin. One notable feature in the ^{1}H NMR spectrum of the

treated lignin containing 2% DMSO is the presence of an intense signal at ~ 3.1 ppm. This signal is likely to belong to either benzylic hydrogens of the $ArC\underline{H}_nCH_nO$ moiety or hydrogens at the lignin β-β linkage.(Kim et al., 2014) The proposed intramolecular alkylation (Fig. 10B) would result in the formation of such a $ArC\underline{H}_nCH_nO$ fragment.

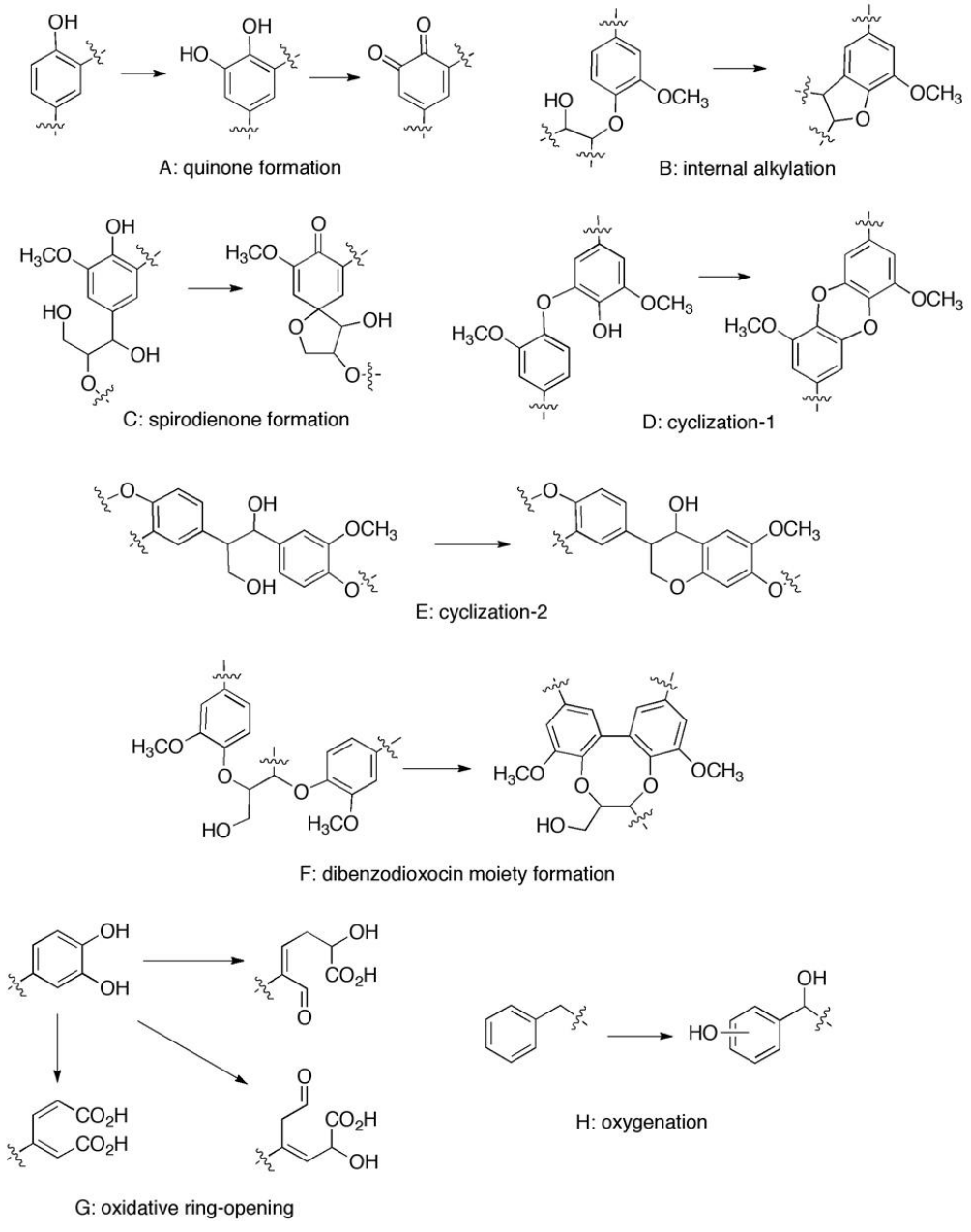


Figure 10. Possible structural changes in the treated lignin.

IV.4.8 Properties of the modified lignin polymer

After a fungal treatment, the APPL formed can be washed with an aqueous acidic solution (pH ~3.5 or lower) to remove DMSO, and then solubilized in water by the addition of NaOH to obtain a lignin-based polymer, which unlike Kraft lignin, remains soluble in water even at neutral pH (pH 6.5 and higher). The APPL produced with 1-3 day fungal treatments can also be solubilized in ethanol, methanol or THF. The acid precipitated polymer obtained with >4 day treatment can be washed with ethanol, methanol or THF and then solubilized in water (pH 6.5 and higher). APPL does not leak any monomers or other chemicals when extracted into either DCM or ethyl acetate.

IV.5 Conclusion

Lignin may be significantly solubilized by co-solvents, e.g., 2-5% DMSO. The main pathway of fungal lignin treatment in the absence of co-substrates and presence of a solubilizing cosolvent (DMSO) was shown to be an intramolecular oxidative cross-linking, accompanied by the removal of low-MW monomeric and oligomeric phenolics. Similar polymers are formed when lignin is treated by fungi without a solubilizing solvent, but the use of DMSO makes such polymers the main products formed in high yield. Significant lignin biotransformation with quasi -immobilized *Coriolus versicolor* (pre-grown on agar) was observed in just 24 h. Given that the 2% DMSO addition also enabled the increase of lignin concentration up to 10g/L (up to 25 g/L with 5% DMSO), this development may enable an economical application of fungi for lignin treatment. This new crosslinked polymer is highly soluble in water at neutral pH, presumably due to a partial oxygenation of phenolic rings, as long as it remains in permanent contact with water. A higher solubility of such polymeric lignin biodegradation products compared to

the untreated lignin is thus not necessarily an indication of lignin biodegradation to smaller MW fragments, as it could also be caused by the opposite process yielding more recalcitrant polymers.

CHAPTER V

Lignin based insoluble polymers (anionic hydrogels) produced by basidiomycetes

V.1 Abstract

Lignin-based polymers, partially soluble in either aqueous or organic solvents, were produced employing a relatively fast (6 days) cultivation of Kraft lignin with basidiomycetes, primarily *Coriolus versicolor*, followed by either vacuum evaporation or acid precipitation. After drying (with an optional acid precipitation followed by washing with water or alcohols) and treatment with alkaline water, this polymer becomes a pH-sensitive anionic hydrogel insoluble in either aqueous or organic solvents. The yield of this polymer increased from 20% to 72% wt%with the addition of 2% DMSO. The mechanical stability and buffering capacity of this polymer at pH 6.5 can be adjusted by using solvents of different polarity (water, methanol or ethanol) to wash the intermediate (acid-precipitated) polymer prior to drying. Yet any of these polymers is characterized by significant thermal stability (assessed as a high thermostable "coked" fraction in thermal carbon analysis), apparently resulting from significant covalent cross-linking occurring during the final treatment of their intermediate polymer precursors.

V.2 Introduction

Lignin, the second most abundant biopolymer on Earth and a large scale by-product of pulp and paper industry and bioethanol production, is a renewable source of organic chemicals. The current focus is on production of phenolic monomers (e.g., vanillin, guaiacol) and other low-MW chemicals, with their application in polymer industry.(Upton and Kasko, 2015) The monomer production from lignin is usually low

(5-35 wt%), with the rest being undesired phenolic oligomers or polymers. Therefore polymers, either designed or undesired, are the primary ultimate products of lignin processing (Fang Richard Smith et al., n.d.).

Hydrogels represent a specific group of polymeric materials whose application is envisioned in many areas, e.g., in medicine as biomimetic scaffolds, wound healing materials or for drug delivery, (Bag and Rao, 2006; Tobergte and Curtis, 2013)in food processing technology as thickening agents, stabilizers or food packaging materials, (Shen et al., 2016) and for waste water treatment.(Shen et al., 2011; Yao et al., 2014) Two main groups of hydrogels are permanent (chemical) and reversible (physical) hydrogels. In physical hydrogels the crosslinking involves hydrogen bonding, polyelectrolyte complexation, molecular entanglement, hydrophobic association and ionic bridging. All of these forces are relatively weak and therefore these hydrogels have a tendency to disintegrate over time. (Bag and Rao, 2006; Tobergte and Curtis, 2013) By contrast, chemical hydrogels, which are characteristic for rigid molecules like lignin, are crosslinked with covalent bonds and thus are significantly more stable.(Gerlach and Arndt, 2009; Passauer, 2012b)

Even though most of the currently used commercial hydrogels are based on synthetic polymers such as acrylic acid, poly(vinyl pyrrolidones) and poly(vinyl alcohols), some of them were recently produced from biobased polymers. (Nagam et al., 2016) Starch and cellulose are already commonly used as a graft base whose properties can be adjusted with crosslinking density. Among the most stable gels are hydroxypropyl, phosphate carboxymethyl and other derivatized polysaccharrides. Derivatives of cellulose and starch are further crosslinked either chemically, with bifunctional reagents such as

dicarboxylic acids or sulfur compounds, or through thermal treatment. (Shen et al., 2015; Thakur and Thakur, 2015)

Hydrogels have been obtained from various kinds of lignin such as kraft lignin, lignosulfonates, and others. (Passauer, 2012b; Thakur and Thakur, 2015) Lignin based hydrogels are produced either through copolymerization with polyacrylamide, isopropyl acrylamide, N,N-methylenebisacrylamide, polyvinylalcohol, polyethylene glycol diglicidyl ether or cellulose, or by crosslinking with formaldehyde, glutaraldehyde, epichlororhydrin or epoxy compounds. (Passauer, 2012b; Thakur and Thakur, 2015)

This study presents an alternative, biological, pathway to produce lignin-based hydrogels. To our best knowledge, no lignin-based hydrogels have been synthesized via biomodification. Recently, we described the production of a water-soluble lignin-based polymer. Here, we report an inexpensive (without the use of any crosslinking agents) relatively fast production scheme for structurally similar hydrogels of varied degree of functionalization, which are insoluble in various solvents, either aqueous or organic. As different hydrogels are formed when the intermediate polymer is washed with varied solvents, we provide their comparison and pertinent characterization, i.e, determination of swelling and buffering capacity and thermal stability.

V.3 Materials and Methods

V.3.1 Feedstock, chemicals and solvents

Kraft lignin (alkali lignin) and all other chemicals except for solvents were purchased from Sigma Aldrich, St. Louis, MO, USA. The non-stabilized (i.e., those without additional chemicals) solvents used, including DMSO, tetrahydrofuran (THF), dioxane and ethylene glycol of either HPLC or spectrophotometric grade, were obtained from VWR International, Radnor, PA, USA.

V.3.2 Modification of insoluble kraft lignin into a soluble polymer

This modification is described in great detail in our previous publication (CHAPTER IV) including the detailed chemical characterization of this biomodified lignin. Below we provide a shorter description; note that in this study a ten-fold larger lignin concentration was used. For samples without DMSO, 10 g of Kraft lignin was transferred into an Erlenmeyer flask together with 100 mL of distilled water. For DMSO containing samples, 10 g of Kraft lignin was first solubilized in 2.0 vol% of DMSO and then distilled water was added. Prior to inoculation of these flasks, fungi (*Coriolus versicolor*) were pre-grown on agar. The agar medium used (300 mL) contained 3.0 g of Kraft lignin, 3.0 g of dry kenaf grass (*Hibiscus cannabinus*) and 4.5 g of agar. This medium, after sterilization in an autoclave (121 °C/ 30 min), was poured on Petri dishes. The solid agar was inoculated with 5×5 mm fragments of fully grown fungal cultures. The plates were incubated for 12 days at 22 °C. These quasi-immobilized fungi were used for the biomodification of the Kraft lignin containing liquid media. The agar with fungi was cut into small fragments (ca. 5×5 mm), with approximately ¼ of the agar plate

used for one Erlenmeyer flask. The Erlenmeyer flasks were placed in a shaker with the temperature set up at 29 °C. Experiments were conducted in triplicate.

V.3.3 Production of intermediate lignin polymer products, hydrogel precursor

The water-soluble polymer which biosynthesis was converted into the corresponding insoluble polymer using 100 mL of the reaction medium with the solubilized Kraft lignin-based polymer described in the previous section. Two separate methods were applied (Fig.1):

1) Acidification to pH ~3.5 with the addition of 1M HCl. This treatment caused the precipitation of acid precipitable polymeric lignin (APPL). To collect the APPL, samples were centrifuged (at 5,000 rpm for 15 min) and the sediment was collected. To remove the remaining DMSO or any other impurities, the samples were washed three times with 100 mL of either distilled water, ethanol, or methanol. After washing, the APPL dried at 75°C for 48 hours (until a constant weight).
2) Some hydrogel precursors were prepared without precipitation by acidification. In this case 100 mL of water soluble lignin polymer (10g/L) evaporated down to ~5mL using vacuum rotary evaporation was used to "wash" and concentrate the water-soluble polymeric lignin then another 100mL of distilled water was added and evaporated. This "washing" step was repeated 3 times to remove DMSO. The last "wash" was continued until the liquid evaporated completely. The solid was then transferred to an oven and dried to a constant weight at 75 °C. This procedure was used for both the APPL and precursors preparation for TCA to

ensure their low percentage of sulfur, which is detrimental for the instruments optics.

While using the first method, washing with water was done repeatedly and for long time period without affecting the formation of the hydrogel. By contrast, the washing step with MeOH and, particularly, EtOH should be done only once and as quickly as possible because long or repeated washing with alcohols will inhibit hydrogel formation.

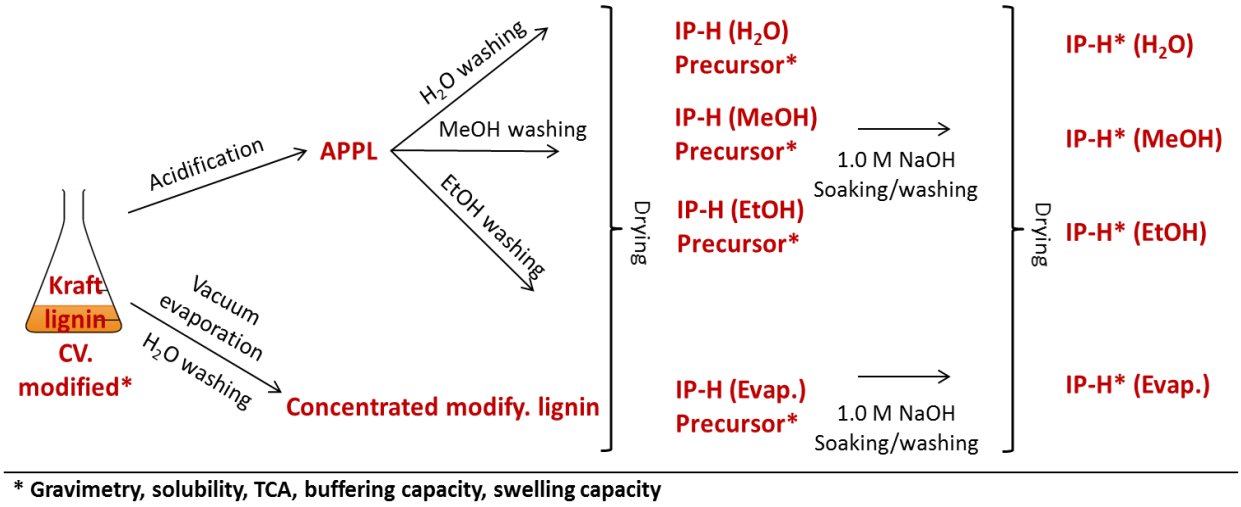


Fig. 1 Experimental and product characterization setup

V.3.4 Production of an insoluble polymer/hydrogel (IP-H)

The hydrogels were formed by the final treatment of their precursors with an aqueous 1.0 M NaOH solution 1 gram of the gel was soaked in 100 mL of 1.0 M NaOH for 48 hours.After this treatment, the hydrogels were returned to their acidic forms by adding 0.1 M HCl, washed with distilled water, and dried to constant weight.

V.3.5 Fungal strains used

Biological treatment of Kraft lignin was performed with 5 fungal strains (*C. versicolor, T. galica, P. ostreatus, P. pulmonarius and G. lucidum*), with only small

differences observed in performance between these strains. Therefore, we present only the results obtained with *C. versicolor*, which consistently produced the highest yield of the lignin based insoluble polymer/hydrogel (IP-H).

V.3.6 GPC analysis

Instrumentation

GPC analyses were performed on an Agilent 1100 Series HPLC with DAD detection (Agilent Technologies, Santa Clara, CA, USA). The PLgel 1000 Å (5 µm, 7.5 mm × 300 mm, 1000 Å) with a guard column (7.5 mm × 50 mm) (Agilent) were utilized and unstabilized THF was used as a mobile phase at a flow rate of 1 mL/min. Polystyrene standards M_p 580–19,760 Da purchased from Varian (Amherst, MA, USA) and poly(methyl methacrylate) standards M_p 550–26,080 Da purchased from Agilent Technologies were used for the GPC column calibration. To prepare samples for GPC analysis, the biomodified lignin filtrate (APPL after filtration) was dissolved in the THF-water system at a 1:1 v/v in a concentrate of 1 mg mL^{-1}. The concentration of standards and lignin was 0.1 and 1.0% dissolved in THF and THF-water 1:1 v/v, with injection volume 100 µL and 50 µL, respectively.

GPC Data Handling

To eliminate DMSO and water artifacts in the chromatogram, the blank sample consisting of THF-water-DMSO 49-49-2 % (v/v) was analyzed. Its chromatogram was subtracted from the chromatograms of the samples prior to molecular weight (MW) calculation. To calculate the number average (M_n) and weight average (M_w) MW of lignin samples, the total absorbance (in au) at wavelengths range of 220–750 nm (A_i) was used. The absorbance at the measurement point *i* had to exceed the baseline noise at least

3 times to be considered an analytical signal. M_n and M_w were calculated in an MS Excel using the standard polymer MW formulas 1 and 2:

$$M_n = \frac{\sum A_i M_i}{\sum A_i} \quad (1)$$

$$M_w = \frac{\sum A_i M_i^2}{\sum A_i M_i} \quad (2)$$

To calculate sample's MW (M_i) at the measurement point *i*, the linear equation derived from the standards' MW plotted vs. retention time was used.

V.3.7 Polymer solubility in solvents

The intermediate lignin polymer products (section 2.3) as well as the final products, IP-Hs (section 2.4), were subjected to a solubility evaluation test. Dried samples were divided into small ~ 1mm pieces and 1.00 g of each sample was subjected to solubilization in 10 mL of either an organic solvent (DMSO, THF, dioxane or ethylene glycol) or aqueous solution at pH 1.5, 7, 9 and 13. 20-mL glass vials containing 1.00 g of this polymer (biologically treated dried lignin) in 10.0 mL of solvent were placed in a shaker incubator at 21 °C. Solubilization was conducted in three 48h steps followed by centrifugation. After centrifugation, the supernatant was removed and replaced with a fresh solvent three times followed by drying of the samples at 75 °C to a constant weight and weighing.

V.3.8 Hydrogel swelling capacity (response to a pH change), and a cursory assessment of mechanical stability

To determine the swelling capacity of the biologically modified lignin polymer, 1.00 g of this polymer was placed into 100.0 mL of 1.0 M NaOH for 72 hours, which was

sufficient to convert the insoluble lignin polymer into the corresponding hydrogel. After 72 hours, the swollen hydrogel was weighed. The swelling capacity was calculated as the amount of water absorbed by 1g of the original polymer.

The pH response was evaluated in subsequent steps (the measurement of the water content in polymer was done after each step). First, when 72hours of swelling in 1.0M NaOH were applied to obtain the hydrogel, the amount water in 1g of this polymer was measured. This hydrogel was placed into an Erlenmeyer flask containing 100mL of a 1.0M NaOH aqueous solution at pH 9.5 for 24 hours. Then the hydrogel was taken out, weighed, then submerged in 100 mL of distilled water (pH 6.5) for 24 hours and weighed again. The water content was determined as the weight difference. This procedure was repeated at pH 3.5 and then at pH 1.5. Then these steps were repeated in the opposite order to check if the hydrogel would swell again.

V.3.9 Thermal stability: TCA analysis

To evaluate the thermal stability of the biotreated polymers in comparison to the original Kraft lignin, Thermal carbon analysis (TCA) was used. The TCA was performed on an organic carbon/elemental carbon analyzer from Sunset Laboratory Inc. (WA, USA). The method used was previously reported by Asina et al. 2015. The TCA thermal evolution profiles were obtained at 200, 300, 400, 500 and 870 °C under an inert helium atmosphere followed by one additional step conducting at 850 °C with oxygen, which allowed for quantification of the non-volatile char portion in samples. TCA thus generates results similar to those of thermogravimetric analysis, TGA, but also allowes mass balance closure on carbon by accounting for the non-volatilizable “coked” fraction.

V.3.10 Polymer buffering capacity

Erlenmeyer flasks containing 25 mL of distilled water were supplemented with 0, 100, 200, 300, 400, 500 and 1000µL of 1M NaOH (or 1M HCl). 1.0 g of either lignin or a biologically modified lignin polymer was added to each of these flasks. The control was a similar sample without lignin. These flasks containing an alkali/acid medium and lignin were placed into an incubator shaker for 1hour. After the incubation, the pH was measured. The buffering capacity was calculated as the number of moles of either a strong base or acid required to change the pH of 6.5 in 100g/L (lignin polymer in water) by one unit.

V.4 Results and Discussion

V.4.1 Production of IP-H and its acceleration by DMSO

The insoluble lignin-based polymer/hydrogel, IP-H, was produced in three steps. First, the corresponding water-soluble polymer, APPL, was synthesized by cultivation of quasi-immobilized (on agar) fungi, primarily, *Coriolus versicolor*, as described in Section 2.2 of Materials and Methods. Then, an IP-H precursor with a limited solubility in solvents was prepared by either vacuum evaporation or acid precipitation, as described in Section 2.3. Finally, IP-H insoluble in both aqueous and organic solvents was produced by drying of this intermediate product followed by its treatment in a highly alkaline solution and the final drying. Table 1 shows the yields of both the soluble polymer (APPL) and insoluble IP-H for different fungal biotreatments. When lignin was not treated with fungi, smaller APPL amounts were obtained but the insoluble hydrogel did

not form at all (Table 1). Apparently, lignin oxidative cross-linking resulting from the fungal treatment is essential to render the polymer insoluble.

The yield of IP-H was rather low while using 0% DMSO (Table 1). However, we discovered that the addition of 2% DMSO to the cultivation medium significantly increased the yield of not only APPL (as described previously in Chapter IV) but also IP-H, from 20 to 72% (Table 1). Thus, DMSO as a solubilizing solvent is not essential for the IP-H formation, but increases the lignin conversion as a result of a fungal biotreatment.

Table 1. APPL and IP-H (washed in distilled water) yields as a result of 6-day fungal (*C. versicolor*) treatments with and without DMSO.

	Yield of APPL (wt. %)	**Yield of IP-H (wt. %)**
2 % DMSO fungal treatment	84 ± 1	72 ± 1
2 % DMSO control (no fungal treatment)	68 ± 2	0
0 % DMSO fungal treatment	27 ± 1	20 ± 1
0 % DMSO control (no fungal treatment)	8 ± 1	0

Besides using DMSO, the key parameter in IP-H production was the composition of the agar blocks used for fungi quasi-immobilization and cultivation, prior to the Kraft lignin biological modification. The best results were obtained with the agar having a 1:1 ratio of lignin and lignocellulose (Kenaf) used as the growth medium for 3 generations. Either smaller or larger amounts of lignin resulted in a lower yield of IP-H. The absence of lignin in the agar cultivation/quasi-immobilization solid medium resulted in a near-zero IP-H yield. Perhaps, the presence of an optimum lignin concentration in the agar growth medium enhances the induction of biosynthesis of specific enzymes. Another important parameter was the cultivation/quasi-immobilization time and storage of the

strains prior to the Kraft lignin modification. The best results were obtained with 12 day old cultures followed by a short (12 to 24hours) storage in a fridge(~7 °C) prior to the lignin modification step.

V.4.2 Intermediate polymer products obtained by acid precipitation or vacuum evaporation of the solvent

Based on the results presented in Table 2, a 6 day cultivation is sufficient for a completion of a lignin treatment with *C. versicolor*. If the Kraft lignin modification with *C. versicolor* was performed for a longer time (12 days), the amount of solubilizable lignin remained virtually the same as on day 6.

Table 2. The fraction removed during the washing of IP-H precursors. 6 days of incubation were selected for the incubation with *C. versicolor*.

Incubation time (days)	Fraction of "solubilizable" lignin polymer removed (wt. %)		
	Distilled water	Methanol	Ethanol
1	55±7	100±1	100±1
2	54±9	100±1	100±1
3	46±3	100±4	100±1
4	32±7	92±7	100±3
5	18±5	50±8	87±10
6	**9±3**	**21±6**	**35±9**
12	7±3	20±7	30±5

Table 2 also reflects the gradual changes in lignin as a result of fungal treatment, which shows as the extent of polymer solubilization as a result of its washing with different solvents. The most apparent trend seen in Table 2 is a gradual shift (with time) of the washed polymer fraction towards a lower polarity as it is preferentially removed with less polar solvents. Yet, prior to precipitation the entire polymer product remained soluble in 2% aqueous DMSO thus showing its significant remaining polarity. Corroborating this statement, the precipitated polymer could still be re-dissolved in

DMSO or in alkaline aqueous solution (pH ≥9.5) either before or after washing with solvents, as long as it was not dried.

An increase in lignin MW upon biomodification with *C. versicolor* was observed (Fig. 2). The modified lignin started eluting earlier in GPC analysis evidencing the presence of higher MW species in the sample. Even though the absolute value of absorbance increased only slightly between 6-8 min of retention time, the impact of this *fraction* on the average MW is significant. The calculated number-average and weight-average MW values corroborated this observation showing an increase from 1,750 Da to 4,780 Da and from 4,690 Da to 28,760 Da, respectively. This observation indicated that intermolecular cross-linking (presumably, oxidative cross-linking) is the major reaction path in lignin biomodification.

The further processed APPL including both the hydrogels and their polymer precursors could not be studied by GPC or any other methods requiring sample to be completely dissolved, owing to their poor solubility in any solvents. However, just their lower solubility compared to APPL indicated their even higher extent of cross-linking and, presumably, MW.

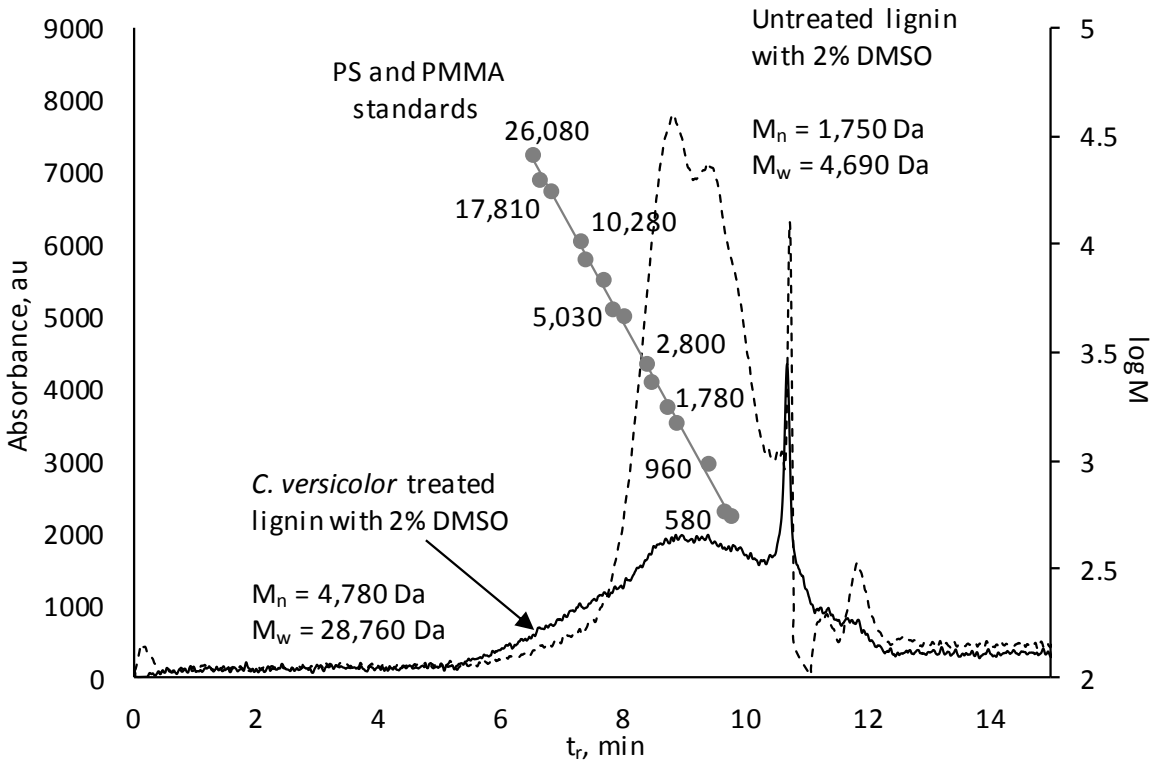


Figure 2. Untreated and *C. vercicolor* treated lignin –APPL before washing or precipitation. Elution profiles demonstrating the induced increase in the MW.

After this biological modification, additional modification changes in the lignin structure were made *via* different separation and purification pathways. The intermediate lignin polymer products (IP-H precursors) can be formed either by 1) immediate air drying (i.e., water evaporation from an APPL-containing solution), or 2) acid precipitation followed by washing with various solvents and air drying at 65 °C, as detailed in the pertinent section of Materials and Methods.

Precipitation (followed by washing with solvents) parenthetically removes DMSO as well as any soluble impurities, including the unreacted lignin. Based on the results presented in Table 2, the extent of mass reduction by applying different solvents correlates with Kraft lignin solubility in these solvents: 7% for water, 10% for methanol

and 12% for ethanol. It is of note that the solubility of Kraft lignin in nonpolar solvents (DCM) is near 0%, so these trends are consistent with its amphiphilic nature. The effects of washing with different solvents on the resulting polymer physical and chemical properties are discussed in the following sections.

V.4.3 Preparation of insoluble polymers-hydrogels (IP-H)

The dried IP-H precursors obtained as described in the previous section were treated with aqueous NaOH. Soaking an IP-H precursor in aqueous solutions at an alkaline pH of 9-13 turned it into a swollen hydrogel. The swelling process was relatively slow, ~2 days. When the pH was returned back to neutral, the polymer remained in the hydrogel form. However, when the pH was decreased to pH 3.5 – 1.5, the hydrogel shrunk back to its un-hydrated form within seconds. Thus, the obtained product may be classified as an anionic hydrogel.

After the final drying step, the final product becomes insoluble in any solvent. Thus, it will be called henceforth an "insoluble polymer-hydrogel," IP-H. The final yields of both this final product and its intermediate precursors washed by various solvents are listed in Table 3. The difference between the yields of IP-H and its precursor is due to the partial solubilization of the latter in aqueous NaOH when making the hydrogel. As expected, the alcohol-washed polymers did not lose much of their weight as a result of this treatment, as most of the unreacted lignin had been washed away. It was also expected that a sizable fraction of the *water*-washed IP-H precursor would be lost in this treatment, just as observed. As a result, the yields of all three IP-Hs obtained with polymer washing were similar.

Table 3. Yields of IP-H precursors and IP-H. (n=3)

	Yield %	
	IP-H precursor	**IP-H**
H_2O washed	72 ± 1	54 ± 1
MeOH washed	64 ± 7	59 ± 2
EtOH washed	54 ± 5	49 ± 5
Vacuum evaporation	83 ± 3	**74 ± 3**

However, the observed much higher IP-H yield obtained by vacuum evaporation was not expected, as we originally assumed that washing with solvents removes only the unreacted lignin. Contrary to this assumption, only about 10% of this polymer dissolved in the aqueous NaOH solution while most of the fraction that would be removed by solvents became incorporated into the IP-H. Apparently, this fraction was significantly modified by fungal treatment contrary to the expectation that the washing solvents removed just the unreacted lignin.

The successful IP-H formation from its precursor obtained by vacuum evaporation shows that acid precipitation and solvent washing are not essential for IP-H formation whereas the drying step is essential. The necessity of drying, in turn, suggests that IP-H precursors undergo additional cross-linking during this procedure; the chemical modification that defines the hydrogel properties. This suggestion was confirmed when studying the polymer properties, as detailed in the subsequent sections.

Table 3. Yields of IP-H precursors and IP-H. (n=3)

	Yield %	
	IP-H precursor	**IP-H**
H_2O washed	72 ± 1	54 ± 1
MeOH washed	64 ± 7	59 ± 2
EtOH washed	54 ± 5	49 ± 5
Vacuum evaporation	83 ± 3	**74 ± 3**

V.4.4 Solubility of insoluble polymer – hydrogel (IP-H) and its precursors

Whenever an IP-H preparation was unsuccessful, e.g., short incubation time, suboptimal inoculation conditions and other cases mentioned in Section 3.1, the yields similar to those reported in Table 3 could be observed for IP-H precursors whereas either zero or near-zero yields of the corresponding IP-Hs were recovered, as these precursors dissolved in the NaOH solution during the final step of the hydrogel preparation. Table 4 emphasizes this difference by showing the solubility of the successful IP-H precursors in comparison with that of lignin. Table 5 expands this information to the rest of IP-H precursors, with the experimental details of solubility measurements shown in Section 2.5. The failure to obtain a hydrogel resulted in the IP-H complete or near-complete solubilization in alkaline water, just as for the control, untreated lignin as shown in Table 4. Either long or repeated polymer precursor washing, particularly with alcohols, also increased its solubility in the NaOH solution thus lowering the hydrogel yield.

The successful IP-H precursors featured reduced solubility not just in alkaline water but also in organic solvents as shown in Table 5, unlike the untreated lignin (Table 4). This observation corroborates the observed gradual reduction of the polymer solubility in all solvents as a result of successful biological modification shown in Table 2. The solubility in various organic solvents of the IP-H precursors produced with and without DMSO was statistically the same, thus confirming that this co-solvent did not affect the product but merely accelerated its formation.

Table 4. Extend of solubilization of the IP-H precursor (obtained with a 6 day *C. versicolor* treatment) and original lignin (control).

	Fraction of solubilized IP-H (wt. %)		Fraction of solubilized IP-H (wt.%)	
Solvents	**Lignin treated by fungi in 2% DMSO**	**Control in 2% DMSO**	**Lignin treated by fungi in 0% DMSO**	**Control in 0% DMSO**
DMSO	15 ± 2	88 ± 5	12 ± 2	96 ± 3
DMF	9 ± 1	92 ± 2	11 ± 1	93 ± 3
NMP	9 ± 2	95 ± 1	10 ± 2	95 ± 3
Dioxane	0 ± 1	84 ± 2	0 ± 2	96 ± 2
Ethylene glycol	0 ± 1	81 ± 2	0 ± 1	90 ± 2
Water pH 1.5	0 ± 1	0 ± 1	0 ± 1	0 ± 1
Water pH7	0 ± 1	3 ± 1	0 ± 1	5 ± 1
Water pH 9.5	3 ± 2	98 ± 3	5 ± 2	100 ± 3
Water pH 13	18 ± 3	100 ± 2	16 ± 3	100 ± 3

Table 5. A comparison of the extend of solubilization of IP-H precursors prepared from APPL washed with distilled water, methanol and ethanol and prepared via vacuum evaporation

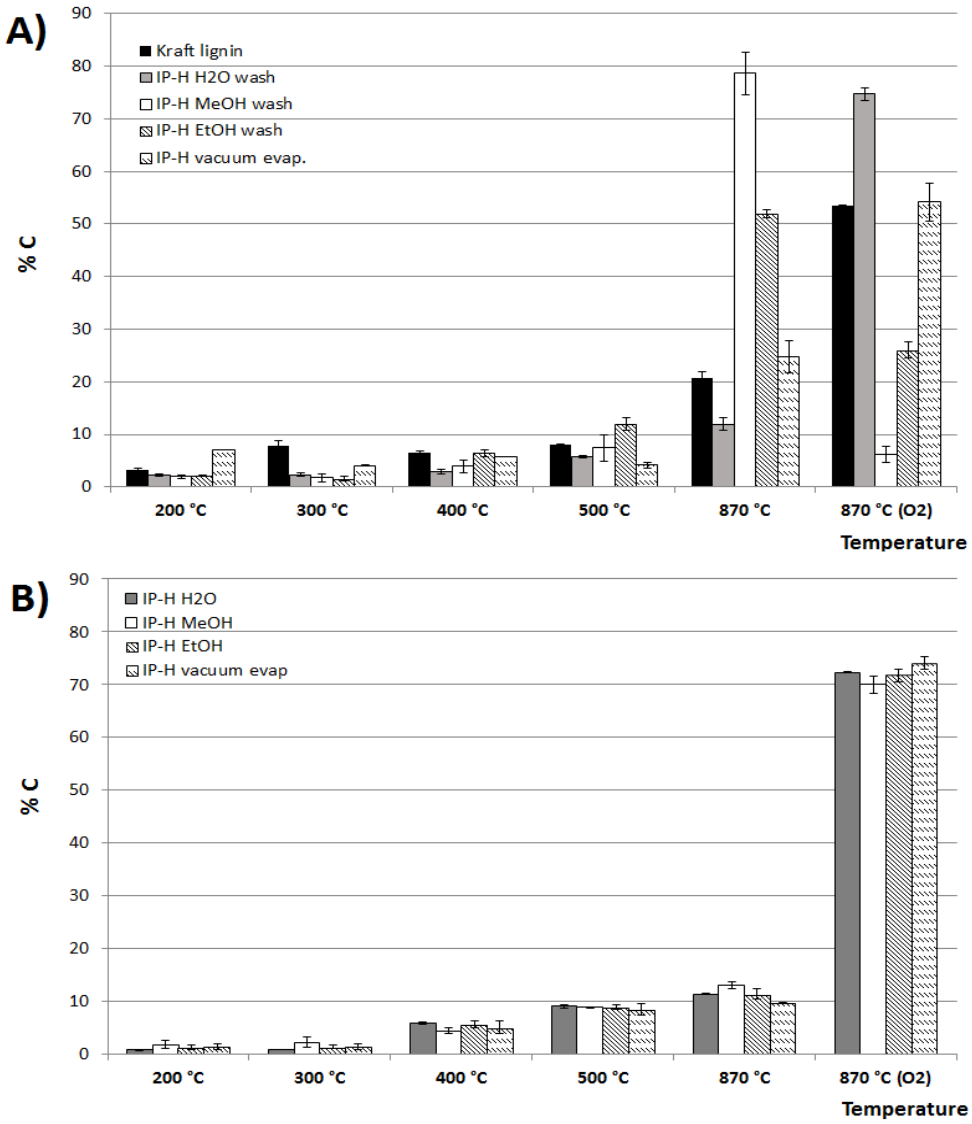
	(wt% of solubilized IP-H precursors prepared with 2% DMSO)			
Solvents	**IP-H precursor washed with distilled water**	**IP-H precursor washed with methanol**	**IP-H precursor washed with ethanol**	**IP-H precursor obtained by vacuum evaporation**
DMSO	15 ± 2	5 ± 1	7 ± 1	7 ± 1
DMF	9 ± 1	5 ± 1	7 ± 2	3 ± 1
NMP	9 ± 2	2 ± 1	4 ± 2	3 ± 1
Dioxane	0	0 ± 1	0 ± 1	0
Ethylene glycol	0	0	0	0
Water pH 1.5	0	0	0	0
Water pH7	0	0	0	0
Water pH 9.5	3 ± 2	2 ± 1	3 ± 1	0 ± 1
Water pH 13	18 ± 3	5 ± 1	5 ± 1	9 ± 1

As for the trends in solubility of IP-H precursors shown in Table 5, only DMSO and high-pH alkaline solutions removed some significant amounts of those polymers, yet these amounts were still small compared to the untreated lignin removed (Table 4). Most of the solubilization occurred during the first day, presumably when the unreacted and insufficiently modified lignin was washed away.

As shown in the two previous sections, washing the intermediate IP-H precursor with water and alcohols altered the solubility of the intermediate IP-H precursor and yield of the final product. We thus investigated whether these differences in IP-H processing could also affect the properties of the resulting polymer. The obtained results are discussed in the subsequent sections.

V.4.5 The thermal stability of lignin polymers

The results of TCA analysis of the IP-H precursors produced in different protocols are shown in Fig. 3A, in comparison to both their precursor, a soluble polymer produced by lignin fungal treatment, and untreated lignin (used as a control). The most important information embodying thermal stability is the amount of the last, "char" fraction that does not evolve at any temperature unless oxygen is added to burn it. The water-washed IP-H precursor showed an increase in this fraction compared to both the untreated and water-soluble lignin, as expected since some of the non-reacted and only slightly modified lignin was removed by washing. The char fraction appeared less pronounced in the non-washed IP-H precursor because the unreacted lignin was not yet removed from this polymer. It was less expected to observe an actual decline of this fraction in the alcohol washed IP-H precursors. Perhaps, alcohol washing removed a less polar and more cross-linked fraction of this polymer. This explanation corroborates the observed lower IP-H yields (Table 3) and lighter colors of the IP-H precursors when they were washed with alcohols.



Firure 3. TCA temperature profiles of A) **IP-H precursors** prepared from APPL washed with distilled water, methanol or ethanol and prepared via vacuum evaporation compared with original Kraft lignin and B) **IP-H after** the final treatment with alkaline water and drying.

However, any differences in the abundance of the coked fraction disappeared when the hydrogels were formed from their polymer precursors (Fig. 3B). A significant increase in this recalcitrant fraction was observed, to near 80%. The increase in the coked fraction for the unwashed polymer can be explained by the removal of its less cross-

linked part with the alkaline solution on the final step of its preparation. However, this explanation cannot be valid for alcohol-washed IP-H precursors, which did not lose much of their mass on the final step of preparation as shown in Tables 3 and 5. Apparently, the IP-H formation by an alkaline treatment followed by drying resulted in additional cross-linking, which corroborates the observed lack of IP-H solubility in both alkaline aqueous and organic solvents. The colors of all four hydrogels also became darker than that of their precursors which indicate additional double bond conjugation apparently results from additional cross-linking.

V.4.6 Extent of swelling and response to changes in pH

Figure 4 shows IP-H swelling curves, i.e., responses in terms of water absorption/desorption to pH changes by adding either an acid (HCl, left and right) or base (NaOH, center). This profile is typical to hydrogels and indicates significant reversible swelling in alkaline media. The swelling occurs only as long as the weakly acidic functional groups remain deprotonated, so the IP-H may be characterized as an anionic hydrogel, as expected for lignin.

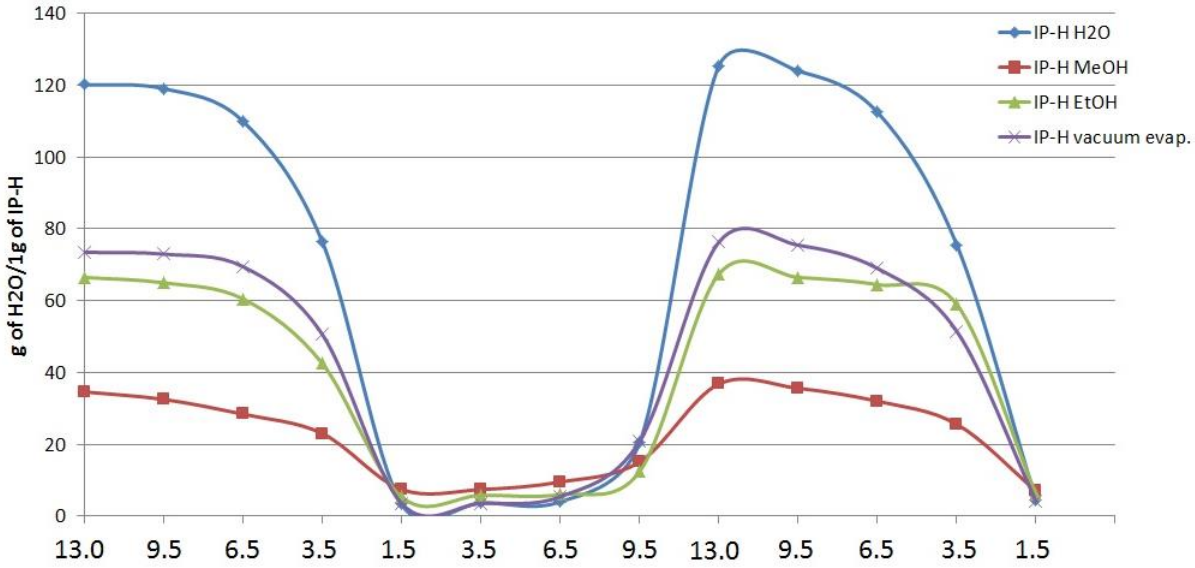


Figure 4. IP-H swelling response to pH changes

A notable feature of the pH response shown in Fig. 4 is that the hydrogel swelling starts at a pH as high as 9.5 and increases with the further NaOH addition; but when an acid is added, the gel remains 100% swollen at pH 9.5 and starts shrinking only at a pH of 7. This hysteresis can be explained by low accessibility of the bulk of deprotonated sites in the swollen gel, which can be overcome only by a large acid concentration causing a massive “unzipping” effect.

The pH response of this biotreated lignin polymer is similar to the behavior of some peptides.(Shen et al., 2005; Tobergte and Curtis, 2013) Protein interactions with lignin were reported earlier, (Yamaguchi et al., 2016) so protein association with lignin might be viewed as a potential cause of IP-H formation. However, the TD-Pyr-GC-MS analysis (CHAPTER IV) showed that protein residues/markers were undetectable in the

produced polymer. Thus, the hydrogel appears to be formed via deprotonation of phenolic functional groups of the lignin-based cross-linked polymer.

The IP-H swelling capacities calculated from the data of Fig. 3 are listed in Table 6. Most of the earlier reported lignin-based hydrogels have swelling capacities around 10g H_2O/g gel but some of them can absorb up to 75 g H_2O/g gel, e.g., the system of lignin - polyethylene glycol diglycidyl ether, where lignin was O_2 activated before crosslinking. The biobased hydrogels reported here thus exhibit a reasonably strong swelling. In particular, the water-washed IP-H precursor yields a hydrogel of a greater swelling capacity (~120 g H_2O with1M NaOH/g gel) than those reported earlier while the alcohol-washed precursors produce the least swelling hydrogels.

Table. 6 Swelling capacity of the hydrogel

IP-H type	Swelling capacity g H_2O with 1M NaOH/1g gel
H_2O washed	120
MeOH washed	34
EtOH washed	66
Vacuum evap.	73

Unfortunately, the observed high swelling capacity of the water-washed hydrogel turned out to be detrimental to its mechanical stability. This hydrogel, unlike the other three, breaks apart into smaller pieces even when simply transferred from one flask to another.

V.4.7 Buffering capacity

The pH changes in response to either HCl or NaOH titration are shown in Figure 5. The hydrogel "buffering" capacity was calculated as the number of moles of either a

strong base or acid required to change the pH 6.5 of a *100g/L (lignin polymer in water)* by one unit. For Kraft lignin, the buffer capacity was 50 mM for NaOH and 25 mM for HCl. This number was statistically similar for the water soluble APPL (data not shown) but decreased with the conversion of lignin into the insoluble polymer, particularly for acid titration.

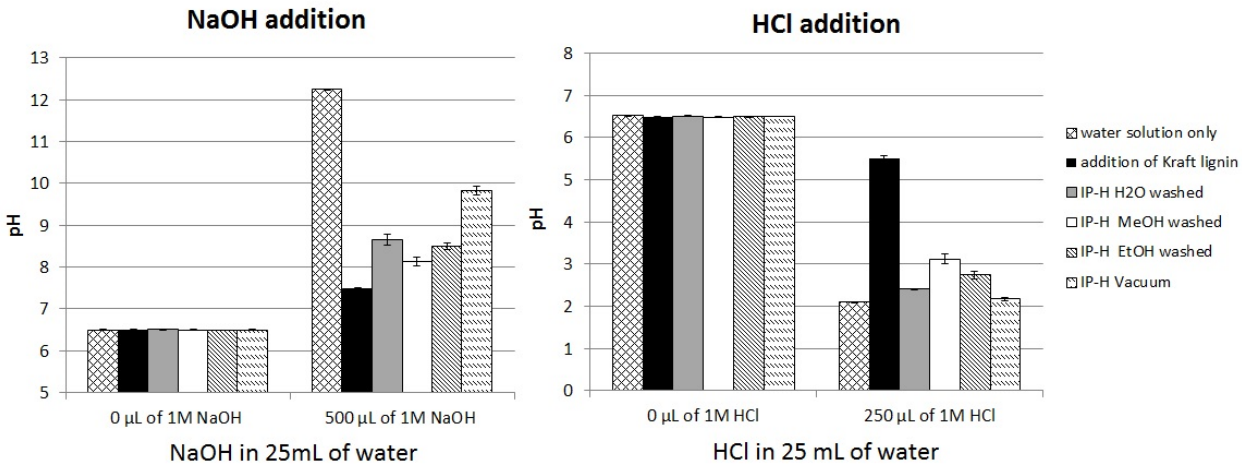


Figure 5. The effect of the preparation protocol, i.e., washing with solvents, on the "buffering" capacity of IP-H precursors

This observation suggests that the removal of functional groups occurs during the drying process, resulting in additional cross-linking. The decrease in buffering capacity continued further washing the APPL with alcohols prior to drying. Apparently, washing with these solvents removed the most functionalized fraction of the polymer formed. The vacuum-dried IP-H precursor showed the smallest buffer capacity followed by the water-washed polymer. This trend is similar to that observed in the TCA where the alcohol-washed samples showed a lower abundance of the coked fraction, Fig. 3. Also, this trend correlates with the IP-H precursor yields (Table 3). It seems that more cross-linking

(partially removing functional groups) occurs when the polymer is not washed with solvents.

V.4.8 Morphological changes (SEM and confocal microscopy)

Based on SEM, the surface area of alcohol washed IP-H precursor appears be to very high compared to the H_2O washed IP-H or the IP-H obtained via vacuum evaporation. The differences between the appearances of xerogels (lyophilized hydrogel) are only minimal. Yet, the xerogel obtained from the hydrogel with the highest swelling capacity (prepared via H_2O washing) seems to have a rather uniform pore size with the walls having a similar thickness compared to the other xerogels. This uniformity might be owing to a more pronounced swelling expressed by this hydrogel, while the other hydrogels might not have reached their maximum swelling capacity before conversion in to the corresponding xerogel, as swelling is slow.

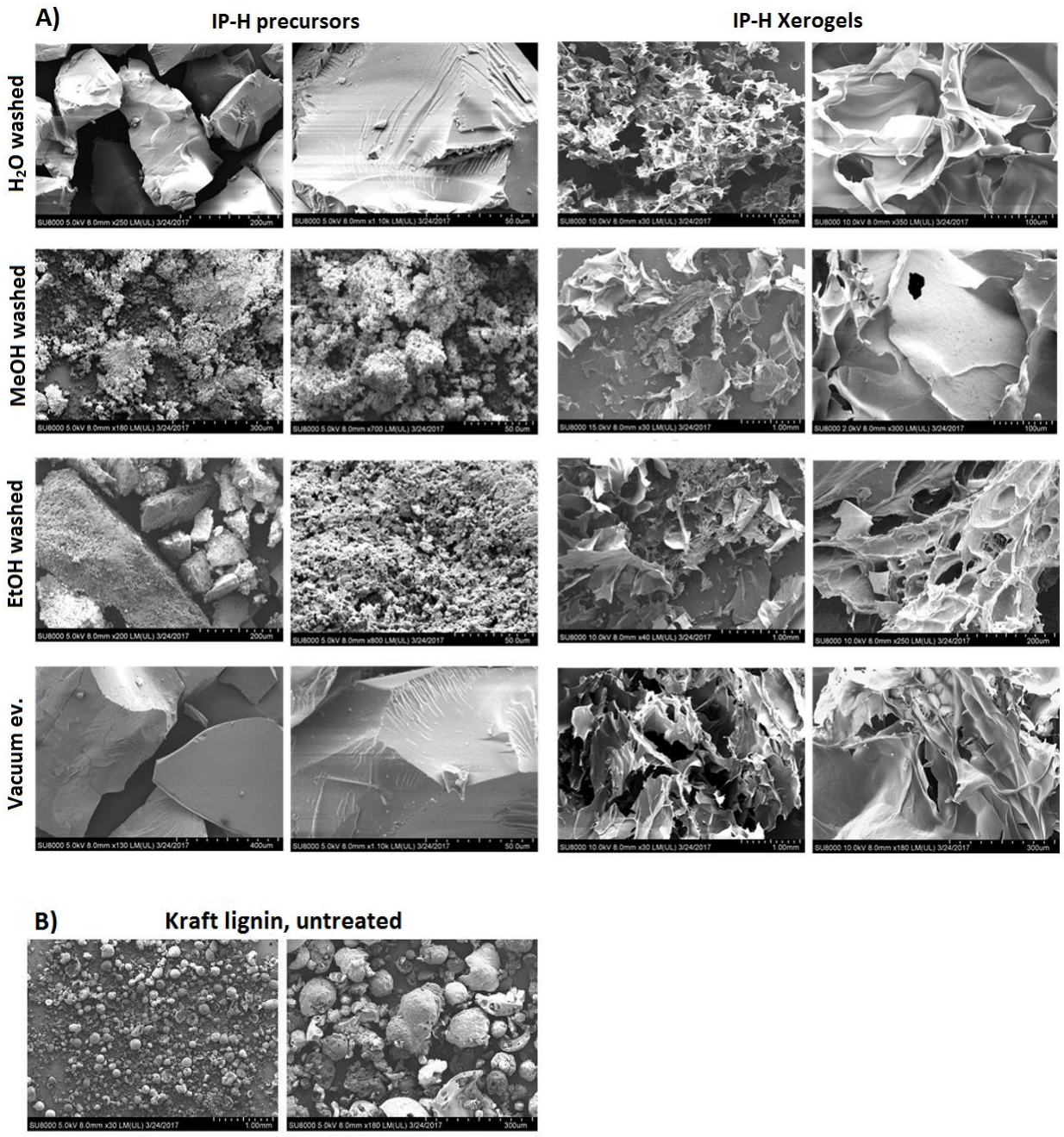


Fig. 3. SEM of A) the IP-H precursors and their responsible xerogels, B) original Kraft lignin

V.4.9 Potential application of IP-H

Broad applications of this bio-modified lignin polymer can be envisioned, similar to other hydrogels. One application may be a treatment of waste waters from textile industries where the dyeing effluent exhibits high alkalinity (usually with pH between 9-

13) and so these waters cannot be treated directly without a pH adjustment. (Shen et al., 2011) Another application of this-lignin based hydrogel may be for water purification and desalination, where it can be used in same way as modified graphene oxide membranes, or as a sorbent for recalcitrant impurities, e.g., metals, prior to neutralization followed by conventional treatment. Medical applications can also be envisioned where swelling under elevated pH can be applied in chronic non-healing wounds which usually feature elevated pH, between 7 and 9. (Gethin, 2007) Given the previously reported antioxidant, antimicrobial and anti-inflammatory properties of lignin, (Sakagami et al., 2005b) the application of a lignin-based hydrogel can improve the healing of those wounds.

V.5 Conclusion

Fungally biomodified lignin, a water-soluble polymeric product obtained by a 6-day lignin treatment in 2% DMSO with agar quasi-immobilized *Coriolus versicolor,* was converted into another polymer product featuring a limited solubility in both aqueous and non-aqueous solvents. By treatment with alkaline water followed by air drying, this polymer was converted into a hydrogel, which is insoluble in either pure organic solvents (DMSO, DMF, NMP, dioxane, ethylene glycol), alcohols or aqueous media over a wide range of pH 1.5 – 13.0. The yield, extent of cross-linking, swelling and buffer capacities, and functionalization of this polymer can be varied by an optional step involving acid precipitation, then washing the acid precipitated product with different solvents, although the greatest yield of a polymer with the highest buffer capacity and a suitable stability can be obtained without any precipitation or washing. This hydrogel has a potential for application such as environmental and medical.

CHAPTER VI

Biologically modified Kraft lignin for water purification

VI.1 Abstract

A lignin-based hydrogel was converted into a nonporous membrane potentially applicable for water desalination. This membrane was prepared through depositions of a thin layer of this modified lignin onto a supporting membrane. The best results were obtained with two depositions of 50 μg of a modified lignin per cm^2. The salt rejection/water desalination efficiency of this membrane was 78% for two hours while using a pressure of 270 kPa. The resulting flux was several orders of magnitudes higher than that conducted by reverse osmosis while using a four times lower pressure.

VI.2 Introduction

There are two main potential applications of lignin for water purification, lignin polymer as a sorbent for toxic ions and lignin polymer in the form of nano-membranes for water desalination. In this study we evaluated both of these applications for biologically modified lignin.

Lignin has a natural ability to capture heavy metals trough ionic or/and coordinate covalent bonding to toxic ions (Fig. 1).(Zhuang et al., 2003) Therefore it can be used as a sorbent. Prior to this application lignin needs to be stabilized to reduce its solubility while retaining the reactivity with heavy metal.

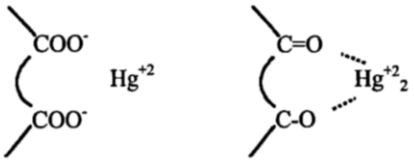


Fig. 1 Ionic or coordinate lignin bonding to a heavy metal (Zhuang et al., 2003)

Modified lignin can also be converted into nano-membranes, where ions larger than water (Na^+Cl^-, K^+Cl^-, etc.) are rejected based on size exclusion. This principle is currently applied during development of hydroxylated or hydrogenated graphene membranes, as one can see in Fig.2

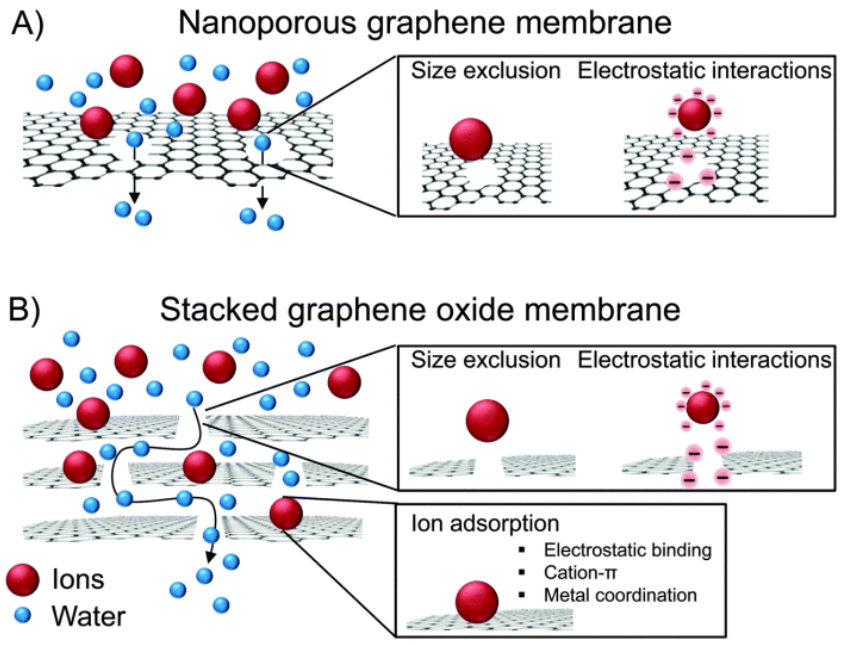


Fig 2. Mechanisms of the action of graphene oxide membranes. (Perreault et al., 2015)

One of many reasons why graphene oxide nano-membranes are becoming popular is that the salt rejection and water flow by these membranes is several orders of magnitude higher than those obtain by reverse osmosis (RO). This reduces the energy costs required for this process. Desalination performance is most sensitive to pore size and pore chemistry and graphene oxide can be modified in many ways. Another

advantage is reduced membrane fouling, thanks to antimicrobial properties of the graphene oxide (compared to traditional RO).

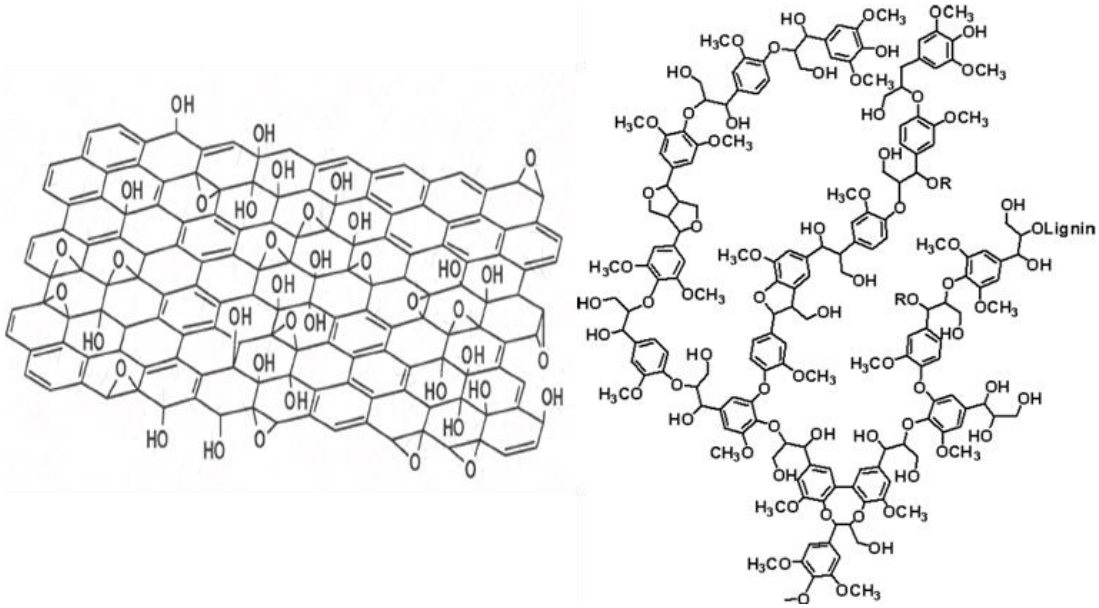


Fig. 3 General structure of graphene oxide (right) and lignin (left)

Lignin shares some of the potential features of graphene oxide it has broad possibilities for modification and the addition of various functional groups, features high thermal stability, has antimicrobial, antiviral, antioxidant properties and if modified, can have high stability within a broad range of pH and organic solvents.(Smolarski, 2012) This makes lignin a great potential candidate for application in water purification systems.

Kraft lignin is known for its chelating activity, it has a significant buffering capacity at pH 6.5, and it can be formed into nano-membranes after modification. However, without modification Kraft lignin is soluble in many organic solvents (DMSO, THF, Dioxane, NMP, etc.), in alkaline solutions with pH >9.5, and partially soluble

(~5%) in water at neutral pH. Therefore stabilization of Kraft lignin is necessary prior to possible water purification applications.

VI.3 Preliminary experiments and results

Common modification and stabilization techniques for lignin involves blending with other polymers, chemical/thermal modifications, or biological modifications. We used the latter to modify Kraft lignin. Based on our analysis we were able to increase the molecular weight from 5,000 Da to at least 29,000 Da, increased crosslinking and decreased the polydispersity of the lignin polymer. Though these changes, which we obtained by a bio-modification with *C. versicolor* (the scheme of the bio-modification process is provided in Fig 4.), we were able to obtain a Kraft lignin-based polymer which cannot be solubilized in organic solvents (DMSO, DMF, NMP, dioxane, etc.) and can be converted into an anionic hydrogel, which can be further cast into membranes (Fig. 5B and Fig 6.) or used as a sorbent.

In order to convert this hydrogel into a membrane ultra-sonication is esential. Vacuum filtration of the ultrasonicated lignin hydrogel with a supportive membrane of a pore size $\leq 0.45\mu m$ (larger pore size would not stop the dispersed/ultrasonicated hydrogel from passing through) followed by drying at 60°C for 1 hour was done in order to prepare the membrane for water filtration.

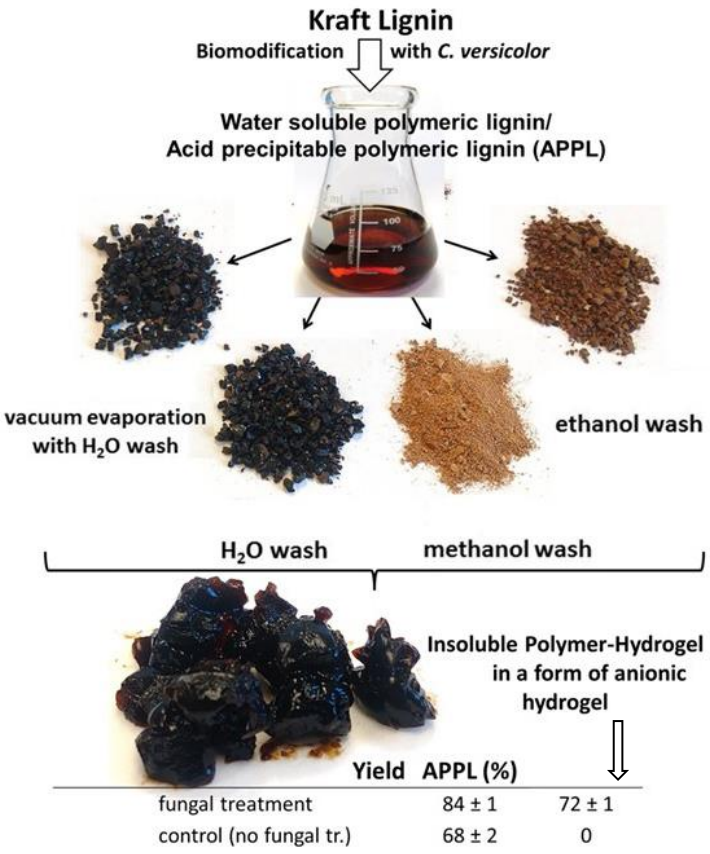


Fig. 4 Kraft lignin bio-modification in order to obtain lignin based hydrogel.

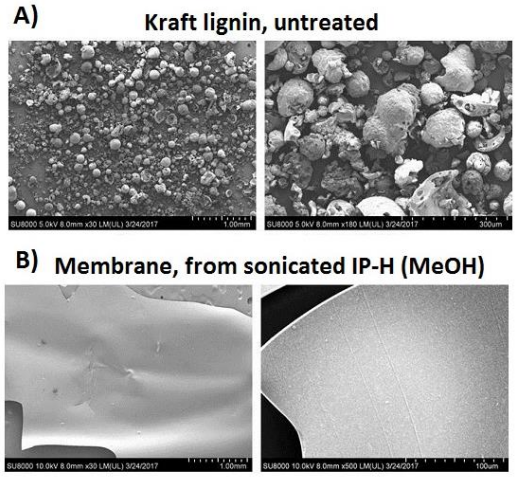


Fig. 5 A) SEM of Kraft lignin without biomodification, B) magnification of the lignin membrane via SEM.

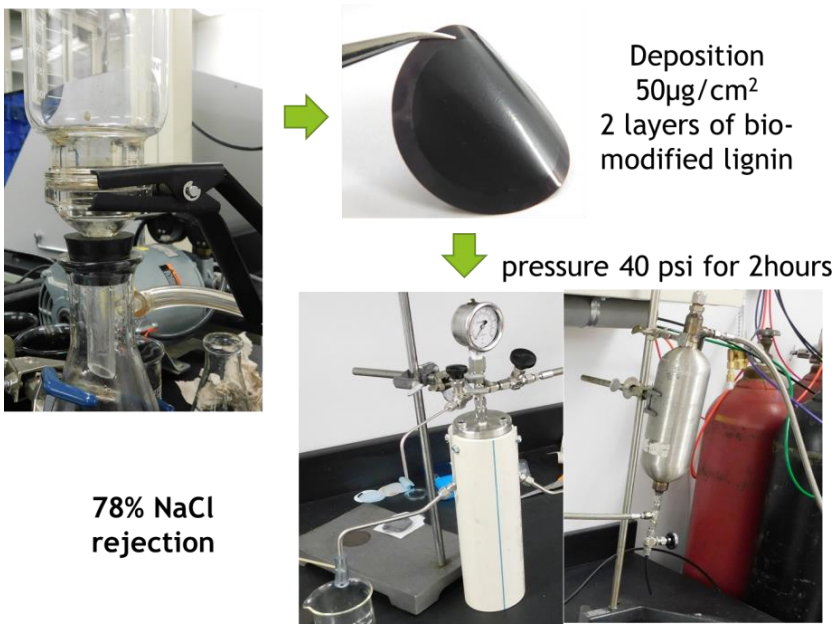


Fig. 6. Preparation of lignin membranes using an ultrasonicated lignin hydrogel

To date, we were able to obtain a 78% salt rejection using a two layers deposition system total 100µg of biomodified lignin per cm^2 (Fig. 7). Based on the previous reports with graphene oxide, membrane architecture plays an important role in salt rejection. In Fig. 7 one can see that only the membrane assembled with two layers of bio modified lignin was able to reject 78% of NaCl for 2 hours. The same deposition amount of bio-modified lignin applied in only one layer failed after less than 2min. This failure might be due to small cracks in the lignin nano-membrane, which are likely to form when a deposition amount is large. Therefore further optimization of the membrane architecture together with increase of elasticity is possible.

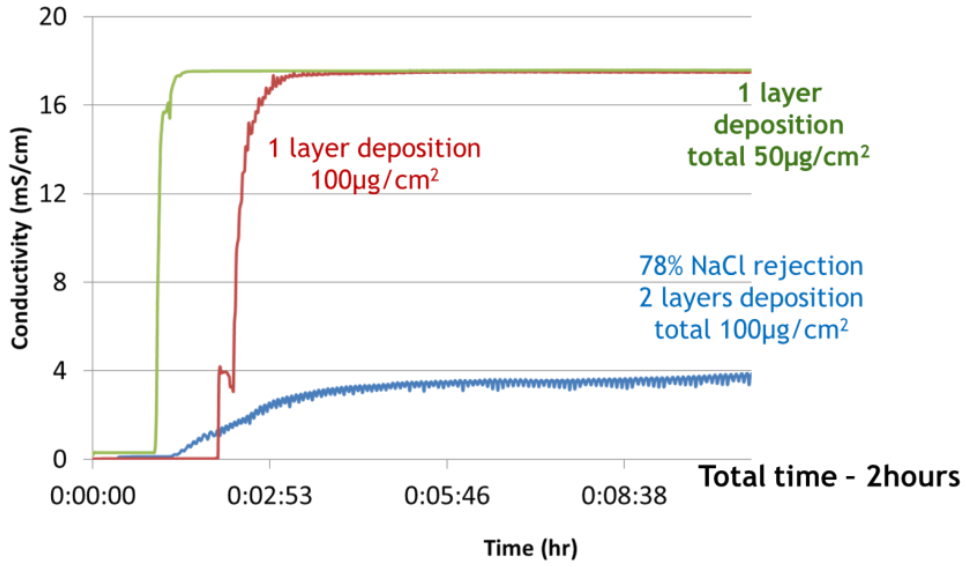


Fig. 7. Salt rejection with different lignin membrane assemblies (the NaCl concentration used was 1 wt% which corresponds to a conductivity of 17.5 mS/cm)

Salt rejection based on the conductivity data in the Fig. 7 suggests that the membrane has two functional phases in the first phase, the salt is attached to the membrane and the conductivity is 0.0 mS/cm.In the second, mechanical phase salt is rejected based on size (Table 1.)

Table 1. Salt rejection and membrane properties.

Type of membrane IP-H (MeOH wash)	Amount IP-H deposited	NaCl is being bond to membrane Conductivity 0.00 mS/cm	NaCl is being rejected Conductivity >0.00 mS/cm	Salt rejection (%) after 2 hours
1 layer	50 µg	~ 45 sec	~ 0 sec	0
1 layer	100 µg	~ 95 sec	~ 0 sec	0
2 layers	2 x 50 µg	~ 60 sec	≥2 hours	78

Comparison of our first attempt with the current research in this area is provided in Fig. 7. We already have a water flux higher than that obtained in reverse osmosis systems while using almost 4 times lover pressure. We believe further optimization can

give results at least comparable to graphene oxide membranes while using a renewable, lignin based polymer.

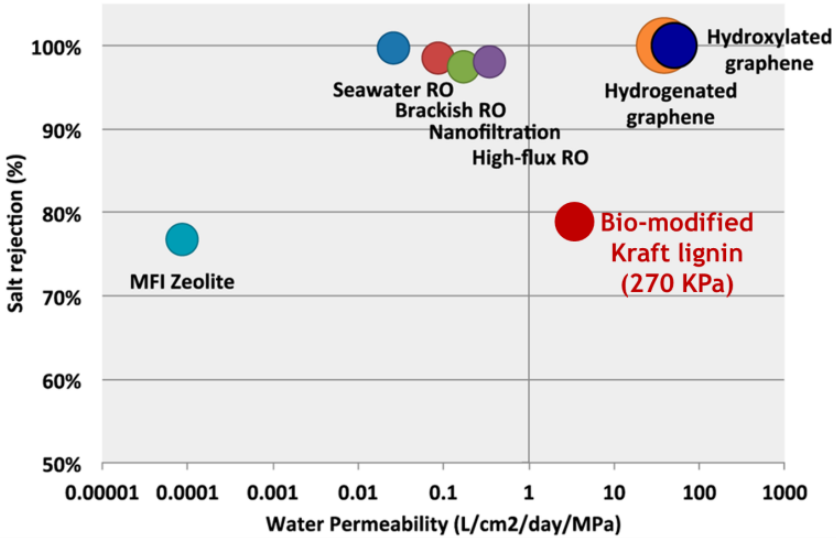


Fig. 8 Performance chart for the comparison of the bio-modified lignin membrane to existing technologies. (Figure adapted from Cohen-Tanugi and Grossman, 2012)

VI.4 Conclusion and future work

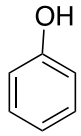
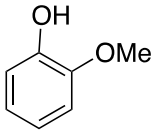
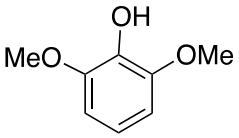
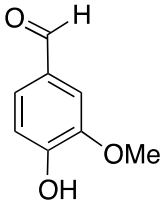
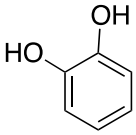
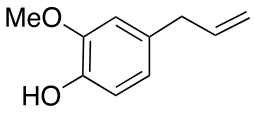
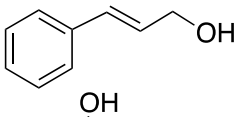
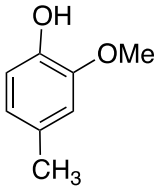
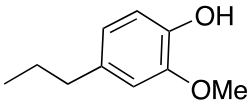
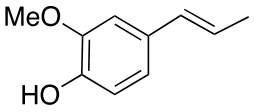
Based on our preliminary data, biologically modified lignin shows a great potential for water filtration systems. With our cursory design of a membrane, we were able to obtain a 78% rejection with the water flux being much higher than that obtained with traditional reverse osmosis while using only 25% of the pressure force as shown in Fig. 8, for two hours. Further optimization and development of these membranes may further increase salt rejection and decrease the water flux.

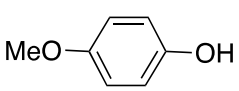
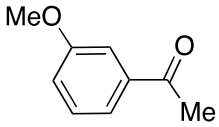
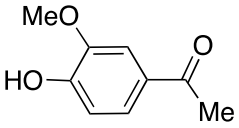
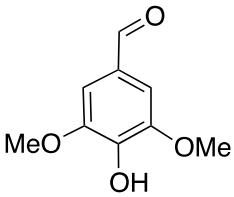
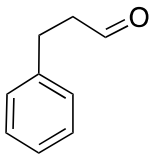
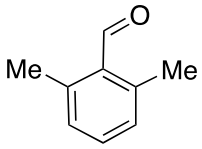
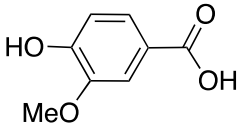
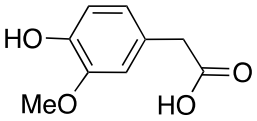
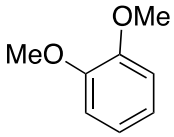
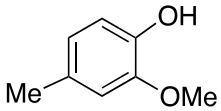
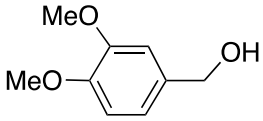
Future work in this area should focus on membrane architecture *via* layer by layer deposition and its optimization, combination of lignin and graphene oxide, evaluation of different support membranes in order to increase the adhesion of modified lignin to the membrane. Addition of plasticizers should also be evaluated, in order to decrease crack

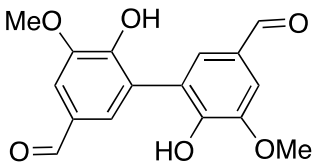
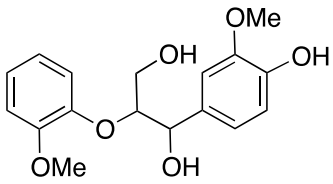
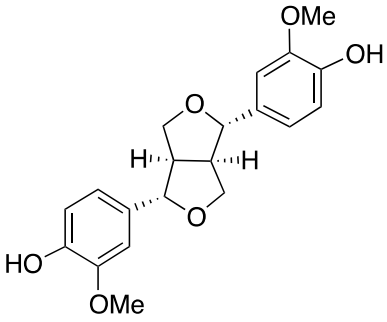
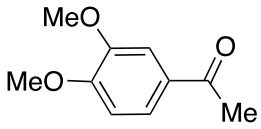
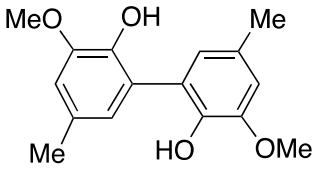
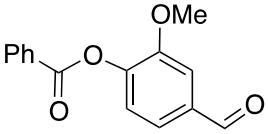
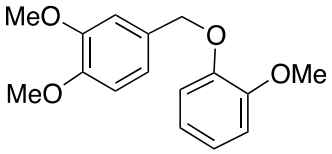
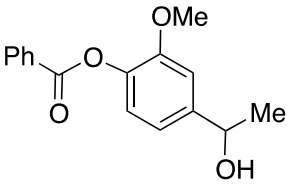
forming due to the brittleness of the modified lignin. Also the properties of membranes obtained with IP-H hydrogels prepared via washing with ethanol, water and vacuum evaporation should be evaluated. Data monitoring salt rejection by the membranes for variation of salts and their concentrations together with adsorption capability for heavy metals should be collected.

APPENDIX

Table 1. Structure and molecular weight of monomers present in industrial lignin after depolymerization

Structure		IUPAC Name	Formula	MW (g/mol)
Phenol		Hydroxybenzene OR phenol	C_6H_6O	94.11
Guaiacol		2-Methoxyphenol	$C_7H_8O_2$	124.24
Syringol		1,3-Dimethoxy-2-hydroxybenzene	$C_8H_{10}O_3$	154.16
Vanillin		4-Hydroxy-3-methoxybenzaldehyde	$C_8H_8O_3$	152.15
Catechol		Benzene-1,2-dioL (2-hydroxyphenol)	$C_6H_6O_2$	110.1
Eugenol		4-Allyl-2-methoxyphenol [2-methoxy-4-(2-propen-1-yl)phenol]	$C_{10}H_{12}O_2$	164.2
Cinnamyl alcohol		(2*E*)-3-Phenylprop-2-en-1-ol	$C_9H_{10}O$	134.17
4-Methylguaiacol		2-Methoxy-4-methylphenol	$C_8H_{10}O_2$	138.16
4-propylguaiacol		2-Methoxy-4-propylphenol	$C_{10}H_{14}O_2$	166.22
Isoeugenol		2-Methoxy-4-propenylphenol	$C_{10}H_{12}O_2$	164.2

p-Guaiacol		4-Methoxyphenol	$C_7H_8O_2$	124.14
3-Methoxy acetophenone		3-Methoxyacetophenone	$C_9H_{10}O_2$	150.17
Acetovanillone		1-(4-Hydroxy-3-methoxyphenyl)ethanone	$C_9H_{10}O_3$	166.17
Syringaldehyde		4-Hydroxy-3,5-dimethoxybenzaldehyde	$C_9H_{10}O_4$	182.17
Hydrocinnamaldehyde		3-Phenylpropanal	$C_9H_{10}O$	134.18
2,5-Dimethyl benzaldehyde		2,5-Dimethylbenzaldehyde	$C_9H_{10}O$	134.18
Vanillic acid		4-Hydroxy-3-methoxybenzoic acid	$C_8H_8O_4$	168.15
Homovanillic acid		2-(4-Hydroxy-3-methoxyphenyl)acetic acid	$C_9H_{10}O_4$	182.15
Veratrole		1,2-Dimethoxybenzene	$C_8H_{10}O_2$	138.16
Creosol		2-Methoxy-4-methylphenol	$C_8H_{10}O_2$	138.16
Veratryl alcohol		3,4-Dimethoxybenzyl alcohol	$C_9H_{12}O_3$	168.19

Divanillin		3-(5-Formyl-2-hydroxy-3-methoxyphenyl)-4-hydroxy-5-methoxybenzaldehyde	$C_{16}H_{14}O_6$	302.07
Guaiacylglycerol-β-guaiacyl ether		1-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol	$C_{17}H_{20}O_6$	320.34
(+)-Pinoresinol		4-[(3*S*,3a*R*,6*S*,6a*R*)-6-(4-Hydroxy-3-methoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-3-yl]-2-methoxyphenol	$C_{20}H_{22}O_6$	358.38
3,4-Dimethoxy acetophenone (acetoveratrone)		3,4-Dimethoxyacetophenone	$C_{10}H_{12}O_3$	180
Bicreosol		3,3'-Dimethoxy-5,5'-dimethyl-(1,1'-biphenyl])-2,2'-diol	$C_{16}H_{18}O_4$	274.31
"Ester dimer"		4-Formyl-2-methoxyphenyl benzoate	$C_{15}H_{12}O_4$	256.07
"Ether dimer"		1,2-Dimethoxy-4-[(2-methoxyphenoxy)methyl]benzene	$C_{16}H_{18}O_4$	274.12
"Alcohol dimer"		4-(1-Hydroxyethyl)-2-methoxyphenyl benzoate	$C_{16}H_{16}O_4$	272.1

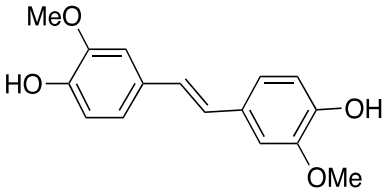
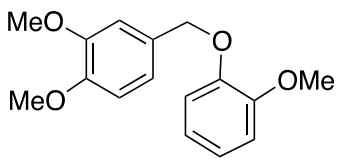
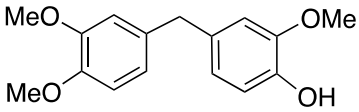
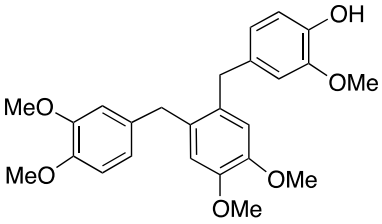
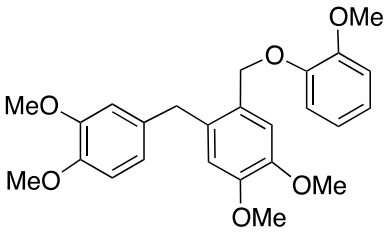
"Alkene dimer"		4,4'-(ethene-1,2-diyl)bis(2-methoxyphenol)	$C_{16}H_{16}O_5$	272.1049
1:1 mixture of the ether dimer and its isomer (TD 12)	 	1,2-Dimethoxy-4-[(2-methoxyphenoxy)methyl]benzene AND 4-(3,4-dimethoxybenzyl)-2-methoxyphenol	$C_{16}H_{18}O_4$	274.12
3:1 (TD-10-2-1) and 2:1 (TD-10-2-2) mixture of two isomeric "ether trimers"	 	4-[2-(3,4-Dimethoxybenzyl)-4,5-dimethoxybenzyl]-2-methoxyphenol AND 1-(3,4-dimethoxybenzyl)-4,5-dimethoxy-2-[(2-methoxyphenoxy)methyl]benzene	$C_{25}H_{28}O_5$	424.19

Figure 2. TCA profile of kraft lignin after sterilization in aqueous solution vs. room temperature.

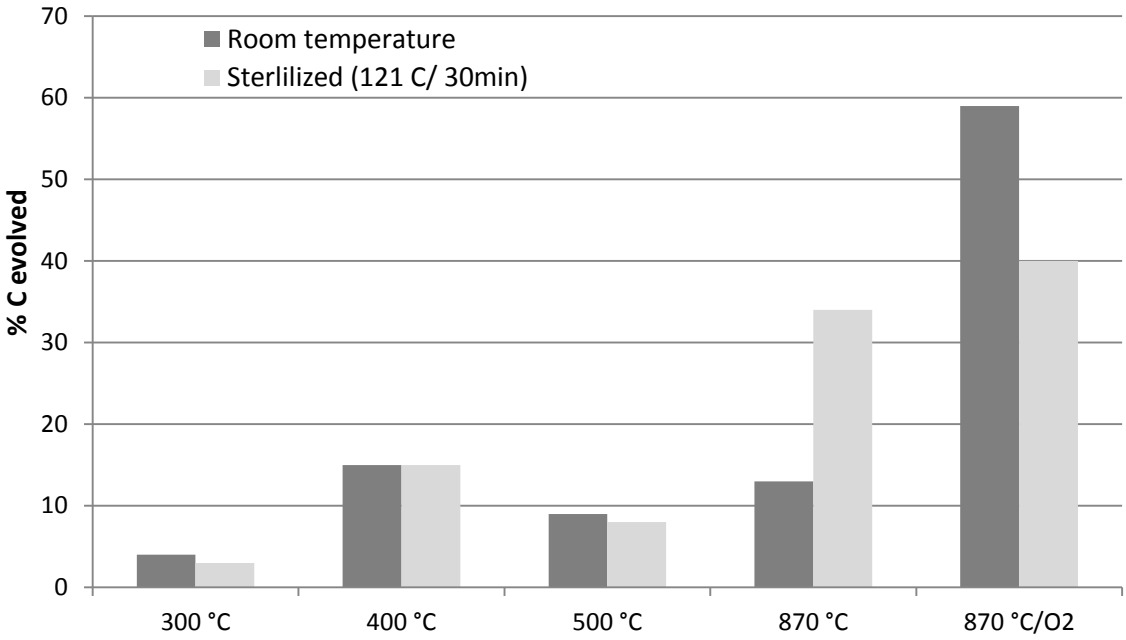


Figure 3. Color change of solubilized lignin obtained during first 12 hours with *C. versicolor* treatment.

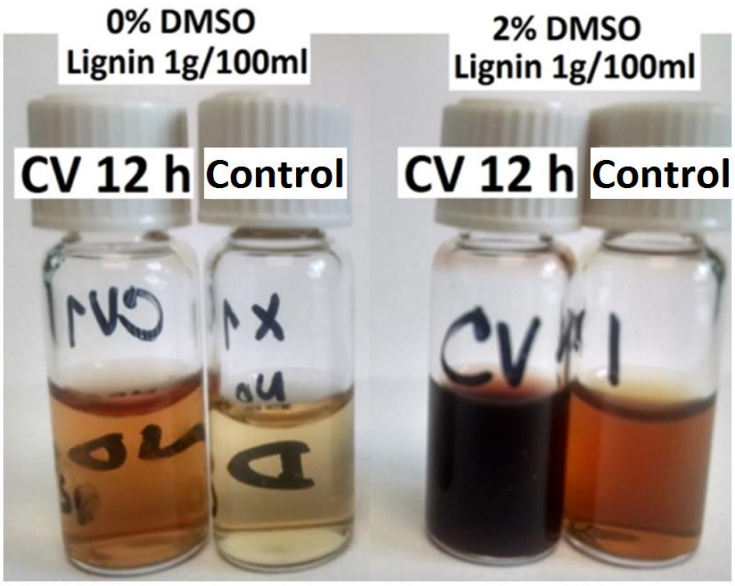


Figure 4. FTIR data of IP-H precursors

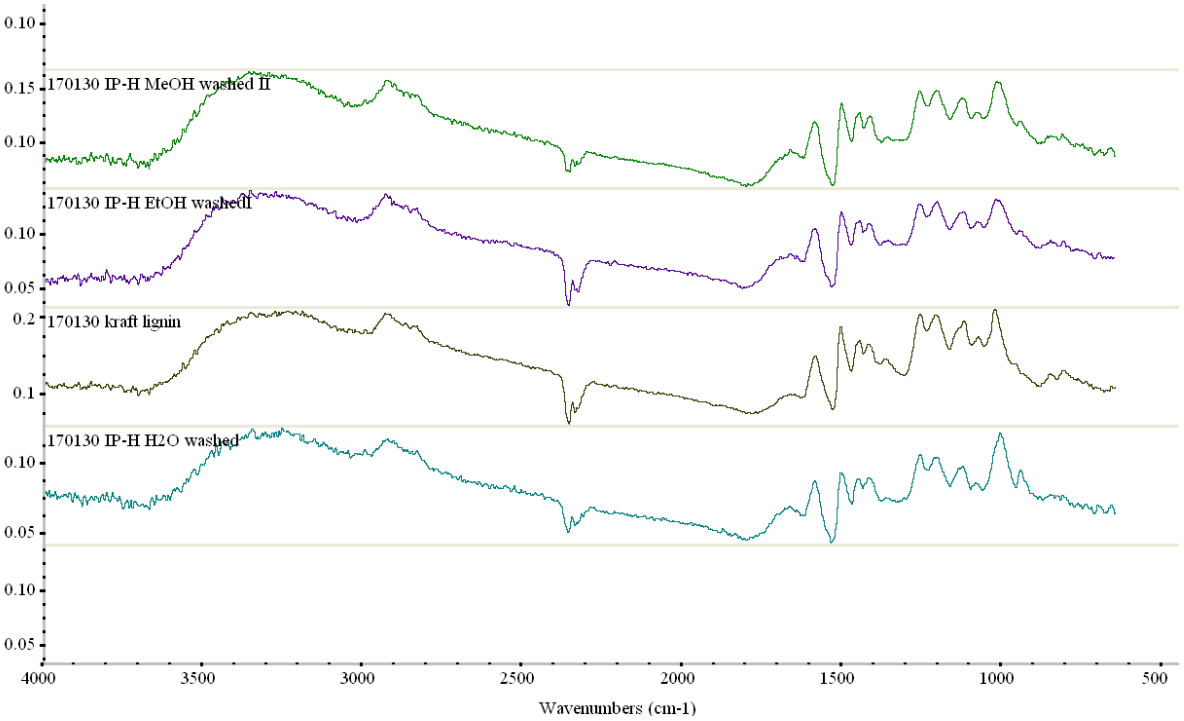


Figure 5. Solid state ^{13}C-NMR of *C. versicolor* treated and untreated kraft lignin

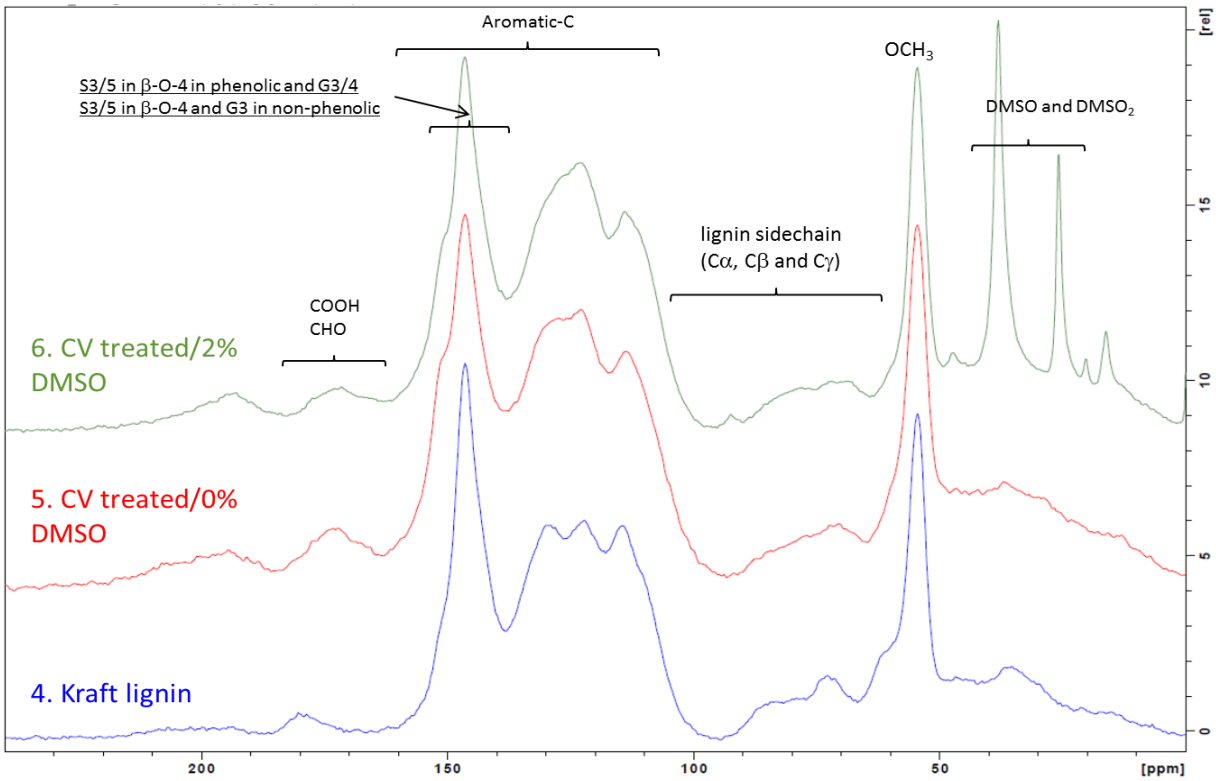


Figure 6. UV-VIS contour plots of *C. versicolor* treated lignin at day 1 and 6 with 2 and 0% DMSO

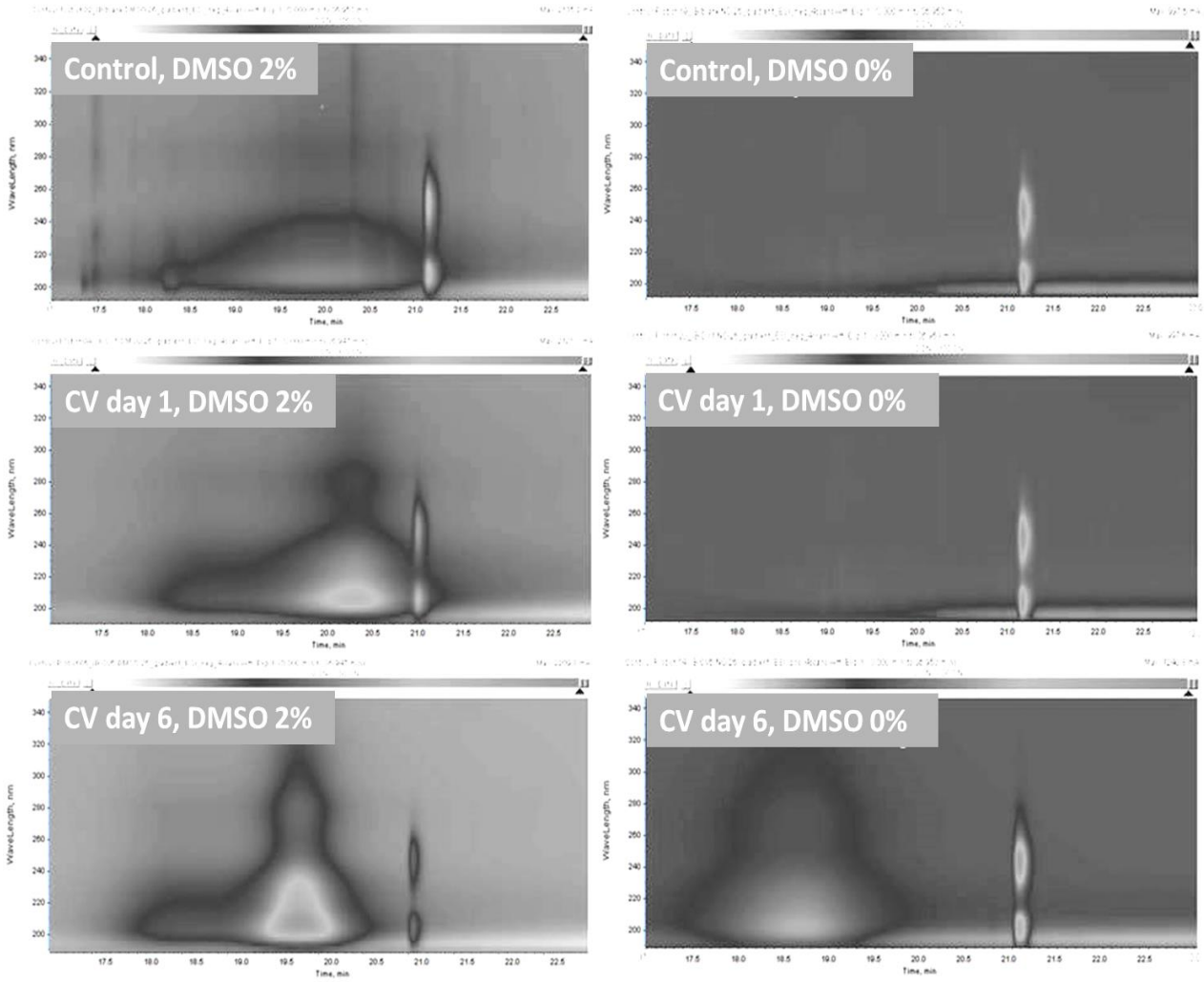


Figure 7. *C. versicolor* behavior in monomer based media *with kraft lignin*, day 1 and day 3, (at the day 6 all monomers were polymerized)

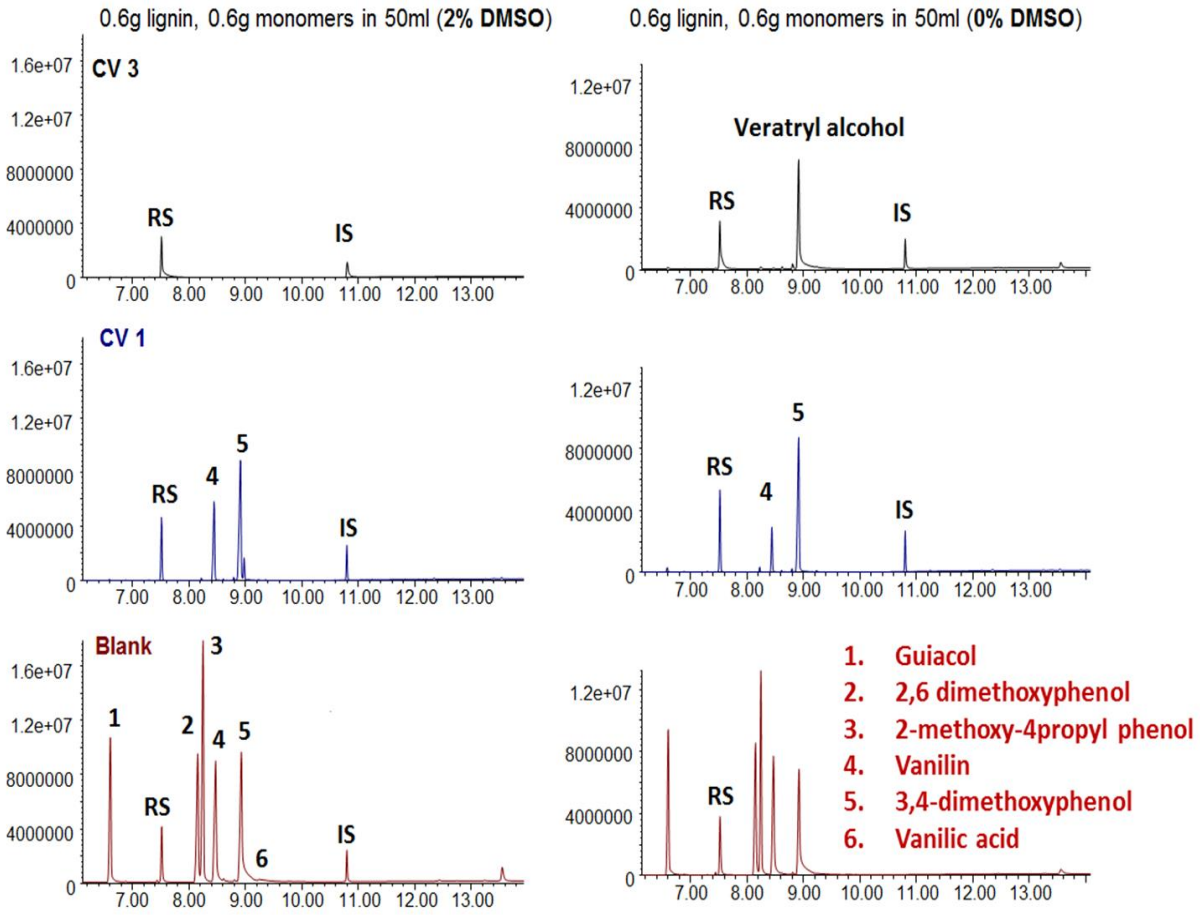


Figure 8. *C. versicolor* behavior in monomer-only based media, day 1 and day 3, at the day 6 all monomers were polymerized

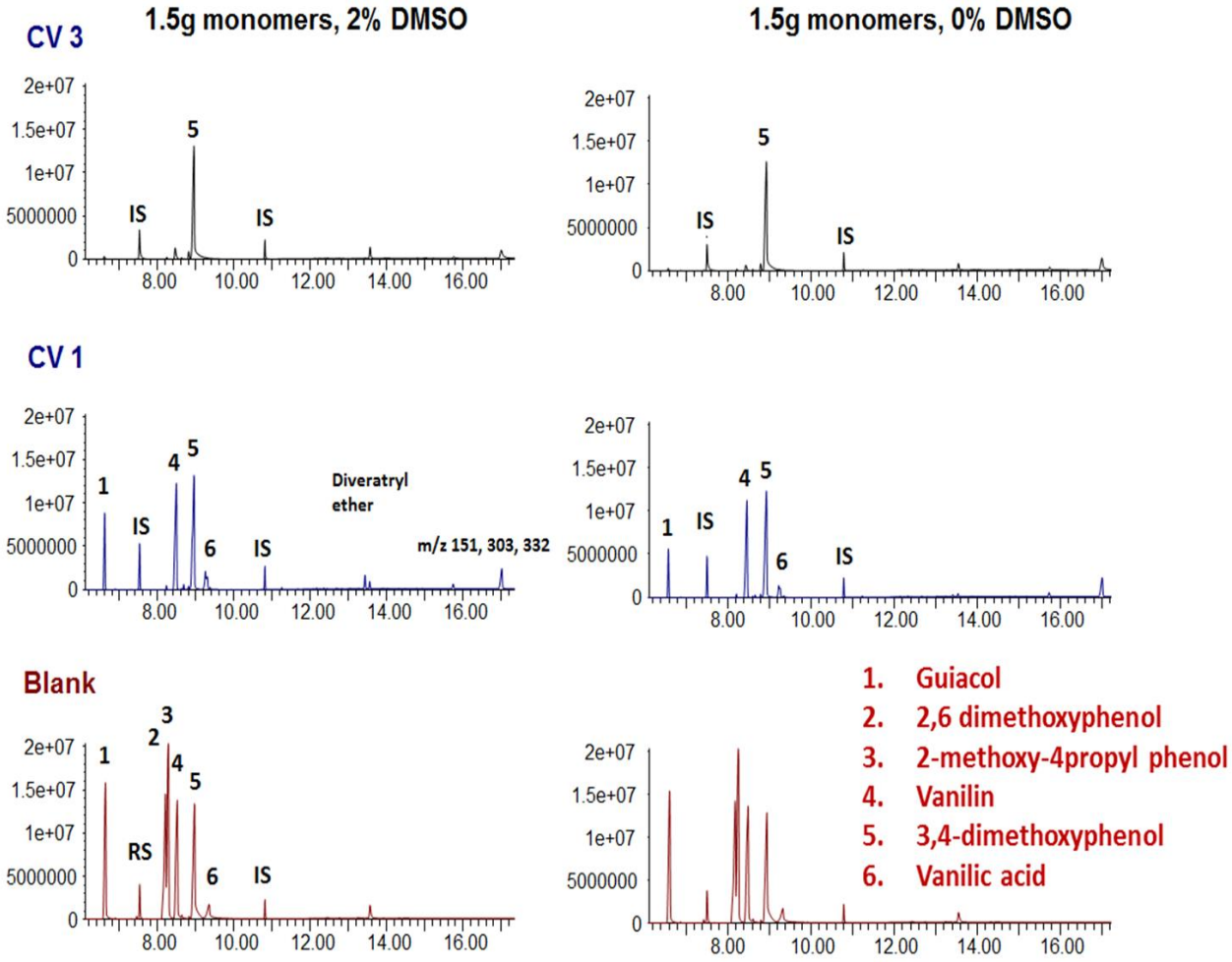


Figure 9. Proposed lignin crosslinking induced by treatment with *C. versicolor*

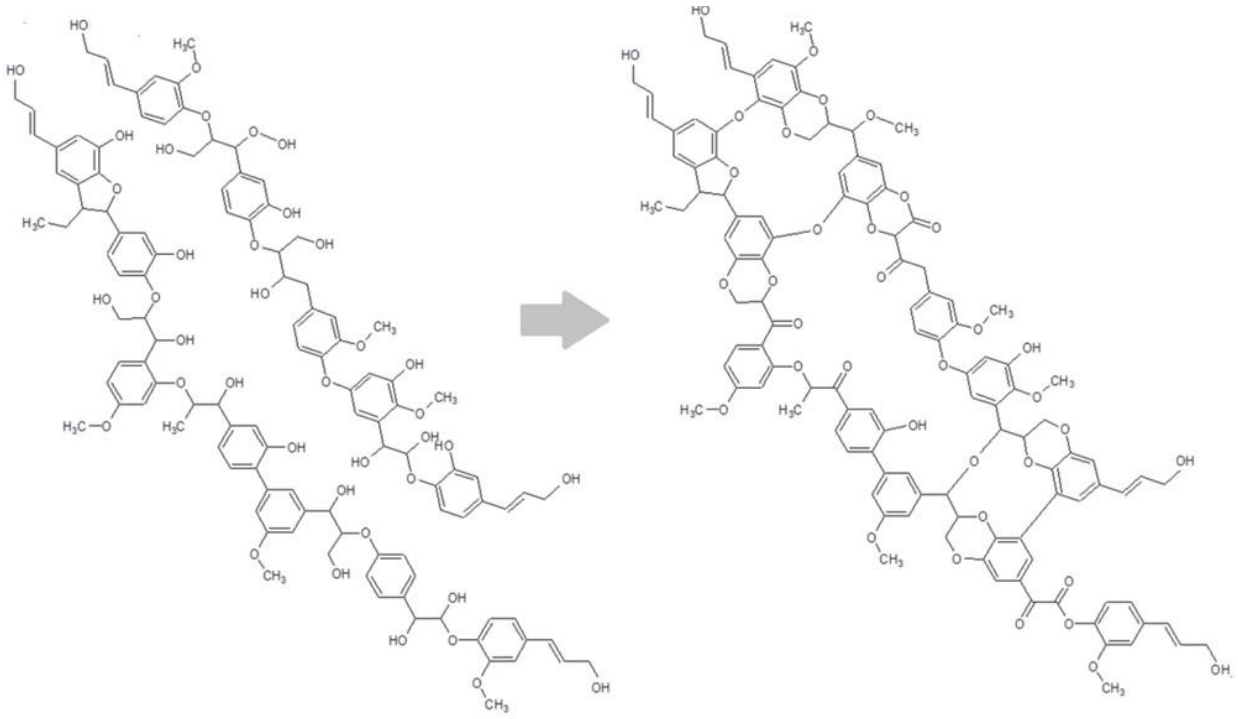


Figure 10. Differences between *C. versicolor* mutants and their ability to produce light colored IP-H precursor

C. versicolor "Short" (left) and "Long" (right) version
Lignin used: 10g/L

Conditions	Washing	IP-H precursor	Washing	IP-H precursor
Low DMSO 3% Short CV	EtOH	Light	EtOH double wash	Dark
	MeOH	Light	MeOH double wash	Dark
	H2O	Dark	H2O double wash	Dark
High DMSO 7% Short CV	EtOH	Light	EtOH double wash	Dark
	MeOH	Light	MeOH double wash	Dark
	H2O	Dark	H2O double wash	Dark
Low DMSO 3% Long CV	EtOH	Dark	EtOH double wash	Dark
	MeOH	Dark	MeOH double wash	Dark
	H2O	Dark	H2O double wash	Dark
High DMSO 7% Long CV	EtOH	Dark	EtOH double wash	Dark
	MeOH	Dark	MeOH double wash	Dark
	H2O	Dark	H2O double wash	Dark

Light IP-H precursor

Dark IP-H precursor

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