Supplementary Information

Enhanced depolymerization of beech wood lignin and its removal with peroxidases through continuous separation of lignin fragments

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Method S1 – Preparation of crude MnP and LiP

The genes encoding MnP of *Ceriporiopsis subvermispora* (Joint Genome Institute (JGI) protein code: 117436) and LiP of *Phanerochaete chrysosporium* (JGI protein code: 2989894) were codon-optimized for *P. pastoris* and synthesized by Thermo Fisher Scientific. The genes were each subcloned into the pPICZαA (Invitrogen, USA) vector to obtain pPICZαA-MnP and pPICZαA-LiP vectors. These vectors were linearized with *Sac*I restriction enzyme and introduced individually into *P. pastoris* X-33 (Invitrogen, USA) by electroporation. Transformed clones for MnP and LiP were confirmed by colony PCR, a small-scale expression check, and activity assaying. The cloned transformants for MnP and LiP were used for the preparation of crude MnP and crude LiP solutions; the same procedure was used for their preparation. The transformant for either MnP or LiP was cultivated on YPDS agar medium (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol, 2% agar, and 100 μg mL^{-1} zeocin) for 3 days at 30 °C. An isolated zeocin-resistant colony was cultured in BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base with ammonium sulfate, 0.1 M potassium phosphate (pH 6), 0.4 μg mL^{-1} biotin, and 1 vol% glycerol) at 30 °C with 200 rpm shaking until OD_{600} reached 6. The cells were collected by centrifugation at 3,500 × g for 5 min, and resuspended to an OD_{600} of 1.0 in BMMY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base with ammonium sulfate, 0.1 M potassium phosphate (pH 6), 0.4 μg mL^{-1} biotin, and 0.5 vol% methanol) to induce protein expression. The induction was conducted at 15 °C. During the induction, methanol and a hemin stock (10 mg mL^{-1} hemin in a 0.1 M ammonia solution) were added to give final concentrations of 0.5% and 0.35%, respectively; this was repeated every twenty-four hours. After three days the culture was centrifuged at 4,400 × g for 30 min to obtain a supernatant containing the secreted enzyme. Then the supernatant was filtered with a 300-kDa cut-off membrane (Pall, USA), concentrated 20-fold, and buffer-exchanged to 50 mM sodium malonate (pH 4.5) with a 10-kDa cut-off membrane (Pall), all using a Minimate TFF system (OAPMP110, Pall). The resulting solution, either the crude MnP or crude LiP, is used for further experiments.

Method S2 – SEC of the products released into the aqueous phase

The products released into the aqueous phase in the batch and membrane bioreactors were subjected to SEC. A sample was diluted with 10 mM NaOH in a 1:1 ratio and then filtered with a 0.2 μm hydrophilic polytetrafluoroethylene (PTFE) membrane. 20 μL of the sample was injected into a high-performance liquid chromatography (HPLC) system (Shimadzu, Japan) equipped with an SPD-20A UV-Vis detector 280 nm absorbance being used for detection. A TSKgel G3000PW$_{XL}$ (7.8 mm I.D. × 300 mm, Tosoh, Japan) analytical column was used, which was held at 35 °C throughout the analysis. The mobile phase was a 10 mM NaOH solution at a flow rate of 0.5 mL min^{-1}.

Method S3 – GC-MS analysis of the depolymerized products in the filtrate

To obtain product M_19, the filtrate fractions were pooled and lyophilized. The resulting solid was dissolved in 10 mM NaOH and injected into an HPLC system (detailed description in Method S2). M_19 was collected based on the elution profile and subsequently neutralized to pH 7 before being lyophilized again. To prepare for GC-MS analysis, the dried M_19 sample was dissolved in ethyl acetate and trimethylsilylated with N,O-Bis(trimethylsilyl)trifluoroacetamide with 1 vol% chlorotrimethylsilane and pyridine. The trimethylsilylated sample was then concentrated under nitrogen gas for analysis by GC-MS.

For the GC-MS analysis, 1 μL of the trimethylsilylated sample was injected in split mode into a GCMS-QP2010SE system (Shimadzu, Japan) equipped with a DB-5MS column (30 m × 0.25 mm id, 0.25 μm film thickness; Agilent Technologies). The injection temperature was set at 250 °C, and the carrier gas was helium at a flow rate of 1.0 mL min^{-1}. The initial column temperature was held at 50 °C for 3 min, then increased to 280 °C at a rate of 6 °C min^{-1}, and held for 3 min. The mass spectrometer was operated in electron impact ionization mode with ionizing energy of 70 eV, an ion source temperature of 250 °C, and an interface temperature of 300 °C. The mass scanning range was set to m/z 40-900.

Method S4 – Chemical composition analysis of the solid residues

The percentages of lignin (LPs) of MBW, RES_{batch}, and $RES_{membrane}$ were determined by both the Klason lignin method and the UV-Vis spectroscopic method. The quantification by both methods was performed in duplicate.

The Klason lignin method was carried out based on a previously reported method. Briefly, 70 mg of a sample was hydrolyzed with 0.7 mL of 72% w/w sulfuric acid for 1 h at 30 °C. Then, it was diluted with 7.7 mL of hot water and further hydrolyzed for 3 h at 100 °C. After hydrolysis, the sample was filtered and washed extensively with Milli-Q, and then dried at 105 °C until a constant weight was reached. The LP was obtained as a percentage of the dried solid remaining after hydrolysis relative to the initial sample weight.

The UV-Vis spectroscopic method was performed based on the method established by Zhang *et al*. recently, with several modifications. Measurement was performed using a UV-Vis spectrophotometer (Shimadzu UV-1900i, Japan) with quartz cuvettes of 1 cm path-length. 5 mg of a sample was incubated in 1 mL of 8% LiCl/DMSO (w/w) at room temperature for 24 h with stirring. After the 24 h incubation, the mixture was diluted to 1 mg/mL with 8% LiCl/DMSO and then stirred at 80 °C for another 48 h. The resultant solution was further diluted accordingly to adjust the absorbance to within the range of 0.2-0.8. With the 8% LiCl/DMSO solvent as a blank control, the diluted samples were scanned in the range of 200-800 nm. The absorbance value of each sample was determined at 276 nm. The LP of the sample was calculated with Eq. (1) where A is the absorbance at 276 nm for the diluted sample, V the total volume of the diluted sample, d the dilution ratio, ε the deduced extinction coefficient of hardwood lignin (13.235 L g^{-1} cm^{-1}), L the length of the light path (1 cm), and m_0 (5 mg) the weight of the sample incubated in 8% LiCl/DMSO.

$$LP = \frac{A \times V \times d}{\varepsilon \times L \times m_0} \times 100\% \quad (1)$$

The content of cellulose and hemicellulose were determined using Eq. (2) and Eq. (3), respectively, from the hydrolysis liquor obtained from the filtration step in the Klason lignin experiment. The content of cellulose (glucan) and hemicellulose (xylan, galactan, arabinan, and mannan) were determined using a HPLC system (Shimadzu, Japan) equipped with a refractive index detector (RID-20A, Shimadzu). The analysis was performed utilizing an Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA, USA) analytical

column maintained at 60 °C. The mobile phase consisted of a 5 mM H_2SO_4 solution at a flow rate of 0.6 ml min^{-1}. To quantify the compounds present in the sample, a calibration curve was established using standards with varying concentration (0.1 to 4 mg mL^{-1}) of glucose, xylose, galactose, arabinose, and mannose. Before analysis, samples were diluted four-fold with 5 mM H_2SO_4 and filtered through a 0.2 µm hydrophilic polytetrafluoroethylene (PTFE) membrane. For analysis, 10 µL of each sample was injected into the HPLC system.

$$\%\ Cellulose = \frac{m_{glucan}}{m_{substrate,\ dry}} \times 100\% \quad (2)$$

$$\%\ Hemicellulose = \frac{m_{xylan} + m_{galactan} + m_{arabinan} + m_{mannan}}{m_{substrate,\ dry}} \times 100\% \quad (3)$$

Method S5 – SEC for solid residues

The molecular weight distributions of lignin in MBW, RES_{batch}, and $RES_{membrane}$ were determined by SEC. Before being subjected to SEC analysis, a sample was pretreated as follows. 10 mg of the sample was treated twice with 5.6 FPU of a cellulolytic enzyme cocktail (CellicCtec2, Novozymes, Denmark) in 50 mM sodium citrate (pH 5) at 50 °C for 72 h to yield cellulolytic enzyme lignin (CEL). 1 mg of CEL was then acetylated by incubation in 500 µL of a 1:1 v/v mixture of anhydrous pyridine and acetic anhydride for 48 h at room temperature with stirring. The acetylated sample was then co-evaporated with toluene to remove the reagents. The obtained acetylated CEL was dissolved in tetrahydrofuran, and the soluble fraction was passed through a 0.2 µm PTFE filter. 10 µL of the filtered sample was injected into a HPLC system equipped with an SPD M20A photo diode array detector and three tandemly connected TSKgel SuperMultiporeHZ-M analytical columns (4.6 mm I.D. × 150 mm, Tosoh). The analysis was performed at 40 °C using tetrahydrofuran as a mobile phase at a flow rate of 0.35 mL min^{-1}.

Table S1 Chemical composition of solid residues based on Klason lignin analysis

Sample	Recoveries[a] (%)	Cellulose (%)	Hemicellulose (%)	Lignin content (%)
MBW	-	36.4 ± 1.6	31.7 ± 2.3	24.5 ± 0.4
Batch bioreactor				
No enzyme	96.5	36.1 ± 1.9	31.4 ± 1.5	22.8 ± 0.4
MnP	92.5	36.7 ± 1.0	31.9 ± 0.8	21.4 ± 0.6
LiP	92.9	36.5 ± 2.2	31.7 ± 1.2	19.8 ± 0.3
Membrane bioreactor				
No enzyme	96.1	36.2 ± 1.7	31.6 ± 0.4	22.9 ± 0.2
MnP	89.6	37.8 ± 1.4	31.5 ± 0.9	16.9 ± 0.6
LiP	88.3	38.6 ± 0.6	32.1 ± 2.1	14.3 ± 0.5

[a]Recoveries (%) of the residues after the reaction relative to the starting material.

Table S2 Assignment of the lignin ^{1}H–^{13}C correlation signals of the HSQC NMR spectrum

Label	δ_C/δ_H (ppm/ppm)	Assignment
B_β	53.3/3.46	C_β–H_β in phenylcoumaran substructures
C_β	53.5/3.06	C_β–H_β in resinol substructures
-OMe	55.6/3.73	C–H in methoxy groups
A_γ	59.5–59.7/3.4–3.7	C_γ–H_γ in β-*O*-4′ substructures
B_γ	62.6/3.73	C_γ–H_γ in phenylcoumaran substructures
A_α (G)	70.9/4.71	C_α–H_α in β-*O*-4′ substructures linked to a guaiacyl unit
C_γ	71.0/3.79 and 4.16	C_γ–H_γ in resinol substructures
A_α (S)	71.8/4.81	C_α–H_α in β-*O*-4′ substructures linked to a syringyl unit
A_β (G)	83.5/4.27	C_β–H_β in β-*O*-4′ substructures linked to a guaiacyl unit
C_α	84.9/4.64	C_α–H_α in resinol substructures
A_β (S)	85.9/4.09 and 86.9/3.97	C_β–H_β in β-*O*-4′ substructures linked to a syringyl unit
B_α	86.8/5.46	C_α–H_α in phenylcoumaran substructures
$S_{2,6}$	103.8/6.71	$C_{2,6}$–$H_{2,6}$ in syringyl unit
$S'_{2,6}$	106.2/7.23 and 7.07	$C_{2,6}$–$H_{2,6}$ in C_α–oxidized syringyl unit
G_2	110.9/6.98	C_2–H_2 in guaiacyl unit
G_5	114.9/6.77	C_5–H_5 in guaiacyl unit
G_6	119.0/6.80	C_6–H_6 in guaiacyl unit

Table S3 Lignin interunit linkages from integration of ^{1}H-^{13}C correlation peaks in the HSQC spectra of the residues after enzymatic reaction in batch and membrane bioreactor[a]

	Batch bioreactor			Membrane bioreactor		
	No enzyme	MnP	LiP	No enzyme	MnP	LiP
Interunit linkages (%)[b]						
β-O-4′ (A)	87.5	91.1	91.5	89.3	100.0	100.0
β-5′ (B)	4.1	3.2	2.5	3.2	nd	nd
β-β′ (C)	8.4	5.7	6.0	7.5	nd	nd

[a] The volume of each signal was normalized as to the signal volume of DSS-d_6

[b] Expressed as a percentage of the total lignin interunit linkage types A-C

[c] nd = not detected

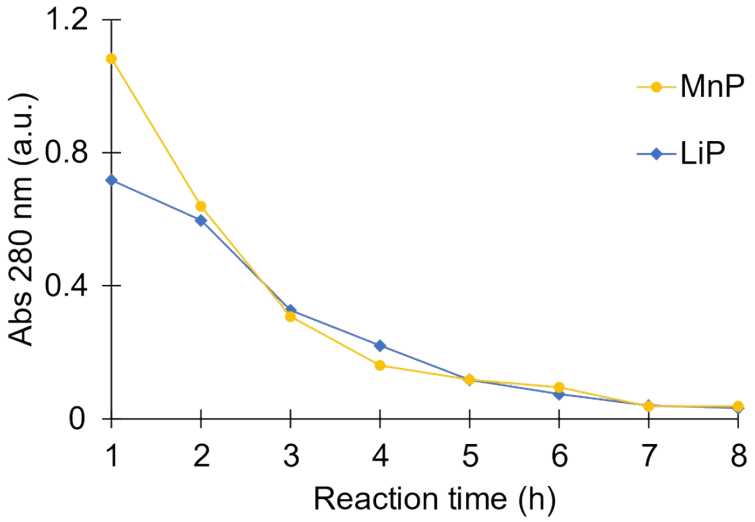

Figure S1 Evolution of product M_19 in the filtrate fraction during the incubation of MBW with either MnP (yellow line) or LiP (blue line) as analyzed by SEC.

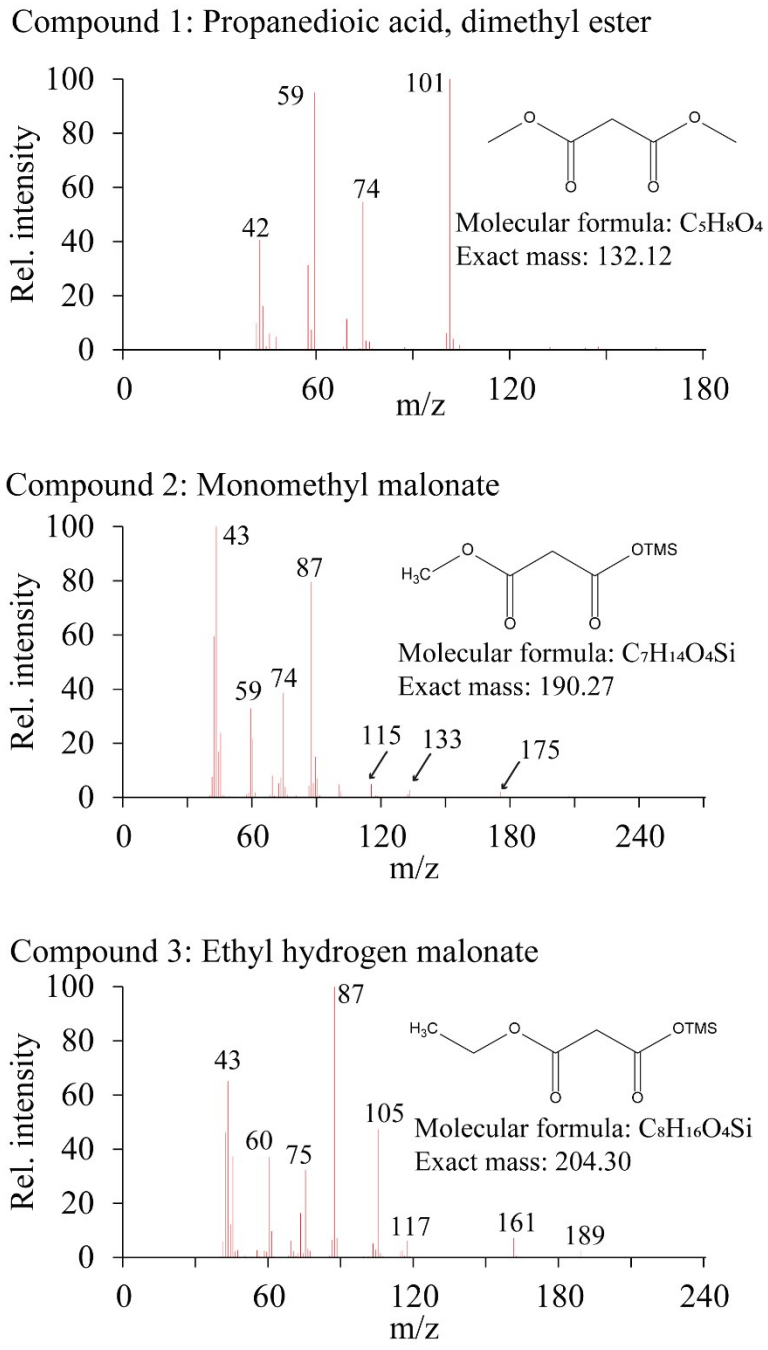

Figure S2 The EI mass spectra of the main depolymerized products of beech wood lignin obtained in the filtrate fraction.

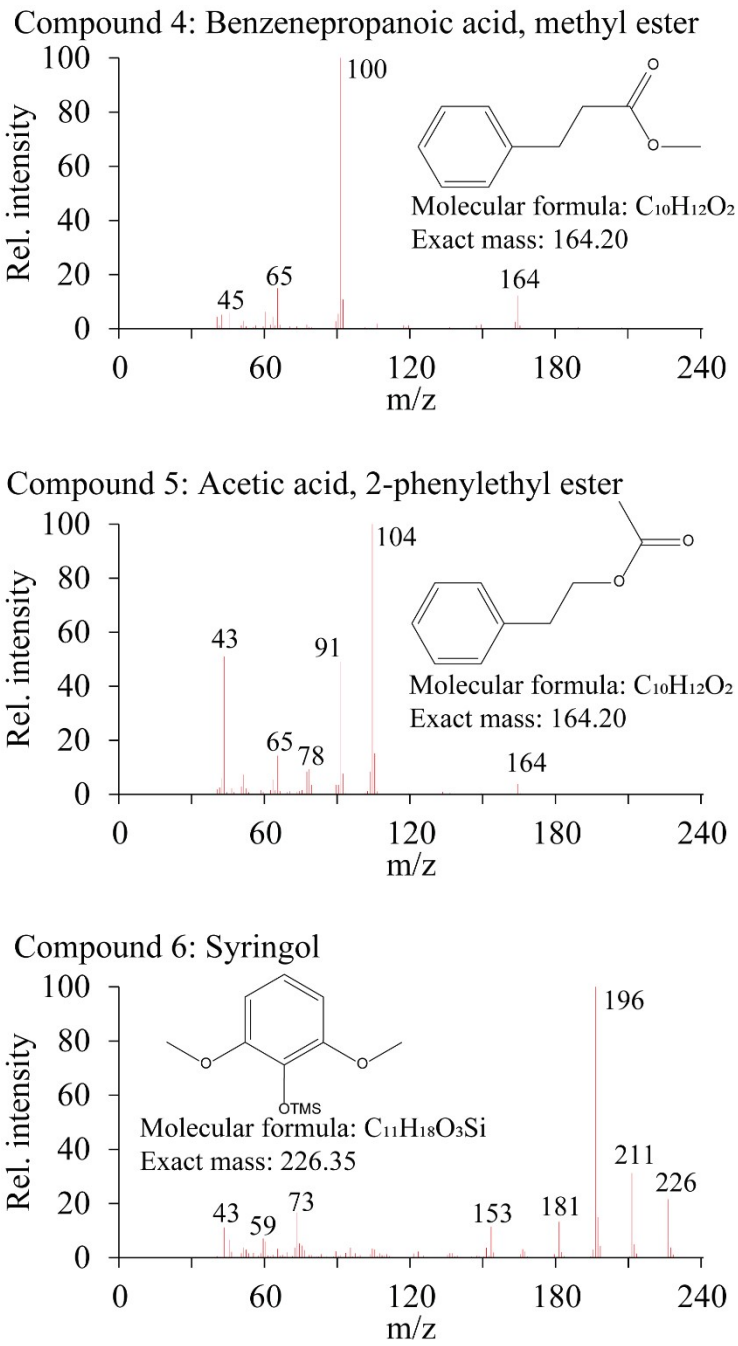

Figure S3 The EI mass spectra of the main depolymerized products of beech wood lignin obtained in the filtrate fraction.

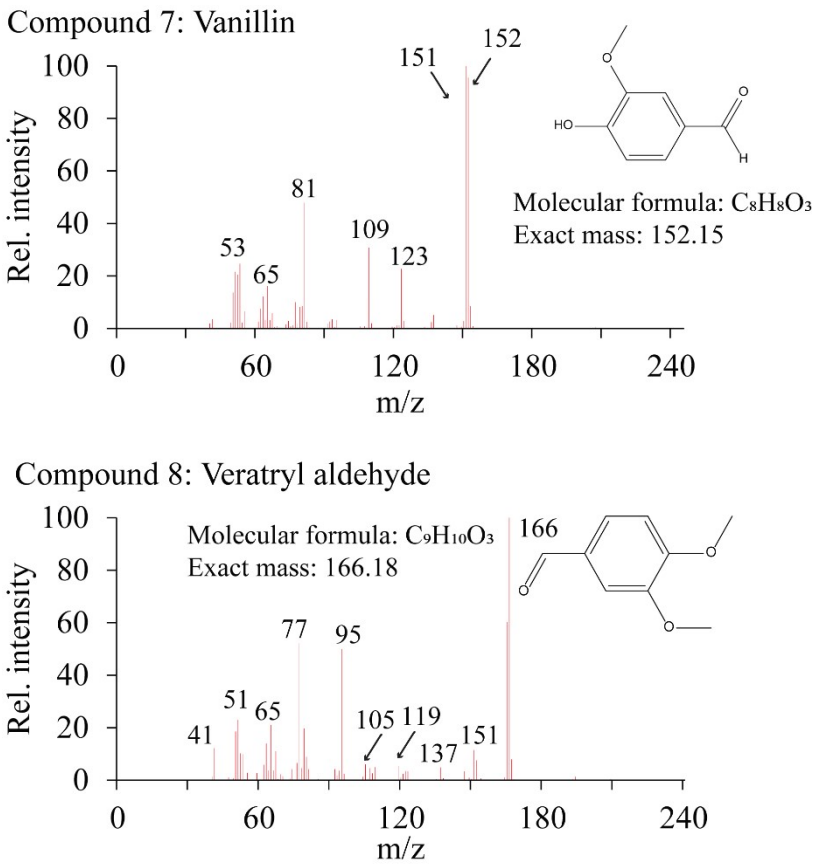

Figure S4 The EI mass spectra of the main depolymerized products of beech wood lignin obtained in the filtrate fraction.