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Biomanufacturing of value-added chemicals from lignin

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Lignin valorization faces persistent biomanufacturing challenges due to the heterogeneous and toxic carbon substrates derived from lignin depolymerization. To address the heterogeneous nature of aromatic feedstocks, plant cell wall engineering and 'lignin first' pretreatment methods have recently emerged. Next, to convert the resulting aromatic substrates into value-added chemicals, diverse microbial host systems also continue to be developed. This includes microbes that (1) lack aromatic metabolism, (2) metabolize aromatics but not sugars, and (3) co-metabolize both aromatics and sugars, each system presenting unique pros and cons. Considering the intrinsic complexity of lignin-derived substrate mixtures, emerging and non-model microbes with native metabolism for aromatics appear poised to provide the greatest impacts on lignin valorization via biomanufacturing.

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Introduction

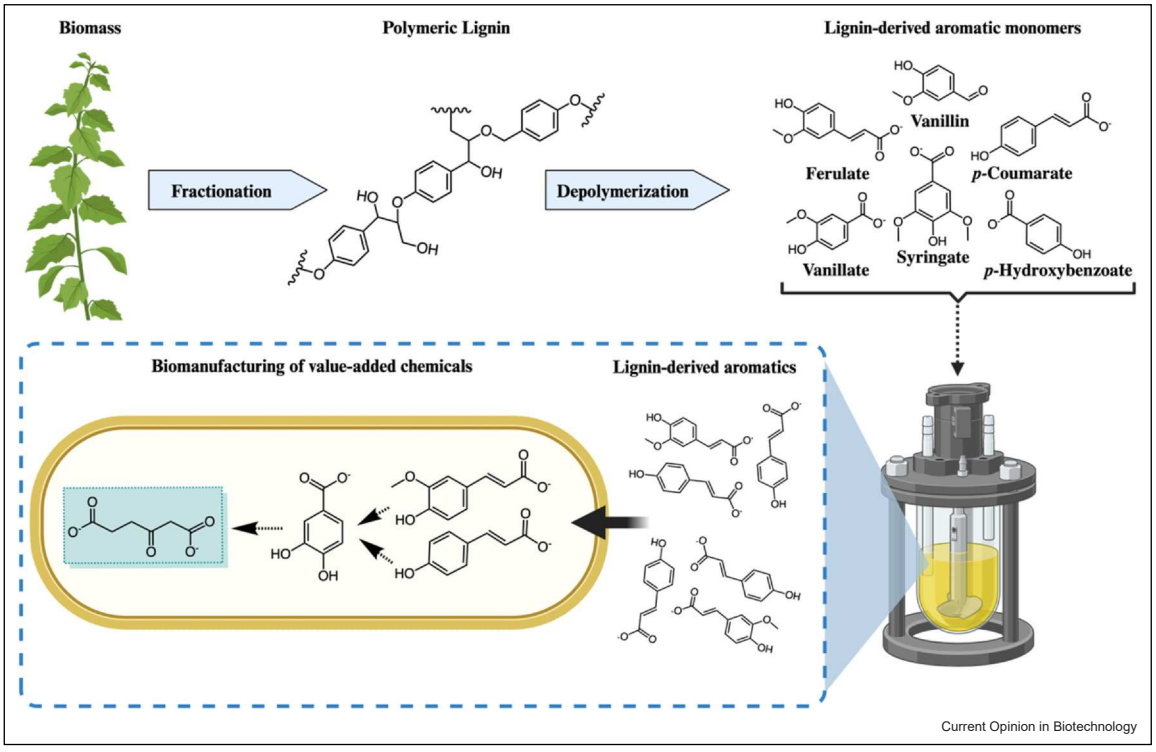
Lignin is the second most abundant natural biopolymer on earth [1]. Of the 50 million tons of lignin generated each year as an agricultural byproduct, only 2% is converted into specialty chemicals, while the rest is used as a crude, combustible fuel [2,3]. However, being composed of phenylpropionate subunits, lignin is well poised to serve as a sustainable feedstock for producing numerous aromatic and other value-added chemicals — an approach that can help reduce global reliance on crude oil [4]. That said, it remains difficult to depolymerize lignin into a narrow stream of industrially useful chemicals, and the released aromatic compounds are often toxic to those microbial cell factories used to convert them into value-added chemicals [5–7]. Accordingly, to enhance lignin valorization, current research efforts have largely focused on (1) biological, chemical, and thermal breakdown of lignin to its monomers [8–12]; (2) engineering plants to preferentially accumulate specific aromatic substrates of interest [13–16]; (3) engineering microbes for direct bioconversion of lignin-derived aromatics into specialty chemicals [17–23]; (4) systems biology studies to guide metabolic engineering and to improve biological lignin valorization [24–26]; and (5) developing translational synthetic biology tools for lignin valorization (Figure 1) [27–29]. In this review, we will summarize the recent advances within these thrusts, with specific emphasis on the importance of metabolic engineering for the valorization of lignin-derived aromatics.

Engineering bioenergy crops for selective accumulation and release of key aromatics

Conventional lignin deconstruction methods render complex, heterogeneous mixtures of aromatics with little selectivity towards any specific aromatic substrate(s) of interest (Figure 2). However, as will be discussed, only a few lignin-derived aromatics (e.g. *p*-coumaric acid, ferulic acid, and protocatechuate) have emerged as key precursors for downstream bioconversion into value-added chemicals. To address this disparity, novel strategies are being developed to enhance the *in planta* production of such aromatics and/or promote their selective release during depolymerization (Figure 3). It has long been known that deleting or down-regulating native cinnamyl alcohol dehydrogenase can enhance the accumulation of vanillin and syringaldehyde in cell walls [30]. These hydroxybenzaldehydes are then readily released during pretreatment [31,32]. Meanwhile, as demonstrated for multiple bioenergy crops, enhanced *in planta* production of free protocatechuate is possible via expression of bacterial 3-dehydroshikimate dehydratase (QsuB) [33–35]. Protocatechuate can then be extracted from biomass during mild alkaline pretreatment for upgrading by microbial biocatalysts [36,37].

As an alternative approach, several 'lignin first' methods have emerged, which allow for the removal of unmodified lignin and the recovery of select aromatic monomers from it. For example, Timokhin et al. recently reported a method involving mild saponification followed by extraction that allows *p*-coumarate to be recovered from corn stover at 4.8% wt. yield and 97% purity [38]. Meanwhile, Mottiar et al. expressed bacterial

Figure 1



Biomass is fractionalized to isolate the polymeric lignin fraction. The polymeric lignin is depolymerized to release a heterologous mixture of aromatic molecules, which can be fed to engineered microbial hosts. These microbes are capable of catabolizing the aromatics and converting them into value-added chemicals. Created with BioRender.com.

chorismate pyruvate lyase (CPL) in poplar to increase its hydroxybenzoate content by >50% [39]. Since it attaches to lignin by a weak ester linkage, p-hydroxybenzoate is readily cleaved off during acid hydrolysis. This latter study demonstrates how, when applied together, these two complementary upstream methods (i.e. enhanced *in planta* accumulation and partial delignification) have the potential to provide narrow yet sizable streams of lignin-derived aromatic feedstocks.

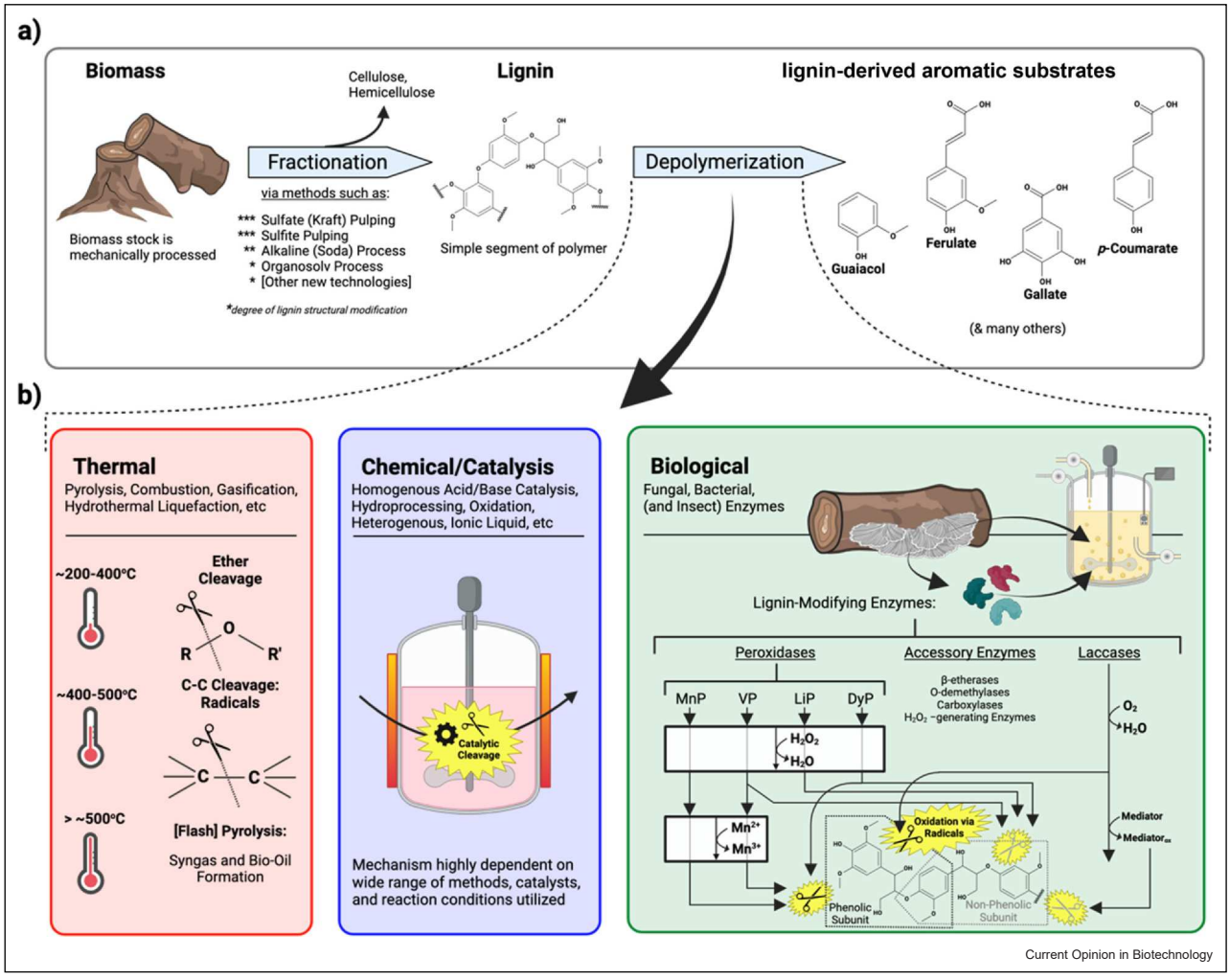
Metabolic engineering of diverse microbial chassis for the biomanufacturing of value-added chemicals from lignin-derived aromatics

Traditionally, the field of metabolic engineering has looked towards nonmodel organisms to discover novel enzymes/pathways to enhance the function of engineered model strains, such as *Escherichia coli* and *Saccharomyces cerevisiae*. However, while model organisms are well-studied and easily manipulatable, they lack the catabolic pathways required to utilize lignin-derived aromatics as substrates [7,40]. While heterologous pathways for aromatic catabolism can be introduced, doing so does not address the next challenge of efficiently utilizing these aromatic substrates, namely their high toxicity at even low concentrations [41]. Alternatively, researchers are increasingly exploring the biocatalytic potential of nonmodel organisms that are natively capable of efficiently (co-)metabolizing aromatics and display increased tolerance towards aromatic substrates [42,43]. Below we summarize recent advancements related to metabolic engineering for upgrading lignin-derived aromatics, including with respect to microbes that (1) lack aromatic metabolism, (2) metabolize aromatics but not sugars, and (3) co-metabolize both aromatics and sugars.

Microbial hosts lacking aromatic metabolism

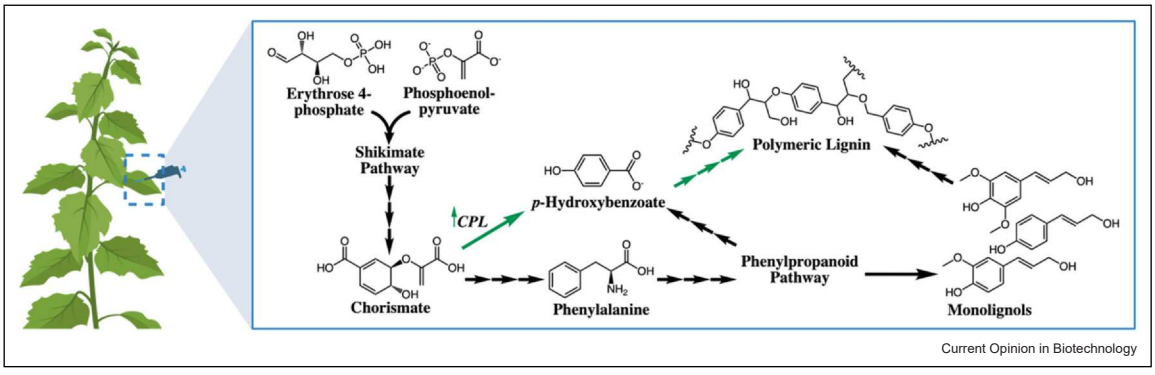
E. coli and S. cerevisiae, the two most well-studied model microbial biocatalysts, both lack native pathways capable of catabolizing lignin-derived aromatics. On the one hand, this absence makes these model organisms advantageous for the characterization and optimization of pathways responsible for converting lignin-derived aromatics into value-added chemicals (Table 1) (Figure 4). For example, when engineered to express a recombinant ligninolytic pathway, *E. coli* produced up to 311 mg/L muconic acid and 6.2 mg/L pyrogallol from lignin-derived vanillin and syringate, respectively [37]. While low initial product titers might be a common occurrence in

Figure 2



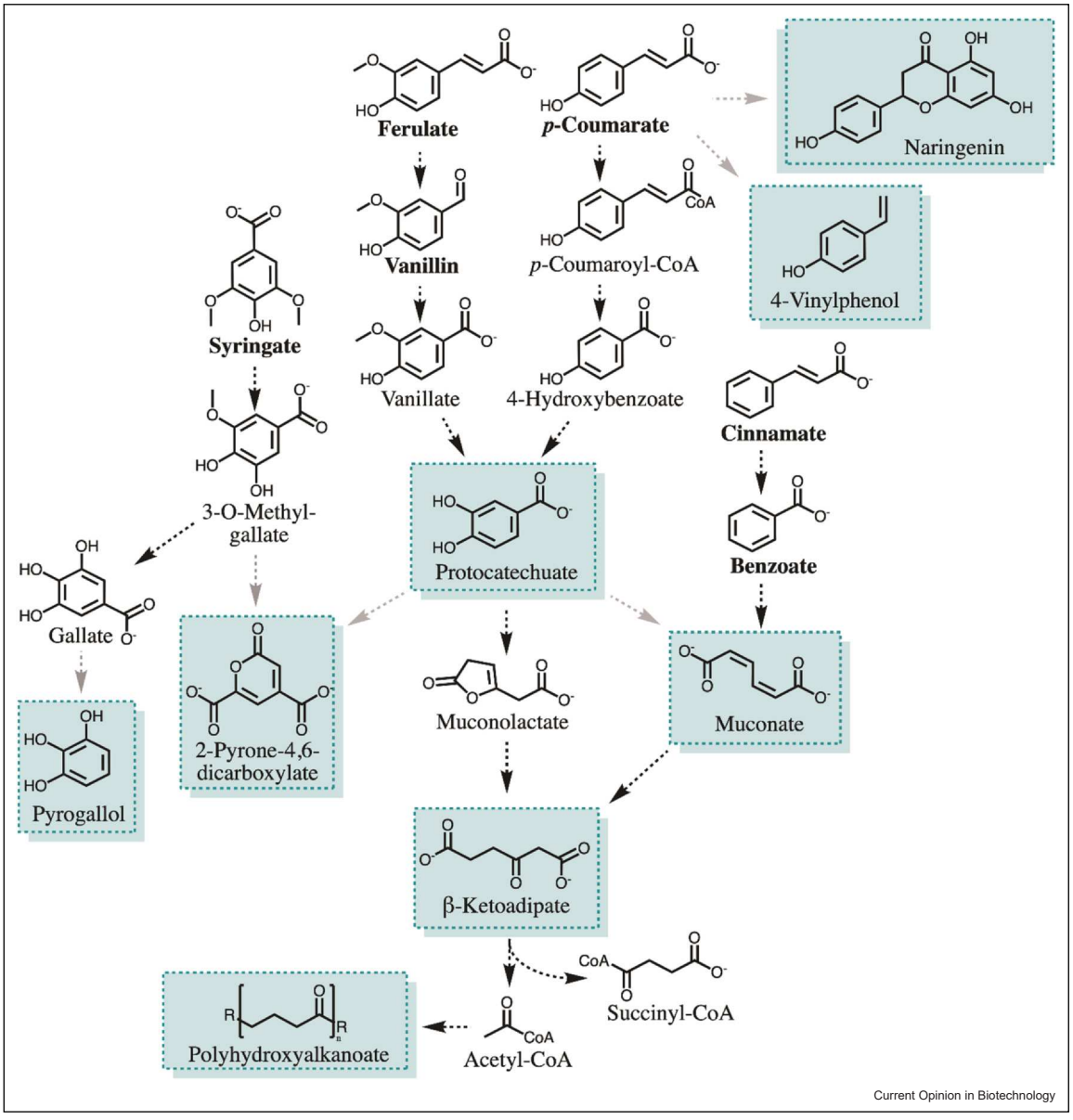
Graphical summary of current lignin depolymerization methods. **(a)** After physical preprocessing of raw biomass, one of many fractionation methods is used to isolate lignin biopolymer from lignocellulose. **(b)** Lignin is then depolymerized to yield several different aromatics. Depolymerization is performed via thermal, chemical/catalytic, or biological methods. Created with BioRender.com.

Figure 3



Plants are capable of being modified through metabolic engineering approaches. By upregulating certain enzymes, such as CPL, an alternative pathway to produce p-hydroxybenzoates (green arrows) can be established to improve their content *in planta*. Created with Biorender.com.

Figure 4



Pathway of aromatic catabolism and the value-added chemicals that can be produced. Value-added chemicals can be accumulated by engineering native reactions in the aromatic catabolism or by heterologous expression. Native reactions (dashed black lines), heterologous reactions (dashed gray lines), lignin-derived aromatics (bold), value-added chemicals (green boxes).

this approach owing to substrate toxicity, this challenge can often be overcome via adaptive laboratory evolution (ALE) and/or additional rational engineering strategies aimed at reducing substrate/intermediate toxicity and improving pathway flux. As an example, Clarkson et al. demonstrated this approach using an *E. coli* strain engineered to express the protocatechuate 3,4-cleavage pathway from *Pseudomonas putida* KT2440 [40]. In this case, while the engineered strain at first grew slowly on protocatechuate, ALE helped the strain to overcome this by acquiring mutations that improved the abundance of protocatechuate 3,4-dioxygenase by 7-fold, thereby enhancing the growth of the resulting mutant by up to 2.4-fold. Through additional rational engineering, the growth rate was later increased by 10-fold using p-hydroxybenzoate as substrate.

In addition to the expression of heterologous pathways for aromatic catabolism, *E. coli* and *S. cerevisiae* have also been engineered to express pathways for converting

Table 1

List of organisms utilized for the valorization of lignin-derived aromatics.

Organism	Aromatic substrate	Product	Titer	Yield	Overexpressed genes	Gene knockouts	Reference #
Acinetobacter baylyi ADP1	*aromatic mixture*[a]	Mevalonate	1014 mg/L	41.4 mmol/mol	*atoB, ERG13, HMG1*	*acr1*	[57]
C. glutamicum ATCC 13 032	*p*-Coumarate	4-Vinylphenol	187 g/L	90% mol/mol	*pad*		[41]
C. glutamicum ATCC 13 032	*aromatic mixture*[a]	Muconate	5 mM	100% mol/mol	*catA, vdh, vanABK, aroY, ecdB, ecdD*	*catB, fudC, vanR*	[58]
C. glutamicum ATCC 21 420	Ferulic acid	Protocatechuate	6.91 mM	62.8% mol/mol	*vanAB*		[59]
C. glutamicum MB001 (DE3) (DelAro3)	Cinnamate	Pinosylvin	121 mg/L		*4CL, CHS, CHI*	*phdBCDE, pcaBCDFGHO, catA1BC, benABCD*	[60]
	Caffeiate	Piceatannol	56.3 mg/L		*4CL, CHS, CHI*	*phdBCDE, pcaBCDFGHO, catA1BC, benABCD*	
	Caffeiate	Eriodictyol	25.4 mg/L		*4CL, CHS, CHI*	*phdBCDE, pcaBCDFGHO, catA1BC, benABCD*	
C. glutamicum MB001 (DE3) (DelAro4)	*p*-Coumarate	Naringenin	3271 mg/L		*4CL, CHS, CHI*	*phdBCDE, pcaBCDFGHO, catA1BC, benABCD, qsuB, pobA*	
	p-Coumarate	Resveratrol	158 mg/L		*STS, CHI*	*phdBCDE, pcaBCDFGHO, catA1BC, benABCD, qsuB, pobA*	
C. glutamicum MB001 (DE3) (DelAro4)	*p*-Coumarate	Naringenin	24 mg/L		*4CL, CHS, CHI*	*phdBCDE, pcaBCDFGHO, catA1BC, benABCD, qsuB, pobA*	[18]
	p-Coumarate	Resveratrol	112 mg/L		*STS, CHI*	*phdBCDE, pcaBCDFGHO, catA1BC, benABCD, qsuB, pobA*	
Escherichia coli BL21	*p*-Coumarate	Resveratrol	2.3 g/L	~1.0 g/g	*4CL, CHS, CHI*		[61]
Escherichia coli DH1	Vanillin	Muconate	314 mg/L	0.63 g/g	*aroY, desA, Lpdc, ligV, ligM, CatA-pmt2*		[37]
	Syringate	Gallate	18 mg/L	59.6 mg/g	*desA, ligM, lpdC*		
	Syringate	Pyrogallol	7.3 mg/L	7.3 mg/g	*desA, ligM, lpdC*		
	Protocatechuate	Muconate	311 mg/L	0.31 g/g	*catA, aroY*		
Pseudomonas putida H	Benzoate	poly (3-hydroxyalkanoate)	6.1 g/L	4.02 mol/mol		*CatA2*	[26]
	Kraft Lignin Hydrolysate	poly (3-hydroxyalkanoate)	1.4 g/L			*CatA2*	
Pseudomonas putida KT2440	Protocatechuate, Catechol	Various value-added chemicals	up to 58 g/L	100% mol/mol		*multiple gene knockouts*	[62]
Pseudomonas putida KT2440	Vanillate, 4-Hydroxybenzoate	Muconate	~100 μM	20% mol/mol	*pcaHG, pdc*	*pcaH, pcaG, catB*	[63]
Pseudomonas putida KT2440	*p*-Coumarate	Bisdemethoxycurcumin	2 mg/L		*CUS*	*Ech*	[19]

Table 1 (*continued*)

Organism	Aromatic substrate	Product	Titer	Yield	Overexpressed genes	Gene knockouts	Reference #
Pseudomonas putida KT2440	Syringate, Ferulate, *p*-Coumarate	2-pyrone–4,6-dicarboxylate	4.3 mM	93% mol/mol	*vanAB, pcaHG*	*galA*	[64]
Rhodococcus jostii RHA1	Vanillate 4-Hydroxybenzoate Wheat Straw Wheat Straw	pyridine–2,4-dicarboxylic acid pyridine–2,5-dicarboxylic pyridine–2,4-dicarboxylic acid pyridine–2,5-dicarboxylic	486 mg/L 810 mg/L 200 mg/L 287 mg/L		*ligAB* *praA*	*pcaGH* *pcaGH*	[65]
Rhodococcus opacus PD630	Corn Stover Lignin	Muconate	1.63 g/L		*aroY, ecdB*	*pcaGH, catB*	[66]
Rhodococcus opacus PD630	Vanillin	Muconate	5.5 mM	97.83 mol/mol	*vdh, vanAB, catA, aroY, ecdB*	*Pd630_LPD06298, Pd630_LPD04406, Pd630_LPD07543, Pd630_LPD04460, Pd630_LPD03722*	[67]
Rhodopseudomonas palustris CGA009	*p*-Coumarate	Polyhydroxybutyrate	0.41 g/L	68.4% carbon conservation efficiency			[68]
Rhodopseudomonas sp. S16-VOGS3	Digested sludge	poly (3-hydroxyalkanoate)	18.5 mg/L				[69]
Saccharomyces cerevisiae BY4742	*p*-Coumarate, Ferulate	Protocatechuate	810 mg/L		*fcs, ech, and vdh*	*DH6, ADH7, BDH2, FDC1*	[7]
Saccharomyces cerevisiae C800	*p*-Coumarate	Naringenin	1.21 g/L		*4CL, CHS, CHI*		[46]
Sphingobium sp. SYK–6	Syringate, Vanillate, 4-Hydroxybenzoate	Muconate	189 μM	45% mol/mol	*vanAB, catA, pdc, kpdB*	*pcaH, pcaG, catB*	[63]
Sphingobium sp. SYK–6	Vanillate	Muconate	1.2 mM	96 mol/mol	*acvABCDEF, vceAB, aroY*		[70]

[a] Mixture of various aromatic substrates.

lignin-derived aromatics into valuable natural biochemicals [44–48]. Gao et al. applied this approach for naringenin biosynthesis where by fine-tuning the expression of the flavonoid pathway in *S. cerevisiae*, up to 1.21 g/L naringenin was obtained from 2.5 g/L *p*-coumarate. One drawback here is that, in addition to the aromatic monomers, flavonoid biosynthesis also requires malonyl coenzyme A (CoA) as co-precursor [49,50]. Thus, further pathway engineering to improve malonyl-CoA levels, combined with additional co-factor balancing, has proven important for enhancing flavonoid production by this approach [50–54]. In addition to pathway engineering, introducing transporter for the aromatic substrates and engineering the strain with aromatics inducible promoter have also resulted in improved product yields [55,56]. The studies discussed above collectively highlight the versatility of model organisms for the valorization of lignin-derived aromatics. As an alternative approach, it is also advantageous to engineer nonmodel microbial hosts that may already contain the aromatic catabolism pathways, which will be discussed next.

Microbial hosts with native aromatic metabolism but lacking sugar metabolism

In nature, microbes have also evolved with the ability to utilize aromatics but lack pathways to catabolize sugars. These aromatic degraders have proven to be a great resource for the discovery of novel or better-performing aromatic-catabolism pathways to improve the performance of the microbial hosts [71]. One such interesting microbe is *Sphingobium* sp. SYK-6 that has the ability to catabolize S-, G-, and H-lignin derivatives [70,72]. Genes from SYK-6 have been integrated into *P. putida* KT2440 to convert S-, G-, and H-lignin derivatives into 2-pyrone-4,6-dicarboxylate (producing 4.3 mM at 93% molar yield) [64]. In addition, SYK-6 can itself serve as a chassis for direct lignin valorization, especially when conventional carbon substrates are limited. This was demonstrated by Sonoki et al., who developed a sugar-free process for the biomanufacturing of muconic acid [63]. By shunting the pathways responsible for the catabolism of G- and H-lignin derivatives, these substrates were instead converted into muconic acid, while S-lignin derivatives were used for growth. Overall, the engineered SYK-6 strain achieved a higher muconic acid yield (5% mol/mol G-lignin derivative) compared to the engineered *P. putida*, which produced muconic acid at a lower yield (20% mol/mol G- and H-lignin derivatives).

In contrast to engineering hosts for product synthesis, aromatic degraders such as *Rhodopseudomonas palustris* can naturally accumulate chemicals of interest [73]. *R. palustris* is a metabolically flexible bacterium that produces polyhydroxyalkanoates naturally as a mode of carbon storage [68,69]. In the study by Brown et al., *R. palustris* CGA009 was shown to produce 0.41 g/L poly (3-hydroxybutyrate) (PHB) anaerobically using *p*-coumarate and sodium bicarbonate as substrates. Furthermore, engineering *R. palustris* represents an opportunity to diversify the products that can be synthesized. Wild-type CGA009 is incapable of producing poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) aerobically, but when it was engineered to overexpress a phasin peptide, it produced PHBV, aerobically, at higher production metrics (0.7 g/L) than anaerobic fermentations (0.41 g/L) [74]. Although the microbial hosts discussed above have contributed immensely towards lignin valorization (Table 1), by doing so, we ignore major carbon substrates such as glucose and xylose that are present in biomass hydrolysates. Moreover, glucose and xylose can contribute both carbon and energy to synthesize cellular building blocks while utilizing aromatics to produce value-added chemicals at a higher titer, rate, and yield (TRY). Therefore, in the next section, we discuss the progress that was made with engineering microbes that have both sugar and aromatic metabolism.

Microbial hosts with both aromatic and sugar metabolism

Many soil-residing microbes have evolved with the ability to (co-)utilize both sugars and aromatic compounds released from the breakdown of plant biomass [75,57,76,77]. What makes these nonmodel organisms especially useful is that many of them have evolved physiological traits that improve their fitness in environments rich in lignin-derived substrates [78–80]. Many of these traits provide the important advantages of being able to (1) assimilate a diverse stream of substrates, and (2) synthesize aromatics-derived chemicals at a very high yield (Table 1) [81,82]. Groups have demonstrated such outcomes using *Pseudomonas putida* KT2440, which is naturally capable of 'funneling' heterogeneous mixtures derived from lignocellulosic biomass (typically composed of glucose, acetate, and aromatics) into various value-added chemicals [83,84,62]. In particular, when Salvachúa et al. engineered KT2440 to overexpress genes in the PHA pathway, the engineered strain produced 116 mg/L PHA, a 3.3-fold improvement compared to KT2440 [85]. Another notable example utilizes *P. putida* to produce muconic acid [86–88]. Through extensive metabolic engineering and bioprocess development, Werner et al. were able to improve muconic acid production by KT2440 to 44.5 g/L with a 100% yield from *p*-coumarate and ferulate mixtures. Alongside pathway engineering, synthetic biology approaches have also been employed to enhance the efficiency of lignin valorization. One noteworthy example is the work by Li et al., in which a *p*-coumarate and ferulate auto-regulatory system was developed in *P. putida* KT2449 for protocatechuate production [89]. Through the engineering of the PadR regulator
and its corresponding promoter (P_{padC}) to overexpress

rate-limiting enzymes, the final system achieved a production of 12.7 g/L protocatechuate, the highest reported titer from *p*-coumarate-ferulate mixtures. This study highlights the unique advancements synthetic biology tools, such as aromatic biosensors [90,91], can make in improving lignin valorization.

In many aromatic degraders, enzyme promiscuity enables them to catabolize structurally similar aromatic molecules, but this can be a challenge in biomanufacturing applications, as unwanted reactions can decrease product titer and yields [58,92]. To circumvent this, Cai et al. first predicted the existence of multiple putative vanillin reductases in the genome of *Rhodococcus opacus* and then deleted them to improve the yield of muconic acid from 10 to 97% (mol/mol *p*-coumarate) [67]. With the reductive branch eliminated, *R. opacus* was then further upgraded to produce muconic acid from lignin. An engineered strain of *R. opacus* was cultured with alkaline-treated corn stover and was capable of catabolizing both the monomeric and polymeric lignin derivatives into muconic acid, improving the yield to 100% (mol/mol total assimilated aromatics) [66]. This approach has proven valuable while engineering other hosts as well, with Weiland et al. discovering and eliminating a similar reductive branch responsible for converting aldehyde-related intermediates into alcohol-analogs in *Corynebacterium glutamicum* [58]. The group then fine-tuned the pathway and eliminated the aromatic regulatory mechanism facilitated by VanR, which improved pathway productivity to also yield 100% conversion of aromatic substrates to muconic acid.

Aromatic feedstocks can also serve as substrates to synthesize chemicals such as polyphenols that have aromatic units in their structure. Utilizing microbial hosts with sugar metabolism allows to knockout catabolic pathways responsible for metabolizing aromatics and instead use them for product synthesis [93]. In one case, *C. glutamicum* was systematically engineered to become incapable of catabolizing lignin-derived aromatics to create a polyphenol-producing strain [60,94]. One shortcoming of this approach is the limited pool of intracellular malonyl-CoA needed to produce many plant polyphenols. *C. glutamicum* was engineered by reducing the flux of acetyl-CoA to the tricarboxylic acid (TCA) cycle, improving naringenin production by 8.8-fold (2.1 mg/L to 18.5 mg/L). The low product yield and titer often seen in lignin valorization can be improved by combining several approaches, such as selecting an appropriate microbial host, narrowing the stream of substrates, and performing minimal engineering [95,96]. This was demonstrated by Rodriguez et al. [41], wherein an engineered strain of *C. glutamicum* produced 187 g/L of 4-vinylphenol from *p*-coumarate at a yield of 90% (mol/mol). All in all, with further developments, co-utilization of sugars with aromatics can achieve high production metrics.

Conclusion and future perspectives

Microbial lignin valorization faces several challenges, from the heterogeneous nature of aromatic substrates present in the lignin hydrolysates to their efficient conversion to value-added chemicals by robust microbial biocatalysts. Cell wall engineering and 'lignin first' approaches designed to selectively accumulate key aromatic substrates of importance in biomanufacturing are promising, but this field is still in its infancy. Among potential microbial biocatalysts, nonmodel hosts have been identified that display greater aromatic tolerance and the ability to 'funnel' diverse aromatics into their metabolism. Among these, those with the native ability to co-utilize both sugars and aromatics have proven to be more promising due to their ability to achieve nearly 100% product yields.

Looking ahead, rapid advancements in biotechnology, such as genome editing technologies like Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), the discovery of novel non-model hosts, and engineered co-cultures, will facilitate significant advances in lignin valorization [97–99]. More specifically, these advancements should aim to enhance the TRY of value-added chemicals produced from lignin-derived aromatics by achieving the following: (1) plant cell wall engineering to accumulate aromatic substrate (s) of interest at higher concentrations, (2) pretreatment methods that render biocompatible lignin hydrolysates with high concentrations of specific aromatics, (3) the development of robust synthetic biology tools for non-model microbial hosts of interest, and (4) systems biology studies on those non-model hosts to identify mechanisms for optimizing carbon and energy flux.

Author contributions

A.L., D.N., and A.M.V. conceptualized the work, drafted the review paper, and handled the review and editing process. D.E., A.M., and J.S. assisted in drafting parts of the review paper. A.L., D.E., and S.B. generated the figures and table. D.N. and A.M.V. did the funding acquisition.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare no conflict of interest.

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- • of special interest
- •• of outstanding interest

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