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Enzymatic delignification of plant cell wall: from nature to mill

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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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Introduction

Central aspects of the enzymatic mechanisms of microbial attack to lignin have been elucidated during recent years [1•]. From their beginning, lignin biodegradation studies have been related to the interest in biotechnological applications for the paper pulp sector, the main nonfood industrial use of plant biomass. The progress attained is especially remarkable when considering that the synthesis and structure of lignin [2,3], a 'black box' until its partial unraveling in the 1970s, is still under investigation and some central issues remain controversial [4•]. This review provides an overview of our current knowledge on the main enzymes involved in lignin degradation, their mechanisms of catalysis, the different 'omics' technologies to identify new enzymes, the use of protein engineering to convert enzymes into industrial biocatalysis, and the combination of biology tools and lignin analytical techniques for the implementation of future biorefineries based on lignocellulosic crops.

The future lignocellulose biorefineries: overcoming the lignin barrier

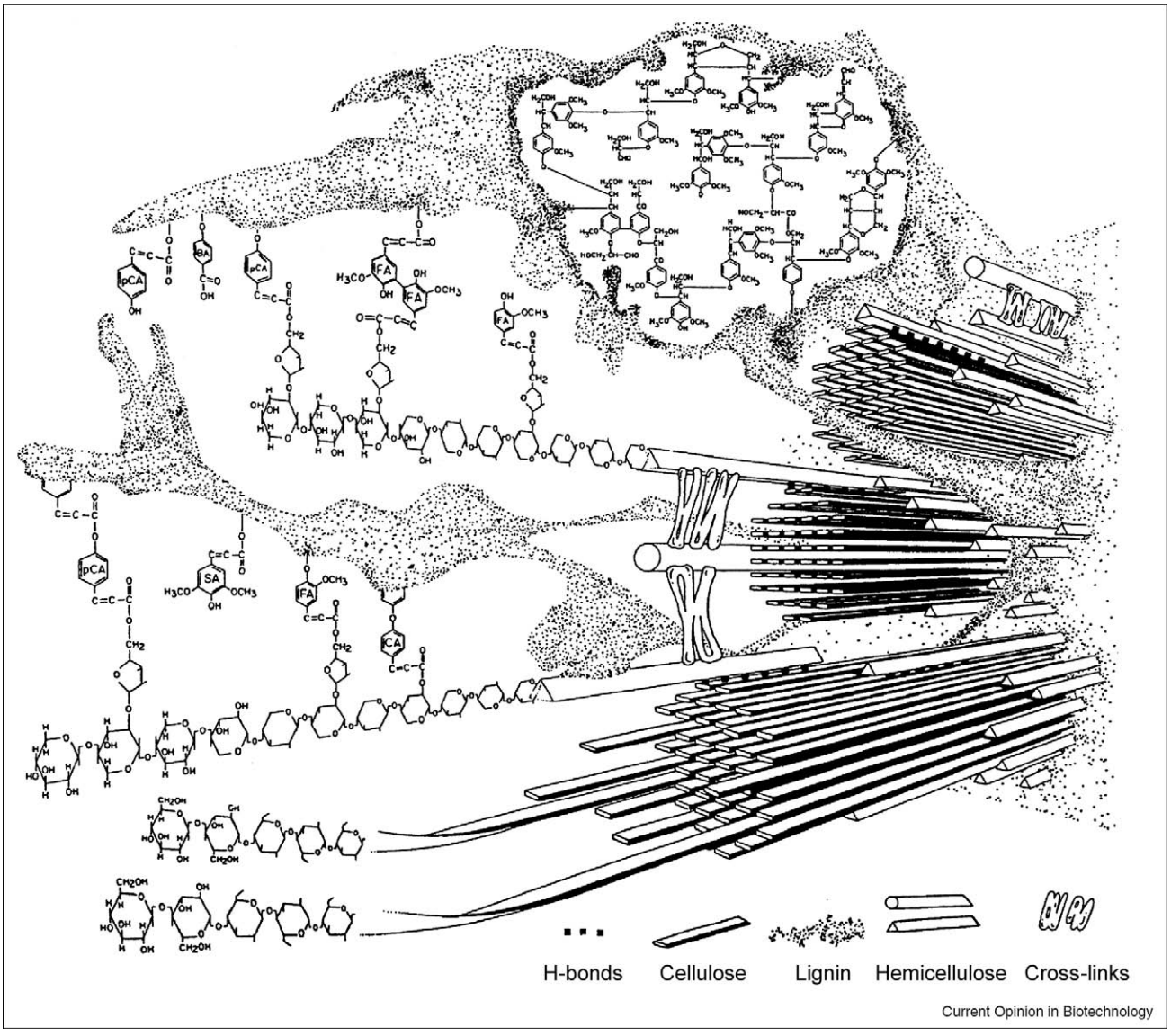
The lignocellulose biorefinery concept [5••,6] is receiving considerable attention as a source of renewable chemicals, materials, and fuels for future sustainable development. Concerns on the growing price of crude oil, which increased during the last two decades (peaking at near 150 US$/barrel in July 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 biosphere [9]. The lignocellulose biorefinery should significantly contribute to reduce this global warming, owing to the neutral balance between the carbon dioxide fixed by plants and that released during the industrial utilization of their biomass.

In the same way that petrol refineries separate oil fractions to obtain different fuels and chemicals, the future biorefineries to be economically feasible should overcome the lignocellulose recalcitrance and separate its different constituents (cellulose, lignin, hemicelluloses, and minor constituents) to obtain different value added products [5••]. Cellulose has a large market in paper manufacture, and its future will also include the production of other chemicals in addition to ethanol. Lignin, and to some extent hemicelluloses, are considered as wastes by the current lignocellulose industries, being burned for energy supply. However, hemicelluloses could be a valuable source of heteropolysaccharides and biofuels, and lignin could be a source of aromatic chemicals and polymers (both chemicals and biodiesel can also be obtained from wood extractives) [10]. Nowadays, the only industrial plants operating like 'forest biorefineries' are some pulp mills that separate lignin from cellulose in such a way that a variety of cellulose products are obtained, at the same time that lignin is modified for different uses, and bioethanol is obtained. We are convinced that this

concept will be adopted by an increasing number of paper pulp and related companies in the future [11•] and that the current technologies will be adapted to more flexible processes to integrate production of both bulk and fine chemicals and biofuels.

Green and white biotechnology can contribute to the successful implementation of biorefineries by providing more amenable raw materials and more efficient biocatalysts [12••]. Owing to the structure of the plant cell wall, the presence of a lignin matrix where polysaccharides are immersed (Figure 1) is the main obstacle in cell-wall deconstruction. For years, plant biologists dedicated their efforts to clone the genes involved in biosynthesis of the three monolignols giving rise to the different lignin units, with the purpose of reducing their production or modifying their balance for easier delignification [13•,14•]. Transgenic trees with improved pulping properties have been obtained [15] but low lignin could also result in altered growth, lower pest resistance, and other environmental problems. Therefore, it has been suggested that the benefits of these 'super trees' should be compared with other alternatives such as improved biocatalysts for biological delignification [16]. These biocatalysts could

Figure 1



Schematic representation of plant secondary wall showing linear cellulose and branched hemicelluloses chains immersed in a lignin matrix formed by dimethoxylated (syringyl), monomethoxylated (guaiacyl), and nonmethoxylated (*p*-hydroxyphenyl) phenylpropanoid units in a variety of substructures containing ether and C–C interunit bonds. Cinnamic acids (CA, *p*-coumaric acid; FA, ferulic acid; and SA, sinapic acid) are also shown (some of them forming lignin–carbohydrate bridges). Reproduced from [81] with the permission of the author and copyright owner.

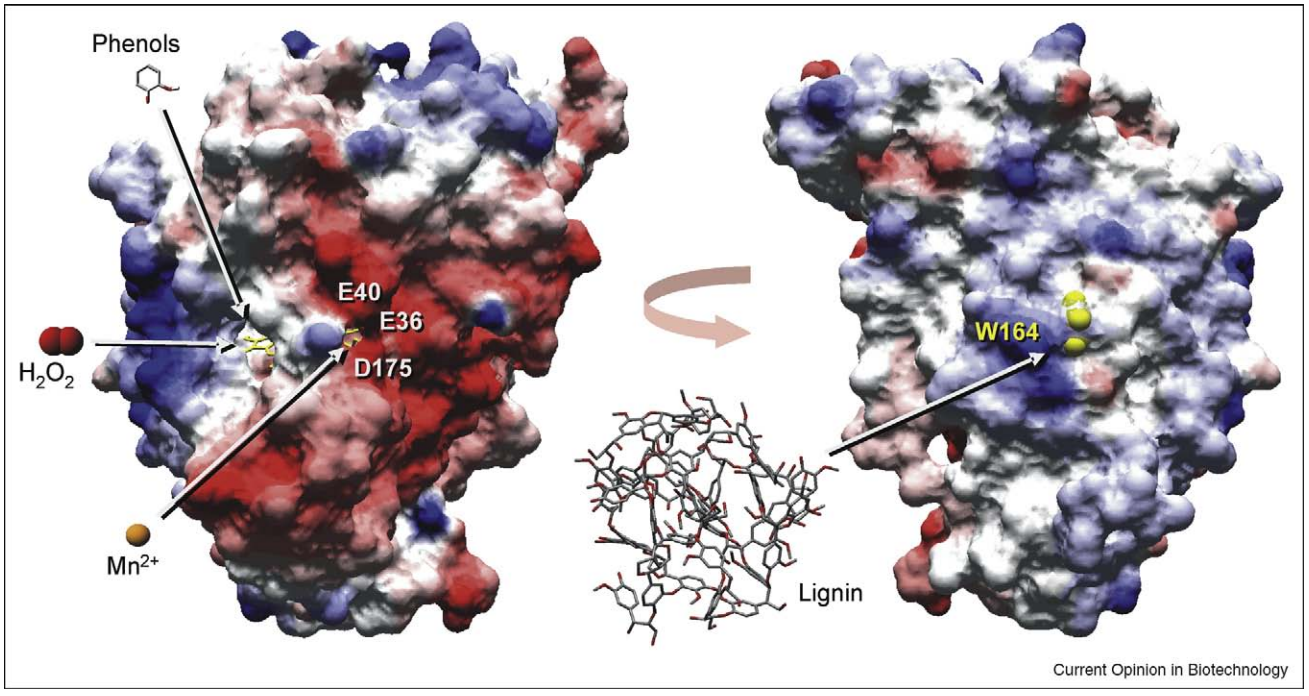
be based on the lignin degradation processes existing in Nature, and should be applied both for taking out the lignin from the cell wall releasing other constituents (enzymatic deconstruction) and to modify the isolated lignin yielding aromatic chemicals and other value added products.

Lignin degradation in Nature: a biotechnological model

Lignin removal is a key step for recycling the carbon fixed by land photosynthesis. Some basidiomycetes (the so-called 'white-rot' fungi because of the whitish color of delignified wood) are the only efficient lignin degraders in Nature [17]. The removal of lignin opens the way for wood colonization by other microbial populations. Lignin attack is an oxidative process, where the extracellular hydrogen peroxide generated by unrelated fungal oxidases, such as glyoxal oxidase, pyranose-2 oxidase, and aryl-alcohol oxidase, oxidizes the polymer in a reaction catalyzed by high redox-potential hemeperoxidases, such as lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP) [17,18••,19•]. Early reports describing the discovery [20] and cloning [21] of LiP in *Phanerochaete chrysosporium* were true landmarks in the field of enzymatic delignification (see [22] for additional references). VP was described more recently as a new peroxidase combining LiP and MnP catalytic properties without some of their drawbacks [23]. Ligninolytic peroxidase genes have been identified in more than 150 agaricomycetes [24] although not all species have the same gene types.

Ligninolytic peroxidases are tailor-made oxidoreductases naturally designed to overcome the recalcitrance and heterogeneity of the lignin polymer, formed by different phenylpropanoid units in a three-dimensional network including a variety of substructures [2]. Their high redox potential, related to heme pocket architecture, enables LiP and VP to directly oxidize the benzenic rings of lignin irrespective of their methoxylation degree and interunit linkages [1•]. The unstable aromatic cation radicals formed, chemically evolve leading to depolymerization and other reactions [17]. Peroxidases were forced to overcome a second difficulty for lignin degradation. The heme group activated by peroxide, which attains the cofactor using an access channel (Figure 2, left), cannot oxidize lignin since this bulky polymer is not able to reach the heme pocket (in contrast with some simple phenols). Therefore, these enzymes oxidize lignin at the protein surface using an exposed tryptophanyl radical (exceptionally a tyrosyl radical) formed by long-range electron transfer to the activated cofactor [1•]. Figure 2 illustrates

Figure 2



Two views of the solvent access surface of a ligninolytic peroxidase (*Pleurotus eryngii* VP; PDB entry 2BOQ) revealing **(left)** the main heme access channel enabling hydrogen peroxide entrance for the activation of the heme cofactor (in yellow) located in a central pocket (phenols can also be oxidized at this channel albeit with low efficiency) and the Mn^{2+}-oxidation channel formed by three acidic residues (Glu36, Glu40, and Asp175) binding the cation in front of the heme internal propionate; and a 180°-rotated view **(right)** showing the partially exposed side-chain (yellow van der Waals spheres including hydrogen atoms) of the catalytic tryptophan (Trp164) involved in the oxidation of lignin, veratryl alcohol (VA), and some phenols (high efficiency) by long-range electron transfer to the heme cofactor (surface colors correspond to electrostatic charge).

how VP would oxidize lignin at the tryptophanyl radical, while Mn^{2+} is directly oxidized by the heme at a second narrow channel [25]. The existence of a catalytic protein radical was indirectly suggested in the 2-electron activated form of LiP, and has been directly demonstrated in VP using low-temperature electron paramagnetic resonance [26••,27]. The role of the tryptophan radical in the whole half-reaction has been recently confirmed by its detection in both the 1-electron and 2-electron oxidized forms of the enzyme [28•].

Most white-rot basidiomycetes and some other fungi [29] and bacteria [30] also produce a multicopper oxidase called laccase. Laccases have a low redox potential that prevents the direct attack on lignin. However, in the presence of small compounds forming stable radicals that act as redox mediators [31], laccases oxidize lignin and other recalcitrant compounds [32]. Although the biological significance of the laccase-mediator system in Nature is to be established, it presents high biotechnological interest. A mediating role is also played by the Mn^{3+} ion formed during MnP and VP oxidation of Mn^{2+} (that acts as an oxidizer of phenolic structures and starter of lipid peroxidation reactions), as well as by some aromatic radicals required for LiP oxidation of lignin, while VP can oxidize lignin directly [1•]. In addition to expanding the substrate range of some enzymes, these mediators can penetrate sound wood, when the size of the cell-wall pores is too small for the penetration of the enzymes.

Searching for lignin-degrading enzymes: the impact of different 'omics'

A large number of plant and microbial genomes relevant for lignocellulose biorefineries are being sequenced [33••] including those of *Trichoderma reesei* [34•], *Pichia stipitis* [35•], and *P. chrysosporium* [36], three fungi of interest in biotechnological transformation of cellulose, hemicellulose, and lignin, respectively. The *P. chrysosporium* genome was the first basidiomycete genome sequenced owing to the interest of the US Department of Energy (DOE) on lignin biodegradation. Its main genes directly involved in lignin degradation were already known [19•], and only one new low redox-potential peroxidase gene was provided by the genome [36]. However, its availability enabled the use of other 'omics' technologies. Moreover, genes of peroxide-producing oxidases, and a large set of P450-type genes, were identified. The *P. chrysosporium* genome also ended a controversy on the production of laccase by this fungus since no laccase genes were present. The genomes of *Pleurotus ostreatus* and *Ceriporiopsis subvermispora*, two white-rot fungi secreting sets of ligninolytic enzymes different from that of *P. chrysosporium* [17], will be soon available with DOE support. Results with biotechnological application are expected since *C. subvermispora* and some *Pleurotus* species were selected some years ago for selective delignification of wood and nonwood lignocellulosic substrates, respectively [37,38].

Table 1

Comparison of genes potentially involved in lignocellulose transformation in the genomes of the model white-rot fungus *P. chrysosporium* [19•,36,43] and the model brown-rot fungus *P. placenta* [39••]

	White-rot genome	Brown-rot genome
Total proteins predicted	10,048	17,173
Proteins potentially involved in *Lignin degradation*		
Lignin peroxidases (LiP)	10	0
Manganese peroxidases (MnP)	5	0
Low redox-potential peroxidases	1	1
Iron reduction		
Multicopper oxidases (Fe-oxidases/laccases)	4 (1/0)	5 (1/3)
Quinone reductases	4	1
Cellobiose dehydrogenases	1	0
Peroxide generation		
Copper-radical oxidases	7	3
Pyranose-2 oxidases (GMC)	1	0
Glucose oxidases (GMC)	1	≥5
Aryl-alcohol oxidases (GMC)	4	3
Methanol oxidases (GMC)	1	1
Total GMC oxidoreductases	35	45
Carbohydrate hydrolysis	282	242
Glycoside hydrolases (GH)	180	144
GH with cellulose-binding domain	30	0
Exocellobiohydrolases	7	0
Endoglucanases	>40	2
β-Glycosidases	9-10	10
Esterases/transferases/expansins/lyases	19/68/11/4	10/75/7/6
Miscellaneous heme-protein reactions		
Cytochrome P450-type enzymes	148	236
Chloroperoxidase-peroxygenases	1–3	8

A second group of wood rotting basidiomycetes causes a preferential removal of cellulose leaving a brown-colored lignin residue. Very recently, DOE supported the sequencing of the first genome of a brown-rot fungus, *Postia placenta*, that was compared with the *P. chrysosporium* genome (Table 1) [39••]. While oxidases and multicopper oxidases were present in both, high redox-potential peroxidases only appeared in the white-rot fungus genome, strongly supporting a central role in lignin degradation. Interestingly, demethoxylation has been reported as the main lignin modification during brown-rot decay [40], and the methanol released seems to be used to generate peroxide for cellulose attack (via Fenton chemistry) since methanol oxidase overexpression was shown in transcriptome and extracellular proteome (secretome) analyses [39••]. Simultaneously, the *P. placenta* genome revealed an unexpected absence of exocellobiohydrolase and cellulose-binding domain genes (and low endoglucanase gene number), and a

collection of P450 genes even larger than that found in *P. chrysosporium* (Table 1).

Proteomic studies aim at identifying the main enzymes involved in the extracellular attack to lignocellulose. LiP and MnP were found on both liquid (using low-N 'ligninolytic' conditions) and solid lignocellulosic cultures of *P. chrysosporium* [41•,42,43] although the isoenzymes varied revealing specific inductions. Numerous proteases recycling limitant nitrogen in wood were also identified. Secretomes of basidiomycetes and ascomycetes have been combined to obtain a toolbox of enzymes of interest, and VP was identified in the *Pleurotus* secretome [44]. The expression of LiP and MnP genes has been detected in transcriptomic analyses using long serial analysis of gene expression libraries of *P. chrysosporium* [45]. Metabolomics has also been combined with proteomics to elucidate degradative routes in *P. chrysosporium* [46].

Metagenomics is a powerful tool for mining genomic resources in complex microbial communities, especially when uncultured organisms are present [47]. Termites are extremely successful wood-degrading organisms, but no genes/transcripts of known lignin-degrading enzymes were identified in their gut metagenome [48••] and metatranscriptome [49]. However, evidence on lignin degradation by termite gut microbiota has been obtained by chemical analysis [50••]. Bovine rumen is another complex community, and a novel laccase has been identified from a metagenomic expression library [51]. The exponentially increasing number of sequenced genomes including oxidoreductases involved in lignin degradation requires specialized databases [52•] and new analysis tools to integrate the data provided by the different 'omics' with the aim of developing synthetic biology approaches.

Engineering lignin biocatalysts for industrial use

'Wild' peroxidases and laccases are not well-suited for industrial use that generally requires particular substrate specificities and application conditions (including pH, temperature, and reaction media) in addition to high expression levels. Therefore, protein engineering is often required to obtain highly expressed and efficient biocatalysts [53,54•].

When the structural basis of the property to be improved is known, a rational approach will be possible. Considering the current structural knowledge on ligninolytic peroxidases [23], engineering their catalytic sites or even transferring them to another enzyme is feasible. Engineering the lignin oxidation site has been recently explored by changing the aromatic residue forming the catalytic radical [28•] or its environment [55]. Other peroxidase regions have been modified by site-directed mutagenesis in a variety of studies aiming to obtain new or improved catalytic properties (see Refs. in [23]), including several patents [56,57]. Improvement of peroxidase resistance toward inactivation by peroxide, a bottleneck in many applications [58], can be addressed by directed evolution (see below); however, directed mutagenesis of oxidizable residues at the distal side of the heme could be an alternative approach.

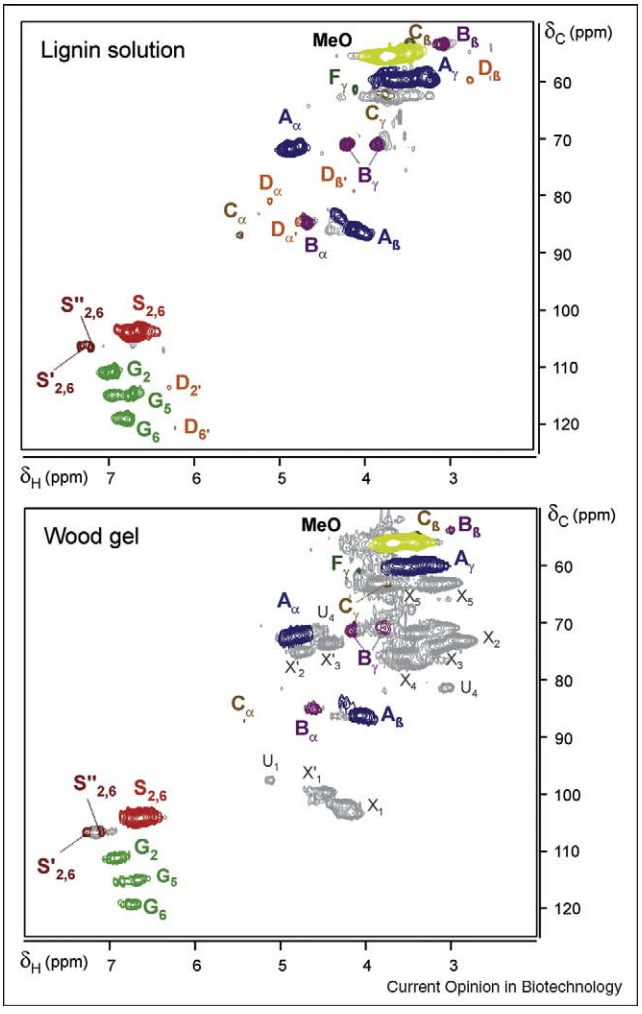
The environment of catalytic coppers has been the target for site-directed mutagenesis of laccases aiming at modifying their redox potential [59]. This seems feasible because a wide range exists between high redox-potential basidiomycete laccases and low redox-potential ascomycete and bacterial laccases. Since the two latter present some biotechnological advantages related to easier expression, increasing their redox potential would result in useful biocatalysts. Among other possible changes, a distal phenylalanine residue has been proved to contribute to high redox potential. A semi-rational approach based on combinatorial saturation mutagenesis at the copper environment, has also been applied to improve the below-mentioned laccase of the ascomycete *Myceliophthora thermophila* [60]. Chimeric enzymes are being constructed, and tailor-made multienzymatic systems could be designed, for example using the cellulosome model [61].

Directed evolution, based on different methods to generate diversity and high-throughput screening, is a very powerful tool for enzyme engineering [62,63]. Evolution of a low redox-potential basidiomycete peroxidase to improve its stability in detergents was one of the first examples [64]. However, no successful evolution of ligninolytic peroxidases (resulting in a significant improvement of the target property) has been reported owing to the lack of sufficient enzyme expression. Nevertheless, detectable levels of expression in yeast have been recently obtained [65], which could potentially be improved by directed evolution. The situation is similar for laccases, and the evolution studies have focused on the low redox-potential enzyme from the ascomycete *M. thermophila* that was first evolved for high-level expression, and then modified for improved properties [66••]. The successful evolution of basidiomycete laccases has not been described yet. Although only modest results have been obtained using wild-type genes [67,68], a basidiomycete laccase gene evolved for high functional expression in yeast has been recently patented [69]. Obviously, the lack of efficient microbial expression systems also limits the industrial production of some of these enzymes [54•], and different alternatives are being explored including their production in the same plants to be used as biorefinery raw materials [14•].

Enzymatic delignification and modern lignin analytical techniques

It is opportune to mention here the strong impact expected from the application of modern lignin analytical

Figure 3



2D NMR spectra of eucalypt lignin solution **(top)** and wood gel **(bottom)** in HSQC (heteronuclear single quantum correlation) experiments. Cross-signals of different lignin substructures are identified: **A**, β-*O*-4′ structures; **B**, resinol structures; **C**, phenylcoumaran structures; **D**, spirodienone structures; **F**, cinnamyl alcohol end-groups; **G**, guaiacyl unit; **S**, syringyl unit; and **S′** and **S″** oxidized syringyl units with conjugated carbonyl or carboxyl groups (^{1}H–^{13}C correlations at positions 2, 5, and 6 of the benzenic ring and α, β and γ of the side-chain; MeO, methoxyls). Some polysaccharide (hemicellulose) cross-signals were also identified: **X** and **X′**, normal and acetylated xylopyranose units, respectively; and **U**, 4-*O*-methylglucuronic acid units. Adapted from [72].

techniques, such as two-dimensional nuclear magnetic resonance (2D NMR), in enzymatic delignification and lignin valorization studies. The availability of 2D NMR spectroscopy based on correlations between ^{1}H and ^{13}C nuclei in lignin represents an extraordinary advance in the structural analysis of this complex polymer (Figure 3, top). Using this technique, signals overlapping in one-dimensional spectra can be resolved, and new lignin precursors and substructures have been discovered [2,3,70••].

During the last years, 2D NMR was successfully used to characterize lignin in plants with modified biosynthesis [3,13•]. In contrast, its use in enzymatic delignification studies is still at the beginning, and a promising future is expected to identify polymer modifications that are not revealed by other analyses. Even more promising is the *in situ* analysis of lignin by NMR of the whole lignocellulosic material at the gel stage [71•,72]. Using this new methodology, problematic and time-consuming isolation protocols are avoided, and the main lignin signals can be analyzed without significant overlapping with the polysaccharide signals (Figure 3, bottom).

Ligninolytic oxidoreductases reaching the mill

Lignin-degrading oxidoreductases are enzymes of industrial interest in lignocellulose biorefineries (for cell-wall delignification in cellulose and ethanol production, functionalization of fibers, production of adhesives, and modification of lignins and other aromatic compounds) including both laccases [73,74•,75] and peroxidases [54•,76].

The laccase-mediator system is commercialized for application in several sectors (including textile) and ready to use for the delignification of paper pulp [32,74•]. The cost and potential toxicity of synthetic mediators, such as –N(OH)– compounds, was a drawback for years. However, recent papers have shown the potential of simple phenols, some of them derived from lignin, as inexpensive and safe (natural) mediators for laccase delignification [77•,78].

Unexpectedly, it has been recently found that the laccase-mediator systems also efficiently degrade pulp lipophilic compounds including sterols, resin acids, glycerides, and fatty acids [79••]. These lipids cause important operational and economic troubles in pulp and paper manufacture (because of pitch deposition) and laccase-mediator remove them irrespective of the raw material and the pulping method. Moreover, the new phenolic mediators are also very efficient in pitch removal. This double effect increases the interest on the laccase-mediator systems, whose use for both industrial applications (delignification and pitch removal) has been recently protected by a new patent [80].

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. • Ruiz-Dueñas FJ, Martínez AT: **Microbial degradation of lignin: How a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this**. *Microb Biotechnol* 2009, **2**:164-177.

A recent review with additional information on microbial degradation of lignin including enzymatic and biochemical aspects and biotechnological applications.

2. Gellerstedt G, Henriksson G: **Lignins: major sources, structure and properties**. In *Monomers, Polymers and Composites from Renewable Resources.* Edited by Belgacem M, Gandini A. Elsevier; 2008:201-224.

3. Ralph J, Lundquist K, Brunow G, Lu F, Kim H, Schatz PF, Marita JM, Hatfield RD, Ralph SA, Christensen JH, Boerjan W: **Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids**. *Phytochem Rev* 2004, **3**:29-60.

4. • Ralph J, Brunow G, Harris PJ, Dixon RA, Schatz PF, Boerjan W: **Lignification: are lignins biosynthesized via simple combinatorial chemistry or via proteinaceous control and template replication?** In *Recent Advances in Polyphenol Research.* Edited by Daayf F, Lattanzio V. Blackwell Pub.; 2008:36-66.

Although the first lignin models were available in the 1970s, our understanding on lignin structure is incomplete, new precursors and substructures are still being described, and other aspects of its biosynthesis are being investigated. This chapter provides an update of the strong polemics caused by an unproved hypothesis suggesting that the last step in lignin biosynthesis (monolignol polymerization) would be under the control of dirigent-type proteins.

5. •• Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD: **Biomass recalcitrance: engineering plants and enzymes for biofuels production**. *Science* 2007, **315**:804-807.

The concept of biomass recalcitrance is used to describe the resistance of natural lignocellulosic materials toward microbial and enzymatic deconstruction to be overcome (by plant and/or enzyme engineering) to separate their individual components and use them as feedstocks for the production of future biofuels and biomaterials in the frame of advanced biorefineries.

6. Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ, Hallett JP, Leak DJ, Liotta CL *et al.*: **The path forward for biofuels and biomaterials**. *Science* 2006, **311**:484-489.

7. Schubert C: **Can biofuels finally take center stage?** *Nat Biotechnol* 2006, **24**:777-784.

8. U.S.DOE: *Breaking the Biological Barriers to Cellulosic Ethanol. A Joint Research Agenda (DOE/SC-0095)*. U.S. Department of Energy Office of Science and Office of Energy Efficiency and Renewable Energy; 2006 www.doegenomestolife.org/biofuels/.

9. U.S.EPA: *Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990–2006 (USEPA #430-R-08-005)* Washington: U.S. Environmental Protection Agency; 2008 www.epa.gov/climatechange/emissions/downloads/08_CR.pdf.

10. Belgacem MN, Gandini A: *Monomers, Polymers and Composites from Renewable Resources*. Amsterdam: Elsevier; 2008.

11. • Ragauskas AJ, Nagy M, Kim DH, Eckert CA, Hallet JP, Liotta CL: **From wood to fuels. Integrating biofuels and pulp production**. *Ind Biotechnol* 2006, **2**:55-65.

The paper pulp industry has developed optimized technologies to obtain a variety of cellulose grades from different woody feedstocks. The biofuel sector should take advantage from these knowledge and facilities. In this article the authors explain which lignocellulose constituents should be used to obtain paper products and which ones to produce biofuels or other biomaterials in an integrated biofuel and paper pulp production (wood biorefinery).

12. •• Weng JK, Li X, Bonawitz ND, Chapple C: **Emerging strategies of lignin engineering and degradation for cellulosic biofuel production**. *Curr Opin Biotechnol* 2008, **19**:166-172.

The authors provide a general overview on how recent advances in lignin structure and biosynthesis suggest new strategies for lignin engineering to obtain more amenable lignocellulosic feedstocks, including plants with highly acylated lignin (see Ref. [70••]), and how new biocatalysts can be obtained, by genomic and other approaches, to be used for enzymatic delignification in the future lignocellulose biorefineries.

13. • Vanholme R, Morreel K, Ralph J, Boerjan W: **Lignin engineering**. *Curr Opin Plant Biol* 2008, **11**:278-285.

A reference review covering several decades of work to elucidate the biosynthesis of classical monolignols (lignin precursors) including system biology approaches, and evaluation of transgenic plants with modified lignin for both paper pulp manufacture and production of bioethanol.

14. • Sticklen MB: **Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol**. *Nat Rev Genet* 2008, **9**:433-443.

The paper reviews several aspects of transgenic plants for lignocellulose biorefineries. In addition to lignin engineering (see also Ref. [13•]) the authors discussed expression of cell-wall deconstructing enzymes in plants. Ideally, the same crop to be used for bioethanol production could include the selected enzyme genes to be activated (induced) after plant growth in such a way that polysaccharide hydrolysis could start in the field.

15. Baucher M, Halpin C, Petit-Conil M, Boerjan W: **Lignin: genetic engineering and impact on pulping**. *Crit Rev Biochem Mol Biol* 2003, **38**:305-350.

16. Talukder K: **Low-lignin wood — a case study**. *Nat Biotechnol* 2006, **24**:395-396.

17. Martínez AT, Speranza M, Ruiz-Dueñas FJ, Ferreira P, Camarero S, Guillén F, Martínez MJ, Gutiérrez A, del Río JC: **Biodegradation of lignocellulosics: microbiological, chemical and enzymatic aspects of fungal attack to lignin**. *Int Microbiol* 2005, **8**:195-204.

18. •• Hammel KE, Cullen D: **Role of fungal peroxidases in biological ligninolysis**. *Curr Opin Plant Biol* 2008, **11**:349-355.

This review focuses on fungal peroxidases playing a key role in lignin degradation, including lignin peroxidases, manganese peroxidases, and versatile peroxidases from *P. chrysosporium* and other basidiomycetes. Their biochemistry and molecular genetics are reviewed, and some future aspects of lignin biodegradation studies are mentioned including the impacts of genomics and modern lignin analytical techniques.

19. • Kersten P, Cullen D: **Extracellular oxidative systems of the lignin-degrading Basidiomycete *Phanerochaete chrysosporium***. *Fungal Genet Biol* 2007, **44**:77-87.

In this paper, the extracellular oxidative system of *P. chrysosporium*, the main model organism in lignin biodegradation studies, is discussed in the light of the sequencing of its complete genome.

20. Tien M, Kirk TK: **Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds**. *Science* 1983, **221**:661-663.

21. Tien M, Tu C-PD: **Cloning and sequencing of a cDNA for a ligninase from *Phanerochaete chrysosporium***. *Nature* 1987, **326**:520-523.

22. Martínez AT: **Molecular biology and structure–function of lignin-degrading heme peroxidases**. *Enzyme Microb Technol* 2002, **30**:425-444.

23. Ruiz-Dueñas FJ, Morales M, García E, Miki Y, Martínez MJ, Martínez AT: **Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases**. *J Exp Bot* 2009, **60**:441-452.

24. Morgenstern I, Klopman S, Hibbett DS: **Molecular evolution and diversity of lignin degrading heme peroxidases in the agaricomycetes**. *J Mol Evol* 2008, **66**:243-257.

25. Ruiz-Dueñas FJ, Morales M, Pérez-Boada M, Choinowski T, Martínez MJ, Piontek K, Martínez AT: **Manganese oxidation site in *Pleurotus eryngii* versatile peroxidase: a site-directed mutagenesis, kinetic and crystallographic study**. *Biochemistry* 2007, **46**:66-77.

26. •• Pogni R, Baratto MC, Teutloff C, Giansanti S, Ruiz-Dueñas FJ, Choinowski T, Piontek K, Martínez AT, Lendzian F, Basosi R: **A tryptophan neutral radical in the oxidized state of versatile peroxidase from *Pleurotus eryngii*: a combined multi-frequency EPR and DFT study**. *J Biol Chem* 2006, **281**:9517-9526.

This paper provided the first direct demonstration on the presence of a catalytic protein radical in a ligninolytic enzyme. Indirect evidence on a long-range electron transfer mechanism to oxidize the bulky lignin polymer at an exposed tryptophan radical had been obtained in other studies, but here the radical was detected by low-temperature electron paramagnetic resonance.

27. Pérez-Boada M, Ruiz-Dueñas FJ, Pogni R, Basosi R, Choinowski T, Martínez MJ, Piontek K, Martínez AT: **Versatile peroxidase oxidation of high redox potential aromatic**

compounds: site-directed mutagenesis, spectroscopic and crystallographic investigations of three long-range electron transfer pathways. *J Mol Biol* 2005, **354**:385-402.

28. • Ruiz-Dueñas FJ, Pogni R, Morales M, Giansanti S, Mate MJ, Romero A, Martínez MJ, Basosi R, Martínez AT: **Protein radicals in fungal versatile peroxidase: catalytic tryptophan radical in both Compound I and Compound II and studies on W164Y, W164H and W164S variants**. *J Biol Chem* 2009, **284**:7986-7994.
Sequel to Ref. [26••] this recent paper reports for the first time the detection of the catalytic tryptophan radical in both the 1-electron (compound I) and 2-electron (compound II) activated forms of a lignino-lytic peroxidase (*P. eryngii* versatile peroxidase) and explores alternative protein radicals using site-directed mutagenesis.

29. Baldrian P: **Fungal laccases — occurrence and properties**. *FEMS Microbiol Rev* 2006, **30**:215-242.

30. Sharma P, Goel R, Capalash N: **Bacterial laccases**. *World J Microbiol Biotechnol* 2007, **23**:823-832.

31. Brogioni B, Biglino D, Sinicropi A, Reijerse EJ, Giardina P, Sannia G, Lubitz W, Basosia R, Pogni R: **Characterization of radical intermediates in laccase-mediator systems. A multifrequency EPR, ENDOR and DFT/PCM investigation**. *Phys Chem Chem Phys* 2008, **10**:7284-7292.

32. Morozova OV, Shumakovich GP, Shleev SV, Yaropolov YI: **Laccase-mediator systems and their applications: a review**. *Appl Biochem Microbiol* 2007, **43**:523-535.

33. •• Rubin EM: **Genomics of cellulosic biofuels**. *Nature* 2008, **454**:841-845.
Near 50 'bioenergy genomes' from potential feedstocks, biomass degraders, and some fuel producers are listed (as completed or in progress). This exponentially increasing genomic information will be vital for the future lignocellulose biorefineries.

34. • Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D *et al.*: **Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*)**. *Nat Biotechnol* 2008, **26**:553-560.
T. reesei is the most investigated cellulolytic organism and the main source of related industrial enzymes. Therefore, sequencing its genome was an obligated step in studies for the enzymatic hydrolysis of cellulose and production of bioethanol.

35. • Jeffries TW, Grigoriev IV, Grimwood J, Laplaza JM, Aerts A, Salamov A, Schmutz J, Lindquist E, Dehal P, Shapiro H *et al.*: **Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis***. *Nat Biotechnol* 2007, **25**:319-326.
Low xylose fermentation by yeast (*Saccharomyces cerevisiae*) is a drawback for the integral use of lignocellulosic biomass in bioethanol production. Genomic information on the mechanisms and regulation of xylose metabolism in this xylose-fermenting yeast (*P. stipitis*) should help to design better strains for an integral use of biomass in future biorefineries.

36. Martínez D, Larrondo LF, Putnam N, Gelpke MD, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F *et al.*: **Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78**. *Nat Biotechnol* 2004, **22**:695-700.

37. Martínez AT, Camarero S, Guillén F, Gutiérrez A, Muñoz C, Varela E, Martínez MJ, Barrasa JM, Ruel K, Pelayo M: **Progress in biopulping of non-woody materials: chemical, enzymatic and ultrastructural aspects of wheat-straw delignification with ligninolytic fungi from the genus *Pleurotus***. *FEMS Microbiol Rev* 1994, **13**:265-274.

38. Messner K: **Biopulping**. In *Forest Products Biotechnology.* Edited by Bruce A, Palfreyman JW. Taylor & Francis; 1998:63-82.

39. •• Martinez D, Challacombe J, Morgenstern I, Hibbett DS, Schmoll M, Kubicek CP, Ferreira P, Ruiz-Dueñas FJ, Martínez AT, Kersten P *et al.*: **Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion**. *Proc Natl Acad Sci U S A* 2009, **106**:1954-1959.
This is the first genome of a brown-rot fungus, compared with the previously sequenced genome of the white-rot fungus *P. chrysosporium* (see ref. [36]). High redox-potential peroxidases (such as LiP and MnP) are present in the genome of the lignin-degrading white-rot fungus but absent from the genome of the polysaccharide-degrading brown-rot fungus, providing indirect confirmation on their involvement in lignin degradation. Moreover, the genome, transcriptome, and secretome analysis in *P. placenta* also indicated a unique mechanism of cellulose attack.

40. Niemenmaa O, Uusi-Rauva A, Hatakka A: **Demethoxylation of [$O^{14}CH_3$]-labelled lignin model compounds by the brown-rot fungi *Gloeophyllum trabeum* and *Poria* (*Postia*) *placenta***. *Biodegradation* 2008, **19**:555-565.

41. • Ravalason H, Jan G, Molle D, Pasco M, Coutinho PM, Lapierre C, Pollet B, Bertaud F, Petit-Conil M, Grisel S *et al.*: **Secretome analysis of *Phanerochaete chrysosporium* strain CIRM-BRFM41 grown on softwood**. *Appl Microbiol Biotechnol* 2008, **80**:719-733.
In this proteomic study lignocellulose-degrading extracellular enzymes are studied during fungal growth using paper pulp as substrate, to investigate those involved in the enzymatic delignification of this industrial material.

42. Sato S, Liu F, Koc H, Tien M: **Expression analysis of extracellular proteins from *Phanerochaete chrysosporium* grown on different liquid and solid substrates**. *Microbiology-Sgm* 2007, **153**:3023-3033.

43. Vanden Wymelenberg A, Minges P, Sabat G, Martinez D, Aerts A, Salamov A, Grigoriev I, Shapiro H, Putnam N, Belinky P *et al.*: **Computational analysis of the *Phanerochaete chrysosporium* v2.0 genome database and mass spectrometry identification of peptides in ligninolytic cultures reveal complex mixtures of secreted proteins**. *Fungal Genet Biol* 2006, **43**:343-356.

44. Bouws H, Wattenberg A, Zorn H: **Fungal secretomes — nature's toolbox for white biotechnology**. *Appl Microbiol Biotechnol* 2008, **80**:381-388.

45. Minami M, Kureha O, Mori M, Kamitsuji H, Suzuki K, Irie T: **Long serial analysis of gene expression for transcriptome profiling during the initiation of ligninolytic enzymes production in *Phanerochaete chrysosporium***. *Appl Microbiol Biotechnol* 2007, **75**:609-618.

46. Matsuzaki F, Shimizu M, Wariishi H: **Proteomic and metabolomic analyses of the white-rot fungus *Phanerochaete chrysosporium* exposed to exogenous benzoic acid**. *J Proteome Res* 2008, **7**:2342-2350.

47. Ferrer M, Beloqui A, Timmis KN, Golyshin PN: **Metagenomics for mining new genetic resources of microbial communities**. *J Mol Microbiol Biotechnol* 2009, **16**:109-123.

48. •• Warnecke F, Luginbuhl P, Ivanova N, Ghassemian M, Richardson TH, Stege JT, Cayouette M, McHardy AC, Djordjevic G, Aboushadi N *et al.*: **Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite**. *Nature* 2007, **450**:560-U17.
This study provides a very good example of metagenomic analysis of an extremely complex microbiota, the hindgut of wood-feeding termites, with the purpose of studying enzymes involved in lignocellulose degradation (resulting in identification of a large variety of glycoside hydrolases).

49. Todaka N, Moriya S, Saita K, Hondo T, Kiuchi I, Takasu H, Ohkuma M, Piero C, Hayashizaki Y, Kudo T: **Environmental cDNA analysis of the genes involved in lignocellulose digestion in the symbiotic protist community of *Reticulitermes speratus***. *FEMS Microbiol Ecol* 2007, **59**:592-599.

50. •• Geib SM, Filley TR, Hatcher PG, Hoover K, Carlson JE, Jimenez-Gasco MD, Nakagawa-izumi A, Sleighter RL, Tien M: **Lignin degradation in wood-feeding insects**. *Proc Natl Acad Sci U S A* 2008, **105**:12932-12937.
Although a related metagenomic study failed to detect genes of known lignin-degrading enzymes in the termite gut (see Ref. [48••]), the authors of the present study confirmed ligninolysis in this microbiota by using specialized techniques for lignin analysis (such as thermochemolysis and NMR spectroscopy) that showed polymer modifications similar to that caused by wood-rotting fungi.

51. Beloqui A, Pita M, Polaina J, Martinez A, Golyshina OV, Zumárraga M, Yakimov MM, García-Arellano H, Alcalde M, Fernandez VM *et al.*: **Novel polyphenol oxidase mined from a metagenome expression library of bovine rumen: biochemical properties, structural analysis and phylogenetic relationships**. *J Biol Chem* 2006, **281**:22933-22942.

52. Levasseur A, Plumi F, Coutinho PM, Rancurel C, Asther M,
• Delattre M, Henrissat B, Pontarotti P, Asther M, Record E: **FOLy: an integrated database for the classification and functional annotation of fungal oxidoreductases potentially involved in the degradation of lignin and related aromatic compounds**. *Fungal Genet Biol* 2008, **45**:638-645.
The FOLy database is a useful tool in lignin biodegradation studies since it contains genes of oxidative enzymes directly or indirectly involved in lignin degradation by fungi (deposited in GeneBank or included in the progressively increasing number of fungal genomes available).

53. Saab-Rincon G, Valderrama B: **Protein engineering of redox-active enzymes**. *Antioxid Redox Signal* 2009, **11**:167-192.

54. Ayala M, Pickard MA, Vazquez-Duhalt R: **Fungal enzymes for**
• **environmental purposes, a molecular biology challenge**. *J Mol Microbiol Biotechnol* 2008, **15**:172-180.
This review on fungal enzymes, with special emphasis on ligninolytic oxidoreductases, identifies three main bottlenecks to be solved for enzyme utilization related to: operational stability (including peroxide inactivation in the case of peroxidases), redox potential (to oxidize more recalcitrant compounds), and heterologous expression (and industrial production).

55. Ruiz-Dueñas FJ, Morales M, Mate MJ, Romero A, Martínez MJ, Smith AT, Martínez AT: **Site-directed mutagenesis of the catalytic tryptophan environment in *Pleurotus eryngii* versatile peroxidase**. *Biochemistry* 2008, **47**:1685-1695.

56. Ruiz-Dueñas FJ, Morales M, Rencoret J, Gutiérrez A, del Río JC, Martínez MJ, Martínez AT: **Improved peroxidases.** *Patent (Spain)* 2008, P200801292.

57. Smith AT, Doyle WA: **Engineered peroxidases with veratryl alcohol oxidase activity**. *Patent (International)* 2006, WO/2006-114616.

58. Valderrama B, Ayala M, Vázquez-Duhalt R: **Suicide inactivation of peroxidases and the challenge of engineering more robust enzymes**. *Chem Biol* 2002, **9**:555-565.

59. Zhukhlistova NE, Zhukova YN, Lyashenko AV, Zaitsev VN, Mikhailov AM: **Three-dimensional organization of three-domain copper oxidases: a review**. *Crystallogr Rep* 2008, **53**:92-109.

60. Zumárraga M, Domínguez CV, Camarero S, Shleev S, Polaina J, Martínez-Arias A, Ferrer M, De Lacey AL, Fernández VM, Antonio B *et al.*: **Combinatorial saturation mutagenesis of the *Myceliophthora thermophila* laccase T2 mutant: the connection between the C-terminal plug and the conserved (509)VSG(511) tripeptide**. *Comb Chem High Throughput Screen* 2008, **11**:807-816.

61. Bayer EA, Lamed R, Himmel ME: **The potential of cellulases and cellulosomes for cellulosic waste management**. *Curr Opin Biotechnol* 2007, **18**:237-245.

62. Zumárraga M, Camarero S, Shleev S, Martinez-Arias A, Ballesteros A, Plou FJ, Alcalde M: **Altering the laccase functionality by in vivo assembly of mutant libraries with different mutational spectra**. *Proteins* 2008, **71**:250-260.

63. Bloom JD, Meyer MM, Meinhold P, Otey CR, MacMillan D, Arnold FH: **Evolving strategies for enzyme engineering**. *Curr Opin Struct Biol* 2005, **15**:447-452.

64. Cherry JR, Lamsa MH, Schneider P, Vind J, Svendsen A, Jones A, Pedersen AH: **Directed evolution of a fungal peroxidase**. *Nat Biotechnol* 1999, **17**:379-384.

65. Ryu K, Kang JH, Wang LS, Lee EK: **Expression in yeast of secreted lignin peroxidase with improved 2,4-dichlorophenol degradability by DNA shuffling**. *J Biotechnol* 2008, **135**:241-246.

66. Zumárraga M, Bulter T, Shleev S, Polaina J, Martinez-Arias A,
•• Plou FJ, Ballesteros A, Alcalde M: **In vitro evolution of a fungal laccase in high concentrations of organic cosolvents**. *Chem Biol* 2007, **14**:1052-1064.
A good example of successful evolution of a laccase gene (*M. thermophila* laccase gene previously evolved for high functional expression in yeast) by mutagenic PCR, *in vivo* assembly, and saturation mutagenesis, combined with high-throughput screening, resulting in one variant with significantly improved activity in the presence of organic solvents.

67. Festa G, Autore F, Fraternali F, Giardina P, Sannia G: **Development of new laccases by directed evolution: functional and computational analyses**. *Proteins* 2008, **72**:25-34.

68. Hu MR, Chao YP, Zhang GQ, Yang XQ, Xue ZQ, Qian SJ: **Molecular evolution of *Fomes lignosus* laccase by ethyl methane sulfonate-based random mutagenesis in vitro**. *Biomol Eng* 2007, **24**:619-624.

69. Camarero S, Alcalde M, Cañas AI, Martínez AT, Martínez MJ, Plou FJ, Ballesteros A, Record E, Asther M.: **High redox-potential laccases obtained by directed evolution**. *Patent (Spain)* 2008, application P200803322.

70. del Río JC, Rencoret J, Marques G, Gutiérrez A, Ibarra D,
•• Santos JI, Jiménez-Barbero J, Martínez AT: **Highly acylated (acetylated and/or *p*-coumaroylated) native lignins from diverse herbaceous plants**. *J Agric Food Chem* 2008, **56**:9525-9534.
Using both spectroscopic (2D NMR) and degradative techniques, the authors showed that lignin in many angiosperms is extensively acylated (see also Ref. [3]). This characteristic would affect both the biosynthesis of lignin (since acylation is produced at the monolignol level) and the biodegradation of the resulting polymer (that will present a less-condensed chemical structure).

71. Kim H, Ralph J, Akiyama T: **Solution-state 2D NMR of ball-milled**
• **plant cell wall gels in DMSO-d_6**. *Bioenerg Res* 2008, **1**:56-66.
The authors show how lignin structure can be directly analyzed in whole lignocellulosic samples (softwood, hardwood, and grass materials) by 2D NMR spectroscopy, using dimethylsulfoxide to form a gel with ball-milled cell walls (directly in the NMR tube). This and related analytical procedures, that avoid lignin isolation, have high potential in enzymatic delignification studies.

72. Rencoret J, Marques G, Gutiérrez A, Nieto L, Santos I, Jiménez-Barbero J, Martínez AT, del Río JC: **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* 2009, **63** (in press, doi:10.1515/HF.2009).

73. Mikolasch A, Schauer F: **Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials**. *Appl Microbiol Biotechnol* 2009, **82**:605-624.

74. Widsten P, Kandelbauer A: **Laccase applications in the forest**
• **products industry: a review**. *Enzyme Microb Technol* 2008, **42**:293-307.
A comprehensive review on the potential of laccases (and laccase-mediator systems) in the forest products industry, including delignification of both wood (biopulping) and paper pulp (biobleaching), improving paper strength by lignin coupling reactions, pitch biocontrol in pulp and paper (see Ref. [79••] for this novel application), deinking of waste paper, effluent treatment, fiber functionalization by grafting low-molecular mass compounds, and fiber/lignin crosslinking in board manufacture.

75. Palonen H, Viikari L: **Role of oxidative enzymatic treatments on enzymatic hydrolysis of softwood**. *Biotechnol Bioeng* 2004, **86**:550-557.

76. Martínez AT: **High redox potential peroxidases**. In *Industrial Enzymes: Structure, Function and Applications.* Edited by Polaina J, MacCabe AP. Springer; 2007:475-486.

77. Camarero S, Ibarra D, Martínez AT, Romero J, Gutiérrez A, del
• Río JC: **Paper pulp delignification using laccase and natural mediators**. *Enzyme Microb Technol* 2007, **40**:1264-1271.
This paper is one of the first reports on biotechnological delignification of paper pulp using laccase in the presence of widely available natural phenols. These compounds could substitute the previously used synthetic mediators that are expensive and potentially risky for the environment.

78. Moldes D, Diaz M, Tzanov T, Vidal T: **Comparative study of the efficiency of synthetic and natural mediators in laccase-assisted bleaching of eucalyptus kraft pulp**. *Bioresour Technol* 2008, **99**:7959-7965.

79. Gutiérrez A, del Río JC, Martínez AT: **Microbial and enzymatic**
•• **control of pitch in the pulp and paper industry**. *Appl Microbiol Biotechnol* 2009, **82**:1005-1018.
Among other aspects, this review refers to a series of four papers of the authors (published in 2006–2008) describing the potential of the laccase-mediator system to degrade pitch-forming lipophilic extractives, the

possibility to use this system for the simultaneous removal of lignin and pitch in paper pulp manufacture, the high potential of 'natural' mediators (see Ref. [77•]) for pitch removal, and the action mechanism of laccase-mediator on model lipids.

80. Gutiérrez A, del Río JC, Rencoret J, Ibarra D, Speranza AM, Camarero S, Martínez MJ, Martínez AT: **Mediator-enzyme system for controlling pitch deposits in pulp and paper production**. *Patent (USA)* 2008, 10080210393/*Patent (European)* 2008, EP 1 908 876 A1.

81. Bidlack J, Malone M, Benson R: **Molecular structure and component integration of secondary cell walls in plants**. *Proc Okla Acad Sci* 1992, **72**:51-56.