Microbial hosts for metabolic engineering of lignin bioconversion to renewable chemicals

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Abstract
This review discusses the use of engineered microbes for bioconversion of lignin from plant biomass to produce renewable chemicals. Existing bacterial hosts for lignin bioconversion are discussed, such as \textit{Pseudomonas putida} KT2440 and \textit{Rhodococcus jostii} RHA1, and case studies where they have been engineered to generate aromatic and non-aromatic products are described, and the different types of lignin substrates used for these studies. Other bacteria identified as lignin degraders are described, and the prospects for using other bacteria as hosts for metabolic engineering of lignin degradation are discussed. Recent advances in genetic modification of fungi are also discussed, which could lead to the metabolic engineering of lignin-degrading fungi for bioproduct formation from lignin. Prospects and challenges in this field are discussed, as the field moves from the laboratory to industrial application, including: choice of chassis organism, choice of lignin feedstock, the complexity of polymeric lignin breakdown, and considerations for scale-up and choice of bioproduct.

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Keywords: lignin; microbial bioconversion; metabolic engineering; β-ketoadipate pathway.

List of abbreviations:
APL  Alkali pretreated lignin
APPL  Acid-precipitable polymeric lignin
CRISPR Clustered regularly interspaced short palindromic repeats
DyP  Dye decolorizing peroxidase
PDC  2-pyrone-4,6-dicarboxylic acid
PDCA  pyridinedicarboxylic acid
PHA  polyhydroxyalkanoate
gRNA  guide RNA
sgRNA  single guide RNA

1. Lignin as a raw material for bioconversion to fuels and chemicals

Fuels and chemicals containing aromatic rings are currently derived from crude oil, via the petrochemical industry, and are used in a wide range of industrial applications such as plastics manufacture, solvents, jet fuel, and fine chemicals production [1]. As supplies of crude oil dwindle, and as the use of fossil fuels currently contributes towards global warming, alternative sustainable sources for production of these chemicals must be found over the next 20-30 years [1]. Lignin, an aromatic heteropolymer found as 15-30% dry weight of lignocellulose, is a major structural component of plant cell walls, and is therefore a major component of plant biomass, and is the most abundant source of renewable aromatic carbon in the biosphere. Therefore, it represents an attractive raw material for conversion to aromatic chemicals, although it is a challenging material for bioconversion. Lignin streams are also produced industrially from pulp/paper production, in the form of Kraft lignin from the Kraft process (60-100 ktons/yr), and lignosulfonate from the Sulphite process (1 Mtonne/yr) [2]. Lignin is also generated from the global bioethanol industry, either as plant bagasse from...
1st generation bioethanol production, or as a pre-treated lignin side-stream from 2nd generation (cellulosic) bioethanol production. The chemical structure of lignins obtained from different methods is variable [2-4], and industrial lignins often contain more “condensed” structures lacking functional groups needed for breakdown, hence they may be more difficult materials for bioconversion, although they are available in large quantities.

2. Lignin structure

Lignin encases the cellulose filaments found in lignocellulose, thereby adding structural rigidity, and giving wood its characteristic strength. It is also covalently attached to hemicelluloses found in lignocellulose. The structure of lignin consists of phenylpropanoid aryl-C₃ units, linked together via a variety of carbon-oxygen ether bonds and carbon-carbon bonds, which are not susceptible to hydrolytic cleavage [2-5]. Lignin is formed in growing plants by radical polymerisation of three precursors: coniferyl alcohol, giving guaiacyl (G) units in lignin; sinapyl alcohol, giving syringyl (S) units in lignin; and p-coumaryl alcohol, giving p-hydroxyphenyl (H) units in lignin [5-7]. Softwoods generally contain G type lignins with mainly G units, while hardwoods generally contain GS-type lignins, with mixtures of G and S units, and grasses contain G type lignins with a higher proportion of H units (see Figure 1A) [2-5].

There are different types of linkages found in lignin, shown in Figure 1B. The most abundant linkage is the β-aryl ether (β-O-4), containing an ether linkage from the β-carbon of the C₃ chain to the phenolic O-4 of another aryl unit, which is found as 45-50% of units in softwoods, and 60% in hardwoods. The biphenyl linkage (5-5), containing an aryl-aryl carbon-carbon bond, is found as 20-25% units in softwoods, but only 3-9% in hardwoods. Other types of linkage include the phenylcoumaran linkage (β-5) and the pinoresinol linkage (β-β). The formation of each of these linkages can be rationalised by radical dimerisation or polymerisation reactions from cinnamyl alcohol precursors [6,7].
Figure 1. Structural units found in polymeric lignin. A. Schematic structures of G, S, and H units. B. Chemical structures of the major chemical linkages found in polymeric lignin (illustrated as G units).

The linkages present in lignin are not susceptible to hydrolytic attack, lignin is inert and difficult to break down chemically. [2]. There is considerable interest in chemocatalytic methods for lignin breakdown [2], but this review will focus on microbial conversion of lignin into renewable chemicals, which has been studied since the 1980s using white-rot basidiomycete fungi, which are known to attack polymeric lignin via production of
extracellular lignin peroxidase and laccase enzymes [8]. Since 2010, there has been a resurgence of interest in the use of soil bacteria that have been identified with lignin degradation activity, which utilise DyP-type peroxidase enzymes and multi-copper oxidases to attack lignin [9]. There is also research interest in the application of lignin-oxidising enzymes \textit{in vitro}, which in practice has been limited by the repolymerisation (or recondensation) of phenoxy radical intermediates formed upon lignin oxidation [9].

The use of whole cell microbial biotransformations for lignin bioconversion has so far proved to be a more effective strategy for the generation of useful bioproducts from lignin [9,10], for several reasons: 1) bacterial aromatic degradation is known to be convergent, so that a complex mixture of aromatic compounds generated from lignin breakdown can be converted to a small number of intermediates in aromatic degradation via a process known as “metabolic funnelling”; 2) lignin-degrading bacteria can be engineered to produce useful products from lignin degradation, while also being able to support growth; 3) microbial cells appear to avoid the problem of repolymerisation of lignin fragments, through the use of accessory enzymes that are only partially understood. This review will compare bacteria and fungi as possible hosts for metabolic engineering of lignin degradation, and illustrate examples of engineered microbes that have been used to generate products from lignin breakdown. A more general review of the bioconversion of lignin by Li and Zheng has recently been published [11].

\textbf{2. Fungi and bacteria for lignin bioconversion}

Lignin degradation has been extensively studied in basidiomycete fungi, from which several extracellular lignin-degrading enzymes have been identified, including heme-dependent lignin peroxidase and manganese peroxidase and multi-copper dependent laccases that can oxidise lignin units, and aryl-alcohol oxidase that generates hydrogen peroxide for use by heme peroxidases [8,12]. White-rot basidiomycetes (\textit{e.g. Phanerochaete chrysosporium, Pleurotus eryngii, Trametes versicolor}) are commonly observed as wood-rotting organisms in the environment. They can degrade lignin, hemicellulose, and cellulose in wood, often leaving
a white residue enriched in cellulose. Brown-rot fungi (e.g. Postia placenta, Gloeophyllum trabeum) grow mainly on softwoods and represent only 7% of wood-rotting basidiomycetes. They partially modify the lignin fraction, and then degrade wood polysaccharides, leaving a brown material consisting of oxidized lignin [13]. Although attractive organisms for lignin degradation, their more complex genetics makes them more challenging organisms for genetic modification, which will be discussed in Section 3. Expression of recombinant fungal enzymes can also be challenging, especially if glycosylated, but expression in filamentous fungi such as Pichia pastoris is often possible [14].

Certain soil bacteria have been known since the 1980s to degrade lignin, of which the best studied organism was Streptomyces viridosporus T7A [15], but until recently, no specific genes or enzymes involved in lignin oxidation had been identified. The potential to carry out genetic modification in bacteria, and more straightforward protein expression of bacterial enzymes, has led to a resurgence of interest in lignin degradation in bacteria, and to the identification of many lignin-degrading bacteria, and several lignin-oxidising enzymes (see Table 1).

<table>
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<th>Enzymes identified</th>
<th>Products from lignin degrn</th>
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Table 1. Bacterial strains reported to break down polymeric lignin. Evidence for lignin degradation: 1, colorimetric assay; 2, delignification via Klason assay; 3, depolymerisation via gel permeation chromatography; 4, aromatic products formed; 5, growth on minimal media containing lignin.

Several different methods have been used to isolate lignin-degrading bacteria, and demonstrate lignin breakdown. In 2010, Ahmad et al reported a colorimetric assay for lignin degradation, using chemically nitrated lignin, which was verified using *Streptomyces viridosporus*, and was used to verify well-studied aromatic degraders, *Rhodococcus jostii* RHA1 and *Pseudomonas putida* mt-2, as lignin degraders [16]. These strains were also found to generate aromatic metabolites upon incubation with lignocellulose [16]. The nitrated lignin assay has been used as a screen on agar plates to identify bacterial lignin degraders from soil [17]. Growth on minimal media containing a lignin as a carbon source has been used to identify a number of strains listed in Table 1, however, caution should be used using this method alone, since lignin preparations often contain low molecular weight aromatics, which are generally utilised more readily as carbon sources for bacterial growth. Therefore,
degradation of polymeric lignin should be confirmed by independent methods. Delignification can be studied using the Klason assay, a gravimetric method to analyse lignin content in lignocellulose, and lignin depolymerisation can be studied via gel permeation chromatography, which reveals more detailed insight into molecular weight changes taking place [18]. The review will discuss strains identified in each bacterial phylum, and relevant lignin-oxidising enzymes identified in those strains.

The Actinobacteria contain the *Streptomyces viridosporus* T7A strain first identified to degrade lignin [15]. Bacterial laccase enzymes have been identified and characterised from *Streptomyces coelicolor* A3(2), *Streptomyces lividans* TK24, *Streptomyces viridosporus* T7A, and *Amycolatopsis* sp. 75iv2 [19]. A lignin-oxidising Dyp2 peroxidase (in DypC class) with high Mn$^{2+}$ oxidation activity has also been reported from *Amycolatopsis* sp. 75iv2 [20]. *Rhodococcus jostii* RHA1, a PCB-degrading bacterium whose genome was sequenced in 2006 [21], was identified in 2010 as a lignin-degrading bacterium [16]. A dye-decolorising peroxidase DypB from *Rhodococcus jostii* RHA1 was identified in 2011 as the first characterised bacterial lignin peroxidase enzyme [22]. This bacterium has been used as a host for metabolic engineering approaches for lignin degradation, as described in the later sections.

Several other actinobacteria have been identified as lignin degraders. *Rhodococcus opacus* strains PD630 and DSM 1069, which accumulate lipids, are able to convert organosolv lignins into lipids [23], and *Rhodococcus opacus* NRRL B-3311 has been reported to convert AFEX corn stover lignin into microbial lipid [24]. Aromatic degrader *Arthrobacter globiformis* has been reported as a lignin degrader on the basis of colorimetric assay [15], and a *Paenarthrobacter* sp has been identified from sugarcane field soil that can grow on alkali lignin [25]. A *Streptomyces* sp S6 strain has been reported that can depolymerise Kraft lignin, and the genome of this strain has been determined, which contains a DyP-type peroxidase and aromatic degradation gene clusters [26]. Three *Microbacterium* isolates that can depolymerise lignin have been identified from screening of soil samples [17], and the genome of lignin-degrading *Microbacterium phyllosphaerae* has been determined, which contains two DyPA genes [27]. A *Leucobacter* sp SHC3 strain has also been reported that can grow on Kraft lignin [28].

In the Firmicutes phylum, although *Bacillus subtilis* has been reported not to degrade lignin [16,18], several other *Bacillus* strains have been reported as degraders of Kraft lignin,
including *Bacillus* sp LD003 [29], *Bacillus pumilus* C6, *Bacillus atrophaeus* B7 [30], *Bacillus ligniniphilus* L1 [31], and *Paenibacillus* sp [32]. A *Lysinibacillus sphaericus* strain has been identified from landfill waste that can depolymerise lignin [32].

Among Gram-negative bacteria, most lignin-degrading bacteria have been reported in the α- and γ-proteobacteria, which are commonly found in soil. In the γ-proteobacteria, as well as the well-studied aromatic degraders *Pseudomonas putida* mt-2 [16] and KT2440 [18], the growth of *P. putida* strain A514 on lignin has been studied via transcriptomic analysis, providing evidence for upregulation of a DyP peroxidase gene during lignin breakdown [33]. *Pseudomonas* sp LD002 [29] and *Pseudomonas* sp Q18 [34] have been reported to grow on Kraft lignin. *Pseudomonas fluorescens* Pf-5 has been found to depolymerise lignin [18], and a dye-decolorising peroxidase Dyp1B has been identified in this strain that can oxidise polymeric lignin [35]. A multi-copper oxidase CopA has been characterised from *P. putida* KT2440 and *P. fluorescens* Pf-5 that has activity for oxidation of polymeric lignin [36]. *Acinetobacter baylyi* ADP1 is also a well-characterised aromatic degrader which has been reported to depolymerise lignin [18]. *Enterobacter lignolyticus* SCF1 has been reported to degrade lignin under aerobic or anaerobic conditions [37], although the precise mechanism of anaerobic lignin degradation is not fully understood. Strains of *Enterobacter ludwigii* and *Enterobacter cloacae* were isolated from landfill soil as lignin degraders, but were found to enhance gas production from lignocellulose under microaerobic conditions, consistent with anaerobic lignin breakdown [32].

In the α-proteobacteria, three *Ochrobactrum* lignin-degrading strains were isolated from woodland soil [17], and a further two *Ochrobactrum* lignin degraders were isolated from landfill soil [32]. A multi-copper oxidase enzyme CueO has been identified in *Ochrobactrum* sp that can oxidise lignosulfonate [38]. *Ochrobactrum* sp SHC2 has also been reported to degrade Kraft lignin [28]. A *Rhizobium* sp YS-1r strain has been reported to depolymerise lignin [39], and an *Agrobacterium* sp strain that can depolymerise lignin and enhance gas production from lignocellulose under microaerobic conditions [32].

Strains from the β-proteobacteria have also been reported to break down lignin. *Comamonas* sp B-9 has been reported to grow on Kraft lignin [40], and *Comamonas testosteroni* and *Comamonas serinivorans* strains have been isolated as lignin degraders from landfill soil [32]. *Cupriavidus basilensis* B8 has been reported as a Kraft lignin degrader [41], *Burkholderia* sp H1 [42] and *Burkholderia* sp CCA53 [43] have been reported as Kraft lignin
degraders, and *Pandoraea norimbergensis* LD001 [29] and *Pandoraea* sp ISTKB [44] are also reported as Kraft lignin degraders. Two Bacteroides strains have also been reported as lignin degrading bacteria: *Sphingobacterium* sp T2 [17], from which an extracellular lignin-oxidising manganese superoxide dismutase enzyme has been identified [45], and *Sphingobacterium* sp HY-H, which can degrade lignosulfonate [46].

Soil metagenomic studies have identified consortia of bacteria involved in lignin degradation in the environment. DeAngelis et al have examined the microbial community formed in tropical forest soil in Puerto Rico amended with Kraft lignin [47]. Analysis of 16S rRNA genes present revealed higher relative abundances in the lignin-amended soil of Actinobacteria, Firmicutes, Acidobacteria and Proteobacteria [47]. Moraes et al have examined a consortium of soil bacteria from sugar cane fields enriched with biorefinery lignin [25]. From 16S rRNA sequencing, the major enriched classes of bacteria were alpha-, beta-, and gamma-proteobacteria, accounting for 60% of operational taxonomic units (OTUs), with actinobacteria and firmicutes also enriched to a lesser extent. However, 19 different families of bacteria were significantly enriched in the consortium, indicating that a complex consortium of soil bacteria is likely to be involved in lignin breakdown in soil. From metagenomic analysis of DNA from enriched soil, the presence of lignin-oxidising enzymes such as DyP-type peroxidases and multi-copper oxidases was observed, together with aromatic degradation genes [25]. Diaz-Garcia et al have carried out metagenomic analysis of soil cultivated on pre-digested plant biomass [48]. They observed enrichment of 20 types of gene potentially linked with lignin breakdown. Taxonomic analysis of the enriched genes revealed that Pseudomonadaceae, Alcaligenaceae, Sphingomonadaceae, Caulobacteraceae, Comamonadaceae and Xanthomonadaceae were key bacterial families in the catabolism of lignin [48]. The observation of gene sequences from the actinobacteria and proteobacteria is consistent with the majority of isolated lignin-degrading bacteria being from these phyla, and also matches bacteria observed in the guts of termites and wood-boring insects [49].

4. Microbial hosts amenable to metabolic engineering of lignin degradation

4.1 Fungal hosts

White-rot basidiomycete fungi are among the most powerful and efficient lignin degrading microorganisms known [50]. The development of genetic engineering tools for
basidiomycetes – white-rot fungi being no exception – has traditionally been hampered by their long life-cycle, complex gene architecture, low efficiency of DNA fragment insertion into the chromosomes and protoplast production from spores [51,52]. Nevertheless, metabolic engineering efforts of white-rot fungi for lignin degradation have been undertaken giving promising results. Key to microbial metabolic engineering is the ability to achieve the transformation of a strain of interest through the stable introduction of exogenous DNA. This has been accomplished for several lignin-degrading fungi via polyethylene glycol-mediated protoplast transformation, such as for Ceriporiopsis subvermispora through the introduction of resistance to hygromycin B [51]; for Phanerochaete chrysosporium through the complementation of adenine auxotrophy [53]; for Schizophyllum commune through the complementation of tryptophan auxotrophy and the introduction of phleomycin and hygromycin B resistance [54-56]; for Trametes versicolor through the insertion of phleomycin resistance [57]; for Dichomitus squalens via the introduction of hygromycin B and G418 resistance [58].

Amongst lignin-degrading fungi, the white-rot P. chrysosporium is a well-known species that secretes a plethora of lignin-degrading peroxidases and oxidases having high redox potential compared to other microorganisms [59]. The metabolism of P. chrysosporium has been engineered to improve its lignin degradation capabilities. It has been subjected to the constitutive high-level expression of the endogenous manganese peroxidase Mnp1 and lignin peroxidase LipH8, as well as of a codon-optimized vpl2 gene from Pleurotus eryngii coding for a versatile peroxidase – which is absent in P. chrysosporium – that can degrade phenolic and non-phenolic aromatics [60]. The overexpression of endogenous peroxidases in white-rot fungi can compensate for the low basal expression of these enzymes, which is normally triggered by nutritional limitations that take place once the secondary metabolism is activated. The heterologous production of a versatile peroxidase in P. chrysosporium is not only contributing to enhancing the ligninolytic abilities of this fungus, but it also provides a potential platform for the purification of recombinant fungal peroxidases that cannot be obtained as efficiently in ascomycete expression hosts, which are often known to fail in the post-translational modifications and correct protein folding of this class of enzymes [61].

Schizophyllum commune is a white-rot fungus that has been used as a model organism to study mating and mushroom development. Plenty of tools are available for its genetic engineering, including for the homologous recombination-mediated gene knockout [62] and
for the CRISPR/Cas9-based gene knockout through pre-assembled Cas9-sgRNA ribonucleoproteins [63]. From the genome analysis of *S. commune*, it appeared that this fungal species contained no peroxidases and had an overall smaller number of lignin-degrading enzymes compared to other white-rot fungi, such as *P. chrysosporium*, and even compared to the coprophilous basidiomycete *C. cinerea* [64]. Given the availability of genetic engineering tools, the short life cycle of approximately ten days that facilitates the quick generation of protoplasts from spores, and the reduced number of native ligninolytic enzymes, *S. commune* appears to be a good canvas that may be used to study and improve the activity of lignin-degrading enzymes, and potentially engineer downstream bioconversion applications.

Metabolic engineering can also be used to expand the lignin degradation capabilities of genetically tractable, industrially relevant microorganisms for which more molecular tools may be available. Unicellular, fast-growing yeasts represent good candidates for such endeavours because of their simpler life cycles and higher availability of genetic engineering tools compared to multicellular, slow-growing white-rot basidiomycetes. Unlike mushroom-forming basidiomycetes, the transformation of yeasts has the advantage that it does not rely on the ability to induce fruiting and sporulation of the fungi in a laboratory setting. Amongst the industrially relevant yeasts, the oleaginous *Yarrowia lipolytica* naturally produces extracellular laccases that can metabolise lignin-derived phenolic compounds [65] and it has been engineered to express further heterologous laccases from white-rot basidiomycetes [66]. *Yarrowia lipolytica* can be efficiently transformed with a variety of methods, including electroporation [67] and a lithium/polyethylene glycol/single-stranded DNA/dithiothreitol-mediated protocol [68]. Plenty of tools are available for the metabolic engineering of *Y. lipolytica* that may aid the further expansion of its lignin degradation pathways, including a modular Golden Gate-based toolkit for multigene expression vector assembly with constitutive and inducible promoters [69] and a CRISPR-Cas12a/Cpf1-based toolkit for multiplexed genome editing [70].

In summary, significant efforts have been made in recent years to optimise the transformation and the genetic engineering tools available for white-rot basidiomycete fungi. Such tools have been used to improve the lignin degradation abilities of selected species and may be translated to other strains within the phylum Basidiomycota upon optimisation, such as through the use of endogenous promoters and the codon-optimisation of selectable
marker and nuclease genes. The metabolic engineering of white-rot basidiomycetes remains more challenging than that of industrially relevant yeasts, the engineering of which can potentially aid researchers in the study and exploitation of ligninolytic enzymes from more complex fungi.

4.2 Bacterial hosts

Metabolic engineering approaches are used primarily to facilitate the creation of an industrially relevant strain that could be used at scale for the production of one or more molecules. However, engineering of lignin degrading strains, including those which may not be obvious candidates for use at industrially relevant scale, can increase our understanding of how these organisms utilise lignin degradation intermediates, the discovery of alternate pathways, and novel ‘parts’ which could be utilised by other, more industrially applicable strains. There are several promising organisms for lignin valorisation, at different stages of development as chassis organisms for synthetic biology or metabolic engineering. Some, such as *P. putida* and *C. glutamicum* have a large, robust toolkit of methods and parts, as well as having been grown on industrial scale for commercial purposes. Other organisms such as *Rhodococcus* and *Amycolatopsis* are still under development for metabolic engineering, and studies on these organisms will benefit from engineering studies on other bacteria.

The question of which is the most efficient lignin degrader is not straightforward to answer, because only relatively few studies have compared the performance of different strains, and different research groups use different methods to monitor lignin breakdown, and different feedstocks. Comparative studies of a panel of 14 lignin-degrading bacteria were reported by Salvachua et al in 2015 [18]. Five strains were found to show enhanced delignification via Klason assay, depolymerisation via GPC, and growth on lignin as carbon source: *Pseudomonas putida* strains mt-2 and KT2440, *Rhodococcus jostii* RHA1, *Amycolatopsis* sp. 75iv2, and *Acinetobacter* sp. ADP1 [18]. A further 4 strains showed intermediate lignin degradation activity: *Cupriavidus necator* H16, *Pseudomonas fluorescens* Pf-5, *Paenibacillus* sp., and *Rhodococcus erythropolis* U23A [18]. Using a colorimetric assay for lignin breakdown, Taylor et al found that *P. putida* mt-2 and *R. jostii* RHA1 showed comparable activity to lignin-degrading *Ochrobactrum* and *Microbacterium* strains [17]. By assessing delignification of milled lignocellulose using Klason assay, Rashid et al observed approximately 20% delignification over 7 days by *P. putida* mt-2 and *P. fluorescens* Pf-5, and 10-20%
delignification by a range of other lignin-degrading bacteria, but highest rates of phenolic compound release were observed using *R. jostii* RHA1, *R. erythropolis*, and *Comamonas testosteroni* [32].

### 4.2.1 *Pseudomonas putida* as a host

The *Pseudomonas* genus contains several strains shown to be versatile host chassis organisms for synthetic biology and metabolic engineering, with several reviews describing their use in biotechnology [71-73]. The genus has many well developed genetic tools and methods for its engineering, as covered in the aforementioned reviews [71-73], including several different implementations of CRISPR/Cas9 systems for the generation of single gene mutations [74], multiplex mutation [75], and CRISPR interference [76].

Numerous examples of products have been produced using engineered *Pseudomonas* strains including: polyketides, non-ribosomal peptides and terpenoids amongst other natural products [77]. Several species have also been engineered to produce aromatic amino acids and other products derived from aromatic amino acids, such as trans-cinnamate [78] and phenol [79], recently reviewed by Schwanemann *et al.* [80]. *Pseudomonas* strains have also been used as industrial biocatalysts, for example in the production of 5-methylpirazine-2-carboxylic acid used by Lonza [81], with further examples reviewed by Poblete-Castro *et al.* [81].

*P. putida* has been shown to degrade high molecular weight lignin [18], as well as being well known as an efficient aromatic degrading bacteria, with species able to metabolise aromatics such as: p-coumarate, caffeate, ferulate, vanillate, coniferyl alcohol, benzoate, p-hydroxybenzoate, benzyamine, phenylacetate, phenylethylamine and phenylhexanoate amongst others [82]. In addition there are several models for the degradation capabilities of *P. putida*, which have been produced using genomic and biochemical data, which allow for a greater ability to design engineering approaches [83,84].

In addition to its degradation ability, *P. putida* is of particular interest as a chassis due to its tolerance to aromatics and solvents. Many factors play a part in this ability, which was reviewed by Ramos *et al.* [85], with the key factors being the ability to change cell membrane permeability, the presence of efflux pumps, and induction of a stress response, which is closely linked to its reductive ability and unusual carbon metabolism strategy [86]. Recently it has been shown that during sub-lethal oxidative stress, *P. putida* shifts its metabolism
towards utilising glucose and the pentose phosphate pathway, which leads to the generation of NADPH, which is used in the removal of H$_2$O$_2$ [87].

At the heart of the synthetic biology concept is the possibility of reducing the genome of an organism to its core components. Many strains of *P. putida* have been produced with a reduced genome: Lieder et al. removed around 4.3% of the genome of *P. putida*, removing elements such as flagella and several prophages, which led to a >50% increase in physiological vigour, based on the output of a *luxCDABE* reporter system [88]. This strain was then shown to perform better than the original strain under bioreactor conditions [89]. *P. putida* can be used for difficult biochemistry, one recent example is the engineering of a strain to undertake fluorination, an important chemical transformation required for the production of many pharmaceuticals, which was achieved by use of a fluorine sensing riboswitch that was optimised for use in *P. putida* [90].

Due to the well characterised nature of genetics parts in *P. putida*, the wider use of biosensors to improve screening or to control biosynthetic pathways could be feasible. Most examples have been developed in *E. coli*, but not used in other organisms [91]. To work optimally in a new host, biosensors need to be characterised and tuned, for example Jha et al. showed that a biosensor for protocatechuate that was built for use in *E. coli* could be repurposed for use in *P. putida* [92]. Once a biosensor system has been established, it can be used for screening libraries of mutant enzymes for changes in activity, such as the loss of feedback activity. This approach was used for the production of 4-hydroxybenzoate from chorismate, which can be inhibited by benzoate [93]. Additionally, a host chassis may have regulatory circuits which could be exploited for expression of novel pathways, for example, the native ferulate degradation operon of *P. putida* was co-opted for the expression of a heterologous pathway for the production of 4-vinylguaiacol from ferulate from lignocellulosic waste [94]. This strategy meant that expression of the biosynthetic pathway only occurred after ferulate was liberated from the wheat straw feedstock, and not during initial growth [94]. The use of biosensors for induction of synthetic pathways could lead to strains capable of complex tuning of gene expression levels based on feedback to external stimuli. The development of *P. putida* could act as a roadmap for development of other less well developed lignin valorising chassis organisms.

4.2.2 Genus Rhodococcus as host organisms
The genus *Rhodococcus* is member of the actinomycetes that exhibits wide genetic diversity amongst its species. *Rhodococci* can be isolated from a wide range of environmental sources and have been shown to degrade a variety of environmental contaminants including benzonitrile [95], polychlorinated biphenyls [96], 4-chlorophenol and 2,4-dichlorophenol [97]. Further examples are also highlighted in the review by Kim *et al.* which discusses additional applications for rhodococci [98].

Several species could be applied towards the utilisation of lignin. *Rhodococcus opacus* (reviewed recently by Winston *et al.* [99]) is an oleaginous species that produces triacylglycerols, unlike most oleaginous bacteria which accumulate polyhydroxyalkanoic acids for storage. It has been shown that these can be formed when grown on both aromatic carbon sources (up to 44% dry cell mass in a strain developed by adaptive evolution [100]) and carbohydrates, including mixed lignocellulosic waste streams [100], and these triacylglycerols can then be converted to biodiesel [101]. *Rhodococcus jostii* RHA1 has been established to be able to degrade polymeric lignin [16,18], and it has been demonstrated that a vanillin dehydrogenase (*vdh*) gene deletion strain can produce vanillin from aromatic carbon sources but also directly from lignocellulosic waste such as wheat straw [102].

Plasmids for gene expression have been developed for *Rhodococcus*, including the pTip series which use thiostrepton as an inducer, and the pNit series which express constitutively [103]. Tools and methods have been developed for the modification of *rhodococci* via the use of suicide vectors and homologous recombination [104,105], however these efforts can often be hampered by a low transformation efficiency, restriction-modification systems, and a low rate of recombination, both for achieving an integrant (first cross) and for achieving the elimination of the plasmid with the second crossover event [105]. Liang et al. implemented several strategies to improve the rate of production of mutant strains of *Rhodococcus ruber* TH [106]: they improved the transformation efficiency through DNA methylation, increased the rate of DNA genome integration via homologous recombination by the introduction of bacteriophage recombinases Che9c60 and che9c61 (characterised for use in *R. opacus* by DeLorenzo et al. [107]) and introduced CRISPR/Cas9 to initiate homologous repair and as an effective counter selection system against the wild type allele [106]. By hosting this system across three different plasmids it was possible to make iterative mutations by first curing the strain of the gRNA plasmid (by omission of the appropriate antibiotic) and then retransforming with a new gRNA sequence.
As well as producing a range of useful synthetic biology ‘parts’ for use in rhodococci such as a curable plasmid backbone, new antibiotic resistance markers and a library of constitutive promoters, DeLorenzo et al. have demonstrated that a catalytically inactive Cas9 can be utilised for CRISPR interference, which can silence gene expression during fermentation [107]. The ability to tune the repression of gene expression is a potentially very useful tool for reducing loss of metabolite to competing pathways that are essential for cell growth. Despite some difficulties with working with this genus, tools and methods are now starting to be developed to improve and refine the ability to engineer rhodococci.

4.2.3 Genus Amycolatopsis as host organisms

*Amycolatopsis* sp. 75iv2 is known to produce a lignin-oxidising Dyp2 peroxidase [20], and this strain has been shown to depolymerise polymeric lignin [18], hence the genus *Amycolatopsis* is a potentially interesting chassis organism for lignin degradation studies. *Amycolatopsis* sp. strain ATCC39116 was previously identified as a microbe for the bioproduction of vanillin from eugenol or ferulic acid [108], and was found to be able to accumulate 6.4 g/L vanillin, in 68% molar yield [109]. Deletion of the vanillin dehydrogenase, *vdh*, was demonstrated to improve yield of vanillin [110]. Constitutive expression of the ferulic acid degradation genes *fcs* and *ech*, and an improved feeding strategy, led to a vanillin titre of 22.3 g/L [110], with a modified bioprocess subsequently giving a more efficient molar yield of 94.9% [111].

There are limited tools developed for *Amycolatopsis*, and potential difficulties in generating mutants. Shuttle and expression vectors, and methods of transformation are available for *Amycolatopsis*, often based on those used in Streptomyces, however, biological parts need to be characterised when being used in a new host [112,113]. Efforts have been made to improve methods for making gene deletions in *Amycolatopsis* via the use of suicide plasmids and homologous recombination. Meyer *et al.* improved off target integration by redesigning the vector, and improved the screening for second cross events by use of *rpsL* from *Saccharopolyspora erythraea*, which confers sensitivity to streptomycin in strains that already have resistance [114]. Barton *et al.* used the introduction of β-glucuronidase to produce a blue-white screen that could be used for the detection of integration and second crossovers, without the need of a strain that was resistant to streptomycin. With this method they found that deletion mutants could be generated in 10 days. Furthermore, they used this new
method to construct a strain capable of producing cis,cis-muconic acid from guaiacol and guaiacol-rich lignin hydrolysate by disruption of the β-ketoadipate pathway, deleting muconate cycloisomerase genes. Following feeding regime improvement, 3.1 g/L muconate was produced from guaiacol in 24 hours (96% molar yield) [115].

4.2.4 Corynebacterium glutamicum as a host

Although not reported to be a lignin depolymerising bacterium, Corynebacterium glutamicum is able to degrade a range of aromatic compounds, reviewed by Shen et al. [116], including phenol, benzoate, 4-hydroxybenzoate, gentisate, protocatechuic acid, ferulate, benzyl alcohol, 2,4-dihydroxybenzoate, resorcinol and naphthalene. The history of the application of Corynebacterium glutamicum has been reviewed [117], and since its isolation in 1956 by the Kyowa Hakko company [118] this strain has become a workhorse for the commercial scale production of L-glutamate and L-lysine. The strains traditionally used for industrial production were improved using classical microbiological techniques, such as chemical mutagenesis, followed by screening for improved production. In recent years researchers have attempted to improve strains using rational approaches, but despite this only the production of L-Lysine has been improved beyond the capability of the origin strain [119]. These approaches have however opened up the range of products produced by C. glutamicum, which have been reviewed by Becker et al. [120], and include compounds such a putrescine [121], isobutanol [122] and pantothenate [123].

C. glutamicum has recently been engineered for the production of cis,cis-muconic acid from aromatics derived from lignin hydrolysate [124]. This approach focused on the use of the catechol degradation pathway to utilise phenol, benzoic acid and catechol produced from hydrothermal depolymerisation of lignin, by deleting muconate cycloisomerase (catB) and constitutively expressing catechol 1,2-dioxygenase (catA) [124], and will be discussed in Section 4. The experience of using this organism on large scale will make further work on new applications easier, since methods, fermentation strategies and tools for genomic manipulation are known. This toolkit makes C. glutamicum a strong candidate for use as a host for utilisation of other lignin fragments.

4.2.5 Sphingobium sp. strain SYK-6 as a host
Sphingobium sp. SYK-6 is able to degrade several lignin-derived fragments, and its catabolic pathways for breakdown of dimeric fragments have been extensively studied by Masai and co-workers [125]. Insertional inactivation of the ligV gene has been reported [126], and recently Sphingobium sp SYK-6 has been engineered to convert S and G lignin monomers into cis,cis-muconate via deletion of catB and pcaHG genes, and expression of protocatechuate decarboxylase pdc gene [127]. Varman et al have reported 13C-metabolic flux analysis of this strain, providing insights into the metabolic behaviour of this organism using different carbon sources, which will aid the development of this strain [128].

4.2.6 Escherichia coli as a host

Escherichia coli K12 is a common host for biotransformation and metabolic engineering, for which multiple genetic tools are available, however, E. coli K12 has no activity for degradation of polymeric lignin [16,18], and only possesses one aromatic degradation pathway, the 3-phenylpropionic acid catabolic pathway [129]. Wu et al have reported the engineering of E. coli with an autoregulatory system containing a vanillin inducible promoter, linked to a CouP coumarate transporter of low molecular weight aromatic compounds, and genes to convert vanillin to catechol [130]. They have also reported the conversion of vanillin and syringate into cis,cis-muconic acid and pyrogallol by engineered E. coli strains [131]. These approaches could be used to convert low molecular weight aromatic compounds arising from chemical lignin depolymerisation [131], but bioconversion of polymeric lignin would require lignin depolymerisation enzymes, uptake of oxidised lignin fragments, and conversion to monocyclic aromatics, which are not fully understood at present.

5. Formation of bioproducts from lignin bioconversion using engineered microbes

This section will discuss examples from the literature where engineered microbes have been used to produce chemical products from lignin bioconversion. We will first introduce different lignin-containing feedstocks that have been used for lignin bioconversion, since the literature studies use different feedstocks, and the method used to prepare the lignin has a major effect on its properties and reactivity. We will then discuss different types of products formed: firstly
aromatic products, then products derived from aromatic ring cleavage, then products arising from microbial metabolism via the citric acid cycle.

5.1 Types of lignin-containing feedstock for microbial conversion

**Lignocellulose biomass.** Some studies utilise native lignocellulose biomass that has been milled. Lignocellulose typically contains 15-30% lignin content, and the lignin structure will be the same as that found in the plant. Lignin valorisation routes using chemocatalysis have often been found to work more effectively on lignocellulose or chemically stabilised lignocellulose, leading to the proposal of the “lignin-first biorefinery” model where lignin is valorised before cellulose and hemicellulose [132]. Grasses such as wheat straw, miscanthus or sugarcane can be easily milled, hence this material is convenient to use as a feedstock on laboratory scale, and many bacteria can utilise the sugar content of lignocellulose for growth on minimal media.

**Pretreated lignocellulose.** Alkaline pretreatment of corn stover [133] or switchgrass [134] generates a partly depolymerised aqueous lignin stream and a solid residue containing cellulose, that shows dramatic changes in cell wall architecture, but only minor changes in cellulose crystallinity [133,134]. The composition of the alkali-pretreated lignin consists mainly of p-hydroxycinnamic acids (p-coumaric acid, ferulic acid) that are liberated from hemicellulose and lignin, and lignin oligomers (500-1000 Da) [135]. The alkali-pretreated lignin can be utilised efficiently by *Pseudomonas putida* KT2440, which consumes the p-hydroxycinnamic acids and approximately 30% of the oligomeric fraction [135]. This material has proved to be an effective feedstock for product formation, as described below, but is quite different to polymeric lignin present in biomass. Ammonia treatment of corn stover, followed by dilute NaOH treatment, also generates a solubilised lignin fraction, containing p-hydroxycinnamic amides [136]. Alkaline treatment of biomass followed by acidification to pH <7 generates what is known as “soda lignin” or alkali lignin (see Figure 2), which is commercially available from wheat straw/sarkanda bagasse (Green Value SA, Orbe, Switzerland), and this material has been characterised as an SGH lignin of M₇ 3270 g/mol [137].

**Solvent-extracted lignin preparations.** Treatment of lignocellulose with alcohol solvents such as ethanol, in the presence of mild acid, leads to the extraction of “organosolv lignin”
Organosolv lignins are high purity, with low amounts of carbohydrates and ash, but contain variable amounts of β-O-4 content [137], and generally exhibit low water solubility. Lignin can also be extracted from lignocellulose by ionic liquids such as 1-ethyl-3-methylimidazolium acetate [139] and protic ionic liquids [140].

**Industrial lignins.** The pulp and paper industry uses either Kraft or sulfite pulping of softwood. The Kraft process uses alkaline sodium sulfide [141], which leads to a condensed lignin structure containing low β-O-4 content and carbon-sulfur bonds [137,141,142]. The sulfite process uses a mixture of sulfites and bisulfites, which leads to a low molecular weight lignosulfonate that has a modified structure [143], and has high water solubility. Although these industrial lignins are available at large scale, and their use would be advantageous in terms of life cycle analysis, they are generally found to be hard to valorise, due to their condensed structure.

There are few studies that compare the conversion of different types of lignins by different types of lignin valorisation process, but a study by Lancefield et al of the conversion of 7 different lignins by two chemocatalytic and three biocatalytic methods found that best conversion by both chemocatalytic and biocatalytic methods was observed using lignins containing high β-O-4 content, and lowest with Kraft lignin containing low β-O-4 content, hence the proportion of β-O-4 linkages present in the lignin appears to be one important determinant in the suitability of lignins for bioconversion [144].

Figure 2. Scheme showing different types of lignin preparations.

5.2 Aromatic products from lignin bioconversion
Since lignin is the most abundant source of renewable aromatic carbon in the biosphere, the formation of aromatic bioproducts has been a major target in lignin valorisation. Using wild-type lignin-degrading bacteria, aromatic metabolites such as vanillic acid can be detected in small amounts [16], but since these microbes are also aromatic degraders, the observed aromatic compounds do not accumulate to high levels. A further complication in microbial bioconversions of lignin is that some lignin preparations contain small amounts of low molecular weight aromatic compounds, so careful control studies are needed in order to demonstrate that any aromatic metabolites observed are due to microbial activity, and are not present due simply to the lignin raw material.

The first application of engineered microbes was the formation of vanillin, a high value chemical used in the food & flavouring industry. Following the identification of genes involved in vanillin metabolism in *Rhodococcus jostii* RHA1 [145], a Δvdh gene deletion mutant was constructed, in which the vanillin dehydrogenase (vdh) gene was deleted [102]. When grown in minimal media containing wheat straw lignocellulose, this mutant was found to generate 96 mg/L vanillin after 7 days (see Figure 3), together with smaller amounts of 4-hydroxybenzaldehyde and ferulic acid [102]. Control studies established that vanillin was formed from lignin rather than hemi-cellulose, and that ferulic acid was converted in *R. jostii* RHA1 into vanillic acid, via β-oxidation, rather than via vanillin [102]. Subsequent testing of the Δvdh *R. jostii* mutant with different lignins as feedstocks showed that syringaldehyde could be formed from two organosolv hardwood lignins, in titres of 243 and 95 mg/L [144].

Aromatic dicarboxylic acids are of considerable interest as bioproducts from lignin, as possible replacements for terephthalic acid (benzene-1,4-dicarboxylic acid) used in the synthesis of polyester plastics. Mycroft et al published in 2015 that 2,4-pyridinedicarboxylic (2,4-PDCA) and 2,5-pyridinedicarboxylic acid (2,5-PDCA) could be formed by *R. jostii* RHA containing copies of the *ligAB* or *praA* genes inserted on an inducible expression vector [146], as shown in Figure 3. Growth on minimal media containing wheat straw lignocellulose over 7 days led to the formation of 90-125 mg/L 2,4-PDCA or 79-106 mg/L 2,5-PDCA, with the ammonia cyclisation probably a non-enzymatic reaction with ammonium chloride present in M9 media [146]. Growth on minimal media containing Kraft lignin led to the formation of 53 mg/L 2,4-PDCA [146], indicating that *R. jostii* RHA1 can metabolise Kraft lignin to some extent. Spence et al have recently reported that deletion of the pcaGH genes encoding the first step
of the β-ketoadipate pathway enhances production of 2,4-PDCA and 2,5-PDCA, and chromosomal integration of the ligAB genes in place of pcaGH, and co-expression of Amycolatopsis sp. dypC, gives a production strain able to generate 330 mg/L 2,4-PDCA in a 40 hr bioconversion [147]. Johnson et al have reported that 2,5-pyridinedicarboxylic acid (referred to as isocinchomeronic acid) can be formed in an engineered Pseudomonas putida KT2440 strain in which protocatechuic acid is generated from glucose, via conversion of 3-dehydroshikimic acid on the shikimate pathway by 3-dehydroshikimate dehydratase (asbF gene) [148].

2-Pyrone-4,6-dicarboxylic acid (PDC) is also a possible replacement for terephthalic acid, and has also been generated from lignin degradation. Otsuka et al reported in 2006 that expression of the Sphingobium sp. SYK-6 ligABC genes in Pseudomonas putida led to the production of PDC from protocatechuic acid in a titre of 10 g/L [149]. Qian et al extended this approach by expressing in addition in P. putida the vanAB and ligV genes responsible for conversion of vanillin and vanillic acid into protocatechuic acid, and this construct was found to efficiently convert vanillin or vanillic acid into PDC [150]. Perez et al have engineered Novosphingobium aromaticivorans, an organism which contains the pathways to convert degraded G and S units into PDC, to accumulate PDC by deletion of ligI and desCD genes [151]. The ΔligIΔdesCD gene deletion mutant was able to convert protocatechuic acid into 2.3 mM PDC, and to efficiently convert other G and S aromatics into PDC, and the strain was able to convert chemically depolymerised poplar lignin into 0.49 mM PDC [151]. PDC has been converted into polyesters via condensation polymerisation with ethane-1,2-diol, propane-1,3-diol, or bis(2-hydroxyethyl)terephthalate, and their properties compared with those of PET plastic [152].

Ferulic acid and p-coumaric acid are both found in the structures of lignin and hemicellulose in grasses. Williamson et al have shown recently that deletion of the ech gene responsible for ferulic acid metabolism in Pseudomonas putida KT2440 leads to the accumulation of ferulic acid and p-coumaric acid when grown on media containing wheat straw or sugarcane bagasse, or a commercially available soda lignin [94]. Chromosomal insertion of the padC gene encoding ferulic acid decarboxylase then leads to the production of aroma chemical 4-vinylguaiacol at 62 mg/L [94], as shown in Figure 3. Tramontina et al have shown that ferulic acid can be converted efficiently into coniferyl alcohol using carboxylic acid reductase (CAR) and aldo-keto reductase (AKR) either using purified enzymes
or via a whole cell biotransformation [153]. Treatment of wheat straw with ferulate esterase XynZ, followed by a whole cell biotransformation with *E. coli* containing overexpressed NiCAR-CgAKR-1 produced 71 mg/L coniferyl alcohol and 58 mg/L 4-hydroxycinnamyl alcohol [153].

![Diagram of lignin degradation and aromatic product formation](image)

**Figure 3.** Aromatic products formed via recombinant microbial transformation of lignin and/or lignocellulose. Key: orange, aromatic dicarboxylic acids; green, vanillin; blue, products derived from ferulic acid.

### 5.3 Products formed via the β-keto adipate pathway and primary metabolism

Protocatechuic acid is metabolised in many soil bacteria via the β-keto adipate pathway [154], shown in Figure 4, proceeding via intradiol cleavage by protocatechuate 3,4-dioxygenase (*PcaGH*), then cyclisation to form carboxy-muconolactone. There is also a related pathway for intradiol cleavage of catechol by catechol 2,3-dioxygenase (*CatA*), followed by cyclisation to form muconolactone, which is then metabolised via the same pathway, via β-keto adipate, to the tricarboxylic acid (TCA) cycle. Since protocatechuic acid is a central
intermediate in microbial lignin breakdown, these pathways could be exploited to generate high-value products from lignin breakdown.

Vardon et al reported in 2015 that muconic acid could be formed in an engineered strain of *Pseudomonas putida* KT2440 in which the *pcaGH* genes were replaced by the *Enterobacter cloacae* *aroY* gene encoding protocatechuate decarboxylase, which converts protocatechuate to catechol [155]. Expression of the *catA* gene was controlled by the inducible *tac* promoter, while the downstream *catBC* genes were deleted [155]. This strain was able to generate cis,cis-muconic acid at 13.5 g/L in a fed-batch bioreactor using p-coumaric acid as carbon source, and 0.7 g/L using alkaline-pretreated lignin (from corn stover) over 24 hr [155]. The cis,cis-muconic acid product could then be chemically converted to adipic acid via catalytic hydrogenation [155]. Johnson et al then reported two further modifications: co-expression of protocatechuate decarboxylase gene *aroY* with genes *ecdBD* that assist in cofactor assembly for this enzyme; and co-expression of 3-dehydroshikimate decarboxylase gene *asbF* which allows the formation of protocatechuate from glucose via the shikimate pathway (see Figure 4) [156]. The re-engineered strain formed 15.6 g/L muconic acid from p-coumaric acid, and 4.9 g/L from glucose [156]. The same group reported in 2017 that, when grown in the presence of glucose, carbon catabolite repression in *Pseudomonas putida* KT2440 causes accumulation of 4-hydroxybenzoate and vanillate, via regulator protein Crc [157]. Deletion of the *crc* gene removed this bottleneck, and led to significant enhancements in muconate production from p-coumarate or ferulate [157]. These improvements were then combined and tested in different bioreactor process formats, and in a constant fed-batch process with high pH feed, a titre of 49.7 g/L muconate was obtained from p-coumarate, and 3.7 g/L muconate from base-catalysed depolymerised corn stover lignin [158]. Kohlstedt et al have reported an alternative engineered *Pseudomonas putida* KT2440 strain in which both native catechol 1,2-dioxygenases, encoded by *catA1* and *catA2* genes, were overexpressed under the control of the *P_{cat}* promoter, which provided enhanced resistance to catechol toxicity [159]. This strain gave 62.4 g/L muconate from catechol in a fed-batch process, and a further engineered strain containing overexpressed phenol hydroxylase generated 13 g/L muconate from hydrothermally depolymerised softwood [159].

Other bacterial strains have also been engineered to produce cis,cis-muconate, using related strategies. *Corynebacterium glutamicum* has been engineered via deletion of the *catB* gene, and overexpression of the *catA* gene using a constitutive promoter, and the resulting
strain gave 85 g/L muconate from catechol in a fed-batch process, and 1.8 g/L muconate from hydrothermally depolymerised softwood [124]. *Amycolatopsis* sp ATCC 39116 has also been engineered via deletion of two muconate cycloisomerase genes [115]. The mutant strain was found to generate 3.1 g/L muconate from guaiacol, and could generate 1.8 mM muconate from hydrothermally depolymerised softwood [115]. *Sphingobium* sp SYK-6 has been engineered to grow on S lignin monomers but convert G lignin monomers into muconate, via deletion of *catB* and *pcaGH* genes, and expression of protocatechuic decarboxylase *pdc* gene [127]. This engineered strain was able to convert vanillic acid to muconate in 45% molar yield, and generate 0.03 g/L muconate from a chemically depolymerised birch lignin [127].

The bioproduct cis,cis-muconic acid can be chemically hydrogenated to form adipic acid, the bulk chemical used for nylon synthesis [155,159], however, Rorrer et al have shown that biologically derived cis,cis-muconic acid can be converted via chemical polymerisation into different types of polymers bearing different levels of unsaturation, and into fiberglass panels [160].

Later intermediates on the β-ketoadipate pathway have also been generated in engineered strains. A Δ*pcaJ* gene deletion mutant of *P. putida* KT2440 was able to convert protocatechuic acid to β-ketoadipic acid, and a Δ*pcaD* gene deletion mutant converted protocatechuic acid to muconolactone, even though β-ketoadipate enol-lactone is substrate for PcaD [161]. Using *P.putida* KT2440 strains engineered to grow on glucose via conversion of 3-dehydroshikimate to protocatechuic acid via gene *asbF*, a Δ*pcaJ* gene deletion mutant was able to generate 41 g/L β-ketoadipic acid, and a Δ*pcaD* gene deletion mutant 24 g/L muconolactone [148].

Products have also been generated from primary metabolism via the β-ketoadipate pathway. *P. putida* KT2440 accumulates medium chain polyhydroxyalkanoate (PHA) biopolymesters from primary metabolism under nitrogen-limiting conditions, and was shown by Linger et al to convert p-coumaric acid to 0.15-0.17 g/L PHAs, and to convert corn stover alkaline pretreated lignin (APL) into 0.25 g/L PHAs [162]. Genetic modification of *P. putida* KT2440 has been reported to improve PHA production: deletion of PHA depolymerase *phaZ* and β-oxidation *fadBA* genes, and overexpression of genes involved in PHA biosynthesis [163]. The engineered strain was found to convert p-coumaric acid to 0.95 g/L PHAs, and to convert corn stover alkaline pretreated lignin (APL) into 0.12 g/L PHAs [163]. *Cupriavidus basilensis* B-
8 has also been reported to convert Kraft lignin into 0.31 g/L PHAs via fed-batch process, without pretreatment of the lignin [164].

*Rhodococcus opacus* DSM1069 and PD630 accumulate triglyceride lipids from primary metabolism under nitrogen-limiting conditions, and are reported to convert ultrasonicated ethanol organosolv lignin to 20 mg/L triglyceride lipids after a 9 day fermentation [23]. These strains can also convert corn stover alkali pretreated lignin into triglyceride lipids, with a titre of 1.3 g/L (42% dry cell weight) by *R. opacus* PD630 [165]. *Rhodococcus opacus* NRRL B-3311 can also convert AFEX corn stover lignin into microbial lipid [24]. *R. opacus* DSM1069 can also grow on media containing Kraft lignin after oxygen pretreatment, generating 67 mg/L triglyceride lipids [166]. Co-culture of *R. opacus* PD630 with a *Rhodococcus jostii ΔvanA* gene deletion mutant was found to enhance the conversion of corn stover alkali lignin into microbial lipid product [167].

Medium chain alkene products have been generated in an engineered strain of *Acinetobacter bayli* ADP1, adapted to grow on ferulate, which is then metabolised by the β-ketoacid pathway [168]. Expression of thioesterase gene *tesA* and fatty acid decarboxylase gene *undA* gave a recombinant strain able to convert glucose to 0.7 mg/L 1-undecene, and ferulate to 0.07 mg/L 1-undecene [168]. The titres of the bioproducts mentioned in Section 5 are summarised in Table 2.
Figure 4. Products generated via β-ketoadipate pathway and primary metabolism. Carboxylic acids, orange; lipids from primary metabolism, green. Orange arrow indicates route from p-coumarate or alkali pretreated lignin; blue arrow indicates route from glucose via the shikimate pathway.

<table>
<thead>
<tr>
<th>Bioproduct</th>
<th>Production strain</th>
<th>Polymeric lignin feedstock</th>
<th>Monomer feedstock</th>
<th>Titre (mg/L)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic products</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanillin</td>
<td><em>R. jostii</em> Δvdh</td>
<td>Wheat straw</td>
<td>Organosolv lignin</td>
<td>96</td>
<td>102</td>
</tr>
<tr>
<td>Syringaldehyde</td>
<td><em>R. jostii</em> Δvdh</td>
<td>Wheat straw</td>
<td>Wheat straw</td>
<td>243</td>
<td>144</td>
</tr>
<tr>
<td>2,4-Pyridine</td>
<td><em>R. jostii</em> p(ligAB)</td>
<td>Wheat straw</td>
<td>Wheat straw</td>
<td>125</td>
<td>146</td>
</tr>
<tr>
<td>dicarboxylic acid</td>
<td><em>R. jostii</em> pcaHG::ligAB p(dypC)</td>
<td>Wheat straw</td>
<td>Wheat straw</td>
<td>330</td>
<td>147</td>
</tr>
<tr>
<td>2,5-Pyridine</td>
<td><em>R. jostii</em> p(praA)</td>
<td>Kraft lignin</td>
<td></td>
<td>106</td>
<td>146</td>
</tr>
<tr>
<td>dicarboxylic acid</td>
<td><em>R. jostii</em> p(praA)</td>
<td></td>
<td></td>
<td>53</td>
<td>146</td>
</tr>
<tr>
<td>2-Pyrene-4,6-</td>
<td><em>P. putida</em> CJ599</td>
<td>glucose</td>
<td>protocatechuate</td>
<td>60</td>
<td>148</td>
</tr>
<tr>
<td>dicarboxylic acid</td>
<td><em>P. putida</em> p(ligABC)</td>
<td></td>
<td></td>
<td>10,000</td>
<td>149</td>
</tr>
<tr>
<td><em>N. aromaticivorans</em></td>
<td>Δlig::desCD</td>
<td></td>
<td></td>
<td>90</td>
<td>151</td>
</tr>
<tr>
<td>4-Vinylguaiacol</td>
<td><em>P. putida</em> ech::padC</td>
<td></td>
<td></td>
<td>62</td>
<td>94</td>
</tr>
<tr>
<td>Coniferyl alcohol</td>
<td><em>E. coli</em> p(NicAR-CgAKR)</td>
<td></td>
<td></td>
<td>71</td>
<td>153</td>
</tr>
<tr>
<td>Polymeric lipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyhydroxybutrate</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1-Undecene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-aromatic products</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis,cis-muconic acid</td>
<td><em>P. putida</em> pcaGH::aroY ΔcatBC</td>
<td>AP lignin</td>
<td>p-coumarate</td>
<td>700</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td><em>P. putida</em> CJ184</td>
<td></td>
<td>glucose</td>
<td>13,500</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4,900</td>
<td>156</td>
</tr>
</tbody>
</table>
Table 2. Bioproduct titres from polymeric lignin and aromatic feedstocks. Key: AP lignin, alkali pretreated lignin; BCD, base-catalysed depolymerisation; HD, hydrothermal depolymerisation; CD, chemically depolymerised; p(x) indicates that gene x was overexpressed on an expression plasmid.

6. Prospects & challenges

Choice of chassis organism. The conversion of lignin to value-added chemicals by microbial biotransformation is now an active area of research, and this article has reviewed several chemical products that have been formed by engineered microbes. Unlike metabolic engineering for biosynthesis of natural products, the well-established chassis organisms for synthetic biology such as Escherichia coli and Saccharomyces cerevisiae are not ideally suited to lignin metabolism, since they are not lignin degraders, nor are they aromatic degraders. Microbes used currently are those which show some activity for lignin breakdown and possess aromatic degradation capability, which can then be engineered to produce target compounds.

Currently, Pseudomonas putida KT2440 has been used for many studies: its genetic tools for metabolic engineering are well established, and a metabolic model is available for this organism. It metabolises p-coumaric acid and alkaline pretreated lignin well [162], but although it can degrade polymeric lignin [18], there are indications that it preferentially uses monomeric & oligomeric lignin fragments [18, 135]. For valorisation of polymeric lignin or industrial lignins, there is interest in other host organisms. Rhodococcus jostii RHA1 and Rhodococcus opacus can metabolise high molecular weight lignin [23,24,102,144,146], and there are several reports of rhodococci being able to utilise industrial Kraft lignin [146,166], but such biotransformations typically take 5-7 days. Hence it may be valuable to develop
some of the organisms mentioned in Table 1 as potential hosts for lignin degradation, although that will in some cases require the development of new genetic tools. A recent survey of genomes of bacterial lignin degraders has identified potential lignin-oxidising enzymes and aromatic degradation gene clusters present in these organisms: there appear to be diverse strategies for lignin oxidation used across different organisms, and although the β-ketoadipate pathway is the prevalent central pathway for aromatic degradation, other aromatic degradation pathways are present, such as the 4-hydroxyphenylacetate catabolic pathway (hpc gene cluster) and homogentisate catabolic pathway (hmg gene cluster), whose involvement in lignin degradation is yet to be confirmed [27].

The arrival of CRISPR/Cas9 has been a boon to the field of genome engineering, providing a counter selection marker, genotypic screen and method to increase the rate of recombination all in one tool [169]. The other powerful aspect of this strategy is the versatility of applying it to use in a wide range of different organisms [170]. Development of CRISPR/Cas9 does require basic underpinning tools, such as transformation methods, and plasmids with inducible expression systems, but has been implemented in industrially relevant bacteria such as Clostridium acetobutylicum [171] and Corynebacterium glutamicum [172]. CRISPR interference technology also offers the possibility of being able to implement more sophisticated control of gene expression, including regulation that results in phenotypic change. In strains where an essential pathway competes with the desired biosynthetic pathway, CRISPR interference could initiate a shift from a growth phase to a biocatalytic phase [172]. However, in strains for which CRISPR interference is not possible, other genetic manipulation tools are available, as described in Sections 4 and 5.

There is potential to use combined biotransformations, co-cultures, or microbial consortia. In the natural environment, microbial consortia are used in soil, and there are reports of consortia active for lignin degradation [173]. However, if there is a target bioproduct, it would be necessary to ensure that the target compound is not degraded further by members of the consortia. Polymeric lignin is heterogeneous, so there could be advantages in using bacteria adapted to utilise different structures present in lignin. There might also be advantages in using an organism able to degrade polymeric lignin, followed by an organism engineered to funnel oxidised lignin fragments to a target compound. One example of a combined fungal/bacterial treatment has been reported by Salvachúa et al, in which the secretome of Pleurotus eryngii was used to depolymerise polymeric lignin, and addition of Pseudomonas
*P. putida* KT2440 was found to enhance lignin breakdown by catabolism of low molecular weight lignin [174]. There are also indications that some facultative anaerobic bacterial strains can degrade lignin under anaerobic or microaerobic conditions [32,37], whose precise mechanism is yet to be understood, but may in future allow lignin degradation under anaerobic conditions.

Among fungi, *Phanerochaete chrysosporium* has the potential to be engineered for product generation from lignin, due to its high lignin degradation ability, and having had multiple endogeneous and heterologous peroxidases expressed [60]. However, further genetic tools such as plasmids for CRISPR/Cas9-based gene knockout would need to be developed for use in this species if more extensive metabolic engineering is to be carried out. On the other hand, there are fungal species such as *Schizophyllum commune* and the oleaginous yeast *Yarrowia lipolytica* for which multiple genetic tools are already available [62,63,69,70] that could potentially be developed as lignin degradation hosts. *Yarrowia lipolytica* further benefits from being unicellular, therefore having a shorter and simpler life cycle, and from having a genome-scale metabolic model available [175]. Although *Y. lipolytica* preferentially uses glucose as carbon source, engineering efforts have already afforded strains that can degrade more complex substrates, including sugarcane bagasse hydrolysate, wheat straw hydrolysate, and molasses [176]. Work towards improving the utilisation of lignin-derived phenolic compounds in this species has been initiated through the expression of laccases from white-rot basidiomycetes [66], hence it can be envisaged that heterologous expression of a wider range of lignin-degrading enzymes in *Y. lipolytica* will be possible.

**Choice of lignin feedstock.** Most studies use monomeric aromatic compounds as proof of principle for validation of metabolic engineering studies, and only some studies then test against polymeric lignin feedstocks. A number of studies use chemically depolymerised lignin as feedstock: alkaline pretreated lignin functions well with *P. putida* KT2440, but consists mainly of p-hydroxycinnamic acids cleaved from lignin and hemi-cellulose [135,162]; material from hydrothermal chemical depolymerisation has also been used, which is mainly monomeric substituted phenols and guaiacols [115,124]. While such feedstocks are derived from biomass, there is a question of whether the chemical depolymerisation step would be feasible on a large scale, and whether the overall process from biomass would be commercially feasible for industrial scale-up. Conversely, the use of lignocellulosic biomass as
feedstock leads to insoluble solid residue in the bioreactor, which is undesirable for large scale bioconversion. Industrial Kraft lignin, although readily available and a by-product of an industrial process, has a condensed structure which is generally hard to convert via chemocatalysis or biocatalysis [144], however, some microbes are able to grow on Kraft lignin as sole carbon source, and some studies have used it for conversion to value-added products, albeit in lower overall titre [144,166]. As the research field moves from the laboratory to industrial scale bioconversion, the technoeconomic feasibility and life cycle analysis of routes from different feedstocks will be put to the test.

**Complexity of polymeric lignin breakdown.** In cases where polymeric lignin has been used, product titres are not as high as using aromatic carbon sources or chemically depolymerised lignin, and conversion times often 5-7 days. There are several aspects of polymeric lignin breakdown that are not fully understood: the pathways for lignin breakdown and degradation of different lignin fragments [177]; the uptake of lignin fragments; and how the process of lignin degradation is regulated. Each of these factors may limit titre from polymeric lignin.

Progress is being made in each of these areas. A 4-hydroxybenzoylformate pathway for utilisation of aryl C2 lignin fragments (which may arise from phenylcoumaran and diarylpropane lignin fragments), leading to vanillin, has been identified in *R. jostii* RHA1 [178]. An alternative pathway for protocatechuic acid metabolism to hydroxyquinol has also been identified in *R. jostii* RHA1 and *Agrobacterium* sp [179]. A protein transporter for biphenyl lignin fragment DDVA has been identified in *Sphingobium* sp SYK-6 [180]. A further transporter for protocatechuic acid in *Sphingobium* sp. SYK-6 has been identified, and overexpression of this transporter leads to a 24% increase in formation of product PDC [181]. Hence, an understanding of pathways, transporters and regulators in lignin-degrading microbes will allow more efficient metabolic engineering, and higher product titres.

In order to achieve high titres and molar conversions at high substrate loadings, there are likely to be further technical issues to solve. The solubility of the lignin raw material may be a limiting factor. The toxicity of some bioproducts may be a problem when products are generated in higher concentrations, which was a limiting factor in the generation of vanillin in *R. jostii* RHA1 [97]. Wang et al have reported that *Cupriavidus necator* is more resistant to toxic intermediates, and can be used for biotransformation of benzoic acid to cis,cis-muconic acid [182]. A copper-dependent oxidase enzyme from *Thermobifida fusca* has been reported
to reduce toxicity due to aromatic aldehydes [183]. Efforts to reduce toxicity of lignin degradation intermediates is an interesting future topic for metabolic engineering.

**Considerations for scale-up and choice of bioproduct.** As this research field moves from the laboratory to industrial scale-up, several factors will influence which bioproducts will be commercially viable to manufacture via the routes described in this article. First, is there a market for the product, and is there a premium in that market for a bio-based product? Several products mentioned here are targeted at the bioplastics market (aromatic dicarboxylic acids, adipic acid from *cis,cis*-muconic acid), where there is public and regulatory pressure around the world to recycle existing plastics more efficiently, to develop new biobased plastics, hence there are good reasons to try to develop routes from biomass to these bioproducts [184]. However, it is important to distinguish between bio-based and biodegradable plastics, and while there are many applications for which compostable plastics would benefit the environment, for other applications society does need non-compostable plastics [184]. There are also other markets (e.g. personal care products, pharmaceutical intermediates) where there might also be a premium for a bio-based product.

Second, is it feasible to make the compound at scale, at a cost that is less than the market price? The cost of the feedstock is an important starting point. Lignocellulosic biomass is relatively inexpensive, but if a pretreatment or catalytic depolymerisation step is needed, then that must be practical on a tonne scale, and without adding a large cost to the process. Aromatic chemical feedstocks are suitable for lab scale experiments, but might not be cost-effective on industrial scale. The value of the final product is also an important consideration: bioplastics are medium value products, and biofuels relatively low value products, so if there are routes to high value pharmaceutical intermediates, there would be more profit margin.

Third, one has to compare with other routes available to these products: if there are existing routes available, is there any commercial advantage in using this route, and is the new bio-based process more sustainable than existing routes? For example, in the case of vanillin, there is a market in the food industry, and a premium for a bio-based product, but there are existing routes to vanillin, from chemocatalysis. In the bioplastics field, the cost of the bioplastic will need to be comparable to the cost of the petrochemical alternative, in order to convince customers to use the bio-based product. The life cycle analysis of a new bio-based process must assess carefully the greenhouse gas emissions of the process. This has been
carried out recently for the conversion of lignin to adipic acid, concluding that this process would lead to 62–78% reduction on GHG emissions, compared with the petrochemical process [185]. Is the feedstock renewable, sustainable, and from a non-food source? One potential advantage of lignin as a feedstock is that it is generated as a side-stream from existing industrial processes (pulp/paper manufacture, biofuel production), however, industrial lignins such as Kraft lignin and lignosulfonate are generally difficult substrates for bioconversion, due to their condensed structure. If some solution to this conundrum could be found, then a process using an industrial lignin as feedstock would be advantageous.

**Conclusion.** In summary, the field of microbial conversion of lignin has accelerated rapidly in the last 10 years, to the point where engineered microbes have been used to generate a range of interesting target compounds, as described above. At the same time, research into the microbiology of lignin degradation has identified a range of possible organisms that might have potential for future metabolic engineering studies, with the goal of developing processes for industrial production of high-value chemicals from lignin breakdown.

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