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# Biotransformation of lignin into 4-vinylphenol derivatives toward lignin valorization

Ruo-Ying Liu,[a,b] Zhi-Hua Liu,*[a,b] Bing-Zhi Li *[a,b] and Ying-Jin Yuan [a,b]

As the most abundant renewable aromatic resource, lignin is an ideal precursor for the production of aromatic fine chemicals. Biotransformation of lignin into 4-vinylphenol (4VP) derivatives holds the promise to contribute to a lignin-based economy. However, the limited bioavailability of lignin and the absence of an effective biotransformation route for lignin to 4VPs have hindered the valorization of lignin and the production efficiency of 4VPs. This review aims to explore an atom-economic biotransformation route for lignin to 4VPs by summarizing the state-of-the-art technologies involved in the entire process, from upstream to downstream. Effective depolymerization technologies have enabled the generation of favorable lignin-derived aromatic precursors for the biosynthesis of 4VPs. Additionally, these technologies also yielded oligomers or monomers that can serve as carbon sources for the cell growth of host strains. Furthermore, the exploration of key enzymes and the construction of effective microbial cell factories have facilitated the assimilation of lignin derivatives and resulted in improved yields and titers of 4VPs. Emerging technologies have further enhanced the biotransformation of lignin by regulating metabolism efficiency, improving the stability and activity of key enzymes, and mitigating inhibitory effects. Overall, by harnessing the inherent aromatic value of lignin, the biotransformation of lignin-derived aromatic precursors into 4VPs offers a promising and sustainable route for 4VPs biosynthesis and lignin valorization. This approach aligns with the atom-economic concept and holds great potential.

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## 1 Introduction

Lignin is the primary constituent of lignocellulosic biomass, providing structural support to plants and aiding in the transportation of nutrients and water within plant tissues. Lignin accounts for about 15–40% of the dry weight and 40% of the energy of terrestrial plant biomass, and it is the largest renewable aromatic resource in nature.[1,2] Approximately 150 billion tons of lignin are produced globally each year, with over 50 million tons being generated as a by-product of the biorefinery, and the pulp and paper industries.[3,4] However, the vast majority of these lignins are discharged as a solid waste or only burned for electricity production.[3,5] With the booming development of lignocellulosic biorefineries, more lignin will be generated. The efficient transformation of lignin will improve the competitiveness of the biorefinery and thus contribute to the bioeconomy.[6]

4-Vinylphenol derivatives (4VPs) such as 4-vinylphenol (4VP), 4-vinylguaiacol (4VG) and 4-vinylsyringol (4VS) are natural aromatic compounds that are widely found in the plant kingdom. These 4VPs have been certified as safe for ingestion by the Flavor and Extract Manufacturer's Association and they have a wide range of applications in the food, perfumery, beverage, flavor, pharmaceutical and polymer industries (Fig. 1). For instance, 4VPs have shown promising biological activities and are increasingly valued in the pharmaceutical industry. Specifically, 4VP has the potential to serve as an effective antifungal agent against the fungus that affects conifer trees.[7] Additionally, 4VG could be a potential candidate for combating colorectal cancer.[8] Furthermore, 4VS has demonstrated superior antioxidant activity compared to common antioxidants like α-tocopherol, vitamin C, and β-carotene.[9] This makes it an excellent natural food preservative and a highly promising agent for diseases associated with oxidative stress. In the materials field, 4VPs play a crucial role as precursors in polymers synthesis. These polymers are extensively utilized in microelectronics, including applications such as chemical sensors, water-stable organic transistors, resistor–capacitor filters, photoresists, and atomic switches.[10–14] Additionally, 4VPs have the potential to serve as bio-based alternatives to petroleum-based styrene. This is particularly significant considering the significant production volume of polystyrene, a major component in plastic production. Given the current production scale of hundreds of millions of tons of

[a]*Frontiers Science Center for Synthetic Biology and Key Laboratory of Systems Bioengineering (Ministry of Education), School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China. E-mail: zhliu@tju.edu.cn, bzli@tju.edu.cn*
[b]*Frontiers Research Institute for Synthetic Biology, Tianjin University, China*

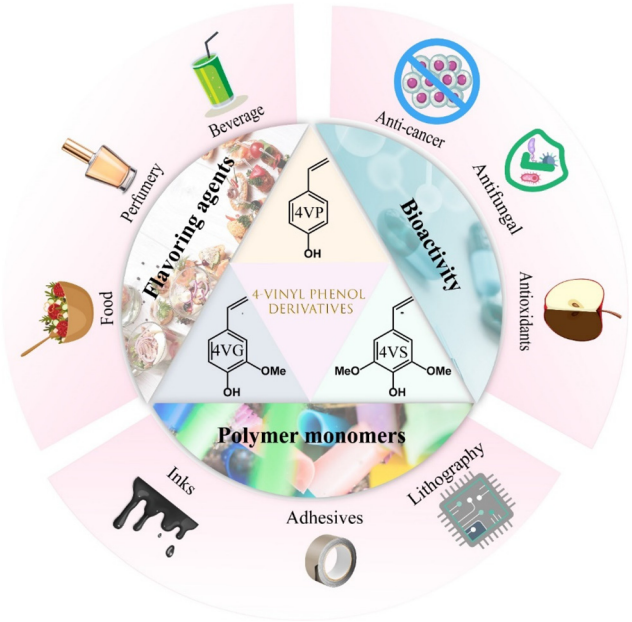


**Fig. 1** Chemical structures of 4-vinylphenol derivatives and their potential applications as flavoring agents, bioactivity, and polymer monomers.

plastics annually and the depletion of petroleum resources, the development of bio-based plastics from renewable sources is essential in reducing carbon footprints.[15,16] Furthermore, poly(4-vinylguaiacol) exhibits excellent biodegradability and strength properties, positioning it as a highly promising bio-based polymer material.[17] As a result, it is crucial to explore environmentally friendly and sustainable manufacturing methods for 4VPs.

Recently, the biological valorization of lignin to value-added products has become a current research hotspot. Ligninolytic microorganisms have evolved the inherent multiple assimilation pathways of lignin derivatives to produce specific fine aromatic chemicals, fuels and materials.[18] Most importantly, biotransformation of lignin derivatives by retaining its inherent aromatic rings is undoubtedly the way forward to open up atomically economic conversion routes for lignin valorization. Fortunately, many microorganisms in nature have evolved enzyme toolboxes for the transformation of lignin-derived aromatic acids to yield aromatic alkenes such as 4VPs. For example, phenolic acid decarboxylase (PAD) can be an excellent biocatalyst to catalyze the non-oxidative decarboxylation of aromatic acids to yield 4VPs.[19] The microbial synthesis of 4VPs from inexpensive lignin-derived aromatic precursors can omit the synthesis process of energy-intensive benzene ring, shortening the metabolic pathway of 4VPs, and improving the transformation efficiency of lignin derivatives. Despite these potentials, the biotransformation efficiency of lignin-derived precursors into 4VPs is still unsatisfactory, and the development of safe and green biocatalysts, coupled with advanced strategies, is still needed to improve the microbial synthesis of 4VPs toward lignin valorization.

This review thus aims to summarize the state-of-the-art biotransformation strategies of lignin derivatives to 4VPs by prospecting the atom economic conversion route. The inherent aromatic ring value of lignin was exploited to synthesize aromatic alkenes by only modifying the side chains of lignin-derived precursors. The emerging biotransformation strategies of lignin to 4VPs had been systematically reviewed from enzyme biocatalyst to microbial cell factory and finally whole-cell biocatalyst. After that, a series of advanced strategies and techniques had been introduced to improve lignin bioavailability and 4VPs production efficiency. The challenges and prospects in the lignin biotransformation toward 4VPs had been discussed, which will guide the design of the conversion route to improve the economic viability of 4VPs biosynthesis.

## 2 Lignin derivatives as ideal precursors for the biosynthesis of 4VPs

Lignin is a complex heterogeneous aromatic polymer, which is formed by the radical coupling reactions of three phenylpropanoid units, namely *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), *via* carbon–carbon and ether bonds (Fig. 2A).[18] Carbon–carbon bonds include β-5 (phenylcoumaran), β-β (resinol), β-1 (spirodienone), *etc*. Ether bonds mainly include β-O-4 (β-aryl ether), 4-O-5 (diaryl ether), α-O-4 (α-arylether), *etc*. Among them, β-O-4 is the most dominant linkage in natural lignin, accounting for 50–80% of the total linkages.[20,21] Due to its complex macromolecular structure, native lignin has limited bioavailability. To overcome this, it is thus essential to employ sustainable and effective lignin depolymerization strategies to depolymerize the lignin polymer and prepare highly bioavailable aromatic derivatives. These derivatives could be further assimilated in a biological funnel pathway toward the biosynthesis of 4VPs.[18]

### 2.1 Lignin depolymerization to give aromatic monomers and dimers

Only aromatic monomers or oligomers can pass through the microbial cellular membranes and be assimilated to synthesize target products.[22] Various physical and chemical strategies have been developed to depolymerize lignin.[23] Recently, more research has gradually shifted toward efficient access to aromatic monomers. For example, *p*-coumaric acid (*p*-CA) was successfully isolated from corn straw using γ-valerolactone extraction, followed by alkali hydrolysis of the lignin fraction.[24] A *p*-CA yield of 4.8% was obtained with a purity of 97% after purification.[24] The integration of pyrolytic pretreatment and enzymatic hydrolysis strategy can significantly increase the yield of *p*-CA and ferulic acid (FA) from pretreated corn straws, ultimately reaching 2.71 and 2.18 g $L^{-1}$, respectively.[25] Moreover, oxidative alkaline depolymerization of lignin directly obtained vanillin from softwoods, while it produced

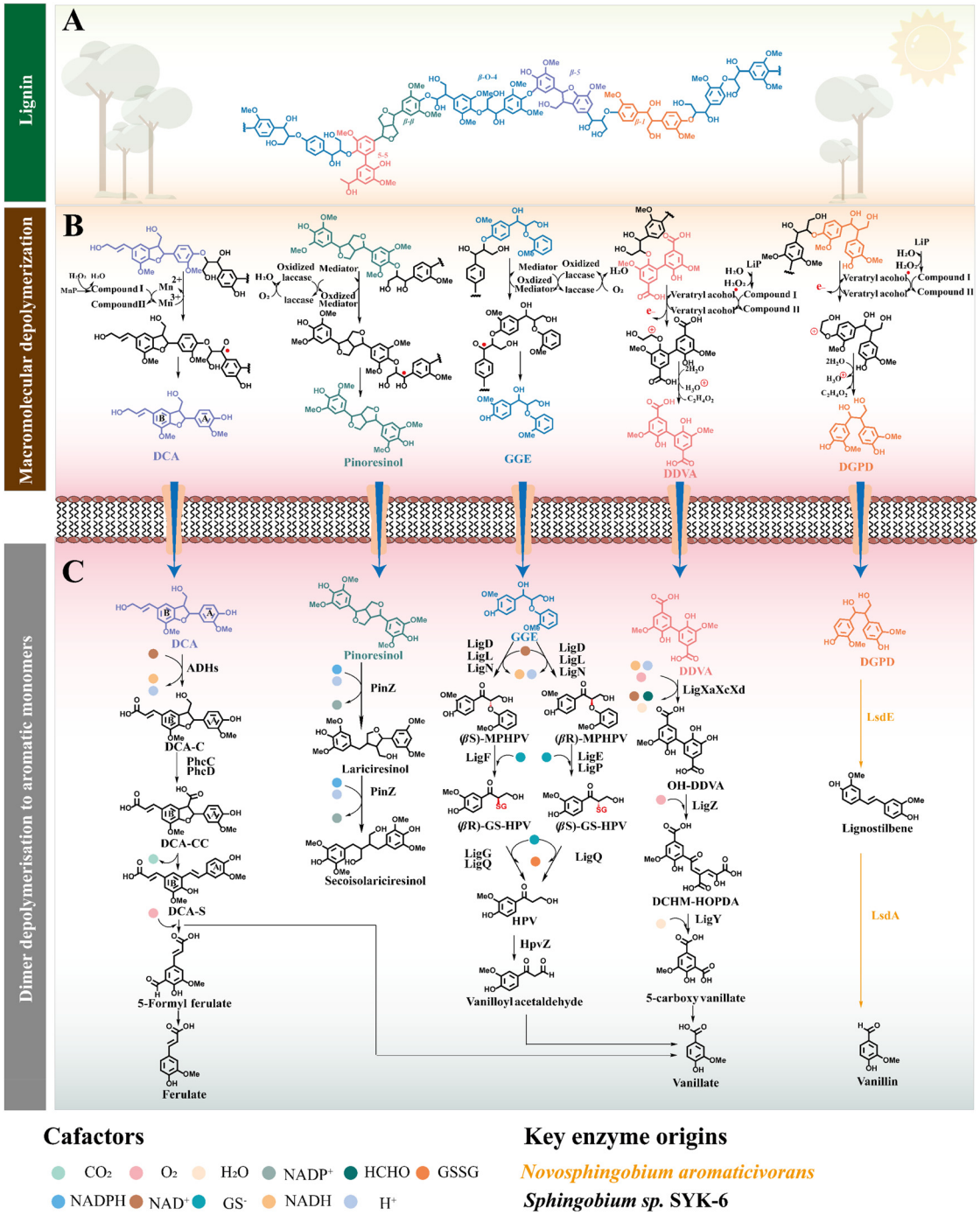


**Fig. 2** Major biodegradation pathways of lignin macromolecule to obtain aromatic monomers. (A) Schematic diagram of chemical structure of lignin. (B) The potential *in vitro* depolymerization of lignin by ligninolytic enzymes. Manganese peroxidases (MnP), lignin peroxidases (LiP), and laccases randomly attack lignin macromolecules through enzymatic oxidation to yield lignin-derived dimers. (C) Metabolic pathways of lignin-derived model dimers *in vivo*. ADH, alcohol dehydrogenase; DCA, dehydrodiconiferyl alcohol; DCA-C, 3-(2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-7-methoxy-2,3-dihydrobenzofuran-5-yl)acrylate; DCA-CC, 5-(2-carboxyvinyl)-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylate; DCA-S, 3-(4-hydroxy-3-(4-hydroxy-3-methoxystyryl)-5-methoxyphenyl)acrylate; DCHM-HOPDA, 4,11-dicarboxy-8-hydroxy-9-methoxy-2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate; DDVA, 5,5′-dehydrodivanillate; DGPD, diguaiacylpropanediol; GGE, guaiacylglycerol-β-guaiacyl ether; GS-HPV, α-glutathionyl-β-hydroxypropiovanillone; HPV, β-hydroxypropiovanillone; HpvZ, β-hydroxypropiovanillone oxidase; LigD, LigL and LigN, Cα-dehydrogenases; LigF, LigE and LigP, β-etherases; LigG and LigQ, glutathione S-transferases; LigXaXcXd, three-component *O*-demethylase; LigY, 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicar-boxybiphenyl *meta*-cleavage compound hydrolase; LigZ, 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicar-boxybiphenyl dioxygenase; LsdA, carotenoid oxygenase; LsdE, *erythro*-1,2-diguaiacylpropane-1,3-diol γ-formaldehyde lyase; MPHPV, α-(2-methoxyphenoxy)-β-hydroxypropiovanillone; OH-DDVA, 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicarboxybiphenyl; PhcC, (+)-3-(2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-7-methoxy-2,3-dihydrobenzofuran-5-yl)-acrylate oxidase; PhcD, (−)-3-(2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-7-methoxy-2,3-dihydrobenzofuran-5-yl)acrylate oxidase; PinZ, pinoresinol/lariciresinol reductase.

 

G- and S-type aromatic derivatives such as vanillin, vanillic acid, syringaldehyde and syringic acid from hardwoods.[26] Therefore, various lignin-derived aromatics can be obtained directly from lignocellulosic biomass using chemical processing. Chemical depolymerization of lignin also yielded various dimers. For example, 36 dimers were identified in the reductive catalytic fractionation of pine wood, accounting for about 16 wt%.[27] However, these depolymerization strategies are often accompanied by the generation of complex heterogeneous mixtures, unnatural products and even condensed structures, which are not suitable for subsequent bioconversion.[28] Therefore, new expertise in lignin depolymerization is urgently being developed to meet the needs of lignin biotransformation.

Biological depolymerization of lignin is a promising method for the acquirement of bioavailable aromatic derivatives due to its specificity, gentleness and environmental friendliness. In natural environments, microorganisms have developed diverse ligninolytic enzymes and metabolic pathways. This allows for efficient degradation of lignin either by individual microorganisms or through the collaborative interactions of microbial communities.[29] Ligninolytic fungi are superior choices for the depolymerization of lignin macromolecules due to their more comprehensive range of powerful oxidative enzymes.[30] Remarkably, white-rot fungi can secrete ligninolytic enzymes to initiate free radical-based oxidation for the cleavage of the bonds in lignin, specifically the C–C and C–O bonds. This process results in the depolymerization of the lignin macromolecule, breaking it down into oligomers and aromatic monomers.[30] The white rot fungus *Phanerochaete chrysosporium* serves as a notable example of a lignin-degrading model. It has been effectively employed in the pretreatment of corn stover, resulting in a significant reduction of lignin content by up to 34.3%.[31]

The involvement of extracellular ligninolytic enzymes, including laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP), plays a vital role in the biodepolymerization of lignin through their own unique catalytic mechanisms (Fig. 2B).[32] Lac utilizes oxygen or small molecules with electron transfer capabilities as a medium to generate a large number of free radicals for the oxidation of both phenolic and non-phenolic structures.[33] MnP oxidizes $Mn^{2+}$ to $Mn^{3+}$ with the help of hydrogen peroxide ($H_2O_2$), non-specifically attacking and oxidizing the phenolic structures in the lignin molecules. In line with MnP, LiP is triggered by $H_2O_2$ and oxidizes phenolic and non-phenolic chemicals in lignin.[34–36] These ligninolytic enzymes boost the depolymerization of the high-molecular-weight lignin polymers into aromatic dimers and monomers.[37] For example, laccase purified from *Bacillus* sp. PCH94 treated kraft lignin and yielded more FA, acetovanillone and three dimers.[38] Fortunately, various ligninolytic microorganisms can metabolize these lignin derivatives toward target products. While strains in nature have shown promising lignin degradation capabilities, there are still gaps in our understanding of lignin biodepolymerization. One of the challenges is deciphering the synergistic interactions and mechanisms of ligninolytic enzymes.[39] Furthermore, the limited molecular manipulation tools available for fungi make it difficult to construct fungal ligninolytic enzyme-mediated biocatalysts for efficient lignin depolymerization. Although bacteria can be genetically modified more easily, it is necessary to engineer bacterial ligninolytic systems for effective lignin depolymerization.[33]

Recently, a series of combined strategies were developed to achieve more efficient depolymerization of lignin.[28] For example, a combined chemical–biological approach was developed to obtain highly bioavailable lignin derivatives.[40,41] The combined depolymerization of alkali and mixed-enzyme yielded 85% soluble lignin, and efficient depolymerization of lignin is achieved by promoting the cleavage of β-O-4 and β-5 bonds.[42] However, the repolymerization reaction could be occurred during lignin depolymerization, presenting an obstacle that limits the biological valorization of lignin.[29] Several effective solutions have been suggested to address this issue. One such solution involves the utilization of the carbonium ion scavenger, 3-hydroxy-2-naphthoic acid, as a blocker during chemical depolymerization. This method can enhance the presence of carboxylic acid groups in the lignin structure and effectively hinder the repolymerization of lignin.[43] Meanwhile, it significantly reduced the molecular weight of lignin by promoting the cleavage of β-β, β-5 and β-O-4 bonds.[43] Additionally, the enzyme-cell system was developed to facilitate the transformation efficiency of depolymerized lignin.[44] The growth of *Rhodococcus opacus* PD630 was significantly enhanced with the assistance of laccase treatment of lignin, indicating the improved conversion efficiency of lignin.[44] Undoubtedly, these strategies offer potential solutions to the current challenges of lignin depolymerization. By efficiently depolymerizing lignin, a significant number of aromatic derivatives can be obtained. These derivatives have the potential to serve as ideal precursors for the biosynthesis of 4VPs.

### 2.2 Intracellular catabolic pathways of lignin-derived dimers

The depolymerization of lignin will generate a variety of aromatic dimers, it is necessary to prospect the biotransformation route of lignin-derived aromatic dimers toward lignin valorization (Fig. 2C). A number of key enzymes and genes involved in the biotransformation of these aromatic dimers have been characterized.[45] *Sphingobium* sp. SYK-6 discovered in pulp effluent is able to employ lignin-derived aromatics as only carbon sources. Especially, it can utilize these aromatic dimers such as β-aryl ether, phenylcoumaran, biphenyl and diarylpropane.[46]

The β-O-4 model compound, guaiacylglycerol-β-guaiacyl ether (GGE), has four stereoisomers. *Sphingobium* sp. SYK-6 is capable of converting them into achiral β-hydroxypropiovanillone (HPV) through a series of three stereospecific reactions: oxidative dehydrogenation, ether bond cleavage, and thioether bond cleavage. The oxidative cleavage is realized by three $C_\alpha$-dehydrogenases encoded by LigD, LigL, and LigN, by which guaiacylglycerol-β-guaiacyl ether (GGE) is converted into α-(2-methoxyphenoxy)-

β-hydroxypropiovanillone (MPHPV) with two stereoisomers. Subsequently, the stereoselective glutathione S-transferases, namely LigF, LigE and LigP, mediate the cleavage of the ether bond by affinity attack of glutathione to generate two different stereoisomers of α-glutathionyl-β-hydroxypropiovanillone (GS-HPV) and guaiacol. Finally, another glutathione S-transferases, LigG and LigQ, break catalyze the thioether bond of α-glutathionyl-β-hydroxypropiovanillone (GS-HPV) to generate non-stereoselective β-hydroxypropiovanillone (HPV), which is eventually converted to vanillic acid easily remitted into the assimilation pathway of aromatic monomers.[47] The β-5 model compound, dehydrodiconiferyl alcohol (DCA), undergoes successive enzymatic oxidation reactions to form 5-(2-carboxyvinyl)-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylate (DCA-CC) by catalyzing two alcohol hydroxyl groups in its structure to generate two carboxyl groups. Such intermediate is decarboxylated to form an alkene bond that is oxidatively cleaved to produce vanillin and 5-formylferulate.[45] The 5-5 model compound of 5,5′-dehydrodivanillate (DDVA) is firstly catalyzed by a three-component monooxygenase LigXaXcXd to give 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicarboxybiphenyl (OH-DDV). Subsequently, an aryl ring of this resulting biphenyl structure was oxidatively cleaved at *meta* position by LigZ-encoded dioxygenase and further underwent LigY-mediated enzymatic hydrolysis to yield 5-carboxyvanillate. 5-Carboxyvanillate is finally converted to vanillic acid by enzymatic decarboxylation.[45] The complete assimilation pathway of β-β model compound of pinoresinol has not been revealed, but a preliminary pinZ-catalyzed two-step reductive cleavage of ethers was explored in *Sphingobium* sp. SYK-6.[48] *Novosphingobium aromaticivorans* had evolved the two-step enzymatic degradation pathway of the β-1 model compound of diguaiacylpropanediol catalyzed by LsdE and LsdA to yield two equivalents of vanillin.[49]

These results highlighted that the prospected metabolism pathways of ligninolytic microorganisms enabled the biotransformation of lignin-derived aromatic dimers into platform aromatic monomers. These monomers can serve as promising precursors of valuable products or suitable carbon sources for cell growth.

### 2.3 Biological funnel pathways of different types of aromatic monomers

Exploiting advanced lignin bioconversion routes is crucial to promote lignin valorization and contribute to lignin-based bioeconomy.[50] Interestingly, lignin depolymerization produces large amounts of aromatic monomers such as *p*-CA, FA and sinapic acid and they are promising precursors for the biosynthesis of 4VPs through biological decarboxylation reaction. Therefore, such a conversion route of lignin derivatives meets the concept of high atom economy by preserving the aromatic ring structure of lignin toward aromatic products of 4VPs (Fig. 3A).

What's more, other lignin-derived aromatic monomers can serve as energy-providing carbon sources to promote microbial cell growth. Wild ligninolytic bacteria in nature such as *Pseudomonas putida* KT2440 and *Sphingomonas* sp. SYK-6 have evolved efficient metabolic systems of lignin-derived aromatic monomers. *P. putida* KT2440 has the intrinsic degradation pathways of H- and G-type aromatic monomers, while *Sphingomonas* sp. SYK-6 has the degradation pathways of H-, G-, and S-type aromatic monomers. They are popular chassis bacteria for the bioconversion of lignin to high value products.[51,52]

Notably, lignin-derived H- and G-type monomers, such as *p*-hydroxybenzaldehyde and vanillic acid, can be first converted to platform compound protocatechuic acid (PCA) *via* "biological funneling". PCA was assimilated *via* three ring-cleavage pathways, including the 2,3-cleavage pathway, the 3,4-cleavage pathway and the 4,5-cleavage pathway.[18] *P. putida* KT2440 cleaves PCA *via* the PCA 3,4-dioxygenase PcaHG-mediated 3,4-cleavage pathway.[53] Artificially designed 3,4-cleavage pathway produced many high-value open-cyclic compounds such as β-ketoadipic acid and muconic acid.[54,55] *Paenibacillus* sp. strain JJ-1b can cleave PCA through PCA 2,3-dioxygenase PraA in the 2,3-cleavage pathway, eventually generating central metabolites.[56] The 4,5-cleavage pathway of PCA has been studied in *Sphingobium* sp. SYK-6 and *N. aromaticivorans*. The PCA 4,5-dioxygenase LigAB mediated the conversion of PCA to 4-carboxy-2-hydroxymuconate-6-semialdehyde.[57,58]

Inconsistently, the lignin-derived S-type monomers, such as syringic acid, is first transformed into the platform compound gallic acid and subsequently entered into the ring cleavage metabolic pathway.[18] The microbial assimilation pathway of S-type monomer has been explored in *Sphingomonas* sp. SYK-6. Syringic acid is first catalyzed by the tetrahydrofolate-dependent *O*-demethylase, namely DesA, to 4-oxalomesaconate, which is directly assimilated into the PCA 4,5-cleavage pathway.[58,59] The ability of the engineered *P. putida* KT2440 to utilize syringic acid had been demonstrated through chromosomal overexpression of VanAB and adaptive laboratory evolution.[60,61] Other aromatic derivatives, such as phenol and guaiacol, can enter the central metabolism through the generation of the platform compound catechol. Catechol is assimilated through *ortho*-cleavage and *meta*-cleavage pathway to support the cell growth (Fig. 3B).[62]

Overall, these results highlighted that an atom-efficient transformation route of lignin derivatives had been prospected for the biosynthesis of 4VPs, providing a green and sustainable way for both lignin valorization and 4VPs production. Lignin polymer can be depolymerized into some promising aromatic compounds of *p*-CA, FA and sinapic acid, which can serve as suitable precursors for the biosynthesis of 4VPs. Other lignin derivatives from depolymerization can be used as energy sources for biocatalysts and carbon sources for cell growth to maintain the biological production of 4VPs. This biotransformation strategy of lignin omitted the energy-intensive synthesis process of the benzene ring and shortened the biosynthetic pathway of 4VPs. However, more efforts are needed to improve the feasibility of the biotransformation of lignin to 4VPs. The lignin fraction produced by chemical depolymeriza-

 

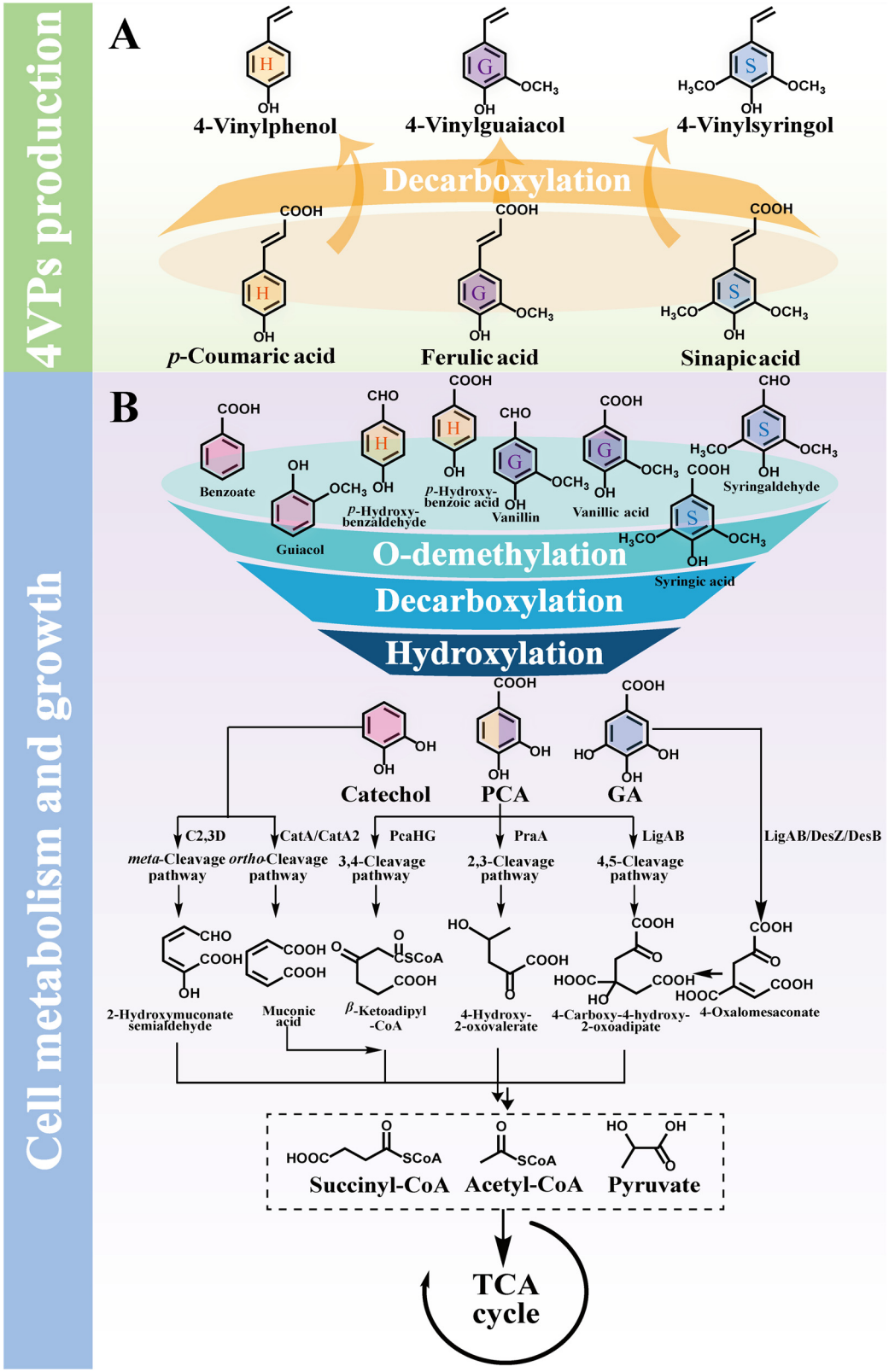


**Fig. 3** An atom-economic biotransformation route of lignin valorization towards 4-vinylphenol derivatives. (A) Lignin-derived *p*-coumaric, ferulic and sinapic acid were enzymatically decarboxylated to yield high values of 4-vinylphenol, 4-vinylguaiacol and 4-vinylsyringol, respectively. (B) Other lignin derivatives can undergo *O*-demethylation, decarboxylation and hydroxylation to produce the platform compounds that subsequently enter the tricarboxylic acid cycle *via* cleavage pathways. CatA/CatA, catechol 1,2-dioxygenase; C2,3D, catechol 2,3-dioxygenase; DesB, gallic acid dioxygenase; DesZ, 3-*O*-methylgallate 3,4-dioxygenase; GA, gallic acid; LigAB, protocatechuate 4,5-dioxygenase; PCA, protocatechuic acid; PraA, protocatechuate 2,3-dioxygenase.

tion still requires additional processing steps to improve the bioavailability of lignin derivatives due to its condensation and low reactivity. In addition to this, the concentration of lignin monomers is still unsatisfactory from the depolymerization and is a limiting factor for lignin valorization. The microbial depolymerization pathways and enzymatic reaction mechanisms are still not fully elucidated, limiting the process coupling of lignin biodegradation and aromatic alkene biosynthesis. It is thus necessary to address several limitations to further maximize the utilization efficiency of lignin and improve the biosynthesis efficiency of 4VPs. The effective depolymerization technology of lignin, such as the chemical–biological depolymerization strategy, is required to release more lignin-derived precursors such as *p*-CA, FA and sinapic acid. More notably, the discovery of new lignin degradation mechanisms and the enrichment of metabolic network information can strengthen the upstream process of biological lignin valorization. For the downstream process, it is important to construct comprehensive metabolic pathways to fully convert the three types of lignin-derived monomers. It is very important to improve the catalytic activity of rate-limiting enzymes in the metabolic pathways of S-type monomers. These together could

**Table 1** The biosynthesis of 4-vinylphenol derivatives from lignin-derived aromatic precursors

| Target products | Precursors | Gene sources | Bioconversion modes | Strategies | Titer ($g\ L^{-1}$) | Conversion yield (%) | Ref. |
|---|---|---|---|---|---|---|---|
| 4-Vinylphenol | *p*-Coumaric acid | *Bl*PAD | Free enzyme | Biphasic organic/aqueous system | 60.63 | 97.02 | 65 |
| | *p*-Coumaric acid | *Bc*PAD | Whole-cell catalysis (*E. coli*) | Biphasic organic/aqueous system | 20.5 | — | 88 |
| | *p*-Coumaric acid | *Bs*PAD | Free enzyme | Application of deep eutectic solvents | — | 75 | 93 |
| | *p*-Coumaric acid | *Ba*PAD | Microbial cell factory (*E. coli*) | Heterologous expression of enzymes<br>Biphasic organic/aqueous system | 31.9 | 88.7 | 64 |
| | Corn cob alkaline hydrolysate | *Lp*PAD | Microbial cell factory (*E. coli*) | Heterologous expression of enzymes<br>Biphasic organic/aqueous system | 1.0035 | — | 81 |
| | Phosphoric acid-swollen cellulose | *Rs*TAL; *Ss*PAD; *Tf*EG | Microbial cell factory (*S. lividans*) | Heterologous expression of enzymes | 0.25 | — | 83 |
| | *p*-Coumaric acid | *Ba*PAD | Microbial cell factory (*C. glutamicum*) | Heterologous expression of enzymes<br>Biphasic organic/aqueous system | 187 | 90 | 19 |
| | *p*-Coumaric acid | *Bl*PAD | Whole-cell catalysis (*E. coli*) | Bacterial surface display system | — | 80.4 | 89 |
| 4-Vinylguaiacol | Ferulic acid | *Bl*PAD | Free enzyme | Biphasic organic/aqueous system | 58.30 | 70.96 | 65 |
| | Ferulic acid | *Ba*PAD | Immobilized enzyme | Affinity-Immobilized enzyme<br>Biphasic organic/aqueous system | 295.8 | 98.95 | 72 |
| | Ferulic acid | *Bs*PAD | Free enzyme | Application of deep eutectic solvents | — | 97 | 93 |
| | Green value protobind lignin | *Bs*PAD | Microbial cell factory (*P. putida* KT2440) | Heterologous expression of enzymes<br>Knockout of endogenous metabolic pathways | 0.0624 | — | 82 |
| | Ferulic acid | *Bl*PAD | Whole-cell catalysis (*E. coli*) | Resting cell system<br>Biphasic organic/aqueous system | 129.9 | 85.6 | 86 |
| | Ferulic acid | *Ba*PAD | Whole-cell catalysis (*E. coli*) | Fed-batch strategy<br>Biphasic organic/aqueous system | 237.3 | 98.9 | 87 |
| | Ferulic acid | *Bl*PAD | Whole-cell catalysis (*E. coli*) | Bacterial surface display system | — | 72.6 | 89 |
| | Ferulic acid | — | Whole-cell catalysis (*B. megaterium* LBI001) | In-Cell crosslinked enzymes | 32.7[a] | — | 84 |
| 4-Vinylsyringol | Sinapic acid | Ile85Ala mutant of *Bp*PAD | Free enzyme | Saturation mutagenesis<br>Biphasic organic/aqueous system | — | 99 | 92 |
| | Sinapic acid | Ile85Ala mutant of *Bs*PAD | Free enzyme | Application of deep eutectic solvents | — | 65 | 93 |

[a] The titer of product represents μmol.

 

make the biotransformation of lignin-derived aromatics to 4VPs feasible, which will contribute to the biological valorization of lignin.

## 3 Biotransformation of lignin into 4-vinylphenol derivatives

The biotransformation of lignin derivatives to 4VPs holds great promise for lignin valorization as it meets to atom economic conversion concept by retaining the inherent aromatic value of lignin. Exploiting state-of-the-art technologies and strategies is crucial to promote the conversion of lignin-derived aromatic precursors into a feasible biological lignin valorization (Table 1).

### 3.1 Enzyme biocatalysis strategies of lignin toward 4-vinylphenol derivatives

Enzyme biocatalysts are natural and highly powerful tools that enable sustainable molecular processing. These catalysts possess several advantages, including high substrate specificity, gentle reaction conditions and remarkable catalytic efficiency.[63] *In vivo*, enzymes play a significant role in defunctionalization and microbial transformation of lignin or its derivatives. *In vitro*, enzymes serve as environmentally friendly and sustainable catalysts, offering immense potential for converting lignin into valuable products in various industrial applications. Therefore, enzyme biocatalyst is one of the promising eco-sustainable solutions for the production of 4VPs from lignin-derived aromatic precursors.

A variety of bacteria and fungi have developed a non-oxidative metabolic pathway of lignin-derived aromatic acids to generate 4VPs through decarboxylation using decarboxylases (Fig. 4).[64] For example, phenolic acid decarboxylases (PADs) play a leading role in the decarboxylation in bacteria,[65,66] while phenylacrylic acid decarboxylase and ferulic acid decarboxylase 1 (FDC1) mainly catalyze the decarboxylation in *S. cerevisiae*.[67] Specifically, FDC1 is entirely responsible for the non-oxidative decarboxylation activity of aromatic substrates in the present of a diffusible cofactor of prenylated flavin. Phenylacrylic acid decarboxylase is responsible for catalyzing the synthesis of the cofactor of prenylated flavin. It utilizes dimethylallyl pyrophosphate as an isoprene donor and facilitates the flavin mononucleotide prenylation reaction.[68,69] The analysis of the atomic resolution crystal structure revealed that FDC1 exhibited the decarboxylation mechanism of

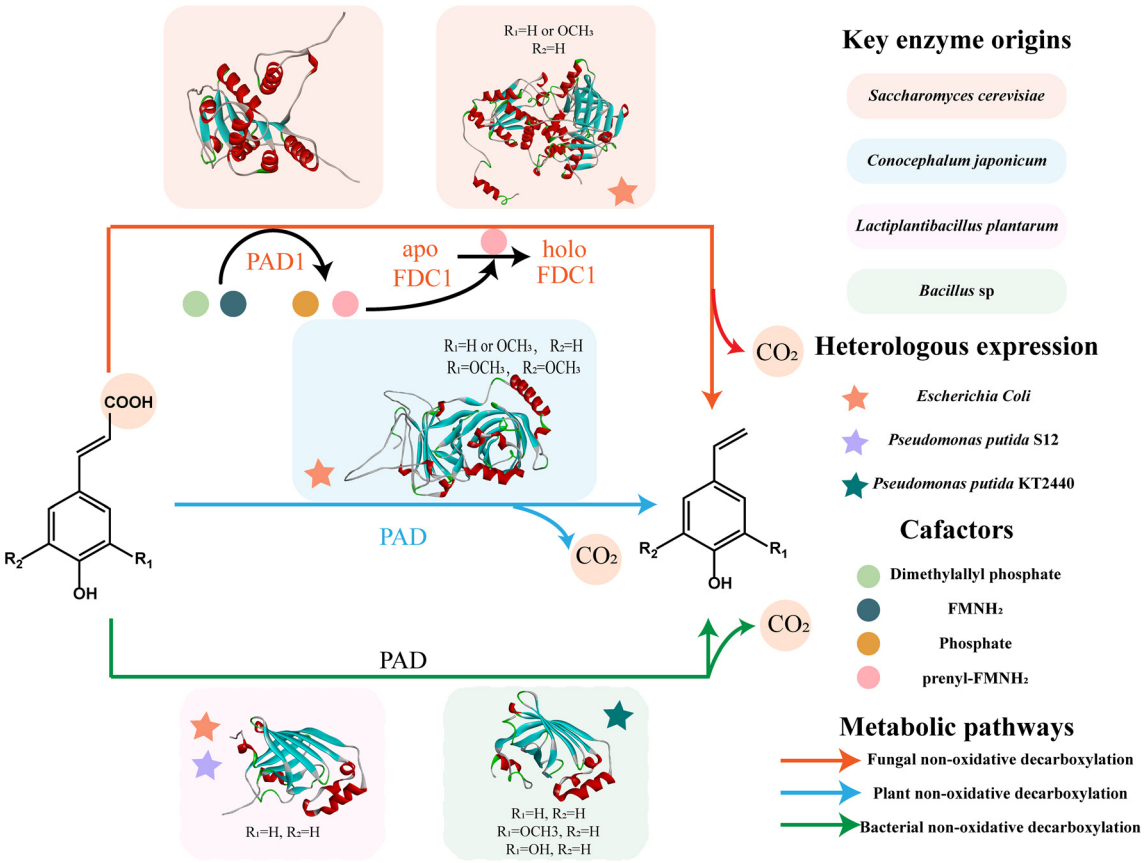


**Fig. 4** Key decarboxylase origins and microbial chasses for the production of 4-vinylphenol derivatives from lignin-derived precursors. Lignin-derived precursors are converted to the corresponding 4-vinylphenols by phenolic acid decarboxylases from fungi, plants and bacteria. The simulated structure of enzymes is obtained from databases Universal Protein (UniProt, https://www.uniprot.org/). FDC1, ferulic acid decarboxylase 1; PAD1, flavin prenyltransferase; PAD, phenolic acid dehydrogenase.

α,β-unsaturated acids through 1,3-dipole cycloaddition reactions.[67,68] Unlike fungal decarboxylases, bacterial PADs necessitate the existence of a phenolic hydroxyl group at the C4 position of the aromatic ring. However, they possess the advantage of high catalytic activity and stability and do not rely on any cofactor.[64] Based on this foundation, the catalytic mechanism of PADs has been hypothesized and confirmed. Initially, the phenolic hydroxyl group at the C4 position is deprotonated, and then a proton is transferred to the carbon at the nucleophilic C2 position, leading to the creation of an intermediate quinone methyl compound. Finally, the C–C bond is cleaved, resulting in the production of 4VPs and the release of one $CO_2$ molecule.[70] Due to their industrial production advantages, bacterial PADs are more commonly utilized for the biosynthesis of 4VPs.

A solvent-resistant *Bl*PAD was cloned from *Bacillus licheniformis* CGMCC 7172 and exhibited broad substrate specificity.[65] It catalyzed the decarboxylation of *p*-CA, FA, caffeic acid and sinapic acid to the corresponding 4VPs, although it had no catalytic activity for vanillic acid and cinnamic acid. This recombinant *Bl*PAD can efficiently catalyze the decarboxylation of 500 mM *p*-CA substrate, with a conversion yield of 97% in a biphasic system with an equal volume ratio of toluene-aqueous buffer. Meanwhile, the recombinant *Bl*PAD catalyzed the decarboxylation of 300 mM FA with a conversion yield of 95%. When the concentration of FA was increased from 300 to 500 mM, the conversion yield decreased from 95% to 71%.[65]

It is crucial to consider the stability and acquisition cost of enzymes in order to achieve an environmentally friendly and cost-effective bioproduction of 4VPs. The concept of green and sustainable manufacturing has spurred the advancement of immobilized enzymes for the synthesis of 4VPs. This approach enhanced the stability and the recycling capacity of the enzyme.[71] *Ba*PAD from *Bacillus atrophaeus* was affinity-immobilized on zeolite to successfully catalyze FA to 4VG.[72] The linker length, fusion orientation, selectivity and affinity were optimized, and the heterogeneous enzyme catalyst formed by a variant of *Ba*PAD fused with a specific linker peptide on Na–Y zeolite was obtained. It retained 73% of its original activity in a biphasic system after 10 cycles of reuse. Finally, it yielded a total of 295.8 g $L^{-1}$ 4VG with a 98.9% conversion of FA after 13 h in a 5 L bioreactor.[72] *Sco*FDC1 from *Schizophyllum commune* was heterologously expressed in *Komagataella phaffii* and immobilized on AminoLink Plus Agarose.[73] *Sco*FDC1 exhibits specific catalytic activity in the decarboxylation of FA to 4VG. When immobilized, the enzyme retained approximately 80% of the applied activity and maintained 20% of the initial activity even after 264 h and 40 000 passed bed volumes.[73]

Enzyme-catalyzed lignin transformation enables specific reactions to be targeted, minimizing the occurrence of side reactions and facilitating precise control over the synthesis process of 4VPs. Biocatalysis of lignin transformation to 4VPs by enzyme not only demonstrates high catalytic efficiency and selectivity but also provides invaluable ecological benefits, including biocompatibility and biodegradability, making it an advantageous approach from an environmental standpoint. Nevertheless, there is room for optimization of enzyme characteristics such as water solubility, optimal catalytic conditions, and substrate specificity to further promote the biotransformation of lignin into 4VPs.[74] These optimizations can contribute to improving the overall process and maximizing the yield of 4VPs. The utilization of high-throughput screening techniques and macrogenomic analysis holds great potential for the discovery of novel and highly efficient decarboxylases as enzyme biocatalysts for lignin transformation into 4VPs. Furthermore, protein engineering techniques can be employed to redesign the enzyme structure and introduce desired characteristics that are suitable for the biotransformation process. Additionally, the implementation of advanced immobilization techniques can enhance the stability and activity of the enzyme biocatalysts, thereby further improving their catalytic efficiency and economic viability for industrial applications.

### 3.2 Microbial cell factories for the synthesis of 4-vinylphenol derivatives

The establishment of microbial cell factories represents a sustainable, secure, and environmentally friendly approach for the biotransformation of lignin into 4VPs. Numerous wild-type microbes, such as *Phomopsis liquidambari*, *Bacillus* spp., *Cupriavidus* sp., have the catalytic decarboxylation activity of lignin derivatives.[75–78] Unfortunately, the yield of 4VPs is not attractive due to their low catalytic activity. The emergence of synthetic biology has introduced an innovative and systematic approach to designing microbial cell factories, enabling the enhancement of decarboxylation biocatalytic efficiency. This advancement could significantly improve the overall efficiency of the biotransforming lignin-derived precursors into 4VPs.

*Escherichia coli* (*E. coli*), a widely used host for heterogeneous gene expression, had been engineered as a crucial microbial cell factory for the efficient production of 4VPs. In 2007, the microbial synthesis of 4VP was successfully achieved by investigating the decarboxylation capacity of *p*-CA using PADs derived from various sources, including *Bacillus subtilis*, *P. fluorescens*, *P. putida*, *Lactobacillus plantarum*, *S. cerevisiae*, and *Rhodotorula rubra*.[79] It is worth noting that bacteria exhibited superior decarboxylation activity compared with yeasts. Recombinant *E. coli* containing PAD from *B. subtilis* and *L. plantarum* successfully converted 1 mM *p*-CA to 4VP at a titer of 0.52 mM and 0.6 mM, respectively.[79] Furthermore, recombinant *E. coli* harboring PAD from *Bacillus amyloliquefaciens* were evaluated to determine the substrate range that could effectively decarboxylation.[80] The microbial cell factory showed a conversion yield of 41%, 27%, 28%, and 3% for 2 mM *p*-CA, caffeic acid, FA, and SA after 36 h, respectively. Notably, the recombinant *E. coli* could not catalyze the decarboxylation of cinnamic acid.[80] Moreover, to enhance the commercial viability of 4VPs produced by microbial cell factories, it is imperative to address the issue of product inhibition and find ways to mitigate its impact. *In situ* extraction strategy had been applied to improve the microbial production of 4VPs by mitigating the toxic effects of the aromatic product on the host strains.

 

Recombinant *E. coli* containing *Ba*PAD successfully produced 31.9 g $L^{-1}$ 4VP from 300 mM *p*-CA with a conversion yield of 88.7% using a biphasic system of 1-octanol/phosphate buffer phase with a three-volume ratio.[64] Alkaline hydrolysate of corn cob biomass has large quantities of lignin-derived aromatic precursors and can serve as an ideal substrate for the synthesis of 4VPs. Recombinant *E. coli* harboring *Lp*PAD obtained 1.0 g $L^{-1}$ 4VP from these detoxified real lignin hydrolysates in a biphasic system consisting of equal volumes of hexane and aqueous phases.[81]

Several microbes possess inherent assimilation pathways of lignin-derived aromatic compounds and biomass-derived oligosaccharides, making them ideal candidates for engineering as microbial cell factories for the production of 4VPs. *P. putida* KT2440 is a pivotal chassis strain for the efficient production of aromatic chemicals, owing to its exceptional solvent tolerance and wide metabolic capacity for utilizing diverse aromatic feedstock. The *Bs*PAD gene from *B. subtilis* was introduced in *P. putida* KT2440 to replace the endogenous hydroxycinnamoyl-CoA hydratase-lyase gene using sacB-mediated homologous recombination. This engineered strain successfully transformed *p*-CA and FA into 4VP and 4VG, respectively, while it produced a 4VG titer of 62.4 mg $L^{-1}$ using 10% protobind lignin.[82] *Corynebacterium glutamicum* has also been constructed as microbial cell factory for the biosynthesis of 4VP.[19] *C. glutamicum* was engineered by deleting the gene encoding *p*-hydroxycinnamoyl-CoA synthetase and heterologously expressing *Ba*PAD. This strain achieved 187 g $L^{-1}$ 4VP with a 90% conversion of *p*-CA using undecanol as the organic phase, while it produced 17 g $L^{-1}$ 4VP with a conversion of 73% from a lignin hydrolysate containing 8 g $L^{-1}$ *p*-CA.[19] *Streptomyces lividans* contained a variety of cellulases, which can synergize with a heterologous endo-glucanase gene from *Thermobifid a fusca* YX to convert the cellulose into cellobiose.[83] The construction of 4VP biosynthetic pathway in *S. lividans* was achieved by co-expressing tyrosine ammonia-lyase from *Rhodobacter sphaeroides* and PAD from *Streptomyces sviceus*, which can produce 4VP at a titer of 250 mg $L^{-1}$.[83]

These findings underscore the importance of assembling "biological parts" with distinct functions into "biological circuits", promoting the biotransformation of lignin-derived aromatic precursors toward 4VPs. These microbial cell factories could act as promising artificial systems with specific functions to be widely used in industrial scale. Nevertheless, it is crucial to address the inhibiting effects of depolymerized lignin derivatives and toxic aromatics products on chassis strains. Advanced depolymerization strategies are necessary to obtain high concentrations of aromatic precursors for efficient synthesis of 4VPs. Additionally, effective detoxification technologies are required to eliminate the potential inhibitors present in the lignin hydrolysate. Enhancing the robustness of microbial cell factories to adapt to the fermentation environment is essential. Furthermore, the screening and expression of key enzymes in microbial cell factories should be optimized to enhance the conversion of lignin-derived aromatic precursors and increase the yields of 4VPs. By implementing these strategies collectively, sustainable production of 4VPs from lignin-derived aromatic precursors can be achieved by microbial cell factories.

### 3.3 Whole-cell biocatalysts of lignin toward 4-vinylphenol derivatives

Whole-cell biocatalyst is a vital technology that involves collecting microbial cells from the culture system and utilizing them as heterogeneous enzyme pools to catalyze the conversion of substrates into the desired target product.[84] This approach eliminates the need for cell lysis, enzyme separation and purification, resulting in significant cost reduction in the process. Moreover, the residual cell wall components provide protection to the resting cells against the detrimental effects of harsh conditions, such as high aromatic concentrations, enabling the proper functioning of the enzyme.[85] Whole-cell biocatalysts provide an environmentally friendly and cost-effective strategy to enhance the biotransformation of lignin-derived aromatic precursors into 4VPs.

The effectiveness of whole-cell biocatalysts relies on the production and activity of key enzymes secreted from the strains. The overall performance of lignin biotransformation can be greatly influenced by comprehensive approaches, such as high-density cell cultivation, optimized induction conditions, and feeding strategies. A recent achievement was the successful development of a high *Bl*PAD-producing *E. coli* strain, which exhibited an impressive activity of 531 U $mL^{-1}$ and productivity of 20.4 U $mL^{-1}$ $h^{-1}$ for *Bl*PAD.[86] The recombinant *E. coli*, as the whole-cell biocatalyst, produced 129.9 g $L^{-1}$ 4VG with a conversion of 85.6% in a 5 L bioreactor containing an equal volume of cyclohexane and buffer system.[86] Genome sequence mining had been applied to identify a PAD with excellent organic solvent tolerance from *Bacillus atrophaeus*.[87] The whole cells of *E. coli*, containing *Ba*PAD, demonstrated enhanced tolerance to FA and 4VG compared to the free enzyme.[87] Such a whole-cell catalytic system converted approximately 310 g $L^{-1}$ FA to 237.3 g $L^{-1}$ 4VG after 13 h with a fed-batch biphasic fermentation strategy with equal volumes of 1-octanol and aqueous phases. It achieved a 98.9% conversion of FA to 4VG and a product productivity of 18.3 g $L^{-1}$ $h^{-1}$.[87] The *Bc*PAD from *Bacillus coagulans* was developed as a coenzyme-free whole-cell biocatalyst for the decarboxylation of phenolic acids.[88] *Bc*PAD exhibits a wide substrate spectrum and displays catalytic activity against *p*-CA, FA, caffeic acid, and sinapic acid. Using a chloroform/water two-phase system, the recombinant *E. coli* containing *Bc*PAD successfully produced 20.5 g $L^{-1}$ of 4VP from 180.0 mM *p*-CA within 18 h.[88]

Bacterial surface display system had been designed for the whole-cell biocatalytic production of 4VPs.[89] *Bl*PAD can be successfully anchored to the surface of *E. coli* cells by replacing the N-terminal signal peptide of the Gly–Asp–Ser–Leu (GDSL) family autotransporter protein from *P. putida* with the PelB signal peptide and the passenger region with *Bl*PAD. The bacterial surface display system enabled the transfer of the enzymatic decarboxylation process to the extracellular. This system successfully overcame the mass transfer barrier imposed by

the cell membrane and mitigated the high cytotoxicity resulting from the intracellular accumulation of substrates and products. The bacterial surface display system obtained conversion yields of 80.4% and 72.6% from 300 mM *p*-CA and FA to 4VP and 4VG, respectively, in a biphasic system. Additionally, the biocatalyst retained over 63% of its activity after 7 cycles.[89] To facilitate the biotransformation of lignin-derived aromatic precursors to 4VPs, it is necessary to enhance the stability of whole-cell biocatalysts. A two-step procedure has been developed to create an effective strategy for in-cell crosslinked enzymes (InCLEs).[84] Ethanol first permeated the cell membranes to facilitate the diffusion of the reactants, followed by crosslinking of the enzyme within *Bacillus megaterium* LBI001 using glutaraldehyde. The biocatalyst was then co-stabilized by internalizing 25 kDa of polyethyleneimine. This approach significantly improved the thermal stability of the biocatalyst and retained 83% and 69% of the decarboxylase activity after 7 cycles at 40 °C and 50 °C, respectively, accumulating 32.7 and 29.8 μmol of 4VG from 26.3 mM FA.[84]

The whole-cell biocatalyst system is highly regarded as a promising technology for the biotransformation of lignin into 4VPs. It eliminated the need for the separation and purification of the enzymes and thereby reducing the cost of enzyme preparation. Additionally, this system does not require the addition of expensive exogenous cofactors and offers an irreplaceable advantage in the application of enzymatic cascade reactions. However, the properties of fermentation medium play a crucial role in the industrial-scale application of whole-cell biocatalysts. This is because many enzymes are inherently sensitive to organic solvents, which can result in their inactivation and loss of functionality. For example, the transfer of hydrophobic products from the aqueous phase to organic solvents is a great challenge in the whole-cell biocatalytic processes. The advanced superabsorber materials are required to improve the on-site separation efficiency of 4VPs. Furthermore, protein engineering has the potential to provide an effective strategy for the efficient biotransformation of lignin-derived aromatic precursors to 4VPs, even in the presence of organic solvents.

# 4 Emerging cutting-edge technologies promoted biological lignin valorization

Significant advancements have been achieved in the biological depolymerization of lignin macromolecules, the metabolism of aromatic derivatives, and the bioproduction of 4VPs.[18,19,90] However, the substrate specificity and catalytic activity of enzymes, as well as the compatibility of chassis bacteria with heterologous genes, and their tolerance to aromatic substrates and products, remain significant challenges in the biological valorization of lignin for 4VPs. Fortunately, advanced strategies are currently available that provide effective solutions to overcome these obstacles.

## 4.1 Protein engineering improved the catalytic performance of ligninolytic enzymes

Protein engineering has emerged as a powerful tool for designing and constructing biocatalysts with desirable properties to enhance the biotransformation of lignin derivatives to 4VPs (Fig. 5A).[91] The catalytic activity, substrate specificity and stability of PADs play crucial roles in the biotransformation of lignin to 4VPs. Several PADs have been identified and characterized for their ability to effectively convert H- and G-type lignin derivatives. However, further improvements are still needed to enhance the catalytic performance of these enzymes for the decarboxylation of S-type lignin monomers to 4VS. Protein engineering had been utilized to expand the substrate profile of PAD, aiming to enable efficient biosynthesis of 4VS. The X-ray crystal structure of *Bp*PAD serves as the foundation for protein engineering. Through saturation mutagenesis and a semi-rational design approach, a single amino acid replacement strategy was employed to engineer an enzyme capable of catalyzing the decarboxylation of sinapic acid.[92] The Ile85Ala mutant had the highest activity for the decarboxylation of 25 g $L^{-1}$ sinapic acid with a conversion rate of 99%.[92] PAD from *B. subtilis* showed lower catalytic activity of sinapic acid compared with *p*-CA and FA.[93] Three *Bs*PAD mutants were generated through targeted mutagenesis, involving the replacement of isoleucine with alanine, valine and leucine, respectively. Among these mutants, the Ile85Ala mutant exhibited the highest decarboxylase activity toward sinapic acid. Nevertheless, it should be noted that all three mutants exhibited decreased catalytic activity toward *p*-CA and FA. This can be plausibly attributed to the fact that expanding the active site of the enzyme creates a more favorable environment for accommodating larger substrates, but at the same time hinders the binding and localization of smaller substrates.[93] Engineering of the N- and C-terminus of the enzyme is also a common strategy for protein engineering.[94] Mutants with an extended C-terminus of *Ba*PAD improved its activity under acidic conditions, while the extended N-terminus helped to improve the resistance of the enzyme to alkalinity and heat.[94]

The depolymerization of lignin is a crucial process for obtaining aromatic derivatives that are suitable for the biosynthesis of 4VPs. Ligninolytic enzymes, which are abundantly present in nature, play a significant role in biotransformation of lignin into valuable products. Computer-assisted directed evolutionary strategies have been employed for protein engineering of laccases, which is one of the important ligninolytic enzymes.[95] A double mutant, namely Ala162Val–Ala458Leu, was generated by saturation mutagenesis and was computationally characterized at the atomic level. The mutation increased the redox potential from 740 mV to 790 mV to enhance the redox catalytic activity of lignin. At the same time, its stability was also improved under thermal and acidic conditions.[95] Similarly, directed evolution and high-throughput screening techniques were employed to design and modify dye-decolorizing peroxidases from *P. putida* MET94.[96] The obtained triple mutation, E188K–A142V–H125Y, showed a

 

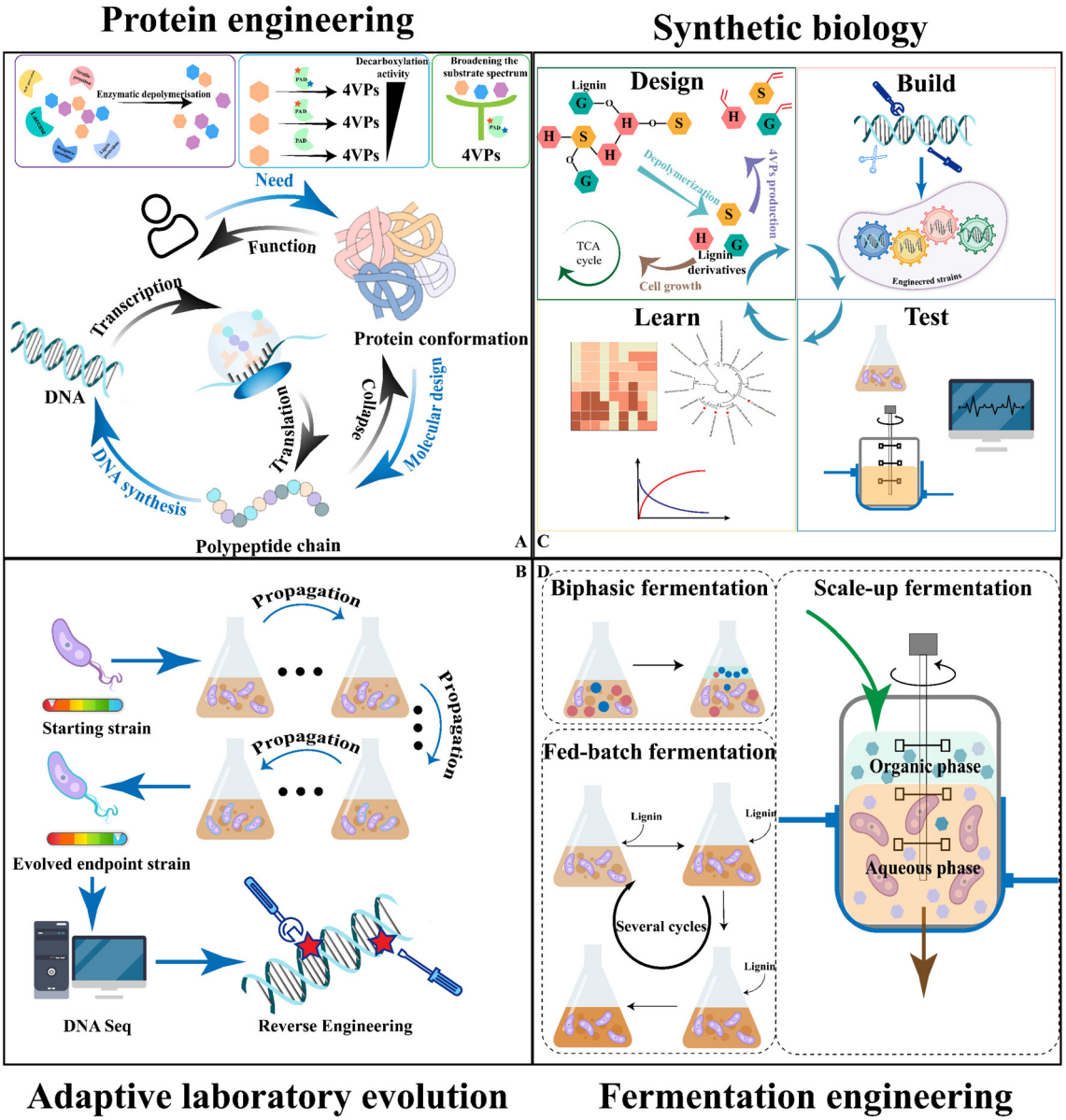


**Fig. 5** Advanced strategies for lignin valorization towards 4-vinylphenol derivatives. (A) Rapid development of protein engineering comprehensively promotes the biological valorization of lignin to 4-vinylphenol derivatives. (B) Adaptive laboratory evolution leads to mutant strains that efficiently synthesize 4-vinylphenol derivatives from lignin. (C) Synthetic biology provides methodological support for the construction of artificially designed metabolic pathways for the redirection of the carbon flows of lignin derivatives. (D) Fermentation engineering addresses common challenges in scale-up processes and facilitates bioproduction of 4-vinylphenol derivatives.

100-fold increase in the catalytic activity of 2,6-dimethoxyphenol.[96] Additionally, the directed evolutionary strategy was also employed to modify a dye-decolorizing peroxidase from *P. fluorescens* Pf-5, resulting in the mutants with higher activity against lignin-derived phenolic and oligomeric substrates.[97] Through saturation mutagenesis near the heme cofactor, two active mutants, H169L and N193L, were obtained. The introduction of non-polar amino acid leucine increased hydrophobicity surrounding the heme and increased the redox potential of the heme chemical complex, leading to an improved catalytic activity. Both mutants showed a significant enhancement, approximately 7–8-fold, in catalytic activity toward 2,4-dichlorophenol.[97]

To achieve atom-economic biotransformation of lignin to 4VPs, biocatalysts with a wide metabolic profile are essential. The depolymerization of lignin typically yields aromatic derivatives that possess various functional groups, with methoxy being one of the predominant groups. *O*-Demethylation is one of the key catalytic steps in the biotransformation of heterogeneous lignin derivatives into central intermediates. The presence of two methoxy groups on the aromatic ring of S-type aromatic monomer makes their biotransformation more

difficult.[29] The structure of the cytochrome P450 enzyme GcoAB was modified through protein engineering, enabling the microbial transformation of syringol.[98] The F169A mutant was obtained on the basis of crystallography and molecular dynamics and it had broader substrate specificity to effectively catalyze the *O*-demethylation of syringol. *P. putida* KT2440 containing this mutant completely converted 1 mM syringol within 6 h.[98] Two mutants, F169S and T296S of GcoAB, were further obtained by rational design to effectively catalyze the *O*-demethylation of the O- and P-isomers of vanillin.[99] Heterologous expression of the T296S mutant in *P. putida* KT2440 resulted in the improved conversion of vanillin.[99]

The inherent structure of natural enzymes grants them unique properties and functions for the conversion of lignin into 4VPs. Nonetheless, the industrial utilization of ligninolytic enzymes is currently constrained by factors such as activity and stability. Therefore, greater emphasis should be placed on enhancing the efficiency of ligninolytic enzymes in depolymerizing lignin macromolecules, thereby improving the bioavailability of lignin. Protein engineering can modify the structure of natural ligninolytic enzymes to obtain the most suitable biocatalysts for lignin biotransformation. The potential of PADs to catalyze the decarboxylation of sinapic acid has not been fully explored. There is still a need for more efficient strategies to screen and engineer the enzyme for effective decarboxylation of sinapic acid. In addition, while the metabolic pathways of H-, G-, and S-type lignin derivatives have been characterized, there is still limited research on the heterologous transfer of metabolic functions to S-type lignin monomers, such as syringic acid. Efforts to achieve atomically economic conversion of all lignin derivatives to 4VPs are crucial. Fortunately, the advent of machine learning technology offers guidance for protein engineering of ligninolytic enzymes, PADs, and biological funnel pathway enzymes. This provides promising tools for constructing biocatalysts capable of catalyzing all lignin derivatives toward the synthesis of 4VPs.

### 4.2 Adaptive laboratory evolution strategies enhanced the strain tolerance

Adaptive laboratory evolution (ALE) is a method that utilizes natural screening to obtain beneficial mutant strains through artificially applied selection pressure.[100] By combining continuous culture techniques, high-throughput sequencing, and bioinformatics, the molecular-level traits of mutations generated through ALE can be clearly elucidated. As a result, ALE proves to be an effective tool in obtaining exceptional chassis strains with high tolerance toward lignin derivatives and 4VPs, thereby facilitating their industrial application (Fig. 5B).

Aromatic acids, such as *p*-CA and FA, serve as ideal precursors for the synthesis of various aromatic fine chemicals. However, high concentrations of aromatic substrates can impede the cell growth of chassis strains, thereby affecting their ability to produce desired products. ALE had been employed to elucidate the mechanisms through which a mutant strain of *S. cerevisiae* demonstrated enhanced tolerance toward *p*-CA and FA at concentrations up to 800 mg $L^{-1}$ when subjected to simulated low pH conditions resembling industrial production settings.[101] Through whole genome sequencing analysis, a common mutation was identified in the transcriptional activator Aro80 gene. Among the genes regulated by Aro80, the presence of an unknown protein called ESBP6 was discovered to be up-regulated in the tolerant strains. It was observed that ESBP6 plays a crucial role in improving tolerance by facilitating the transport of aromatic acids out of the cell. Moreover, overexpressing ESBP6 also increased the production of *p*-CA from 0.89 g $L^{-1}$ to 1.23 g $L^{-1}$.[101]

Similarly, ALE facilitated the catabolism and tolerance of *P. putida* KT2440 to *p*-CA and FA.[102] Compared with the wild-type strain, the mutant strain had a reduced lag phase at 20 g $L^{-1}$ *p*-CA, while its growth rate increased by 2.4 times at 30 g $L^{-1}$ FA. Through whole gene sequencing analysis of the mutant strains, it was found that *PP_3350* and *ttgB* were the most frequently mutated genes. It was confirmed that the *PP_3350*-deficient bacteria showed an increased tolerance to high concentrations of *p*-CA. Although the exact function of *PP_3350* is yet to be determined, it is hypothesized that the deletion of this porin, which is responsible for the update of *p*-CA or FA, would result in lower concentrations of these compounds in the cytoplasm, thereby reducing their toxic effects. In contrast, the ALE-derived strains that had *ttgB* knockout (a part of the TtgABC efflux pump) were severely inhibited by a complex substrate consisting of *p*-CA and FA. This indicates the presence of epistatic interactions among these mutations.[102]

Some lignin derivatives have been observed to act as inhibitors, disrupting the structure of the cell membrane. This disruption can increase the permeability of intracellular components and potentially affect the cell growth, especially at a high concentration.[103] Due to their high hydrophobicity, lignin-derived aromatic aldehydes have a greater tendency to interact with the cell membranes of microorganisms. Even at a low concentration of 0.25 g $L^{-1}$, all three aromatic aldehydes (vanillin, syringaldehyde and 4-hydroxybenzaldehyde) exhibited strong inhibitory effects on the growth of the *Yarrowia lipolytica* $XYL^{+}$ strains.[103] Through ALE, the mutant strains were obtained that exhibited high tolerance to three aromatic aldehydes. These mutant strains acquired the ability to convert aromatic aldehydes into less toxic alcohols and/or acids, which contributed to their increased tolerance. However, it was observed that apart from aromatic alcohols and acids, there are no other degradation products detected, indicating that the downstream degradation pathway may not be present in *Yarrowia lipolytica*. Furthermore, thorough the analysis of transcriptome profiling, enzymatic properties, and reverse engineering revealed that the aldehyde ketone reductase gene YALI0_B07117g and aldehyde dehydrogenase gene YALI0_B01298g played a significant role in the conversion of aromatic aldehydes.[103]

Due to the high partitioning in organic solvents, 4VPs are usually separated using an *in situ* extraction fermentation strategy. The ability of the engineered strains to tolerate organic solvents becomes a crucial factor in determining the pro-

 

duction of 4VPs. *P. putida* S12 harbors a megaplasmid called pTTS12, which contains gene clusters associated with solvent tolerance. The removal of this megaplasmid resulted in a significant decrease in solvent tolerance. However, through ALE, the solvent tolerance of the plasmid-cured *P. putida* S12 was successfully restored.[104] The obtained ALE-derived strains can maintain cell growth at a high toluene concentration of 10%. The whole genome sequencing of the mutant strains showed that mutations in *arpR*, which acts as a negative regulator of the efflux pump of resistance–nodulation–division family, led to the up-regulation of the expression level of the multifunctional efflux pump (ArpABC). At the same time, many genes associated with cell motility, energy conversion and biofilm synthesis were down-regulated. These findings provided a possible genetic engineering target for improving solvent tolerance in host strains for the biosynthesis of 4VPs.

ALE serves as an advanced strategy to apply Darwinian theory in laboratory settings. By subjecting organisms to continuous evolution, ALE enables the acquisition of mutant strains with optimized phenotypes to better adapt to high concentrations of lignin derivatives and aromatic products. However, ALE has not been extensively utilized for specific applications in the biotransformation of lignin into 4VPs. Given the diverse and complex metabolic pathways involved in lignin derivatives, ALE holds great potential in obtaining strains capable of efficiently converting a wide range of lignin derivatives into 4VPs, enabling economically sustainable bioconversion of lignin. Additionally, ALE can be exploited as a viable solution for enhancing bacteria tolerance to 4VPs. When combined with advanced technologies like automated serial batch cultivations and multi-omics analysis, ALE will undoubtedly become an indispensable tool for obtaining industrial-scale strains suitable for lignin transformation.

### 4.3 Synthetic biology technologies boosted the construction of ligninolytic strains

Synthetic biology offers a powerful toolkit for obtaining microbial cell factories and effective biocatalysts for lignin biotransformation toward 4VPs (Fig. 5C). This is achieved through the construction of product synthesis pathways, regulating metabolic networks, and enhancement of tolerance in chassis strains.[29] Synthetic biology has the potential to drive the biotransformation of lignin into a wide range of aromatic chemicals and valuable platform compounds.[105]

The numerous benefits and potential applications of synthetic biology have spurred the development of various gene editing and assembly strategies, which have been extensively employed to construct cell factories for the efficient biotransformation of lignin into 4VPs. Based on the concept of synthetic biology, a *sacB*-based suicide vector was used in *C. glutamicum* for genome editing to knock out the gene encoding an acyl:CoA ligase in order to block the endogenous metabolic pathway of aromatic acids.[19] Gibson assembly had introduced an exogenous decarboxylation pathway of aromatic acids. This microbial cell factory allowed a maximum conversion of lignin-derived aromatic precursors to 4VP with a titer of 187 g $L^{-1}$ in a biphasic system.[19] Synthetic biology has also been applied to enhance the synthesis and mass transfer of the pathway intermediate, namely tyrosine, to facilitate the *de novo* biosynthesis of 4VP.[106] The endogenous toxin gene *hipA* in *E. coli* was utilized as a selection element, with its expression controlled by the aroP promoter. The tyrosine sensor protein TyrR was applied to detect varying concentrations of tyrosine and regulate the expression of *hipA* gene. Strains that exhibit high tyrosine production are able to grow normally as a result of the suppression of *hipA* gene, whereas strains with low tyrosine production are eliminated through the expression of *hipA*. Furthermore, the overexpression of the amino acid exporter protein PhpCAT facilitated the mass transfer of tyrosine between the upstream tyrosine-producing strain and the downstream 4VP-producing strain. Finally, the biosynthetic pathway of 4VP was constructed by introducing tyrosine ammonia-lyase and *Sc*FDC1 into the downstream production strain. This microbial cell factory yielded 298 mg $L^{-1}$ of 4VP from 5 g $L^{-1}$ glucose, which was the highest titer from the *de novo* synthesis of 4VP known so far.[106]

Transformation of all lignin-derived aromatics is an effective strategy to enhance the utilization efficiency of lignin and drive lignin valorization toward 4VPs. *Rhodococcus opacus* PD630 harbored three biocatalytic reactions, namely aryl side-chain oxidation; hydroxylation and *O*-demethylation, by introducing exogenous catalytic pathways.[107] It can convert three types of lignin derivatives into gallic acid. Among them, H- and G-type monomers could be converted into protocatechuic acid, while gallic acid was produced through the hydroxylation process of protocatechuic acid by a designed hydroxylase system. For S-type monomers, syringic acid was successfully converted to gallic acid by introducing a heterologous tetrahydrofolate-dependent *O*-demethylation pathway and a tetrahydrofolate recycling system. The engineered strain produced 0.41 g $g^{-1}$ and 0.63 g $g^{-1}$ of gallate from alkali-pretreated lignin and ammonia fiber explosion lignin, respectively.[107] The catabolic pathway of syringic acid had been introduced in *P. putida* KT2440 by overexpressing LigABC from *Sphingobium* sp. and VanAB from *Pseudomonas* sp. HR199, and deleting endogenous PcaHG and VanAB. These strategies created an efficient biological funnel for the utilization of lignin, while this engineered strain fully converted three types of lignin monomers.[60] These findings open a new perspective for the conversion pathway design toward the biotransformation of full lignin-derived aromatics.

Synthetic biology had been employed to improve the solvent tolerance of host bacteria, enabling them to withstand the toxicity of both products and substrates during the bioconversion of lignin-derived aromatics. For instance, synthetic biology has been employed to engineer various crucial factors that influence the solvent tolerance of microorganisms, including resistance–nodulation–cell division (RND) efflux pump, cell membrane fluidity, and stress response regulation.[108] The solvent tolerance of *P. putida* S12 was conferred by the RND family efflux pump SrpABC, which actively removed organic solvent molecules such as styrene and toluene from cells.[109]

The SrpABC gene from *P. putida* S12 was introduced into the solvent-sensitive *E. coli*, enabling the tolerance of saturated concentrations of toluene and improving tolerance to the decanol and hexane at 1%.[110] The heat shock proteins of GrpE, GroESL and ClpB were introduced in *E. coli* with a modular semi-synthetic stress reaction system, which significantly improved its tolerance to organic solvents.[111] These findings highlighted that synthetic biology strategies provided promising opportunities to construct bacteria with improved solvent tolerance. These strains are suitable for biphasic fermentation in the production of 4VPs.

Synthetic biology shows the promise to construct chassis strains in overcoming lignin heterogeneity and promoting lignin biotransformation. Nevertheless, there is a need to further explore gene engineering tools and regulatory patterns to facilitate the construction of the strains for lignin transformation and biosynthesis of 4VPs. Additionally, it is essential to engineer the host strain to facilitate the development of both the upstream biodepolymerization module and the downstream conversion module for lignin. The integration of synthetic biology with advanced DNA sequencing, genome editing techniques, and transposon libraries can offer a more comprehensive understanding of genetic mechanisms involved in lignin transformation and biosynthesis of 4VPs.

### 4.4 Fermentation strategies facilitated the production of 4VPs

Given the distinct features of lignin-derived aromatic substrates and 4VPs, it is of utmost importance to explore and design the appropriate and advanced fermentation strategies for the biotransformation of lignin-derived aromatic precursors to 4VPs. It is crucial to identify the optimal intersection points among cell growth, lignin derivative consumption, and 4VP synthesis to achieve a balance between product yield, energy consumption, and economic benefits. Especially, the successful biotransformation of lignin-derived aromatic monomers into valuable aromatic fine chemicals necessitates the implementation of effective fermentation strategies to mitigate the inhibitory effects of both aromatic substrates and products on bacteria during fermentation, thereby enhancing overall fermentation performance (Fig. 5D).[65]

The fermentation process with *in situ* product recovery is extensively employed to alleviate the inhibitory effects of products, particularly in the case of 4VPs.[112] The biphasic system of liquid–liquid extraction involves using the aqueous phase as the fermentation medium and the water-insoluble organic solvent as the extraction phase of 4VPs. The product, such as 4VPs, is selectively extracted into the organic phase according to the different distribution between these two phases.[113] Biocompatibility is the foremost factor to consider when choosing organic solvents. In general, solvent toxicity can be evaluated by the $\log P$ parameter.[114] Additionally, selecting organic solvents should also take into consideration the partition coefficient of the product in the two phases, ease of subsequent purification, potential safety and health risks, as well as the environmental impact.[115]

To determine the optimal conditions of biphasic fermentation, nine different organic phases were investigated.[65] Factors such as enzyme stability, substrate and 4VPs partition coefficients, and other relevant parameters were taken into consideration.[65] The results demonstrated that toluene and 1-octanol were effective extractants for 4VP, while the extraction of 4VP in other organic phases was suboptimal. Interestingly, 4VG tended to partition strongly into these nine organic phases, especially toluene and 1-octanol. Considering biocompatibility, partition coefficient and purification efficiency, toluene and cyclohexane were optimal extraction organic phases of 4VP and 4VG, resulting in the titers of 60.6 g $L^{-1}$ 4VP and 58.3 g $L^{-1}$ 4VG, respectively.[65] In addition, the effects of four organic phases on the biological production of 4VG were investigated, including toluene, cyclohexane, *n*-octanol and *n*-hexane.[86] Cyclohexane was selected as the optimal organic phase for evaluating two different forms of biphasic systems, phase-separation and emulsification, in the 4VG production. Interestingly, the phase-separated biphasic system obtained 61.05 g $L^{-1}$ 4VG and a conversion yield of 40.7%, while the biphasic emulsion system is more attractive for industrial production, obtaining a 4VG titer of 129.9 g $L^{-1}$ and a conversion yield of 85.6%. One possible explanation for this could be that the contact between the organic phase and the cells enhances the permeability of the cell membrane, thereby facilitating the transmembrane transport of FA and 4VG.[86]

Fed-batch strategies have proven to be an effective approach for the biotransformation of lignin-derived aromatic precursors into 4VPs. This method allows for the attainment of high cell density and high enzyme expression, while also alleviating substrate inhibition.[116] The implementation of fed-batch culture resulted in the production of high-density recombinant host bacteria and high-activity PAD, ultimately leading to enhanced biosynthesis of 4VPs. The feeding strategies had been designed for the high-density culture of recombinant *E. coli* containing *Bl*PAD.[86] Exponential-feeding strategy yielded the highest production and activity of *Bl*PAD, corresponding to 20.4 U $mL^{-1}$ $h^{-1}$ and 531 U $mL^{-1}$, respectively. It is noteworthy that, in the scale-up production of 4VG, lactose has been identified as a suitable substitute for isopropyl β-D-thiogalactoside (IPTG) as an inducer. This substitution could reduce the cost associated with using IPTG in the fed-batch cultivation process. Therefore, a continuous lactose feeding strategy induced the expression of recombinant *Bl*PAD at an activity of 512 U $mL^{-1}$. Combined with the biphasic system in a 5 L bioreactor, this system produced 129.9 g $L^{-1}$ 4VG with a conversion yield of 85.6%.[86] Moreover, the design of fed-batch fermentation strategies was integrated with pH control during fermentation. The performance of whole-cell catalysis in FA decarboxylation was notably enhanced by feeding FA to maintain the pH value of the aqueous phase at 6.5.[87] These findings indicate that effective fed-batch fermentation strategy is necessary to enhance the biotransformation of lignin-derived aromatic precursors into 4VPs.

Fermentation strategies play a crucial role in various applications in enzyme production, lignin fermentation, and aro-

 

matic production. However, the distinctive characteristics of heterogeneous lignin derivatives and aromatic products necessitate the utilization of advanced fermentation strategies to enhance the biotransformation of lignin into 4VPs and achieve higher product yields. To enhance the biotransformation of lignin derivatives into 4VPs, it is essential to screen new organic solvents with excellent biocompatibility for designing a suitable biphasic system that can improve the on-site separation efficiency of 4VPs. Additionally, effective fed-batch or continuous fermentation strategies are necessary to reduce the inhibition of aromatic substrate and improve its conversion performance. Complex process control and online monitoring technologies are also crucial in guiding the design of the feed profile, maximizing lignin conversion, and improving product yield. Ultimately, the implementation of advanced fermentation strategies can greatly facilitate the biotransformation of lignin derivatives toward the production of 4VPs.

## 5 Conclusions and future prospects

The above-mentioned research emphasizes the significance of utilizing the biotransformation of lignin into aromatic fine chemicals as a green and sustainable approach for lignin valorization, aligning with the concept of atom economy. Emerging strategies and technologies, including synthetic biology and protein engineering, offer powerful tools for constructing microbial cell factories and screening effective biocatalysts to improve the conversion of lignin into 4VPs.

Despite these potentials, the complex structural properties of lignin and the absence of satisfactory biocatalytic system have hindered the performance of lignin biotransformation toward 4VPs. Several emerging strategies and technologies have been developed as promising tools to tackle these challenges. First, as lignin is a heterogeneous biopolymer that cannot be directly converted into valuable products by microbes, the development of efficient depolymerization technologies is crucial for the upstream process. These technologies aim to deconstruct the lignin polymer and obtain suitable lignin derivatives that can be further transformed. One approach is to enhance the production of ligninolytic enzymes from fungi or bacteria, which can improve the depolymerization efficiency of lignin by ligninolytic microorganisms. It is important to note that while ligninolytic microorganisms are natural producers of ligninolytic enzymes, other non-ligninolytic microorganisms, such as *E. coli* and yeast, can also be employed to produce ligninolytic enzymes due to their powerful enzyme expression systems. This approach expands the availability of highly bioavailable lignin derivatives. Additionally, exploring the compatible effects of multiple ligninolytic enzymes enables the construction of efficient ligninolytic systems, such as multi-enzyme systems and synthetic consortia. A comprehensive understanding of lignin depolymerization mechanisms is crucial in guiding the design of advanced depolymerization strategies that produce sufficient levels of lignin derivatives suitable for biotransformation.

Second, lignin derivatives are complex mixtures of aromatic compounds, which can impede the efficiency of converting them into 4VPs. During the midstream process of constructing biocatalysts, genome mining can be employed to identify key enzymes and pathways involved in biologically mediated lignin conversion. This approach can also help discover regulatory loci that are effective in converting lignin. Bioinformatics strategies, such as genomics and proteomics, combined with gene editing techniques, can be explored to identify metabolism pathway genes for different types of lignin derivatives and incorporate them into dominant microorganisms. It is essential to design an effective conversion route that can transform aromatic acid precursors into 4VPs and other derivatives, serving as carbon sources for cell growth.

Both ligninolytic microorganisms and non-ligninolytic microorganisms can serve as chassis microorganisms for the biological valorization of lignin towards 4VPs. Ligninolytic microorganisms possess natural pathways to metabolize lignin derivatives, while non-ligninolytic microorganisms could offer the advantage of easily accessible genetic manipulation tools and a clear genetic background. In the process of selecting the optimal microorganism for lignin valorization, it is important to carefully consider the advantages and disadvantages of each type to improve the overall performance of biocatalysts. Furthermore, the metabolic pathways of different lignin oligomers have not been fully elucidated, which limits the carbon utilization efficiency of lignin. However, identifying potential conversion routes for these oligomers holds the potential for constructing efficient biocatalysts that can effectively utilize a wide range of lignin derivatives. This improvement in carbon atom utilization efficiency is crucial for the current biosynthesis of 4VPs from lignin.

Third, both lignin substrates and 4VPs products can exhibit high toxicity toward biocatalysts or host strains. To address this challenge in the downstream process of 4VPs production, synthetic biology technology can be employed to engineer the host strains and enhance their tolerance to these potential inhibitors. Advanced technologies like protein engineering and ALE also show promise in improving strain tolerance and overcoming inhibitory effects during the biotransformation of lignin into 4VPs. Furthermore, fermentation strategies, such as biphasic systems, novel bioreactors and effective fermentation modes, hold potential as solutions to mitigate the inhibitory effects of aromatic compounds and enhance the overall biotransformation performance of lignin into 4VPs.

Overall, the integration of these emerging technologies and strategies has the potential to significantly enhance the biotransformation efficiency of lignin derivatives in the biosynthesis of 4VPs. The successful biosynthesis of 4VPs from heterogeneous lignin source aligns with the concept of atom economic conversion and holds great promise for advancing lignin valorization and enhancing the market competitiveness of 4VPs. This progress has the potential to make a significant contribution to the development of the lignin-based economy.

## Abbreviations

| | |
|---|---|
| 4VG | 4-Vinylguaiacol |
| 4VP | 4-Vinylphenol |
| 4VS | 4-Vinylsyringol |
| ALE | Adaptive laboratory evolution |
| FA | Ferulic acid |
| FDC1 | Ferulic acid decarboxylase 1 |
| Lac | Laccase |
| LigAB | PCA 4,5-dioxygenase |
| LigC | CHMS dehydrogenase |
| LigD, LigL and LigN | $C_\alpha$-dehydrogenases |
| LigF, LigE and LigP | β-Etherases |
| LigG and LigQ | Glutathione S-transferases |
| LigXaXcXd | Three-component *O*-demethylase |
| LigY | 2,2′,3-Trihydroxy-3-methoxy-5,5′-dicar-boxybiphenyl *meta*-cleavage compound hydrolase |
| LigZ | 2,2′,3-Trihydroxy-3′-methoxy-5,5′-dicar-boxybiphenyl dioxygenase |
| LiP | Lignin peroxidase |
| LigZ | 2,2′,3-Trihydroxy-3′-methoxy-5,5′-dicar-boxybiphenyl dioxygenase |
| LsdA | Carotenoid oxygenase |
| LsdE | *erythro*-1,2-Diguaiacylpropane-1,3-diol γ-formaldehyde lyase |
| Mnp | Manganese peroxidase |
| PAD | Phenolic acid decarboxylase |
| PCA | Protocatechuic acid |
| *p*-CA | *p*-Coumaric acid |
| PinZ | Pinoresinol/lariciresinol reductase |
| VanAB | Vanillate *O*-demethylase oxygenase |

## Conflicts of interest

The authors declare that they have no competing interests.

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