Towards microbial platforms for lignin valorization: Pseudomonas putida cell factories and Bacillus synthetic communities

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Chapter 6

Application and bottlenecks of microbial lignin valorization; overview and general discussion

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Abbreviations

GGE: 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenyl) propane-1,3-diol
DDVA: 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-dicarboxybiphenyl
FA: Ferulic acid
CA: *p*-Coumaric acid
SA: Syringic acid
MB: Methylene blue
AB: Azure B
DNP: 2,4-dinitrophenylhydrazine
SynCom: Synthetic Community
MnP: Manganese peroxidase
LiP: Lignin peroxidases
VP: Versatile peroxidases

Why Lignin?

Lignin is the second most abundant natural terrestrial polymer after cellulose. Together with cellulose and hemicellulose, lignin constitutes lignocellulose which represents a major fraction of plant biomass (Selvam et al., 2021). Yearly, 50-70 million tons of lignin are generated from the forestry and pulp and paper industries (Bajwa et al., 2019). Nevertheless, lignin remains largely underutilized due to its structural complexity, rigidity, and inherent chemical recalcitrance, which limits efficient depolymerization and conversion into value-added products (Shrestha et al., 2024). The main use of lignin currently resides in application as structural component in fibrous materials, resins and asphalt, or as energy source via incineration (Mariana et al., 2021). However, and importantly, lignin is a valuable renewable resource of natural carbon and various aromatic and aliphatic compounds rich in functional groups. This renders lignin as a highly promising feedstock for the production of renewable aromatics, polymer precursors, and various functional materials (Sheldon & Woodley, 2018; Jia et al., 2026).

The development of more effective strategies enabling the utilization and valorization of lignin has become a major research focus in recent years. In the natural environment, the ability to decompose lignified plant biomass is widespread among microorganisms. Mostly recognized in lignin-degrading fungi, but also in bacterial genera that contribute to lignin transformation and conversion in decaying wood (Janusz et al., 2017a). Biological degradation of lignin indeed originally deserved some attention in the 1970s and in recent years has regained attention focusing on the importance of lignin as a valuable and renewable feedstock and its use as an essential part of a circular economy (Atiwesh et al., 2022).

Challenges in lignin biodegradation

Despite the growing number of studies building on and claiming microbial lignin biodegradation, fundamental challenges remain largely unaddressed. Increasing numbers of metabolic engineering work have focused on and succeeded in converting

lignin-derived compounds into valuable products - an important and highly impactful direction (He et al., 2024; Liu et al., 2024). However, the challenge of the upstream step – to transform the original lignin polymeric network in an efficient and controllable manner into lignin-derived compounds that microbes can actually uptake - has often been overlooked or neglected. This challenge represents a central theme of this thesis and was discussed in **Chapter 2**, which highlights controlled depolymerization of polymeric lignin as a major bottleneck for microbial lignin valorization. This omission implicitly reflects the difficulty as well as our lack of understanding of the required conversion of the complex lignin polymeric network into monomeric and oligomeric derivatives, via fungal or bacterial enzymatic systems.

The majority of studies aimed at screening and identification of “lignin-degrading enzymes” rely heavily on so-called lignin model substrates and low-molecular-weight aromatics such as 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-dicarboxybiphenyl (DDVA) and 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenyl) propane-1,3-diol (GGE) (Fig.1), rather than testing activity on “real” lignin directly (Fanitsios et al., 2025; Voß et al., 2020). While model compounds enable throughput and mechanistic clarity, they do not represent the heterogeneity and complexity of the natural lignin. In **Chapter 3** and **Chapter 4**, methylene blue, a lignin mimicking dye, was also used, but only as complementary activity readouts rather than direct measures of lignin depolymerization.

Whether results on increased-value lignin utilization, obtained with lignin-derived compounds or with lignin model substrates, can be translated and industrially implemented on natural lignin is not a trivial question but a central bottleneck to the field. Compared with these model substrates, “real” lignin typically has a vastly higher molecular weight, is highly heterogeneous, display poor solubility, and much greater structural complexity (Fig. 1), making polymer-level biodegradation a fundamentally tougher task for microbes and their enzymes. Initial “attack” of the lignin polymeric network has to be done by secreted enzymes, scavenging the lignin complex for access and binding.

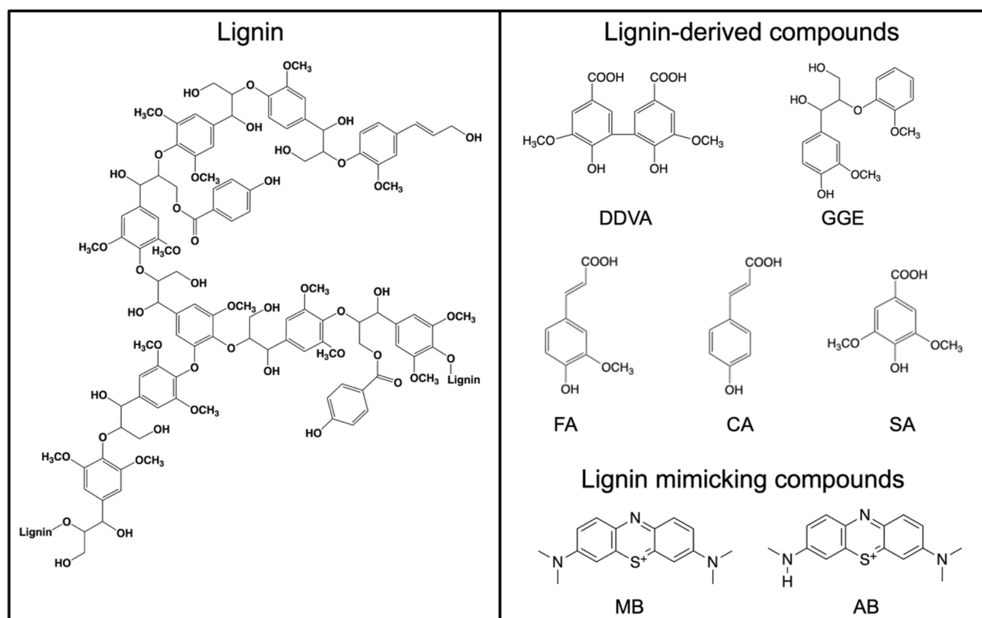

Figure 1. Comparison between lignin polymer and commonly used lignin-related model compounds. The left panel illustrates the complex, heterogeneous, and irregular polymeric structure of lignin, characterized by diverse interunit linkages and functional groups. The right panel shows representative lignin-derived and lignin mimicking compounds.

Equally challenging are the limitations of current measurement approaches used to infer lignin degradation. UV absorbance at 280 nm (A280) remains one of the most used methods to track lignin degradation due to its simplicity and low cost. However, A280 is highly nonspecific and sensitive to interference from soluble microbial products (Anthis & Clore, 2013; Hatfield & Fukushima, 2005), aromatic metabolites, and extracellular proteins, which can lead to apparent increases rather than decreases in absorbance during cultivation and presumed lignin degradation. Similar to A280, other assays targeting low-molecular-weight aromatic structures (Rashid & Bugg, 2021), such as the Folin–Ciocalteu assay, DNP assay, and Purpald assay, are also nonspecific. These approaches only report the changes in total phenols and therefore cannot directly quantify the extent of overall lignin degradation (Rashid & Bugg, 2021). Chromatography- and spectroscopy-based techniques such as GC-MS, LC-MS, HPLC, and NMR provide valuable insights into lignin-derived products, molecular weight distributions, and structural features, offering higher chemical specificity than bulk optical assays. However, these methods are often limited by destructive sample preparation, long

analysis times, low throughput, and difficulties in handling lignin heterogeneity and polydispersity, making them challenging to apply as routine, definitive measures of true lignin depolymerization (Lupoi et al., 2015). In **Chapter 4**, A280 was initially used for measuring lignin degradation, but the readout increased over time, likely due to nonspecific absorbance contributions from soluble microbial products, such as secreted proteins and nucleic acids, which would misleadingly suggest an “increase” in lignin in the medium. Therefore, in **Chapter 4** and **Chapter 5** we used the Folin assay to assess lignin degradation by quantifying phenols released upon lignin breakdown. However, these approaches remain indirect measures of lignin depolymerization. In contrast to polymeric lignin assays, **Chapter 3** focused on defined lignin-derived compounds. This enabled direct, compound-specific quantification of consumption and conversion by HPLC. Together, these limitations highlight the urgent need for more accessible, rapid, and informative analytical approaches to assess microbial lignin biodegradation, in particular for methods that can bridge the gap between simple proxy assays and detailed structural analyses.

Beyond lignin’s intrinsic structural complexity and the limitations of current analytical methods, enzyme discovery itself has been incomplete and unevenly explored, especially in bacteria (**Chapter 2** and **Chapter 3**). Although a wide range of bacteria have been announced as lignin-degrading organisms, the identity and specific mechanistic roles of the responsible enzymes remain largely unclear. Since the identification of DypB in *Rhodococcus jostii* RHA1 as the first bacterial lignin degrading enzyme in 2011 (Chapter 5; Ahmad et al., 2011a), only a small number of enzyme have been reported to participate directly in lignin degradation (Zhao et al., 2022). Apparently, identification and characterization of lignin-degrading enzymes remain intrinsically challenging for several reasons. First, lignin is a heterogeneous and irregular polymer with no single defined structure, meaning that its breakdown does not proceed via a single enzymatic step but rather through multienzyme, multistep, and largely nonspecific oxidative processes (Bugg et al., 2011a; Bugg, 2024b). Therefore, enzyme function is difficult to define, and many reported candidates may only participate in lignin modification or oxidation rather than true polymer depolymerization. Second, current enzyme screening and validation strategies rely heavily on lignin model compounds, which provide limited insight into enzyme activity on the lignin polymer itself. When extrapolated to native or industrial lignins, enzymatic mechanisms often remain unverified at the polymer level.

Lessons from Nature: Lignin Breakdown as a Community Process

In nature, the death of a tree represents a massive and long-lasting carbon reservoir for surrounding microorganisms (Röllig et al., 2025). Rather than being degraded by a single organism or enzyme, deadwood is processed by complex, dynamic microbial communities inhabiting decaying wood, where fungi and bacteria interact, compete, and cooperate (Tláskal et al., 2021; Zhao et al., 2022). Extensive research has shown that deadwood decomposition is driven primarily by fungi, while bacteria play distinct and complementary roles (Tláskal et al., 2021). As the most recalcitrant component of lignocellulose, lignin is nevertheless decomposed in nature by those microbial communities, where it is progressively transformed into oligomers and monomers and ultimately mineralized into carbon dioxide, water, and minerals (Janusz et al., 2017b; Xia et al., 2018). In natural lignin degradation, fungi such as white-rot fungi initiate the breakdown of the lignin polymer, while bacteria play well-defined complementary roles in the downstream metabolism of lignin-derived compounds, highlighting the importance of microbial division of labor in natural lignin turnover (Haq et al., 2022; Wilhelm et al., 2019). These observations underscore that lignin breakdown in nature is fundamentally a community-driven process, providing important design principles for engineering synthetic consortia and enzyme combinations for lignin valorization.

From Communities to Design: SynCom and Enzyme Cocktail Concept

Inspired by the lignin degradation process in nature, synthetic microbial communities (SynCom) or microbial co-cultures may provide novel, controllable models to study and engineer lignin degradation (Mee & Wang, 2012; Zuroff & Curtis, 2012; Chapter 4). Given the current bottlenecks in lignin-degrading enzyme discovery, a key advantage of SynCom or Co-culture approaches is that they can be assembled based on metabolic phenotypes without initial detailed insight into the relevant enzymes (Xu et al., 2025).

Initially proposed by Minty and colleagues (Minty et al., 2013) the interest in constructing synthetic communities for lignocellulose is growing rapidly (Chen et al., 2025; Liu et al., 2025, Chapter 4). Lignocellulosic degradation can be enhanced by a fungal SynCom, however, in this study main focus still was on the utilization of the (hemi-)cellulose fractions(Liu et al., 2025). Many soil- and deadwood-associated

microorganisms, including species of white-rot fungi, and *Bacillus*, *Streptomyces*, and *Pseudomonas* bacteria have been reported to exhibit lignin degradation ability while also possessing strong environmental robustness (Abdel-Hamid et al., 2013; Reshmy et al., 2022; Xu et al., 2018). Using these potential candidates to assemble a SymCom may enable efficient lignin degradation (**Chapter 4**). Building on these insights, we explored a community-based strategy for lignin degradation in **Chapter 4** by constructing a defined *Bacillus* synthetic community. In lignin-containing cultures, we observed that *Bacillus* strains could attach to lignin particles, form biofilm, and support lignin degradation. This finding provides an experimentally model to study division-of-labor-driven lignin degradation.

Enzyme mixtures or enzyme cocktails may offer an alternative strategy for *in vitro* lignin degradation. One major constraint is that during lignin depolymerization mediated by ligninolytic enzymes (such as laccases, VP, LiP, MnP), lignin may spontaneously repolymerize through radical coupling reactions involving phenoxy radicals or quinone intermediates (Liang et al., 2025; Majeke et al., 2021). When multiple ligninolytic enzymes are combined with specific auxiliary enzymes in an enzyme cocktail, lignin repolymerization can be inhibited, enabling more effective lignin degradation (Cajnko et al., 2021; Ferreira et al., 2009). Such synergistic interactions among multiple enzymes has been shown to enable comprehensive and scalable lignin degradation (Liang et al., 2025). For example, in **Chapter 5** we explored heterologous expression of DyP-type peroxidases in *P. putida*, as an initial step toward deploying lignin-degrading enzymes in a bacterial chassis. Extending this approach to express multiple lignin-degrading enzymes or combining engineered *P. putida* strains that each express a specific enzyme in co-culture, may provide a route toward more systematic, standardized, and controllable lignin depolymerization.

We strongly believe that SynCom and enzyme cocktail concepts can contribute to overcoming current bottlenecks in lignin degradation by enabling functional integration beyond single-organism or single-enzyme approaches.

The promising host for lignin valorization: *Pseudomonas putida*

Biorefinery can be achieved by engineering a single microorganism, as shown

by the example of engineered *Escherichia coli* converting lignocellulosic substrates into advanced biofuels (Bokinsky et al., 2011). While lignocellulosic biorefineries are expanding, increasing attention has turned toward the valorization of residual lignin, with researchers actively exploring strategies to convert this underutilized fraction into a broader range of higher-value products (Ragauskas et al., 2014). For lignin valorization, the choice of a suitable and robust microbial chassis becomes critical. Among available hosts, *Pseudomonas putida* has emerged as a particularly promising platform due to its broad native aromatic metabolism, high robustness and tolerance to toxic lignin-derived aromatics and industrial stresses, and strong genetic amenability for pathway engineering and product synthesis (Nikel & de Lorenzo, 2018). Numerous studies have demonstrated that *P. putida* can efficiently convert lignin-derived monomeric compounds into a wide range of valuable products, exemplified by established routes from lignin aromatics to materials such as nylon precursors (Kohlstedt et al., 2018; Linger et al., 2014a; Liu et al., 2024). However, moving upstream to directly address polymeric lignin depolymerization remains substantially challenging. Achieving a system in which lignin is depolymerized at the source, with part of the resulting carbon used for microbial growth and another fraction funneled into value-added products, represents a major bottleneck. We propose that heterologous overexpression of a cocktail of ligninolytic enzymes in *P. putida* may offer a promising strategy to overcome this limitation, enabling closer integration of lignin depolymerization and downstream valorization within a single microbial platform.In **Chapter 5**, the DyP from *Thermobifida fusca* was successfully expressed in *P. putida* KT2440 and showed peroxidase activity on substrates such as ABTS. This work provides a solid starting point and foundation for further developing *P. putida* KT2440 as a chassis for deploying lignin-active enzymes.

Taken together, this thesis is setting the stage for integrating lessons and approaches derived from natural lignin-degrading communities with SynComs, enzyme cocktails, and *P. putida*-based cell factory approaches towards accelerating the rational development of efficient and scalable lignin valorization platforms (Fig. 2).

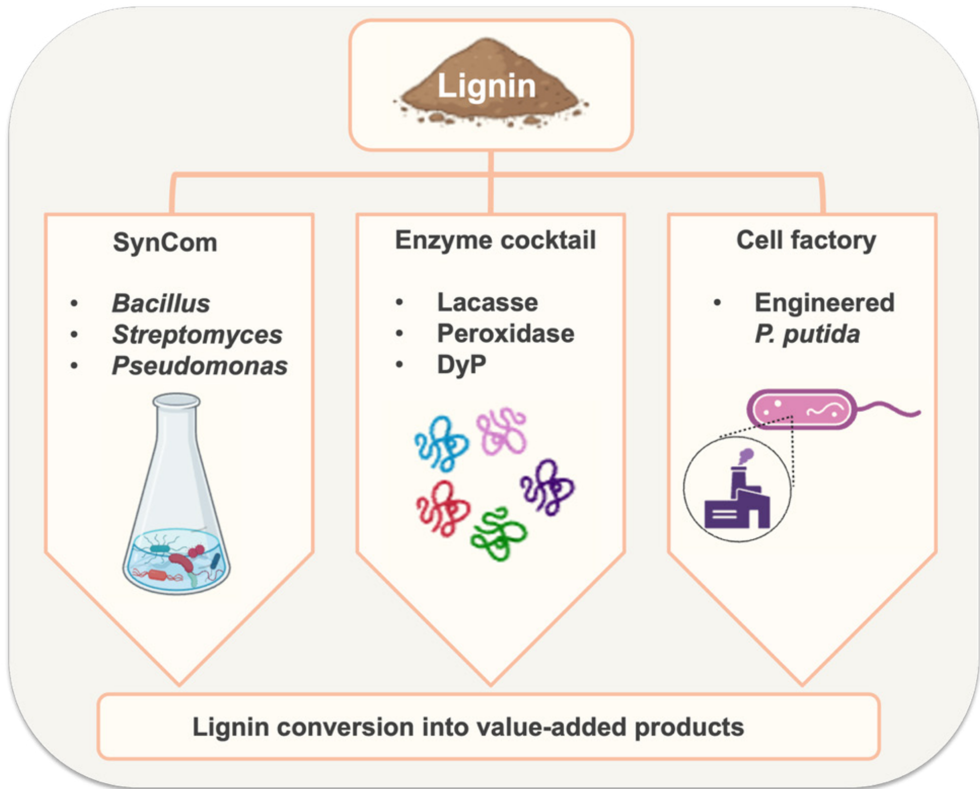

Figure 2. Schematic illustration of strategies for lignin conversion into value-added products: SynCom, enzyme cocktail for lignin depolymerization, and engineered *P. putida* as a lignin-valorizing cell factory.