Supplementary material

Recombinant laccase biosynthesis for efficient polydopamine coating

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1. Materials and Methods

1.1 Recombinant plasmid expressing *Trametes versicolor* Lcc2 gene

Laccase was expressed in its native form using *K. phaffii* X33 (Invitrogen) under the control of AOX promoter. The plasmid was commercially obtained from Genscript (Piscataway, New Jersey, USA) and contains the lcc2 gene of 520 amino acids from *T. versicolor* (GenBank Accession No. CAA77015.1). The diagram of the cloning site of the recombinant plasmid expressing *T. versicolor* Lcc2 gene (named as pPICZ B_Lcc2) in *K. phaffii* is shown in Fig. S1. The target protein was expressed in *K. phaffii* X33 using the native signal peptide.

GAATTCATGTCGAGGTTTCACTCTCTTCTCGCTTTCGTCGTTGCTTCCCTTGCGGCTGTGGCCCACGCT
GGTATCGGTCCTGTCGCCGACCTCACCATCACCAACGCAGCGGTCAGCCCCGACGGGTTTTCTCGCC
AGGCCGTCGTCGTGAACGGTGGCACCCCTGGCCCTCTCATCACCGGTAACATGGGGGATCGCTTCCA
GCTCAATGTCATCGACAACCTCACGGACCACACGATGCTGAAGAGCACCAGTATTCACTGGCACGGT
TTCTTCCAGAAGGGCACCAACTGGGCCGACGGTCCCGCCTTCATCAACCAGTGCCCGATCTCATCTGG
CCACTCGTTCCTGTATGACTTCCAGGTTCCTGACCAGGCTGGCACCTTCTGGTACCACAGTCACTTGTC
CACGCAGTACTGTGATGGTCTGAGGGGTCCGTTCGTTGTTTACGACCCGAATGACCCGGCCGCCGAC
CTGTACGACGTCGACAACGACGACACGGTCATTACCCTTGCGGATTGGTACCACGTCGCCGCGAAGC
TGGGCCCCGCATTCCCTCTCGGCGCCGACGCCACTCTCATCAACGGTAAGGGACGCTCCCCCAGCAC
GACCACCGCGGACCTCACTGTTATCAGCGTCACTCCGGGTAAACGTTACCGTTTCCGCCTGGTGTCCC
TGTCGTGCGACCCCAACCACACCTTCAGCATCGATGGCCACAACATGACGATCATCGAGACCGACTC
GATCAACACGGCGCCCCTCGTGGTCGACTCCATTCAGATCTTCGCTGCCCAGCGTTACTCCTTCGTGC
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CACCGGCGGCATCAACTCGGCTATCCTCCGCTATGATGGCGCCGCTGCCATCGAGCCCACCACCACG
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GGTCGTCCGCAGCGCCGGCAGCACGGTCTACAACTACGACAACCCCATCTTCCGCGACGTCGTCAGC
ACGGGGACGCCTGCGGCCGGTGACAACGTCACCATCCGCTTCCGCACCGACAACCCCGGCCCGTGG
TTTCTCCACTGCCACATCGACTTCCACCTCGAGGCCGGCTTCGCCGTCGTGTTTGCGGAGGACATCCC
CGATGTCGCATCGGCGAACCCCGTCCCCCAGGCGTGGTCCGACCTCTGCCCGACCTACGACGCGCGC
GACCCGAGCGACCAGTAATCTAGA

Figure S1. Diagram of the cloning site of recombinant plasmid pPICZ B_Lcc2 used in this work for laccase expression in *K. phaffii* X33. The EcoR I and Xba I restriction sites used for cloning are highlighted in grey, the methionine initiation codon in blue and the stop codon in yellow. The native signal peptide which also contains the initial methionine is highlighted in purple. The mature Lcc2 gene sequence is shown in green.

1.2 *K. phaffii* X33 manipulation, storage, and transformation with pPICZ B_Lcc2 plasmid

K. phaffii X33 was maintained in glycerol stocks at a 1:1 ratio with 50 % (v/v) glycerol in YPD medium and stored at -80 °C. To reactivate the microorganism, the glycerol stock microorganism was grown on YPD agar plates with 2 % (w/v) agar (along with 1 µL/mL zeocin for transformed strains). The plates were then incubated at 30 °C for three days.

K. phaffii X33 cells were transformed by electroporation with the recombinant plasmid pPICZB_Lcc2 according to the manufacturer's instructions and as previously described by Pedro et al [1]. Commercially acquired pPICZB_Lcc2 was linearized with Sac, and then introduced into freshly made *K. phaffii* X33 competent cells via electroporation (2.5 kV, 25 µF, 1000 Ω) using an Eppendorf 2510 electroporator. After the pulse, the cells were immediately resuspended in 1 mL of ice-cold 1 M sorbitol. For screening of high expression transformants, cells were plated on YPDS containing 200 µg/mL zeocin and incubated at 30 °C for 4 days.

1.3 Cell lysis and intracellular protein recovery

For the cell lysis procedure, the reagents used were phenylmethylsulphonyl fluoride (PMSF) from Sigma-Aldrich, Pierce protease inhibitor, urea and dithiothreitol (DTT) from Acros Organics-Thermo Fisher Scientific and sodium chloride (NaCl) from LabKem. Protein precipitation was achieved by using ammonium sulfate (99.5 % purity) from Panreac (Barcelona, Spain).

Cell lysis was performed on samples from fermentation in Shake-flasks. The pellet was recovered after centrifugation at 1500 g for 10 min at 4 °C. 0.75 g of pellet was lysate by adding in 25 µL PMSF 100 mM, 50 µL Pierce protease inhibitor, 3.75 g glass beads and 2.5 mL lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM DTT). This means a proportion of cells:beads:buffer of 0.75g:3.75g:2.5 mL, respectively. The mixture was vortexed 7 times for 1 min each, with 1 min on ice in between. The glass beads were removed by decantation, and the sample pellet was recovered by centrifugation at 5000 g for 10 min at 4 °C. The pellet was then washed twice at a ratio of 1:2 (v/v) initial mixture solution: wash solution (first wash solution was Tris 10 mM pH 8 and second wash solution was 1 % Tritton X-100, 10 mM Tris pH

8 and 4 M urea). Afterward, it was centrifuged at 15000 g for 10 min at 4 °C, and the pellet was recovered. The pellet was then incubated for 1h at room temperature in a ratio of 1:2 (v/v) initial mixture solution: solution of 10 mM Tris pH 12, 8 M urea and 20 mM DTT. The mixture was then centrifuged (15000 g for 10 min), and the supernatant was subjected to overnight dialysis in 10 mM Tris pH 8 to remove urea from the medium. The protein profile was analyzed by SDS-Page, and Semi-Native Page evaluated the enzymatic activity (described in section 1.7 in the SM).

1.4. Effect of temperature and pH on the laccase activity

The effect of pH on the enzymatic activity of the produced laccase was studied at 25 °C resorting to citrate phosphate buffer (150 mM) and potassium phosphate buffer (150 mM), respectively, in pH intervals ranging from 3.0 to 6.0 and 7.0 and 8.0. To this end, 100 µL of the extracellular fraction obtained from *K. phaffii* X33 cultivation in shake-flasks under standard conditions was diluted in 900 µL of buffer, and the enzymatic activity measured immediately (t = 0 min), as well as after an incubation period of 15 min and 24 h.

Additionally, the effect of temperature on the laccase enzymatic activity was studied by incubating the fraction containing the enzyme at 4, 25, 30, 40 and 50 °C. Similar to the study of the influence of pH, 100 µL of the extracellular fraction containing laccase was diluted in 900 µL of potassium phosphate buffer (150 mM, pH 7.0), incubated for the same period and the enzymatic laccase activity measured.

Relative laccase activity (%) was calculated according to Eq. S1.

$$Relative\ laccase\ activity(\%) = \frac{Enzymatic\ activity\ in\ sample\ (U/L)}{Enzymatic\ activity\ in\ control\ (U/L)} \times 100 \quad \text{Eq. S1}$$

where the enzymatic activity in control is the activity of the enzyme solution in water measured at t=0 min at room temperature.

1.5. Laccase production in bioreactor

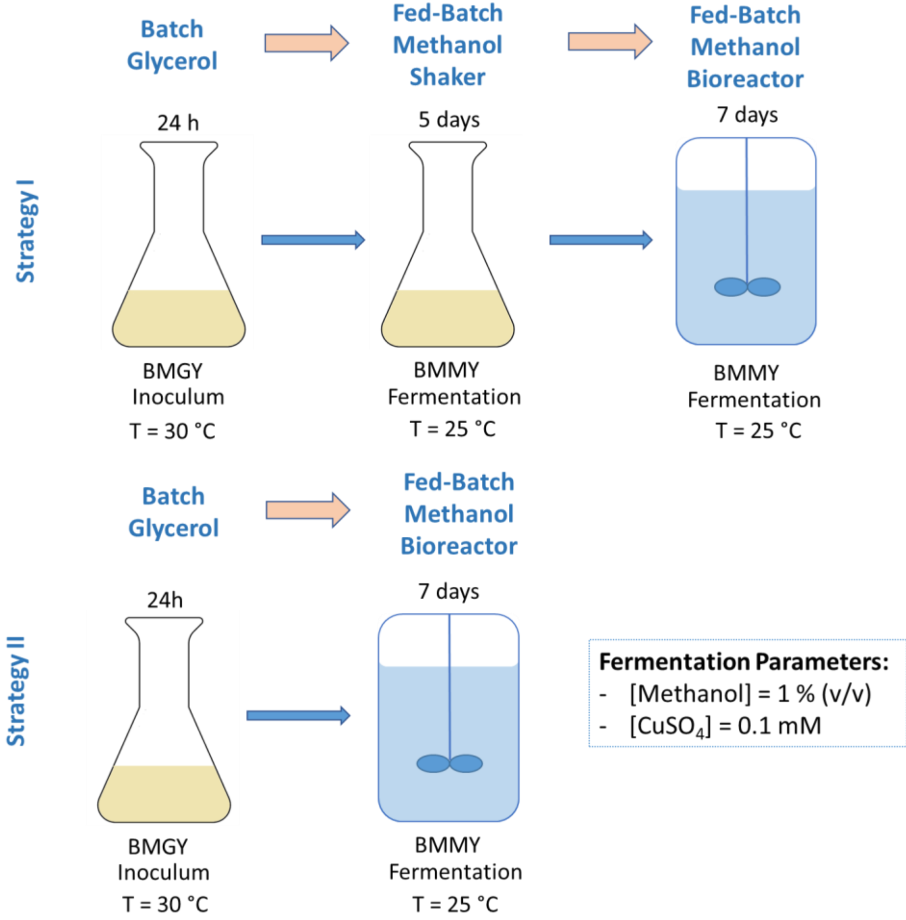

Figure S2. Schematic diagram representing the two strategies investigated for laccase production in the 6 L bioreactor (working volume 4 L).

1.6. Total protein concentration

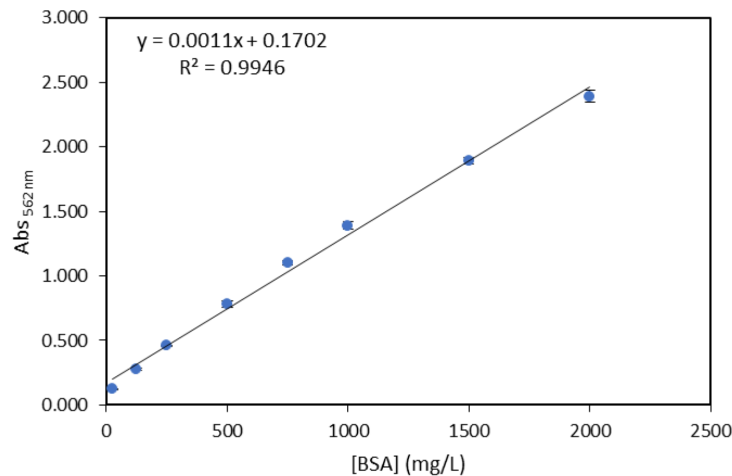

Figure S3. Standard BSA curve used for the total protein quantification using the BCA protein assay kit (Thermo Scientific, Waltham, USA).

1.7 Polyacrylamide gel electrophoresis

For SDS-PAGE, samples were diluted 1:1 (v/v) in a sample buffer containing Tris-HCl (0.5 mM, pH 6.8) 1:4 (v/v), SDS 10% (w/v) 2:5 (v/v), glycerol 1:5 (v/v), bromophenol blue 1:5 (w/v) and DTT (31 mg/mL). After dilution, the samples were heated at 95 ◦C for 5 min. All samples were loaded (20 µL) onto Novex™ WedgeWell™ 16 %, Tris-Glycine, 1.0 mm polyacrylamide gels (Invitrogen™). Electrophoresis was performed at 100 V for 90 min. The running buffer used was Novex™ Tris-Glycine SDS running buffer 10× (Invitrogen™). Gels were stained with BlueSafe from NZYtech (Lisbon, Portugal). NZYColour Protein Marker I was used as the molecular weight marker, and commercial laccase from *T. versicolor* (> 96.0 % pure) applied as the standard.

The enzymatic activity was also evaluated by semi-native PAGE. Samples obtained from the fermentation broth and the lysate were concentrated by precipitation using ammonium sulfate. In this procedure, ammonium sulfate (5 g) was slowly added to 8 mL of sample and incubated at 4 °C for 30 h under orbital stirring 90 rpm [2]. Then, the mixture was centrifuged at 10000 rpm for 10 min at 4 °C, and the resulting pellet was dissolved in sodium phosphate buffer (100 mM, pH 7.5). To obtain the electrophoresis gel, the same protocol as described for SDS-PAGE was followed, with some modifications: (i) DTT was not added to the samples;

(ii) the samples were not heated, and (iii) the gel was stained using an ABTS solution (1.7 mM ABTS in 150 mM citrate phosphate buffer pH 4.5).

2. Results and Discussion

2.1. Laccase production by *K. phaffii* X33 in shake-flask

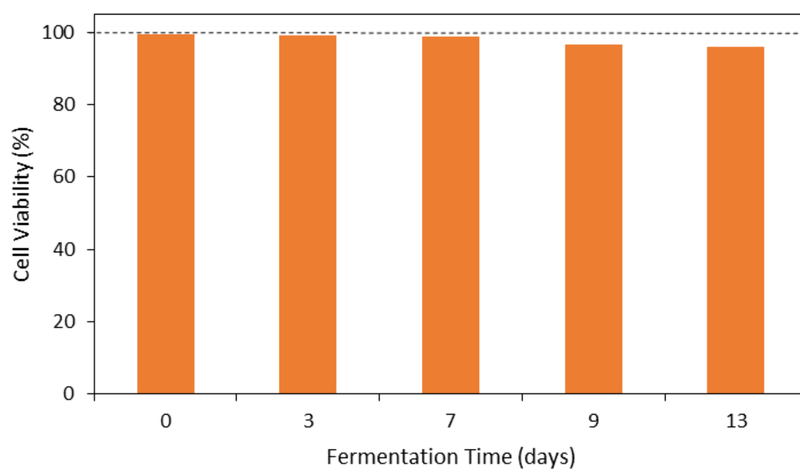

Figure S4. Cell viability (%) assessed by the trypan blue dye exclusion method by fermentation time (days) during laccase production by *K. phaffii* X33 in shake-flask.

2.2. Optimization of laccase production

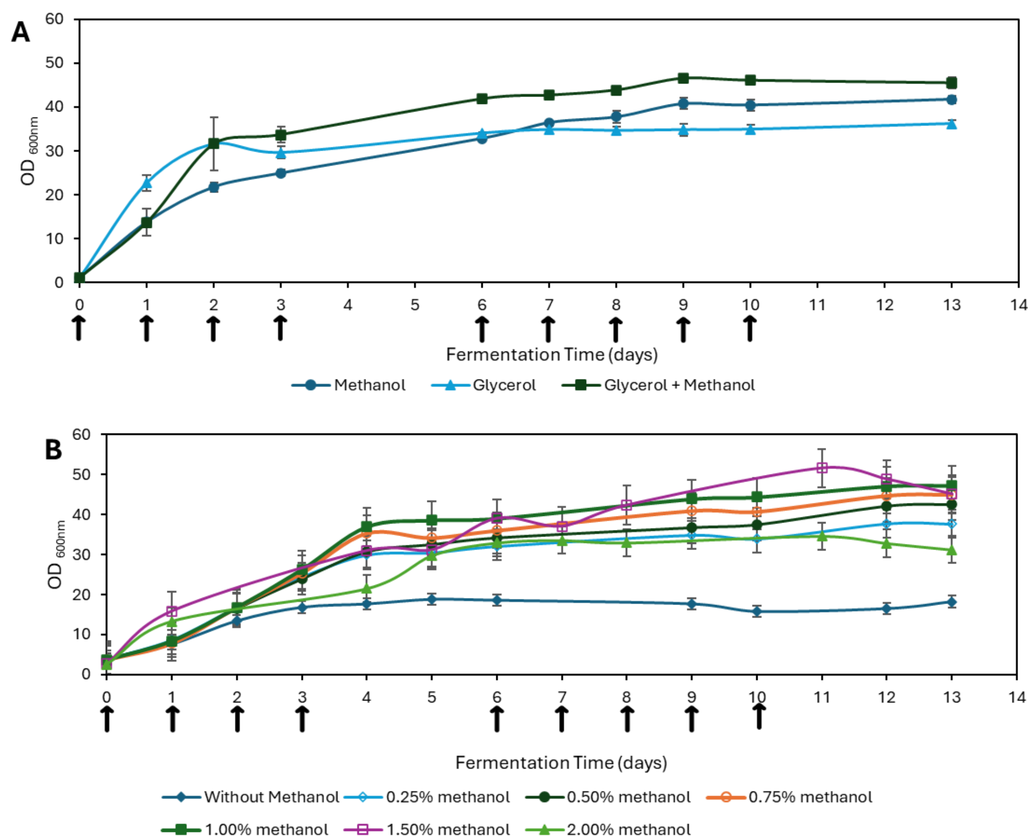

Figure S5. Effect of the type and concentration of the carbon source on recombinant *K. phaffii* X33 growth, measured by OD_{600} at different fermentation days. A) Effect of carbon source: glycerol (1 % (v/v)), methanol (1 % (v/v) daily feeding), and glycerol 1 % (v/v) + daily feeding of 1 % (v/v) methanol. B) Effect of the methanol daily feeding (0-2.00 % (v/v)) in cultures solely grown in methanol. Arrows represent days where methanol supplementation occurred in working days.

Table S1. Enzymatic activity (U/L) and Relative laccase production (%) to standard conditions: daily feeding of 1 % (v/v) methanol, supplementation of 0.1 mM CuSO4 and 30 °C about effect of carbon source: glycerol (1 % (v/v)), methanol (1 % (v/v) daily feeding), and glycerol 1 % (v/v) + daily feeding of 1 % (v/v) methanol.

Condition	**Enzymatic activity (U/L)**	**Relative laccase production (%)**
Glycerol	0 ± 0	0 ± 0
Glycerol + methanol	1.57 ± 0.55	11.32 ± 3.98
Methanol	13.83 ± 1.19	100.00 ± 8.60

Table S2. Enzymatic activity (U/L) and relative laccase production (%) to standard conditions: daily feeding of 1 % (v/v) methanol, supplementation of 0.1 mM $CuSO_4$ and 30 °C about effect of the methanol daily feeding (0-2.00 % (v/v)) in cultures solely grown in methanol.

Methanol concentration % (v/v)	**Enzymatic activity (U/L)**	**Relative laccase production (%)**
0	0 ± 0	0 ± 0
0.25	0.78 ± 0.01	4.24 ± 0.04
0.50	5.44 ± 0.14	29.59 ± 0.75
0.75	12.30 ± 0.20	66.92 ± 1.09
1.00	18.38 ± 1.74	100.00 ± 9.47
1.50	8.14 ± 0.02	44.28 ± 0.12
2.00	0.46 ± 0.08	2.48 ± 0.47

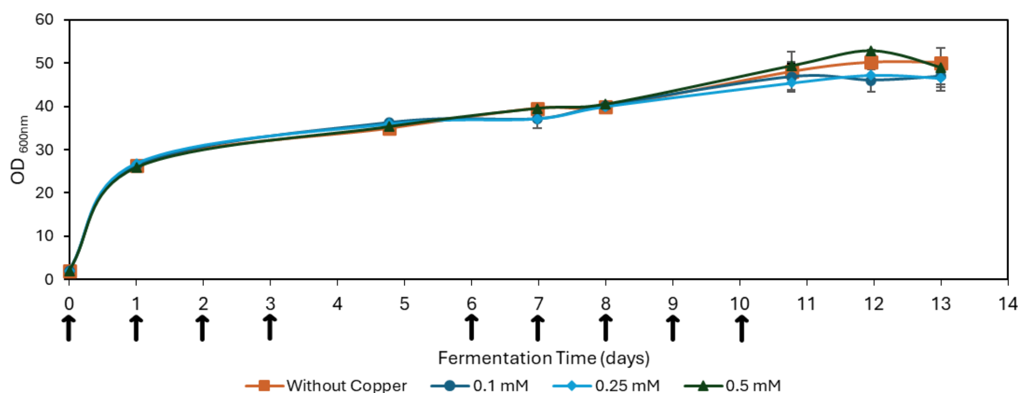

Figure S6. Effect of the $CuSO_4$ concentration on recombinant *K. phaffii* X33 growth, measured by OD_{600} at different fermentation days. Arrows represent days where methanol supplementation occurred in working days.

Table S3. Enzymatic activity (U/L) and Relative laccase production (%) to standard conditions: daily feeding of 1 % (v/v) methanol, supplementation of 0.1 mM $CuSO_4$ and 30 °C about effect of $CuSO_4$ on relative laccase production (%).

$CuSO_4$ concentration (mM)	**Enzymatic activity (U/L)**	**Relative laccase production (%)**
0	2.67 ± 0.40	16.87 ± 2.55
0.10	17.03 ± 0.53	100.00 ± 3.13
0.25	15.81 ± 0.14	92.83 ± 0.83
0.50	14.82 ± 0.61	87.06 ± 3.59

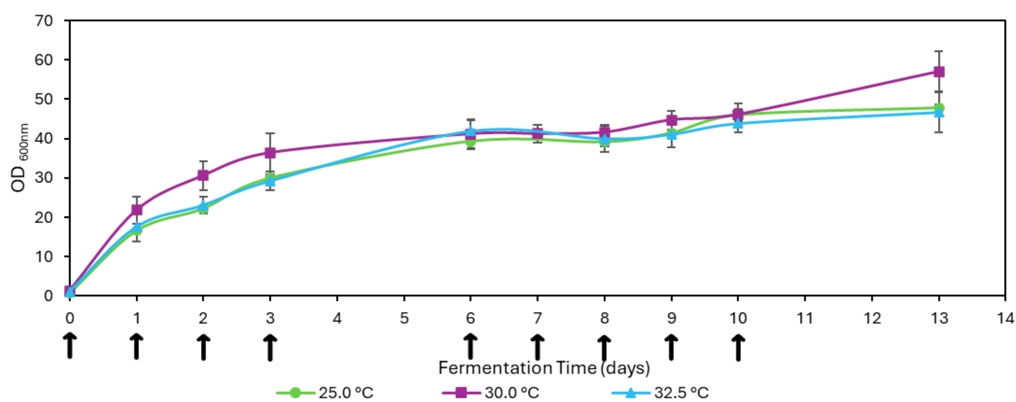

Figure S7. Effect of temperature on recombinant *K. phaffii* X33 growth, measured by OD_{600} at different fermentation days. Arrow represents days where methanol supplementation occurred in working days.

Table S4. Enzymatic activity (U/L) and Relative laccase production (%) to standard conditions: daily feeding of 1 % (v/v) methanol, supplementation of 0.1 mM $CuSO_4$ and 30 °C about effect of incubation temperature.

Temperature (°C)	Enzymatic activity (U/L)	Relative laccase production (%)
25.0	51.81 ± 1.03	294.15 ± 5.83
30.0	17.61 ± 1.25	100.00 ± 7.09
32.5	2.35 ± 0.03	13.35 ± 0.17

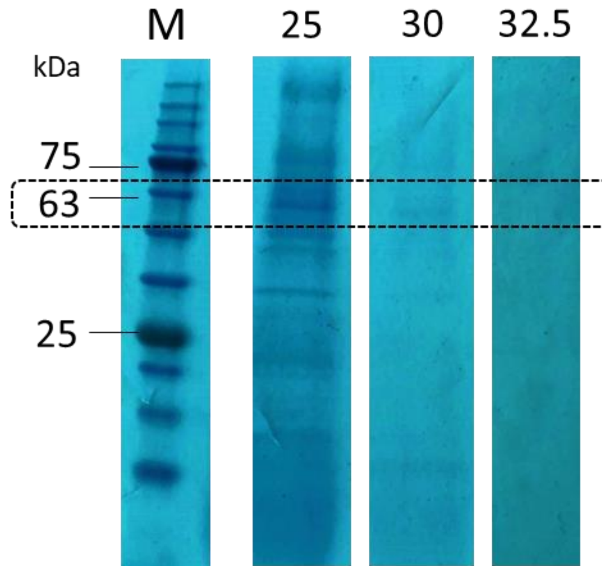

Figure S8. SDS-PAGE of proteins present in the fermentation broth about the effect of incubation temperature. Samples: 25 – incubation temperature of 25.0 °C; 30 – incubation temperature of 30.0 °C; 32.5 – incubation temperature of 32.5 °C; M - Molecular weight marker.

2.3. Production of laccase in bioreactor

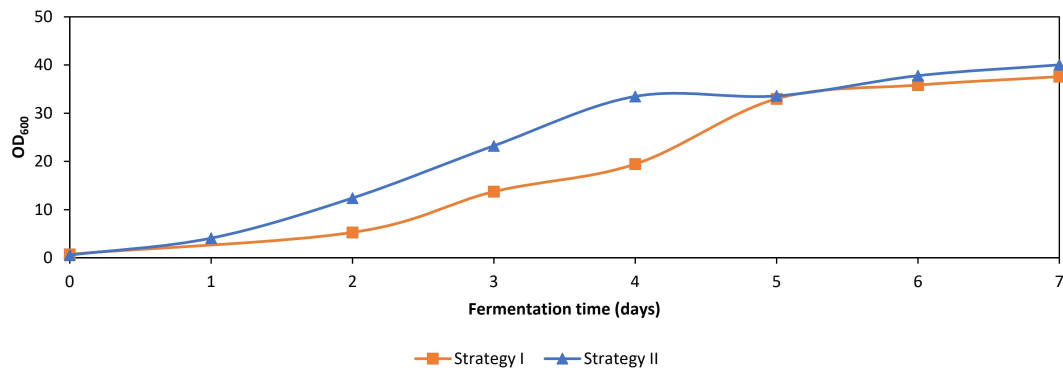

Figure S9. Evolution of OD_{600} by fermentation time (days) in the bioreactor in two different strategies: Strategy I) Start BMMY adaptation in a shaker before inoculating the bioreactor; Strategy II) Inoculate the bioreactor directly with inoculum growth in BMGY.

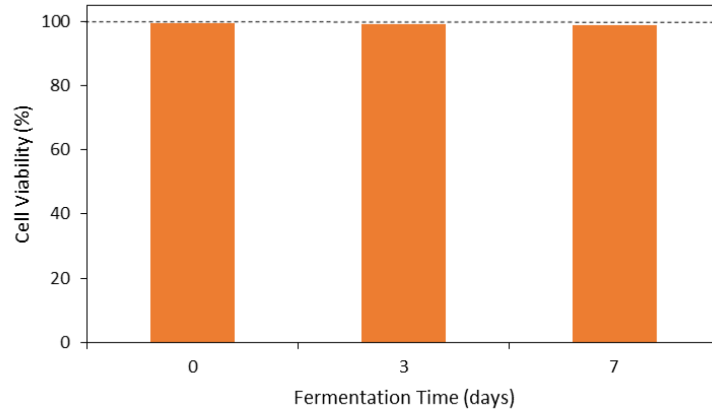

Figure S10. Cell viability (%) assessed by the trypan blue dye exclusion method by fermentation time (days) during laccase production by *K. phaffii* X33 in bioreactor.

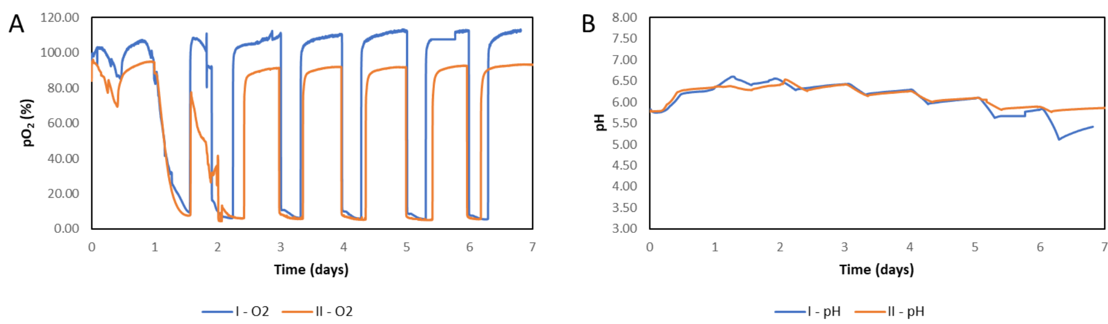

Figure S11. A) pO_2 (%) and B) pH by fermentation time (days) in the bioreactor in two different strategies: Strategy I) Start BMMY adaptation in a shaker before inoculating bioreactor; Strategy II) Inoculate the bioreactor directly with inoculum growth in BMGY.

2.4. Laccase recovery from the fermentation broth

Table S5. Enzymatic activity (U/L), Protein concentration (g/L), specific enzymatic activity and Purification factor of samples after protein precipitation with methanol (using the crude supernatant as initial sample) and acetone (using the crude supernatant and Vivaspin® sample as initial samples). Precipitation with acetone was studied with time (1, 2 and 4 h) and concentration variation (2:1 and 4:1 v/v solvent: sample). Data presented represent the average of 3 replicates.

Initial Sample	Sample		Enzymatic activity (U/L)	Protein concentration (g/L)	Total Protein (g)	Specific activity (U/g)	Purification factor
Broth	Broth		112.0±2.1	3.061±0.036	(9.18±0.11) x10^{-3}	36.59±0.35	1.00±0.01
	Methanol precipitate	2h 2:1	0.0±0.0	1.312±0.216	(0.79±0.05) x10^{-3}	0.00±0.00	0.00±0.00
	Acetone precipitate	1h 2:1	100.6±0.6	3.833±0.216	(1.92±0.11) x10^{-3}	26.35±1.54	0.72±0.04
		2h 2:1	122.0±4.4	3.739±0.060	(2.24±0.04) x10^{-3}	32.64±1.07	0.89±0.03
		2h 4:1	102.6±4.6	6.573±0.136	(3.94±0.08) x10^{-3}	15.59±1.03	0.43±0.03
		4h 2:1	175.1±6.2	4.165±0.494	(2.50±0.29) x10^{-3}	42.58±3.76	1.16±0.10
	VivaSpin		541.3±6.0	5.922±0.404	(5.92±0.40) x10^{-3}	95.10±8.77	2.51±0.18
Vivaspin	Acetone precipitate	2h 2:1	1356.8±95.4	12.244±0.339	(2.45±0.07) x10^{-3}	107.91±8.43	3.02±0.13

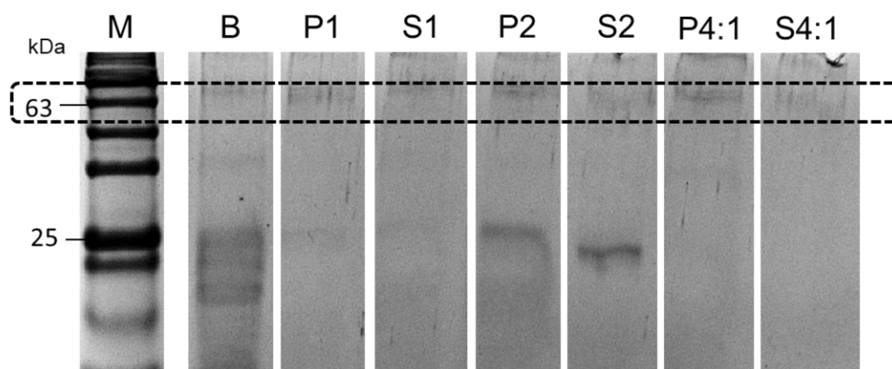

Figure S12. SDS-PAGE of proteins present in the fermentation broth (B), acetone precipitate 1h 2:1 v/v solvent/sample (P1), acetone sobrenatant 1h 2:1 v/v solvent/sample (S1), acetone precipitate 2h 2:1 v/v solvent/sample (P2), acetone sobrenatant 2h 2:1 v/v solvent/sample (S2), acetone precipitate 2h 4:1 v/v solvent/sample (P4:1), acetone sobrenatant 2h 4:1 v/v solvent/sample (S4:1). Molecular weight marker (M).

2.4 Kinetic Parameters

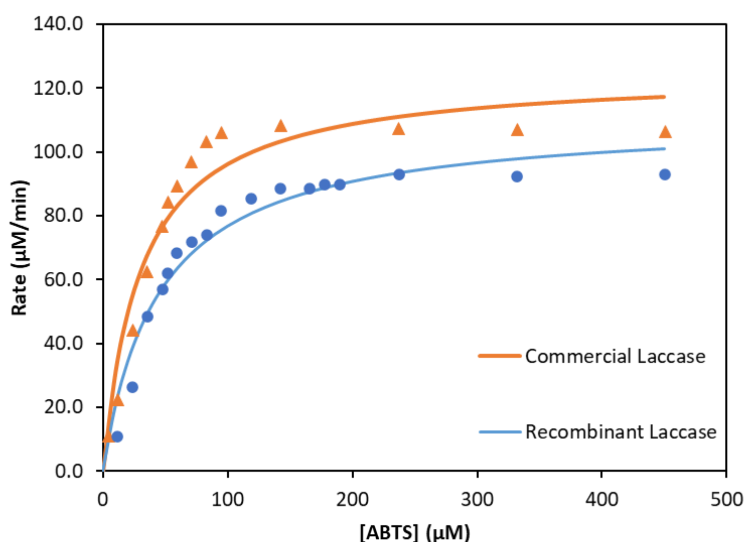

Figure S13. Experimental data for the determination of kinetic parameters K_M and V_{max} of commercial laccase and recombinant laccase using ABTS as substrate. Triangles and circles represent experimental data. Lines represent the data fitting according to Michaelis-Menten equation (Eq. 6). Commercial laccase: K_M = 30.0 µM; V_{max} = 125.2 µM/min; R^2=0.962. Recombinant laccase: K_M = 44.5 µM; V_{max} = 110.9 µM/min; R^2=0.972.

3. References

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[2] T.C. Ezike, A.L. Ezugwu, J.O. Udeh, S.O.O. Eze, F.C. Chilaka, Purification and characterization of new laccase from *Trametes polyzona* WRF03, Biotechnology Reports 28 (2020) e00566. https://doi.org/10.1016/j.btre.2020.e00566.