Supplementary Materials

Supplementary Materials and Methods

Gene cloning

Transformation

The recombinant plasmids containing dye-decolorizing peroxidase (DyP) genes were transformed by electroporation into One Shot *E. coli* TOP10 competent cells (ThermoFisher Scientific, USA). The transformed cells were selected on an LB-agar plate containing appropriate antibiotics (37 °C overnight). The recombinant plasmids were transformed into *E. coli* BL21(DE3) strain by electroporation, and the transformed cells were grown in LB supplemented with the appropriate antibiotics at 37 °C overnight. Colonies containing the plasmid were confirmed by restriction digestion with NdeI and Xho and by Sanger sequencing (University of Tartu, Institute of Genomics, Estonia).

Enzyme Assays

Optimal pH

The optimal pH for 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,6-dimethoxyphenol (2,6-DMP) oxidation was determined for each DyP from *Streptomyces coelicolor* (*Sc*DyP) using 1 mM ABTS for *Sc*DyP1A and *Sc*DyP2A, 0.1 mM ABTS for *Sc*DyPB, and sodium citrate (50 mM, pH 2.5–3.5), sodium acetate (50 mM, pH 4.0–5.0), HEPES (50 mM, pH 7.0–8.0), Tris-HCl (50 mM, pH 8.5), and glycine (50 mM, pH 9.0–9.5) buffers. 2 mM 2,6-DMP, 1 mM H_2O_2, 500 nM *Sc*DyP2A, and 100 nM *Sc*DyPB were used for 2,6-DMP oxidation.

Temperature Dependence & Thermostability

Substrates were added to sodium citrate buffer (50 mM, pH 3.5) that was adjusted to a desired temperature and the reaction was started by the addition of the enzyme. The final concentrations of enzymes and substrates in each of the reaction mixtures were as follows: 100 nM *Sc*DyP1A (1 mM ABTS), 50 nM *Sc*DyP2A (1 mM ABTS), 15 nM *Sc*DyPB (0.1 mM ABTS), and 1 mM H_2O_2. For assessing the stability of DyPs, the enzymes were pre-incubated in Tris-HCl (20 mM, pH 7.5), 100 mM NaCl buffer at 30 °C. At selected times, aliquots were drawn and the ABTS oxidizing activity was measured using the concentration of the reagents outlined above. Concentrations of the enzymes in pre-incubation were 10 μM, 5 μM, and 1.5 μM for *Sc*DyP1A, *Sc*DyP2A, and, *Sc*DyPB, respectively.

Enzyme Kinetics

100 nM *Sc*DyP1A (0.1−5 mM ABTS), 50 nM *Sc*DyP2A (0.1−5 mM ABTS), 15 nM *Sc*DyPB (0.01−0.5 mM ABTS), 1 mM H_2O_2, sodium citrate buffer (50 mM, pH 3.5) were used for determining the apparent kinetic constants with ABTS. 500 nM *Sc*DyP2A (0–10 mM 2,6-DMP) and 100 nM *Sc*DyPB (0–10 mM 2,6-DMP), 1 mM H_2O_2, Tris-HCl buffer (50 mM, pH 8.5) were utilized for enzyme kinetics with 2,6-DMP. The observed rates were plotted against substrate concentrations and V_{max}, K_M, and K_i values were found using non-linear regression according to equation 1.

$$\frac{v}{E_0} = \frac{k_{cat}^{app}[S]}{K_M^{app} + [S] + \frac{[S]^2}{K_i^{app}}} \qquad \text{(Eq. 1)}$$

The curve fit and data analysis were carried out using OriginPro 2021 software.

Enzymatic Treatment of Organosolv Lignin

Gel Permeation Chromatography

Gel permeation chromatography (GPC) was used to follow changes in the molecular weight distribution of lignins. Prior to the GPC analysis, the samples were dissolved in tetrahydrofuran (THF). GPC analysis was carried out with a Prominence LC-20A Modular HPLC System (Shimadzu, Japan) equipped with a photodiode array detector. A GPC/SEC Guard Column (50 x 7.5 mm, Agilent Technologies, USA) and two Organic GPC/SEC PLgel MesoPore columns connected in tandem (300 mm × 7.5 mm, inner diameter, 3 μm, Agilent Technologies) were calibrated using the EasiVial PS-L polystyrene standards for GPC/SEC (Agilent Technologies). The following conditions were used – mobile phase: THF stabilized with 250 ppm butylated hydroxytoluene in isocratic mode; flowrate: 1 mL/min; column oven temperature: 40 °C and UV detection: 254 nm. Each analysis was performed in triplicate. The number average molecular weight (Mn), weight average molecular weight (Mw), and the molar-mass dispersity ($Đ_M$) were determined with GPC postrun software (Shimadzu). Statistical significance was determined by analysis of variance (ANOVA), which was established at $p \leq 0.05$.

Gas Chromatography-Mass Spectrometry

To analyze low molecular weight compounds (LMW) in *Mxg*- and aspen lignins treated with *Sc*DyPs, and without enzymatic treatment, gas chromatography-mass spectrometry (GC-MS) was used. The reactions were centrifuged, and the supernatant was extracted with ethyl acetate, lyophilized, and dissolved in methanol. 1 μL of the sample was injected in splitless mode at 250 °C to a 7890A gas chromatograph coupled to a 5975C mass spectrometer (Agilent Technologies) with an electron ionization source and a quadrupole mass analyzer. The flow rate of carrier gas (helium, 6.0 purity) was kept constant at 1.3 mL/min. LMW compounds in lignin were separated in a ZB-5plus capillary column (30 m x 0.25 mm x 0.25 μm, Agilent Technologies). The following oven temperature program was used: the initial temperature was 35 °C (1 min), then increased to 300 °C (10 °C min^{-1}, held for 2 min). The total run time was 29.5 min. The analyte ionization was performed in electron ionization mode using the electron energy of 70 eV. The interface, ion source, and mass analyzer temperatures were set at 280, 230, and 150 °C, respectively. Scan mode in the range of 30–500 m/z was used for monitoring all analytes. LMW compounds of lignin were determined by the National Institute of Standards and Technology 17 (NIST 17) library. Agilent MassHunter Qualitative Analysis was used for data analysis. The fragment (m/z) with the highest intensity characteristic for each compound was used for quantification.

Table S1. Oligonucleotide primers used for gene amplification.

	forward	reverse
SCO2276 (*Sc*DyP1A)	5'-GAC CGC GCC GGT GCC CAT ATG GAC CCG GCC GGT GCC-3'	5'-GAT CAG GTA GCT CGA GAA CAC CGT TAC GCC TCC TTG-3'
SCO3963 (*Sc*DyP2A)	5'-CGC AGC CGC CCC CTC GCA TAT GGC GAC CCC CCT CAC-3'	5'-CCG CGG GGT GAT ACG CCC CTC GAG TCA CCC CTC CAG-3'
SCO7193 (*Sc*DyPB)	5'-GGA GGA TGT TCG CAT ATG GGC GGA GAA GTC-3'	5'-CGG GTC GGG TCG CTC GAG GCT CAG GGC CGA G-3'

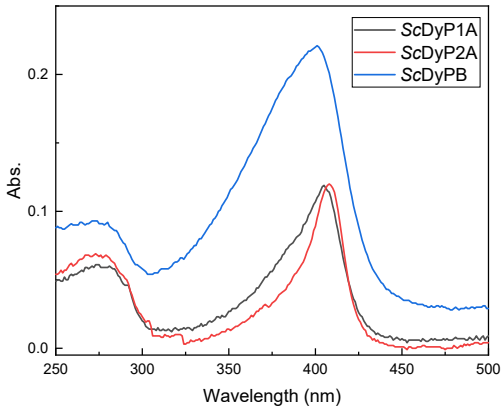

Figure S1. Spectra of *Sc*DyPs. The enzymes were diluted in Tris-HCl (20 mM, pH 7.5), 100 mM NaCl buffer, and the spectrum of each *Sc*DyP was recorded (250–500 nm). *Sc*DyP1A showed the Soret band maximum at 408 nm, *Sc*DyP2A at 405 nm, and *Sc*DyPB at 401 nm.

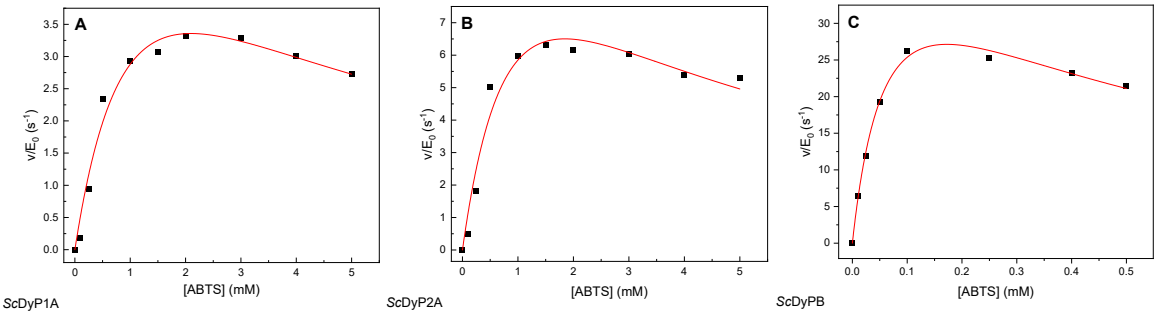

Figure S2. Steady-state kinetics of *Sc*DyPs against ABTS as a substrate: *Sc*DyP1A (panel A), *Sc*DyP2A (panel B), and *Sc*DyPB (panel C). Experiments were performed using sodium citrate (50 mM, pH 3.5) buffer and 1 mM H_2O_2 as a co-substrate. In red, fitted curves are shown employing the substrate inhibition equation (Eq 1).

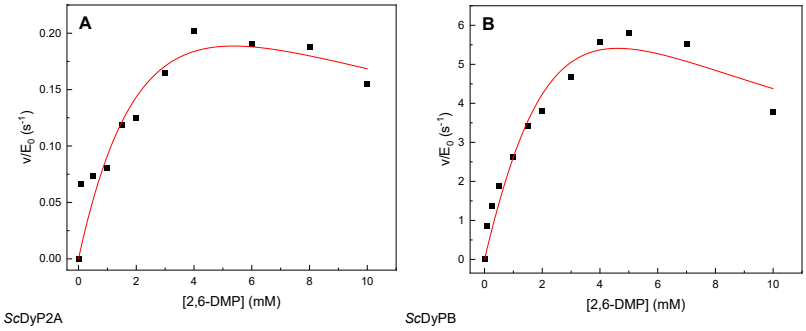

Figure S3. Steady-state kinetics of *Sc*DyP2A and *Sc*DyPB against 2,6-DMP: *Sc*DyP2A (panel A), *Sc*DyPB (panel B). Experiments were performed using Tris-HCl (50 mM, pH 8.5) buffer and 1 mM H_2O_2 as a co-substrate. In red, fitted curves are shown employing substrate inhibition equation (Eq 1).