2D-NMR of Lignin in the Eucalypt Pulp Mill Biorefinery: General Aspects and Enzymatic Delignification Studies

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• Chemistry and Biotechnology for lignocellulose (CSIC)
• Lignin (and lignocellulose) analysis by 2D-NMR
• 2D-NMR of lignins from different eucalypt species
• Lignin modification in enzymatic bleaching of eucalypt pulp
• Lignin modification in enzymatic delignification of wood
• The potential of commercial laccases as shown by 2D-NMR
• Past EU funding (and H2020 possibilities)
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Our scientific **objectives** are related to the use of **microorganisms** (mainly filamentous fungi) and their enzymes in **industrial** processes to obtain fuels, materials and chemicals (White Biotechnology) from **renewable** plant resources. The final aim is to contribute to the sustainable development of our society and reduce the biosphere warming by a reduced consumption of fossil resources. These objectives are well in agreement with those of the new CIB Department of "Environmental Biology".

The work of the group has provided important contributions to the knowledge of the **enzymatic system** involved in the **degradation of lignin** (and other recalcitrant compounds) by **fungi**, which represents a key step for C recycling in land ecosystems and a central issue in the industrial use of plant biomass in agreement with the **Biorefinery concept** (for the integrated production of fuels, chemicals and other products). According to these results, the most recent studies combine **basic** and more **applied aspects** (this dual approach in the field of the enzymatic degradation of lignin has been discussed in a review of the group published in *Curr Opin Biotechnol*, see [Martínez et al., 2008]):

i) **Basic** projects on **structure-function** of key enzymes involved in lignocellulose biodegradation (to improve their catalytic properties) including:
- Ligninolytic hemeperoxidases, like **versatile peroxidase** (VP)
- Flavooxidases providing peroxide, like **aryl-alcohol oxidase** (AAO)
- Multicopper oxidases like **laccases** (and their redox mediators)
- **Esterases** with different substrate specificities
Enzymes can be used as industrial biocatalysts (as an alternative to harsh chemicals) for the development of clean technologies.
Research lines

The main research activity of the Group is aimed at the characterization of the main components of agro-forest crops and the study of the mechanisms of their microbial transformation, with special emphasis in the most recalcitrant components such as lignin and extractives, whose removal is a key step in the recycling of cellulose in terrestrial ecosystems as well as in their industrial utilization. These investigations have also important environmental applications, as the development of environmentally-friendly technologies for reducing the environmental impact of the pulp and paper industry (that use lignocellulosics as raw materials and often contaminant technologies for producing the cellulose products). These studies are intended to get a wider and more rational use of this important agro-forest resource as is the cultivated plant biomass used as raw material for the manufacturing of pulp and paper (the main non-food industrial use of the plant biomass) by the use of environmentally friendly procedures. For this reason, our Group will continue to cooperate with other research groups (including the technological centers of leader companies in the pulp and paper as well as in the biotechnological sectors) in the course of National and European projects as well as with research agreements with private companies.
Enzymatic delignification of plant cell wall: from nature to mill
Ángel T Martínez¹, Francisco J Ruiz-Dueñas¹, María Jesús Martínez¹, José C del Río² and Ana Gutiérrez²

Lignin removal is a central issue in paper pulp manufacture, and production of other renewable chemicals, materials, and biofuels in future lignocellulose biorefineries. Biotechnology can contribute to more efficient and environmentally sound deconstruction of plant cell wall by providing tailor-made biocatalysts based on the oxidative enzymes responsible for lignin attack in Nature. With this purpose, the already-known ligninolytic oxidoreductases are being improved using (rational and random-based) protein engineering, and still unknown enzymes will be identified by the application of the different ‘omics’ technologies. Enzymatic delignification will be soon at the pulp mill (combined with pitch removal) and our understanding of the reactions produced will increase by using modern techniques for lignin analysis.

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The future lignocellulose biorefineries: overcoming the lignin barrier
The lignocellulose biorefinery concept [5,6] is receiving considerable attention as a source of renewable chemicals, materials, and fuels for future sustainable development. Concerns on the growing price of crude oil, which increased during the last two decades (peaking at near 150 US$/barrel in July 2008), relaunched the interest in the development of cheap and widely available second-generation biofuels [7,8]. The potential of lignocellulose as a biofuel source was already considered during the first oil crises in the 1970s, although the interest decreased with the fall of oil prices. The exhaustion of crude reserves will be accelerated by the incorporation of the Asian emerging economies, and by the increasing amount of chemicals and materials obtained from petrochemical resources. This increased consumption of fossil fuels is also the main source of the greenhouse gases that are changing climate, causing a global warming of the bio-
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2D-NMR as a powerful tool to characterize chemical (and enzymatic) modification of lignin during eucalypt deconstruction in the pulp mill biorefinery.
NMR has been classically used to analyze lignin but signal overlapping was a major problem in 1D NMR.

2D-NMR solved the problem (in heteronuclear correlation spectra) providing an invaluable tool for understanding the complex structure of lignin.
2D-NMR enables determination of lignin units and inter-unit linkages after its isolation (MWL, CEL, etc) from wood.

- p-coumaryl
- coniferyl
- sinapyl

The two latter discovered by 2D NMR! →

- resinol
- phenylcoumaran
- β-O-4 ether
- dibenzodioxocin
- spirodienone
Lignin 2D-NMR: Whole HSQC spectrum

Information about lignin:
- Composition (aromatic region)
- Linkages (aliphatic oxygenated region)

Aliphatic region
Aliphatic oxygenated region
Aromatic region

HSQC (heteronuclear single quantum correlation) spectrum of Eucalyptus globulus MWL
Lignin 2D-NMR: Aliphatic oxygenated region
Lignin 2D-NMR: Aliphatic oxygenated region (β-O-4' linkages)
Lignin 2D-NMR: Aliphatic oxygenated region (resinols)
Lignin 2D-NMR: Aliphatic oxygenated region (phenylcoumarans)
Lignin 2D-NMR: Aliphatic oxygenated region (spirodienones)
Lignin 2D-NMR: Aliphatic oxygenated region (cinnamyl ends)
Lignin 2D-NMR: Aromatic region (S units)
Lignin 2D-NMR: Aromatic region ($C_\alpha$-oxidized S units)
Lignin 2D-NMR: Aromatic region (G units)
Lignin 2D-NMR: Aromatic region (spirodienone aromatic signals)
Lignin 2D-NMR: Whole spectrum

E. globulus MWL

δC

δH

ppm

δC

δH

ppm

δC

δH

ppm

δC

δH

ppm

δC

δH

ppm

A

B

C

D

F

I

G

S

S'
2D-NMR analysis of lignin structure in five eucalypt species (evaluated as pulp mill feedstocks)

<table>
<thead>
<tr>
<th>Species</th>
<th>Density (Kg/m³)</th>
<th>Active alkali (%)</th>
<th>Kappa index</th>
<th>Yield (%)</th>
<th>Viscosity (ml/g)</th>
<th>Residual alkali (g/L)</th>
<th>Klason lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. globulus</td>
<td>600</td>
<td>13.0</td>
<td>16.1</td>
<td>59.5</td>
<td>1413</td>
<td>3.6</td>
<td>18.7</td>
</tr>
<tr>
<td>E. nitens</td>
<td>450</td>
<td>17.5</td>
<td>16.3</td>
<td>50.4</td>
<td>1177</td>
<td>6.2</td>
<td>22.5</td>
</tr>
<tr>
<td>E. maidenii</td>
<td>600</td>
<td>18.0</td>
<td>16.5</td>
<td>50.8</td>
<td>1093</td>
<td>1.3</td>
<td>22.6</td>
</tr>
<tr>
<td>E. grandis</td>
<td>435</td>
<td>17.0</td>
<td>15.7</td>
<td>49.7</td>
<td>1148</td>
<td>9.2</td>
<td>21.1</td>
</tr>
<tr>
<td>E. dunnii</td>
<td>595</td>
<td>20.0</td>
<td>16.1</td>
<td>48.7</td>
<td>931</td>
<td>15.5</td>
<td>21.6</td>
</tr>
</tbody>
</table>

Syringyl-to-guaiacyl (S/G) ratio, inter-unit linkages and end units (percentage of side-chains) in lignin (MWL) isolated from five eucalypt species as shown by 2D-NMR

<table>
<thead>
<tr>
<th></th>
<th>E. globulus</th>
<th>E. nitens</th>
<th>E. maidenii</th>
<th>E. grandis</th>
<th>E. dunnii</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-O-4´ aryl ether</td>
<td>69.3</td>
<td>71.7</td>
<td>69.7</td>
<td>66.9</td>
<td>65.9</td>
</tr>
<tr>
<td>Resinol</td>
<td>18.2</td>
<td>16.1</td>
<td>16.4</td>
<td>16.5</td>
<td>19.0</td>
</tr>
<tr>
<td>Phenylcoumaran</td>
<td>2.9</td>
<td>4.0</td>
<td>3.6</td>
<td>6.8</td>
<td>4.0</td>
</tr>
<tr>
<td>Spirodienone</td>
<td>2.8</td>
<td>1.3</td>
<td>3.6</td>
<td>2.9</td>
<td>4.2</td>
</tr>
<tr>
<td>β-O-4´-Cα=O</td>
<td>2.0</td>
<td>1.3</td>
<td>1.7</td>
<td>1.7</td>
<td>1.9</td>
</tr>
<tr>
<td>Cinnamyl end-groups</td>
<td>4.7</td>
<td>5.7</td>
<td>4.9</td>
<td>5.3</td>
<td>4.9</td>
</tr>
<tr>
<td>S/G ratio</td>
<td>2.9</td>
<td>2.7</td>
<td>2.4</td>
<td>1.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

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The next examples concern 2D-NMR to better understand the potential of Biotechnology for the removal of *E. globulus* lignin in the pulp mill biorefinery.

Among enzymes involved in lignin degradation, laccases (in presence of mediators) have often been assayed for delignification (due to stability, availability and lack of co-substrates).
In eucalypt TCF pulp, a **laccase-mediator** stage modifies residual lignin (**NMR**) and improves delignification (and bleaching) when followed by a peroxide stage.

1) L-O-O-Q-PoP (control: O-O-Q-PoP) $\leftarrow$ discarded
2) O-O-L-Q-PoP (control: O-O-a-Q-PoP)
3) O-O-LQ-PoP (control: O-O-Q-PoP) \[ \text{other analyses (including 2D-NMR)} \rightarrow \]
Kappa number and brightness analyses of enzymatic and control bleaching sequences

Eucalypt lignin modification during O-O-L-Q-PoP sequence as shown by 2D-NMR of isolated lignin

Aliphatic signals (lignin linkages)
Eucalypt lignin modification during O-O-L-Q-PoP sequence as shown by 2D-NMR of isolated lignin
Eucalypt lignin modification during O-O-L-Q-PoP sequence as shown by 2D-NMR of isolated lignin

- Eucalypt residual lignin is rich in S units (>70%) and β-O-4' bonds (>75% side-chains)

- Enzymatic stage causes oxidation of S units (>60%)

- Enzyme-altered lignin is removed by alkali in PoP stage

- Therefore, the kappa number decreases

- However, peroxide is necessary for good brightness (>90% ISO)

Interestingly, it is possible to analyze changes in lignin (and polysaccharides) by 2D-NMR without its isolation (whole wood swelled in dimethylsulfoxide-$d_6$) of interest in enzymatic delignification →

Rencoret et al. 2009. HSQC-NMR analysis of lignin in woody (Eucalyptus globulus and Picea abies) and non-woody (Agave sisalana) ball-milled plant materials at the gel state. Holzforschung 63:691-698
**Laccase** is not only able to attack lignin in pulp but also when applied directly on ground wood, as a biorefinery pretreatment.

<table>
<thead>
<tr>
<th>Doses laccase</th>
<th>10 – 50 U/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doses HBT</td>
<td>2.5 %</td>
</tr>
</tbody>
</table>

*Trametes villosa* laccase (and HBT as mediator)

<table>
<thead>
<tr>
<th>4 Cycles LEp</th>
<th>Lignin content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.0</td>
</tr>
<tr>
<td>Laccase (10 U g(^{-1}))-HBT</td>
<td>12.2</td>
</tr>
<tr>
<td>Laccase (25 U g(^{-1}))-HBT</td>
<td>11.9</td>
</tr>
<tr>
<td><strong>Laccase (50 U g(^{-1}))-HBT</strong></td>
<td><strong>9.4</strong></td>
</tr>
<tr>
<td>Laccase (50 U g(^{-1}))</td>
<td>17.5</td>
</tr>
</tbody>
</table>

Control Ep - LEp treatment: △ 8.6 % KL  **Nearly 50% lignin reduction!**

This resulted in improved **hydrolyzability** of wood →
Eucalypt samples pretreated with laccase and mediator were further evaluated for saccharification and fermentation at VTT.

... lignin modifications were "in situ" analyzed by 2D-NMR.
2D-NMR of the treated eucalypt (gel stage) revealed removal of lignin without changes in polysaccharides.
2D-NMR of the treated eucalypt (gel stage) revealed removal of lignin without changes in polysaccharides.
However, the most noticeable change is the increase of Cα-oxidized S units

✓ S' units have been identified (by HMBC NMR) as aromatic acids and ketones

Recently, wood (and pulp) lignin removal by a commercial laccase (MtL from Novozymes) and a phenolic mediator (MeS) is investigated to increase the industrial feasibility.

<table>
<thead>
<tr>
<th>Eucalypt samples</th>
<th>Lignin (%)</th>
<th>Glucose (%)</th>
<th>Xylose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial eucalypt wood</td>
<td>22.3 ± 0.3</td>
<td>39.5 ± 1.1</td>
<td>6.7 ± 0.1</td>
</tr>
<tr>
<td>Control</td>
<td>21.1 ± 1.0</td>
<td>43.7 ± 0.2</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>Laccase (10 U·g⁻¹)-MeS (1%)</td>
<td>13.3 ± 0.1</td>
<td>54.8 ± 1.0</td>
<td>9.2 ± 0.2</td>
</tr>
<tr>
<td><strong>Laccase (50 U·g⁻¹)-MeS (3%)</strong></td>
<td>11.2 ± 0.3</td>
<td>55.7 ± 0.4</td>
<td>9.1 ± 0.1</td>
</tr>
<tr>
<td>Laccase (10 U·g⁻¹)</td>
<td>18.5 ± 0.4</td>
<td>46.3 ± 0.8</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>Laccase (50 U·g⁻¹)</td>
<td>16.8 ± 0.3</td>
<td>47.8 ± 1.2</td>
<td>8.1 ± 0.2</td>
</tr>
</tbody>
</table>

The effect of MtL-Ms was being investigated by 2D-NMR directly on the treated (and control) wood and on the lignin (CEL) isolated from the treated (and control) wood.
Towards industrially-feasible delignification by treating eucalypt pulp with *Myceliophthora thermophila* laccase (MtL) and a phenolic mediator (MS)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MtL</th>
<th>MtL-SA</th>
<th>MtL-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Ep Ep</td>
<td></td>
<td></td>
<td>Ep Ep</td>
<td></td>
</tr>
<tr>
<td>Kappa number</td>
<td>13.3</td>
<td>10.7</td>
<td>12.9</td>
<td>9.8</td>
</tr>
<tr>
<td>Brightness (% ISO)</td>
<td>43.5</td>
<td>57.0</td>
<td>41.5</td>
<td>60.1</td>
</tr>
<tr>
<td>Intrinsic viscosity (mL·g⁻¹)</td>
<td>1230</td>
<td>1030</td>
<td>1240</td>
<td>1020</td>
</tr>
<tr>
<td>Lignin SiG ratio</td>
<td>1.45</td>
<td>0.92</td>
<td>1.10</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Low-cost MtL can be used for eucalypt pulp delignification in combination with methyl syringate (MS)

Interesting results were obtained after lowering the doses of both MtL and MS, which made enzymatic bleaching with laccase-mediator industrially-feasible

Towards industrially-feasible delignification by treating eucalypt pulp with *Myceliophthora thermophila* laccase (MtL) and a phenolic mediator (MeS)

Pilot-scale trials showed that an enzymatic stage using low-cost laccase and unexpensive phenolic mediator can be implemented in eucalypt pulp bleaching providing improvements in:

1) consumption of bleaching agents
2) control of pitch lipids

Process scale-up at CTP pilot-plant presented → 5th ICEP*

2D-NMR results not yet available...

*Burnet et al. 2011. Upscaling TCF bleaching of *Eucalyptus globulus* pulp using an enzyme-catalyzed oxygen delignification. Proc.5th ICEP (International Colloquium on *Eucalyptus* Pulp), Porto Seguro (Brazil), 9-12 May.

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Conclusions:

- **2D-NMR** is a powerful tool to analyze lignin composition (in terms of aromatic units) and structure (including different interunit linkages).
- Structural information on lignin in five eucalypt species correlated with their pulp-making properties (including yield and viscosity).
- Biotechnology can benefit from 2D-NMR availability to develop most efficient enzymatic delignification and bleaching processes (information on the oxidative attack to the lignin polymer).
- In addition to isolated lignins (wood MWL/CEL and pulp residual lignin), whole lignocellulosic material can also be analyzed by 2D-NMR (as gels).
- The latter approach was used to show for the first time the enzymatic delignification of whole eucalypt wood (with polysaccharides unaffected).
- The use of commercial laccases and phenolic mediators provides an industrially-feasible combination for pulp bleaching and enzymatic delignification of wood in mill biorefineries.
Members of the CIB group

Thanks you for your attention!