UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

ENGINEERING OF A FUNGAL LACCASE AS BIOCATALYST FOR ORGANIC SYNTHESIS
INGENIERÍA DE UNA LACASA FÚNGICA COMO BIOCATALIZADOR PARA SÍNTESIS ORGÁNICA

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

FELIPE DE SALAS DE LA CUADRA

DIRECTOR

SUSANA CAMARERO FERNÁNDEZ

A mi familia

A Teresa y Fernando

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ABBREVIATIONS

Nucleotides

A	Adenine	**K**	G/T
C	Cytosine	**M**	A/C
T	Thymine	**B**	C/G/T
G	Guanine	**D**	A/G/T
R	A/G	**H**	A/C/T
Y	C/T	**V**	A/C/G
S	G/C	**N**	A/C/G/T
W	A/T		

Amino acids

A/Ala	Alanine	**M/Met**	Methionine
C/Cys	Cysteine	**N/Asn**	Asparagine
D/Asp	Aspartic acid	**P/Pro**	Proline
E/Glu	Glutamic acid	**Q/Gln**	Glutamine
F/Phe	Phenylalanine	**R/Arg**	Arginine
G/Gly	Glycine	**S/Ser**	Serine
H/His	Histidine	**T/Thr**	Threonine
I/Ile	Isoleucine	**V/Val**	Valine
K/Lys	Lysine	**W/Trp**	Tryptophan
L/Leu	Leucine	**Y/Tyr**	Tyrosine

Other

4-AAP	4- aminoantipyrine
4-HBA	4-hydroxybenzoic acid
7D5	Laccase from the recombination of the evolved variants of PcL and PM1L
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ANL	Aniline
CD	Circular dichroism
CDS	Coding sequence
C-terminal	Carboxyl end
DAD	Donor-acceptor distance
DM	Double mutant from 7D5
DMP	2,6-dimethoxyphenol
DMPD	N,N-dimethyl-p-phenylenediamine

dNTP	Deoxyribonucleotide triphosphate
E^0	Standard redox potential
EM	Expression mutant from 7D5 laccase
Endo-H	Endoglycosydase H
***ep*PCR**	Error-prone PCR
EPR	Electronic Paramagnetic Resonance
ET	Electron transfer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRAS	Generally recognized as safe
HBT	1-hydroxybenzotriazole
HPLC	High performance liquid chromatography
HRPL	High redox potential laccase
HTS	High-throughput screening
k_{cat}	Catalytic constant
kET	Electron transfer constant
K_m	Michaelis constant
LMS	Laccase mediator system
LRPL	Low redox potential laccase
MCO	Multicopper oxidase
MD	Molecular dynamics
MRPL	Medium redox potential laccase
Mw	Molecular weight
N-terminal	Amino end
PANI	Polyaniline
PcL	*Pycnoporus cinnabarinus* laccase
PDB	Protein data bank
PELE	Protein energy landscape exploration
PK2	Evolved variant 7D5 laccase
PM1L	PM1 basidiomycete laccase
PPD	p-phenylenediamine
QM/MM	Quantum mechanics/molecular mechanics
RY2	Final evolved variant of 7D5 laccase
SASA	Solvent accessible surface area
SDBS	Sodium dodecyl-benzenesulfonate
SLACs	Smal laccases
T_{50}	Temperature at which initial enzyme activity is reduced by half
TAI	Total activity increase
TEMPO	2,2,6,6-Tetramethylpiperidine 1-oxyl
TNC	Trinuclear cluster

RESUMEN/SUMMARY

Resumen

1. Antecedentes

Las lacasas (EC 1.10.3.2) son oxidasas multicobre capaces de oxidar una gran variedad de compuestos aromáticos y ciertos metales. Su versatilidad de sustrato y sus bajos requerimientos catalíticos hacen de estas enzimas biocatalizadores de interés para distintos sectores. Una de las aplicaciones más interesantes de las lacasas es la síntesis de compuestos orgánicos de alto valor añadido, ofreciendo una mayor selectividad que las reacciones químicas y con menor generación de residuos tóxicos. Para muchas de estas reacciones, se requiere el empleo de lacasas fúngicas de alto potencial redox debido a su mayor poder oxidativo. Tal es el caso de la polimerización de anilina para obtener polianilina (PANI), un polímero electroconductor con una amplia variedad de aplicaciones. Sin embargo, las condiciones de reacción (pH ácido y presencia de surfactantes) y el alto potencial redox de la anilina a pH ácido dificultan la acción de la enzima, haciendo necesaria su ingeniería para optimizar su actividad y estabilidad en dichas condiciones. En estudios previos, dos lacasas fúngicas de alto potencial redox fueron evolucionadas en el laboratorio para su expresión en *Saccharomyces cerevisiae*. Posteriormente, se recombinaron dichas enzimas para obtener una librería de lacasas quiméricas con diferentes actividades y estabilidades. De entre ellas, se seleccionó la lacasa 7D5 como punto de partida de esta Tesis Doctoral.

2. Objetivos

El principal objetivo de esta Tesis Doctoral fue obtener una lacasa fúngica de alto potencial redox que actuase como biocatalizador para reacciones de síntesis orgánica, con especial interés en la síntesis de polianilina electroconductiva. Para ello se llevaron a cabo diferentes tareas:

1. Optimización de las condiciones de reacción para la síntesis de polianilina conductiva, incluyendo la selección de la lacasa y del compuesto que actuará como molde dopante para obtener la máxima producción de polianilina con las mejores características.
2. Diseño de la enzima por evolución dirigida y diseño computacional para mejorar la actividad lacasa hacia aminas aromáticas sin reducir su versatilidad de oxidación sobre otros sustratos y, simultáneamente, aumentar la estabilidad de la enzima en las condiciones de reacción de síntesis de polianilina.
3. Mejora de la secreción de la enzima por *S. cerevisiae* y expresión de las nuevas variantes de lacasa en una cepa industrial de *Aspergillus oryzae*
4. Caracterización bioquímica, cinética, computacional y estructural de las nuevas variantes de lacasa para mejorar el conocimiento estructura-función de estas oxidasas multicobre.

3. Resultados

3.1. Síntesis de polianilina catalizada por lacasa

De la comparación de diferentes lacasas (comerciales y obtenidas en el laboratorio), se seleccionó la lacasa 7D5 para su empleo como biocatalizador en la síntesis de polianilina debido a su mayor actividad sobre aminas aromáticas y mayor estabilidad a pH ácido. Se ensayaron diferentes concentraciones de enzima, diferentes concentraciones y tipos de surfactantes aniónicos y distintas condiciones de reacción. Tras una exhaustiva caracterización de los polímeros resultantes, se determinaron las condiciones óptimas de síntesis de sal de esmeraldina (forma electroconductiva de PANI) con SDBS como surfactante aniónico. La polianilina resultante es soluble en agua, presenta una estructura supramolecular en forma de nanofibras (ofreciendo una gran superficie de contacto), una excelente electroactividad y electroconductividad, y se obtiene con un notable rendimiento.

3.2. Comparación estructura-función de la lacasa 7D5 y la enzima salvaje

La lacasa 7D5 fue híper-expresada en una cepa industrial de *A. oryzae*. Esto permitió obtener su estructura cristalina (PDB: 6H5Y) y realizar un estudio estructura-función comparado con la lacasa salvaje del basidiomiceto PM1 (PDB: 5ANH), con la que 7D5 comparte un 98 % de identidad de secuencia. La lacasa obtenida en el laboratorio mostró una mejora significativa en la actividad catalítica frente a todos los sustratos ensayados con respecto a la lacasa salvaje. Mediante simulaciones computacionales de PELE y QM/MM se determinó que una mutación en el bolsillo catalítico de 7D5 mejoraba el posicionamiento y transferencia electrónica de los sustratos al cobre catalítico. Aunque ambas enzimas mostraron buena estabilidad a temperatura y pH, la menor estabilidad de la lacasa 7D5 se relacionó en estudios computacionales con la pérdida de flexibilidad en algunos lazos superficiales de la proteína provocada por las mutaciones adquiridas.

3.3. Ingeniería de la lacasa 7D5

Mediante evolución dirigida de la secuencia codificante de la enzima 7D5 unida a la secuencia evolucionada del pre-prolíder del factor α, se obtuvieron dos nuevas mutaciones en la secuencia señal que incrementaron más de cinco veces la expresión de la enzima en *S. cerevisiae.* Posteriormente, por simulaciones de PELE y QM/MM, se identificaron dos mutaciones en el bolsillo catalítico de la enzima que incrementaron significativamente la constante catalítica para la oxidación deanilinas debido una mejor interacción lacasa-sustrato. Esta nueva variante se sometió a mutagénesis saturada en un residuo próximo al cobre catalítico cuya mutación al azar en este estudio y en estudios previos se asociaba con mejoras significativas en la actividad catalítica pero con pérdida de estabilidad. Combinando ensayos experimentales y computacionales se seleccionó una

mutación en esta posición que incrementaba significativamente la expresión de la enzima sin pérdidas importantes de estabilidad. La sustitución del extremo C-terminal de esta última variante por el de otra lacasa termoestable, dio lugar a la variante final RY2 con cuatro nuevas mutaciones. RY2 presentó una mejora en las constantes catalíticas frente a todos los sustratos probados. La mayor flexibilidad de su C-terminal, que ahora interacciona con el canal de entrada de O_2/H_2O, podría explicar estas mejoras, así como el incremento en la estabilidad de la enzima, gracias a una mayor capacidad de absorber el impacto de las altas temperaturas protegiendo el resto de la estructura proteica. Varias de las variantes obtenidas durante la ingeniería de 7D5 fueron exitosamente expresadas en *A. oryzae*. Esto facilitó su empleo para catalizar la síntesis de polianilina a mayor escala, así como de un nuevo colorante orgánico cuyas excelentes propiedades como tintes textiles fueron demostradas en un entorno industrial.

4. Conclusiones

Una vez demostrada la viabilidad de la síntesis enzimática de polianilina conductiva con excelentes propiedades, la ingeniería de la enzima permitió incrementar los rendimientos de obtención del polímero. Además, la variante final de lacasa obtenida mediante diferentes técnicas de ingeniería de proteínas es estable a alta temperatura y diferentes pH, y presenta una elevada actividad catalítica frente a diversos sustratos como fenoles, aminas aromáticas o mediadores redox. Al mismo tiempo esta nueva lacasa se expresa en *S. cerevisiae* en niveles superiores a los descritos hasta la fecha. La lacasa de partida (7D5) y algunas de sus variantes obtenidas durante su evolución en el laboratorio fueron expresadas en *A. oryzae* a escala de relevancia industrial. Esto ha permitido obtener la primera estructura cristalina de una lacasa de basidiomiceto diseñada en el laboratorio y llevar a cabo un exhaustivo estudio estructura función, comparándolo con la lacasa salvaje PM1. Por otro lado, una de las variantes evolucionadas en *S. cerevisiae* e hiper-expresadas en *A. oryzae* se aplicó con éxito como biocatalizador de la síntesis de PANI y de otro compuesto orgánico cuya caracterización como colorantes textiles se llevó a cabo en un entorno industrial.

Summary

1. Background

Laccases (EC 1.10.3.2) are multicopper oxidases with the capability to oxidize a great variety of aromatic compounds and some metals. Their substrate versatility and their low catalytic requirements turn these enzymes into biocatalysts of interest for different sectors. One of the most promising applications of laccases is the synthesis of high value organic compounds, providing higher selectivity than chemical reactions and generating less toxic residues. For many of these reactions fungal high redox potential laccases are required due to their higher oxidation capabilities. One of these reactions is the synthesis of polyaniline (PANI), an electroconductive polymer with a large number of applications. However, the reaction conditions (acid pH and surfactants presence) and the high redox potential of aniline at acid pH hinder laccase activity, requiring the engineering of the enzyme to optimize their activity and stability under these conditions. In previous works, two high redox potential fungal laccases were evolved in the laboratory for their expression in *Saccharomyces cerevisiae*. These enzymes were thereafter recombined to obtain a library of chimeric laccases with different activities and stabilities. Among them, 7D5 laccase was selected as starting point for this Doctoral Thesis.

2. Objectives

The main objective of this Doctoral Thesis was to develop a high redox potential laccase as biocatalyst of industrial interest for organic synthesis, with a special emphasis in the synthesis of electroconductive polyaniline. For this, different tasks were considered:

1. Optimization of the reaction conditions for the enzymatic synthesis of conductive polyaniline, including selection of laccase and doping template, to obtain a polymer with best performing properties at high production yields.
2. Enzyme directed evolution and computational design to enhance laccase activity towards aromatic amines without reducing its substrate versatility and, simultaneously, improve the stability of the enzyme to the reaction conditions of polyaniline synthesis.
3. Improvement of laccase secretion by *S. cerevisiae* and assessment of the over-expression of the new laccase variants in an *Aspergillus oryzae* industrial strain.
4. Biochemical, kinetical, computational and structural characterization of the laccase variants obtained to contribute to improve the structure-function knowledge of these multicopper oxidases.

3. Results

3.1. Synthesis of polyaniline catalyzed by laccase

Among a set of lacase (commercial or developed in the lab), laccase 7D5 was selected as biocatalyst for the synthesis of polyaniline due to its superior activity towards aromatic amines and stability at acid pH. Different enzyme doses, different concentrations and types of anionic surfactants, and different reaction conditions were assayed. After a deeply characterization of the resulting polymers, the optimal conditions for the synthesis of emeraldine salt (the electroconductive form of PANI) with SDBS as anionic surfactant were set. The resulting polianiline is water-soluble, displays a nanofibered supramolecular structure (yielding high contact surface) and shows excellent electroactivity and electroconductivity, all of it at a high production yield.

3.2. Structure-function comparison of 7D5 laccase and the wild-type laccase

Laccase 7D5 was over-expressed in an *A. oryzae* industrial strain. This allowed to obtain the crystal structure of the enzyme (PDB: 6H5Y) and carry out a structure-function comparison study with the wild laccase from basidiomycete PM1 (PDB: 5ANH), the parental laccase of 7D5 with which it shares the highest sequence identity. The engineered laccase showed a significantly improved catalytic activity towards all the substrate assayed. By PELE and QM/MM simulations it was identified a mutation in the catalytic pocket of 7D5 that improved the substrate positioning and the electron transfer in the catalytic pocket. Even though both enzymes showed high thermal and pH stabilities, the lower stability of 7D5 laccase was related, by computational studies, with the loss of flexibility of certain superficial loops due to the acquired mutations.

3.3. Engineering of 7D5 laccase

Through directed evolution of 7D5 CDS fussed to the α–factor pre-proleader sequence, two new mutations in the signal sequence that improved the enzyme expression in *S. cerevisiae* more than five times were discovered. Subsequently, by PELE and QM/MM simulations, two mutations in the catalytic pocket of the enzyme that increased the catalytic constant towards anilines due to a better substrate-laccase interaction were identified. This new variant was subjected to saturation mutagenesis in a residue close to the catalytic copper whose mutation in this and previous studies was associated with significant activity improvements but with concomitant loss of stability. A mutation in this position that improved significantly the enzyme expression without significant loss in stability was selected by combining experimental assays and computational simulations. The substitution of the C-terminal tail of this last variant by other from a more stable laccase lead to the final variant (RY2), with four new mutations. RY2 displayed improved catalytic constants towards all the substrates assayed due to a higher

flexibility of the C-terminal, which now interacts with the O_2/H_2O channel. This higher flexibility of the C-terminal enables the terminal tail to absorb the impact of high temperatures protecting the protein structure, thus increasing the stability of the enzyme. Some of the laccase variants obtained during the engineering of 7D5 were successfully over-expressed in *A. oryzae*. This make easier its use for the catalysis of the synthesis of polyaniline and of a new organic dye whose excellent properties as textile dyes were tested in an industrial environment.

4. Conclusions

Once proven the enzymatic synthesis of conductive polyaniline with excellent properties, the engineering of the enzyme allowed improving the polymer's production yields. Furthermore, the final laccase variant obtained by means of different protein engineering techniques is stable to high temperature and extreme pH, and it shows high catalytic activity towards different substrates as phenols, aromatic amines or redox mediators. Simultaneously, this new laccase is secreted by *S. cerevisiae* at higher levels than those reported in the literature. The parental laccase (7D5), and some of the variants obtained during its design in the laboratory, were expressed in *A. oryzae* at relevant industrial scale. This allowed to obtain the first crystal structure of a basidiomycete laccase engineered in the laboratory and to perform a deep structure-function comparison study with the wild-type laccase from PM1. Furthermore, one of the *S. cerevisiae* evolved variant over-expressed in *A. oryzae* was successfully applied as biocatalyst in the synthesis of PANI and other organic compound which characterization as textile dyes was carried out in an industrial environment.

THESIS STRUCTURE

Thesis structure

This Doctoral Thesis is structured in three chapters, each of them belonging to a publication in an indexed scientific journal. In addition, this Thesis includes a general introduction and discussion that connects the published works. The content of the published works has been kept unaltered together with the supplementary information. All the chapters have been adjusted to maintain a homogenous structure of the Thesis and each of them have their own abstract, introduction, materials and methods, results and discussion, conclusions and references sections. The published content has been reproduced under an open access license.

List of publications included in this Doctoral Thesis

Chapter 1: Felipe de Salas, Isabel Pardo, Horacio J. Salavagione, Pablo Aza, Eleni Amougi, Jesper Vind, Angel T. Martínez and Susana Camarero. 2016. Advanced Synthesis of Conductive Polyaniline using Laccase as Biocatalyst. *Plos One*. (DOI:10.1371/journal.pone.0164958).

Chapter 2: Felipe de Salas, Rubén Cañadas, Gerard Santiago, Alicia Virseda-Jerez, Jesper Vind, Patrizia Gentili, Angel T. Martínez, Víctor Guallar, Inés G. Muñoz, Susana Camarero. 2019. Structural and Biochemical Insights Into an Engineered High-Redox Potential Laccase Overproduced in *Aspergillus*. *International Journal of Biological Macromolecules*. (DOI: 10.1016/j.ijbiomac.2019.09.05)

Chapter 3: Felipe de Salas, Pablo Aza, Joan F. Gilabert, Gerard Santiago, Sibel Kilic, Mehmet Sener, Jesper Vind, Víctor Guallar, Angel T. Martínez and Susana Camarero. 2019. Engineering of a Fungal Laccase to Develop a Robust, Versatile and Highly-Expressed Biocatalyst for Sustainable Chemistry. *Green Chemistry*. (DOI. /10.1039/C9GC02475A)

A fourth scientific article on related studies carried out during this Doctoral Thesis was published as well. However, this article could not be included as a chapter here because it was previously included in the Doctoral Thesis of other first author, Gerard Santiago

Gerard Santiago[┴], Felipe de Salas[┴], M. Fátima Lucas[┴], Emanuele Monza, Sandra Acebes, Ángel T. Martinez, Susana Camarero and Víctor Guallar. 2016. Computer-Aided Laccase Engineering: Toward Biological Oxidation of Arylamines. *ACS Catalysis*. [┴]These authors contributed equally. (DOI: 10.1021/acscatal.6b01460).

GENERAL INTRODUCTION

1. Laccases

1.1. General aspects

Laccases are phenol oxidases (EC 1.10.3.2) of the multicopper oxidases (MCO) superfamily. First discovered in 1883 in the exudates of the lacquer tree *Toxicodendron vernicifluum* (Yoshida, 1883), laccases have been isolated from plants, fungi (ascomycetes, basidiomycetes, and deuteromycetes), prokaryotes and arthropods. Laccases are implicated in different biological processes. Their main function in insects is related with the synthesis of the exoskeleton (cuticle sclerotization). In bacteria, laccases are involved in morphogenesis processes and spore pigmentation, while in plants they are involved in the synthesis of lignin. Fungal laccases are implicated in intra- and extracellular physiological processes including morphogenesis, pigmentation, pathogenesis, delignification and detoxification (Claus, 2004; Hoegger *et al*., 2006; Morozova *et al*., 2007). White-rot basidiomycetes, responsible for wood decay in nature are one of the main laccase producers. They secrete laccases as part of an array of oxidoreductases, with ligninolytic peroxidases as the main players, enabling the efficient biodegradation of the lignin polymer (Eggert *et al*., 1996; Lundell *et al*., 2010).

Four copper ions act as cofactors for the catalytic activity of laccases. Depending on their UV/visible and electron paramagnetic resonance (EPR) spectroscopy properties, these copper ions are classified as follows: Type 1 copper (T1), is EPR detectable and has a strong absorption at 600 nm, being responsible of the blue color of laccases; Type 2 (T2) copper, is colorless but detectable by EPR; and a pair of type 3 (T3) copper ions with no EPR signal due to an antiferromagnetic coupling mediated by a bridging hydroxyl ligand, and weak absorbance at 330 nm (Jones and Solomon, 2015). T2 and T3 coppers form the tri-nuclear cluster (TNC) where O_2 is reduced to water by the four electrons taken from four molecules of substrate at the T1 site (Sekretaryova *et al*., 2019). According to the reduction potential of the T1 site, laccases can be classified as low (LRPLs, E^0 < +500 mV, most plant and prokaryotic laccases), medium (MRPLs, E^0 +500 to around +700 mV) and high (HRPLs, E^0 from +720 to +800 mV) redox potential laccases (Pardo and Camarero, 2015). The latter are mainly produced by the white-rot and the litter decomposing basidiomycete fungi, and they are of great biotechnological interest due to their high oxidation versatility (Hoegger *et al*., 2006).

Laccases are capable to oxidize a wide range of different compounds, preferably o- and p- substituted phenols and aromatic amines, together with N-heterocycles (indole, benzothiazol, tetrahydroquinoline, hydroxyphthalimide, naphthol, etc), heterocyclic thiols, as well as some inorganic/organic metals. The oxidation of these compounds by laccase only requires O_2 from the air as co-substrate and produces water as sole by-product (Gianfreda *et al*., 1999; Polak and Jarosz-Wilkolazka, 2012; Mogharabi and Faramarzi, 2014). Their promiscuous activity

and low catalytic requirements turn laccases in biocatalysts of choice for many different applications.

Most of laccases are extracellular proteins, but intracellular laccases have been detected in several fungi and insects as well. Fungal laccases are mainly monomeric although some basidiomycete (e.g. *Pleurotus ribis*, *P. pulmonarius*, *Trametes villosa, Cantharelus cibarius*) or ascomycete (*Rhizoctonia solani*) laccases consist of homodimers with each subunit with M_W similar to monomeric laccases (Morozova *et al*., 2007). Laccases are usually constituted by three cupredoxin-like domains each of them with a typical β-barrel topology, being the structure stabilized by two disulfide bonds, the first one located between domains one (D1) and three (D3) and the other one between domains one (D1) and two (D2) (Fig. 1) (Rivera-Hoyos *et al*., 2013; Hakulinen and Rouvinen, 2015).

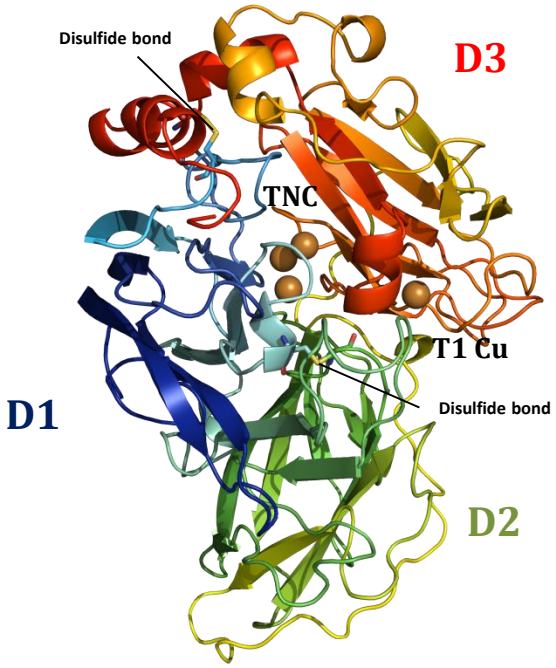

Figure 1. Cartoon representation of *Pycnoporus cinnabarinus* laccase structure (PDB 2XYB) showing the three cupredoxin-domain (D1-D3) folding, the catalytic coppers as spheres, and the two disulphide bridges in yellow.

There are also two-domain enzymes with laccase activity (Small laccase, SLAC) displayed in dimers, trimers and tetramers with different subgroups depending of the position of the coppers (Komori *et al*., 2009; Skálová *et al*., 2009). SLACs are mainly from bacterial origin and they have been proposed to be the common ancestor of three-domain laccases (Skálová *et al*., 2009). However, SLACs have been also described in fungal species such as *Cantharellus cibarius*, *P. eryngii*, and *Tricholoma gianteum* (Nakamura and Go, 2005).

Eukaryotic laccases show molecular masses between 60-130 kDa of which 10-50% may be attributed to glycosylation (mainly N-glycosylation), showing plant

laccases the highest glycosylation degrees (Xu *et al*., 2019). N-glycosylation takes place in the lumen of the endoplasmic reticulum and finalizes in the Golgi (Burda and Aebi, 1999; Herscovics, 1999) and might influence laccase´s secretion, proteolytic susceptibility, catalytic activity or thermal stability (Madhavi and Lele, 2009).

1.2. Catalytic site and reaction mechanism

The dramatically changes in laccase's redox potential is attributed to a perturbation of the geometry of the T1 site (Augustine *et al*., 2008), in part modulated by the presence of the axial ligand. In bacteria and plant laccases (LRPLs), the T1 site presents a distorted tetrahedral geometry due to the presence of a Met residue that acts as a weak fourth axial ligand (Durão *et al*., 2006). By contrast, fungal laccases (MRPLs and HRPLs) present a trigonal planar geometry with a non-coordinating residue (Leu or Phe) in the axial position (Hakulinen *et al*., 2002; Piontek *et al*., 2002). The influence of the axial amino acid in T1 redox potential has been demonstrated in several studies (Hall *et al*., 1999; Xu, 1999). For instance, substitution of the axial ligand Met 502 in CotA laccase from *Bacillus subtillis* (E^0= 455 mV) by non-coordinating Leu or Phe increases E^0 to 515 or 548 mV, respectively (Durão *et al*., 2006). The hydrophobicity of the axial amino acid and the backbone dipoles exert a positive effect in the redox potential of T1 site while the presence of side chain and solvent dipoles decreases the E^0 of T1 site (Hong *et al*., 2011; Jones and Solomon, 2015). In addition, the distance between T1 Cu and the Nδ of His455 (PM1 laccase numbering), one of the His coordinating T1 copper that constitutes the entrance of electrons, accounts for a more electron-deficient copper and, consequently, for an increase of the redox potential of the T1 site (Piontek *et al*., 2002). In addition to this, substrate oxidation at the T1 site strongly depends on the positioning of the substrate in the catalytic pocket (Monza *et al*., 2015). The residues delimiting the pocket exert a great influence in the enzymatic activity by determining the positioning and binding of the substrate at the active site (Lucas *et al*., 2017), being these residues important targets for laccase engineering (Galli *et al*., 2011; Pardo and Camarero, 2015, Pardo *et al* 2016; Khodakarami *et al*., 2018).

T1 site is located in the D3 of the protein, while the tri-nuclear cluster (T2/T3) is embed between D1 and D3 with both domains providing residues for the coordination of the three coppers (situated in a regular triangle). The T1 copper is coordinated by one Cys and two His. In the TNC, each of the T3 coppers have three His ligands and both are connected by an OH bridge, while the T2 copper is coordinated by two His (Fig. 2) and by an aquo-derived hydroxide oriented away from the cluster (Fig. 3) (Jones and Solomon, 2015). The highly conserved Cys-His superexchange pathway (around 13 Å) serves as via to transfer the electrons from T1 site to the TNC (Piontek *et al*., 2002; Jones and Solomon, 2015). The eleven

amino acids coordinating the four copper ions are organized in four separated motifs within the primary amino acid sequence that are strictly conserved in laccases (Rivera-Hoyos *et al*., 2013). In addition a conserved Asp residue (Asp 206 according to PM1 numbering) assists with His455 (PM1 laccase numbering) the concerted electro-proton transfer at the T1 site (Galli *et al*., 2013). Besides, there are two conserved acid residues in the vicinity of the TNC (Asp 77 and Asp 453 according to PM1 laccase numbering) that aid O_2 binding and provide protons for O_2 reduction (Jones and Solomon, 2015).

The catalytic cycle starts with the oxidation of the substrate at T1 site (Fig. 3). It is assumed that laccase acts as a battery, storing the electrons from four monovalent oxidation reactions, which are transferred to the TNC to reduce one O_2 molecule to two H_2O molecules (Jones and Solomon, 2015). Consequently, the enzyme is transformed from the resting oxidized state into the fully reduced state (Fig. 3). Then, the interaction of the TNC with O_2 proceeds in two consecutive steps. In the first one, two electrons, one from the T2 Cu and the other from one of the T3 Cu ions of the fully reduced enzyme are donated to the O_2 molecule generating the peroxy intermediate. In the second step, the bond O-O is broken with the donation of two more electrons from T1 and T3 coppers, rendering the fully oxidized native intermediate (NI). The proton transfer required for the reduction of molecular O_2 to water in the TNC takes place at the same time of the electron transfer. The total reduction of NI by the oxidation of a new set of substrate molecules and the release of two water molecules will start a new catalytic cycle or, in case of lack of more substrate, the slow decay to the resting oxidized form will take place (Yoon and Solomon, 2007; Jones and Solomon, 2015).

The redox potential of the T1 site determines the oxidation capability of the enzyme, only compounds with ionization potential not higher or slightly higher than the redox potential of the T1 Cu can be directly oxidized. However, in contrast to LRPLs where T1 reduction by substrate is the rate limiting step (Jones and Solomon, 2015), in HRPLs the limiting step is the intramolecular electron transfer (IET) from T1 site to the TNC in the fully oxidized intermediate. The increase of the redox potential in T1 Cu derives in a decrease in the driving force for IET to the NI in HRPLs compared with LRPLs, which derives in slower IET rate. However the IET rate is faster enough compared with the decay rate of the NI, which makes this state the catalytically relevant fully oxidized form of HRPLs (Heppner *et al*., 2013; Sekretaryova *et al*., 2019).

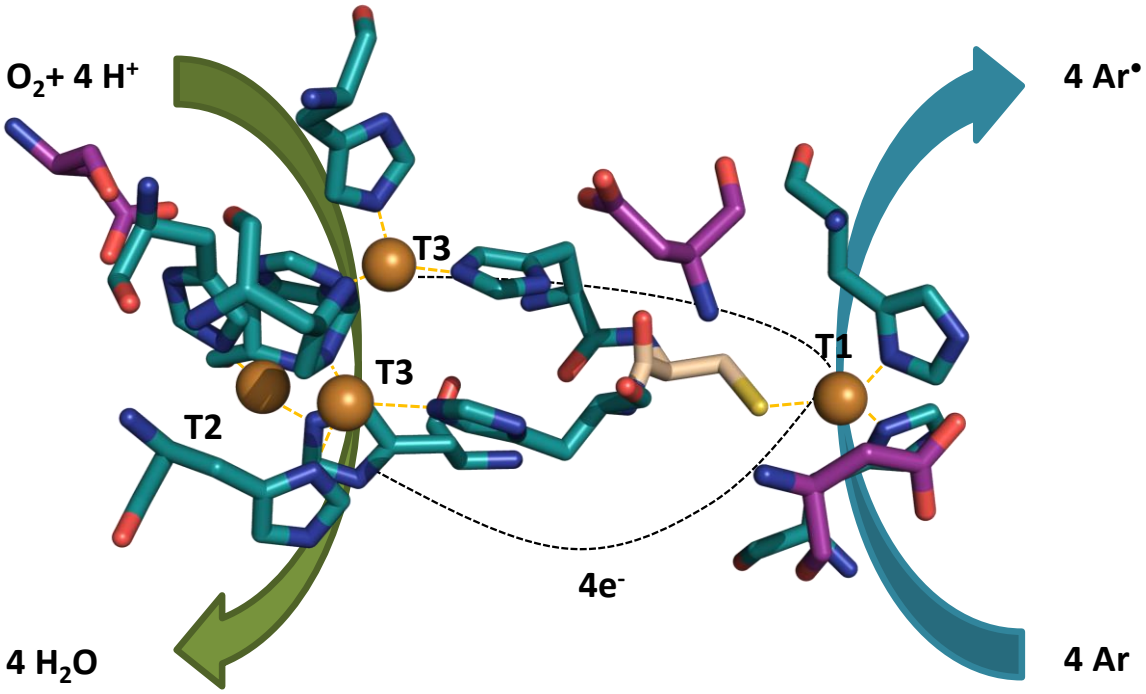

Figure 2. Catalytic site and catalytic mechanism of laccase (based on PM1 laccase, PDB:5ANH). His (blue) and Cys (wheat) residues coordinating the catalytic coppers and conserved Asp residues involved in proton transfer (purple) are shown. The four coppers are depicted as spheres. Electron transfer from T1 site to TNC through the triplet H-C-H is represented as dashed lines.

The catalytic activity as well as the temperature and pH range activity differ greatly among laccases. Bacterial laccases usually have more extremophilic properties (higher thermal stability and more alkalophilic optimum pH) but they display significant lower (≥10-fold) catalytic efficiencies than fungal laccases (Toscano *et al*., 2013; Martins *et al*., 2015; Pollegioni *et al*., 2015). Nevertheless, some fungal laccases show wide pH activity profiles and high thermal stabilities as well as optimal temperatures up to 75 C° (Pardo *et al*., 2018; Mateljak *et al*., 2019).

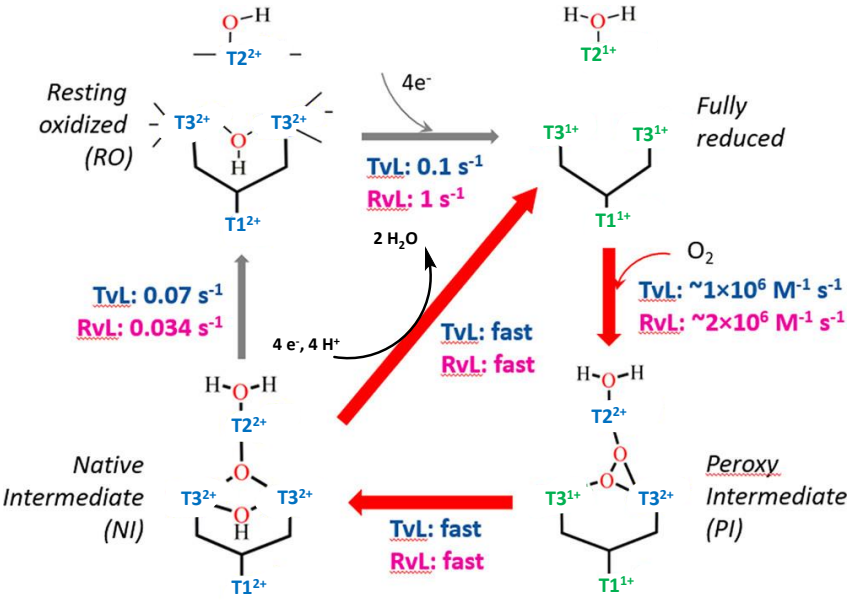

Figure 3. Redox mechanism of the HRPL from *T. versicolor* (TvL) *vs* the LRPL from *Rhus vernicifera* (RvL) (adapted from Sekretaryova *et al*., 2019).

2. Laccase mediator systems

The oxidation capabilities of laccase can be enhanced in the presence of low molecular weight compounds that act as redox mediators once oxidized by the enzyme. In the so-called laccase mediator systems (LMS), the oxidized mediator ("stabilized radical") acts as a diffusible electron carrier, overcoming steric hindrances, to enable the oxidation of bulky substrates such as lignin, cellulose or starch inaccessible to the enzyme or the oxidation of substrates with higher redox potentials that are not oxidized by the enzyme alone (Baiocco *et al*., 2003; Camarero *et al*., 2004; Kunamneni *et al*., 2008; Cañas and Camarero, 2010). The 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), was first described in the 90's as laccase redox mediator for the enzymatic oxidation of non-phenolic lignin model compounds and the delignification and bleaching of paper pulps (Bourbonnais and Paice, 1990, 1992). Since then, other synthetic compounds such as 1-hydroxybenzotriazole, violuric acid, or TEMPO have been described as redox mediators of laccase (Call and Mücke, 1997; Galli and Gentili, 2004; Xu *et al*., 2009; Benzina *et al*., 2012; Rostami *et al*., 2018). Due to their different oxidation mechanisms, the use of different mediators may lead to different products (Baiocco *et al*., 2003). The two major drawbacks of the use of a LMS are the cost of synthetic mediators and the possible generation of toxic intermediates. Substitution of synthetic mediators by low-cost natural mediators like certain phenolic compounds released during degradation of lignin constitutes an efficient and eco-friendly alternative for dye decolorization, detoxification of aromatic

pollutants, pitch removal, delignification and bleaching of paper pulps or to enhance saccharification in biofuel production (Camarero *et al*., 2005, 2007, 2008; Cañas *et al*., 2007; Cañas and Camarero, 2010; Gutiérrez *et al*., 2007; Kunamneni *et al*., 2008; Babot *et al*., 2011; Hollmann and Arends, 2012; Rico *et al*., 2014; Pardo and Camarero, 2015). Ultimately, laccase mediator system adds to the enzyme higher versatility to act as multipurpose biocatalyst in a wide range of industrial processes (Fig. 4.).

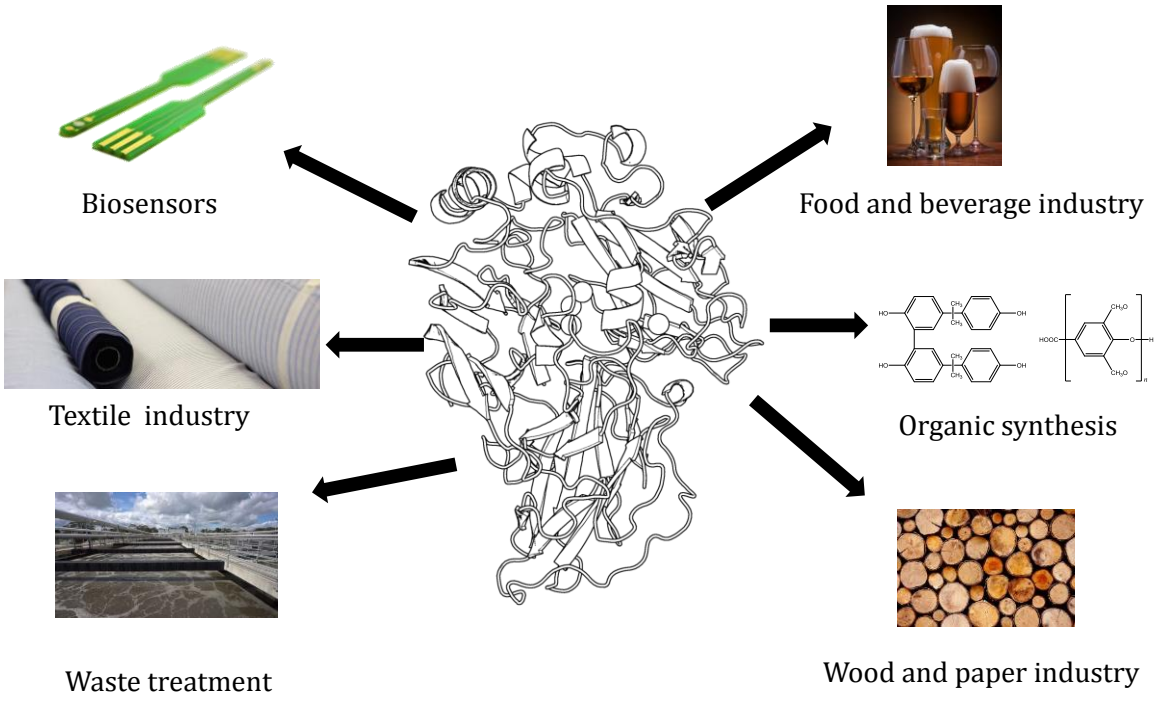

Figure 4. Laccase application examples.

3. Biotechnological applications of laccases

Due to the natural activity of laccases towards lignin phenols, one of the first applications studied was related with the pulp and paper industry. Laccase mediator systems can be applied for pulp bleaching and delignification to remove the residual lignin remaining in the paper pulp after cooking. The integration of laccases in the bleaching sequences contribute to reduce or even avoid the use of chlorine-based harsh chemicals and, simultaneously, enhance pulp strength properties while reducing the energy consumption during refining of the pulp (García *et al*., 2003; Camarero *et al*., 2004; Ibarra *et al*., 2006). Other applications of laccases in the paper industry are: the coupling of low molecular weight compounds to enhance paper strength or improve the resistance to water absorption, modification of the lipophilic extractives to control pulp pitch, deinking of secondary fibber or enzymatic treatment of the effluents from paper mills

(Gutiérrez *et al*., 2007; Widsten and Kandelbauer, 2008; Madhavi and Lele, 2009; Garcia-Ubasart *et al*., 2011; Fillat *et al*., 2012). Laccases can be used also in textile bleaching to avoid back-staining by degrading the released dyestuff after dyeing or for bleaching the textile fibers from natural dyes or impurities (Vasil'eva *et al*., 2008). Some of the first commercial laccase formulations were developed for denim fabric finishing (fading of indigo dyed denim) to give denim the characteristic grey cast (Galante and Formantici, 2003; Pezzella *et al*., 2015). In addition, laccases can improve the dyeing efficiency and reduce the cost of the dyeing process by *in situ* oxidation of inexpensive precursors after their adsorption by the fabric. They have also been used for functional modification of molecules on textile fabrics such as cotton or wool, improving properties as water repellence, antimicrobial features or resistance (Lantto *et al*., 2004; Guimarães *et al*., 2011; Pezzella *et al*., 2015). More demanding environmental regulations have pushed new application opportunities for laccases in waste treatment and disposal (Pezzella *et al*., 2015). Laccase can transform toxic compounds into less toxic derivatives by direct dechlorinating, cleavage of aromatic rings or oxidative transformation of heterocycles and polycyclic aromatic hydrocarbons (Schultz *et al*., 2001; Cañas *et al*., 2007). Waste waters from the food, textile, dye or printing industries that are enriched in phenols and aromatic amines can be detoxified with laccases (Lante *et al*., 2000; Madhavi and Lele, 2009).

Synthetic organic dyes are very stable to temperature, light and microbial attack, making them harsh to degrade. The world total dye production per year is around 800,000 tons and at least a 10% enters in the environment through waste waters where they are a source of eutrophication and can originate toxic by-products through oxidation, hydrolysis or other chemical reactions (Konstantinou and Albanis, 2004). Traditional methods for color removal from these waste waters, such filtration, adsorption or coagulation-flocculation, are expensive and have operational problems. Laccases can catalyze the decolorization of a wide range of synthetic organic dyes alone or in the presence of redox mediator compounds (Claus, 2002; Gubitz *et al*., 2002; Camarero *et al*., 2005). The decolorization is most frequently linked to detoxification. For instance, detoxification of phenolic azo dyes by laccase was proved by the asymmetrical breakdown of azo linkage releasing molecular nitrogen and prohibiting aromatic amine formation (Chivukula and Renganathan, 1995).

As regards the application of laccases in food and beverage processing, one relevant example is found in the wine industry. Laccases substitute the use of SO_2 for the degradation of phenolic groups involved in the madeirization of wine, a process that causes turbidity, color intensification and aroma and flavor alterations. The polyphenols are oxidized by the enzyme, polymerized and removed by clarification. Similar uses have been proposed for the treatment of fruit juices and beer (Minussi *et al*., 2002; Pezzella *et al*., 2015).

Laccases can be used to detect reducing substrates by the changes of O_2, in the absorbance at 600 nm or in voltage current when an electrode is used as final electron acceptor (Xu, 1999). By laccase coupling to electrodes, these enzymes are used in biofuel cell devices with applications in biosensors, bioreactors and bioenergy conversion (Sekretaryova *et al*., 2016; Zhang *et al*., 2017; Rodríguez-Padrón *et al*., 2018; Alba-Molina *et al*., 2019).

3.1. Laccases in organic synthesis

One of the most interesting applications of laccases is their use as biocatalysts for the synthesis of chemical organic compounds, as a sustainable alternative to the use of expensive metal catalysts or toxic reagents, which has attracted the attention of a number of recent research studies.

Various phenolic and aromatic compounds can be oxidized by laccase in order to produce colorful compounds that can be used as dyes. Catechol or indole derivatives (Ganachaud *et al*., 2008; Kim *et al*., 2011; Sousa *et al*., 2016), phenoxiazinone dyes like cinnabarinic acid from 3-hydroxyanthranilic acid or 3-hydroxyorthanilic derived dye (Eggert *et al*., 1995; Bruyneel *et al*., 2008) and several azo dyes have been obtained with laccase as biocatalyst (Polak and Jarosz-Wilkolazka, 2012). Azo dyes are the most used dyes in paper printing and textile dyeing (accounting around a 50 % of the world total dye production) due to their color variety (Konstantinou and Albanis, 2004). Some examples of laccase-synthesized azo dyes are: the synthesis of azobencene dyes using CotA laccase (Sousa *et al*., 2019), a novel nontoxic azo-dye with two anthraquinoinic sulfonated chromophores obtained by immobilized *Perenniporia ochroleuca* MUCL 41114 laccase (Enaud *et al*., 2010), the synthesis of SIC-RED using *Pleorotus ostreatus* laccase POXA1b using p-phenylenediamine and α-naphtol as precursors, or the synthesis of a new azo dye using resorcinol and 2,5–diaminobenzenenesulfonic acid (Pezzella *et al*., 2016; Giacobelli *et al*., 2018). In this PhD thesis, we present a new laccase-synthesized acidic azo dye obtained from 1-naphtol and 1-naphthol-8-amino-3,6-disulfonic acid as precursors.

Laccases have been also used to transform organic compounds into pharmaceutical compounds of high value. Some examples are the enzymatic oxidation of 4-methyl-3-hydroxyanthranilic acid yielding actinocin, a proven antitumoral compound (Osiadacz *et al*., 1999; Burton, 2005), or the synthesis of the powerful antitumoral drug Vinblastine by oxidative coupling of katarantine and vindoline (Sagui *et al*., 2009). These enzymes have been use to synthesize new cyclosporine derivatives (Molino *et al*., 2004), oxidize catechins and synthetize hormone derivatives such β-estradiol (Nicotra *et al*., 2004). The regioselectivity of some LMS in oxidation reactions save time and money compared with chemical organic synthesis. Laccase and 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) can selectively oxidize the primary hydroxyl group in monosaccharides and

disaccharides under mild conditions for their application in different technical areas (Marzorati *et al*., 2005). A laccase of *Trametes pubescens* and TEMPO were used for the oxidation of the primary hydroxyl group of natural glycosides as amygdalin or colchicoside to the corresponding carbonyl groups. Glycerol can be also selectively oxidized using LMS (TEMPO) to generate glyceraldehyde and glyceric acid using low and high TEMPO concentrations respectively (Liebminger *et al*., 2009).

Laccase amination reactions have been also studied (Niedermeyer *et al*., 2005) focusing, for example, in the interesting antibacterial, antifungal, antiallergenic, anti-inflammatory, and anticonvulsant activities of aniline derivatives. For example, reaction of anilines and 2,5-dihydroxybenzoic acid derivatives catalyzed by laccase renders N-analogous of corollosporines (Mikolasch *et al*., 2008) It has been recently reported also the synthesis of 5-alkylaminobenzoquinone and 2,5-bis(alkylamino)-1,4benzoquinone by the coupling of 2-methoxy-3-methylhydroquinone and primary amines, such as octylamine, cyclooctylamine, and geranylamine (Herter *et al*., 2011), or the dimerization of salicylic esters or bisphenol A (Ciecholewski *et al*., 2005). 2,5-dihydroxybenzoic acid derivatives have been also used as precursor of different cyclic products together with aromatic and heteroaromatic amines through oxidative C–N bond formation followed by cyclization catalyzed by laccases (Hahn *et al*., 2010).

3.2. Oxidative coupling and polymerization reactions catalyzed by laccase

The enzyme-initiated radical coupling of phenols and aromatic amines is one of the most interesting organic synthesis applications for laccase learned from nature. In fact, lignification of the plant cell walls consists on the oxidative *p*- and *o*-coupling of cinnamyl alcohols catalyzed by plant laccases and peroxidases. The electronic delocalization enables the radicals to couple at different sites and yield diverse polymeric products (type of lignins) depending on the abundance of precursors available in the plant or tissue, and the linkages formed (Boerjan *et al*., 2003). Accordingly, two different products (with C-O or C-C bonds) are obtained in vitro from dimerization of ferulic acid catalyzed by laccase, while *in vitro* dimerization of sinapic acid leads to a single product (C-C bond) (Tranchimand *et al*., 2006; Mogharabi and Faramarzi, 2014).

While the enzymatic polymerization of syringic acid or DMP is regioselective, yielding chains of poly-phenylene oxide units (Ikeda *et al*., 1996, 1996), other oxidative coupling reactions can lead to non-controllable polymer structures with undesirable characteristics. Different strategies have been developed for gaining control of the process and obtaining the desired polymer structure. Organic co-solvents such as 1,4-dioxane or methanol have been used to increase the solubility of the aromatic substrates achieving in some cases an important control over the

polydispersity and polymer size (Hollmann and Arends, 2012). In addition to classical organic solvents, ionic liquids have been use as co-solvents for polymerization of phenols (Sgalla *et al*., 2007; Eker *et al*., 2009; Khlupova *et al*., 2016). These chemicals increase substrate solubility or act as enzyme immobilization matrix, enabling enzyme recycling during the reaction. It has also been observed that ionic liquids stabilize the enzyme in the presence of an anionic surfactant for the synthesis of polyaniline, thus increasing polymer yields (Zhang *et al*., 2014). Regioselectivity is crucial for most polymerization reactions and it can be achieved by substrate engineering. For example, linear conductive polyaniline can be obtained without templates by blocking the *o*-position of aniline. However, this strategy yields a less conductive and electroactive polymer than the standard chemical synthesis of polyaniline (Kim *et al*., 2007). On the other hand some compounds can act as scaffold during the polymerization reaction to avoid branch formation. These templates are molecules generally with long chain structures that direct the proper alignment of the monomers. In some cases, they are negatively charged, what serves as counter ion (dopant) for the synthesized polymer (Hollmann and Arends, 2012). While templates are often used to achieve polymerization of phenolic precursors, polyaniline synthesis is one of the most significant examples of the use of templates to aid the synthesis of a linear polymer (Kim *et al*., 2005).

4. Polyaniline

The high processability of conductive organic polymers compared with other conducting elements such as metals provides these molecules with an important applicability potential, thus awakening the interest of industry in these kind of polymers (Otero, 2016).

One of the most studied conductive polymer is polyaniline (PANI). The great importance of PANI lies in its electronic conductivity, redox ion-exchange and environmental stability properties in addition to the low cost of the monomer (aniline) needed for its synthesis. PANI offers a number of advanced applications most of them related with eco-friendly processes such energy generation and storage (Boeva and Sergeyev, 2014). Conductive PANI has been successfully assayed as part of solar panels, pseudo-supercapacitors assembly, biosensors, electrostatic and electromagnetic insulating materials, dyestuff (electrochromics), graphene composite, corrosion protection, etc (Feng *et al*., 2011; Hu *et al*., 2014). As regards pharmaceutical applications, PANI has been explored for cancer treatment. Once injected directly in the tumor, its outstanding conductive characteristics make it suitable to be activated by microwave radiation. The heat generated by PANI vibration would lead to destruction of the tumor (Li *et al*., 2015).

Nowadays, industrial synthesis of PANI is carried out through chemical or electrochemical reactions. Chemical synthesis of PANI requires a very acid media (pH 1) and the presence of high quantities of ammonium persulfate as oxidant, whereas the electrochemical process is difficult to perform at industrial scale because of the high energy consumption. Both processes yield an electroactive polyaniline soluble only in some organic solvents (Boeva and Sergeyev, 2014). Besides, the industrial polymerization of aniline produces high quantities of pollutants (Shumakovich *et al*., 2011). Polyaniline can be obtained in very different structures. However, branched (*o*-substituted) polymerization severely limits the degree of conjugation and hence the electrical and optical properties of the resulting polymer. Conductive PANI is formed only by linear *p*-coupling (head to tail) of aniline monomers (Liu *et al*., 1999). Branch formation in chemical synthesis is prevented by the very acid pH of the reaction where head to tail polymerization of aniline prevails.

The combination of benzenoid (amine N) and quinoid (imine N) rings leads to the three different oxidation states of PANI: lecuoemeraldine (totally reduced), emeraldine (half oxidized and half reduced aniline units) and pernigraniline (totally oxidized). Emeraldine can be found protonated (emeraldine salt) or deprotonated (emeraldine base) depending of the pH of the medium. Emeraldine base is blue and non-conductive, while emeraldine salt, which name refers to its emerald green color, is the conductive form of PANI and it is found in a polaron state (Fig. 5) (Sapurina and Stejskal, 2008). Three spectrophotometric absorption bands are characteristic of the emeraldine salt: a band at 325 nm, due to π-π* transition of the benzenoid ring, and absorption peaks at 414 and around 800 nm caused by polaron band transitions (Liu *et al*., 1999; Sapurina and Shishov, 2012).

Polyaniline may yield different supramolecular nanostructures as spheres, microtubules, fibers, sticks or granular powder. These morphologies depend on multiple factors being the most important the nature of solvent and template and the template/substrate concentration ratio. As an example of how different templates can yield different structures, the use of cetyl-trimethyl-amonium bromide for the chemical synthesis of PANI yields nanofibers, while nanofibers and nanotubes with controlled diameters may be prepared with the use of β-naphthalenesulfonic acid (Wei *et al*., 2002; Li and Zhang, 2004). Conductivity of PANI ranges from 10^{-8} to 10^{2} S cm^{-1} and the cyclic voltammograms can display the two or four redox oxidation states (leucoemeraldine-emeraldine-pernigraniline) depending of the conditions used for its synthesis. (Stejskal *et al*., 2008; Tran *et al*., 2011; Boeva and Sergeyev, 2014)

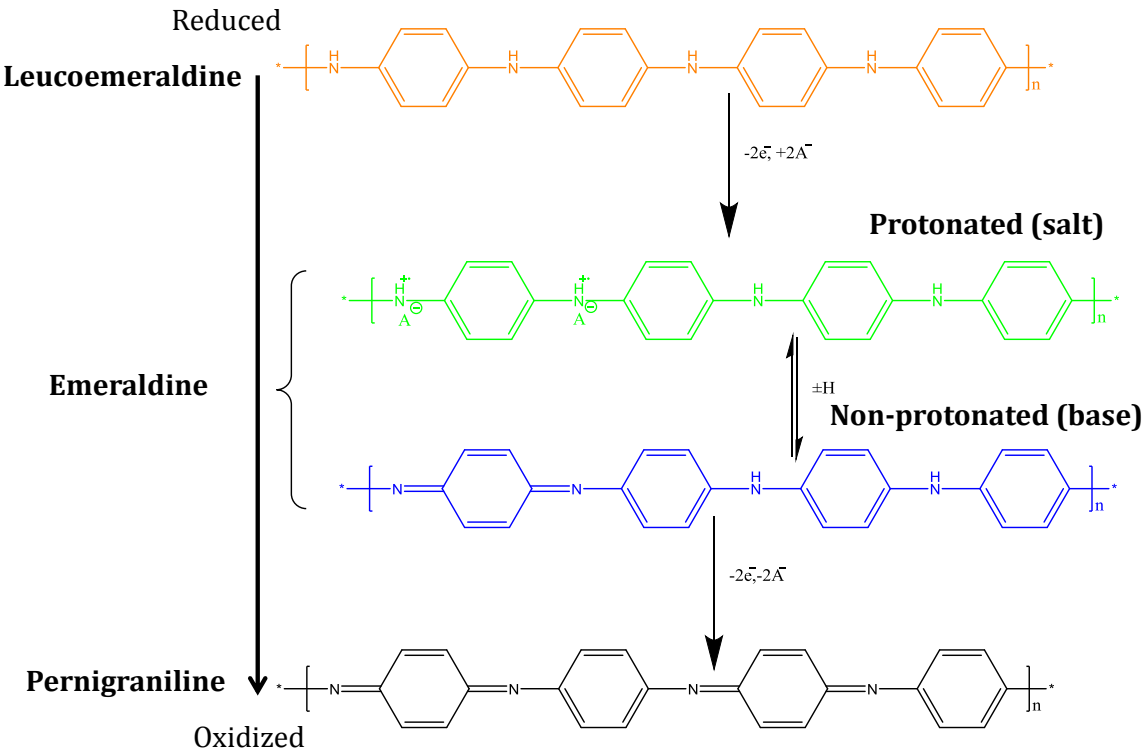

Figure 5. Chemical structures of the different oxidation and protonation states of polyaniline.

4.1. Synthesis of polyaniline catalyzed by laccase

Chemical synthesis of conductive PANI is relative cheap due to the low cost of aniline and the high product yields. However this synthesis is far from being environmentally friendly (Shumakovich *et al.*, 2011). Even more, since the PANI obtained from the chemical polymerization of aniline is soluble only in some organic solvents, the processability of the product is difficult and, hence, the cost of its application is high (Huang and Kaner, 2006).

On the other hand, the enzymatic polymerization of aniline to produce conductive PANI avoids the use of chemical oxidizers and is carried out at milder pH, around 3-3.5, (pH must be below the pKa of aniline to have a prevalence of anilinium cations in the reaction). Higher pH values implicate the prevalence of neutral aniline that yields PANI oligomers with low or none electrocondutive capabilities (Sapurina and Stejskal, 2008) (Fig. 6). As aforementioned only linear polyaniline displays conductive capabilities. To obtain head to tail polymers and avoid parasitic branch formation, a co-solvent or template must be present during the reaction.

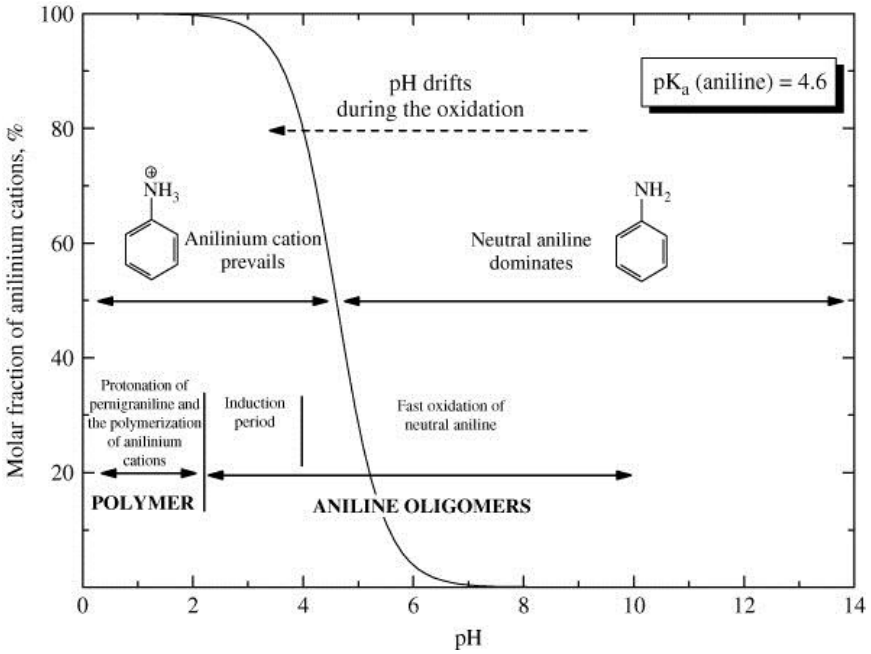

Figure 6. Chemical oxidation of aniline in aqueous media at different pH values. Enzymatic synthesis of conductive PANI can be performed in the induction period (pH range 2-4). However neutral/basic pH yields polyaniline oligomers (from Sapurina and Stejskal, 2008).

Polyelectrolytes like anionic surfactants, poly(vinylphosphonic acid), sulfonated polystyrene or even DNA are used as templates for the synthesis of electroactive PANI (Liu *et al*., 1999; Nagarajan *et al*., 2000, 2001; Karamyshev *et al*., 2003). Anionic surfactants are preferred due their commercial availability and capability to maintain the water solubility of PANI, thus increasing its processing. They form micelles over a critical micellar concentration, providing a suitable local environment for aniline *p*-coupling (Hino *et al*., 2006). The polymerization of aniline associated to this micellar structures yields water soluble PANI that can be precipitated by adding an organic solvent as ethanol or acetone to disaggregate the anionic surfactant micelles (Streltsov *et al*., 2008). As mentioned before, different macromolecular structures can be obtained by changing the anionic surfactant during the enzymatic synthesis of PANI. Anionic surfactants usually inhibits enzyme activity by protein denaturalization, however the presence of aniline protects the enzyme from the action of the surfactant (Otzen, 2011).

The presence of an anionic surfactant in the reaction, the acid pH at which most laccases are unstable, and the high redox potential of the anilinium cation (E^0 = 1.05 V) by contrast to the lower redox potential of aniline at neutral pH (E^0 = 0.63 V), are the main hurdles for the enzymatic polymerization of PANI (Junker *et al*., 2014). The high redox potential of the anilinium cation makes mandatory the use of a high redox potential laccase for its oxidation. In addition, the demanding polymerization reaction conditions might make necessary the engineering of the enzyme to improve its intrinsic properties (activity on aniline and stability) at the reaction conditions (Fig. 7).

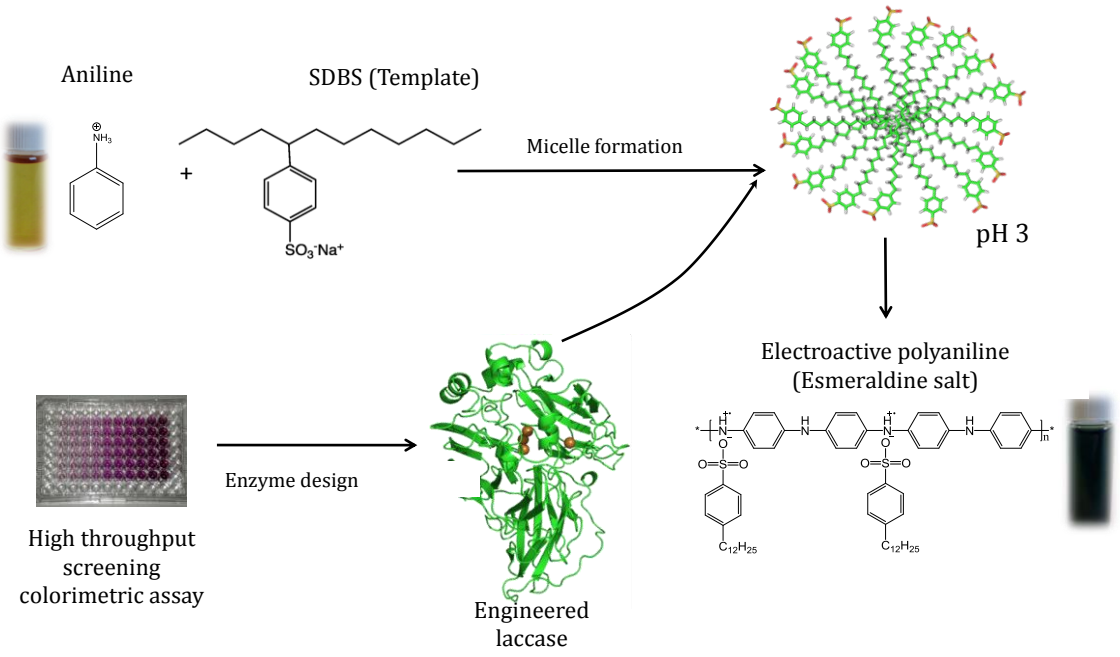

Figure 7. Scheme of the synthesis of conductive PANI carried out in our lab using an engineered laccase and SDBS as template.

5. Enzyme engineering to develop industrial biocatalysts

Enzymes as biocatalysts offer numerous advantages over chemical processes with respect to higher catalytic selectivity, reduced processing time, lower energy input and milder reaction conditions that result into cost-efficient, sustainable and eco-friendly processes. Hence, applications of microbial enzymes in the pharmaceutical, detergents, food and beverage, textile, leather, pulp and paper and other industries are numerous and increasing rapidly over less sustainable conventional methods (Singh *et al.*, 2016). To this end, recombinant microbial enzymes are engineered to meet the operational conditions (extreme pH or temperature, presence of organic solvents, low water concentration) and to adjust their catalytic activity on recalcitrant or non-natural substrates. The production of the developed enzymes is thereafter scaled up in industrial strains to enable their commercialization as tailor made biocatalysts by world-leader companies (Novozymes, BASF, DuPont Danisco, Royal DSM, Codexis). Today, the demand of enzymes for industrial applications is experiencing a continuous increase. Fungal laccases are commercialized as industrial enzymes by Novozymes (Denmark), Jena Biosciences (Germany), Creative Enzymes (USA), Ecostar (India), USBiological (USA), ASA Spezialenzyme (Germany) etc. Bacterial laccases are produced and commercialized by MetGen (Finland). So far, there are several laccases formulated and commercialized for target-applications: denim fabric finishing (DeniLite®),

paper pulp delignification and bleaching (Novozyme NS-51003, MetZyme® LIGNO™), O_2 depletion to preserve flavors from food and beverages (Flavourstar®) or treatment of wine cork stoppers (Suberzyme®). Nevertheless, these old enzymes first discovered in the XIX century, are yet poorly implemented in industrial processes. Protein engineering allows to develop new tailor made laccases for new or specific applications.

5.1. Enzyme directed evolution

In the 90's, directed molecular evolution arose as a powerful alternative to rational approaches to design biocatalysts with null or very little structural knowledge of the enzyme required. According to the Darwinian Theory, the fantastic diversity of life was created by random mutation and natural selection. Over many generations, beneficial mutations accumulate resulting in a successively improved phenotype. The power and simplicity of the evolution algorithm tempted scientists and engineers to try to implement this same approach to the molecular design. Frances H. Arnold (CALTECH, US) was the pioneer to put into practice the concept of evolutionary engineering at the molecular level (Fig. 8). She reported the first iterative random mutagenesis and selection of an enzyme in the lab (Chen and Arnold, 1993). One year later, Willem P. Stemmer described the recombination of homologous genes (DNA-shuffling) as a breakthrough technology to accelerate enzyme directed evolution (Stemmer, 1994). Since then, Arnold's group and many others refined directed evolution to design new enzymes working on unnatural conditions (Zhao and Arnold, 1999), to produce biofuels (Bastian *et al*., 2011) or for doing all kinds of complex chemistry (Kan *et al*., 2016, 2017). Due to her contribution in enzyme directed evolution to design biocatalysts with broad applications from pharmaceuticals to renewable fuels, Frances H. Arnold was awarded the Nobel Prize in Chemistry in 2018. The emergence of advance directed evolution techniques at the protein, pathway and genome level have sped up evolutionary engineering at outstanding levels during the last years (Wang *et al*., 2019).

Today enzyme directed evolution is an essential part of many industrial processes for manufacturing new enzymes for the pharmaceutical, chemical or food industries. From its beginnings to date, the directed evolution of enzymes has greatly expanded the repertoire of biocatalysts useful in the chemical and biotechnology industry. For instance, Novozymes, the world leader producer of industrial enzymes, which introduced the use of proteases and lipases in laundry detergents, was the first to market improved variants of these enzymes obtained by directed evolution in the 90's.

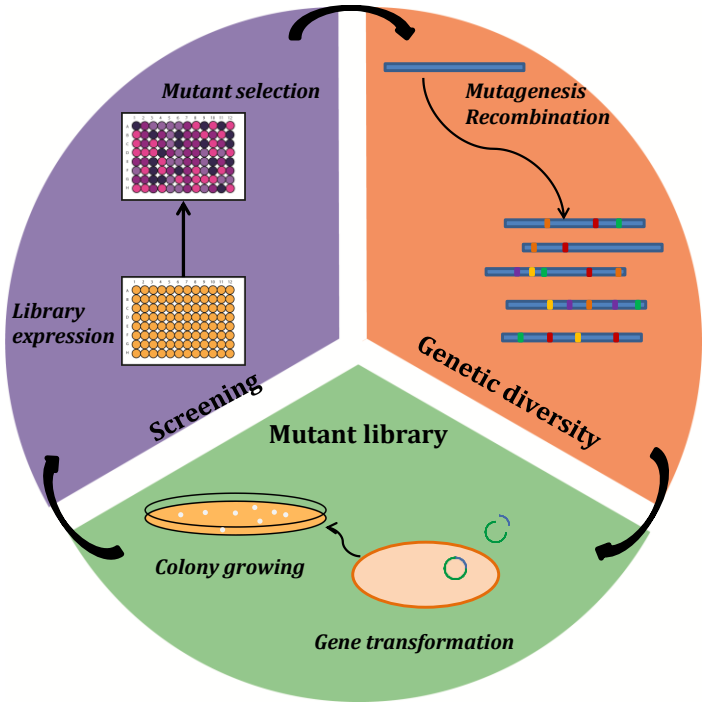

Figure 8. Schematic representation of a directed evolution cycle

5.2. Fundamentals of directed evolution

5.2.1. Heterologous host selection

The thoroughly described physiology, fast growth, easy manipulation and great number of standard protocols available make *Escherichia coli* the preferred host for enzyme directed evolution in directed evolution studies (Pourmir and Johannes, 2012) (Fig. 9). However, the different post-translational routes followed by eukaryotic proteins often result in non-functional proteins, what heavily hinders the use of *E. coli* for expression and engineering of eukaryotic enzymes (Kraševec and Benčina, 2016). By contrast, eukaryotic hosts as *S. cerevisiae* or *Pichia pastoris* are capable of performing the post-translational modifications required to obtain fully active secreted enzymes. The latter yeast provides high protein secretion yields. However, most vectors available are integrative and display low integrative efficiency, what is reflected in low transformation efficiencies and difficult gene recovery, thus limiting their application as host for directed evolution (Mate *et al*., 2013; Chumnanpuen *et al*., 2016). On the contrary, *S. cerevisiae* provides high transformation efficiencies with a range of episomal multi-copy vectors available that enable the easy and efficient genetic manipulation of the laboratory strains (Gnügge and Rudolf, 2017). Besides, the homologous DNA recombination machinery of *S. cerevisiae,* with proof-reading activity, allows the *in vivo* recombination and cloning of PCR products in the linearized shuttle vector in one single step (Alcalde, 2006). On the other hand, the yeast provides low protein yields and frequently hyperglycosylated enzymes (up

to 50 %) what discourage its use as industrial expression host (Gonzalez-Perez *et al*., 2012).

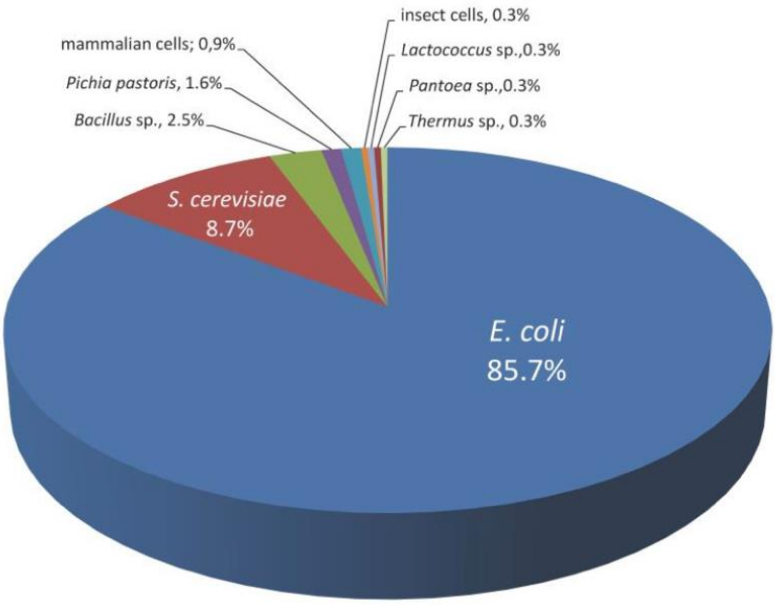

Figure 9. Preferred directed evolution hosts based on studies from PubMed database (from Pourmir and Johannes, 2012).

One approach to improve the secretion of recombinant proteins by *S. cerevisiae* is the replacement of the protein´s native signal peptide by the signal sequences from own yeast proteins, such as those from the alpha-factor mating pheromone or the K1 killer toxin from *S. cerevisiae* (Cartwright *et al*., 1992; Mate *et al*., 2010; Camarero *et al*., 2012; Garcia-Ruiz *et al*., 2012; Inokuma *et al*., 2016). The prepro-leader of the alpha-factor contains a pre-region of 19 amino acids and a pro-region of 64 amino acids. The pre-region acts as signal peptide, leading the translocation of the nascent protein from the ribosome to the endoplasmic reticulum, where it is cleaved by the action of a signal peptidase starting the glycosylation. Then, the protein packed inside vesicles is transported to the Golgi apparatus where protein glycosylation and folding continue aided by the chaperone-like action of the α-factor pro-leader before its cleavage by the action of proteases KEX2, STE13 and KEX1. The folded enzyme is thereafter secreted to the extracellular medium (Fuller, 1988; Fitzgerald and Glick, 2014). The directed evolution of the alpha-factor prepro-leader attached to the enzyme gene has been successfully used for the heterologous expression of different fungal oxidoreductases in *S. cerevisiae*, including important secretion improvements of different laccases (Mate *et al*., 2010; Camarero *et al*., 2012; Garcia-Ruiz *et al*., 2012; Pardo *et al*., 2012; Viña-Gonzalez *et al*., 2015; Mateljak *et al*., 2017).

5.2.2. Creating genetic diversity

In natural evolution, genetic diversification of the offspring enables the natural selection of the most fitted phenotypes and the inheritance of their genotypes during the evolution of the species. In lab evolution, mutagenesis and recombination of the parent genes enable to create the genetic diversity that results in the fitness difference that is subsequently explored and selected.

Conventionally, when there is no or little structure-function knowledge of the enzyme under study, genetic diversity is created by random mutagenesis of the whole gene using error-prone PCR (epPCR), either with Taq polymerase under mutagenic conditions (Cadwell and Joyce, 2016) or using commercial polymerase mixtures like Mutazyme II® specially developed to reduce mutation biases (Packer and Liu, 2015). In addition, recombination of homologous genes allows the accumulation of neutral or beneficial mutations from different parent genes without jeopardizing the functionality of the enzyme. DNA shuffling (Stemmer, 1994) consists in random reassembly by PCR of the gene fragments obtained after DNase digestion of the parent genes (Fig. 10). Thanks to the homologous recombination machinery of *S. cerevisiae*, its use as host in enzyme directed evolution provides additional recombination of PCR products and their *in vivo* cloning in a single step only by creating overlapping fragments with the linearized vector (Alcalde *et al.*, 2006).

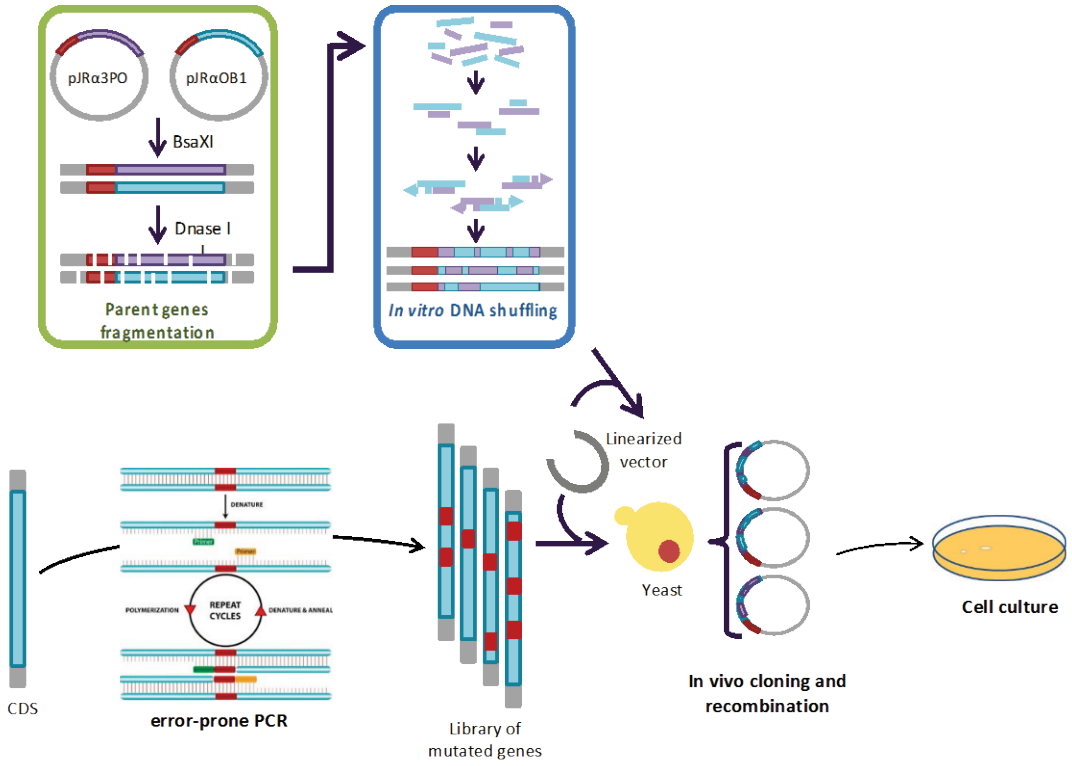

Figure 10. In vivo cloning and recombination in *S. cerevisiae* of the mutant libraries obtained by random mutagenesis or recombination of parent genes.

Other recombination technique that avoids DNase fragmentation is the staggered extension process (StEP) (Zhao *et al*., 1998), in which a PCR with an interrupted elongation step allows to switch primers permitting template recombination in one amplicon. Related methodologies based in homologous recombination are the random chimeragenesis of transient templates (RACHITT)(Pelletier, 2001) that uses a different reassembly method, or the nucleotide exchange and excision technology (NExT) (Müller *et al*., 2005) which incorporates deoxyuridine triphosphate (dUTP) during PCR, allowing to determine the size distribution of random fragments after treatment with uracil deglycosylases. Other methodologies allow the design of libraries of non-homologous sequences such as the incremental truncation for the creation of hybrid enzymes (ITCHY) (Benkovic *et al*., 1999) or the overlap extension PCR (Horton *et al*., 1989). However, non-homologous recombination methods have high probabilities to yield non active domain-disrupted enzymes. Some algorithms as SCHEMA (Voigt *et al*., 2002) arose to identify these breakpoints in proteins, and minimize the number of disrupted inter-domain interactions. When there is previous structure-function knowledge of the enzyme, the directed evolution experiment can be significantly improved by targeting mutagenesis to specific hotspot positions associated with enzyme stability or catalytic activity (focused mutagenesis). The design of small libraries enriched in functional proteins (smart libraries) significantly reduces the screening effort. The most simple focused evolution strategy is the saturation mutagenesis of a single position to explore all possible amino acid substitutions. Combinatorial saturation mutagenesis (CSM) of different positions at the same time exponentially increase the number of possible mutation combinations and, consequently, increases the screening effort to numbers impossible to manage. This problem can be partially overcome by using codons with reduced degeneration (Wells *et al*., 1985), or following iterative saturation mutagenesis (ISM) strategies that facilitate the systematic exploration of different regions and discover possible epistatic effects among mutations (Reetz and Caballeira, 2007; Pardo *et al*., 2016). I*n silico* analyses and computational calculation greatly facilitate de design of smart libraries providing the identification of hotspots for enzyme engineering or predicting beneficial amino acid substitutions. Several software and web servers such as GLUE-It, CAVER CASTER, HotSpot Wizard, Popmusic are public available for scientists working on enzyme design (Sebestova *et al*., 2014) (see section below).

5.2.3. High-throughput screening

One of the main bottlenecks of enzyme directed evolution is the development of a suitable selection or screening method to explore the mutant libraries preserving the linkage between genotype and phenotype. Screening methods rely on the inspection of individual phenotypes by spatial separation of clones and the rapid assessment of optical features such as color, fluorescence, luminescence or

turbidity. However, most biomolecules are not associated with directly observable phenotypes and require a readily detectable reporter (GFP, luciferase, beta-galactosidase, etc) to enable the detection of gene expression, or the use of surrogate substrates to generate a fluorescent, luminescent or colorimetric signal that is proportional to the enzymatic activity of interest (Romero and Arnold, 2009). The optical signal can be directly screened in the colonies grown agar plates or after expression of the enzyme library in liquid culture (microtiter plates). Different HTS colorimetric methods have been developed in our lab for the directed evolution of laccases using different subrogate substrates (Fig. 11) (Camarero *et al.*, 2012; Pardo *et al.*, 2013; Rodríguez-Escribano *et al.*, 2017; and this Doctoral Thesis). The selective pressure has to be correctly applied during the screenings according to the enzyme properties shought ("*you get what you screen for*", Frances Arnold). Since the increment in activity towards a particular substrate often entails the loss of other beneficial properties as enzyme stability or substrate promiscuity, multiple-screening assays are commonly performed (Pardo *et al.*, 2013; Pardo and Camarero, 2015) and combined with stability assays (Garcia-Ruiz *et al.*, 2012).

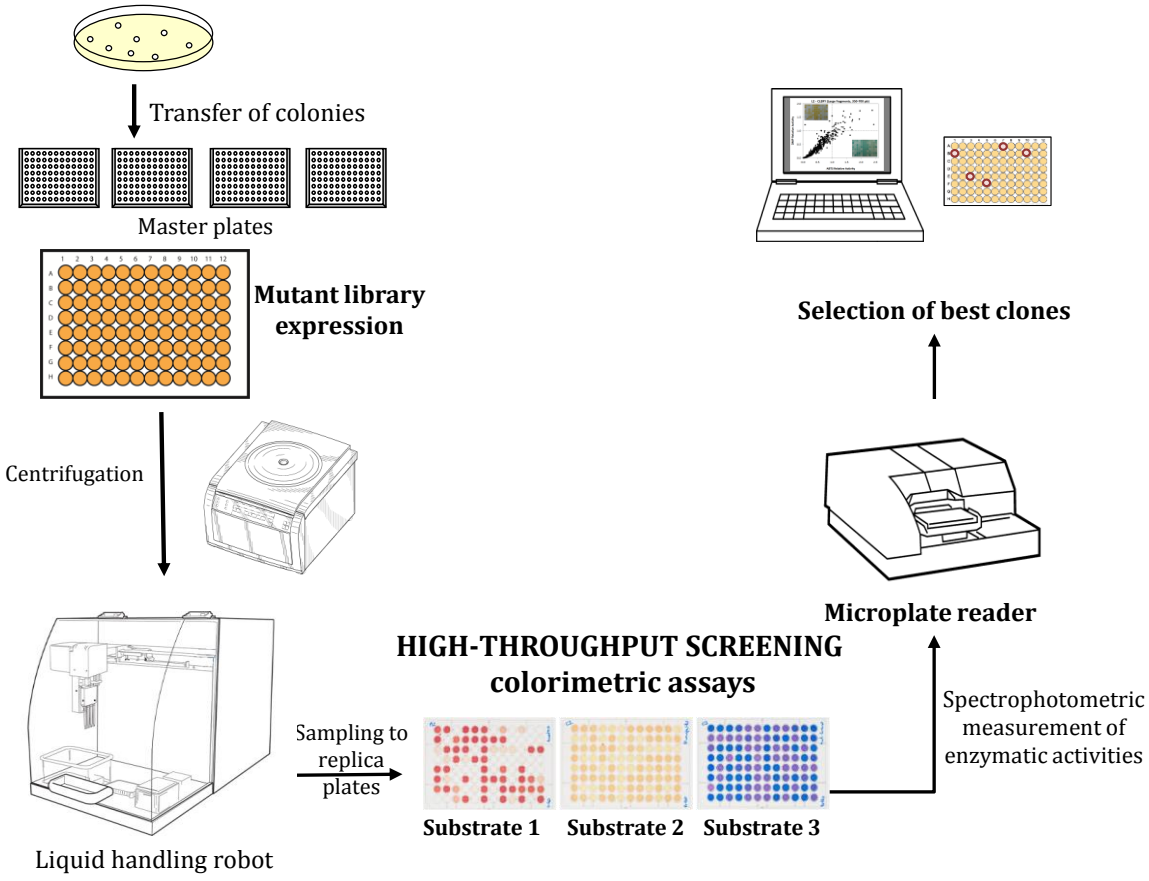

Figure 11. Schematic representation of a multiple HTS colorimetric assay carried out in our lab.

The above described screening methods have the limitation of high-throughput measurement that hinders the exploration of the great variability possibly

obtained in the mutant libraries. To surpass this, instead of spatially separation of the clones, a mass population can be interrogated at the level of individual cells using the cell membrane or cell wall to maintain the genotype-phenotype association. High-throughput screening by flow cytometry enables the classification of fluorescence activated cells (FACS) by measuring the fluorescence of individual cells and the separation of subpopulations by electrostatic deflection (Ostafe *et al*., 2013). More recently this methodology has been applied to miniaturized directed evolution experiments in artificial reaction compartments composed of uniform water-in-oil droplets produced by microfluidics. The fluorescence-activated droplet sorting (FADS) offers an ultrahigh-throughput screening platform that has revolutionized the scale and speed of screening as compared with microtiter-plates based systems (Agresti *et al*., 2010). The system has been recently used to evolve enzymes acting on synthetic nucleic acids encapsulated in drop-based microfluidics (Vallejo *et al*., 2019).

5.3. Computational simulation

A main hurdle in enzyme directed evolution is the impossibility to cover the entire mutational space of a typical protein and to explore the massive number of possible mutations obtained. The design of small high-quality libraries help to focus the mutagenesis in hotspot regions to raise the frequency of improved phenotypes (Lutz, 2011). The exponential progress in computational power over the last years offers a powerful tool to reveal new protein hotspots for mutagenesis and predict beneficial amino acid substitutions. Two main computational approaches can support enzyme design to highly improve the results obtained by directed evolution in the lab: i) sequence, structure and/or phylogenetic-based computational analysis and ii) physics-based computational analysis. Both methodologies can be combined to speed up and improve the results of directed evolution experiments (Monza *et al*., 2017).

Data-driven computational methods are based on either evolutionary information encapsulated in homologous protein sequences from phylogenetic analysis or provided by previous directed evolution rounds, or in previous protein structure-function knowledge. PoPMuSiC (Dehouck *et al*., 2009),3DM (Kuipers *et al*., 2010) and HotSpot Wizard (Sumbalova *et al*., 2018) tools combine sequence and structure databases using statistical potentials to yield mutability maps in the target protein. They and the above mentioned SCHEMA software have been proven to be very effective to identify protein regions with evolvability potential.

Multiple sequence alignments and phylogenetic analyses are standard tools for exploration amino acid conservation and ancestral relationships among groups of homologous protein sequences and structures. Consensus enzyme design is based on the hypothesis that at a given position, the respective consensus amino acid contributes more than average to the stability of the protein than non-conserved

amino acids (Porebski and Buckle, 2016). Involving ancestral phylogenetic relations, the reconstructing evolutionary adaptive paths (REAP) method, is capable to identify mutations emerged during functional divergence from a common universal ancestor (Cole *et al*., 2013). A recent successful example of phylogeny-based ancestral design is the development of a thermostable variant of *Agaricus brasiliensis* laccase. The replacement of sixteen amino acid residues by those found in the phylogenetically inferred ancestral sequence, not only notably increased the thermal stability of the enzyme but also enabled its heterologous expression in *P. pastoris* (Hamuro *et al*., 2017).

While directed evolution is a powerful tool for obtaining desired protein properties, it can be resource-intensive. Moreover, information from all but the highest-performing variants is commonly ignored in the lab. Machine Learning methods can support enzyme directed evolution taking advantage of the huge amounts of potential training data produced in the directed evolution campaigns (positive and negative results). Thus, machine-learning allows to efficiently explore sequence space by learning patterns from each round of evolution to guide subsequent rounds (Yang *et al*., 2019).

On the other hand, computational design combining sequence or structural elements with physics-based methods can assist the development of three mayor aspects in enzyme design: catalytic rate, protein stability and protein-ligand binding processes. Two of the most popular computational tools used to simulate stability changes produced by mutations, are Rosetta (Damborsky and Brezovsky, 2014) and FoldX (van Durme *et al*., 2011). Both software's estimate the folding free energy for the mutated variant depending on physic-based terms (van der Waals, hydrogen bonds and solvation and electrostatic energies) and knowledge-based contributions which determine the probability of a given rotamer (Monza *et al*., 2015, 2017). To date, FoldX (Komor *et al*., 2012), Rosetta-ddG (Sammond *et al*., 2018), and PoPMuSiC (Zhang and Wu, 2011) have been successfully employed to improve protein stability.

The computational design of protein-ligand recognition is a slow and complex process that requires extensive sampling for protein-ligand dynamics including in many cases induced-fit protein conformational changes. These studies are not only reduced to the catalytic pocket as some mutations along the substrate channel could obstruct the ligand entrance or exit process. PELE (Borrelli *et al*., 2005), HTMD (Doerr and De Fabritiis, 2014), RAMD (Lüdemann *et al*., 2000) and directed MD (Grubmuller *et al*., 2008) algorithms help us to explore the most favorable ligand-receptor interactions. Quantum mechanics (QM) can be used to validate beneficial mutations, and combined with molecular mechanics (MM) can significantly improve protein-ligand binding prediction through explicit energy calculations. QM/MM methods treat the active region with QM and with MM the rest of the system (Monza *et al*., 2017). Exploration of ligand diffusion using PELE and QM/MM calculations to evaluate the electronic transfer have been successfully

combined to understand the oxidation mechanism and to design different types of oxidoreductases; first, to rationalize the effect of mutations selected experimentally and validate the methodology, and then to predict beneficial mutations to improve the enzymes (Monza *et al.*, 2015; Linde *et al.*, 2016; Pardo *et al.*, 2016; Santiago *et al.*, 2016; Acebes *et al.*, 2017; Lucas *et al.*, 2017; Carro *et al.*, 2019; Mateljak *et al.*, 2019; Serrano *et al.*, 2019; Viña-Gonzalez *et al.*, 2019) (Fig. 12).

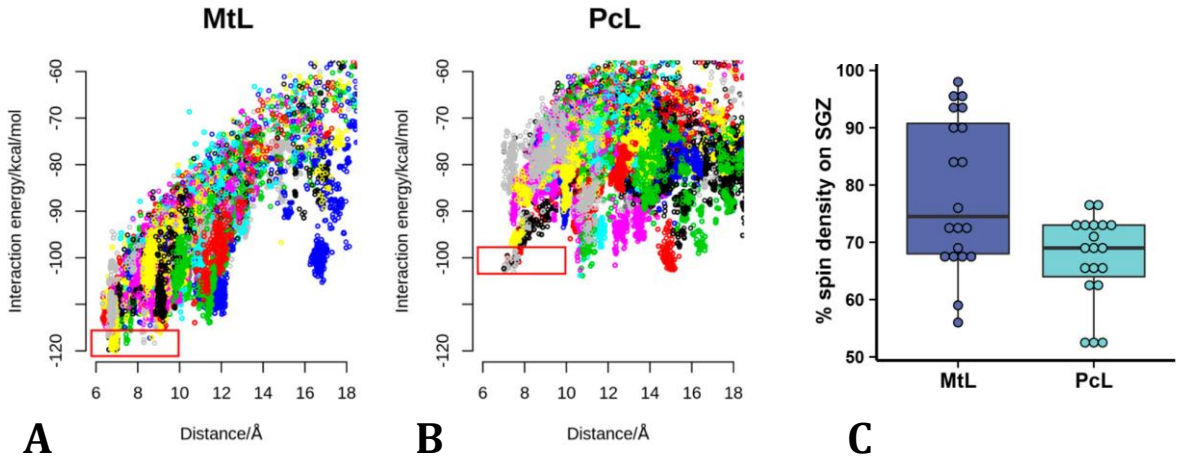

Figure 12. Interaction energies vs distance between the center of mass of syringaldazine and the T1 Cu atom obtained with PELE (**A**, **B**) and distribution of spin densities (**C**) for two different fungal laccases from *Myceliophthora thermophila* and *Pycnoporus cinnabarinus* (from Lucas *et al.*, 2017).

6. Heterologous expression of fungal laccases

Overexpression of basidiomycete laccases constitutes a challenging research field. Examples of overproduction of wild basidiomycete laccases are barely found in the literature. One outstanding example is the gram-scale production of *P. cinnabarinus* wild type laccase by the monokaryotic *P. cinnabarinus* ss3 strain (1.5 g/l) using ethanol as inducer (Lomascolo *et al.*, 2003). The homologous over-production of the recombinant laccase was later achieved (1.2 g/l of native enzyme) by transforming a laccase-deficient *P. cinnabarinus* monokaryotic strain with the homologous laccase gene under the control of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (Alves *et al.*, 2004). The homologous overexpression of laccase III from *Coriolus versico*lor (= *Trametes versicolor*) under GAPDH promoter was also reported, but no enzyme yields were provided (Kajita *et al.*, 2004).

The difficult genetic manipulation of the natural producing strains, together with the lack of GRAS (generally recognized as safe) status and optimized scale-up protocols for industrial fermentation of wild laccases, discouraged the use of basidiomycete strains to engineer and produce laccases so far, drawing the

attention to heterologous expression hosts (Otterbein *et al*., 2000). To this end, optimization of laccase CDS according to the codon usage of the heterologous host, selection of adequate expression vectors and optimization of the expression conditions are carried out with dissimilar results.

Escherichia coli is successfully used for the expression, engineering and scale up production of bacterial laccases (Alessandra *et al*., 2010; Hämäläinen *et al*., 2018), but the expression of active fungal laccases in *E. coli* has not been achieved due to the different post-translational processing machinery of the bacterium (Salony *et al*., 2008; Ma *et al*., 2018).

By contrast, ascomycete expression systems (both yeasts and filamentous fungi), well established for industrial production of enzymes, are well suited as heterologous hosts for the functional expression of basidiomycete laccases. However, the levels of basidiomycete enzymes produced in these systems are often much lower than those of ascomycete enzymes. Some of the reasons behind this fact maybe related to differences in basidiomycete and ascomycete gene models (basidiomycete genes often have more introns with less conserved start and stop sequences) or to sensitivity to ascomycete proteases (Casado López *et al*., 2016).

As aforementioned, the low expression yields and frequent hyperglycosylation of recombinant proteins hinder the use of *S. cerevisiae* as industrial host (Herscovics, 1999). The powerful methanol-inducible alcohol oxidase (AOX1) promoter and the high density growth of *P. pastoris* (up to OD_{600} = 500 in bioreactor) provide higher enzyme yields than those obtained in *S. cerevisiae* (Cereghino, 2002; Mate *et al*., 2013; Wang *et al*., 2016). The highest enzyme yields ever reported (550 mg/l) for a basidiomycete laccase produced by *P. pastoris* in fed-batch fermentation correspond to *T. versicolor* laccase using AOX1 promoter (Hong *et al*., 2002). Constitutive promoters such as the glyceraldehyde-3-phosphate dehydrogenase promoter (GAPDH) have also been assayed, sometimes with even better results. That is the case of fed-batch fermentation of *P. pastoris* producing POXA1b laccase (from *Pleurotus ostreatus* basidiomycete), where constitutive production under GAP outperformed the enzyme levels obtained in AOX induced cultures (Pezzella *et al*., 2017), in agreement with results obtained for the ascomycete *Botrytis aclada* laccase (Kittl *et al*., 2012) the enzyme levels were about (40-60 mg/l). A drawback of the use of *P. pastoris* is the variable enzyme expression yields obtained depending on the characteristics of the heterologous protein, which makes this expression system weak predictable (Mate *et al*., 2013).

Among all the host systems used for protein expression, filamentous fungi show the highest expression levels especially when the secreted protein is homologous. Even if the genetic manipulation of filamentous fungi is more complex than yeasts', the high expression yields of active enzyme and the low cost of the growth media (filamentous fungi show and enormous nutrition flexibility) make them the preferred hosts for production of industrial enzymes (Fig. 13). There are several

examples of basidiomycete laccases heterogously expressed in *Aspergillus*, *Trichoderma* or *Penicillium* (Abianova *et al*., 2010). The highest expression yields (close to gram per litter) have been reported in *Aspergillus* (Couto and Toca-Herrera, 2007; Alessandra *et al*., 2010). For instance, gram-scale production of a laccase from *Trametes sp*. C30 has been achieved in *A. niger* (Mekmouche *et al*., 2014). The heterologous production of other native basidiomycete laccases and their variants engineered in vitro has been also attained in this species although at a lower scale: *P. cinnabarinus* laccase, wild-type (145 mg/l, Record, 2002) and an evolved variant (23 mg/l, Camarero *et al*., 2012), and wild POXA1b and its 1H6C variant (13 mg/l and 20 mg/l respectively, Macellaro *et al*., 2014). *Aspergillus oryzae* is employed at Novozymes for the industrial production of a recombinant high-redox potential laccase from *T. villosa* commercialized as NS 51002 (no longer available) and the ascomycete *Myceliphtora thermophila* laccase (NS 51003). This expression system has been used as well for the production of different basidiomycete laccase variants developed in our lab during this Doctoral Thesis at industrial relevant scale in Novozymes.

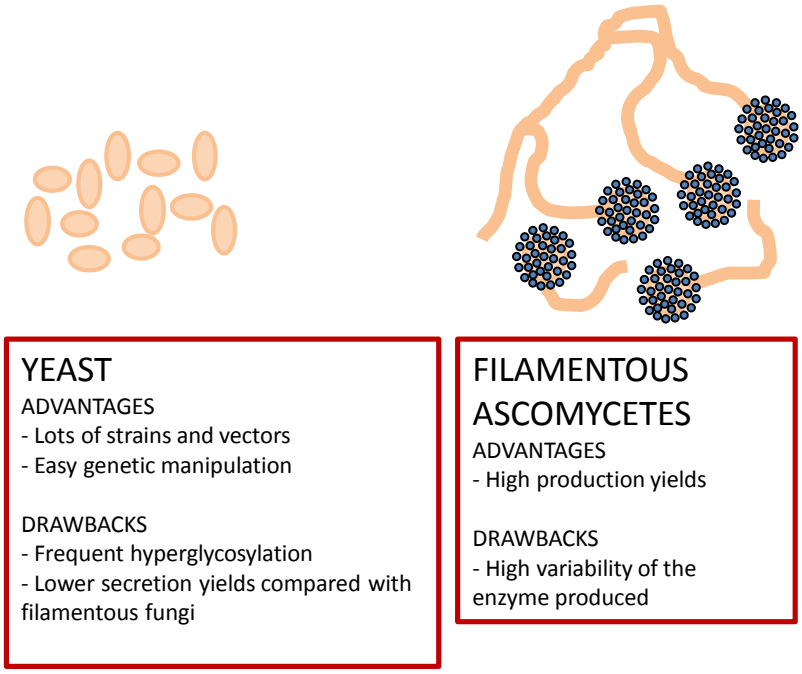

Figure 13. Comparison of yeast and filamentous ascomycetes as heterologous hosts for laccase production.

7. Directed evolution of high-redox potential laccases

There are quite a few studies on directed evolution of basidiomycete laccases in *S. cerevisiae* towards different goals: enhanced expression, improved catalytic activity or stability, shift of optimal pH or substrate specificity (Mate *et al*., 2010,

2013; Robert *et al*., 2011; Camarero *et al*., 2012; Pardo *et al*., 2012, 2016, 2018; Mateljak *et al*., 2017, 2019, 2019; Vicente *et al*., 2019). This work aims at developing a tailor-made robust biocatalyst for organic synthesis, especially for the synthesis of electroconductive PANI, using laccase directed evolution in combination with computer-aided design.

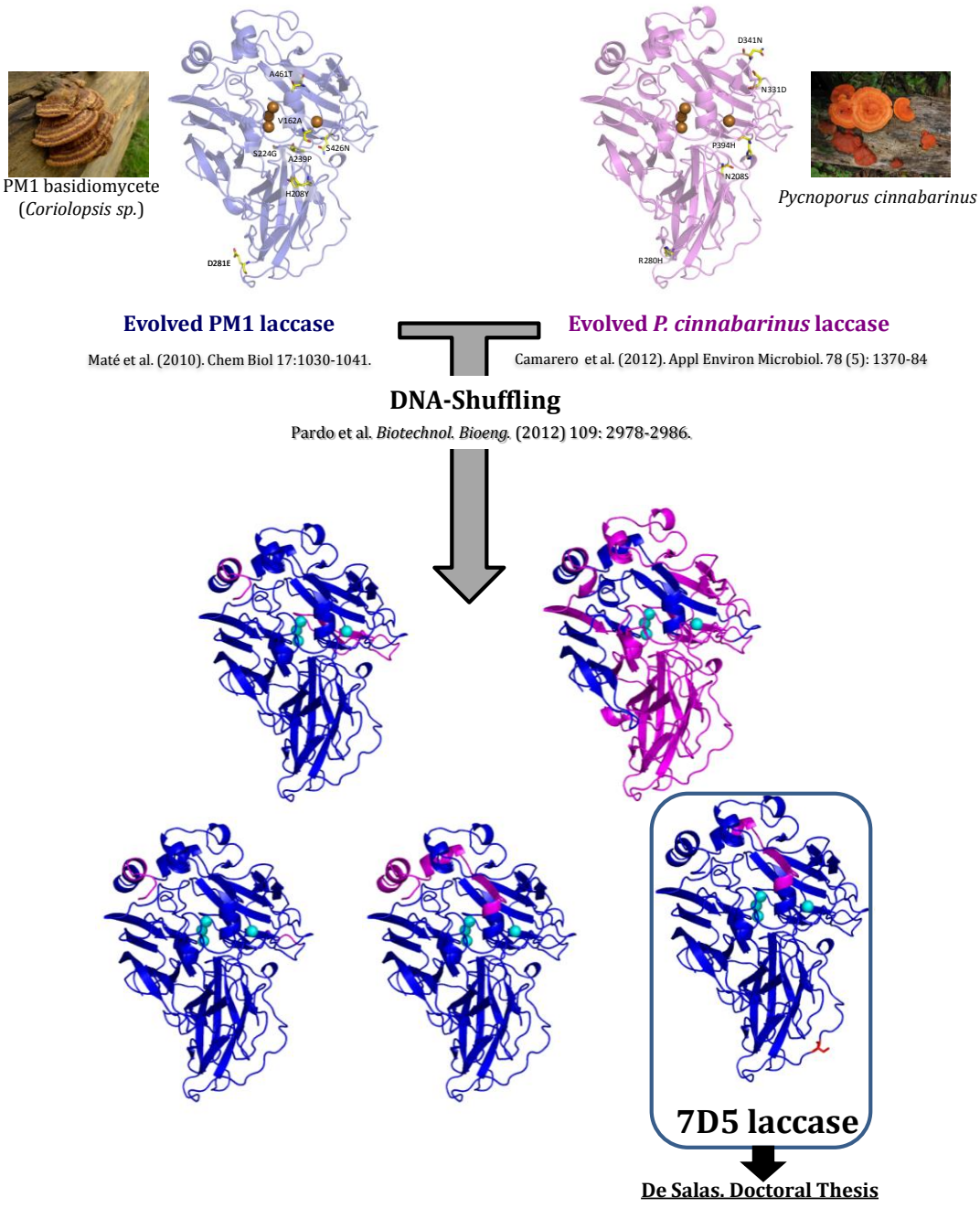

Figure 14. Evolutionary pathway to obtain 7D5 laccase. The enzyme was obtained from the DNA-shuffling of two fungal laccases from *P. cinnabarinus* and basidiomycete PM1 previously evolved in *S. cerevisiae*

The starting point of this Doctoral Thesis is a high-redox potential laccase, namely 7D5 laccase, obtained in our lab by DNA shuffling of two fungal laccases, from *P. cinnabarinus* and PM1 basidiomycete (Pardo *et al.,*.2012), that had been previously evolved for their functional expression in *S. cerevisiae* (Mate *et al*., 2010; Camarero *et al*., 2012) (Fig. 14). The fungus PM1 was isolated from the waste water of a paper factory and its laccase was isolated by Coll and coworkers (Coll *et al*., 1993, 1993). The laccase of *P. cinnabarinus* is the predominant extracellular phenol oxidase produced by this fungus and it shows interesting capabilities in terms of stability and activity towards natural and synthetic substrates (Eggert *et al*., 1996; Lomascolo *et al*., 2003). After several rounds of lab evolution of each laccase CDS fused to the prepro-leader of the alpha-mating factor of *S. cerevisiae*, both laccases were secreted by the yeast in active form (2-8 mg/l). In addition, the catalytic activity towards phenolic and non-phenolic compounds was notably increased in the evolved *P. cinnabarinus* laccase, whereas the evolved PM1 laccase recovered the outstanding catalytic properties of the wild type (Maté *et al.*, 2010; Camarero *et al.*, 2012).

Recombination of homologous genes is an easy way to introduce neutral mutations in the enzyme to produce functional variants with maybe improved robustness or promiscuous activities (Arnold, 1998). With this aim, the two aforementioned evolved fungal laccases were subjected to DNA shuffling to obtain a library of chimeric laccases functionally secreted by the yeast. The selected enzymes displayed modified pH activity profiles, different substrate affinities and enhanced thermostability respecting the parent laccases (Pardo *et al*., 2012). One of these stable chimeric laccases is 7D5 laccase, which was selected as starting point for this Doctoral thesis due to its superior ability to oxidize aromatic amines and stability to acid pH.

OBJECTIVES

Objectives

The main objective of this Doctoral Thesis was to develop a high redox potential laccase as biocatalyst of industrial interest for organic synthesis, with a special emphasis in the synthesis of electroconductive polyaniline. For this, different tasks were considered:

1. Optimization of the reaction conditions for the enzymatic synthesis of conductive polyaniline, including the selection of laccase and doping template, to obtain a polymer with best performing properties at high production yields.

2. Enhancement of laccase activity towards aromatic amines without reducing its substrate versatility and, simultaneously, improving the stability of the enzyme to the reaction conditions of polyaniline synthesis by combining enzyme directed evolution and computational design.

3. Improvement of laccase secretion by *Saccharomyces cerevisiae* and assessment of over-expression of the engineered laccase variants in *Aspergillus oryzae*.

4. Biochemical, kinetical, computational and structural characterization of the laccase variants obtained to contribute to improve the structure-function knowledge of these multicopper oxidases.

CHAPTER 1

Advanced Synthesis of Conductive Polyaniline using Laccase as Biocatalyst

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Abstract

Polyaniline is a conductive polymer with distinctive optical and electrical properties. Its enzymatic synthesis is an environmentally friendly alternative to the use of harsh oxidants and extremely acidic conditions. 7D5L, a high-redox potential laccase developed in our lab, is the biocatalyst of choice for the synthesis of green polyaniline (emeraldine salt) due to its superior ability to oxidize aniline and kinetic stability at the required polymerization conditions (pH 3 and presence of anionic surfactants) as compared with other fungal laccases. Doses as low as 7.6 nM of 7D5L catalyze the polymerization of 15 mM aniline (in 24 h, room temperature, 7% yield) in the presence of different anionic surfactants used as doping templates to provide linear and water-soluble polymers. Aniline polymerization was monitored by the increase of the polaron absorption band at 800 nm (typical for emeraldine salt). Best polymerization results were obtained with 5 mM sodium dodecylbenzenesulfonate (SDBS) as template. At fixed conditions (15 mM aniline and 5 mM SDBS), polymerization rates obtained with 7D5L were 2.5-fold the rates obtained with commercial *Trametes villos*a laccase. Moreover, polyaniline yield was notably boosted to 75% by rising 7D5L amount to 0.15 μM, obtaining 1g of green polyaniline in 1L-reaction volume. The green polymer obtained with the selected system (7D5L/SDBS) holds excellent electrochemical and electro-conductive properties displayed in water-dispersible nanofibers, which is advantageous for the nanomaterial to be readily cast into uniform films for different applications.

Introduction

Organic polymers capable of conducting electricity upon partial oxidation-reduction have attracted increasing attention in recent years to replace metals and semiconductors as conductors in storage devices, electromagnetic screens and conducting fibers. In particular, polyaniline (PANI) is attractive among the polymeric materials available due to a unique combination of easy processability and highly stable conductivity, with diverse optical and mechanical properties accessible in a wide range of nanostructures (Tran *et al*., 2011; Y. Y. Zhang *et al*., 2014). Conductivity/resistance can be rapidly changed by acid doping and base dedoping providing PANI with many of the properties desired for a chemical sensor (Huang *et al*., 2003). In addition, PANI-based composite materials are also opening new dimensions in polymer electronics. PANI-graphene supercapacitors provide high specific capacitance and stability during recharging (Wu *et al*., 2010; Wang *et al*., 2013). Thermal and conductive composites recently obtained from a combination of PANI and thermosensitive hydrogel might be useful to build electronic sensors of pressure and switch-controlled by temperature (Martínez *et al*., 2015).

Polyaniline consists of lineal chains of *p*-coupled aniline units. The combination of benzenoid (amine N) and quinoid (imine N) rings leads to three different oxidation states of PANI: leucoemeraldine, emeraldine and pernigraniline (S1 Fig.). The redox state of the polymer and the degree of protonation are responsible for different optical and electrical properties. The emeraldine salt (green-colored polymer) is the electro-conductive form of PANI (polaron) (Shumakovich *et al*., 2011). Aniline polymerization is conventionally achieved by chemical oxidation under harsh conditions using ammonium peroxydisulfate, potassium dichromate or ferric chloride as oxidant in highly acidic solutions and usually results in complex by-products. The formation of a polymer chain starts with the oxidation of aniline monomer. Emeraldine base is formed in the course of the growth of the *p*-coupled chain, and emeraldine salt is later obtained by protonation of the imine nitrogen atoms of emeraldine base with strong acids. This process is referred to as "doping" (Cruz-Silva *et al*., 2005).

The enzymatic oxidation of aniline for the synthesis of conducting PANI constitutes an environmentally friendly alternative to the chemical polymerization because it is carried out under milder conditions. Even if peroxidases and laccases have been both explored as biocatalysts for aniline polymerization (Liu *et al*., 1999; Vasil'eva *et al*., 2007), laccases offer important operational advantage over peroxidases as they do not require stepwise addition of hydrogen peroxide to catalyze the reaction. Besides, peroxidases are sensitive to inactivation by hydrogen peroxide (Baynton *et al*., 1994) whereas laccases only require oxygen from the air to oxidize the arylamine, releasing water as the only by-product.

Laccase oxidizes aniline monomers, dimers and oligomers. After that, the polymerization apparently proceeds by non-enzymatic coupling of the oxidized products (Junker *et al*., 2014). The mixture of aniline polymers might be as complex as varied are the conditions used for the synthesis. Thus, a particular challenge is to control the reaction conditions to attain the desired product, avoiding over-oxidized or side-effect products. The use of templates favors the desired (linear head-to-tail) aniline polymerization over unwanted (side-chain branching) coupling reactions. Anionic surfactants serve as doping templates and also as amphiphilic systems to solubilize PANI by forming micelles or vesicles (Streltsov *et al*., 2008; Junker *et al*., 2014). From a practical point of view, water soluble or dispersible conducting PANI is more promising by contrast to the poor solubility of the chemically-obtained polymer in common organic solvents (Liu *et al*., 1999).

In this study, we use a high-redox potential laccase developed in our laboratory (7D5L) as the biocatalyst of choice for the synthesis of green polyaniline due to its superior ability to oxidize aniline and high stability at preferred reaction conditions (Pardo *et al*., 2012). Different anionic surfactants are assayed as doping templates and the resulting polymers are fully characterized. Thus, we set up the

conditions for the enzymatic synthesis of electro-conductive emeraldine easily processable in water with reliable conversion yields.

Material and Methods

Reagents

Citrate-phosphate buffer was prepared with Na_2HPO_4 and citric acid purchased from Merck Millipore. N,N'-dimethyl-p-phenylenediamine (DMPD), N-methyl-2-pyrrolidone (NMP), aniline, sodium dodecyl sulfate (SDS), docusate sodium salt (AOT) and sodium dodecylbenzenesulfonate (SDBS) were all from Sigma Aldrich. Sodium lauryl ether sulfate (SLES) (40%) was obtained from Gran Velada. Tetrahidrofuran (THF) and N,N-dimethylformamide (DMF) were obtained from LabsScan. 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from Roche. Chemical synthesized emeraldine salt (average Mw>15,000 Da) was purchased from Sigma Aldrich. All chemicals were of reagent-grade purity.

Strains and culture media

The protease deficient *Saccharomyces cerevisiae* BJ5465 strain (LGCPromochem) transformed with the shuttle pJRoC30 vector carrying either 3A4, 7A12 or 7D5 chimeric laccases under the control of the GAL1 promoter were obtained in a previous work (Pardo *et al*., 2012). Transformed yeast cells were grown for 3 days, in flasks, in laccase expression medium supplemented with ethanol and copper to obtain the crude laccases used in this study (Camarero *et al*., 2012). PM1 laccase was produced in GAE medium (Mate *et al*., 2013), *Pycnoporus cinnabarinus* laccase (PcL) was provided by INRA-Marseille and *Myceliophtora thermophila* (Novozym 51003) and *Trametes villosa* (Novozym 51002) laccases (MtL and TvL, respectively) were provided by Novozymes (Denmark).

Enzyme characterization

Determination of laccase activity

Laccase activity was measured with 20 µl samples and 180 µl of 3 mM ABTS (ABTS cation radical ε418 = 36000 M^{-1} $cm^{-1,}$ (Alcalde and Bulter, 2003) or 5 mM DMPD (ε550= 4134 M^{-1} cm^{-1}, determined in this study) in triplicate, using a SpectramaxPlus (Molecular Devices) plate reader in kinetic mode. One activity unit (U) was defined as the amount of enzyme needed to transform 1 µmol substrate/minute at room temperature. Oxidation of 300 mM aniline was followed at 410 nm (ε410 = 1167 M^{-1} cm^{-1} determined in this study) in 50 mM citrate-phosphate buffer pH 3.0.

Laccase activity in the presence of templates was measured by adding 20 µl of enzyme diluted to 0.1 U/mL of activity (measured with ABTS) to 180 µl of 3 mM ABTS in 50 mM citrate-phosphate buffer pH 3.0 and different concentrations of SDS, SDBS, AOT or SLES (0.25- 3.2 mM).

Enzyme stability at pH 3

Laccase samples with 0.1 U/mL activity (measured with ABTS) were incubated in 50 mM citrate-phosphate buffer, pH 3.0 in the presence or absence of template (15 mM SDS), for 24 h, at room temperature. Laccase activity was measured at different time points with ABTS as aforementioned. Residual activities were calculated as a percentage of the initial activity.

Enzyme thermostability

Laccase samples with 0.1 U/mL activity (measured with ABTS) were incubated at 70 °C for 10 minutes. Then, aliquots of 20 µl were chilled on ice for 10 min and incubated at room temperature for another 5 min before adding 180 µL of 3 mM ABTS in 50 mM citrate-phosphate buffer pH 3.0. Residual activities were calculated as aforementioned.

Enzymatic polymerization of aniline

Evaluation of different anionic surfactants as doping templates

15 mM Aniline was polymerized with 0.1 U/mL of laccase (measured with ABTS) in aqueous medium buffered with 50 mM citrate-phosphate, pH 3.0, in the presence of SDS, SDBS, AOT or SLES in a concentration range of 0.6–15 mM. The reaction was carried out for 24 h at room temperature and constant stirring, in 12.5 mL reaction volume (Pyrex bottles), maintaining a liquid:air 1:1 v/v ratio. Samples were precipitated and washed with absolute ethanol to purify the polymer (Streltsov *et al*., 2008). Purified and non-purified polyaniline were lyophilized in a Telstar lyophilizer.

Polymerization assays with pure 7D5 laccase

Assays were carried out as aforementioned with 5 mM SDBS as template and 0.1 U/mL (7.6 nM enzyme concentration), 1 U/mL (76 nM) or 2 U /mL (0.15 µM) of pure 7D5 laccase produced in *Aspergillus oryzae*. Samples were taken at different time-points and measured in triplicate by following the increase of absorbance at 800 nm typical for the emeraldine salt. The enzyme was produced in *Aspergillus oryzae* (Matsui *et al*., 2016), in standard MDU-2BP media containing $CuSO_4$ and purified by two ion-exchange and one size-exclusion chromatographic steps: i) anion-exchange chromatography using a Q-sepharose column and a 75 mL gradient of 0 – 100 % elution buffer (20mM Tris pH 7 + 0.5M NaCl pH 7); ii) molecular exclusion chromatography using a HiLoad 16/600 Superdex 75 pg column (20 mM Tris-HCl + 150 mM NaCl, pH 7); iii) an anion-exchange chromatography using a Mono Q HR 5/5 column and a 30 mL gradient of 0 – 25 %

elution buffer (20 mM Tris-HCl + 1 M NaCl, pH 7). All columns are from GE Healthcare. Fractions containing laccase activity were pooled, dialyzed and concentrated between each chromatographic step.

Polymerization assays at fixed conditions

15 mM of aniline was polymerized with either 0.1 or 2 U/mL of crude enzyme (activity measured with ABTS) in the presence of 5 mM of SDBS as template, in 50 mM citrate-phosphate buffer pH 3.0. The reaction was carried out in 100 mL-flask (50 mL final volume) or 2 L flask (1 L final volume), at room temperature and constant stirring for 24 h.

Characterization of polymers

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS)

Lyophilized samples were resuspended in water, 10% ethanol, DMF or THF before the analysis. The best spectra were acquired with DMF as solvent. The measurements were taken in a MALDI-TOF-TOF Autoflex III from Bruker calibrated with Bruker peptide. The matrix used was 10 mg/mL 2,5-dihydroxybenzoic acid in DMF.

Spectroscopic analyses

UV-visible absorbance spectra of water-soluble PANI samples were acquired in a UV 1800 spectrophotometer (Shimadzu) using quartz cuvettes. Commercial emeraldine salt was diluted in 50% dimethylformamide. FTIR spectra of PANI were obtained in a Jasco FTIR-4200 spectrophotometer from KBr pellets of samples previously dried in an aeration oven.

Cyclic voltammetry

The electrochemical measurements were conducted using Dropsens screen printed electrodes (DRP-110) in a DropSens μStat400 potentiostat. For sample preparation, 0.01 g of PANI was dispersed in 5 mL of NMP. Then, 2 μL of each solution were drop-casted onto the carbon working electrode and dried under vacuum. The electrolyte employed was 1 M HCl.

Scanning electron microscopy (SEM)

Lyophilized PANI samples were metalized with an alloy of Au/Pd in an 80:20 ratio and a plasma current of 5-10 mA by a SC7640 Polaron sputter coater from Quorum Technologies (East Sussex, United Kingdom). The images were taken in a FE-SEM Hitachi model SU8000 (Tokio, Japan) with an acceleration voltage of 1.5 kV.

Conductivity

Direct current (DC)-conductivity measurements were carried out using the four-probe method on pellets obtained from dried purified PANI pressed into a disk. The measurements were carried out using a four-probe setup equipped with a DC

current source (LCS-02) and a digital micro-voltmeter (DMV-001) from Scientific Equipment and Services. Prior to conductivity measurements, the polymers were re-doped with 1 M HCl.

Dynamic light scattering (DLS)

DLS experiments were carried out in a Protein Solutions DynaPro MS/X instrument (Protein Solutions, Piscataway, NJ) at 20 °C using a 90° light scattering cuvette. Prior to the analysis, the samples were centrifuged during 20 min at 8,000 g and 20 °C. Data were collected and analyzed with Dynamics V6 Software.

Results and discussion

Selection of biocatalyst

From a set of high-redox potential laccases engineered and expressed in *S. cerevisiae* (Pardo *et al*., 2012), three thermostable laccases, namely 3A4, 7A12 and 7D5, were evaluated at the preferred conditions for the synthesis of polyaniline (acid pH and presence of anionic surfactant). Of these, 7D5 laccase (7D5L) resulted the most stable (Fig. 1a) and it was selected for further studies. Then we compared the oxidation of aniline by 7D5L and other fungal laccases such as the wild-type laccases from *P. cinnabarinus* (PcL), the basidiomycete PM1 (PM1L), or the commercial laccases from *M. thermophila* (MtL) or *T. villosa* (TvL) (Fig. 1b). The activity of 7D5L on aniline was notably superior to the rest of the enzymes tested. Further comparison of 7D5L with TvL (the second best laccase oxidizing aniline) showed the stability of both enzymes at pH 3 (room temperature) and the higher stability of 7D5L at high temperature (70 °C). Also, 7D5L has twice as high relative activity on aromatic amines (DMPD), respecting the activity with ABTS, than TvL (Table 1).

Table 1. Comparison of 7D5L and T. villosa laccase (TvL) for stability at acid pH and high temperature, and relative activity on aromatic amines (DMPD) respecting the activity with ABTS**.**

	Half-life pH 3 (h)	10 min at 70 °C (%)	DMPD/ABTS activity
7D5L	22	76	1.4
TvL	20	32	0.6

The oxidation of aniline by laccase at pH 3 is hampered because at this pH aniline is mostly protonated, in the form of anilinium cation (pKa = 4.6), and the cation (E^0 = 1.05 V) is much less oxidizable than neutral aniline (E^0 = 0.63 V) (Sapurina and Stejskal, 2008; J. Zhang *et al*., 2014). Hence, the use of high-redox potential laccases

such as 7D5L, PcL, PM1L or TvL ($E^0 \sim +0.8$ V) is required to overcome the high potential barrier for oxidizing aniline in acidic medium (Yang *et al*., 2007); whereas MtL, with a lower redox potential, is unable to catalyze the reaction. Even so, the polymerization reaction with high-redox potential laccases proceeds slowly, in several hours. The superior ability of 7D5L to oxidize aniline over other high-redox potential counterparts is likely related to its higher relative activity on aromatic amines, but its optimum pH (pH 3) for aniline oxidation (compared to pH 4.5 for TvL) and kinetic stability at the working conditions might also contribute to this enhancement. Consequently, 7D5L is the biocatalyst of choice to synthesize polyaniline in this study.

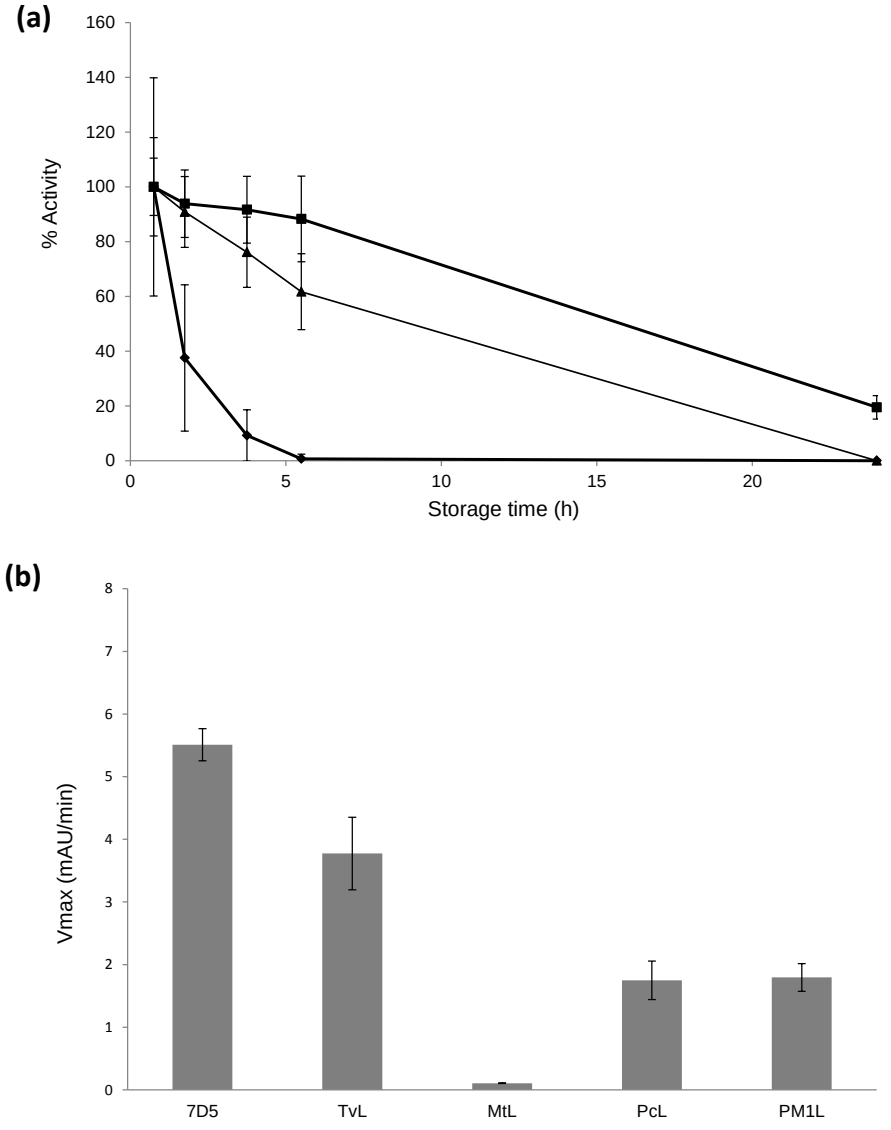

Figure 1. Stabilities of the engineered laccases expressed in *S. cerevisiae* in citrate-phosphate buffer pH 3 with 15 mM SDS, in the presence of 15 mM aniline; 7D5L (squares), 7A12L (diamonds), 3A4L (triangles) (**a**). Oxidation of 100 mM aniline (at pH 3.5) by 7D5L, the wild type laccases PM1L and PcL, and TvL and MtL commercial laccases (**b**).

Enzymatic polymerization of aniline

Our final goal is the biosynthesis of water-soluble PANI with electrochemical and electro-conductive capabilities (emeraldine salt). To achieve these properties, we used 50 mM citrate-phosphate buffer pH 3, as doping agent to maintain the aniline monomer protonated. We also assayed different anionic surfactants, SDS, SDBS, AOT and SLES, to serve as i) templates to facilitate the *p*-directed coupling of the monomers, ii) as anionic dopants to get PANI in its conductive state, and iii) to make the polymer soluble in water by aggregation in micelles (S2 Fig.). First, since the critical micelle concentrations of anionic surfactants are high and their negative effect on the activity of enzymes is large (Zhang *et al*., 2009), we evaluated the activity of 7D5L in the presence of the different anionic surfactants. The residual enzyme activity was as follows: SDS >AOT> SLES> SDBS (Fig. 2a). On the other hand, SDBS displays a lower critical micelle concentration (1.3 mM) than AOT (2.5 mM) or SDS (8.3 mM) in aqueous solution (Lin, 2004; Chauhan and Sharma, 2014), which can be beneficial for the synthesis of soluble PANI at low template concentrations. By contrast to the important loss of activity observed in the absence of a reducing substrate, it is worth mentioning the "protective" effect that aniline plays on the enzyme against the presence of anionic surfactants, which allows 7D5L to be active for hours even if high concentrations of anionic surfactants are used (Fig. 1a).

After 24 h of reaction with crude laccase (0.1 U/mL), the type and amount of template relative to a fixed amount of aniline (15 mM) determined the product's properties as regards color, polymerization degree, structure and electrochemical properties. Soluble green PANI was obtained with different templates when using template/aniline ratios from 1 to 0.2, whereas a large excess of aniline respecting the template (e.g. 0.6 mM AOT) led to dark-colored precipitates due to the collapse of micelles (Y. Y. Zhang *et al*., 2014). UV-visible spectroscopy analysis of the green PANI synthesized using template/aniline molar ratio of 1 evidenced the absorption bands typical for emeraldine salt (Fig. 2b) at 420 nm, characteristic of the semiquinoid radical cation (Zhang *et al*., 2011), and 800 nm, the distinctive signal of doped PANI due to π–Polaron electronic transitions (Junker *et al*., 2014). However, the magnitude of the latter varied with the different templates used as follows: SDBS>SLES>AOT>SDS. What is more, only the polymer obtained with SDBS showed the maximum at 800 nm (Fig. 2b), whereas the rest showed the peak around 750 nm, suggesting partial doping (Wen *et al*., 2012). Then, we decreased SDBS content (relative to 15 mM aniline) to determine the minimum amount of this template required for the synthesis of emeraldine salt. The polaron absorption at 800 nm reached its maximum with 5 mM SDBS in the reaction, whereas higher SDBS concentrations provided lower absorbance values and 1 mM of template was not sufficient to detect the polaron signal (Fig. 3a). By comparison with other templates, the absorbance at 800 nm was 4-fold higher for PANI obtained with 5 mM SDBS than for PANI obtained with 5 mM AOT (data not shown).

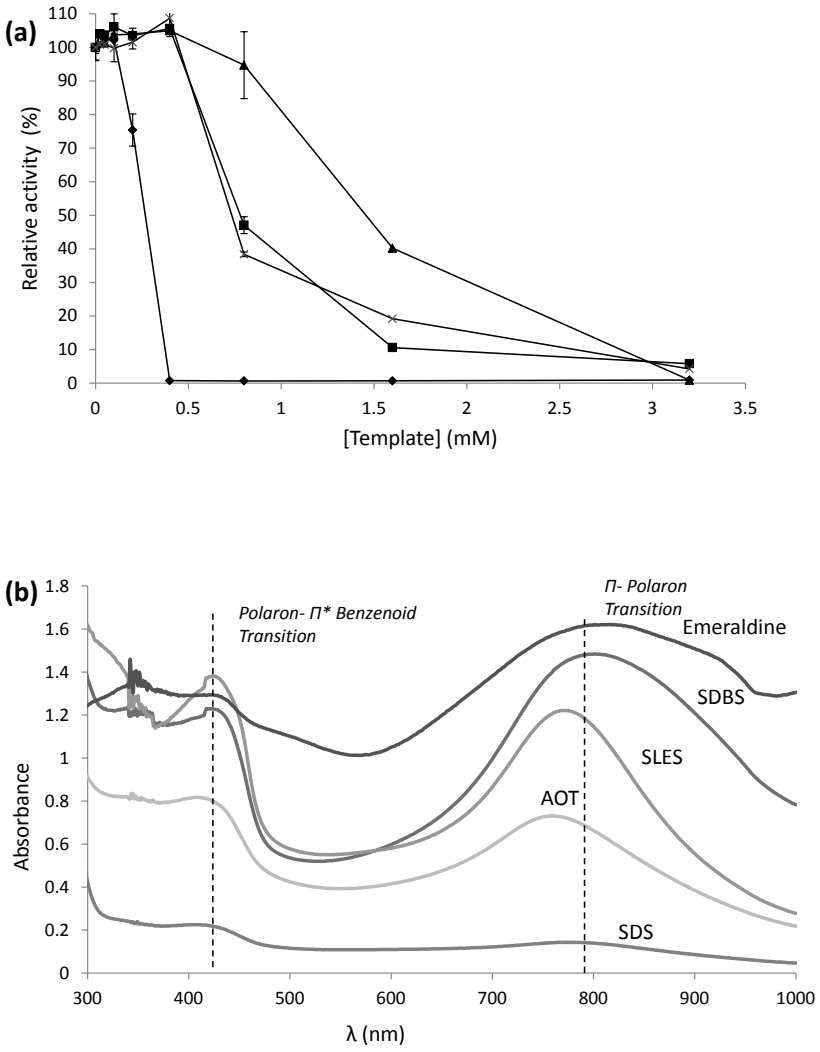

Figure 2. Activity of 7D5L in the presence of SDS (triangles), SLES (crosses), AOT (squares) or SDBS (diamonds) (**a**). UV-visible absorbance spectra of green PANI synthesized after 24 h of reaction with 7D5L (0.1 U/mL) using different anionic surfactants as templates (15 mM aniline and template were used in all cases). The spectrum of commercial emeraldine salt is provided for comparison (**b**).

To sum up, even though SDBS was the anionic surfactant producing the strongest loss of enzyme activity, it endowed the best performance as doping template for the enzymatic synthesis of green PANI. In addition to the aforementioned "protective" effect of aniline that increases the enzyme's tolerance to every anionic surfactant, this apparent contradiction is most likely related to the lower critical micelle concentration of SBDS respecting the other surfactants tested, thus offering better polymerization results with less template. Hence, 5 mM SDBS as template was fixed for the next polymerization assay with increasing amounts of 7D5 laccase and 15 mM of aniline. The maximum polymerization rate (measured by the increase of polaron signal at 800 nm) was remarkably raised (0.37 < 6.37 < 14.64 mUA/min) in direct correlation with the amount of enzyme used (0.1 < 1 < 2 U/mL), suggesting the catalytic role of the enzyme in the polymerization (Fig 3b).

In all cases, polymerization slowed down from roughly 8-12 h onwards, most probably due to the inactivation of the enzyme. It is worth mentioning that this assay was carried out with pure 7D5L produced in *Aspergillus oryzae* using enzyme concentrations between 7.6 nM (0.1 U/mL) and 0.15 μM (2 U/mL). The fact that 7D5L can be produced in industrial relevant scale by the host *A. oryzae*, maintaining its outstanding features for the synthesis of emeraldine, is of significance for the potential use of the biocatalyst at higher scale.

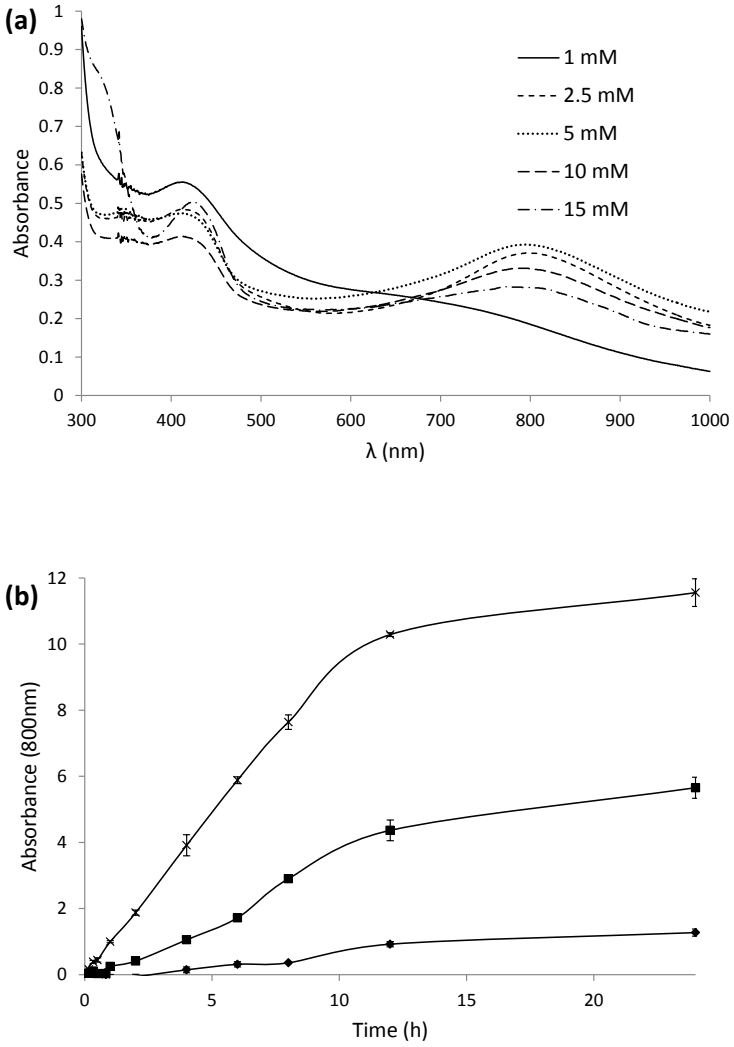

Figure 3. UV-visible spectra of aniline polymerized for 24 h with 0.1 U/mL of 7D5L and different concentrations of SDBS (10-fold diluted) (**a**). Aniline (15 mM) polymerization followed by the increase of absorbance at 800 nm, using 5 mM SDBS and 0.1 (diamonds), 1 (squares) or 2 U/mL (crosses) of pure 7D5L produced in *A. oryzae* (**b**).

Finally, we compared 7D5L and TvL for aniline polymerization at the established conditions, using 0.1 U/mL of crude enzymes. Polymerization rates were 2.5-fold higher for 7D5L (1.8 mUA/min) than for TvL (0.7 mUA/min) (Fig 4). Besides, the final yield in emeraldine salt (isolated with ethanol) obtained after 24 h of reaction

with crude 7D5L was boosted from about 7% to 75% when the enzyme dose was raised from 0.1 U/mL to 2 U/mL.

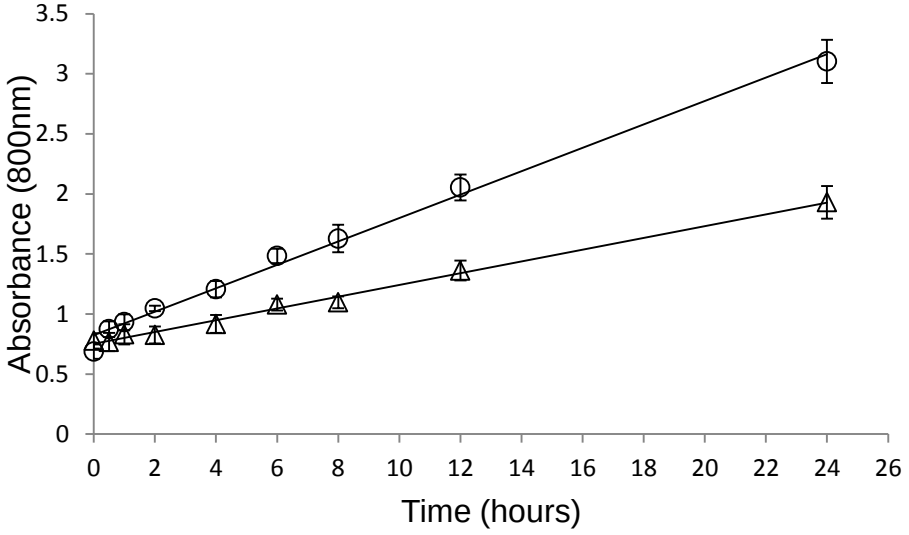

Figure 4. Polymerization rates (followed by the increase of absorbance at 800 nm) during 24 h of reaction with 0.1 U/mL of 7D5L and TvL using 15mM aniline and 5 mM SDBS.

Characterization of PANI

Oligomers/polymers

Oligomer components were detected by MALDI-TOF-MS analysis in several of the PANI samples obtained, conforming to the co-existence of oligomers and polymers during aniline polymerization (Stejskal *et al*., 2008). Indeed, high molecular mass polymeric fractions were not detected, most probably because of instrumental limitations. We found oligomerization degrees of up to 19 residues in the dark PANI obtained with 0.6 mM AOT and of 7–8 residues in the soluble green products obtained with 15 mM template (S3a-c Fig.). By contrast, no oligomers were detected in the green PANI synthesized with 2.5 mM or 5 mM of SDBS as template, suggesting greater polymerization yields under these conditions (data not shown). In line with this hypothesis, oligomers were neither observed in commercial emeraldine salt (S3d Fig.).

Emeraldine salt features revealed by FTIR

In concordance with UV-visible spectroscopy results, PANI obtained with 7D5L in the presence of SDBS clearly exhibited the characteristic FTIR bands of emeraldine salt (Fig. 5a). Bands around 1560 and 1490 cm^{-1} respectively correspond to stretching vibrations of the quinoid ring (Q) and benzenoid ring (B), whereas 1300 cm^{-1} band reveals C–N stretching vibration of secondary amines in the doped form (Karamyshev, 2003), and 1245 cm^{-1} band is characteristic of the conducting

protonated form of PANI (assigned to C–$N^{\bullet+}$ stretching vibration in the polaron lattice). The ratio of the maximum intensity of the first two bands (IQ/IB) represents an estimation of the oxidation degree of polyaniline: when it approaches one, it is assumed that PANI is in the emeraldine form. In our case, the IQ/IB ratio of PANI obtained with 5 mM SDBS as template was apparently near to 1. The typical absorbance band around 1140 cm^{-1}, indicating the protonation of the PANI backbone (B–NH^{+}=Q or B–$NH^{\bullet+}$–B vibration), was more evident after removal of template with ethanol (Fig. 5b). However, the appearance of a small band around 1380 cm^{-1} suggested partial de-doping to the base form after the template's removal (Fig. 5b). The sulfonate groups of the template contributed to 1130 cm^{-1} (with a characteristic band around 1180 cm^{-1}) and 1037 cm^{-1} signals (Fig. 5a) (Hino *et al*., 2006).

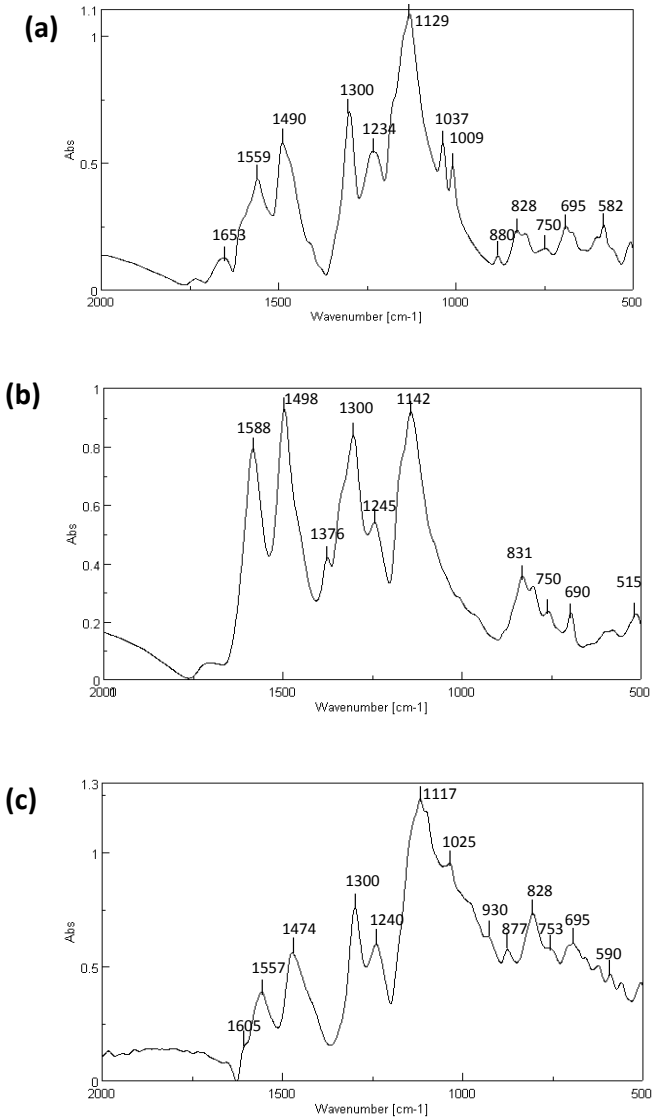

Figure 5. FTIR spectroscopy of PANI showing the representative bands for emeraldine salt. PANI obtained with 7D5L, 15 mM aniline and 5 mM SDBS (**a**). Same polymer after removal of SDBS with ethanol (**b**). Commercial emeraldine salt (**c**).

In the substitution region (900 to 650 cm^{-1}), 828 cm^{-1} band confirmed the dominating *p*-coupled chains (due to the out-of-plane deformation of C–H in 1,4-disubstituted benzene ring and Q ring deformation), whereas bands at 753 and 695 cm^{-1} respectively correspond to the out-of-plane C–H deformation and ring bending of the monosubstituted phenylene ring. On the other hand, bands at 880 and 582 cm^{-1} can be attributed to sulfonate counterions. In particular the peak at 582 cm^{-1} assigned to SO_3^- group from SDBS (Hino *et al*., 2006), disappeared after PANI isolation with ethanol (Karamyshev *et al*., 2003; Trchová and Stejskal, 2011).

The use of the other anionic surfactants rendered dissimilar FTIR spectra, with significant differences in the absorbance peaks respecting the typical signals of the emeraldine salt (Fig. 5c).

PANI morphology

Polyaniline structure greatly depends on the external templates that direct the nano-structural growth of the polymer in or around self-assembled micelles (Tran *et al*., 2011). The number of nano- and micro-scale structures for polyaniline has no paragon in other organic nano-materials (X. Zhang and Xu, 2014). Given that structure determines nanomaterial's properties, we examined the structure and size of the polymers synthesized here by SEM and DLS.

Oxidation of aniline by 7D5L in the absence of template gave no structured product due to the irregular branched polymerization of aniline (data not shown). On the contrary, enzymatic polymerization in the presence of SDBS as template led to a nanofiber-structured PANI (Fig. 6a). In theory, nanofibers are formed during the initial polymerization stages and they serve as a scaffold for the growth of new PANI particles. If homogeneous nucleation occurs, well-dispersed PANI nanofibers are obtained, whereas heterogeneous nucleation leads to particle aggregation (Huang and Kaner, 2006). Similarly, after template's removal, PANI structure was lost and the particles aggregated in micron-sized agglomerates (Fig. 6e) due to strong inter-molecular H-bonding between the chains' backbones (Wang *et al*., 2014). Other PANI nano- and micro-scale structures were obtained by varying the doping template: granular spherical particles with AOT, splintered PANI with SDS, and amorphous PANI with SLES (Fig. 6b-d), whereas commercial PANI showed a granular morphology of irregular shaped micron-sized agglomerates (Fig. 6f) as described for PANI obtained by chemical synthesis with ammonium peroxydisulfate in a strong acid environment (Wudl *et al*., 1987).

The uniform size and morphology of the nanofibers obtained with laccase and SDBS provide higher surface area that result in superior performance of the polymer to be readily cast into uniform films. Also, the outstanding water dispersibility of nanofibers facilitates their interaction with ions in solution, enabling nanofibers to be uniformly modified, thus giving rise to superior

functionalities in environmentally friendly processing and biological applications (Huang *et al*., 2003; Huang and Kaner, 2006).

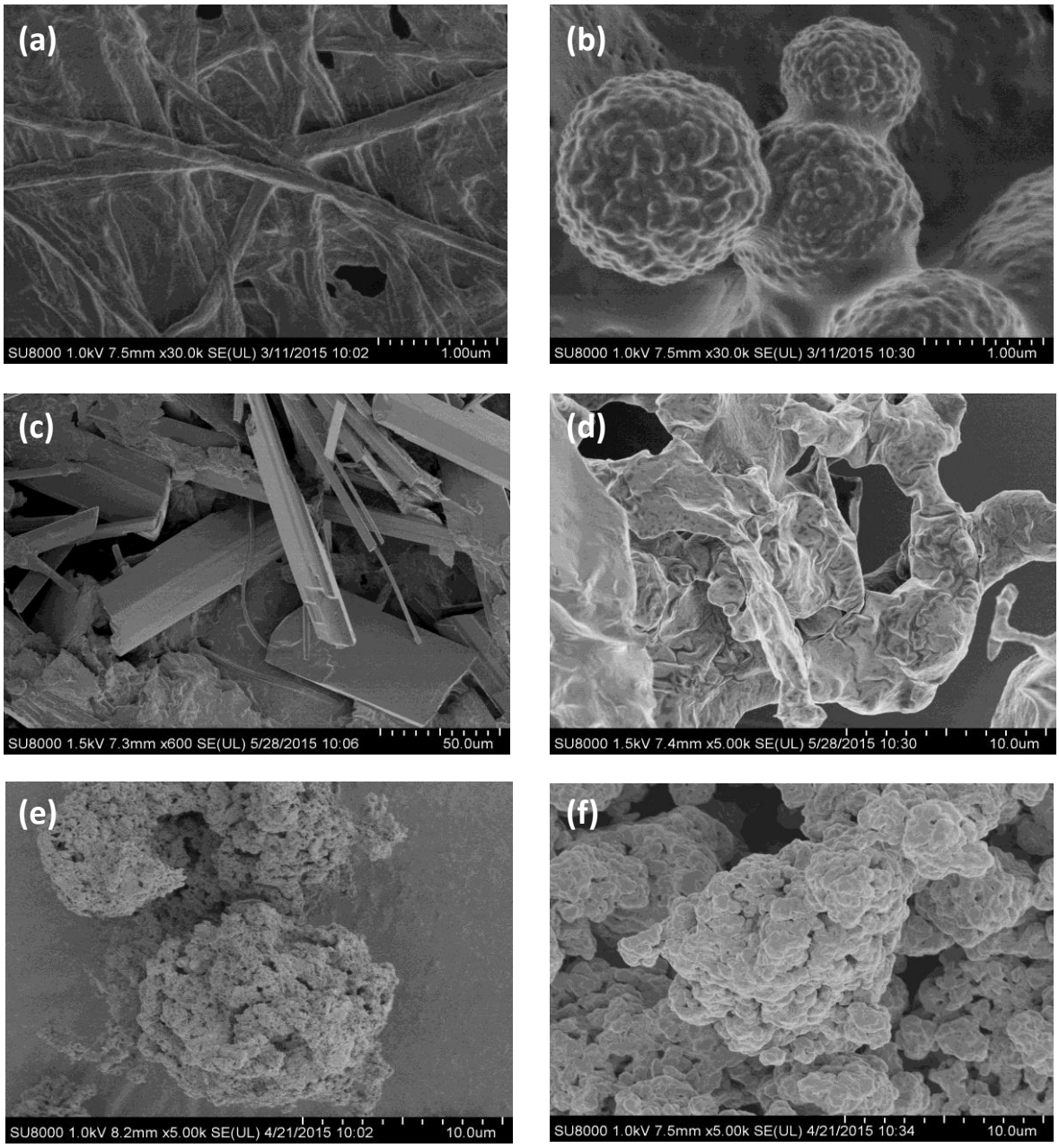

Figure 6. Polyaniline structures observed by SEM. Polymerization was carried out with 7D5L, 15 mM aniline and 5mM of different templates. Nanofibers obtained with SDBS (**a**). Granular spheres obtained with 5 mM AOT (**b**). Splinters obtained with 5 mM SDS (**c**). Amorphous agglomerates obtained with 5 mM SLES (**d**). Amorphous agglomerates obtained after washing (**a**) with ethanol (**e**). Micro-sized granular agglomerates of commercial PANI (**f**).

On the other hand, PANI size-distribution profiles detected by light-scattering are in agreement with their respective structures observed by SEM. Green PANI synthesized with 7D5L and SDBS showed an hydrodynamic radius ranging between 10 and 290 nm (S4a Fig.), whereas the radius varied between 14 and 80 nm in the green PANI synthetized in the presence of AOT (S4b Fig.). The remarkably broader size distribution profile of PANI obtained using SDBS as template correlates with its nanofibered structure, whereas the narrow size-distribution profile of PANI obtained with AOT is in concordance with the uniformity of PANI particles (around 1μM-size spheres).

Electrochemistry of PANI

The electrochemical responses of green PANI synthetized enzymatically were evaluated by cyclic voltammetry. The voltammetric behaviors of thin films of the green polymers obtained with SDBS or AOT as templates were very similar to that of commercial emeraldine salt (Fig. 7). Two redox processes were observed, which correspond to the leucoemeraldine/emeraldine transition (A/A') and emeraldine/pernigraniline (B/B') forms. However, some differences were observed with increasing number of voltammetric cycles. While the response was maintained in the PANI obtained in the presence of SDBS (Fig. 7a), a new redox pair (c/c') (Liu *et al*., 1999) appeared in the polymer obtained with AOT as template (Fig. 7(b)). This is an evidence of higher oxidation stability for the former as the c/c' peaks are attributed to the double-electron redox transition between *p*-benzoquinone and the *p*-hydroquinone through hydrolysis of PANI (Yang *et al*., 1992). We corroborated their origin as degradation products of PANI (by using potentials higher than 0.7 V (Zhang *et al*., 2010)) when we used a short range potential (from -0.2 V to +0.6 V) and these peaks were missing (data not shown). The redox peaks for PANI synthesized with 7D5L and SDBS were notably sharper and more intense than those for PANI obtained with AOT (at same reaction conditions), most likely due to the polymer's morphology. In conclusion, the use of SDBS as template resulted in better PANI redox electrochemical properties as regards the rest of anionic surfactants tested (data not shown) or even the chemically synthesized emeraldine salt (Fig. 7c).

Finally, we evaluated the conductivity of the green PANI synthesized with 7D5L and SDBS by the four-probe method. The polymer was electro-conductive, with conductivity values around 1.1×10^{-5} S/cm after removing the template with ethanol and water. Since dedoping of the sample occurs during washing, we re-doped the washed sample with 1 M HCl and the polymer's conductivity was raised two orders of magnitude to 2.4×10^{-3} S/cm. Even if studies on enzymatic polymerization of aniline scarcely illustrate the electro-conductivities of the resulting polymers (Guo *et al*., 2013; Y. Y. Zhang *et al*., 2014), this value is three orders of magnitude higher than that reported for PANI obtained after 18 days of reaction with *Trametes versicolor* laccase and AOT (after re-doping with camphor-10-sulfonic acid) (Junker *et al*., 2014). We also use milder reaction conditions

(much less enzyme, room temperature, shorter reaction times) than other polymerization reactions catalyzed by laccase (Vasil'eva *et al*., 2008; Shumakovich *et al*., 2010; Junker *et al*., 2014). On the other hand, conductivities of PANI obtained by conventional means range from 10^{-10} to 27 S/cm (Stejskal and Gilbert, 2002; Blinova *et al*., 2007), although conductivity values above 10^{-3} S/cm are only attained in strongly acidic media.

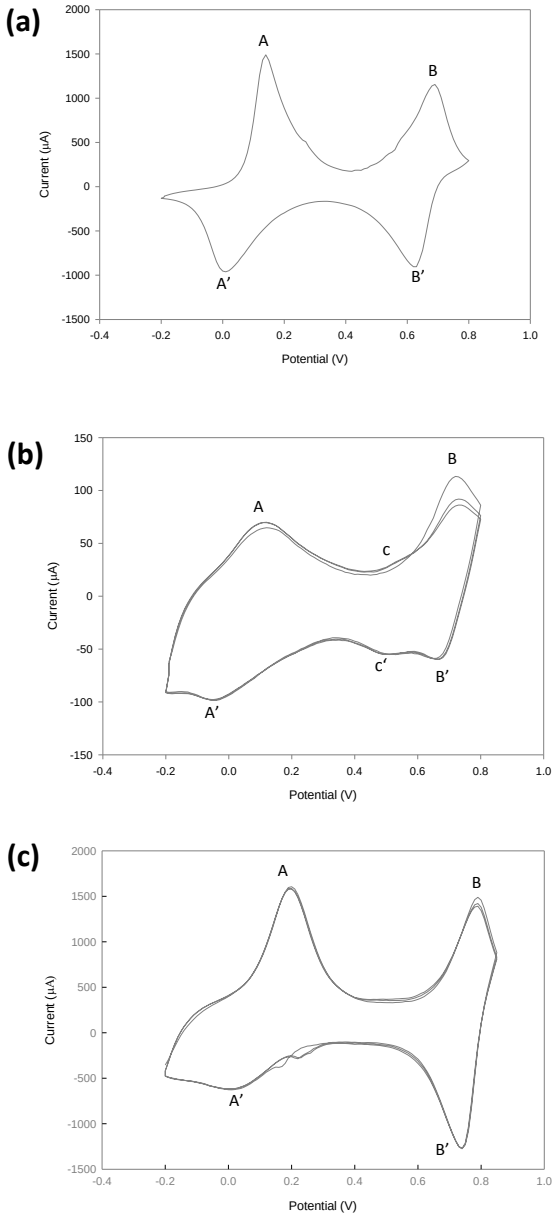

Figure 7. Cyclic voltammetry curves of PANI obtained with 7D5L and SDBS (**a**) or AOT (**b**) as templates (after isolation with ethanol) compared with commercial emeraldine salt (**c**).

Finally, the nanofibered green PANI synthesized here might be very useful as chemical sensor, since uniform nanofibered films respond fast and with high sensitivity to an acid, by becoming more conducting, and the doped form responds to a base by becoming more insulating (Wang *et al*., 2014).

Conclusions

The laccase used here (7D5L) displays outstanding activity on aniline and stability at the conditions required for the synthesis of green polyaniline (emeraldine salt), as compared with other fungal laccases, enabling the use of low amounts of biocatalyst. The systematic characterization of the polymers obtained in the presence of different anionic surfactants revealed the profound effect of the doping template in polyaniline's properties and permitted the adjustment of the reaction conditions to boost the enzymatic synthesis of emeraldine salt. By using a first-class laccase and SDBS as template, we developed an environmentally-friendly method to produce water-soluble conductive PANI with excellent electrochemical properties in up to 75% conversion yield. Its nanofibered structure provides additional advantages like a porous structure and a large surface-to-volume ratio.

Acknowledgements

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Supplementary material

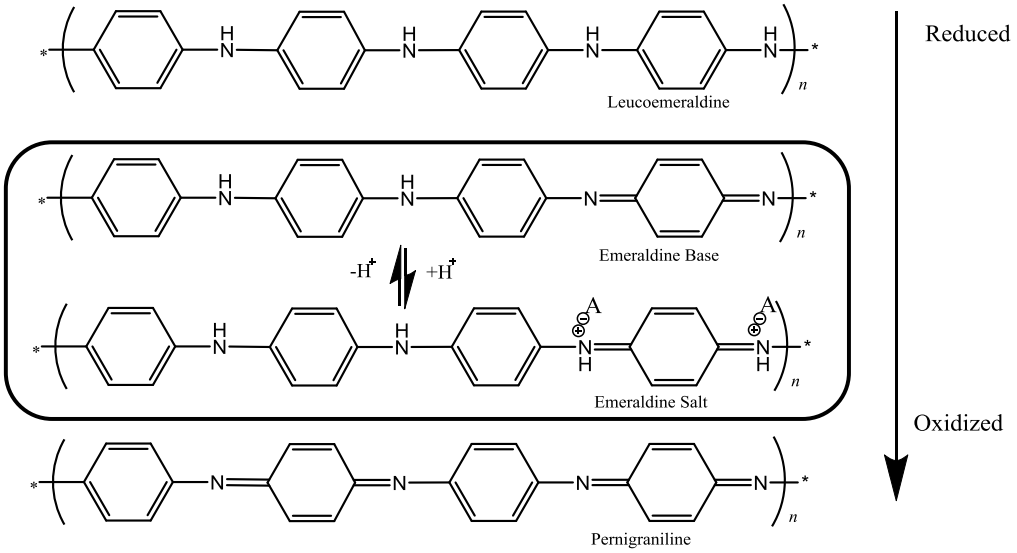

Figure S1. Structural formulas of the different oxidation and protonation states of polyaniline: Leucoemeraldine, Emeraldine base, Emeraldine salt (bipolaron form) and Pernigraniline.

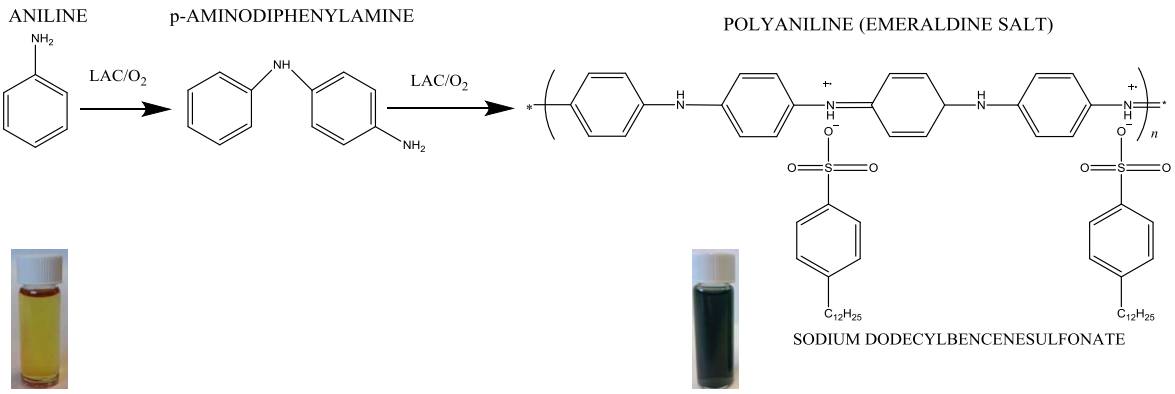

Figure S2. Scheme of the synthesis of polyaniline catalyzed by laccase in the presence of SDBS

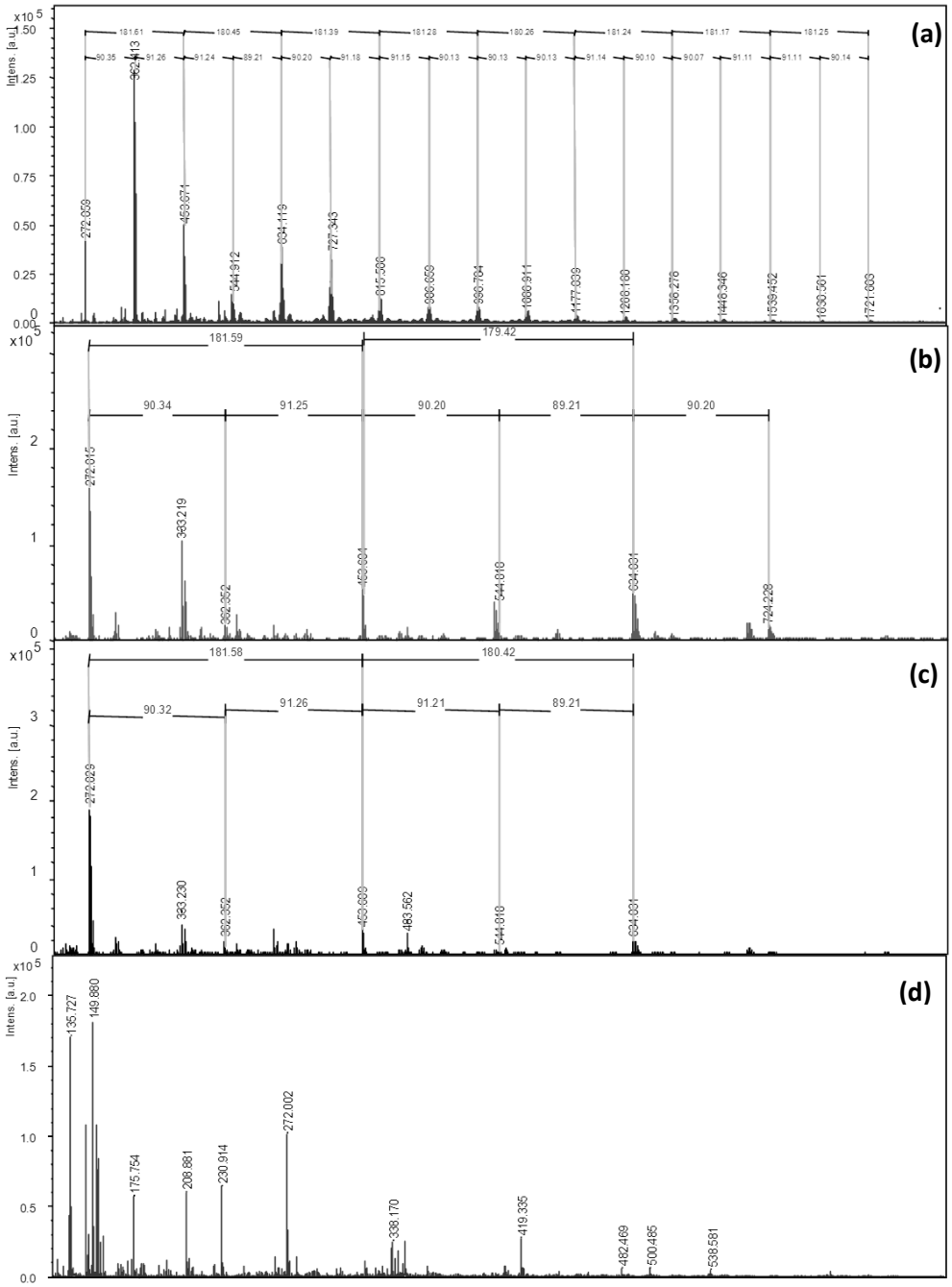

Figure S3. MALDI-TOF spectra of enzymatic PANI synthesized with 0.1 U/mL of 7D5 laccase, 15 mM aniline and 0.6 mM AOT (**a**), 15 mM SDS (**b**) or 15 mM SDBS (**c**) compared with commercial Emeraldine salt (**d**).

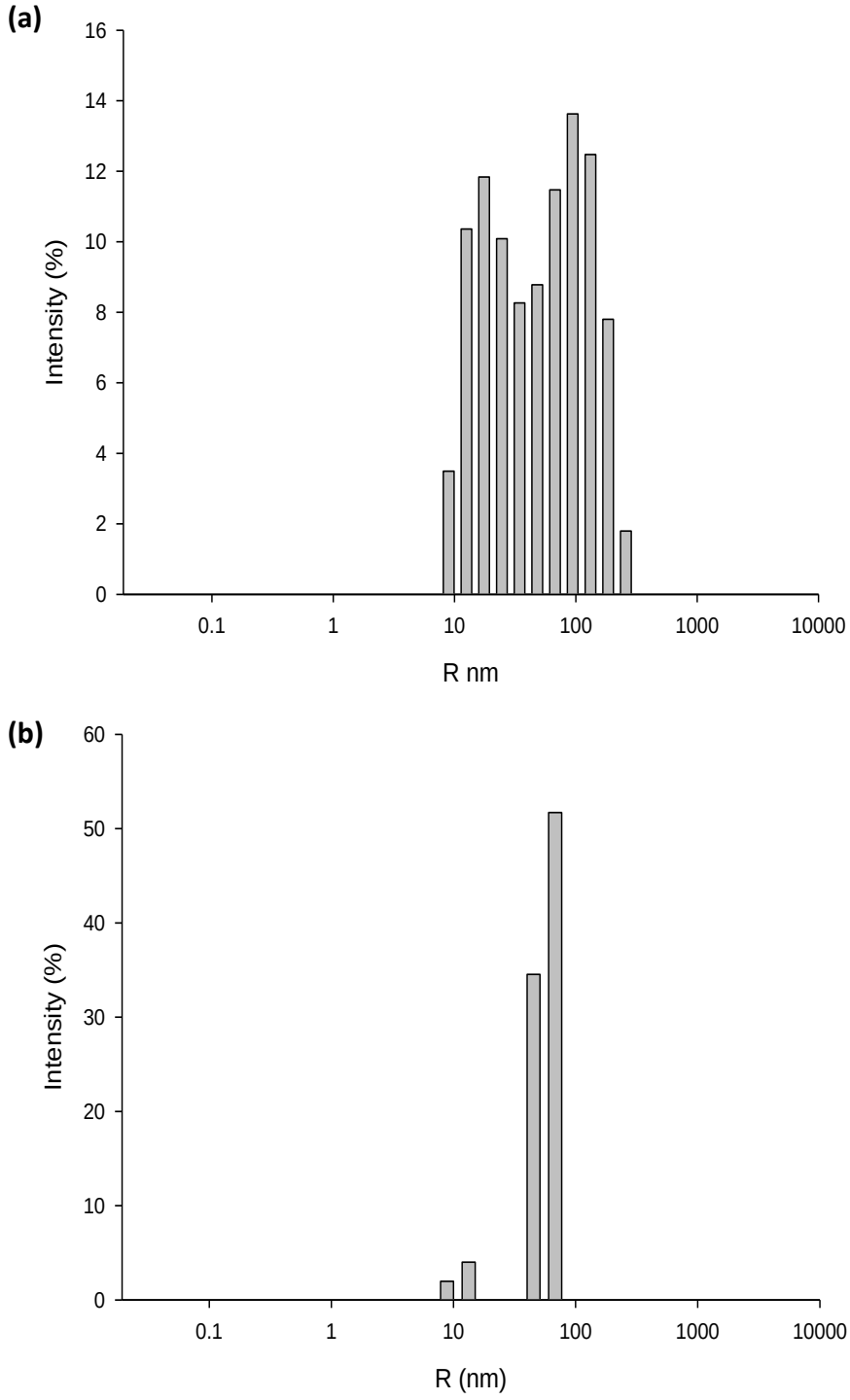

Figure S4. DLS analysis of PANI synthesized with 7D5 laccase in the presence of 5 mM SDBS (**a**) and 5 mM AOT (**b**)

CHAPTER 2

Structural and Biochemical Insights Into an Engineered High-Redox Potential Laccase Overproduced in *Aspergillus*

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Abstract

Fungal laccases have great potential as biocatalysts oxidizing a variety of aromatic compounds using oxygen as co-substrate. Here, the crystal structure of 7D5 laccase (PDB 6H5Y), developed in *Saccharomyces cerevisiae* and overproduced in *Aspergillus oryzae*, is compared with that of the wild type produced by basidiomycete PM1 (*Coriolopsis sp.*), PDB 5ANH. SAXS showed both enzymes form monomers in solution, 7D5 laccase with a more oblate geometric structure due to heavier and more heterogeneous glycosylation. The enzyme presents superior catalytic constants towards all tested substrates, with no significant change in optimal pH or redox potential. It shows noticeable high catalytic efficiency with ABTS and dimethyl-4-phenylenediamine, 7 and 32 times better than the wild type, respectively. Computational simulations demonstrated a more favorable binding and electron transfer from the substrate to the T1 copper due to the introduced mutations. PM1 laccase is exceptionally stable to thermal inactivation ($t_{1/2}$ 70 °C =1.2 h). Yet, both enzymes display outstanding structural robustness at high temperature. They keep folded during 2 h at 100 °C though, thereafter, 7D5 laccase unfolds faster. Rigidification of certain loops due to the mutations added on the protein surface would diminish the capability to absorb temperature fluctuations leading to earlier protein unfolding.

Introduction

Laccases (EC 1.10.3.2) are blue-multicopper oxidases capable of oxidizing multiple aromatic compounds such as substituted phenols, aromatic amines, N-heterocycles (indole, benzothiazol, tetrahydroquinoline, hydroxyphthalimide, etc), heterocyclic thiols and others, as well as some inorganic/organic metals (Fabbrini *et al*., 2002; Camarero *et al*., 2005; Abdel-Mohsen *et al*., 2013; Saadati *et al*., 2018). The paramagnetic blue copper at the T1 site is the responsible of subtracting one electron from the substrate and transferring it to the trinuclear T2/T3 copper cluster, were molecular oxygen is reduced to water (Solomon *et al*., 1996; Giardina *et al*., 2010). Laccases are widespread in nature and can be classified, according to their T1 site as low (< +500 mV, most plant and prokaryotic laccases), medium (+500 to around +700 mV) and high (from +720 to +800 mV) redox potential laccases (Pardo and Camarero, 2015). The high-redox potential laccases (HRPLs) are only produced by certain fungi such as the white-rot basidiomycetes responsible for lignin decay in nature. In addition to the low requirements of laccases (use of oxygen from the air and release of water as the sole by-product) as compared with peroxidases, HRPLs are of particular interest as biocatalyst for certain oxidation reactions (to replace harsh chemicals and metal catalysts) due to their higher catalytic promiscuity. For instance, they directly oxidize high-redox

potential compounds such as aniline, synthetic organic azo-dyes, technical lignins or synthetic redox mediators like 1-hydroxybenzotriazole (HBT) (Claus *et al*., 2002; Camarero *et al*., 2005; Hirai *et al*., 2006; De Salas *et al*., 2016; Hämäläinen *et al*., 2018). The latter has been widely assayed to promote the enzymatic oxidation of recalcitrant compounds (Camarero *et al*., 2004; Cambria *et al*., 2008). With or without mediators, HRPLs can catalyze a huge range of polymerization and degradation oxidative reactions valuable for different industrial sectors such as pulp and paper, textile and food industry, waste decontamination and detoxification and organic synthesis (Cañas and Camarero, 2010).

However, their difficult heterologous expression together with low activity or stability at the required operating conditions is one of the main obstacles for the industrial application of HRPLs, which must be addressed by protein engineering. In recent years, the progress in computational power and the increasing availability of protein crystal structures offered stimulating structure-function knowledge to enzyme engineers. This, combined with the use of directed evolution tools, have notably aid in the design of tailor-made industrial biocatalysts (Arnold, 2015). Enzyme directed evolution allows to discover new hotspots for protein engineering otherwise not revealed by rational strategies, while computational design permits to analyze the mutational space of these hotspots much faster than they can be explored in the lab, thus contributing to reduce the screening effort (Monza *et al*., 2017; Moore *et al*., 2018). In previous works, simulation studies helped us to understand the beneficial effect of the mutations accumulated through directed evolution on laccase activity (Monza *et al*., 2015; Pardo *et al*., 2016). Moreover, they predicted beneficial mutations to enhance laccase activity towards a particular substrate (Santiago *et al*., 2016), or envisaged the oxidation of a target substrate by different laccases, according to differences in their substrate-binding pockets (Lucas *et al*., 2017). These studies mainly consisted of protein-substrate exploration using the Protein Energy Landscape Exploration (PELE) software, in combination with quantum mechanics/molecular mechanics (QM/MM) simulations. In redox systems, such a combination can map the donor-acceptor distance (DAD), the solvent-accessible surface area (SASA) and spin densities of the substrate. In addition, several bioinformatics tools, such as HotSpot Wizard 3.0, RING and CABS-flex 2.0, allow stability predictions associated to position mutability according to evolution conservation, intra-protein contacts and potential protein motions (Kuriata *et al*., 2018; Sumbalova *et al*., 2018).

In previous works, two wild HRPLs from basidiomycetes PM1, *Coriolopsis sp*., and *Pycnoporus cinnabarinus*, sharing a 76% of sequence identity, were subjected to parallel directed evolution campaigns for expression in *Saccharomyces cerevisiae* (Mate *et al*., 2010; Camarero *et al*., 2012). Both laccases were actively secreted by the yeast upon lab evolution of their CDS fused to the prepro-leader of the alpha-mating factor of *S. cerevisiae.* In addition, the catalytic activity towards phenolic and non-phenolic compounds was notably increased in the evolved *P. cinnabarinus*

laccase, whereas the evolved PM1 laccase recovered the catalytic properties of the wild type. In a further directed evolution step, both evolved enzymes were subjected to DNA shuffling to obtain chimeric laccases functionally secreted by the yeast with combined properties (in terms of optimal pH, substrate affinity or stability) (Pardo *et al*., 2012). One of them is 7D5 laccase, which was later used as biocatalyst for the synthesis of conductive polyaniline due to its outstanding capabilities to oxidize aromatic amines (De Salas *et al*., 2016).

In this work, we fully characterize 7D5 laccase once over expressed in *Aspergillus oryzae* (Novozymes) (De Salas *et al*., 2016). Given its higher sequence identity with parent PM1 laccase (as compared to *P. cinnabarinus* laccase), the former was used as the reference wild type enzyme (pdb 5ANH) to evaluate the structural and biochemical modifications observed in the engineered enzyme. Changes in k_{cat} values or enzyme stability were rationalized by computational analyses to assess the contribution of the mutations accumulated in the engineered laccase to the activity and stability of the enzyme.

Materials and methods

Reagents

2,6-dimethoxyphenol (DMP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), N,N-dimethyl-p-phenylenediamine (DMPD), aniline, 1-hydroxybenzotriazole (HBT), 4-hydroxybenzoic acid (4-HBA), 4-aminoantipyrine (4-AAP) and 2-metoxyphenol (guaiacol) were purchased from Sigma-Aldrich (Madrid, Spain). Endoglycosydase-H (Endo-H) was purchased from Roche (Basel, Switzerland).

Enzyme production and purification

7D5 laccase was produced in *A. oryzae* (Matsui *et al*., 2016) at Novozymes, in standard MDU-2BP media containing $CuSO_4$ as described in De Salas *et al*. 2016. The enzyme was purified in one step using an anion exchange Mono Q HR 5/5 column attached to a FPLC (AKTA purifier, GE Healthcare) in a 30 mL gradient of 0-25% elution buffer (20 mM Tris-HCl + 1 M NaCl, pH 7). PM1 laccase was produced by PM1 fungus grown in 1 L flasks with 300 mL GAE medium (Coll *et al*., 1993) at 30 °C and 180 rpm. After 11 days of incubation, liquid extracts were filtrated (first with filter paper and then using a 0.45-µm cutoff membrane) and concentrated and ultra-diafiltrated using a Pellicon tangential filtration membranes (Merck Millipore, Germany) and Amicon stirred cells (Merck Millipore, Germany), both with a 10 kDa cutoff. PM1 laccase was purified by FPLC in two anion exchange steps: i) HiPrep Q FF 16/10 column in a 100 mL gradient of 0–40% elution buffer; and ii) Mono Q HR 5/5 column in a 30 mL gradient of 0–25%

elution buffer. All columns were purchased from GE Healthcare. Fractions containing laccase activity were pooled, dialyzed in Tris-HCl pH 7 and concentrated after each chromatographic step. Enzyme purification was confirmed by the A280/A600 nm ratio of the purified enzymes and their electrophoretic mobility in SDS-PAGE (12% acrylamide) stained with Coomassie brilliant blue.

Determination of molecular weight and glycosylation degree

Laccases were deglycosylated with Endo-H (0.5 U/ 5mg purified laccase, added in two steps) in sodium acetate buffer 50 mM pH 5.5, at 37 °C for 24 h. Enzyme deglycosylation was confirmed by SDS-PAGE electrophoresis (12% acrylamide).

MALDI-TOF-TOF analyses of glycosylated and deglycosylated samples were performed in an Autoflex III instrument (Bruker Daltonics, Bremen, Germany) with a smartbeam laser. The spectra were acquired using a laser power just above the ionization threshold. Samples were analysed in the positive ion detection and delayed extraction linear mode. Typically, 1000 laser shots were summed into a single mass spectrum. External calibration was performed, using the Protein Standard II from Bruker, covering the range from 15000 to 70000 Da. The 2,5-Dihydroxy-acetophenone (2,5-DHAP) matrix solution was prepared by dissolving 7.6 mg (50 µmol) in 375 µl ethanol followed by the addition of 125 µl of 80 mM diammonium hydrogen citrate aqueous solution. For sample preparation, 2.0 µl the sample were diluted with 2.0 µl of 2% trifluoroacetic acid aqueous solution and 2.0 µl of matrix solution. A volume of 1.0 µl of this mixture was spotted on the 800 µm AnchorChip target (Bruker-Daltonics) and allowed to dry at room temperature. The molecular weight analysis by MS-MALDI TOF was carried out in Proteomics and Genomics Facility (CIB-CSIC), a member of ProteoRed-ISCIII network.

X-ray crystallography

X-ray crystallography analysis of 7D5 laccase was carried out after deglycosylation of the enzyme and removal of Endo-H by using a Mono-Q column in a 30 mL gradient of 0−25% elution buffer (20 mM sodium acetate 150 mM NaCl pH 5.7). The enzyme was dialyzed and concentrated in 20 mM Tris-HCl pH 7.

Crystallization trials were set up at 293 K, starting with a condition already published (Sáez-Jiménez *et al*., 2015). The best diffracting crystals were obtained by the hanging-drop vapour-diffusion method, in 24-well plates (Hampton Research), after seeding fresh drops consisted on 2 ul of protein at 5 mg/ml and 1 ul of reservoir solution (100 mM NaAc, 200 mM Li_2SO_4, 20% (v/v) polyethylenglycol 4000 and 100 mM HEPES pH 7.5). The crystals grew in ~5 days and reached final dimensions of 0.07× 0.07 × 0.07 mm^3. Prior data collection, they were immersed in the precipitant solution containing 20% (v/v) glycerol, followed by rapid flash cooling in liquid nitrogen. A complete X-ray diffraction data set was collected at the beam line I02 at Diamond Light Source (Harwell Campus, UK). Data

were indexed and integrated using XDS (Kabsch, 2010) and scaled with SCALA from the CCP4 suite (Evans, 2006). The crystals belonged to the space group I23 with cell dimensions a=b=c= 202.61 Å and α=β=γ= 90°. Matthews coefficient and self-rotation function indicated the presence of two molecules in the asymmetric unit, with a solvent content of 62.23%. Molecular replacement was performed with Phaser (McCoy *et al*., 2007) using the Protein Data Bank entry 1GYC as model, and the refinement was carried out with PHENIX (Adams *et al*., 2010) including rigid body refinement as the first step. Several rounds of iterative refinement and manual building steps were done with Coot (Emsley and Cowtan, 2004). Coordinates and structure factors have been deposited at the PDB with accession code 6H5Y. Model quality was checked using MolProbity implemented within the PHENIX suite. Figures were prepared with Pymol (Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.). Details of data collection and processing, refinement statistics and quality indicators of the final model are summarized in Table S2.

Small-angle X-ray scattering

SAXS measurements of laccase in solution were performed at Diamond Light Source beam line B21 (Harwell Campus, UK), using a BioSAXS robot for sample loading, from solutions of the glycosylated and deglycosylated forms of 7D5 laccase at different concentrations in 20 mM Tris-HCl pH 7 at 277 K. Samples of 40 µl corresponding to PM1 laccase glycosylated at 17 mg/ml and to PM1 deglycosylated at 15 mg/ml, were delivered via an in-line Agilent 1200 HPLC system in a Shodex Kw-403 column using the same running buffer. During the experiment the samples were exposed for 300 s in 10 s acquisition blocks using a sample to detector distance of 3.9 m and X-ray wavelength of 1 Å. The data were analyzed, buffer-subtracted, scaled, and merged using the Scåtter software package (www.bioisis.net). This software was also used to check possible radiation damage of the samples by visual inspection of the Guinier region as a function of exposure time during data collection. R_G and D_{max} values were calculated with PRIMUS and GNOM, and shape estimation was carried out with DAMMIF/DAMMIN, all these programs included in the ATSAS package (Petoukhov *et al*., 2012). The radius of gyration (R_g) can be obtained from the P(r) function by integrating the function with r^2 over all values of r. The P(r) distribution function is used to describe the paired-set of distances between all of the electrons within the macromolecular structure and is a useful tool for visibly detecting conformational changes within a macromolecule. Real-space scattering profiles of atomic models were calculated using FoxS (Schneidman-Duhovny *et al*., 2016) and the final *ab initio* models were superimposed with the high-resolution structure using the program SUPCOMB from the ATSAS package. The proteins molecular mass was estimated with SAXSMoW (Fischer *et al*., 2010). Figures were prepared with Pymol (Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.). Details of data collection and processing are summarized in Table S1.

Determination of redox potential

Laccase redox potential was determined by the poised potential method using the redox couple $Fe(dipyridyl)_2Cl_2/Fe(dipyridyl)_2Cl_3$ in 8 mM MES buffer (pH 5.3). Oxidation of $Fe(dipyridyl)_2Cl_2$ at each titration point was followed by the decrease in absorbance at 522 nm (ε_{522} = 5974 M^{-1} cm^{-1}) until equilibrium was reached. The concentration of reduced laccase at equilibrium was considered to be 1/4 of the oxidized $Fe(dipyridyl)_2Cl_2$ concentration.

Optimal pH and stability assays

Laccase pH profiles were determined as shown in Pardo *et a*l 2012 using purified enzymes. pH stability assays were carried out in 2 ml 0.1 mM Britton and Robinson buffer adjusted to pH 2-9, using 0.1 U/ml purified laccase (final activity with 3 mM ABTS pH 3). Then, samples were incubated at 25 °C for 24 h and 20-µl aliquots of each sample were taken at 0, 3, 6 and 24 h and transferred to a 96-well plate to measure the residual activity with 3 mM ABTS pH 3. Relative activities were calculated as a percentage of the initial laccase activity at each experimental pH. All reactions were measured in triplicate. Determination of T_{50}, defined as the temperature at which the enzyme retains 50% of its activity after 10 min of incubation, was carried out as shown in Pardo *et a*l 2012. Laccase half-life values at 50, 60, 70 and 80 °C, thermal inactivation constants and activation energies (E_a) were obtained as shown in Pardo *et a*l 2018. Circular dichroism (CD) analyses by far-UV CD spectroscopy were performed in a spectropolarymeter Jasco J815 associate to Jasco PTC-4235/15 peltier (JASCO Corporation, Japan). The enzyme was diluted to a concentration of 10 µM in buffer Tris-HCl 20 mM pH 7. Denaturalization ramps were set from 50 °C to 95 °C with a slope of 60 °C/h and measured at 220 nm. Besides, 10 µM of the two enzymes were incubated at 100 °C for 24 h, and CD spectra of samples taken at different incubation times were recorded and compared with the CD spectra of the enzymes at room temperature. The CD spectra were collected between 190 and 250 nm with a scanning speed of 10 nm min^{-1}, using a spectral bandwidth of 1 nm and 0.1 cm path length quartz cell (Hellma, Germany). The protein signal was obtained by subtracting buffer spectrum and represented the average of 5 accumulations.

Kinetic assays

The oxidation of different substrates by purified laccase was carried out in triplicate, in 96-well plates using 0.0013 µM enzyme for DMPD and DMP, 0.0001 µM enzyme for ABTS, 0.013 µM enzyme for guaiacol, 0.13 µM enzyme for HBT and 0.145 µM enzyme for 4-HBA, in 50 mM citrate phosphate buffer pH 3 (for the assay with ABTS), 100 mM sodium acetate buffer pH 5 (for DMP, HBT and guaiacol) or 100 mM sodium acetate buffer pH 4 (for DMPD). Reactions were measured by the increment of absorbance at 418 nm for ABTS (ε_{418}= 36000 M^{-1} cm^{-1}), 550 nm for

DMPD (ε_{550}= 4134 M^{-1} cm^{-1}), 470 nm for DMP (ε_{470}= 27500 M^{-1} cm^{-1}), 409 nm for HBT (ε_{409} = 321 M^{-1} cm^{-1}) and 470 nm for guaiacol (ε_{470}= 26600 M^{-1} cm^{-1}) in a plate reader Spectramax Plus (Molecular Devices, CA, USA), in kinetic mode. Kinetic constants for the oxidation of 4-HBA were determined using 4-AAP. For that, increasing equimolar concentrations of 4-HBA and 4-AAP were added to the reaction and the increment of absorbance from the coupling of 4-HBA to 4-AAP (ε_{500}= 12200 M^{-1} cm^{-1}) was monitored at 500 nm (Ettinger *et al*., 1951). Initial oxidation rates were plotted against substrate concentration and fitted to a single rectangular hyperbola function using SigmaPlot 10.0 software. Parameter *a* was the k_{cat} and the parameter *b* was equal to the K_m.

Computational analysis

Protein-ligand interactions were analyzed using HBT and DMPD as substrates with the PELE software. Ligands were optimized in implicit solvent with the density functional M06 and 6-31G* basis set level of theory using Jaguar from Schrodinger; ESP charges were then extracted for ligand parameterization. The enzymes were prepared with the protein wizard from Schrodinger, at pH 4. PELE is a Monte-Carlo (MC) based software that was developed for mapping protein-ligand interactions, both at the global and local level. Each MC step includes a perturbation and a relaxation phase, before a Metropolis acceptance test accepts or rejects the proposed new pose. Binding energies are then scored using an OPLS-AA protein-ligand interaction energy, which includes a generalized surface born implicit solvent. All simulations involved 100 processors for 48 h, were we applied distance harmonic restraints to all copper's coordination bonds. QM/MM calculations were also performed on 5 randomly-selected structures to estimate the amount of substrate oxidation. This technique partition the system into a classical region (MM) and a quantum one (QM), allowing to describe electronic effects in the frame of the enzyme. The QM region, including the T1 copper, its coordination residues and the substrate, was computed at the DFT (Density Functional Theory) level of theory, with the M06-L functional and the LACVP* basis set. The MM region was treated using the OPLS-2005 force field; residues beyond 10 angstroms from the catalytic center (T1 copper) were kept frozen. For each selected structure, 5 optimization steps were run, in order to relax the system before the atomic spin densities were extracted. The sum of all atomic spin densities in the substrate depicts the total amount of the unpaired electron (radical) as a result of its oxidation.

Structure stability modeling has also been analyzed by 40-nanosecond MD simulations using CABS-flex, an efficient coarse grained modelling tool for fast simulations of protein structure flexibility (Kmiecik *et al*., 2016).

Results and discussion

Physico-chemical and structural characterization

PM1 and 7D5 laccases were purified to homogeneity as confirmed by SDS-PAGE and A280/A600 ratios of 17-19 (Fig. S1A). According to MALDI/TOF-TOF analyses, MW of PM1L is 57491 whereas it is 57770-65000 for 7D5, suggesting a heterogeneous glycosylation of laccase by *A. oryzae.* Glycosylation degrees around 5% for PM1L and 5-17% for 7D5 were found after deglycosylation with Endo-H (Fig. S1B, C).

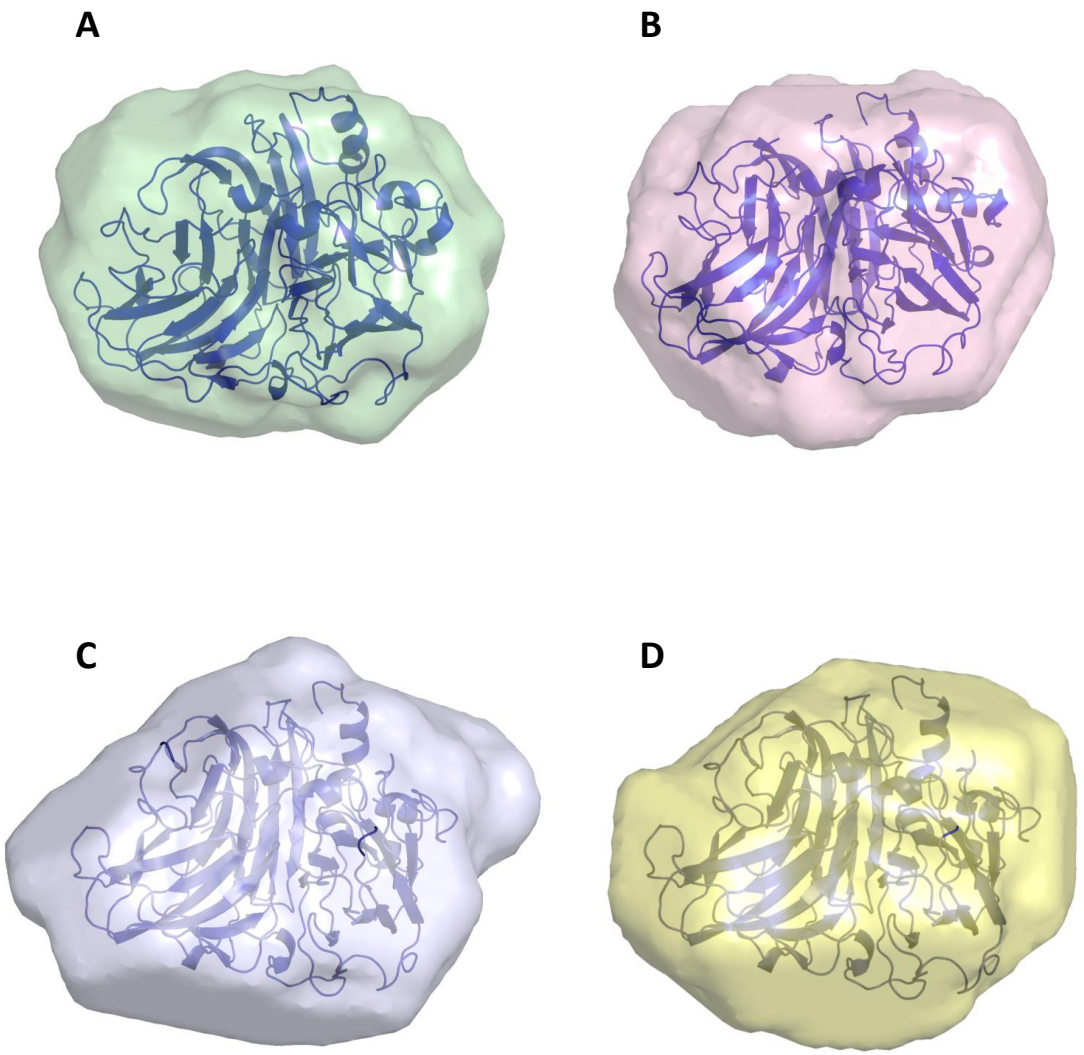

Figure 1. SAXS structures of glycosylated (**A, C**) and Endo-H-deglycosylated (**B, D**) forms of wild type (PM1) laccase (top) and engineered (7D5) laccase expressed in *A. oryzae* (bottom).

We explored the glycosylation effect on the structural stability of the enzymes in solution by small-angle X-ray scattering (SAXS), to check how the elimination of the glycans could affect the three-dimensional structure of the protein and interparticle interactions due to the biophysical changes correlated with the bulk of the glycan (Fig. 1). Both enzymes perform as monomeric proteins in solution like the majority of basidiomycete laccases (Rivera-Hoyos *et al*., 2013), with some exceptions, exhibiting homodimeric structures (Yaver *et al*., 1996; Wang and Ng, 2006). Enzyme deglycosylation decreases the maximum dimension (Dmax) of both monomeric proteins. Nevertheless, N-glycosylation seems to have a special impact on 7D5 laccase where the glycosylated form adopts a geometrical oblate structure that is converted to a somehow spherical form after deglycosylation. By contrast, PM1L shows a more spherical structure before and after deglycosylation. Clearly, the size of 7D5_deglyco is higher than that found for PML1_deglyco (Fig. 1, Table S1), most probably due to the heavier O-glycosylation of 7D5, in line with what we observed by MALDI/TOF-TOF (Fig. S1C).

To evaluate the influence of the glycan on the intermolecular interactions of 7D5 laccase, SAXS-measurements were done at different concentrations. No indication of strong aggregation was detected during data collection, with data showing linear relationships within the Guinier region (Table S1). The presence of glycans in 7D5 (and PM1 laccase) is easily observed in Fig. S2A-D, which illustrates their corresponding pair distance distribution (P(r)) and clearly shows the presence of additional interatomic distances. This is also reflected in the radius of gyration (Rg) of glycosylated and deglycosylated forms (Table S1). This effect is probably due to a reduction of backbone dynamics due to the presence of the bulky glycans. When comparing the relative shift of heights of the parabola shapes curves in the Kratky plots, it is observed that the deglycosylation forms maintain compact structures similar to the glycosylation forms in both proteins (Fig. S2E-H).

7D5 laccase, derived from the DNA shuffling of evolved PM1L and PcL (Pardo *et al*., 2012), differs in nine mutations of the wild PM1L (Fig. 2). Mutations V162A, H208Y, S224G, A239P, D281E and S426N accumulated in the protein sequence during the evolution pathway of PM1L for expression in yeast (Mate *et al*., 2010), while T291S, E457D and I468T come from the shuffling with evolved PcL parent (Camarero *et al*., 2012).

The crystal structure of 7D5 laccase (PDB code, 6H5Y) was obtained at 2.3 Å resolution by molecular replacement, using as search model 1GYC. The real space group I23, led us to a solution with two molecules in the asymmetric unit, a Mathews coefficient of 3.26 $Å^3$/Da and a solvent content of ~63%. The structure confirmed the common three-cupredoxin-like-domain folding of fungal laccases (Orlikowska *et al*., 2018), quite similar to that of PM1L (PDB code, 5ANH); with a backbone (Ca atoms) rmsd of 0.46 Å and same topology (Fig. 3, table S2). Both crystal structures show equal number of β-strands (30) and α-helixes (7), although

mutation I468T enlarges the sixth α-helix in three residues: from VAAT sequence in PM1L, to TPDVAAT sequence in 7D5 (Fig. 2).

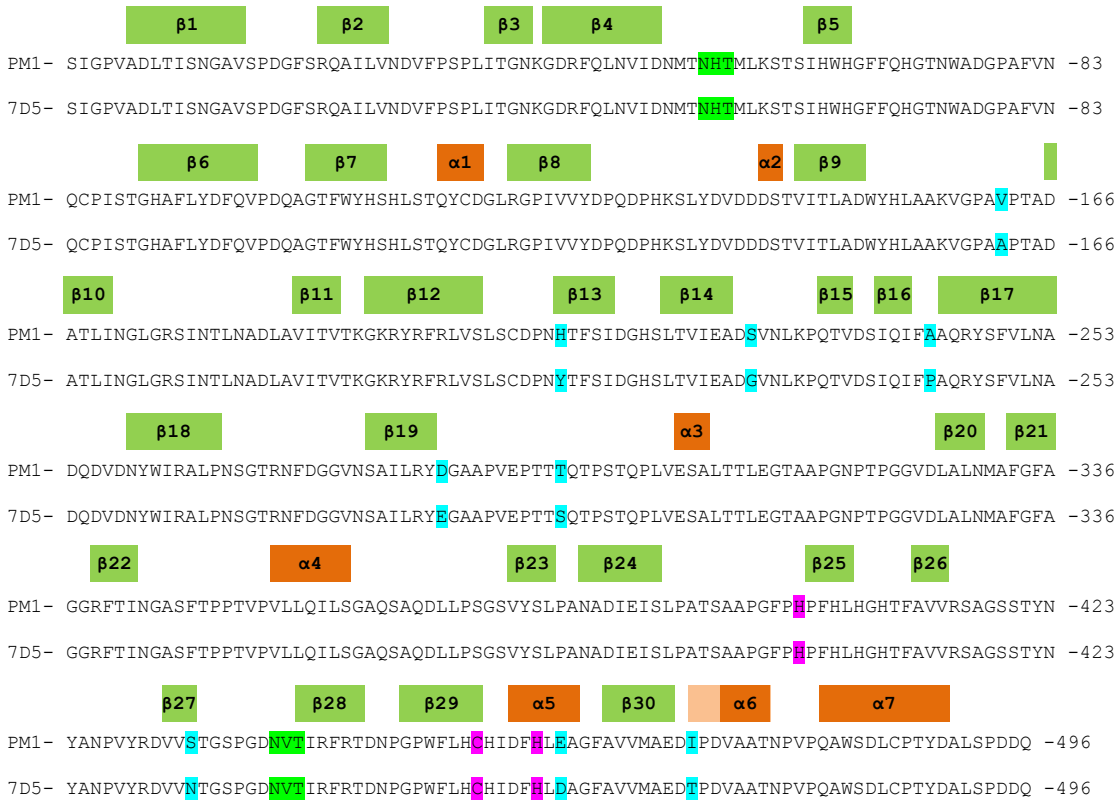

Figure 2. Comparison of amino acid sequences and numbering of secondary structures in wild PM1 and engineered 7D5 laccases. Location of β-strands are depicted in green and α-helixes in orange (elongation of α-6 in 7D5 laccase due to I468T mutation is shown in light orange). Amino acid replacements between PM1 and 7D5 laccases are shown in cyan, T1-copper coordinating residues in magenta and N-glycosylation sites in bright green.

Two N-glycosylation sites, Asn54 and Asn433, were found in both molecules, present in the asymmetric unit with well-defined electron densities (Fig. 3). Both sites are highly conserved amongst basidiomycete laccases, and are considered to play an important role during nascent protein folding, stabilization and secretion (Christensen and Kepp, 2013; Orlikowska *et al.*, 2018). However, the higher Dmax and less spherical structure of 7D5 observed by SAXS and the glycosylation percentages deducted from MALDI-TOF/TOF indicate a heavier and more heterogeneous carbohydrate moiety in the enzyme expressed in *A. oryzae*. This fungus possesses two kinds of alpha- 1,2- mannosidases, one located in the ER and the other in the Golgi (Deshpande *et al.*, 2008) that would be responsible for highly diverse glycan processing. In addition, and as aforementioned, O-glycosylation seems to be especially important in 7D5 laccase, as suggested by the larger Dmax

of the protein after deglycosylation with Endo-H, and in concordance with the formation of branched O-glycans by *Aspergillus* (Deshpande *et al*., 2008).

Up to 72 structures of fungal laccases are deposited in Protein Data Bank, including laccases from Ascomycetes and Basidiomycetes. However, the majority of the 54 basidiomycete laccase structures (from 21 different species) correspond to wild enzymes because of the difficulty to obtain recombinant basidiomycete laccases at high yields (Kunamneni *et al*., 2008). In fact, only two basidiomycete laccases produced heterologously have been crystallized: one from *Coprinus cinereus*, expressed in *A. oryzae* (1HFU and 1A65), and other laccase from *Trametes hirsuta*, expressed in *Penicillium canescens* (5LDU); both of them correspond to native enzymes (Table S3). Conversely, the structural characterization of new variants engineered in the lab might shed light on the protein determinants responsible for modified catalytic activity or robustness, thus providing high-quality information for laccase engineering. Indeed, rational design and enzyme directed evolution techniques are converging in protein science delivering new data for machine learning to accelerate the engineering process (Li *et al*., 2019).

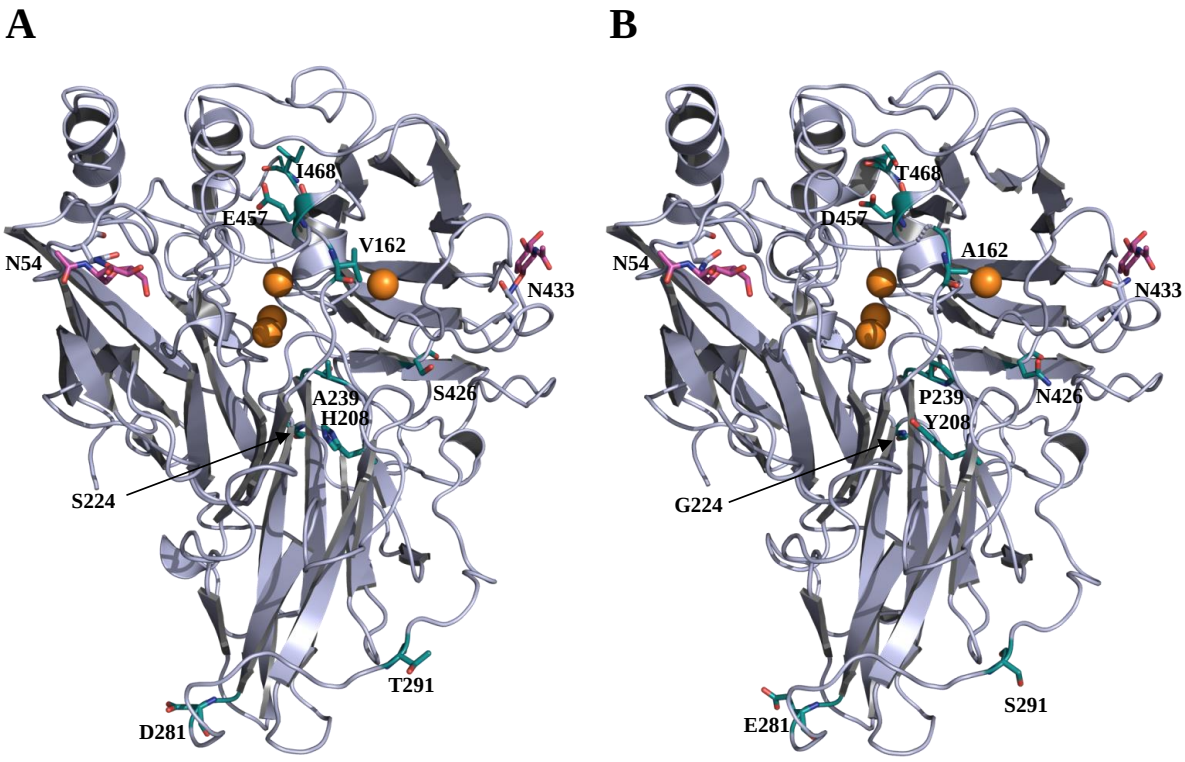

Figure 3. Cartoon representation for 3D crystal structures of wild PM1 laccase (PDB code 5ANH, **A**) and engineered 7D5 laccase produced in *A. oryzae* (PDB code 6H5Y, **B**), showing the catalytic copper ions as orange spheres and the residues mutated during laccase evolution as green-C sticks. N-glycosylation sites (Asn residue) are also depicted as grey-C sticks (with first GlcNAc sugar in magenta-C).

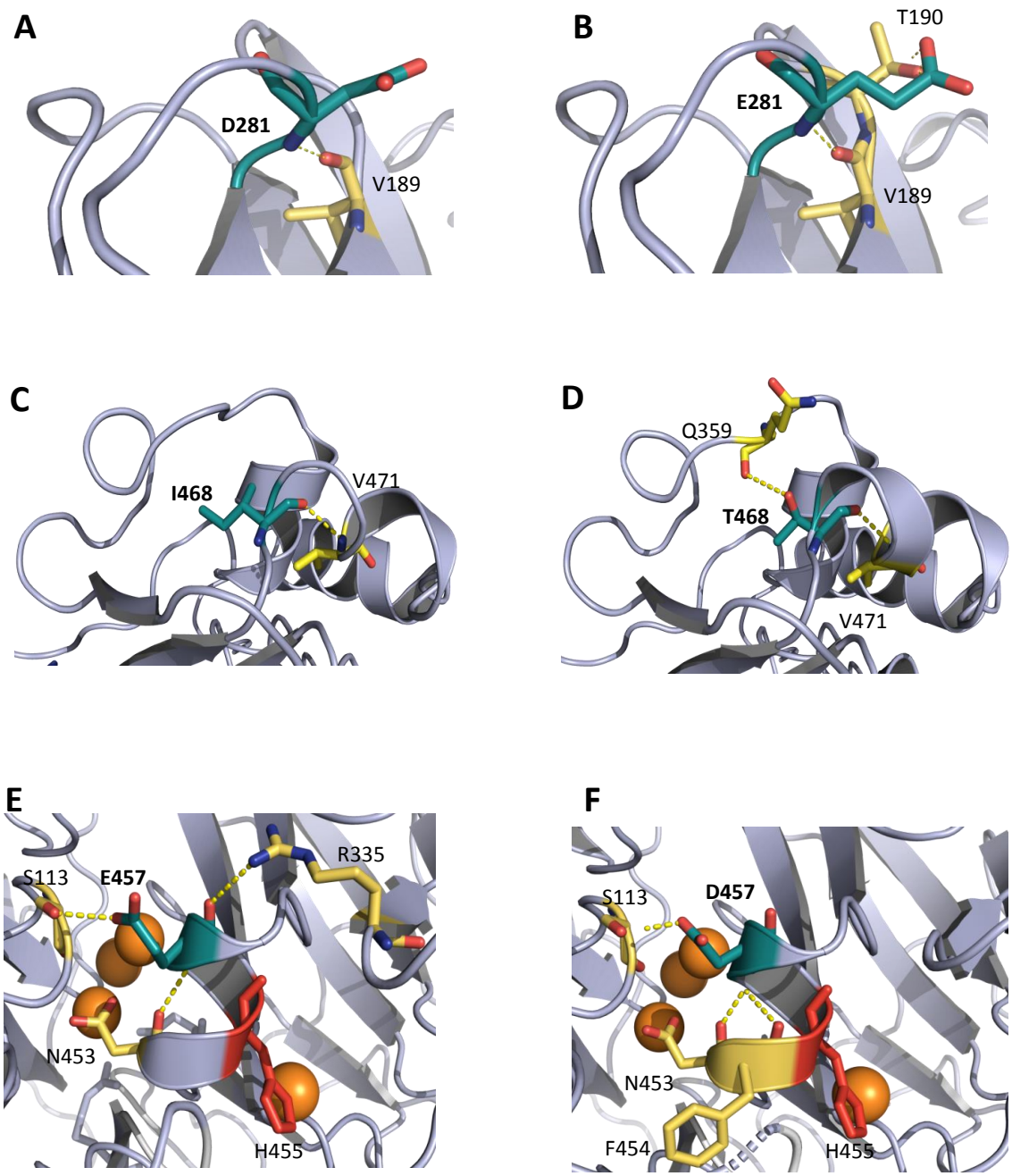

Figure 4. Close-up of PM1 (left) and 7D5 (right) laccase structures showing the H-bonding of residues 468 (**A, B**), 208 (**C, D**), 457 (**E, F**) and 281 (**D, E**)

Residues 281, 291 and 468 are located in surface loops far away from the catalytic site, whereas residues 162, 239, 426 and 457 are in the vicinity of the substrate binding pocket (distance to T1 site < 10 Å) (Fig. 3). As regards mutations on distal loops of the protein, D281E causes a new H-bond with T190, keeping the interaction with V189 (Fig. 4A, B). In mutation I468T, the introduction of Thr induces a separation from the opposite loop, thus affecting the side-chain conformation of Q359, which rotates to form a salt bridge between its carboxyl oxygen and hydroxyl of T468. Besides, both OE1 and OG1 from Q359, now lie towards the pyrrolidine ring of P469 at a distance of near 3 Å, thus producing a re-structuration of the chain from random coil to alpha helix (Fig. 4C, D). On the other

hand, residue 162 is delimiting the substrate binding pocket and residue 457 is in the same α-helix than H455 which coordinates T1 copper. E475 is H-bonded to S113, R335 and D453 in PM1L. Contact with R335 is lost in 7D5 laccase due to E457D mutation and a new H-bond is formed with F454 (Fig. 4E, F).

Catalytic activity

Substituted phenols 4-hydroxybenzoic acid (4-HBA), guaiacol and 2, 6-dimethoxyphenol (DMP), aromatic amines N,N-dimethyl-p-phenylenediamine (DMPD) and two heterocyclic substrates: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT) were used to assess laccase's substrate promiscuity. ABTS and DMP are used as standard substrates to assay laccase activity (Pardo *et al*., 2012). Besides, ABTS and HBT have been applied as redox mediators, improving the oxidation capabilities of laccase in many biotechnological studies (Camarero *et al*., 2004; Moilanen *et al*., 2014). For all the substrates assayed, the evolved 7D5 laccase displayed higher k_{cat} (2-9 fold) values than the wild type (Table 1).

Basidiomycete PM1 it's related to *Coriolopsis gallica* and *Trametes trogii* and PM1L is closely related to other HRPLs produced by the above species (Coll *et al*., 1993). When PM1 and 7D5 laccases were compared with these and other *Trametes* laccases, we observed a remarkably superior catalytic activity of 7D5 for ABTS (Nyanhongo *et al*., 2007). Although it matches the turnover numbers of *Trametes* laccases at room temperature, its outstanding affinity for this substrate, provides it with a value of catalytic efficiency with ABTS not reported in the literature (Jordaan *et al*., 2004; Nyanhongo *et al*., 2007; Yan *et al*., 2014). 7D5 laccase also presents better affinity for DMP although lower k_{cat}. Comparison with other HRPLs like *P. cinnabarinus* laccase confirmed the superior k_{cat} of 7D5 with both substrates (Camarero *et al*., 2012). It is worth mentioning that the k_{cat} values obtained here for PM1 and 7D5 laccases with DMP can be underestimated because they were obtained at pH 5 instead at the optimal pH (4).

To sum up, the most remarkable differences in k_{cat} between the wild type and the engineered laccase are obtained for the oxidation of DMP, ABTS and DMPD. The catalytic efficiency was raised 7-fold for ABTS and 3.5-fold for DMP in the engineered enzyme. This would be a consequence of the use of both compounds as substrates for screening the mutant libraries generated during laccase evolution to 7D5 (Mate *et al*., 2010; Camarero *et al*., 2012). On the other hand, the significant better oxidation of DMPD by 7D5 laccase (with a catalytic efficiency 32 times higher than that of the wild type) was not sought during its design. However, this is not an unexpected result. Due to its superior capability to oxidize aromatic amines, the enzyme had been selected among other counterparts evolved in our lab to carry out the enzymatic synthesis of conducting polyaniline (De Salas *et al*., 2016)

and thereafter subjected to computational design to improve aniline oxidation at the conditions required for polymerization (Santiago *et al*., 2016).

The improved catalytic activity of 7D5 is not related to changes in laccase optimal pH or redox potential, given the similar pH profiles and redox potentials of both laccases. The high-redox potential of PM1 laccase (E° = 0.77 ± 0.01 V, referred to NHE standard electrode) was kept in the engineered enzyme (E° = 0.76 ± 0.01 V *vs* NHE). Both laccases also showed same optimum pH values, pH 2 for ABTS and pH 4 for DMP (Fig. S3) coinciding with the acidic activity profiles characteristic of HRPLs, except for some particular cases (Jordaan *et al*., 2004; Nyanhongo *et al*., 2007). In general, laccases display maximum activities at pH 2-3 for oxidation of ABTS, and slightly less acidic and bell-shaped activity profiles for the oxidation of phenolic compounds due to the counteracting effects: i) decrease in the redox potential of phenol by increasing the pH and ii) inactivation of laccase at alkaline pH because OH^- prevent intramolecular electron transfer (Gunne and Urlacher, 2012). The 20% decrease of activity at pH 2-3 for DMP found in the engineered enzyme would be most probably due to the selective pressure applied during the evolution pathway where mutant libraries were screened with DMP at pH 5 (Mate *et al*., 2010; Camarero *et al*., 2012).

Simulation analysis

To better study the effect that mutations accumulated in 7D5 laccase have on the improvement of its catalytic activity, PELE simulations were carried out for the wild type and engineered laccase with DMPD and HBT, the substrates with higher and lower k_{cat} increase, respectively. In the case of DMPD, PELE calculations displayed similar binding energies for both enzymes but with a significant decrease of the best catalytic distances in 7D5 (Fig. S4A). For the mediator HBT the binding energy profile shows fewer differences for both enzymes (Fig. S4B). Importantly, 7D5 has a significantly higher number of catalytic events for both substrates, defining them as those structures where the substrate adopts a distance below 4 Å to His455 (the T1 copper ligand responsible for electron subtraction from the substrate) (Pardo and Camarero, 2015). The increment was more important for DMPD. Comparing the catalytic events populations, we appreciated a large relative increase in 7D5 (418 and 1663 catalytic events for DMPD and HBT) as compared with PM1 laccase (19 and 445, respectively). These results correlate with the kinetic results obtained in the lab and suggest a better positioning of the substrate in the catalytic site. The analysis of these catalytic poses indicates that mutation V162A, one of the hydrophobic residues in the loop that delimits the substrate pocket at the T1 site (Bertrand *et al*., 2002), improves the catalytic poses by opening an additional space in the copper cavity. This would decrease the distance of DMPD substrate to the Cu-H455 moiety (Fig. 5A, B), thus improving the electronic coupling and increasing the electron transfer rate, which correlates with the increase in k_{cat} and decrease in K_m (Table 1).

Table 1. Kinetic constants for the oxidation of different substrates by the engineered (7D5) and wild type (PM1) laccases.

		k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
ABTS (pH 3)	PM1L	44.4 ± 1.7	0.002 ± 0.0004	26106 ± 6222
	7D5L	240.0 ± 11.2	0.0013 ± 0.0002	184500 ± 29663
DMP (pH 5)	PM1L	13.2 ± 0.3	0.01 ± 0.001	1325 ± 136
	7D5L	45.3 ± 0.7	0.05 ± 0.003	905 ± 56
Guaiacol (pH 5)	PM1L	2.85 ± 0.05	0.24 ± 0.02	11.9 ± 1.0
	7D5L	10.5 ± 0.2	1.04 ± 0.06	10.1 ± 0.6
HBA (pH 5)	PM1L	3.02 ± 0.12	1.96 ± 0.28	1.54 ± 0.23
	7D5L	8.6 ± 0.2	1.41 ± 0.14	6.1 ± 0.6
DMPD (pH 4)	PM1L	108.6 ± 3.1	1.06 ± 0.06	102 ± 6.5
	7D5L	938.0 ± 29.6	0.29 ± 0.03	3253 ± 393
HBT (pH 5)	PM1L	15.5 ± 1.0	47.7 ± 6.99	0.33 ± 0.05
	7D5L	28.8 ± 2.4	34.1 ± 6.95	0.84 ± 0.18

The positive effect of mutation V162A by opening the entrance to the binding cavity was already suggested during the evolution of PM1 laccase (Mate *et al*., 2010). It is worth noting that previous studies carried by our group (Monza *et al*., 2015; Pardo *et al*., 2016; Santiago *et al*., 2016) have confirmed the crucial role of catalytic pocket residues in substrate orientation and binding which determine the enzyme activity. Besides due to the crucial location of residue 162, it has been targeted in several focused evolution studies to improve laccase activity by favoring the binding of selected substrates (Pardo and Camarero, 2015; Pardo *et al*., 2016; Mateljak *et al*., 2019).

We also computed substrates spin density using QM/MM techniques. Spin densities provide the amount of unpaired electrons and thus, they can be used to monitor the extent of electron transfer between the donor and the acceptor, that is, the oxidation. The percentage of spin density in DMPD and HBT for 7D5 is, however, the same as for PM1 laccase (Fig. S4C). This might reflect the fact that, while the substrate gets closer to the catalytic His455 (increase in electronic coupling) its pocket environment remains quite similar (invariance in the substrate oxidation potential). Due to the different nature of ABTS with respect to the other substrates, we performed additional binding and spin density calculations. Interestingly, for this bulkier substrate we find a significant larger conformational change as a result of the reduction in side chain size in the V162A mutation. As seen in Fig. 5C, D, about half of the substrate rearranges its position towards the catalytic His455, considerably reducing its exposure to the solvent and, importantly, increasing its spin density in 7D5 (Fig. S4C) as a result of a local shift in its redox potential.

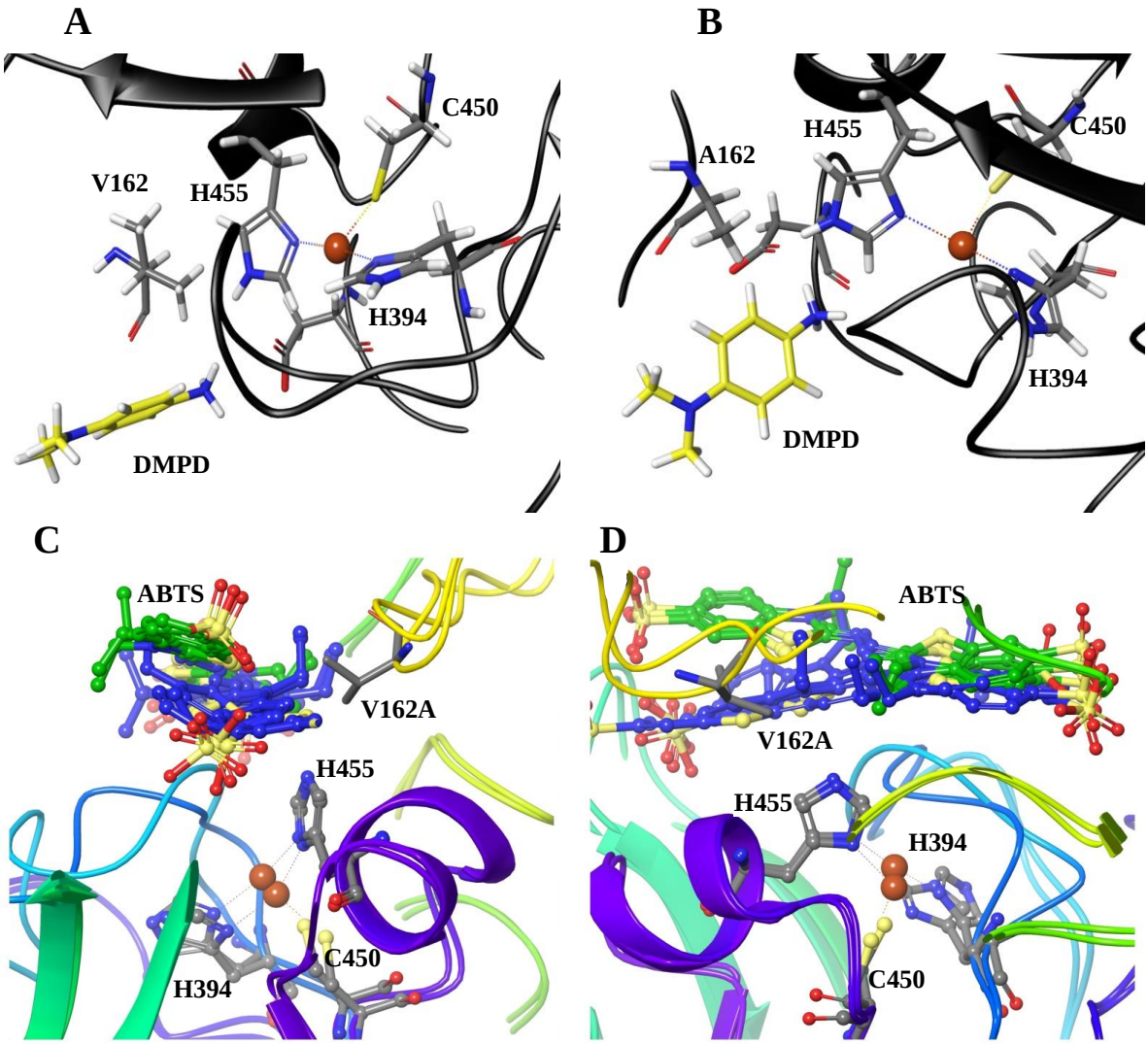

Figure 5. A representative snapshot of DMPD interaction with the catalytic cavities of PM1 (**A**) and 7D5 (**B**) laccases, and two different views (**C**, **D**) for ABTS interactions with the binding pocket of PM1 and 7D5 laccases (as green and blue-C colored sticks, respectively).

Previous studies have suggested a change in the substrate oxidation potential due to (mutation induced) rearrangements in the binding site (Monza *et al.*, 2015). Our results indicate that this effect might not be important in the smaller substrates, where we observed only a small repositioning (a better approach to the catalytic His) and no significant change in spin densities. In ABTS, however, we observed an almost 20% increase in spin density. Thus, for this mediator, both an increase in the electronic coupling and a local shift in its oxidation potential seem to be responsible for the changes in kinetic parameters.

Qualitatively, we can model if the increase in electronic coupling can derive in a ~8 fold increase in k_{cat} for DMPD. Assuming that such an increase will originate mainly

by the change in kET, and using the alternative form of the Marcus equation kET ≈ exp[-β(r-r0)], we can derive $kET_{7D5}=kET_{PM1L}$ exp(Δr), assuming the medium constant, β, to be the same in both species and where Δr represents the reduction in the distance the electron must travel. If we take this difference to be between 1.5 Å and 2 Å (based on Fig. S4A) we obtain a change in kET on the 4.5 to 7.4 range, in close agreement with the change observed in k_{cat}. In addition to mutation V162A that opens an additional space in the copper cavity and has a main effect in the outstanding catalytic improvement for oxidizing DMPD, mutation E457D located in the same α-helix than H455, produces a new interaction with contiguous F454. This residue has been described to modulate the enzymatic activity and have also a significant influence on the stability of the enzyme (Mate *et al*., 2010, 2013). Furthermore, the tripeptide L456-E457-A458 (PM1 laccase numbering) located in this α-helix is highly conserved in HRPLs. The hydrogen bonding between E457 and S113 is characteristic of HRPLs, causing an elongation of the Cu1-N (His455) bond at the T1 site and, therefore raising the E^0 of these laccases (Piontek *et al*., 2002). This bond is maintained in 7D5 laccase variant (Fig. 4E,F) due to the conservative nature of mutation E457D, which would explain the preservation of the high-redox potential of the enzyme.

As regards phenolic compounds, we can observe similar behavior of both enzymes for guaiacol and HBA than for DMP. The three molecules have close similar structure and it would be expected that the increase of activity towards DMP obtained for 7D5 during the evolution pathway would be valid for other phenolic compounds. Hence, all the catalytic increment should be associated with a change in the conformation and charge of the catalytic pocket that may favor the substrates oxidation as suggested by the increase of catalytic events and spin densities observed by PELE and QM/MM.

Enzyme stability

Stabilities of PM1 and 7D5 laccases at pH 2-9 were monitored during 24 h at room temperature. Both were quite stable over pH 6. At pH 6, the wild type maintained its initial activity after 24 h, whereas 7D5 retained 60% of the initial activity. Below pH 6, both enzymes were less stable, although the wild type to a lesser extent. In fact, PM1 laccase displays high stability at extreme pH values. It retains near 100% and 60% of the initial activity after 24 h at pH 9 or pH 2, respectively, whereas 7D5 retained 75% activity at pH 9, but the stability at acidic pH was dramatically diminished (Fig. 6).

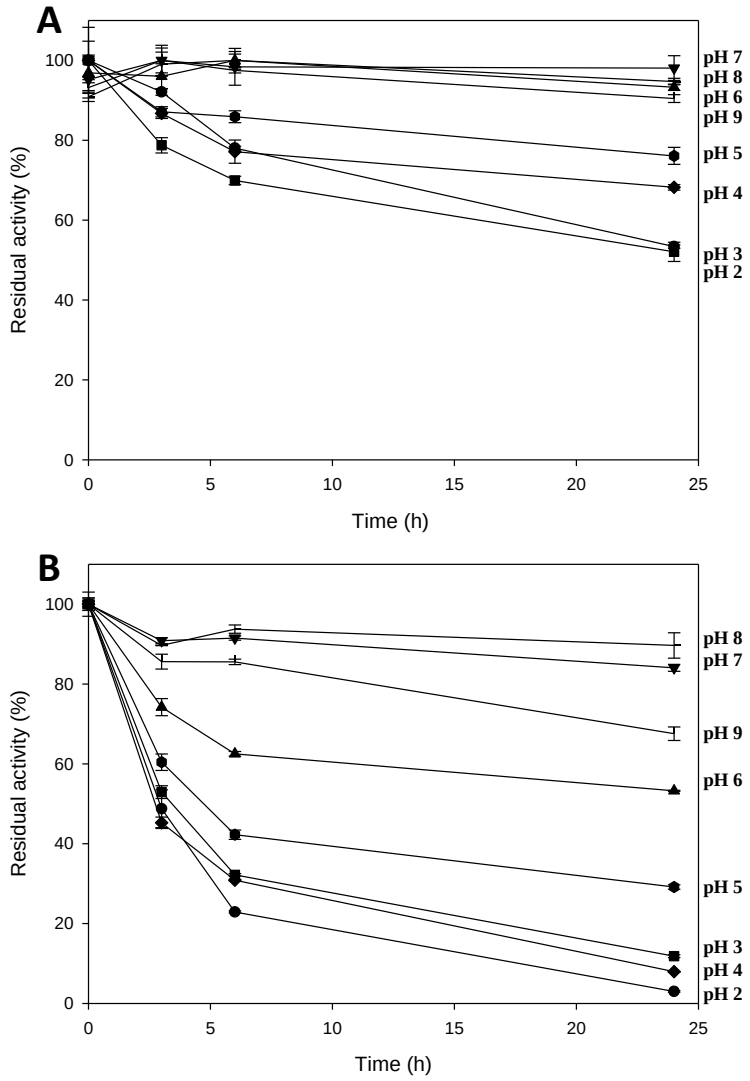

Figure 6. Stabilities of wild type (PM1) (**A**) and engineered (7D5) (**B**) laccases at pH 2-9. Activities at different incubation times were measured with 3 mM ABTS, pH 3.

On the other hand, the wild type has an outstanding stability to high temperature, displaying a T_{50} (10 min) value of 79 °C (with no decrease of activity until 75 °C), whereas the engineered laccase showed a T_{50} (10 min) value of 65 °C (Fig. 7 A). It is worth mentioning that 7D5 laccase was notably activated at high temperature (laccase activity increased around 60% during the first 5 min of incubation at 75 °C) whereas PM1 laccase was not. The long term kinetic stability proved to be very high for both laccases, with outstanding half-life values for PM1 laccase at 50-80 °C, and with lower but still elevated values for 7D5 (Table 2). For instance, when these data were compared with those from 55 fungal thermotolerant laccases from different basidiomycete and ascomycete strains, only a few showed higher half-lives than PM1 laccase or even its evolved variant, proving the stability of both laccases (Hildén *et al*., 2009).

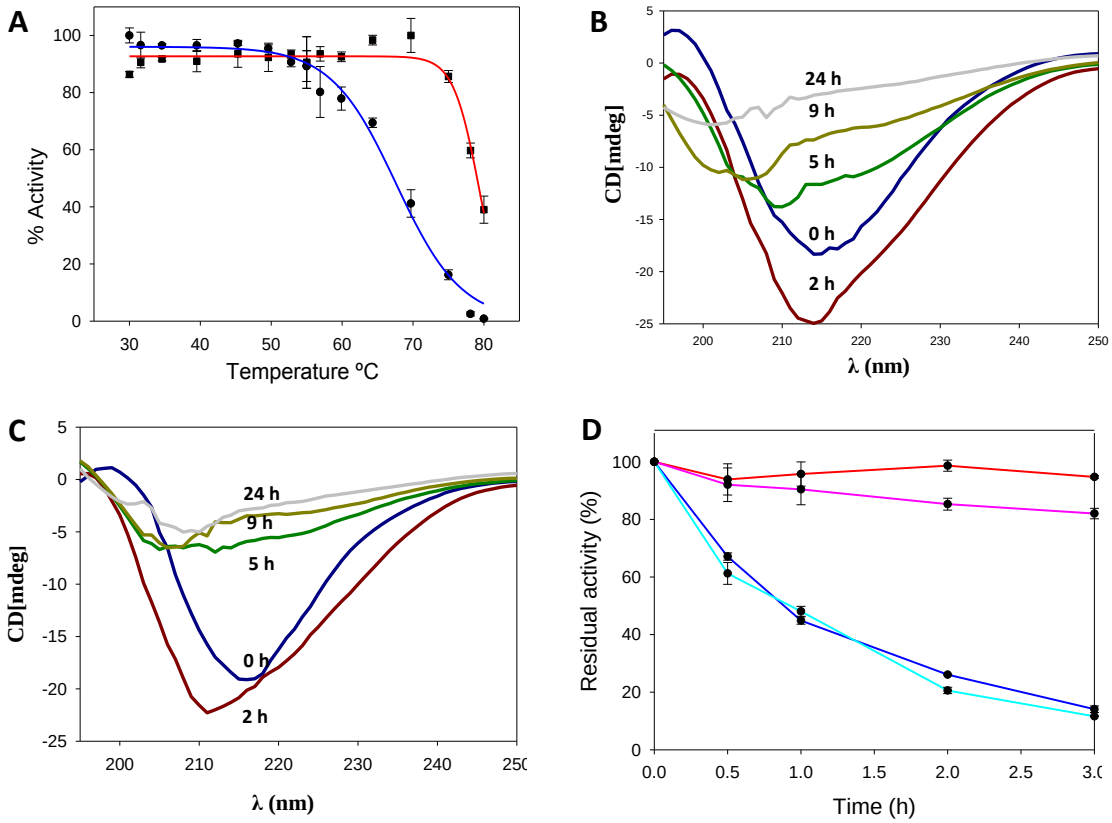

Figure 7. Comparison of thermal stabilities for PM1 and 7D5 laccases: T50 (10 min) curves for PM1L (red) and 7D5 (blue) (**A**). CD spectra for thermal denaturation of PM1L (**B**) and 7D5 (**C**) at 100 °C at different incubation times. Residual activities of PM1L-glyco (red) and PM1L-deglyco (pink) and 7D5_glyco (blue) and 7D5_deglyco (cyan) during incubation at 65 °C (**D**).

The slow thermal inactivation of the wild type was evidenced by its notably low inactivation constants (Table 2). Also, the lower E_a for thermal inactivation of PM1 laccase (175 kJ/mol) than for 7D5 laccase (214 kJ/mol), calculated from Arrhenius the plots (Fig. S5A, B), confirmed the lesser sensitivity to temperature changes of the wild type. Thereafter, we evaluated denaturation of both enzymes by monitoring the changes in typical absorption circular dichroism (CD) bands due to perturbations in secondary structures. First, the enzymes were subjected to a temperature ramp between 50 and 95 °C and ellipticity was monitored at 220 nm, the characteristic band for α-helixes (Fig. S5C, D). The intensity of this band didn't decrease and, consequently, no apparent Tm could be calculated for any of the enzymes. In fact, ellipticity values for both enzymes increased once the temperature reached 70 °C. Next, the enzymes were incubated at 100 °C for 24 h and far UV CD spectra were recorded at different incubation times (Fig. 7B,C). Their initial spectra revealed the presence of two dichroic bands: a single negative band with a strong minimum at 216 nm and a positive band with maximum around 196 nm, both typical of antiparallel β-sheet proteins (Greenfield, 2006). No

protein denaturation was observed during the first 2 h at 100 °C. In fact, even more marked negative band at 216 nm was observed, in particular for PM1 laccase. The initial increments of ellipticity observed in the two CD assays (50-95 °C ramp and 100 °C incubation) suggests the existence of intermediate conformations as a protein adaptation to high temperature. This trend has also been observed in other thermostable laccases, and may be associated to a high structural flexibility at high temperature (Bonomo *et al*., 2001; Ferrario *et al*., 2015; Karshikoff *et al*., 2015; Kikani and Singh, 2015; Mukhopadhyay and Banerjee, 2015; Pardo *et al*., 2018). Above 2 h of incubation at 100 °C, the gradual lessening of ellipticity, with the reduction and shift of 216 nm band to lower wavelengths indicative of random-coil polypeptides, and the disappearance of the positive band at 196 nm, evidenced the progress of protein denaturation. Loss of secondary structures was more pronounced and earlier produced in 7D5 laccase, whereas PM1 laccase was not completely unfolded after 5 h of incubation at 100 °C, which is an extremely long incubation time according to data obtained with other stable enzymes (Ferrario *et al*., 2015; Sammond *et al*., 2018).

Table 2. Half-lives and thermal inactivation constants of wild type (PM1) and engineered (7D5) laccases at different temperatures.

Temperature	PM1 laccase		7D5 laccase	
	$t_{1/2}$ (h)	k_d (h^{-1})	$t_{1/2}$ (h)	k_d (h^{-1})
50 °C	40.30	0.02	21.70	0.03
60 °C	4.85	0.14	2.80	0.25
70 °C	1.60	0.43	0.22	3.11
80 °C	0.12	5.73	0.03	26.20

To study if the distinct glycosylation of 7D5 and PM1 laccases due to the expression system may influence protein stability, glycosylated and deglycosylated forms of both enzymes were incubated at 65 °C, and residual activities were measured at different incubation times (Fig. 7D). While some differences were found between glycosylated and deglycosylated forms in PM1 laccase, in 7D5, both forms showed close similar thermostability. The main differences were obtained when comparing the wild type and the engineered enzyme, regardless of their glycosylation state (82-94% residual activities for PM1 laccase vs 12-14% for 7D5 variant after 3 h at 65 °C).

The wild type enzyme used in this study comes from a fungal strain (PM1) isolated from the water streams of a paper pulp mill (Coll *et al*., 1993) that would explain the outstanding stability to high temperature and alkaline pH of PM1 laccase. Conversely, common activity-stability tradeoff during enzyme evolution would explain the decrement of thermal and pH stability observed in the engineered

variant (Romero and Arnold, 2009; Tokuriki and Tawfik, 2009; Kurahashi *et al*., 2018). Still, 7D5 laccase maintains high thermostability as compared to other fungal laccases (Hildén *et al*., 2009), thus confirming DNA-shuffling of homologous genes as an effective strategy to obtain robust enzymes by accumulation of neutral mutations (Bloom *et al*., 2007; Pardo *et al*., 2012, 2018).

Using PyMOL (Delano Scientific LLC) and B-Fitter software to calculate the B-factors of laccase structures we can observe a reduction of the flexibility of some of the surface loops were the mutated residues are present. The loop where the mutation D281 is located shows up less flexibility in 7D5 than in PM1 laccase, where more interactions have been found for the mutant variant. Furthermore, loop 356-364 is rigidified by the mutation of the residue I468T that adds and hydrogen bond with residue Q359, as mentioned during the structural analysis of 7D5 (Fig. S4C, D). This interaction also appears in coarse-grained simulations with a high representation frequency (~90% of the simulation). It seems that the reduction of flexibility could affect the enzyme capability to adapt to changes, perturbing the protein thermostability. These results contrast with the traditional assessment of "the higher protein rigidity, the better enzyme thermostability", but they agree with other studies highlighting the fact that rigidity and thermostability are not necessarily correlated (Plana *et al*., 2019) or proving the correlation between kinetic stability and thermal flexibility (Risso *et al*., 2017). So far, a variety of alterations in dynamic behavior of thermophilic enzymes, with reductions in certain types of motions and increases in others, have been observed using different techniques (Wintrode *et al*., 2003).

We also performed coarse-grained MD simulations to analyze the overall intra-protein contacts by using CABS-flex. The number of high-frequency contacts (those being formed at least 75% of the simulation length) is closely similar in PM1L and 7D5 (1753 and 1720, respectively). Taking all this into account, the rigidification of certain surface loops in the mutated laccase might affect the thermostability of the enzyme. The CD spectra obtained here for PM1 and 7D5 laccases during first hours at 100 °C seem to corroborate this hypothesis. The ß-sheet folding would be maintained longer in PM1L because the flexible loops would better absorb the impact of high temperatures, keeping longer intact the secondary structures of the backbone. In line with this, two thermostable variants of p-nitrobenzyl esterase generated by directed evolution, showed mutations conferring more flexibility to surface loops (Wintrode et al., 2003). The gain in thermal stability of mutated hemoglobine has been also correlated with the improved flexibility of a certain loop allowing the protein to concentrate its fluctuations in this single loop and avoid unfolding (Bustamante et al., 2014). Also, a larger flexibility in the CD loop of the globin family has been recently correlated with higher thermostability (Plana et al., 2019). Finally, high structural flexibility is characteristic of ancestral proteins which are adapted to high temperature conditions (Risso et al., 2017).

Conclusions

The structure of the laccase solved in this study is, so far, the first crystal structure obtained from a basidiomycete laccase engineered in the lab. Its production at a relevant industrial scale by a hyper-secretory *A. oryzae* strain enabled the deep structural and biochemical characterization of the enzyme. Simultaneously, computational simulations revealed how certain mutations of the catalytic pocket can provide a better positioning of the substrate and improved electronic coupling, thus increasing the electron transfer, or how mutations on the protein surface can affect enzyme stability by reducing the flexibility of the loops. The enzyme studied here holds noteworthy properties such as high-redox potential, overall improved activity and remarkable catalytic efficiency for ABTS and aromatic amines, good stability to high temperature and feasible heterologous overexpression. All together opens new and promising scenarios for its development as an industrial biocatalyst.

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Supplementary material

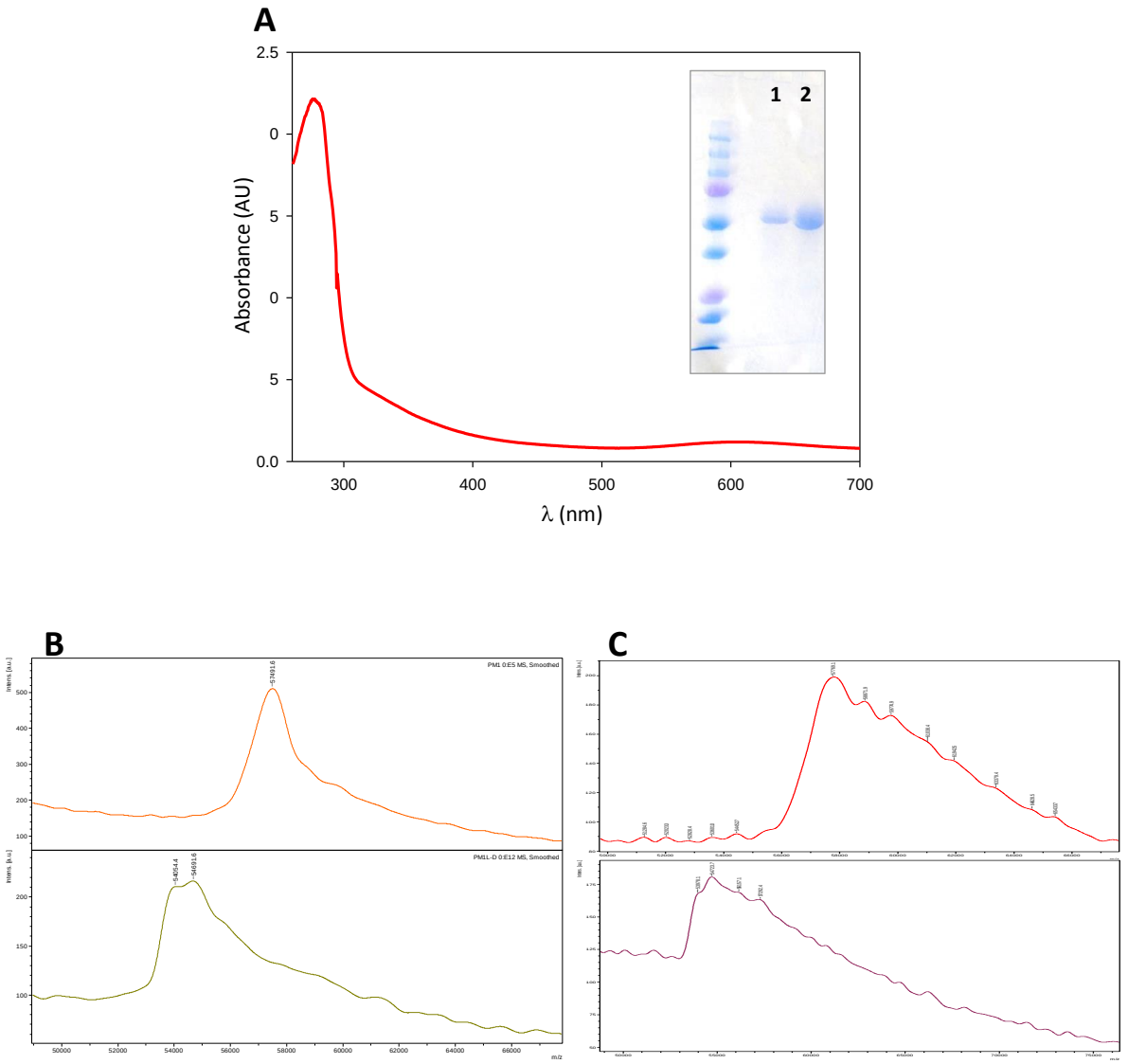

Figure S1. UV/Vis spectra and 12 % SDS-PAGE (inset) of purified wild PM1 (red, lane 1) and engineered 7D5 (blue, lane 2) laccases (**A**); and MALDI-TOF/TOF analysis of glycosylated (up) and deglycosylated (bottom) PM1 (**B**) and 7D5 (**C**) laccases.

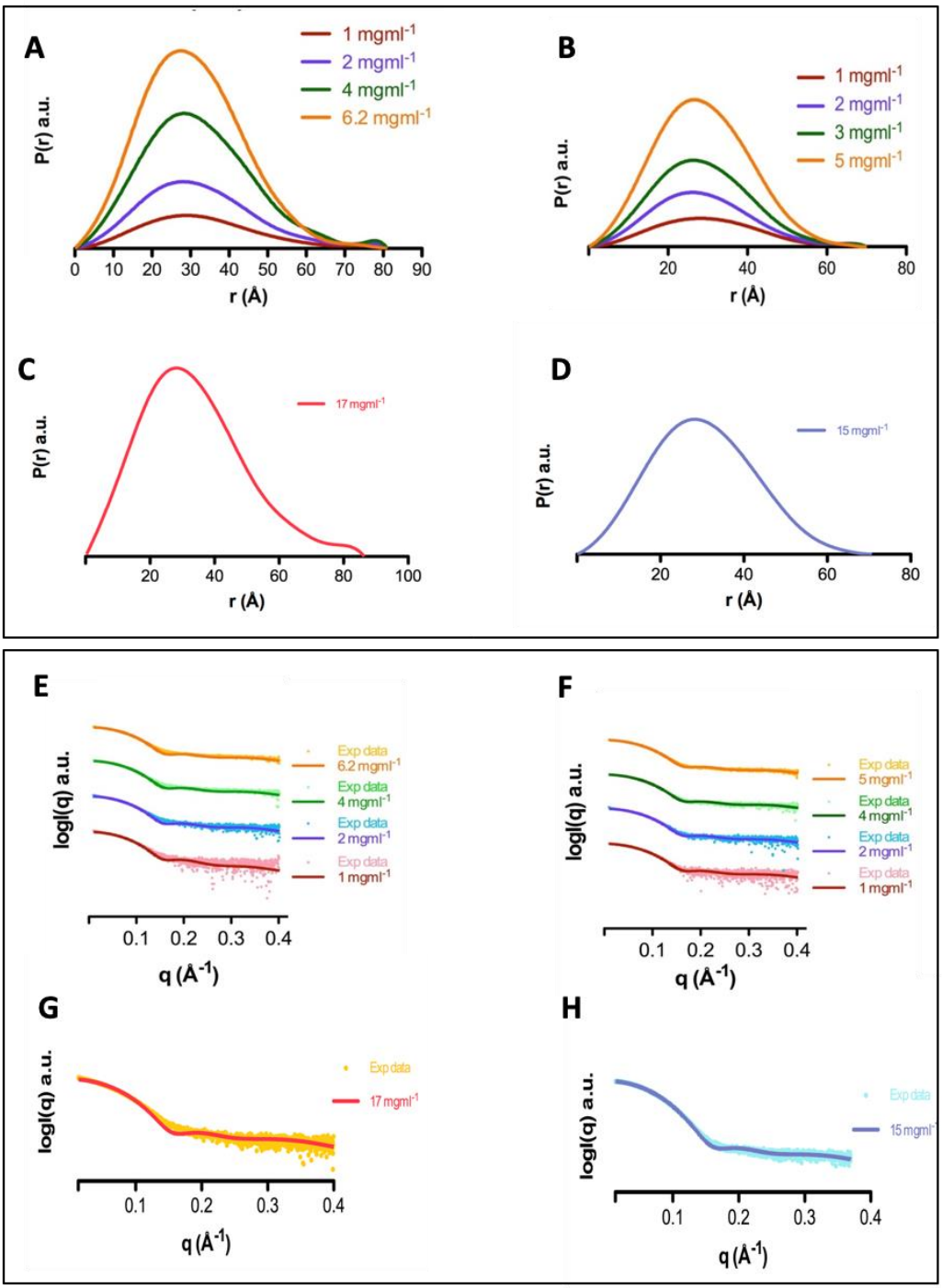

Figure S2. Normalized pair-distance distribution function P(r) for glycosylated (**A**) and deglycosylated 7D5 laccase (**B**) and for glycosylated (**C**) and deglycosylated PM1 laccase (**D**) at their different experimental concentrations. Data are offset vertically for clarity; and SAXS experimental scattering data (dots) and theoretical scattering computed from the models (smooth curves) for glycosylated (**E**) and deglycosylated (**F**) 7D5 laccase, and glycosylated (**G**) and deglycosylated (**H**) PM1 laccase. a.u., arbitrary units.

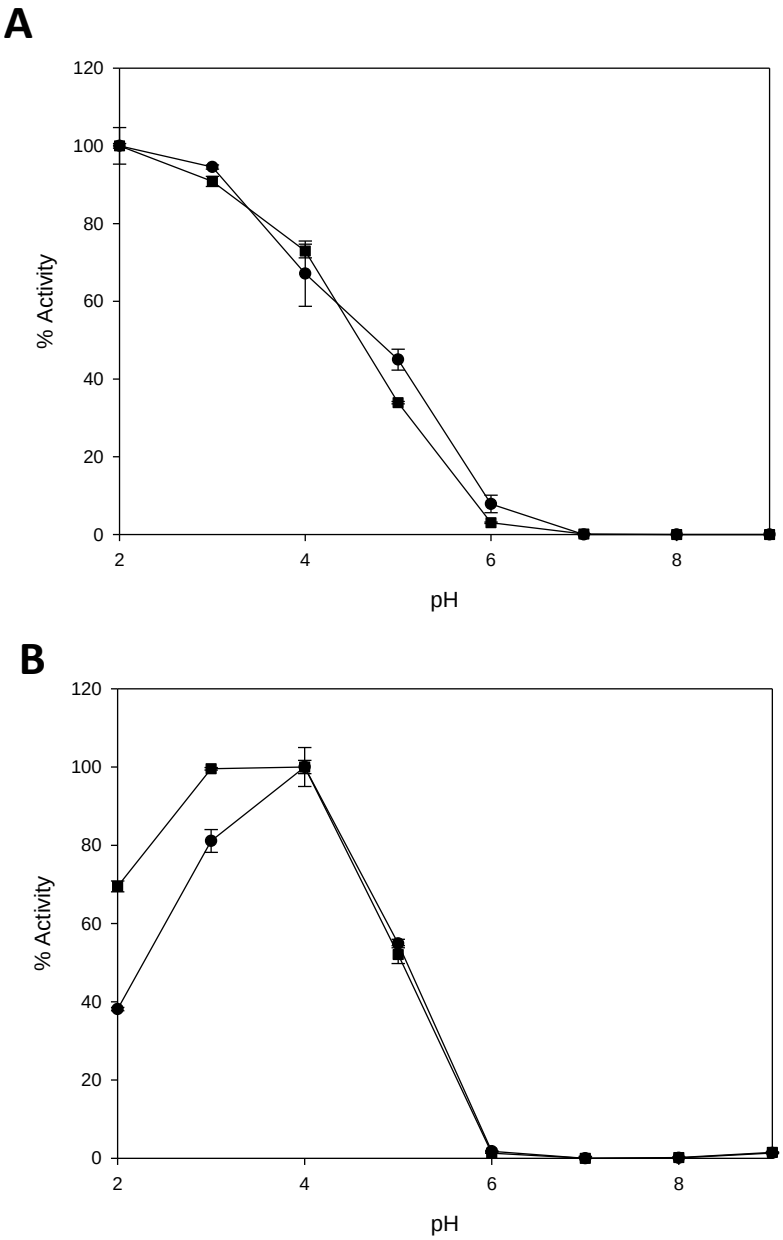

Figure S3. Optimum pH for oxidation of ABTS (**A**) and DMP (**B**) by PM1 (squares) and 7D5 (circles) laccases.

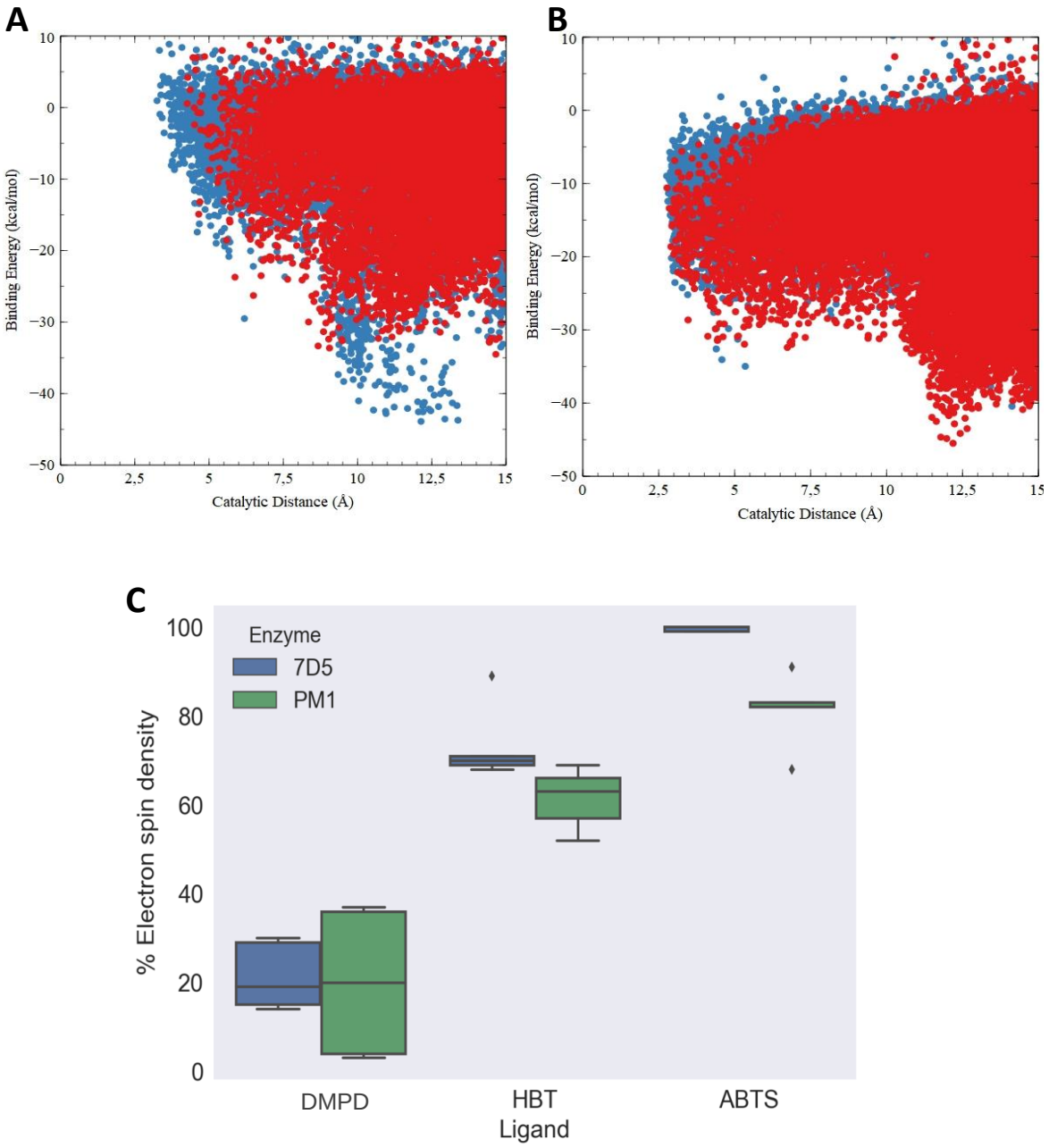

Figure S4. Binding energy versus T1 copper-substrate (center of mass) distance for PM1 (red) and 7D5 (blue) laccases towards DMPD (**A**) and HBT (**B**) as substrates; and spin densities for 7D5 (blue) and PM1 (green) laccases with DMPD, HBT and ABTS substrates (**C**).

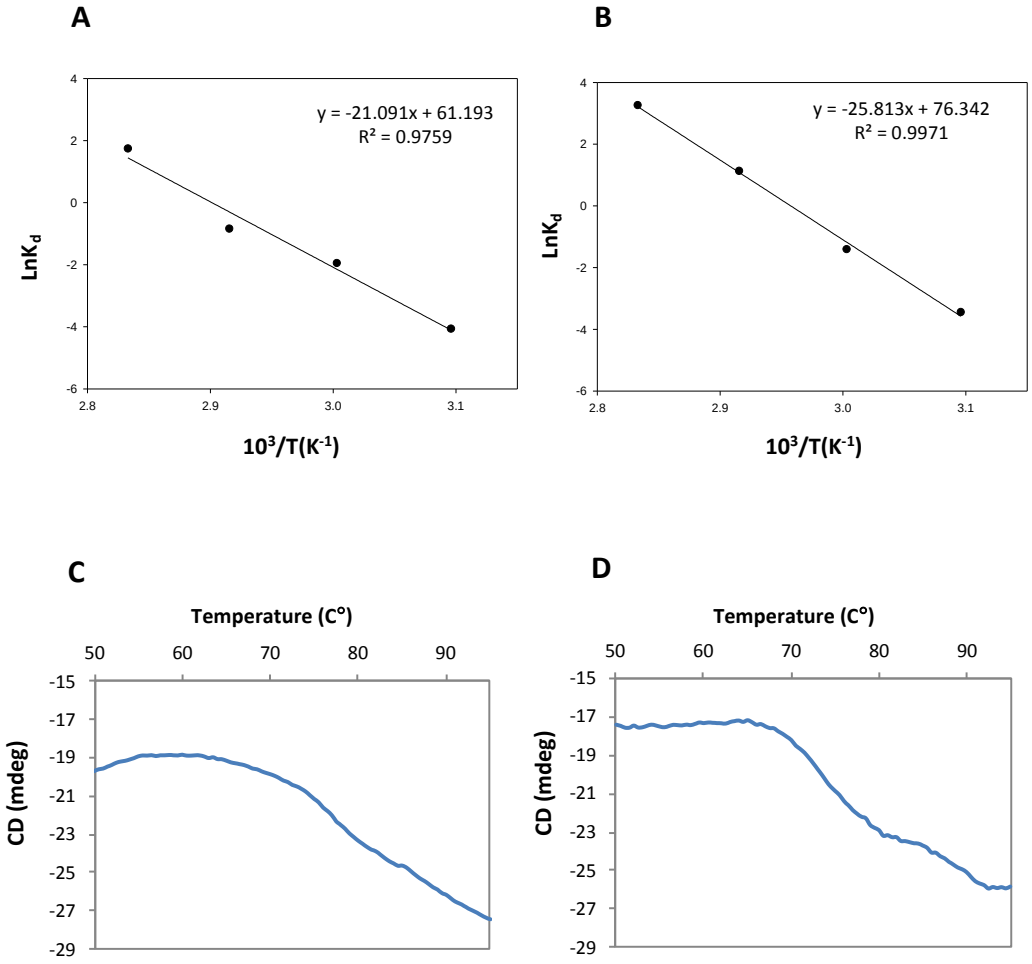

Figure S5. Arrhenius plots from the thermal inactivation curves of PM1 (**A**) and 7D5 (**B**) laccases at different temperatures (residual activities measured with 3 mM ABTS pH 3); and CD analysis of the ellipticity changes at 220 nm observed as a function of temperature in PM1 (**C**) and 7D5 (**D**) laccases (temperature ramp from 50 to 95 °C).

Table S1. SAXS data collection and derived parameters

Data collection parameters				
Instrument	Diamond Light Source (Harwell Campus, UK)			
Wavelenght (Å)	1			
q-range ($Å^{-1}$)	0.01-0.6			
Exposure time (s)	300			
Concentration range	7D5L glycosylated, 1-6.2 mg/ml 7D5L deglycosylated, 1-5 mg/ml			
Temperature (K)	277			
Structural parameters				
7D5L glycosylated				
Concentration (mgml-1)	1	2	4	6.2
Rg (Å) (from Guinier)	27.55±0.12	26.77±0.06	25.08±0.04	23.93±0.02
Rg (Å) (from P(r))	25.71±0.01	25.75±0.01	24.78±0.02	24.07±0.03
Dmax (Å)	81±8	84±8	81±8	83±8
Molecular mass determination				
MM (kDa) from Porod Volume	51±5	55±5	50±5	46±5
Calculated MM (kDa) from sequence	53.21			
7D5L deglycosylated				
Concentration (mgml-1)	1	2	3	5
Rg (Å) (from Guinier)	24.34±0.09	24.08±0.06	23.22±0.03	22.49±0.02
Rg (Å) (from P(r))	23.47±0.01	23.48±0.01	22.98±0.01	22.53±0.02
Dmax (Å)	70±7	74±7	73±7	72±7
Molecular mass determination				
MM (kDa) from Porod Volume	47±5	47±5	46±5	43±4
Calculated MM (kDa) from sequence	53.21			
PML1 glycosylated				
Concentration (mgml-1)	17			
Rg (Å) (from Guinier)	25.85±0.08			

Rg (Å) (from P(r))	25.91±0.02
Dmax (Å)	83±8
Molecular mass determination	
MM (kDa) from Porod Volume	44±4
Calculated MM (kDa) from sequence	53.23
PML1 deglycosylated	
Concentration (mgml-1)	15
Rg (Å) (from Guinier)	21.92±0.09
Rg (Å) (from P(r))	21.91±0.02
Dmax (Å)	67.25±7
Molecular mass determination	
MM (kDa) from Porod Volume	40±4
Calculated MM (kDa) from sequence	53.23
Software employed	
Data processing	SCÅTTER, PRIMUS, GNOM
Ab initio analysis	DAMMIF, DAMMIN
Validation and averaging	SUPCOMB, DAMAVER
Computation of model intensities	FoXS
Computation of molecular weight	SAXSMoW
3D graphics representations	PyMOL

Table S2. Data collection and refinement statistics. Statistics for the highest-resolution shell are shown in parentheses.

Protein	7D5L
PDB ID.	6H5Y
Data collection	
Beam Line	I02 DLS
Wavelength (Å)	0.9795
Resolution range	143.27-2.3 (2.36 - 2.3)
Space group	I23
Unit cell (Å/º)	202.610 202.610 202.610 90 90 90
Total reflections	1553587 (69678)
Unique reflections	31706 (2298)
Multiplicity	49 (30.3)
Completeness (%)	100 (100)
Mean I/sigma(I)	19.4 (2.0)
Wilson B-factor	61.43
Rmeas	0.227 (2.829)
CC1/2	99.9 (0.935)
Refinement	
R-work	0.1627 (0.2417)
R-free	0.1983 (0.2861)
Number of non-hydrogen atoms	7486
protein	7326
solvent	91
Cu^{+2}	8
NAG	61
Protein residues	972
RMS(bonds)	0.008
RMS(angles)	1.13
Ramachandran favored (%)	95.4
outliers (%)	0.63
Average B-factor	57.06

Table S3. Basidiomycete laccases with crystal structures deposited in the Protein Data Bank

PDB ID	Species	Type	Expression host	Reference
5EHF	*Antrodiella faginea*	wild	-	(Glazunova, 2018)
3DIV	*Cerrena maxima*	wild	-	
2H5U	*Cerrena (Trametes) maxima*	wild	-	(Zhukova, 2006)
4JHU	*Coriolopsis caperata*	wild	-	
2HZH	*Coriolus zonatus*	wild	-	(Lyashenko, 2006)
3X1B	*Lentinus sp.*	wild	-	
2QT6	*Lentinus Tigrinus*	wild	-	(Ferraroni, 2007)
5E9N	*Steccherinum murashkinskyi*	wild	-	(Polyakov, 2017)
5MEJ				
5MEW				
5MHU				
5MHV				
5MHW				
5MHX				
5MHY				
5MHZ				
5MI1				
5MI2				
5MIA				
5MIB				
5MIC				
5MID				
5MIE				
5MIG				
3FPX	*Trametes hirsuta*	wild	-	(Polyakov, 2009)
3V9C				
3PXL				

5LDU	*Trametes hirsuta*	native	*Penicillium canescens*	-
2HRH	*Trametes trogii*	wild	-	(Matera, 2008)
2HRG				
5Z1X	*Cerrena sp. RSD1*	wild	-	(Wu, 2018)
1A65	*Coprinus cinereus*	native	*A. oryzae*	(Ducros, 2001)
1HFU				
4JHV	*Coriolopsis caperata*	wild	-	(Glazunova, 2015)
5A7E	*Coriolopsis gallica*	wild	-	(De La Mora, 2012)
4A2F				
4A2G				
4A2E				
4A2H				
4A2D				
5ANH	*PM1 (Coriolopsis sp.)*	wild	-	(Pardo, 2016)
2XYB	*Pycnoporus cinnbarinus*	wild	-	
5NQ7	*Pycnoporus sanguineus*	wild	-	(Orlikowska, 2018)
5NQ8				
5NQ9				
1V10	*Rigidoporus lignosus*	wild	-	(Garavaglia, 2004)
3T6W	*Steccherinum ochraceum*	wild	-	(Ferraroni, 2012)
3T6X				
3T6Z				
3T71				
3KW7	*Trametes sp. AH28-2*	wild	-	(Ge, 2010)
1KYA	*Trametes versicolor*	wild	-	(Piontek, 2002)
1GYC				

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CHAPTER 3

Engineering of a Fungal Laccase to Develop a Robust, Versatile and Highly-Expressed Biocatalyst for Sustainable Chemistry

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Abstract

Fungal laccases can play an important role as biocatalysts in organic chemistry to replace chemical synthesis. In a previous work we synthesized conductive polyaniline using a high-redox potential laccase from our collection of recombinant fungal variants. Still, the oxidation of aniline is hindered by the reaction conditions (low pH and presence of anionic surfactants). Thus, we tackle here the directed evolution of the enzyme asisted by computational simulation aiming at improving aniline oxidation at the required polymerization conditions while maintaining the enzyme´s substrate promiscuity. Simultaneously, its secretion by the host used for the engineering (*Saccharomyces cerevisiae*) was enhanced. Then, the improved laccase variant was overproduced in the industrial host *Aspergillus oryzae* and assayed for one-pot synthesis of polyaniline and naphtol-derived dyes whose textile dyeing properties were verified in an industrial environment. Finally, modification of its C-terminal tail further enhanced laccase stability by flexibilization of the region. The resulting biocatalyst displays noticeable stability at high temperature and extreme pH while shows improved k_{cat} values on the different substrates tested. Moreover, it is remarkably produced in *S. cerevisiae* at rates not formerly reported in the literature. These facts, together with the overexpression in *A. oryzae* opens new scenarios for its further development and application.

Introduction

Laccases (EC 1.10.3.2, benzenediol: O_2 oxidoreductase) are multicopper oxidases that contain four catalytic copper ions involved in the one-electron oxidation of substrates coupled to the four-electron reduction of molecular oxygen to water. The T1 copper, responsible for the characteristic blue color of laccases (absorbance 600 nm), catalyzes the oxidation of the reducing substrate. The electrons are sequentially transferred through cysteine and histidine ligands to the other three copper ions (one T2/ two T3) arranged in a trinuclear cluster (TNC) where the reduction of O_2 takes place (Mehra *et al*., 2018; Sekretaryova *et al*., 2019). Laccases are widely distributed in fungi, plants, bacteria and some insects, playing diverse physiological roles. In fungi they are involved in lignin degradation, morphogenesis, pathogenesis (fungal-plant interaction) and stress defense (Baldrian, 2006). The redox potential of laccases at the T1 site ranges from near 0.4 V in plant and bacterial laccases to up to 0.8 V in some fungal laccases. Saprobic basidiomycetes degrading lignin during decay of wood and leaf litter produce high redox potential laccases (HRPLs), with (E^0 = 0.720- 0.790 V) (Pardo *et al*., 2012; Rivera-Hoyos *et al*., 2013). Laccases are promiscuous oxidizing a broad spectrum of phenols, aryl amines, substituted N-heterocycles, thiols and some metal ions

(Riva, 2006; Rivera-Hoyos *et al*., 2013; Rodríguez-Padrón *et al*., 2018). The use of atmospheric oxygen for their activation, the production of water as sole by-product, and the possibility to catalyze either degradation reactions (such as lignin depolymerization) or synthesis reactions depending on the conditions used, make these enzymes ideal biocatalysts for a number of applications (Mate and Alcalde, 2017; Franco *et al*., 2018; Puente-Santiago *et al*., 2019). Their use in single or multistep biocatalytic processes for organic synthesis has drawn increasing interest due to the considerable advantages obtained from the milder reaction conditions and lower environmental impact than equivalent chemical methods (Mogharabi and Faramarzi, 2014).

Polyaniline (PANI) is a conducting polymer discovered over 150 years ago. Because of its rich chemistry, high electrical conductivity and attractive processing properties, it is one of the most studied conducting polymers of the past 50 years. Polyaniline shows high stability, simple production, low cost of the monomer and the ability to change its optic, structural and conductivity capabilities depending on the synthesis conditions and protonation state (Shumakovich *et al*., 2011). Broad range high-value applications of PANI include supercapacitors, solar panels, biosensors, static insulators etc. Electroconductive PANI is formed after oxidative *p*-coupling (without branching) of aniline, an aromatic amine with applications in dye, rubber or urethane production. Besides linearity, a half oxidized (imine) - half reduced (amine) protonated state of the polymer is required to obtain electroconductive PANI (Emeraldine salt). To obtain this green PANI, aniline polymerization has to be performed at acidic conditions (below the pKa 4.6 of aniline), which increase the redox potential of the monomer (from E^0 = 0.63 V of non-protonated aniline to E^0 = 1.05 V of anilinium cation) (Zhang *et al*., 2014). Nowadays, the industrial synthesis of PANI follows chemical processes with ammonium peroxydisulfate as oxidizer and extremely acidic conditions. By contrast, the enzymatic synthesis of PANI catalyzed by laccase allows the use of milder conditions and reduces pollution (Vasil'eva *et al*., 2007). The addition of anionic surfactants as doping templates in PANI synthesis prevents polymer branches while, acting as amphiphilic systems, they solubilize the polymer in water by forming micelles or vesicles (Hino *et al*., 2006). Besides, the use of different templates results in diverse nano-structured polymers (Wei *et al*., 2002; De Salas *et al*., 2016).

In a previous work, we set the optimal conditions for the enzymatic synthesis of electroconductive polyaniline, obtaining a nano-fibered water-soluble polymer with excellent electrochemistry and conductivity (De Salas *et al*., 2016). The laccase used as biocatalyst had been developed by DNA shuffling of two fungal laccases expressed in *Saccharomyces cerevisiae* (Pardo *et al*., 2012), and it was selected for this target due to acidic pH profile and better activity on aromatic amines than commercial laccases (De Salas *et al*., 2016). Nevertheless, the reaction is still demanding for laccases due to their poor stability to acid pH and anionic

surfactants, and the difficult oxidation of the protonated aniline. Protein engineering can help us to improve the catalytic activity or stability of the enzyme at target conditions (Zumárraga *et al*., 2007; Scheiblbrandner *et al*., 2017; Pardo *et al*., 2018; Wallraf *et al*., 2018).

The easy manipulation, high recombination frequency and feasible secretion of heterologous proteins, make *S. cerevisiae* the preferred host for the directed evolution of fungal oxidorreductases (Zumárraga *et al*., 2007; Mate *et al*., 2010; Camarero *et al*., 2012; Gonzalez-Perez *et al*., 2012; Mateljak *et al*., 2019). However, the low protein yields provided by *S. cerevisiae* as expression system is a major bottleneck to evaluate the biotechnological potential of the enzymes engineered in the lab. Consequently, their up-scale production in other fungal hosts such as *Pichia pastoris* or *Aspergillus* is commonly pursued (Kunamneni *et al*., 2008; Alessandra *et al*., 2010).

In this work, laccase directed evolution and computational design have been combined to improve the acidic synthesis of conductive polyaniline, while maintaining the generalist catalytic activity of the enzyme, and to increase laccase expression in *S. cerevisiae*. Then, the improved laccase variant was overexpressed in the industrial host *Aspergillus oryzae* (Novozymes A/S, Denmark), and used as biocatalyst for the synthesis of polyaniline and dyes to be tested on textiles at an industrial environment (SETAŞ AS, Turkey). Finally, engineering of the C-terminal tail significantly raised laccase stability and activity.

Materials and methods

Reagents and culture media

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), N,N-dimethyl-p-phenylenediamine (DMPD), Sodium dodecylbenzenesulfonate (SDBS), 2,6-Dimethoxyphenol (DMP), aniline, the Yeast transformation kit and the primers used in this study were all purchased from Sigma-Aldrich (Madrid, Spain). Pfu-ultra high fidelity polymerase was purchased from Agilent (Santa Clara, EEUU). Endoglycosilase-H (Endo-H) and the High Pure Plasmid Isolation Kit were both purchased from Roche (Basel, Switzerland). The Gel Extraction Kit was purchased from Qiagen (Hilden, Germany), Zymoprep Yeast Plasmid Miniprep II from Zymo Research (Irvine, USA) while Genemorph II random mutagenesis kit was obtained from Stratagene (La Jolla, EEUU). Protease-deficient *S. cerevisiae* strain BJ5465 comes from LGCPromochem (Barcelona, Spain). Mixture of dNTPs was acquired from Roche and minimal medium, yeast extract-peptone medium (YP), expression medium and synthetic complete (SC) dropout plates without uracil were prepared as seen in Camarero 2012. pJRoC30 containing 7D5 laccase gene insert was obtained in previous works (Pardo *et al*., 2012). To linearize pJRoC30 vector, the

gene was removed using the restriction enzymes BamHI and XhoI purchased from New England BioLabs (Hertfordshire, United Kingdom).

High-Throughput Screening (HTS) assays

HTS colorimetric assay with DMPD

To avoid handling aniline during the screening of the laccase mutant libraries (thousands of clones) we selected a less toxic analogue, DMPD, as substrate to test laccase activity on aromatic amines. The extinction coefficient of oxidized DMPD (Würsten dye) was calculated by measuring the absorbance of increasing concentrations of oxidized DMPD at 550 nm, pH 3, in a Shimazdu UV-1900 Spectrophotometer and applying the Beer-Lambert equation. The reliability of the colorimetric assay in high-throughput format was tested with the supernatants of *S. cerevisiae* cells transformed with laccase gene and cultured in 96-well plates. To test reproducibility of the assay, a certain clone was cultured in the 96 wells of the same microplate and the coefficient of variation of the colorimetric responses given in each well was determined. The linearity of the assay was evaluated by the response obtained with increasing volumes of the same laccase supernatant. Finally, the sensitive of the colorimetric assay was evaluated by screening a 1800-clone mutant library. Changes in DMPD absorbance were measured in end point in a SpectraMax 384 Plus plate reader (Molecular Devices, USA).

Screening of laccase mutant libraries

HTS of the mutant libraries were carried out as previously described (Camarero *et al*., 2012) except for some modifications here specify. The libraries were screened with 5 mM DMPD and 3mM ABTS as substrates, in 100 mM citrate-phosphate buffer, pH 3. In addition, stability assays to acid pH (3) were carried out during the first and second re-screenings. In each generation, the best laccase variants were selected and used as parents for the next round of evolution as follows: higher activity with DMPD> higher stability to pH3 > higher activity with ABTS. Aniline oxidation and aniline polymerization (15 mM aniline, 5 mM SDBS, same buffer, see Application study section) was assayed in the variants selected in each evolution round. In the first case, 300 mM aniline in 190 µl of the same buffer, were stirred until aniline was totally dissolved. Then, 10 µl of crude enzyme (10 µg/ml concentration) were added, and the increment of Absorbance 410 nm was monitored in kinetic mode for 20 min using the plate reader (triplicate samples).

Laccase engineering

First evolution round, random mutagenesis with Taq/$MnCl_2$

epPCR over the whole construction (7D5 laccase CDS fused to the corresponding mutated alpha-factor pre-proleader) (Pardo *et al*., 2012) inserted in the vector pJRoC30 was carried out using Taq/$MnCl_2$ in a gradient thermocycler T100 Bio-Rad (CA, USA), using the following protocol: 95 ºC (2 min), 1 cycle; 94 ºC (45 s), 53 ºC (45 s), 74 ºC (3 min), 28 cycles; and 74 ºC (10 min), 1 cycle. For 50 µl reaction, 3

µl $MgCl_2$, 0.5 µl $MnCl_2$, 3 µl DMSO, 0.3 mM dNTPs mix, 90 nm each primer, 0.5 µl Taq polymerase and 4.6 ng of DNA template were added. The primers used RMLN and RMLC are depicted in Table S1. Purification and transformation of the PCR products were carried out as already described (Camarero *et al*., 2012).

Second evolution round, random mutagenesis with Mutazyme II

epPCR was carried out with the Genemorph II random mutagenesis kit (medium mutagenic degree, according to kit protocols) over the winner of the first evolution round. The reaction mix contained 2000 ng of the plasmid pJRoC30 with the laccase mutant gene, 5 µl of Mutazyme II buffer, 1 mM dNTPs, 125 ng of each RMLN and RMLC primers (Table S1) and 1 µl of Mutazyme II in 50 µl volume. The amplification was carried out under the conditions aforesaid and purification and transformation of the PCR products were carried out as already described (Camarero *et al*., 2012).

Reversion of F454S mutation

Reversion of mutation F454S was carried out using primers pJro30 9H2 F and pJro30 9H2 R combined with RMLC and RMLN, respectively (Table S1). For 50 µl reaction, 5 µl buffer PFU, 3 µl DMSO, 1 mM dNTPs mix, 2.5 µl each primer (0.25 µm), 1 µl PFU polymerase and 100 ng of DNA template were added. PCR was carried out under the following conditions: 95 ºC (2 min), 1 cycle; 94 ºC (30 s), 55 ºC (30 s), 74 ºC (2 min), 28 cycles; and 74 ºC (10 min), 1 cycle. The two purified PCR products were recombined and cloned by *in vivo* overlap extension (Alcalde *et al*., 2006) (IVOE), taking advantage of the DNA recombination machinery of *S. cerevisiae*.

Site directed mutagenesis N263D and N207S

The site directed mutagenesis of N263D and N207S was performed as described in Santiago *et al* 2016.

Saturated mutagenesis of residue 454

Saturated mutagenesis of the position 454 was carried out using degenerated primers 454DFw and 454DRv respectively combined with RMLC and RMLN (Table S1), to obtain two PCR products that were recombined and cloned by IVOE. The PCR protocol and reaction mix was done as for the reversion of F454S.

C-terminal substitution

The ten last amino acids of the C-terminal from 3A4 laccase was extracted from the gen using primers C-terminal3A4Fw and RMLN (Table S1) and the following PCR protocol: 95 ºC (2 min), 1 cycle; 94 ºC (30 s), 55 ºC (30 s), 74 ºC (30 s), 28 cycles; and 74 ºC (10 min), 1 cycle. The PK2 laccase gen was extracted without the last 10 amino acids using primers C-terminal3A4Rv and RMLN (Table S1) and the same PCR protocol as for the 1st evolution round. In both cases the above described PFU reaction mixtures were used. Both fragments were recombined and cloned by IVOE.

Enzyme production and purification

Laccase was produced by *S. cerevisiae* 1 L-flask cultures and purified as described before (Santiago *et al*., 2016). Laccase activity in the culture was measured spectrophotometrically with 3 mM ABTS in 100 mM citrate-phosphate buffer, pH 3 by the increase of Absorbance 418 nm ($\varepsilon 418 = 36000\ M^{-1}\ cm^{-1}$). One activity unit (U) was defined as the amount of enzyme needed to transform 1 µmol substrate/minute. To estimate enzyme production, enzyme concentration of a purified laccase variant was measured by the A280 (Nanodrop 2000, Thermofisher, USA) and the specific activity (U/mg) was calculated and used to deduce the mg of enzyme/l obtained in the culture.

Enzyme characterization

All characterization assays were performed with purified enzymes.

Thermal stability

T_{50} assays were performed using ABTS as substrate and following already described protocol (Camarero *et al*., 2012).

Laccase half-life values at 50, 60, 70 and 80 ºC and thermal inactivation constants were obtained as shown in (Pardo *et al*., 2018). Activation energies (Ea) were calculated from the Arrhenius plots.

The effect of glycosylation in the thermal stability of the enzyme was evaluated after deglycosylation with Endo-H enzyme (0.5 U/ 5 mg purified laccase in 50 mM sodium acetate buffer pH 5.5, 24 h, 37 ºC, gentle agitation in thermoblock). Glycosylated samples were also incubated for 24 h at 37 ºC to avoid errors due to the possible stability decrease in the desglycosylated variants after treatment. Enzyme deglycosylation was confirmed by SDS-PAGE electrophoresis (12% acrylamide). Then, 0.2 U/ml of glycosylated and deglycosylated samples were incubated at 65 ºC for 5 h. Aliquots of 20 µl were taken at different times and its residual activity was measured in microtiter plates, in triplicate, with 3 mM ABTS, pH 3.

Far-UV CD spectroscopy analysis were carried out in a spectropolarymeter Jasco J815 associate to Jasco PTC-4235/15 peltier (JASCO Corporation, Japan). Enzyme samples were diluted to a concentration of 10 µM in 20 mM buffer Tris-HCl pH 7. Denaturalization ramps were set from 50 °C to 95 °C with a slope of 60 °C/h and measured at 220 nm. CD spectra were collected after incubation of the enzyme in a thermoblock at 100 ºC for 24 h (except for the first sample taken at room temperature). The spectra were obtained between 190 and 250 nm with a scanning speed of 10 nm min^{-1}, using a spectral bandwidth of 1 nm and 0.1 cm path length quartz cell (Hellma, Germany). The protein signal was obtained by subtracting buffer spectrum and represented the average of 5 accumulations.

Optimal pH and pH stability

The pH profiles of the different laccase variants was determined in microtiter plates by adding 10 µl of 0.1 U/ml (20 µl of 4 U/ml in the assay with aniline) enzyme activity measured with ABTS to 180 µl (170 µl for aniline) 0.1 mM Britton and Robinson (B&R) buffer pH range 2-9. Reactions were started immediately by adding 10 µl 60 mM ABTS/DMP (3 mM final concentration) or 10 µl of 300 mM aniline (15 mM final concentration). All reactions were measured in triplicate.

To test enzyme stability to pH, laccase samples were diluted in 2 ml 0.1 mM B&R buffer adjusted to pH 2-9 to attain 0.1 U/ml final activity (with ABTS) and incubated at 25 ºC during 24 h. Aliquots of each sample (20 µl) were taken at 0, 1, 2, 3, 6 and 24 h and transferred to a microtiter plate to measure the residual activity in the plate reader as shown before. Relative activities were calculated as a percentage of the maximum laccase activity of each variant. All reactions were measured in triplicate.

MALDI-TOF-TOF analysis

MALDI-TOF-TOF analyses of glycosylated and deglycosylated laccase samples were performed on an Autoflex III instrument (Bruker Daltonics, Bremen, Germany) with a smartbeam laser. The spectra were acquired using a laser power just above the ionization threshold. Samples were analysed in the positive ion detection and delayed extraction linear mode. Typically, 1000 laser shots were summed into a single mass spectrum. External calibration was performed, using the Protein Standard II from Bruker, covering the range from 15000 to 70000 Da. The 2,5-Dihydroxy-acetophenone (2,5-DHAP) matrix solution was prepared by dissolving 7.6 mg (50 µmol) in 375 µl ethanol followed by the addition of 125 µl of 80 mM diammonium hydrogen citrate aqueous solution. For sample preparation, 2.0 µl samples were diluted with 2.0 µl 2% trifluoroacetic acid aqueous solution and 2.0 µl matrix solution. Aliquots of this mixture (1.0 µl) were spotted on the 800 um AnchorChip target (Bruker-Daltonics) and allowed to dry at room temperature. The molecular weight analysis by MS-MALDI TOF was carried out in Proteomics and Genomics Facility (CIB-CSIC), a member of ProteoRed-ISCIII network.

Kinetic constants

Kinetic constants for the oxidation of ABTS (ε418 = 36000 M^{-1} cm^{-1}), DMPD (ε550 = 4134 M^{-1} cm^{-1}), DMP (ε470 = 27500 M^{-1} cm^{-1}), and aniline (ε410 = 1167 M^{-1} cm^{-1}) in laccase variants were measured in a microtiter plate, in triplicate. Twenty µl enzyme were added to 50 mM citrate phosphate buffer pH 3 (ABTS, DMPD, and aniline assays) or 100 mM sodium acetate buffer pH 5 or 100 mM sodium acetate buffer pH 4 (for DMP assays) to a final 250 µl reaction volume. To calculate K_m and k_{cat} values the average V_{max} was represented versus substrate concentration and fitted to a single rectangular hyperbola function in SigmaPlot (version 10.0) software, where parameter *a* was equal to k_{cat} and parameter *b* was equal to K_m. To calculate K_m and k_{cat} for aniline the average V_{max} was represented versus substrate

concentration and fitted to a 3 parameter sigmoidal function in SigmaPlot software, where parameter *a* was equal to k_{cat} and parameter x0 was equal to K_m.

Computational analysis

System preparation

The laccase structure used in this study corresponds to the 7D5 laccase crystal (PDB entry 6H5Y). Mutations in Phe454 were introduced manually using Schrödinger's Maestro and prepared with assistance from Protein Preparation Wizard. Aniline was modelled as an anilinium cation and optimized using Jaguar at the M06 (density functional) with the 6-31G** basis set level of theory; electrostatic potential charges were used for the parameterization.

Aniline activity with different mutations on 454 position

Possible mechanistic explanation for the increase in activity for the four 454-mutated variants was studied by PELE simulations. In these, aniline was initially placed at approximately 10 Å distance to the T1 copper and allowed to diffuse freely. Briefly, the PELE algorithm consists of a combination of perturbation and relaxation phases. In the first, the ligand is randomly rotated and translated, and the protein backbone is perturbed via an anisotropic network model (ANM). Then, side chain prediction techniques are applied to alleviate possible clashes and finally an energy minimization improves the acceptance probability of the Monte Carlo step, which is accepted or rejected according to the Metropolis criterion (refer to Gilabert *et al*., 2018) for a more detailed explanation of the method).

Five PELE simulations were run in total, one for DM variant (Phe 454) and one for each 454 mutation. Each simulation consisted of an ensemble of 240 trajectories run in parallel, where several metrics were monitored, such as the distance between the N1 atom of aniline and the NE2 atom of His455, the ligand's interaction energy or the substrate's relative solvent accessible surface area.

Role of C-Terminal tail on thermal stability and activity

To study the gain in stability introduced by the four C-terminal mutations to PK2 (obtaining RY2 variant), we run molecular dynamics (MD) simulations at 27 and 67 °C. The simulations were run using OpenMM (Eastman *et al*., 2017) as the MD engine. Simulations were run using Amber ff14SB for proteins (Maier *et al*., 2015). Four trajectories were run for PK2 and RY2 at 27 and 67 °C, for a total of 16 trajectories, each consisting of 500 ns, with an aggregated simulated time of 8 μs. The initial structures were minimized for 2000 steps using the L-BFGS optimization algorithm implemented by OpenMM, followed by a 400 ps NVT equilibration at the corresponding temperature with restraints to the protein and ligand heavy atoms of 5 kcal mol^{-1} $Å^{-2}$, and by a final 4ns NPT equilibration at the corresponding temperature and 1 atm with restraints to protein alpha carbons and heavy atoms of 0.5 kcal mol^{-1} $Å^{-2}$. Furthermore, distance constraints between the four copper ions and the coordinating residues were applied, as well as distance

constraints between the copper ions in the trinuclear cluster. The 500 ns production simulations were run with a water box set up with 10 Å buffer from the closest solute atom. During all phases of equilibration and production a time step of 2 fs was used, using periodic boundary conditions and PME electrostatics with a cutoff radius of 8 Å.

Application case studies

Synthesis of PANI and acid dye

Aniline (15 mM) polymerization with crude (unpurified) parent type (EM) and the engineered laccase variants DM, PK2 and RY2 was carried out in the presence of 5 mM SDBS in 250 ml flask, with liquid:air ratio of 0.25 under vigorous shaking. Samples were taken at different times and the polymerization was followed by A800 nm increase.

Enzymatic synthesis of PANI performed at SETAŞ AS (Turkey) by the laccase variant engineered in the lab expressed in *Aspergillus oryzae* was carried out in 2 L final volume under the conditions previously described (De Salas *et al*., 2016).

Enzymatic synthesis of the acid dye (1 L final volume) was carried out with 125 mM 1-naphthol and 25 mM 1-amino-8-hydroxy-3,6-naphtalenedisulfonic acid monosodium salt in 50 mM Tris HCl pH 8 at 25-30 ℃ for 24 h using 10 U/ml (activity measured with ABTS) of the engineered laccase or Novozyme 51003 commercial laccase. The synthesized dye was concentrated by nano-filtration equipment (handmade equipment by Setas) using a polyamide membrane (AFC40, PCI membrane) to a final volume of 250 ml.

Dyeing tests

Standard industrial dyeing tests of the enzymatically-synthesized PANI and acid dye were carried out at SETAŞ AS. The CIELAB color space coordinates (L*, a*, b*) and dyeing efficiency (% STR-WSUM) of the samples were evaluated using a reflectance measuring apparatus Datacolor SF600 plus. Nyloset Brown N2R dye from SETAŞ dye range was used as a reference to evaluate the new acid dye.

Multifiber dyeing tests on acetate, cotton, nylon, polyester, acrylic and wool were carried out with 8% v/v concentrated dyes and 5 g of each textile (1:1 ratio) in 100 mL water containing 0.2 g/l Setacid VS-N, 10 g/l Setalan PM71, 20 g/l Na_2CO_3. Nyloset Brown N2R was used as a reference dye (Setas). Dyeing was performed for 1 h at 102 ℃ in an IR Dyeing Machine (Copower Technology, LTD, Taipei Taiwan).

For the PANI and the 1-naphthol derived dye fiber dyeing tests, two different fabrics were used. Acrylic fiber (5 g textile) was dyed using different concentrations (2 ÷ 8% v/v) of the concentrated PANI dye, in 100 mL water containing 0.3-1.0 g/l SetalanIK-200, 0.3-1 % Migrasist ACM, 2 g/l Na_2CO_3 and 1.0-2.0 g/l Hydrosulfide. Dyeing was performed for 1 h at 102 ℃ in an IR Dyeing Machine. After the first dyeing procedure, the same bath was used to dye another 5 g textile piece (second bath). In the case of the acid dye, nylon fibers (5 g textile)

were dyed using different concentrations (2 ÷ 8% v/v) of each concentrated dye (enzyme synthesized dye and Nyloset Brown N2R), in 100 mL water containing 0.2 g/l Setacid VS-N, 10 g/L Setalan PM71 and 20 g/L Na_2CO_3. Dyeing was performed for 1 h at 102 °C in an IR Dyeing Machine. After the first dyeing procedure, the same bath was used to dye another 5 g textile piece (second bath).

The color fastness of the dyed fabrics were evaluated following established test procedures: ISO 105-B02:1994-Color fastness to artificial light: Xenon arc fading lamp test (Blue scale 1–8); ISO 105-CO6:1998-Color fastness to domestic and commercial washing (Grey scale 1–5) and ISO/DIS 105-X12:2001-Color fastness to rubbing (Grey scale 1–5).

Results and discussion

Laccase directed evolution and semi-rational design

The laccase used here as the starting point for enzyme engineering was selected among a set of fungal laccase variants previously evolved in *S. cerevisiae* in our group. This enzyme, laccase 7D5, possesses high redox potential (E° = 0.76 V referred to NHE standard electrode), activity towards anilines, acidic pH profile, and better stability to the reaction conditions than other counterparts developed in the same directed evolution campaign, (Pardo *et al*., 2012) being, therefore, selected for PANI synthesis (De Salas *et al*., 2016).

To direct laccase evolution toward better activity on anilines, we used a high throughput screening (HTS) colorimetric assay with N,N-dimethyl-p-phenylenediamine (DMPD) as a surrogate substrate less-toxic than aniline to explore laccase activity in the mutant libraries expressed in yeast. Laccase oxidizes DMPD to the stable Würstern dye at pH 3, with ε_{550}= 4134 M^{-1} cm^{-1} (Fig. S1A). The low coefficient of variation (CV=12 %) of the response given by a certain clone cultured in the 96 wells of the same microplate proved the reproducibility of the colorimetric assay (Fig. S1B). Also, the direct correlation of the response with increasing volumes of the same supernatant confirmed the linearity of the HTS assay (Fig. S1C). Finally, its sensitivity was verified on a laccase mutant library of 1800 clones generated by epPCR and expressed in the yeast (Fig. S1D).

Besides, a stability assay at pH 3 was performed during the HTS of laccase libraries to avoid a significant loss of enzyme stability during the evolution pathway. The oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was also used as a reference assay for laccase activity to maintain the generalist activity of the enzyme while increasing its activity on aromatic amines. In each evolution round, improvements in total activity (TAI) for the oxidation of DMPD and ABTS and in stability to pH 3 were calculated for each clone compared with the parent

laccase. Finally, the oxidation of aniline in the presence of the anionic surfactant SDBS was used to evaluate the activities and stabilities of the new variants to the reaction conditions for PANI synthesis.

Laccase engineering started with two rounds of random mutagenesis over the laccase CDS fused to the mutated *S. cerevisiae* alpha mating factor pre-proleader, as signal sequence (Pardo *et al*., 2012). After screening over 4000 clones, the highest activity improvement (8.5-fold TAI) was obtained with variant 9H2 which held two mutations in the α pre-proleader (Aα20T and Qα32H) plus F454S mutation in the mature protein (Fig. 1). The new variant was produced in flask cultures and though laccase activity detected in the yeast culture broth was significantly higher than that of parent 7D5, the stability at pH 3 was remarkably low (Fig. 2). Mutation F454S had appeared likewise during the evolution pathway of PM1 basidiomycete laccase (one of the 7D5 parents) for expression in *S. cerevisiae*, although it was reverted due to its destabilizing effect (Mate *et al*., 2010, 2013). Molecular dynamics (MD) simulation of Phe454 and Ser454 in 7D5 laccase showed only 4 hydrogen bonds in the T1 coordination site for the F454S mutant, 2 less than the parental protein. This correlates with the result obtained from the server ENCoM (Frappier *et al*., 2015) that shows a flexibility increase in this protein region, opening the T1 site into solvent and affecting the hydrogen bond network. This reduction in the number of hydrogen bonds correlates with an increase in activity and loss of stability (Kataoka *et al*., 2011).

Our attempts to offset the destabilizing effect in 9H2 variant were unsuccessful. Thus, we reverted F454S mutation maintaining the two mutations of the signal sequence (Aα20T and Qα32H), giving rise to the expression mutant (EM). The EM variant produced in *S. cerevisiae* flasks cultures (Fig. 2A) recovered the stability of 7D5, although the total activity improvement (TAI) was lowered respecting 9H2. TAI is the result of joint contribution of enhanced secretion or/and activity in crude extracts. By contrast, the 5-fold TAI detected for EM respecting 7D5 is just the result of better secretion due to the two new mutations in the α pre-proleader, which increased laccase production from 3 mg/l (7D5) to 16 mg/l (EM).

Next, to enhance the difficult oxidation of aniline at pH 3, we focused the engineering of laccase on the catalytic pocket. With this purpose, we took advantage of computational simulation by using PELE (Protein Energy Landscape Exploration) and QM/MM (quantum mechanics /molecular mechanics) calculations (Monza *et al*., 2015). Two mutations, N207S and N263D, were predicted to lower the interaction energy of the protonated aniline in the binding pocket, and increase the spin density and electron transfer from the anilinium cation to the catalytic T1 through His455. Both mutations were introduced in EM to obtain the double mutated variant (DM) which was produced in *S. cerevisiae*, purified and characterized (Santiago *et al*., 2016). The latter showed improved catalytic activity towards aromatic amines, without jeopardizing laccase stability.

By contrast, N263D mutation alone decreased 4-fold the production of the enzyme, and reduces in 27 % the activity with aniline respecting DM (Fig. S2) confirming the synergism between both mutations.

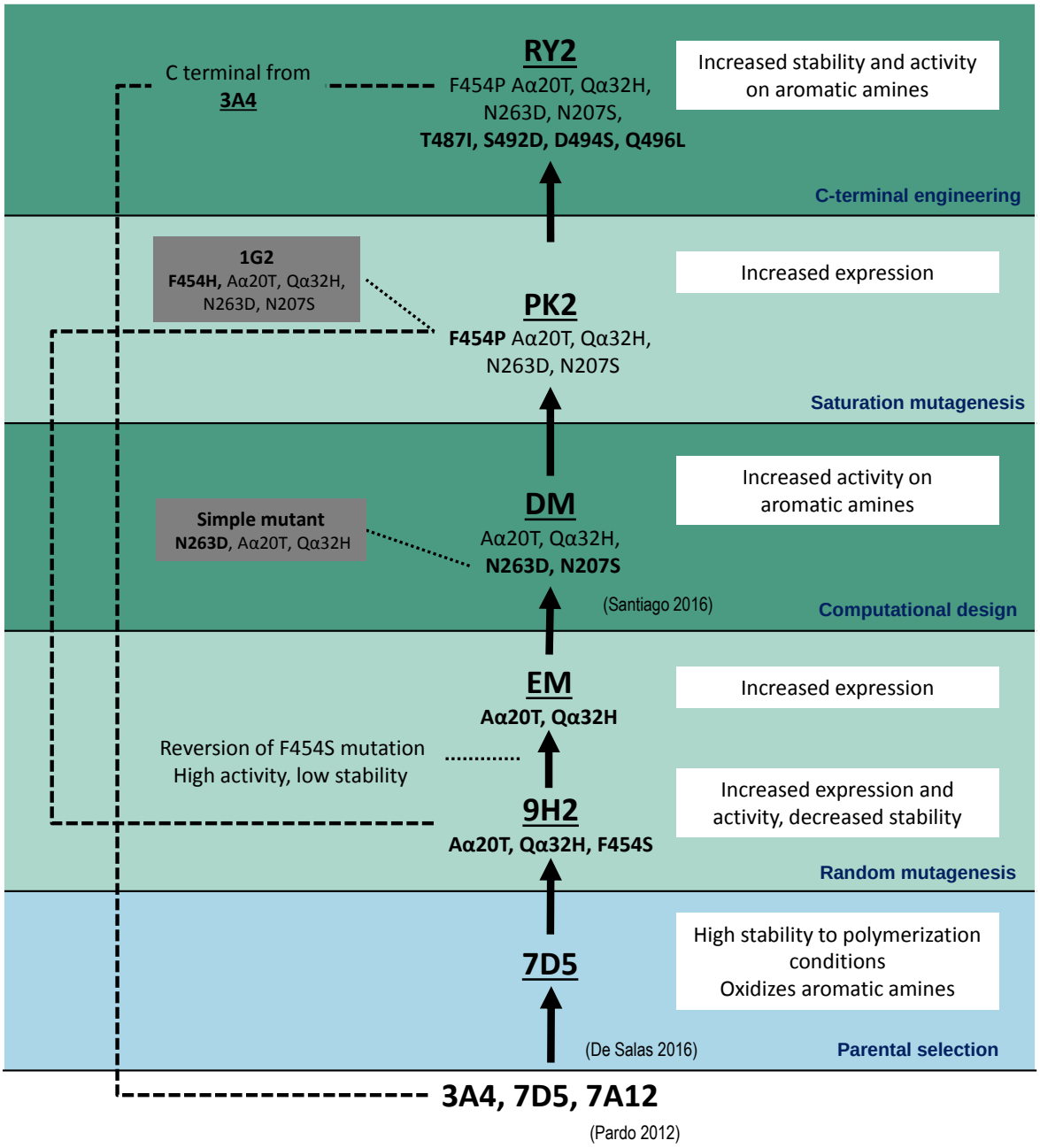

Figure 1. Laccase engineering pathway consisting of: i) selection of parent laccase 7D5; ii) random mutagenesis; iii) computational design; iv) saturation mutagenesis; and v) C-terminal engineering. The mutations accumulated in the improved variants and the main results attained in each consecutive mutational step are shown.

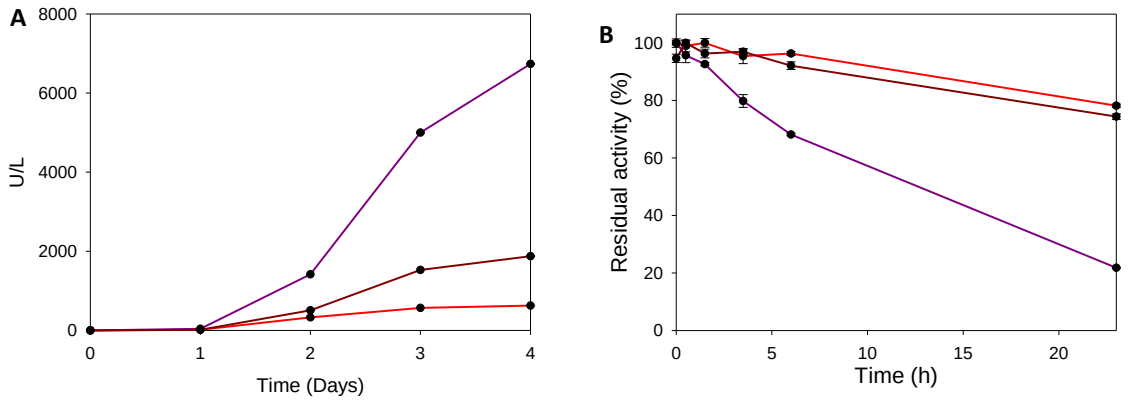

Figure 2. Laccase production in flask cultures of recombinant *S. cerevisiae* (**A**), and acidic (pH 3) stability (**B**) of laccase 7D5 (red) compared with 9H2 (purple) and EM (brown) variants from random mutagenesis (crude enzymes).

Thereafter, the repeated occurrence of mutations in residue 454 during this and related evolution campaigns led us to explore this position to enhance the catalytic activity towards anilines at pH 3 (Fig. 3) (Mate *et al*., 2010, 2013). Residue 454 is adjacent to His 455 that coordinates T1 copper and it is involved in the binding of the reducing substrate and electron withdrawal and transfer to T1 copper (Pardo and Camarero, 2015). As aforementioned, mutation F454S (appeared in 9H2) induced an important activity improvement in 7D5 laccase, although it entailed a significant decrease of stability (Fig. 2). Saturation mutagenesis of Phe 454 in DM variant led to selection of mutations F454H, F454T, F454P and, again, F454S, with significant TAI values on aromatic amines at pH 3. These 454-mutated variants were produced in flasks and the activities and acidic stabilities of the crude enzymes were compared with aniline as substrate (Fig 3A). Mutation F454H (variant 1G2) followed by F454S (variant 2H8) enhanced the most the oxidation of aniline at acid pH. However, the 12-fold increment of activity of 1G2 variant respecting DM significantly jeopardized the stability to acid pH and thermostability (T_{50}) (Fig 3B, D). This destabilizing trend was even more pronounced in variants 2H8 (F454S) and 1E2 (F454T). Conversely, mutation F454P in PK2 variant induced a 6-fold increment of activity, while maintained the stability to pH 3 and kept the T_{50} value closer to that of DM.

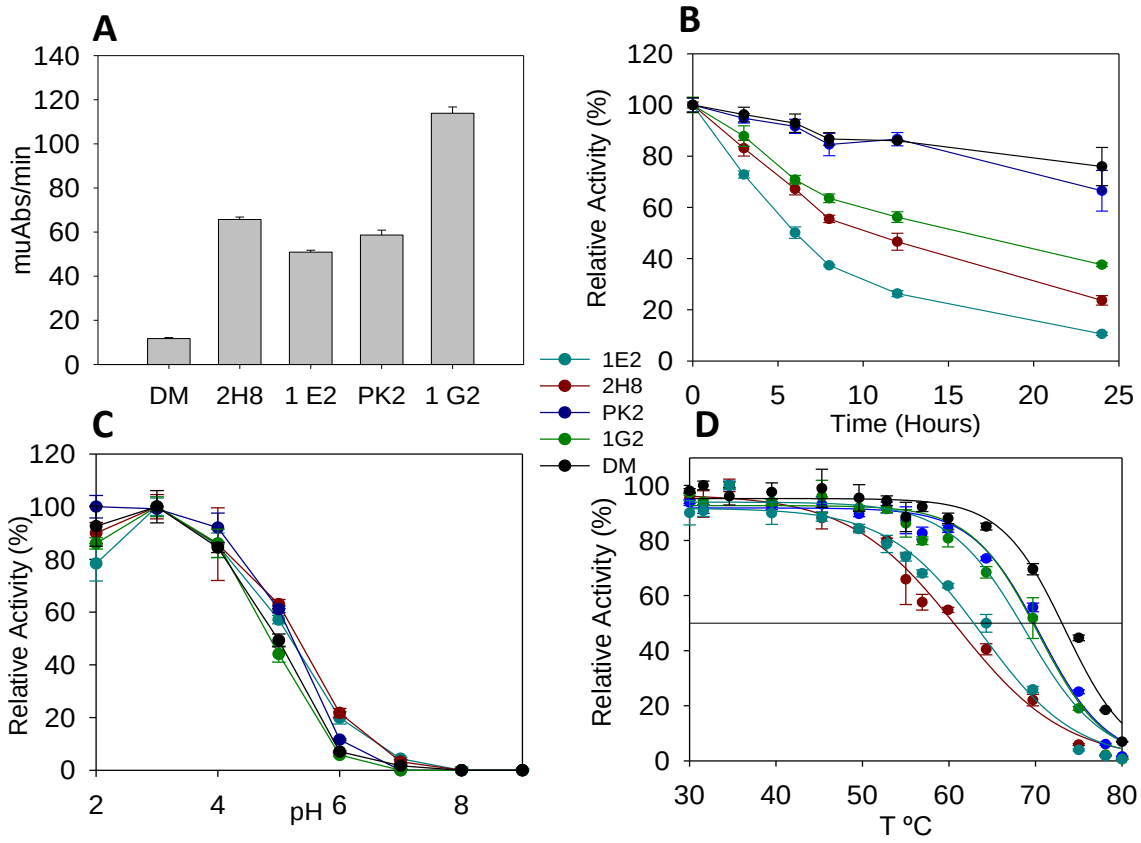

Figure 3. Oxidation of 300 mM aniline at pH 3 (**A**), residual activity after incubation at pH 3 (**B**), optimum pH with ABTS (**C**), and T_{50} (10 min) curves (**D**) for DM (F454) and its 454-mutated variants 2H8 (F454S), 1E2 (F454T), PK2 (F454P) and 1G2 (F454H) (crude enzymes).

To sum up, serine and threonine polar residues in position 454 heavily jeopardize the enzyme stability at acid pH and at high temperature, while basic histidine or nonpolar proline, especially the latter, scarcely affect protein stability. Previous studies have demonstrated that thermophilic enzymes usually are mostly made of hydrophobic and charged residues, showing smaller proportion of uncharged polar residues (Gromiha *et al*., 2013). Recently, a highly stable laccase towards acid pH and high temperature has been developed by directed evolution through increasing the hydrophobicity of the T1 copper environment (Mateljak *et al*., 2019).

Variant PK2 was selected as parent for the next evolution round. In order to improve its performance as biocatalyst, we put the attention on former results obtained during the development of 7D5 laccase (Pardo *et al*., 2012). In that study, most of the stable chimeras obtained from the DNA shuffling of PM1 and *Pycnoporus cinnabarinus* laccases shared a number of residues from the C-terminal tail of *P. cinnabarinus* parent, although they had higher total sequence identity with PM1. Since the C-terminal tail of 7D5 laccase differs from those of other counterparts obtained in the same directed evolution campaign, we modified it accordingly. Specifically, the C-terminal tail of the stable 3A4 laccase differs in four

mutations from 7D5 (T487I, S492D, D494S, Q496L). Therefore, we introduced these mutations in PK2 by replacing the last ten amino acids by those from 3A4 using in vivo overlap extension, IVOE (Fig. 4) (Alcalde *et al*., 2006). The RY2 new variant showed a remarkable stability at the target reaction conditions and also at high temperature compared with its parent PK2. In line with these finding, a small library of chimeric laccases has been recently generated from three fungal laccases, two of which are the 7D5 parents, using SCHEMA RASPP to guide the recombination of protein blocks. Most of the stable variants selected shared also the C-terminal block from *P. cinnabarinus* laccase (Mateljak *et al*., 2019).

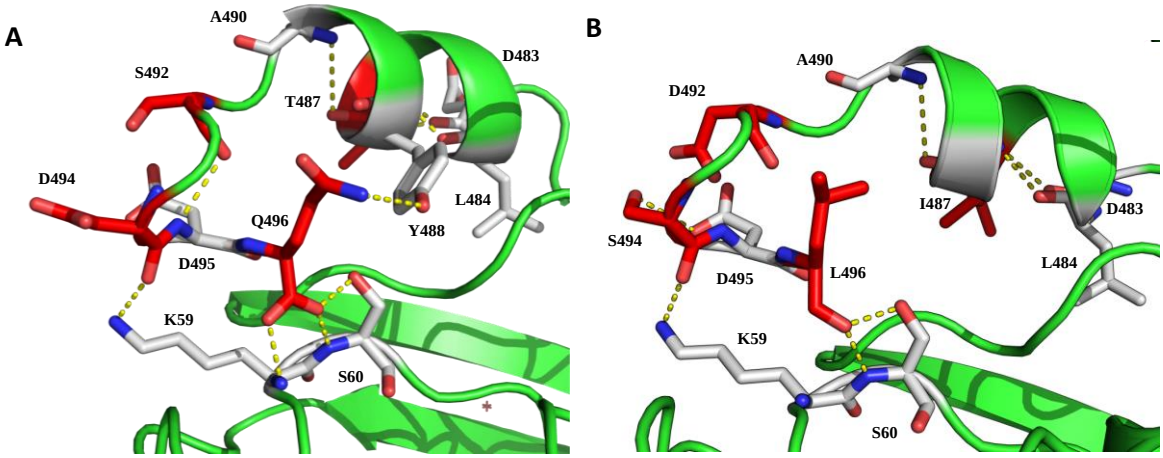

Figure 4. Detail of the C-terminal tail in parent laccase 7D5 (**A**) and final variant RY2 engineered in this study (**B**). Mutated residues are shown as red-carbon sticks and residues with which they establish polar contacts are depicted as white-carbon sticks. Based on PDB entry 6H5Y.

Laccase production was significantly raised through 7D5 engineering pathway. To the major contribution of mutations Aα20T and Qα32H (in the α pre-proleader), which upgraded secretion in flask cultures from 3 mg/l of 7D5 parent to 16 mg/l of EM and DM variants, the added effect of mutation F454P further raised enzyme production up to 25 mg/l in PK2 and RY2 variants. This production rate is, as far as we know, the highest ever reported for the heterologous expression of basidiomycete laccases in *S. cerevisiae* (Kunamneni *et al*., 2008; Mate *et al*., 2013). The yeast offers remarkably advantages as host for the engineering of eukaryotic enzymes (Gonzalez-Perez *et al*., 2012), but the expression yields are noticeably poor. Thanks to elevated cell densities under the control of strong promoters, *P. pastoris* offers superior expression yields and is frequently used as expression system for the production of fungal enzymes (Hartner *et al*., 2008; Kunamneni *et al*., 2008; Alessandra *et al*., 2010), including up-scaling of those engineered in *S. cerevisiae* (Mate *et al*., 2013). By contrast, the protein yields obtained here in *S. cerevisiae* are similar or even greater than some reported for *P. pastoris* (Otterbein *et al*., 2000; Soden *et al*., 2002; Hartner *et al*., 2008; Mate *et al*., 2013). Moreover, 7D5 laccase and evolved variants can be overproduced at relevant scale in an industrial strain of *A. oryzae* (De Salas *et al*., 2016).

In total 9 new mutations were selected during the 7D5 engineering pathway: N207S, N263D, F454P, T487I, S492D, D494S and Q496L in the mature laccase sequence (Fig. 5), together with Aα20T and Qα32H in the signal peptide. Altogether, these mutations remarkably boost the activity, stability and production of the enzyme by *S. cerevisiae* and, consequently, its biotechnological value as biocatalyst (see sections below).

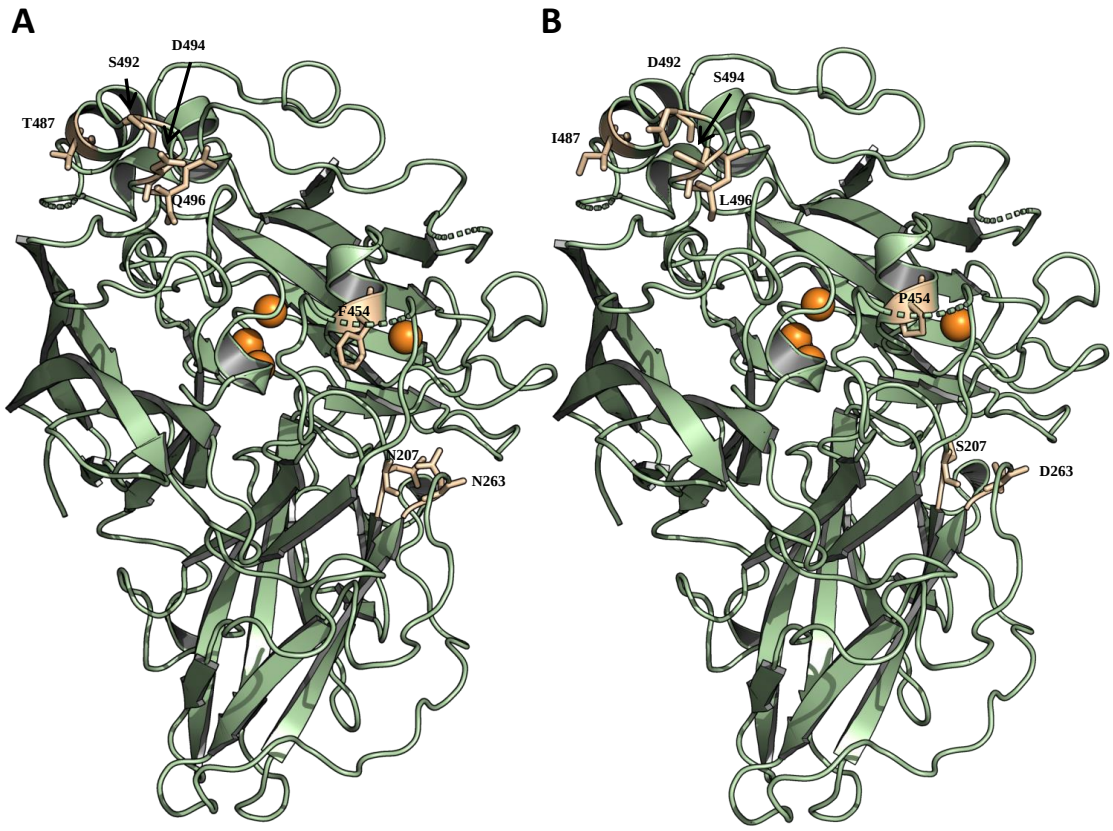

Figure 5. Cartoon representation of the 3D-structures of the parent laccase 7D5 (**A**) and the final variant RY2 engineered in this study (**B**) showing the four copper ions as orange spheres and the residues mutated during the evolution pathway as wheat-colored sticks. Based on PDB entry 6H5Y.

Characterization of the engineered variants

The final laccase RY2, together with PK2, DM and EM variants, were produced in *S. cerevisiae*, purified to homogeneity, and characterized. EM was used as reference of the parent type, because it has the same mature sequence as 7D5 but it is 5-fold better produced (due to the two mutations of the signal peptide). According to the NetNGlyc 1.0 server the parent laccase and the engineered variants hold two conserved N-glycosylation sites, N54 and N433 (Christensen and Kepp, 2013; Orlikowska *et al*., 2018). SDS-PAGE of purified enzymes before and after Endo-H deglycosylation showed around a 10 % N-glycosylation for all variants (Fig. S3).

However, some hyperglycosylation of RY2 was evidenced by a faint smear at 150-100 kDa that disappeared after treatment with Endo-H, resulting in a MW for deglycosylated RY2 (55187 Da) similar to that of deglycosylated EM (54658 Da) according to MALDI/TOF-TOF analysis.

Catalytic activity

Laccase activity was evaluated with different substrates: aniline, DMPD and ABTS (used during the screening of the mutant libraries), and 2,6-dimethoxyphenol -DMP- (a phenolic substrate not targeted during the engineering of the enzyme).

Optimal pH for the oxidation of ABTS, aniline and DMP by DM, PK2 and RY2 variants were compared with those of the parent type (EM) (Fig. S4 A-C, respectively). The shift in maximum absorbance of Würsten dye with pH precluded the use of DMPD in this comparison. In general, the parent type showed a more acidic profile than the rest. Nevertheless, all variants maintained the maximum ABTS activity at the lowest pH, with some increment of activity at pH 3-5 for the engineering variants (Fig. S4A). The optimal pH with aniline was also shifted from 4 to 5 by mutations N263D and N207S first introduced in DM, although the activity at pH 3 was maintained (Fig. S4B). Changes in pH profile were more pronounced with DMP, with a clear shift of the optimum pH from 4 to 5 through enzyme evolution. Mutations of DM notably raised laccase activity at pH 5, and mutation F454P, first selected in PK2, further shifted and narrowed the pH profile, with a clear maximum at pH 5 (Fig. S4C). By contrast, mutations of C-terminal tail did not modify the pH profile for DMP of RY2 respecting PK2 variant.

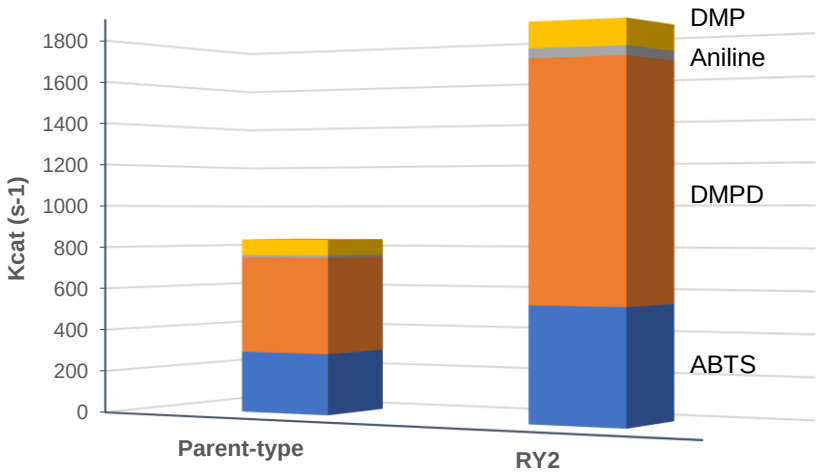

Figure 6. Enhancement of the catalytic activity (-k_{cat}- for the different substrates) from the parent laccase (EM) to the last engineered variant (RY2).

The final variant RY2 displayed improved catalytic constants with all the substrates tested as compared with the parent type (and also with the other variants) (Fig. 6, Table S1). The oxidation rate (k_{cat} values) of aromatic amines was

3-5 fold enhanced, and around 2 fold for ABTS. Taking into account the shift in optimal pH for DMP through the engineering pathway, reactions were carried out at pH 4 (parent type's optimum) and pH 5 (optimum for PK2 and RY2). RY2 maintained the oxidation rate of the parent type with DMP, which widens its applicability as biocatalyst. In general, K_m was not improved during the enzyme engineering (the use of saturation concentrations of substrate during the screenings is likely to contribute to this). However, this is not crucial for the industrial application of biocatalysts taking into account substrate is added in excess. Finally, none of the modifications observed in the kinetic constants were related to changes in the laccase's redox potential, given the equal E^0 of the T1 copper (0.76 ± 0.01 V *vs* NHE) of the parent laccase and DM and PK2 variants (RY2's redox potential is supposed to be also unaltered due to the distal location of C-terminal mutations respecting the T1 site).

The active-site mutations of DM and the C-terminal mutations of RY2 made the most important contributions to expand the oxidation of aromatic amines (pH 3) by laccase. Surprisingly, we found no progress in the k_{cat} values of PK2 variant with respect to DM for DMPD and aniline (or ABTS), although we had observed important improvement on laccase activity during the screening of the saturation mutagenesis library (on Phe454 of DM) and also during the comparison of the selected 454-mutated variants produced in flask cultures (Fig. 3)

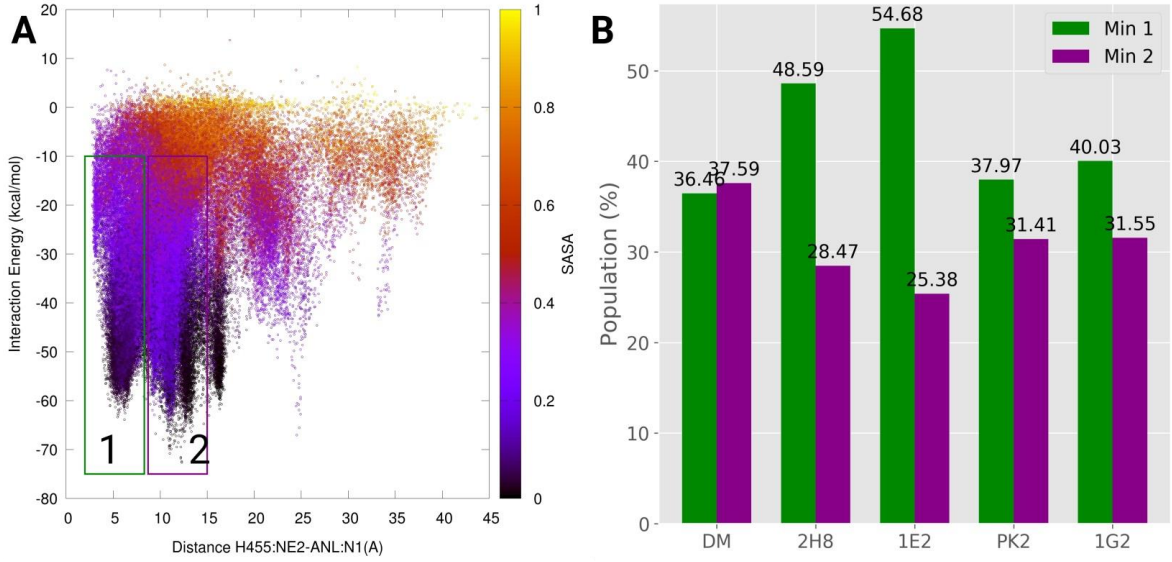

Figure 7. Analysis of minima during PELE diffusion of aniline on the 454-laccase variants. Interaction energy vs distance between aniline N1 and His455 NE2 atoms in laccase DM. Region 1 includes 0-8.5 Å distances (catalytic and close to catalytic poses) while region 2 includes poses at 8.5-15 Å distances. The two regions are delimited by interaction energies between -10 and -80 kcal/mol. Similar minima were obtained for the 2H8 (F454S), 1E2 (F454T), PK2 (F454P) and 1G2 (F454H) variants (**A**). Percentage population of each region in the number of simulation frames for laccase DM and the above variants (**B**).

To rationalize the molecular mechanism behind the activity of PK2 and the other 454-mutated variants, we used PELE to study the substrate migration and binding. Substrate positioning has been previously shown to correlate with increases in activity of laccases (Monza *et al*., 2015). From PELE simulations we extracted a profile of the ligand binding energy with respect to the distance between the N1 atom of aniline and the NE2 atom of His455, which we will refer as N-N distance (Fig. 7A). This shows the profile for DM simulation, but those from the 454 mutations show a remarkable similarity in terms of minima values and topology. The profiles for the five laccase variants feature two main minima (marked as 1 and 2 in Fig. 7A). The first one consists of those poses with a low N-N distance that correspond to catalytic-like conformation, with a peak in interaction energy of -60 kcal/mol. The second minimum, a non-catalytic one, is located at approximately 12 Å of N-N distance and a peak in interaction energy of -70 kcal/mol. While the shape and energy values of these regions are very similar for both DM and the 454-mutated variants, the population of each minimum, measured as the number of simulation frames included in each region, is different from DM to the mutated systems. In Fig. 7B, the percentage of the simulation frames located in regions 1 and 2 is shown for all the enzyme variants. For DM, the two percentages are almost exactly equal, with a difference of 1%. This ratio is shifted in the 454-mutated variants, favoring the catalytic minimum with varying values. The notable shift in population for 2H8 (F454S) and 1E2 (F454T) variants indicates an important increase in catalytic conformations. By contrast, these mutations are the most deleterious for enzyme stability (as shown in Fig. 3B and D). The poor increment in population in the catalytic minima for PK2 correlates with the null progress of their k_{cat} values respecting DM, but it does not correlate with the activity improvement observed with crude enzymes (Fig 3A) These differences are explained by the effect that mutations on Phe454 may exert on laccase production (together with laccase activity). In fact, laccase yields rose from 16 mg/l in DM to 25 mg/l in PK2 (F454P), thus explaining the selection of PK2 during the HTS.

Enzyme stability

The stability to pH 2-9 of the final variant RY2 was compared with those of the parent type (EM) and former variant PK2 (Fig. 8, purified enzymes). The loss of stability at basic pH observed in PK2 was recovered in RY2 variant to values even higher than those of the parent type. Besides, the final variant showed superior stability at pH 3.

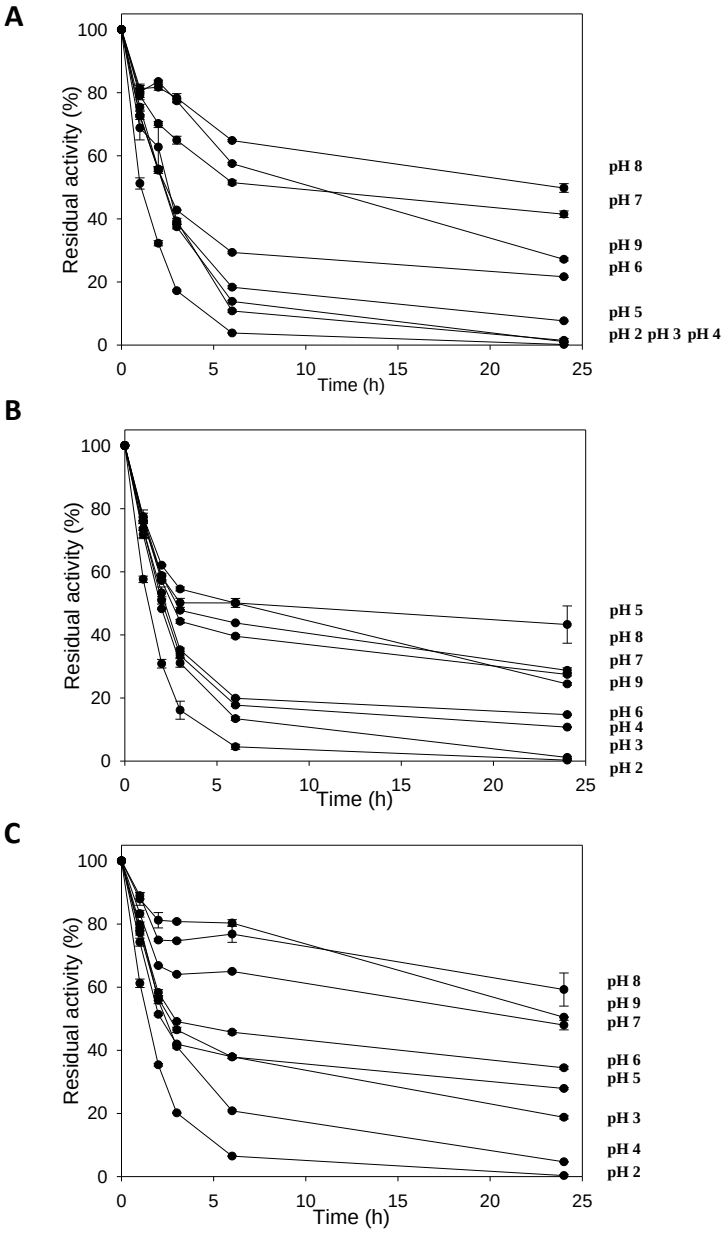

Figure 8. Residual activities of parent type EM (**A**), and PK2 (**B**) and RY2 (**C**) variants during 24 h incubation at pH 2-9. Activities of the purified enzymes at different incubation times (triplicates measured with ABTS, pH 3) are depicted as percentages of the initial activity at pH 3.

Table 1. Half-lives, thermal inactivation constants and E_a of purified parent type (EM) and DM, PK2 and RY2 variants at different temperatures.

T (°C)	EM			DM			PK2			RY2		
	$t_{1/2}$ (h)	*k*d (h^{-1})	E_a (kJ/mol)	$t_{1/2}$ (h)	*k*d (h^{-1})	E_a (kJ/mol)	$t_{1/2}$ (h)	*k*d (h^{-1})	E_a (kJ/mol)	$t_{1/2}$ (h)	*k*d (h^{-1})	E_a (kJ/mol)
50 °C	13.05	0.05	201.1	19.25	0.04	209.8	13.59	0.05	204.3	22.43	0.03	162.1
60 °C	2.88	0.24		3.04	0.23		2.01	0.34		2.03	0.34	
70 °C	0.29	2.43		0.31	2.23		0.18	3.76		0.36	1.93	
80 °C	0.02	29.60		0.03	27.35		0.02	30.56		0.04	19.30	

The engineering of laccase C-terminal tail also produced a noteworthy increment of T_{50} (10 min) in RY2 variant with T_{50} = 73 °C, whereas the rest of purified variants (EM, DM and PK2) displayed T_{50} values around 65 °C (Fig. 9). Thermal inactivation assays at 50-80 ºC for the four variants showed slightly improvement of half-lives from EM to DM that were decreased in PK2 variant (Table 1). Thereafter, the mutations introduced in PK2 C-terminal tail seemed to stabilize the enzyme, increasing the half-lives and lowering the inactivation constants in RY2 variant. The significantly lower activation energy (E_a), calculated from the slope of Arrhenius plot, for the last evolved variant, indicates a lesser sensitivity to temperature changes as compared with the parental and the intermediate variants (Table 1).

We evaluated the possible contribution of protein hiperglycosylation to enhanced thermostability in RY2 variant by comparing the stability at 65 °C of the glycosylated and deglycosylated forms of this variant with those from the glycosylated and deglycosylated forms of the parent type (EM) (Fig. S5). The deglycosylated form of RY2 was slightly less stable than the glycosylated form, exactly the same as observed in the parent type (which is not hyperglycosylated), thus evidencing hyperglycosylation is not the main responsible for RY2's thermostability.

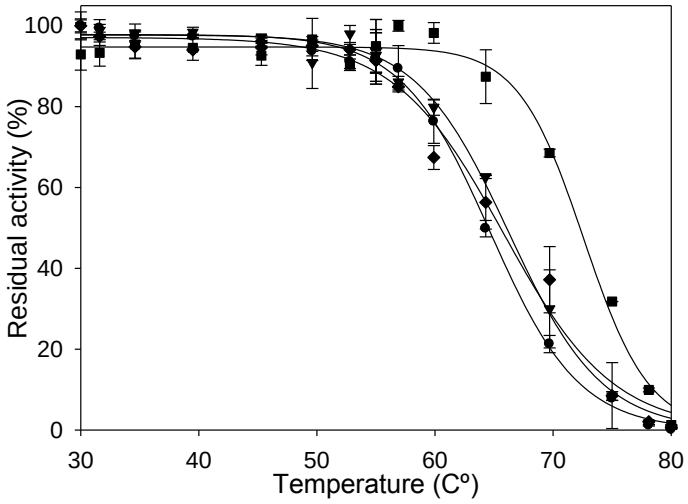

Figure 9. T_{50} (10 min) curves of purified parent type EM (circles), and DM (triangles), PK2 (diamonds) and RY2 (squares) variants.

The effect of temperature on laccase structure was analyzed by circular dichroism (CD), by monitoring the changes in the ellipticity spectrum of the protein that are produced by perturbations in the secondary structures (Chattopadhyay and Mazumdar, 2000). First, a ramp temperature between 50 and 95 °C was recorded at 220 nm (characteristic band of α-helices) for RY2 and PK2 variants and parent type (EM). No loss of secondary structures could be observed and consequently no apparent Tm could be calculated for any of the laccase variants assayed. Therefore, we incubated the enzymes at 100 °C during 24 h and measured the changes in their far UV CD spectra at different times (Fig. 10). The initial spectra presented two dichroic bands, a strong minimum at 216 nm and a maximum around 196 nm,

typical of antiparallel β-sheet proteins (Greenfield, 2006). The conformation of the parent type (EM) was maintained during the first 2h of incubation at 100 °C. Thereafter, the gradual loss of ellipticity revealed the loss of secondary structures until total denaturalization of the enzyme after 24 h at 100 °C. The drop in ellipticity observed in PK2 CD spectrum after 2 h at 100 °C and its total denaturalization after 5 h, denotes the significantly diminished structural stability of this variant. On the contrary, an important recovery of stability was observed in the final variant, RY2 whose ellipticity CD spectrum was slightly modified during the first 8 h of incubation at 100 °C (Fig. 10C). These results suggest that the four mutations of RY2 C-terminal tail contribute to the structural stabilization of the enzyme hindering the prompt denaturalization of the protein.

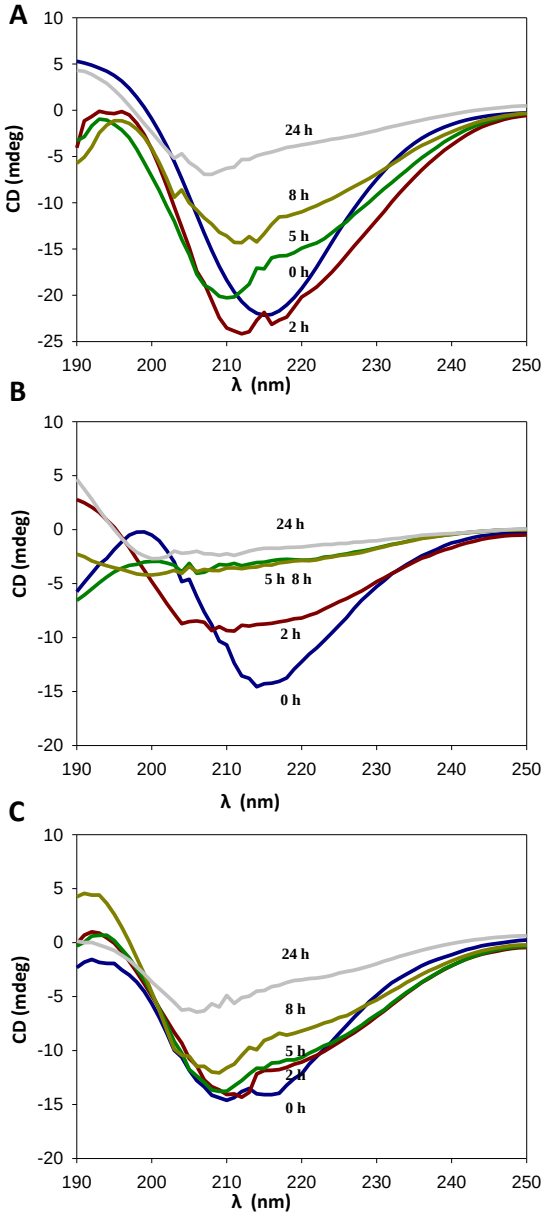

Figure 10. CD spectra for the thermal denaturation assay of parent type EM (**A**), and PK2 (**B**) and RY2 (**C**) variants after different incubation times at 100 °C.

To understand the reason for this stability improvement, we run MD simulations using OpenMM as the MD engine and Amber ff14SB for proteins. Using these simulations we analyzed the flexibility of the C-terminal residues (defined as the residues between Ala480 and Gln496, or Leu496 in the mutated variant) by calculating the root-mean square fluctuation (RMSF) of those residues in PK2 and RY2 variants, at 27 °C or 67 °C (Fig 11).

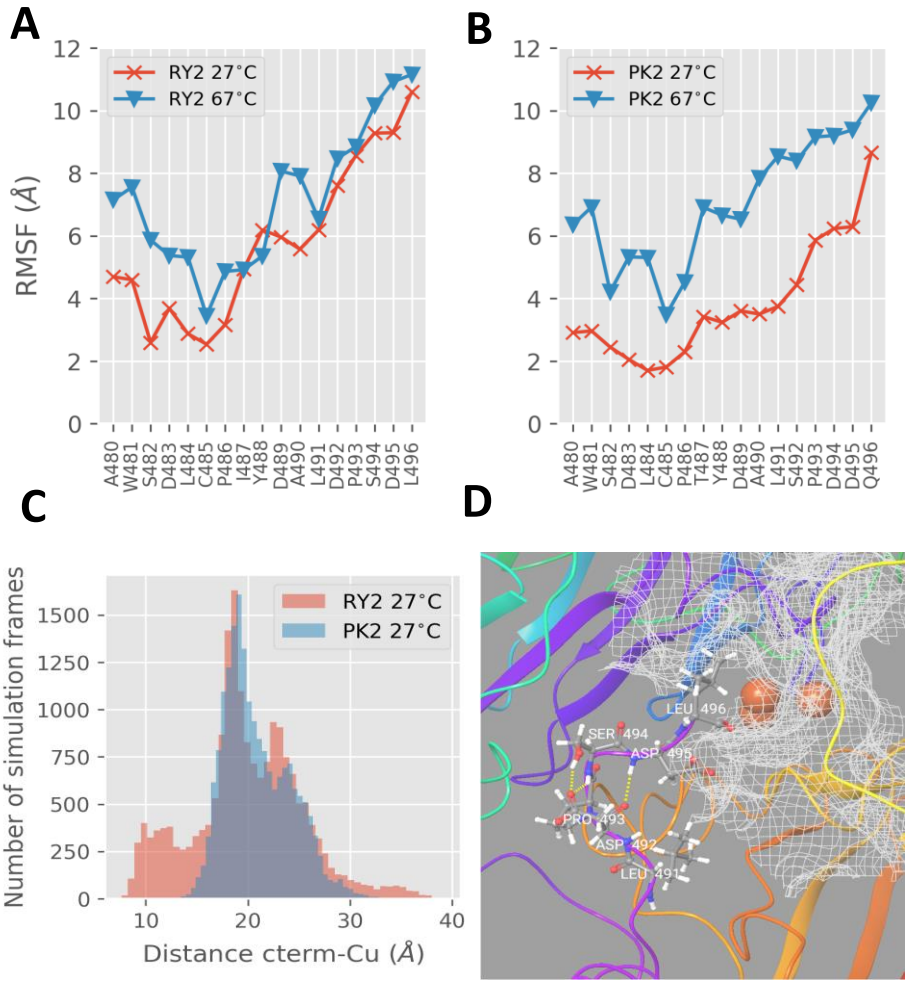

Figure 11. RMSF per residue of the C-terminal region, defined as the last 16 residues, from Ala480 to Gln496 (Leu496 in the mutated variant). The RMSF values are shown for each variant at temperatures of 27 and 67 °C. (**A**) RMSF for RY2 variant (**B**) RMSF for PK2 variant. Histogram of the minimum distance between residues 494, 495 or 496 to the TNC for PK2 and RY2 at 27 °C (**C**). Snapshot showing the closer position of the C-terminal tail for RY2 system, copper ions are shown in VDW representation, the TNC channel is shown in a white mesh surface and C-terminal residues are shown in ball-and-stick representation and labeled (**D**).

To calculate the RMSF values we: i) discarded the first 100 ns of each trajectory, avoiding possible biases imposed by the initial structures; and ii) used the average

structure as a representative reference. The RMSF plot of RY2 variant showed significantly larger mobility of C-terminal residues at 27 °C, which did not increase too much when moving to 67 °C. PK2, however, shows a large increase in mobility when increasing the temperature. The large increase in RY2's mobility at 27 °C seems to originate the loosening of the secondary structure of the C-terminal tail, most likely as a result of the Thr487 mutation. When monitoring the three hydrogen bonds of this short alpha helix: Ala480-Leu484, Asp483-Thr487 (Ile487 in the RY2 variant) and Pro486-Ala490, the original PK2 variant maintains the three H bonds 24.1% of the time, while for RY2 species it only happens 3.7 % of the simulation time. In addition, the mutations of the C-terminal entail the loss of H bonds with neighbor residues (Fig. 4), contributing to increase the mobility of the region. Such an increase in mobility in flexible regions has recently been observed when analyzing other thermostable variants (Plana *et al*., 2019). Thus, higher flexibility of the C-terminal region in RY2 helps in neutralizing the destabilization caused by the larger thermal fluctuations at higher temperatures, which could allow the rest of the protein to maintain the native structure and remain active. The effect of C-terminal on the enzymatic thermal stability was described in 1985 by Arnold and collaborators (Giver *et al*., 1998). In this study, the thermostability of an esterase was enhanced due to six different mutations clustered in the C-terminal region of the enzyme.

As regards the increment of activity showed by RY2 towards all the substrates assayed, the analysis of the MD trajectories reveal that the new C-terminal region directly interacts with the entrance of the water channel that forms the access route to the type-3 copper sites in the TNC (Fig. 11C) (Piontek *et al*., 2002; Sekretaryova *et al*., 2019). In particular, we observed how the hydrophobic substitution at position 496 partially occupies the channel (Fig. 11D), which significantly modifies its hydrophobicity and could affect O_2/H_2O traffic (Sekretaryova *et al*., 2019). In fact, we observed a better interaction between the mutated C-terminal and Asp 457 (Fig S6). Mutation D494S changes the charge of the distal region of the C-terminal from negative to neutral, which might lead to modifications in the electrostatic environment of TNC. A more positive charge of TNC environment has been recently described to increase the redox potential of the catalytic native intermediate (NI), strongly influencing the overall laccase activity (Sekretaryova *et al*., 2019).

The relationship of the C-terminal tail with the enzyme kinetic behavior has been observed in other fungal laccases. Ascomycete laccases are characterized by elongated C-terminal tails, which block the TNC tunnel if they are not correctly processed, leading to dramatic changes in the catalytic behavior of these enzymes (Hakulinen *et al*., 2002; Andberg *et al*., 2009). On the other hand, substitution of the 11 amino acids of the C-terminal region of *T. versicolor* basidiomycete laccase with a single cysteine residue significantly reduces the redox potential of the copper T1 (Gelo-Pujic *et al*., 1999). While, the last 18 amino acids in the C-terminal

tail of other basidiomycete laccase from *P. eryngii* seem to play a critical role in the activity, stability and kinetics of the enzyme (Bleve *et al*., 2013). Although the above laccases hold elongated C-terminal tails that lie closer to the entrance of TNC than in our case, mutations added to PK2 C-terminal tail make it more flexible and may entail a displacement that could improve the access to the TNC channel (Autore *et al*., 2009; Hu *et al*., 2014). This hypothesis will be verify when the RY2 variant is crystallized, taking advantage of the high production yields obtained in yeast.

Application case studies

Crude (unpurified) variants of laccase 7D5 developed and produced in *S. cerevisiae* were tested for the synthesis of green polyaniline and compared with the parent laccase using described conditions (Fig 12A) (De Salas *et al*., 2016). The synthesis of the polymer was monitored by the increase of absorbance at 800 nm, typical for the conductive form (Emeraldine salt) (Liu *et al*., 1999). The use of EM entailed an important increment of polymerization (respecting the use of 7D5 laccase) due to its superior secretion by the yeast. Variant DM also raised the polymerization rate attained with EM after 8 h reaction due to the better oxidation of aniline. Finally, PK2 and RY2 variants slightly increased the polymerization rates obtained with DM.

In a next step, the last variant engineered towards aniline oxidation, that is PK2, was expressed in the industrially relevant host *A. oryzae* (Novozymes), under the same conditions used for the parent 7D5 laccase (De Salas *et al*., 2016). The purified engineered variant expressed in *A. oryzae* augmented the PANI yields from 75 % to 87 % after 24 h of polymerization reaction.

Aniline is an important precursor for the synthesis of different dyestuff as indo dyes, aniline black (pernigraline), mauveine or aniline blue (Michaelson, 1993; O'Neill *et al*., 2000; Sousa *et al*., 2016). Then, performance as biocatalyst of the enzyme overproduced in *A. oryzae* for the synthesis of polyaniline and a naphtol-derived compound as dyes was assessed at relevant industrial conditions at SETAŞ A.S (Turkey), a leader company in chemistry and industrial color processes. The textile dyeing capacities of the resulting colorants were evaluated using standard industrial tests. The type of dye, the reproducibility of the color and color depth, exhaustion of the bath at the end of the dyeing process and fastness properties of the new dyes were determined.

According to the multifiber dyeing test, the enzymatically synthesized polyaniline produced diverse color strengths on different fabrics. The best dyeing efficiency was obtained on acrylic, nylon and acetate fabrics (Fig. 12B). A subsequent fiber dyeing test on acrylic fiber demonstrated the excellent dyeing efficiency of the enzymatic synthesized PANI, with high exhaustion of the dyeing bath and strong color fastness to light (Fig. S7A).

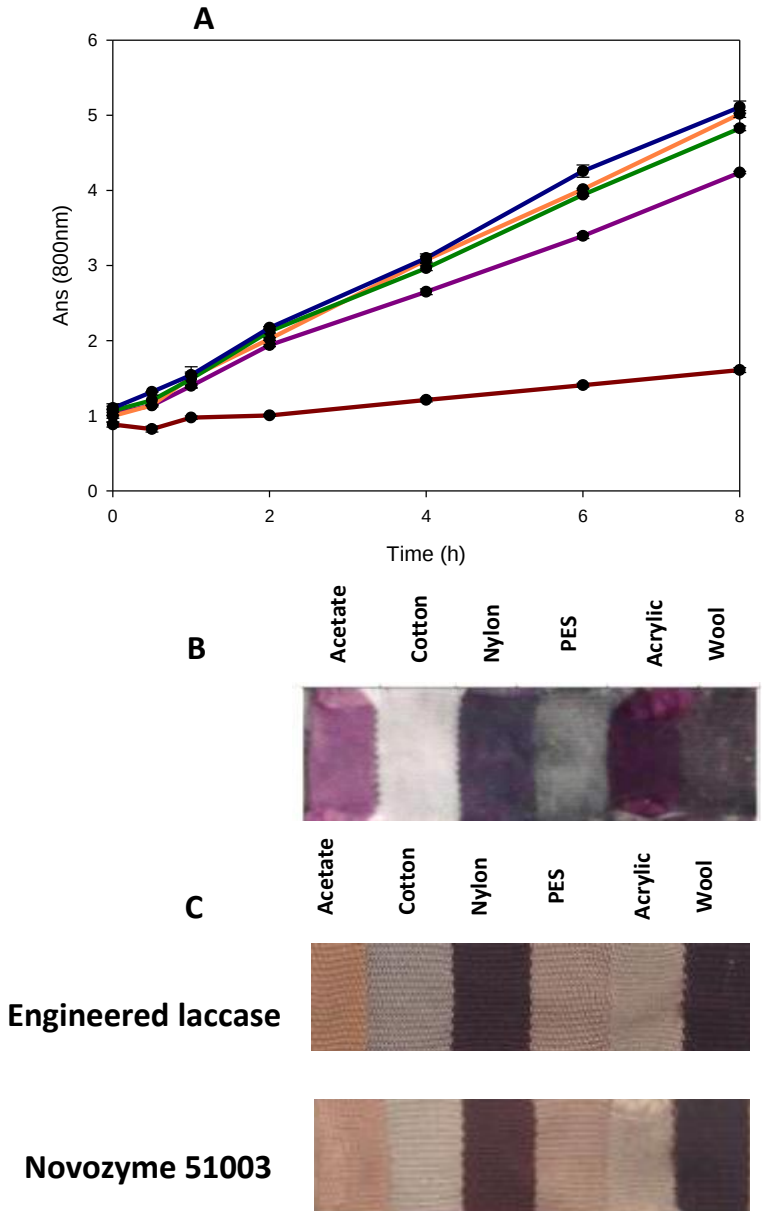

Figure 12. Polymerization of 15 mM aniline by 0.594 mg/ml of 7D5 (red), EM (purple), DM (orange), PK2 (green) and RY2 (blue) crude enzymes in the presence of 5 mM SDBS as template (**A).** Multifiber test from PANI synthesized with the engineered laccase variant (**B**), and multifiber test of the acid dye synthesized by the engineered laccase variant and Novozyme 51003 laccase with 1-naphthol as precursor (**C**).

As regards the synthesis of naphtol-derived dye, the performance of the engineered laccase variant was compared with that of the commercial laccase Novozyme 51003. The new dye is in the yellow scale of the spectra (maximum absorbance at 470 nm) and it's defined as acid dye by its dyeing properties. The new acid dye obtained with both laccases rendered an intense dye fixation for wool and nylon fabrics in the multifiber test (Fig 12C). Due to the color shade similarity with the new dye, Nyloset Brown N2R from SETAŞ dye range was used

as reference to compare the dyeing efficiency of the new colorant. The exhaustion of the bath at the end of the second dyeing process of nylon fibers with the dye synthesized with the engineered laccase showed a 55% STR-SUM, which indicates lower dye efficiency compared with PANI (Fig. S7B). The color fastness to light was also lower than the observed for polyaniline. However, the dye efficiency of the new acid dye was higher when it was synthesized by the engineered enzyme than by the commercial laccase, and very close to the dye efficiency of Nyloset Brown N2R (Fig. S7C). Also, the color strengths of the new dye and the Nyloset Brown N2R are comparable, even when the new dye are not concentrated enough. It is worth mentioning that Nyloset Brown N2R synthesis requires very acid pH (<1) and very toxic reagents as $NaNO_2$ (Mao and Tang, 2010). By contrast, we demonstrate here the enzymatic synthesis of similar performing dyes using notably milder conditions.

Conclusions

The intrinsic low catalytic requirements, broad substrate promiscuity, high redox potential and stability of certain basidiomycete laccases make them excellent candidates to develop new tailor-made biocatalysts for different oxidation reactions. However, their difficult heterologous expression constitutes a main bottleneck first for engineering and then for application. So far, they are not actively expressed in *E. coli* and the enzyme yields rendered by *S. cerevisiae*, our preferred host for laccase engineering, are very low. By contrast, the variants engineered here are produced by the yeast at remarkable levels and can be overexpressed by *A. oryzae* at relevant industrial scale. This allowed us to prove their excellent properties as biocatalysts for feasible synthesis of high-performing organic dyes without adding any redox mediators. The viable application of the engineered laccases in an industrial environment to replace toxic chemical catalysts and harsh industrial conditions by milder ones is an important advance towards the development of green chemistry industrial processes.

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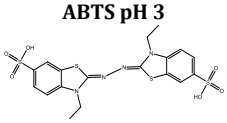
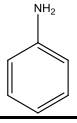
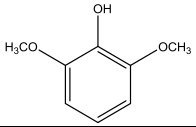
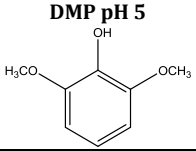
Supplementary material

Primers used for the engineering of 7D5 laccase.

Primer Name	Sense/Antisense	Sequence 5´→3´
RMLN	Sense	CCTCTATACTTTAACGTCAAGG
RMLC	Antisense	GGGAGGGCGTGAATGTAAGC
pJro30 9H2 F	Sense	TTCCTCCACTGCCACATTGA**TTC**CACCTTGACGCAGGCTT
pJro30 9H2 R	Antisense	AAAGCCTGCGTCAAGGTG**GAA**GTCAATGTGGCAGTGGAGGAA
454DFw	Sense	CACATTGAC**NNK**CACCTTGACG
454DRv	Antisense	CGTCAAGGTG**MNN**GTCAATGTG
C-terminal3A4Fw	Sense	GCAAGCATGGTCGGATCTGTGCCCG
C-terminal3A4Rv	Antisense	CGGGCACAGATCCGACCATGCTTGC

Results

Table S1. Kinetic constants for the oxidation of different substrates by the variants obtained during the evolution of 7D5 laccase. (* Data from Santiago *et al* 2016).

		EM	DM*	PK2	RY2
ABTS pH 3 	k_{cat} (s^{-1})	291 ± 18	570 ± 26	454 ± 13	543 ± 17
	K_m (mM)	0.004 ± 0.001	0.01 ± 0.002	0.016 ± 0.002	0.01 ± 0.001
	k_{cat} /K_m (mM^{-1} s^{-1})	69254	40975	28350	52240
DMPD pH 3 	k_{cat} (s^{-1})	459 ± 18	741 ± 48	688 ± 36	1126 ± 47
	K_m (mM)	1.72 ± 0.17	1.2 ± 0.2	3.83 ± 0.46	4.59 ± 0.53
	k_{cat} / K_m (mM^{-1} s^{-1})	265	617	179	245
Aniline pH 3 	k_{cat} (s^{-1})	10 ± 1.1	23 ± 3.4	24 ± 0.6	44.6 ± 0.6
	K_m (mM)	28 ± 7.2	59 ± 16	27 ± 0.7	118 ± 1.8
	k_{cat} / K_m (mM^{-1} s^{-1})	0.36	0.38	0.88	0.37
DMP pH 4 	k_{cat} (s^{-1})	105 ± 3.2	-	54 ± 2.8	83 ± 1.4
	K_m (mM)	0.1 ± 0.01	-	1.52 ± 0.24	2.74 ± 0.15
	k_{cat} / K_m (mM^{-1} s^{-1})	1095	-	35	61
DMP pH 5 	k_{cat} (s^{-1})	76 ± 2	-	74 ± 6	123 ± 6
	K_m (mM)	0.05 ± 0.004	-	0.36 ± 0.09	0.26 ± 0.04
	k_{cat} / K_m (mM^{-1} s^{-1})	1519	-	206	479

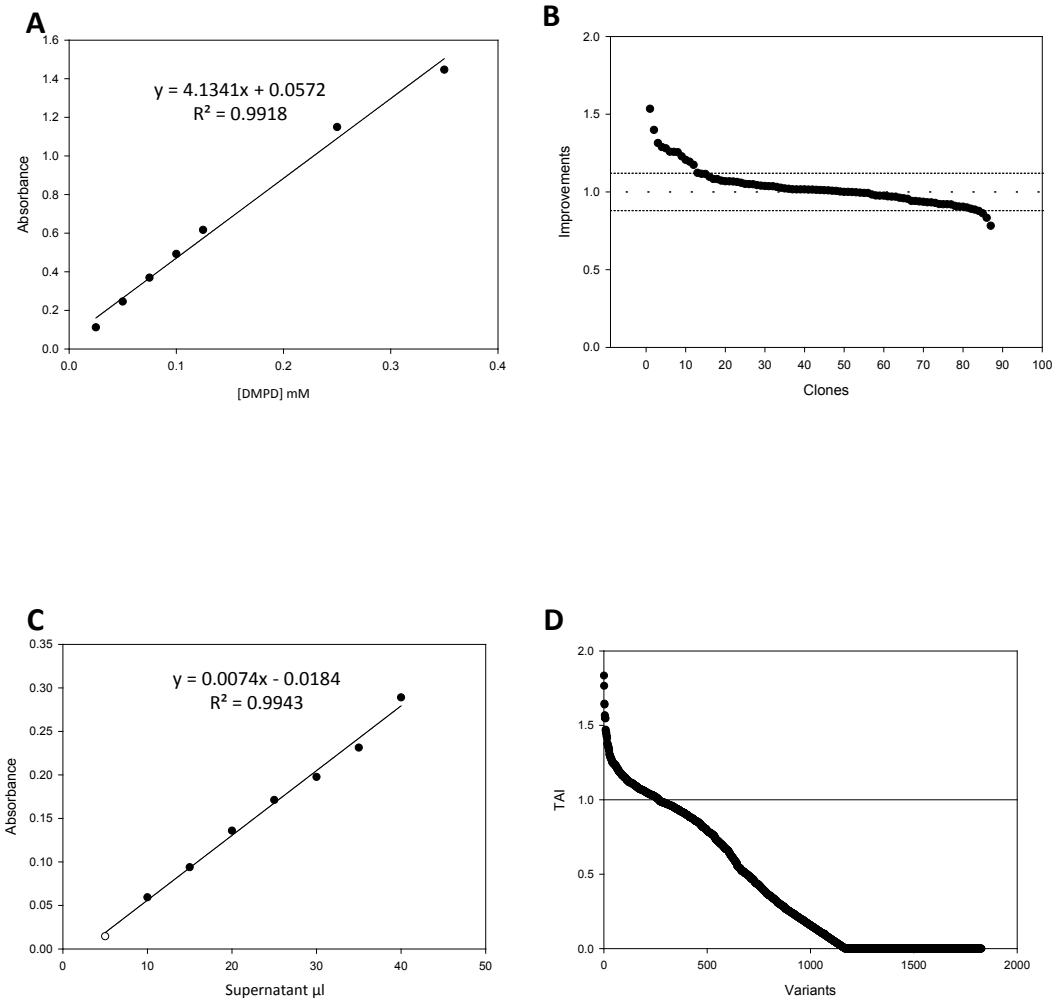


Figure S1. Extinction coefficient for oxidized DMPD -Würsten dye- (**A**), reproducibility (**B**), linearity (**C**), and sensitivity (**D**) of HTS assay with DMPD.

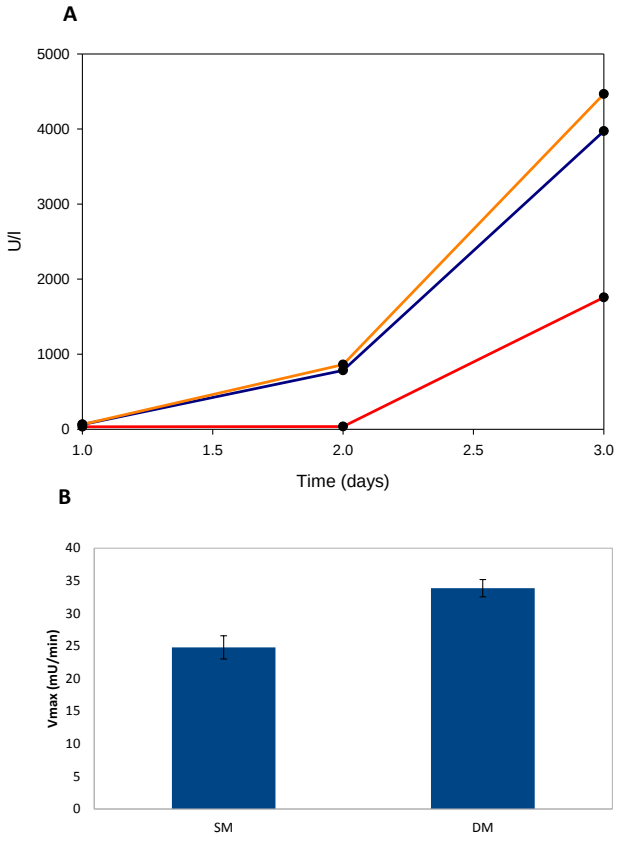


Figure S2. Laccase activity in *S. cerevisiae* flask cultures producing SM (red), EM (blue) or DM (orange) laccase variants (**A**). Oxidation of 300 mM aniline (pH 3) by equal ABTS activity units of SM and DM crude enzymes (**B**).

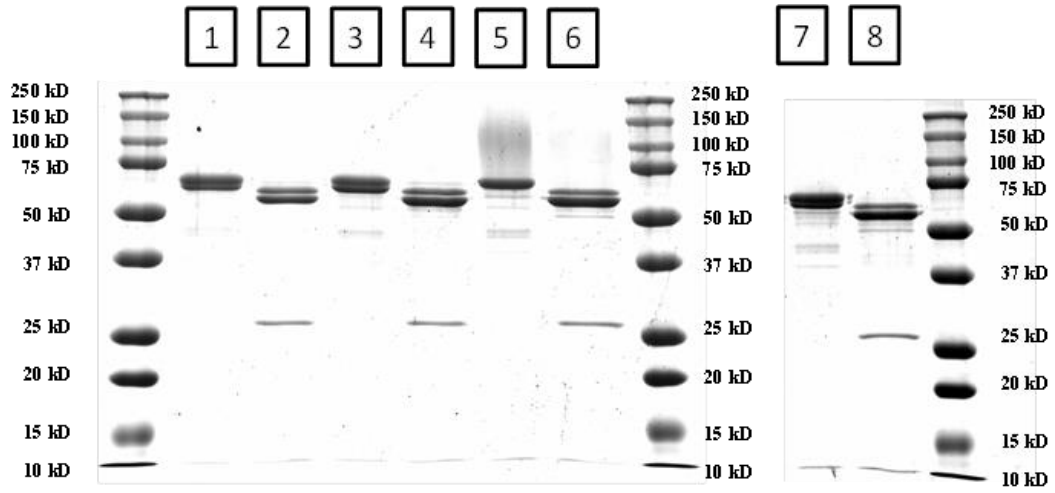


Figure S3. 12 % SDS-PAGE of glycosylated (1) and deglycosylated DM laccase (2), glycosylated (3) and deglycosylated 7D5 laccase (4), glycosylated (5) and deglycosylated RY2 laccase (6) and glycosylated (7) and deglycosylated (8) PK2 laccase. All enzymes were purified.

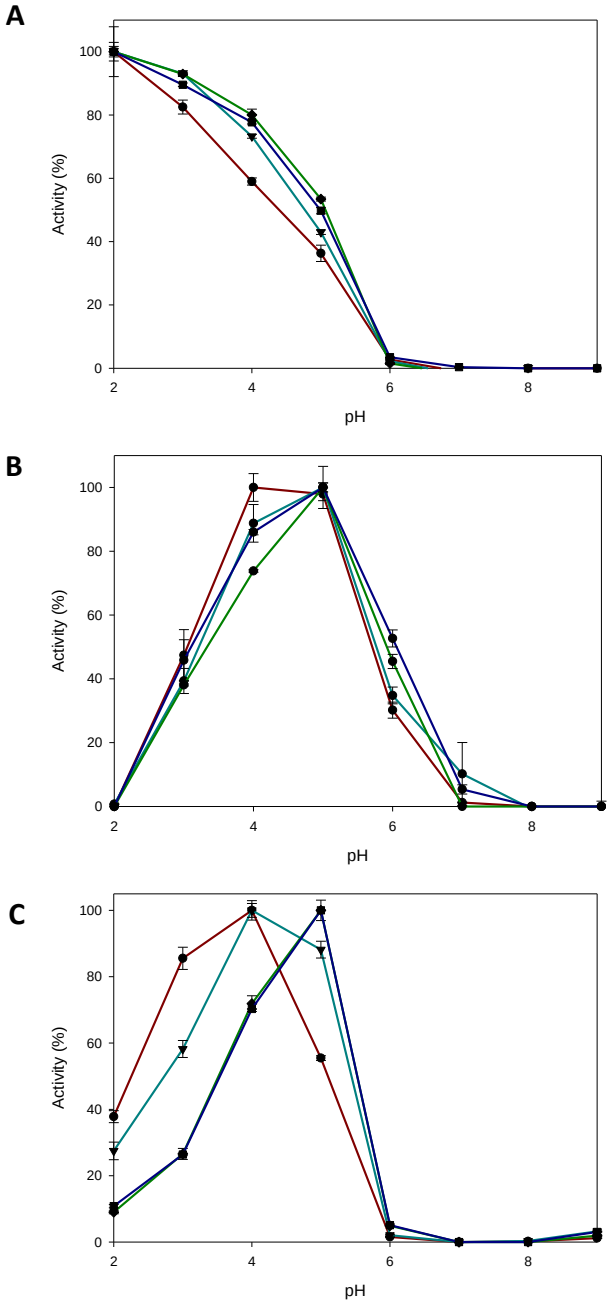


Figure S4. Optimal pH for ABTS (**A**), aniline (**B**) and DMP (**C**) oxidation by the parent type EM (red) and DM (cyan), PK2 (green) and RY2 (blue) variants (purified enzymes).

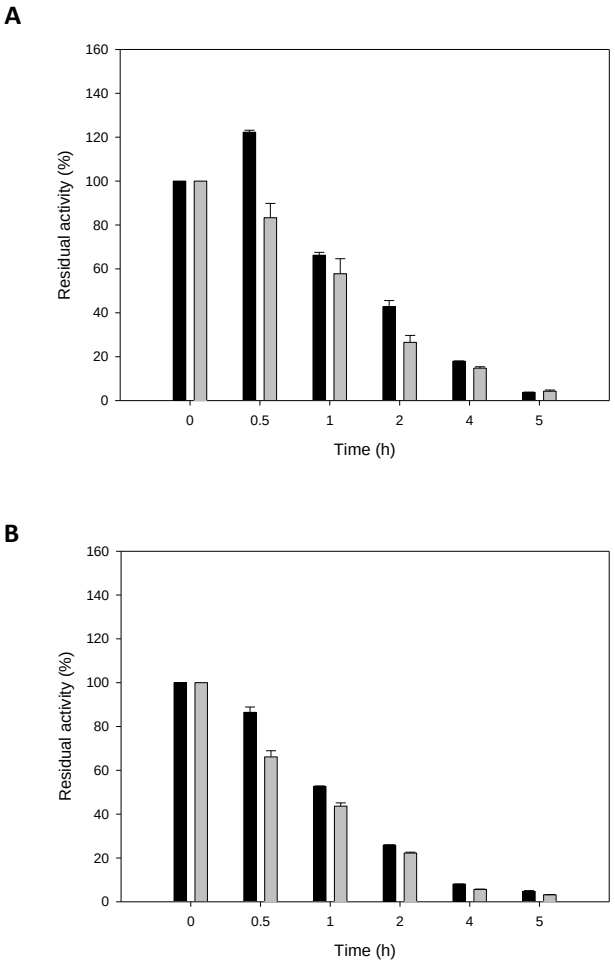


Figure S5. Residual activity of glycosylated (black bars) and Endo-H desglycosylated (grey bars) forms of purified parent type EM (**A**) and RY2 final variant (**B**) after different incubation times at 65 °C.

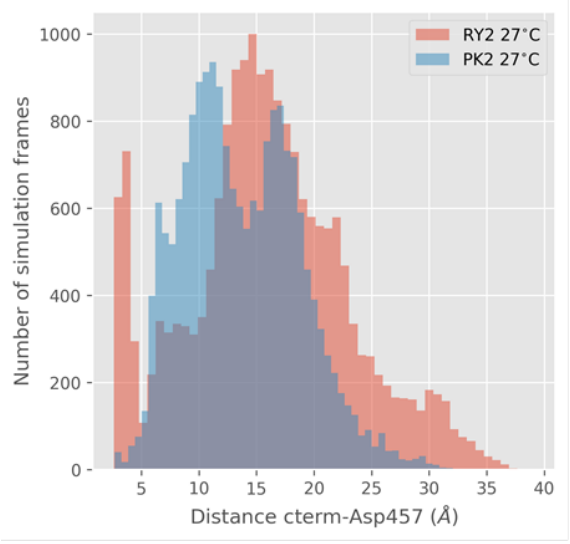


Figure S6. Histogram of the minimum distance between C-terminal residues to the Asp 457 for PK2 and RY2 variants at 27 °C.

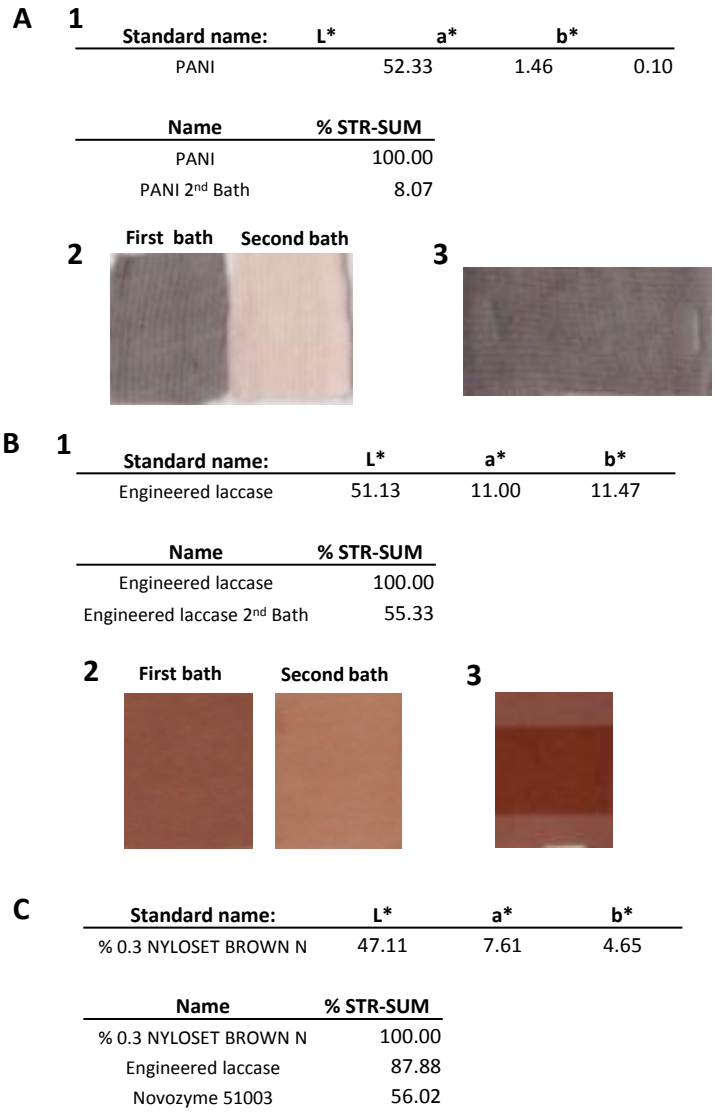


Figure S7. Dyeing tests of enzymatically synthesized PANI (**A**) and naphtol-derived acid dye (**B**). Dye properties (CIELAB color space coordinates, L*, a*, b*) and dyeing efficiency (% STR-WSUM) by comparison of first dye bath and second dye bath (**1**); Exhaustion of the bath at the end of the dyeing process (**2**); and Light fastness properties (**3**). Properties of Nyloset Brown commercial dye and comparison of dyeing efficiency (% STR-WSUM) with the acid dye obtained with the engineered or the commercial laccases (**C**).

GENERAL DISCUSSION

General discussion

The laccase used as starting point for this Doctoral Thesis, named 7D5, was obtained by homologous recombination of two high-redox potential laccases from basidiomycetes PM1 and *P. cinnabarinus* (Pardo *et al*., 2012) that had been previously evolved in the lab for their expression in *S. cerevisiae* (Mate *et al*., 2010; Camarero *et al*., 2012). The different laccase variants obtained by recombination showed remarkable variability in activity at different pH and affinity for different substrates, as well as in stability (Pardo *et al*., 2012). Among them, 7D5 laccase was selected for the synthesis of polyaniline (Chapter 1 of this Thesis) due to its superior capability to oxidize aromatic amines and higher stability to the reaction conditions. Its overexpression in *Aspergillus oryzae*, allowed to carry out a deep biochemical and structural characterization of the enzyme (Chapter 2 of this Thesis). Finally, since the high redox potential of protonated aniline (anilinium cation) and the conditions required to obtain polyaniline in its electroconductive form -emeraldine salt- (Junker *et al*., 2014) hinder the enzymatic oxidation of aniline, the engineering of the enzyme was carried out by computational design (Santiago *et al*., 2016) and directed evolution (Chapter 3 of this Thesis) to confer the laccase greater activity and stability at the reaction conditions (Fig. 1).

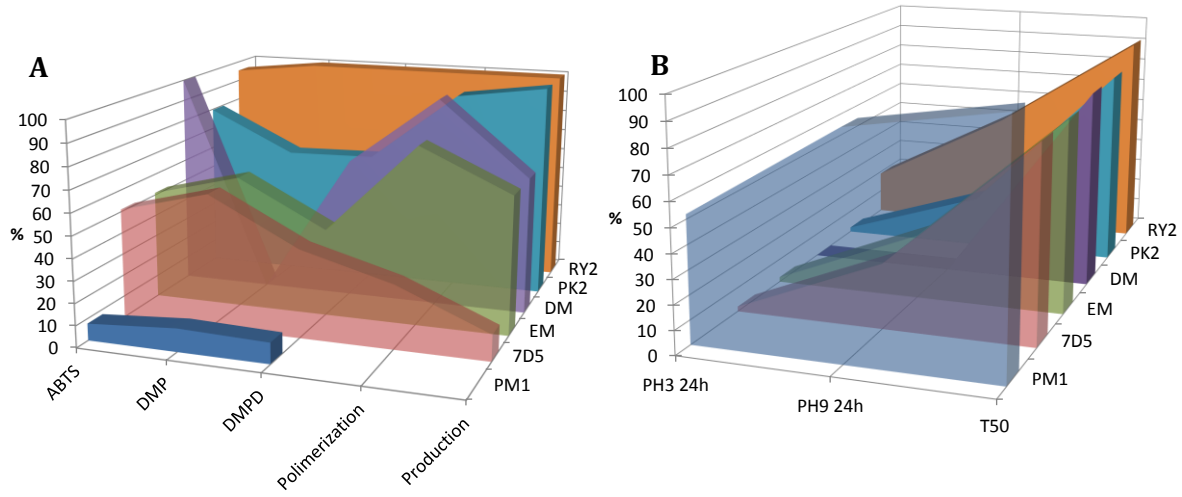


Figure 1: Summary of the properties of the wild-type laccase (PM1), the starting point for enzyme engineering (7D5 laccase) and the selected laccase variants obtained in this Thesis (EM, DM, PK2 and RY2). Activities with ABTS, DMP, and DMPD, aniline polymerization rates and enzyme production yields in *S. cerevisiae* (**A**); as well as T50 (10 min) values and residual activities after 24 h incubation at pH 3 and 8 (**B**) of the different laccase variants are shown as relative values.

1. Polyaniline synthesis by 7D5 laccase

First, 7D5 laccase was compared with other fungal laccases available in the laboratory: wild-type (from basidiomycetes PM1 and *P. cinnabarinus*), engineered

in the same directed evolution campaign (Pardo *et al*., 2012) and commercial laccases (from *Trametes villosa* and *Myceliophtora thermophila*). The activity of 7D5 laccase towards aniline was higher than any of the other laccases assayed, with a V_{max} 2 times higher than *T. villosa* laccase (the second best enzyme). The stability of 7D5 laccase to acid pH and high temperature was also superior to that of *T. villosa* laccase, which had been used as biocatalyst for polyaniline synthesis in previous studies (Junker *et al*., 2014). The enzymatic synthesis of polyaniline was then assayed with 7D5 and the optimal conditions to obtain a linear electroconductive polymer were determined.

The polymers resulting from different reaction conditions were characterized by UV/Vis and FTIR spectrophotometry, MALDI-TOF-TOF, cyclic voltammetry, and scanning electron microscopy (SEM). The results obtained from mass spectrometry analysis (which only detects polyaniline oligomers) evidenced that the concentration and type of doping template (anionic surfactant) in the reaction strongly determine the polymerization degree. The maximum polymerization yield was obtained with a 1:3 surfactant/aniline ratio. In addition, SEM analysis revealed different supramolecular structures (micro-spheres, micro-rods, and nano-fibers) depending on the concentration and type of template used, while PANI synthesized without template and commercial PANI (Sigma-Aldrich) show amorphous structures. The nano-fibers obtained with SDBS as template are of special interest because they provide larger contact surfaces, which give the possibility of extending the polymer directly into uniform films of great applicability in biological sensors. Such nano-fibered films are capable to react faster and with higher sensitivity to pH changes of the medium resulting in conductivity changes (Huang *et al*., 2003; Wang *et al*., 2014). In addition, as compared to polymers obtained with other anionic surfactants as templates, the use of SDBS rendered the highest production yields of emeraldine and with the best properties. Conversely, among the anionic surfactants assayed, SDBS hinders laccase activity the most. These type of compounds form hydrophobic and electrostatic interactions with the enzyme breaking down the secondary and tertiary structures (Zhang *et al*., 2009). Probably, the lower critical micellar concentration required for the synthesis of emeraldine with SDBS (compared to other anionic surfactants) allows to attain better polymerization efficiencies with lower concentrations of surfactant, which reduces the enzyme denaturalization by SDBS (Lin, 2004; Chauhan and Sharma, 2014).

Once the reaction conditions for the enzymatic synthesis of polyaniline were optimized, a 75% conversion yield of aniline into emeraldine salt was achieved (1 g/l of polyaniline from 1,4 g/l of aniline) by using 7D5 laccase produced in *A. oryzae* as biocatalyst. The resulting polymer displayed in water-soluble nanofibers, showed the characteristic absorbance maximum at 800 nm in the visible spectrum and similar intensity of the quinoids and benzenoids bands in the FTIR spectrum (Karamyshev *et al*., 2003), together with an excellent electroactivity and

electroconductivity (2.4 x 10^{-3} S/cm) in the range of other electroconductive polyanilines (Blinova *et al*., 2007; Junker *et al*., 2014). Taking into account the excellent properties and production yields of the polymer, its water solubility, and the mild reaction conditions used in comparison with those of chemical synthesis (Huang *et al*., 2003), the enzymatic synthesis of polyaniline carried out here can be considered as an efficient process of green chemistry. The reaction conditions set up in this chapter were later used by SETA*Ş* color company (Turkey) for the enzymatic synthesis of polyaniline to evaluate its capabilities as textile dye (Chapter 3).

2. Structure-function studies of 7D5 and PM1 laccases

The successful overexpression in *A. oryzae* of 7D5 laccase (carried out by the doctoral student at Novozymes A/S, Denmark) allowed to obtain the first crystal structure of a basidiomycete laccase engineered in the laboratory (PDB, 6HY5) and to perform SAXS analysis. A deep molecular and biochemical study of this laccase variant was carried out in comparison with the wild-type laccase from basidiomycete PM1 (PDB. 5ANH, Pardo *et al*., 2016), one of the two parent fungal laccases from which 7D5 derives and the one with which it shares the highest sequence identity (Pardo *et al*., 2012). SAXS analysis of both laccases glycosylated and deglycosylated (with Endo-H) showed a more oval and larger structure of 7D5 compared with PM1 laccase due to a greater and more heterogeneous glycosylation of the former. This is related to the great variety of glycosylations (N- and O- glycosylations) produced by *Aspergillus* (Deshpande *et al*., 2008). These results correlate with the higher glycosylation rates observed in 7D5 (5-17 %) than in PM1 laccase (5 %) by MALDI/TOF-TOF. This new laccase structure was of great help during the design of new improved variants of this enzyme (see Chapter 3).

Laccase 7D5 showed a catalytic activity (k_{cat}) superior to PM1 laccase towards all the substrates assayed (ABTS and HBT, phenols, and aromatic amines). It is worth noting the outstanding catalytic efficiency towards ABTS of the engineered laccase, the highest ever described for laccases (Jordaan *et al*., 2004; Nyanhongo *et al*., 2007; Yan *et al*., 2014). The increase in catalytic efficiency with ABTS respecting the two wild-type laccases from which 7D5 comes (36 and 7 times superior than *P. cinnabarinus* and PM1 laccases, respectively) would be partially associated with the use of this substrate in the screenings of the mutant libraries obtained in the evolutionary campaigns that led to 7D5 variant (Mate *et al*., 2010, Camarero 2012 *et al*., Pardo *et al*., 2012). It is also noteworthy the high capability of 7D5 to oxidize aromatic amines (oxidizes DMPD with an efficiency 32 times higher than that of the laccase from PM1).

Since the catalytic improvements of 7D5 were not associated to optimal pH or redox potential changes with respect to the wild-type laccase from basidiomycete PM1, the effect of the mutations accumulated in 7D5 was studied using computational tools. By using PELE, a notable increment of the catalytic events with DMPD (structures where the substrate adopts a distance below 4 Å to His455) was observed in 7D5 laccase. These studies showed that mutation V162A opens an additional space in the catalytic pocket of the enzyme, causing an approach of the DMPD molecule to the active center (Cu T1-H455). However, no increment in spin density respecting PM1 laccase was observed by QM/MM calculations. This would indicate that while the substrate is better positioned in 7D5 laccase, the redox potential of the substrate in the catalytic pocket hardly varies between both laccases. The significant improvement in k_{cat} (8 times) was however associated with an increment in the electron transfer constant (kET), from 4.5 to 7.5, due to the reduction of the electronic transfer distance in 7D5 generated by the substrate reposition (between 1.5 -2 Å). In the case of larger substrates like ABTS, PELE and QM/MM simulations showed as well a better repositioning of the substrate due to the reduction of the side chain of the mutated residue V162A, reducing its exposure to the solvent and increasing the spin density in the catalytic pocket of 7D5 due to a local redox potential change, in correlation with the k_{cat} improvements (Monza *et al*., 2015).

Both laccases, especially the wild-type enzyme, displayed good stability at high temperature and different pH values. 7D5 laccase showed high stability at basic pH but lower stability at acid pH compared whit PM1 laccase. It also turned out to be more unstable at high temperature. Even so, stability values of the engineered enzyme are higher than those described for other thermo-tolerant basidiomycetes and ascomycetes laccases (Hildén *et al*., 2009). The two laccases showed outstanding structural stability at high temperature, as demonstrated in CD experiments by the absence of protein denaturalization in a temperature ramp from 50 to 95 °C or even after two hours of incubation at 100 °C. This adaptation of the protein structure to high temperatures has been also described in other thermostable laccases (Bonomo *et al*., 2001; Ferrario *et al*., 2015; Karshikoff *et al*., 2015; Kikani and Singh, 2015; Mukhopadhyay and Banerjee, 2015). Longer incubation of the enzymes at 100 °C showed the faster unfolding of 7D5 laccase. By calculating the displacement of the Cα of the protein residues using B-fitter, it was detected the high rigidity of a superficial loop in 7D5 due to I468T mutation. It seems that while β-sheets folding of PM1 laccase would remain stable for longer time due to the presence of flexible loops that would better absorb the impact of high temperatures (Plana *et al*., 2019; Vicente *et al*., 2019), the loss of flexibility of some of these loops caused by mutations in the engineering enzyme would diminish its overall ability to adapt to thermal changes, reducing its thermostability. The outstanding stability displayed by PM1 laccase maybe related to the fact that PM1 fungus was isolated from the wastewaters of a paper mill (Coll

*et a*l., 1993). Whereas, the lower stability of 7D5 laccase is related to the common destabilizing effect mutations accumulated during *in vitro* evolution exert in the enzyme (Tokuriki *et al*., 2008).

3. Engineering of 7D5 laccase

3.1. Adaptive directed evolution

The replacement of the native signal peptide of the enzyme by the signal sequences of own *S. cerevisiae* proteins such as the pre-proleader of the alpha-mating factor (α pre-proleader) or the signal sequence of the killer toxin (K1), is a strategy commonly used to increase the secretion of heterologous proteins by the yeast (Zsebo *et al*., 1986; Cartwright *et al*., 1992; Viña-Gonzalez *et al*., 2015). For instance, the α pre-proleader was used as signal sequence of PM1 and *P. cinnabarinus* laccases during their directed evolution campaigns for their functional expression in *S. cerevisiae*. The different mutations accumulated in the α pre-proleader improved the expression of each laccase (Mate *et al*., 2010; Camarero *et al*., 2012). It is particularly worth noting the efficiency of the mutated α pre-proleader sequence obtained during the directed evolution of *P. cinnabarinus* laccase to increase the secretion by the yeast of other laccases. This is the case of 7D5 laccase used in this study and of the other chimeric laccases obtained by DNA shuffling of the two fungal laccases mentioned above. Although their mature sequences are more similar to that of PM1 laccase, all of them inherited the evolved α pre-proleader from *P. cinnabarinus* parent laccase (Pardo *et al*., 2012). Recombination of the same two fungal laccases with another basidiomycete laccase later confirmed these findings (Mateljak *et al*., 2017).

In order to improve the catalytic activity of 7D5 laccase on aromatic amines, while maintaining its versatile activity against other substrates, and improving at the same time its expression in yeast, the engineering of the enzyme was carried out in this thesis. After two adaptive directed evolution cycles in *S. cerevisiae* of 7D5 coding sequence plus its evolved α pre-proleader (Pardo *et al*., 2012), and using DMPD and ABTS for screening the laccase activities of the mutant libraries, two mutations were selected in the signal sequence (Aα20T and Qα32H) and one in the mature laccase (F454S). They notably increased the laccase activity detected in microplate (TAI = 8.5 times). Despite the contribution of the F454S mutation to this increment in total activity, it produced a significant loss of stability at acidic pH and high temperature, and, it was consequently removed to obtain EM variant. Mutation F454S had been already selected and its deleterious effect for the stability of the enzyme described in previous directed evolution campaigns of PM1 laccase (Mate *et al*., 2010, 2013).

The two mutations of the α pre-proleader produce a notable enhancement in laccase secretion by the yeast. In fact, the secretion by *S. cerevisiae* of the new variant, the expression mutant (EM) was increased to 16 mg/l compared to the 3 mg/l obtained of laccase 7D5.

3.2. Computational design

Previous simulation studies had demonstrated the importance that the substrate binding event has in the overall catalytic activity of the enzyme, so that the positioning of the substrate and the interaction energy in the active site determines the electronic transfer between the substrate and the T1 copper (Monza *et al*., 2015, 2017; Pardo *et al*., 2016). In order to select those residues of the catalytic pocket of 7D5 laccase that could improve the substrate-enzyme interaction and electron transfer without the need of exploring a large number of clones, it was carried out the computational design of the enzyme combining PELE and QM/MM. PELE allows rapid mapping of the enzyme-substrate interaction dynamics and select those positions that favor catalysis (shorter substrate-T1 distance, SASA or interaction energy). Then the electronic transfer between the substrate and the enzyme can be evaluated by calculating the spin densities and the kET using QM/MM (Monza *et al*., 2017).

The goal was to find amino acid substitutions which would increase the activity of laccase 7D5 on aniline at the polymerization conditions (pH 3), aiming at improving the difficult extraction of the electron from the anilinium cation. It was observed that residues N207 and N263, located at the entrance of 7D5 catalytic pocket, directly interacted with the aniline and that their substitution with negatively charged residues improved the electrostatic conditions of the oxidized aniline in the catalytic pocket, following the strategy of modify the substrate redox (transient) potential at the active site. However, the mutation of both residues by others with a negative charge was avoided since this would affect the stability of the enzyme. The Asn from 263 position was replaced by Asp while the Asn from residue 207 was replaced by Ser, which would favor substrate binding without compromising enzyme stability. The viability of a simple mutant (N263D) was studied, but the resulting variant showed significantly reduced expression and stability, demonstrating the synergy between both mutations. Therefore, mutations N207 and N263 were introduced in EM variant. The resulting laccase variant (DM) displayed a better positioning of aniline in the binding pocket and higher spin density with aromatic amines and ABTS, which directly correlates with the increases in the k_{cat} towards these substrates without affecting the stability towards acid pH (Santiago *et al*., 2016). The efficacy of these computational tools in the design of other oxidoreductases has been demonstrated in other studies (Acebes *et al*., 2016; Carro *et al*., 2019; Mateljak *et al*., 2019; Serrano *et al*., 2019).

3.3. Focused mutagenesis on the catalytic site

The increment in activity observed with F454S mutation, its repeated occurrence in previous laccase directed evolution campaigns (Mate *et al*., 2010, 2013) and the proximity of this residue to the T1 copper and His455 (which coordinates T1 and constitutes the "entry" of substrate's electrons, Galli *et al*., 2013), led us to study 454 position by saturation mutagenesis, looking forward to replace Phe454 by another residue that would increase the catalytic activity without diminishing the enzyme stability. Four mutations were selected, F454H, F454T, F454P and, again, F454S, with significant activity improvements (TAI 3-2 times). Substitution of Phe454 by polar residues (F454T and F454S) caused an important destabilization of the laccase at acidic pH and high temperature. The substitution by a basic residue (F454H) also displayed a notable loss of stability, while a Pro in this position (F454P, PK2 variant) was the only one that did not lead to a significant loss of enzyme stability.

The influence of residue 454 in the enzymatic activity at the molecular level was evaluated by PELE, using the structure of 7D5 (PDB. 6H5Y) as a template. Simulations on the different 454 mutated variants were compared with DM (Phe454). The four mutants showed a similar distance profile between the N1 atom of the aniline and the NE2 atom of His455, presenting two main minima, one at a catalytic distance from the T1 copper and the other, non-catalytic, at a distance of 12 Å. The catalytic profiles showed great similarity between mutations. However, the event population of the catalytic minimum increased significantly in the variants with F454T and F454S mutations while in the variants with F454H or F454P mutation (PK2) remained similar to that of the non-mutated variant (DM). Once PK2 variant was purified, an increase in the secretion of this laccase was observed (from 16 mg/l to 25 mg/l), but it showed similar kinetic constants than DM variant. All this indicates that the F454P mutation is responsible for the increased secretion of the enzyme, in correlation with the results obtained by simulation, and not with a better catalytic activity as firstly thought due to its location in the T1 copper environment.

3.4. C-terminal engineering

As afore mentioned, the catalytic activity improvements obtained during enzyme directed evolution often lead to a loss of stability (Bloom *et al*., 2004). During the evolution of 7D5 laccase, the stability at acidic pH was not compromised due to the use of pH 3 stability tests during the screening of the mutant libraries. However, thermal stability decreased significantly from 7D5 to PK2 variant. In order to increase laccase stability at high temperatures and thereby enhance its applicability as biocatalyst, the C-terminal (last 10 amino acids) of PK2 variant was replaced by that of another highly thermostable chimeric laccase (3A4) obtained in the same directed evolution campaign than 7D5 (Pardo *et al*., 2012). The objective

was to introduce the 4 mutations of this region in which these enzymes differ. These mutations came from *P. cinnabarinus* parent laccase and they are present in most of the C-terminal ends of the thermostable chimeric laccases obtained in that work (Pardo *et al*., 2012). Recently, it has been described the development of chimeric laccases by SCHEMA from three fungal laccases, being one of them *P. cinnabarinus* (Mateljak *et al*., 2019). Most of the higher thermostable laccases obtained also shared the C-terminal end of *P. cinnabarinus* laccase, verifying the results obtained in our laboratory.

The final variant RY2 (Fig. 2) obtained by engineering of PK2 variant C-terminal end showed a significant improvement in kinetic and structural stability at high temperatures, keeping its secondary structures largely intact after 5 h of incubation at 100 °C. The four mutations of the C-terminal end also significantly improved the stability of the enzyme at different pH, especially at basic pH (RY2 presents twice as high residual activity after 24 h at pH 8-9 than PK2). Unexpectedly, the replacement of the C-terminal end also improved the catalytic activity of RY2 towards all the substrates tested (ABTS, DMPD, aniline, and DMP). Compared to the starting enzyme, the k_{cat} towards ABTS was improved 2 times, 3-5 times towards aromatic amines and maintained against DMP (despite this substrate was not used during the enzyme engineering).

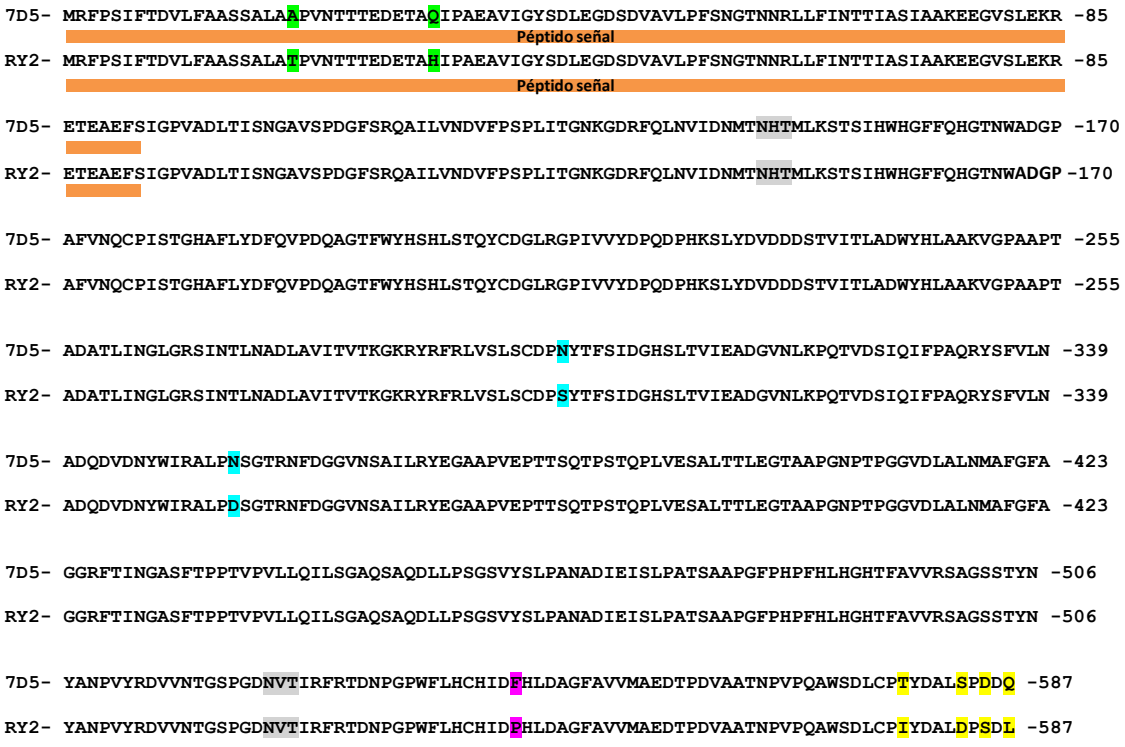


Figure 2. Comparison of the mature sequences of 7D5 laccase and its final variant (RY2) obtained in this Thesis together with their respective evolved signal peptides (α pre-proleader, orange underline). N-glycosylation sites are shown in gray, and the mutations acquired are shown in different colors: signal peptide mutations, Aα20T and Qα32H, first selected in EM variant (green); catalytic site

mutations, N207S and N263D, of DM variant (cyan) and F454P of PK2 variant (in magenta); and C-terminal mutations, T487I, S492D, D494S, Q496L, of RY2 variant (in yellow).

4. Heterologous expression of 7D5 laccase and its variants

4.1. *S. cerevisiae* expression

During the evolutionary route of 7D5 laccase carried out in this Doctoral Thesis the secretion of the enzyme by the yeast has been increased from 3 mg/l (starting laccase, 7D5) to 25 mg/l (final variant, RY2). Aα20T and Qα32H mutations in the signal peptide and F454P mutation in the mature laccase are the main factors of this remarkable improvement in enzyme production. These values are the highest ever reported for the production of basidiomycete laccases in *S. cerevisiae* (Kunamneni *et al*., 2008; Mate *et al*., 2013) and are similar to some values obtained in *Pichia pastoris* (Mate *et al*., 2013). So far, no crystallographic structure of a basidiomycete laccase expressed in *S. cerevisiae* has been solved. Thanks to the high levels of functional expression of laccase in *S. cerevisiae* reached in this thesis, the crystal structure of RY2 variant can be achieved in a future work. The resolution of its structure will allow the verification of the results obtained by computational calculations. In particular, it is of special interest to clarify the interaction of the C-terminal with the TNC and how it may affect oxygen reduction, since the role of the TNC seems decisive in the catalytic activity of high redox-potential laccases (Sekretaryova *et al*., 2019). In addition, the new structure will determine the position and behavior of the N-terminal end in the laccases expressed in *S. cerevisiae* which, due to a failure of the STE13 protease in the processing of the α pre-proleader, presents 6 extra amino acid residues, which have been associated with improvements in the secretion of the enzyme (Mate *et a*l., 2013).

4.2. Expression in *A. oryzae* and industrial applications

Several of the laccase variants engineered in this Doctoral Thesis have been successfully produced in *A. oryzae* in collaboration with Novozymes A/S company. The final variant RY2 was not expressed in *A. oryzae* since it was obtained after the doctoral student's stay in this company, but the successful expression of 7D5 laccase, the starting point of this Thesis, PK2 variant and 3A4 laccase (to which the C-terminal of RY2 belongs) suggests that RY2 variant could also be expressed successfully by *A. oryzae*. As mentioned before, this overexpression allowed us to obtain the crystal structure of 7D5 laccase (PDB: 6H5Y) and to calculate the redox potentials of the T1 copper of several variants.

The feasible production of these laccases at an industrial scale would allow their application in different industrial sectors. As an example, this Thesis presents the use of these enzymes as biocatalysts of reactions of organic synthesis, specifically of the synthesis of Esmeraldine salt (the electroconductive form of polyaniline, Chapter 1), and of polyaniline and an industrial dye derivate from 1-naphthol (Chapter 3). The synthesis of the latter catalyzed by one of the laccase variants engineered in the laboratory and expressed in *A. oryzae*, and its characterization following textile industry standards, was carried out at SETAŞ Color Company, a leading company in the synthesis of textile dyes. The synthetized polyaniline as dye showed a high dyeing efficiency and a significant resistance to light degradation. On the other hand, the dye derived from 1-naphthol proved to be an acid dye with intense fixation properties in wool and nylon fabrics. This dye was compared with Nyloset Brown N commercial dye, a chemically synthesized dye with similar characteristics, and with the dye obtained using the commercial Novozyme 51003 laccase as biocatalyst. The dye synthesized with the evolved laccase showed a greater dyeing efficiency than that synthesized with the commercial laccase, but slightly less than the commercial dye (88% staining efficiency for the evolved laccase, and 56% for Novozyme 51003 compared to 100% commercial dye).

This Doctoral Thesis is an example of how enzyme engineering by directed evolution and computational design enable to develop biocatalysts with new or improved capabilities adapted to the operating conditions. While the engineering of the enzyme can significantly improve its heterologous expression in laboratory strains, the use of industrial strains is essential for its production at a relevant scale and industrial application. The use of tailor made biocatalysts allows the substitution of conventional chemical synthesis by new, more efficient, sustainable and environmentally friendly processes.

CONCLUSIONS

Conclusions

1. The optimization of the reaction conditions for the enzymatic synthesis of polyaniline revealed the profound influence the reaction conditions, in general, and the type and concentration of doping template, in particular, exert in the properties of the resulting polymer in terms of degree of polymerization, macromolecular structure, electroactivity and electroconductivity.

2. Water-soluble electroactive and electroconductive PANI (emeraldine salt) displayed in nanofibers have been synthetized at high yields (ranging from 75 % to 87%) in a green and easy manner, using high-redox potential laccases designed in the laboratory as biocatalyst and SDBS as doping template.

3. Laccase 7D5 and some of the new laccase variants engineered in this thesis have been successfully over-expressed in *A. oryzae* at an industrial relevant scale.

4. The structure of 7D5 laccase (PDB: 6H5Y) expressed in *A. oryzae* solved in this study is the first crystal structure reported so far of a basidiomycete laccase designed in the laboratory.

5. Comparison of this 7D5 laccase and the wild-type laccase PM1 showed the superior catalytic activity of the former with all the substrates tested, with outstanding catalytic efficiency with DMPD and ABTS due to mutation V162A, among other reasons. On the contrary, 7D5 laccase shows a diminished stability to high temperature most likely related to the rigidification of certain superficial loops after mutation.

6. The semi-rational design of 7D5 laccase combining directed evolution and computational design rendered a set of new laccase variants with different properties. The mutations accumulated in the final variant RY2 resulted in an efficient, versatile and robust biocatalyst that is secreted by *S. cerevisiae* at very good levels.

7. Functional secretion of laccase by *S. cerevisiae* has been notably improved during the engineering of 7D5 laccase. The two mutations of the alfa pro-leader (Aα20T and Qα32H form EM variant) and a third mutation in T1 Cu environment (F454P, accumulated in PK2 variant) increased laccase secretion from 3 mg/l to 25 mg/l which is one of the highest yields ever reported for the heterologous expression of basidiomycete laccases in *S. cerevisiae.*

8. The four mutations incorporated to the laccase C-terminal tail in the final variant RY2 (T487I, S492D, D494S, Q496L) are responsible for significant

improvements in the catalytic activity and enzyme stability. New interactions of this tail with the O_2/H_2O channel entrance and the increased flexibility of this region (better absorbing the perturbations caused by high temperature) seem respectively responsible for these improvements.

9. PANI and a new organic acid-dye were enzymatically synthesized and their excellent textile dyeing properties were proved in a relevant industrial environment.

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APPENDIX

In addition to the publications included in this work, Felipe de Salas has participated in three more articles during the development period of this Doctoral Thesis:

Felipe de Salas and Susana Camarero. 2020. Applications of fungal laccases. Book chapter in *Encyclopedia of Mycology* (Elsevier). In press

Isabel Pardo, David Rodríguez-Escribano, Pablo Aza, Felipe de Salas, Angel T. Martínez and Susana Camarero. 2018. A Highly Stable Laccase Obtained by Swapping the Second Cupredoxin Domain. *Scientific Reports*. (DOI:10.1038/s41598-018-34008-3)

David Rodríguez-Escribano, Felipe de Salas, Isabel Pardo and Susana Camarero. 2017. High-Throughput Screening Assay for Laccase Engineering toward Lignosulfonate Valorization. *International Journal of Molecular Science*. (DOI:10.3390/ijms18081793)