

UNIVERSIDAD COMPLUTENSE DE MADRID
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TESIS DOCTORAL

Diseño e ingeniería de lacasas quiméricas por evolución dirigida para el tratamiento de biomasa vegetal

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**DISEÑO E INGENIERÍA DE LACASAS
QUIMÉRICAS POR EVOLUCIÓN DIRIGIDA PARA
EL TRATAMIENTO DE BIOMASA VEGETAL**

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TABLA DE CONTENIDO

ABREVIATURAS (ABBREVIATIONS)	I
RESUMEN / SUMMARY	1
ESTRUCTURA DE LA TESIS	11
INTRODUCCIÓN GENERAL	13
1. Aspectos generales de las lacasas.....	15
1.1. Estructura de las lacasas.....	15
1.2. Mecanismo de reacción de las lacasas	17
2. Las lacasas y la biomasa vegetal	20
2.1. Composición química de la lignina.....	21
2.2. Degradación de la lignina por hongos.....	22
2.3. Mediadores naturales de lacasas	22
3. Aplicaciones industriales y biotecnológicas de las lacasas	24
4. Ingeniería de lacasas mediante evolución dirigida.....	27
4.1. Principios de la evolución dirigida	27
4.2. Distintas estrategias de evolución dirigida.....	32
4.3. Evolución dirigida de lacasas de alto potencial redox	35
BIBLIOGRAFÍA	40
OBJETIVOS	51
CHAPTER 1. Development of chimeric laccases by directed evolution	55
ABSTRACT	57
INTRODUCTION	57
MATERIAL AND METHODS.....	58
Reagents and enzymes	58
Strains and culture media.....	58
Construction of chimeric libraries using CLERY	59
High-throughput screening.....	60
Flask production of chimeras.....	61
Characterization of chimeric laccases	61
RESULTS.....	62
Constructing and screening chimeric libraries	62

Chimera characterization.....	65
DISCUSSION	66
Family shuffling and sequence analysis.....	66
Chimeric laccases with combined features	68
CONCLUSIONS.....	70
ACKNOWLEDGEMENTS.....	70
REFERENCES	71
SUPPLEMENTARY INFORMATION	74
CHAPTER 2. New colorimetric screening assays for the directed evolution of fungal laccases to improve the conversion of plant biomass	75
ABSTRACT	77
BACKGROUND.....	78
RESULTS AND DISCUSSION	80
Oxidation of natural phenolic compounds of biotechnological interest.....	80
Violuric acid as reporter assay for assessing redox potentials during protein engineering.....	87
Decolorization of synthetic organic dyes	89
CONCLUSIONS.....	93
METHODS.....	94
Reagents and enzymes	94
Culture media.....	94
Oxidation assays with commercial laccases.....	94
Micro-cultures of <i>S. cerevisiae</i> cells expressing laccase mutants	95
Validation of the HTS colorimetric assays	95
Construction of the mutagenic libraries	95
High-throughput screening of laccase libraries	96
ABBREVIATIONS.....	96
COMPETING INTERESTS	96
AUTHORS' CONTRIBUTIONS.....	96
ACKNOWLEDGEMENTS.....	97
REFERENCES	97

CHAPTER 3. Re-designing the substrate binding pocket of laccase for enhanced oxidation of sinapic acid 101

ABSTRACT	103
INTRODUCTION	103
EXPERIMENTAL SECTION.....	104
Construction of mutant libraries	104
Screening of mutant libraries.....	105
Production and purification of mutated laccases	105
Biochemical characterization of laccase variants.....	106
Synthesis of DAD.....	107
Systems setup for computational simulations.....	107
PELE sampling	108
Quantum mechanics/molecular mechanics (QM/MM)	108
RESULTS AND DISCUSSION	109
Construction and screening of mutant libraries	109
Biochemical characterization of selected ISM variants	111
Hammett and Marcus correlations	113
Computational analysis of substrate binding and oxidation	114
Oxidation of related syringyl-type phenols.....	119
CONCLUSIONS.....	120
ABBREVIATIONS	120
ACKNOWLEDGEMENTS.....	121
REFERENCES.....	121
SUPPLEMENTARY MATERIAL.....	126

CHAPTER 4. Exploring the oxidation of lignin-derived phenols by a library of laccase mutants 137

ABSTRACT	139
INTRODUCTION	139
RESULTS AND DISCUSSION	141
Production of laccase mutants.....	142
Multi-screening with phenolic compounds.....	143
Comparison of laccase mutants.....	147

EXPERIMENTAL SECTION.....	150
Construction and screening of mutant libraries	150
Production of laccase mutants.....	150
Multi-screening with phenolic compounds.....	151
CONCLUSIONS.....	151
ACKNOWLEDGMENTS.....	152
AUTHOR CONTRIBUTIONS	152
CONFLICTS OF INTEREST.....	152
REFERENCES	152
SUPPLEMENTARY MATERIAL	156
CHAPTER 5. A highly stable hybrid laccase obtained by domain swapping ...	159
ABSTRACT	161
INTRODUCTION	161
RESULTS.....	163
Construction and expression of domain-swap mutants.....	163
Activity of domain-swap laccases.....	164
Stability of domain-swap laccases.....	165
Evaluation of the factors determining thermostability in the domain-swap laccases.....	167
DISCUSSION	168
CONCLUSIONS.....	172
MATERIAL AND METHODS.....	172
Generation of 3D-structure protein models.....	172
Construction of domain-swap mutants and site-directed mutagenesis	172
Flask production of chimeras	173
Characterization of chimeric laccases.....	174
ACKNOWLEDGEMENTS.....	175
REFERENCES	175
SUPPLEMENTARY MATERIAL	178
DISCUSIÓN GENERAL.....	181
1. Obtención de lacasas quiméricas.....	183
1.1. Estrategias de quimeragénesis aleatoria y racional.....	183

1.2. Expresión heteróloga de las lacasas quiméricas en levadura.....	184
1.3. Actividad de las lacasas quiméricas.....	185
1.4. Estabilidad de las lacasas quiméricas.....	188
2. Ingeniería de lacasas para mejorar el tratamiento de la biomasa lignocelulósica.....	189
2.1. Desarrollo de nuevos ensayos de HTS.....	190
2.2. Diseño semi-racional del bolsillo de unión a sustrato.....	191
2.3. Oxidación de otros compuestos fenólicos derivados de la lignina.....	192
2.4. Estudios computacionales de las mutaciones del bolsillo enzimático.....	193
BIBLIOGRAFÍA.....	195
CONCLUSIONES / CONCLUSIONS.....	199
ANEXO.....	205

ABREVIATURAS (*ABBREVIATIONS*)

Nucleótidos (<i>Nucleotides</i>)	
A	Adenina (<i>Adenine</i>)
C	Citosina (<i>Cytosine</i>)
G	Guanina (<i>Guanine</i>)
T	Timina (<i>Thymine</i>)
R	A /G
Y	C/T
S	G/C
W	A/T
K	G/T
M	A/C
B	C/G/T
D	A/G/T
H	A/C/T
V	A/C/G
N	A/C/G/T
Aminoácidos (<i>Amino acids</i>)	
A/Ala	Alanina (<i>Alanine</i>)
C/Cys	Cisteína (<i>Cysteine</i>)
D/Asp	Ácido aspártico (<i>Aspartic acid</i>)
E/Glu	Ácido glutámico (<i>Glutamic acid</i>)
F/Phe	Fenilalanina (<i>Phenylalanine</i>)
G/Gly	Glicina (<i>Glycine</i>)
H/His	Histidina (<i>Histidine</i>)
I/Ile	Isoleucina (<i>Isoleucine</i>)
K/Lys	Lisina (<i>Lysine</i>)
L/Leu	Leucina (<i>Leucine</i>)
M/Met	Metionina (<i>Methionine</i>)
N/Asn	Asparragina (<i>Asparagine</i>)
P/Pro	Prolina (<i>Proline</i>)
Q/Gln	Glutamina (<i>Glutamine</i>)
R/Arg	Arginina (<i>Arginine</i>)
S/Ser	Serina (<i>Serine</i>)
T/Thr	Treonina (<i>Threonine</i>)
V/Val	Valina (<i>Valine</i>)
W/Trp	Triptófano (<i>Tryptophane</i>)
Y/Tyr	Tirosina (<i>Tyrosine</i>)
Otros (<i>Other</i>)	
3PO	Variante evolucionada de P _{CL} (<i>P_{CL} evolved variant</i>)
ABTS	Acido 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfónico) (<i>2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)</i>)
CLERY	<i>Combinatorial Libraries Enhanced by Recombination in Yeast</i>
CSM	Mutagénesis combinatorial saturada (<i>Combinatorial Saturation Mutagenesis</i>)
C-terminal	Extremo carboxilo (<i>Carboxylic end</i>)
Cu T1, T2 y T3	Cobre de tipo I, II y III (<i>Type I, II and III Copper</i>)
D1, D2 y D3	Primer, segundo y tercer dominio cupredoxina (<i>First, second and third cupredoxine domain</i>)
DMP	2,6-dimetoxifenol (<i>2,6-dimethoxyphenol</i>)
DNA	Ácido desoxirribonucleico (<i>Deoxyribonucleic acid</i>)
dNTP	Desoxirribonucleótido trifosfato (<i>Deoxyribonucleotide triphosphate</i>)
E°	Potencial redox estándar (<i>Standard redox potential</i>)
epPCR	PCR propensa a error (<i>Error-prone PCR</i>)
EPR	Resonancia paramagnética electrónica (<i>Electronic Paramagnetic Resonance</i>)
ET	Transferencia electrónica (<i>Electron Transfer</i>)
H, G y S	Unidad de tipo <i>p</i> -hidroxifenilo, guayacilo y siringilo (<i>Phenylpropane-, guaiacyl- and syringyl-type unit</i>)
HTS	Cribado de alto rendimiento (<i>High-throughput screening</i>)
ISM	Mutagénesis saturada iterativa (<i>Iterative Saturation Mutagenesis</i>)
k_{cat}	Constante catalítica (<i>Catalytic constant</i>)
K_m	Constante de Michaelis (<i>Michaelis constant</i>)

LRPL, MRPL y HRPL	Lacasa de bajo, medio y alto potencial redox (<i>Low-, Medium- and High-redox potential laccase</i>)
MCO	Oxidasa multicobre (<i>Multicopper oxidase</i>)
MD	Dinámica molecular (<i>Molecular Dynamics</i>)
N-terminal	Extremo amino (<i>Amino end</i>)
OB1	Variante evolucionada de PM1L (<i>PM1L evolved variant</i>)
PcL	Lacasa de <i>Pycnoporus cinnabarinus</i> (<i>Pycnoporus cinnabarinus laccase</i>)
PCR	Reacción en cadena de la polimerasa (<i>Polymerase Chain Reaction</i>)
PELE	<i>Protein Energy Landscape Exploration</i>
PM1L	Lacasa de Basidiomiceto PM1 (<i>PM1 basidiomycete laccase</i>)
QM/MM	Mecánica cuántica/Mecánica molecular (<i>Quantum Mechanics/Molecular Mechanics</i>)
T₅₀	Temperatura a la cual la actividad enzimática es la mitad de la inicial (<i>Temperature at which initial enzyme activity is reduced by half</i>)
TAI	Incremento de actividad total (<i>Total Activity Increase</i>)
TCF	Totalmente libre de cloro (<i>Totally Chlorine Free</i>)
TNC	Centro trinuclear (<i>Trinuclear Cluster</i>)

RESUMEN / SUMMARY

RESUMEN

1. Antecedentes

Las lacasas son oxidasas multicobre que se encuentran ampliamente distribuidas en la naturaleza aunque los principales productores de estas enzimas son los hongos basidiomicetos de la podredumbre blanca de la madera, los únicos organismos capaces de degradar completamente la lignina. Las lacasas catalizan la oxidación de una gran variedad de compuestos aromáticos acoplada a la reducción de oxígeno a agua. Además, su capacidad catalítica puede ampliarse en presencia de mediadores redox, compuestos de bajo peso molecular que, una vez oxidados por la lacasa, pueden oxidar otros compuestos que en principio no son sustrato de la enzima.

Debido a su amplio rango de sustratos y a sus bajos requerimientos catalíticos, las lacasas y los sistemas lacasa-mediador tienen un gran potencial biotecnológico, particularmente en procesos de tratamiento de la biomasa vegetal como son la producción de pasta de papel o de biocombustibles de segunda generación. Además, en estos procesos se generan una gran cantidad de residuos ricos en fenoles derivados de la lignina que pueden actuar como mediadores de lacasa, y cuyo uso podría reducir los costes y el impacto medioambiental del proceso. Sin embargo, para la aplicación industrial de las lacasas puede ser necesario recurrir a la ingeniería de proteínas para ajustar sus propiedades catalíticas a las condiciones de operación. En trabajos previos se ha descrito la evolución dirigida de dos lacasas de alto potencial redox procedentes de los basidiomicetos PM1 (PM1L) y *Pycnoporus cinnabarinus* (PcL). Las variantes evolucionadas de estas lacasas, OB1 y 3PO, respectivamente, son el punto de partida de esta tesis doctoral.

2. Objetivos

El objetivo general de esta tesis doctoral fue la ingeniería de lacasas de alto potencial redox mediante técnicas de evolución dirigida para su aplicación en el tratamiento de la biomasa vegetal. Para ello, se plantearon tres grandes tareas:

- i. Obtener lacasas quiméricas a partir de las variantes evolucionadas de PM1L (OB1) y PcL (3PO), con el fin de combinar y/o mejorar sus propiedades de interés y obtener información sobre los determinantes estructurales de la actividad y estabilidad de estas enzimas.
- ii. Desarrollar nuevos ensayos de determinación de actividad lacasa en formato de *high-throughput screening* (HTS) para la exploración de las librerías de mutantes generadas por evolución dirigida.
- iii. Mejorar la actividad lacasa sobre mediadores fenólicos derivados de la lignina mediante el diseño semi-racional del bolsillo de unión a sustrato.

3. Resultados

3.1. Obtención de lacasas quiméricas

En primer lugar se empleó una estrategia de quimeragénesis al azar mediante la combinación del barajado *in vitro* e *in vivo* (CLERY) de los genes de OB1 y 3PO, seleccionando aquellos mutantes que presentaban mayor actividad. Los segmentos de secuencia intercambiados dieron lugar a la introducción de 3–31 sustituciones aminoacídicas en las secuencias de las lacasas maduras. Entre las lacasas quiméricas obtenidas se observaron perfiles de pH óptimo desplazados respecto a los de ambos parentales, y algunas resultaron ser también más termoestables. De la batería de quimeras generadas, se seleccionaron cinco como posibles puntos de partida para estudios posteriores de evolución dirigida.

Con el fin de obtener variantes con un mayor número de mutaciones, se construyeron nuevas lacasas mediante el intercambio del segundo dominio cupredoxina entre OB1 y 3PO. Las dos nuevas quimeras adquirieron 65 sustituciones aminoacídicas cada una. Una de ellas mostró un perfil de pH óptimo desplazado, mientras que la otra presentó un importante incremento de estabilidad a temperatura, pH y solventes.

3.2. Desarrollo de nuevos métodos de high-throughput screening

Se desarrollaron varios métodos de HTS para analizar librerías de mutantes de lacasa generadas por evolución dirigida. En primer lugar, se diseñaron tres ensayos basados en la oxidación de fenoles derivados de la lignina de tipo siringilo que actúan como mediadores de lacasa (ácido sinápico, siringaldehído y acetosiringona). En segundo lugar, se desarrolló un ensayo específico para lacasas de alto potencial redox basado en la oxidación del mediador sintético ácido violúrico, con el fin de asegurar que el potencial redox de la enzima no se veía afectado durante la evolución dirigida. Por último, se diseñaron tres ensayos basados en la oxidación de colorantes sintéticos de tipo azoico (azul de Evans y naranja de metilo) y antraquinoide (azul brillante de remazol), los tipos de colorantes más empleados en la industria textil y cuya biodegradación sería de interés para el tratamiento de efluentes.

3.3. Diseño semi-racional del bolsillo de unión a sustrato

Utilizando como punto de partida una de las lacasas quiméricas obtenidas por CLERY, se realizó mutagénesis saturada iterativa (ISM) sobre seis residuos del bolsillo de unión a sustrato con el fin de mejorar su actividad sobre compuestos fenólicos de interés. Para la exploración de las librerías de mutantes se empleó el ensayo de HTS con ácido sinápico desarrollado en esta tesis. De los mutantes obtenidos, se seleccionaron dos para su caracterización bioquímica. Las nuevas

variantes de lacasa presentaron perfiles de pH óptimo desplazados hacia valores más neutros y constantes de reacción mayores que el parental para el ácido sinápico y el sinapato de metilo, si bien su actividad hacia otros fenoles de tipo siringilo no mejoró significativamente. Con el fin de racionalizar el efecto de las mutaciones en las variantes de ISM, se realizaron simulaciones computacionales de PELE y QM/MM. Éstas revelaron que las mutaciones introducidas permitían un nuevo modo de unión del ácido sinápico que favorecía una transferencia electrónica más eficiente al cobre catalítico de la lacasa. Por último, también se evaluó la oxidación de otros fenoles derivados de la lignina por parte de otros mutantes obtenidos por ISM, con el objetivo de encontrar si alguno mejoraba su actividad hacia otros sustratos distintos del ácido sinápico. Entre ellos, se encontraron algunos que oxidaban mejor fenoles de tipo guayacilo y otros compuestos de interés como el resveratrol.

4. Conclusiones

Empleando distintas técnicas de quimeragénesis se han obtenido nuevas lacasas de alto potencial redox con propiedades mejoradas respecto a ambos parentales. Una de estas quimeras, más estable y con mayor actividad sobre fenoles, ha servido como punto de partida para mejorar la oxidación del ácido sinápico, empleando estrategias de diseño semi-racional y ensayos de HTS desarrollados *ad hoc*. Por otro lado, el uso de simulaciones computacionales ha aportado valiosa información sobre los factores estructurales que determinan la oxidación eficiente del sustrato por las lacasas.

SUMMARY

1. Background

Laccases are multicopper oxidases widely distributed in nature although the main producers of these enzymes are the white-wood rot basidiomycete fungi, the only organisms capable of completely degrading lignin. Laccases catalyze the oxidation of a large variety of aromatic compounds coupled to the reduction of oxygen to water. In addition, their catalytic capacities can be enhanced in the presence of redox mediators, low molecular weight compounds that, once oxidized by laccase, can oxidize other compounds that originally are not substrates of the enzyme.

Due to their wide substrate range and their low catalytic requirements, laccases and laccase-mediator systems have a huge biotechnological potential, especially in processes of plant biomass treatment such as the production of paper and pulp or second generation biofuels. Also, these processes generate great amounts of residues enriched with lignin derived phenols that can act as laccase mediators, and their application could help reduce the process's costs and environmental impact. However, for the industrial application of laccases, one may need to adjust their catalytic properties to the operation conditions by protein engineering. Previous works have described the directed evolution of two high-redox potential laccases from the basidiomycetes PM1 (PM1L) and *Pycnoporus cinnabarinus* (PcL). The evolved variants of these laccases, OB1 and 3PO, respectively, are the departure point for this doctoral thesis.

2. Aims

The main goal for this doctoral thesis was to engineer high-redox potential laccases by directed evolution techniques for their application in plant biomass treatment. To this end, we established three major tasks:

- i. To obtain chimeric laccases from the evolved variants of PM1L (OB1) and PcL (3PO), in order to combine and/or enhance their properties of interest and to obtain information on the structural determinants for the activity and stability of these enzymes.
- ii. To develop new laccase activity assays in high-throughput screening (HTS) format in order to explore the mutant libraries generated by directed evolution.
- iii. To enhance laccase activity over lignin-derived phenolic mediators by the semi-rational design of the substrate binding pocket.

3. Results

3.1. Construction of chimeric laccases

First, we applied a random chimeragenesis strategy that combined the *in vitro* and *in vivo* DNA shuffling (CLERY) of OB1 and 3PO genes, selecting those mutants that presented higher activity. The exchanged sequence segments gave way to 3–31 amino acid substitutions in the mature laccase sequences. Among the chimeric laccases obtained, we observed shifted pH activity profiles respecting parent types, and some were also more thermostable. From the battery of generated chimeras, we chose five as possible departure points for future directed evolution studies.

With the aim to obtain variants with a higher number of mutations, we constructed two new laccases by exchanging the second cupredoxin domain between OB1 and 3PO. The two new chimeras presented 65 amino acid substitutions each. One of them presented a shifted pH activity profile, while the other presented an important increase of stability towards temperature, pH and solvents.

3.2. Development of new high-throughput screening methods

We developed several HTS methods to analyze laccase mutant libraries generated by directed evolution. First of all, we designed three assays based on the oxidation of syringyl-type phenols derived from lignin that act as laccase mediators (sinapic acid, syringaldehyde and acetosyringone). Second, we developed an assay specific for high-redox potential laccases based on the oxidation of the synthetic mediator violuric acid, so as to assure that the enzyme's redox potential was not affected during directed evolution. Finally, we designed three assays based on the oxidation of azoic (Evans Blue and Methyl Orange) and anthraquinoid (Remazol Brilliant Blue) synthetic dyes, which are the group of dyes most used in the textile industry and whose biodegradation would be of interest for effluent treatment.

3.3. Semi-rational design of the substrate binding pocket

Taking one of the chimeric laccase obtained by CLERY as departure point, we performed iterative saturation mutagenesis (ISM) over six residues from the substrate binding pocket in order to enhance its activity over phenolic compounds of interest. We used the sinapic acid HTS assay developed in this thesis for the exploration of the mutant libraries. Of all the mutants obtained, two were selected for their biochemical characterization. The new laccase variants presented shifted pH activity profiles towards more neutral values and higher reaction constants than parent type towards sinapic acid and methyl sinapate, although their activity towards other syringyl-type phenols was not significantly enhanced. With the purpose of rationalizing the effect of the mutations in the ISM variants, we

performed PELE and QM/MM computational simulations. These revealed that the mutations introduced allowed a new binding mode of sinapic acid that favored a more efficient electron transfer to the laccase's catalytic copper. Finally, we also evaluated the oxidation of other lignin derived phenols by different ISM mutants, in order to find whether any of them presented increased activity towards other substrates different than sinapic acid. Some of them were able to better oxidize guaiacyl-type phenols and other compounds of interest such as resveratrol.

4. Conclusions

Using different chimeragenesis techniques, we have obtained new high-redox potential laccases with better properties than both parent types. One of this chimeras, more stable and with better activity over phenols, was used as departure point in order enhance the oxidation of sinapic acid by applying semi-rational design strategies and HTS assays developed *ad hoc*. On the other hand, computational simulations have provided valuable information on the structural factors that determine the efficient oxidation of substrate by laccase.

ESTRUCTURA DE LA TESIS

La presente memoria para la obtención del título de Doctor se estructura en cinco capítulos, correspondientes a cuatro artículos científicos ya publicados en revistas indexadas y a uno en preparación. Además, incluye una introducción y una discusión general que relaciona todos ellos. El contenido de los trabajos publicados se ha mantenido íntegramente y el material suplementario, en el caso de que lo hubiera, se ha incluido al final de cada capítulo. Todos los capítulos han sido formateados para mantener una estructura homogénea, y cada uno incluye su propia sección de introducción, materiales y métodos, resultados, discusión y bibliografía. El contenido de los artículos publicados ha sido reproducido con permiso de la editorial (capítulo 1) o bajo la licencia de Acceso abierto (*Open access*).

Listado de publicaciones:

A continuación se enumeran las publicaciones que se han incluido en la presente memoria:

Capítulo 1: Pardo I, Vicente AI, Mate DM, Alcalde M, Camarero S. 2012. Development of chimeric laccases by directed evolution. *Biotechnol. Bioeng.* **109**:2978–2986. (DOI: 10.1002/bit.24588).

Capítulo 2: Pardo I, Chanagá X, Vicente AI, Alcalde M, Camarero S. 2013. New colorimetric screening assays for the directed evolution of fungal laccases to improve the conversion of plant biomass. *BMC Biotechnol.* **13**:90. (DOI: 10.1186/1472-6750-13-90).

Capítulo 3: Pardo I, Santiago G, Gentili P, Lucas F, Monza E, Medrano FJ, Galli C, Martínez AT, Guallar V, Camarero S. 2016. Re-designing the substrate binding pocket of laccase for enhanced oxidation of sinapic acid. *Catal. Sci. Technol.* (DOI: 10.1039/C5CY01725D).

Capítulo 4: Pardo I, Camarero S. 2015. Exploring the oxidation of lignin-derived phenols by a library of laccase mutants. *Molecules* **20**:15929–15943. (DOI: 10.3390/molecules200915929).

Capítulo 5: Pardo I, Camarero S. 2016. A highly stable hybrid laccase obtained by domain swapping. *En preparación*.

INTRODUCCIÓN GENERAL

1. Aspectos generales de las lacasas

Las lacasas son enzimas con actividad fenoloxidasa (EC 1.10.3.2) pertenecientes a la superfamilia de las oxidasas multicobre (MCOs). Catalizan la oxidación de una gran variedad de compuestos, utilizando oxígeno como co-sustrato y generando agua como único subproducto. Aunque actúan principalmente sobre compuestos aromáticos, especialmente fenoles sustituidos y aminas aromáticas, también tienen una actividad limitada sobre iones inorgánicos. Estas enzimas fueron descritas por primera vez a finales del siglo XIX, primero en el árbol de la laca japonés (tradicionalmente *Rhus vernicifera*) y posteriormente como enzimas fúngicas (Yoshida, 1883; Bertrand, 1896). Desde entonces, las lacasas se han encontrado ampliamente distribuidas en la naturaleza, participando principalmente en procesos de polimerización y despolimerización. En plantas, participan en la formación de la pared celular y la lignificación; en bacterias, en la melanización y formación de pigmentos de espora; y en insectos, en la esclerotización de la cutícula en el exoesqueleto. Sin embargo, los mayores productores de lacasas son los hongos, en los que estas enzimas tienen un papel importante en la formación del cuerpo fructífero, en la síntesis de pigmentos, en procesos de patogénesis y en la degradación y reciclado de la lignocelulosa. Dentro de los hongos, destacan especialmente los basidiomicetos degradadores de la madera, los únicos organismos capaces de degradar eficientemente el polímero de lignina (Thurston, 1994; Claus, 2004).

1.1. Estructura de las lacasas

Las lacasas suelen ser proteínas monoméricas con un peso molecular de entre 50 y 100 kDa. Presentan una estructura altamente conservada formada por tres dominios cupredoxina (D1, D2 y D3) y cuatro cobres catalíticos, aunque en los últimos años se han descrito varias lacasas de dos dominios, de origen bacteriano, que forman trímeros activos. En el caso de las lacasas fúngicas, éstas suelen estar N-glicosiladas y presentan dos puentes disulfuro entre dominios, lo cual favorece la estabilidad estructural de la proteína (Hakulinen y Rouvinen, 2015) (**Fig. 1**). El plegamiento de tipo cupredoxina, con una arquitectura de barril- β , se encuentra conservado en otras proteínas de cobre de uno (azurina), dos (nitrito reductasa), tres (ascorbato oxidasa, ferroxidasa Fet3p, bilirrubina oxidasa) o seis dominios (ceruloplasmina), sugiriendo un ancestro común entre ellas (Nakamura y Go, 2005). En el caso particular de las MCOs de tres dominios, el alto grado de homología en el plegamiento global de estas proteínas las hace virtualmente indistinguibles. Sin embargo, es el entorno del cobre de tipo I, significativamente diferente entre las distintas enzimas, el que proporciona la base para las diferentes especificidades de sustrato (Quintanar et al., 2007).

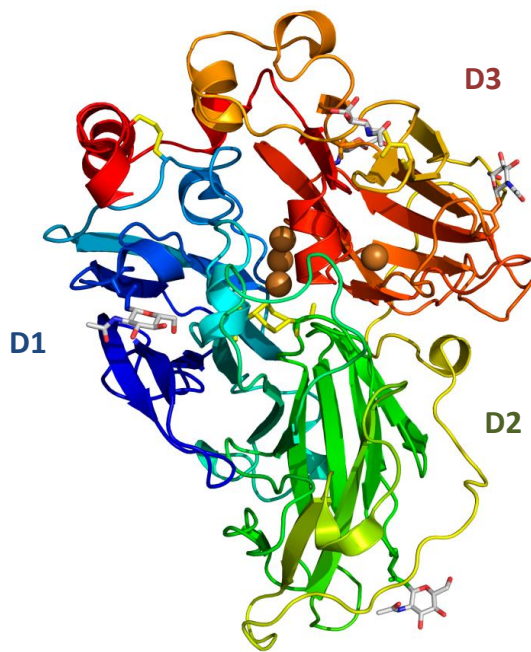


Figura 1. Estructura de la lacasa de *Pycnoporus cinnabarinus* (PDB 2XYB). Los cobres catalíticos están representados como esferas. Los residuos de azúcares están representados como barras blancas (por simplicidad solo se muestra el primer residuo de N-acetil-D-glucosamina). También se muestran los puentes disulfuro en amarillo.

En las lacasas los cuatro átomos de cobre catalíticos se encuentran agrupados en tres sitios redox (Jones y Solomon, 2015):

- Un cobre de tipo I (Cu T1), responsable del color azul de la enzima, que presenta un pico de absorbancia alrededor de los 600 nm y una constante de acoplamiento de resonancia paramagnética electrónica (EPR) de $40\text{--}90 \times 10^{-4} \text{ cm}^{-1}$.
- Un cobre de tipo II (Cu T2), que no da señal en el espectro de absorbancia pero presenta una constante de acoplamiento de EPR de $140\text{--}200 \times 10^{-4} \text{ cm}^{-1}$.
- Dos cobres de tipo III (Cu T3), que presentan un pico de absorbancia alrededor de los 330 nm y no ofrecen señal de EPR debido al acoplamiento antiferromagnético generado al unir un grupo hidroxilo entre ambos.

Los Cu T2 y T3 se encuentran agrupados en el denominado centro trinuclear (TNC), coordinados por ocho residuos de histidina de los dominios D1 y D3, mientras que el Cu T1 está coordinado por dos residuos de histidina y uno de cisteína del D3. El Cu T1 puede estar coordinado además por una metionina como cuarto ligando axial en lacasas de plantas y bacterias, mientras que las lacasas fúngicas presentan un residuo de leucina o fenilalanina en esa posición (ligando no coordinante). De esta forma, el Cu T1 presenta una geometría de coordinación tetragonal o planar trigonal, respectivamente (**Fig. 2**).

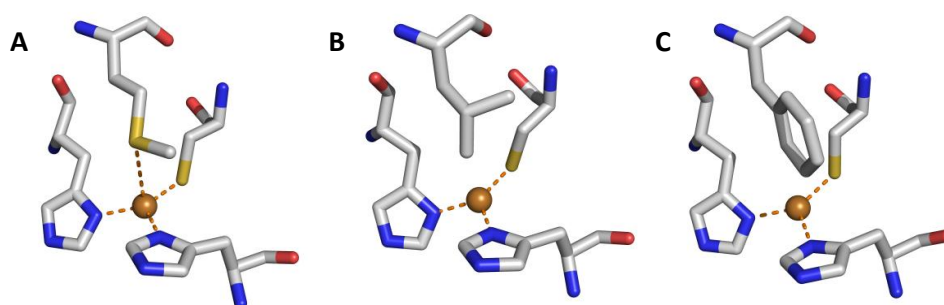


Figura 2. Geometrías de coordinación del Cu T1 en las lacasas de **A)** la bacteria *Bacillus subtilis* (PDB 1GSK), **B)** el ascomiceto *Melanocarpus albomyces* (PDB 1GW0) y **C)** el basidiomiceto *Pycnoporus cinnabarinus* (PDB 2XYB).

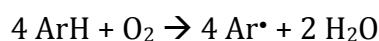
Todos los residuos que participan en la coordinación de los cobres catalíticos se encuentran en cuatro segmentos de secuencia de entre 8 y 24 aminoácidos altamente conservados en lacasas (Kumar et al., 2003), denominados L1–L4 (**Tabla I**).

Tabla I. Motivos de secuencia conservados en lacasas. Los residuos que actúan como ligandos de los cobres catalíticos se muestran en negrita. Adaptado de Kumar et al., 2003.

L1	H -W- H -G-X9-D-G-X5-Q-C-P-I
L2	G-T-X-W-Y- H -S- H -X3-Q-Y-X-D-G-L-X-G-X-(FLIM)
L3	H -P-X- H -L- H -G-H
L4	G-(PA)-W-X-(LFV)- H -C- H -I-(DAE)-X- H -X3-G-(LMF)-X3-(LFM)

1.2. Mecanismo de reacción de las lacasas

Las lacasas catalizan la oxidación monovalente del sustrato aromático acoplada a la reducción de oxígeno molecular a agua según la reacción:



El sustrato es oxidado en el sitio del Cu T1, desde donde los electrones sustraídos son transferidos rápidamente a través del tripéptido His-Cys-His al TNC, donde tiene lugar la reducción del O₂ (**Fig. 3**). Aunque el mecanismo exacto de reacción no se conoce en detalle, comúnmente se acepta que la lacasa actúa a modo de batería, acumulando los electrones de la oxidación secuencial de cuatro moléculas de sustrato y transfiriéndolos de dos en dos al O₂, antes de liberar dos moléculas de agua como sub-producto.

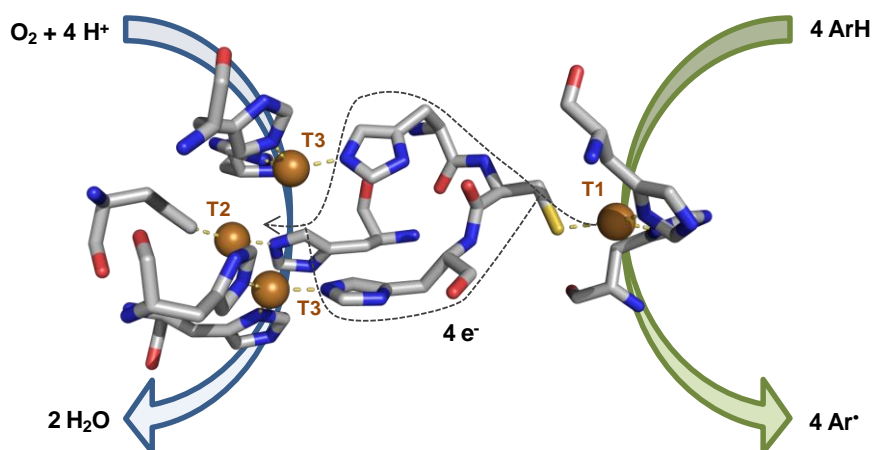


Figura 3. Mecanismo catalítico de las lacasas. Se muestran los residuos que coordinan los cobres catalíticos y la transferencia de electrones desde el Cu T1 al TNC a través del triplete H-C-H. Basado en la estructura de la lacasa de *Pycnoporus cinnabarinus* (PDB 2XYB). Reproducido de Pardo y Camarero, 2015.

El paso limitante de la reacción catalizada por las lacasas es la transferencia electrónica (ET) desde el sustrato al Cu T1, a través de una de las histidinas que actúan como ligandos. Esta ET está determinada principalmente por la diferencia de potencial redox (E°) entre el sustrato y el Cu T1. En función del potencial redox del Cu T1, las lacasas se pueden clasificar como de bajo ($< +500$ mV), medio ($+500$ – 700 mV) o alto ($> +750$ mV) potencial redox (LRPL, MRPL y HRPL, respectivamente). En el primer grupo se encuentran lacasas de plantas y bacterias, mientras que las HRPL son producidas exclusivamente por hongos basidiomicetos. Las MRPL pueden encontrarse tanto en ascomicetos como en basidiomicetos. El potencial redox del Cu T1 es altamente dependiente de la presencia o no de un residuo de metionina como cuarto ligando axial. En las LRPLs, que presentan esta metionina, se postula que la fuerza del enlace Cu–S(Met) modula el potencial redox de las lacasas, siendo mayor cuanto más débil es esta interacción. Sin embargo, existen otros factores que modulan el potencial redox del Cu T1, tales como la hidrofobicidad de los residuos cercanos (especialmente en la posición del cuarto ligando axial), el establecimiento de puentes de hidrógeno con la Cys que coordina el Cu T1 e interacciones electrostáticas o de dipolos entre residuos de la segunda esfera de coordinación, que pueden aumentar o disminuir significativamente el potencial redox (Hong et al., 2011).

Una vez sustraídos cuatro electrones de cuatro moléculas de sustrato, éstos son transferidos al TNC. El modelo del ciclo catalítico de reducción del O_2 a agua comúnmente aceptado se detalla de forma esquemática en la **Fig. 4**. En el estado de reposo los cuatro átomos de cobre están oxidados, con un grupo hidroxilo uniendo ambos Cu T3. La oxidación secuencial de cuatro moléculas de sustrato da paso a la forma reducida de la enzima, que reacciona con el O_2 y da lugar al intermediario

peróxido, en el que dos átomos de cobre del TNC pasan a estar oxidados. A continuación tiene lugar la rotura del enlace O–O con la transferencia de dos electrones desde los otros dos cobres reducidos, obteniendo el intermediario nativo en el que, a diferencia de la forma oxidada inicial, los tres átomos de cobre del TNC están interconectados. Al mismo tiempo, dos residuos ácidos conservados y localizados cerca del TNC asisten en la transferencia de protones al O₂, la cual se ve favorecida a pH bajo. El intermediario nativo puede volver rápidamente a la forma reducida con la oxidación de otras cuatro moléculas de sustrato y liberando dos moléculas de agua, mientras que en ausencia de más sustrato reductor el intermediario nativo decae lentamente a la forma oxidada inicial.

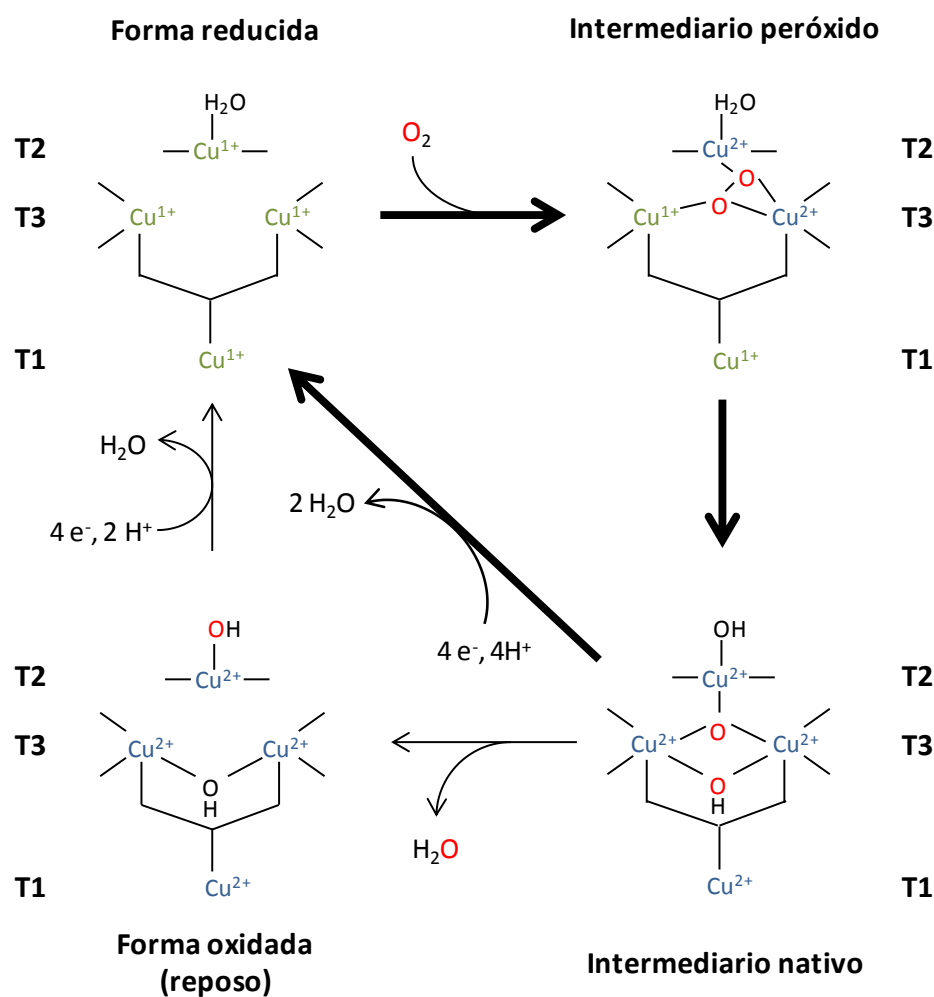


Figura 4. Mecanismo de reducción del O₂ catalizado por las cascas. Las flechas más gruesas indican las reacciones que tienen lugar en el ciclo catalítico en presencia del sustrato reductor. Adaptado de Jones y Solomon, 2015.

Un factor esencial que determina la actividad de las cascas es la unión favorable del sustrato a la enzima. A pesar de la alta similitud estructural de las cascas, existe una gran variabilidad en el tamaño y polaridad de los residuos que delimitan la entrada del bolsillo de unión a sustrato, pertenecientes al D2 y D3. Sin embargo, las

estructuras disponibles de lacasas co-cristalizadas con sustratos fenólicos o inhibidores muestran una disposición parecida (Bertrand et al., 2002; Matera et al., 2008; Kallio et al., 2009). En ellas se aprecia como el sustrato interacciona con una de las histidinas que coordina el Cu T1, que actuaría como aceptor primario del electrón. Además, en las lacasas fúngicas aparece un residuo ácido conservado en el fondo del bolsillo enzimático, generalmente un aspártico, localizado cerca del Cu T1. La presencia de un grupo carboxilato (pK_a 3.0–5.0) en esta posición jugaría un papel importante durante la oxidación de sustratos fenólicos, anclando el sustrato y asistiendo en la sustracción de un protón del grupo hidroxilo. Se postula que la transferencia del protón ocurre de forma concertada a la transferencia electrónica, dando lugar al radical fenoxilo (**Fig. 5**). De esta forma se evitaría la formación de intermediarios de alta energía, como un intermediario oxidado fuertemente ácido, en el caso de que el electrón fuese transferido primero; o un intermediario desprotonado fuertemente reductor, si el protón fuese el primero en ser abstraído (Galli et al., 2013).

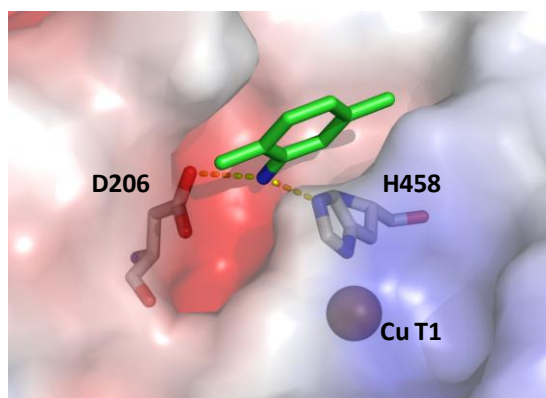


Figura 5. Detalle de la unión de una molécula de 2,5-xilidina (en verde) a la lacasa de *Trametes versicolor* (PDB 1KYA). Se muestran los residuos de ácido aspártico y de histidina aceptores de protón y electrón, respectivamente.

2. Las lacasas y la biomasa vegetal

Como se comentó en el apartado anterior, los principales productores de lacasas en la naturaleza son los hongos degradadores de la madera y saprófitos del suelo. Estos hongos juegan un papel esencial en ciclo del carbono en la Tierra, permitiendo el acceso y la degradación de los polisacáridos presentes en la biomasa vegetal. La biomasa vegetal o lignocelulósica, como su nombre indica, está compuesta principalmente por celulosa, hemicelulosa y lignina. Mientras que la celulosa y la hemicelulosa son polímeros de azúcares más o menos ordenados que sirven como fuente de carbono para otros organismos, la lignina es un polímero aromático formado por distintas unidades de tipo fenilpropano unidas por diversos tipos de enlace, lo que le confiere un carácter altamente heterogéneo y recalcitrante a la degradación. La lignina aporta rigidez a la pared celular, actúa como pegamento entre los polisacáridos y las fibras de la madera, recubre los haces vasculares que transportan el agua por la planta y sirve de barrera frente al ataque de patógenos (Boerjan et al., 2003).

2.1. Composición química de la lignina

La lignina está formada por distintas unidades fenilpropanoides derivadas de la polimerización oxidativa de tres monolignoles básicos: el alcohol *p*-cumarílico (unidades *p*-hidroxifenilo o H), el alcohol coniferílico (unidades guayacilo o G) y el alcohol sinapílico (unidades siringilo o S). Durante la lignificación, la oxidación de los monolignoles genera radicales que se acoplan entre sí, dando lugar a una gran variedad de enlaces éter y C-C de los cuales el mayoritario es el β -O-4 (**Fig. 6**). La proporción de cada tipo de unidad fenilpropanoide varía entre especies de plantas e incluso entre tejidos de un mismo individuo. No obstante, como norma general las gimnospermas presentan un contenido mayoritario de unidades de tipo G con algunas unidades de tipo H, las angiospermas dicotiledóneas poseen unidades de tipo G y S y trazas de tipo H, y las monocotiledóneas presentan unidades de tipo G y S y una mayor proporción de unidades tipo H que las dicotiledóneas (Boerjan et al., 2003). Además de los ya mencionados, se han descrito otros monolignones "no-clásicos" como el acetato de sinapilo, el *p*-cumarato de sinapilo o el *p*-hidroxibenzoato de sinapilo (Lu et al., 2004; Lu y Ralph, 1999; Lu y Ralph, 2002). La esterificación de los monolignoles actuaría como un mecanismo de control de la estructura de la lignina, siendo responsable de la presencia de ligninas altamente aciladas en algunas angiospermas (Martínez et al., 2008).

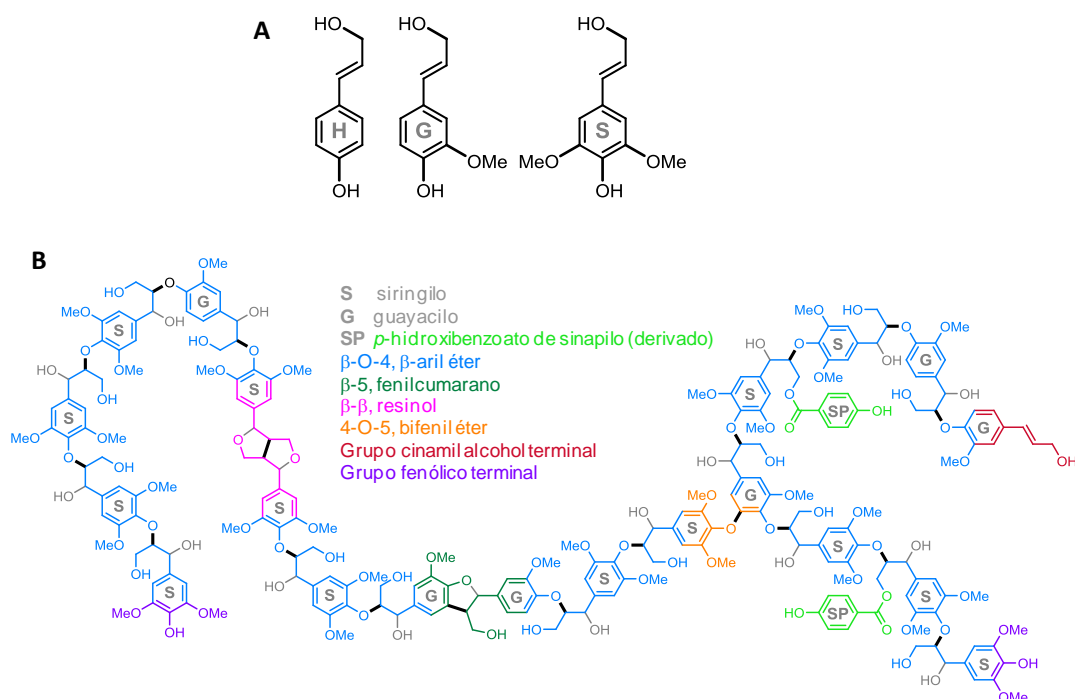


Figura 6. A) Estructura química de los monolignoles clásicos alcohol *p*-cumarílico, alcohol coniferílico y alcohol sinapílico. B) Modelo de la estructura del polímero de lignina de álamo (dicotiledónea) predicha por 2D-NMR. Los distintos tipos de enlaces se muestran con un código de colores. En negrita se muestran los enlaces formados por el acoplamiento de los monolignoles durante la lignificación, mientras que en gris se muestran enlaces y grupos añadidos en reacciones posteriores a la polimerización. Adaptado de Stewart et al., 2009.

2.2. Degradación de la lignina por hongos

Aunque existen varios organismos capaces de modificar o degradar parcialmente la lignina para acceder a los polisacáridos vegetales, los hongos basidiomicetos son los únicos capaces de degradarla completamente y, en algunos casos, de manera selectiva (Camarero et al., 1998; Fernández-Fueyo et al., 2012). En concreto destacan los hongos de la podredumbre blanca de la madera, denominados así por el residuo blanquecino rico en celulosa que generan al eliminar la lignina. La biodegradación de la lignina es un proceso oxidativo inespecífico, catalizado por enzimas y agentes oxidantes de bajo peso molecular. El sistema ligninolítico de los hongos degradadores de la madera incluye peroxidasas ligninolíticas, lacasas y enzimas auxiliares generadoras de peróxido (aril-alcohol oxidasa, glioxal oxidasa). Además intervienen iones metálicos (Fe^{3+} , Mn^{3+}), radicales aromáticos y especies activas de oxígeno (OH^\bullet) (Martínez et al., 2005).

Debido a su alto potencial redox ($\leq +1.0$ V), las peroxidasas ligninolíticas (manganeso peroxidasa, lignina peroxidasa y peroxidasa versátil) son consideradas las principales responsables de la oxidación de la lignina. Las lacasas, con un potencial redox más limitado ($\leq +0.8$ V), sólo serían capaces de oxidar las unidades fenólicas de la lignina, que constituyen alrededor del 10–20% del polímero (Kawai et al., 1987). Sin embargo, el descubrimiento de que ciertos compuestos pueden actuar como mediadores redox una vez oxidados por la lacasa, oxidando a su vez otros compuestos que no serían sustratos naturales de la enzima, ha incentivado el estudio de las lacasas y los sistemas lacasa-mediador en este proceso (Kawai et al., 1989; Bourbonnais y Paice, 1990). La descripción de fenoles derivados de la lignina como mediadores redox de las lacasas (Camarero et al., 2005), además del alto número de copias de genes que codifican para estas enzimas en genomas de hongos degradadores de madera, sugieren que también cumplen un papel importante en la biodegradación de la lignina (Floudas et al., 2012).

2.3. Mediadores naturales de lacasas

Los mediadores de lacasa son moléculas de bajo peso molecular que, una vez oxidados por la lacasa, generan radicales estables que pueden difundir lejos del bolsillo enzimático y oxidar otros compuestos que no pueden ser oxidados por la propia lacasa, debido a su alto potencial redox o a impedimentos estéricos. Los mediadores ideales deben ser reciclables, pudiendo ser nuevamente oxidados por la lacasa sin polimerizar o sin ser degradados (**Fig. 7**).

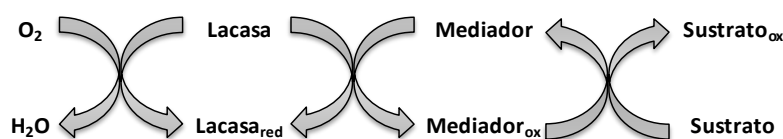


Figura 7. Representación esquemática del sistema lacasa-mediador.

Tradicionalmente, los mediadores de lacasa más estudiados han sido compuestos sintéticos como el 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonato) (ABTS), el 1-hidroxibenzotriazol (HBT), el ácido violúrico (VA) o el radical (2,2,6,6-tetrametilpiperidin-1-il)oxilo (TEMPO) (**Fig. 8**). Existen numerosos trabajos que demuestran la capacidad de estos compuestos para mediar la actividad lacasa sobre compuestos modelo de lignina no fenólicos, contaminantes aromáticos recalcitrantes, colorantes industriales y pastas de papel (Claus et al., 2002; Barreca et al., 2004; Camarero et al., 2004; Bajpai et al., 2006; Husain y Husain, 2007; Morozova et al., 2007).

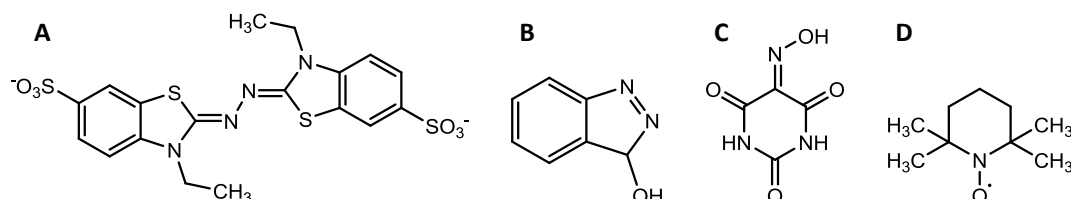


Figura 8. Estructura química de algunos mediadores sintéticos: **A)** ABTS, **B)** HBT, **C)** VA y **D)** TEMPO.

Sin embargo, el elevado coste de los mediadores sintéticos, así como la posible formación de derivados tóxicos, ha propiciado la búsqueda de otros compuestos más baratos y respetuosos con el medio ambiente. Estos incluyen compuestos fenólicos procedentes del metabolismo de los hongos, como el ácido 4-hidroxibenzoico y el alcohol 4-hidroxibencílico (Johannes y Majcherczyk, 2000), o fenoles relacionados con la lignina (aldehídos, cetonas y ácidos) como la acetosiringona, el siringaldehído, la vainillina, el metil vainillato y el ácido *p*-cumárico (Camarero et al., 2005) (**Fig. 9**). Este último grupo de fenoles relacionados con la lignina es de especial interés, ya que estos compuestos se encuentran en las plantas herbáceas estableciendo puentes entre la lignina y las fibras de polisacáridos (en el caso de los ácidos *p*-hidroxicinámicos como el ácido *p*-cumárico) y/o son liberados durante la biodegradación de la lignina (Camarero et al., 1994; Camarero et al., 1997), por lo que pueden ser los verdaderos mediadores redox de las lacasas en la naturaleza (Cañas y Camarero, 2010). Además, estos compuestos fenólicos se pueden obtener fácilmente de los residuos generados durante el pretratamiento de la biomasa vegetal en la producción de bioetanol (Klinke et al., 2004; Adeboye et al., 2014) o de la producción de pasta de papel (Camarero et al., 2007), por lo que se puede disponer de ellos a bajos costes.

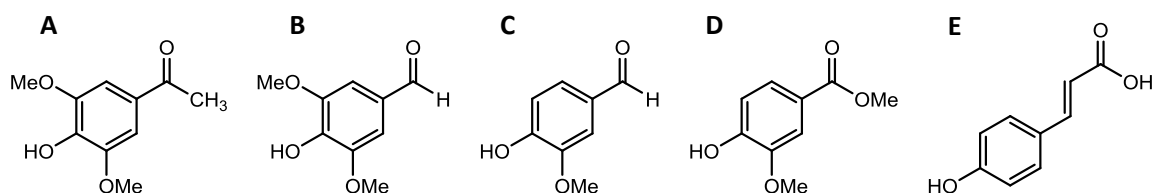


Figura 9. Estructura química de algunos mediadores fenólicos derivados de la lignina: **A)** acetosiringona, **B)** siringaldehído, **C)** vainillina **D)** vainillato de metilo y **E)** ácido *p*-cumárico.

3. Aplicaciones industriales y biotecnológicas de las lacasas

Frente a los problemas acuciantes que suponen el calentamiento global y la limitada disponibilidad de los recursos fósiles, se hace indispensable el desarrollo de tecnologías limpias y el empleo de recursos renovables que reduzcan o incluso eliminen la dependencia del petróleo. La biomasa lignocelulósica es la materia prima renovable más abundante en la Tierra, y en los últimos años se han hecho enormes esfuerzos para aprovechar esta biomasa en la producción de biocombustibles de segunda generación. Sin embargo, en éste y otros procesamientos de biomasa como es la producción de pasta de papel, se genera un exceso de residuos de lignina que tradicionalmente se han infrautilizado (generalmente se queman en planta para obtener la energía necesaria para el proceso). No obstante, la lignina es una valiosa fuente de productos químicos, por lo que su valorización constituye uno de los grandes retos para la viabilidad económica de las biorefinerías de lignocelulosa (Zakzeski et al., 2010; Ragauskas et al., 2014). De forma análoga a la refinería del petróleo, el concepto de biorefinería lignocelulósica tiene por objeto el aprovechamiento integral de todos los componentes de la biomasa vegetal para la generación de combustibles, materiales y otros productos de valor añadido, reduciendo a la vez el balance de CO₂ y la generación de residuos a cero (**Fig. 10**). Con este fin se están dedicando considerables esfuerzos para el desarrollo de rutas catalíticas específicas para la oxidación/despilimerización de la lignina y su transformación, y, en este particular, el empleo de oxidorreductasas ligninolíticas como biocatalizadores surge como una opción muy prometedora (Camarero et al., 2014).

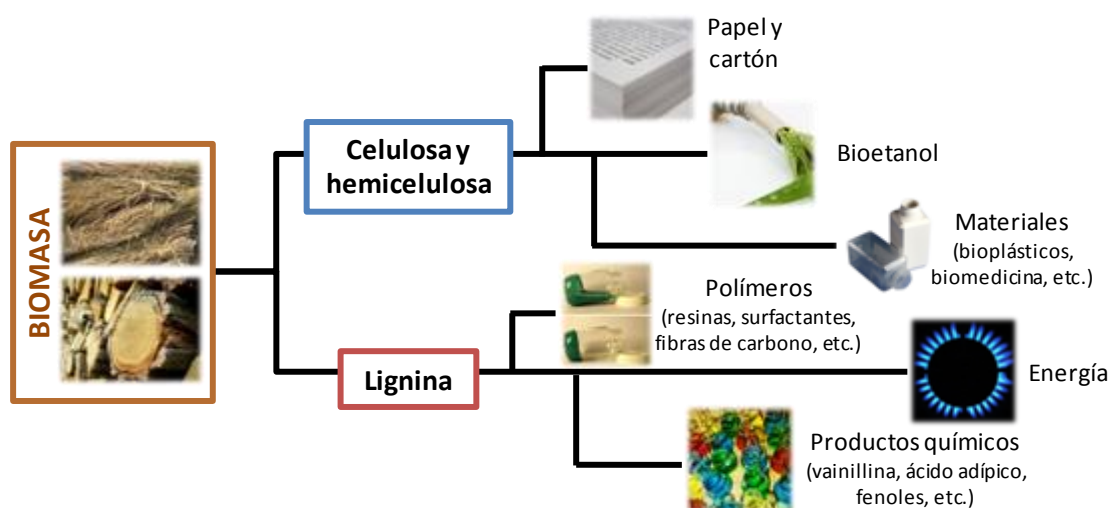


Figura 10. Representación esquemática del concepto de aprovechamiento integral de la biomasa vegetal en las biorefinerías lignocelulósicas.

Es evidente que las lacasas y los sistemas lacasa-mediador tienen un gran potencial de uso en las biorefinerías lignocelulósicas (Cañas y Camarero, 2010; Roth y Spiess, 2015), pero además, gracias a su versatilidad catalítica, encuentran aplicación en muchos otros procesos industriales y biotecnológicos (Pezzella et al., 2015). A continuación se enumeran algunas posibles aplicaciones de las lacasas que se han descrito en la literatura.

- Industria papelera:

Las fábricas de pasta de papel pueden ser el punto de partida para el lanzamiento de las biorefinerías lignocelulósicas. Actualmente, la celulosa se utiliza para hacer pasta de papel, los reactivos se reciclan y los residuos no aprovechables se queman para proporcionar energía a la fábrica. Sin embargo, las ligninas “técnicas” se podrían aprovechar para la producción de productos de valor añadido, y de los licores negros se podrían extraer compuestos fenólicos de interés como mediadores redox de lacasa para su empleo en el blanqueo de las pastas (Camarero et al., 2007; Cañas y Camarero, 2010). De hecho, muchos estudios con lacasas y sistemas lacasa-mediador se han realizado con vistas a la deslignificación y el blanqueo de las fibras de celulosa: desde los primeros estudios sobre sistemas lacasas-mediador (Bourbonnais y Paice, 1992) hasta la integración de etapas enzimáticas en secuencias de blanqueo respetuosas con el medio ambiente para mejorar la calidad de las pastas obtenidas en procesos TCF (del inglés *Totally Chlorine Free*) (Camarero et al., 2004; Ibarra et al., 2006a; Ibarra et al., 2006b). Más recientemente también se ha descrito el uso de lacasas en el destintado de papel reciclado (Fillat et al., 2012) y en la funcionalización de las fibras del papel gracias al acoplamiento de distintos fenoles (Aracri et al., 2010).

- Producción de bioetanol:

Como alternativa al bioetanol de primera generación, obtenido a partir de azúcares fácilmente fermentables como el azúcar (sacarosa) de caña o remolacha o el almidón del grano de maíz, el etanol de segunda generación se basa en el aprovechamiento de los azúcares polimerizados en la lignocelulosa, ya sea de residuos forestales o agrícolas o de cultivos energéticos que no compiten con el sector alimentario. Sin embargo, este proceso requiere de un pretratamiento (generalmente físico-químico) de la biomasa que haga accesible los polímeros de celulosa y hemicelulosa, seguido de una hidrólisis enzimática de dichos polisacáridos para liberar los azúcares fermentables. Durante el paso de pretratamiento se liberan compuestos fenólicos (entre otros compuestos) que inhiben la posterior fermentación alcohólica llevada a cabo por la levadura (Klinke et al., 2004; Adeboye et al., 2014). Numerosos trabajos muestran que la adición de lacasas a la biomasa pretratada, bien tras la hidrólisis enzimática o en un paso de sacarificación y fermentación simultáneas, mejoran los rendimientos de producción

de etanol (Jurado et al., 2009; Moreno et al., 2012; Alvira et al., 2013). Nuevamente, en este proceso se genera un exceso de residuos de lignina que también puede emplearse para la obtención de productos de alto valor añadido (Zakzeski et al., 2010; Ragauskas et al., 2014).

- Industria textil:

Al igual que con la pasta de papel, las lacasas y los sistemas lacasa-mediador pueden utilizarse para el blanqueo de las fibras textiles gracias a su capacidad de actuar sobre una gran variedad de colorantes orgánicos sintéticos. De hecho, existen preparados comerciales de lacasa-mediador para obtener el efecto lavado en el tejido vaquero basados en la decoloración del tinte índigo (DeniLite, Novozymes). Además, pueden emplearse para el tratamiento y decoloración de las aguas residuales generadas en el proceso de tinción (Claus et al., 2002; Camarero et al., 2005). En el sentido contrario, las lacasas también pueden catalizar la síntesis de colorantes derivados de compuestos fenólicos y aminas aromáticas (como los colorantes de tipo azo, los más utilizados a nivel industrial) (Polak y Jarosz-Wilkolazka, 2012). Por último, destaca también el uso de lacasas para la funcionalización de las fibras, aportándoles propiedades antioxidantes, antimicrobianas o hidrófobas (Hossain et al., 2009).

- Industria alimentaria:

El tratamiento con lacasas sobre masas para pan genera entrecruzamientos de ésteres de ácido ferúlico con la matriz de arabinoxilanos, mejorando su fuerza y estabilidad y reduciendo su adherencia, lo cual facilita el manejo de las masas con maquinaria. Las lacasas también se emplean para el tratamiento de otros alimentos, ralentizando su deterioro o mejorando sus propiedades organolépticas. En bebidas como vinos, cervezas o zumos, las lacasas permiten la eliminación de polifenoles y evitan la formación de precipitados, y al tratar los tapones de corcho también se evita la transferencia de sabor al vino (Osma et al., 2010).

- Síntesis orgánica:

Las lacasas pueden utilizarse en la transformación de compuestos y en la síntesis de polímeros como alternativa a procesos químicos más agresivos o tóxicos. Se han descrito varias reacciones de síntesis de compuestos de interés farmacéutico catalizadas por lacasas o sistemas lacasa-mediador, tales como los antitumorales actinocina y vinblastina y derivados de antibióticos β -lactámicos. En cuanto a la síntesis de polímeros, se ha descrito el acoplamiento oxidativo de flavonoides para obtener polímeros con mayor actividad biológica (antioxidante, antitumoral, antiviral, etc.) que los flavonoides libres; la síntesis de polifenoles que pueden emplearse como lacas, resinas o adhesivos “verdes” libres de formaldehído para la

manufactura de tableros y aglomerados de madera; y la síntesis de polímeros conductores como polianilina, politiofeno y polipirrol (Karamyshev et al., 2003; Song y Palmore, 2005; Kunamneni et al., 2008; Shumakovich et al., 2012).

- Biorremediación:

Además del tratamiento de efluentes procedentes de la industria papelera y textil, así como de efluentes ricos en fenoles de las industrias cerveceras y aceiteras, las lacasas y los sistemas lacasas-mediador son capaces de degradar xenobióticos como hidrocarburos aromáticos policíclicos, clorofenoles, o compuestos fitosanitarios (contaminantes emergentes) presentes en agua o suelos (Strong y Claus, 2011).

- Nanobiotecnología:

Por último, destaca el creciente interés en el uso de lacasas en nanodispositivos como biosensores para la detección de fenoles, azidas, flavonoides, etc., o en biopilas de combustibles, dada la capacidad de las lacasas (de alto potencial redox) para sustraer electrones directamente de un cátodo (Le Goff et al., 2015).

4. Ingeniería de lacasas mediante evolución dirigida

Dados su amplio rango de sustratos reductores y sus bajos requerimientos catalíticos, las lacasas tienen un gran potencial biotecnológico, tal y como se ha comentado en el apartado anterior. Sin embargo, para su aplicación industrial se necesitan grandes cantidades de enzima que sea activa y estable en las condiciones de operación. Esto ha motivado que en los últimos 20 años numerosos estudios se hayan orientado a mejorar las lacasas, tanto por diseño racional como por evolución dirigida (Mate y Alcalde, 2015; Pardo y Camarero, 2015). La disponibilidad de varias estructuras de lacasas en las bases de datos ha permitido que se hayan llevado a cabo diversas aproximaciones de mutagénesis dirigida, destinadas tanto a la comprensión del mecanismo catalítico de estas enzimas como a mejorar su actividad. No obstante, es mediante la evolución dirigida de estas enzimas que se han obtenido lacasas mucho más activas y robustas que las encontradas en la naturaleza, con mejoras que habrían sido difíciles de obtener por diseño racional.

4.1. Principios de la evolución dirigida

La evolución dirigida es una herramienta muy potente para la ingeniería de proteínas que consiste en reproducir en el laboratorio los principales pasos de la evolución natural (**Fig. 11**). En primer lugar se genera variabilidad genética, bien mediante la mutagénesis aleatoria de un gen parental o mediante la recombinación de varios genes homólogos. La librería de genes mutantes obtenida se transforma y expresa en un hospedador heterólogo adecuado. A continuación, se mide la actividad o propiedad que se quiere mejorar mediante técnicas de cribado de alto rendimiento

o *high-throughput screening* (HTS). Aquellos mutantes ganadores que presentan las características deseadas son seleccionados y sometidos a una nueva ronda de mutagénesis y/o recombinación. Este ciclo se repite durante varias generaciones, acumulando mutaciones beneficiosas en la proteína hasta que se alcanza la propiedad deseada o ésta no se puede mejorar más.

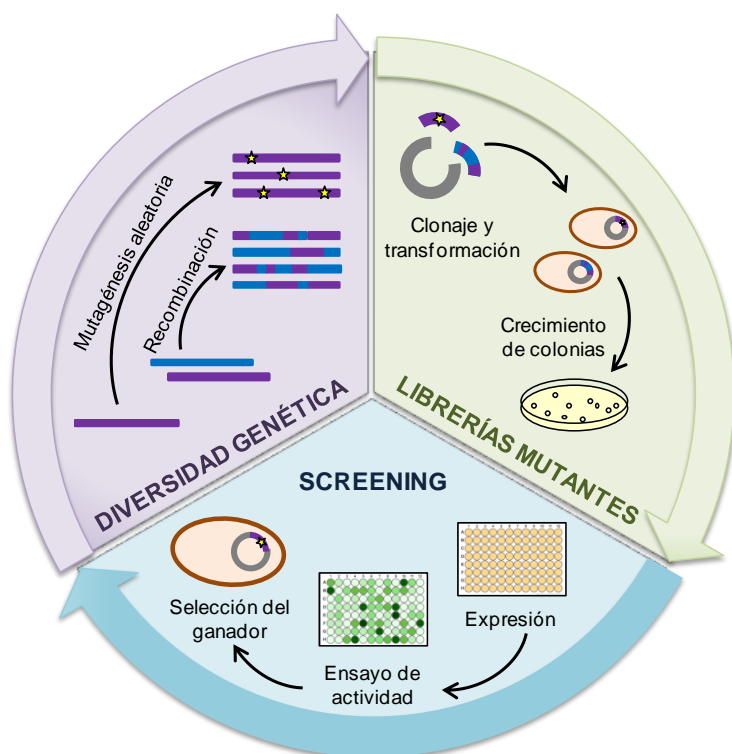


Figura 11. Representación esquemática de un ciclo de evolución dirigida. Adaptado de Pardo y Camarero, 2015.

A diferencia del diseño racional de enzimas mediante mutagénesis sitio-dirigida, la evolución dirigida presenta la ventaja de que no se necesita un conocimiento previo de la estructura o del mecanismo catalítico de la proteína. Sin embargo, cada uno de los tres pasos básicos de un ciclo de evolución en el laboratorio representa un cuello de botella que debe tenerse en cuenta para el éxito de este método.

- Selección del hospedador heterólogo:

Se considera un hospedador heterólogo apropiado un organismo de rápido crecimiento, cuya manipulación genética sea fácil y que exprese la proteína de forma activa. Comúnmente, se emplean la bacteria *Escherichia coli* o la levadura *Saccharomyces cerevisiae*, para los cuales existe un gran abanico de vectores episómicos que pueden resultar apropiados para la expresión de distintas proteínas. Sin embargo, debido a su capacidad de llevar a cabo las modificaciones post-traduccionales necesarias para la secreción de la enzima activa, tales como el procesamiento del péptido señal, la formación de puentes disulfuro o la glicosilación de la proteína, *S. cerevisiae* es el hospedador más apropiado para la expresión heteróloga de glicoproteínas eucariotas como las lacasas fúngicas. Además, su

eficiente maquinaria de reparación de DNA y su alta tasa de recombinación homóloga convierten a la levadura en una herramienta muy útil de ingeniería genética molecular, tanto para el clonaje como para la recombinación *in vivo* de DNA (González-Pérez et al., 2012).

- Creación de variabilidad genética:

Existen numerosos métodos de mutagénesis que abarcan un menor o mayor espacio de secuencia proteica y que generan más o menos variantes que deben ser exploradas en el posterior paso de *screening*. En primer lugar destaca por su facilidad la PCR propensa a error (*epPCR*), consistente en emplear DNA polimerasas de baja fidelidad o en utilizar condiciones de reacción que favorezcan la introducción de mutaciones aleatorias durante la amplificación del gen de interés, tales como altas concentraciones de MgCl₂, la adición de MnCl₂, concentraciones no equilibradas de los distintos dNTPs o bajas concentraciones de DNA molde. Habitualmente la tasa de mutación se ajusta a 1–3 mutaciones por kilobase para obtener una librería con una proporción adecuada de mutantes activos, ya que se estima que en torno al 50–70% de las mutaciones resultan deletéreas para la función de la proteína. Por otro lado, estas condiciones de mutagénesis suelen ser más propensas a introducir transiciones (A↔G y C↔T) que transversiones (A/G↔C/T), lo cual, a causa de la degeneración del código genético, da lugar a una alta proporción de mutaciones silenciosas y conservativas, reduciendo el espacio de secuencia proteica que se puede explorar. Ambos hechos implican que, en teoría, se necesite analizar vastas librerías de mutantes para poder encontrar una mutación beneficiosa. A pesar de ello, un gran número de trabajos exitosos han puesto de manifiesto la validez de la *epPCR* como método de mutagénesis, dando lugar a variantes de proteína mejoradas con tan solo unas cuantas mutaciones puntuales acumuladas tras varios ciclos de evolución. No obstante, cabe destacar que en los últimos años se han desarrollado varias técnicas para enriquecer el espectro mutacional y aumentar el espacio de secuencia proteica explorado (Shivange et al., 2009), tales como TriNEx (*TriNucleotide Exchange*, Baldwin et al., 2008), TIM (*Transposon Integration mediated Mutagenesis*, Hoeller et al., 2008) o SeSaM-Tv (*Transversion-enriched Sequence Saturation Mutagenesis*, Wong et al., 2008).

La mutagénesis puede realizarse sobre todo el gen (*random mutagenesis*) o focalizarse sobre ciertas “zonas calientes” o residuos concretos de la proteína que se sepa de antemano pueden afectar a la propiedad deseada (*focused mutagenesis*). Esto permite la creación de librerías inteligentes que aumenten la proporción de mutantes activos y permitan reducir el esfuerzo de *screening*. Algunos ejemplos de técnicas que limitan la mutagénesis a zonas concretas elegidas de antemano incluyen OSCARR (*One-pot Simple methodology for Cassette Randomization and Recombination*, Hidalgo et al., 2008) y MORPHING (*Mutagenic Organized*

Recombination Process by Homologous In vivo Grouping, González-Pérez et al., 2014). En caso de querer mutar algún residuo en concreto se puede aplicar la mutagénesis saturada, consistente en sustituir dicho residuo por todos los demás aminoácidos posibles, empleando cebadores mutagénicos que incluyan codones degenerados en la posición deseada. Esta estrategia se puede aplicar sobre varios residuos a la vez en la llamada mutagénesis combinatorial saturada (CSM). Sin embargo, el número de clones a analizar para poder explorar todas las posibles combinaciones aumenta de forma exponencial con cada residuo mutado (**Tabla II**). Con el fin de reducir el esfuerzo de *screening*, es común el empleo de codones con degeneración reducida o de estrategias como ISM (*Iterative Saturation Mutagenesis*, Reetz y Carballeira, 2007), la cual permite explorar combinaciones de mutaciones con el fin de descubrir posibles relaciones de epistasia.

Tabla II. Codones degenerados para mutagénesis saturada y número de clones que deben ser analizados para obtener una cobertura de la librería del 95% (sobremuestreo). Modificado de Reetz y Carballeira, 2007.

Degeneración	Nº de codones	Aminoácidos codificados	Nº clones (1 posición)	Nº clones (2 posiciones)	Nº clones (3 posiciones)
NNN	64	Todos (20)	192	12270	785200
NNK/NNS	32	Todos (20)	96	3066	98163
NDT	12	RNDCGHILFSYV (12)	36	430	5175
DBK	18	ARCGILMFSTWV (12)	54	969	17470
NRT	8	RNDCGHSY (8)	24	190	1532

La recombinación de los genes de las distintas variantes obtenidas por las técnicas de mutagénesis descritas arriba permite acelerar la evolución dirigida mediante la incorporación de varias mutaciones beneficiosas en un mismo gen y la eliminación de mutaciones perjudiciales (Stemmer, 1994a), o combinar librerías con distintos espectros mutacionales (Zumárraga et al., 2008a). Sin embargo, la variante de proteína final resultante por lo general no presentará más de 10–15 mutaciones. En este sentido, la recombinación de genes homólogos resulta particularmente interesante (Cramer et al., 1998). Ésta permite la introducción de un alto número de mutaciones en un único ciclo de evolución y la exploración de un gran espacio de secuencia sin que el plegamiento y la funcionalidad nativos de la proteína se vean comprometidos. Al igual que con la mutagénesis, la recombinación de DNA puede realizarse de forma aleatoria o racional. Dentro de la primera estrategia se incluyen técnicas dependientes de secuencia, como el barajado de DNA o *DNA shuffling in vitro* (Stemmer, 1994b) e *in vivo* (CLERY, *Combinatorial Libraries Enhanced by Recombination in Yeast*, Abécassis et al., 2000), StEP (*Staggered Extension Process*, Zhao et al., 1998) o RACHITT (*Random Chimeragenesis on Transient Templates*, Coco et al., 2001), que limitan los puntos de recombinación entre genes a zonas muy conservadas (identidad de secuencia superior al 60%); y técnicas independientes de secuencia que tratan de sortear este sesgo, tales como ITCHY (*Incremental*

Truncation for the Creation of Hybrid Enzymes, Ostermeier et al., 1999), SCRATCHY (Lutz et al., 2001) o SHIPREC (*Sequence Homology-Independent Protein Recombination*, Sieber et al., 2001). Como ejemplo de recombinación racional cabe destacar SCHEMA, un algoritmo que permite detectar puntos óptimos de entrecruzamiento basándose en la estructura de la proteína minimizando la pérdida de interacciones entre aminoácidos al intercambiar los fragmentos de secuencia entre los parentales (Voigt et al., 2002).

- High-throughput screening (HTS):

El mayor cuello de botella en evolución dirigida es el análisis de las librerías de mutantes, principalmente por la capacidad de procesamiento. Por ejemplo, la sustitución de un único aminoácido por cualquier otro en todas las posiciones de una proteína de 300 residuos puede dar lugar a 5700 variantes distintas, mientras que la sustitución de dos aminoácidos ya genera 32381700 variantes posibles (Tracewell y Arnold, 2009). En algunas ocasiones se pueden relacionar directamente el fenotipo y el genotipo, empleando técnicas como el *phage display* y el *ribosome display* o bien asociando la supervivencia de la célula hospedadora a la actividad de la proteína de interés. Estas estrategias permiten la selección directa entre millones de clones, aunque su aplicación está limitada a solo unas pocas proteínas. En el caso de la evolución dirigida de enzimas como las lacasas es necesario recurrir al aislamiento y análisis de clones individuales mediante ensayos de actividad en formato *high-throughput screening* (HTS), generalmente en placas de multipocillo, que sólo permiten analizar unos miles de clones ($< 10^4$) por generación (Packer y Liu, 2015). Estos ensayos de HTS deben ser rápidos, sensibles y reproducibles, permitiendo detectar pequeñas diferencias de actividad entre mutantes. De este modo son de especial interés los ensayos colorimétricos, en los se puede seguir la desaparición de un sustrato o la formación de un producto coloreados de manera sencilla en un espectrofotómetro para placas multipocillo (**Fig. 12**).

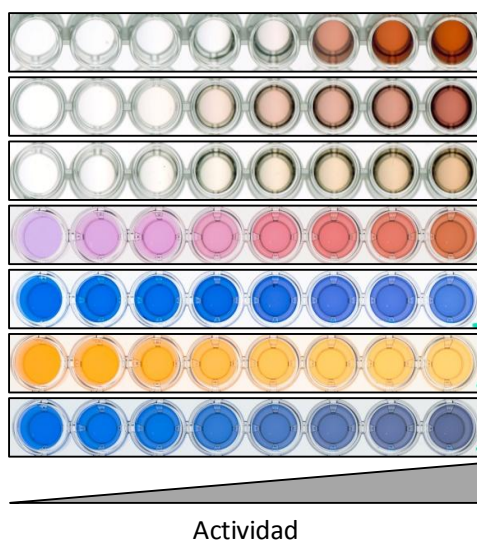


Figura 12. Ejemplos de ensayos HTS colorimétricos con distintos sustratos de lacasa.

Otro aspecto esencial de los ensayos de HTS son las condiciones de presión selectiva que se imponen. Una de las máximas de la evolución dirigida se resume en la frase “*you get what you screen for*”, entendiéndose que, en principio, sólo se podrá mejorar la proteína en aquellas características que se están evaluando con el método de *screening* empleado. Por tanto, si se quiere mantener un balance entre mejorar la propiedad de interés y no perjudicar otras características de la proteína que resulten beneficiosas, se hace necesario realizar ensayos de HTS múltiples, tales como el empleo de varios sustratos para no perder la versatilidad catalítica de la enzima, la introducción de ensayos de estabilidad o utilizar distintas condiciones de reacción (pH, temperatura, co-solventes, etc.) simultáneamente (**Fig. 13**).

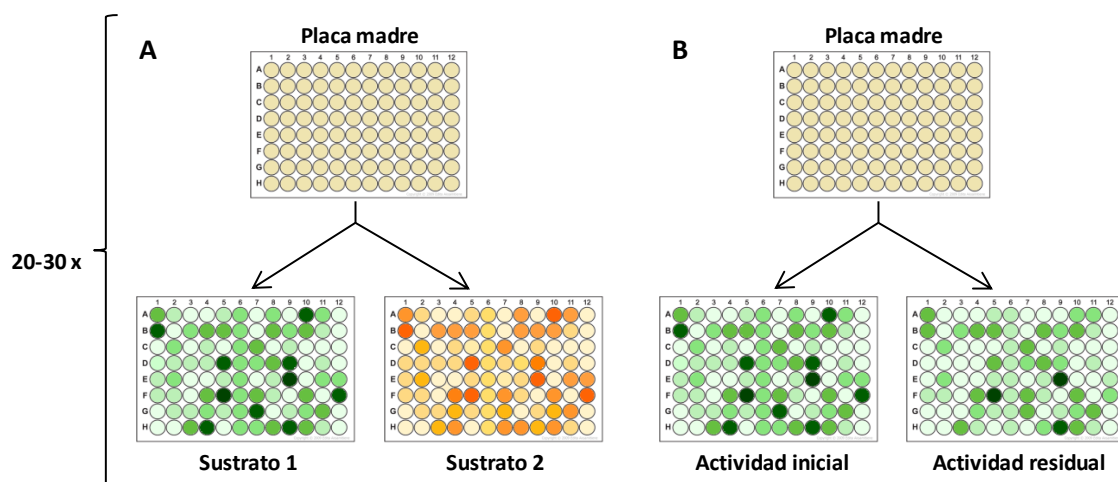


Figura 13. Ejemplos de ensayos múltiples de HTS colorimétricos: **A)** Ensayo con dos sustratos distintos. **B)** Ensayo de estabilidad en el que se compara la actividad inicial y la residual tras un tiempo de incubación en unas condiciones dadas.

4.2. Distintas estrategias de evolución dirigida

Aunque la evolución dirigida clásica es una herramienta muy potente para la ingeniería de proteínas, pueden emplearse estrategias mixtas que permiten obtener enzimas con mayores incrementos en actividad y/o estabilidad o incluso con actividades nuevas, además de generar una gran cantidad de información valiosa sobre las relaciones estructura-función de las proteínas.

- Diseño semi-racional asistido por computación:

Los avances en computación que se han dado de forma paralela a la expansión de la evolución dirigida han permitido adoptar estrategias semi-racionales en la ingeniería de proteínas, combinando herramientas de evolución dirigida aleatoria con el diseño racional asistido por simulación. El diseño semi-racional permite reducir el esfuerzo de *screening* y aumentar el espacio de secuencia explorado, pero requiere de unas nociones mínimas de las relaciones estructura-función de la proteína de interés (Cheng et al., 2015). Por ejemplo, en caso de querer mejorar la

actividad enzimática habría que emplear mutagénesis focalizada sobre zonas del bolsillo de unión a sustrato o sobre residuos cercanos a los catalíticos, mientras que si se quiere mejorar la estabilidad de la proteína habría que mutagenizar zonas lábiles o ciertos residuos para introducir interacciones estabilizantes (**Fig. 14**).

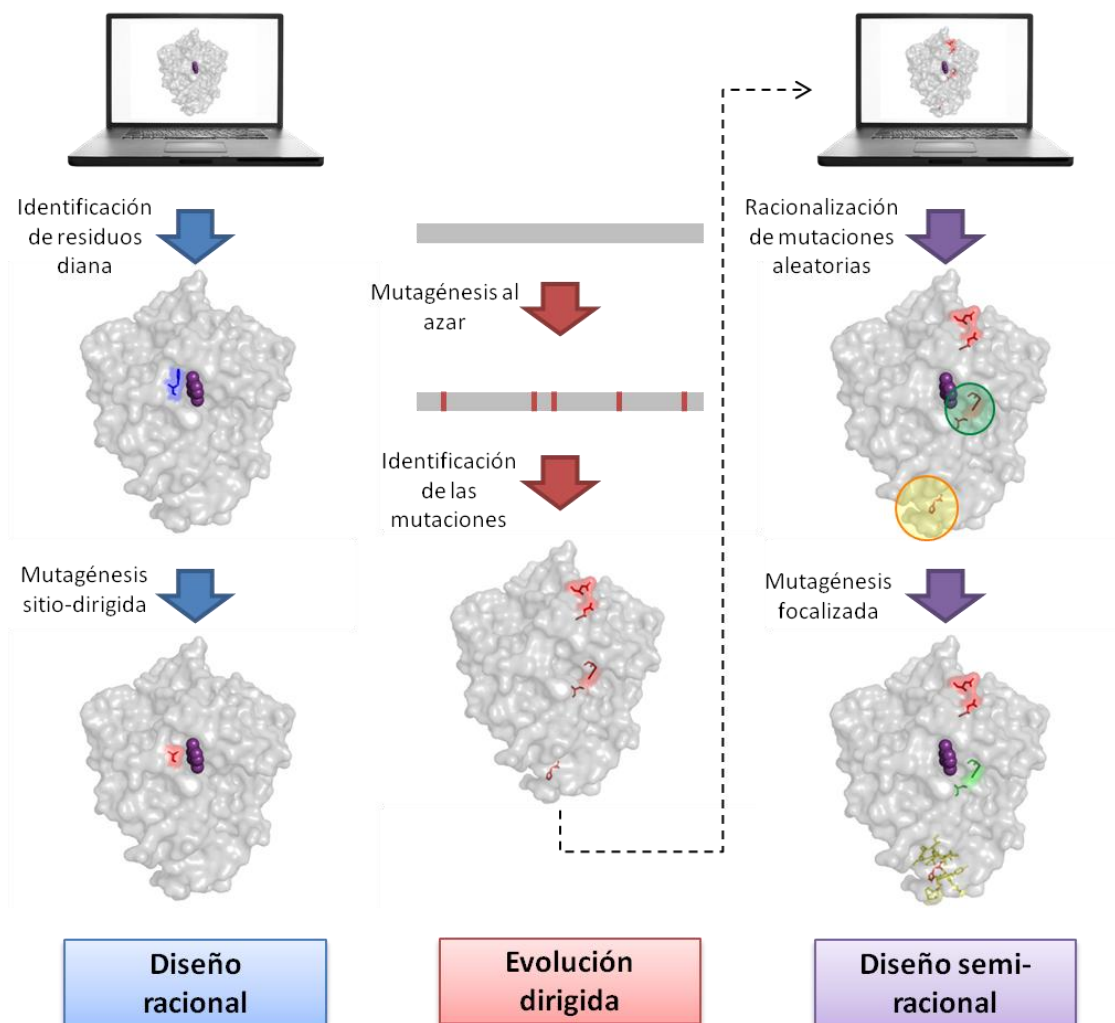


Figura 14. Distintas estrategias de ingeniería de proteínas. En el diseño racional se seleccionan residuos diana y se diseñan mutaciones basándose en conocimientos de la estructura-función de la proteína. En el ejemplo, se muta un residuo del bolsillo enzimático (azul) que interactúa con el sustrato (morado). En la evolución dirigida se introducen mutaciones aleatorias y se seleccionan aquellas que resultan beneficiosas para la propiedad que se desea mejorar, sin necesidad de tener un conocimiento previo de las relaciones estructura-función de la proteína. En el diseño semi-racional se seleccionan determinados residuos (en el ejemplo mostrados en verde) o zonas de interés (en el ejemplo mostrada en amarillo) bien conocidas de antemano o reveladas por mutagénesis aleatoria y selección, que pueden volver a someterse a una mutagénesis focalizada.

En los últimos años se han desarrollado numerosas herramientas bioinformáticas para asistir a la ingeniería de proteínas, tanto en el diseño de librerías inteligentes como en la identificación de zonas o residuos críticos para mejorar la propiedad de interés, basándose en alineamientos de secuencias y/o en la estructura tridimensional de la proteína (Voigt et al., 2001; Chaparro-Riggers et al., 2007).

Algunos ejemplos de estos programas incluyen PoPMuSiC (*Prediction of Proteins Mutations Stability Changes*, Kwasigroch et al., 2002), FRESCO (*Framework for Rapid Enzyme Stabilization by Computational libraries*, Wijma et al., 2014), REAP (*Reconstructed Evolutionary Adaptive Path*, Chen et al., 2010), HotSpot Wizard (Pavelka et al., 2009) o 3DM (Kuipers et al., 2010).

Por otro lado, el análisis *a posteriori* de las mutaciones aleatorias que han dado lugar a variantes de proteína mejoradas tras varios ciclos de evolución dirigida puede ofrecer información valiosa sobre las relaciones estructura-función de la proteína de interés, revelando nuevas dianas para la mutagénesis que hubiesen sido difíciles de predecir por diseño puramente racional. En este sentido, la simulación por computación basada en estudios de dinámica molecular (MD), ensayos de difusión de ligando (PELE) y cálculos híbridos de mecánica cuántica y mecánica molecular (QM/MM) ha resultado de gran ayuda tanto para la racionalización del efecto de las mutaciones obtenidas experimentalmente (Molina-Espeja et al., 2015; Monza et al., 2015) como para la selección de nuevas dianas para el diseño de enzimas novedosas (Lucas et al., 2016).

- Evolución adaptativa frente a deriva genética:

En una aproximación clásica de evolución dirigida, también conocida como evolución adaptativa, se selecciona en cada generación el clon o clones que presenten una mejora con respecto al parental, de manera que con los sucesivos ciclos se van acumulando mutaciones beneficiosas y la característica de interés va mejorando de forma escalonada (*fitness up-hill walk*). Sin embargo, tras varios ciclos de mutagénesis, es posible que no se consiga mejorar más la propiedad de interés, por lo general debido a la descompensación entre la actividad y la estabilidad de la proteína (Bloom et al., 2006). En contraposición a esta estrategia, la deriva genética consiste en introducir mutaciones neutras en la secuencia de la proteína. Las mutaciones neutras en principio no tienen efecto sobre el plegamiento ni la actividad nativa de la proteína, pero, a través de relaciones epistáticas, pueden favorecer la aparición de actividades “promiscuas” distintas a la original y/o generar variantes polimórficas más robustas y “evolucionables” que permitan la asimilación de un mayor número de mutaciones que el parental (Bloom y Arnold, 2009) (**Fig. 15**). Existen varios trabajos en los que se lleva a cabo de forma exitosa la deriva genética mediante mutagénesis aleatoria, seleccionando en cada ciclo los clones que mantienen la actividad parental y generando un *pool* genético variado antes de proseguir con una estrategia de evolución adaptativa (Amitai et al., 2007; Bloom et al., 2007a; Bloom et al., 2007b; Smith et al., 2011). No obstante, la recombinación de genes homólogos es sin duda la forma más rápida y sencilla de generar gran variabilidad de secuencia, al permitir introducir un gran número de mutaciones neutras en un único ciclo (Cramer et al., 1998).

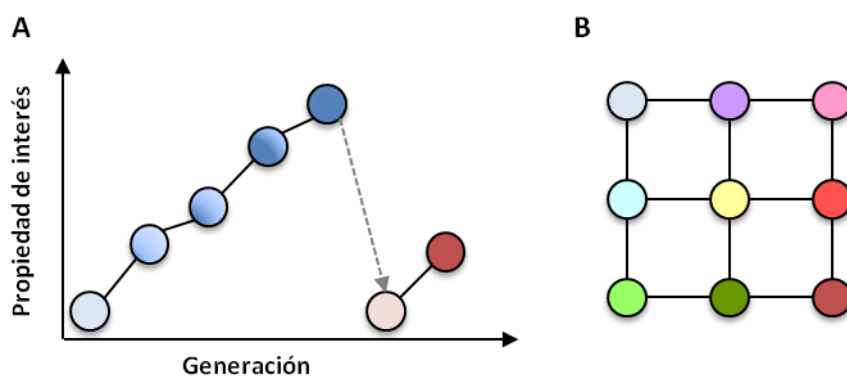


Figura 15. Representación esquemática de distintas estrategias de evolución dirigida. **A)** En una estrategia de evolución adaptativa se selecciona en cada generación aquel clon que mejora la propiedad de interés (en azul). Sin embargo, para obtener otra propiedad distinta, como actividad sobre un sustrato no natural (en rojo), puede ser necesario pasar por un mínimo local del *fitness* de la proteína a través de una mutación deletérea. **B)** En una estrategia de deriva genética se obtiene la nueva propiedad de interés a través de varios pasos neutrales intermedios que no suponen cambios sustanciales en la función nativa de la proteína, pero que en cambio revelan actividades promiscuas y/o la preparan para una más fácil evolución hacia nuevas funciones.

4.3. Evolución dirigida de lacasas de alto potencial redox

La primera lacasa que fue sometida a un proceso de evolución dirigida fue la MRPL del hongo ascomiceto *Myceliophthora thermophila* (Bulter et al., 2003). Desde entonces, se han publicado varios trabajos sobre la evolución dirigida de ésta (Zumárraga et al., 2007; Zumárraga et al., 2008b; Torres-Salas et al., 2013) y otras lacasas como la del hongo *Pleurotus ostreatus* (POXA1b, Festa et al., 2008; Miele et al., 2010a; Miele et al., 2010b), las de las bacterias *Bacillus subtilis* (CotA, Gupta et al., 2010; Gupta and Farinas, 2010) y *Bacillus licheniformis* (Koschorreck et al., 2009) o incluso una lacasa bacteriana cuyo gen codificante fue aislado de un metagenoma (Liu et al., 2011). Sin embargo, debido a su mayor potencial de aplicabilidad y a su difícil expresión heteróloga, cabe destacar la evolución dirigida las HRPLs de los basidiomicetos PM1 (PM1L, Maté et al., 2010) y *Pycnoporus cinnabarinus* (PcL, Camarero et al., 2012), cuyas variantes evolucionadas son el punto de partida de esta tesis doctoral.

Dados los bajos niveles de secreción de PM1L y PcL en *S. cerevisiae*, la mejora de su expresión funcional en la levadura constituyó un objetivo en sí mismo en los primeros ciclos de evolución dirigida de estas enzimas, complementario a la mejora de su actividad. Por tanto, la selección de los ganadores en ambas rutas evolutivas se basó en los incrementos de actividad total (TAI), que incluían tanto mejoras catalíticas como mejoras en la secreción de dichas enzimas. Además, para evitar perder la versatilidad catalítica propia de las lacasas, los ensayos de HTS se realizaron de forma paralela con dos sustratos de lacasa: ABTS y 2,6-dimetoxifenol (DMP). En primer lugar, se sustituyeron los péptidos señales nativos de cada lacasa por el péptido señal del factor alfa de *S. cerevisiae*. Ambas construcciones de fusión

fueron sometidas a varias rondas de *ep*PCR y barajado de DNA *in vivo*, de modo que se seleccionaron mutaciones tanto en el péptido señal como en las lacasas maduras (**Fig. 16**). En el caso de PM1L se consiguieron valores de TAI de 34000 veces y niveles de producción de 8 mg L⁻¹ tras ocho ciclos de evolución (Maté et al., 2010), mientras que para P_cL se consiguieron valores de TAI de 8000 veces y niveles de producción de 2 mg L⁻¹ tras seis ciclos de evolución (Camarero et al., 2012).

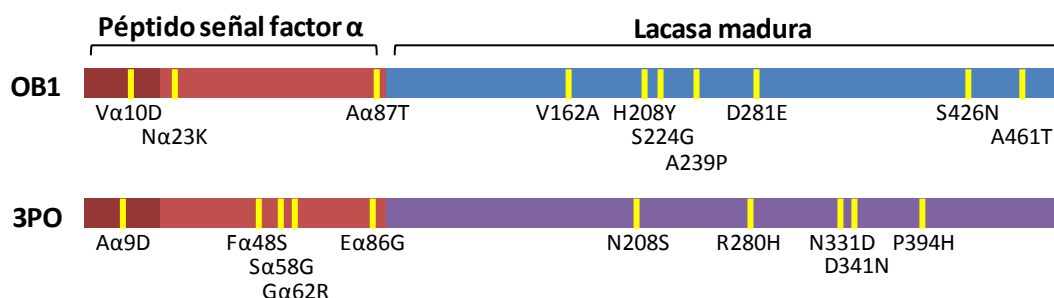


Figura 16. Representación esquemática de los genes de las variantes evolucionadas de αPM1L (OB1) y αP_cL (3PO). En rojo se muestra el péptido señal del factor alfa, con la secuencia correspondiente al pre-líder en rojo oscuro y la del pro-líder en rojo claro. Las secuencias de las lacasas maduras se muestran en azul para OB1 y en violeta para 3PO. Las mutaciones seleccionadas durante la evolución dirigida se muestran en amarillo.

- Mutaciones en el péptido señal del factor alfa:

La variante evolucionada de PM1L (OB1) presentaba tres mutaciones en el péptido señal del factor alfa, mientras que la variante evolucionada de P_cL (3PO) presentaba cinco. En el caso de P_cL se comprobó que el péptido señal evolucionado mejoraba por sí solo 40 veces la secreción de la lacasa nativa en levadura (α*-P_cL). Sin embargo, cabe destacar que las mejoras de secreción alcanzadas no se debían únicamente a las mutaciones en el péptido señal, sino que también podían ser causadas por mutaciones silenciosas que daban lugar a codones de mayor frecuencia de uso en la levadura o por mutaciones no sinónimas en la lacasa madura que favorecían su plegamiento y/o secreción. Curiosamente, ambas variantes presentaban dos mutaciones muy similares entre sí en el péptido señal. Por un lado, estaban las mutaciones Vα10D en OB1 y Aα9D en 3PO. Estas mutaciones están localizadas en el núcleo hidrofóbico en el pre-líder, involucrado en la translocación de la proteína al retículo endoplásmico durante su traducción (Nothwehr y Gordon, 1990). Ambas mutaciones podían ser intercambiadas entre las dos lacasas, dando lugar a incrementos similares de secreción en la levadura. Por otro lado, estaban las mutaciones Aα87T en OB1 y Eα86G en 3PO, localizadas en el péptido espaciador entre el péptido señal y la lacasa madura en las construcciones de fusión. Este péptido espaciador (EAEA) es un sitio de procesamiento reconocido por la proteasa STE13 (Romanos et al., 1992). Ambas mutaciones provocan un fallo de procesamiento que genera una extensión de seis aminoácidos extra en el extremo N-terminal de las lacasas maduras (**Fig. 17**). Cuando se hizo una nueva construcción

para OB1 en la que se eliminó por completo el péptido espaciador, se comprobó que la lacasa madura tenía las mismas propiedades con y sin la extensión de seis aminoácidos en el N-terminal, pero que la secreción se reducía un 40% (Mate et al., 2013a).

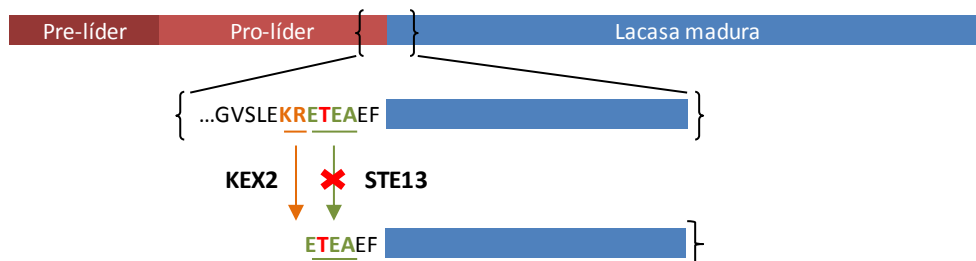


Figura 17. Representación esquemática del procesamiento del factor alfa durante la maduración de la lacasa evolucionada OB1 (adaptado de Mate et al., 2013).

- Mutaciones en la lacasa madura:

La variante evolucionada OB1 presentaba siete sustituciones aminoacídicas respecto al parental PM1L en la secuencia de la lacasa madura (**Fig. 18A**). A pesar de ello, OB1 presentaba constantes cinéticas similares a las de la lacasa silvestre producida por el hongo. Al comparar OB1 con los ganadores de generaciones anteriores, se comprobó que los valores de k_{cat} fueron mejorando significativamente generación tras generación. Esto sugería que la lacasa α -PM1L recombinante presentaba características catalíticas peores que las de la lacasa silvestre. Sorprendentemente, sólo tres de las mutaciones seleccionadas en OB1 se encontraban cerca del centro catalítico y del bolsillo de unión a sustrato (**Fig. 18C**). La mutación V162A afectaba a uno de los residuos que delimita la entrada del bolsillo enzimático. La mutación S426N podría cambiar la orientación del residuo adyacente T427, que a su vez establece puentes de hidrógeno con una de las histidinas ligando del Cu T1 (H394). Por último, la mutación A461T se encontraba adyacente a F460, posición que correspondería al cuarto ligando axial del Cu T1 en lacasas de plantas y bacterias. Esta mutación podría provocar una alteración de la geometría local del Cu T1. Las otras cuatro mutaciones (H208Y, S224G, A239P y D281E) se encontraban alejadas del centro catalítico, por lo que su posible efecto en la actividad de la lacasa es difícil de racionalizar. Tampoco se puede descartar que estén implicadas en mejorar la secreción de la lacasa en la levadura.

En el caso de la evolución dirigida de P_cL, se seleccionaron cinco mutaciones en la secuencia de la lacasa madura (**Fig. 18B**). La variante evolucionada 3PO presentaba mejoras catalíticas importantes (k_{cat} 10–20 veces mayores) con respecto tanto a P_cL silvestre producida por el hongo como a α^* -P_cL expresada por la levadura. Además, debido a la presión selectiva del ensayo de HTS realizado a pH 5, el perfil de pH óptimo de la lacasa se fue desplazando hacia valores más neutros en cada

generación. Tres de las mutaciones seleccionadas se encontraban cerca del centro catalítico (**Fig. 18D**). Las mutaciones N208S y N331D se encontraban en *loops* (lazos) que conforman el bolsillo enzimático, cercanos a residuos que están directamente involucrados en el reconocimiento enzima-sustrato (D206 y F332). Por otro lado, la mutación P394H se encontraba adyacente al residuo ligando del Cu T1 H395. Al introducir esta mutación en PM1L por mutagénesis sitio-dirigida también se lograron importantes incrementos de actividad, aunque finalmente se descartó debido a que provocaba una pérdida de termoestabilidad. Por último, las mutaciones R280H y D341N se encontraban en superficie, lejos del centro catalítico.

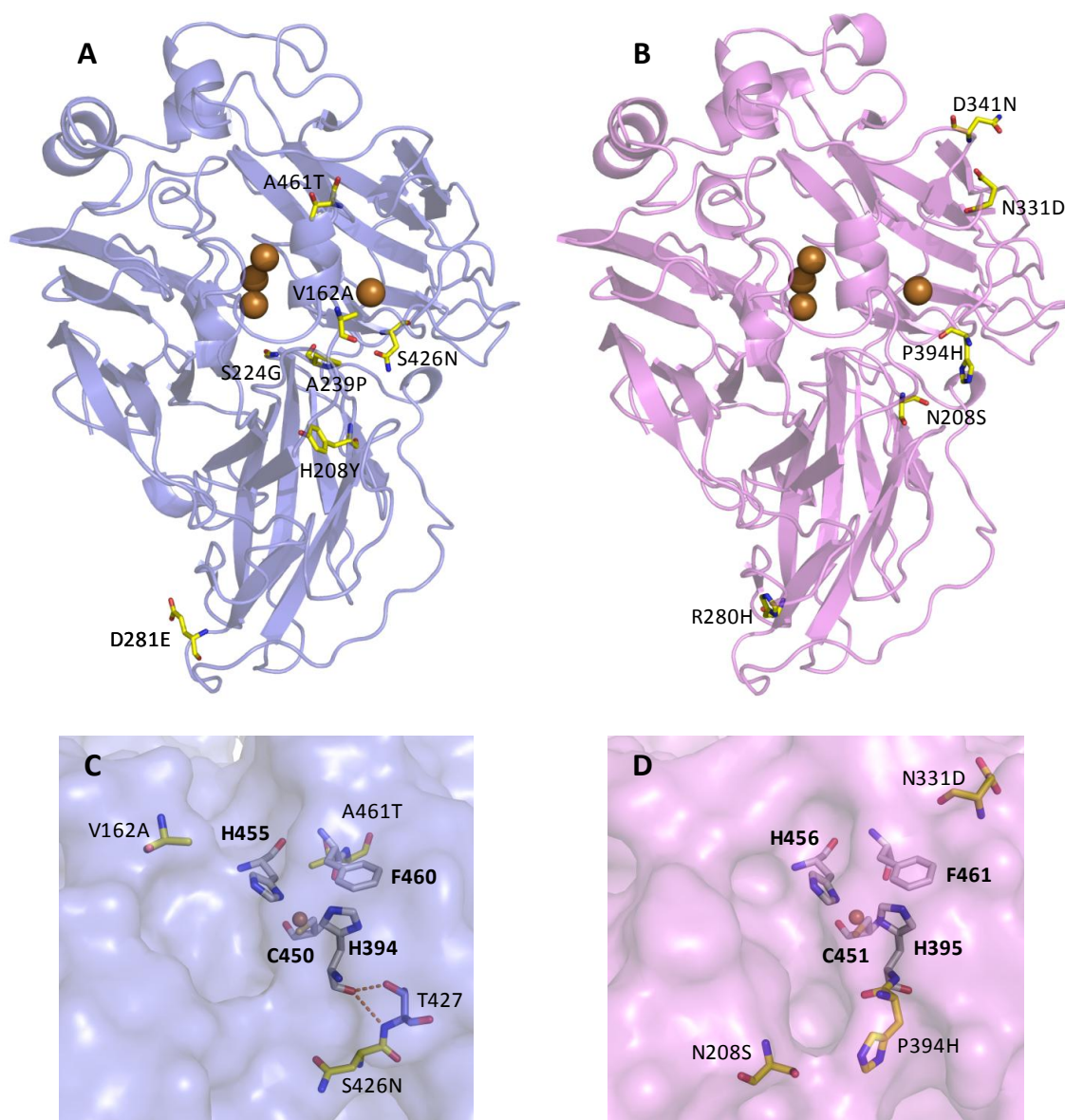


Figura 18. En la parte superior, modelos de las variantes evolucionadas **A)** OB1 y **B)** 3PO. En la parte inferior, detalle del entorno del Cu T1 de **C)** OB1 y **D)** 3PO. Las mutaciones seleccionadas en cada variante se muestran en amarillo. Los ligandos del Cu T1 (junto con F460/F461) se muestran en blanco.

Nuevamente, se observaron algunas similitudes en las mutaciones seleccionadas en las secuencias de las lacasas maduras. Curiosamente, tanto en OB1 como en 3PO no se seleccionó ninguna mutación beneficiosa en el D1 de las lacasas. También es interesante destacar que dos de las mutaciones seleccionadas en PM1L y PcL están en sitios parecidos. Por un lado, H208Y en OB1 y N208S en 3PO, que se encuentran en el mismo *loop* que el residuo de aspartato responsable de la abstracción del protón del sustrato (D205 en OB1 y D206 en 3PO); y por otro, D281E en OB1 y R280H en 3PO, pertenecientes al mismo *loop* distal y que podrían estar relacionadas con una mejor secreción. Estos resultados son un ejemplo de cómo la evolución dirigida puede revelar puntos calientes para la ingeniería de proteínas, aportando información valiosa sobre relaciones estructura-función que puede ser aplicada en posteriores estudios de diseño racional.

Debido al gran interés biotecnológico de las variantes evolucionadas de lacasas de alto potencial redox obtenidas en estos trabajos, por su eficiencia catalítica, estabilidad y niveles de expresión en levadura, éstas se han empleado como punto de partida para trabajos posteriores de evolución dirigida. Por un lado OB1 ha sido evolucionada para ser activa en sangre para su uso en nanodispositivos (Mate et al., 2013b), mientras que por otro, se han obtenido lacasas quiméricas con nuevas propiedades a partir de OB1 y 3PO, tal y como se describe en esta tesis doctoral.

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OBJETIVOS

OBJETIVOS

El objetivo general de esta tesis doctoral fue la ingeniería de lacasas de alto potencial redox mediante técnicas de evolución dirigida para su aplicación en el tratamiento de la biomasa vegetal. Para ello, se plantearon tres grandes tareas:

- i. Obtener lacasas quiméricas a partir de las variantes evolucionadas de PM1L (OB1) y PcL (3PO) con el fin de combinar y/o mejorar sus propiedades de interés y obtener información sobre los determinantes estructurales de la actividad y estabilidad de estas enzimas. Para llevar a cabo este objetivo, se plantearon dos estrategias de recombinación, aleatoria o racional, de los genes parentales.
- ii. Desarrollar nuevos ensayos de determinación de actividad lacasa en formato de *high-throughput screening* (HTS) para la exploración de las librerías de mutantes generadas por evolución dirigida, con especial interés en el diseño de métodos colorimétricos basados en la oxidación de compuestos fenólicos relacionados con la biomasa vegetal.
- iii. Mejorar la actividad lacasa sobre fenoles derivados de la lignina mediante el diseño semi-racional del bolsillo de unión a sustrato, con la creación de librerías inteligentes y el estudio computacional de las mutaciones seleccionadas.

CHAPTER 1

DEVELOPMENT OF CHIMERIC LACCASES BY DIRECTED EVOLUTION

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ABSTRACT

DNA recombination methods are useful tools to generate diversity in directed evolution protein engineering studies. We have designed an array of chimeric laccases with high-redox potential by *in vitro* and *in vivo* DNA recombination of two fungal laccases (from *Pycnoporus cinnabarinus* and PM1 basidiomycete), which were previously tailored by laboratory evolution for functional expression in *Saccharomyces cerevisiae*. The laccase fusion genes (including the evolved α -factor prepro-leaders for secretion in yeast) were subjected to a round of family shuffling to construct chimeric libraries and the best laccase hybrids were identified in dual high-throughput screening (HTS) assays. Using this approach, we identified chimeras with up to six crossover events in the whole sequence, and we obtained active hybrid laccases with combined characteristics in terms of pH activity and thermostability.

INTRODUCTION

Laccases (EC 1.10.3.2) are blue-multicopper oxidases capable of acting on a wide range of aromatic compounds, especially phenolic compounds and aromatic amines. These enzymes were first discovered in the 19th century and they are widely distributed in nature, where they participate in synthesis and degradation processes (Claus, 2004; Thurston, 1994). White-rot basidiomycete fungi, the only organisms capable of complete degradation of lignin, are the most important producers of laccases. Laccases use molecular oxygen as the final electron acceptor and they release water as the only by-product. They contain four copper atoms arranged in three oxidation-reduction sites. The reducing substrate is oxidized at the T1 Cu site and the electrons from four monovalent oxidations are transferred to the trinuclear Cu cluster, formed by one T2 and two T3 Cu sites, where one molecule of oxygen is reduced to two molecules of water (Morozova et al., 2007). Depending on the redox potential at the T1 Cu site, laccases are sorted as low- (< 470 mV), medium- (470-700 mV) or high- (> 700 mV) redox potential enzymes (Shleev et al., 2005).

Due to their oxidative versatility and low catalytic requirements, fungal laccases, especially the high-redox potential laccases (HRPLs), are of particular interest as potential biocatalysts in different industrial sectors. However, for successful biotechnological application, the intrinsic properties of the enzyme usually need to be adjusted by protein engineering to comply with operational requirements. Unfortunately, white-rot fungi, the only known HRPL producers, are not amenable to genetic manipulation and it is difficult to heterologously express HRPLs (Rodgers et al., 2010). Recently, we described the *in vitro* evolution of HRPLs from PM1 basidiomycete (PM1L) and *Pycnoporus cinnabarinus* (PcL), which share 76%

sequence identity, resulting in their successful functional expression in *S. cerevisiae* (Maté et al., 2010; Camarero et al., 2012). To accelerate directed evolution for improved secretion, native signal leaders were replaced by the prepro-leader of the α -mating factor from *S. cerevisiae* and the whole α -laccase constructs were subjected to random mutagenesis and recombination. Laccase secretion by yeast was significantly increased in the final evolved mutants (2–8 mg L⁻¹), which also showed improved kinetic values for phenolic and non-phenolic compounds. Moreover, the pH activity profile of evolved P_cL shifted upwards and the evolved PM1L had greater thermal stability.

DNA shuffling has become the most commonly used method to create chimeric genes since it was described by Stemmer (1994). In the present study, we sought to build complex chimeric libraries through DNA shuffling of HRPL genes to generate new rearranged proteins with modified/improved characteristics over the parent laccases. Using the combinatorial libraries enhanced by recombination in yeast (CLERY) strategy (Abécassis et al., 2000), the two evolved PM1L and P_cL genes (Maté et al., 2010; Camarero et al., 2012) were recombined *in vitro* and *in vivo*, and a new collection of active chimeras was obtained after high-throughput screening (HTS) of the chimeric libraries. The selected laccase hybrids with combined properties constitute the first successful example of chimeragenesis of HRPLs from different fungi.

MATERIAL AND METHODS

Reagents and enzymes

DMP (2,6-dimethoxyphenol) and the *S. cerevisiae* transformation kit were purchased from Sigma-Aldrich (Madrid, Spain). ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DNase I from bovine pancreas lyophilisate and the high pure plasmid isolation kit were purchased from Roche. Zymoprep yeast plasmid miniprep II kit was purchased from Zymo Research (Orange, CA), and the Plasmid midi kit and QIAquick gel extraction kit from QIAGEN (Valencia, CA). The restriction enzymes BamHI, NotI and BsaXI were obtained from New England Biolabs (Hertfordshire, UK), and Pfu Ultra High-Fidelity DNA polymerase was purchased from Strategene (La Jolla, CA).

Strains and culture media

The protease deficient *S. cerevisiae* BJ5465 strain was purchased from LGCPromochem (Barcelona, Spain). The uracil-independent and ampicillin-resistance shuttle pJRoC30 vector carrying the 3P₀ (evolved P_cL) and OB1 (evolved PM1L) constructs (including their evolved α prepro-leaders) under the control of the GAL1 promoter were generated as described previously (Camarero et al., 2012; Maté et al., 2010). The BJ5465 yeast strain was grown in YPD medium. Specific

uracil-deficient media used to grow yeast transformants and the laccase expression media supplemented with ethanol and copper have been described previously (Camarero et al., 2012). The DH5 α *E. coli* strain was used to amplify the plasmids and it was grown in LB medium.

Construction of chimeric libraries using CLERY

General aspects:

All PCR products and/or digested fragments were purified by loading the reaction products into 0.8% medium EEO agarose gels and using the QIAquick gel extraction kit. The fragments were cloned into the pJRoC30 vector (previously linearized with NotI and BamHI to remove the native gene) by designing overhangs that specifically annealed with the ends of the linearized vector (annealing regions of 40 bp and 66 bp).

Optimization of *in vitro* DNA shuffling:

In vitro DNA shuffling was conducted as described previously (Joern, 2003; Zhao and Arnold, 1997) with some modifications. Parent DNA was obtained by digesting pJR3PO and pJROB1 with BsaXI, as the plasmid contains two restriction sites flanking the complete fusion gene. Thus, 2.8 kbp fragments were generated, purified and concentrated. Then, a mixture of 1.25 μ g of each parent gene was digested with 0.02 units of DNase I in a volume of 50 μ L for 5 minutes at 15 $^{\circ}$ C in the thermocycler (Eppendorf Mastercycler pro S). Digestion was stopped by adding 5 μ L of 0.5 M EDTA on ice and the reaction products were separated in an agarose gel, producing a typical smear of DNA fragments ranging from 80 to 1500 bp. The 80–350 bp and 350–700 bp fragments were purified and processed separately to create libraries L1 and L2, respectively. The PCR reassembly reaction mixture contained 25 μ L of fragments (of either 80–350 or 350–700 bp), 5 μ L of 10X PfuUltra HF buffer, 1 μ L of 40 mM dNTP mix and 2.5 units of PfuUltra HF DNA polymerase in a final volume of 50 μ L. PCR cycles were carried out as described previously (Abécassis et al., 2000) and fragments were amplified with primers designed to generate the aforementioned overhangs: RMLN sense (5'-CCTCTATACTTTAACGTCAAGG-3') which binds to nucleotides 160–180 of the plasmid; and RMLC antisense (5'-GGGAGGGCGTGAATGTAAGC-3'), which binds to nucleotides 2031–2050 of the plasmid. The reaction mixture contained 1, 5 or 10 μ L of the reassembly product, 5 μ L of 10X PfuUltra HF buffer, 3% DMSO, 1 mM dNTP mix, 0.25 μ M of each primer and 2.5 units of PfuUltra HF DNA polymerase in a final volume of 50 μ L. Reactions were performed as follows: 95 $^{\circ}$ C for 2 min; 28 cycles at 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 2 min; and a final elongation step at 72 $^{\circ}$ C for 10 min. Maximum amplification yield was obtained with 10 μ L of the reassembly product derived from small fragments and 5 μ L derived from large fragments. Bands of 1.9 kb (1.8 kb of α -

laccase plus the overhangs generated with RMLN and RMLC) were purified and subjected to *in vivo* DNA shuffling.

- *In vivo* DNA shuffling:

Of the purified PCR products generated by *in vitro* DNA-shuffling, 400 ng was mixed with 100 ng of the linearized vector (the molar insert:vector ratio was 27:1) and used to transform *S. cerevisiae*-competent cells. Transformed cells were then plated on synthetic complete (SC) drop-out plates and the colonies of cells containing the whole autonomously replicating vector were grown for 48 h at 30 °C. Up to 3000 transformants per µg of DNA were obtained for L2 and 1400 transformants per µg of DNA for L1. Of these, 550 individual colonies were picked from each library and screened, using transformed cells with plasmids harboring the parent genes for reference during the library screening.

High-throughput screening

- Laccase activity screening:

Individual clones were picked and cultured in 50 µL of minimal medium in sterile 96-well plates (Falcon BD, Meylan Cedex, France), in which columns 6 and 7 were inoculated with the parent types, and one uninoculated well (H1) served as a negative control. The plates were sealed and incubated at 30 °C in 60% humidity with agitation (200 rpm). After 48 hours, 160 µL of expression medium (Camarero et al., 2012) was added and the plates were incubated for a further 24 hours at 20 °C, after which they were centrifuged at 2200 x *g* for 15 minutes at 4 °C (Eppendorf centrifuge 5430 R). A 20 µL volume of supernatant was transferred to two replica plates (Greiner Bio-One) using a Quadra 96-320 Liquid Handler (Tomtec, Hamden, CT, USA). To each replica plate 180 µL of 3 mM ABTS or DMP was added in 100 mM acetate buffer (pH 5.0) and the absorption was measured at 418 nm ($\epsilon_{\text{ABTS}^{*+}} = 36000 \text{ M}^{-1} \text{ cm}^{-1}$) and 469 nm ($\epsilon_{\text{DMP}} = 27500 \text{ M}^{-1} \text{ cm}^{-1}$) using a SPECTRAMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA). Plates were incubated in the darkness at room temperature (RT) until the color fully developed and the endpoint absorptions were measured. Relative activities were calculated from the differences between the final and initial absorption and normalized against each parent type in the corresponding plate.

- First re-screening:

Aliquots (5 µL) from microcultures of the selected clones (21 clones from L1 and 47 from L2) were removed from the master plates and used to inoculate 50 µL of minimal medium in columns 2 and 7 of new sterile 96-well plates. Wells D7 and E7 were inoculated with the two parent types. Columns 1 and 12, and rows A and H, were not used. Plates were sealed and incubated for 24 hours at 30 °C with agitation

at 200 rpm. Next, 5 μ L of growth medium was transferred to the four adjacent wells and the plates were incubated for another 24 hours. Finally, 160 μ L of expression medium was added to each well and the plates were incubated for an additional 24 h at 20 °C. Laccase activity was measured as described above.

- Second re-screening:

Aliquots (100 μ L) from the selected clones (12 clones from L1 and 29 from L2) were used to inoculate 3 mL of YPD medium, which was then incubated for 24 hours at 30 °C. The plasmids isolated from these cultures were transformed into *E. coli*, and single colonies of these cells grown on LB-Amp plates were expanded overnight at 37 °C in 5 ml of LB-Amp medium and the amplified plasmids were recovered. *S. cerevisiae* was transformed with the purified plasmids and the parent types, and five single colonies were picked and screened for each clone, as described above. Plasmids from selected mutants were sequenced with the RMLN, RMLC, LAC-F and LAC-R primers, the latter of which were designed to anneal to stretches of identity between parent laccases, except for one mismatch (underlined). LAC-F (5'-AGGGCAAGCGCTACCGCTTCCGC-3') binds to nucleotides 1057-1079 of pJR3PO, and LAC-R (5'-CGGATCCAGTAGTTGTCCAC-3') binds to nucleotides 1242-1261 of pJR3PO (the positions for pJROB1 are three nucleotides less).

Flask production of chimeras

Single colonies of the *S. cerevisiae* clones transformed with plasmids containing the selected genes were picked from SC drop-out plates, used to inoculate 3 mL of minimal medium in duplicate and incubated for 48 hours at 30 °C with agitation (220 rpm). An aliquot of the cells was used to inoculate a final volume of 5 mL in 100 mL flasks, adjusting the OD₆₀₀ to 0.25, which were then incubated for two complete growth phases (6–8 hours). Thereafter, the cells were diluted to OD₆₀₀ = 0.1 in a final volume of 10 mL of expression medium (supplemented with 4 mM CuSO₄) in 100 mL flasks. When maximum activity was reached, the cells were separated by centrifugation at 13000 rpm at 4 °C and the supernatants were concentrated in 10000 MWCO Amicon-Ultra Centrifugal filters (Millipore Iberica S.A.U., Madrid, Spain).

Characterization of chimeric laccases

- pH activity profiles:

Appropriate dilutions of the concentrated crude extracts were prepared such that 10 μ L aliquots produced a linear response in the kinetic mode in the plate reader. Plates containing 10 μ L of laccase samples and 180 μ L of 100 mM Britton and Robinson buffer were prepared at pH values of 2, 3, 4, 5, 6, 7 and 8. The assay commenced when 10 μ L of 60 mM ABTS or DMP was added to each well to give a

final substrate concentration of 3 mM. Activities were measured in triplicate in kinetic mode and the relative activity was calculated as a percentage of the maximum activity of each variant in the assay.

- Thermostability:

T₅₀ values were assessed in the gradient thermocycler, defining the T₅₀ as the temperature at which the enzyme retains 50% of its activity after a 10 min incubation. Appropriate dilutions were prepared such that 20 µL aliquots produced a linear response in the kinetic mode in the plate reader. Subsequently, 35 µL aliquots were used in triplicate at each point of the following temperature scale (°C): 39.9, 40.4, 41.7, 43.5, 45.8, 48.4, 51.1, 53.8, 56.2, 58.1, 59.5, 61.4, 63.3, 65.6, 68.2, 70.9, 73.6, 76.0, 77.9, 79.3 and 79.9. After a 10 min incubation, samples were chilled on ice for 10 min and incubated at RT for another 5 min. Thereafter, 20 µL of each sample was added to 180 µL of 100 mM acetate buffer (pH 5) containing 3 mM ABTS and the activity was measured in the kinetic mode. The thermostability values were deduced from the ratio between the residual activities incubated at different temperature points and the maximum activity for each variant in the assay.

- K_m values for ABTS and DMP:

Oxidation of increasing substrate concentrations was analyzed by measuring absorption at 418 nm (ABTS) and 469 nm (DMP) in the plate reader. Reactions were conducted in triplicate by adding 20 µL of the crude extract dilutions to 100 mM acetate buffer (pH 5.0) to a final volume of 250 µL. To calculate the K_m value, the relative activity (relative to maximum activity in each assay) was plotted against the substrate concentration and fitted to a single rectangular hyperbola function in SigmaPlot software, where $b = K_m$.

RESULTS

Constructing and screening chimeric libraries

The evolved laccase variants finally obtained through the directed evolution of PM1L and PcL, the OB1 and 3PO mutants (Maté et al., 2010; Camarero et al., 2012), were used as the parent lacasses for chimeragenesis. These mutants accumulate several different beneficial mutations in their evolved α -factor prepro-leaders that promote their secretion by yeast, along with other mutations in the mature protein that enhances their activity/stability. These two evolved fusion genes were shuffled by CLERY, which combines the PCR-dependent reassembly of the fragmented genes (*in vitro* DNA shuffling) with the recombination and cloning of the PCR products in *S. cerevisiae* (*in vivo* DNA shuffling, Abécassis et al., 2000). Gene fragments of different sizes (80–350 bp and 350–700 bp) were tested to create libraries with distinct crossover profiles, given that *in vitro* DNA shuffling of smaller fragments generates

more crossover events and larger fragments are easier to reassemble (Abécassis et al., 2000; Moore et al., 2001). The higher probability of correct PCR-reassembly using large fragments was confirmed by the enrichment of the desired 1.9 kb band in the final amplification step (see Materials and Methods for details). Two chimeric libraries were tailored by *in vitro* DNA shuffling, one of small fragments (L1) and the other of large fragments (L2), and they were separately transformed in the yeast to optimize the final laccase family shuffling process.

The laccase activity in the chimeric libraries was screened independently by the oxidation of ABTS and DMP (Maté et al., 2010; Camarero et al., 2012). We sought active laccase variants that performed better than the parent laccases in our experimental conditions (due to either improved secretion, enhanced catalytic activity or altered optimum pH). Since the OB1 parent exhibited higher activity for both substrates than the 3PO parent, the best clones were selected based on their activity with respect to OB1. More specifically, the best hybrids were chosen based on the Total Activity Improvement (TAI) value, defined as the combined improvement in secretion and k_{cat} relative to the OB1 parent type. In general, we observed a direct correlation of laccase activities with the two substrates (**Fig. 1**), which allowed us to select clones with the best TAI values for both compounds. The protocol used to build L2 seemed to be more efficient in generating active hybrids, given that 5.2% of the clones screened in L2 exhibited higher laccase activity than the parent types, as opposed to 1.9% in L1. These data indicate that the higher recombination frequency among small fragments might limit the generation of active chimeras in L1. After two consecutive re-screenings, 7 clones from L1 and 19 clones from L2 were selected and further characterized, having TAI values for both substrates approximately two-fold those of the OB1 parent.

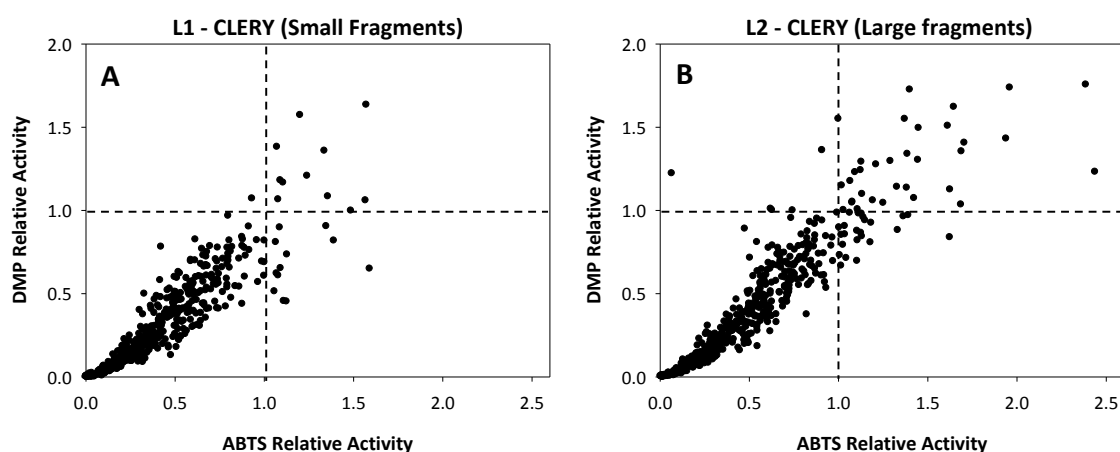


Figure 1. Landscapes obtained from the HTS of chimeric libraries A) L1 and B) L2, showing the laccase activities of clones for ABTS and DMP in comparison with that of the OB1 parent type (1).

DNA sequencing revealed that all selected clones were chimeric (**Fig. 2**), although some were overrepresented in the same library as commonly occurs in DNA shuffling (Chaparro-Riggers et al., 2007), and three clones acquired a single mutation, in concordance with the rate of inadvertent point mutations for *in vitro* DNA shuffling using *Pfu* polymerase (Zhao and Arnold, 1997; Chaparro-Riggers et al., 2007). Most genes selected combined beneficial mutations from the two evolved α -factor prepro-leaders. In general, the new hybrid products included three different types of chimera: shuffled prepro-leaders fused to the unaltered OB1 mature laccase, mature protein chimeras with parent prepro-leaders, and global chimeras (**Fig. 2**).

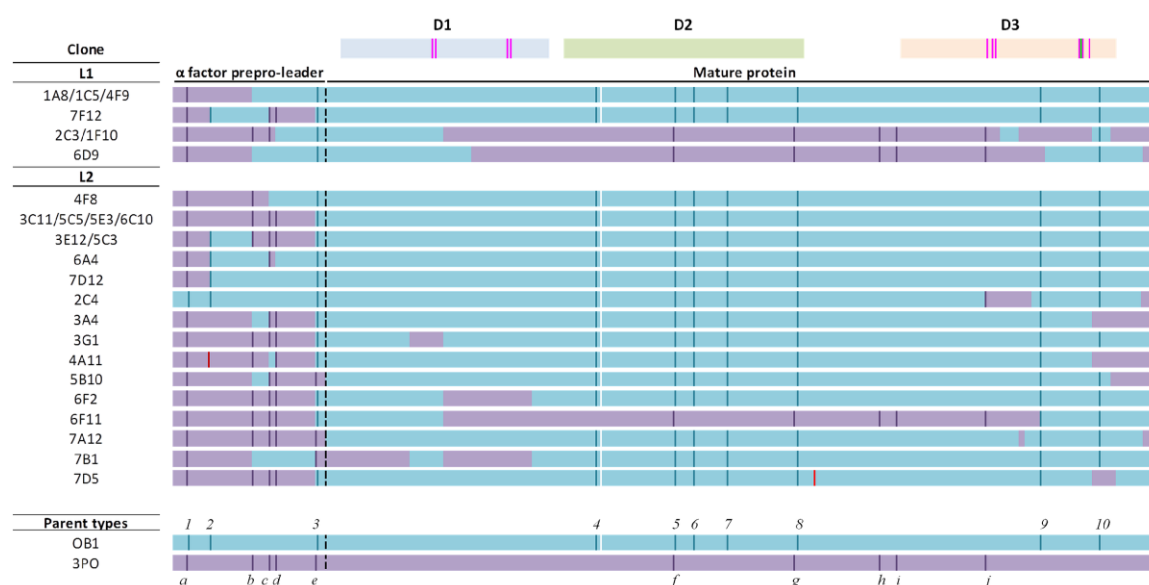


Figure 2. Schematic illustration of the sequence block exchanges produced in the selected hybrid sequences. Light blue and purple boxes represent the OB1 and 3PO parental types, respectively. The distinct mutations (dark blue and purple lines) accumulated in both the mature laccase and the α -factor prepro-leader sequences during the parallel evolution pathways are indicated. For simplification, block exchange is represented in the first residue that differs between the two laccases. Mutations in OB1 are: (1) V α 10D, (2) N α 23K, (3) A α 87T, (4) V162A, (5) H208Y, (6) S224G, (7) A239P, (8) D281E, (9) S426N, and (10) A461T. Mutations in 3PO are (a) A α 9D, (b) F α 48S, (c) S α 58G, (d) G α 62R, (e) E α 86G, (f) N208S, (g) R280H, (h) N331D, (i) D341N and (j) P394H. The T1, T2 and T3 Cu ligands are depicted as magenta lines at the laccase domains (D1, D2 and D3). The white line represents a 1 residue gap introduced for sequence alignment, and the red lines represent inadvertent point mutations introduced during PCR amplification.

DNA shuffling was produced at stretches of identity of 3–93 bp. Many of the crossover events in the mature protein sequence occurred at the four conserved signature laccase motifs that contain the preserved ligands of the T1, T2 and T3 Cu sites. Other crossover events occurred repeatedly in distinct regions of high homology between both parent sequences (**Fig. S1**). In both libraries, the number of crossover events in the whole chimeric sequence ranged from 1 to 6. The *in vivo* shuffling may have contributed to the similar number of crossover events observed in L2 and L1, as some recombined fragments found in L2 were very small and yeast favors crossovers within homologous stretches as small as 2–20 bp (Mézard et al.,

1992). However, the higher recombination frequency following *in vitro* shuffling of small fragments (L1) was evident through the higher proportion of chimeras with 6 crossovers selected from L1 (29%) than from L2 (5%).

Chimera characterization

Chimeras were grown in flasks to obtain sufficient secreted laccase for preliminary characterization studies. The pH activity profiles for oxidation of ABTS and DMP (**Fig 3A, B**) and the thermostability (**Table I**) of the mature laccase hybrids were measured to identify possible changes in laccase properties due to sequence block exchange. The 2C4 variant (containing a higher proportion of OB1) exhibited the most significant differences in its pH activity profile to all the other laccase chimeras analyzed. Its pH profile shifted considerably towards more neutral values, with a sharp maximum at pH 5.0 for both substrates, and there was a significant decrease in activity at pH 3.0 and 4.0, values at which other laccase hybrids displayed maximal activity. The activity of the chimeras 2C3, 6D9 and 6F11, whose protein sequences shared greater identity with the 3PO parent (**Fig. 2**), was enhanced at more neutral pHs, retaining 40–50% activity at pH 6.0 for both substrates (as opposed to ~10–20% for the parent types). By contrast, 3A4, 7D5 and 4A11 chimeras (holding a higher proportion of OB1) exhibited narrower pH profiles, which unpredictably shifted towards more acidic values. Only 40% ABTS activity was retained at pH 5 compared to the 70–80% of the parent types (**Fig. 3A**).

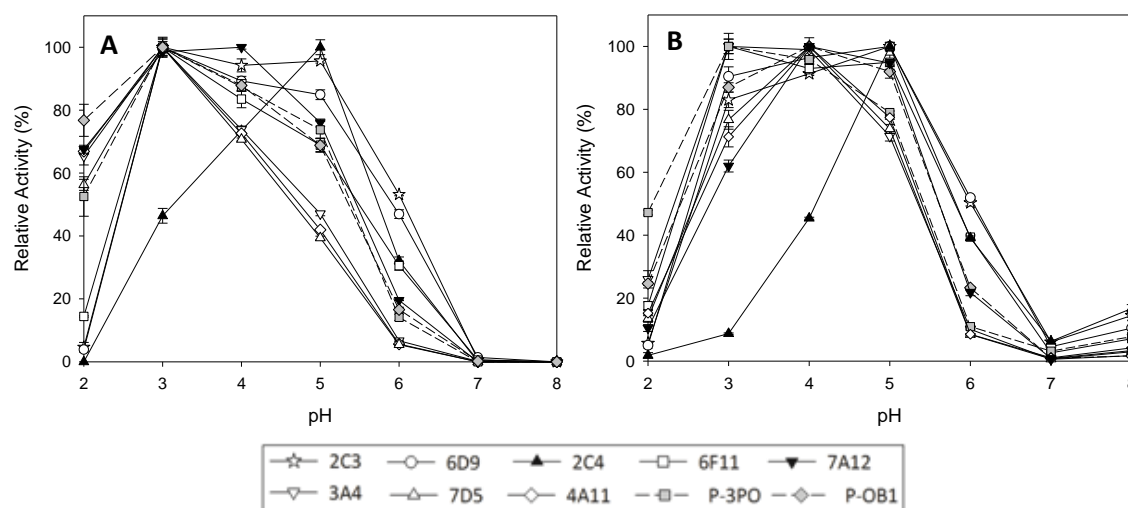


Figure 3. pH activity profiles of chimeric laccases for the oxidation of **A)** ABTS and **B)** DMP. For simplification, only laccase hybrids with activity profiles distinct from the parent forms are depicted. Each point represents the average of three independent experiments \pm standard deviation.

Unexpectedly, five laccase hybrids (2C4, 3A4, 4A11, 7A12 and 7D5) exhibited significantly higher T_{50} values than both parent types, with increases of up to 3 °C over that of OB1 (**Table I**). The most unstable variants ($T_{50} \leq 62$ °C) were 2C3 and 6F11, which shared the highest sequence content with the 3PO parent. However,

variant 6D9 (also containing a 3PO scaffold) retained a T_{50} value similar to that of 3PO.

Table I. T_{50} values of chimeric laccases and parent types.

Library	Clone	T_{50} (°C)
L2	6F11	59.1
L1	2C3	61.9
L2	7B1	65.0
L2	3G1	67.1
L2	6F2	68.1
L2	5B10	68.7
L1	6D9	69.5
Parent	3PO	70.9
Parent	OB1	73.3
L2	7D5	74.9
L2	2C4	75.5
L2	3A4	76.1
L2	4A11	76.6
L2	7A12	76.6

The substrate affinities for most promising chimeras (with improved stability or modified pH activity profiles) were determined (**Table II**) and as expected, the K_m values of the 6D9 chimera were comparable to those of the 3PO parent. Similarly, the 3A4, 7D5 and 7A12 chimeras, in which the OB1 sequence was largely retained, exhibited a similar K_m for ABTS as the OB1 parent, and an even higher affinity for DMP.

Table II. Substrate affinities for ABTS and DMP of selected hybrid and parental laccases.

Laccase	K_m (μM)	
	ABTS	DMP
6D9 chimera	46.5 \pm 1.0	361.4 \pm 12.0
3A4 chimera	9.8 \pm 1.1	61.8 \pm 4.8
7D5 chimera	7.6 \pm 0.5	53.4 \pm 2.5
7A12 chimera	8.1 \pm 0.4	86.2 \pm 2.9
2C4 chimera	8.0 \pm 0.4	777.8 \pm 22.2
OB1 parent	9.1 \pm 0.5	93.1 \pm 3.3
3PO parent	42.9 \pm 1.3	243.5 \pm 6.7

DISCUSSION

Family shuffling and sequence analysis

DNA recombination by family shuffling is based on the use of distinct parent genes with sufficient sequence homology to allow the assembly of whole genetic products without jeopardizing enzyme folding and function. The present study

describes the family shuffling of two fungal HRPLs that are functionally expressed in *S. cerevisiae* to generate chimeric laccases. We selected the CLERY strategy that combines *in vitro* and *in vivo* DNA shuffling (Abécassis et al., 2000) in order to create as much diversity as possible through the high recombination frequency between the parent types. The generation of chimeric laccases has been described previously using isoenzymes from the same fungal strain (Cusano et al., 2009; Nakagawa et al., 2010). Here, we used laccase genes from different basidiomycete fungi, which in conjunction with the DNA recombination strategy employed, allows access to different regions of the sequence space and promotes the exchange of sequence blocks, thereby yielding chimeras with larger sequence divergence (Arnold and Volkov, 1999; Crameri et al., 1998) than those described previously (Cusano et al., 2009; Nakagawa et al., 2010).

Laccases are organized into three cupredoxin-like domains (D1, D2 and D3) and when the mature hybrid protein sequences generated by chimeragenesis were analyzed no crossover events were detected in the second laccase domain (D2). All crossover events were observed at D1 and D3, where the four highly conserved laccase signature motifs are located (Kumar et al., 2003), although many crossovers occurred outside of these conserved regions. In fact, the number of crossover events at D3 doubled that observed at D1, indicating a biased window of recombination in the 3' region (C-terminal), consistent with previous findings (Cusano et al., 2009). Crossovers preferably occur in regions of high homology and at positions that minimize disruptions of interactions (Joern et al., 2002). However, the random fragmentation of genes used in the present study reduced such bias in recombination when compared with other reported methods. The bias in the *in vivo* gap-repaired ligation method (Cusano et al., 2009) is introduced by linearizing the receptor fragment at the targeted restriction sites, where the eukaryotic machinery repairs the gap by recombination with the donor gene. Likewise, by using the DNA swapping method, protein block exchange can only occur at the site at which the genes have been cut (Nakagawa et al., 2010).

DNA shuffling facilitates a markedly broader exploration of protein sequences by increasing the recombination frequency (Abécassis et al., 2000; Stemmer, 1994; Volkov and Arnold, 2000) and indeed, screening the chimeric libraries revealed active laccase hybrids with up to six crossover events. By contrast, only one protein block exchange and two crossovers were obtained by DNA swapping (Nakagawa et al., 2010) and *in vivo* gap-repaired ligation (Cusano et al., 2009), respectively. We do not dismiss that even higher recombination frequencies might have occurred generating less active chimeras that would have not been detected by the screening procedure, as only clones with enhanced activity with respect to parent types were chosen. The HTS protocol used also allowed us to get rid of the parent background

usually produced during DNA shuffling (Abécassis et al., 2000; Joern et al., 2002; Stemmer, 1994).

Of the 12 mature chimeric sequences selected, 9 retained a predominant fraction of the parent OB1 laccase, in good agreement with previous studies of shuffled gene libraries (Joern et al., 2002). In principle, chimeras are inactivated by disrupting interactions that contribute to proper folding, stability or activity. Hence, chimeric clones with a higher proportion of a single parent are less prone to inactivation as more interactions are conserved (Crameri et al., 1998). Most hybrid sequences exhibited shuffled signal peptides, including the A α 9D and A α 87T mutations from the 3PO and OB1 evolved α -factor prepro-leaders, respectively. Interestingly, other mutations at similar locations were also evident in both variants (V α 10D in OB1 and E α 86G in 3PO). A α 9 and V α 10 are non-polar amino acids in the hydrophobic core of the canonical pre-leader that is involved in the orientation and insertion of the nascent polypeptide during translocation to the endoplasmic reticulum (Nothwehr and Gordon, 1990). Their substitution by aspartic acid suggests that a reduction in hydrophobicity in this area may be beneficial for secretion. However, the A α 9D mutation, which led to the greatest improvement during the directed evolution of *P. cinnabarinus* laccase (Camarero et al., 2012), appears to be more beneficial than the V α 10D mutation (A α 9D was found in 18 out of the 19 chimeras analyzed). The A α 87T mutation (and E α 86G) is located in the spacer dipeptide (Glu-Ala-Glu-Ala) that lies between the α -factor prepro-leader and the mature laccase, which is the processing site of the dipeptidyl aminopeptidase STE13 in the Golgi compartment (Romanos et al., 1992). This mutation may be involved in the alternative processing that promotes the secretion of active laccases containing a 6 amino acid extension at the N-terminal, without compromising activity or stability (unpublished data). In general, the shuffled prepro-leaders fused to the unaltered OB1 mature laccase increased their secretion by yeast. Along with the observed prevalence of certain beneficial mutations from the evolved prepro-leaders, these findings provide new evidence to support the hypothetical design of a "universal" prepro-leader for laccase expression in yeast.

Chimeric laccases with combined features

The dual colorimetric assay used to screen the chimeric libraries allowed the selection of active hybrids that maintained a broad oxidation range for phenolic (DMP) and non-phenolic polar compounds (ABTS). The increase in laccase activity at neutral pHs may be of interest for certain biotechnological applications, such as enzyme-aided TCF bleaching of paper pulp or decolorization of textile effluents (Abadulla et al., 2000; Ibarra et al., 2006). A predictable increase in laccase activity at pH 5 was obtained for some laccase hybrids, although other unexpected features were also observed following chimeragenesis, such as increased thermostability and more acidic activity profiles. Laccase thermostability was significantly enhanced in

several chimeras (3A4, 4A11, 7D5, 2C4 and 7A12), and in some cases this increase in thermostability was accompanied by a sharper optimum pH. On the other hand, the recombination of OB1 and 3PO genes gave rise to some laccase hybrids with a broader pH activity profile than parent types, and a significant increase in laccase activity at pH 6.0. These included chimeras 2C3, 6D9 and 6F11, which largely retained the 3PO scaffold while incorporating portions of the OB1 protein, although 2C3 and 6F11 exhibited severely diminished thermostability. The activity of the 2C4 hybrid also improved at more neutral pH values but unlike the aforementioned chimeras, its pH profile narrowed notably and its activity was maximal at pH 5.0. 2C4 is mainly composed of the OB1 parent type and two small segments of 3PO (**Fig. 4**). The first segment (residue 394–421 in 3PO) includes the third highly conserved laccase motif (with T1, T2 and T3 Cu ligands plus the P394H mutation from 3PO). The P394H mutation is contiguous with the His395 ligand of the T1 copper (Valderrama et al., 2003) and there is a noticeable mismatch between both parent laccases in this signature motif. The second segment introduced in 2C4 belongs to the last 10 C-terminal (C_t) residues of the 3PO parent enzyme. Interestingly, the 7A12 chimera shares the same scaffold and sequence block exchange at the C_t with 2C4, although it does not contain the 394–421 segment (**Fig. 4**). The 7A12 chimera was the most thermostable variant and besides the substrate affinities for DMP or ABTS were not impaired by recombination. Comparing the biochemical features of the 2C4 and 7A12 chimeras revealed the contribution of the 394–421 segment to the regulation of substrate affinity and optimum pH.

It should be noted that other chimeric laccases with combined properties also incorporate several residues of the C_t from 3PO. Chimeras 3A4, 4A11 and 7D5 contain the C_t of 3PO in the OB1 scaffold and displayed sharper acidic optimal pH, yet they were very thermostable with improved K_m values for phenolic compounds. These properties may be of interest for processes such as the enzymatic detoxification of thermo-chemically pretreated lignocellulosic biomass (Jurado et al., 2009). The C-terminal tail of ascomycete laccases plays a key role in enzyme stability and activity, containing a plug that regulates the traffic of oxygen to the trinuclear Cu cluster (Andberg et al., 2009; Zumárraga et al., 2008a; Zumárraga et al., 2008b). Although the case of basidiomycete laccases is different (they only share ~33 % of sequence identity with ascomycete laccases), as there are several mismatches between OB1 and 3PO in this region, the role of the C_t in the global folding and function of HRPLs cannot be underestimated and requires further investigation.

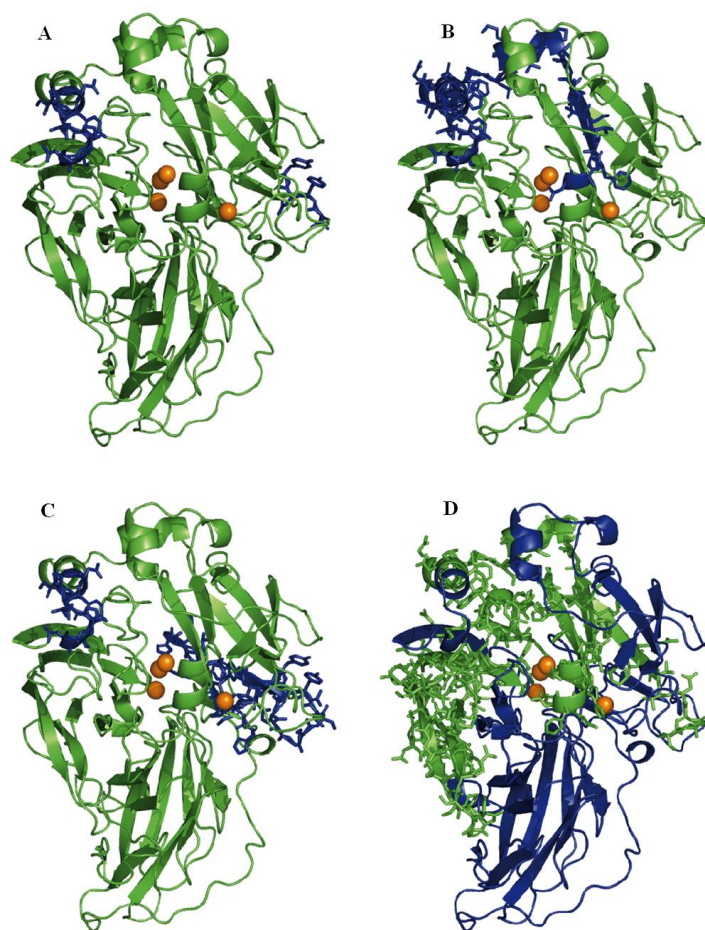


Figure 4. 3D models of selected laccase hybrids showing the exchange of protein fragments from the two parent laccases. The protein scaffold of the OB1 parent (green) was retained in chimeras **A**) 7A12, **B**) 3A4 and 4A11 and **C**) 2C4, and combined with different 3PO fragments (blue). **D**) 6D9 retained the scaffold of the 3PO parent and contained two blocks from OB1. Chimeras were modeled using the structures of *P. cinnabarinus* (PDB: 2XYB, from K. Piontek) and *T. troggi* (PDB: 2HRG) laccases as templates using PyMol software (DeLano, 2002).

CONCLUSIONS

In summary, we describe the engineering of chimeric laccases *via* family shuffling of two HRPLs from different fungi. The exchange of diverse protein blocks were obtained by *in vitro* plus *in vivo* recombination of the parent genes, and hybrid proteins active on phenolic and non-phenolic polar substrates were identified by dual high-throughput screening of the chimeric libraries. Selected chimeric laccases displayed modified pH activity profiles, enhanced thermostability or improved substrate affinities. This collection of chimeras may provide a suitable starting point for tailoring HRPLs with novel properties.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

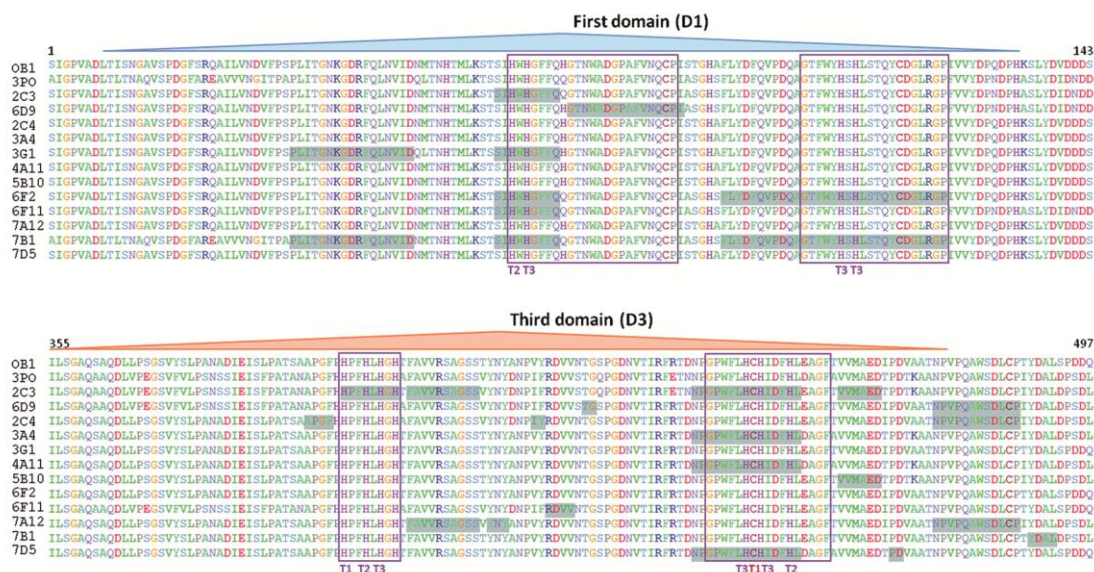


Figure S1. Suggested crossover events in the selected chimeric sequences. Areas where crossover should occur based on the block sequence exchanges observed in the mature chimeras are depicted in grey. The four conserved laccase signature motifs containing the ligands of the catalytic coppers are also depicted (represented as T1, T2 and T3: pink boxes).

CHAPTER 2

NEW COLORIMETRIC SCREENING ASSAYS FOR THE DIRECTED EVOLUTION OF FUNGAL LACCASES TO IMPROVE THE CONVERSION OF PLANT BIOMASS

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ABSTRACT

Background

Fungal laccases are multicopper oxidases with huge applicability in different sectors. Here, we describe the development of a set of high-throughput colorimetric assays for screening laccase libraries in directed evolution studies.

Results

Firstly, we designed three colorimetric assays based on the oxidation of sinapic acid, acetosyringone and syringaldehyde with λ_{\max} of 512, 520 and 370 nm, respectively. These syringyl-type phenolic compounds are released during the degradation of lignocellulose and can act as laccase redox mediators. The oxidation of the three compounds by low and high-redox potential laccases evolved in *Saccharomyces cerevisiae* produced quantifiable and linear responses, with detection limits around 1 mU mL⁻¹ and CV values below 16%. The phenolic substrates were also suitable for pre-screening mutant libraries on solid phase format. Intense colored-halos were developed around the yeast colonies secreting laccase. Furthermore, the oxidation of violuric acid to its iminoxyl radical (λ_{\max} of 515 nm and CV below 15%) was devised as reporter assay for laccase redox potential during the screening of mutant libraries from high-redox potential laccases. Finally, we developed three dye-decolorizing assays based on the enzymatic oxidation of Methyl Orange (470 nm), Evans Blue (605 nm) and Remazol Brilliant Blue (640 nm) giving up to 40% decolorization yields and CV values below 18%. The assays were reliable for direct measurement of laccase activity or to indirectly explore the oxidation of mediators that do not render colored products (but promote dye decolorization). Every single assay reported in this work was tested by exploring mutant libraries created by error prone PCR of fungal laccases secreted by yeast.

Conclusions

The high-throughput screening methods reported in this work could be useful for engineering laccases for different purposes. The assays based on the oxidation of syringyl-compounds might be valuable tools for tailoring laccases precisely enhanced to aid biomass conversion processes. The violuric assay might be useful to preserve the redox potential of laccase whilst evolving towards new functions. The dye-decolorizing assays are useful for engineering *ad hoc* laccases for detoxification of textile wastewaters, or as indirect assays to explore laccase activity on other natural mediators.

BACKGROUND

Laccases catalyze the oxidation of a variety of substituted phenols and many other aromatic compounds without any other requirement than oxygen from air. As a result, these multicopper oxidases are promising green biocatalysts for several industrial sectors such as textile, food, wood and pulp, bioremediation, organic synthesis or electrocatalysis (Rodríguez Couto and Toca Herrera, 2006; Shleev et al., 2006; Kunamneni et al., 2008). Fungal laccases and, in particular, the high-redox potential laccases (HRPLs) secreted by white-rot basidiomycetes, exhibit high biotechnological applicability due to the wider range of reducing substrates that can be oxidized at the T1 copper site ($E^\circ \approx +0.8$ V, Xu et al., 1999).

Lignin biodegradation is an oxidative process carried out by white-rot fungi in which the breakdown of aryl-ether (β -O-4) linkages and the oxidative degradation of the side chains from the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units, releases a set of phenolic compounds (acids, ketones and aldehydes) (Camarero et al., 1994). Some of them (e.g. acetosyringone, syringaldehyde, *p*-hydroxycinnamic acids) show remarkable activity as laccase redox mediators. Once oxidized by fungal laccases, they act as diffusible electron shuttles, promoting the oxidation of the own lignin polymer and a variety of different recalcitrant aromatic pollutants (Camarero et al., 2005; Cañas et al., 2007; Nousiainen et al., 2009; Torres-Duarte et al., 2009). In recent findings we have highlighted the biotechnological potential of fungal laccases and their natural redox mediators for improving the conversion of plant biomass in the modern integrated lignocellulose biorefineries (Cañas and Camarero, 2010). Moreover, sinapic acid, acetosyringone and other bioactive compounds with anti-bacterial and antioxidant properties (Kikuzaki et al., 2002; Baker et al., 2005) can be used to add new properties to cellulose or wood fibers by grafting reactions catalyzed by laccase (Aracri et al., 2010). Other phenolic compounds that can be extracted from wood lignin like syringaldehyde or vanillin provide flavor and fragrance or are used as chemical precursors for pharmaceuticals (Wu et al., 1994; Rodrigues Pinto et al., 2011). Indeed, the occurrence of lignin-derived phenolic compounds has been profusely described during the processing of lignocellulosic materials. Black liquors from pulp cooking constitute low-cost sources of natural mediators which can be applied in laccase-mediator systems for Totally-Chlorine-Free bleaching of paper pulps (Camarero et al., 2007). Lignin-related phenolics are also released during the thermo-chemical pretreatment of lignocellulose for bioethanol production, inhibiting the subsequent fermentation step (Parawira and Tekere, 2011). Detoxification of these slurries can be achieved by the polymerization of these phenols catalyzed by laccase (Jurado et al., 2009), although some of them (vanillin) are somehow resistant (Alvira et al., 2013). Engineering robust laccases with improved activities/specificities towards the abovementioned compounds represents a valuable step forward to implement these

enzymes in white –industrial– biotechnology processes for conversion of lignocellulosic biomass into chemicals, materials and biofuels (Cañas and Camarero, 2010).

In this scenario, directed molecular evolution constitutes a powerful strategy to adjust the stability and catalytic efficiency of the enzyme to the restrictive industrial operational conditions. On the other hand, it is well known that the availability of high-throughput screening (HTS) assays is mandatory for exploring the enzyme libraries created by random mutagenesis and recombination of the parent gene(s) (Arnold and Volkov, 1999). Indeed, one of the main bottlenecks in directed evolution originates from the lack of reliable HTS assays specific for the targeted enzyme, and laccase is not an exception. Another major difficulty for engineering fungal laccases, in particular those from basidiomycete fungi, is their tricky heterologous expression. Nevertheless, we have recently reported the successful functional expression of two HRPLs (from *Pycnoporus cinanbarinus* and PM1 basidiomycetes) in *S. cerevisiae* by directed evolution (Camarero et al., 2012; Maté et al., 2010). We have also obtained a set of chimeric HRPLs, secreted by yeast, with improved thermostability, diverse pH activity profiles and high-rate oxidation activity as generalist biocatalysts (Pardo et al., 2012). These platforms are good starting points to face up to new challenges such as the design of laccases with improved efficiency towards substrates of biotechnological interest and stable under specific industrial conditions. Promising laccase engineering targets would be the first-order oxidation rate of certain phenolic compounds derived from lignocellulose, to contribute to the integral conversion of plant biomass, or of synthetic organic dyes, for enzymatic removal of color from textile effluents. The development of new HTS assays based on the oxidation of phenolic compounds and organic dyes (under preferred pH and temperature conditions) is of high relevance for the aforementioned purposes.

The current work describes the design and validation of an array of novel HTS assays based on natural compounds derived from lignocellulose and synthetic organic dyes to explore mutant libraries of fungal laccases. Specifically, we developed colorimetric assays based on the oxidation of phenolic compounds related to the S lignin units. These compounds, which are natural substrates of laccases (and ligninolytic peroxidases, Ruiz-Dueñas et al., 2009), might constitute a key step in the enzymatic deconstruction of lignocellulose due to their role as linkages between carbohydrates and lignin in the secondary cell wall of grasses (Bidlack et al., 1992); or they may act as efficient laccase redox mediators promoting the removal of pollutants or complex polymers (Camarero et al., 2005; Gutiérrez et al., 2007; Camarero et al., 2008). In addition, the oxidation of the artificial mediator violuric acid was devised as reporter assay for the preservation of the redox potential of HRPLs through the evolution procedure. Finally, we performed the

development of HTS assays based on the enzymatic oxidation of synthetic dyes either directly or indirectly (in the presence of mediators).

RESULTS AND DISCUSSION

Oxidation of natural phenolic compounds of biotechnological interest

Among lignin-related phenolic compounds, we chose three S-type phenolic compounds whose enzymatic oxidation generates colored products (acetosyringone, sinapic acid and syringaldehyde) to develop the HTS assays. S-type compounds are easily oxidized by both high- and low-redox potential laccases (LRPLs), as we confirmed here by using the commercial HRPL from *Trametes villosa* (TvL) and the LRPL from *Myceliophthora thermophila* (MtL). The changes in the UV-visible spectra of sinapic acid, acetosyringone and syringaldehyde during their oxidation by laccase showed similar patterns: a rapid decrease of maximum absorbance at 300 nm along with the appearance of absorbance peaks in the visible spectrum (**Fig. 1**). In the case of sinapic acid, we detected a rapid pinkish response (with maximum absorbance around 515 nm) resulting from oxidized dimeric products derived from the dehydrodisinapic acid dilactone (Lacki and Duvnjak, 1998). Once sinapic acid is oxidized by laccase, the high tendency of its phenoxyl radicals for β - β' coupling is responsible for the accumulation of phenolic dimeric products, which are again oxidized by the enzyme. The oxidation of acetosyringone and syringaldehyde generated an immediate increase of absorbance around 370 nm (yellow color). The color kept stable for syringaldehyde but turned to red in the case of acetosyringone, whose maximum wavelength shifted to 520 nm and was maintained for several hours. Syringaldehyde oxidation finally rendered a strong absorption maximum at 284 nm with a smaller peak at 370 nm, in concordance with the yellow product 2,6-dimethoxy *p*-benzoquinone. The latter has been reported as end product from the enzymatic oxidation of syringaldehyde, acetosyringone, syringic acid or sinapic acid, depending on the reaction conditions (Shin, 1995; Lacki and Duvnjak, 1998; Rosado et al., 2012).

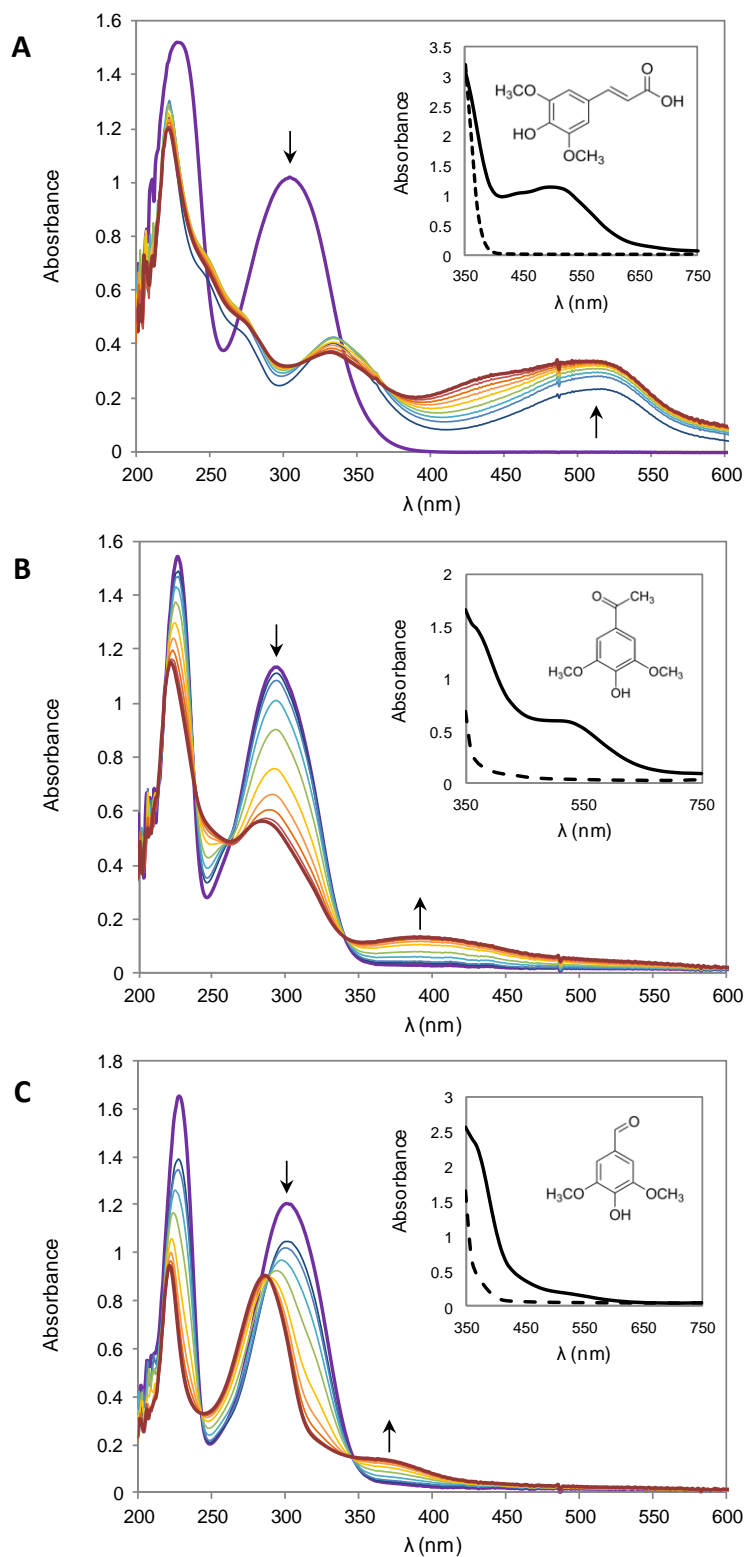


Figure 1. Oxidation spectra of S-type phenolic compounds. Changes in the absorption UV-Vis spectra of **A)** sinapic acid, **B)** acetosyringone and **C)** syringaldehyde during oxidation by *M. thermophila* laccase (50 mU) at 0, 1, 2, 5, and 10–60 min (in 10 min intervals) reaction times. Insets show the visible initial spectra (dashed lines) and after 10h (continuous lines).

The λ_{\max} for measuring the oxidation of the S-type substrates were established as follows: 512 nm for the sinapic acid's pinkish product, 370 nm for the syringaldehyde's yellow product and 520 nm for the acetosyringone's reddish product. Laccase oxidation rates showed the typical Michaelis-Menten kinetics for the three compounds with K_m values of 85, 120 and 93 μM , respectively, for TvL (**Fig. 2A-C**). The concentrations used in the HTS assays (providing stable response without precipitation) were 2 mM acetosyringone or syringaldehyde and 250 μM sinapic acid. The assays were validated using fresh supernatants from the micro-fermentations of *S. cerevisiae* transformed cells secreting laccase (in 96-well plate format). In particular, to check the reproducibility and linearity of the assays, we used *S. cerevisiae* cells expressing either a LRPL, R2 (obtained from the directed evolution of MtL, Zumárraga et al., 2007), or a HRPL, 3A4 (a chimeric laccase engineered by family shuffling of evolved PcL and PM1L, Pardo et al., 2012). The colored responses were feasibly quantified by the increment of absorbance with time (**Fig. 2D-F**), although in the case of the sinapic acid assay an initial lag time was observed due to the multiple oxidation, coupling and cyclization steps required to provide the colored product (oxidized dimer, Lacki and Duvnjak, 1998). Regardless of the compound used, the colored responses were linear (absorbance increased with increasing volumes of supernatant) with both laccases, the LRPL R2 and the HRPL 3A4, expressed by *S. cerevisiae* cells (**Fig. 3A-C**). The lowest detection limits for the acetosyringone and syringaldehyde endpoint assays (carried out in 5 h) were around 0.6 laccase mU mL^{-1} (0.15 mU in the well, referred to ABTS activity), whereas, due to the initial lag phase of the sinapic acid assay, 1 mU mL^{-1} (0.25 mU in the well) was the lowest activity detected during the 1–2 h of reaction. However, it is worth noting that for longer reaction times, lower laccase activities may also be detected with sinapic acid. The validation of the assays was completed by replicating the same clone in a test 96-well plate and measuring the laccase activities of each well with the target substrate. In all cases, the CV values ranged from 11 to 16% (**Fig. 3D-F**), which is satisfactory to guarantee the reliability of the assays for directed evolution studies.

Finally, the assays were tested for screening mutant libraries of HRPLs secreted by yeast. It is important to highlight that the sinapic acid assay has been recently used to screen mutant libraries generated during the directed evolution of *P. cinnabarinus* laccase (PcL, Camarero et al., 2012). In the present study, we used this assay (together with ABTS and DMP assays) to screen a laccase library obtained by random mutagenesis and *in vivo* DNA shuffling of chimeric HRPLs recently engineered in our lab (Pardo et al., 2012). The 3D landscape obtained from the multi-screening of this library demonstrated that most of the 2000 clones kept the characteristic substrate promiscuity of laccases and some of them showed slight activity improvements respecting the parent types (**Fig. 4**).

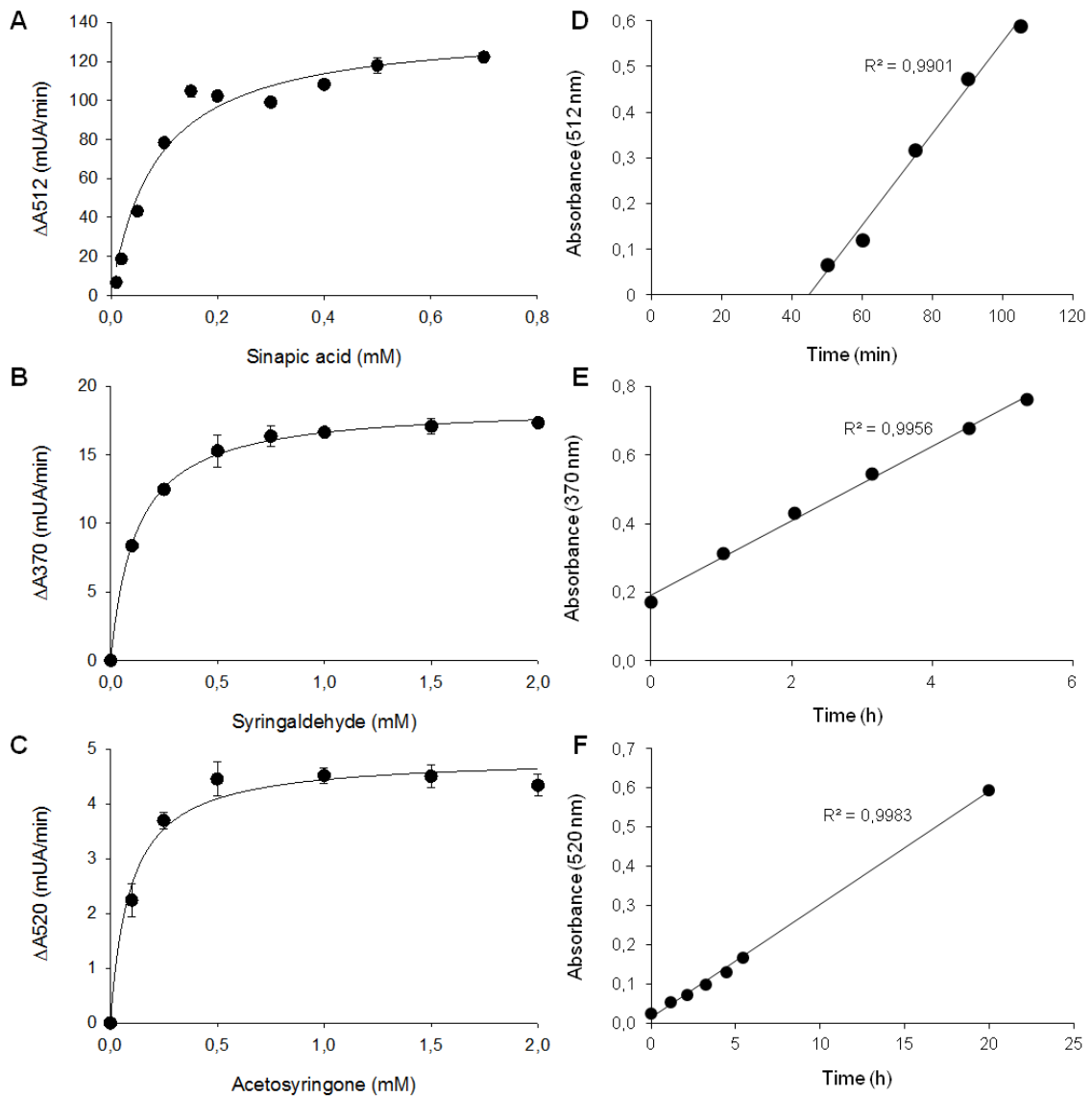


Figure 2. Determination of conditions for the HTS assays with S-phenolic compounds. Oxidation rates of TvL (10 mU) for **A**) sinapic acid, **B**) syringaldehyde and **C**) acetosyringone measured at 512 nm, 370 and 520 nm, respectively; and color responses of the endpoint HTS assays with time using 250 μ M of **D**) sinapic acid and 2 mM of **E**) syringaldehyde and **F**) acetosyringone with 15 μ L of crude extracts from *S. cerevisiae* micro-cultures secreting 3A4 HRPL. Each point represents the mean and SD derived from three independent experiments.

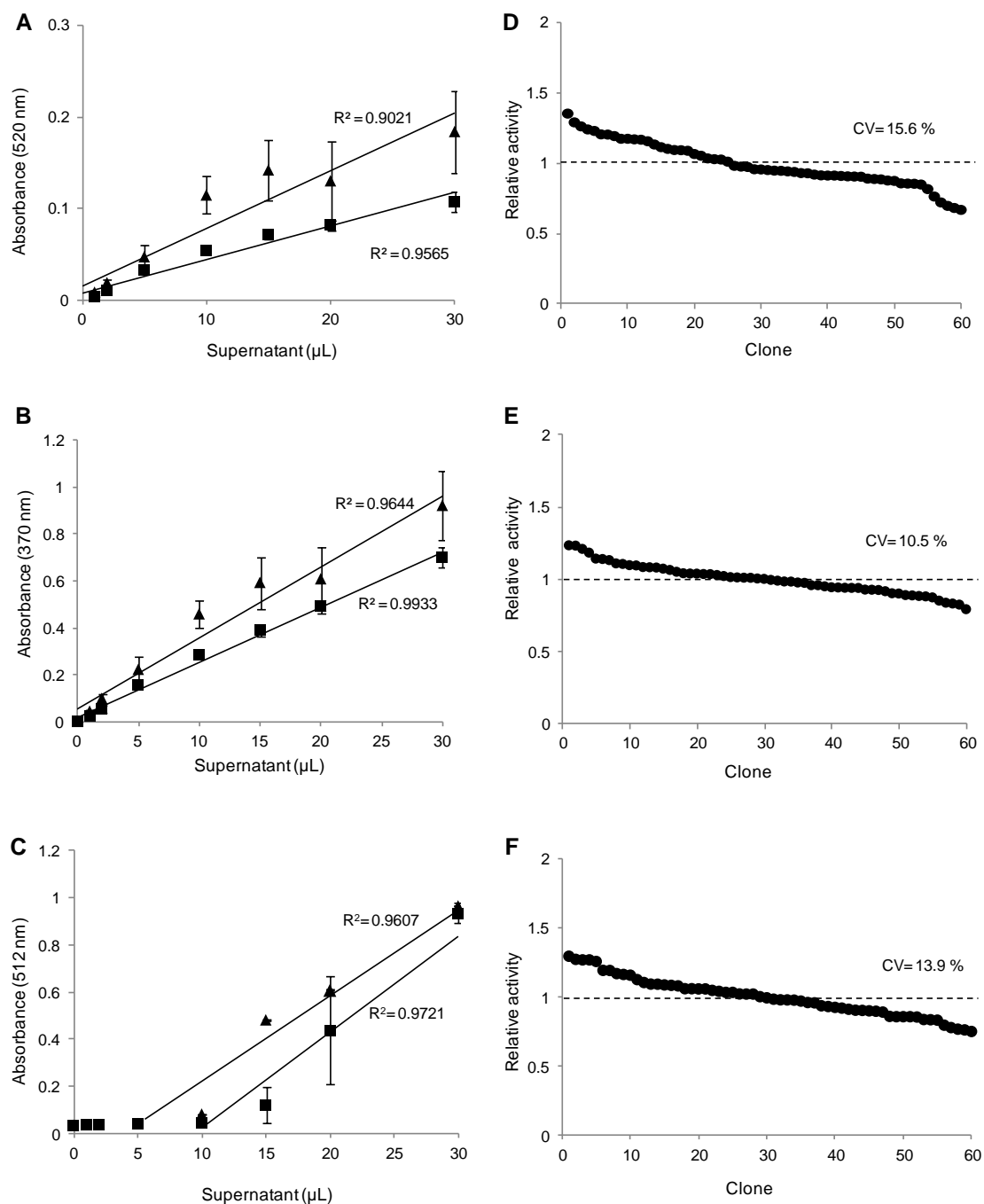


Figure 3. Validation of HTS colorimetric assays with S-phenolic compounds. Linearity of the assays based on the oxidation of **A**) acetosyringone and **B**) syringaldehyde after 5 h, and **C**) sinapic acid after 2 h, using crude extracts from *S. cerevisiae* micro-cultures secreting 3A4 HRPL (triangles) or R2 LRPL (squares). Coefficient of variation (CV) of the HTS colorimetric assays based on the oxidation of **D**) acetosyringone, **E**) syringaldehyde and **F**) sinapic acid. Laccase activities from different replicates of the same clone are plotted in descending order.

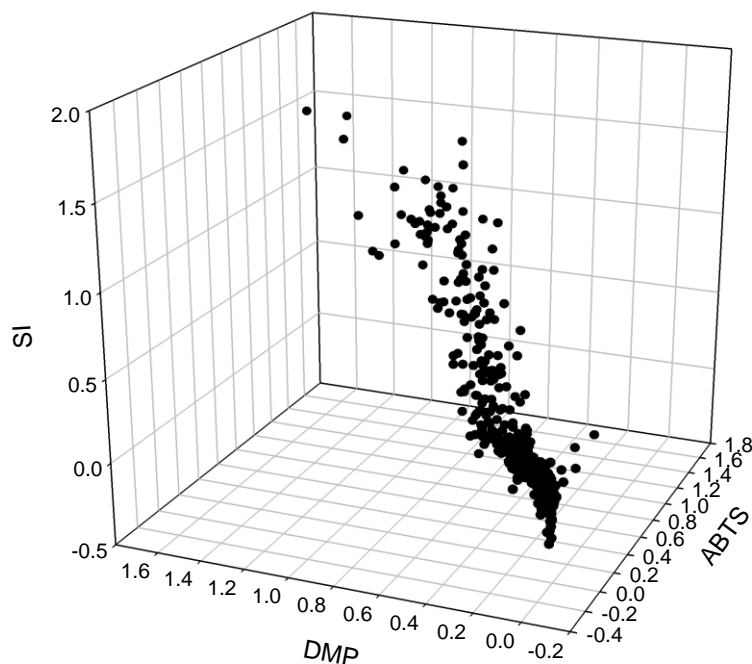


Figure 4. 3D Landscape from the multi-screening with sinapic acid, ABTS and DMP of the laccase mutant library obtained by random mutagenesis and *in vivo* shuffling of HRPLs. Laccase activities of the clones are depicted in descending order respecting the activity of the best parent (3A4 laccase). Sinapic acid (SI), 2,6-dimethoxyphenol (DMP).

To complete the study, small libraries of around 250 clones were constructed by error-prone PCR of 3A4 HRPL (with a mutational rate of 1–3 amino acid substitutions per protein sequence) and explored with acetosyringone and syringaldehyde. Landscapes from the dual screening were similar and the data were quite consistent for the two assayed protocols. Approximately 100 clones were inactivated by the mutagenesis and no notable activity increases respecting the parent type were observed (**Fig. 5A-B**). The small size of the mutagenic library probably precludes the selection of remarkable mutants (typically, a directed evolution generation comprises the screening of 2000–3000 clones). Even so, as we could detect slight differences in laccase activity among the different clones, the sensitivity of the colorimetric assays was confirmed.

It is worth mentioning that the abovementioned S-phenolic substrates might also be used for pre-screening of mutant laccase libraries in solid format. We cultured fresh *S. cerevisiae* laccase transformants on agar-SC-expression plates supplemented with acetosyringone, syringaldehyde or sinapic acid. Laccase secretion was detected by the presence of intense colored halos around the colonies grown in plates supplemented with syringaldehyde or sinapic acid, as compared with the negative control. The intensity of the halos when using acetosyringone was, however, much less intense (**Fig. 6**).

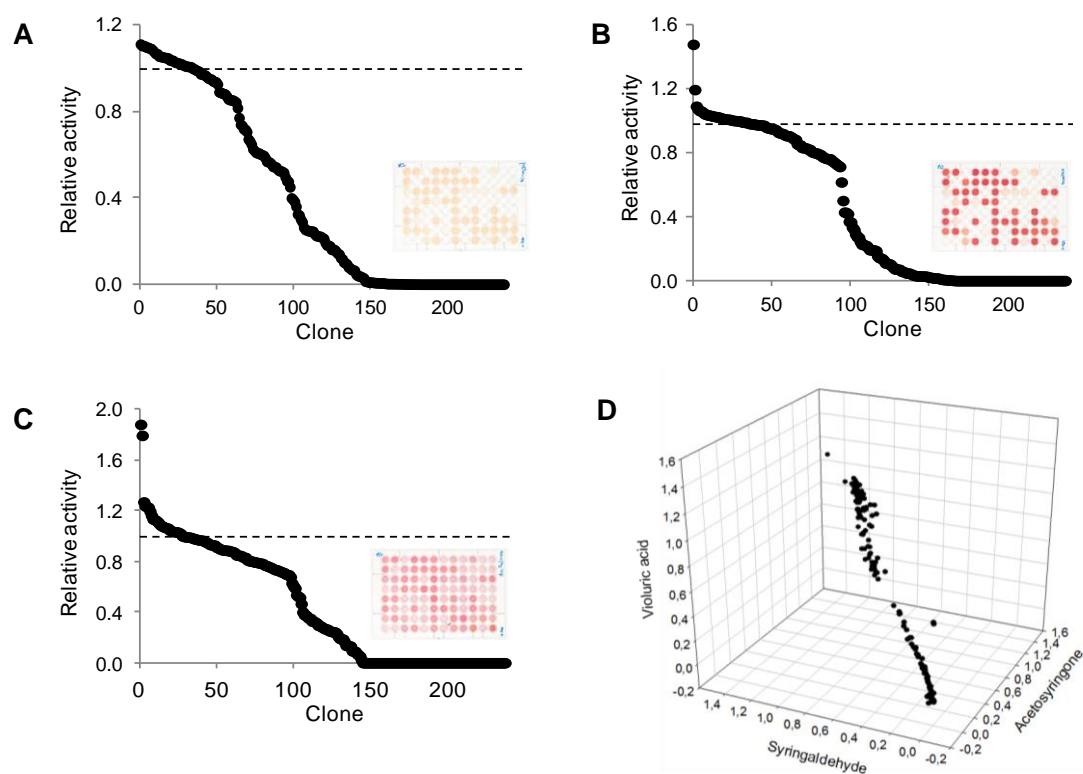


Figure 5. Multi-screening with mediator compounds. Landscapes from the HTS of the mutagenic library obtained from 3A4 HRPL using **A)** 2 mM syringaldehyde, **B)** 2 mM acetosyringone and **C)** 20 mM violuric acid. Laccase activities of the clones are depicted in descending order respecting the activity of the parent type. **D)** Direct correlation of the activities of the clones explored by the triple screening is shown in the 3D landscape.

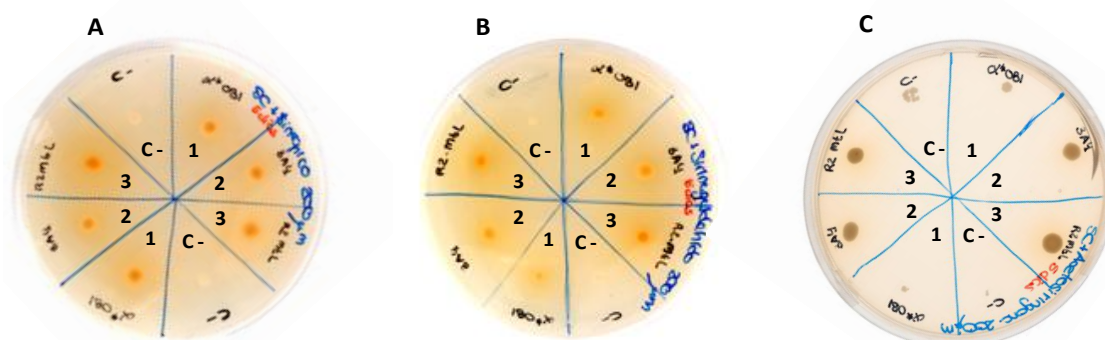


Figure 6. Screening in solid format. *Saccharomyces cerevisiae* cells secreting different laccase mutants grown for 5 days in SC plates supplemented with **A)** sinapic acid, **B)** syringaldehyde or **C)** acetosyringone. The colored halo around the colonies demonstrated the secretion of laccase (1 and 2 correspond to HRPL mutants, and 3 to LRPL mutant) as compared with the negative control (C-).

Lignin-related phenolics have been proved to mediate the *in vitro* degradation of recalcitrant aromatics by laccase (Cañas et al., 2007; Nousiainen et al., 2009), and they constitute an alternative for expensive artificial mediators such as 1-hydroxybenzotriazol (HBT) or violuric acid. Moreover, by contrast to the restricted use of HBT or violuric acid as mediators of HRPLs (Li et al., 1999), S-type phenolic mediators from lignocellulosic feedstock could be applied with other laccases.

Indeed, S-phenolic compounds notably promote oxidative reactions catalyzed by LRPLs such as MtL from the ascomycete *M. thermophila* (Babot et al., 2011; Fillat et al., 2012) or even by bacterial laccases with lower redox potentials (Rosado et al., 2012). This fact is of high interest for the biotechnological application of ascomycete or bacterial laccases that have the advantage of being more easily amendable by protein engineering than basidiomycete laccases. The prompt oxidation of the S-type phenolic compounds (due to the presence of two methoxyl substituents in the aromatic ring) make feasible the use of the new HTS assays for directed evolution studies of LRPLs or other phenoloxidases depicted in bacterial genome databases (Sharma et al., 2007).

Violuric acid as reporter assay for assessing redox potentials during protein engineering

Unlike the straightforward oxidation of S-type phenolic compounds, violuric acid is effectively oxidized only by HRPLs due to its high-redox potential ($E^\circ \approx +1.1$ V) (Li et al., 1999). The distinct oxidation rates of violuric acid by low (MtL) and high-redox potential (TvL) fungal laccases confirmed this assessment (**Fig. 7A**). When engineering fungal laccases a single amino acid change in the coordinating sphere of T1 copper may alter the complex modulation of laccase redox potential (Cambria et al., 2012). Thus, it would be helpful to have an assay to check if the high redox potential of the parent type is being maintained in the selected mutants. We devise here a HTS colorimetric assay based on the oxidation of violuric acid as an easy method to initially evaluate the redox potential of the laccase mutants generated through directed evolution of fungal laccases.

The oxidation of violuric acid generates very stable iminoxyl radicals (without dimerization products) whose purple color can be detected and quantified in the visible spectrum (λ_{\max} around 515 nm, Kim et al., 2003). The color turned to reddish when using crude extracts from *S. cerevisiae* micro-cultures due to coupling of violuric radicals to the Cu^{2+} ions from the expression medium, which produces an increment of absorbance at 420 nm (Leermakers and Hoffman, 1958). Nevertheless, the increment of absorbance at 515 nm could be measured without interferences (**Fig. 7B**). Crude extracts of *S. cerevisiae* cells secreting laccase in microplate wells were used to validate the assay. We used 20 mM violuric acid because, though it was not a saturating concentration, it rendered soluble and quantifiable colored response with absorbance values within the plate reader's detection limit. With a CV around 15% and high linearity, the assay worked for the evolved HRPL (3A4), whereas, as expected, no oxidation of violuric acid was obtained with the evolved LRPL (R2), even when both crude cell extracts showed closely similar activity on ABTS (around 120 mU mL⁻¹ supernatant, **Fig. 7C, D**). The lowest detection limit for this assay was around 0.6 mU mL⁻¹ supernatant (0.15 mU in the well).

Finally, the mutagenic library obtained by error-prone PCR of the evolved HRPL 3A4 was also screened with violuric acid as substrate (**Fig. 5C**). We observed a direct correlation among the activities of the clones with violuric acid, acetosyringone and syringaldehyde (**Fig. 5D**). In other words, active mutants on S-type phenolic compounds were also capable of oxidizing violuric acid. Thus, by using this reporter assay, we can assess whether the high redox potential of a parental laccase is preserved in all the active mutants generated through the evolution pathway or not.

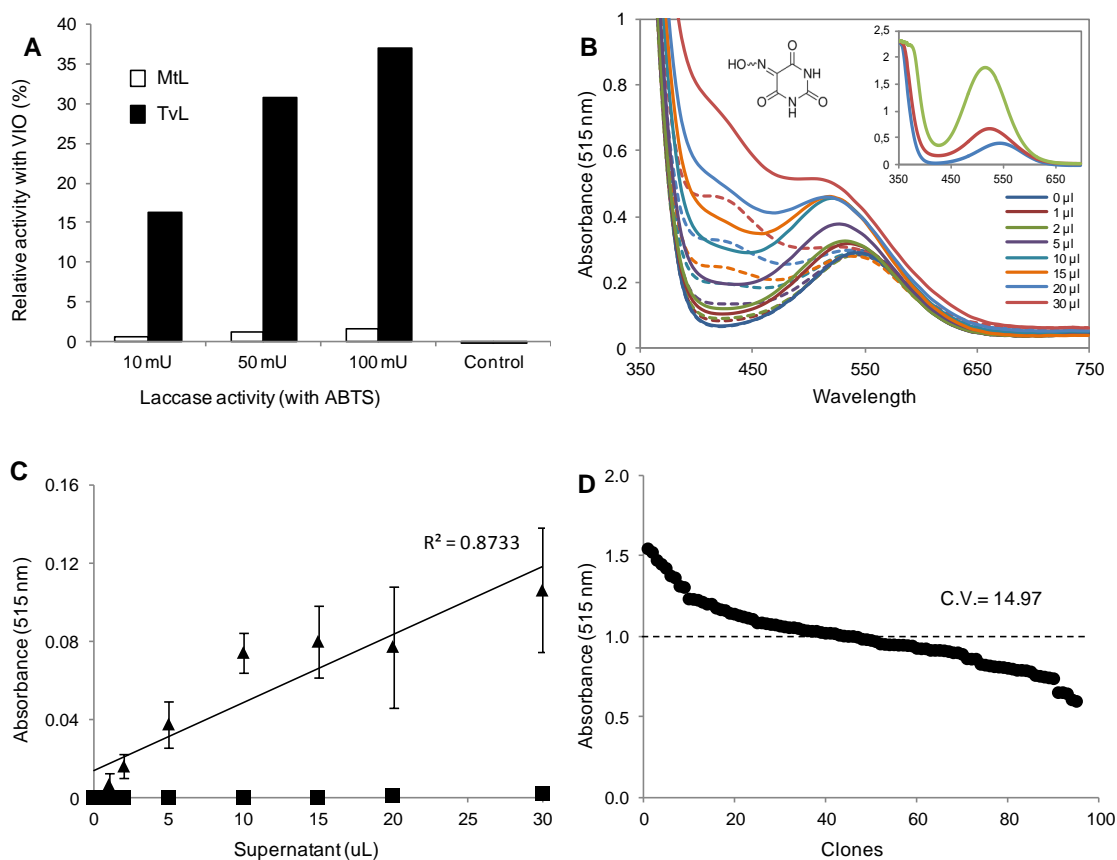


Figure 7. Validation of violuric acid HTS assay. **A**) Oxidation of 20 mM violuric acid (VIO) by MtL (white bars) and TvL (black bars) using equal laccase activity units respecting ABTS. **B**) Changes in the visible spectrum of 20 mM VIO after oxidation (t_0 , dashed lines; 20 h, continuous lines) by different volumes of crude cell extracts from *S. cerevisiae* micro-cultures secreting 3A4 HRPL (the inset shows the oxidation of VIO by 100 mU TvL in 0, 1 and 20 h). **C**) Linearity of the HTS assay using crude extracts from *S. cerevisiae* micro-cultures secreting 3A4 HRPL (triangles) or R2 LRPL (squares). **D**) Reproducibility of the endpoint HTS assay based on the oxidation of 20 mM VIO using crude cell extracts from replicates of the same clone expressing 3A4 HRPL (20 µL supernatant). The activities are plotted against the clones in descending order.

Decolorization of synthetic organic dyes

Three synthetic organic dyes, Methyl Orange (MO), Evans Blue (EB) and Remazol Brilliant Blue (RBB), were assayed as substrates for the HTS of laccase libraries. The three dyes were selected among a set of different dyes on the basis of their chemical structure since azoic (MO and EB) and anthraquinoid (RBB) dyes are the most common chromophores utilized in the dyeing industry (Drumond Chequer et al., 2011). Besides, they were directly oxidized by commercial HRPL (TvL). Changes in the absorption visible spectra carried out during the enzymatic oxidation of the three dyes provided the following λ_{\max} for measuring their decolorization: 470, 605 and 640 nm for MO, EB and RBB, respectively.

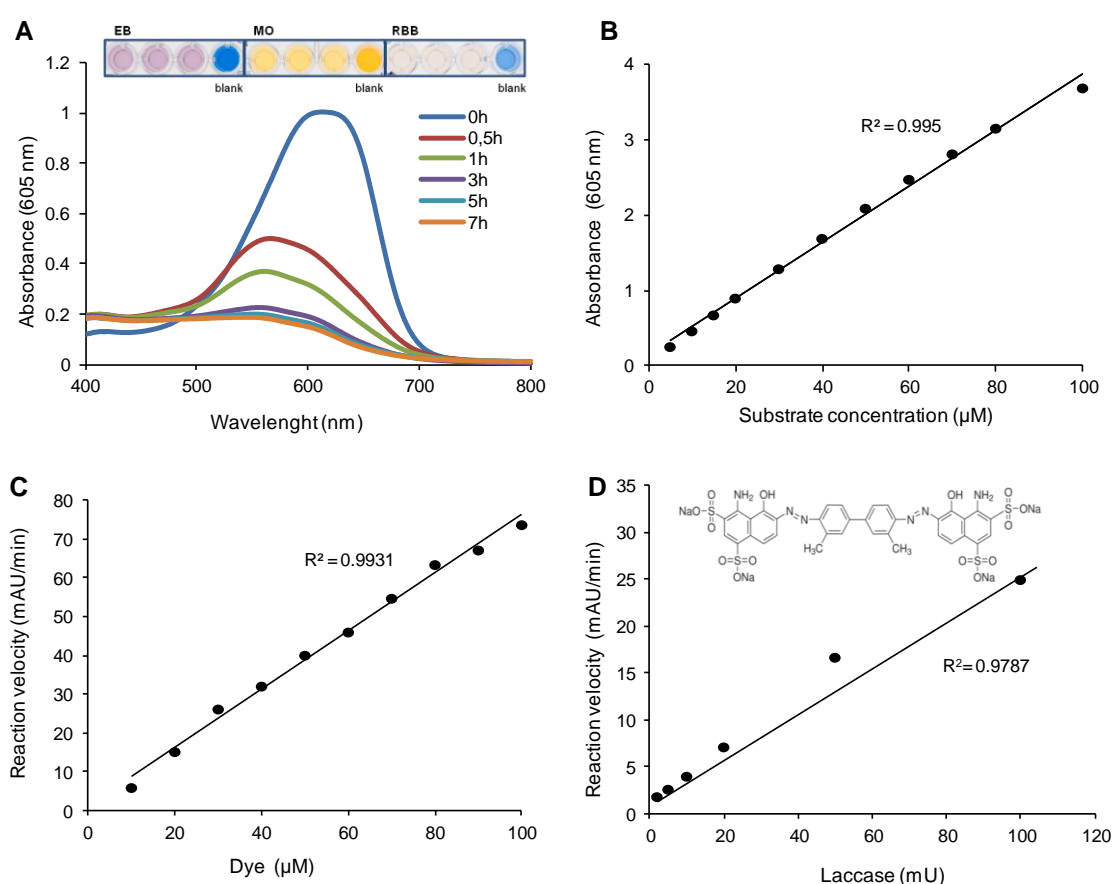


Figure 8. Oxidation of Evans Blue by laccase. **A**) Changes in the absorbance visible spectrum of Evans Blue dye (EB) during its oxidation by 20 mU *T. villosa* laccase (TvL). The inset shows the colorimetric (decolorizing) response obtained using 50 μM EB, 50 μM Methyl Orange (MO) and 200 μM Remazol Brilliant Blue (RBB) after 30 min of reaction with 10 mU TvL in triplicate. **B**) Correlation between concentration of EB and maximum peak absorbance. **C**) Oxidation velocities of EB by TvL (200 mU) measured by the decrease of absorbance at 605 nm using dye concentrations within the plate reader's detection limit. **D**) Decolorization of EB (50 μM) as absorbance decrease/min using different amounts of TvL.

Fig. 8 illustrates the oxidation of EB as an example. The elevated initial absorbance values of high dye concentrations were beyond the plate-reader's detection limit (**Fig. 8B**), thus precluding the calculation of maximum velocities during decolorization of the three dyes by TvL (**Fig. 8C**). However, we fixed 200 μM RBB and 50 μM MO and EB for the HTS assays because these concentrations provided perceptible responses (see **Fig. 8A**, inset, for the *de visu* responses given by three replicates of the same laccase sample with each dye) and quantifiable decolorization rates (**Fig. 8D** illustrates the linear response obtained with 2–100 mU laccase and 50 μM EB). Decolorization percentages of 49% for EB, 24% of RBB and 10% of MO were obtained after 3 h of reaction with TvL (10 mU). The presence of two hydroxyl substituent groups probably favors the rapid oxidation of Evans Blue (diazio) by laccase as compared to Methyl Orange (azo), whose redox potential is around +1 V ($E^\circ = +0.961$ V vs NHE, Zille et al., 2004).

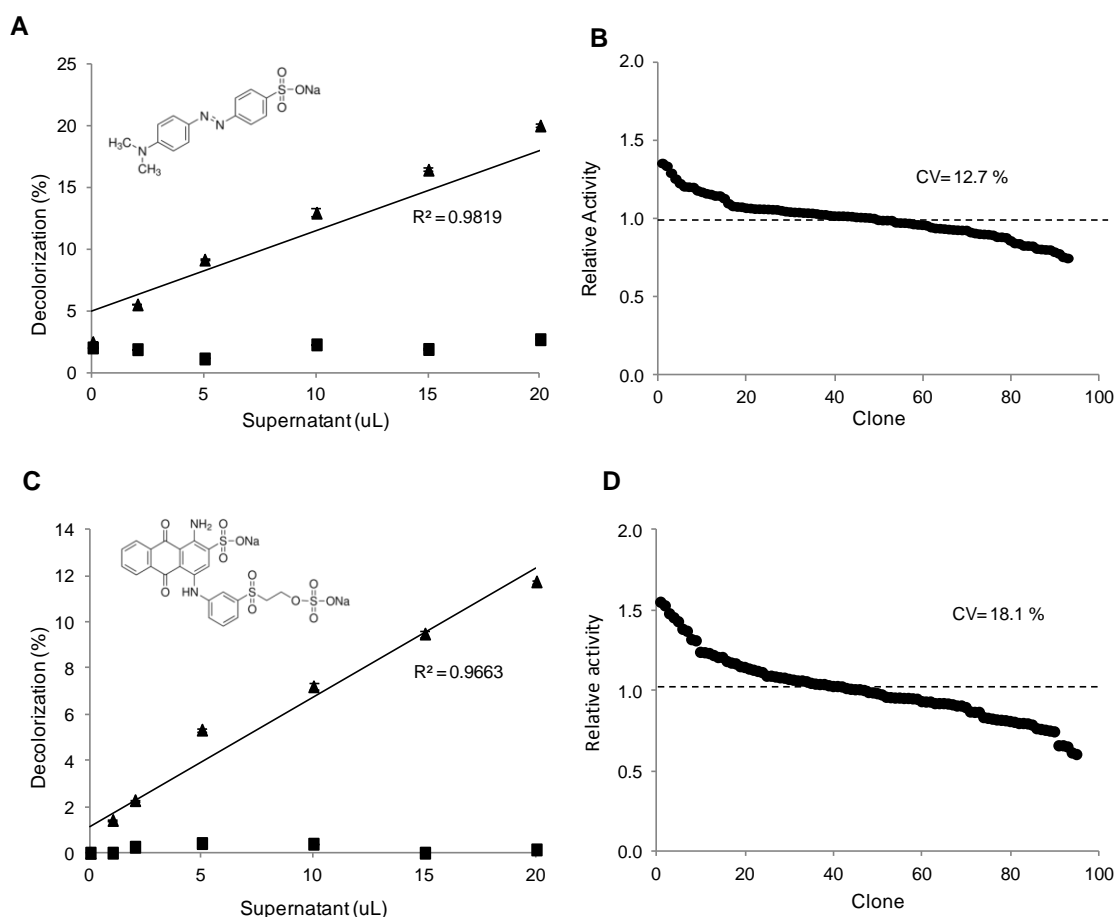


Figure 9. Validation of HTS assay with **A, B**) Methyl Orange and **C, D**) Remazol Brilliant Blue. **A, C**) Linearity and **B, D**) reproducibility of the decolorization assays with 50 μM Methyl Orange (20 h) and 200 μM Remazol Brilliant Blue (5 h), using crude extracts of *S. cerevisiae* micro-cultures expressing 3A4 HRPL (triangles) or R2 LRPL (squares). Reproducibility was tested with replicates of the same clone expressing 3A4 HRPL. The activities are plotted against the clones in descending order.

The oxidation of the three dyes was assayed in high-throughput format using crude extracts from *S. cerevisiae* cells secreting the evolved HRPL 3A4 or the evolved LRPL R2. The latter was unable to oxidize any dye under the conditions used, whereas the HRPL decolorized the three dyes. The decolorizing yields obtained with 3A4 HRPL followed the same patterns as those obtained with TvL for the three dyes: EB > MO > RBB. Decolorization yields were around 39%, 11% and 6%, for EB, RBB and MO, respectively, after 6 h of reaction using 15 μ l of supernatant from the well. The direct correlation between the volume of supernatant used and the decolorization values attained demonstrated the linearity of the three endpoint assays. Moreover, the CV values for the three dye-decolorizing assays were on average around 15%, which are acceptable to start directed evolution studies. The linearity and reproducibility of MO and RBB-based HTS assays are illustrated in **Fig. 9**.

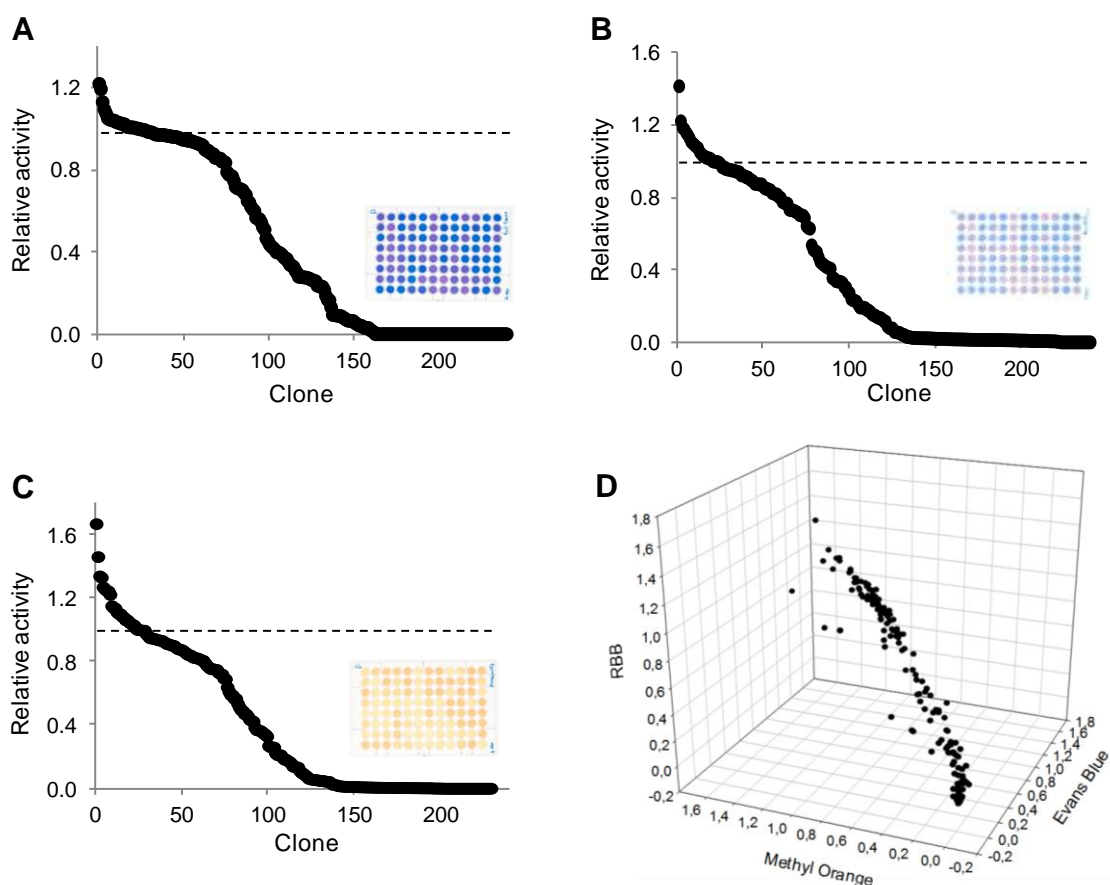


Figure 10. Multi-screening with synthetic organic dyes. Landscapes from the HTS of the mutagenic library from 3A4 HRPL using **A)** 50 μ M Evans Blue, **B)** 200 μ M Remazol Brilliant Blue and **C)** 50 μ M Methyl Orange. Laccase activities of the clones are depicted in descending order respecting the activity of the parent type. **D)** Direct correlation of the activities of the clones explored by the triple screening is shown in the 3D landscape.

The three dye-decolorizing HTS assays were finally tested for screening a mutagenic library created by error prone PCR of the HRPL 3A4. Landscapes showed quantifiable differences among the decolorizing activities of the different clones and some slight laccase activity improvements respecting the parent type (**Fig. 10A-C**). In general, we observed a direct correlation among the activities of the different laccase mutants for the three dyes (**Fig. 10D**).

Textile wastewaters contain high concentrations of unfixed dyes (mostly azo and anthraquinone dyes) which cause great pollution problems due to their recalcitrance against conventional aerobic treatments and the generation of toxic aromatic intermediates during anaerobic treatment (Drumond Chequer et al., 2011). By contrast, oxidative decolorization of azo dyes by laccase produces a detoxifying effect (Chivukula and Renganathan, 1995; Champagne and Ramsay, 2010). These dye-decolorizing HTS methods can be useful for engineering laccases for decolorization and detoxification of synthetic organic dyes. In addition, the dye-decolorization assays can be used as indirect methods to evaluate the oxidative capability of laccase-mediator systems (Brissos et al., 2009) or, more in particular, for screening laccase activity on natural mediators whose oxidation by laccase does not render colored products. This is the case of H-type phenolic compounds such as *p*-coumaric acid or methyl coumarate (Cañas et al., 2007; Gutiérrez et al., 2007). We tested the decolorization of MO by HRPL (TvL) and LRPL (MtL) in the absence or presence of phenolic mediators related to H- or S-lignin units (**Fig. 11**). In general, decolorization was significantly improved in the presence of S-type and H-type phenolic compounds, demonstrating their capability to mediate the enzymatic oxidation of the dye. Both laccases rendered similar decolorization values with the S-type mediators, whereas the decolorization attained in the presence of H-type mediators were much lower with MtL than with TvL. The low redox potential of S-type phenolic compounds (sinapic acid $E^\circ = +590$ mV, Lin et al., 1998; syringaldehyde $E^\circ = +660$ mV, Moodley et al., 2011) facilitates their oxidation by both enzymes. By contrast, the oxidation of methyl coumarate and *p*-coumaric acid ($E^\circ \approx +700$ mV, Aaby et al., 2004), although thermodynamically feasible, is limited for the LRPL MtL (E° at T1 copper site $\approx +470$ mV), but not for the HRPL TvL ($E^\circ \approx +780$ mV) (Xu et al., 1998), thus explaining the better decolorization values attained with the latter when using H-type mediators. Nonetheless, the decolorization assay might still be useful as an indirect method for the *in vitro* evolution of fungal laccases towards H-type mediators whose oxidation cannot be detected by the naked-eye.

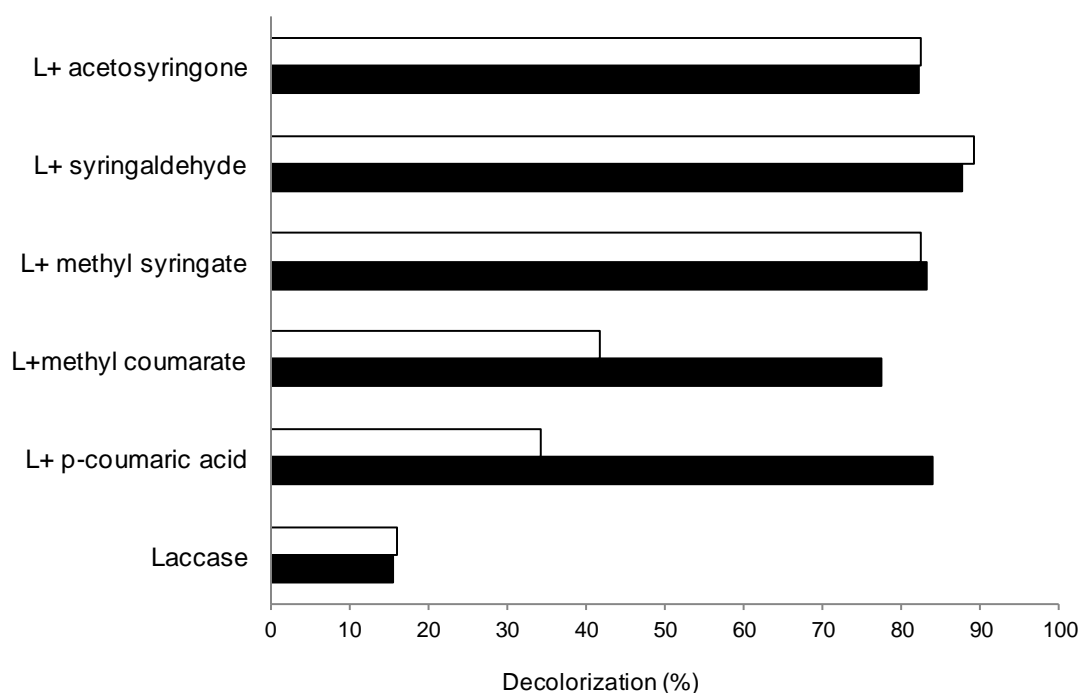


Figure 11. Decolorization of 50 μ M Methyl Orange by 10 mU of laccase from *M. thermophila* (white bars) or *T. villosa* (black bars), with or without natural phenolic mediators. Mean values from two replicates after blank subtraction are shown (molar ratio of mediator/dye = 4; 5 h reaction time).

CONCLUSIONS

We have devised and validated a set of colorimetric activity assays in high-throughput format for exploring laccase activity in mutant libraries generated by directed evolution. The assays are based on the enzymatic oxidation of natural redox mediators derived from lignocellulose and synthetic organic dyes. Besides, the use of violuric acid assay as reporter of laccase redox potential can be useful to preserve this significant property whilst evolving towards new functions. As we demonstrate here, these new colorimetric HTS assays are reproducible and reliable enough for contributing to face up to new evolution challenges. The engineering of laccase variants with better catalytic efficiencies towards key natural phenolic compounds, under preferred conditions, might be of relevance for the application of these enzymes in industrial processes of conversion of plant biomass. The dye-decolorizing HTS assays can be used for engineering *ad hoc* laccases to be applied in detoxification of textile industrial wastewaters. In addition, they can be used as indirect HTS assays for searching for better oxidation activities on phenolic mediators of interest, whose enzymatic oxidation cannot be detected in the visible spectrum.

METHODS

Reagents and enzymes

Crude laccases from *Trametes villosa* (TvL) (NS-51002) and *Myceliophthora thermophila* (MtL) (NS-51003) were purchased from Novozymes (Denmark). Reagents Methyl Orange (MO), Evans Blue (EB), Remazol Brilliant Blue (RBB), sinapic acid, acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone), syringaldehyde (3,5-dimethoxy-4-hydroxybenzaldehyde), violuric acid (5-(hydroxyimino)-2,4,6(1H,3H,5H)-pyrimidinetrione) and ABTS were purchased from Sigma-Aldrich.

Culture media

Minimal medium contained 100 mL 67 g L⁻¹ sterile yeast nitrogen base, 100 mL 19.2 g L⁻¹ sterile yeast synthetic drop-out medium supplement without uracil, 100 mL sterile 20% raffinose, 700 mL sterile double-distilled H₂O (ddH₂O), and 1 mL 25 g L⁻¹ chloramphenicol. Yeast extract-peptone (YP 1.55X) medium contained 10 g yeast extract, 20 g peptone, and ddH₂O to 650 mL. Expression medium contained 720 mL YP 1.55X, 67 mL 1 M KH₂PO₄, pH 6.0, buffer, 110 mL 20% galactose, 2 mM CuSO₄, 25 g L⁻¹ ethanol, 1 mL 25 g L⁻¹ chloramphenicol, and ddH₂O to 1000 mL. The yeast extract-peptone-dextrose (YPD) solution contained 10 g yeast extract, 20 g peptone, 100 mL 20% sterile glucose, 1 mL 25 g L⁻¹ chloramphenicol, and ddH₂O to 1000 mL. Synthetic complete (SC) dropout plates contained 100 mL 67 g L⁻¹ sterile yeast nitrogen base, 100 mL 19.2 g L⁻¹ sterile yeast synthetic drop-out medium supplement without uracil, 20 g bacto agar, 100 mL 20% sterile glucose, 1 mL 25 g L⁻¹ chloramphenicol, and ddH₂O to 1000 mL. The SC drop-out plates to test the screening assays in solid format contained 10 μM CuSO₄, 2 g L⁻¹ galactose instead of glucose and 200 μM syringaldehyde, acetosyringone or sinapic acid.

Oxidation assays with commercial laccases

Standard assay with 3 mM ABTS was used for initial measurement of laccase activities by recording the increase of absorption with time at 418 nm ($\epsilon_{\text{ABTS}^{*+}} = 36000 \text{ M}^{-1} \text{ cm}^{-1}$). Changes in the UV-VIS spectra of S-type phenolic substrates, dyes and violuric acid during oxidation by TvL or MtL (20 mU) in 100 mM tartrate buffer pH 4.0, were recorded in the spectrophotometer (Shimadzu UV-1800) to determine the corresponding λ_{max} and concentrations to be used in the HTS assays. Then, oxidation of S-type phenolics was measured by the increase of absorbance at 370 nm for syringaldehyde (2 mM), 520 nm for acetosyringone (2 mM) and 512 nm for sinapic acid (250 μM); decolorization of dyes was measured by the decrease of absorbance at 470 nm for MO (50 μM), 605 nm for EB (50 μM) and 640 nm RBB (200 μM); and oxidation of violuric acid (20 mM) was measured by the increase in absorbance at 515 nm. All measurements were carried out in sodium tartrate buffer

pH 4.0 (250 μ L final volume) in a plate reader (SPECTRAMax Plus 384, Molecular Devices).

Micro-cultures of *S. cerevisiae* cells expressing laccase mutants

Colonies from yeast transformed cells were picked and transferred to 96-well plates where they were cultured in 50 μ L of minimum medium for two days. Then, 160 μ L of expression medium (Camarero et al., 2012) were added and the plates were incubated during another three days. Micro-fermentations (210 μ L) were carried out at 30 °C and 200 rpm in a humidity shaker.

To determine laccase activity in the wells, plates were centrifuged and aliquots of the supernatant were transferred to new plates with the help of a liquid handler (Quadra96, Tomtec, USA). Target substrates in tartrate buffer pH 4.0 were added to a final volume of 250 μ L and endpoint absorbances at the corresponding λ_{\max} were measured in the plate reader, except for ABTS, which was measured in kinetic mode.

Validation of the HTS colorimetric assays

- Linearity of the endpoint assay:

To test the linearity and sensitiveness of the assays, wells were inoculated with yeast cells expressing the evolved laccases R2 or 3A4. Un-inoculated wells were used as negative control. Micro-fermentations were carried out as mentioned above and, after centrifugation, different volumes of supernatant (1–30 μ L) were transferred to new plates. Next, target substrates were added in tartrate buffer to a final volume of 250 μ L and laccase activities were measured in the plate reader as described above.

- Reproducibility of the endpoint assay (CV):

Yeast transformed cells expressing the evolved HRPL 3A4 were cultured in each well of the same 96-well plate and incubated as above mentioned. Thereafter, 30 μ L of the supernatants containing the secreted laccase were transferred to new plates and 220 μ L of the different substrates in tartrate buffer pH 4.0 were added. The reactions were kept during 24 h and the oxidation products were measured at the corresponding λ_{\max} using the plate reader in endpoint mode.

Construction of the mutagenic libraries

A small mutagenic library was created by error-prone PCR of the chimeric HRPL 3A4 (Pardo et al., 2012) to test the HTS-assays. Reaction mix contained 5 μ L 10X Taq buffer, 3% DMSO, 1.5 mM MgCl₂, 0.01 mM MnCl₂, 0.3 mM dNTP mix, 90 nM of primers RMLN and RMLC, 4.6 ng parent plasmid DNA (pJR α 3A4) and 2.5 units of Taq DNA polymerase in a final volume of 50 μ L. PCR cycles were 95 °C for 2 min; 28 cycles of 94 °C for 0.45 min, 53 °C for 0.45 min, 74 °C for 3 min; and 74 °C for 10 min.

Reaction products were loaded into 0.8% agarose gels and 1.9 kb bands were cut and purified. 400 ng of this purified product was used to transform yeast together with 100 ng of the pJRoC30 expression vector previously linearized with BamHI and NotI. A larger laccase library created by error-prone PCR and *in vivo* shuffling of selected chimeric laccases (Pardo et al., 2012) was used for testing the sinapic acid assay. The error-prone PCR reactions of five chimeric laccases were the same as described above. Then, the amplified products were purified and jointly transformed in the yeast, using 133 ng of each parent insert and 200 ng of the linearized plasmid.

High-throughput screening of laccase libraries

The endpoint colorimetric assays were tested in the abovementioned mutagenic libraries. Two hundred colonies were picked from SC-dropout plates and individual clones were grown in wells of 96-well plates as described above. Column 6 from each plate was inoculated with parent type, while well H1 was not inoculated (blank). After centrifugation, 30 μ L supernatants were transferred to replica plates where 220 μ L of 2 mM acetosyringone, 2 mM syringaldehyde, 20 mM violuric acid, 50 μ M MO, 50 μ M EB or 200 μ M RBB in tartrate buffer pH 4 were added. The plates were briefly stirred, and the absorption at the corresponding λ_{\max} (see above) were measured. The plates were incubated at room temperature in darkness and laccase activities were measured by the increase (mediators) or decrease (dyes) of color. Relative activities were calculated from the difference in absorption over time normalized against the parent type in the corresponding plate. The colorimetric assay with sinapic acid as substrate (250 μ M) was evaluated with two thousand colonies from a larger laccase library, following the same procedure.

ABBREVIATIONS

HTS, High-throughput screening; H, *p*-hydroxyphenyl lignin units; S, Syringyl lignin units; HRPL, High-redox potential laccase; LRPL, Low-redox potential laccase; TvL, *Trametes villosa* laccase; MtL, *Myceliophthora thermophila* laccase; R2, Evolved LRPL from MtL; 3A4, Evolved HRPL from shuffling of PM1 and *P. cinnabarinus* laccases; MO, Methyl orange; EB, Evans blue; RBB, Remazol brilliant blue.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

IP and XC have equally contributed to this study. IP constructed the mutagenic libraries, and contributed to the development and validation of the colorimetric assays in high-throughput format and to figures design. XC carried out the assays

with commercial enzymes, developed the dye-decolorizing assays in high-throughput format and performed the screening of mutant libraries. AIV developed the screening assays in solid format. MA made critical revision of the manuscript. SC coordinated the conception and design of the study and wrote the manuscript, which was read and approved by all the authors.

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CHAPTER 3

RE-DESIGNING THE SUBSTRATE BINDING POCKET OF LACCASE FOR ENHANCED OXIDATION OF SINAPIC ACID

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ABSTRACT

Iterative saturation mutagenesis was performed over six residues delimiting the substrate binding pocket of a high-redox potential chimeric laccase with the aim of enhancing its activity over sinapic acid, a lignin-related phenol of industrial interest. In total, more than 15000 clones were screened and two selected variants, together with the parent-type laccase, were purified and characterized. The new variants presented shifted pH activity profiles and enhanced turnover rates on sinapic acid and its methyl ester, whereas the oxidation of related phenols was not significantly enhanced. Neither the enzyme's redox potential nor the mechanism of the reaction were affected. Quantum mechanics and molecular dynamics calculations were done to rationalize the effect of the selected mutations, revealing the critical role of the residues of the enzyme pocket to provide the precise binding of the substrate that enables an efficient electron transfer to the T1 copper. The results presented highlight the power of combining directed evolution and computational approaches to give novel solutions in enzyme engineering and to understand the mechanistic reasons behind them, offering new insights for further rational design towards specific targets.

INTRODUCTION

Laccases are multi-copper oxidases capable of oxidizing a wide range of compounds, especially substituted phenols and aromatic amines, coupled to the reduction of molecular oxygen to water. Some laccases secreted by white-rot fungi involved in lignin degradation (typically belonging to the order Polyporales) stand out for their high-redox potential at the T1 copper site ($E^0 \sim 0.8$ V), compared to laccases from other fungi, plants or bacteria (from 0.4 to 0.7 V). Moreover, the use of redox mediators, which are small molecules that can act as diffusible electron shuttles between the substrate and laccase, can further extend the oxidative capabilities of these enzymes. In previous studies we described the ability of certain phenolic compounds related to lignin to act as laccase mediators (Camarero et al., 2005; Camarero et al., 2008). The combined application of laccase and these natural mediators might be a key factor for the integrated use of plant biomass in lignocellulosic biorefineries for the sustainable production of chemicals, materials and fuels (Camarero et al., 2014; Cañas and Camarero, 2010).

The potential of laccases as industrial biocatalysts has led to numerous efforts to engineer these enzymes with the aim of enhancing their activity and/or stability for industrial application (Pardo and Camarero, 2015a). In this sense, directed evolution has proven to be a powerful tool to obtain laccases *a la carte*. In a previous work we described the construction of chimeric laccases by random DNA shuffling of two high-redox potential laccases (HRPLs) from basidiomycetes PM1 and *Pycnoporus*

cinnabarinus expressed in *Saccharomyces cerevisiae* (Camarero et al., 2012; Maté et al., 2010; Pardo et al., 2012). The new laccases presented improved properties with respect to both parent types, including better substrate affinities and increased stability towards temperature and pH.

Using one chimeric laccase (3A4) with increased affinity towards phenols as scaffold, we aim here to enhance its activity towards sinapic acid (SA). This *p*-hydroxycinnamic acid is of biotechnological interest for: a) its ability to act as laccase mediator (Camarero et al., 2008); b) the biological activity (as antioxidant, antimicrobial, immunomodulatory, antitumoral or UV-B screening agents) of SA itself, its esters or other derivatives (Landry et al., 1995; Gaspar et al., 2010; Nićiforović and Abramovič, 2014); and c) the role of sinapate dehydrodimers or heterodimers (with ferulate) in cross-linking polysaccharides in grass cell walls (Bunzel et al., 2003). Indeed, *p*-hydroxycinnamic acids are one of the main inhibitory phenolic compounds released during physico-chemical pretreatment of lignocellulose for ethanol production. Detoxification of wheat straw slurries by laccase, after the enzymatic hydrolysis or during the simultaneous saccharification and fermentation, has been described to enhance yeast fermentation performance and raise the final ethanol yields (Jurado et al., 2009; Moreno et al., 2012), allowing also to work at higher substrate consistencies (Moreno et al., 2013).

To this end, we describe here the re-design of the laccase's substrate binding pocket by Iterative Saturation Mutagenesis (ISM) (Reetz and Carballeira, 2007) of six residues limiting the cavity. This is the first time that the systematic exploration of all possible amino acid substitutions and their combinations is attempted in laccases, as compared to previous works (Gupta et al., 2010; Galli et al., 2011; Toscano et al., 2013). At the same time we address the biochemical and mechanistic characterization of two of these engineered laccase variants selected for their increased activity towards SA, together with the computational simulations performed to rationalize the effect of the inserted mutations.

EXPERIMENTAL SECTION

Construction of mutant libraries

Sequences of laccases from basidiomycetes of the order Polyporales were retrieved from UniProt KB, filtering for a minimum length of 450 amino acids and clustering identical sequences. Selected sequences were aligned using T-Coffee (Notredame et al., 2000) and sequence logos were generated with WebLogo3 server (Crooks et al., 2004). The most variable residues delimiting the substrate binding pocket (six in total) were selected for mutagenesis. Mutant libraries were obtained by IVOE (Alcalde, 2010), using the pJRoC30 plasmid containing 3A4 coding sequence fused to an evolved alpha-factor prepro-leader as template (Pardo et al., 2012).

Complementary mutagenic primers were designed so that degenerate codons (NNK, where N = A/C/G/T, and K = G/T) were flanked by ~20 bp (**Table S1**). For each mutagenesis site two PCRs were performed: one with RMLN and the reverse mutagenic primer, and another with RMLC and the forward mutagenic primer. RMLN and RMLC primers anneal with pJRoC30 plasmid, flanking the α -3A4 CDS. PCR conditions, product purification and *in vivo* cloning in competent *S. cerevisiae* BJ5465 cells have been described previously (Pardo et al., 2012).

Screening of mutant libraries

For each mutant library, at least 3066 clones were analyzed in order to obtain 95% library coverage (two positions with NNK degeneracy). Culture media for cell growth and laccase expression in 96-well plates, and the general procedure for library screening and the two re-screenings are described previously (Camarero et al., 2012; Pardo et al., 2012). In this work, libraries were screened for activity towards 250 μ M SA (at 512 nm) in 100 mM acetate buffer, pH 5 (Camarero et al., 2012; Pardo et al., 2013). A thermostability screening assay was also performed with some selected clones (García-Ruiz et al., 2010).

Production and purification of mutated laccases

Individual colonies of selected clones were grown in minimal medium (6.7 g L⁻¹ YNB, 1.92 g L⁻¹ drop-out media supplements without uracil, 2% raffinose and 25 mg L⁻¹ chloramphenicol) for 48 h at 30 °C, and 220 rpm. Aliquots of these cultures were used to inoculate 30 mL of minimal medium in 250 mL flasks to an OD₆₀₀ = 0.25, which were incubated until an OD₆₀₀ = 1 was reached. Then, cells were diluted tenfold to a final volume of 300 mL with laccase expression minimal medium (6.7 g L⁻¹ YNB, 1.92 g L⁻¹ drop-out media supplements without uracil, 2% galactose, 67 mM phosphate buffer, pH 6, 1 mM CuSO₄, and 25 mg L⁻¹ chloramphenicol) in 1 L flasks. Cultures were grown at 30 °C, 220 rpm, until maximum laccase activity was reached (3–4 days). Standard laccase activity assay consisted in measuring the oxidation of 3 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 100 mM acetate buffer (pH 5) in the spectrophotometer (ABTS⁺, $\epsilon_{418} = 36000 \text{ M}^{-1} \text{ cm}^{-1}$), defining one unit of laccase as the amount of enzyme needed to transform 1 μ mol ABTS per minute.

Cultures were centrifuged at 10000 *g* for 15 min at 4 °C, and supernatants were consecutively filtered through 0.8 and 0.45 μ m pore-size membranes. Then, crude extracts were concentrated by ultra-filtration through 10000 MWCO membranes and dialyzed against 20 mM Tris-HCl buffer, pH 7. Laccases were purified by HPLC (AKTA purifier, GE Healthcare) in two anion-exchange and one molecular exclusion steps: first, using a HiPrep Q FF 16/10 column and a 100 mL gradient of 0–40% elution buffer (20 mM Tris-HCl + 1 M NaCl, pH 7); second, using a Mono Q HR 5/5

column and a 30 mL gradient of 0–25% elution buffer; and finally using a HiLoad 16/600 Superdex 75 pg column and 20 mM Tris-HCl + 150 mM NaCl, pH 7 (all columns from GE Healthcare). Fractions containing laccase activity were pooled, dialyzed and concentrated between each chromatographic step.

Biochemical characterization of laccase variants

Microplate assays to determine T_{50} (10 min), pH activity profiles, and kinetic constants of purified laccase variants are described previously (Camarero et al., 2012). Kinetic constants for SA ($\epsilon_{312} = 17600 \text{ M}^{-1} \text{ cm}^{-1}$), methyl sinapate (MS, $\epsilon_{320} = 18855 \text{ M}^{-1} \text{ cm}^{-1}$) and 2,6-dimethoxyphenol (DMP, product $\epsilon_{469} = 27500 \text{ M}^{-1} \text{ cm}^{-1}$, referred to DMP concentration) were determined in 96-well plates, using UV-transparent microplates for SA and MS (UV-Star, Greiner Bio-One). Kinetic constants for dehydrodisinapic acid dilactone (DAD, product $\epsilon_{530} = 17000 \text{ M}^{-1} \text{ cm}^{-1}$, referred to DAD concentration) were determined in the spectrophotometer using 1 cm path-quartz cuvettes. In the case of syringic acid (SyA, $\epsilon_{260} = 9035 \text{ M}^{-1} \text{ cm}^{-1}$) and methyl syringate (MSy, $\epsilon_{275} = 11660 \text{ M}^{-1} \text{ cm}^{-1}$), reactions were carried out in 0.1 cm path-quartz cuvettes. Due to the high initial absorbance of MSy, reactions for higher concentrations (mM range) were followed discontinuously, taking aliquots at different time-points and diluting tenfold prior to measuring A_{275} . The chemical structure of phenolic compounds for which kinetic constants were obtained is shown in **Fig. S1**.

Competition reactions with *p*-X-substituted phenols were carried out as described previously (Galli et al., 2013), with slight modifications: 2 or 5 units of the different variants were added to a 600 μL mixture of O_2 -saturated 100 mM citrate buffer (pH 5) and dioxane (v:v ratio 2:1) containing 6 μmol of each phenolic substrate. Reactions proceeded at room temperature with stirring and under O_2 atmosphere. After addition of 4-methoxyacetophenone as internal standard and extraction with ethyl acetate, consumption of substrates at end-point was determined with an Agilent 6850 Series II GC equipped with a methyl silicone capillary column. Hammett plots were obtained by representing relative reaction constants $\log(k_X/k_H)$ versus σ^+ for the different *p*-X-substituted phenols and fitting to a straight line.

Redox potentials of the different variants were determined by the poised potential method using the redox couple $\text{Fe}(\text{dipyridyl})_2\text{Cl}_2/\text{Fe}(\text{dipyridyl})_2\text{Cl}_3$ in 8 mM MES buffer (pH 5.3) (Xu et al., 1996; Galli et al., 2013). Oxidation of $\text{Fe}(\text{dipyridyl})_2\text{Cl}_2$ at each titration point was followed by the decrease in absorbance at 522 nm ($\epsilon_{522} = 5974 \text{ M}^{-1} \text{ cm}^{-1}$) until equilibrium was reached. Concentration of reduced laccase at equilibrium was considered to be 1/4 of the oxidized $\text{Fe}(\text{dipyridyl})_2\text{Cl}_2$ concentration.

Marcus plots were obtained by representing $\ln(k_X/k_H)$ versus ΔG° , calculated from the difference in redox potentials between the laccase and the different *p*-X-substituted phenols. Reorganization energies for electron transfer were calculated by fitting data to the following equation (Galli et al., 2013): $\ln(k_X/k_H) = \ln(k_B T/h) - \ln k_H - \Delta G^\ddagger/RT$, where $\Delta G^\ddagger = (\lambda/4)(1 + \Delta G^\circ/\lambda)^2$.

Synthesis of DAD

SA was dissolved in 80 mL of acetonitrile (27.5 mM concentration), and 80 mL of a 27.5 mM aqueous solution of CuSO₄ was added. The mixture was stirred for 6 h at room temperature and evaporated to dryness at 40 °C under reduced pressure. The solid residue was redissolved in water and extracted with ethyl acetate. The organic layers were washed with brine, dried over anhydrous Na₂SO₄, and concentrated under vacuum to give the SA lactone dimer (308 mg, 0.69 mmol) (Fan et al., 2009). Crystallization from aqueous acetone yielded dilactone as yellow prisms (m.p. 212–214 °C, lit 208 °) (Freudenberg and Schraube, 1955; Lu and Ralph, 2008). Structure was confirmed by NMR in acetone-*d*₆. ¹H-NMR spectrum (300 MHz, δ (ppm): 7.60 (brs, 1H, OH), 6.74 (s, 4H), 5.76 (s, 2H), 4.12 (s, 2H) and 3.84 (s, 12H). ¹³C-NMR spectrum (75 MHz) δ (ppm): 176.8, 149.7, 138.1, 130.6, 105.0, 84.2, 57.5 and 49.8.

Systems setup for computational simulations

The initial structure was taken from the coordinates of PM1L (PDB 5ANH), which shares 98% sequence identity with the chimeric variant 3A4. The 3A4 model contains a single mutation in the substrate binding pocket regarding PM1L (V162A), selected during the directed evolution of PM1L (Maté et al., 2010). The C14F12 model additionally introduces F392N, while the CA32F1 model includes mutations V162R, T164E and F392N. In the first two proteins mutations were modeled with Maestro (version 10.0, Schrodinger, LLC, New York, NY, 2014), allowing for the modified amino acid and its neighbors (5 Å distance) to relax with IMPACT (version 6.5, Schrodinger, LLC, New York, NY, 2014). In the case of CA32F1 however, the introduction of a large side chain in position 162 (V to R) required further attention, and 20 ns of molecular dynamics (MD) simulation with DESMOND (Shivakumar et al., 2010) was performed to assure the model's stability (see supplementary material).

The protonation state of titratable residues was estimated with the Protein Preparation Wizard (PROPKA) and the H++ server (<http://biophysics.cs.vt.edu/H++>), followed by visible inspection (Anandakrishnan et al., 2012; Madhavi Sastry et al., 2013; Olsson et al., 2011). At pH 5 (in which most of the experiments were performed) histidines 55, 71, 91, 133, 153 and 401 were established to be fully protonated (positively charged); 66, 394 and 455 were ϵ -protonated; and all others were δ -protonated. Aspartic acids 50, 77, 101, 453 and

467 and glutamic acids 308 and 470 were also found to be protonated at pH 5. Further experiments were conducted at pH 3 and predicted pK_a values showed no changes in protonation states for both 3A4 and C14F12 (in particular in the binding pocket, where D205 is found to have a pK_a of 1.2 and 1.8, respectively). In CA32F1, however, D205 has an increased pK_a of 3.8 and for this reason extra simulations for this system were also performed with protonated D205.

With an experimentally determined pK_a of 4.9 (Ragnar et al., 2000), SA is expected to have the protonated (SAH) and deprotonated (SA⁻) species in equilibrium at pH 5, and so all simulations have been performed with both models. Structures for SA, DAD and MS were fully optimized with Jaguar (Bochevarov et al., 2013) in an implicit solvent and electrostatic potential charges computed with the density functional M06 at 6-31G* level of theory. From these, ligand parameters were extracted for the classic simulations.

PELE sampling

To sample the binding modes of the three computationally studied substrates (SA, DAD and MS) in the chimeric proteins, we have used the Protein Energy Landscape Exploration (PELE) software (Borrelli et al., 2005). PELE is a Monte Carlo algorithm composed of a sequence of perturbation, relaxation, and a Metropolis acceptance test. In the first step, the ligand is subjected to random rotations and translations while the protein is perturbed based on the anisotropic network model (ANM) (Atilgan et al., 2001). For ligand perturbation the maximum allowed translation was 2.2 Å and the maximum rotation 20°. For the protein perturbation all atoms are displaced (a maximum of 0.5 Å) by moving α -carbons to follow a random linear combination of the 6 lowest eigenvectors obtained in the ANM model. The relaxation step includes the repositioning of all amino acid side chains within 6 Å of the ligand and the 5 side chains with the highest energy increase along the previous ANM step. The relaxation stage ends with a truncated Newton minimization using the OPLS all-atom force field and an implicit surface-generalized Born continuum solvent (Bashford and Case, 2000; Kaminski et al., 2001). New proposed minima are then accepted or rejected based on a Metropolis test. Substrate binding plots contain all accepted conformations for three 48 h-simulations using 40 processors.

Quantum mechanics/molecular mechanics (QM/MM)

After applying an energy filter, randomly selected structures from PELE simulations were further optimized through five cycles of geometry optimization using Qsite (Murphy et al., 2000), and the atomic Mulliken spin density of the substrate was computed (Mulliken, 1955). The protein is modeled classically using the OPLS force field while the substrate, the T1 copper atom, and residues H394, C450, H455, I452 and F460 are included in the quantum region. The density functional method employed was the M06-L functional, with the LANL2DZ effective

core basis set for the copper atom and the 6-31G* for all other atoms (Hehre et al., 1972; Hay and Wadt, 1985).

RESULTS AND DISCUSSION

Construction and screening of mutant libraries

Inspection of the molecular model for the parent 3A4 laccase revealed that the substrate binding pocket was delimited by amino acids in positions 162–164, 263, 264, 331, 336 and 390–392. In order to select the target residues for mutagenesis, we performed a multiple sequence alignment of laccases from fungi of the order Polyporales (including *P. cinnabarinus* and basidiomycete PM1) (Fig. 1).

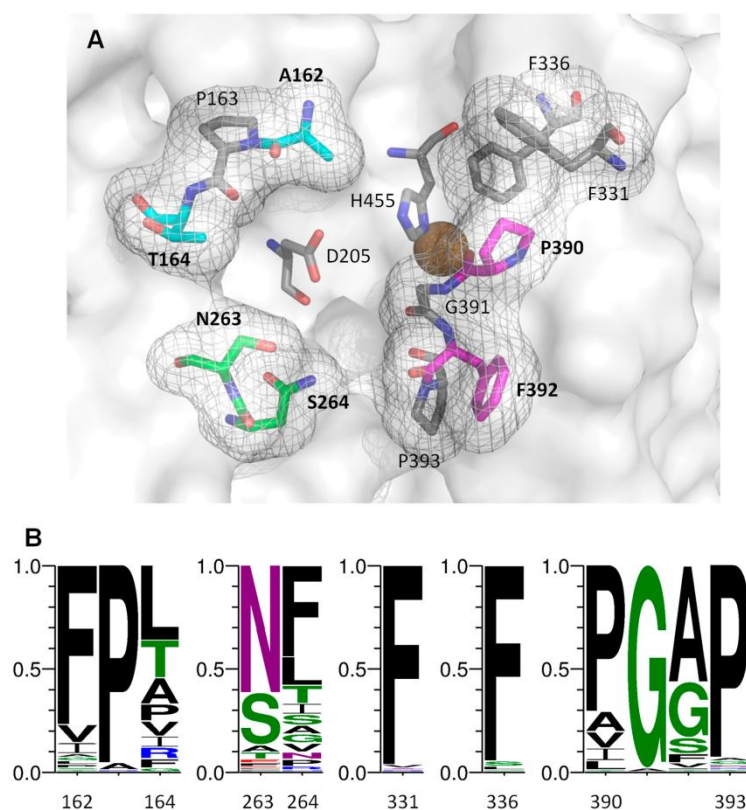


Figure 1. A) Residues delimiting the substrate binding pocket of 3A4 laccase. Residues selected for mutagenesis are shown in cyan (region A), green (region B) and magenta (region C). Conserved residues His 455 and Asp 205, respectively involved in electron and proton transfer, are also shown. B) Sequence logo for residues delimiting the substrate binding pocket in laccases of Polyporales. Heights indicate relative frequencies. Hydrophobic residues are shown in black, polar residues in green, acid residues in red, basic residues in blue and neutral residues in purple.

Residues in positions 162, 164, 263, 264, 390 and 392 (according to 3A4 laccase numbering) were the most variable and, therefore, were chosen for randomization. As simultaneous combinatorial saturation mutagenesis (CSM) of the six sites would imply screening more than 10^9 clones for 95% library coverage, we used the ISM approach in order to diminish the screening effort (Reetz and Carballeira, 2007). In

our case, we defined three regions, each comprising two target residues for mutagenesis: region A, for residues 162 and 164; region B, for residues 263 and 264; and region C, for residues 390 and 392. Individual libraries were constructed for each region and, this way, the number of clones to be screened for 95% coverage was reduced to 3066 per library.

The mutant libraries were screened with SA at pH 5. This pH is described as the working pH for the enzymatic saccharification and yeast fermentation of prehydrolyzates from wheat straw biomass to produce ethanol (Alvira et al., 2013). Owing to the common acidic pH of laccases, the availability of recombinant enzymes with more neutral optimum pH is of interest for biomass conversion processes. Activity landscapes of first generation mutant libraries A, B and C revealed different plasticity among regions, *i.e.*, the admission of amino acidic variability in the selected positions (**Fig. 2A**). Region B was the least flexible of the three studied, with 83% of clones presenting less than 5% activity respecting the parent laccase, followed by region A (48%) and region C (29%). The latter presented a high proportion of clones with better activity than parent type. A first re-screening was performed to rule out false positives, and plasmids from selected clones from each library were extracted and sequenced (**Table S2**). In the case of library A, the selected clones mutated A162 for polar residues. Notably, several included basic amino acids, even though the sequence alignment of laccases showed conserved hydrophobic residues in this position (mostly phenylalanine). All five clones from library B that were sequenced maintained N263, indicating its essential role for activity of these laccase variants (even though this position is quite variable among polyporal laccases). Finally, mutations in library C were the most variable, although the most frequent were the substitution F392N and the conservation of P390.

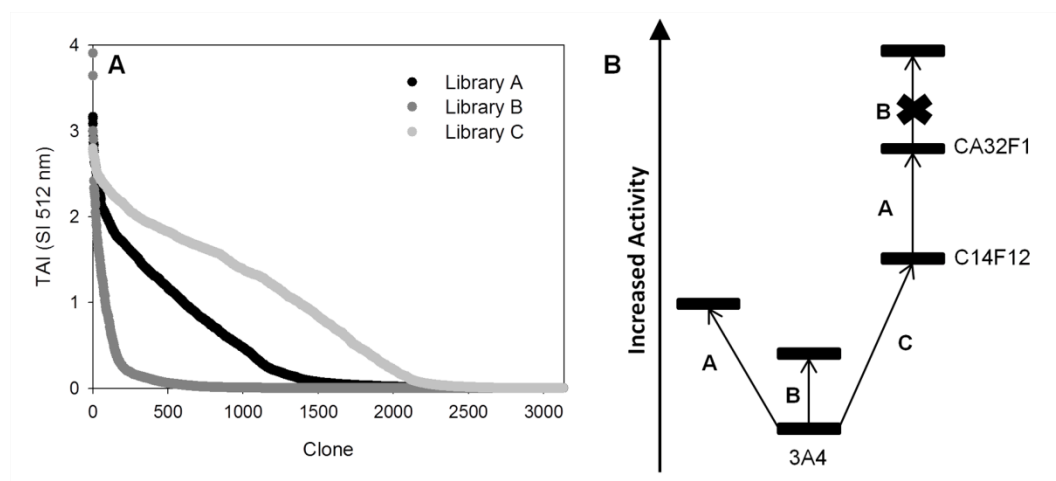


Figure 2. A) Activity landscapes for mutant libraries A (black), B (dark grey) and C (light gray). B) Representation of the ISM pathway followed in this work.

Next, a second re-screening, in which plasmids were re-transformed in yeast, was performed simultaneously for selected mutants from the three libraries. In general, total activity increase (TAI) values decreased in the order $C > A > B$, so the ISM pathway was defined as shown in **Fig. 2B**: the winner from library C would be used as scaffold for mutagenesis of region A (library CA), and the winner of this library would then be used as scaffold for the mutagenesis of region B (library CAB). In order to select the clone from library C to be used as parent for the next round of mutagenesis, ten clones showing the highest TAI values (2.5- to 4-fold higher than 3A4) were further subjected to a thermostability assay to discard any destabilizing mutations (**Fig. S2**). In general, we observed that clones that maintained P390 were the most stable. Hence, C14F12 mutant (F392N), with around 4-fold TAI and increased stability respecting 3A4 parent type, was selected as the winner.

The activity landscape of library CA was similar to that observed for library A, although apparently the number of clones that retained parent activity was somewhat lower (**Fig. S3A**). Sequencing of selected clones revealed a possible epistatic relationship between mutations in region A and mutation F392N. This substitution seemed to favor the appearance of an acid-basic residue pair in positions 162 and 164 (**Table S2**), a bias that was not observed in library A. It is worth noting that available structures of fungal laccases co-crystallized with different substrates (Bertrand et al., 2002; Kallio et al., 2009), as well as site-directed mutagenesis studies (Koschorreck et al., 2008; Galli et al., 2011), suggest that non-polar residues in these positions are essential for substrate-enzyme recognition, establishing hydrophobic interactions with the substrate's aromatic ring. This apparent contradiction could be explained by the increased polarity of the phenolic ring of SA due to the presence of two methoxyl groups, which could interact with the polar residues in positions 162 and 164.

As in the first generation, a thermostability screening was performed for selected clones from library CA, but no significant changes in stability were found. Therefore, the most active mutant CA32F1 (A162R/T164E/F392N) was selected as parent type for the third generation. In accordance with what was observed for libraries A and CA, activity landscape for library CAB was similar to library B, although maximum TAI values were not as high (**Fig. S3B**). Moreover, after screening over 5000 clones, DNA sequencing revealed that all clones selected held no new mutations.

Biochemical characterization of selected ISM variants

Parent laccase 3A4 and variants C14F12 and CA32F1 were produced and purified to homogeneity for their characterization. While 3A4 and C14F12 presented T_{50} values of ~ 70 °C, it decreased to 67 °C for CA32F1. ISM variants presented similar pH activity profiles for SA oxidation, more neutral compared to parent type (**Fig. 3**): optimum pH was 4 for all laccases, but the second best value (relative activity >

80%) was obtained at pH 3 for 3A4 and at pH 5 for both C14F12 and CA32F1. This is a consequence of the selective pressure applied during library screening, performed at pH 5, as has been described in other laccase directed evolution studies (Bulter et al., 2003; Festa et al., 2008; Camarero et al., 2012; Torres-Salas et al., 2013).

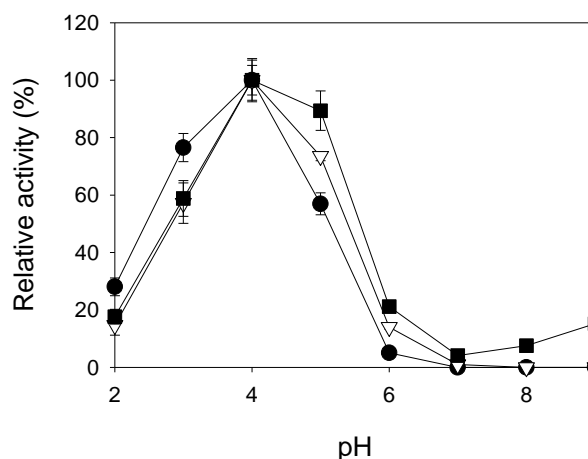


Figure 3. pH activity profiles with SA for laccase variants 3A4 (circles), C14F12 (triangles) and CA32F1 (squares). Means were obtained from triplicates; error bars represent standard deviations.

Concerning reaction kinetics for SA at pH 5 (**Table I**), ISM variants presented increased k_{cat} values, around 1.6-fold respecting parent type. Although these increments are not as high as desirable, it should be taken into account that SA is a natural substrate of fungal laccase and, hence, an enhancement of several folds in k_{cat} is difficult to achieve. For instance, during the active site re-design of a small bacterial laccase towards 2,6-dimethoxyphenol, several-fold improvements were attained by punctual mutations, but the wild-type activity on this substrate was extremely low (k_{cat} 0.87 s^{-1}) (Toscano et al., 2013). Concerning K_{m} , C14F12 laccase presented a 2-fold increase respecting parent 3A4, whereas affinity was somewhat recovered in CA32F1. In all, catalytic efficiencies for SA at pH 5 are not significantly affected in the new variants. Nevertheless, it should be mentioned that from an industrial point of view, increasing the turnover rates is the aim, independently of the changes obtained in K_{m} , since substrate concentration does not represent a limiting factor in industrial enzymatic transformations (where high amounts of substrate are generally applied). It is also worth noting that despite the shifted pH activity profiles, CA32F1 still presents a higher turnover rate (and catalytic efficiency) towards SA than 3A4 parent type at pH 3.

In the HTS assay used for the screening of mutant libraries, oxidation of SA was followed by the development of a pinkish color ($\lambda_{\text{max}} = 512 \text{ nm}$) (Pardo et al., 2013). This colored product is a result of the oxidation of DAD, a phenolic dimeric compound formed from the β - β' coupling of two phenoxyl radicals of SA that is also a substrate for laccase (**Scheme S1**) (Lacki and Duvnjak, 1998; Koschorreck et al.,

2008). DAD was synthesized and the corresponding kinetic constants for its oxidation by the three laccase variants were obtained to verify whether TAI values observed in the library screenings were due to enhanced activity over SA alone or also to a better oxidation of DAD. Surprisingly, k_{cat} values were not enhanced, and affinity towards DAD was significantly worse for the ISM variants.

Table I. Kinetic constants for the oxidation of SA and DAD by the parent type laccase 3A4 and the ISM variants (C14F12 and CA32F1). Reactions at pH 5 were performed in 100 mM acetate buffer, while reactions at pH 3 were performed in 100 mM tartrate buffer.

Substrate		3A4	C14F12	CA32F1
SA (pH 5)	k_{cat} (s ⁻¹)	156 ± 3	251 ± 4	257 ± 3
	K_{m} (μM)	7.0 ± 0.7	14.2 ± 0.7	9.9 ± 0.5
	$k_{\text{cat}}/K_{\text{m}}$	22 ± 3	17.7 ± 1.1	26 ± 2
SA (pH 3)	k_{cat} (s ⁻¹)	313 ± 8	338 ± 3	429 ± 9
	K_{m} (μM)	92 ± 5	137 ± 13	80 ± 4
	$k_{\text{cat}}/K_{\text{m}}$	3.4 ± 0.3	2.5 ± 0.3	5.3 ± 0.4
DAD (pH 5)	k_{cat} (s ⁻¹)	68 ± 1	68 ± 3	73 ± 3
	K_{m} (μM)	21.6 ± 1.3	59 ± 7	66 ± 9
	$k_{\text{cat}}/K_{\text{m}}$	3.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.2

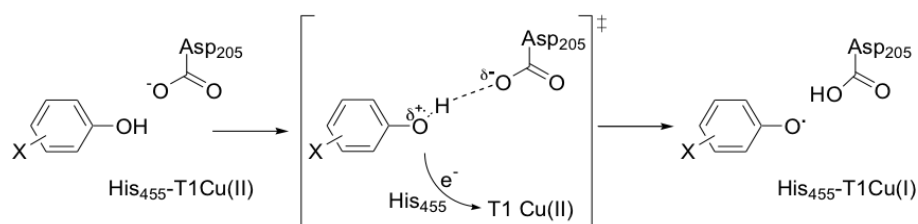
Hammett and Marcus correlations

In order to ascertain whether mutations in ISM variants C14F12 and CA32F1 affected the reaction mechanism, Hammett and Marcus plots were obtained for the three laccases. Competition reactions at pH 5 with different *p*-X-substituted phenols (*p*-methoxyphenol, *p*-methylphenol, *p*-phenylphenol, *p*-chlorophenol and phenol) were carried out and $\log(k_{\text{x}}/k_{\text{H}})$ values were plotted against σ^+ values for each substrate (**Fig. S4A-C**). Calculated ρ -values for the three laccases were all around -2 , indicating that all three laccase variants are similarly dependent on the nature of the *p*-X-substituent group (**Table II**). These values are slightly lower than those obtained for other HRPLs from *Trametes villosa* and *Trametes versicolor* (Tadesse et al., 2008; Galli et al., 2013). Redox potentials for the three laccases were also obtained, but no significant differences were found. The same occurred for total reorganization energies (λ) for electron transfer calculated according to Marcus equation (**Fig. S4D-E**), with values ranging from 45 to 47 kcal mol⁻¹ (**Table II**), similar to those described for *T. villosa* and *T. versicolor* laccases (Tadesse et al., 2008; Galli et al., 2013).

Table II. Redox potentials (V vs NHE), ρ -values for Hammett plots and reorganization energies (kcal/mol) for Marcus plots calculated at pH 5 for 3A4 laccase and ISM variants (C14F12 and CA32F1).

Laccase	E^0	ρ (Hammett)	λ (Marcus)
3A4	0.7887 ± 0.0004	-1.9 ± 0.3	44.6 ± 0.3
C14F12	0.783 ± 0.002	-2.1 ± 0.2	47.4 ± 0.4
CA32F1	0.7752 ± 0.0006	-2.2 ± 0.1	45.6 ± 0.5

Similar to these two laccases, these results suggest that 3A4 laccase and ISM variants oxidize phenols by an asynchronous concerted electron–proton transfer mechanism, where the electron transfer to T1 copper site leads to the formation of a fleeting radical cation reactive intermediate (in agreement with the observed negative ρ -values) and the concomitant proton transfer to D205 residue leads to a phenoxy radical product. This feature is appropriately described as an unbalanced transition state, according to Muller (**Scheme 1**) (Muller, 1994). In all, these results suggest that the selected mutations do not affect the mechanism of the reaction, and thus the increased turnover rates observed in the ISM laccase variants are caused by other factors.



Scheme 1. Concerted electron proton transfer mechanism for the oxidation of phenols by 3A4 laccase and ISM variants.

Computational analysis of substrate binding and oxidation

To rationalize the effect of mutations on substrate oxidation, molecular simulations were performed (Monza et al., 2015). The first step involved the identification of the main binding modes with PELE, which was performed for both protonation forms of SA (calculations were made at pH 5, **Fig. 4**). Then, SA oxidation was estimated through spin densities from QM/MM simulations (**Table III**).

Table III. Average spin density (%) on the substrate at site 1 for the different QM/MM calculations. Standard deviations are indicated, as well as the proportion of structures corresponding to site 1 of all those evaluated.

	3A4		C14F12		CA32F1	
	Poses	Spin density (%)	Poses	Spin density (%)	Poses	Spin density (%)
SAH	0.95	88 ± 6	0.65	85 ± 5	0.89	83 ± 20
SA[•]	0.33	87 ± 8	0.75	82 ± 10	0.95	86 ± 9
DAD	0.45	80 ± 8	0.69	88 ± 6	0.29	100 ± 1

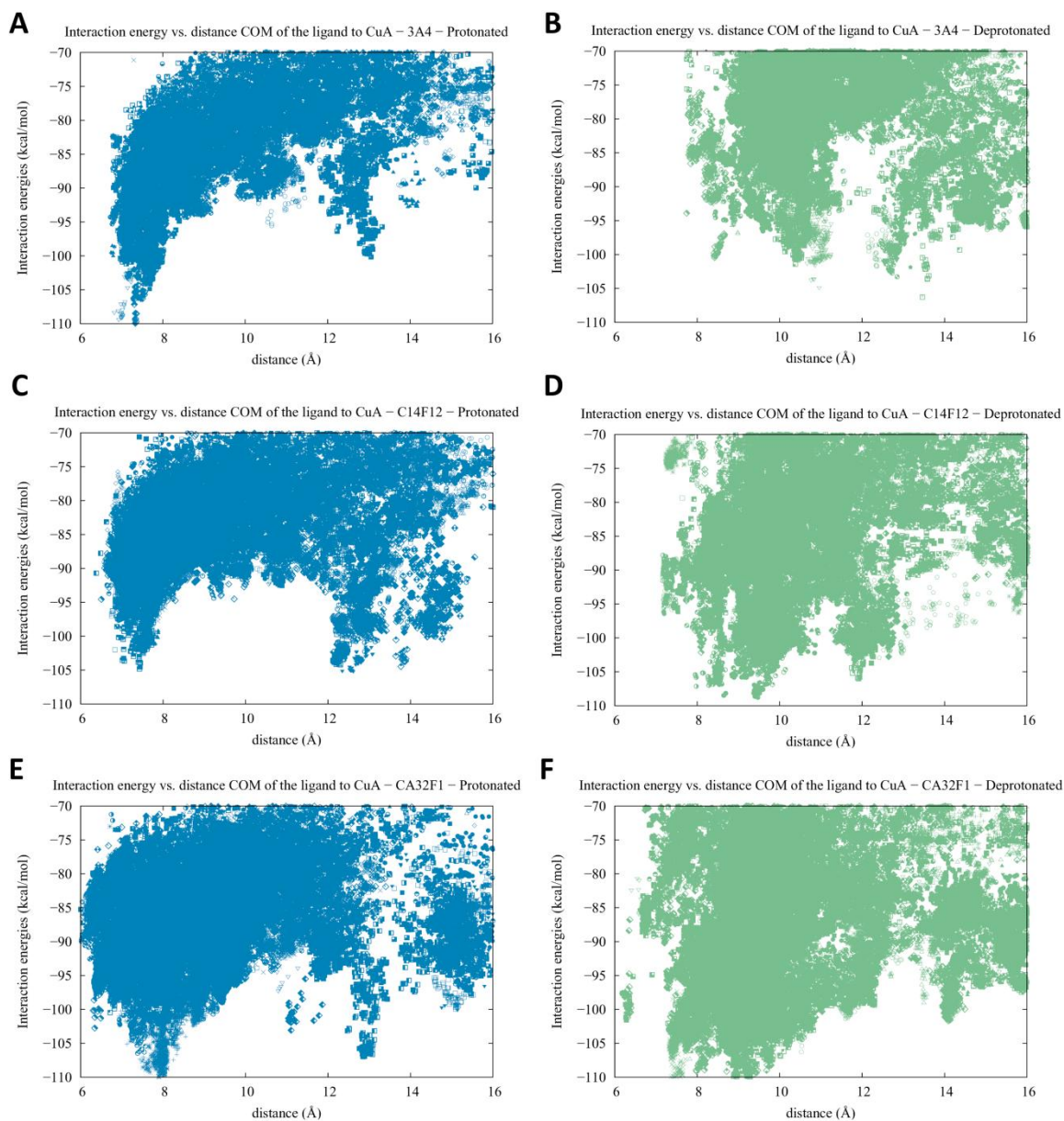


Figure 4. Interaction energy versus the T1 copper–substrate (center of mass) distance for **A, B** 3A4, **C, D** C14F12 and **E, F** CA32F1 variants conformational search of SAH (blue) and SA⁻ (green).

PELE results indicate that protonated SA (SAH) binds with higher affinity in the vicinity of the T1 site in 3A4 laccase (**Fig. 4A**): inspection of structures with interaction energy below $-100 \text{ kcal mol}^{-1}$ confirms that 19 out of 20 SAH binding modes were found inside the T1 cavity (hereafter named site 1, **Fig. 5A**). At this site the substrate's carboxylic acid is interacting with P393 and N207, while the phenolic group is surrounded by A162, P163 and the side chain of S264. In this site we find an average substrate spin density of 88%. For deprotonated SA (SA⁻), however, site 1 is less populated and a second minimum appears (**Fig 4B**). Site 1 seen in **Fig. 5B** shows the substrate in a different orientation and further away from the metal center due to the favorable interaction between the negatively charged carboxylate group and

the backbone of G391 and F392 and the hydroxyl group of S387. Nevertheless, site 1 shows an average 87% spin density (although only one third of the analyzed structures correspond to this site), while the second minimum is less reactive (61% spin density, **Table III**). Furthermore, SA⁻ is in average less protected from solvent than SAH, as seen by the higher solvent accessible surface area (SASA) at the best interaction energies (**Fig. S5**). All these data indicate the preference of 3A4 laccase for SAH oxidation over SA⁻.

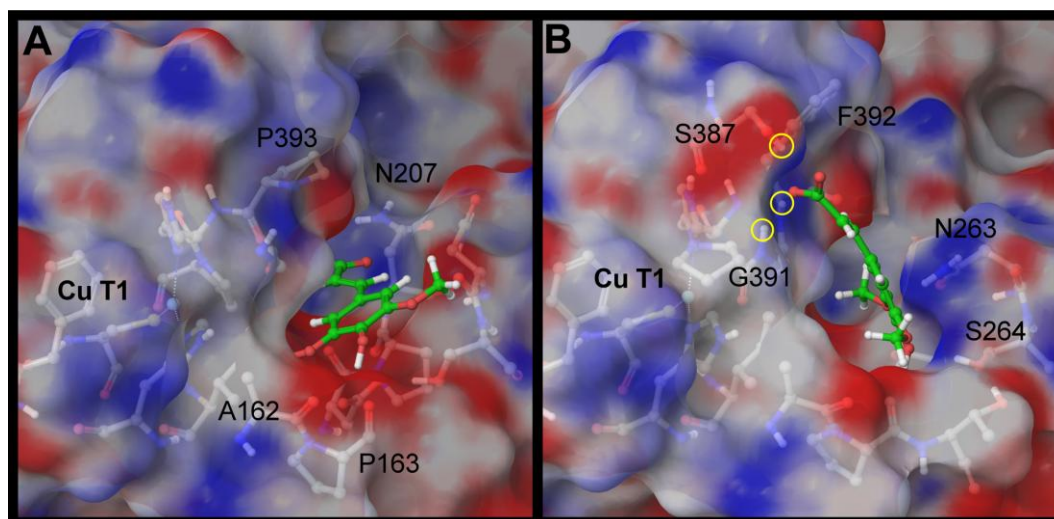


Figure 5. Representative binding modes for SA in 3A4. **A)** Most reactive mode for SAH and **B)** SA⁻. The main residues participating in the backbone amine site are identified with yellow circles.

In C14F12, SAH presents two degenerate minima, at 7 Å and at 12 Å (**Fig 4C**); both sites are also present in 3A4, although C14F12 has an increased population at the second minimum. Spin density for SAH at site 1 (85%) is similar to that of 3A4, but the second minimum shows reduced spin density (40%, **Table III**). Conversely, SA⁻ binds mainly in site 1 (**Fig 4D**). As in the case of 3A4, the substrate's carboxylate group interacts with the backbone of G391 and N392 and S387's side chain. However, mutation F392N causes the rotation of the backbone, creating a favorable binding site for the negatively charged substrate with the N392 side chain. Despite the anchored carboxylate group, the substrate's phenolic group shows two main orientations with different oxidation rates: inside the T1 Cu site, which is more frequent, interacting with P163 backbone and near D205 (93% spin density, **Fig. 6A**), or pointing directly to the solvent (48% spin density, **Fig. 6B**). These results suggest that the shifted pH activity profile and the improved activity seen at pH 5 for C14F12 ($k_{\text{cat}} = 251 \text{ s}^{-1}$) relative to 3A4 ($k_{\text{cat}} = 156 \text{ s}^{-1}$) could be mainly due to improved oxidation of SA⁻, which is more abundant at this pH than at pH 3. In agreement with this is the fact that the k_{cat} for 3A4 and C14F12 at pH 3 are very similar (313 and 338 s⁻¹, respectively).

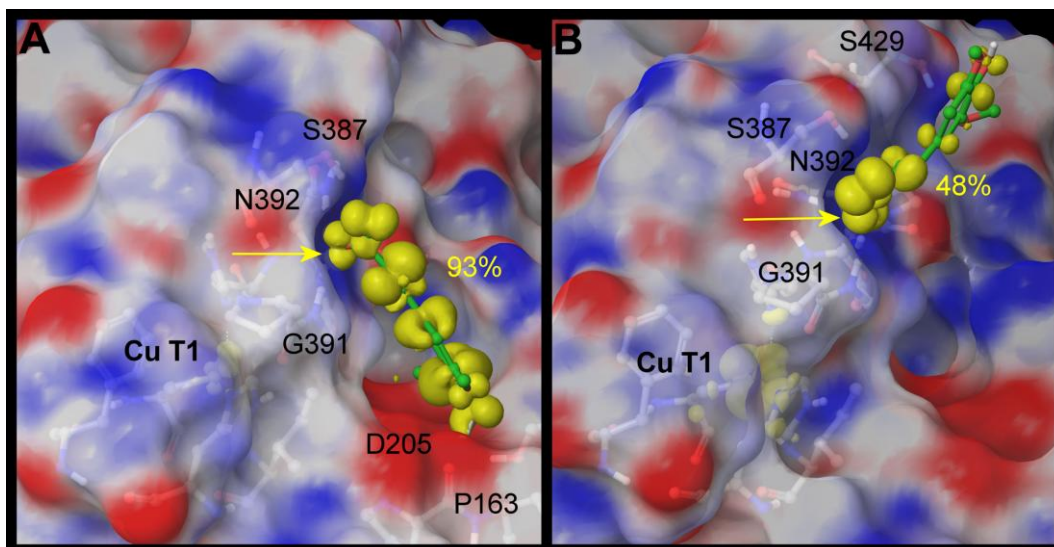


Figure 6. Representative binding modes for SA⁻ with C14F12 protein. **A)** Most reactive mode (in yellow the spin density isosurface on the substrate). **B)** A less active orientation with the carbonyl group exposed to the solvent. Arrows locate the carboxylate anchor point.

MD simulation performed for CA32F1 laccase (**Fig. S6, S7**) shows that R162 is mostly interacting with E164, but in ~25% of the time R162 is also interacting with D205, as a result of the flexibility observed in the loop hosting R162 and E164. Following MD relaxation, PELE simulations further confirmed the existence of these two conformations. For SAH, site 1 is highly populated (**Fig. 4E**) and two possible binding modes are observed: i) one very reactive (99% spin density) due to the favorable stacking of substrate between the two negatively charged residues (D205 and E164, **Fig. 7A**) and; ii) a less reactive one (75%) due to simultaneous interaction of D205, R162 and E164 (**Fig. 7B**). In the case of SA⁻, again it is found almost exclusively in site 1 (**Fig. 4F**). In fact, 19 out of 20 structures were in this site with an average 86% spin density (**Table III**). SA⁻ is anchored by G391, F392 and S387, similar to the most reactive binding mode found in C14F12, although not as well positioned due to the presence of the large side chain of R162. This indicates that the relative orientation of the side chains from R162, E164 and D205 affects the oxidation of the substrate. In fact, an arginine residue in the substrate binding pocket of CotA laccase has recently been described to be crucial for SA oxidation. The crystal structure reveals that its side chain rotates 10° upon SA binding, sandwiching the substrate between itself and the T1 site (Xie et al., 2015). Finally, the SASA analysis shows a more buried interaction of the substrate at the T1 copper site in CA32F1, particularly in the case of SAH (**Fig. S5E, F**).

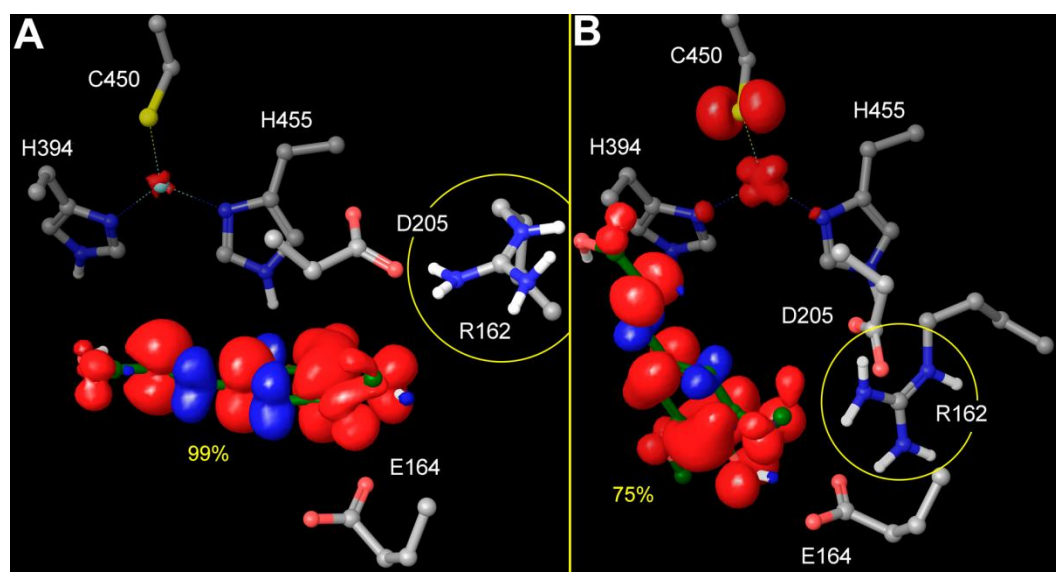


Figure 7. Representative protein-substrate complexes for CA32F1 and SAH. Spin density isosurfaces are shown in red and blue. **A)** example of a conformation where the substrate is stacked between the two acids. **B)** A less favorable oxidation position with simultaneous D205–R162–E164 interaction.

We have also investigated the effect of lowering pH to 3 but predictions indicate that only CA32F1 could undergo changes in the binding pocket. With an estimated pK_a of 3.8, D205 could be protonated, so calculations were repeated with SAH and both protonation states for D205. In this case PELE results indicate that SAH also binds in site 1, but the orientation of the ligand is different from that observed when D205 is not protonated. Also, low spin densities (18–65%) are obtained when using the neutral form of D205, while values up to 100% are obtained for deprotonated D205. Hence, the improved catalytic efficiency of CA32F1 on SA at pH 3 can be attributed to the favorable oxidation of SAH (the dominant species at this pH) and not to changes occurring in the protein. Although estimated pK_a show that catalytic D205 coexists in protonated and deprotonated forms in this variant at pH 3, binding SAH would displace the equilibrium, shifting D205 population towards the carboxylate form and allowing proton transfer from the substrate to the protein. Indeed, mutation of the equivalent residues for non-acidic amino acids in laccases from *T. versicolor* (D206A) and *Melanocarpus albomyces* (E235T) severely affected oxidation of phenolic substrates, especially at acidic pH (Madzak et al., 2006; Galli et al., 2013; Kallio et al., 2009).

Taking all these results into account, we can conclude that the increased turnover rates for SA at pH 5 are caused by the enhanced oxidation of SA⁻, thanks to the anchoring of the substrate's carboxylate group by mutation F392N. However, in C14F12 variant this is at the expense of SAH oxidation. On the other hand, CA32F1 is very active on both protonation forms of SA, presenting also increased k_{cat} at pH 3 respecting parent 3A4, although the relative position of the new acid–basic pair formed by R162 and E164 is determinant for efficient binding of the substrate.

Finally, simulations were also performed for DAD with the three proteins. They all presented more or less similar binding in site 1, although it's worth noting that in CA32F1 variant the interaction between R162 and E164 is observed in several structures, blocking the entrance of DAD into the substrate pocket (**Fig. S8**). Overall, QM/MM results (**Table III**) are in agreement with the similar turnover rates obtained for DAD with the three laccases. The CA32F1 variant showed up to 100% spin density, but due to the low proportion of site 1 structures used, these results were inconclusive.

Oxidation of related syringyl-type phenols

We also evaluated the oxidation of other syringyl-type phenols (with two *o*-methoxyl substituents) by the three laccase variants, determining their kinetic constants towards methyl sinapate (MS), 2,6-dimethoxyphenol (DMP), syringic acid (SyA) and methyl syringate (MSy) at pH 5 (**Table IV**).

Table IV. Kinetic constants for the oxidation of MS, DMP, SyA and MSy (pH 5).

Substrate		3A4	C14F12	CA32F1
MS	k_{cat} (s^{-1})	195 \pm 5	250 \pm 5	309 \pm 7
	K_{m} (μM)	12.0 \pm 1.1	16.0 \pm 0.9	18.6 \pm 1.2
	$k_{\text{cat}}/K_{\text{m}}$	16 \pm 2	15.6 \pm 1.2	16.6 \pm 1.4
DMP	k_{cat} (s^{-1})	90 \pm 1	131 \pm 3	128 \pm 2
	K_{m} (μM)	46 \pm 2	320 \pm 30	191.7 \pm 1.1
	$k_{\text{cat}}/K_{\text{m}}$	2.0 \pm 0.1	0.41 \pm 0.05	0.668 \pm 0.014
SyA	k_{cat} (s^{-1})	57 \pm 1	71 \pm 3	63 \pm 1
	K_{m} (μM)	32.9 \pm 2.5	189 \pm 4	102 \pm 5
	$k_{\text{cat}}/K_{\text{m}}$	1.7 \pm 0.2	0.38 \pm 0.02	0.61 \pm 0.04
MSy	k_{cat} (s^{-1})	71 \pm 2	66 \pm 1	68 \pm 1
	K_{m} (μM)	132 \pm 14	890 \pm 30	710 \pm 30
	$k_{\text{cat}}/K_{\text{m}}$	0.54 \pm 0.07	0.082 \pm 0.004	0.096 \pm 0.005

These substrates, differing in the presence or not of a carboxylic group and the length of the aliphatic side chain, would help us understand the importance of the new binding mode created by mutation F392N, which was solely responsible for the increased k_{cat} obtained towards SA in the first generation. The kinetic constants obtained for MS were similar to those of SA, with a 1.5-fold increase in k_{cat} and also in K_{m} , resulting in a similar efficiency among the different laccase variants. However, catalytic efficiencies for DMP, SyA and MSy were severely impaired due to similar k_{cat} values and an important increase in K_{m} values (3- to 7-fold respecting parent type). Additional simulations were performed to elucidate the improved oxidation of MS in C14F12. The minimum with highest spin density (ranging from 65 to 73%) for parent 3A4 is very different from that found in variant C14F12 (with spin density ranging from 78 to 90%, **Fig. S9**). In fact, C14F12 minima resemble the previously seen site 1 for SA⁻, with a favorable interaction between MS's carbonyl and the

backbone of N392 and, more importantly, with the phenolic group well positioned for proton abstraction at D205. This is not seen in 3A4, where the substrate is in a different orientation, probably due to steric hindrance of F392 on the extra methyl group. These results suggest that the opening of the T1 cavity by replacement of F392 by N could be the main responsible for the increased activity towards MS in C14F12, contributing also to the enhancement of SA oxidation. On the other hand, the lower catalytic efficiencies towards DMP, SyA and MSy shown by ISM variants suggest that the length of the side chain present in SA and MS could also be an important factor for favorable substrate binding in the engineered variants, providing better interaction between the phenolic group and D205. Indeed, in a related work we evaluated the oxidation of a battery of lignin-derived phenols by the selected laccase mutants from libraries A, B and C enumerated in **Table S2**. In this screening we observed a more pronounced increase in activity for mutants with F392N mutation towards SA, MS, ferulic acid and methyl ferulate than towards SyA, MSy, vanillic acid and methyl vanillate, respectively, which differ in the presence or not of the propenyl chain (Pardo and Camarero, 2015b).

CONCLUSIONS

Combinatorial saturation mutagenesis on the substrate binding pocket together with experimental characterization and computational analysis of the resulting variants provided valuable information on the structural determinants for the oxidation of sinapic acid and related phenols by laccase. The results obtained put in evidence that the diversity of substrate binding pockets of fungal laccases in nature stands for the varied oxidation capabilities found in these generalist enzymes even if they hold similar redox potentials. Although the latter will be limiting the electron transfer between the substrate and the T1 copper, the binding event plays a crucial role in the overall rate of the reaction by creating an appropriate environment and substrate positioning that allows the oxidation to proceed. Concerning the engineering of laccases by rational design, it is shown that educated guesses may be misleading even for assessing the effect of a single mutation. The oxidation of each ligand is a unique combination of many factors. Thus, to engineer the enzyme, it is decisive to define the particular substrate and conditions for which the biocatalyst will be used, stressing the usefulness of atomic simulations in unveiling the molecular determinants for the efficient oxidation of a target molecule.

ABBREVIATIONS

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); **CDS**, coding DNA sequence; **CSM**, combinatorial saturation mutagenesis; **DAD**, dehydrodisinapic acid dilactone; **DMP**, 2,6-dimethoxyphenol; **HRPL**, high-redox potential laccase; **ISM**, iterative saturation mutagenesis; **IVOE**, in vivo overlap extension; **MD**, molecular

dynamics; **MS**, methyl sinapate; **MSy**, methyl syringate; **PELE**, protein energy landscape exploration; **QM/MM**, quantum mechanics/molecular mechanics; **SA**, sinapic acid; **SAH**, protonated SA; **SA⁻**, deprotonated SA; **SASA**, solvent-accessible surface area; **SyA**, syringic acid; **TAI**, total activity increase.

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SUPPLEMENTARY MATERIAL

Supplementary material and methods

- Computational details:

Molecular Dynamics (MD) simulation was performed for the system CA32F1 to investigate the effect of the introduction of the large side chain of Arg162. After appropriate preparation of the system as explained in the main manuscript an orthorhombic water box with a minimum distance of 10 Å was introduced. The system was then neutralized and 150 mM NaCl added. Equilibration using the default protocol was performed followed by 20 ns NPT simulation at 300 K and 1 atm using DESMOND software with the OPLS-2005 force field. The temperature was regulated with the Nosé-Hoover chain thermostat, with a relaxation time of 1.0 ps, while the pressure was controlled by the Martyna–Tobias–Klein barostat with isotropic coupling and a relaxation time of 2.0 ps. The RESPA integrator was employed with bonded, near, and far time steps of 2.0, 2.0, and 6.0 fs, respectively. A 9 Å cutoff is used for non-bonded interactions together with the smooth particle mesh Ewald method.

To minimize the effects of manually introduced mutation V162AR (given the significant change in side chain size) in the model system of CA32F1 we have performed a 20 ns MD simulation. The simulation, shown to stabilize after about 5 ns (**Fig. S6**) contains an initial orientation of the R162 side chain, placed with Maestro software, that changes along the simulation. Initially, the CZ atom of the guanidino group is about 8 Å away from the carboxylic group of both D205 and E164, as can be seen in **Fig. S7**. Throughout the simulation it is seen that the loop in which both R162 and E164 are located is very flexible allowing for a diversity of possible arrangements. Two main conformations are observed: one where the R162 is interacting with E164 (between 4 to 11 ns and 13 until 15 ns) and R162 and D205 are between 6 and 11 Å away, and a second where the three amino acids are interacting from 15ns until the end of the simulation. We have selected the last configuration from the MD simulation to use in PELE calculations and have observed that also in PELE simulations this loop is very flexible and all conformations observed in MD are reproduced.

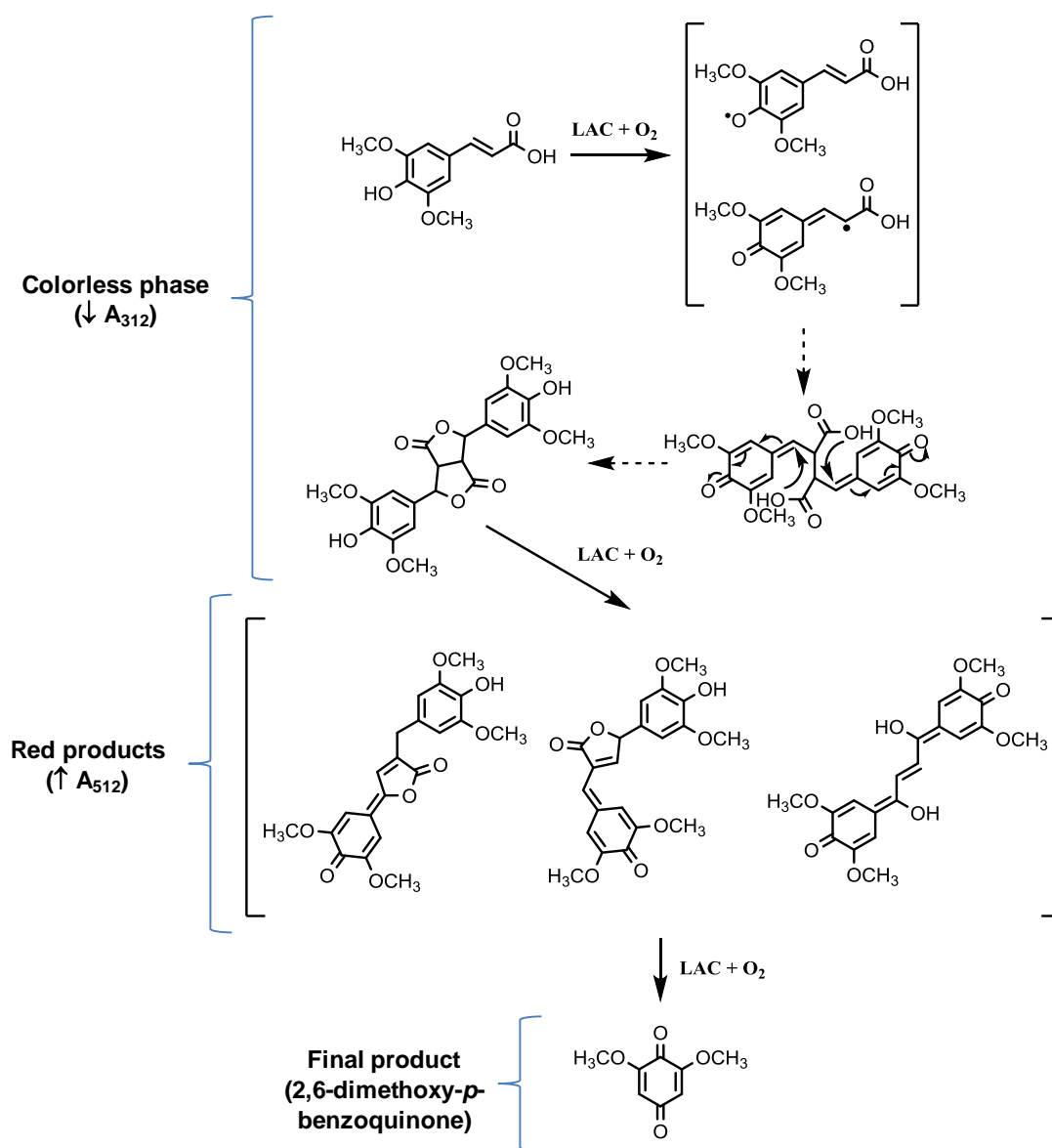
Supplementary Tables**Table S1.** Primers used for mutagenesis of the six residues of the substrate binding pocket of 3A4 laccase.

Primer	5'-3' sequence
mut162-164-F	GCTGCCAAAGTCGGCCCGGCGNNKCCGNNKGGCGATGCTACTCTTATCAAC
mut162-164-R	GTTGATAAGAGTAGCATCGGCMNNCGGMNNGCCGGGCCGACTTTGGCAGC
mut263-264-F	CTACTGGATCCGTGCCCTTCCCNNKNNKGGGACCAGGAAGTTCGACG
mut263-264-R	CGTCGAAGTTCCTGGTCCCMNNMNNNGGGAAGGGCACGGATCCAGTAG
mut390-392-F	CTCCCGCCACCTCCGCCGCCNNKGGCANNKCCGCACCCCTTCCACTTG
mut390-392-R	CAAGTGGAAAGGGGTGCGGMNNGCCMNNGGCGGCGGAGGTGGCGGGGAG
RMLN	CCTCTATACTTTAACGTCAAGG
RMLC	GGGAGGGCGTGAATGTAAGC

Table S2. Mutations selected during ISM of the binding pocket of 3A4 laccase.

		A		B		C	
		162	164	263	264	390	392
3A4		Ala	Thr	Asn	Ser	Pro	Phe
Library A	A3F12	Lys	Val	-	-	-	-
	A12A11	Lys	Ile	-	-	-	-
	A15F1	His	Ser	-	-	-	-
	A18F3	Arg	Thr	-	-	-	-
	A22A12	Asn	Glu	-	-	-	-
	A29B3	Asn	Gly	-	-	-	-
	A29G7	Ser	Gly	-	-	-	-
Library B	B1A1/B25H1	-	-	Asn	His	-	-
	B31G12	-	-	Asn	Arg	-	-
	B33E5	-	-	Asn	Thr	-	-
	B35A8	-	-	Asn	Glu	-	-
Library C	C1H5	-	-	-	-	Pro	Thr
	C2A11	-	-	-	-	Arg	Met
	C2F11	-	-	-	-	Met	Asn
	C8B12	-	-	-	-	Leu	His
	C11C1	-	-	-	-	Val	Met
	C13E1	-	-	-	-	Pro	Gln
	C14F12/C19H10	-	-	-	-	Pro	Asn
	C16G1	-	-	-	-	Pro	Ser
	C18A1/C18F12	-	-	-	-	Lys	Asn
	C18C11	-	-	-	-	Pro	Val
	C18D11	-	-	-	-	Ser	Met
C30D12	-	-	-	-	Val	Asn	
Library CA	CA26H5	Thr	Gln	-	-	Pro	Asn
	CA30E7	Leu	Glu	-	-	Pro	Asn
	CA32F1	Arg	Glu	-	-	Pro	Asn
	CA35A2	Leu	Thr	-	-	Pro	Asn
	CA38A3	Ile	Glu	-	-	Pro	Asn
	CA38H12	Arg	Asp	-	-	Pro	Asn
	CA39D1	His	Glu	-	-	Pro	Asn

Supplementary Schemes



Scheme S1. Reaction pathway for SA oxidation with the main oxidation intermediates as proposed by Lacki and Duvnjak (1998). Solid arrows indicate reaction steps catalyzed by laccase. Dashed arrows indicate non-enzymatic steps.

Supplementary Figures

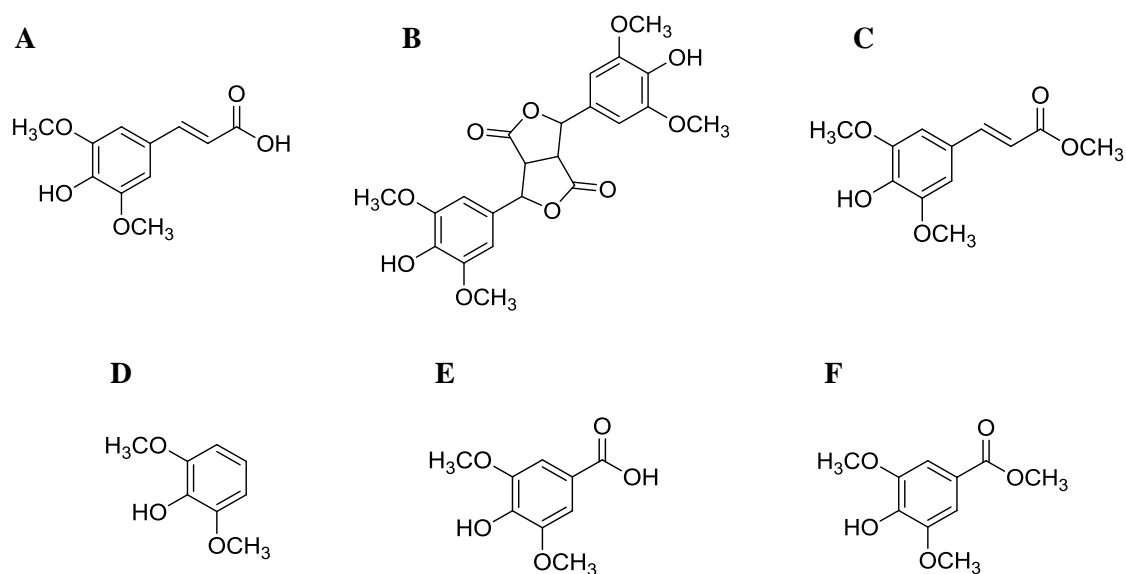


Figure S1. Chemical structures of A) SA, B) DAD, C) MS, D) DMP, E) SyA and F) MSy.

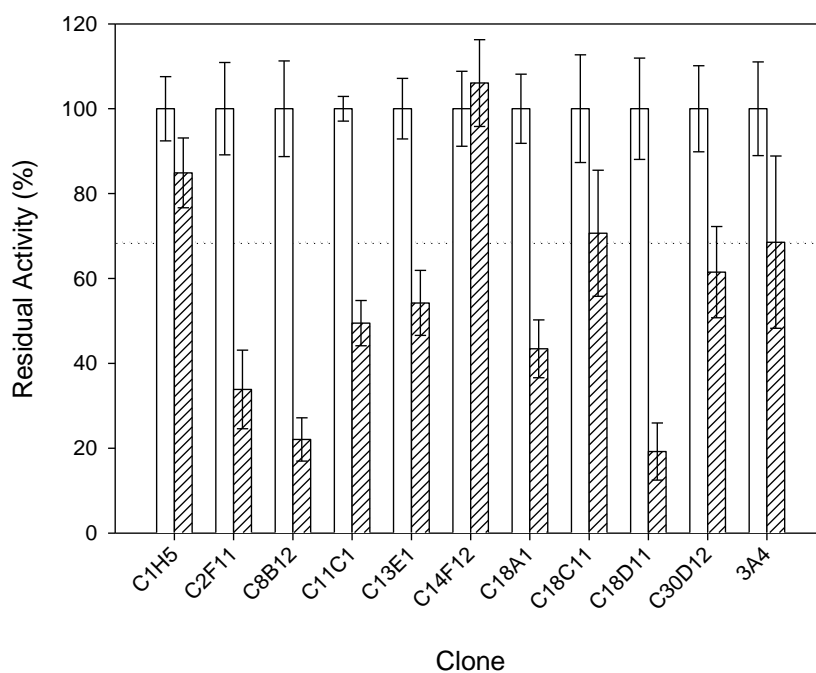


Figure S2. Thermostability screening assay for selected clones from library C. Initial activity and residual activity after 10 min incubation at 71 °C are shown in white and striped bars, respectively. Mean values were obtained from five replicates, error bars represent standard deviations.

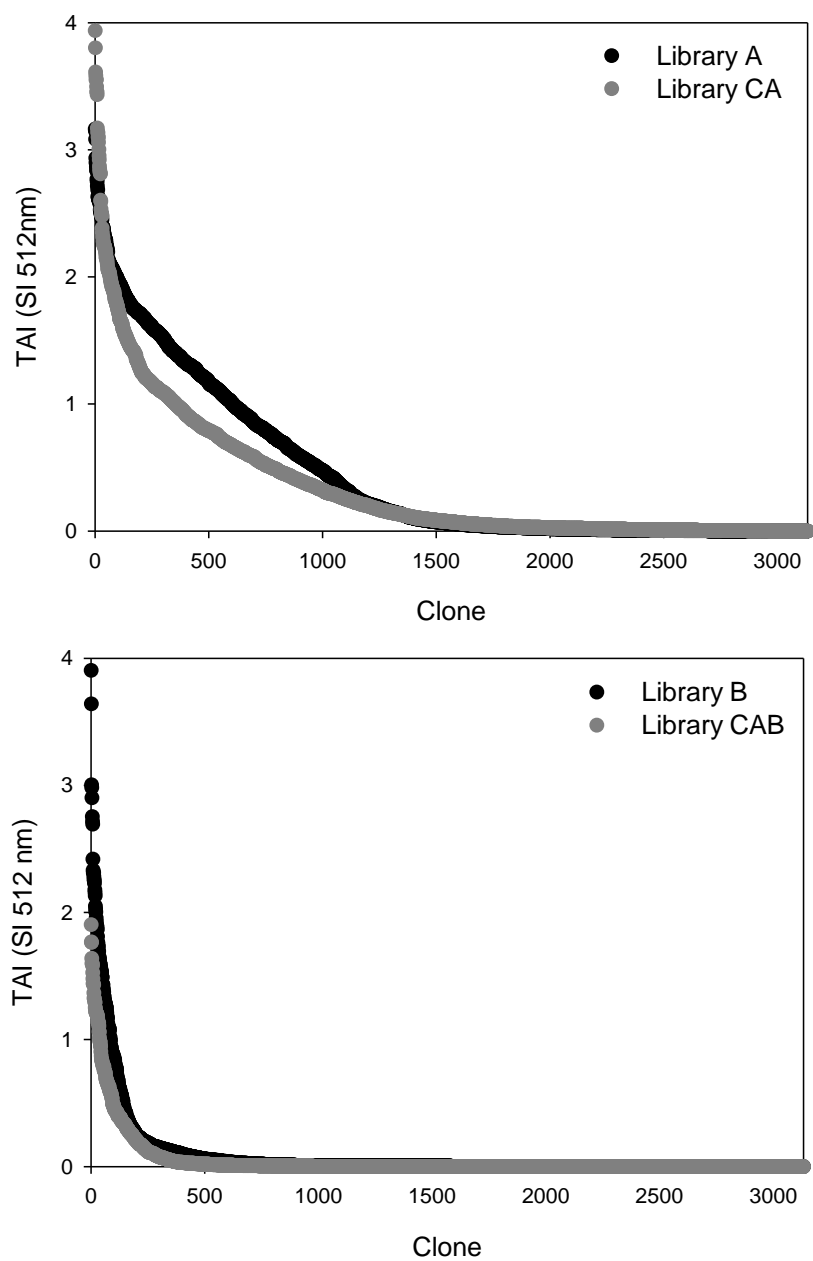


Figure S3. Activity landscapes for mutant libraries **A)** A vs CA and **B)** B vs CAB.

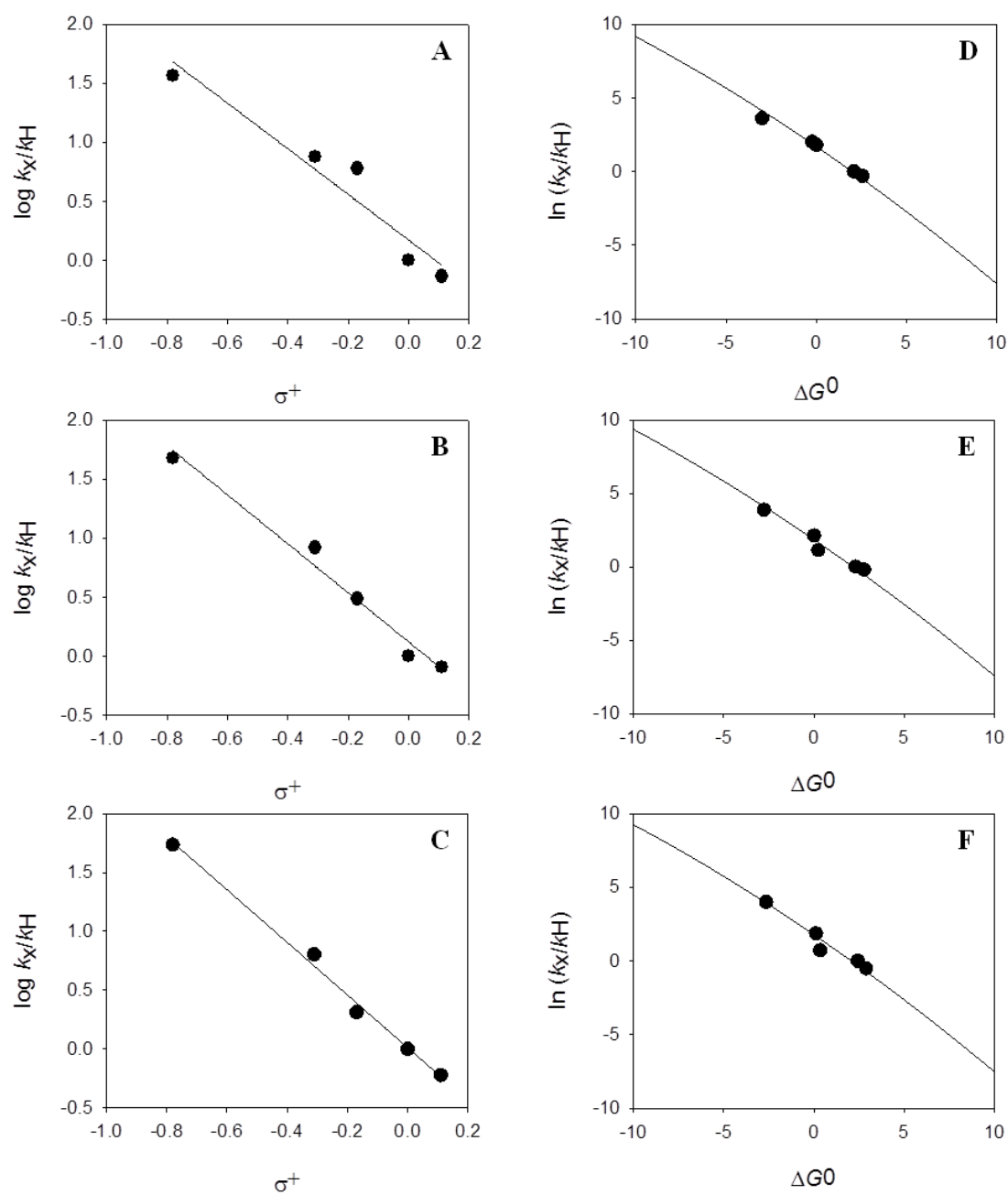


Figure S4. A-C) Hammett and D-F) Marcus plots obtained from competition reactions with different *p*-substituted phenols for laccase variants **A, D)** 3A4, **B, E)** C14F12 and **C, F)** CA32F1.

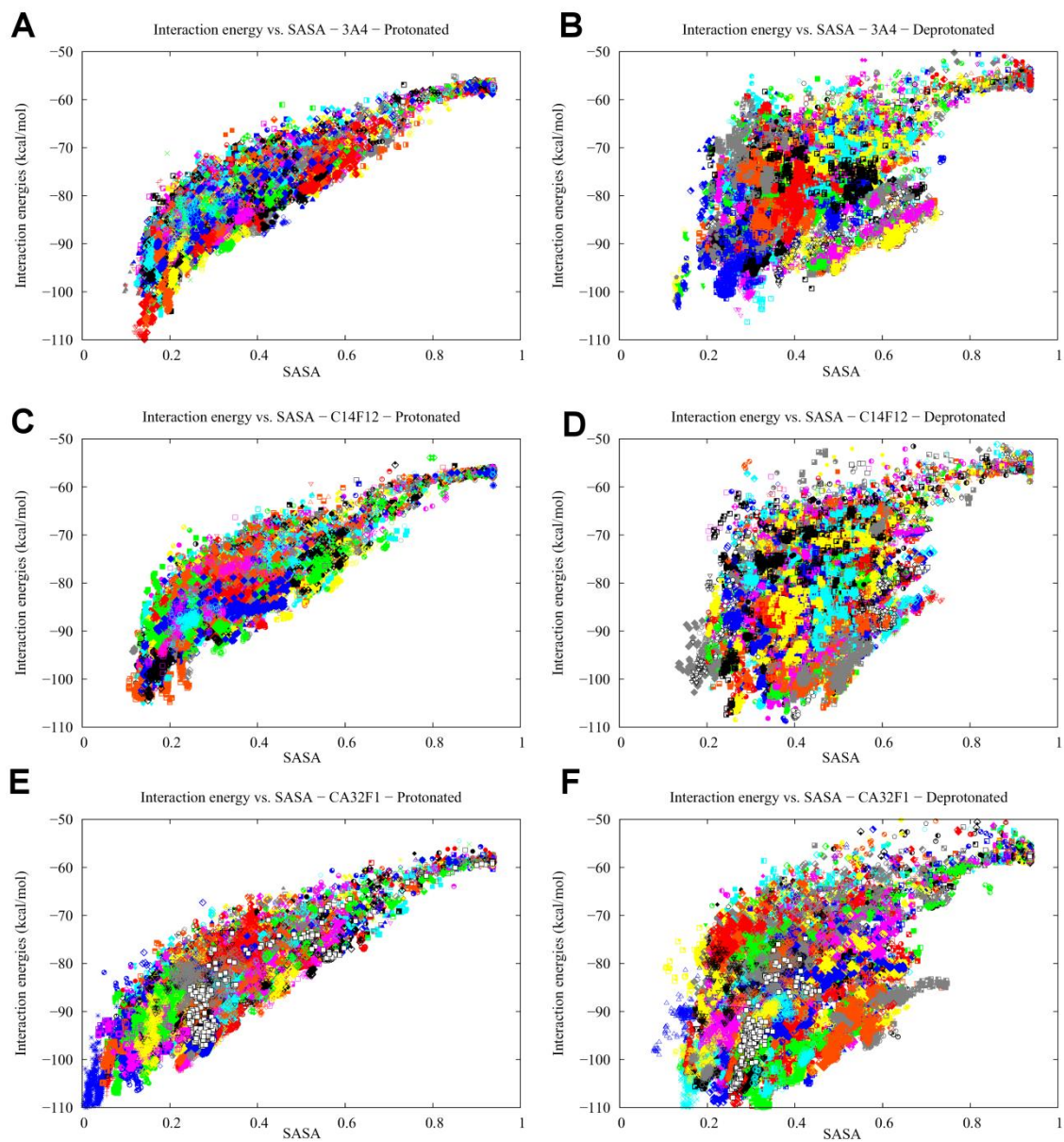


Figure S5. PELE results for the **A, B**) 3A4, **C, D**) C14F12 and **E, F**) CA32F1 variants conformational search of SAH (left) and SA⁻ (right).

RMSD variation along MD simulation

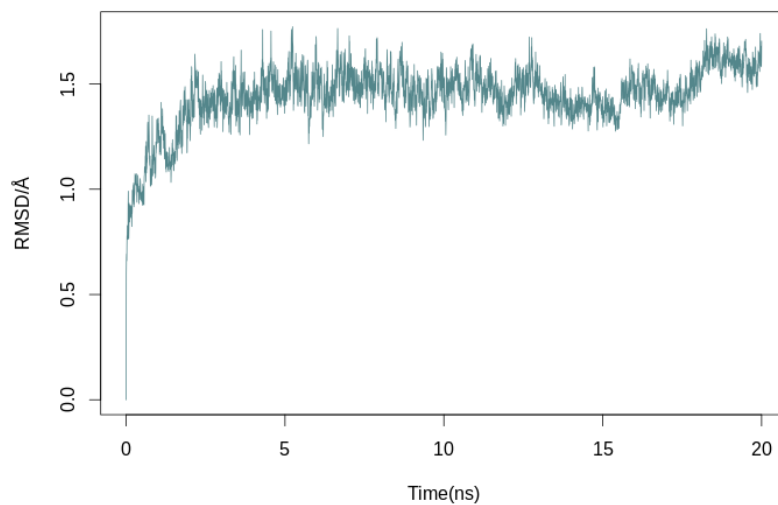


Figure S6. RMSD vs. time for MD simulation of CA32F1

Distance variation along MD simulation

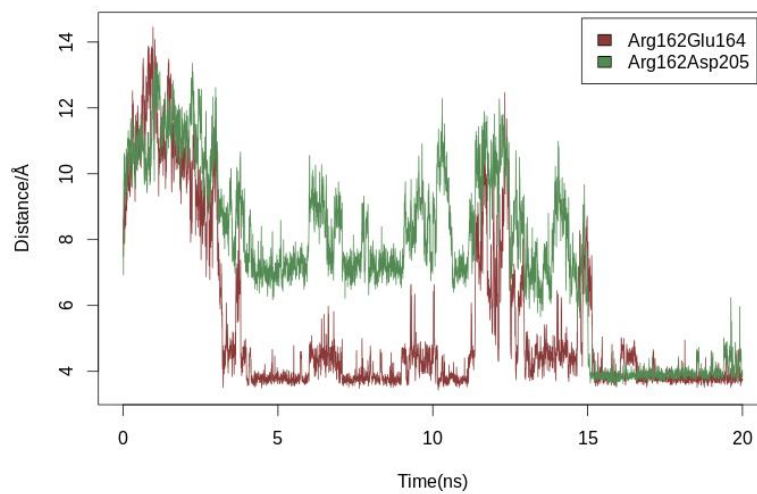


Figure S7. Plot showing how the distance between R162-E164 and R162-E205 vary along the MD simulation of CA32F1.

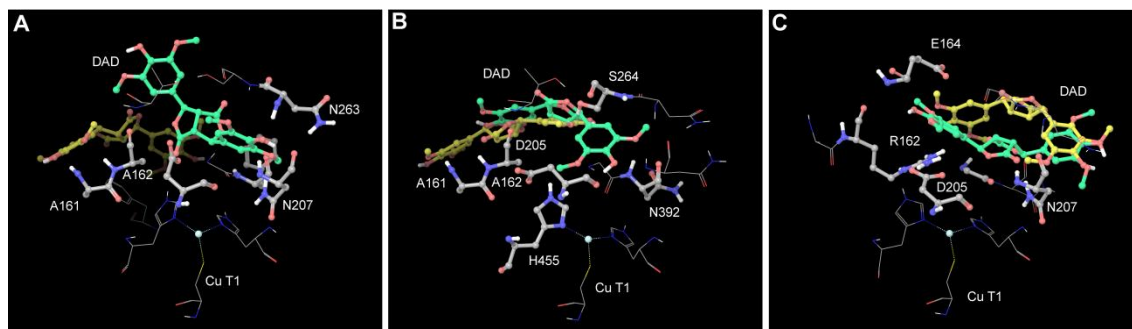


Figure S8. Representative protein–substrate complexes for **A)** 3A4, **B)** C14F12 and **C)** CA32F1 variants with DAD. Each plot represents the best (green) and worst (yellow) position for electron transfer computed with QM/MM.

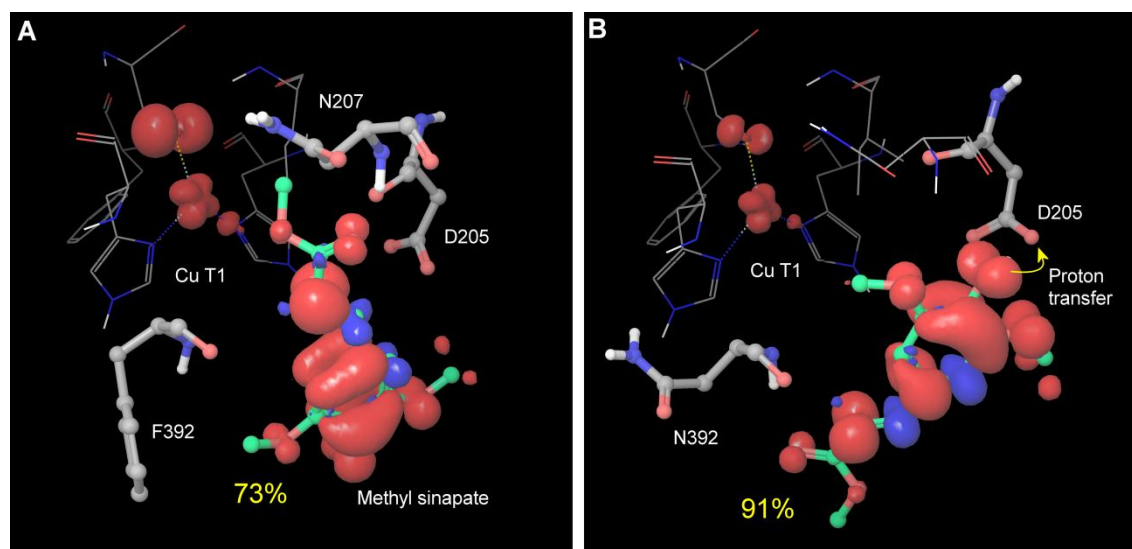


Figure S9. Representative protein–substrate complexes for **A)** 3A4 and **B)** C14F12 variants with MS. Spin density isosurfaces shown in red and blue.

CHAPTER 4

EXPLORING THE OXIDATION OF LIGNIN-DERIVED PHENOLS BY A LIBRARY OF LACCASE MUTANTS

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ABSTRACT

Saturation mutagenesis was performed over six residues delimiting the substrate binding pocket of a fungal laccase previously engineered in the lab. Mutant libraries were screened using sinapic acid as a model substrate, and those mutants presenting increased activity were selected for exploring the oxidation of lignin-derived phenols. The latter comprised a battery of phenolic compounds of interest due to their use as redox mediators or precursors of added-value products and their biological activity. The new laccase variants were investigated in a multi-screening assay and the structural determinants, at both the substrate and the protein level, for the oxidation of the different phenols are discussed. Laccase activity greatly varied only by changing one or two residues of the enzyme pocket. Our results suggest that once the redox potential threshold is surpassed, the contribution of the residues of the enzymatic pocket for substrate recognition and binding strongly influence the overall rate of the catalytic reaction.

INTRODUCTION

Laccases are blue-multicopper oxidases that are widely distributed in nature. They primarily catalyze the oxidation of substituted phenols and aromatic amines, among other aromatic compounds, coupled to the reduction of O₂ to water. Laccases participate in processes of polymerization, such as lignin formation in plants, cuticle sclerotization in insects, and sporulation and pigmentation in bacteria and fungi; and depolymerization, mainly lignin oxidation by fungi and bacteria (Giardina et al., 2010). Wood-rotting fungi are the most important producers of laccases, and white-rot basidiomycetes in particular are the only organisms capable of completely degrading lignin, thanks to the coordinated action of a battery of oxidoreductases (ligninolytic peroxidases and laccases), auxiliary enzymes (H₂O₂-producing enzymes, cellobiose dehydrogenase, etc.) and oxidized species of low-molecular weight compounds (e.g., Mn³⁺, Fe³⁺, reactive oxygen species and phenoxyl radicals). Although white-rot basidiomycetes produce the laccases with the highest redox potential in nature, with up to +0.8 V (vs. +0.4 to +0.7 V of laccases from bacteria, plants and other fungi), this is still insufficient to oxidize certain components of lignin or other substrates of higher redox potential. However, laccases' oxidative capabilities can be extended thanks to the presence of redox mediator compounds that, once oxidized by the enzyme, can in turn oxidize a recalcitrant substrate on which laccases cannot act on their own. In addition, mediators may act as electron shuttles, diffusing far away from the laccase active site and oxidizing complex compounds that cannot fit into the substrate binding pocket (Camarero et al., 2014).

Due to their broad substrate range and low requirements, the use of laccases and laccase-mediator systems (LMS) is of great interest for different processes using

lignocellulose as renewable raw material, including pretreatment and detoxification of straw materials for the production of second generation bioethanol, biobleaching of fibers in paper and textile industries, deinking of recycled paper or functionalization of lignocellulosic polymers (Ibarra et al., 2006; Kudanga et al., 2008; Jurado et al., 2009; Aracri et al., 2010; Božić et al., 2012; Fillat et al., 2012;). Laccases are also endowed with a great potential in organic chemistry for the synthesis of different types of polymers and dyes, naphthoquinones, benzofurans, lignans and other compounds with antioxidant, antimicrobial or antitumoral activities (Riva, 2006; Witayakran et al., 2007; Witayakran and Ragauskas, 2007; Kunamneni et al., 2008; Polak and Jarosz-Wilkolazka, 2012; Mogharabi and Faramarzi, 2014).

Although many LMS applications have been described, these typically rely on synthetic mediators such as ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), HBT (1-hydroxybenzotriazole) or violuric acid, which, apart from being expensive, may generate toxic species, hampering their utilization at an industrial scale. For this reason, the search for natural laccase mediators has become increasingly appealing. Indeed, we have described the ability of certain phenolic compounds derived from lignin to act as redox mediators in several works (Camarero et al., 2005; Camarero et al., 2007; Camarero et al., 2008), which is of special interest since these could be easily obtained from lignocellulosic residues or as a byproduct from biomass transformation processes. Some of the most recurrently studied are syringaldehyde, acetosyringone, methyl syringate, vanillin, acetovanillone and *p*-hydroxycinnamic acids (*p*-coumaric, ferulic and sinapic acids) (Cañas and Camarero, 2010; Fillat et al., 2010; Babot et al., 2011; Fillat et al., 2012; Rosado et al., 2012).

Another bottleneck limiting the industrial use of laccases is the need of high amounts of enzyme, which also needs to be active and stable under the harsh operational conditions. This has encouraged numerous protein engineering efforts in order to heterologously express laccases in different hosts and to obtain tailor-made biocatalysts presenting the desired properties (Pardo and Camarero, 2015). In previous works, we have described the engineering of two high-redox potential laccase variants from basidiomycete PM1 (PM1L) and *Pycnoporus cinnabarinus* (PcL), by means of directed molecular evolution in *Saccharomyces cerevisiae* (Maté et al., 2010; Camarero et al., 2012). The new laccase variants were actively expressed and secreted by yeast, presented enhanced catalytic efficiencies and retained the stability of the wild-type enzymes. Later on, the two evolved laccase variants were recombined by random DNA shuffling, and selected chimeras presented higher stability, shifted pH activity profiles and better substrate affinities respecting both parents (Pardo et al., 2012). One of the aforementioned chimeric laccases, variant 3A4, was chosen for its higher affinity towards 2,6-

dimethoxyphenol as departure point for further engineering of its activity over lignin derived phenols. In this occasion, saturation mutagenesis was performed over six residues delimiting the substrate binding pocket, and mutant libraries were screened with sinapic acid as model substrate (unpublished data). In the present work, we investigate the oxidation of 28 phenols by selected laccase mutants in order to compare their oxidation according to their different chemical structure and to evaluate how point mutations at the substrate binding pocket might affect laccase activity. The compounds included in this multi-screening assay were chosen for their prevalence in lignin, their ability to act as redox mediators, their use as precursors for added-value products and/or their biological activity as antioxidants, antimicrobials, antitumorals, immunomodulators, etc. (Niwa et al., 2002; Camarero et al., 2005; Mathew and Abraham, 2006; Camarero et al., 2007; Camarero et al., 2008; Aracri et al., 2010; Babot et al., 2011; Božić et al., 2012; Fillat et al., 2012; Rosado et al., 2012; Yamanaka et al., 2012).

RESULTS AND DISCUSSION

Laccase mutants studied in this work come from the saturation mutagenesis of six residues of the substrate binding pocket of a high-redox potential laccase (3A4) previously engineered in the lab by recombination of PcL and PM1L (Pardo et al., 2012). In particular, the residues selected for randomization included Ala162, Thr164, Asn263, Ser264, Pro390 and Phe392 (**Fig. 1** and **Fig. S1**). These positions were chosen since they were the most variable among the residues delimiting the enzyme pocket in the multiple sequence alignment of laccases from polyporal fungi, group to which *P. cinnabarinus* and basidiomycete PM1 belong to (data not shown).

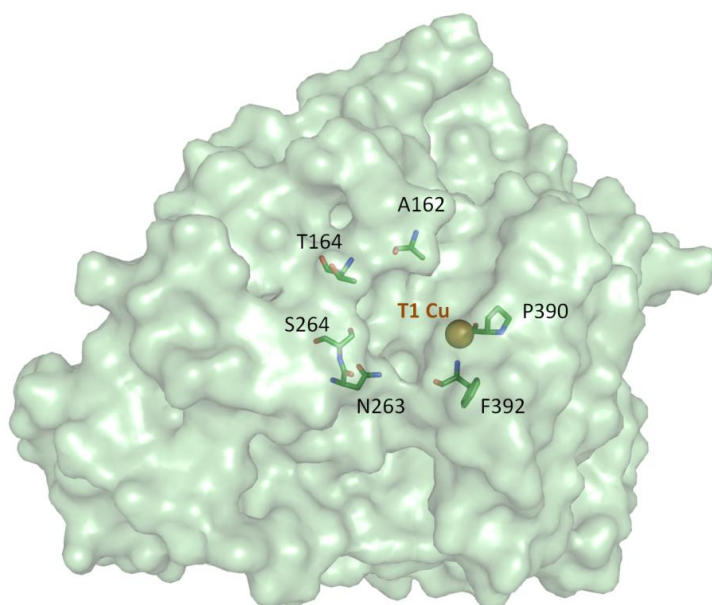


Figure 1. Structure model for 3A4 laccase generated with Swiss-Model server (Arnold et al., 2006), based on the crystal structure of laccase from *Trametes trogii* (PDB 2HRG), with 94% sequence identity. Target residues for mutagenesis are shown as sticks. Catalytic T1 copper is shown as a sphere.

Three mutant libraries were constructed, each for the combinatorial mutagenesis of two positions: library A, for residues 162 and 164; library B, for residues 263 and 264; and library C, for residues 390 and 392. At least 3066 clones from each library were screened, in order to explore all possible codon-pair combinations with 95% library coverage. Mutant libraries were submitted to a high-throughput screening (HTS) assay based on the activity towards sinapic acid (**14** in **Table I**), a lignin related phenol whose oxidation renders a pinkish colored product (Camarero et al., 2012; Pardo et al., 2013). Clones presenting the highest increases in activity for sinapic acid respecting parent laccase were submitted to a re-screening prior to plasmid extraction, amplification and re-transformation in yeast cells. Thus, 23 laccase mutants in total (seven from library A, four from library B and 12 from library C) and 3A4 parent type were produced in flask cultures in order to obtain sufficient amount of enzyme to evaluate activity towards other phenolic substrates.

Production of laccase mutants

Yeast cells expressing individual laccase mutants were grown in flasks in expression media for four days, after which cultivations were stopped and laccase activity in the culture media was measured using ABTS as substrate. Surprisingly, several mutants presented remarkably lower activities than the parent type. Protein concentration in the supernatant, however, was more similar between clones (variance coefficient of 14% vs. 48% for activity, **Fig. 2**).

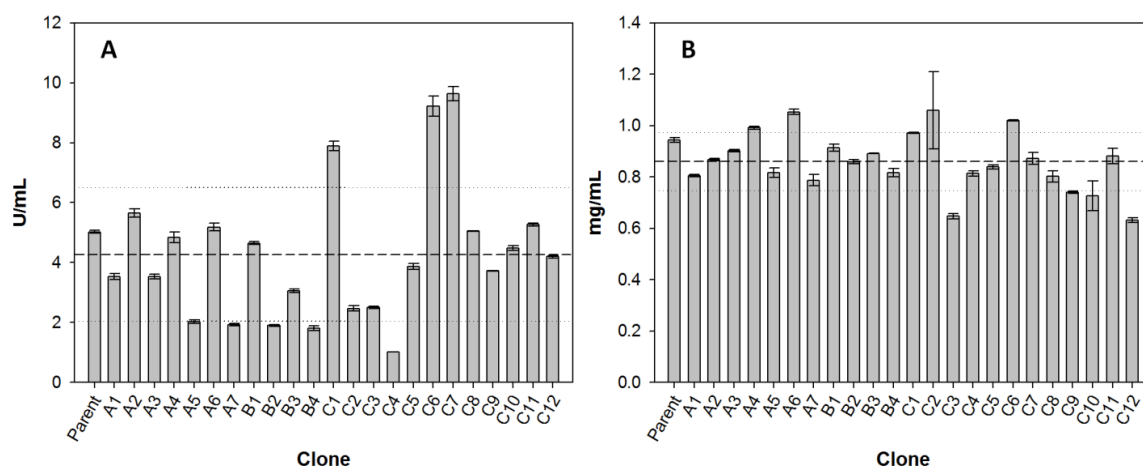


Figure 2. A) Activity measured with ABTS (U mL^{-1}) and B) protein concentration (mg mL^{-1}) in the concentrated supernatants of the different laccase mutants produced in flask cultures. Error bars represent standard deviation for duplicate measures for each mutant. Dashed lines indicate mean values and dotted lines indicate standard deviation between mutants.

Due to the mutagenesis strategy used for library construction, the mutations introduced were not expected to significantly affect laccase secretion levels by yeast (as it was lately confirmed, **Fig. 2B**). Hence, the low activities observed in several mutants could be due to (i) a specific enhancement of oxidation of sinapic acid but

not of other substrates; or (ii) lower stability of some mutants, which lost activity after longer cultivation times (HTS with sinapic acid was performed two days after inducing laccase expression in microcultures while it was induced for four days in flasks).

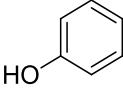
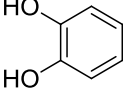
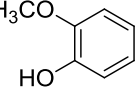
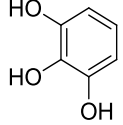
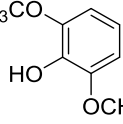
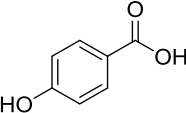
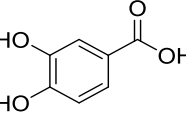
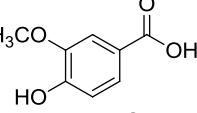
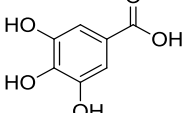
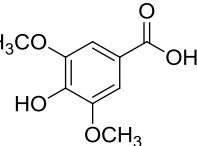
Concerning the first point, one of the key principles of directed evolution is that “you get what you screen for” (Schmidt-Dannert and Arnold, 1999). For the engineering of PM1L and PcL and their chimeric variants, multi-screening assays based on the oxidation of phenolic (2,6-dimethoxyphenol, sinapic acid) and non-phenolic (ABTS) substrates were used in order to preserve the versatility of the parent laccases’ activity (Maté et al., 2010; Camarero et al., 2012; Pardo et al., 2012). In this study, however, our goal was to specifically enhance laccase activity towards lignin-derived phenolic substrates. In addition, the high number of clones screened (over 3000 per mutant library) limited the use of high-throughput multi-screening assays for the simultaneous comparison of the three mutant libraries. This way, some selected mutations that are beneficial for activity on sinapic acid may be detrimental for activity on ABTS or other substrates with dissimilar chemical structure. As for the second point, it is well known that in directed evolution approaches there is generally a trade-off between enhanced activity and stability. For this reason, it is recommended to introduce stability assays in order to discard destabilizing mutations. For example, during the directed evolution of PM1L, a single mutation (F454S) increased parent-type activity fivefold, but it also caused a reduction of T_{50} (10 min) of 5 °C. For this reason, it was removed from the final evolved variant (García-Ruiz et al., 2010; Maté et al., 2010). In summary, the differences in activity observed when the selected laccase mutants were produced in flask cultures can be justified by one or both of the explanations accounted for above. In order to compare the activity of the different mutants, we set protein concentration (which was less variable among clones) as the parameter for normalizing the results of the screening of laccase activity with the different phenols.

Multi-screening with phenolic compounds

We selected a battery of 28 compounds as substrates to study their oxidation by chosen laccase mutants produced in flask, following a systematic approach in order to evaluate the effect of the different chemical structures on their feasible oxidation by laccase (**Table I**). These comprise phenols with and without substituents in *para*, including carboxyl (free or as methyl esters), ketone, aldehyde and propenoic groups. Besides these substitutions, they can hold different substituents in *ortho* respecting the hydroxyl: one or two hydroxyl groups, one or two methoxyl groups (guaiacyl or G-type and syringyl or S-type, respectively), or none (*p*-hydroxyphenyl or H-type). In addition, three phenolic compounds of renowned interest (eugenol, resveratrol and quercetin) were also evaluated. All these substrates are of plant

origin and can be obtained as plant extracts or released during degradation of the lignin polymer either by physicochemical or enzymatic treatment of lignocellulose.

Table I. Substrates used for the laccase activity screening of selected mutants. Wavelengths and time-points chosen for each substrate for the calculation of relative activities are indicated.

Code	Name	Structure	Wavelength (nm)	Time (h)
Phenols				
1	Phenol		420	24
2	Catechol		400	2
3	Guaiacol		470	2
4	Pyrogallol		450	2
5	2,6-Dimethoxyphenol		470	0.25 ^a
<i>p</i>-Hydroxybenzoic Acids				
6	4-Hydroxybenzoic acid		260	24
7	3,4-Dihydroxybenzoic acid		410	2
8	Vanillic acid		350	4
9	Gallic acid		450	2
10	Syringic acid		360	2

Exploring the oxidation of lignin derived phenols

Table I. Cont.

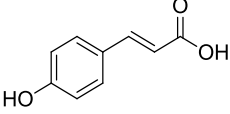
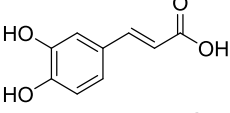
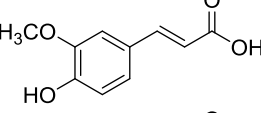
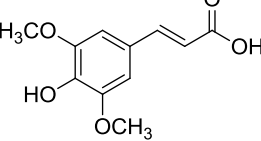
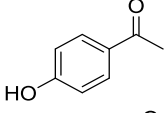
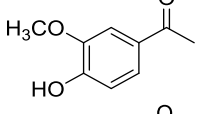
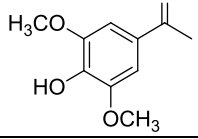
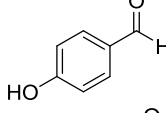
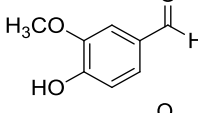
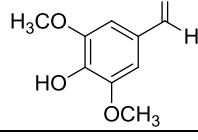
Code	Name	Structure	Wavelength (nm)	Time (h)
<i>p</i>-Hydroxycinnamic Acids				
11	<i>p</i> -Coumaric acid		260	24
12	Caffeic acid		390	2
13	Ferulic acid		430	2
14	Sinapic acid		520	2
<i>p</i>-Hydroxyacetophenones				
15	4-Hydroxyacetophenone		260	24
16	Acetovanillone		300	24
17	Acetosyringone		520	8
<i>p</i>-Hydroxybenzaldehydes				
18	4-Hydroxybenzaldehyde		260	24
19	Vanillin		410	24
20	Syringaldehyde		370	4

Table I. Cont.

Code	Name	Structure	Wavelength (nm)	Time (h)
Methyl <i>p</i>-Hydroxybenzoates				
21	Methyl vanillate		370	24
22	Methyl syringate		390	1
Methyl <i>p</i>-Hydroxycinnamates				
23	Methyl <i>p</i> -coumarate		400	24
24	Methyl ferulate		370	1
25	Methyl sinapate		410	4
Others				
26	Eugenol		370	1
27	Resveratrol		400	1
28	Quercetin		290	4

^a Determined in kinetic mode.

Changes in the absorption spectra of the different phenols in the presence of parent 3A4 laccase along 24 h are shown in **Fig. S2**. In accordance with previous studies, we observed that, in general, substitutions in *ortho* favor phenol oxidation by laccase, being the *o*-di-substituted phenols more easily oxidized than the mono- and non-substituted phenols (the latter were the most recalcitrant). In fact, 4-hydroxybenzoic acid (**6**) was not oxidized at all in the assayed conditions. This is due to the fact that the hydroxyl and methoxyl substituents in *ortho* act as electron donating groups in the aromatic ring (making the π system more nucleophilic) and generally lower the substrate's redox potential, thus allowing an easier oxidation by laccase. Furthermore, the parent laccase seemed to oxidize phenols with *o*-methoxyl substituents better than with hydroxyl substituents, even though hydroxyls are more activating substituents of the aromatic ring. The same has been described for

laccases from *Trametes villosa* and *Lentinula edodes* (D'Annibale et al., 1996; Xu, 1996), which present higher catalytic efficiency (k_{cat}/K_m) for guaiacol (**3**) than for catechol (**2**). Similarly, laccase from *Gaeumannomyces graminis* var. *tritici* presented higher affinity (lower K_m) for guaiacol over catechol and for 2,6-dimethoxyphenol (**5**) over pyrogallol (**4**) (Edens et al., 1999). This highlights the fact that, apart from a redox potential threshold that must be overcome by laccase, other factors must account for the enzyme's preference to oxidize certain compounds over others of lower redox potential, mainly a more favorable binding of the substrate. In this sense, the importance of the binding event in the overall efficiency of the enzymatic reaction is influenced not only by steric features that limit the entrance of the substrate to the enzymatic pocket (Tadesse et al., 2008), but also by an optimal docking that assures an efficient electron transfer to the T1 Cu site (Monza et al., 2015).

On the other hand, respecting the effect of the groups/side chains in *para* position, a general tendency (essentially for G- and S-type compounds) was found based on how rapidly the changes in the UV-Vis spectra were observed. The reactivity decreased in the following order: phenols without *p*-substituent > methyl *p*-hydroxycinnamates > *p*-hydroxycinnamic acids > *p*-hydroxybenzoic acids > methyl *p*-hydroxybenzoates > *p*-hydroxyketones > *p*-hydroxyaldehydes. This correlates well with the fact that all the *p*-substituents tested are moderately deactivating, except for the propenoic chain of cinnamic acids and cinnamic esters due to the presence of the double bond. In fact, during fungal degradation of the lignin polymer, the oxidation of the side chains of the phenylpropane lignin units (C α -C β cleavage) takes place in the initial stages, giving rise to vanillic and syringic acid as main degradation products from G- and S-type units, respectively (Camarero et al., 1997).

Comparison of laccase mutants

In order to compare the activity of the different laccase mutants, an appropriate wavelength was chosen for each substrate. When changes in the absorption spectra were observed in more than one peak, increase in the visible wavelength range was selected, since these could be useful for developing future colorimetric high-throughput screening (HTS) assays. To detect differences between clones, time points were selected so that the change in absorbance (ΔAbs) observed for the parent type at the given wavelength was around 50% of the maximum ΔAbs . Relative activity for each clone respecting parent type was calculated according to the formula:

$$\frac{((\Delta Abs_x - \Delta Abs_{blank})/[protein]_x)}{((\Delta Abs_{parent} - \Delta Abs_{blank})/[protein]_{parent})} \quad (1)$$

Results for relative activities were represented in a heat map and clustered according to substrate (columns) and laccase mutant (rows) (**Fig. 3**). *o*-Di-substituted phenols, for which the highest increases in activity were found, were grouped together at the right side of the map. In fact, sinapic acid and methyl sinapate (**14** and **25**, respectively) formed a distinct cluster, separate from the rest of substrates tested. The utmost enhancement of laccase activity on **14** and **25** is expected because laccase mutants were selected based on their activity towards sinapic acid, and methyl sinapate is the most structurally similar compound used in the screening. *o*-Monosubstituted phenols, on the other hand, were more evenly represented in the different clusters. It is worth noting that vanillin related compounds (**16**, **19** and **21**) clustered at the far left side of the map, with laccase mutants presenting even worse activity for these substrates than towards the theoretically more difficult to oxidize H-type phenols (except for **6**). Interestingly, greater increases in activity were found for substrates with hydroxyl substitutions in *ortho* than for those with methoxyl substitutions (except for **7** and **8**), even though parent laccase was more efficient oxidizing the latter. The mutations introduced in the enzyme binding pocket are not expected to significantly affect the laccases' redox potential, since none of the target residues coordinate the T1 Cu (the closest are Pro390 and Phe392, located in the same loop as His395 that coordinates T1 Cu). For this reason, the observed differences between mutants are most probably caused by different affinities. In fact, it is possible that not all substrates were used in saturating concentrations in the screening assay and, consequently, oxidation rates would be affected by the mutants' affinity towards the different compounds.

Respecting laccase mutants, roughly three main clusters were formed. Cluster 1 was formed by clones that performed worse than parent type laccase towards most substrates (upper part of the map). Except for the case of clone C9, mutants grouping in this cluster are those that presented lower activity towards ABTS in the culture supernatant (**Fig. 2A**), meaning that these laccase variants were generally less active than the parent type. The second cluster included clones that retained similar activity values than parent type, and it could be subdivided into a group which presented a general enhanced oxidation of phenols with two substitutions in *ortho* (Cluster 2a, mutants from library C) and another group that did not (Cluster 2b, mutants from libraries A and B). Finally, Cluster 3 was formed by four clones presenting the highest increases in activity towards several compounds (lower part of the map). These included three mutants from library C and one from library A. They showed enhanced oxidation over all *o*-di-substituted phenols tested and several *o*-monosubstituted phenols (including eugenol) and, interestingly, also increased activity on resveratrol.

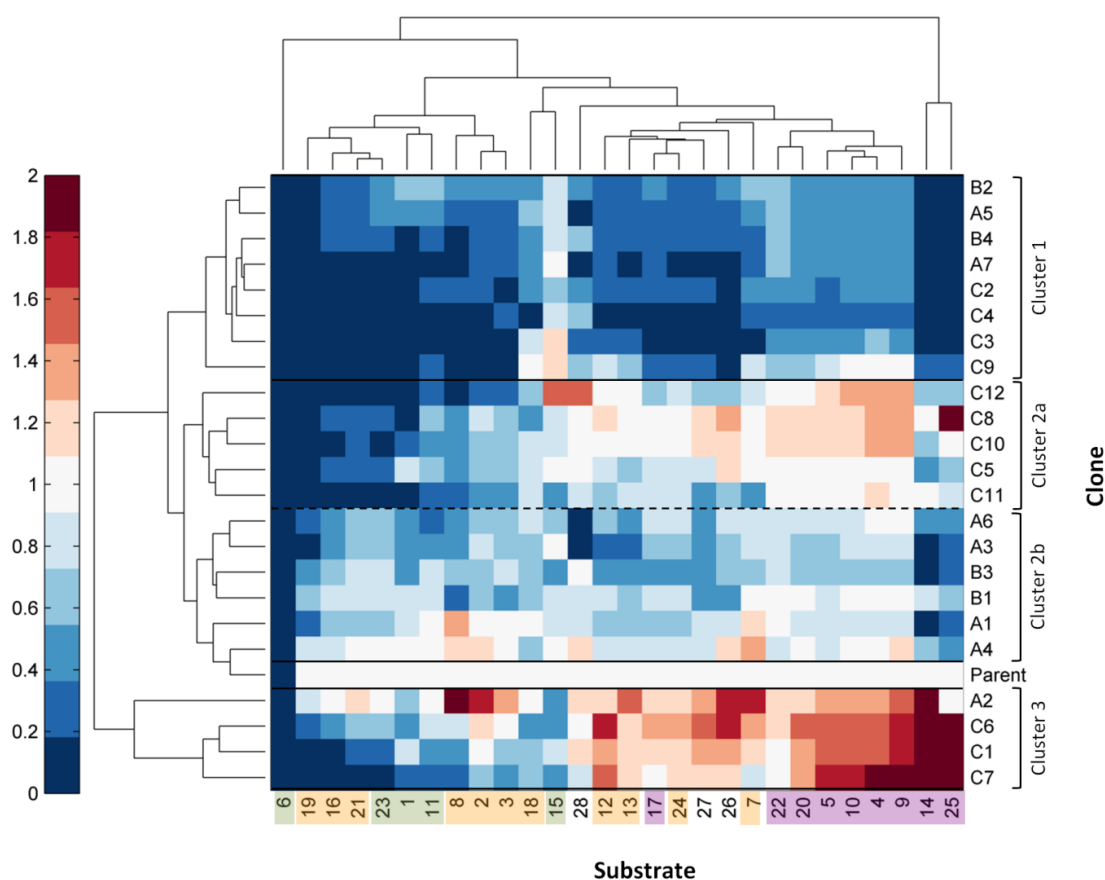


Figure 3. Heat map with hierarchical clustering based on the relative activity values (respecting parent type laccase) of the library of laccase mutants (rows) towards the different phenols under study (columns). Substrate labels are highlighted in purple for *o*-di-substituted phenols, orange for *o*-monosubstituted phenols, green for H-type phenols, and non-highlighted for others. The three main clusters grouping the laccase mutants are indicated.

Analysis of DNA sequences revealed that all clones from library C mutated Phe392 for other residues. In particular, clones belonging to Cluster 3, namely C7, C1 and C6, mutated Phe392 for Asn, Thr and Gln, respectively, but maintained Pro390 from parent type. It is worth noting that the best mutants from Cluster 2a, C8 (F392S) and C10 (F392V), which also presented better activity towards *o*-di-substituted phenols but to a lesser extent, also maintained parent Pro390. The mutations found in these clones revealed that while position 392 is quite flexible, accommodating variable residues, position 390 is more restrictive. As for A2 mutant, substitutions A162K and T164I were found. Remarkably, clone A2 was significantly better at oxidizing *o*-monosubstituted phenols (e.g., **2**, **3**, **8**, **7** and **13**) than di-substituted phenols, in comparison to clones from library C. The same occurred for clones A1 (A162K T164V) and A4 (A162R). This suggests that the substitution of Ala162 for basic residues Lys or Arg could be beneficial for the oxidation of this type of compounds. Interestingly, several studies on laccase structure–function relationships hypothesize that a nonpolar residue in position 162 is important to

establish correct hydrophobic interactions with the phenolic substrate (Bertrand et al., 2002; Galli et al., 2011). However, our results point in the opposite direction, at least for the oxidation of phenolic substrates with one or two hydroxyl or methoxyl substituents in *ortho*. This highlights the often-difficult prediction of amino acid substitutions to enhance enzyme activity by rational design, and by contrast, the potential of directed evolution and random approaches to reveal unexpected mutations that might provide the solution to a problem.

EXPERIMENTAL SECTION

Construction and screening of mutant libraries

General considerations for DNA manipulation and library screening are described elsewhere (Maté et al., 2010; Camarero et al., 2012; Pardo et al., 2012). Mutant libraries were obtained by In Vivo Overlap Extension (IVOE, Alcalde, 2010). Amino acids in positions 162, 164, 263, 264, 390 and 392 were randomized using mutagenic primers with NNK degeneracy. Each pair of mutagenic primers simultaneously mutated two nearby residues, so that three individual libraries were obtained: library A (positions 162 and 164), library B (positions 263 and 264) and library C (positions 390 and 392). The 5'-end gene fragments were amplified with RMLN and reverse mutagenic primers, and the 3'-end fragments with RMLC and forward mutagenic primers, using pJR α 3A4 as template (Pardo et al., 2013). Purified PCR products were co-transformed in yeast together with the linearized expression vector (pJR α C30) using Yeast Transformation Kit (Sigma, St. Louis, MO, USA). Individual colonies were picked and cultured in 96-well format in minimal media for two days and in expression media for another two days (Maté et al., 2010; Camarero et al., 2012; Pardo et al., 2012). Then, plates were centrifuged and 20 μ L aliquots of supernatant were transferred to new plates for screening, which was performed by adding 180 μ L of 250 μ M sinapic acid in 100 mM acetate buffer, pH 5.0. Transformation of sinapic acid by laccase was followed in end-time mode in the plate reader, measuring the increase in absorption at 512 nm (Camarero et al., 2012; Pardo et al., 2013). Mutants presenting higher activity were submitted to a re-screening, in which activity of each mutant was measured in quadruplicate. Plasmids from clones selected in the re-screening were extracted and transformed in *E. coli* strain DH5 α in order to obtain sufficient copies for DNA sequencing and for re-transformation in yeast cells.

Production of laccase mutants

Re-transformed yeast cells expressing laccase mutants selected in the re-screening were produced in duplicate in 100 mL flasks containing 30 mL of expression media (Maté et al., 2010; Camarero et al., 2012; Pardo et al., 2012), growing the cells at 30 °C, 220 rpm, for 4 days. Afterwards, cultures were

centrifuged at 5000 *g* for 20 min and supernatants were sequentially filtered through 0.8 and 0.45 μm pore-size membranes. Filtered supernatants were then concentrated tenfold in Amicon Ultra 10000 MWCO centrifugal units (Millipore). Laccase activity in the concentrated extracts was measured with 3 mM ABTS ($\epsilon_{418} = 36000 \text{ M}^{-1} \text{ cm}^{-1}$) in 100 mM acetate buffer, pH 5.0, and protein concentration was determined with the Bio-Rad Protein Assay, using the microassay procedure for microtiter plates.

Multi-screening with phenolic compounds

All the assayed substrates were purchased from Sigma-Aldrich, except for methyl syringate, which was purchased from Alpha Aesar (Karlsruhe, Germany), and methyl coumarate, methyl ferulate and methyl sinapate, from Apin Chemicals (Abingdon, UK). Stock solutions for the different phenols were routinely prepared at 10 mM in 20% ethanol, except for phenol, catechol, pyrogallol, guaiacol and 2,6-dimethoxyphenol, which were dissolved in water; and resveratrol and quercetin, which were dissolved in absolute ethanol. Reactions were carried out in duplicate for each mutant in UV-Star 96-well plates (Biogen), by adding 10 μL of laccase crude extracts, 180 μL of 100 mM acetate buffer, pH 5.0, and 20 μL of stock substrate solution in each well. Control reaction without enzyme was included in order to subtract non-enzymatic substrate oxidation. UV-Vis absorption spectra (250–700 nm, with steps of 10 nm) were recorded in a SpectraMax Plus 384 plate reader (Molecular Devices) at reaction times 0, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h. In the case of 2,6-dimethoxyphenol, reaction was too fast, so it was measured in kinetic mode at 469 nm ($\epsilon_{469} = 27500 \text{ M}^{-1} \text{ cm}^{-1}$). For each substrate, absorption peaks and appropriate time points were selected. Average ΔAbs per mg of protein was calculated for each mutant and normalized against parent type. Results were represented in a heat map and hierarchical clustering analysis was performed using the clustergram algorithm with Matlab software (MathWorks, Natick, MA, USA).

CONCLUSIONS

In this work, we have evaluated the oxidation of a battery of phenols with different chemical structures by a library of laccase mutants obtained by saturation mutagenesis of six residues of the substrate binding pocket. This has given us some insight into the structural factors, both from the substrate and the enzyme, that modulate laccase activity. Even though the substrates tested are structurally related to sinapic acid, high variability in laccase activity was found only by changing one or two residues of the enzyme pocket. This indicates that besides the difference in redox potential between laccase and substrate, which limits the electron transfer, the efficient binding of the substrate to the enzymatic pocket determines the oxidative capabilities of laccases. These results also highlight once more the

importance of the screening assay for directed evolution, which will define the fate of the protein subject of the study. The use of either a single or a multi-screening assay will determine whether we obtain a specialist or a generalist biocatalyst, respectively.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

S.C. conceived and planned the work; I.P. designed and performed the experiments; I.P. and S.C. analyzed the data; and I.P. and S.C. wrote the paper.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

SIGPVADLTISNGAVSPDGFSRQAILVNDVFPSPLITGNKGDRFQLNVIDNMTNHTMLKS	60
TSIHWGFFQHGTNWADGPAFVNQCPISTGHAFLYDFQVPDQAGTFWYHSHLSTQYCDGL	120
RGPIVVYDPQDPHKSLYDVDDDSTVITLADWYHLAAKVGPAAPTADATLINGLGRSINTL	180
NADLAVITVTKGKRYRFRLVSLSCDPNYTFSIDGHSLTVIEADGVNLKPQTVDSIQIFPA	240
QRYSFVLNADQVDVNYWIRALPNSGTRNFDGGVNSAILRYEGAAPVEPTTTQTPSTQPLV	300
ESALTTLEGTAAPGNPTPGGVDLALNMAFGFAGGRFTINGASFPTPPTVPVLLQILSGAQS	360
AQDLLPSGSVYSLPANADIEISLPATSAAPGFPHPHLHGHTFAVVRSAQSSTYNYANPV	420
YRDVVNTGSPGDNVTIRFRFDNPGPWLHCHIDFHLDAGFAVMAEDTPDTKAANPVPQA	480
<u>WSDLCPITYDALDPSDL</u>	496

Figure S1. Amino acid sequence of chimeric 3A4 laccase used as parent type in this work. Sequence inherited from Pcl evolved variant during chimeragenesis is underlined (residues 457–496), while the rest corresponds to PM1L evolved variant. Copper coordinating residues are highlighted in cyan. Mutated residues located at the substrate binding pocket are shown in magenta.

Exploring the oxidation of lignin derived phenols

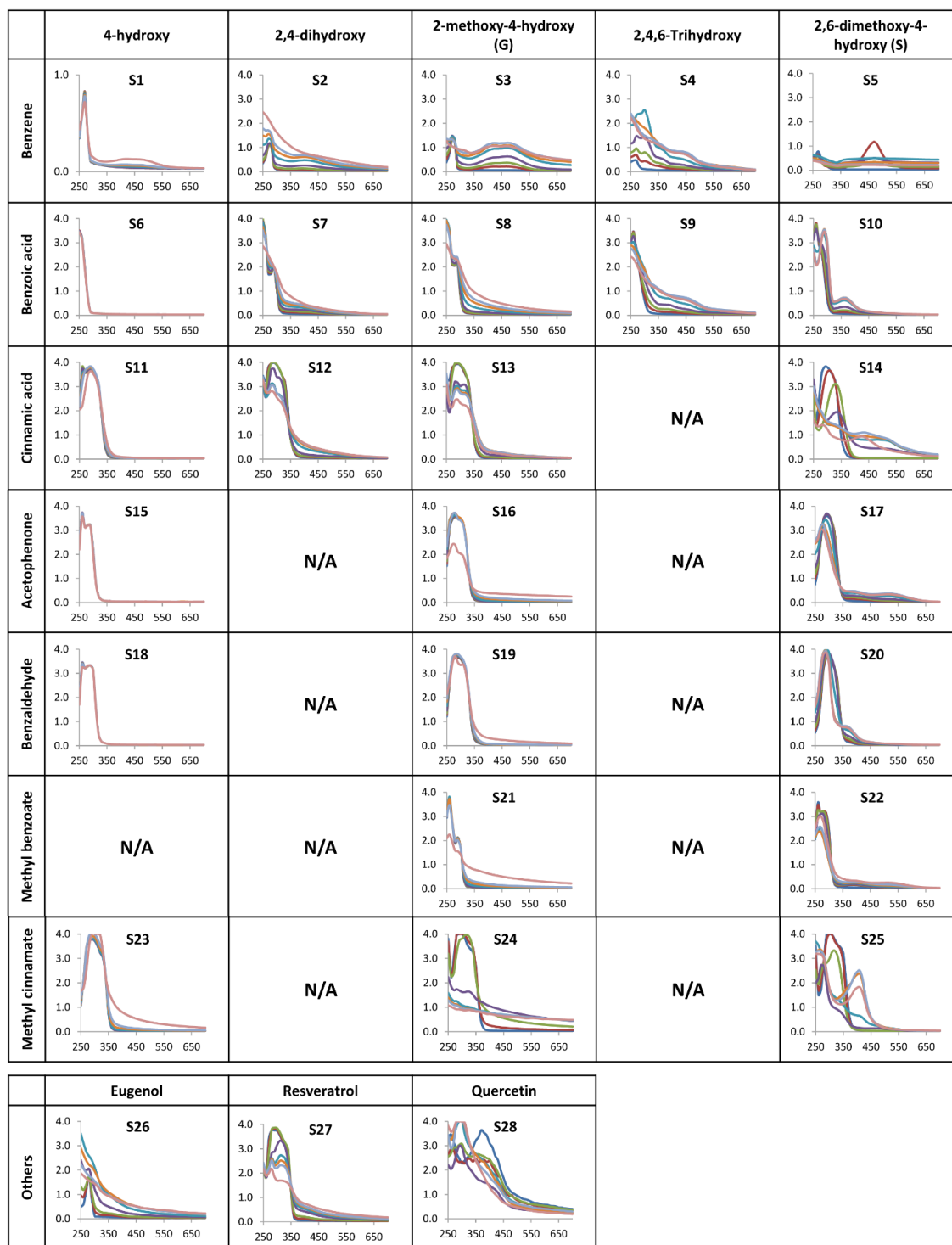


Figure S2. Changes in UV-Vis absorption spectra of the different phenols upon oxidation by laccase. Spectra shown correspond to initial time (blue), 30 min (red), 1 h (green), 2 h (purple), 4 h (cyan), 6 h (orange), 8 h (light blue) and 24 h (pink).

CHAPTER 5

A HIGHLY STABLE HYBRID LACCASE OBTAINED BY DOMAIN SWAPPING

Isabel Pardo, Susana Camarero

(In preparation)

ABSTRACT

Two hybrid laccases were obtained by exchanging the second cupredoxin domain between two high-redox potential laccases previously engineered in the lab by directed evolution. The new swapped variants were functionally expressed by *Saccharomyces cerevisiae* and presented different properties to those of both parent types, including shifted pH activity profiles and altered substrate affinities. Besides, one of the swapped variants showed an outstanding increase in stability to temperature and also to acid pH and organic solvents. Possible causes for the increased thermostability of this variant, such as N-glycosylation and salt bridges, were evaluated by site-directed mutagenesis. However, they were not found to be the main determinants for stability. The results presented here confirm the power of DNA recombination in protein engineering to give way to better protein robustness by the introduction of a pool of neutral mutations, providing high evolvability for further engineering challenges.

INTRODUCTION

Laccases, belonging to the superfamily of blue-multicopper oxidases, are enzymes capable of oxidizing a broad range of phenols, aromatic amines and other substrates with the concomitant reduction of molecular oxygen to water. They are widely distributed in nature and participate in a variety of physiological functions, although basidiomycete fungi, mediating wood decay, are the most important producers of these enzymes. Laccases are typically monomeric proteins that consist of three cupredoxin domains (D1, D2 and D3) and present four catalytic copper ions: one T1 copper, which accepts one electron from the reducing substrate; and one T2 and two T3 coppers, which form the trinuclear cluster (TNC) and transfer four electrons to O₂ to render two molecules of H₂O. Highly conserved residues (ten His and one Cys) located at the D1 and D3 coordinate these copper atoms and provide a fast electron transfer pathway from the T1 Cu to the TNC (Hakulinen and Rouvinen, 2015). The redox potential at the T1 Cu site limits to some extent whether a certain compound may be oxidized by laccase, but effective oxidation is also determined by steric limitations and favorable interactions between the substrate and residues delimiting the enzyme's substrate binding pocket, located in D1 and D2.

In recent years, the engineering of laccases for their application in different fields has raised a great interest. In order to be used as industrial biocatalysts, highly efficient enzymes that are active and stable under harsh operational conditions are needed (Pardo and Camarero, 2015). Directed evolution is a powerful tool for tailoring enzymes *a la carte*, allowing the attainment of desired properties in a few rounds of mutagenesis, screening and selection. However, after several generations of uphill walk on protein fitness landscape, one may fail to obtain new single

beneficial mutations, frequently caused by a trade-off between protein activity and stability (Bloom et al., 2006; Tracewell and Arnold, 2009). Introduction of neutral mutations on the other hand may increase the protein's robustness, while maintaining its function, and allow the accommodation of new beneficial mutations by an epistatic effect (Bloom and Arnold, 2009). Indeed, the introduction of neutral mutations in directed evolution experiments, resembling natural genetic drift, has been successfully used to obtain more active proteins with increased stability or to enhance promiscuous enzymatic activities (Amitai et al., 2007; Bloom et al., 2007; Smith et al., 2011). However, the simplest way to obtain functional sequences with an elevated number of neutral mutations (which may be hard to find and select in libraries generated by error-prone PCR) is the recombination of related sequences, rendering a rapid exploration of a large sequence space (Cramer et al., 1998). The recombination of DNA sequences can be performed randomly, either by sequence dependent (DNA shuffling, StEP) (Stemmer, 1994; Zhao et al., 1998) or independent approaches to avoid biases in crossover points (ITCHY, SCRATCHY, SHIPREC) (Lutz et al., 2001; Ostermeier et al., 1999; Sieber et al., 2001); and rationally, selecting the cross-over points based on the inspection of protein structure and trying to minimize the disruption of interactions between residues (SCHEMA) (Otey et al., 2004; Smith et al., 2012; Voigt et al., 2002).

Previously we have described the engineering of chimeric laccase variants by random DNA shuffling of two high-redox potential laccases actively expressed in *Saccharomyces cerevisiae* (Pardo et al., 2012). Parent types used came from the directed evolution of laccases from basidiomycetes *Pycnoporus cinnabarinus* (evolved variant 3PO) and PM1 (evolved variant OB1) (Camarero et al., 2012; Maté et al., 2010). The selected chimeras presented an array of different properties respecting parent types, even though mutants had only exchanged relatively small sequence blocks located in D1 and D3. In the present work we describe the construction of two new hybrid laccases by domain-swapping, *i.e.* the exchange of D2 between OB1 and 3PO variants. Again, differences are observed between swapped laccases and parent types, including shifted pH activity profiles, different substrate affinities and, notably, an important stability increase (to temperature, acid pH and organic solvents) for one of the new variants. Finally, we discuss here the possible causes for this enhanced stability, which were evaluated by a series of site-directed mutagenesis approaches.

RESULTS

Construction and expression of domain-swap mutants

For the design of domain-swap laccases we chose to exchange D2 because of the lower sequence identity of the parent laccases 3PO and OB1 at this domain (69% amino acid sequence identity in D2 versus 81% in D1 and 79% in D3), being this fact the reason behind the absence of crossover events in D2 during the previous random chimeragenesis of both high-redox potential laccases (Pardo et al., 2012). Based on the structure models for 3PO-PcL (using PDB 2XYB as template) and OB1-PM1L (using PDB 2HRG as template), cross-over points were selected in loops between domains in order to maintain the integrity of each cupredoxin domain. Swapped mutants were constructed by amplifying individual domains and reassembling them by PCR overlap extension, obtaining mutants OB1::3PO (with D1 and D3 from OB1 and D2 from 3PO) and 3PO::OB1 (with D1 and D3 from 3PO and D2 from OB1) (**Fig. 1**).

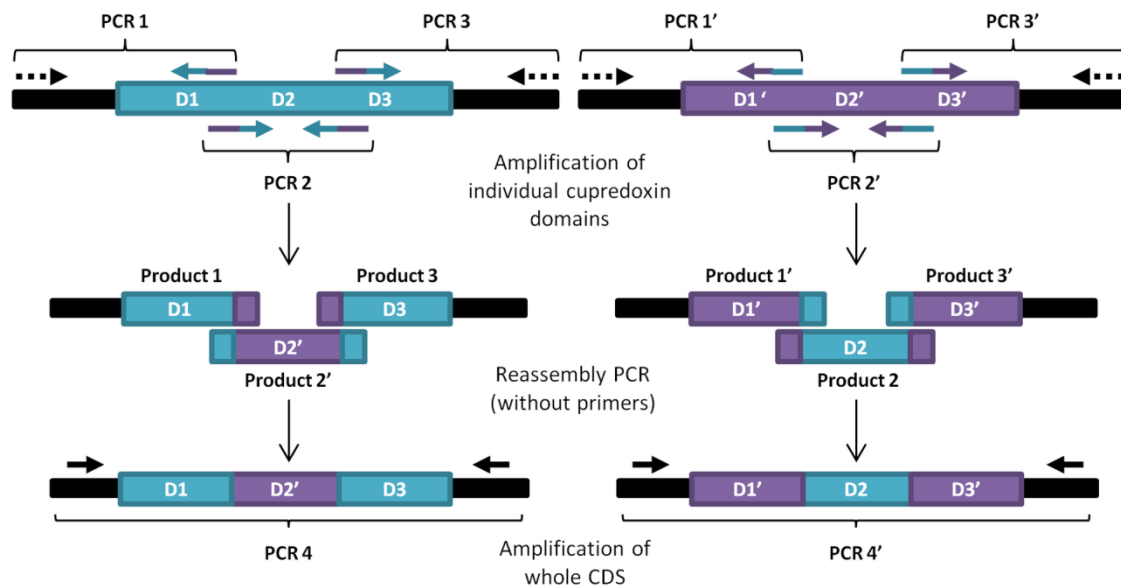


Figure 1. Schematic representation of the strategy used for the construction of domain-swap mutants. The sequence belonging to OB1 is shown in blue, to 3PO in purple and to the expression vector in black.

Once the correct construction of the swapped mutants was confirmed by DNA sequencing, the new laccases were produced together with OB1 and 3PO parents. The parent laccases present different optimal temperatures for their heterologous expression and secretion by yeast: maximum production for the first (around 800 U L⁻¹) is achieved after 3–4 days at 30 °C, while the latter reaches maximum activity (350 U L⁻¹) after 5–6 days at 20 °C. Taking this into consideration, both temperatures were tested for the production of the swapped variants. OB1::3PO and 3PO::OB1 were more efficiently secreted at 20 °C, although maximum activity

achieved was much lower than for parent types: 150 U L⁻¹ and 50 U L⁻¹, respectively (**Fig. 2**). Due to the low levels of laccase activity obtained, unpurified concentrated samples were used for the preliminary characterization of the swapped variants.

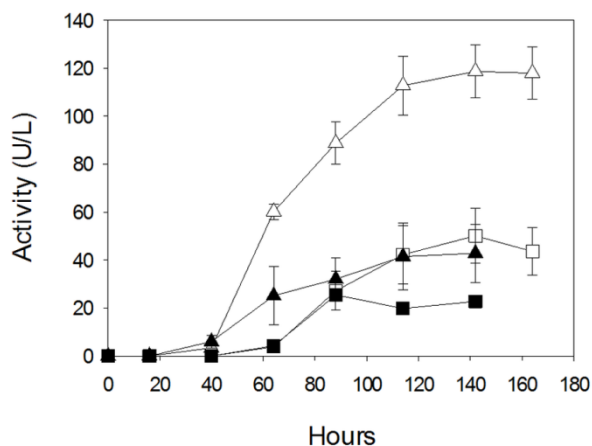


Figure 2. Laccase production curves of swapped variants OB1::3PO (triangles) and 3PO::OB1 (squares) at 20 (white symbols) and 30 °C (black symbols). Error bars indicate standard deviation for at least two replicates.

Activity of domain-swap laccases

Firstly, the oxidation of ABTS and DMP by the swapped variants was evaluated (**Fig. 3**). 3PO::OB1 presented a hampered activity at low pH values for both substrates. While parent laccases present 70–80% relative activity for ABTS at pH 2, it is almost zero for this variant. A drastic change in the case of DMP oxidation was also observed: the pH profile was much narrower, with a marked maximum at pH 5 and an important loss of activity at lower pH values. As for variant OB1::3PO, it presented a slightly more acidic activity profile towards ABTS than parent laccases, with a maximum at pH 2, while the profile towards DMP was the same as OB1. Next, K_m values for both substrates were estimated (**Table I**). Variant 3PO::OB1 presented an important loss of affinity towards both substrates, with increases in K_m values of approximately 5-fold for ABTS and 12-fold for DMP, respecting the more similar 3PO parent. On the other hand, K_m values for OB1::3PO variant were not so different from those of parent types. In the case of ABTS, it presented a K_m more similar to 3PO parent, whereas K_m for DMP was closer to OB1 parent.

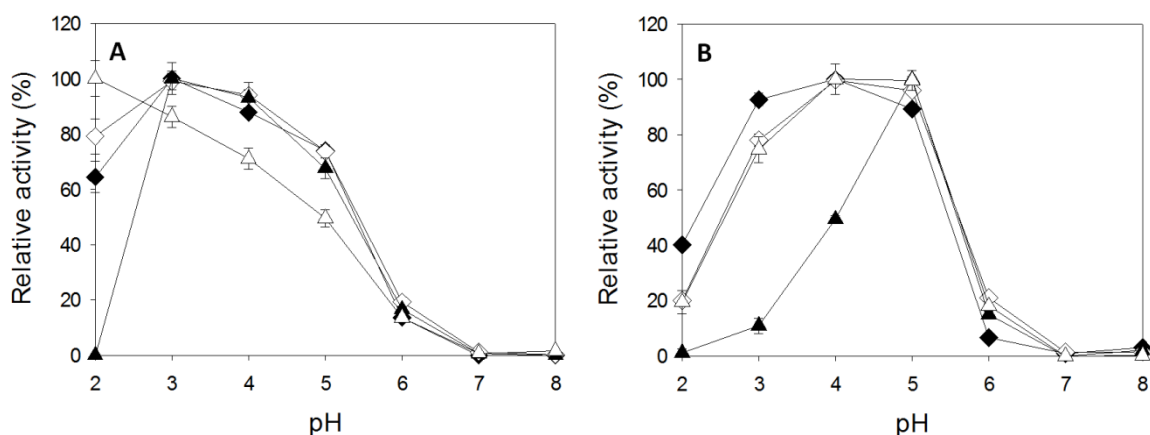


Figure 3. pH activity profiles for the oxidation of **A)** ABTS and **B)** DMP by laccases OB1 (white diamonds), 3PO (black diamonds), OB1::3PO (white triangles) and 3PO::OB1 (black triangles). Error bars indicate standard deviation for three replicates.

Table I. K_m values (μM) towards ABTS and DMP for the different laccase variants calculated at pH 5.

	OB1	3PO	OB1::3PO	3PO::OB1
ABTS	9.1 ± 0.5	42.9 ± 1.3	53.2 ± 5.2	204.7 ± 13.0
DMP	93.1 ± 3.3	243.5 ± 6.7	133.8 ± 4.6	3005 ± 162

Stability of domain-swap laccases

An initial T_{50} (10 min) assay was performed to assess the stability of the swapped variants. Variant OB1::3PO showed an increase in T_{50} value of 7 °C ($T_{50} = 78.1 \pm 0.6$), whereas for 3PO::OB1 it decreased in 5 °C ($T_{50} = 65.7 \pm 0.2$) respecting parent type laccases (**Fig. 4**). In order to see if these changes in T_{50} correlated with stability to other factors, long-term (6 hours) stability to temperature, pH and solvents was then evaluated. Respecting stability to temperature, all laccases retained initial activity after 6 hours at temperatures up to 50 °C. At 60 °C, however, OB1::3PO still retained 90% initial activity, while OB1 (the most thermostable parent) presented 60% residual activity. 3PO and 3PO::OB1 on the other hand were almost completely inactivated. Outstandingly, OB1::3PO still presented more than 10% residual activity after 6 hours at 70 °C (**Fig. 5**). Swapped variant 3PO::OB1 was also the most unstable at extreme pH, presenting an important loss of activity at pH values 2, 3 and 9. OB1::3PO on the other hand was quite more stable at acidic pH (**Fig. 6**). As for solvents, all laccases proved to be stable to acetone and DMSO up to 50% (v/v) concentration (data not shown). Surprisingly, for the rest of solvents 3PO parent was more stable than OB1, which was severely affected (despite being the most thermostable of both parents). Swapped variant OB1::3PO was again the most stable in almost all the solvent concentrations tested, presenting the highest residual activity of all laccases at 50% (v/v) solvent concentration. In the case of 10–20% BMIM otf, 3PO parent seemed to present a slight activation (**Fig. 7**).

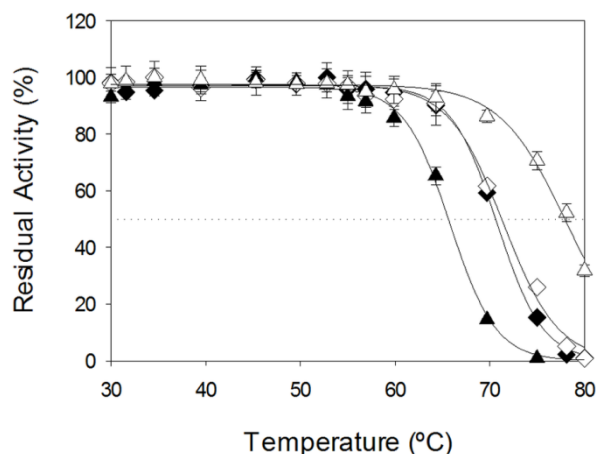


Figure 4. T_{50} (10 min) curves for laccases OB1 (white diamonds), 3PO (black diamonds), OB1::3PO (white triangles) and 3PO::OB1 (black triangles). Error bars indicate standard deviation for three replicates.

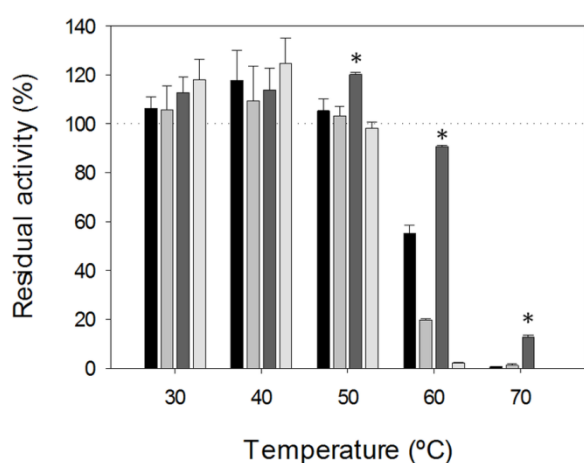


Figure 5. Residual activity of the different laccase variants after 6 hours incubation at different temperatures. From left to right: OB1, 3PO, OB1::3PO and 3PO::OB1. Error bars indicate standard deviation for three replicates. Variants showing significantly higher residual activity respecting the most stable parent OB1 ($p < 0.05$) are marked with an asterisk.

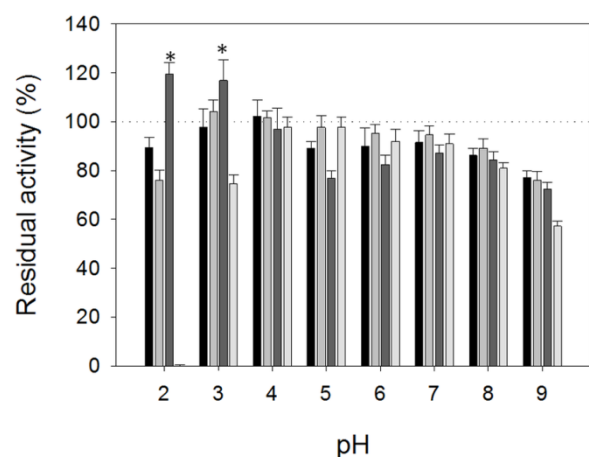


Figure 6. Residual activity of the different laccase variants after 6 hours incubation at different pH. From left to right: OB1, 3PO, OB1::3PO and 3PO::OB1. Error bars indicate standard deviation for three replicates. Variants showing significantly higher residual activity respecting the most stable parent OB1 ($p < 0.05$) are marked with an asterisk.

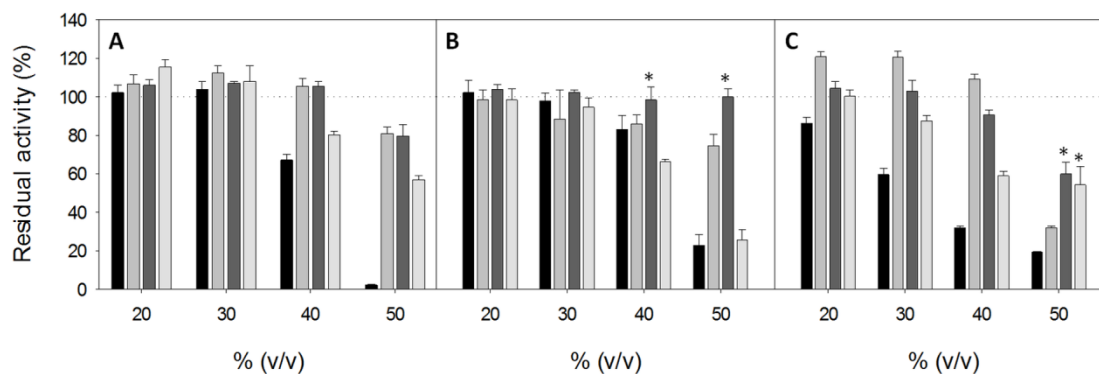


Figure 7. Residual activity of the different laccase variants after 6 hours incubation in the presence of different concentrations of **A)** ethanol, **B)** methanol and **C)** BMIM otf. From left to right: OB1, 3PO, OB1::3PO and 3PO::OB1. Error bars indicate standard deviation for three replicates. Variants showing significantly higher residual activity respecting the most stable parent 3PO ($p < 0.05$) are marked with an asterisk.

Evaluation of the factors determining thermostability in the domain-swap laccases

In order to determine what factors could cause the increased stability of OB1::3PO, we examined and compared the structure models for both swapped variants. No differences in cysteine bonding were expected, as both parent types present two disulfide bonds (connecting D1-D2 and D1-D3) whose positions are extremely conserved in all fungal laccases. In particular, we searched for differences in N-glycosylation and superficial salt-bridges, known to have a positive effect on the stability of proteins, including laccases (Christensen and Kepp, 2013). Concerning glycosylation we found that, as a consequence of D2 exchange, OB1::3PO had inherited an N-glycosylation site from 3PO (**Fig. 8A**). To elucidate whether this fact could be responsible for the enhanced stability of OB1::3PO variant, residue Asn215 was mutated for the original Gly found in OB1 parent. Gly is in fact the most conserved amino acid in this position for basidiomycete laccases with a known crystal structure (data not shown). However no change in the T_{50} value was observed for OB1::3PO-N215G mutant (**Table II**).

Table II. T_{50} (10 min) values for the different mutants obtained by site-directed mutagenesis of the swapped laccases.

Variant	T_{50} (10 min) (°C)
OB1::3PO-N215G	78.6 ± 0.2
OB1::3PO-E232T/K298Q	77.3 ± 0.2
OB1::3PO-D379S/R440E	78.4 ± 0.2
OB1::3PO-E232T/K298Q/D379S/R440E	77.0 ± 0.2
3PO::OB1-T231E/Q297K	65.5 ± 0.2
3PO::OB1-S378D/E439R	65.6 ± 0.2
3PO::OB1-T231E/Q297K/S378D/E439R	65.7 ± 0.2

Next, putative salt-bridges on the protein surface were inspected, searching for acid-basic amino acid pairs with $< 5 \text{ \AA}$ distance between them in the protein models. We found 13 putative salt bridges in parent laccases OB1 and 3PO, 14 in OB1::3PO variant and 12 in 3PO::OB1 variant (**Table S3**). The most remarkable difference between both swapped variants was that OB1::3PO inherited acid-basic pairs E232–K298 (from 3PO-D2) and D379–R440 (from OB1-D3), while they were lost in 3PO::OB1 variant (T231–Q297 and S378–E439) (**Fig. 8A**). Taking this into account, we exchanged these residues between both mutants by site-directed mutagenesis, obtaining double mutants E232T/K298Q and D379S/R440E for OB1::3PO, and T231E/Q297K and S378D/E439R for 3PO::OB1, together with the respective quadruple mutants. When we determined their T_{50} values, we found that all 3PO::OB1 salt-bridge mutants presented the same T_{50} as the original variant. As for OB1::3PO, T_{50} was reduced in only $1 \text{ }^\circ\text{C}$ for the E232T/K298Q and quadruple mutants, while it was maintained for D379S/R440E mutant (**Table II**).

DISCUSSION

In this work we describe for the first time the construction of hybrid laccases by structure-guided recombination to exchange the cupredoxin domains of two high-redox potential laccases. The strategy used here allowed us to obtain active enzymes despite the high number of mutations introduced (65 substitutions in a 185 amino acid sequence-length exchange), as the integrity of three-domain multicopper oxidase architecture was maintained. However, the new variants were not as efficiently secreted by yeast as the parent types, and the maximum activity achieved in the cultures was much lower. For the directed evolution of PM1 and *P. cinnabarinus* laccases, the respective cDNAs were fused to the coding sequence of the signal peptide from the *S. cerevisiae* alpha-mating factor, and the whole fusion genes were submitted to random mutagenesis. As a consequence, the signal peptides of the evolved laccases OB1 and 3PO hold different mutations in the prepro-leader sequence, accumulated throughout their respective evolution routes (Maté et al., 2010; Camarero et al., 2012). Due to the strategy followed for the construction of the swapped mutants described in this work, each one inherited the signal peptide that was fused to the corresponding parent D1. When we attempted to exchange the signal peptide of OB1::3PO variant (from OB1) for that of 3PO parent, expression was not enhanced (data not shown). In this sense, future approaches to increase OB1::3PO secretion, such as further directed evolution of the alpha-factor prepro-leader or engineering of other signal peptides, would be of great interest (Rakestraw et al., 2009; Viña-Gonzalez et al., 2015).

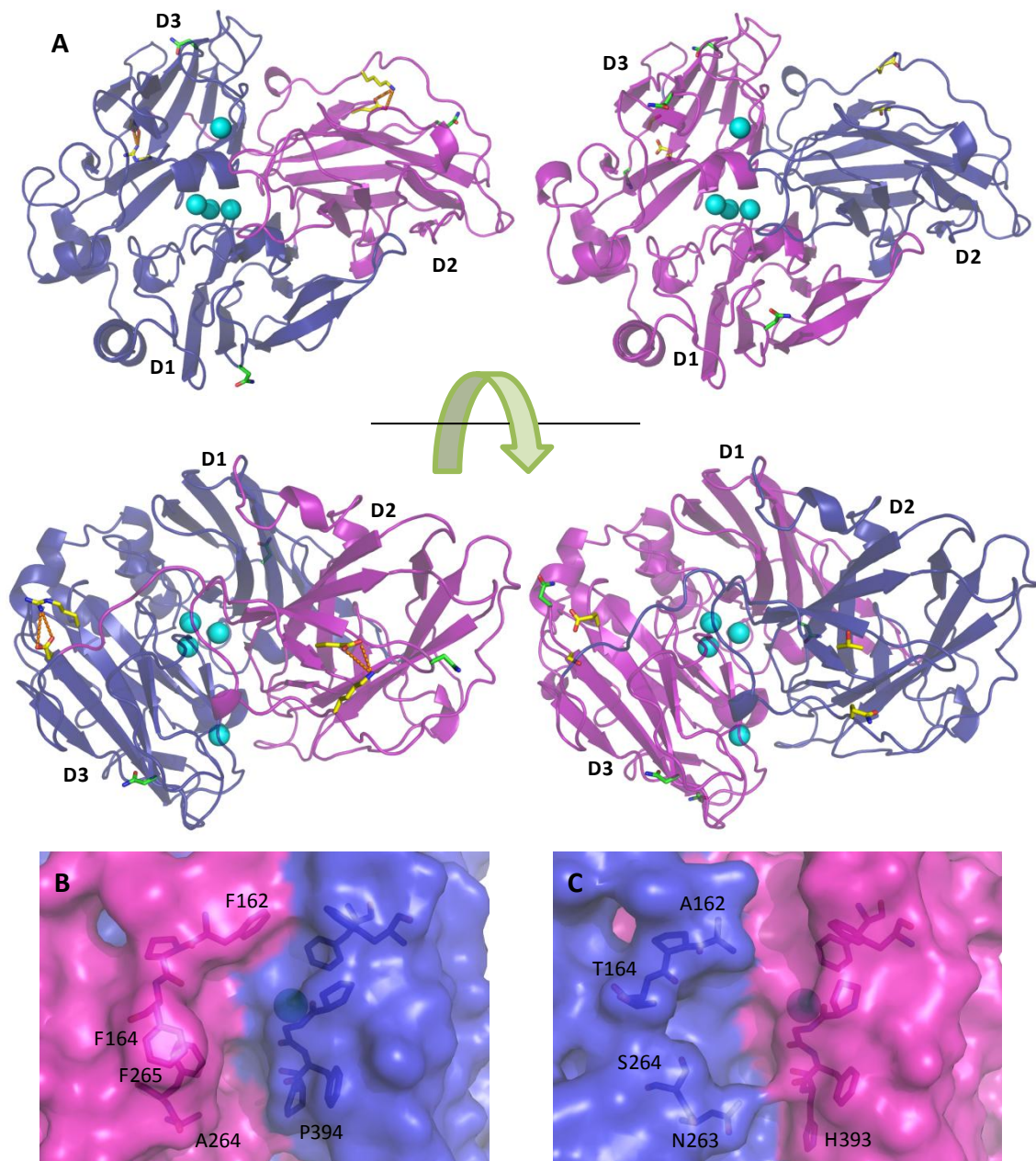


Figure 8. A) Structure models for domain-swap laccase variants OB1::3PO (left) and 3PO::OB1 (right). Putative N-glycosylation sites inherited from parent laccases are shown as green sticks. Residues establishing salt bridges (in OB1::3PO) selected for site-directed mutagenesis are shown as yellow sticks. Bottom panels show close-ups of the substrate binding pockets of B) OB1::3PO and C) 3PO::OB1 laccases. Residues delimiting the substrate binding pocket that differ between both laccases are labeled.

The new domain-swap laccases presented different properties respecting parent types, as has been previously described for other engineered chimeric laccases (Cusano et al., 2009; Nakagawa et al., 2010; Pardo et al., 2012; Bleve et al., 2014;). The pH activity profile was severely affected for swapped laccase 3PO::OB1, especially in the case of DMP oxidation. With an important decrease in activity at acidic pH, it presents a similar behavior to a chimeric laccase obtained by random

DNA shuffling of the same parent genes used in this work (Pardo et al., 2012). This chimera, namely 2C4, was in its greater part identical to OB1 parent, and it incorporated two small fragments from 3PO in D3. The shift in pH optimum was attributed to a small sequence block exchange in residues 394–421 (according to 3PO numbering), which included P394H mutation inherited from 3PO parent. P394H is located near the T1 Cu site, contiguous to the coordinating His395. This mutation appeared in the first generation during the directed evolution of Pcl, providing a 5-fold increase in total laccase activity at pH 5, and it was maintained in the following generations (Camarero et al., 2012). The presence of a basic His could change the local pK_a at the substrate binding site and, therefore, it can be concluded that this mutation is responsible for the observed shift towards more neutral pH values in chimeric laccases 2C4 and 3PO::OB1. However, it seems that residues from 3PO-D2, located in the surroundings of P394H in the protein's tridimensional structure, may buffer the effect of this mutation in such a way that the loss of activity at acidic pH is not so severe in the parent laccase 3PO, as opposed to chimeric laccases 2C4 and 3PO::OB1.

Mutation P394H could also be responsible for the important increase in K_m in 3PO::OB1 swapped laccase, which again is more pronounced in the case of DMP. Evolved variant 3PO and chimeric variant 2C4 also present high K_m values towards DMP (0.2 and 0.8 mM, respectively) when compared to their respective parents Pcl (0.01 mM) and OB1 (0.1 mM). Recently we evaluated the effect of mutation P394H in ABTS and DMP oxidation by means of molecular simulations in 3PO laccase (Monza et al., 2015). This mutation allowed an alternative DMP-laccase binding mode that could coexist simultaneously with the original binding mode found in the wild-type Pcl. It is possible that this could also be taking place in 3PO::OB1 and 2C4 variants. Interestingly, OB1::3PO variant on the other hand presented K_m values intermediate to those of 3PO and OB1 parents. It is worth noting that, due to the swapping of D2, the residues delimiting the entrance of the substrate binding pocket are exchanged between the new variants (**Fig. 8B, C**). However, taking into account the high K_m values (20 to 30-fold increment) observed for 3PO::OB1 variant respecting OB1, and the elevated number of mutations introduced, it is unlikely that the changes in affinity are merely caused by the substitutions in the residues delimiting the binding pocket alone. Indeed, directed evolution studies have demonstrated that even mutations located far from the active site can affect enzyme activity by altering the geometry, electrostatic properties or dynamics of the residues involved in substrate binding and catalysis (Tracewell and Arnold, 2009).

The most relevant feature obtained by domain swapping was a remarkable increase in the thermostability of OB1::3PO variant and also to acid pH and high concentrations of organic solvents and the ionic liquid BMIM otf. It should be noted that the mechanisms of protein inactivation by the aforementioned factors are

different. High temperature causes the breakage of hydrogen bonds and the deamination of Arg and Glu residues, while extreme pH leads to the titration of ionizable groups and therefore to the loss of electrostatic interactions between amino acids. On the other hand, organic solvents may strip water molecules from the protein surface that are needed to maintain the protein structure or, in the case of polar protic solvents, compete with these water molecules in the formation of hydrogen bonds with superficial polar residues. In accordance with the latter, we found that the extent to which our laccase variants were inactivated correlated with the higher tendency of the solvents to form hydrogen bonds (acetone < DMSO < ethanol < methanol) (Reichardt, 2002).

Several protein features determine its stability to extreme conditions of temperature, pH or organic solvents, mainly the burying of hydrophobic residues in the folded protein core, the formation of disulfide bonds and the occurrence of polar interactions in the protein surface, such as salt bridges and hydrogen bonding between amino acids or between amino acids and sugar moieties in glycoproteins (Christensen and Kepp, 2013). However, the extent to which each of these individual interactions contributes to total stability is unclear, depending largely on the protein studied.

It is known that the stabilization introduced by glycosylation is achieved basically by increasing the enthalpy of the unfolded protein state (Shental-Bechor and Levy, 2008). The introduction of the thermodynamic stabilization correlates with the degree of glycosylation but it also depends on the position of the glycosylation sites. Indeed, N-glycosylation sites may be more or less conserved amongst fungal laccases, and in particular those in Asn54 and Asn433 (the only ones present in OB1) are highly conserved. Therefore, these are considered to play an important role during nascent protein folding and protein stabilization (Christensen and Kepp, 2013; Kallio et al., 2011; Maestre-Reyna et al., 2015). Even so, the removal of glycosylation by site-directed mutagenesis has different effects depending on the position and the laccase under study. For example, when N-glycosylation site in Asn238 in laccase from *Lentinus* sp., which would be located in the same loop as Asn215 in OB1::3PO, was removed by site-directed mutagenesis, the activity was only 3% respecting that of wild-type (Maestre-Reyna et al., 2015), an effect which was not observed in OB1::3PO-N215G mutant obtained in the present work.

As with glycosylation, it is generally accepted that electrostatic interactions confer thermostability to proteins, and hyperthermostable proteins clearly confirm this trend (Jelesarov and Karshikoff, 2009). In the present study, however, the removal of salt bridges from OB1::3PO variant did not notably reduce its stability. Only when residues E232 and K297 (inherited from 3PO parent) were mutated, a slight decrease in T₅₀ (10 min) could be observed. At the same time, the attempt to

stabilize 3PO::OB1 variant by the introduction of charged residues that could form salt bridges in the same positions was unsuccessful. However, considering that the formation of salt bridges depends on many factors, including not only the distance between residues and their orientation, but also their degree of solvation and the local environment in which these residues are introduced (Makhatadze et al., 2003), we cannot assure that these new salt bridges were indeed formed.

Taking into account these results as a whole, it seems that the outstanding stability of OB1::3PO variant is due to new stabilizing interactions between amino acids introduced by domain swapping, that are difficult to pin-point by site-directed mutagenesis approaches. More complex calculations, such as those obtained by Molecular Dynamics simulations, could help find possible hot-spots for further evaluation. Nevertheless, considering that the stability was assayed by measuring residual activity after incubation under different conditions (e.g. T_{50}), it is likely that the loss of activity we observed in our experiments is due to small conformational changes located near the catalytic coppers, affecting catalysis but not the overall protein folding (*i.e.* T_m). In order to confirm this, unfolding kinetics would need to be determined by circular dichroism or fluorescence spectroscopy with purified laccase samples (Fernandes et al., 2012; Ferrario et al., 2015).

CONCLUSIONS

The introduction of a pool of neutral mutations accomplished by the exchange of cupredoxin domains between laccases rendered active enzymes with new properties, in particular a notable increment of stability in OB1::3PO variant that could not have been predicted by rational design. This makes OB1::3PO laccase a promising scaffold for future directed evolution approaches, as now it would be more tolerant to new random mutations in an uphill walk strategy.

MATERIAL AND METHODS

Generation of 3D-structure protein models

Protein models of swapped laccase variants were generated with Modeller in the UCSF Chimera graphical interface (Pettersen et al., 2004; Sali and Blundell, 1993). Crystal structures of laccases from *Trametes trogii* (PDB 2HRG, 97% sequence identity with OB1) and *P. cinnabarinus* (PDB 2XYB, 99% sequence identity with 3PO) were used as templates. Generated models with lowest Z-score were selected and visually inspected with PyMol Molecular Graphics System (Schrödinger, LLC).

Construction of domain-swap mutants and site-directed mutagenesis

General considerations regarding PCR conditions, purification of amplification products and *in vivo* cloning in competent *S. cerevisiae* BJ5465 cells have been

described previously (Pardo et al., 2012). The pJRoC30 plasmids carrying the 3PO (evolved PcL) and OB1 (evolved PM1L) constructions, fused to their respective evolved alpha-factor prepro-leaders, were obtained in previous works (Camarero et al., 2012; Maté et al., 2010). Primers with overhangs of 10–20 bp complementary to the adjacent cupredoxin domain from the different parents were used for the amplification of each domain (**Table S1, Fig. 1**). The PCR products were mixed in equimolar proportion for a reassembly PCR and subsequent amplification as described previously (Pardo et al., 2012). Cloning of the whole reassembled coding DNA sequences (CDS) into the pJRoC30 vector (previously linearized with NotI and BamHI) was done *in vivo* by designing overhangs which specifically anneal with the ends of the linearized vector (annealing regions of 40 bp and 66 bp). CDS products (400 ng) were co-transformed with linearized pJRoC30 vector (100 ng) into competent BJ5465 cells with the Yeast Transformation Kit (Sigma). Transformed cells were then grown on Synthetic Complete (SC) drop-out plates (6.7 g L⁻¹ YNB, 1.92 g L⁻¹ drop-out media supplements without uracil, 20 g L⁻¹ glucose, 20 g L⁻¹ agar, 25 mg L⁻¹ chloramphenicol) at 30 °C for 2–3 days, until colonies containing the whole autonomously replicating vector appeared. Plasmids were then extracted, amplified in DH5 α and purified for sequencing in order to confirm correct construction. Site-directed mutants were obtained by In Vivo Overlap Extension (Alcalde, 2010), designing complementary mutagenic primers with ~20 bp flanking the desired mutations (**Table S2**).

Flask production of chimeras

Single colonies from the yeast clones transformed with plasmids containing the different laccase variant genes were picked from SC drop-out plates, inoculated in 3 mL of minimal medium (6.7 g L⁻¹ YNB, 1.92 g L⁻¹ drop-out media supplements without uracil, 20 g L⁻¹ raffinose, 25 mg L⁻¹ chloramphenicol) and incubated for 48 hours at 30 °C and 220 rpm in duplicate. The OD₆₀₀ was then adjusted to 0.25 in 5 mL minimal medium in 100 mL flasks, which were incubated to OD₆₀₀ = 1. Thereafter, cells were diluted ten-fold in a final volume of 30 mL of expression medium (20 g L⁻¹ peptone, 10 g L⁻¹ yeast extract, 20 g L⁻¹ galactose, 60 mM phosphate buffer pH 6.0, 4 mM CuSO₄, 25 g L⁻¹ ethanol, 25 mg L⁻¹ chloramphenicol) in 100 mL flasks. Laccase activity in the culture supernatant was followed spectrophotometrically with 3 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, $\epsilon_{418} = 36000 \text{ M}^{-1} \text{ cm}^{-1}$) in 100 mM acetate buffer pH 5.0, defining one activity unit as the amount of enzyme needed to transform 1 μmol substrate/minute. When maximum activity was reached, cells were separated by centrifugation at 13000 rpm, 4 °C, and supernatants were concentrated in 10000 MWCO Amicon-Ultra Centrifugal filters (Millipore).

Characterization of chimeric laccases

- pH activity profiles:

96-well plates were prepared by adding 180 μL of 100 mM Britton and Robinson buffer at pH values of 2–9, 10 μL of laccase samples diluted to $\sim 0.1 \text{ U L}^{-1}$ and 10 μL of 60 mM ABTS or 2,6-dimethoxyphenol (DMP). Activities were measured in triplicate in kinetic mode in the plate reader at 418 nm for ABTS and at 469 nm for DMP ($\epsilon_{469} = 27500 \text{ M}^{-1} \text{ cm}^{-1}$). The relative activity was calculated based on the maximum activity in the assay for each variant.

- K_m values for ABTS and DMP:

Laccase activity over growing concentrations of substrate was followed in triplicate in kinetic mode in the plate reader. Reactions were carried out in a final volume of 250 μL in 100 mM acetate buffer, pH 5, by addition of 20 μL of laccase sample dilutions. To calculate the value of K_m , relative activity (referred to maximum activity in each assay) was represented vs substrate concentration and fitted to a single rectangular hyperbola function in SigmaPlot (Systat Software, San Jose, CA), where parameter b was equal to K_m .

- T_{50} (10 min) assay:

Concentrated laccase samples were diluted to $\sim 0.1 \text{ U mL}^{-1}$ and aliquots of 35 μL were transferred to 96-well PCR plates, which were sealed and incubated for 10 min in a gradient thermocycler with temperatures that ranged from 30 to 80 $^{\circ}\text{C}$. Samples were then chilled on ice for 10 min and incubated at room temperature for another 5 min. Residual activity was determined by transferring 20 μL to a 96-well plate and adding 180 μL of 3 mM ABTS in acetate buffer pH 5. Activities were measured in triplicate in kinetic mode in the plate reader.

- Long-term stability assays:

Concentrated laccase samples were diluted to $\sim 0.1 \text{ U mL}^{-1}$ in 100 mM Britton and Robinson buffer with pH values of 2–9 for pH stability assays, and in 20 mM Tris-HCl buffer pH 7 for temperature and solvent stability assays. In the solvent stability assay, ethanol, methanol, acetone, dimethyl sulfoxide (DMSO) or 1-butyl-3-methylimidazolium trifluoromethanesulfonate (BMIM otf) were added to a final concentration of 20, 30, 40 or 50% (v/v). Laccase aliquots of 40 μL were transferred to 96-well plates, sealed and incubated for 6 hours at room temperature for pH and solvent stability assays and at 30, 40, 50, 60 and 70 $^{\circ}\text{C}$ in the thermocycler for the temperature stability assays. In the case of stability assay with BMIM otf, plates were under constant agitation during incubation time to assure homogenous mixture of the sample. After 6 hours, 20 μL aliquots were used to measure residual activities in

triplicate in kinetic mode in the plate reader, after adding 180 μ L of 3 mM ABTS in acetate buffer pH 5.

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SUPPLEMENTARY MATERIAL

Table S1. Primers used for construction of swapped laccases and for amplification of reassembled products. Sequences annealing with pJRoC30 are shown in italics, with 3PO underlined, and with OB1 in normal font. PCR indicates reactions in which the different primers are used according to the scheme shown in Fig. 1.

Primer	Sense/Antisense	5' → 3' Sequence	PCR
extpJR-F	Sense	<i>CTGGGGTAATTAATCAGCGAAGC</i>	1, 1'
extpJR-R	Antisense	<i>CCAAAACCTTCTCAAGCAAGG</i>	3, 3'
D1-OB1 rev	Antisense	<u>GTGAGGATCGTT</u> AGGGTCATAGACAAC	1
D2-3PO fwd	Sense	GTCTATGACCCTA <u>ACGATCCTCACGC</u>	2
D2-3PO rev	Antisense	GTCGGGTGCC <u>AGGCACAGGCATAG</u>	2
D3-OB1 fwd	Sense	<u>CCTGTGCCTGGCAACCCGACCC</u>	3
D1-3PO rev	Antisense	GTGGGGATCTTGGGGT <u>CGTAGACGAC</u>	1'
D2-OB1 fwd	Sense	<u>TCGTCTACGACCCCAAGATCCCCACAAG</u>	2'
D2-OB1 rev	Antisense	<u>CTCGGGCTGCCGGGCGCAG</u>	2'
D3-3PO fwd	Sense	<u>CTGCGCCCGGCAGCCCGAG</u>	3'
RMLN	Sense	<i>CCTCTATACTTTAACGTC AAGG</i>	4, 4'
RMLC	Antisense	<i>GGGAGGGCGTGAATGTAAGC</i>	4, 4'

Table S2. Primers used for site-directed mutagenesis by IVOE. Mutated codons are shown in bold and underlined. RMLN was used as sense primer for reactions with mutagenic antisense primers and RMLC was used as antisense primer for reactions with mutagenic sense primers.

Primer	Sense/ Antisense	5' → 3' Sequence
mut3PON215G-F	Sense	GCCATACATTGATGAT <u>GGT</u> CACACAATGACTATAATTGAGGC
mut3PON215G-R	Antisense	GCCTCAATTATAGTCATTGTGTG <u>ACC</u> ATCAATGCTGAATGTATGGC
OB1_3PO-E232T-F	Sense	GGACTCGATCAACACTCAACCCCTA <u>ACT</u> GTTGATTCAATCCAGATTTTTCG
OB1_3PO-E232T-R	Antisense	GCAAAAATCTGGATTGAATCAAC <u>AGT</u> TAGGGGTTGAGTGTGATCGAGTCC
OB1_3PO-K298Q-F	Sense	CGTCTGTCCAGACTACTCCTACG <u>CAG</u> CCTCTGAACGAGGTCGACTTGC
OB1_3PO-K298Q-R	Antisense	GCAAGTCGACCTCGTTCAGAGG <u>CTG</u> CGTAGGAGTAGTCTGGACAGACG
OB1_3PO-D379S-F	Sense	CGCTCCCTGCGAACCGG <u>TCC</u> ATTGAGATCTCCCTCCCCGC
OB1_3PO-D379S-R	Antisense	GCGGGGAGGAGATCTCAAT <u>GGAC</u> GCGTTCGCAGGGAGCG
OB1_3PO-R440E-F	Sense	GGACAACGTCACGATCCGGTTC <u>GAG</u> ACGGACAACCCCGGCCCG
OB1_3PO-R440E-R	Antisense	CGGGCCGGGTTGTCCGT <u>CTC</u> GAACCGGATCGTGACGTTGTCC
3PO_OB1-T231E-F	Sense	GGCGTGAATCTCAAGCCCAG <u>GAG</u> GTCGACTCCATCCAGATCTTCC
3PO_OB1-T231E-R	Antisense	GGAAGATCTGGATGGAGTCGAC <u>CTC</u> CTGGGGCTTGAGATTCACGCC
3PO_OB1-Q297K-F	Sense	CCACGACCCAGACCGGTCGACG <u>AAG</u> CCTTTGGTGGAGTCCGC
3PO_OB1-Q297K-R	Antisense	GCGGACTCCACCAAAGG <u>CTT</u> CGTCGACGGCGTCTGGGTCGTGG
3PO_OB1-S378D-F	Sense	CGTCTTCCAGCAACTCG <u>GAC</u> ATTGAGATATCCTTCCCTGCC
3PO_OB1-S378D-R	Antisense	GGCAGGAAGGATATCTCAAT <u>GTC</u> CGAGTTGCTGGGAAGAACG
3PO_OB1-E339R-F	Sense	CGACAACGTCACGATTCGCTTC <u>AGG</u> ACCAATAACCCAGGCCCG
3PO_OB1-E339R-R	Antisense	CGGGCCTGGGTTATTGGT <u>CCT</u> GAAGCGAATCGTGACGTTGTCC

Table S3. Putative surface salt bridges in parent and swapped laccases as predicted by PyMol. “<5 Å” indicates amino acid pairs for which PyMol does not predict hydrogen bonding but whose sidechains are less than 5 Å apart. The two acid-basic pairs inherited by OB1::3PO (and lost in 3PO::OB1) are highlighted.

OB1			3PO			OB1::3PO			3PO::OB1		
ACID	BASIC	Bonded	ACID	BASIC	Bonded	ACID	BASIC	Bonded	ACID	BASIC	Bonded
D96	R43	YES	D96	R43	YES	D96	R43	YES	D96	R43	YES
D118	R22	YES	D118	R22	YES	D118	R22	YES	D118	R22	YES
D128	K40	<5 Å	D128	K40	<5 Å	D128	K40	<5 Å	D128	K40	<5 Å
D131	R196	YES	D131	R197	YES	D131	R197	YES	D131	R196	YES
D138	R194	YES	D138	R195	NO	D138	R195	NO	D138	R194	<5 Å
D140	R198	YES	D140	R199	YES	D140	R199	YES	D140	R198	YES
D213	R259	YES	D214	R260	YES	D214	R260	YES	D213	R259	YES
D223	R422	YES	D224	R423	YES	D224	R423	YES	D223	R422	YES
T231	Q297	NO	E232	K298	<5 Å	E232	K298	<5 Å	T231	Q297	NO
E287	R175	YES	E288	R176	YES	E288	R176	YES	E287	R175	YES
D378	R439	YES	S379	E440	NO	D379	R440	YES	S378	E439	NO
E380	R437	YES	E381	R438	YES	E381	R438	YES	E380	R437	YES
D423	R242	YES	D424	R243	YES	D424	R243	YES	D423	R242	YES
E457	K157	NO	D458	K157	<5 Å	D458	K157	<5 Å	E457	K157	NO
D495	K59	YES	D496	K59	YES	D495, D496	K59	YES	D495	K59	<5 Å

DISCUSIÓN GENERAL

1. Obtención de lacasas quiméricas

En trabajos previos a esta tesis doctoral se ha descrito la evolución dirigida de dos lacasas fúngicas de alto potencial redox (HRPL), procedentes de los basidiomicetos PM1 (PM1L) y *Pycnoporus cinnabarinus* (PcL) (Maté et al., 2010; Camarero et al., 2012). Las variantes evolucionadas de estas lacasas, denominadas OB1 y 3PO respectivamente, se expresan heterológamente en *Saccharomyces cerevisiae* de manera efectiva, mantienen la alta estabilidad de las lacasas nativas y presentan una alta eficiencia catalítica. Además, durante la evolución dirigida de PcL, el pH óptimo de actividad se fue ampliando hacia valores más neutros generación tras generación, aunque de manera destacada la eficiencia catalítica mejoró no sólo al pH al que se realizaron los ensayos de HTS (pH 5) sino también al pH óptimo de la enzima salvaje (pH 3). La evolución dirigida de ambas lacasas fue llevada a cabo de forma paralela, lo cual permitió descubrir similitudes entre las mutaciones acumuladas en las variantes OB1 y 3PO (tanto en el péptido señal del factor alfa como en la lacasa madura) y estudiar, mediante mutagénesis sitio-dirigida, el efecto de intercambiar mutaciones entre ellas. Con el fin de obtener nuevas variantes de lacasa que combinaran o mejoraran las propiedades de interés de OB1 y 3PO, se planteó como objetivo de esta tesis doctoral la construcción de lacasas quiméricas. Su diseño nos ha permitido a su vez profundizar en el estudio de los determinantes estructurales que modulan la actividad de estas enzimas.

1.1. Estrategias de quimeragénesis aleatoria y racional

En un primer momento se optó por una estrategia de quimeragénesis al azar mediante la combinación del barajado *in vitro* e *in vivo* (CLERY, Abécassis et al., 2000) de los genes de OB1 y 3PO. La mayoría de las quimeras seleccionadas eran más parecidas al parental OB1 que al parental 3PO, lo cual podría deberse a que el primero es expresado de manera más eficiente por la levadura. Los eventos de recombinación tuvieron lugar en el péptido señal y en el primer y tercer dominio cupredoxina (D1 y D3), concretamente alrededor de los motivos de secuencia conservados (L1–L4) donde se encuentran los ligandos de los cobres catalíticos. La falta de eventos de recombinación en el segundo dominio cupredoxina puede explicarse por la menor identidad de secuencia nucleotídica entre ambos genes en el D2 (67%) respecto al D1 y D3 (75%), un sesgo común en los métodos de recombinación de DNA dependientes de secuencia (Chaparro-Riggers et al., 2007; Joern et al., 2002). Los segmentos de secuencia intercambiados tenían un tamaño de 3–93 pares de bases, dando lugar a la introducción de 3–31 sustituciones aminoacídicas totales en las secuencias de las lacasas maduras. De la batería de lacasas quiméricas obtenidas, se seleccionaron cinco de ellas (2C4, 3A4, 6D9, 7A12 y 7D5) como posibles puntos de partida para estudios posteriores de evolución dirigida hacia distintos fines, en base a su termoestabilidad (similar o mayor que la

de los parentales OB1 y 3PO) y a presentar perfiles de pH óptimo diversos (**Fig. 1A-E**). Posteriormente, se construyeron nuevas lacasas quiméricas mediante el intercambio del D2 entre OB1 y 3PO con el fin de evaluar el efecto de introducir un mayor número de mutaciones. De este modo, se obtuvieron dos nuevas lacasas que presentaban 65 sustituciones aminoacídicas cada una (**Fig. 1F-G**). 3PO::OB1 presentaba un importante desplazamiento del perfil de pH óptimo, mientras que OB1::3PO presentaba un inesperado incremento de estabilidad.

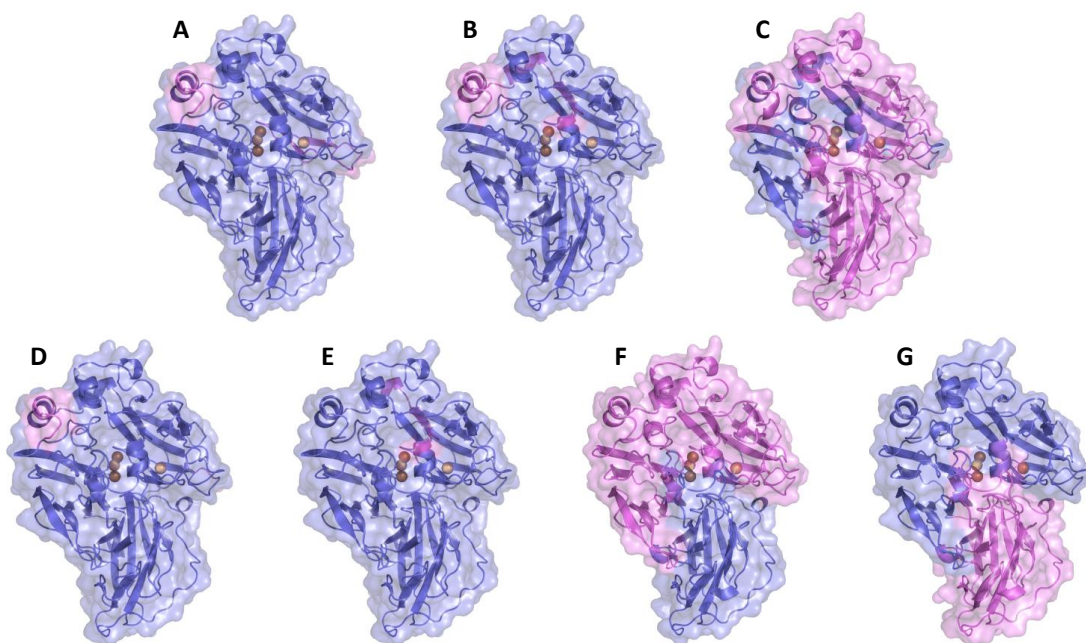


Figura 1. Modelos tridimensionales de las quimeras de CLERY **A)** 2C4, **B)** 3A4, **C)** 6D9, **D)** 7A12 y **E)** 7D5; y de intercambio de dominios **F)** 3PO::OB1 y **G)** OB1::3PO. Los fragmentos heredados de los parentales OB1 y 3PO se muestran en azul y magenta, respectivamente.

1.2. Expresión heteróloga de las lacasas quiméricas en levadura

Las lacasas parentales OB1 y 3PO tienen distintas condiciones óptimas de producción en *Saccharomyces cerevisiae*. Mientras que OB1 alcanza la máxima producción en cultivo tras 3–4 días a 30 °C (~800 U L⁻¹), 3PO se produce mejor a los 5–6 días a 20 °C (~350 U L⁻¹). En cuanto a las lacasas quiméricas obtenidas por CLERY, por lo general éstas se producían mejor a 20 o a 30 °C en función de si eran más parecidas a los parentales 3PO u OB1, respectivamente. Cabe destacar que entre los ganadores obtenidos por CLERY había varias secuencias que presentaban eventos de recombinación en el péptido señal pero mantenían la secuencia de la lacasa madura del parental OB1. Sin embargo, a la hora de aumentar la escala de producción a matraz, ninguno mejoró la actividad máxima conseguida por OB1. Por otro lado, ambos mutantes obtenidos por intercambio de dominios se producían mejor a 20 °C que a 30 °C, a pesar de que heredaban íntegramente los péptidos señal de los parentales OB1 (en el caso de OB1::3PO) y 3PO (en el caso de 3PO::OB1).

Estos resultados sugieren que la secreción de las HRPLs en levadura depende tanto de las secuencias del péptido señal como de la proteína madura, debiendo optimizarse las condiciones de producción para cada caso. Consecuentemente, el diseño de un péptido señal universal para la secreción eficiente de lacasas fúngicas en la levadura, inicialmente concebido por las similitudes encontradas en los péptidos señales evolucionados de OB1 y 3PO, es improbable. En este sentido, la evolución dirigida focalizada sobre el péptido señal puede ser una buena aproximación para mejorar la expresión de una proteína concreta de interés (Rakestraw et al., 2009; Viña-Gonzalez et al., 2015). Esta estrategia se está empleando actualmente en el caso de la variante de intercambio de dominios OB1::3PO, que presenta características deseables para su aplicación pero que se produce a muy bajos niveles en levadura.

1.3. Actividad de las lacasas quiméricas

Entre las lacasas quiméricas obtenidas tanto por CLERY como por intercambio de dominios se observaron perfiles de pH óptimo de actividad y afinidades por sustrato distintos de los de los parentales. Sorprendentemente, estas diferencias se daban incluso en quimeras que habían intercambiado fragmentos de secuencia relativamente pequeños, muchos de ellos en la zona C-terminal de la proteína. La comparación de los eventos de recombinación encontrados en las lacasas quiméricas permite obtener algunas conclusiones sobre el efecto de determinadas mutaciones sobre la actividad de estas enzimas.

En primer lugar, las quimeras 3A4, 4A11 y 7D5 (las dos primeras idénticas a nivel de secuencia de lacasa madura) presentan perfiles de pH óptimo más ácidos que ambos parentales y mejor afinidad hacia DMP (valores de K_m ~0.5 veces la de OB1). Estas quimeras son en su mayor parte como OB1 pero presentan segmentos de secuencia de 3PO en el extremo C-terminal. En la **Tabla I** se muestra un alineamiento de secuencia del C-terminal de estas quimeras y de los parentales. Se observa que, debido a los eventos de recombinación, las quimeras 3A4, 4A11 y 7D5 tienen en común las mutaciones E457D, T461A y T468I respecto a OB1. Cabe destacar que la mutación T461A revierte una mutación seleccionada en la segunda generación de la evolución dirigida de PM1L (A461T) que se mantuvo en las generaciones siguientes (Maté et al., 2010).

Tabla I. Secuencias del C-terminal de los parentales OB1 y 3PO y de las quimeras obtenidas por CLERY 3A4, 4A11 y 7D5. En magenta se muestran los residuos que actúan como ligandos de los cobres catalíticos, y en azul y morado los residuos que difieren entre OB1 y 3PO, respectivamente.

OB1	⁴⁴⁹ HCHIDF ⁴⁹⁶ HLEAGFTVVMAEDIPDVAATNPVPQAWS ⁴⁹⁶ DLCPTYDALSPDDQ ⁴⁹⁶
3PO	⁴⁵⁰ HCHIDF ⁴⁹⁷ HLDAGFAVVMAEDTPD ⁴⁹⁷ TKAANPVPQAWS ⁴⁹⁷ DLCPIYDALDPSDL ⁴⁹⁷
3A4/4A11	⁴⁴⁹ HCHIDF ⁴⁹⁶ HLDAGFAVVMAEDTPD ⁴⁹⁶ TKAANPVPQAWS ⁴⁹⁶ DLCPIYDALDPSDL ⁴⁹⁶
7D5	⁴⁴⁹ HCHIDF ⁴⁹⁶ HLDAGFAVVMAEDTPDVAATNPVPQAWS ⁴⁹⁶ DLCPTYDALSPDDQ ⁴⁹⁶

En el extremo opuesto se encuentran las quimeras 2C3, 6D9 y 6F11. Estas son en su mayor parte similares a 3PO y presentan perfiles de pH óptimo ampliados respecto al de los parentales. Por otro lado, 2C4 y 3PO::OB1 presentan perfiles de pH óptimo más estrechos y desplazados hacia valores más básicos, con máximos de actividad muy marcados a pH 5, sobre todo en el caso de DMP. Las variantes 2C4 y 3PO::OB1 presentaban además un importante aumento de la K_m hacia DMP (10–20 veces) respecto a sus parentales más cercanos (OB1 y 3PO, respectivamente). Todas las quimeras mencionadas, con un importante aumento de la actividad relativa a pH 5, tienen en común la mutación P394H heredada del parental 3PO. Esta mutación fue seleccionada en la primera generación de la evolución dirigida de Pcl y se mantuvo en todas las generaciones siguientes (Camarero et al., 2012). Sin embargo, las diferencias en los perfiles de pH óptimo entre 3PO, 2C3, 6D9 y 6F11 (más amplios) en comparación con 2C4 y 3PO::OB1 (más estrechos) indican que esta mutación tiene distintos efectos según los residuos de su entorno (**Tabla II**), que pueden evitar la pérdida de actividad a pH ácido que se observa en las dos últimas quimeras.

Tabla II. Residuos del entorno (< 5 Å) de P394H (o P393H) en el parental 3PO y las quimeras 2C3, 6D9, 6F11, 2C4 y 3PO::OB1. En colores, los residuos que difieren entre los parentales OB1 (azul) y 3PO (morado). Entre paréntesis, residuos a > 5 Å de la posición 394/393.

3PO	2C3	6D9	6F11	2C4	3PO::OB1
S208	S208	S208	S208	N207	N207
Q237	Q237	Q237	Q237	Q236	Q236
F239	F239	F239	F239	F238	F238
(A264)	(A264)	(A264)	(A264)	N263	N263
G392	G392	G392	G392	G391	G391
F393	F393	F393	F393	F392	F392
H395	H395	H395	H395	H394	H394
P396	P396	P396	P396	P395	P395
S427	S427	S427	N427	N426	S426
T428	T428	T428	T428	T427	T427
G429	G429	G429	G429	G428	G428
Q430	Q430	S430	S430	S429	Q429
I453	I453	I453	I453	I452	I452

Los únicos residuos del entorno de la mutación P394H (o P393H) que diferencian a 2C4 y 3PO::OB1 del resto son N207 y N263, heredados del parental OB1 y localizados en el D2 (**Fig. 2**). El primero se corresponde con S208 en 3PO, que fue mutado (N208S) en el tercer ciclo de evolución dirigida de Pcl. Por otro lado, N263 se corresponde con A264 en 3PO y las demás quimeras, que al ser un residuo apolar más pequeño no llega a interactuar con P394H. Además, cabe destacar que los residuos del D2 que rodean a N263/A264 son muy variables entre 2C4 y 3PO::OB1 y las demás variantes, por lo que también podrían influir en el efecto que ejerce la mutación P394H sobre la actividad de estas quimeras.

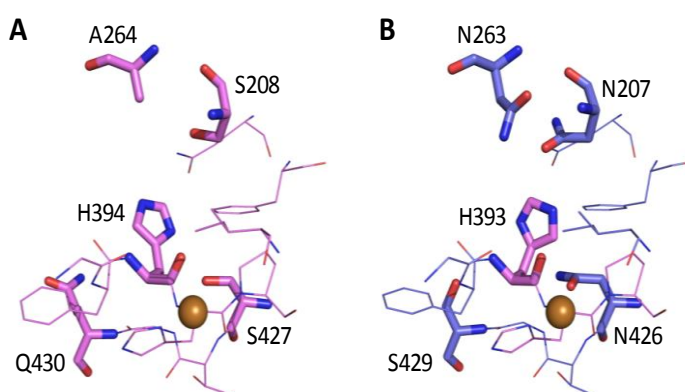


Figura 2. Detalle del entorno ($< 5 \text{ \AA}$) de la mutación P394H (o P393H) **A**) en el parental 3PO y **B**) en la quimera 2C4, respectivamente. Los residuos heredados de los parentales OB1 y 3PO se muestran en azul y magenta, respectivamente. Aquellos que son distintos entre 3PO y 2C4 se muestran como barras. El Cu T1 se muestra como una esfera.

El efecto de la mutación P394H en el desplazamiento del perfil de actividad de la enzima se ha evidenciado en experimentos realizados en el laboratorio de forma complementaria a esta tesis doctoral. Se introdujo la mutación P394H en α^* -PcL, observándose que el mutante α^* -PcL P394H presentaba un perfil de pH óptimo notablemente menos ácido que la enzima nativa PcL y similar al de 3PO (**Fig. 3A**). Además, los valores de k_{cat} y K_{m} para DMP también eran intermedios entre los de la lacasa nativa y la evolucionada (datos no publicados). El efecto de esta mutación se ha estudiado también mediante simulaciones computacionales de PELE y QM/MM (Monza et al., 2015). Éstas revelaron que la mutación P394H, junto con la mutación N208S, permitía un modo de unión alternativo del DMP al bolsillo enzimático que podía coexistir con el observado en PcL, lo cual explicaría los incrementos de K_{m} observados. Además, se apreciaba un aumento de la transferencia de *spin* entre el sustrato y el Cu T1, justificando la mejora de k_{cat} obtenida en 3PO (**Fig. 3B-C**).

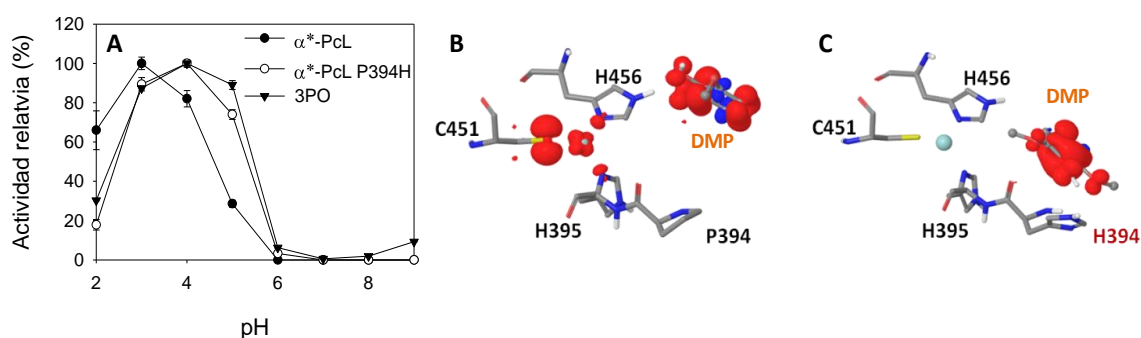


Figura 3. **A**) Perfil de pH óptimo para la oxidación de DMP de α^* -PcL, α^* -PcL P394H y 3PO. **B-C**) Resultados de las simulaciones computacionales para la oxidación del DMP por parte de **B**) PcL y **C**) 3PO.

1.4. Estabilidad de las lacasas quiméricas

Además de los cambios en actividad observados en las lacasas quiméricas, se obtuvieron también mejoras considerables en termoestabilidad. Es el caso de las quimeras 2C4, 3A4, 7A12 y 7D5, obtenidas por CLERY, y de la quimera de intercambio de dominios OB1::3PO, que presentan valores de T_{50} de entre 2 y 7 °C mayores que el más estable de los parentales (OB1). Las cuatro quimeras obtenidas por CLERY, todas ellas con mayor identidad de secuencia con OB1, tienen en común haber sufrido eventos de recombinación en el C-terminal. El extremo C-terminal suele ser una zona lábil en la estructura de las proteínas, por lo que se esperaría que algunos de los residuos procedentes de 3PO e introducidos por recombinación aleatoria diesen lugar a nuevas interacciones estabilizantes. De hecho, las estructuras cristalográficas de las lacasas silvestres Pcl (PDB 2XYB) y PM1L (PDB 5ANH) indican que el C-terminal de esta última es más flexible (mayor *B-factor*). Varios trabajos han demostrado de forma experimental y mediante simulaciones de dinámica molecular la importancia del C-terminal en la estabilidad de las lacasas de basidiomicetos, siendo la zona más propensa a desestructurarse a altas temperaturas (Autore et al., 2009; Blevé et al., 2013; Christensen y Kepp, 2013; Ferrario et al., 2015; Festa et al., 2008). Sin embargo, llama la atención que los modelos estructurales de las quimeras no indican que haya un mayor número de puentes de hidrógeno entre los residuos del C-terminal y/o residuos adyacentes por efecto de la recombinación respecto a OB1 (**Fig. 4**). Esto hace suponer que las mutaciones introducidas con la recombinación puedan tener un efecto secundario en otras zonas de la proteína que dé lugar a una mayor estabilidad cinética, sin afectar necesariamente a su plegamiento global.

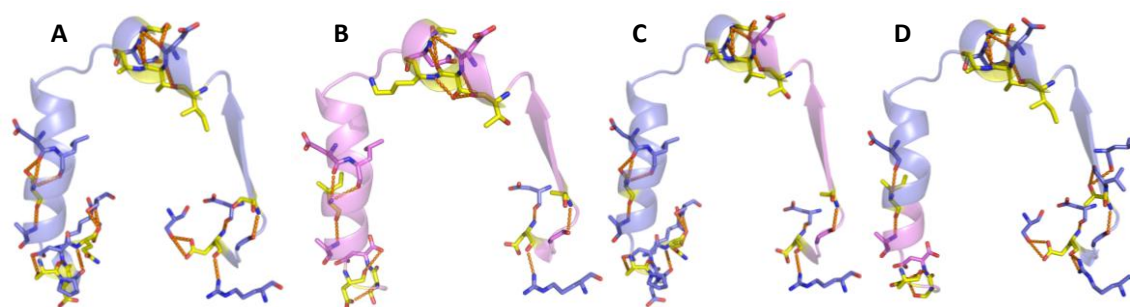


Figura 4. Detalle del extremo C-terminal de los modelos tridimensionales de A) el parental OB1 y las quimeras B) 3A4, C) 7D5 y D) 7A12. El último residuo de 3A4 y 7A12 (Leu496) no pudo ser modelado. En azul se muestran los residuos heredados de OB1 y en magenta los de 3PO. En amarillo se indican los residuos que difieren entre las dos lacasas. Los posibles puentes de hidrógeno entre los residuos del C-terminal y/o residuos adyacentes se muestran en naranja.

Por otro lado, el estudio más en profundidad de la lacasa de intercambio de dominios OB1::3PO tampoco llevó a dilucidar los determinantes estructurales responsables de la notable estabilidad cinética de esta enzima. Ni el nuevo sitio de glicosilación respecto a OB1 ni los dos puentes salinos extras respecto a 3PO::OB1

parecen los responsables últimos del aumento en termoestabilidad, si bien debido al gran número de mutaciones introducidas es difícil analizar todas las posibles nuevas interacciones experimentalmente. Estos resultados ponen de manifiesto la dificultad para diseñar enzimas más estables mediante mutaciones puntuales (Eijsink et al., 2004), ya que la estabilidad de las proteínas se debe al conjunto de muchos factores. El estudio computacional de estas enzimas, con análisis de dinámica molecular, puede resultar de gran ayuda para esclarecer este asunto y avanzar en el diseño racional de lacasas altamente estables. Para ello, sería de gran interés la resolución de la estructura cristalográfica de estas lacasas quiméricas, para lo cual se hace necesaria una mayor producción. Con este fin, una de las quimeras estables obtenidas por CLERY ya ha sido producida a mayor escala en *Aspergillus oryzae* y está siendo cristalizada.

Por último, debe resaltarse que una alta termoestabilidad no implica necesariamente una mayor estabilidad de la proteína a otros factores, ya que, tal y como se comenta en el capítulo 5 de esta tesis, los mecanismos de inactivación/desnaturalización en condiciones de altas temperaturas, pH extremo o presencia de solventes orgánicos difieren. Por ejemplo, el parental OB1 es muy sensible a la presencia de solventes orgánicos a pesar de ser el más termoestable de los parentales. Sin embargo, destaca que la quimera OB1::3PO es también bastante estable a pH y solventes orgánicos, lo cual refuerza la idea de que su mayor estabilidad cinética no se debe a un único factor, sino al conjunto de nuevas interacciones que se establecen con el intercambio de dominios. Cabe mencionar además que las quimeras de CLERY con T_{50} mayor que la de los parentales por lo general también resultaron tener mayor estabilidad en otras condiciones (datos no publicados). El hecho de que las lacasas quiméricas sean estables en diversas condiciones extremas es un ejemplo de cómo la deriva genética, mediante la introducción de mutaciones neutras, puede dar lugar a variantes más robustas y, en principio, más evolucionables (Bloom et al., 2006; Bloom y Arnold, 2009). Por tanto, las lacasas quiméricas obtenidas en estos estudios constituyen excelentes puntos de partida para la ingeniería de lacasas hacia nuevos objetivos.

2. Ingeniería de lacasas para mejorar el tratamiento de la biomasa lignocelulósica

Las lacasas y los sistemas lacasa-mediador encuentran un gran nicho de aplicación en las biorefinerías lignocelulósicas. Sin embargo, es conveniente ajustar las características de estas enzimas a las condiciones del proceso, que en muchos casos no coinciden con las condiciones óptimas para que se produzca la catálisis enzimática. Las lacasas quiméricas obtenidas en el laboratorio presentan una alta estabilidad y distintos perfiles de actividad que las hacen interesantes como puntos de partida para la aplicación de lacasas de alto potencial redox en distintos procesos.

Por ejemplo, para la destoxificación de la biomasa procedente de un pretratamiento físico-químico con H_2SO_4 o SO_2 se requieren enzimas con un perfil ácido de pH óptimo (Jurado et al., 2009), mientras que para el blanqueo de pasta de papel o para el tratamiento de efluentes de la industria textil son de interés lacasas con un perfil básico (Abadulla et al., 2000; Ibarra et al., 2006).

En el caso concreto de la destoxificación de la biomasa pretratada para la producción de bioetanol, se ha demostrado que el tratamiento con lacasa, bien tras la hidrólisis enzimática de los polisacáridos o en un paso de hidrólisis y fermentación simultáneas, aumenta el rendimiento de la fermentación y los niveles de producción final de etanol, permitiendo además trabajar con cargas de sustrato más altas (Alvira et al., 2013; Moreno et al., 2012). Esto se consigue gracias a la oxidación y polimerización de los inhibidores fenólicos liberados durante el pretratamiento que afectan al crecimiento del microorganismo fermentativo (Adeboye et al., 2014; Jurado et al., 2009; Klinke et al., 2004). Además, algunos de estos compuestos fenólicos pueden actuar como mediadores redox de las lacasas, por lo que serían de aplicación en este u otros procesos industriales. Así, uno de los objetivos de esta tesis fue la obtención de lacasas con actividad mejorada sobre mediadores fenólicos derivados de la lignina, para lo cual se desarrollaron nuevos ensayos de *screening* y se empleó una estrategia semi-racional para re-diseñar el bolsillo de unión a sustrato.

2.1. Desarrollo de nuevos ensayos de HTS

Un factor crucial para la ingeniería de enzimas mediante evolución dirigida es la disponibilidad de ensayos de detección de actividad enzimática en formato *high-throughput* que permitan la exploración sistemática de miles de clones y que sean sencillos, sensibles y reproducibles. En esta tesis se han desarrollado nuevos ensayos colorimétricos de HTS para la exploración de librerías de mutantes de lacasas basados en la oxidación de fenoles relacionados con las unidades de tipo siringilo (S) de lignina (ácido sinápico, siringaldehído y acetosiringona), del mediador sintético ácido violúrico y de colorantes orgánicos sintéticos con cromóforos de tipo azo (naranja de metilo y azul de Evans) o antraquinona (azul brillante de Remazol).

Los compuestos fenólicos de tipo S empleados para estos ensayos han sido descritos como mediadores redox de lacasa, presentando varias ventajas sobre los mediadores sintéticos empleados habitualmente: en primer lugar, pueden obtenerse a bajo coste a partir de los residuos de lignina generados en los procesos de producción de pastas de papel o de bioetanol de segunda generación; segundo, no generan residuos tóxicos; y, por último, debido a su bajo potencial redox (~ 500 mV), pueden ser oxidados por lacasas de bajo, medio y alto potencial redox. Además, los mediadores fenólicos de tipo S seleccionados presentan la ventaja sobre otro tipo de

fenoles de que generan productos de oxidación coloreados, resultantes del acoplamiento de los radicales fenoxilo (Lacki y Duvnjak, 1998; Rosado et al., 2012).

En el caso concreto de las lacasas de alto potencial redox, resulta interesante disponer de ensayos HTS que permitan verificar que su alto E° se mantiene durante la evolución, ya que los factores que lo modulan son variados y una única mutación en el entorno del Cu T1 puede afectarlo (Cambria et al., 2012). Teniendo esto en cuenta, se diseñó un ensayo con el mediador sintético ácido violúrico como sustrato ($E^\circ = +1.1$ V), que únicamente puede ser oxidado por lacasas de alto potencial redox (Li et al., 1999). Por último, los colorantes sintéticos naranja de metilo, azul de Evans y azul brillante de remazol se escogieron como sustratos para los ensayos de HTS en base a su estructura química (los azoicos y los antraquinoides son los más empleados en la industria textil). Estos colorantes también son oxidados exclusivamente por lacasas de alto potencial redox, por lo que los ensayos de HTS podrían ser útiles para la ingeniería de estas enzimas para el destintado de fibras y el tratamiento de efluentes de textiles (Drumond Chequer et al., 2011). Además, se podrían utilizar en ensayos acoplados para detectar la capacidad como mediadores redox de nuevos compuestos cuya oxidación con lacasa no genere un producto coloreado, como se ha demostrado con fenoles de tipo H como el ácido *p*-cumárico, un mediador de origen natural (Cañas et al., 2007).

2.2. Diseño semi-racional del bolsillo de unión a sustrato

La lacasa quimérica 3A4, obtenida por CLERY, fue seleccionada como punto de partida para mejorar la oxidación de compuestos fenólicos derivados de la lignina debido a su alta estabilidad y a su mayor afinidad hacia DMP. En esta ocasión se empleó una estrategia semi-racional, aplicando mutagénesis saturada iterativa (ISM, Reetz y Carballeira, 2007) sobre seis residuos del bolsillo de unión a sustrato: A162 y T164 (librería A), N263 y S264 (librería B) y P390 y F392 (librería C). Para la exploración de las librerías de mutantes se empleó el método de HTS descrito en el capítulo 2 basado en la oxidación del ácido sinápico a pH 5. Además de su capacidad de actuar como mediador redox de lacasa, el ácido sinápico es un compuesto de interés biotecnológico por ser un posible precursor de las unidades siringilo de la lignina, por encontrarse estableciendo puentes entre la lignina y la hemicelulosa en plantas herbáceas, y por la actividad biológica (antioxidante, antimicrobiana y antitumoral) del propio ácido sinápico y de sus productos de oxidación (Camarero et al., 2008; Nićiforović y Abramovič, 2014; Yamauchi et al., 2002).

Los paisajes de actividad de las tres librerías mutagénicas mostraron diferencias en la tolerancia de cada región para aceptar mutaciones, siendo la C la más plástica y la B la más restrictiva. Además, el análisis de las mutaciones seleccionadas indicaba algunas tendencias: la posición 162 siempre se mutaba (normalmente por residuos básicos), la posición 263 no se mutaba nunca, la posición 390 también tendía a no

mutarse y la posición 392 se mutaba siempre. Como ensayos adicionales de termoestabilidad revelaron que la mutación del residuo P390 daba lugar a variantes más inestables, en la primera generación se seleccionó el mutante C14F12, que únicamente presentaba la mutación F392N. En cuanto a la segunda generación (librería CA), llamaba la atención la selección de varios mutantes con pares de residuos básico-ácido en las posiciones 162 y 164, respectivamente, una tendencia que no se observó en la librería A y que indicaba cierta relación de epistasis con la mutación F392N seleccionada en la primera generación. De hecho, la variante de lacasa seleccionada en esta generación (mutante CA32F1) presentaba las mutaciones A162R, T164E y F392N respecto al parental 3A4. Las relaciones epistáticas son propias de los paisajes evolutivos de las proteínas, pero pueden ser difíciles de obtener en el laboratorio si una de las mutaciones por sí sola resulta deletérea para la propiedad de interés (Romero y Arnold, 2009). Sin embargo, el diseño de librerías inteligentes y la mutagénesis focalizada, como en el caso del ISM, puede aumentar la probabilidad de encontrarlas al explorar las posibles combinaciones de mutaciones. En línea con esta idea, cabe destacar que, a pesar de emplear un sustrato natural de lacasa para el *screening* de las librerías de mutantes, los residuos seleccionados en la variante final son muy poco frecuentes en sus respectivas posiciones entre las lacasas de hongos poliporales. Por último, no se obtuvieron nuevos mutantes mejorados al mutagenizar la región B sobre el mutante CA32F1 (librería CAB).

A continuación, las variantes de lacasa C14F12 y CA32F1 fueron producidas y purificadas para su caracterización. Se observó que la mutación F392N, seleccionada en la primera generación, provocaba un leve desplazamiento del perfil de pH óptimo hacia valores más neutros y daba lugar a un aumento tanto de k_{cat} como de K_m para el ácido sinápico a pH 5 (valor de pH al cual se realizaron los ensayos de HTS). Por otro lado, las mutaciones A162R y T164E desplazaban aún más el perfil de pH hacia valores neutros y mejoraban la afinidad (menor K_m) para el ácido sinápico respecto a C14F12. Además, daban lugar a un aumento inesperado de la k_{cat} a pH 3, a pesar del desplazamiento del perfil de pH óptimo.

2.3. Oxidación de otros compuestos fenólicos derivados de la lignina

A continuación, se evaluó la oxidación de otros fenoles de tipo S por parte de la quimera 3A4 y sus variantes C14F12 y CA32F1, obtenidas por ISM. Se observó que la constante de reacción para sinapato de metilo había aumentado de forma similar que para el ácido sinápico, mientras que para otros sustratos como el DMP, el siringato de metilo o el ácido sirínico no sólo no aumentaba la k_{cat} sino que además la K_m era significativamente mayor, disminuyendo por tanto la eficiencia catalítica. Estos sustratos se diferencian por la presencia o no de una cadena lateral propenoide y/o de un grupo carboxilo libre o esterificado. Los resultados obtenidos indicaban que con el método de *screening* empleado para analizar las librerías de

mutantes de ISM se había aumentado de forma específica la actividad sobre aquellos sustratos que presentaban una cadena propenoide (ácido sinápico y sinapato de metilo).

Con el fin de encontrar si los demás mutantes obtenidos por ISM habían mejorado la oxidación de otros compuestos fenólicos, se realizó un *multi-screening* con distintos fenoles de origen vegetal, incluyendo algunos derivados de la lignina y otros con propiedades de interés, como el eugenol, el resveratrol o la quercetina. En el *screening* se incluyeron los mutantes seleccionados de las librerías A, B y C construidas en la primera generación. Se observó que la actividad de los mutantes de la librería C había aumentado preferentemente hacia fenoles con dos sustituciones en *orto*, incluyendo fenoles de tipo S. De éstos, destacaban nuevamente el ácido sinápico y el sinapato de metilo. Todos los clones seleccionados de la librería C habían mutado el residuo F392, si bien los que mantenían el residuo P390 sin mutar eran los que presentaban mayores mejoras. Por otro lado, varios mutantes de la librería A, con el residuo A162 sustituido por residuos básicos como lisina y arginina, mejoraban también la actividad sobre fenoles con una única sustitución en *orto*. Estos incluían fenoles de tipo guayacilo (G) como el ácido ferúlico, muy abundante en la lignocelulosa de herbáceas, y compuestos como el eugenol o el resveratrol, de gran interés farmacológico. Por tanto, estos mutantes serían un punto de partida interesante en la obtención de biocatalizadores mejorados para la transformación de estos compuestos.

2.4. Estudios computacionales de las mutaciones del bolsillo enzimático

Por último, el efecto de las mutaciones seleccionadas en las variantes de ISM C14F12 (F392N) y CA32F1 (A162R/T164E/ F392N) sobre la oxidación del ácido sinápico fue evaluado mediante cálculos computacionales de PELE y QM/MM. Las simulaciones revelaron que la mejor oxidación del sustrato a pH 5 se debía a que la mutación F392N permitía un nuevo modo de unión de la especie desprotonada del ácido sinápico a través del grupo carboxilato, lo cual justificaba el desplazamiento del pH óptimo observado experimentalmente. Este modo de unión también se observaba en las simulaciones realizadas con sinapato de metilo. Junto con los datos cinéticos para la oxidación de los otros fenoles de tipo S (DMP, ácido siríngico y siringato de metilo), estos resultados indican que la longitud de la cadena propenoide del ácido sinápico y el sinapato de metilo es determinante para la oxidación eficiente de estos dos sustratos, permitiendo la orientación favorable del grupo fenólico para la transferencia protón-electrón. En cuanto a las mutaciones A162R y T164E de la variante final, se comprobó que estos residuos podían interactuar entre sí y con el residuo aceptor de protones D205. La orientación relativa de estos residuos era decisiva para que la unión del sustrato fuese favorable para la transferencia protón-electrón. Además, se vio que la mejor eficiencia catalítica a pH 3 de la variante CA32F1 se debía a que era capaz de unir y oxidar

eficientemente tanto la especie desprotonada como la especie protonada del ácido sináptico.

Estos resultados, en combinación con los resultados experimentales de la oxidación de otros compuestos fenólicos, muestran que el evento de unión enzima-sustrato es crucial para la reacción, más allá de la diferencia de potencial redox entre el sustrato y el Cu T1. De este modo, para el diseño de lacasas y otras enzimas oxidoreductasas, es esencial crear un entorno para la unión de sustrato que favorezca una transferencia electrónica eficiente. En este evento juegan un importante papel la distancia entre el dador-aceptor de electrones, el reconocimiento proteína-ligando y la superficie del sustrato accesible al solvente (Monza et al. 2015). Además, ponen de manifiesto que el diseño de proteínas mediante una aproximación puramente racional es muy complicado, ya que la predicción del efecto de una mutación en ocasiones puede resultar errónea. Por ello, la combinación de la evolución dirigida con simulaciones computacionales es una herramienta muy potente para la ingeniería de proteínas. Por un lado, la evolución dirigida puede revelar soluciones inesperadas a un problema, mientras que las simulaciones computacionales pueden explicar esa solución y tener en cuenta la nueva información para diseñar enzimas mejoradas.

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CONCLUSIONES / CONCLUSIONS

CONCLUSIONES

1. Se han obtenido lacasas quiméricas por recombinación aleatoria (CLERY) o racional (intercambio de dominios) de las variantes evolucionadas de las lacasas del basidiomiceto PM1 (OB1) y *Pycnoporus cinnabarinus* (3PO). Algunas quimeras seleccionadas (2C4, 3A4, 6D9, 7A12, 7D5 y OB1::3PO) presentan perfiles desplazados de pH óptimo, mayor estabilidad y/o mejor afinidad por sustrato, por lo que son buenos puntos de partida para el diseño de lacasas hacia nuevos retos.
2. El estudio comparado de las secuencias intercambiadas en algunas de las quimeras obtenidas por CLERY ha permitido identificar algunos residuos clave en el cambio de las propiedades de la lacasa (p. ej. H394 en el desplazamiento del pH óptimo). Sin embargo, los factores que determinan la estabilidad de la enzima son más complejos y el estudio mediante mutagénesis sitio-dirigida de la variante OB1::3PO, obtenida por intercambio de dominios, no permitió atribuir la mayor estabilidad cinética de la enzima a la adquisición puntual de nuevos sitios de N-glicosilación o de puentes salinos en superficie.
3. La recombinación de genes homólogos permite obtener mutaciones neutras que no afectan a la funcionalidad de la proteína pero que le pueden conferir mayor robustez y potencial de evolución. Sin embargo, las sustituciones aminoacídicas obtenidas por recombinación aleatoria son más escasas y restringidas a ciertas zonas de la proteína debido al propio sesgo del método, que limita los eventos de recombinación a las regiones de mayor identidad de secuencia nucleotídica. Por el contrario, la recombinación racional permite el intercambio de mayores bloques de secuencia y en zonas de la proteína elegidas de antemano.
4. Se han desarrollado métodos colorimétricos de cribado de alto rendimiento (HTS) basados en la oxidación de fenoles derivados de la lignina para su empleo durante la evolución dirigida de lacasas para el tratamiento de biomasa vegetal. Así mismo, se han desarrollado otros métodos colorimétricos específicos para la exploración de librerías de mutantes de lacasas de alto potencial redox basados en la oxidación de ácido violúrico y de colorantes orgánicos sintéticos. Estos últimos también pueden ser empleados en métodos acoplados para detectar la capacidad mediadora de otros compuestos.
5. Tomando como punto de partida la lacasa 3A4 obtenida por CLERY, se ha mejorado específicamente la oxidación de ácido sinápico y sinapato de metilo a valores de pH más neutros (pH 5) mediante mutagénesis saturada iterativa (ISM) de seis residuos que delimitan el bolsillo de unión a sustrato. Esta estrategia de mutagénesis reveló la distinta plasticidad de las zonas mutadas y permitió encontrar ciertas relaciones de epistasis entre residuos.

6. Estudios computacionales sobre las variantes de ISM seleccionadas han confirmado la importancia del reconocimiento enzima-sustrato, de forma que la sustitución de un único residuo del bolsillo (p. ej. F392N) puede afectar significativamente a la velocidad de reacción. Así mismo, las simulaciones permitieron explicar la mejora específica de actividad sobre el ácido sinápico y el sinapato de metilo (y no sobre otros fenoles relacionados) debido a la presencia de la cadena lateral propenilo; así como la mejor oxidación del ácido sinápico a pH 5, gracias a una unión más favorable de su forma desprotonada. El conocimiento generado al combinar la evolución dirigida con simulaciones computacionales puede ser muy útil para un futuro diseño racional de lacasas.
7. Por último, la exploración de los mutantes generados en las distintas librerías de ISM en un *multi-screening* con distintos compuestos fenólicos ha permitido encontrar variantes con mejor actividad sobre otros fenoles de interés biotecnológico como el ácido ferúlico, el ácido vainillico, el eugenol o el resveratrol, por lo que pueden ser buenos puntos de partida para la ingeniería de lacasas hacia este tipo de compuestos.

CONCLUSIONS

1. We have obtained chimeric laccases by random (CLERY) or rational (domain exchange) recombination of the evolved laccase variants from basidiomycete PM1 (OB1) and *Pycnoporus cinnabarinus* (3PO). Some of the selected chimeras (2C4, 3A4, 6D9, 7A12, 7D5 and OB1::3PO) presented shifted optimum pH profiles, greater stability and/or better substrate affinities, which makes them appropriate departure points to design laccases towards new challenges.
2. The comparative study of the exchanged sequences in some of the chimeras obtained by CLERY has allowed us to identify some of the key residues involved in the modification of laccase properties (e.g. H394 in the shifted optimum pH). However, the factors that determine enzyme stability are more complex and the study by means of site-directed mutagenesis of the most stable variant OB1::3PO, obtained by domain exchange, did not allow us to attribute the enzyme's greater kinetic stability to the sole acquisition of new N-glycosylation sites or superficial salt bridges.
3. The recombination of homologous genes allows obtaining neutral mutations that do not affect protein function but can provide greater robustness and evolution potential to the protein. However, the amino acid substitutions obtained by random recombination are fewer and restricted to certain regions of the protein due to the method's own bias, which limits recombination events to stretches of high nucleotide sequence identity. On the other hand, rational recombination allows exchanging larger sequence blocks in regions of the protein selected beforehand.
4. We have developed colorimetric high-throughput screening (HTS) assays based on the oxidation of lignin-derived phenols for their application in the directed evolution of laccase towards plant biomass treatment. Also, we have developed other colorimetric methods specific for the exploration of high-redox potential laccase mutant libraries based on the oxidation of violuric acid and synthetic organic dyes. The latter can also be used in coupled methods to detect the mediating capacity of other compounds.
5. Using the chimeric laccase 3A4 obtained by CLERY as departure point, we have specifically enhanced the oxidation of sinapic acid and methyl sinapate at more neutral pH values (pH 5) by means of the iterative saturation mutagenesis (ISM) of six residues delimiting the substrate binding pocket. This mutagenesis strategy revealed the different plasticity of the mutated regions and allowed us to find certain epistatic relationships between residues.
6. Computational studies on the selected ISM variants have highlighted the importance of the enzyme-substrate recognition event, in the sense that the

substitution of a sole residue from the pocket (e.g. F392N) can significantly affect the reaction rate. Also, the simulations have allowed to explain the specific increase in activity towards sinapic acid and methyl sinapate (and not towards other related phenols) due to the presence of the propenyl side chain; as well as the enhanced oxidation of sinapic acid at pH 5, thanks to a more favorable binding of its deprotonated form. The knowledge generated by combining directed evolution and computer simulations can be very useful for the future rational design of laccases.

7. Finally, the exploration of the mutants obtained in the different ISM libraries in a multi-screening assay with different phenolic compounds has allowed us to find variants with better activity towards phenols of biotechnological interest such as ferulic acid, vanillic acid, eugenol or resveratrol, which makes them appropriate departure points for the engineering of laccases towards this type of compounds.

ANEXO

Además de los artículos recogidos en esta memoria, durante el periodo de desarrollo de esta tesis doctoral se han publicado los siguientes trabajos:

1. Camarero S, Pardo I, Cañas AI, Molina P, Record E, Martínez AT, Martínez MJ, Alcalde M. 2012. Engineering platforms for directed evolution of laccase from *Pycnoporus cinnabarinus*. *Appl. Environ. Microbiol.* **78**:1370–1384. (DOI: 10.1128/AEM.07530-11).

2. Pardo I, Camarero S. 2015. Laccase engineering by rational and evolutionary design. *Cell. Mol. Life Sci.* **72**:897–910. (DOI: 10.1007/s00018-014-1824-8).