Final technical report
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Specific Aim 1. Identify the minimal set of genes from sequenced wood-rotting fungal species necessary and sufficient for the early breakdown of lignin biomass.

In this project, we demonstrate that expression of fungal genes in Nicotiana benthamiana enables production of members from seven major classes of enzymes associated with lignin degradation (23 of 35 tested) in soluble form for direct use in lignin activity assays. We combinatorially characterized a subset of these enzymes in the context of model lignin dimer oxidation, revealing that fine-tuned coupling of peroxide-generators to peroxidases results in more extensive C-C bond cleavage compared to direct addition of peroxide (**Figure 1, 2**). Comparison of peroxidase isoform activity revealed that the extent of C-C bond cleavage depends on peroxidase identity, suggesting that peroxidases are individually specialized in the context of lignin oxidation. We anticipate the use of *N. benthamiana* as a platform to rapidly produce a diverse array of fungal lignin-degrading enzymes will facilitate a better understanding of their concerted role in nature and unlock their potential for lignin valorization, including within the plant host itself (**Figure 3**).

FIGURE 1:

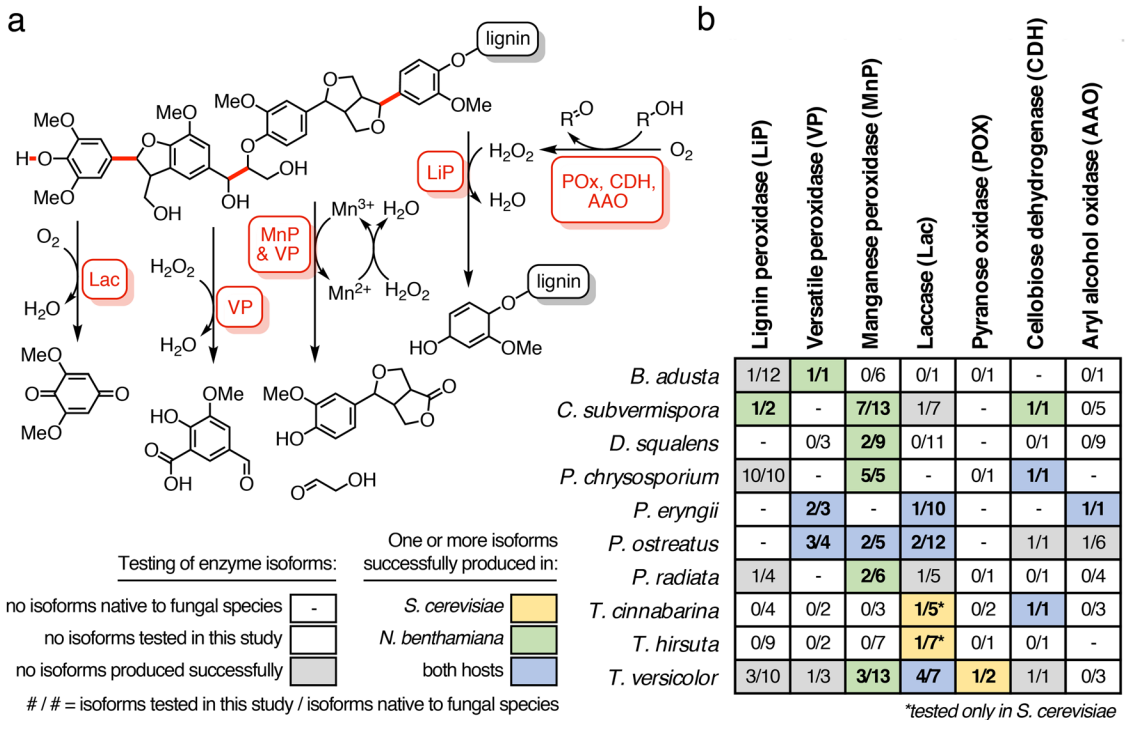

Figure 1. Overview of fungal enzymatic lignin deconstruction in nature. a A representative schematic of bond cleavage catalyzed by different families of ligninases. Featured in red are carbon–carbon bonds that can be cleaved by different types of heme peroxidases (MnP, VP, LiP), as well as phenolic bonds that can be cleaved by laccases (Lac). Auxiliary enzymes such as aryl alcohol oxidase (AAO), cellobiose dehydrogenase (CDH), and pyranose oxidase (POX) are shown as examples that can be coupled to peroxidase activity on lignin bonds. b An

overview of ligninases tested in this study. Each entry indicates the number of isozymes tested out of the total number of isozymes native to each fungal species. Entries bolded and highlighted in yellow indicate successful heterologous production in S. cerevisiae, those in green for N. benthamiana, and those in blue for both species of one or more isozymes in that category. See Fig. 2 for more detailed activity analysis of individual enzymes. Entries with dashes indicate the absence of that enzyme type in the fungal species, and those having zero as the first number indicate that no representative genes from that species and enzyme class were synthesized or tested. Laccases from T. cinnabarina and T. hirsuta were tested only in S. cerevisiae and are denoted with an asterisk.

FIGURE 2:

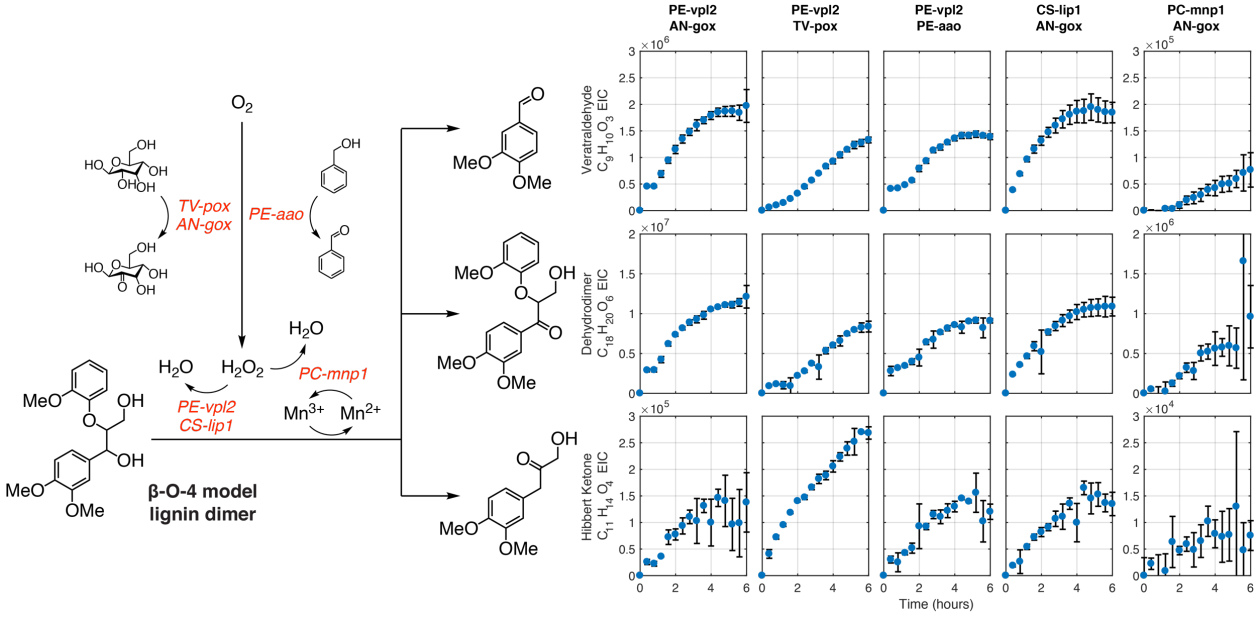

Fig. 2 Cleavage of a model β-O-4 lignin dimer using heterologous lignin-degrading enzymes produced by N. benthamiana. a Diafiltrated extracts of a versatile peroxidase (PE-vpl2), a lignin peroxidase (CS-lip1), or a manganese peroxidase (PC-mnp1) were coupled with peroxide-generating sugar oxidases (TV-pox or AN-gox) or aryl alcohol oxidase (PE-aao(FX9)) in the presence of corresponding co-substrates and under the indicated pH conditions. PE-aao but not PE-vpl2 was found to have activity toward benzyl alcohol (Bn-OH), meaning this coupling reaction could proceed without interference between the two enzymes (Supplementary Fig. 15). Decomposition products (veratraldehyde, dehydrodimer, and Hibbert ketone) were tracked over the course of the reaction using LC-MS. b Dimer oxidation by GOx-coupled diafiltrated extracts of two isoforms of versatile peroxidase from P. ostreatus (PO-vp1 and PO- vp3) were compared to PE-vpl2, CS-lip1, and PC-mnp1 under direct oxidation and Mn(III)-mediated oxidation conditions. c Activity phase diagram of white- rot lignin-degrading peroxidases. The extent of dimer conversion to veratraldehyde and dehydrodimer in reactions containing peroxidase isozymes coupled to glucose oxidase was measured under direct-oxidation conditions (pH 3.5) and Mn(III)-mediated conditions (pH 4.5 with 1 mM MnSO4). The extent of dimer cleavage was calculated as the proportion of veratraldehyde EIC relative to the sum of veratraldehyde EIC and dehydrodimer EIC. The maximum dimer cleavage extent under either condition is displayed in the peroxidase isozyme labels and represented by marker size. Data points represent the

average of three independent reaction replicates with error bars calculated as one standard deviation.

FIGURE 3:

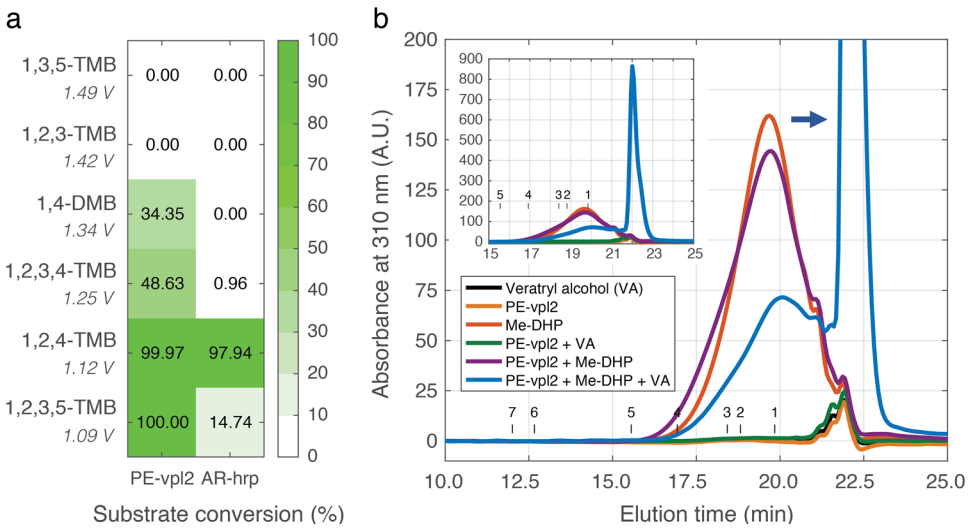

Fig. 3 Heterologous lignin-degrading enzymes catalyze in vitro oxidation and depolymerization of lignin-related substrates. a Comparison of methoxybenzene oxidation by heterologous FPLC-purified PE-vpl2 extracted from N. benthamiana as compared to peroxidase from horseradish. Values represent substrate consumption relative to a no-enzyme control and are the average of three independent reaction replicates. DMB dimethoxybenzene, TMB trimethoxybenzene or tetramethoxybenzene. Redox potentials previously reported19,50 for methoxybenzenes are shown for each substrate. b Gel permeation chromatography (GPC) of methylated DHP lignin (Me-DHP) subjected to depolymerization by PE-vpl2 purified by FPLC after heterologous production in N. benthamiana. Arrows indicate a decrease in lignin molecular weight by PE-vpl2 relative to a no-enzyme control. In vitro conditions involved 200 µg ml−1 methylated DHP lignin, 10 mM veratryl alcohol, 0.25% v/v Tween-20, and 0.66 µM purified enzyme in 10 mM sodium acetate, pH 4.5. 100 µM hydrogen peroxide was added every 1.5 h for a total of 600 µM. The entire reaction contents were lyophilized and reconstituted in dimethylformamide containing 0.1 M lithium bromide for analysis by GPC. Control reactions had veratryl alcohol omitted (PE-vpl2 + Me-DHP), enzyme omitted (Me-DHP alone), lignin omitted (veratryl alcohol + PE-vpl2), both enzyme and lignin omitted (veratryl alcohol alone), and both lignin and veratryl alcohol omitted (PE-vpl2 alone). Absorbance traces shown here are representative of reaction duplicates. The molecular weights of polystyrene standards used for calibration were as follows: (1) 1.37 kg mol−1; (2) 2.93 kg mol−1; (3) 4.43 kg mol−1; (4) 10.1 kg mol−1; (5) 21.7 kg mol−1; (6) 139 kg mol−1; (7) 281 kg mol−1.

Specific Aim 3. Engineer a microbial strain with tunable metabolism for lignin utilization that efficiently converts lignin waste into valuable carbon feedstocks.

In efforts towards this aim, we have tested *in planta* heterologous expression of the fungal ligninases characterized in Aim 1 and attempted to quantify changes in lignin amount and cellular structure. As part of this effort, we have also transient expressed lignin-biosynthesis TFs (see: https://doi.org/10.1021/acssynbio.2c00289) to reinitiate lignin deposition in the plant pavement cells of interest. While to date these results have not revealed changes in lignin content or structure as a result of expression of funal ligninases in planta, we think further optimization could result in lignin breakdown in planta given the ability of plant cells to express fungal ligninases in active form.