Oxidoreductases for medium and large-scale industrial biotransformations

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Footnotes:

[#] *In memoriam*

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[¶] Abbreviations: AAD, aryl-alcohol dehydrogenase; AAO, aryl-alcohol oxidase; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); API, active pharmaceutical ingredients; 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 superfamily; GOX, glucose oxidase; HMF, 5-hydroxymethylfurfural; HSQC, heteronuclear single quantum correlation (NMR experiment); HTP, hemethiolate peroxidases; 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

ABSTRACT

Lignin-degrading basidiomycetes and related fungi produce heme-containing peroxidases and peroxygenases, flavin-containing oxidases and dehydrogenases, and different copper-containing oxidoreductases. Heme peroxidases comprise classical ligninolytic peroxidases and new dye-decolorizing peroxidases, while heme peroxygenases belong to a still largely unexplored superfamily of heme-thiolate peroxidases. 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 source of oxygen and final electron acceptor. Flavooxidases are involved in both lignin and cellulose

decay generating H_2O_2 that activates peroxidases and generates hydroxyl radical, respectively. 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. However, the recently described lytic polysaccharide monooxygenases attract the highest attention among copper oxidoreductases, since they are able to break down (oxidatively) crystalline cellulose, a major bottleneck in lignocellulose biorefineries (together with lignin degradation by peroxidases and laccases). Interestingly, some flavin-containing dehydrogenases also play a key role "fueling" (directly or indirectly) electrons for polysaccharide monooxygenase activation. Many of the above oxidoreductases have been engineered, using both rational design and directed evolution, to attain the selectivity, catalytic efficiency and operational properties (stability included) required for their industrial utilization. Using *ad hoc* software and current computational capabilities, it is now possible to predict substrate access to the active site (biophysical simulations) and electron transfer efficiency (biochemical simulations), reducing in orders of magnitude the time of experimental work in enzyme screening and engineering. The above is illustrated by a series of oxyfunctionalization and oxidation reactions (e.g. enzymatic synthesis of 1-naphthol, 25-hydroxyvitamin D_3, drug metabolites, chiral alcohols, furandicarboxylic acid, indigo dyes and conductive polyaniline, terminal oxygenation of alkanes, biomass delignification and lignin oxidation) as successful case stories demonstrating the potential of oxidoreductases in medium and large-scale industrial biotransformations.

Keywords:
- Heme peroxidases/peroxygenases
- Oxidases and dehydrogenases
- Laccases
- Lytic polysaccharide monooxygenases
- Biophysical and biochemical computational modeling
- Rational design
- Directed evolution
- Enzyme cascades
- Selective oxyfunctionalizations
- Lignocellulose biorefinery

1. Fungal oxidoreductases

Oxidoreductases take advantage from the incorporation of different cofactors (such as heme, flavin and metal ions) to catalyze redox reactions, using a variety of electron acceptors and a great number of electron-donating substrates, and yielding many products of industrial interest (Gygli and van Berkel, 2015). Fungi, including wood-rotting 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., 2016). Fungal oxidoreductases of biotechnological interest typically include: **i)** heme-containing peroxidases and peroxygenases, being activated by H_2O_2 as electron acceptor; **ii)** flavin-containing oxidases and dehydrogenases, being activated by O_2 and other oxidizers (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 catalytic mechanism) (**Fig. 1**).

Classical fungal oxidoreductases include basidiomycete ligninolytic peroxidases, and ascomycete and basidiomycete multicopper oxidases (MCO¶, mainly laccases) with different

redox potentials and abilities to act on lignin-derived products (optionally using redox mediators). 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- stereo-selective oxyfunctionalizations with H_2O_2 working as the oxygen source and final electron acceptor; and **ii)** copper-containing lytic polysaccharide monooxygenases (LPMOs), which turned out to be the "missing" enzymes in the microbial attack to crystalline cellulose and other polysaccharides.

Enzymes of the glucose-methanol-choline oxidase/dehydrogenase (GMC) and copper-radical oxidase (CRO) superfamilies have been classically investigated as the source of H_2O_2 for: **i)** ligninolytic peroxidase in white-rot (lignin-degrading) basidiomycetes; or **ii)** hydroxyl radical generation *via* Fenton reaction in brown-rot (cellulose-degrading) basidiomycetes. However, the preferential or optional use of other electron acceptors by some of them (dehydrogenase activity) suggests additional functions, e.g. preventing lignin repolymerization or fueling electrons to LPMOs. These and other fungal flavooxidases are also emerging industrial biocatalysts.

2. Oxidoreductases as industrial biocatalysts

The above oxidoreductases are biocatalysts of interest for establishing a circular economy with the highest potential in: **i)** the production of renewable building blocks from plant biomass; and **ii)** their use for the manufacture of sustainable chemicals and materials in lignocellulose biorefineries (**Fig. 2**). However, the chemical industry (some specialties excluded) is not yet embracing enzymatic oxidation reactions to a significant extent primarily due to lack of biocatalysts with the required selectivity, availability and compatibility with the rigorous process conditions (high substrate concentrations, use of solvents, and strongly oxidative conditions).

The above bottleneck has been addressed through enzyme engineering and process optimization using state-of-the-art technologies, and some recent (2014-2017 period) representative results are reviewed here. The following oxidation and oxyfunctionalization target reactions formed the basis for the screening and optimization of new biocatalysts in this study, whose selection was based on proof of reaction at laboratory scale, industrial interest of the corresponding products and possibility for broadening the application field to similar substrate classes: **i)** Intermediates for agrochemicals and active pharmaceutical ingredients (APIs), flavors and fragrances, chiral alcohols, epoxidation products and drug metabolites; **ii)** Precursors for specialty polymers including 5-hydroxymethylfurfural (HMF) products such as 2,5-furandicarboxylic acid (FDCA), diols, hydroxyacids and diacids (from alkanes and fatty acids), and functionalized plant polymers including oxidized cellulosic fibers and lignin; and **iii)** Intermediates for dye-stuff production including phenolic and amine derivatives, indole and indole derivatives, and aniline polymers (**Fig. 3**).

The work performed comprises: **i)** Recovery of selective oxidative biocatalysts from fungal genomes and other sources for target reactions from the groups of heme-peroxidases/ peroxygenases, flavo-oxidases and copper-oxidoreductases; **ii)** Improvement of their oxidation activity and stability by protein engineering using rational design (based on structural-functional information), directed evolution and hybrid approaches (in yeast and other expression systems), combined with computer simulations speeding the engineering process, to better fulfill the operational and catalytic conditions required by the chemical industry; and **iii)** Optimization of reaction conditions including enzyme cascade reactions.

The above multidisciplinary approach was possible by creating a highly-specialized consortium of SMEs, large companies and research/academic institutions in the frame of the INDOX project "Optimized oxidoreductases for medium and large scale industrial biotransformations" (http://indoxproject.eu). Production of the new optimized biocatalysts, and their introduction into the chemical market will take advantage from the participation of the world-leading company in the sector of industrial enzymes (Novozymes) together with several chemical companies (such as Cheminova, Rhodia, Setas and Chiracon) willing to implement new medium- and large-scale enzymatic biotransformation processes. Moreover, these successful case stories demonstrate the potential of oxidative biotransformations in related chemical sectors.

3. Heme-containing peroxidases/peroxygenases

Classical peroxidases and heme-containing peroxygenases are members of the peroxidase-catalase and hemethiolate peroxidase (HTP) superfamilies, respectively. Although these enzymes share a heme cofactor, the phylogenetic connection between them would be remote or null. Classical ligninolytic peroxidases are known for near forty years, and representatives for their three families – lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP) – have been extensively characterized due to biotechnological interest (**Fig. 1A**) (Martínez et al., 2016). Distribution of the corresponding genes in white-rot and brown-rot fungal genomes sequenced at JGI (http://genome.jgi.doe.gov/programs/fungi) provides strong evidence on their involvement in lignin degradation (Barrasa et al., 2016). In this way, genes of the ligninolytic peroxidase families are found in all the white-rot (ligninolytic) basidiomycete genomes sequenced to date, but absent from all the brown-rot (cellulolytic) sequenced genomes. Recently, additional information on these enzymes has been obtained in post-genomic studies where the complete inventory of peroxidase genes from different basidiomycete genomes was heterologously expressed and the different LiP, MnP and VP products, among others, were analyzed providing the first demonstration of: **i)** VP ability to degrade lignin; and **ii)** C-terminal tail contribution to long MnP stability, among other relevant information (Fernández-Fueyo et al., 2014d; 2014a).

In contrast to well-known ligninolytic peroxidases, the first basidiomycete peroxygenase (currently known as unspecific peroxygenase, UPO) was reported only twelve years ago from *Agrocybe aegerita* (**Fig. 1C**). Ascomycete chloroperoxidase also belongs to the HTP superfamily but it has low oxygenase activity. Unrelated vanadium-containing (and H_2O_2 resistant) chloroperoxidase also catalyzes reactions of biotechnological interest (Fernández-Fueyo et al., 2015b; 2016b). Interestingly, HTP genes are well represented in most basidiomycete genomes sequenced at JGI, as well as in additional genomes sequenced in the search for HTP genes (Kellner et al., 2016). Despite this wide genomic presence and the biotechnological interest of mono(per)oxygenation reactions, which resulted in several recent patents (Landvick et al., 2016a; 2016b), just a few UPOs have been purified and characterized to date, and central aspects of their catalytic mechanism have been only recently solved (Wang et al., 2015).

UPOs share the same active site and reaction chemistry of cytochrome P450 monooxygenases (P450s) resulting in highly versatile oxidation/oxygenation reactions, which can be classified as follows: **a)** two-electron oxidations with O-transfer; **b)** two-electron oxidations with O-transfer and bond cleavage; **c)** two-electron oxidations with O-transfer to heteroatoms; and **d)** one electron oxidations (as typical peroxidases) (**Fig. 4A**) (Hofrichter et al., 2015). However,

compared with P450s that need a flavin-containing reductase or protein 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**). Nevertheless, UPOs exhibit some catalase activity, along with an oxidative instability, that must be taken into account for correct H_2O_2 dosage (Karich et al., 2016). In addition, UPOs are secreted enzymes and, therefore, more stable than P450s that are generally intracellular membrane-bound proteins.

Also recently, the so-called dye-decolorizing peroxidases (DyPs) have been described in basidiomycetes (Strittmatter et al., 2015) (**Fig. 1B**). Their classification in the CDE superfamily (including chlorite dismutase, DyP and EfeB protein from *Escherichia coli*) reveals a different phylogenetic origin (Linde et al., 2015b). In this case, the convergence with ligninolytic peroxidases not only includes a histidine residue as heme iron ligand (while a cysteine occupies this position in HTPs) but also a long-range electron transfer (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 electron paramagnetic resonance and directed mutagenesis studies revealed that such pathway starts at a radical-forming catalytic tryptophan, as previously described in LiP and VP (Baratto et al., 2015; Linde et al., 2015a). Simultaneously, the first fungal DyP oxidizing Mn^{2+} to Mn^{3+}, as MnP and VP do, has been described (Fernández-Fueyo et al., 2015a), providing another example of evolutionary convergence between unrelated enzymes oxidizing lignin products.

In the search for new peroxidases/peroxygenases (and enzymes of the other oxidoreductase superfamilies discussed below) the genomic inventories have been complemented with enzyme screening in fungi with different lifestyles and transcriptomic and secretomic studies using natural substrates under laboratory conditions (Barrasa et al., 2014; Fernández-Fueyo et al., 2014b; 2016a; Hori et al., 2014).

4. Flavin-containing oxidases/dehydrogenases

The GMC superfamily includes flavin-containing **(i)** oxidases, such as aryl-alcohol oxidase (AAO), methanol oxidase (MOX), pyranose 2-oxidase (P2O) and glucose oxidase (GOX), and **(ii)** dehydrogenases, such as cellobiose dehydrogenase (CDH, which contains flavin and heme domains) and glucose dehydrogenase (GDH). Their distribution, phylogenetic relationships and potential role in lignocellulose degradation has been recently reviewed based on the analysis of 10 sequenced Polyporales genomes (Ferreira et al., 2015a).

Opposite to that described above for ligninolytic peroxidases, a strict distribution of genes of different H_2O_2-generating oxidases was not observed in wood-rotting fungi. However, AAO appears as the most frequent GMC oxidase in the white-rot species, where H_2O_2 activates ligninolytic peroxidases. In contrast, MOX genes are more abundant in the brown-rot species, where H_2O_2 is reduced by Fe^{2+} yielding hydroxyl radical involved in the initial attack on cellulose by these fungi. AAO and P2O are secreted proteins and, therefore, can be more easily involved in the extracellular degradation of lignocellulosic materials. However, several pieces of evidence indicate that oxidases lacking such secretion mechanism, such as GOX and especially MOX, can be also involved in lignocellulose decay thanks to an alternative secretion process or simply by hyphal lysis. AAO can be considered as the model GMC oxidase in lignocellulose decay, with recent studies on the catalytic mechanism of the best known *Pleurotus eryngii* enzyme (Ferreira et al., 2015b) (**Fig. 1F**), and isolation of new AAOs of interest (Couturier et al., 2016).

Among GMC dehydrogenases, CDH is involved in cellulose decay by white-rot fungi, in agreement with gene distribution in 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 (**Fig. 1E**), where cellobiose is oxidized to cellobionolactone, to the heme (or cytochrome) 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 will be explained below, other dehydrogenases can also promote the action of LPMO by quinone redox cycling, as in the case of the first basidiomycete GDH and the first aryl-alcohol dehydrogenase (AAD) reported to date (both identified in the *Pycnoporus cinnabarinus* genome) (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 access channel, compared with that of AAO that only enables the access of O_2 (**Fig. 5**).

Another two microbial flavooxidases of biotechnological interest in organic synthesis are vanillyl-alcohol oxidase (VAO; **Fig. 1D**) and eugenol oxidase, which belong to a different protein superfamily, 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

Three different copper-containing oxidoreductases are considered in this section: **i)** CRO; **ii)** MCO (laccases); and **iii)** LPMOs. The CRO family is characterized by the presence of a copper ion and a protein radical involved in catalysis, and includes glyoxal oxidase, the first H_2O_2-producing enzyme described in the model white-rot fungus *Phanerochaete chrysosporium*, and galactose oxidase that, in addition to oxidize monosaccharides, has some activity on benzylic alcohols (Kalum et al., 2014b). CROs are present in most basidiomycete genomes, and some of them showed new catalytic properties of interest after their heterologous expression (Daou et al., 2016). Among MCOs, laccases are characterized by the presence of four copper ions (**Fig. 1G**) and, together with peroxidases, are the most thoroughly studied oxidoreductases in wood rotting fungi and, by far, the largest number of biotechnological applications have been reported for these multicopper enzymes (Mate and Alcalde, 2016; Pezzella et al., 2015). Due to the high number of well characterized laccases with different redox potentials and other properties, recent work on these enzymes focused on tailoring the catalytic properties of commercially available, and other laccases of interest, for target reactions by rational design, often guided by computational simulations, and directed evolution, as described in the next sections.

In contrast with the above copper-containing enzymes that are known for many years (fungal laccase was for the first time reported in the early 1960's), LPMOs (**Fig. 1H**) were recognized as a new oxidoreductase family, playing a crucial role in cellulose degradation, only six years ago (Martínez, 2016). 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 family GH61. This striking confusion originated from: **i)** whole sequence similarities with glycoside hydrolases, including the presence of carbohydrate binding domains in some of them; **ii)** weak hydrolase activity of some LPMOs or their contaminating proteins; and **iii)** requirement of a reducing

(electron providing) substrate for enzyme activation. Such 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 importance for lignocellulose biorefineries.

As summarized in **Fig. 6**, different mechanisms can operate fueling electrons for LPMO activation in wood-rotting 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 routes for LPMO activation, 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). Another LPMO activating routes could involve photosynthetic pigments and lignin-derived phenols whose radicals after LPMO oxidation would be reduced back by some lignin fractions/domains (Martínez, 2016)

Due to the relatively recent reports on LPMO structure and activity, several important aspects of its reaction mechanism are still to be fully understood: from the reactive oxygenation species to the interaction with substrates. Concerning the latter aspect, recent evidence from 2D nuclear magnetic resonance (NMR) spectroscopy suggests that reductants, such as CDH, and cellulose bind the same region of the LPMO molecule (**Fig. 7**), in contrast with the alternative 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 different 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, enabling significant reduction of time dedicated to experimental engineering work.

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. The state-of-the-art technology for modeling substrate and product diffusion in enzymes is PELE (an acronym for Protein Energy Landscape Exploration) being capable of accurately reproducing long time scale processes in only few hours of CPU (https://pele.bsc.es).

The PELE algorithm, which combines a steered stochastic approach with protein structure prediction methods capable of projecting the migration dynamics of ligands in proteins, is based on three main steps: **i)** ligand and protein local perturbation, including translation and rotation of the ligand, and protein α-carbon displacement following an anisotropic network model approach; **ii)** side-chain sampling, by placing all side chains local to the ligand; and **iii**) energy minimization of a region including, at least, all residues local to the atoms involved in **(i)** and **(ii)**. Typically, a simulation involves several processors running multiple steps and sharing information towards addressing a common task.

As shown in different studies described in next sections, PELE modeling properly informs about substrate access and positioning at the enzyme active site (Babot et al., 2015a) including distances and angles between the redox centers (Lucas et al., 2016; Molina-Espeja et al., 2016a) but, in oxidoreductase reactions, this is often followed by electron transfer estimation by quantum calculations, as described below. Furthermore, a computational methodology accumulating beneficial interactions between a laccase and a target substrate has resulted, through a repurposing strategy, useful to design a novel polar binding scaffold to anchor negatively charged groups (Giacobelli et al., 2017).

Following the biophysical study (PELE simulation of substrate diffusion into the active site) it is possible to perform the quantum biochemical characterization of the electronic coupling involved in the oxidation process. In a model study with a basidiomycete peroxidase, the values obtained showed a striking correlation with the oxidation turnover values determined experimentally validating the computational approach (**Fig. 8**) (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, being able 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 *in silico* mutated variants, 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. This methodology has been applied for engineering laccase for aniline oxidation, as described below, and the calculations validated by experimental determination of the kinetic constants of the best predicted variant (Santiago et al., 2016). In this way, a binding focused general strategy based on QM/MM reactivity scoring has been proposed for laccase engineering (Monza et al., 2015).

A similar approach has been used to rationalize the improvements observed in: **i)** laccase oxidation of phenols (sinapic acid) after saturation mutagenesis at the substrate binding site (Pardo et al., 2016b); and **ii)** stereoselective sulfoxidation after directed mutagenesis at the DyP active site (Linde et al., 2016). Mixed QM/MM calculations were also used to identify the LRET pathways that characterize peroxidase oxidation of bulky substrates, using 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) as shown for anthraquinoid dye oxidation by DyP (Linde et al., 2015a).

7. Engineering oxidative enzymes

Oxidoreductase engineering for industrial application considers both directed mutagenesis (rational design) and directed molecular evolution, as well as combinations of both (semi-rational approaches) on the whole protein or focused on target regions (Maté et al., 2016; Molina-Espeja et al., 2016b; Pardo and Camarero, 2015b; Viña-González et al., 2016), including the development of new directed evolution methods (González-Pérez et al., 2014b). A prerequisite for all protein engineering methodologies is the availability of an expression system to generate improved variants. Therefore, the heterologous expression (in *Saccharomyces cerevisiae*, *Escherichia coli* or other systems) of oxidoreductase genes was first 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) engineering.

In rational engineering studies, VP has been used as a model peroxidase and both oxidative and alkaline inactivation have been addressed to develop 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 (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 and, consequently, the accumulation of compound I, whose reaction with H_2O_2 excess inactivates the enzyme *via* non-catalytic compound III (Sáez-Jiménez et al., 2015a).

While the 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, based on: **i)** selection of a naturally-stable peroxidase (MnP) from genome screening (and heterologous expression); **ii)** identification of the structural determinants for this stability (such as H-bonds, salt bridges and basic residues exposed to the solvent in the crystal structure); and **iii)** introducing them in the target enzyme (VP) by directed mutagenesis (Sáez-Jiménez et al., 2015d).

Rational design has been also used to create a peroxidase (VP) with strong ligninolytic activity due to its ability to act at extremely acidic pH (that increases the redox potential of the heme iron). With this purpose, the catalytic tryptophan of VP (and LiP) was introduced in a peroxidase (MnP) scaffold from genome screening 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 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 (Linde et al., 2016).

Paralleling the rational design described above, directed evolution and hybrid strategies have been applied to improve the H_2O_2 (González-Pérez et al., 2014a) and alkaline (González-Pérez et al., 2016) stability of VP. On the other hand, polyvalent UPO was submitted to directed evolution to enhance its mono(per)oxygenase activity (and reduce competing one-electron oxidation peroxidase activity) for oxyfunctionalizations of biotechnological interest, such as naphthalene hydroxylation to 1-naphthol (**Fig. 9**) (Gómez de Santos et al., 2016; Molina-Espeja et al., 2015b; 2016a). Moreover, directed evolution and other engineering approaches (such as domain swapping) have also been applied to laccase variants expressed in yeast to improve properties of interest, such as oxidation of different phenolic substrates (Maté and Alcalde, 2016; 2015; Pardo et al., 2016a; Pardo and Camarero, 2015a; 2015b; Vicente et al., 2016) and *ad hoc* high-throughput screening strategies have been developed (Pardo and Camarero, 2017).

The engineered enzymes were expressed using a *Saccharomyces cerevisiae-Pichia pastoris* tandem system (Molina-Espeja et al., 2015a) and the industrial expression technology of Novozymes (Vind et al., 2015) for the applications described below (together with *Escherichia coli* expression and *in vitro* activation for some laboratory-scale reactions). The industrial applicability often improved by enzyme immobilization (Lettera et al., 2016; Poraj-Kobielska et al., 2015b).

8. Enzymatic oxyfunctionalization

Basidiomycete UPOs appear as a 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 oxygenation reactions have also been developed with engineered variants of other peroxidases, such as DyP sulfoxidation mentioned above (Linde et al., 2016). Several oxyfunctionalization reactions of biotechnological interest are described below.

1-Naphthol - a large market chemical (40,000 tons/year) used in the production of herbicides, insecticides, pharmaceuticals and dye precursors - can be obtained by naphthalene hydroxylation using an UPO variant evolved with this purpose through an *ad hoc* screening protocol (**Fig. 9**) (Molina-Espeja et al., 2016a). Epoxidation of non-cyclic alkenes and terpenes of interest for the chemical sector using different UPOs has also been obtained (Lund et al., 2016).

25-Hydroxyvitamin D_3 - of interest in feeding chickens and other farm animals to reduce skeleton problems caused by rapid growth and reduced mobility - can be produced using the *Coprinopsis cinerea* UPO due to the *ad hoc* active-site architecture for vitamin D_3 regioselective hydroxylation (**Fig. 10**) (Babot et al., 2015b; Lucas et al., 2016). Related hydroxylations are those catalyzed by different basidiomycete peroxygenases on a variety of steroidal substrates (mainly at their 25 position) yielding products with antimicrobial properties, among others (Babot et al., 2015a).

Desmethylation in the synthesis of human metabolites of the bile acid reabsorption inhibitor SAR548304, a seven-step chemical reaction using palladium catalysis and laborious chromatographic purification with an overall yield of only 27%, can be obtained by one-pot selective (and high yield) *N*-desmethylation using the *Marasmius rotula* UPO (**Fig. 11**) (Kiebist et al., 2015). Also of pharmaceutical interest is the use of the same UPO for the enzymatic removal of corticoid side-chain, involving hydroxylation and C-C bond cleavage (Poraj-Kobielska et al., 2015a).

Stereoselective hydroxylation of ethylbenzene into (*R*)-1-phenylethanol, an example of enzymatic production of chiral secondary alcohols, can be achieved in a methanol supported and by-product free multi-enzymatic cascade using: **a)** *Agrocybe aegerita* UPO; **b)** methanol oxidase for H_2O_2 supply; **c)** formaldehyde dismutase, converting each two formaldehyde molecules into one molecule of methanol (reacting again with methanol oxidase) and one molecule of formic acid; **d)** formate dehydrogenase converting formic acid into CO_2 and H_2O at expenses of $NAD(P)^+$; and **e)** 3-hydroxybenzoate-6-hydroxylase regenerating $NAD(P)^+$ from the NAD(P)H formed in the previous dehydrogenase reaction (**Fig. 12**) (Hollmann and Ni, 2016; Ni et al., 2016). Concerning the last step in the enzyme cascade, other NAD(P)H regeneration systems have also been reported (Pham et al., 2015).

Last but not least, enzymatic oxygenation of long-chain *n*-alkanes at their unreactive terminal position has been very recently reported using the *Marasmius rotula* UPO, yielding dicarboxylic acids and other oxygenated products from these largely inert compound family (Olmedo et al., 2016). Note that all other basidiomycete peroxygenases and wild-type P450s mostly produce subterminal diols, among other products from alkane oxygenation (Lund et al., 2014).

9. Enzymatic oxidations

A series of oxidation reactions of biotechnological interest are catalyzed by different oxidoreductases, UPOs included, for the production of polymer building blocks, dyeing molecules, and other compounds of interest, as described below.

According to the US Department of Energy, HMF is one of the top-ten renewable chemicals for a sustainable bioeconomy, as the precursor of FDCA and other platform chemicals, such as 2,5-diformylfuran (DFF). HMF-derived FDCA is the bio-based alternative to fossil-based terephthalic acid for the production of polyester-type plastics. HMF is an aromatic (benzylic) alcohol and, therefore, a substrate of AAO, and this oxidase also acts on hydrated aldehydes (*gem*-diols). Therefore, an enzyme cascade has been developed where the limiting step in HMF oxidation by AAO, i.e. the 2,5-formylfurancarboxylic acid (FFCA) oxidation to FDCA, is catalyzed by UPO at expenses of the H_2O_2 generated by AAO in the two initial oxidation steps (**Fig. 13**) (Carro et al., 2015). On the other hand, a galactose oxidase variant has been claimed for the conversion of HMF into DFF, since it selectively catalyzes the first oxidation step (Kalum et al., 2014b).

A variety of indigo dyes, differing in the benzene ring substituents, can be obtained by oxidative coupling of the 3-hydroxyindol precursors obtained by indole hydroxylation by UPO (**Fig. 14A**) (Herold-Majumdar et al., 2016; Kalum et al., 2014a). In this way enzymatic *in situ* dyeing of fabrics is possible, with the indigo dye becoming insoluble once oxidized, and the color remaining after fabric washing (**Fig. 14B-D**). Additionally, green routes for the production of polymeric dyes can be developed by the enzymatic copolymerization of aniline-type and phenolic precursors, such as 2,5-diaminebenzenesulfonic acid and resorcinol, by laccase (Pezzella et al., 2016) and *ad hoc* evolved variants (Vicente et al., 2016). Such reactions are milder and 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 (such as Nyloset Brown N2R) (**Fig. 15**).

Synthesis of conductive polyaniline has been widely investigated during the last decade for a range of applications (sensor devices, rechargeable batteries, etc). However, the conducting polyaniline polymer is industrially produced under strongly acidic conditions and using strong oxidants. Recently, a laccase variant that exhibits improved aniline oxidation has been obtained by a combination of computational simulations and directed evolution tools (Santiago et al., 2016) as a green alternative for polyaniline synthesis under industrially relevant conditions (de Salas et al., 2016) (**Fig. 16**).

Lignin removal is a bottleneck for biomass conversion into fuels and chemicals in lignocellulose biorefineries that is overcome using strong physicochemical treatments. Mimicking those changes caused by lignin-degrading white-rot fungi (van Kuijk et al., 2016), isolated oxidoreductases (especially laccases in the presence/absence of redox mediators) have been applied as milder and more environmentally-friendly pretreatments of woody and nonwoody lignocellulosic feedstocks (Rencoret et al., 2016). In these studies, changes in lignin structure during the enzymatic treatment have been followed using 2D-NMR, the state-of-the-art technology for structural characterization of lignin (Rico et al., 2015).

Concerning technical lignins from lignocellulose biorefineries (with variable phenolic content), treatment with fungal oxidoreductases (as laccases and peroxidases) can be followed by growth of selected bacteria acting as a microbial sink for the synthesis of products of

interest through metabolic engineering (Salvachúa et al., 2016). Interestingly, using a water soluble lignin (lignosulfonate) as substrate, and a combination of 2D-NMR and stopped-flow rapid spectrophotometry, it has been possible to demonstrate that: **i)** ligninolytic peroxidases are able to abstract 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 nonphenolic lignin (Sáez-Jiménez et al., 2015b; Sáez-Jiménez et al., 2015c; Sáez-Jiménez et al., 2016).

In the near future, advanced processing of biomass in lignocellulose biorefineries could take advantage from synthetic biology tendencies and tools to combine the extracellular lignin and cellulose degrading machineries (ligninosome and cellulosome) and the intracellular fermentative capabilities of microorganisms to develop biofuel-producing "white-rot yeasts" and other tailor-made microorganisms (**Fig. 17**) (Alcalde, 2015; González-Pérez, 2016; González-Pérez and Alcalde, 2014).

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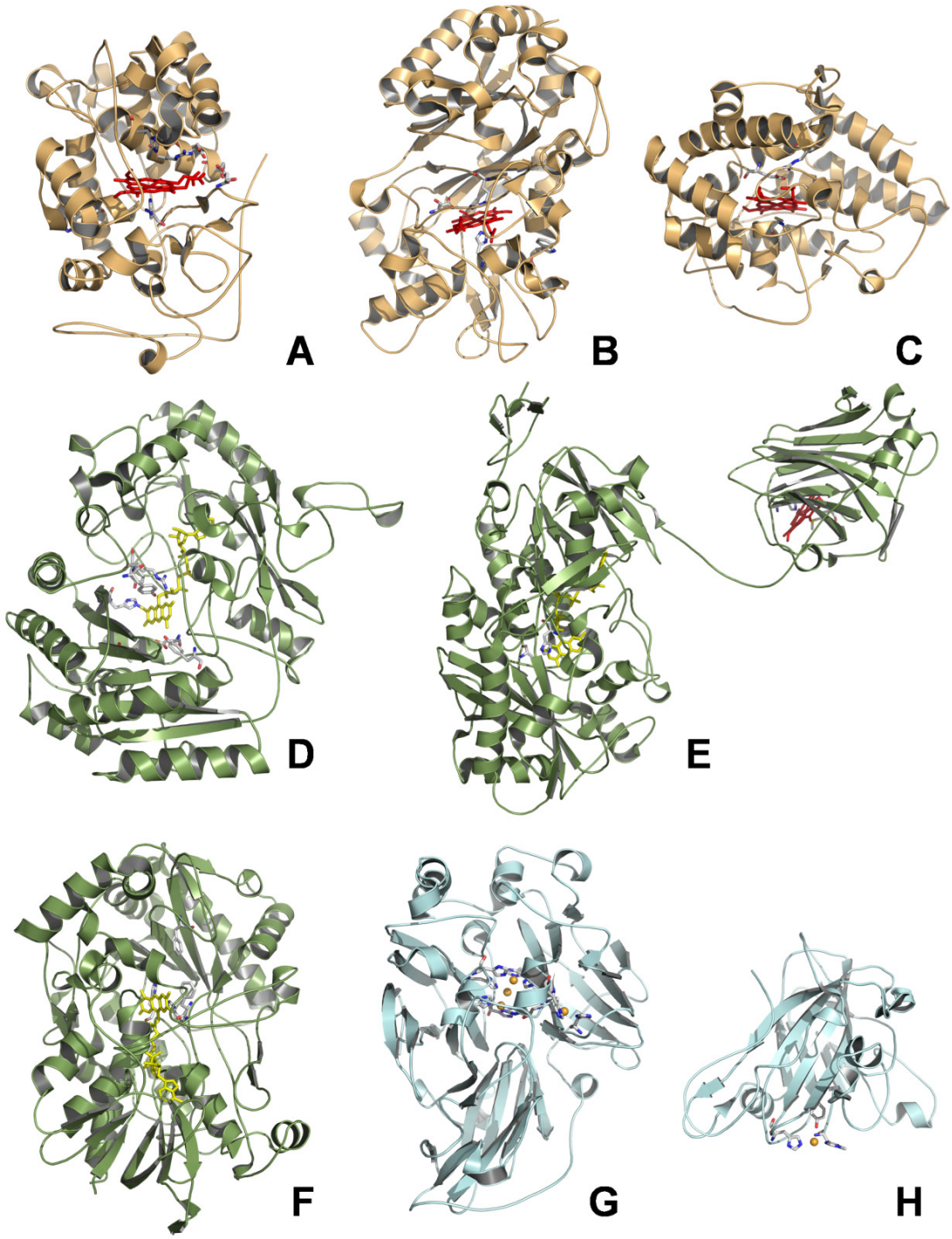

Fig. 1. Classical and new oxidoreductase families involved in lignocellulose degradation and other biotransformations of interest: **A.** *Pleurotus eryngii* VP (3FJW); **B.** *Auricularia auricula-judae* DyP (4W7J); **C.** *Agrocybe aegerita* UPO (2YP1); **D.** *Penicillium simplicissimum* VAO monomer (1VAO); **E.** *Neurospora crassa* CDH with flavin and heme domains (4QI7); **F** *Pleurotus eryngii* AAO (3FIM); **G.** *Pycnoporus cinnabarinus* laccase (2XYB); and **H.** *Thermoascus aurantiacus* LPMO (2YET). Cofactors (heme/FAD and copper ions as red and yellow sticks and orange spheres, respectively) and residues relevant for catalysis (Corey/Pauling/Koltun, CPK, colored sticks) such as: **i)** His, Cys and Met/His ligands of heme iron in **A/B**, **C** and **E**, respectively; **ii)** His/Arg, Asp/Arg and Glu/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 **B**; **v)** active site residues in **D-F**, including His linked to FAD in **D**; **vi)** 4 copper ions in **G**; and **vii)** 2 His and 1 Tyr copper ligands in **H**.

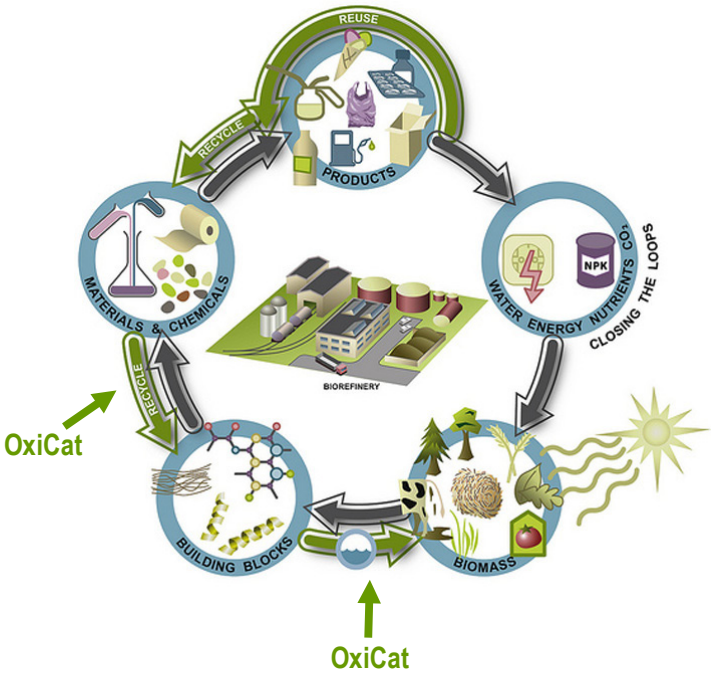

Fig. 2. Oxidative biocatalysts for circular economy. Production of renewable building blocks and manufacture of sustainable chemicals and materials are two steps where oxidative biocatalysts (OxiCats) can exert the most positive impact enabling greener and more efficient biotransformation routes. Adapted from http://biconsortium.eu/news/bioeconomy-circular-nature.

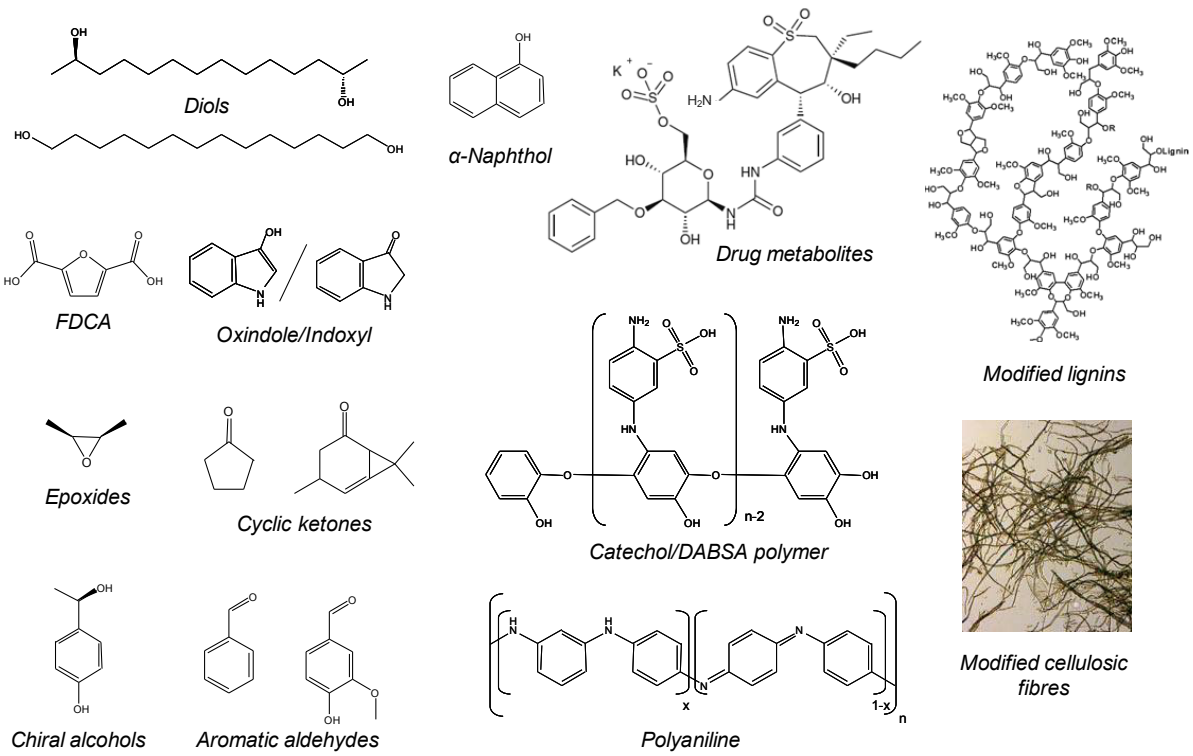

Fig. 3. Some model target chemicals whose production using oxidative enzymes has been evaluated.

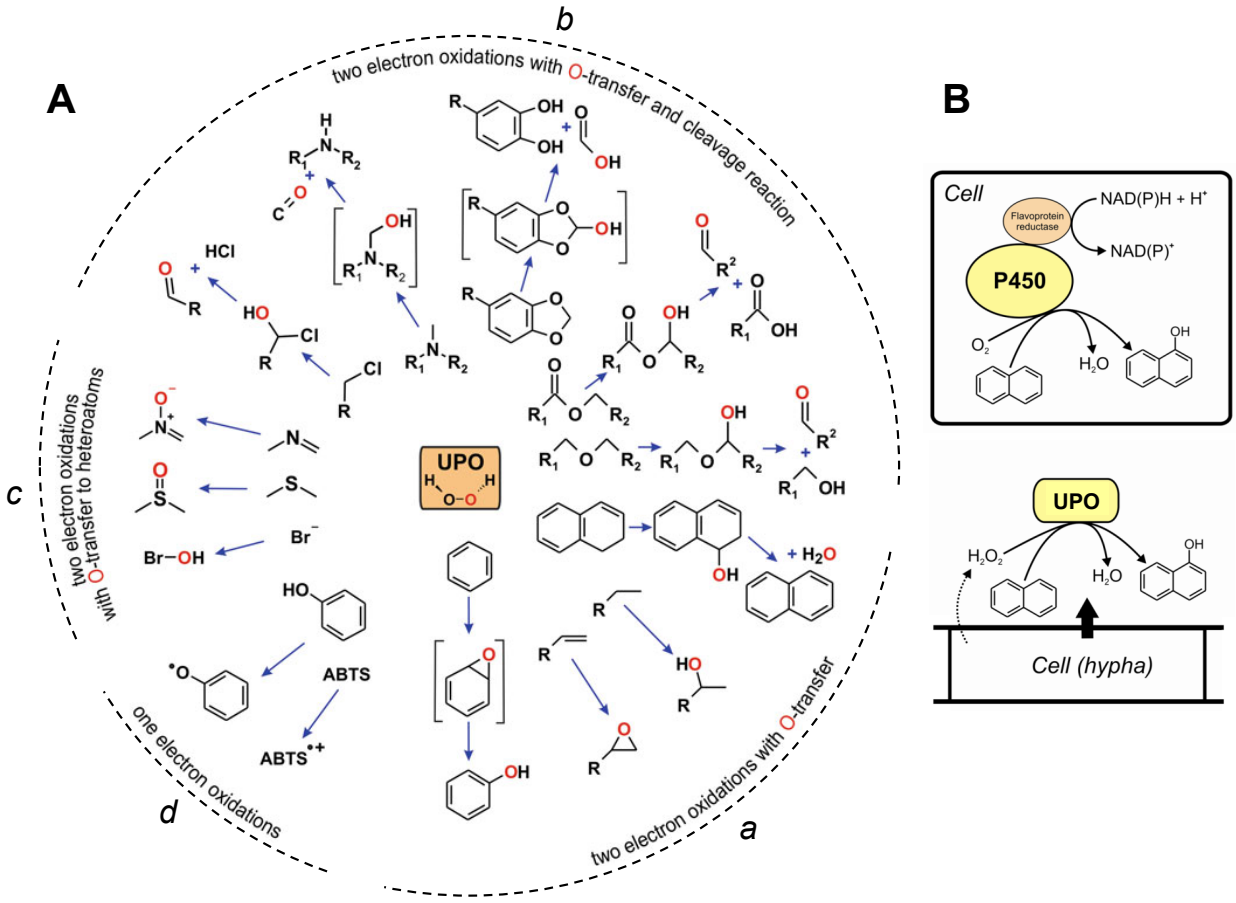

Fig. 4. Basidiomycete UPO catalyzing a variety of monooxygenation reactions (and others) with advantages over P450s. A. UPO oxidation and oxyfunctionalization reactions, at expenses of H_2O_2, include: **a)** two-electron oxidations with O-transfer; **b)** two-electron oxidations with O-transfer and cleavage reaction; **c)** two-electron oxidations with O-transfer to heteroatoms; and **d)** one-electron oxidations. **B.** While intracellular P450s (top) require a source of reducing power (NAD[P]H) and an auxiliary flavin-containing reductase or protein domain (often wasting 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). Partially adapted from Hofrichter et al. (2015).

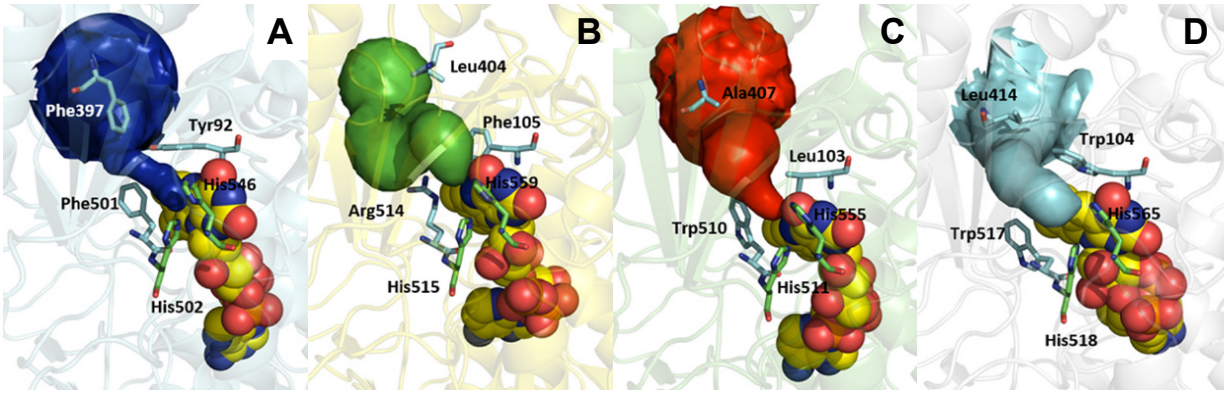

Fig. 5. Wider FAD-access channels in LPMO-activating AADs compared with AAO. The channels connecting the active-site cavity to solvent in *Pleurotus eryngii* AAO (PDB 3FIM) (**A**) and three AADs (AAQO1-AAQO3) from the *Pycnoporus cinnabarinus* genome and secretome (**B-D**, homology models) are shown. 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**). Adapted from Mathieu et al. (2016).

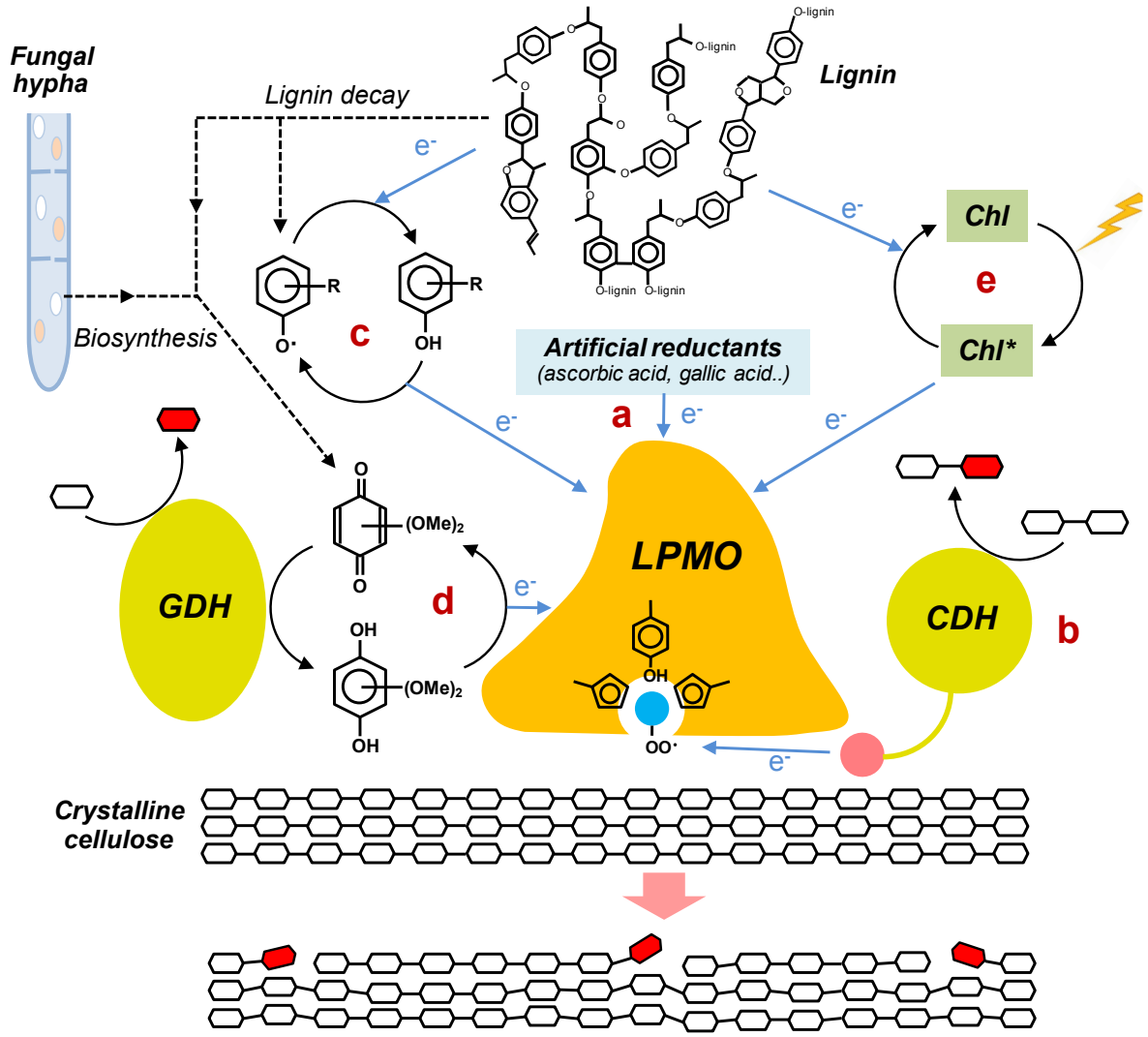

Fig. 6. Different LPMO activation routes. After initial studies, where LPMO activity was detected using artificial reductants, several mechanisms have been shown to operate "fueling" electrons to the LPMO copper cofactor for the oxidative breakdown (red units) of crystalline cellulose. These alternative mechanisms involve other enzymes, such as CDH (being directly oxidized by LPMO) and GDH (acting through redox cycling of quinones derived from lignin decay or fungal metabolism), as well as lignin-derived phenoxy radicals (being reduced by lignin) and light-activated photosynthetic pigments (Chl). Adapted from Kracher et al. (2016) and Martínez (2016).

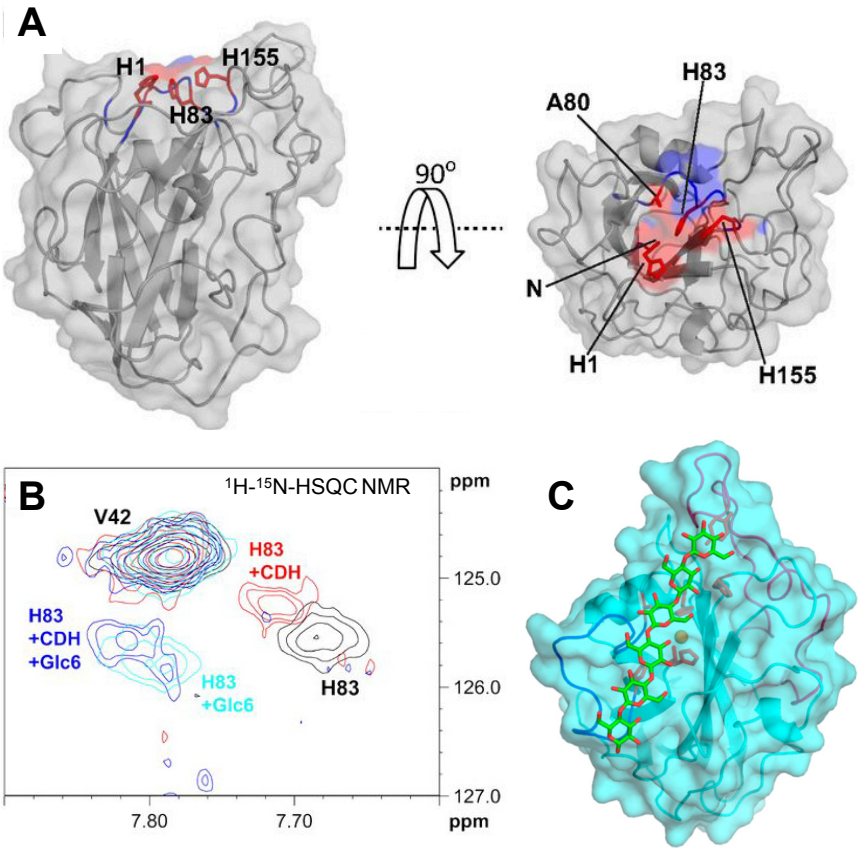

Fig. 7. 2D-NMR study on LPMO binding to substrates. His83, one of the copper ligands together with His155 and His1 side-chain and terminal amine (N) (**A**), participates in cellulose binding on LPMO (**C**) as shown by strong signal displacement in the ^{1}H-^{15}N heteronuclear single quantum (HSQC) spectra acquired in the absence (black contour) and the presence (cyan contour) of a cellulose hexasaccharide (Glc6) (the unaffected signal of Val42 is shown as reference) (**B**). More interestingly, a smaller but significant displacement of the above NMR signal in the presence of CDH suggests that the same site, near the catalytic copper, is also involved in binding of CDH, or other copper-reducing molecules, for LPMO activation. The strongest interactions (NMR displacements) correspond to red surface in **A**, followed by blue surface. Adapted from Courtade et al. (2016).

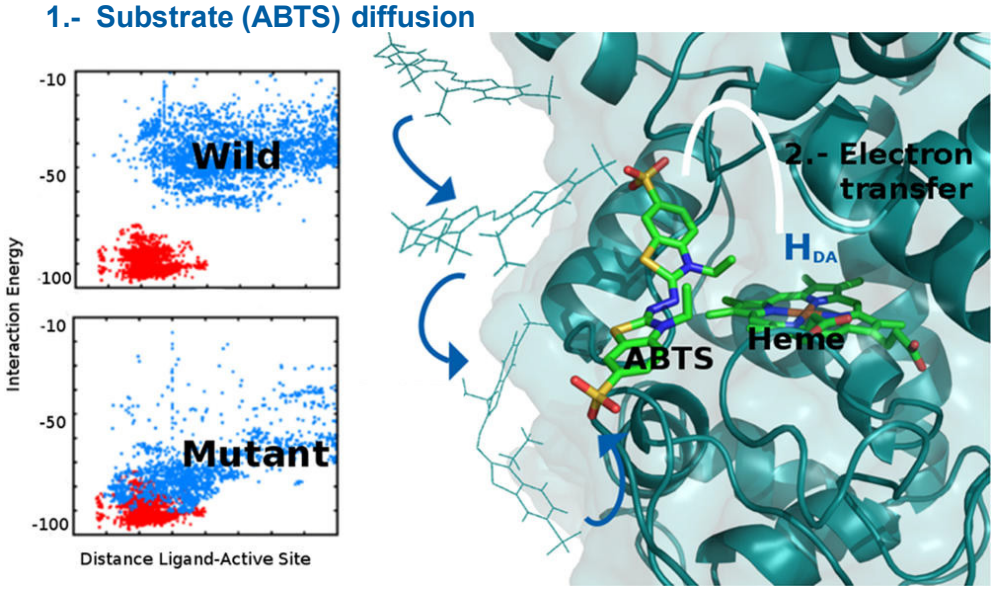

Fig. 8. Simulations guiding introduction of a new peroxidase activity. A highly-stable peroxidase was engineered for oxidation of a new substrate (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid], ABTS) using PELE simulations for substrate diffusion in wild and mutant enzyme (left) and electron transfer estimation by quantum calculations after proper docking at the active site (right). The substrate binding residues to be introduced were identified by previous substrate diffusion on an active enzyme, and the new activity of the engineered enzyme was experimentally confirmed by PCR mutagenesis, heterologous expression and estimation of kinetic constants. ABTS-heme distances *vs* interaction energies in the model active 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. Adapted from Acebes et al. (2016).

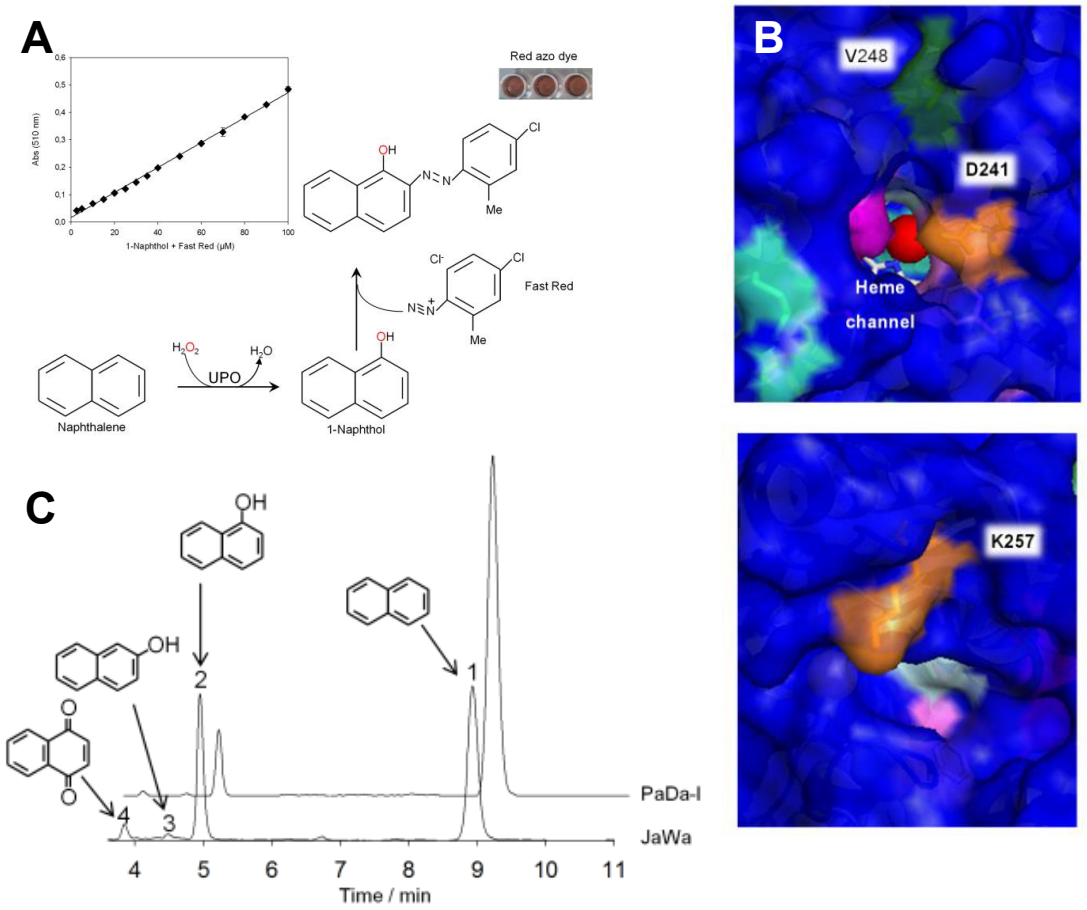

Fig. 9. Directed evolution and enzymatic production of 1-naphthol. A. Fast red-based screening resulting in several UPO variants hydroxylating naphthalene. **B.** Mutations in the best UPO variant (JaWa) located at the heme channel (top) and the surface (bottom) of the UPO crystal structure. **C.** HPLC profile of 1-naphtol production by JaWa compared with a second variant (PaDa-I). Adapted from Molina-Espeja et al. (2016a).

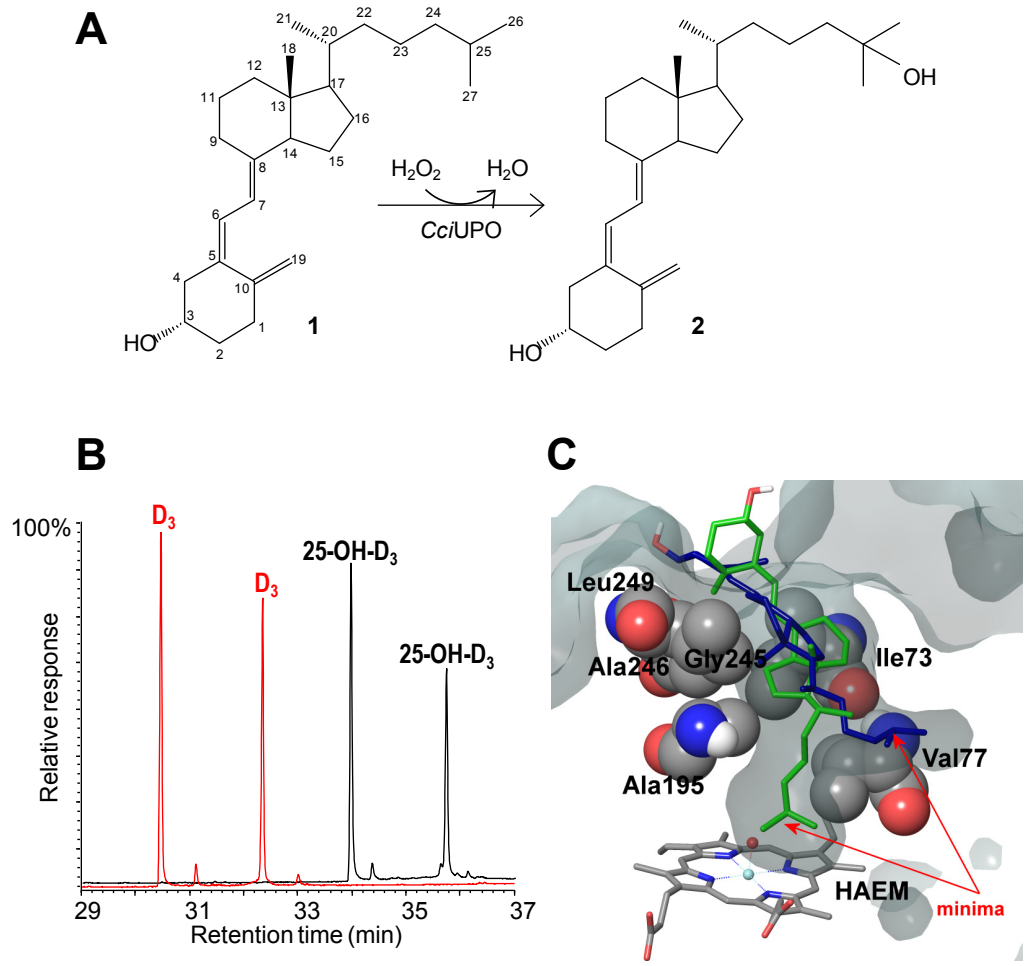

Fig. 10. Regioselective hydroxylation by UPO. Regioselective conversion of vitamin D_3 (**1**) into 25-hydroxyvitamin D_3 (**2**) by *Coprinopsis cinerea* UPO (**A**) was shown by GC-MS profiles of reactions (**B**, black line) compared with controls (**B**, red line), and explained by PELE simulations 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 amino acid residues are shown as CPK spheres (part of the solvent access surface is also shown). Adapted from Lucas et al. (2016).

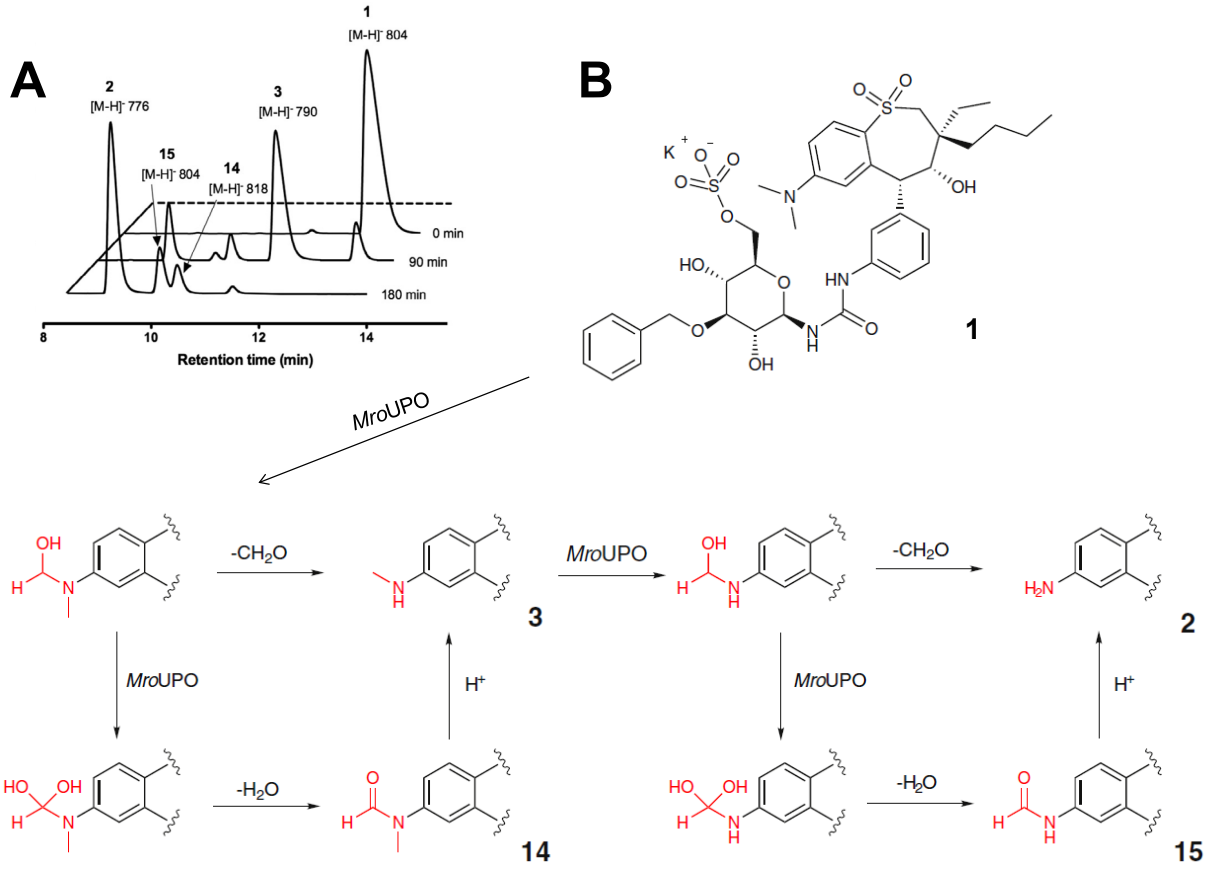

Fig. 11. Synthesis of human metabolites by basidiomycete peroxygenase. A. The bile acid reabsorption inhibitor SAR548304 is N-desmethylated by *Marasmius rotula* UPO, as shown by HPLC analysis at different reaction times. **B.** The postulated *N*-desmethylation mechanism (from 1 to 2) may proceed *via* an unstable hemiaminal intermediate and formation of *N*-formyl derivatives (14 + 15) arising from a geminal alcohol intermediate. Adapted from Kiebist et al. (2015).

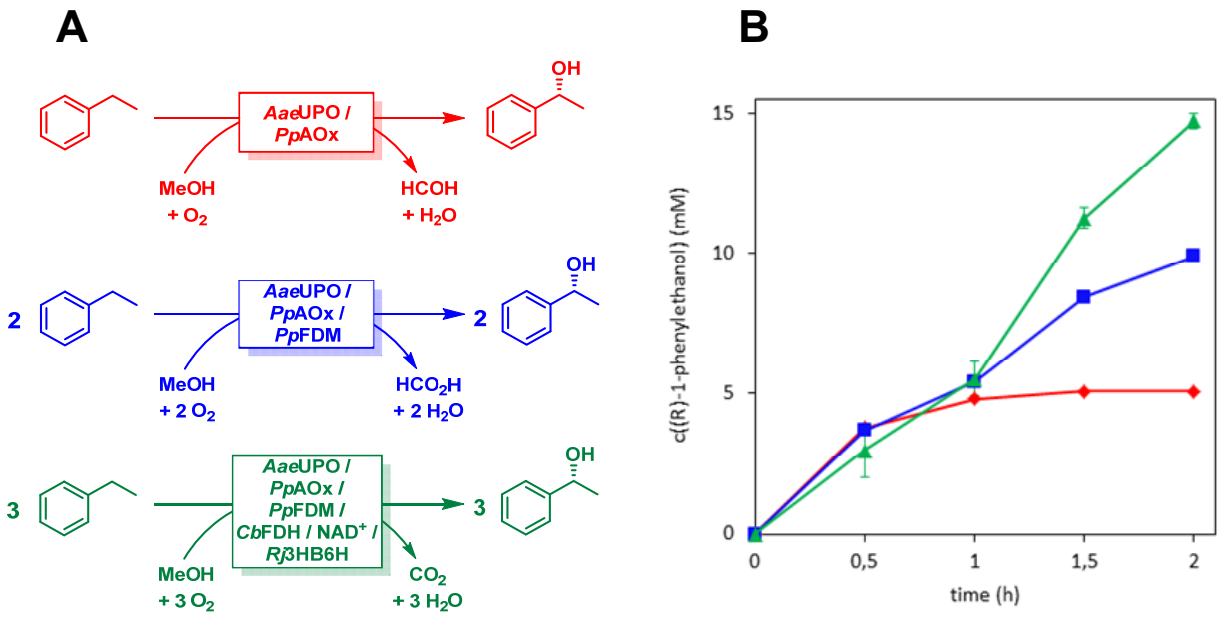

Fig. 12. Stereoselective hydroxylation in a byproduct-free enzyme cascade. Ethylbenzene conversion into (*R)*-1-phenyethanol by UPO, at expenses of methanol oxidation to CO_2 and H_2O, was obtained by increasing the number of cascade steps (**A**) resulting in higher product yield (**B**) as shown by 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*. Adapted from Ni et al. (2016).

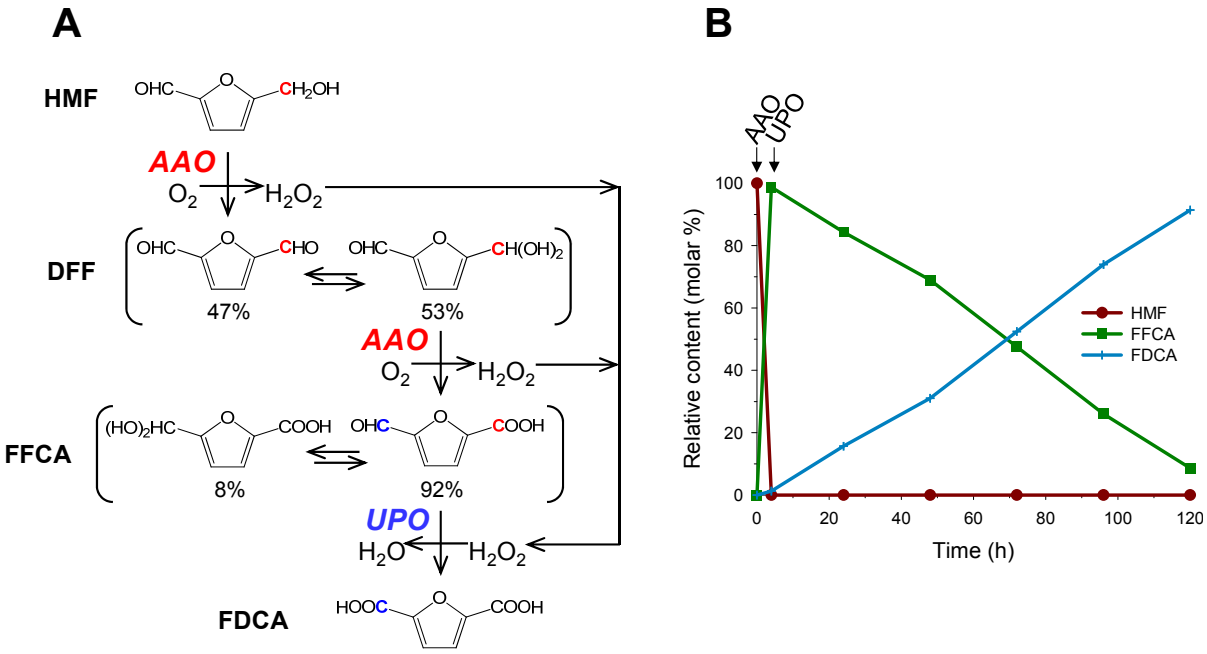

Fig. 13. Enzymatic production of FDCA from renewable HMF. A. Scheme for a cosubstrate-free cascade including**: i)** HMF oxidation by AAO to DFF, whose hydrated (*gem*-diol) form undergoes a second AAO oxidation yielding FFCA that is not substrate of AAO due to its low hydration degree; and **ii)** Conversion of unhydrated FFCA into FDCA by UPO at expenses of the H_2O_2 previously formed by AAO, with H_2O as the only by-product. **B.** Time course of HMF products by the above cascade (GC-MS analysis) with AAO and UPO additions at times 0 and 4 h, respectively (DFF was not detected due to its rapid conversion by AAO). Adapted from Carro et al. (2015).

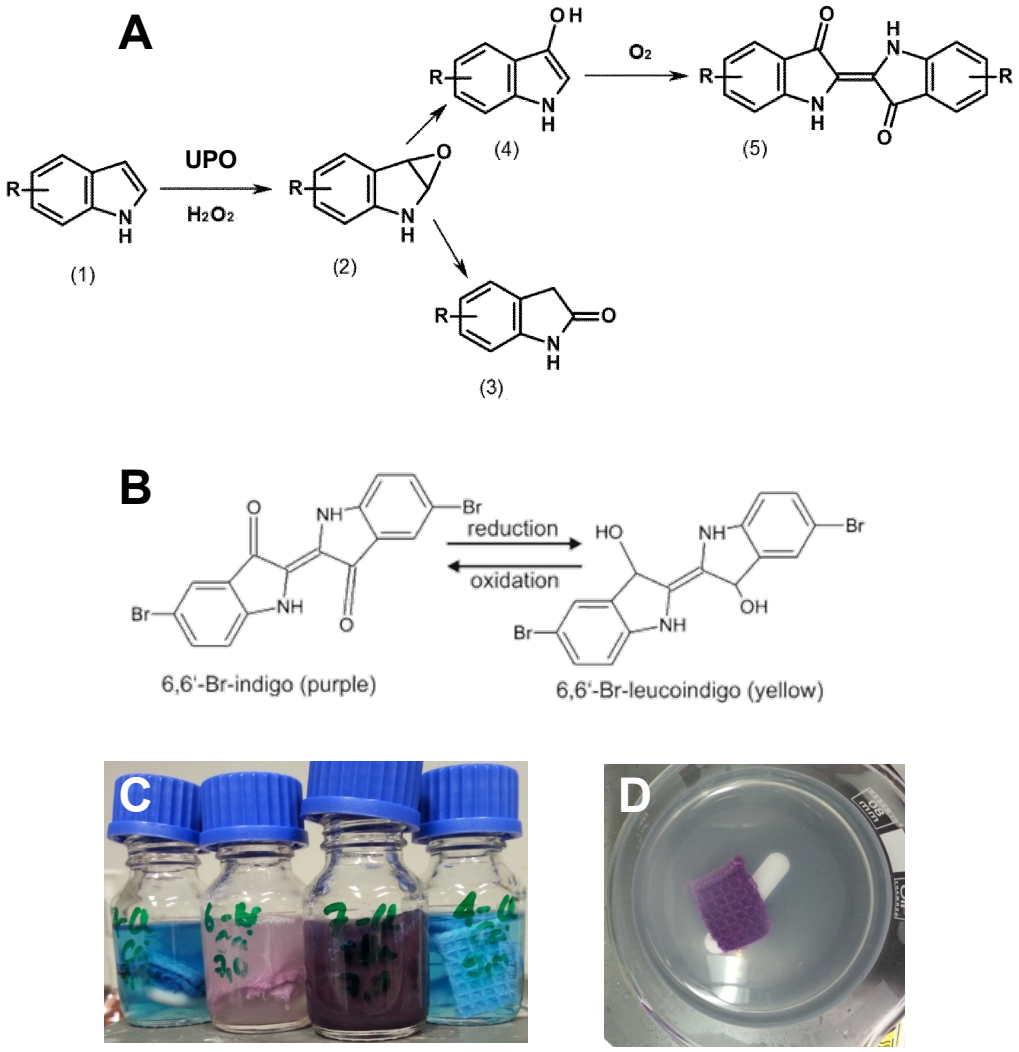

Fig. 14. Enzymatic indigo dyes for fabric dyeing. A. Proposed reaction mechanism for the UPO-catalyzed formation of different indigo dyes. (1) 1*H*-indoles; (2) 2,3-epoxy-1*H*-indoles; (3), 3,3-dihydro-1*H*-indol-2-ones; (4) 3-hydroxy-1*H*-indoles; (5) indigos: R= H (indigo), R=Br (6,6'-dibromoindigo) or R= Cl (6,6'-dichloroindigo). **B.** Oxidized and reduced (soluble) forms of Tyrian purple (6,6'-bromoindigo) during the dyeing process. **C.** Enzymatic *in situ* dyeing of cotton fabrics. **D.** Dyeing persistence after washing one of the above fabrics dyed with enzymatic Tyrian purple. In part (**A** and **B**) adapted from Kalum et al. (2014a) and Herold-Majumdar et al. (2016).

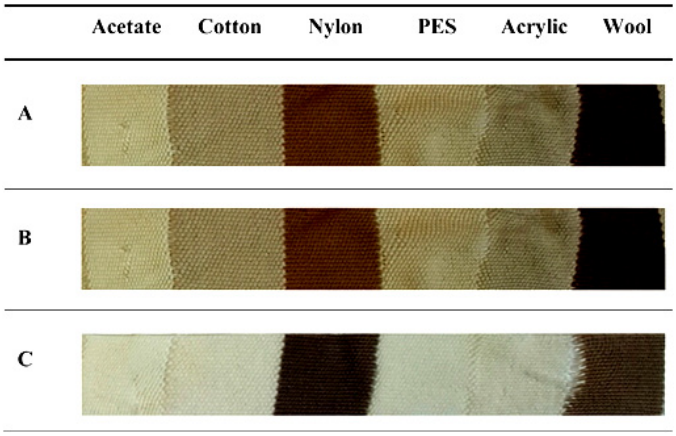

Fig. 15. Multifiber test of enzymatic biodyes. Two dyes obtained by laccase copolymerization of 2,5-diaminobenzenesulfonic acid and resorcinol in different ratios (**A,B**) yielded results on natural and synthetic fibers comparable to a commercial dye (**C**). Adapted from Pezzella et al. (2016).

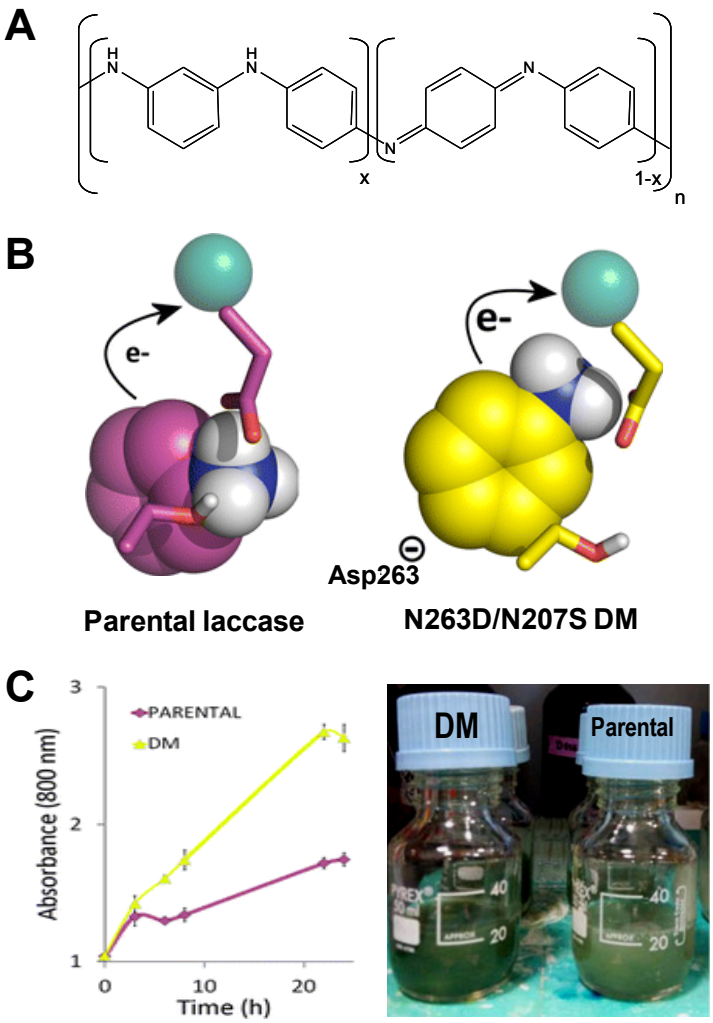

Fig. 16. Polyaniline production with a laccase double mutant (DM). A. Formula of conductive polyaniline. **B.** Two mutations predicted by PELE simulations enabled efficient electron transfer (estimated by QM/MM calculations) from docked aniline (CPK-colored spheres) to laccase copper-1 (cyan spheres) 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. Adapted from Santiago et al. (2016).

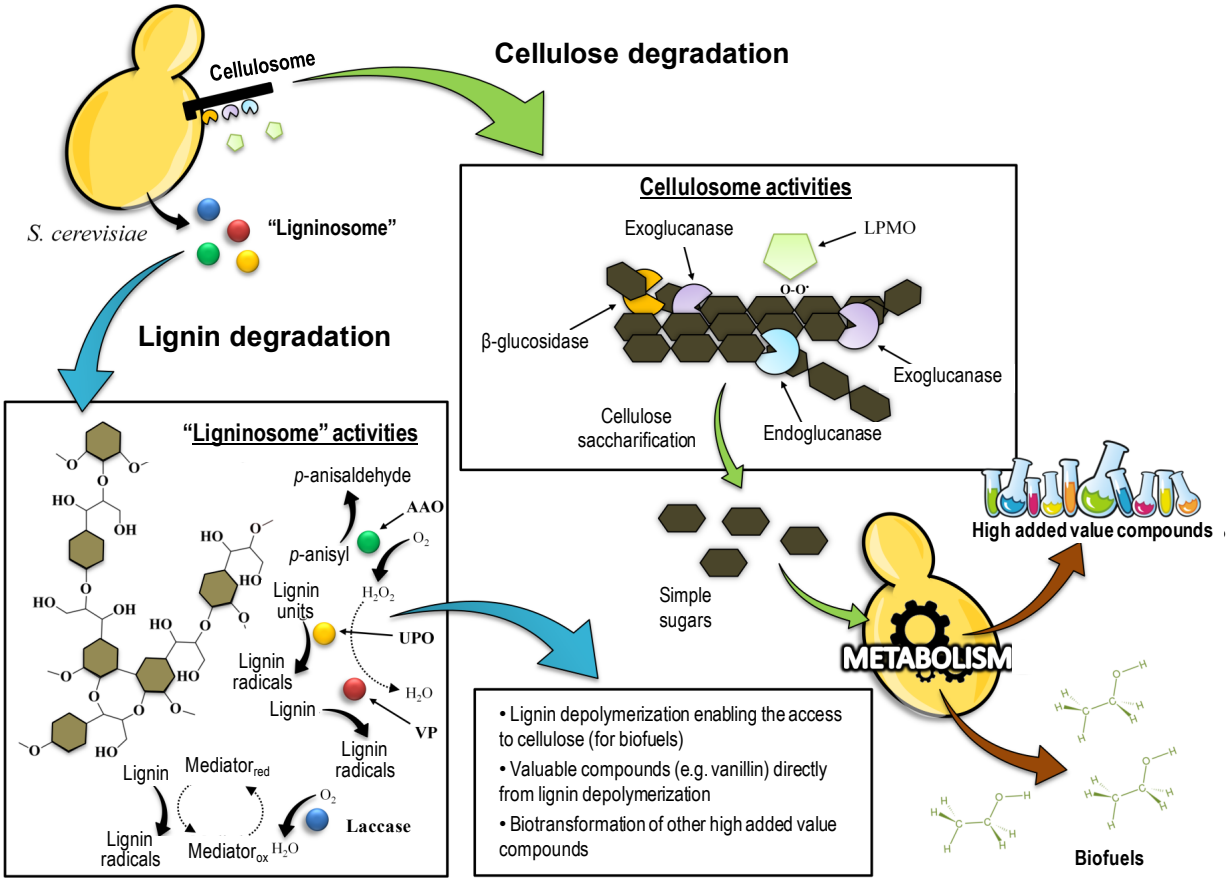

Fig. 17. Scheme of proposed *Saccharomyces cerevisiae* engineering for its use in lignocellulose biorefinery as a "white-rot yeast". Adapted from González-Perez (2016).