Prospecting Ligninolytic Enzymes Toward Lignin Valorization

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The enzymatic breakdown of lignin generates a spectrum of aromatic monomers — including vanillin, guaiacol, syringaldehyde, vanillic acid, ferulic acid, and p-coumaric acid — that serve as platform chemicals for pharmaceuticals, fragrances, resins, and bio-based polymers. However, its complex and recalcitrant structure necessitates highly efficient enzymatic systems for depolymerization. This review systematically classifies ligninolytic enzymes into five functional categories: laccases, peroxidases (including manganese peroxidases, lignin peroxidases, and versatile peroxidases), cytochrome P450s, dye-decolorizing peroxidases (DyPs), and auxiliary enzymes, evaluating their distinct roles and synergies in lignin breakdown. Laccases emerge as particularly versatile biocatalysts due to their widespread occurrence, broad substrate specificity, and operational flexibility under diverse conditions. Peroxidases drive critical oxidative reactions, while DyPs represent a functionally robust peroxidase class with superior stability under extreme pH, temperature, and pressure. Complementary enzymes such as etherases and lignin-mimetic systems further expand the toolbox for lignin valorization. To overcome inherent limitations of native enzymes, protein engineering strategies were highlighted to enhance catalytic efficiency, stability, and substrate affinity. Additionally, enzyme immobilization on advanced matrices (*e.g.*, metal-organic frameworks) is discussed as a breakthrough approach to improve reusability and reaction scalability. These integrated advancements pave the way for sustainable lignin valorization.

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INTRODUCTION

Lignocellulosic biomass stands as one of the most critical renewable resources on Earth, offering distinct advantages over non-renewable fossil fuels, including extensive availability, cost-effectiveness, environmental compatibility, and biodegradability (Yoo *et al*. 2020; Subbotina *et al*. 2021; Leynaud Kieffer Curran *et al*. 2022; Hu *et al*. 2025). The rational utilization of lignocellulosic biomass holds strategic significance in accelerating energy structural transitions, fostering rapid development of the bioeconomy, and advancing global carbon neutrality objectives (De Gonzalo *et al*. 2016a; Erickson *et al*. 2022). Lignin—one of the three primary components of lignocellulose-ranks as the second most abundant biopolymer after cellulose, with an

annual regeneration rate of 50 billion tons (Ren *et al*. 2019). As the sole natural macromolecule containing aromatic rings, lignin exhibits a structurally heterogeneous framework characterized by intricate aromatic and aliphatic backbones, along with diverse functional groups amenable to chemical modifications (Li *et al*. 2022). Recently, the targeted conversion of lignin into value-added chemicals represents a viable pathway for achieving dual-carbon objectives while aligning with principles of atomic economy (Janusz *et al*. 2017). Lignin can serve as a bio-based alternative to phenolic polymers and holds significant market value. The price of kraft lignin has increased from 7.327 million USD in 2015 to 9.131 million USD in 2025, with a compound annual growth rate of 2.2%. Lignin-derived products demonstrate competitive advantages in fields such as battery materials, carbon fibers, and dispersants. For example, the production cost of lignin-based carbon fibers is 5% lower than that of traditional PAN-based carbon fibers, and carbon emissions are reduced by 22% (Moretti *et al*. 2021). However, in contrast to holocellulose, which has undergone first- and second-generation industrial upgrades—with bioethanol production from cellulose and hemicellulose achieving commercial scalability—lignin structural complexity and heterogeneity pose significant challenges to its precise valorization (Yoo *et al.* 2020; Singhania *et al*. 2022). Efficient lignin depolymerization is thus pivotal for enhancing the high-value utilization of agricultural byproducts and driving sustainable circular economic models (Gesing *et al*. 2024).

Lignin is a three-dimensional, highly branched polymer formed through the random polymerization of highly substituted phenylpropane units interconnected by an extensive network of C-O (*β*-*β*, *β*-5) and C-C (*β*-O-4, *α*-O-4) bonds, in addition to aryl-glycerol-*α*/*β*-aryl ether bonds, and C-C bonds (Pollegioni *et al*. 2015). The macromolecular structure of lignin is composed of three monolignol monomers: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, with their relative proportions varying significantly depending on the biomass source (De Gonzalo *et al*. 2016b). The presence of reactive functional groups in lignin's molecular structure—including aromatic rings, phenolic hydroxyl groups, aliphatic hydroxyl groups, and conjugated carbonyl systems—enables diverse chemical reactions such as oxidation, reduction, hydrolysis, and graft copolymerization (Taha *et al*. 2016a; Salvachúa *et al*. 2018). Reactions enable targeted structural modifications of lignin, thereby broadening the potential pathways for its depolymerization and valorization.

Although conventional lignin depolymerization methods can produce aromatic fuels, aldehydes, and phenolic compounds, their scalability and efficiency within biological conversion systems remain unproven (Tonin *et al*. 2017a). Model compounds, including dimers, GGE, veratryl alcohol, and ABTS, have been studied at the lab scale. The vast majority of published enzyme characterization data derives from simple dimeric or monomeric model substrates. While essential for mechanistic studies and enzyme benchmarking, model-compound data overestimate catalytic performance by factors of 5 to 50 relative to real lignin substrates, and therefore must be interpreted with caution when projecting industrial applicability.

Bioconversion strategies, leveraging environmentally benign processes and selective advantages, have emerged as a research frontier for lignin valorization. Strategies are grounded in the theoretical framework of 'biological funneling,' which comprises two critical stages: first, the microbial enzyme-mediated depolymerization of heterogeneous lignin macromolecules into a restricted set of aromatic intermediates, followed by the synthetic biology-driven conversion of these intermediates into target products. Through simplifying lignin's structural complexity and establishing

standardized conversion workflows, this approach significantly enhances feedstock utilization efficiency and economic feasibility (Bugg *et al*. 2011a). In nature, lignin-degrading biological systems are predominantly found in fungi, bacteria, and insects. The lignin-depolymerizing enzymes secreted by these organisms are systematically classified into five categories: laccases, peroxidases, P450 enzymes, dye-degrading enzymes, and auxiliary enzymes. Notably, over the past decade, a paradigm shift has occurred in research focus (Biko *et al*. 2020a).

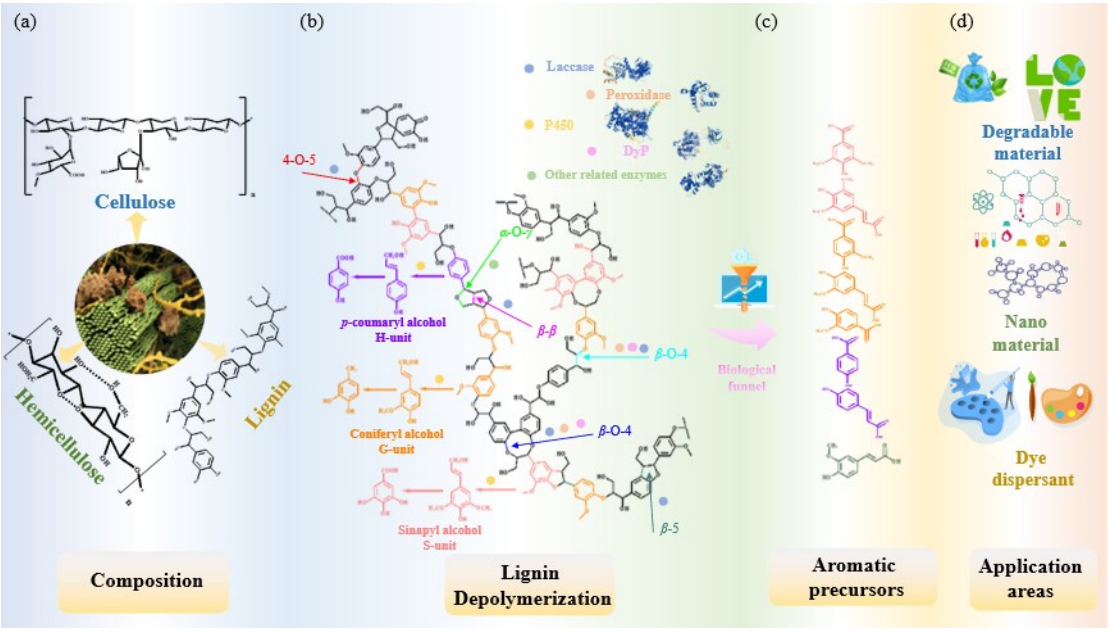

Fig. 1. The high-value utilization of lignin with the key depolymerization of lignin by ligninolytic enzymes: (a) The sources and classification of lignin and lignin-depolymerizing enzymes; (b) Structure of lignin and bonds targeted by depolymerizing enzymes; (c) Aromatic compounds generated *via* bio-funneling strategies; (d) Aromatic compounds enable multidisciplinary applications including green chemical synthesis, chemical recycling, and dye manufacturing

While fungal systems — particularly white-rot basidiomycetes — have historically served as the primary source of ligninolytic enzymes, their industrial deployment is constrained by several inherent limitations: slow growth rates, complex mycelial morphology that hinders mass transfer, strict nutritional and environmental requirements, and challenging genetic manipulation. In contrast, bacterial enzyme systems offer compelling practical advantages: rapid growth and high-density cultivation, ease of heterologous expression in established hosts such as *E. coli*, broader pH and temperature tolerance (particularly toward alkaline and thermophilic conditions), and simpler genetic tractability for protein engineering. This review therefore prioritizes bacterial ligninolytic enzymes while contextualizing them against their well-studied fungal counterparts.

The main technical challenges are primarily manifested in the complex structure of lignin, enzyme performance limitations, and difficulties in large-scale application (De Gonzalo *et al*. 2016b). Detailed challenges include high heterogeneity of lignin; repolymerization phenomena during depolymerization; radical intermediates that easily lead to condensation reactions, reducing monomer yields; poor stability (most lignin enzymes have a half-life of less than 24 h under industrial conditions, requiring frequent supplementation); low catalytic efficiency (the oxidation efficiency of natural laccase towards non-phenolic lignin units is only 1/1000 that of chemical catalysts); and

difficulties in product separation, as enzymatic hydrolysis products have complex compositions and high purification costs (Salvachúa *et al*. 2018). Therefore, elucidating the mechanisms of lignin-depolymerizing enzymes and discovering novel enzymes are critical to addressing these challenges (Liu *et al*. 2024). Examples include: the cloning, expression, and biochemical characterization of DyP-type peroxidases from *Bacillus* species for lignin degradation; the cloning, expression, and functional identification of laccase from *Bacillus licheniformis* NS2324 (Singh *et al*. 2013a); the application of *Bacillus licheniformis* NS2324-derived laccase in *Escherichia coli* systems, including its heterologous expression and characterization for exploring its role in dye decolorization; and the discovery of a novel DyP-type peroxidase from marine actinomycetes (Ruxandra Leontieș *et al*. 2022). Lignin conversion lacks cost competitiveness, and the cost increases significantly after high-value processing. For example, the minimum selling price of catalytic hydrodeoxygenation to produce aromatic chemicals is 30% higher than that of petrochemical counterparts. There are also significant challenges in extraction and conversion technologies, as biological conversion is difficult to scale up (Ullah *et al*. 2022). Furthermore, enhancing enzymatic activity remains a pivotal research direction in lignin depolymerization. Current strategies to improve enzyme activity primarily involve enzyme modification through protein engineering and genetic manipulation (Linde *et al*. 2015a). However, maintaining high enzymatic activity under stringent reaction conditions poses a significant challenge in biocatalytic processes (Martínez *et al*. 2009a; Bugg *et al*. 2011b; Lin *et al*. 2018a; Biko *et al*. 2020a).

Thus, the purpose of this article is to present the current research status and challenges in lignin depolymerization, while systematically reviewing bacteria and bacterial enzymes capable of lignin bioconversion. The article first details the discovery of each enzyme category, emphasizing bacterial sources and heterologous expression systems. The text then clarifies the catalytic mechanisms and optimal operational parameters of these enzymes. Subsequently, it reviews enzyme modification strategies, encompassing protein engineering and genetic optimization. Emerging metal-organic framework/covalent organic frameworks (MOF/COF)-based immobilization techniques are highlighted to improve enzyme reusability, expand operational windows (pH/ temperature), and enable continuous-flow bioreactor applications. Through integrating mechanistic understanding with cutting-edge approaches in enzyme engineering, immobilization science, and computational biology (metagenomics, synthetic biology, and machine learning), this work provides a strategic roadmap to overcome lignin valorization bottlenecks and develop industrially viable bioconversion technologies.

The high-value utilization of lignin is still in a critical stage of transitioning from the laboratory to industrialization. It follows that interdisciplinary collaboration will be needed to address core challenges, such as structural complexity, process economic viability, and environmental sustainability, to truly achieve the cyclic utilization of this renewable resource. Throughout this review, Fig. 1 serves as the organizational framework. The figure illustrates how enzymes can drive depolymerization (Figs. 1a–b) and how aromatic precursors can be generated *via* biological funnels (Fig. 1c).

LACCASE AS A COMMONLY USED ENZYME IN LIGNIN DEPOLYMERIZATION

Laccase is a member of the multi-copper oxidase family and is widely distributed among species including fungi, plants, bacteria, and insects, providing a broad source for the screening of this enzyme (Agustin *et al*. 2021; Zhou *et al*. 2023). Laccases (Fig. 2a) employ a multi-copper active center (T1/T2 Cu ions) to catalyze one-electron oxidation of phenolic lignin units *via* phenoxy radical formation (Fig. 2b). While fungal laccases have historically dominated research, bacterial laccases (*e.g.*, CotA from *B. subtilis*, CueO from *E. coli*) offer distinct advantages: exceptional thermostability (CotA retains activity at 60–80 °C), broad pH tolerance (pH 2 to 10 for *Bacillus* laccases), and ease of heterologous overexpression in *E. coli*, making them more suitable for industrial applications. Laccase uses oxygen as an electron acceptor to completely degrade phenolic substrates into carbon dioxide, and the intermediate process does not require hydrogen peroxide. Because laccase is a non-specific enzyme, its substrate range is broad, including o- and *p*-diphenols, aminophenols, polyphenols, lignin, aromatic diamines, and inorganic ions (Zhao *et al*. 2022). Additionally, laccase can form a laccase mediator system (LMS) together with a mediator, such as 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), which is reported to significantly enhance enzyme activity and broaden the range of oxidized substrates (Koschorreck *et al*. 2008).

In the laccase mediator system, the small-molecule mediator, once oxidized by laccase through electron abstraction, diffuses out of the enzyme's catalytic pocket to oxidize the non-phenolic subunits of lignin. Furthermore, non-phenolic subunits can be catalyzed as the oxidative mediator becomes a highly reactive intermediate with a more significant redox potential than the non-phenolic lignin subunits (Xu *et al*. 2024). In general, mediators can help overcome steric hindrance between substrate and enzyme as well as the high reduction potential of the substrate. Currently, laccase research encompasses various fields such as biochemistry, molecular biology, and genetics, including strain growth characteristics, synthesis regulation, separation and purification, crystal structure, catalytic mechanism, and gene cloning and expression (Ihssen *et al*. 2015).

Among them, laccase produced by white-rot fungi is considered the most effective, and as a result, much of the research on laccase is focused on fungi. Laccase-producing fungi are primarily found in the phyla Basidiomycetes, Ascomycetes, and Deuteromycetes. Among these, the white-rot fungi of the Basidiomycetes are particularly significant. Interestingly, bacterial laccase (such as CotA laccase) has higher catalytic rates than fungal laccase when redox mediators are absent (Merk *et al*. 2015). Co-cultivating aromatic decomposing bacteria with fungal secretions enhances the depolymerization of lignin by laccase or peroxidases. Co-cultivation of *Phanerochaete chrysosporium* and *Pseudomonas putida* increased lignin degradation rate 42% compared to single fungal cultivation, and enhanced laccase activity by 2.3 times (Van Der Made *et al*. 2023). Co-cultivation of *Trametes versicolor* and *Streptomyces viridosporus* increased the degradation rate of lignosulfonate from 57% to 89%, with bacterial removal of inhibitory aromatic products being crucial in the co-culture system. Research has demonstrated that in co-culture systems, the peak activity of laccase and peroxidase is extended 3 to 5 days due to bacterial metabolism alleviating feedback inhibition on fungi from intermediate products. This is because phenolic monomers produced by fungi when they degrade lignin, such as vanillic acid and 4-

hydroxybenzoic acid, inhibit laccase activity (Azubuike *et al*. 2022). Furthermore, bacterial laccase possesses additional desirable properties, including a wider pH range, thermal stability, exceptional tolerance to various inhibitors, and ease of genetic manipulation. Therefore, bacterial laccase plays a crucial role in overcoming the resistance of lignin to depolymerization and offers significant opportunities for the value-added utilization of lignin (Wu *et al*. 2005).

In laccase-mediated lignin depolymerization, solvent selection critically influences process efficiency. Although water is the traditional medium, its poor lignin solubility significantly limits reaction kinetics (Zhu *et al*. 2020). Deep eutectic solvents (DESs) have emerged as superior alternatives, combining excellent lignin solubility with environmental and economic advantages. These low-eutectic mixtures form through specific hydrogen-bonding interactions between acceptors (*e.g*., choline chloride, betaine) and donors (glycerol, lactic acid, urea), resulting in room-temperature liquids with tunable properties. The integration of DESs with laccase catalysis synergistically combines the sustainability of green solvents with enzymatic precision, offering a promising approach for efficient lignin valorization (Liu *et al*. 2022).

Bacillus ligninus L1

Bacillus ligninus L1 has been demonstrated to degrade lignin effectively (Zhu *et al*. 2017). Laccase was significantly upregulated, resulting in an 11.3-fold increase in response to lignin as the sole carbon source. This indicates that laccase plays a crucial role in the depolymerization of lignin by *B. ligninus* L1. Furthermore, it is widely accepted that *Bacillus laccases* exhibit higher temperature and alkaline resistance compared to other bacterial laccases, making them more suitable for lignin depolymerization. *B. ligninus* L1 DSM 26145T and *E. coli* were used as cloning and expression hosts and cultivated at 37 °C. The effects of temperature, pH, metals, solvents, and NaCl were assessed using ABTS as a substrate (Tonin *et al*. 2017a).

Enzymatic activity analysis showed that laccase is active within a wide temperature range from 25 to 90 ℃ and pH 2 to 10. Furthermore, when guaiacol was used as a substrate, laccase displayed remarkable heat resistance, retaining nearly 88% of its maximum activity at 90 °C. Metal ions, such as Cu^{2+}, Mg^{2+}, and Ca^{2+}, significantly enhanced laccase enzyme activity (Zhu *et al*. 2020). Interestingly, the addition of 300 mmol NaCl resulted in a significant increase in enzyme's activity by up to 235%. Additionally, laccase exhibited excellent tolerance to organic solvents, especially in 0.5 mM isopropanol and n-hexane solvents with activities > 90%. This tolerance to solvents and metal ions is advantageous for the dissolution of lignin using the ionic liquid method (Koschorreck *et al*. 2008). Moreover, laccase demonstrated exceptional thermal stability. Analysis of kinetic parameters, including K_m values and K_{cat}/K_m values, revealed that ABTS was the optimal substrate. Furthermore, as a mediator, ABTS can interact with lignin subunits and inhibit the polymerization of lignin subunits by laccase (Merk *et al*. 2015).

Monomeric aromatic and small molecular organic acid compounds were identified from the alkali lignin (AL) samples. Among these compounds, vanillyl alcohol, guaiacol, and acetosyringin were only observed following the enzymatic hydrolysis of the samples. Meanwhile, aromatic compounds and four small molecular organic acids were detected in the milled wood lignin (MWL) samples (Lyu *et al*. 2018). Coniferaldehyde, syringaldehyde, hydroxyphenylacetic acid, benzoic acid, succinic acid, and furancarboxylic acid were detected exclusively in the enzymatic hydrolysis samples. The study demonstrated that laccase and the laccase-mediator

system (LMS) can break linkages between lignin subunits and cleave side chains, yielding aromatic compounds and organic acids. These findings were supported by Fourier Transform Infrared Spectrometer analysis (FTIR) (Huang *et al*. 2011). The increase in the peak area of small organic acid molecules was significantly higher than that of aromatic compounds, which may be attributed to the formation of carboxylic acids through the cleavage of aliphatic side chains. Therefore, it was confirmed that laccase is more likely to act on side chain excision rather than breaking the bonds between lignin units, which aligns with the results of FTIR analysis. Additionally, laccase catalyzed the conversion of 4-hydroxybenzoic acid to 4-hydroxyphenylacetic acid through a C-C coupling reaction. The self-assembly of phenol radicals *via* C-C and C-O couplings has been observed in many fungal and bacterial laccase systems (Du *et al*. 2013). Unlike other laccase, no dimers or oligomers were observed in the monomeric reactions catalyzed by laccase.

A Novel Microbial Laccase *Ct*-lac

The extracellular assimilation mechanism of lignin requires an enzyme secretion system. To accelerate the depolymerization of lignin and improve its bioavailability, it is crucial to discover and express novel lignin-decomposing enzymes that exhibit high stability and excellent catalytic performance. A novel microbial laccase, Ct-Lac, was isolated from *Caldalkali bacillus thermarum* (Ghatge *et al*. 2018). Ct-Lac has a wide range of substrates and exceptional thermal stability. It can break down organic solvent-fractionated lignin and produce seven aromatic monomers, including vanillin and *p*-hydroxybenzaldehyde. Another noteworthy enzyme is LacZ1, which is a novel bacterial laccase screened from a microbial community (Chopra and Sondhi 2022). LacZ1 demonstrates excellent ability to decompose lignin, primarily by breaking *β*-O-4, *β*-5, and *β*-*β* bonds and generating various aromatic monomers through gentisic acid, benzoic acid, and protocatechuic acid. Laccase secreted by *Amycolatopsis* spp. induce monoaryl oxidation, leading to the production of phenoxy radicals. They also mediate the catalysis of carbon-carbon and *β*-aryl bonds (Zhou *et al*. 2023), resulting in a 6-fold increase in low molecular weight lignin. Through employing proteomics-guided biodesign, heterologous laccase expressed and secreted in *Rhodococcus lactis* PD630 achieved record protein yields. This approach depolymerized 81.1% of *β*-5 structures and cleaved more 4-O-5 and 5-5 linkages, ultimately producing low molecular weight lignin. Results demonstrated that bacterial laccases' discovery and heterologous expression significantly enhance lignin depolymerization, likely due to their small molecular weight and high activity.

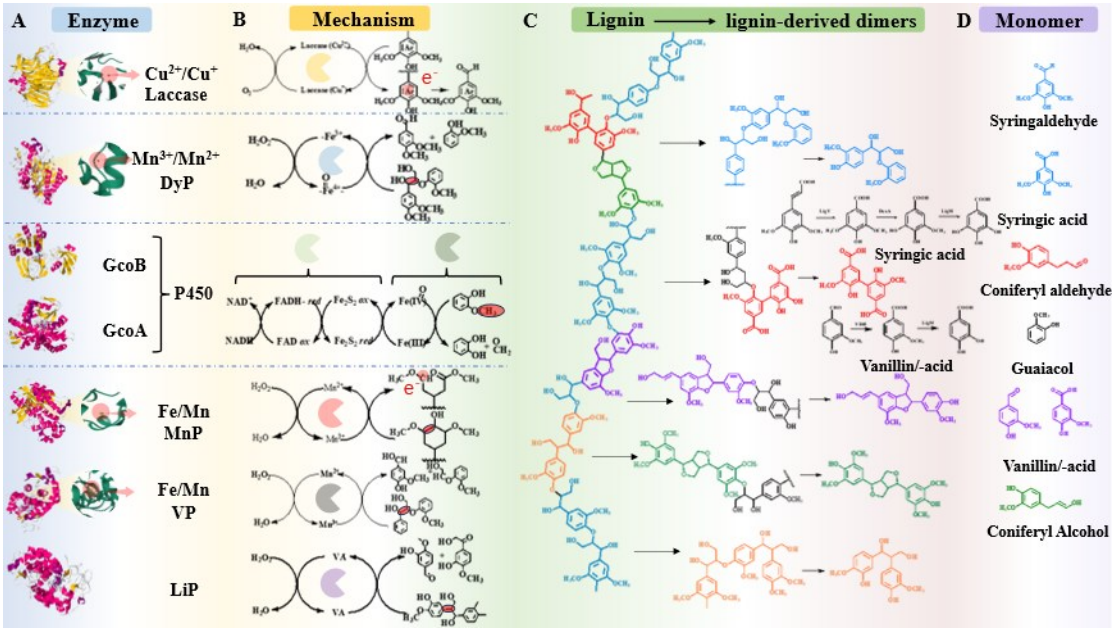

Fig. 2. The depolymerization mechanisms of lignin by four types of ligninolytic depolymerase: (a) The structure and active metal of enzymes; (b) The basic mechanism of enzymatic depolymerization of lignin; (c) The basic mechanism of enzymatic depolymerization of lignin; (d) Monomers resulting from enzymatic depolymerization of dimer derivatives: MnP represent manganese peroxidase, VP represent versatile peroxidase, LiP represent lignin peroxidase, DyP represent dye decolorizing peroxidase, GcOA represent glyoxal oxidase A, and GcoB represent glyoxal oxidase B

CotA of *Bacillus subtilis*

Another fascinating enzyme from the family Bacillaceae is the CotA of *Bacillus subtillis*, which functions as a copper-dependent and profoundly active laccase. CotA, unlike other laccases, demonstrates remarkable thermostability even at 60 °C, making it a suitable enzyme for industrial applications (Zhu *et al*. 2020). The CotA laccase of *Bacillus subtilis* with the substrate ABTS has been studied by the binding of the substrate to the T1 site and the binding of oxygen. The results showed that the reduction order of copper ions in the presence of the substrate was T1→T3→T2. The X-ray diffraction patterns of *Bacillus subtilis* CotA in the presence of copper chloride, hydrogen peroxide, enzyme inhibitors, and reduced states, indicated a hypothesized pathway for electron transfer of laccase. The first step is the movement of molecular oxygen to the central sites of the three copper ions facilitated by the acidic amino acids (Hullo *et al*. 2001). In the first step, three copper ions are divalent. The second step is to speculate that there may be an intermediate, and the electron is transmitted through two T3 copper ions (Choolaei *et al*. 2021). CotA has shown potential in the decolorization of complex aromatic dyes, including anthraquinonic and azo types, degrading pollutants, and the lignin aromatics vanillin and syringaldehyde, highlighting its broad-spectrum in the degradation of aromatic compounds including lignin. This has been made possible *via* protein engineering aimed at optimizing and enhancing the enzyme's versatility for the decomposition of aromatic compounds.

Peroxidases Used for Lignin Depolymerization

Ligninolytic peroxidases can be categorized into three groups: manganese peroxidases (MnP), lignin peroxidases (LiP), and multifunctional peroxidases (VP) (Kong *et al*. 2017; Li *et al*. 2019). The latter two can degrade intact lignin, while VP

has the broadest range of oxidizing refractory substrates. One of the main limitations of using these two enzymes in lignocellulosic biorefining is their gradual inactivation under acidic pH conditions, where they exhibit the highest oxidative activity (Weng *et al*. 2021). A fundamental principle often underappreciated outside the field is that laccases and peroxidases function synergistically rather than in isolation. In white-rot fungi, these two enzyme classes are co-secreted and operate interdependently. This synergy rests on three interconnected mechanisms: (i) complementary redox potentials – laccases (0.4 to 0.8 V) oxidize phenolic units but cannot access non-phenolic linkages (>80% of lignin), a gap filled by LiP's high-potential Compound I/II cycle (>1.2 V) (Pollegioni *et al.* 2015; Janusz *et al.* 2017); (ii) an H_2O_2 supply chain – laccase-generated phenoxy radicals undergo spontaneous dismutation to release H_2O_2, supplemented by co-secreted auxiliary oxidases, sustaining peroxidase activity; and (iii) repolymerization suppression – peroxidases and quinone reductases consume reactive radical intermediates that would otherwise re-condense, preserving monomer yields (De Gonzalo et al. 2016). This natural division of labor explains why enzyme cocktails consistently outperform isolated enzymes in lignin depolymerization.

Lignin peroxidase oxidizes non-phenolic units, such as syringyl lignin, and specifically cleaves *β*-O-4 bonds, while manganese peroxidase oxidizes phenolic units such as hydroxyphenyl lignin. Versatile peroxidase has dual active sites, including a Mn^{2+} binding site and a tryptophan residue channel, which determines its ability to catalyze the depolymerization of both phenolic and non-phenolic units. Versatile peroxidase also has a broad substrate spectrum, capable of depolymerizing low molecular weight phenolics, such as guaiacol, to high molecular weight lignin fragments. LiP requires a highly acidic environment, typically at pH of 2 to 3, whereas MnP has a broader pH range, generally between pH 4 and 5. Studies have shown that by designing a cascade catalysis system involving all three enzymes, the conversion of lignin to monophenolic aromatic compounds can be increased to 76% (Dillies *et al*. 2020). LiP, due to its extremely high oxidation capability, is irreplaceable in the degradation of recalcitrant structures. MnP has become the preferred choice for large-scale wastewater treatment due to its environmental adaptability. VP, as a 'universal' catalyst, demonstrates potential in depolymerization within the field of biomanufacturing. In the future, zero-waste goals for lignin resource utilization might be achieved through the design of enzyme molecular machines and optimization of microbial community metabolic division.

Manganese Peroxidase

MnP enzymes generate Mn (III) by oxidizing phenolic compounds, including phenolic dyes, amines, and lignin derivatives. Mn (III) functions as a chelating agent, and under specific physiological conditions, it oxidizes phenolic lignin structures. The oxidation of these lignin compounds involves the conversion of the compound (1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-(hydroxymethyl)-2-methoxyphenoxy)1,3-dihydroxy-propane) to a complex with the substrate Mn (III) chelating agent (Raj *et al*. 2007). This complex acts as a diffusible oxidant, leading to the formation of an intermediate phenoxy radical. The phenoxy radical then undergoes non-enzymatic depolymerization, bond cleavage, and rearrangement, resulting in the production of various products such as (1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-(hydroxymethyl)-2-ethoxyphenoxy)-1-oxo-3-hydroxypropane, 2,6-dimethoxy-1,4-benzoquinone, and 2,6-diethoxy-1,4-hydroxy-benzene) (Wong 2009). In the presence of a secondary mediator, Mn (III) can also catalyze the oxidation of non-phenolic lignin derivatives.

This process involves electrons to passing through the aromatic ring and generating reactive radicals/phenoxy cations.

Furthermore, Mn (III) can mediate the oxidation of substituted diarylpropane and benzyl alcohol structures to their respective ketones and aldehydes in the presence of thiols such as glutathione. In these reactions, Mn (III) is oxidized by thiols to generate sulfhydryl groups, thereby forming benzylic substrates. Mn (III)-containing enzymes also couple lipid peroxidation to catalyze the cleavage of *β*-aryl ethers (Zhang *et al*. 2015), C_α-C_β cleavage of non-phenolic diarylpropanes, and *β*-O-4 lignin and its derivatives, respectively. MnP has made significant breakthroughs in engineering. Firstly, through rational design of mutants, thermal stability has been improved. The half-life at 70 °C was increased from 0.8 h in the wild type to 11.3 h. Alkaline tolerance has also been enhanced, with 85% activity retained at pH 8.0 (Garcia-Ruiz *et al*. 2012).

Lignin Peroxidase

Lignin peroxidase (LiP), also known as diarylpropane oxygenase, contains heme and catalyzes the oxidative depolymerization of lignin using hydrogen peroxide (Barber-Zucker *et al.* 2022). LiP belongs to the oxidoreductase family and breaks down lignin and its derivatives in the presence of H_2O_2. These enzymes, which contain heme, are primarily secreted by higher fungi and some bacteria and facilitate the depolymerization of polymers through an oxidative process.

Additionally, insects, such as the yellow chest beetle and the eastern subterranean termite, utilize this enzyme to digest woody debris. The molecular structure of lignin peroxidase (LiP) consists of a monomeric glycosylated enzyme with a molecular weight of 40 to 68 kDa (Taha *et al*. 2016b). It contains four carbohydrates, 370 water molecules, 343 amino acid residues, two calcium ions, and a heme group. Furthermore, the heme iron is associated with the amino acid His, and the enzyme exhibits a high redox potential. The distance between each heme group and the His amino acid contributes to the enzyme's redox potential and creates an electron deficiency in the iron porphyrin ring. Lignin peroxidase by its multiple active sites that directly oxidize Mn (II) and phenolic and non-phenolic substrates (such as Reactive Black, and Direct Blue) in the absence of Mn (II).

In addition to white-rot and brown-rot fungi, certain bacteria—particularly actinomycetes, *α*-proteobacteria, and *γ*-proteobacteria—have also been reported to decompose lignin and may produce ligninases (Ruxandra Leontieș *et al*. 2022). However, research on bacterial lignin peroxidases (LiPs) remains limited, highlighting the need for bioprospecting to identify novel bacterial LiPs with broad substrate specificity and high enzymatic activity. Structurally, LiPs differ from other heme peroxidases, most notably in their low optimal pH, which distinguishes them from related enzymes.

LiP catalysis is a two-step reaction involving the native enzyme in the iron-resting state, the radical cation oxyferroyl unstable intermediate compound I, and the unbiased oxyferroyl intermediate compound II (Romero *et al*. 2019). In the presence of co-substrates, such as H_2O_2 and resveratrol, LiP can also degrade various phenolic compounds and lignin. Reducing resveratrol forms compound II and resveratrol radical cation, and the enzyme then returns to the native state through 1e reduction, completing the catalytic cycle. LiP-I with the reduced substrate to form LiP-II is pH-dependent, and the rate decreases with increasing pH. The pH dependence of the reduction of LiP-I to LiP-II rather than LiP-I dictates the unusually low pH and optimization of the enzyme. The porphyrin *π*-cation radical first accepts an electron from the substrate in

the first reduction step, accompanied by a proton transfer to the distal His, so that the LiP-II formed from the donor substrate together with the porphyrin field is an electron oxidation equal to that of native LiP.

Multifunctional Peroxidase

Multifunctional peroxidases (VP) are a new class of heme peroxidases, mainly derived from white-rot fungi, belonging to class II lignifying peroxidases. VP was first described in *Pleurotus eryngii* and is distinguished from manganese peroxidase (Woo *et al.* 2014; Li *et al.* 2019). Although VP exhibits functional diversity, the yield and purity of VP from wild-type fungi cannot meet industrial needs. Heterologous expression of VP using prokaryotic and eukaryotic hosts, such as *Saccharomyces cerevisiae*, *Escherichia coli*, and *E. acidophilus*, may help overcome this limitation. A new VP from the white-rot fungus *P. vitreus* PF18 has been obtained, with extraordinary potential in engineering value-added lignin products (Vardhan *et al*. 2019). The substrate channel diameter of VP is 45% larger than that of LiP, which is one reason for its fifth range light. Recently, there have been significant breakthroughs in the engineering of VP, with different effects depending on the breakthrough at various key sites. Some modifications can enhance thermal stability by up to 400% at 80 °C, while others improve alkaline tolerance up to pH 9 (Granja-Travez *et al*. 2018a).

Catalytic oxidation of lignin with rVP1 was performed with 40 mg of alkaline lignin. The total yield of rVP1 reached 18 mg/L, which is significantly higher than that of *P. albinoa* VPL3 (1.5 mg/L) and *P. abalone* VPL2 (5.5 mg/L). Therefore, the expression of fungal VP in prokaryotic cells was successful, with good yield and *in vitro* activity. Moreover, the optimal pH and temperature were 5.0 and 40 °C, respectively. The pH values showed that rVP1 exhibited the same properties as wild-type VP from *Pleurotus* species and *Bjerkandera* species, which were 4.5 and 5.0, respectively (Liu *et al.* 2019a).

Dye Decolorization Peroxidase for Lignin Depolymerization

Dye decolorizing peroxidases (DyP) are a family of heme peroxidases with a substrate preference for xenobiotic azo and anthraquinone-type dyes, which are poor substrates for other peroxidases (De Gonzalo *et al.* 2016c; Tonin *et al.* 2017b). However, their broad spectrum as an oxidizing agent of aromatic compounds makes them a potential depolymerizer of lignin compounds, though this ability will depend on DyP type, source, and reaction conditions. DyP is divided into four types based on sequence similarity: A, B, C, and D. Type D DyP are found only in fungi and share a tertiary structure (ferredoxin-like fold) with types A, B, and C (Linde *et al.* 2015b). An attractive property of DyP is its resistance to high temperatures, pressures, and acidic conditions. The catalytic cycle of DyP-type enzymes begins with their activation by peroxides. DyPs are a recently recognized family of heme peroxidases (Fig. 2a) that oxidize lignin via a peroxidase-type mechanism (Fig. 2b). Originally characterized for xenobiotic dye degradation, DyPs also depolymerize kraft lignin and β-aryl ether model compounds. Critically, bacterial DyPs from *Pseudomonas*, *Rhodococcus*, *Bacillus*, and *Amycolatopsis* exhibit remarkable thermostability (Rh_DyPB optimal at 65 °C; whereas amycolatopsis DyP retains 75% activity at 50 °C for 2 h), can function across a broad pH range (3.0 to 8.0), and are routinely expressed as soluble active proteins in *E. coli* at high yields (>100 mg/L).

DyP of Gram-negative Bacteria

Gram-negative *Pseudomonas* species exhibit lignin-oxidizing activity and also contain DyP-type peroxidase genes (Rahmanpour and Bugg 2015). *Pseudomonas fluorescens* Pf-5 contains three DyP-type peroxidases (35, 40, and 55 kDa), which have been overexpressed in *E. coli*, purified, and characterized. Each of the three enzymes showed activity in oxidizing phenolic substrates, but the 35 kDa DyP1B enzyme also showed activity in oxidizing Mn (II) and kraft lignin (Brown *et al.* 2012). Treatment of powdered lignocellulose with DyP1B in the presence of Mn (II) and hydrogen peroxide resulted in the release of low molecular weight lignin fragments that have been identified as *β*-aryl ether lignin dimers containing one G unit and one H unit with a benzyl ketone (Vignali *et al.* 2018).

Streptomyces virulosus has been shown to degrade lignin; however, until recently, the enzymatic mechanisms underlying bacterial lignin depolymerization remained poorly understood. The *Jostii* RHA1 can oxidize a model compound of *β*-aryl ether lignin and attack kraft lignin or wheat straw lignocellulose in the presence of Mn^{2+}. The catalytic activity of *R. jostii*-DyPB was improved by site-directed mutagenesis. In addition, the DyP-type peroxidase TfuDyP derived from the moderately thermophilic bacterium *Thermobifida fusca* exhibited dye decolorization activity and showed activity on substrates such as guaiacol and 2,6-dimethoxyphenol. In the presence of H_2O_2, it can be efficiently oxidized. When reacted with guaiacol for 30 min, the conversion rate reached up to 95%. Its specific activity with 2,6-dimethoxyphenol (DMP) can attain a maximum of 27.9 U/g. The enzyme from this strain has the advantage of thermal stability, as the strain is thermophilic, growing at 55 °C, and this enzyme can retain 80% of its activity at 60 °C (Rahmanpour and Bugg 2015). The study confirmed that Gram-negative bacterial strains, such as *P. fluorescens*, also contain DyP-type peroxidases that can oxidize lignin. Homologs of DyP1B are present in many *Pseudomonas* strains and *Burkholderia* and *Bordetella*. These bacterial DyP-type peroxidases towards lignin, Mn (II), and a range of aromatic substrates further demonstrate the potential of DyP peroxidases for biotechnological applications, including valorization of lignin and the potential of *Pseudomonas* to convert lignin into aromatic products.

DyP of Gram-positive Bacteria

Dye-decolorizing peroxidases from Gram-positive bacteria can oxidize lignin model compounds. For instance, the lignin-degrading bacterium *Rhodococcus jostii* can oxidize a *β*-aryl ether lignin model compound, degrade solvent-derived kraft lignin fractions or wheat straw lignocellulose in the presence of Mn^{2+}. To facilitate practical applications, the N246A variant of DyPB Rh-DyPB was overexpressed in *E. coli* using a designed synthetic gene (De Gonzalo *et al.* 2016a). Adopting optimized conditions, the enzyme was produced entirely as a folded holoenzyme, thus avoiding further time-consuming and expensive reconstitution steps. The peroxidase showed higher activity in the presence of Mn^{2+}. It was able to degrade wheat straw lignocellulose and catalyze the cleavage of the C_α-C_β bonds of the lignin model molecule *β*-aryl ether (Tonin *et al.* 2017b).

A single chromatographic purification step yielded > 100 mg/L of enzyme culture fluid with a purity of > 90%. Rh_DyPB, an extremely thermophilic enzyme with an optimum temperature of 65 °C, exhibited classical peroxidase activity with maximum peroxidase activity occurring in the 45 to 75 °C temperature range. The activity was significantly enhanced after adding Mn^{2+}, in which H_2O_2, Mn^{2+}, ABTS,

and 2,6-DMP(2,6-dimethoxyphenol) kinetic parameters were determined. The recombinant enzyme also showed good thermal stability (melting temperature 63 to 65 °C) and was stable at pH 6 to 7. Rh_Dyp B maintained about 50% of the initial activity in the 3 to 9 pH range, and ≥ 80% of the initial activity in the pH 6 to 7 range after incubation at 25 °C for 24 h, and most of the initial activity was retained after incubation at 25 to 37 °C for 24 h (De Eugenio *et al.* 2021). A total of 1 M NaCl, 10% DMSO, and 5% Tween-80 did not affect the activity of Rh_DyPB. In particular, various aromatic monomers from lignin were oxidized in the presence of Mn^{2+}. The dimerized lignin model compound guaiacylglycerol-*β*-guaiacyl ether (GGE) was used as a substrate, and 2 mM $MnCl_2$ and 1 mM H_2O_2 were added to 50 mM sodium malonate buffer (pH 6.0) to investigate the ability of Rh_DyPB to act on lignin. Under the optimized conditions, 2 mM GGE was completely cleaved by recombinant Rh_DyPB in only 10 min to generate guaiacol at a rate of 12.5 μmol/min·mg enzyme. The results showed that Rh_DyPB can also oxidize various monomeric lignin model compounds and effectively break the C_α-C_β bond of GGE (dimeric lignin model of *β*-O-4 bond). *Streptomyces virulosus* has been shown to degrade lignin; however, until recently, the enzymatic mechanisms underlying bacterial lignin depolymerization remained poorly understood.

DyP of Fungal Source

Dye decolorizing peroxidase was cloned from *Lactobacillus* and heterologously expressed in *E. coli* as inclusion bodies, activated against ABTS, 2,6-dimethoxyphenol (DMP), anthraquinone, and azo dyes, as reported for other fungal DyP (Singh *et al.* 2013b). Beyond its interest in dye decolorization, findings from the conversion of softwood and hardwood lignin sulfonates indicate that this enzyme plays a well-established biological role in the depolymerization of phenolic lignin. The overall structure of *Lactobacillus* DyP consists of two domains with a ferredoxin-like fold corresponding to a typical DyP. IrlacDyP activity was studied in the range of 1.5 to 9.0 using ABTS, DMP, vinyl acetophenone (VA), and $MnSO_4$ as substrates (Dhankhar 2021). The enzyme was more active at pH 1.5 to 6.0, but the optimal pH varied, depending on the substrate. The oxidation of DMP and VA was maximal at pH 2.5. Regarding pH stability, the enzyme was remarkably stable between pH 3 to 6 and at 4 °C, retaining 90% of its activity after 168 h of incubation. Enzyme stability was lower at 25 °C, retaining 90% of its activity between pH 5 and 6 but decreasing to 60% and 20% after 70 h of incubation at pH 3 and 4, respectively (Dhankhar 2021). Approaches such as protein engineering techniques can also be used to design proteins with improved stability, as has been reported for VP of *P. albinoa*. Recombinant DyP from *Irpex* is the only fungal DyP described to oxidize high redox potential dyes such as VA and $MnSO_4$.

Bacillus subtilis

The structure of the *Bacillus subtillis* (Bs) DyP monomer comprises nine *β*-strands and fifteen *α*-helices with ferredoxin-like folds, similar to other DyP structures. The formerly cloned BsDyP was expressed and purified, as mentioned previously (Dhankhar 2021). Enzyme activity of BsDyP against different substrates, such as VA, DMP, and guaiacol, was measured spectrophotometrically at 25 °C by observing the changes in the absorbance at their specific wavelength. The results revealed that the optimum pH is 4.0 for the maximum enzyme activity towards phenolic compounds (DMP and guaiacol) and non-phenolic compounds. However, the enzyme is active in a

pH range of pH 2.0 to 9.0. The BsDyP retained 40% and 10.9% of peak activity to oxidize VA at pH 2.0 and 9.0, respectively.

A DyP-type peroxidase enzyme DyPB from *R. jostii* RHA1 has been identified that can oxidize a *β*-aryl ether lignin model compound and can attack kraft lignin or wheat straw lignocellulose in the presence of Mn^{2+}. The catalytic activity of *R. jostii* DyPB has been enhanced by site-directed mutagenesis, and a further DyP2 enzyme has been identified in *Amycolatopsis sp.* 75iv2 (Vuong *et al*. 2021). The DyP2 has higher activity towards Mn^{2+} oxidation and exhibits peroxidase activity against phenols, azo dyes, and anthroquinone dye. A DyP-type peroxidase enzyme, TfuDyP from *Thermobifida fusca*, a moderate thermophile bacterium, exhibits dye-decolourising activity and shows activity towards substrates such as guaiacol and 2,6-dimethoxypheno.

P450 Enzymes Used for Lignin Depolymerization

The P450 enzyme group is a superfamily of multifunctional redox reductases characterized by heme as the reaction center and molecular oxygen as the oxidant. P450 enzymes catalyze a variety of stereoselective and regioselective oxidation chemistries such as hydroxylation, epoxidation, dealkylation, and sulfur oxidation (Ichinose 2013; Mallinson *et al.* 2018; Nelson 2018; Wolf *et al.* 2022). Based on the similarity of amino acid sequences, P450 has been divided into more than 2,200 families. Members of the same family have at least 40% sequence identity and biological function (Sutherland 1986). Several studies have found P450 to be involved in the biosynthesis and depolymerization of lignin in nature. This is due to the generation of O-methylated Lewis acid catalysts (LDACs) from lignin fractionation and depolymerization, potential substrates for P450.

Aryl-O-demethylation is an important biochemical reaction that ultimately degrades aromatic compounds derived from coniferyl alcohol and is often a key bottleneck in both natural and engineered bioconversion pathways. Therefore, all lignified compounds must be O-demethylated to diols before they can be oxidatively cleaved to generate ring-opened compounds that ultimately enter central carbon metabolism. The importance of O-demethylation has prompted a strong effort to identify and characterize enzymes that can demethylate methoxy substituents of different lignin-derived substrates. For example, the VanAB O-demethylase from *Acinetobacter baylyi* ADP1 was described, which converts vanillin to the central intermediate protocatechuate *via* a Rieske non-heme iron monooxygenase mechanism (Zhang *et al.* 2014). The cytochrome P450 aromatic-O-demethylase in *Amycolatoposis* sp. plays an important role in bacterial depolymerization of lignin by converting the lignin monomer guaiacol into catechol, which then enters the *β*-ketoadipate pathway (Mallinson *et al.* 2018).

In wood-destroying basidiomycetes, P450 of the CYP53D family catalyzes the O-demethylation of resveratrol and related stilbenes. The best characterized P450 involved in lignin valorization are bacterial CYP255A and CYP199A enzymes (Harlington *et al.* 2022). The exceptional range of stereo-selective and regio-selective chemistry catalyzed by P450 and their amenability to protein engineering make them ideal systems for developing microbial cell factories. Thus, in designing microbial cell factories, wild-type and engineered P450 can be used to expand the LDAC conversion capacity of host strains, as well as functionalize pathway metabolites to produce difficult-to-access chemicals from biomass. For example, both GcoABJ3 and GcoABAmyc from *R. rhodochrous* J3 have been produced in *P. putida* to confer growth

on guaiacol, a compound that this platform organism does not naturally catabolize (Machovina *et al.* 2019).

Flexible combinations of P450 components may have important implications for function and specificity. Engineering P450 redox partners *via* fusions similar to those of class VII systems could enable more efficient biocatalytic transformations. For example, P450BM3 fusion proteins have been used in a synthetic peroxidase system to selectively O-demethylate several aromatic ethers (Ali *et al.* 2020). Additional studies are needed to evaluate the advantages of expressing one larger enzyme *versus* two or more smaller enzymes.

The cytochrome P450 reductase gene pair GcoAB was recently isolated from the robust aromatic-decomposing bacterium, *P. putida* KT2440, that was able to confer guaiacol (2-methoxyphenol) growth *via* plasmid-based expression (Reisky 2018). Unlike other known tetrahydrofolate- or non-heme iron-dependent demethylases, which have comparable substrate specificity, the P450 reductase pair characterized here demethylates diverse aromatic substrates, which could offer important advantages in both natural and biotechnological contexts. First, guaiacol, the natural substrate of GcoA, is the primary decomposition product of plant lignin (Jiang *et al.* 2020). Demethylation of guaiacol produces catechol, which can be ring-opened by catechol dioxygenases that cleave either endo- or exo-diol. Second, in contrast to other known O-aryl-demethylases, the substrate preference of GcoA is broad, allowing for a variety of guaiacol analogs, also known as lignin decomposition products.

Biofunneling of lignin-derived aromatic compounds is a promising approach to catalytic depolymerization product pricing. Industrial processes for aromatic bioconversion will require efficient enzymes to carry out key reactions, including the demethylation of O-methoxy-aryl groups, which is an essential and often rate-limiting step. The recently characterized GcoAB cytochrome P450 system comprises a coupled monooxygenase (GcoA) and reductase (GcoB) that catalyze the oxidative demethylation of the O-methoxy-aryl groups in guaiacol (Karlson *et al.* 1993). At least three classes of enzymes, Rieske non-heme iron monooxygenases, tetrahydrofolate-dependent O-demethylases, and cytochrome P450 enzymes, are known to catalyze aromatic O-demethylation. P450 are powerful redox catalysts that serve as particularly useful scaffolds for bioconversion. The core P450 system was first identified and demonstrated the O-demethylation of guaiacol. The simplest compound with a G-type substitution pattern on the aromatic ring reported the N-terminal sequence of the P450 oxygenase. The decomposition of *p*-aniline was further demonstrated by heterologous expression of the variant enzyme in *P. putida* KT2440, where the same variant was able to efficiently demethylate the reduction product of *p*-vanillin (*p*-vanillyl alcohol) (Jiang *et al.* 2020).

Table 1. Key Enzymes, Reaction Pathways and Mechanism of the Bio-depolymerisation Strategy of Lignin Polymer

Ligninolytic Enzymes	Gene Origin	Key Enzymes	Strains	Main Strategies	Substrates	Aromatic Products	Reaction Temperature and pH	References
Laccases	Endogenous	CopA-I, CopA-II	*Pseudomonas putida* KT2440	Heterologous expression and characterization	Lignin model compounds	Giving oxidized dimerized products	50 °C 2.3 to 10.4	Granja-Travez and Bugg 2018
	Ochrobactrum sp.	CueO	*Escherichia coli*	Heterologous expression	*β*-Aryl ether and biphenyl lignin dimer model compounds and lignosulfonate	Vanillic acid and low molecular weight lignin	/ 3.0 to 5.0	Granja-Travez *et al.* 2018b
	Streptomyces coelicolor A3	Small laccase	*Streptomyces coelicolor*	Small laccases from four different *Streptomyces* species	Lignin model compounds and ethanosolv lignin	Corresponding ketone derivative	/ 8.0	Majumdar *et al.* 2014
	Bacillus pumilus	CotA-laccase SF	*E. coli*	Heterologous overexpression; Co-expression of phospholipase C.	Malachite green	4-minophenol and 4-aminobenzophenone	80 °C 3.5	Xu *et al.* 2020
	Bacillus sp.	LacZ1	*E. coli* BL21	Heterologous overexpression	Lignin in rice straw	Nine monophenolic compounds	75 °C 4.5	Zhang *et al.* 2021
	B. subtilis	CotA	*E. coli*	Heterologous overexpression	Kraft lignin	Lower molecules; A lower S/G ratio	30 to 50 °C /	Mayr *et al.* 2021
	Bacillus licheniformis	rLacNS2324	*E. coli*	Heterologous overexpression	Methylene blue	/	/	Chopra and Sondhi 2022
Dye-decolorizing peroxidases (DyPs)	*P. fluorescens*	DyP1B	*E. coli*	Heterologous overexpression and purification	Kraft lignin wheat straw and lignin model compounds	Low molecular weight lignin fragment	25 °C 5.5	Rahman-pour and Bugg 2015
	Bacillus sp. BL5	DyP	*E. coli* BL21	Heterologous overexpression and purification	ABTS and alkali lignin	Low molecular weight compounds, vanillin, and methoxyphenol	25 °C 4.0	Dhankhar 2021

	R. jostii	DypB	*E. coli* BL21	Heterologous overexpression and purification	Lignin model compound: ABTS, 2,6-DMP, and GGE	Guaiacol	25 to 50 °C 3.0 to 8.0	Khan *et al.* 2021
	Comamonas testosteroni TK102	DypB	*E. coli* BL21	Heterologous overexpression and purification	ABTS, 2,4-dichlorophenol (DCP) and guaiacol	Low molecular weight aldehyde and ketone	25 to 37 °C 6.0	Vignali *et al.* 2018
	Amycolatopsis sp. ATCC 39116	*Asp*DyP2	*E. coli* BL21	Expressed as soluble and active His-tagged proteins	Phenolic lignin model dimer GGE	Veratraldehyde formation	/	Rashid and Bugg 2021
	Bacillus subtilis	*Bs*DyP	*E. coli*	Heterologous expression; Molecular docking and simulation	Malachite green and methyl violet, reactive black 5	/	25 °C 3.0 to 4.0	Dhankhar *et al.* 2020b
	Irpex lacteus	DyP	*E. coli*	Heterologously expressed as inclusion bodies purified in one chromatographic step	ABTS, 2,6-dimethoxyphenol (DMP), and anthraquinoid azo dyes, veratryl alcohol, and lignosulfonates	/	/	De Eugenio *et al.* 2021
Lignin peroxidase (LiP)	*Phanerochaete chrysosporium*	LiP	*E. coli* BL21	Rational design, engineering and introducing salt bridge	Model dimeric lignin compound	Guaiacol and 3,4-dimethoxy benzaldehyde	25 °C 2.5	Pham *et al.* 2018
	Aspergillus sp.	LiP	*Pichia pastoris*	Heterologous overexpression, purification, and characterization	Azo dyes	/	/	Liu *et al.* 2020b
Manganese peroxidase (MnP)	*Phanerochaete chrysosporium*	MnP	*E. coli*	Co-expression of MnP with chaperone	ABTS	/	/	Alfi *et al.* 2019

	B. aryabhattai	MnP	*B. aryabhattai*	Strain screening; Bacterial identification	Kraft lignin	Almost 84% kraft lignin degradation	/	Singh *et al.* 2022
Versatile peroxidases (VP)	*Pleurotus eryngii*	VP	*Saccharomyces cerevisiae*	Random mutagenesis libraries; Expressed on the surface of yeast cells; Flow cytometry-based high-throughput screening system	RB5	/	25°C 3.5	Đurđić 2020
	Physisporinus vitreus	rVP1	*E. coli*	Overexpressed as inclusion body	Alkali lignin	More G-type lignin; Less S-type lignin	25 °C 4.5	Liu *et al.* 2019b
	Pleurotus eryngii	VP	*S. cerevisiae*	Saturation mutagenesis and VP-immobilization	Azo dyes	/	25 °C 3.5	Ilić Đurđić *et al.* 2020
P450	*Amycolatopsis* sp. ATCC 39116	GcoAB	*Pseudomonas putida* KT2440	Heterologous expression	Guaiacol Ferulate vanillate	Catechol	/	Mallinson *et al.* 2018

Abbreviations: DyP, dye-decolorizing peroxidase; LiP, lignin peroxidase; MnP, manganese peroxidase; VP, versatile peroxidases; DypB, DyP1B, are members of the Dyp peroxidase family; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); 2,6-DMP, 2,6-dimethoxyphenol; GGE, guaiacylglycerol-β-guaiacyl ether; VA, veratryl alcohol; VGE, veratrylglycerol-guaiacyl ether; G/S ratio, the ratio of guaiacyl and syringyl units

These studies expanded the known substrate specificity of P450 to lignin-related compounds. Further, it demonstrated the potential applicability of the versatile demethylation system to the biofunneling of lignin-derived aromatic compounds.

O-dealkylation of substrates by P450 has been observed with multiple isozymes and is currently part of the depolymerization and metabolism of drug molecules. Thus, the O-demethylation reaction shows similarities to aliphatic hydroxylations by P450 enzymes, which usually proceed *via* an initial hydrogen atom abstraction from compound I to form an anionic (IV)-hydroxy complex that rebounds its OH group onto the substrate as an alcohol product. P450-mediated O-demethylation also produces formaldehyde, a toxic compound that microorganisms detoxify through multiple pathways. Engineering enhanced formaldehyde detoxification pathways and robust microbial cell factories may be critical to improving the utility of P450-based biocatalysts. Mitigating this intermediate toxicity may require dynamic metabolic control and tolerance adaptive laboratory evolution, which uses natural selection to identify targets that improve growth in the presence of toxic compounds (Ali *et al.* 2020; Ellis *et al.* 2021). Ultimately, the most exciting application of P450 in lignin valorization may lie in functionalizing pathway metabolites to generate bioprivileged compounds from them.

Other Ligninolytic Enzymes used for Lignin Depolymerization

The bacterial *β*-etherase system can also degrade *β*-aryl ether structures (*β*-O-4). The *β*-etherase family was first identified in *Sphingobacteria.* SYK-6 is a bacterium that degrades lignin dimers with *β*-O-4 structures to produce intermediate compounds such as vanillic acid and syringic acid. *Sphingobium sp.* SYK-6 showed a significant effect on the depolymerization of lignin in *β*-O-4 bonds, including C_α dehydrogenase (Ligd, Ligl, Ligo, and Lign), etherase (Lige, Ligf, and Ligp), and glutathione lyase (Ligg) (Mathieu *et al.* 2016). The Ligdfg system catalyzes the following reactions, divided into three steps. First, in the presence of NAD^+, Ligd oxidizes C_α, the hydroxyl group of lignin, to form the ketone group, which makes the lignin substrate oxidized from alcohol to the corresponding ketone. Second, Ligf attacks C *via* reduced glutathione *β* while destroying the ether bond. Third, with the assistance of another reduced glutathione, the glutathione transferase Ligg releases oxidized dimeric glutathione and aromatic monomers from the substrate to generate the final product.

The copper complex is the active center of laccase. The hydrochloride complex of copper ions and ethylenediamine (without hydrogen peroxide) can mimic laccase and oxidatively degrade lignin monomer and dimer model compounds at room temperature. In addition to promoting the lignin model compound C, the complexes of copper ions and ethylenediamine in the reaction α and C_β or C_α not only destroy the connecting bond between the position and the benzene ring (Liu *et al.* 2020a), but they also promote the ring-opening reaction of the aromatic compound 4,6-di-tert-butyl-2-methoxyphenol. The hydrochloride complex of Cu^{2+} and ethylenediamine qualitatively simulates the depolymerization mechanism of laccase in the reaction process. As a new type of non-porphyrin catalytic oxidation system, the Gattermann-Iron formulation (GIF) system is primarily composed of acetic acid or another acid (proton source), pyridine (solvent), iron powder (electron source), and iron catalyst precursor. Among the various enzyme mimicry systems currently under study, the GIF enzyme mimicry oxidation system composed of Cu^{2+}, pyridine, and peroxide is more promising. It is one of the enzyme mimicry systems closest to laccase. The GIF system can directly destroy C-H bonds (Fujita *et al.* 2020), so phenolic and non-phenolic lignin structures are degraded in the Cu^{2+}/Pyr/H_2O_2 enzyme

mimicry. In this system, the Cu^{2+}/pyridine complex is first reduced to Cu^{+} by hydrogen peroxide while releasing oxygen. The Cu^{3+}/pyridine complex reacts with oxygen to form dioxide in excess of hydrogen peroxide. This dioxide has a strong oxidizing ability. It can directly attack C-H bonds. Studies have shown that this dioxide can selectively oxidize hydrocarbons containing carbon-hydrogen bonds to ketones and carboxylic acids, which is the core part of the entire mechanism.

In addition to LIP, MnP, and laccase, some other enzymes are closely related to the lignin depolymerization process (Mathieu *et al.* 2016; Fujita *et al.* 2020; Liu *et al.* 2020a). Aromatic alcohol oxidase is an enzyme that provides extracellular H_2O_2. It plays an important role in the enzymatic depolymerization of lignin, especially in the oxidation of lignin polymers. Aromatic alcohol oxidase substrates can include lignin-derived compounds and aromatic fungal metabolites. They can also reduce the phenoxy groups laccase produces during lignin depolymerization, preventing lignin from polymerizing under the action of the enzyme. Aromatic alcohol oxidase can convert aromatic alcohols into corresponding aldehydes and provide extracellular H_2O_2 to degrade lignin. Among several lignin-degrading fungi, aromatic alcohol oxidase activity was detected in *Trichoderma reesei* strains. Two extracellular manganese superoxide dismutases from *Sphingobacterium* T2 were identified, which catalyze the interconversion of dioxygen with reactive oxygen species superoxide and peroxide. They are highly active in oxidizing recombinant forms of organosolv lignin, producing various reaction products, with *Sphingobacterium* sp. T2 having a higher lignin oxidation activity in the presence of dioxygen than H_2O_2.

Laccase exhibits the widest temperature adaptation range and highest thermal stability. For example, laccase from *Trametes versicolor* retains 60% activity after treatment at 65 °C for 2 h (Arregui *et al.* 2019). The thermal stability of DyP is second. DyP from *Amycolatopsis* retains 75% activity after 1 h at 60 °C (Dhankhar *et al.* 2020a). P450 enzymes exhibit the poorest thermal stability, which limits their potential for industrial applications. Peroxidases and laccases prefer acidic environments, whereas P450 enzymes show highest activity under neutral to slightly alkaline conditions (Wolf *et al.* 2024). This difference mainly arises from variations in the metal coordination environment of the enzyme active site: peroxidases contain heme iron, while laccases contain copper redox centers.

The efficient depolymerization of lignin relies on the spatiotemporal coordination of the aforementioned enzymes, which can be primarily categorized into two collaborative patterns. The first is the oxidative relay system, where laccase/peroxidase initiates the cleavage of macromolecules, generating medium-sized fragments; DyP further degrades these medium-sized fragments, while P450 performs refined modifications. Throughout this process, auxiliary enzymes, such as quinone reductase prevent the repolymerization of intermediate products. The second pattern is complementary substrate specificity: laccase primarily targets phenolic units, LiP attacks non-phenolic structures, DyP processes dye-like domains, and P450 modifies low-molecular-weight fragments. Co-culture studies of white-rot fungi have shown that when Trametes versicolor is co-cultured, the activities of laccase and peroxidase are increased by 5.94-fold, respectively, confirming the strong synergistic effect of enzyme systems from different sources (Singh *et al.* 2025).

The economic rationale for enzymatic lignin depolymerization is best illustrated by three representative products spanning the value spectrum. Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the most established lignin-derived aromatic, commanding $50–200/kg (lignin-sourced) to the range $1,200 to 1,500/kg (natural) in a $600 million global

market; enzymatic production *via* laccase-mediator oxidation of ferulic acid achieves 60–80% molar yields, though product toxicity above 1 to 2 g/L necessitates *in situ* removal strategies (Fache *et al.* 2016; Mallinson *et al.* 2018). Bridging specialty and commodity applications, the guaiacol–catechol pair – generated *via* a two-enzyme cascade of laccase/peroxidase depolymerization followed by P450 O-demethylation (>95% selectivity) – provides versatile platform intermediates ($5 to 15/kg) for pharmaceuticals (L-DOPA), agrochemicals, and flavors, with the added advantage of distillation-compatible separation (Schutyser *et al.* 2018; Harlington *et al.* 2022). Together, these cases underscore that the economic viability of lignin valorization hinges not solely on enzyme performance but equally on product separation cost, market price, and downstream synthetic versatility.

Table 2. Comprehensive Comparison of Four Major Ligninolytic Enzyme Classes

Feature	Laccases	Peroxidases (LiP/MnP/VP)	DyPs	P450s
Active Center	Multi-copper (4 Cu ions: T1, T2, T3, T4)	Heme (Fe-protoporphyrin IX)	Heme (Fe-protoporphyrin IX)	Heme (Fe-protoporphyrin IX)
Cofactor / Redox Mediator	O_2 (no H_2O_2 needed); mediator often required for non-phenolics	H_2O_2 essential	H_2O_2 essential	O_2 + NAD(P)H (electron donor) / redox partner
pH Optimum	2–10 (highly variable; fungal laccases acidic, bacterial broader)	LiP: 2.5; MnP: 4–5; VP: 3–5	3.0–6.0 (typically acidic)	Neutral to slightly alkaline (7–8)
Temperature Optimum	25–90 °C (broadest; CotA up to 80 °C)	Moderate (25–50 °C)	25–65 °C (Rh_DyPB up to 65 °C)	Moderate (25–37 °C)
Thermal Stability	Highest (T. versicolor retains 60% at 65 °C, 2 h)	Moderate	Second to laccase (Amycolatopsis DyP retains 75% at 50 °C, 2 h)	Lower

ENZYME MODIFICATION TOWARD LIGNIN VALORIZATION

Wild-type ligninolytic enzymes are often ill-suited to industrial conditions not by defect, but by evolutionary history. These enzymes evolved in rotting wood – a cool (15 to 25 °C), moist, mildly acidic (pH 4 to 6) niche where degradation occurs over weeks. There was no selective pressure to develop the thermostability (>40 °C) or extreme-pH tolerance demanded by bioreactors operating at 40 to 80 °C and pH <3 to >10. This ecological mismatch underpins the performance limitations reviewed here and motivates the engineering strategies that follow. Thus, wild-type enzymes generally are unsuitable for industrial reagent applications. The oxidation of non-phenolic lignin model compounds by VP enzymes represents the most commonly utilized challenging substrate for degradation. One limitation of using this enzyme in the delignification of lignin into value-added

products has to do with the gradual loss of activity under acidic pH conditions. However, it exhibits the highest oxidative activity under acidic conditions (Valderrama *et al.* 2002; Gonzalez-Perez *et al.* 2016). In addition, a lower pH environment is conducive to lignin decomposition. Sequence direct design and protein engineering technology have been used in enzyme modification technology to enhance the activities of enzymes involved in lignin deconstruction.

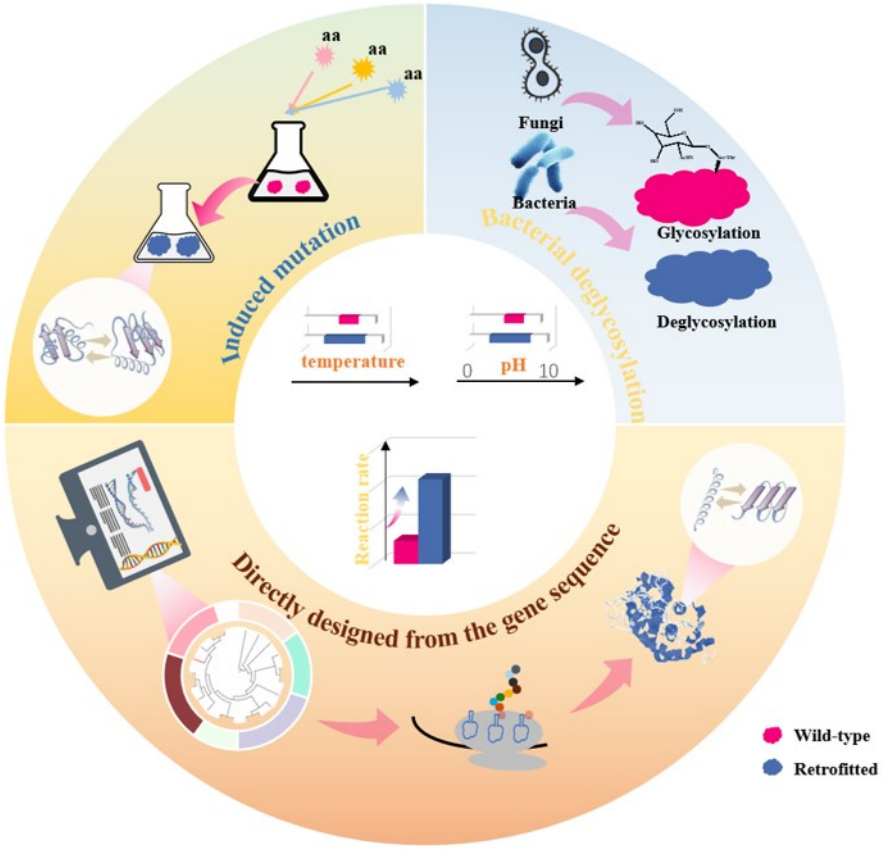

Fig. 3. Methods for transforming lignin depolymerase

Direct Design Based on Gene Sequence

Computational enzyme optimization methods can be applied to many enzymes in parallel (Martínez *et al.* 2009b; Ayuso-Fernández *et al.* 2018). The reliability of a new generation of structure predictors and design methods will increase the scale and scope of computational enzyme optimization, enabling efficient discovery and exploitation of the functional diversity of natural enzyme families directly from genomic databases.

It is confirmed that model structures calculated by deep learning-based *ab initio* structure prediction methods are reliable starting points for one-off PROSS stability design calculations (Popelier 2022). VP was structurally characterized and optimized for recombinant functional expression, stability, and activity. Four designed VP, encoding up to 43 mutations relative to the wild-type enzyme, were functionally expressed in yeast, with three showing substantial functional diversity in their reactivity profiles. In the natural environment, lignin is degraded in an acidic environment, and the activity of VP is strongly dependent on acidity. In particular, a low pH 2 to 3 promotes the formation of tryptophan radicals at highly oxidative sites, which is determined by the enzyme's stability and activity. Additionally, H_2O_2 is the terminal electron acceptor in VP, but at high

concentrations, it also inactivates the enzyme. Among the four mutants mentioned above, 11 h showed the highest stability to hydrogen peroxide (Singh *et al.* 2013b; Lin *et al.* 2018b). The VP was also designed to have higher thermostability. Although the enzyme lost half of its maximal activity after 15 min of incubation, it did not completely lose activity at high temperatures for 5 h; during shorter incubations, the activity at 45 to 60 °C for 5 h was significantly enhanced compared with the activity at room temperature (Gao *et al.* 2017).

The lignin-degrading fungus *Rhodococcus jostii* was recently shown to degrade solvent-derived kraft lignin fractions. To facilitate practical applications, the N246A variant of DyPB was overexpressed in *E. coli* using a designed synthetic gene. Under optimized conditions, the enzyme was produced entirely as a folded holoenzyme, thus avoiding further time-consuming and expensive reconstitution steps (Biko *et al.* 2020b).

Protein Engineering Technology for Ligninolytic Enzymes

Peroxidases from the *Echinops* spp. genus exhibit remarkable acid stability and are the most acidic pH-stable lignin-degrading peroxidases described to date. This peroxidase isozyme was used as a robust protein scaffold to obtain a VP-type peroxidase by introducing an exposed catalytic tryptophan in two different protein environments. The engineered peroxidase would be an industrial biocatalyst due to its promiscuity in oxidizing different recalcitrant aromatic compounds and dyes, as well as its high stability at acidic pH, improving its action on lignin (Fernández-Fueyo *et al.* 2014).

There are two approaches to confer VP-type activity on aromatic substrates and dyes to MnP6 by protein engineering (Barber-Zucker *et al.* 2022). The first strategy is to enter only the tryptophan residue to obtain the S168W variant. In contrast, the second approach mimicks a tryptophan environment similar to Trp164 in *Pleurotus ostreatus* VP1, generating the S168W environmental variant. The introduced mutations did not affect the Mn^{2+} binding capacity. More importantly, the kinetic constants showed that both strategies for introducing a functional catalytic tryptophan succeeded, as both variants could oxidize VA and RB5. With respect to the pH 3.0 used in the standard assay, the activity increased by four-, six-, and eight-folds at pH 2.5, 2.0, and 1.6, respectively.

The different stabilities of native and recombinant DyP forms may be due to the lack of glycosylation in the enzyme produced in *E. coli*. Therefore, a eukaryotic expression system would be required to improve the properties of IrlacDyP. However recombinant active fungal heme peroxidases production in eukaryotic systems shows two significant disadvantages. First, low heterologous expression levels are usually obtained. Second, highly glycosylated proteins are usually obtained when yeast is used as an expression system, affecting the catalytic properties and stability of the recombinant peroxidase. It is worth noting that the expression of eukaryotic recombinant proteins may simplify protein folding and present high secretion rates. However, genetic manipulation can be more challenging, and highly glycosylated proteins show lower recovery and specific activity. In contrast, the expression of complex proteins in prokaryotic systems can accumulate as inclusion bodies. Nonetheless, the formation of inclusion bodies can be reduced by co-expression of chaperones or continuous addition of heme, which has been successfully used to express blood peroxidase to produce more stable proteins. In both cases, protein engineering techniques promote the protein design with improved stability, as reported for *P. eryngii* VP.

IMMOBILIZATION OF LIGNINOLYTIC ENZYMES USING MOFS AND COFS

Before the advent of MOFs and COFs, several conventional materials have been extensively applied for ligninolytic enzyme immobilization, each with distinct industrial suitability profiles. These are sol-gel silica matrices and magnetic nanoparticles.

Sol-gel Silica Matrices

Silica sol-gel encapsulation, pioneered by the entrapment of laccase in tetramethoxysilane (TMOS)-derived gels, offers excellent mechanical stability and resistance to microbial degradation. Encapsulated laccase typically retains 60 to 85% of free enzyme activity with 5 to 10 fold improvement in thermal stability ($t_{1/2}$ at 60 °C extended from 0.5 h to the range 3 to 5 h). However, mass transfer limitations imposed by the silica network reduce apparent activity toward macromolecular lignin substrates by 30 to 50%, and the sol-gel process requires careful pH control to avoid enzyme denaturation during gelation.

Magnetic Nanoparticles (MNPs)

Fe_3O_4-based magnetic nanoparticles functionalized with amino, epoxy, or carboxyl groups enable rapid enzyme recovery *via* external magnetic fields, which can be a decisive advantage for continuous processing. MNP-immobilized laccase achieves 70 to 90% activity retention, can be reused for 8 to 15 cycles, and reduces enzyme loss during product separation to near zero. Surface-functionalized MNPs achieve enzyme loadings of 50 to 200 mg/g (Oraby *et al.* 2025).

Metal Organic Frameworks

Metal organic frameworks (MOFs) and covalent organic frameworks (COFs) materials offer various opportunities for their application in different fields due to their unique network properties and huge composite space (Xu 2024). Due to their large surface area, ligand adjustability, stable framework structure, and porosity, MOFs have a higher enzyme loading capacity. They can be a good carrier for immobilized enzymes, significantly improving their catalytic activity and reusability (Liu *et al.* 2025). The main technical challenges in enzymatic lignin valorization can be categorized into some quantifiable barriers. Most wild-type ligninolytic enzymes exhibit limited operational stability under industrial conditions. Fungal laccases typically lose >50% activity within 2 to 6 h at pH <3 or >50 °C (*e.g.*, *T. versicolor* laccase retains only 60% after 2 h at 65 °C; Arregui *et al.* 2019). In laccase-mediator systems, up to 30 to 50% of depolymerized fragments can re-condense into higher-molecular-weight products within minutes unless continuously extracted or trapped (*e.g.*, by organic solvent overlay or *in situ* derivatization). This repolymerization is a primary cause of the low net monomer yields observed in one-pot enzymatic lignin processing. Combining MOFs and COFs with lignin-degrading enzymes may lead to novel discoveries in lignin depolymerization research (Park *et al.* 2007). For example, the Cu-ZIF8 nanoenzyme exhibits laccase-like activity and significant catalytic performance in the oxidation of a wide range of phenolic pollutants such as 2,4-dichlorophenol (Safavi-Mirmahaleh and Moradi-Shoeili 2025).

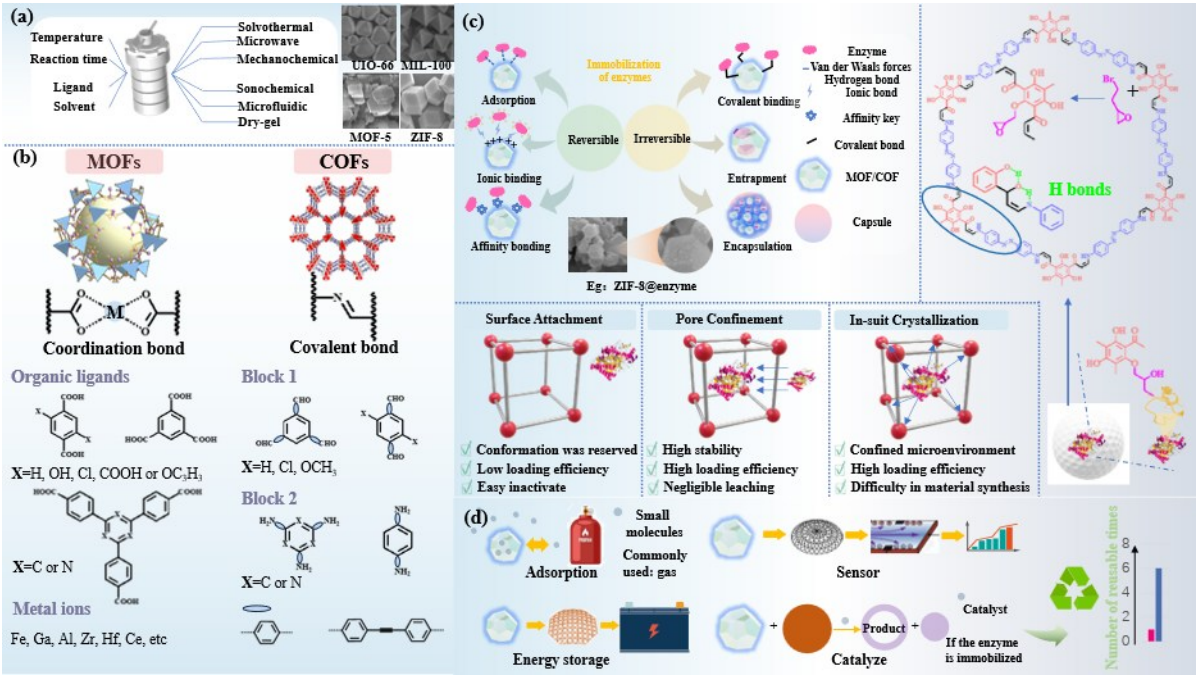

Fig. 4. Modifying lignin depolymerase using MOFs and COFs materials: (a) Synthetic methodologies and critical governing factors for MOFs and COFs; (b) Spatial architecture and structural topology of MOFs and COFs; (c) Immobilization strategies and mechanistic principles of enzymes on MOFs and COFs; (d) Functional applications and technological deployment of MOFs and COFs; MOFs represent metal-organic frameworks and COFs represent covalent organic frameworks

The MOFs are porous crystalline organic-inorganic hybrid materials formed by metal nodes and organic ligands, also known as porous coordination polymers (Cavka *et al.* 2008; Ma *et al.* 2022; Xu *et al.* 2022). COFs comprising carbon atoms and other light elements are non-metallic porous materials integrated by selected organic blocks and strong covalent bonds. The ultra-high surface area and porosity enable them to have higher enzyme loading. Likewise, the structural and functional diversity of MOFs and COFs can simplify the enzyme immobilization process. With the deepening and development of MOFs, MOFs immobilized enzymes with different topological structures have been widely reported (Feng *et al.* 2022). Enzyme-MOFs composite fabrication strategies include surface biocoupling (physical adsorption or covalent bonding), pore infiltration, and *in situ* encapsulation (employing liquid- or solid-phase approaches). Compared to MOFs-based immobilization, enzyme immobilization on COFs offers comparable advantages while avoiding potential enzyme inactivation caused by toxic metal ions in MOFs.

Both MOFs and COFs were successfully used to immobilize laccase, and the immobilized laccase had better resistance to alkaline pH, high temperature, longer storage time, and higher repeated catalytic activity (Wen *et al.* 2016). A new bimodal micro-mesoporous Zr MOFs (Zr-MOFs, MMU) with a particle size of about 200 nm was synthesized. The specific surface area of nano-MMU was 453.8 mg/g, and the pore size was 3.5 to 7 nm. Laccase was immobilized on MMU by physical adsorption, yielding a large 221.8 mg/g adsorption capacity. Mesoporous Zr-MOFs (MMU) similar to UIO66 (UiO from the University of Oslo) were synthesized using a surfactant template method. Zirconium was chosen as the central metal ion instead of copper because a series of MOFs

are easily decomposed in the aqueous phase and the adsorbed laccase is inactivated due to the release of copper ions (Aghaee *et al.* 2024).

Although the surface area of MMU is lower, when compared with other types of MOFs, MMU has sufficient pore size, stability in solution, and carboxyl groups to help immobilize laccase is a suitable support for immobilization (Pang *et al.* 2016). The study also optimized the adsorption conditions of immobilized laccase. When the initial concentration of laccase reached 5 mg/mL, the maximum activity of immobilized laccase moved from 18263 to 18369 IU/g, and the amount of laccase adsorbed on MMU also reached the maximum value. The fixation time of 1 h was considered the ideal time to achieve the maximum fixed activity from 17833 to 18023 IU/g and the activity recovery of about 93.9% (Pang *et al.* 2016). The activity of free laccase reached a maximum value from 13946 to 15134 IU/g at pH 3.0, then decreased rapidly with the increase in pH and was almost wholly inactivated under neutral conditions. The immobilized laccase exhibited distinct properties compared to its free counterpart: its optimal pH shifted from 3.0 to 4.0, while demonstrating significantly higher activity across a broader pH range (3.0 to 7.0). This enhanced performance suggests improved stability through the immobilization process.

The activity of the immobilized laccase also showed an increasing trend from 20 °C until it reached the maximum activity of 15687 IU/g at 40 °C and then decreased rapidly (Aghaee *et al.* 2024). In contrast, free laccase reached its highest activity at 30 °C. In order to reduce the cost of use in industry, the study also measured its activity after repeated use. The results indicated that immobilized laccase activity decreased rapidly in the first five cycles, maintained 65.5% of the initial activity, and remained stable after five cycles. The results showed that the immobilized laccase maintained more than 55% of its activity after 3 weeks, while the free laccase was completely inactivated. The adsorption of MMU is achieved through physical adsorption, which mainly relies on the hydrophilicity, electrostatic interaction, and intermolecular forces between the enzyme and MMU. Therefore, due to the crowded internal environment, high concentration is not conducive to improving activity (Gao *et al.* 2022).

Stability studies have shown that immobilized laccase is superior to free laccase in terms of pH, temperature, and recyclability. Laccase was immobilized on the COFs TpPa-1 through an *in situ* loading process to obtain immobilized laccase Lac@TpPa-1. The study adopted a mild *in situ* encapsulation method with the COFs TpPa-1 as the immobilization matrix to immobilize laccase under mild conditions. The *in situ* growth of COFs materials with laccase as the encapsulation site significantly improved laccase loading stability and reduces the risk of enzyme shedding (Ma *et al.* 2022). The modified Coomassie Brilliant Blue method was used to test the laccase loading. After testing, the immobilization efficiency was about 49.8% and the loading amount was 175.9 mg/g.

The immobilized laccase also had the highest pH shift under alkaline conditions and showed the highest activity at pH 5 (Li *et al.* 2018). The optimum temperature of the immobilized laccase shifted to a high temperature, reaching 35 °C, and the activity of the immobilized laccase decreased more slowly than that of the free enzyme as the temperature increased. At 45 °C, the immobilized laccase retained 91.9% of its original activity, while the free laccase only retained 65.7%. It showed the highest activity stability in an aqueous solution, indicating that immobilized laccase is more suitable for an aqueous medium reaction system. After storage at 4 °C for 31 days, the activity of free laccase began to decline after 16 days. By the end of the storage period, the relative activity dropped to 81.6% of the initial maximum. In contrast, Lac@TaPa-1 maintained its initial maximum

activity without significant decline during storage. Lac@TpPa-1 retained its initial highest activity even after six cycles of repeated use. The relative activity of laccase remained at 98.4% of the initial maximum value, indicating the excellent cyclic stability of immobilized laccase Lac@TpPa-1. This superior performance ensures its suitability for repeated use, effectively reducing the overall production cost of Lac@TpPa-1. (Tüzmen *et al.* 2012).

CONCLUSIONS

Lignin valorization can proceed *via* two fundamentally different strategies: macromolecular utilization (*e.g*., lignosulfonates as dispersants, kraft lignin as carbon fiber precursor) and depolymerization to aromatic monomers. This review has focused on the latter – adopting as its working assumption that enzymatic depolymerization, despite its current challenges, unlocks a broader spectrum of high-value chemical products (vanillin, muconic acid, catechol) and enables integration with biological funneling and synthetic biology platforms.

While fungi have dominated ligninolytic enzyme research, bacterial systems remain underexplored despite offering distinct advantages over fungal platforms. Fungal applications face inherent industrial challenges: mycelial morphology impedes mass transfer, strict culture requirements increase operational complexity, and extended lag phases reduce productivity. Furthermore, fungal enzymes often demonstrate limited robustness under industrial-relevant conditions (*e.g.*, alkaline pH, oxygen limitation, and high lignin loads) while demanding costly nutrient supplementation.

The recalcitrant nature of lignin necessitates synergistic enzyme systems or microbial consortia for effective depolymerization. Current reliance on model compounds creates a significant gap between laboratory studies and industrial implementation. Scaling ligninase applications faces additional barriers including low production yields, operational instability, and prohibitive recovery costs – challenges that bacterial heterologous expression systems may help overcome.

Future advancements should prioritize the optimized enzyme cocktails with compatible reaction parameters, protein engineering (directed evolution, rational design) to tune catalytic properties, and tailored solvent systems that maintain metalloenzyme functionality. Immobilization technologies present particular promise for enhancing operational stability and enabling continuous bioprocessing.

Conflict of Interest

The authors declare that there is no conflict of interest.

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