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Multicopper oxidases with laccase-ferroxidase activity: Classification and study of ferroxidase activity determinants in a member from *Heterobasidion annosum s. l.*

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ABSTRACT

Multi-copper oxidases (MCO) share a common molecular architecture and the use of copper ions as co-factors to reduce O_2 to H_2O, but show high sequence heterogeneity and functional diversity. Many new emerging MCO genes are wrongly annotated as laccases, the largest group of MCOs, with the widest range of biotechnological applications (particularly those from basidiomycete fungi) due to their ability to oxidise aromatic compounds and lignin. Thus, comprehensive studies for a better classification and structure-function characterisation of MCO families are required. Laccase-ferroxidases (LAC-FOXs) constitute a separate and unexplored group of MCOs with proposed dual features between laccases and ferroxidases. We aim to better define this cluster and the structural determinants underlying putative hybrid activity. We performed a phylogenetic analysis of the LAC-FOXs from basidiomycete fungi, that resulted in two subgroups. This division seemed to correlate with the presence or absence of some of the three acidic residues responsible for ferroxidase activity in Fet3p from *Saccharomyces cerevisiae*. One of these LAC-FOXs (with only one of these residues) from the fungus *Heterobasidion annosum s. l.* (HaLF) was synthesised, heterologously expressed and characterised to evaluate its catalytic activity. HaLF oxidised typical laccase substrates (phenols, aryl amines and N-heterocycles), but no Fe (II). The enzyme was subjected to site-directed mutagenesis to determine the key residues that confer ferroxidase activity. The mutated HaLF variant with full restoration of the three acidic residues exhibited efficient ferroxidase activity, while it partially retained the wide-range oxidative activity of the native enzyme associated to laccases sensu stricto.

1. Introduction

The multicopper-oxidase (MCO) superfamily comprises oxidoreductase enzymes that use copper as cofactor to reduce O_2 to H_2O. This group includes ascorbate oxidases (EC 1.10.3.3), laccases (EC 1.10.3.2) and ferroxidases (EC 1.16.3.1) among others, according to their oxidative capabilities. Despite being widely distributed in different organisms (bacteria, fungi, plants or even insects), MCOs share a common molecular folding and conserved elements such as the catalytic coppers and the copper ligand residues involve in the electron transfer from the reducing substrate to the molecular oxygen [1–3]. Typically, the protein (monomeric) harbours four catalytic coppers classified according to their spectroscopic and magnetic features: one Type 1 (T1) copper, and the trinuclear cluster consisting of a nuclear Type 2 (T2) and the coupled-binuclear Type 3 (T3) copper centre. The electrons from the one-electron oxidation of substrate at the T1 site are transferred via a conserved amino acid bridge (His-Cys-His) to the trinuclear cluster T2/T3, where the four-electron reduction of O_2 to H_2O takes place [1,3,4]. Concerning substrate specificity, MCOs such as laccases, generally act on a broad spectrum of aromatic molecules, except for ferroxidases like Fet3p or ceruloplasmin, which are mostly specific for Fe (II) oxidation [3].

The wide distribution in nature and non-specific activity remark the pivotal role of MCOs in many physiological processes. For instance, well-known is the function of laccases in different organisms. Fungal laccases can be involved in lignin biodegradation,

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morphogenesis, pigment production or plant pathogenesis [5–7], whereas bacterial laccases facilitate resistance and pigment formations [8], and those found in plants are associated to wound response and lignin synthesis [9]. Ferroxidase activity found in ceruloplasmin and Fet3p is critical for iron uptake and metal homoeostasis in mammals [10] and fungi [11], respectively, whereas ascorbate oxidase seems to influence growth and regulate the redox state in some plants [12]. However, none of these MCOs has concerted as much interest as laccases, especially those from fungal sources.

Fungal laccases are the deepest studied MCO enzymes due to their ability to oxidase a broad range of aromatic substrates, including lignin, substituted phenols, aryl amines or N-heterocycles, making them versatile biocatalysts for a range of biotechnological purposes over other MCO members. Their applicability potential ranges from the pulp & paper industry for pulp bleaching and pitch control, to bioremediation and green chemistry [13–15]. In nature, they take part in the complex protein toolkit of most plant polymer decomposer fungi, widespread among different eco-physiological groups. They are found in wood-rotting fungi able to overcome the oxidation of recalcitrant lignin, as well as encoded in the genomes of soil-litter fungi which inhabit grasslands or forests [16]. Unlike their bacterial and plant counterparts, many basidiomycete laccases are high-redox potential enzymes (E° > 720 mV). To some extent, this is due to the trigonal coordination of T1 copper (by one cysteine and two histidine residues) found in fungal laccases, with a non-coordinating phenylalanine or leucine in the position of the fourth-axial methionine ligand that appears in lower redox potential laccases of bacterial or plant sources [17,18]. However, this is not the only determinant in the redox potential of laccases [19–21].

In the last years, significant efforts have been made to classify MCO superfamily, either differentiating the MCO families by their substrate oxidation activity [22], or using sequence homology-based methodologies [2, 16, 23, 24]. However, due to the overlapping activities and the low sequence homology among some MCO enzymes, a comprehensive classification of this enzyme superfamily is still a challenge. Access to the ever-increasing amount of fungal genome data generates many new MCO entries that are automatically annotated as laccases, even though they await for experimental verification. In a pioneering comparative genomic and phylogenetic work, the enzymatic toolkits of 52 basidiomycete genomes from the Joint Genome Institute (JGI) MycoCosm portal were correlated with their fungal lifestyles [16]. In that work, the large diversity of MCO sequences assembled in diverse emerging clusters, some of them according to already-reported functional families, but also to new ones that we described for the first time.

One of the already known MCO groups corresponds to laccase-ferroxidase (LAC-FOX) cluster that have some of the metal binding determinants found in ferroxidases [16]. LAC-FOX family was already described in previous phylogenetic studies [2,24] and some of their members were functionally expressed [25–27]. LAC-FOXs were proposed as hybrid enzymes with dual catalytic properties between laccase and ferroxidase, which were first found in a MCO gene from the opportunistic fungal pathogen *Cryptococcus neoformans*, whose ferroxidase activity was associated to a virulence factor [25,28]. Then, Larrondo and co-workers shed light on these catalytic combined features when they found the MCO1 gene in the polyporal fungal *Phanerochaete chrysosporium,* which, apart from oxidising some typical laccase substrates, also displayed a strong ferroxidase activity [26]. In addition, a similar hybrid activity was found in the fungal *Phanerochaete flavido-alba*, with a weaker reactivity toward Fe (II) than MCO1, but showing higher activity towards phenols and aryl amines [27]. Apparently, some of the catalytic determinants that contribute to Fe (II) oxidation in the extensively studied ferroxidase Fet3p from *S. cerevisiae* are harboured by these characterised LAC-FOXs. Fet3p is a transmembrane ferroxidase essential for a high affinity iron uptake system in the yeast [11]. Its efficient ferroxidase activity underlies in three acidic residues: E185, D283 and D409. Their negative charged side chains are orientated towards the T1 copper, contributing to both the substrate binding and the electron transfer to the T1 site [29]. The complementary role of these amino acids in ferroxidase activity has been confirmed by the detrimental effect that their removal had on the catalytic constants of Fet3p [29,30]. Additionally, a fourth residue (Y354) has been proposed as responsible for iron binding to a lesser extent [31,32].

With the aim of deepening in the structure-function of LAC-FOX family, we carry out here a phylogenetic analysis of reported basidiomycete LAC-FOXs and we give a comprehensive classification of this cluster based on signature residues responsible for ferroxidase activity. Moreover, we addressed the characterisation of a new LAC-FOX from *Heterobasidion annosum s. l.* genome, annotated with JGI ID: Hetan2–157048 [33]. This basidiomycete species was classified in the eco-physiological group of white-rot fungi [16], organisms capable of efficiently degrading the complex lignin polymer to access sugars of cellulose and hemicellulose [34]. The *H. annosum s. l.* LAC-FOX (HaLF) was found to be highly up-regulated in the transcriptome of the fungus grown on lignin and heartwood wood [35]. Moreover, HaLF seemed to have a possible role as a virulence factor during Scots pine seedling colonisation [36]. In this study, the HaLF coding sequence (CDS) was synthesised, mutated, and the corresponding variants were heterologously expressed and characterised.

2. Material and methods

2.1. Reagents and strains

Yeast Transformation Kit, 2,6-Dimethoxyphenol (DMP), Guaiacol (GUA), Vanillin (VAI), 4-Hydroxybenzaldehyde (HBA), Syringaldehyde (SYR), p-Coumaric acid (PCA), Ferulic acid (FA), Sinapic acid (SA), N,N-Dimethyl-1,4-phenylenediamine (DMPD), p-Phenylenediamine (PPD), 1,2-Phenylenediamine (OPD), Aniline (ANL), o-Dianisidine (ODN), Remazol Brilliant Blue R (RBB), Evans Blue (EB), Aniline Blue (AB), Methyl Orange (MO), Reactive Black 5 (RB5), Iron (II) Sulphate, Manganese (II) Sulphate and FerroZine Iron Reagent were purchased from Sigma-Aldrich. High Pure Plasmid Isolation Kit and 2,20-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were obtained from ROCHE. Phusion High Fidelity DNA polymerase and Restriction enzymes were purchased from New England Biolabs. QIAquick gel extraction kit from Qiagen. Zymoprep Yeast Plasmid Miniprep II was purchased from Zymo Research. *S. cerevisiae* BJ5465 strain was purchased from LGC Promochem and *A. oryzae* strain from Novozymes A/S. *H. annosum s. l.* genome, belongs to the *Heterobasidion irregulare* TC31–2 strain accessible in MycoCosm portal from JGI.

2.2. Multiple sequence alignment and phylogenetic tree

The predicted amino acid sequences of LAC-FOXs were selected from 52 Basidiomycete genomes studied by Ruiz Dueñas and co-workers [16] (available at the DOE JGI MycoCosm portal) and from previously characterised LAC-FOXs [25–27]. Laccases from *Pycnoporus Cinnabarinus* (JGI: Pycci1-8672, PDB 2XYB), *Trametes versicolor* (JGI: Trave1-138261, PDB 1KYA), and *Pleurotus ostreatus* (JGI: PleosPC15-2-1067328, POXA3) were used as outgroups. The sequences were aligned with MAFFT v7 [37] (default options) and trimmed by removing poorly aligned regions using trimAI v1.4 (gappyout method) [38]. The multiple sequence alignment (MSA) was tested with ProtTest 3.4.2 [39] to obtain the evolutionary model that best fits the data based on the Akaike Information Criterion (AIC). Then, RAxML-NG was used for maximum-likelihood analysis [40] applying Whelan and Goldman evolution model with gamma-distributed rate of heterogeneity, empirical amino acid frequencies

from alignment and invariant sites (WAG + I + G + F). Best-scoring mL tree was found after conducting 20 tree searches (10 random and 10 parsimony-based starting trees) and branch supports of the best mL tree were estimated by 500 bootstrap replicates.

2.3. 3D-model structure

The CDS of LAC-FOX from *H. annosum s. l* (JGI ID: Hetan2-157048) without the native predicted signal peptide (predicted by SignalP 5.0, [41]) (Fig. S1) was used to model the 3D-structure of the protein via ColabFold web [42], using the AlphaFold2_mmseqs2 notebook, powered by AlphaFold2 [43] and combined with the fast multiple sequence alignment generator, MMseqs2.

2.4. Cloning and variants construction

The HaLF CDS (Fig. S1) was synthesised substituting the predicted signal peptide by the preproleader sequence of the α-mating factor of *S. cerevisiae*. The gene was cloned in the pJRoC30 bifunctional vector by ligation reaction using *Bam*HI/ *Not*I cleavage sites. For single, double and triple mutant variants, successive site-directed mutagenesis were carried out, using native HaLF sequence as template for single constructions (A227E, F344D, R422Y and D475A) and adding subsequent mutations for the double (A227E, F344D) and triple (A227E, F344D, R422Y) variants. For each mutated site, two fragments were obtained by PCR: one with the ExtFw sense and specific mutation antisense primers and the second with the specific mutation sense and ExtRv antisense primers (Table S1). Products were co-cloned in *S. cerevisiae* by in vivo overlap extension (IVOE) [44].

2.5. Flask fermentation and purification

Enzyme production in *S. cerevisiae* flask cultures was performed as previously reported [45]. Enzyme overexpression in *A. oryzae* [46] was carried out at Novozymes A/S, Denmark, in standard MDU media supplemented with 2 mM $CuSO_4$.

Enzyme purification was performed in 4 steps in FPLC (AKTA purifier, GE Healthcare); (i) desalting column G25 (20 mM Tris-HCl, pH 7); (ii) anion exchange Source 15 (37 mL) using a 0–50 % salt gradient (20 mM Tris-HCl, 1 M NaCl, pH 7) to elute the LAC-FOXs; (iii) anion exchange Mono Q 5/50 GL column (GE Healthcare), using a 0–50 % salt gradient (20 mM Tris-HCl, 1 M NaCl, pH 7) to elute the enzymes; (iv) molecular exclusion column HiLoad 16/600 Superdex 75 pg (GE Healthcare).

2.6. Ferroxidase assay

Ferroxidase activity was evaluated by ferrozine-based colorimetric assay (Absorbance at 560 nm) following the decrease in free Fe (II) iron as proportional indicator of Fe (III) production.

First, the molecular extinction coefficient of ferrozine–ferrous ion complex was determined and the conditions of the assay were established. The non-enzymatic oxidation of Fe (II) (substrate as Iron (II) Sulphate) was tested under different conditions, being Acetate Phosphate (AcNa) pH 5 selected as buffer for assays (data not shown). The Molecular extinction coefficient of ferrozine–ferrous ion complex (27,300 M^{-1} cm^{-1}) and linearity of the assay were calculated from range 2.7–164 μM Fe (II) (Fig. S2) in 96-well plates by adding a different Fe (II) concentrations to a final volume of 180 μL (100 mM AcNa pH 5). Then, 20 μL of ferrozine (20 mM) were quick added and, after 1 min, absorbance at 560 nm was measured by SpectraMax M2 microplate reader.

Activity assay: Prior to ferroxidase activity assay, crude/purified enzyme was dialysed in H_2O Milli-Q in order to remove chemical contaminants or other chelating agents from the sample. Additionally, aliquots of HaLF and variants were denatured under heat treatment and used as negative controls to evaluate possible remaining chemical oxidation of Fe (II) during the assay. After this, 20 μL of enzyme solution stock at 10–50 U/mL ABTS activity were added to a mixture reaction of 160 μL AcNa at pH 5 (final concentration in well: 100 mM) and Fe (II) (final concentration in well: 100 μM). For calculating the decrement of absorbance at 560 nm along time (ΔAbs/min), 20 μL of ferrozine (stock 20 mM) are put on, stopping ferrous iron oxidation at different times. Absorbance was measured at end-point mode after 1 min with SpectraMax M2 reader. Afterwards, slope decrements (ΔAbs/min) were obtained in triplicate.

Kinetic constant associated to ferroxidase activity were calculated in 96-well plates using a substrate range from 5 μM to 150 μM of Fe (II) and 100 mM AcNa buffer at pH 5. Different substrate concentrations were set to a final volume of buffer 160 μL. Then 20 μL of purified enzyme was added stopping reaction with 20 μL ferrozine (stock 20 mM) at different times to record absorbance decrement. To calculate K_M and k_{cat} values the average V_{max} was represented versus substrate concentration and fitted to a single rectangular hyperbola using SigmaPlot software (version 14.5).

2.7. Activity and stability assays

2.7.1. Pre-characterisation of non-purified enzymes produced in S. cerevisiae

Enzymatic assays with non-purified enzymes produced in *S. cerevisiae* were carried out in 96-well plates and measured in a SpectraMax M2 plate reader. ABTS activity (ε_{418} = 36,000 M^{-1} cm^{-1}) was calculated in 100 mM Citrate-Phosphate (CP) pH 3 and 20 mM of substrate concentration for every LAC-FOX. Activities with DMP (ε_{469} = 27,500 M^{-1} cm^{-1}) and DMPD (ε_{550} = 4134 M^{-1} cm^{-1}) were determined using 20 μL of enzyme solution at 1 U/mL with ABTS added to 180 μL of 20 mM of DMP at 100 mM CP pH 5 or of 20 mM of DMPD at 100 mM CP pH 4. Respective activities were calculated by the increments in absorbance and normalised to the activity with ABTS (taken as 100 %). T50 (10 min) assay, the temperature at which the enzyme retains 50 % of the initial activity after incubation for 10 min, was performed as previously described in Aza and co-workers [45] but using 20 mM ABTS.

2.7.2. Characterisation of purified enzymes produced in A. oryzae

Determination of long-term pH stability and optimal pHs activity were performed as described in Aza et al. [45], excepting for some details as follows: the substrates concentration for ABTS was 20 mM, long-term pH stability was performed at 20 °C and ABTS activity was measured in Citrate-Phosphate (CP) pH 4.

For the long-term thermostability assays, dilutions were prepared in 20 mM AcNa buffer, pH 5 to a final concentration of 1 U/L ABTS at 30 or 40 °C. At times 0.5, 1, 3 and 6 h, 20 μL of enzyme stocks are added by triplicate to 96-well plates and after being tempered to RT, 180 μL of 20 mM ABTS in CP pH 4 is added to measured residual activity.

Kinetic constants were determined using a range from 0.01 mM to 70 mM for ABTS; 0.15–20 mM for DMP; 0.15–80 mM for GUA (ε_{470} = 26,600 M^{-1} cm^{-1}); 0.2–40 mM for DMPD; 0.2–70 mM for OPD (ε_{460} = 11,300 M^{-1} cm^{-1}) and in 100 mM CP buffer at pH 4 for ABTS and amines, and pH 5 for phenols. To calculate K_M and k_{cat} values the average V_{max} was represented versus substrate concentration and fitted to a single rectangular hyperbola or Hill sigmoid function in SigmaPlot (version 14.5). In all assays three replicates of each LAC-FOX variant were used.

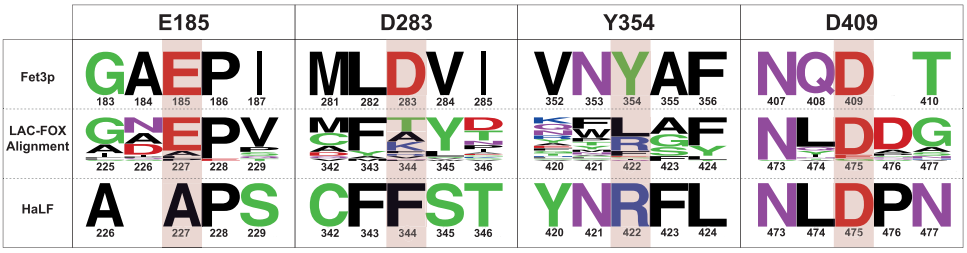

Fig. 1. Amino acid frequency plot of selected regions of the multiple sequence alignment of 34 fungal LAC-FOX compared with the sequences of Fet3p ferroxidase from *S. cerevisiae*, and the LAC-FOX from *H. annosum s. l.* (HaLF). Residues E185, D283, Y354 and D409 correspond to Fet3p numbering.

2.8. Multi-screening assay

Stock solutions were routinely prepared in 0–100 % ethanol and Milli-Q H_2O. All reactions were performed in the same fashion excepting for Fe (II) (described in Section 2.6) and Mn (II) oxidation [47]. Reactions in triplicate were carried out in 96-well plates (UV-Star 96-well plates when needed) by adding 20 µL of pure diluted enzyme to a mixture reaction of 160 µL of CP buffer (final concentration in well: 100 mM) at pH 4 for azo dyes and aromatic amines, and pH 5 for phenols and Mn (II). Then 20 µL of stock substrate solution were added in each well. The non-enzymatic oxidation of every substrate was also tested as negative controls. Absorbance changes for different substrates were measured in kinetic mode in SpectraMax M2 reader at the corresponding wavelengths (Table S2). The (ΔAbs/min)/protein (mg) was calculated for every substrate and enzyme, and later normalised to parental values. Results were represented in a heat map using R program (ggplot2 package).

2.9. Protein amount

Protein was quantified by using a Qubit 3.0 fluorometer of Sigma-Aldrich.

3. Results

3.1. Phylogenetic analysis of LAC-FOX enzymes

We performed a multiple sequence alignment of the 34 basidiomycete LAC-FOXs described so far, including the 31 sequences we previously identified from 52 fungal genomes [16], among which were the four MCO genes from *P. chrysosporium* [26]. We also incorporated sequences of functionally expressed enzymes like the LAC-FOX from *C. neoformans* [25], together with another sequence encoded in the same fungus with which shared high homology, and the *P. flavido-alba* LAC-FOX [27]. Then, a sequence frequency plot was constructed for all LAC-FOXs (Fig. 1) to explore the amino acid conservation at positions equivalent to the residues that allow Fe (II) oxidation in the canonical ferroxidase Fet3p, E185, D283 and D409 [29], and additionally Y354 [31,32]. About 28 LAC-FOXs harboured a glutamic acid residue equivalent to E185 or in an adjacent position (Table S3), whereas the other sequences had a non-polar residue occupying this position. Like E185, D409 was highly conserved in every LAC-FOX excepting for PfaL (*P. flavido-alba*) and MCO4 (*P. chrysosporium*) genes (Table S3). By contrast, none of the 34 sequences studied had equivalent residues to D283 or Y354, showing high variability in the amino acid distribution on these two positions.

The phylogenetic analysis of LAC-FOX sequences showed two main branches (Fig. 2). When analysing the residues occupying the positions related to ferroxidase activity, we found that a notable difference between both subgroups was the presence or absence of the residue equivalent to E185. Every sequence classified in subgroup 1 held this glutamic acid, in contrast to the sequences clustered in subgroup 2 in which this position was occupied by an alanine or a proline (Fig. 2 and Table S3). In general, sequences clustered in subgroup 1 had E185 and D409 equivalents (only the close-related sequences PfaL and MCO4 lack the D409 equivalent), whereas sequences from subgroup 2 only harboured the residue equivalent to D409 (Fig. 2 and Table S3).

As the most well-described LAC-FOX enzymes belong to subgroup 1, e.g MCO1 from *P. chrysosporium* [26] and PfaL from *P. flavido-alba* [27], we selected for further characterisation an enzyme member from the unexplored subgroup 2. The sequence with JGI ID: Hetan2–157048, encoded in the genome of the white-rot fungus *H. annosum s. l.* was selected as a representative member of the emerging LAC-FOX subgroup 2. In addition, this species belongs to the poorly studied Russulales order [16].

3.2. Preliminary evaluation of HaLF and ferroxidase activity determinants

The CDS of HaLF, without its predicted signal peptide (Fig S1), was synthesised fused to the α-factor preproleader of *S. cerevisiae* for expression in the yeast. The molecular modelling of HaLF was obtained by AlphaFold2 algorithm, via ColabFold web [42]. The modelled structure exhibited an average per-residue confidence of 88, measured as predicted local distance difference test (pLDDT), indicating the accuracy of the model.

According to the multiple LAC-FOX alignment (Fig. 1), and the comparison of the 3D-structure model of HaLF with the crystal structure of Fet3p (PDB: 1ZPU), residues A227, F344, R422 and D475 of HaLF were respectively identified as equivalent to E185, D283, Y354 and D409 in Fet3p (Fig. 3).

Given native HaLF already got one acidic amino acid, D475 (D409 in Fet3p numbering), but lacked the residues equivalent to E185, D283, Y354, new mutated variants were designed to test the contribution of all these residues to ferroxidase activity in the enzyme, while laccase activity was also evaluated. A total of six mutated HaLF variants were obtained in *S. cerevisiae*: $HaLF_{A227E,\ F344D}$, harbouring the three acidic residues; $HaLF_{A227E,\ F344D,\ R422Y}$, holding the four ferroxidase catalytic determinants; $HaLF_{D475A}$, with none of them; and three single-mutated variants introducing individually the acidic residues ($HaLF_{A227E}$ and $HaLF_{F344D}$) or the tyrosine ($HaLF_{R422Y}$).

Native HaLF and its mutated variants were produced in *S. cerevisiae* flask cultures at 28 °C. After 96 h, maximum laccases activities were detected in the liquid extracts with ABTS. Laccase activity greatly varied among the different HaLF variants. Native HaLF and variant $HaLF_{F344D}$ showed the highest activities (around 800 U/L), but for the rest of the variants the activities detected were very low (below 36 U/L) or even null (Table S4). Therefore, a second set of fermentations was carried out at 20 °C in order to enhance laccase expression/secretion (Table S4). Laccase activity and cell growth were monitored for 144 h (Fig. 4). The highest activity levels were found for native HaLF (3208 U/L), followed by the single mutated

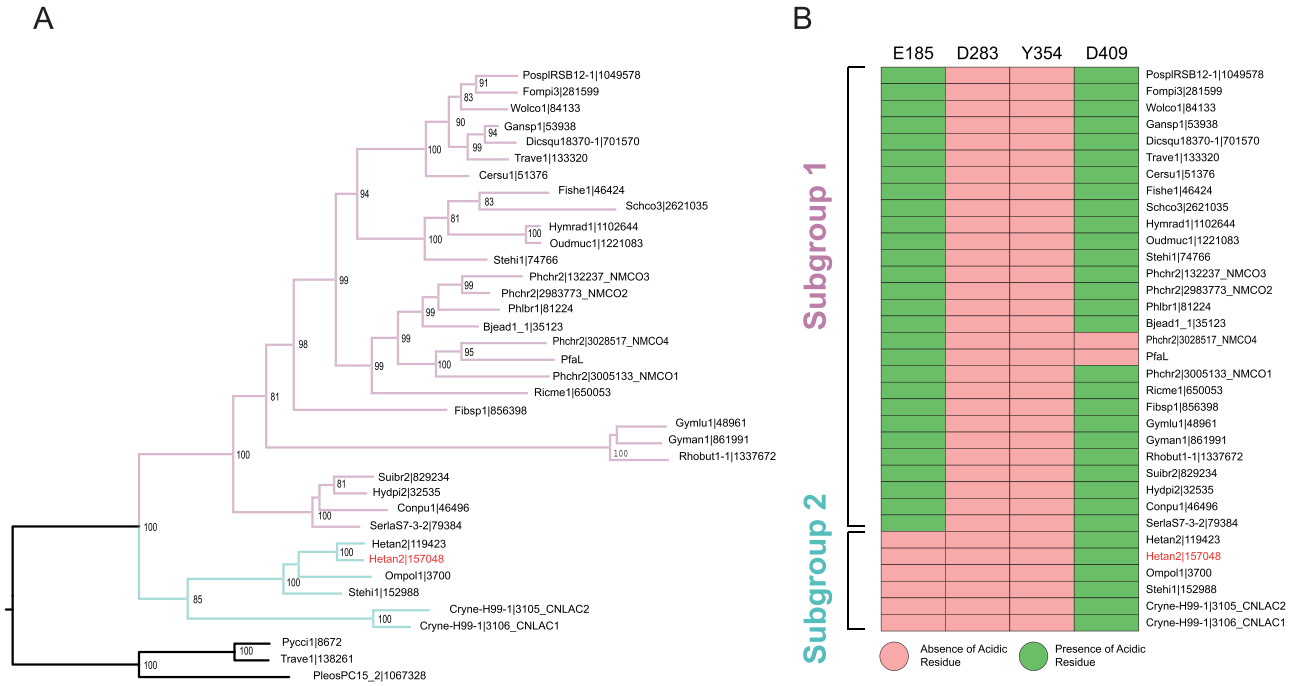

Fig. 2. Phylogenetic tree (**A**) and amino acid residues equivalent to E185, D283, Y354 and D409 in ferroxidase Fet3p (**B**) of the 34 basidiomycete LAC-FOX sequences studied here. LAC-FOX cluster is spliced in two: subgroup 1 comprises sequences mostly holding the two acidic residues equivalent to E185 and D409, and subgroup 2 comprises sequences with only the D409 equivalent (see Table S3). The selected *H. annosum s. l.* enzyme from subgroup 2 is highlighted in red. *P. cinnabarinus*, *T. versicolor* and *P. ostreatus* laccases were used as outgroups.

variants $HaLF_{F344D}$ (1607 U/L) and $HaLF_{R422Y}$ (713 U/L). Variants $HaLF_{A227E,\ F344D}$ (with the three acid residues), $HaLF_{A227E}$ (with the two main acidic residues equivalent to E185 and D409) and $HaLF_{D475A}$ (with no acidic residue) showed similar lower activities (around 300 U/L). Finally, variant $HaLF_{A227E,\ F344D,\ R422Y}$ had no activity with ABTS. All HaLF variants showed a similar growth rate, pointing out there was no influence of yeast growth on the expression/secretion of the different enzyme variants (Fig. 4).

Cultures were filtered and the liquid extracts concentrated to an equal final volume for the pre-characterisation of the enzymatic crudes. Laccase activities with DMP (as a model phenolic compound), and DMPD (as a model aryl amine), were referred to the activity of the enzyme with ABTS to rule out the influence of the amount of enzyme produced (Table 1). In general, the enzyme variants oxidised DMP considerably worse than ABTS, except for variant $HaLF_{R422Y}$. As for DMPD, while native HaLF and variant $HaLF_{D475A}$ showed similar activities with DMPD, the activity of variant $HaLF_{A227E}$ was 6 fold higher with DMPD, and those of $HaLF_{A227E,\ F344D}$ and $HaLF_{R422Y}$ were 3 fold higher. Only the activity of the $HaLF_{F344D}$ variant halved with DMPD with respect to its activity with ABTS. Variant $HaLF_{A227E,\ F344D,\ R422Y}$ did not show activity with any substrate. Finally, ferroxidase activity was measured using the colorimetric ferrozine assay, in which changes in the absorbance of the ferrozine-ferrous ion complex are followed at 560 nm (Table 1). Because of the difficulties that underlie the measurement of ferroxidase activity due to Fe (II) auto-oxidation, an exhaustive protocol with different controls was carried out to eliminate the background produced by the non-enzymatic oxidation of iron (see Section 2). No Fe (II) oxidation was detected in any HaLF variant except for $HaLF_{A227E,\ F344D}$, which had fully restored the three acidic residues. Despite $HaLF_{A227E,\ F344D,\ R422Y}$ had also the three acidic amino acids, no ferroxidase activity could be observed, probably due to its inefficient recombinant expression/secretion.

A T50 (10 min) assay was carried out to test the influence of the aforementioned substitutions in the thermotolerance of HaLF (Table 1). All mutations had a detrimental effect on the enzyme thermotolerance. Only, $HaLF_{F344D}$ exhibited a similar T50 to the native enzyme.

3.3. Overproduction in *A. oryzae* and characterisation of purified HaLF variants

Since the protein yields obtained in *S. cerevisiae* were insufficient to carry out the purification of laccases, native HaLF and mutated variants were produced in *A. oryzae* in flask cultures, except for $HaLF_{A227E,\ F344D,\ R422Y}$ and $HaLF_{D475A}$ variants. The first variant was not included because it showed no functional expression, while $HaLF_{D475A}$ was discarded because it showed similar activity profile to native HaLF (no ferroxidase activity and similar laccase activity), but notably lower production levels and reduced thermotolerance.

The activity levels (measured with ABTS) detected in the liquid extracts after 96 h largely surpassed (over 10 fold) those found in *S. cerevisiae* (Table S4). The enzymes were purified to homogeneity and characterised.

First, we examined the activities of the different HaLF variants as a function of pH. All of them showed a clear peak of activity at pH 4 for oxidation of ABTS (Fig. 5A), and maximum activity at pH 5 for oxidation of DMP, except for variant $HaLF_{F344D}$ with a broad maximum at pH 3–5 (Fig. 5B). Only, $HaLF_{F344D}$ displayed maximum activity at pH 4 with DMP.

Native HaLF and mutated variants were subjected to a multi-activity assay with 20 compounds (Fig. 6). These compounds are representative substrates to evaluate laccase and ferroxidase activities: model derived-lignin phenols, aryl amines and azo dyes, some of which have high redox potential, and metal ions (Table S2). Substrate oxidation was expressed as the change in maximum absorbance along time divided by the amount of protein added in the reaction (ΔAbs/mg). Then, the activities of the mutated variants were normalised to the activity of the native HaLF for each compound to assess the increases and decreases in activity due to the introduction of the different mutations. The oxidation capabilities of

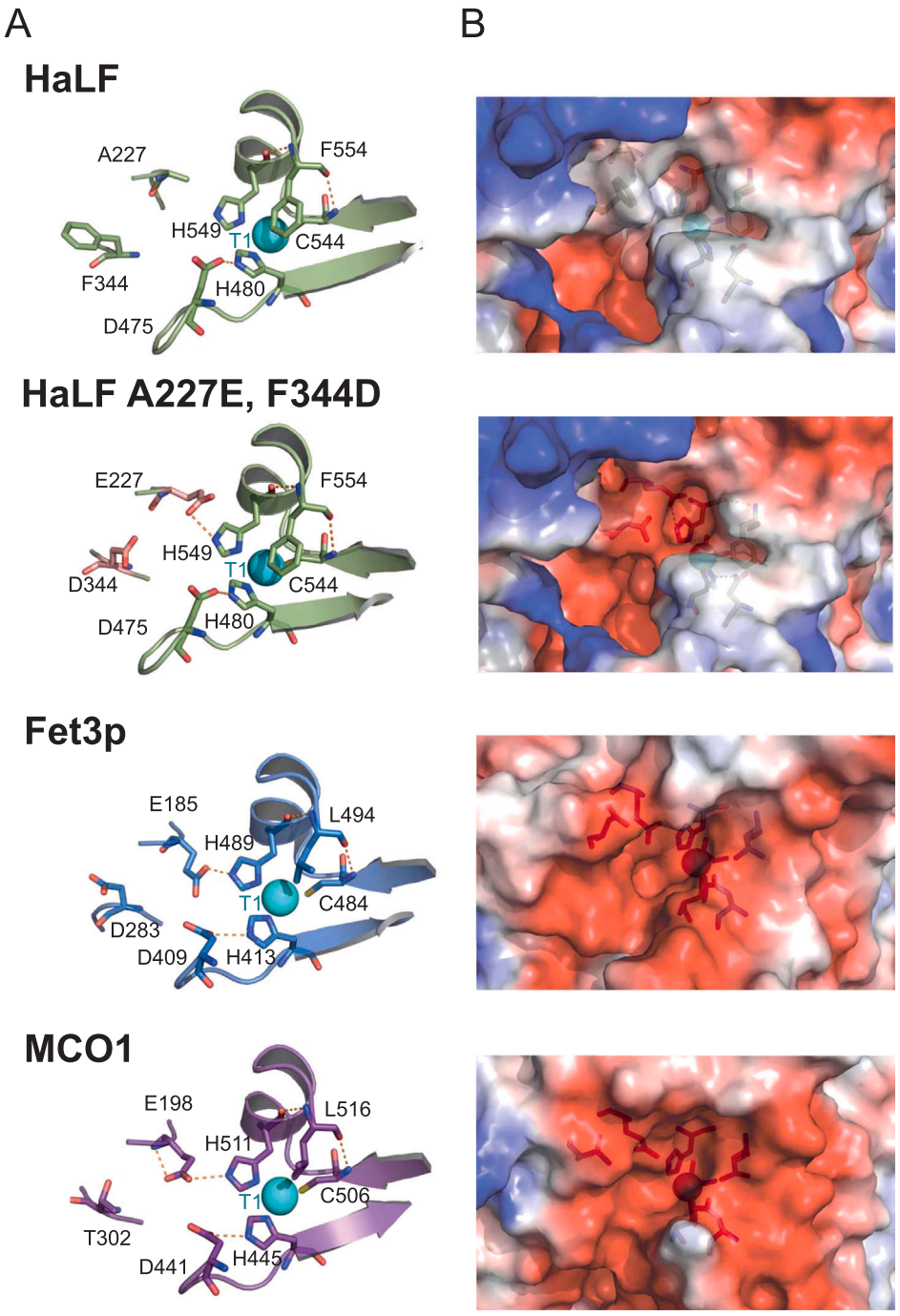

Fig. 3. Close-up of the binding pockets of HaLF, the LAC-FOX from *H. annosum s. l.*, its mutated variant, HaLF $_{A227E,\ F344D,}$ the Fet3p ferroxidase from *S. cerevisiae* (PDB: 1ZPU), and the LAC-FOX from *P. chrysosporium* MCO1. Shown are the residues equivalent to those responsible for ferroxidase activity in Fet3p (E185, D283, Y354 and D409), and the two histidines and one cysteine coordinating T1 copper at the catalytic site (**A**); together with the electrostatic surface of the binding pockets (**B**), with acid amino acids highlighted in red and basic amino acids in blue.

HaLF variants were also compared with those of a high-redox potential sensu stricto laccase, 7A12, engineered in our lab [48].

Under the assayed conditions, native HaLF was capable to oxidise all laccase substrates except for the two non-substituted phenols, p-coumaric acid (PCA) and 4-hydroxybenzaldehyde (HBA), and aniline (ANL). We observed a reduced oxidation of phenols by the mutated variants compared to native HaLF, being the differences minor with *p*-hydroxybenzaldehydes, in particular with vanillin (VAI), than with the *p*-hydroxycinnamic acids ferulic acid (FA) and sinapic acid (SA). The mutated variants also oxidised aryl amines worse than the native enzyme, although the differences were somehow smaller than with phenols, particularly for PPD. Most enzyme variants were also able to oxidise different azo dyes, including the high-redox potential RB5, although at a lower extend than the native HaLF; the exception was EB that was better oxidised by some of the mutated variants. In accordance with results from previous section, the variant harbouring the three acidic determinants (HaLF$_{A227E,\ F344D}$) was the only enzyme able to oxidise Fe (II), and it also exhibited activity towards other typical laccase substrates. With regards to manganese activity, it was not detected in any variant. In summary, native HaLF showed good laccase-like activity, with capacity for oxidising 15 out of the 18 laccase substrates assayed, whereas it showed no ferroxidase

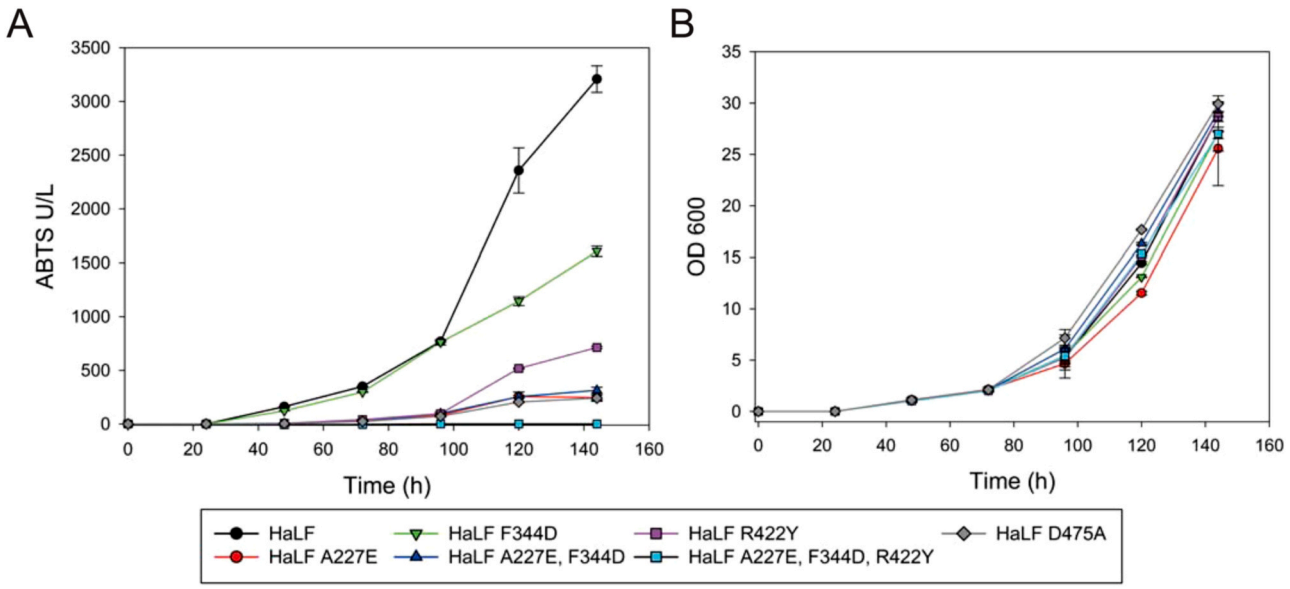

Fig. 4. Time course for production of HaLF variants, monitored by the laccase activity with ABTS **(A)**, and cell growth, represented as OD 600 (**B**), in *S. cerevisiae* flask cultures at 20 °C. Error bars show the standard deviation from three flask replicates.

activity. HaLF oxidised different recalcitrant azo dyes better than 7A12, although its activity towards phenols and aryl amines was lower. The mutated HaLF variants also showed laccase activity with many of the compounds assayed, but in general with reduced oxidation capabilities due to the substitutions of A227, F344 and R422 residues. The $HaLF_{A227E}$ variant was the one that better retained the activity of the native enzyme with several of the laccase substrates. Ferroxidase activity was exclusively found in $HaLF_{A227E,\ F344D}$, which also retained activity with laccase substrates, in particular with aryl amines.

The stability of the purified HaLF variants at pH 3–9 were monitored for 24 h (Fig. 7). In general, the enzymes roughly retained almost maximal activity at more neutral pHs (5 and 7), while under more acidic (pH 3) or basic conditions (pH 9), the enzymes were less stable. Native HaLF exhibited 80 % of activity at pH 5 and 7 but almost no activity after 24 h at pH 3. The single-mutated variants were fully stable at pH 5 and 7 and showed even better residual activity at pH 3 and pH 9 than the native enzyme. By contrast, the double-mutated variant $HaLF_{A227E,\ F344D}$ was the most unstable of all, respectively retaining only 40 % and 30 % of activity after 24 h at pH 5 and 7, and almost no activity after 6 h of incubation at pH 3 and 9.

Given the low T50 (10 min) values observed for the native HaLF and mutated variants produced in *S. cerevisiae*, a thermostability assay was carried out at 30 and 40 °C for 6 h with the purified enzymes produced in *A. oryzae* (Fig. 8). Variant $HaLF_{A227E,\ F344D}$ displayed the lowest stability at 30 °C, with barely 7 % of activity after 1 h. The rest of variants showed similar stabilities, retaining around 20–15 % of residual activity after 6 h at 30 °C. All purified HaLF variants showed no activity after 1 h at 40 °C (Fig. 8 B).

3.4. Kinetic constants

Kinetics of native HaLF and $HaLF_{A227E,\ F344D,}$ the only variant with detected ferroxidase activity, were compared for oxidation of a battery of substrates: ABTS, phenols (DMP and GUA) and aryl amines (DMPD and OPD), to better assess the impact that the presence of the three acidic residues responsible for ferroxidase activity had on the oxidation of typical laccase substrates. Kinetics for Fe (II) oxidation were also determined. In general, HaLF showed notably higher k_{cat} values and lower K_M for the oxidation of the laccase substrates and, consequently, better catalytic efficiencies than the variant with ferroxidase activity (Table 2). Nonetheless, the affinities of $HaLF_{A227E,\ F334D}$ for aryl amines were not severely reduced, and it was even improved in the case of OPD. According to the ferroxidase activity, variant $HaLF_{\ A227E,\ F334D}$ exhibited good affinity for Fe (II) (K_M 0.018 mM) although a low turnover rate (k_{cat} of 0.001 s^{-1}).

4. Discussion

Multicopper oxidases with dual laccase and ferroxidase activity have been described in different fungi [25–27], and identified by genomic and phylogenetic analysis as a well-differentiated cluster [2,16,24].

Table 1
Laccase and ferroxidase activities, and thermotolerance as T50 (10 min) assay of the native HaLF and mutated variants. The activities with DMP and DMPD are relative to the activity with ABTS. Ferroxidase activity is based on the ferrozine assay (changes in Abs 560 nm with time are given as positive number). No activity (n.a.); non determined (n.d.). * Laccase activities (with ABTS) detected in *S. cerevisiae* liquid cultures after 144 h at 20 °C.

	Laccase activity (U/L)*	Relative laccase activity (%)			Ferroxidase Activity	T50 (10 min)
		ABTS	DMP	DMPD	(ΔA_{560} /min)	(°C)
HaLF	3208	100	7 ± 0.4	108 ± 8	n. a.	46.4 ± 0.2
$HaLF_{A227E}$	247	100	21 ± 1.6	676 ± 52	n. a.	40.3 ± 0.4
$HaLF_{\ F344D}$	1607	100	20 ± 0.7	50 ± 1	n. a.	45.1 ± 0.2
$HaLF_{\ A227E,\ F344D}$	315	100	8 ± 0.5	374 ± 52	58×10^{-3}	42.2 ± 0.3
$HaLF_{\ R422Y}$	713	100	82 ± 6	354 ± 36	n. a.	37.6 ± 0.8
$HaLF_{\ A227E,\ F344D,\ R422Y}$	0	n.a.	n.a.	n.a.	n. a.	n. d.
$HaLF_{\ D475A}$	241	100	16 ± 0.6	97 ± 4	n. a.	36.5 ± 0.4

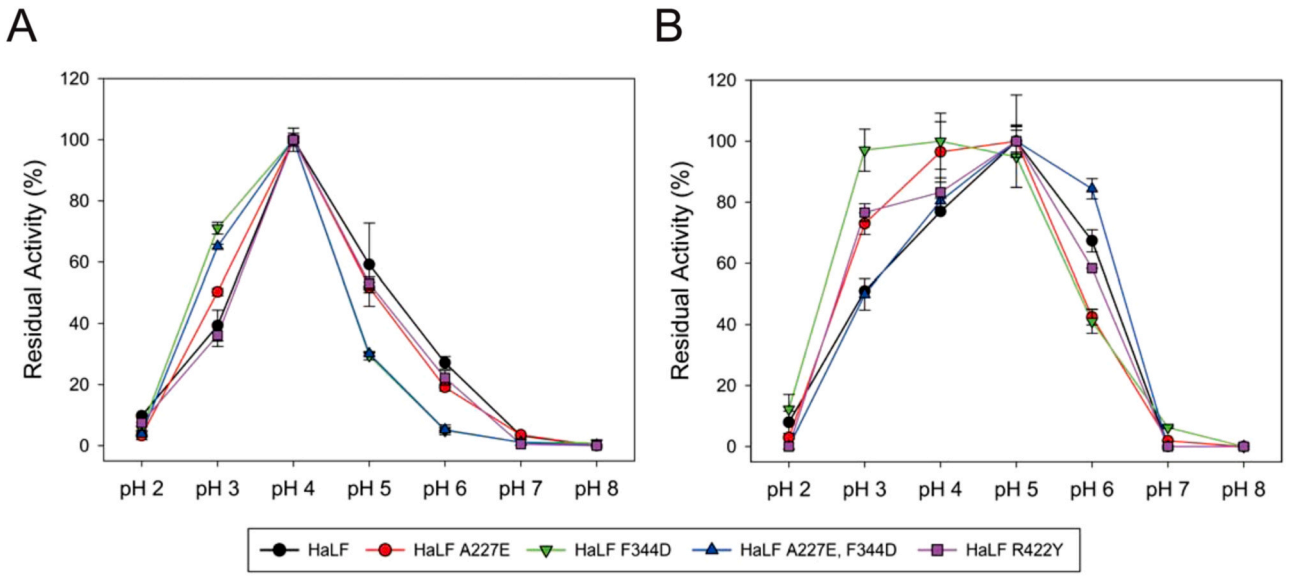

Fig. 5. Laccase activity as a function of pH for the oxidation of ABTS (**A**) and DMP (**B**) in native HaLF and mutated variants produced in *A. oryzae*. Error bars indicate standard deviation for triplicates.

Here, the phylogenetic analysis of 34 LAC-FOXs encoded in 28 basidiomycete genomes resulted in an evolutionary tree split into two congruent subgroups. We found, for the first time, a correlation between the two LAC-FOX subgroups and the presence of some acidic residues equivalent to those determining the ferroxidase activity in Fet3p [29]. Subgroup 1 was mostly comprised of enzymes that conserved two acidic residues in the active site equivalent to E185 and D409 of Fet3p, whereas enzymes from the subgroup 2 only had the equivalent to D409. In agreement with this classification, the two already characterised LAC-FOXs from *Phanerochaete spp*, MCO1 and PfaL, which have demonstrated ferroxidase activity (k_{cat} of 40.8 s^{-1} for MCO1 [26] and around 0.26 s^{-1} for PfaL [27]), were clustered in subgroup 1. Both enzymes exhibited also laccase activities, MCO1, that has the strongest ferroxidase activity found in a LAC-FOX so far, with reactivity towards aryl amines but poorer oxidation of phenols than some sensu stricto laccases [26], while PfaL displayed an overall better laccase-like activity on aryl amines, phenols and ABTS than MCO1 [27]. On the other hand, the MCO from *C. neoformans*, clustered in the subgroup 2, has reported activity on polyphenolic compounds [28] but a presumably weaker ferroxidase activity than MCO1 and PfaL (a large amount of laccase activity units were needed to detect Fe (II) oxidation) [25]. Furthermore, HaLF, the LAC-FOX from *H. annosum s. l.* studied here, which is also classified in subgroup 2 (and therefore has only the residue equivalent to D409), presents laccase-like activity but null ferroxidase activity. All this would suggest that the two evolutionary LAC-FOX branches divide enzymes with different catalytic profiles and functionalities: subgroup 1 with some laccase-like activity and a strong ferroxidase activity, and subgroup 2 with laccase activity, but without efficient ferroxidase activity. However, the information available is still reduced to draw final conclusions.

HaLF was selected as a LAC-FOX from subgroup 2, from which no enzyme members had been thoroughly characterised. *Heterobasidion annosum s. l.* refers to a collection of five species that besides being considered as root-rot coniferous pathogens [49] they are also wood decayers [33]. Among them, HaLF sequence was encoded in the genome of *H. irregulare*, one of the members of this species complex sequenced by JGI [33]. To evaluate the possible hybrid activity of HaLF due to presence of D475, equivalent to D409 of Fet3p, the enzyme was synthesised and heterologously expressed. In addition, different HaLF variants were designed to introduce and complete the repertoire of acidic residues equivalent to those of Fet3p responsible for Fe (II) binding and oxidation (E185, D283 and D409) [29], and Y354 [31,32]. First, we developed and produced the different HaLF variants in *S. cerevisiae*, the host of choice for engineering fungal laccases, due to its high-frequency DNA recombination machinery,

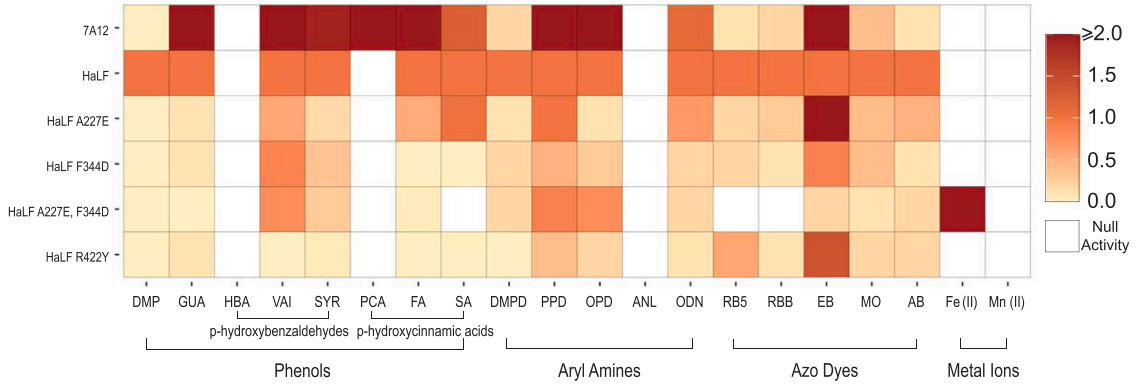

Fig. 6. Heat map of the activities of HaLF mutated variants and 7A12 laccase [48] relative to the activity of native HaLF for the oxidation of different phenols, aryl amines, azo dyes and metal ions. Increments or decrements in activity with respect to native activity are indicated by colour gradient, except for null activity that is represented in white. To better visualise and discriminate between smaller differences, activity increments ≥ 2 fold are represented with same red colour. See Table S2 for description of substrate abbreviations.

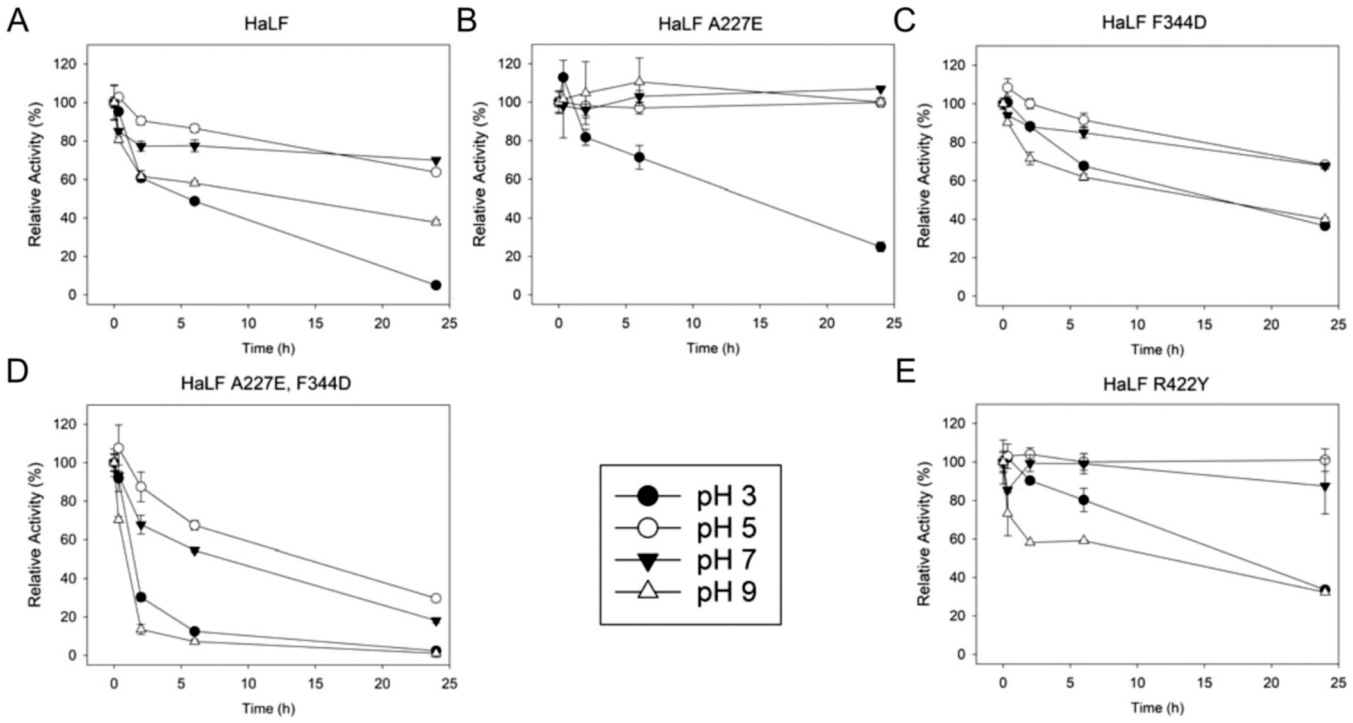

Fig. 7. Long-term stability of native HaLF (**A**) and variants holding mutations A227E (**B**), F344D (**C**), A227E, F344D (**D**) and R422Y (**E**) at pH 3 (black circles), pH 5 (white circles), pH 7 (black triangles) and pH 9 (white triangles). Residual activities were measured with ABTS pH 4 at room temperature. Error bars indicate standard deviation for triplicates.

easier genetic manipulation for the rapid obtainment of mutants, and ability to secrete active enzymes [45,48,50–53]. Except for the native enzyme and $HaLF_{F344D}$ variant, the activities detected in the liquid extracts from flask cultures were insufficient for carrying out the full characterisation of the purified enzymes. Therefore, since filamentous fungi are able to secrete large amounts of proteins [54], the enzymes were later produced in *A. oryzae*, reaching laccase activity levels that greatly surpassed those found in yeast.

The fact that the highest and lowest activity levels found in both hosts corresponded to the same LAC-FOX variants evidenced the higher enzyme production yields by the filamentous fungus. Nevertheless, differences in intrinsic enzymatic properties depending on the host producer cannot be completely ruled out. For instance, dissimilarities in glycosylation patterns between yeast and filamentous fungi [55] can ultimately influence enzyme activity [45,56]. However, variations found here when comparing the relative activities with different substrates of HaLF variants obtained in *S. cerevisiae* or *A. oryzae* can be explained by the fact that they were normalised to the activity with ABTS in the case of yeast-produced enzymes, or to the native activity in the case of *A. oryzae*-produced enzymes. On the other hand, discrepant results found in the thermotolerance of HaLF and variants depending on the host producer

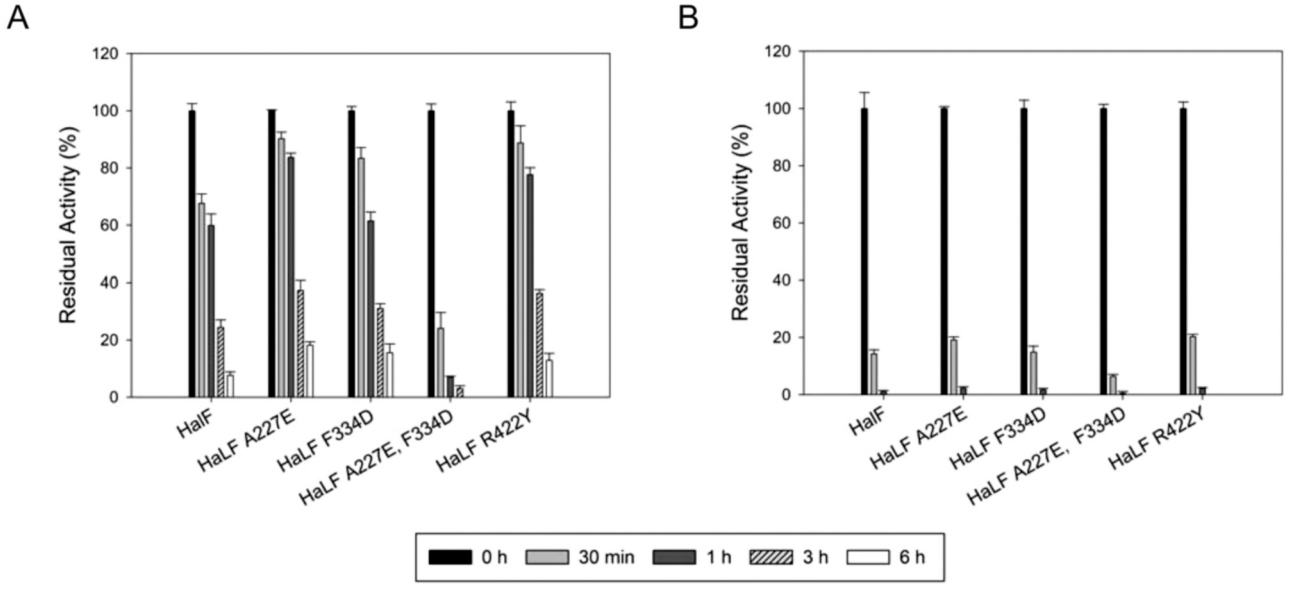

Fig. 8. Stabilities of native HaLF and mutated variants through 6 h of incubation at 30 °C (**A**) and 40 °C (**B**). Residual activity was measured with ABTS pH 4 at room temperature. Error bars indicate standard deviation for triplicates.

Table 2
Kinetic constants for the oxidation of laccase and ferroxidase substrates by the native HaLF and HaLF$_{A227E,\ F344D}$ variant. Oxidation of ABTS, DMPD and OPD was measured at pH 4, and DMP, GUA and Fe (II) at pH 5.

	HaLF			HaLF$_{A227E,\ F344D}$		
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\ s^{-1}$)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\ s^{-1}$)
ABTS	4 ± 0.6	428 ± 14	107 ± 16	11 ± 1.5	16 ± 1	1.4 ± 0.2
DMP	0.5 ± 0.05	122 ± 4	247 ± 25	3.8 ± 0.3	0.87 ± 0.03	0.23 ± 0.02
GUA	20 ± 5	3.7 ± 0.4	0.18 ± 0.05	31 ± 4	0.3 ± 0.02	0.002 ± 0.0005
DMPD	8 ± 0.4	192 ± 4	23 ± 1.3	9 ± 0.8	29 ± 1	3.3 ± 0.3
OPD	1.6 ± 0.3	26 ± 2	17 ± 3	0.91 ± 0.2	6.6 ± 0.5	7.2 ± 1
Fe (II)	n.a.	n.a.	n.a.	0.018 ± 0.001	0.001 ± 0.00005	0.0116 ± 0.0011

might be related to the different glycosylation patterns which can affect enzyme stability [57].

Native HaLF exhibited an acidic optimal pH similar to basidiomycete laccases, with a sharp maximum of activity with ABTS and a more opened profile for DMP [58,59]. The enzyme was stable at neutral pH like most fungal laccases. In general, purified HaLF displayed a notably lower thermotolerance than some sensu stricto laccases from basidiomycete fungi. The enzyme barely showed any activity after 1 h of incubation at 40 °C, whereas the half-lives of some *Pycnoporus sp* laccases at this temperature surpassed 1000 h [60] and the residual activity of *M. thermophila* laccase after 34 h at 40 °C is over 40 % [61]. In addition, T50 (10 min) value of HaLF was lower than those from other recombinant laccases heterologously expressed in *S. cerevisiae*, such as the sensu stricto laccases of *Agrocybe pediades* [45] and *P. cinnabarinus* [50], both around 70 °C. On the other hand, the lack of information about resistance to high temperatures of other LAC-FOX members impairs concluding whether this is a general feature of these MCOs or not.

Alike sensu stricto laccases, native HaLF showed a broad oxidation catalytic profile. It oxidised 15 out of 18 typical laccase substrates assayed, including recalcitrant azo dyes with high redox potential such as RB5, and poorly oxidisable phenols and aryl amines. The fact that HaLF overcame the redox threshold imposed by some of these substrates may suggest the enzyme possesses a high-redox potential. According to this, in HaLF a non-coordinating phenylalanine occupies the position of the 4th axial ligand of T1 copper, a structural feature strongly associated with high-redox potential of certain fungal laccases [62,63], as opposed to the presence of leucine in MCO1 and PfaL. Only the non-substituted phenols HBA and PCA, and the aryl-amine ANL were not oxidised by HaLF. Methoxy-substituted compounds such as the phenols VAI, SYR, FA or SA are more easily oxidised due to the presence of the electron-donating substituents to the aromatic ring, thus lowering the redox potential of the compounds in comparison to non-substituted ones [64]. HaLF showed the broadest substrate spectrum observed for a LAC-FOX so far, compared to MCO1 [26], PfaL [27] and *C. neoformans* MCO [25,28], although it did not oxidise Fe (II). Moreover, HaLF had a notably superior k_{cat} for some model substrates than those observed in basidiomycete laccases, with special mention to the turnover rates with DMP (k_{cat} 122 s^{-1}) and DMPD (k_{cat} 192 s^{-1}), outperforming the results observed for sensu stricto laccases from *A. pediades* (k_{cat} of 6 and 56 s^{-1} for DMP and DMPD, respectively) [45], *Coprinopsis cinerea* (k_{cat} 2.3 s^{-1} for DMP) [65], *T. versicolor* (k_{cat} 0.51 s^{-1} for DMP) [66], basidiomycete PM1 (k_{cat} 13.2 s^{-1} for DMP and k_{cat} 108.6 s^{-1} for DMPD) [67] or *Trametes trogii* (k_{cat} 1.8 s^{-1} for DMP) [68]. Nevertheless, the affinity of HaLF for phenols (DMP K_M of 0.5 mM) and amines (DMPD, K_M of 8 mM) was inferior than those of most of these laccases; *A. pediades* (K_M of 0.016 and 0.53 mM for DMP and DMPD, respectively) [45], *T. versicolor* (K_M of 0.19 mM for DMP) [66], PM1 (K_M of 0.01 and 1.06 mM for DMP and DMPD, respectively) [67] and *T. trogii* (K_M of 0.41 mM for DMP) [68].

The completion in HaLF$_{A227E,\ F344D}$ variant of the full set of acidic residues equivalent to E185, D283 and D409 in Fet3p, conferred ferroxidase activity to the enzyme, confirming their role in the oxidation of Fe (II). Moreover, the mutated enzyme retained laccase-like activity, although notably reduced. Single-mutated variants HaLF$_{A227E}$ and HaLF$_{F344D}$, having two out of the three acidic ferroxidase determinants, showed the same laccase-like oxidative spectrum than the native HaLF, but with reduced oxidation rates, except for EB in HaLF$_{A227E}$. This detrimental effect was further enlarged when the three acidic residues were included in the variant HaLF$_{A227E,\ F344D}$, indicating the direct correlation between the enzymatic activity and the amino acids occupying these positions.

Interestingly, native HaLF, with only D475 (D409 in Fet3p), and variants with two acidic determinants, HaLF$_{A227D}$ (D409 and E185 in Fet3p), and HaLF$_{F344D}$ (D409 and D283 in Fet3p), were not able to oxidise Fe (II). These results contrast with those reported for other LAC-FOXs or even Fet3p, in which a complete set of these three determinants is not needed for an efficient Fe (II) oxidation, even if only counts with one acidic residue like PfaL [26,27,29,30]. Residues E185 and D409 have been described to drive the electron transference from Fe (II) to T1 site in Fet3p, via two independent hydrogen-bonded pathways E185-H489 or D409-H413 involving the two T1 histidine ligands [29] (Fig. 3). The ferroxidase activity of LAC-FOXs PfaL could be accordingly explained. However, in our case, Fe (II) oxidation was only observed when the three acidic residues E227, D344 and D475 (equivalent to E185, D283 and D409 in Fet3p) were fully restored in HaLF$_{A227E,\ F344D}$ variant (Fig. 3) when using the aforementioned exhaustive protocol to eliminate non-enzymatic Fe (II) oxidation background.

Variant HaLF$_{A227E,\ F344D}$ displayed a remarkable affinity to Fe (II), with K_M value orders of magnitude lower than those for laccase-like substrates, and in the range of that of MCO1 (K_M = 2.05 μM) [26] or secreted Fet3p (around K_M = 2 μM) [31,69], and much better than PfaL LAC-FOX (K_M = 416 mM) [27]. This low K_M is a signature feature of metalloxidases, which, in contrast to laccases, exhibit a high affinity for their substrates due to the favourable Fe (II) binding sites at the acidic residues [70]. In addition, HaLF$_{A227E,\ F344D}$ partly retained the notable broad substrate oxidation observed in native HaLF, pointing out the strong dual activity expected for LAC-FOXs. Variant HaLF$_{A227E,\ F344D}$ oxidised 12 out of the 18 typical laccase substrates tested, although with a general worsening of the oxidation rates compared to the native enzyme. As for kinetics, only the affinity towards amines was maintained or even improved for OPD in the double-mutated variant, in agreement with observed for MCO1, whose binding site seems to favour aryl amine coupling for oxidation as occurs for Fet3p [26,69]. The more negative electrostatic environment at HaLF$_{A227E,\ F344D}$ binding site, which mimics those observed in Fet3p and MCO1 (Fig. 3), defines its activity/substrate specificity. In this sense, empirical experiments had also demonstrated the structural/substrate affinity relationship between E185, D283 and D409 residues and Fe (II) reaction rate in Fet3p, in which different substitutions can varied K_M significantly [30,32,71]. Compared to laccases, the spatially more constrained binding pocket of ferroxidases, with negative charge on the surface, may mask the binding of some organic substrates as bulky azo-dyes or phenols at the T1 site [72]. Subtle changes in polarity or size of the binding pocket of laccases have also a significant impact on the activity of

these enzymes [73,74], together with residues conforming the so-called T1 "outer-sphere" which determine the turnover rate of laccases [58,75,76].

Before crystal structure for Fet3p was elucidated, a sequence homology approach identified Y354 as a Fe (II) ligand in Fet3p [77], afterwards its active implication in Fe (II) reaction rate was demonstrated [31,71]. Based on the multiple sequence alignment, the equivalent Y354 seems not to be a signature residue in LAC-FOX, despite this, we studied the R422Y substitution in HaLF. The poorer oxidation profile of the purified $HaLF_{R422Y}$ variant compared to the native enzyme, evidenced the implication of this residue on the catalytic activity of the enzyme. In this sense, the triple variant $HaLF_{A227E,\ F344D,\ R422Y,}$ designed to assess the combined effect of the most relevant residues in ferroxidase activity, did not show activity towards any substrate, including Fe (II). We hypothesised that the substitutions introduced in the binding site close to His T1 ligands may completely disrupt the enzyme secretion/activity or stability.

$HaLF_{D475A}$ variant was discarded for further characterisation as, because of the absence of Fe (II) oxidation in HaLF, substituting D475A did not add information about the role of this residue in ferroxidase activity. In addition, the mutated variant had similar activity on laccase substrates than the native enzyme. Moreover, D475A mutation seemed to severely impair laccase secretion/expression, given the low or null activity detected in *S. cerevisiae* flask cultures compared to native HaLF. The latter could be related to the notable detrimental effect of D475A on the thermotolerance of the enzyme. In view of these results, $HaLF_{A227E,\ F344D,\ R422Y}$ and $HaLF_{D475A}$ variants were excluded for overexpression in *A. oryzae* and further characterisation.

Despite HaLF was identified as a LAC-FOX, its lack of Fe (II) oxidation but efficient laccase-like activity toward a broad spectrum of substrates, adds questions about its biological function. Up to 6 manganese peroxidases and 20 MCOs were found as the ligninolytic oxidoreductases in *H. annosum s. l.* described as wood-decayer species [16], 12 are sensu stricto laccases and two LAC-FOXs, which would indicate laccases are major contributors for lignin degradation in these species. In accordance with this supposition, a transcript level profile of *H. annosum s. l.* showed that the transcripts of LAC-FOX Lcc15, equivalent to HaLF, was up-regulated in lignin and heartwood cultures in comparison to enriched cellulose media, with laccase transcripts being more overrepresented than other gene families in lignin and heartwood cultures [35]. Moreover, *H. annosum s. l.* is a pathogen able to act during saprotrophic phases and living tissue colonisation, in this line a Scot pine infection study by *H. annosum s. l.* remarked the up-regulation of HaLF gene and its possible role in host/pathogen interaction [78]. Likewise, MCO from *C. neoformans,* another characterised LAC-FOX classified in subgroup 2, has been proposed as a virulence factor for protection from macrophages [25]. Based on these reports and on the oxidation of lignin-derived phenols by HaLF demonstrated in this study, it is possible that the role of this LAC-FOX is related to the degradation processes underlying fungal colonisation in wood.

5. Conclusions

Saprotrophic basidiomycete fungi play a key role in carbon cycle in nature and constitute valued sources of enzymes for industrial biomass conversion. Laccases are well-known multicopper oxidases secreted by wood-rot and litter-decomposing fungi that play a key role in the degradation of lignin and other aromatic substrates. However, MCO superfamily includes other families of enzymes with different physiological functions not sufficiently explored. This is the case of the LAC-FOX family, with putative hybrid laccase-ferroxidase activity.

The phylogenetic analysis of the LAC-FOX family carried out here, seems to indicate that the division in two differentiated subgroups is related to the putative hybrid ferroxidase/laccase activity of the enzymes. Subgroup 1 comprises enzymes harbouring two of the three determinants of ferroxidase activity, E185 and D409 (Fet3p numbering), and they show efficient ferroxidase activity and some laccase-like activity. By contrast, enzymes from subgroup 2 only have one acidic residue, D409, and show an insufficient or null ferroxidase activity but good laccase-like activity, as confirmed here by the characterisation of a member of this subgroup from the fungus *H. annosum s. l.*

Similar to sensu stricto laccases, HaLF shows a wide-range oxidation capability on aromatic substrates (phenols, aryl-amines, synthetic organic dyes) with kinetic constants comparable to certain laccases sensu stricto. On the other hand, HaLF does not have ferroxidase activity. Only, the variant $HaLF_{A227E,\ F344D}$, with the full restoration of the three acidic determinants E185, D283 and D403 (Fet3p numbering), exhibits an efficient oxidation of Fe (II). The mutated variant also retains laccase-like activity on a broad spectrum of substrates, remarking its hybrid ferroxidase/laccase activity, although with poorer kinetic constants when compared to the native HaLF. This is partly explained by the more acidic binding pocket of the $HaLF_{A227E,\ F344D}$ variant, which may hinder the binding of some typical laccase substrates.

CRediT authorship contribution statement

S. Camarero: Conceptualization, Supervision, Writing – review & editing, Funding acquisition. **P. Aza:** Investigation, Methodology, Data curation, Writing – original draft. **G. Molpeceres:** Data curation, Software, Validation. **J. Vind:** Supervision, Investigation, Methodology. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors of the work entitled "Multicopper oxidases with laccase-ferroxidase activity: classification and study of ferroxidase activity determinants in a member from *Heterobasidion annosum s. l.",* declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2023.01.030.

References

[1] Solomon EI, Sundaram UM, Machonkin TE. Multicopper oxidases and oxygenases. Chem Rev 1996;96(7):2563–605. https://doi.org/10.1021/cr950046o
[2] Hoegger PJ, Kilaru S, James TY, Thacker JR, Kües U. Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. FEBS J 2006;273(10):2308–26. https://doi.org/10.1111/j.1742-4658.2006.05247.x

[3] Sakurai T, Kataoka K. Structure and function of type I copper in multicopper oxidases. Cell Mol Life Sci 2007;64(19–20):2642–56. https://doi.org/10.1007/s00018-007-7183-y
[4] Sekretaryova A, Jones SM, Solomon EI. O2 reduction to water by high potential multicopper oxidases: contributions of the T1 copper site potential and the local environment of the trinuclear copper cluster. J Am Chem Soc 2019;141(28):11304–14. https://doi.org/10.1021/jacs.9b05230
[5] Baldrian P. Fungal laccases-occurrence and properties. FEMS Microbiol Rev 2006;30(2):215–42. https://doi.org/10.1111/j.1574-4976.2005.00010.x
[6] Claus H. Laccases: structure, reactions, distribution. Micron 2004;35(1–2):93–6. https://doi.org/10.1016/j.micron.2003.10.029
[7] Thurston C. The structure and function of fungal laccases. Microbiology. 140. Nomos Verlagsgesellschaft mbH & Co. KG; 1994. p. 19–26.
[8] Singh G, Bhalla A, Kaur P, Capalash N, Sharma P. Laccase from prokaryotes: a new source for an old enzyme. Rev Environ Sci Biotechnol 2011;10(4):309–26. https://doi.org/10.1007/s11157-011-9257-4
[9] Mayer AM, Staples RC. Laccase: new functions for an old enzyme. Phytochemistry 2002;60(6):551–65. https://doi.org/10.1016/S0031-9422(02)00171-1
[10] Harris ZL, Durley AP, Man TK, Gitlin JD. Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. Proc Natl Acad Sci USA 1999;96(19):10812–7. https://doi.org/10.1073/pnas.96.19.10812
[11] Stearman R, Yuan DS, Yamaguchi-Iwai Y, Klausner RD, Dancis A. A permease-oxidase complex involved in high-affinity iron uptake in yeast. Science (80-) 1996;271(5255):1552–7. https://doi.org/10.1126/science.271.5255.1552
[12] Pignocchi C, Foyer CH. Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. Curr Opin Plant Biol 2003;6(4):379–89. https://doi.org/10.1016/S1369-5266(03)00069-4
[13] Riva S. Laccases: blue enzymes for green chemistry. Trends Biotechnol 2006;24(5):219–26. https://doi.org/10.1016/j.tibtech.2006.03.006
[14] Rodríguez Couto S, Toca Herrera JL. Industrial and biotechnological applications of laccases: a review. Biotechnol Adv 2006;24(5):500–13. https://doi.org/10.1016/j.biotechadv.2006.04.003
[15] Moreno AD, Ibarra D, Eugenio ME, Tomás-Pejó E. Laccases as versatile enzymes: from industrial uses to novel applications. J Chem Technol Biotechnol 2020;95(3):481–94. https://doi.org/10.1002/jctb.6224
[16] Ruiz-Dueñas FJ, Barrasa JM, Sánchez-García M, Camarero S, Miyauchi S, Serrano A, et al. Genomic analysis enlightens agaricales lifestyle evolution and increasing peroxidase diversity. Mol Biol Evol 2021;38(4):1428–46. https://doi.org/10.1093/molbev/msaa301
[17] Mot AC, Silaghi-Dumitrescu R. Laccases: complex architectures for one-electron oxidations. Biochemistry 2012;77(12):1395–407. https://doi.org/10.1134/S0006297912120085
[18] Hakulinen N, Rouvinen J. Three-dimensional structures of laccases. Cell Mol Life Sci 2015;72(5):857–68. https://doi.org/10.1007/s00018-014-1827-5
[19] Piontek K, Antorini M, Choinowski T. Crystal structure of a laccase from the fungus trametes versicolor at 1.90-Å resolution containing a full complement of coppers. J Biol Chem 2002;277(40):37663–9. https://doi.org/10.1074/jbc.M204571200
[20] Rodgers CJ, Blanford CF, Giddens SR, Skamnioti P, Armstrong FA, Gurr SJ. Designer laccases: a vogue for high-potential fungal enzymes. Trends Biotechnol 2010;28(2):63–72. https://doi.org/10.1016/j.tibtech.2009.11.001
[21] Hong G, Ivnitski DM, Johnson GR, Atanassov P, Pachter R. Design parameters for tuning the type 1 Cu multicopper oxidase redox potential: insight from a combination of first principles and empirical molecular dynamics simulations. J Am Chem Soc 2011;133(13):4802–9. https://doi.org/10.1021/ja105586q
[22] Reiss R, Ihssen J, Richter M, Eichhorn E, Schilling B, Thöny-Meyer L. Laccase versus laccase-like multi-copper oxidase: a comparative study of similar enzymes with diverse substrate spectra. PLoS One 2013;8(6). https://doi.org/10.1371/journal.pone.0065633
[23] Kumar SVS, Phale PS, Durani S, Wangikar PP. Combined sequence and structure analysis of the fungal laccase family. Biotechnol Bioeng 2003;83(4):386–94. https://doi.org/10.1002/bit.10681
[24] Kues U, Ruhl M. Multiple multi-copper oxidase gene families in basidiomycetes – what for? Curr Genom 2011;12(2):72–94. https://doi.org/10.2174/138920211795564377
[25] Liu L, Tewari RP, Williamson PR. Laccase protects cryptococcus neoformans from antifungal activity of alveolar macrophages. Infect Immun 1999;67(11):6034–9. https://doi.org/10.1128/iai.67.11.6034-6039.1999
[26] Larrondo LF, Salas L, Melo F, Vicuña R, Cullen D. A novel extracellular multicopper oxidase from phanerochaete chrysosporium with ferroxidase activity. Appl Environ Microbiol 2003;69(10):6257–63. https://doi.org/10.1128/AEM.69.10.6257-6263.2003
[27] Rodríguez-Rincón F, Suarez A, Lucas M, Larrondo LF, De La Rubia T, Polaina J, et al. Molecular and structural modeling of the phanerochaete flavido-alba extracellular laccase reveals its ferroxidase structure. Arch Microbiol 2010;192(11):883–92. https://doi.org/10.1007/s00203-010-0616-2
[28] Williamson PR. Biochemical and molecular characterization of the diphenol oxidase of cryptococcus neoformans: identification as a laccase. J Bacteriol 1994;176(3):656–64. https://doi.org/10.1128/jb.176.3.656-664.1994
[29] Taylor AB, Stoj CS, Ziegler L, Kosman DJ, Hart PJ. The copper-iron connection in biology: structure of the metallo-oxidase Fet3p. Proc Natl Acad Sci USA 2005;102(43):15459–64. https://doi.org/10.1073/pnas.0506227102
[30] Stoj CS, Augustine AJ, Zeigler L, Solomon EI, Kosman DJ. Structural basis of the ferrous iron specificity of the yeast ferroxidase, Fet3p. Biochemistry 2006;45(42):12741–9. https://doi.org/10.1021/bi061543
[31] Bonaccorsi Di Patti MC, Felice MR, Camuti AP, Lania A, Musci G. The essential role of Glu-185 and Tyr-354 residues in the ferroxidase activity of Saccharomyces cerevisiae Fet3. FEBS Lett 2000;472(2–3):283–6. https://doi.org/10.1016/S0014-5793(00)01435-6
[32] Wang TP, Quintanar L, Severance S, Solomon EI, Kosman DJ. Targeted suppression of the ferroxidase and iron trafficking activities of the multicopper oxidase Fet3p from Saccharomyces cerevisiae. J Biol Inorg Chem 2003;8(6):611–20. https://doi.org/10.1007/s00775-003-0456-5
[33] Olson Å, Aerts A, Asiegbu F, Belbahri L, Bouzid O, Broberg A, et al. Insight into trade-off between wood decay and parasitism from the genome of a fungal forest pathogen. New Phytol 2012;194(4):1001–13. https://doi.org/10.1111/j.1469-8137.2012.04128.x
[34] Floudas D, Binder M, Riley R, Barry K, Blanchette R, Henrissat B, et al. The paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science (80-) 2012;336(June):1715–9.
[35] Yakovlev IA, Hietala AM, Courty PE, Lundell T, Solheim H, Fossdal CG. Genes associated with lignin degradation in the polyphagous white-rot pathogen heterobasidion irregulare show substrate-specific regulation. Fungal Genet Biol 2013;56:17–24. https://doi.org/10.1016/j.fgb.2013.04.011
[36] Kuo HC, Détry N, Choi J, Lee YH. Potential roles of laccases on virulence of Heterobasidion Annosum s.S. Microb Pathog 2015;81:16–21. https://doi.org/10.1016/j.micpath.2015.03.004
[37] Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 2013;30(4):772–80. https://doi.org/10.1093/molbev/mst010
[38] Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. TrimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics 2009;25(15):1972–3. https://doi.org/10.1093/bioinformatics/btp348
[39] Abascal F, Zardoya R, Posada D. ProtTest: selection of best-fit models of protein evolution. Bioinformatics 2005;21(9):2104–5. https://doi.org/10.1093/bioinformatics/bti263
[40] Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics 2019;35(21):4453–5. https://doi.org/10.1093/bioinformatics/btz305
[41] Almagro Armenteros JJ, Tsirigos KD, Sønderby CK, Petersen TN, Winther O, Brunak S, et al. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 2019;37(4):420–3. https://doi.org/10.1038/s41587-019-0036-z
[42] Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, Steinegger M. ColabFold: making protein folding accessible to All. Nat Methods 2022;19(6):679–82. https://doi.org/10.1038/s41592-022-01488-1
[43] Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, et al. Highly accurate protein structure prediction with alphaFold. Nature 2021;596(7873):583–9. https://doi.org/10.1038/s41586-021-03819-2
[44] Alcalde M, Zumarraga M, Polaina J, Ballesteros A, Plou F. Combinatorial saturation mutagenesis by in vivo overlap extension for the engineering of fungal laccases. Comb Chem High Throughput Screen 2006;9(10):719–27. https://doi.org/10.2174/138620706779026079
[45] Aza P, Molpeceres G, Ruiz-Dueñas FJ, Camarero S. Heterologous expression, engineering and characterization of a novel laccase of agrocybe pediades with promising properties as biocatalyst. J Fungi 2021;7(5). https://doi.org/10.3390/jof7050359
[46] Matsui T, Udagawa H, Kishishita S, Skovlund D, J Q. Recombinase-Mediated Integration of a Polynucleotide Library. WO2016026938A1; 2016.
[47] Sánchez-Ruiz MI, Ayuso-Fernández I, Rencoret J, González-Ramírez AM, Linde D, Davó-Siguero I, et al. Agaricales mushroom lignin peroxidase: from structure––function to degradative capabilities. Antioxidants 2021;10(9):1–23. https://doi.org/10.3390/antiox10091446
[48] Pardo I, Vicente AI, Mate DM, Alcalde M, Camarero S. Development of chimeric laccases by directed evolution. Biotechnol Bioeng 2012;109(12):2978–86. https://doi.org/10.1002/bit.24588
[49] Garbelotto M, Gonthier P. Biology, epidemiology, and control of heterobasidion species worldwide. Annu Rev Phytopathol 2013;51:39–59. https://doi.org/10.1146/annurev-phyto-082712-102225
[50] Camarero S, Pardo I, Cañas AI, Molina P, Record E, Martínez AT, et al. Engineering platforms for directed evolution of laccase from Pycnoporus cinnabarinus. Appl Environ Microbiol 2012;78(5):1370–84. https://doi.org/10.1128/AEM.07530-11
[51] Maté D, García-Burgos C, García-Ruiz E, Ballesteros AO, Camarero S, Alcalde M. Laboratory evolution of high-redox potential laccases. Chem Biol 2010;17(9):1030–41. https://doi.org/10.1016/j.chembiol.2010.07.010
[52] Molina-Espeja P, Garcia-Ruiz E, Gonzalez-Perez D, Ullrich R, Hofrichter M, Alcalde M. Directed evolution of unspecific peroxygenase from Agrocybe aegerita. Appl Environ Microbiol 2014;80(11):3496–507. https://doi.org/10.1128/AEM.00490-14
[53] Viña-Gonzalez J, Alcalde M. Directed evolution of the aryl-alcohol oxidase: beyond the lab bench. Comput Struct Biotechnol J 2020;18:1800–10. https://doi.org/10.1016/j.csbj.2020.06.037
[54] Piscitelli A, Pezzella C, Giardina P, Faraco V, Sannia G. Heterologous laccase production and its role in industrial applications Alessandra. Bioeng Bugs 2010;1(4):252–62.
[55] Deshpande N, Wilkins MR, Packer N, Nevalainen H. Protein glycosylation pathways in filamentous fungi. Glycobiology 2008;18(8):626–37. https://doi.org/10.1093/glycob/cwn044
[56] Vite-Vallejo O, Palomares LA, Dantán-González E, Ayala-Castro HG, Martínez-Anaya C, Valderrama B, et al. The role of N-glycosylation on the enzymatic

activity of a Pycnoporus sanguineus laccase. Enzym Microb Technol 2009;45(3):233–9. https://doi.org/10.1016/j.enzmictec.2009.05.007
[57] Han C, Wang Q, Sun Y, Yang R, Liu M, Wang S, et al. Improvement of the catalytic activity and thermostability of a hyperthermostable endoglucanase by optimizing N-glycosylation sites. Biotechnol Biofuels 2020;13(1):1–11. https://doi.org/10.1186/s13068-020-1668-4
[58] Xu F. Effects of redox potential and hydroxide inhibition on the PH activity profile of fungal laccases. J Biol Chem 1997;272(2):924–8. https://doi.org/10.1074/jbc.272.2.924
[59] Wu MH, Lee CC, Hsiao AS, Yu SM, Wang AHJ, Ho THD. Kinetic analysis and structural studies of a high-efficiency laccase from Cerrena Sp. RSD1. FEBS Open Bio 2018;8(8):1230–46. https://doi.org/10.1002/2211-5463.12459
[60] Ramírez-Cavazos LI, Junghanns C, Ornelas-Soto N, Cárdenas-Chávez DL, Hernández-Luna C, Demarche P, et al. Purification and characterization of two thermostable laccases from Pycnoporus sanguineus and potential role in degradation of endocrine disrupting chemicals. J Mol Catal B Enzym 2014;108:32–42. https://doi.org/10.1016/j.molcatb.2014.06.006
[61] Bulter T, Alcalde M, Sieber V, Meinhold P, Schlachtbauer C, Arnold FH. Functional expression of a fungal laccase in Saccharomyces cerevisiae by directed evolution. Appl Environ Microbiol 2003;69(8). https://doi.org/10.1128/aem.69.8.5037.2003. [5037–5037].
[62] Xu F, Palmer AE, Yaver DS, Berka RM, Gambetta GA, Brown SH, et al. Targeted mutations in a Trametes villosa laccase: axial perturbations of the T1 copper. J Biol Chem 1999;274(18):12372–5. https://doi.org/10.1074/jbc.274.18.12372
[63] Durão P, Bento I, Fernandes AT, Melo EP, Lindley PF, Martins LO. Perturbations of the T1 copper site in the CotA laccase from Bacillus subtilis: structural, biochemical, enzymatic and stability studies. J Biol Inorg Chem 2006;11(4):514–26. https://doi.org/10.1007/s00775-006-0102-0
[64] Pardo I, Camarero S. Exploring the oxidation of lignin-derived phenols by a library of laccase mutants. Molecules 2015;20(9):15929–43. https://doi.org/10.3390/molecules200915929
[65] Wang B, Wang L, Lin Y, Han Q, Han J, Gao J, et al. Purification and characterization of a laccase from Coprinopsis cinerea in Pichia pastoris. World J Microbiol Biotechnol 2014;30(4):1199–206. https://doi.org/10.1007/s11274-013-1540-9
[66] Madzak C, Mimmi MC, Caminade E, Brault A, Baumberger S, Briozzo P, et al. Shifting the optimal PH of activity for a laccase from the fungus trametes versicolor by structure-based mutagenesis. Protein Eng Des Sel 2006;19(2):77–84. https://doi.org/10.1093/protein/gzj004
[67] De Salas F, Cañadas R, Santiago G, Virseda-Jerez A, Vind J, Gentili P, et al. Structural and biochemical insights into an engineered high-redox potential laccase overproduced in Aspergillus. Int J Biol Macromol 2019;141:855–66. https://doi.org/10.1016/j.ijbiomac.2019.09.052
[68] Garzillo AMV, Colao MC, Caruso C, Caporale C, Celletti D, Buonocore V. Laccase from the white-rot fungus Trametes trogii. Appl Microbiol Biotechnol 1998;49(5):545–51. https://doi.org/10.1007/s002530051211
[69] De Silva D, Davis-Kaplan S, Fergestad J, Kaplan J. Purification and characterization of Fet3 protein, a yeast homologue of ceruloplasmin. J Biol Chem 1997;272(22):14208–13. https://doi.org/10.1074/jbc.272.22.14208
[70] Jones SM, Solomon EI. Electron transfer and reaction mechanism of laccases. Cell Mol Life Sci 2015;72(5):869–83. https://doi.org/10.1007/s00018-014-1826-6. Electron
[71] Quintanar L, Gebhard M, Wang TP, Kosman DJ, Solomon EI. Ferrous binding to the multicopper oxidases Saccharomyces cerevisiae Fet3p and human ceruloplasmin: contributions to ferroxidase activity. J Am Chem Soc 2004;126(21):6579–89. https://doi.org/10.1021/ja049220t
[72] Quintanar L, Stoj C, Taylor AB, Hart PJ, Kosman DJ, Solomon EI. Shall we dance? How a multicopper oxidase chooses its electron transfer partner. Acc Chem Res 2007;40(6):445–52. https://doi.org/10.1021/ar600051a
[73] Pardo I, Santiago G, Gentili P, Lucas F, Monza E, Medrano FJ, et al. Re-designing the substrate binding pocket of laccase for enhanced oxidation of sinapic acid. Catal Sci Technol 2016;6(11):3900–10. https://doi.org/10.1039/c5cy01725d
[74] Lucas MF, Monza E, Jørgensen LJ, Ernst HA, Piontek K, Bjerrum MJ, et al. Simulating substrate recognition and oxidation in laccases: from description to design. J Chem Theory Comput 2017;13(3):1462–7. https://doi.org/10.1021/acs.jctc.6b01158
[75] Xu F, Shin W, Brown SH, Wahleithner JA, Sundaram UM, Solomon EI. A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. Biochim Biophys Acta - Protein Struct Mol Enzymol 1996;1292(2):303–11. https://doi.org/10.1016/0167-4838(95)00210-3
[76] Xu F. Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochemistry 1996;35(23):7608–14. https://doi.org/10.1021/bi952971a
[77] Bonaccorsi Di Patti MC, Pascarella S, Catalucci D, Calabrese L. Homology modeling of the multicopper oxidase Fet3 gives new insights in the mechanism of iron transport in yeast. Protein Eng 1999;12(11):895–7. https://doi.org/10.1093/protein/12.11.895
[78] Raffaello T, Chen H, Kohler A, Asiegbu FO. Transcriptomic profiles of Heterobasidion annosum under abiotic stresses and during saprotrophic growth in bark, sapwood and heartwood. Environ Microbiol 2014;16(6):1654–67. https://doi.org/10.1111/1462-2920.12321