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# Oxidoreductases on their way to industrial biotransformations

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## ABSTRACT

Fungi produce heme-containing peroxidases and peroxygenases, flavin-containing oxidases and dehydrogenases, and different copper-containing oxidoreductases involved in the biodegradation of lignin and other recalcitrant compounds. Heme peroxidases comprise the classical ligninolytic peroxidases and the new dye-decolorizing peroxidases, while heme peroxygenases belong to a still largely unexplored superfamily of heme-thiolate proteins. Nevertheless, basidiomycete unspecific peroxygenases have the highest biotechnological interest due to their ability to catalyze a variety of regio- and stereo-selective monooxygenation reactions with $H_2O_2$ as the source of oxygen and final electron acceptor. Flavo-oxidases are involved in both lignin and cellulose decay generating $H_2O_2$ that activates peroxidases and generates hydroxyl radical. The group of copper oxidoreductases also includes other $H_2O_2$ generating enzymes - copper-radical oxidases - together with classical laccases that are the oxidoreductases with the largest number of reported applications to date. However, the recently described lytic polysaccharide monooxygenases have attracted the highest attention among copper oxidoreductases, since they are capable of oxidatively breaking down crystalline cellulose, the disintegration of which is still a major

*Abbreviations:* AAD, aryl-alcohol dehydrogenase; AAO, aryl-alcohol oxidase; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); CDH, cellobiose dehydrogenase; CPK, Corey/Pauling/Koltun (atom coloring convention); CRO, copper-radical oxidase; DFF, 2,5-diformylfuran; DyP, dye-decolorizing peroxidase; FDCA, 2,5-furandicarboxylic acid; FFCA, 2,5-formylfurancarboxylic acid; GDH, glucose dehydrogenase; GMC, glucose-methanol-choline oxidase/dehydrogenase; GOX, glucose oxidase; HMF, 5-hydroxymethylfurfural; HSQC, heteronuclear single quantum correlation (NMR experiment); HTP, heme-thiolate peroxidase; LiP, lignin peroxidase; LPMO, lytic polysaccharide monooxygenase; LRET, long-range electron transfer; MCO, multicopper oxidase; MnP, manganese peroxidase; MOX, methanol oxidase; NMR, nuclear magnetic resonance; P2O, pyranose 2-oxidase; PELE, protein energy landscape exploration (software); QM/MM, mixed quantum mechanics/molecular mechanics; UPO, unspecific peroxygenase; VAO, vanillyl-alcohol oxidase; VP, versatile peroxidase

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bottleneck in lignocellulose biorefineries, along with lignin degradation. Interestingly, some flavin-containing dehydrogenases also play a key role in cellulose breakdown by directly/indirectly "fueling" electrons for polysaccharide monooxygenase activation. Many of the above oxidoreductases have been engineered, combining rational and computational design with directed evolution, to attain the selectivity, catalytic efficiency and stability properties required for their industrial utilization. Indeed, using *ad hoc* software and current computational capabilities, it is now possible to predict substrate access to the active site in biophysical simulations, and electron transfer efficiency in biochemical simulations, reducing in orders of magnitude the time of experimental work in oxidoreductase screening and engineering. What has been set out above is illustrated by a series of remarkable oxyfunctionalization and oxidation reactions developed in the frame of an intersectorial and multidisciplinary European RTD project. The optimized reactions include enzymatic synthesis of 1-naphthol, 25-hydroxyvitamin $D_3$, drug metabolites, furandicarboxylic acid, indigo and other dyes, and conductive polyaniline, terminal oxygenation of alkanes, biomass delignification and lignin oxidation, among others. These successful case stories demonstrate the unexploited potential of oxidoreductases in medium and large-scale biotransformations.

## 1. Fungal oxidoreductases

Oxidoreductases take advantage from the incorporation of different cofactors - such as heme, flavin and metal ions - to catalyze redox reactions. In these reactions, they use a variety of electron acceptors and a large number of electron-donating substrates yielding many products of industrial interest (Gygli and van Berkel, 2015). Fungi, in first place wood-rot basidiomycetes, are involved in the oxidative degradation of lignocellulosic biomass, recycling the carbon fixed by plant photosynthesis through a battery of secreted and robust high redox-potential oxidoreductases (Martínez et al., 2017). Fungal oxidoreductases of biotechnological interest typically include: i) heme-containing peroxidases and peroxygenases, being activated by $H_2O_2$ as sole electron acceptor; ii) flavin-containing oxidases and dehydrogenases, being activated by $O_2$ and other oxidants - such as $Fe^{3+}$ and quinones - respectively; and iii) copper-containing oxidases and monooxygenases, being activated by $O_2$, the latter with a more complicated activation mechanism.

Classical fungal oxidoreductases comprise basidiomycete ligninolytic peroxidases, and ascomycete and basidiomycete multicopper oxidases (MCO, mainly laccases) with different redox potentials and abilities to act on lignin-derived products. Moreover, new heme- and copper-containing oxidoreductases of high biotechnological interest have been recently discovered including: i) unspecific peroxygenases (UPOs) catalyzing a variety of regio- and stereo-selective oxyfunctionalizations with $H_2O_2$ acting as the oxygen source (peroxygenation reaction) and terminal electron acceptor; ii) other still unexplored peroxidases, such as the so-called dye-decolorizing peroxidases (DyPs); and iii) copper-containing lytic polysaccharide monooxygenases (LPMOs), which turned out to be the "missing" enzymes in the microbial attack of crystalline cellulose and other recalcitrant polysaccharides.

Enzymes of the glucose-methanol-choline oxidase/dehydrogenase (GMC) and copper-radical oxidase (CRO) superfamilies have been typically investigated as the source of $H_2O_2$ for: i) ligninolytic peroxidases in white-rot (*i.e.* lignin-degrading) basidiomycetes; or ii) hydroxyl radical generated *via* Fenton chemistry in brown-rot (*i.e.* cellulose-degrading) basidiomycetes. However, the preferential or optional use of other electron acceptors by some of them (dehydrogenase activity) has suggested additional functions, *e.g.* preventing lignin re-polymerization or "fueling" electrons to LPMOs. These and other fungal flavin-oxidases are also of emerging industrial relevance.

## 2. Oxidoreductases as industrial biocatalysts

The above oxidoreductases are biocatalysts of interest for establishing a bio-based economy (Fig. 1) with the highest potential in the production of polymer building blocks, sustainable chemicals and materials from plant biomass within lignocellulose biorefineries. However, the chemical industry, specially bulk chemicals' production, has not yet been embracing enzymatic oxidation reactions to a large extent. This is primarily due to lack of biocatalysts with the required selectivity, commercial availability and compatibility with the rigorous process conditions in terms of high substrate concentrations, use of solvents, and strongly oxidative conditions. Nowadays, oxidoreductases are most often employed in specific segments of the chemical industry and often in the form of whole-cell catalysts (*e.g.* P450 monooxygenases for selective hydroxylations) and not as isolated protein biocatalysts in medium and large scale biotransformations.

The main bottlenecks for implementing oxidative enzymatic biotransformations mentioned above have been addressed through protein engineering and process optimization using state-of-the-art technologies. The work performed comprised: i) recovery of selective oxidative biocatalysts, from the groups of heme-peroxidases and peroxygenases, flavo-oxidases and copper-oxidoreductases, from fungal genomes and other sources; ii) tailoring the catalytic and operational properties of the enzymes to fulfill the industry needs, by enzyme engineering based on structural-functional information, directed evolution or a combination of both, aided by computational simulations to reduce the experimental work; and iii) optimizing the process conditions including enzyme cascade reactions.

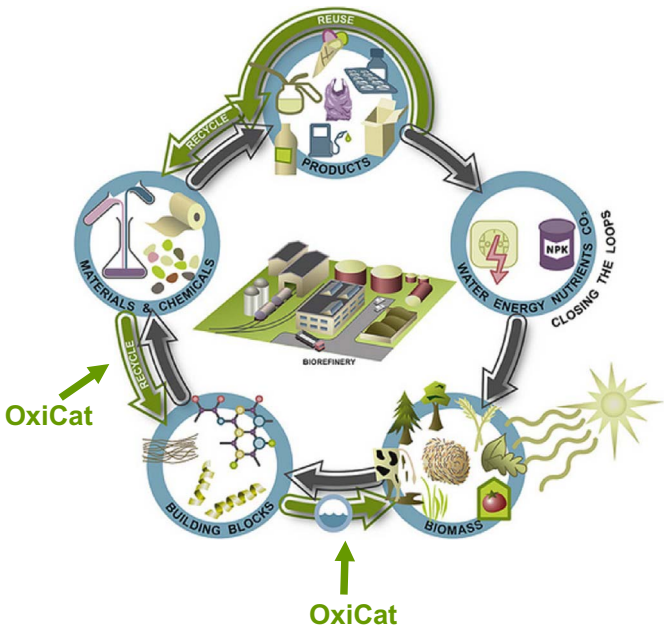


**Fig. 1.** Oxidative biocatalysts for a bio-based economy. Production of renewable building blocks and manufacture of sustainable chemicals and materials are the steps where oxidative biocatalysts (OxiCats) can exert the most positive impact for greener and more efficient biotransformation routes in a bio-based (and circular) economy.
Adapted from http://biconsortium.eu/news/bioeconomy-circular-nature.

During the next few pages, we will describe the search and engineering recently performed on the above fungal oxidoreductases to attain several industrial target reactions that include: i) intermediates for agrochemicals and active pharmaceutical ingredients, flavors and fragrances, epoxidation products and drug metabolites; ii) precursors for specialty polymers including 5-hydroxymethylfurfural (HMF) products such as 2,5-furandicarboxylic acid (FDCA), diols/hydroxy-acids/di-acids from alkanes/fatty acids, and functionalized plant polymers including oxidatively-modified cellulosic fibers and lignin; and iii) intermediates for dye-stuff production including phenolic and amine derivatives, indole and indole derivatives, and aniline polymers (Fig. S1). Their selection was based on proof of reaction at laboratory scale, industrial interest regarding the corresponding products and possibility for broadening the application field to similar substrate classes.

Accordingly, this review presents the main achievements attained in a recently concluded RTD project on "Optimized oxidoreductases for medium and large scale industrial biotransformations" (INDOX; http://indoxproject.eu) funded by the European Union. This project was an unprecedent coordinated effort for engineering oxidoreductases of different families and developing a repertoire of medium and large-scale oxidative biotransformations of industrial interest. It was preceded by other research efforts on oxidative enzymes funded by previous EU projects, and by several EU biotechnology companies that occupy a world-leading position in the sector of industrial enzymes. Screening of new biocatalysts in genomes was performed in collaboration with the Joint Genome Institute (JGI, Walnut Creek, CA, USA) of the US Department of Energy (DOE) that has already sequenced a large number of fungal genomes. The most representative results obtained in this European-level initiative (2013–2017 period) are summarized throughout the review while the new upcoming trends within this

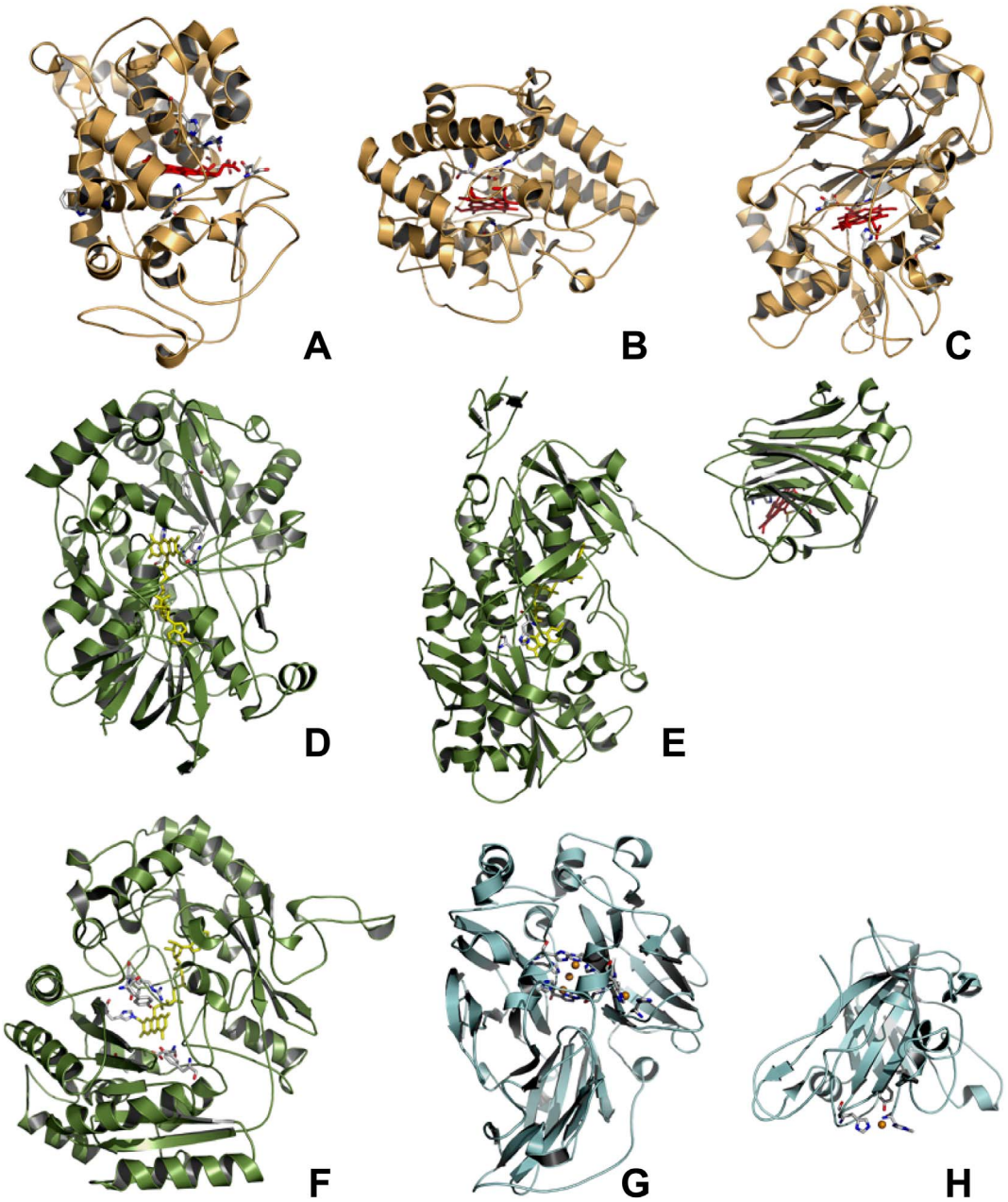


**Fig. 2.** 3D structures from representatives for classical and new oxidoreductase families involved in lignocellulose degradation and other biotransformations of interest: General crystal structures. A. *Pleurotus eryngii* VP (3FJW). B. *Agrocybe aegerita* UPO (2YP1). C. *Auricularia auricula-judae* DyP (4W7J). D. *Pleurotus eryngii* AAO (3FIM). E. *Neurospora crassa* CDH with flavin and heme (also called cytochrome) domains (4QI7). F. *Penicillium simplicissimum* VAO monomer (1VAO). G. *Pycnoporus cinnabarinus* laccase (2XYB). H. *Thermoascus aurantiacus* LPMO (2YET). Active site details are shown in Fig. 3.

fascinating research field are also commented.

For the sake of clarity, the review is structured in several sections. First, we introduce the main actors of the project (heme-containing peroxidases/peroxygenases, flavin-containing oxidases/dehydrogenases, and copper-containing oxidoreductases) paying special attention to the most novel enzyme systems represented by UPOs and LPMOs. Then, the significance of new computational tools applied in enzyme engineering is outscored and an ensemble of case studies combining both rational and directed evolution approaches is addressed, including both oxidations and oxyfunctionalizations. Finally, a large number of studies funded by the INDOX project are included in the list of references.

## 3. Heme-containing peroxidases/peroxygenases

Classical peroxidases (EC 1.11.1) and heme-containing peroxygenases (EC 1.11.2) are members of the peroxidase-catalase and heme-thiolate peroxidase (HTP) superfamilies, respectively. Although these enzymes share a heme cofactor (protoporphyrin IX), the phylogenetic connection between them is remote.

Ligninolytic peroxidases have been known for some forty years, and representatives of the three main types – lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and versatile peroxidase (VP, 1.11.1.16, Fig. 2A) – have been extensively studied due to their biotechnological potential for the chemical modification and degradation of lignin and other recalcitrant compounds (Martínez et al., 2017). All of them have a classical peroxidase cycle including two-electron oxidation of the $Fe^{3+}$-containing cofactor by $H_2O_2$, aided by distal histidine and arginine residues (Fig. 3A), forming an oxo-ferryl ($Fe^{4+}=O$) porphyrin cation radical complex, which successively oxidizes two substrate molecules *via* one-electron abstraction. They differ in the site where substrate oxidation takes place (Fig. 3A) including: i) a $Mn^{2+}$-binding site formed by three acidic residues near one of the heme propionates in MnP and VP yielding $Mn^{3+}$ acting as a diffusible oxidizer; and ii) a solvent exposed tryptophan abstracting electrons from lignin and transferring them to the heme *via* a long-range electron transfer (LRET) pathway, as recently shown using stopped-flow rapid spectrophotometry (Sáez-Jiménez et al., 2015c).

Distribution of the corresponding genes in white-rot and brown-rot fungal genomes sequenced at DOE Joint Genome Institute (JGI, http://genome.jgi.doe.gov/programs/fungi) provides strong evidence on their involvement in lignin degradation (Barrasa et al., 2016). Ligninolytic

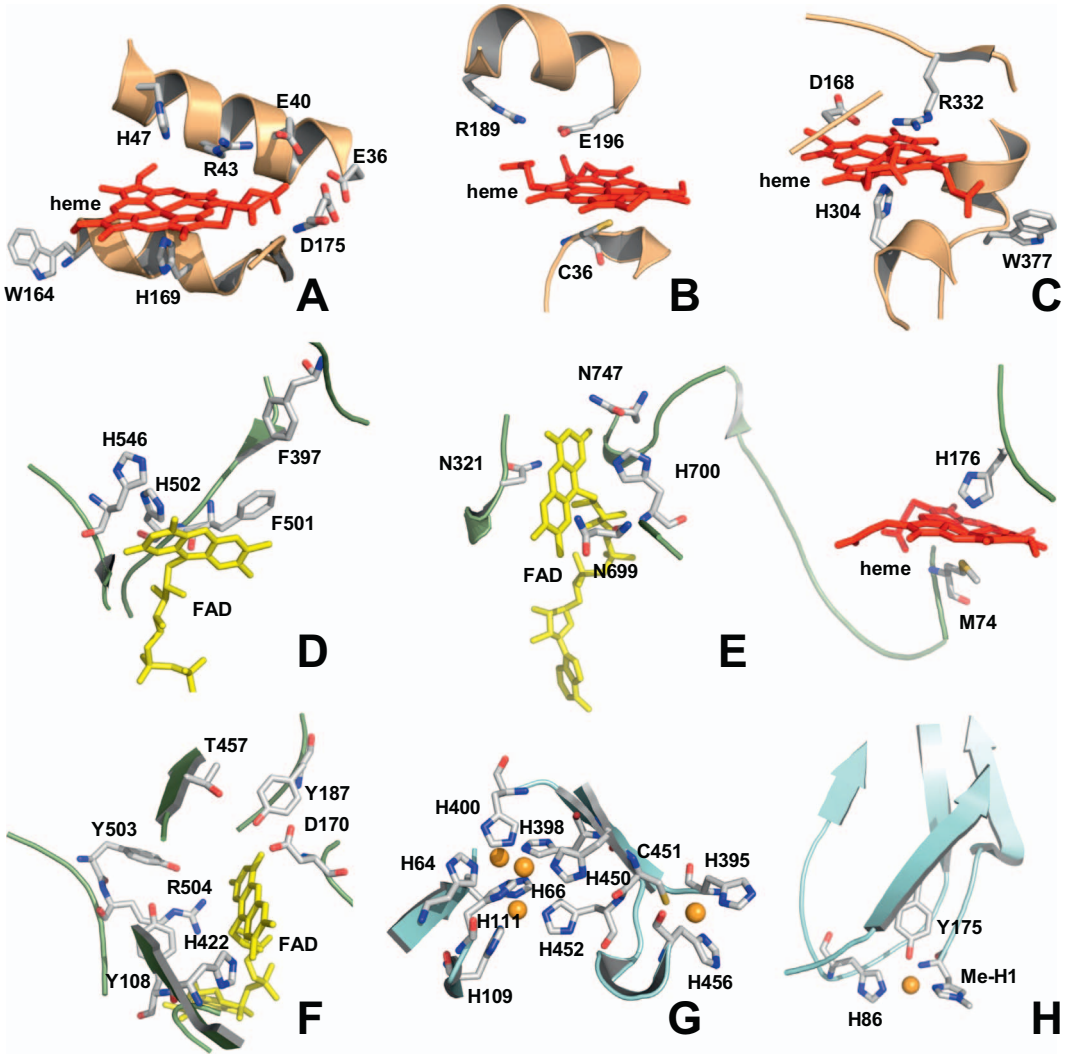


**Fig. 3.** 3D structures from representatives for classical and new oxidoreductase families involved in lignocellulose degradation and other biotransformations of interest: Active site architectures. A. VP. B. UPO. C. DyP. D. AAO. E. CDH flavin and heme domains. F. VAO monomer. G. Laccase. H. LPMO. Heme/FAD and copper ion cofactors (as red and yellow sticks and orange spheres, respectively) and residues relevant for catalysis (Corey/Pauling/Koltun, CPK, colored sticks) such as: i) His, Met/His and Cys ligands of heme iron in A/C, B and E, respectively; ii) His/Arg, Glu/Arg and Asp/Arg involved in activation by $H_2O_2$ in A, B and C, respectively; iii) 2 Glu and 1 Asp forming the Mn binding site in A; iv) catalytic Trp in A and C; v) active site residues in D-F, including His linked to FAD in F; vi) 4 copper ions in G; and vii) 2 His and 1 Tyr copper ligands in H. See Fig. 2 for the origin (species) of the different enzymes and the PDB references for their atomic coordinates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

peroxidase genes are present in all the white-rot (ligninolytic) basidiomycete genomes sequenced to date, but absent from all the brown-rot (cellulolytic) sequenced genomes, as well as from all the ascomycete (soft-rot) genomes. Additional information on these enzymes has been obtained in post-genomic studies where the complete inventory of peroxidase genes from some basidiomycete genomes has been heterologously expressed, and the different LiP, MnP and VP products, among others, were analyzed. In this way, the first demonstration of the VP ability to break down non-phenolic dimers and degrade lignin, playing in some Agaricales the same role that LiP plays in many Polyporales, was obtained (Fernández-Fueyo et al., 2014d). In a similar way, the contribution of C-terminal tail to the remarkable stability of long MnPs and their lack of Mn-independent activity on low redox-potential substrates has been shown (Fernández-Fueyo et al., 2014a).

In contrast to well-known ligninolytic peroxidases, the first basidiomycete peroxygenase (Fig. 2B), currently known as unspecific peroxygenase (UPO, EC 1.11.2.1), was reported only twelve years ago from *Agrocybe aegerita* (as an aromatic peroxygenase). Ascomycete chloroperoxidase (CPO, 1.11.1.10) that had been an "orphan enzyme" for decades, also belongs to the HTP superfamily, but it exhibits just moderate oxygenating activity towards organic substrates. Interestingly, HTP genes are well represented in most fungal genomes sequenced at JGI, as well as in additional genomes sequenced in the search for related HTP genes, including the genome of the CPO producer *Caldariomyces fumago* (= *Leptoxyphium fumago*) by Kellner et al. (2016). Despite this wide genomic presence, and the biotechnological relevance of mono(per)oxygenation reactions that has resulted in recent patents on UPO sequences (Landvick et al., 2016a, 2016b), just a few UPOs have been purified and characterized to date. Moreover, central aspects of UPO's catalytic mechanism have been only recently solved including the characteristics of the reactive compound I and the key role of protonated compound II (Wang et al., 2015).

Unlike ligninolytic (and other) peroxidases, UPOs use a glutamate as acid-base catalyst for activation by peroxide; and they share a proximal cysteine acting as ligand of heme iron (Fig. 3B) as well as reaction chemistry with cytochrome P450 monooxygenases (P450s). This results in highly versatile oxygenation and oxidation reactions, which can be classified as follows: a) two-electron oxidations with oxygen (*O*)-transfer; b) two-electron oxidations with *O*-transfer and subsequent bond cleavage; c) two-electron oxidations with *O*-transfer to heteroatoms (N or S); and d) one electron oxidations, as typical peroxidases do (Fig. 4A) (Hofrichter et al., 2015). The catalytic cycle of these enzymes differs from that of other peroxidases in the nearly simultaneous abstraction of the two electrons from the substrate, with the second one being associated to the transfer of the oxygen atom of the iron complex (monooxygenation activity). However, compared with P450s that need an electron-donating partner - flavin-containing protein or domain - and a source of reducing power, UPOs can be considered as "self-sufficient" monooxygenases only requiring a source of $H_2O_2$ to be activated (Fig. 4B). Not least, UPOs are secreted enzymes and, therefore, *per se* more stable than P450s or other monooxygenases, which are generally intracellular membrane-bound or cytosolic proteins. Unfortunately, UPOs exhibit some catalase activity along with oxidative instability against high amounts of $H_2O_2$, which must be taken into account for correct peroxide dosage (Karich et al., 2016).

Furthermore, the so-called dye-decolorizing peroxidases (DyPs, EC

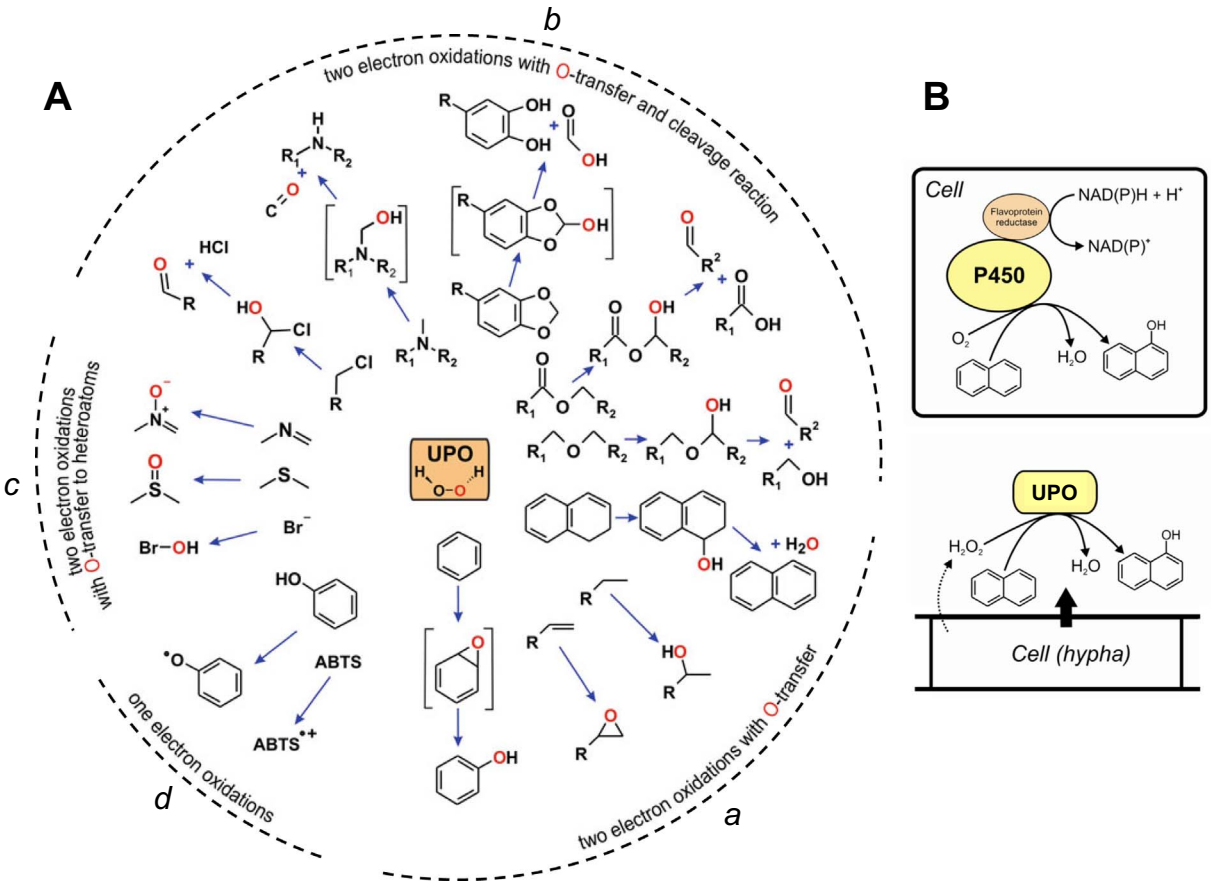


**Fig. 4.** Basidiomycete UPO catalyzing a variety of monooxygenation and other reactions with advantages over P450s. A. UPO oxidation and oxyfunctionalization at expenses of $H_2O_2$, include: a) two-electron oxidations with *O*-transfer; b) two-electron oxidations with *O*-transfer and cleavage reaction; c) two-electron oxidations with *O*-transfer to heteroatoms (S or N); and d) one-electron oxidations. Modified according to Hofrichter et al. (2015). B. While intracellular P450s (top) require a source of reducing power (NAD[*P*]H) and an auxiliary flavin-containing reductase or protein domain (and often waste a significant part of the reducing power in unproductive $H_2O_2$ formation), secreted UPO just needs a source of $H_2O_2$ to be activated (being also more robust due to its extracellular nature).

1.11.1.19) have been recently described and structurally-characterized in basidiomycetes (Strittmatter et al., 2015) (Fig. 2C). Their classification in the CDE superfamily - including chlorite dismutase, DyP and *Efe*B protein from *Escherichia coli* - reveals a phylogenetic origin different from classic peroxidases and HTPs (Linde et al., 2015b). In this case, the convergence with ligninolytic peroxidases not only includes a histidine residue as proximal heme iron ligand, and one arginine involved in activation by peroxide (as charge stabilizer) together with an aspartic acid that acts as acid-base catalyst (as distal histidine in classical peroxidases) (Fig. 3C), but also an LRET mechanism for oxidation of bulky lignin-derived and dye substrates. Although some surface tyrosines have been suggested as the beginning of LRET pathways in fungal DyP (Strittmatter et al., 2015), combination of computational, electron paramagnetic resonance and directed mutagenesis studies revealed that the main pathway may start at a radical-forming catalytic tryptophan (Fig. 3C), as previously described for LiP and VP (Baratto et al., 2015; Linde et al., 2015a). Interestingly, DyP is also able to oxidize and nitrate mononitrophenols (Büttner et al., 2015), as previously reported for some of the high-redox potential peroxidases mentioned above. At the same time, the first fungal DyP being able to oxidize $Mn^{2+}$ to $Mn^{3+}$, as MnP and VP do, was described (Fernández-Fueyo et al., 2015a) providing another example of evolutionary convergence between phylogenetically unrelated enzymes oxidizing recalcitrant structures including lignin.

In the search for new peroxidases/peroxygenases and other oxidoreductases of interest, the genomic inventories have been complemented by enzyme screenings in basidiomycete cultures, as well as by transcriptomic and secretomic studies using natural substrates under laboratory conditions. In the former studies, different enzyme secretion patterns were recognized in humus/wood Agaricales (Barrasa et al., 2014). This resulted in a genome sequencing project, including over 30 species of this basidiomycete order, for the analysis of enzymes associated to different lifestyles (JGI CSP-2015-1609). Transcriptomic studies using quantitative PCR have contributed to understand the duplication of oxidoreductase genes, since their differential regulation is produced in response to environmental conditions, and secretomic studies have confirmed that oxidoreductases - laccases, oxidases and peroxidases - are among the main proteins in lignocellulosic cultures of white-rot fungi (Fernández-Fueyo et al., 2014b, 2016a). Finally, combined transcriptomic and secretomic studies have shown the additional expression of genes putatively involved in extractives decay during basidiomycete growth on fresh pine for biological control of early wood decay (Hori et al., 2014).

## 4. Flavin-containing oxidases/dehydrogenases

The GMC oxidoreductase superfamily includes flavin-containing (i) oxidases, such as aryl-alcohol oxidase (AAO, EC 1.1.3.7), methanol oxidase (MOX, also known as alcohol oxidase, EC 1.1.3.13), pyranose 2-oxidase (P2O, EC 1.1.3.10) and glucose oxidase (GOX, 1.1.3.7), and (ii) dehydrogenases, such as cellobiose dehydrogenase (CDH, EC 1.1.99.18) that contains flavin and heme separate domains, and glucose dehydrogenase (GDH, 1.1.99.35). All of them abstract two electrons from alcohol substrates that the reduced flavin passes to $O_2$ forming $H_2O_2$ (oxidases) or to other oxidizing substrates (dehydrogenases). The distribution, phylogenetic relationships and potential role of GMC oxidoreductases in lignocellulose degradation has been recently reviewed based on the analysis of ten sequenced Polyporales genomes (Ferreira et al., 2015a) whose results are commented below.

Unlike ligninolytic peroxidases, a wide distribution of genes of $H_2O_2$-generating oxidases was observed among the different types of wood-rot fungi. Interestingly, AAO appears to be the most frequent GMC oxidase in the white-rot species, where $H_2O_2$ is required to activate ligninolytic peroxidases. By contrast, MOX genes are more abundant in the brown-rot species, where $H_2O_2$ is reduced by $Fe^{2+}$, yielding $Fe^{3+}$ and hydroxyl radical ($HO^{\bullet}$) involved in the incipient attack on cellulose by these fungi. AAO and P2O are secreted proteins involved in the extracellular degradation of lignocellulosic materials, compared to other oxidases located in the cytosol or peroxisomes. However, several pieces of evidence indicate that oxidases lacking a typical secretion mechanism, such as GOX and especially MOX, can be also involved in lignocellulose decay thanks to an alternative secretion process (*via* vesicles) or simply by hyphal lysis (Ferreira et al., 2015a).

AAO (Fig. 2D) can be considered as the model GMC oxidase in lignocellulose decay studies and biorefinery applications, such as biopulping and biobleaching, flavor biosynthesis, deracemization of chiral alcohols and oxidation of furfurals (Carro et al., 2016). The most recent studies on its catalytic mechanism are based on computational simulations (see Section 6) of substrate interactions with the active-site residues of the best known AAO from *Pleurotus eryngii*, which includes two histidine and several aromatic residues (Fig. 3D) (Ferreira et al., 2015b). Simultaneously, new enzymes of this family have been isolated from other fungi, such as *Ustilago maydis* showing dehydrogenase activity (Couturier et al., 2016) of interest for reduction of quinones and phenoxy radicals, as described below.

Among GMC dehydrogenases, CDH (Fig. 2E) is involved in cellulose decay by white-rot fungi, in agreement with gene distribution in

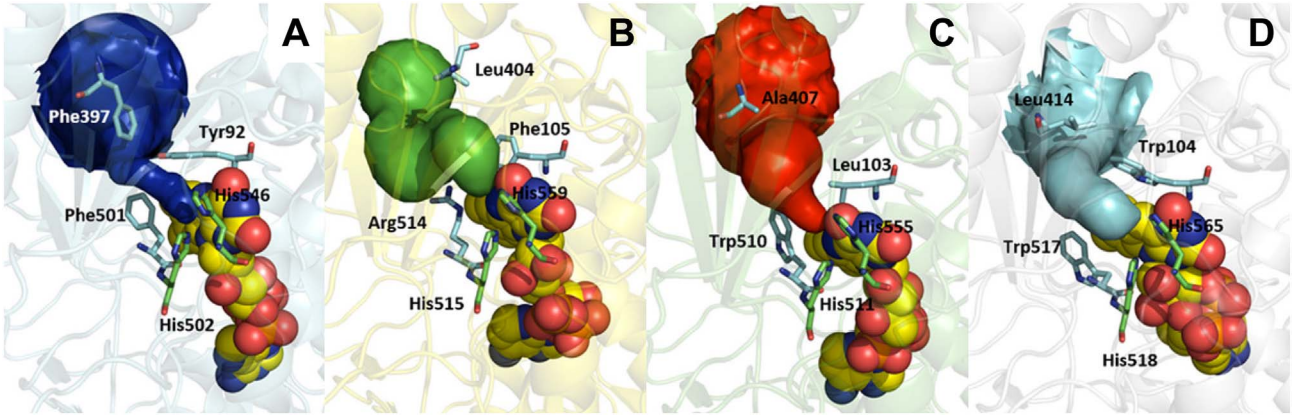


**Fig. 5.** Wider FAD-access channels in LPMO-activating AADs compared with AAO. The channels connecting the active-site cavity to solvent in *Pleurotus eryngii* AAO (PDB 3FIM) (A) and three AADs (AAQO1-AAQO3) from the *Pycnoporus cinnabarinus* genome and secretome (B-D, homology models) are shown. Channels were depicted by CAVER, with FAD as spheres and several active-site residues as sticks (CPK colored), including two catalytic histidines (green carbons) and other residues (cyan carbons) affecting the size and shape of the FAD access channel (see bottleneck in A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Adapted from Mathieu et al. (2016).

sequenced genomes (Kracher et al., 2016). The effect of CDH is related to its synergistic action with LPMO, which is described in the next section. Such reaction implies electron transfer from the flavin domain, where cellobiose is oxidized to cellobionolactone with contribution of catalytic histidine and other residues (Fig. 3E), to the heme domain in an intramolecular reaction that has been characterized from a structural and mechanistic point of view (Kracher et al., 2015; Tan et al., 2015). As it will be explained below, other dehydrogenases can also promote the action of LPMO by quinone redox cycling. These are the cases of the first basidiomycete GDH and the first aryl-alcohol dehydrogenase (AAD) reported to date, both identified in the genome of the white-rot fungus *Pycnoporus cinnabarinus* (Mathieu et al., 2016; Piumi et al., 2014). The structural basis for the quinone-reducing ability of these AADs seems related to a wider active-site channel. Such channel would enable the access of quinones and phenoxy radicals, compared with that of AAO that only permits the access of $O_2$ due to a narrow bottleneck formed by two phenylalanine and one tyrosine residues (compare Fig. 5B–D with Fig. 5A). The above quinone-reducing GMCs could also play a role preventing repolymerization of phenoxy radicals from the peroxidase or laccase degradation of lignin, which the above enzymes would reduce to the corresponding phenols.

Other microbial flavo-oxidases of biotechnological interest in organic synthesis (such as vanillin production) are vanillyl-alcohol oxidase (VAO, EC 1.1.3.38; Fig. 2F) and eugenol oxidase (EC 1.17.99.1), which belong to a different flavo-protein superfamily. With a different active site (see Fig. 3F compared with Fig. 3D), VAO and eugenol oxidase have ascomycete and bacterial origins, respectively, and differ in oligomerization degree due to a single loop identified in the dimer interface (Ewing et al., 2016).

## 5. Copper-containing oxidoreductases

Different copper-containing oxidoreductases have been related to lignocellulose degradation, including: i) CRO; ii) MCO (laccases); and iii) LPMOs. The CRO family is characterized by the presence of a metaloradical complex, involved in the redox catalysis, including a copper ion coordinated to the phenolic side chain of a tyrosylcysteine adduct formed by post-translational modification. The family includes glyoxal oxidase (EC 1.2.3.15), the first $H_2O_2$-producing enzyme described in the model white-rot fungus *Phanerochaete chrysosporium*, and galactose oxidase (EC 1.1.3.9) that, in addition to oxidizing monosaccharides and terminal galactose in polymers, has activity on benzylic alcohols enabling HMF conversion (see Section 4) into 2,5-diformylfuran (DFF), a valuable chemical (Kalum et al., 2014b). CROs are present in most

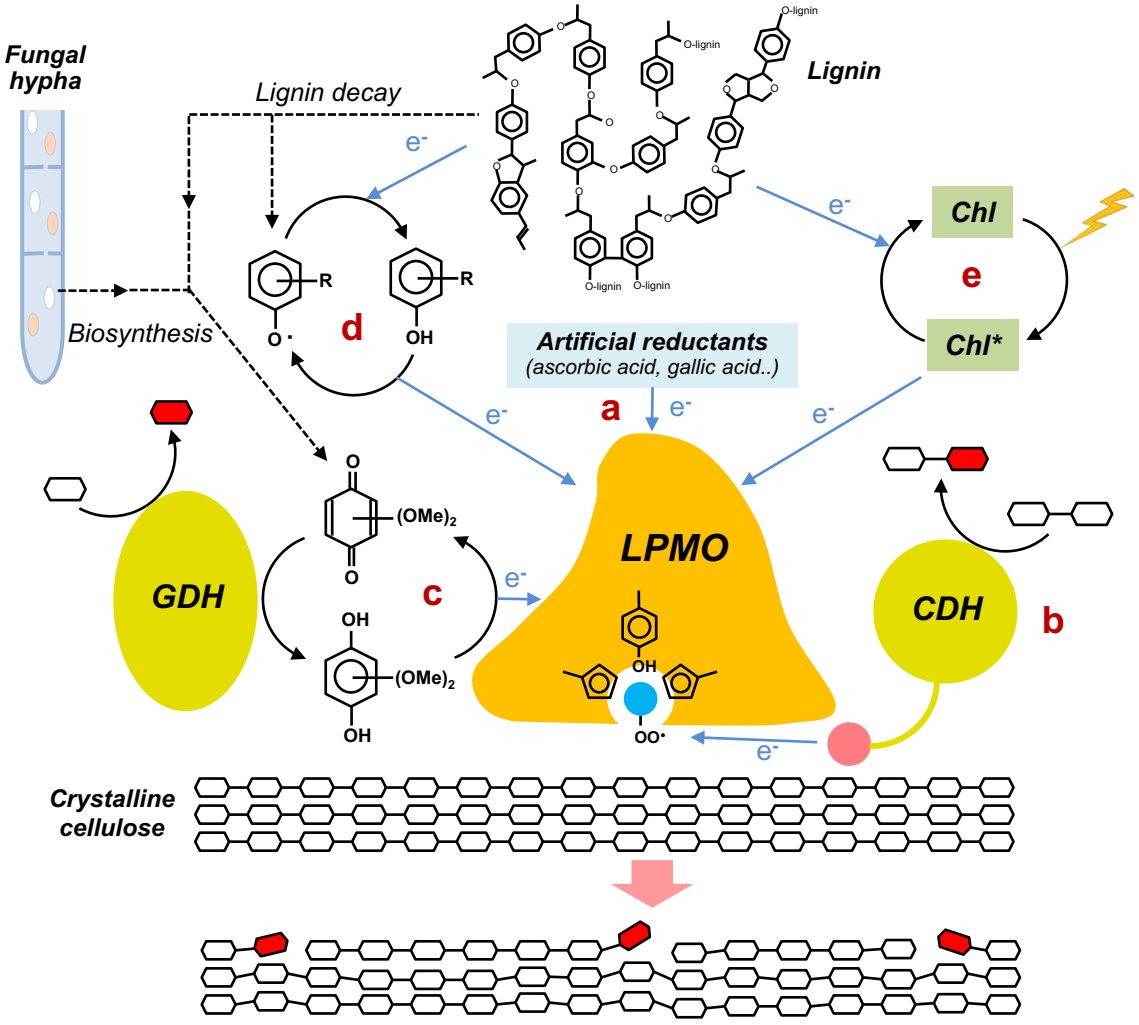


**Fig. 6.** Different LPMO activation routes. After initial studies, where LPMO activity was detected using artificial reductants (*route-a*), several mechanisms have been shown to operate fueling electrons to the LPMO copper cofactor for the oxidative breakdown (red units) of crystalline cellulose. These alternative mechanisms involve other enzymes, such as CDH being directly oxidized by LPMO (*route-b*) and GDH acting through redox cycling of quinones derived from lignin decay or fungal metabolism (*route-c*), as well as lignin-derived phenoxy radicals being reduced by lignin (*route-d*) and light-activated photosynthetic pigments (Chl, *route-e*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Adapted from Kracher et al. (2016) and Martínez (2016).

basidiomycete genomes, and some of them showed new catalytic properties of interest after their heterologous expression (Daou et al., 2016). Among MCOs, laccases (EC 1.10.3.2, Fig. 2G) are the best known copper oxidases, being characterized by the presence of four copper ions with their histidine and cysteine ligands (Fig. 3G). Their catalytic cycle combines four one-electron abstractions from phenolic and other substrates with reduction of one $O_2$ molecule to water. Opposite to peroxidases, laccase genes are present in genomes of both white-rot and brown-rot fungi sequenced at JGI. Together with peroxidases, laccases are the most thoroughly studied oxidoreductases in wood-rot fungi and, by far, the largest number of biotechnological applications have been reported for these MCOs (Maté and Alcalde, 2016b; Pezzella et al., 2015). There is a high number of well characterized, often commercially available, laccases with different redox potentials, although these are always lower than those of ligninolytic peroxidases. Therefore, recent work on these enzymes has focused on tailoring the catalytic properties for target reactions by rational design and directed evolution, often guided by computational simulations, as described in the next sections.

In contrast to the above copper-containing enzymes that have been known for many years, the first fungal laccase was reported in the early 1960's, LPMOs (Fig. 2H) have been recognized as a new oxidoreductase family playing a crucial role in cellulose degradation only seven years ago (Harris et al., 2010; Vaaje-Kolstad et al., 2010). More recently, LPMOs with different activities have been reported from several fungi (Isaksen et al., 2014; Patel et al., 2016). Paradoxically, the first sequences of this new oxidoreductase family, from genomes and other sources, were stored for years in databases (such as CAZY, http://www.cazy.org) as corresponding to glycoside hydrolase family GH61. This striking confusion originated from: i) the presence of carbohydrate binding domains and other sequence similarities in some of them; ii) weak hydrolase activity of LPMOs or their contaminating proteins; and iii) requirement of a reducing substrate for LPMO activation, which is not included in glycoside hydrolase reaction mixtures. LPMO activity requires reduction of a catalytic cupric ion, which has a methylated terminal histidine as ligand together with a second histidine and a tyrosine (Fig. 3H). This activation, initially obtained with artificial reductants such as ascorbic acid, reduces $Cu^{2+}$ to $Cu^{+}$, which reacts with $O_2$ forming a reactive copper-superoxide complex. The resulting monooxygenase activity causes the oxidative breakdown of crystalline polysaccharide chains (lytic oxygenase activity). The search for the natural LPMO reductants described below is a hot topic in lignocellulose degradation with an enormous implication for lignocellulose biorefineries.

As summarized in Fig. 6, different mechanisms can operate fueling electrons for LPMO activation in lignocellulose-decaying fungi. Among them, CDH is able to transfer electrons from cellulose products to LPMO using its heme domain, after intramolecular electron transfer from the flavin domain where the reaction with cellobiose takes place (Kracher et al., 2015; Loose et al., 2016; Tan et al., 2015). Other GMCs provide alternative activation routes, as shown for GDH (Garajova et al., 2016) that can redox-cycle quinones from lignin degradation or fungal metabolism for a continuous supply of easily oxidizable hydroquinones to LPMO (Kracher et al., 2016). Other LPMO activating routes may involve photosynthetic pigments and lignin-derived phenols whose phenoxy radicals from LPMO oxidation would be reduced back by some lignin fractions or domains (Martínez, 2016).

Due to the relatively recent reports on LPMO structure and activity, several important aspects of its reaction mechanism have not been fully understood yet: from the interaction with substrates to the reactive oxygenation species generated at the active site. Concerning the first aspect, recent findings from 2D nuclear magnetic resonance (NMR) spectroscopy suggest that reductants and cellulose bind the same region of the LPMO molecule. This is shown by displacement of NMR signals of the same amino-acid residues when LPMO interacts with CDH and with a cellulose oligosaccharide (Fig. S2), and contrasts with the alternative

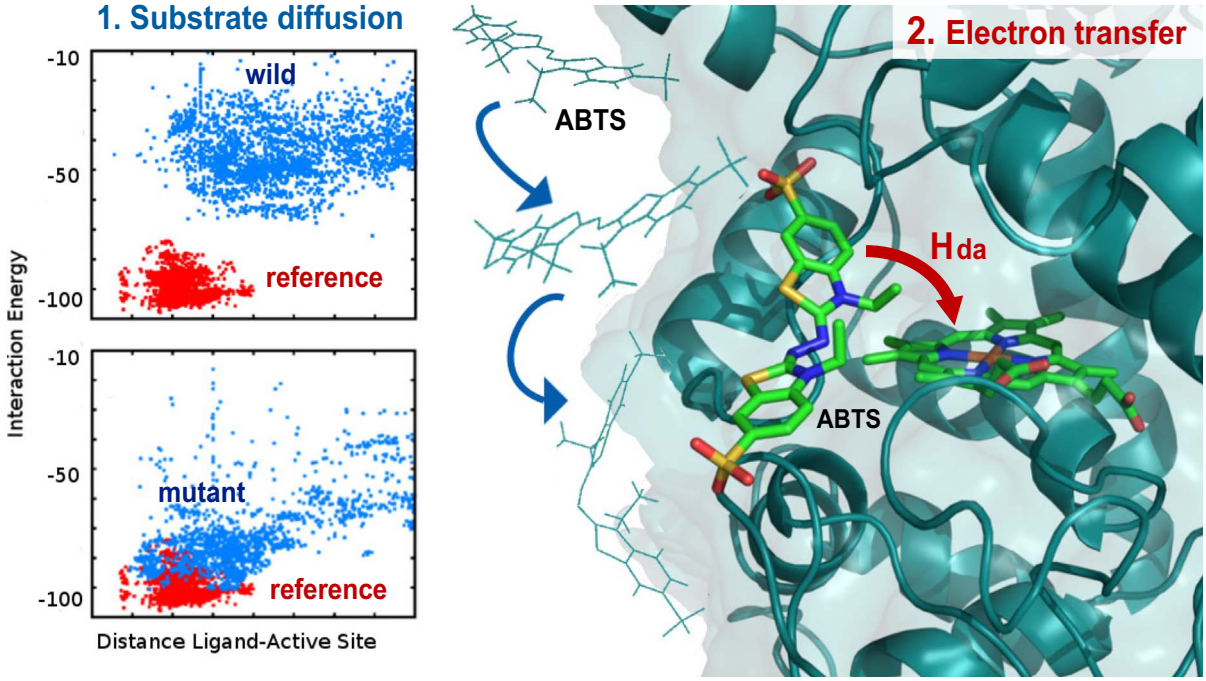


**Fig. 7.** Simulations guiding introduction of a new peroxidase activity. A highly-stable peroxidase was engineered for oxidation of a new substrate (ABTS) using PELE simulations in wild and mutated enzyme (*left*) and electron transfer estimation (Hda) by quantum calculations after docking at the active site (*right*). The substrate binding residues to be introduced were identified by substrate diffusion on an active enzyme (reference), and the new activity was confirmed by PCR mutagenesis, heterologous expression and estimation of kinetic constants. ABTS-heme distances *vs* interaction energies in the reference enzyme (red dots) and the wild and mutated target enzyme (blue dots) during PELE simulations are shown in left. Detail of ribbon-type structure with docked ABTS and heme as CPK-colored sticks (other ABTS molecules during PELE diffusion as blue lines) and solvent access surface in grayish blue are shown in right. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Adapted from Acebes et al. (2016).

hypothesis that suggested the existence of a LRET pathway for enzyme activation ($Cu^{2+}$ reduction) starting at a different region of the protein surface (Courtade et al., 2016).

## 6. Biophysical and biochemical computational modeling

Oxidoreductase engineering has benefited from computational simulations, where the target reaction to be achieved, or the enzyme property to be improved, was explored with *in silico* biophysical and biochemical tools. In this way, the time dedicated to experimental engineering work can be significantly reduced.

Biophysical modeling typically included dynamic simulations of substrate diffusion to the enzyme active site, optimized docking and, if it can represent a limiting step, analysis of product diffusion to the solvent region. To this aim, the Protein Energy Landscape Exploration (PELE) program, an state-of-the-art software for modeling substrate and product migration in enzymes that is capable of accurately mapping long time scale processes in only few hours of CPU (https://pele.bsc.es), has been used.

The PELE algorithm is built on top of a Monte Carlo procedure, combining a substrate steered stochastic approach with techniques for protein structure prediction. The overall algorithm, designed to study ligand diffusion and binding, is composed by three main steps: i) an initial ligand and protein perturbation step, based on a translation and rotation of the ligand, and protein backbone displacement following an anisotropic network model approach; ii) a side-chain sampling step, using experimental rotamer libraries to place all side chains local to the ligand; and iii) an energy minimization step, including all residues involved in the previous steps. A typical PELE run involves on the order of tens to hundred processors running multiple steps and optionally sharing information towards a common goal.

As shown in different studies described in the next sections, PELE simulations properly inform about substrate access and positioning at the enzyme active site including distances and angles between the redox centers, as shown for different UPO reactions (Lucas et al., 2016; Molina-Espeja et al., 2016a). Furthermore, a computational methodology accumulating beneficial interactions between a laccase and a target substrate has proved to be useful, through a repurposing strategy, to design a novel polar binding scaffold to anchor negatively charged groups (Giacobelli et al., 2017).

Following the biophysical analyses by PELE simulation of substrate diffusion, it is possible to perform the quantum biochemical characterization of the electronic coupling involved in the oxidation process. Interestingly, in a model study with a basidiomycete peroxidase, the predicted electron transfer values showed a striking correlation with the turnover numbers determined experimentally, validating the mixed biophysical/biochemical computational approach (Fig. 7) (Acebes et al., 2016). Quantum calculations have been also used to estimate stacking interaction energies of different alcohol substrates at the active site of AAO. In this way, it is possible to successfully predict changes in the enzyme reaction mechanism from ping-pong to ternary complex interactions with reducing and oxidizing substrates (Ferreira et al., 2015b).

In other cases, *e.g.* when the substrate can adopt different oxidation poses at the active site of the native enzyme or for *in silico* mutagenesis, mixed quantum mechanics/molecular mechanics (QM/MM) calculations can be performed to predict the best active site mutations from the estimated average spin density on the substrate molecule. In this way, a binding focused general strategy based on QM/MM reactivity scoring has been proposed for laccase engineering (Monza et al., 2015). This methodology has been applied for engineering laccase for aniline oxidation, as described below, with the calculations being validated by the experimentally-determined kinetic constants of the best predicted variant. Indeed, a flawless correlation between the turnover ($k_{cat}$) improvement in the engineered enzyme and the estimated increment of substrate's spin density was observed (Santiago et al., 2016).

A similar approach has been used to rationalize the improvements observed experimentally in: i) laccase oxidation of bioactive phenols, such as sinapic acid and methyl sinapate, after combinatorial saturation mutagenesis at the substrate binding site (Pardo et al., 2016); and ii) stereoselective sulfoxidation after directed mutagenesis at the DyP active site (Linde et al., 2016), as described in the next section. In an interesting application, mixed QM/MM calculations can be also used to identify the LRET pathways that characterize peroxidase oxidation of bulky substrates. The “e-pathway” approach - where relevant residues are successively included in the QM region, while the rest of the protein is in the MM region - has been used with this purpose, as shown for anthraquinoid dye oxidation by DyP (Linde et al., 2015a) and very recently for non-phenolic aromatic substrate oxidation by VP and LiP (Acebes et al., 2017). In the latter study, the main predicted pathway, which includes several conserved aromatic residues and represents a central aspect in peroxidase action on lignin, has been confirmed by directed mutagenesis.

## 7. Engineering oxidative enzymes

Engineering oxidoreductases for industrial application considers both rational design and directed molecular evolution, as well as combinations of both (semi-rational approaches) on the whole protein or focused on target regions/domains, with a variety of recent examples (Maté et al., 2016; Molina-Espeja et al., 2016b; Pardo and Camarero, 2015b; Viña-González et al., 2016). New library creation methods have been developed with this purpose, such as MORPHING (mutagenic organized recombination process by homologous *in vivo* grouping) (González-Pérez et al., 2014b). A prerequisite for all protein engineering methodologies is the availability of a suitable expression system to generate improved variants. Therefore, the heterologous expression of oxidoreductase genes in *Saccharomyces cerevisiae*, *Escherichia coli* or other systems was optimized for UPO (Alcalde et al., 2014; Molina-Espeja et al., 2014), DyP (Linde et al., 2014), AAO (Viña-González et al., 2015), ligninolytic peroxidases (García-Ruiz et al., 2014) and VAO (Gygli and van Berkel, 2017) enabling their subsequent engineering. In several cases, significant increases in the oxidoreductase gene expression were obtained after several rounds of directed evolution, which were followed by additional rounds to improve the target catalytic properties.

In rational engineering studies, VP has been used as a model peroxidase, and both oxidative and alkaline inactivation have been investigated to obtain better variants for industrial application. Two different strategies have been successfully combined to improve the VP oxidative stability against $H_2O_2$: i) substitution of easily oxidizable residues, such as methionines located between the cofactor and the surface catalytic tryptophan; and ii) mutation of distal heme pocket residues for reducing the efficiency of peroxidase reaction with $H_2O_2$ forming compound I, whose reaction with $H_2O_2$ excess inactivates the enzyme (Sáez-Jiménez et al., 2015a). In this way, inactivation *via* non-catalytic compound III and oxygen radical formation at the heme pocket can be limited. While improvement of oxidative stability was based on our knowledge on VP structure-function relationships, a different strategy was successfully applied for rational improvement of alkaline stability. This was based on: i) selection of a naturally-stable peroxidase (in this case a MnP) by genome screening and heterologous expression; ii) identification of the structural determinants for this stability, such as H-bonding patterns, salt bridges and basic residues exposed to the solvent in the crystal structure; and iii) introducing them into the target enzyme (in this case a VP) by directed mutagenesis (Sáez-Jiménez et al., 2015d). Rational design has been also used to create a peroxidase (VP) with strong ligninolytic activity based on the ability to act at extremely acidic conditions (< pH 3) that increases the redox potential of the heme iron. With this purpose, the catalytic tryptophan characterizing VP and LiP was introduced in another peroxidase scaffold from genome screening (also corresponding to a MnP)

that was stable under these acidic conditions (Fernández-Fueyo et al., 2014c). In another example of rational design, the active site of DyP has been broadened resulting in stereoselective sulfoxidation reactions (Linde et al., 2016). This selective activity is due to efficient substrate docking near the reactive oxygen of enzyme compound I, as shown by computational modeling using the crystal structure of the engineered variant.

Paralleling the rational design described above, directed evolution and hybrid strategies have been applied to improve the $H_2O_2$ and alkaline stability of VP. In the first case, the best variant accumulated eight mutations that increased the VP half-life from 3 (parental type) to 35 min in the presence of 3000 equivalents of $H_2O_2$ (González-Pérez et al., 2014a). More recently, a VP variant was obtained that oxidizes substrates at alkaline pH both at the heme channel and at the $Mn^{2+}$ site, while the catalytic tryptophan was not operational under these conditions (González-Pérez et al., 2016). Moreover, UPO was submitted to directed evolution to enhance its mono(per)oxygenase activity, and reduce competing one-electron oxidation, *i.e.* classic peroxidative activity. The evolved variants were used in oxyfunctionalizations of biotechnological interest, such as the naphthalene oxygenation to 1-naphthol described below. Rational and evolutionary design has been applied in the last past decades to laccase engineering. However, directed evolution of the whole gene and focused evolution by mutagenesis or recombination of specific residues/regions, have better improved the laccase features for specific targets, such as the oxidation of phenolic substrates of biotechnological interest (Maté and Alcalde, 2016a; Pardo et al., 2016; Pardo and Camarero, 2015b; Vicente et al., 2016).

For the applications described below, engineered enzymes were finally expressed in a *Saccharomyces cerevisiae-Pichia pastoris* tandem system (Molina-Espeja et al., 2015) or in the industrial expression host *Aspergillus oryzae* (Vind et al., 2015). *Escherichia coli* expression followed by *in vitro* activation was used for some laboratory-scale reactions. The industrial applicability of oxidoreductases could be often improved by enzyme immobilization, as shown for juice clarification and production of drug metabolites (Lettera et al., 2016; Poraj-Kobielska et al., 2015b).

## 8. Enzymatic oxyfunctionalization

Basidiomycete UPOs appear as highly promising biocatalysts for a variety of aromatic and aliphatic oxyfunctionalization reactions of industrial interest (Bormann et al., 2015; Hofrichter and Ullrich, 2014). Some selective oxygenations have also been developed with engineered variants of other peroxidases, such as the stereoselective DyP sulfoxidation mentioned above (Linde et al., 2016). Several relevant UPO reactions with interesting biotechnological background are described below.

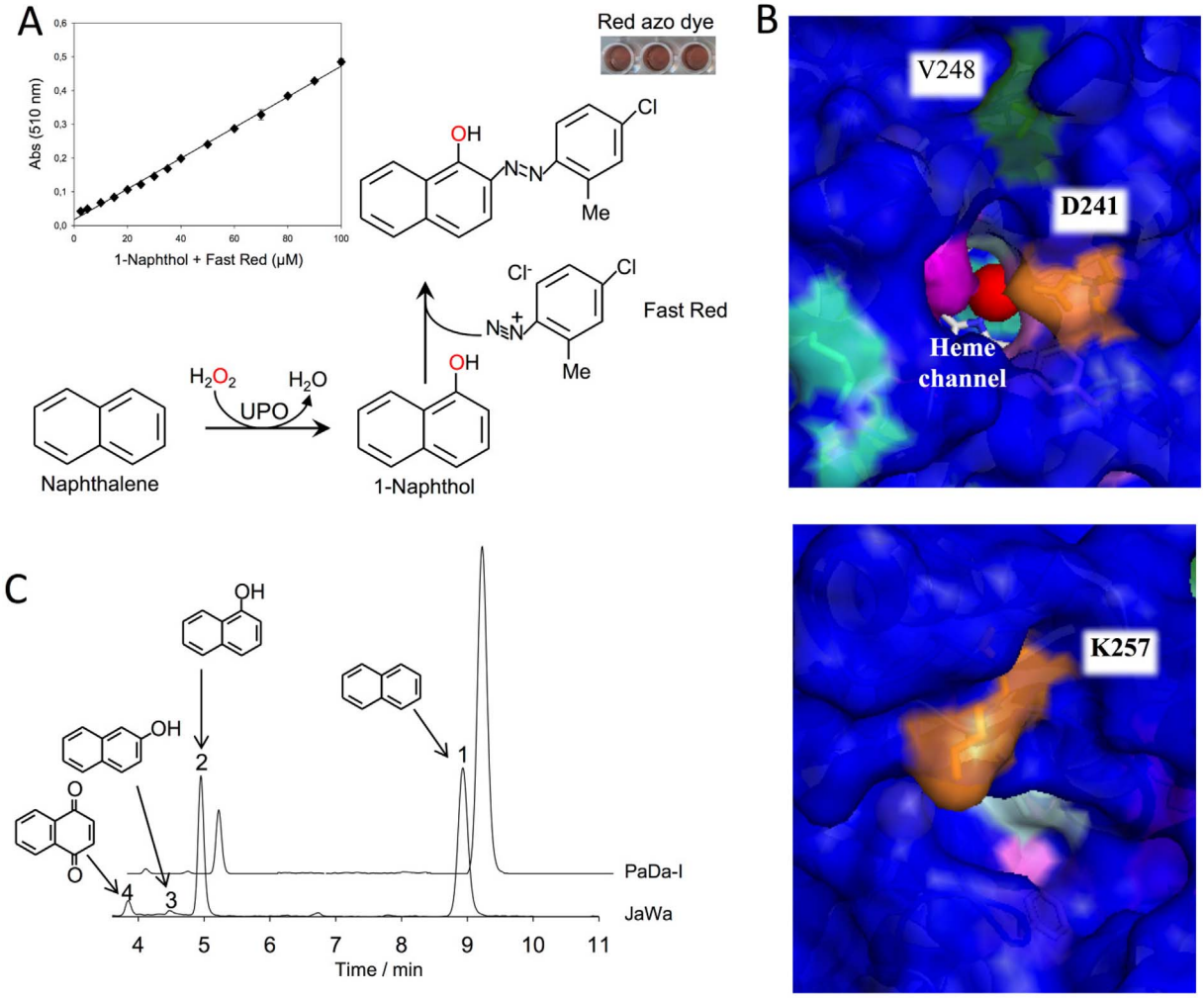


**Fig. 8.** Directed evolution and enzymatic production of 1-naphthol. A. Fast Red-based screening resulting in several UPO variants hydroxylating naphthalene. B. Mutations in the best UPO variant (JaWa) located at the heme channel (top) and the surface (bottom) of the UPO crystal structure. C. HPLC profile of 1-naphthol production by JaWa compared with a second variant (PaDa-I).
Adapted from Molina-Espeja et al. (2016a).

1-Naphthol - a 40,000 tons/year market chemical used in the production of herbicides, insecticides, pharmaceuticals and dye precursors - can be obtained by naphthalene hydroxylation (actually an epoxidation followed by spontaneous re-aromatization) using a UPO variant evolved for this purpose. The variant, obtained through an *ad hoc* screening assay using Fast Red for enhanced detection of α-naphthol (Fig. 8A), the formation of which was confirmed by HPLC (Fig. 8B), only included two mutations located at the heme channel and at the enzyme surface (Fig. 8C) (Maté and Alcalde, 2015; Molina-Espeja et al., 2016a; Pardo and Camarero, 2015a). Epoxidation of non-cyclic alkenes and terpenes of interest for the chemical sector has also been achieved using different wild-type and recombinant UPOs (Lund et al., 2016).

25-Hydroxyvitamin $D_3$ - of interest in feeding chickens and other farm animals to reduce skeletal problems caused by rapid growth and reduced mobility - can be optimally produced using the recombinant *Coprinopsis cinerea* UPO from Novozymes (Fig. 9A and B). This is due to the *ad hoc* active-site architecture for vitamin $D_3$ regioselective hydroxylation, as shown by computational simulations (Fig. 9C) (Babot et al., 2015b; Lucas et al., 2016). Related hydroxylations are those catalyzed by different basidiomycete UPOs on a variety of steroidal substrates, mainly at their 25 position, yielding products with antimicrobial properties, among others (Babot et al., 2015a).

Human drug metabolites are required for pharmacological studies prior to the authorization of new pharmaceuticals. *N*-demethylation in the synthesis of drug metabolites of the bile acid reabsorption inhibitor SAR548304 (Sanofi) requires a seven-step chemical reaction using palladium catalysis and laborious chromatographic purification with an overall yield of only 27%. However, the same reaction can be accomplished in a one-pot system with a yield of 60% using the *Marasmius rotula* UPO (Fig. S3) (Kiebist et al., 2015). Also of pharmaceutical interest is the use of the same UPO for the enzymatic removal of corticoid side-chains, involving hydroxylation and C—C bond cleavage (Poraj-Kobielska et al., 2015a).

Oxyfunctionalization of saturated hydrocarbons under mild conditions is a major challenge of modern chemistry. Among the thousands of reagents for organic synthesis, few have been developed that are capable of carrying out selective oxidation of alkanes. In this context, enzymatic oxygenation of long-chain *n*-alkanes at their unreactive terminal position has been very recently reported using the *Marasmius rotula* UPO (Olmedo et al., 2016). Indeed, this enzyme catalyzes a cascade of mono- and di-terminal oxidations from long-chain *n*-alkanes yielding dicarboxylic acids and other oxygenated products from these largely inert compound family (the proposed reaction pathway is shown in Fig. S4). Note that all other basidiomycete peroxygenases and wild-type P450s mostly produce subterminal diols, and ketones, from alkane oxygenation (Lund et al., 2014).

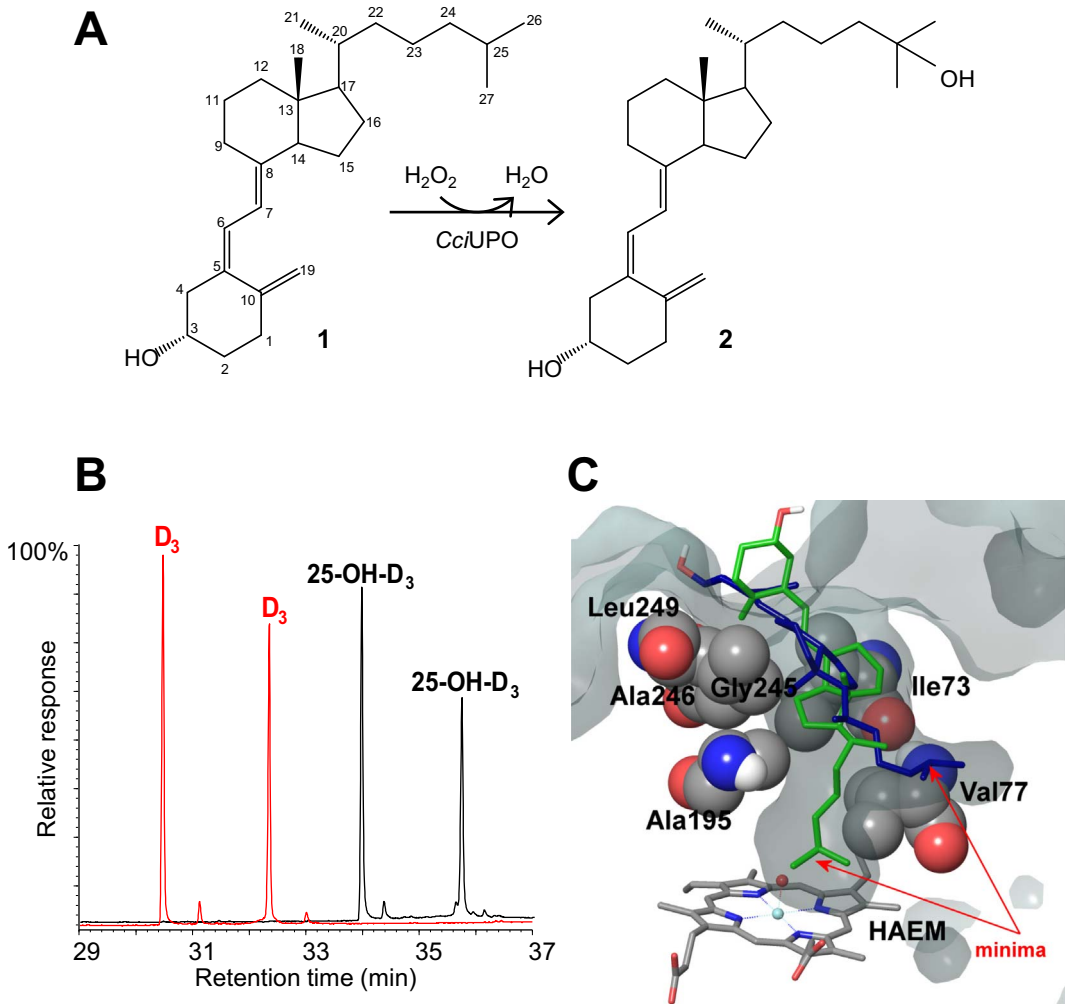


**Fig. 9.** Regioselective hydroxylation by UPO. Regioselective conversion of vitamin $D_3$ (1) into 25-hydroxyvitamin $D_3$ (2) by *Coprinopsis cinerea* UPO (A) was shown by GC–MS profiles of reactions (B, black line) compared with controls (B, red line), and explained by PELE simulations revealing an optimal minimum for oxygen transfer from heme compound I to substrate $C_{25}$ after its diffusion at the heme access channel (C). Double peaks in B are pyro-isomers formed during GC–MS analysis. Substrate and cofactor in C are shown as CPK-colored sticks, while relevant residues are shown as CPK spheres (part of the solvent access surface is also shown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Adapted from Lucas et al. (2016).

Stereoselective hydroxylation of hydrocarbons is also a challenging task for chemical catalysts. P450s have been considered for decades as possible catalysts but the disappointingly low turnover numbers obtained so far seem to limit their application to high-value added products. The *Agrocybe aegerita* UPO, and other basidiomycete peroxygenases, may be a relevant alternative, although their efficient use requires careful provision with $H_2O_2$. For this type of reactions, an enzymatic cascade has been investigated, including two (Fig. 10A, *top*), three (Fig. 10A, *middle*) or five enzymes (Fig. 10A, *bottom*) for the waste-free, complete and stereoselective conversion of ethylbenzene into (*R*)-1-phenylethanol as a model reaction (Fig. 10B). The final cascade resulted in full oxidation of methanol to $CO_2$ and thereby utilizes all reducing equivalents stored in methanol productively, yielding three $H_2O_2$ molecules and enabling complete conversion of ethylbenzene. It is worth mentioning that the turnover numbers obtained, despite the rather early stage of development of the above reaction, already are in the range of 500,000 thereby becoming interesting for preparative application (Hollmann and Ni, 2016; Ni et al., 2016). Similar strategies can be applied to other oxyfunctionalization reactions described above. Many dehydrogenase reactions, as the last step of the above cascade (Fig. 10A), require *in situ* regeneration of the oxidized cofactor (NAD[P]$^+$). To accomplish this reaction, $O_2$ is the ideal oxidant, providing sufficient thermodynamic driving force to shift the reaction equilibrium and yielding only water as waste-product. For similar reactions, a laccase-mediator system has been set-up and characterized for NAD(P)H$^+$ regeneration with favorable characteristics for at-scale applications (Pham et al., 2015).

## 9. Enzymatic oxidations

A series of oxidation reactions of biotechnological interest were found to be catalyzed by different oxidoreductases for the production of polymer building blocks, dyeing molecules, and other compounds of interest. Some exemplary reactions are described below.

According to the US Department of Energy, HMF is one of the top-ten renewable compounds for a sustainable bioeconomy, as the precursor of FDCA and other platform chemicals, such as DFF. HMF-derived FDCA is the bio-based alternative to fossil-based terephthalic acid for the production of polyester-type plastics. Its synthesis from HMF involves three consecutive oxidations to obtain DFF, 2,5-formylfurancarboxylic acid (FFCA) and finally FDCA. HMF is an aromatic (benzylic) alcohol and, therefore, a substrate of AAO, and this oxidase also acts on hydrated aldehydes (*gem*-diols), such as hydrated DFF, as shown in HMF reactions (Carro et al., 2015). Therefore, an enzyme cascade has been developed where the limiting step in HMF oxidation by AAO, *i.e.* the FFCA oxidation to FDCA, is catalyzed by UPO at expenses of the $H_2O_2$ generated by AAO in the two initial oxidation steps with a yield over 90% (Fig. S5). Furthermore, a galactose oxidase variant has been claimed for the production of DFF, since it selectively catalyzes the first HMF oxidation step (Kalum et al., 2014b).

A variety of indigo dyes, differing in the benzene ring substituents,

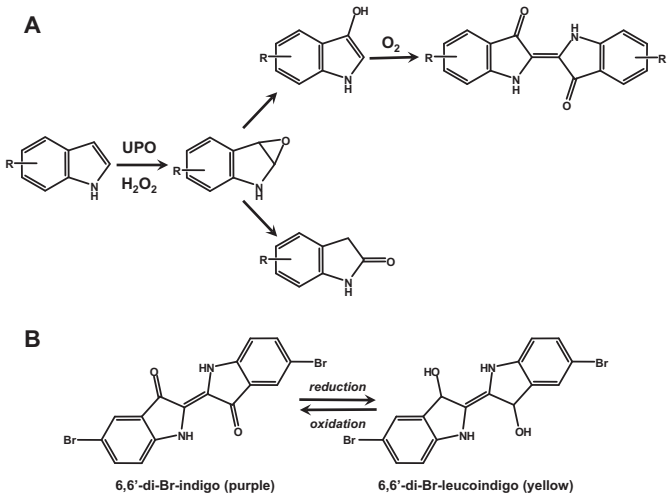


**Fig. 11.** Enzymatic indigo dyes for fabric dyeing. A. Proposed reaction mechanism for the UPO-catalyzed formation of different indigo dyes, followed by oxidative coupling. (1) 1*H*-indoles; (2) 2,3-epoxy-1*H*-indoles; (3), 3,3-dihydro-1*H*-indol-2-ones; (4) 3-hydroxy-1*H*-indoles; (5) indigos: R = H (indigo), R = Br (6,6′-dibromoindigo) or R = Cl (6,6′-dichloroindigo). B. Oxidized and reduced (soluble) forms of Tyrian purple (6,6′-bromoindigo) during the dyeing process.
Adapted from Kalum et al. (2014a).

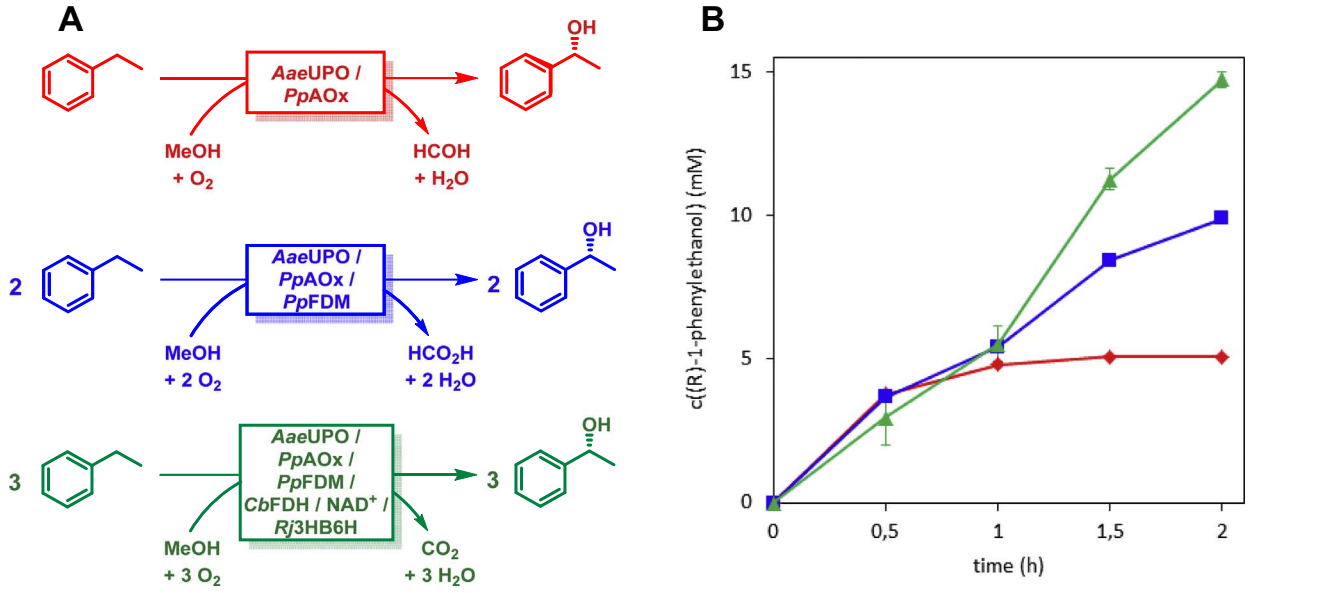


**Fig. 10.** Stereoselective hydroxylation in a byproduct-free enzyme cascade. Ethylbenzene conversion into (*R*)-1-phenyethanol by UPO, at expenses of methanol oxidation to $CO_2$ and $H_2O$, was obtained by increasing the number of cascade steps (A) resulting in higher product yield (B) as shown by green *vs* blue and red lines and reactions. *Aae*UPO, *Agrocybe aegerita* UPO; *Pp*AOx, *Pichia pastoris* MOX; *Pp*FDM, formaldehyde dismutase from *Pseudomonas putida*; *Cb*FDH, formate dehydrogenase from *Candida boidinii*; *Rj*3HB6H, 3-hydroxybenzoate-6-hydroxylase from *Rhodococcus jostii*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Adapted from Ni et al. (2016).

can be obtained by oxidative coupling of the 3-hydroxyindol precursors from indole hydroxylation by UPO (Fig. 11A) such as Tyrian Purple in its oxidized/reduced forms (Fig. 11B) (Kalum et al., 2014a). Additionally, green routes for the production of polymeric dyes can be developed by the enzymatic *co*-polymerization of aniline-type and phenolic precursors, such as 2,5-diaminebenzenesulfonic acid and resorcinol, by wild-type laccase (Pezzella et al., 2016) and *ad hoc* evolved variants (Vicente et al., 2016). Such reactions are milder and may be more environmentally-friendly than the chemical counterparts, and dyeing of natural and synthetic fabrics is comparable to that obtained with commercial dyes from chemical synthesis (Fig. S6).

Peroxidase-catalyzed formation of hypohalogenites has for a long time been neglected for preparative oxidation chemistry. There are, however, a range of applications where its mild conditions offer significant advantages over the stoichiometric use of bleach. The vanadium-containing, and $H_2O_2$ resistant, chloroperoxidase from *Curvularia inaequalis* has been applied for the catalytic halogenation of thymol yielding antimicrobial chlorothymol (Fernández-Fueyo et al., 2015b). The same enzyme was also used to initiate the so-called Achmatowicz reaction (Fernández-Fueyo et al., 2016b). In the latter case, the biocatalytic *in situ* generation of hypohalogenites (*e.g.* from seawater) led to a significant reduction in halogenated wastes as compared to the 'traditional method'. It is particularly worth mentioning that in both cases enzyme turnover numbers of more than 1,000,000 were obtained.

Synthesis of conductive polyaniline (Fig. 12A) has been widely investigated during the last decade for a range of applications in sensor devices, rechargeable batteries, *etc.* However, the polymer is industrially produced under extremely acidic conditions and using strong oxidants. Recently, a green alternative for polyaniline synthesis under industrially relevant conditions has been developed using a high redox-potential laccase as biocatalyst (de Salas et al., 2016). The enzyme has been further engineered by computer-aided design to improve aniline oxidation (Santiago et al., 2016) (Fig. 12B). The engineered variant, produced in yeast, causes over 35% increment in the synthesis of green polyaniline with respect to the parental laccase (Fig. 12C).

Lignin removal is a bottleneck for biomass conversion into fuels and chemicals in lignocellulose biorefineries that so far is overcome using strong physic/chemical treatments. Mimicking those changes caused by lignin-degrading fungi (van Kuijk et al., 2016), isolated oxidoreductases have been applied as milder and more environmentally-friendly pretreatments of woody (Rico et al., 2015) and non-woody (Rencoret et al., 2016) lignocellulosic feedstocks. In both cases, near 50% lignin removal was attained with laccase (from *Pycnoporus cinnabarinus* and *Myceliophthora thermophila*) and a mediator (1-hydroxybenzotriazole and methyl syringate, respectively) followed by alkaline peroxide extraction, resulting in higher saccharification degrees. Lower delignification rates were attained by laccase alone or in the absence of the alkaline extraction. Modification of lignin structure during the enzymatic treatments has been followed by 2D-NMR (Fig. S7), the state-of-the-art technology to get information on lignin aromatic units and inter-unit linkages and other lignocellulose constituents. In this way, the delignification process could be followed at the molecular level, and evidence for Cα-oxidation in lignin degradation was obtained, even in the treatments with laccase alone.

Concerning technical lignins from lignocellulose biorefineries, with variable phenolic content, treatment with laccases and peroxidases can be combined with growth of selected bacteria acting as a microbial sink for the synthesis of products of interest through metabolic engineering (Salvachúa et al., 2016). Moreover, using water soluble lignosulfonates and a combination of 2D-NMR and stopped-flow rapid spectrophotometry, it has been recently possible to demonstrate that: i) ligninolytic peroxidases are capable of abstracting electrons directly from the lignin polymer; ii) the minor phenolic moiety of lignin is preferentially oxidized by these enzymes; and iii) the catalytic tryptophan of VP and LiP is strictly required to oxidize the main non-phenolic lignin moiety (Sáez-Jiménez et al., 2015b, 2015c, 2016).

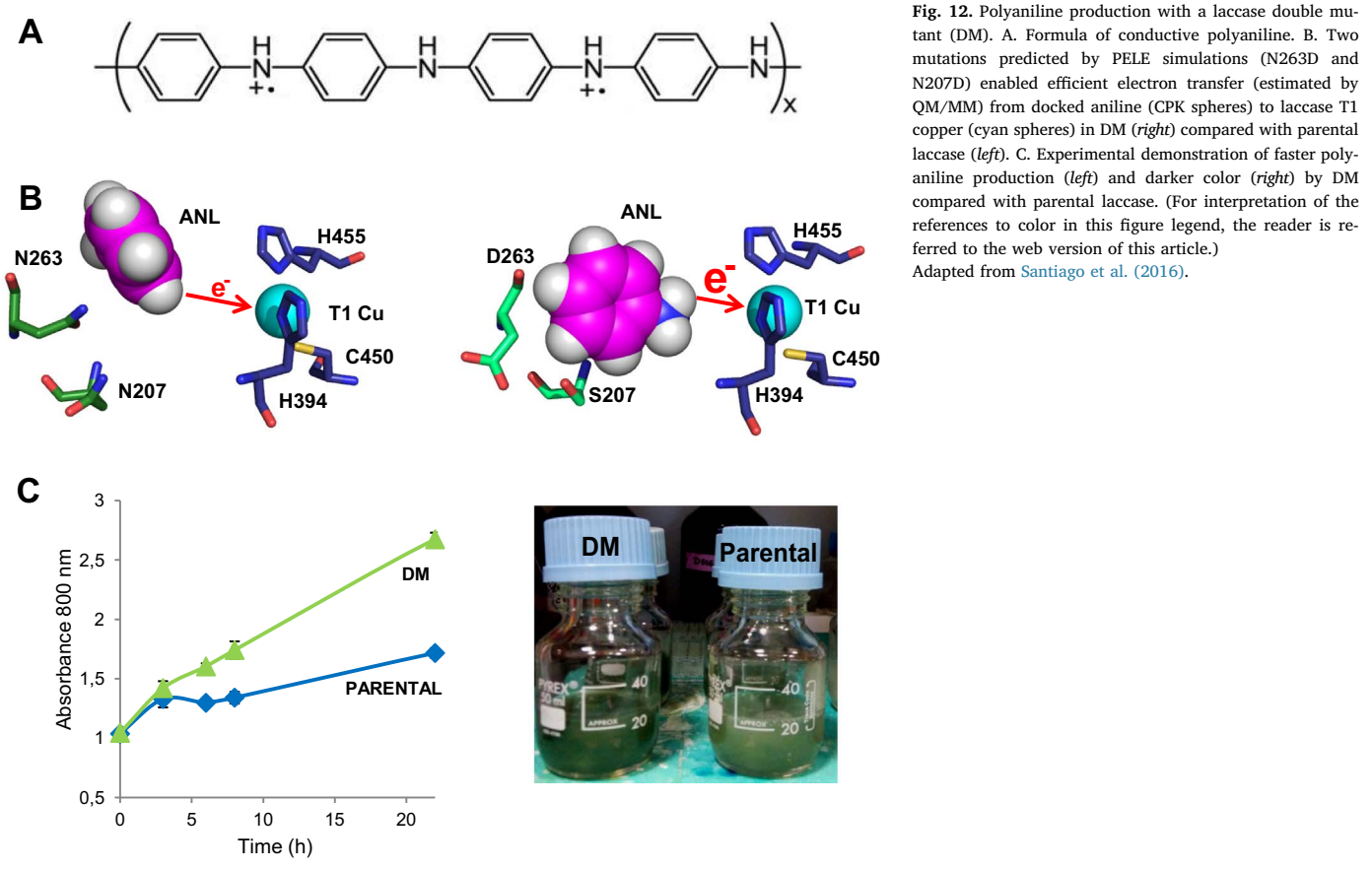


**Fig. 12.** Polyaniline production with a laccase double mutant (DM). A. Formula of conductive polyaniline. B. Two mutations predicted by PELE simulations (N263D and N207D) enabled efficient electron transfer (estimated by QM/MM) from docked aniline (CPK spheres) to laccase T1 copper (cyan spheres) in DM (*right*) compared with parental laccase (*left*). C. Experimental demonstration of faster polyaniline production (*left*) and darker color (*right*) by DM compared with parental laccase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Adapted from Santiago et al. (2016).

## 10. Outlook

In addition to classical oxidoreductases - including fungal laccases, ligninolytic peroxidases and several oxidases - new oxidative enzymes of biotechnological interest have been recently (re)discovered attracting considerable attention as industrial biocatalysts: from improving already in place biotransformations to opening new routes for the chemical industry. The successful case stories described in the present review are not only of direct interest for a variety of polymer, dye, specialty chemicals and pharmaceutical companies, but they aim to show to the chemical sector that medium and large scale biotransformations with oxidative enzymes can be successfully implemented in many other industrial processes.

Among different oxidoreductases, copper-containing LPMOs are already being incorporated to the new cellulases cocktails by enzyme producers (Novozymes included) since the action of hydrolytic enzymes is significantly boosted by the oxidative breakdown of crystalline cellulose by LPMOs (Kracher et al., 2016; Martínez, 2016). In the same scenario, CDH, whose role in lignocellulose decay was in debate for years, and other flavooxidases have been recently identified as important partners of LPMOs for their activation by efficient electron fueling (Tan et al., 2015).

The basidiomycete UPOs deserve a particular mention among the new oxidoreductases. These amazing heme proteins catalyze with advantages a variety of stereoselective and/or regioselective oxygen transfer reactions, previously assigned exclusively to P450s, acting as "self-sufficient" monooxygenases (Bormann et al., 2015; Hofrichter et al., 2015; Hofrichter and Ullrich, 2014). A noteworthy oxygenation ability, recently reported for a basidiomycete UPO, is the terminal hydroxylation of largely inert alkanes at the less reactive terminal position up to dicarboxylic acids (Olmedo et al., 2016). Other examples of interesting reactions by different UPOs are in the synthesis on renewable chemical building blocks, dye precursors, and drug ingredients and metabolites.

In most of the studied reactions, the oxidative enzymes were not suitable for industrial application as they are produced in nature, and their catalytic and/or operational properties need to be optimized by application of rational design and/or directed evolution tools (Molina-Espeja et al., 2016b; Pardo and Camarero, 2015b). The protein engineering work can be helped by biophysical/biochemical simulations using current computational facilities, significantly reducing the time of experimental work (Acebes et al., 2016; Monza et al., 2015).

It is also important that, in addition to their interest for sustainable bioeconomy, the above biotransformations also present additional economic and environmental benefits for the industry thanks to developing by-product and/or cosubstrate free multienzymatic cascades, such as those including peroxygenases, oxidases and other oxidative enzymes recently optimized (Carro et al., 2015; Ni et al., 2016).

Currently, most of the above oxidoreductase families are either commercial or under active development by biotechnology companies. For example, LPMO is an ingredient of the last generation cellulolytic cocktails as explained above, laccases are commercialized for phenols' removal in different applications (such as juice clarification and cork treatment, among others) and peroxidases for detergents and analytical tools, several oxidases are also produced for analytical tools and other uses, and peroxygenases are actively investigated at the enzyme industry and demanded by chemical companies for selective oxygenation reactions (in fact nine of the eleven patents cited in the present review concern peroxygenase reactions).

From a perspective of future, advanced processing of biomass in lignocellulose biorefineries should also take advantage from synthetic biology tendencies and tools to combine the extracellular lignin and

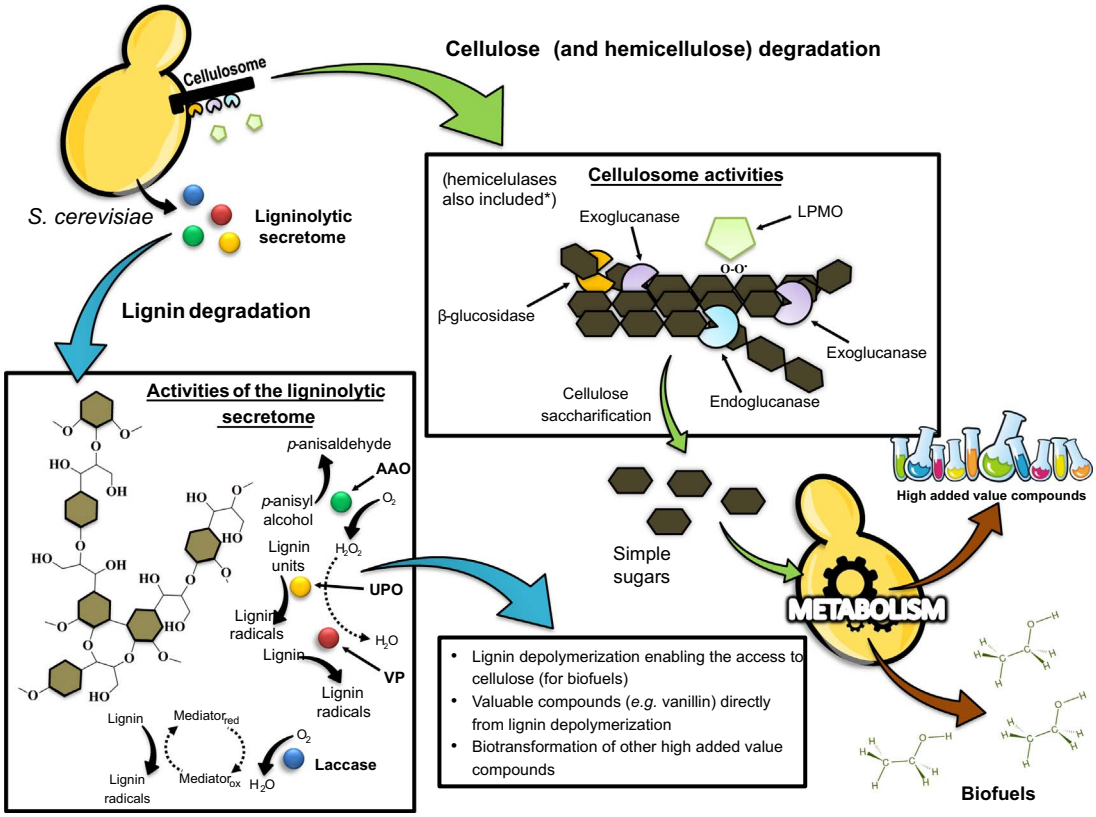


**Fig. 13.** Scheme of proposed *Saccharomyces cerevisiae* engineering for its use in lignocellulose biorefinery as a "white-rot yeast". The cellulolytic/hemicellulolytic machinery is presented here as a cellulosome with the immobilized enzymes but could also be envisaged as a free secretome of cellulases and hemicellulases, as in the case of ligninolytic secretome combining free enzymes involved in lignin degradation. *For clarity the action of hemicellulases is not shown.
Adapted from González-Pérez (2016).

cellulose/hemicellulose degrading machineries (ligninolytic secretome and cellulosome) within *Saccharomyces cerevisiae*. This artificial soluble secretome is formed by the most efficient members of the extracellular ligninolytic enzyme consortium after engineering them in the laboratory, including evolved versions of heme peroxidases, UPO, AAO and high-redox potential laccases (Alcalde, 2015; González-Pérez and Alcalde, 2014). Therefore, both ligninolytic secretome and cellulosome (or also as plausible option a secretome of free cellulose/hemicellulose-acting enzymes) could work in a synergetic manner in yeast for lignocellulose deconstruction. The combined action of both enzymatic machineries along with the intracellular fermentative capabilities of *S. cerevisiae* aims at developing a sugar-fermenting and biofuel-producing "white-rot yeast" that can act onto lignocellulosic raw material (Fig. 13) (González-Pérez, 2016).

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## Appendix A. Supplementary data

Additional information in Appendix A includes some target chemicals whose production using oxidative enzymes has been evaluated (Fig. S1), 2D-NMR study on LPMO binding to substrates (Fig. S2), synthesis of human drug metabolites by basidiomycete peroxygenase (Fig. S3), pathways for terminal oxygenation of n-alkanes to dicarboxylic acids by UPO (Fig. S4), enzymatic production of FDCA from renewable HMF (Fig. S5), multifiber test of enzymatic biodyes (Fig. S6), and 2D-NMR analysis of wheat straw after laccase and laccase-mediator treatment (Fig. S7). Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.biotechadv.2017.06.003.

## References

Acebes, S., Fernández-Fueyo, E., Monza, E., Lucas, F., Almendral, D., Ruiz-Dueñas, F.J., et al., 2016. Rational enzyme engineering through biophysical and biochemical modeling. ACS Catal. 6, 1624–1629.

Acebes, S., Ruiz-Dueñas, F.J., Toubes, M., Sáez-Jiménez, V., Pérez-Boada, M., Lucas, F., et al., 2017. Mapping the long-range electron transfer route in ligninolytic peroxidases. J. Phys. Chem. B 121, 3946–3954.

Alcalde, M., 2015. Engineering the ligninolytic enzyme consortium. Trends Biotechnol. 33, 155–162.

Alcalde, M., Molina-Espeja, P., García-Ruiz, E., González-Pérez, D., Ullrich, R., Hofrichter, M., 2014. Unspecific peroxygenase with high monooxygenase activity. Patent (Spain) P201430595-1.

Babot, E.D., del Río, J.C., Cañellas, M., Sancho, F., Lucas, F., Guallar, V., et al., 2015a. Steroid hydroxylation by basidiomycete peroxygenases: a combined experimental and computational study. Appl. Environ. Microbiol. 81, 4130–4142.

Babot, E.D., del Río, J.C., Kalum, L., Martínez, A.T., Gutiérrez, A., 2015b. Regioselective hydroxylation in the production of 25-hydroxyvitamin D by *Coprinopsis cinerea* peroxygenase. ChemCatChem 7, 283–290.

Baratto, M.C., Sinicropi, A., Linde, D., Sáez-Jiménez, V., Sorace, L., Ruiz-Dueñas, F.J., et al., 2015. Redox-active sites in *Auricularia auricula-judae* dye-decolorizing peroxidase and several directed variants: a multifrequency EPR study. J. Phys. Chem. B 119, 13583–13592.

Barrasa, J.M., Blanco, M.N., Esteve-Raventós, F., Altés, A., Checa, J., Martínez, A.T., et al., 2014. Wood and humus decay strategies by white-rot basidiomycetes correlate with two different dye decolorization and enzyme secretion patterns on agar plates. Fungal Genet. Biol. 72, 106–114.

Barrasa, J.M., Ruiz-Dueñas, F.J., Fernández-Fueyo, E., Pacheco, R., Martínez, A.T., 2016. Lignin Biodegradation in the Genomic Era. Proc. LignoBiotech 4, Madrid, 19–22 June. (http://lignobiotech.es/wp-content/uploads/2016/10/Lignobiotech_IV_Madrid_2016_Conference_Book.pdf) pp. P11.

Bormann, S., Baraibar, A.G., Ni, Y., Holtmann, D., Hollmann, F., 2015. Specific oxyfunctionalisations catalysed by peroxygenases: opportunities, challenges and solutions. Catal. Sci. Technol. 5, 2038–2052.

Büttner, E., Ullrich, R., Strittmatter, E., Piontek, K., Plattner, D.A., Hofrichter, M., et al., 2015. Oxidation and nitration of mononitrophenols by a DyP-type peroxidase. Arch. Biochem. Biophys. 574, 86–92.

Carro, J., Ferreira, P., Rodríguez, L., Prieto, A., Serrano, A., Balcells, B., et al., 2015. 5-Hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase. FEBS J. 282, 3218–3229.

Carro, J., Serrano, A., Ferreira, P., Martínez, A.T., 2016. Fungal aryl-alcohol oxidase in lignocellulose degradation and bioconversion. In: Gupta, V.K., Tuohy, M.G. (Eds.), Microbial Enzymes in Bioconversions of Biomass. Springer, Berlin, Germany, pp. 301–322.

Courtade, G., Wimmer, R., Røhr, A.K., Preims, M., Felice, A.K.G., Dimarogona, M., et al., 2016. Interactions of a fungal lytic polysaccharide monooxygenase with β-glucan substrates and cellobiose dehydrogenase. Proc. Natl. Acad. Sci. U. S. A. 113, 5922–5927.

Couturier, M., Mathieu, Y., Li, A., Navarro, D., Drula, E., Haon, M., et al., 2016. Characterization of a new aryl-alcohol oxidase secreted by the phytopathogenic fungus *Ustilago maydis*. Appl. Microbiol. Biotechnol. 100, 697–706.

Daou, M., Piumi, F., Cullen, D., Record, E., Faulds, C.B., 2016. Heterologous production and characterization of two glyoxal oxidases from *Pycnoporus cinnabarinus*. Appl. Environ. Microbiol. 82, 4867–4875.

Ewing, T.A., Gygli, G., van Berkel, W.J.H., 2016. A single loop is essential for the octamerisation of vanillyl alcohol oxidase. FEBS J. 283, 2546–2559.

Fernández-Fueyo, E., Acebes, S., Ruiz-Dueñas, F.J., Martínez, M.J., Romero, A., Medrano, F.J., et al., 2014a. Structural implications of the C-terminal tail in the catalytic and stability properties of manganese peroxidases from ligninolytic fungi. Acta Crystallogr. D. Biol. Crystallogr. 70, 3253–3265.

Fernández-Fueyo, E., Castanera, R., Ruiz-Dueñas, F.J., López-Lucendo, M.F., Ramírez, L., Pisabarro, A.G., et al., 2014b. Ligninolytic peroxidase gene expression by *Pleurotus ostreatus*: differential regulation in lignocellulose medium and effect of temperature and pH. Fungal Genet. Biol. 72, 150–161.

Fernández-Fueyo, E., Ruiz-Dueñas, F.J., Martínez, A.T., 2014c. Engineering a fungal peroxidase that degrades lignin at very acidic pH. Biotechnol. Biofuels 7, 114.

Fernández-Fueyo, E., Ruiz-Dueñas, F.J., Martínez, M.J., Romero, A., Hammel, K.E., Medrano, F.J., et al., 2014d. Ligninolytic peroxidase genes in the oyster mushroom genome: heterologous expression, molecular structure, catalytic and stability properties and lignin-degrading ability. Biotechnol. Biofuels 7, 2.

Fernández-Fueyo, E., Linde, D., Almendral, D., López-Lucendo, M.F., Ruiz-Dueñas, F.J., Martínez, A.T., 2015a. Description of the first fungal dye-decolorizing peroxidase oxidizing manganese(II). Appl. Microbiol. Biotechnol. 99, 8927–8942.

Fernández-Fueyo, E., van Wingerden, M., Renirie, R., Wever, R., Ni, Y., Holtmann, D., et al., 2015b. Chemoenzymatic halogenation of phenols by using the haloperoxidase from *Curvularia inaequalis*. ChemCatChem 7, 4035–4038.

Fernández-Fueyo, E., Ruiz-Dueñas, F.J., López-Lucendo, M.F., Pérez-Boada, M., Rencoret, J., Gutiérrez, A., et al., 2016a. A secretomic view of woody and nonwoody lignocellulose degradation by *Pleurotus ostreatus*. Biotechnol. Biofuels 9, 49.

Fernández-Fueyo, E., Younes, S.H.H., van Rootselaar, S., Aben, R.W.M., Renirie, R., Wever, R., et al., 2016b. A biocatalytic aza-Achmatowicz reaction. ACS Catal. 6, 5904–5907.

Ferreira, P., Carro, J., Serrano, A., Martínez, A.T., 2015a. A survey of genes encoding $H_2O_2$-producing GMC oxidoreductases in 10 polyporales genomes. Mycologia 107, 1105–1119.

Ferreira, P., Hernández-Ortega, A., Borrelli, K., Lucas, F., Herguedas, B., Guallar, V., et al., 2015b. Aromatic stacking interactions govern catalysis in aryl-alcohol oxidase. FEBS J. 282, 3091–3106.

Garajova, S., Mathieu, Y., Beccia, M.R., Bennati-Granier, C., Biaso, F., Fanuel, M., et al., 2016. Single-domain flavoenzymes trigger lytic polysaccharide monooxygenases for oxidative degradation of cellulose. Sci Rep 6, 28276.

García-Ruiz, E., Maté, D.M., González-Pérez, D., Molina-Espeja, P., Camarero, S., Martínez, A.T., et al., 2014. Directed evolution of ligninolytic oxidoreductases: from functional expression to stabilization and beyond. In: Riva, S., Fessner (Eds.), Cascade Biocatalysis: Integrating Stereoselective and Environmentally Friendly Reactions. Wiley-VCH.

Giacobelli, V.G., Monza, E., Lucas, M.F., Pezzella, C., Piscitelli, A., Guallar, V., et al., 2017. Repurposing designed mutants: a valuable strategy for computer-aided laccases engineering. The case of POXA1b. Catal. Sci. Technol. 7, 515–523.

González-Pérez, D., 2016. Directed Evolution of Versatile Peroxidase for the Design of a White-rot Yeast. PhD thesis UAM, Madrid.

González-Pérez, D., Alcalde, M., 2014. Assembly of evolved ligninolytic genes in *Saccharomyces cerevisiae*. Bioengineered 5, 254–263.

González-Pérez, D., García-Ruiz, E., Ruiz-Dueñas, F.J., Martínez, A.T., Alcalde, M., 2014a. Structural determinants of oxidative stabilization in an evolved versatile peroxidase. ACS Catal. 4, 3891–3901.

González-Pérez, D., Molina-Espeja, P., García-Ruiz, E., Alcalde, M., 2014b. Mutagenic organized recombination process by homologous in vivo grouping (MORPHING) for directed enzyme evolution. PLoS One 9, e90919.

González-Pérez, D., Mateljak, I., García-Ruiz, E., Ruiz-Dueñas, F.J., Martínez, A.T., Alcalde, M., 2016. Alkaline versatile peroxidase by directed evolution. Catal. Sci. Technol. 6, 6625–6636.

Gygli, G., van Berkel, W.J.H., 2015. Oxizymes for biotechnology. Curr. Biotechnol. 4, 100–110.
Gygli, G., van Berkel, W.J.H., 2017. Vanillyl alcohol oxidases produced in *Komagataella phaffii* contain a highly stable non-covalently bound anionic FAD semiquinone. Biocatalysis 3, 17–26.
Harris, P.V., Welner, D., McFarland, K.C., Re, E., Poulsen, J.C.N., Brown, K., et al., 2010. Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. Biochemistry 49, 3305–3316.
Hofrichter, M., Ullrich, R., 2014. Oxidations catalyzed by fungal peroxygenases. Curr. Opin. Chem. Biol. 19, 116–125.
Hofrichter, M., Kellner, H., Pecyna, M.J., Ullrich, R., 2015. Fungal unspecific peroxygenases: heme-thiolate proteins that combine peroxidase and cytochrome P450 properties. Adv. Exp. Med. Biol. 851, 341–368.
Hollmann, F., Ni, Y., 2016. Enzymatic conversion using hydrogen peroxide. Patent (NL) NL2013351A.
Hori, C., Ishida, T., Igarashi, K., Samejima, M., Suzuki, H., Master, E., et al., 2014. Analysis of the *Phlebiopsis gigantea* genome, transcriptome and secretome gives insight into its pioneer colonization strategies of wood. PLoS Genet. 10 (12), e1004759.
Isaksen, T., Westereng, B., Aachmann, F.L., Agger, J.W., Kracher, D., Kittl, R., et al., 2014. A C4-oxidizing lytic polysaccharide monooxygenase cleaving both cellulose and cello-oligosaccharides. J. Biol. Chem. 289, 2632–2642.
Kalum, L., Lund, H., Hofrichter, M., Ullrich, R., 2014a. Enzymatic preparation of indigo dyes and intermediates. Patent (International) WO2014122109A1.
Kalum, L., Morant, M. D., Lund, H., Jensen, J., Lapainaite, I., Soerensen, N. H. et al., 2014b. Enzymatic oxidation of 5-hydroxymethylfurfural and derivatives thereof. Patent (International)WO2014-015256A2.
Karich, A., Scheibner, K., Ullrich, R., Hofrichter, M., 2016. Exploring the catalase activity of unspecific peroxygenases and the mechanism of peroxide-dependent heme destruction. J. Mol. Catal. B-Enzym. 134, 238–246.
Kellner, H., Pecyna, M.J., Buchhaupt, M., Ullrich, R., Hofrichter, M., 2016. Draft genome sequence of the chloroperoxidase-producing fungus *Caldariomyces fumago* Woronichin DSM1256. Genome Announc 4, e00774-16.
Kiebist, J., Holla, W., Heidrich, J., Poraj-Kobielska, M., Sandvoss, M., Simonis, R., et al., 2015. One-pot synthesis of human metabolites of SAR548304 by fungal peroxygenases. Bioorg. Med. Chem. 23, 4324–4332.
Kracher, D., Zahma, K., Schulz, C., Sygmund, C., Gorton, L., Ludwig, R., 2015. Interdomain electron transfer in cellobiose dehydrogenase: modulation by pH and divalent cations. FEBS J. 282, 3136–3148.
Kracher, D., Scheiblbrandner, S., Felice, A.K.G., Breslmays, E., Preims, M., Ludwicka, K., et al., 2016. Extracellular electron transfer systems fuel oxidative cellulose degradation. Science 352, 1098–1101.
van Kuijk, S., del Río, J.C., Rencoret, J., Gutiérrez, A., Sonnenberg, A.S.M., Baars, J.J.P., et al., 2016. Selective ligninolysis of wheat straw and wood chips by the white-rot fungus Lentinula edodes and its influence on in vitro rumen degradability. J. Anim. Sci. Biotechnol. 7, 55.
Landvick, S., Ostergaard, L. H., Kalum, L., 2016a. Polypeptides having peroxygenase activity. Patent (International) WO2014056916A3.
Landvick, S., Ostergaard, L. H., Kalum, L., 2016b. Polypeptides having peroxygenase activity and polynucleotides encoding same. Patent (USA) US20160244731A1.
Lettera, V., Pezzella, C., Cicatiello, P., Piscitelli, A., Giacobelli, V.G., Galano, E., et al., 2016. Efficient immobilization of a fungal laccase and its exploitation in fruit juice clarification. Food Chem. 196, 1272–1278.
Linde, D., Coscolín, C., Liers, C., Hofrichter, M., Martínez, A.T., Ruiz-Dueñas, F.J., 2014. Heterologous expression and physicochemical characterization of a fungal dye-decolorizing peroxidase from *Auricularia auricula-judae*. Protein Express. Purif. 103, 28–37.
Linde, D., Pogni, R., Cañellas, M., Lucas, F., Guallar, V., Baratto, M.C., et al., 2015a. Catalytic surface radical in dye-decolorizing peroxidase: a computational, spectroscopic and directed mutagenesis study. Biochem. J. 466, 253–262.
Linde, D., Ruiz-Dueñas, F.J., Fernández-Fueyo, E., Guallar, V., Hammel, K.E., Pogni, R., et al., 2015b. Basidiomycete DyPs: genomic diversity, structural-functional aspects, reaction mechanism and environmental significance. Arch. Biochem. Biophys. 574, 66–74.
Linde, D., Canellas, M., Coscolín, C., Davó-Siguero, I., Romero, A., Lucas, F., et al., 2016. Asymmetric sulfoxidation by engineering the heme pocket of a dye-decolorizing peroxidase. Catal. Sci. Technol. 6, 6277–6285.
Loose, J.S.M., Forsberg, Z., Kracher, D., Scheiblbrandner, S., Ludwig, R., Eijsink, V.G.H., et al., 2016. Activation of bacterial lytic polysaccharide monooxygenases with cellobiose dehydrogenase. Protein Sci. 25, 2175–2186.
Lucas, F., Babot, E.D., del Río, J.C., Kalum, L., Ullrich, R., Hofrichter, M., et al., 2016. Molecular determinants for selective C25-hydroxylation of vitamins D2 and D3 by fungal peroxygenases. Catal. Sci. Technol. 6, 288–295.
Lund, H., Brask, J., Kalum, L., Gutiérrez, A., Babot, E. D., Ullrich, R. et al., 2014. Enzymatic preparation of diols. Patent (USA) US20140234917A1.
Lund, H., Kalum, L., Hofrichter, M., Peter, S., 2016. Epoxidation using peroxygenase. Patent (USA) US 9458478 B2.
Martínez, A.T., 2016. How to break down crystalline cellulose. Science 352, 1050–1051.
Martínez, A.T., Camarero, S., Ruiz-Dueñas, F.J., Martínez, M.J., 2017. Biological lignin degradation. In: Beckham, G.T. (Ed.), Lignin Valorization: Emerging Approaches. Royal Society of Chemistry Chapter 8.
Maté, D.M., Alcalde, M., 2015. Laccase engineering: from rational design to directed evolution. Biotechnol. Adv. 33, 25–40.
Maté, D., Alcalde, M., 2016a. Directed evolution of fungal laccases: an update. In: Rahman, A. (Ed.), Advances in Genome Science. Genes in Health and Disease, vol 4. Bentham Books, pp. 91–112.
Maté, D., Alcalde, M., 2016b. Laccase: a multi-purpose biocatalyst at the forefront of biotechnology. Microbial Biotechnol. online. http://dx.doi.org/10.1111/1751-7915.12422.
Maté, D., González-Pérez, D., Mateljak, I., Gómez de Santos, P., Vicente, A.I., Alcalde, M., 2016. The pocket manual of directed evolution: tips and tricks. In: Brahmachari, G., Demain, A., Adrio, J.L. (Eds.), Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications. Elsevier, Amsterdam, pp. 185–214.
Mathieu, Y., Piumi, F., Valli, R., Aramburu, J.C., Ferreira, P., Faulds, C.B., et al., 2016. Activities of secreted aryl alcohol quinone oxidoreductases from *Pycnoporus cinnabarinus* provide insights into fungal degradation of plant biomass. Appl. Environ. Microbiol. 82, 2411–2423.
Molina-Espeja, P., Garcia-Ruiz, E., Gonzalez-Perez, D., Ullrich, R., Hofrichter, M., Alcalde, M., 2014. Directed evolution of unspecific peroxygenase from *Agrocybe aegerita*. Appl. Environ. Microbiol. 80, 3496–3507.
Molina-Espeja, P., Ma, S., Maté, D.M., Ludwig, R., Alcalde, M., 2015. Tandem-yeast expression system for engineering and producing unspecific peroxygenase. Enzym. Microb. Technol. 73-74, 29–33.
Molina-Espeja, P., Canellas, M., Plou, F.J., Hofrichter, M., Lucas, F., Guallar, V., et al., 2016a. Synthesis of 1-naphthol by a natural peroxygenase engineered by directed evolution. Chembiochem 17, 341–349.
Molina-Espeja, P., Viña-Gonzalez, J., Gomez-Fernandez, B.J., Martin-Diaz, J., Garcia-Ruiz, E., Alcalde, M., 2016b. Beyond the outer limits of nature by directed evolution. Biotechnol. Adv. 34, 754–767.
Monza, E., Lucas, F., Camarero, S., Alejaldre, L.C., Martínez, A.T., Guallar, V., 2015. Insights on laccase engineering from molecular simulations: towards a binding focused strategy. J. Phys. Chem. Lett. 6, 1447–1453.
Ni, Y., Fernández-Fueyo, E., Baraibar, A.G., Ullrich, R., Hofrichter, M., Yanase, H., et al., 2016. Peroxygenase-catalyzed oxyfunctionalization reactions promoted by the complete oxidation of methanol. Angew. Chem. Int. Ed. 55, 798–801.
Olmedo, A., Aranda, C., del Río, J.C., Kiebist, J., Scheibner, K., Martínez, A.T., et al., 2016. From alkanes to carboxylic acids: terminal oxygenation by a fungal peroxygenase. Angew. Chem. Int. Ed. 55, 12248–12251.
Pardo, I., Camarero, S., 2015a. Exploring the oxidation of lignin-derived phenols by a library of laccase mutants. Molecules 20, 15929–15943.
Pardo, I., Camarero, S., 2015b. Laccase engineering by rational and evolutionary design. Cell. Mol. Life Sci. 72, 897–910.
Pardo, I., Santiago, G., Gentili, P., Lucas, F., Monza, E., Medrano, F.J., et al., 2016. Redesigning the substrate binding pocket of laccase for enhanced oxidation of sinapic acid. Catal. Sci. Technol. 6, 3900–3910.
Patel, I., Kracher, D., Ma, S., Garajova, S., Haon, M., Faulds, C.B., et al., 2016. Salt-responsive lytic polysaccharide monooxygenases from the mangrove fungus *Pestalotiopsis* sp. NCi6. Biotechnol. Biofuels 9 (108).
Pezzella, C., Guarino, L., Piscitelli, A., 2015. How to enjoy laccases. Cell. Mol. Life Sci. 72, 923–940.
Pezzella, C., Giacobbe, S., Giacobelli, V.G., Guarino, L., Kylic, S., Sener, M., et al., 2016. Green routes towards industrial textile dyeing: a laccase based approach. J. Mol. Catal. B-Enzym. 134, 271–279.
Pham, N.H., Hollmann, F., Kracher, D., Preims, M., Haltrich, D., Ludwig, R., 2015. Engineering an enzymatic regeneration system for NAD(P)H oxidation. J. Mol. Catal. B-Enzym. 120, 38–46.
Piumi, F., Levasseur, A., Navarro, D., Zhou, S.M., Mathieu, Y., Ropartz, D., et al., 2014. A novel glucose dehydrogenase from the white-rot fungus *Pycnoporus cinnabarinus*: production in *Aspergillus niger* and physicochemical characterization of the recombinant enzyme. Appl. Microbiol. Biotechnol. 98, 10105–10118.
Poraj-Kobielska, M., Gröbe, G., Kiebist, J., Grün, M., Ullrich, R., Scheibner, K., et al., 2015a. Verfahren zur Deacylierung von Corticoiden. Patent (Germany), DE102014005371.
Poraj-Kobielska, M., Peter, S., Leonhardt, S., Ullrich, R., Scheibner, K., Hofrichter, M., 2015b. Immobilization of unspecific peroxygenases (EC 1.11.2.1) in PVA/PEG gel and hollow fiber modules. Biochem. Eng. J. 98, 144–150.
Rencoret, J., Pereira, A., del Río, J.C., Martínez, A.T., Gutiérrez, A., 2016. Laccase-mediator pretreatment of wheat straw degrades lignin and improves saccharification. Bioenerg. Res. 9, 917–930.
Rico, A., Rencoret, J., del Río, J.C., Martínez, A.T., Gutiérrez, A., 2015. In-depth 2D NMR study of lignin modification during pretreatment of *Eucalyptus* wood with laccase and mediators. Bioenerg. Res. 8, 211–230.
Sáez-Jiménez, V., Acebes, S., Guallar, V., Martínez, A.T., Ruiz-Dueñas, F.J., 2015a. Improving the oxidative stability of a high redox potential fungal peroxidase by rational design. PLoS One 10, e0124750.
Sáez-Jiménez, V., Baratto, M.C., Pogni, R., Rencoret, J., Gutiérrez, A., Santos, J.I., et al., 2015b. Demonstration of lignin-to-peroxidase direct electron transfer. A transient-state kinetics, directed mutagenesis, EPR and NMR study (vol 290, pag 23201, 2015). J. Biol. Chem. 290, 30268.
Sáez-Jiménez, V., Baratto, M.C., Pogni, R., Rencoret, J., Gutiérrez, A., Santos, J.I., et al., 2015c. Demonstration of lignin-to-peroxidase direct electron transfer: a, transient-state kinetics, directed mutagenesis, EPR and NMR study. J. Biol. Chem. 290, 23201–23213.
Sáez-Jiménez, V., Fernández-Fueyo, E., Medrano, F.J., Romero, A., Martínez, A.T., Ruiz-Dueñas, F.J., 2015d. Improving the pH-stability of versatile peroxidase by comparative structural analysis with a naturally-stable manganese peroxidase. PLoS One 10, e0140984.
Sáez-Jiménez, V., Rencoret, J., Rodríguez-Carvajal, M.A., Gutiérrez, A., Ruiz-Dueñas, F.J., Martínez, A.T., 2016. Role of surface tryptophan for peroxidase oxidation of non-phenolic lignin. Biotechnol. Biofuels 9, 198.
de Salas, F., Pardo, I., Salavagione, H.J., Aza, P., Amougi, E., Vind, J., et al., 2016. Advanced synthesis of conductive polyaniline using laccase as biocatalyst. PLoS One

11, e0164958.
Salvachúa, D., Katahira, R., Cleveland, N.S., Khanna, P., Resch, M.G., Black, B.A., et al., 2016. Lignin depolymerization by fungal secretomes and a microbial sink. Green Chem. 18, 6046–6062.
Santiago, G., de Salas, F., Lucas, M.F., Monza, E., Acebes, S., Martínez, A.T., et al., 2016. Computer-aided laccase engineering: toward biological oxidation of arylamines. ACS Catal. 6, 5415–5423.
Strittmatter, E., Serrer, K., Liers, C., Ullrich, R., Hofrichter, M., Piontek, K., et al., 2015. The toolbox of *Auricularia auricula-judae* dye-decolorizing peroxidase. Identification of three new potential substrate-interaction sites. Arch. Biochem. Biophys. 574, 75–85.
Tan, T.-C., Kracher, D., Gandini, R., Sygmund, C., Kittl, R., Haltrich, D., et al., 2015. Structural basis for cellobiose dehydrogenase action during oxidative cellulose degradation. Nat. Commun. 6, 7542.
Vaaje-Kolstad, G., Westereng, B., Horn, S.J., Liu, Z.L., Zhai, H., Sorlie, M., et al., 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 330, 219–222.
Vicente, A.I., Viña-González, J., Santos-Moriano, P., Marquez-Alvarez, C., Ballesteros, A.O., Alcalde, M., 2016. Evolved alkaline fungal laccase secreted by *Saccharomyces cerevisiae* as useful tool for the synthesis of C-N heteropolymeric dye. J. Mol. Catal. B-Enzym. 134, 323–330.
Viña-González, J., González-Pérez, D., Ferreira, P., Martínez, A.T., Alcalde, M., 2015. Focused directed evolution of aryl-alcohol oxidase in *Saccharomyces cerevisiae* by using chimeric signal peptides. Appl. Environ. Microbiol. 81, 6451–6462.
Viña-González, J., González-Pérez, D., Alcalde, M., 2016. Directed evolution method in *Saccharomyces cerevisiae*: mutant library creation and screening. J. Vis. Exp. 110, e53761.
Vind, J., Ostergaard, L. H., de Leonardo, M., Kalum, L., Amougi, E., 2015. Peroxygenase variants. Patent (International) WO2015079064A3.
Wang, X., Ullrich, R., Hofrichter, M., Groves, J.T., 2015. Heme-thiolate ferryl of aromatic peroxygenase is basic and reactive. Proc. Natl. Acad. Sci. U. S. A. 112, 3686–3691.