Supporting Information (SI)

A loop engineering strategy improves laccase lcc2 activity in ionic liquid and aqueous solution

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1. Analysis of FoldX calculations of ΔΔG upon in silico SSM of loop L1 (amino acids 284-320) in lcc2

In order to explore the best positions on loop region, we have exchanged to all possible 20 amino acids in each position of loop L1 (amino acids 284-320) of lcc2 in two different ionic strengths (0.05 and 0.5 M). **Figs. S1** and **S2** show the distribution of ΔΔG upon *in silico* SSM of each position. As can be seen in **Fig. S1** we found that the amino acid positions 285, 310, 312, and 318 lead to stablizing substitutions in comparison to lcc2 WT. In addition, comparison of **Figs. S1** and **S2** shows that generally mutations in loop are more stabilized in ionic strengths 0.5 than 0.05 M.

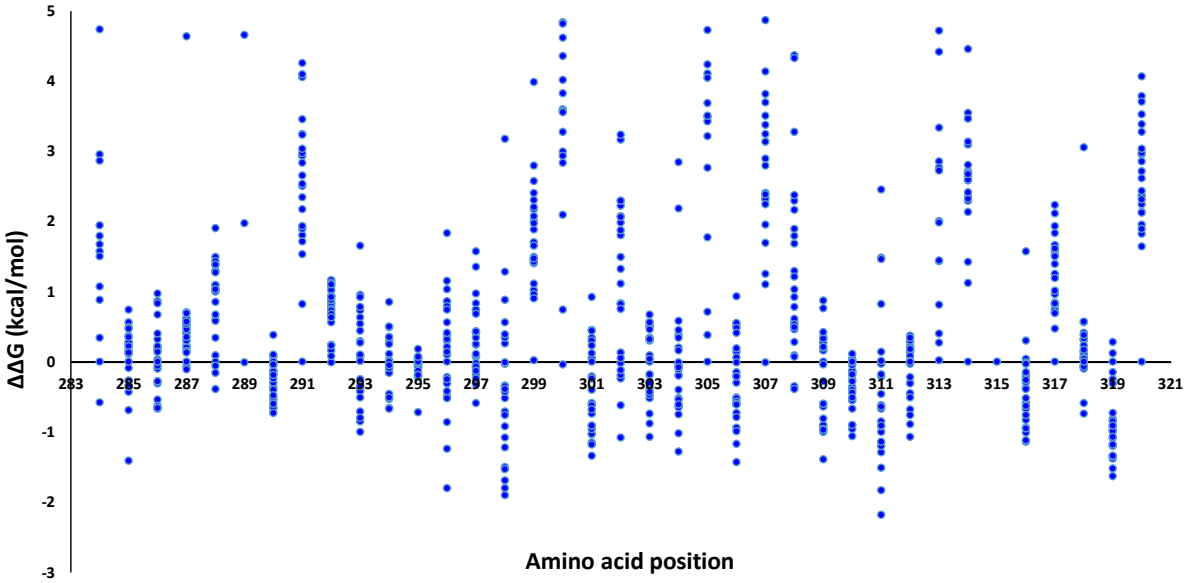

Fig. S1: Calculated stabilization energy (ΔΔG) in kcal/mol for 3 independent runs of lcc2 WT loop variants (amino acid positions 284-320) with respect to lcc2 WT by using the FoldX method ; ΔΔG=ΔG(variant) – ΔG(WT). Ionic strength= 0.05 M. Values above 5 kcal/mol were not shown.

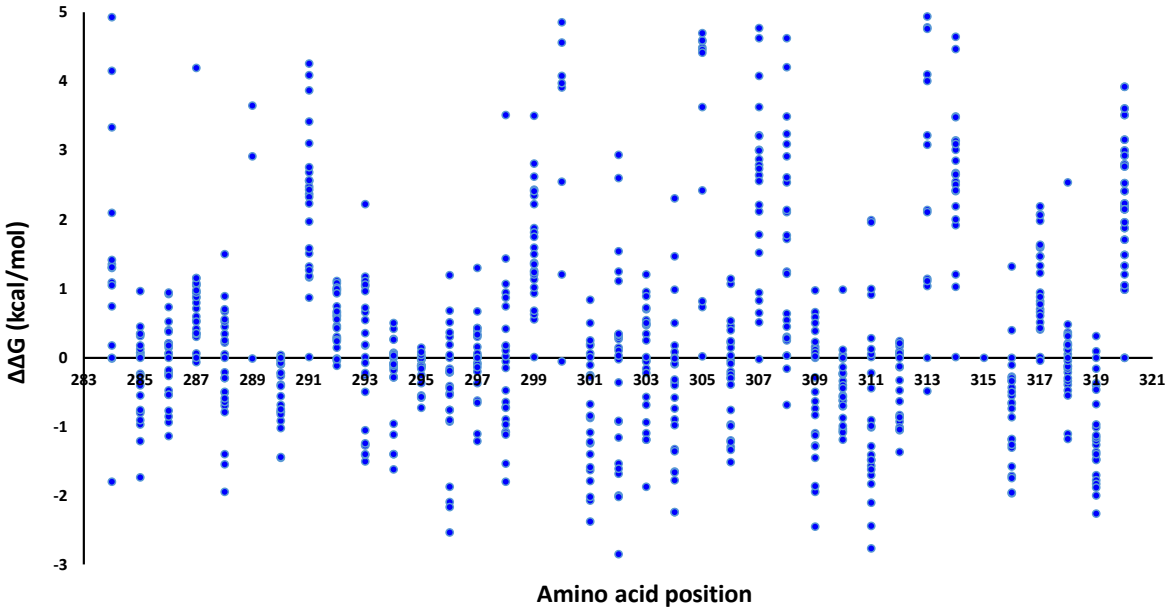

Fig. S2: Calculated stabilization energy (ΔΔG) in kcal/mol for 3 independent runs of lcc2 WT loop variants (amino acid positions 284-320) with respect to lcc2 WT by using the FoldX method [1] ; ΔΔG=ΔG(variant) – ΔG(WT). Ionic strength= 5 M. Values above 5 kcal/mol were not shown.

2. Media for agar plate and competent cells recovery

Table S1: SC-U medium composition, *[1]: adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan, *[2]: aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine

SC-U medium	
Agar	15 g/L (optional)
Amino acids-1*[1]	0.1 g/L
Amino acids-2*[2]	0.05 g/L
Glucose	2 g/L
Yeast nitrogen base without amino acids	6.7 g/L

3. Media for preculture and expression

*10*x Hartwell's complete (HC) dropout solution (1000 mL): 200 mg L-Met, 150 mg L-Trp, 500 mg L-Ile, 500 mg L-Phe,1000 mg L-Thr, 500 mg L-Asp, 500 mg L-Val, 500 mg L-Ser, 200 mg L-

Arg, 1000 mg L-Leu, 200 mg L-His and 150 mg Ade. 10 × YNB stock solution (1000 mL): yeast nitrogen base (without amino acids, 57.1 g), ammonium sulfate (7.4 g). Optimized HC medium for preculture (preculture medium, 1000 mL): 200 ml 10 % (w/v) sterile raffinose, 100 ml 10 × YNB, 100 ml 10 × HC dropout solution, 100 ml 1 M KPi-buffer (pH 6.2). Optimized HC medium for expression (expression medium, 1000 mL): 100 ml 10 % (w/v) sterile galactose, 100 ml 10 × YNB stock solution, 100 ml 10 × HC dropout solution, 100 ml 1 M KPi-buffer (pH 6.2), 2 ml sterile 0.25 M $CuSO_4$.

4. *Long-term resistance of lcc2 WT, OM1, and OM3 towards EMIM $EtSO_4$*

Lcc2 variants (OM1, and OM3) were incubated in presence of 10% (v/v) EMIM $EtSO_4$ (0.11 µM laccase, 0.1 M KPi buffer, pH 6.2) at RT. The $R_{IL/B}$ was determined at regular time intervals for five days. 20 µl incubated enzyme was mixed with 80 µl assay buffer, 30 µl 10 mM ABTS, 20 µl EMIM $EtSO_4$ (10 v/v % reaction system) and 50 µl ddH_2O. Absorbance at 420 nm was immediately measured continuously for 120 min. The relative activity of variants compared to the activity of lcc2 WT in 10% (v/v) EMIM $EtSO_4$ at 0 h is shown in **Fig. S3**.

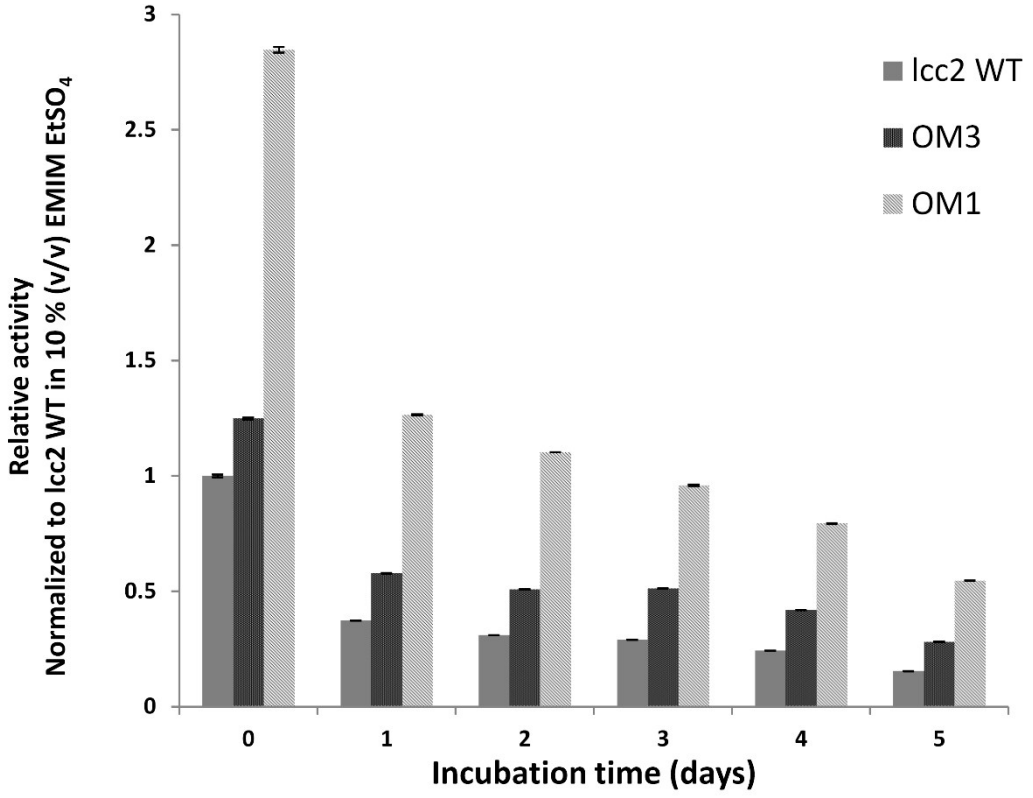

Fig. S3 Long-term IL resistance of lcc2 WT and OmniChange variants OM1 and OM3. The relative activity of lcc2 WT, OM1 and OM3 (normalized to the activity of lcc2 WT in 10%

(v/v)) was determined via the ABTS assay after incubation in presence of 10% (v/v) EMIM $EtSO_4$ over 5 days in triplicates.

5. Thermal resistance of lcc2 WT, OM1, and OM3

The thermal resistance of lcc2 WT, OM1, and OM3 was determined by activity measurements after heat treatment. All variants were incubated at a range of five temperatures (25 to 60°C) for 30 min. Afterwards, the residual activity was determined with ABTS assay. The relative activity in (%) was calculated by the ratio of activity at 25°C divided by activity after incubation at elevated temperatures. The T_m is defined as the temperature at which only 50% of the activity remains upon heat treatment.

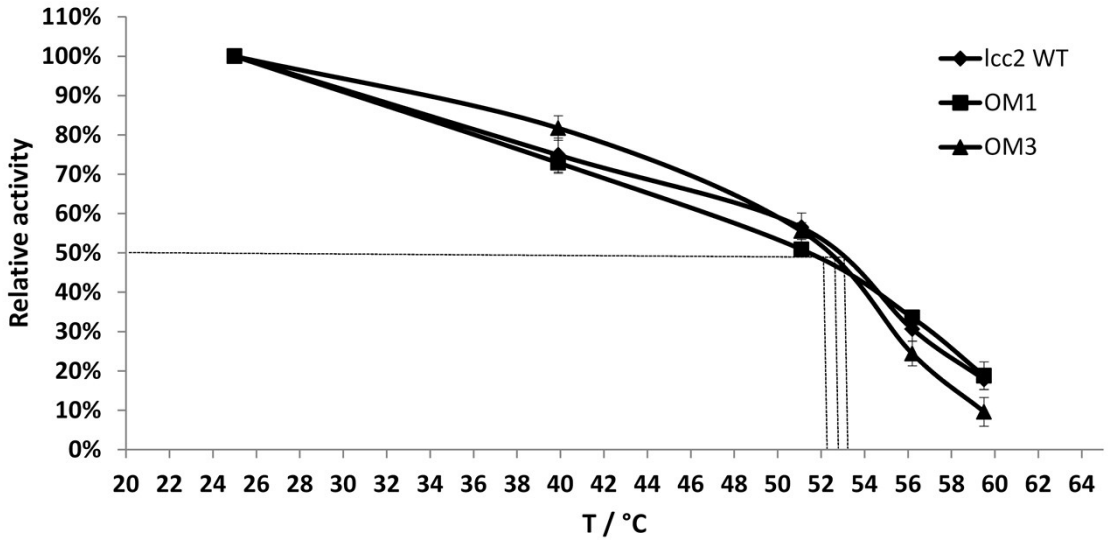

Fig. S4 Thermal resistance of lcc2 WT and OmniChange variants OM1 and OM3. The relative activity of lcc2 WT, OM1, and OM3 (normalized to the activity at 25°C) was determined via the ABTS assay after incubation at elevated temperatures in triplicates (25 to 60°C for 30 min). The T_m values of lcc2 WT, OM1, and OM3 are indicated by dotted lines.

Table S2 Sequence identity of fungal laccases with lcc2 WT and amino acid positions of loop structures

Laccase (PDB ID)	Organism	Sequence identity with lcc2 WT	Amino acid positions forming the domain connecting loop	Ala in the domain connecting loop
2XYB	*Pycnoporus cinnabarinus*	79.7%	282-323	1
3DIV	*Cerrena maxima*	84.6%	282-323	2
3FPX[2]	*Trametes hirsuta*	85.6%	282-323	2
5E9N	*Steccherinum murashkinskyi*	64.6%	285-327	4
3T6V[3]	*Steccherinum ochraceum*	64.2%	285-327	3
3X1B	*Lentinus sp.*	73.0%	303-346	7
4A2F[4]	*Coriolopsis gallica*	78.4%	302-343	6
4JHV[5]	*Coriolopsis caperata*	79.1%	281-323	5
4X4K[6]	*Botrytis aclada*	26.3%	313-350	4
3PPS[7]	*Thielavia arenaria*	23.0%	313-356	4

6. Surface entropy analysis

The surface entropy reduction prediction (SERp) server was originally developed for identifying mutation candidates with high conformational entropy that are likely to enhance a protein's crystallizability via the generation of crystal contacts. The profile contains high scores for residues with high conformational entropy and high probability of significant solvent exposure. A relative scale with the range of 0.1 for Ala and 1.0 for Lys was used hereby[8]. By analyzing lcc2 WT, OM1, and OM3 with SERp server we could show that the four targeted Ala (285,310, 312, and 318) formed a low-entropy patch within the loop region (284-320). This explains why substituting Ala at these four positions with amino acid residues having higher entropy (e.g. Asp) improved the resistance of lcc2 towards IL.

7. B-Factor analysis of amino acids in loop L1 of lcc2 WT

The B-Factors of amino acid residues of lcc2 WT were extracted from the PDB structure by using the YASARA Structure version 13.9.8[9]. This tool calculates the residue B-Factor as an

average of B-Factor of all the atoms of a residue in a given protein excluding hydrogen. **Fig. S3** shows the B-factor of amino acid residues in loop L1 of lcc2 WT. According to this figure, there are more fluctuations in chosen positions of OM1 and OM3 variants (amino acid residues 285, 310, 312, and 318), so they probably have significant role in loop flexibility and function.

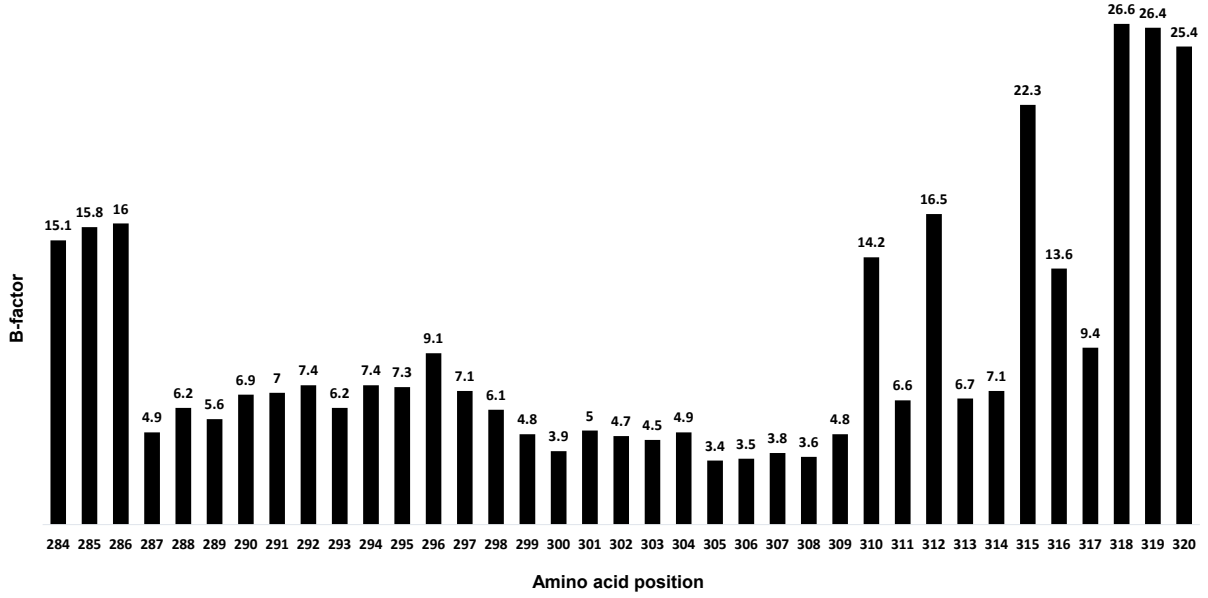

Fig. S5: B-factor of amino acid residues in loop L1 of lcc2 WT.

References

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