Synthesis of bio-polymers starting from deconstructed lignin

Lionel Longe
MSc

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Abstract

Lignin is a widely available biopolymer in nature, only second in volume to cellulose. It is a major component of nearly any vegetal, located in the plant cell walls. In wood, it makes up about 30% of the total dry mass. As global environmental issues arise and long term raw material feedstock for chemical and polymers production need to be secured, its exploitation becomes a major stake. At the moment, lignin is produced as a side product from pulp and paper industry and is usually burnt on site for energy production. However, lignin is the only major renewable source of aromatic structure available in nature. Therefore it represents the only potential feedstock to produce bio-based renewable aromatic materials. Above all, production of renewable aromatic and phenolic polymers depends on the development of efficient depolymerisation techniques.

Lignin is inherently recalcitrant to degradation due to its heterogeneous and crosslinked structure. Different techniques have been developed for lignin depolymerisation which often require harsh condition such as strong acid or base, high pressure and high temperature. However, degradation product under such conditions often loose most of their functional groups and thus become less promising material for monomer synthesis. Pyrolysis methods have been developed to collect volatile compounds from lignin by thermal degradation, but isolated products lack potential for innovative functionalisation. In nature, some microorganisms can degrade lignin such as certain bacteria found in herbivore animals or insect guts, or some wood fungi. Those organisms have possess efficient ligninolytics enzymes to break down lignin structure. Enzymatic degradation of lignin would represent a greener alternative to current depolymerisation processes to yield degradation products with more functional groups should be expected.

In the first part of the presented work, we study the enzymatic depolymerisation of lignin to produce small aromatic molecules. Different enzymes are considered along with different substrates to optimise degradation. Horseradish peroxidase, Lignin peroxidase, Manganese peroxidase and laccase were selected for their ligninolytic behaviour. Furthermore, solvent and additional small chemicals were found to influence the overall efficiency of the depolymerisation. Laccase was one of the best ligninolytic enzyme, and its efficiency was further improved by the use of small phenolic compounds called mediators. Good overall depolymerisation was obtained but production of small monomers could not be reached. Therefore, oligomers repolymerisation will be part of the second section of the thesis in addition to phenolic monomers.

The second half of the work is complementary with the first section. Repolymerisation of lignin degradation products have been studied in literature. In particular vanillin have been extensively considered as building block for monomer synthesis. However some small phenolic compound have been much less studied and have great potential for polymerisation. Different strategies were considered. Radical polymerisation is a robust method for quick polymer synthesis. Furthermore, the resulting polystyrene-like structure is expected to yield polymers promising with properties. Several monomers were tested to create different linear polymers. Polycondensation of protocatechuic acid allows the synthesis of a new hyperbranched polyester. Such polymer synthesis proved to be challenging and involved several side reactions that yielded improvement of the thermal stability. Finally, a reversible polymerisation of lignin oligomers was designed to finish linking both thesis section. By demethoxylation reaction, some catechol group were added into the oligomers structure. In presence of metallic ion and in basic conditions, weak bonding were created between the oligomers through catechol-iron complexe formation. This method was used to create lignin thin film on solid substrates and will be investigated further in the future.

Overall this project yielded improvement of the procedure for enzymatic depolymerisation of lignin compared to literature. Furthermore, several renewable polymers with different properties have been successfully synthesized from known lignin degradation product that can be used in different field of industry.

Declaration

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Signature: ……

Print Name: …Lionel Longe…….

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Publications

1- Lionel Longe, Gil Garnier. Kei Saito, Lignin Biodegradation with Fungi, Bacteria and Enzymes for Producing Chemi-cals and Increasing Process Efficiency, In Production of Biofuels and Chemicals from Lignin. Springer, Singapore, 2016. 147-179
2- Lionel F. Longe, Julien Couvreur, Mathilde Leriche Grandchamp, Gil Garnier, Florent Allais and Kei Saito, Importance of Mediators for Lignin Degradation by Fungal Laccase,:ACS *Sustainable Chemistry and Engineering*, 2018, **6**, 8, 10097-10107
3- Lionel Longe, Gil Garnier. Kei Saito, Linear bio-based aromatic polymers from syringic acid, S type degradation fragment from lignin (submitted to Green Chemistry)
4- Lionel Longe, Gil Garnier. Kei Saito, Synthesis of lignin-based phenol terminated hyperbranched polymer (to be submitted to ChemChom)

Conferences Presentations

1- 2018 ACS National Meeting & Expo New Orleans, Nexus of Food, Energy & Water Oral talk and poster presentation. SciMix presentation
2- 2017 EPTS 2017 (Emerging Polymer Technologies Summit 2017) Poster presentation
3- 2017 Appita 2017 Fibre Value Chain Conference & Expo Oral talk
4- 2017 RACI Centenary Congress Oral talk and poster presentation
5- 2016 The sixth Asia-Oceania Conference on Sustainable and Green Chemistry (AOC-SGC6) Poster presentation
6- 2015 Appita 2015 Fibre Value Chain Conference & Expo Oral talk

Thesis including published works declaration

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This thesis includes (2) original paper published in peer reviewed journal and (1) submitted publications, in addition to (1) paper format drafts to be submitted. The core theme of the thesis is (Lignin biodegradation and repolymerisation). The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the school of chemistry under the supervision of Dr. Kei Saito and Prof. Gil Garnier from Chemical Engineering.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of *four experimental chapters* my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
1	*Lignin Biodegradation with Fungi, Bacteria and Enzymes for Producing Chemi-cals and Increasing Process Efficiency*	*Published*	*80%. Labwork, data collection and redaction*	*1) Dr Kei Saito, key idea and manuscript editing 10%* *2) Prof. Gil Garnier, key idea and manuscript editing 10%*	*Y*
2	Importance of mediators for lignin degradation by fungal enzymes	*Published*	*55%. Labwork, data collection and redaction*	*1) Dr Kei Saito, key idea and manuscript editing 10%* *2) Prof. Gil Garnier, key idea and manuscript editing 10%* *3) Prof Florent Allais, key idea and manuscript editing 10%* *4) Julien Couvreur, labwork 10%* *5) Mathilde Leriche Grandchamp, key ideas 5%*	*N*
3	Linear bio-based aromatic polymers from syringic acid,	*Submitted*	*80%. Labwork, data collection and redaction*	*1) Dr Kei Saito, key idea and*	*Y*

	S type degradation fragment from lignin			*manuscript editing 10%* *2) Prof. Gil Garnier, key idea and manuscript editing 10%*	
4	Synthesis of lignin-based phenol terminated hyperbranched polymer	*Not submitted*	*80%. Labwork, data collection and redaction*	*1) Dr Kei Saito, key idea and manuscript editing 10%* *2) Prof. Gil Garnier, key idea and manuscript editing 10%*	*Y*

I have / have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: **Date:** 31/12/18

Acknowledgements

The following research is the result of a 3 years and a half journey that saw me evolve from a naive, idealistic master graduate to a more mature, equally idealistic young professional. The voyage was demanding, sometimes stressful and emotionally straining but often exhilarating and always thrilling.

*This thesis subject is rooted in a will to be an active part of the growing effort to create today solutions to tackle tomorrow's environmental challenges in industry *insert I'm-doing-my-part meme*. Before graduating my master/engineering degree in polymer chemistry I applied and joined a research team in Bordeaux focussing on synthesis of bio based polymers and Green chemistry in general. During those 5 month I gradually discovered, understood and finally completely adhered to this type of chemistry and the general philosophy. I want to believe that my project is now a tiny gear in a growing machine that we can rely on, in the future, for a more sustainable world.*

I would like to thanks everyone who supported me during this time, obviously first my parents, my sister and the rest of my family, as they always supported me anytime I needed it even though I was literally halfway across the world. I also would like to deeply thank both my supervisor, Prof. Kei Saito and Prof. Gil Garnier, as they provided a constant scientific and moral support when needed. I also thank my academic panel that oversight my project at each milestones and gave me thorough feedback: Prof. Toni Patti and Prof. Victoria Harritos. As a PhD student, you might be alone on your project, but you are never alone in the lab. I thank all of my research group for the everyday support. Weather it was for lending you a spatula, organising birthday cakes, helping you chase down free food events or just for grabbing a beer at the end of the week, you could always count on Kei's group and Co. Special thanks to our favourite post-doc Sepa N for keeping all of us more or less in line and avoiding several catastrophe. Special thanks to my fume-hood-mate Vu Dao for dealing with my less than ideal sense of tidiness and weird way to keep things "ordered". Special thanks to Jinhuo Dai for helping me from day 1 by helping me with the paperwork while I was just a mess with jet-lag still clouding my brain, and fora sharing my struggle with lignin during the rest of my project. Special thanks to Ahmed for teaching me some Arabic and in return learning French slang. Special thanks to Mustafa for being the nicest person I ever met, you seriously restored my faith in the human race. Special thanks to to Gavin for sharing my enthusiasm for ethyl hydroxyl aqueous solutions, even though sometimes you "have to be in bed by midnight". Special thanks to Parijat for being my green chartreuse buddy: I promise, it's an acquired taste. Special thanks to Tim, I'll take you to the bus station anytime. Thanks also to Yannalah, Temma, Hussain, Ruchi, Sinuo, Poornima and Abodi, good luck with your PhD if you are not done yet, and thanks for everything. Finally I would like to also thank everyone that got involve in my PhD at any time: Anna, Sarah and Janett for helping me deal with paperwork, Peter and Roger for NMR analysis, Scott in APPI for his thorough help in facilitating my interaction with the infamous chemical engineering OHSE department, and any PhD student from BAMI or other research group, post-doc, technicians or PI.

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Chapter 1

Introduction

1.1 Literature review:

With an estimate annual growth of $6x10^{10}$ tons[1], wood is the biggest renewable resource non-competitive with food stream. As two of its main components – namely cellulose and hemicellulose- already have clear and established market, lignin has yet to find commercial application, other than being burnt for energy. Lignin structure is nevertheless a gold mine, that contains the only renewable and sustainable source of aromatic and phenolic compounds. At the moment, the vast majority of high volume commercial material based on aromatic structure is produced from fossil reserves. Oil supplies said to be running out, fluctuant prices and ecological considerations make lignin break down a critical stake for future chemical industry. Isolation of small phenolic compounds would enable synthesis of a wide variety of biobased materials and polymers. However lignin depolymerisation is hindered by its crosslinked and highly heterogenous structure. In nature, bioorganisms use combination of ligninolytic enzymes to efficiently degrade lignin to monomeric compounds. If this biological degradation can be transferred to lab or industry scale, cheap and abundant source of small phenolic compound will allow for the synthesis of a wide range of monomers and bio-renewable polymers. Below is a review of the biodegradation of lignin by fungi, bacteria and enzymes in nature. It was published as a book chapter in “Production of Biofuels and Chemicals from Lignin” edited by Zhen Fang and Richard L. Smith and published by Springer.

Chapter 6
Lignin Biodegradation with Fungi, Bacteria and Enzymes for Producing Chemicals and Increasing Process Efficiency

Lionel Longe, Gil Garnier, and Kei Saito

List of Abbreviation

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
Acetyl COA	Acetyl coenzyme A
Aff.	"affinis", Latin form of "related"
CTMP	Chemithermomechanical Pulping
DPM	Diphenyl Methane bond
EC	Enzyme Commission number
GFC	Gel Filtration Chromatography
1-HBT	1-Hydroxybenzotriazole
Lac	Laccase
LC-MS	Liquid Chromatograph-Mass Spectrometry
LiP	Lignin Peroxidase
mcl-PHA	Medium Chain Length Polyhydroxyalkanoate
MnP	Manganese Peroxidase
NMR	Nuclear Magnetic Resonance
Sp.	Species (sing.)
Spp.	Species (plur.)

L. Longe
School of Chemistry, Bioresource Processing Research Institute of Australia (BioPRIA), Monash University, Clayton, VIC 3800, Australia
e-mail: lionel.longe@monash.edu

G. Garnier (✉)
Department of Chemical Engineering, Bioresource Processing Research Institute of Australia (BioPRIA), Monash University, Clayton, VIC 3800, Australia
e-mail: gil.garnier@monash.edu

K. Saito (✉)
School of Chemistry, Monash University, Clayton, VIC 3800, Australia
e-mail: kei.saito@monash.edu

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TCA cycle	Tricarboxylic Acid cycle
TEMPO	2,2,6,6-Tetramethyl-1-piperidinyloxy
VLA	Violuric acid
VP	Versatile peroxidase

6.1 Introduction

The word "Lignin" comes from Latin, "lignum", that can be literally translated as tree, timber or even firewood. Evolving from this, the word "lignin" was specifically used in 1813 by A.P. de Candolle, a Swiss botanist, to denote the product obtained after wood treatment with solvents and mild acid. Twenty-five years later, Anselme Payen reported on two products in wood: cellulose, and what would be later called "lignin" [1]. During the following decades, scientists investigated this peculiar product to identify its structure. The NMR studies [2] of Ludwig [3] and Nimz [4] have led to the commonly admitted representation of the lignin structure: a 3D polymer network, highly cross-linked, and resulting from the co-polymerisation of three different phenol derivative monomers (Fig. 6.1a, b). The reaction of these three monomers leads to the creation of a wide variety of linkages, among which six common bonds: the β-O-4, 5–5, β-5, 4-O-5, β-1 and β-β (Fig. 6.1b and Table 6.1). This large range of bonds and the high cross-linking density render lignin extremely recalcitrant to degradation [5]. It is this high degree of bonding and chemical heterogeneity that has prevented science from upgrading lignin into fine chemicals or bio-based polymers on industrial scale. As a result, 98 % of the lignin world production at present is currently simply burned for energy [6].

Numerous studies have described approaches to depolymerise lignin. Many approaches apply pretreatment and rely on chemical methods to depolymerise lignin. However, results are often difficult to compare as a consequence of the complicated 3D structure of lignin and the lack of standard analytical methods. In nature, organisms naturally degrade wood by different catabolic pathways involving enzymes.

The present chapter primarily focuses on how these organisms have been investigated and utilised to break down lignin into valuable chemicals and by-products. This review first analyses fungi as catalyst to synthesise fine chemicals from lignin. Second, the application of bacteria and the mechanism by which they operate is studied; last, the role of enzymes is analysed. It is the objective of this chapter to review the biodegradation approaches for selectively breaking down and converting lignin into fine chemicals, to provide a perspective on the promising strategies and to map the current scientific frontiers.

6.2 Fungal Degradation

When considering decaying wood, the first organisms to come to mind are fungi. Wood degrading fungi are mostly divided into three broad categories: white-rot, brown-rot and soft-rot fungi [10] (Table 6.2). Biomass degrading fungi described in

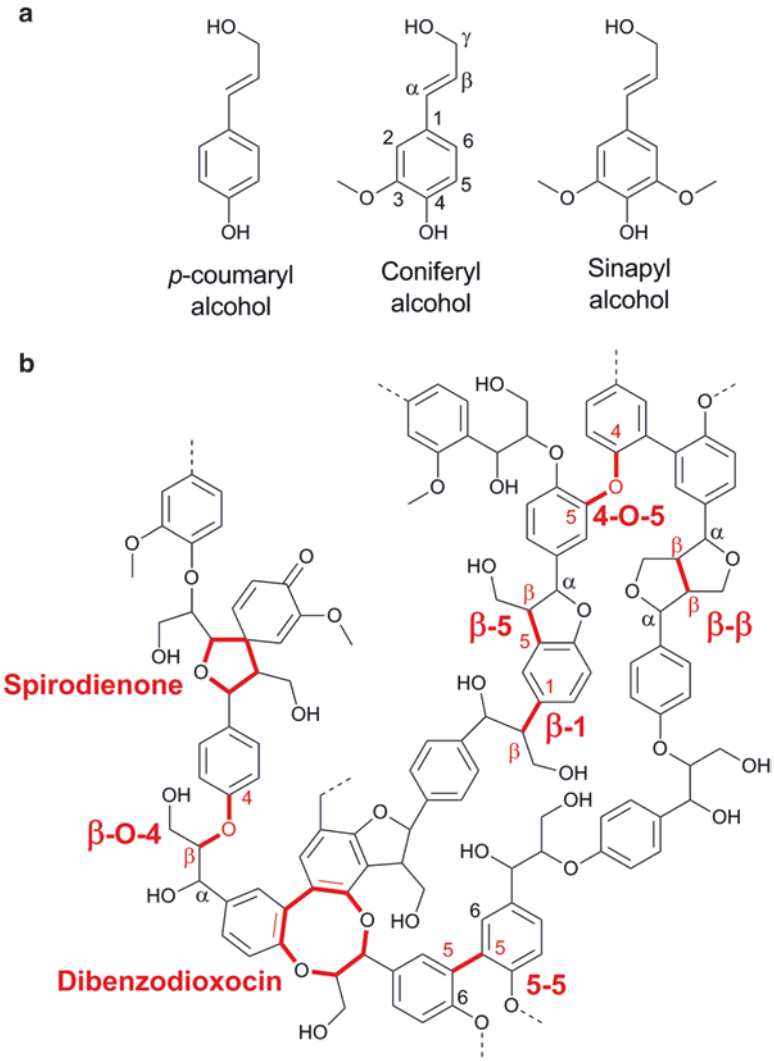


Fig. 6.1 (**a**) Three constitutive monomers of lignin: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (**b**) Structure illustrating the principal bonds in lignin, a three dimensional highly cross-linked biopolymer

the literature are mostly from two phyla (subdivision in biological classification): Basidiomycota and Ascomycota, both from the subkingdom of fungi Dikarya [11]. Although, other phyla are sometimes considered, these two are the most commonly reported for biomass degradation. In the literature, basidiomycetous white-rot fungi are the most studied, since they can selectively degrade lignin, leaving the cellulose and hemicellulose fraction rather intact. However, lignin degradation can also result from the action of other strains and even marine-derived fungi [12]. The term

Table 6.1 Repartition of the different bonds found in lignin from two different woods. *Spruce*, a softwood, has more 5-positions available, enabling more 5–5 and β-5 linkages, than *Eucalyptus grandis*, a hardwood [1]

Abundance per 100 C9-units	*Spruce* [7, 8]	*Eucalyptus grandis* [9]
β-O-4	45–50	61
5-5	19–22	3
β-5	9–12	3
4-O-5	4–7	9
β-1	7–9	2
β-β	2–4	3
Spirodienone	ND	5
Dibenzodioxocin	ND	ND

ND not determined

Table 6.2 Different categories of wood degrading fungi and their action on lignin [10]

Fungi (Subdivision)	Main examples	Action	Preferred substrate
White-rot fungi (Basidiomycota)	*Phanerochaete, Pleurotus, Trametes, Phlebia spp.*	Active degradation of lignin and possibly cellulose	Hardwood
Soft-rot fungi (Ascomycota)	*Chaetomium, Ceratocystis, Kretzschmaria deusta spp.*	Lignin modification	Hardwood
			Softwood
Brown-rot fungi (Basidiomycota)	*Serpula lacrymans, Piptoporus betulinus, Gloeophyllum trabeum, Postia Placenta, Fomitopsis cajanderi*	Slight lignin modification	Softwood

white-rot fungi results from the colour of the degraded wood; as lignin is being digested by the organism, the only remains are the white cellulose and hemicellulose. This selectivity is particularly important for applications that upgrade cellulose, such as pulp or bioethanol production.

White-rot fungi represent a large group of species, each featuring a different mechanism to attack the lignin barrier directly with a cocktail of enzymes [13, 14]. Fungi produces various enzymes, some to degrade lignocellulose, and others with different roles but with essential role for the fungi to prosper on complex biomass. It has been shown that inhibition of the production of these enzymes can highly hinder or even prevent any growth of the fungi on ligneous and cellulosic mediums [15–17].

For the last 50 years, lignin degrading fungi have been studied from a microbiological perspective, focussing on the mechanisms of lignin degradation and identifying the enzymes involved. Three different types of fungi application have emerged [18]: (a) conversion of lignocellulosic biomass into animal feed and food [19–21] (b) pretreatment agent for delignification (c) biodegradation agent for some phenolic contaminants or various wastes. Transformation of woody biomass into edible mushrooms falls beyond the scope of this study. This review focuses on the two latter applications.

It is worthwhile to clarify some of the nomenclature that can be confusing. There is a clear distinction between delignification, which is the actual removal of lignin from biomass, and lignin degradation/depolymerisation, which represents the cleavage of C-C bonds or ether bonds within the lignin structure, yielding lignin oligomers [22]. Delignification, characterised, for example, by a smaller kappa number,[1] is often due to increased solubility of lignin, either by modification or by radical grafting [24, 25]. Consequently, it is possible that lignin degradation also contributes to increased solubility through decreased molecular weight; however delignification does not imply lignin depolymerisation. Both delignification and lignin bio-degradation are reviewed in this chapter. These two phenomena are critical for unlocking economically feasible bio-refineries capable of producing a full range of marketable chemicals and materials.

6.2.1 Delignification

The delignification process has two main domains of application: pulp and paper industry, and biofuel production. The pulp and paper industry converts wood into fibres for paper. There are two main pulping processes: mechanical and chemical pulping. In mechanical pulping, lignocellulosic fibres are separated from the wood structure by applying stress. An example is Thermo-Mechanical Pulping (TMP) which relies on steam to plasticise and heat wood above the glass transition temperature (T_g) of lignin while applying defibrillating shear, thus reducing fibre damage and energy consumption. Chemical pulping relies on chemical agents and heat to dissolve the ligning-rich fraction of the lumen lamella binding fibres. There are two main chemical processes: Kraft pulping (alkaline) and sulphite pulping (acidic). Kraft pulping is the most important pulping process producing 70 % of all pulp. Pulping aims at developing good quality fibres; full delignification is not always required as it decreases pulp yield. Full fibre delignification or whitening requires bleaching, increasing cost and environmental impact [27–31]. White-rot fungi, with their mild reaction condition, have been investigated as low energy and environment-friendly treatment to reduce lignin content or to replace an existing step of the chemical/thermomechanical pretreatment; this process is referred to as biopulping. In biofuel production processes, sugars from diverse lignocellulosic resources are fermented by microorganisms to yield targeted products. Hence, the first step of biomass processing is its conversion into simple sugars (glucose, xylose), usually by an enzymatic pathway. Although this process is easy for starch (glucose units linked by α-1,4 and α-1,6 glycosidic bonds) compounds, it is much more complicated for cellulose due to its regular structure (glucose unit linked by β-1,4 glycosidic bonds) which makes it highly crystalline and compact leading to a high

[1] Kappa number is a titration process representative of the pulp colour and approximatively proportional to lignin content at low concentration [23]; it can be used to assess delignification efficiency.

resistance to biological degradation [26]. Moreover, the intertwining between cellulose, hemicellulose and lignin results in recalcitrance which hinders the action of the microorganisms [5]. Fungi represent a promising pretreatment agent to reduce this intertwining considering its mild reaction conditions [27, 28]. White-rot strains are of special consideration because of their low uptake of cellulose sugars.

It is estimated that enzymes account (sing.) for 4.5 % of the cost for biofuel production from corn starch and up to 20 % when the whole plant is involved [29–31]. Reviews note that mechanical and chemical pretreatment of biomass for biofuel production can become costly [5, 32–37]. Therefore pretreatment is a critical step for the economics of a process. Different approaches with fungi have been considered to promote delignification by pretreatment combinations.

Single Fungus Treatment Physical and chemical pretreatment of biomass are notorious for the harsh reaction conditions required. On the contrary, biological pretreatment involves much milder conditions, representing a perfect substitute or complementary degrading agent for delignification [38]. Ge and co-workers have reported on the use of *Ceriporiopsis subvermispora*, a white-rot fungus, as treatment for *Albizia moluccana* (Albizia), a widespread invasive tree species in tropical and subtropical regions [39]. The white-rot fungus efficiently decreased the lignin content by 24 % while degrading cellulose and hemicellulose only by half this value. As a consequence, pretreatment with *Ceriporiopsis subvermispora* allowed a jump in cumulative methane yields from 33.9 L/kg of volatile solid for raw albizia to 123.9 L/kg for pretreated wood chips.

For biofuel applications, fungi could serve not only as delignification agents, but also as production vectors for various products. Indeed, some fungi can degrade lignin along with cellulose which can be useful for production of sugars (Table 6.2) White-rot fungus *Phlebia* sp. were shown by Kamei et al. to exhibit such combined behaviour for the direct production of bioethanol from cellulosic materials [40]. The same group later reported on the direct conversion of lignocellulosic biomass to bioethanol using this same fungus [41], then proving *Phlebia* sp. MG-60 to have the combined abilities of lignin degradation, cellulose saccharification, and ethanol fermentation.

Sugar cane bagasse was treated to improve bioethanol yield. Biomass and process variables such as moisture content, additives and presence of metallic cations were shown to greatly affect delignification efficacy [42]. Growth medium addition improved both delignification and ethanol production. Addition of Fe^{2+}, Mn^{2+} or Cu^{2+} slightly decreased delignification but bioethanol production was improved by reducing bagasse carbohydrate degradation.

Following the same strategy, Xie et al. reported on the use of an oleaginous fungus strain as lignin degrading microorganism [43]. *Cunninghamella echinulate FR3* was determined to be able to degrade cell wall lignin as efficiently as most Basidomycetes fungi. Two strains of sorghum, the wild-type and reduced-lignin-content type (genetically modified to feature improved saccharification efficiency), were submitted to enzymatic hydrolysis followed by fungal biodegradation. Lignin

loss reached 31 % for the wild-type sorghum, while toping up to 46 % for the mutant strain. However, up to 35 % wt of cellulose was degraded during the process. This oleaginous fungus was not targeted as pretreatment to increase sugar production, but was used for lipid bioaccumulation, potentially leading to a new way to provide feedstock for biodiesel refineries [43].

Fungus as Co-treatment Even though the current chemical and thermomechanical pulping pretreatments are efficient, an additional bio-treatment stage could further increase delignification yield, decrease energy consumption, increase selectivity or fibre quality while decreasing environmental impact. With those benefits in mind, Baker et al. reported on the synergistic effect combining pressure refining, a common process for the pulp and paper industry, with three different white-rot fungi [44]. *Ceriporiopsis subvermispora*, *Phlebiopsis gigantea* and *Phlebia radiata* were fed with pressure refined Miscanthus wood chips. In this strategy, pressure refining concentrates lignin in pellets on the surface of the cellulose fibres [45]. This configuration was tested to increase delignification efficiency by improving the lignin accessibility to the degrading enzymes. After 28 days, the three strains reached their white-rot fungi expectation by decreasing the relative amount of lignin in Miscanthus medium by 10–20 %. However, it is the *Ceriporiopsis subvermispora* strain that exhibited the best lignin degrading properties by decreasing lignin by 70–75 % of the original content. Un-pressure refined Miscanthus could only reach 10 % of lignin content reduction, highlighting the cooperative action of physical and biological pretreatments.

Kamei et al. reported on the decrease of lignin content and increase in ethanol production when *Phlebia* sp. MG-60 was fed with alkaline-pretreated sugarcane bagasse compared to untreated sugarcane bagasse [46]. The final results depended on the initial alkaline concentration. After 10 days, the total production of ethanol was 210 mg/g of treated bagasse, providing an ethanol yield (production of ethanol compared to theoretical maximum) of 66 % for the bagasse pretreated with 0.8 % NaOH. Untreated bagasse only reached ca. 2 % of ethanol yield these demonstrating the synergistic effect of the two pretreatments.

Fungi Co-culture Fungi co-cultures have been investigated to improve lignocellulosic ethanol production efficiency. The cumulative effects of pretreating biomass with two fungi can either improve delignification in the case of two lignolytic fungi [47], or significantly improve the release of reducing sugars by incubating white-rot and brown-rot fungi together [48]. Co-culture of white-rot *Ceriporiopsis subvermispora* and brown-rot *Postia placenta* fungi on *Liriodendron tulipifera* wood chips was reported by Parrow et al. [49]. Although each species increased reducing sugar production during the post saccharification process compared to sterile conditions, rising from 75 to 250 mg/g, the co-culture showed no benefits. Evaluation of interspecific growth interactions showed that an “inhibition” zone was created at the intersection of each taxon growth domain. The two fungi can coexist in the same medium, but did not feature interspecific stimulatory (or inhibitory) interactions. Better understanding of how different species can cooperate in the same biomass is required to improve delignification and saccharification efficiency.

Ma and Ruan reported that *Coprinus comatus* (producing lignolytic enzymes) and *Trichoderma reesei* (producing hemi and cellulolytic enzymes) have synergistic interaction on corn stover [50]. No inhibition was observed at the intersection of the two growth domains, even with intertwining. The co-culture improved delignification by 10 % compared with the lignolytic fungus alone, giving a maximum of 66.5 % delignification after 72 h at optimum temperature conditions. Weight loss, glucan and xylan degradation were also synergistically improved by the co-cultivation of the two strains. A total reducing sugar yield of 82 % was achieved.

Table 6.3 summarises the current critical studies on fungal delignification. Comparison of results remains a challenge because of the different methods used to measure delignification; methods used range from lignin content measurement to IR spectroscopy [51] and even include wettability testing [52].

6.2.2 Waste Treatment

Lignin degrading fungi have been investigated for their waste degrading properties. Indeed, many fungi have phenol degrading properties suitable to treat a wide range of phenolic pollutants [69, 70]. This field is investigated not only for the treatment of oil -or paper-mill waste water for lignin removal, but also for the textile industry waste water which has a high content of toxic aromatic dyes [71, 72]. Martin and Manzanares reported on the delignification of straw alkaline-pulping liquors by *Trametes versicolor* [73]. The white-rot fungus was able to remove 75 % of the lignin in the effluent, bringing it from 2 g/L to 0.5 g/L. An important simultaneous decolourisation of the effluent resulted from the delignification.

6.2.3 Chemical Production

Some studies have considered fungi for lignin chemical modification to change its properties and also to produce chemicals from lignin derivatives. Falconnier et al. reported on the production of vanillin from ferulic acid by white-rot fungus *Pycnoporus cinnabarinus* I-937 [74]. Vanillin concentration up to 64 mg/L was achieved for a molar yield of 27.5 % w/w. However, recovering vanillin from this mixture proved to be challenging as the fungus produces laccase that repolymerises ferulic acid into a lignin-like polymer.

Work from Zou and co-workers detail lignin demethylation by two fungal strains, *Cylindrocladium* sp. and *Aspergillus* sp. [75]. Demethylation of lignin is of high interest for functionalising lignin by liberating reactive groups. The new hydroxyl groups can serve for grafting new functionalities, dangling chains, or even for resins [76, 77]. After 3 weeks in culture, 40 % of the methoxy groups were removed from lignin without causing significant degradation (10 % decrease in content). This work

Table 6.3 Overview of lignin degradation process by fungi and their performance

Measurement method	Fungi	Substrate	Delignification amount	Ref.
Klason lignin weight loss: TAPPI [53], NREL [54] or Kirk and Obst [55]	*Arthrinium. phaeospermum* and *Phanerochaete chrysosporium*	*Miscanthus* leaves and sugarcane	11 % and 15 %	[31]
	Coprinus comatus and *Trichoderma reesi* (synergistic interaction)	Corn stover	66.5 %	[50]
	Ceriporiopsis subvermispora	*Albizia moluccana*	24 %	[39]
	Ceriporiopsis subvermispora	Pressure refined *Miscanthus sacchariflorus* and *giganteus*	25–30 %	[44]
	Coriolopsis caperata RCK 2011, *Ganoderma* sp. rckk-02 and *Pleurotus florida*	Sugarcane bagasse	5.5 %, 5.6 % and 7.9 %	[56]
	Cunninghamella echinulate FR3	Wild-type and mutant lines of *Sorghum bicolor*	46 % and 31 %	[43]
	Dichomytus squalens, Formitopsis pinicola, Ganoderma lucidum, Lenzites betulinus, Pleurotus ostreatus, Pleurotus eryngii, Trametes versicolor	Wheat straw	34 %, 32 %, 20 %, 28 %, 7 %, 14 % and 21 %	[57]
	Phlebia sp.	Oak wood	40.7 %	[41]
	Phlebia sp.	Sugarcane bagasse	44 %	[42]
	Pleurotus ostreatus	Sugarcane bagasse	34.8 % (compared to 19.6 % for CTMP)	[58]
	Trametes multicolor and *Trametes pubescens*	Wheat straw and oak sawdust	6 % and 50 %	[59]
Lignin weight loss, Van Soest method [60]	*Phanerochaete flavido-alba*	Wood fibre, corn stover and wheat straw	20 %	[61]
	Phanerochaete chrysosporium	Corn stover and corn stover silage	60 % and 20 %	[62]

(continued)

Table 6.3 (continued)

Measurement method	Fungi	Substrate	Delignification amount	Ref.
Kappa number (TAPPI [63])	*Pleurotus ostreatus*	Sugarcane bagasse	70 % (dat)	[58]
	Trametes versicolor	Oil palm trunk chips	35 % (dat)	[64]
Acetyl bromide soluble lignin loss by quantitative spectrophotometry [65]	*Trichoderma viride* and surfactant	Rice straw	74 %	[66]
Reducing sugars released from enzymatic degradation of cellulose (saccharification efficacy)	*Ceriporiopsis subvermispora* and *Postia placenta*	*Liriodendron tulipifera* wood chips	330 % (isr)	[49]
	Ceriporiopsis subvermispora	Wheat straw	60 % (acs)	[67]
	Coriolopsis caperata RCK 2011, *Ganoderma* sp. rckk-02 and *Pleurotus. Florida*	Sugarcane bagasse	150–240 % (isr)	[56]
	Pleurotus ostreatus and *Pleurotus pulmonarius*	*Eucalyptus grandis* sawdust	17 % and 15 % (acs) (3 % for blank)	[38]
	Myrothecium roridum	Rice straw	37 % better than chemical pretreatment (dilute acid)	[68]
Ethanol released during fermentation of free sugars (ethanol yield)	*Phlebia* sp.	Sugarcane bagasse	39 % (mey)	[42]
		Oak wood	43.9 % (mey)	[41]
		Alkaline-pretreated sugarcane bagasse	66 % (mey)	[46]

CTMP Chemithermomechanical Pulping, *dat* decrease after treatment, *isr* increase in sugar release, *acs* available cellulose saccharified, *mey* maximum ethanol yield

represents a promising start for using fungi as biological modifiers in industrial applications.

6.2.4 Perspectives

Even though engineering fungi is in its infancy, the current trend to engineer fungi as reactant or catalyst to convert lignin into valued products is very promising. Fungus can be genetically modified as has been proven in the past [15–17, 78], but there are presently only a small number of applications compared with those for bacteria.

White-rot fungi in general, and the *Pleurotus ostreatus* strain in particular, are of interest for their high delignification rate. However, most of the previous studies have been limited either to basic pretreatment process for lignocellulosic biomass or to waste biodegradation. Using fungi as individual species, co-culture or even combined with chemical reactions has tremendous potential, including the synthesis of fine chemicals and functionalisation of lignin into value added polymers. A clear understanding of the fungi reaction mechanisms is needed, along with their kinetics and the adoption of standard lignin analytical (for yield and content) methods for enabling unbiased comparisons among processes and studies.

6.3 Bacterial Degradation

In contrast to fungal lignin degradation, enzymology of bacterial lignin breakdown is currently not well understood [79]; extracellular peroxidase and laccase enzymes also appear to be involved. These bacteria can be found in soils, where decaying wood is present, but also in the digestive systems of herbivores, like cows rumens, or in xylophage insects guts, like termites [80]. These bacteria belong to three phyla, actinomycetes, α-proteobacteria and γ-proteobacteria [79]. One review reports on some lignin-degrading prokaryotes in the following phyla: firmicutes, β-proteobacteria, δ-proteobacteria, bacteroidetes and archaea [81].

As a lignin degrading organism, bacteria have been studied for delignification or bioremediation, just as fungi, but to a smaller extent, which is probably because bacterial enzymes have shown to have a lower redox potential [82, 83]. However bacteria have some interesting properties over those of fungi: they are stable over a wide range of pH [84], they can feed on lignin as the sole source of carbon and energy [85], and they are easy to genetically modify [86].

6.3.1 Delignification

Although considered less effective than fungi, lignin degrading bacteria are also able to utilise lignocellulosic biomass. They are used in delignification processes, either for biopulping or for the biofuel production to increase accessibility of cellulose, but also for bioremediation and waste management. Hacq et al. reported that

Serratia liquefaciens could detoxify pulp and paper mill effluent by removing contaminants and lignin by up to 58 % [87]. Agricultural residues can also create environmental pollution, and as a lignin degrading organism, bacteria have received wide interest for treatment [88, 89].

Bacteria delignification research has focused onto Kraft pulp applications because of its high annual production [90]. Shi and co-workers showed that different bacteria strains could successfully degrade Kraft lignin with good yields [91–93]. *Cupriavidus basilensis* B-8 and *Pandaroea* sp. B6, two protobacteria, expressed high lignolytic enzymes (manganese peroxidase and laccase) activity when reacted with Kraft lignin. After 7 days in optimum concentration and pH, a total lignin removal of ca. 45 % was achieved. By degrading Kraft lignin with no other source of carbon required, these two bacteria strains showed good potential for industrial delignification.

Priyadarshinee et al. reported improvement of eucalyptus Kraft pulping by bacterial treatment, resulting in a decrease in kappa number [23]. The raw eucalyptus Kraft pulp was inoculated with two different bacteria strains, *Pseudomonas fluorescens* NITDPY and *Planococcus* sp.TRC1. The two bacteria decreased the kappa number by 32 and 37 % in 7 days, respectively. The total amount of phenolic compounds was also reduced. The two different strains react differently to their new carbon source, with *P. fluorescens* NITDPY degrading lignin faster in the early stage of the experiment through phenolic compound release. Both strains released reducing sugar, but only in a small amount: 0.32 mg/g and 0.15 mg/g for *Planococcus* sp.TRC1 and *P. fluorescens* NITDPY, respectively. This indicates that cellulose is not much affected much by the bacteria which is important for pulp and paper applications. The *Pseudomonas fluorescens* NITDPY and *Planococcus* sp.TRC1 bacteria show good industrial potential, more than some fungi and they provide non-negligible cellulose degradation. Example of lignin degrading bacteria studied for delignification can be found in Table 6.4.

6.3.2 Chemical Production

Bacteria are considered to be merely as effective as fungi for delignification; however, they are much easier to genetically modify than fungi [86]. The ability of bacteria to accumulate some of the lignin degradation products as carbon source or energy is an interesting property for chemical production [98]. In the last few years, applications for lignin degrading bacteria have changed. Initially, microorganisms were mostly investigated to degrade and remove lignin from biomass to facilitate recovery of other compounds. Now some research groups are reporting their use for lignin valorisation. While procedures can be complex, the principles are simple. By genetically modifying some bacteria strains, disruption in the metabolic pathway is created, leading to the accumulation of a compound of interest in the medium.

Sainsbury and co-workers reported on the conversion of lignocellulose to vanillin by deleting one enzyme coding gene in *Rhodococcus jostii* RHA1 [99]. *R. jostii*

Table 6.4 Overview of lignin degradation process by bacteria and their performance

Measurement method	Bacteria	Substrate	Delignification amount	Ref.
Klason lignin weight loss: TAPPI [53]	*Pandoraea* sp. *ISTKB*	Sugarcane bagasse	10.4 % after 20 days	[94]
Lignin weight loss	*Cupriavidus basilensis* B-8	Kraft lignin	44.4 %	[92]
	Pandoraea sp. B-6		45.5 %	[91]
Lignin loss by quantitative GFC	*Bacillus pumilus* and *Bacillus atrophaeus*		50 % and 70 % for high molecular lignin	[95]
Lignin weight loss, Van Soest [60]	*Escherichia coli* from beef cattle rumen	Maize stover	36.8 % after 4 days	[96]
Measurement of lignin loss by quantitative UV spectroscopy	*Bacillus* sp. extracted from soil	Alkali lignin	40 % after 24 h, 80 % after 48 h	[86]
Kappa number (TAPPI method [63])	*Cryptococcus albidus*	Bagasse	22 % smaller than control, still 4.5 % smaller when further Kraft pulping is applied	[97]
	Planococcus sp. *TRC1* and *Pseudomonas fluorescens NITDPY*	Raw eucalyptus Kraft pulp	37 % and 32 % decrease	[23]

GFC Gel Filtration Chromatography

degrades lignin derivatives using a biological funnelling process, creating a few intermediates from multiple substrates. The genetic material coding the mechanism responsible for the degradation of vanillin and vanillic acid intermediates were removed from the bacterium DNA. The resulting suppression of *vanillin dehydrogenase* production causes vanillin to accumulate along with by-products in the reaction medium (Fig. 6.2). After 144 h in a medium containing 2.5 % wheat straw lignocellulose and 0.05 % glucose, the genetically modified bacterium accumulated vanillin up to 96 mg/L. An important amount of ferulic acid could also be observed after 168 h of reaction. When the substrate was changed to Kraft lignin, which is a major industrial by-product of the pulp and paper, an amount of 13 mg/L of vanillin could still be obtained, indicating, however, that lignocellulose remains a better substrate than Kraft lignin. With a similar objective, Graf et al. reported the identification of the gene coding *vanillin dehydrogenase* in *Bacillus subtilis* 3NA [100], proving the versatility of this technique.

Linger et al. reported on the biological funnelling behaviour of a bacteria, *Pseudomonas putida* KT2440. This organism converts a heterogeneous substrate, such as lignin derivatives, into a sole product, in this case a medium chain length polyhydroxyacid (mcl-PHA). The process was tested on alkaline pretreated liquor (APL), a highly concentrated depolymerised lignin mixture: 32 % lignin made mostly of monomers, dimers and trimers. The result was an accumulation of mcl-

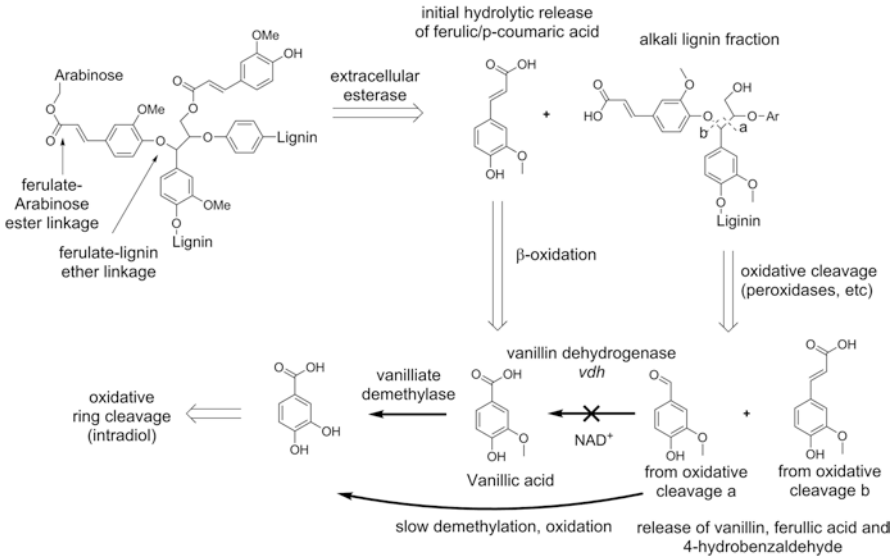


Fig. 6.2 Catabolic pathways of *R. jostii* RHA1 used for lignin depolymerisation. Italicised names are gene coding the elementary reaction involved. Vanillin accumulation is due to the aldehyde dehydrogenase gene *vdh* deletion (Adapted with permission from Sainsbury et al. [99]. Copyright 2013 American Chemical Society. NAD: Nicotinamide adenine dinucleotide)

PHA into *P. putida* at high concentration (0.252 g/L at 32 % cell dry weight). Mcl-PHA has a wide range of possible use, from depolymerisation to alkenoic acids, via the thermal pathway, to alkane productions; these reactions were reported in the article [101].

Vardon et al. highlighted an innovative pathway to produce fine chemicals from lignin [102]. Through genetic modification, the natural degradation pathway of bacterium *Pseudomonas putida* KT2440 was reshaped to yield the production of muconate. *P. putida* was engineered to transform lignin derived aromatics into catechol and to prevent muconate degradation (Fig. 6.3). Basically, the *pcaHG* gene that encodes the degradation of protocatechuate is replaced by *aroY*, a gene from another bacterium, *Enterobacter cloacae*, which allows decarboxylation instead. The genomic portion that promotes degradation of muconate is then deleted and the transformation of phenol to catechol is allowed through the addition of genomic material *dmpKLMNOP* from *Pseudomonas* sp. *CF600*. The overall production of muconate was reported to reach 0.70 g/L after 24 h [102].

Similarly, Johnson and Beckham reported on the genetic modification of *P. putida* to create pyruvate from lignin derivatives [103]. This was achieved by removing the undesired endogenous reactions in *P. putida*, the ortho (intradiol) degradation pathway of catechol and protocatechuate and replacing it with the meta (extradiol) cleavage, from another bacterium, *Sphingobium* sp., to increase the production of pyruvate which is ultimately converted into L-lactate. To avoid the pyruvate from undergoing side reactions, the gene encoding pyruvate dehydrogenase

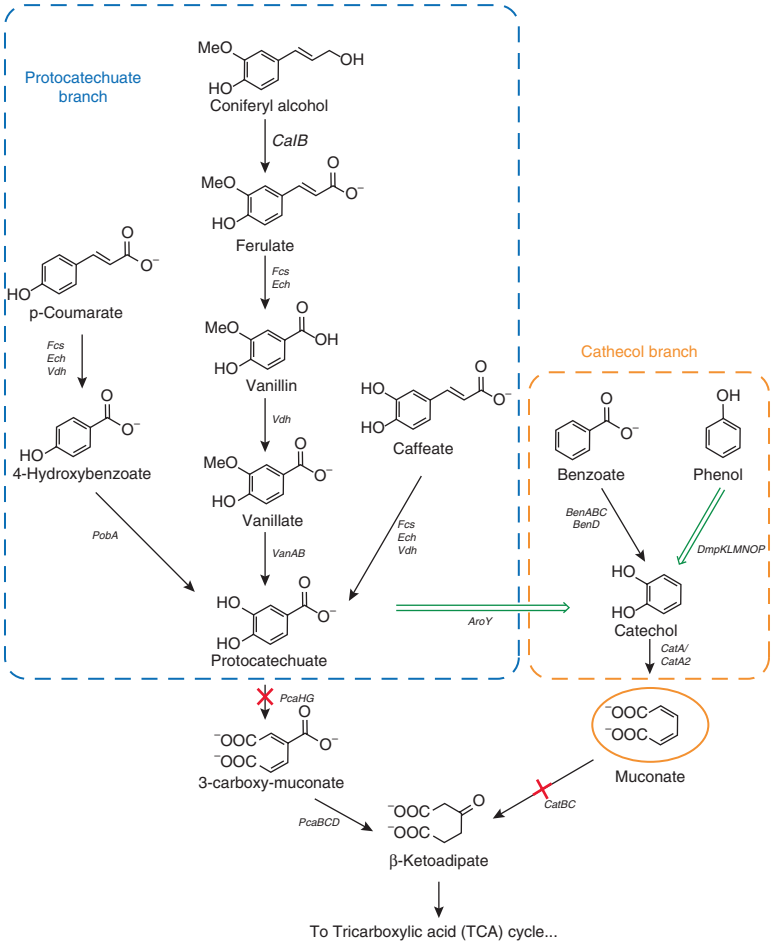


Fig. 6.3 Protocatechuate and catechol branch of the β-ketoadipate pathway in *P. putida* KT2440 disrupted by deletion of the genes encoding *PcaHG* and *CatBC* (*crossed arrow*). Italicised names are genes coding the elementary reaction involved. Insertion of genes encoding *AroY* and *DmpKLMNOP* (*double line arrow*) yielded muconate accumulation (Adapted from Vardon et al. 2015 [102] with permission of The Royal Society of Chemistry)

was deleted, preventing any reaction into acetyl-CoA. Moreover, the addition of an external genetic material coding bovine lactate dehydrogenase into *Pseudomonas putida* was part of a strategy to provide greater competition for pyruvate that might otherwise react within the TCA cycle. However, the efficiency of this method was only tested with lignin model compounds such as benzoate or *p*–coumarate. No experiments have been performed with lignin.

6.3.3 Perspectives

Delignification by bacteria is still very poorly understood and less studied than fungal degradation. An attractive strategy currently being explored is to rely on microbial consortia consisting of mixtures of bacteria and fungi to synergistically further lignin biodegradation [104, 105]. Another promising avenue to explore is the bacteria's bio-funnelling behaviour for chemical production. Through advances in genetic engineering, the synthesis of valuable products from lignin is now achievable; reactions rates, control of competitive reaction and selectivity will determine the economics.

6.4 Enzymatic Degradation

Both fungi and bacteria involve complex mechanisms of enzymes and intermediates to degrade lignin [13]. However, there is a major drawback for their deployment toward bio-refineries. The control over the inner process is poor and often non-existent, making it difficult to recover the intermediate species of interest. Using enzymes directly and individually in a controlled way is an attractive alternative to deconstructing the degradation process into elementary reactions, allowing a better understanding of the depolymerisation mechanisms and control, which can improve the isolation of the selected chemicals [106].

Enzymes are macro-proteins which can be described as biological catalysts of high selectivity. Each enzyme accepts a very defined range of substrates. Hence, to degrade the different lignin bonds, organisms require multiple types of enzymes classified into two families: peroxidases and laccases. Peroxidases represent a large group of enzymes involving hydrogen peroxide as the electron acceptor for the specific oxidative reaction they catalyse, such as lignin peroxidases (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP, EC 1.11.1.16), and horseradish peroxidase (HRP, EC 1.11.1.7). Peroxidases belong to the super family of heme-dependent peroxidases. Laccases represents the other major enzyme family for lignin degradation. (Lac, EC 1.10.3.2) Laccase forms its own group by itself belonging to the family of multidomain cupredoxin, and the superfamily of cupredoxins. Laccases use oxygen as electron acceptor. That process avoids any deactivation by hydrogen peroxide that sometimes happens with peroxide enzymes.

Lignolytic enzymes have complex mechanisms. Their origin, structure of substrate (type of lignin) and exterior condition play a major role in their actions. Laccase native from plants, for example, tend to polymerise lignin while fungal or bacterial laccase rather catalyse lignin degradation [107]. Laccases by themselves tend to polymerise low phenolic lignin [108–111] while depolymerisation is favoured with the presence of mediator molecules or with high phenolic ratio lignin [110–114]. Versatile peroxidase from *Pleurotus eryngii* have been demonstrated to polymerise low molecular weight compounds by inducing cross-linking [115].

Lignolytic enzymes can be produced by bacteria or fungi in a stimulating medium, then extracted, purified and concentrated. The optimisation of this costly

production influences industrial viability of enzyme processes. It has been well investigated along with improving enzymes performance (temperature stability, pH sensitivity, etc.) [116–121].

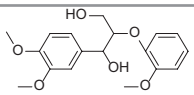
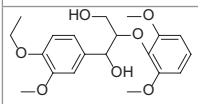
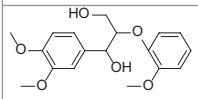
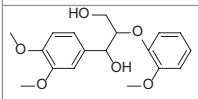
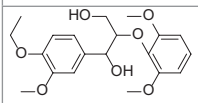
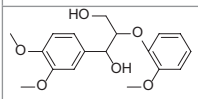
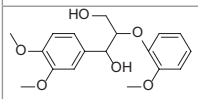
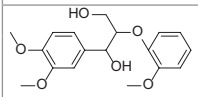
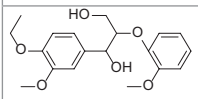
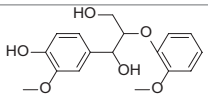
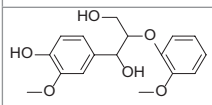
Enzymes can be considered for similar application as those of fungi or bacteria, such as delignification or waste treatment [122–127], but they also have been widely used to better understand the mechanism of lignin degradation by organisms. A series of model lignin molecules, each representing a specific bond in lignin (Fig. 6.1) have been investigated [128, 129].

6.4.1 Laccases

Laccase represents one of the most reported lignolytic enzymes in the literature [22, 130, 131]. For a long time, laccase was ignored for lignin degradation for two reasons: (i) *Phanerochaete chrysosporium*, considered a model lignin degrading organism was thought to be unable to produce laccase (ii) its low redox potential (0.5–0.8 V versus normal hydrogen electrode [6]) only allows it to oxidise a small portion of lignin components (the phenolic parts). The role of laccase in lignin degradation is now well demonstrated [17, 22, 128, 132, 133]. The laccase reaction happens around four different copper ions explaining its blue colour [6]. The indirect mechanism of degradation was highlighted in 1990 by Bourbonnais and Paice [132] which who showed the importance of small intermediate molecules, called mediators. Laccase uses molecular oxygen to oxidise the mediator, which then acts as chemical oxidant for lignin. This process allows enzymes to overcome their steric limitation that otherwise eliminates bulky molecules, such as lignin, as potential substrates. These intermediates allow laccases to overcome their phenolic-substrate restriction, thus expanding the range of potential oxidation of the enzyme. Laccase mediators are usually small phenolic compounds such as lignin phenolic components, vanillin, syringaldehyde, veratryl alcohol, or even synthetic mediators, 1-hydroxybenzotriazole (1-HBT), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS), 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), violuric acid (VLA) [134]. The choice of mediator can influence the oxidative potential of laccase and also induce stereo-preference in the substrate. Bohlin et al. showed laccase to exhibit different behaviours with a mixture of diastereoisomers of a β-O-4 model bond molecule [135]. The oxidation is maximum with HBT and shows no preferential isomer, while it is slightly lower with ABTS which exhibits the threo diastereoisomer to be less reactive than the erythro isomer.

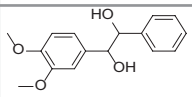
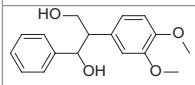
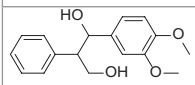
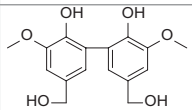
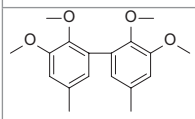
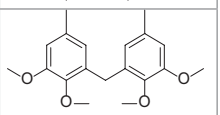
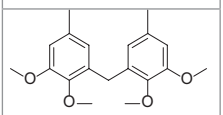
The source of laccase, the mediator and the substrate significantly influence the products and reaction rate. Heap et al. reported on the reaction between laccase from *Trametes versicolor* (white-rot fungus) and various model compounds of β-O-4 bonds with and without mediator [136]. For phenolics, dimerisation can happen with or without the mediator 1-HBT, while Cα oxidation (oxidation of the hydroxyl group on the α position to ketone, see Fig. 6.1b) proceeded with the two non-phenolic compounds (Table 6.5). Bond degradation can happen through ring cleavage for the most electron-rich aromatic ring dimer due to the addition of a

Table 6.5 Lignin model bond degradation conversion by enzymes

Bond	Molecule	Enzymes and conditions	Results	Ref.
Non phenolic β-O-4	 diastereomer erythro and threo	Laccase with 1-HBT, TEMPO, ABTS and VLA as mediators	HBT 55 % (o)	[135]
			TEMPO 41 % (o)	
			VLA 51 % (o)	
			ABTS 43 % (o) preferentially erythro	
		Laccase 1-HBT as mediator	Aromatic ring (c), β-ether (c), Cα-Cβ (c), Cα (o)	[129]
		Laccase 1-HBT as mediator	50 % (o).	[169]
		Laccase with or without 1-HBT as mediator	Cα (o) with 1-HBT	[136]
			Cα (o) and (c) with 1-HBT	
		Lignin peroxidase H_2O_2	27 % (o).	[143]
		Versatile peroxidase H_2O_2 and Mn^{2+}	19 % (c) & (o)	[148]
		Versatile peroxidase	3 % Cα (o)	[170]
		Manganese peroxidase	4.5 % Cα (o)	
		Manganese *meso*-tetra(*N*-methylpyridino) porphyrin pentaacetate	81 % (c). & (o).	[160]
Phenolic β-O-4		Laccase with or without 1-HBT as mediator	(d) w/and w/o 1-HBT	[136]
		Versatile peroxidase H_2O_2 and Mn^{2+}	65 % (c) & (o)	[148]

(continued)

Table 6.5 (continued)

Bond	Molecule	Enzymes and conditions	Results	Ref.
β-1		Laccase ABTS as mediator	60 % (o)	[169]
		Lignin peroxidase H_2O_2	46 % (c)	[141]
			39 % (c)	
5-5		Manganese *meso*-tetra(*N*-methylpyridino) porphyrin pentaacetate	93 % (c) & (o)	[160]
			73 % (c) & (o)	[165]
DPM			48 % (c) & (o)	[160]
			15 % (c) & (o)	[165]

ABTS 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), *1-HBT* 1-Hydroxybenzotriazole, *TEMPO* 2,2,6,6-Tetramethyl-1-piperidinyloxy, *VLA* Violuric Acid, *DPM* Diphenyl Methane bond
(c): cleavage, (d): dimerisation, (o): oxidation

methoxy group; for both these non-phenolic molecules, the suppression of mediator prevents any reaction and yields unreacted dimers. The same couple laccase-HBT improved the saccharification of wheat straw. Combining 150 U/g of laccase with 5 % w/w 1-HBT as mediator increased the dilute acid pretreated wheat straw saccharification process by increasing the glucose concentration by 35 % (6.6–8.9 g/L) [136].

The degradation of the β-O-4 bonds were extensively studied as it is the most prevalent lignin bond. Kawai et al. reviewed the mechanism of a non-phenolic β-O-4 model bond, 1,3-dihydroxy-2-(2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)propane, to identify the resulting products [129, 137, 138]. After characterisation of the products, multiple reactions were identified. From 1000 nmol of initial β-O-4 model bond molecule, 120 nmol of products from Cα-Cβ cleavage (cleavage of the bond between carbon α and carbon β, see Fig. 6.1) were collected, along with 99 nmol from β-ether cleavage, 64 nmol from aromatic ring degradation and 43 nmol from Cα oxidation highlighting the complexity of enzymatic lignin degradation.

6.4.2 Peroxidases

Peroxidases represent the second main category of lignolytic enzymes. Lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and horseradish peroxidase (HRP) belong to this group, with a respective redox potential of 1.2 V, 0.8 V, 1.4 V and 0.95 V versus standard hydrogen electrode [6]. Their reaction mechanism are extensively described in two reviews [6, 139].

The high redox potential of lignin peroxidase allows it to oxidise both the phenolic and non-phenolic moieties of lignin. Its heme ion is a ferric one contained inside a ferric protoporphyrin [6]. In the past, lignin peroxidase was shown able to cleave the C-C bond in synthetic lignin[2] [140]. Lim and co-workers presented the C-C cleavage in different β-1 model bonds [141]. The different model bonds had various substituent groups on the two aromatic rings, and showed different reactivity towards lignin peroxidase, with a conversion ranging from 46 % for the less substituted dimer to 14 % for the dimer with two methoxy groups on each ring. A slight stereo-preference was also highlighted with erythro dimers preferred for degradation. Product analysis suggests a single electron transfer on one of the aromatic rings, followed by a Cα-Cβ cleavage.

Lignin peroxidase can degrade ether model bonds [142]. Lim et al. studied the action of lignin peroxidase of β-O-4 dimers and tetramers with different amounts of methoxy substituent on aromatic rings [143]. Similar to the β-1 bond models, the less substituted model underwent faster bond cleavage. The mechanism combines single electron transfer from dimer or trimer followed by bond cleavage. These two studies highlight different lignin degradation for the two different structures of lignin.

Manganese peroxidase is one of the most common lignolytic enzymes in organisms. It uses hydrogen peroxide just as LiP. Although it can function without [144], MnP requires manganese ions Mn^{2+} in solution to be fully efficient. In acidic medium, Mn(II) is oxidised by hydrogen peroxide, creating free Mn^{3+} ions which diffuse and oxidise the lignin phenolic moieties (Eq. 6.1). MnP structure is very similar to LiP, both having ferric ions in their heme group [6].

$$2\mathrm{Mn}(\mathrm{II}) + 2\mathrm{H}^{+} + \mathrm{H_2O_2} \rightleftarrows 2\mathrm{Mn}(\mathrm{III}) + 2\mathrm{H_2O} \tag{6.1}$$

After reporting biobleaching abilities of *Bjerkandera* sp. strain BOS55 with a high concentration of MnP, Moreira et al. isolated this enzyme and tested it for delignification properties [145]. After 6 h, and under optimal conditions of pH, H_2O_2 and Mn^{2+}, a maximum reduction of kappa number of 13 % was obtained for eucalyptus

[2] Synthetic polymer is a polymer with structure similar to lignin. It can vary from simple polyphenol to more elaborate structure.

unbleached Kraft pulp. This work highlights the potential application of manganese peroxidase as a bio-bleaching additive.

Versatile peroxidase (VP) is another lignolytic enzyme that can be found in some lignin degrading fungi such as *Pleurotus eryngii* or *Bjerkandera* spp. [146]. Its high redox potential (E^0>+1.4 V versus standard hydrogen electrode) allows it to accept a wide range of potential substrate compared to other lignolytic enzymes [6]. VP can degrade β-O-4 model bonds and synthetic lignin [147, 148]. Fernández-Fueyo and co-workers successfully isolated versatile peroxidase from *Pleurotus ostreatus* [147]. It was shown that this fungus lacked any lignin peroxidase activity; VP assumed this role instead. A phenolic β-O-4 model bond was exposed to VP and both Cα-Cβ cleavage and Cα oxidation occurred. A few products from Cβ-O-C_4 were detected. A significant depolymerisation was measured by gel permeation chromatography when the same enzyme was fed to synthetic lignin (dehydrogenation polymer).

Horseradish peroxidase (HRP) is considered to be part of the phenoloxidase responsible for lignin degradation [13]. However, it is also an important lignin and aromatic compounds polymerisation promoter [149, 150]. For that reason, HRP is usually considered as a polymerisation biocatalyst or as a lignin modifier agent [151, 152]. Xia et al. reported horseradish peroxidase to depolymerise two synthetic highly phenolic lignin-based polymers (lignophenols) [153]. Upon continuous addition of hydrogen peroxide, HRP degraded products at a yield of 17 % for lignocatechol and 33 % for lignocresol. Average molecular weight was significantly decreased by about 4–8 fold. For comparison laccase was able to degrade the two lignophenols but at lower conversions, probably due to a low activity of laccase on the lignin moieties in lignophenols.

6.4.3 Cocktails

From the research perspective, using only one type of enzyme with one well-defined substrate is ideal to elucidate the fundamental mechanism behind delignification and lignin degradation. However, it is a costly operation since it requires production and isolation of the enzyme. Millions of years of natural evolution have lead microorganisms to use not a single enzyme but a cocktail of enzymes to degrade lignin. Mixture of lignolytic enzymes might represent a cheaper and more effective alternative to achieve lignin degradation.

Schroyen and co-workers investigated peroxide enzyme VP from Bjerk*andera adusta* and laccase from *Trametes versicolor* to improve production of phenolic compounds and biomethane potential from various lignocellulosic substrates [154]. The two enzymes created a significant increase in phenolic compounds and showed potential as enzymatic pretreatment.

Afrida et al. studied extracellular enzymes from two fungi, *Irpex lacteus* KB-1.1 and *Lentinus tigrinus* LP-7 for biobleaching [155]. No separation, isolation or purification of those enzymes mixture was performed, allowing the full enzyme broth to

participate to biobleaching. After 4 days, the kappa number was reduced by 4.4 % and 6.7 %, respectively, while the combination of the two enzymes reduced the kappa number by up to 7.4 %. This small improvement demonstrates a synergistic interaction between the extracellular enzymes and has applicability on the industrial scale to significantly decrease chlorine dioxide for bleaching. A similar study showed the use of xylanase and laccase to save 15 % and 25 % of ClO_2 respectively, even reaching 35 % when used one after the other (xylanase then laccase) [127]. However, the co-operation of the two enzymes has not been investigated.

6.4.4 Bioinspired Enzyme-Like Synthetic Compounds

Enzyme production is often complex and costly, thus limiting industrial applications. After identification of lignin degrading enzymes in early 1980s in *Phanerochaete chrysosporium*, numerous research groups tried to biomimic these "ligninases", by synthesising competitive enzyme-like complexes. Zucca et al. brought these compounds back to the front scene with an extensive review [156]. Natural metalloporphines, such as hemin or heme group in hemoglobin, are well known to exhibit peroxidase or catalase-like activity and inspired research on new oxidation catalyst. The ferriheme in lignolytic peroxidases was shown to degrade high-potential lignin structure [156]. After several generations of synthetic heme groups, the stability became higher than enzymes with higher catalytic activity [156–159]. Immobilisation on clay or other substrate can significantly increase its stability [160, 161]. Metalloporphines quickly proved similar efficiency than enzymes at degrading common lignin model bond molecules [162–164], and even more. Compared to enzymes, the absence of protein scaffold reduced hindrance, allowing a wider range of substrate. Some lignin model bonds usually not considered for enzymatic degradation (e.g. 5–5, β-5, or diphenyl methane DPM bond) can be cleaved, oxidised or undergo ring cleavage reaction with metalloporphines [160, 165, 166].

Farell and Skerker analysed four metalloporphines/metalloporphyrin (Fig. 6.4) and their catalytic degradation of different model bonds [166]. Similar to natural enzymes, these biomimic catalysts were deactivated by high H_2O_2 concentration, and their activity was pH dependent. Veratryl alcohol, a typical substrate for lignolytic enzymes, could be readily oxidised by the two catalysts. Metalloporphine compound with iron metallic ion can oxidise β-1 and β-O-4 model bonds, but also induce C-C cleavage in β-5 model bond and aromatic ring cleavage in 5–5 model bonds, which has never been observed with enzyme of fungal systems. For metalloporphyrin with manganese for active centre, clear delignification was identified, with a reduction of 40 % in the kappa number in just 15 min.

Crestini and co-workers highlighted the catalytic activity between manganese and iron porphyrin [165]. As Kraft lignin contains a significant amount of DPM and 5–5 substructure, two model molecules representing these bonds were studied for degradation. The manganese porphines exhibited higher conversions than the iron one, with a particular high activity for manganese *meso*-tetra(*N*-

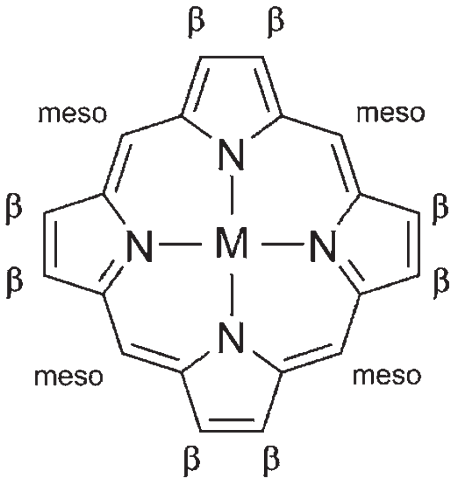


Fig. 6.4 Structure of a metalloporphine, M being the metallic ion, with the eight β positions and four meso one. IUPAC nomenclature [168] defines porphyrins as porphine derivatives where organic side chains are substituted for all the eight hydrogen atoms in the porphine pyrrole rings (the β positions). Although most of the synthetic heme catalysts are porphines by definition, they are nevertheless they are usually misleadingly referred as porhyrins [156]

methylpyridinio)porphine pentaacetate. Higher activity of Mn porphines was attributed to higher stability of these complexes. When residual Kraft lignin was submitted to the different catalysts, results indicated that manganese and iron porphines oxidised the lignin, but the latter induced a high amount of coupling reactions probably yielding higher molecular weight lignin. In general, manganese porphines proved superior to iron porphines for delignification and model bond cleavage.

A delignification of wood sawdust by metalled phthalocyanine or porphyrin was reported by Barbat and co-workers [167]. This pretreatment is considered for replacement of chemical process, sodium chlorite solution followed by alkaline extraction, for holocellulose recovery. Although this is a promising technique, with 1 % w/w phenolic compounds release from biomass, quantitative delignification amount has not been measured.

6.4.5 Perspectives

An emerging pathway for enzymes (natural or synthetic) is the catabolic treatment of biomass for delignification. To this end, enzymes represent a more manageable alternative to fungi or bacteria as their degradation mechanism is much simpler. Despite significant progress, there is still no commercial application of lignolytic enzymes for lignin degradation. The lack of efficient production systems and the poor understanding of the degradation pathways have prevented the development of efficient systems implementable on the industry industrial scale. A good

comprehension of the interaction mechanism between enzymes and the role of the different mediators (natural and synthetic) is needed for critical breakthrough in lignin bio-treatment. This knowledge can enable engineering microorganisms with high efficient lignin degradation properties tailored for specific industrial application, including fine chemical production.

6.5 Conclusion and Future Outlook

Lignin is the second most abundant polymer on earth, just behind cellulose. It represents a widely available, low cost and sustainable feedstock offering tremendous opportunities for the production of phenolic bio-based fine chemicals and monomers. Despite numerous studies devoted to its degradation, lignin still remains a most recalcitrant polymer to break down into oligomers and reproducible monomers. This is because of the multitude of chemical bonds involved and the variability of lignin chemical composition which is a function of the lignocellulosic source and the extraction process. This chapter has highlighted many of the shortcomings in fundamental knowledge restricting development; a methodical and comprehensive study on the principles and mechanism of biodegradation is required to unleash lignin as a controlled source for conversion into fine chemicals. Alternatives mimicking nature are of special interest. Many fungi and bacteria can degrade lignin, inspiring a plethora of schemes and processes for delignification and production of fine chemicals. Of those currently studied, white-rot fungi appear as the most promising delignification microorganism. The choice to genetic engineer – or not – has to be considered as it offers new routes for increased yields and selectivities needed for fine chemical production. Enzymatic degradation of lignin still faces high costs, low reaction rates, and poorly known bond selectivity. The inhibition mechanisms typical to enzymatic degradation are not well understood for "ligninases", as well as the effect of temperature resistant enzymes and reactions in a solvent. A promising avenue is to rely on enzyme cocktails or sequential enzymatic reaction schemes, because purification is typically an important cost of the process, and a wide array of products can be expected from lignin biodegradation into monomers/short oligomer due to the multitude of bonds. As lignin represents the best and most renewal natural source of phenol, it is well worth investing into the fundamental biodegradation studies that will lead to breakthroughs for process commercialization.

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1.2 Research objectives:

The aim of the project relies on connecting two objectives. Firstly, lignin feedstock is to be used to create a wide panel of options for repolymerisation. To respect the principles of Green Chemistry, biological pathways will be investigated to create recoverable degradation products. To optimise our process different lignin feedstock and different micro-organisms needs to be investigated to maximise the depolymerisation efficiency. Lignin being well known to yield very heterogeneous degradation products, analysis of the substrate after reaction is identified as key element of this work. Accurate identification of the post-reaction substrates will allow to determine the main factors that affect the depolymerisation process and will lay the foundation of the repolymerisation study in part two of this thesis.

In a second time, a list of different repolymerisation options from lignin degradation products will be established. With the help of the literature review, the different polymerisation strategies already in use in the field will be identified in order to create new pathways towards biopolymers from lignin. Some small phenolic molecules being already well known to be possibly obtained from lignin, our work will focus on their functionalisation in order to synthesize novel monomer and polymerise to create a series of polymer with different properties. Ultimately, the objective of this work is then to connect both parts by isolating degradation fragments in the first section and polymerising them back into innovative macromolecules in the second section.

The main objectives of this work are then:

1. The study of lignin degradation by enzymatic systems and the identification of the parameters playing a key role in the depolymerisation process, in order to optimise the decrease in molecular weight of the remaining substrate.
2. The synthesis of novel monomer from small phenols that are known to be obtained from lignin, such as vanillin or syringic acid.
3. To establish new polymerisation strategies to create bio-based polymer. Polymer with industrial application will be favoured.
4. Joining both thesis section together by creating a robust system that can handle enzyme degraded lignin despite of non-complete depolymerisation of purification.

1.3 Thesis outline:

The thesis report will be organised in 5 main chapters.

Chapter 1: Introduction

This chapter brings background information on the state of literature regarding lignin biodegradation. It highlights the reasons of the choices we made in later chapter in particular chapter 2. A quick overview of the current state of the art in lignin repolymerisation is also given. Clear objectives of the project are stated and quick overview of the thesis is offered.

Chapter 2: Importance of mediators for lignin degradation by fungal enzymes

The biodegradation of lignin is detailed and optimised to yield and collect degradation products. Mechanism and justification for the choice of the enzyme system are provided. This chapter was published in ACS Sustainable chemistry and Engineering.

Chapter 3: Linear bio-based aromatic polymers from syringic acid, S type degradation fragment from lignin

In this chapter syringic acid, a lignin degradation monomer, is functionalised to undergo radical polymerisation. The resulting polymer exhibit a polystyrene-like structure. Several polymer with interesting properties such as water solubility are synthesized. This work was submitted for publication in ACS Sustainable chemistry and Engineering in 2019.

Chapter 4: Synthesis of lignin-based phenol terminated hyperbranched polymer

In this chapter aromatic monomer is synthesized from vanillin. Its AB_2 structure create a novel hyperbranched polymer upon polycondensation and yield to ultra-high molecular weight. The obtained polyphenol is studied for its radical scavenging and antioxidant properties. Side reactions are discussed and their effect on the structure characterised. This work was submitted for publication in Macromolecules in 2019.

Chapter 5: Preparation of lignin films for coating by coordination complexes

In this final chapter some lignin oligomers are treated to be able to create reversible 3D structure through the formation of weak bond. Catechol groups are created inside the oligomers structure, which can then coordinate with metallic ions in solution. Lignin film were created and their properties were quickly overviewed.

Chapter 6: Conclusions & future work

In this chapter we review the project results and provide suggestion for future research directions

Annex:

Includes supporting information for the chapter 2, 3 and 4

1.4 References:

1 Higuchi, Takayoshi, ed. *Biosynthesis and biodegradation of wood components*. Elsevier, 2012

Chapter 2

Importance of mediators for lignin degradation by fungal enzymes

2 Enzymatic degradation of lignin

In this chapter we investigate on the degradation of lignin by ligninolytic enzymes. Three known enzymes were selected and depolymerisation condition are optimised to create small oligomers from lignin. Reaction was closely followed by NMR spectroscopy to conclude on the mechanism.

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Research Article

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Importance of Mediators for Lignin Degradation by Fungal Laccase

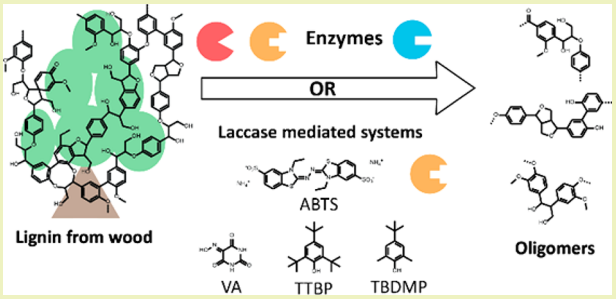
Lionel F. Longe,[†] Julien Couvreur,[§] Mathilde Leriche Grandchamp,[§] Gil Garnier,[*,‡] Florent Allais,[*,§] and Kei Saito[*,†]

[†]School of Chemistry, Bioresource Processing Research Institute of Australia (BioPRIA), and [‡]Department of Chemical Engineering, Bioresource Processing Research Institute of Australia (BioPRIA), Monash University, Clayton 3800, Australia

[§]Chaire ABI, AgroParisTech, CEBB, 3 rue des Rouges Terres, Pomacle 51110, France

Supporting Information

ABSTRACT: Three of the major ligninolytic enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (LA), as well as the secretome of a white-rot fungi, *Grammothele fuligo*, are tested on three industrial lignins (organosolv, alkali, and Kraft), to investigate and study the differences in biodegradation reactions and mechanism of these three lignins. Strategies involving additives in laccase mediated systems were also considered to produce small phenolic compounds. Three new or underreported additives including 2,4,6-tri-*tert*-butylphenol (TTBP), 4-*tert*-butyl-2,6-dimethylphenol (TBDMP), and 3-hydroxyanthranilic acid (HAA) are compared to three classic laccase mediators violuric acid (VA), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 1-hydroxybenzotriazole (1-HBT). Decrease of molecular weight by up to 73% could be obtained on organosolv lignin with LA−VA systems, and by 49%, 43%, and 39% when LA was used with ABTS, TBDMP, and 1-HBT, respectively. In-depth analysis of the degradation products by quantitative 2D HMQC NMR indicated that the oxidation is mediator-dependent and provides new insights on the enzymatic mechanism.



KEYWORDS: *Lignin, Enzymes, Mediator, Biomass, Depolymerization, Lignin peroxidase, Manganese peroxidase, Laccase, Degradation, Biodegradation*

INTRODUCTION

Lignin is the second most abundant renewable biopolymer, which, combined with cellulose, represents over 70% of the worldwide biomass. While cellulose has a well-defined market, lignin remains an industrial waste stream with few value-added applications. As a result, 98% of the world's lignin production is simply burned for energy.[1] As the most important source of phenols and aromatics in the biosphere, lignin has tremendous potential. Its depolymerization in small oligomers or phenolic molecules is critical for the implementation of biorefineries and to deliver new biobased materials. However, lignin recalcitrance has significantly hindered its valorization.

Lignin results from the enzymatic copolymerization of three phenolic monomers (monolignols), leading to a wide variety of chemical bonds and cross-linking either by stable carbon−carbon (β−β, β-1, 5-5, β-5) or more labile ether (β-*O*-4, 4-*O*-5, spirodienone, dibenzodioxocin) linkages (Figure 1). That heterogeneous complex structure has proved to be a significant obstacle to chemical depolymerization and has prevented upgrading lignin feedstock into fine phenolic chemicals. Some industrial processes exist but involve either extreme conditions of pH, temperature, or pressure or toxic chemicals.[2−4] Greener alternatives are required.

In nature, micro-organisms such as bacteria or wood-rot fungi have developed strategies to degrade and even catabolize the resulting aromatic compounds into carbon and energy.[5−7] These natural depolymerization processes usually combine internal and external enzymes. Different groups of enzymes involved in this process have been identified. The main group includes phenol-oxidases, which comprise lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (LA), and versatile peroxidase. However, it also has been demonstrated that some of these enzymes can oxidize nonphenolic moieties of lignin in certain conditions, a key behavior to drastically increase the biodeterioration of the entire lignin structure.[7−10] It is still unclear whether these ligninolytic enzymes can function independently or if mutual cooperation is required.

Microorganisms can degrade lignin down to small aromatic compounds or even smaller molecules.[11−13] Therefore, a combination of enzymes interacting cooperatively is necessary to cleave the various bonds of lignin. This suggests that the combination of multiple enzymes or their sequential use could improve depolymerization as compared to single enzyme biodegradation, to yield smaller aromatic molecules. A particular enzyme may selectively cleave a specific lignin bond, while another enzyme will cleave another bond. There

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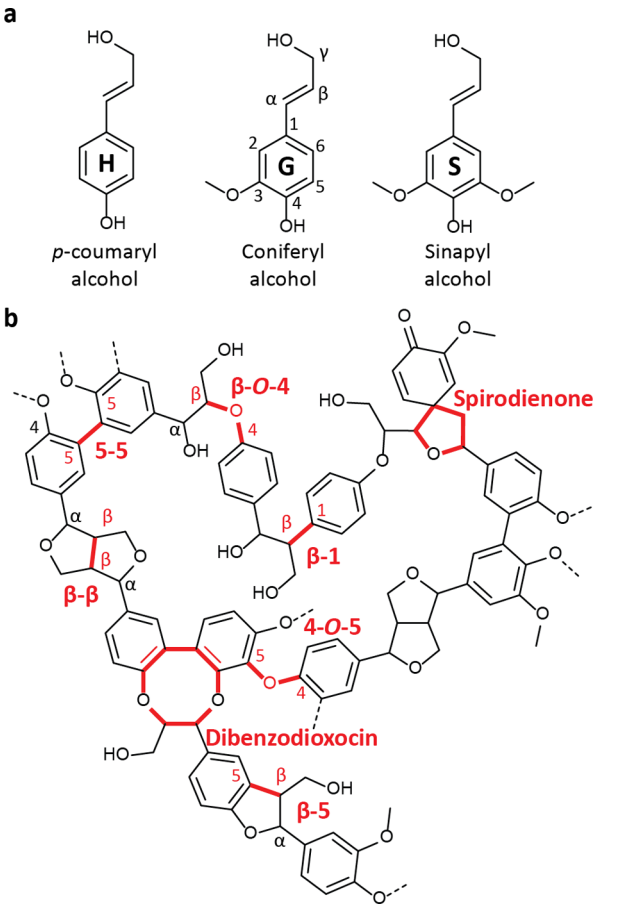

Figure 1. (a) Three constitutive monomers of lignin: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol; and (b) structure illustrating the principal bonds in lignin, a three-dimensional highly cross-linked biopolymer.

are a few studies in the literature describing enzymes preferentially degrading some types of bonds rather than others in lignin small model.[14−17]

While peroxidases can work alone in lignin oxidation, LA tends to polymerize lignin when by itself, especially in the case of plant laccases. In the presence of small aromatic molecules defined as "mediators", this phenomenon reverses, which improves lignin degradation.[18] A wide range of mediators, natural or synthetic, have been identified.[19] It was also reported that laccases from different microorganisms interact differently with mediators.[20] However, the mechanism remains identical for all strains, with LA using molecular oxygen to oxidize the mediator, which then acts as a chemical oxidant for lignin (Figure 2). It was hypothesized that mediators can diffuse into the intricate lignin structure, thanks to their small size. The presence of mediators also allows LA to oxidize the type of bonds that would otherwise remain untouched due to the size of laccase and selectivity.[18] The addition of mediators should further improve lignin depolymerization by cleaving some new bonds of the heterogeneous structure.

In this study, we select three of the major ligninolytic enzymes, two peroxidases and LA, as well as a white rot fungi external enzyme cocktail from *Grammothele fuligo*,[21] to compare the differences in lignin depolymerization. A set of three industrial lignins, Kraft, alkali, and organosolv, are used to represent the different structures and bonds in lignin. Finally, six mediators, including two new, are investigated for laccase mediated systems.

In this study, we aim at improving our understanding of the mechanism underlying the enzymatic degradation of lignin by monitoring changes in its structure by 2D $^{1}H-^{13}C$ quantitative heteronuclear multiple-quantum correlation (Q-HMQC) NMR analysis. Three new and three well reported mediators will be investigated to study the different interactions and mechanism involved in laccase mediated systems. Furthermore, the impact of lignin structure and composition on its enzymatic depolymerization will be determined to optimize a biocatalytic process for the production of small phenolic molecules from lignins. To the best of our knowledge, this is the first report comparing the effect of different types of mediators for lignin enzymatic degradation using different types of lignin and analyzing the depolymerized structures using Q-HMQC NMR to understand the oxidation reaction on the different bonds of lignin and the degradation mechanism.

EXPERIMENTAL SECTION

Materials. Enzymes: Lignin peroxidase (LiP) from *Phanerochaete chrysosporium* (EC: 1.11.14), manganese peroxidase (MnP) from *Nematoloma frowardii* (EC: 1.11.1.13), and laccase (LA) from *Cerrena unicolor* (EC: 1.10.3.2) were purchased from Jena Bioscience (Jena, Germany). Laccase from *Trametes versicolor* was purchased from Sigma-Aldrich (Sydney, NSW, Australia).

Chemicals and solvents: Chemicals were purchased from Sigma-Aldrich (Sydney, NSW, Australia) (hydrogen peroxide, manganese sulfate, citric acid monohydrate, sodium phosphate dibasic dehydrate, succinic acid, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate), violuric acid monohydrate, 1-hydroxybenzotriazole, KF, triton X, bovine serum albumine, potassium fluoride, 2,6-dimethoxyphenol (DMP), lithium bromide, sodium hydroxide, 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP)), from Acros Organics (Geel, Belgium) (3-hydroxyanthranilic acid), and from TCI (Tokyo, Japan) (aluminum chloride, DMP, *tert*-butyl chloride, 2,4,6-tri-*tert*-butylphenol) and used as received. Reagent grade solvents (propanol, dimethylformamide, acetonitrile, hexane, chloroform) were purchased from Thermo Fisher Scientific (Melbourne, VIC, Australia).

Fungus strain: *Grammothele fuligo* strain was obtained from UMR BBF (Biodiversité et Biotechnologie Fongiques, Marseille, France). The cultures were grown at 30 ± 1 °C during 7 days on potato dextrose agar (PDA) medium.

Lignins: Organosolv lignin from corn cob from *Stigmata maydis* was purchased from Chemical Point (Munich, Germany) and was washed with methanol before use to remove low molecular weight fraction. This precaution aimed at reducing dispersity of the lignin sample so that degradation detection by GPC analysis is facilitated: the pre-emptive removal of small molecular weight fraction allows the

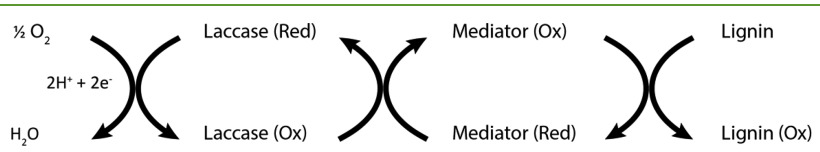

Figure 2. Catalytic cycle of laccase and mechanism of laccase mediated biodegradation of lignin.

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future detection of small oligomers to be identified as an indicator of bond cleavage and degradation of the studied sample. Lignin was set in methanol at 1 g/100 mL overnight under vigorous stirring. The resulting insoluble part was filtered and dried to yield about 20% high molecular weight lignin. Gel permeation chromatography analysis indicated M_n = 6000 and $Đ$ = 7.3. Alkali lignin was purchased from Sigma-Aldrich (Sydney, NSW, Australia) and was washed by the same process to remove low molecular weight. GPC analysis indicated M_n = 5600 and $Đ$ = 7.2. Kraft lignin was isolated through the process described later from black liquor provided by the Visy paper mill from Tumut, NSW, Australia, which mainly uses *Pinus radiata* (softwood). After isolation, the lignin is washed with methanol to yield some Kraft lignin with M_n = 6000 and $Đ$ = 7.2. ^{31}P NMR analysis has been performed on the lignins to determine the aliphatic hydroxyl, phenolic, and carboxylic acid content. Results can be found in Figure S1 and Table S1.

Methods. Buffers: Each enzyme was associated with its best buffer solvent to improve activity as follows: LiP in 300 mM citric acid 400 mM sodium phosphate dibasic adjusted to pH = 4.5, MnP in 500 mM succinic acid adjusted to pH = 4.5, and LA in 300 mM citric acid 400 mM sodium phosphate dibasic adjusted to pH = 5. Experiments involving several enzymes used the latter buffer.

Isolation of lignin from black liquor: Kraft lignin was isolated from black liquor according to the following procedure. Water was added to dilute the black liquor to 12 w/w% solid content to reduce viscosity. Under stirring, hydrochloric acid was added to acidify the solution to pH around 1. The resulting solid was filtered out, washed/rinsed with deionized water, and then dried overnight at 40 °C under vacuum. The dried lignin was dissolved in 3 w/w% sodium hydroxide solution to a total concentration of 12 w/w% lignin. The solution was heated at 100 °C under reflux for an hour to further break down, dissolve, and remove remaining hemicellulose from the lignin sample. After being cooled, the solution was once again acidified, filtered, rinsed with water, and dried overnight to isolate lignin. Elemental analysis of carbon, hydrogen, nitrogen, and sulfur gave the following result (average over three measurements): carbon 65.30%, hydrogen 6.07%, nitrogen <0.3% (detection limit), sulfur 2.57%.

Isolation of fungal external secretome: *Grammothele fuligo* was chosen as it exhibited activity of LiP, MnP, and LA. Production of extracellular enzyme cocktail from *Grammothele fuligo* was carried out using a solid-state fermentation. Experiments were performed in 500 mL Erlenmeyer flasks containing 10 g of ground *Miscanthus* straw. Flasks were autoclaved, impregnated to 80% moisture content with deionized water, and inoculated with five agar plugs (diameter, 0.7 cm). The Erlenmeyer flasks were incubated at 30 ± 1 °C without shaking. The samples extractions were performed after 7 days of incubation by adding 100 mL of deionized water per flask. Flasks were incubated on a rotary shaker (200 rpm) for 1 h. The mixture was then centrifuged at 5000 rpm for 15 min at 4 °C to isolate the secretome and stored at −20 °C.

Peroxidases assays: Enzyme assays were performed by following the procedure of Zhou et al.[22] Peroxidase (LiP and MnP) activities were measured regarding the oxidation of DMP. One unit (1 U) of these enzymes is the amount of enzyme that will oxidize one micromole (1 μmol) of DMP per minute.

To a UV cuvette were added 1 mL of buffer, 524 μL of enzyme solution, 256 μL of 17.2 mM KF solution, and 200 μL of 18 mM DMP solution, and the reaction was started by addition of 20 μL of 0.1 M H_2O_2 solution. Absorbance at λ = 468 nm was then followed for 5 min, while the temperature was maintained at 30 °C. Oxidized DMP has an extinction coefficient of ε = 49 600 M^{-1} cm^{-1}.[23] Blank experiment with no enzyme was also used as reference. For MnP, 40 μL of the buffer solution was replaced with 40 μL of 5 mM manganese sulfate solution. The KF solution inhibited the activity of laccase; it is mostly useful when dosing the peroxidase activity in *Grammothele* enzyme cocktail.

Laccase assays: LA activity were measured regarding the oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). One unit (1 U) of laccase is the amount of enzyme that will oxidize one micromole (1 μmol) of ABTS per minute.

To a UV cuvette were added 1.5 mL of buffer and 1.2 mL of enzyme solution, and the reaction was started by addition of 300 μL of 5 mM ABTS solution. Absorbance at λ = 405 nm was then followed for 5 min, while the temperature was maintained at 30 °C. Oxidized ABTS has an extinction coefficient of ε = 36 800 M^{-1} cm^{-1}.[24] Blank experiment with no enzyme was also used as reference.

Lignin degradation: Degradation experiments of lignin by peroxidases (LiP, MnP, and *Grammothele* cocktail) were performed as follows. 50 mg of the selected lignin was added to a test tube, along with 0.5 mL of isopropanol, 100 μL of 5 mM $MnSO_4$ solution in the case of MnP, the enzyme solution, and was topped up to 4.95 mL with buffer. Degradation was started by addition of 50 μL of 0.1 M hydrogen peroxide. Reaction mixture was stirred gently and maintained at 30 °C. All experiments were at least performed in duplicate.

Degradation experiments of lignin by LA were performed as follows. 50 mg of selected lignin was added to a test tube along with 0.5 mL of isopropanol. Additives were added in different amounts. For ABTS, violuric acid (VA), 1-hydroxybenzotriazole (1-HBT), and 3-hydroxyanthranilic acid (HAA), the ratio mediator to lignin was chosen according to research from Bohlin et al. on degradation of β-*O*-4 model bond by LA and a mediator. A ratio of lignin:mediator = 1:1 where lignin was regarded to the molecular weight of 334.37.[25] The optimum amount of mediator was also confirmed by changing the ratio of LA/mediator to higher and lower values and by measuring lignin degradation over 24 h (Supporting Information Figure 5). For both TBDMP and its tri-*tert*-butyl equivalent, TTBP (2,4,6-tri-*tert*-butylphenol), ratios were chosen similarly as if lignin was PPO and with a ratio of 1:1 regarding the repeating unit.[26]

Degradation experiments involving LiP were performed with a total of 0.06 U, MnP with a total of 0.02 U, *Grammothele* cocktail exhibited an activity of 0.01 U of laccase, 0.25 U MnP, and 0.03 U of LiP, and laccase experiment with a total of 0.3 U. Yields ranged between 95% and 99%.

The synthesis of *tert*-butyl-dimethyl-phenol (TBDMP) is described in the Supporting Information.

Characterization. The molecular weights of lignin samples were analyzed at 40 °C by gel permeation chromatography (GPC) performed on a Tosoh EcosHLC-8320 equipped with double detectors (RI, UV 280 nm) using Tosoh alpha 4000 and 2000 columns. DMF/LiBr 0.1 M was used as a mobile phase (flow rate 1 mL/min). Polystyrene standards were used for the calibration.

UV measurements for enzyme assays were interchangeably performed on a Thermo Scientific MultiskanFC microplate photometer or an Agilent Cary 60 UV−vis cell reader spectrophotometer.

2D HMQC NMR experiments were performed on a Bruker Avance 500 MHz NMR spectrometer equipped with a CryoProbe Prodigy 5 mm ^{1}H/2H−^{13}C/^{15}N probe. NMR experiments were performed with the sample held at 25 ± 0.1 °C. Chemical shifts for all experiments are referenced using the Unified Scale relative to 0.3% tetramethylsilane in deuterochloroform.[27,28] Multiplicity edited HSQC experiments used the standard hsqcedetgpsisp2.2 Bruker pulse program. There were 512 experiments with 64 scans collected over 6010 Hz in the ^{1}H dimension and 20 139 Hz in the ^{13}C dimension with a 1.5 s relaxation delay. The data were processed in phase-sensitive (echo-antiecho) mode to 2048 × 2048 data points using a π/2-shifted sine-squared window function in both dimensions.

^{31}P NMR experiments were performed on a Bruker Advance III 400 (9.4 T magnet) with a 5 mm broadband probe with *z*-gradient and BACS 60 tube autosampler. TMDP was used as derivatizating agent for phosphylation of hydroxyl groups, following procedures reported in the literature.[29] Experiments were performed with 180 scans and a 25 s relaxation delay.

RESULTS AND DISCUSSION

Degradation by Ligninolytic Enzymes. LiP, MnP, LA, and an extracellular enzyme cocktail from *Grammothele fuligo* were investigated for the degradation of three different sources of lignin: organosolv, alkali, and Kraft lignin. As shown in

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Figure 3, lignins' molecular weight tended to decrease rapidly in the first minutes of the reaction, followed by a slow repolymerization over time, reaching even higher molecular weight than the original sample for reaction time over 48 h. This recondensation happening for longer reaction times was expected as ligninolytics enzymes can both depolymerize and polymerize lignin.[30] It is also believed that the oxidation of some lignin bonds leads to a homolytic cleavage, as observed on numerous model bond degradation studies.[31−33] After some time, the resulting free radicals can react together or form a more stable bond.

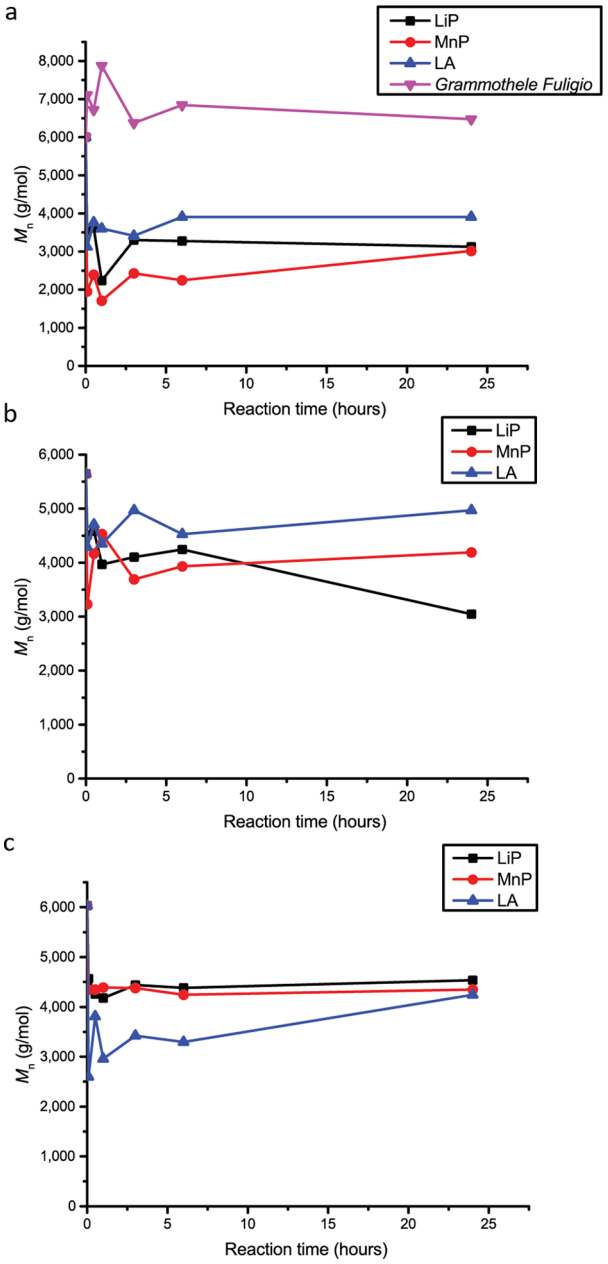

Figure 3. Evolution of average molecular weight by number versus reaction time, measured by GPC. Three sources of technical lignin are compared: (a) organosolv, (b) alkali, and (c) Kraft, degraded by LiP, MnP, LA, and *Grammothele fuligo*.

LiP is reported in the literature as the enzyme with the highest redox potential (1.2 V versus standard hydrogen electrode[1]) followed by MnP (0.8 V) and LA (0.5−0.8 V versus SHE). Therefore, oxidation and degradation of lignin should be more easily achieved by LiP, then MnP, and finally LA. However, lignin depolymerization with MnP reveals the best overall bonds' cleavage ability, followed by LiP and LA. The extracellular enzyme cocktail extracted from *Grammothele fuligo* exhibits activity of both peroxidases and LA. However, when exposed to the enzyme cocktail, organosolv lignin showed a rapid polymerization (Figure 3a).

These results highlight the complexity of the lignin enzyme degradation mechanism by enzyme cocktails. The combination of multiple enzymes could not improve the depolymerization efficiency. The three different lignins reacted differently to enzymatic degradation. Overall, organosolv was much easier to degrade than the Kraft or Alkali lignin; the organosolv lignin was thus chosen for the subsequent studies. Literature recently highlighted how different processes such as Kraft and alkali pulping can change the amount of β-*O*-4 bonds in treated lignins.[34] 2D HMQC analysis of the organosolv, alkali, and Kraft lignin used for our depolymerization indicated a β-*O*-4 content of 62%, 67%, and 37%, respectively. Although this β-ether bond is considered to be the easiest lignin bond cleavable through oxidation,[4,35] the β-*O*-4 richest lignin, alkali lignin, is actually the least prone to degradation. Other factors must influence the enzymatic depolymerization. Besides the structure difference, Kraft lignin also possesses a non-negligible amount of sulfur (2.5 w/w%, measured by elemental analysis), about 30% of which is in the sulfate form and the remaining as organically bound sulfur.[36] The higher degradation recalcitrance of Kraft lignin as compared to organosolv might be partially due to enzyme inhibition by these species.

Mediators for Enhanced Enzymatic Activity. The use of additives to enhance LA lignin-degradation properties has been vastly reported.[18,37−39] It was hypothesized that these agents facilitate the degradation mechanism by improving diffusion into the lignin network and by widening the range of lignin bonds that can be cleaved. Bourbonnais and Paice highlighted the indirect mechanism of degradation showing the importance of small intermediate molecules, called mediators.[40] Molecular oxygen is used by LA in the catalytic cycle to oxidize the mediator, which then acts as chemical oxidant for lignin. This mechanism eliminates the steric limitation of enzymes that could otherwise highly hinder oxidation of bulky molecules. The mediated process also allows laccases to overcome their phenolic-substrate restriction, expanding the range of lignin units that can be oxidized. Typical laccase mediators are small phenolic compounds such as vanillin, syringaldehyde, veratryl alcohol, but also synthetic mediators such as 1-hydroxybenzotriazole (1-HBT), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), or violuric acid (VA).[41] The choice of mediator can influence the oxidative potential of laccase and also induce stereopreference in the substrate.[42] 3-Hydroxyanthranilique acid (HAA) was investigated by our group for the degradation of lignin-like polymer (unpublished results). In nature, this mediator can favor lignin degradation by laccase and hinder the repolymerization process.[43] 2,4,6-Tri-*tert*-butylphenol (TTBP) and 4-*tert*-butyl-2,6-dimethylphenol (TBDMP) are involved in the redistribution mechanism to cleave the 4-*O*-5 bonds in lignin.[26] The initial step is an oxidation and the creation of a phenoxy radical, cleaving the bond by replacing the aromatic at the C4 position. If laccase can oxidize TBDMP and activate it as a mediator, a strong decrease in 4-*O*-5 bonds and concomitant drop in M_n would be

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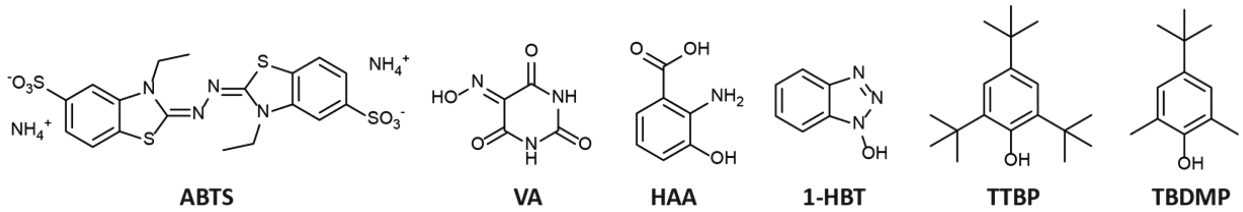

Figure 4. Different agents used to improve laccase degradation of lignin.

expected. Those six compounds (see Figure 4) were used as a mediator for screening reaction and to determine which could best improve lignin depolymerization (Figure 5).

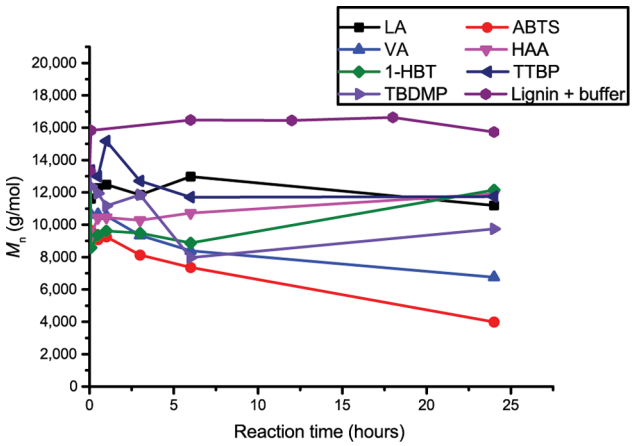

Figure 5. Lignin degradation by LA and different mediators. Evolution of average molecular weight by number versus reaction time, measured by GPC. Molecular weights are overestimated due to experimental constraint.

Organosolv lignin has a high amount of β-*O*-4 linkages and proved to be a better substrate for oxidative degradation (previous section). Thus, organosolv lignin was selected for the following study. Table 1 summarizes the changes in molecular weight of lignin with the different mediators. Molecular weights were overestimated as compared to the first section due to a cut off of the low molecular weight fraction as the residual mediator area was removed from the molecular weight analysis using GPC (Figure S2).

Table 1. Summary of Lignin Degradation Experiment by LA Enzyme with the Different Mediators

entry	enzyme	mediator	M_n at max degradation (g/mol)	time at max degradation	$Đ$
0	untreated original lignin		14000[a]		3.8
1	none	none	15700[a]	24 h	9.3
2	LA	none	11200[a]	24 h	3.2
3	LA	ABTS	4000[a]	24 h	1.5
4	LA	VA	6800[a]	24 h	3.9
5	LA	HAA	9400[a]	5 min	4.5
6	LA	1-HBT	8600[a]	5 min	4.2
7	LA	TTBP	11700[a]	6 h	3.6
8	LA	TBDMP	8000[a]	6 h	3.5
9	none	ABTS	15500[a]	24 h	9.9
10	none	VA	14600[a]	24 h	7.3

[a]Overestimation due to analysis constraints; see Figure S2.

The six mediators were compared altogether and versus LA alone. Molecular weight was clearly reduced with VA and ABTS (Figure 5 and Table 1, entries 3 and 4). In comparison, other mediators had less effects on lignin molecular weight, but still provided a better degradation than LA alone. VA and ABTS presented a slow degradation process as compared to the four other mediators, with maximum depolymerization reached at 24 h. Some repolymerization of the lignin fragments was also observed. Higher reaction time led to molecular weight higher than that of the untreated original lignin by recondensation reaction. Full information is shown in Figure S3. TBDMP, a new synthetic agent for laccase mediated systems, was more effective to depolymerize lignin than was 1-HBT, a well-reported mediator (Table 1, entries 6 and 8). HAA had an effect similar to that of 1-HBT, with a rapid depolymerization within the first 5 min, followed by recondensation. As for TTBP, no significant improvement of depolymerization was observed. TTBP and TBDMP required a longer reaction time than HAA and 1-HBT, suggesting a different pathway or slower mechanism. TTBP has however a much weaker impact on lignin degradation; this is likely due to the additional steric hindrance around the phenol group caused by the two *tert*-butyl side groups. After this screening, ABTS and VA showed the best improvement of lignin degradation by LA. The biodegradation products of these two systems require further study to elucidate the degradation mechanism.

Analysis of Degradation Products. From the previous screening reactions, we were able to identify VA and ABTS as the two best candidates for a more thorough investigation. GPC analysis data through lignin depolymerization using both mediators with LA are shown in Figure 6.

The clear shift of the lignin peak to right indicates a change toward lower molecular weight. At this point, as the commercial production of LA from *Cerrena unicolor* had been discontinued, LA from *Trametes versicolor* was used instead. Although both LA have similar redox potentials (Table 3), they present a similar trend but slight differences in lignin degradation. Overall depolymerization had similar efficiency, with lignin M_n decreased by 49% (7200 g/mol) and 73% (3800 g/mol) from the original value (14 000 g/mol) for ABTS and VA, respectively, using LA from *C. unicolor*. 2D Q-HMQC NMR analysis was conducted to investigate the structural changes in lignin during the LA action. Four regions in 2D proton-carbon NMR allow insight on the lignin structure: (a) the aliphatic oxygenated side chain region (δ_C/δ_H 50−90/2.5−5.8), (b) the aromatic/unsaturated region (δ_C/δ_H 90−150/5−8.5), (c) the aliphatic side chain region (δ_C/δ_H 5−38/0.5−2.8), and (d) the aldehyde region (δ_C/δ_H 170−210/9−10), although only the first two are of interest for this study.[39] Correlations regions were compared to previous literature and peaks were assigned accordingly.[34,44−48] Q-HMQC NMR allows quantification of carbon bonded protons

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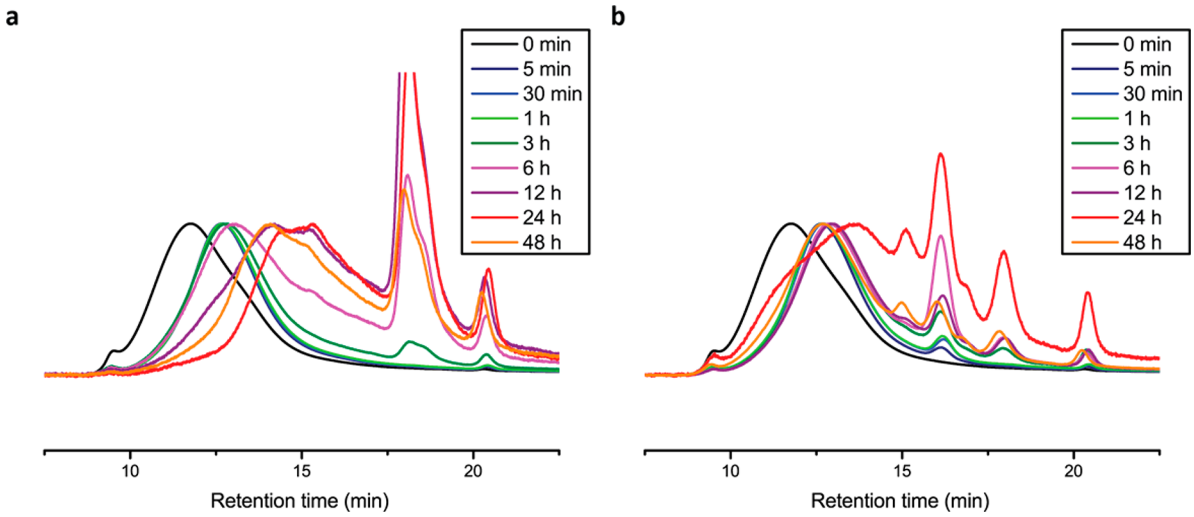

Figure 6. GPC analysis of organosolv lignin degraded by Laccase from *Trametes versicolor* and (a) ABTS and (b) violuric acid, at different reaction times. The clear shift to the right indicates a decrease in lignin molecular weight. The peak at 16.5 min retention time corresponds to ABTS, while that at 17.5 min is violuric acid. The peak at 20.5 min retention time represents the solvent. Other peaks appearing in high retention time area may be lignin oligomers or side products from reaction of the mediator with enzyme.

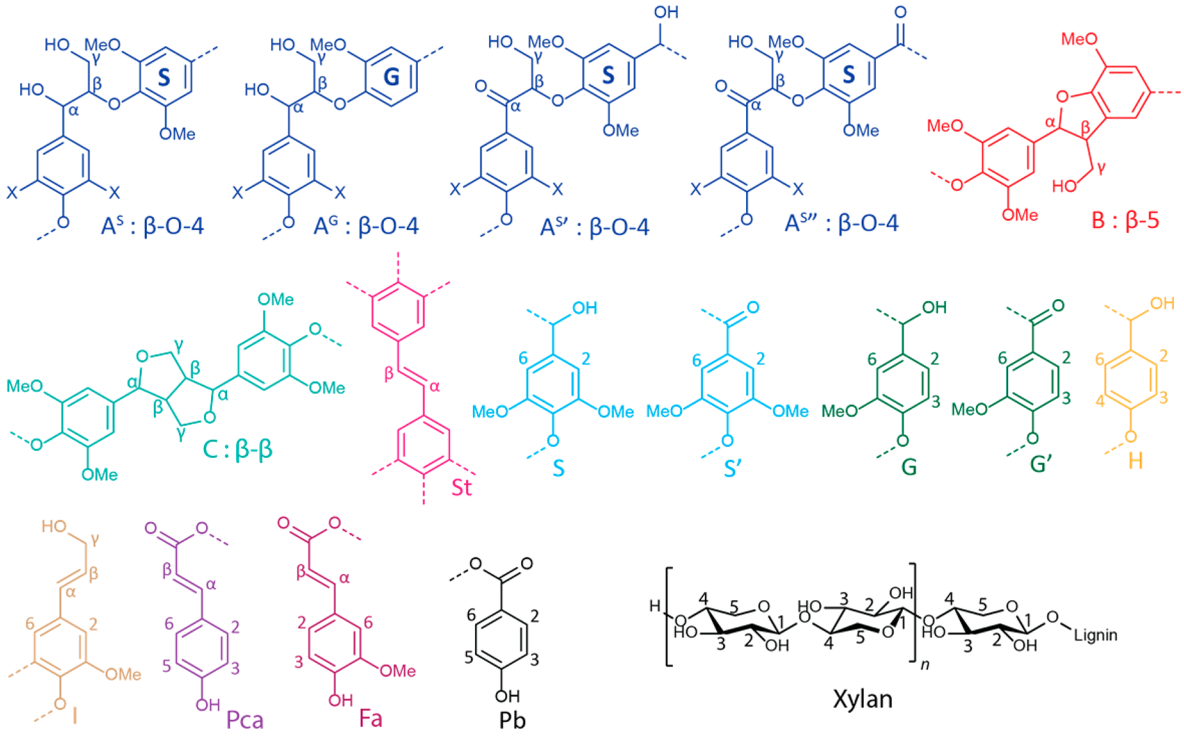

Figure 7. Summary of the different lignin linkages and other structures identified by $^{13}C/^{1}H$ correlation 2D NMR. X = H/OCH_3.

by integration of the C−H correlation patterns. Examination of the aromatic/unsaturated region allows distribution of aromatic units inside lignin between the three possible forms: syringyl (S), guaiacyl (G), or *p*-hydroxyphenyl (H) units. These units differ in structure by the presence of two (S), one (G), or the absence (H) of methoxy group on the aromatic ring (Figure 7). The ratio between these units can vary from one lignin to another and can allow the determination of its origin (hardwood, softwood, plant).

In the aliphatic oxygenated side chain region, correlation patterns allow quantification of several bonds between the aromatic units in lignin, including the *β*-*O*-4, the *β*-5, and the *β*−*β* bond (Figure 7). Furthermore, in the case of *β*-*O*-4 bonds, the type of unit at the position C4 can also be differentiated between S (A^S) or G (A^G) units. Finally, oxidation of those bonds can be monitored in both aliphatic oxygenated ($A^{S'}$, $A^{S''}$) and aromatic regions (G′, S′).

The initial organosolv lignin structure was characterized (Figure 8a and d). The analysis of the aliphatic oxygenated side chain region indicates the presence of *β*-*O*-4 and a few *β*-5 bonds; however, no *β*−*β* bonds could be detected. Integration and percentage of the bonds forming lignin (that can be detected with HMQC NMR) are summarized in Table 2. Peaks in the aromatic region show evidence of the presence of

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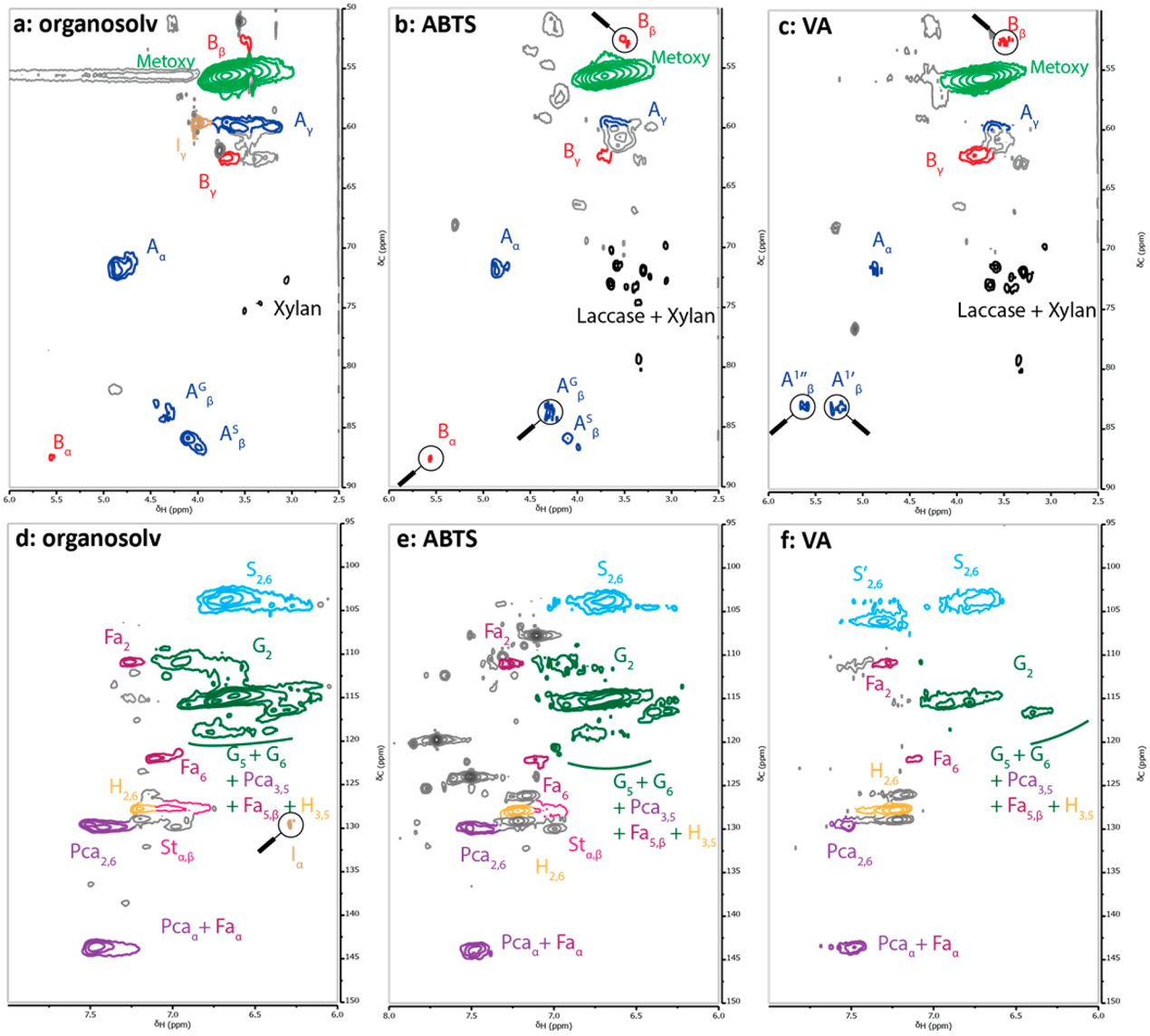

Figure 8. $^{13}C/^{1}H$ correlation HMQC 2D NMR analysis. (a−c) Aliphatic oxygenated side chain region of original lignin, ABTS-LA degraded lignin, and VA-laccase degraded lignin, respectively. (d−f) Aromatic hydrogen region of the same samples, respectively.

Table 2. Characterization of Lignin Sample Structures from GPC and 2D NMR Integration

	original lignin	treated LA + ABTS	treated LA + VA
M_n (g/mol)	14000	7200	3800
S/G/H units ratio	44%/53%/3%	45%/44%/11%	58%/26%/16%
oxidized S unit/S unit[a]	n.d.[b]	9%	47%
oxidized G unit/G unit[a]	n.d.	n.d.	n.d.
β-5[a]	8%	10%	12%
β−β[a]	n.d.	n.d.	n.d.
non ox. β-O-4[a]	62%	59%	50%
β-O-4 (A^G)	22%	21%	20%
β-O-4 (A^S)	34%	33%	22%
β-O-4 ox. ($A^{S'}$)[a]	n.d.	n.d.	14%
β-O-4 ox. ($A^{S''}$)[a]	n.d.	n.d.	15%
St[a]	13%	13%	n.d.
DC	0.00	0.10	0.53

[a]Per aromatic circle. [b]n.d., not detected.

S, G, and H units, with a ratio of 44/53/3. The origin (grass lignin) of the organosolv lignin was confirmed by the HMQC analysis. Syringyl units (S, δ_C/δ_H 102−108/6.1−6.8) can be found in both hardwood and herb lignin. The presence of *p*-hydroxyphenyl units (H, δ_C/δ_H 128/7.2, non-negligible amount in herb lignin) and the absence of *p*-hydroxy benzoate group (Pb, δ_C/δ_H 132/7.5−7.9, typical of hardwood) also confirm that observation.[38] The presence of some protein residue was found from plant cell wall typical of many grasses. Kim et al. reported that two proteins (phenylalanine and tyrosine) have a distinct correlation pattern in the same area as *p*-hydroxyphenyl units, therefore causing its slight overestimation.[49] Organosolv process involving mild acid ethanol/water treatment was reported to create β-O-4 ethoxy groups in the Cα position (instead of hydroxyl), which could hinder these bond oxidations; however, no corresponding peak could be observed in this case (δ_C/δ_H 81/4.55 and 64/3.3), suggesting an even milder treatment was used for this lignin.[43] Furthermore, no polysaccharide peaks could be observed in the carbohydrate region, nor the usual ether bond between C_α in a

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β-O-4 and a C6 of polysaccharide (δ_C/δ_H 82/4.6), indicating the absence of cellulose from the lignin sample.

Next, depolymerized lignin products using both mediators ABTS and VA with LA were characterized by 2D HMQC NMR (Figure 8b,e and c,f). After the enzymatic treatment of lignin samples, some clear changes were observed in their structure. These changes appear to be mediator-dependent, suggesting a more complex mechanism from the mediator than a simple intermediate. A common behavior observed was a general decrease in the relative amount of G units in both lignins. This decrease suggests that some new bonding was created, such as condensation reaction in position 5 of the aromatic ring, for example. Such reactions would cause a shift in the resonance frequency of protons in positions 2 and 6. Previous literature proved that 4-O-5 condensation reaction can induce a shift of G_6 protons (C6 protons of G structure) into the $S_{2,6}$ (C2 and C6 protons of S structure) correlation area (Figure 8), which would explain a slight increase in the calculated relative amount of S units.[50−52] An increase in H units' amount could be a result of superimposition with signal from phenylalanine protein, which is present in LA from T. *versicolor*.

Furthermore, calculation for a condensation degree was formulated by modifying the Capanema equation on ^{13}C quantitative NMR spectroscopy.[53] The degree of condensation (DC) refers to the average number of aromatic protons per aromatic ring being substituted by condensation reactions. It can be calculated from integrating the correlation region δ_C/δ_H 100−125/6−7.5. The contribution of the S, G, and H units in the region of interest is 2 protons for syringyl units, 3 for guaiacyl units, and 2 for H units.

$$DC = \frac{Ar{-}H_{theory} - Ar{-}H_{measured}}{Ar{-}H_{theory}} \quad (1)$$

$$Ar{-}H_{theory} = 2 \times S + 3 \times G + 2 \times H \quad (2)$$

The condensation degree of the original and degraded lignins can be found in Table 2 with detailed calculations in the Supporting Information. As expected, a clear increase in condensation degree follows the drop in relative amount of G units, thus corroborating the expected recondensation phenomenon by action of ligninolytics enzymes, also observed in the first section of this study. Initially, organosolv lignin samples contained about 62% of β-O-4 bonds, 8% of β-5 bonds, and an amount of β−β bonds below detection levels (Table 2); this is typical of organosolv herb lignin.[32] 2D HMQC analysis can differentiate if a β-O-4 bond involves an S (syringyl) or G (guaiacyl) unit in the C_4 position (Figure 7). When a β-O-4 bond involving a syringyl unit at the C_4 position (A^S) gets oxidized (C_α ketone) during depolymerization, the β proton shifts to lower fields, thus allowing quantification of oxidized β-O-4. In the case of ABTS mediated degradation, no oxidized β-O-4 ($A^{S\prime}$ nor $A^{S\prime\prime}$) bonds were observed in $^1H-^{13}C$ NMR, while action of the mediator VA oxidized many β-O-4 bonds with a high amount of $A^{S\prime}$ and $A^{S\prime\prime}$, 14% and 15%, respectively (Table 2). The concomitant decrease of the overall S units involved in nonoxidized β-O-4 bonds (A^S and A^G units) and the increase of oxidized S units (δ_C/δ_H 104−110/7.1−7.5) confirm the ability of VA to efficiently mediate lignin oxidation. In both cases, no oxidized G units were observed, suggesting that β-O-4 involving S units are easier to oxidize/cleave than that involving G. This corroborates literature on β-O-4 model bonds' degradation by LA and mediator.[5,16,25,39] An overview of S/G type simple phenolic compounds and mediators redox potential is shown in Table 3.

Table 3. Redox Potential of Mediators and Different S/G Model Molecules[a]

ox	red	E^0 (V)	S/G type molecule	ref
O_2	H_2O	1.2−0.8		
$ABTS^{+\bullet}$	$ABTS^{+2}$	1.09		37
VA	VA_{Ox}	0.91		37
ferulic $acid_{Ox}$	ferulic $acid_{Red}$	0.79[b]	G1	56
conyferyl $aldehyde_{Ox}$	conyferyl $aldehyde_{Red}$	0.79[b]	G2	56
LA $T.V._{Ox}$	LA $T.V._{Red}$	0.78		57
LA $C.U._{Ox}$	LA $C.U._{Red}$	0.75		58
$guaiacol_{Ox}$	$guaiacol_{Red}$	0.71	G3	59
ABTS	$ABTS^{+\bullet}$	0.68		37
synapic $acid_{Ox}$	synapic $acid_{Red}$	0.68[b]	S1	56
synapyl $aldehyde_{Ox}$	synapyl $aldehyde_{Red}$	0.65[b]	S2	56
$syringol_{Ox}$	$syringol_{Red}$	0.56	S3	59

[a]Potentials are reported versus standard hydrogen electrode. [b]Anodic potential. S/Gx are aromatic with the same structure with one (G) or two (S) methoxy groups.

As a general trend, two simple phenols with similar structure differing only by the substitution of one (G) or two (S) methoxy groups on the aromatic ring have different redox potentials, with the disubstituted (S) phenol having the lowest. This seems to indicate that G units are more stable toward oxidation than S units. With a lower redox potential by 0.2 V as compared to VA, ABTS is less efficient in oxidizing S type units. VA could oxidize S units easier, thus depolymerizing lignin further. However, it is known that ABTS can undergo a second oxidation (from $ABTS^{+\bullet}$ to $ABTS^{+2}$) at a higher redox potential of 1.09 V. It was suggested that due to the Nernst law, some $ABTS^{+2}$ and oxidized VA should be present in the medium as LA from *Trametes versicolor* and have a strong redox potential around 0.8 V.[54] Some VA and, to a less extent, $ABTS^+$ might have been oxidized by LA. Both mediators will then oxidize lignin proportionally to their concentration. Through this mechanism, laccase could perform the thermodynamically unfavored oxidation of mediator, which in turn led to lignin bonds' oxidation and cleavage. S units being seemingly easier to oxidize, lignin with a high amount of syringyl units should therefore be favored as a feedstock for enzymatic degradation of lignin and products recovery. Although β-O-4 bonds have proved to be targeted by the LA mediated systems, it is not a sufficient condition for high performance lignin depolymerization.

In addition, not only β-O-4 bonds but also stilbene bonds (St, see Figure 7) also seem to be oxidized during the depolymerization. Stilbene bonds are believed to be linkages resulting from reaction of β-1 or β-5 bonds during the pulping process, and are present in the original lignin. With LA−VA treatment, stilbene bonds disappeared from NMR, suggesting an oxidation reaction of the alkene bond or a condensation reaction. Enzymatic oxidation of resveratrol, a lignin model stilbene compound, was previously reported. Crestini et al. observed that oxidation of stilbene on model bond led to ketone with up to 29% yield by the action of ABTS mediated laccase; a higher yield can be expected from VA.[55]

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In addition, infrared spectroscopy analysis was performed to confirm the overall oxidation of the structure (Figure 9).

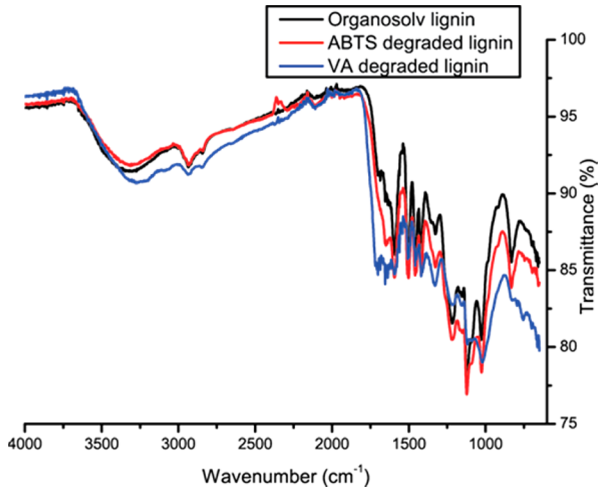

Figure 9. Infrared spectroscopy of organosolv lignin (black) and the degradation products of the laccase ABTS mediated (red) and violuric acid mediated (blue) biodegradation of lignin.

Increase of absorption in the region $\nu \approx 1600-1800$ cm^{-1}, corresponding to the C═O stretch vibration, indicates an overall increase in ketones and carboxylic acids in lignin after enzymatic treatment.

CONCLUSION

While both LA and peroxidases could degrade lignin individually, the enzyme cocktail from *Grammothele fuligo*, which contained both enzymes, induced straight repolymerization of lignin, highlighting the complexity of enzymes' synergistic interactions that still require a better understanding. Of the three lignins, organosolv is the most prone to biodegradation even though it presented an amount of *β-O*-4 bonds similar to that of alkali lignin. The amount of syringyl (S) units in lignin was identified as one of the main factors affecting the efficiency of enzymatic depolymerization, thus explaining the weak results with the Kraft and alkali lignins.

TBDMP, a novel mediator for LA mediated systems, has an efficiency in lignin degradation similar to that of ABTS, the most used LA mediator. ABTS and VA are the best agents for lignin depolymerization in this study, with up to a 73% decrease in molecular weight as compared to original lignin. 2D HMQC NMR analysis highlighted that the mediator that depolymerized lignin the most also induced more oxidation. Lignin S units were clearly targeted by the enzymatic oxidation, as well as the *β-O*-4 bonds involving S units in the C_4 position. Therefore, when considering chemical production from lignin biodegradation, a lignin feedstock with both high amounts of S units and *β-O*-4 bonds should be considered; hardwood and herb lignin are working best, while softwood lignin would be the most recalcitrant. A strong correlation was established between the redox potential of lignin model molecules and the lignin oxidation by LA mediated systems.

Highly purified and concentrated enzymes are costly, and single enzymatic systems have limited efficiency in degrading lignin. Enzymatic cocktail from white fungi secretome is a cheaper alternative for biorefineries implementation, but complex interactions between the different agents highly hinder the depolymerization process. LA mediated systems present another approach for lignin valorization in biorefineries. VA and TBDMP are good candidates as mediator for depolymerization. The future of lignin valorization by enzymatic degradation might lie in a combination of mediators instead of a combination of enzymes, the mechanisms of which are still too poorly understood.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b01426.

Description of GPC analysis of organosolv lignin degraded by LA mediated systems, different lignin linkages, structures identified by $^{13}C/^{1}H$ correlation 2D NMR, $^{13}C/^{1}H$ correlation HMQC 2D NMR analysis, calculation of S/G/H ratio and degree of condensation, and analysis of three lignin structures (PDF)

AUTHOR INFORMATION

Corresponding Authors

*Tel.: +61(0)399054600. E-mail: kei.saito@monash.edu.
*Tel.: +33(0)352620462. E-mail: florent.allais@agrosparistech.fr.
*Tel.: +61(0)399059180. E-mail: gil.garnier@monash.edu.

ORCID

Gil Garnier: 0000-0003-3512-0056
Florent Allais: 0000-0003-4132-6210
Kei Saito: 0000-0002-5726-8775

Notes

The authors declare no competing financial interest.

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Chapter 3

Linear bio-based aromatic polymers from syringic acid, S type degradation fragment from lignin

3 Enzymatic degradation of lignin

In this chapter syringic acid, a S type degradation fragment from lignin, is studied to create different monomers. Two options are considered: functionalisation through the phenol or the acid moieties. Both strategies are tried out to yield polymers with polystyrene like structure.

Linear bio-based aromatic polymers from syringic acid, S-type degradation fragment from lignin

Lionel F. Longe[a], Gil Garnier[b] and Kei Saito*[a].*

[a] School of chemistry, Bioresource Processing Research Institute of Australia (BioPRIA), 17 Rainforest walk, Monash University, Clayton 3800, Australia

[b] Department of Chemical Engineering, Bioresource Processing Research Institute of Australia (BioPRIA), 15 Alliance Lane, Monash University, Clayton 3800, Australia

* Corresponding authors email: kei.saito@monash.edu, tel: +61(0)399054600, email: gil.garnier@monash.edu, tel: +61(0)399059180

KEYWORDS: biomass – aromatic polymer – renewable - monolignol – syringic acid – syringaldehyde – radical polymerization -.

ABSTRACT: Degradation products of lignin, a widely available biopolymer, represent the most abundant feedstock for aromatics in nature. In this work, we focused on syringic acid (SA), derived from lignin S-type monolignol, to synthesise different monomers for radical polymerisation. The resulting polymers have molecular weight spanning over a wide range which depends on the monomer, with up to M_n = 1,100,000 and $Đ$ = 2.8. The novel polymers exhibit a thermal stability higher than 350°C and differentiated glass transition temperatures, ranging from 105°C to 120°C.

One of the monomers synthesised with a free carboxyl group was able to polymerise in water - instead of organic solvent - and resulted in a water soluble polymer. Monomers from syringic acid offer a unique bio-based alternative to oil-based polystyrene in industry for a wider range of applications.

INTRODUCTION:

Bio-based polymers are of growing interest as alternatives to petroleum plastics. Polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), polyvinyl chloride (PVC) and polystyrene (PS) constitute the top five polymers produced worldwide[1] and their renewable alternatives have been widely studied[2-5]. However, the emergence of aromatic biopolymers as polystyrene replacement has been hindered by the restricted availability of aromatic compounds in biomass. Lignin, the second most abundant biopolymers on earth, is a polyphenol highly recalcitrant to depolymerisation due to its extremely heterogeneous and crosslinked structure. This natural macromolecule results from the oxidative polymerisation of three monomers also known as monolignols, namely p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol; their ratio depends on the type of plant and environmental factors (Figure 1). Once polymerised, those monolignols form three different types of monomeric units labelled H, G or S units, respectively. By extension, after lignin degradation, the small depolymerised products can also be labelled S, G or H-type fragments based on the number of methoxy group in positions 3 and 5 of the aromatic. Recent advances in depolymerisation technics and purification processes now enables the use of a large range of aromatic compounds as bio-sourced platform chemicals.

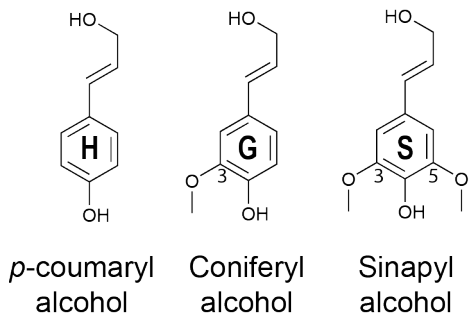

Figure 1. Three lignin monolignols responsible for the creation of (from left to right) *p*-coumaryl, guiacyl and syringyl units. After depolymerisation, those units will yield H-, G- or S-type lignin degradation fragments, respectively.

Several phenols have already been successively isolated from lignin and considered for polymerisation. The main studied strategy is generally poly-condensation. The resulting polymers are typically studied as bio-renewable alternatives to polycarbonate or polyethylene terephthalate[6-8]. With this approach, inclusion of the arene structure in the polymer chain and the addition of comonomer offers great tunability of the final polymer properties. However, functionalisation and copolymerisation often largely increase the ultimate petroleum-based fraction in the final composition.

Another approach to aromatic polymer synthesis is to use the aromatic ring as the side chain, as in polystyrene (PS). Bio-based polymers with PS-like configuration offer several advantages as compared to the polycondensation method. Simple one-step monomer functionalisation combined with the elimination of comonomer allows to synthesize aromatic-rich polymers with an overall much greener process and very high loading of renewable material. Furthermore, monomer synthesis typically leaves one functional group unreacted (phenol or side chain)

allowing for wider application such as post functionalisation. Such a configuration permits free radical polymerisation or other chain growth polymerisation.

S and G-type lignin degradation fragments include small phenol compounds such as vanillin and syringic acid. Many studies have focussed on vanillin as platform chemical for synthesising bio-based aromatic polymers[8-14]. Fache et al. extensively reviewed different functionalisation strategies to create different monomers for polycondensation and radical polymerisation. The 22 monomers can then be used for the synthesis of bio-based polyester, polyurethanes, non-isocyanate polyurethanes (NIPU) and epoxy polymers[15]. S-type phenols, despite being a major degradation product of lignin from hardwood, have only recently attracted attention as bio-renewable feedstock. Epps et al. studied the polymerisation of monomer from syringol[16]. This work further highlighted that similar monomer synthesised from G-type compound – vanillin, guaiacol, 4-methylguaiacol and 4-ethylguaiacol – result in polymers with different properties. Copolymerisation allows fine tuning of physicochemical or thermal properties, such as glass transition temperature or intrinsic viscosity to fit applications. Phenolic compounds with aldehyde or aliphatic side chains have been identified as products of lignin pyrolysis[17-19]. However, as milder and greener depolymerisation processes gain traction, phenolic depolymerisation products tend to be less degraded and retain most of their functional group. Phenolic compounds from oxidative degradation with acid or aldehyde group now offer more possibilities for monomer synthesis and polymer functionalisation.

In this work, we present the synthesis of several novel linear polymers with PS-like structures from biomass feedstock. To the best of our knowledge, functionalisation and polymerisation of S type degradation fragments with carboxylic acid functional group has never been reported. Two different strategies were considered for functionalisation by attaching an alkene group on either

the acid group or the phenol function. Although the former strategy required further protection, polymerisation yielded a pure white and high molecular weight polymer. Functionalisation through the phenol group allowed the resulting monomer to be polymerised in aqueous medium and yielded a water soluble polymer. Thermal analysis showed high thermal stability and a glass transition temperature ranging from 105°C to 120°C. Application in water soluble paint to replace the polymeric binder and possible use in water treatment will be studied in a future study. Three important principles of green chemistry - safer solvent, benign chemical and atom economy - were applied.

MATERIALS AND METHODS

Chemicals and solvents: Chemicals were purchased from Sigma Aldrich (Sydney, NSW, Australia) (syringic acid, vinyl benzyl chloride, pyridine, acetic anhydride, benzoyl chloride, 4-dimethylaminopyridine (DMAP), trimethylamine (TEA), methacryloyl chloride, methacrylic anhydride, 2,2′-Azobis(2-methylpropionitrile) (AIBN), 1,1′-Azobis(cyclohexanecarbonitrile) (ACCN), dicumyl peroxide, benzoyl peroxide, ammonium persulfate (APS), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), sodium formaldehyde sulfoxylate, sodium dodecyl sulphate, span 80, lithium bromide), from Merck PTY (Melbourne, VIC, Australia) (Sodium bicarbonate, sodium nitrate, potassium phosphate monobasic, sodium chloride, anhydrous manganese sulphate, sodium hydroxide, deuterated chloroform, deuterium oxide, sodium deuteroxide), from TCI (Tokyo, Japan) (deuterated dimethyl sulfoxide or DMSO) and from FUJIFILM Wako Chemicals (Richmond, VA, USA) (2,2'-Azobis[2-methyl-*N*-(2-hydroxyethyl)propionamide] or VA-086) and used as received. Reagent grade solvents (anhydrous dimethylformamide or DMF, diethyl ether, hydrochloric acid, xylene, ethylene glycol, chloroform, toluene, methanol) were purchased from Thermo Fisher Scientific

(Melbourne, VIC, Australia). Poly(vinyl alcohol) (PVA) was synthesized by our group and had molecular weight of *ca*. 20,000 $g.mol^{-1}$.

The molecular weight of polymer samples was analysed by size exclusion chromatography (SEC) in DMF or water. SEC in DMF was performed at 40°C on a Tosoh EcosHLC-8320 equipped with double detectors (RI, UV 280 nm) using Tosoh alpha 4000 and 2000 columns. DMF/LiBr 0.1 M was used as a mobile phase (flow rate 1 mL/min). Polystyrene standards were used for the calibration. Similarly, aqueous SEC was performed on a Tosoh EcosHLC-8320 equipped with double detectors (RI, UV 305 nm) using three TSKgel PWXL columns (TSKgel G5000PWxL, TSKgel G6000PWxL and TSKgel MPWxL) connected in series. The mobile phase solution was prepared by mixing sodium nitrate and sodium bicarbonate (0.1 M $NaNO_3$ 0.1 M $NaHCO_3$), and the flow rate was set to 1 mL/min. Polyacrylic acid standards were used for calibration.

^{1}H experiments were performed in deuterated DMSO on a Bruker Avance 400 MHz NMR spectrometer equipped with a CryoProbe Prodigy 5 mm ^{1}H/^{1}H-^{13}C/^{15}N probe. NMR experiments were performed with the sample held at 25±0.1 °C. Chemical shifts for all experiments are referenced using the Unified Scale relative to 0.3 % tetramethylsilane in deuterated chloroform[20, 21].

Degradation temperatures were measured by thermogravimetric analysis (TGA) performed on a Simultaneous Thermal Analyser (STA) 8000 from PerkinElmer. Heating rate was fixed to 30°C/min under nitrogen flow.

Glass transition temperatures were measured by differential scanning calorimetry (DSC) on a DSC 8000 from PerkinElmer fitted with a dual-stage heat-exchanger cooling system Intracooler

2. Samples were analysed in aluminium pans and under nitrogen atmosphere. The heating rate was set to 10°C/min and cooling rate 150°C/min.

An Agilent Technologies Cary 630 FTIR was used for IR spectrum acquisition. Data analysis was carried on Respro software.

Mass analysis was coupled with LC-UV equipment with the following parameters. An Agilent 1260 Infinity liquid chromatograph system was coupled with 6120 series quadrupole mass spectrometer. A Kinetex 5 µm C18 100 Å column (250 mm x 4.6 mm ID particle size 5 µm) was used with 0.2% HCOOH in water and 0.2% HCOOH in acetonitrile as gradient of mobile phase (0.8 mL/min) maintained at 30°C. Mass parameters were set as follow: drying gas flow of 9.8 L/min, nebuliser pressure of 28 psi, drying gas temperature of 300°C, and capillary voltage of 3000 V. Mass scan range was set to 100-900 Da with a cycle time of 1.57 sec per cycle.

Compound **1** synthesis

Functionalisation of syringic acid by vinyl benzyl chloride was conducted in anhydrous DMF. 10 g of syringic acid (50.5 mmol) were added in a dried round bottom flask to 5.35 g of anhydrous sodium carbonate (50.5 mmol) along with the solvent (50 mL). After a few minutes of stirring and N_2 flushing, vinyl benzyl chloride (0.7 mL, 5.05 mmol) was added slowly through a septum. After addition, the solution was heated at 60°C and let to react overnight. For work up, the reaction mixture was allowed to cool down. 150 mL of water were added to the solution, which was then extracted five times with 30 mL of diethyl ether. The combined organic phases were then washed successively with water (30 mL, three times) and brine (30 mL). After drying on $MgSO_4$, the organic phase was evaporated to yield compound **1**, yield 52%. More than 95% of unreacted syringic acid could be recovered by acidification and filtration of aqueous phase to be reused in future reactions. ^{1}H-NMR (400 MHz, d6-DMSO): δ 7.49 (d, 2H, ArH), δ 7.44 (d,

2H, ArH), δ 7.24 (s, 2H, ArH), 6.73 (m, H, HC=), 5.86 (d, H, H_2C=), 5.31 (s, 2H, CH_2), 5.26 (d, H, H_2C=), 3.81 (s, 6H, CH_3). ^{13}C-NMR (100 MHz, d6-DMSO): δ 166 (OO-C-Ar), δ 148 (Ar), δ 138 (Ar), δ 134 (Ar), δ 129 (Ar), δ 126 (Ar), δ 114 (Ar-CH=), δ 108 (CH_2=), δ 67 (Ar-CH_2-O), δ 56 (O-C-H_3). IR (KBr): ν_{O-H} = 3432 cm^{-1}, ν_{Ar-H} = 3077 cm^{-1}, $\nu_{=C-H}$ = 3059 cm^{-1}, ν_{C-H} = 2950 cm^{-1}, $\nu_{C=O}$ = 1746 cm^{-1}, $\nu_{C=C}$ = 1674 cm^{-1}, ν_{Ar-Ar} = 1602 cm^{-1}, ν_{C-H} = 1473 cm^{-1}, ν_{C-H} = 1369 cm^{-1}, ν_{C-O} = 1254 cm^{-1}, ν_{C-O} = 1137 cm^{-1}. m/z: 314.0 (6.3%), 199.0 (13.5%), 181.0 (19.5%), 153.0 (100.0%), 131.0 (12.4%), 117.0 (25.8%), 103.0 (7.7%), 43.0 (14.5%).

Compounds **2** and **3** synthesis

Protection of compound **1** (5 g, 15.8 mmol) by acetate group was conducted in anhydrous pyridine (50 mL). After flushing for about 15 min with nitrogen acetic anhydride (1.8 mL, 19.11 mmol) was added through a septum. Heating was set to achieve reflux and the solution was let to react overnight. After cool-down 200 mL of diethyl ether was added and the organic phase was washed successively with 1 M HCl (three times, 100 mL), 1 M NaOH (three times, 100 mL) and brine (150 mL). After drying on $MgSO_4$, the solvent was evaporated under reduced pressure to yield compound **2** in high yield (>99%). ^{1}H-NMR (400 MHz, d6-DMSO): δ 7.50 (d, 2H, ArH), δ 7.40 (d, 2H, ArH), δ 7.33 (s, 2H, ArH), 6.79 (m, H, HC=), 5.87 (d, H, H2C=), 5.38 (s, 2H, CH2), 5.27 (d, H, H2C=), 3.83 (s, 6H, CH3), 2.29 (s, 3H, OOCCH3). ^{13}C-NMR (100 MHz, d6-DMSO): δ 172 (ArOO-C-CH3), δ 167 (OO-C-Ar), δ 152 (Ar), δ 137 (Ar), δ 136 (Ar), δ 133 (Ar), δ 128 (Ar), δ 126 (Ar), δ 115 (Ar-CH=), δ 108 (CH2=), δ 69 (Ar-CH2-O), δ 59 (O-C-H3), δ 21 (OOC-CH3). IR (KBr): ν_{Ar-H} = 3053 cm^{-1}, $\nu_{=C-H}$ = 3064 cm^{-1}, ν_{C-H} = 2943 cm^{-1}, ν_{C-H} = 2932 cm^{-1}, $\nu_{C=O}$ = 1758 cm^{-1}, $\nu_{C=C}$ = 1673 cm^{-1}, ν_{Ar-Ar} = 1662 cm^{-1}, ν_{C-H} = 1488 cm^{-1}, ν_{C-H} = 1357 cm^{-1}, ν_{C-O} = 1243 cm^{-1}, ν_{C-O} = 1173 cm^{-1}. m/z: 314.0 (6.3%), 241.0(19.6%), 223.0 (23.3%), 195.0 (100%), 131.0 (14.1%), 117.0 (16.7%), 103.0 (6.5%), 43.0 (34.3%).

Similarly, protection by benzoate group was performed in anhydrous pyridine. Compound **1** (5,0 g, 15.8 mmol) was dissolved in pyridine (50 mL) while flushing for about 15 min with nitrogen. The reaction flask was maintained on ice while vinyl benzyl chloride (2.2 mL, 18.9 mmol) was slowly added through a septum. The reaction was maintained on ice for 1 h, then allowed to continue at room temperature (RT) for a further 4 h. 200 mL of diethyl ether were then added to the flask and the solution was washed successively with 1 M HCl (three times, 100 mL), 1 M NaOH (three times, 100 mL) and brine (150 mL). After drying on $MgSO_4$, the solvent was evaporated under reduced pressure to yield compound **3** in high 90% yield. ^{1}H-NMR (400 MHz, d6-DMSO): δ 8.11 (d, 2H, ArH), δ 7.77 (t, H, ArH), δ 7.61 (d, 2H, ArH), δ 7.48 (d, 4H, ArH), δ 7.38 (s, 2H, ArH), 6.76 (m, H, HC=), 5.88 (d, H, H_2C=), 5.40 (s, 2H, CH_2), 5.27 (d, H, H_2C=), 3.83 (s, 6H, CH_3). ^{13}C-NMR (100 MHz, d6-DMSO δ 168 (OO-C-Ar), δ 163 (ArOO-C-Ar), δ 152 (Ar), δ 138 (Ar), δ 136 (Ar), δ 133 (Ar), δ 130 (Ar), δ 129 (Ar), δ 128 (Ar), δ 127 (Ar), δ 116 (Ar-CH=), δ 107 (CH_2=), δ 67 (Ar-CH_2-O), δ 56 (O-C-H_3). IR (KBr): ν_{Ar-H} = 3073 cm^{-1}, $\nu_{=C-H}$ = 3058 cm^{-1}, ν_{C-H} = 2957 cm^{-1}, $\nu_{C=O}$ = 1763 cm−1, $\nu_{C=C}$ = 1622 cm^{-1}, ν_{Ar-Ar} = 1675 cm^{-1}, ν_{C-H} = 1493 cm^{-1}, ν_{C-H} = 1335 cm^{-1}, ν_{C-O} = 1242 cm^{-1}, ν_{C-O} = 1178 cm^{-1}. m/z: 314.0 (8.9%), 303.0 (26.7%), 285.0 (23.4%), 257.0 (100%), 131.0 (17.1%), 117.0 (23.8%), 103.0 (8.2%), 43.0 (5.4%).

Compound **4** synthesis

Syringic acid (10,0 g, 50.5 mmol) was added in anhydrous DMF (50 mL), along with TEA (17.5 mL, 0.125 mol) and DMAP (616 mg, 5.05 mmol) and stirred until complete dissolution. The reaction mixture was then kept on ice and flushed with nitrogen for 1 h. Methacryloyl chloride (4.5 mL, 46.1 mmol) was carefully and slowly added through a septum over ~20 min. The reaction mixture was further kept on ice for 3 h, then let overnight at room temperature. 500

mL of water were added to the mixture at the end of the reaction, and extracted five times with 50 mL chloroform fractions. The combined organic phases were then washed with pH = 6 buffer solution (five times, 50 mL), 1 M HCl (50 mL) and brine (50 mL) successively. The organic phase was then dried on $MgSO_4$ and solvent was removed under reduced pressure. Compound **4** was obtained with 73% yield. ^{1}H-NMR (400 MHz, d6-DMSO): 7.30 (s, 2H, ArH), 6.28 (s, H, H_2C=), 5.91 (s, H, H_2C=), 3.81 (s, 6H, OCH_3), 1.99 (s, 3H, CH_3). ^{13}C-NMR (100 MHz, d6-DMSO): δ 166 (OO-C-Ar), 148 (Ar), 138 (Ar), 134 (Ar), 129 (Ar), 126 (Ar), 114 (Ar-CH=), 108 (CH_2=), 67 (Ar-CH_2-O), 56 (O-C-H_3). IR (KBr): ν_{O-H} = 3598 cm^{-1}, ν_{Ar-H} = 3023 cm^{-1}, $\nu_{=C-H}$ = 3084 cm^{-1}, ν_{C-H} = 2943 cm^{-1}, ν_{C-H} = 2926 cm^{-1}, $\nu_{C=O}$ = 1743 cm^{-1}, $\nu_{C=C}$ = 1645 cm^{-1}, ν_{Ar-Ar} = 1595 cm^{-1}, ν_{C-H} = 1474 cm^{-1}, ν_{C-H} = 1401 cm^{-1}, ν_{C-O} = 1234 cm^{-1}, ν_{C-O} = 1269 cm^{-1}. m/z: 249.0 (13.7%), 221.0 (35.9%), 198.0 (100%), 69.0 (43.5%), 43.0 (23.6%).

Alternatively, compound **4** can be obtained by the addition of methacrylic anhydride to syringic acid. Syringic acid (10.0 g, 50.5 mmol) was dissolved in anhydrous DMF (50 mL) along with DMAP (123 mg, 1.01 mmol). The reaction mixture was flushed with nitrogen for about 1h. Methacrylic anhydride (11.3 mL, 75.7 mmol) was added through a septum and the solution was stirred for one hour at room temperature before increasing heating to 50°C and allowing the reaction to proceed overnight. After cooldown, water and chloroform were added in the reaction flask. The organic phase was collected and the aqueous phase was further extracted four times. All organic phases were combined and washed five times with pH = 6 buffer, 1 M HCl and brine successively. The organic phase was then dried on $MgSO_4$ and the solvent removed under reduced pressure. Compound **3** was obtained with 68% yield.

Polymerisation of compounds **1**, **2**, **3** and **4**

Radical polymerisations in bulk or organic solvent were conducted in the following conditions. Monomer was dissolved in solvent (if any) at concentration 0.4 $M.L^{-1}$ then heated to polymerisation temperature: 70°C (AIBN), 90°C (ACCN), 110°C (benzoyl peroxide) or 140°C (dicumyl peroxide). Finally, the initiator was added to the reaction and heating was maintained for 6 hours. After cool down reaction mixture was added dropwise to acetone or diethyl ether to crush out and recover the polymer.

For emulsion polymerisation, the monomer was dissolved in toluene or xylene. PVA and sodium dodecyl sulphate (10 $\%_{w/w}$ to monomer each) were then added and well mixed with a small amount of water while bubbling nitrogen for about 15 min. Finally, initiators were added and heating was turned on as required. After 6 hours the reaction mixture was collected and crushed out in acetone to recover the polymer.

Aqueous polymerisation for compound 4 was conducted in alkaline condition (NaOH/compound **3** = 1:1 molar ratio). Nitrogen was bubbled for about 15 min. Heating was set to polymerisation temperature and initiator were added to reaction: APS (80°C), AAPH (80°C), AIBN (80°C), VA-086 (90°C) and *tert*-butyl hydroperoxide (95°C). After 6 hours, the solution was collected and purified by dialysis on Snakeskin molecular weight cut off 3,000 Da. The remaining product was then isolated by lyophilisation.

Reverse emulsion polymerisation was performed by dissolving the monomer in alkaline solution (NaOH/compound **3** = 1:1 molar). The monomer solution was then added dropwise in a vigorously stirred solution of toluene and PVA plus span 80 (10 $\%_{w/w}$ to monomer each). Finally, heating was turned on. After about 6 hours the reaction the mixture was collected and crushed out in acetone to recover the polymer.

RESULTS AND DISCUSSION

Functionalisation through acid group

As a first step to create aromatic polymer from syringic acid, a monomer with unsaturation has to be synthesized. Two clear pathways for functionalisation are to make use of either the phenolic group on C4 or the carboxyl group on C1. Phenol reactivity is known to be low and syringic acid furthermore showcases risk of deactivation by steric hindrance from the two adjacent methoxy groups. For these reasons the vinyl group was added by reaction with the carboxylic acid as shown in Figure 2.

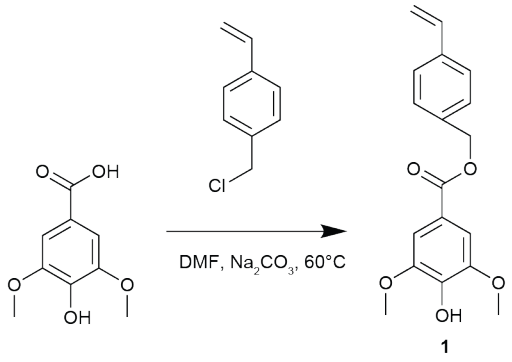

Figure 2. Synthesis of compound **1** by reaction of syringic acid with vinyl benzyl chloride in DMF.

Benzoyle chloride reaction in DMF with syringic acid yielded compound **1** with 84% yield. However a high amount of di-reacted syringic acid was also formed. Formation of ether bond in the phenol position was confirmed by NMR and masss spectroscopy (Figures SI1 and SI2). Di-reacted syringic acid com during radical polymerisation. Ten times excess of syringic acid successfully and entirely removed the side reaction (purity >99%) while more than 95% of the unreacted SA could be recovered and be reused.pound is not desired as even a small amount will lead to crosslinking reaction

Screening of solvent and initiator for compound **1** could not point out suitable conditions for free radical polymerisation (Table 1). The resulting molecular weight after reaction could not be obtained higher than M_n = 1,700. NMR analysis indicated that more than 86% of the treated monomer still exhibited an intact vinyl group. Phenolic compound are well known for their anti-oxidant and radical scavenging properties[22-25] due to the resonance in the aromatic ring and the formation of quinone stabilising the overall structure (Figure 3). Thus it is highly probable that although the two adjacent methoxy groups were thought to hinder that effect, the phenol captures radical after the initiation step, quickly leading to termination of the polymerisation.

Table 1. Polymerisation screening for compound **1**.

Polymerisation medium	Initiator	M_n (g.mol^{-1})
Bulk	AIBN	1,650
Bulk	ACCN	1,700
Bulk	Benzoyl Peroxide	2,300
Bulk	Dicumyl Peroxide	1,400
Ethylene Glycol	AIBN	800
Ethylene Glycol	ACCN	800
Ethylene Glycol	Benzoyl Peroxide	800
Ethylene Glycol	Dicumyl Peroxide	1,600
Xylene	AIBN	800
Xylene	ACCN	800
Xylene	Benzoyl Peroxide	800

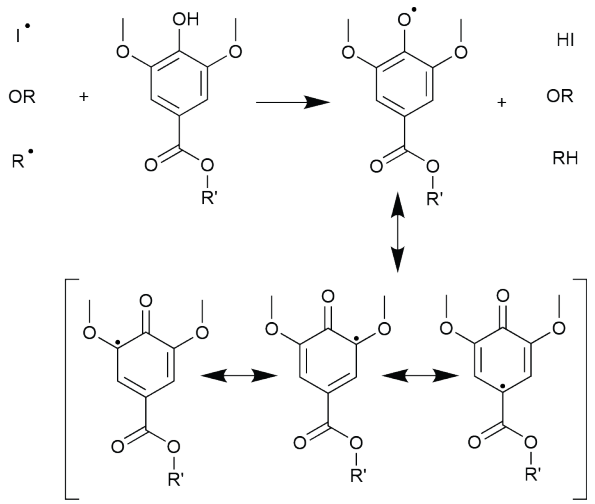

Figure 3. Radical scavenging behaviour of compound 1. Activated initiator species (I•) and growing chain (R•) are deactivated by the capture of radical. The resulting structure is stabilised by resonance and can no longer participate in the polymerisation.

To favour the propagation reaction during polymerisation, the phenol group has to be protected. Two pathways were explored to eliminate the radical scavenging properties of compound **1** and enable free radical polymerisation. Protection by acetic anhydride and benzoyl chloride lead to compounds **2** and **3** respectively with high yield and high purity (Figure 4).

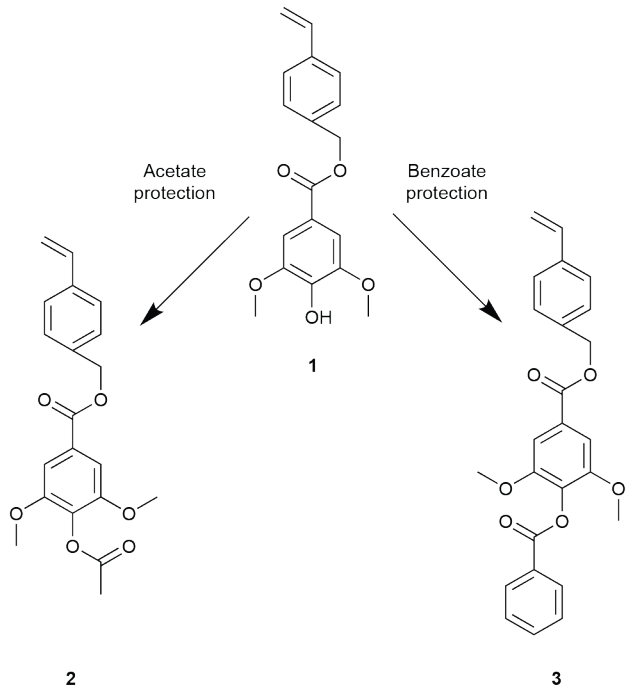

Figure 4. Protection of phenol group by acetic anhydride or benzoyl chloride, to yield compounds **2** and **3**.

After screening for different polymerisation conditions compounds **2** and **3** proved to exhibit two contrasting polymerisation behaviours. The acetyl protected compound **2** was polymerised up to M_n = 2,000-3,000 while compound **3** reached up to M_n = 1,088,000, (Table 2, Figures 5 and 6). The resulting polymer formed a surprisingly pure white fluffy powder, while the high content in aromatic rings was rather expected to yield an orange/yellowish compound.

Table 2. Compounds **2** and **3** free radical polymerisation molecular weight characterisation. Polymerisation of compound **3** yields high molecular weight polymer while compound **2** only produces small oligomers.

Protecting group	Initiator	M_n (g.mol^{-1})	$Đ$
Acetate (**2**)	AIBN[a]	2,400	3.7
	ACCN[a]	2,300	5.1
	Benzoyl Peroxide[a]	2,800	3.6
	Dicumyl Peroxide[a]	2,000	2.6
Benzoate (**3**)	AIBN[b]	9,500	25.8
	ACCN[b]	41,000	4.3
	Benzoyl Peroxide[b]	1,100,000	2.8
	Dicumyl Peroxide[a]	10,000	11.0

[a]: bulk polymerisation, 1%$_{mol}$ initiator

[b]: bulk polymerisation, 0.1%$_{mol}$ initiator

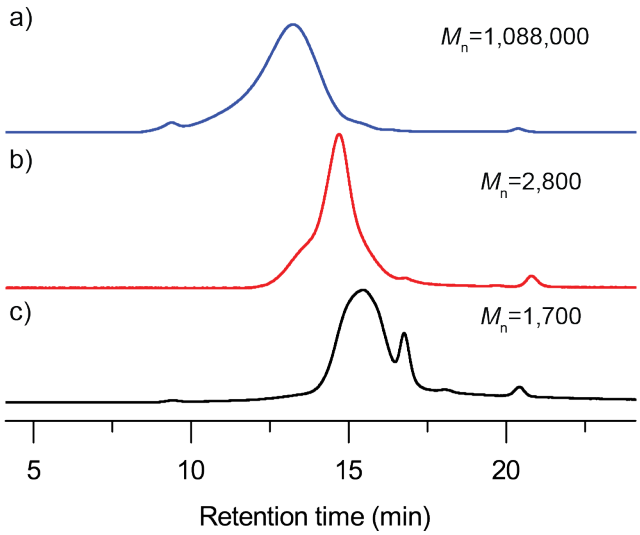

Figure 5. SEC analysis of the polymer synthesized by free radical polymerisation of a) compound 3, b) compound 2 and c) compound 1. Signal below 10 min retention time are disregarded.

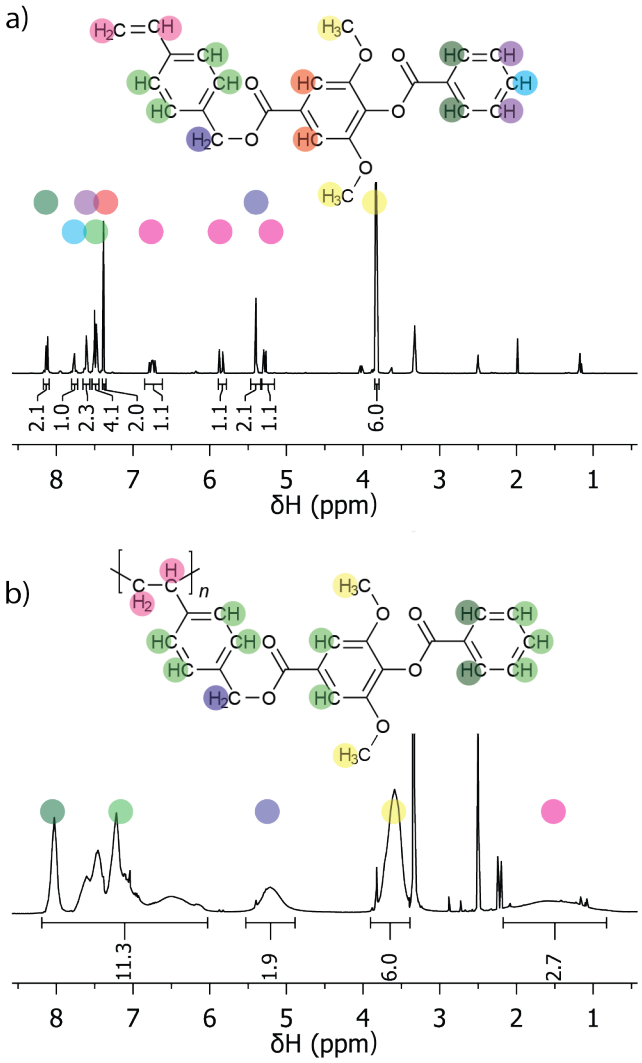

Figure 6. ^{1}H NMR in d6-DMSO analysis of a) compound 3 and b) polymer synthesised by free radical polymerisation.

Upon heating, the purified polymer showed high stability towards high temperature with a degradation temperature measured by TGA around 350°C. DSC indicated a clear T_g at 118°C (Figure 7). Biopolymers with high thermal stability and processable properties are not widespread in industry. Compound **3** proves that aromatic biopolymers could become a platform for performant and high value added industrial polymer.

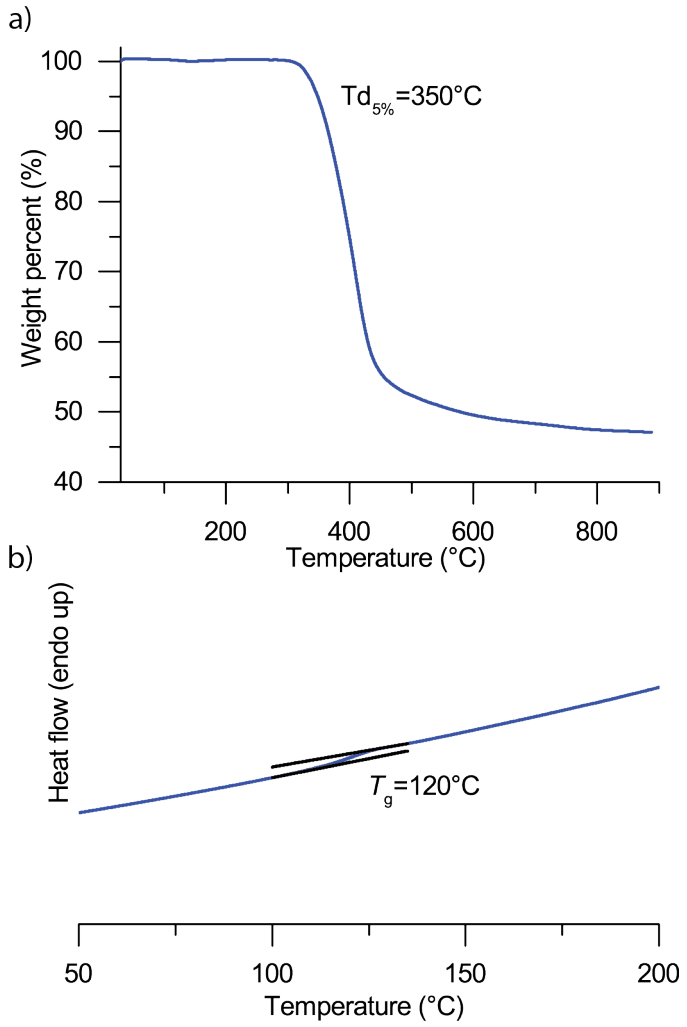

Figure 7. Polymerisation of compound 3 yields a polymer with thermal stability higher than 350°C. High glass transition temperature is also detected by DSC.

Functionalisation through phenol

The phenol radical scavenging properties hinder polymerisation. However, protecting reaction decreases our overall bio-based content. By considering the phenol moieties instead of the carboxylic group for addition of alkene group both functionalisation and protection are combined to increase efficiency. Methacryloyl chloride reacted with syringic acid to yield compound **4** in high yield and high purity. Methacrylic anhydride was also considered as a greener alternative to

methacrylic acid. Atom economy is therefore reduced by side production of methacrylic acid (Figure 8). Furthermore, complete purification is only achieved after intensive purification. However, as acid chloride requires energy intensive processes and involves toxic chemicals to be synthesized, the use of the anhydride is preferred.

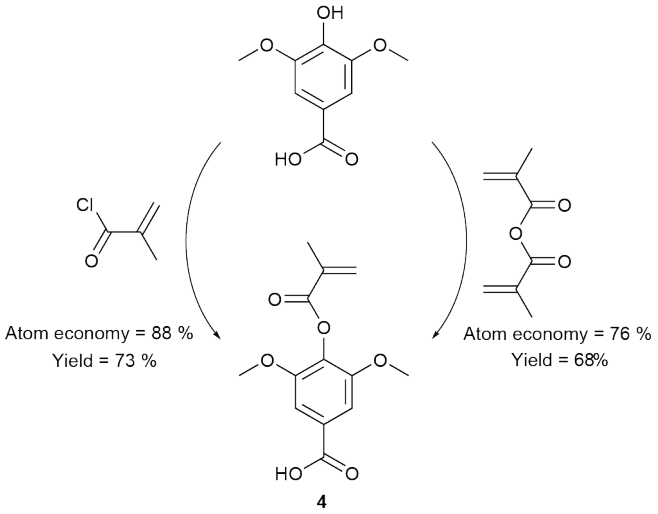

Figure 8. Synthesis of compound **4** can be achieved by methacryloyl chloride or methacrylic anhydride.

Monomer **4** polymerised readily in aqueous solvent while organic solvent only yielded low molecular weight oligomers (Table 3 and supporting information Table SI2). VA-086 proved to be the best initiator for solution polymerisation with molecular weight up to M_n = 23,500. In reverse-emulsion conditions AIBN initiator increased the final molecular weight up to 34,000 g.mol^{-1}. However, a simple aqueous polymerisation is preferred as it offers a reduced waste process. TGA analysis of the obtained polymer indicated that thermal stability diminished compared to the polymer from compound **3**, with a degradation temperature of 300°C. The glass transition temperature was measured by DSC at T_g = 105°C (Figure 9).

Table 3. Compounds **2** and **3** free radical polymerisation molecular weight characterisation. Polymerisation of compound **3** yields high molecular weight polymer while compound **2** only produces small oligomers.

Initiator	$\frac{[\text{initiator}]}{[\text{monomer}]}$	M_n (g.mol^{-1})	$Đ$
APS	2%	11,300	2.8
AAPH	2%	1,400	2.0
AIBN	2%	1,500	1.8
VA-086	2%	23,500	2.6
ACCN	2%	3,700	3.0
tert-butyl peroxide	2%	6,800	2.9
AIBN (reverse emulsion)	2%	34,000	3.6
ACCN (reverse emulsion)	2%	1,500	1.5

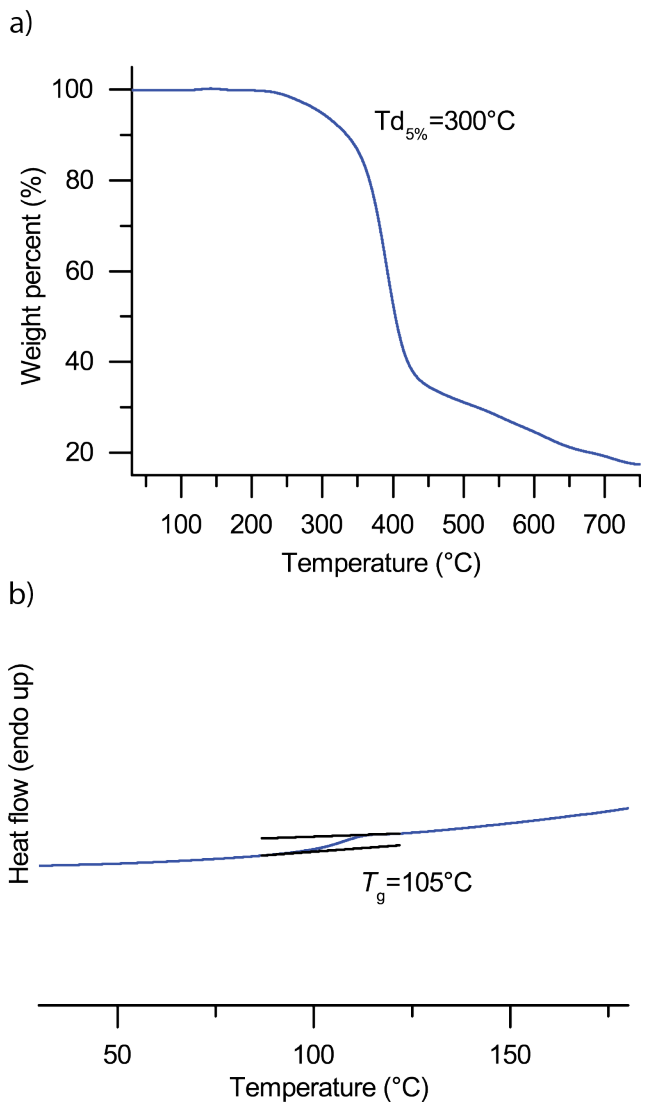

Figure 9. a) Thermogravimetric analysis of polymer synthesised by free radical polymerisation of monomer **4** and b) its glass transition temperature measured by differential scanning calorimetry.

The high amount of free carboxylic acid functional group allowed the polymer from compound **4** to be water soluble. Future application such as post functionalisation reaction can be considered in greener condition without the use of organic solvent.

Conclusions

Monomers from S-type lignin fragment were successfully synthesised from syringic acid by two different functionalisation strategies. Phenol moieties, although expected to be inactivated by steric hindrance from two neighbouring methoxy groups, exhibited intense anti-oxidant properties. This radical scavenging behaviour proved to greatly hinder free radical polymerisation and the use of protecting group was required. Depending on the protection strategies, resulting polymer could be either obtained in high yield and low molecular weight (compound **2**), or super high molecular weight but low yield (compound **3**). The polymer was found to have great thermal stability and a glass transition temperature around 120°C.

A second functionalisation strategy was designed in order to increase the overall bio-based material loading in the final product. As a result of free carboxylic acid group, a water soluble polymer was synthesized in organic-solvent free condition. The polymer glass transition temperature was found to be very similar to polystyrene. It is clear that this biopolymer now offers a reliable green alternative for numerous application over a wide range of industries, and is bound to witness a quick development in the future.

ASSOCIATED CONTENT

The following files are available free of charge.

Supporting information, includes some NMR, mass spectrometry and SEC data (PDF)

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AUTHOR INFORMATION

Corresponding Author:

Kei Saito, email: kei.saito@monash.edu, tel: +61(0)399054600,

Gil Garnier, email: gil.garnier@monash.edu, tel: +61(0)399059180

AUTHOR CONTRIBUTION:

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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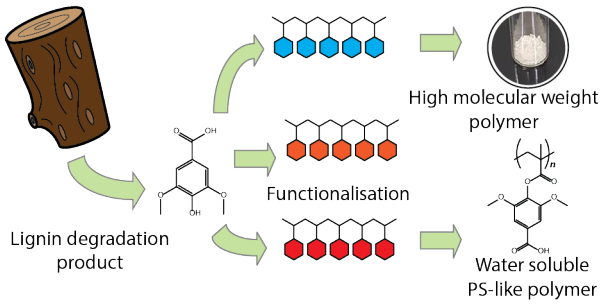

SYNOPSIS: The presented work review the use of wood biomass feedstock to design linear aromatic polymers with great range of physical and thermal properties in order to offer renewable alternative to high volume oil-based plastics.

Chapter 4

Synthesis of lignin-based phenol terminated hyperbranched polymer

4 Synthesis of lignin-based phenol terminated hyperbranched polymer

In this chapter protocatechuic acid monomer is tested for polycondensation to create a new hyperbranched polymer. Side reaction with solvent are detected and monitored by NMR spectroscopy.

molecules

MDPI

Article

Synthesis of lignin-based phenol terminated hyperbranched polymer

Lionel Longe [1], Gil Garnier [2,*] and Kei Saito [1,*]

[1] Monash University, School of Chemistry, Bioresource Processing Research Institute of Australia (BioPRIA), Clayton 3800, Australia

[2] Monash University, Department of Chemical Engineering, Bioresource Processing Research Institute of Australia (BioPRIA) Clayton 3800, Australia

* Correspondence: kei.saito@monash.edu ; gil.garnier@monash.edu

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Abstract: In this work we proved the efficient synthesis of a bio-based hyper-branched polyphenol from a lignin degradation fragment. Protocatechuic acid was readily obtained from vanillin via alkaline conditions, and further polymerised to yield super high molecular weight hyperbranched phenol terminated polyesters. Vanillic acid was also subjected to similar polymerisation conditions in order to compare polymerisation kinetics and differences between linear and hyperbranched polymers. Overall, hyperbranched polymer was faster to polymerise and was more thermostable with a degradation temperature well above linear vanillic acid polyester. Both polymers exhibited important radical scavenging activity and present tremendous potential for antioxidant applications.

Keywords: bio-based, hyperbranched, polyphenol, antioxidant

1. Introduction

Hyperbranched polymers (HBP) have received increasing attention over the past two decades, as their field of application keeps expanding every year [1,2]. Hyperbranched polymer structure is very similar to dendrimers with a high amount of end group, often used for post functionalisation or grafting reactions. However HBP are often cheaper and are easier to synthesize. For this reason HBP have replaced dendrimers in a lot of applications, and are now commonly used in therapeutics, drug delivery, coatings, and as rheological or curing agents [3-6]. Recently, interest in the synthesis of bio-based hyperbranched polymer has quickly grown as the logical consequence of the success of green chemistry in the polymer field [7-14]. However, to our knowledge, synthesis from lignin degradation products has yet to be reported.

Polyphenols are well known for their antioxidant properties. The synthesis of efficient biobased polymers with radical scavenging properties have seen a growing interest in the past few years [15,16]. The most common strategy involves grafting highly antioxidant molecule on a polymer backbone [17,18]. Phenol terminated molecules such as caffeic acid are often investigated. To increase the phenol content, hyperbranched polymers and dendrimers are also starting to draw attention and should drastically improve the final radical scavenging activity [19-23].

In this work, we present the monomer preparation and synthesis of a phenol-terminated hyperbranched polymer. High antioxidant properties are expected and radical scavenging activity will be monitored to determine the efficacy of the synthesized polymer. Biomass feedstock will be used for the monomer synthesis in order to create a bio-renewable polymer. Vanillin can be readily obtained from lignin degradation and already has well established in industrial production and commercial markets [24]. In strong caustic conditions, vanillin will yield protocatechuic acid (PA) in high yield and high purity. We report here the synthesis of a hyperbranched polymer by

polycondensation of PA. Furthermore, similar conditions were employed with vanillic acid (VA) to compare final polymer properties. We also report the possible interaction of the monomer and polymerisation solvent, yielding side reactions and structure modification.

PA was readily obtained by caustic fusion of vanillin (Figure 1). Reaction temperature determines whether demethylation occurs in addition to the oxidation of the aldehyde group. With temperature below 250°C, vanillic acid (VA) is formed, while a higher temperature yields PA. Purity is highly dependent on mixing method and homogeneous heating.

2. Results and discussion

After isolation and purification, PA and VA were polymerised by polycondensation using Steglich esterification reaction. Polymerisation kinetics proved to be slow, possibly due to the low reactivity of phenol group. This issue can be partially circumvented by increasing the polymerisation temperature. Therefore, high boiling point polar solvent proved to be best suited for this reaction (Figure 2). Dimethyl sulfoxide (DMSO) was also considered as a high boiling point solvent for this reaction and results are discussed in the last section of this article. Steglish esterification reaction is well known for the intensive associated work-up to remove dicyclohexylurea (DHU), a side product from the acid activation by *N*,*N*'-dicyclohexylcarbodiimide (DCC) (Figure SI 1). In the present case, DCC was efficiently removed overnight by soxhlet extraction in acetone. GPC analysis only indicated a small difference between PA and VA polymers. Overall, PA polymer reached slightly higher molecular weight than VA and, correspondingly moderately faster polymerisation kinetics (Table 1). The remaining methoxy group in VA is expected to reduce the phenol by steric hindrance and can be the cause of that difference. However, GPC equipment was calibrated versus linear polystyrene standards and branched structures are known to reduce the accuracy of molecular weight determination [25]. Therefore, no major difference in polymerisation behaviour is to be noted at this stage.

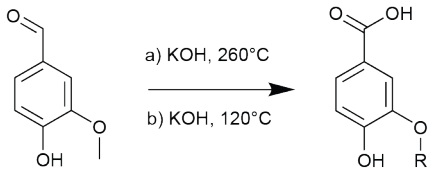

Figure 1: Synthesis of a) protocatechuic acid (PA) and b) vanillic acid (VA) from vanillin. a) R=H / b) R=CH_3

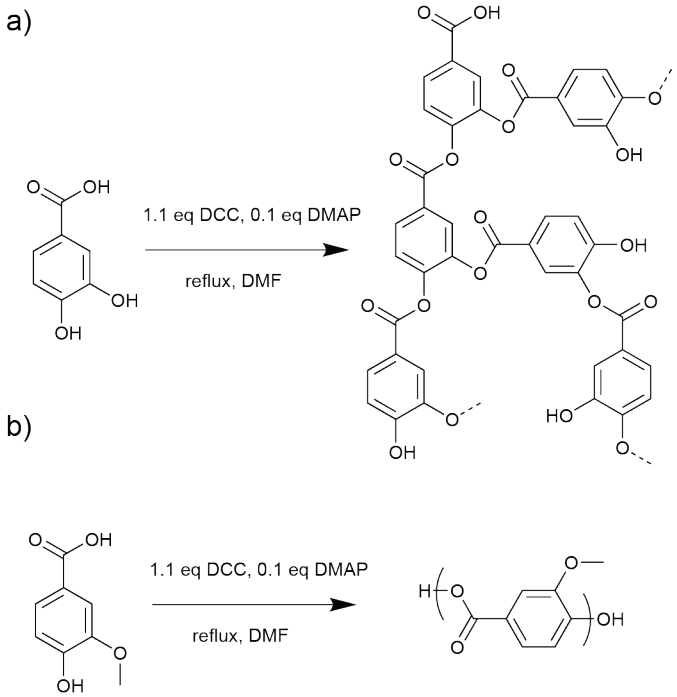

Figure 2: Polycondensation of a) protocatechuic acid (PA) and b) vanillic acid (VA) by Steglish esterification

Thermal behaviour highlighted a clear difference between both polymers, with PA-based polymer proving to be stable up to 165°C, while VA polymer started to deteriorate above 110°C. Analysis by differential scanning calorimetry (DSC) of both polymer samples could not point out any clear glass transition temperature presumably due to hyperbranched structure of PA-polymer and the low degree of polymerisation of VA polymer.

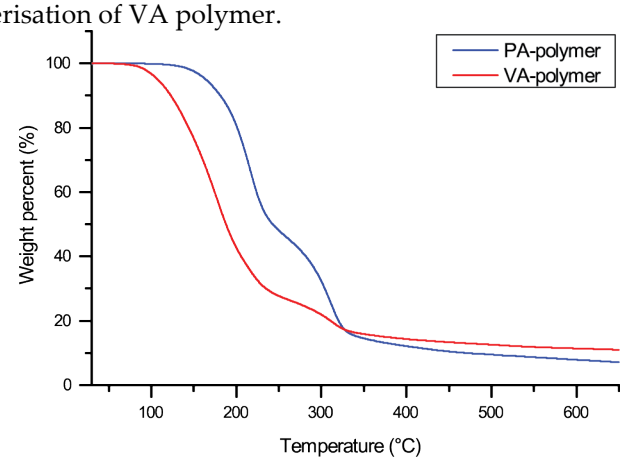

Figure 3: Thermogravimetric analysis of the decomposition of both PA and VA-based polymer

	M_n (g/mol)	$Đ$	$T_{d5\%}$
PA-based polymer	7,900	3.1	165°C
VA-based polymer	6,400	2.6	108°C

Table 1: Physical properties of polymers from protocatechuic acid and vanillic acid

One of the limiting factors to higher polymerisation was determined to be reaction temperature. DMSO was elected to allow stronger heating. All other conditions were kept constant (Figure 2). Polymerisation kinetics proved to be slow but, with long reaction time, ultra-high molecular weight

polymers could be obtained (Figure 4). Samples with M_n of up to 2,000,000 g.mol^{-1} were successfully isolated. However, ^{1}H NMR analysis of the synthesized polymer evidence a side reaction due to interaction with the solvent (Figure 5). Moffat and Burdon previously reported that DCC and DMSO can react in the presence of acid catalyst - also known as Pfitzner–Moffatt conditions – and react with phenolic compounds [26]. Several compounds can be created from this reaction. The main reported behaviour was the alkylation of the phenols in the available *ortho* positions with thiomethoxymethyl groups. Binding of dicyclohexylurea to the molecules was also reported in the same position. Formation of cyclic hemithioacetal structures was also reported. In ^{13}C NMR analysis, signal in the δ = 25-50 ppm clearly indicated the binding of DHU molecules to the polymer structure. Presence of DHU structure was further confirmed by 2D HMQC analysis. By integration of proton signals in NMR the relative amount of each of the three structure can be calculated. Alkylation in *ortho* position with thiomethoxymethyl group is shown to be highly favoured, with 79% of each aromatic ring undergoing that side reaction (Table 2). Binding of DHU is also non negligible as it is present in 33% of PA structures. The cyclic hemithioacetal structure is less present but can still be found in every 15 out of 100 aromatic rings.

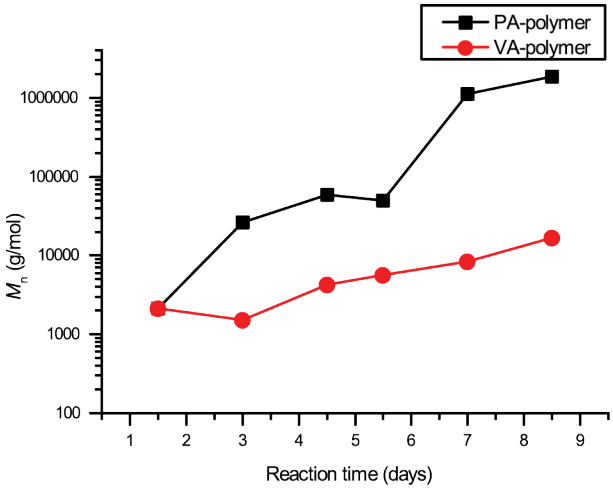

Figure 4: Compared polymerisation kinetics of protocatechuic acid and vanillic acid by Steglish esterification in DMSO

Measured by	Arom. unit	Thiomethoxymethyl group	Cyclic hemithioacetal	Binded DHU
^{1}H NMR	100	79	15	33
^{13}C NMR	100	83	11	35

Table 2: Relative amount of three possible structures arising from side reaction with activated DMSO and DHU, per 100 aromatic units

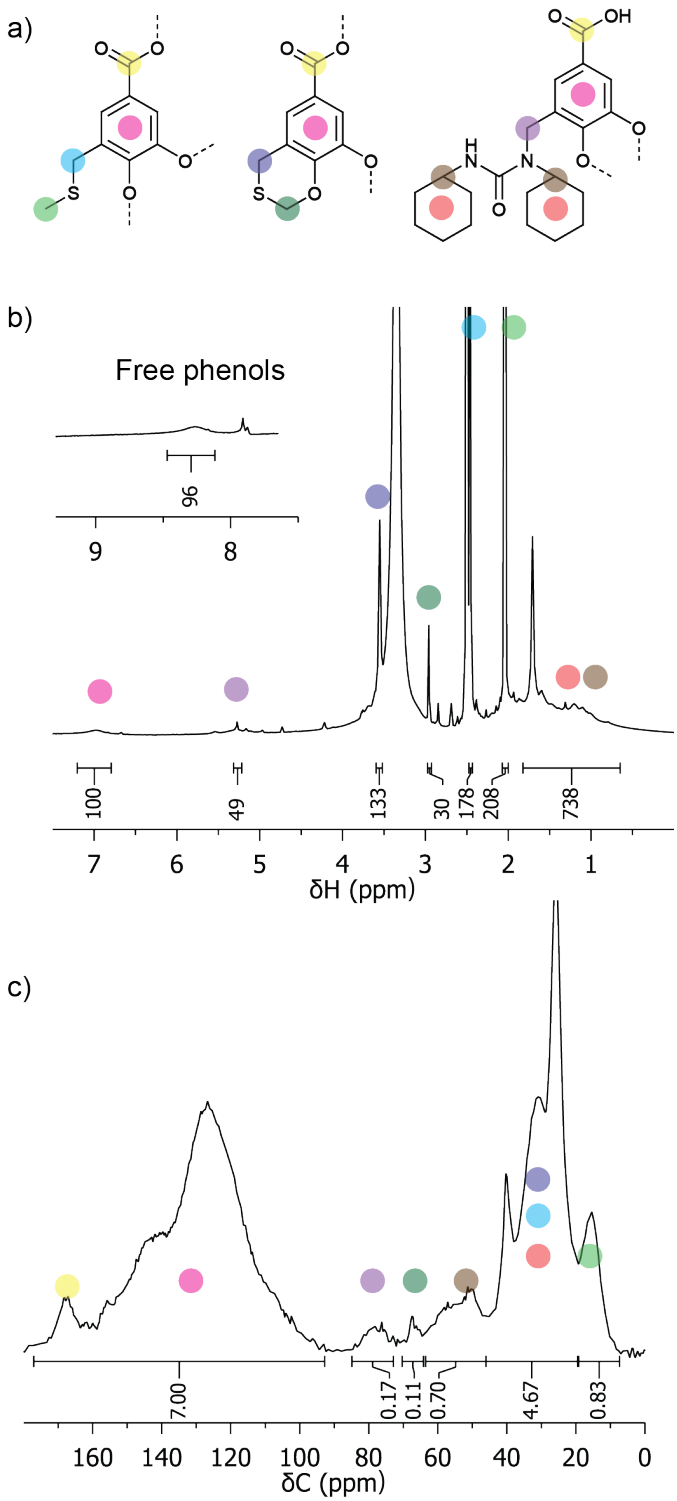

Figure 5: a) Three different structures that can arise from DMSO activation by DCC and reaction with PA b) 1H NMR of PA-polymer highlighting evidence of side reactions in d6-DMSO c) 13C NMR of PA-polymer in d6-DMSO

The formation of methylthiomethyl esters and N-acylureas by reaction of the carboxylic acid group has been reported in literature [27] (Figure 6). However, considering the overall molecular weight of the final polymer sample this reaction either a) was not favourable or b) was reversible in a way that does not prevent polycondensation. The latter case could however explain the overall slow kinetics of the polymerisation. Nevertheless, due to the high polymerisation degree, this reaction cannot be detected by NMR analysis.

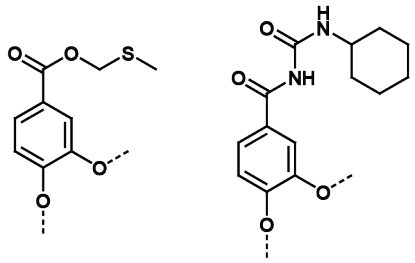

Figure 6: Carboxylic acid can react with activated DMSO or bind with DHU to form methylthiomethyl ester (left) or N-acylurea (right) structures

The thermal stability of both polymers synthesized in DMSO was measured by thermogravimetric analysis (Figure 7). Polymerisation in DMSO didn't change the degradation temperature for VA polymer but remaining ash content greatly increased from 10% to 50%. Similarly, degradation of PA-polymer now also yields about 50% of mass of ashes. The degradation temperature for PA-polymer however now greatly increased, and is now thermostable to up to 310°C, probably partially due to the 200 fold increase in molecular weight. During differential scanning calorimetry (DSC) analysis over a large range of temperatures (-70°C to $Td_{5\%}$ - 20°C) no clear glass transition temperature could be detected. Glass transition temperature of branched polymers can be hard to detect using DSC so other detection methods should be used in future work.

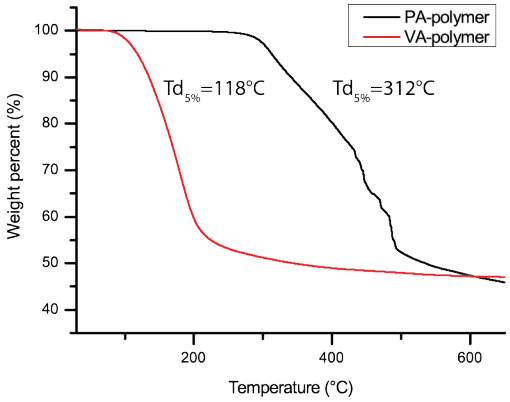

Figure 7: Thermogravimetric analysis of polymer from protocatechuic acid and vanillic acid

Due to the presence of phenolic group, antioxidant properties are expected from both polymers. Radical scavenging activity is measured by the determination of the amount of substrate that will bleach half the amount of a stable radical compound, 2,2-diphenyl-1-picrylhydrazyl (DPPH). The procedure is adapted from the recommendation of Kedare et al [28] (Figure 8). As expected, due to the higher amount of phenol group due to its hyperbranched structure, PA based polymer is almost twice as efficient as VA-polymer, with an EC_{50} (efficient concentration) of 0.08 mg/mol (Table 2). Antioxidant biobased polymer represent a high interest in research and for industrial application. Chitosan grafted polymer are especially well investigated. By grafting an increasing amount of phenol on the polymer backbone, the antiradical efficacy can be radically improved (Table 2). Overall, our HBP have higher antiradical efficacy than all other biopolymer but one, and is also two to four time more efficient than the commercial non biobased option (Irganox® 1010 and Irganox® 1098).

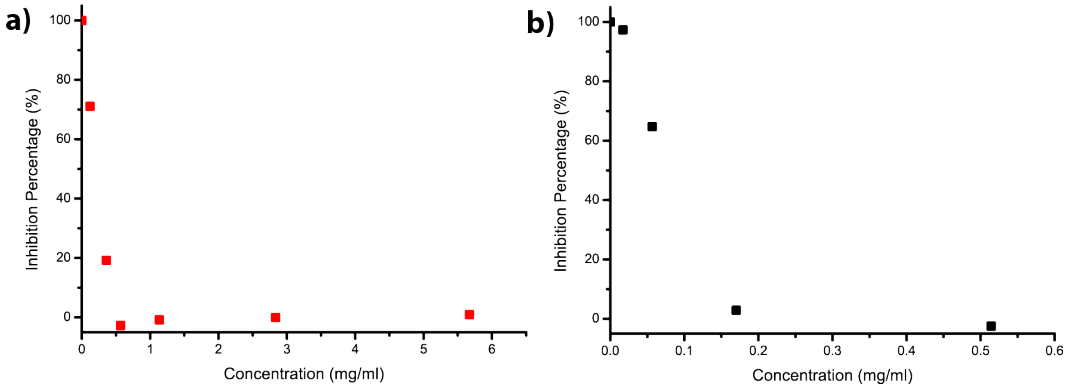

Figure 8: Inhibition percentage of a) PA-polymer and b) VA-polymer versus concentration. Measured by bleaching of DPPH at 515 nm during radical scavenging assays.

	EC_{50} (mg/ml)	Antiradical efficacy (mmol) [a]
PA-polymer	0.08	0.76
VA-polymer	0.22	0.30
Chitosan	/	n.d. [17]
Chitosan graft caffeic acid	/	0.16 [17]
Chitosan graft tannic acid	/	5.8 [18]
Dendritic phenol	/	0.36 [19]
Irganox® 1010	/	0.18 [29]
Irganox® 1098	/	0.36 [19]

[a]: DPPH equivalents per gram of material

n.d.: not detected

Table 2: Antioxidant properties of synthesized polymer compared to literature and commercially available antioxidant

4. Materials and Methods

Chemicals and solvents: Chemicals were purchased from Sigma Aldrich (Sydney, NSW, Australia) (vanillin, 4-dimethylaminopyridine (DMAP), N,N'-Dicyclohexylcarbodiimide (DCC), lithium bromide), from Merck PTY (Melbourne, VIC, Australia) (Potassium hydroxide, anhydrous manganese sulphate) and from TCI (Tokyo, Japan) (deuterated dimethyl sulfoxide or DMSO) and used as received. Reagent grade solvents (anhydrous dimethylformamide or DMF, diethyl ether, hydrochloric acid, Tetrahydrofuran or THF) were purchased from Thermo Fisher Scientific (Melbourne, VIC, Australia).

The molecular weight of polymers samples was analysed by size exclusion chromatography (SEC) in DMF or water. SEC in DMF was performed at 40°C on a Tosoh EcosHLC-8320 equipped with double detectors (RI, UV 280 nm) using Tosoh alpha 4000 and 2000 columns. DMF/LiBr 0.1 M was used as a mobile phase (flow rate 1 mL/min). Polystyrene standards were used for the calibration.

^{1}H and ^{13}C NMR experiments were performed in deuterated DMSO on a Bruker Avance 400 MHz NMR spectrometer equipped with a CryoProbe Prodigy 5 mm $^{1}H/^{1}H$-$^{13}C/^{15}N$ probe. NMR experiments were performed with the sample held at 25±0.1 °C. Chemical shifts for all experiments are referenced using the Unified Scale relative to 0.3 % tetramethylsilane in deuterated chloroform.

Radical scavenging activity was measured through the measurement of the bleaching of DPPH at 515 nm by UV-visible spectroscopy. A fresh solution of DPPH at 2.65 mg/ml in methanol is

prepared and kept away from light in fridge. Solution of the substrate in DMSO are also prepared at known concentration. In a test tube, 60 μl of the DPPH solution is added with a known volume of the substrate solution. The volume is then adjusted to 3 ml with DMSO. The test tube is then sealed closed and kept to react away from light for 3 hours. After that time the absorption of the solution at λ = 315 nm is measured. The absorption of the substrate alone is also measured to subtract its effect on the measurement. This is repeated as much as needed with different volume of the substrate solution in order to gather data before and after the total quenching of DPPH. The residual absorption of totally quenched DPPH is also measured for more accurate results. Ascorbic acid was selected as standard and its antiradical efficacy was also measured and compared to literature to confirm measurements (Figure SI1, table SI1) [16]. UV measurements were performed on an Agilent Cary 60 UV-Vis cell reader spectrophotometer in quartz cells.

Degradation temperatures were measured by thermogravimetric analysis (TGA) performed on a Simultaneous Thermal Analyser (STA) 8000 from PerkinElmer. Heating rate was fixed to 30°C/min under nitrogen flow.

Glass transition temperatures were measured by differential scanning calorimetry (DSC) on a DSC 8000 from PerkinElmer fitted with a dual-stage heat-exchanger cooling system Intracooler 2. Samples were analysed in aluminium pans and under nitrogen atmosphere. The heating rate was set to 10°C/min and cooling rate 150°C/min.

PA synthesis

Protocatechuic acid was obtained by the caustic fusion of vanillin. In a nickel crucible potassium hydroxide (33 g, 0.59 mol) is added with a small volume of water (4 ml). A crucible is placed in an oil bath and an overhead mechanical stirrer is installed. Heating is set to 255°C and stirring is set to medium speed. After a few minutes the salt mixture becomes a homogeneous viscous mixture. Vanillin (12 g, 0.079 mol) is then carefully added into the solution. After 45 min the reaction is stopped and allowed to cool down. The reaction mixture is dissolved in water (200 ml) and the product is then recovered by acidification/precipitation with hydrochloric acid. Filtration in a Buchner funnel yields 9.5 g of protocatechuic acid (0.061 mol). The aqueous phase is further extracted three times with diethyl ether (50 ml). The organic phase in then dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to recover an additional 2.1 g of protocatechuic acid (0.013 mol). ^{1}H-NMR (400 MHz, d6-DMSO): δ 7.32 (s, H, ArH), δ 7.27 (d, 1H, ArH), δ 6.79 (d, 1H, ArH).

VA synthesis

Synthesis of vanillic acid is achieved by identical procedure only maintaining reaction temperature at 150°C. Potassium hydroxide (33 g, 0.59 mol) and water (4 ml) mixture are brought to a gel in the nickel crucible by maintaining heating at 150°C and constant stirring. Vanillin (12 g, 0.079 mol) is then added and let to react for 45 min. After allowed to cool down, the mixture is dissolved in water (200 ml) and vanillic acid is crushed out by acidification with HCl. 10.3 g of vanillic acid car be recovered (0.067 mol). Extraction of the aqueous phase with diethyl ether further yields 1.9 g of vanillic acid (0.012 mol). ^{1}H-NMR (400 MHz, d6-DMSO): δ 7.44 (s, 1H, ArH), δ 7.43 (d, 1H, ArH), δ 6.84 (d, 1H, ArH), δ 3.92 (s, 3H, CH_3).

Polymerisation of VA and PA

Vanillic acid and protocatechuic acid are polymerised by polycondensation using Steglish esterification reaction. The phenol compound (3 mmol) is added to a 50 ml round bottom flask and dissolved in 30 ml of solvent. 3.6 mg of DMAP (0.03 mmol) and 680 mg of DCC (3.3 mmol) are further added to the flask. Magnetic stirring is set up until complete homogenisation of the solution. The round bottom flask is then fitted with a condenser and the reaction is allowed to carry on in reflux condition. After reaction, the mixture is allowed to cool down and is then filtered to remove insoluble side product dicyclohexylurea. The soluble fraction is then added dropwise to a large volume of THF to crush out high molecular weight fraction.

5. Conclusions

In this work we reported the synthesis and polymerisation of a bio-based monomer from lignin, PA. Its AB_2 configuration was compared to another monomer with simple AB structure, VA. During the polymerisation study side reaction with the solvent was detected. However, in those conditions the final synthesised polymer gained better thermal properties and was able to reach high degree of polymerisation. Both polymers exhibited high antioxidant properties due to the presence of phenolic groups in the structure. NMR spectroscopy was used to characterise the different side reactions and their effect on the polymer structure. In future work, we ought to improve the PA-polymer characterisation, focussing on the structure (degree of branching) and physico-mechanical properties. This polymer is expected to have great impact as a 100% bio-based polymer with antioxidant properties. Application are expected for paint, packaging, emulsion, formulation or drug delivery application. In addition, the phenol terminated structure allows easy post modification and will be considered in future work to create further applications for this polymer.

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Conflicts of Interest: The authors declare no conflict of interest

Supporting information: DPPH analysis of ascorbic acid standard are available in supporting information.

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Chapter 5

Preparation of lignin films for coating by coordination complexes

5 Preparation of lignin films for coating by coordination complexes

5.1 Abstract

In this work we present the preparation of lignin film for coating applications. Iron catechol complexes will be used to create soft bonding between different lignin oligomers to yield a reversible matrix on the substrate surface. Catechol group were added to lignin by demethoxylation of a range of different samples with different composition and structure. Small model molecules were first used as proof of concept. Demethoxylation was monitored by HMQC and ^{31}P NMR and complexes formation was confirmed by UV-Vis spectroscopy. Finally, coating tests were performed on glass slides and physicochemical properties were assessed by some preliminary measurement. Future work will see complete characterization of lignin films and process optimization.

5.2 Introduction

Lignin is the second most abundant biopolymer in nature and represents a vast feedstock for industry. Furthermore, it is essentially benign for human health and can be considered in several health or food applications. As society and industry adapt to new economic, politic and environmental global issues, efficient ways to exploit this renewable resource become critical. One of the main focuses of research is the complete depolymerisation of lignin to create bio-based chemicals and new renewable polymers. However, depolymerisation processes are facing some challenges and total depolymerisation is laborious and grinding.

In most cases heterogeneous mixtures of small oligomers are obtained and purification then becomes a challenge. As stated in the introductory chapter, we will focus our work in this chapter on the use of lignin oligomers obtained from depolymerisation to create a new bio-polymer and characterise it.

Catechols groups are well known structures that can create complexes with different ionic species[1]. Furthermore, their formation is quick and reversible. Recently, Caruso et al. provided intensive study on the coordination complexes in tannic acid (TA), a catechol rich molecule from biomass [2]. Tannic acid have previously been reported for hydrogel formation as it is easy to crosslink. Indeed, iron and other metal ions can create a *mono*, *bis* or *tris* complexes with catechol groups depending on pH (Figure 1). As tannic acid molecules exhibit multiple catechol group, the creation of *bis* or *tris* complexes allows the formations of a crosslinked 3D structure.

In their work, Carusso et al. efficiently provide coating on different substrates with iron-TA complexes by control over the solution pH[3]. They report the successful coating on different scales, from nanoscale polystyrene beads to macroscale glass substrate. Iron-catechol complexes behaviour depends on the solution pH and can be found to complex once, twice or three times with the metal ion (Figure 1). Therefore, in basic conditions tannic acid will tend to create a crosslinked structure while if turned back to acidic medium each molecule will be essentially separated from the others. TA-iron systems can efficiently create a reversible network.

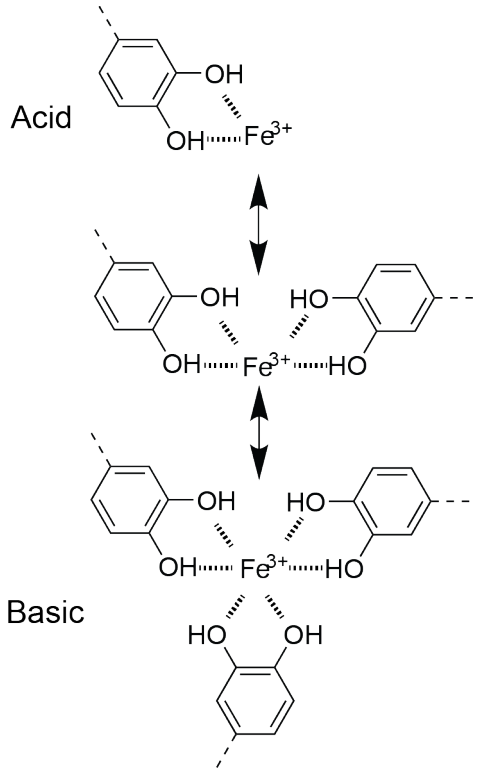

Figure 1: Iron ions will complexes with one, two or three catechol groups with increased pH.

Catechols groups do not naturally occur in lignin, nore in its depolymerized products. Phenols, methoxy and aliphatic alcohol are yet the most common groups in native and depolymerized lignin. However phenol groups often have an adjacent methoxy group in ortho position. This is due to the three lignin natural phenolic monomers, p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 2). Chemical demethylation of the lignin methoxyl group will efficiently create several catechol groups in the structure. The total number of created catechol groups depends on the total amount of methoxyl groups and therefore on the lignin monomers ratio, referred as S/G/H ratio (Figure 2). H units do not provide methoxyl groups, thus high G or S ratio lignin are the best candidates. Upon presence of metallic ions in solution (Iron or Vanadium) soft and reversible bonding should

be created by the formation of metal-organic complexes between the lignin oligomers. The resulting macrostructure will be studied for coating application and drug delivery use.

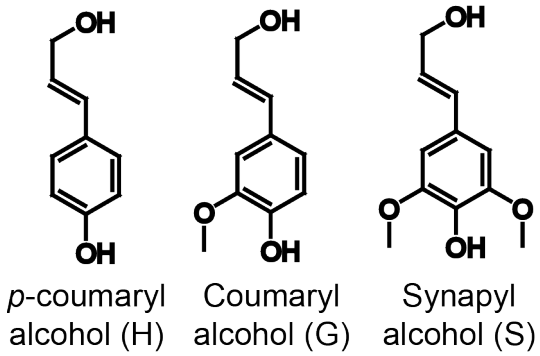

Figure 2: Three lignin monolignol responsible for the creation of (from left to right) p-coumaryl, guiacyl and syringyl units. After depolymerisation, those units will yield H, G or S type lignin degradation fragments, respectively

In this chapter, we will report the demethoxylation of three different type of lignin with different S/G/H ratio and the corresponding changes in structure. The best candidate will be tested for coordination complexes in solution and on solid substrate.

5.3 Result and discussion

5.3.1 Lignin and model molecule demethylation

For lignin demethylation two different methods were tested. The first method, demethylation by boron tribromide, have recently seen increased interest in organic chemistry as an efficient and easy to handle reaction compared to other methods. For this reaction we followed the procedure described by K. Rice[4]. The reaction is almost instantaneous with total conversion. Furthermore, it is also less toxic or dangerous to handle than some previous method. This approach was first tested on a model molecule, vanillin (Figure 3). Demethylation efficiency was assessed by ^{1}H NMR by monitoring the

disappearance of the protons in the methyl group (Figure 4). Demethylation was found to be complete, yielding protocatechuic aldehyde with no remaining vanillin after only an hour of reaction.

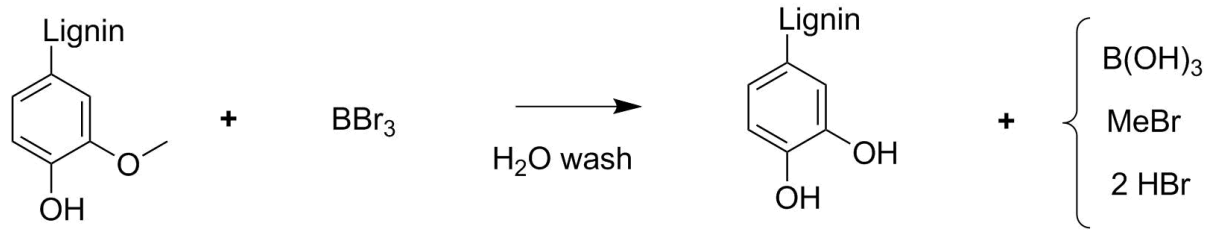

Figure 3: Lignin demethylation by boron tribromide

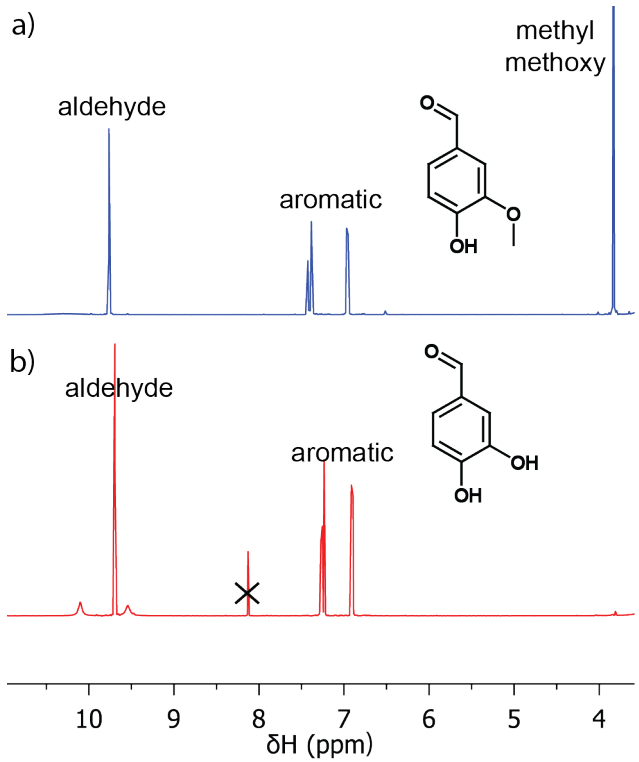

Figure 4: ^{1}H NMR analysis of a) vanillin and b) the demethylated produced after boron tribromide treatment.

One of the main requirement for BBr_3 demethylation reaction is that very few solvent are suitable. While vanillin is soluble in chloroform or dichloromethane, only a very small fraction of lignin is. A second method was developed following a 1979 US Patent[5]. Instead of BBr_3, amine hydrochloride are used to perform the demethylation. The reaction can be performed in water, in alkaline condition, in which all lignin sample are soluble. *n*-

butylamine hydrochloride was synthesized in a straightforward way, with high yield, from hydrochloric acid and *n*-butyl amine (Figure 5). Demethylation in aqueous condition were performed following the patent procedure to yield demethylated lignin oligomers.

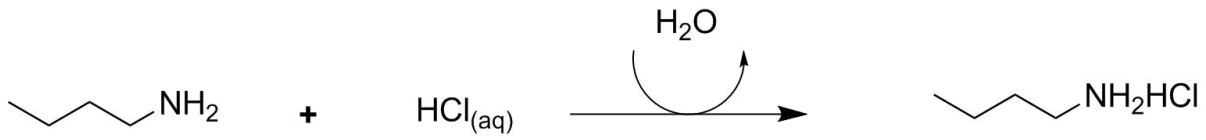

Figure 5: Synthesis of *n*-butylamine hydrochloride

For this study, three type of lignin were selected. Organosolv herbaceous (OL), kraft hardwood (KhL) and kraft softwood lignins (KsL) have different structure and may react differently to demethoxylation or complexations. S/G/H ratio was determined by 2D HMQC NMR (Table 1).

Table 1: Lignin sample characterization by HMQC NMR spectroscopy.

	Organosolv	Kraft hardwood	Kraft softwood
S/G/H ratio	44/53/3	86/14/0	0/99.5/5
Protons in methoxy group per lignin unit in original sample	4.95	6.01	2.87
Protons in methoxy group per lignin unit in treated sample	2.35	1.95	1.12

As expected, hardwood lignin has the highest ratio of S units, followed by herbaceous lignin, while softwood mainly exhibit G units. Lignin with high S ratio can be more suitable for demethylation as they will be overall more methoxy group in the structure. 2D HMQC NMR on the demethoxylated lignin oligomers allows to measure the ratio of protons in methoxy group per lignin unit. After boron tribromide treatment, the steep decrease in the ratio indicates that the demethylation is at least partially successful. Demethoxylated organosolv (D-OL), kraft hardwood (D-KhL) and kraft softwood (D-KsL) reduced by 53%, 68% and 61% the amount of methoxy group per lignin unit. The non-total demethoxylation of lignin samples was expected as lignin is inherently more resilient to chemical degradation. Optimisation of reaction conditions may yield better demethylation in future work. However only partial demethoxylation can still create suitable samples for coordination with metal ion. From observations, D-KhL is the best candidate for metal-catechol complexes formation, as it showed good demethoxylation, high content in S units and featured important amount of free phenols on S units before demethoxylation.

^{31}P NMR spectroscopy enable to precisely measure the total loading in phenolic group per gram of sample, as previously described in chapter 2. The presence of phenolic group is essential as catechol group consist of two adjacent free phenols. KhL exhibit an important content of free phenols located on S units. Demethoxylation of at least one of the adjacent methoxy group will create a viable catechol group.

Interestingly, after demethoxylation phosphorous NMR indicates that the overall phenol loading decreased compared to the methylated sample (Table 2). With conflicting results we move on to the metal-catechol complex study to provide more information on the oligomers structure.

Table 2: Lignin sample characterization by ^{31}P NMR spectroscopy.

	Organosolv	Kraft hardwood	Kraft softwood
Free phenols on S unit (mmol/g)	0.28	1.18	n.d.
Free phenols on G unit (mmol/g)	0.54	0.21	1.33
Free phenols on H unit (mmol/g)	1.07	n.d.	n.d.
Free phenols on S unit (mmol/g), demethylated	0.15	n.d.	n.d.
Free phenols on G unit (mmol/g), demethylated	0.04	n.d.	n.d.
Free phenols on H unit (mmol/g), demethylated	0.24	n.d.	n.d.

5.3.2 Metal catechol complexes in UV-vis

Metallic complexes formation with the model molecule, protocatechuic aldehyde was monitored by UV-vis spectroscopy over a wide range of pH (Figure 6). Three different behavior can be observed: acid conditions (pH<6), basic condition (pH>11) and in between. In the acid condition, the *mono* complex iron-protocatechuic aldehyde is the most abundant species, while the *tris* and *bis* complexes are favoured in the alkaline or intermediary domain respectively. Each complex has a distinct absorption spectrum than can be observed in Figure 6. The oxidized molecule, protocatechuic acid, was also tested to measure to potential effect of side chain functional group on the self-assembly behaviour (Figure 7). Similarly, three different behavior were observed, with a change of configuration at pH = 6 and pH =9.

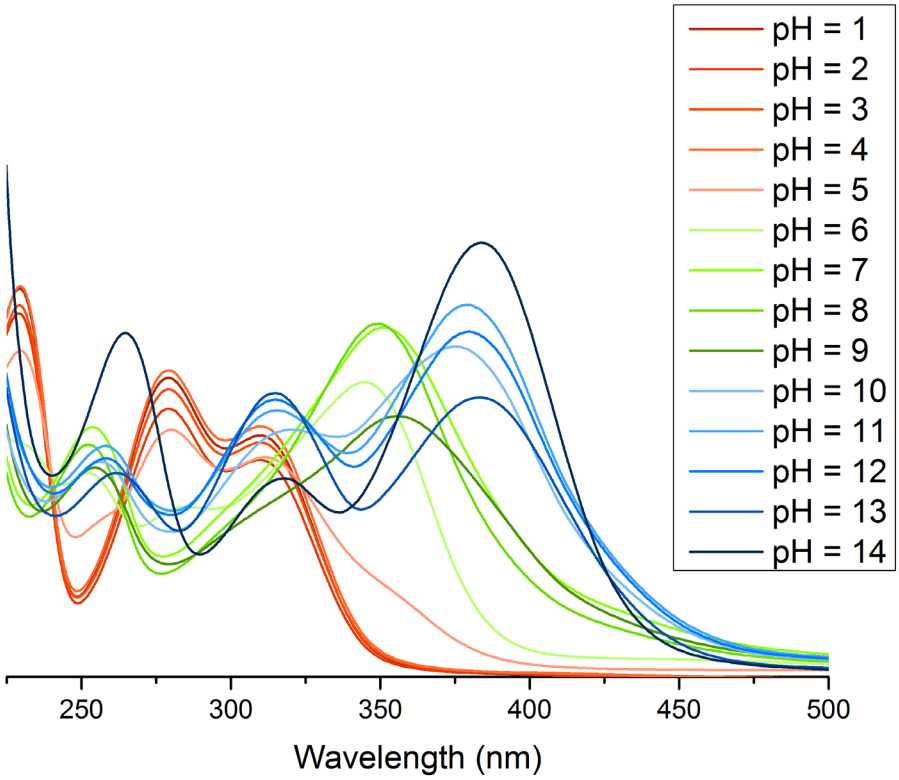

Figure 6: UV-Vis spectroscopy of protocatechuic aldehyde-ferric ion at different pH. Three different behavior can be observed: below pH = 6, pH between 7 and 10, and above pH = 11.

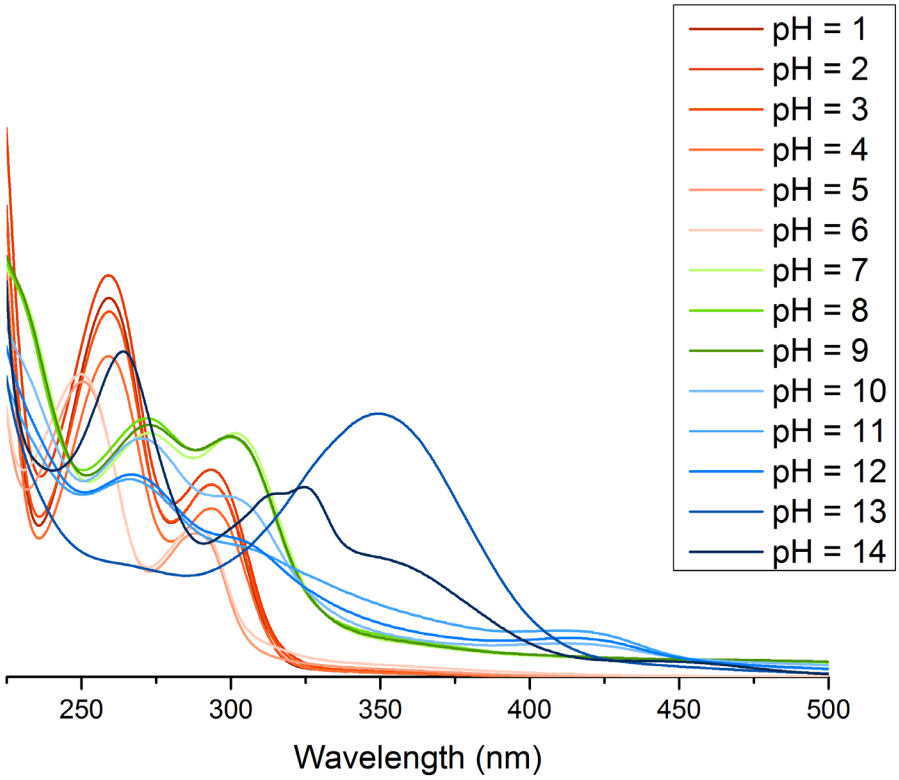

Figure 7: UV-Vis spectroscopy of protocatechuic acid-ferric ion at different pH. Three different behavior can be observed: below pH = 5, pH between 6 and 9, and above pH = 10.

D-KhL, D-OL and D-KsL are all tested in solution to observe the expected complex formation. However, out of the three samples only D-KhL proved soluble enough to finish the test. UV-vis spectroscopy was used to follow the iron-catechol complexation in a large range of pH. However no clear trend could be established. Furthermore, lignin naturally absorbs in the region UV-600 nm so clear measurement are difficult (Figure 8).

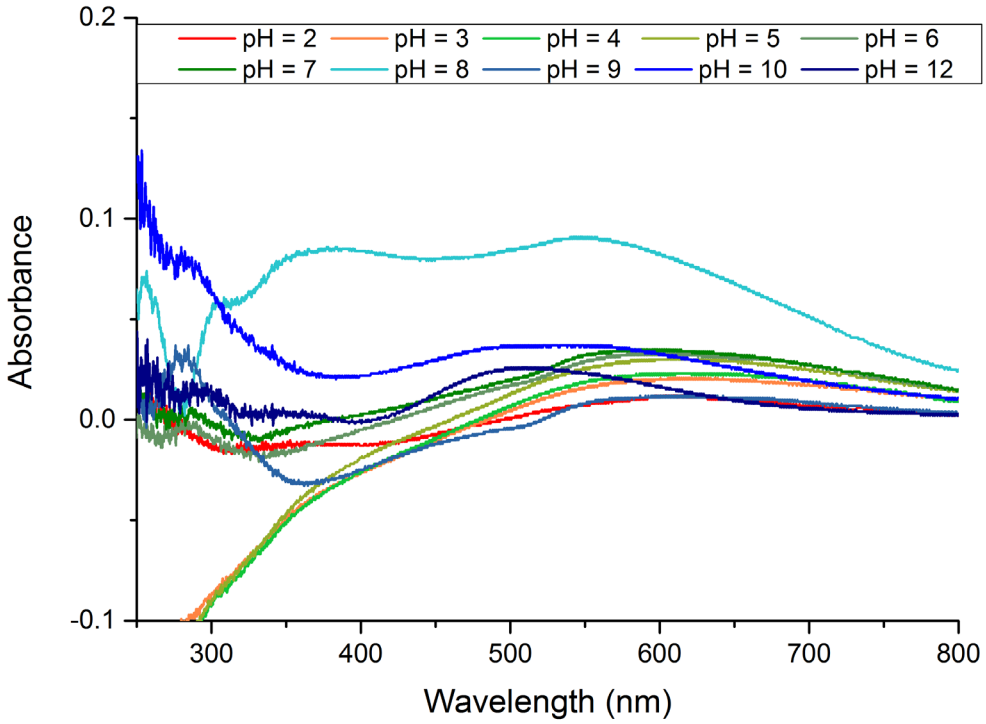

Figure 8: UV-Vis spectroscopy of D-KhL-ferric ion at different pH. No clear trend can be observed.

Catechol groups can also combine with other ions in solution similarly to ferric ions. Cr^{3+} and V^{+3} have been reported in literature to create complexes in such fashion. Vanadium proved

to be the best candidate as it allowed the formation of different complexes detectable by UV-visible spectrometry (Figure 9). From observations the *tris* complex is formed above pH ≈ 8.

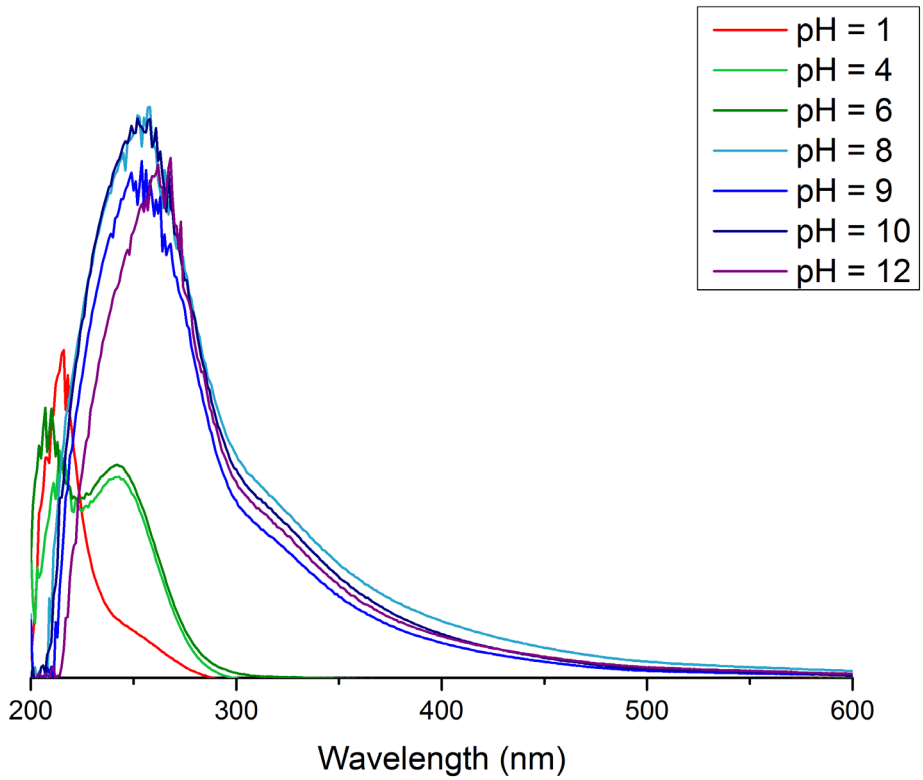

Figure 8: UV-Vis spectroscopy of D-KhL-V^{3+} ion at different pH. Three clear behaviour can be observed in acidic, alkaline and intermediary conditions.

5.3.3 Coordination complexes on solid substrate

D-KhL was tested for coating on glass substrates with the method described by Carusso et al. After several cycle of coating in acid condition / fixation in basic conditions / washing with water a light brown film started to be visible. After several additional cycles (15 total) a film of similar aspect to what can be obtained with tannic acid was observed (Figure 9). The film proved to be slightly resistant to water but could be easily removed by mechanical friction. However, compared to tannic acid, the lignin film had much less adherence to the substrate. It is reasonable to think that D-KhL does not have as much catechol group as tannic acid and

the created network is therefore much weaker. Contact angle test indicated no noticeable change in hydrophilicity compared to naked glass. Additional characterization are required to conclude on the film structure.

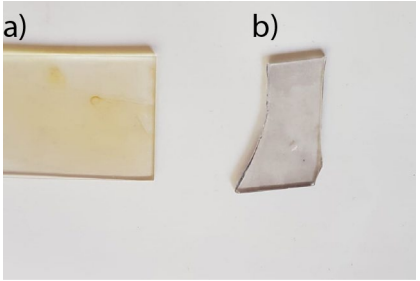

Figure 9: a) Lignin film obtained after 15 coating cycles, compared to b) tannic acid coordination film after 5 coating cycles.

The film sample was also analysed by scanning electron microscope for closer analysis (Figure 10). The resulting images indicate a good coating on the substrate with good uniformity.

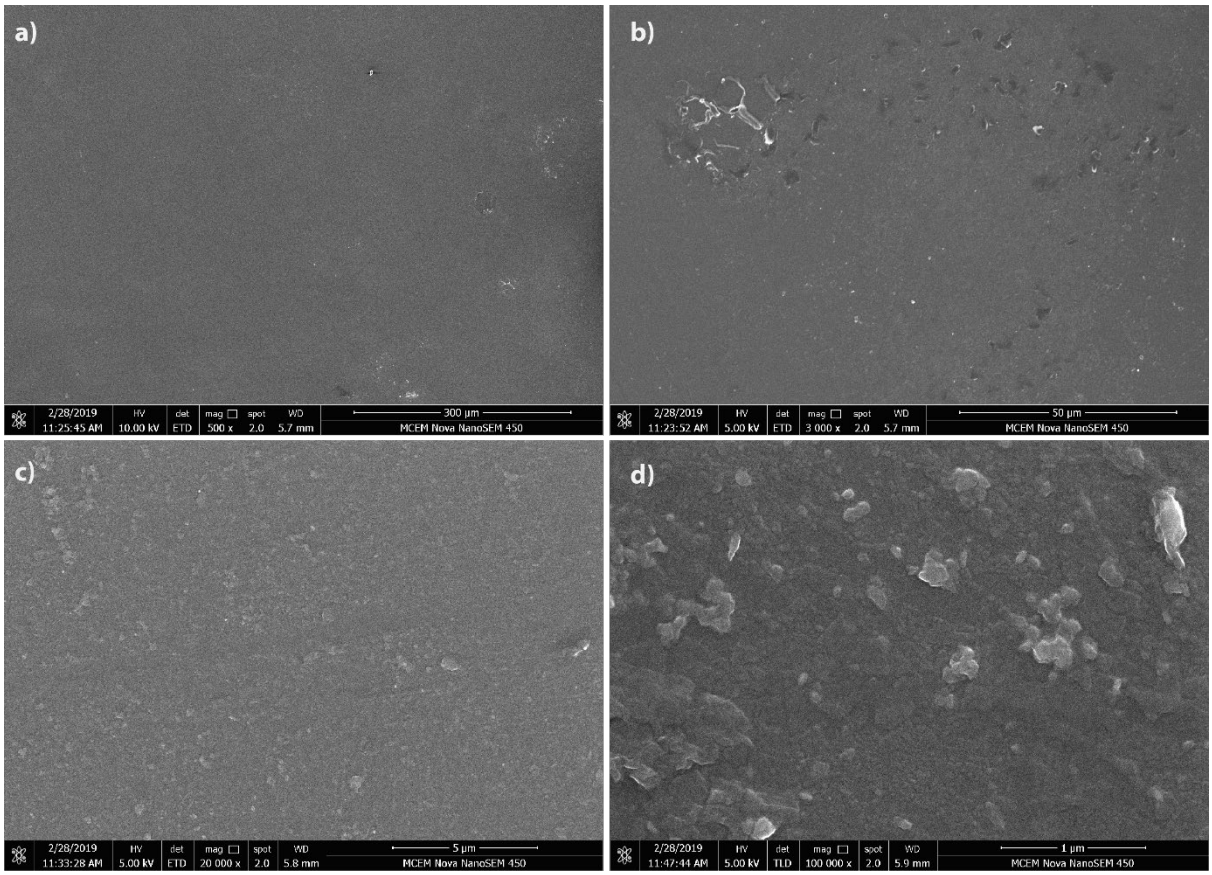

Figure 10: Scanning electron microscope (SEM) analysis of the Iron-D-KhL film coated on a glass substrate.

5.4 Conclusion

In this chapter we reported the successful demethoxylation of different lignin samples. Reaction was monitored by 2D NMR and up to 68% of methoxy group could be demethylated. Measurement of free phenol by phosphorus was unsuccessful but the formation of catechol-vanadium complexes in solution seems to indicate the presence of several catechol group in the sample. The coating process on solid substrate proved tedious compared to reported work on tannic acid. After several repeated coating cycles, a lignin thin film was created on glass substrate. However characterization is poor and should be completed in future work.

5.5 Material and methods

Demethylation by boron tribromide

In a 250 ml 3 neck flask 75 ml of chloroform are added with the lignin sample (2.5 g). The top neck is then fitted with a condenser equipped on top with a rubber seal and a NaOH moisture filter. The two other necks are fitted with rubber seal and argon is flushed in the system for 30 min. Boron tribromide (about 10-12 ml) is then carefully collected with a dried glass syringe from the commercial vial equipped with argon flushed and transferred to the reaction mixture. The addition should be slow (≈ 5min). The reaction is then allowed to continue at room temperature for 20 min. The reaction mixture is further poured carefully onto ice and ammonia mixture (80 g of ice and 20 ml of ammonia for 15 g of BBr_3). Once the ice is totally melted, the whole mixture is transferred into a separation funnel to separate

organic and aqueous phase. Depending on the lignin source, the product will be differently distributed between the two phases. Aqueous phase can be acidified to crush out and recover lignin oligomers by filtration.

Amine hydrochloride synthesis:

In a round bottom flask 10 g of *n*-butylamine (136.7 mmol) are added. 15.6 ml of concentrated hydrochloric acid (37%$_{w/w}$, 136.9 mmol) is then carefully and slowly added to the amine. The mixture is mixed by hand for a few second before the excess water is removed under reduced pressure with rotary evaporator. The process is repeated 2-3 times. 10.206 g of a white hygroscopic solid is then recovered on the round bottom flask wall. Yield = 68 %.

NMR spectroscopy:

2D HMQC NMR experiments were performed on a Bruker Avance 500 MHz NMR spectrometer equipped with a CryoProbe Prodigy 5 mm ^{1}H/2H-^{13}C/^{15}N probe. NMR experiments were performed with the sample held at 25±0.1 °C. Chemical shifts for all experiments are referenced using the Unified Scale relative to 0.3 % tetramethylsilane in deuterated chloroform. Multiplicity edited HSQC experiments used the standard hsqcedetgpsisp2.2 Bruker pulse program. There were 512 experiments with 64 scans collected over 6010 Hz in the ^{1}H dimension and 20139 Hz in the ^{13}C dimension with a 1.5 s relaxation delay. The data were processed in phase-sensitive (echo-antiecho) mode to 2048 × 2048 data points using a π/2-shifted sine-squared window function in both dimensions.

^{31}P NMR experiment were performed on a Bruker Advance III 400 (9.4 Tesla magnet) with a 5mm broadband probe with z-gradient and BACS 60 tube autosampler. TMDP was used as

derivatisating agent for phosphylation of hydroxyl groups following procedures reported in chapter 2. Experiments were performed with 180 scans and a 25 s relaxation delay.

Metal-catechol UV-visible study:

A solution of VCl_3 or $FeCl_3$ is first freshly prepared in water, as well as a solution of the substrate. If the substrate doesn't fully dissolve alkaline condition can be used instead of water. In the cuvette, 1 ml of water is first added, followed by the metal ion solution and the substrate solution. The cuvette is then filled up to 3 ml with acid or basic water in order to reach desired pH. Absorption between 800 and 200 nm is then measured in an Agilent Cary 60 UV-Vis cell reader spectrophotometer in quartz cuvettes. The absorption of the substrate alone is also measured for baseline correction.

Solid substrate coating:

To coat a solid substrate, the glass slide is first placed in a 50 ml falcon tube with a solution of the lignin oligomer. The falcon tube is well shacked to entirely soak the glass slide. The solution of the metal ion is then added. The ratio of lignin unit/metal ion is fixed to 5. The total volume does not need to be more than $1/4^{th}$ of the total volume. After soaking the glass slide in the solution, the pH is instantly increased to pH ≈ 9 by addition of 1 N NaOH solution and shacked vigorously in vortex shaker. After 10 - 20 s of shaking, the glass slide is recovered and carefully rinsed with deionise water. The process is repeated as much as needed to enhance the color on the slide.

Scanning Electron Microscopy:

Preparation: The coated glass sample is placed on SEM stub and held in place by carbon tape to insure conductivity. The sample is then coated by palladium by sputtering.

Analysis: The analysis was performed on a FEI Nova NanoSEM 450 FEGSEM.

The voltage was maintained between 5kV and 10kV.

5.6 References

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Chapter 6

Conclusion

6.1 Conclusions

Lignin biodegradation by enzymatic system did not prove to be completely efficient. Contemporary literature equally confirm that enzymatic degradation of lignin is promising, there is no clear industrial application in the close future. Several technical lock first have to be addressed before commercial viability of the system:

1) Cheap and reproducible enzyme production. Current market price prohibit any widespread use of enzymes, beside research. Furthermore, during this project the different supplier but also different enzyme batch proved to have highly variable purity and efficiency. A more reliable production first need to be created
2) Purification and separation techniques for degradation product. After enzymatic degradation of lignin heterogeneous mixtures are hard to work with due to no clear purification method. Furthermore, small degraded fragment can deactivate enzyme in solution as they are created. An optimum system would remove degradation product as they are created.
3) A different approach for lignin production. At the moment lignin is just a side product of another industry and to which we want to give value. A different approach, called lignin first, dictates that the type of wood and pulping process should be chosen having in mind the lignin first, to favour a desired structure or prevent other reaction. Production of cellulose and hemicellulose would then become a consequence of the lignin production.

Despite being not complete, depolymerisation of lignin samples was still good enough to successfully create several oligomers for future polymerisation.

Synthesis of monomers from small phenolic compounds proved very successful. Multiple new monomers were created and yielded several interesting renewable polymers. From syringic acid we successfully synthesised a water soluble polymer with thermal properties close to polystyrene. Several commercial applications can be considered, such as coating, water soluble paint, rheological agent or water treatment and filtration.

Polymer from protocatechuic acid was at first hard to understand due to side reaction with solvent. However, those side reaction helped to create a highly thermostable polymer. Furthermore, both this polymer and the polymer from polycondensation of vanillic acid exhibit high antioxidant properties Application for those polymers are numerous including packaging or formulation. Finally, the hyperbranched end groups offers a lot more possibilities as it will now be easy to post functionalise to fit desired applications.

6.2 Future work

In future work complete characterisation of lignin film is required. The current film formation is still not entirely understood. Furthermore, physicochemical properties of the film should be assessed to fit with commercial application.

Chapter 4 and chapter 5 will be completed and turned into article for publication in close future.

Annex

Supporting information

Annex 1: Supporting information for chapter 2

Importance of mediators for lignin degradation by fungal enzymes

Lionel F. Longe[a], Julien Couvreur[b], Mathilde Leriche Grandchamp[b], Gil Garnier[c], Florent Allais*[b] and Kei Saito*[a]*

14 pages, 6 figures, 2 tables

[a] School of chemistry, Bioresource Processing Research Institute of Australia (BioPRIA), 17 Rainforest walk, Monash University, Clayton 3800, Australia

[b] Chaire ABI, AgroParisTech, CEBB, 3 rue des Rouges Terres, 51110 Pomacle, France

[c] Department of Chemical Engineering, Bioresource Processing Research Institute of Australia (BioPRIA), 15 Alliance Lane, Monash University, Clayton 3800, Australia

* Corresponding authors email: kei.saito@monash.edu, tel: +61(0)399054600, email: florent.allais@agrosparistech.fr, tel: +33(0)352620462, email: gil.garnier@monash.edu, tel: +61(0)399059180

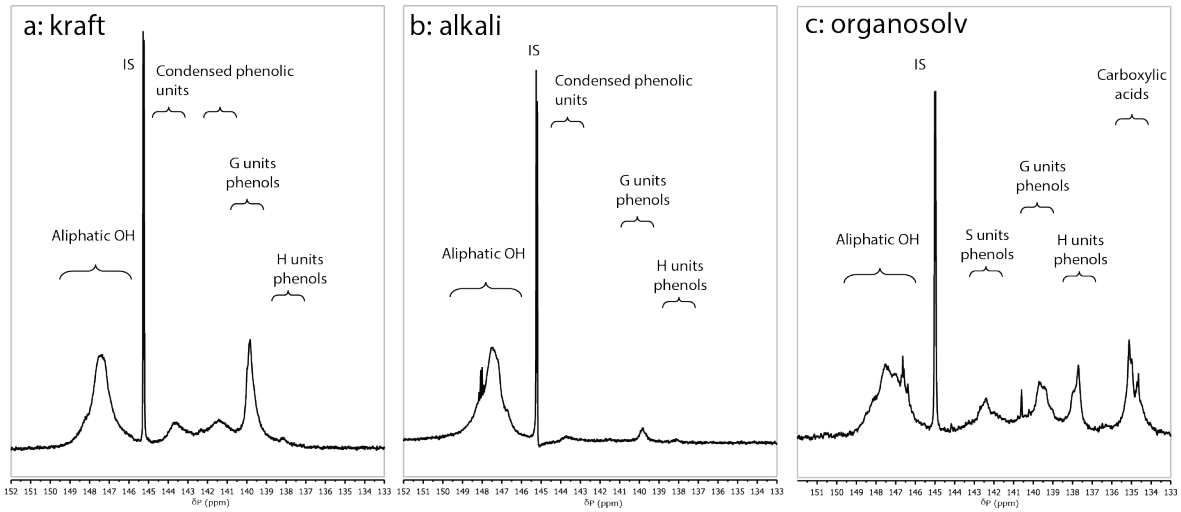

Figure S1. 31P NMR analysis of the three lignins a) kraft b) alkali c) organosolv. 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP) was used as derivatisating agent for phosphylation of hydroxyl groups.

	Aliphatic OH (mmol/g)	S units phenol (mmol/g)	G units phenol (mmol/g)	H units phenol (mmol/g)	Carboxylic acid (mmol/g)	Condensed units (mmol/g)
Kraft lignin	1.64	0.13	1.00	0.06	0	0.94
Alkali lignin	2.16	0	0.12	0.02	0	0.08
Organosolv lignin	1.35	0.47	0.58	0.44	0.68	0

Table S1. Hydroxyl group contents of the different lignins determined by 31P NMR analysis.

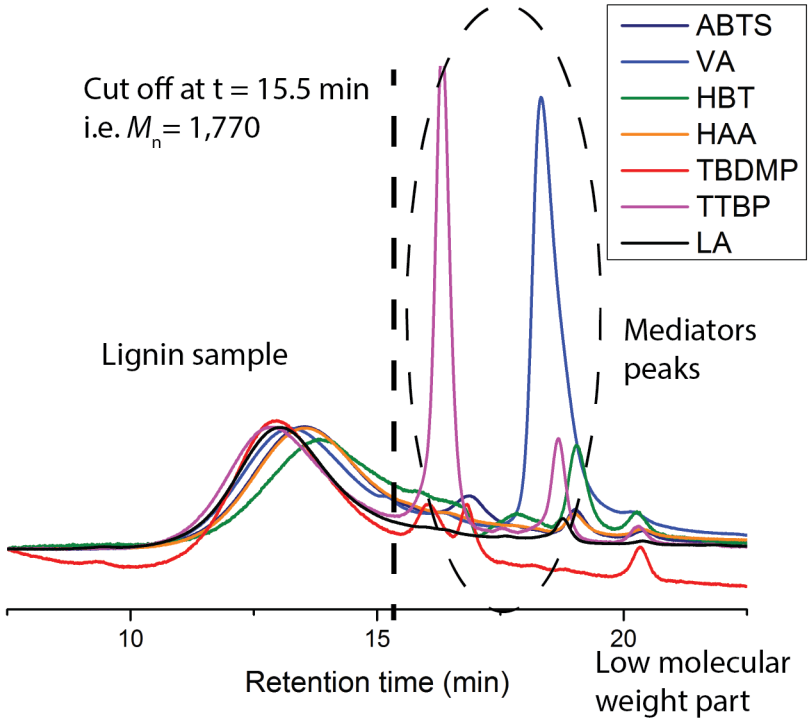

Figure S2. GPC analysis of organosolv lignin degraded by different LA mediated systems. Peaks at retention time higher than 15.5 min are due to presence of mediators in the sample, and are not representative of the lignin molecular weight. A cut off at t = 15.5 min for all GPC measurement allows to overcome the issue, but removes all low molecular weight lignin oligomers from the measurement. Therefore, lignin M_n are slightly overestimated.

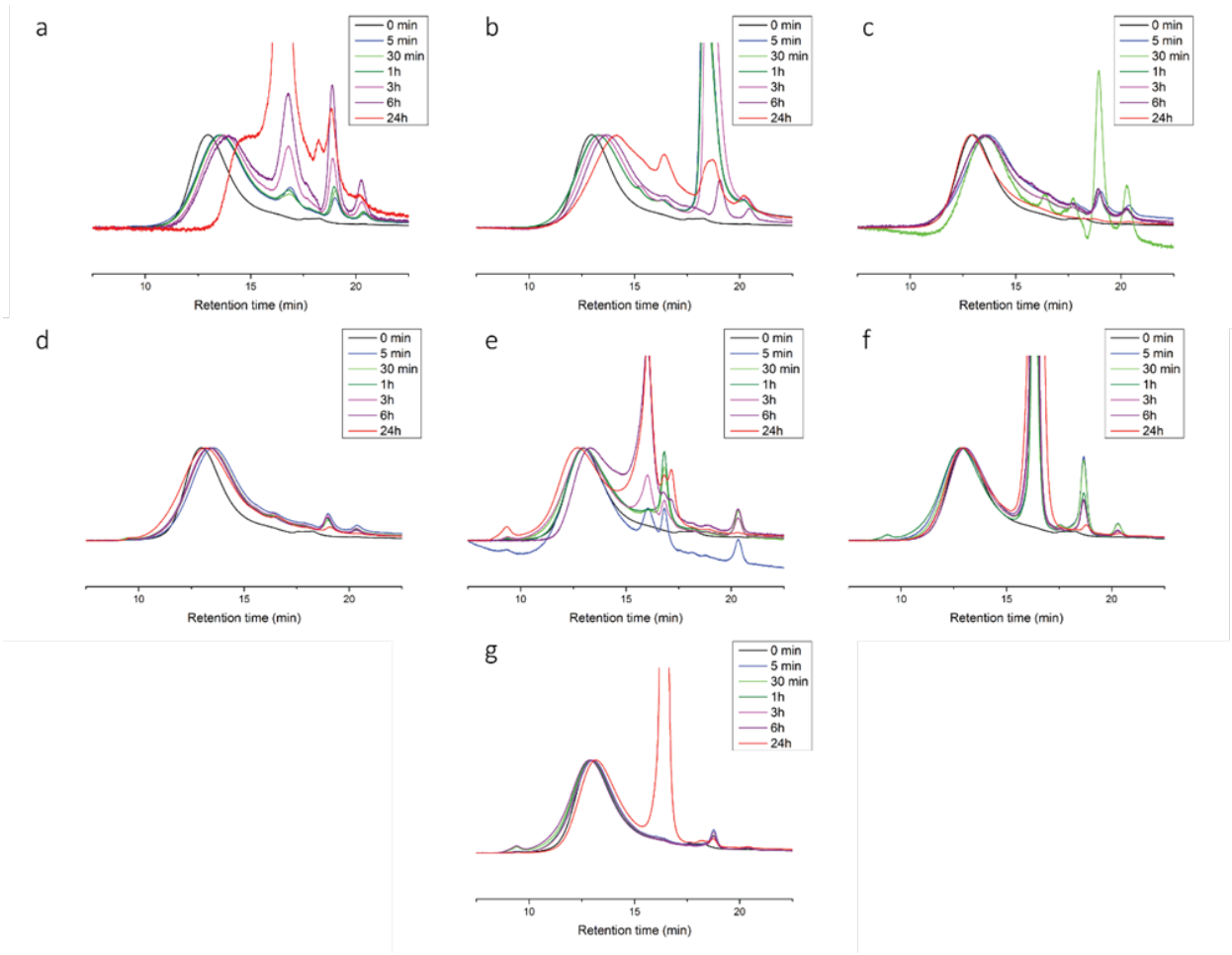

Figure S3. GPC analysis of organosolv lignin degraded by LA mediated systems with a) ABTS b) violuric acid c) HBT d) HAA e) TBDMP f) TTBP g) laccase alone, at different reaction time.

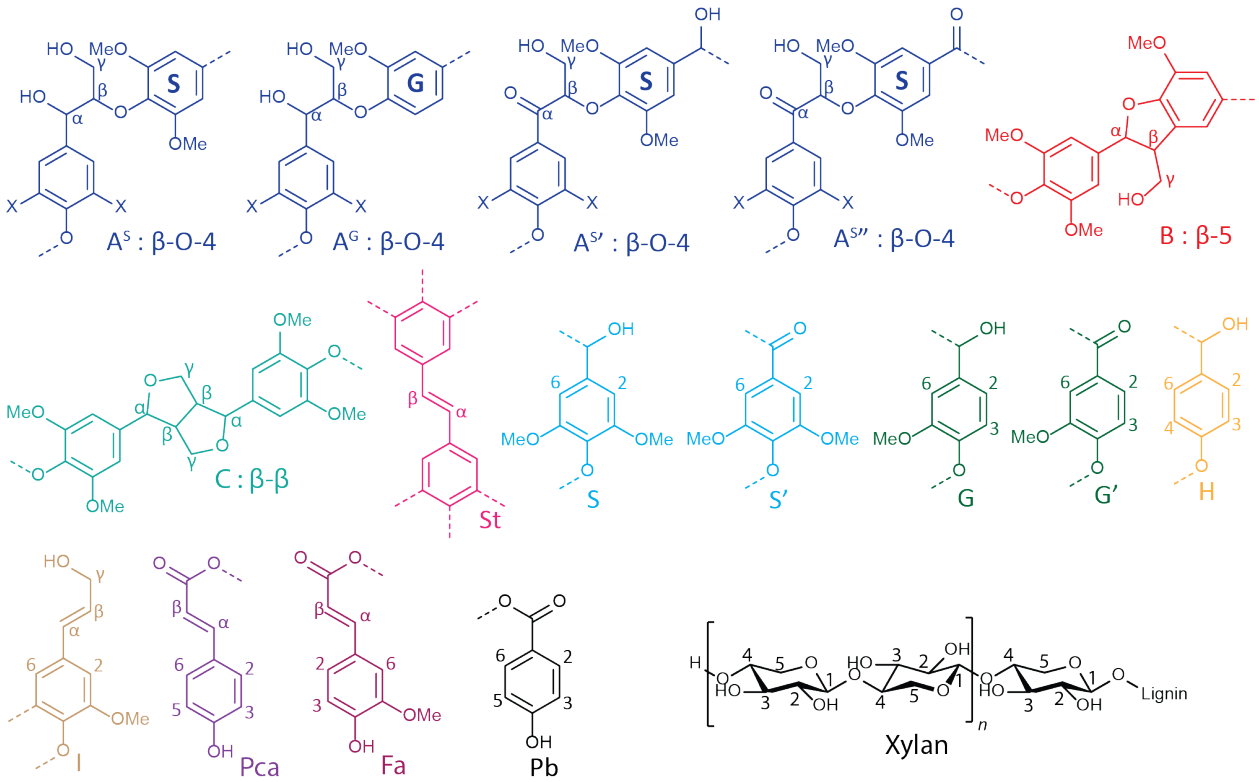

Figure S4. Different lignin linkages and structures identified by $^{13}C/^{1}H$ correlation 2D NMR. X=H/OCH_3

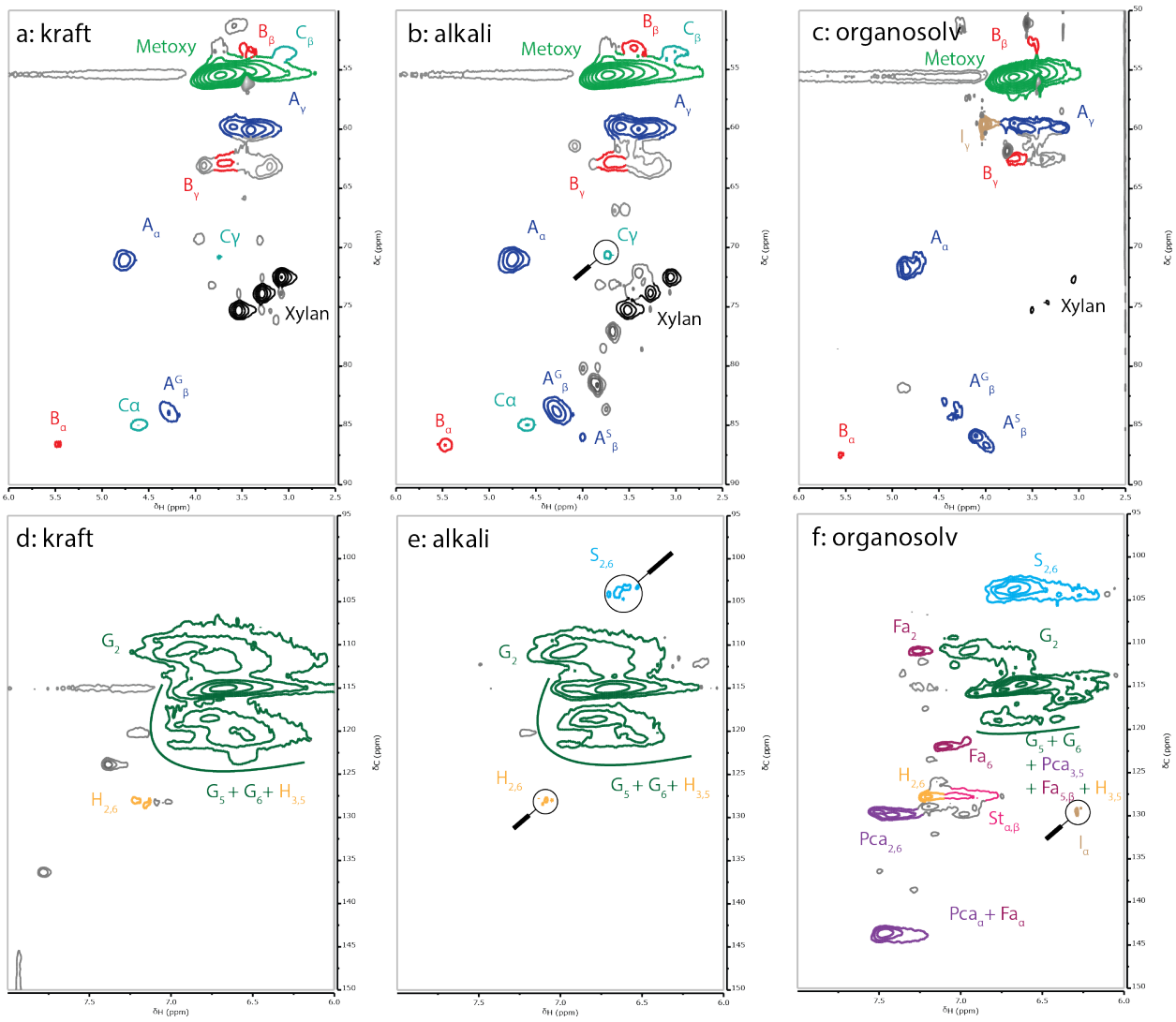

Figure S5. $^{13}C/^{1}H$ correlation HMQC 2D NMR analysis. a), b), c): aliphatic oxygenated side chain region of kraft, alkali and organosolv lignin respectively. d), e), f): aromatic hydrogen region of the same samples, respectively.

Calculation of S/G/H ratio:

In quantitative HMQC of lignin, S, G and H units are often used as internal standards for the integration. Integration (I) of the proton 2 of the syringyl unit, S_2 and S_2' area, represent the total amount of S units in lignin.

$$I_S = \frac{I_{S_{2,6}}}{2} + \frac{I_{S'_{2,6}}}{2}$$

Similarly, total of G units are represented by G_2 and G_2'. H units are represented by H_2.

$$I_G = I_{G_2} + I_{G'_2}$$

$$I_H = \frac{I_{H_{2,6}}}{2}$$

The percentage of each unit in lignin is thus easy to calculate

$$\%S = \frac{I_S}{I_S + I_G + I_H} \times 100$$

Calculation of linkages ratio:

Lignin structure exhibit all three aromatic units, S, G and H. Therefore, the average aromatic unit in a lignin sample is an aromatic unit with:

$$average\ protons\ per\ aromatic = \frac{\%S \times 2 + \%G \times 3 + \%H \times 4}{100}$$

And:

$$bondings\ or\ functional\ group = 6 - average\ protons\ per\ aromatic$$

This theoretical average aromatic lignin is chosen as a reference for the determination of the different linkages ratio inside the structure. The integration of one aromatic proton of this average unit (I_{proton}) can then be calculated from the two following equation:

$$I_{total\ aromatic\ protons} = average\ protons\ per\ aromatic\ \times I_{proton}$$

$$I_{total\ aromatic\ protons} = I_{S_{2,6}} + I_{S'_{2,6}} + I_{G_2} + I_{G'_2} + I_{H_{2,6}} = 2I_S + 3I_G + 4I_H$$

Thus:

$$I_{proton} = \frac{2I_S + 3I_G + 4I_H}{average\ protons\ per\ aromatic}$$

Finally, linkages ratio can be calculated from the integration of one of their specific protons. In the case of non-oxidized β-*O*-4 bonds:

$$\%_{non-ox.\ \beta-O-4} = \frac{I_{A_\alpha}}{I_{proton}}$$

Degree of condensation (DC):

The degree of condensation measure the number of aromatic protons that have been replaced by new linkages during condensation reaction. Theoretical amount of aromatic protons from lignin units correlating in the upper aromatic area (δ_C/δ_H 100-135/6-8.7) can be calculated from the S/G/H ratio considering the contribution of two protons from S units (H2 and H6), three protons from G units (H2, H3 and H6) and four protons from H units (H3, H5, H2 and H6).

$$Aromatic\ protons_{theory} = 2I_S + 3I_G + 4I_H$$

$$Aromatic\ protons_{experimental} = I_{S_{2,6}} + I_{S'_{2,6}} + I_{G_2} + I_{G'_2} + I_{G_5} + I_{G_6} + I_{G'_6} + I_{H_{2,6}} + I_{H_{3,5}}$$

Thus:

$$DC = \frac{Aromatic\ protons_{theory} - Aromatic\ protons_{experimental}}{average\ protons\ per\ aromatic}$$

Table S2. Analysis of three lignin structure

	Kraft lignin	Alkali lignin	Organosolv lignin
S/G/H units ratio	n.d. / 99 % / 1 %	2% / 97 % / 1 %.	44 %/53 %/3 %

Oxidized S unit/S unit*	n.d.	n.d.	n.d.
Oxidized G unit/G unit*	n.d.	n.d.	n.d.
β-5*	8 %	11 %	8 %
β- β*	6 %	6 %	n.d.
Non ox. β-*O*-4*	37 %	67 %	62 %
β-*O*-4 (A^G)*	27 %	49 %	22 %
β-*O*-4 (A^S)*	n.d.	16 %	34 %
β-*O*-4 ox. ($A^{S'}$)*	n.d.	n.d.	n.d.
β-*O*-4 ox. ($A^{S''}$)*	n.d.	n.d.	n.d.
St*	n.d.	n.d.	13 %
DC	0.93	0.94	0.00

* per aromatic circle

n.d. not detected

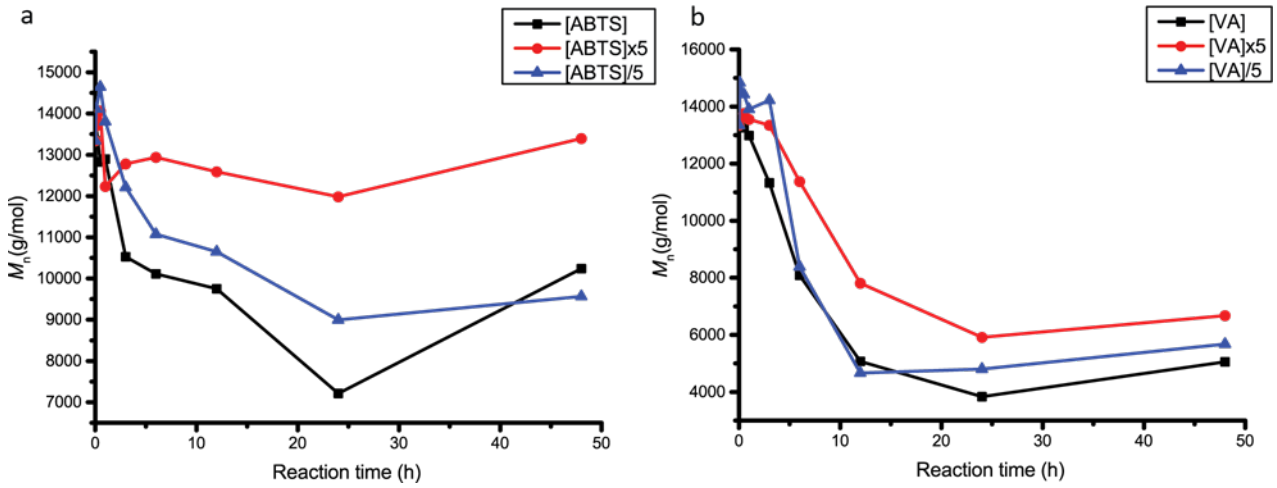

Figure S6. Evolution of average molecular weight by number versus reaction time, measured by GPC. Three different ratio [mediator]/[LA] have been studied to determine optimal conditions for LA mediated degradation of lignin: a) ABTS, b) VA

Synthesis of TBDMP:

Synthesis of *tert*-butyl-di-methyl-phenol (TBDMP) was performed as follow: dried acetonitrile (200 mL) was added into a dried round bottom flask, along with aluminium chloride (16.25 g, 175.5 mmol) and *tert*-butyl chloride (2.3 g, 24.8 mmol). The reaction mixture was stirred for 15 min then 2,6-dimethyl-phenol (2.5 g, 20.4 mmol) was slowly added. The reaction was allowed to proceed for 36 hours at 50 °C in reflux conditions. After reaction, mixture was cooled down and acetonitrile was removed under reduce pressure. The resulting solid was dissolved in chloroform (20 mL) and washed with water (20 mL). The chloroform phase was recovered while aqueous phase was extracted an additional two times with chloroform (20 mL). The organic layer were combined and dried over $MgSO_4$, then chloroform was removed under reduced pressure. Finally, the resulting solid was dissolved in cyclohexane and recrystallized in freezer after 48 hours to yield a solid white crystal (1.82 g, 10.2 mmol). Yield was 50 % but further recrystallisation steps will

yield additional product. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.03 (s, 2H, ArH), 2.28 (s, 6H, CH_3), 1.32 (s, 9H, CH_3). ^{13}C-NMR (400 MHz, $CDCl_3$): δ 16.3, 31.7, 34.0, 122.5, 125.7, 143.0, 150.0. IR (KBr): $\nu_{O–H}$ = 3403 cm^{-1}, $\nu_{C–H}$ = 2917 cm−1, $\nu_{C=C}$ = 1 617 cm^{-1}.

Annex 2: Supporting information for chapter 3

Linear bio-based aromatic polymers from syringic acid, S-type degradation fragment from lignin

Lionel F. Longe[a], Gil Garnier[b] and Kei Saito*[a].*

[a] School of chemistry, Bioresource Processing Research Institute of Australia (BioPRIA), 17 Rainforest walk, Monash University, Clayton 3800, Australia

[b] Department of Chemical Engineering, Bioresource Processing Research Institute of Australia (BioPRIA), 15 Alliance Lane, Monash University, Clayton 3800, Australia

* Corresponding authors email: kei.saito@monash.edu, tel: +61(0)399054600, email: gil.garnier@monash.edu, tel: +61(0)399059180

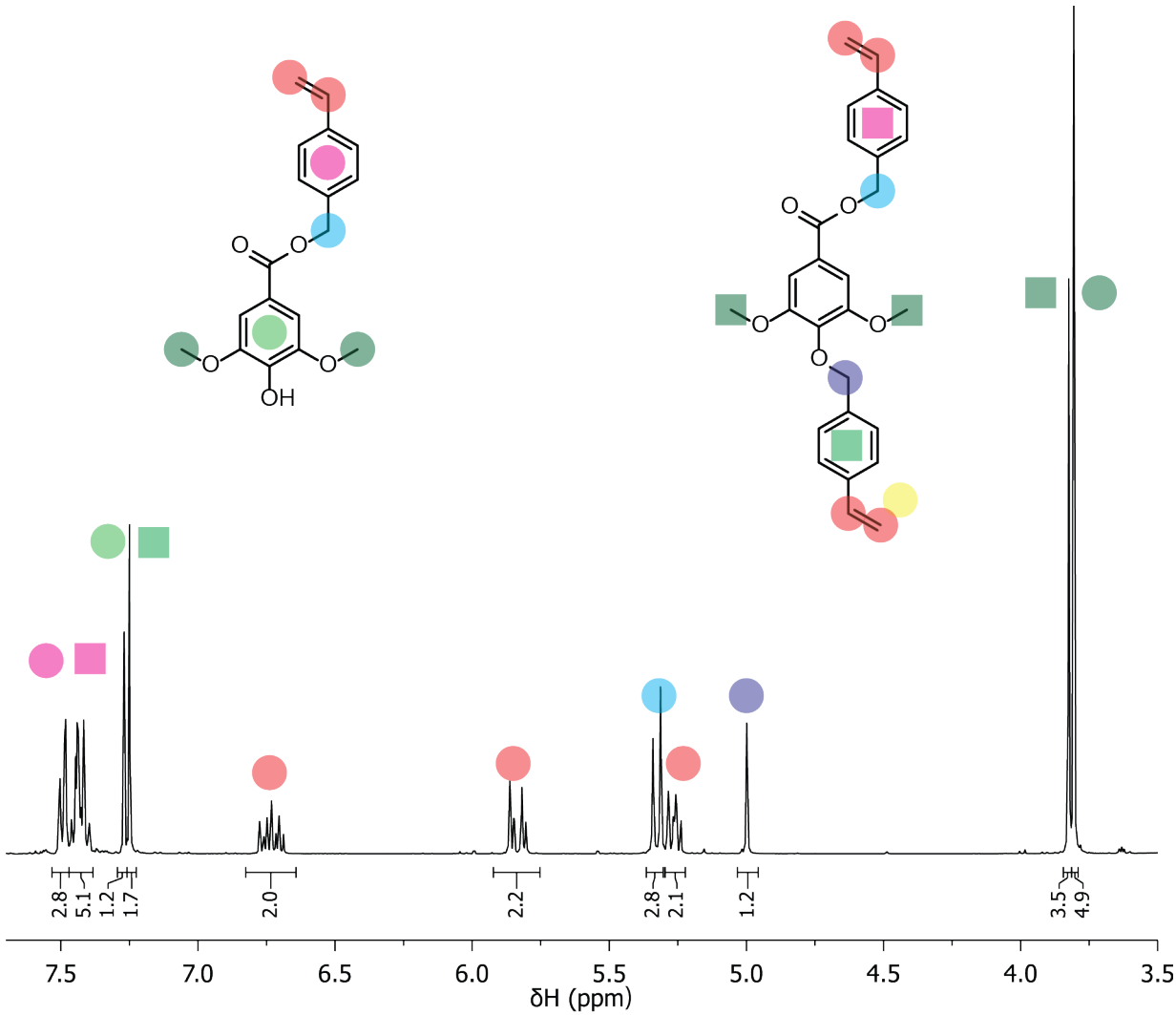

Figure SI1. ^{1}H NMR of mono- and di-reacted syringic acid with vinyl benzyl chloride.

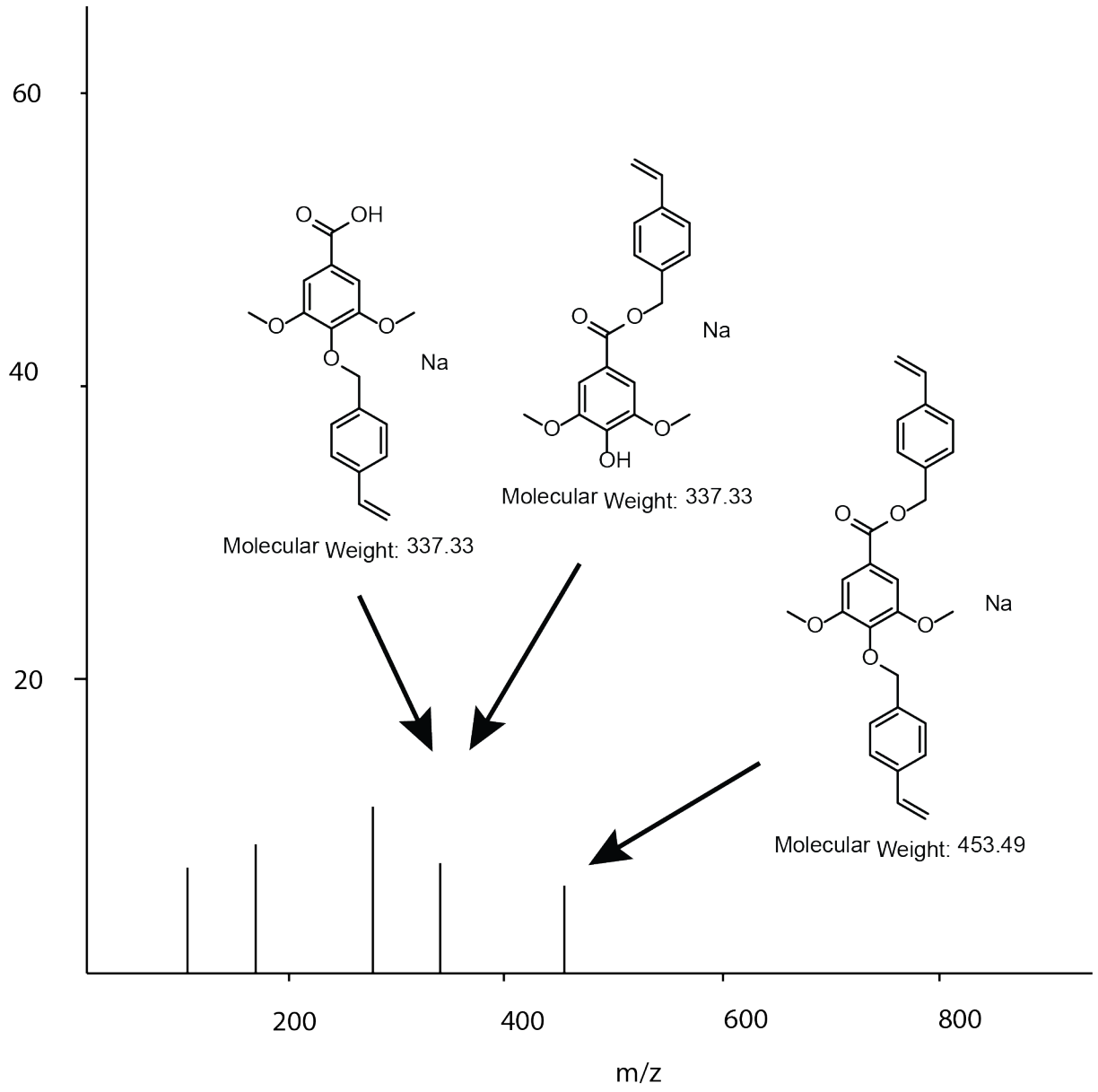

Figure SI2. Mass spectrometry of mono- and di-reacted syringic acid with vinyl benzyl chloride.

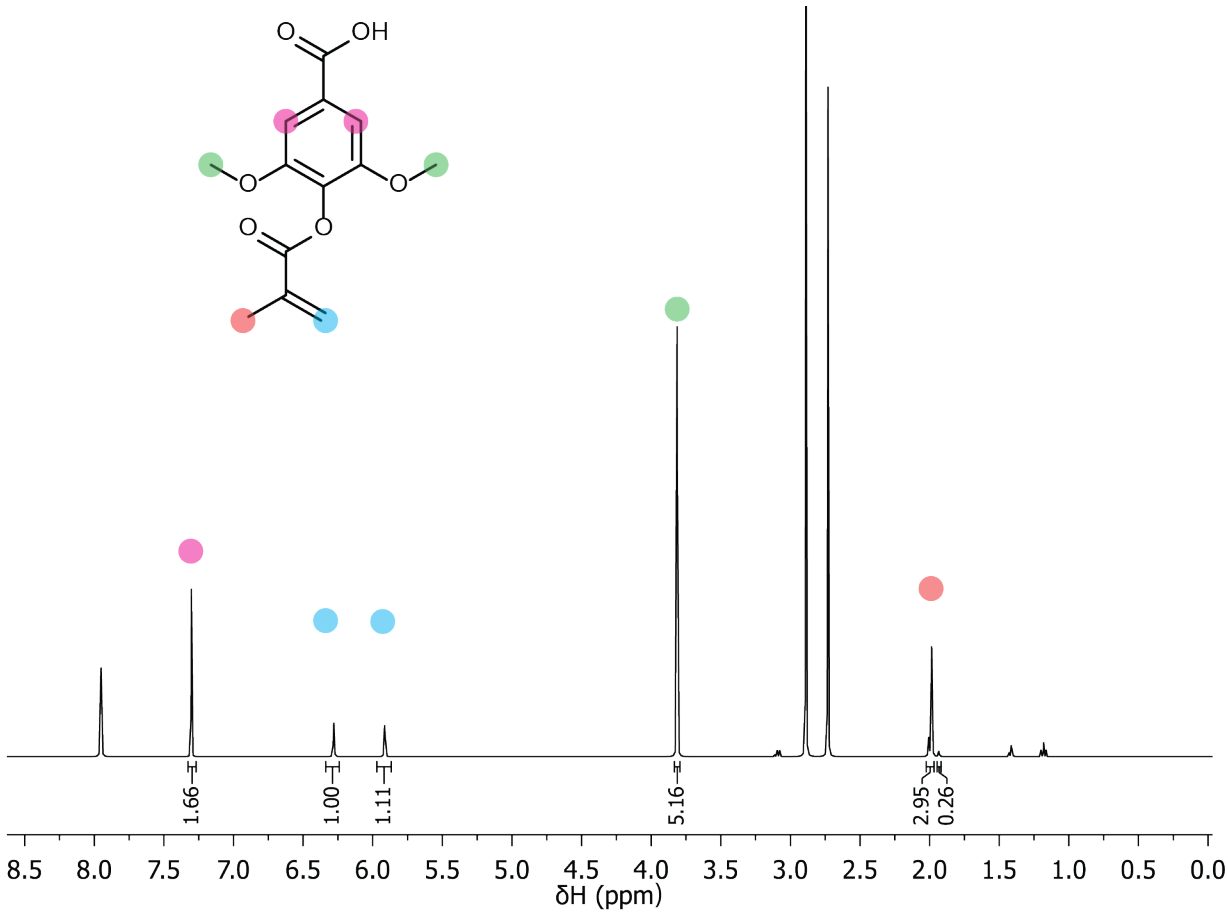

Figure SI3. ^{1}H NMR of compound **4**.

Table SI1. Diffusion coefficient of compound **4** and different side product measured by DOSY confirm efficient conversion.

Compound	Chemical shift	Assignment	D ($m^2.s^{-1}$)
SA	δ= 7.21 ppm	Ar-H	$2.5 .10^{-10}$
SA	δ= 3.80 ppm	O-CH_3	$2.4 .10^{-10}$
MA	δ = 5.96 & 5.54 ppm	C=CH_2	$4.2 .10^{-10}$
MA	δ = 1.81 ppm	C-CH_3	$4.2 .10^{-10}$
Mon. 4	δ = 7.31 ppm	Ar-H	$1.4 .10^{-10}$
Mon. 4	δ = 3.80 ppm	O-CH_3	$1.4 .10^{-10}$

Mon. 4	δ = 6.28 & 5.87 ppm	$C{=}CH_2$	$1.4 .10^{-10}$
Mon. 4	δ = 1.97 ppm	$C\text{-}CH_3$	$1.4 .10^{-10}$

Table SI2. Polymerisation condition screening for compound **4** in different solvents and with different initiators.

Initiator	Solvent system	M_n (g.mol^{-1})	*Đ*
Dicumyl peroxide	organic (DMF)	700	1.8
Benzoil peroxide	organic (DMF)	1,300	1.5
APS	organic (DMF)	600	1.5
AAPH	organic (DMF)	800	1.8
AIBN	organic (DMF)	5,300	1.6
VA-086	organic (DMF)	800	1.6
ACCN	organic (DMF)	4,300	1.4
APS	aqueous	11,300	2.8
AAPH	aqueous	1,400	2.0
AIBN	aqueous	1,500	1.8
VA-086	aqueous	23,500	2.6
ACCN	aqueous	3,700	3.0
tert-butyl peroxide	aqueous	6,800	2.9
AIBN	reverse emulsion	34,000	3.6
ACCN	reverse emulsion	1,500	1.5

AUTHOR INFORMATION

Corresponding Author:

Kei Saito, email: kei.saito@monash.edu, tel: +61(0)399054600,

Gil Garnier, email: gil.garnier@monash.edu, tel: +61(0)399059180

Annex 3: Supporting information for chapter 4

molecules

MDPI

Article

Synthesis of lignin-based phenol terminated hyperbranched polymer

Lionel Longe [1], Gil Garnier [2,*] and Kei Saito [1,*]

[1] Monash University, School of Chemistry, Bioresource Processing Research Institute of Australia (BioPRIA), Clayton 3800, Australia

[2] Monash University, Department of Chemical Engineering, Bioresource Processing Research Institute of Australia (BioPRIA) Clayton 3800, Australia

* Correspondence: kei.saito@monash.edu ; gil.garnier@monash.edu

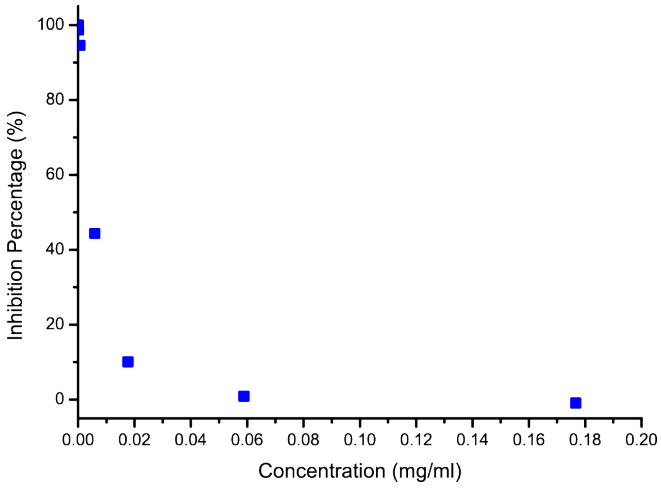

Figure SI1: Inhibition percentage of ascorbic acid versus concentration. Measured by bleaching of DPPH at 515 nm during radical scavenging assays

	EC_{50} (mg/ml)	Antiradical efficacy (mmol) [a]
ascorbic acid	0.0052	12.7
ascorbic acid [1]	/	12.0

[a]: DPPH equivalents per gram of material

Table SI1: Antiradical efficacy of ascorbic acid compared to literature

References

1. Omidi, S., Kakanejadifard, A. Modification of chitosan and chitosan nanoparticle by long chain pyridinium compounds: Synthesis, characterization, antibacterial, and antioxidant activities. *Carbohydr. polym.*, **2019** *15*; 477-485

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