Review

Biofpr Biofuels Bioproducts & Biorefining

Understanding lignin biodegradation for the improved utilization of plant biomass in modern biorefineries

Susana Camarero, María Jesús Martínez and Angel T. Martínez, Centro de Investigaciones Biológicas, Madrid, Spain

Received September 20, 2013; revised November 26, 2013 and accepted November 28, 2013
View online at Wiley Online Library (wileyonlinelibrary.com); DOI: 10.1002/bbb.1467;
***Biofuels, Bioprod. Bioref.* (2014)**

Abstract: Wood-rotting fungi are the sole organisms in nature able to degrade the lignin polymer making the polysaccharide components of lignocellulose fully accessible. This process has been investigated for decades as a model for biotechnological application in the pulp and paper industry, animal feeding, and ethanol production. In the current lignocellulose biorefinery concept, ligninolytic fungi and the oxidoreductases (laccases and peroxidases) secreted by these fungi constitute powerful biotechnological tools for the complete utilization of plant biomass. The evolution of molecular biology, which brings into play specifically designed biological systems and on-demand enzymes, together with the technological advances in processing of plant biomass, smoothes the way for a sustainable conversion of renewable feedstocks to new added-value products, with lower energy costs and less environmental impact. The present study reviews some of the main achievements attained by our group in the field of lignin biodegradation that have contributed to: (i) better understanding of the mechanisms by which fungi delignify the lignocellulosic materials; and (ii) assessing the applicability of these ligninolytic systems to increase the efficiency of some industrial processes and to develop new means for sustainable and environmentally sound production of chemicals, materials, and fuels. © 2014 Society of Chemical Industry and John Wiley & Sons, Ltd

Keywords: lignin biodegradation; white biotechnology; plant biomass; biorefinery; white-rot fungi; laccases; ligninolytic peroxidases

Introduction

Development of more competitive economies by way of a more efficient use of resources has become mandatory to face current global problems such as shortage of raw materials, energy and water, and global warming. In 2010, the European Commission launched a ten-year Strategy (Europe 2020) for smart, sustainable, and inclusive growth of the economy based on bio-industrial processes and alternative energies. Biotechnology can contribute to building a knowledge-driven European economy providing by more efficient and cleaner production methods in different sectors including agriculture, energy, and industry. In particular, white biotechnology – promoting the use of micro-organisms and their enzymes to generate industrially useful products – offers innovative solutions with true economic benefits such as reduction of the energy costs and low environmental impact. Simultaneously, it provides new added-value products.

Correspondence to: Susana Camarero, Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28040 Madrid, Spain.
E-mail: susanacam@cib.csic.es

SCI

The unparalleled progress of molecular biology and the boost given by the international policies (particularly in the field of biofuels) are responsible for the huge evolution of white biotechnology during the last few years. The design of specifically directed biological systems and on-demand enzymes is now feasible thanks to the new *omics* and protein engineering tools. Concurrently, the technological advances in processing of plant biomass as a renewable alternative to the use of eventually dwindled fossil resources smooth the way to reach industrial sustainability, with reduced energy costs and fewer contaminant residues. All of this will bring the optimization of already existing technologies and the development of new means of production for chemicals, materials, fuels, and energy. Processes and equipments for conversion of plant biomass will be integrated in multifunctional lignocellulose biorefineries, by contrast to the low processing flexibility of most of the existing facilities using grain feedstock. Lignocellulose biorefineries will combine various feedstock (woody and non-woody energy crops with agricultural, forestry, industrial or municipal residues) and different conversion technologies to produce second-generation biofuels, added-value chemicals and materials, simultaneously with electricity and heat energy for self-consumption.[1] Biotechnology can contribute to this biorefinery concept providing higher integration of the facility, increasing the yield and sustainability of the processes by a better use of biomass, enabling more selective transformations, and reducing the generation of residues.[2]

However, sugars polymerized in lignocellulose are not easily hydrolyzed due to the natural resistance of the plant cell walls to microbial degradation. The compact architecture of cellulose, hemicelluloses, and lignin polymers, and the rate of lignification are responsible for this recalcitrance. Arrangement of cellulose microfibrils (straight chains of β-D-glucose), firmly packed together side-by-side and linked by H-bonds, confer high rigidity to the cellulose core. By contrast, hemicelluloses are branched heterogeneous polysaccharides constituted by different pentoses and hexoses. Hemicelluloses from herbaceous plants mostly consist of a xylan scaffold with ramifications of arabinose and estherified by *p*-hydroxycinnamic acids (mainly ferulic acid), which establish bridges with lignin. In hardwood, hemicelluloses are also mainly composed of xylan branched with glucuronic acid and glucomannan. The high content in pentoses (from xylan) and their limited fermentability are other constraints to produce high-yield cellulosic ethanol. Finally, the lignin matrix covers cellulose and hemicelluloses in the plant cell wall and acts as an adhesive between wood fibers,[3] forming some direct lignin-carbohydrate linkages.[4] Lignin polymer is the result of a biosynthetic pathway that leads to the oxidative coupling of three monolignols: the *p*-coumaryl, coniferyl and sinapyl alcohols giving rise, respectively, to the *p*-hydroxyphenylpropane (H), guaiacylpropane (G) and syringylpropane (S) lignin units linked by different ether and C-C bonds.[5] Lignin composition is very heterogeneous, varying within organisms, tissues, and even cell wall layer. Secondary cell wall is mainly S-lignin whereas middle lamella is rich in G units. Gymnosperm lignin consists almost entirely of G units (G-lignin), dicotyledonous angiosperm lignin is a mixture of G and S (GS-lignin) and monocotyledonous lignin is constituted by the three units (GSH-lignin).

Lignin biodegradation carried out by wood-rotting fungi constitutes a key step for carbon cycling in nature, as well as for the industrial use of plant biomass by increasing accessibility to cellulose. It is an oxidative process that has been investigated for decades as a model for biotechnological application in the pulp and paper industry, animal feeding and bioethanol production. Ligninolytic oxidoreductases (laccases and different types of peroxidases) secreted by wood-rotting fungi are the sole enzymes able to oxidize the phenylpropane lignin units. The use of these biocatalysts might aid the enzymatic hydrolysis of the structural polysaccharides from biomass, improve the quality or add new properties to cellulose products (e.g. paper pulps), or provide new lignin-based products. Production of high-added-value products from the non-fermentable components of biomass is precisely one of the main challenges of biotechnology to reach the complete conversion of plant biomass and launch the modern lignocellulose feedstock biorefineries. Moreover, the design of *ad hoc* enzymes with the desired properties under preferred application conditions is now feasible, being a significant advance toward the implementation of ligninolytic oxidoreductases at industrial scale.[6] On the other hand, the numerous projects for fungal genomes sequencing currently in progress can confirm previous know-how and provide new clues about wood-decay, while they constitute a valuable tool for searching for new enzymes. In the last two decades, achievements attained in the research field of lignin biodegradation have contributed to a better understanding of the mechanisms by which fungi degrade lignocellulose, as well as to depicting the potential of ligninolytic systems to be applied in white biotechnology processes. Here we revise the main contributions of our group on lignin biodegradation (including some case studies) to illustrate the relevance of wood-rotting fungi and their ligninolytic oxidoreductases as biotechnological tools for

Table 1. Fungal pre-treatment of wheat straw for ethanol production.

	Degradation (%)			Sugar yields (%)		Yield (%)
	Cellulose	Hemicellulose	Lignin	Glucose	Xylose	Ethanol
Irpex lacteus	17	26	34	66	58	62
Control (untreated)	0	0	0	36	35	35

the conversion of plant biomass to useful products in the current lignocellulose biorefinery framework.

White-rot decay: Diverse patterns suitable for different biomass conversion applications

Different fungal degradation patterns were disclosed by chemical, histological, and enzymatic analyses of lignocellulosic materials treated with white-rot fungi under solid-state fermentation (SSF) which aims to resemble natural conditions.[7,8] According to the pattern of lignin removal from lignocellulose, different wood-rotting fungi could be selected as models for bio pre-treatment of lignocellulose for different purposes.

We investigated first the delignification of agricultural residues by white-rot fungi as pre-treatment for manufacturing corrugated board from wheat straw. In pulp manufacture, the integrity of cellulose fibers has to be preserved while lignin is removed, so we searched for selective lignin degraders (mainly toward the middle lamella). *Phanerochaete chrysosporium* (the model white-rot fungus at that moment) degraded simultaneously cellulose, hemicelluloses and lignin, whereas *Pleurotus eryngii* was the most selective in removing lignin from wheat straw. It produced a ratio of lignin removal to weight loss triple (67%) the one obtained with *P. chrysosporium* (21%). Histological and ultrastructural studies confirmed these two fungal degradation patterns: simultaneous degradation of all cell wall components by *P. chrysosporium*[9] contrasted with the separation and preservation of straw sclerenchymatic fibers that occurred during lignin removal by *P. eryngii*. Moreover, biopulping assays aimed to reduce the energy costs of the industrial process – consisted of alkaline cooking to dissolve lignin followed by mechanical refining – corroborated *P. eryngii* as a biotechnological model for pulp manufacture owing to its capability for producing significant energy savings during pulping while preserving cellulose integrity. The energy for refining bio-semi chemical pulp was 319 kW/h compared to 576 kW/h needed for refining standard semi chemical pulp.[10]

On the other hand, the pre-treatment stage for producing cellulosic ethanol aims to break down the rigid macromolecular structure of lignocellulose to remove lignin and solubilize hemicellulose, making cellulose more accessible to hydrolytic enzymes. Thermochemical methods are commonly used, but the recycling of acids is expensive, the sugar yields can be improved, and inhibitor compounds (such as phenolic compounds and furfurals), negatively affecting the subsequent stages, are released. Bio pre-treatment of the lignocellulosic material with white-rot fungi can aid lignin removal and favor the action of carbohydrate hydrolases. In fact, bio pre-treatment combined with mild alkali treatment might replace or complement conventional methods for ethanol production from wheat straw.[11]

Irpex lacteus produced the most significant enhancement of saccharification and fermentation yields (Table 1). The fungus partially degraded cellulose and hemicellulose at the same time it removed lignin, resulting in a notable enhancement of the digestibility of the treated material. The sugar yields attained after saccharification were significantly higher and the fermentation yields almost doubled those obtained after 21-day pre-treatment with *P. eryngii*.[11] Moreover, sugar yields could be further improved by optimization of culture and nutritional parameters during pre-treatment of wheat straw with *I. lacteus*.[12]

Different peroxidase types involved in lignin biodegradation

White-rotting fungi are the sole organisms in nature able to mineralize lignin. Consequently, their ligninolytic capacity can be assessed *in vitro* by measuring the CO_2 released from the complete degradation of lignin. We cultured different fungi on wheat straw whose lignin had been labeled *in vivo* with ^{14}C. *Pleurotus* produced high mineralization yields (over 80%). Laccase, manganese peroxidase (MnP), and aryl-alcohol oxidase (an assisting enzyme which produces hydrogen peroxide required by peroxidases) were detected during this process. Lignin mineralization values were significantly raised in the presence of Mn^{2+} (from 20% to 50%,

after 30 days of SSF) and the maximum $^{14}CO_2$ release correlated with maximum peroxidase activity, suggesting the involvement of the ligninolytic peroxidase in the process.[13] Conclusive evidence for the capability of *P. eryngii* peroxidase for *in situ* modification of lignin in wheat straw was subsequently provided by pyrolysis-gas chromatography-mass spectrometry analysis. Up to 90% of the phenolic lignin units were degraded after the enzymatic treatment.[14]

Of the three peroxidases secreted by *P. eryngii* during SSF of wheat straw, one of them showed not only the typical Mn-oxidizing activity but also additional activities on phenolic and non-phenolic compounds in reactions without Mn^{2+}. These findings led to the description of a new type of peroxidase.[15,16] The enzyme, initially reported in submerged cultures as a Mn-oxidizing enzyme with activity on veratryl alcohol and dimethoxyphenol,[17,18] was called versatile peroxidase (VP) due to its oxidative versatility. VP, described for the first time in *P. eryngii* by our group, combines properties from LiP (oxidation of non-phenolic lignin model compounds like veratryl alcohol) and MnP (oxidation of Mn^{2+} to Mn^{3+} which oxidizes phenols and initiates lipid peroxidation reactions).[19–21] In addition, VP shows activity on phenolic compounds in the absence of Mn, similarly to plant peroxidases.[22,23]

More recently, another type of ligninolytic peroxidase, known as dye-decolorizing peroxidase (DyP),[24] was found to be secreted by *I. lacteus* during fungal pre-treatment of wheat straw.[25] DyP-like enzymes can degrade non-phenolic lignin compounds.[26] The enzyme secreted by *I. lacteus* under SSF and submerged culture conditions holds attractive features such as high stability (pH, temperature and H_2O_2) and high catalytic efficiency oxidizing recalcitrant organic dyes and veratryl alcohol. In addition, *I. lacteus* DyP displayed a synergistic action with cellulases during the hydrolysis of wheat straw, increasing significantly the yield of fermentable glucose recovered from this substrate.[25] Recent studies on the *I. lacteus* secretome provided some clues on its suitability for wheat straw pre-treatment for ethanol production. The combination of lignin-modifying enzymes (DyP and MnP) and polysaccharide-depolymerizing cellulases, and the nearly complete absence of secreted cellobiose hydrolases, results in greater availability of hydrolyzable and fermentable sugars.[27]

Lignin removal and biotechnological applications of laccase and mediators

Laccases oxidize a wide range of substituted phenols, aromatic amines, and other aromatic compounds coupled to the reduction of oxygen to water. The applicability potential of laccases can be notably enhanced by the presence of certain compounds that, once oxidized by the enzyme, act as redox mediators, oxidizing recalcitrant substrates which are not oxidized by laccase alone. The mediator radicals behave as electron shuttles: they diffuse far away from the active site and reach complex molecules that cannot enter the substrate binding pocket.

Due to their oxidative versatility, low catalytic requirements, and ability to catalyze polymerization or degradation reactions, fungal laccases possess huge applicability in different sectors. The enzymatic detoxification of steam-exploded wheat straw, by polymerization of the phenolic compounds from the slurry, is one example of oxidative coupling reactions catalyzed by laccase that are useful for biomass conversion processes.[28] Conversely, oxidative degradation reactions catalyzed by laccase (in the presence of mediators) have been extensively studied for paper pulp manufacture or detoxification of aromatic pollutants since the ability of laccase-ABTS to oxidize non-phenolic lignin models was first described.[29]

We demonstrated the efficient bleaching of wood and non-wood fibers by the laccase-mediator system, over other ligninolytic enzymes,[30] and the feasibility of developing laccase-aided industrial sequences for environmentally friendly production of high-quality bleached paper pulps. Annual plants such as flax, sisal, or hemp are used to manufacture high-quality pulps for specialty papers. The optimization of a short totally clorine free (TCF) bleaching sequence using laccase-mediator and hydrogen peroxide resulted in non-wood pulps with outstanding brightness and higher integrity of cellulose, and paper with better mechanical properties.[31,32] Likewise, the integration of an enzymatic step in the industrial TCF bleaching sequence of eucalypt kraft pulp produced an important reduction of lignin content and notable enhancement of pulp brightness and brightness stability (Table 2).[33,34]

Moreover, chemical studies using 2D-NMR allowed us to follow the changes produced in lignin structure and composition during the process.[35] Laccase-mediator degradation of pulp lignin proceeds by Cα oxidation of lignin units and subsequent Cα-Cβ breakdown, in agreement with previous results obtained during fungal degradation of lignin.[36] We could also demonstrate simultaneous delignification of pulp fibers and pitch removal by laccase in the presence of mediators using different techniques,[37,38] as well as the degradation/transformation of aromatic pollutants,[39–41] or the bio-deinking of recycled paper by laccase-mediator systems.[42]

Table 2. Laccase-aided TCF bleaching of wood (eucalypt) and non-wood (flax) pulps, compared with industrial-type chemical sequences.

	Eucalypt		Flax	
	O-O-L-Q-P_OP	O-O-Q-P_OP	L-P_O	A-O-Z-R-P-A
Kappa number (lignin content)	5.2	6.8	1.3	3
Brightness (% ISO)	91.2	87.9	81.5	82.5
Brightness reversion (%)	29.2	36.0	–	–
Viscosity (mL/g)	693	758	640	565
Refining energy (Wh)	–	–	249	254

Bleaching stages with: oxygen (O), laccase-mediator (L), chelating agent (Q), peroxide (P), oxygen pressurized peroxide (P_O), ozone (Z), reductive agent (R) or acidic washing (A). Refining energy: energy needed to attain 92 °SR refining degree.

Laccase technology is extremely useful for organic synthesis, ranging from the oxidation of functional groups to the synthesis of complex natural products.[43] In particular, the synthesis of a variety of new added-value products from cellulose and lignin can be obtained using laccase as biocatalyst.[44] The polymerization of technical lignins by laccase to produce lignin-based adhesives or dispersants for polymer blends,[45] and the enzymatic functionalization of cellulosic pulp to confer new properties to paper by grafting of phenolic derivates to fibers surface,[46] are research issues of topical interest.

Natural mediators

Even when the applicability potential of laccase-mediator systems has been comprehensively demonstrated, the expensive synthesis of chemical mediators and the generation of possible toxic species hamper the utilization of laccases at industrial scale. Consequently, we aimed to search for low cost and environmentally friendly laccase mediators. In preceding studies, we had observed a preferential degradation of phenolic units as the initial stage of lignin degradation by white-rot fungi, no matter the type of lignocellulose degradation pattern the fungus produced.[47] During this process, oxidative cleavage of lignin side-chains (at the Cα-Cβ linkage) occurred, rendering phenolic compounds such as vanillic and siringic acid.[36] On the other hand, we had reported the ability of an enzymatic crude from *P. eryngii* (with laccase as single ligninolytic enzyme) to oxidize non-phenolic substrates. This ability disappeared after ultrafiltration, suggesting the presence of a natural low molecular mass mediator.[48] Taking all these data into account, we searched for alternative mediators of laccase among aromatic compounds (aldehydes, ketones and acids) that could be found in nature and be related to the phenylpropane lignin units. We selected a set of lignin-derived phenolic compounds, namely acetosyringone, syringaldehyde, vanillin and *p*-hydroxycinnamic acids (Fig. 1), which promote oxidative reactions catalyzed by laccase.[40] These compounds might be released during degradation of lignocellulose by white-rot fungi (*p*-hydroxycinnamic acids being also lignin precursors) and are, seemingly, the natural redox mediators of fungal laccases during lignin biodegradation.[49]

The mediating capabilities of these compounds were demonstrated when they promoted or made feasible the degradation of recalcitrant compounds or complex substrates otherwise not oxidized by the enzyme, such as synthetic organic dyes, polycyclic aromatic hydrocarbons, or lignin and lipid deposits from paper pulps.[40,41,49–52] The mechanism by which the phenoxyl radicals of these mediators oxidize the target substrate (H atom transfer) was closely similar to that of the artificial mediator HBT (1-hydroxybenzotriazol), and the efficiency of *p*-coumaric acid to promote laccase removal of anthracene and benzopyrene (over 95%) was also alike.[41] The peroxidation of lipids by laccase in the presence of *p*-hydroxycinnamic acids was also feasible,[51] being able to produce powerful oxidants as described for MnP and Mn^{2+}. These findings suggest that laccase may contribute to fungal lipid peroxidation *in vivo,* thus expanding its role in the biodegradation of lignin and other recalcitrant aromatic compounds.

The interest of these findings was huge given these natural compounds can be easily obtained from lignocellulosic residues (e.g. from the black liquors of paper pulp industry[50]) and might significantly promote the use of laccases in the pulp and paper industry and in a variety of biomass conversion processes integrated in the lignocellulose biorefinery.[49,53]

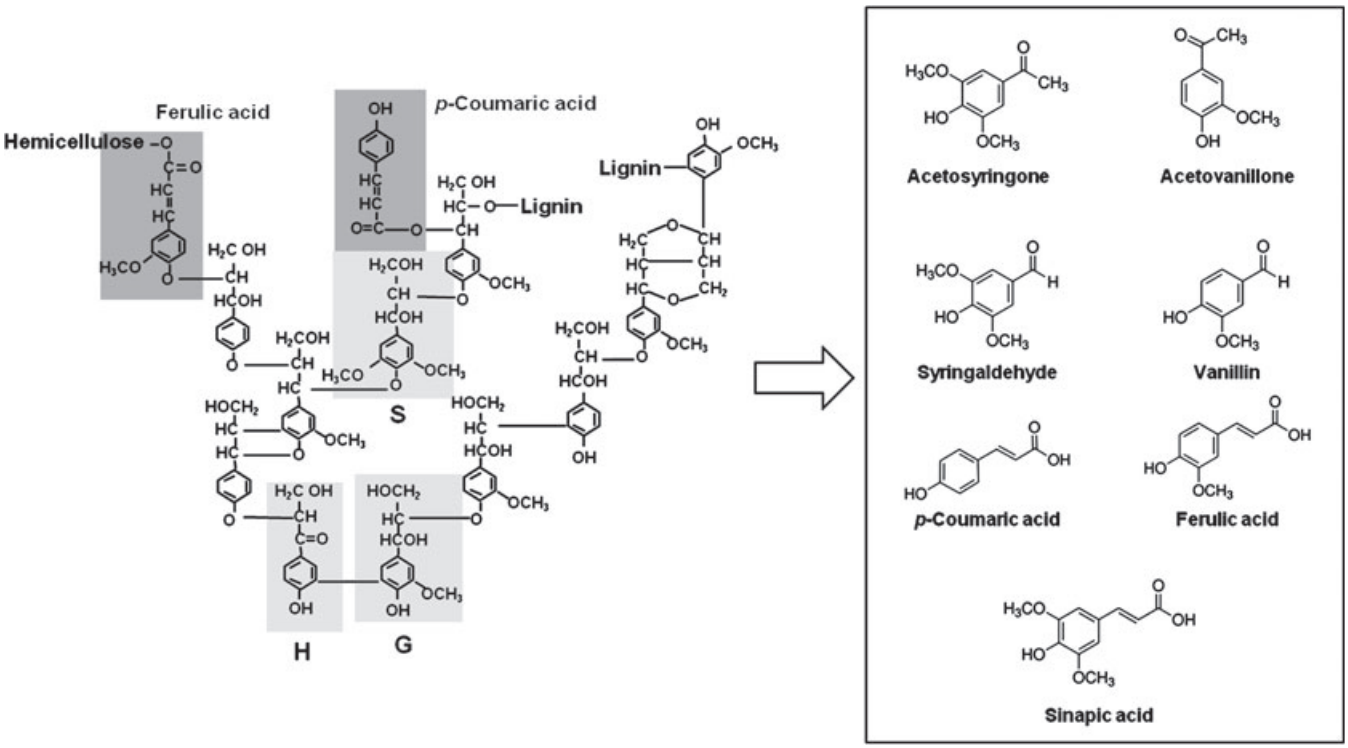


Figure 1. Natural redox mediators of laccase derived from lignin polymer.

Fungal genomes: Enzyme inventories and new clues on lignocellulose biodegradation

The study of fungal genomes, together with transcriptomes and secretomes, provides an inventory of known and unknown enzymes to confirm previous results and give new evidence for better comprehension of wood degradation. Wood-rotting basidiomycetes attack wood through two main processes called white-rot and brown-rot decay. White-rot fungi can degrade all components of plant cell walls and have the unique ability to degrade lignin completely to CO_2 and H_2O, making the structural polysaccharides exposed. By contrast, brown-rot fungi produce an incomplete ligninolysis and the modified lignin remains *in situ* as a polymeric residue after polysaccharide removal.[8]

The genome of the model white-rot fungus *P. chrysosporium* confirmed the presence of the enzymatic machinery required for efficient ligninolysis and cellulose degradation (Table 3).[54] Up to 15 ligninolytic peroxidases and 32 glycosyl-hydrolase genes were found together with H_2O_2-producing enzymes, cellobiose deshydrogenases, etc. Conversely, the genome from the brown-rotting *P. placenta* revealed the lack of ligninolytic peroxidases and

Table 3. Comparison of genes encoding lignocellulose degradation enzymes in the white-rot (*Phanerochaete chrysosporium*) and brown-rot (*Postia placenta*) fungal genomes.

	P. chrysosporium	*P. placenta*
Ligninolytic oxidoreductases		
Peroxidases (LiP/MnP)	10/5	0/0
MCOs (laccase/ ferroxidase)	0/1	3/1
Peroxide generating GMCs		
Aryl-alcohol oxidases	4	3
Methanol oxidases	1	1
Glyoxal oxidase and others	7	3
Carbohydrolases		
Glycoside hydrolases (GH)	180	144
GH with cellulose binding domain	30	0
Exocellulases	7	0
Endoglucanases	>40	2

a unique extracellular enzyme system responsible for the efficient degradation of cellulose. The typical exo-cellobiohydrolases and cellulose-binding domain were absent, whereas a complete enzymatic machinery for generation of Fenton chemistry was found. The highly reactive hydroxyl radicals generated are supposed to contribute for the oxidative depolymeryzation of cellulose and a partial oxidation of lignin.[55]

Up to now, several genome mining projects have been developed to offer new insights into the diversification of lignocellulose degrading mechanisms in white-rot fungi. Recently, the genome of *Ceriporiopsis subvermispora* has been published, and its secretome has been studied under ligninolytic conditions in order to investigate the selective ligninolysis process. The repertory of genes and their expression patterns showed an increase of Mn peroxidases (up to thirteen genes) and laccases (seven genes) and diminished cellulolytic machinery respecting *P. chrysosporium*.[56] The number of genes potentially involved in the lipid metabolism was also augmented and significantly overexpressed in wood cultures together with MnP and laccase genes. This fact supports the hypothesis of a significant role for Mn-mediated peroxidase activity in lignin degradation, and corroborates our previous results obtained for another selective ligninolytic fungus (*Pleurotus*). The lipid peroxidation products, generated by the action of Mn^{3+} from MnP (or by laccase and natural mediators) would act as powerful oxidants producing the breakdown of the more recalcitrant non-phenolic lignin units. Additionally, two new ligninolytic peroxidases, phylogenetically and catalytically intermediates between classic LiPs and VPs, were discovered in the *C. subvermispora* genome, offering new insight into selective lignin degradation.[57] Lately, the extensive sequencing of basidiomycete genomes is providing a huge number of peroxidase-encoding genes,[58] not only from the best known superfamily of plant-fungal-prokaryotic peroxidase superfamily (where LiP, MnP and VP genes are included), but also from the new DyP and heme-thiolate peroxidase superfamilies, which are still to be explored and present high biotechnological interest for oxidation and oxygenation biotransformations.

Engineering ligninolytic enzymes: From structure-function to biocatalyst improvement

Since its discovery, structure-function studies of VP have revealed its oxidation versatility as a result of the coexistence of different catalytic sites in the same protein scaffold that are reminiscent of those individually present in other peroxidase families.[23] Mn^{2+} oxidation ability is provided by three acidic residues forming the typical Mn^{2+} binding site.[59] A solvent-exposed tryptophan is the catalytically active residue for veratryl alcohol oxidation, forming a tryptophanyl radical after VP activation by peroxide and initiating an electron transfer pathway to heme. Finally, some phenols and dyes are oxidized at the edge of the main heme access channel (Fig. 2(a)).[60]

The different catalytic sites of VP can be modified to adjust the catalytic activity of the enzyme. Indeed, tailor-made VP variants of industrial interest have been designed by site- directed mutagenesis for the removal of phenolic compounds from mill effluents by modifying the heme channel.[61] In addition to rational design, directed molecular evolution constitutes a powerful protein engineering tool for adjusting the intrinsic enzyme properties to the harsh industrial operational conditions. This methodology has been used for developing new VP variants with improved stability towards temperature and alkaline pH.[62]

Fungal laccases are excellent targets for directed evolution studies owing to their robustness and substrate promiscuity which provide a high evolvability potential to be exploited in the lab. During the last few years we performed the directed evolution of high-redox potential laccases from white-rot fungi. Diversity was generated by random mutagenesis using error-prone PCR and *in vivo* approaches for DNA recombination using the homologous recombination machinery of *S. cerevisiae,*[63] all combined with semi-rational approaches. The difficult heterologous expression of basidiomycete laccases was overcome by the joint evolution of the recombinant prepro-leader (from the α mating factor) plus the laccase sequence. At the end of the evolution processes, a remarkable increment of laccase secretion by *S. cerevisiae* was obtained (e.g. up to 8 mg/l for basidiomycete PM1 laccase).[64] The accumulation of selected mutations resulted in active and stable evolved laccases with improved kinetic values for oxidizing phenolic and non-phenolic aromatic substrates. In addition, the optimum pH of *P. cinnabarinus* laccase was significantly broadened.[65] Some of the amino acid substitutions of the evolved laccases were not located in the vicinity of the active site or the binding pocket, thus revealing targets for protein engineering that cannot be easily predicted by rational design (Fig. 2(b)). The two aforementioned fungal laccases were used to obtain chimeric proteins by DNA shuffling. The exchange of diverse protein blocks provided soluble and active hybrid enzymes that conserved the generalist

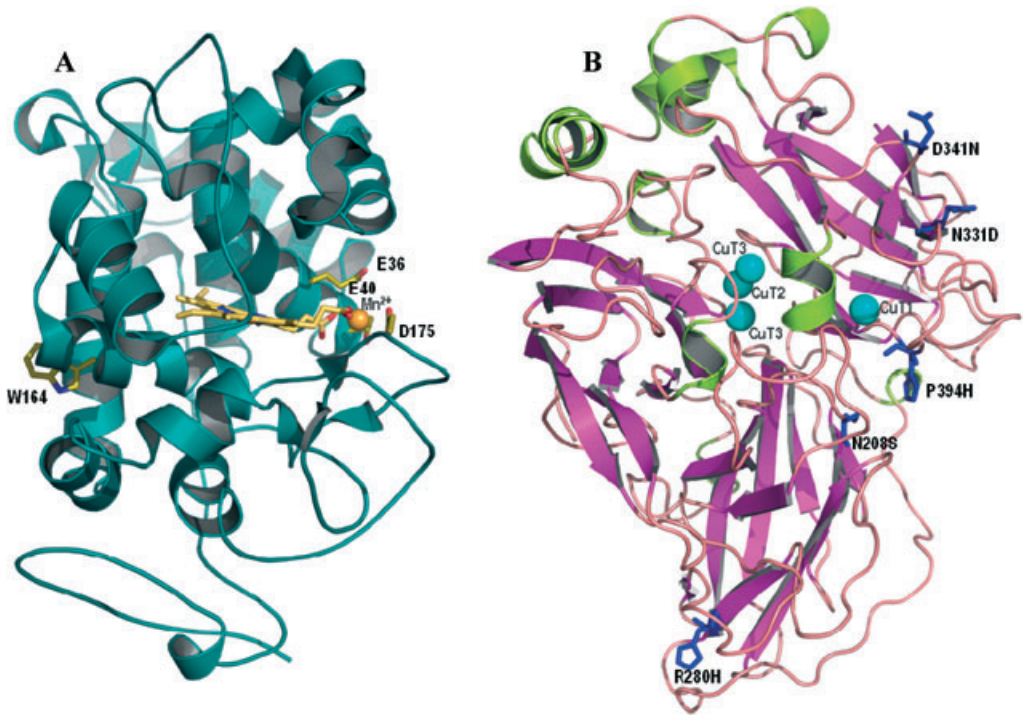


Figure 2. *Pleurotus eryngii* versatile peroxidase showing the different catalytic sites (A), and *Pycnoporus cinnabarinus* laccase with the amino acid substitutions from the *in vitro* evolution (B).

oxidative activity characteristic of laccases. Besides, they displayed modified pH activity profiles, higher thermostability, or improved affinity for phenolic compounds.[66] Using these platforms as starting point, new challenges for laccase engineering can be faced. Promising targets would be the enzymatic detoxification of thermo-chemically pretreated lignocellulosic biomass for ethanol production, the enzyme-aided TCF bleaching of paper pulps, or the enzymatic decolorization of textile effluents, for which we have recently developed *ad hoc* high-throughput screening methods based on our experience in this field.[67]

Conclusion

For years, understanding the mechanism by which wood-rotting fungi delignify lignocellulose has been essential to disclose the biotechnological potential of these fungi and their enzymatic systems. Today, the wide-range of oxidative capabilities of the ligninolytic oxidoreductases have been thoroughly demonstrated and their intrinsic properties can be upgraded by protein engineering. Moreover, the huge amount of information provided by the new *omics* tools lets us confirm previous knowledge on lignocellulose degradation and discover new enzymes of interest as industrial biocatalysts. Considering the above scenario, the present review provides a brief picture of the possible utilization of the lignin degrading systems to aid industrial processes to be cleaner and more efficient, to manufacture new added-value products from lignocellulose and to contribute to the biorefinery and sustainable development concepts.

Acknowledgements

Funding from the EU projects PEROXICATS (KBBE-2010-4-265397) and BIORENEW (NMP2-CT-2006-026456), and from the Spanish projects EVOFACEL (BIO2010-19697), HIPOP (BIO2011-26694) and PRI-PIBAR-2011-1402 is acknowledged. The authors thank CIB collaborators (particularly, F.J. Ruiz-Dueñas, A. Prieto, M. Alcalde, I. Pardo, D. Salvachúa and E. Fernández-Fueyo) for results mentioned in the present review.

References

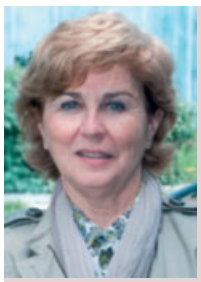
1. Kamm B and Kamm M, Biorefineries - Multi product processes, in *White Biotechnology*, *Vol. 105*, (Eds.: R. Ulber, D. Sell), Springer, Berlin (2007) pp. 175–204.
2. Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA *et al.*, The path forward for biofuels and biomaterials. *Science* **311**:484–489 (2006).
3. Fengel D and Wegener G, *Wood: Chemistry, ultrastructure, reactions*, De Gruyter, Berlin, 1984.

4. Koshijima T and Watanabe T, *Association between lignin and carbohydrates in wood and other plant tissues*, Springer, Berlin, 2003.
5. Ralph J, Lundquist K, Brunow G, Lu F, Kim H, Schatz PF *et al.,* Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochem Rev* **3**:29–60 (2004).
6. Martínez AT, Ruiz-Dueñas FJ, Martínez MJ, del Río JC and Gutiérrez A, Enzymatic delignification of plant cell wall: from nature to mill. *Curr Opin Biotechnol* **20**:348–357 (2009).
7. Martínez AT, Camarero S, Guillén F, Gutiérrez A, Muñoz C, Varela E *et al.*, 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* **13**:265–274 (1994).
8. Martínez AT, Speranza M, Ruiz-Dueñas FJ, Ferreira P, Camarero S, Guillén F *et al.,* Biodegradation of lignocellulosics: Microbiological, chemical and enzymatic aspects of fungal attack to lignin. *Int Microbiol* **8**:195–204 (2005).
9. Barrasa JM, Camarero S, Martínez AT and Ruel K, Ultrastructural aspects of wheat straw degradation by *Phanerochaete chrysosporium* and *Trametes versicolor. Appl Microbiol Biotechnol* **43**:766–770 (1995).
10. Camarero S, Barrasa JM, Pelayo M and Martínez AT, Evaluation of *Pleurotus* species for wheat-straw biopulping. *J Pulp Paper Sci* **24**:197–203 (1998).
11. Salvachúa D, Prieto A, Lopez-Abelairas M, Lú-Chau T, Martínez AT and Martínez MJ, Fungal pretreatment: An alternative in second-generation ethanol from wheat straw. *Bioresource Technol* **102**:7500–7506 (2011).
12. Salvachúa D, Prieto A, Vaquero ME, Martínez AT and Martínez MJ, Sugar recoveries from wheat straw following treatments with the fungus *Irpex lacteus*. *Bioresource Technol* **131**:218–225 (2013).
13. Camarero S, Böckle B, Martínez MJ and Martínez AT, Manganese-mediated lignin degradation by *Pleurotus pulmonarius*. *Appl Environ Microbiol* **62**:1070–1072 (1996).
14. Camarero S, Bocchini P, Galletti GC, Martínez MJ and Martínez AT, Compositional changes of wheat lignin by a fungal peroxidase analyzed by Pyrolysis-GC/MS. *J Anal Appl Pyrolysis* **58/59**:413–423 (2001).
15. Camarero S, Sarkar S, Ruiz-Dueñas FJ, Martínez MJ and Martínez AT, Description of a versatile peroxidase involved in natural degradation of lignin that has both Mn-peroxidase and lignin-peroxidase substrate binding sites. *J Biol Chem* **274**:10324–10330 (1999).
16. Camarero S, Ruiz-Dueñas FJ, Sarkar S, Martínez MJ and Martínez AT, The cloning of a new peroxidase found in lignocellulose cultures of *Pleurotus eryngii* and sequence comparison with other fungal peroxidases. *FEMS Microbiol Lett* **191**:37–43 (2000).
17. Martínez MJ, Ruiz-Dueñas FJ, Guillén F and Martínez AT, Purification and catalytic properties of two manganese-peroxidase isoenzymes from *Pleurotus eryngii*. *Eur J Biochem* **237**:424–432 (1996).
18. Ruiz-Dueñas FJ, Martínez MJ and Martínez AT, Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus *Pleurotus eryngii*. *Mol Microbiol* **31**:223–236 (1999).
19. Tien M and Kirk TK, Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. *Science* **221**:661–663 (1983).
20. Gold MH, Kuwahara M, Chiu AA and Glenn JK, Purification and characterization of an extracellular H_2O_2-requiring diarylpropane oxygenase from the white rot basidiomycete *Phanerochaete chrysosporium*. *Arch Biochem Biophys* **234**:353–362 (1984).
21. Kuwahara M, Glenn JK, Morgan MA and Gold MH, Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. *FEBS Lett* **169**:247–250 (1984).
22. Ruiz-Dueñas FJ, Camarero S, Pérez-Boada M, Martínez MJ and Martínez AT, A new versatile peroxidase from *Pleurotus*. *Biochem Soc Trans* **29**:116–122 (2001).
23. Ruiz-Dueñas FJ, Morales M, García E, Miki Y, Martínez MJ and Martínez AT, Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. *J Exp Bot* **60**:441–452 (2009).
24. Sugano Y, Muramatsu R, Ichiyanagi A, Sato T and Shoda M, DyP, a unique dye-decolorizing peroxidase, represents a novel heme peroxidase family. *J Biol Chem* **282**:36652-36658 (2007).
25. Salvachúa D, Prieto A, Martínez AT and Martínez MJ, Characterization of a novel DyP-type peroxidase from I*rpex lacteus* and its application in the enzymatic hydrolysis of wheat straw. *Appl Environ Microbiol* **79**:4316–4324 (2013).
26. Liers C, Bobeth C, Pecyna M, Ullrich R and Hofrichter M, DyP-like peroxidases of the jelly fungus *Auricularia auricula-judae* oxidize nonphenolic lignin model compounds and high-redox potential dyes. *Appl Microbiol Biotechnol* **85**:1869–1879 (2010).
27. Salvachúa D, Martínez AT, Tien M, López-Lucendo MF, García F, de los Ríos V *et al.,* Differential proteomic analysis of the secretome of *Irpex lacteus* and other white-rot fungi during wheat straw pretreatment. *Biotechnol Biofuels* **6**:115 (2013).
28. Jurado M, Prieto A, Martínez-Alcalá MA, Martínez AT and Martínez MJ, Laccase detoxification of steam-exploded wheat straw for second generation bioethanol. *Bioresource Technol* **100**:6378–6384 (2009).
29. Bourbonnais R and Paice MG, Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Lett* **267**:99–102 (1990).
30. Sigoillot C, Camarero S, Vidal T, Record E, Asther M, Pérez-Boada M, Martínez MJ, Sigoillot J-C, Asther M, Colom J and Martínez AT, Comparison of different fungal enzymes for bleaching high-quality paper pulps. *J Biotechnol* **115**:333–343 (2005).
31. García O, Camarero S, Colom J, Martínez AT, Martínez MJ, Monje R and Vidal T, Optimization of a laccase-mediator stage for TCF bleaching of flax pulp. *Holzforschung* **57**:513–519 (2003).
32. Camarero S, García O, Vidal T, Colom J, del Río JC, Gutiérrez A, Gras JM, Monje R, Martínez MJ and Martínez AT, Efficient bleaching of non-wood high-quality paper pulp using laccase-mediator system. *Enzyme Microb Technol* **35**:113–120 (2004).
33. Ibarra D, Romero J, Martínez MJ, Martínez AT and Camarero S, Exploring the enzymatic parameters for optimal delignification of eucalypt pulp by laccase-mediator. *Enzyme Microb Technol* **39**:1319–1327 (2006).
34. Ibarra D, Camarero S, Romero J, Martínez MJ and Martínez AT, Integrating laccase-mediator treatment into an industrial-type sequence for totally chlorine free bleaching eucalypt kraft pulp. *J Chem Technol Biotechnol* **81**:1159–1165 (2006).
35. Ibarra D, Chávez MI, Rencoret J, del Río JC, Gutiérrez A, Romero J, Camarero S, Martínez MJ, Jiménez-Barbero J and Martínez AT, Structural modification of eucalypt pulp lignin in a totally chlorine free bleaching sequence including a laccase-mediator stage. *Holzforschung* **61**:634–646 (2007).

36. Camarero S, Galletti GC and Martínez AT, Demonstration of *in situ* oxidative degradation of lignin side-chains by two white-rot fungi using analytical pyrolysis of methylated wheat straw. *Rapid Commun Mass Spectrom* **11**:331–334 (1997).
37. Gutiérrez A, del Río JC, Ibarra D, Rencoret J, Romero J, Speranza M, Camarero S, Martínez MJ and Martínez AT, Enzymatic removal of free and conjugated sterols forming pitch deposits in environmentally sound bleaching of eucalypt paper pulp. *Environ Sci Technol* **40**:3416–3422 (2006).
38. Speranza M, Ibarra D, Romero J, Martínez AT, Martínez MJ and Camarero S, Microscopy studies reveal delignification and sterol removal from eucalypt kraft pulps by laccase-HBT. *Biocatal Biotransform* **25**:251–259 (2007).
39. Rodríguez E, Nuero O, Guillén F, Martínez AT and Martínez MJ, Degradation of phenolic and non-phenolic aromatic pollutants by four *Pleurotus* species: the role of laccase and versatile peroxidase. *Soil Biol Biochem* **36**:909–916 (2004).
40. Camarero S, Ibarra D, Martínez MJ and Martínez AT, Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Appl Environ Microbiol* **71**:1775–1784 (2005).
41. Cañas A, Alcalde M, Plou FJ, Martínez MJ, Martínez AT and Camarero S, Transformation of polycyclic aromatic hydrocarbons by laccase is strongly enhanced by phenolic compounds present in soil. *Environ Sci Technol* **41**:2964–2971 (2007).
42. Fillat U, Prieto A, Camarero S, Martínez AT and Martínez MJ, Biodeinking of flexographic inks by fungal laccases using synthetic and natural mediators. *Biochem Eng J* **67**:97–103 (2012).
43. Kunamneni A, Camarero S, Garcia-Burgos C, Plou FJ, Ballesteros A and Alcalde M, Engineering and applications of fungal laccases for organic synthesis. *Microb Cell Fact* **7**:32 (2008).
44. Widsten P and Kandelbauer A, Laccase applications in the forest products industry: A review. *Enzyme Microb Technol* **42**:293–307 (2008).
45. Prasetyo EN, Kudanga T, Ostergaard L, Rencoret J, Gutiérrez A, del Río JC *et al.,* Polymerization of lignosulfonates by the laccase-HBT (1-hydroxybenzotriazole) system improves dispersibility. *Bioresource Technol* **101**:5054–5062 (2010).
46. Aracri E, Fillat A, Colom JF, Gutiérrez A, del Río JC, Martínez AT and Vidal T, Enzymatic grafting of simple phenols on flax and sisal pulp fibres using laccases. *Bioresource Technol* **101**:8211–8216 (2010).
47. Camarero S, Galletti GC and Martínez AT, Preferential degradation of phenolic lignin-units by two white-rot fungi. *Appl Environ Microbiol* **60**:4509–4516 (1994).
48. Martínez MJ, Muñoz C, Guillén F and Martínez AT, Studies on homoveratric acid transformation by the ligninolytic fungus *Pleurotus eryngii*. *Appl Microbiol Biotechnol* **41**:500–504 (1994).
49. Cañas AI and Camarero S, Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. *Biotechnol Adv* **28**:694–705 (2010).
50. Camarero S, Ibarra D, Martínez AT, Romero J, Gutiérrez A and del Río JC, Paper pulp delignification using laccase and natural mediators. *Enzyme Microb Technol* **40**:1264-1271 (2007).
51. Camarero S, Cañas AI, Nousiainen P, Record E, Lomascolo A, Martínez MJ and Martínez AT, *p*-Hydroxycinnamic acids as natural mediators for laccase oxidation of recalcitrant compounds. *Environ Sci Technol* **42**:6703–6709 (2008).
52. Gutiérrez A, Rencoret J, Ibarra D, Molina S, Camarero S, Romero J *et al.,* Removal of lipophilic extractives from paper pulp by laccase and lignin-derived phenols as natural mediators. *Environ Sci Technol* **41**:4124–4129 (2007).
53. Babot ED, Rico A, Rencoret J, Kalum L, Lund H, Romero J *et al.,* Towards industrially feasible delignification and pitch removal by treating paper pulp with *Myceliophthora thermophila* laccase and a phenolic mediator. *Bioresource Technol* **102**:6717–6722 (2011).
54. Martinez D, Larrondo LF, Putnam N, Gelpke MD, Huang K, Chapman J *et al.,* Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. *Nat Biotechnol* **22**:695–700 (2004).
55. Martinez D, Challacombe J, Morgenstern I, Hibbett DS, Schmoll M, Kubicek CP *et al.,* Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. *Proc Natl Acad Sci USA* **106**:1954–1959 (2009).
56. Fernández-Fueyo E, Ruiz-Dueñas FJ, Ferreira P, Floudas D, Hibbett DS, Canessa P *et al.,* Comparative genomics of *Ceriporiopisis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. *Proc Natl Acad Sci USA* **109**:5458–5463 (2012).
57. Fernández-Fueyo E, Ruiz-Dueñas FJ, Miki Y, Martínez MJ, Hammel KE and Martínez AT, Lignin-degrading peroxidases from genome of selective ligninolytic fungus *Ceriporiopsis subvermispora*. *J Biol Chem* **287**:16903–16906 (2012).
58. Floudas D, Binder M, Riley R, Barry K, Blanchette RA, Henrissat B *et al.,* The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. *Science* **336**:1715–1719 (2012).
59. Ruiz-Dueñas FJ, Morales M, Pérez-Boada M, Choinowski T, Martínez MJ, Piontek K and Martínez AT, Manganese oxidation site in *Pleurotus eryngii* versatile peroxidase: A site-directed mutagenesis, kinetic and crystallographic study. *Biochemistry* **46**:66–77 (2007).
60. Morales M, Mate MJ, Romero A, Martínez MJ, Martínez AT and Ruiz-Dueñas FJ, Two oxidation sites for low redox-potential substrates: A directed mutagenesis, kinetic and crystallographic study on *Pleurotus eryngii* versatile peroxidase. *J Biol Chem* **287**:41053–41067 (2012).
61. Ruiz-Dueñas FJ, Morales M, Rencoret J, Gutiérrez A, del Río JC, Martínez MJ and Martínez AT, Improved peroxidases. *Patent (Spain)* P200801292 (2008).
62. García-Ruiz E, González-Pérez D, Ruiz-Dueñas FJ, Martínez AT and Alcalde M, Directed evolution of a temperature-, peroxide- and alkaline pH-tolerant versatile peroxidase. *Biochem J* **441**:487–498 (2012).
63. Maté D, García-Ruiz E, Camarero S and Alcalde M, Directed evolution of fungal laccases. *Curr Genomics* **12**:113–122 (2011).
64. Maté D, García-Burgos C, García-Ruiz E, Ballesteros A, Camarero S and Alcalde M, Laboratory evolution of high redox potential laccases. *Chem Biol* **17**:1030–1041 (2010).
65. Camarero S, Pardo I, Cañas AI, Molina P, Record E, Martínez AT, Martínez MJ and Alcalde M, Engineering platforms for directed evolution of laccase from *Pycnoporus cinnabarinus*. *Appl Environ Microbiol* **78**:1370–1384 (2012).
66. Pardo I, Vicente AI, Alcalde M and Camarero S, Development of chimeric laccases by directed evolution. *Biotechnol Bioeng* **109**:2978–2986 (2012).
67. Pardo I, Chanagá X, Vicente AI, Alcalde M and Camarero S. New colorimetric screening assays for the directed evolution of fungal laccases to improve the conversion of plant biomass. *BMC Biotechnology* **13**:90 (2013).

Susana Camarero

Susana Camarero is Staff Scientist at CIB, Madrid where she currently leads the research on laccase engineering and natural redox mediators at the Biotechnology for Lignocellulosic Biomass group. She received her Master's degree in Biotechnology (1990) from the Faculty of Chemical Sciences and her PhD in Biology (1995) from the Department of Microbiology at the Complutense University of Madrid. She worked on lignin-degrading enzymes as postdoctoral fellow in the Center of Biological Research (CIB) and the Institute of Catalisis (ICP) from the Spanish Research Council (CSIC).



María Jesús Martínez

María Jesús Martínez is currently Senior Researcher and leads the Biotechnology for Lignocellulosic Biomass group together with Prof. A.T. Martínez. Her research is focused on biochemical and molecular characterization of fungal enzymes involved in lignocellulose degradation, and biotechnological applications of fungi and their enzymes for future sustainable development. She obtained her PhD in Biology and Biochemistry at the Complutense University of Madrid (1980). After different postdoctoral fellowships, she got a permanent position at CIB, CSIC (1986).

Angel T. Martínez

Angel T. Martínez is Research Professor and leads the group of Biotechnology for Lignocellulosic Biomass together with Dr M.J. Martínez. His main research area is biochemistry and molecular biology of lignin-degrading enzymes, lignin structure, and biotechnology for pulp and paper and lignocellulose biorefineries. He obtained his PhD in Microbial Ecology at the University of Navarre (1976). After postdoctoral stays at the CNRS (France) and CBS (the Netherlands) he got a position at CIB, CSIC (1981).

