PEROXICATS Report Summary

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Final Report Summary - PEROXICATS (Novel and more robust fungal peroxidases as industrial biocatalysts)

Executive Summary:
Today most industrially used enzymes are hydrolases, such as glycosidases and esterases. However, oxidoreductases have an unexploited potential as industrial biocatalysts to substitute harsh (and scarcely selective) chemical processes in the production of fine and bulk chemicals. These applications are dependent on the availability of high redox-potential peroxidases being able to oxidize the target industrial substrates, including different aromatics. In nature, several fungi from the group of basidiomycetes are the only organisms degrading the recalcitrant (aromatic) polymer of lignin, enabling the subsequent use of plant polysaccharides. Therefore, these organisms and the enzymes involved in lignin attack (that is, some peroxidases in the superfamily of non-animal peroxidases) would be the biocatalysts of choice for industrial delignification and other oxidative biotransformations. The latter also include oxygenation reactions, which are catalyzed with exquisite regio and stereo selectiveness by a group of basidiomycete peroxidases (peroxygenases) recently discovered by one of the project partners. In search for novel and more robust peroxidases and peroxygenases, basidiomycetes from unexplored habitats were screened, and many hundred genes were identified in basidiomycete genomes (in collaboration with the DOE Joint Genome Institute in California; and the INRA in Marseille) and grouped in three superfamilies (the classical non-animal peroxidases, and two new superfamilies of heme-thiolate peroxidases including peroxygenases, and dye-decolorizing peroxidases, respectively). Many of the most interesting genes (after "in silico" analyses) were heterologously expressed in laboratory and industrial hosts, and the corresponding enzymes structurally-functionally characterized and evaluated for oxidative biotransformations (in terms of activity and stability). Moreover, the structural-functional information obtained enabled us to further improve the enzyme operational (increasing pH stability, resistance against peroxide inactivation, etc) and catalytic properties for target transformations by rational design using directed mutagenesis. However, the structural bases for some of the above properties are unknown and, therefore, their improvement was addressed by "non-rational" design using directed evolution and combinatorial libraries. In this way, higher functional expression, thermal stability, peroxygenase-to-peroxidase activity ratio and other desirable properties were obtained. Then, more than one hundred target biotransformations, based on the new or improved oxidative biocatalysts, were analyzed (by general chromatographic/spectroscopic and more specific methods) including aliphatic and aromatic oxygenations, oxidative degradations, and related reactions. From one side, it is possible to emphasize, the regio and stereo selective hydroxylation of long/short-chain alkanes (a challenging reaction given the inert nature of the alkane C-H bond), the epoxidation of a variety of alkenes (e.g. yielding dimethyloxiranes) and the production of hydroxy-fatty acids. Interestingly, the possibility of inverting the reaction stereo and regio selectivities, by using different members from the same enzyme family, was also shown. Concerning aromatic oxygenations, regioselective hydroxylation of flavonoids, and stereoselective hydroxylation/epoxidation of alkyl/alkenyl benzenes were among the most remarkable reactions, together with enzymatic production of pyridine N-oxide. Most of the above reactions represent small-medium scale biotransformations of interest in the chemical or pharmaceutical sectors. Moreover, the feasibility of upscaled enzymatic hydroxylation of benzene was shown as a mild alternative for current harsh chemical processes with a high potential interest for large chemical markets (bulk chemicals). Finally, peroxidases/peroxygenases, and their improved variants, also showed a potential as delignification catalysts for lignocellulosic materials and for cellulose pulp bleaching, as well as in the decolorization of contaminant dyes from textile industries.
Project Context and Objectives:
The final objective of PEROXICATS is to supply the EU industry with new peroxidase-type robust biocatalysts of outstanding and diverse catalytic properties to substitute harsh 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.

Enzymes catalyze a huge amount 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. Simultaneously, protein engineering using rational and non-rational designs permits to adapt these enzymes to the industrial application conditions, and to increase their expression yields. This situation represented a real breakthrough for industrial biotechnology enabling the production of commercial enzymes at very low costs. The enzymatic biocatalysts obtained are often very competitive and more environmentally-friendly than the corresponding chemical reagents, at the same time that their substrate specificity permits to obtain specific products that are difficult and/or very expensive to obtain by pure chemical synthesis. 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 catalyse 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 80's. Therefore, although very important progresses in the understanding of these and other associated microbial oxidoreductases have been produced from these dates, our current knowledge level is by far much lower than on many hydrolases, a situation that leads to three main consequences: i) The complete repertoire of basidiomycete oxidoreductases has not been attained (as demonstrated by the discovery of new types widely represented in recently completed genomes); ii) Only the catalytic mechanism and structure-function relationships of a few of them have been elucidated; and iii) General systems for their heterologous expression are not available. The above issues not only limit the industrial production of these enzymes, but also the application of protein engineering tools to adapt their catalytic and operational properties to the specific requirements of industrial bio-transformations.

In the above context, PEROXICATS focuses on a dual approach (general objective) including: i) Screening fungal cultures from specific/extreme habitats and the huge genomic resources available to obtain enzymes with oxidoreductase activities of interest (including also the development of different heterologous expression systems); and ii) Developing rational design, directed evolution and related tools for engineering this particular type of enzymes and provide them with the catalytic and/or application properties required for their industrial utilization.

Among the different types of microbial peroxidases, a group of basidiomycete enzymes with the highest redox-potentials (i.e. better catalytic capabilities) enabling transformation of the widest range of chemical compounds, including the most recalcitrant ones, has been selected. Special attention was paid also to some basidiomycete peroxidases catalyzing selective mono-oxygenations acting as robust and self-sufficient biocatalysts, as well as to reactions catalyzed by other new peroxidase (super)families of industrial interest, some of them just recently discovered. Therefore, higher industrial use of such oxidoreductases will be promoted in PEROXICATS by the increased availability of novel enzymes (among the wide variability shown by the analysis of genomic resources) and a better adaptation of their properties to the industrial needs by both rational and non-rational protein engineering tools.

As a final result of the research carried out in the project, a universal “toolbox” of both novel and more robust peroxidase-type
enzymes will be available to the biotechnology companies to be offered to the chemical sector as an efficient alternative, in terms of energy consumption, environmental friendliness and/or reaction selectiveness, in specific chemical oxidation and oxyfunctionalization reactions of industrial interest.

According to the general concept described above, the following six specific S&T objectives were defined to be developed in the different workpackages of the PEROXICATS workplan:

- The search for peroxidases with novel properties of industrial interest (such as self-sufficient mono-oxygenation, and oxidation of recalcitrant and chemically inaccessible compounds) by screening fungal cultures from specific/extreme habitats, and the exponentially increasing genomic resources made available by the current and future use of massive sequencing tools (together with transcriptomic studies).
- The structural-functional characterization of the most interesting peroxidases/ peroxygenases selected to understand the bases of their catalytic properties, and to engineer these or some already-known peroxidases (e.g. commercial enzymes already expressed at higher yields) by a rational design using the above information to provide the properties required by site-directed mutagenesis and other techniques.
- To perform directed evolution, and apply related tools, as a non- or semi-rational alternative to rational design with the aim of solving some of the peroxidase drawbacks that could represent general bottlenecks for their industrial utilization and cannot be rationally addressed because a simple and direct structure-function relationship does not exist or cannot be easily established.
- To optimize different strategies for the production (homologous and heterologous expression) of peroxidases/peroxygenases of interest, adapted to the different needs of the gene screening, rational or non-rational design as well as the use of industrial hosts for large-scale production enabling the subsequent commercialisation of the selected enzyme candidates.
- The detailed chemical analysis of the reaction products of the new peroxidases/peroxygenases to characterize their catalytic activities and evaluate their industrial interest in biodegradation and oxyfunctionalization reactions using modern analytical techniques and both aromatic and lipophilic substrates including simple models.

Project Results:
The most outstanding non-confidential S&T results obtained in the course of the PEROXICATS EU-project are described, with a mention of those already disseminated by scientific publications (and other means) as they are listed in Point-E of the next Section of this Report (note that a number of results corresponding to the last points of this description will be disseminated in future publications, still in preparation).

A) SCREENING OF CULTURES AND GENOMES FOR ENZYMES OF INTEREST

A1) Enzymatic screening and secretomic analysis

The screening of fungal cultures included (i) the search for new extracellular enzymatic activities in over one hundred basidiomycete strains from unique and extreme environments and (ii) the identification of specific heme peroxidases of interest in secretomes of previously isolated basidiomycetes. The isolation and growth of the new fungal strains used culture media with complex carbon and nitrogen sources 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 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 members of the class-II of the classic superfamily of plant-fungal-prokaryotic peroxidases (hereinafter PODs) - namely lignin peroxidases (LiPs), versatile peroxidases (VPs), manganese peroxidases (MnPs) and generic peroxidases (GPs) - as well as auxiliary enzymes were identified, produced, characterized and evaluated as industrial biocatalysts, as described below. Among the novel peroxidases discovered, one from a lichenized ascomycete has a completely different phylogenetic origin (7), while a new hydrolase acting on both polysaccharide and cinnamic acid linkages
Xylaria polymorpha was among the new auxiliary enzymes that help peroxidases in their attack to lignified materials (8).

A2) Screening of basidiomycete genomes

The screening of basidiomycete genomes (in collaboration with the Joint Genome Institute, JGI, in California, and the INRA in Marseille) provided a huge information on the distribution of genes htp, dyp and pod (after a structural-functional classification of the predicted peroxidase sequences in genomes) 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 forty 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).

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 the structure-function relationships of the different catalytic sites appeared during the evolution of these enzymes, and will also permit to predict 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.

A3) Transcriptomic, metatranscriptomic and related studies

The search for peroxidases of industrial interest was completed by transcriptomic, metatranscriptomic and related studies. In this way, 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/secretion patterns and the ability of white-rot basidiomycete to grow on wood and 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.

B) STRUCTURE-FUNCTION STUDIES AND RATIONAL DESIGN OF ENZYMES

These studies included (i) biochemical characterization, (ii) stability studies, and (iii) molecular structures of the already-known and new enzymes (from genomes and other sources) followed by (iv) tailoring the above biochemical and stability properties by rational design based on the structural-functional information available.

B1) Evaluation of the whole peroxidase repertoire (in Pleurotus ostreatus)

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 ligninolytic peroxidases in a ligninolytic basidiomycete, after their
heterologous expression. It is important to mention that all the ligninolytic peroxidase gene models annotated in these genomes are operational (i.e. they are not pseudogenes) as shown by parallel transcriptional studies, although different proteins are over/expressed under different culture conditions, as mentioned below (9,10).

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, a 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 cofactor making the exposed tryptophan non-functional, as shown by reversed 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 lignin labeled with carbon-14 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 significant 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 was analyzed by quantitative PCR, by varying the temperature and pH conditions of cultures grown in a lignocellulose medium, combined with secretomic and activity 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 bases of the above properties. The high abundance of basic residues and salt-bridge/H-bond interactions at the surface of the protein was related to the high stability of one of these peroxidases, as confirmed by directed mutagenesis (see below).

B2) In the search for the 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 short and long subfamilies, an extralong MnP subfamily is considered in recent surveys of basidiomycete genomes (10). The three MnPs share a binding site (formed by three acidic residues near the internal propionate of heme) where Mn(II) is oxidized to Mn(III), acting 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(II)-oxidation site. It was concluded that the short forms represent a true MnP subfamily,
whose different catalytic and stability properties are related to the absence of the tail, while the so-called long and extralong MnPs did not show enough differences for two separate subfamilies. A highly stable extralong MnP (maintaining its activity at pH 2 that inactivates other PODs) was crystallized and, after structural-functional characterization, used as a robust protein scaffold to obtain stable high redox-potential peroxidases of interest as biocatalysts by introducing an exposed catalytic tryptophan by directed mutagenesis.

B3) 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 that a third oxidation site could exist in this enzyme. 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 all 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 evidence revealing the binding of guaiacol, whose oxidation was improved in the above variants, at the main heme access channel. This is the first time that a ligninolytic peroxidase is crystallized in complex with an aromatic substrate. Moreover, the VP variants 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 confer 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 (see above) were transferred to P. eryngii VP resulting in a significant stability improvement (22). These results confirm that stabilizing motifs found by 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: i) eliminated oxidizable residues; and ii) slowed-down the formation of compound-I, whose accumulation leads to peroxidase inactivation.

B4) 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, which had been isolated in Japan 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 electronic paramagnetic resonance (EPR) of the peroxide-activated enzyme (23).

Interestingly, a short initial delay during veratryl alcohol oxidation, which could have gone unnoticed, started further studies that resulted in the discovery of a novel activation mechanism for 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 (a fungal metabolite) 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 protease 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 oxidoreductase, and opens new possibilities in the design of high redox potential oxidative biocatalysts.

B5) Structure-function of the first self-sufficient heme oxygenase
The A. aegerita and related HTPs were extensively investigated here because of their monoxygenase activity on different types of substrates, acting as an unspecific peroxygenase (UPO). The catalytic cycle of the 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 compound-I, reminiscent to that recently found in cytochrome P450, as the sole intermediate (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 (an Fe(IV)-oxo porphyrin radical complex, which catalyzes a two electron oxidation of the substrate during transfer of its oxygen atom). In contrast, P450s need a second flavoprotein or flavin domain, and a source or reducing power, 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 oxygen-18 labeled hydrogen peroxide, which resulted in the incorporation of one oxygen-18 atom from the peroxide in 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 picoseconds and an oxygen rebound rate of 100 billion per second. Because of the above characteristics, together with its extracellular nature and stability, UPO behaves as a robust and self-sufficient (peroxide-activated) monoxygenase with a huge biotechnological potential.

To obtain structural-functional information helping to improve the UPO properties as an industrial biocatalyst, the first two crystal structures of a UPO (from A. aegerita) were solved (Fig. 2) (28). Interestingly, one of the UPO structures includes one 4(5)-hydroxymethylimidazole molecule at the active site, which would originate from hydroxylation of 4(5)-methylimidazole, a UPO substrate present in the culture medium. As expected, the active site is similar to that of P450s, with a cysteine as the fifth ligand of the heme iron and an axial access channel directly on the position of the reactive Fe(IV) oxo group in compound-I (occupied by a water molecule in the 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 the UPO 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, which serves as acid-base catalyst in peroxide activation in UPOs, is a glutamic acid residue and not a histidine.

The structural-functional information was useful in the “ad hoc” modulation of UPO catalytic properties for different target substrates by: i) selecting other HTPs from genomes; and ii) engineering the already available enzymes by semi-rational (see below) 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.

B6) New structural-functional studies on the “enigmatic” DyP superfamily

As in the case of the HTP superfamily, the structural-functional characteristics of the 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 distal side residues activating hydrogen peroxide. Among them, a mobile aspartic acid seems to play a key role by acting 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 propionate 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 a 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 peptide), 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. A variety of simple and multiple variants were obtained resulting in different tryptophanyl and tyrosyl radicals in the peroxide-activated enzyme that could be directly detected 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.

B7) Peroxidase "auxiliary" enzymes

The action of peroxidases in nature is absolutely dependent on the action of auxiliary enzymes providing the hydrogen peroxide required for enzyme 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.

First, diffusion of both the enzyme oxidizing (molecular oxygen) and reducing (different aryl alcohols) substrates into the active site, an intriguing aspect because of its buried nature, was shown to involve interaction of the aromatic rings of substrates with two aromatic residues and side-chain reorientations, while these changes were not required for oxygen diffusion (32). Then, it was shown 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 applications, as shown using chiral mixtures of aryl alcohols (35).

C) DIRECTED EVOLUTION TO DEVELOP INDUSTRIAL BIOCATALYSTS

C1) Laboratory directed evolution

One of the election expression hosts in laboratory directed evolution experiments, the bakery yeast Saccharomyces cerevisiae, was used for directed peroxidase evolution. New high-throughput protocols, or adaptations of those previously developed for improving 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" 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.

C2) Evolving the first ligninolytic peroxidase

The model VP from P. eryngii was the first peroxidase to be evolved for functional expression, activity and thermal stability. The best secretion variant (22 mg/L) was obtained after four generations (resulting in incorporation of four mutations) and two more generations under stronger temperature 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 (resulting in 60% residual activity after 120 h at pH 9, while the parental VP 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 combined 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, an important problem to be overcome in peroxidase biocatalysts (and also addressed by rational design). Since oxidative stability of peroxidase is an intrinsic mechanism based process, a variant with significantly higher half-life (at high peroxide concentrations) than the parental variant 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).

C3) 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 expression level and activity, using a new selective and sensitive colorimetric assay which allowed us to assess both peroxidative and oxygen-transfer activities (42). After five generations of evolution and hybrid approaches, 9 mutations were introduced providing a total activity improvement of 3,250-fold (without jeopardizing the protein stability). Expression levels of ~8 mg/L were achieved with activities of 6,500 ABTS-U/L and 1,300 NBD-U/L. Moreover, the evolved UPO was active and highly stable in the presence of organic cosolvents. 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. Mutations at the hydrophobic core of the signal peptide enhanced secretion levels whereas some mutations placed in the neighborhood of the heme access cavity increased the catalytic efficiencies for peroxidative and oxygen transfer reaction. Due to the high interest in UPOs for the different oxyfunctionalization reactions described below, 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 (see above), 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.

D) PRODUCT ANALYSIS AND INDUSTRIAL BIOCATALYST EVALUATION

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 P450 biocatalysts, the basidiomycete peroxygenases have the advantage of being self-sufficient (i.e. independent of a second flavin-containing enzyme or domain, and of a source of reducing power) and more robust enzymes, while yielding similar products.

D1) 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 3-1 and 3-2
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 continues with further hydroxylations yielding the [−1]- and [−2]-ketones (by dehydration of the gem-diol intermediates). In the case of terminal fatty alcohols, the oxygenation also takes place on the alcohol group first yielding the corresponding aldehyde (by gem-diol dehydration). The final products from these compounds are the [−1]- and [−2]-ketone 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 oxygen-18 in reactions with oxygen-18 labeled hydrogen peroxide, 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. Significantly 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 into cyclohexanone) (48); ii) linear, branched and cyclic alkenes into the corresponding epoxides, which constitute building blocks of high relevance (isobutene epoxide) or into allylic alcohols (e.g. limonene alcohols) (49); and iii) 1,2-dihyronaphthalene into naphthalene hydrates that desaturate to naphthalene (followed by oxygenation to 1-naphthol and 2-naphthol) (50).

D2) Selective oxygenations of aromatic target compounds

Regioselective and stereoselective oxygenations were also obtained in UPO reactions on aromatic compounds, affecting both the aromatic rings and the aliphatic substituents. Among them, a variety of flavonoids are hydroxylated by UPO, often 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 (95%), total turnover numbers (over 10 000) and enantiomeric excesses (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 investigated are related to 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 two reactions selected for industrial feasibility evaluation, 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), which would reduce the phenoxyl/semiquinone radicals, 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 two reactions to be evaluated: benzene-to-phenol and phenol-to-hydroquinone. 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 cumene process (that is energetically costly, operates under harsh conditions and generates equivalent amounts of acetone, whose demand affects the economy of the whole 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). However, the phenol yield was very low based on the peroxide added. This fact, together with the observed lost of enzyme activity during the first minutes of incubation, indicated 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 of this inactivation). Fortunately, the parallel trials of phenol hydroxylation showed no enzyme inactivation (no epoxide intermediate in this biotransformation) and yielded high hydroquinone production that, interestingly, was close to commercial viability of the process.

D3) Oxidative degradation reactions and related peroxidase applications

The new and improved peroxidases were also evaluated in a series of 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 and correlated with their capability for oxidative depolymerization of synthetic lignin (DHP) (5,18,20). In the case of VP, we were able to analyze its action of softwood/hardwood lignosulfonates showing that it is associated to the presence of the exposed catalytic tryptophan. 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 (Fig. 6). Redox mediators 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.

Pulp bleaching using peroxidases had already been reported, therefore, we investigated the potential of UPO in the presence of three mediators. In all cases the brightness increase was higher at pH 4 than at pH 7, suggesting that the "peroxidase" more than the "peroxygenase" activity of the enzyme was involved. This was supported by the similar brightness increases obtained with three UPO variants designed for increased peroxygenase activity. Interestingly, wild-type UPOs produced the highest brightness increase in the presence of 1-hydroxybenzotriazole, while the three variants consistently yielded the highest brightness in the presence of methyl syringate, a potentially cheap and safe mediator. On the other hand, a bleach boosting effect was also found with a new hydrolase (from Xylaria polymorpha) sharing different enzymatic activities.

Concerning whole lignocellulose delignification, milled eucalypt and Elephant grass (Pennisetum purpureum), a fast growing plant of interest for biofuel production, were treated with VP and veratryl alcohol without any previous chemical deconstruction. The 2D NMR spectra of the treated wood showed lignin modification in terms of aromatic units (with respect to polysaccharide units) and inter-unit linkages per aromatic unit, while the grass was more recalcitrant towards the treatment. Moreover, the existence of new veratraldehyde NMR signals after the enzymatic treatment suggested its partial incorporation to the lignin during VP oxidation of veratryl alcohol. This shows that VP (in the presence of veratryl alcohol) is able to modify lignin in whole lignocellulosic materials, although the changes are less drastic than obtained with the laccase-mediator system on the same substrates. 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 condensation 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 coupling reactions (60). These reactions included the successful homogeneous polymerization of molecules of low molecular
mass, such as lignans and peptides, and high molecular mass, such as proteins and polysaccharides. Heterogeneous polymerization of lignans and peptides were also observed, as well as gelation of feruloylated arabinoxylans and casein polymerization 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, e.g., enables oxidation of intercalary tyrosine residues in proteins and peptides).

Finally, the UPOs were 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 require large volumes of water for rinsing. Some of the UPO variants, among a total of more than thirty tested, were also able to decolorize the partially hydrolyzed dye Reactive Black 5 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.

Potential Impact:

A) SHORT-TERM IMPACT OF THE NEW BIOCATALYSTS

The aim of the PEROXICATS project was to identify new peroxidases/peroxygenases with a potential interest as industrial biocatalysts through fungal culture, genomic, transcriptomic and metatranscriptomic screenings, to improve their performances by rational design and directed evolution, and to study their oxygenation/oxidation activity on a variety of simple aliphatic and aromatic compounds, lignocellulosic materials and recalcitrant dyes. These tasks have been successfully performed by a small consortium constituted by two biotechnology companies (the world-leading enzyme producer Novozymes, and the specialized SME BIOS) and two research organizations (the Spanish CSIC, represented here by three institutes, and the International Graduate School of Zittau, IHIZ, since January 2013 part of the Dresden University of Technology, TUD, in Germany).

The results obtained will already have a direct impact, since several patents were submitted in the course of the project (or are currently in preparation) that will permit to the project companies to produce and commercialize the new or improved biocatalysts, or to be licensed by third parties. Moreover, specific aspects of the project results were further developed in several national projects and contracts with companies. However, to fully exploit the results obtained, further collaboration between some of the biotechnology companies and research organizations involved in PEROXICATS and additional companies representative for different areas of the chemical sector (including fine and bulk chemical producers), together with additional research groups (from the biotechnology, chemistry and chemical engineering sectors), has been necessary.

This is underway in the frame of the new INDOX large collaborative project (17 participants) on "Optimized oxidoreductases for medium and large scale industrial biotransformations" (KBBE-2013-7-613549) that started just one month before the end of PEROXICATS to minimize the delay in continuing and exploiting the promising results already available. While the main aim of PEROXICATS was the development and optimization of biocatalysts, the aim of INDOX will be to develop and optimize a limited number of bioprocesses, selected because of the interests of the chemical companies involved, and the availability of peroxidase/peroxygenase biocatalysts. In a wider frame, it is expected that the new bioprocesses will not only result in new commercial bioproducts by the companies involved, but will represent selected case stories for convincing the European chemical sector (of fine and, especially, bulk chemicals) on the potential advantages (see below) of introducing enzymatic biotechnologies in their production processes.

B) GENERAL IMPACT OF OXIDATIVE BIOCATALYSTS/BIO PROCESSES

The PEROXICATS results have the potential of contributing to a wider implementation of biotechnological processes in the European chemical sector, by incorporating oxidative biocatalysts that only represent a minor percentage of the industrial enzymes currently in use (that today are mainly represented by different types of hydrolases). The new oxidative biocatalysts (from the groups of peroxidases and peroxygenases) can occupy a variety of industrial niches to catalyze both oxidation and oxygenation reactions in the manufacture of fine chemicals and pharmaceuticals, and also in the production of different bulk chemicals of both petrochemical and renewable origin (from lignocellulosic biomass).

The potential impact of a wider use of biotechnology in the chemical sector will be both: i) economic, since the use of
biocatalysts can reduce the costs of the industrial processes due to their high selectivity (reducing the number of steps in bioprocesses compared with the corresponding chemical processes) and mild application conditions (resulting in reduced operation expenses); and ii) environmental, since the oxidative bioprocesses can substitute different harsh chemical processes that exert negative impacts in terms of both energy consumption (and carbon dioxide release) and generation of contaminant wastes and side-streams.

The European biotechnological industry still maintains a world leading position in the sector of industrial enzymes that will be reinforced against competitors (in North America, Japan and emerging countries) by the incorporation of new oxidative biocatalysts to their enzyme portfolios. More important, the introduction of innovative and more efficient technologies, as the new enzymatic biocatalysts developed by our biotechnological sector, could be a key factor to increase the competitiveness of the European Chemical sector, whose position in the world markets is declining in favor of Asian countries (with pessimistic forecasts for the near future). Finally, given the large dimension of the Chemical industrial sector in Europe, any competitiveness increase should have a very important economic and social (employment) impact in Europe.

In summary, the development of new oxidative biocatalysts, and the corresponding industrial bioprocesses, will contribute to maintain the world leading position of our Biotechnological sector, and can help the large European Chemical sector (producing different bulk and fine chemicals and pharmaceuticals) to recover its, currently declining, competitiveness resulting in positive economic, environmental and social impacts.

C) RESULT DISSEMINATION

In spite of the small size of the PEROXICATS project, the results obtained have been efficiently disseminated though Scientific publications, Congress presentations, Press releases and notes (including "popular press" and other general diffusion means), and the Website of the project. Forty-six scientific publications have been produced reporting results from studies funded by PEROXICATS. These publications include high impact journals where general aspects of the results obtained are described (such as Science, J. Amer. Chem. Soc. and Proc. Natl. Aca. Sci. USA), and specialized journals in the areas of Biotechnology (such as Biotechnol. Biofuels, Biofpr, Enzyme Microb. Technol., and J. Biotechnol.), Chemistry (such as Green Chem., ChemBioChem., and Angew. Chem.), Biochemistry (such as J. Biol. Chem., Biochem. J., Biochemistry, FEBS J., Arch. Biochem. Biophys. and Anal. Biochem.), Genetics (such as Genome Biol., and Fungal Genet. Biol.) and Microbiology (such as Appl. Environ. Microbiol., FEMS Microbiol. Ecol., Appl. Microbiol. Biotechnol., AMB Express, and Mycologia).

Eleven additional publications (in some of the above scientific journals plus Tetrahedron, Holzforschung, J. Mol. Catalysis-B, Biochem. Pharmacol., Sensor Actuat.-B, and Anal. Bioanal. Chem.) report results from studies performed by the project partners with other fundings, but directly based on or related to the results obtained with the PEROXICATS project funding. The national and international Congress presentations where PEROXICATS results were disseminated by the different project partners attained a total of forty-eight (such as 14th, 15th and 16th Meetings of the Spanish Lignocellulose Network in Madrid, Salamanca and Santiago de Compostela, respectively, 17th and 18th Intern. Conf. on Cytochrome P450 in Manchester and Seattle, respectively, 1st Eur. Congr. on Applied Biotechnology in Berlin, 1st World Congr. on Microbes in Beijing, 5th and 6th ICEP Conf. in Porto Seguro and Colonia de Sacramento, respectively, 6th and 7th JGI User Meeting in Walnut Creek, 6th Conf. Biocatalysts in Hamburg, 9th Conf. on Protein Stabilization in Lisbon, COST FP0901 Meeting-2012 in Espoo, EWLP-2012 in Espoo, FEMS Conf. on Ecology of Soil Microorganisms in Prague, FEMS-2013 Symp. in Leipzig, Lignobiotech-II in Fukuoka, MECP-2012 in Graz, Seminar of International Year of Chemistry in Madrid, Meeting on Functional P450-monoxygenases hybrid enzymes in Frankfurt, Oxizymes-2012 in Marseille, SEBBM-2011 in Barcelona, SEBBM-2013 in Madrid, SEM-2013 in Hospitalet de Llobregat, Symp. on Flavins and Flavoproteins in Berkeley, Workshop on Biomimetic and Bioanalytic Systems in Luckenwald, Workshop on Humans & Environment in Erfurt, and Biotrans-2011 in Sicilia). In the above congresses, twenty-three oral presentations and twenty-six poster presentations disseminated the project results. In addition to the above dissemination to the scientific community, some of the main PEROXICATS results were also disseminated to broader audiences and citizens in general. With this purpose, a total of twenty-one press releases and notes were published in the course of the project in different internet media including popular press (such as ABC Science, Agencia SINC, Biomass Magazine, CORDIS Research EU Results, CORDIS Technology Marketplace, CSIC website, DOE-JGI website,
As explained above, the PEROXICATS project was developed by a consortium constituted by two Biotechnology companies and two Research organizations, and its R&T objectives included the search and optimization of new/improved peroxidases and peroxygenases with a potential interest as oxidative industrial biocatalysts. Although many of the new findings and enzyme improvements obtained in the course of the project were already covered by previous patents of Novozymes and other partners (on peroxidases/peroxygenases), the following patents covering specific applications (such as the enzymatic production of diols from linear or branched substituted or unsubstituted aliphatic hydrocarbons, and N-oxides from N-containing heterocycles) were submitted in the course of the project:


Moreover, four additional patents are in preparation, or under consideration, covering several specific reactions with a potential industrial interest catalyzed by wild-type and/or engineered peroxidase/peroxygenase (by ICP and other partners, IRNAS and other partners, and Novozymes and other partners) and hydrolases (by Novozymes and IHIZ-TUD), as described in Table B2 (confidential information).

E) LIST OF SCIENTIFIC PUBLICATIONS AND PATENTS

The list includes all the forty-eight publications or patents (manuscript in preparation excluded) that were total or partially supported by the PEROXICATS project. Eleven publications (numbers 6, 8, 29-31, 47, 50, 53-55 and 58) of the project partners supported by other funding, but directly based or related to PEROXICATS publications, are also included, together with a congress presentation corresponding to a PEROXICATS manuscript in preparation (number 22).


aromatic peroxygenase by the agaric fungus Marasmius rotula. AMB Express 1:31-42.


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