Supplementary data

Engineering of a fungal laccase to develop a robust, versatile and highly-expressed biocatalyst for sustainable chemistry

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The Supplementary information contains:

- Materials and Methods (including cited references and a table with the sequences of primers used).
- One table and seven figures supporting the results described in the main text.

Materials and methods

Reagents and culture media

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), N,N-dimethyl-p-phenylenediamine (DMPD), Sodium dodecylbenzenesulfonate (SDBS), 2,6-Dimethoxyphenol (DMP), aniline, the Yeast transformation kit and the primers used in this study were all purchased from Sigma-Aldrich (Madrid, Spain). Pfu-ultra high fidelity polymerase was purchased from Agilent (Santa Clara, EEUU). Endoglycosilase-H (Endo-H) and the High Pure Plasmid Isolation Kit were both purchased from Roche (Basel, Switzerland). The Gel Extraction Kit was purchased from Qiagen (Hilden, Germany), Zymoprep Yeast Plasmid Miniprep II from Zymo Research (Irvine, USA) while Genemorph II random mutagenesis kit was obtained from Stratagene (La Jolla, EEUU). Protease-deficient *Saccharomyces cerevisiae* strain BJ5465 comes from LGCPromochem (Barcelona, Spain). Mixture of dNTPs was acquired from Roche and minimal medium, yeast extract-peptone medium (YP), expression medium and synthetic complete (SC) dropout plates without uracil were prepared as seen in Camarero 2012.[1] pJRoC30 containing 7D5 laccase gene insert was obtained in previous works.[2] To linearize pJRoC30 vector, the gene was removed using the restriction enzymes BamHI and XhoI purchased from New England BioLabs (Hertfordshire, United Kingdom).

High-Throughput Screening (HTS) assays

HTS colorimetric assay with DMPD

To avoid handling aniline during the screening of the laccase mutant libraries (thousand of clones) we selected a less toxic analogue, DMPD, as substrate to test laccase activity on aromatic amines. The extinction coefficient of oxidized DMPD (Würsten dye) was calculated by measuring the absorbance of increasing concentrations of oxidized DMPD at 550 nm, pH 3, in a Shimazdu UV-1900 Spectrophotometer and applying the Beer-Lambert equation. The reliability of the colorimetric assay in high-throughput format was tested with the supernatants of *S. cerevisiae* cells transformed with laccase gene and cultured in 96-well plates. To test reproducibility of the assay, a certain clone was cultured in the 96 wells of the

same microplate and the coefficient of variation of the colorimetric responses given in each well was determined. The linearity of the assay was evaluated by the response obtained with increasing volumes of the same laccase supernatant. Finally, the sensitive of the colorimetric assay was evaluated by screening a 1800-clone mutant library. Changes in DMPD absorbance were measured in end point in a SpectraMax 384 Plus plate reader (Molecular Devices, USA).

Screening of laccase mutant libraries

HTS of the mutant libraries were carried out as previously described[3] except for some modifications here specify. The libraries were screened with 5 mM DMPD and 3mM ABTS as substrates, in 100 mM citrate-phosphate buffer, pH 3. In addition, stability assays to acid pH (3) were carried out during the first and second re-screenings. In each generation, the best laccase variants were selected and used as parents for the next round of evolution as follows: higher activity with DMPD> higher stability to pH3 > higher activity with ABTS. Aniline oxidation and aniline polymerization (15 mM aniline, 5 mM SDBS, same buffer, see Application study section) was assayed in the variants selected in each evolution round. In the first case, 300 mM aniline in 190 µl of the same buffer, were stirred until aniline was totally dissolved. Then, 10 µl of crude enzyme (10 µg/ml concentration) were added, and the increment of Absorbance 410 nm was monitored in kinetic mode for 20 min using the plate reader (triplicate samples).

Laccase engineering

First evolution round, random mutagenesis with Taq/$MnCl_2$

epPCR over the whole construction (7D5 laccase CDS fused to the corresponding mutated alpha-factor pre-proleader)[2] inserted in the vector pJRoC30 was carried out using Taq/$MnCl_2$ in a gradient thermocycler T100 Bio-Rad (CA, USA), using the following protocol: 95 ºC (2 min), 1 cycle; 94 ºC (45 s), 53 ºC (45 s), 74 ºC (3 min), 28 cycles; and 74 ºC (10 min), 1 cycle. For 50 µl reaction, 3 µl $MgCl_2$, 0.5 µl $MnCl_2$, 3 µl DMSO, 0.3 mM dNTPs mix, 90 nm each primer, 0.5 µl Taq polymerase and 4.6 ng of DNA template were added. The primers used RMLN and RMLC are depicted in Table S1. Purification and transformation of the PCR products were carried out as already described.[3]

Second evolution round, random mutagenesis with Mutazyme II

epPCR was carried out with the Genemorph II random mutagenesis kit (medium mutagenic degree, according to kit protocols) over the winner of the first evolution round. The reaction mix contained 2000 ng of the plasmid pJRoC30 with the laccase mutant gene, 5 µl of Mutazyme II buffer, 1 mM dNTPs, 125 ng of each RMLN and RMLC primers (Table S1) and 1 µl of Mutazyme II in 50 µl volume. The amplification was carried out under the conditions aforesaid and purification and transformation of the PCR products were carried out as already described.[3]

Reversion of F454S mutation

Reversion of mutation F454S was carried out using primers pJro30 9H2 F and pJro30 9H2 R combined with RMLC and RMLN, respectively (Table S1). For 50 µl reaction, 5 µl buffer PFU, 3 µl DMSO, 1 mM dNTPs mix, 2.5 µl each primer (0.25 µm), 1 µl PFU polymerase and 100 ng of DNA template were added. PCR was carried out under the following conditions: 95 ºC (2 min), 1 cycle; 94 ºC (30 s), 55 ºC (30 s), 74 ºC (2 min), 28 cycles; and 74 ºC (10 min), 1 cycle. The two purified PCR products were recombined and cloned by *in vivo* overlap extension[4] (IVOE), taking advantage of the DNA recombination machinery of *S. cerevisiae*.

Site directed mutagenesis N263D and N207S

The site directed mutagenesis of N263D and N207S was performed as described in Santiago *et al* 2016.[5]

Saturated mutagenesis of residue 454

Saturated mutagenesis of the position 454 was carried out using degenerated primers 454DFw and 454DRv respectively combined with RMLC and RMLN (Table S1), to obtain two PCR products that were recombined and cloned by IVOE. The PCR protocol and reaction mix was done as for the reversion of F454S.

C-terminal substitution

The ten last amino acids of the C-terminal from 3A4 laccase was extracted from the gen using primers C-terminal3A4Fw and RMLN (Table S1) and the following PCR protocol: 95 ℃ (2 min), 1 cycle; 94 ℃ (30 s), 55 ℃ (30 s), 74 ℃ (30 s), 28 cycles; and 74 ℃ (10 min), 1 cycle. The PK2 laccase gen was extracted without the last 10 amino acids using primers C-terminal3A4Rv and RMLN (Table S1) and the same PCR protocol as for the 1st evolution round. In both cases the above described PFU reaction mixtures were used. Both fragments were recombined and cloned by IVOE.

Enzyme production and purification

Laccase was produced by *S. cerevisiae* 1 L-flask cultures and purified as described before.[5] Laccase activity in the culture was measured spectrophotometrically with 3 mM ABTS in 100 mM citrate-phosphate buffer, pH 3 by the increase of Absorbance 418 nm ($\varepsilon 418 = 36000\ M^{-1}\ cm^{-1}$). One activity unit (U) was defined as the amount of enzyme needed to transform 1 µmol substrate/minute. To estimate enzyme production, enzyme concentration of a purified laccase variant was measured by the A280 (Nanodrop 2000, Thermofisher, USA) and the specific activity (U/mg) was calculated and used to deduce the mg of enzyme/l obtained in the culture.

Enzyme characterization

All characterization assays were performed with purified enzymes.

Thermal stability

T_{50} assays were performed using ABTS as substrate and following already described protocol.[6]

Laccase half-life values at 50, 60, 70 and 80 ℃ and thermal inactivation constants were obtained as shown in Pardo 2018.[7] Activation energies (Ea) were calculated from the Arrhenius plots.

The effect of glycosylation in the thermal stability of the enzyme was evaluated after deglycosylation with Endo-H enzyme (0.5 U/ 5 mg purified laccase in 50 mM sodium acetate buffer pH 5.5, 24 h, 37 ℃, gentle agitation in thermoblock). Glycosylated samples were also incubated for 24 h at 37 ℃ to avoid errors due to the possible stability decrease in the desglycosylated variants after treatment. Enzyme deglycosylation was confirmed by SDS-PAGE electrophoresis (12% acrylamide). Then, 0.2 U/ml of glycosylated and deglycosylated samples were incubated at 65 ℃ for 5 h. Aliquots of 20 µl were taken at different times and its residual activity was measured in microtiter plates, in triplicate, with 3 mM ABTS, pH 3.

Far-UV CD spectroscopy analysis were carried out in a spectropolarymeter Jasco J815 associate to Jasco PTC-4235/15 peltier (JASCO Corporation, Japan). Enzyme samples were diluted to a concentration of 10 µM in 20 mM buffer Tris-HCl pH 7. Denaturalization ramps were set from 50 °C to 95 °C with a slope of 60 °C/h and measured at 220 nm. CD spectra were collected after incubation of the enzyme in a thermoblock at 100 ℃ for 24 h (except for the first sample taken at room temperature). The spectra were obtained between 190 and 250 nm with a scanning speed of 10 nm min^{-1}, using a spectral bandwidth of 1 nm and 0.1 cm path length quartz cell (Hellma, Germany). The protein signal was obtained by subtracting buffer spectrum and represented the average of 5 accumulations.

Optimal pH and pH stability

The pH profiles of the different laccase variants was determined in microtiter plates by adding 10 µl of 0.1 U/ml (20 µl of 4 U/ml in the assay with aniline) enzyme activity measured with ABTS to 180 µl (170 µl for aniline) 0.1 mM Britton and Robinson (B&R) buffer pH range 2-9. Reactions were started immediately by adding 10 µl 60 mM ABTS and DMP (3 mM final concentration) or 10 µl of 300 mM aniline (15 mM final concentration). All reactions were measured in triplicate.

To test enzyme stability to pH, laccase samples were diluted in 2 ml 0.1 mM B&R buffer adjusted to pH 2-9 to attain 0.1 U/ml final activity (with ABTS) and incubated at 25 ℃ during 24 h. Aliquots of each sample (20 µl) were taken at 0, 1, 2, 3, 6 and 24 h and transferred to a microtiter plate to measure the residual activity in the plate reader as shown before. Relative activities were calculated as a percentage of the maximum laccase activity of each variant. All reactions were measured in triplicate.

MALDI-TOF-TOF analysis

MALDI-TOF-TOF analyses of glycosylated and deglycosylated laccase samples were performed on an Autoflex III instrument (Bruker Daltonics, Bremen, Germany) with a smartbeam laser. The spectra were acquired using a laser power just above the ionization threshold. Samples were analysed in the positive ion detection and delayed extraction linear mode. Typically, 1000 laser shots were summed into a single mass spectrum. External calibration was performed, using the Protein Standard II from Bruker, covering the range from 15000 to 70000 Da. The 2,5-Dihydroxy-acetophenone (2,5-DHAP) matrix solution was prepared by dissolving 7.6 mg (50 µmol) in 375 µl ethanol followed by the addition of 125 µl of 80 mM diammonium hydrogen citrate aqueous solution. For sample preparation, 2.0 µl samples were diluted with 2.0 µl 2% trifluoroacetic acid aqueous solution and 2.0 µl matrix solution. Aliquots of this mixture (1.0 µl) were spotted on the 800 um AnchorChip target (Bruker-Daltonics) and allowed to dry at room temperature. The molecular weight analysis by MS-MALDI TOF was carried out in Proteomics and Genomics Facility (CIB-CSIC), a member of ProteoRed-ISCIII network.

Kinetic constants

Kinetic constants for the oxidation of ABTS (ε418 = 36000 M^{-1} cm^{-1}), DMPD (ε550 = 4134 M^{-1} cm^{-1}), DMP (ε470 = 27500 M^{-1} cm^{-1}) and aniline (ε410 = 1167 M^{-1} cm^{-1}) in laccase variants were measured in a microtiter plate, in triplicate. Twenty µl enzyme were added to 50 mM citrate phosphate buffer pH 3 (ABTS, DMPD and aniline assays) or 100 mM sodium acetate buffer pH 5 or 100 mM sodium acetate buffer pH 4 (for DMP assays) to a final 250 µl reaction volume. To calculate K_m and k_{cat} values the average V_{max} was represented versus substrate concentration and fitted to a single rectangular hyperbola function in SigmaPlot (version 10.0) software, where parameter *a* was equal to k_{cat} and parameter *b* was equal to K_m. To calculate K_m and k_{cat} for aniline the average V_{max} was represented versus substrate concentration and fitted to a 3 parameter sigmoidal function in SigmaPlot software, where parameter *a* was equal to k_{cat} and parameter x0 was equal to K_m.

Computational analysis

System preparation

The laccase structure used in this study corresponds to the 7D5 laccase crystal (PDB entry 6H5Y). Mutations in Phe454 were introduced manually using Schrödinger's Maestro and prepared with assistance from Protein Preparation Wizard. Aniline was modelled as an anilinium cation and optimized using Jaguar at the M06 (density functional) with the 6-31G** basis set level of theory; electrostatic potential charges were used for the parameterization.

Aniline activity with different mutations on 454 position

Possible mechanistic explanation for the increase in activity for the four 454-mutated variants was studied by PELE simulations. In these, aniline was initially placed at approximately 10 Å distance to the T1 copper and allowed to diffuse freely. Briefly, the PELE algorithm consists of a combination of perturbation and relaxation phases. In the first, the ligand is randomly rotated and translated, and the protein backbone is perturbed via an anisotropic network model (ANM). Then, side chain prediction techniques are applied to alleviate possible clashes and finally an energy minimization improves the acceptance probability of the Monte Carlo step, which is accepted or rejected according to the Metropolis criterion (refer to [8] for a more detailed explanation of the method).

Five PELE simulations were run in total, one for DM variant (Phe 454) and one for each 454 mutation. Each simulation consisted of an ensemble of 240 trajectories run in parallel, where several metrics were monitored, such as the distance between the N1 atom of aniline and the NE2 atom of His455, the ligand's interaction energy or the substrate's relative solvent accessible surface area.

Role of C-Terminal tail on thermal stability and activity

To study the gain in stability introduced by the four C-terminal mutations to PK2 (obtaining RY2 variant), we run molecular dynamics (MD) simulations at 27 and 67 °C. The simulations were run using OpenMM[9] as the MD engine. Simulations were run using Amber ff14SB for proteins.[10] Four trajectories were run for PK2 and RY2 at 27 and 67 °C, for a total of 16 trajectories, each consisting of 500 ns, with an aggregated simulated time of 8 µs. The initial structures were minimized for 2000 steps using the L-BFGS

optimization algorithm implemented by OpenMM, followed by a 400 ps NVT equilibration at the corresponding temperature with restraints to the protein and ligand heavy atoms of 5 kcal mol^{-1} $Å^{-2}$, and by a final 4ns NPT equilibration at the corresponding temperature and 1 atm with restraints to protein alpha carbons and heavy atoms of 0.5 kcal mol^{-1} $Å^{-2}$. Furthermore, distance constraints between the four copper ions and the coordinating residues were applied, as well as distance constraints between the copper ions in the trinuclear cluster. The 500 ns production simulations were run with a water box set up with 10 Å buffer from the closest solute atom. During all phases of equilibration and production a time step of 2 fs was used, using periodic boundary conditions and PME electrostatics with a cutoff radius of 8 Å.

Application case studies

Synthesis of PANI and acid dye

Aniline (15 mM) polymerization with crude (unpurified) parent type (EM) and the engineered laccase variants DM, PK2 and RY2 was carried out in the presence of 5 mM SDBS in 250 ml flask, with liquid:air ratio of 0.25 under vigorous shaking. Samples were taken at different times and the polymerization was followed by A800 nm increase.

Enzymatic synthesis of PANI performed at SETAŞ AS (Turkey) by the laccase variant engineered in the lab expressed in *Aspergillus oryzae* was carried out in 2 L final volume under the conditions previously described.[11]

Enzymatic synthesis of the acid dye (1 L final volume) was carried out with 125 mM 1-naphthol and 25 mM 1-amino-8-hydroxy-3,6-naphtalenedisulfonic acid monosodium salt in 50 mM Tris HCl pH 8 at 25-30 °C for 24 h using 10 U/ml (activity measured with ABTS) of the engineered laccase or Novozyme 51003 commercial laccase. The synthesized dye was concentrated by nano-filtration equipment (handmade equipment by Setas) using a polyamide membrane (AFC40, PCI membrane) to a final volume of 250 ml.

Dyeing tests

Standard industrial dyeing tests of the enzymatically-synthesized PANI and acid dye were carried out at SETAŞ AS. The CIELAB colour space coordinates (L*, a*, b*) and dyeing efficiency (% STR-WSUM) of the samples were evaluated using a reflectance measuring apparatus Datacolor SF600 plus. Nyloset Brown N2R dye from SETAŞ dye range was used as a reference to evaluate the new acid dye.

Multifibre dyeing tests on acetate, cotton, nylon, polyester, acrylic and wool were carried out with 8% v/v concentrated dyes and 5 g of each textile (1:1 ratio) in 100 mL water containing 0.2 g/l Setacid VS-N, 10 g/l Setalan PM71, 20 g/l Na_2CO_3,. Nyloset Brown N2R was used as a reference dye (Setas). Dyeing was performed for 1 h at 102 °C in an IR Dyeing Machine (Copower Technology, LTD, Taipei Taiwan).

For the PANI and the 1-naphthol derived dye fibre dyeing tests, two different fabrics were used. Acrylic fiber (5 g textile) was dyed using different concentrations (2 ÷ 8% v/v) of the concentrated PANI dye, in 100 mL water containing 0.3-1.0 g/l SetalanIK-200, 0.3-1 % Migrasist ACM, 2 g/l Na_2CO_3 and 1.0-2.0 g/l Hydrosulfide. Dyeing was performed for 1 h at 102 °C in an IR Dyeing Machine. After the first dyeing procedure, the same bath was used to dye another 5 g textile piece (second bath). In the case of the acid dye, nylon fibers (5 g textile) were dyed using different concentrations (2 ÷ 8% v/v) of each concentrated dye (enzyme synthesized dye and Nyloset Brown N2R), in 100 mL water containing 0.2 g/l Setacid VS-N, 10 g/L Setalan PM71 and 20 g/L Na_2CO_3. Dyeing was performed for 1 h at 102 °C in an IR Dyeing Machine. After the first dyeing procedure, the same bath was used to dye another 5 g textile piece (second bath).

The colour fastness of the dyed fabrics were evaluated following established test procedures: ISO 105-B02:1994-Colour fastness to artificial light: Xenon arc fading lamp test (Blue scale 1–8); ISO 105-CO6:1998-Colour fastness to domestic and commercial washing (Grey scale 1–5) and ISO/DIS 105-X12:2001-Colour fastness to rubbing (Grey scale 1–5).

References

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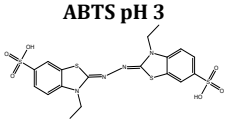
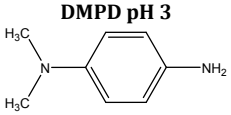
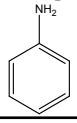
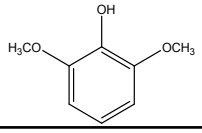
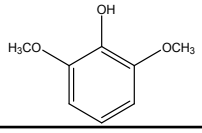
Table S1. Primers used for the engineering of 7D5 laccase.

Primer Name	Sense/Antisense	Sequence 5´→3´
RMLN	Sense	CCTCTATACTTTAACGTCAAGG
RMLC	Antisense	GGGAGGGCGTGAATGTAAGC
pJro30 9H2 F	Sense	TTCCTCCACTGCCACATTGA**TTC**CACCTTGACGCAGGCTT
pJro30 9H2 R	Antisense	AAAGCCTGCGTCAAGGTG**GAA**GTCAATGTGGCAGTGGAGGAA
454DFw	Sense	CACATTGAC**NNK**CACCTTGACG
454DRv	Antisense	CGTCAAGGTG**MNN**GTCAATGTG
C-terminal3A4Fw	Sense	GCAAGCATGGTCGGATCTGTGCCCG
C-terminal3A4Rv	Antisense	CGGGCACAGATCCGACCATGCTTGC

Results

Table S1. Kinetic constants for the oxidation of different substrates by the variants obtained during the evolution of 7D5 laccase. (* Data from Santiago et al 2016).

		EM	DM*	PK2	RY2
ABTS pH 3 	k_{cat} (s^{-1})	291 ± 18	570 ± 26	454 ± 13	543 ± 17
	K_m (mM)	0.004 ± 0.001	0.01 ± 0.002	0.016 ± 0.002	0.01 ± 0.001
	k_{cat} / K_m (mM^{-1} s^{-1})	69254	40975	28350	52240
DMPD pH 3 	k_{cat} (s^{-1})	459 ± 18	741 ± 48	688 ± 36	1126 ± 47
	K_m (mM)	1.72 ± 0.17	1.2 ± 0.2	3.83 ± 0.46	4.59 ± 0.53
	k_{cat} / K_m (mM^{-1} s^{-1})	265	617	179	245
Aniline pH 3 	k_{cat} (s^{-1})	10 ± 1.1	23 ± 3.4	24 ± 0.6	44.6 ± 0.6
	K_m (mM)	28 ± 7.2	59 ± 16	27 ± 0.7	118 ± 1.8
	k_{cat} / K_m (mM^{-1} s^{-1})	0.36	0.38	0.88	0.37
DMP pH 4 	k_{cat} (s^{-1})	105 ± 3.2	-	54 ± 2.8	83 ± 1.4
	K_m (mM)	0.1 ± 0.01	-	1.52 ± 0.24	2.74 ± 0.15
	k_{cat} / K_m (mM^{-1} s^{-1})	1095	-	35	61
DMP pH 5 	k_{cat} (s^{-1})	76 ± 2	-	74 ± 6	123 ± 6
	K_m (mM)	0.05 ± 0.004	-	0.36 ± 0.09	0.26 ± 0.04
	k_{cat} / K_m (mM^{-1} s^{-1})	1519	-	206	479

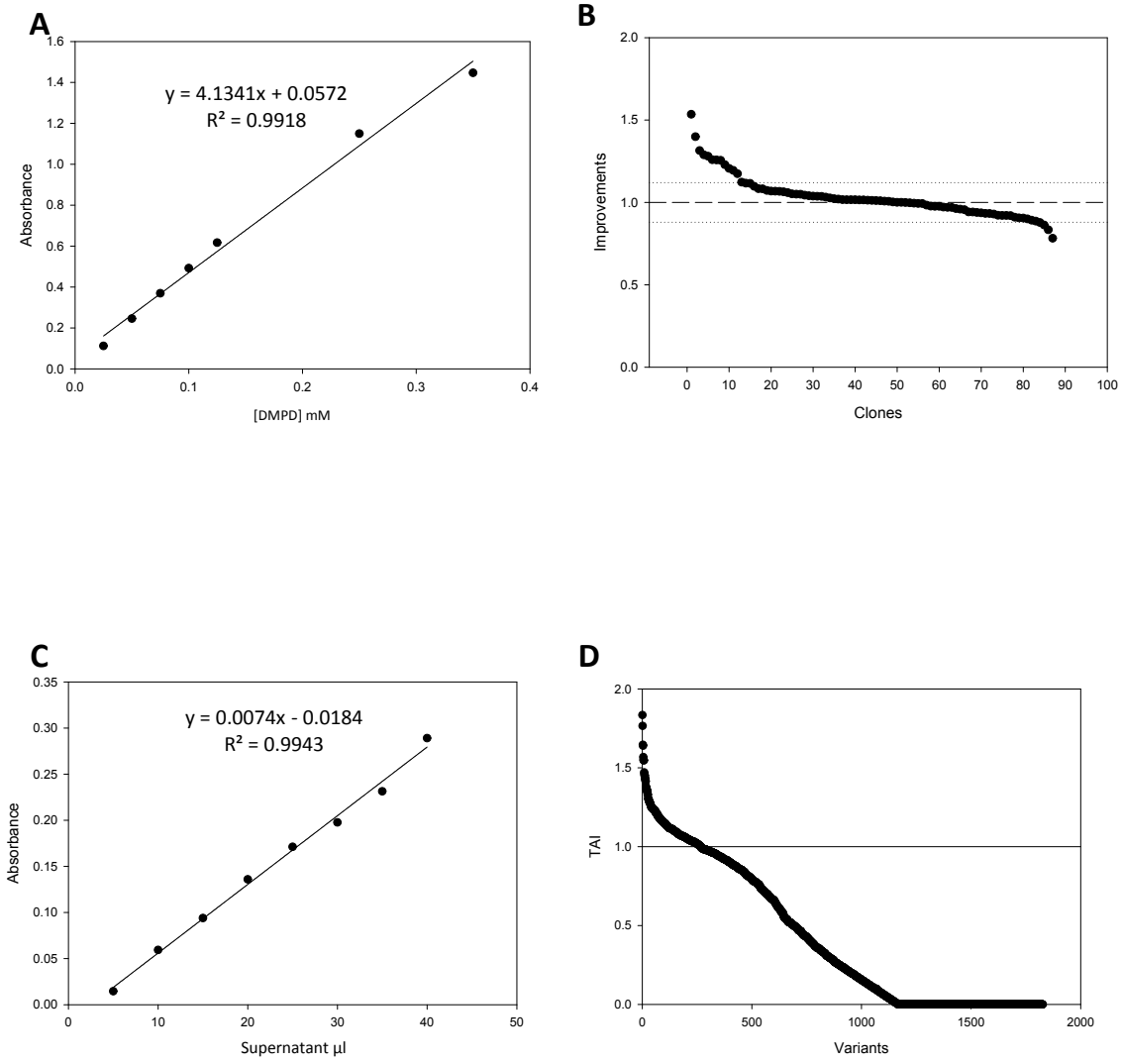


Figure S1. Extinction coefficient for oxidized DMPD -Würsten dye- (**A**) and reproducibility (**B**), linearity (**C**) and sensitivity (**D**) of HTS assay with DMPD.

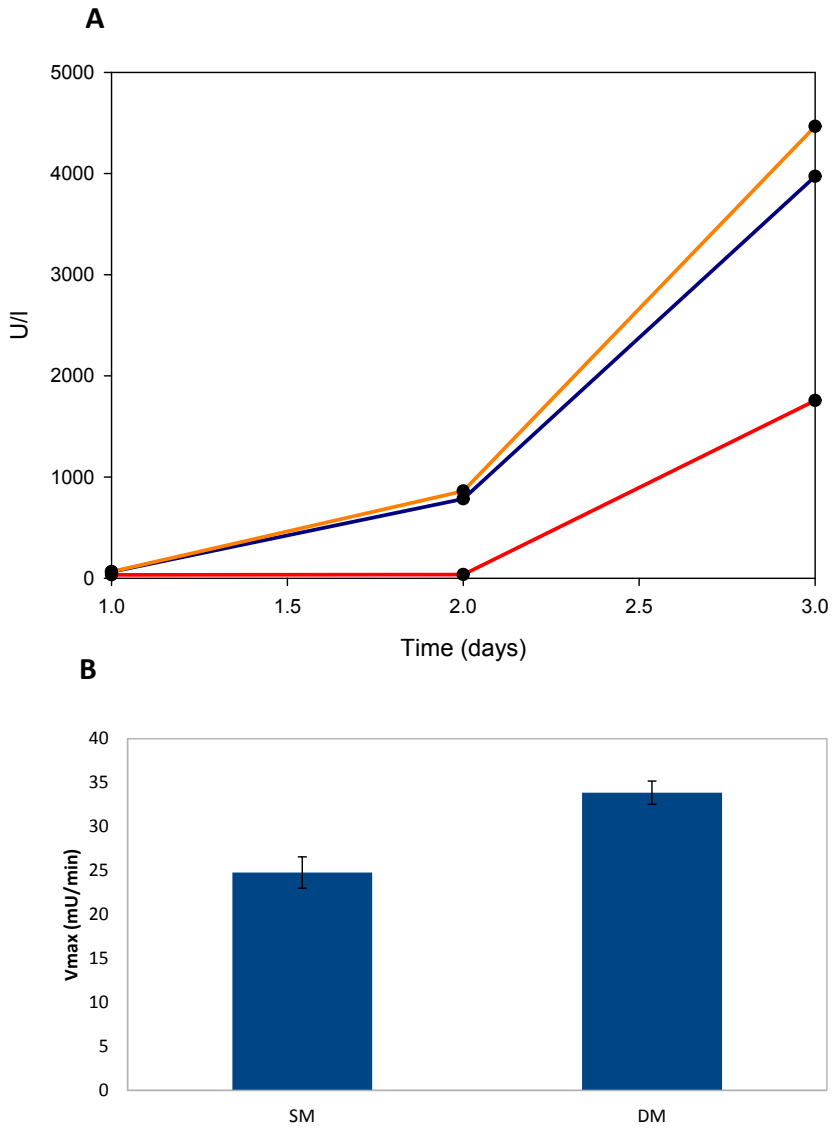


Figure S2. Laccase activity in *S. cerevisiae* flask cultures producing SM (red), EM (blue) or DM (orange) laccase variants (**A**). Oxidation of 300 mM aniline (pH 3) by equal ABTS activity units of SM and DM crude enzymes (**B**).

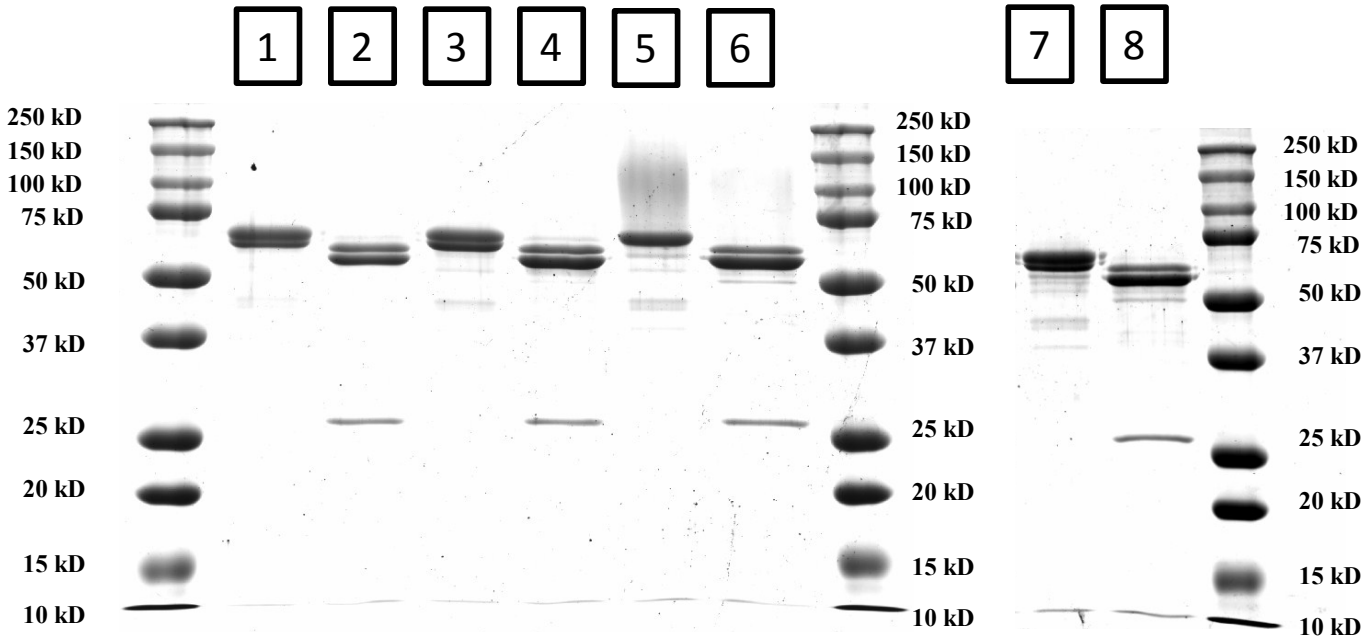


Figure S3. 12 % SDS-PAGE of glycosylated (1) and deglycosylated DM laccase (2), glycosylated (3) and deglycosylated 7D5 laccase (4), glycosylated (5) and deglycosylated RY2 laccase (6) and glycosylated (7) and deglycosylated (8) PK2 laccase. All enzymes were purified.

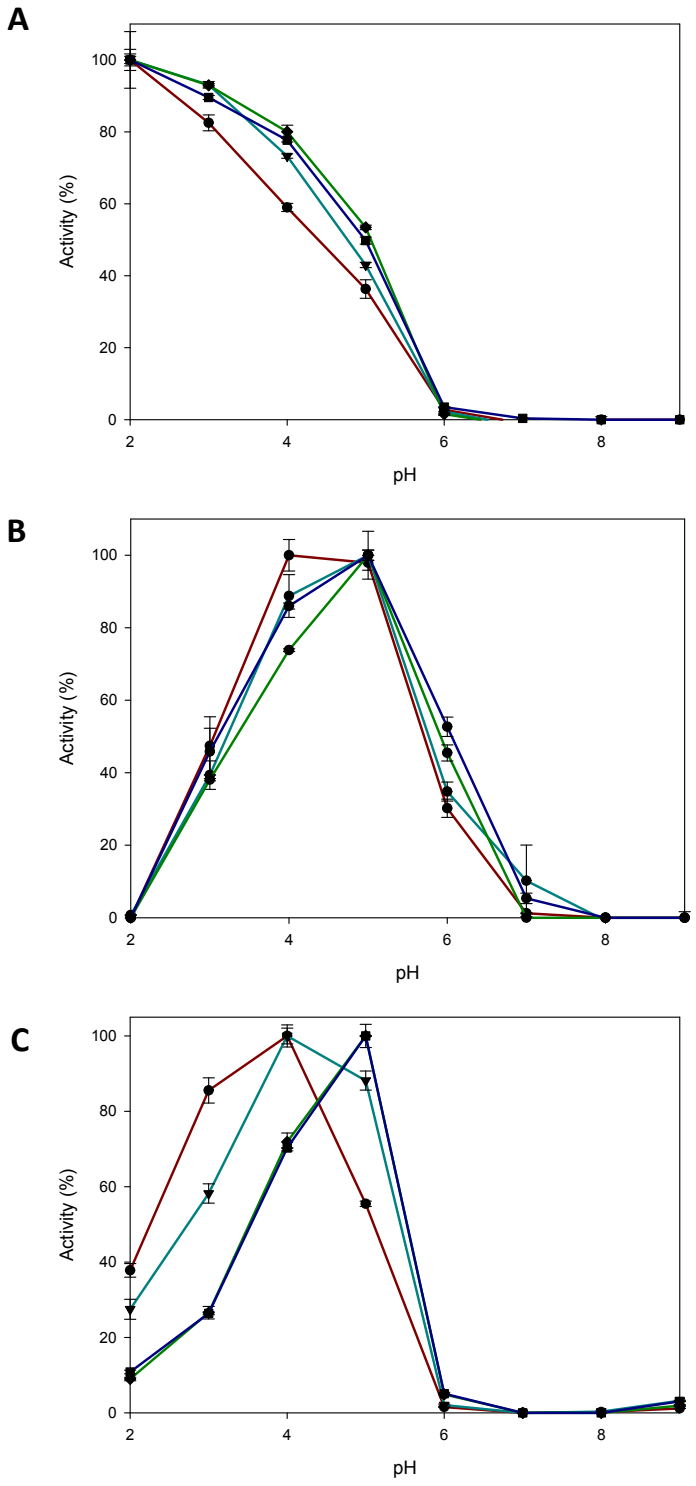


Figure S4. Optimal pH for ABTS (**A**), aniline (**B**) and DMP (**C**) oxidation by the parent type EM (red) and DM (cyan), PK2 (green) and RY2 (blue) variants (purified enzymes).

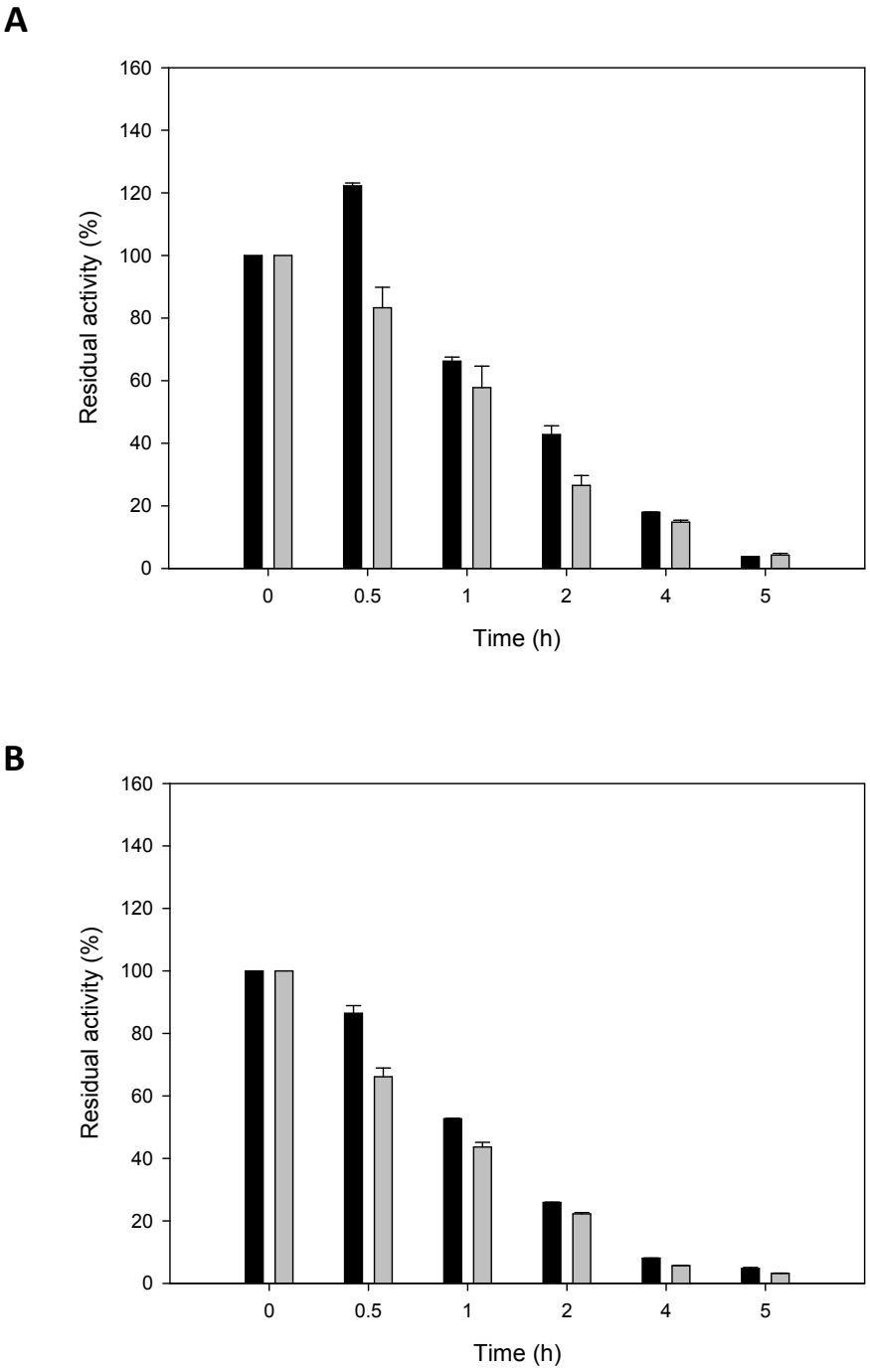


Figure S5. Residual activity of glycosylated (black bars) and Endo-H desglycosylated (grey bars) forms of purified parent type EM (**A**) and final variant RY2 (**B**) after different incubation times at 65 °C .

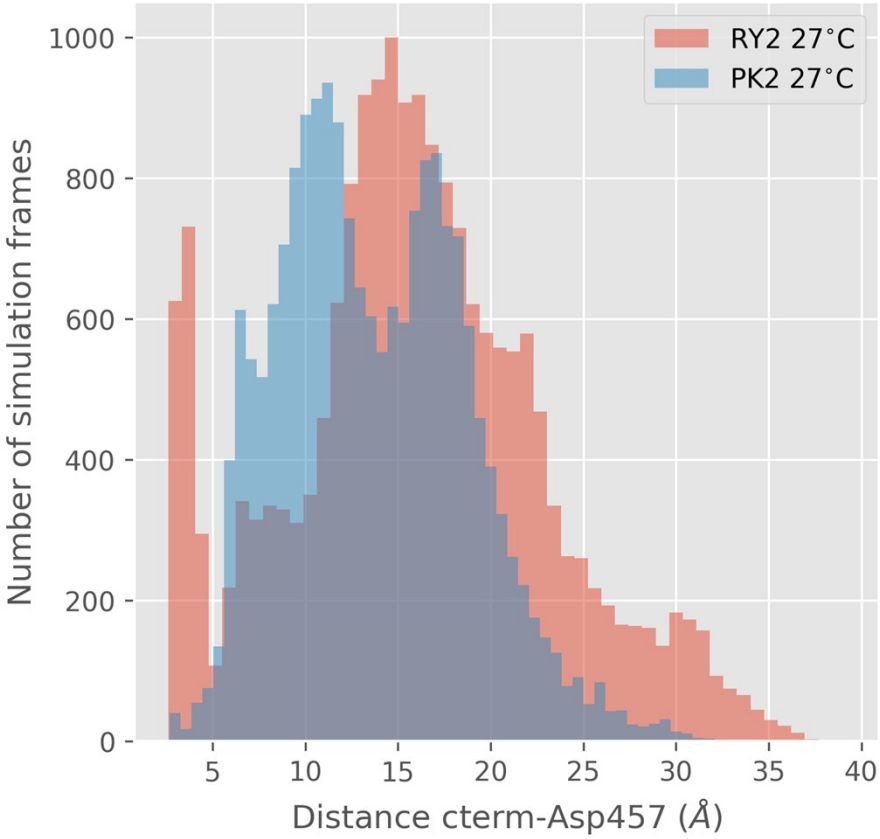


Figure S6. Histogram of the minimum distance between C-terminal residues to the Asp 457 for PK2 and RY2 variants at 27 °C.

A **1**

Standard name:	L*	a*	b*
PANI	52.33	1.46	0.10

Name	% STR-SUM
PANI	100.00
PANI 2nd Bath	8.07

2

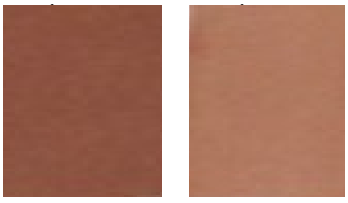
3

B **1**

Standard name:	L*	a*	b*
Engineered laccase	51.13	11.00	11.47

Name	% STR-SUM
Engineered laccase	100.00
Engineered laccase 2nd Bath	55.33

2



3

C

Standard name:	L*	a*	b*
% 0.3 NYLOSET BROWN N	47.11	7.61	4.65

Name	% STR-SUM
% 0.3 NYLOSET BROWN N	100.00
Engineered laccase	87.88
Novozyme 51003	56.02

Figure S7. Dyeing tests of enzymatically synthesized PANI (**A**) and naphtol-derived acid dye (**B**). Dye properties (CIELAB colour space coordinates, L*, a*, b*) and dyeing efficiency (% STR-WSUM) by comparison of first dye bath and second dye bath (**1**); Exhaustion of the bath at the end of the dyeing process (**2**); and Light fastness properties (**3**). Properties of Nyloset Brown commercial dye and comparison of dyeing efficiency (% STR-WSUM) with the acid dye obtained with the engineered or the commercial laccases (**C**).