SUPPLEMENTARY DATA

Rational engineering of the lccβ *T. versicolor* laccase for the mediator-less oxidation of large polycyclic aromatic hydrocarbons

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Site specific mutagenesis

The WT laccase gene (D13372)[6] was synthesized by Genscript (Genscript USA Inc.) and cloned into the vector pPICZαA (Invitrogen). The QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) was used to mutate this WT sequence in the vector pPICZαA by means of the following primers:

F162A/L164A Fwd: GCTTGGTCCAGCTGCTCCTGCGGGAGCAGATGC

F162A/L164A Rev: GCATCTGCTCCCGCAGGAGCAGCTGGACCAAGC

D206N Fwd: GGTTTCACTTAGTTGTAATCCAAACTATACCTTCTCTATTGACGG

D206N Rev: CCGTCAATAGAGAAGGTATAGTTTGGATTACAACTAAGTGAAACC

F332A Fwd: GCTATTAACATGGCCTTTAACGCCAACGGTACTAACTTTTTC

F332A Rev: GAAAAAGTTAGTACCGTTGGCGTTAAAGGCCATGTTAATAGC

The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation) and used to transform DH5α *E. coli* cells. The transformed DH5α cells carrying WT or mutated laccase gene were grown overnight in 5 mL LB medium (10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract) supplemented with 100 µg/mL zeocin at 37 °C and 180 rpm. DNA minipreps were made with a GenElute Plasmid Miniprep Kit (Sigma-Aldrich) with an overnight bacterial culture. DNA quantifications were done with a NanoDrop 2000c (Thermo Scientific). Each mutated sequence was checked by means of DNA sequencing (Microsynth AG, Balgach, CH) and BLAST alignment[7].

***P. pastoris* transformation and optimization of culture conditions for heterologous laccase expression**

The WT and mutated laccases were produced and purified from *P. pastoris* to measure their activity experimentally. To this aim, yeast competent *P. pastoris* X33 cells were grown in 250 mL Yeast Peptone Dextrose (YPD, 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) at 30 °C and 200 rpm. The pPICZαA vectors carrying the laccase genes (5-20 µg/µL) were digested with PmeI (New England Biolabs Inc.), purified with the Wizard SV Gel and PCR Clean-Up System and resuspended in dH_2O to avoid salt failure during electroporation. The cleaved DNA was used to transform the competent cells (40-80 µL of cells with 0.5-2 µL of linearized DNA) by electroporation (25 µF, 200 ohm, 1.5-2 kV) with a GenePulser apparatus (Bio-Rad Laboratories Inc.). Immediately after electroporation, 1 mL of ice-cold 1 M sorbitol was added to the cuvette. The content was then transferred to a sterile 15 mL tube and incubated for 2 hours at 30 °C without shaking. Afterwards, the electroporated cells were spread onto Yeast Peptone Dextrose Sorbitol (YPDS) agar plates (10 g yeast extract, 20 g peptone, 20 g glucose, 186 g sorbitol, 15 g agar) supplemented with 100 µg/mL zeocin and incubated at 30 °C for 72-96 h.
Yeast transformants (carrying WT and mutated laccase gene) were selected on Minimal Methanol (MM) agar plates supplemented with copper and ABTS (MM/Cu/ABTS, 13.4 g/L YNB, 0.5% (v/v) methanol, 0.4 mg/L biotin, 0.3 mM $CuSO_4$, 0.2 mM ABTS, 15 g/L agar) to determine the phenotype (Mut^S or Mut^+) and screened for production of laccase via a simple colorimetric assay based on the chromogenic substrate ABTS. Briefly, colonies were patched on Minimal Dextrose (MD) agar plates (13.4 g/L Yeast Nitrogen Base (YNB), 20 g/L glucose, 0.4 mg/L biotin, 15 g/L agar) and on MM/Cu/ABTS agar plates. Afterwards, the growth of the colonies was compared. Interestingly, the green halos on the plates, indicative of laccase activity, had different size and color intensity for the various mutants (Figure S1), suggesting that a rough estimation of the enzymatic activity of mutated laccases can be made from the size and intensity of halos on the MM/Cu/ABTS agar plates and from the time they appear. The colonies that produced the largest green halo (indicative of the secretion of active laccase) were chosen.

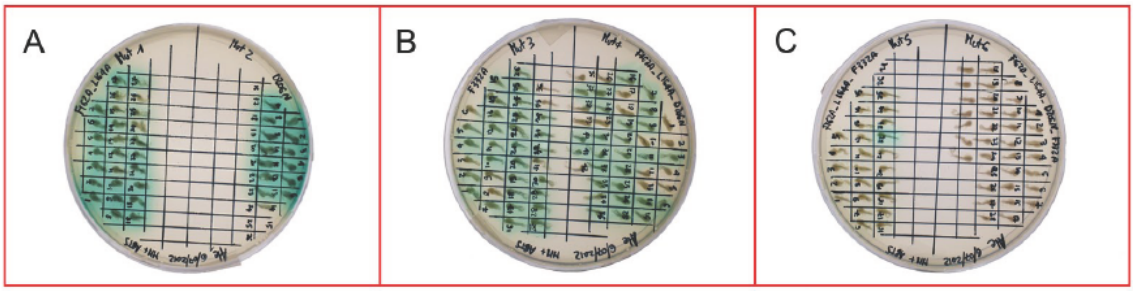


Figure S1. Selection of yeast transformants carring the laccase variants on MM/Cu/ABTS agar plates.

Laccase production was verified by growing the transformants (Mut^+) in 250 mL flasks and analyzing the supernatant from methanol-induced cultures by enzyme activity assays (see below), SDS-PAGE (10% (w/v), under reducing and denaturing conditions) and in-gel activity assays (zymogram obtained by developing the SDS-PAGE by using 0.4 mM ABTS in 50 mM acetate buffer, pH 5.0). The best laccase producers (WT and mutants) were grown in 50 mL Buffered Glycerol-complex Medium (BMGY, 10 g/L yeast extract, 20 g/L peptone, 0.1 M sodium phosphate buffer (NaP) pH 6.0, 10% (v/v) glycerol, 0.4 mg/L biotin) to an optical density (OD) of ~1.0 at 600 nm (OD_{600}) (Biophotometer, Eppendorf AG) of 20. Then, the biomass was recollected by centrifugation, washed twice with phosphate buffer saline pH 7.4 (PBS) (Gibco®, Life Technologies) and resuspended in 7.5 mL BMGY and 2.5 mL of glycerol. This cells stock solution was dispensed, frozen in a liquid nitrogen bath and stored at -80 °C.

A *P. pastoris* transformant with high expression of the WT *T. versicolor* lccβ was selected and cultured to find the most suitable conditions for the expression of this enzymatic isoform in flask cultures. The effect of copper concentration (0-0.5 mM), pH control (buffered or unbuffered culture medium), recovery time (12-96 hours), temperature (20-28 °C), methanol concentration (0.5-2% (v/v)) and feeding time (once or twice per day) on laccase expression level was sequentially studied. The activity of the supernatants was evaluated over time for each different condition and used to compare the results. A wild type X33 strain transformed with an empty pPICZαA vector was also included in these experiments as a control. Regarding copper, cultures with 0, 0.3 and 0.5 mM of $CuSO_4$ were set up in the buffered BMM medium and cultured at 30 °C. The higher activity value (5.69 ± 0.07 UI/L) was shown at the end of the test (72 hours of induction) in the culture supplemented with 0.3 mM of $CuSO_4$. On the contrary, cultures containing 0 and 0.5 mM of copper reached a maximum value of 3.49 ± 0.09 and 5.27 ± 0.12 UI/L, respectively. No laccase activity was detected in *Pichia* cultures with empty pPICZαA vector. These results confirm that Cu^{2+} is required during laccase production to increase the enzymatic activity [8,9]. However, the biomass concentration of cultures decreased slightly (30-35%) as the copper concentration increased, as previously reported by Hong and co-workers [10], especially if $CuSO_4$ was already present during the preculture phase. For this reason, the copper was omitted in the preculture medium, differently from previous literature [10,11].

In order to study the effect of pH on laccase expression, the WT transformant was precultured in BMGY and then induced at 30 °C in BMM (pH 6.0) or in MM media, both containing 0.3 mM $CuSO_4$, and the enzymatic activity of culture supernatants was daily assayed. A higher activity was determined in BMM medium: at 48 and 72 hours of induction, the measured value were almost double in comparison with those obtained with the MM medium (Figure S2-A). Furthermore, at the end of the test (72 h), the whole cultures (MM and BMM) were centrifuged and the enzyme was partially purified by a single Hi-trap step. The activity of the purified fractions revealed higher values for BMM laccase (26.65 ± 0.13 UI/L, 0.23 ± 0.01 UI/mg), in comparison with values obtained from the MM culture (16.49 ± 0.09 UI/L, 0.17 ± 0.01 UI/mg). The better results obtained with a buffered medium are not surprising. The pH of the BMM culture at 48 h was near to 6.0, while it was in the acidic range in the MM culture. Guo and collaborators reported that the optimal pH for laccase production is 6.5, while very little laccase activity is produced at pH 4.0, 4.5 and 5.0 [12]. This effect could be due to the presence of proteolytic activity, higher at pH 5.0 than at pH 6.5 [10], or to an inhibitory effect of the acidic pH on the yeast protein expression system. Therefore, for the following expression tests the BMM medium was used. Once the culture

medium was chosen, the optimal time point for enzyme recovery was investigated. The culture supernatants were daily assayed for 96 hours, and at the end of the test it was clear that the highest amount of active laccase was reached around 72-84 hours after induction (Figure S2-B).

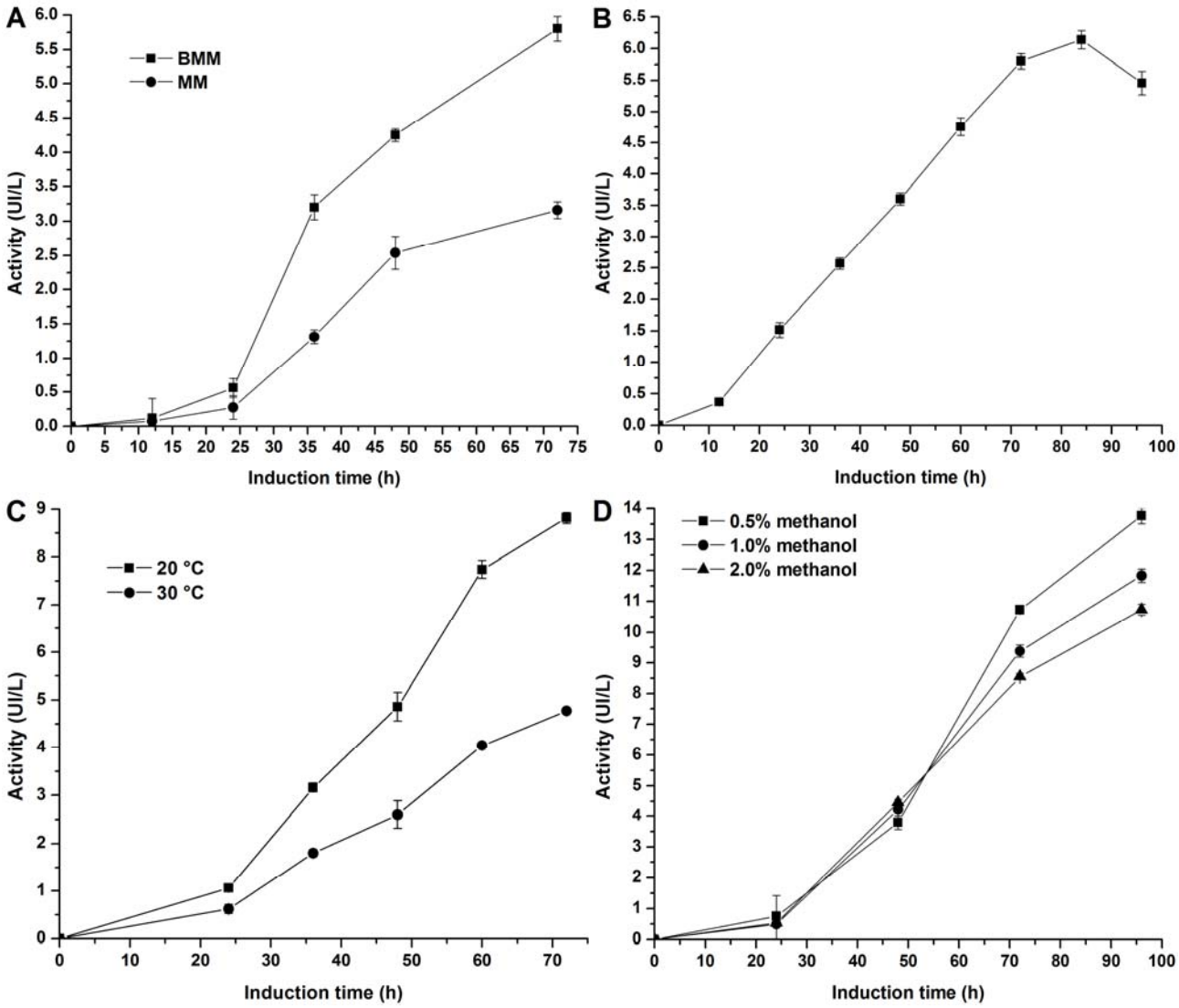


Figure S2: A) Laccase activity of supernatants of cultures operated in BMM and MM media. B) Evaluation of the optimal time of recovery after methanol induction of cultures conducted in BMM. C) Laccase activity of supernatants of cultures conducted at 20 and 28 °C. D) Laccase activity in supernatants of cultures daily fed with 0.5-2.0% (v/v) methanol.

In order to find the optimal temperature for laccase expression, the WT was grown at 30 °C in BMGY medium (preculture phase), then, 50 mL of biomass was transferred to BMM medium containing 0.3 mM $CuSO_4$ (induction phase) and cultured at 20 or 28 °C. During the whole tests the enzyme activity was almost double in the culture maintained at 20 °C in comparison with the one kept at 28 °C (Figure S2-C), even if a slower growth was recorded for the former (data not shown). The protein content was very similar in both temperature conditions, but the cultures kept at 20 °C provided about two-fold specific activity than those kept at 28 °C. Therefore, it can be concluded that 28 °C is more beneficial for the growth of the yeast cells than 20 °C, but not for obtaining high laccase specific activity, which is improved by a lower culture temperature. The mechanisms behind the influence of temperature should be related as well to a higher amount of proteases released from dead cells, combined with poor stability, aggregation and folding problems at temperature higher than 20 °C. Other authors reported the improvement of *T. versicolor* laccase expression in *S. cerevisiae* by lowering the temperature (Cassland and Jönsson, 1999; Hong et al., 2002; Zhang and Meagher, 2000).

Various papers showed that methanol could negatively affect the production of active heterologous laccase [10,12], even if the Invitrogen guidelines recommend to induce the protein expression by daily feeding methanol up to 1% (v/v) for Mut^S strains and up to 3% (v/v) for Mut^+ [13], without any detrimental effect. Indeed, an accurate regulation of methanol concentration in *P. pastoris* cultures is mandatory not only to regulate the gene expression, but also to prevent the raise of methanol to levels that are harmful to the cells [13]. A fed-batch strategy is frequently used to obtain high cell density cultures while keeping a low level of methanol. In order to identify the optimal methanol concentration for laccase expression, diverse feeding strategies have been tested: methanol 0.5, 1.0, 2.0% (v/v), every 12-24 hours. In Figure S2-D the laccase activities measured from supernatants of cultures fed daily with different methanol concentration are reported. All the tested concentrations (0.5-2.0% (v/v)) allowed the yeast to grow regularly and without any significant difference in cell growth, ensuring that the microorganism was not inhibited. Furthermore, at 72 h of induction, the laccase activity in the supernatants were 10.73 ± 0.08, 9.39 ± 0.04 and 8.55 ± 0.04 UI/L for 0.5, 1.0 and 2.0% (v/v), respectively. Hence, at the end of the test (96 h) the whole cultures were centrifuged and the supernatant was roughly purified by a single Hi-trap step. The activity (UI/L) and specific activity (UI/mg) of the eluted fractions revealed the following values for the three methanol feeding strategies: 0.5% (v/v) (68.75 ± 0.15 UI/L, 0.24 ± 0.01 UI/mg); 1.0% (v/v) (62.2 ± 0.12 UI/L, 0.28 ± 0.02 UI/mg); 2.0% (v/v) (44.0 ± 0.10 UI/L, 0.22 ± 0.01 UI/mg). A similar trend was obtained by feeding the cultures every 12 hours with the same range of methanol concentrations (data not shown), but the highest laccase activity value was achieved by the culture daily fed with 0.5% (v/v) methanol. Therefore, this feeding strategy was adopted for the following tests, including the expression of mutants.

Overall, the optimization process resulted in 89% increased enzymatic activity and showed that in flask cultures the WT Iccβ laccase is ideally produced at 20 °C in BMM pH 6.0 containing $CuSO_4$ 0.3 mM and daily supplemented with methanol 0.5% (v/v); the highest yield of active enzyme was obtained after 72 hours of induction. Temperature and methanol feeding strategy were the most important parameters that control the expression of Iccβ laccase in *P. pastoris*, as summarized in Table S1.

Table S1: Optimization of the expression of the WT laccase in P. pastoris (sampling time: 72 hours)

Parameter	Optimal Condition	Enzymatic activity [UI/L]	Specific enzymatic activity [UI/mg]	Increase of enzymatic activity [%]*
$CuSO_4$	0.3 mM	5.69 ± 0.07	0.03 ± 0.002	0
pH	pH 6.0 (BMM medium)	5.80 ± 0.09	0.03 ± 0.003	1.93
Recovery time	72 h	6.13 ± 0.04	0.04 ± 0.002	7.73
Temperature	20 °C	8.81 ± 0.11	0.08 ± 0.001	54.83
Feeding strategy	Me-OH 0.5% (v/v) /24 h	10.73 ± 0.03	0.08 ± 0.002	88.57

* Increase of enzymatic activity in comparison to the conditions in the first row.

All the mutants were subsequently produced and purified with the conditions established for the WT enzyme; the growth curves of all the yeast cultures did not differ significantly from those of the WT protein (data not shown).

After expression, the enzymes were recovered from supernatants and purified through affinity and size exclusion chromatography. The molecular weight of the purified recombinant laccases was estimated as ~85 kDa by SDS-PAGE and ~100 kDa by size-exclusion chromatography, in agreement with values previously reported[14].

Preliminary decolorization tests with a commercial WT *T. versicolor* laccase

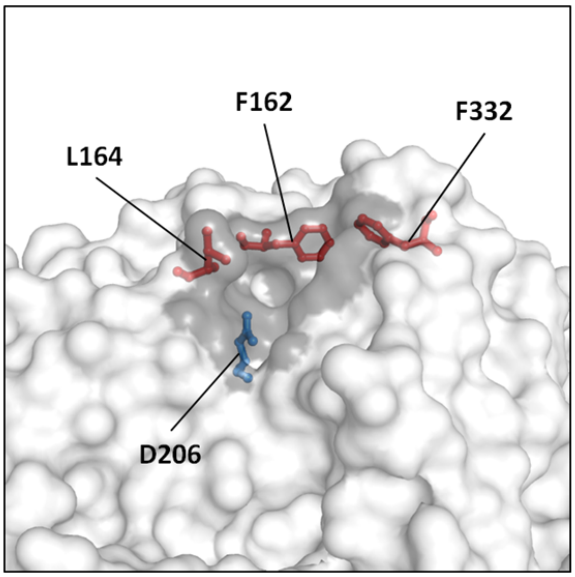
Table S2. Decolorization activity of the WT T. versicolor laccase towards selected dyes listed by structural class.

Dye	Class	MW [g/mol]	Max OD λ [nm]	Tested µM concentration	Decolorization*
Sudan black B *C.I. 26150 (SBB)*	Di-Azo	456.54	600	1,000	No
Ponceau S *C.I. 27195 (PS)*	Di-Azo	760.57	500	26.3	No
Eosin Y *C.I. 45380 (EY)*	Xanthene	691.85	500	23.1	No
Alcian blue 8G *C.I. 74240 (AB)*	Copper Phthalocyanine	1,298.86	580	577.4	No
Coomassie brilliant blue R250 *C.I. 42655 (CBBR)*	Triphenylmethane	825.97	600	12,107	B
Xylene cyanol *C.I. 42135 (XC)*	Triphenylmethane	538.61	600	28.5	B
Ethyl green *C.I. 42590 (EG)*	Triphenylmethane	653.24	600	20	B
Safranin O *C.I. 50240 (SO)*	Phenazine	350.84	500	74	B
Toluidine blue *C.I. 52040 (TB)*	Phenothiazine	270.374	660	31.3	B
Methylene blue *C.I. 52015 (MB)*	Phenothiazine	319.85	675	25	B
Poly R-478 *C.I. / (R-478)*	Polymeric	n.d.	520	25	B
Bromophenol blue *C.I. / (BPB)*	Brominated phtalein	669.96	600	271.3	A, B
Hematoxylin *C.I. 75290 (HT)*	Natural (Flavone)	302.28	500	306.2	A, B, C
Remazol brilliant blue R *C.I. 61200 (RBBR)*	Anthraquinone	626.54	600	1,596	A, B

*A: dye alone; B: HBT 1 mM; C: Glycine 1 mM

The decolorization of a selection of dyes belonging to different structural classes was evaluated with a commercial *T. versicolor* laccase (Table S2). These dyes, responsible for serious environmental pollution, were tested at different pH (3.0-6.0), with or without a mediator (HBT, glycine, cysteine or imidazole). The WT laccase was not able to decolorize Azo, Xanthene and Phthalocyanine dyes (SBB, PS, EY and AB) in none of the tested conditions, even in the presence of the mediators. The decolorization of mono-Azo and di-Azo dyes has been extensively confirmed in *Phanerochaete chrysosporium* [1] and *Trametes versicolor* [2]. However, these fungi express not only laccase, but also many different ligninolytic enzymes, such as peroxidases.

The WT was able to decolorize all the other tested dyes. SO, XC, TB, MB, CBBR, EG and R-478, were transformed only if 1 mM HBT was present in the reaction mixture, while BPB, HT, and RBBR were also oxidized by the enzyme alone. The optimal pH was always in the acidic range (3.0-5.0), confirming previous results [3] and HBT gave better results if used as a mediator. HT turns from dark blue to pale yellow also if 1 mM glycine was used as a mediator instead of HBT, probably because of the low pH of glycin, whereas cysteine was found to be useless as a mediator, differently from what previously reported [4,5].



	F162	L164	F332	D206
M1	A	A	wt	wt
M2	wt	wt	wt	N
M3	wt	wt	A	wt
M4	A	A	wt	N
M5	A	A	A	wt
M6	A	A	A	N

Figure S3: Laccase binding pocket (grey) of the WT laccase (displayed as white surface) showing the residues changed by rational design (red and blue sticks). The mutations are indicated in the aside table.

Further comparison between the activity of the mutants towards phenolic and non-phenolic compounds

A comparison between ABTS and 2,6-DMP, given by calculating the activity ratio (ABTS/2,6-DMP, Figure S3), indicates that the WT showed similar specific activity values for both substrates (ratio of about 1), but in the reaction mixture a ten-fold higher 2,6-DMP concentration was used. This result agrees with Jolivalt et al., that determined ten-fold higher K_M values for 2,6-DMP than for ABTS [15]. Concerning mutants, they retained a higher reactivity against ABTS in comparison to 2,6-DMP. M1 has a specific activity of 10.21 ± 0.02 UI/mg against ABTS, and of 7.05 ± 0.13 UI/mg against 2,6-DMP at pH 5.0. This effect is even enhanced by D206N mutants (M2, M4, M6), which presented the greater change in substrate specificity. This result is probably due to the different oxidation mechanisms of these substrates, as far as for a phenolic substrate such as 2,6-DMP a proton transfer from the substrate is required by the reaction, in contrast to the non-phenolic substrate ABTS [16].

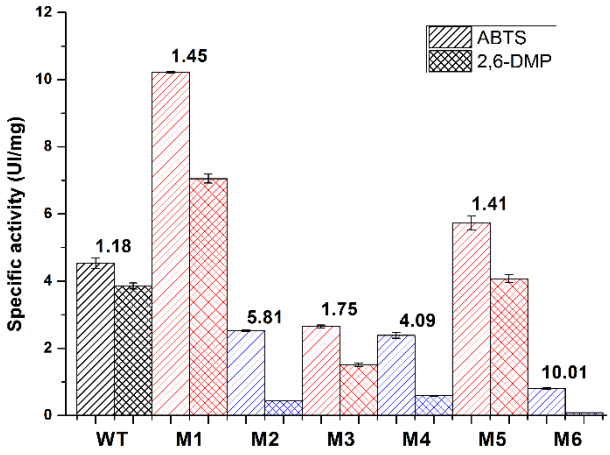


Figure S4: Direct comparison of the enzymatic activity of WT and mutated enzymes against ABTS and 2,6-DMP at pH 5.0. WT laccase is in black; mutants carrying the D206N substitution are in blue, those with a larger binding pocket are in red. The ABTS/2,6-DMP activity-ratio is reported on top of the column bars.

It is instructive to compare the results of computational simulations to the experimentally measured enzymatic activity (Figure S4), keeping in mind that computational docking can only estimate the free energy of binding (related to binding affinity) for each ligand/enzyme complex. The algorithm predicted that ABTS and 2,6-DMP would bind more strongly to M1 than WT laccase (Table 1); experimental assays showed that M1 has also increased activity. Similarly, M2 and M3 has calculated impaired binding and measured impaired activity. M5

has both similar binding and similar activity to WT. Therefore, the experimental specific activity qualitatively agrees with the estimated free energy of binding calculated during computational docking. There was a sharp contrast, instead, between the estimated binding (stronger than WT) and measured activity (smaller than WT) of M6 with both substrates. This may simply point to a lack of accuracy in the simulated ΔG calculations. However, binding affinity is not necessarily correlated with activity. A ligand may bind strongly to the binding pocket but sit in an orientation not suitable to chemical activity due to the lack of specific contacts between catalytic residues and ligand.

Overall, the obtained results encourage further use of computational simulations to design rational protein mutants but also recommend to always experimentally verify the outcomes.

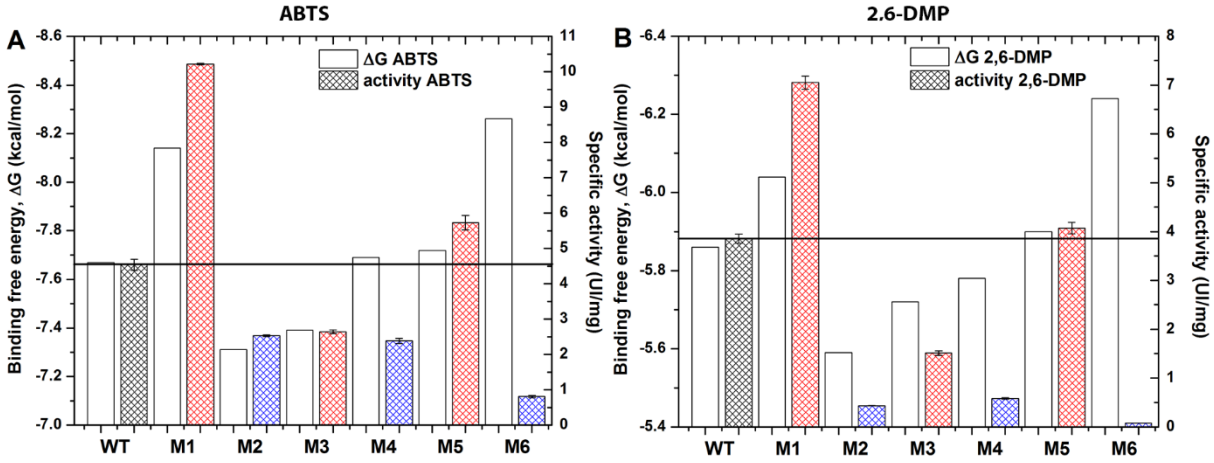


Figure S5: Comparison between the free energy of binding estimated during computational docking (white bars) and the experimental enzymatic activity of WT and mutants (shaded bars) against ABTS (A) and 2,6-DMP (B) at pH 5.0 (maximum WT activity). The black line represents the WT threshold value.

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