

Material and methods S1: Media for preculture and expression culture

10 × 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 media for preculture (preculture media, 1000 mL): 200 ml 10% sterile raffinose, 100 ml 10 × YNB stock solution, 100 ml 10 × HC dropout solution, 100 ml 1 M KPi-buffer (pH 6.2). Optimized HC media for expression (expression media, 1000 mL): 100 ml 10% 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 CuSO₄.

Material and methods S2: Cultivation and expression of lcc2 in 96-well plates

Colonies grown on HC preculture agar plates (ampicillin 100 µg/mL) were transferred, by using toothpicks, into 96-well microtiter plates (flat bottom, polystyrene plates; Greiner Bio-One GmbH, Frickenhausen, Germany), containing HC preculture medium (150 µl) supplemented with ampicillin (100 µg mL⁻¹). After 48 h of cultivation in a microtiter plate shaker (Multitron II, Infors GmbH, Einsbach, Germany; 37 °C, 900 rpm, 70% humidity), each well was replicated by using a replicator (EnzyScreen BV, Leiden, Netherlands) into a second series of 96-well microtiter plates containing HC expression medium (150 µl). The first set of plates was stored at -80 °C after addition of glycerol. The clones in the second set of plates were cultivated for 72 h (Multitron II, Infors GmbH, Einsbach, Germany; 19 °C, 900 rpm) and used for screening.

Material and methods S3: Expression of lcc2 in shaking flask and purification procedure

3 ml HC preculture media in the tube was inoculated with glycerol stocks of 5 µl laccase variants and incubated (30 °C, 250 rpm, 2 days). 80 ml HC preculture media in 250 ml flask was inoculated with 2-day culture and incubated (30 °C, 250 rpm, overnight).

Shaking flasks (1 L) containing HC expression media (500 mL) were inoculated with a dilution (final OD₆₀₀ is 0.4) of the preculture (*Saccharomyces cerevisiae* pYES2 harboring lcc2) grown in HC preculture medium. After 72 h of expression (19 °C with agitation 150 rpm), *S. cerevisiae* cells were centrifuged (Eppendorf 5810R 4 °C, 2800 g, 10 min) and the clear supernatant was concentrated and desalted by ultra filtration (Regenerated cellulose membrane with cutoff size of 10 kDa, Stirred Cell 8200, Millipore). Lcc2 and mutants were subsequently purified by a three-step chromatography procedure: The concentrated and desalted supernatant was cleared by filtration through a low-protein-binding filter (0.45 µM) and was applied to anion exchange column (Toyopearl SuperQ-650C, Tosoh, Stuttgart, Germany) previously equilibrated with buffer A (20 mM Tris-HCl, pH 7.0). At a flow rate of 3 ml/min, bound protein was firstly eluted with 8% buffer B (20 mM Tris-HCl, 0.5 M NaCl, pH 7.0) to remove some impurities. Buffer B was increased to 12%, this elution fraction collected and concentrated to 0.5 ml. The 0.5 ml eluted fraction from anion exchange was loaded onto a hydrophobic interaction column (Phenyl sepharose HP, GE Healthcare Europe, Freiburg, Germany) equilibrated with buffer C (20 mM Tris-HCl, 1.8 M (NH₄)₂SO₄, pH 7.0). The proteins were eluted with a linear gradient (120 ml) from 1.8 to 0 M (NH₄)₂SO₄ at a rate of 0.5 ml/min. Fractions with laccase activity were pooled, concentrated and subjected to a gel filtration column (Sephacryl S-200 HR, GE Healthcare Europe, Freiburg, Germany) and eluted with buffer A at a flow rate of 0.1 ml/min. The elution fractions were collected for the enzymatic assay. Throughout the purification protocol the fractions were analyzed by 8% SDS gel.

Material and methods S4: Long term ionic liquid resistance of lcc2 WT and variant M3

Lcc2 WT and M3 were incubated in presence of 25% (v/v) [EMIM] [EtSO₄] (0.2 µM laccase, 75% (v/v) 0.1 M KPi-buffer, pH 6.2) at room temperature. The residual activities were determined at regular time intervals, up to five days. The assay procedure as following: 1.3 µL incubated enzyme solutions were supplemented to 96-well MTP containing assay buffer (50 µL), ABTS (5 mM, 70 µL), [EMIM] [EtSO₄] (50 µL, final concentration 25%, v/v) and ddH₂O (28.7 µL). Absorbance at 420 nm was immediately measured with a microtiter plate reader (Tecan Sunrise, Tecan Group AG). Laccase residual activity was calculated based on ABTS conversion. Fig. S1 shows the residual activity of lcc2 WT and M3 after

incubation for 0, 1, 2, 3, 4 and 5 days. The residual activity was shown after comparing to the activity of lcc2 WT incubation in presence of 25% (v/v) [EMIM] [EtSO₄] for 0 day.

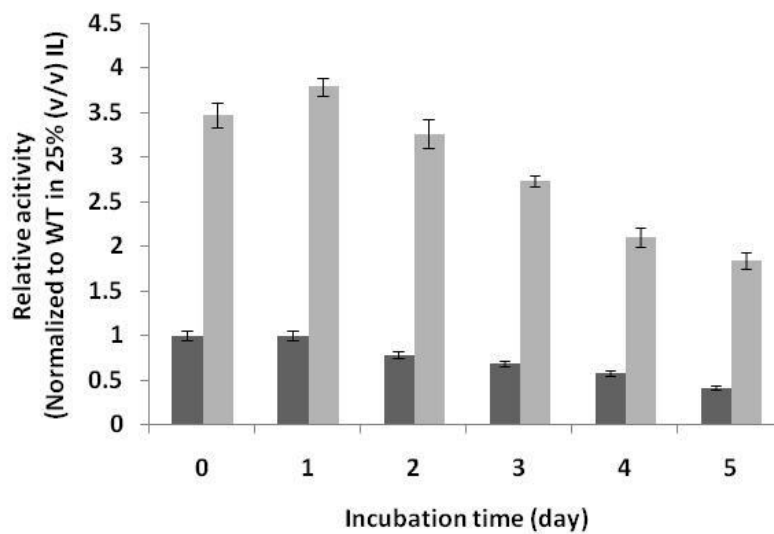


Fig. S1. Residual activity of lcc2 WT and M3 was determined with the ABTS detection system after incubation in presence of 25% (v/v) [EMIM] [EtSO₄] over 5 days. Residual activities were determined once per day in triplicates.