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Evolved alkaline fungal laccase secreted by *Saccharomyces cerevisiae* as useful tool for the synthesis of C-N heteropolymeric dye

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Abstract

Enzymatic production of C-N heteropolymeric dyes at alkaline pHs is an attractive process for the textile industry. In this work, we have designed a fungal laccase by directed evolution so that it may be used at alkaline pHs for the synthesis of C-N heteropolymeric dyes (C-N polydye) from catechol and 2,5-diaminobenzenesulfonic acid (2,5-DABSA). Firstly, several medium- and high-redox potential fungal laccases from previous laboratory evolution campaigns were benchmarked for the synthesis of the C-N polydye at pH 8.0, choosing an alkaline laccase mutant from *Myceliophthora thermophila* as the departure point for further engineering. Mutant libraries were then constructed, expressed in *Saccharomyces cerevisiae* and screened using a high-throughput colorimetric assay for the detection of the C-N polydye. By combining directed and focused molecular evolution, a novel, strongly expressed alkaline laccase variant was identified. This laccase was secreted at 37 mg/L and its catalytic efficiency for the oxidation of catechol and 2,5-DABSA at pH 8.0 was enhanced 3.5-fold relative to that of the wild-type, promoting the synthesis of the C-N polydye at basic pHs. While the improved expression was mostly the result of accumulating mutations that favor the yeast's codon usage together with the recovery of a secretion mutation, the enhanced C-N polydye synthetic activity of the mutant laccase was dependent on the alkaline mutations it inherited. Readily secreted, this laccase mutant would appear to be a valuable platform for organic synthesis at basic pHs.

1. Introduction

Textile manufacturing currently represents one of the world most global and profitable industries, with an approximate turnover of 18 billion € in the last year alone. Accordingly, the global production of dye-stuff for textiles is estimated to be around 34 million ton/year, with global sales of aprox. 6 billion €/year [1]. With more than 15,000 synthetic dyes produced on an industrial scale, there is ever enhanced interest in implementing eco-friendly biocatalytic methods to replace the traditional and energy demanding processes that are associated with the use of toxic precursors, low production yields and a harmful impact on the environment.

Fungal laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are among the best known and greenest biocatalysts in the biotechnology rainbow [2]. Their activity is fueled simply by oxygen from the air, oxidizing a wide range of compounds and releasing water as the only by-product. In addition to their potential in several biotechnological areas, including pulp and paper, food, furniture manufacture, biofuels or nanobiodevices (biosensors and biofuel cells), the use of fungal laccases and/or laccase mediator systems (LMS) for organic synthesis has been studied in some depth, specifically in processes ranging from the production of pharmacological compounds (e.g. antibiotics, antitumor and antiviral agents) to that of complex polymers [3-6]. The latter is a particularly interesting area of research and it is directly related to the generation of a palette of different colorants through homo- and heteromolecular coupling (including C-C, C-O, C-N and C-S coupling reactions) [5]. Indeed, the synthesis of C-N heteropolymeric dyes from catechol and 2,5-diaminobenzenosulfonic acid (2,5-DABSA) by laccases has been documented

exhaustively for the *in situ* dyeing of protein and cellulose fibers [7-10]. This process involves three main steps (**Figure 1**): i) enzymatic oxidation of catechol to produce highly reactive phenoxyl radicals and quinones, which is followed by spontaneous non-enzymatic homopolymerization to polycatechol; ii) activation of 2,5-DABSA (promoted by enzymatic oxidation and basic pHs); and iii) non-enzymatic cross coupling between the activated donor and the polycatechol to generate the C-N heteropolymeric dye (in short, C-N polydye). It is well known that pH is a limiting factor in this process, not only because the C-N cross-coupling through 1,4 Michael additions requires basic conditions to activate the reaction donor [11] but also, basic pHs enhance the formation of polycatechol [12]. Finally, alkaline pHs help solubilize substrates, which is important when working with high substrate loads for the *in situ* dyeing of textiles [8, 9].

All studies into the synthesis of the C-N polydye from catechol and 2,5-DABSA described to date have been carried out at acid pHs given the lack of fungal laccases that perform well in alkaline environments [7-10]. Here, we report the development of a fungal laccase that can be used in the synthesis of C-N polydye at basic pHs. A strict benchmarking for the oxidation of catechol and 2,5-DABSA was performed with several laccase mutants from different evolutionary campaigns [13-15]. An alkaline laccase variant was chosen and further evolved for the synthesis of the C-N polydye at basic pHs. The final mutant was characterized biochemically, and the role of the mutations in terms of enhanced secretion in *Saccharomyces cerevisiae* and alkalophilicity was considered.

2. Material and methods

2.1. Materials

Purified wildtype Mtl (produced in *Aspergillus oryzae* [16]) was kindly donated by Dr. Jesper Vind from Novozymes (Denmark) whereas the remaining laccase mutants for benchmarking experiments were obtained as described elsewhere [13-15, 20]. All chemicals were reagent-grade purity. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), 2,5-DABSA (2,5-diaminobenzenesulfonic acid), *Taq* polymerase and the *S. cerevisiae* transformation kit were purchased from Sigma-Aldrich (Madrid, Spain) while catechol was from Fisher Scientific (Madrid, Spain). The iProof High Fidelity DNA polymerase was obtained from Bio-Rad (Hercules, CA, USA), and the Zymoprep Yeast Plasmid Miniprep kit and Zymoclean Gel DNA Recovery kit from Zymo Research (Orange, CA, USA). The *Escherichia coli* XL2-Blue competent cells and the GeneMorph II Random mutagenesis kit were from Stratagene (La Jolla, CA, USA) and the protease-deficient *S. cerevisiae* strain BJ5465 (*α ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*) was from LGCPromochem (Barcelona, Spain). The episomal shuttle vector pJRoC30 was from the California Institute of Technology (CALTECH, USA). The NucleoSpin® Plasmid kit was acquired from Macherey-Nagel (Düren, Germany), the restriction enzymes BamHI and XhoI from New England Biolabs (Hertfordshire, UK) and the oligonucleotides were synthesized by Isogen Life Science (Barcelona, Spain). High Density Glyoxal 4BCL was from Agarose Bead Technologies (FL, USA).

2.2. Laboratory evolution

All PCR fragments were cleaned, concentrated, and loaded onto a low melting point preparative agarose gel (0.75 % w:v) and purified using the Zymoclean gel DNA Recovery kit. Mutant libraries were cloned under the

control of GAL1 promoter of the pJRoc30 expression shuttle vector replacing the parent gene. The pJRoc30 plasmid was linearized with BamHI and XhoI, and the linear plasmid was concentrated and purified as described above for the PCR fragments. Parental type IG-88 mutant was previously obtained as described elsewhere [15].

First generation: A mutant library of ~1,700 mutants was built by *in vivo* assembly of mutant libraries (lvAM, [17]). The PCR reactions (final volume 50 μ L) were performed in a gradient thermocycler (MyCycler, BioRad, USA). The Taq/MnCl₂ reaction contained 90 nM of each primer, 5 ng of template, dNTPs (0.075 mM each), 3% dimethyl sulfoxide (DMSO), 1.5 mM MgCl₂, 0.01 mM MnCl₂ and 2.5 units of Taq polymerase. The Genemorph II reaction contained 0.4 μ M of each primer, 2 μ g of template, dNTPs (0.2 mM each), 3% DMSO, and 2.5 U Mutazyme II DNA polymerase. PCR reaction was performed as follow: 95°C for 2 min (1 cycle); 94°C for 0.45 min, 53°C for 0.45 min and 74°C for 3 min (28 cycles); 74°C for 10 min (1 cycle). The following primers were used for amplification: RMLN sense (5'-CCTCTATACTTTAACGTCAAGG-3', which binds to bp 160-180 of pJRoc30-MtL); and RMLC antisense (5'-GGGAGGGCGTGAATGTAAGC-3', which binds to bp 2127-2146 of pJRoc30-MtL). To promote *in vivo* cloning, overhangs of 40 and 66 bp homologous to the linear vector were designed. An equimolar mixture of Taq/MnCl₂ and Mutazyme II fragments (400 ng each) were mixed with the linearized vector (100 ng) and transformed into competent cells using the yeast transformation kit (ratio equimolar library: vector = 8:1). Transformed cells were plated on SC drop-out plates and incubated for 3 days at 30°C. Colonies containing the whole

autonomously replicating vector were selected and screened as described below.

Second generation: Two error-prone PCR libraries (~1000 mutants each) with different mutational loads were constructed, independently transformed in yeast and screened as described below. The PCR reaction conditions were as described in the first round of evolution.

Third generation: Two independent libraries (~1,000 mutants each) were built as in the second generation but enhancing the $[MnCl_2]$ to 0.03mM for the Taq/ $MnCl_2$. In a parallel experiment, focused evolution by MORPHING [18] was carried out targeting the following stretches for mutagenesis and recombination: Ala159-Thr261 (MORPHING-I), Leu351-His436 (MORPHING-II), and both combined segments (MORPHING-I-II). The PCR reaction for MORPHING-I and MORPHING-II contained 90 nM of each primer, 46 ng of template, dNTPs (0.075 mM each), 3% DMSO, 1.5 mM $MgCl_2$, 0.1 and 0.15 mM $MnCl_2$ for MORPHING-I and MORPHING-II, respectively, and 2.5 units of Taq polymerase. PCR was performed as follows: 95°C for 2 min (1 cycle), 94°C for 0.45 min, 58 and 55°C for 0.45 min in MORPHING-I and MORPHING-II, respectively, 74°C for 0.45 min (28 cycles) and 74°C for 10 min (1 cycle). The primers used for MORPHING-I were FORWARD1REGION1 sense (5'-CCTCGCTGCCGTACGACACC-3', which binds to bp 826-845 of pJRoc30-MtL) and REVERSE2REGION1 antisense (5'-CGTCATGGCGTTGACGGGCACCATG-3', which binds to bp 1,109-1,133 of pJRoc30-MtL); for MORPHING-II we used FORWARD1REGION2 sense (5'-CTCGCCAAGCGGCCCGACAACACG-3', which binds to bp 1,401-1,424 of pJRoc30-MtL) and REVERSE2REGION2 antisense (5'-

GTGCAGGTGCATCGGATGCG-3', which binds to bp 1,639-1,658 of pJRoC30-MtL). The PCRs for the four high-fidelity regions were carried out in a final volume of 50 μ L, containing 0.5 μ M of each primer, 10 ng of template, dNTPs (0.2 mM each), 3% DMSO and 1 unit of iProof High Fidelity DNA polymerase. PCR was performed at 98°C for 0.5 min (1 cycle), 98°C for 0.17 min, 50 °C and 53 °C for the different high fidelity segments for 0.5 min and 72°C for 1 min (28 cycles); 72°C for 10 min (1 cycle). The primers used for amplifications were: RMLN and REVERSE1REGION1 antisense (5'-GCTGATGGGGAACACGCCAGGTCG-3', which binds to bp 845-869 of pJRoC30-MtL); post-MORPHING-I: FORWARD2REGION1 sense (5'-GACCATCATCGCCGCCGACATGG-3', which binds to bp 1,091-1,113 of pJRoC30-MtL) and RMLC; before-MORPHING-II: RMLN and REVERSE1REGION2 antisense (5'-CCATGGTGTGCGAGGGTGACG-3', which binds to bp 1,430-1,449 of pJRoC30-MtL); and post-MORPHING-II: FORWARD2REGION2 sense (5'-CGGCGCACCTTTCACCGTACCG-3', which binds to bp 1,619-1,640 of pJRoC30-MtL) and RMLC. Additionally, a library of ~2.100 clones using IvAM was carried out in the same conditions as described above.

High-throughput screening assay: As a strong background was detected in *S. cerevisiae* supernatants in the presence of catechol and 2,5-DABSA, a new medium (selective expression medium, SEM) was developed for expression in yeast. SEM composition: 100 mL yeast nitrogen base 67 g/L, 100 mL yeast synthetic drop-out medium supplement without uracil 19.2 g/L, 100 mL galactose 20%, 67 mL KH₂PO₄ buffer 1 M [pH 6.0], 5 mL CuSO₄ 2 mM, 1 mL chloramphenicol 25 g/L and ddH₂O to 1000 mL. Individual clones from mutant

libraries were picked and cultured in 96-well plates (Greiner Bio-One GmbH, Germany) containing 200 μL of SEM. In each plate, column 6 was inoculated with the parental type and one well (H1-control) was not inoculated as a negative control. The plates were sealed to prevent evaporation and incubated at 30°C, 225 rpm and 80% relative humidity in a humidity shaker (Minitron-INFORS, Biogen, Spain). After 72 h, the plates were centrifuged for 10 min at 4°C and 3,500 $\times g$ (Eppendorf 5810R centrifuge, Germany). Each master plate was quadruplicated with the help of the liquid handler robotic station (Freedom EVO, Tecan, Switzerland) (20 μL of supernatant each). Subsequently, 180 μL 100 mM McIlvaine buffer [pH 8.0], containing 25 mM catechol and 10 mM 2,5-DABSA were added with the Multidrop robot (Multidrop Combi, ThermoFischer Scientific, Vantaa, Finland). Plates were stirred briefly and absorption was recorded at 550 nm in the plate reader (SPECTRAMax Plus 384, Molecular Devices, Sunnyvale, CA, USA). The plates were incubated at room temperature until a brownish color was developed and the plates were measured again. Three control assays were performed with i) 100 mM sodium acetate buffer [pH 5.0] containing 3 mM ABTS, ii) 100 mM McIlvaine buffer [pH 8.0] containing 10 mM 2,5-DABSA and iii) 100 mM Bis-Tris HCl buffer [pH 6.5] containing 25 mM catechol. The absorptions were measured in the plate reader at 418 nm for ABTS, 450 nm for 2,5-DABSA and 400 nm for catechol.

First re-screening: aliquots (5 μL) of the best clones were removed from the master plates to inoculate minimum media (50 μL) in new 96-well plates. Columns 1 and 12 (rows A and H) were not used to prevent the appearance of false positives. The plates were incubated for 24 h at 30°C and 225 rpm with 80% humidity; after that an aliquot (5 μL) was transferred to the adjacent wells

and further incubated for 24 h. Finally, SEM (200 μ L) was added and the plates were incubated for 72 hours. Thus, each mutant was grown in 4 independent wells. The parental types were subjected to the same procedure (lane D, wells 7-11) and the plates were subjected to the screening assay as described above.

Second re-screening: an aliquot from the wells with the best clones from the first re-screening was used to inoculate 3 mL of YPD (10 g yeast extract, 20 g peptone, 100 mL 20% glucose (w:v), 1 mL 25 g/L chloramphenicol and *ddH*₂O to 1000 mL) and incubated for 24 h at 30°C and 225 rpm. The plasmids contained in these clones were recovered by Zymoprep Yeast Plasmid Miniprep kit. As the product of the zymoprep was very impure and the DNA concentration extracted was very low, the shuttle vectors were transformed into super-competent *Escherichia coli* XL2-Blue cells and plated onto LB with ampicillin (LB-amp) agar plates. Single colonies were picked, used to inoculate LB-amp liquid media (5 mL) and grown overnight at 37°C and 250 rpm. The plasmids from the best mutants and the parental type were then extracted with NucleoSpin® Plasmid kit and used to transform *S. cerevisiae*. Five colonies for each mutant and parental type were picked and re-screened.

2.3. Enzyme production and kinetic characterization

Selected mutants were produced, purified and characterized biochemically as described elsewhere [15]. The kinetics parameters were estimated at two different pH values for each substrate. Reactions were carried out by triplicate in a final volume of 200 μ L containing the corresponding substrate in 100 mM Britton and Robinson buffer at pH 4.0 or pH 8.0. Substrate oxidation was followed by measuring the absorption at 392 nm for catechol (ϵ_{392}

1456 $M^{-1}cm^{-1}$), and 475 nm for 2,5-DABSA (ϵ_{475} 2372 $M^{-1}cm^{-1}$) using the plate reader. To calculate the values of K_m and k_{cat} , the average V_{max} was represented versus substrate concentration and fitted to a single rectangular hyperbola function in SigmaPlot 10.0, where parameter a was equaled to k_{cat} and parameter b was equaled to K_m .

2.4. Synthesis of the C-N polydye

Enzyme immobilization: Laccase mutant was covalently immobilized on glyoxal agarose beads. Laccase mutant supernatants were double-filtered using both glass membrane filter and a nitrocellulose membrane of 0.45 μm pore size (Merck Millipore, Billerica, MA), precipitated with ammonium sulfate at 95 % and centrifugated at 10,000 g for 20 min at 4 $^{\circ}C$ (Avanti J-E centrifuge, Beckman Coulter, CA, USA). The pellet was recovered in 20 mM phosphate buffer [pH 6.0]. Afterwards, the pH of the enzyme solution was adjust to pH 9.0 with sodium bicarbonate and added to 0.6 g of glyoxal agarose beads (previously washed with distilled water to remove any preservatives). The sample was incubated for 1 hour at room temperature with rotational agitation (roller shaker, JP Selecta S.A. Spain). After the addition of 6 mg of $NaBH_4$, the mixture was incubated for 30 min for the reductive amination of the Schiff base bonds. The resulting mixture was filtrated with acetate/nitrate cellulose filters 0.45 μm , washed three times with 20 mM phosphate buffer [pH 6.0] to remove any weakly adsorbed enzyme and stored at 4 $^{\circ}C$.

Synthesis of the C-N polydye: 0.5 g of immobilized laccase mutant were mixed with precursor 2,5-DABSA (10 mM) and modifier Catechol (25 mM) in 100 mM McIlvaine buffer [pH 8.0] and incubated at room temperature on a roller shaker

for 3 hours. The immobilized enzyme was removed by filtration with acetate/nitrate cellulose filters. The dark product was evaporated to dryness and suspended in ethyl acetate:ethanol (1:1 v/v). The dye suspension was transferred to a glass column (1,5 x 30 cm) packed with 40 g of silica gel 60, particle size 0.06-0.2 mm, and eluted with ethyl acetate:ethanol:water 40 ml 1:1:1 v/v and 40 ml 1:1:2 v/v). Different fractions were collected and the most colored ones evaporated to dryness. The sample was analyzed by FTIR as follows: attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra of powder samples were acquired using a Nicolet Nexus 670 spectrometer provided with a MCT detector and a GladiATR single-bounce monolithic diamond ATR accessory. The spectra was recorded in the 4000-400 cm^{-1} wavenumber range, at 4 cm^{-1} resolution, by averaging 256 scans.

3. Results and Discussion

3.1 Laccase benchmarking and directed evolution

Initially, an array of medium and high-redox potential laccases that had been previously evolved in our laboratory for different purposes were tested for C-N polydye synthesis at pH 8.0 and pH 5.0 (the standard pH value used in former studies for the synthesis of C-N polydyes [7-10]). This benchmarking focused on the following enzymes: i) the OB-1 variant (a high-redox potential laccase mutant evolved from the basidiomycete PM1 laccase for functional expression, activity and stability [13]); ii) the ChU-B variant that was evolved from OB-1 to be active in human blood [14]; iii) the 7D3 and 12A8 variants derived from ChU-B by introducing new stabilizing mutations (unpublished data); iv) the R2 variant that was evolved from the medium-redox potential *Myceliophthora thermophila* laccase (MtL) to improve its functional expression

in yeast [19] and its activity in the presence of organic co-solvents [20]; and iv) the IG-88 variant that was evolved from R2 to be active at alkaline pHs [15]. With the help of a specific colorimetric assay for cross-coupling polymerization between catechol and 2,5-DABSA (see below), this activity was assessed (**Figure 2**). The OB-1 laccase mutant best synthesized the C-N polydye at pH 5.0, yet as expected, the ChU-B variant that had been evolved for tolerance to human blood (blood pH 7.4) was more active than OB-1 at alkaline pHs, as were its more stable 7D3 and 12A8 variants. Of all the laccase variants, the alkaline IG-88 mutant was of particular interest at pH 8.0 and as such, it was chosen as the departure point for further engineering.

The high-throughput screening assay for directed evolution was based on the oxidative cross-coupling between 2,5-DABSA and (poly)-catechol initiated by the laccase at pH 8.0. Upon enzyme oxidation, the colorless catechol and 2,5-DABSA generate a colorimetric response that is strongly dependent on pH and that differs in color from that obtained with the single oxidized substrates (**Supplementary Figure 1**). A selective expression medium (SEM) for laccase production in yeast was prepared to dampen the background while preserving vector stability during fermentation in 96-well plates. Galactose was included as single carbon source, as was a gene promoter to reduce the noise in this specific reaction otherwise produced in conventional yeast expression medium. The screening assay was reliable, linear and sensitive, with a detectable response at 550 nm and a coefficient of variance around 10 % (**Figure 3**). Over 11,500 clones were explored in 3 rounds of directed and focused evolution combining IvAM, *ep*PCR, DNA shuffling and MORPHING (see Materials and Methods for details). The ultimate variant of the evolution

route, the KyLO mutant harboring G385D-H552N beneficial mutations plus two silent mutations G96G-Y132Y, improved the synthesis of C-N polydye at pH 8.0 ~2-fold after a second re-screening of the culture supernatants.

3.2 Biochemical Characterization

The KyLO mutant, the parental type IG-88 (both expressed in *S. cerevisiae*) and the wild-type MtL expressed in *A. oryzae* (its weak expression in yeast precluded its production and characterization) [16] were purified to homogeneity and characterized biochemically. The specific activities of the purified IG-88 and KyLO mutants were similar, although there was a significant difference in the total activity of the culture supernatants (69 U/mg and 1104 U/L as opposed to 62 U/mg and 2322 U/L, respectively). This enhanced activity reflected the 2.3-fold improvement in KyLO secretion (16 vs 37 mg/L for IG-88 and KyLO, respectively), apparently the best expression reported for a laccase in *S. cerevisiae*. The pH activity profile of these two variants was similar, irrespective of the substrate tested, maintaining up to ~25 % of their activity for catechol oxidation at pH 9.0 under conditions in which wild-type MtL activity was negligible (**Figure 4**). Given that the synthesis of the C-N polydye depends on the independent oxidation of catechol and 2,5-DABSA by the laccase, the kinetic parameters of these compounds were measured at both pH 4.0 and 8.0 (**Table 1**). The kinetics of the IG-88 and KyLO mutants were similar regardless of the substrate and pH, indicating that the 2-fold improved activity detected for the synthesis of the C-N polydye mainly reflected its enhanced secretion. Nevertheless, the catalytic efficiency (k_{cat}/K_m) for catechol of the KyLO mutant was 2.7 and 3.5-fold higher than that of wild-type MtL at pH 4.0 and 8.0, respectively, indicating that the alkaline mutations inherited from a previous

evolution campaign [15] were beneficial to this process (see the mutation analysis, below).

Independently of the laccase tested, there was a noticeable decrease in affinity for catechol at acidic pH (over 1 order of magnitude with respect to that at basic pH), in good agreement with previous studies for the oxidation of phenolic compounds by laccases at different pHs [13, 15, 21]. A 2.6-fold improvement in the k_{cat}/K_m during the oxidation of 2,5-DABSA at pH 4.0 was detected for KyLO relative to the wild-type MtL, although the poor laccase affinity towards 2,5-DABSA at pH 8.0 (well into the mM range) was beyond the threshold of substrate solubility, precluding reliable kinetic parameters from being obtained at this pH. We estimated a 2-fold increase for the initial turnover rates in the synthesis of the C-N polydye at pH 8.0 for KyLO vs wild-type-MtL.

Finally, the C-N polydye was produced, purified and preliminary characterized. To avoid enzyme contamination, the KyLO mutant was covalently bound to glyoxal agarose beads (46 ABTS U/g of carrier; operational stability of 93% of the initial activity after 7 batches of synthesis of C-N polydye) and used for the batch production of the C-N polydye (see Material and Methods for details). The biocatalyst was separated by filtration and the C-N polydye was purified for preliminary characterization. We isolated reaction products with a characteristic FTIR spectrum with indicative peaks of the C-N polydye, in good agreement with the literature [8, 9]).

3.3 Analysis of the mutations

In general terms laccases are structurally organized into three cupredoxin domains (D1, D2 and D3), with the main catalytic sites located

between D1 and D3 [22]. The enzyme contains four copper atoms organized at the T1Cu site (where the reducing substrate binds), and a trinuclear copper cluster -located 12 Å away from the T1Cu site- that is formed by one T2Cu and two T3Cu atoms, where O₂ is reduced to two molecules of H₂O [23].

The KyLO mutant is the genetic product of 23 rounds of directed evolution for secretion by yeast, organic co-solvent tolerance, alkalophilicity and now for the synthesis of C-N polydyes [24]. The 18 mutations introduced into KyLO in the course of these evolutionary campaigns were mapped in a homology model that is based on the crystal structure of the laccase from *Melanocarpus albomyces* (75% sequence identity to MtL, PDB 1GW0; **Figure 5A** [25-28]). In particular, two of the four mutations introduced in the current study were silent, while they favored codon usage that benefits expression by modifying the elongation rate (**Table 2**) [29]. The His552Asn mutation appears to arise at a mutational hot spot given the whole directed evolution history of MtL (**Figure 5B**) [24]. Indeed, the original Tyr552 was first replaced by an Asn during the directed evolution of MtL for functional expression in *S. cerevisiae* [19]. Subsequently, in the course of evolution for organic co-solvent tolerance [20], we introduced an Asn552His mutation, although this has since been reverted to Asn highlighting the significance of this substitution for functional laccase expression. The Gly385Asp mutation was located in the D3 domain, far from the relevant catalytic sites and without establishing any new relevant contact with surrounding residues (**Figure 5C**). We cannot find a reasonable explanation for the improved secretion associated to this change although a more adequate folding upon mutation cannot be ruled out.

As mentioned above, the evolved alkalophilicity of the IG-88 parental type was conserved in the KyLO variant, which was fundamental for the synthesis of the C-N polydye. Fungal laccases have a strong but reversible inhibitory effect at basic pHs which is thought to occur through the attachment of hydroxide ions to the trinuclear Cu cluster, abolishing intramolecular electron transfer from the T1Cu site [30]. A detailed inspection of our structural model showed that the N109S alkaline mutation inherited from IG-88 is located in the surroundings of the T2/T3 trinuclear Cu cluster (**Figure 5D**). The latter is connected to the protein surface via two opposite channels (at the back and front of the protein) involved in the entrance and exit of O₂ and H₂O [25, 26]. Any subtle modification in these environments could affect the transit of hydroxide ions to the T2/T3 cluster, enabling the enzyme to work at basic pHs. In this regard, computational simulations may shed light on hydroxide inhibition, which is currently under study.

4. Conclusions

In this study we have explored C-N heteropolymeric dye synthesis from catechol and 2,5-DABSA using an evolved alkaline fungal laccase secreted strongly by yeast. The alkaline pHs used in such a process permit high substrate loads to be used for dye-synthesis *in situ*, while promoting the activation of precursors for C-N hetero-coupling. Very recently, the laccase mutant was successfully transferred to the industrial host *Aspergillus oryzae* for overproduction with high titers while conserving its main biochemical properties (Dr Jesper Vind, Novozymes A/S, personal communication). As such, this mutant is becoming an attractive point of departure to address challenging reactions at alkaline pHs [6].

5. Acknowledgements

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FIGURE LEGENDS

Figure 1. Suggested reaction route for C-N polydye synthesis by laccase. (adapted from [9]).

Figure 2. Laccase benchmarking for C-N polydye synthesis. Reactions were performed in 100 mM Britton and Robinson buffer containing 25 mM catechol and 10 mM 2,5-DABSA at pH 5.0 and 8.0. The activity was normalized to that of the best variant at each pH value and the results shown are the means \pm S.D. from three independent experiments.

Figure 3. Validation of the colorimetric assay. **(A)** CV estimation and **(B)** linearity of the assay for the cross-coupling between catechol and 2,5-DABSA mediated by IG-88. The experiment was performed with individual clones of IG-88 parent in 96-well plates containing SEM and over 72 h. The activity was recorded at 550 nm in 100 mM Britton and Robinson buffer containing 25 mM catechol and 10 mM 2,5-DABSA at pH 8.0. **(C)** 96-well plate of a laccase mutant library screened using a catechol-2,5-DABSA colorimetric assay. Column 6 contained the parental IG-88 (internal standard) and well H1 the negative control (non-transformed *S. cerevisiae* cells).

Figure 4. The pH activity profiles for ABTS **(A)**, Catechol **(B)** and 2,5-DABSA **(C)**. The activity was measured in 100 mM Britton and Robinson buffer containing 2 mM ABTS, 25 mM catechol or 10 mM 2,5-DABSA at different pH values. Laccase activity was normalized to the maximum activity value and the results represent the mean \pm S.D. from three independent experiments.

Figure 5. The location of the mutations in a structural laccase model. **(A)** Model of the KyLO mutant based on *Melanocarpus albomyces* (75% sequence identity

to MtL: PDB 1GW0) [25-28]. Mutations to improve functional expression in *S. cerevisiae* are represented in pink [19], those for tolerance to organic co-solvents in blue [20], alkaline mutations in orange [15] and mutations introduced in the current study in green. **(B)**, **(C)** represent the mutations H552N and G385D, and their interactions with surrounding residues through H-bonds. **(D)** Location of the N109S mutation and distances to the T2/T3 trinuclear cluster (highlighted as yellow dotted lines that range from 9 to 13 Å). Cu atoms are depicted as blue spheres.

Supplementary Figure 1. Spectrum of reactions for catechol and 2,5-DABSA catalyzed by the IG-88 parental type at pH 5.0 **(A)** and 8.0 **(B)**. **(C)** Aspect of the reactions with laccase and the corresponding controls (in the absence of enzyme). Reactions were followed for 1 min at pH 5.0 or 8.0 in 100 mM Britton and Robinson buffer containing 25 mM catechol and 10 mM 2,5-DABSA.

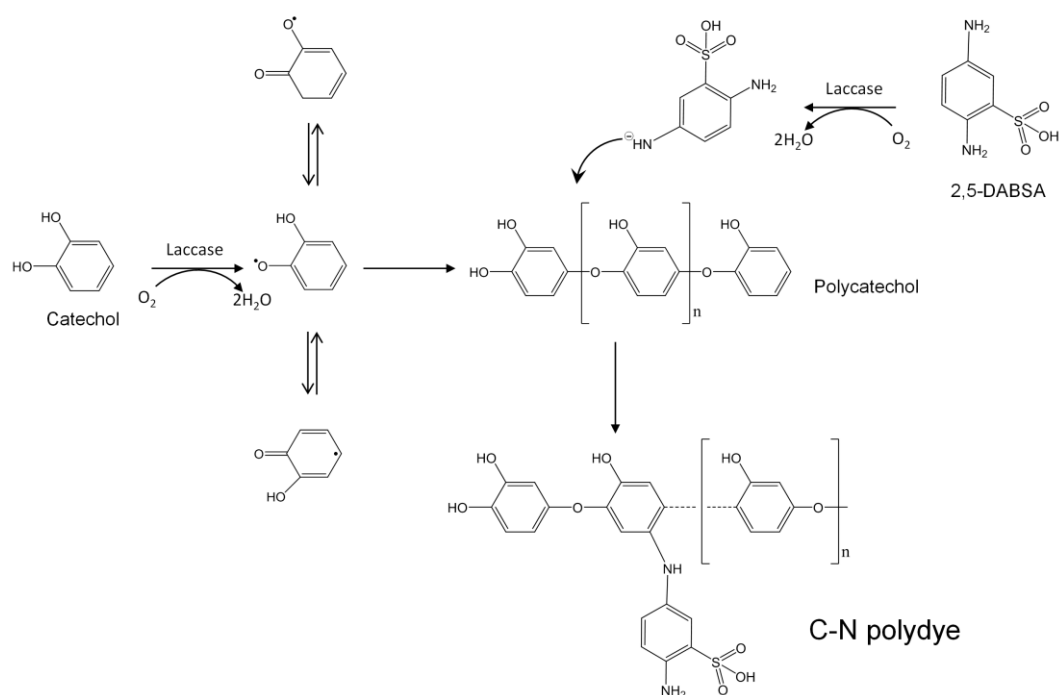


Fig. 1

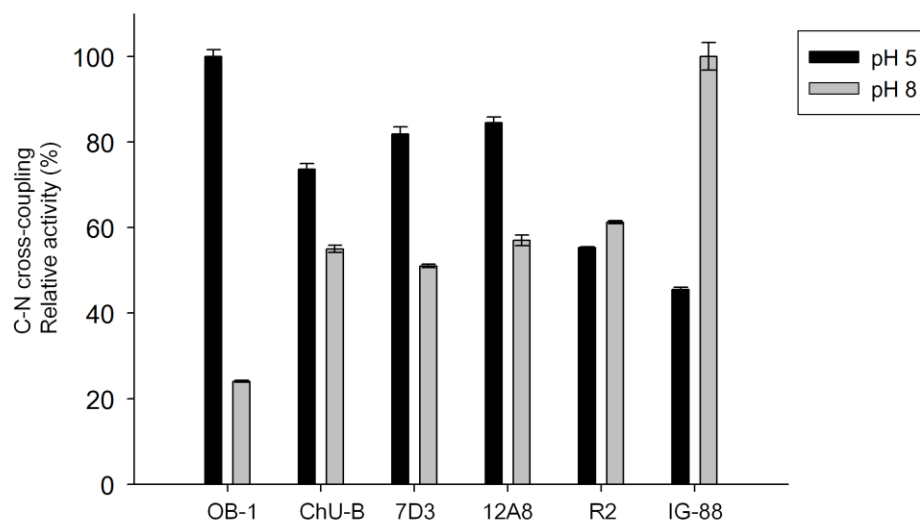


Fig. 2

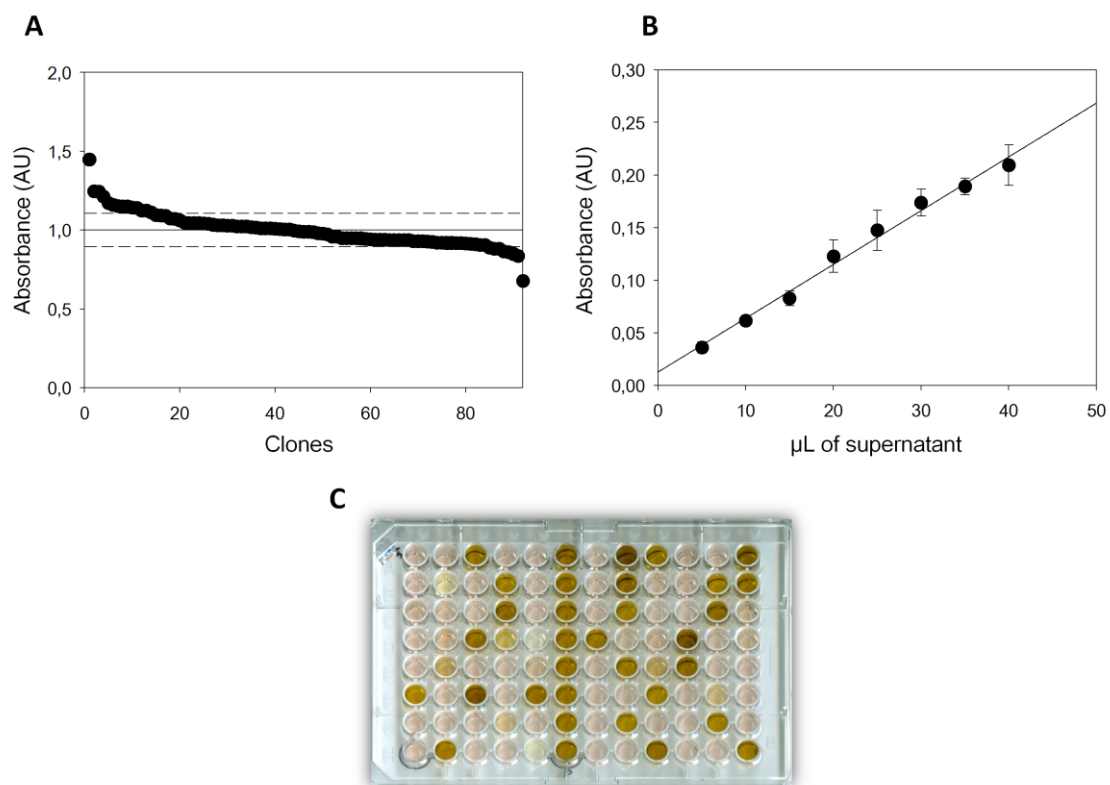


Fig. 3

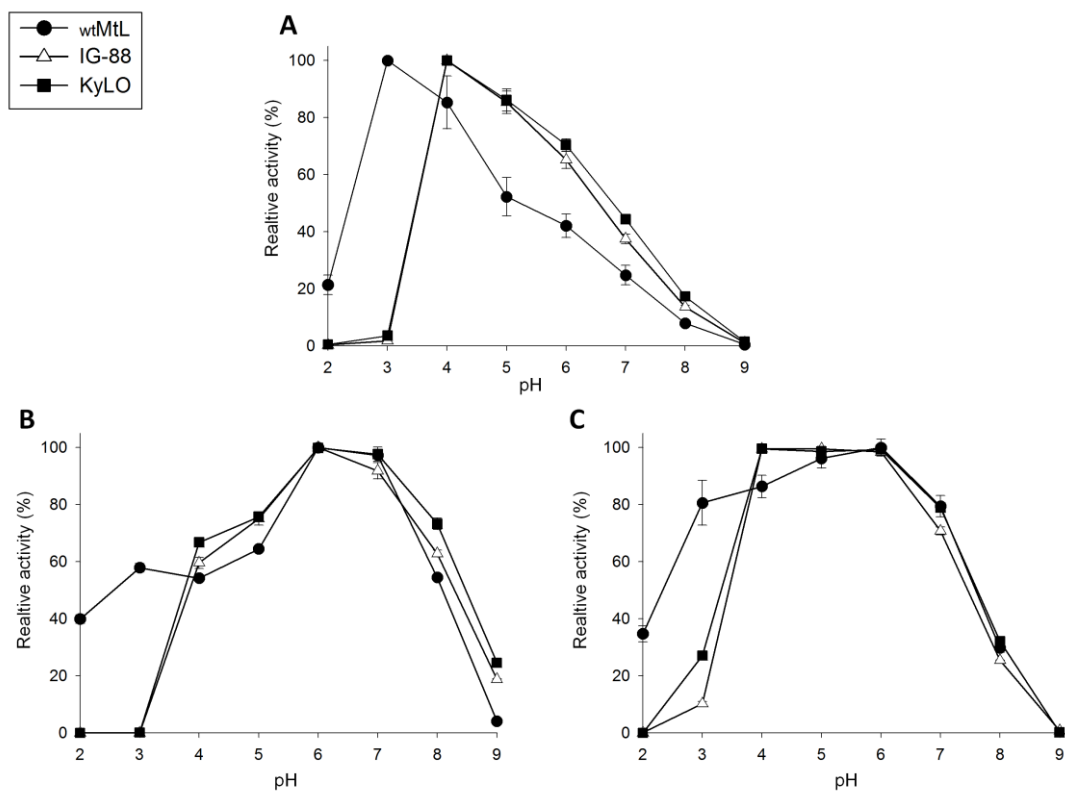


Fig. 4

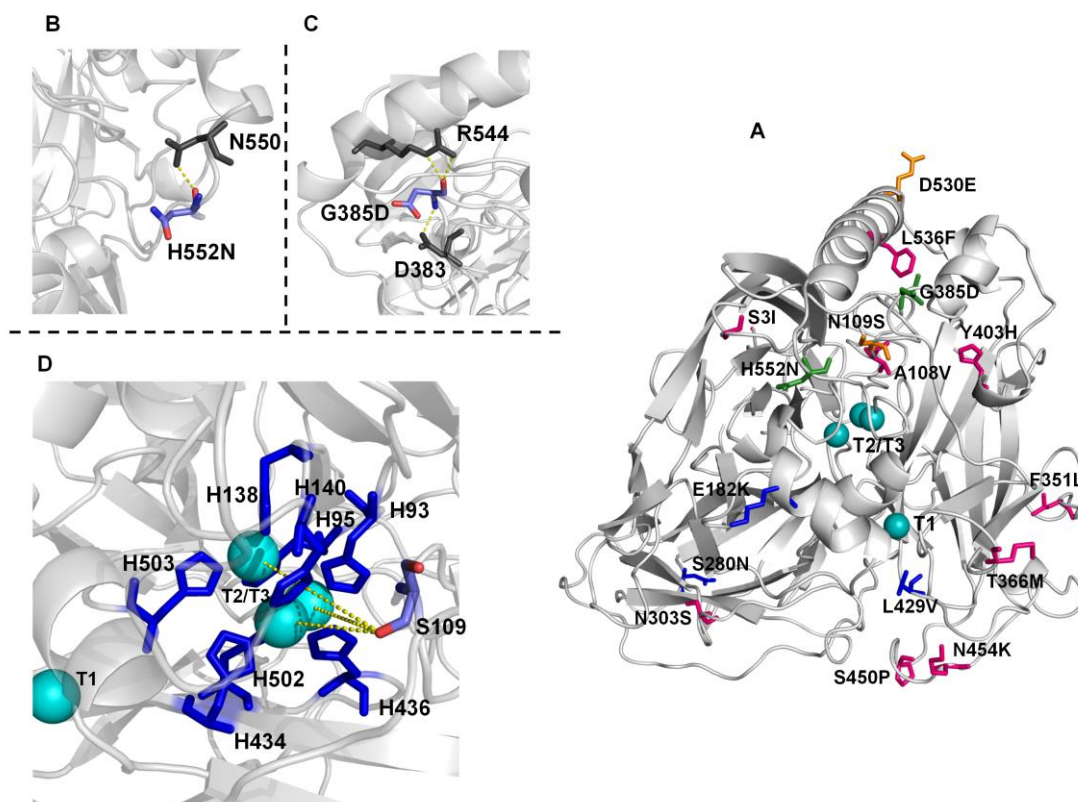
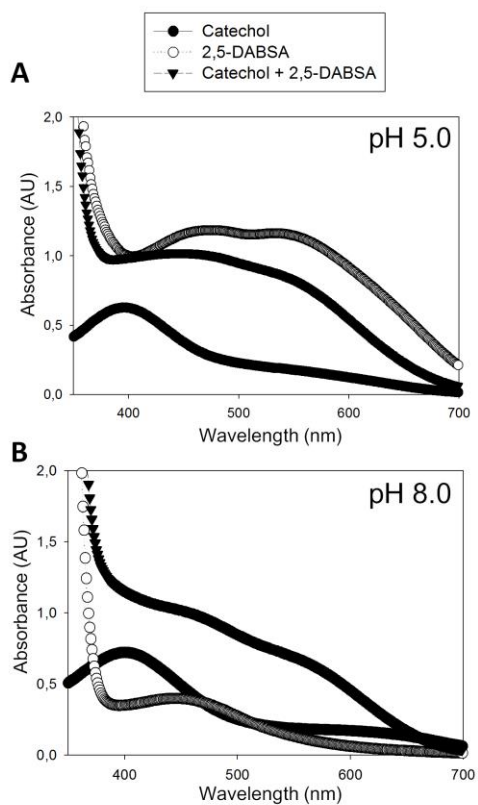


Fig. 5



Supplementary Figure 1

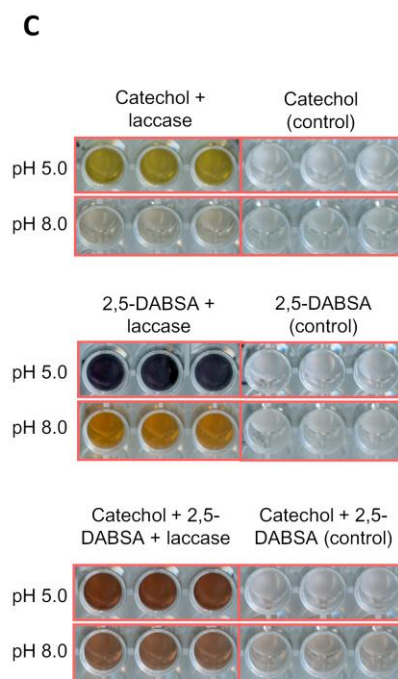


Table 1. Kinetic parameters of wild-type and evolved MtL variants

| pH | Substrate | Kinetic constants | w_t MtL | IG-88 | KyLO |
|-----|-----------|------------------------------------|----------------|----------------|----------------|
| | | K_m (μ M) | 2826 \pm 271 | 3200 \pm 333 | 3090 \pm 252 |
| 4.0 | Catechol | k_{cat} (s^{-1}) | 12.3 \pm 0.3 | 38 \pm 1 | 37 \pm 1 |
| | | k_{cat}/K_m ($mM^{-1} s^{-1}$) | 4.35 | 12 | 12 |
| | | K_m (μ M) | 951 \pm 32 | 291 \pm 137 | 284 \pm 118 |
| 8.0 | Catechol | k_{cat} (s^{-1}) | 4.5 \pm 0.1 | 5.1 \pm 1 | 4.8 \pm 1 |
| | | k_{cat}/K_m ($mM^{-1} s^{-1}$) | 4.8 | 17.6 | 16.9 |
| | | K_m (μ M) | 1145 \pm 147 | 1987 \pm 115 | 1638 \pm 125 |
| 4.0 | 2,5-DABSA | k_{cat} (s^{-1}) | 17 \pm 0.5 | 81 \pm 2 | 64 \pm 1 |
| | | k_{cat}/K_m ($mM^{-1} s^{-1}$) | 14.8 | 40 | 39 |
| | | K_m (μ M) | n.m. | n.m. | n.m. |
| 8.0 | 2,5-DABSA | k_{cat} (s^{-1}) | n.m. | n.m. | n.m. |
| | | k_{cat}/K_m ($mM^{-1} s^{-1}$) | n.m. | n.m. | n.m. |

*Kinetic constants were estimated in 100 mM Britton and Robinson buffer at the corresponding pH value. w_t MtL, MtL wild-type expressed in *A. oryzae*; n.m., not measurable. Catechol and 2,5-DABSA kinetics were measured at pH 4.0 since it is the optimum pH value for ABTS oxidation -used as control- by MtL variants.

Table 2. Mutations introduced in KyLO mutant.

| Amino acid substitution | Nucleotide substitution and codon usage | Domain | Secondary structure motif | Relative Position | Distance to the T1 Site (Å) | Distance to the T2/T3 (Å) | Interactions by hydrogen bonds with surrounding residues |
|-------------------------|---|--------|---------------------------|--|-----------------------------|---------------------------|--|
| G96G* | ₁₁ GGA/GGT ₂₅ | --- | --- | --- | --- | --- | --- |
| Y132Y* | ₁₅ TAC/TAT ₁₈ | --- | --- | --- | --- | --- | --- |
| G385D | GGC/GAC | D3 | Coil | Surface | 24.2 | 19.6 | R544, D383 |
| H552N | CAC/AAC | D3 | Coil | Surface, close to the H ₂ O and O ₂ channels | 25.6 | 25.1 | N550 |

*Silent mutation