

**Original citation:**

Lancefield, Christopher S., Rashid, Goran M. M., Bouxin, Florent, Wasak, Agata, Tu, Wei-Chien, Hallett, Jason P., Zein, Sharif, Rodriguez, Jaime, Jackson, S. David, Westwood, Nicholas J. and Bugg, Timothy D. H.. (2016) An investigation of the chemocatalytic and biocatalytic valorisation of a range of different lignin preparations : the importance of  $\beta$ -O-4 content. ACS Sustainable Chemistry & Engineering .

**Permanent WRAP URL:**

<http://wrap.warwick.ac.uk/81998>

**Copyright and reuse:**

The Warwick Research Archive Portal (WRAP) makes this work by researchers of the University of Warwick available open access under the following conditions. Copyright © and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners. To the extent reasonable and practicable the material made available in WRAP has been checked for eligibility before being made available.

Copies of full items can be used for personal research or study, educational, or not-for profit purposes without prior permission or charge. Provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.

**Publisher's statement:**

This document is the Accepted Manuscript version of a Published Work that appeared in final form in ACS Sustainable Chemistry & Engineering, copyright © American Chemical Society after peer review and technical editing by the publisher.

To access the final edited and published work see

<http://dx.doi.org/10.1021/acssuschemeng.6b01855>

**A note on versions:**

The version presented here may differ from the published version or, version of record, if you wish to cite this item you are advised to consult the publisher's version. Please see the 'permanent WRAP url' above for details on accessing the published version and note that access may require a subscription.

For more information, please contact the WRAP Team at: [wrap@warwick.ac.uk](mailto:wrap@warwick.ac.uk)

# **An Investigation of the Chemocatalytic and Biocatalytic Valorisation of a Range of Different Lignin Preparations: the Importance of $\beta$ -O-4 Content**

Christopher S. Lancefield<sup>b§</sup>, Goran M.M. Rashid<sup>a§</sup>, Florent Bouxin<sup>c</sup>, Agata Wasak<sup>a</sup>, Wei-Chien Tu<sup>d</sup>,  
Jason Hallett<sup>d</sup>, Sharif Zein<sup>e</sup>, Jaime Rodríguez<sup>f</sup>, S. David Jackson<sup>c</sup>, Nicholas J. Westwood<sup>b</sup>,  
Timothy D.H. Bugg<sup>a\*</sup>

<sup>a</sup>Department of Chemistry, University of Warwick, Coventry CV4 7AL, U.K. Email  
T.D.Bugg@warwick.ac.uk

<sup>b</sup>School of Chemistry and Biomedical Sciences Research Complex, University of St Andrews and  
EaStCHEM, North Haugh, St Andrews, Fife, KY16 9ST, U.K.

<sup>c</sup>Centre for Catalysis Research, WestCHEM, School of Chemistry, University of Glasgow, Glasgow  
G12 8QQ, U.K.

<sup>d</sup>Department of Chemical Engineering, Imperial College London, Exhibition Road, London SW7  
2AZ, U.K.

<sup>e</sup>School of Engineering, University of Hull, Cottingham Road, Hull HU6 7RX, U.K.

<sup>f</sup>Biotechnology Center, Universidad de Concepción, Casilla 160-C Concepcion, Chile.

<sup>§</sup>These authors contributed equally to this work.

Abstract: A set of 7 different lignin preparations was generated from a range of organosolv (acidic, alkaline, ammonia-treated and dioxane-based), ionic liquid, autohydrolysis and Kraft pretreatments of lignocelluloses. Each lignin was characterised by 2D HSQC NMR spectroscopy, showing significant variability in the  $\beta$ -O-4 content of the different lignin samples. Each lignin was then valorised using three biocatalytic methods (microbial biotransformation with *Rhodococcus jostii* RHA045, treatment with *Pseudomonas fluorescens* Dyp1B or *Sphingobacterium sp.* T2 manganese superoxide dismutase) and two chemocatalytic methods (catalytic hydrogenation using Pt/alumina catalyst, DDQ benzylic oxidation/Zn reduction). Highest product yields for DDQ/Zn valorisation were observed from poplar ammonia percolation-organosolv lignin, which had the highest  $\beta$ -O-4 content of the investigated lignins and also gave the highest yield of syringaldehyde (243 mg/L) when using *R. jostii* RHA045, and the most enzymatic products using *P. fluorescens* Dyp1B. The highest product yield from the Pt/alumina hydrogenation was observed using oak dioxasolv lignin, which also had a high  $\beta$ -O-4 content. In general, highest product yields for both chemocatalytic and biocatalytic valorisation methods were obtained from preparations that showed highest  $\beta$ -O-4 content, while variable yields were obtained with preparations containing intermediate  $\beta$ -O-4 content, and little or no product was obtained with preparations containing low  $\beta$ -O-4 content.

Synopsis. The study examines the conversion of biomass-derived lignin preparations into renewable chemicals using chemocatalytic and biocatalytic methods.

Keywords: lignin valorization; catalytic hydrogenation; microbial biotransformation; *Rhodococcus jostii* RHA1; Dyp peroxidase; superoxide dismutase

## Introduction

The aromatic heteropolymer lignin is an important component of plant cell walls, comprising 15-30% of lignocellulosic plant biomass. It therefore represents an abundant renewable raw material for conversion to aromatic chemicals, provided that efficient conversion methods can be found. Since the aryl-C<sub>3</sub> units present in the lignin structure are linked via alkyl-aryl ether linkages and carbon-carbon bonds (see Figure 1B), lignin is difficult to depolymerise, and there is considerable interest in methods for lignin valorisation.<sup>1,2</sup> Several new chemocatalytic approaches for lignin valorisation have been published in recent years, that are able to generate aromatic monomers from lignin feedstocks.<sup>3-9</sup> There is also renewed interest in novel microbial enzymes for lignin oxidation,<sup>10</sup> and the use of engineered microbial hosts for the generation of aromatic products via microbial biotransformation.<sup>11-14</sup>

A complicating factor in the field is that there are a number of different methods for the fractionation of lignocellulose that generate lignin preparations of very different structure, physical properties, and reactivity. Some lignin isolation methods such as those based on mechanical milling and dioxane extraction are designed to preserve the structure of lignin as found in plant lignocellulose,<sup>15</sup> while organosolv fractionation methods use a mild organic acid or solvent treatment to solubilise lignin and hemicellulose.<sup>16</sup> Industrially, technical lignins are generated in potentially large quantities from the industrial Kraft and sulfite processes for pulp and paper manufacture, but Kraft lignin<sup>17</sup> and liginosulfonate<sup>18</sup> generated from the sulfite process are both extensively structurally modified. In addition new lignin streams are now being generated in commercial lignocellulosic biorefineries, mostly as enzymatic hydrolysis residues produced from either steam explosion or dilute acid-pretreated substrates. Alternative extraction methods such as ionic liquid pretreatments seek to combine cellulose isolation with high quality lignin production.<sup>19,20</sup>

In spite of a range of lignin valorisation methods noted above, there are only a few observations in the literature concerning the advantages or disadvantages of different lignin isolation methods for downstream lignin valorisation. Kraft lignin has been found to give lower yields of lignin valorisation products in some biocatalytic processes,<sup>11,14</sup> and Bouxin *et al.* have found variable yields of chemocatalytic reduction products using four different lignin preparations.<sup>21</sup> *From observations of groups in this consortium, our hypothesis was that the method used to prepare the lignin sample, and the molecular structure of that lignin sample, are likely to have major effects on its value for subsequent valorisation.* Since lignin valorisation is an important part of any future lignocellulose-based biorefinery, we felt that this was an important research question, and we have carried out a short collaborative project to study the consequences of different lignin isolation methods on lignin valorisation. We have assembled a collection of seven

different lignins prepared by different research groups in the consortium, that are representative of methods currently published in the field: organosolv methods using mildly acid or mildly alkaline conditions; a lignin prepared via ionic liquid extraction; an autohydrolysis lignin and a Kraft lignin. We have tested this collection of lignins against five recently published valorisation methods developed by research groups in the consortium: two chemocatalytic methods, involving either catalytic hydrogenation or oxidative modification followed by reductive C-O cleavage; and three biocatalytic methods, involving either a microbial biotransformation, or two enzymatic biotransformations (see Figure 1); and have analysed the low molecular weight aromatic products from these treatments. The results of this study could then be useful in choosing appropriate pretreatment methods to maximise the value of lignin streams in future biorefinery designs.

Figure 1. Design of experimental study.

## Experimental Section

### *Materials.*

Poplar ammonia organosolv lignin was prepared from hybrid poplar (*Populus robusta*) sawdust using a 15% ammonia percolation method at 180 °C followed by a mild organosolv treatment in EtOH/H<sub>2</sub>O containing 0.1 M H<sub>2</sub>SO<sub>4</sub>, as previously described.<sup>22</sup> Poplar alkaline organosolv lignin was prepared from hybrid poplar (*Populus robusta*) sawdust as previously described.<sup>21,22</sup> Oak dioxasolv lignin was prepared from English oak (*Quercus robur*) sawdust using dioxane/H<sub>2</sub>O (9:1) containing 0.2 M HCl for 1 hr under reflux, as previously described.<sup>6</sup> Eucalyptus Kraft lignin was prepared from *Eucalyptus globulus* wood chips as previously described.<sup>23</sup> Eucalyptus organosolv lignin was prepared from *Eucalyptus globulus* wood chips by acidic ethanol/water extraction as previously described.<sup>24</sup> Eucalyptus autohydrolysis lignin was prepared from *Eucalyptus globulus* wood chips by autohydrolysis pretreatment at 175 °C followed by alkaline extraction, as described previously.<sup>25</sup> Miscanthus ionosolv lignin was prepared from *Miscanthus x giganteus* by treatment with the ionic liquid triethylammonium hydrogen sulfate ([TEA][HSO<sub>4</sub>]) containing 20 wt% water at 20% solids loading and 150 °C for 1 h, as described previously.<sup>26</sup>

<sup>1</sup>H-<sup>13</sup>C NMR spectra were recorded in d<sub>6</sub>-DMSO on a Bruker Avance III spectrometer, using the protocols previously described.<sup>21,27</sup> Assignment of <sup>1</sup>H NMR signals was based upon considerable literature work on NMR analysis of isolated lignin preparations<sup>28,29</sup> and intact plant cell wall lignin<sup>30,31</sup>, including assignment of NMR signals for tricetin flavonoid.<sup>32</sup> Gel permeation chromatography and thioacidolysis were carried out using the previously described method.<sup>22</sup>

### *DDQ/Zn valorisation method.*

Lignin samples (300 mg, 0.05-0.2 mmol, see molecular weight data in Table 2) were oxidised using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 400 mg, 1.7 mmol) in a mixture of 2-methoxyethanol (3 mL) and 1,2-dimethoxyethane (4 mL) at 60 °C for 16 hours. The samples were then precipitated in EtOAc and collected by filtration. The samples were then re-suspended in methanol (x3) to remove excess DDQ/DDQH<sub>2</sub> and then collected by filtration. Typical recovery ~65%. 100 mg was used for NMR analysis, and 100 mg was used for Zn depolymerisation. Oxidised lignin samples (~50 mg) were dissolved in 2-methoxyethanol (0.8 mL) and water (0.2 mL) at 80 °C. NH<sub>4</sub>Cl (65 mg) was then added followed by Zn powder (75 mg) and the mixture stirred for 1 hour. The reaction mixture was then diluted with water (3 mL), the pH adjusted to ~7 with NaH<sub>2</sub>PO<sub>4</sub> and then extracted with DCM (3 x 5 mL). The samples (10 µL injection in MeOH) were separated on a YMC C<sub>18</sub> column 5 µm (120 Å, 250 mm, 4.6 mm) on a Gilson HPLC system, at a flow rate of 0.8 mL/min, monitoring at 280 nm. The solvents were water as solvent A and MeOH as solvent B. A gradient of 10-35% solvent B from 0-35 min; 35-100% from 35-39 min; 100-10% from 39-48 min; 10% from 48-50 min was used for separation of products.

### *Hydrogenolysis over Pt/alumina*

The catalyst used throughout was a 1 wt% Pt/alumina catalyst supplied by Johnson Matthey (reference number 1074). A platinum dispersion of 56 % was determined from carbon monoxide chemisorption, giving a particle size of ~2 nm. The support was confirmed as principally  $\theta$ -alumina from XRD analysis. The catalyst had a BET surface area of 119 m<sup>2</sup>.g<sup>-1</sup>, a pore volume of 0.49 cm<sup>3</sup> g<sup>-1</sup> and an average pore diameter of 11 nm.

The catalytic reactions were conducted in a 300 mL, 316 stainless steel, Parr batch autoclave reactor, equipped with a digital temperature controller ( $\pm$  1 K). The reactant mix within the reactor was stirred using a Parr magnetic driven stirrer and pressure was monitored during the reaction using a standard pressure gauge. Prior to reaction the catalyst was pre-reduced by heating to 523 K in 2 % H<sub>2</sub>/N<sub>2</sub> at a ramp rate of 10 K.min<sup>-1</sup> with a dwell time at 523 K of 2 h. After the reduction step, the catalyst was cooled to room temperature in flowing argon then passivated in 2 % O<sub>2</sub>/Ar.

Hydrogenolysis was carried out in a Parr autoclave reactor followed by analysis of the products by GC-MS (full analysis methodology given in reference 33). Briefly, 0.5 g of lignin (0.1-0.3 mmol, see molecular weight data in Table 2) and 0.1 g of catalyst were added to a 100 mL methanol-water mix (50/50, v/v) and loaded into the reactor. The system was purged with hydrogen and pressurised to 20 barg before heating to 573 K at 10 deg.min<sup>-1</sup>. The mixture was stirred at 1000 rpm throughout. The reactor was held at this temperature for 2 h. At reaction temperature the typical pressure recorded was 145 barg. The reaction mixture was filtered to remove the catalyst and any

insoluble products. The methanol-water soluble fraction was processed and an aliquot analysed by GC-MS. For GC-MS analysis the products were derivatised with trimethylsilyl chloride (TMS). Chemical composition was determined using a Shimadzu GC-MS-QP2010S coupled to a Shimadzu GC-2010 equipped with a ZB-5MS capillary column (30 m x 0.25 mm x 0.25  $\mu$ m) with He as the carrier gas.

#### *Microbial biotransformation of lignin samples using *Rhodococcus jostii* $\Delta$ vdh mutant*

The procedure is adapted from that in reference 11. Lignin (25 mg) was added to 5 mL M9 salts and autoclaved, to which filter sterilised solutions of 0.1% glucose, 2 mM MgSO<sub>4</sub> and 0.4 mM CaCl<sub>2</sub> were added, then 100  $\mu$ L of overnight culture of *Rhodococcus jostii* RHA1 strains RHA045 ( $\Delta$ vdh) was added to inoculate the media. The solution was left to shake at 30°C for 6 days at 180 rpm. The samples were acidified with 1 mL HCl (1 M) and frozen overnight, then cells disrupted by freeze-thawing three times. The products were extracted with 5 mL EtOAc and the organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and analysed as described below.

#### *Enzymatic treatment of lignin samples*

Recombinant *P. fluorescens* Dyp1B was expressed and purified as previously described.<sup>34</sup> Lignin (5 mg) was added to 3 mL succinic buffer (50 mM, pH 5.5), and then 100  $\mu$ L of DyP1B (1 mg/ mL) was added, followed by addition of 30  $\mu$ L of 100 mM H<sub>2</sub>O<sub>2</sub> and 3  $\mu$ L of 1 M MnCl<sub>2</sub>. The resulting solution was incubated at 30°C for 60 min, then acidified with 1 mL HCl (1 M) and extracted with 4 mL EtOAc. The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and analysed as described below.

Recombinant *Sphingobacterium* sp. T2 manganese superoxide dismutase SOD1 was expressed and purified as previously described.<sup>35</sup> To 3 mL phosphate buffer (pH 7.8, 50 mM), 5 mg of lignin was added, followed by 200  $\mu$ L of SOD1 (1 mg/ mL) and 1 mL of saturated KO<sub>2</sub> in dry DMSO. The resulting solution was incubated at 30°C for 60 min, then acidified with 1 mL HCl (1 M) and extracted with 4 mL EtOAc. The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and analysed as described below.

#### *Metabolite analysis by LC-MS*

The organic residues from microbial biotransformation of lignin samples and enzyme treatments were re-suspended in 200  $\mu$ L of MeOH/water 1:1 prior to LC/MS analysis. The samples (50  $\mu$ L) were separated on a C<sub>18</sub> column using Phenomenex Luna 5  $\mu$ m (100 Å, 50 mm, 4.6 mm) on an Agilent 1200 and Bruker HCT Ultra mass spectrometer, at a flow rate of 0.5 mL/min, monitoring at 310 and 270 nm. The solvents were water/ 0.1% formic acid as solvent A and MeOH/0.1% formic acid as solvent B. Two gradients were used for separation of products: method A starts with 15%

solvent B for 5 min; 15-25% B from 5-15 min; 25-70% B from 15-23 min; 70-100% from 23-30 min. Method B starts with 5% solvent B; 5-30% B from 0-20 min; 30-100% from 30-45 min, 100% solvent B from 45-55 min; 100-5% solvent B 55-65 min. Peaks were compared where possible with authentic standards in order to confirm structural assignments.

## Results

### *Preparation of lignin samples*

From samples of poplar hardwood, two methods were used to prepare lignin samples: a 15% ammonia percolation method at 180 °C in 0.1 M EtOH/H<sub>2</sub>O<sup>21</sup> and an alkaline organosolv method.<sup>21,22</sup> Oak hardwood was treated with dioxane/H<sub>2</sub>O (9:1) containing 0.2 M HCl for 1 hr under reflux, to give an organosolv lignin,<sup>6</sup> and a standard organosolv method was used to prepare lignin from eucalyptus, which was compared with autohydrolysis lignin and Kraft lignin also from eucalyptus. Lignin was also prepared using ionic liquid triethylammonium hydrogen sulfate ([TEA][HSO<sub>4</sub>]) from *Miscanthus x giganteus* as described previously.<sup>26</sup>

### *Structural characterisation of lignin samples*

Each lignin sample was analysed by two-dimensional <sup>1</sup>H-<sup>13</sup>C HSQC NMR spectroscopy,<sup>28-32</sup> allowing a semi-quantitative estimation of the relative proportions of S, G and H units, the β-O-4, β-5 and β-β linkages, and ferulate, *p*-coumarate and *p*-hydroxybenzoate ester linkages (see Footnote 1). This analysis revealed that both the compositional and structural features of the examined lignins varied greatly (see Table 1).

The poplar ammonia lignin (Figure 2A), a typical hardwood S-G lignin, had the highest β-O-4 content of all the samples examined and also contained a small number of α-ethoxylated β-O-4 units presumably resulting from the mild acid catalysed ethanol-water organosolv treatment used to purify the crude carbohydrate rich ammonia lignin. This lignin also contained a smaller number of β-β and β-5 linkages as well as a small number of *p*-hydroxybenzoate units, typical of poplar lignins, which appear to survive the ammonia treatment. Similarly, the oak dioxasolv lignin, also an S rich S-G lignin, had a high proportion of β-O-4 units (see Figure 2) and also contained some Hibbert ketones formed by acid catalysed hydrolytic reactions (see Supporting Information Figures S1, S2).

The wheat straw microwave-organosolv and *Miscanthus* ionosolv lignins were more complex, containing S, G, H, ferulate and *p*-coumarate groups as well as significant amounts of tricetin units in the case of the wheat lignin. Both contained significant amounts of β-O-4 units, although less than the poplar ammonia and oak dioxasolv lignins. The *Miscanthus* lignin also contained Hibbert ketones consistent with the aqueous acidic ionic liquid pretreatments. This lignin

also contained relatively few  $\beta$ - $\beta$  units, consistent with the high degree of  $\gamma$ -acylation which precludes the formation of such units, and the lower proportion of S units present in grass lignins.

The three eucalyptus lignins examined were particularly S rich. Both the organosolv and autohydrolysis eucalyptus lignins contained some  $\beta$ -O-4 and  $\beta$ - $\beta$  units but almost no  $\beta$ -5 units, consistent with the S rich nature of these lignins. Interestingly, the Kraft lignin contained no detectable amounts of  $\beta$ -O-4 or  $\beta$ -5 units but contain significant amounts of  $\beta$ - $\beta$  units and the hemicellulose xylan.

Interestingly, with the exception of the wheat lignin, in all these lignins detectable amounts of epimerised  $\beta$ - $\beta$  units are present resulting from either acid or base catalysed processes. We have previously shown that such units are not detectable in a softwood Kraft lignin (G type),<sup>27</sup> so their occurrence appears to be both process and feedstock dependent, but they may be present in other S-G type technical lignins.

Figure 2. Analysis of lignin structure by  $^1\text{H}$ - $^{13}\text{C}$  NMR spectroscopy, showing (A) poplar ammonia organosolv lignin containing high  $\beta$ -O-4 content; (B) miscanthus ionic liquid lignin containing medium  $\beta$ -O-4 content; (C) Eucalyptus Kraft lignin containing no  $\beta$ -O-4 units.

#### *Chemical valorisation methods*

Lancefield *et al.* have previously shown that  $\beta$ -O-4 units can be selectively oxidised using cat. DDQ/O<sub>2</sub>, and then the oxidised  $\alpha$ -keto- $\beta$ -aryl ether linkages can be reductively cleaved by Zn to yield 2-hydroxyethyl-aryl ketones (see Figure 3).<sup>6</sup> Based on this method, three lignin samples were oxidised by stoichiometric DDQ in 2-methoxyethanol/1,2-dimethoxyethane at 60 °C for 16 hr to give lignin<sup>ox</sup>. NMR spectroscopic analysis of the oxidised lignin samples verified that the  $\beta$ -O-4 units had been either completely oxidised (for oak dioxasolv lignin and eucalyptus autohydrolysis lignin, see Supporting Information Figures S9 and S11) or largely oxidised (for ammonia organosolv lignin, see Supporting Information Figure S10). Treatment of the lignin<sup>ox</sup> samples with zinc in 2-methoxyethanol/water/NH<sub>4</sub>Cl at 80 °C for 1 hr was found to generate a mixture of 2-hydroxyethyl-aryl ketones **1** and **2** which were quantified by HPLC analysis (see Supporting Information).

The highest total yield (5.3 wt%) was obtained from the poplar ammonia lignin, with a 5:1 ratio of S:G products. This lignin sample also showed the highest proportion (40 per 100 C<sub>9</sub> units) of  $\beta$ -O-4 units by NMR analysis, which rationalises why this lignin would perform well via this method, since this method requires the presence of two consecutive  $\beta$ -O-4 units.<sup>6</sup> Excluding the effects of end groups, *p*-hydroxybenzoylated and condensed units, a theoretical maximum yield of

approximately 16 wt% would be expected from this lignin (see Footnote 2). Yields of 1.3 wt% and 1.4 wt% were obtained from the oak dioxosolv lignin and eucalyptus autohydrolysis lignin respectively, with 7:1 and 19:1 ratios of S:G products respectively. These two lignins showed similar or slightly lower proportions (40% and 27% respectively) of  $\beta$ -O-4 units by NMR analysis.

Figure 3. Conversion of lignin samples to 2-hydroxyethyl aryl ketones

Lignin samples were also chemically treated via high-pressure hydrogenation (20 bar) at 300 °C in a Parr reactor in methanol/water (1:1), over a Pt/alumina catalyst.<sup>21</sup> The products were analysed by GC-MS giving a collection of guaiacyl (G) or syringyl (S) products with n = 0-3 carbon sidechains, and some products containing demethylated G and S units. As shown in Figure 4, best product yields were obtained with the oak dioxasolv lignin, which gave 14% w/w yield of S 3-carbon products, 9% of 3-methoxyguaiacol, and 22% of other S products, and 9-10% of a mixture of G products. Miscanthus ionic liquid lignin was also processed efficiently via this method, giving 10% 3-methoxyguaiacol and 12-13% of a mixture of S products and 9-10% of a mixture of G products. Poplar ammonia organosolv lignin gave 7-8% of a mixture of S products and 3-4% of a mixture of G products, similar to the yields reported previously for this lignin preparation, which was found to give higher product yield than the poplar alkaline organosolv lignin preparation (6.6% S C3 products).<sup>21</sup>

Figure 4. Products obtained from catalytic hydrogenation at 300 °C/20 bar hydrogen over a Pt/alumina catalyst.

#### *Biocatalytic valorisation using Rhodococcus jostii gene deletion mutant RHA045*

Sainsbury *et al* have reported that microbial biotransformation of a *Rhodococcus jostii*  $\Delta$ vdh gene deletion mutant lacking vanillin dehydrogenase on M9 minimal media containing 1% (w/v) wheat straw lignocellulose led to the accumulation of vanillin (96 mg/L), 4-hydroxybenzaldehyde (50 mg/L) and ferulic acid (23-86 mg/L) after 6 days.<sup>11</sup> This *R. jostii* mutant strain was grown on M9 minimal media containing 1% (w/v) of each of the set of lignin samples, and samples of culture supernatant were removed after 6 days microbial biotransformation at 30 °C, products extracted into ethyl acetate, and analysed by LC-MS (see Table 3).

Aldehyde product syringaldehyde was observed in microbial biotransformations of poplar ammonia organosolv lignin (243 mg/L) and oak dioxasolv lignin (95 mg/L). Both of these lignins are rich in S units, so these incubations might be expected to accumulate syringaldehyde rather than vanillin. Small amounts of vanillin (1-5 mg/L yields) were observed, but were also observed at

similar concentrations in control samples with no bacteria, suggesting that the lignin preparations themselves contain small amounts of vanillin, as observed previously for Kraft lignin.<sup>11</sup> Also observed were high yields of vanillic acid (330-630 mg/L), obtained from poplar ammonia organosolv lignin, poplar alkaline organosolv lignin, and oak organosolv lignin, and vanillic acid was also observed using eucalyptus organosolv lignin and eucalyptus autohydrolysis lignin. The latter two samples also yielded substantial quantities of protocatechuic acid (230-300 mg/L), which had been observed as a metabolite from wheat straw lignocellulose and is thought to be a shunt metabolite.<sup>11</sup> Small amounts of reduced vanillyl alcohol were observed in 3 cases, and this was the only metabolite observed from the miscanthus ionic liquid lignin. Ferulic acid was also observed as a metabolite from oak dioxasolv lignin (yield 74 mg/L). No products were observed from eucalyptus Kraft lignin.

#### *Treatment of lignin samples with bacterial lignin-oxidising enzymes*

Bacterial Dyp-type peroxidases have been discovered in *Rhodococcus jostii*<sup>36</sup> and *Pseudomonas fluorescens* Pf-5<sup>34</sup> that can oxidise lignin model compounds, and in the presence of Mn<sup>2+</sup> can oxidise polymeric Kraft lignin and lignocellulose. In the case of *P. fluorescens* Dyp1B, an oxidised lignin dimer bio-product was isolated from treatment of wheat straw lignocellulose with hydrogen peroxide and Mn<sup>2+</sup>.<sup>34</sup>

Each lignin sample was treated with *P. fluorescens* Dyp1B in 50 mM succinate buffer pH 5.5 containing 1 mM hydrogen peroxide and 1 mM MnSO<sub>4</sub> for 1 hr at 30 °C, and then products were extracted into ethyl acetate and analysed by LC-MS. The largest number of product peaks (6 peaks, see Figure 5) were observed upon treatment of poplar ammonia lignin with Dyp1B, including S-containing products syringyl C<sub>3</sub> triol (MNa<sup>+</sup> 277), syringyl hydroquinone (MNa<sup>+</sup> 193) and syringyl alcohol (MNa<sup>+</sup> 207), and G-containing vanillyl alcohol (MH<sup>+</sup> 155). Syringyl products were also observed for poplar alkaline organosolv lignin and eucalyptus organosolv lignin. No assigned product peaks were observed using eucalyptus Kraft lignin or autohydrolysis lignin.

Figure 5. Products obtained by treatment of lignin preparations with *Pseudomonas fluorescens* Dyp1B (PfDyp1B) and *Sphingobacterium* sp. T2 manganese superoxide dismutase 1 (SpSOD1).

A novel manganese superoxide dismutase enzyme has recently been discovered in *Sphingobacterium* sp. T2 that can oxidise polymeric organosolv and Kraft lignin, generating multiple oxidation products.<sup>35</sup> Each lignin sample was treated with *Sphingobacterium* MnSOD1 in 50 mM phosphate buffer pH 7.8 containing 1 mM KO<sub>2</sub> in DMSO for 5 hr at 30 °C, and then products were extracted into ethyl acetate and analysed by LC-MS. The largest number of product

peaks were observed upon treatment of poplar alkaline organosolv lignin (6 peaks, including syringyl hydroquinone, guaiacyl hydroquinone, and guaiacol) and poplar ammonia lignin (5 peaks, including vanillic acid, syringaldehyde, and vanillin). Treatment of oak dioxasolv lignin gave 4 product peaks including syringaldehyde and syringyl alcohol, and miscanthus ionic liquid lignin gave guaiacol and a second unidentified peak. No product peaks were observed using eucalyptus Kraft lignin, eucalyptus organosolv lignin, or eucalyptus autohydrolysis lignin.

## Discussion

Despite extensive interest in lignin valorisation around the world, using both chemocatalytic and biocatalytic approaches, there is currently no clear consensus about what type of lignin preparation is optimum for subsequent lignin valorisation. This is an important question for the design of a lignocellulose-based biorefinery that could generate high-volume cellulosic biofuels, but also generate low-volume, high-value aromatic chemicals from the lignin fraction. For this reason, we undertook a study to assemble a small collection of different lignin preparations, to characterise their structures, and to study their valorisation via both chemocatalytic and biocatalytic methods. We have found considerable variation in lignin structure, product distribution and yield, verifying the original hypothesis that the method used to prepare lignin can have a large effect on its chemical and biological reactivity.

Of the lignin samples studied, the poplar ammonia organosolv lignin and oak dioxasolv lignin both show high levels of  $\beta$ -O-4 content, and both lignins show consistently good levels of lignin valorisation products (see Figure 6). The poplar ammonia organosolv lignin gave the highest yield of arylketone products from DDQ/Zn valorisation, a 5.3% yield compared with a theoretical maximum yield of 16% via this method (calculated from the incidence of adjacent  $\beta$ -O-4 units in the structure), and also gave the highest yield of syringaldehyde from *R. jostii* RHA045 microbial biotransformation, and the largest number of products from treatment with *P. fluorescens* Dyp1B. The oak dioxasolv lignin gave the highest yield of products from reductive catalytic hydrogenation, and also generated syringaldehyde from *R. jostii* RHA045 microbial biotransformation, and products from enzymatic oxidation. The observation that the poplar ammonia organosolv lignin gave highest yield for DDQ/Zn valorisation, while the oak dioxasolv lignin gave highest yield for reductive catalytic hydrogenation, implies that the two chemical methods are highly dependent on the method used for lignin fractionation.

Lignin samples with intermediate levels of  $\beta$ -O-4 content gave variable types and amounts of valorisation products. The miscanthus ionic liquid lignin gave good product yields for the reductive catalytic hydrogenation method, but not for the DDQ/Zn valorisation method; it yielded no bioproducts from *R. jostii* RHA045 microbial biotransformation, but generated some enzymatic

oxidation products. The eucalyptus autohydrolysis lignin gave some ketone products from DDQ/Zn valorisation; it gave no syringaldehyde product from *R. jostii* RHA045 microbial biotransformation, but did produce other bio-products vanillic acid and protocatechuic acid.

Lignin preparations with low levels of  $\beta$ -O-4 content showed either no product in the case of eucalyptus Kraft lignin, or products from 2 methods for eucalyptus organosolv lignin. *Hence an important conclusion of our study there is a correlation between the proportion of  $\beta$ -O-4 content and the value of the lignin for valorisation.* There are a few studies in the literature that have compared different lignin preparations with individual lignin valorization methods. In the lignin pyrolysis field, Lin *et al* have compared the fast pyrolysis of four lignins from different isolation processes, observing variable product ratios;<sup>37</sup> and Liu *et al* have studied the pyrolysis of organosolv lignin vs soda alkali lignin, finding that organosolv lignin with higher  $\beta$ -O-4 content is pyrolysed more readily.<sup>38</sup> In the lignin chemocatalysis field, Bouxin *et al* have noted differences in yield between different lignin preparations using catalytic hydrogenation,<sup>21</sup> and Jongerius *et al* have observed differences in yield between three lignin preparations using liquid-phase reforming and hydrodeoxygenation,<sup>39</sup> in each case highest yield was obtained with the lignin with highest  $\beta$ -O-4 content. In this study we have examined a larger collection of lignin preparations across five different chemocatalytic and biocatalytic lignin valorization methods. It would be interesting to study a greater range of different lignin preparations from the same feedstock (e.g. poplar, oak, miscanthus), to see if there are differences in behaviour within individual feedstocks. For individual valorisation methods there may also be a preference for particular feedstock, as found for example by van den Bosch *et al* for reductive fractionation of lignocellulosic biomass.<sup>40</sup>

Figure 6. Summary of the products of biocatalytic and chemocatalytic valorisation methods applied to different lignin preparations.

The *R. jostii* RHA045 gene deletion strain used in this study contains a gene deletion in vanillin dehydrogenase responsible for oxidation of vanillin to vanillic acid, and was previously shown to accumulate vanillin (96 mg/L) and *p*-hydroxybenzaldehyde when grown on minimal media containing wheat straw lignocellulose, containing G and H lignin units. From hardwood lignins containing predominantly S lignin units, one might expect to accumulate syringaldehyde rather than vanillin, as observed from poplar ammonia organosolv lignin (95 mg/L) and oak dioxasolv lignin (243 mg/L), thus it appears that syringaldehyde is also metabolised via the vanillic acid catabolic pathway in *Rhodococcus jostii*. The yield of syringaldehyde obtained from poplar ammonia organosolv lignin is 2.5-fold higher than the yield of vanillin obtained previously by Sainsbury *et al* from wheat straw lignocellulose,<sup>11</sup> although in this experiment the growth substrate

was a lignin preparation, whereas there is only 20-25% lignin content in wheat straw lignocellulose. However, the overall product yields from *R. jostii* RHA045 treatment of poplar ammonia organosolv lignin and oak dioxasolv lignin are 6.3% and 5.1% respectively, higher than product yields of 3-4% obtained for wheat straw lignocellulose.<sup>11</sup>

The appearance of protocatechuic acid as a bio-product was observed from wheat straw lignocellulose, and was interpreted as a shunt metabolite formed by slow demethylation of vanillin to give 2,3-dihydroxybenzaldehyde, which is exported and oxidised to form protocatechuic acid (see Figure 7),<sup>11</sup> and this metabolite was observed in significant quantities in the bioconversions of eucalyptus autohydrolysis and organosolv lignin. The appearance of vanillic acid as a bio-product is unexpected and puzzling, since the enzymatic oxidation of vanillin to vanillic acid has been disrupted by gene knockout. In these cases it is possible that vanillic acid is formed from  $\beta$ -oxidation of ferulic acid via the pathway elucidated by Otani *et al*,<sup>41</sup> or from another unidentified pathway, but one would expect that the vanillic acid would be further metabolised via demethylation and subsequently via the  $\beta$ -keto adipate pathway, as shown in Figure 7. One possible rationalisation is that the vanillate demethylase gene, which is normally induced by the presence of vanillin,<sup>42</sup> is not induced by syringaldehyde, which would result in a shortage of vanillate demethylase enzyme in the host organism, thereby accumulating the substrate vanillic acid.

Figure 7. Formation of syringaldehyde, protocatechuic acid and vanillic acid in *Rhodococcus jostii* RHA045.

A number of product peaks were generated by enzyme-catalysed oxidation by *Pseudomonas fluorescens* Dyp1B and *Sphingobacterium sp.* T2 manganese superoxide dismutase, and once again the product identities and ratios varied between lignin substrates. In the case of *P. fluorescens* Dyp1B, most products were obtained upon treatment of poplar ammonia lignin (6 product peaks), including a syringyl C<sub>3</sub> triol, structurally related to lignin dimer observed previously upon treatment of wheat straw lignocellulose.<sup>35</sup> 3-Methoxyhydroquinone had previously been observed as a metabolite from treatment of wheat straw organosolv lignin with *Sphingobacterium* MnSOD1,<sup>35</sup> so the appearance of syringyl hydroquinone can be rationalised by the predominance of S units in the poplar and oak hardwood lignins.

The development of new methods for chemocatalytic or biocatalytic conversion of lignin into feedstock or high-value aromatic chemicals will add value to the generation of biofuels from lignocellulose, provided that a compatible pre-treatment method can be developed that allows both biofuel generation and lignin valorisation. At present the majority of lignin preparations generated commercially in pulp/paper or biofuel manufacture involve either the Kraft process or acidic

pretreatments that lead to condensation of the lignin structure and reduction in  $\beta$ -O-4 content. Our studies suggest that in order to generate a lignin fraction that can be efficiently valorised for chemicals production, the pre-treatment should ideally leave the majority of  $\beta$ -O-4 lignin units intact, such as mild organosolv treatments, or methods such as  $\gamma$ -valerolactone fractionation.<sup>43</sup>

**Acknowledgements.** This project was supported by a grant from the Lignocellulosic Biorefinery Network, a BBSRC-funded network in Biotechnology and Bioenergy. We thank Sofia Valenzuela (University of Concepcion, Chile) for assistance with lignin materials for the project.

### Footnotes

1. The volume integral of a cross peak in a HSQC spectrum is a semi-quantitative measure and detailed error assessment methods remain challenging. However, the values presented in Tables 1 and 2 are reasonable based on the obtained spectra (see ESI Figures S2-S8).
2. Since this method requires the presence of two consecutive  $\beta$ -O-4 units, the probability of obtaining two consecutive  $\beta$ -O-4 units in a lignin with 40%  $\beta$ -O-4 units is  $0.4 \times 0.4 = 0.16$ , therefore 16% theoretical yield of monomers.

**Supporting Information.** ESI comprises  $^1\text{H}$ - $^{13}\text{C}$  NMR spectra for lignin preparations (Figures S1-S8) and DDQ-oxidised lignin samples (Figures S9-S11); analysis of 2-hydroxyethyl-aryl ketone products from DDQ oxidation/Zn reduction of lignin samples (Figures S12, S13); and LC-MS data for enzymatic products using *P. fluorescens* Dyp1B (Figure S14) and *Sphingobacterium* sp. SOD1 (Figure S15).

### References

1. Zakzeski, J.; Bruijninx, P.C.; Jongerius, A.L.; Weckhuysen, B.M. The catalytic valorization of lignin for the production of renewable chemicals. *Chem. Rev.* **2010**, *110*, 3552–3599.
2. Ragauskas, A.J.; Beckham, G.T.; Bidd, M.J.; Chandr, R.; Chen, F.; Davis, M.F.; Davison, B.H.; Dixon, R.A.; Gilna, P.; Keller, M.; Langan, P.; Naskar, A.K.; Saddler, J.N.; Tschaplinski, T.J.; Tuskan, G.A.; Wyman, C.E. Lignin valorization: improving lignin processing in the biorefinery. *Science* **2014**, *344*, 1246843.
3. Rahimi, A.; Azarpira, A.; Kim, H.; Ralph, J.; Stahl, S.S. Chemoselective metal-free aerobic alcohol oxidation in lignin. *J. Am. Chem. Soc.* **2013**, *135*, 6415-6418.
4. Rahimi, A.; Ulbrich, A.; Coon, J.J.; Stahl, S.S. Formic acid-induced depolymerization of oxidised lignin to aromatics. *Nature* **2014**, *515*, 249-252.

5. Sturgeon, M.R.; O'Brien, M.H.; Ciesielski, P.N.; Katahira, R.; Kruger, J.S.; Chmely, S.C.; Hamlin, J.; Lawrence, K.; Hunsinger, G.B.; Foust, T.D.; Baldwin, R.M.; Bidy, M.J.; Beckham, G.T. Lignin depolymerisation by nickel supported layered-double hydroxide catalysts. *Green Chem.* **2014**, *16*, 824-835.
6. Lancefield, C.S.; Ojo, O.S.; Tran, F.; Westwood, N.J. Isolation of functionalized phenolic monomers through selective oxidation and C-O bond cleavage of the  $\beta$ -O-4 linkages in lignin. *Angew. Chem.* **2015**, *127*, 260-264.
7. Deuss, P.J.; Scott, M.; Tran, F.; Westwood, N.J.; de Vries, J.G.; Barta, K. Aromatic monomers by in situ conversion of reactive intermediates in the acid-catalyzed depolymerization of lignin. *J. Am. Chem. Soc.* **2015**, *137*, 7456-7467.
8. Rinaldi, R.; Jastrzebski, R.; Clough, M.T.; Ralph, J.; Kennema, M.; Bruijninx, P.C.A.; Weckhuysen, B.M. Paving the way for lignin valorisation: recent advances in bioengineering, biorefining, and biocatalysis. *Angew. Chem. Intl. Ed.* **2016**, *55*, 8164-8215.
9. Kärkäs, M.D.; Matsuura, B.S.; Monos, T.M.; Magallanes, G.; Stephenson, C.R.J. Transition-metal catalysed valorisation of lignin: the key to a sustainable carbon-neutral future. *Org. Biomol. Chem.* **2016**, *14*, 1853-1914.
10. Bugg, T.D.H.; Rahmanpour, R. Enzymatic conversion of lignin into renewable chemicals. *Curr. Opin. Chem. Biol.* **2015**, *29*, 10-17.
11. Sainsbury, P.D.; Hardiman, E.M.; Ahmad, M.; Otani, H.; Seghezzi, N.; Eltis, L.D.; Bugg, T.D.H. Breaking down lignin to high-value chemicals: the conversion of lignocellulose to vanillin in a gene deletion mutant of *Rhodococcus jostii* RHA1. *ACS Chem. Biol.* **2013**, *8*, 2151-2156.
12. Linger, J.G.; Vardo, D.R.; Guarneri, M.T.; Karp, E.M.; Hunsinger, G.B.; Franden, M.A.; Johnson, C.W.; Chupka, G.; Strathmann, T.J.; Pienkos, P.T.; Beckham, G.T. Lignin valorization through integrated biological funnelling and chemical catalysis. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 12013-12018.
13. Vardon, D.R.; Franden, M.A.; Johnson, C.W.; Karp, E.M.; Guarneri, M.T.; Linger, J.G.; Salm, M.J.; Strathman, T.J.; Beckham, G.T. Adipic acid production from lignin. *Energy Environ. Sci.* **2015**, *8*, 617-628.
14. Mycroft, Z.; Gomis, M.; Mines, P.; Law, T.D.H. Bugg, T.D.H. Biocatalytic conversion of lignin to aromatic dicarboxylic acids in *Rhodococcus jostii* RHA1 by re-routing aromatic degradation pathways. *Green Chem.* **2015**, *17*, 4974-4979.
15. Samuel, R.; Pu, Y.; Raman, B.; Ragauskas, A.J. Structural characterization and comparison of switchgrass ball-milled lignin before and after dilute acid pretreatment. *Appl. Biochem. Biotechnol.* **2010**, *162*, 62-74.

16. Bauer, S.; Sorek, H.; Mitchell, V.D.; Ibanez, A.B.; Wemmer, D.E. Characterization of *Miscanthus giganteus* lignin isolated by ethanol organosolv process under reflux condition. *J. Agr. Food Chem.* **2012**, *60*, 8203-8212.
17. Balakshin, M.Y.; Capanema, E.A.; Loung, C.; Gracz, H.S. Elucidation of the structures of residual and dissolved pine Kraft lignins using an HMQC NMR technique. *J. Agric. Food Chem.* **2003**, *51*, 6116-6127.
18. Marques, A.P.; Evtuguin, D.V.; Magina, S.; Amado, F.M.L.; Prates, A. Structure of lignosulfonates from acidic magnesium-based sulphite pulping of *Eucalyptus globulus*. *J. Wood Chem. Technol.* **2009**, *29*, 337-357.
19. Mora-Pale, M.; Meli, L.; Doherty, T.V.; Linhardt, R.J.; Dordick, J.S. Room temperature ionic liquids as emerging solvents for the pretreatment of lignocellulosic biomass. *Biotechnol. Bioeng.* **2011**, *108*, 1229-1245.
20. Wen, J.L.; Sun, S.L.; Xue, B.L.; Sun, R.C. Quantitative structures and thermal properties of birch lignins after ionic liquid pretreatment. *J. Agr. Food Chem.* **2013**, *61*, 635-645.
21. Bouxin, F.P.; McVeigh, A.; Tran, F.; Westwood, N.J.; Jarvis, M.C.; Jackson, S.D. Catalytic depolymerisation of isolated lignins to fine chemicals using a Pt/alumina catalyst: Part 1-Impact of the lignin structure. *Green Chemistry* **2015**, *17*, 1235-1242.
22. Bouxin, F.P.; Jackson, S.D.; Jarvis, M.C. Isolation of high quality lignin as a by-product from ammonia percolation treatment of poplar wood. *Bioresource Technol.* **2014**, *162*, 236-242.
23. Guerra, A.; Elissetche, J.P.; Norambuena, M.; Freer, J.; Valenzuela, S.; Rodriguez, J.; Balocchi, C. Influence of lignin structural features on *Eucalyptus globulus* Kraft pulping. *Ind. Eng. Chem. Res.* **2008**, *47*, 8542-8549.
24. Castillo, R.P.; Baeza, J.; Rubilar, J.; Rivera, A.; Freer, J. Infrared spectroscopy as alternative to wet chemical analysis to characterise *Eucalyptus globulus* pulps and predict their ethanol value for a simultaneous saccharification and fermentation process. *Appl. Biochem. Biotechnol.* **2012**, *168*, 2028-2042.
25. Araya, F.; Troncoso, E.; Mendonça, R.T.; Freer, J.; Rencoret, J.; Del Rio, J.C. Structural characteristics and distribution of lignin in *Eucalyptus globulus* pulps obtained by a combined autohydrolysis/alkaline extraction process for enzymatic saccharification of cellulose. *J. Chilean Chem. Soc.* **2015**, *60*, 2954-2960.
26. George, A.; Brandt, A.; Tran, K.; Zahari, S.M.S.N.S.; Klein-Marcuschamer, D.; Sun, N.; Sathitsuksanoh, N.; Shi, J.; Stavila, V.; Parthasarathi, R.; Singh, S.; Holmes, B.M.; Welton, T.; Simmons, B.A.; Hallett, J.P. Design of low-cost ionic liquids for lignocellulosic biomass pretreatment. *Green Chemistry*, **2015**, *17*, 1728-1734.

27. Tran, F.; Lancefield, C. S.; Kamer, P. C. J.; Lebl, T.; Westwood, N. J. Selective modification of the  $\beta$ - $\beta$  linkage in DDQ-treated Kraft lignin analysed by 2D NMR spectroscopy. *Green Chemistry* **2015**, *17*, 244-249.
28. Ralph, J.; Landucci, L. NMR of lignins, p. 137-244 of "Lignin and lignans", eds. Heitner, C.; Dimmel, D.R.; Schmidt, J.A., **2010**, CRC Press, Taylor & Francis, Boca Raton, Florida.
29. Rencoret, J.; Prinsen, P.; Gutierrez, A.; Martinez, A.T.; del Rio, J.C. Isolation and structural characterization of the milled wood lignin, dioxane lignin, and cellulolytic lignin preparations from brewer's spent grain. *J. Agric. Food Chem.* **2015**, *63*, 603-613.
30. Mansfield, S.D.; Kim, H.; Lu, F.; Ralph, J. Whole plant cell wall characterization using solution-state 2D NMR. *Nat. Protocols* **2012**, *7*, 1579-1589.
31. Del Rio, J.C.; Rencoret, J.; Prinsen, P.; Martinez, A.T.; Ralph, J.; Gutierrez, A. Structural characterization of wheat straw lignins revealed by analytical pyrolysis, 2D-NMR, and reductive cleavage methods. *J. Agric. Food Chem.* **2012**, *60*, 5922-5935.
32. Lan, W.; Lu, F.; Regner, M.; Zhu, Y.; Rencoret, J.; Ralph, S.A.; Zakai, U.I.; Morreel, K.; Boerjan, W.; Ralph, J. Tricin, a flavonoid monomer in monocot lignification. *Plant Physiol.* **2015**, *167*, 1284-1295.
33. Bouxin, F.P.; McVeigh, A.; Jarvis, M.C.; Jackson, S.D. Catalytic depolymerisation of isolated lignin to fine chemicals using a Pt/alumina catalyst: Part 2 – Process optimisation. *Catalysis Science & Technology*, **2016**, in press DOI:10.1039/c5cy01896j.
34. Rahmanpour, R.; Bugg, T.D.H. Characterisation of Dyp-type peroxidases from *Pseudomonas fluorescens* Pf-5: oxidation of Mn(II) and polymeric lignin by Dyp1B. *Arch. Biochem. Biophys.* **2015**, *574*, 93-98
35. Rashid, G.M.M.; Taylor, C.R.; Liu, Y.; Zhang, X.; Rea, D.; Fülöp V.; Bugg, T.D.H. Identification of manganese superoxide dismutase from *Sphingobacterium sp.* T2 as a novel bacterial enzyme for lignin oxidation. *ACS Chem Biol* **2015**, *10*, 2286-2294.
36. Ahmad, M.; Roberts, J.N.; Hardiman, E.M.; Singh, R.; Eltis, L.D.; Bugg, T.D.H. Identification of DypB from *Rhodococcus jostii* RHA1 as a lignin peroxidase. *Biochemistry* **2011**, *50*, 5096-5107.
37. Lin, X. N.; Sui, S. J.; Tan, S.; Pittman, C. U.; Sun, J. P.; Zhang, Z. J. Fast pyrolysis of four lignins from different isolation processes using Py-GC/MS. *Energies* **2015**, *8*, 5107-5121.
38. Liu, C.; Hu, J.; Zhang, H. Y.; Xiao, R. Thermal conversion of lignin to phenols: relevance between chemical structure and pyrolysis behaviors. *Fuel* **2016**, *182*, 864-870.
39. Jongerius, A. L.; Bruijninx, P. C. A.; Weckhuysen, B. M. Liquid-phase reforming and hydrodeoxygenation as a two-step route to aromatics from lignin. *Green Chem.* **2013**, *15*, 3049-3056.

40. Van den Bosch, S.; Schutyser, W.; Vanholme, R.; Driessen, T.; Koelewijn, S-F.; Renders, T.; de Meester, B.; Huijgen, W.J.; Dehaen, W.; Courtin, C.M.; Lagrain, B.; Boerjan, W.; Sels, B.F. Reductive lignocellulose fractionation into soluble lignin-derived phenolic monomers and dimers and processable carbohydrate pulps. *Energy Environ. Sci.* **2015**, *8*, 1748-1763.
41. Otani, H.; Lee, Y.E.; Casabon, I.; Eltis, L.D. Characterization of p-hydroxycinnamate catabolism in a soil Actinobacterium. *J. Bacteriol.* **2014**, *196*, 4293-4303.
42. Chen, H.P.; Chow, M.; Liu, C.C.; Lau, A.; Liu, J.; Eltis, L.D. Vanillin catabolism in *Rhodococcus jostii* RHA1. *Appl. Environ. Microbiol.* **2012**, *78*, 586-588.
43. Luterbacher, J.S.; Azarpira, A.; Motagamwala, A.H.; Lu, F.; Ralph, J.; Dumesic, J.A. Lignin monomer production integrated into the  $\gamma$ -valerolactone sugar platform. *Energy Environ. Sci.* **2015**, *8*, 2657-2663.

## Figure Legends.

Figure 1. A. Design of experimental study. B. Structural units found in polymeric lignin.

Figure 2. Analysis of lignin structure by  $^1\text{H}$ - $^{13}\text{C}$  NMR spectroscopy, showing (A) poplar ammonia organosolv lignin containing high  $\beta$ -O-4 content; (B) miscanthus ionic liquid lignin containing medium  $\beta$ -O-4 content; (C) Eucalyptus Kraft lignin containing low  $\beta$ -O-4 content. Key: A (light blue),  $\beta$ -O-4 linkages between S/G units and either G (S/G-G) or S (S/G-S) units or containing  $\alpha$ -ethoxy groups (OEt); B (green),  $\beta$ -5 units; C (purple),  $\beta$ - $\beta$  units; red, Hibbert ketones; grey, condensed units. Signals indicative of  $\beta$ -O-4 linkages indicated in box.

Figure 3. Chemocatalytic conversion of lignin samples to 2-hydroxyethyl aryl ketones

Figure 4. Products obtained from catalytic hydrogenation at 300 °C/20 bar hydrogen over a Pt/alumina catalyst. Yield estimated by GC-MS. Key: G, guaiacyl; S, syringyl; 0-3 indicate the number of carbon atoms in the alkyl side-chain at C-1; G(OH), 3,4-dihydroxybenzene aromatic unit; S(OH), 3,4-dihydroxy-5-methoxy aromatic; 3(i), 3-carbon side-chain containing alkene; 3(OMe), 3-carbon side-chain containing OMe; 3(OH), 3-carbon side-chain containing OH.

Figure 5. Products obtained by treatment of lignin preparations with *Pseudomonas fluorescens* Dyp1B (PfDyp1B) and *Sphingobacterium* sp. T2 manganese superoxide dismutase 1 (SpSOD1). Products identified by LC-MS. Black shading, major peak; grey shading, minor peak. Key: S, syringyl; G, guaiacyl; H, hydroxybenzyl; 0-3 indicate the number of carbon atoms in the alkyl side-chain at C-1.

Figure 6. Summary of the products of biocatalytic and chemocatalytic valorisation methods applied to different lignin preparations. Key: SA, syringaldehyde; VA, vanillic acid; PCA, protocatechuic acid; FA, ferulic acid; NT, not tested. Colour coding: orange, highest product yield ; pale orange, good product yield; yellow, some products observed; grey, no products observed.

Figure 7. Formation of syringaldehyde, protocatechuic acid and vanillic acid in *Rhodococcus jostii* RHA045. + indicates up-regulation of vanillate dehydrogenase gene by vanillin.

Table 1. Ratio of S/G/H units determined by  $^1\text{H}$ - $^{13}\text{C}$  NMR spectroscopy and by thioacidolysis. % condensed S units ( $S_{\text{con}}$ ) is also shown. ND, not determined.

Lignin	% S	% G	% H	% $S_{\text{con}}$	S:G:H	Thioacidolysis S:G ratio
Oak dioxasolv	53	19	0	28	4.2:1:0	2.65 $\pm$ 0.04
Poplar ammonia organosolv	62	38	0	0	1.6:1:0	1.86 $\pm$ 0.03
Poplar alkaline organosolv (ref 19)	48	52	0	ND	0.9:1:0	1.32 $\pm$ 0.05
Miscanthus ionic liquid	16	52	15	17	0.6:1:0.33	0.73 $\pm$ 0.01
Eucalyptus autohydrolysis	60	10	0	30	9.0:1:0	ND
Eucalyptus organosolv	46	18	0	36	4.6:1:0	ND
Eucalyptus Kraft	29	12	0	59	7.1:1:0	ND

Table 2. Characterisation of lignin samples. Percentage of  $\beta$ -O-4,  $\beta$ -5 and  $\beta$ - $\beta$  units based upon alkyl hydrogen signals observed by  $^1\text{H}$ - $^{13}\text{C}$  NMR spectroscopy (calculation of these data is illustrated in Figure S8 in Supporting Information);  $M_w$  and  $M_n$  values of acetylated lignins by gel permeation chromatography. Notes: a, excluding  $\alpha$ -ethoxy units, arising from ethanol used in lignin fractionation.

Lignin	$\beta$ -O-4 (%)	$\beta$ -5 (%)	$\beta$ - $\beta$ (%)	$M_w$	$M_n$
Oak dioxasolv	40	3	8	5267	734
Poplar ammonia organosolv	49 (40 <sup>a</sup> )	3	7	4013	1072
Poplar alkaline organosolv (ref 19)	12	5	4	ND	ND
Miscanthus ionic liquid	10	5	2	3061	894
Eucalyptus autohydrolysis	27	2	8	4364	822
Eucalyptus organosolv	16 (13 <sup>a</sup> )	2	5	1765	750
Eucalyptus Kraft	0	0	5	1764	664

Table 3. Bioproduct yields (in mg/L) obtained via microbial biotransformation of *Rhodococcus jostii* RHA045 using different lignins as carbon source at 1% (w/v) in minimal M9 media after 6 days microbial biotransformation at 30 °C. nac, small amounts (1-5 mg/L) not above those of control incubation lacking bacteria; nd, not detected.

Lignin	S:G:H ratio	vanillin	syring-aldehyde	vanillic acid	vanillyl alcohol	Proto-catechuic acid	ferulic acid	Total yield (%)
Oak dioxasolv	3:1:0	nac	95	340	nac	nd	74	5.1
Poplar ammonia organosolv	1.6:1:0	nac	243	360	23	nac	nac	6.3
Poplar alkaline organosolv	1.3:1:0	nac	nac	630	42	nd	nac	6.7
Miscanthus ionic liquid	0.8:1:0.9	nac	nac	nac	nac	nd	nac	<1
Eucalyptus autohydrolysis	5.9:1:0	nd	nd	130	nd	230	nac	3.6
Eucalyptus organosolv	5.9:1:0	nac	nd	105	20	300	nac	4.2
Eucalyptus Kraft	3.4:1:0	nac	nac	nac	nac	nac	nac	<1
Wheat straw lignocellulose <sup>9</sup>		96	nd	3-120	nd	nd	23-86	3-4

Figure 1

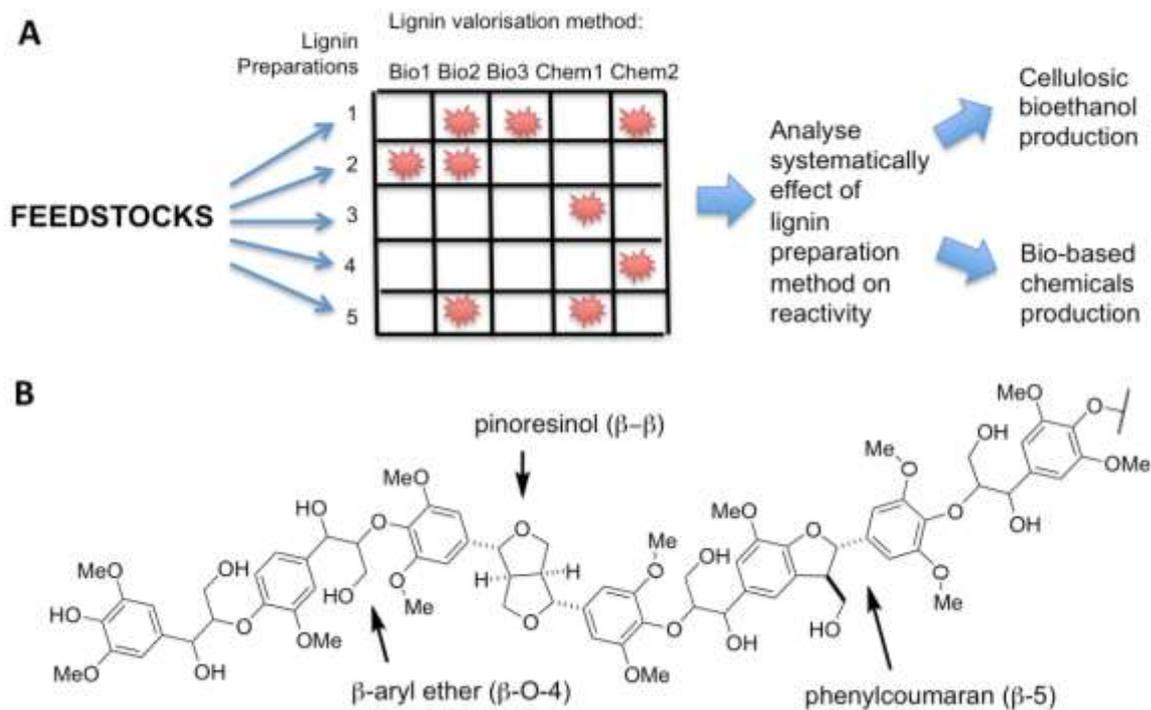


Figure 2

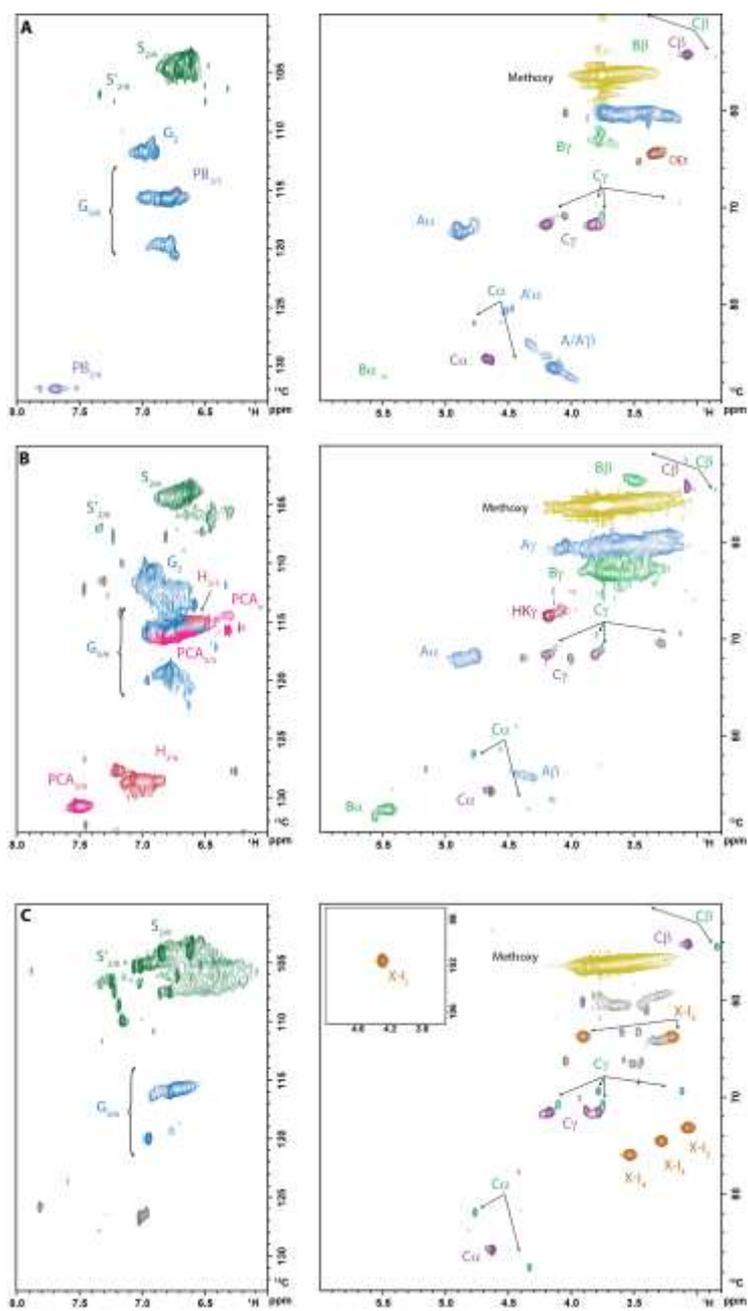


Figure 3.

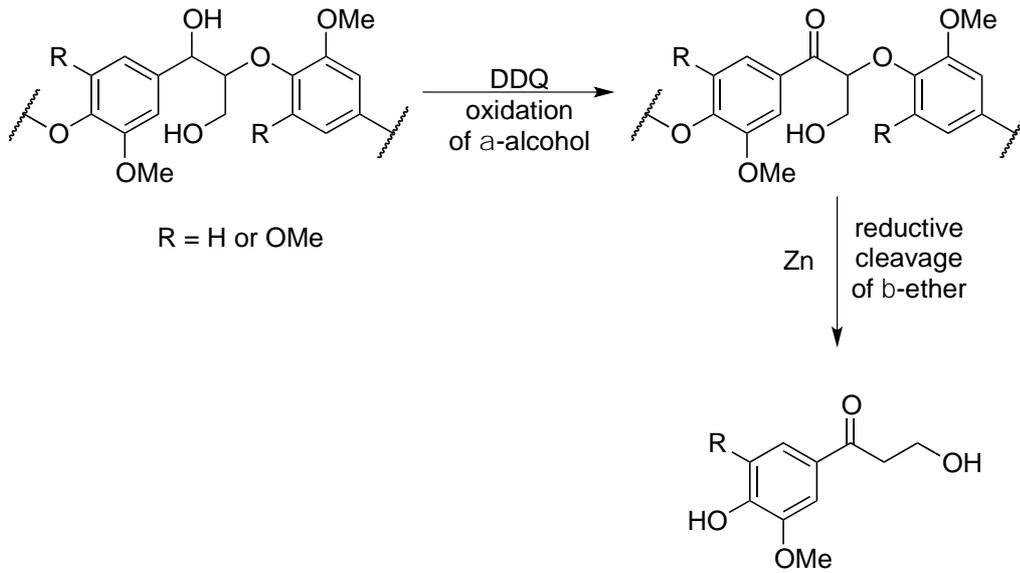


Figure 4

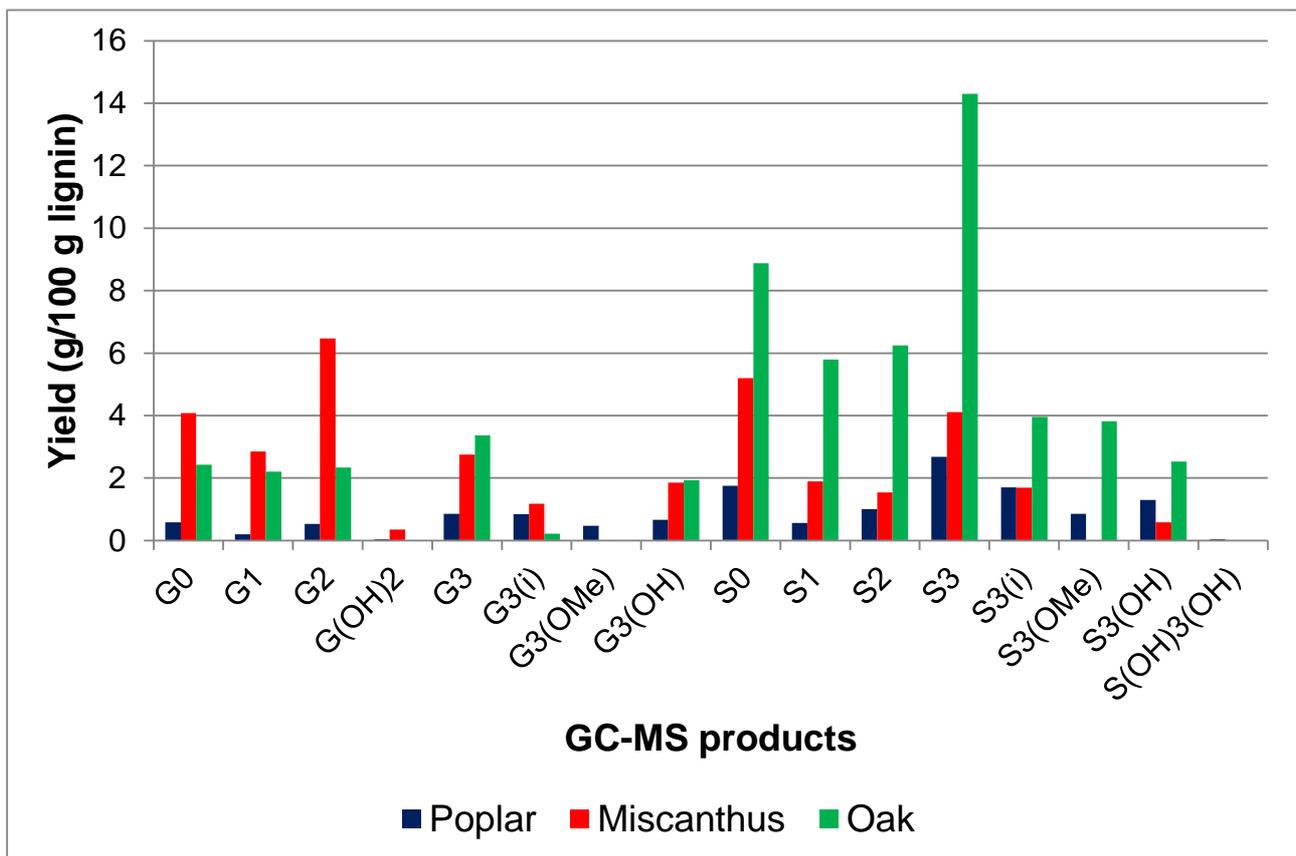
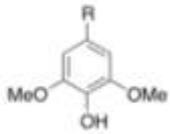


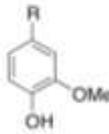
Figure 5

	Content	Enz	R	Syringyl (S) products					Guaiacyl (G) products				H product	Other products	Total products
				S0	S1	S3		G0	G1		G3	H1			
				OH	CO <sub>2</sub> H	CHO	CH <sub>2</sub> OH	C <sub>2</sub> (OH)	H	OH	CO <sub>2</sub> H	CHO	CH <sub>2</sub> OH		
Poplar ammonia lignin	S>G	PfDyp1B		■		■	■							1	6
		SpSOD1			■				■	■				3	6
Poplar alkali organosolv	S>G	PfDyp1B		■	■				■	■				1	3
		SpSOD1		■					■	■				1	5
Oak dioxasolv lignin	S>G	PfDyp1B				■	■							2	2
		SpSOD1				■	■							1	3
Eucalyptus organosolv lignin	S>G	PfDyp1B	■	■	■					■				2	6
Eucalyptus autohydrolysis lignin	S>G	PfDyp1B												1	1
Eucalyptus Kraft lignin	S>G	PfDyp1B												0	0
Miscanthus ionosolv lignin	S,G,H	PfDyp1B							■		■	■		3	5
		SpSOD1							■					1	2



syringyl (S) products



guaiacyl (G) products

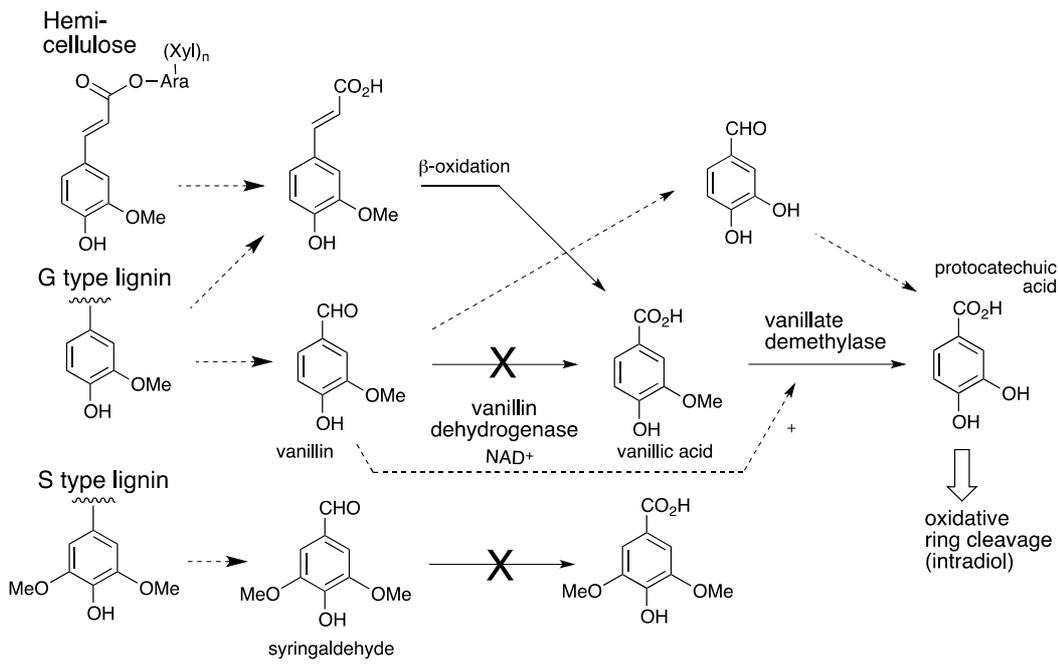


hydroxybenzyl (H) products

Figure 6

		Lignin valorisation method				
		Chem1	Chem2	Bio1	Bio2	Bio3
Lignin substrate	$\beta$ -O-4 content	Pt/H <sub>2</sub> /alumina	DDQ/Zn	<i>R. jostii</i> <i>Avdh</i>	PfDyp1B peroxidase	SpSOD1
Poplar ammonia lignin	High	3% S3	5.3% (theor 16%)	SA 243 mg/L VA 360 mg/L	S3,S0,G1 6 products	S1,G1 6 products
Oak dioxasolv lignin	High	14% S3 9% S0	1.3% (theor 16%)	SA 95 mg/L VA 340 mg/L	2 products	S1 3 products
Poplar alkaline organosolv	Medium	6.6% S3 (ref 19)		VA 630 mg/L	S1,S0 3 products	S0,G0,G1 5 products
Miscanthus ionosolv lignin	Medium	10% S0			H1, FA 5 products	G0 2 products
Eucalyptus autohydrolysis lignin	Medium	NT	1.4% (theor 7%)	VA 130 mg/L PCA 230 mg/L	1 product	
Eucalyptus organosolv lignin	Medium	NT		VA 105 mg/L PCA 300 mg/L	S1,S0,G1 6 products	
Eucalyptus Kraft lignin	Low	NT				

Figure 7.



For Table Contents Use Only

## An Investigation of the Chemocatalytic and Biocatalytic Valorisation of a Range of Different Lignin Preparations: the Importance of $\beta$ -O-4 Content

Christopher S. Lancefield, Goran M.M. Rashid, Florent Bouxin, Agata Wasak, Wei-Chien Tu, Jason Hallett, Sharif Zein, Jaime Rodríguez, S. David Jackson, Nicholas J. Westwood, Timothy D.H. Bugg\*

Synopsis: A set of 7 lignins prepared from biomass using a range of fractionation methods was characterised, and tested against 2 chemocatalytic and 3 biocatalytic valorisation methods.

