Emerging Strategies for Modifying Lignin Chemistry to Enhance Biological Lignin Valorization

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Abstract: Biological lignin valorization represents a promising approach contributing to sustainable and economic biorefineries. The low level of valuable lignin derived products remains a major challenge hindering the implementation of microbial lignin conversion. Lignin properties play a significant role in determining the efficiency of lignin bioconversion. In this work, emerging and state-of-the-art strategies for biomass pretreatment and lignin fractionation are summarized to elaborate their roles in modifying lignin structure for bioconversion. Fermentation strategies aimed at enhancing lignin depolymerization for microbial utilization are systematically reviewed as well. To date, despite the significant progress in the development of biomass pretreatment, lignin fractionation, and fermentation over the last few decades, little efforts have gone into identifying the ideal lignin substrates for an efficient microbial metabolism. With an improved understanding of the ideal lignin structure elucidated by comprehensive metabolic pathways and/or big data analysis, modifying lignin chemistry could be more directional and effective. Ultimately, together with the progress of fermentation process optimization, biological lignin valorization will become more competitive in biorefineries.

1. Introduction

Lignin valorization is essential for achieving profitable and sustainable biorefineries based on techno-economic analyses.[1] Recently, biological conversion of lignin has begun to open alternative product pathways with significant potential, such as overcoming the heterogeneity of lignin and representing a relatively green process.[2] Similar to cellulose utilization in biorefinery,[3] biological lignin valorization generally consists of four steps: lignocellulose pretreatment/fractionation, lignin depolymerization, aromatic compound catabolism, and biosynthesis of valuable products (Figure 1).[4] Substantial efforts have been directed at improving the above four processes efficiency to enhance biological lignin valorization.[5b,6] Despite significant progress, the levels of valuable products generated from lignin substrates are still not fully satisfactory, and hence hinder the economic performance of lignin bioconversion.[6c,7]

In a fundamental sense, biological lignin valorization is the process that involves the engineering selection of lignin streams as a feedstock to produce value-added compounds, such as lipids,[4a,8] polyhydroxyalkanoate (PHA),[9] muconic acid,[10] and vanillin by microorganisms.[2a,4b] The properties of lignin substrates play a significant role in determining the efficiency of microbial conversion. Herein this minireview focuses on the modification of lignin chemistry to enhance biological lignin valorization. In detail, biomass pretreatment and lignin fractionation to modify lignin structure for bioconversion are reviewed. Meanwhile, emerging fermentation strategies that facilitate lignin depolymerization to make it more accessible to microbial cells are systematically summarized. Furthermore, the significance and potential strategies to elucidate the ideal lignin structure for microbial conversion are highlighted and discussed towards further guiding lignin bioconversion processes.

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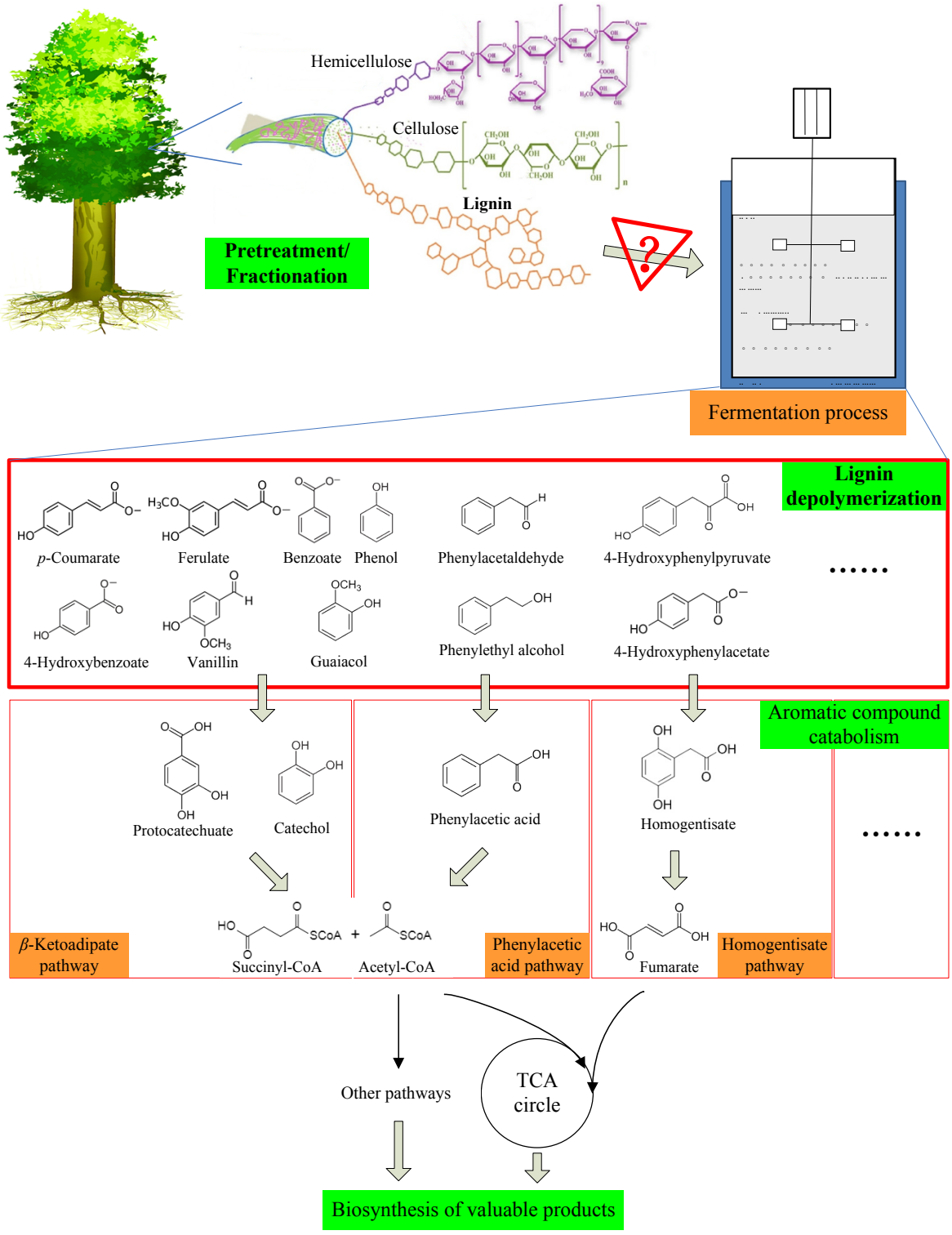

Figure 1. General schemes for biological lignin valorization.[2b,4,5] Adapted with permission from Ref. [5c].

2. Biomass pretreatment and lignin fractionation to modify lignin structure

2.1. Acid-alkali combinatorial pretreatment of lignocellulosic biomass

To facilitate lignin valorization, future biorefinery requires a pretreatment step that should not only focus on carbohydrate outputs but also lignin processability in an integrated way.[2b,11] Acid-alkali combinatorial pretreatment is an effective strategy to improve lignin utilization.[4a,12] In the first phase, dilute acid (i.e., 1 % w/v H_2SO_4) or hydrothermal pretreatment is applied to primarily remove hemicelluloses, extractives, ash, and other non-structural compositions from biomass.[12,13] In the second phase, dilute alkali (i.e., 1 % w/v NaOH) is employed to depolymerize and dissolve lignin into the liquid stream and thereby separating it from cellulose.[9b,12-14] As shown in Table 1, this combinatorial pretreatment increased glucose and xylose percent yield by 9.8% and 8.1%, respectively, compared with the single alkali pretreatment control. Meanwhile, lignin output in the liquid stream from this combinatorial pretreatment increased by 33.4%.[13] These strategies not only significantly improved the lignin output to produce a comparable level of coproducts but also synergistically enhanced the release of fermentable sugars from carbohydrates in a biorefinery.

Table 1. Comparison of glucose and xylose percent yield and fermentation performance utilizing the lignin isolated by acid-alkali combinatorial pretreatment versus single alkali pretreatment.[9b,13]

	Acid-alkali combinatorial pretreatment	Single alkali pretreatment
Glucose percent yield [%][a]	88.4±2.8	80.5±2.1
Xylose percent yield [%]	72.6±3.2	67.2±1.8
Cell dry weight [CDW, g L^{-1}]	5.3±0.1	3.5±0.5
PHA concentration [g L^{-1}]	1.0±0.2	0.30±0.05
PHA content [g g^{-1} CDW]	0.19±0.04	0.08±0.01

[a] Percent yield = $\frac{\text{Actural yield}}{\text{Theoretical yield}} \times 100\%$.

Moreover, lignin structure was altered to improve its subsequent processability through combinatorial pretreatments.[4a,12a,13] HSQC nuclear magnetic resonance (NMR) characterization of the fractionated lignin demonstrated that guaiacyl (G) and *p*-hydroxyphenyl (H) units were increased whereas β-β and β-O-4 linkages were disrupted after the optimal combinatorial pretreatment.[13] Meanwhile, ^{31}P NMR results indicated that combinatorial pretreatments led to fewer aliphatic OH but more phenolic OH and carboxylic acid groups. Most importantly, aromatic monomers were generated during the optimal combinatorial pretreatment to facilitate lignin bioconversion[12a,13] These modified lignin chemistry results suggested the improved lignin processability for microbial conversion.[9b,13] Table 1 showed that both cell growth and PHA production were significantly improved when the lignin from the combinatorial pretreatment was used as a carbon source.[4a,13] Therefore, acid-alkali combinatorial pretreatment enhanced biological lignin valorization by increasing lignin output and improving lignin processability and thereby enhanced the performance of biorefineries.

2.2. Cosolvent enhanced lignocellulosic fractionation (CELF) process

A CELF pretreatment involves a dilute acid treatment of lignocellulosic biomass in a THF-water mixture directed at overcoming biomass recalcitrance.[15] In a one-pot reaction (generally at 150-180 °C), over 90% of the lignin from biomass was extracted into the THF media and recovered as a fine lignin powder (Figure 2).[16] Through this process, percent yields of glucose, xylose, and arabinose reached up to 95% after enzymatic hydrolysis at even low enzyme loadings (2 mg enzyme g^{-1} glucan).[17d] Moreover, lignin chemistry was significantly modified during the CELF pretreatment. Meng et al. systematically investigated the chemical transformations of poplar lignin during CELF process.[17a] Gel permeation chromatography (GPC) results showed that CELF lignin presented a dramatic reduction in its molecular weight by up to 90% compared with untreated native lignin. HSQC NMR analysis illustrated that the β-O-4 interunit linkages were extensively cleaved after CELF pretreatment at elevated temperatures (>160 °C). ^{31}P NMR analysis of phosphitylated lignin indicated that the aliphatic OH content in CELF lignin decreased due to the loss of γ-methylol group as formaldehyde and OH groups on $C_{\alpha,\beta}$ or the whole side chain in general to form stilbene structures.[17a,18] Total phenolic OH content in CELF lignin significantly increased which was attributed to the cleavage of interunit ether lignin linkages, while the amount of carboxylic acid group increased due to the hydrolysis of ester bonds or oxidation of aliphatic OH groups.[17a,19] Since lignin is generally not soluble in aqueous medium,[20] it is critical to disperse lignin uniformly in the liquid fermentation medium to provide easier access for microbial cells. Given the obvious reduction in molecular weight and increase of carboxylic acid groups content, CELF pretreatment should improve lignin distribution uniformity in fermentation medium and benefit to the fermentation performance. In addition, other efforts have also been devoted to improve lignin dispersion in aqueous medium. Lin et al.[6a] dissolved lignin powder through adjusting medium to pH 12.5 and then adjusting to pH 7.0-7.5 prior to fermentation to improve the lignin distribution uniformity. Besides, recent studies[21] reported the significant role of lignin-derived material as a surfactant and/or emulsion stabilizer based on the potential self-surfactivity of lignin. Therefore, to unleash the self-surfactivity of lignin through further modification approaches represents a promising strategy to enhance lignin dispersion in fermentation medium and thus to improve biological lignin valorization.

Other lignin-target pretreatments such as supercritical fluid,[22] ionic liquid,[23] and deep eutectic solvents[24] pretreatment have been thoroughly investigated in recent years and are summarized in several critical reviews elsewhere in literature. After pretreatment, the complex structure and occasionally high polydispersity of the obtained depolymerized lignin still represent key challenges that restrict the value-added biological applications. Therefore, fractionation technique has been developed and applied to technical lignins to further decrease its structural complexity.

2.3. Lignin fractionation to produce functionally distinct lignin cuts

Lignin exhibits inhomogeneous physicochemical properties due to the complexity of bond cleavage, functional group modification, and repolymerization reactions.[25] Lignin chemistry determines its reactivity and functions while the structural heterogeneity significantly restricts lignin valorization.[26] Fractionation approach provides an effective tool to obtain lignin fractions with relatively uniform properties, which is beneficial to their rational utilization.[27] At present, there are mainly three strategies for lignin fractionation, including membrane/ultrafiltration technology, sequential precipitation, and organosolv sequential extraction.[27,28] Membrane/ultrafiltration technology typically fractionates lignin based on lignin molecular size (i.e., molecular weight), while sequential precipitation and organosolv sequential extraction methods are determined by both lignin molecular weight and functional groups.[29] Meng et al. applied sequential precipitation to fractionate CELF lignin extracted from poplar biomass.[17b] As shown in Figure 2, ethanol was applied firstly to extract the low molecular weight portion from CELF lignin. The ethanol soluble CELF lignin was then fully solublized into 40 wt% THF aqueous solutions. By reducing the concentration of THF sequentially via adding water into the lignin solution, five lignin fractions with different molecular weights were recovered (Table 2). Wang et al. developed a simple sequential precipitation method utilizing hexane as an antisolvent to fractionate CELF lignin dissolved in THF-methanol cosolvent (Figure 2).[17c] Through tuning the solvent/antisolvent ratio via the gradual addition of hexane into the lignin solution, lignin fractions with well-defined characteristics, such as narrowly distributed molecular weights and tunable chemical structures were obtained. The lignin fractions with the highest and lowest molecular weight are shown in Table 2.

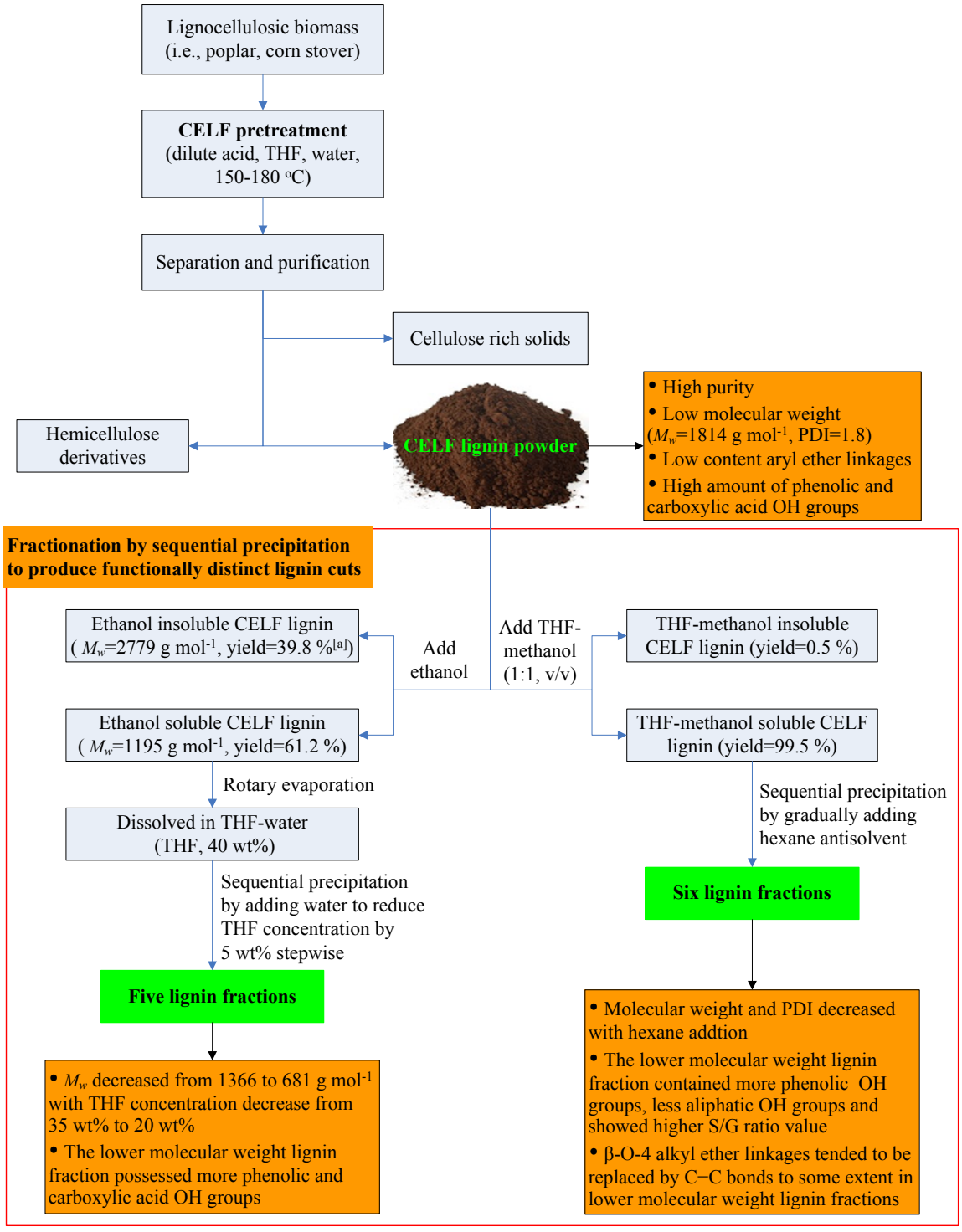

Figure 2. Flowcharts of CELF process and CELF lignin fractionation by sequential precipitation.[16,17] CELF: cosolvent enhanced lignocellulosic fractionation, M_w: weight-average molecular weight, PDI: polydispersity index. [a] Yield in this Figure indicates the weight percentage of lignin fraction to the parent lignin.

Table 2. Molecular weight and yield of different CELF lignin fractions.

	CELF lignin fractions subdivided by reducing THF concentration by 5 wt% sequentially[a][17b]					CELF lignin fractions subdivided by gradually adding hexane antisolvent[b][17c]		
	F_{35}	F_{30}	F_{25}	F_{20}	$F_{<20}$	F_6	F_{20}	$F_{>20}$
M_w [g mol^{-1}]	1366	1168	750	681	560	14500	2300	1200
PDI	1.6	1.8	1.3	1.6	1.8	5.5	1.6	1.5

Yield [%][c]	18.0	51.0	17.5	3.2	7.5	29.5	9.8	31.1

[a] F_{35}, the number 35 means that THF concentration is 35 wt%. [b] F_6, the number 6 means that hexane addition volume is 6 mL. [c] Yield here indicates the weight percentage of lignin fraction to the parent lignin.

Similarly, ionic liquid[30] and other organic solvents including ethanol,[31] acetone,[32] γ-Valerolactone,[33] acetic acid[34] have been all extensively used to decrease lignin heterogeneity by the gradient precipitation technique using water as the antisolvent. Holtz et al. further demonstrated the effect of antisolvent on the lignin fractionation in 2-methyl-tetrahydrofuran solutions by screen 13 different antisolvents based on the precipitation yield, solvent recovery, and energy efficiency.[35] Among the test solvents, maximum lignin precipitation yields around 70 wt% were obtained using n-pentane as the antisolvent, while efficient solvent recovery could be achieved keeping minimum energy consumption. Besides using organic solvent/water mixture, successive extraction using organic solvents with different salvation power (i.e., hydrogen bonding capacity, cohesive energy) was also widely employed to obtain lignin with different molecular weights and functional groups.[36] In conclusion, lignin isolation/depolymerization by pretreatment followed by solvent fractionation represents an effective way to obtain a more uniform structure of lignin that favors its biological valorization.

As described above, functionally distinct lignin cuts can be obtained through lignin fractionation.[17b,17c,37] Specific applications of the lignin fractions based on their structure-function relationships promise to improve lignin valorization efficiency.[38] Wang et al. synthesized lignin-based polyurethanes (PUs) from three different softwood Kraft lignin fractions prepared through sequential precipitation.[37a] The PUs were characterized systematically to examine the effects of different lignin fractions on properties of PUs. Table 3 showed that as the Kraft lignin fraction molecular weight decreased from 53625 (F1) to 3790 g mol^{-1} (F3), the aliphatic OH content decreased by 16.4%, the phenolic OH content increased by 32.2%, and the total OH content increased by 12.7%. However, the change in molecular weight was more than 10-fold and had an overwhelming impact on the material properties of lignin-based PUs. By comparing the mechanical properties of lignin-based PUs synthesized with different fractions, it was found that parameters including ultimate tensile strength, elongation at break, and Young's modulus increased with the increasing of lignin molecular weight. On the other hand, it was noted that mechanical properties of PU from lignin fraction F4 (the final soluble fraction in fractionation process) had not been determined because F4 with lower molecular weight and higher phenolic OH content failed to form PU networks with adequate mechanical strength for tensile testing. Therefore, it can be concluded that utilizing the high molecular weight fraction of lignin was able to improve the material stiffness or resistance to deformation of lignin-based PUs.[37a] Whereas Figure 1 suggested that lignin with lower molecular weight should be suitable for microbial conversion. Therefore, fractionation improves uniformity of lignin through rationally separating them into different fractions, which could be subsequently utilized in different ways based on their structure-function relationships, representing a value-added lignin valorization process.[39]

Table 3. Properties of different softwood Kraft lignin fractions subdivided by sequential precipitation and characterization of lignin-based polyurethanes.[37a]

	Lignin fraction	F1	F2	F3	F4
	M_w [g mol^{-1}]	53625	14488	3790	1322
	PDI	15.2	6.2	2.0	1.6
	Yield [%][a]	21.5	14.0	25.5	26.5
OH content [mmol g^{-1}]	Aliphatic OH	2.14	1.88	1.79	1.22
	Phenolic OH	2.83	3.16	3.74	4.30
	Carboxylic acid OH	0.32	0.39	0.43	0.64
	Total OH	5.29	5.43	5.96	6.16
Mechanical properties of lignin-based polyurethanes	Ultimate tensile strength [σ_{max}, MPa]	43.2±6.6	38.1±4.5	35.3±4.1	N.D.[b]
	Elongation at break [ε_{break}, %]	6.6±1.1	5.2±1.1	5.4±1.6	N.D.
	Young's modulus [E_{Young}, MPa]	961±5	839±51	750±21	N.D.

[a] Yield here indicates the weight percentage of lignin fraction to the parent lignin. [b] N.D.: not detected.

2.4. Lignin fractionation to screen lignin with specific biological activities

Lignin structure determines its functions as well as biological activities.[40] Wang et al. applied organosolv sequential dissolution to fractionate lignin extracted with ethanol from corn stover.[41] As shown in Table 4, the heterogeneous parent lignin (M_w=8317 g mol^{-1}, PDI=1.82) was fractionated into four relatively homogeneous parts with gradually increasing molecular weight and decreasing phenolic OH content. The four lignin fractions exhibited different anti-tyrosinase activities. Interestingly, it was found that the phenolic OH content of lignin showed a positive correlation with anti-tyrosinase activity. Fraction F1 with the lowest molecular weight but highest phenolic OH content presented the strongest inhibition on tyrosinase, which was comparable to the effect of positive control *p*-hydroxybenzaldehyde. The results suggested that lignin fractionation was an effective tool to screen lignin fractions with high anti-tyrosinase activity, showing potential application as an tyrosinase inhibitor in food, medicinal and cosmetic areas.[41,42] Moreover, Wang et al. employed a simple ethanol-water dissolution to fractionate enzymatic hydrolysis lignin (EHL) and examined antimicrobial activities of the three obtained lignin fractions.[37b,43] It was noted that the antimicrobial activities of EHL were centralized in the fraction which was extracted by the 95% ethanol-water solution while the other two fractions barely revealed inhibitory effects on the microbial growth.[37b] Therefore, lignin fractionation is effective to separate lignins with different biological activities, which could provide lignin substrates more

suitable for microbial conversion and thus enhance biological lignin valorization.

Table 4. Molecular weight and anti-tyrosinase activity of different lignin fractions subdivided by organosolv sequential dissolution method.[41,42]

	F1 (dichloro-methane fraction)	F2 (acetic ether fraction)	F3 (*n*-butyl alcohol fraction)	F4 (residue fraction)
M_w [g mol^{-1}]	4071	4598	5302	10308
PDI	1.58	1.48	1.52	1.71
Yield [%][a]	34.40±0.04	13.31±1.26	14.94±0.73	37.36±1.34
Anti-tyrosinase activity coefficient[b]	0.71	0.57	0.48	0.23

[a] Yield here indicates the weight percentage of lignin fraction to the parent lignin. [b] Anti-tyrosinase activity was determined at the lignin concentration of 0.4 mg mL^{-1}. The largrer anti-tyrosinase activity coefficient represents the greater inhibition on tyrosinase.

2.5. Elucidating the ideal lignin structure for microbial conversion

Even various methods have been employed to alter and modify lignin structure,[44] however, the ideal lignin substrate for microbial conversion have not been completely identified, limiting the directional modification of lignin substrates. Analysis of the biological lignin valorization process in Figure 1 suggests that lignin with low molecular weight that can be easily degraded into reactive monomers should be preferred by microbial metabolism.[2b] Reactive monomers including *p*-coumarate, ferulate, benzoate, phenol, 4-hydroxybenzoate, vanillin, guaiacol, phenylacetaldehyde, phenylethyl alcohol, 4-hydroxyphenylpyruvate, and 4-hydroxyphenylacetate are summarized in Figure 1.[4a,8a] Besides these monomers, there are still other reactive intermediates that need to be further identified.[2b,6b,45] Furthermore, biomass pretreatment could depolymerize lignins to some derivatives that are easy to be converted into these known reactive monomers, but these molecules have not been exactly identified. That is, the target lignin structure we want to produce through pretreatment and further fractionation is still not that clear, although it is critical for guiding the lignin modification for bioconversion.[46] There are two general ways to identify the ideal lignin structure: 1) metabolic pathways of microorganisms should be further studied to clarify the lignin metabolism, although this systematic approach is complicated and time consuming. 2) Another relatively simple way is portraying the ideal lignin substrates through big data analysis. Various kinds of modified lignin can be applied as substrates for microbial conversion. By analyzing the properties of lignins that show good fermentation performance, the lignin structure-function relationships could become more evident, eventually revealing the ideal lignin substrates for microbial conversion.

Genetic modifications of lignin provide a promising approach from the starting point by modifying lignin biosynthesis, which could weaken the dependence on pretreatment/fractionation to some extent.[47] Wilkerson et al. incorporated increased amounts of ferulate into the lignin polymer within poplar through transgenic technology.[48] Presumably, lignin of this nature would generate notably increased amounts of ferulate into the lignin stream after pretreatment/fractionation, which could be an ideal substrate for microbial metabolism.[2b,48] Consolidated bioprocessing (CBP) technology, which combines enzyme production, hydrolysis, and fermentation stages into a single step,[3,49] represents another effective approach to tailor lignin by linking it back to the native lignin either by natural variants or genetic modified plants.[50] Akinosho et al. showed that *Clostridium thermocellum* in CBP was found to largely preserve the basic lignin structure in *Populus*, and the resultant lignin from CBP is desirable for numbers of applications.[51] To conclude, in-depth understanding of the properties of ideal lignin substrates that are engineered for microbial conversion could guide modifying lignin chemistry, which needs to be studied further.

3. Fermentation strategies to enhance lignin depolymerization

It was pointed out that the lignin depolymerization capacity of bacteria is relatively weak, often resulting in an insufficient supply of reactive aromatics monomers for microbial cells.[4b,5b,6c] This inefficient lignin depolymerization capacity might be the limiting step for bacterial lignin conversion.[6] To address this challenge, efforts have been paid to develop advanced fermentation strategies to enhance lignin depolymerization and thus to improve biological lignin valorization.

3.1. Synergistic enzymatic and microbial lignin conversion

Mixing ligninolytic enzymes with microbial fermentation could be an effective strategy to enhance fermentation performance of lignin-rich substrates.[52] Zhao et al. investigated the effect of adding exogenous, commercial laccase to *Rhodococcus opacus* PD630 fermentation utilizing commercial Kraft lignin as a sole carbon source.[53] As a result, lignin consumption was obviously enhanced with the laccase treatment. Microbial cell growth increased exponentially in response to the laccase concentration from 0 to 2 U mL^{-1}. Moreover, lipids production increased by 17-fold to 145 mg L^{-1} compared with the fermentation without laccase addition. Further NMR characterization suggested that laccase treatment promoted the cleavage of different types of lignin linkages, providing sufficient lignin monomers for *R. opacus* cells growth and accumulation of lipids.

At present, the optimization of enzyme-microbe synergy strategy is still needed based on extensive experimental work.[54] For example, what the optimal combination of ligninolytic enzymes will be employed and how much these enzymes will be needed.[2b] Combining powerful systems biology techniques as

well as advanced chemical characterization and analytics holds promise to improve understanding of complex lignin substrate utilization by natural systems.[2b,6c] This fundamental understanding should further guide the design of enzyme-microbe interactions to optimally depolymerize lignin and catabolize the resulting monomers into target compounds.[2b,54,55] A recent study by Salvachúa and coworkers reported that *Pseudomonas putida* KT2440 secreted a striking amount of outer membrane vesicles (OMVs) in lignin-rich medium.[56] Many enzymes involved in the *β*-ketoadipate pathway which exhibit activity toward lignin-derived aromatic compounds were enriched in OMVs. The OMVs-mediated catabolism provided a means to access carbon sources that cannot translocate the microbial cell membrane and/or mitigate substrate toxicity by restricting cytoplasmic encounters or altering toxic compounds to less toxic molecules extracellularly, thus finally improving the lignin bioconversion performance.[10a,56] These findings gained a deeper understanding of how natural systems utilize complex lignin substrates and could provide guidance on the optimization of enzyme-microbe synergy and the rational design of lignolytic microbes through synthetic biology.[56,57]

3.2. Co-fermentation by consortium strains

Another effective strategy to enhance lignin depolymerization and bioconversion is co-fermentation by consortium strains. Yang's research group systematically examined the efficiency of lignin bioconversion process by a co-fermentation strategy.[5b,8a,58] It was found that co-culture of *R. opacus* PD630, *R. jostii* RHA1, and *R. jostii* RHA1 VanA$^-$ achieved the highest lignin degradation and highest lipids biosynthesis capacities compared with single culture of each strain or co-culture of *R. opacus* PD630 and *R. jostii* RHA1 VanA$^-$.[5b] As shown in Figure 3, lignin-degrading related enzymes including catalase-peroxidase, dye peroxidase (DyP), glutathione peroxidase, superoxide dismutase, and multicopper oxidase were detected during the co-fermentation process. Except that catalase-peroxidase was generated by *R. opacus* PD630 and *R. jostii* strains, other ligninolytic enzymes were originated from *R. jostii*. These results suggested that *R. opacus* PD630 had lower extracellular depolymerization activity to produce reactive lignin intermediates compared with *R. jostii* RHA1 and *R. jostii* RHA1 VanA$^-$. The co-fermentation with *R. opacus* PD630 and other two strains may help *R. opacus* PD630 get easier access to lignin-derived aromatics as a carbon source for cell growth and lipids accumulation.[5b,8a] Besides the effect of enhancing lignin depolymerization, co-fermentation by the consortium strains should also enrich metabolic pathways for lignin conversion, which contribute to a more efficient utilization of lignin.[5b,6c] Therefore, the co-fermentation methodology showed an effective strategy to improve biological lignin valorization. However, more in-depth studies are still needed to reveal the detailed mechanisms of synergic effects and thus to guide the optimization of strains combination and fermentation processes design to unleash the potential of this strategy.[5b,8a]

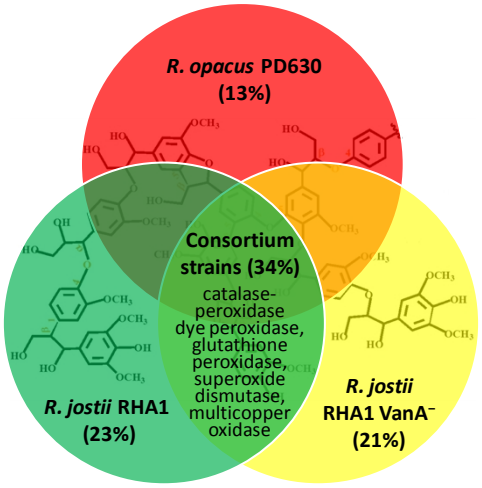

Figure 3. Co-fermentation by consortium strains enhanced lignin degradation compared with a single strain fermentation. Numbers in parentheses indicate the percentage of lignin degradation.[5b,6c,8a] Adapted with permission from Ref. [6c].

3.3. Systems biology guided genetic design of microbial strains

Genetic engineering of lignin utilization stains is a promising approach to improve lignin depolymerization and bioconversion efficiency.[59] A proteomics study by Lin et al. suggested that DyP is a key enzyme for lignin depolymerization in *P. putida* A514.[6a] Based on this discovery, an effective functional module for overexpressing DyP enzyme was introduced to *P. putida* A514 via genetic engineering. NMR analysis showed that degradation of lignin structure, such as β-5 and β-O-4, were enhanced by the DyP-engineered strain, validating the chemical function of DyP on lignin degradation.[6a,60] Correspondingly, the DyP-engineered strain showed a significant improvement in cell growth on Kraft lignin, representing a 2.1-fold growth increase compared with the control strain. The integration with two other functional modules for aromatic compound catabolism and PHA synthesis by genetic engineering provided PHA content reaching 0.73 g g^{-1} CDW with engineered *P. putida* A514.[6a]

Xie et al. systematically investigated lignin degradation and consumption by *R. opacus* PD630_FL, which is an engineered strain integrating laccase secretion module and lipids production module via genetic engineering.[6b] Moreover, when acetosyringone was added to the fermentation medium as a laccase mediator, lignin structure was further degraded. Table 5 showed that *R. opacus* PD630_FL with laccase mediator led to more efficient degradation of lignin structure than the control strain *R. opacus* PD630. Therefore, the synergistic activity of the engineered strain and laccase mediator enabled the efficient depolymerization of lignin. Based on the mechanistic study and the engineering of microorganism, a strategy coupling genetic engineering with co-fermentation was developed to utilize lignin-containing biorefinery waste from ammonia fiber expansion (AFEX) pretreated corn stover. Two strains engineered with laccase secretion module (*R. opacus* PD630_La)/lipids synthesis module (*R. opacus* PD630_Fa) were co-cultivated. As a result, lipids concentration reached 0.95 g L^{-1} by the consortium of the engineered strains, representing an increase of 48.4% compared

with the control strain *R. opacus* PD630.[6b] Therefore, genetic design of microbial cells enabled a high titer in converting lignin to valuable products including lipids and PHA. Meanwhile, the results also highlighted the synergic effect in co-fermentation. In the future, more powerful tools should be exploited to modify the microbial strains with the rapid development of systems and synthetic biology.[6c,61]

Table 5. Lignin structure degradation by engineered strain *R. opacus* PD630_FL with laccase mediator versus the control strain *R. opacus* PD630.[6b]

Lignin structure	*R. opacus* PD630_FL + laccase mediator	*R. opacus* PD630
β-5	81.1% degradation	33.9% degradation
4-O-5, 5-5, guaiacyl and carboxylic acid	18.6~21.7% degradation	2.7~6.7% degradation
Aliphatic hydroxyl group content	decrease by 40.8%	decrease by 15.5%
Guaiacyl hydroxyl group content	decrease by 26.3%	decrease by 8.9%

4. Summary and Outlook

Various pretreatment/fractionation methods are emerging to modify lignin chemistry and improve carbohydrate outputs. Fundamental understanding of lignin derivation during fermentation is critical for guiding process optimization and strain design to improve lignin bioconversion. Several effective fermentation strategies have been developed to improve lignin depolymerization, which includes enzyme-microbe synergy, co-fermentation by consortium strains, and genetic engineering of microbial strains. These strategies showed significant improvement in lignin valorization efficiency. In the future, substantial research and development effort going forward through metabolic pathways and/or big data analysis are required to figure out what is the ideal lignin substrate for microbial conversion. With the improved understanding of the ideal lignin structure, pretreatment/fractionation for modifying lignin chemistry could be guided and more advanced fermentation strategies are going to be developed for effectively harnessing the intrinsic capabilities of microbes to valorize lignin.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biological lignin valorization • biomass • fermentation • lignin chemistry • structure elucidation

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