Perspective on Lignin Conversion Strategies that Enable Next Generation Biorefineries

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Abstract

The valorization of lignin, a currently underutilized component of lignocellulosic biomass, has attracted attention to promote a stable and circular bioeconomy. Successful approaches including thermochemical, biological, and catalytic lignin depolymerization have been demonstrated, enabling opportunities for lignino-refineries and lignocellulosic biorefineries. Although significant progress in lignin valorization has been made, this review describes unexplored opportunities in chemical and biological routes for lignin depolymerization and thereby contributes to economically and environmentally sustainable lignin-utilizing biorefineries. This review also highlights the integration of chemical and biological lignin depolymerization and identifies research gaps while also recommending future directions for scaling processes to establish a lignino-chemical industry.

Keywords

Lignin valorization, chemical depolymerization, computational biology, microbial consortia, extremophiles

Introduction

Lignin, accounting for 15-35% by weight of the dry lignocellulosic biomass, is a complex aromatic heteropolymer composed of three aromatic units; *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), which are derived from phenylpropanoids - *p*-coumaryl, coniferyl, and sinapyl alcohols, respectively.[1,2] These units are cross-linked together by a variety of chemically stable C-O and C-C bonds including β-aryl ether (β-O-4), phenylcoumaran (β-5), biphenyl (5-5'), resinol (β-β), and diaryl ether (4-O-5) (Figure 1). The composition of lignin varies depending on the plant and growing conditions, among other factors, resulting in unique and non-identical polymeric structures.

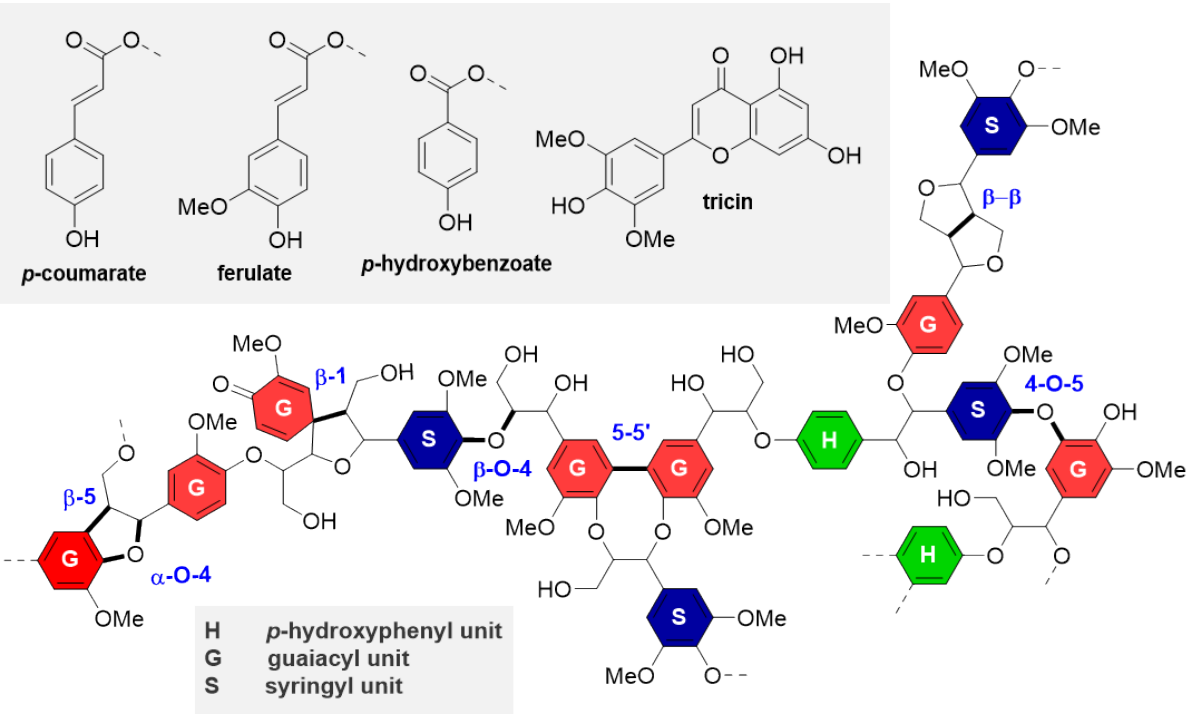

Figure 1. Representative structure of lignin that includes various known units and interunit linkages in a range of grassy and woody lignocellulosic biomass. These units and interunit linkages are neither shown in proportions nor expected to all be present together in native lignin for any given lignocellulosic biomass.

Large quantities of lignin are generated in different environments as a component of agricultural and forestry residues, municipal solid waste, animal waste, and as a by-product from paper and pulping processes and lignocellulosic biorefineries. Agricultural

waste generates 225 million tons of lignin per year while paper and pulp mills produce 50-70 million tons of lignin annually.[3,4] The second-generation cellulosic ethanol biofuel plants are also projected to produce 62 million tons of waste lignin.[5] The high carbon/oxygen ratio and the aromatic skeleton of lignin render it a sustainable alternative to petrochemical-based aromatic feedstocks. Lignin can be used to produce adhesives, resins, lubricants, textiles, fertilizers, and fuels, and thus address the energy and environmental concerns associated with products sourced from petrochemicals.[6–12] Despite its widespread availability and high potential for industrial applications, lignin is still underutilized due to its inherent recalcitrance and structural heterogeneity. Currently, 98% of the lignin isolated, predominantly from the paper and pulp industry, is used in heat generation while only 1-2% is used for commercial applications.[3] This has led to focused research on lignin utilization through (bio)chemical processes.[11,13,14]

Due to the environmental impacts of fossil fuel-based approaches and increasing energy demand, biorefineries are gaining attention as a solution for decarbonizing our economy.[15–19] To improve the cost-effectiveness, carbon efficiency, and energy efficiency of lignocellulosic biorefineries, all its components including lignin need to be utilized.[20] Several lignin depolymerization methods including thermal, microwave-assisted, chemical, and biological, among others, are known and are performed at various technology readiness levels and scales.[6,21–24] Each method has unique advantages and disadvantages. For example, energy-intensive thermal depolymerization (including gasification and pyrolysis) results in mixtures of liquid (phenolics, guaiacols, acetone, methanol), gaseous (CO_2, CH_4, H_2, C_2H_4), and solid (biochar, recondensed lignin) products. On the other hand, cost-effective and high specificity biological depolymerization methods suffer from slower kinetics and poor efficiency given the complexity of the polymeric lignin. While lignin depolymerization and valorization using chemical and biological approaches have been studied and demonstrated,[9,23,25–31] there is still a need to develop efficient, cost-effective, and environmentally sustainable chemical and biological depolymerization and valorization methods guided by the life-cycle assessment (LCA) and techno-economic assessment (TEA) studies for commercialization of such processes.[32–38]

As discussed above, excellent reviews focused on existing lignin depolymerization technologies have been compiled in the last decade.[3,6,9,23,30,39,40] Nevertheless, there is still a lack of discussion on steps required to integrate chemical and biological approaches with a focus on addressing research gaps and challenges of upscaling lignino-chemical industries while being cost-competitive to existing petrochemical industries. This review encompasses both chemical and biological lignin depolymerization with a focus on providing current perspectives, highlighting the research gaps, and recommending future directions. This review first presents the chemical building blocks of lignin and chemo-catalytic depolymerization approaches. Next, biological (fungal, bacterial, and enzymatic) lignin depolymerization and valorization of lignin-derived monomers by aerobic and anaerobic fungi and bacteria with a focus on host engineering are also discussed. Furthermore, the review also emphasizes employing extremophiles, designing synthetic microbial consortia, and using computational biology as future research directions to improve lignin conversion. Finally, we provide perspectives from process engineering to highlight the issues related to the translation of the bench scale research.

Chemical Pathways for Lignin Depolymerization to Enable Bioconversion

The chemical valorization of lignin, as mentioned earlier, is an important step and opportunity to increase the global market share of renewable chemicals. Especially, in the case of biorefineries, lignin valorization and utilization will serve as an additional carbon-feedstock closing the carbon-loop while also reducing the carbon-rich waste stream that is otherwise disposed of or burned. However, the variation in lignin along with dense reticulated and crosslinked aromatic structure sometimes requires severe chemical depolymerization conditions that result in the generation of a mixture of product streams, complicating separation and purification processes.[21,39] The design of cost- and energy-efficient and environmentally benign chemical depolymerization processes (e.g., generating uniform or bioavailable product stream) will enhance the feasibility of such lignino-biorefineries.

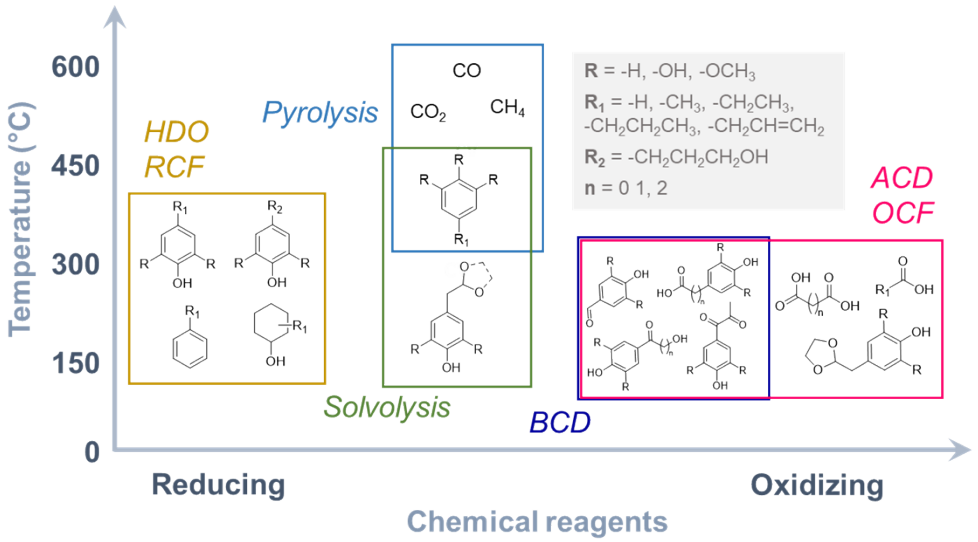

Figure 2. Typical products obtained after chemical depolymerization of lignin. HDO is hydrodeoxygenation, RCF is reductive catalytic fractionation, BCD is base-catalyzed depolymerization, ACD is acid-catalyzed depolymerization, and OCF is oxidative catalytic fractionation.

Different technologies to chemically depolymerize lignin can be grouped as solvolysis, pyrolysis, and catalytic depolymerization including hydrogenolysis, hydrodeoxygenation, acid-catalyzed depolymerization (ACD), base-catalyzed depolymerization (BCD), and oxidation (Figure 2).[9,25,39,41] As shown in Figure 2, the product-type and the corresponding yields are highly dependent on the nature of the reagents and the severity of the process. For instance, the use of aqueous acidic or alkaline catalysts cleaves the dominant ether linkages producing aromatic molecules. The presence of an oxidant such as hydrogen peroxide under such conditions often results in the further breakdown of these aromatics into aliphatic acids or even CO_2. The use of (heterogeneous) catalysts in the presence of reducing or oxidizing agents has the advantages of higher efficiency and product selectivity even at moderate reaction conditions. The use of high temperature-based techniques under neutral or inert conditions, including pyrolysis or solvolysis, have been often performed for maximizing solubility and/or conversion of lignin. Nevertheless, this leads to the formation of a complex mixture of solid, liquid, and

gaseous products. Each depolymerization methodology offers a unique suite of advantages and disadvantages and has been discussed in detail elsewhere.[22,29,39,42–46]

From the viewpoint of a biorefinery, facilitating the bioconversion of a chemically depolymerized stream would be beneficial by a) producing a mixture of products that can be biologically funneled,[47] b) eliminating the need for energy-intensive and tedious separations, and c) generating additional revenue streams. However, major products obtained from the technologically mature processes with higher conversions including: hydrodeoxygenation, hydrogenolysis, or pyrolysis often result in a product profile (e.g., phenols, guaiacols, aromatic hydrocarbons, etc.) with either limited or negligible biocompatibility. This necessitates the development of processes such as BCD or ACD that produce biocompatible products including aromatic and aliphatic carboxylic acids. However, the yields of aromatic acids produced via BCD are still low, therefore optimization to enhance the yields is necessary. Aside from optimizing the existing chemical routes, novel routes to generate highly biocompatible streams should be explored and demonstrated. It has already been demonstrated that integrating chemical depolymerization of lignin followed by bioconversion has great prospects in bio- and lignino-refineries. While the integrated processes pave their path – upstream chemical depolymerization processes, especially catalytic depolymerization that usually employs precious/rare metals such as ruthenium (Ru), palladium (Pd), or rhenium (Re),[9,48–50] should be designed for scalability and cost-compatibility by focusing on the development of efficient, robust, and reusable catalysts including earth-abundant metals such as iron (Fe), nickel (Ni), or copper (Cu). The key challenges in the design of earth-abundant metal catalysts include control of reactivity patterns and lattice environments for long-term stability and high active-site density.

Biological Pathways for Lignin Depolymerization and Catabolism of Lignin-derived Monomers

The biological lignin conversion routes have been extensively explored in terms of lignolytic microbes (namely fungi and bacteria), their metabolic pathways, and enzyme libraries. The most commonly employed biological pathway for the conversion of lignin-derived chemicals is aromatic catabolism. In the following sections, we discuss the

available biological tools and methodologies to not only improve the high rates of aromatic catabolism, but also the incorporated pathways to convert aromatics to chemicals and biomaterials unlocking the full potential of an aromatic biopolymer i.e., lignin (Figure 3).

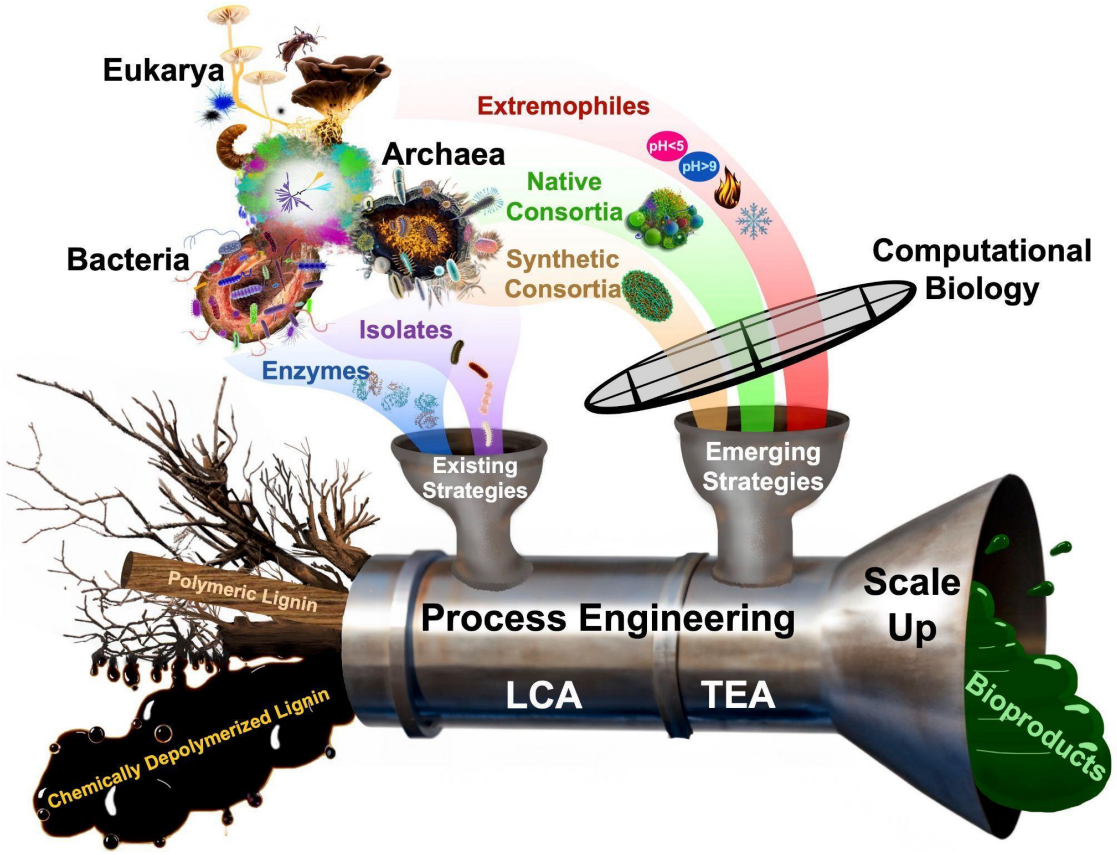

Figure 3. Schematic representation of existing and emerging biological approaches for lignin conversion to high-value bioproducts guided by process engineering, life cycle assessment, and techno-economic analysis to enable next-generation biorefineries.

Fungal lignin depolymerization

Lignin polymers are naturally degraded by many known fungal species. White-rot species, in particular, have been shown to depolymerize lignin. Characterized phenotypically by the white appearance of the decaying wood they grow on, white-rot fungi are able to fully depolymerize lignin through numerous physical and enzymatic mechanisms. The most abundant lignin-degrading enzymes within these species are peroxidases and laccases.[30,51] These are nonspecific enzymes that are secreted from fungi to extracellularly break down lignin and can produce aromatic monomers.[52] Low molecular weight compounds are similarly excreted to aid in lignin breakdown.[30] In some white-rot

species, lignin-derived aromatic monomers can then be taken up and are intracellularly catabolized.[53] Most of the mechanistic knowledge of lignin depolymerization mediated by white-rot fungi is generated through genomics studies and other uncharacterized enzymes likely play a role in depolymerization.[54,55] While genetic engineering is possible and forward genetic screens using random mutagenesis and phenotype screening have been performed,[56,57] these approaches have yet to be widely deployed in these species.[58] As such, the most explored applications of white-rot fungi for lignocellulosic biomass treatment[59] and bioremediation[60] (not the focus of this review) take advantage of white-rot fungal species' innate abilities where genetic modification could improve efficiency but is not required to perform the function.

Another group of lignin-interacting fungi being explored is found in the digestive tracts of herbivores. Unlike white-rot fungi, gut microbes are primarily anaerobic, allowing for the possibility of anaerobic conversion of lignocellulosic biomass for bioproduction.[61] In part due to a lack of a good model system and also the lack of methods for determining the products of lignin depolymerization, lignin depolymerization mechanisms by gut microbes are also largely studied through multi-omics approaches that associate transcriptomic and proteomic differences to bulk changes in overall lignin composition.[62–64]

Many of the more genetically tractable lignin-degrading fungi have been identified in lignin-rich environmental isolates.[65,66] Within filamentous fungi, species of *Aspergillus* have long been studied for their ability to consume lignin-derived aromatics.[67,68] While many of these species are studied similarly to white-rot and anaerobic fungi through multi-omics approaches, *Aspergillus niger* has emerged as a model system that can be developed for bioproduction purposes.[69] Similarly, yeast species have been identified that natively produce laccases and peroxidases or that can catabolize lignin monomers.[70] Many oleaginous yeasts, in particular, have been found to catabolize lignin-derived aromatics.[71–73] *Yarrowia lipolytica*[74–76] and *Rhodosporidium toruloides*[77,78] are both being pursued as genetically tractable bioproduction hosts due to their ability to grow in inhibitor-rich feedstocks and their abilities to break down or catabolize aromatic compounds, including *p*-coumaric acid with 4-hydroxybenzoic acid (4-HBA) as an intermediate and ferulic acid.[77,79]

Many fungal species do not directly catabolize sugar but are able to grow in lignocellulosic-derived feedstocks despite the inhibitors that are often present.[80] As a few of these species are highly amenable to genetic engineering, they are also being explored as potential candidates for lignin depolymerization. *Pichia pastoris*, for example, has emerged as an ideal candidate for genetically engineering lignin depolymerization pathways into a model host system[81–83] due to its relative ease in engineering and its ability to secrete recombinant proteins.[84]

One of the largest challenges for addressing the fungal depolymerization of lignin is that there are so many microbes that interact with it in unique ways. Even within a single microbial species, there are multiple metabolic pathways related to lignin depolymerization. By biasing the studies to what seems the most feasible, many interesting enzymes, metabolites, and pathways with great potential get missed. Using computational and predictive tools on a wide variety of species from diverse lignin rich environments will help give an idea of the breadth of lignin degrading fungi. In addition, exploring which of these species can be cultivated within a laboratory or industrial environment can help determine what opportunities are present to discover novel metabolic pathways and enzymes.

Bacterial lignin depolymerization

Lignin depolymerization has recently been well characterized in some bacteria. There are examples of lignin-degrading enzymes such as peroxidases from the DyP family, a few MnPs, and putative LiP sequences in bacterial lignin-degrading isolates.[30,85–87] The upstream depolymerization methods produce a mixture of high and low-molecular-weight lignin species ranging from oligomers and dimers to monomers. The bacterial catabolism of high-molecular-weight lignin is not well understood. On the other hand, there are well-defined pathways in bacterial hosts such as *Pseudomonas putida* KT2440,[88] *Pseudomonas putida* M2,[89] *Rhodococcus jostii* RHA1,[90,91] *Sphingobium* sp. SYK-6,[92] *Novosphingobium aromaticivorans*,[93] *Acinetobacter baylyi* ADP1[94] for catabolism of a wide range of lignin-derived aromatic monomers and oligomers.[95]

Bacteria can biologically funnel a wide range of monomers present in depolymerized lignin via common central metabolism intermediates such as pyruvate and acetyl CoA, to a single desired product under aerobic conditions.[31,40] There have been several bacterial host engineering efforts to utilize lignin derived monomers into desirable bioproducts including: *R. opacus* for lipid production, *P. putida* for pyruvate,[96] lactic acid,[96] polyhydroxyalkanoate (PHA),[97] indigoidine,[98,99] and muconic acid[100] production, and *Corynebacterium glutamicum* for muconic acid production[101]. Among other bioproducts, the production of muconic acid has been explored extensively from lignin monomers such as *p*-coumaric acid to afford up to 13-15 g/L.[104] In most cases, bacterial lignin depolymerization ranged from 20% to 65% of monomeric product yield with feedstocks including alkaline and kraft lignin.[102,103] The yield and range of products of such lignin depolymerization methods have been studied comprehensively in recent reviews.[102,105–107] However, the current challenges are that most of these studies used only a few model aromatic monomers which do not fully represent the diversity of monomers present in a depolymerized lignin stream and also the toxicity due to the depolymerized lignin hydrolysate often results in low growth and low product titers.

Anaerobic lignin depolymerization is far less understood and unexplored compared to aerobic metabolism. Lignin depolymerization occurs in several anaerobic and anoxic environments such as marshes, swamps, mangroves, and paddy fields.[108,109] Previous studies have shown that only chemically modified lignin such as kraft lignin, soluble lignin, and lignin with a high degree of methoxylation (composed of S-structures) undergo depolymerization in anaerobic conditions but not the native lignin.[110,111] Lignin monomers such as vanillin, syringic acid, syringaldehyde, and ferulic acid are also amenable to anaerobic ring reduction and fission reactions.[108,112,113] Lignolytic enzymes (and encoding genes) involved in aromatic catabolism via an anaerobic mechanism have been identified in microbes such as *Azoarcus* sp. CIB, *Thauera aromatica*, *Rhodopseudomonas palustris, Tolumonas lignolytica* sp. Nov, *Enterobacter lignolyticus* SCF1, *Klebsiella* sp. strain BRL6-2.[113–118] Some preliminary studies that explored micro-aeration[119] as well as anaerobic microorganisms from gastrointestinal tracts of termites and herbivores that have a lignin-rich diet[120,121] seem promising and should be further investigated for anaerobic lignin depolymerization in engineered fermentative systems.

Enzyme engineering to improve activity and stability, for lignin depolymerization

Enzymatic lignin depolymerization avoids the use of harsh thermochemical processes and provides a sustainable mode of breaking down recalcitrant lignin biopolymer. There are several known lignin-degrading enzymes, such as Heme Peroxidases (Lignin peroxidase (LiP)), Manganese Peroxidase (MnP), Versatile Peroxidase (VP), Dye-decolorizing Peroxidases (DyP-type), Laccases, Lytic Polysaccharide Monooxygenases (LPMOs), and 5-Carboxyvanillate Decarboxylase (LigW), that are discussed in detail elsewhere.[122–125] Recently, advances in enzyme engineering have shown promising results related to lignin depolymerization, including rational engineering, semi-rational engineering, random mutagenesis, and directed evolution have led to improved activity and stability of enzymes as shown in Table 1. Although enzyme engineering has provided leads, most of the engineering efforts are based on commercial substrates. Thus, improving enzyme properties on actual lignin biomass is needed.

Enzymatic depolymerization becomes costlier as some enzymes get inactivated by lignin-derived aromatic compounds.[126] Microbial enzymes are also often inhibited by the solvents used to dissolve lignin[123] therefore focusing on solvents that offer a better platform for microbial enzymes would provide a better degree of depolymerization. Ionic liquids can be a good solvent to dissolve lignin, but the depolymerization efficacy of various enzymes and the engineered microbial strains need to be tested in such ionic liquids. Furthermore, large-scale cultivation using lignolytic enzymes under optimal conditions can potentially bring down the cost of lignin-derived products and provide a sustainable option to source a variety of bio-based chemicals.

Table 1. List of lignin-degrading enzymes and their engineering methods to achieve desired properties.

Organism	Enzyme	Substrate	Enzyme Engineering Technique	Improved Features	Reference
Phanerochaete chrysosporium	Lignin peroxidase isozyme H8 (LiPH8)	VA, dimeric lignin	Rational Design	12.5-fold increase in half-life at pH 2.5, 9.9-fold increased catalytic efficiency (VA), 7.8-fold enhanced lignin model dimer conversion	[127]
Ceriporiopsis subvermispora	Manganese Peroxidase (MnP6)	VA, RB5, Mn^{2+}	Directed mutagenesis	Ability to oxidize VA and RB5, 7-fold increase in activity at optimal pH	[128]
Pleurotus eryngii	Versatile Peroxidase (VPL2)	ABTS, RB5, VA, Mn^{2+}, DMP	Directed Evolution	129-fold increase in activity, improved peroxide stability	[129]
Pleurotus ostreatus	Versatile Peroxidase (VP1)	ABTS, DMP, RB5, VA, Mn^{2+}	Rational Design	Increased temperature and pH stability	[130]
Escherichia coli	Quadruple mutant myoglobin (YRW2 Mb)	Guaiacol, ABTS, GGE, RB5, RB19	Rational Design	Enhanced peroxidase activity and catalytic efficiency comparable to that of the most efficient natural enzyme	[131]
Rhodococcus jostii	Dye-decolorizing Peroxidase	Mn^{2+}	Rational Design	80-fold increase in activity for Mn(II) oxidation	[132]

	(RHA1 DyPB)				
Pseudomonas fluorescens	Dye-decolorizing Peroxidase (PfDyP)	Mn^{2+}, DCP, alkali Kraft Lignin, ABTS	Rational Design	~ 10-fold improved catalytic efficiency for 2-chlorophenol and Mn (II) oxidation; enhanced thermostability	[133]
Pseudomonas putida	Dye-decolorizing Peroxidase (MET94 PpDyP)	DMP, ABTS, various phenolic and aromatic compounds	Directed Evolution	100-fold enhanced activity towards DMP, improved H_2O_2 resistance, up-shift of pH optimum to pH 8	[134]
Vibrio cholerae	Dye-decolorizing Peroxidase (VcDyP)	RB19	Rational Design	Optimum pH for degradation of RB19 up-shifted to pH 7	[135]
Bacillus subtilis	Dye-decolorizing Peroxidase (BsDyP)	DMP	Directed Evolution	~10-fold higher activity towards DMP and improved protein yields	[136]
Pseudomonas fluorescens	Dye-decolorizing Peroxidase (Dyp1B)	Mn^{2+}, DCP, ABTS, polymeric lignin substrates	Rational Design	5-fold improved catalytic efficiency for Mn^{2+} oxidation	[122]
Saccharomyces cerevisiae	Myceliophthora thermophila Laccase (MtL)	ABTS, SGZ	Directed Evolution	22-fold increase in k_{cat}, 170-fold increase in total activity	[137]

Basidiomycete	OB-1 mutant Laccase	ABTS, DMP, Guaiacol	Directed Evolution	Functional expression and activity in S. cerevisiae	[138]
Pleurotus ostreatus	Laccase (POXA1b)	ABTS	Semi-rational mutagenesis	5-fold increase in specific activity, higher stability and activity in wide temperature and pH ranges	[139]
Escherichia coli	Copper efflux oxidase (CueO)	ABTS, DAT, DMP, other phenolic compounds	Site-directed mutagenesis	Increased redox potential by ~150 mV, 140-fold increase in catalytic activity	[140]
Pleurotus ostreatus	Laccases (1M9B, 3M7C)	ABTS, SGZ, DMP	Random mutagenesis	1.8-fold increase in specific activity, increased stability	[141]
Tinea versicolor	Laccase (LAC3)	ABTS	Synthetic biology, immobilization	Improved laccase activity, stability, and reusability	[142]
Bacillus subtilis LS03	CotA-laccase	ABTS, SGZ	Random mutagenesis, DNA shuffling	Increased catalytic activity, dye-decolorizing ability	[143]
Saccharomyces cerevisiae	OB-1 mutant High-redox potential laccase (HRPL)	ABTS, DMP, $K_4Mo(CN)_8$	Directed Evolution	32-fold increase in thermal inactivation half-life at 75°C, increased pH stability	[144]

Cirripectes polyzona	Laccase (Cplcc1)	ABTS	Heterologous expression	Increased redox potential and thermal stability	[145]
Camponotus japonicus	Lytic polysaccharide monooxygenase (CjLPMO10A)	Shrimp shell chitin	Rational Design	3-fold increase in half-life at 60°C, increased resistance to chemical denaturation, 150% increase in enzyme activity	[146]
Integrated Microbial Genomes & Microbiomes system[147]	Lytic polysaccharide monooxygenase (mgLPMO10)	Avicel PH-101, PASC	Rational Design	Functional activity at 80°C	[148]
NA	Self-assembled peptoid/hemin nanomaterials	ABTS, TMB	Peptoid-based crystalline nanomaterial construction	Mimetic function on lignin models and mediators	[149]

ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); DAT: 2,5-diaminotoluene; DCP: 2,4-dichlorophenol; DMP: 2,6-dimethoxyphenol; GGE: guaiacylglycerol-β-guaiacyl ether; PASC: phosphoric acid-swollen cellulose; SGZ: syringaldazine; TMB: 3,3’,5,5’-tetramethylbenzidine; RB5: Reactive black 5; RB19: Reactive blue 19; VA: Veratryl alcohol, NA: Not Applicable

Emerging and Future Research Opportunities to Enable Biochemical Conversion of Lignin

Viable future strategies to fully depolymerize and utilize lignin will likely be a combination of chemical and biological methods, as both have a complementary set of advantages and disadvantages in a biorefinery context. Bioconversion of the residue streams from multiple types of chemical (or even biological) depolymerization of lignin, for instance, is an attractive approach to realize a circular bioeconomy (Figure 3). We discuss below some emerging and future research directions that would enable efficient microbial conversion following upstream lignin depolymerization.

Extremophiles: Bioprospecting for Novel Lignin Metabolizing Microorganisms

For circular bioeconomy processes, it is desirable to limit the amount of feedstock pretreatment and conversion additives to reduce chemical use, cost, and time. Many target feedstocks are waste streams with chemical or physical properties beyond the range that support the growth of typical, industrial production microbes. Extremophiles are microorganisms, including fungi, bacteria, and archaea, with physiologies that enable life in extreme environments such as very high (>50 °C) or very low (<10 °C) temperatures, strongly acidic or basic, high salt concentrations, high toxin concentrations, etc. Many target feedstocks, including the chemically depolymerizedn lignin discussed above, have properties resembling extreme environments. Extremophilic microorganisms, like *Sulfolobus solfataricus, Thermus aquaticus,* and *Halomonas spp.*, have already been used and engineered successfully in several biotechnology applications as sources of stable enzymes to prepare enzyme cocktails or to produce methane and other biofuels.[150,151] With new advances in sequencing and culturing methods for diverse microorganisms and through targeted bioprospecting,[152] we have the potential to find and harness microbial strains better capable of depolymerizing and converting lignin residues.[153] These environmental microbes from extreme environments could be applied in next generation biorefineries in different capacities. One existing use would be the production of enzymes such as cellulases or laccase. Another potential extremophile role would be in a two-stage conversion where in the first stage, an extremophile would be grown to detoxify or pretreat the waste lignin biomass to make it

habitable for the production microbe in the second stage. Additionally, the extremophile may be a production microbe itself and able to produce a bioproduct from growth directly on the stream.[154] Due to microbial physiological factors like regulation, stress response pathways, cell wall architecture, and proton motive force direction, we postulate that a microbe or microbial community that natively tolerates extreme conditions and metabolizes unusual substrates will be a more robust production microbe.

Extremophilic microorganisms and their enzymes have been employed to depolymerize and convert lignin to valuable molecules.[155] However, these processes are inefficient or represent a small range of feedstocks. Therefore, obtaining and evolving microbes or microbial communities from natural environments that are chemically similar to the target stream in a lignin biorefinery, which includes both chemically and biologically depolymerized lignin, could be a better approach rather than extensively engineering a mesophilic model strain such as *E. coli, Saccharomyces cerevisiae* or *P. putida*.[156,157] Some examples of currently used microorganisms obtained from targeted lignin-degradation bioprospecting are discussed in the above sections such as wood-eating insect guts or white rot fungi. We suggest additional locations for extremophile bioprospecting to obtain novel lignin metabolizing microorganisms that will tolerate biorefinery conditions.

For lignin depolymerization, locations or environments with rapid *in situ* lignin depolymerization should be explored including tropics, rainforests, and carbon-starved environments stimulated by the presence of lignin, such as lower layers of soils or vadose zone sediments.[158,159] These samplings should include anaerobic fungi which have many unique and untapped metabolisms.[62] It is also possible that lignin depolymerization would be enhanced by consortia of microorganisms from multiple domains,[160] as discussed below. For lignin-depolymerizing microbes, biofilm-forming microbes may also have enhanced extracellular enzyme degradation rates through attachment to lignin.

For the conversion of residue streams generated from various chemical and biological lignin depolymerization approaches, extremophiles could be mined from environments that match the chemical composition of the stream. The salt, solvent content, pH, and

types of molecules vary by lignin breakdown method. For high salt solutions, halophiles should be targeted from places like oceans, the Great Salt Lake, and saltwater marshes.[151] Many species of halotolerant soil and sediment microbes could be targeted as well, but it should be considered if microbes that grow in a biofilm or attached lifestyle versus planktonic growth would be preferable for a conversion process in a biorefinery. Similarly, microbes from pH and temperature extremes should be tested in lignin residue streams as many alkaliphiles, acidophiles, thermophiles, and microbes with antifreeze properties or psychrophiles are now culturable.[153] A challenge to lignin conversion is tolerating the toxic aromatic, phenolic, or solvent content. Hydrocarbon-degrading microbes or those isolated from near oil wells, refineries, retention ponds, or chemical plants may display the desired qualities.[161] Therefore, the use of extremophiles is an emerging strategy for lignin depolymerization and conversion.

Top-down and bottom-up approaches to designing lignin-degrading microbial consortia

In natural or manmade lignocellulosic biomass-rich environments such as soil, sludge of pulp and paper mill, compost, gastrointestinal tracts of termites, and decomposing woods, microbes live in consortia and take advantage of microbial interactions where each member works synergistically to perform efficient breakdown of lignocellulose including lignin.[120,121,162,163] The use of microbial consortia has shown enhanced substrate utilization due to synergistic effects, improved enzymatic activities, and higher enzyme diversity than when a single species is used.[160,164–166] Compared to single species, microbial consortia are robust and resilient to environmental perturbations.[167] Microbial hosts have high substrate specificity, but a narrow substrate spectrum. As discussed earlier, each combination of feedstock, chemical and biological depolymerization method, and target product, will create different environmental conditions, and no single microbe even after engineering will be optimal in all such conditions. Additionally, heterologous expression of multiple catabolic pathways is possible through metabolic engineering but as the feedstock composition changes, it will require expression of different pathway combinations which can cause metabolic burden on a single organism. Consortia, on the other hand, distribute metabolic reactions among strains reducing the metabolic burden

and increasing process efficiency to achieve maximal conversion of a heterogeneous stream obtained from lignin depolymerization. [167,168]

Top-down enrichment and bottom-up synthetic consortia are the two major ways to develop a consortium with lignin-degrading properties.[168] Enrichment method such as dilution-to-stimulation is the most commonly employed strategy to develop a lignin-degrading consortium.[169–172] To date, lignin-degrading microbial consortia have been enriched from environmental samples such as soil, compost, seawater, decomposing wood, etc.[162,169–171,173] The enrichment step can be followed by isolation, identification, and metabolic characterization with multi-omics methods to elucidate its microbial composition. However, not all the members in the enriched consortia can be cultivated or genetically engineered. Also, typical enrichment methods using environmental samples still result in a complex consortium with a high diversity level making it difficult to untangle the microbial populations responsible for the conversion process. In a previous study, dilution-to-stimulation and dilution-to-extinction were combined to construct a simplified consortium with less complexity enriched with key microbial players for lignocellulose conversion from forest soil.[174] A similar approach can be used to obtain a minimal consortium selected for efficient lignin depolymerization and conversion.

It is challenging for a single monoculture system to concurrently depolymerize lignin, metabolize the diverse monomers to a high-value product, and tolerate the toxic degradation products. Designing microbial consortia such as co-culture using a bottom-up approach allows us to rationally pair complementary microbes with native or engineered metabolism, where each member performs one task as a part of the overall lignin conversion function (Figure 4). It is possible to design three different kinds of co-culture systems to improve lignocellulose or lignin depolymerization and conversion: bacteria-bacteria,[165,175,176] fungi-fungi,[177,178] and bacteria-fungi,[179–181] co-culture systems (Figure 4). Cai et al developed a coculture system of *Sphingobium* sp. and *R. opacus,* where *Sphingobium* sp. cleaved lignin-derived dimers to monomers which were further converted to *cis*, *cis*-muconate, and gallate by *R. opacus*.[175] Such consortia allow precise control of the individual function, metabolic networks, and their interactions to construct a simple, yet efficient lignin-metabolizing consortium (Figure 4).[182,183]

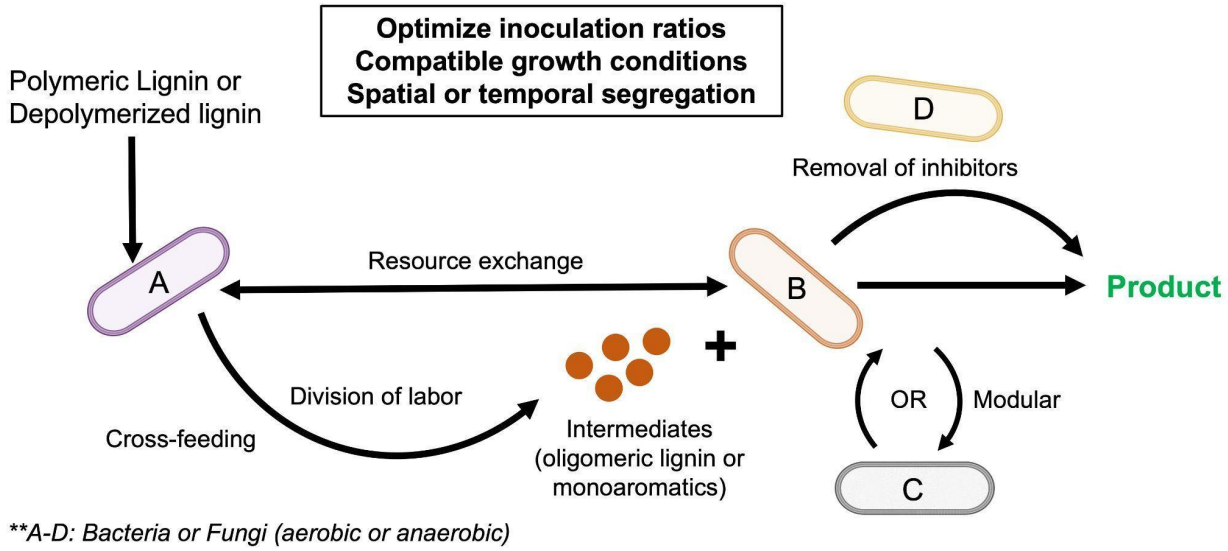

Figure 4. Designing synthetic microbial consortia for biological valorization of lignin and/or lignin-derived oligomers and monomers.

Controlling and maintaining the functional stability and productivity of a consortium remains a challenge. Instability might arise due to resource competition, differences in growth rates and conditions, or the production of toxic intermediates. In several instances, depolymerized lignin often has residual sugars and other assimilable substrates such as ionic liquid,[88] which can favor opportunistic organisms such as sugar cheaters.[184] In such case, sugar cheaters can dominate and outcompete other members and interfere with community stability. A thorough screening needs to be done to evaluate different microbial combinations and develop a strategy to avoid any competition for which the metabolism of individual strains should be thoroughly understood. However, combinatorial evaluation experiments can be laborious and costly. *In silico* models can be used to predict composition over time or simulate microbial interactions between different combinations of microbes in different environmental conditions and optimize parameters needed for the stable coexistence of the consortium members.[185–187] It is equally important to characterize growth conditions such as pH, temperature, and oxygen for each species in the consortium to control the consortia, and quite often they are different for each member. For instance, Wen et al[176] used metabolic and evolutionary engineering to overcome differences in pH between *Clostridium cellulovorans* (pH 6.4–

7.5) and *Clostridium beijerinckii* (pH 4.5–5.5) in a coculture system designed for butanol production from cellulose.[176]

Spatial and temporal segregation can be used to address physiological or metabolic incompatibilities such as differences in oxygen affinity. Several studies have shown added benefits of temporal segregation by sequential inoculation to minimize growth competition and spatial organization by utilizing a biofilm system, cell immobilization techniques (such as beads, hydrogel entrapment), microfluidics, and microwell platforms to compartmentalize microbes during lignocellulose conversion and similar strategies can be applied for lignin bioconversion.[166,179,180,182,188] For instance, a tubular membrane-aerated bioreactor was used to develop a spatial ecological niche where an oxygen gradient was formed in the biofilm. This allowed for the growth of an aerobic fungus *Trichoderma reesei* which enzymatically hydrolyzed cellulose to generate sugars while the anaerobic conditions in the bulk phase enabled the facultative bacterium *Lactobacillus pentosus* to produce lactic acid.[179]

To avoid the accumulation of inhibitory intermediates, promoting two-way mutualism through metabolite cross-feeding[189] can be a viable strategy to achieve stable coexistence. In such approach, the toxic lignin depolymerization byproduct of one consortium member serves as a carbon source for another member to generate a desired product. Furthermore, interactions between consortia members can also be engineered via signaling molecules such as quorum-sensing molecules that induce desired functional outcomes.[190–192]

There are limited studies on the use of natural or engineered consortia for lignin depolymerization and conversion, most of which are proof-of-concept, and several challenges discussed above need to be addressed before they can be scaled up. The implementation of integrated automation technology, systems biology, multi-omics methods, metabolic engineering, and quantitative and predictive computational tools will improve our ability to predict and control lignin-degrading consortia for efficient lignin valorization.

Role of Computational Biology

There have been a number of recent advances in computational tools, software, and workflows for lignin and lignocellulosic biomass valorization (Figure 5).[193] The latest genome scale metabolic model (GSMM) for the bacterium *Novosphingobium aromaticivorans*, a well-known lignin degrading bacterial chassis, was recently used to demonstrate specific enzymes that were thermodynamically favored with carbon and energy efficient pathways for ferulic acid utilization and co-metabolism of a mixture of vanillic acid, 4-HBA, and syringic acid.[194] The latest GSMMs developed include the oleaginous yeast, *R. toluroides* with desirable lignolytic properties and an extremophile *Microbacterium* strain with C1 and lignin-related aromatic compounds (4-HBA, vanillate, and 3,4-hydroxybenzoate or protocatechuate) utilization pathways.[77,195] Advances in GSMM based strain engineering for bioproduction using model aromatic substrates (e.g. *p*-coumaric acid) may also provide a starting point for more complex lignolysates.[99] Historically labeled lignin models have been used to discover lignin degrading pathways, but recently ^{13}C kinetic profiling and ^{13}C metabolic flux analysis were utilized to understand multilevel regulation during the conversion of lignin-derived aromatic compounds (4-HBA and vanillic acid) in *Comamonas testosteroni* KF-1.[196]

The latest *de novo* pathway design tool, Novostoic, was used to demonstrate the biological funneling of mono- and biaryl representative aromatics and explore the cleavage pathways of β-1 and β-β dimers, discover energy and carbon-efficient pathways with the addition of a few heterologous enzymes, and identify bottlenecks during the bioconversion process.[197] The tool was utilized for monomers including ferulic acid, *p*-coumaric acid, and hydroxypropiovanillone and dimers pinoresinol and 1,2-bis(4-hydroxy-3-methoxyphehyl)1,3-propanediol (HMPPD). Novostoic holds promise to test other non-canonical aliphatics and aromatics recovered from depolymerized lignin. Similarly, the retrobiosynthesis tools[198–203], that predict optimized *de novo* biosynthetic pathways for the production of high-value bioproducts using natural or engineered enzymes, can be harnessed in a similar fashion to explore the unknown native biological funneling of lignin-derived monomers, dimers, and oligomers.

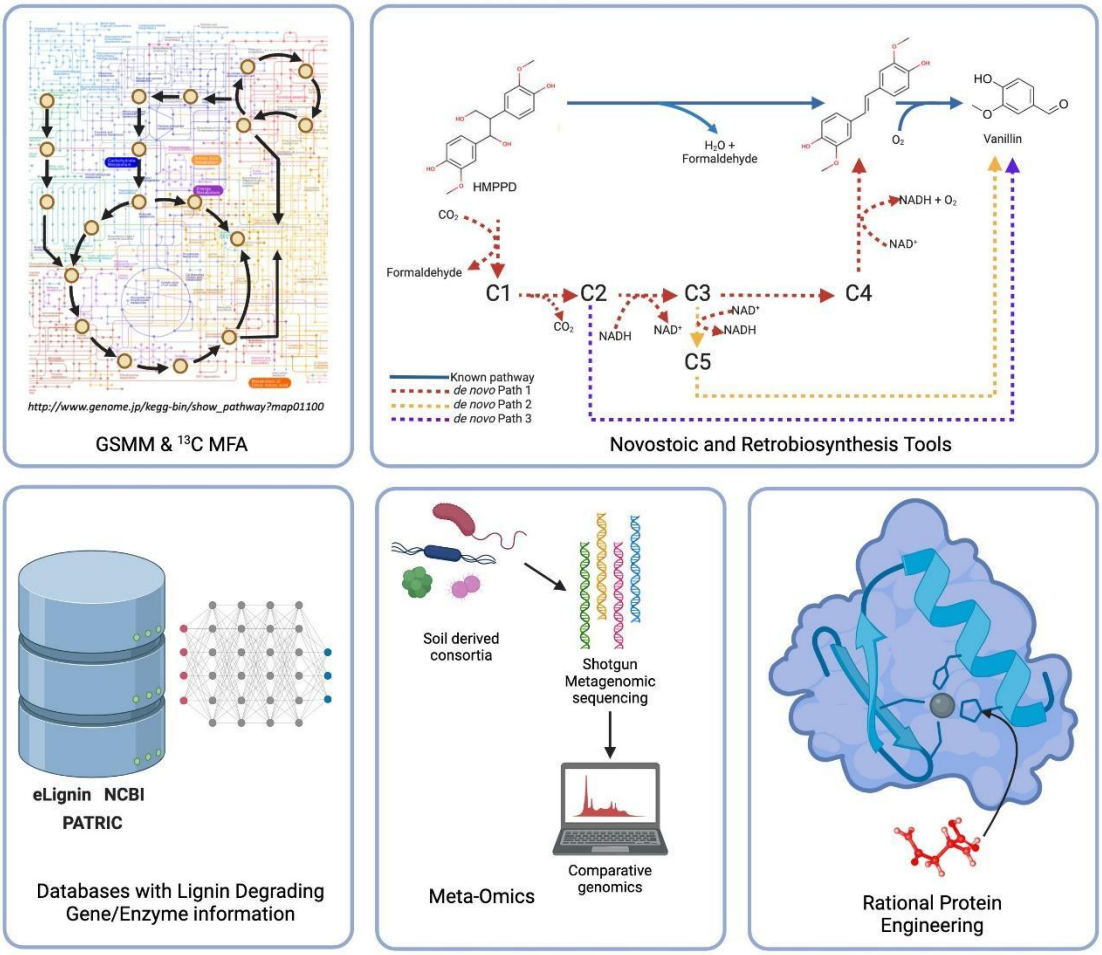

Figure 5. Advances in computational tools, software, and workflows for lignin valorization. Top left - recent application of Genome-scale metabolic modeling (GSMM) and ^{13}C metabolic flux analysis (MFA), top right - application of retrobiosynthesis tools and software like Novostoic, bottom left - databases such as the eLignin microbial database aid in bioprospecting for lignin degrading genes or enzymes, bottom middle - advances in metagenomics and metatranscriptomics using soil derived consortia to discover lignin degrading functions and properties and bottom right - computational protein engineering via molecular dynamics simulations have helped tailor enzyme binding pockets for improved function. Created with BioRender.com

Several databases might help with computational bioprospecting for pathways as well as functional lignin-degrading genes. These databases include the eLignin microbial database[204] and genome databases including NCBI[205] and PATRIC.[206] Unfortunately, such databases still lack well-annotated bacterial sequences of lignin-degrading enzymes

while the genome databases are still limited by gene annotations via annotation algorithms solely based on homology.

Strain-agnostic bioprospecting methods use metagenomics and metatranscriptomics.[207] ^{13}C-labeled lignin, coupled with shotgun metagenomics, has demonstrated that species from the *Caulobacteraceae* family are relevant microbes for not only lignin but all three lignocellulosic polymers.[162] Metagenomics has also been used to predict specific lignolytic functional roles of members of soil-derived microbial consortia[170] and predicted that *Pseudomonadaceae* have broad metabolic capacities while *Caulobacteraceae* could act on specific aromatic compounds. Integration of all meta-omics approaches into a single workflow could be an interesting step forward to discovering novel gene functions for lignin valorization.

Computational methods have also been used to improve substrate specificity for different carbon sources that are derived from lignin. Rational protein engineering via molecular dynamic simulation was used to redesign the binding pocket of a chimeric laccase to improve the oxidation of sinapic acid, an important lignin-derived phenol.[208] This tool was also employed to characterize a new promiscuous cytochrome P450 aromatic O-demethylase in apo, guaiacol-bound, and catechol-bound configurations.[209]

A big challenge associated with such computationally predicted putative sequences of key lignolytic enzymes is the functional confirmation by experimental expression and enzymatic assay, which is a bottleneck at high throughput scales. Although there has been a significant advancement in computational efforts towards utilizing aliphatic compounds as well as certain representative aromatic monomers and dimers in bacterial as well as fungal systems,[210] guaiacols/phenols, and benzoquinones still are understudied mostly owing to the associated toxicity.

Processing Challenges and Commercial-scale Feasibility

Given the heterogeneous polymeric structure of lignin, the lignin conversion process cannot be designed in an isolated context. The structure and functional groups of lignin are altered during the lignin fractionation from biomass which directly affects the bioconversion efficiency of lignin.[211] At the same time, the presence of certain

compounds generated during lignin fractionation could be toxic to microorganisms, so the biomass pretreatment and lignin fractionation methods should be chosen carefully considering the overall process. Liu et al. investigated the bioconversion of corn stover lignin for lipid production using *R. opacus* and observed that the use of combinatorial pretreatment compared to the conventional pretreatment process could result in up to 75% higher lipid concentration during fermentation.[211] Another approach to address this challenge would be careful strain screening and characterization to select robust strains to assemble a microbial consortium that can tolerate the inhibitory compounds. At the same time, designing the lignin bioconversion process considering the integrated biorefinery could provide possibilities of co-utilizing non-lignin molecules that are underutilized in conventional biorefineries (e.g., acetate and C5 sugars), enhancing the overall process efficiency.[47]

Chemical depolymerization of lignin offers fast reaction rates and a wider range of products. However, the effectiveness and feasibility of chemical conversion technologies hinge upon the design of high-performance catalysts. The inherent poor stability of many heterogeneous catalysts and the complex mechanisms underlying homogeneous catalytic reactions necessitate further investigation to successfully implement these technologies.[212] Furthermore, as previously discussed, minimal alterations to the lignin structure are necessary to achieve high product yields. However, the loss of β-O-4 linkages and lignin condensation are common occurrences during lignin separation, presenting significant challenges during lignin processing. Integrating chemical depolymerization with microbial conversion could be a sustainable approach due to its mild operating conditions, greater selectivity, and lower environmental impact, as it avoids the use of toxic chemicals. However, as most biological lignin valorization processes are at the proof-of-concept stage or demonstrated only at the laboratory scale, there is high risk involved and a high level of investment is needed to deploy these technologies at a commercial scale. Future research demands a systematic and integrated approach to using biological techniques, processing technologies, and engineering models, which will require interdisciplinary knowledge and collaborative efforts. To mitigate this scale-up risk and understand the economic viability of large-scale applications, process simulation models and TEA along with LCA are needed. Process simulation models are commonly

used to assess the economic feasibility and carbon footprint of early-stage bioprocesses. Process models for lignin bioprocessing using newly engineered microbial systems can be developed and applied to predict the performance of new microbes, understand process economics, and optimize the process conditions. In addition to determining the process economics, the process simulation models can be used as screening tools to identify the hot spots in the process, determine the minimum yield required, and provide targeted modifications (expression levels) to the microbiologists. For example, in a recent study on engineered sugarcane that metabolizes carbon to produce triacylglycerides instead of sucrose, it was concluded that although the fuel yields per unit of land were significant using this engineered crop, a minimum of 10% triacylglycerides was required in the stem to match the jet-fuel production with other oil-based feedstocks.[213] This kind of analysis can guide the research to achieve a sustainable biological system and utilization of under-utilized components in a given feedstock.[33,214]

Conclusions

The valorization of underutilized lignin is a critical step in the realization of sustainable bio- and lignino-refineries for renewable chemicals and materials. Nevertheless, the heterogeneity of lignin structure among other traits offers significant technical challenges restricting commercial applications. A significant research effort has paved the path for lignin valorization to an extent and there are still several un(der)-explored pathways. For instance, an urgent need lies in the design of an upstream chemical depolymerization process to achieve a higher titer of biocompatible and bioavailable molecules with a narrow product distribution profile. From the bioconversion perspective, it is important to engineer existing enzymatic and microbial processes to enhance the utilization of a wider variety of lignin-derived monomers and/or oligomers. Additionally, engineering of existing microbial host strains to tolerate, consume, and assimilate carbon from toxic molecules (e.g., phenols) is required. We highlight the possibility of using synthetic co-culture and microbial hosts from extreme environmental conditions (extremophiles). We also emphasize dedicating significant research to the complex lignin-derived streams rather than using model compounds to better understand the behavior and metabolic pathways of the microbial host under actual considerations. Finally, through this review, we draw

attention to the scalability issues related to the transition from laboratory-scale to industrial-scale processes.

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Conflict of Interest

BAS has a financial interest in Illium Technologies, Caribou Biofuels, and Erg Bio. All other authors declare the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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