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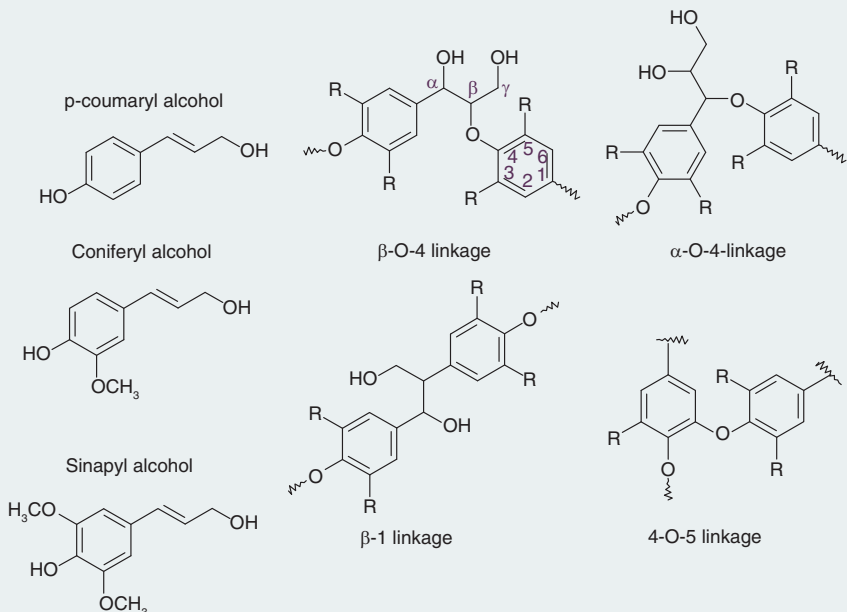
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Chapter 2

Enzymatic deconstruction and metabolism of lignin for biofuels

Mark A Blenner

Lignin is a phenolic biopolymer synthesized by plants to provide structure for plant biomass. Worldwide interest in cellulosic biofuels has created a large wastestream of lignin, which is normally burned, can potentially be converted into biofuels and other renewable chemicals. The recalcitrance of lignin to degradation is well described; however, an increasing number of microorganisms are being discovered that can depolymerize lignin and subsequently metabolize the liberated aromatic monomers. Combining these new microbial systems with recent advances in protein engineering has allowed more genetically and industrially tractable hosts the ability to produce lignin-degrading enzymes. Metabolic engineering efforts are rewiring the metabolic pathways of lignin degradation and making possible higher yields and production rates. And last, we described recent work using oleaginous microorganisms to convert lignin to biofuels and biofuel precursors. Lignin is far too cheap and abundant to be ignored as a potential source of biofuels to meet global energy demand.

Figure 2.1 Lignin structural units and heterogeneity.

p-coumaryl alcohol

Coniferyl alcohol

Sinapyl alcohol

β-O-4 linkage

α-O-4-linkage

β-1 linkage

4-O-5 linkage

Lignin is polymerized from three monomer units: p-coumaryl, coniferyl and sinapyl alcohols. The free-radical polymerization results in many β-O-4-aryl ether bonds, with varying amounts of other C-O and C-C linkages.

Introduction

Lignin is one of the most abundant biopolymers in the world. Its phenolic polymeric structure serves to provide structure for plants and protect cellulose and hemicellulose, which form the balance of plant biomass. Comprising up to 30% of biomass worldwide [1], lignin offers great potential for the production of biofuels. Lignin is already an abundant waste stream of the paper and pulp industry and will become increasingly available as a byproduct of cellulosic biofuels production. Its heterogeneous aromatic structure makes lignin degradation and conversion into biofuels extremely challenging. Degradation of lignin requires depolymerization of aromatic rings, and ring-opening reactions that form chemical building blocks (**Figure 2.1**). These reactions are common among various soil fungi, yeasts and bacteria; however, these microorganisms do not produce biofuels. With recent advances in systems biology, synthetic biology and metabolic engineering, lignin may become a viable feedstock for biofuels and other renewable chemicals. This chapter is broken into three sections: a description of the major pathways and enzymes used to depolymerize lignin, and to break the ring of

Table 2.1. Representative group of microorganisms with lignin depolymerization capabilities.

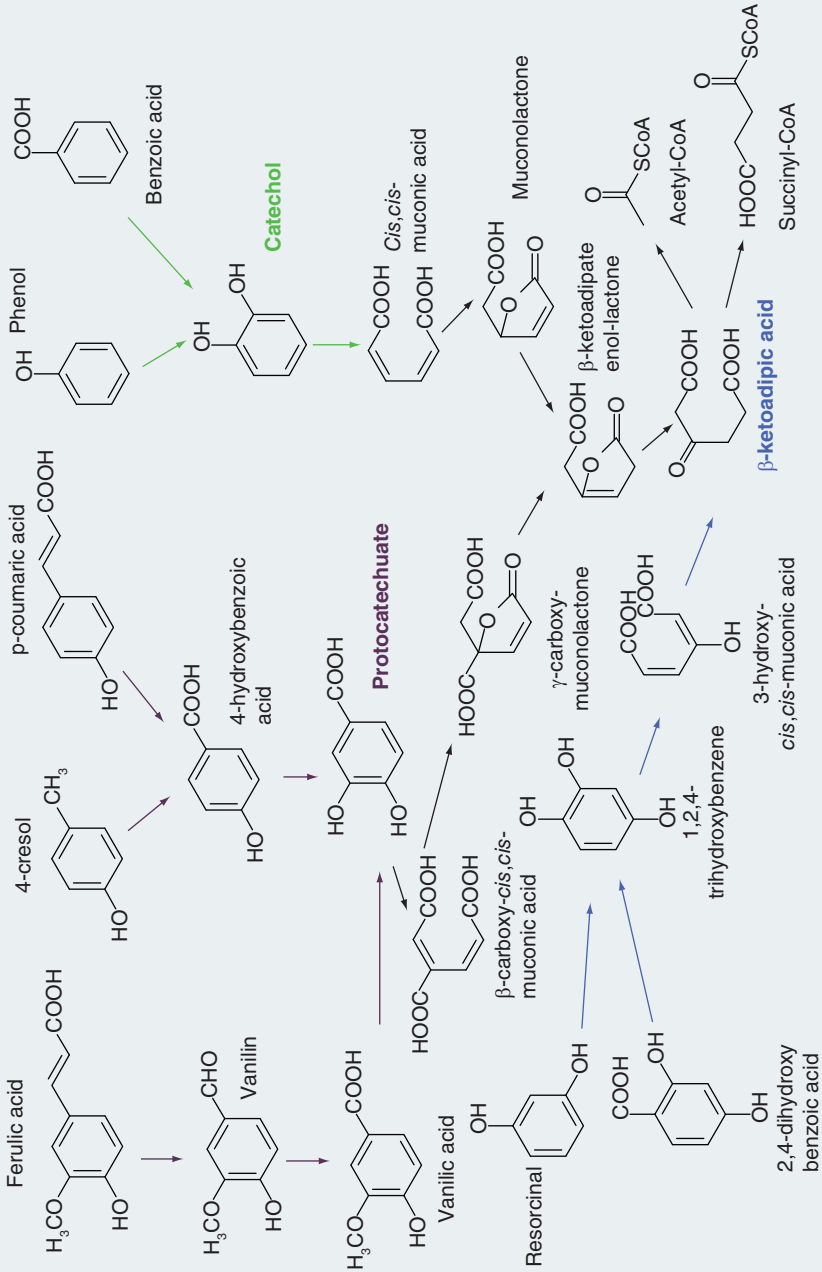| Microorganism	Type
Phanerochaete chrysosporium	Fungi
Pleurotus ostreatus	Fungi
Trametes versicolor	Fungi
Streptomyces viridosporus T7A	Gram-positive bacteria
Rhodococcus jostii RHA1	Gram-positive bacteria
Pseudomonas putida	Gram-negative bacteria

mono-aromatic depolymerized lignin to generate substrates for central metabolism, recent advances in engineering microorganisms for metabolism of lignin-like compounds, the future of lignin-derived biofuels.

Pathways for lignin degradation & metabolism

Lignin degradation has been extensively reviewed [2–5]. Here, we discuss the basic enzymes and pathways involved to suggest new directions for research. Lignin is highly recalcitrant to depolymerization; hence it is used as a structural component of plant biomass. Still, there are limited number of microorganisms that are known to depolymerize lignin. *Phanerochaete chrysosporium*, is the archetypical species of the so-called white-rot fungi. These fungi secrete three major peroxidase: lignin peroxidases (LiP, EC 1.11.1.14), manganese-dependent peroxidase (MnP, EC 1.11.1.13) and versatile peroxidase (VP, EC 1.11.1.16). Another important secreted enzyme is the multicopper phenol oxidase (Laccase, EC 1.10.3.2). These enzymes are supported by diverse oxidoreductases, which reduce methoxy radicals leading to degradation instead of repolymerization. The degradation rates of these enzymes have been reported as high as 1 g/day-g-cell; however, white-rot fungi are extremely sensitive to environmental condition and are difficult to culture, rending their use in industrial biotechnology nearly impracticable. Recently, several bacteria have been described that secrete these enzymes, but the activity of bacterial peroxidases is significantly lower than fungal peroxidases. A list of microorganisms with well-studied lignin depolymerization ability is listed in **Table 2.1**. Nevertheless, the prospect of engineering more industrialized microoganisms to express these lignin depolymerizing enzymes is increasingly enabled by modern biotechnology (described in the second section).

Depolymerization of lignin liberates a majority phenolic and minority non-phenolic aromatic compounds. Here, we focus on metabolism of the main phenolic products. As microorganisms have evolved to utilize the diverse

Figure 2.2 Pathways for aromatic ring-opening and metabolite funneling.

Diverse aromatic substrate are metabolized through metabolite funneling through protocatechuate (purple), catechol (green) or directly to β-ketoadipate (blue).
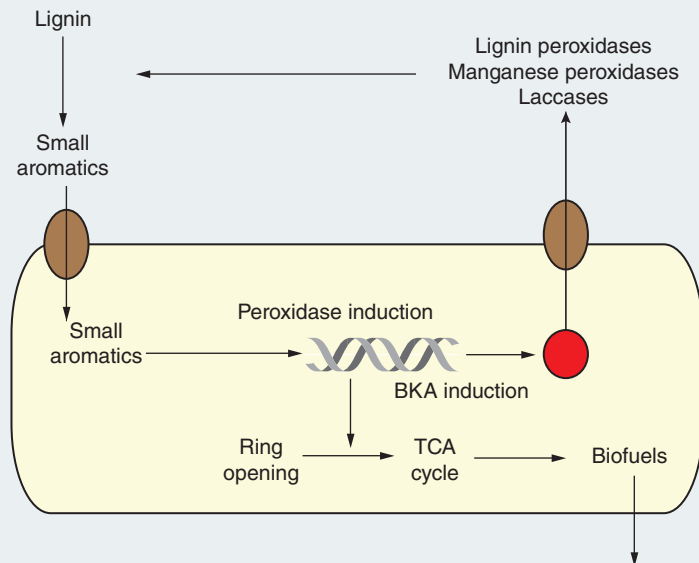
phenolic products of lignin, promiscuous enzymes are able to convert lignin products to central metabolites through by creating a small set of intermediates. This process of converting a diverse feedstock to just a few species that can easily enter a catabolic pathway is known as metabolic funneling. As phenolic compounds are oxidized, they are funneled into protocatechuate, catechol or 1,2,4-trihydroxybenzene (**Figure 2.2**). These intermediates are then catabolized to form β-ketoadipic acid through intradiol ring cleavage also known as the ortho cleavage. Finally, β-ketoacdipic acid is activated with acetyl CoA and converted into tricarboxylic acid cycle metabolites, succinyl-CoA and acetyl-CoA. The β-ketoadipate pathway has been studied extensively in Gram-negative bacteria, and to a lesser extent in Gram-positive bacteria [6]. Additionally, the white-rot fungi also have well studied β-ketoadipate pathways. A list of microorganisms with well-studied β-ketoadipate pathways is shown in **Table 2.2**. The archetypical microorganism for ortho ring-opening catabolism is the Gram-negative bacterium *Pseudomonas putida*. Since the β-ketoadipate pathway is quite prevalent in different microorganisms, it suggests great diversity is available for protein and metabolic engineering to convert lignin into biofuels.

Advances in engineering microorganisms for metabolism of lignin-like compounds

This chapter focuses largely on the most recent advances in biotechnology related to conversion of lignin into biofuels. A full picture of the lignin to biofuels process might involve the depolymerization of lignin, transport into the cell, catabolism into central metabolites and anabolism into biofuels (**Figure 2.3**). We focus on four topical areas: identification of enzymes and microbes, protein engineering, tolerance & transport, metabolic

Table 2.2. Representative group of microorganisms with well-characterized β-ketoadipate pathways.

Microorganism	Type
Pseudomonas putida	Gram-negative bacteria
Acinetobacter	Gram-negative bacteria
Corynebacterium glutamicum	Gram-positive bacteria
Streptomyces sp.	Gram-positive bacteria
Rhodococcus opacus	Gram-positive bacteria
Phanerochaete chrysosporium	Fungi
Rhodotorula sp.	Fungi (yeast)
Candida tropicalis	Fungi (yeast)

Figure 2.3 Lignin biofuels concept.

Biomass is treated to separate the lignin from cellulose and hemicellulose. Lignin is then depolymerized by peroxidases and metabolized through the β-ketoadipate pathway. Transcription of these enzymes is regulated by the presence of lignin substrates. The central metabolites are then engineered to produce biofuel.

engineering. This is not meant to be an exhaustive review of the recent literature. Instead, this section is meant to highlight technological advances that enable research in lignin degradation.

Identification of new enzymes & microbes

Despite the abundance of well-studied and characterized lignin depolymerization and metabolizing enzymes, there is a still an urgent need to accelerate the discovery of ligninases, laccases and oxygenases that utilize lignin compounds as substrates. Traditional enrichment studies employing classical microbiology and biochemistry was used to discover a strain of *Comamonas* sp. B-9 that could degrade kraft lignin into small aromatic compounds [7]. The advent of massively paralleled DNA sequencing has been an enabling tool for genomic and metagenomic analysis of lignin-degrading microorganisms. Next-gen sequencing and proteomics of *Amycolatopsis* 75iv2 quickly identified a major secreted lignin degrading enzyme that is a bifunctional peroxidase-catalase [8]. Proteomics approaches can also be used to identify pathways of enzymes involved in aromatic metabolism simultaneously, supporting genomics-based predictions. The induction of catabolic enzymes was

confirmed *Pseudomonas putida* KT 2440 using MS [9]. These new biochemical tools will find further application in identifying and rapidly characterizing novel sources of lignin degradation enzymes.
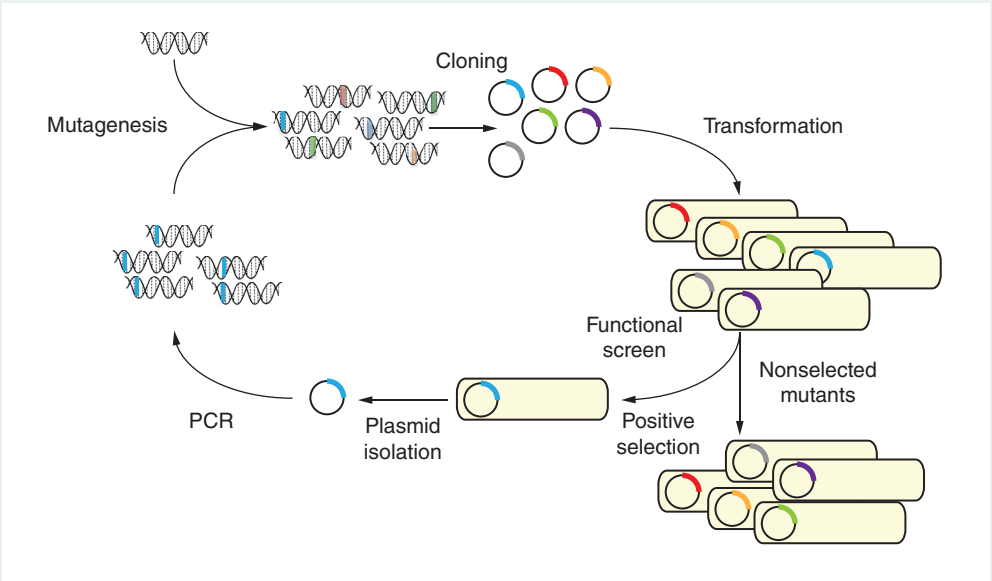
Protein engineering

Even though nature provides an incredible amount of biodiversity from which novel and interesting biocatalysts can be obtained, these enzymes often do not have properties compatible with industrial application. Intrinsic catalytic and biogenesis limitations hinder broader use of these enzymes at industrial scale. Therefore, protein engineering techniques are often employed to improve the secretion level, specific activity, substrate specificity, solubility, pH optimum or solvent-specific activity. Without changing the actual protein sequence, these enzymes are often expressed in heterologous hosts, such as *Escherichia coli* or *Saccharomyces cerevisiae*. The goal is to improve the costs of enzyme production by using cheap, rapid growing and industrial hosts. Recent advances in the production of lignin peroxidase and laccases in heterologous hosts includes expression of *P. chrysosporium* LiP H2 in *Pichia pastoris* [10], and laccase in *Yarrowia lipolytica* [11] – both yeasts. In both cases, the yield and specific activity of the peroxidases were comparable to *P. chrysosporium*, but with the added benefit of industrial scalability.

Rational and computational protein engineering have their appeal, however, the still incomplete understanding of protein structure-function relationships leaves protein engineers with few options for improving enzyme function – the most successful of which is known as directed evolution. Directed evolution enables engineering of protein properties (e.g., substrate specificity, specific activity and optimal pH), without extensive biochemical or structural characterization. The key elements of directed evolution are repetitions of creating genetic diversity and functional screening or selection for improved properties. A schematic of the process is showing in **Figure 2.4**.

Genetic diversity is introduced by several means and on various scales. Random mutagenesis through the entire genome can be accomplished through treatment with radiation or by chemical mutagen N-methyl-N′-nitro-N-nitrosoguanidine (NMG), either throughout the entire genome, or to a specific gene through more directed mutagenesis including error-prone PCR, and ligation of randomized synthetic oligonucleotides. Methods for generating diversity are widely standard now. The technically challenging part of directed evolution lies in the functional selection of improvement of enzyme activity. If an appropriate colorimetric screen is available, then individual microbial clones (containing individual mutations) can be screened in high-throughput 96-well plate format. The availability of such an assay has become more common recently, including screens for fungal laccase

Figure 2.4 Directed evolution.

Protein engineering using directed evolution mimics Darwinian evolution. Random mutations are created and screened or selected for functional improvements. The process is repeated until the desired functional improvement is achieved.

activity [12], and general lignin degradation [13]. Even better than a high-throughput screen is a selection, where the survival of the microorganism hosting the mutant enzyme is directly linked to survival. Unfortunately, selections are more rare than screens, and are more technically difficult to implement. Growth of microorganisms on lignin as the sole carbon source is a reasonable selection method, however, it has not yet been applied in the context of directed evolution.

Directed evolution has been used to improve the activity of laccases for over a decade, for a variety of purposes. Ionic liquids are used in biomass pretreatment and to solubilize lignin; however laccase activity is severely compromised in these solvents. Heterologous expression in *S. cerevisiae* of a *T. versicolor* laccase was performed resulting in a nearly fivefold improvement in ionic liquid activity [14]. In another study, versatile peroxidases from *Pleurotus eryngii* were heterologously expressed in *S. cerevisiae* and subjected to six rounds of directed evolution, leading to mutations that achieved the highest level of secretion reported for fungal lignolytic peroxidases to date (21 mg/l) [15]. The catalytic efficiency was maintained despite

the introduction of four mutations, and the overall peroxidase stability was also improved.

Tolerance/transport

The use of natural or engineered microorganisms for lignin bioconversion into biofuels requires strains that are aromatic and solvent tolerant. We consider here the challenge of engineering greater tolerance to growth on and in the presence of aromatic substrates. A related but distinctly different challenge that is also address through protein engineering is the issue of transporting aromatic metabolites into the cell for ring opening reactions involved in the β-ketoadipate pathway.

The issue of solvent tolerance has primarily been addressed through standard microbiological isolation from highly aromatic environments, such as wastewater streams, and effluents from paper mills. Natural aromatic metabolizing microbes have evolved tolerance to these compounds, however, most industrial organisms are not as tolerant. *Saccharomyces cerevisiae* was grown in medium containing phenol (and other growth inhibitors) and the cells rapidly developed tolerance [16]. Overexpression of laccase also confers phenol tolerance to *S. cerevisiae* [17]. This result suggests heterologous expression of lignin degrading enzymes might be an effective strategy for simultaneously engineering lignin degradation and tolerance.

An often overlooked but essential step of lignin metabolism is the import of small aromatics into cells for ring opening reactions, and therefore the transporters involved are poorly elucidated. ATP-binding cassette (ABC) transporters are integral membrane proteins that recognize molecules through a solute-binding subunit. Benzoic acid like molecule transporters have been identified in *Rhodococcus jostii* [18], homogentisic acid transporters in *P. putida* [19] and broadly specific aromatic transporters in *Rhodopseudomonas palustris* [20]. Structural studies of these transporters have provided several high-resolution X-ray crystal structures that might lead to rational protein engineering of transporter for improved import rates and altered specificity.

Metabolic engineering

After lignin is depolymerized and catabolized to central metabolites, it must then be converted to a biofuel. The possibility of engineering synthetic pathways to make advanced biofuels such as fatty acid ethyl esters has been successful in *E. coli* and butanol production has been successful in *E. coli*, and *Clostridium*. However, the feedstock for these processes is typically glucose. No studies have addressed the production of biofuels using lignin feedstocks. What has been of growing interest are microorganisms that metabolize lignin-like compounds and produce fatty acids in the form of

triacylglycerides (TAGs). These oleaginous microorganisms can accumulate large amounts of TAG that are precursors to transesterified biodiesel, and alkane fuels. Another facet of metabolic engineering for is the design of promoters to control gene expression. This section focuses on advances in aromatic metabolism in oleaginous bacteria and yeast, and the characterization of aromatic-induced transcription regulation.

Oleaginous microorganisms are defined by their ability to synthesize over 20% w/w of their mass as storage lipids in the form of TAGs. The intersection of oleaginous bacteria and aromatic degrading bacteria is small, with *Rhodococcus opacus*, *Acinetobacter baylyi* and *Streptomyces coelicolor* being of great interest due to the existence of molecular cloning techniques. *Rhodococcus opacus* has long been appreciated as a oleaginous as well as containing the β-ketoadipate pathway. Kosa and Ragauska recently extended the study of *R. opacus* aromatic degradation and TAG accumulation to lignin model compounds, including 4-hydroxybenzoic acid, vanillic acid and syringic acid. Unfortunately, the yield coefficient ($Y_{lipid/substrate}$) is quite low still (<10%); however, after significant metabolic engineering, better yields might make this approach more viable [21].

The recent interest in microbial lipid production for biofuels, alkanes and other chemicals has rekindled studies of oleaginous yeasts. Among these yeasts, *Candida tropicalis* has been the most well studied. However, due to biosafety concerns raised in the European Union, research on the industrialization of *C. tropicalis* has waned. Instead, researchers have been focused on *Yarrowia lipolytica* – also an oleaginous yeast that was previously classified as Candida lipolytica, and has been designated by the US Food & Drug Administration as Generally Recognized As Safe (GRAS). *C. tropicalis* and *Y. lipolytica* have robust metabolism for hydrophobic substrates, including alkanes and neutral lipids. *C. tropicalis* has been widely reported as a phenol degrader. With a focus on wastewater treatment, *C. tropicalis* has been reported to grow on phenol as the sole carbon source at concentrations of 2.4 g/l with substrate consumption nearly complete after just 16 h [22,23]. Interestingly, the same work described a strain of *Y. lipolytica* capable of similar growth on phenol with delayed growth kinetics, even though *Y. lipolytica* has generally not been considered a phenol degrader. Earlier work identified a strain of *Y. lipolytica* capable of degrading phenol, 4-chlorophenol, catechol and benzoic acid [24].

Metabolic engineering efforts rely on the ability to genetically modify microorganisms in order to regulate gene transcription. Overproduction of enzymes at times when the cell does not need to consume a particular substrate is wasteful of the cellular resources and is known to lead to loss of yield and productivity. Therefore, creating a toolkit of regulatory genes that can coordinate and induce gene expression at the appropriate time

is paramount for efficient bioprocesses. In order to prevent unnecessary gene induction by nondegradable or nonpreferred aromatic compounds, gene-regulating proteins often work by sensing the pathway metabolites rather than substrates, as was observed for *S. macrogolitabida* [25]. Much of the transcriptional regulation of aromatic compound metabolism is based on histidine kinase two component systems, such as the TodS regulator of *P. putida* [26].

The future outlook for lignin-derived biofuels

While there are clearly many challenges remaining to make lignin a more viable feedstock for biofuels production, the vast quantity and availability of this waste is too tempting to ignore. Advances in protein engineering, synthetic biology and metabolic engineering are sure to begin making a more significant impact in engineering microbial systems capable of lignin depolymerization, catabolism and synthesis of biofuels. Based on the vast amount of engineering required to add the β-ketoadipate pathway into a microbial host that does not naturally have one, advances in lignin biofuels will likely come from a known aromatic degrader, such as *R. opacus* or *C. tropicalis*. The effort required to make lignin biofuels using these microorganisms will require the addition of lignin degrading peroxidases, improving transport rates and tolerance to aromatic compounds, metabolic engineering to improve overall product yield and production rate and improved tools for regulating gene expression to enable advanced metabolic engineering strategies. Lignin degrading peroxidases from *P. chrysosporium* are currently the most active of this class of enzymes. Since heterologous expression of these enzymes in bacteria has had little success, it suggests that a yeast host will be required; however, as researchers continue to discover and engineer improvements to bacterial LiPs, this may make bacterial processes more viable. The abundance of genetic engineering tools in bacterial systems makes them more attractive for protein and metabolic engineering. There is great interest in developing new genetic engineering tools for eukaryotic systems and this technology will be enabling for yeast-based lignin biofuels processes.

In principle, the production of lignin based biofuels can benefit from biofuel production pathways established using glucose as the feedstock, since both the β-ketoadipate pathway and glycolysis lead to the same central metabolites. For example, an *E. coli* strain has been engineered to produce ethanol aerobically, fatty acids and their esterification into fatty acid ethyl ester biodiesel. Such consolidated bioprocessing removes the need for intermediate purification and reduces the process costs. Furthermore, new gene regulation tools will be instrumental in a lignin biofuels process. The enzymes for lignin degradation are complex and require energy intensive

prosthetic groups. The induction of these enzymes will need to be carefully regulated to prevent toxicity associated with enzyme overexpression. Such autoinduction strategies have been shown repeatedly to produce improvements in both the yield and productivity of engineered metabolic pathways.

Continued interest in cellulosic biofuels will undoubtedly result in an abundance of cheap waste lignin that could be converted into biofuel, chemicals and materials. Those interested in lignin biofuels would benefit greatly from advances in protein and metabolic engineering. Synergy between engineers, biochemists and microbiologists will be critical for future developments in this field.

Financial & competing interests disclosure

Summary

- Lignin will be a cheap abundant feedstock for biofuels production.
- Depolymerized lignin heterogeneity can be accommodated by a bioprocess.
- The β-ketoadipate pathway is a funneling pathway for aromatic substrates.
- Solvent and substrate tolerance is a critical barrier.
- Protein engineering, metabolic engineering and synthetic biology will be instrumental in turning lignin into a viable biofuels feedstock.
- There is great potential for engineering oleaginous microorganisms to metabolize lignin.

Key terms

Lignin:	a structural phenolic biopolymer comprising between 20 and 30% of all biomass.
Metabolic engineering:	the intentional altering of natural cellular biochemical pathways to alleviate bottlenecks and improve the production of a product.
Synthetic biology:	the re-imagination of biochemical systems to meet a desired outcome.
β-ketoadipate pathway:	a funneling pathway used by cells to convert diverse carbon substrates into common metabolites, acetyl-CoA and succinyl-CoA.

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