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Alkaline versatile peroxidase by directed evolution

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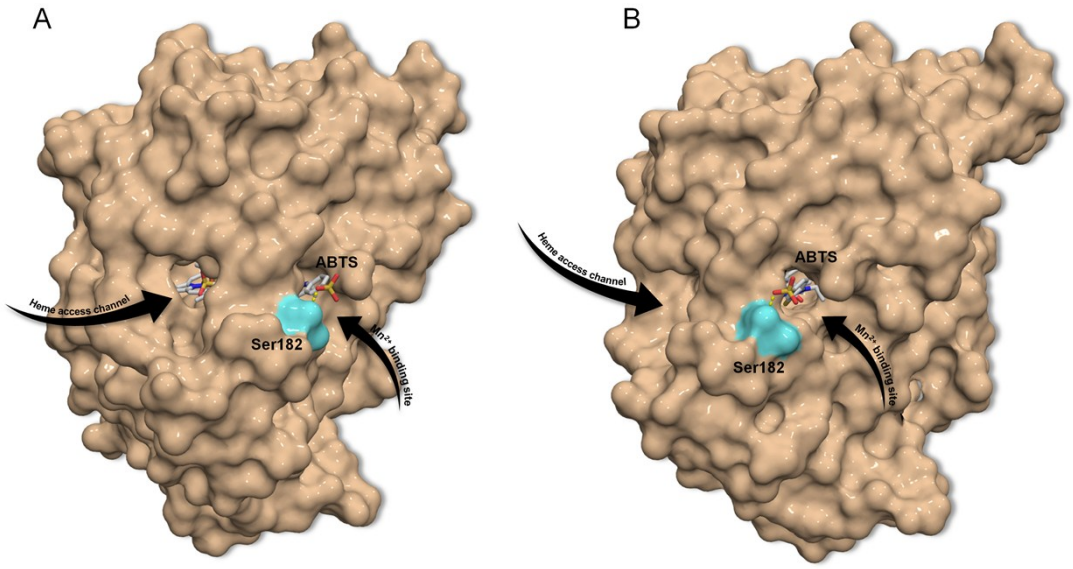

Fig. S1 Molecular Docking for ABTS at the Mn^{2+} site.

The protein is shown in the surface mode, the heme cofactor and ABTS are highlighted as stick models (CPK colors). The hydrogen bond between the SO^{3-} group of ABTS and Ser182 is represented as a yellow dash. The PDB file used for molecular docking was 3FKG. (**A**) Front view. (**B**) Side view.

Multiple structural alignment (MSA)

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                                                                E37K  H39R
AURDE-GP11          ----RPAASVCADGVTTVNNPQCCFWANVRDRFINEIFL-GVCQENVHSLVRIAFHDAIG        55
PHLRA-MnP2          -------KTTCSNG-VVVPDAVCCDFVPLASALQSEVLM-GDCGEDAHELVRLIFHDAIA        51
PHACH-MnP1          --------AVCPDG-TRVSHAACCAFIPLAQDLQETIFQ-NECGEDAHEVIRLTFHDAIA        50
BJEAD-LiP           ---AIIKRVACPDGRHTAINAACCNLFTVRDDIQRNMFDGGKCNDIAHQALRLTFHDAVA        57
PHLRA-LiP3          --ASVTRRATCPDG-TQLMNAECCALLAVRDDLQNNMFN-NECGDEAHEALRLTFHDAIA        56
PHACH-LiPH8         --------ATCSNG-KTVGDASCCAWFDVLDDIQQNLFHGGQCGAEAHESIRLVFHDSIA        51
PLEOS-MnP4          ---VPAHRAKCSKG-RTASNDACCVWFDVLDDIQENLFDGGECGEEVHESLRLTFHDAIG        56
BJEAD-VP            ---AITRRVACPDGVNTATNAACCALFAVRDDIQQNLFDGGECGEEVHESLRLTFHDAIG        57
BB8-PLEER-VP        --------ATCDDG-RTTANAACCILFPILDDIQENLFDGAQCGEKVRESLRLTFHDAIG        51
PLEOS-MnP5          -VSLPQKRATCAGG-QVTANAACCVLFPLMEDLQKNLFDDGACGEDAHEALRLTFHDAIG        58
PLEOS-VP3           -VTLPQKRATCAGG-QVTANAACCVLFPILEDLQQNLFDGGECGEEVHESLRLTFHDAIG        58
Consensus                     *  *     .  **    : . :   .     *   .:. :*: ***::.

AURDE-GP11          FSLTD----PSKGGGADGSIIMFGDTELNFHANEGIDFITAFLQPFADTVG-VTYGDAIQ        110
PHLRA-MnP2          ISQSMG---PSAGGGADGSMLIFPTVEPAFFPNLGIADSVNNLIPFLSQFPTISAGDLVQ        108
PHACH-MnP1          ISRSQG---PKAGGGADGSMLLFPTVEPNFSANNGIDDSVNNLIPFMQKHNTISAADLVQ        107
BJEAD-LiP           FSPALEAEGKFGGNGADGSIITFGNIETNFHPNIGLDEIVEIEKPFIARHN-MTPGDFLH        116
PHLRA-LiP3          ISPAMEATGQFGGGGADGSIMIFSDIETKFHPNIGLDEVVESFRPFQQRSG-MGVADFIQ        115
PHACH-LiPH8         ISPAMEAQGKFGGGGADGSIMIFDDIETAFHPNIGLDEIVKLQKPFVQKHG-VTPGDFIA        110
PLEOS-MnP4          FSPALTRQGKFGGGGADGSIMLFSDIETNFAANNGVDDIVEQQKPIAIKHQ-VSFGDFIQ        115
BJEAD-VP            ISPSIAATGKFGGGGADGSIMIFDDIEPNFHANNGVDEIINAQKPFVAKHN-MTAGDFIQ        116
BB8-PLEER-VP        FSPTLG-----G-GGADGSIIAFDTIETNFPANAGIDEIVSAQKPFVAKHN-ISAGDFIQ        104
PLEOS-MnP5          FSPSRG-----VMGGADGSVITFSDTEVNFPANLGIDEIVEAEKPFLARHN-ISAGDLVH        112
PLEOS-VP3           FSPTKG-----G-GGADGSVLTFSDPEVNFPANLGIDEIVEAQKPFLARHN-ISAGDLVQ        111
Consensus           :* :          *****:: .   *  *  * *:        *:      :  .* :
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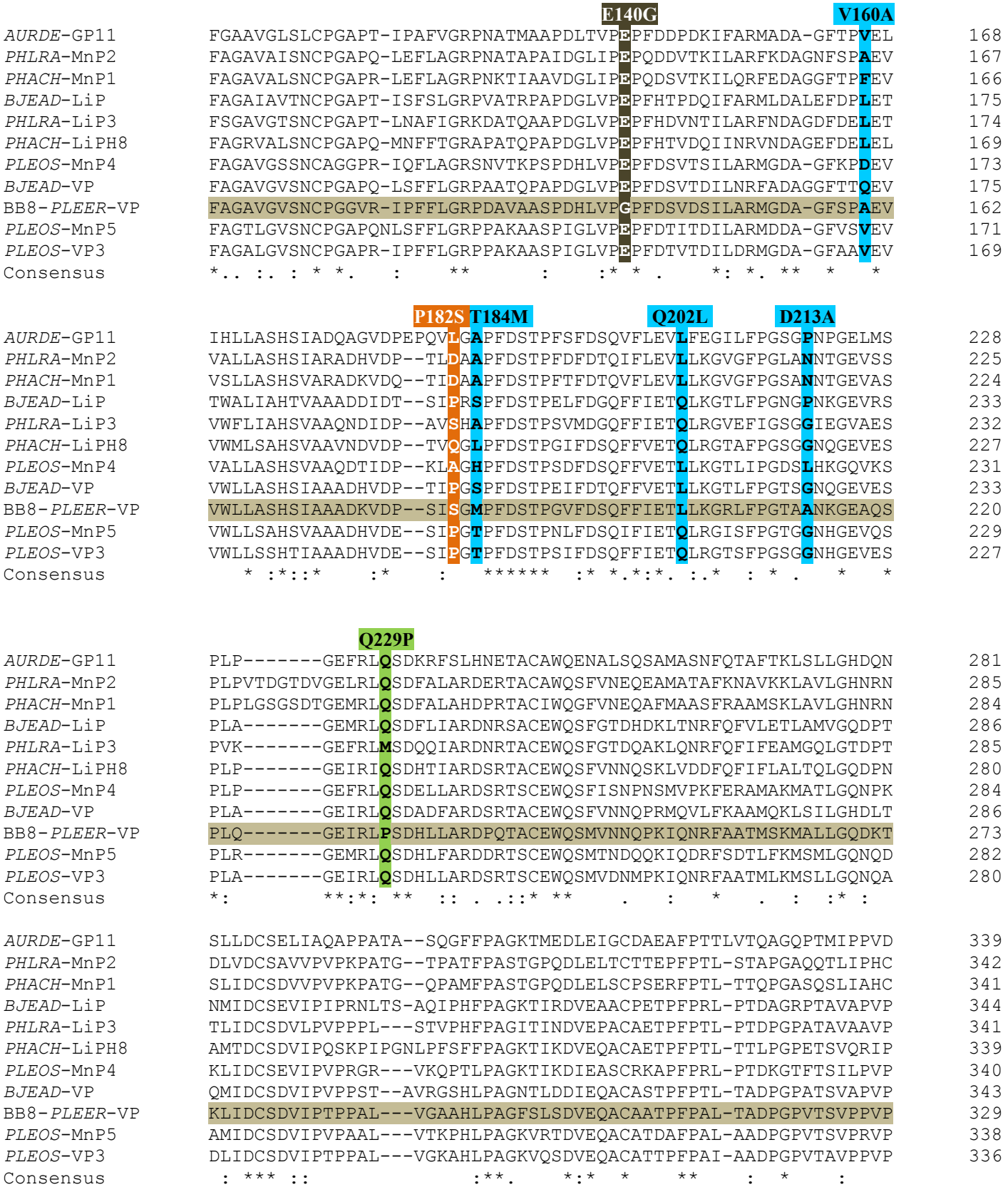
E140G
V160A
P182S
T184M
Q202L
D213A
Q229P
AURDE-GP11 FGAAVGLSLCPGAPT-IPAFVGRPNATMAAPDLTVPEPFDDPDKIFARMADA-GFTPVEL 168
PHLRA-MnP2 FAGAVAISNCPGAPQ-LEFLAGRPNATAPAIDGLIPEPQDDVTKILARFKDAGNFSPAEV 167
PHACH-MnP1 FAGAVALSNCPGAPR-LEFLAGRPNKTIAAVDGLIPEPQDSVTKILQRFEDAGGFTPFEV 166
BJEAD-LiP FAGAIAVTNCPGAPT-ISFSLGRPVATRPAPDGLVPEPFHTPDQIFARMLDALEFDPLET 175
PHLRA-LiP3 FSGAVGTSNCPGAPT-LNAFIGRKDATQAAPDGLVPEPFHDVNTILARFNDAGDFDELET 174
PHACH-LiPH8 FAGRVALSNCPGAPQ-MNFFTGRAPATQPAPDGLVPEPFHTVDQIINRVNDAGEFDELEL 169
PLEOS-MnP4 FAGAVGSSNCAGGPR-IQFLAGRSNVTKPSPDHLVPEPFDSVTSILARMGDA-GFKPDEV 173
BJEAD-VP FAGAVGVSNCPGAPQ-LSFFLGRPAATQPAPDGLVPEPFDSVTDILNRFADAGGFTTQEV 175
BB8-PLEER-VP FAGAVGVSNCPGGVR-IPFFLGRPDAVAASPDHLVPGPFDSVDSILARMGDA-GFSPAEV 162
PLEOS-MnP5 FAGTLGVSNCPGAPQNLSFFLGRPPAKAASPIGLVPEPFDTITDILARMDDA-GFVSVEV 171
PLEOS-VP3 FAGALGVSNCPGAPR-IPFFLGRPPAKAASPIGLVPEPFDTVTDILDRMGDA-GFAAVEV 169
Consensus *.. :. : * *. : ** : :* * . *: *. ** * *
AURDE-GP11 IHLLASHSIADQAGVDPEPQVLGAPFDSTPFSFDSQVFLEVLFEGILFPGSGPNPGELMS 228
PHLRA-MnP2 VALLASHSIARADHVDP--TLDAAPFDSTPFDFDTQIFLEVLLKGVGFPGLANNTGEVSS 225
PHACH-MnP1 VSLLASHSVARADKVDQ--TIDAAPFDSTPFTFDTQVFLEVLLKGVGFPGSANNTGEVAS 224
BJEAD-LiP TWALIAHTVAAADDIDT--SIPRSPFDSTPELFDGQFFIETQLKGTLFPGNGPNKGEVRS 233
PHLRA-LiP3 VWFLIAHSVAAQNDIDP--AVSHAPFDSTPSVMDGQFFIETQLRGVEFIGSGGIEGVAES 232
PHACH-LiPH8 VWMLSAHSVAAVNDVDP--TVQGLPFDSTPGIFDSQFFVETQLRGTAFPGSGGNQGEVES 227
PLEOS-MnP4 VALLASHSVAAQDTIDP--KLAGHPFDSTPSDFDSQFFVETLLKGTLIPGDSLHKGQVKS 231
BJEAD-VP VWLLASHSIAAADHVDP--TIPGSPFDSTPEIFDTQFFVETLLKGTLFPGTSGNQGEVES 233
BB8-PLEER-VP VWLLASHSIAAADKVDP--SISGMPFDSTPGVFDSQFFIETLLKGRLFPGTAANKGEAQS 220
PLEOS-MnP5 VWLLSAHSVAAADHVDE--SIPGTPFDSTPNLFDSQIFIETQLRGISFPGTGGNHGEVQS 229
PLEOS-VP3 VWLLSSHTIAAADHVDE--SIPGTPFDSTPSIFDSQFFIETQLRGTSFPGSGGNHGEVES 227
Consensus * :*::* :* : ****** :* *.*:*. :.* : * . * *
AURDE-GP11 PLP-------GEFRLQSDKRFSLHNETACAWQENALSQSAMASNFQTAFTKLSLLGHDQN 281
PHLRA-MnP2 PLPVTDGTDVGELRLQSDFALARDERTACAWQSFVNEQEAMATAFKNAVKKLAVLGHNRN 285
PHACH-MnP1 PLPLGSGSDTGEMRLQSDFALAHDPRTACIWQGFVNEQAFMAASFRAAMSKLAVLGHNRN 284
BJEAD-LiP PLA-------GEMRLQSDFLIARDNRSACEWQSFGTDHDKLTNRFQFVLETLAMVGQDPT 286
PHLRA-LiP3 PVK-------GEFRLMSDQQIARDNRTACEWQSFGTDQAKLQNRFQFIFEAMGQLGTDPT 285
PHACH-LiPH8 PLP-------GEIRIQSDHTIARDSRTACEWQSFVNNQSKLVDDFQFIFLALTQLGQDPN 280
PLEOS-MnP4 PLP-------GEFRLQSDELLARDSRTSCEWQSFISNPNSMVPKFERAMAKMATLGQNPK 284
BJEAD-VP PLA-------GEIRLQSDADFARDSRTACEWQSFVNNQPRMQVLFKAAMQKLSILGHDLT 286
BB8-PLEER-VP PLQ-------GEIRLPSDHLLARDPQTACEWQSMVNNQPKIQNRFAATMSKMALLGQDKT 273
PLEOS-MnP5 PLR-------GEMRLQSDHLFARDDRTSCEWQSMTNDQQKIQDRFSDTLFKMSMLGQNQD 282
PLEOS-VP3 PLA-------GEIRLQSDHLLARDSRTSCEWQSMVDNMPKIQNRFAATMLKMSLLGQNQA 280
Consensus *: **:*: ** :: . .::* ** . : * . : :* :
AURDE-GP11 SLLDCSELIAQAPPATA--SQGFFPAGKTMEDLEIGCDAEAFPTTLVTQAGQPTMIPPVD 339
PHLRA-MnP2 DLVDCSAVVPVPKPATG--TPATFPASTGPQDLELTCTTEPFPTL-STAPGAQQTLIPHC 342
PHACH-MnP1 SLIDCSDVVPVPKPATG--QPAMFPASTGPQDLELSCPSERFPTL-TTQPGASQSLIAHC 341
BJEAD-LiP NMIDCSEVIPIPRNLTS-AQIPHFPAGKTIRDVEAACPETPFPRL-PTDAGRPTAVAPVP 344
PHLRA-LiP3 TLIDCSDVLPVPPPL---STVPHFPAGITINDVEPACAETPFPTL-PTDPGPATAVAAVP 341
PHACH-LiPH8 AMTDCSDVIPQSKPIPGNLPFSFFPAGKTIKDVEQACAETPFPTL-TTLPGPETSVQRIP 339
PLEOS-MnP4 KLIDCSEVIPVPRGR---VKQPTLPAGKTIKDIEASCRKAPFPRL-PTDKGTFTSILPVP 340
BJEAD-VP QMIDCSDVIPVPPST--AVRGSHLPAGNTLDDIEQACASTPFPTL-TADPGPATSVAPVP 343
BB8-PLEER-VP KLIDCSDVIPTPPAL---VGAAHLPAGFSLSDVEQACAATPFPAL-TADPGPVTSVPPVP 329
PLEOS-MnP5 AMIDCSDVIPVPAAL---VTKPHLPAGKVRTDVEQACATDAFPAL-AADPGPVTSVPRVP 338
PLEOS-VP3 DLIDCSDVIPTPPAL---VGKAHLPAGKVQSDVEQACATTPFPAI-AADPGPVTAVPPVP 336
Consensus : *** :: :**. *:* * ** : * :

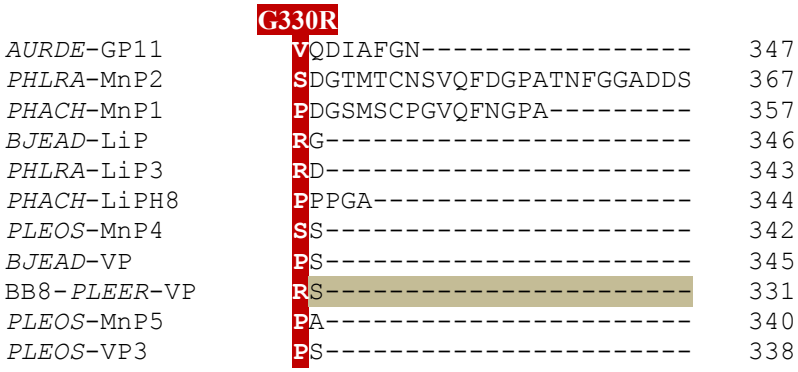

Fig. S2 MSA of representative ligninolytic peroxidases.

Sequences were obtained from the Uniprot database and aligned together with BB-8-*PLEER*-VP (the VP mutant from *Pleurotus eryngii*, O94753; obtained in the present study) using the Clustal Omega server (http://www.ebi.ac.uk/Tools/msa/clustalo/). The accession numbers used in the MSA were: *AURDE*-GP11 (GP11 from *Auricularia delicata;* J0WUI3); *PHLRA*-MnP2 (MnP2 from *Phlebia radiata;* Q70LM3); *PHACH*-MnP1 (MnP1 from *Phanerochaete chrysosporium;* Q02567); *BJEAD-LiP* (LiP from *Bjerkandera adusta;* W8YN06); *PHLRA*-LiP3 (LiP3 from *Phlebia radiata;* Q53WT9); *PHACH*-LiPH8 (LiPH8 from *Phanerochaete chrysosporium;* P06181); *PLEOS*-MnP4 (MnP4 from *Pleurotus ostreatus;* A0A067NYV2); *BJEAD*-VP (VP from *Bjerkandera adusta;* W8YE46); *PLEPU*-MnP5 (MnP5 from *Pleurotus pulmonarius;* Q2VT17); *PLEOS*-VP3 (VP3 from *Pleurotus ostreatus;* A0A067NKY1). The mutations in the stabilizing backbone are highlighted in red, those mutations that promote functional expression in yeast in cyan, and the mutations driving activity at alkaline pHs in dark brown, orange and green.

	AURDE-GP11	*PHLRA*-MnP2	*PHACH*-MnP1	*BJEAD*-LiP	*PHLRA*-LiP3	*PHACH*-LiPH8	*PLEOS*-MnP4	*BJEAD*-VP	BB8-*PLEER*-VP	*PLEOS*-MnP5	*PLEOS*-VP3
AURDE-GP11	**100**	45.9	46.3	43.5	43.3	45.2	46.0	48.5	47.0	43.0	44.6
PHLRA MnP2	45.9	**100**	72.8	41.7	45.0	47.6	48.0	52.7	48.6	49.5	49.4
PHACH-MnP1	46.3	72.8	**100**	43.6	47.4	47.3	50.6	56.8	52.9	50.6	51.0
BJEAD-LiP	43.5	41.7	43.6	**100**	61.1	58.5	54.4	59.9	56.8	57.0	56.0
PHLRA-LiP3	43.3	45.0	47.4	61.1	**100**	63.2	50.4	58.8	54.2	52.2	53.3
PHACH-LiPH8	45.2	47.6	47.3	58.5	63.2	**100**	55.2	64.3	55.9	54.2	56.5
PLEOS-MnP4	46.0	48.0	50.6	54.4	50.4	55.2	**100**	62.6	63.1	53.7	59.5
BJEAD-VP	48.5	52.7	56.8	59.9	58.8	64.3	62.6	**100**	69.5	62.0	67.3
BB8-*PLEER*-VP	47.0	48.6	52.9	56.8	54.2	55.9	63.1	69.5	**100**	66.8	75.5
PLEOS-MnP5	43.0	49.5	50.6	57.0	52.2	54.2	53.7	62.0	66.8	**100**	81.0
PLEOS-VP3	44.6	49.4	51.0	56.0	53.3	56.5	59.5	67.3	75.5	81.0	**100**

Identity Matrix from MSA

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reagents and strains

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), 2,6-DMP (2,6-Dimethoxyphenol), Reactive black 5 (RB5), sinapic acid, guaiacol, veratryl alcohol, syringaldazine, catechol, Manganese sulphate, *Taq* DNA polymerase, bovine hemoglobin, hemin from bovine, Horseradish peroxidase (HRP) and the *S. cerevisiae* transformation kit were purchased from Sigma-Aldrich (Madrid, Spain). Hydrogen peroxide 30 % (v/v) was obtained from Merck Millipore (Darmstadt, Germany). *Pfu*-Ultra polymerase, *Escherichia coli* XL1-Blue chemocompetent cells and the GeneMorph II Kit (mutazyme II polymerase) were from Stratagene (La Jolla, CA, USA). The Zymoprep Yeast Plasmid Miniprep Kit was obtained from Zymo-Research (Orange, CA, USA). The Low melting point agarose was from Bio-Rad (Hercules, CA), the NucleoSpin and PCR clean-up kit from Macherey-Nagel (Düren, Germany) and the *Bam*HI and *Xho*I restriction enzymes from New England Biolabs (Hertfordshire, U.K.). The protease-deficient *S. cerevisiae* strain BJ5465 (α ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL) was from LGCPromochem (Barcelona, Spain). The oligonucleotides used along the evolutionary process were purchased from Isogen Life Science (De Meern, The Netherlands). All chemicals were of reagent-grade purity.

Culture media

Synthetic complete (SC, minimum medium) contained 0.67% (w/v) yeast nitrogen base, 1.92 g/L yeast synthetic drop-out medium supplement without uracil, 2% (w/v) D-raffinose and 25 μg/mL chloramphenicol. SC-Hemin medium contained the recipe for SC plus bovine hemin (25 g/L) and 67 mM KH_2PO_4 buffer (pH 6.0). YP (1.55x) medium contained 10 g yeast extract, 20 g peptone and ddH_2O to 650 mL. Microplate expression medium (YP-Hb)

contained 720 mL YP (1.55x), 67 mL 1 M KH_2PO_4 buffer (pH 6.0), 111 mL 20% (w/v) D-galactose, 100 mg/L bovine hemoglobin, 1 ml 25 g/L chloramphenicol and ddH_2O to 1000 mL. Microplate YP-EtOH expression medium contained 720 mL YP (1.55x), 67 mL 1 M KH_2PO_4 buffer (pH 6.0), 111 mL 20% (w/v) D-galactose, 25 g/L ethanol absolute, 1 ml 25 g/L chloramphenicol and ddH_2O 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. Flask expression medium contained 720 mL YP (1.55x), 67 mL 1 M KH_2PO_4 buffer (pH 6.0), 111 mL 20% (w/v) D-galactose, 25 g/L ethanol absolute, 500 mg/L bovine hemoglobin, 1 mM $CaCl_2$, 1 mL 25 g/L chloramphenicol and ddH_2O to 1,000 mL. 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 and in the case of drop-out plates LB/amp plus 2% (w/v) bacto agar.

Re-screenings

First re-screening: Aliquots of 5 µL of the best clones were removed from the master plates and used to inoculate 50 µL of SC minimal medium (SC-Hemin 1st generation) in new 96-well plates. Columns 1 and 12, and rows A and H, were not used to prevent the appearance of false positives. After incubating for 24 h at 30 °C, 225 rpm, and 80% relative humidity, 5 µL was transferred to the adjacent wells and incubated for a further 24 h. Finally, 160 µL of YP-Hb microplate expression medium (YP-EtOH for 1st generation) was added and the plates were incubated for another 24 h. Accordingly, each mutant was grown in 4 wells. The parental types were subjected to the same procedure (row D, wells 7-11) and the plates were assessed using the same protocols as those used for the HTS-screening protocol.

Second re-screening: An aliquot from the wells with the best clones in the first re-screening 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 extracted very low, the zymoprep mixtures containing shuttle vectors were transformed into super-competent *E. coli* cells (XL1-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, Germany) and *S. cerevisiae* suspension was transformed with plasmids from the best mutants as well as with the parental type. Five colonies for each mutant were selected and re-screened as described above.

Third re-screening (determination of pH profiles, kinetic thermo-stabilities and pH stabilities): A single colony from the *S. cerevisiae* clone containing the parental 2-1B, the new mutants and untransformed yeast were picked from a SC drop-out plate (SC supplemented with uracil for untransformed cells), inoculated into 5 mL of minimal medium and incubated for 48 h at 30 °C and 225 rpm (Orbitron-INFORS, Biogen, Spain). An aliquot of cells was removed and used to inoculate a final volume of 5 mL of minimal medium in a 50 mL falcon tube (optical density, OD_{600} = 0.3), and they were incubated until two growth phases were completed (6-8 h, OD_{600} = 1). Thereafter, 9 mL of flask expression medium (500 mg/L bovine hemoglobin) was inoculated with 1 mL of this pre-culture in a 100 mL flask (OD_{600} = 0.1). After incubating for ~48 h at 30 °C and 225 rpm (maximal VP activity; OD_{600} = 25-30), the cells were separated by centrifugation for 15 min at 3,000 rpm and 4 °C (Eppendorf 5810R Centrifuge with F-34-6-38 rotor, Germany), and the supernatants were collected and stored at 4 °C.

pH activity profile: Parental type (2-1B) and the mutant winners of each round of evolution were assayed for activity in a pH range from 2 to 9. Aliquots (20 µL) of crude supernatants from *S. cerevisiae* were diluted to give a linear response in kinetic mode. Then, 180 µL of reaction mixture were added using a Multidrop station (Multidrop Combi, ThermoFischer Scientific,Vantaa, Finland). The final concentrations of reaction mixture per well were 100 mM citrate-phosphate-borate buffer at different pH (2-10), 0.1 mM of H_2O_2 and 2 mM ABTS. The absorbance was recorded at 418 nm in kinetic mode using the plate reader.

pH stability assay: Appropriate dilutions of the supernatants were prepared such that aliquots (20 µL) produced a linear response in kinetic mode. Each variant was diluted in 100 mM citrate-phosphate-borate buffer with pH ranging from 3.0-10.0. Aliquots of 20 µL were removed during 144h and measured in the presence of 180 µL of reaction. The final concentrations in the well were 100 mM citrate-phosphate-borate buffer pH 4.0, 0.1 mM of H_2O_2 and 2 mM ABTS. The absorbance was monitored at 418 nm in kinetic mode using a plate reader.

Thermostability assay (T_{50}): A gradient profile was constructed using a thermocycler (Mycycler, Bio-Rad, USA) for the selected mutants and the parental type, using 50 µL for each point in a gradient scale ranging from 30 to 80 °C. After a 10 min incubation, samples were removed and chilled on ice for 10 min. Thereafter, 20 µL samples were removed and incubated for 5 min at room temperature. Finally, 180 µL of 100 mM sodium tartrate buffer (pH 4.0), 2 mM ABTS and 0.1 mM H_2O_2 was added to the samples to measure activities. The thermostability values were calculated as the ratio between the residual activity at different temperature points and the initial activity at room temperature.

Production and Purification of VP variants

A single colony from transformed yeast cells was used to inoculate 20 mL of SC minimal medium (in a 100 mL flask) and incubated for 48h at 30 °C and 220 rpm (Orbitron-INFORS, Biogen, Spain). Then, OD_{600} was measured and this pre-culture used to inoculate 120 mL of minimal medium in a 250 ml flask (OD_{600}= 0.3). They were incubated until two growth phases had been completed (6-8 h, OD_{600} = 1) and thereafter, 450 mL of flask expression medium (500 mg/L bovine hemoglobin) was inoculated with 50 mL of this pre-culture in a 2 litre baffled flask (OD_{600}= 0.1). The cultures were incubated for 48h at 30 °C and 230 rpm (Micromagmix shaker) and the maximal VP activity reached (OD_{600} =25–30); the cells were recovered by centrifugation at 5,000 rpm for 30 min at 4 °C (Avanti J-E centrifuge Beckman Coulter with JA-14 rotor, Fullerton, CA). The supernatant was collected and triple filtered (through filter paper, a glass fibre filter and then a nitrocellulose membrane of 0.45 µm pore size).

Purification protocol: VP crude extracts were first submitted to a fractional precipitation with ammonium sulphate (50-75%). Samples were pelleted at 12,000 rpm, 15 min and 4 °C (Avanti J-E centrifuge Beckman Coulter with JA-14 rotor). The final pellet was recovered and dialyzed in 20 mM piperazine buffer (buffer P, pH 5.5), then the sample was filtered and loaded on to the FPLC (Äkta Purifier; GE Healthcare Uppsala, Sweden) coupled with a strong anion-exchange column (HiTraP QFF; GE Healthcare Uppsala, Sweden) pre-equilibrated with buffer P. The proteins were eluted with a linear gradient from 0 to 1 M of NaCl in two phases at a flow rate of 1 ml/min: from 0 to 25% over 60 min and from 25 to 100% over 5 min. Fractions with VP activity were pooled, concentrated and dialysed against buffer P with a stirred ultrafiltration cell and an ultracell 10 kDa ultrafiltration Disc of 44.5 mm (Amincon cell, Merck

Millipore, Germany). Thereafter, samples were loaded onto a HPLC–PDA column coupled with a 10 µm high resolution anion-exchange Biosuite Q (Waters) pre-equilibrated with buffer P. The proteins were eluted on a linear gradient from 0 to 1 M NaCl at a flow rate of 1 ml/min in two phases: from 0 to 6% in 30 min, and from 6 to 100% in 5 min. The fractions with VP activity were pooled, dialysed against 10 mM sodium tartrate buffer (pH 5.0), concentrated and stored at 4 °C. Throughout the purification protocol, the fractions were analysed by SDS/PAGE on 12% gels and the proteins were stained with colloidal Coomassie Blue (Protoblue Safe, National Diagnostics). Purified VP concentrations were determined spectrophotometrically (SHIMADZU UV-1800 spectrophometer, Columbia, MD, USA) in 1 mL quartz cuvettes with molar extinction coefficient for VP at 407nm (ε_{VP} =150,000 M^{-1}. cm^{-1}).The Reinheitszahl values (R_z: Abs_{407}/Abs_{280}) obtained were above 2.