Trends in Biotechnology

CellPress

Review

Synthetic Biology towards Engineering Microbial Lignin Biotransformation

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Lignin is the second most abundant biopolymer on earth and is a major source of aromatic compounds; however, it is vastly underutilized owing to its heterogeneous and recalcitrant nature. Microorganisms have evolved efficient mechanisms that overcome these challenges to depolymerize lignin and funnel complex mixtures of lignin-derived monomers to central metabolites. This review summarizes recent synthetic biology efforts to enhance lignin depolymerization and aromatic catabolism in bacterial and fungal hosts for the production of both natural and novel bioproducts. We also highlight difficulties in engineering complex phenotypes and discuss the outlook for the future of lignin biological valorization.

Highlights

A sustainable lignocellulosic bioeconomy will not be realized without overcoming hurdles associated with the structural complexity associated with lignin waste streams.

Metabolic engineers capitalize on robust, naturally occurring funneling pathways that convert a wide spectrum of substrates to a few key intermediates for ring cleavage and conversion to central metabolites.

Expanding the reaction conditions and the range of substrates for lignin depolymerization and funneling enzymes should improve lignin valorization by microorganisms.

Enabling the Lignocellulosic Bioeconomy through Lignin Valorization

Lignin (see Glossary) is the second most abundant biopolymer on Earth and acts as the structural component of plants. It is a recalcitrant, highly branched aromatic polymer composed of three main monomer subunits – guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units – connected through diverse linkages that vary among plant species. Two primary sources of lignin are agricultural waste and Kraft lignin from paper mills. Kraft pulping plants generate ~130 million tons per year [1]. Agricultural waste streams have the potential to produce an additional 225 million tons of lignin feedstock per year [2]. Despite this generous feedstock potential, lignin is mostly used for its process heat value [2–4]. Economic evaluations demonstrate lignin valorization is vital for a viable biobased economy.

Biological systems are better suited to handling heterogeneous mixtures than physiochemical systems because lignin-degrading microbes utilize **funneling pathways** (Figure 1) to channel multiple **lignomonomers** through key intermediates. Many organisms exhibiting this phenotype are non-conventional and difficult to engineer. Although **synthetic biology** tool development is becoming more common for several organisms, non-conventional organisms remain largely genetically inaccessible. While many reviews have covered decades of lignin research, this review addresses the role that synthetic biology has started to play in biotransforming lignin by bacteria and fungi, and discusses the strengths and weaknesses of each host. It should be noted that we differentiate between unicellular fungi, hereafter called yeast, and filamentous fungi, hereafter called fungi, because of their vastly different potential for engineering and large-scale cultivation. In addition, we remark on recent developments in sequencing and computational biology and how their combination can not only identify new engineering targets but progress our understanding of lignin biotransformation at a genetic, protein, and systems level. Lastly, we provide our perspectives on the future of biological lignin valorization that will be enabled by the transformative impact of synthetic biology.

Lignin Enzymatic Depolymerization

Although several excellent reviews discuss common biological and catalytic methods for lignin depolymerization [5–10], this review focuses on advances in synthetic biology efforts towards microbial lignin depolymerization, as summarized in Table 1. White-rot fungi are the most efficient

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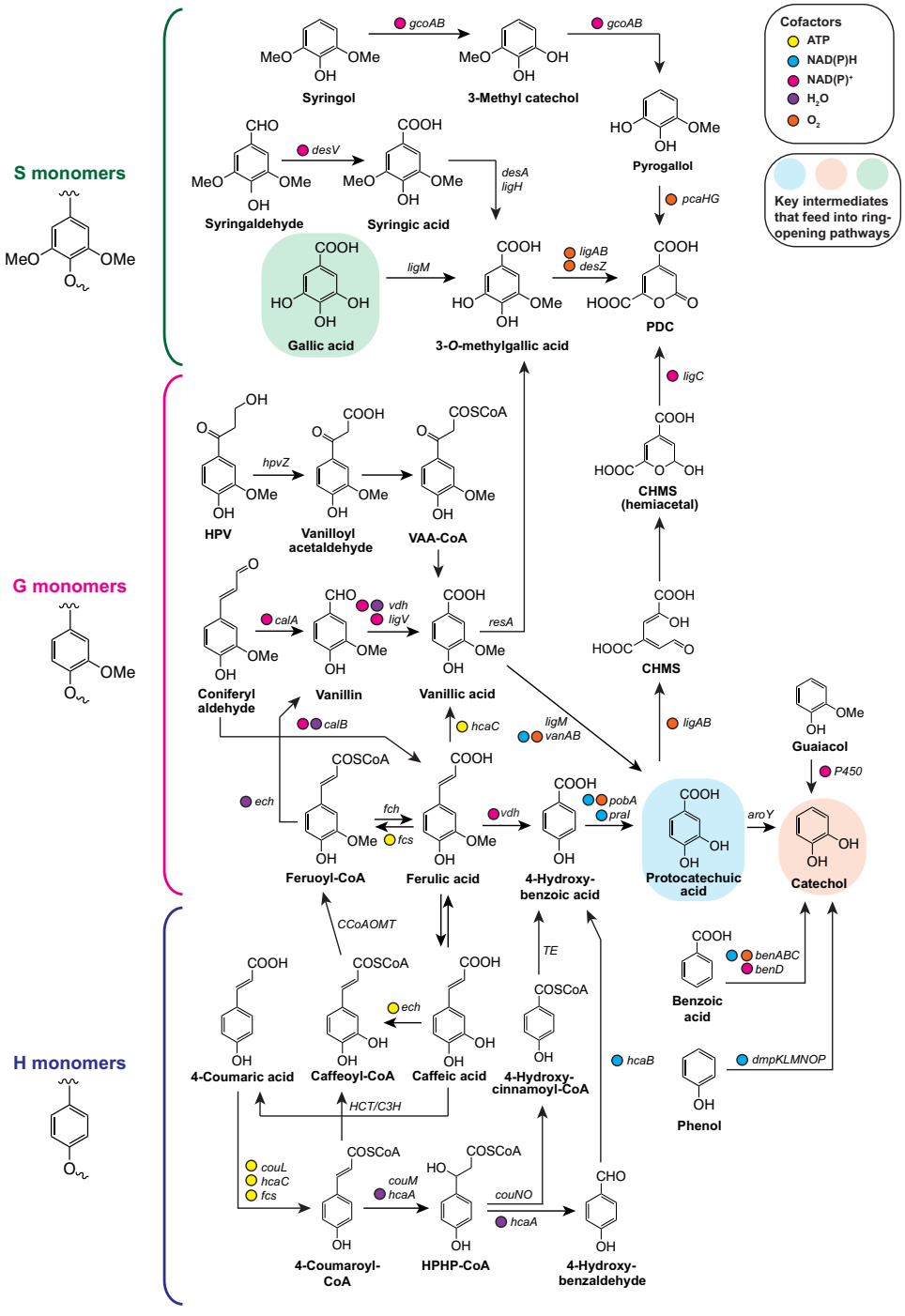

Figure 1. Upper Branches of Aromatic Catabolism of S (Syringyl, Green), G (Guaiacyl, Pink), and H (*p*-Hydroxyphenyl, Purple) Lignomonomers. The upper branches funnel through three main intermediates, gallate (green shaded box), protocatechuate (blue shaded box), and catechol (orange shaded box). Cofactor utilization is indicated by colored dots proximal to each enzyme name. Abbreviations: CHMS, carboxy-2-hydroxymuconoate-semialdehyde; HPHP-CoA, 4-hydroxyphenyl-β-hydroxy-propionyl-CoA; HPV, β-hydroxypropiovanillone; PDC, 2-pyrone-4,6-dicarboxyl acid; VAA-CoA, vanilloyl acetic acid-CoA.

Glossary

Adaptive evolution: laboratory evolution driven by artificial selection pressure exerted by the researcher to change host phenotype.
Catabolite repression: when the presence of a substrate, such as a sugar, inhibits the expression of another catabolic pathway.
Catalytic efficiency: calculated from $K_{cat}\,K_m^{-1}$; the measure of how efficiently an enzyme converts substrates to products.
Chaperones: proteins that facilitate correct protein folding.
Chemical oxygen demand: the amount of oxygen required to oxidize organic carbon.
Directed evolution: a method mimicking natural selection to steer the evolution of biomolecules, in this case proteins, toward a user-defined goal.
Dry cell weight (DCW): the dry weight of cells after their water content has been removed; a measure of biomass concentration.
Funneling pathways: pathways that funnel the multitude of lignomonomers into a few key intermediates preceding ring cleavage.
Genetic engineering: the deliberate manipulation of DNA material to intentionally change a host phenotype.
Heterologous: using a host to express at least one gene from a different organism.
Hyperglycosylation: an increased degree of sugars, or glycans, that are post-translationally added to a protein.
***In silico* design:** protein engineering based on computational, structure-guided models
***In vitro*:** experiments performed in a non-cellular environment. Examples include cell-free extracts or purified proteins.
Intergenic region: the region of DNA between coding sequences
Kraft lignin: lignin released from the cellulose and hemicellulose fractions during the Kraft pulping process.
Lignin: the structural component of plant biomass; the second most abundant biopolymer on Earth after cellulose.
Ligninase: the class of enzymes that depolymerize lignin.
Lignocellulosic hydrolysate: the aqueous fraction resulting from biomass hydrolysis, typically consists of a mixture of low molecular weight lignin and lignomonomer chemical species.

lignin depolymerizers in nature. They use numerous laccases and peroxidases to break different linkages connecting the S, G, and H lignin monomers. Other enzymes implicated in lignin depolymerization include glutathione *S*-transferases, etherases, thiolases, and cytochrome P450s. This review focuses on synthetic biology related to the major lignin degrading enzymes: laccases, manganese-dependent peroxidases (MnP), lignin peroxidases (LiP), dye-decoloring peroxidases (DyP), and versatile peroxidases (VP).

Fungal enzymes have high **catalytic efficiencies** for lignin degradation; however, slow growth rates and genetic intractability of fungal systems prevent common use. Bacterial systems are more amenable to **genetic engineering**, and clever expression methods in *Escherichia coli* combining **chaperones** and ***in vitro*** maturation have resulted in bacterially expressed peroxidases with activity near commercial level [11–13]. Active, soluble MnP from *Ceriporiopsis subvermispora* was successfully folded with a properly incorporated heme group when coexpressed with chaperone DsbC [11]. Despite coexpression with DsbC, DnaK, DnaJ, and GrpE, MnP from *Phanerochaete chrysosporium* required *in vitro* maturation with hemin, ATP, and an ATP-regeneration system [12]. Although homologous proteins, the conditions required to express active forms of the *P. chrysosporium* MnP were much more elaborate than for the *C. subvermispora* MnP. It can be difficult to anticipate the additional conditions necessary to fold active heterologous **ligninases**, and host optimization is often required.

Other groups have added more robust secretion tags to enhance bacterial laccase expression in non-*E. coli* bacterial systems. Lignin degradation by *Rhodococcus opacus* PD630 was enabled by **heterologous** expression of a laccase from *Streptomyces coelicolor* [14]. Engineering the promoter, ribosome binding site (RBS), and secretion tag upstream of the laccase, in conjunction with a Tat secretory system, led to a 7.1-fold increase in total protein production, the majority of which was laccase, compared to the wild-type strain containing no laccase. Production in optimized glucose media led to 13.7 g/l of secreted protein. Engineered PD630 grown in 1% insoluble **Kraft lignin** degraded 81.1% of the β5 linkage, 40.8% of the aliphatic hydroxyl group, and 26.3% of the guaiacyl hydroxyl group, whereas the wild-type strain containing no laccase only degraded 33.9%, 15.5%, and 8.9%, respectively. Similarly, a DyP from *Amycolatopsis* sp. 75iv2 was overexpressed in *Pseudomonas putida* A514 using a strong, constitutive promoter (P_{1099}) and an endogenous secretion tag (PelB) [15]. The engineered strain showed a 2.1-fold enhancement in cell growth while utilizing insoluble Kraft lignin. The lignin showed a >40% reduction in the β5 phenolic groups compared to the wild-type strain.

Bacterial expression of fungal enzymes poses problems owing to insufficient secretion mechanisms, lack of post-translational modifications (PTMs), and lack of chaperones and cofactors for successful folding. Yeast can be a better platform, although yeast-derived fungal enzymes often have other limitations, such as lower catalytic efficiencies than native fungal enzymes. There has been significant work engineering laccases and peroxidases in yeast to improve protein **titer**, enzyme kinetics, and optimized protein performance in different reaction conditions (Table 1).

Recently, a body of work focused on using *Pichia pastoris* as a host for fungal enzyme expression. Rivera-Hoyos and colleagues expressed POXA 1B laccase from *Pleurotus ostreatus* and compared **chemical oxygen demand** (COD) removal and decolorization of **synthetic black liquor** (SBL) by the recombinant and natively expressed enzyme coupled to CuO/TiO_2/visible light photocatalysis [16]. SBL treated with engineered *P. pastoris* showed 63.7% COD removal and 46% decolorization. Although impressive, it was unable to outperform the native fungal *P. ostreatus*-treated biomass, which showed 80.3% COD removal and 70.6% decolorization.

Lignomonomer: aromatic monomers released from lignin depolymerization.
Passages: when cells are transferred, or passaged, from old media into fresh media.
Putative: a predicted gene usually based on sequence similarity to known genes.
Single-nucleotide polymorphism (SNP): a genetic variation in which a single nucleotide differs between sequences.
Synonymous: a mutation in the nucleotide sequence of DNA that does not confer a change in amino acid sequence.
Synthetic biology: the application of engineering principles to biology to redesign and/or enhance existing biological systems for useful purposes.
Synthetic black liquor (SBL): the aqueous solution of lignin residues that is produced from the Kraft pulping process.
Titer: a concentration; units are mass per volume.
Total enzyme activity: enzyme activity achieved through the combined effects of secreted protein titers and specific activity.

Table 1. Summary of Engineering Strategies To Express Soluble, Active Enzymes for Lignin Depolymerization Broken Down by Bacteria, Yeast, and Fungal Hosts[a]

Host	Gene(s)	Origin	Substrate	Engineering strategy	Outcome	Refs
Bacterial expression host						
Escherichia coli BL21(DE3)	*MnP50297*, *MnP117436*, *MnP124076*, *MnP157986*	*Ceriporiopsis subvermispora*	2,6-DMP	Expression of codon-optimized genes under cold-induced promoter *cspA* and coexpression of chaperone DsbC	Active, soluble fungal MnP with properly incorporated heme with a protein titer of 30 mg/l	[11]
Escherichia coli SHuffle T7 Express	*MnP*	*Phanerochaete chrysosporium*	ABTS	Expression chaperones coupled with *in vitro* maturation with ATP and hemin	Active, soluble fungal MnP with high activity comparable to commercial MnP	[12]
Escherichia coli BL21(DE3)	*ScLac*	*Streptomyces cyaneus* (CECT 3335)	ABTS, 2,6-DMP, guaiacol	Heterologous expression with induction of native chaperones with benzyl alcohol	Protein titer of 104 mg/l with activity across a broad pH and temperature range	[13]
Rhodococcus opacus PD630	*SLSC*	*Streptomyces coelicolor*	Insoluble Kraft lignin	Omics-guided engineering of promoter, RBS, secretion tag of laccase	Over 7.1-fold improvement of total laccase activity and enhanced metabolism of kraft lignin	[14]
Pseudomonas putida strain A514	DyP2	*Amycolatopsis* sp. IV2	Insoluble Kraft lignin	Overexpression using a strong constitutive promoter and a secretion tag	2.1-fold growth improvement over WT strain	[15]
Yeast expression host						
Saccharomyces cerevisiae	*OB1*	*Coriolopsis* sp. PM1	ABTS, 2,6-DMP, guaiacol, sinapic acid	Structure-guided engineering using SCHEMA	Variants with higher catalytic efficiency, acidic pH stability, or thermostability	[28]
	3PO	*Pycnoporus cinnabarinus*				
	Lac3	*Trametes* sp. strain C30				
Pichia pastoris	POXA 1B	*Pleurotus ostreatus*	Synthetic kraft black liquor	rWT followed by light photocataylsis	63.7% COD removal, 46% decolorization when using the *P. pastoris* system. This compares to 80.3% COD removal and 70.6% removal by the *P. ostreatus* system	[16]
Pichia pastoris	*lac1*	*Grifola frondosa*	ABTS, 2,6-DMP, guaiacol	Targeted genome integration of cDNA from *G. frondosa*	More stable (thermal and acidic pH) expression, but decreased catalytic efficiency than native Lac1 owing to hyperglycosylation	[17]
Pichia pastoris GS115	*lcc9*	*Pleurotus ostreatus*	ABTS, 2,6-DMP, guaiacol, syringaldazine	Genomic integration	Increased secretion, alkaline pH stability, and thermostability compared to native Lcc9	[18]
Pichia pastoris X-33	*Lac-2*	*Pleurotus ostreatus*	ABTS, 2,6-DMP, guaiacol, corn stover lignin	Genomic integration of cDNA from *P. ostreatus*	The recombinant laccase had higher catalytic efficiency towards ABTS and 2,6-DMP than the native *P. ostreatus* enzyme. Recombinant strain was able to degrade 18.36% of the corn stover lignin whereas *P. ostreatus* degraded 14.05% in the same conditions	[19]

Table 1. (continued)

Host	Gene(s)	Origin	Substrate	Engineering strategy	Outcome	Refs
Fungal expression host						
Aspergillus nidulans	*pslcc*	*Pycnoporus sanguineus*	ABTS	Used RsmA transcription factor to control transcription by P_{aflR} coupled to a double-knockout of the proteases DppV and PepA	15-fold higher expression; 13-fold higher activity in the double KO strain	[21]
Aspergillus niger	LAC3	*Trametes* sp. C30	ABTS, syringaldazine, 2,6-DMP	Heterologous expression	Industrially relevant titers, though lower efficiency than the same gene expressed in *S. cerevisiae*	[20]
Trichoderma reesei	*lac*	*Pycnoporus sanguineus*	ABTS; Bisphenol A	Non-targeted genome integration	Maximum production of 17.7 U/ml from fed-batch fermentation with greatly enhanced thermostability	[22]
Phanerochaete chrysosporium	MnP1, LiPH8	Endogenous	ABTS, 2,6-DMP, guaiacol, sugarcane bagasse, wheat bran	Non-targeted genome integration	25% more lignin depolymerization, 2.6-fold higher peroxidase activity, 4-fold higher laccase activity compared to the WT strain	[23]
	Vpl2	*Pleurotus erygnii*				
	LacIIIb	*Trametes versicolor*				
Protein engineering						
Saccharomyces cerevisiae	OB1	Previously evolved *Coriolopsis* sp. PM1 (OB1) laccase	ABTS, 2,6-DMP, eucalyptic kraft lignin	Swapped the cupredoxin domain D2 in OB1 for a more stable domain from a laccase from *Pycnoporus cinnabarinus* 3PO and added a better secretion tag	Increased stability to high temperature, acidic pH, and organic co-solvents. Increased solubilization, decreased phenolic content, and increased carbonyl content of Kraft lignin	[33]
Saccharomyces cerevisiae	*lcc2*	*Trametes versicolor*	ABTS	Rational engineering of loop L1	Increased resistance to 35% (v/v) EMIM $EtSO_4$ by 8.4-fold	[34]
Saccharomyces cerevisiae INVSc1	*MaL*	*Melanocarpus albomyces*	ABTS, 2,6-DMP, 4-aminoantipyrine, syringaldazine	Directed evolution (KnowVolution)	Increased activity twofold at pH ≥ 9.0 and K_{cat} threefold at pH 9.8. Increased optimum pH from 4.0 to 6.0	[35]
Saccharomyces cerevisiae	-	*Cerrena unicolor* BBP6	ABTS	Random mutagenesis	Increased TAI 37-fold compared to rWT	[36]
Pichia pastoris	*lcc9*	*Coprinopsis cinerea*	ABTS, guaiacol, 2,6-DMP	Random mutagenesis	Increased the optimum pH from 6.5 to 8.5 with 70% of activity retention	[26]
Pichia pastoris X-33	*MrMnP1*	*Moniliophthora roreri*	2,6-DMP, GGE, VGE, veratryl alcohol, 3,4,5-trimethoxycinnamic acid, and 3-(3,4-dimethoxyphenyl) propionic acid	*In silico* design based on ViP from *Pleurotus ostreatus*	Mutants had activity on listed substrates when WT MnP did not. Produced 132 mg/l of active protein	[31]
Escherichia coli Rosetta DE3	*cotA*	*Bacillus pumilus*	ABTS, 2,6-DMP, guaiacol	Structure-guided engineering and DNA shuffling	Improved guaiacol turnover rate by 1.4-fold and catalytic efficiency by 4.5-fold. Improved thermostability	[27]
Escherichia coli BL21(DE3)	*dypB*	*Psuedomonas fluorescens* Pf-5	ABTS, DCP, DNP, alkaline Kraft lignin	Structure-guided engineering based on MD simulation	Increased alkaline Kraft lignin oxidation by 3–4-fold compared to the WT	[25]

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Table 1. (continued)

Host	Gene(s)	Origin	Substrate	Engineering strategy	Outcome	Refs
Escherichia coli BL21(DH3)	*LiPH8*	*Phanerochaete chrysosporium*	Veratryl alcohol, VGE	*In silico* design based on MnP6 from *Ceriporiopsis subvermispora*	12.5-fold increased stability at pH 2.5, 9.9-fold increased catalytic efficiency toward veratryl alcohol, 7.8-fold enhanced VGE catalytic efficiency compared to native LiPH8	[29]
Escherichia coli BL21(DE3)	*VP1*	*Pleurotus ostreatus*	ABTS, 2,6-DMP, veratryl alcohol	*In silico* design based on royal palm tree peroxidase (RPTP) from *Roystonea regia*	1.5-fold improvement of activity retention and structural stability after 1 h incubation at optimal pH and temperature compared to the WT	[30]
Escherichia coli Tuner DE3	*ppDyP*	*Pseudomonas putida* MET94	ABTS, 2,6-DMP, GGE, guaiacol, syringaldehyde, methyl syringate, kraft lignin	Random mutagenesis	Improved catalytic efficiency for 2,6-DMP by 100-fold, improved catalytic efficiency for lignin related phenolics, GGE, and Kraft lignin. Increased optimal pH to 8.5, improved H_2O_2 resistance, and produced twofold higher yields than rWT.	[24]
In vitro expression						
Escherichia coli B834	*ligDNEFG*	*Sphingobium* sp. strain SKY-6	GGE, GTE, hybrid poplar lignin	Minimal set of secreted enzymes for *in vitro* depolymerization of lignin oligomers	Release of guaiacyl, syringyl, and tricin units from lignin oligomers and depolymerization of a high-syringyl transgenic hybrid poplar lignin	[37]
	AvGR	*Allochromatium vinosum* (DSM180)				
	$NaGST_{NU}$	*Novosphingobium aromaticivorans* (DSM12444)				

[a]Key and abbreviations: –, not reported; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ADBP/DMA, 4-amino-2,6-dibromophenol/3,5-dimethylaniline; DCP, 2,6-dichlorophenol; 2,6-DMP, 2,6-dimethylphenol; DMPPDA, *N*,*N*-dimethyl-p-phenylenediamine sulfate; DNP, 2,4-dinitrophenylhydrazine; GGE, guaiacylglycerol-β-guaiacyl ether; GTE, guaiacylglycerol-β-tricin ether; KO, knockout; rWT, recombinant expression of wild-type enzyme; TAI, total activity improvement (enhancement of specific activity and secretion); VGE, veratrylglycerol β-guaiacyl ether.

Hyperglycosylation by *P. pastoris* may be partly responsible for the decreased catalytic efficiency and increased thermal and pH stability of the *Grifola frondosa* Lac1 and *P. ostreatus* Lcc9 laccase proteins [17,18]. Conversely, when Lac2 from *P. ostreatus* was expressed in *P. pastoris*, it had a higher catalytic efficiency towards 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,6-dimethoxyphenol (2,6-DMP) [19]. In addition, it showed higher degradation of corn stover lignin than when homologously expressed. These examples underscore our lack of predictive understanding of PTM influences on enzyme activity.

Although yeasts have significant advantages over fungi and bacteria, ligninases are particularly challenging to recombinantly express. This motivates engineering fungal systems, despite their associated challenges. *Aspergillus niger* was used to express *lac3* from *Trametes* sp. C30 [20]. Although the catalytic efficiencies towards ABTS, syringaldazine, and guaiacol were worse than the *S. cerevisiae*-produced Lac3 by 71%, 57%, and 54%, respectively, the dramatic 200-fold increase in titer overcame catalytic deficiencies. Expression of a laccase gene from *Pycnoporus sanguineus* using a strongly activated promoter, P_{alfR}, in an *Aspergillus nidulans* strain deficient in two proteases (DppV and PepA) achieved a 15-fold increased expression and a 13-fold

increased **total enzyme activity** compared to the wild-type *A. nidulans* [21]. Combined deletion of a mannotransferase, *mnn9*, and protease, *pepA*, also had a significant positive effect on enzyme activity. The authors do not report simultaneous deletion of *dppV*, *pepA*, and *mnn9*. The same *P. sanguineus* laccase was expressed in *Trichoderma reesei* resulting in a final titer of 17.7 U/ml from a fed-batch bioreactor system [22]. The enhanced thermostability of the recombinantly expressed enzyme was attributed to differences in glycosylation, as has been reported with other heterologous hosts. Overexpression of several ligninases and associated enzymes from different fungi (MnP1, LiPH8, Vpl2, LacIIIb) in *P. chrysosporium* resulted in 25% more lignin depolymerization than the wild-type fungi owing to the 2.6-fold higher peroxidase activity and fourfold higher laccase activity [23]. Although less work has been done on fungal systems, the native activities, on the order of U/ml, and catalytic efficiencies, on the order of 10^3 mM^{-1} s^{-1}, are significantly greater than many heterologous bacterial and yeast systems, warranting serious consideration of an intensive focus on building the synthetic biology tool set for filamentous fungi.

Various types of protein engineering have been used to enhance total activity of an enzyme and improve on heterologous expression methods mentioned earlier. Random mutagenesis generated by error-prone PCR on a DyP from *P. putida* MET94 (ppDyP) resulted in improved catalytic efficiency towards 2,6-DMP by 100-fold and secretion by twofold compared to the ppDyP expressed in *E. coli* [24]. In addition, the evolved enzyme was more resistant to hydrogen peroxide, a necessary cofactor, and had a more alkaline optimal pH, useful for solubilizing lignin. The best-performing mutant had a catalytic efficiency of 110 mM^{-1} s^{-1}, a similar value to peroxidases from *P. chrysosporium*, *Pleurotus eryngii*, and *Irpex laceus*. The mutant H169L of DyP from *P. fluorescens* Pf-5 (DypB) expressed in *E. coli* showed 7–8-fold improved catalytic efficiency to 2,4-dichlorophenol, which resulted in a remarkably high catalytic efficiency of 42 × 10^5 mM^{-1} s^{-1}. It also showed enhanced depolymerization of alkaline Kraft lignin [25]. Randomly mutagenized Lcc9 from *Coprinopsis cinerea* expressed in *P. pastoris* achieved a catalytic efficiency of 180 mM^{-1} s^{-1} at a pH of 8.5 [26]. Another example demonstrated **directed evolution** and structure-guided mutagenesis of CotA, a laccase-like multi-copper oxidase from *Bacillus pumilus* [27]. Although an order of magnitude less than fungal enzymes, the best mutant obtained showed a catalytic efficiency towards ABTS, 2,6-DMP, and guaiacol of 11.3 mM^{-1} s^{-1}, 27.1 mM^{-1} s^{-1}, and 28.5 mM^{-1} s^{-1}, respectively. *In vivo* shuffling in *S. cerevisiae* to create a mutant library of laccases derived from OB1 from *Coriolopsis* sp. SP1, 3PO from *Pycnoporus cinnabarinus*, and Lac3 from *Trametes versicolor* sp. C30 all showed enhanced catalytic efficiency, acidic pH stability, or thermostability compared to parent enzymes [28]. Therefore, protein engineering is a powerful way to improve ligninase activity in heterologous systems.

Several studies have used a structure-guided approach based on ***in silico*** **designs** and generated mutants with high catalytic efficiency in favorable processing conditions, such as high temperature, high alkalinity to facilitate lignin solubilization, high acidity to prevent lignin repolymerization, and the presence of solvents and cofactors such as organic solvents, ionic liquids, and hydrogen peroxide [24,27,29–32]. LiPH8 from *P. chrysosporium* was mutated based on the structure of acid-stable manganese peroxidase isozyme 6, MnP6, from *Ceriporiopsis subvermispora* [29]. The mutant had a catalytic efficiency of 157.5 mM^{-1} s^{-1} towards veratryl alcohol at a pH of 2.5, which is 90.2% higher than the recombinant wild-type enzyme. The mutant also demonstrated 7.8-fold higher veratrylglycerol β-guaiacyl ether conversion efficiency than the recombinant wild-type LiPH8. Several studies utilized *S. cerevisiae* as an expression host and achieved similar results [28,33–36]. Rational engineering of loop L1 of the Lcc2 protein from *T. versicolor* increased resistance to 35% EMIM $EtSO_4$, an ionic liquid beneficial for lignin depolymerization, by 8.4-fold [34]. Pardo and coworkers swapped the D2 cupredoxin

domain in OB1 for a more stable domain from a *P. cinnabarinus* 3PO laccase, which increased stability to high temperatures, acidic pH, and organic co-solvents [33]. The catalytic efficiencies of the chimeric laccase towards ABTS and 2,6-DMP were 6160 mM^{-1} s^{-1} and 986 mM^{-1} s^{-1}, respectively. The chimera retained ~90% of its activity at pH 2, whereas both parent laccases, OB1 and 3PO, had <10% residual activity. The mutant also showed nearly full activity at 60 °C, whereas the OB1 mutant showed slightly less than 60% activity, and 3PO showed ~20% activity. Altogether, these improved characteristics allowed Kraft lignin to be enzymatically treated at 65°C and a pH of 5 for increased lignin solubilization, decreased phenolic content, and increased carbonyl content compared to lignin processed in the same conditions without enzyme. Given the acidic pH stability of the enzyme, a new processing condition at low pH could be explored to prevent reactions competing with depolymerization.

In lignin depolymerization, there is often competition between microorganism health and enzyme optimization. Efficient lignin depolymerization can result in toxic conditions that cell-free systems are inherently immune to. *E. coli* B834 was used as a host to express *ligDNEFG* from *Sphingobium* sp. SKY-6, *avGR* from *Allochromatium vinosum*, and $NaGST_{NU}$ from *Novosphingobium aromaticivorans* [37]. All the heterologously expressed enzymes were secreted, purified, and combined *in vitro* to treat lignin extracted from an engineered poplar with high syringyl content. The authors were able to show release of guaiacyl, syringyl, and tricin units from relevant lignin oligomers and depolymerization of the hybrid poplar lignin extract. The enzymatic pretreatment can be diluted to a tolerable concentration and used as feedstock.

Although purified enzymes can alleviate toxicity limits, an increasing body of work focused on consolidated biomanufacturing is gaining traction. Enhancing aromatic metabolism provides a microbial sink for released lignin depolymerization products and helps to alleviate toxicity. Although the field is still discovering catabolic genes in bacteria, yeast, and fungi [38–41], a very comprehensive review paper comparing natural catabolic pathways was recently published and can provide a roadmap for future engineering efforts [42].

Enhancing and Expanding Monoaromatic Catabolism in Bacteria

Lignocellulosic hydrolysates are complex feedstocks. Because all depolymerization techniques release a mixture of monomers whose composition changes based on the lignin source and depolymerization method, many strategies for utilizing depolymerized lignin entail engineering organisms to consume multiple lignin-derived monomers. Table 2 (Key Table) summarizes engineering efforts in bacteria, yeast, and fungi to add branches of aromatic catabolism, expand the range of substrates utilized, or improve the production of value-added products using structure-guided engineering and **adaptive evolution** methods. Cofeeding with sugar substrates and scale-up techniques were also explored as ways to increase product yields and titers.

E. coli is extensively studied and easy to engineer, motivating its frequent use for introducing aromatic catabolic pathways [43–50]; however, there are increasing examples of enhancing organisms that are naturally capable of aromatic metabolism. *Actinetobacter baylyi* ADP1 regulates benzene and phenol catabolism pathways through BenM and CatM, respectively. Removal of the regulators, coupled to constitutive expression of the *ben* operon, allowed coutilization of benzene and ferulic acid [51]. *Pseudomonas putida* can naturally metabolize several monoaromatic compounds but cannot naturally metabolize guaiacol as a sole carbon source. Heterologous expression of GcoAB, a cytochrome P450 monooxygenase isolated from *Amycolatopsis* sp. ATCC 39116, was insufficient for guaiacol metabolism in *P. putida* KT2440 [52]. Growth on 6 mM guaiacol as the sole carbon source was enabled when *catA*, encoding a catechol 1,2-dioxygenase, was fused to *gcoA*. However, the strain exhibited a 72 h lag phase. In another

Key Table

Table 2. Summary of Synthetic Biology Methods To Enhance Aromatic Metabolism or Aromatic-Derived Production by Bacteria, Yeast, and Fungal Hosts[a]

Host	Gene(s)	Origin	Substrate	Glucose supplementation	Product	Engineering strategy	Outcome	Refs
Bacterial expression host								
Acinetobacter baylyi ADP1	*ΔbenM*	Endogenous	4-HBA, BA	No	NA	Several strains were created with constitutive BenM and CatM expression, PcaK expression, and/or the *benABCD* and *catABCIJFD* operons	Upregulation of PcaK transporter did not enhance 4-HBA catabolism Constitutive expression of BenM and CatM did not alleviate regulation of *ben* and *cat* operons; knockouts of *benM* and *catM*, coupled with constitutive expression of the *ben* operon, facilitated coutilization of BA and 4-HBA	[51]
	ΔcatM	Endogenous						
	pcaK	Endogenous						
	benABCD	Endogenous						
	catABCIJFD	Endogenous						
Acinetobacter baylyi ADP1	*undA*	*Pseudomonas putida* KT2440	FA	No	1-Undecene	*'tesA* and *undA* were expressed under a cyclohexanone-inducible promoter *ChnR*/P_{chnB}, originally isolated from *A. johnsonii*	The engineered adapted strain produced 72 μg/l of 1-undecene from 110 mM of ferulate as the sole carbon source, resulting in a yield of 7.2 μg/g). The engineered wild-type strain did not survive the conditions	[71]
	'tesA	*Escherichia coli* MG1655						
Amycolatopsis sp. ATCC 39116	*ΔAATC3_020100009302*	Endogenous	Guaiacol, softwood Kraft lignin hydrolysate	Yes	*Cis*,*cis*-muconic acid	Two **putative** *catB* genes were deleted from the *Amycolatopsis* genome to accumulate muconic acid	The double-knockout strain produced 25 mM muconic acid from guaiacol, a 96% molar yield, in a fed-batch reactor with glucose cofeeding. The same strain produced 1.8 mM muconic acid from depolymerized softwood Kraft ligin hydrolysate, resulting in 75% molar yield. It was found that 2-methyl-muconic acid was produced from the *O*-cresol in the hydrolysate, as opposed to muconic acid	[86]
	ΔAATC3_020100018510	Endogenous						

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Table 2. (continued)

Host	Gene(s)	Origin	Substrate	Glucose supplementation	Product	Engineering strategy	Outcome	Refs
Corynebacterium glutamicum MA-2	*ΔcatB*	Endogenous	Phenol, catechol, BA, depolymerized softwood lignin	Yes	*Cis*,*cis*-muconic acid	*catA* was genomically integrated under a constitutive promoter, P_{tuf}, whereas *catB* was deleted to allow accumulation of muconic acid	Deletion of *catB* necessitates glucose cofeeding. Constitutive expression of CatA increased tolerance to catechol threefold. Cultures fed with catechol and glucose produce 85 g/l muconic acid with a molar yield from catechol of 100%. Cells also produced 1.8 g/l muconic acid from softwood lignin hydrolysate	[87]
	catA	Endogenous						
Escherichia coli DH1	*couP*	*Rhodopseudomonas palustris*	Vanillin	No	Catechol	Expressed a catechol degradation pathway and aromatic transporter gene, *couP*, under a vanillin autoinducible promoter, P_{ADH7}	The authors do not comment on increased tolerance for vanillin, vanillic acid, or catechol. The inclusion of the aromatics transporter gene, *couP*, in the catechol operon increased catechol production by 40% and vanillic acid production by 10% as compared to the strain without the transporter	[45]
	ligV	*Sphingobium* sp. SKY-6						
	ligM	*Sphingobium* sp. SKY-6						
	aroY	*Klebsiella pneumoniae* sp. *pneumoniae*						
Escherichia coli	P_{vtac}	*Escherichia coli*	Vanillin	No	NA	The spacer regions from P_{tac}, P_{trc}, and P_{tic} were used to replace that of the phenolic-inducible promoter, P_{emrR}, native to *E. coli*	Three hybrid, phenolic-inducible promoters of high but variable strength were constructed and characterized with vanillin as the inducer. The promoters P_{vtac}, P_{vtrc}, and P_{vtic} increased protein expression by 4.6-, 3.0-, and 1.5-fold, respectively, compared to P_{emrR}	[46]
	P_{vtrc}	*Escherichia coli*						
	P_{vtic}	*Escherichia coli*						
Escherichia coli	*aroY*	*Klebsiella pneumoniae* sp. *pneumoniae*	Vanillin	No	*Cis*,*cis*-muconic acid	*catA*, *vanAB*, and *vdh* were episomally expressed as a single operon. *aroY* and *kpdB* were episomally expressed as a second operon	Expression of KpdB enhanced AroY levels and increased Pdc activity. Interestingly, KpdD impaired AroY levels and product titer. Accumulation of	[47]
	kpdBD	*Klebsiella pneumoniae* NBRC14940						

	vdh	*Pseudomonas putida* KT2440					intermediate PCA was eliminated, but VA accumulation remained the same between strains. Alleviation of the PCA bottleneck created higher muconic acid production	
	vanAB	*Pseudomonas putida* KT2440						
	catA	*Pseudomonas putida* KT2440						
Escherichia coli	*fcs*	*Pseudomonas fluorescens* BF13	FA	No	Vanillin	The two genes were cloned as an operon under the P_{fer} promoter and chromosomally integrated. A two-stage growth period separated growth and vanillin production. Ferulic acid was slowly fed using sol-gel technology	Host cells containing solely the *Pseudomonas* genes produced only 3.5 mM of vanillin. The combined strain, culturing, and feeding strategies improved vanillin production to 28 mM	[48]
	ech	*Pseudomonas fluorescens* BF13						
Escherichia coli DH1	*ligV*	*Sphingobium* sp. SKY-6	Vanillin extracted from alkaline H_2O_2 pretreated tobacco lignin, PCA extracted from engineered tobacco lignin with MeOH:H_2O	No	*Cis*,*cis*-muconic acid	Express *ligV*, *ligM*, and *aroY* on a single operon under P_{Trc} control. Express CatA (either *pmt2* or *ac*) under P_{T7} control	Enabled 69% yield (g/g) of *cis*,*cis*-muconic acid from vanillin and 0.73% yield (g/g) of pyrogallol from syringate in a strain that is natively unable to metabolize aromatics. Expressing two copies of the *aroY* and *catA* operon under a P_{T7} decreased muconic acid titers by 50%. Yield of pyrogallol was significantly lower than muconic acid; however, addition of cofactor THF increased gallic acid yields to 5.96% from syringate	[49]
	ligM	*Sphingobium* sp. SKY-6						
	aroY	*Klebsiella pneumoniae* sp. *pneumoniae*						
	catA-pmt2	*Pseudomonas putida* mt-2						
	catA-ac	*Acinetobacter calcoaceticus*						
Escherichia coli DH1	*desA*	*Sphingobium* sp. SKY-6	SA extracted from alkaline H_2O_2 pretreated tobacco lignin	No	Pyrogallol, gallic acid	Express *desA*, *ligM*, and *lpdc* as a single operon under P_{trc}	Enabled 31% yield (g/g) of *cis*,*cis*-muconic acid from PCA in a strain natively unable to metabolize aromatics	[49]
	lpdc	*Lactobacillus plantarum* WCFS1						
	ligM	*Sphingobium* sp. SKY-6						
Escherichia coli BW25113	*pcaHGBDCIJFK*	*Pseudomonas putida* KT2440	PCA	No	NA	Heterologous expression of nine genes followed by adaptive laboratory evolution. Genes were expressed as two operons, both under $P_{T5\text{-}lac}$	Expression of the synthetic catabolic pathway facilitated growth on 1 g/l PCA as the sole carbon source	[50]

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Table 2. (continued)

Host	Gene(s)	Origin	Substrate	Glucose supplementation	Product	Engineering strategy	Outcome	Refs
Escherichia coli JME17	*pcaHGBDCIJFK*	*Pseudomonas putida* KT2440	4-HBA	No	NA	Addition of PraI allows conversion of 4-HBA to PCA; an RBS point mutation was included to maintain enhanced PcaH expression	Addition of *praI* to the evolved strain JME17 allowed growth on 1 g/l 4-HBA. The RBS mutation seen in the strain above increased the growth rate by nearly 10-fold as compared to the non-engineered strain containing only the synthetic PCA pathway + PraI	[50]
	praI	*Paenibacillus* sp. JJ-1B						
Escherichia coli JME17 [49]	*pcaHGBDC*	*Pseudomonas putida* KT2440	4-HBA, PCA	No	NA	The *pcaHGBDC* operon was integrated chromosomally. Either *praI* or *pobA* was integrated alongside the *pca* operon	Inclusion of PraI or PobA allowed growth on 4-HBA as a sole carbon source, but cells were unable to be passaged into fresh media containing 4-HBA. Cells were adapted (Table 3)	[43]
	praI	*Pseudomonas putida* KT2440						
	pobA	*Paenibacillus* sp. JJ-1B						
Escherichia coli JME82 [42]	*couLHTMNO*	*Rhodococcus jostii*	4-CA	No	NA	A S134Y mutation in CouL and frameshift mutation in NadR that deletes residues 326–410, discovered during adaptive evolution, were reconstructed in *E. coli* JME82 [42]	Strains with WT *pobA*, *nadR*, and *couL* genes could utilize PCA, but not 4-CA. The strain containing mutated genes was the only strain able to grow on 1 g/l 4-CA	[44]
	nadR (*Δ*326-410)	Endogenous						
Escherichia coli JME82 [42]	*hcaABCK*	*Acinetobacter baylyi* ADP1	4-CA	No	NA	A single G-to-A mutation in the intergenic region 47 bp upstream of *hcaC* and a D243G mutation in *guaB*, discovered during adaptive evolution, were reconstructed in *E. coli* JME82 [42]	The strain containing a mutated *pobA* and intergenic region upstream of *hcaC*, but WT *guaB*, showed growth on 4-CA growth, whereas the strain containing all WT genes did not. Addition of the mutated *guaB* enhanced the growth rate of cells grown with 1 g/l 4-CA ~threefold, but did not enhance PCA growth rate	[44]
	guaB-D243G	Endogenous						
Novosphingobium aromaticivorans	*ΔdesCD*	Endogenous	Vanillin, VA, 4-HBA, FA, 4-hydroxybenzaldehyde,	Yes	PDC	Knockout of *desCD* and *ligI* from the *N. aromaticivorans*	Bioreactor cultivation of this strain intermittently fed with a vanillin, VA,	[63]
	ΔligI	Endogenous						

			syringaldehyde, SA, S-diketone, G-diketone			genome to reroute flux into G and H monomer metabolism and force accumulation of PDC	and glucose resulted in 26.7 mM PDC after 48 h	
Pseudomonas putida KT2440	*ΔpcaHG*	Endogenous	Catechol, phenol, BA, PCA, coniferyl alcohol, FA, vanillin, caffeic acid, 4-CA, 4-HBA, alkaline pretreated corn stover lignin	Yes	*cis*,*cis*-muconic acid	AroY replaced PcaHG to force PCA branch metabolism through the catechol branch. *dmpKLMNOP* and *catA* were expressed as a single operon under strong, constitutive promoter, P_{tac}	Engineered cells produced 13.5 g/l of muconic acid from 4-CA delivered by fed-batch over 78.5 h. Glucose inhibited 4-CA catabolism and was fed at 1 mM. Engineered cells cultured with APL converted the major monomers, 4-CA and ferulate, with 67% molar yield	[64]
	ΔcatBCR	Endogenous						
	catA	Endogenous						
	aroY	*Enterobacter cloacae*						
	dmpKLMNOP	*Pseudomonas putida* CF600						
Pseudomonas putida	*dmpKLMNOP*	*Pseudomonas putida* CF600	Phenol, catechol, softwood lignin hydrothermally treated with supercritical water	Yes	*Cis*,*cis*-muconic acid, 3-methyl--muconic acid, nylon	Engineered promoter to increase expression of *catA*; inclusion of a phenol hydroxylase to enable phenol to catechol conversion; cofactor regeneration was facilitated through glucose cofeeding	1.5 kg of muconic acid and 3-methyl-muconic acid (*cis*,*cis* and *cis*,*trans* mixtures) were produced from a 50 l bioreactor using treated lignin as feedstock. Muconic acid was hydrogenated to adipic acid and polymerized into nylon 6,6. This is the first report to generate biobased nylon from lignin	[67]
	catA2	Endogenous						
Pseudomonas putida	*aroY*	*Enterobacter cloacae*	4-CA, FA, corn stover APL and BCDL	Yes	*Cis*,*cis*-muconic acid	Overexpress *aroY* and *ecdBD* under the P_{tac} promoter to convert PCA to catechol; knockout of *crc* to facilitate glucose cofeeding without catabolite repression	Additional introduction of VanAB resulted in 100% conversion of ferulic acid to muconic acid, but the low levels of ferulate in lignin streams discouraged pursuit of this strain	[65]
	ecdBD	*Enterobacter cloacae*						
	Δcrc	Endogenous						
Pseudomonas putida PDH	*vanAB*	*Pseudomonas putida KT2440*	Vanillin, softwood lignin extract, alkaline nitrobeneze oxidation extracts of softwood lignin (Japanese cedar) and hardwood lignin (birch)	Yes	PDC	One plasmid containing *vanAB* and *ligVABC* and a second plasmid containing *ferAB* and *desZ* were expressed in *P. putida* PDH	Growth on softwood and hardwood lignin extracts released more monoaromatic and led to higher PDC production than Kraft lignin extracts. The highest titer PDC achieved was 655 mg/l from Japanese cedar extracts and cells fully metabolized the released vanillin	[59]
	ligVABC	*Sphingomonas paucimobilis*						
	ferAB	*Sphingomonas paucimobilis*						
	desZ	*Sphingomonas paucimobilis*						

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Table 2. (continued)

Host	Gene(s)	Origin	Substrate	Glucose supplementation	Product	Engineering strategy	Outcome	Refs
Pseudomonas putida	*ΔpcaHG*	Endogenous	VA + 4-HBA, alkaline nitrobenzene oxidation extracts of softwood lignin (Japanese cedar)	No	*Cis*,*cis*-muconic acid	Remove funneling of PCA into the TCA cycle by knocking out *pcaHG*; accumulate *cis*,*cis*-muconic acid by knocking out *catB*; convert PCA to catechol through overexpression of *pdc*	20% molar yield of *cis*,*cis*-muconic acid from VA and 4-HBA as dual carbon sources and 12% molar yield from treated softwood lignin as the sole carbon source	[66]
	ΔcatB	Endogenous						
	pdc	*Klebsiella pneumoniae* sp. *pneumoniae*						
Pseudomonas putida A514	*Dyp2*	*Amycolatopsis* sp. 75iv	VA, Kraft lignin, lignin-containing biorefinery waste from AFEX pre-treatment	No	PHA	Fuse endogenous secretion tags to DyP2 to enhance endogenous lignin depolymerization; overexpression of *vanAB* to enhance flux through the β-ketoadipate pathway; expression of the PHA synthesis module (*phaJ4* and *phaC1*) under a P_{van} promoter to make it responsive to aromatics	The engineered strain grown in nitrogen starvation conditions with 1% biorefinery waste as the sole carbon source produces 161 mg/l of PHA in batch fermentation, a sixfold improvement over WT A514 cells, and accumulated 73.5% PHAs per CDW when utilizing VA. Table 1 for additional engineering efforts towards Dyp2 activity	[15]
	vanAB	*Pseudomonas putida* A514						
	phaJ4	*Pseudomonas putida* A514						
	phaC1	*Pseudomonas putida* A514						
Pseudomonas putida	*Δcrc*	Endogenous	FA, 4-CA	Yes	*Cis*,*cis*-muconic acid	Global catabolite repressor, *crc*, was knocked out of the *P. putida* genome to enhance aromatic conversion while cofeeding with glucose or acetate. Knockouts of *catAB* and *pcaHG* allow accumulation of muconic acid	Protein abundances of PobA and VanAB increased upon deletion of Crc. A molar yield of muconic acid from 20 mM 4-CA increased from 56% to 94.6% in the presence of glucose and from 40.7% to 47.7% in the presence of acetate. Molar yields from 20 mM FA increased from 12% to 28.3% in the presence of glucose and from 10.3% to 16.9% in the presence of acetate	[68]
	ΔcatAB	Endogenous						
	ΔpcaHG	Endogenous						
Pseudomonas putida	*ΔaceEF*	Endogenous	BA, 4-CA	No	Pyruvate, L-lactate	*aceEF* was deleted across all strains. Catechol *meta*-cleavage strains	The catechol *meta*-cleavage pathway outperformed the *ortho*-cleavage pathway	[57]
	ΔcatABC	Endogenous						
	ΔpcaHG	Endogenous						

	xylEGFJQKIH	*Pseudomonas putida* mt-2				had *ligABCDUJK* genomically integrated as a single operon under the strong, constitutive promoter, P_{tac}, to replace CatABC. PCA *meta*-cleavage strains had *xylEGFJQKIH* genomically integrated as a single operon under P_{tac} to replace PcaHG. Lactate producing strains genomically expressed LDHA from P_{tac}	by achieving 10% higher of the maximum theoretical maximum of pyruvate + L-lactate. The PCA 4,5-*meta*-cleavage pathway achieved 75.1% of the maximum theoretical maximum of pyruvate + L-lactate, 45.1% higher than the *ortho*-cleavage strain	
	ligABCIUJK	*Sphingobium* sp. SKY-6						
	LDHA	*Bos taurus*						
Pseudomonas putida	*ΔfabBA*	Endogenous	4-CA, enzymatically hydrolyzed corn stover lignin	No	Mcl-PHA	*phaG*, *alkK*, *phaC1*, and *phaC2* were genomically overexpressed under strong constitutive promoter, P_{tac}, in a strain deficient for PhaZ, FadBA1, and FadBAE2	The engineered strain produced 953 g/l of mcl-PHAs at 54.2% per dry cell weight under the best culturing conditions with 4-CA as the sole carbon source. The same strain produced 116 mg/l mcl-PHAs, leading to 17.7% accumulation per DCW, while utilizing corn stover hydrolysate. *phaZ* deletion did not improve titer or yields	[74]
	ΔphaZ	Endogenous						
	phaG	Endogenous						
	phaC1	Endogenous						
	alkK	Endogenous						
Pseudomonas putida KT2440	*catA*	Endogenous	4-CA	Yes	*Cis*,*cis*-muconic acid	*catBC* and *pcaHG* were knocked out to accumulate muconic acid. *catA* was genomically expressed under a constitutive, strong promoter, P_{tac}. *aroY* and *ecdBD* were genomically expressed as a single operon under P_{tac}	Cells expressing EcdBD and AroY produced 15.59 g/l muconic acid from 4-CA at a specific productivity of 90.46 mg/g cells/h with 100% molar yield. Addition of AsbF, required for muconic acid production from glucose, alongside AroY and EcdB produced 4.92 g/l muconic acid. Inclusion of *ecdD* in the operon reduced the titer to 0.59 g/l, cell productivity dropped from 9.50 mg/g cell/h to 1.63 mg/g cell/h and molar yield dropped from 7.7% to 0.8%	[69]
	ecdBD	*Enterobacter cloacae*						
	aroY	*Enterobacter cloacae*						
	asbF	*Bacillus cereus*						
	ΔpcaHG	Endogenous						
	ΔcatBC	Endogenous						

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Table 2. (continued)

Host	Gene(s)	Origin	Substrate	Glucose supplementation	Product	Engineering strategy	Outcome	Refs
Pseudomonas putida PpY1100	*vceAB*	*Sphingobium* sp. SKY-6	HPV	Yes	PDC	Express *vceAB* and *hpvZ* as an operon under P_m to enable conversion of HPV to PDC	Cells could convert 1 mM HPV to PDC with 100% molar yield after 24 h. Increasing HPV concentration to 2 mM resulted in a higher titer, but lower molar yield (70%) owing to accumulation VA, an intermediate compound	[60]
	hpvZ	*Sphingobium* sp. SKY-6						
Pseudomonas putida KT2440	*ΔpcaHG*	Endogenous	4-HBA	Yes	PDC	Overexpressed genes were expressed chromosomally from a strong, constitutive promoter, P_{tac}	The engineered strain accumulated 58.0 g/l PDC at a productivity of 0.2 g/l/h with a molar yield of 80.7% from 4-HBA	[61]
	ligAB	*Sphingobium* sp. SKY-6						
Pseudomonas putida KT2440	*ΔpcaD*	Endogenous	4-HBA	Yes	β-Ketoadipate--enol-lactone	*pcaD* was knocked out of the chromosome	This single-knockout strain produced 24.3 g/l of β-ketoadipate-enol--lactone, a 94.4% molar yield, at a productivity of 0.4 g/l/h from 4-HBA	[61]
Pseudomonas putida KT2440	*ΔpcaIJ*	Endogenous	4-HBA	Yes	β-Ketoadipic acid	*pcaIJ* was knocked out of the chromosome	This double-knockout strain accumulated 41.1 g/l β-ketoadipate at a productivity of 0.8 g/l/h. The molar yield from 4-HBA as 107.8%	[61]
Pseudomonas putida KT2440	*ΔpcaHG*	Endogenous	BA	Yes	4-Hydroxy-2-oxovaleric acid			
	ΔcatA2	Endogenous				Overexpressed genes were expressed chromosomally from a strong, constitutive promoter, P_{tac}	4-Hydroxy-2-oxovaleric acid was produced at 0.2 g/l/h for a final titer of 13.3 g/l. The molar yield from benzoate was 98.6%	[61]
	ΔcatBCA	Endogenous						
	praAH	*Paenibacillus* sp. JJ-1b						
	xylEGFJIH	*Pseudomonas putida mt-2*						
Pseudomonas putida KT2440	$gcoAB_{F169A}$	*Amycolatopsis* sp. ATCC 39116	Syringol	Yes	Pyrogallol	Engineered GcoA protein allows conversion of syringol to pyrogallol. PcaHG converts pyrogallol to PDC, a more stable product than pyrogallol	Cells expressing rWT GcoAB accumulated ~0.2 mM PDC and did not fully metabolize all of the syringol. Cells expressing GcoAB-F169A metabolized all of the syringol and produced	[88]
	pcaHG	Endogenous						
	ΔcatBCA	Endogenous						
	ΔcatA2	Endogenous						

							approximately the same amount of PDC. More pyrogallol, the product of interest, accumulated early during culturing	
Pseudomonas putida KT2440	*paaH* *paaF* *ter* *ΔpcaF*	*Escherichia coli* *Escherichia coli* *Treponema denticola* Endogenous	4-HBA	Yes	Adipic acid	*paaFH* and *ter* were expressed as a single operon under the P_{tac} promoter and chromosomally integrated	The engineered strain produced 17 mM of adipic acid (17.4% molar yield) under two-phase bioreactor conditions. 4-HBA was fed at 100 mM with constant glucose feeding	[72]
Pseudomonas putida PpY1100	*ligVABC* *vanAB*	*Sphingobium* sp. strain SKY-6 *Pseudomonas putida* KT2440	Desulfonated, depolymerized Vanilex	Yes	PDC	Genes were episomally expressed as two different operons	The engineered strain was able to accumulate ~50 mM of PDC. It was not reported how much of the released aromatic monomers were metabolized	[62]
Pseudomonas putida A514	*phaJ4* *phaC1*	Endogenous Endogenous	VA	No	PHA	*phaJ4* and *phaC1* were expressed as a single operon under control of strong, xylose-induced promoter, P_{xylA}	Cells were able to fully utilize 15 mM VA as the sole carbon source (2 mM xylose provided as inducer). 246 mg/l mcl-PHAs were produced (PHA content of 34.4 wt% DCW). The same operon under P_{van}, the promoter for *vanAB*, produced 23 mg/l mcl-PHAs at 3.4 wt%, likely because it is a weaker promoter than that of *xylA*. WT cells produced 11 mg/l PHA at 1.7 wt% per cell	[73]
Pseudomonas putida EM42	WP_085469912 WP_085469913 WP_020416430	*Rhodococcus rhodochrous* strain J3 *Rhodococcus rhodochrous* strain J3 *Amycolatopsis* sp. ATCC 39116	Guaiacol	No		The host line EM42 was chosen because it has higher ATP and NAD(P)H availability and genetic stability than KT2440. All genes were codon optimized and episomally expressed as a single operon from the strong, IPTG-inducible promoter, P_{trc}	Engineered strains utilized 5 mM guaiacol as sole carbon source and simultaneously with 10 g/l glucose. Metabolism of guaiacol as the sole carbon source required a ferredoxin reductase. The strain expressing a ferredoxin from *Amycolatopsis* showed the highest guaiacol consumption rates	[53]

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Table 2. (continued)

Host	Gene(s)	Origin	Substrate	Glucose supplementation	Product	Engineering strategy	Outcome	Refs
Rhodococcus jostii RHA1	*ligAB*	*Sphingomonas paucimobilis*	VA, 1% wheat straw lignocellulose	No	Pyridine 2,4-dicarboxylic acid	Convert native *ortho*-cleavage pathways to *meta*-cleavage pathways. Extradiol products are cyclized with ammonium to pyridine 2,4-dicarboxylic acid	Yields of 90 mg/l of product from minimal media containing 1% wheat straw lignocellulose after 7 days. Growth in a bioreactor for 9 days resulted in 125 mg/l pyridine 24-dicarboxyl acid	[58]
Sphingobium sp. SKY-6	*ΔligAB*	Endogenous	VA + syringic acid, alkaline nitrobenzene oxidation extracts of hardwood lignin (birch)	No	*Cis*,*cis*-muconic acid	Enhance native *O*-demethylation of SA and VA with vanAB, convert PCA to *cis*,-*cis*-muconic acid by expression of *pdc* and *catA*. Enhance *pdc* expression with *kpdB*	45% molar yield of *cis*,*cis*-muconic acid from VA and SA as dual carbon sources and 40% molar yield from treated hardwood lignin as sole carbon source. It was observed that the engineered strain required addition of tryptone	[66]
	vanAB	*Pseudomonas putida* KT2440						
	pdc	*Klebsiella pneumoniae* sp. *pneumoniae*						
	kpdB	*Klebsiella pneumoniae* sp. *pneumoniae*						
	catA	*Pseudomonas putida* KT2440						
Yeast expression host								
Saccharomyces cerevisiae CEN.PK 102-3A	*CALDH*	*Pseudomonas* sp. HR199	Coniferyl aldehyde	Yes	NA	*CALDH* was expressed from P_{TDH3} and chromosomally integrated into the *S. cerevisiae* genome	In 1.1 mM coniferyl aldehyde, the engineered strain had a much longer lag phase (36 h) than the control strain (14 h), but had a much higher specific conversion of coniferyl aldehyde (0.03 g/g/h) than the control (0.0011 g/g/h)	[77]

Saccharomyces cerevisiae CEN.PK 102-3A	*ALD5*	Endogenous	Coniferyl aldehyde, FA, PCA	Yes	NA	All genes were individually expressed from P_{TDH3} and genomically integrated	The engineered strain could grow in 1.4 mM coniferyl aldehyde whereas the control strain could not grow above 1.18 mM	[78]
	PAD1	Endogenous						
	ATF1	Endogenous						
	ATF2	Endogenous						
Rhodosporidium toruloides IFO 0880	*BIS*	*Abies grandis*	IL-derived corn stover hydrolysate, 4-CA	Yes and No	Bisabolene	Both genes were codon optimized and genomically integrated randomly with P_{GAPDH} and T_{NOS}	Engineered cells produced higher titers of bisabolene in the hydrolysate (261 mg/l) than the analogous synthetic medium containing glucose, xylose, and 4-CA (127 mg/l). Cells utilizing 0.5 wt% 4-CA as a sole carbon source produced ~75 mg/l bisabolene. Fed-batch fermentations with the corn stover hydrolysate resulted in 628 mg/l bisabolene	[79]

[a]Abbreviations: AFEX, ammonia fiber expansion; APL, alkaline pre-treated lignin; BA, benzoic acid; BCDL, base-catalyzed depolymerized lignin; 4-CA, 4-coumaric acid; CDW, cell dry weight; FA, ferulic acid; 4-HBA, 4-hydroxybenzoic acid; HPV, β-hydroxypropiovanillone; IL, ionic liquid; IPTG, isopropyl β-D-thiogalactopyranoside; μ_{max}, maximum specific growth rate (h^{-1}); mcl-PHA, medium chain length polyhydroxyalkanoate; NA, not applicable; PCA, protocatechuic acid, PDC, 2-pyrone-4,6-dicarboxylic acid; RBS, ribosome binding site; rWT, recombinant wild-type; SA, syringic acid; TCA, tricarboxylic acid; THF, tetrahydrofolate; VA, vanillic acid; WT, wild type.

study, integrated cytochrome P450 monooxygenase from *Rhodoccocus rhodochrous* strain J3 in *P. putida* EM42 showed the necessity for coexpression of a ferredoxin reductase from *R. rhodochorus* J3 and a ferredoxin from *Amycolatopsis* sp. ATCC 39116 [53]. The engineered *P. putida* strain fully consumed 5 mM guaiacol by 70 h without a growth lag. It would be interesting to see these two studies blended, where the GcoA–CatA fusion is expressed with GcoB, ferredoxin, and ferredoxin reductase in *P. putida* or another suitable expression host.

Table 3 details protein engineering efforts towards enhanced or altered aromatic catabolism. For example, point mutations in GcoAB from *Amycolatopsis* increased catalytic efficiency 2-fold towards syringol without compromising its ability to demethylate guaiacol [54]. Similarly, LigAB, a protocatechuate 4,5-dioxygenase from *Sphingobium* sp. strain SKY-6, was engineered to allow binding of 3-*O*-methyl gallate (3OMG) [32]. The best-performing mutants have catalytic efficiencies towards 3OMG 12–31-fold higher than wild-type LigAB enzyme and outperform DesZ, the native 3OMG dioxygenase. These examples demonstrate the effectiveness of protein engineering to expand the range of substrates utilized as feedstock without extra enzymes or pathways.

Adaptive evolution is another common method to enhance aromatic catabolism (Table 4). Close and coworkers evolved *E. coli* strains containing two different heterologous pathways for 4-coumaric acid utilization: the Hca pathway from *Acinetobacter baylyi* ADP1 and the Cou pathway from *Rhodococcus jostii* [44]. Both strains included PobA, a 4-hydroxybenzoate 3-monooxygenase, from *Paenibacillus* sp. JJ-1B. After 300 **passages** in minimal media containing 4-coumaric acid as the sole carbon source, a genomic evaluation found two different evolutionary paths for the two strains. Both strains showed a **synonymous** T3T mutation in PobA that enhanced expression levels by ninefold. The same mutation was reported by Standaert and colleagues [43]. A mutation in an **intergenic region** before *hcaC* caused a twofold expression increase of HcaB and HcaC [44]. A conserved mutation in GuaB, an inosine monophosphate dehydrogenase (IMPDH), relieved uncompetitive inhibition from 4-hydroxybenzaldehyde, an intermediate of the Hca pathway. The strain containing the Cou pathway showed less consistent mutations compared to the strain containing the Hca pathway; however, two strains showed mutations in *couL*, decreasing enzyme expression similarly to *hcaC*, which is also located at the end of its operon. Evolution of GcoAB in *A. baylyi* ADP1 led to discovery that the GcoA–CatA fusion enabled growth on guaiacol in *P. putida* [52]. Two separate studies passaged

Table 3. Summary of Protein Engineering To Improve Aromatic Catabolism[a]

Enzyme	Function	Origin	Mutation	Substrate	Engineering strategy	Outcome	Refs
GcoAB	*O*-aryl-demethylase	*Amycolatopsis* sp. ATCC 39116	F169A	Guaiacol, syringol	Increase size of the active site to allow syringol binding	GcoA-F169A had doubled K_{cat}/K_m (mM^{-1} s^{-1}) values for syringol conversion over guaiacol conversion. Lower K_D (μM) for syringol over guaiacol imply syringol is the preferred substrate. K_{cat}/K_m for guaiacol conversion of the mutant improved nearly twofold over the GcoA-WT	[54]
LigAB	Protocatechuate 4,5-dioxygenase	*Sphingobium* sp. strain SKY-6	F103V, F103T, F103H	PCA, gallate, 3OMG	Site-directed mutagenesis to F103, which is located in the allosteric binding pocket and associates with the C5 position of PCA	Mutants F103V, F103T, and F103H have 12–31-fold higher activity catalytic efficiency to 3OMG than WT LigAB. These values are higher than for DesZ, the native 3OMG dioxygenase	[32]

[a]Abbreviations: 3OMG, 3-*O*-methyl gallate; PCA, protocatechuic acid.

Table 4. Summary of Adaptive Evolution Strategies for Enhancing Aromatic Catabolism in Bacteria and Yeast[a]

Organism	Evolution method	Selection substrate	Glucouse supplementation	Number of passages	Analysis	Outcome	Refs
Bacterial hosts							
Acinetobacter baylyi ADP1	Serial passaging	FA	No	362	NA	The evolved strain grew robustly in 180 mM FA, whereas WT growth rate is significantly decreased in 80 mM. The evolved strain showed additional tolerance to up to 160 mM VA, but not 4-CA	[71]
Acinetobacter baylyi ADP1	EASy	Guaiacol	No	1000	G	One strain fused *gcoAB* to *catA*. Another strain created a 34 amino acid linker between *gcoAB* and *catA*. Other point mutations were discovered, but the effects of these were difficult to probe	[52]
Escherichia coli BW25113	Serial passaging	PCA	No	500	G	Growth rate improved by nearly 2.5-fold compared to the non-evolved strain, which is largely attributed to improved expression of PcaH through an RBS point mutation. Additional RBS strength increases do not improve growth on PCA	[50]
Escherichia coli JME17 [49]	Serial passaging	4-HBA	No	300	G, P	+PobA mutant strains showed 5′ silent mutations in PobA and *pcaHGBDC* duplication. +PraI mutant strains showed 5′ silent mutations in *praI* and mutations in *pcaK* which likely improved expression. The *pcaK* mutations only arose in tandem with growth on 4-HBA, whereas *pca* operon duplication occurred much more frequently	[43]
Escherichia coli JME38 [42] + *hca* operon	Serial passaging	4-CA	No	300	G	Five of the six mutants showed mutations in *guaB*, an IMPDH, and in the intergenic region upstream of *hcaC*. Mutations to *hcaC* created a twofold increased expression of HcaB and HcaC. The mutation in *guaB* relieved inhibition from 4-hydroxybenzaldehyde, an intermediate of the *hca* pathway. This is comparable to the IMPDH of *Acinetobacter baumannii*, which is not inhibited by 4-hydroxybenzaldehyde	[44]
Escherichia coli JME38 [42] + *cou* operon	Serial passaging	4-CA	No	300	G	Mutations were less consistent than those seen in the strains with the Hca pathway. In two of the mutants, mutations were seen in *nadR* and *couL*. The effect of *nadR* gene mutation is uncertain, whereas the *couL* mutation caused a decrease in expression	[44]
Rhodococcus opacus PD630	Serial passaging	Phenol	No	40	G, T	Evol40 had a 24% higher IC_{50} value than the WT after 45 h of growth, and a 102% increase in OD and 34% decrease in lag time in 1.5 g/l phenol. When grown with phenol, the evolved strains accumulated significantly more lipids than the WT strain. Adapted strains showed SNPs in two transporter/permease genes. Transcriptomic analysis shows a decreased stress response by evolved strains, perhaps due to upregulation of catabolic and transport genes	[55]
Rhodococcus opacus PD630	Serial passaging	PCA, guaiacol, BA, 4-HBA, phenol	No	100	G, T, M	The evolved strain was compared across glucose and all listed aromatics individually and in combinations. There was up to 1900% improvement in OD. 36% of non-synonymous SNPs in the aromatic evolved strain were in genes related to redox reactions. Improvements were attributed to upregulated catabolic genes and tolerance mechanisms such as redox genes and transporters	[56]

(continued on next page)

Table 4. (continued)

Organism	Evolution method	Selection substrate	Glucouse supplementation	Number of passages	Analysis	Outcome	Refs
Yeast hosts							
Saccharomyces cerevisiae CEN. PK113-7D [77]	EMS mutagenesis	Coniferyl aldehyde	Yes	113	G, T	The evolved strain improved coniferyl aldehyde tolerance from 0.25 mM to 1.7 mM. It also showed cross-resistance to vanillin and FA	[82]
Saccharomyces cerevisiae	EMS + ALE	Ext_{SECS}	Yes	180	T	The evolved strain grew in 2 g/l vanillin with a μ_{max} of 0.104 hr^{-1}, a 176% improvement of WT μ_{max}. There was also enhanced tolerance to furfural and acetic acid	[84]
Saccharomyces cerevisiae CEN-TE	MBC + ALE	Vanillin	Yes	1	G	Aneuploid mutants showed enhanced vanillin tolerance over triploid and euploid strains. The best variant, TE15, consumed 6.7 mM vanillin, with significant accumulation of vanillyl alcohol and slight accumulation of VA	[83]

[a]Abbreviations: ALE, adaptive laboratory evolution; 4-CA, 4-coumaric acid; EASy, evolution by amplification and synthetic biology; EMS, ethyl methanesulfonate; Ext_{SECS}, extract of steam exploded corn stover; FA, ferulic acid; G, genomic; IMPDH, inosine monophosphate dehydrogenase; M, metabolomic; MBC, methyl benzimadol-2-ylcarbamate; N, none; OD, optical density; P, proteomic; T, transcriptomic; VA, vanillic acid; WT, wild type.

Rhodococcus opacus PD630 and discovered **single nucleotide polymorphisms** (SNPs) in redox, transport, and stress-response genes [55,56]. Together, these works present a strong case for reverse engineering of elusive phenotypes through adaptive evolution.

Bacterial Bioproduction from Monoaromatic Feedstocks

Once lignomonomers are converted to key intermediates, they are cleaved at either the *ortho* or the *meta* position (Figure 2). The decision to pursue *meta*- versus *ortho*-cleavage pathways depends on several factors including how the final product is derived, the importance of product yield, and necessity of cofactor regeneration. *Meta*-cleavage pathways produce CO_2, reducing overall yield, but generate more NADH.

Johnson and coworkers compared *meta*- and *ortho*-cleavage pathways to produce pyruvate and L-lactate, a pyruvate-derived compound [57]. For this particular application, the *meta*-cleavage pathways outperformed the *ortho*-cleavage pathways for both catechol and protocatechuate metabolism because the *meta*-cleavage pathway produces pyruvate, whereas the *ortho* pathway produces acetyl-CoA and succinyl-CoA. Several studies converted existing *ortho*-pathways into *meta*-pathways to obtain pyridine 2,4-dicarboxylic acid (PDC) through *meta*-cleavage of protocatechuate [58–62]. Among these studies, the best strain produced 58 g/l of PDC and had a molar yield of 80.7% from 4-HBA in *P. putida* KT2440 by knocking out *pcaHG* and including *ligAB* from *Sphingobium* sp. SKY-6 [61]. This engineered *P. putida* strain outperformed PDC production from *Novosphigobium aromaticivorans*, a species that natively uses the *meta* pathway [63].

Cis,*cis*-muconic acid (referred to as muconic acid) is a precursor for biobased nylon, and is a commonly targeted product from *ortho* cleavage of monoaromatic substrates. To facilitate muconic acid accumulation, β-ketoadipate genes such as *catBC* and *pcaHG* are typically knocked out in hosts such as *E. coli*, *P. putida*, and *Sphingobium* sp. SKY-6 [47,49,64–69]. To date, the highest reported titer is by engineered *Corynebacterium glutamicum* MA-2 which produced 85 g/l of muconic acid from cofeeding catechol and glucose; however, there was a large drop in titer to 1.8 g/l when softwood lignin hydrolysate was used as the feedstock [70]. Several studies describe a build-up of protocatechuate, a key intermediate in funneling pathways [47,64,65]. In *E. coli*, overexpression of the protocatechuate decarboxylase, AroY, and the

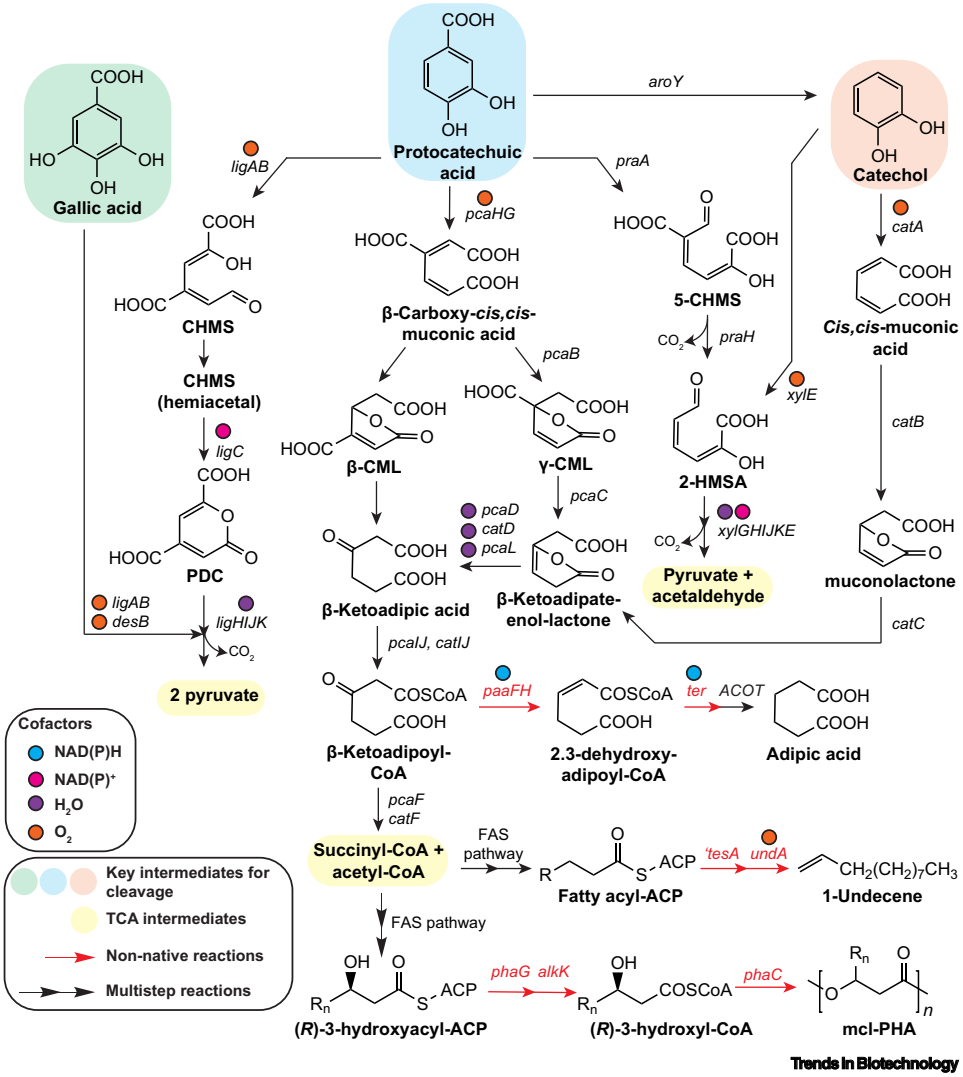

Figure 2. Cleavage and Conversion Pathways for Gallate (Green Shaded Box), Protocatechuate (Blue Shaded Box), and Catechol (Orange Shaded Box) through TCA Intermediates (Yellow Shaded Words). Non-native reactions are indicated with red arrows and text. Cofactor utilization is indicated by colored dots proximal to each enzyme name. Abbreviations: ACP, acyl carrier protein; CHMS, carboxy-2-hydroxymuconoate-semialdehyde; 5-CHMS, 5-carboxy-2-hydroxymuconoate-semialdhyde; β-CML, β-carboxymuconolactone; γ-CML, γ-carboxymuconolactone; 2-HMSA, 2-hydroxymuconoate-semialdehyde; mc-PHA, medium chain length polyhydroxyalkanoate; PDC, 2-pyrone-4,6-dicarboxylic acid; TCA, tricarboxylic acid cycle.

B-subunit of the 4-hydroxybenzoate (4-HBA) decarboxylase, KpdB, from *Klebsiella pneumonia* eliminated the protocatechuate bottleneck measured during muconic acid production from vanillin [47]. Counterintuitively, overexpression of the D-subunit of the 4-HBA decarboxylase, KpdD, alongside AroY and KpdB inhibited AroY activity. A similar approach was used in *P. putida* KT2440 using EcdB and EcdD, KpdBD homologs, from *Enterobacter cloacae* [69]. When both EcdB and EcdD are coexpressed, AroY activity and muconic acid production are enhanced. The cell-specific effects of decarboxylase-associated proteins remains an open question.

Cofeeding glucose can facilitate high molar conversions of aromatic compounds to products because glucose can provide building blocks and energy carriers, such as acetyl-CoA and NADH, but aromatic pathways often suffer **catabolite repression** [64,65,68]. Deletion of a catabolite repression regulator gene, *crc*, facilitated cofeeding with glucose and acetate [65,68]. Engineered *P. putida* cells deficient in the Crc regulator increased the molar yield of muconic acid derived from 4-coumaric acid from 50% to 96.4% in the presence of glucose [68]. The same strain cultured with ferulic acid and glucose had a lower overall molar yield, but still improved the molar yield of muconic acid from 12% to 28.3%. A follow-up study introduced EcdBD and AroY into the *P. putida Δcrc* strain to convert protocatechuate to catechol [65].

Other studies have sought products not directly related to ring-cleavage products. *Acinetobacter baylyi* ADP1 produced 72 μg/l of 1-undecene using 110 mM of ferulic acid as the sole carbon source when coexpressing 'TesA, a thioesterase from *E. coli,* and UndA, a fatty acid decarboxylase from *P. putida* [71]. *Pseudomonas putida* KT2440 fed 100 mM 4-HBA with constant glucose in a two-stage bioreactor produced 17 mM adipic acid with a 17.4% molar yield when expressing PaaHG, an isomerase-hydratase involved in β-oxidation, from *E. coli* and Ter, a *trans*-2-enoyl-CoA reductase, from *Treponema denticola* [72]. *Pseudomonas putida* is a known aromatic metabolizer and robust producer of medium chain length polyhydroxyalkanoates (mcl-PHAs). Endogenous genes encoding PhaJ4, an enoyl-CoA hydratase, and PhaC1, a PHA synthase, were expressed under a weak vanillic acid-inducible promoter, P_{van}, resulting in *P. putida* producing 161 mg/l PHAs with a lipid content of 73.5% per **dry cell weight** (DCW) from 6 mM vanillic acid in a nitrogen-limited medium [15]. In a subsequent study, the same genes were expressed under a strong, xylose-inducible promoter, P_{xylA} [73]. This strain completely consumed 15 mM vanillic acid in a nitrogen-rich medium and produced 246 mg/l PHA at a PHA content of 34.4% DCW. Nitrogen-limited conditions produced a much higher PHA content of 66.3%, but biomass titers were lower, negatively impacting on PHA titer. Overexpression of an 3-hydroxyacyl-acyl carrier protein (ACP):CoA transferase (PhaG), acyl-CoA synthetase (AlkK), and PHA synthase (PhaC1) under P_{xylA} was designed to encourage flux through PHA precursors; however, both titer and PHA content suffered in both nitrogen-rich and -poor conditions. By comparison, Salvachúa and colleagues constructed a similar strain but used a fed-batch culturing method to improve biomass and PHA titers [74]. In this case a strong, constitutive promoter, P_{tac}, drove the expression of PhaG, AlkK, PhaC1, and PhaC2. Fed-batch culturing with 4-coumaric acid overcame biomass limitations and toxicity limits, whereas the low-nitrogen conditions maintained high cellular PHA content, producing 953 mg/l mcl-PHAs with a 54.3% PHA content per DCW. The same strain produced 116 mg/l mcl-PHAs, a 17.7% PHA content per DCW, when utilizing corn stover hydrolysate, containing 4-coumaric acid and ferulic acid, as the sole carbon source.

Enhancing and Expanding Monoaromatic Catabolism in Yeast and Fungi

Growing numbers of yeast and fungi are known to metabolize monoaromatic compounds [75,76]. Aromatic catabolism in yeast and fungi has been extensively studied biochemically; however, little is known about the underlying genetics, making it difficult to engineer native aromatic metabolism [42]. There are two studies where bacterial aromatic catabolism genes were used to engineering coniferyl aldehyde metabolism in *S. cerevisiae*, but both strains require glucose supplementation [77,78]. In one, a coniferyl aldehyde dehydrogenase, CALDH, from *Pseudomonas* sp. HR199 was overexpressed and facilitated the uptake of 1.1 mM coniferyl aldehyde [77]. Separately, overexpression of endogenous *atf1*, *atf2*, *pad1*, and *ald5* in *S. cerevisiae* led to conversion of 1.1 mM coniferyl aldehyde, 1.8 mM ferulic acid, and 9.7 mM 4-coumaric acid [78]. *Rhodotorula toruloides* IFO 0880 naturally metabolizes aromatics and was engineered to produce bisabolene using a *BIS* gene from *Abies grandis* [79]. When using

0.5% 4-coumaric acid as the sole carbon source, the engineered strain produced approximately 75 mg/l bisabolene. We expect an increase in future publications as developing genetic toolkits for non-conventional organisms becomes more commonplace [80,81].

Organisms utilizing lignin-derived feedstocks balance tolerance and metabolism – mechanisms difficult to parse and transfer to model organisms. Adaptive evolution can introduce and improve both traits on a scale that targeted engineering strategies cannot achieve without deep *a priori* knowledge. Several notable studies on *S. cerevisiae* have evaluated genomic and transcriptomic changes resulting from phenotypes evolved by chemical mutagenesis [82–84]. Hacisalihoglu and coworkers performed ethyl methanesulfonate (EMS) mutagenesis and increased coniferyl aldehyde tolerance from 0.25 mM to 1.7 mM and also established vanillin and ferulic acid cross-resistance [82]. Thorough transcriptomic analysis and genomic resequencing implicate several genes for enhanced resistance; however, lack of validation by forward genetics leaves open the possibility tolerance is caused by some so far unknown factors. Zheng and colleagues used methyl benzimadol-2-ylcarbamate to mutate *S. cerevisiae* and showed that aneuploid mutants were more vanillin-tolerant than polyploid strains [83]. Shen and coworkers mutated *S. cerevisiae* with EMS and evolved it over the course of 113 passages in corn stover hydrolysate containing 0.2 g/l vanillin [84]. The evolved strain had a higher antioxidant capacity and higher vanillin tolerance and metabolism. A follow-up study investigated the genome and transcriptome of the wild-type and evolved strains [85]. A wild-type strain was engineered to achieve the same tolerance of the evolved strain by knocking out a transcription factor gene, *yrr1p*, which increased expression of ABC transporters, rRNA processing, and ribosome biogenesis.

Concluding Remarks and Future Perspectives

The future economic success of lignocellulosic-based biorefineries is tied to the valorization of lignin. Microbes are particularly well suited to handle the inherent recalcitrant properties of lignin, its heterogeneity, and its toxicity. Many groups have engineered bacteria, yeast, and fungi alike to produce value-added products, enhance lignin depolymerization, enhance the metabolism of aromatic monomers, or a combination thereof. We expect to see continued development of model hosts for lignin valorization, especially as the synthetic biology advances; however, toxicity is likely to limit the usefulness of these systems. Cell-free systems inherently avoid cellular toxicity but would present a huge challenge for functional protein expression. A possible solution would be for cell-free systems to either depolymerize or metabolize the most toxic components, effectively detoxifying lignin and its depolymerization products. Future work should explore the balance between the two approaches.

Non-model and non-conventional organisms typically have robust, niche phenotypes that can be difficult to replicate in model organisms; however, their genetic immaturity makes it difficult to engineer them to produce value-added products (see Outstanding Questions). The field must carefully consider whether to pursue these uniquely adapted strains and develop their genetic accessibility or stay with model organisms whose availability of genetic tools allows extensive rewiring. The growing number of publications in non-model organisms suggests that the value of starting with naturally evolved complex phenotypes outweighs the investment in tool development.

To facilitate further genetic and protein engineering in both model and non-model organisms, the discovery of novel genes driving the complex lignin tolerance and valorization phenotype in higher organisms, such as yeast and fungi, should be sought. We anticipate growing interest in yeast and fungal functional genome annotation of lignin tolerance and metabolism. Enabled by advanced synthetic biology tools, such as genome-scale CRISPR/Cas9 and transposon libraries,

Outstanding Questions

Often, lignin depolymerization and catabolism of released low molecular weight compounds are considered as two separate steps. Is consolidated bioprocessing a feasible route for lignin valorization from depolymerization to production?

Would enzymatic pretreatment of the lignin by cell-free systems before monomer uptake and conversion to high-value products by suitable microorganisms be cost-effective in biorefineries?

Would fermentative setups based on coculturing organisms with distinct and complementary abilities to depolymerize lignin and catabolize its various monomers be of added value in lignin valorization, particularly to produce high-value products?

What non-model organisms exist that have improved lignin depolymerization and catabolism of released monomers?

What are the genes utilized in yeast species for lignin depolymerization and lignomonomer catabolism? An enhanced understanding of these processes will facilitate the design of lignin-degrading microbes for efficient lignin valorization.

Should future research focus on establishing genetic tractability of non-model organisms or transporting lignin-relevant pathways to model organisms?

What transcription factors regulate the funneling and cleavage pathways, and how might they be engineered or eliminated to enhance aromatic catabolism?

Fungi have a consortium of accessory enzymes that generate hydrogen peroxide and regenerate cofactors. How important is it to consider balancing the cyclic catalytic nature of laccases and peroxidases when engineering a lignin-depolymerization pathway?

What are key features of fungal secretion systems that enable high-titer production of active lignin-degrading enzymes?

will be central to elucidating the genetics behind biochemically characterized lignin-metabolism biochemical pathways found in some eukaryotes, and the genetics underlying uncharacterized tolerance and metabolic pathways. Furthermore, we anticipate that unlocking the distinctive features of fungal secretion systems will enable expression of highly active lignin-degrading enzymes in non-fungal (yeast and perhaps bacterial) systems.

How do PTMs effect protein activity, folding, and secretion? Can structure-guided engineering facilitate favorable PTMs?

Declaration of Interests

The authors declare no conflicts of interest.

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