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Directed Evolution of Ligninolytic Oxidoreductases: from Functional Expression to Stabilization and Beyond

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1.1

Introduction

The ligninolytic enzymatic consortium, formed mainly by nonspecific oxidoreductases (laccases, peroxidases, and H₂O₂-supplying oxidases), is a potentially powerful multipurpose tool for industrial and environmental biotechnology. In nature, these enzymes are typically produced by basidiomycete white-rot fungi that are involved in lignin decay. Thanks to their broad substrate specificity, high redox potential, and minimal requirements, these enzymes have many potential applications in the field of green chemistry, including the production of biofuels, bioremediation, organic syntheses, pulp biobleaching, food and textile industries, and the design of bionanodevices. The implementation of this enzymatic armoury in different biotechnological sectors has been hampered by the lack of appropriate molecular instruments (including heterologous hosts for directed evolution) with which to improve their properties. Over the last 10 years, a wealth of directed evolution strategies in combination with hybrid approaches has emerged in order to adapt these oxidoreductases to the drastic conditions associated with many biotechnological settings (e.g., high temperatures, the presence of organic co-solvents, extreme pHs, the presence of inhibitors). This chapter summarizes all efforts and endeavors to convert these ligninolytic enzymes into useful biocatalysts by means of directed evolution: from functional expression to stabilization and beyond.

1.2

Directed Molecular Evolution

Enzymes are versatile biomolecules that exhibit a large repertory of functions acquired over millions of years of natural evolution. Indeed, they are the fastest known catalysts (accelerating chemical reactions as much as 10¹⁹-fold) and are environmentally friendly molecules, working efficiently at mild temperatures, in water, and releasing few by-products. Moreover, they can exhibit high enantioselectivity and chemoselectivity. Nonetheless, when an enzyme is removed

from its natural environment and introduced into a specific biotechnological location (e.g., the transformation of a hydrophobic compound in the presence of co-solvents or at high temperatures), its molecular structure may not tolerate the extreme operational conditions and may unfold becoming inactive. Unfortunately, the enzymes that cells use to regulate strict metabolic pathways and that promote fitness and survival in nature are not always applicable to the harsh requirements of many industrial processes.

The development of the polymerase chain reaction (PCR) in the early 1980s heralded a biotechnological revolution for protein engineers, allowing us for the first time to manipulate and design enzymes by site-directed mutagenesis supported by known protein structures: the so-called rational design. However, further advances were frustrated owing to the limited understanding of protein function and the lack of protein structures available at the time. Nevertheless, the following decade saw a second biotechnological revolution with the development of directed molecular evolution. This powerful protein engineering tool does not require prior knowledge of protein structure to enhance the known features or to generate novel enzymatic functions, which are not generally required in natural environments. The key events of natural evolution (random mutation, DNA recombination, and selection) are recreated in the laboratory, permitting

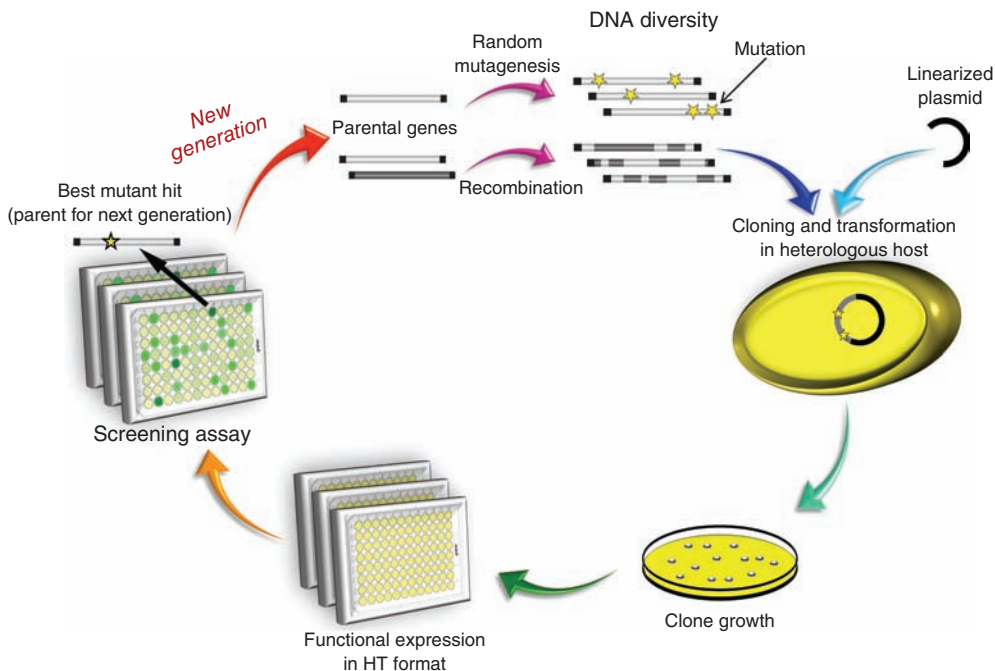


Figure 1.1 Directed molecular evolution. The basic premises to carry out a successful directed evolution experiment are (i) a robust heterologous expression system

(typically *S. cerevisiae* or *E. coli*); (ii) a reliable high-throughput (HT)-screening assay; and (iii) the use of different molecular tools for the generation of DNA diversity.

scientifically interesting and technologically useful enzymes to be designed [1–3]. Diversity is generated by introducing random mutations and/or recombination in the gene encoding a specific protein [4, 5]. In this process, the best performers in each round of evolution are selected and used as the parental types in a new round, a cycle that can be repeated as many times as necessary until a biocatalyst that exhibits the desired traits is obtained: for example, improved stability at high temperatures, extreme pHs, or in the presence of nonconventional media such as organic solvents or ionic fluids; novel catalytic activities; improved specificities and/or modified enantioselectivities; and heterologous functional expression [6–8] (Figure 1.1). Of great interest is the use of directed evolution strategies to engineer ligninolytic oxidoreductases while employing rational approaches to understand the mechanisms underlying each newly evolved property.

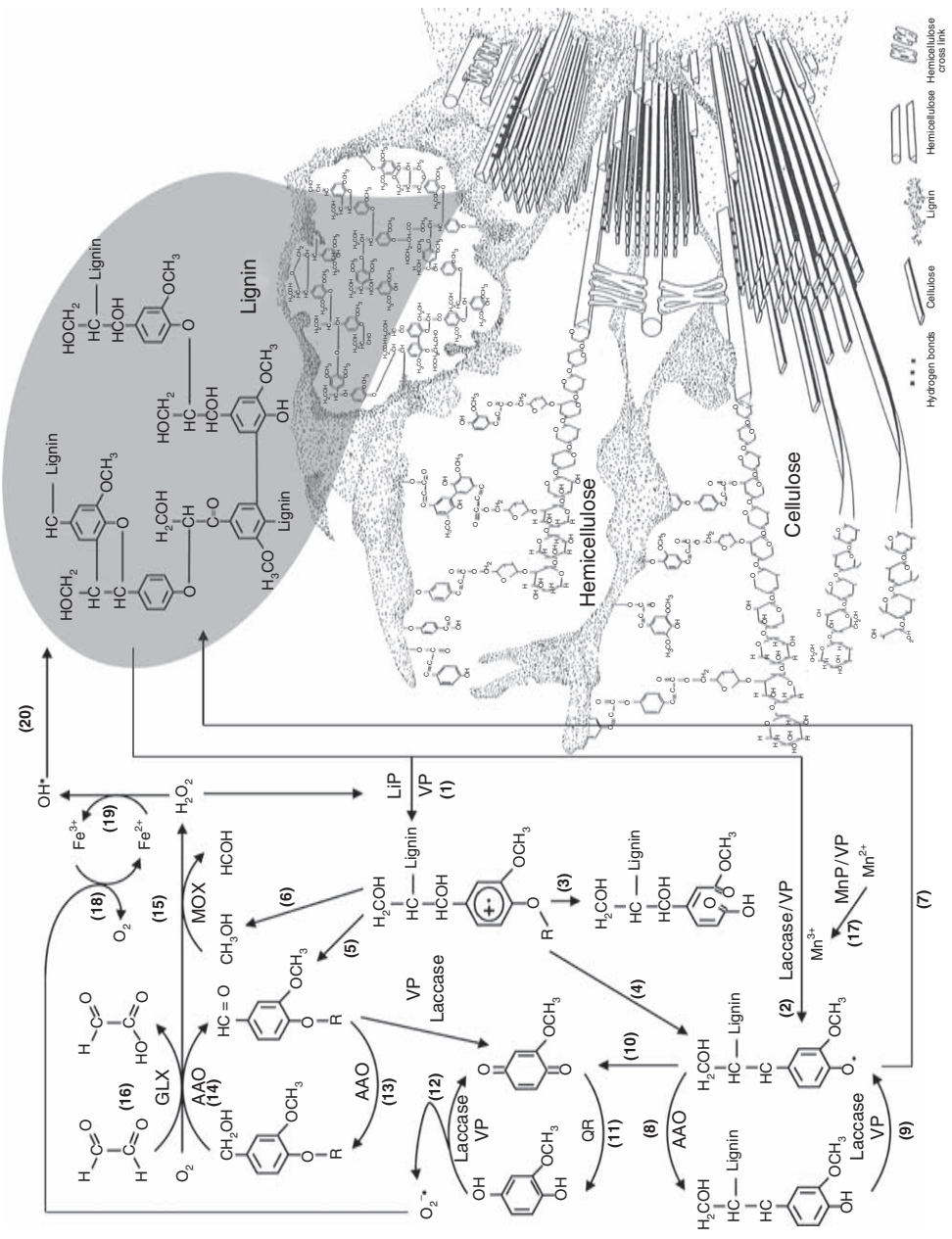
1.3

The Ligninolytic Enzymatic Consortium

Lignin is the most abundant natural aromatic polymer and the second most abundant component of plant biomass after cellulose. As a structural part of the plant cell wall, lignin forms a complex matrix that protects cellulose and hemicellulose chains from microbial attack and hence from enzymatic hydrolysis. This recalcitrant and highly heterogeneous biopolymer is synthesized by the dehydrogenative polymerization of three precursors belonging to the *p*-hydroxycinnamyl alcohol group: *p*-coumaryl, coniferyl, and sinapyl alcohols [9]. As one-third of the carbon fixed as lignocellulose is lignin, its degradation is considered a key step in the recycling of carbon in the biosphere and in the use of the plant biomass for biotechnological purposes [10, 11]. Lignin is modified and degraded to different extents by a limited number of microorganisms, mainly filamentous fungi and bacteria. Lignin degradation by bacteria is somewhat limited and much slower than that mediated by filamentous fungi [12, 13]. Accordingly, the only organisms capable of completing the mineralization of lignin are the white-rot fungi, which produce a white-colored material upon delignification because of the enrichment in cellulose [14, 15].

Through fungal genome reconstructions, recent studies have linked the formation of coal deposits during the Permo-Carboniferous period (~260 million years ago) with the nascent and evolution of white-rot fungi and their lignin-degrading enzymes [16]. Lignin combustion by white-rot fungi involves a very complex extracellular oxidative system that includes high-redox potential laccases (HRPLs), peroxidases and unspecific peroxygenases (UPOs), H₂O₂-supplying oxidases and auxiliary enzymes, as well as radicals of aromatic compounds and oxidized metal ions that act as both diffusible oxidants and electron carriers [12, 13, 15, 17]. Although the role of each component of the consortium has been studied extensively, many factors remain to be elucidated (Figure 1.2).

Laccases typically oxidize the phenolic units of lignin. Lignin peroxidases (LiPs) oxidize both nonphenolic lignin structures and veratryl alcohol (VA), a metabolite synthesized by fungi that helps LiP to avoid inactivation by H₂O₂ and whose radical



cation may act as a redox mediator [20]. Manganese peroxidases (MnPs) generate Mn^{3+} , which upon chelation with organic acids (e.g., oxalate synthesized by fungi) attacks phenolic lignin structures; in addition, MnP can also oxidize nonphenolic compounds via lipid peroxidation [21]. Versatile peroxidases (VPs) combine the catalytic activities of LiP, MnP, and generic peroxidases to oxidize phenolic and nonphenolic lignin units [22]. Some fungal oxidases produce the H_2O_2 necessary for the activity of peroxidases. Among them, aryl-alcohol oxidase (AAO) transforms benzyl alcohols to the corresponding aldehydes; glyoxal oxidase (GLX) oxidizes glyoxal producing oxalate, which in turn chelates Mn^{3+} ; and then methanol oxidase (MOX) converts methanol into formaldehyde; all the above oxidations are coupled with O_2 reduction of H_2O_2 . Other enzymes such as cellobiose dehydrogenase (CDH) have been indirectly implicated in lignin degradation. This is because of CDH ability to reduce both ferric iron and O_2 -generating hydroxyl radicals via Fenton reaction. These radicals are strong oxidizers that act as redox mediators playing a fundamental role during the initial stages of lignin polymer decay, when the small pore size of the plant cell wall prevents the access of fungal enzymes [23]. The same is true for laccases, whose substrate spectrum can be broadened in the presence of natural mediators to act on nonphenolic parts of lignin [24].

High-redox potential laccases and peroxidases/peroxygenases are of great biotechnological interest [25, 26]. With minimal requirements and high redox potentials (up to +790 mV for laccases and over +1000 mV for peroxidases), these enzymes can oxidize a wide range of substrates, finding potential applications in a variety of areas, which are as follows:

Figure 1.2 General view of the plant cell wall and the action of the ligninolytic enzymatic consortium. The lignin polymer is oxidized by white-rot fungi laccases and peroxidases, producing nonphenolic aromatic radicals (1) and phenoxy radicals (2). Nonphenolic aromatic radicals can suffer nonenzymatic modifications such as aromatic ring cleavage (3), ether breakdown (4), $C_\alpha-C_\beta$ cleavage (5), and demethoxylation (6). The phenoxy radicals (2) can repolymerize on the lignin polymer (7) or be reduced to phenolic compounds by AAO (8) (concomitantly with aryl alcohol oxidation). These phenolic compounds can be re-oxidized by fungal enzymes (9). In addition, phenoxy radicals can undergo $C_\alpha-C_\beta$ cleavage to produce *p*-quinones (10). Quinones promote the production of superoxide radicals via redox cycling reactions involving QR, laccases, and peroxidases (11, 12). The aromatic aldehydes released from $C_\alpha-C_\beta$ cleavage, or synthesized by fungi, are involved in the production of H_2O_2 via another redox cycling reaction involving

AAO and AAO (13, 14). Methanol resulting from demethoxylation of aromatic radicals (6) is oxidized by MOX to produce formaldehyde and H_2O_2 (15). Fungi also synthesize glyoxal, which is oxidized by GLX to produce H_2O_2 and oxalate (16), which in turn chelate Mn^{3+} ions produced by MnP (17). The Mn^{3+} chelated with organic acids acts as a diffusible oxidant for the oxidation of phenolic compounds (2). The reduction of ferric ions present in wood is mediated by the superoxide radical (18) and they are re-oxidized by the Fenton reaction (19) to produce hydroxyl radicals, which are very strong oxidizers that can attack the lignin polymer (20). AAO, aryl-alcohol oxidase; AAD, aryl-alcohol dehydrogenase; GLX, glyoxal oxidase; LiP, lignin peroxidase; MnP, manganese peroxidase; MOX, methanol oxidase; QR, quinone reductase; VP, versatile peroxidase. (Figure adapted from [18, 19].) (Source: Bidlack, J.M. *et al.* 1992 [18], Fig. 1, p. 1. Reproduced with permission of the Oklahoma Academy of Science.)

- The use of lignocellulosic materials (e.g., agricultural wastes) in the production of second-generation biofuels (bioethanol, biobutanol) or the manufacture of new cellulose-derived and lignin-derived value-added products.
- The organic synthesis of drugs and antibiotics, cosmetics and complex polymers, and building blocks.
- In nanobiotechnology as (i) biosensors (for phenols, oxygen, hydroperoxides, azides, morphine, codeine, catecholamines, or flavonoids) for clinical and environmental applications; and (ii) biofuel cells for biomedical applications.
- In bioremediation: oxidation of polycyclic aromatic hydrocarbons (PAHs), dioxins, halogenated compounds, phenolic compounds, benzene derivatives, nitroaromatic compounds, and synthetic organic dyes.
- The food industry: drink processing and bakery products.
- The paper industry: pulp biobleaching, pitch control, manufacture of mechanical pulps with low energy cost, and effluent treatment.
- The textile industry: remediation of dyes in effluents, textile bleaching (e.g., jeans), modification of dyes and fabrics, detergents.

A few years ago, the engineering and improvement of ligninolytic oxidoreductases was significantly hampered by the lack of suitable heterologous hosts to carry out directed evolution studies. Fortunately, things have changed and several reliable platforms for the directed evolution of ligninolytic peroxidases, peroxygenases, and several medium-redox potential laccases and high-redox potential laccases (HRPLs) have been developed using the budding yeast *Saccharomyces cerevisiae*. These advances have allowed us, for the first time, to specifically tailor ligninolytic oxidoreductases to address new challenges.

1.4

Directed Evolution of Laccases

Laccases (EC 1.10.3.2) are extracellular glycoproteins that belong to the blue multicopper oxidase family (along with ascorbate oxidase, ceruloplasmin, nitrite reductase, bilirubin oxidase, and ferroxidase). Widely distributed in nature, they are present in plants, fungi, bacteria, and insects [27, 28]. Laccases are *green* catalysts, which are capable of oxidizing dozens of compounds using O₂ from air and releasing H₂O as their sole by-product [29–31]. These enzymes harbor one type I copper (T1), at which the oxidation of the substrates takes place, and a trinuclear copper cluster (T2/T3) formed by three additional coppers, one T2 and two T3s, at which O₂ is reduced to H₂O. The reaction mechanism resembles a battery, storing electrons from the four monovalent oxidation reactions of the reducing substrate required to reduce one molecule of oxygen to two molecules of H₂O. Laccases catalyze the transformation of a wide variety of aromatic compounds, including *ortho*- and *para*-diphenols, methoxy-substituted phenols, aromatic amines, benzenothiols, and hydroxyindols. Inorganic/organic metal compounds are also substrates of laccases, and it has been reported that Mn²⁺ is oxidized by laccase to form Mn³⁺, and organometallic compounds such

$[\text{Fe}(\text{CN})_6]^{2-}$ are also accepted by the enzyme [32]. The range of reducing substrates can be further expanded to nonphenolic aromatic compounds, otherwise difficult to oxidize, by including redox mediators from natural or synthetic sources. Upon oxidation by the enzyme, such mediators act as diffusible electron carriers in the so-called laccase-mediator systems [24].

Later we summarize the main advances made in the directed evolution of this interesting group of oxidoreductases, paying particular attention to fungal laccases.

1.4.1

Directed Evolution of Low-Redox Potential Laccases

Several directed evolution studies of bacterial laccase CotA have successfully improved its substrate specificity and functional expression, modifying its specificities by screening mutant libraries through surface display [33–37]. The advantages of some bacterial laccases include high thermostability and activity at neutral/alkaline pH, although a low-redox potential at the T1 site often precludes their use in certain sectors.

1.4.2

Directed Evolution of Medium-Redox Potential Laccases

The first successful example of the directed evolution of fungal laccase involved the laccase from the thermophile ascomycete *Myceliophthora thermophila* laccase (MtL). This study led to subsequent directed evolution experiments in *S. cerevisiae* with several high-redox potential ligninolytic oxidoreductases (see below). MtL was subjected to 10 cycles of directed evolution to enhance its functional expression in *S. cerevisiae* [38]. The best performing variant of this process (the T2 mutant that harbored 14 mutations) exhibited a total improvement of 170-fold in activity: its expression levels were enhanced 8-fold and the k_{cat}/K_m around 22-fold. The H(c2)R mutation at the C-terminal tail of MtL introduced a recognition site for the KEX2 protease of the Golgi compartment, which facilitated its appropriate maturation and secretion by yeast. Using this laccase expression system as a departure point, five further cycles of evolution were performed to make the laccase both active and stable in the presence of organic co-solvents, a property that makes it suitable for many potential applications in organic syntheses and bioremediation [39–42]. The stability variant (the R2 mutant) functioned in high concentrations of co-solvents of different chemical natures and polarities (a promiscuity toward co-solvents that was promoted during the directed evolution [40]). Most of the mutations introduced in the evolutionary process were located at the surface of the protein, establishing new interactions with surrounding residues, which resulted in structural reinforcement. In the course of these 15 generations of evolution for functional expression in yeast [38] and stabilization in the presence of organic co-solvents [40], the laccase shifted its optimum pH toward less acidic values. Fungal laccases that are active at neutral/alkaline pHs are highly desirable for many applications, such as detoxification, pulp biobleaching, biomedical uses,

and enzymatic co-factor regeneration. Recently, the MtL-R2 mutant was converted into an alkalophilic fungal laccase [43]. Accordingly, a high-throughput screening (HTS) assay based on the activity ratio at pH 8.0 to 5.0 was used as the main discriminatory factor. Screening the laccase mutant libraries at alkaline pH while conserving activity at acidic pHs led to a shift in the pH activity profiles that was accompanied by improved catalytic efficiency at both pH values (31-fold at pH 7.0 and 12-fold at pH 4.0). The final variant obtained in this evolution experiment (the IG-88 mutant) retained 90% of its activity at pH 4.0–6.0 and 50% at pH 7.0, and some activity was even detected at pH 8.0.

After 20 generations, the successful *in vitro* evolution of MtL can be attributed to the plasticity and robustness of this thermostable protein, highlighting that there may be an additional margin for further engineering.

1.4.3

Directed Evolution of Ligninolytic High-Redox Potential Laccases (HRPLs)

Two HRPLs from the basidiomycete PM1 laccase (PM1L) and *Pycnoporus cinnabarinus* laccase (PcL) were subjected to parallel comprehensive directed evolution in order to achieve functional expression in *S. cerevisiae* while conserving their thermostability [44]. PM1L was tailored during eight cycles of directed evolution combined with semirational/hybrid approaches [45]. The native laccase signal sequence was replaced by the α -factor prepro-leader from *S. cerevisiae* and it was evolved in conjunction with the mature protein, adjusting both elements for a successful exportation by yeast. After screening over 50 000 clones, this approach led to the generation of a highly active, soluble, and thermostable HRPL. The total improvement in activity achieved was as high as 34 000-fold relative to the parental type, an effect brought about by the synergies established between the evolved prepro-leader and the mature laccase. Several strategies were employed to maintain the stability of the laccase while enhancing its activity and secretion during evolution: (i) screening for stabilizing mutations [46]; (ii) mutational exchange with beneficial PcL mutations; and (iii) mutational recovery of beneficial mutations with a low likelihood of recombination [44, 47].

The final mutant generated in this process (the OB-1 variant with 15 mutations accumulated both in the prepro-leader and in the mature protein) exhibited secretion levels of $\sim 8 \text{ mg l}^{-1}$, and it was very active and stable over a range of temperatures ($T_{50} \sim 73^\circ\text{C}$) and pH values, as well as in the presence of organic co-solvents [45]. OB-1 was recently subjected to four further rounds of directed evolution and saturation mutagenesis in order to achieve activity in human blood, a milestone that will allow it to be used in a wide array of exciting biomedical and bioanalytical applications [48]. The inherent inhibition of laccase by the combined action of high NaCl concentrations ($\sim 140 \text{ mM}$) and the alkaline pH (~ 7.4) of blood was overcome by using an *ad hoc* HTS assay in a buffer that simulated blood but lacked coagulating agents and red blood cells. Bearing in mind that HRPLs are not active at neutral pH, the selective pressure was enhanced in successive rounds of evolution, starting at pH 6.5 and finishing at physiological pH. The final

laccase mutant obtained (the ChU-B variant) was comprehensively characterized and tested in real human blood samples, revealing the mechanisms underlying this unprecedented improvement. The ChU-B variant conserved a high-redox potential at the T1 site and exhibited the highest tolerance to halides reported for any HRPL (with an increase in the I_{50} for Cl^- from 176 mM to over 1 M with -2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as the substrate), and it displayed significant activity at neutral pH (retaining 50% and 20% of its activity for 2,6-dimethoxyphenol (DMP) and ABTS, respectively). This was the first successful example of the use of laboratory evolution to optimize an oxidoreductase for enhanced catalysis in blood for biomedical purposes. From a more general point of view, this development is of considerable importance for a wide range of biotechnological sectors (e.g., bioremediation, pulp-kraft biobleaching), and especially in biocatalysis to develop novel green syntheses. With respect to the parental type, the ChU-B variant presented only two extra mutations in the mature protein (F396I and F454E), which were responsible for its activity in blood but compromised its stability (a 10 °C decrease in the T_{50} , Figure 1.3). By individually analyzing F454E and F396I mutations, a shift in the pH profile from 4.0 to 5.0 (with DMP as substrate) was detected along with a considerable increases in the I_{50} for

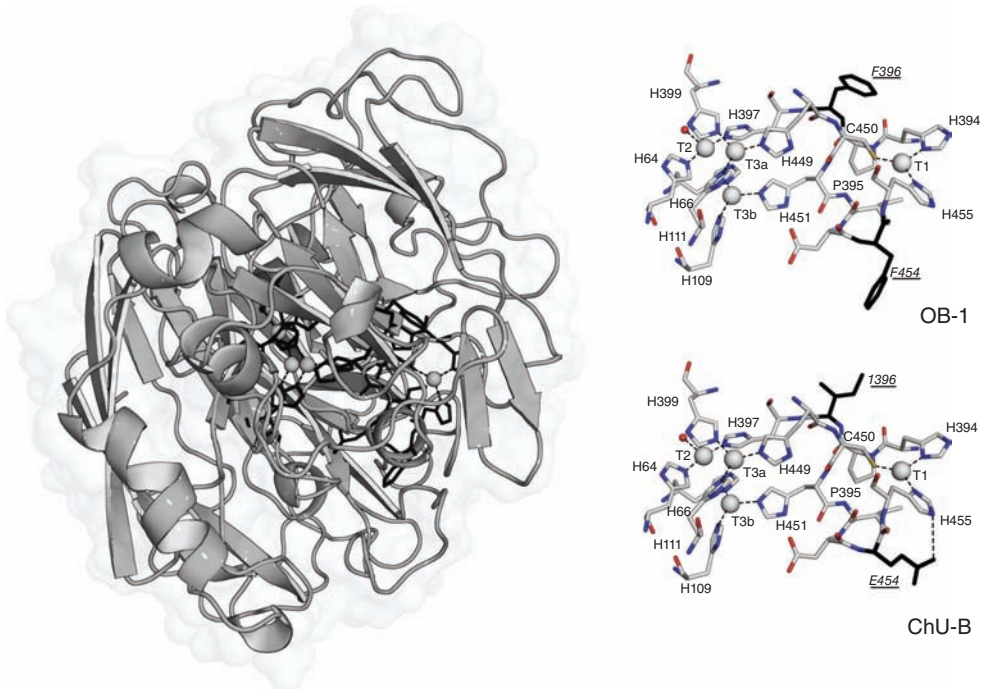


Figure 1.3 General structure and details of the blood-tolerant laccase (ChU-B mutant). The F396I and F454E mutations are located 7.6 Å away from the T1 Cu site (in the

second coordination sphere). The 3D structure model is based on the crystal structure of the *Trametes trogii* laccase (97% identity, PDB: 2HRG).

halides and decreases in T_{50} values (4.8 and 3.6 °C for both mutations, respectively). When a crossroad is reached between activity and stability, it is difficult to further evolve the protein as it does not tolerate the introduction of new sets of beneficial but destabilizing mutations without compromising its structure and function. We are currently attempting to improve the stability of this variant by introducing new stabilizing mutations, such as A361T and S482L from the 16B10 mutant of PM1L [46]. These results reflect the subtle equilibrium between activity and stability when evolving enzymes in the laboratory for nonnatural functions, consistent with the observations in earlier directed evolution studies. For example, a decrease by 10 °C in the T_{50} was obtained following the directed evolution of P450 BM-3 from *Bacillus megaterium* to convert it into an alkane monooxygenase [49–51].

To conclude this mutational pathway, PM1L was sculpted by 12 rounds of directed evolution, in which it accumulated 22 mutations (8 silent) throughout the entire fusion gene. Beneficial mutations that enhanced secretion or activity were located in the signal prepro-leader (5 mutations) and the mature protein (7 mutations), respectively. Significantly, only two mutations located in the second coordination sphere of the T1 copper site conferred tolerance to blood. Therefore, the re-specialization required to adapt the PM1L to such inclement conditions affected only 0.4% of the amino acid sequence.

The evolution of the HRPL from P_{CL} was tackled using a similar approach to that described for PM1L (i.e., joint evolution of the α -factor prepro-leader and the mature protein). Six cycles of directed evolution were performed to obtain an enzyme that could be readily expressed by yeast (with secretion levels of $\sim 2 \text{ mg l}^{-1}$ [52]). A multiple HTS assay based on the oxidation of natural and synthetic redox mediators was employed to discriminate between mutants with improved activities against phenolic and nonphenolic compounds. The final variant of this process (the 3PO mutant, containing 14 mutations) retained its thermostability while significantly broadening its pH activity profile. Notably, the breakdown in secretion and activity was accomplished by fusing the evolved prepro-leader to the native P_{CL}. The evolved signal sequence improved secretion 40-fold, while the mutations that accumulated in the evolved mature protein were responsible for a ~ 14 -fold enhancement in the k_{cat} , together with an improved secretion/folding of the enzyme (~ 14 -fold improvement). The directed evolution of signal peptides to enhance secretion and their additional attachment to nonevolved proteins is a valuable strategy for the directed evolution of other ligninolytic oxidoreductases (unspecific peroxigenases, see below [53]).

The sequence identity between P_{CL} and PM1L is over 77%, which facilitated mutational exchange between the two parallel evolution pathways and allowed us to switch protein sequence blocks to create chimeric proteins of HRPLs with hybrid or even enhanced features. To favor multiple crossover events between laccase scaffolds, *in vitro* and *in vivo* DNA recombination methods were combined in a single evolutionary step (see Section 1.6). Chimeras with up to six crossover events per sequence were identified, which generated active laccase hybrids with combined characteristics in terms of substrate affinity, pH activity, and thermostability [54]. Interestingly, some chimeras showed higher thermostabilities than the original

laccases, demonstrating the importance of accumulating neutral mutations to create an artificial genetic drift that is beneficial to stabilize the protein structure. Other laccase chimeragenesis experiments have been performed using laccase isoenzymes from *Trametes* sp. C30, but employing a low-redox potential laccase backbone to construct the chimeric libraries [55].

PcL and PM1L evolution aside, the *lcc1* gene from *Trametes versicolor* laccase (TvL) was evolved in the yeast *Yarrowia lipolytica*, demonstrating the potential for directed evolution in this host [56]. More recently, the *lcc2* gene from TvL expressed by *S. cerevisiae* was subjected to two rounds of random mutagenesis for improved ionic liquid resistance [57]. In addition, directed evolution experiments have been carried out with HRPLs from *Pleurotus ostreatus* to enhance the laccase activity in combination with computational approaches [58–60], and with HRPLs from *Rigidoporus lignosus* to increase functional expression in *Pichia pastoris* [61]. Recently, the evolved PM1L was analyzed using a computational algorithm to elucidate the physical forces that govern the thermostability of the variant [62]. Indeed, the combination of *in silico* computational methods (based on Monte Carlo simulations and molecular dynamics) and directed evolution may offer new directions to study evolved enzymes.

1.5

Directed Evolution of Peroxidases and Peroxygenases

Ligninolytic peroxidases (EC 1.11.1) are high-redox potential oxidoreductases belonging to Class II of the plant-fungal-prokaryotic peroxidase superfamily, and they correspond to fungal secreted heme-containing peroxidases. These enzymes contain ~300 amino acids distributed in 10–12 α -helix and 4–5 short β -structures that are located in two domains. The heme-prosthetic group contains an Fe^{3+} in the resting state, and the overall structure is supported by four or five disulfide bridges and two structural Ca^{2+} ions that confer stability to the protein. The general catalytic cycle of ligninolytic peroxidases begins with the oxidation of the enzyme by one molecule of H_2O_2 . This activates the enzyme to Compound I (a two-electron-deficient intermediate), which under turnover conditions is reduced back to the resting state via two successive one-electron oxidation steps. Ligninolytic peroxidases are divided into three types [12, 13, 15, 26, 63, 64]:

- (i) Lignin peroxidases (LiP, EC 1.11.1.13) are capable of directly oxidizing model lignin dimers and nonphenolic aromatic compounds, as well as other high-redox potential substrates (including dyes) using VA as redox mediator, through a catalytic tryptophan located at the surface of the protein.
- (ii) Mn peroxidases (MnP, EC 1.11.1.14) oxidize Mn^{2+} to form Mn^{3+} , which upon chelation with organic acids can act as a diffusible oxidant for the oxidation of phenolic compounds.
- (iii) Versatile peroxidases (VP, EC 1.11.1.16) combine the catalytic properties of LiP and MnP, and they exhibit great versatility and biotechnological potential. VP oxidizes typical LiP substrates (e.g., VA, methoxybenzenes, and

nonphenolic model lignin compounds), as well as Mn^{2+} (the classical MnP substrate). VP contains a manganese binding site similar to that of MnP, and a surface catalytic Trp similar to that of LiP that is involved in the oxidation of high- and medium-redox potential compounds but that also oxidizes azo-dyes and other nonphenolic compounds with high-redox potential in the absence of mediators. VP also contains a third catalytic site, located at the entrance to the heme channel, involved in the oxidation of low- to medium-redox potential compounds (similar to generic (low-redox potential) peroxidases).

As described earlier for ligninolytic laccases, the directed evolution of high-redox potential peroxidases has also been hindered by the absence of suitable heterologous expression systems. Most attempts at directed peroxidase evolution have to date been carried out using generic peroxidases. The *Coprinopsis cinerea* peroxidase (CIP) was evolved to enhance its operational stability versus temperature, H_2O_2 , and alkaline pH. *S. cerevisiae* was used as the expression system in the evolution process, and the mutated variants were subsequently overexpressed in *Aspergillus oryzae* [65]. These *in vitro* evolution studies were complemented by the resolution of the crystal structures of both wild type and evolved CIP [66]. A few years later, the evolution of horseradish peroxidase (HRP) for functional expression in *S. cerevisiae* and overexpression in *P. pastoris* was described, using this system to improve the thermal stability and resistance to H_2O_2 [67]. A recent report described the evolution of *de novo* designed proteins with peroxidase activity [68]. With regard to ligninolytic peroxidases, using an *in vitro* expression system based on *Escherichia coli*, preliminary attempts were made to enhance the oxidative stability of MnP [69]. Some years later, LiP was evolved to enhance its catalytic rate and stability by both yeast surface display and secretion to the extracellular medium [70, 71].

VP was recently evolved for secretion, thermostabilization, and H_2O_2 resistance ([72, 73] and Gonzalez-Perez, D., *et al.*, unpublished material). First, a fusion gene formed by the α -factor prepro-leader and the mature VP from *Pleurotus eryngii* was subjected to four cycles of directed evolution to favor functional expression in *S. cerevisiae*, achieving secretion levels of $\sim 22 \text{ mg l}^{-1}$. The secretion mutant (R4 variant) harbored four mutations in the mature protein and increased its total VP activity 129-fold relative to the parental type, together with a marked improvement in catalytic efficiency at the heme channel. Although the catalytic Trp was unaltered after evolution, the Mn^{2+} site was negatively affected by the mutations. Notably, signal leader processing by the STE13 protease at the Golgi compartment was altered as a consequence of the levels of VP expression, retaining the additional N-terminal sequence EAEA (Glu-Ala-Glu-Ala, Figure 1.4). A similar effect was detected with the evolved prepro-leader of the laccase OB-1 [74]. With both enzymes, the engineered N-terminal truncated variants displayed similar biochemical properties to those of their nontruncated counterparts, although their secretion levels were negatively affected, probably owing to the modifications in the acidic environment close to the KEX2 cleavage site. The R4 secretion mutant was used as the departure point to improve thermostability [46, 72] and three additional cycles of evolution led to a more thermostable variant (2-1B), harboring three

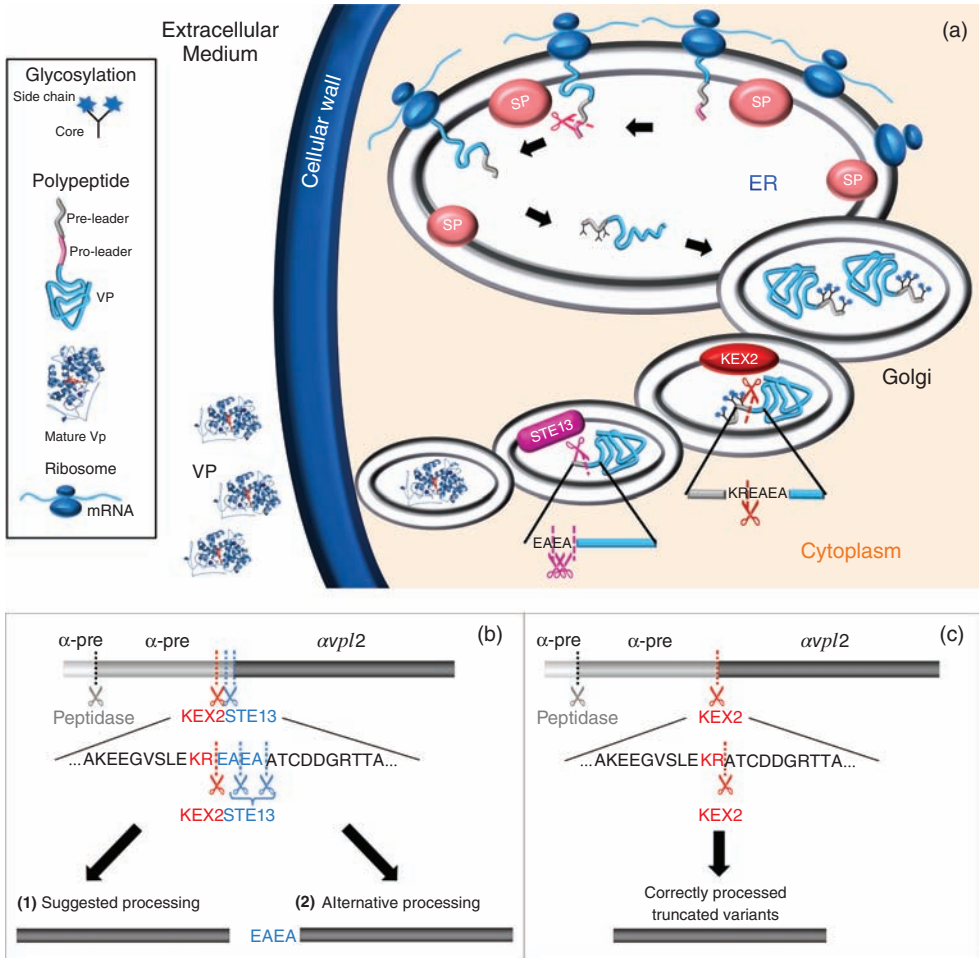


Figure 1.4 (a) General overview of protein processing, maturation and exocytosis in yeast. (b,c) Processing of the α -factor prepro-leader with/without N-terminal extension (EAEA).

additional stabilizing mutations. The 2-1B mutant exhibited a T_{50} 8 °C higher than that of the parental type, together with a broader thermoactivity range (from 30 to 45 °C in the parent to 30–50 °C for 2-1B). However, some unexpected side-effects as a consequence of the whole laboratory evolution process were observed:

- (i) The enzyme's stability at alkaline pH increased significantly, with ~60% of its residual activity retained at pH 9.0. Bearing in mind that all known ligninolytic peroxidases are unstable at neutral/alkaline pHs (because of the loss of structural Ca^{2+} ions), this represents a suitable departure point for further engineering of VPs that act at basic pHs (Gonzalez-Perez, D., Garcia-Ruiz, E., and Alcalde, M., unpublished material).

- (ii) The K_m for H_2O_2 was enhanced up to 15-fold while the catalytic efficiency was maintained. We are currently working on evolving oxidative stability (i.e., resistance to H_2O_2) using the R4 mutant as the initial variant. All peroxidases are inhibited by catalytic concentrations of H_2O_2 (so-called suicide inactivation), a mechanism-based phenomena that has provoked great

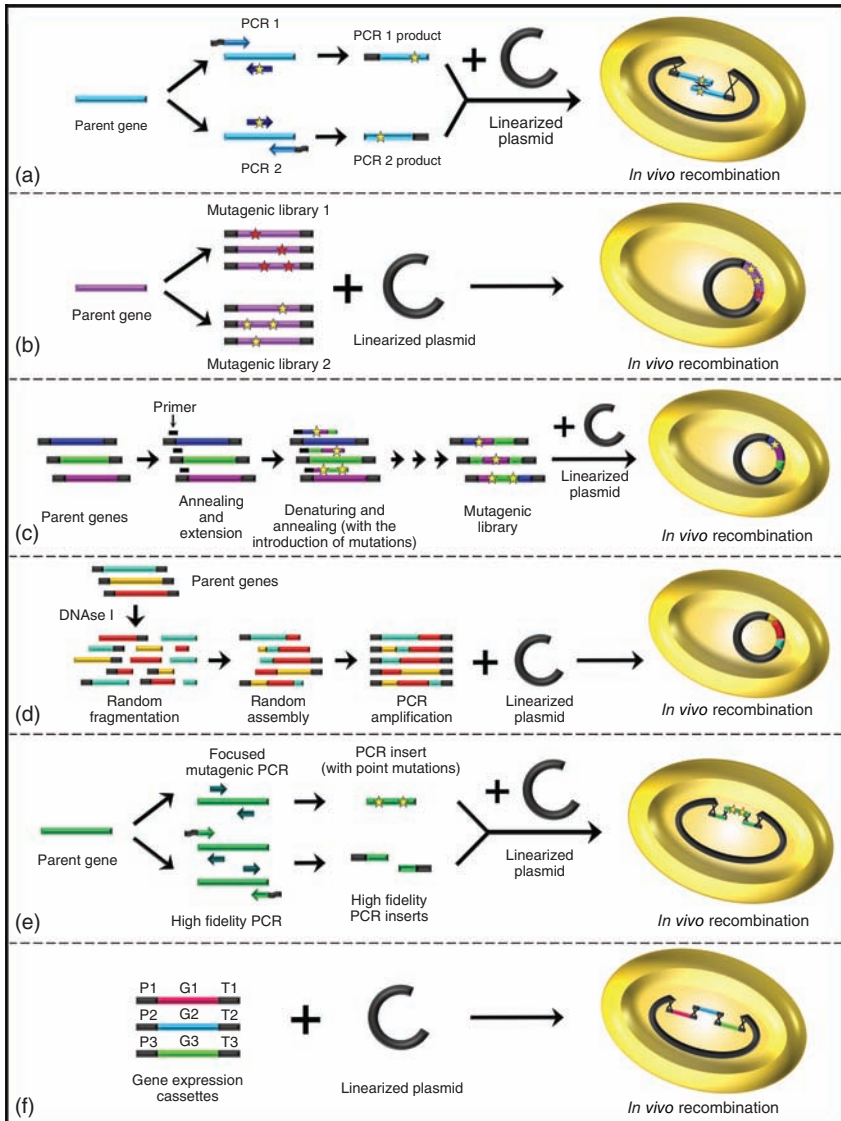


Figure 1.5 Different genetic methods for library creation in *S. cerevisiae*: (a) IVOE; (b) IvAM; (c) STEP + *in vivo* DNA shuffling; (d) CLERY; (e) MORPHING; and (f) DNA assembler.

interest. To face this challenge, we have developed a new domain-random mutagenesis/recombination method based on *S. cerevisiae* physiology known as MORPHING (Mutagenic Organized Recombination Process by Homologous IN vivo Grouping) [73]. This method has also been useful to evolve the signal peptide of UPOs for functional expression (Figure 1.5). MORPHING is highly suited to the exploration of limited targeted regions (even those <30 residues long), which has allowed us to reveal certain structural determinants involved in H₂O₂ inhibition. The combination of MORPHING with classical DNA-diversity evolution methods (e.g., random mutagenesis, DNA recombination, saturation mutagenesis), as well as with the mutational recovery of certain positions by site-directed mutagenesis, has enabled us to obtain final VP variants with longer half-lives after several rounds of evolution and screening (Gonzalez-Perez, D., *et al.*, unpublished material).

Our laboratory is also studying the directed evolution of a new type of potentially ligninolytic peroxidase classified as unspecific peroxygenase, UPO (EC 1.11.2.1). UPO was initially defined as a heme-thiolate peroxidase, exhibiting both peroxidative and peroxygenative activities toward aromatic compounds (aromatic peroxygenase, also referred to as APO [26, 75]). However, more recent studies have described the monooxygenase activity of UPO toward aliphatic compounds (UPO, [76, 77]). The peroxygenative (oxygen-transfer) activity is of particular significance as UPO can behave as a self-sufficient monooxygenase performing regio- and enantio-specific oxyfunctionalizations that are of great interest for organic syntheses. UPO, like other ligninolytic oxidoreductases described in this chapter, was not functionally expressed in heterologous hosts for directed evolution. Very recently, this problem was tackled by subjecting it to five cycles of evolution in *S. cerevisiae*, which enhanced its secretion up to 6500 ABTS units l⁻¹ (8 mg/L) with the help of a dual colorimetric HTS assay to protect the synthetic abilities of the enzyme (i.e., both peroxidative and peroxygenative activities were simultaneously screened, [53]). In the same study, MORPHING was applied to independently evolve the native signal sequence, which was finally attached to the native mature UPO to be able to consistently establish the secretion and catalytic efficiencies by comparing the native and evolved UPO secreted by yeast [53, 73].

1.6

***Saccharomyces cerevisiae* Biomolecular Tool Box**

Most of the directed evolution studies described in this chapter have been carried out in *S. cerevisiae* because it is not only important for the functional expression of evolved mutants but also is fundamental to create methods to generate DNA diversity, which introduces and recombines new point mutations [78]. In addition to providing an efficient secretory machinery that permits complex post-translational processing, *S. cerevisiae* offers a high frequency of homologous DNA recombination. This feature is extremely useful to devise *in vivo* diversity strategies, from DNA shuffling to the assembly of cassette mutant genes for

metabolic engineering and synthetic biology studies (some of the DNA diversity methods based on *S. cerevisiae* physiology for library creation are summarized in Figure 1.5). In all cases, the methodology involves engineering overlapping regions that favor *in vivo* splicing and recombination in yeast. Some of the most versatile methods are outlined as follows:

- (i) *In vivo* overlap extension (IVOE) is a fast and simple protocol to perform site-directed mutagenesis, combinatorial (saturation) mutagenesis, and insertion and deletion mutagenesis [79, 80] (Figure 1.5a). It has been used to construct combinatorial saturation mutagenesis libraries of MtL in order to explore the functionality of the C-terminal plug [81]; to engineer truncated variants of VP and PM1L to analyze the processing of the α -factor prepro-leader [72, 74]; to replace native signal peptides in Pcl and UPO ([52, 53]; to perform site-directed mutagenesis and saturation mutagenesis of MtL for evolution toward organic co-solvent tolerance and activity at an alkaline pH [40, 43]; and during directed PM1L evolution for secretion in yeast and activity in blood [45, 48].
- (ii) *In vivo* assembly of mutant libraries (IvAM) combines the mutational profiles of different polymerases in a single round of evolution [42] (Figure 1.5b). It has been used comprehensively during the evolution of MtL, PM1L, UPO, and VP [40, 43, 45, 48, 53, 72].
- (iii) StEP (staggered extension process) + *in vivo* DNA shuffling was useful to enhance the thermostability of VP without compromising activity [46] (Figure 1.5c).
- (iv) *In vitro* DNA shuffling + *in vivo* DNA shuffling (also known as *CLERY* (*combinatorial libraries enhanced by recombination in yeast*) [82]) has been used to generate chimeric laccases with large sequence divergence [54] (Figure 1.5d). Both (iii) and (iv) are combinations of *in vivo* and *in vitro* recombination methods for library creation.
- (v) MORPHING is a focused directed evolution method used to introduce random mutations and recombination of specific regions (Figure 1.5e). It was highly suited to the directed evolution of VP for oxidative stability and for the directed evolution of the signal peptide of UPO for its secretion in yeast [73].
- (vi) DNA assembler allows the construction of complex metabolic pathways by assembling whole genes and promoters with common overlapping regions [83] (Figure 1.5f). This approach is currently being used in our laboratory to construct synthetic ligninolytic pathways in yeast (Gonzalez-Perez D. and Alcalde M., unpublished material).

1.7

Conclusions and Outlook

Some years ago, the engineering of the ligninolytic consortium of oxidoreductases was too littered with hurdles to apply these enzymes to aggressive industrial

environments. However, this situation has changed with the development of directed evolution. In the last 10 years, a plethora of ideas for the construction of ligninolytic mutant libraries in *S. cerevisiae* has arisen to improve secretion, activity, and thermostability to broaden pH activity profiles and even to induce activity in blood or in organic co-solvents. And this is just the beginning. However, many challenges remain despite the great potential of these evolutionary platforms. We will have to deal with serious bottlenecks, such as the poor production of the mutants in different heterologous hosts (practical alternatives may exploit tandem expression systems with *S. cerevisiae* as a host for directed evolution and *P. pastoris* or *Aspergillus niger* for overexpression of the evolved variants [84]); the delicate balance between activity and stability when introducing beneficial (but destabilizing) mutations; and the difficulty of maintaining the redox potential of mutants after several rounds of evolution. In this context, the use of neutral genetic drift for the creation of polymorphic mutant libraries will open new adaptive pathways to enhance the stability or even the catalytic promiscuity of these oxidoreductases. Finally, the combination of directed evolution with both hybrid and rational approaches, including computational studies, will permit to convert this versatile array of high-redox potential oxidoreductases into real biotechnological products.

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Abbreviations

| | |
|----------|--|
| AAD | aryl-alcohol dehydrogenase |
| AAO | aryl-alcohol oxidase |
| ABTS | 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) |
| CDH | cellobiose dehydrogenase |
| CIP | <i>Coprinopsis cinerea</i> peroxidase |
| CLERY | combinatorial libraries enhanced by recombination in yeast |
| DMP | 2,6-dimethoxyphenol |
| EDTA | ethylenediamine-tetra-acetic acid |
| GLX | glyoxal oxidase |
| HRP | horseradish peroxidase |
| HRPLs | high-redox potential laccases |
| HTS | high-throughput screening |
| I_{50} | Concentration of an inhibitory substance at which the enzyme retains 50% of its initial activity |

| | |
|-----------------------|---|
| IvAM | <i>in vivo</i> assembly of mutant libraries with different mutational spectra |
| IVOE | <i>in vivo</i> overlap extension |
| LiP | lignin peroxidase |
| MnP | manganese peroxidase |
| MORPHING | Mutagenic organized recombination process by homologous <i>in vivo</i> grouping |
| MOX | methanol oxidase |
| MtL | <i>Myceliophthora thermophila</i> laccase |
| PAHs | Polycyclic aromatic hydrocarbons |
| PcL | <i>Pycnoporus cinnabarinus</i> laccase |
| PM1L | PM1 laccase |
| QR | quinone reductase |
| StEP | staggered extension process |
| $t_{1/2}$ vs H_2O_2 | half-life in the presence of hydrogen peroxide |
| T_{50} | temperature at which the enzyme retains 50% of its activity after incubation for 10 min |
| TvL | <i>Trametes versicolor</i> laccase |
| UPO | unspecific peroxygenase |
| VA | veratryl alcohol |
| VP | versatile peroxidase |

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