Assembly of evolved ligninolytic genes in *Saccharomyces cerevisiae*

David Gonzalez-Perez and Miguel Alcalde*

Department of Biocatalysis; Institute of Catalysis, CSIC; Madrid, Spain

**Keywords:** white-rot fungi, ligninolytic oxidoreductases, directed evolution, *Saccharomyces cerevisiae*, laccase, versatile peroxidase, secretome

**Abbreviations:** AAO, aryl alcohol oxidase; ABTS, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); BSA, Bovine Serum Albumin; dNTP, deoxyribonucleotide triphosphate; HRPL, high-redox potential laccase; HTS, high-throughput screening; IVOE, in vivo overlap extension; Lac, laccase gene; LiP, lignin peroxidase; MCS, Multiple Cloning Site; MnP, manganese peroxidase; PCR, polymerase chain reaction; PM1L, laccase from *Coriolopsis* PM1; T50, the temperature at which the enzyme retains 50% of its activity after a 10 minute incubation; UPO, unspecific peroxygenase; Vp, versatile peroxidase gene; VP, versatile peroxidase

The ligninolytic enzymatic consortium produced by white-rot fungi is one of the most efficient oxidative systems found in nature, with many potential applications that range from the production of 2nd generation biofuels to chemicals synthesis. In the current study, two high redox potential oxidoreductase fusion genes (laccase -Lac- and versatile peroxidase -Vp-) that had been evolved in the laboratory were re-assembled in *Saccharomyces cerevisiae*. First, cell viability and secretion were assessed after co-transforming the Lac and Vp genes into yeast. Several expression cassettes were inserted in vivo into episomal bi-directional vectors in order to evaluate inducible promoter and/or terminator pairs of different strengths in an individual and combined manner. The synthetic white-rot yeast model harboring Vp(GAL1/CYC1)-Lac(GAL10/ADH1) displayed up to 1000 and 100 Units per L of peroxidase and laccase activity, respectively, representing a suitable point of departure for future synthetic biology studies.

**Introduction**

The study and engineering of the ligninolytic enzymatic consortium is generating much interest due to its enormous potential in several biotechnological applications, from bioremediation, the production of biofuels (bioethanol, biobutanol) or its use in organic synthesis, to name just a few. In nature, these enzymes (mostly laccases, peroxidases and H2O2 supplying enzymes) are secreted by basidiomycete white-rot fungi that carry out the degradation of the lignin in plant cell walls through a complex (and still not well defined) multi-enzymatic cascade. Understanding the synergies and interactions in this plastic array of high-redox potential enzymes during lignin combustion has been littered with many obstacles, including the lack of suitable functional heterologous hosts in which to perform detailed biomolecular studies.

During last decade there has been much effort to develop robust and reliable platforms for the directed evolution of ligninolytic laccases and peroxidases in the baker’s yeast, *Saccharomyces cerevisiae*. This approach has allowed us to design enzymes with improved properties of biotechnological interest, including: new activities and specificities; enhanced thermostability; or even tolerance to unnatural media, such as human blood or organic co-solvents. Through these experiments, we have witnessed the wealth of possibilities offered by *S. cerevisiae* in terms of creating DNA diversity by its physiological homologous recombination machinery and more generally, the outstanding role this yeast can play in synthetic biology studies.

A critical issue when considering Lignocellulose Biorefineries, for the sustainable production of biofuels and biomaterials, is the need to engineer artificial microorganisms with attributes tuned to the efficient use of raw plant biomass, and their subsequent exploitation as a renewable source with an industrial and energetic fate. Important advances in the field include the transformation of chemicals (e.g., that of xylose from hemicellulose to xylitol, a C5 platform chemical) or the engineering of recombinant yeast strains able to ferment pentose sugars to produce ethanol from plant biomass. However, in all these approaches it is indispensable to remove the recalcitrant biopolymer and obtain lignin-free hemicellulose. Typically, lignocellulosic material is pre-treated by high energy consuming and non-environmentally friendly physicochemical processes, such that the potential use of lignin derivatives in multiple sectors is misspent (including lignin-based products for manufacturing...
materials, surfactants and adhesives). Hence, the engineering of customized microorganisms capable of cost-effectively producing industrial chemicals and biofuels from raw materials (i.e., using whole plant biomass as the feedstock) is one of the most important goals in modern synthetic biology and metabolic engineering.

In this study, two high potential ligninolytic genes encoding a laccase (EC 1.10.3.2) and a versatile peroxidase (VP) (EC 1.11.1.16), which were previously evolved in the laboratory toward improved secretion, activity and stability, were assembled in vivo in S. cerevisiae using the eukaryotic biomolecular toolbox. Expression cassettes were constructed and inserted into uni- and bi-directional episomal vectors under the control of different promoters. Secretion yields and cell viability were evaluated in a simplified white-rot yeast model, thereby opening up new avenues for the engineering of a full-synthetic ligninolytic secretome for future synthetic biology studies.

Results and Discussion

Ligninolytic genes are proving to be complex targets when attempting to functionally express them in heterologous systems for metabolic engineering and synthetic biology studies (typically performed in Escherichia coli and/or S. cerevisiae). In bacteria, the main bottlenecks encountered are associated with misfolding due to the strong differences found in codon usage, missing chaperones, and the lack of post-translational modifications, all of which lead to the formation of inclusion bodies. Although the machinery of S. cerevisiae is closer to that of the basidiomycete white-rot fungi, serious and still unidentified shortcomings arise during the inner secretory pathway and the final exocytosis of the enzymes by yeast.

In recent years, we have circumvented many of these hurdles by undertaking exhaustive protein engineering work, including signal peptide switching and directed evolution of whole fusion proteins in order to adapt foreign genes to the subtleties of yeast. Using these approaches, we have individually expressed medium- and high-redox potential laccases (HRPL),

unique feature of these two evolved genes is the capacity to tolerate neutral and/or alkaline pHs, at which most ligninolytic laccases and/or peroxidases reported to date are inactive. Moreover, it is worth pointing out that combining these VP and Lac genes in the same microorganism guarantees a broader oxidative potential and substrate promiscuity: while the laccase mutant has a high redox potential (~760 mV vs NHE) and wide substrate specificity; the VP mutant comprises three catalytic sites for the oxidation of low-, medium-, and high-redox potential compounds, sharing common catalytic properties with lignin-peroxidases (LiP), manganese-peroxidases (MnP) and generic peroxidases.

In the first place, the culture medium was optimized by combining the different ingredients required for the individual expression of these genes (i.e., copper sources for Lac, Ca<sup>2+</sup> and heme supplies for VP), such that the health of yeast cells was not significantly affected. Thus, the VP and Lac were co-transformed in independent high-copy episomal pJRoC30 vectors under the control of the GAL1/CYC1 promoter/terminator pair, (Fig. S1). From microtiter fermentations, individual clones were analyzed by means of their capacity to express both VP and Lac. The same experiment was performed using competent cells harboring either of the two constructs and co-transforming the gene missing, however the strong background precluded the isolation of positive clones due to the limitations imposed on the screening process (data not shown). After screening the supernatants, several clones with higher, lower or similar VP and Lac expression were found (with secretion levels up to ~100 U/L), indicating that co-expression was not harmful to yeast (Fig. 2A). It is likely that the transcription and translation of VP and Lac, although energetically expensive, did not produce an important metabolic burden on the cell with associated nucleotide and amino acid leakage.

Encouraged by these results, we employed in vivo overlap extension (IVOE)<sup>7</sup> to generate an array of vectors containing single and double expression cassettes (VP (GAL1/CYC1), VP (GAL10/ADH1), Lac (GAL1/CYC1), Lac (GAL10/ADH1), VP (GAL1/CYC1)-Lac (GAL10/ADH1), and Lac (GAL1/CYC1)-VP (GAL10/ADH1)), using a pESC bi-directional vector as it harbors promoter/terminator pairs with different strengths (GAL1/CYC1, GAL10/ADH1, Fig. 3 and 4; Fig. S2). First, individual expression was evaluated by inserting the VP and Lac genes from pJRoC30 into pESC (Fig. 4A and B; Fig. S2). However, the secretion of these enzymes proved to be lower in pESC than in pJRoC30, even for the same GAL1/CYC1 pair (Fig. 2B and C). Apart from the bi-directional characteristics of pESC, the only other difference of note between both vectors is that pJRoC30 comprises the FLP/FRT recombinase system associated with the stability of the copies of the 2 µm plasmid (Fig. S1).<sup>31</sup> This could represent an important difference in microfermentations, yet it may have only minor consequences in larger scale fermentations (see below). Despite the weaker secretion in pESC, sufficient quantities were available as to readily detect activity when assembling both VP and Lac genes in the same vector (i.e., the VP (GAL1/CYC1)-Lac (GAL10/ADH1) and Lac (GAL1/CYC1)-VP (GAL10/ADH1) double constructs (Figs. 2B and C and 4C and D). Indeed, combined VP–Lac constructs gave rise to similar levels of secretion for both
promoter/terminator pairs (around 30 Units/L), albeit lower than those obtained for the individual counterparts.

At this point, it is important to highlight that this set of experiments was performed in microtiter plates (i.e., ~200 µL microfermentations where stirring conditions and oxygen availability were limiting, impeding reaching the stationary phase [see Materials and Methods for details]). Since we could expect higher secretion yields when fermentations are translated to a larger scale, individual, and combined constructs were subjected to larger scale fermentation (in flask) to accurately monitor cell growth, as well as VP and laccase activity (Fig. 5). In all cases, the stationary phase was reached after around 50 h growth and hemoglobin was added to the expression media as a heme supply for VP as it was practically consumed by yeast after ~30 h (at the earliest logarithmic stage, when protein secretion became evident, Fig. 5I). Regardless of whether the plasmids harbored single or double constructs, maximal secretion was detected by the middle and/or final stages of the logarithmic phase. Indeed, the evolution of the activity profiles was quite similar in all cases, achieving a plateau at ~40 h after which VP activity slowly increased while laccase activity fell slightly. It is likely that the presence of certain metabolites released by yeast cells could negatively affect laccase activity/stability at this stage of fermentation. Independent of the promoter and vector used, the levels of expression from single constructs were quite similar (with strong secretion of 750–1500 and 600 Units/L for VP and laccase, respectively). The best double construct (Vp[GAL1/CYC1]-Lac[GAL10/ADH1]) produced strong secretion of ~1000 and 100 units/L for VP and laccase, respectively, Figure 5A. Nevertheless, secretion from single constructs of Vp were 1.25 to 2.5-fold higher than for Lac, which might partially explain the significant 10-fold drop in laccase activity upon co-expression with VP. It seems plausible that the pathway involved in processing the α-factor prepro-leader in yeast cannot support the heterologous co-expression of these two ligninolytic genes without sacrificing some level of secretion. Although VP and laccase joint secretion is sufficient for synthetic

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**Figure 1.** General overview of the evolved VP (in green) and laccase (in magenta) variants. The active sites (heme domain and copper coordination spheres) are highlighted in the CPK stick diagram. In both models, the mutations involved in secretion are shown in yellow, while the mutations that enhance thermostability (VP) are depicted in cyan and the mutations for blood tolerance (laccase) in red. In the laccase model Cu²⁺ ions are shown as blue spheres, whereas in the VP model, structural Ca²⁺ ions are shown as light-yellow spheres, with Mn²⁺ and Fe³⁺ as gray spheres. Homology models were created from PDBs ID:3FJW and ID:2HRG for the VP and laccase variants, respectively.
biology studies, possible approaches to enhance secretion could include the replacement of signal peptides by others that do not require the KEX2-StE13 proteases, the inclusion of KEX2-StE13 genes in the same pESC vector, or the engineering of chimeric peptides based on the combination of different regions of the α-factor prepro-leader and the prepro-toxin K1 killer from S. cerevisiae (unpublished material).

Conclusions

The current study is the first successful attempt to re-assemble a ligninolytic secretome in S. cerevisiae, representing a suitable starting point to develop an autonomously recombinant yeast strain capable of working directly on raw plant biomass. In this context, the most extensively investigated white-rot fungi (Phanerochaete chrysosporium and Pleurotus eryngii) have distinct extracellular enzymatic machineries to degrade lignin. While P. chrysosporium secretes LiP, MnP and the copper-radical glyoxal oxidase, P. eryngii produces MnP, VP, laccase, and aryl-alcohol oxidase. Future studies will assess the construction of a more complex artificial secretome harboring laboratory evolved versions of VP, HRPL, UPO, and AAO genes. This synthetic white rot yeast will constitute a new model organism for future synthetic biology and directed evolution developments aimed at improving the production of chemicals and biofuels, while

Figure 2. Secretion of Vp and Lac in the pJRoc30 and pESC shuttle vectors containing distinct expression cassettes (values obtained from microfermentations in 96-well plates). (A) Activities of isolated clones from co-transformation of pJRoc30-Vp and pJRoc30-Lac in yeast. (B) Expression of Vp and Lac from single and double constructs with different promoter/terminator pairs and vectors. Laccase activity in black, VP activity in white. Each bar represents the average of three independent fermentations including the standard deviation. (C) 96-well plate showing the activities of different constructs. VP and Lac expression was assessed using the ABTS screening assay (100 mM citrate-phosphate buffer [pH 4.0] containing 3 mM ABTS). To determine the VP activity, 0.1 mM H2O2 was added to the ABTS assay solution. VP activities were deduced from the difference of the activities assessed in the presence (VP + laccase activity) and absence (laccase activity) of 0.1 mM H2O2. Each lane represents the data from 8 independent microfermentations (see Materials and Methods for details).
providing a plastic vehicle to understand the function of the ligninolytic enzymatic consortium during natural wood decay.

**Materials and Methods**

*Vp* (2–1B variant) and *Lac* (ChU-B variant) evolved genes were obtained from previous laboratory evolution projects. ABTS (2,2′-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]), Bovine hemoglobin, *Taq* polymerase and the *S. cerevisiae* transformation kit were purchased from Sigma-Aldrich. The iProof High Fidelity DNA polymerase was purchased from Bio-Rad. The Zymoprep Yeast Plasmid Miniprep Kit and Zymoclean Gel DNA Recovery Kit were obtained from Zymo Research. The *Escherichia coli* XL2-Blue competent cells and shuttle vector pESC containing two multiple cloning site (MCS1, MCS2) under the control of different promoter/terminator pairs (GAL1/CYC1, GAL10/ADH1) were from Stratagene. The uracil independent and ampicillin resistance pJRoc30 vector was obtained from the California Institute of Technology (Caltech). The *ura3*-deficient *S. cerevisiae* strain BJ5465 (α ura3–52 trpl leu2Δ1 his3Δ200 pep4::His2 prb1Δ1.6R can1 GAL1) was obtained from LGCPromochem, the NucleoSpin Plasmid kit was purchased from Macherey-Nagel, and the restriction enzymes BamH1, Nhe1, Spe1, SacI, and NotI from New England Biolabs. All chemicals were of reagent-grade purity.

**Culture media**

Minimal medium (SC) contained 0.67% (w/v) yeast nitrogen base, 1.92 g/L yeast synthetic drop-out medium supplement without uracil, 2% (w/v) D-rafifinose and 25 µg/mL chloramphenicol.YP medium contained 10 g yeast extract, 20 g peptone and 500 mg/L bovine hemoglobin and 1 mM CaCl₂ for *Vp* expression, 2 mM CuSO₄ for *Lac* expression, 1 mL 25 g/L chloramphenicol and ddH₂O to 1000 mL. Microplate expression medium contained 720 mL YP, 67 mL 1 M KH₂PO₄ buffer (pH 6.0), 111 mL 20% (w/v) D-galactose, 25 g/L ethanol, 500 mg/L bovine hemoglobin and 1 mM CaCl₂ for *Vp* expression, 2 mM CuSO₄ for *Lac* expression, 1 mL 25 g/L chloramphenicol and ddH₂O to 1000 mL. Microplate expression medium contained 720 mL YP, 67 mL 1 M KH₂PO₄ buffer (pH 6.0), 111 mL 20% (w/v)}
D-galactose, 100 mg/L bovine hemoglobin for Vp, 2 mM CuSO₄ for Lac, 1 ml 25 g/L chloramphenicol and ddH₂O to 1000 mL. YPD solution contained 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) D-glucose and 25 µg/mL chloramphenicol. SC drop-out plates contained 0.67% (w/v) yeast nitrogen base, 1.92 g/L (w/v) yeast synthetic drop-out medium supplement without uracil, 2% (w/v) bacto agar, 2% (w/v) D-glucose and 25 µg/mL chloramphenicol. Luria-Bertani (LB) medium was prepared with 1% (w/v) peptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, and 100 µg/mL ampicillin.

**Engineering of single and double constructions**

PCR products and linearized vectors were cleaned, concentrated and loaded onto a low melting-point preparative agarose gel and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research). DNA concentrations were estimated spectrophotometrically at 260 nm in 100 µL quartz cuvettes. Vp and Lac including their respective signal peptides (α factor prepro-leader) were amplified by PCR and in vivo cloned into S. cerevisiae. Crossover areas were engineered with overhangs of ~50 bp flanking each segment between Vp, Lac and linearized pESC.

**Figure 4.** In vivo assembly of synthetic genes by IVOE. Each overlapping region allowed crossover events to occur between fragments giving rise to an autonomously repaired vector containing single and double expression cassettes in the correct orientation. (A) and (B) Single expression cassettes are constructed in pESC by engineering specific primers containing overhangs to foster in vivo cloning with the linearized plasmid in yeast (pJRoc30 was used as template for Vp or Lac amplifications). (C) and (D) The pESC constructs obtained in (A) and (B) were used as scaffolds to assemble Lac and Vp genes under the control of different promoter/terminator pairs. Primers used: (1)-MCS1-Vp/Lac-α-BamHI, (2)-MCS2-Vp-ter-NheI, (3)-MCS2-Lac-ter-Nhel, (4)-MCS1-Vp/Lac-α-SpeI, (5)-MCS1-Lac-ter-Sacl and (6)-MCS1-Vp-ter-Sacl. Black arrows indicate the direction of the transcription process.
**PCRs for in vivo cloning in MCS1-pESC (MCS1, Multiple cloning site 1)**

Reaction mixtures were prepared in a final volume of 50 μL containing: DNA template (0.2 ng/μL, pJRoC30-Vp or pJRoC30-Lac), 0.5 μM MCS1-Vp/Lac-α-Spel primer (5′-AATTTTTTGAA AAATCGAATT CAACCCTCAC TAAAGGGCGG CCGCACTAGT ATGAGATTTT CCCTCATTTT TACTGCTG-3′), 0.5 μM MCS1-Vp/Lac-ter-Sacl primer (5′-GATTGGAGAC TTGACCAAAC CTCTGGCGAA GAATTGTTAA TTAAGAGCTC TTACGATCTA GGGACGGGAG-3′ for Vp and 5′-GATTGGAGAC TTGACCAAAC CTCTGGCGAA GAATTGTTAA TTAAGAGCTC TTACGATCTA GGGACGGGAG-3′ for Lac), 0.8 mM dNTPs (0.2 mM each), 3% (v/v) dimethylsulfoxide (DMSO), and 0.02 U/μL iProof polymerase. High fidelity PCRs were performed on a gradient thermocycler using the following conditions:

**Figure 5.** Large scale fermentation of single and double expression cassettes in pJRoc30 and pESC, showing the OD_{600} vs. Laccase or VP activity over time (both measured with ABTS): black circles, cell growth (OD_{600}); black triangles, VP activity; white triangles, laccase activity; white squares, background generated by the heme source (hemoglobin). Each point represents the average of three independent measurements including the standard deviation.
conditions: 98 °C for 30 s (1 cycle); 98 °C for 10 s, 55 °C for 25 s, 72 °C for 1 min 30 s (28 cycles); and 72 °C for 10 min (1 cycle). Underlined are highlighted crossover areas between genes and pESC plasmid one each other.

**PCR for in vivo cloning in MCS2-pESC (MCS2, Multiple cloning site 2)**

Reaction mixtures were prepared in a final volume of 50 μL containing: DNA template (0.2 ng/μL, pJRoC30-Vp or pJRoC30-Lac), 0.5 μM MCS2-Vp/Lac-α-BamHI primer (5′-TGTTAAATATA CCTCTATACT TTAACGTCAA GGAGAAAAAA CCCCATGCC ATGAGATTTTC TTTCAATTTT TACTGCTG-3′), 0.5 μM MCS2-Vp/Lac-ter-NheI primer (5′-TTCAGGTTGT CTAACTCCTT CCTTTTCGGT TAGAGCGGAT CTTAGCTAGC TTACTGGTCG TTAGCTAGC TGGAAGCAGGAG-3′ for Vp and 5′-TTCAGGTTGT CTAACCTCCTT CCTTTTCGGTT TAGAGCGGAT CTTAGCTAGC TTACTGGTCG TCAGGGGAGGAG-3′ for Lac), 0.8 mM dNTPs (0.2 mM each), 3% (v/v) dimethylsulfoxide (DMSO), and 0.02 U/μL iProof polymerase. High fidelity PCRs were performed on a gradient thermocycler using the following conditions: 98 °C for 30 s (1 cycle); 98 °C for 10 s, 55 °C for 25 s, 72 °C for 1 min 30 s (28 cycles); and 72 °C for 10 min (1 cycle). Underlined are highlighted crossover areas between genes and pESC plasmid one each other.

**pESC digestion**

pESC was linearized in final volume of 20μL containing: 2 μg of empty pESC vector, 100μg/mL BSA, 20 total Units of SpeI and SacI for opening MCS1 (GAL10/ADH1), or 20 total Units of BamHI and NheI for MCS2 (GAL1/CYC1) (Figs. 3 and 4). Digestion reactions were incubated for 1 h at 37 °C, and then enzymes heat-inactivated at 80 °C during 20 min. The products of inactivation were cleaned and purified.

**Reassembly in S. cerevisiae by IVOE**

Constructs were reassembled in vivo by transformation into *S. cerevisiae* cells using the Yeast Transformation Kit. The DNA transformation mixture contained 100 ng of linearized plasmid (MCS1 or MCS2) mixed with single Vp or Lac genes (400 ng per gene, ratio 1:4 vector:gene). Transformed cells were plated on SC drop-out plates and incubated for 3 d at 30 °C. Subsequently, the cloning libraries were subjected to the high-throughput protocol for laccase/VP activity.

**Microfermentations and high-throughput screening (HTS) protocol for laccase/VP activity**

Individual clones were selected and cultured in sterile 96-well plates (Greiner Bio-One GmbH) containing 50 μL per well of SC minimal medium. In each plate well-H1 was inoculated with *S. cerevisiae* containing pESC empty vector as a negative control. Plates were sealed to prevent evaporation and incubated at 30 °C, 225 rpm, and 80% relative humidity in a humidity chamber.
shaker (Minitron-INFORS, Biogen). After 48 h, 160 μL of expression medium was added to each well and the plates were incubated for a further 24 h. The plates (master plates) were centrifuged for 15 min at 3000 rpm and 4 °C (Eppendorf 5810R centrifuge) and the master plates were duplicated with the help of a robot (EVO Freedom-100, Tecan) by transferring 20 μL of crude supernatant. Next, 180 μL of ABTS screening assay solution were added using a Multidrop robot (Multidrop Combi, ThermoFischer Scientific). The assay solution was prepared in 100 mM citrate-phosphate buffer (pH 4.0) containing 3 mM ABTS. For the determination of VP activity the assay solution was supplemented with 0.1 mM H₂O₂. The plates were stirred briefly and the absorption at 418 nm (εABTS•+ = 36000 M⁻¹ cm⁻¹) was recorded in kinetic mode on a plate reader (SPECTRAMax Plus 384, Molecular Devices). VP activities were deduced from the difference of the activities assessed in the absence (laccase activity) and in the presence (VP + laccase activity) of 0.1 mM H₂O₂.

Isolation of constructions

A 100 μL aliquot from the wells with the positive clones was inoculated in 3 mL of YPD and incubated at 30 °C and 225 rpm for 24 h, recovering the plasmids from these cultures (Zymoprep Yeast Plasmid Miniprep Kit). As the product of the zymoprep was very impure and the concentration of DNA was low, the zymoprep mixtures containing shuttle vectors were transformed into super-competent E. coli cells (XL2-Blue, Stratagene) and plated on LB/amp plates. Single colonies were picked and used to inoculate 5 mL LB/amp media, and they were grown overnight at 37 °C and 225 rpm. The plasmids were then extracted (NucleoSpin Plasmid kit, Macherey-Nagel). Plasmids and their containing cassettes were linearized with 10 units of NotI in a final volume of 20 μL containing 0.5 μg and 100μg/mL BSA of the enzyme was heat-inactivated and the volume of 20 μL containing 0.5 μg and 100μg/mL BSA of the enzyme was heat-inactivated and the resulting DNA (Zymoprep Yeast Plasmid Miniprep Kit). As the product of the zymoprep was very impure and the concentration of DNA was low, the zymoprep mixtures containing shuttle vectors were transformed into super-competent E. coli cells (XL2-Blue, Stratagene) and plated on LB/amp plates. Single colonies were picked and used to inoculate 5 mL LB/amp media, and they were grown overnight at 37 °C and 225 rpm. The plasmids were then extracted (NucleoSpin Plasmid kit, Macherey-Nagel). Plasmids and their containing cassettes were analyzed by digestion and conventional PCR (Fig. S2). The plasmids were linearized with 10 units of NotI in a final volume of 20 μL containing 0.5 mM forward primer, 0.09 μM reverse primer, 0.2 mM dNTPs (0.05 mM each), 3% (v/v) dimethylsulfoxide (DMSO), and 0.05 U/μL Taq DNA polymerase. Constructs including VP or Lac in MCS1 were amplified using forward primer GAL10p-Binding (5′-CGGTTCATCC AAAAAAAAAG TAAGAATT-3′) and reverse primer ADH1t-binding (5′-CGACAACCTT GATTGGAGAC TTG-3′) and CYC1t-binding (5′-AATAAATAGG GACCTAGACT TCAGG-3′). PCRs were performed on a gradient thermocycler using the following conditions: 95 °C for 2 min (1 cycle); 94 °C for 45 s, 51 °C for 30 s, 72 °C for 1 min 30 s (28 cycles); and 72 °C for 10 min (1 cycle) (Fig. S2B). Whole expression vectors isolated from positive clones that contained the expected PCR products were verified by DNA sequencing using an ABI 3730 DNA Analyzer-Applied Biosystems Automatic Sequencer at the Secugen (CIB). The primers used were: GAL10p Seq (5′-GGTTGAATATG CCATGTAATA TG-3′) and ADH1t Seq (5′-TATAGAAAG CGCAACACCTT G-3′), for MCS1; GAL1p Seq (5′-CAACATTTTG GTTGTATT ACTTC-3′) and CYC1t Seq (5′-GGACCTAGAC TTCAGGTGTG C-3′), for MCS2. Internal primers for VP were 3R-direct (5′-GTTCATCAT CGCGTTCG-3′) and 5F-reverse (5′-GGATTCCTTT CTTCTGTAATG C-3′). Internal primers for Lac were PM1FS (5′-ACGATTTCCA GGTCCCTGAC CAAGC-3′), and PM1RS (5′-TCAATGTCGG CTTCGCCAG A-3′).

VP and Lac expression

Microfermentations

Individual clones were picked from a SC drop-out plate and culture in sterile 96-well plates (Greiner Bio-One GmbH) containing 50 μL per well of SC minimal medium. The following constructs were fermented: (VP-(GAL1/CYC1), Lac-(GAL1/CYC1), VP-(GAL10/ADH1), Lac-(GAL10/ADH1), VP-(GAL1/CYC1)-Lac-(GAL10/ADH1), Lac-(GAL1/CYC1)-VP(GAL10/ADH1), pJRoC30-VP, pJRoC30-Lac and pESC empty vector). For each plate individual columns were inoculated with S. cerevisiae containing the different constructions. Plates were sealed to prevent evaporation and incubated at 30 °C, 225 rpm and 80% relative humidity in a humidity shaker (Minitron-INFORS, Biogen, Spain). After 48 h, 160 μL of expression medium was added to each well and the plates were incubated for a further 24 h. The plates were centrifuged for 15 min at 3000 rpm and 4 °C (Eppendorf 5810R centrifuge) and supernatants subjected to the abovementioned HTS-protocol.

Larger scale fermentations

A single colony of S. cerevisiae containing VP-(GAL1/CYC1), Lac-(GAL1/CYC1), VP-(GAL10/ADH1), Lac-(GAL10/ADH1), VP-(GAL1/CYC1)-Lac-(GAL10/ADH1), Lac-(GAL1/CYC1)-VP(GAL10/ADH1), pJRoC30-VP, pJRoC30-Lac, and empty pESC was picked from a SC drop-out plate and used to inoculate 5 mL of minimal medium in a 50 mL tube. The cultures were incubated for 48 h at 30 °C and 225 rpm (Micromagmix shaker) up to saturation. An aliquot of cells was removed and used to inoculate a final volume of 5 mL of minimal medium in a 50 mL tube (Optical Density, OD₆₀₀ = 0.3), and they were incubated until two growth phases had been completed (6–8 h, OD₆₀₀ = 1). Thereafter, 9 mL of expression medium was inoculated with 1 mL of this pre-culture in a 100 mL flask (OD₆₀₀ = 0.1). The flask cultures were incubated during 70 h at 30 °C and 225 rpm (OD₆₀₀ = 45–50). Aliquots of culture were collected every 4 h to determine OD₆₀₀ and VP/laccase activity. Activity was measured from crude supernatants, cells were separated by centrifugation for 5 min at 3000 rpm at room temperature (Eppendorf 5424 Centrifuge). The VP/laccase activities were measured with
20 µL of supernatant in a final volume of 200 µL containing 100 mM citrate-phosphate buffer (pH 4.0) with 3 mM ABTS. For VP activity, the assay solution was supplemented with 0.1 M H₂O₂. The absorption at 418 nm (εABTS⁺⁺ = 36 000 M⁻¹ cm⁻¹) was recorded in kinetic mode on a plate reader (SPECTRAMax Plus 384, Molecular Devices).

**Protein and homology modeling**

PyMOL Molecular Visualization System (Schrödinger) was used to generate protein models using as templates the VP12 from *P. eryngii* (PDB ID: 3FJW) and *Trametes trogii* laccase (PDB: 2HRG, 97% sequence identity with the PM1 laccase) for VP and laccase mutants, respectively.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Materials**

Supplemental materials may be found here: www.landesbioscience.com/journals/bioe/article/29167/