Review

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Search, engineering, and applications of new oxidative biocatalysts

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Abstract: Most industrial enzymes are hydrolases, such as glycosidases and esterases. However, oxidoreductases have an unexploited potential for substituting harsh (and scarcely selective) chemical processes. A group of basidiomycetes are the only organisms degrading the aromatic lignin polymer, enabling the subsequent use of plant polysaccharides. Therefore, these fungi and their ligninolytic peroxidases are the biocatalysts of choice for industrial delignification and oxidative biotransformations of aromatic and other organic compounds. The latter also include oxygenation reactions, which are catalyzed with high regio/stereo selectivity by fungal peroxygenases. In search for novel and more robust peroxidases/peroxygenases, basidiomycetes from unexplored habitats were screened, and hundreds of genes identified in basidiomycete genomes (in collaboration with the DOE JGI). The most interesting genes were heterologously expressed, and the corresponding enzymes structurally-functionally characterized. The information obtained enabled us to improve the enzyme operational and catalytic properties by directed mutagenesis. However, the structural-functional relationships explaining some desirable properties are not established yet and, therefore, their introduction was addressed by 'non-rational' directed evolution. Then, over 100 oxidative biotransformations were analyzed. Among them, it is noteworthy to mention the regio/stereo selective hydroxylation of long/short-chain alkanes (a chemically challenging reaction), epoxidation of alkenes, and production of hydroxy-fatty acids. Concerning aromatic oxygenations, the regioselective hydroxylation of flavonoids, and stereoselective hydroxylation/epoxidation of alkyl/alkenyl-benzenes were among the most remarkable reactions, together with enzymatic hydroxylation of benzene (as an alternative for harsh chemical process). Finally, peroxidases and peroxygenases also showed a potential as delignification biocatalysts and in the decolorization of contaminant dyes from textile industries. © 2014 The Authors. Biofuels, Bioproducts and Biorefining published by Society of Chemical Industry and John Wiley & Sons, Ltd.

Keywords: peroxidases; peroxygenases; oxidative industrial biocatalysts; enzyme rational design; directed enzyme evolution; selective oxygenation; lignin degradation

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Introduction

Enzymes catalyze a huge number of chemical reactions in living organisms, which take place under mild conditions compatible with life, and with exquisite substrate specificity. Nowadays, the availability of genetic engineering tools permits large-scale production of enzymes and other proteins at low cost by isolating (or synthesizing) the corresponding genes and introducing them into adequate expression hosts after their cloning in expression vectors. At the same time, protein engineering using rational and non-rational approaches permits the adaptation of these enzymes to the industrial application conditions, and to increase their expression yields. This situation represents a real breakthrough for industrial biotechnology enabling the production of commercial enzymes at low costs. In the last decades, enzymes have entered different industrial sectors, such as detergents, food and beverages, textiles, pulp and paper, leather and personal care, and have recently started to occupy an important position in the fuel markets. They are also entering the market of fine chemicals that, although initially less receptive against biotechnology, is recognizing the potential advantages of using enzymes that can catalyze highly specific transformations.

The main enzymes currently used in the above processes are of microbial origin and belong to the group of hydrolases, together with other enzymes occupying quantitatively less important industrial niches. Among them, enzymes catalyzing redox reactions – oxidoreductases – represent an environmentally friendly alternative to harsh chemicals in different industrial processes that include oxidative transformations for the production of bulk and fine chemicals, including pharmaceuticals, and other value-added products. However, the industrial penetration of oxidoreductases is still low (compared with hydrolases) due to different circumstances. High redox-potential basidiomycete peroxidases, the key enzymes in the attack of plant biomass by micro-organisms (that make possible the natural recycling of most of the carbon in land ecosystems by removing the lignin polymer protecting plant polysaccharides) were only discovered in the 1980s. Therefore, although important progress has been made in understanding these and other microbial oxidoreductases, our current knowledge level is much lower than on many hydrolases.

This work presents the main achievements attained in the course of a recently concluded RTD project on 'Novel and more robust fungal peroxidases as industrial biocatalysts' (PEROXICATS; www.peroxicats.org) funded by the European Union (EU). The aim of the PEROXICATS project was to supply European industry with new robust peroxidase-type biocatalysts of outstanding and diverse catalytic properties. Such biocatalysts will contribute to substitute harsh chemical oxidizers in more sustainable and environmentally-friendly oxidation (and oxygenation) processes for the bulk and fine chemicals sectors, including pharmaceuticals, as well as for the development of new bio-products in the above sectors. This was preceded by years of research efforts on peroxidases and other oxidative enzymes of industrial interest funded by previous EU projects, and by the EU biotechnology companies that occupy a world-leading position in the sector of industrial enzymes. For the screening of new biocatalysts in fungal genomes, the project took advantage from the increasing number of basidiomycete genomes sequenced at the Joint Genome Institute (JGI, Walnut Creek, CA, USA) with the financial support of the US Department of Energy (DOE). The most important results obtained in this European-level effort are summarized in the different points constituting the present review.

Screening of fungal cultures and genomes for enzymes of interest

The search for new peroxidases/peroxygenases of industrial interest included culture and genomic screenings combined with transcriptomic, metatranscriptomic and related studies.

Enzymatic screening and secretomic analysis

The screening of fungal cultures included (i) the search for new extracellular enzymatic activities in over 100 basidiomycete strains from unique and extreme environments and (ii) the identification of specific heme peroxidases of interest in secretomes of previously isolated basidiomycetes. For isolation and growth of the new fungal strains, culture media with complex carbon and nitrogen sources were used to satisfy the special nutritional requirements of many of them. The secretomic analyses were performed by sequencing tryptic peptides using nano-liquid chromatography coupled with tandem mass spectrometry (nLC-MS/MS).[1,2]

The enzymes of interest were purified by ion-exchange and other chromatographic methods, and characterized including both general physico-chemical properties, substrate specificity, and estimation of the redox potential

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of some of them.[3–6] In this way, a large number of interesting members of the new heme-thiolate peroxidase (HTP) and dye-decolorizing peroxidase (DyP) superfamilies, together with new class-II peroxidases (hereinafter PODs) belonging to the classic superfamily of plant-fungal-prokaryotic peroxidases – namely lignin peroxidases (LiPs), versatile peroxidases (VPs), manganese peroxidases (MnPs) and generic peroxidases (GPs) – and new auxiliary enzymes were identified, produced, characterized and evaluated as industrial biocatalysts, as described below. Among the novel peroxidases discovered, one from a lichenized ascomycete was interesting because of its different phylogeny,[7] while a new hydrolase (from *Xylaria polymorpha*) acting on both polysaccharides and cinnamic acid linkages was among the new auxiliary enzymes that help peroxidases in their attack on lignified materials.[8]

Screening of basidiomycete genomes

The screening of basidiomycete genomes in collaboration with the JGI (California) and the INRA (Marseille) provided huge information on the distribution of HTP, DyP, and POD encoding genes (after a structural-functional classification of the predicted peroxidase sequences) up to a total of more than four hundred (Fig. 1), together with additional genes encoding auxiliary enzymes.[9–13] This was especially relevant in the case of genes encoding HTP and DyP proteins, since these new superfamilies were previously known in basidiomycetes by less than half a dozen isolated enzymes (such as *Agrocybe aegerita* and *Coprinellus radians* HTPs and *Bjerkandera adusta* and *Auricularia auricula-judae* DyPs) and currently several hundred genes are known from basidiomycete genomes (and up to 978 and 319, respectively, when all fungal genomes available were analyzed). Among auxiliary enzymes, aryl-alcohol oxidase (AAO), which provides the hydrogen peroxide required by ligninolytic peroxidases in several lignin-degrading fungi (such as *Pleurotus* and *Bjerkandera* species), were also considered and 40 AAO sequences from basidiomycete genomes and other sources were analyzed, together with other peroxide-producing enzymes up to a total of more than one hundred genes.[14]

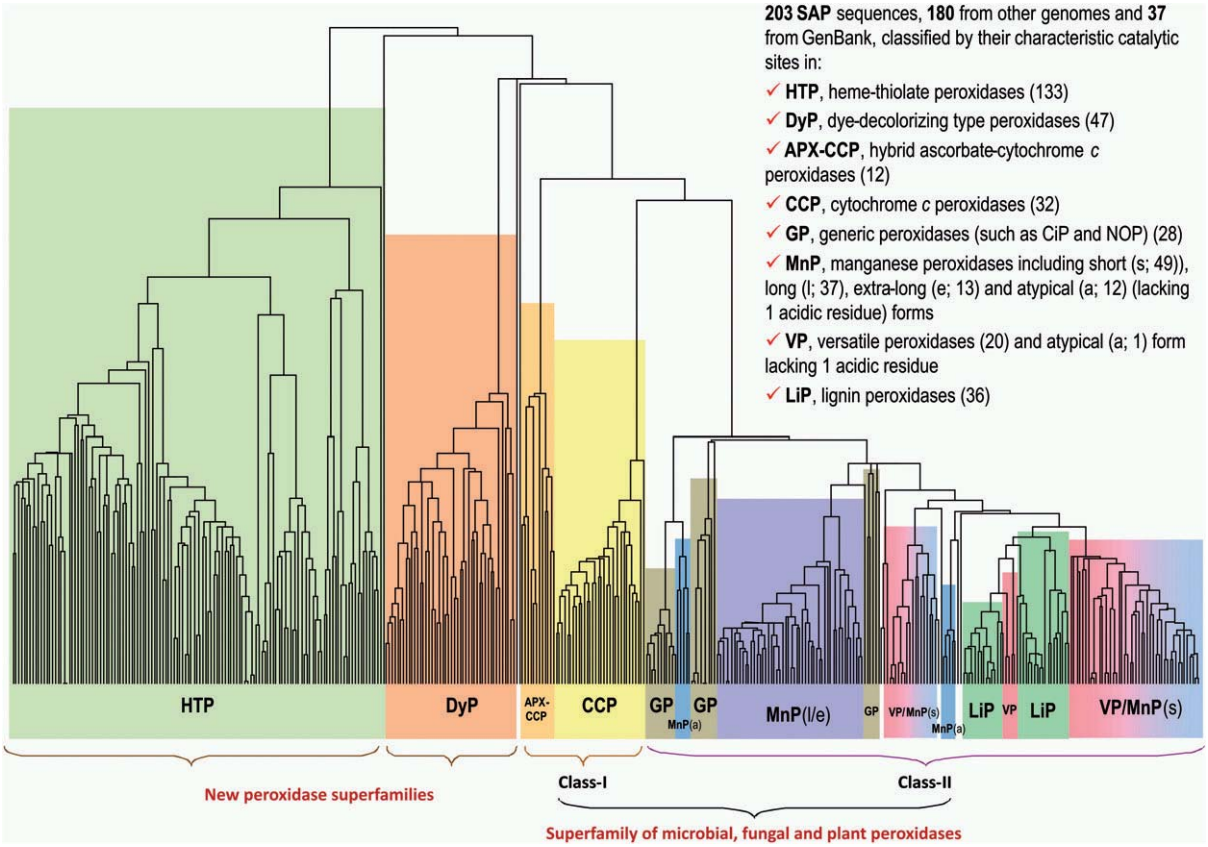

Figure 1. Evolutionary relationships between 420 basidiomycete peroxidases from SAP (JGI Saprotrophic Agaricomycotina Project) genomes and other sources classified in different superfamilies. Based on Floudas *et al.*[10]

As a result of the above genomic studies, it was possible to establish the evolutionary history of ligninolytic peroxidases, originating from a GP, and to date the appearance of the first lignin-degrading basidiomycete equipped with the above enzymes at the end of the Carboniferous period.[10] This information was useful to understand structure-function relationships of the different catalytic sites appeared during the evolution of these enzymes, and will also permit to reconstruct by ancestral inference and 'resurrect' ancestral peroxidases, characterized by higher robustness and evolvability to be exploited by directed evolution. It is interesting to mention that the basidiomycete genomes annotated in the course of the project included those of the strong lignin-degraders *Ceriporiopsis subvermispora* and *Pleurotus ostreatus*, most of whose peroxidase genes were then expressed and the corresponding enzymes evaluated for industrial applicability, among other peroxidases and auxiliary enzymes.

Transcriptomic, metatranscriptomic, and related studies

The transcriptome of *A. aegerita*, grown on different culture media, informed on the repertoire of HTPs expressed by this fungus, whose genome is not (yet) sequenced. Moreover, peroxidase production under environmental conditions was investigated by different techniques. These included a new method enabling the spatio-temporal analysis of wood degradation by fungi,[15] the purification and characterization of different peroxidases secreted by lignocellulose-degrading ascomycetes and basidiomycetes in a wood microcosm,[16] and a plate assay establishing a correlation between the oxidoreductase type and secretion patterns and the ability of white-rot basidiomycetes to grow on wood and in leaf litter habitats.[17] Finally, a metatranscriptomic study on wood and soil litter samples showed the feasibility of identifying those peroxidases expressed during the natural decay process in the forest.

Structure-function studies and rational design of enzymes

The sequenced genomes of *P. ostreatus* and *C. subvermispora* provided a unique opportunity to compare the biochemical, structural and operational properties of the complete set of PODs in a ligninolytic basidiomycete, after their heterologous expression, and to identify those directly involved in lignin degradation. It is important to mention that all the POD gene models annotated in these genomes are operational (i.e. they are not pseudogenes) as shown in transcriptional studies, although different proteins are (over)expressed under different culture conditions, as mentioned below.[9,10]

Additional structural-functional and protein engineering studies were performed on both model VP and new peroxidases, including a unique LiP together with representatives for the HTP and DyP superfamilies, as well as on auxiliary enzymes. The above studies included biochemical characterization, stability studies, and molecular structures of the already-known and new enzymes (from genomes and other sources) followed by tailoring the above biochemical and stability properties by rational design based on the structural-functional information obtained.

Evaluation of the whole peroxidase repertoire (in *Pleurotus ostreatus*)

The kinetic constants of the different PODs from the *P. ostreatus* genome (expressed in *Escherichia coli*) revealed a repertoire formed by three VPs and six MnPs, and confirmed the absence of LiP in this model fungus.[18] The study also showed, for the first time, an MnP with a tryptophan residue homologous to the exposed tryptophan responsible for oxidation of the bulky lignin polymer at the surface of VPs and LiPs. This anomaly was explained by a natural mutation in peroxidase evolution that interrupts the electron transfer pathway from the surface to the activated heme making the exposed tryptophan non-functional, as shown by reverse mutagenesis. The structural-functional study of the six MnPs revealed that they correspond to a novel subfamily characterized by their Mn-independent activity on some substrates, and the presence of a C-terminal tail shorter than in typical (long) MnPs from *Phanerochaete chrysosporium*. Moreover, studies using ^{14}C-labeled lignin and model dimers showed for the first time the lignin degrading ability of VPs. The above findings indicated that the role of LiP in *P. chrysosporium* and other species of the order Polyporales is played by VP in *P. ostreatus* and other species of Agaricales, where the VP-to-LiP transition in peroxidase evolution did not take place.[10]

Although the different isoenzymes of MnP (six forms) and VP (three forms) from the *P. ostreatus* genome showed, with a few exceptions, no important differences in their kinetic constants on selected substrates, they exhibited surprisingly large differences in their temperature and pH stabilities.[18] To investigate the biological meaning of these differences, specific primers were designed for each isoenzyme and their differential transcription

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was analyzed by quantitative polymerase chain reaction (PCR) after varying the temperature and pH conditions of cultures grown in a lignocellulose medium, combined with secretomic studies.[19] Although the genes of some of the most stable isoenzymes showed higher relative transcription levels at the most extreme temperature and pH conditions assayed, no correlation between the transcriptomic and secretomic results was observed for the most expressed MnP gene. This was due to impaired secretion as shown by the abundance of this isoenzyme in the intracellular proteome. These results showed the environmental regulation of isoenzyme gene expression, but also evidenced the need for careful studies to establish correlations between transcriptomic data and production of extracellular enzymes by these fungi.

To take advantage from all the above findings, the most temperature and pH stable isoenzymes from the *P. ostreatus* genome were crystallized with the aim of identifying the structural basis of the above properties. The high abundance of basic residues and salt-bridge/H-bond interactions at the protein surface was related to the high stability of one of these peroxidases, as confirmed by directed mutagenesis (see below).

In the search for lignin-degrading enzymes (of *Ceriporiopsis subvermispora*)

Although *P. chrysosporium* has been the model ligninolytic fungus for years, the simultaneous degradation of wood lignin and polysaccharides prevents its use in biotechnological applications, where the use of cellulose is intended. In contrast, *C. subvermispora* is a selective lignin degrader of interest in wood delignification (similar to *Pleurotus* species in delignification of agricultural wastes) but its production of lignin-degrading peroxidases (LiPs and VPs) remained unclear. With the aim of clarifying this paradox, several peroxidase candidate genes identified in its sequenced genome were expressed, biochemically characterized and its lignin-degrading ability evaluated on polymeric lignin and model dimers.[20] In this way, two previously unknown *C. subvermispora* peroxidases were identified as functionally competent LiPs (probably representing VP-to-LiP transition forms).

In addition to the above mentioned subfamilies of short and long MnPs, a subfamily of extralong MnPs is considered in recent surveys of basidiomycete genomes.[10] The three MnP types share one binding site (formed by three acidic residues near the internal propionate of heme) where Mn^{2+} is oxidized to Mn^{3+}, which acts as a diffusible oxidizer of phenolic lignin or lignin-derived products, but it has been suggested that they can differ in their catalytic and stability properties. Interestingly, the *C. subvermispora* genome revealed the joint presence of short, long and extralong MnPs (in addition to the above mentioned LiPs) offering a unique opportunity to compare the three proposed subfamilies. After their heterologous expression, we performed biochemical and structural characterization and directed mutagenesis studies of the C-terminal tail, which is placed at the vicinity of the conserved Mn-oxidation site. These results, together with the first crystal structure of an extralong MnP obtained, enabled the structural-functional validation of the three MnP subfamilies proposed.

Further insights and engineering of model versatile peroxidase

VPs share the substrate oxidation sites of typical LiPs and MnPs (exposed tryptophan and three acidic residues near the heme propionate, respectively). However, some pieces of evidence, including the double kinetics for oxidation of some aromatic substrates, suggested a third oxidation site. This was investigated in the best characterized VP from *Pleurotus eryngii* by directed mutagenesis of the main heme access channel, which constitutes the entrance for hydrogen peroxide in heme peroxidases and the site for substrate oxidation in plant and fungal GPs. The steady-state and transient-state kinetic constants of several single and multiple variants at the heme channel (combined with removal of the exposed catalytic tryptophan for better analyzing the effect of mutations) showed that a wider main heme channel strongly increases the VP efficiency to oxidize phenols and dyes.[21]

The above location of the third substrate oxidation site in VP was also supported by crystallographic data revealing the binding of guaiacol, whose oxidation was improved in the variants mutated at the main heme access channel. This is the first time that a ligninolytic peroxidase has been crystallized in complex with an aromatic substrate. The VP variants mutated at the heme channel were more efficient than native VP, not only in oxidizing simple phenols but also in removing a complex phenolic mixture from an industrial effluent, a fact that confers them an undeniable biotechnological interest.

On the other hand, some of the surface interactions and charged residues potentially related to the high pH stability of one of the *P. ostreatus* MnPs were transferred to *P. eryngii* VP resulting in a significant stability improvement.[22] These results confirm that stabilizing motifs from genomic screenings can be used for engineering proteins of interest in the development of industrial biocatalysts. Also for improving the industrial applicability of these

peroxidases, their inactivation by peroxide (the so-called suicide inactivation) was reduced by directed mutations at the vicinity of the heme that eliminated oxidizable residues, and slowed-down the formation of compound-I, whose accumulation leads to peroxidase inactivation. This is an important problem in peroxidase biocatalysts, which was also tackled by directed evolution.

A unique LiP with a novel peroxidase activation mechanism

In the search for new peroxidases, we investigated the only known LiP (or VP) with a catalytic tyrosine instead of a catalytic tryptophan from the basidiomycete *Trametes cervina*. Crystallographic, steady-state and transient-state kinetic, biochemical and spectroscopic studies, combined with directed mutagenesis, showed that a tyrosine residue located near the internal propionate of heme was involved in catalysis forming a tyrosyl radical, which was detected by low-temperature electron paramagnetic resonance (EPR) of the peroxide-activated enzyme.[23]

Interestingly, a short initial delay during oxidation of veratryl alcohol (a fungal metabolite and LiP/VP substrate), which could have gone unnoticed, started further studies that resulted in the discovery of a novel activation mechanism for peroxidase oxidation of high redox-potential substrates. The need for such activation is because tyrosine radicals normally lack the high redox potential required for oxidation of non-phenolic aromatics, such as veratryl alcohol and lignin. For these studies, 'activated' LiP was obtained after several turnovers with the above substrate, its molecular mass was estimated (using matrix-assisted laser desorption and ionization-time of flight, MALDI-TOF) and the peptides from a specific proteolytic hydrolysis were sequenced (using MALDI-TOF and nLC-MS/MS).[24] We found that the tyrosine forms an adduct with veratryl alcohol during activation, as shown by the molecular mass increase of both the whole protein and one of the peptides (whose sequence revealed the specific modification of this residue). Moreover, the EPR spectrum of the activated enzyme showed the disappearance of the tyrosine radical, and its substitution by the tyrosine adduct radical. This novel activation mechanism could also operate in other tyrosine-containing oxidoreductases, and opens new possibilities in the design of high redox potential oxidative biocatalysts.

Structure-function of the first self-sufficient heme (per)oxygenase

The *A. aegerita* peroxidase and related HTPs were extensively investigated here because of their monooxygenase activity on different types of substrates, acting as an unspecific peroxygenase (UPO). The catalytic cycle of UPO purified from *A. aegerita* cultures, in the presence of peroxides and reducing substrates, was investigated by stopped-flow rapid spectrophotometry and other techniques resulting in the description of a (single) compound-I intermediate, reminiscent to that recently found in a cytochrome P450 enzyme.[25] The advantages of UPO, compared with P450s catalyzing similar reactions, are illustrated by its catalytic cycle, since it just needs peroxide to be activated to reactive compound-I (a Fe[IV]-oxo·porphyrin radical complex, which catalyzes a two electron oxidation of the substrate during transfer of the oxygen atom). In contrast, P450s need a second flavoprotein or flavin domain, and a source or reducing power (e.g. NAD[P]H), to be activated by molecular oxygen. Then, the high redox-potential of the activated UPO (compound-I) has been deduced from the driving force for oxygen transfer.[26] The true peroxygenase mechanism of UPO on different substrates was shown using labeled hydrogen peroxide ($H_2{}^{18}O_2$) as co-substrate, which resulted in the incorporation of one ^{18}O atom from the peroxide into the new hydroxyl group.[27] Simultaneously, the existence of a transient heme-associated substrate radical (as found in P450 catalysis) was shown using the "radical clock" substrate norcarane, whose oxidation into different products allowed the calculation of a radical lifetime of only 9 ps and an oxygen rebound rate in the range of 10^{11} s^{-1}. Due to the above characteristics, together with its extracellular nature and stability, UPO behaves as a robust and self-sufficient (peroxide-activated) mono-(per)oxygenase with a huge biotechnological potential.

To obtain structural-functional information helping to improve the UPO properties as an industrial biocatalysts, the first UPO (from *A. aegerita*) crystal structure was solved (Fig. 2).[28] The UPO active site is similar to that of P450s in terms of a cysteine as the fifth ligand of the heme iron and an axial access channel directly leading to the position of the reactive Fe(IV) oxo group in compound-I (occupied by a water molecule in the ferric resting state crystal). However, this channel in UPO is wider (and funnel shaped) in agreement with the bulkier nature of many of its substrates, and includes five phenylalanine residues. The three lower phenylalanines would cooperate in the anchoring and orientation of aromatic substrates (as shown by molecular docking of different polycyclic aromatic hydrocarbons), while the two upper phenylalanines would impose an upper limit on the longitudinal dimension of the substrates. In contrast to PODs, the amino acid that serves as acid-base catalyst in UPO activation by peroxide is a glutamic acid residue and not a histidine.

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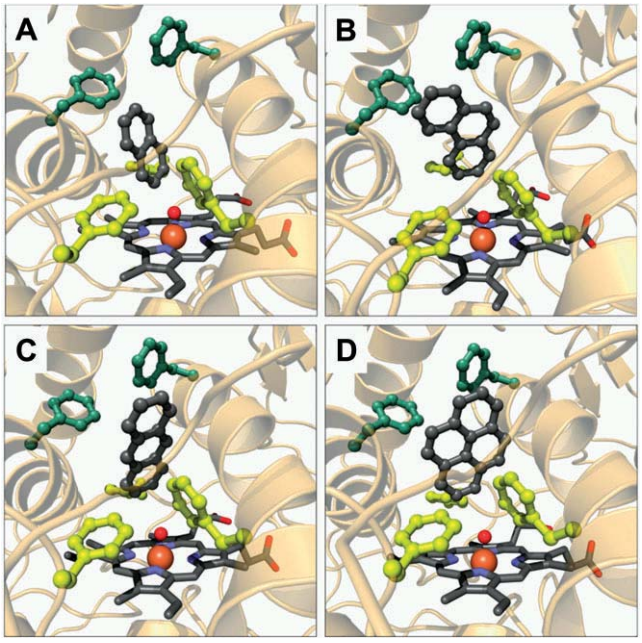

Figure 2. Docking of naphthalene (A), phenanthrene (B), anthracene (C) and pyrene (D) on the *A. aegerita* UPO crystal structure (see text for comments). Adapted from Piontek *et al.*[28]

The structural-functional information was useful in the *ad hoc* modulation of UPO's catalytic properties for different target substrates by (i) selecting other HTPs from genomes, and (ii) engineering the already available enzymes by semi-rational and rational approaches. In this way, a large number of UPO variants, expressed in the industrial host *Aspergillus oryzae*, were prepared by directed mutagenesis of oxidizable residues, resulting in stability improvements that were incorporated in the development of commercial peroxygenases.

New structural-functional studies on the 'enigmatic' DyP superfamily

As in the case of the HTP superfamily, the structural-functional characteristics of the new DyP superfamily are still largely to be investigated, although the structures of *B. adusta* DyP (initially referred to as *Thanatephorus cucumeris* DyP) and one bacterial DyP were available at the beginning of the project. The same happens with the catalytic activities and reaction mechanisms of these enzymes, whose natural role remains unknown. Here the crystal structure of the DyP from *A. auricula-judae*, a wood-rotting jelly fungus (order Auriculariales) phylogenetically distant from *B. adusta* (order Polyporales) was solved.[29] The enzyme shares with PODs the proximal histidine acting as heme iron ligand (which in HTPs and P450s is a cysteine) but differs in the residues supporting the reaction with hydrogen peroxide. Among them, an aspartic acid seems to play the role of distal histidine in PODs (or glutamic acid in HTPs), at the same time that its mobile side-chain would act as a partial gate of the heme access channel. As found in UPO, the heme channel of DyP has an axial orientation providing access to the distal side of the heme pocket, while the main and manganese channels in PODs are lateral giving access to the edge and the propionates of the heme, respectively.

The *A. auricula-judae* DyP is able to oxidize non-phenolic lignin model dimers,[5] and could also act on lignin.[3] This characteristic suggests the existence of long-range electron transfer from the protein surface to the heme, as found in LiP and VP. Several exposed tryptophan and tyrosine residues were localized in the *A. auricula-judae* DyP crystal structure, and formation of a tyrosine radical was shown by trapping with 2-methyl-2-nitrosopropane (followed by nLC-MS/MS analysis of the peptides from enzymatic hydrolysis) whose catalytic nature was suggested by activity decrease after tyrosine modification with tetranitromethane.[30] Since the involvement of both tyrosine and tryptophan residues in DyP catalysis cannot be excluded,[6] the enzyme was heterologously expressed in *E. coli*. Then, a variety of simple and multiple variants were obtained resulting in different tryptophanyl and tyrosyl radicals in the peroxide-activated enzymes that could be directly detect by low-temperature EPR, together with significant changes in the kinetic constants for the assayed dyes and other substrates. In this way, a definitive evaluation of the contribution of each of the exposed aromatic residues to the enzyme's catalytic activity could be performed.

Peroxidase 'auxiliary' enzymes

The action of peroxidases in nature is absolutely dependent on the activity of auxiliary enzymes providing the hydrogen peroxide required for their activation (i.e. formation of compound-I). These enzymes are oxidases (such as AAO) that in turn are activated by oxygen (freely available in the atmosphere), which is reduced to the hydrogen peroxide released to the medium. They are, therefore, an alternative to exogenous peroxide addition or its *in situ* photocatalytic generation using flavin nucleotides, a 'biomimetic' reaction that has also been used for UPO activation.[31] Due to the importance of the above enzymes, the key aspects of AAO catalytic cycle and reaction mechanisms were elucidated, after heterologous expression of the *P. eryngii* enzyme.

The diffusion of both the enzyme oxidizing (molecular oxygen) and reducing (different aryl alcohols) substrates into the active site is an intriguing aspect because of its buried nature. By using 'in silico' computational methods in combination with site-directed mutagenesis, it was shown that this involves interaction of the aromatic rings of substrates with two aromatic residues, together with side-chain reorientation, while these changes were not required for oxygen diffusion.[32] Then, it was found that one active site histidine plays a key role in substrate oxidation by acting as a catalytic base helping a hydride transfer reaction from the alcohol to the flavin cofactor.[33] With relevance for biocatalyst development, it was shown for the first time in an oxidase that its reactivity with molecular oxygen can be modulated by reducing the volume of the active site cavity by directed mutagenesis.[34] Finally, and also of interest for biotechnological application of this enzyme, it was found (using deuterated alcohols) that the hydride transfer reaction is stereoselective and can be used for deracemization purposes, as shown using chiral mixtures of aryl alcohols.[35]

Directed evolution and related approaches for industrial biocatalysts

Laboratory directed evolution was applied for engineering those peroxidase/peroxygenase properties where a direct structural-functional relationship could not be previously established. After designing laboratory evolution protocols, functional expression and different catalytic and operational properties of one ligninolytic peroxidase, and one peroxygenase from the HTP superfamily were successfully evolved, the latter together with use of combinatorial libraries.

Laboratory-directed evolution protocols

One of the election expression hosts in laboratory-directed evolution experiments, the baker's yeast *Saccharomyces cerevisiae*, was used for directed evolution of ligninolytic genes. New high-throughput protocols, or adaptations of those previously developed for improving

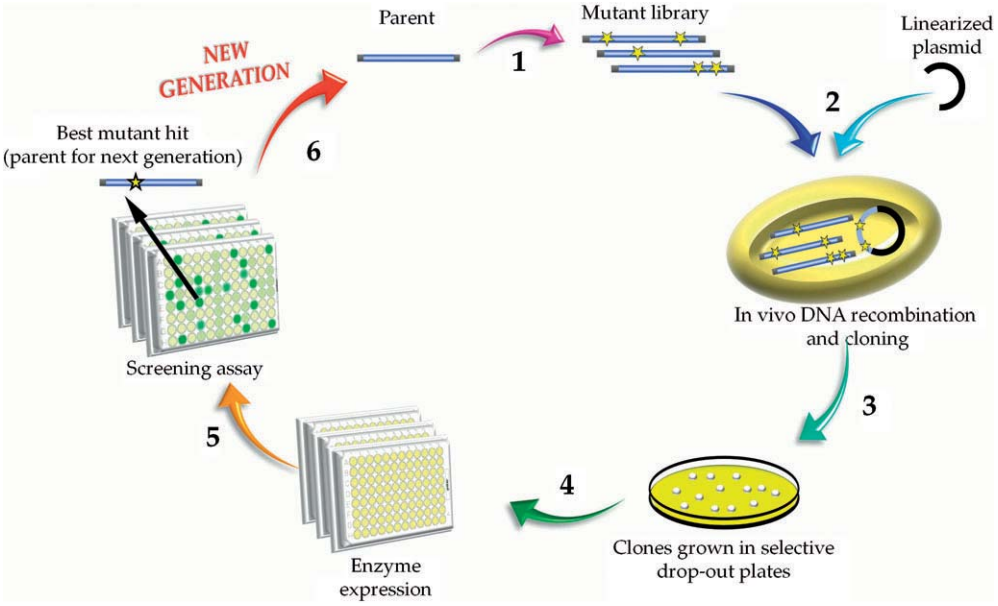

Figure 3. A typical directed peroxidase evolution experiment using *S. cerevisiae* as a host. The cycle begins with diversity generation by mutagenic PCR (1) (stars indicate single mutations). The mutagenic library is transformed into *S. cerevisiae* (2) and the pool of templates is recombined by *in vivo* DNA shuffling. Each template contains adequate overhangs (in black) that overlap with the linearized plasmid, facilitating *in vivo* cloning to generate the autonomously replicating vector. The clones are grown on selective drop-out plates (3) and transferred to 96-well plates, where the expression is induced. After secretion, the supernatants are subjected to a high throughput assay (4) to select the best enzyme variants. Finally, the best hits are recovered, characterized and their genes subjected to a further generation of directed evolution (5). Adapted from González-Pérez *et al.*[36]

other oxidoreductases of interest,[36–41] were used. In these experiments, we took advantage from the *S. cerevisiae* genetic machinery for DNA recombination and *in vivo* DNA shuffling, combined with mutagenic PCR and adequate screening methods to select the best enzyme variants (Fig. 3).

The first step for this 'non-rational' improvement of basidiomycete peroxidases was the design of the high-throughput protocols to be used, and the development of methods for enzymatic activity estimation suitable to be incorporated into these protocols.[42] The directed evolution experiments started increasing the level of functional expression in yeast, and continued improving the catalytic and/or stability properties of interest. Several substrates were used for selection of the best variants with the purpose of maintaining the wide specificity of many peroxidases.

Evolving the first ligninolytic peroxidase

The model VP from *P. eryngii* was the first peroxidase to be evolved for functional expression, as well as for activity and thermal stability improvement. The best secretion variant (22 mg/L) was obtained after four generations (resulting in incorporation of four mutations) and two more generations under stronger selective pressure (up to 90ºC) yielded a thermostable variant with 8ºC higher inactivation temperature (incorporating three more mutations).[43] Interestingly, several evolutionary side-effects were detected, including a strongly enhanced stability at alkaline pH (60% residual activity after 120 h at pH 9 compared to the parental VP that was completely inactivated after 1 h at pH 9). The improvements in pH stability attained by directed evolution could be rationalized by expressing the evolved variant in *E. coli* and analyzing its crystal structure, in combination with the stability results obtained from several single and multiple directed mutagenesis variants harboring the same mutations recruited by directed evolution.

The first VP variant with improved expression in yeast was taken as scaffold to increase the oxidative stability of the enzyme in the presence of hydrogen peroxide (also addressed by rational design). Since oxidative stability of peroxidase is an intrinsic mechanism based process, several variants with significantly higher half-life (at high peroxide concentrations) than the parental type could be obtained by a strategy that combined classical with focused directed evolution using two new methods for generation of DNA diversity (called MORPHING and DNA-puzzle) in a five-generation process.[44]

Evolving the first basidiomycete peroxygenase

An even more challenging task was to evolve UPO for industrial applicability, since basidiomycete HTPs had never been improved by this protein engineering technique before. The previous experience from VP evolution was exploited, and the *A. aegerita* UPO was evolved in *S. cerevisiae* for functional expression and activity, using a new selective and sensitive colorimetric assay.[42] Significant improvements of both properties were obtained without a detrimental effect on the enzyme stability. The effect of the mutations introduced was discussed considering the position of the affected residues in the previously solved crystal structure of the *A. aegerita* UPO.

Due to the high interest on UPOs for the different oxyfunctionalization reactions described, these studies also included the improvement of the *Coprinopsis cinerea* UPO properties by a semi-rational approach using the industrial expression host *A. oryzae*. With this purpose, multiple substitutions were introduced at a series of positions, which were selected using a homology model of the *C. cinerea* UPO based on the *A. aegerita* UPO crystal structure, multiple alignments of HTP predicted sequences, and other reasons. Using this strategy, a high number of interesting variants with (i) altered relative substrate specificity (enabling identification of residues affecting specificity), (ii) improved oxidative stability against peroxide, and (iii) reduced catalase and peroxidase *vs* peroxygenase activities (two important issues for industrial application, with the aim of reducing the peroxide consumption and limiting undesirable one-electron oxidation reactions, respectively) were obtained, which were used in different oxygenation reactions as described below.

Product analysis and enzyme evaluation as industrial biocatalysts

A variety of oxygenation reactions of industrial interest were investigated by applying the new and/or improved peroxidases/peroxygenases on different aliphatic and aromatic substrates. Compared with similar chemical reactions, the enzymatic reactions have the advantage of their regioselectivity and stereoselectivity, two objectives that are difficult to be attained by other means. Compared with P450s, the basidiomycete peroxygenases have the advantage of being self-sufficient (that is, independent of a second flavin-containing enzyme or domain, and of an expensive source of reducing power) and more robust enzymes, while yielding similar products. Finally, several

oxidative degradation and related peroxidase reactions of industrial interest are described.

Selective oxygenations of aliphatic target compounds

The (stereo and regio) selectivity of the enzymatic hydroxylations investigated was shown using the *A. aegerita* UPO acting on long[45] and short/medium chain[27] linear aliphatic substrates that yielded the (*R*)-enantiomers at the ω-1 and ω-2 positions. Using the long-chain substrates, it was shown that monohydroxylation reaction takes place on alkanes, fatty acids and fatty alcohols, always with similar selectivity, and that the reactions continue with further hydroxylations yielding the ω-1 and ω-2 ketones (by dehydration of the *gem*-diol intermediates). In the case of the terminal fatty alcohols, oxygenation takes place on the alcohol group first yielding the corresponding aldehyde (by *gem*-diol dehydration). The final products from these compounds are the ω-1/ω-2 keto-fatty acids. In this way, alkanes can be selectively converted into subterminal diols (and ketones) and terminal fatty alcohols into subterminal hydroxy (or keto) fatty acids. In all these transformations, the exact chemical nature of the products obtained could be determined by diagnostic ions in mass spectra, and the oxygenase nature of the reaction was shown by the selective incorporation of ^{18}O in reactions with $H_2{}^{18}O_2$ as cosubstrate, also analyzed by mass spectrometry.

An unexplored diversity of UPOs (and other HTPs) was revealed by the genomic and culture screenings, among others. Among UPOs with predicted divergent molecular structures (compared with the solved *A. aegerita* structure), the *Marasmius rotula* enzyme provided different conversion rates and stereoselectivities on aliphatic compounds including oxygenation reactions of industrial relevance. Different transformation rates of steroidal compounds to products of interest were also obtained when the two above UPOs and a third one obtained from the *C. cinerea* genome were compared. The commercial interest of the enzymatic oxygenation of aliphatic compounds was increased when studies using this recombinant UPO from *C. cinerea* expressed in high-yield in the *A. oryzae* industrial system (Fig. 4) showed the same transformation reactions described for the *A. aegerita* UPO with comparable or even better yields, and selectivity rates.[46] The enzymatic production of diols from alkanes is a very attractive reaction given the very low chemical reactivity of these substrates, and the high potential of diols for different applications, that was covered by a new patent including the *C. cinerea* and other seven UPOs, together with a variety of recombinant forms and mutated variants.[47]

Additional studies expanded the range of oxygenation reactions of aliphatic compounds, and the industrial interest of UPOs for transformation of: (i) cyclic alkanes into cyclic ketones (e.g. cyclohexane *via* cyclohexanol

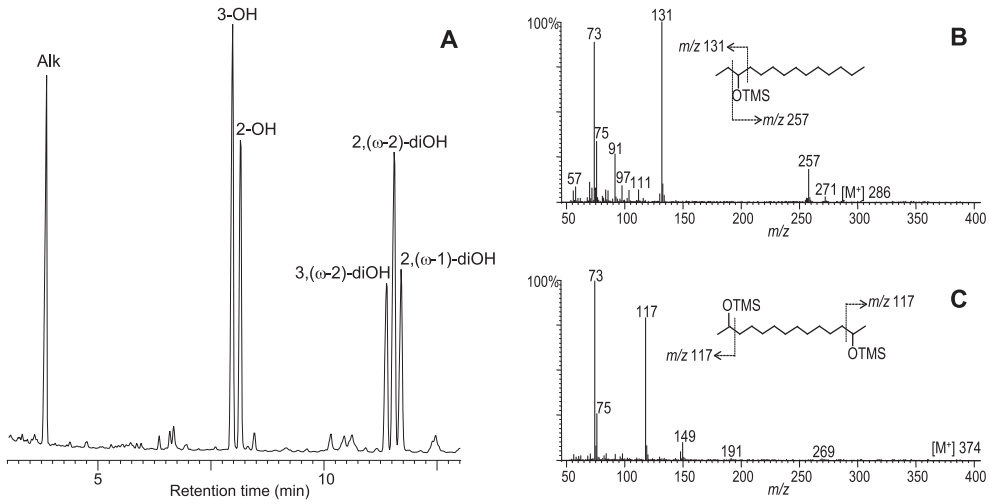

Figure 4. GC-MS analysis of recombinant *C. cinerea* UPO reaction (in 40% acetone) with tetradecane (Alk). A) Chromatogram showing all the possible subterminal monohydroxy (2-OH and 3-OH) and dihydroxy (2[ω-1]-diOH, 2[ω-2]-diOH and 3[ω-2]-diOH) derivatives. B and C) Example of mass spectra of one monohydroxy (3-OH) and one dihydroxy (2[ω-1]-diOH) derivative, respectively, showing characteristic ions and fragmentation patterns. Adapted from Babot *et al*.[46]

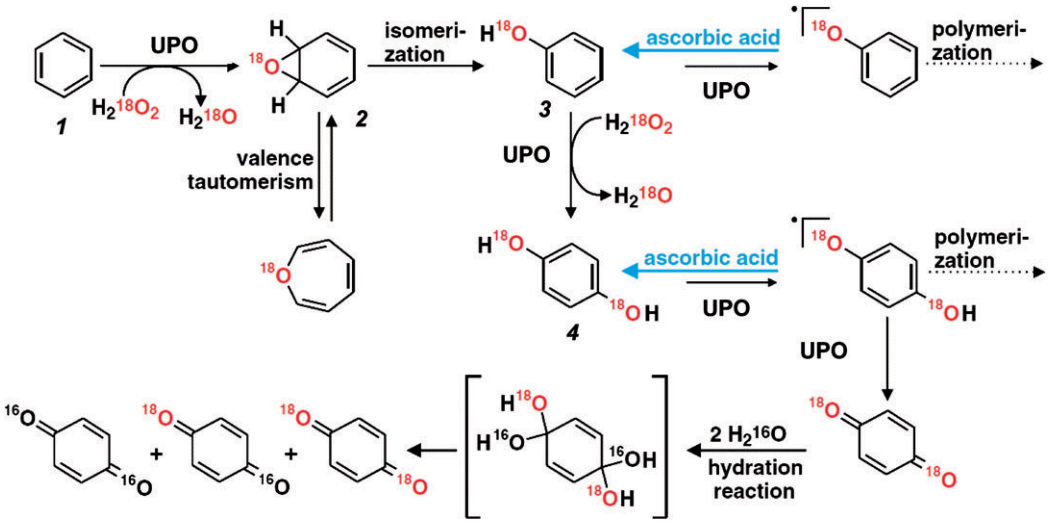

Figure 5. Scheme for the epoxidation/hydroxylation and further oxidation of benzene *(1)* and its following products phenol *(3)* and *p*-hydroquinone *(4)* by UPO *via* the benzene epoxide intermediate *(2)* (in the same way, catechol may be oxidized to *o*-benzoquinone). Adapted from Karich *et al.*[57]

into cyclohexanone);[48] (ii) linear, branched and cyclic alkenes into the corresponding epoxides, which constitute building blocks of high relevance (e.g. isobutene oxide) or into allylic alcohols (e.g. limonene alcohols);[49] and (iii) 1,2-dihydronaphthalene into naphthalene hydrates that desaturate to naphthalene (followed by oxygenation to 1-naphthol and 2-naphthol).[50]

Selective oxygenations of aromatic target compounds

Regioselective and stereoselective oxygenations were also obtained in UPO reactions with aromatic compounds, affecting both the aromatic rings and the aliphatic substituents. Among them, a variety of flavonoids were hydroxylated by UPO preferably at the C6 position (aromatic ring-A) *via* initially formed epoxide intermediates.[51] A group of remarkable reactions catalyzed by UPO is the stereoselective hydroxylation of *n*-alkyl (and cycloalkyl) benzenes and the epoxidation of styrenes (and cycloalkenyl benzenes), which exhibited high conversion rates (over 95%), total turnover numbers (over 10 000) and enantiomeric excesses (over 98%) in near half of all the reactions assayed.[52] Hydroxylation of pyridine and other *N*-heterocycles to the corresponding *N*-oxides is another UPO reaction of industrial interest, which is covered by a specific patent.[53] Additional UPO applications were investigated in drug synthesis,[54] and preparation of biosensors and electrodes.[55,56]

Benzene hydroxylation by UPO to obtain phenol, and subsequent phenol hydroxylation to obtain *p*-hydroquinone (and catechol) were evaluated for their industrial feasibility, given the large markets of both compounds in plastic manufacture, photographic chemicals, and other products. The monohydroxylation and dihydroxylation reactions by the peroxygenase activity of UPO were analyzed in detail, starting with the epoxide that rearomatizes yielding phenol, together with the competing one-electron oxidation (due to the additional peroxidase activity of UPO) yielding phenoxyl radicals, which can disproportionate to undesirable quinones and form coupling products[57] (Fig. 5). It was expected that this could be prevented by addition of radical scavengers (like ascorbic acid) and the use of *C. cinerea* UPO variants with high peroxygenase-to-peroxidase activity ratios. Before the upscaling trials, the reaction parameters were optimized by the partners. Native (wild-type) UPOs from *A. aegerita* and *C. cinerea*, and a series of variants of the latter enzyme with improved aromatic hydroxylation activity, stability and other properties (all recombinant enzymes produced by the industrial expression system) were compared in the benzene-to-phenol and phenol-to-hydroquinone reactions. From the results obtained, one of the UPO variants was selected for larger trials, based on high total yield, product selectivity, and enzyme availability. Then, pH, hydrogen peroxide, substrate and organic cosolvent concentrations, and enzyme dose were optimized for the two

biotransformations to be evaluated at the CEPSA Research Center (Alcalá de Henares, Madrid, Spain).

CEPSA, an integrated energy company operating at every stage of the oil value chain, is interested in the enzymatic hydroxylation of benzene for the production of phenol, as a future alternative to substitute the energetically-costly cumene process, as well as in the selective production of hydroquinone by mild enzymatic methods. Several enzyme reactor trials were performed, where the remaining enzymatic activity was followed together with the amount of the main reaction products (i.e. phenol, hydroquinone, catechol and benzoquinone). During benzene hydroxylation, it was found that the enzyme activity was lost during the first minutes of incubation, resulting in a low phenol yield (based on the peroxide added). This revealed that the enzyme stability in the reaction mixture was the critical issue to be solved under industrially-relevant conditions (the initial epoxide intermediate could be responsible for this inactivation). However, the parallel trials of phenol hydroxylation showed no enzyme inactivation (suggesting a very short-lived epoxide intermediate in this biotransformation) and yielded high hydroquinone production that, interestingly, was close to commercial viability of the process.

Oxidative degradation reactions and related peroxidase applications

The new and improved peroxidases were also evaluated in different biodegradation and related reactions with a potential industrial interest. Since many of the basidiomycete peroxidases investigated are involved in natural degradation of lignin, several of their applications are related to lignocellulose processing as a renewable industrial feedstock.[58] The peroxidase ability for oxidative degradation of lignin was evaluated using dimeric compounds including the most frequent inter-unit linkages in the polymer, which experienced characteristic Cα oxidation and Cα-Cβ breakdown when incubated with LiPs, VPs and DyPs that correlated with their capability for oxidative depolymerization of synthetic lignin (DHP).[5,18,20] UPOs can also degrade lignin model dimers but the different reaction mechanism (initial *O*-demethylation) would explain their apparent inability to depolymerize DHP.[59] Then, the above enzymes were evaluated for lignin removal from whole (lignocellulose) and partially delignified (paper pulp) materials in laboratory reactors. Redox mediators (diffusible electron carriers) were added to favor the access inside the plant cell wall, and the modification of lignin in some of the experiments was compared with that obtained using the well-known laccase-mediator system.[60,61]

Pulp bleaching using peroxidases had already been reported, therefore, we investigated the potential of UPO in the presence of mediators. Interestingly, wild-type UPOs produced the highest brightness increase in the presence of 1-hydroxybenzotriazole, while some of the variants yielded the highest brightness in the presence of methyl syringate, a potentially cheap and safe mediator. A bleach boosting effect was also found with a new hydrolase (from *Xylaria polymorpha*) sharing different enzymatic activities. Concerning whole lignocellulose delignification, the 2D NMR spectra of the treated milled eucalypt wood showed lignin modification in terms of aromatic units (with respect to polysaccharide units) and inter-unit linkages per aromatic unit. This shows that VP (in the presence of veratryl alcohol) is able to modify lignin in whole lignocellulosic materials, without any previous chemical deconstruction, although the changes are less drastic than obtained with the laccase-mediator system. Moreover, wheat straw treatment with a wild DyP improved both the saccharification and bioethanol production yields,[3] in agreement with the previously shown action on synthetic lignin.[5]

Radical coupling and polymerization are among the 'secondary' reactions taking place after oxidation of aromatic compounds by peroxidases. In this context, VP was successfully used for generation of new biomolecules of interest by specific coupling reactions.[62] These reactions included the homogeneous polymerization of lignans, peptides and high molecular mass proteins and polysaccharides. Heterogeneous polymerization (of lignans and peptides) was also observed, as well as gelation of feruloylated arabinoxylans by VP. In these reactions, VP behaves better than laccases and plant peroxidases due to its higher redox potential and the presence of an exposed catalytic residue (that, for example, enables oxidation of intercalary tyrosine residues in proteins and peptides).

UPOs were also evaluated for their ability to degrade hydrolyzed reactive dyestuffs with a view to improving the sustainability of textile dyeing operations, where removal of non-fixed dyestuffs requires large volumes of water for rinsing. Some of the UPO variants, among a total of more than thirty tested, were able to decolorize partially hydrolyzed Reactive Black 5 (a recalcitrant dye) in the presence of redox mediators. However, the most efficient results were obtained with DyP that successfully decolorized intact (non-hydrolyzed) Reactive Black 5 without any mediator.

Conclusions

The PEROXICATS project has demonstrated that enzymes acting on peroxide as electron acceptor (EC 1.11; i.e.

peroxidases and peroxygenases) represent useful biocatalysts for the chemical modification of diverse organic substrates, including regioselective and stereoselective oxygenations that are very difficult to be obtained by chemical reactions, and other oxidative biotransformations. Simultaneously, it has created a broad platform for the further development of peroxidases/peroxygenases as powerful industrial biocatalysts, in the chemical and lignocellulose biorefinery sectors, on the basis of molecular engineering and improvement of their process performance.

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References

1. Carabajal M, Kellner H, Levin L, Jehmlich N, Hofrichter M and Ullrich R, The secretome of *Trametes versicolor* grown on tomato juice medium and purification of the secreted oxidoreductases including a versatile peroxidase. *J Biotechnol* **168**:15–23 (2013).
2. Salvachúa D, Martínez AT, Tien M, López-Lucendo MF, García F, de los Ríos V *et al.,* Differential proteomic analysis of the secretome of *Irpex lacteus* and other white-rot fungi during wheat straw pretreatment. *Biotechnol Biofuels* **6**:115 (2013).
3. Salvachúa D, Prieto A, Martínez AT and Martínez MJ, Characterization of a novel DyP-type peroxidase from *Irpex lacteus* and its application in the enzymatic hydrolysis of wheat straw. *Appl Environ Microbiol* **79**:4316–4324 (2013).
4. Gröbe G, Ullrich M, Pecyna M, Kapturska D, Friedrich S, Hofrichter M and Scheibner K, High-yield production of aromatic peroxygenase by the agaric fungus *Marasmius rotula*. *AMB Express* **1**:31–42 (2011).
5. Liers C, Pecyna MJ, Kellner H, Worrich A, Zorn H, Steffen KT *et al.,* Substrate oxidation by dye-decolorizing peroxidases (DyPs) from wood- and litter-degrading agaricomycetes compared to other fungal and plant heme-peroxidases. *Appl Microbiol Biotechnol* **87**:5839–5849 (2013).
6. Liers C, Aranda E, Strittmatter E, Piontek K, Plattner DA, Zorn H, Ullrich R and Hofrichter M, Phenol oxidation by DyP-type peroxidases in comparison to fungal and plant peroxidases. *J Mol Catal B-Enzym* **103**:41–46 (2014).
7. Liers C, Ullrich R, Hofrichter M, Minibayeva FV and Beckett RP, A heme peroxidase of the ascomyceteous lichen *Leptogium saturninum* oxidizes high-redox potential substrates. *Fungal Genet Biol* **48**:1139–1145 (2011).
8. Nghi dH, Bittner B, Kellner H, Jehmlich N, Ullrich R, Pecyna MJ *et al.,* The wood rot ascomycete *Xylaria polymorpha* produces a novel GH78 glycoside hydrolase that exhibits a-L-rhamnosidase and feruloyl esterase activities and releases hydroxycinnamic acids from lignocelluloses. *Appl Environ Microbiol* **78**:4893–4901 (2012).
9. Fernández-Fueyo E, Ruiz-Dueñas FJ, Ferreira P, Floudas D, Hibbett DS, Canessa P *et al.,* Comparative genomics of *Ceriporiopisis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. *P Natl Acad Sci USA* **109**:5458–5463 (2012).
10. Floudas D, Binder M, Riley R, Barry K, Blanchette RA, Henrissat B *et al.,* The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. *Science* **336**:1715–1719 (2012).
11. Ruiz-Dueñas FJ, Fernández E, Martínez MJ and Martínez AT, *Pleurotus ostreatus* heme peroxidases: An *in silico* analysis from the genome sequence to the enzyme molecular structure. *C R Biol* **334**:795–805 (2011).
12. Ruiz-Dueñas FJ, Lundell T, Floudas D, Nagy LG, Barrasa JM, Hibbett DS and Martínez AT, Lignin-degrading peroxidases in Polyporales: An evolutionary survey based on ten sequenced genomes. *Mycologia* **105**:1428–1444 (2013).
13. Levasseur A, Lomascolo A, Chabrol O, Ruiz-Dueñas FJ, Uzan E, Piumi F *et al.,* The genome of the white-rot fungus *Pycnoporus cinnabarinus*: A new basidiomycete model with a versatile arsenal for lignin deconstruction. *Genome Biol* in press (2014).
14. Hernández-Ortega A, Ferreira P and Martínez AT, Fungal aryl-alcohol oxidase: A peroxide-producing flavoenzyme involved in lignin degradation. *Appl Microbiol Biot* **93**:1395–1410 (2012).
15. Hahn F, Ullrich R, Hofrichter M and Liers C, Experimental approach to follow the spatiotemporal wood degradation in fungal microcosms. *Biotechnol J* **8**:127–132 (2013).
16. Liers C, Arnstadt T, Ullrich R and Hofrichter M, Patterns of lignin degradation and oxidative enzyme secretion by different wood- and litter-colonizing basidiomycetes and ascomycetes grown on beech-wood. *FEMS Microbiol Ecol* **78**:91–102 (2011).
17. Barrasa JM, Blanco MN, Esteve-Raventós F, Altés A, Checa J, Martínez AT and Ruiz-Dueñas FJ, 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*. [Online]. (2014). Available at: http://dx.doi..org/10.1016/j.fgb.2014.03.007 [Accessed April 11, 2014].
18. Fernández-Fueyo E, Ruiz-Dueñas FJ, Martínez MJ, Romero A, Hammel KE, Medrano FJ and Martínez AT, Ligninolytic peroxidase genes in the oyster mushroom genome: Heterologous expression, molecular structure, catalytic and stability properties and lignin-degrading ability. *Biotechnol Biofuels* **7:**2 (2014).
19. Fernández-Fueyo E, Castanera R, Ruiz-Dueñas FJ, López-Lucendo MF, Ramírez L, Pisabarro AG and Martínez AT, Ligninolytic peroxidase gene expression by *Pleurotus ostreatus*: Differential regulation in lignocellulose medium and effect of temperature and pH. *Fungal Genet Biol*. [Online]. (2014). Available at: http://dx.doi.org/10.1016/j.fgb.2014.03.003 [Accessed February 18, 2014].
20. Fernández-Fueyo E, Ruiz-Dueñas FJ, Miki Y, Martínez MJ, Hammel KE and Martínez AT, Lignin-degrading peroxidases from genome of selective ligninolytic fungus *Ceriporiopsis subvermispora*. *J Biol Chem* **287**:16903–16906 (2012).

21. Morales M, Mate MJ, Romero A, Martínez MJ, Martínez AT and Ruiz-Dueñas FJ, Two oxidation sites for low redox-potential substrates: A directed mutagenesis, kinetic and crystallographic study on *Pleurotus eryngii* versatile peroxidase. *J Biol Chem* **287**:41053–41067 (2012).
22. Sáez-Jiménez V, Fernández-Fueyo E, Medrano FJ, Romero A, Martínez AT and Ruiz-Dueñas FJ, Improving pH-stability of versatile peroxidase through search for stabilizing motifs in stable peroxidases from genomes. *Res XXXVI Congr SEBBM,* P09r-2*,* Madrid, September 3–6 (2013).
23. Miki Y, Calviño FR, Pogni R, Giansanti S, Ruiz-Dueñas FJ, Martínez MJ *et al.*, Crystallographic, kinetic, and spectroscopic study of the first ligninolytic peroxidase presenting a catalytic tyrosine. *J Biol Chem* **286**:15525–15534 (2011).
24. Miki Y, Pogni R, Acebes S, Lucas F, Fernández-Fueyo E, Baratto MC *et al.,* Formation of a tyrosine adduct involved in lignin degradation by *Trametopsis cervina* lignin peroxidase: A novel peroxidase activation mechanism. *Biochem J* **452**:575–584 (2013).
25. Wang XS, Peter S, Kinne M, Hofrichter M and Groves JT, Detection and kinetic characterization of a highly reactive heme-thiolate peroxygenase compound I. *J Am Chem Soc* **134**:12897–12900 (2012).
26. Wang XS, Peter S, Ullrich R, Hofrichter M and Groves JT, Driving force for oxygen-atom transfer by heme-thiolate enzymes. *Angew Chem* **52**:9238–9241 (2013).
27. Peter S, Kinne M, Wang X, Ulrich R, Kayser G, Groves JT and Hofrichter M, Selective hydroxylation of alkanes by an extracellular fungal peroxygenase. *FEBS J* **278**:3667–3675 (2011).
28. Piontek K, Strittmatter E, Ullrich R, Grobe G, Pecyna MJ, Kluge M *et al.,* Structural basis of substrate conversion in a new aromatic peroxygenase: Cytochrome P450 functionality with benefits. *J Biol Chem* **288**:34767–34776 (2013).
29. Strittmatter E, Liers C, Ullrich R, Wachter S, Hofrichter M, Plattner DA and Piontek K, First crystal structure of a fungal high-redox potential dye-decolorizing peroxidase: Substrate interaction sites and long-range electron transfer. *J Biol Chem* **288**:4095–4102 (2013).
30. Strittmatter E, Wachter S, Liers C, Ullrich R, Hofrichter M, Plattner DA and Piontek K, Radical formation on a conserved tyrosine residue is crucial for DyP activity. *Arch Biochem Biophys* **537**:161–167 (2013).
31. Churakova E, Kluge M, Ullrich R, Arends I, Hofrichter M and Hollmann F, Specific photobiocatalytic oxyfunctionalization reactions. *Angew Chem* **50**:10716–10719 (2011).
32. Hernández-Ortega A, Borrelli K, Ferreira P, Medina M, Martínez AT and Guallar V, Substrate diffusion and oxidation in GMC oxidoreductases: An experimental and computational study on fungal aryl-alcohol oxidase. *Biochem J* **436**:341–350 (2011).
33. Hernández-Ortega A, Lucas F, Ferreira P, Medina M, Guallar V and Martínez AT, Role of active site histidines in the two half-reactions of the aryl-alcohol oxidase catalytic cycle. *Biochemistry* **51**:6595–6608 (2012).
34. Hernández-Ortega A, Lucas F, Ferreira P, Medina M, Guallar V and Martínez AT, Modulating O2 reactivity in a fungal flavoenzyme: Involvement of aryl-alcohol oxidase Phe-501 contiguous to catalytic histidine. *J Biol Chem* **286**:41105–41114 (2011).
35. Hernández-Ortega A, Ferreira P, Merino P, Medina M, Guallar V and Martínez AT, Stereoselective hydride transfer by aryl-alcohol oxidase, a member of the GMC superfamily. *ChemBioChem* **13**:427–435 (2012).
36. González-Pérez D, García-Ruiz E and Alcalde M, *Saccharomyces cerevisiae* in directed evolution. *Bioengineered* **3**:172–177 (2012).
37. Camarero S, Pardo I, Cañas AI, Molina P, Record E, Martínez AT *et al.,* Engineering platforms for directed evolution of laccase from *Pycnoporus cinnabarinus*. *Appl Environ Microbiol* **78**:1370–1384 (2012).
38. García-Ruiz E, Mate DM, González-Pérez D, Molina-Espeja P, Camarero S, Martínez AT *et al.,* Directed evolution of ligninolytic oxidoreductases: From functional expression to stabilization and beyond, in *Cascade Biocatalysis*, ed by S. Riva S and Fessner WD. Wiley-VCH, Weinheim, Germany (2014).
39. Torres-Salas P, Mate DM, Ghazi I, Plou FJ, Ballesteros AO and Alcalde M, Widening the pH activity profile of a fungal laccase by directed evolution. *ChemBioChem* **14**:934–937 (2013).
40. Mate DM, Gonzalez-Perez D, Kittl R, Ludwig R and Alcalde M, Functional expression of a blood tolerant laccase in *Pichia pastoris*. *BMC Biotechnol* **13:**38 (2013).
41. Mate DM, Gonzalez-Perez D, Falk M, Kittl R, Pita M, De Lacey AL *et al.,* Blood tolerant laccase by directed evolution. *Chem Biol* **20**:223–231 (2013).
42. Poraj-Kobielska M, Kinne M, Ullrich R, Scheibner K and Hofrichter M, A spectrophotometric assay for the detection of fungal peroxygenases. *Anal Biochem* **421**:327–329 (2012).
43. García-Ruiz E, González-Pérez D, Ruiz-Dueñas FJ, Martínez AT and Alcalde M, Directed evolution of a temperature-, peroxide- and alkaline pH-tolerant versatile peroxidase. *Biochem J* **441**:487–498 (2012).
44. González-Pérez D, Molina-Espeja P, García-Ruiz E and Alcalde M, Mutagenic organized recombination process by homologous *In vivo* grouping (MORPHING) for directed enzyme evolution. *PlosOne* **9**:3–e90919 (2014).
45. Gutiérrez A, Babot ED, Ullrich R, Hofrichter M, Martínez AT and del Río JC, Regioselective oxygenation of fatty acids, fatty alcohols and other aliphatic compounds by a basidiomycete heme-thiolate peroxidase. *Arch Biochem Biophys* **514**:33–43 (2011).
46. Babot ED, del Río JC, Kalum L, Martínez AT and Gutiérrez A, Oxyfunctionalization of aliphatic compounds by a recombinant peroxygenase from *Coprinopsis cinerea*. *Biotechnol Bioeng* **110**:2323–2332 (2013).
47. Gutiérrez A, Babot ED, Ullrich R, Hofrichter M, Martínez AT, del Río JC *et al.,* Enzymatic preparation of diols. International Patent WO2013-004639A2 (2011).
48. Peter S, Karich A, Ullrich R, Gröbe G, Scheibner K and Hofrichter M, Enzymatic one-pot conversion of cyclohexane into cyclohexanone: Comparison of four fungal peroxygenases. *J Mol Catal B-Enzym* **103**:47–51 (2014).
49. Peter S, Kinne M, Ullrich R, Kayser G and Hofrichter M, Epoxidation of linear, branched and cyclic alkenes catalyzed by unspecific peroxygenase. *Enzyme Microb Technol* **52**:370–376 (2013).
50. Kluge M, Ullrich R, Scheibner K and Hofrichter M, Formation of naphthalene hydrates in the enzymatic conversion of 1,2-dihydronaphthalene by two fungal peroxygenases and subsequent naphthalene formation. *J Mol Catal B-Enzym* **103**:56–60 (2014).
51. Barkova K, Kinne M, Ullrich R, Hennig L, Fuchs A and Hofrichter M, Regioselective hydroxylation of diverse flavonoids by an aromatic peroxygenase. *Tetrahedron* **67**:4874–4878 (2011).

52. Kluge M, Ullrich R, Scheibner K and Hofrichter M, Stereoselective benzylic hydroxylation of alkylbenzenes and epoxidation of styrene derivatives catalyzed by the peroxygenase of *Agrocybe aegerita*. *Green Chem* **14**:440–446 (2012).
53. Pecyna MJ, Schnorr KM, Ullrich R, Scheibner K, Kluge MG and Hofrichter M, Fungal peroxygenases and methods of application. European Patent EP2468852A1 (2012).
54. Poraj-Kobielska M, Kinne M, Ullrich R, Scheibner K, Kayser G, Hammel KE and Hofrichter M, Preparation of human drug metabolites using fungal peroxygenases. *Biochem Pharmacol* **82**:789–796 (2011).
55. Wu YH, Wollenberger U, Hofrichter M, Ullrich R, Scheibner K and Scheller FW, Direct electron transfer of *Agrocybe aegerita* peroxygenase at electrodes modified with chitosan-capped Au nanoparticles and its bioelectrocatalysis to aniline. *Sensor Actuat B-Chem* **160**:1419–1426 (2011).
56. Yarman A, Grobe G, Neumann B, Kinne M, Gajovic-Eichelmann N, Wollenberger U *et al.,* The aromatic peroxygenase from *Marasmius rotula*-a new enzyme for biosensor applications. *Anal Bioanal Chem* **402**:405–412 (2012).
57. Karich A, Kluge M, Ullrich R and Hofrichter M, Benzene oxygenation and oxidation by the peroxygenase of *Agrocybe aegerita*. *AMB Express* **3:**5 (2013).
58. Camarero S, Martínez MJ and Martínez AT, Understanding lignin biodegradation for the improved utilization of plant biomass in modern biorefineries. *Biofuels Bioprod Bioref* (2014), DOI:10.1002/bbb.1476.
59. Kinne M, Poraj-Kobielska M, Ullrich R, Nousiainen P, Sipila J, Scheibner K *et al.*, Oxidative cleavage of non-phenolic beta-O-4 lignin model dimers by an extracellular aromatic peroxygenase. *Holzforschung* **65**:673–679 (2011).
60. Gutiérrez A, Rencoret J, Cadena EM, Rico A, Barth D, del Río JC and Martínez AT, Demonstration of laccase-mediator removal of lignin from wood and non-wood plant feedstocks. *Bioresource Technol* **119**:114–122 (2012).
61. Rico A, Rencoret J, del Río JC, Martínez AT and Gutiérrez A, Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of *Eucalyptus* feedstock. *Biotechnol Biofuels* **7:**6 (2014).
62. Salvachúa D, Prieto A, Mattinen ML, Tamminen T, Liitiä T, Lille M *et al.,* Versatile peroxidase as a valuable tool for generating new biomolecules by homogeneous and heterogeneous cross-linking. *Enzyme Microb Technol* **52**:303–311 (2013).

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Dr Scheibner is professor for Enzyme Technology at the Brandenburg University of Technology (Senftenberg, Germany) and has worked for more than ten years as scientific manager of the JenaBios Biotechnologien und Serviceleistungen GmbH, a biotechnology SME in Jena (Germany). Her activities are related to microbial enzymes of industrial and biomedical interest. In the PEROXICATS project, she was in charge of the screening for new basidiomycetes producing wild-type peroxygenases and peroxidases, and she was responsible for the JenaBios contribution to the project.

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Dr Kalum is a senior researcher at Novozymes A/S in Bagsvaerd (Denmark), specialized in oxidoreductase for lignocellulose conversion, and advanced oxidation reactions. In PEROXICATS, she was responsible for the evaluation of a wide range of peroxygenase variants obtained at Novozymes on a wide range of oxygenation reactions and other biotransformations of industrial interest.

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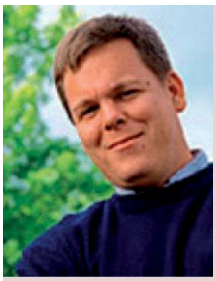
Dr Vind is Senior Science manager at Novozymes A/S. He has made numerous enzyme-variants that have turned into products that are being sold for various industrial applications. He is an inventor on more than 50 patents and patent applications. In the PEROXICATS project, he has been responsible for the fungal expression of the genes of interest, as well as the generation of the *Coprinopsis cinerea* peroxygenase variants.



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Henrik Lund is an R&D Director of Novozymes A/S in Bagsvaerd (Denmark), where he heads up the application R&D within the Forest Products and wood composites sector. His main expertise and interest is on lignocellulosic fiber modifying enzymes – and in particular on the transfer of novel enzyme products and processes from laboratory scale to commercially viable solutions at industrial scale. In PEROXICATS, he acted as the project Industrial Manager, promoting the exploitation of all the project results, and was also responsible for the Novozymes contribution to the project.

