



Cite this: *Chem. Commun.*, 2024, 60, 14360

## Sustainable production of aromatic chemicals from lignin using enzymes and engineered microbes

Victoria Sodré and Timothy D. H. Bugg \*

Lignin is an aromatic biopolymer found in plant cell walls and is the most abundant source of renewable aromatic carbon in the biosphere. Hence there is considerable interest in the conversion of lignin, either derived from agricultural waste or produced as a byproduct of pulp/paper manufacture, into high-value chemicals. Although lignin is rather inert, due to the presence of ether C–O and C–C linkages, several microbes are able to degrade lignin. This review will introduce these microbes and the enzymes that they use to degrade lignin and will describe recent studies on metabolic engineering that can generate high-value chemicals from lignin bioconversion. Catabolic pathways for degradation of lignin fragments will be introduced, and case studies where these pathways have been engineered by gene knockout/insertion to generate bioproducts that are of interest as monomers for bioplastic synthesis or aroma chemicals will be described. Life cycle analysis of lignin bioconversion processes is discussed.

Received 28th September 2024,  
Accepted 11th November 2024

DOI: 10.1039/d4cc05064a

rsc.li/chemcomm

### 1. Introduction

The petrochemical industry is the source of most of the chemicals that we rely on for modern society, to make plastics, solvents, pharmaceuticals and specialty chemicals, but chemical production contributes to greenhouse gas (GHG)

Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK.  
E-mail: T.D.Bugg@warwick.ac.uk



**Victoria Sodré**

*Victoria Sodré is a postdoctoral researcher working on the biocatalytic production of pyridines from lignin in Prof Tim Bugg's group, at the University of Warwick, UK. She obtained her Bachelor's degree in Biological Sciences (2016) from the University of Campinas (UNICAMP), Brazil, and PhD in Biochemistry (2021) from the same institution. During her doctoral studies, Victoria explored diverse microbial and enzymatic strategies for valorisation of lignin and lignocellulosic hydrolysates. Her research interests are in developing new sustainable routes to produce chemicals from renewable feedstocks. She is interested in sustainability and enjoys spending time in nature, cycling, and exploring historical places.*

emissions that must be reduced to meet global sustainability targets. The use of plant biomass to generate both fuels and chemicals is in principle a carbon-neutral strategy, since biomass is ultimately derived from CO<sub>2</sub>, although in practice energy demands for all production processes need to be assessed carefully *via* life cycle analysis.<sup>1</sup> Plant cell wall lignocellulose contains three polymers: cellulose, hemi-cellulose, and lignin. Cellulose and hemi-cellulose polysaccharides can be converted *via* enzymatic saccharification into C<sub>6</sub> and C<sub>5</sub>



**Timothy D. H. Bugg**

*Tim Bugg is a Professor of Biological Chemistry at the University of Warwick. His academic career started at the University of Southampton in 1991, where his group studied enzymes involved in the bacterial degradation of aromatic compounds and enzymes involved in bacterial peptidoglycan assembly. After moving to Warwick in 1999, his group more recently studied enzymes involved in bacterial degradation of lignin and the application of biocatalysis to convert lignin into renewable aromatic chemicals. He is the author of the undergraduate textbook "Introduction to Enzyme and Coenzyme Chemistry".*



sugars, which can then be converted *via* fermentation into biofuels such as bioethanol or biobutanol or into aliphatic carboxylic acids.<sup>2,3</sup> Hence there is considerable interest in the “biorefinery” concept of using plant biomass to make a range of sustainable fuels and chemicals, as an alternative to petrochemicals.<sup>2,3</sup> The third polymer lignin is an aromatic heteropolymer and is the most abundant renewable source of aromatic carbon in the biosphere, comprising 15–25% of lignocellulosic biomass; hence there is interest in lignin conversion to chemicals. However, lignin is a much more refractory polymer than cellulose and hemi-cellulose, for reasons that will be explained, and in pulp/paper and biofuel industries that generate lignin, it is often burnt to generate power.

As well as harvested biomass (*e.g.* wheat straw), there is a large amount of agricultural waste from food production (*e.g.* corn stover, sugarcane bagasse, and rice husks) that contains lignocellulose, which could be used for biorefinery applications.<sup>4</sup> Lignin is also produced in large quantities from pulp/paper manufacture and as a by-product of cellulosic bioethanol production.<sup>5</sup> The use of a waste or commercial byproduct improves the sustainability of the process for renewable chemical production, termed the “circular economy”.<sup>1</sup> Conversion processes using biocatalysis such as enzymatic treatment or microbial bioconversion employ less harsh reaction conditions and solvents, and hence they are considered green technologies for biomass conversion.<sup>4,5</sup> In this article we will describe advances since 2010 in the enzymology of lignin degradation and the use of metabolically engineered microbial hosts for conversion of lignin to renewable chemicals, featuring case studies from the author’s research group and linking to research efforts around the world.

## 2. Introduction to lignin structure

Lignin is a high molecular weight heteropolymer, made up of oxygenated aryl-C<sub>3</sub> units linked together *via* ether C–O bonds or C–C bonds, linkages which are not susceptible to cleavage using aqueous acid or alkali.<sup>6,7</sup> Lignin is biosynthesised in plants *via* radical polymerisation of three monolignol precursors (*p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol), which are oxidised by peroxidase or laccase enzymes to form phenoxy radical intermediates, which undergo radical polymerisation in different ways.<sup>6</sup>

Softwood lignin (*e.g.* pine) contains predominantly disubstituted G units derived from coniferyl alcohol; hardwood lignin (*e.g.* oak and poplar) contains trisubstituted S and disubstituted G units; and grass lignins (*e.g.* wheat straw and miscanthus) contain a mixture of G, S and monosubstituted H units. The most abundant type of linkage is the  $\beta$ -aryl ether ( $\beta$ -O-4) linkage, in which the  $\beta$ -carbon of the C<sub>3</sub> alkyl sidechain is linked *via* an ether bond to O-4 of the next aryl unit, which is normally found in 45–60% of the linkages found in native lignin.<sup>6,7</sup> Biphenyl units (5-5 linkage) are found in 20–25% of softwood lignin, but are much less abundant in hardwood lignin. The phenylcoumaran ( $\beta$ -5) linkage and pinoresinol ( $\beta$ - $\beta$ ) linkage both contain C–C bond linkages to the  $\beta$ -position of the C<sub>3</sub> alkyl chain, with the former involving a fused

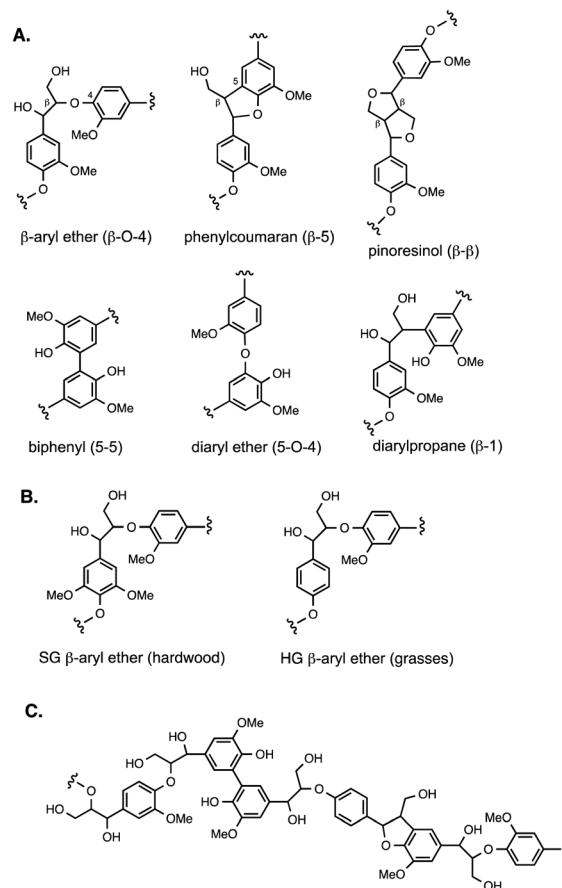


Fig. 1 (A) Lignin sub-structures. (B) S, G and H units in polymeric lignin. (C) Representative partial lignin structure.

dihydrofuran ring. The same group of substructures are found in different types of plant biomass; therefore, depolymerisation strategies often work with lignin from different types of plant biomass. Grass lignins are acylated at the  $\gamma$ -position with ferulic acid and/or *p*-coumaric acid and ferulic acid, which are also found in hemi-cellulose, and comprise 0.5–4% dry weight of lignocellulose. The proportion of different substructures present in lignin preparations can be studied by 2D NMR, which has been reviewed (Fig. 1).<sup>7</sup>

Literature studies on lignin bioconversion use different types of feedstocks; in some cases milled lignocellulose is used and in other cases an isolated lignin substrate is used. There are several different types of lignin preparations, whose chemical composition and physical properties vary. Organosolv lignin is prepared from lignocellulose using organic solvent/organic acid treatment at elevated temperature, and this preparation retains much of the structure of native plant lignin<sup>8</sup> and is soluble in organic solvents but has low water solubility; therefore, it is usually not used for biocatalytic conversions. Soda lignin is obtained by treatment of lignocellulose with sodium hydroxide, followed by acidification to pH 7 and precipitation.<sup>9,10</sup> The residual aqueous fraction from this process is termed “alkali pretreated lignin”, although it contains mainly *p*-hydroxycinnamic acids rather than lignin oligomers.<sup>11</sup>



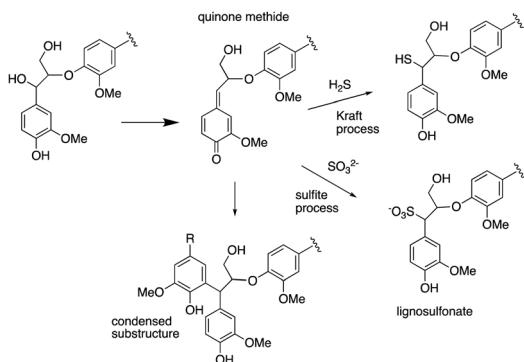


Fig. 2 Condensed units found in Kraft lignin and lignosulfonate.

Kraft lignin is produced industrially by the Kraft process for pulp/paper manufacture, involving treatment of lignocellulose with  $\text{H}_2\text{S}$  under alkaline conditions.<sup>12</sup> Although available industrially (and commercially from Sigma-Aldrich), Kraft lignin has a condensed structure resulting from loss of the  $\alpha$ -hydroxyl group,<sup>13,14</sup> to form a quinone methide intermediate, which reacts with sulfide ions to form an  $\alpha$ -thiol. However, the quinone methide also reacts with adjacent aromatic units to form “condensed” units containing additional C–C bonds or eliminates to form stilbene units (see Fig. 2).<sup>14</sup> The condensed units are generally more difficult to depolymerise, so although the use of Kraft lignin as an industrial waste would be advantageous for sustainability reasons, conversion yields are generally low.<sup>15</sup> Lignosulfonate is a by-product of the industrial sulfite process for pulp/paper manufacture and is also chemically modified with sulfonate groups, giving it high water solubility.<sup>13</sup> Unlike cellulose and hemi-cellulose preparations, each type of lignin is structurally different and hence its reactivity is different.<sup>15</sup>

### 3. Microbial enzymes for degradation of lignin

The molecular structure of lignin described above explains why lignin is inert towards depolymerisation, since its C–O ether and C–C linkages are not susceptible to cleavage under acidic or basic conditions. Lignin therefore degrades slowly in the environment, but there are several lignin-degrading microbes that produce remarkable enzymes to degrade lignin.

Microbial degradation of lignin was first elucidated in Basidiomycete (white-rot) fungi, notably *Phanerochaete chrysosporium*, which produces an arsenal of extracellular peroxidases to degrade lignin.<sup>16</sup> Lignin peroxidase (LiP) is a heme-containing peroxidase enzyme, which was shown to cleave lignin model compounds *via*  $\text{C}_\alpha\text{--C}_\beta$  oxidative cleavage.<sup>17</sup> The *P. chrysosporium* genome contains 10 lignin peroxidase genes, explaining its high activity for lignin breakdown.<sup>18</sup> This organism also produces an extracellular manganese peroxidase (MnP), which oxidises  $\text{Mn}^{2+}$  to  $\text{Mn}^{3+}$  as a diffusible oxidant to degrade polymeric lignin.<sup>19</sup> The major cleavage reaction by fungal MnP is *via* aryl- $\text{C}_\alpha$  cleavage, to give

methoxyhydroquinone and methoxyquinone products.<sup>20</sup> Other lignin-degrading white-rot fungi such as *Trametes versicolor* produce an extracellular laccase, containing four Cu centres, which can oxidise lignin.<sup>21</sup> Fungal laccase has been shown to catalyse oxidation of the  $\alpha$ -hydroxyl group present in lignin  $\beta\text{-O-4}$  units, but also catalyses aryl- $\text{C}_\alpha$  cleavage.<sup>22</sup> Hence different types of oxidative cleavage reactions are possible, resulting in different classes of products, which are summarised in Table 1. A further type of oxidative cleavage observed using fungal LiP is cleavage of the distal  $\text{C}_\alpha\text{--O}_\beta$  ether bond, to generate a  $\text{C}_3$  triol product,<sup>23</sup> which has also been observed by NMR spectroscopy during lignin processing by white-rot fungus *Ceriporiopsis subvermispora*.<sup>24</sup> The catalytic mechanisms of microbial lignin-degrading peroxidases and laccases are thought to involve one-electron oxidation of the phenolic rings present in lignin and have been recently reviewed.<sup>25</sup>

Although it was reported in the 1980s that certain soil bacteria such as *Streptomyces viridosporus* had the ability to depolymerise lignin,<sup>35</sup> the identification of a bacterial lignin-degrading enzyme was not reported until 2011, when a dye-decolorizing peroxidase DypB was identified in *Rhodococcus jostii* RHA1.<sup>26</sup> This enzyme showed activity for oxidative cleavage of lignin model compounds and oxidation of  $\text{Mn}^{2+}$ , and in the presence of  $\text{Mn}^{2+}$ , it showed activity towards polymeric lignin.<sup>26</sup> A Dyp2 dye-decolorizing peroxidase has been identified in *Amycolatopsis* sp 75iv2, which shows high  $\text{Mn}^{2+}$  oxidation activity,<sup>36</sup> and a lignin-oxidising Dyp1B enzyme derived from *Pseudomonas fluorescens* has also been characterised<sup>28</sup> and tested against a range of different lignin substrates.<sup>15</sup> Products arising from  $\text{C}_\alpha\text{--C}_\beta$  cleavage, aryl- $\text{C}_\alpha$  cleavage, distal aryl- $\text{O}_\beta$  cleavage, and  $\text{C}_\beta\text{--O}_\beta$  cleavage were observed,<sup>15</sup> which are summarised in Table 1.

Several *Streptomyces* soil bacteria in the Actinobacteria phylum produce small laccase enzymes that have activity for lignin oxidation<sup>37</sup> and show similar activities to fungal laccase enzymes. *S. coelicolor* also contains extracellular peroxidase enzymes, which have also been shown to be involved in lignin breakdown.<sup>38</sup> The two bacteria that have been used as hosts for metabolic engineering of lignin degradation (described below) are *Rhodococcus jostii* RHA1 and *Pseudomonas putida* KT2440, both shown to break down polymeric lignin.<sup>39,40</sup> *R. jostii* RHA1 contains two dye-decolorising peroxidases, of which DypB has been shown to be active for lignin degradation,<sup>26</sup> and this microbe also contains three multi-copper oxidase genes, whose overexpression has recently been shown to enhance the titre of lignin degradation products.<sup>41</sup> *P. putida* KT2440 contains a B-type dye-decolorising peroxidase implicated in lignin breakdown,<sup>42</sup> and two CopA pseudo-laccases, which have been shown to be active for lignin degradation.<sup>43</sup> The presence of multiple lignin-oxidising enzymes may explain the utility of these microbes as lignin degradation hosts. Moreover, the genomes of 10 lignin-degrading bacteria were shown to contain either dye-decolorizing peroxidases or multi-copper oxidases, or both, and most of them involved the  $\beta$ -ketoadipate pathway for metabolism of protocatechuic acid, whose significance is discussed below.<sup>44</sup>



**Table 1** Products arising from different enzyme-catalysed oxidative bond cleavage processes for lignin degradation. The last column indicates the downstream catabolic pathways in which these products are likely to be degraded (see Section 4)

Types of bond cleavage observed in $\beta$ -aryl ether units for lignin-degrading enzymes				
Bond cleavage	Primary product	Other related metabolites	Enzyme	Pathway
$C_\alpha-C_\beta$ cleavage			Fungal LiP <sup>17</sup> Bacterial DyP <sup>26</sup> MnSOD <sup>27</sup>	PCA and BKA
Aryl- $C_\alpha$ cleavage			Fungal MnP <sup>20</sup> Bacterial DyP <sup>15</sup> Laccases <sup>22</sup> MnSOD <sup>27</sup>	HQ
Distal aryl- $O_\beta$ cleavage			Fungal LiP <sup>23,24</sup> PfDyp1B <sup>15,28</sup>	Not known
$C_\beta-O_\beta$ cleavage			Bacterial $\beta$ -ether-ases <sup>29-33</sup> PfDyp1B <sup>15</sup>	BFD
Demethyl-ation			MnSOD1 <sup>27,34</sup>	HQ

Abbreviations: PCA, protocatechuic acid; BKA, beta-ketoadipate pathway; HQ, hydroxyquinol pathway; BFD, benzoylformate decarboxylase pathway.

Other types of enzymes that can degrade lignin have also been found in other bacterial lignin degraders. A class of glutathione-dependent beta-etherase enzymes have been discovered in *Sphingobium* SYK-6<sup>29-32</sup> and *Novosphingobium* sp.<sup>33</sup> These bacteria are able to degrade  $\beta$ -aryl ether lignin dimers *via* oxidation of the  $\alpha$ -hydroxyl group to a ketone, *via* dehydrogenase LigD, and *via* stereospecific nucleophilic cleavage of the  $\beta$ -ether bond by the thiol group of glutathione by LigE and LigF.<sup>31,32</sup> Glutathione is then removed by LigG to give an aryl 3'-hydroxypropiophenone product,<sup>30</sup> as shown in Table 1. This product has also been observed as a product of oxidative DyP-catalysed reactions,<sup>15</sup> for which a possible mechanism has been discussed.<sup>25</sup> A LigE-type enzyme has been recently identified in lignin-degrading *Agrobacterium* sp. that can attack the phenylcoumaran substructure in lignin, resulting in stilbene reaction products.<sup>45</sup> Lignin-degrading bacterium *Sphingobacterium* sp. T2 has been shown to produce two extracellular manganese superoxide dismutase enzymes that can degrade polymeric lignin and lignin model compounds.<sup>27,34</sup> Unlike normal

manganese superoxide dismutase enzymes, these enzymes are able to reduce hydrogen peroxide to the hydroxyl radical, which can degrade lignin,<sup>27,34</sup> and two mutations near the Mn centre have been found to be essential for this unusual reactivity.<sup>34</sup> The major reaction catalysed by these enzymes is demethylation, but also aryl- $C_\alpha$  oxidative cleavage is observed, as shown in Table 1. Recently a thermostable catalase-peroxidase enzyme derived from *Thermobacillus xylanolyticus* has been characterised, which can oxidise lignin-derived phenolic compounds.<sup>46</sup> A group of fungal polyphenol oxidases have also been identified, which can hydroxylate lignin-derived phenolic compounds.<sup>47</sup>

In spite of the fact that a number of recombinant lignin-degrading enzymes have been identified and studied, the use of lignin-degrading enzymes *in vitro* for generation of monomeric aromatic products generally does not result in high yield or selectivity, for several reasons. Firstly, as shown in Table 1, mixtures of different cleavage products are obtained from enzyme-catalysed lignin oxidation. Secondly, lignin repolymerisation is observed *in vitro*, because the radical intermediates



formed in lignin oxidation can polymerise to form higher molecular weight species, which are often the major products of such reactions *in vitro*.<sup>16,26</sup> However, this repolymerisation phenomenon is generally not observed in lignin-degrading microbes,<sup>16</sup> so they must have mechanisms to combat this problem. A flavin-dependent dihydrolipoamide dehydrogenase derived from *Thermobifida fusca* has been identified, which can prevent repolymerisation of lignin model compounds *in vitro*,<sup>48</sup> and combinations of bacterial DyPs with dihydrolipoamide dehydrogenase enzymes have been shown to be more effective for product generation from lignin,<sup>49</sup> and hence there is interest in the use of enzyme combinations for lignin conversion.

Another issue for lignin conversion *in vitro* is the supply of hydrogen peroxide for lignin-oxidising peroxidase enzymes, since peroxidases are often inactivated by millimolar concentrations of hydrogen peroxide. Accessory enzymes have been identified in Basidiomycete fungi that can generate hydrogen peroxide, of which aryl alcohol oxidase<sup>50</sup> and glyoxal oxidase<sup>51,52</sup> are thought to be involved in lignin degradation. A bacterial glycolate oxidase has been identified in *Rhodococcus jostii* RHA1 that can function effectively in combination with bacterial DyPs *in vitro*.<sup>53</sup>

In view of the technical problems described here for *in vitro* conversion of polymeric lignin by enzymes, the alternative approach is to use an engineered lignin-degrading microbe to break down lignin, but produce a useful bioproduct. This approach has the advantage that microbial lignin degradation is convergent, transforming multiple oxidised metabolites into a small number of key intermediates, which are then metabolised by common degradation pathways, such as the  $\beta$ -ketoadipate pathway. As described below, this approach has been successful in generating several different types of bioproducts from breakdown of lignin, lignocellulose, or pretreated lignin.

## 4. Microbial hosts for lignin valorisation

Degradation of lignin in Nature, while necessary for carbon cycling, is only performed by a relatively small group of micro-organisms, often working synergistically. The most conspicuous microorganisms are probably basidiomycetes and filamentous fungi such as white-rot and brown-rot fungi. These were the first lignin degraders to be studied and to this day gain importance due to the impressive catalytic activity of their secreted lignin-degrading enzymes. However, the modification of fungal strains for industrial lignin valorisation faces several challenges, such as longer cultivation times, complex life cycles, and large and convoluted genomes recalcitrant to engineering efforts, as well as a paucity of genetic tools available to modify white-rot fungi (reviewed in detail elsewhere<sup>54</sup>), including the introduction of selection markers and overexpression of heterologous proteins that improved lignin degradation.

One alternative to engineering white-rot fungi would be the use of yeasts, for which a plethora of genetic tools are available. For instance, *Yarrowia lipolytica* is a non-conventional

oleaginous yeast, “generally regarded as safe” (“GRAS”) for industrial processes. This yeast is naturally capable of producing lignin depolymerising enzymes such as laccases and has been successfully engineered to overexpress a heterologous laccase from the basidiomycete *Pycnoporus cinnabarinus*.<sup>55</sup> A new species of red yeast, *Rhodospiridium fluviale* LM-2, was recently isolated from a lignin-degrading microbial consortium and its metagenomic profiling revealed some lignin-degrading genes, such as  $\beta$ -etherases, as well as genes for aromatic catabolism, such as phenolic acid decarboxylases.<sup>56,57</sup> The latter were successfully applied to generate 4-vinyl guaiacol from ferulic acid in a cofactor-independent manner.<sup>57</sup> The overexpression of relevant genes in non-lignolytic *Saccharomyces cerevisiae* has also been employed to produce high-value molecules such as scopoletin,<sup>58</sup> vanillin,<sup>59</sup> and protocatechuic acid<sup>60</sup> from lignocellulose-derived p-hydroxycinnamic acids.

Compared to fungi, bacteria are easier to grow and are more amenable to genetic modification. A number of bacteria known to degrade aromatic compounds have been shown to be capable of degrading lignin, including *Rhodococcus jostii* RHA1, *Amycolatopsis* sp. 75iv2, *Pseudomonas putida* KT2440, *Pseudomonas fluorescens* Pf-5, *Ochrobactrum* sp., and *Sphingobacterium* sp. T2.<sup>39,40</sup> While bacterial aromatic catabolism had been studied for many years, the relatively new understanding of lignin depolymerisation mechanisms allowed the following two processes – depolymerisation of lignin into lower molecular weight fragments and their catabolism by upper aromatic degradation pathways – to be directed towards lignin valorisation.

The use of lignin-degrading bacteria as hosts for metabolic engineering for the production of high-value molecules from lignin has been a particularly successful strategy, for the following reasons: firstly, because these organisms act as “biological funnels” by effectively converting the many different lignin oxidised fragments arising from depolymerisation into a few key metabolic intermediates, mainly protocatechuic acid and catechol, partially overcoming the issue of lignin heterogeneity. Secondly, the simultaneous depolymerisation and biotransformation capacities of these bacteria allow for “consolidated bioprocessing” of lignin and lignocellulosic hydrolysates, which is a more cost-effective and direct biotechnological strategy. Finally, the targeted interruption of aromatic catabolism *via* genetic engineering of downstream pathways allows for accumulation of desirable intermediates, which may be of high value themselves, or could be further converted into other valuable compounds.

The bacterial hosts that are most commonly used for metabolic engineering of lignin degradation are *Rhodococcus jostii* RHA1 and *Pseudomonas putida* KT2440. *Rhodococcus* species are well known for their ability to degrade a wide range of xenobiotic compounds and tolerate harsh environmental conditions.<sup>61</sup> *R. jostii* RHA1 is a powerful degrader of polymeric lignin and untreated lignocellulosic biomass, containing both dye-decolorizing peroxidases and multi-copper oxidases (see Table 2), and has been used for several case studies described in Section 6. The genome of *R. jostii* RHA1 contains 15 gene clusters encoding aromatic catabolic pathways,<sup>62</sup>



Table 2 Lignin-degrading enzymes found in lignin-degrading fungi and bacteria used for metabolic engineering of lignin degradation

Microbe	Strain	Peroxidases	Laccases	Other enzymes
Basidio-mycete fungi	<i>Phanerochaete chrysosporium</i>	LiP <sup>16,17</sup> MnP <sup>19,20</sup>	—	Aryl alcohol oxidase <sup>50</sup>
	<i>Trametes versicolor</i>	LiP	Lcc <sup>21</sup> SLAC <sup>37</sup>	Glyoxal oxidase <sup>51,52</sup>
Actino-bacteria	<i>Streptomyces coelicolor</i>	MnP	McoA, McoB, McoC <sup>41</sup>	Glycolate oxidase <sup>53</sup>
	<i>Rhodococcus jostii</i> RHA1	DyP <sup>38</sup> DypA, DypB <sup>26</sup>	SLAC <sup>37</sup>	
$\gamma$ -Proteo-bacteria	<i>Amycolatopsis</i> sp 75iv2	Dyp2 <sup>36</sup>	McoA <sup>37</sup>	
	<i>Pseudomonas putida</i> KT2440	DypB <sup>28,42</sup>	SLAC <sup>37</sup>	
	<i>Sphingobacterium</i> sp. T2	—	CopA <sup>43</sup>	MnSOD1 and MnSOD2 <sup>27,34</sup>

several of which are involved in the degradation of lignin fragments, which will be discussed in Section 5. Genetic engineering of *Rhodococcus* sp. has traditionally relied on homologous recombination using suicide vectors, such as pK18mobsacB,<sup>63</sup> and *E. coli*-*Rhodococcus* shuttle vectors for protein expression, such as the pNit/pTip plasmid series.<sup>64</sup> In the past few decades, several new molecular biology tools have been published for use in *Rhodococcus* sp., including libraries of promoters, ribosome-binding sites, reporter genes, and tools for gene expression studies.<sup>65–68</sup> New strategies for gene editing and expression control have also been published, including the use of viral recombinases and the CRISPR/Cas9 system in *R. ruber* TH<sup>69</sup> and development of CRISPRi tools in *R. opacus* PD630.<sup>70</sup>

*Pseudomonas putida* KT2440 is perhaps the lignin-degrading bacterium with the most well-developed set of molecular biology tools for metabolic engineering. This species is highly tolerant to oxidative stress and capable of degrading polymeric lignin and aromatic compounds and grows very effectively on “alkali pre-treated lignin” derived from alkaline treatment of lignocellulose. Moreover, this bacterium is capable of accumulating polyhydroxy-alkanoate (PHA) under nutrient-limiting conditions.<sup>40</sup> The genetic toolbox available for this species includes *I-SceI* and CRISPR/Cas9 systems for targeted genome engineering,<sup>71–74</sup> reduced genome strains,<sup>75–77</sup> and a library of plasmids, promoters, transposons and viral recombinases.<sup>78,79</sup> Several biosensors relevant to lignin catabolism have been developed using *P. putida* KT2440, including a PCA-catechol biosensor derived from *Escherichia coli*,<sup>80</sup> a 4-hydroxybenzoate biosensor,<sup>81</sup> and several transcription factors responsive to phenolic acids, which have been recently characterised.<sup>82</sup>

The actinomycete *Amycolatopsis* sp. 75iv2 (ATCC 39116) is also a powerful lignin degrader that contains both dye-decolorizing peroxidases and multi-copper oxidases (see Table 2) and has been used in case studies described in Section 6.

While using lignin-degrading bacteria for consolidated bioprocessing of lignin certainly has its advantages, there are challenges associated with the use of non-conventional microorganisms. Actinobacteria such as *R. jostii* RHA1 and *Amycolatopsis* sp. 75iv2 have less well developed genetic tools for engineering and also have lower transformation and recombination efficiencies, longer cultivation times, and a more complex catabolism. Thus, non-lignolytic bacteria that can be readily engineered and used for industrial-scale production have also been explored for conversion of feedstock compounds that can be generated from lignin degradation. *Corynebacterium*

*glutamicum* is a biotechnological powerhouse well-known for its industrial-scale production of amino acids. *C. glutamicum* lacks lignin depolymerising enzymes; however it can degrade a wide range of aromatic molecules, such as hydroxycinnamates, protocatechuate, catechol, benzoate, 4-hydroxybenzoate, 4-hydroxyphenylpropionate, gentisate, phenol, and naphthalene, among others,<sup>83,84</sup> and has been applied to the conversion of chemically depolymerised lignin, as described in Section 6.

## 5. Pathways for degradation of oxidised lignin fragments

If we wish to convert lignin into a high value chemical by metabolic engineering of lignin catabolism, then we need to understand the catabolic pathways used by lignin-degrading microbes to mineralise the fragments generated from lignin oxidation (see Table 1). Our knowledge of the intermediate steps of lignin breakdown is still limited: we do not know what size fragments are imported into microbes, nor exactly which steps take place extracellularly vs. intracellularly. However, the aromatic degradation pathways responsible for utilisation of aromatic monomers are well understood, and information is emerging about which pathways are responsible for conversion of different types of lignin degradation fragments. We will introduce the pathways that are known to degrade the different types of fragments illustrated in Table 1. Some of these pathways have then been used to generate high-value products, which will be described in Section 6.

### 5.1. Pathways for aryl C<sub>1</sub> fragments

As described in Section 3, C<sub>α</sub>-C<sub>β</sub> oxidative cleavage of  $\beta$ -aryl ether units found in G-type lignin generates vanillin and/or vanillic acid. Vanillin is oxidised to vanillic acid, which is then demethylated to form protocatechic acid (PCA), a key intermediate in lignin degradation. Protocatechic acid can also be generated from 4-hydroxybenzoic acid (formed by oxidation of H-type lignin) by flavin-dependent 4-hydroxybenzoate hydroxylase (*pobA* gene). PCA is then degraded via the  $\beta$ -ketoadipate pathway, as shown in Fig. 3. This pathway proceeds via intradiol cleavage of PCA catalysed by protocatechuate 3,4-dioxygenase, to give carboxy-*cis,cis*-muconic acid, which is converted via lactonization into  $\beta$ -ketoadipate. There is also a related pathway from catechol to  $\beta$ -ketoadipate, as shown in Fig. 3. The  $\beta$ -ketoadipate pathway gene cluster is found in the



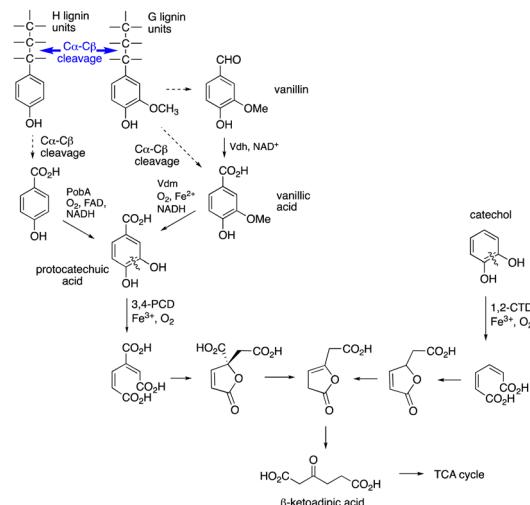


Fig. 3  $\beta$ -Ketoadipate pathways for degradation of protocatechualic acid and catechol.

genomes of nearly all bacterial lignin degraders<sup>44</sup> and appears to be the major pathway used to degrade lignin fragments in lignin-degrading bacteria. This pathway has been engineered to generate high-value products, as will be described in Section 6.

S-lignin units found in hardwood are converted *via* C $\alpha$ -C $\beta$  oxidative cleavage into syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), which is processed *via* the 3-O-methylgallate pathway in certain bacteria, which will be discussed in Section 6.

The fate of the two-carbon fragment released from C $\alpha$ -C $\beta$  oxidative cleavage has been studied in *R. jostii* RHA1 using synthetic [ $\beta$ -<sup>13</sup>C]-labelled DHP lignin as a substrate for bioconversion.<sup>85</sup> The formation of <sup>13</sup>C-labelled oxalic acid was observed, and a glycolate oxidase enzyme was identified that can convert glycolaldehyde *via* successive oxidation to glycolic acid, glyoxylic acid, and finally, oxalic acid.<sup>85</sup>

## 5.2. Pathways for aryl C<sub>3</sub> fragments

p-Hydroxycinnamic acids are attached to grass lignins (e.g. corn stover and wheat straw) and hemi-cellulose *via* ester linkages and can be released *via* treatment with aqueous alkali. The soluble fraction remaining after neutralisation is termed “alkali pre-treated lignin” and is used as a feedstock in some case studies described in Section 6, especially using *P. putida* KT2440 as a microbial treatment system.<sup>86</sup> These p-hydroxycinnamic acids, primarily ferulic acid (4-hydroxy-3-methoxycinnamic acid) and p-coumaric acid (4-hydroxycinnamic acid), therefore represent a valuable renewable feedstock for conversion to a range of high-value products.<sup>87</sup>

Generally, the catabolism of phenylpropanoids involves shortening of the C<sub>3</sub> side chain, which can be performed *via* a few different routes,<sup>88</sup> as shown in Fig. 4:

(a) Cofactor-independent decarboxylation, resulting in the corresponding styrenes, which are then converted to the respective 4-hydroxybenzaldehydes or 4-ethyl derivatives – present in *Bacillus* sp., *Cupriavidus* sp. B-8, and several yeasts;<sup>89</sup>

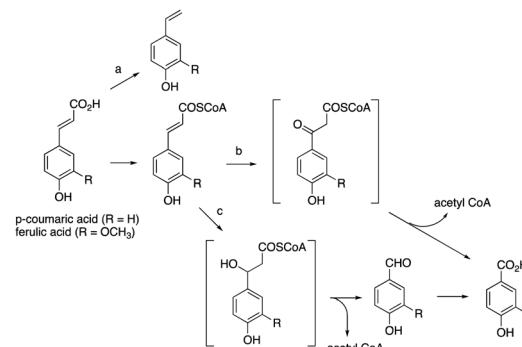


Fig. 4 Pathways for catabolism of p-hydroxycinnamic acids. Route a, cofactor-independent decarboxylation; route b, CoA-dependent  $\beta$ -oxidation; route c, CoA-dependent hydration-retroaldol cleavage.

(b) CoA-dependent  $\beta$ -oxidation, yielding the corresponding hydroxybenzoic acids – present in *R. jostii* RHA1<sup>90</sup> and *C. glutamicum* ATCC 13032;<sup>84</sup>

(c) CoA-dependent cleavage *via* hydration/retroaldol steps, yielding the corresponding hydroxybenzaldehydes – present in *P. putida* KT2440,<sup>91</sup> *Amycolatopsis* sp. ATCC 39116,<sup>92</sup> and *Sphingobium* sp. SYK-6.<sup>93</sup>

In routes (b) and (c), the end product is converted to protocatechualic acid *via* the steps shown in Fig. 3 and then metabolised *via* the  $\beta$ -ketoadipate pathway. Notably, the enzymes responsible for these initial steps display some promiscuity towards different hydroxycinnamates, which greatly contributes to the “funnelling” capacity of these microorganisms and could be an adaptation to the large number of different aromatics arising from lignin depolymerisation.<sup>88,94</sup>

It is uncertain how the fragments containing glycerol side-chains (or oxidised versions) shown in Table 1 are metabolised, but oxidation of the  $\gamma$ -hydroxyl group to a carboxylic acid, followed by  $\beta$ -oxidation, is feasible.

## 5.3. Pathways for aryl C<sub>2</sub> fragments

A pathway in *R. jostii* RHA1 for the degradation of aryl-C<sub>2</sub> oxidised lignin fragments arising from the cleavage of phenylcoumaran ( $\beta$ -5) and diarylpropane units has been verified experimentally.<sup>95</sup> These fragments were found to be oxidised into substituted phenylglyoxals, which are further oxidised by an FMN-dependent glycolate oxidase into the respective phenylglyoxylic acids, such as 4-hydroxybenzoylformate and 4-hydroxy-3-methoxybenzoylformate. The latter are then decarboxylated by a thiamine-diphosphate dependent decarboxylase into vanillin or 4-hydroxybenzaldehyde, which are then oxidised into vanillic acid or 4-hydroxybenzaldehyde by *vdh*, a vanillate dehydrogenase, as shown in Fig. 5.<sup>95</sup> Notably, this glycolate oxidase was later found to oxidise a wide range of  $\alpha$ -ketoaddehyde and  $\alpha$ -hydroxyacid substrates, with generation of H<sub>2</sub>O<sub>2</sub>, which acts synergistically with lignin-degrading Dyp peroxidases.<sup>96</sup>

Feeding of <sup>13</sup>C-labelled DHP lignin to *R. jostii* RHA1 was shown to generate <sup>13</sup>C-labelled 4-hydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid as metabolites.<sup>85</sup> These

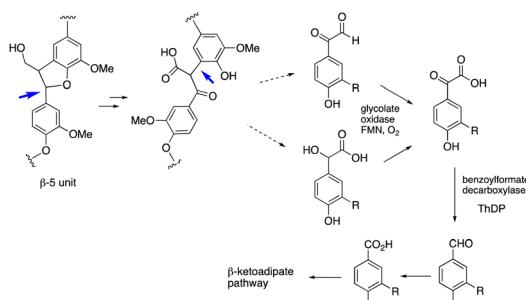


Fig. 5 4-Hydroxybenzoylformate catabolic pathway in *R. jostii* RHA1, responsible for degradation of aryl C<sub>2</sub> lignin fragments, which may be derived from β-5 units in polymeric lignin.<sup>95</sup> R = H or OCH<sub>3</sub>.

compounds were also used as substrates for *R. jostii* glycolate oxidase, yielding the corresponding phenylglyoxylic acids, which are then catabolised by the 4-hydroxybenzoylformate pathway.<sup>85,95</sup> Related gene clusters are present in *P. fluorescens* Pf-5, *Comamonas testosteroni*, and *Burkholderia multivorans*.<sup>95</sup>

#### 5.4. A pathway for aryl C<sub>0</sub> fragments

An alternative pathway for the catabolism of PCA has been verified in *R. jostii* RHA1, in which PCA is converted into hydroxyquinol (benzene 1,2,4-triol) by the action of decarboxylase and mono-oxygenase enzymes.<sup>97</sup> Hydroxyquinol is then cleaved by an intradiol catechol dioxygenase to yield maleylacetate, which is converted to β-ketoadipate, as shown in Fig. 6.<sup>97</sup> Hydroxyquinol can also be formed by demethylation of 4-methoxyhydroquinone, which is formed from aryl-C<sub>2</sub> cleavage of lignin units (see Table 1) and from 1,4-hydroquinone. The hydroxyquinol pathway gene cluster is also present in lignin-degrading *Agrobacterium* sp.<sup>97</sup> and has been identified in fungi *Aspergillus niger*<sup>98</sup> and *Trametes versicolor*.<sup>99</sup> This pathway is therefore likely used by several lignin-degrading bacteria and fungi.

Pathways for degradation of other lignin substructures have also been elucidated, as well as *meta*-cleavage pathways from protocatechic acid and catechol,<sup>100</sup> but in this article we will focus on pathways that have been engineered to produce high-value chemicals from lignin, which will be described in the following section.

## 6. Conversion of lignin by engineered microbes into high-value products

As shown in the previous section, the richness of aromatic degradation pathways in lignin-degrading bacteria offers several opportunities for valorisation. Aromatic bioproducts can be obtained by interruption of upper catabolic pathways prior to ring fission and occasionally by introducing further biotransformation steps. We will illustrate several case studies, some from the author's laboratory, considering first aromatic bioproducts and then non-aromatic bioproducts. The production of high-value molecules from lignin using engineered microbes has also been reviewed recently by Weng *et al.*<sup>101</sup> and Rosini *et al.*<sup>102</sup>

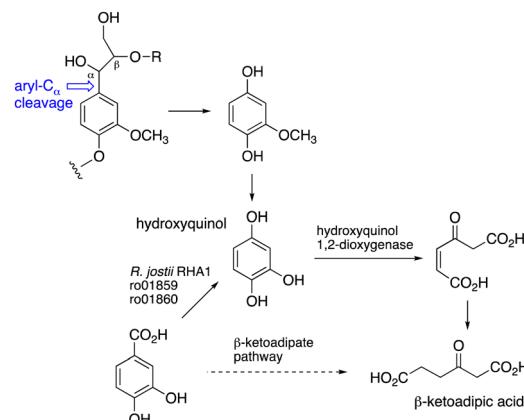


Fig. 6 Hydroxyquinol degradation pathway.

### 6.1. Aromatic bioproducts

**6.1.1. Vanillin, a flavour/aroma chemical.** The first example of the production of a high-value aromatic product from polymeric lignin and untreated lignocellulosic biomass was the production of vanillin by engineered *R. jostii* RHA1.<sup>103</sup> Vanillin is an aroma compound used in the food/flavour industry, traditionally obtained from the pods of the orchid *Vanilla planifolia* – a labour-intensive process that greatly contributes to the high market price of natural vanillin. In *R. jostii*, vanillin is a product of β-aryl ether cleavage by peroxidase DypB, and an intermediate in the catabolism of phenylcoumaran and diaryl propane lignin-derived oxidised fragments.<sup>95</sup> Importantly, vanillin produced by biological routes is considered natural vanillin, which is preferred by consumers over synthetic vanillin (which can be produced from petrochemicals).<sup>104</sup> Aiming for vanillin accumulation from wheat straw biomass, Sainsbury and colleagues knocked-out the gene encoding vanillin dehydrogenase (*vdh*) in *R. jostii* RHA1.<sup>103</sup> This interrupted the conversion of vanillin into vanillic acid (and further metabolism towards PCA) in the *Δvdh* strain and led to accumulation of 96 mg L<sup>-1</sup> vanillin after 6 days from minimal media supplemented with 2.5% wheat straw lignocellulose (corresponding to an overall yield of 1.9% from polymeric lignin), as shown in Fig. 7.<sup>103</sup> A lower titre of 13 mg L<sup>-1</sup> vanillin was obtained from minimal media supplemented with 0.5% (w/v) Kraft lignin,<sup>103</sup> but still significant as a proof-of-concept of high-value molecule production from untreated polymeric industrial lignin.

Zhao and colleagues recently produced vanillin from alkaline lignin (soda lignin), prepared from dissolved corn stalks, using a novel *Arthrobacter* sp. C2 strain.<sup>105</sup> The authors identified that catabolism of G-derived lignin units in this species occurs *via* vanillyl alcohol, which is oxidised by alcohol dehydrogenase PchF to vanillin. Vanillin is then oxidised to vanillic acid by *vdh*-like aldehyde dehydrogenase *xyIC*. Vanillic acid is finally demethylated by LigM and VanAB homologues to yield PCA. To promote vanillin accumulation, the authors knocked-out *xyIC* and overexpressed PchF, achieving 57 mg L<sup>-1</sup> vanillin from cultivation in an alkaline lignin mineral salt medium.



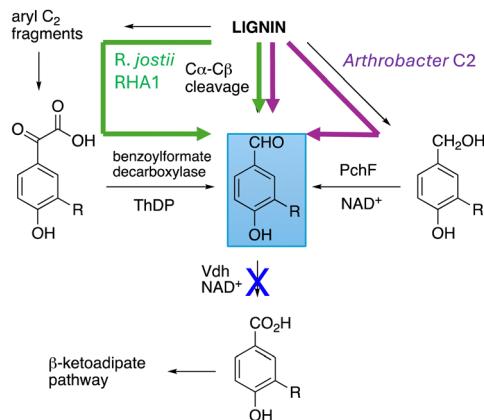


Fig. 7 Production of vanillin from polymeric lignin using *R. jostii* RHA1  $\Delta$ Vdh (green) or *Arthrobacter* C2  $\Delta$ xyIC (magenta). Gene knockout labelled with blue cross. R = H or OCH<sub>3</sub>.

**6.1.2. Pyrone- and pyridine-dicarboxylic acids as bioplastic monomers.** Aromatic dicarboxylic acids are of considerable biotechnological interest as monomers for the production of polyester bioplastics. The plastics PET (non-biodegradable, but can be re-cycled) and PBAT (biodegradable) both contain terephthalic acid, which is derived from petrochemicals (see Fig. 8A). The other components of PBAT, adipic acid and 1,4-butanediol can both be produced by microbial production from renewable feedstocks.<sup>106</sup> Therefore, new routes to aromatic dicarboxylic acid would enable the production of entirely bio-based versions of PET and PBAT.

Mycroft *et al.* have successfully established the consolidated bioproduction of pyridine-dicarboxylic acids (PDCAs) from wheat straw and Kraft lignin.<sup>107</sup> Preceded by studies that demonstrated the generation of picolinic acids from hydroxymuconate semialdehyde (generated from the *meta*-cleavage of catechol) and ammonia,<sup>108</sup> the authors sought to apply this strategy to carboxyhydroxymuconate semialdehyde precursors. The protocatechuate 4,5-dioxygenase LigAB derived from *Sphingobium* sp. SYK-6 or the protocatechuate 3,4-dioxygenase PraA derived from *Paenibacillus* sp. JJ-1b was overexpressed in *R. jostii* RHA1, to generate new extradiol ring cleavage products from PCA. By supplementing the culture medium with ammonium chloride, the semialdehyde precursors cyclised into 2,4- or 2,5-PDCA. Using 2.5 L bioreactors, a total of 125 mg L<sup>-1</sup> 2,4-PDCA was obtained by the LigAB-expressing strain after 9 days from 1% (w/v) wheat straw, and 53 mg L<sup>-1</sup> from 0.5% (w/v) Kraft lignin. Likewise, the PraA-expressing strain produced 106 mg L<sup>-1</sup> 2,5-PDCA after 9 days from 1% (w/v) wheat straw (corresponding to an overall yield of 4.0–6.3% from polymeric lignin).<sup>107</sup>

Further genetic modification of *R. jostii* RHA1 showed improvements in PDCA production.<sup>109</sup> Firstly, deletion of the *pcaHG* genes, responsible for PCA *ortho*-cleavage, was performed, re-routing PCA catabolism towards the heterologous *meta*-cleavage pathway. Secondly, this gene deletion was achieved by successfully knocking-in the *ligAB* genes under the control of the constitutive promoter *P<sub>tpc5</sub>*, allowing stable and robust expression of LigAB. Finally, lignin depolymerisation was enhanced by

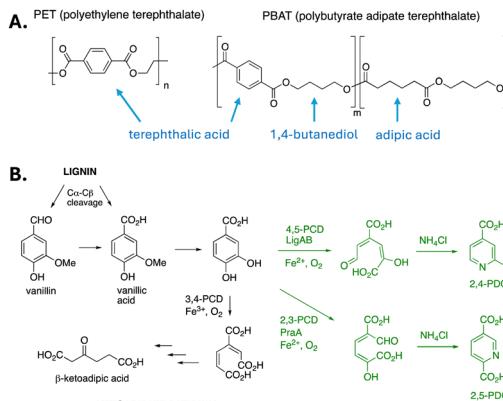


Fig. 8 (A) Structures of PET and PBAT bioplastics. (B) Pathways for production of 2,4-pyridinedicarboxylic acid and 2,5-pyridinedicarboxylic acid in engineered *R. jostii* RHA1.

overexpressing the *dyp2* peroxidase from *Amycolatopsis* sp. 75iv2. The final strain presented improved yields of 2,4-PDCA, with a maximum of 330 mg L<sup>-1</sup> after only 40 hours from minimal media supplemented with 1% wheat straw (corresponding to an overall yield of 16% from polymeric lignin) and 240 mg L<sup>-1</sup> from 1% Green Value Protobind lignin (GVPL), a soda lignin obtained from wheat straw and sarkanda grass biomass.<sup>109</sup> Overexpression of *dyp2* also reduced the time for peak PDCA production (40 h), improving the productivity by >10-fold. Further improvement in PDCA titre has been reported recently by overexpression of *R. jostii* *mcoA* or *mcoC* genes.<sup>41</sup> Pellis *et al.* have shown that 2,4-PDCA and 2,5-PDCA can be converted into polyester bioplastics with similar material properties to commercial Ecoflex plastic.<sup>110</sup>

More recently, 2,4-PDCA production was achieved in engineered *P. putida* KT2440 resting cells.<sup>111</sup> The engineered strain had the *ligAB* genes, under the control of the *lacI* repressor and the *P<sub>tac</sub>* promoter, knocked-in to replace the *pcaG* gene, effectively re-routing PCA *ortho*-cleavage to *meta*-cleavage. Furthermore, the authors overexpressed a PCA transporter, PcaK, to increase the uptake of aromatics by the cell. The final strain was able to produce a maximum of 0.24 mM (0.04 g L<sup>-1</sup>) 2,4-PDCA from minimal media supplemented with 1.5% GVPL after 20 hours. The productivity of PDCA in *P. putida* was >10-fold higher than in wild-type *R. jostii* RHA1, but the titre was slightly lower.<sup>111</sup>

A pyrone dicarboxylic acid bio-product has also been generated microbially. In *Sphingobium* sp. SYK-6 and *N. aromaticivorans*, the 4,5-extradiol dioxygenase LigAB catalyses the ring fission of PCA into 4-carboxy-2-hydroxy-*cis*,*cis*-muconate-6-semialdehyde (CHMS), which spontaneously cyclises into the hemiacetal form, as shown in Fig. 9. The latter is then oxidised by the NADP<sup>+</sup>-dependent dehydrogenase LigC to yield 2-pyrone-4,6-dicarboxylic acid (PDC). PDC is converted into 4-oxalomesaconate (OMA) by hydrolase LigI, which undergoes further reactions to yield pyruvate. Additionally, LigAB1 and LigAB2 (in *N. aromaticivorans*) and DesZ (*Sphingobium* sp. SYK-6) also catalyse the ring fission of 3MGA, ultimately converging into PDC.



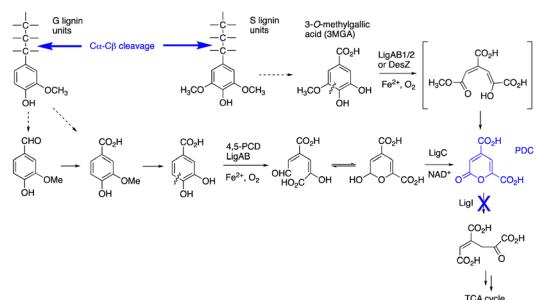


Fig. 9 Generation of pyrone-dicarboxylic acid (PDC) in *Novosphingobium aromaticivorans*. Gene knockout is shown by a blue cross.

Working with *N. aromaticivorans*, Perez and collaborators performed modification of its catabolic pathway by knocking-out *ligI* and successfully accumulating PDC from the PCA 4,5-*meta*-cleavage branch.<sup>112</sup> Furthermore, the *desC* and *desD* genes, responsible for conversion of the LigAB1/2 ring cleavage product into a different product, were also knocked-out. These genetic modifications allowed the engineered strain to produce PDC from the parallel catabolism of S-, G- and H-lignin units and led to production of 0.49 mM PDC from a chemically depolymerised poplar lignin stream.<sup>112</sup> In a recently published follow-up study, the production of PDC using the engineered *N. aromaticivorans* strain was dramatically increased by using high-density cultures and flow-through membrane bioreactors, resulting in a maximum PDC productivity of 1.53 g L<sup>-1</sup> h<sup>-1</sup> from alkaline-pretreated poplar hydrolysates.<sup>113</sup>

PDC production has also been achieved in *P. putida* PpY1100, a strain that is capable of degrading low-molecular weight aromatic compounds. This bacterium was engineered to heterologously express all the genes required to convert vanillin, vanillic acid, and ferulic acid into PDC from *Sphingobium* sp. SYK-6. This allowed production of PDC from kraft, Japanese cedar (*Cryptomeria japonica*), and birch (*Betula platyphylla*) lignin extracts.<sup>114</sup> More recently, the engineered strain was also successfully applied to produce PDC from desulphonated and depolymerised lignosulfonate extracts.<sup>115</sup>

**6.1.3. 4-vinyl guaiacol, an aroma chemical.** 4-Vinyl guaiacol (4VG) can be produced by the decarboxylation of ferulic acid and has applications as an aroma and flavouring agent in food and cosmetic products. Williamson *et al.* have successfully generated 4-VG and 4-vinyl phenol from lignin-derived ferulic and *p*-coumaric acid, respectively, using an engineered *P. putida* KT2440 strain.<sup>116</sup> The authors knocked-out the *ech* gene, which encodes an enoyl-CoA hydratase/lyase that catalyses the second step in hydroxycinnamate catabolism in this species, as shown in Fig. 10. In its place, the *padC* gene encoding phenolic acid decarboxylase from *Bacillus subtilis* was inserted. Interestingly, the *ech* gene is located within an operon regulated by repressor *ferR*, which is inactivated in the presence of feruloyl-CoA, allowing expression. Thus, expression of PadC was independent of the addition of external inducers and instead responded adequately to the presence of ferulic acid (and consequently, feruloyl-CoA) in the medium. The engineered strain was able to produce a maximum of 62 mg L<sup>-1</sup> 4VG from minimal media supplemented

with 10% GVPL. Furthermore, the authors demonstrated the polymerisation of 4VG and 4-vinyl catechol, generated *in vitro* by PadC, to biopolystyrenes, catalysed by *Trametes versicolor* laccase.<sup>116</sup>

Ferulic acid has also been converted successfully to 4VG in the lignolytic yeast *Rhodosporidium fluviale* LM-2,<sup>55</sup> and *p*-coumaric acid has been converted in high yield to 4-vinylphenol in *Corynebacterium glutamicum*.<sup>117</sup>

**6.1.4. Coniferyl alcohol.** An efficient bioconversion of biomass-derived ferulic acid to coniferyl alcohol has also been engineered.<sup>118</sup> The biocatalyst of choice was *E. coli*, overexpressing a carboxylic acid reductase from *Nocardia iowensis* (Ni-CAR) and an aldo-keto reductase from lower termite *Coptotermes gestroi* (Cg-AKR), which allowed the production of coniferyl alcohol from ferulic acid *via* coniferyl aldehyde (see Fig. 10). Since *E. coli* is not capable of depolymerising lignin or lignocellulosic biomass, the authors expressed and purified the chimeric xylanase/feruloyl esterase XynZ from *Clostridium thermocellum*, which was applied to release ferulic acid from wheat straw. The final cascade containing XynZ and whole-cell biocatalyst expressing Cg-AKR and Ni-CAR was able to produce a maximum of approximately 25 mg L<sup>-1</sup> coniferyl alcohol from wheat straw biomass.<sup>118</sup>

## 6.2. Non-aromatic bioproducts

**6.2.1. *Cis,cis*-muconic acid, a bio-privileged molecule.** *cis,cis*-Muconic acid (MA) is a product of catechol *ortho*-cleavage in species such as *R. jostii* RHA1, *P. putida* KT2440, *Amynolatopsis* sp. ATCC 39116, and *C. glutamicum* ATCC 13032<sup>88</sup> and is considered a “bio-privileged molecule” due to its potential use as a chemical building block for an extensive list of molecules relevant to biomaterial production, such as adipic acid, caprolactam, unsaturated polyesters, hexamethylene diamine, caprolactone, terephthalic acid, and 3-hexenedioic acid.<sup>119</sup> Vardon and colleagues demonstrated one of the first microbial engineering efforts to produce MA from an alkaline pretreated liquor (APL) from corn stover.<sup>120</sup> Using *P. putida* KT2440, the authors first redirected the aromatic catabolism from PCA towards catechol by replacing the PCA 3,4-dioxygenase gene *pcaHG* by the *aroY* gene from *Enterobacter cloacae* encoding a PCA decarboxylase, which redirected metabolic flux into PCA towards catechol and MA (see Fig. 11). The engineered strain was cultivated in minimal media

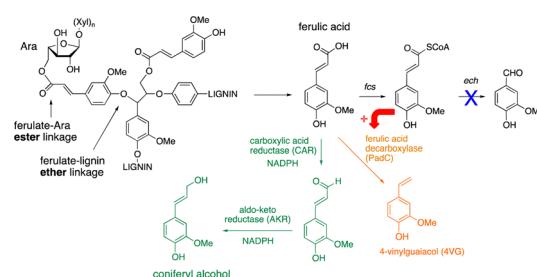


Fig. 10 Production of 4-vinylguaiacol in engineered *P. putida* KT2440 (in orange) and coniferyl alcohol (in green) via enzymatic conversion of ferulic acid, released from grass lignocellulose. The red arrow indicates auto-induction of *padC* expression by feruloyl CoA.

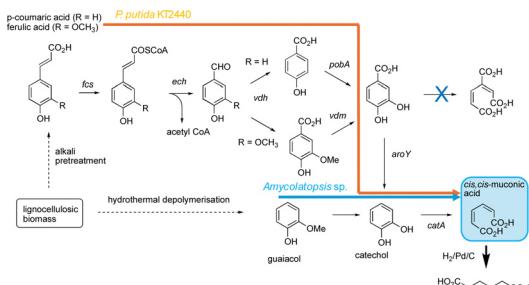


Fig. 11 Production of *cis,cis*-muconic acid in engineered bacterial strains, showing the routes engineered in *P. putida* KT2440 (in orange, also for *C. glutamicum* and *N. aromaticivorans*) and *Amycolatopsis* sp. (in blue).

supplemented with alkali pretreated lignin, containing significant levels of *p*-coumarate and ferulate, which were converted into  $0.7 \text{ g L}^{-1}$  MA after 24 h, representing a molar yield of 67%.<sup>120</sup> The authors then converted MA, produced from a fed-batch cultivation with *p*-coumarate, into adipic acid *via* catalytic hydrogenation using a Pd/C catalyst.<sup>120</sup> Other works have creatively addressed some of the bottlenecks associated with MA production in *P. putida*, including PCA decarboxylase activity and catabolite repression,<sup>121</sup> catechol sensitivity and substrate range,<sup>122</sup> and hydroxylation of 4-hydroxybenzoic acid into PCA and the resulting nicotinamide cofactor ratios.<sup>123</sup>

While *P. putida* is the host of choice for MA production in several studies, other microorganisms have the potential to produce MA, notably from modified native pathways. In a recent work, Vilbert and colleagues investigated a pathway for MA production in *N. aromaticivorans*.<sup>124</sup> They verified that this strain catabolises PCA not only *via* 4,5-*meta*-cleavage (initiated by LigAB1 and LigAB2), but also through decarboxylation towards catechol, catalysed by the gene products of the novel *NadBCD* gene cluster. Overexpression of the native *NadCD* proteins was achieved by placing new copies of the genes in the *ligAB1* locus, simultaneously disrupting the PCA *meta*-cleavage pathway and promoting the conversion of accumulated PCA into catechol. Finally, the authors replaced the *xylE* gene, which encodes a catechol 2,3-dioxygenase, by an additional copy of the native *catA* gene. The genes *catB* and *catC*, which promote further MA degradation towards the  $\beta$ -ketoadipate pathway, were also knocked-out, effectively accumulating MA. The final strain, engineered using only native genes and promoters, had the same conversion efficiency as that of the *N. aromaticivorans* strain engineered using foreign homologues. Furthermore, the engineered strains were able to produce MA from poplar alkaline pretreated lignin streams, in quantitative yield.<sup>124</sup>

MA has been successfully produced by an engineered *Amycolatopsis* sp. ATCC 39116 strain, which is capable of tolerating and utilising a wide range of lignin-derived aromatics, notably guaiacol, but also catechol, phenol, toluene, *p*-coumarate, and benzoate.<sup>125</sup> The authors deleted two *catB* genes encoding muconate cycloisomerases, and the double knockout strain produced  $3.1 \text{ g L}^{-1}$  MA from guaiacol in 96% yield. Furthermore, the strain successfully converted a hydrothermally-depolymerised softwood Kraft

lignin (IndulinAT) stream into MA at 72% yield, as well as *o*-cresol into methyl-MA.<sup>125</sup>

Similarly, the elimination of the muconate cycloisomerase *catB* gene and constitutive overexpression of *catA* in *C. glutamicum* led to  $85 \text{ g L}^{-1}$  MA being produced from catechol in 60 h. The authors also tested the same hydrothermally-depolymerised IndulinAT lignin stream with the engineered strain *C. glutamicum* MA-2, obtaining as much as  $1.8 \text{ g L}^{-1}$  MA.<sup>126</sup> In a follow-up study, this strain was further modified to convert vanillin and vanillate – obtained in high amounts from the catalytic alkaline oxidation of softwood lignin – into MA.<sup>127</sup> Using a systems metabolic engineering approach, the authors performed extensive modifications in the *C. glutamicum* MA-2 strain, such as: (i) eliminating the reductive vanillin catabolic branch; (ii) promoting the oxidative vanillin catabolism by overexpressing native *vdh*; (iii) de-repressing and overexpressing the *vanABK* operon, which encodes vanillate *O*-demethylase VanAB and vanillate/PCA transporter VanK; (iv) and finally, screening different *aroY*-homologues and overexpressing the most efficient catalyst (*E. cloacae* *aroY* and its two associated proteins EcdBD) in the *pcaG* gene locus. The final strain, named *C. glutamicum* MA-9, produced stoichiometric amounts of MA from vanillin, including vanillin derived from alkaline-oxidised softwood lignin. Using this substrate, the MA-9 strain produced  $0.35 \text{ mmol g}^{-1} \text{ h}^{-1}$  MA.<sup>127</sup>

**6.2.2. Polyhydroxalkanoate bioplastics.** Certain species of lignin-degrading bacteria, such as *P. putida* KT2440, are capable of accumulating storage compounds known as medium chain length polyhydroxalkanoates (*mcl*-PHAs). PHAs are stored as intracellular inclusion bodies under nutrient limiting conditions. Generated *via* the fatty acid biosynthesis pathway, these molecules have diverse applications as biothermoplastics and adhesives and can be further valorised into fuels and chemicals. Since the  $\beta$ -ketoadipate pathway leads to the TCA cycle, it is possible to generate PHAs from lignin-containing feedstocks (see Fig. 12). *mcl*-PHAs have been produced in *P. putida* KT2440 from corn stover alkaline pretreated lignin (APL), which consists mostly of a mixture containing aromatic monomers such as *p*-coumaric acid, ferulic acid, vanillic acid, acetate, and trace amounts of glucose.<sup>128</sup> The strain accumulated  $0.252 \text{ g L}^{-1}$  *mcl*-PHAs from undiluted APL, the equivalent of 32% cell dry weight (CDW). The produced compounds were extracted and subjected to thermal depolymerisation, yielding alkenoic acids, which were further processed *via* catalytic deoxygenation to produce fuel-range hydrocarbons.<sup>128</sup>

*mcl*-PHA accumulation was increased in *P. putida* KT2440 by knocking-out the *phaZ* gene encoding a PHA depolymerase and *fadBA1* and *fadBA2*  $\beta$ -oxidation genes.<sup>129</sup> Carbon flux was further enhanced towards *mcl*-PHAs by promoting chromosomal overexpression of genes involved in PHA biosynthesis, such as *phaH*, *phaG*, *alkK*, *phaC1*, and *phaC2*. The final strain *P. putida* AG2162 had a 100% yield improvement compared to the parental strain, with a final *mcl*-PHA production of  $116 \pm 35 \text{ mg L}^{-1}$  from depolymerised lignin liquor (containing mostly *p*-coumarate, some ferulates, and some high molecular weight lignins).<sup>129</sup>

Another study engineered the peripheral aromatic catabolism of *P. putida* strain H to enhance the conversion of



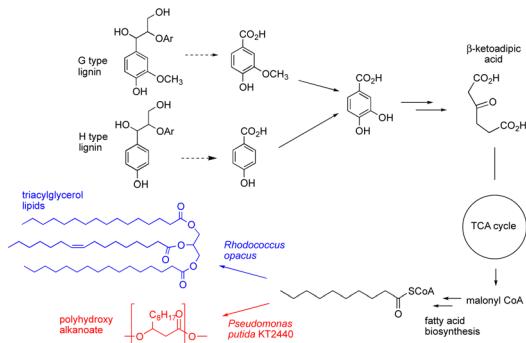


Fig. 12 Pathways for production of polyhydroxyalkanoates and triacylglycerol lipids from lignin-containing feedstocks, via primary metabolism.

lignin-derived monomers towards acetyl-CoA and, ultimately, malonyl-CoA – the main precursor for PHA biosynthesis.<sup>130</sup> The *P. putida* H strain had both *ortho*-cleavage and *meta*-cleavage pathways for catechol, initiated by two catechol 1,2-dioxygenases and one catechol 2,3-dioxygenase, respectively. Knocking-out one of the catechol 1,2-dioxygenases, *cata2*, resulted in improved PHA production from aromatic sources. The authors devised a fed-batch strategy using as the substrate an IndulinAT Kraft lignin hydrolysate, consisting mainly of catechol (50 mM), with feed addition of pure catechol, which, elegantly, was coupled to oxygen demand resulting from the action of catechol dioxygenases, to prevent catechol overload and cell toxicity. Under this regime, up to 1.4 g L<sup>-1</sup> PHA was produced, consisting mainly (78%) of polymerised C<sub>10</sub> units of 3-hydroxydecanoate.<sup>130</sup>

**6.2.3. Triacylglycerols from lignin feedstocks.** Certain oleaginous bacteria accumulate triacylglycerol lipids as storage compounds, which are also biosynthesised by fatty acid biosynthesis (see Fig. 12). Triacylglycerols (TAGs) are precursors for biodiesel, so accumulation of TAGs by lignin-degrading microbes would offer a potential route from biorefinery lignin to biodiesel.

Among lignin degraders, *Rhodococcus* sp. are well-known lipid producers, with the potential of accumulating TAGs simultaneously from carbohydrates and lignin-derived carbon present in lignocellulosic hydrolysates. Production of TAGs from lignin aromatic monomers such as 4-hydroxybenzoic acid (4-HBA) and vanillic acid has been shown in *R. opacus* strains DSM 1069 and PD630,<sup>131</sup> as well as from oxygen-pretreated Kraft lignin.<sup>132</sup> *R. opacus* NRRL B-3311 accumulated up to 32 mg L<sup>-1</sup> lipids from lignin obtained from ammonia fiber expansion (AFEX) pretreatment of corn stover.<sup>133</sup> By simultaneously utilising sugars and aromatics present in a pine organosolv pretreatment effluent as a substrate, *R. opacus* DSM 1069 accumulated a maximum of 26.9% of its CDW as oleic, palmitic, and stearic fatty acids.<sup>134</sup> In another study, *R. opacus* PD630 accumulated a maximum of 1.3 g L<sup>-1</sup> lipids, corresponding to 42% CDW, from a hydrolysate obtained from a multi-stage pretreatment process of corn stover biomass, which included firstly a mild alkaline pre-extraction process, followed by an alkali-hydrogen peroxide post-extraction

process.<sup>135</sup> The generated TAGs were successfully transesterified to yield biodiesel.<sup>135</sup>

A novel lignin-degrading species, *Rhodococcus pyridinivorans* CCZU-B16, identified from screening of environmental samples, accumulated 0.52 g of lipids per g CDW using a commercial alkali lignin at 4 g L<sup>-1</sup> as a sole carbon source.<sup>136</sup> Employing an innovative co-culture strategy, He and colleagues produced lipids from dilute alkali corn stover lignin. The co-culture consisted of *R. jostii* RHA1 VanA<sup>-</sup>, which degraded lignin and accumulated vanillate and lipids, and *R. opacus* PD630, which used the accumulated vanillate to generate lipids. An incremental lignin loading of up to 10 g L<sup>-1</sup> led to a lipid production of 0.39 g lipid/CDW using the co-fermentation strategy.<sup>137</sup>

## 7. Life-cycle assessment of processes for high-value chemical production from lignin feedstocks

While the environmental impact of using a renewable source of aromatics instead of petrochemicals seems obvious, there are only a limited number of published life-cycle assessments (LCAs) that address lignin valorisation processes.

Kylili *et al.* performed a review of studies published in the past few decades regarding lignin valorisation LCAs, in the context of a circular bioeconomy – a concept that exists at the interface of circular economy and bioeconomy models and focuses on sustainably generating bio-based products, while effectively “closing the loop” to minimise waste and reuse and recycle the biomass.<sup>138</sup> The authors highlighted several challenges associated with LCAs of lignin valorisation processes, including: (1) a lack of consistency in the LCA methodologies used in different studies; (2) difficulties in modelling processes involving powder *vs.* liquid lignin streams at the industrial scale; (3) undefined market value of possible products; and (4) confidentiality issues around life cycle inventories. While each process had their specific bottlenecks, the most common sources of negative environmental impacts were: high energy consumption (for production and transportation of materials, as well as processes requiring high pressure/temperature) and use of solvents and harsh chemicals (and subsequent disposal). Accordingly, this review highlighted that lignin-derived products, while achieving small improvements in greenhouse gas (GHG) emissions, global warming mitigation, fossil fuel depletion, and ecotoxicity, may in some cases present increased environmental burden, compared with routes based on petrochemicals.<sup>138</sup> A similar observation was made in a recent report on processes for chemical production from biomass.<sup>1</sup>

Conversely, a review of 42 LCAs for lignin-based products found that, in most cases, lignin valorisation presents a better environmental impact than fossil-based processes, especially regarding climate change.<sup>139</sup> Moretti *et al.* also identified that the assessment of the environmental impact is hindered by a lack of standard LCA methodologies. Furthermore, the heterogeneity of lignin percentage and composition, as well as the



question of which fuel should replace lignin (in biorefineries that use lignin for energy generation), was also highlighted. Based on their analysis, the authors outlined ten “lessons learned” and respective recommendations going forward for new LCA analyses for lignin valorisation.<sup>139</sup>

Corona *et al.* have performed a LCA for their previously described bio-based production of adipic acid from lignin.<sup>120,140</sup> The conventional petrochemical route for adipic acid production generates 0.3 kg of N<sub>2</sub>O per kg of adipic acid. Worryingly, N<sub>2</sub>O generates 300-fold higher greenhouse gas emissions compared with CO<sub>2</sub>.<sup>140</sup> The new process for production of renewable adipic acid was proposed in the context of a lignocellulosic ethanol biorefinery, where lignin can be sourced from the solid residue obtained after ethanol fermentation. The authors proposed that the lignin residue, named HLFB (high lignin fermentation by-product), is depolymerised into aromatic monomers in NaOH at 2 wt% at 160 °C. The resulting mixture is separated by filtration, where the liquid stream is sent for microbial biotransformation, and the solids are sent to the boiler to generate heat and electricity. The microbial biotransformation step is performed at 32 °C using an engineered *P. putida* KT2440 strain<sup>120</sup> capable of accumulating *cis,cis*-muconic acid (MA) in stoichiometric amounts. The fermentation broth is filtered, and MA is precipitated from the liquid fraction by the addition of sulfuric acid at 5 °C, 1 atm. The residual broth is neutralised with NaOH and recycled by anaerobic digestion in the in-house wastewater treatment facility, while the resulting sodium sulphate salts are sent to landfill. The recovered MA is dissolved in ethanol and subjected to catalytic hydrogenation at 33 bar and 75 °C, with the ethanol and hydrogen being partially recycled. The LCA for this process revealed substantial improvements compared to petrochemical-based adipic acid across all analysed impact categories, except for respiratory effects. Hotspot analysis identified NaOH consumption and heating costs as the highest burdens of the process.<sup>140</sup>

Van Duuren and colleagues performed a limited LCA for a different process for production of lignin-derived adipic acid using microbial biotransformation.<sup>141</sup> Using pyrolysis, soft-wood lignin is depolymerised into a biphasic bio-oil containing aromatic monomers and oligomers. A hydro-deoxygenation step is applied to the bio-oil, aiming to convert guaiacol into catechol and phenol. The bio-oil aqueous fraction, containing high amounts of catechol, is used as the substrate for the biotransformation step, performed using an engineered *P. putida* KT2440-BN6 strain. The resulting MA is purified using steam distillation and hydrogenated to yield adipic acid. The authors propose that byproducts, including NaOH and HCl, can be recycled through bipolar membrane electrodialysis and that the organic fraction from the bio-oil can be valorised into other products, such as phenol-formaldehyde resins. The limited LCA revealed that the proposed process can reduce CO<sub>2</sub>-equivalent emissions by 58% compared to the traditional petrochemical route and decrease energy consumption by 23%. The main environmental burdens were high energy use, especially during fermentation and rectification steps, use of hydrogen, which could present a negative environmental

impact if obtained from fossil fuels, and the use of harsh chemicals, such as NaOH and HCl, and toxic byproducts, such as phenol and guaiacol.<sup>141</sup>

A recent work on production of vanillin using a recombinant *Arthrobacter* sp. C2 performed a LCA of their laboratory-based process, and compared it to other lab-scale vanillin bioproduction processes.<sup>105</sup> Vanillin was produced from corn alkaline lignin by an engineered *Arthrobacter* sp. C2 strain, which was then isolated from the medium by extracting into ethyl acetate and rotary evaporation. The LCA revealed that the major environmental impact is “human toxicity” (55%), which consists mainly of electricity consumption (98%). More than half (57%) of the electricity demand of the process was consumed during microbial biotransformation. Thus, the authors stress the importance of using renewable electricity to power this bioprocess.<sup>105</sup>

Choice of lignin feedstock is an important consideration for future lignin-based processes. Although the use of industrial Kraft lignin is attractive, since it is a by-product of an existing industrial process: (1) its condensed structure makes it much more difficult to valorise and (2) Kraft lignin has a calorific value of 26.5 MJ kg<sup>-1</sup>, which is used to power biorefinery boilers, and hence whatever high-value chemical is produced from a new process, it must have added value, compared with use of lignin as a fuel.<sup>142</sup> A report from an academic-industrial consortium in The Netherlands in 2016 highlighted the need to consider the full value chain from plant biomass to products, so that all components of the lignocellulosic biomass can be utilised effectively for chemical production.<sup>143</sup> Often processes that are optimised for cellulose production generate low-quality lignin that cannot be effectively valorised for chemical production (e.g. Kraft lignin and lignosulfonates), whereas processes optimised for high quality lignin production (e.g. organosolv lignin) are not commercially viable in the current climate. New types of biomass pretreatment may be able to deliver both high quality lignin and sugar streams.<sup>143</sup> Alternatively, lignin-degrading microbes could be engineered to utilise cellulose as well as lignin, which was recently reported for *R. jostii* RHA1,<sup>144</sup> which may in the future allow chemical production from both lignin and cellulose fractions.

## 8. Conclusions

In this Feature Article, we highlight the potential of using engineered lignin-degrading microbes for the production of a range of high-value molecules. As our understanding of the catabolic pathways for lignin degradation improves, new routes to different high-value products emerge, using polymeric lignin and biomass as substrates for biotransformation, which are important proof-of-concepts towards consolidated bioprocessing of raw lignin and lignocellulose. Importantly, as highlighted by the LCAs above, the use of energy-intensive processes, harsh chemicals, and a large volume of solvents greatly contributes to the negative environmental impacts of some lignin valorisation processes. At least some of these

impacts could be alleviated by novel biotechnologies using microbes directly in untreated lignin streams, circumventing the need to purify and depolymerise lignin prior to valorisation. Accordingly, research efforts could be directed towards the discovery and improvement of microbial lignin-based cell factories with increased lignin depolymerisation capacities, efficient aromatic catabolic flux, tolerance towards high concentrations of potential inhibitors, and shorter cultivation times.

## Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

Research on lignin valorisation conducted by the authors' group is supported by the BBSRC research grant BB/Y004027/1.

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