Widening the pH activity profile of a fungal laccase by directed evolution

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Introduction

Due to their broad substrate specificity and minimal requirements, laccases (EC 1.10.3.2) have become one of the best studied oxidoreductases. These members of the blue-multicopper oxidase family contain four catalytic coppers in two active sites and they only require O_2 from air to oxidize dozens of different compounds, producing H_2O as the sole by-product. The T1 site is where the blue T1 Cu oxidizes the reducing substrate, and the trinuclear copper cluster (with one T2 Cu and two T3 Cu), is where molecular oxygen is reduced to water.[1]

Laccases constitute a reliable biotechnological platform for the efficient transformation of a wide range of aromatic molecules (*e.g.,* phenols, polyphenols, benzenothiols and anilines). Moreover, with the aid of redox mediators (of natural or of synthetic origin) the oxidative capabilities of lacasses can be expanded considerably to include more recalcitrant molecules - such as PAHs, non-phenolic components from lignin matrix, industrial dyes and pesticides- through a redox cascade mechanism of action.[2] Given their catalytic versatility, laccases have found applications in several areas, from bioremediation to novel green processes.[3] Although widely distributed in nature (fungi, plants, bacteria, insects and even lichens), to date biotechnological applications have only been demonstrated for laccases secreted by fungi. This is mainly due to their high redox potential, the availability of several suitable hosts for their heterologous overexpression and the wide variety of molecular tools available to tailor new properties through rational and non-rational approaches.[4]

One of the most intriguing characteristics of fungal laccases is their pH-dependent catalysis. Although fungal laccases are highly stable above pH 7.0, their activity is negligible close to a neutral pH. The optimum pH for activity lies in the range of pH 2.0-4.0 (depending on the substrate), albeit at the cost of stability. It is thought that the lack of activity at neutral/basic pH's is due to the strong intrinsic inhibitory effect of hydroxide ions that abolish the intramolecular electron transfer between the catalytic coppers, thereby inactivating the biocatalyst.[5] Fungal laccases that are active at neutral/basic pH values are highly desirable since they can be used for the decolorization of recalcitrant dyes, pulp biobleaching, the engineering of bionanodevices, organic synthesis and enzymatic cofactor regeneration.[6] Thus, the engineering of fungal laccases with catalytic activity at pH ≥ 7.0 is an interesting issue worthy of further study.

In previous works, we described the directed evolution of the medium redox potential laccase from the ascomycete *Myceliophthora thermophila* (MtL). These studies aimed to attain functional expression in *Saccharomyces cerevisiae* (giving rise to the MtL-T2 variant with 11 beneficial mutations)[7] and to enhance the tolerance to organic co-solvents (generating the MtL-R2 variant harboring 4 beneficial mutations).[8] The departure point in those experiments were laccases with almost undetectable activity under the corresponding selective pressure. To find active mutants even after several hours of incubation in the assay mixture, the pH of the corresponding screening assays was set at 5.0, in order to reach a compromise between activity and stability. Interestingly, in the course of these evolutionary routes, the pH activity profile of the laccase shifted, with the final MtL-R2 mutant switching its optimal pH for activity from 3.0 to 4.0. This unexpected side-effect was a consequence of exploring the mutant libraries at pH 5.0, and indicates that the pH profiles of the selected variants were modified, as well as enhancing their secretion and tolerance to co-solvents.

Results and Discussion

In the present study, we describe the engineering of a fungal laccase with activity at neutral/alkaline pH by directed evolution, using the MtL-R2 mutant as a scaffold and performing 5 cycles of *in vitro* evolution. To generate diversity, we combined error-prone PCR with different *in vitro* and *in vivo* DNA recombination methods (StEP, IvAM, IVOE), as well as saturation mutagenesis (**Supplementary Fig. 1**). Over 12,000 clones were analyzed using an *ad-hoc* screening assay to shift and/or broaden the pH profile of the laccase (see Methods for details). The ratio of activity at pH 8.0 to that at pH 5.0 was used as the main discriminatory factor (*i.e.,* only clones with improved activity at pH 8.0 and that retained a similar activity at pH 5.0 as the corresponding parental type were selected, and characterized further). To rule out the presence of false positives, three consecutive re-screenings were carried out, including an analysis of the pH activity profile *vs* the non-phenolic and phenolic substrates 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,6-dimethoxyphenol (DMP).

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The final mutant of the directed evolution experiment (IG-88 variant) was purified and characterized biochemically. Regardless of the substrate, the pH activity profile shifted towards more alkaline values in response to the selective pressure applied in the screening (**Fig. 1**). In fungal laccases, pH profiles for phenolic substrates are typically bell-shaped, whereas those for non-phenolic compounds decrease monotonically from pH 2.0-3.0 upwards. The oxidation of phenols by fungal laccases is dependent on the redox potential difference between the substrate and the T1 Cu site: at acidic pH values, the release of one proton upon oxidation increases the difference between the redox potentials of the enzyme and the substrate, finally reaching a maximum at which the enzyme activity decreases due to the inhibitory effect of the OH^- attached to the T2/T3 copper cluster site.[5] By contrast, the redox potential of non-phenolic substrates is independent of pH (*i.e.,* no H^+ release occurs upon oxidation), resulting in monotonic pH profiles that are exclusively dependent on the increasing concentrations of OH^-.

The native MtL expressed by yeast exhibited maximum activity against the non-phenolic ABTS at pH 3.0, decreasing monotonically as the pH value increased. However, the activity of the MtL-T2 variant at pH 3.0 was 40% lower than that of the native MtL,[7] and after 5 subsequent rounds of evolution, the MtL-R2 mutant only retained 10% of its activity at pH 3.0.[8] The IG-88 mutant obtained in the current study showed similar levels of activity for ABTS at pH 3.0 as the parental MtL-R2, but with a broadly extended pH range rather than a monotonic decrease. Thus, IG-88 retained ~90% of its activity at pH 4.0-6.0, whereas the MtL-R2 parent type conserved only ~30% of its activity at pH 6.0. Notably, ~50% of IG-88 activity was retained at pH 7.0 and some activity could even be detected at pH 8.0 (**Fig. 1A**). The effect of evolution on the pH activity profile for the phenolic DMP was even more marked. The maximal activity shifted from pH 4.0 to 6.0, whilst the relative activity at pH 7.0 and 8.0 increased 2-fold, with over 20% activity retained at pH 8.0 (**Fig. 1B**).

The kinetic constants for ABTS and DMP were determined at pH 4.0 (**Table 1**) and 7.0 (**Table 2**). Regardless of the substrate used, the k_{cat} was increased notably: ~8-fold at pH 4.0 and from 14 to 18-fold at pH 7.0 over the parental type for ABTS and DMP, respectively. At both pH values, the K_m decreased for ABTS from 1.4 to 2-fold, although it increased for DMP ~2-fold. It is highly likely that the use of ABTS as a substrate in the screening led to the increased affinity for this compound. Finally, k_{cat}/K_m values improved at neutral pHs 31-fold for ABTS and 9-fold for DMP, and at pH 4.0 these were enhanced 12- and 4-fold, respectively. These data demonstrate that screening mutant libraries for activity at alkaline pH values while conserving the activity at acidic pH allowed us to shift the pH profiles while improving the catalytic efficiency at both pH values.

The IG-88 mutant harboured two beneficial mutations in the mature protein (N109S, D530E) and one mutation in the pro-leader (E38G), which was removed upon maturation in the Golgi compartment and therefore, exerted no effect on the biochemical properties of the laccase. The mutations were mapped in a laccase homology model based on the crystal structure of the laccase from *Melanocarpus albomyces* (75% sequence identity to MtL; PDB 1GW0),[9] including the 15 mutations introduced in the previous generations of evolution for secretion and organic co-solvent tolerance (**Supplementary Fig. 2**).

The D530E mutation is located at the protein surface in a helix far from the catalytic coppers (**Fig. 2**; **Supplementary Table 1**). It is highly unlikely that this mutation could have been anticipated by structure-function studies, and we have no reasonable explanation for the effect that this amino acid change exerted on laccase activity at different pH values. The N109S mutation is located close to the T2/T3 trinuclear Cu cluster where molecular oxygen binds and is reduced to two molecules of H_2O upon the concomitant mono-oxidation of four molecules of substrate at the T1 Cu site. At neutral/alkaline pH values, the T2/T3 cluster can accept hydroxyl ions, which interrupt the trafficking of electrons from the T1 site and hence, the catalytic activity. According to our model, the interaction of Asn109 with the surrounding Ser91, His140 and Gly558 residues through a complex network of H-bonds (**Fig. 2A**) is likely to be abolished following mutation (**Fig. 2B**). His140 is one of the three co-ordinating residues of the Cu T3a[9] and the general increase in activity of the IG-88 mutant might indicate a possible conformational rearrangement of the T2/T3 coordination sphere. Gly558 forms part of the C-terminal plug of MtL ($_{556}$DSGL$_{559}$), which is involved in regulating the entry of O_2 to the trinuclear copper cluster.[10] It is feasible that OH^- uses the same route to access the T2/T3 site and thus, any subtle change in this environment may affect the activity at neutral/alkaline pH values. Finally, the adjacent Leu559 of the plug also interacts with His140, emphasising the importance of this region in improving the kinetics of IG-88 at acidic and neutral/basic pH values. Our results agree well with those from a previous comprehensive rational study of the C-terminal plug of the *Melanocarpus albomyces* laccase, further supporting the view that the highly conserved Asn109 (**Supplementary Table 2**) is a key determinant of the overall geometry of the T2/T3 cluster and, consequently, of laccase catalysis.[11]

We recently generated a high-redox potential basidiomycete laccase by directed evolution that was active in human blood.[12] Unlike mutations discovered in the IG-88 mutant, all the mutations that conferred activity in blood samples (at pH ~7.4 with [NaCl] ~140 mM) were mapped to the second coordination sphere of the T1 Cu. Inhibition by OH^- and halides is a very complex process, which involves at least two distinct mechanisms whereby anions bind to the T1 Cu site (Cl^- and Br^-) or the T2/T3 cluster (F^- and OH^-, and possibly also Cl^-).[12 and references herein]

MtL is a medium redox potential ascomycete laccase with a wide range of applications in different industrial settings. This laccase is easily over-produced in *Aspergillus orizae*[13] and it is one of the most commonly used laccases in several biotechnological areas.[14] The engineering of MtL relies on a well-established directed evolution trajectory based on the *S. cerevisiae* machinery.[15] The directed evolution approach used in the present study produced an enzyme active over a broader pH range. Accordingly, this biocatalyst is now ready to face new challenges at neutral/alkaline pHs.

Experimental Section

Materials: All chemicals were of the highest purity commercially available. The pJRoC30-MtL-R2 parental type and the culture media were prepared as described previously.[8] The protease deficient *S. cerevisiae* strain BJ5465 was from LGCPromochem (Barcelona, Spain)

Laboratory evolution: For each generation, PCR fragments were cleaned and concentrated, loaded onto a low-melting point preparative agarose gel, and purified using the Zymoclean gel DNA recovery kit (Zymo Research). The PCR products were cloned under the control of the GAL1 promoter of the expression shuttle vector pJRoC30, replacing the R2 gene in the plasmid. To remove the parental gene, the plasmid was linearized with XhoI and BamHI and the linearized vector was concentrated and purified as previously described for the PCR fragments.

First generation: mutagenic PCR and *in vivo* shuffling. A library of ~2,000 mutants was generated by mutagenic PCR with Mutazyme I DNA polymerase (Stratagene). Error prone PCR was carried out in a gradient thermocycler in a final volume of 50 µL, containing: 0.4 µM of each primer, 2 µg of template, dNTPs (0.2 mM each), 3% dimethyl sulfoxide (DMSO), and 2.5 U Mutazyme I. PCR was performed as follows: 95ºC for 2 min (1 cycle); 94ºC for 0.45 min, 53ºC for 0.45 min and 74ºC for 3 min (28 cycles); 74ºC for 10 min (1 cycle). The following primers were used for amplification: RMLN sense (5'-CCTCTATACTTTAACGTCAAGG-3', which binds to bp 420-441 of pJRoC30-MtL); and RMLC antisense (5'-GGGAGGGCGTGAATGTAAGC-3', which binds to bp 2399-2418 of pJRoC30-MtL). To promote *in vivo* cloning and DNA shuffling, overhangs of 40 and 66 bp homologous to the linear vector were designed. The PCR fragments (400 ng) were mixed with the linearized plasmid (100 ng) and transformed into competent cells using a yeast transformation kit (Sigma). Transformed cells were plated on SC drop-out plates and incubated for 3 days at 30ºC. Colonies containing the whole autonomously replicating vector were selected and screened as described below (this protocol was used to screen each generation of evolution).

Second generation: Mutagenic PCR and *in vivo* shuffling. A library of ~4,000 clones was built following the same procedure described for the first round of evolution but using Mutazyme II DNA polymerase (Stratagene) to vary the mutational bias.

Third generation: *In vivo* assembly of mutant libraries (IvAM). A library of ~4,000 clones was built by *in vivo* assembly of two independent libraries with different mutational spectrums.[16] An equimolar mixture of *Taq*/$MnCl_2$ and Mutazyme II libraries was added to 100 ng of the linearized vector and transformed into yeast competent cells (ratio equimolar library: vector = 8:1).

Fourth generation: Staggered Extension Process (StEP) and *in vivo* shuffling. Error prone PCR was carried out in a gradient thermocycler in a final volume of 50 µL, containing: 90 nM of each primer, 4 µg of template, dNTPs (0.075 mM each), 3% DMSO, 0.01 mM $MnCl_2$ and 2.5 U of *Taq* DNA polymerase. PCR was performed as follows: 95ºC for 5 min (1 cycle); 94ºC for 30 s and 55ºC for 20 s (90 cycles). The DNA products were purified and co-transformed into yeast along with the linearized vector to promote *in vivo* cloning and DNA shuffling. A library of ~2,000 clones was screened.

Fifth generation: Saturation mutagenesis at position 554. A library of ~500 clones was constructed to explore position 554 by saturation mutagenesis using the *in vivo* overlap extension (IVOE) method[17] with the 9G1 mutant as the parental template. Two separate PCR reactions were performed simultaneously to amplify the two DNA fragments that overlapped at the specific position corresponding to the region targeted for saturation mutagenesis of the parental sequence. PCR reactions were carried out in a final volume of 50 µL containing 0.25 µM of each primer, 100 ng of template, dNTPs (0.25 mM each), 3% DMSO and 2.5 U of PfuUltra High-Fidelity DNA polymerase (Agilent Technologies). The primers used for PCR1 were RMLN and 9G1REV (5'-CGCTTGAGGCCCGAGTCGGA**SNN**GGGGTGGGGGTTGGTAGGCC-3', which binds to bp 2246-2288 of pJRoC30-MtL). The primers used for PCR2 were 9G1FOR (5'-GGCCTACCAACCCCCACCCC**NNS**TCCGACTCGGGCCTCAAGCG-3', which binds to bp 2246-2288 of pJRoC30-MtL) and RMLC. The codon subjected to mutagenesis is highlighted in bold, where N is A/T/G/C and S is G/C.

HTP-screening protocol: Individual clones were picked and cultured in 96-well plates (Sero-well, Staffordshire, UK) containing 50 µL of minimal medium per well. In each plate, column number 6 was inoculated with the parental type, and one well (H1-control) was left uninoculated. The plates were sealed to prevent evaporation and incubated at 30ºC with 80% relative humidity and constant agitation at 225 rpm (Minitron-INFORS, Biogen, Spain). After 48 h, 160 µL of expression medium was added to each well and the plates were incubated for 24 h. The plates (master plates) were centrifuged (Eppendorf 5810R, Germany) for 5 min at 4ºC and 3000 x *g*, and 20 µL of supernatant was transferred from the master plate onto two replica plates using a robot (Liquid Handler Quadra 96-320, Tomtec, Hamden, CT, USA). The first replica plate was filled with 180 µL of 100 mM B&R buffer (pH 5.0) containing 3 mM ABTS, and the second with 180 µL of 100 mM B&R buffer (pH 8.0) containing 3 mM ABTS. The plates were stirred briefly and absorption at 418 nm ($\varepsilon_{ABTS}^{\bullet+}$=36,000 M^{-1} cm^{-1}) was recorded on a plate reader (SPECTRAMax Plus 384, Molecular Devices, Sunnyvale, CA). The plates were incubated at room temperature until the color developed and absorption was measured again. Relative activities were calculated as the difference between the absorption values recorded before and after incubation, normalized to the parental type that was used as the reference value in the corresponding plate. Clones with relative activity at pH 5.0 ≥ 1 and with a ratio of relative activity at pH 8.0/relative activity at pH 5.0 ≥ 1 were subjected to two consecutive re-screenings[8]. A third re-screening was performed to determine the full pH activity profile *vs* ABTS and DMP from large scale cultures of the best mutant *hits*. Selected mutants were produced, purified and characterized biochemically as described elsewhere.[8]

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Keywords: alkaline pH, laccase, directed evolution, *Saccharomyces cerevisiae,* C-terminal plug

Figure 1

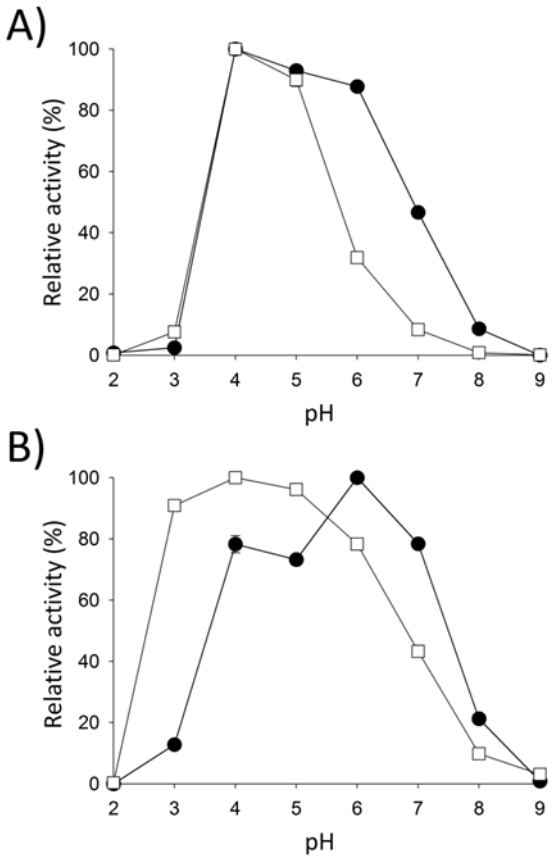

Figure 1. pH-activity profiles for the parental MtL-R2 (white squares) and the IG-88 mutant (black circles) using ABTS (A) and DMP (B) as substrates. Laccase activity was normalized to the maximum activity value and the results represent the mean ± S.D. from three independent experiments.

Figure 2

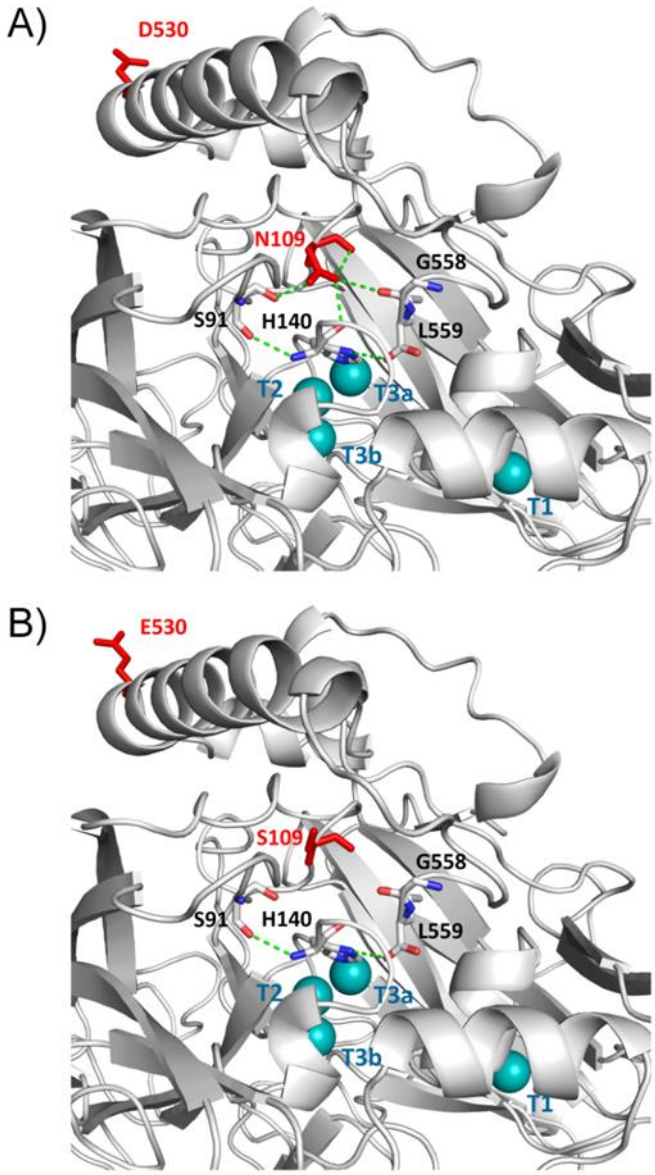

Figure 2. Location of the mutations (red sticks) and their interactions with surrounding residues. (A) Parental MtL-R2, (B) IG-88 mutant. Blue spheres represent Cu atoms; H-bonds are represented by green dashes.

Table 1. Kinetic constants at pH 4.0 for the parental and evolved laccases.

Laccase	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m (mM^{-1} s^{-1})
MtL-R2 (Parent)	ABTS	0.036 ± 0.004	93 ± 2	2583
	DMP	0.098 ± 0.007	20.0 ± 0.3	204
IG-88	ABTS	0.025 ± 0.005	785 ± 35	31400
	DMP	0.21 ± 0.03	171 ± 6	814

Table 2. Kinetic constants at pH 7.0 for the parental and evolved laccases.

Laccase	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m (mM^{-1} s^{-1})
MtL-R2 (Parent)	ABTS	0.34 ± 0.03	31.2 ± 0.7	91
	DMP	0.0073 ± 0.0008	9.7 ± 0.2	1329
IG-88	ABTS	0.16 ± 0.01	450 ± 8	2813
	DMP	0.014 ± 0.003	171 ± 8	12214

Figure S1

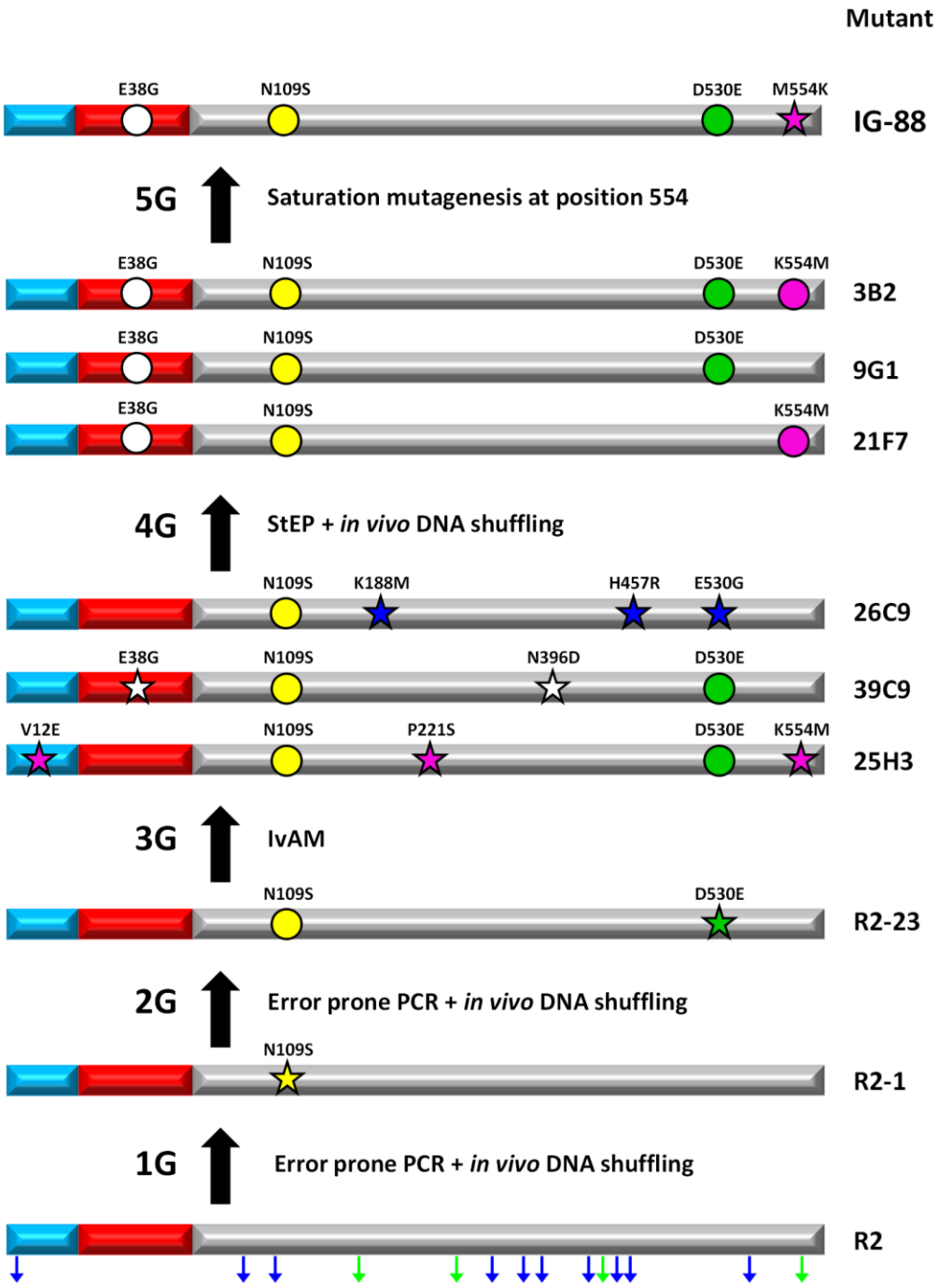

Figure S1. Directed laccase evolution. The parental type gene (R2 mutant) (Zumarraga et al., 2007) is formed by a prepro-peptide (in blue and red) and the mature laccase (in grey). The mutations introduced for functional expression in mature R2 and tolerance in co-solvents are indicated by blue and green arrows, respectively. The new mutations are labelled with stars and accumulated mutations with circles.

Figure S2

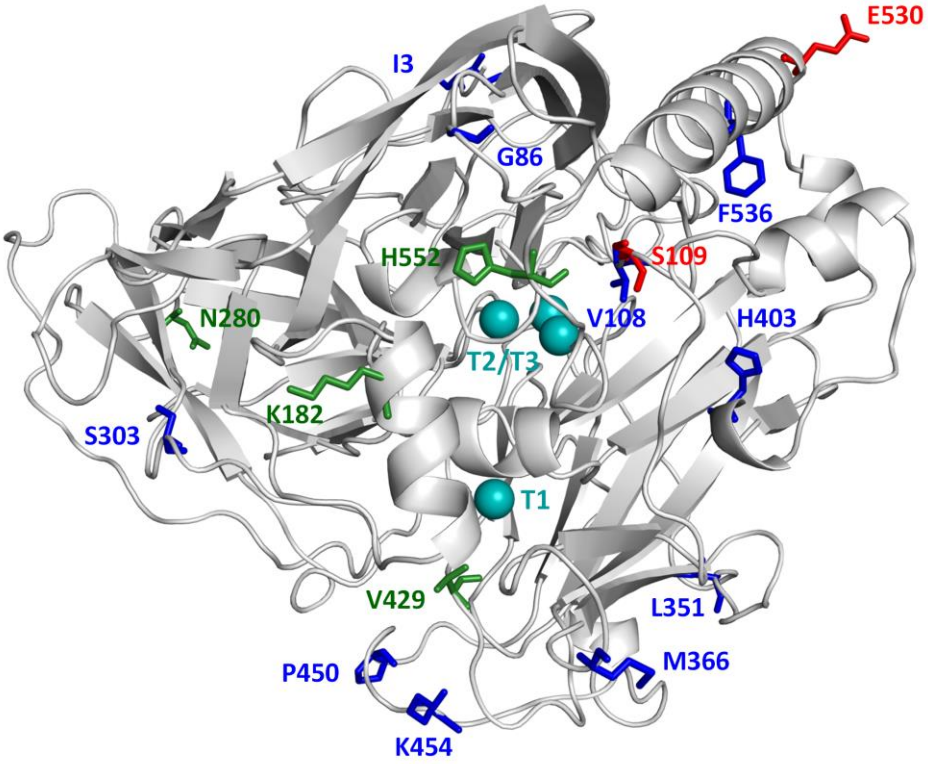

Figure S2. Model of the MtL IG-88 mutant based on the crystal structure of the *Melanocarpus albomyces* laccase (76% sequence identity; PDB 1GW0). Mutations to improve functional expression in *S. cerevisiae* are represented in blue (Bulter *et al.*, 2003), those for tolerance to co-solvents in green (Zumárraga *et al.*, 2007), and those for widening the pH activity profile in red. Copper atoms are represented by blue spheres.

Supplementary Table 1. Mutations in the mature IG-88 variant. Distances were measured between the Cα of the amino acid residue and the corresponding Cu atom.

Mutation	Distance to the T1 Cu (Å)	Distance to the T2 Cu (Å)	Distance to the T3a Cu (Å)	Distance to the T3b Cu (Å)	Domain	Secondary structure motif
N109S	19.7	12.4	10.0	13.3	I	Loop (G96-K119)
D530E	37.0	26.4	25.3	27.5	III	α-helix (D530-T549)

Supplementary Table 2. Partial alignment of the amino acid sequences of IG-88 mutant (including N109S mutation, highlighted in grey) with other known ascomycete laccases.

	IDENTITY		
IG-88	-	89	GTSIHWHGLHQKGTNLHDGVSGITECPIPPKGGRKVYRFKAQQYGTSWYH 138
M. thermophila	96%	89	GTSIHWHGLHQKGTNLHDGANGITECPIPPKGGRKVYRFKAQQYGTSWYH 138
C. globosum	78%	136	GTSIHWHGMHQKDTNLHDGANGVTECPIPP-GGRRVYRFKAQQYGTSWYH 184
M. albomyces	74%	89	GTSIHWHGIHQKDTNLHDGANGVTECPIPPKGGQRTYRWRARQYGTSWYH 138
T. terrestris	73%	129	GTSIHWHGLRQLNNNLNDGVNGVTECPIPPKGGKKLYRFRAVQYGTTWYH 178
P. anserina	69%	134	GTSIHWHGLHQKGTNMHDGANGVTECPIPPKGGSRIYRFRAQQYGTSWYH 183
N. crassa	65%	140	GTSIHWHGMHQRNSNIQDGVNGVTECPIPPRGGSKVYRWRATQYGTSWYH 189
G. clavigera	57%	122	GTSMHWHGIRQLNNNLQDGVNGVTECPIAP-GSSRTYTFLAEQYGTTWYH 170
M. oryzae	54%	136	GTSFHWHGIRMLNNCVNDGANGITECPIAPN-KSRKYRFRAQQYGTSWYH 184
G. graminis	53%	113	GTSMHWHGVRMLNNNPNDGANGITECPIPPN-ASKVYRFRVEQYGTAWYH 161

The numbering of the IG-88 mutant sequence does not include the preproleader sequence. The sequences were aligned in the NCBI-BLAST server, with the COBALT (Constraint-based Multiple Alignment Tool) algorithm. IG-88, MtL mutant obtained in the present study; M. thermophila, laccase from *M. thermophila* (XP_003663741); C. globosum, laccase from *Chaetomium globosum* CBS 148.51 (XP_001228806); M. albomyces, laccase from *Melanocarpus albomyces* (1GW0); T. terrestris, laccase from *Thielavia terrestris* NRRL 8126 (XP_003654809); P. anserina, laccase from *Podospora anserina* (XP_001904217); N. crassa, laccase from *Neurospora crassa* (AAA33591); G. clavigera, laccase from *Grosmannia clavigera* kw1407 (EFX02122); M. oryzae, laccase from *Magnaporthe oryzae* 70-15 (XP_003711640); G. graminis, laccase from *Gaeumannomyces graminis var. tritici* (CAD10749).