Chapter 7

THE POTENTIAL ROLE OF ENZYMATIC CATALYSIS AND METABOLIC ENGINEERING IN LIGNIN VALORIZATION

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ABSTRACT

Lignin, the second richest biomass, is considered to be the potential resource of high value-added chemicals with the rapid development of lignocellulose biorefinery. During the past decades, physical and chemical methods have been used widely to degrade lignin into chemicals; however, they are lagged by several challenges, such as the fewer yields of low-weight molecules, higher energy consumption and non-specific cleavage. A large number of metabolic pathways in organism have been discovered for the lignin degradation and bioconversion, which was consist of lignolytic enzymes including radical lignolytic enzymes and non-radical lignolytic enzymes. The metabolic engineering of non-radical lignolytic enzymes shows a promising value for the conversion of lignin into aromatic chemicals or other high value-added chemicals. In the present mini-review, recent developments on enzyme catalysis and metabolic engineering of lignin valorization will be

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summarized and discussed, including already discovered non-radical lignolytic enzymes, their metabolic pathways and molecular mechanism for lignin conversion, their recent application in lignin biorefinery and the possible combination of bio-catalyst and physical/chemical methods for lignin refinery.

**Keywords:** lignin valorization, non-radical lignolytic enzymes, enzyme catalysis, metabolic engineering, high value-added chemicals

## INTRODUCTION

Lignin is a major component of lignocellulose and is also the most abundant aromatic polymer on earth. Lignin has a highly branched, three-dimensional, poly-phenolic structure that includes three phenylpropane units, namely p-coumaryl, coniferyl and sinapyl, joined by ether and C–C linkages. While lignin’s structural heterogeneity and poly-phenolic composition results in its inherent stability and recalcitrance, lignin can ultimately be converted into CO₂ by microorganisms (bacteria, fungi, actinomycetes, etc.), maintaining the carbon balance in nature. The biological degradation of lignin by microorganisms is a complex process, involving the extracellular oxidative degradation of natural lignin into low-molecular-weight aromatics, followed by their intracellular metabolic degradation, and their bioconversion as carbon and energy sources. The enzymes involved in these processes can be classified, on the basis of their reaction mechanism, as radical-dependent and non-radical lignolytic enzymes [1, 2], which constitute the metabolic pathways of lignin degradation in nature. Generally, the radical-dependent lignolytic enzymes are secreted extracellularly to produce the free radicals that degrade lignin into low-molecular-weight products. These radical-dependent lignolytic enzymes have been studied extensively over the past few decades, and have been thoroughly reviewed [3, 4]. With the rapid development of lignocellulose biorefinery, the intracellular metabolic degradation and bioconversion of lignins with a low-molecular weight aromatics has provided a new approach for the lignin valorization.

## THE BIO-DEGRADATION OF LIGNIN-DERIVED AROMATIC

Currently, the detailed metabolic pathways, transportation and regulation of lignin-derived aromatics are mainly associated with bacterial resources. Corresponding research into fungi have lagged behind, and the metabolic pathways for aromatics in fungi have been mostly proposed on the basis of discovered intermediates. Early studies indicated that the production of ligninolytic enzymes in fungi occurred during secondary metabolism and were mainly triggered by limited nutrient levels, including carbon and nitrogen limitations.
These studies suggest that the large scale production of ligninolytic enzymes in fungi is not directly related to the catabolism of aromatics [7]. Thus, the metabolism and regulation of lignin-derived aromatics in fungi is far more complicated than those in bacteria.

Lignin is a highly branched three-dimensional poly-phenolic structure, consisting of \( p \)-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. In addition, \( \beta-O-4 \) linkages account for 40\% to 70\% of chemical bonds in natural lignin. Consequently, the metabolism of lignin-derived aromatics would necessarily involve enzymatic hydrolysis of \( \beta-O-4 \) linkages and the metabolic pathways of lignin derivatives from G-/S-/H-type units.

The Enzymatic Degradation of \( \beta \)-Ethers

It has been discovered that enzymes acting on \( \beta \)-ethers can cleave \( \beta-O-4 \) bonds in lignin model compounds. The \( \beta \)-etherase system of *Sphingobium* sp. SYK-6 is most well-known among these enzymes. The degradation of \( \beta-O-4 \) bond by *Sphingobium* sp. SYK-6 includes \( \text{C}_n \) dehydrogenase (LigD, LigL, LigN, LigO), etherase enzyme (LigE, LigF, LigP) and glutathione-lyase (LigG) (Figure 1). The genes encoding these three kinds of enzymes have a number of family members, which have been reviewed by Wang [8] and Kamitama [9]. The mining of these three kinds of enzymes was undertaken for the National Center for Biotechnology Information (NCBI) by Kamitama and coworkers to identify additional \( \beta \)-ether enzymes [9]. The \( \beta \)-etherase and \( \text{C}_n \)-dehydrogenase gene orthologs are mainly distributed in *Sphingomonadaceae* with the exception of LigE orthologs that are somewhat widely distributed in \( \alpha \)-proteobacteria, in which *Sphingomonadaceae* represents 53\%. The orthologs of the glutathione-removing enzyme gene were mainly distributed in \( \alpha \)-proteobacteria, however, the \( \text{Na} \) class of Glutathione S-transferase (GST) from *Novosphingobium* sp. MBES04 (GST3), with stereospecificity for both R and S substrates, is distributed in \( \alpha \)-proteobacteria, \( \beta \)-proteobacteria and \( \gamma \)-proteobacteria [9]. These results indicate that in nature *Sphingomonadaceae* is specialized for \( \beta \)-aryl ether catabolism, which is in agreement with the reports of the Bugg and coworkers [10]. The different family members of the \( \beta \)-etherase systems show a variety of pH optima, thermal stabilities and substrate specificities. In addition to their enantioselective differences, the chemical functionality of their substrates can significantly impact the activity of these enzymes. These functionalities include groups on the aromatic rings, linkages between two aromatic rings, groups at \( \text{C}_n \), the side chain of \( \text{C}_p \), and the positions of methoxy groups on the aromatic rings. In particular, the presence of a hydroxyl group at the \( \text{C}_n \) position of lignin model compounds makes the \( \beta-O-4 \) bond resistant to the \( \beta \)-etherase. When the group at this \( \text{C}_n \) position is replaced by carbonyl, the \( \beta \)-etherase can effectively cleave the \( \beta \)-ether bond linking the aromatic groups in dimeric compounds [11, 12].
The large number of gene family members identified might have evolved to adapt to the environment and the intrinsic heterogeneity of lignin in order to effectively utilize lignin as a carbon and an energy source. However, from the point of industrial application, the presence of a large number of enzymes makes a process unduly complex. Thus, it is important to study the catalytic mechanisms of these enzymes to discover the enzymes that act on a broad range of substrates. Recently, the X-ray crystal structures of \( \beta \)-ether degrading enzymes have been reported [13-15] and these will undoubtedly be used in combination with gene mining and protein engineering to promote studies on \( \beta \)-ether degrading enzymes.

The Metabolic Pathways of Lignin Derivatives from G-/S-/H-Units

In practice, natural lignin can be depolymerized into a large number of chemical products after physical, chemical or biological treatment. The type and number of these chemical products are dependent on the operating conditions. In the present paper, the metabolic pathways of depolymerizing products only focus on the aromatic slurry. The metabolic pathways of \( \beta \)-coumaric acid (pCA), ferulic acid (FA) and sinapic acid (SA) have been studied extensively. G-/S-/H- units and many lignin-derived aromatics share the common chemical structure with pCA, FA and SA. Consequently, the metabolic pathways of pCA, FA and SA have been chosen to introduce the catabolic pathways for lignin-derived aromatics.

pCA and FA can be catabolized to protocatechuate (PCA) or catechol (CA) through CoA-dependent \( \beta \)-oxidation and CoA-dependent non-\( \beta \)-oxidation pathways (Figure 2A). In the CoA-dependent \( \beta \)-oxidation pathway, CoA is first catalytically added, followed by double bond hydrolysis, further oxidation and sulfurylation to remove the ethyl group. pCA
and FA are eventually hydrolyzed to p-hydroxybenzoic acid (pHBA) and vanillic acid, respectively [8, 16, 17] (Figure 2B1, B2). The CoA-dependent non-β-oxidation pathway is similar to the β-oxidation pathway with the exception that in the CoA-dependent β-oxidation pathway further thiolysis and oxidation were not observed after the first removal of acetyl-CoA [16, 18] (Figure 2B3, B4).

Additionally, there is also a side chain reduction pathway in the metabolism of pCA and FA. In the plants *Gleditsia* sp. and *Vanilla* sp., a CoA-independent pathway produces pHBA from pHHA, which proposed to involve one reductase (pHBAL) and one decarboxylase (pHBALS) (Figure 2B5) [16, 19]. In *Corynebacterium glutamicum*, FA is reduced by aromatic reductase (RE) to form dihydroferulic acid and decarboxylase (DCL) catalyzes dihydroferulic acid to produce acetic acid and vanillic acid. This pathway is generally present in bacteria and fungi grown under anaerobic conditions [8]. In addition, FA can also be reduced to coniferyl alcohol, which is further degraded to vanillic acid [20, 21] (Figure 2B6).

Notably, FA can also be degraded by non-oxidative decarboxylation [8, 22, 23], which has not been observed in pCA catabolism. FA, in the non-oxidative decarboxylation pathway, is catalytically decarboxylated to vanillin and vanillic acid (Figure 2B7), which has been found in *Fusarium solani* (Mart) Sacc., [24] *Bacillus coagulans* [25], and *Bacillus cereus* strain PN24 [26].

Most catabolic microorganisms for pCA and FA cannot degrade SA, which indicates the adverse impact of aromatic methoxyl groups on SA catabolism [27]. The initial step from SA to syringic acid is proposed to be catalyzed by radical lignolytic enzymes (laccases) [27, 28] or non-radical lignolytic enzymes (decarboxylases) [29, 30] (Figure 2A). No genes corresponding to the decarboxylases involved in non-radical catalysis have yet been reported. SA is transformed into syringic acid through the removal of two carbon atoms from its side chain. In *Sphingomonas paucimobilis* SYK-6, syringic acid is O-demethylated by a tetrahydrofolate-dependent O-demethylase (DesA) to produce 3-O-methylgallate (3MGA), and then the 3MGA is O-demethylated by another O-demethylase (LigM) to generate gallic acid. The aromatic ring of 3MGA and gallic acid can be cleaved by dioxygenase, moving into the TCA cycle [31-33]. There is currently no evidence to support the transformation of syringic acid to PCA or CA prior to ring opening.

**THE TRANSPORTATION OF LIGNIN DERIVATIVES FROM G-/S-/H-UNITS AND THEIR METABOLIC REGULATION**

The genes involved in aromatic metabolism are usually physically assembled in operons or in clusters within bacteria. Around the aromatic metabolizing genes there are always transporting genes responsible for the uptake of the aromatic substrates and
regulating genes, which act as trans-elements to regulate the aromatic metabolism. Metabolizing genes, transporting genes, and regulating genes have developed to efficiently assimilate the substrate and catabolize it for carbon and energy demands.

In bacteria, there are four transporting systems for the uptake of lignin-derived aromatic compounds: ATP-binding cassette (ABC) transporters, major facilitator superfamily (MFS) transporters, a tripartite ATP-independent periplasmic (TRAP) transporter, and an ion transporter (IT) superfamily. The four transporting systems show signs of binding affinity towards pCA, FA, SA or their derivatives [9].

ABC transporters are widely distributed and are well-studied transporters. They can transport an enormous variety of substrates, ranging from small ions to large organic molecules [34]. When Enterobacter lignolyticus SCF1 and Bacillus ligninophilus L1 was cultivated in media with lignin as sole carbon source, the production of ABC transporters increased [35], demonstrating that the ABC transporters were involved in the assimilation of lignin-derived aromatics. A fluorescence thermal shift-based assay indicated that the component of ABC transporters can interact with pCA, FA and SA with different binding affinities [36]. In Rhodopseudomonas palustris, the co-crystallization of ABC transporter (CouT) and FA indicates that H-bond interactions occur between the 4-OH group of the aromatic ring and His309/Gln305 and also between the carboxyl group on the FA side chain and Arg197, Ser222, and Thr102 [37]. CouT in R. jostii RHA1 is believed to be a MFS transporter for p-hydroxybenzoate. At the mRNA level, couT is upregulated in a strain cultivated on FA and pCA. The growth status indicates that pCA is better than FA as the growth substrate, reflecting the substrate specificity of couT [38]. In addition, there are also porins, substrate-specific channels or TonB-dependent receptors at the outer membrane of Gram-negative bacteria, involved in the uptake of aromatics [9]. There are not direct reports of their involvement in the transport of pCA, FA, and SA.

The metabolizing genes of aromatics are usually clustered in the operon, around which regulating genes were recruited to manipulate the metabolic pathway and transportation for the effective catabolism. While the regulators of many aromatic monomers have been discovered, no regulators of lignin-derived biaryl have been reported. The FerC from Sphingobium sp. SYK-6 recognizes the thioester product of pCA, FA, and SA as effectors [9]. This suggests that FerC and its cognate FA catabolic regulon in SYK-6 are involved in the metabolism of lignin derivatives from G/S/H-type units. Other reported regulators only bind a portion of feruloyl-CoA, p-coumaroyl-CoA and sinapoyl-CoA. For example, CouR from R. jostii RHA1 and HcaR from Acinetobacter sp. ADP1 recognizes p-coumaroyl-CoA and feruloyl-CoA as effectors [39, 40], and CouR from R. palustris CGA009 and FerR from P. fluorescens BF13 bind p-coumaroyl-CoA and feruloyl-CoA, respectively [41, 42]. In addition to the regulators of the metabolic pathways of pCA, FA, and SA, the regulating genes for protocatechuate [9], vanillate [9], benzoate [43], and
catechol [44], the intermediates of pCA, FA, and SA catabolism, have also been discovered. This suggests that the regulation mechanism of lignin-derived aromatics is a complicated system involving substrate competition, the integration of different metabolic pathways, and environmental factors. In *Pseudomonas putida*, the catabolite repressor (Crc) binds to and inhibits the translation of benR mRNAs, and benR encodes the transcriptional activator inducing the expression of benzoate degradation genes. Further research indicates that Crc can also bind to the translation initiation regions of the mRNA of several structural genes in benzoate degradation and the benzoate transporter gene, which suggests that Crc may also control benzoate degradation and uptake. All this evidence demonstrates that Crc can regulate benzoate metabolism at multi-tier levels, including uptake, induction and degradation [45]. The catA2 gene in *Pseudomonas putida* mt-2 works as an enzymatic safety valve for excess of catechol, the intermediate of benzoate catabolism, to alleviate the toxicity of catechol. The catA2 gene is located downstream of the ben operon and the ben operon is regulated by the benR. CatA is in the cat operon for the normal catechol catabolism, which is activated by catR [44]. PeaY is an aromatic acid chemoreceptor in *P. putida* F1, which belongs to the pca operon under the control of PcaR. PeaK, a transporter protein, is also in the pca operon. Data suggests that PeaK facilitates the uptake of 4-hydroxybenzoate, resulting in the increase of PcaY expression. Consequently, the chemotaxis, transport, and metabolism of aromatic compounds are integrated by PcaR in *P. putida* [46]. Recent studies show the existence of an aerobic-anaerobic metabolism switch [43] and a regulator coupled with dioxygenase [47] in aromatic catabolism. This provides many potential tools for increasing lignin valorization with microbiology by metabolic engineering.

**BIOTRANSFORMATION FOR THE PRODUCTION OF CHEMICALS FROM DEPOLYMERIZED LIGNIN AND LIGNIN MODEL COMPOUNDS**

There are two approaches for the biotransformation of depolymerized lignin or lignin model compounds into chemicals. The first is *in vitro* enzymatic conversion and the second is *in vivo* metabolic conversion. Given that 40% to 70% of the chemical linkages in lignin are β-O-4, researchers have tried to degrade the lignin using a β-etherase system to obtain value-added chemicals or chemical precursors for the further metabolic engineering (Table 1). However, improving degradation efficiency is a big challenge for *in vitro* enzymatic catalysis. Moreover, although some value-added chemicals have been produced from lignin using microorganisms, most reports still rely on lignin model compounds to study the metabolic conversion *in vivo*.
Figure 2. Metabolic pathways of p-coumaric acid, ferulic acid and sinapic acid. (A) The brief outline of metabolic pathways; (B) Detailed metabolic pathways. BADH, benzaldehyde dehydrogenase; ECH, enoyl-CoA hydratase; HADH, 3-hydroxyacyl-CoA dehydrogenase; HDE, hydroxylase; pHBAL, p-hydroxybenzaldehyde synthase; pHCI, p-hydroxyphenyllactic-CoA hydratase/lyase; pHBDC, p-hydroxybenzoate decarboxylase; pHBDH, p-hydroxybenzoate-3-hydroxylase; pHCS, p-hydroxyphenylacetyl-CoA synthetase; KAT, 3-ketocarbonyl-CoA thiolase; PCADC, protocatechuate decarboxylase; DCL, decarboxylase; DHG, dehydrogenase; ECH, enoyl-CoA hydratase; ECH/A, enoyl-CoA hydratase/1,4-dioxygenase; FCS, feruloyl-CoA synthetase; FDC, ferulic acid decarboxylase; β-KTE, β-keto thiolase; 0-DML, O-demethylase; PCADC, protocatechuate decarboxylase; POG, phenol oxidase; RE, reductase; TF, thiolase; VD, vanillin decarboxylase; VGDH, 4-vinylguaiacol dehydrogenase; 3MGA, 3-O-methylgalactose; PDC, pyrolyse-4,6-dicarboxylic acid.
Table 1. The enzymatic conversion *in vitro* of natural lignin

<table>
<thead>
<tr>
<th>Lignin resource</th>
<th>Involved enzymes</th>
<th>Process</th>
<th>The reacting conditions</th>
<th>Products and yield</th>
<th>The organic solution for lignin dissolution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Softwood/hardwood alkali-lignin and bagasse organosolv-lignin</td>
<td>UlgD/LigF/LigG/AVR</td>
<td>One pot</td>
<td>0.1M ammonium acetate buffer (pH 9.5); NAD⁺, GSH</td>
<td>Guaiacol/Tertiarc acid/Eugenol/ Acetovanillione/Vanillin (Softwood); Guaiacol/Vanillin (Hardwood); No products detected (organosolv-lignin)³</td>
<td>NA-</td>
<td>[1]</td>
</tr>
<tr>
<td>C-MWL and E-MWL³</td>
<td>SDR3/SDR5/ GSI4/ GSI5/ GSI3</td>
<td>One pot</td>
<td>0.1M TAPS (pH 8.5); NAD⁺, GSH; 15°C</td>
<td>GSH from C-MWL (2.4 ± 0.053 wt %); [2] 0.05 mg g⁻¹ lignin); GSH/SH from E-MWL (1.9 ± 0.012 and 4.7 ± 0.025 wt %); [19] 0.12 and 47 ± 0.25 mg g⁻¹ lignin, respectively</td>
<td>N,N- dimethylformamide</td>
<td>[48]</td>
</tr>
<tr>
<td>Organocat lignin from beach</td>
<td>Linacero/LigE/LigF-NA/LigG-TD</td>
<td>Three steps</td>
<td>Step 1: sulfuric acid; 0.1M sodium acetate buffer (pH 5); [EMIM] [ESO]; room temperature, 250 rpm; 3 days; Step 2: 50 mM glycine/NaOH buffer (pH 9.5); DMSO (25%), GSH 25°C overnight; Step 3: DMSO in step II was diluted to 10% with water, 20°C overnight</td>
<td>GSH</td>
<td>DMSO</td>
<td>[49]</td>
</tr>
<tr>
<td>HP lignin and MCS lignin</td>
<td>UlgD/LigW/LigG/LigY/ MBG5/THU (or LigG)/AeGR</td>
<td>One pot</td>
<td>25 mM Tris buffer (pH 8.0); DMSO (2.0%), NAD⁺, GSH; room temperature, 4 hours</td>
<td>1.0 mM HFS from HP lignin (12.5 wt% yield); 0.4 mM HPV, 0.1 mM HPS and trim from MCS lignin 55% yield (HPV - HPS)³</td>
<td>DMSO</td>
<td>[50]</td>
</tr>
</tbody>
</table>

a. Yield referred to Reiser [1]
b. Milled lignin from *Cayratia japonica* (C-MWL) and milled lignin from *Eucalyptus globulus* (E-MWL).
c. HP - the high-syringyl hybrid poplar; MCS - maize corn stover.
d. HPV - hydroxypropionic acid; HPS - hydroxypropiorylglycol.
Enzymatic Conversion in Vitro

Given the complexity of lignin structures, the large scale depolymerization lignin, into value-added functional aromatic compounds, is difficult using chemical or physical treatment [2]. Enzymatic methods, particularly ones using non-radical lignolytic enzymes, represent an alternative approach for lignin valorization under environmentally friendly and substrate-specific conditions [2].

The cleavage of natural lignin with the β-etherase system was first carried out by Reiter [1] (Table 1). The β-etherase system included LigD/E/G and AvGR, and softwood/hardwood alkali-lignin and bagasse organosolv-lignin were selected as substrates. GPC analysis showed that softwood and hardwood alkali-lignin were only slightly degraded, but this did not occur with bagasse-organosolv lignin even after 7 days of treatment [1]. Subsequently, Picart prepared a fluorescently labeled synthetic lignin model (DHP-MUAV), a multiple polymer of coniferyl alcohol and α-O-(β-methylumbelliferyl) acetovanillone (MUAV), to assess whether β-etherases could cleave β-O-4 aryl ether linkages present in lignin-like polymers. The results showed that DHP-MUAV was converted into different fragments of smaller mass, suggesting that it was possible for the β-etherase to catalyze cleavage of lignin-like polymers [12]. Afterwards, three different research groups [48-50] reported the enzymatic degradation of natural lignin with improved β-etherase systems (Table 1). The highest yield of enzymatic degradation was 12.5 wt% and different depolymerization products were obtained (Table 1). Based on data from the in vitro enzymatic conversion, the efficiency of β-etherase systems towards natural lignin or lignin-like polymers was far lower than towards lignin model dimers. This might result from the problems of enzymatic enantioselectivity, substrate availability, or the inhibitory effect of lignin on enzyme activity [51]. Consequently, more research is still required to understand the mechanism of β-etherase catalysis and the interaction between these enzymes and lignin. In addition, from the view of industrial application, the yield of enzyme-hydrolyzing products still needs to be improved. Further studies need to be carried out on the β-etherase system, such as mining or engineering enzymes to broaden their range of substrates and improve their efficiency, simplifying the process, reducing its cost.

Metabolic Conversion in Vivo

The “funneling pathway” includes both the upper and lower pathways, present in many microorganisms, for the metabolism of aromatic compounds [52] (Figure 3). In the upper pathways, aromatic molecules are catabolized into several conserved intermediates, including catechol (CA) in bacteria and protocatechuate (PCA) in most fungi and some bacteria [52]. In the lower pathways, the aromatic rings of these conserved intermediates

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are cleaved by dioxygenases, producing ring-opened species that are metabolized through the β-ketoacidipate pathway into the TCA cycle [52, 53].

Recently, researchers have used metabolic engineering to explore the biotransformation of lignin and lignin-derived monomers into different chemicals for the purpose of lignin valorization (Table 2). These target chemicals include aromatics, ring-opened chemicals, TCA cycle chemicals, lipids and lipid derived polymers (polyhydroxalkanoate, PHA). These chemicals are distributed within upper pathways, lower pathways, TCA cycle, and lipid synthesis pathways (Figure 3).

Figure 3. Lignin valorization by combining enzymatic catalysis and metabolic engineering and using a multi-disciplinary approach.
Table 2. The reported chemicals biotransformed from lignin or lignin model compounds

<table>
<thead>
<tr>
<th>Molecular</th>
<th>Substrate</th>
<th>Host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate, Lactate</td>
<td>benzoate and p-coumarate</td>
<td><em>P. putida</em> KT2440</td>
<td>[72]</td>
</tr>
<tr>
<td>β-Ketoaspartic acid, Macrodictyon</td>
<td>protocatechelic acid</td>
<td><em>P. putida</em> KT2440</td>
<td>[55-59]</td>
</tr>
<tr>
<td>Muconic acid</td>
<td>catechol, p-coumarate, benzoic acid, ferulate, guaiacol, vanillin, lignin</td>
<td><em>E. coli</em>, <em>E. coli</em> XL-1 Blue, <em>P. putida</em> KT2440, <em>Sphingobium sp.</em> SYK-6, <em>Ansiculodesmus sp.</em> ATCC 39116</td>
<td>[61-68, 97]</td>
</tr>
<tr>
<td>Picolinic acid, HEMS</td>
<td>catechol</td>
<td><em>Pseudomonas putida</em> (arvilia) cm-2 ATCC 23973</td>
<td>[78]</td>
</tr>
<tr>
<td>3-Carboxy-muconate</td>
<td>vanillin</td>
<td><em>Escherichia coli</em> BL21-Gold (DE3)</td>
<td>[66]</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>corn stover hydrolysate</td>
<td><em>Rhodopseudomonas palustris</em></td>
<td>[57]</td>
</tr>
<tr>
<td>2,4-PDCA</td>
<td>wheat straw lignocelluloses</td>
<td><em>Rhodococcus Jostii</em> RHA1</td>
<td>[71]</td>
</tr>
<tr>
<td>Fatty acid, PHA</td>
<td>alkylate protected lignin, organosolv lignin, Kraft lignin</td>
<td><em>Aspergillus fumigatus</em>, <em>P. putida</em> KT2440 and Y541, <em>C. saccharolyticus</em> B-8, <em>Fusarium sp.</em> ISTVR</td>
<td>[53, 76, 83-85]</td>
</tr>
<tr>
<td>Lipid</td>
<td>4-hydroxybenzoic acid, resorcinol, vanillic acid, lignin, sugarcane bagasse</td>
<td><em>Rhodococcus opacus</em> DSM 1059 and FD830, <em>Rhodococcus jostii</em> RHA1 Van-, <em>P. palustris</em> 4, <em>P. palustris</em> 5, <em>C. paucivorans</em> commensima, <em>C. cirulinata</em> FLR3</td>
<td>[77-82]</td>
</tr>
<tr>
<td>Vanillin</td>
<td>eugenol, isoeugenol, ferulic acid, vanillic acid, lignin</td>
<td><em>E. coli</em>, <em>Pseudomonas putida</em>, <em>Rhodococcus</em>, <em>Pseudomonas</em></td>
<td>[55-56, 101-104]</td>
</tr>
<tr>
<td>PDC</td>
<td>protocatechuate</td>
<td><em>P. putida</em> Pyr1100</td>
<td>[69]</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>DHP (Synthetic Lignin)</td>
<td><em>Phanerochaete chrysopogon</em></td>
<td>[70]</td>
</tr>
</tbody>
</table>

Note: a: 2,3-Dihydroxymuconic semialdehyde (HMS), h: Pyridine-2,3-alucarboxylic acid (2,3-PDCA) Pyridine 2,4 dicarboxylic acid (2,4-PDCA), and c. 2-Pyrene-4,5-dicarboxylic acid (PDC).

Over the past several decades, various biotechnology-based approaches have been developed for the production of vanillin. A number of specialized microorganisms have been used to produce vanillin from aromatic molecules such as eugenol, isoeugenol, FA, vanillic acid [8, 54, 55]. Lignin has also been studied as a starting material for the biocatalytic production of vanillin. When a vanillin-dehydrogenase deletion strain of *R. jostii* RHA1 was grown for 144 h on pretreated wheat straw lignocelluloses, it was found to accumulate vanillin with yields of up to 96 mg/L [56]. In addition, ferulate catabolic pathways and β-aryl ether cleavage produces vanillin as an intermediate metabolite within different lignin degradation pathways, providing valuable approaches for the bioproduction of vanillin from lignin. When *Rhodopseudomonas palustris* is cultivated in corn stover hydrolysate, the bioconversion of aromatics can be observed. The deletion of *badE* gene of *R. palustris*, encoding benzoyl-CoA reductase, results in the accumulation of benzoic acid from aromatics in hydrolysate and the deletion of *hbaBCD* gene, encoding 4-hydroxybenzoyl-CoA reductase, accumulates 4-hydroxybenzoic acid. The concentration
of both benzoates was at more than 1 mM. It is interesting that the *R. palustris* can only utilize the aromatics in lignocellulose hydrolyzate, while leaving sugars unaltered. Thus, this approach provides an effective method to remove bio-toxic factors from biomass hydrolyzate before further biotransformation [57].

Dicarboxylic acid and its derivatives originate from the cleavage of aromatic ring in central intermediates. Currently dicarboxylic acid and its derivatives account for most kinds of microbial biotransformation products coming from lignin or lignin model compounds. These chemicals include β-ketoadipic acid [58], muconolactone [58, 59], 3-carboxy-muconate [60], muconic acid [60-68], 2-pyrene-4,6-dicarboxylic acid (PDC) [69], picolinic acid [70], 2-hydroxymuconic semialdehyde [70], pyridine 2,5-dicarboxylic acid (2,5-PDCA) and 2,5-dicarboxylic acid (2,4-PDCA) [71]. All these chemicals share common structures (two or more carboxylic acid groups), making these compounds, or their derivatives, useful raw materials for the synthesis of bio-based polymers.

The cleavage of aromatic ring has two modes: ortho ring-cleavage and meta ring-cleavage, which was carried out by intradiol dioxygenases or extradiol dioxygenases, respectively [72]. Ring cleavage can be conducted by two enzymes, a 1,2-catechol dioxygenase or a 2,3-catechol dioxygenase in catechol, and when in protocatechuic acid can be cleaved by three enzymes in the 2,3 (meta), 4,5 (meta), and 3,4 (ortho) positions [73].

Muconic acid, the recent focus of dicarboxylic acids, can be converted into the dicarboxylic acid, adipic acid, through hydrogenation. Adipic acid is an important precursor of nylon, plasticizers, lubricants and polyester polyols. The production of muconic acid through the aromatic catabolic pathway results in higher atom efficiency than its production through the sugar pathway. When benzoate and glucose were fermented in a DO stat fed-batch culture of KT2440-CJ102 and the pH value was maintained at 7.0 for 124 h, muconic acid was produced at a titer of 34.5 g L⁻¹ [64]. By deleting *Crc* gene, encoding catabolite repression control protein, from *P. putida* KT 2440, muconate production is enhanced and the yield of muconate produced from pCA after 36 h was increased nearly 70% and the yield from FA after 72 h was more than doubled [66]. Moreover, pCA and FA from alkaline pretreated lignin were converted by engineered *P. putida* KT2440 to 0.70 g L⁻¹ of muconic acid in 24 h and the molar yield was 67% [64]. Barton [67] and Sonoki [68] developed an engineered strain that could produce muconic acid with lignin or lignin hydrolysate. Recent research indicates that insufficient protocatechuic acid decarboxylase activity is considered to be the bottleneck in muconic acid production. Studies on the increase of protocatechuic acid decarboxylase activity have been performed for the improvement of muconic acid production. The results indicate that the muconic acid production could be increased by 50% with pCA as substrate after the improvement of protocatechuic acid decarboxylase activity [65].

The “upper pathways” in aromatic-compounds degrading organisms are utilized to integrate or funnel the heterogeneous lignin-depolymerizing slurry into a few common intermediates, such as CA (1,2-dihydroxybenzene) or pCA (3,4-dihydroxybenzoate).
Cleaving the aromatic rings gave rise to different products through the ortho (intradiol) or meta (extradiol) patterns, which are funneled into TCA cycle. The metabolic pathways of various ring-opened chemicals enter the central metabolism with different carbon efficiency and redox balance [73]. The replacement of the protocatechuate (PCA) ortho pathway in *P. putida* K12440 with a meta-cleavage pathway from *Sphingobium* sp. SYK-6 results in a nearly five-fold increase in the yield of pyruvate, which indicates that the catabolic pathway from ring-cleavage to TCA cycle could be selected to optimize the yield of a desired product [74]. With synthetic lignin, *Phanerochaete chrysosporium* (white rot fungus) was demonstrated to accumulate succinic acid by the short-cut TCA cycles, providing a potential strain for the future lignin biorefinery [75].

The recent and rapid progress in prokaryotic lignin depolymerizing enzymes suggests that lignin valorization, involving fatty acid metabolism, might result in potential applications in the preparation of biofuels and biodegradable materials. Utilizing lignin through the fatty acid and lipid synthesis pathway began with the study on lignin model compounds (4-hydroxybenzoic acid, resorcinol, vanillic acid), and then has led to use of natural lignin in different microorganisms, such as *Aspergillus fumigatus* [76], *Cunninghamella echinulata* FR3 [28], *Rhodococcus opacus* [77, 78], *Rhodococcus jostii* [79], and *Trichosporon oleaginosus* [80]. The synergy between laccase and microbial lignin conversion and co-fermentation of two *R. jostii* strains have been examined in lipid production [79, 81]. Lipid accumulation by *Rhodococcus opacus* DSM 1069 reached 26.99 ± 2.88% of its cellular dry weight by utilizing pine organosolv pretreatment effluent [82]. Linger [53] reported that the alkaline depolymerized lignin could be converted by *Pseudomonas putida* KT2440 into medium chain length (mcl)-PHAs through integrated biological funneling pathway, and another *Pseudomonas putida* strain A514 was engineered to improve the yield of PHA on the basis of genomic and proteomic analysis, in which PHA content reached 73% per cell dry weight [83]. Shi [84] and Kumar [85] reported that *Cupriavidus basilensis* B-8 and *Pandoraea* sp. ISTKB could also bioconvert Kraft lignin into PHA. All these metabolic conversions provide a roadmap for the further research on lignin biotransformation and suggest that more value-added products will one day be produced from lignin.

**COMBINING ENZYMATIC CATALYSIS AND METABOLIC ENGINEERING WITH A MULTI-DISCIPLINARY APPROACH FOR LIGNIN VALORIZATION**

Given the environmentally friendly and substrate-specific characterization, lignin valorization with enzymatic catalysis and metabolic engineering for the production of high value-added chemicals represent an attractive target. However, the economic feasibility
and efficiency of such processes remains a major challenge. Consequently, a multidisciplinary approach relying on biology, chemistry and physics will be needed to develop practical process for lignin biological valorization (Figure 3).

The “funneling pathway” in microorganisms represents an practical approach for integrating different aromatic substances, obtained through lignin depolymerization, into some central intermediates for high-value chemical generation [52, 53]. The tools of synthetic biology and systems biology, such as biosensors [86-89], “-omic” technologies [90], and tolerance engineering [91], can be applied to reconstruct the metabolic pathways to further improve the yield of target products. Next generation industrial biotechnologies (NGIB) represent a promising method for improving the economic feasibility of fermentation processes. Costs can be reduced by continuous bioprocessing under non-sterile (open) conditions using ceramic, cement or plastic bioreactors. Most lignin-utilizing microorganisms are aromatic-toxicity tolerant matching the NGB requirements [92].

It is necessary to generate as many different lignin monomers and oligomers as possible using suitable pretreatment methods to improve the biotransformation efficiency of lignin. Unfortunately, the degradation of lignin using radical lignolytic enzymes requires weeks [93-94] and, thus, is unsuitable for the industrial applications. Chemical depolymerization usually results in the ring opening or can cause the competing re-polymerization of lignin-depolymerized products, thus, it is inefficient for lignin monomer production. More recently, the formic acid [95] and formaldehyde [96] processes, developed to produce lignin monomer in high yield, have recently become available. These pretreatments or depolymerization technologies may afford promising processes for lignin valorization through the combination of chemical depolymerization and biotransformation.

In addition to combining biotransformation and chemical catalysis in lignin depolymerization, industrial demands for saving energy and resources will also require the combination of the biotransformation and chemical or physical methods in other process steps. These combinations leverage the specificity of microorganisms and advantages of continuous reactions associated with chemical catalysis, thus, overcoming the drawbacks of either a purely chemical or biological approach for the industrial production of high-value chemicals from lignin. Studies by Linger [53] and Vardon [97] provide successful examples for the application of combination technologies in lignin bio-valorization, in which ncl-PHA and cis, cis-muconic acid from lignin bioconversion were used to produce alkenoic acids and adipic acid by chemical catalysis. Furthermore, the synthesis of picolinic acid, 2,5-PDCA and 2,4-PDCA [71] using in vivo metabolic conversion have also involved in such combinational technologies. Furthermore, besides the application in microorganism, the “funneling pathway” strategy can also be used to pool the high-value alkylenols from pyrolysis bio-oil, generated from lignocellulosic biomass, which is referred to as the chemical “funneling pathway” [98]. The biotransformation of cellulose sugar could also provide many valuable clues to new approaches for lignin biological valorization, such as one pot conversion of platform molecules [99-100].
CONCLUSION

The insolubility and structural heterogeneity inherent to lignin and the complexity of its depolymerization provides a major challenge for lignin valorization. Recently, the enzymatic catalysis and metabolic engineering of lignin valorization provide some approaches for lignin bioconversion into high-value-added chemicals. These new approaches promise the generation of more varieties of lignin-derived high-value chemicals in the near future. However, enzymatic conversion in vitro shows its lowest efficiency towards natural lignin. This calls for studies on the mechanism of enzymatic catalysis and the identification of more β-etherases through protein engineering and gene mining. In addition, the chemical yields from natural lignin using metabolic conversion cannot meet the demands of industry. Thus, natural lignin will need to be depolymerized into more aromatic monomers or oligomers for the efficient bioconversion. Ultimately, the economic feasibility and effectiveness of industrial production will undoubtedly require the combination of biological, physical and chemical technologies for lignin biological valorization.

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REFERENCES


[40] Parke, D. & Ornston, L. N. (2003). “Hydroxycinnamate (hca) catabolic genes from Actinobacter sp. strain ADP1 are repressed by HcaR and are induced by hydroxycinnamoyl-coenzyme A thioesters.” Appl Environ Microbiol, 69(9), 5398-5409.


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