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Research review paper

Laccase engineering: From rational design to directed evolution

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ABSTRACT

Laccases are multicopper oxidoreductases considered by many in the biotechonology field as the ultimate "green catalysts". This is mainly due to their broad substrate specificity and relative autonomy (they use molecular oxygen from air as an electron acceptor and they only produce water as by-product), making them suitable for a wide array of applications: biofuel production, bioremediation, organic synthesis, pulp biobleaching, textiles, the beverage and food industries, biosensor and biofuel cell development. Since the beginning of the 21st century, specific features of bacterial and fungal laccases have been exhaustively adapted in order to reach the industrial demands for high catalytic activity and stability in conjunction with reduced production cost. Among the goals established for laccase engineering, heterologous functional expression, improved activity and thermostability, tolerance to non-natural media (organic solvents, ionic liquids, physiological fluids) and resistance to different types of inhibitors are all challenges that have been met, while obtaining a more comprehensive understanding of laccase structure–function relationships. In this review we examine the most significant advances in this exciting research area in which rational, semi-rational and directed evolution approaches have been employed to ultimately convert laccases into high value-added biocatalysts.

Contents

Introduction

Laccases (EC 1.10.3.2, benzenediol:oxygen oxidoreductases) catalyse the oxidation of a wide array of compounds coupled with the four-electron reduction of oxygen to water (Morozova et al., 2007a). They belong to the multicopper oxidase family, which also comprises ceruloplasmin, ascorbate oxidase, bilirubin oxidases and ferroxidases

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(Solomon et al., 1996). These enzymes contain four copper atoms: one paramagnetic type 1 copper (T1 Cu) that is responsible for their characteristic blue colour and where the oxidation of the reducing substrate occurs, one type 2 copper (T2 Cu)and two type 3 coppers (T3 Cu) that conform a trinuclear cluster in which molecular oxygen is reduced to two molecules of water (Davies and Ducros, 2006; Mot and Silaghi-Dumitrescu, 2012) (Fig. 1).

Since a laccase was first extracted from the exudate of the Japanese lacquer tree *Toxicodendron vernicifluum* in the late nineteenth century (Yoshida, 1883), these enzymes have been identified in more than 20 bacterial species (Santhanam et al., 2011), in several higher plant species (Mayer and Staples, 2002) and in lichens (Laufer et al., 2009). Moreover, polyphenol oxidases with laccase-like activity have also been described in insect cuticles (Lang et al., 2012), in oysters (Luna-Acosta et al., 2010) and in metagenomic libraries of bovine rumen (Beloqui et al., 2006). However, laccases are particularly abundant in fungi, having been found in almost all wood-rotting fungi analysed to date (Brijwani et al., 2010). While bacterial laccases are intracellular or periplasmic enzymes, fungal laccases are typically extracellular proteins that show different glycosylation degrees.

Laccases play diverse biological roles that are determined by their origin and the life stage of the organism that produces them. In bacteria they participate in morphogenesis, pigmentation, oxidation of toxic compounds, and protection against ultraviolet radiation and oxidizing agents (Singh et al., 2011). In addition, plant laccases are involved in wound responses and lignin polymerization (Mayer and Staples, 2002), while the polyphenol oxidases with laccase activity discovered in insect cuticles participate in the sclerotisation (Miessner et al., 1991). In fungi, diverse functions are fulfilled by laccases, including morphogenesis, stress defence, fungal plant-pathogen/host interactions and lignin degradation (Alcalde, 2007).

The substrate range of laccases is very broad as they can oxidize aromatic compounds (*ortho*- and *para*-diphenols, methoxysubstituted phenols, diamines, benzenethiols), metal ions (Mn^{2+}), organometallic compounds (*e.g.* $[W(CN)_8]^{4-}$-, $[Fe(EDTA)]^{2-}$), organic redox compounds (*e.g.* 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ABTS; 1-hydroxybenzotriazole, HBT) and the iodide anion (Morozova et al., 2007b; Xu, 1996). Furthermore, in the presence of both natural and synthetic redox mediators, the catalytic activity of these enzymes may be expanded to non-phenolic substrates that are very recalcitrant and hardly oxidized by laccase alone (*e.g.* polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls, azo-dyes or organophosphate pesticides) (Cañas and Camarero, 2010).

From an electrochemical viewpoint and based on the analysis of their molecular structures, these enzymes are classified into three different groups according to the redox potential of the T1 site ($E^{0'}_{T1}$): low-, medium-, and high-redox potential laccases (Mot and Silaghi-Dumitrescu, 2012) (Table 1). The $E^{0'}_{T1}$ of laccases is not determined by a single structural feature but it is rather the result of the combination of various factors, such as copper–ligand interactions, the effects of desolvation around the T1 site, the intermolecular electrostatic interactions, and the restrictions in protein folding (Li et al., 2004). Bacterial and plant laccases constitute the group of low-redox potential laccases, with an $E^{0'}_{T1}$ below +460 mV *vs.* NHE (Normal Hydrogen Electrode) and a Met residue as the T1 Cu axial ligand. Fungal laccases fall into the medium- and high-redox potential classes. The group of medium-redox potential laccases includes enzymes from ascomycetes and basidiomycetes fungi, with an $E^{0'}_{T1}$ ranging from +460 to +710 mV *vs.* NHE, and typically with a Leu as the non-coordinating axial ligand. High-redox potential laccases (HRPL) are mainly produced by basidiomycete white-rot fungi, with an $E^{0'}_{T1}$ ranging from +730 to +790 mV *vs.* NHE, and with a Phe as the non-coordinating axial ligand. This latter group is

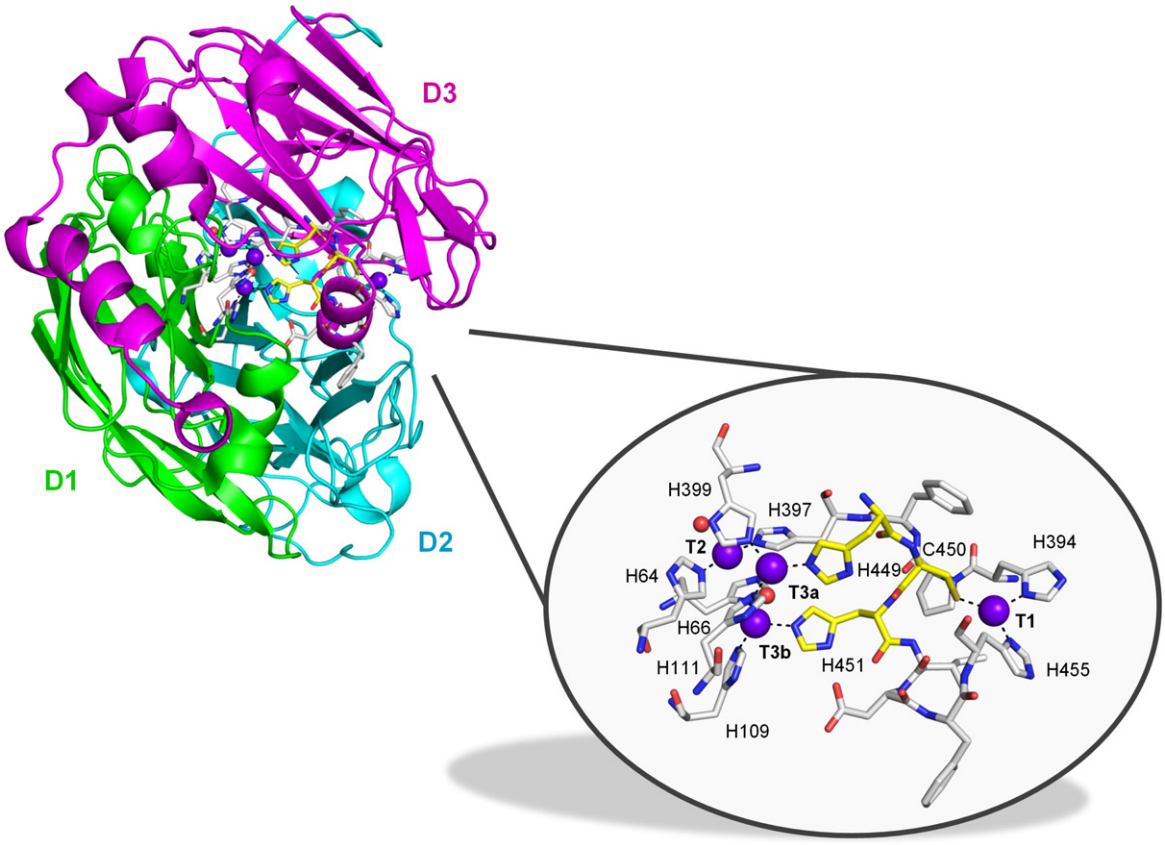

Fig. 1. General structure and details of the active site of laccase (*Trametes trogii* laccase, PDB ID: 2HRG). The three cupredoxin-like domains (D1, D2 and D3) are shown in green, cyan and magenta, respectively. Purple blue spheres represent copper ions and red spheres depict coordinating water molecules. The residues of the internal transfer pathway from T1 Cu to the T2/T3 trinuclear cluster are colored in yellow. Residues involved in the first coordination sphere of the catalytic coppers and their interactions (as black dashes) are also represented.

Table 1
Redox potential at the T1 site ($E^{0'}_{T1}$) of laccases from different organisms and the corresponding sequence alignments.

Laccase source	Organism	Sequence source	$E^{0'}_{T1}$, mV (*vs.* NHE)	Sequence alignment	Reference
High redox potential					
Trametes ochracea	Basidiomycete	PDB 2HZH	+790 ± 10	^{452}H C *H* I D F H L E A G **F**^{463}	Shleev et al. (2004)
Trametes trogii	Basidiomycete	PDB 2HRH	+790	^{449}H C *H* I D F H L E A G **F**^{460}	Garzillo et al. (2001)
Trametes versicolor	Basidiomycete	PDB 1GYC	+785	^{452}H C *H* I D F H L E A G **F**^{463}	Reinhammar (1972)
Botrytis cinerea	Ascomycete	CCD45861.1	+780	^{508}H C *H* I A W H A S E G **L**^{519}	Li et al. (1999)
Coriolopsis fulvocinerea	Basidiomycete	n.d.	+780 ± 10	n.d.	Shleev et al. (2004)
Trametes hirsuta	Basidiomycete	AAA33103.1	+780 ± 10	^{452}H C *H* I D F H L D A G **F**^{463}	Shleev et al. (2004)
Trametes villosa	Basidiomycete	AAC41686.1	+780	^{452}H C *H* I D F H L E A G **F**^{463}	Li et al. (1999)
Basidiomycete PM1	Basidiomycete	CAA78144.1	+759	^{449}H C *H* I D F H L E A G **F**^{460}	Mate et al. (2013a)
Cerrena maxima	Basidiomycete	UP D0VWU3.1	+750 ± 5	^{452}H C *H* I D F H L E G G **F**^{463}	Shleev et al. (2004)
Pycnoporus cinnabarinus	Basidiomycete	AAC39469.1	+750	^{450}H C *H* I D F H L E A G **F**^{461}	Li et al. (1999)
Trametes pubescens (LAC1)	Basidiomycete	AAM18408.1	+746 ± 5	^{457}H C *H* I D F H L E A G **F**^{468}	Shleev et al. (2007)
RL5	Metagenomic	n.d.	+745	n.d.	Beloqui et al. (2006)
Pleurotus ostreatus (POXC)	Basidiomycete	PRF 1587216	+740	^{460}H C *H* I D W H L E I G **L**^{471}	Garzillo et al. (2001)
Trametes pubescens (LAC2)	Basidiomycete	AAM18407.1	+738 ± 5	^{452}H C *H* I D F H L E A G **F**^{463}	Shleev et al. (2007)
Trametes sp. C30 (LAC1)	Basidiomycete	AAF06967.1	+730	^{449}H C *H* I D F H L E A G **F**^{460}	Klonowska et al. (2002)
Botrytis aclada	Ascomycete	AFC76164.1	+720	^{525}H C *H* I A W H A S E G **L**^{536}	Osipov et al. (2014)
Medium redox potential					
Rhizoctonia solani	Basidiomycete	UP Q02081.1	+710	^{459}H C *H* I D W H L E A G **L**^{470}	Xu et al. (1998)
Rigidoporus lignosus	Basidiomycete	PDB 1V10	+700	^{472}H C *H* I D W H L E A G **L**^{483}	Bonomo et al. (1998)
Trichoderma harzianum WL1	Ascomycete	n.d.	+692	n.d.	Sadhasivam et al. (2008)
Pleurotus ostreatus (POXA1b)	Basidiomycete	CAA06292.1	+650	^{450}H C *H* I D W H L D L G **F**^{461}	Garzillo et al. (2001)
Trametes sp. C30 (LAC2)	Basidiomycete	AAM66348.1	+560	^{452}H C *H* I D F H L E A G **F**^{463}	Klonowska et al. (2002)
Coprinus cinereus	Basidiomycete	PDB 1A65	+550	^{451}H C *H* I E F H L M N G **L**^{462}	Schneider et al. (1999)
Trichophyton rubrum LKY-7	Ascomycete	EGD86557.1	+540	^{530}H C *H* I A W H S S Q G **L**^{541}	Jung et al. (2002)
Trametes sp. C30 (LAC3)	Basidiomycete	AAR00925.1	+530	^{452}H C *H* I D F H L D A G **F**^{463}	Klonowska et al. (2005)
CueO from *Escherichia coli*	Bacteria	PDB 2FQG	+500[a]	^{471}H C *H* L L E H E D T G **M**^{482}	Miura et al. (2009)
Scytalidium thermophilum	Ascomycete	Berka et al. (1995)	+510	^{506}H C H I A W H V S G G **L**^{517}	Xu et al. (1998)
Melanocarpus albomyces	Ascomycete	PDB 1GW0	+470	^{502}H C *H* I A W H V S G G **L**^{513}	Andberg et al. (2009)
Myceliophthora thermophila	Ascomycete	ADA41449.1	+470	^{502}H C *H* I A W H V S G G **L**^{513}	Xu et al. (1998)
Low redox potential					
CotA from *Bacillus subtilis*	Bacteria	PDB 1GSK	+455	^{491}H C *H* I L E H E D Y D **M**^{502}	Melo et al. (2007)
CueO from *Escherichia coli*	Bacteria	PDB 2FQG	+440[b]	^{471}H C *H* L L E H E D T G **M**^{482}	Miura et al. (2009)
Rhus vernicifera	Plant	BAB63411.2	+434	^{495}H C *H* F E R H T T E G **M**^{506}	Reinhammar (1972)
SLAC from *Streptomyces coelicolor*	Bacteria	UP Q9XAL8	+430	^{287}H C *H* V Q S H S D M G **M**^{298}	Gallaway et al. (2008)
McoP from *Pyrobaculum aerophilum*	Bacteria	PDB 3AW5	+398	^{430}H C H N L E H E D G G **M**^{441}	Fernandes et al. (2010)
Ssl1 from *Streptomyces sviceus*	Bacteria	PDB 4M3H	+375 ± 8	^{284}H C *H* V Q S H S D M G **M**^{295}	Gunne et al. (2014)

The isoenzyme is in parentheses. The T3 Cu ligands are in italics, T1 Cu ligands are underlined and the T1 Cu axial ligand is in bold. Unless otherwise specified (PDB, Protein Data Bank, UP, UniProt database, PRF, Protein Research Foundation), the sequence codes are from GenBank. n.d.: sequence not deposited.
[a] $E^{0'}_{T1}$ determined at pH 5.0.
[b] $E^{0'}_{T1}$ determined at pH 7.0.

the most relevant for distinct industrial applications, since their $E^{0'}_{T1}$ allows HRPL to oxidize a much wider range of substrates than their low- and medium-redox potential counterparts (Rodgers et al., 2010).

There are many potential applications for laccases in different industrial and technological sectors, the most prominent of which include:

- Food industry: in beverage processing (wine, fruit juice and beer), in the determination of ascorbic acid and in the gelation of sugar beet pectin (Osma et al., 2010).
- Paper industry: in the chlorine-free bleaching of paper pulp and in effluent treatment (Couto and Herrera, 2006).
- Textile industry: in fibre modification, fabric bleaching and in the degradation of wastewater dyes (Couto and Herrera, 2006).
- Furniture and construction industry: in the cross-linking of lignin-based materials to obtain medium density fibreboards (Alcalde, 2007).
- Paint industry: laccase-mediator systems for the drying of alkyd resins widely employed in paints and coatings (Greimel et al., 2013).
- Organic chemistry: in the oxidative coupling of radical intermediates to obtain antitumor compounds (*e.g.* actinocin or vinblastine), cyclosporin derivatives (*e.g.* cyclosporin A), hormones (*e.g.* β-estradiol) and phytoalexins (*e.g.* resveratrol), (Kunamneni et al., 2008a) as well as in the enzymatic derivatization of amino acids such as L-tryptophane, L-phenylalanine or L-lysine (Mogharabi and Faramarzi, 2014).
- Biofuels: to remove phenolic compounds that inhibit the fermentation of the sugars present in the hydrolysate of lignocellulosic materials (Kudanga and Le Roes-Hill, 2014).
- Soil bioremediation: degradation of 2,4,6-trinitrotoluene (TNT) and PAH (Couto and Herrera, 2006).
- Cosmetics industry: in the preparation of hair dyes and skin lightening preparations (Couto and Herrera, 2006).
- Biomedical industry: laccase immobilization in strips or bandages to be used for the diagnosis of fungal infections (Schneider et al., 2012).
- Nanobiotechnology: in the development of biofuel cells and biosensors to detect different compounds and metabolites (Kunamneni et al., 2008b).

Laccases have been reviewed extensively in recent years and the reader is referred to other reviews about these enzymes, addressing general aspects (Alcalde, 2007; Gianfreda et al., 1999; Giardina et al., 2010), industrial applications (Kunamneni et al., 2008a), heterologous production (Piscitelli et al., 2010), laccases in organic synthesis (Kunamneni et al., 2008a; Mogharabi and Faramarzi, 2014; Riva, 2006), laccase mediator systems (Call and Mucke, 1997; Cañas and Camarero, 2010; Morozova et al., 2007b), laccase design (Maté et al., 2011; Rodgers et al., 2010), laccase electronic pathways (Wherland et al., 2014), fungal laccases (Baldrian, 2006) and bacterial laccases (Santhanam et al., 2011; Singh et al., 2011).

In the current review, we provide a comprehensive update of all the studies focused on the production and engineering of this versatile group of oxidoreductases. First of all, the heterologous laccase expression systems reported to date will be reviewed and thereafter, we will focus on laccase engineering by rational approaches. We will then address the semi-rational strategies employed to improve these enzymes involving saturation mutagenesis of multiple or specific residues in the laccase and finally, we will analyse the most recent advances in directed laccase evolution combined with hybrid approaches. For the sake of clarity, the studies on bacterial and fungal laccases are described independently due to their different properties in terms of redox potential, thermostability, pH activity profiles and halide inhibition.

Heterologous laccase expression

The biotechnological application of laccases requires industrial-scale production of active and stable enzymes. However, achieving this goal is generally impeded by poor expression in homologous microorganisms, as well as by the growth conditions. This hurdle has been addressed in two different ways: i) by optimizing the components of the culture medium in order to increase homologous expression (*e.g.* adjusting the presence of metal ions, aromatic compounds derived from lignin, or the nitrogen and carbon sources) (Collins and Dobson, 1997; Lee et al., 1999; Terrón et al., 2004); and ii) by enhancing heterologous expression through the use of strong promoters, multicopy vectors and signal peptides capable of directing laccase secretion to the extracellular medium, as well as by directed evolution (see below). Indeed, with the exception of the homologous overexpression of the laccase from *Pycnoporus cinnabarinus*, which augmented laccase secretion to 1.2 g/L (Alves et al., 2004), most effort has focused on the development of heterologous expression systems (Kunamneni et al., 2008a; Piscitelli et al., 2010).

The heterologous expression of laccases has been achieved in different bacterial species (*Escherichia coli* and *Streptomyces lividans*), plants (*Arabidopsis thaliana*, *Lycopersicon esculentum*, *Nicotiana tabacum*, *Oryza sativa* and *Zea mays*), yeasts (*Kluyveromyces lactis*, *Pichia methanolica*, *Pichia pastoris*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*) and filamentous fungi (*Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus sojae, Penicillium canescens* and *Trichoderma reesei*). In particular, fungal laccases have been expressed in yeast, filamentous fungi and plants. Moreover, functional expression of the laccase from the ligninolytic fungus *Cyathus bulleri* was recently described in *E. coli*, becoming the first fungal laccase expressed in a bacterial host (Garg et al., 2008). Tables 2, 3 and 4 give a complete list of all heterologously expressed laccases to date, both from bacterial (Table 2) and fungal origin (Tables 3 and 4).

In many cases, the expression of fungal laccases can be achieved by replacing the native signal peptide with the signal sequence of proteins that are secreted in large amounts by the host microorganism. Thus, heterologous production of fungal laccases has been described using different signal peptides, among them: the α factor prepro-leader and the signal peptide of the invertase from *S. cerevisiae*; the signal sequence of the XPR2 alkaline protease from *Y. lipolytica*; the leader of *A. niger* glucoamylase; the leader of the β-galactosidase from *P. canescens*; the signal peptide of glutelin B1 from *O. sativa*; the signal sequence of the cellobiosehydrolase 1 from *T. reesei* and the α-amylase leader from *Hordeum vulgare*. The use of filamentous fungi as expression systems has led to the strongest heterologous production of fungal laccases. *T. reesei* secretes up to 920 mg/L of *Melanocarpus albomyces* laccase (Kiiskinen et al., 2004), and between 800 and 1000 mg/L of *Trametes versicolor* laccase (Baker and White, 2001), while *A. niger* produces 840 mg/L of the LAC3 laccase from *Trametes* sp. C30 (Mekmouche et al., 2014) (Table 4). However, optimization of gene expression through different mutagenic techniques (especially directed evolution) is difficult to achieve in filamentous fungi (Robert et al., 2011; Pourmir and Johannes, 2012). For this reason, the cloning and expression of fungal laccases is often carried out in yeast, since they can grow as individual colonies, they do not produce endogenous laccases and the recombinant products are directly secreted into the extracellular medium facilitating the detection of laccase activity without complex lysis steps. Among yeast species, *S. cerevisiae* and *P. pastoris* are the most commonly used for laccase production, since there are a wide variety of molecular biology techniques that facilitate their manipulation (*e.g.* replicative plasmids, constitutive or inducible promoters and simple transformation protocols) and they are easily cultured (Piscitelli et al., 2010).

Laccase engineering

Rational approaches

Site-directed mutagenesis of specific residues chosen on the basis of prior structural information is a strategy that has been commonly used to study laccase structure–function relationships (Table 5).

Bacterial laccases

Two different site-directed mutagenesis studies have been carried out using the endospore-coat laccase CotA from *Bacillus subtilis* (Durão

Table 2
Bacterial laccases and multicopper oxidases heterologously expressed in bacteria (*E. coli* and *Streptomyces lividans*).

Expression host	Laccase source	Expression yield[a]	Reference
E. coli	*Aquifex aeolicus* VF5 (McoA)	n.r.	Fernandes et al. (2007)
E. coli	*Bacillus sp.* HR03 (CotA)	n.r.	Mohammadian et al. (2010)
E. coli	*Bacillus clausii* KSM-K16 (CotA)	n.r.	Brander et al. (2014)
E. coli	*Bacillus licheniformis* DSM 13 (CotA)	410 U/L and 26 mg/L for the wild-type (ABTS); 3400 U/L and ~300 mg/L for the K316N/D500G mutant (ABTS)	Koschorreck et al. (2009)
E. coli	*Bacillus halodurans*	2600 U/L (SGZ), 400 U/L (ABTS) and 0.54 U/L (DMP)	Ruijssenaars and Hartmans (2004)
E. coli	*Bacillus pumilus*	n.r.	Reiss et al. (2011)
E. coli	*Bacillus subtilis* (CotA)	n.r.	Martins et al. (2002)
E. coli	Lac15 from a metagenome library of marine microbes	n.r.	Fang et al. (2011)
E. coli	Lac591 from a metagenome library of mangrove soil microbes	380 mg/L (guaiacol)	Ye et al. (2010)
E. coli	RL5 from a metagenome library of bovine rumen microflora	n.r.	Beloqui et al. (2006)
E. coli	*Pyrobaculum aerophilum* (McoP)	n.r.	Fernandes et al. (2010)
E. coli	*Streptomyces coelicolor* M145 (SLAC)	n.r.	Machczynski et al. (2004)
E. coli	*Streptomyces griseus* IFO 13350 (EpoA)	n.r.	Endo et al. (2003)
E. coli	*Streptomyces ipomoea* CECT 3341	n.r.	Molina-Guijarro et al. (2009)
E. coli	*Streptomyces lavendulae* REN-7	76 U/L, 10 mg/L (catechol)	Suzuki et al. (2003)
Streptomyces lividans	*Streptomyces coelicolor* A3(2) (SLAC)	350 mg/L (ABTS)	Dubé et al. (2008)

The expressed laccase is in parentheses. n.r. not reported. SGZ: syringaldazine; DMP: 2,6-dimethoxyphenol.
[a] The substrate used for determination of laccase activity is indicated in parentheses.

Table 3
Fungal laccases heterologously expressed in yeast (*Kluyveromyces lactis*, *Pichia pastoris*, *Pichia methanolica*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*).

Expression host	Laccase source	Signal peptide (SP) used	Expression yield*	Reference
K. lactis	*Pleurotus ostreatus*[b] (POXA1b)	Native SP	2030 U/L, 1.6 mg/L	Piscitelli et al. (2005)
K. lactis	*Pleurotus ostreatus*[b] (POXC)	Native SP	100 U/L, 1.9 mg/L	Piscitelli et al. (2005)
P. pastoris	*Botrytis aclada*[a]	Native SP	53,300 U/L[1], 517 mg/L[1]	Kittl et al. (2012a)
P. pastoris	*Botrytis aclada*[a]	Native SP	51,000 U/L[2], 495 mg/L[2]	Kittl et al. (2012b)
P. pastoris	*Ganoderma lucidum*	Native SP	686 U/L, 6 mg/L	You et al. (2014)
P. pastoris	*Pleurotus sajor-caju*[b]	Native SP	10,200 U/L, 4.85 mg/L	Soden et al. (2002)
P. pastoris	*Pycnoporus cinnabarinus*[b]	Native SP/*S. cerevisiae* α-factor prepro-leader	8 mg/L	Otterbein et al. (2000)
P. pastoris	*Pycnoporus coccineus*[b]	Native SP/*S. cerevisiae* α-factor prepro-leader	Solid phase	Hoshida et al. (2001)
P. pastoris	*Fomes lignosus*[b]	Native SP	9030 U/L, 144 mg/L	Liu et al. (2003); Hu et al. (2007)
P. pastoris	*Trametes sanguínea* M85-2	Native SP	Solid phase	Hoshida et al. (2001)
P. pastoris	*Trametes* sp. 420[b]	*S. cerevisiae* α-factor prepro-leader	239,000 U/L, 136 mg/L	Cui et al. (2007)
P. pastoris	*Trametes* sp. AH28-2[b] (LacB)	*S. cerevisiae* α-factor prepro-leader	32,000 U/L, 31.6 mg/L	Li et al. (2007)
P. pastoris	*Trametes trogii*[b]	SP nativo	2520 U/L, 17 mg/L	Colao et al. (2006)
P. pastoris	*Trametes versicolor*[b]	SP nativo	140,000 U/L	Hong et al. (2002)
P. methanolica	*Trametes versicolor*[b]	*S. cerevisiae* α-factor prepro-leader	12,600 U/L	Guo et al. (2006)
S. cerevisiae	*Melanocarpus albomyces*[a]	*S. cerevisiae* α-factor prepro-leader	270 U/L, 7.4 mg/L	Andberg et al. (2009)
S. cerevisiae	*Myceliophthora thermophila*[a]	*S. cerevisiae* α-factor prepro-leader	18 mg/L	Bulter et al. (2003)
S. cerevisiae	*Pleurotus eryngii*[b]	Native SP	146 U/L	Bleve et al. (2008)
S. cerevisiae	*Pleurotus ostreatus*[b] (POXA1b)	Native SP	200 U/L	Piscitelli et al. (2005)
S. cerevisiae	Basidiomycete PM1[b]	*S. cerevisiae* α-factor prepro-leader	~8 mg/L	Maté et al. (2010)
S. cerevisiae	*Pycnoporus cinnabarinus*[b]	*S. cerevisiae* α-factor prepro-leader	300 U/L, ~2 mg/L	Camarero et al. (2012)
S. cerevisiae	*Pycnoporus coccineus*[b]	Native SP	Solid phase	Hoshida et al. (2005)
S. cerevisiae	*Trametes sanguínea* M85-2	Native SP	Solid phase	Hoshida et al. (2001)
S. cerevisiae	*Trametes* sp. C30[b] (LAC3)	*S. cerevisiae* invertase SP	2 mg/L	Klonowska et al. (2005)
S. cerevisiae	*Trametes hirsuta* [b]	Native SP	Solid phase	Kojima et al. (1990)
Y. lipolytica	*Pycnoporus cinnabarinus*[b]	*Y. lipolytica* XPR2 prepro-sequence	1026 U/L, 19.8 mg/L	Madzak et al. (2005)
Y. lipolytica	*Trametes versicolor*[b] (Laccase IIIb)	Native SP	230 U/L, 2.5 mg/L	Jolivalt et al. (2005)
Y. lipolytica	*Trametes versicolor*[b] (Lcc1)	Native SP	250 U/L[3], 1000 U/L[4]	Theerachat et al. (2012)

The expressed isoenzyme is in parentheses. [a]Ascomycete. [b]Basidiomycete. *Activity measured with ABTS in all cases, with the exception of: *Trametes* sp. C30 LAC3 laccase produced in *S. cerevisiae*, where activity was determined with syringaldazine; *P. coccineus* laccase expressed in *P. pastoris* and *S. cerevisiae*, *T. sanguinea* M85-2 produced in *P. pastoris* and *S. cerevisiae*, and *T. hirsuta* laccase expressed in *S. cerevisiae*, with the activity confirmed in the three cases by using agar plates containing guaiacol.[1]Expression under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter from *P. pastoris*. [2]Expression under control of the methanol-inducible alcohol oxidase 1 promoter from *P. pastoris*. [3]Expression levels achieved by single-copy integration into the genome of *Y. lipolytica*. [4] Expression levels achieved by multiple-copy integration into the genome of *Y. lipolytica*.

et al., 2006, 2008). The main goal was to evaluate how the redox potential and catalytic efficiency of this enzyme are influenced by modifying residues involved in the coordination and stabilization of the T1 Cu site. Initially, the weakly coordinating Met of the T1 Cu was substituted with non-coordinating Leu and Phe residues (Durão et al., 2006). The geometry of the T1 copper centre in these two variants (M502L and M502F) was similar to that of the wild-type, yet both the redox potential and the catalytic activity were significantly altered. The $E^{0'}_{T1}$ of the

Table 4
Fungal laccases heterologously expressed in filamentous fungi (*Aspergillus nidulans, Aspergillus niger*, *Aspergillus oryzae*, *Penicillium canescens* and *Trichoderma reesei*), plants (*Nicotiana tabacum*, *Oryza sativa* and *Zea mays*) and bacteria (*Escherichia coli*).

Expression host	Laccase source	Signal peptide (SP) used	Expression yield[a]	Reference
A. nidulans	*Ceriporiopsis subvermispora* (Lcs-1)	Native SP	200 U/L	Larrondo et al. (2003)
A. niger	*Ceriporiopsis subvermispora* (Lcs-1)	Native SP	200 U/L	Larrondo et al. (2003)
A. niger	*Phanerochaete flavido-alba*	*A. niger* glucoamylase SP	2500 U/L, 30 mg/L	Benghazi et al. (2013)
A. niger	*Pleurotus eryngii* (PEL3)	*A. niger* glucoamylase SP	2.4 U/mg protein	Rodríguez et al. (2008)
A. niger	*Pleurotus ostreatus*[b] (POXA1b)	*A. niger* glucoamylase SP	60,000 U/L, 20 mg/L	Macellaro et al. (2014)
A. niger	*Pycnoporus cinnabarinus*[b]	*A. niger* glucoamylase SP	7000 U/L, 70 mg/L	Record et al. (2002)
A. niger	*Trametes* sp. C30[b] (LAC3)	*A. niger* glucoamylase SP	42,000 U/L, 840 mg/L	Mekmouche et al. (2014)
A. niger	*Trametes versicolor*[b]	Native SP	2700 U/L	Bohlin et al. (2006)
A. oryzae	*Coprinus cinereus*[b] (Lcc1)	Native SP	135 mg/L	Yaver et al. (1999)
A. oryzae	*Myceliophthora thermophila*[c]	Native SP	850 U/L, 19 mg/L	Berka et al. (1997)
A. oryzae	*Rhizoctonia solani*[b]	Native SP	n.d.	Wahleithner et al. (1996)
A. oryzae	*Pycnoporus coccineus*[b]	Native SP	3000 U/L	Hoshida et al. (2005)
P. canescens	*Trametes hirsuta*[b]	*P. canescens* β-galactosidase SP	3000 U/L	Abyanova et al. (2010)
T. reesei	*Melanocarpus albomyces*[c]	Native SP	46,800 U/L, 920 mg/L	Kiiskinen et al. (2004)
T. reesei	*Phlebia radiata*[b]	Native SP	462 U/L, 19.5 mg/L	Saloheimo and Niku-Paavola (1991)
T. reesei	*Trametes versicolor*[b]	*T. reesei* cellobiohydrolase I SP	800-1000 mg/L	Baker and White (2001)
N. tabacum	*Schizophyllum commune*	Native SP	0.79 U/g dry weight	Hirai et al. (2008)
N. tabacum	*Trametes versicolor*[b]	Native SP	n.r.	Sonoki et al. (2005)
O. sativa	*Melanocarpus albomyces*[c]	*O. sativa* glutelin B1 SP	13 ppm	de Wilde et al. (2008)
O. sativa	*Pycnoporus cinnabarinus*[b]	*O. sativa* glutelin B1 SP	39 ppm	de Wilde et al. (2008)
Z. mays	*Trametes versicolor*[b]	*Hordeum vulgare* α-amylase SP	>50 ppm	Bailey et al. (2004)
E. coli	*Cyathus bulleri*[b]	Native SP	n.r.	Garg et al. (2008)

The expressed isoenzyme is in parentheses.
[a] Activity measured with ABTS in all cases, with the exception of *M. thermophila* and *Trametes* sp. C30 LAC3 laccases produced in *A. oryzae* and *A. niger*, respectively, where activity was quantified with syringaldazine; *P. coccineus* laccases expressed in *A. oryzae* (activity determined with N,N-dimethyl-1,4-phenylenediamine); *T. hirsuta* laccase expressed in *P. canescens* (activity determined with catechol); and *S. commune* laccase produced in *N. tabacum* (activity measured with DMP).
[b] Basidiomycete. n.r. not reported.
[c] Ascomycete.

Table 5
Laccases engineered by site-directed mutagenesis.

Laccase source	Property in study	Characterization	Main results[a]	Reference
CotA from *Bacillus subtilis*	$E^{0'}_{T1}$ and catalytic efficiency	Redox titration, EPR, CAA[b]	Increase of $E^{0'}_{T1}$ by ~100 mV and decrease in the catalytic efficiency	Durão et al. (2006, 2008)
Bacillus sp. HR03	Thermal stability	CD spectroscopy, intrinsic fluorescence analysis, CAA[b]	3-fold higher thermal activation and 3 °C higher T_{50}[c]	Mollania et al. (2011)
Bacillus sp. HR03	Organic solvent tolerance	Colorimetric assays	Increase in C_{50}[d] and decrease in thermoinactivation rates in the presence of organic solvents	Rasekh et al. (2014)
CueO from *Escherichia coli*	$E^{0'}_{T1}$ and catalytic efficiency	EPR, CD spectroscopy, CAA[b]	Significant variations in $E^{0'}_{T1}$ and activity compared to the wild-type	Kurose et al. (2009)
CueO from *Escherichia coli*	$E^{0'}_{T1}$ and catalytic efficiency	EPR, CD spectroscopy, cyclic voltammetry, CAA[b]	$E^{0'}_{T1}$ between 150 mV lower and 100 mV higher than the wild-type, catalytic efficiencies up to 140 fold higher	Kataoka et al. (2013)
SLAC from *Streptomyces coelicolor*	Role of Tyr108 in the catalytic mechanism	Absorption spectroscopy, CAA[b]	~2.5-fold lower turnover numbers for Y108F and Y108A mutants	Gupta et al. (2012)
SLAC from *Streptomyces coelicolor*	Redesign of the putative substrate binding pocket	CAA[b]	Higher catalytic efficiencies for DMP and better mediated-decolorization of indigo carmine	Toscano et al. (2013)
SLAC from *Streptomyces coelicolor*	Activity	CAA[b]	Determination of amino acid residues important for activity	Sherif et al. (2013)
Ssl1 from *Streptomyces sviceus*	$E^{0'}_{T1}$ and catalytic efficiency	Redox titration, CAA[b]	Improved $E^{0'}_{T1}$, lower kinetic constants	Gunne et al. (2014)
Myceliophthora thermophila	$E^{0'}_{T1}$ and catalytic efficiency	Redox titration, EPR, CAA[b]	Structural perturbation at the T1 Cu and important changes in the catalytic efficiencies	Xu et al. (1998); Palmer et al. (2003)
Rhizoctonia solani	$E^{0'}_{T1}$ and catalytic efficiency	Redox titration, EPR, CAA[b]	Structural perturbation at the T1 Cu and important changes in the catalytic efficiencies	Xu et al. (1998)
Trametes villosa	$E^{0'}_{T1}$ and catalytic efficiency	Redox titration, EPR, CAA[b]	F463M mutant showed a decrease of 0.1 V in the $E^{0'}_{T1}$, altered EPR and UV–visible spectra, higher *k*cat and *K*m values and a more basic optimal pH	Xu et al. (1999)
Melanocarpus albomyces	Role of Glu235 in laccase catalysis	CD spectroscopy, redox titration, mass spectrometry, CAA[b]	D235T variant preferred DMP over ABTS and showed pH activity profiles for phenolic substrates significantly altered	Kallio et al. (2009)
Melanocarpus albomyces	Role of the C-terminus in laccase catalysis	CD spectroscopy, redox titration, CAA[b]	Mutations in the C-terminal resulted in significant decrease on the laccase secretion levels in *T. reesei* and *S. cerevisiae*	Andberg et al. (2009)
Trametes versicolor	Catalytic efficiency towards phenolic and non-phenolic compounds	HPLC and HPSEC analysis, CAA[b]	3-fold increase in k_{cat} for ABTS and the optimal pH for DMP was increased 1.4 units	Madzak et al. (2006)
Trametes versicolor	Catalytic efficiency towards bulky phenolic compounds	GC and HPLC analysis, CAA[b]	Diverse results in function of the mutations tested	Galli et al. (2011)
Botrytis aclada	Role of Leu499 in the overall structure and redox potential	Redox titration, CAA[b]	No significant changes in the overall structure; $E^{0'}_{T1}$ 140 mV lower than that from the wild-type; higher K_m values both for ABTS and DMP	Osipov et al. (2014)

[a] Main results are described in comparison with the wild-type laccase.
[b] CAA: Colorimetric (spectrophotometric) activity assays.
[c] T_{50}: Temperature at which 50% of the initial laccase activity is retained after 30 min incubation.
[d] C_{50}: Organic solvent concentration at which the enzyme retains 50% of its initial activity (*i.e.* activity in the absence of co-solvent).

two mutants increased by around 100 mV with respect to that of the native laccase but the k_{cat} was negatively affected. The M502L mutant displayed a 2 to 4-fold decrease in the k_{cat} for phenolic and non-phenolic substrates while in the M502F variant the effect was even more pronounced: the k_{cat} values were 10 to 1840-fold lower to those of the wild-type enzyme. However, it was not found a direct correlation between the enhancement in the redox potentials and the decrease in activity. Subsequently, two hydrophobic residues in the vicinity of the T1 Cu (Leu386 and Ile494) were mutated to Ala, generating two mutants with strongly altered spectral properties of the copper centres compared to the wild-type (Durão et al., 2008). Additionally, these mutants showed a decrease in the $E^{0'}_{T1}$ (approx. 60 and 100 mV for L386A and I494A mutants, respectively). According to the crystal structures of these two site-directed mutants, the replacement of hydrophobic residues by Ala in the neighbourhood of the T1 Cu increased the solvent accessibility affecting the $E^{0'}_{T1}$.

Laccase from *Bacillus* sp. HR03 was subjected to rational design in order to increase its thermal stability (Mollania et al., 2011). The key Glu188 residue – located in the connecting loop between domains 1 and 2 – was replaced by one hydrophobic (Ala) and two positive (Lys and Arg) residues. These three variants had enhanced thermal stability as well as thermal activation with respect to the wild-type. The most stable mutant (E188K) displayed a 3-fold improvement in thermal activation and a 5 °C higher T_{50} -the temperature at which 50% of the initial laccase activity is retained after a given period of incubation- than that of the wild-type, while its K_m for syringaldazine was reduced 1.4-fold. This increase in thermal stability and substrate affinity might suggest an enhanced robustness of the laccase structure. Similarly, the tolerance of this laccase to organic solvents was improved by directed mutagenesis when Glu188 was substituted with non-polar (Ala, Ile, Leu and Val) and positively charged (Lys and Arg) residues (Rasekh et al., 2014). Although all variants had higher C_{50} values – the organic solvent concentration at which the enzyme retains 50% of its activity – than the native laccase, mutants containing non-polar substitutions exhibited the most pronounced increase in C_{50}, as well as the highest decrease in the thermoinactivation rate in the presence of organic solvents.

The laccase CueO from *E. coli* has been also subjected to site-directed mutagenesis. The Met axial ligand of the T1 center (Met510) was replaced by: i) Leu, to generate the three-coordinated T1 Cu typical of fungal laccases with medium-redox potential (see Table 1); ii) Ala and Thr, to evaluate the effect of bulkiness and polarity of the side chain on the redox potential; and iii) Gln, to obtain a T1 Cu site with lower redox potential found in other blue copper containing proteins, such as stellacyanin or mavicyanin (Kurose et al., 2009). Compared with the wild-type laccase, the mutants displayed significant changes in the

activity and $E^{0'}_{T1}$, which were in correlation with the characteristics of their circular dichroism, absorption and electron paramagnetic resonance spectra. More recently, CueO variants with single, double and triple mutations in the first and second coordination spheres of the T1 Cu have been studied (Kataoka et al., 2013). Specifically, four single mutants (D439A, P444A, M510L and M510Q), five double mutants (D439A/P444A, D439A/M510L, D439A/M510Q, P444A/M510L and P444A/M510Q) and two triple variants (D439A/P444A/M510L and D439A/P444A/M510Q) were evaluated. These mutants showed catalytic efficiencies for ABTS up to 140-fold higher than the wild-type, while their $E^{0'}_{T1}$ were between 150 mV lower and 100 mV higher than that of the native enzyme.

In the small laccase SLAC from *Streptomyces coelicolor*, the Tyr108 located at the interface of two subunits of the trimeric form of the enzyme was replaced by Phe and Ala to study the role of this residue in the catalytic mechanism (Gupta et al., 2012). The mutation reduced ~2.5-fold the turnover numbers without affecting the catalytic efficiency. Lately, new studies have focused on redesigning the substrate binding pocket of SLAC for phenolic compounds by replacing residues close to the T1 site that seem to be involved in binding the reducing substrate (Toscano et al., 2013). In particular, five single (M168G, M168A, Y199W, M266A and M266W) and two double mutants (M168G/M266A and M168G/M266W) were characterized. All the variants had improved catalytic efficiencies for 2,6-dimethoxyphenol (DMP) compared to the wild-type, together with better mediator-assisted decolorization of indigo carmine. This laccase has also been studied rationally to reveal the residues that are important for activity, 10 of these being His residues that coordinate the copper sites (Sherif et al., 2013).

Recently, the two-domain laccase Ssl1 from *Streptomyces sviceus* was subjected to site-directed mutagenesis (several Met residues located in the putative substrate-binding site of the enzyme were replaced by Leu) (Gunne et al., 2014). The mutagenesis studies involved the axial Met ligand of the T1 Cu (Met295) as well as the three Met located nearby the T1 site (Met195, Met220 and Met293). Moreover, a truncated mutant without the 17 residues corresponding to the C-terminus of the laccase was evaluated. All the variants showed higher redox potentials (ranging from 16 and 81 mV over the wild-type). Among them, the strongest effect was observed in the M295L mutant whose $E^{0'}_{T1}$ was risen up to +456 mV but at the cost of jeopardizing the kinetic parameters highlighting the delicate balance between redox potential and substrate binding.

Fungal laccases

The late 1990's saw the first attempts to engineer fungal laccases by rational approaches when several residues surrounding the catalytic copper centres of laccases from *Myceliopthora thermophila*, *Rhizoctonia solani* and *Trametes villosa* were subjected to site-directed mutagenesis to determine the parameters responsible for the catalytic activity and redox potential (Palmer et al., 2003; Xu et al., 1998, 1999). First, four different mutants of the medium-redox potential laccases from *R. solani* and *M. thermophila* were designed, carrying either a single or a triple mutation in a highly conserved pentapeptide which corresponds to the sequence $_{512}$HLHMGM$_{517}$ of the *Zucchini* ascorbate oxidase (zAO) (Xu et al., 1998). More specifically, the L470F and L466V/E467S/A468G variants from *R. solani* laccase and the L513F and V509L/S510E/G511A mutants of *M. thermophila* were evaluated. The single mutations neither significantly alter the spectro-electrochemical properties (redox potential, cooper geometry), nor the biochemical ones (kinetic parameters, pH activity profiles, fluoride inhibition). By contrast, the triple mutant showed a different behaviour: shifted pH activity profiles, lower k_{cat} values for both syringaldazine and ABTS, and higher tolerance against inhibition by fluoride ions at low pH values. In a later study, Xu and co-workers reported the mutagenesis of two specific residues of the HRPL from *T. villosa*: i) the Phe463 – corresponding to the Cu axial Met ligand in zAO – was replaced by a non-coordinating Leu – mimicking medium-redox potential laccases – (Table 1) or by Met; and ii) the Ala461 – near the T1 Cu site and corresponding to the Met515 in the zAO – by Glu (Xu et al., 1999). The F463L and A461E mutations hardly modified the general features of the enzyme in terms of redox potentials, EPR and UV–visible spectra, kinetic values and pH-activity profiles. On the contrary, the F463M did showed several significant changes: a decrease of 100 mV in the $E^{0'}_{T1}$, altered EPR and UV–visible spectra, and an increase in the k_{cat} and K_m for syringaldazine and ABTS shifting the optimum pH value for oxidation of syringaldazine (but not for ABTS). Finally, the electronic structure of the L513H variant of the *M. thermophila* laccase was analysed by spectroscopic methods and density functional estimations (Palmer et al., 2003). This mutation gave rise to significant alterations of the structural arrangement of the T1 Cu, which even switched from blue to green colour at the resting oxidized state.

More recently, structure–function studies were performed on the laccase from the ascomycete *Melanocarpus albomyces* (MaL) to study the catalysis on phenolic and non-phenolic substrates (Kallio et al., 2009). Based on the MaL crystal structure, a conserved carboxylic acid (specifically Glu235) located at the bottom of the substrate-binding pocket was mutated. E235D and E235T variants were constructed and expressed in *S. cerevisiae*. The E235D variant showed higher preference for DMP than the parent type, while E235T showed improved affinity for ABTS. Additionally, the pH profiles of E235T for DMP, syringic acid and methyl syringate were altered, but not for the non-phenolic ABTS, suggesting that the oxidation of phenolic and non-phenolic compounds likely proceeds through distinct catalytic pathways. The same researchers also evaluated the role of the C-terminus on the MaL features (Andberg et al., 2009). Three different MaL mutants were constructed: the delDSGL$_{559}$ mutant (a truncated C-terminal version of MaL), the L559G and the L559A variants. Overall changes in the C-terminal plug addressed the significance of this region in the MaL activity and stability. Indeed, the MaL crystal structure revealed that the L559A mutation certainly altered the overall geometry of the trinuclear Cu cluster.

The role of the C-terminus was also studied in the ERY4 laccase from *Pleurotus eryngii* to find out why the recombinant enzyme was not active in *S. cerevisiae* (Bleve et al., 2013). A total of six deletion variants (carrying C-terminal deletions of 2, 5, 8, 11, 14 and 18 consecutive amino acids) and four site-specific mutants at the C-terminus (K532R, K532A, K532E and P530A) were designed, being all of them expressed in active form in yeast (except for the K532R mutant). It was concluded that this region may be important for the inactivation/activation mechanism of the enzyme since it lacks of proper processing in yeast.

The HRPL from *T. versicolor* expressed in *Y. lypolytica* was rationally engineered to enhance the capacity to oxidize phenolic and non-phenolic substrates (Madzak et al., 2006), and bulky phenolic compounds (Galli et al., 2011). On the one hand, after replacing Asp206 (which strongly interacts with the phenolic substrate 2,5-xylidine) with Glu, Ala and Asn, the oxidation of several substrates was tested, revealing a 3-fold increase in the k_{cat} towards ABTS for the most efficient D206N mutant when compared to the wild-type (Madzak et al., 2006). Interestingly, this substitution also led to a significant shift in the optimum pH value for DMP (a 1.4 unit increase). The same mutation was assayed during the laboratory evolution of the HRPL from basidiomycete PM1 for its activity in human blood, resulting in a comparable improvement (Mate et al., 2013b): a 2 unit increase in pH optimum over the parental type with DMP as substrate (see below). On the other hand, mutants were also generated in which four individual Phe residues at key positions at the entrance to the substrate binding pocket (specifically Phe162, Phe265, Phe332 and Phe337) were replaced by Ala (Galli et al., 2011). The F162A variant displayed higher oxidation efficiency towards the bulky phenols than the wild-type, and the F265A and F332A mutants consumed less substrate, possibly due to the absence of adequate hydrophobic interactions. The F337A variant was not active, which might be explained by the fact that this residue belongs to the second coordination sphere of T1 Cu and a change in this position gives rise to dramatic destabilization. As the F162A and

F332A mutants showed enhanced oxidative capabilities towards bulky substrates, a double variant F162A/F332A was constructed behaving more efficient than the single mutant counterparts in transforming bisphenol A.

Very recently, the HRPL from the ascomycete *Botrytis aclada* has been rationally studied (Osipov et al., 2014). The non-conserved Leu499 residue in the axial position of the T1 site was substituted by Me and the crystal structures, the redox potential and the kinetic constants were determined. While the overall structures of the wild-type and the mutant were very similar, the mutation led to a decrease of 140 mV in the $E^{0'}_{T1}$ (+720 and +580 mV for the wild-type and the L499M variant, respectively) as well as to higher K_m values both for ABTS and DMP.

Semi-rational approaches

Most semi-rational approaches performed in laccases are experimentally supported by saturation mutagenesis, a reliable technique commonly employed to explore the characteristics of enzymes at hot-spot residues identified by rational analysis or directed evolution. With this method, a single codon can be replaced by all the codons that will generate the 20 naturally occurring amino acids, or a representative selection of those, depending on the degree of the degeneration chosen in the mutagenized codon (Lutz, 2010). This approach can be performed to simultaneously mutate several codons (*i.e.* combinatorial saturation mutagenesis — CSM), and sometimes performing iterative cycles so that optimal interactions and synergies between residues are revealed (Chica et al., 2005; Reetz and Carballeira, 2007).

Bacterial laccases

The CotA laccase from *B. subtilis* was recently subjected to saturation mutagenesis in order to assess the role of an acidic residue at the water exit channel (Asp116) in the mechanism of O_2 reduction to water (Brissos et al., 2012; Silva et al., 2012) (Table 6). The five selected mutants (D116E, D116A, D116N, D116T and D116L) only showed small changes in the geometry of the copper centres. Nonetheless, turnover rates were drastically reduced and the optimal pH for the oxidation of substrates was downshifted around 1–2 units when compared with the native laccase. Furthermore, the crystal structures of the D116E, D116A and D116N variants were determined (Silva et al., 2012). Asp116 seems to be essential in the modulation of Glu498 protonation, — the latter being a structurally conserved residue placed at the dioxygen entrance channel. CSM was also employed to narrow the substrate specificity of the CotA laccase from *B. subtillis* (Gupta and Farinas, 2009; Gupta et al., 2010). The simultaneous randomization of 19 amino acids involved in the ABTS-bound, and the subsequent recombination of the best variants, gave rise to a double mutant (G417L-L386W) with strong preferences for ABTS over syringaldazine than the wild-type. Notably, this mutant also showed enhanced thermal stability (the half-life at 80 °C was enhanced by 62 min), despite the fact that the screening assay was designed for substrate specificity and not for thermostability.

Fungal laccases

CSM was employed to increase turnover rates in the T2 mutant from *Myceliopthora thermophila* laccase (obtained by laboratory evolution for functional expression in *S. cerevisiae*; Bulter et al., 2003) focusing on the highly conserved $_{509}$VSG$_{511}$ tripeptide located in the neighbourhood of the T1 Cu site (Zumárraga et al., 2008a) (Table 6). Over 180,000 clones were screened, among which the S510G mutant showed about 3- and 8-fold higher catalytic efficiencies for ABTS and DMP, respectively. Moreover, the T1 site geometry of this laccase variant was altered but without having significant effects on the redox potential. The S510G mutation directly interacts with the C-terminal plug, which seems to modulate the entrance of O_2 to the T2/T3 trinuclear cluster in ascomycete laccases (Andberg et al., 2009).

Directed evolution and hybrid approaches

Directed molecular evolution has become a powerful tool to develop biocatalysts with improved features or new functions (Cobb et al., 2012; Tracewell and Arnold, 2009). This approach recreates Darwinian principles of natural evolution (random mutation, gene recombination and selection), enabling the design of enzymes with properties of particular biotechnological interest in the absence of structural or mechanistic information (Arnold, 2009). In directed evolution the selection pressure is controlled by the researcher searching to improve a specific enzymatic trait, compressing the evolutionary time scale to just months or even days of work in the lab (Dalby, 2011; Esvelt et al., 2011). Of particular interest is the synergistic use of directed evolution and semi-rational approaches applied to laccases in order to improve or create enzymatic features, such as: functional heterologous expression and activity; performance in non-natural environments like organic solvents, human blood or ionic liquids; and the design of chimeric laccases with combined properties (García-Ruiz et al., 2014) (Tables 6 and 7).

Directed evolution for functional expression and activity

Bacterial laccases. The CotA laccase from *Bacillus licheniformis* was subjected to random and site-directed mutagenesis in order to improve its functional expression in *E. coli* (Koschorreck et al., 2009). After one round of error-prone PCR and 6000 clones analysed, laccase variants containing the independent K316N and D500G mutations showed up to a 2-fold increase in ABTS activity with respect to that of the wild-type. The double mutant K316N/D500G was then constructed to study the combinatorial effect of both mutations on laccase expression. This variant displayed 11.4-fold higher expression level than the wild-type, also being more efficient in the conversion of ferulic acid and in the decolorization of industrial dyes. The metagenome-derived alkaline laccase Lac591 was evolved in *E. coli* with the aim of achieving better performing laccases for textile dye decolorization (Liu et al., 2011). After three rounds of error-prone PCR and screening, a mutant (the Lac3T93 variant) with 4.8-fold increased specific activity towards DMP and higher decolorization efficiency for several dyes was obtained. The CotA laccase from *B. subtilis* was engineered using directed evolution and structure-based methods in order to increase the specificity for ABTS over syringaldazine (Gupta and Farinas, 2010). A library of CotA laccase genes was expressed in the bacterial spore coat and the corresponding laccase mutants were screened for substrate specificity. The best variant of this study (the CotA-ABTS-SD1 mutant) exhibited 120-fold higher specificity for ABTS than the wild-type.

Fungal laccases. During the last 10 years, both medium- and high-redox potential laccases have been heterologously expressed by means of laboratory evolution in yeast, mainly *S. cerevisiae* (Maté et al., 2011). There are four main reasons to use *S. cerevisiae* for the directed evolution of fungal laccases: i) it has a high transformation efficient (10^7–10^8 CFU - colony forming units-per μg of DNA), comparable to that of *E. coli* (10^8–10^{10} CFU per μg of DNA); ii) it can secrete laccases to the extracellular medium, avoiding the cumbersome steps of cell lysis required in bacteria; iii) there are a wide variety of episomal uni- and bi-directional vectors available on the market that facilitates the recovery of improved variants; and iv) it shows a high frequency of homologous DNA recombination, which permits the *in vivo* shuffling of mutant libraries and the development of new tools for the creation of genetic diversity (Gonzalez-Perez et al., 2012, 2014).

The first successful study about directed evolution of a fungal laccase was performed with the medium-redox potential laccase from the thermophilic ascomycete *M. thermophila* (MtL) (Bulter et al., 2003). The full laccase gene subjected to evolution comprised the mature sequence joined to the native prepro-leader sequence and the C-terminal tail, the latter two being cleaved from the mature protein during maturation. In total 20,000 clones were explored in 10 rounds of random

Table 6
Laccases engineered by saturation mutagenesis (SM), combinatorial saturation mutagenesis (CSM) and directed evolution.

Laccase source	Approach	Property in study	Characterization techniques	Main results[a]	Reference
CotA from *Bacillus subtilis*	SM	Mechanism of reduction of O_2 to water	Redox titration, EPR, CD spectroscopy, CAA[b]	Small changes in the geometry of the Cu sites. Turnover rates highly reduced and optimal pH downshifted 1–2 units	Brissos et al. (2012)
CotA from *Bacillus subtilis*	SM	Mechanism of reduction of O_2 to water	Simulated pH titrations	Asp116 appears to be crucial in modulating Glu498 protonation	Silva et al. (2012)
CotA from *Bacillus subtilis*	CSM	Substrate specificity	CAA[b]	The CotA-ABTS-10 mutant was 132-fold more specific for ABTS	Gupta et al. (2010)
Myceliophthora thermophila	CSM	Catalytic efficiency	CAA[b]	3- and 8-fold higher catalytic efficiencies for phenolic and non-phenolic compounds	Zumárraga et al. (2008a)
CotA from *Bacillus licheniformis*	Directed evolution	Functional expression in *E. coli*	CAA[b]	K316N/D500G mutant showed 11.4-fold higher expression levels	Koschorreck et al. (2009)
Lac591 (metagenomic)	Directed evolution	Efficiency in textile dyes decolorization	CAA[b]	4.8-fold increased specific activity for DMP. Higher decolorization efficiency for several dyes	Liu et al. (2011)
CotA from *Bacillus subtilis*	Directed evolution	Substrate specificity	Bacterial surface display	The CotA-ABTS-SD1 mutant was 120-fold more specific for ABTS	Gupta et al. (2010)
Myceliophthora thermophila	Directed evolution	Functional expression and activity in *S. cerevisiae*	CAA[b]	Expression levels in *S. cerevisiae* of 18 mg/L (T2 mutant)	Bulter et al. (2003)
Basidiomycete PM1	Directed evolution	Functional expression and activity in *S. cerevisiae*	CAA[b]	Expression levels in *S. cerevisiae* of ~8 mg/L (OB-1 mutant)	Maté et al. (2010)
Pycnoporus cinnabarinus	Directed evolution	Functional expression and activity in *S. cerevisiae*	CAA[b]	Expression levels in *S. cerevisiae* of ~2 mg/L (3PO mutant)	Camarero et al. (2012)
Pleurotus ostreatus	Directed evolution	Activity in *S. cerevisiae*	CAA[b], molecular dynamics	Mutants with enhanced specific activity and/or improved stability	Festa et al. (2008)
Pleurotus ostreatus	Directed evolution	Activity in *S. cerevisiae*	CAA[b]	Mutants with enhanced specific activity and/or improved stability	Miele et al. (2010a,b)
Trametes versicolor (Lcc1)	Directed evolution	Activity in *Y. lipolytica*	CAA[b]	5.8-fold increase in total activity (rM-4A mutant)	Theerachat et al. (2012)
Fomes lignosus	Directed evolution	Activity in *P. pastoris*	CAA[b]	Expression levels in *P. pastoris* of 144 mg/L (PPM5 mutant)	Hu et al. (2007)
Myceliophthora thermophila	Directed evolution	Organic solvent tolerance	redox titration, EPR, cyclic voltammetry, CAA[b]	Remarkable resistance to high concentrations of organic solvents (R2 mutant)	Zumárraga et al. (2007)
Myceliophthora thermophila	Directed evolution	Activity at alkaline pH	CAA[b]	pH activity profiles widely shifted towards alkaline pH values (IG-88 mutant)	Torres-Salas et al. (2013)
Basidiomycete PM1	Directed evolution	Blood tolerance	CAA[b], cyclic voltammetry	Activity in human blood and plasma (ChU-B mutant)	Mate et al. (2013b)
Trametes versicolor (Lcc2)	Directed evolution	Ionic liquid tolerance	CAA[b]	4.5-fold higher activity in 15% (v/v) of [EMIM][$EtSO_4$][c] (M3 mutant)	Liu et al. (2013)

[a] Main results are described in comparison with the wild-type laccase.
[b] CAA: Colorimetric (spectrophotometric) activity assays.
[c] [EMIM][$EtSO_4$]: 1-ethyl-3-methylimidazolium ethyl sulfate.

mutagenesis combined with staggered extension process (StEP) and *in vivo* shuffling, giving rise to the highest expression levels of a laccase in *S. cerevisiae* reported so far (18 mg/L). The best mutant generated in this process (the T2 variant) accumulated 14 mutations that were responsible of a 170-fold improvement in the total activity, 8-fold in the expression levels and 22-fold in the k_{cat} for ABTS. It is worth noting that the most beneficial mutation (H(c2)R, 10-fold increase in total activity) introduced a Kex2 protease recognition site at the C-terminal tail, adjusting the laccase sequence to the different protease specificities of the heterologous host.

The success with MtL led to subsequent laboratory evolution for functional expression in *S. cerevisiae* of two HRPL: those from the ligninolytic basidiomycetes PM1 and *Pycnoporus cinnabarinus* (Camarero et al., 2012; Maté et al., 2010). PM1 laccase (PM1L) was subjected to eight rounds of directed evolution combined with semi-rational design (Maté et al., 2010) (Figs. 2A and 3A). The native signal sequence was replaced by the α-factor prepro-leader from *S. cerevisiae* (commonly used to express heterologous proteins in yeast; Zsebo et al., 1986), and the whole fusion gene was evolved to enhance the total laccase activity 34,000-fold over the parental type. Mutagenic

Table 7
Chimeric laccases.

Parental types	Sequence identity	Heterologous host	Main results	Reference
Lcc1 and Lcc4 from *Lentinula edodes*	60%	Tobacco BY-2 cells	Chimeric laccases with similar expression levels than Lcc1, K_m values similar to those of Lcc4 and pH and temperature profiles intermediate between those of both parents	Nakagawa et al. (2010)
LAC1, LAC2, LAC3 and LAC5 from *Trametes* sp. C30	65–71%	*S. cerevisiae*	Chimeric laccases with similar apparent kinetic parameters at acidic pH than those for LAC3. LAC131 and LAC232 chimeras exhibited higher tolerance to alkaline pH than LAC3	Cusano et al. (2009)
OB-1 and 3PO mutants[a]	76%	*S. cerevisiae*	Chimeric laccases with combined characteristics in terms of pH activity, substrate affinity and thermal stability	Pardo et al. (2012)
Ery4 and Ery3 from *Pleurotus eryngii*	n.r.	*S. cerevisiae*	The 4NC3 chimera showed the highest enzymatic activities, substrate affinities and stability over a broad pH and temperature range. It was also successfully displayed on the cell wall of *S. cerevisiae*	Bleve et al. (2014)

[a] OB-1 and 3PO are, respectively, the final mutants of the directed evolution campaigns of PM1 and *P. cinnabarinus* laccases for expression in *S. cerevisiae* (Camarero et al., 2012; Mate et al., 2013b). n.r. not reported.

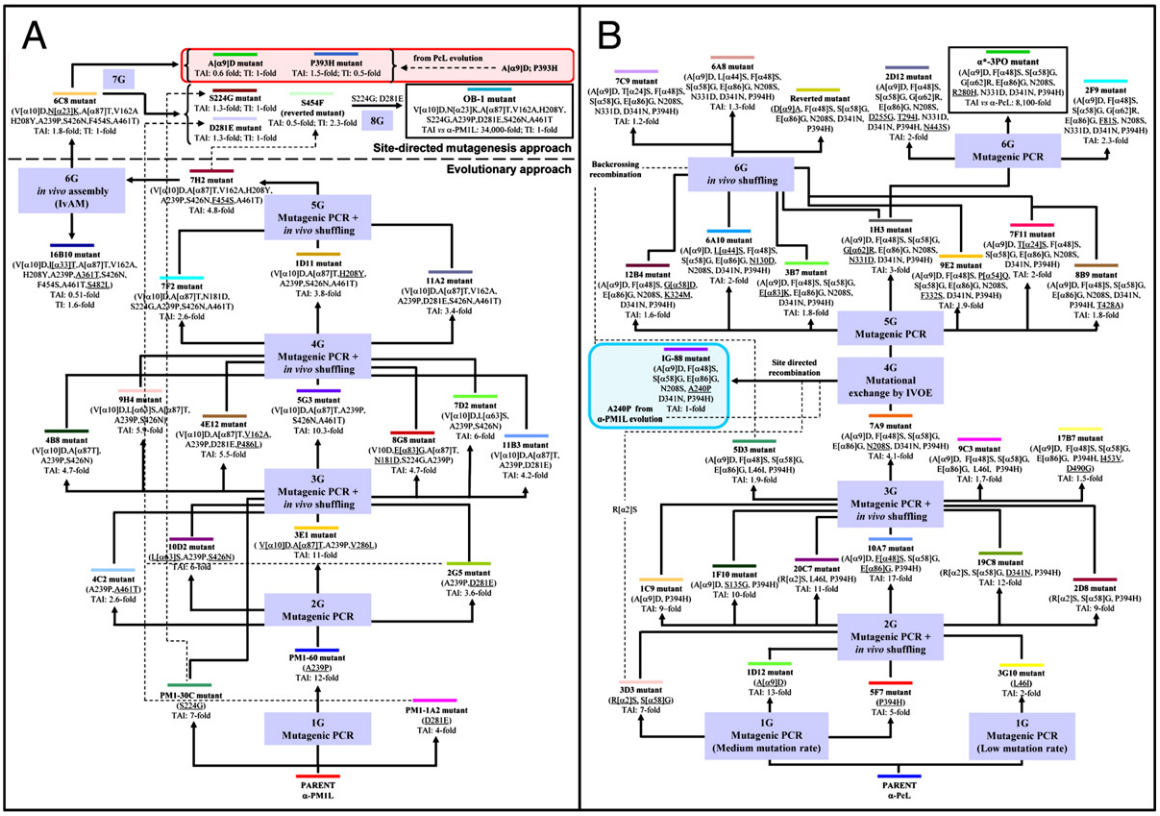

Fig. 2. Artificial evolution pathways for functional secretion in *S. cerevisiae* of HRPL (PM1L (A) and PcL, (B)). Mutational exchange between the two parallel evolutionary trees is indicated by the red and blue shaded box for PM1L and PcL, respectively. The new point mutations are underlined. TAI: total activity improvement detected in *S. cerevisiae* microcultures for each mutant compared to the best parental type of the corresponding generation. TI: thermostability improvement in reference to the parental type of the corresponding generation. IVOE: *in vivo* overlap extension. IvAM: *in vivo* assembly of mutant libraries with different mutational spectra.

libraries created with different DNA polymerases were recombined by *in vivo* DNA shuffling and/or *in vivo* assembly of mutant libraries with different mutational spectra (IvAM) (Zumárraga et al., 2008b). After screening over 50,000 clones, 15 beneficial mutations were located at the signal prepro-leader (five mutations) and the mature protein (ten mutations), while the stability of the protein was preserved by a combined strategy that included: i) a high-throughput screening (HTS) for kinetic thermostability (García-Ruiz et al., 2010); ii) the recovery of beneficial mutations lost along the evolutionary process; and iii) the incorporation of beneficial mutations discovered in the parallel evolutionary study of the *Pycnoporus cinnabarinus* laccase (PcL) into the PM1L scaffold. The best performing mutant of this process (the OB-1 variant) had the highest levels of secretion ever reported for a HRPL in *S. cerevisiae* (~8 mg/L), enhanced kinetics values for phenolic and non-phenolic substrates, as well as high stability in terms of pH, organic co-solvent tolerance and temperature. Indeed, PM1L evolution was recently chosen as model to validate computational protocols (based on several FoldX algorithms and molecular dynamics simulations) which permit the quantification of the relative stability of mutants of HRPL (Christensen and Kepp, 2012). The protocols developed were applied to nine different PM1L mutants, in all cases showing good correlation with the experimental values. In a parallel directed evolution study, PcL was tailored using a similar strategy to that employed for PM1L (*i.e.*, substitution of the native signal peptide by the α-factor prepro-leader and joint evolution of the α-PcL fusion gene (Camarero et al., 2012) (Fig. 2B). After six rounds of evolution coupled to a HTS assay based on the oxidation of natural and synthetic mediators, the total laccase activity was improved 8000 times over the parent α-PcL. The final mutant of this study (the 3PO variant) accumulated a total of 15 mutations in the fusion gene. The five mutations located in the α-factor prepro-leader were responsible for a 40-fold enhancement in secretion by *S. cerevisiae* (~2 mg/L), while the ten beneficial mutations in the mature protein led to a 13.7-fold increase in the k_{cat} for ABTS. Notably, the pH activity profile was shifted to more neutral pH values throughout this evolution, while thermostability was retained.

The POXA1b laccase from *Pleurotus ostreatus* was evolved with the aim of increasing its activity using *S. cerevisiae* as a host (Festa et al., 2008; Miele et al., 2010a). A library of 2300 randomly mutated variants was screened by assaying activity towards ABTS (Festa et al., 2008) and DMP (Miele et al., 2010a), yielding laccase mutants with enhanced specific activity and/or improved stability. Moreover, molecular dynamics simulations of the wild-type enzyme and two mutants allowed the changes observed in laccase properties to be understood at the molecular level (Festa et al., 2008). Among the set of evolved variants, the most stable mutant and the one with the highest catalytic efficiency were selected as parental types to further improve laccase activity *vs.* ABTS (Miele et al., 2010b). The strategy involved the incorporation of the mutations of both parental genes into the same laccase gene scaffold, which was subsequently subjected to random mutagenesis and screening. The best performing POXA1b laccase mutant (the 1H6C variant) had 5-fold higher specific activity towards ABTS compared to the wild-type, as well as increased stability across the whole pH range (Miele et al., 2010b). Very recently, POXA1b wild-type and 1H6 mutant were produced in high levels in *A. niger* by attaching the signal peptide of *A. niger* glucoamylase to the mature laccase (Macellaro et al., 2014, Table 4).

Besides laboratory evolution in *S. cerevisiae*, there are few examples in the literature where fungal laccases have been engineered for heterologous expression in other yeast species. The *lcc1* gene coding for *T. versicolor* laccase was cloned into the genome of *Y. lipolytica* using

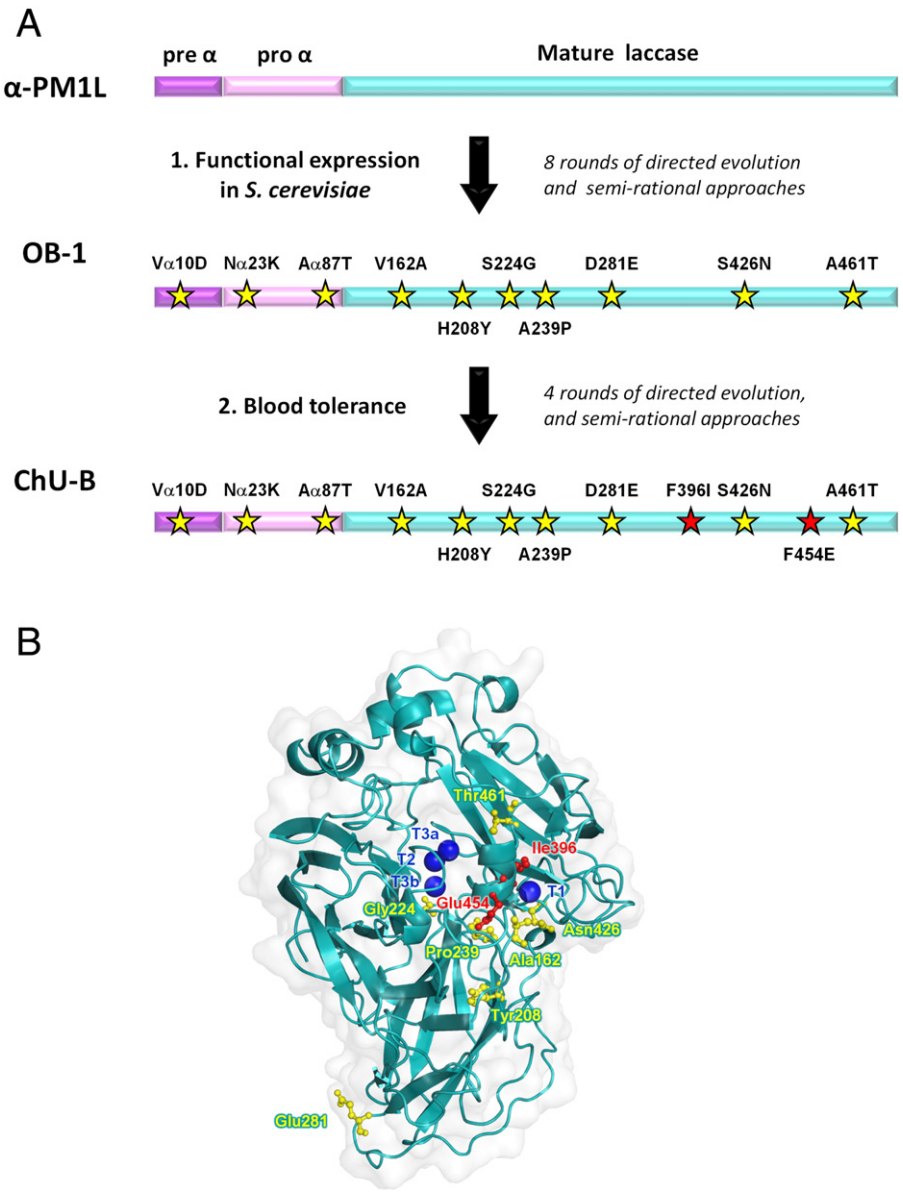

Fig. 3. Laboratory evolution history of the PM1L. (A) Combination of directed evolution, site-directed mutagenesis (SDM) and saturation mutagenesis (SM) for functional expression in *S. cerevisiae* and blood tolerance. The α-factor pre-leader is represented in purple, the α-factor pro-leader is in pink, and the mature laccase is in cyan. The amino acid mutations responsible for secretion in *S. cerevisiae* and blood tolerance are depicted as yellow and red stars, respectively. Silent mutations are not included. (B) Model of ChU-B mutant. The copper ions are depicted as blue spheres. The amino acid substitutions responsible for functional expression and activity in *S. cerevisiae* and blood tolerance are highlighted as yellow and red sticks, respectively.

either single or multiple integration sites (Theerachat et al., 2012). After checking for successful secretion into the culture media, the strain with a single integration was selected for expression and subsequent screening of mutant libraries. The best variant (the rM-4A mutant) contained two mutations (L185P/Q214K), which gave rise to a 5.8-fold increase in the total activity compared to the wild-type, together with a 2.4 and 2.8-fold enhancement of the catalytic efficiency towards ABTS and DMP, respectively. Also, the laccase from the plant pathogen *Fomes lignosus* was functionally expressed in *P. pastoris* by constructing mutant libraries through ethyl methane sulfonate-based random mutagenesis (Hu et al., 2007). After screening 20,000 colonies, the best laccase variant (the PPM5 mutant) harboured four mutations that were responsible for a 3.7-fold enhancement in expression (144 mg/L), as well as a 1.4-fold higher k_{cat} for ABTS. Two of the mutations (specifically G160D and A167T) were found in the proximity of the water channel and they became hydrophilic amino acids, which might help water molecules to exit the trinuclear cluster, thereby explaining the increase of the k_{cat}.

Directed evolution in non-natural environments

One of the most attractive aspects of the combined use of directed evolution with rational and semi-rational strategies is the fact that it allows protein engineers to design new enzymes with functions not previously required in nature (Lutz and Borscheuer, 2012). In particular, in recent years several evolution studies have been published on the improvement of laccase catalysis in non-natural media as diverse as organic solvents, alkaline media, human blood or ionic liquids (Table 6).

In terms of MtL, the aforementioned T2 mutant was selected as template to improve activity and stability in the presence of organic co-solvents, a required feature to employ laccases in organic syntheses or bioremediation processes (Zumárraga et al., 2007). After validation of the HTS assay in 20% (v/v) acetronitrile and 30% (v/v) ethanol (Alcalde et al., 2005), five rounds of evolution were performed that led to the final variant, the R2 mutant. R2 accumulated six additional mutations (four in the mature protein and two in the C-terminal tail) and displayed a remarkable initial activity in the presence of high concentrations of co-solvents (*e.g.* in 50% ethanol (v/v), R2 kept around 20% of the activity showed in aqueous solution). Interestingly, in the course of the 15 generations of evolution for functional expression in yeast and tolerance against organic co-solvents, the pH profile of MtL shifted progressively towards more alkaline pH, clearly as a consequence of screening the mutant libraries at pH 5.0. This unexpected side-effect, combined with the fact that fungal laccases are highly attractive to use in processes where basic pH is a requisite (*i.e.* pulp biobleaching, organic syntheses or cofactor regeneration), made the R2 mutant a suitable departure point to tackle the evolution towards activity at basic pH (Torres-Salas et al., 2013). The R2 mutant was then subjected to five further cycles of error-prone PCR combined with different *in vivo* and *in vitro* DNA recombination methods, being the ratio of activity at pH 8 to that at pH 5 used as selection pressure factor. After screening over 12,000 clones, the final mutant (the IG-88 variant) harboured two beneficial mutations in the mature protein. IG-88 exhibited pH activity profiles both for ABTS and DMP that were widely shifted towards an alkaline pH values (retaining ~90% of its activity at pH 4.0–6.0, 50% at pH 7.0, and even some activity detectable at pH 8.0). Additionally, it showed k_{cat}/K_m values significantly improved over the MtL-R2 parent at neutral pH (31- and 9-fold with ABTS and DMP, respectively), while at pH 4.0 the improvement was slightly less pronounced (12- and 4-fold with ABTS and DMP, respectively). IG-88 variant accumulated two additional mutations with respect to the parental type, one of them being a highly conserved residue (Asp109) previously reported to be involved in keeping the overall geometry of the trinuclear site in *M. albomyces* laccase (Andberg et al., 2009). To sum up, a total of 20 generations of directed MtL evolution was performed in three different studies: heterologous expression in yeast (10 rounds, T2 mutant), stabilization in organic co-solvents (5 rounds, R2 mutant) and activity at alkaline pH (5 rounds, IG-88 mutant). The success of the *in vitro* evolution of MtL can be ascribed to the plasticity and robustness of this protein, able to accumulate 17 amino acid mutations in the mature laccase without compromising its overall stability.

In terms of HRPL, the secretion variant of the PM1L evolution (OB-1 mutant) was recently used as the parental type to design a laccase that could function in human blood, so that it may be incorporated into 3D-nanobiodevices for biomedical purposes (Mate et al., 2013b) (Fig. 3A). Like all other HRPL, the OB-1 mutant was not active at neutral/alkaline pHs and it was strongly inhibited by modest concentration of halides, hampering its performance in human physiological fluids since human blood has a pH of 7.4 and it contains around 150 mM NaCl. Accordingly, we established a HTS assay based on a surrogate blood (named blood buffer) that imitated the biochemical composition of human blood but that lacked coagulating factors and blood cells. OB-1 was subjected to four rounds of directed evolution combined with site-directed and saturation mutagenesis, the selection pressure being gradually increased from pH 6.5 to physiological pH during the evolution. The final mutant obtained (the ChU-B variant) showed a 40,000-fold increase in total activity in blood buffer with respect to the parental type, as well as the highest tolerance to chloride yet reported for a basidiomycete HRPL, with an increase in the I_{50} for Cl^- from 176 mM to 1025 mM (I_{50} was the concentration of Cl^- at which the laccase keeps 50% of its activity). In addition, it displayed significant activity at neutral pH (retaining ~50% and ~20% of its activity for DMP and ABTS, respectively), while conserving a high-redox potential at the T1 Cu. It was also tested on real human blood and plasma after over-expression in *P. pastoris*, revealing the mechanisms underlying this unprecedented improvement (Mate et al., 2013c). These unique features were a consequence of the accumulation of only two additional mutations in the mature protein (specifically F396I and F454E) (Fig. 3B), which led to activity in blood but at the expense of the thermal stability, a phenomenon similar to that observed throughout the evolutionary history of cytochrome $P450_{BM3}$ from *Bacillus megaterium* (Fasan et al., 2008). Very recently, the ChU-B mutant was reported to be the first laccase able to catalyse the electro-oxidation of water to molecular oxygen by immobilization onto chemically modified electrodes (Pita et al., 2014). These results open promising perspectives for the sustainable production of hydrogen as a renewable source of energy. Furthermore, the blood tolerant laccase has been successfully incorporated into a self-powered biodevice with wireless signal transmission (Falk et al., 2014).

The latest example of directed evolution of HRPL towards tolerance in non-natural media is that of the laccase Lcc2 from *T. versicolor*, which was subjected to two rounds of random mutagenesis and screening towards improved ionic liquid resistance (Liu et al., 2013). The best variant from this study (the M3 mutant) displayed 4.5-fold stronger activity than the wild-type in the presence of 15% (v/v) of the ionic liquid [EMIM][$EtSO_4$] (*i.e.* 1-ethyl-3-methylimidazolium ethyl sulfate). These findings show the potential of using HRPL as efficient catalysts for lignin degradation in homogeneous ionic solutions and consequently, for second-generation biofuel production.

Chimeric laccases

DNA recombination methods can be used to generate novel genes by combining DNA fragments sharing certain sequence identity, irrespective of their genetic background (Miyazaki and Arnold, 2004). In recent years, family shuffling has been proposed as an efficient strategy to construct chimeric laccases with improved features over the parental types (Table 7).

Two different laccases from the basidiomycete *Lentinula edodes* sharing less than 60% identity in their cDNA sequences (Lcc1 and Lcc4) were recombined using the N-terminus of the *lcc4* cDNA and the C-terminus of the *lcc1* cDNA (Nakagawa et al., 2010). The resulting chimeric laccase cDNA (*lcc4/1*) was expressed in tobacco BY-2 cells, giving rise to the Lcc4/1 laccase. As expected, Lcc4/1 was a chimera of the two parental laccases, being produced at similar levels as Lcc1, with K_m values for phenolic and non-phenolic substrates similar to those of Lcc4, and with an intermediate pH and temperature profile between the two parental types.

Despite the good results obtained in the creation of the *L. edodes* chimeric laccase, the expression system based on tobacco cell culture cannot be used to screen a large number of recombinant proteins, dramatically reducing the chances of obtaining chimeric enzymes that perform better than the parental types. This drawback can be overcome by taking advantage of the homologous recombination in *S. cerevisiae* (Gonzalez-Perez et al., 2012). Laccase chimeras of the *Trametes* sp. strain C30 were constructed by yeast-mediated homologous recombination of four cDNAs (*lac1*, *lac2*, *lac3* and *lac5*), which shared 65–71% DNA sequence identity (Cusano et al., 2009). The best chimeras (LAC131, LAC232 and LAC 535) apparently exhibited similar kinetic parameters at acidic pH to those obtained for the LAC3 reference laccase (a low-redox potential laccase previously expressed in yeast; Klonowska et al., 2005). However, LAC131 and LAC232 chimeras were more tolerant to alkaline pH, showing 5- and 12-fold higher catalytic efficiencies, respectively, than LAC3 at pH 8.0, as well as pH activity profiles shifted towards basic pH values.

DNA shuffling in *S. cerevisiae* has also been employed to generate chimeric HRPL by directed evolution (Pardo et al., 2012). The cDNA of the final variants of the evolution for expression in yeast of PM1L and PcL (OB-1 and 3PO mutants, respectively: 51% DNA sequence identity) were recombined *in vitro* and *in vivo* by CLERY (combinatorial libraries enhanced by recombination in yeast; Abécassis et al., 2000). Laccase hybrids with up to six crossover events per sequence were identified,

which generated active chimeric laccases with combined features in terms of pH activity, substrate affinity and thermal stability. Unexpectedly, several laccase chimeras exhibited stronger thermal stabilities than both parent types. This effect may be attributed to the accumulation of neutral mutations known to be beneficial for the stabilization of the protein structure (Bloom et al., 2006).

Very recently, the ERY4 laccase from *P. eryngii* has been modified with the N-, C- and both N- and C-terminal regions of ERY3 laccase, another laccase isoform of *P. eryngii*, to get the enzyme expressed in active form in *S. cerevisiae* (Bleve et al., 2014). Among the set of chimeric laccases, the best performing enzyme in terms of activity and stability was the result of substituting both the N- and the C-terminal regions. Significantly, this chimera was successfully displayed on the cell wall of *S. cerevisiae* by attaching either the Pir2 or the Flo1 anchor proteins to the N-terminal of the laccase.

Conclusions and outlook

Over the years, laccases have become very attractive candidates for protein engineering as a consequence of their broad oxidative capabilities and consequently, their strong potential application in different industrial sectors. Incorporating targeted mutations into the laccase scaffold has permitted researches to inquire into the influence of 'hot spot' amino acid residues on several laccase features, such as the redox potential, catalytic efficiency or thermal stability. The creation of 'smart libraries' coupled to *ad-hoc* HTS assays has helped to surpass the difficulties encountered when using strict rational design. Thus, directed evolution combined with hybrid strategies have proven to be successful in achieving functional heterologous expression and activity, adaptation to non-natural environments and for the design of laccase chimeras with combined traits. In most of these experiments, homologous recombination in *S. cerevisiae* has permitted detrimental mutations to be sorted out, while accelerating the full *in vitro* evolution approach.

Among the remaining challenges in laccase engineering are the increasing of the redox potential at the T1 Cu site beyond the nature limits (above +800 mV) without sacrificing neither the stability nor the catalysis (Abdellaoui et al., 2013) and the design of *ad-hoc* high efficient laccases acting on redox mediators while surpassing the intrinsic inhibitory constraints (Sayut and Sun, 2010). Even though these milestones could be achieved, laccase large-scale production (at the scale of g/L) is the key to access to the industry with competitive costs. Therefore, suitable heterologous expression systems with tolerance to high laccase expression levels (*i.e.* with low metabolic drains and/or high resistance to laccase toxicity) must be first accomplished.

In the forthcoming years, it is expected that the combination of laboratory evolution with both rational and semi-rational strategies, including molecular dynamics and quantum mechanics/molecular mechanics simulations, will lead to the development of laccases with exciting biotechnological properties and produced at high titers while enhancing our understanding, at the molecular level, of the mechanisms that govern the behaviour of this thrilling group of oxidoreductases.

Acknowledgements

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