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

Zhou, Q.

Chapter 2

Lignin-Degrading Enzymes and the Potential of *Pseudomonas putida* as a Cell Factory for Lignin Degradation and Valorization

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Abstract: Efficient utilization of lignin, a complex polymer in plant cell walls, is one of the key strategies for developing a green and sustainable bioeconomy. However, bioconversion of lignin poses a significant challenge due to its recalcitrant nature. Microorganisms, particularly fungi and bacteria, play a crucial role in lignin biodegradation, using various enzymatic pathways. Among bacteria, Pseudomonas putida is considered a promising host for lignin degradation and valorization, due to its robust and flexible metabolism and its tolerance to many noxious and toxic compounds. This review explores the various mechanisms of lignin breakdown by microorganisms, with a focus on *P. putida*'s metabolic versatility and genetic engineering potential. By leveraging advanced genetic tools and metabolic pathway optimization, *P. putida* can be engineered to efficiently convert lignin into valuable bioproducts, offering sustainable solutions for lignin valorization in industrial applications.

Keywords: Lignin degradation; Fungi; Bacteria; Enzyme; *Pseudomonas putida*

1 Introduction

Lignocellulose, composed of intertwined cellulose, hemicellulose, and lignin, is the most abundant renewable material on the Earth (Liu et al., 2018; Prakram Singh, 2020). Lignin represents a class of complex and rigid organic polymers that form important structural and strengthening support in vascular plants and algae tissues. Lignin is the second most abundant terrestrial polymer on Earth after cellulose. Because of its stability and high recalcitrance, lignin has long been regarded as an industrial byproduct in pulp and paper waste, agricultural residues, and other hydrolytic industries (Karthäuser et al., 2021; Palazzolo & Kurina-Sanz, 2016).

The complex aromatic polymer is synthesized in plants mainly from three basic building blocks: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Consequently, the complex heterogenic polymeric lignin network mainly consists of three recognizable basic units: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) moieties (Faix, 1991). Gymnosperms (softwood, i.e., conifer, pine, cedar, spruce, etc.) mostly contain guaiacyl-based lignin (G), whereas dicotyledonous plants (legumes, beans, sunflower, tomato, etc.) mainly contain guaiacyl-syringyl-based lilac lignin (G-S), and monocotyledonous plants (maize, wheat, rice, cane, etc.) mainly contain guaiacyl-syringyl-hydroxyphenyl-based lilac lignin (G-S-H) (Mansfield et al., 2012) (Figure 1).

Lignin polymerization in plants occurs via the formation of oxidative radicals of these structural units, followed by combinatorial radical coupling (Vanholme et al., 2010). Among the variety of linkages, the -O-4 bond is the most prominent one (40–50%), followed by -*β*, *β*-5, 5-5, 5-O-4, and -O-4 linkages (Kai et al., 2016; Lahive et al., 2020; Parthasarathi et al., 2011; Picart et al., 2014) (for details, see Figure 1).

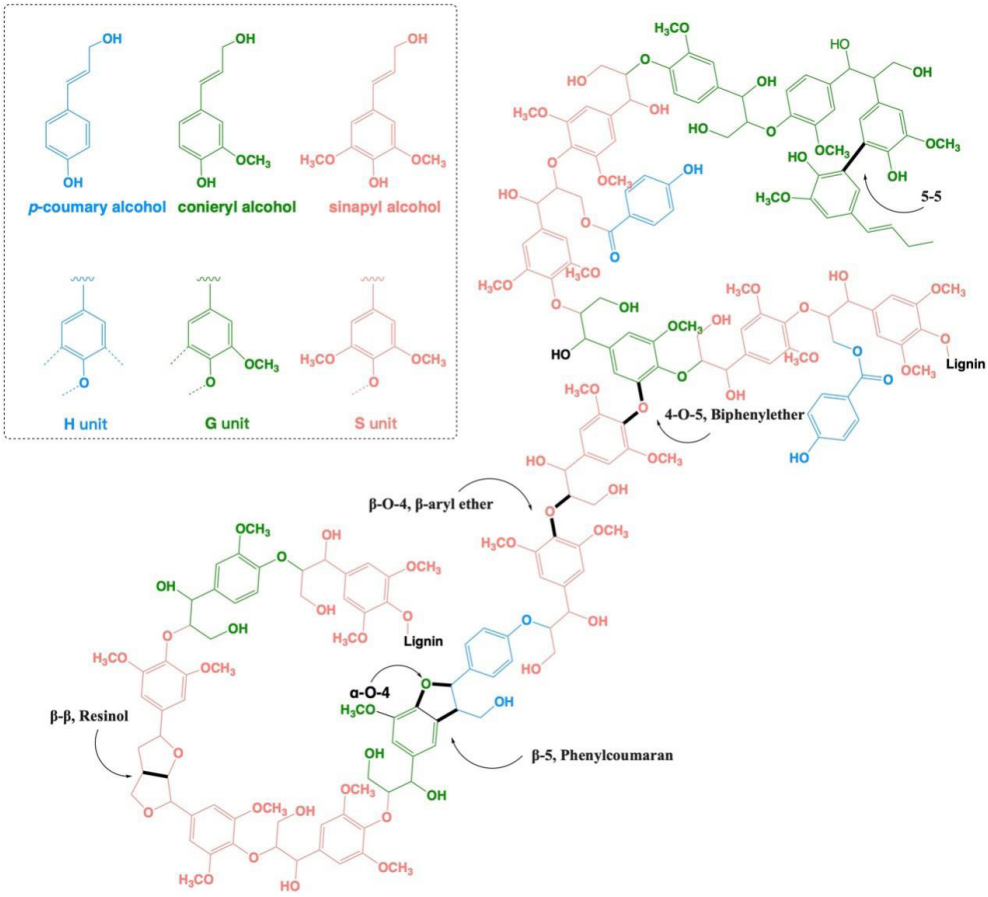

Figure 1. A structural representation of the complex lignin polymeric network (adapted from (Faix, 1991; Vanholme et al., 2010)). The three basic units constituting lignin are *p*-coumaryl alcohol (blue), coniferyl alcohol (green), and sinapyl alcohol (red). These monomeric alcohols are linked to form lignin mainly by the following linkages: *β*-O-4 (β-aryl ether) linkages, *β*-*β* (resinol), *β*-5 (phenylcoumaran), 5-5 (biphenyl), and 4-O-5 (biphenylether) bonds.

With the advent in recent years of biorefineries, millions of tons of lignin are available annually as a side product of industrial lignocellulose hydrolysis and utilization (Irmer, 2019). In the pulp and paper industry alone, around 100 million tons of lignin are

becoming available as valuable but limited-use feedstock (Beckham et al., 2016; Zhang et al., 2020). Cellulose and hemicellulose fractions are readily used as feedstock in subsequent biorefinery and fermentation scale-ups for the biotechnological production of various biofuels and biochemicals. In contrast, most lignin cannot be utilized efficiently. The utilization of lignin still largely resides in heat and energy production through combustion and the implementation of raw lignin in the production of glues, resins, and asphalt (Demuner et al., 2019; Lora & Glasser, 2002; Stewart, 2008). The global lignin market size was estimated at USD 1.08 billion in 2023 and is expected to grow at a compound annual growth rate (CAGR) of 4.5% from 2024 to 2030 (Research, 2024). Consequently, a more efficient and valuable utilization of lignin has recently gained growing interest.

Lignin degradation is important for the recycling and valorization of plant biomass and plays a crucial role in carbon cycling and nutrient cycling in ecosystems. The first and most important step in the valorization of lignin is the efficient decomposition and depolymerization of the complex and recalcitrant polymer. The chemical decomposition of lignin has been described (Kleine et al., 2013; van Erven et al., 2024; vom Stein et al., 2013); however, it yields a complex and toxic mixture with a difficult-to-define composition. Such chemical hydrolysates are not very useful in subsequent added-value utilization (Linger et al., 2014b). The Organosolv process, effectively reducing lignin molar mass and heterogeneity was recently published as an interesting example (Smit & Huijgen, 2017; Smit et al., 2023; Smit et al., 2024). However, obtained fractions still contain relatively undefined lignin polymers. Combined and integrated chemical and biochemical approaches may be much more favorable (Bugg et al., 2020). Biological decomposition by microorganisms may provide a promising route toward added-value utilization of lignin. Microbial enzymes may specifically target lignin for efficient degradation into aromatic monomers and oligomers. These can subsequently be converted into valuable biochemicals through microbial metabolic pathways, providing a sustainable route for lignin utilization in biotechnology.

White-rot fungi have been known for several decades to naturally degrade lignin, whereas brown-rot fungi are only capable of modifying the lignin network to a limited extent (Kirk & Farrell, 1987; Tien & Kirk, 1983). These microbes produce oxidative enzymes like laccases and various types of peroxidases, which play a significant role in the aspecific breakdown of lignin (Atiwesh et al., 2022; Martínez et al., 2005; Weng et

al., 2021). In addition, many bacteria have been reported to degrade lignin. Moreover, bacterial lignin degradation involves enzymatic cleavage that is geared toward specific lignin linkages, suggesting a targeted approach to lignin breakdown (Atiwesh et al., 2022). In this critical review, we summarize and discuss the current knowledge and understanding of lignin-degrading microorganisms, their enzymes, reported to be active in lignin degradation, as well as aspects of proposed catalytic mechanisms. Despite the large volume of literature on microbial lignin degradation existing to date, relatively little insight has been gained on enzyme specificity and catalytic mechanisms operating to yield defined degradation products. We propose and discuss the potential of the robust soil bacterium *Pseudomonas putida* as a suitable host and cell factory for industrial lignin valorization, genetic and enzyme engineering strategies to enhance the synthesis of value-added bioproducts derived from lignin, and subsequent industrial applications of these bioproducts.

2 Microbial Degradation of Lignin

2.1 Lignin Degradation by Fungi

Various pioneering studies have identified fungi as effective lignin-degrading microorganisms, secreting a variety of non-specific but relatively efficient lignin-decomposing enzymes. Typical fungi implicated with lignin degradation are listed in Table 1.

White-rot fungi are commonly associated with hardwood and are renowned for their high potency to degrade lignin (Nurul-Aliyaa et al., 2023). Lignin degradation by white-rot fungi leads to the bleaching of wood, exposing cellulose and hemicellulose fibers. This degradation enables more efficient enzymatic hydrolysis of polysaccharides, facilitating the subsequent conversion of these fibers into fermentable sugars for bioethanol production (Paul et al., 2023). This selective degradation makes white-rot fungi interesting for many biotechnological applications since they remove lignin and leave the valuable cellulose intact.

Brown-rot fungi typically grow on softwoods and constitute only 7% of wood-rotting basidiomycetes (Bugg et al., 2011c). In contrast to white-rot fungi, they preferentially

hydrolyze the cellulose component of lignocellulose while only partially oxidizing lignin. This involves Fenton oxidation chemistry, during which hydroxyl radicals are produced that may partly be independent of specific enzyme activity (Arantes et al., 2011).

In 1984, the white-rot fungus *Phanerochaete chrysosporium*, was found to produce an extracellular lignin-degrading enzyme, an oxygenase, which catalyzes several oxidations in the alkyl side chains of lignin-related compounds (Tien & Kirk, 1984). Subsequently, *Phlebia radiata* was reported to degrade lignin, and three peroxidases and one laccase were purified and characterized from this fungus. These enzymes were shown to modify kraft lignin and phenolic compounds containing hydroxyl and methoxy groups (Niku-Paavola et al., 1988). In addition, *Pleurotus eryngii* was shown to remove lignin from cereal straw (Martínez et al., 1994), and two isoenzymes of manganese peroxidase were purified from this fungus (Martínez et al., 1996). Several other white-rot fungi have been reported to degrade different types of lignin. The white-rot fungus *Trametes hirsuta* has been shown to secrete several laccases and peroxidases to degrade kraft lignin. The decrease in kraft lignin molecular weight is clearly correlated with the activities of these enzymes (Moiseenko et al., 2021). *Trametes versicolor* (Bourbonnais et al., 1995) and *Pycnoporus cinnabarius* operated through laccase as the major phenoloxidase (Eggert et al., 1996).

Table 1. Lignin degradation by fungi: overview of strains, sources, and references. This table summarizes various fungi species known for their lignin-degrading capabilities. It includes the specific strains studied, the lignin sources they were tested on, the year of publication, and the corresponding references.

Microorganism	Strain	Lignin Source	Year	Ref.
White-Rot Fungi	*Phanerochaete chrysosporium* BKM-1767	Lignin model compounds	1984	(Tien & Kirk, 1984)
White-Rot Fungi	*Phlebia radiata*	Kraft lignin	1988	(Niku-Paavola et al., 1988)
White-Rot Fungi	*Pleurotus eryngii*	Cereal straw	1994	(Martínez et al., 1994)
White-Rot Fungi	*Trametes versicol*or	Kraft lignin	1995	(Bourbonnais et al., 1995)
White-Rot Fungi	*Pycnoporus cinnabarius*	Pine wood	1996	(Eggert et al., 1996)
White-Rot Fungi	*Ceriporiopsis subvermispora*	Pinus taeda Wood chips	2004	(Guerra et al., 2004)
White-Rot Fungi	*Ganoderma lucidum* IBL-06	Lignocellulosic substrates	2010	(Asgher et al., 2010)

Microorganism	Strain	Lignin Source	Year	Ref.
White-Rot Fungi	*Phlebia sp.* MG-60	Oak wood	2012	(Kamei et al., 2012)
White-Rot Fungi	*Dichomytus squalens*	Wheat straw Lignin	2013	(Knežević et al., 2013)
White-Rot Fungi	*Pleurotus ostreatus*	Palm midrib	2018	(Metri et al., 2018)
White-Rot Fungi	*Trametes hirsuta*	Kraft lignin	2021	(Moiseenko et al., 2021)
Brown-Rot Fungi	*Gloeophyllum trabeum (Lenzites trabea)* (Pers. ex Fr.) 83	Lignin model compounds	2008	(Niemenmaa et al., 2008)
Brown-Rot Fungi	*Postia placenta* MAD-698-R	Aspen (Modification of Lignin)	2009	(Martinez et al., 2009)
Brown-Rot Fungi	*Fomitopsis pinicola*	Wheat straw Lignin	2013	(Knežević et al., 2013)
Soft-Rot Fungi	*Aspergillus fumigatus*	Kraft lignin	1986	(Kadam & Drew, 1986)
Soft-Rot Fungi	*Podospora anserina*	Wheat straw Lignin	2020	(van Erven et al., 2020)
Fungi	*Aspergillus sp.*	Alkali lignin	2011	(Yang et al., 2011)

For the brown-rot fungus *Gloeophyllum trabeum,* a lignin degradation redox cycling process was proposed, involving two extracellularly produced quinones that reduce Fe^{3+} to Fe^{2+} (Bugg et al., 2011c). This was supported by research on *Postia placenta* indicating up-regulation of genes associated with iron acquisition (Wymelenberg et al., 2010). These Fe^{3+}-reducing compounds play an important role since their low molecular weight enables them to access the cell wall structure in wood and initiate decay so that the larger lignin-degrading enzymes can access and act upon lignin (Atiwesh et al., 2022). Release of $^{14}CO_2$ was observed when *Gloeophyllum trabeum* and *Postia placenta* were cultured with a non-phenolic, ($O^{14}CH_3$)-labeled lignin *β-O*-4 dimer model compound. Hence, these brown-rot fungi may produce enzymes that may specifically cleave the *β-O*-4 linkage in lignin (see Figure 1).

Apart from white-rot and brown-rot fungi, soft-rot fungi, such as *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus sp.* LPB5*,* generally demonstrate only limited lignin degradation abilities and tend to be less efficient than other fungi proficient in lignin degradation (Betts & Dart, 1988). However, *Aspergillus fumigatus* was reported to degrade kraft lignin through demethoxylation and dehydroxylation five times better

compared to the white-rot fungus *C. Versicolor* (Kadam & Drew, 1986). Moreover, the ascomycete *Podospora anserina* could cause 24% (*w*/*w*) of substantial lignin removal during the 7 days of growth (van Erven et al., 2020), unambiguously confirming its ligninolytic activity.

2.2 Lignin Degradation by Bacteria

Several species of bacteria have been described to possess enzymes that can degrade lignin (Table 2). The following describes important examples of reported lignin-degrading bacterial species.

Rhodococcus spp.

Certain strains of *Rhodococcus* bacteria, such as *Rhodococcus jostii* RHA1 (Ahmad et al., 2010), are known for their lignin-degrading capacity. *R. jostii* RHA1 degrades lignin in lignocellulose as well as kraft lignin to a low-molecular-weight phenolic byproduct, as monitored by spectrophotometric assays (Ahmad et al., 2010). *R. jostii* RHA1 encodes two putative so-called dye-decolorizing peroxidases, or DyP peroxidases (see below for description of enzymes). One was characterized as lignin peroxidase DypB, active in lignin breakdown (Ahmad et al., 2011b). A genetically modified *R. jostii* RHA1 was able to produce 330 mg/L 2,4-PDCA (pyridine-dicarboxylic acid) in 40 h from 1% wheat straw lignocellulose, corresponding to a conversion yield of approximately 16% of the available lignin fraction (Spence et al., 2021). *Rhodococcus pyridinivorans* CCZU-B16 (Chong et al., 2018), isolated from soil, could under optimized conditions degrade 30.2% of alkali lignin (4 g/L) in 72 h.

Bacillus spp.

Bacteria of the genus *Bacillus* isolated from pulp and paper mill effluent exhibited the potential to degrade lignin (Chandra et al., 2007). For example, *Bacillus altitudinis* SL7 reduced lignin content by 44% when grown with alkali lignin (Khan et al., 2021). *Bacillus pumilus* LSSC3 and *Bacillus atrophaeus* CL29 exhibited high oxidative laccase activity in kraft lignin degradation, measured by the oxidation of the lignin model compound guaiacol (Huang et al., 2013a). *Bacillus flexus* RMWW II showed lignin degradation by 20% at a lignin concentration of 400 mg L^{-1} (Kumar et al., 2019). *Bacillus ligniniphilus* L1 can utilize alkaline lignin as a sole carbon source, producing 15 types of

aromatic compounds as identified via GC-MS analysis (Zhu et al., 2017). Transcriptomic data indicate at least four pathways putatively involved in lignin degradation and metabolization of breakdown products, including the Gentisate pathway, Benzoic acid pathway, and β-ketoadipate pathway. *Bacillus* sp. (CS-1 and CS-2) can degrade alkali lignin with high laccase activities detected in crude enzyme extracts (Chang et al., 2014). Nevertheless, the specific lignin-degrading enzymes remain to be characterized.

***Pseudomonas* spp.**

Several *Pseudomonas* strains, including *Pseudomonas putida* and *Pseudomonas fluorescens,* have been found to possess lignin-degrading enzymes. These bacteria are often investigated for their applications in bioremediation and lignocellulosic biomass conversion. *P. putida* A514 was able to grow with alkali-insoluble lignin as the sole carbon source (Lin et al.). Recently, *P. putida* NX-1, isolated from leaf mold samples, could grow on kraft lignin and was engineered for PHA production (Xu et al., 2018; Xu et al., 2021). Genome analysis of *P. putida* NX-1 revealed putative enzymes involved in lignin decomposition, including dyp-type peroxidases, versatile peroxidases, manganese peroxidases, and laccases. However, their functions and contributions to lignin decomposition have not yet been experimentally characterized. The ability to catabolize a wide range of natural aromatinds (Eberlein et al., 2018; Salvachúa et al., 2020) indicates that *P. putida* KT2440 holds potential to be an excellent host for lignin degradation. *P. putida* KT2440 could utilize alkaline pretreated liquor (APL), primarily composed of lignin, to produce mcl-PHA in relatively good yield under nitrogen depletion (Linger et al., 2014b). Furthermore, outer membrane vesicles (OMVs) from *P. putida* KT2440 have been implicated in the biodegradation of lignin-derived aromatic compounds (Eberlein et al., 2018; Salvachúa et al., 2020). The copper-dependent oxidase CopA from *P.* putida KT2440 was shown to be involved in extracellular lignin oxidation (Granja-Travez & Bugg, 2018a). Moreover, *P. putida* was recently shown to produce cis,cis-muconic acid from PCA, which is an intermediate product of lignin degradation (He et al., 2023; Johnson et al., 2016). Hence, *P. putida* appears highly promising as a biotechnology host strain to produce valuable compounds from lignin (Belda et al., 2016b).

***Streptomyces* spp.**

Several actinobacterial species, such as members of the *Streptomyces genus*,

have shown lignin-degrading potential. *Streptomyces viridosporus* T7A is an example of an actinobacterium with ligninolytic activity (Crawford et al., 1983). *Streptomyces spp*. F-6 and *Streptomyces spp*. F-7 can remove around 38% of lignin, after 12 days of culture (Yang et al., 2012). Recently, *Streptomyces thermocarboxydus* DF3-3 was isolated for alkali lignin degradation (Tan et al., 2022), secreting ligninolytic enzymes, such as manganese peroxidase, laccase, and specific small laccases (Sidar et al., 2024). For this species, a total of seven lignin-based derivatives metabolic pathways were predicted: the *β*-ketoadipate pathway and peripheral reactions; the gentisate pathway; the anthranilate pathway; the homogentisic pathway; the catabolic pathway for resorcinol; the phenylacetate–CoA pathway; and the 2,3-dihydroxyphenylpropionic acid pathway (Tan et al., 2022). *Streptomyces sp.* S6 isolated from a decaying oil palm empty fruit bunch can grow on kraft lignin as the sole carbon source. After 7 days of incubation with *Streptomyces sp.* S6, the loss of the molecular weight of kraft lignin was up to 55.3% (Riyadi et al., 2020).

Sphingomonas spp.

Sphingomonas species, and more specifically *Sphingomonas paucimobilis* SYK-6, have been shown to degrade lignin-related aromatic model compounds (Masai et al., 1999). These bacteria are known for their ability to break down various lignin-related structures. SYK-6 was the first bacterium shown to harbor several functional lignin-degrading enzymatic routes, involving glutathione peroxidases and etherases (see below).

Other Proteobacteria

Proteobacteria like *Pandoraea sp.*, *Enterobacter*, or *Ochrobactrum* have been confirmed can utilize lignin or lignin model compounds. *Pandoraea sp.* B-6 secreted extracellular ligninolytic enzymes to degrade kraft lignin (Shi et al., 2013b). The low-molecular-weight compounds of kraft lignin were detected by GC-MS. Proteomics suggested *Enterobacter lignolyticus* SCF1 was able to use lignin in both assimilatory and dissimilatory pathways (DeAngelis et al., 2013). *Ochrobactrum* was first reported to depolymerize and utilize lignin in 2018 (Chong et al., 2018).

Table 2. Lignin degradation by bacteria: overview of strains, sources, and references. This table summarizes various species of bacteria known for their lignin-degrading capabilities. It includes the specific strains studied, the lignin sources they were tested on, the year of publication, and the corresponding references.

Microorganism	Strain	Lignin Source	Year	Ref.
Actinobacteria	*Rhodococcus jostii* RHA1	Kraft lignin	2011	(Ahmad et al., 2011b)
	Rhodococcus erythropolis	Alkali lignin	2012	(Taylor et al., 2012)
	Rhodococcus opacus DSM 1069	Lignin	2013	(Kosa & Ragauskas, 2013)
	Rhodococcus opacus PD630	Alkali Corn Stover Lignin	2017	(He et al., 2017)
	Rhodococcus pyridinivorans CCZU-B16	Alkali lignin	2018	(Chong et al., 2018)
	Amycolatopsis sp. 75iv2	Acid-precipitable, polyphenolic, polymeric lignin (APPL)	2011	(Brown et al., 2011)
	Streptomyces viridosporus T7A	APPL	1983	(Crawford et al., 1983)
	Streptomyces spp. F-6	Alkali lignin	2012	(Yang et al., 2012)
	Streptomyces spp. F-7	Alkali lignin	2012	(Yang et al., 2012)
	Streptomyces coelicolor A3(2)	Lignin model compounds	2014	(Majumdar et al., 2014)
	Streptomyces sp. S6	Kraft lignin	2020	(Riyadi et al., 2020)
	Streptomyces thermocarboxydus DF3-3	Alkali lignin	2022	(Tan et al., 2022)
	Micromonospora sp.	Kenaf	2014	(Brzonova et al., 2014)
	Thermobifida fusca YX	Untreated biomass	2011	(Deng & Fong, 2011)
Anaerobic Microorganisms	*Clostridium thermocellum*	Populus Lignin	2017	(Akinosho et al., 2017)
Brevibacillus	*Brevibacillus thermoruber*	Lignin	2021	(Niu et al., 2021)
	Caldicellulosiruptor bescii DSM 6725	Untreated switchgrass	2013	(Kataeva et al., 2013)

Microorganism	Strain	Lignin Source	Year	Ref.
Bacteroidetes	*Sphingobacterium sp.* HY-H	Sodium lignosulfonate	2013	(Wang et al., 2013)
	Sphingobacterium sp. T2	Wheat straw Organosolv lignin, alkali kraft lignin	2015	(Rashid et al., 2015)
	Sphingomonas paucimobilis SYK-6	dimeric lignin compounds	1999	(Masai et al., 1999)
Proteobacteria	*Citrobacter sp.* (HQ873619)	Black liquor	2011	(Chandra et al., 2011)
	Citrobacter sp. (FJ581023)	Black liquor	2011	(Chandra & Abhishek, 2011)
	Citrobacter freundii (FJ581026)	Black liquor	2011	(Chandra & Abhishek, 2011)
	Comamonas sp. B-9	Kraft lignin	2012	(Chen et al., 2012)
	Comamonas testosterone KF-1	Lignin-associated monomers	2023	(Wilkes et al., 2023)
	Klebsiella pneumoniae (GU193983)	Black liquor	2011	(Chandra et al., 2011)
	Klebsiella pneumoniae NX-1	Kraft lignin	2018	(Xu et al., 2018)
	Pseudomonas aeruginosa (DSMZ 03504)	Pulp mill effluents	2010	(Tiku et al., 2010)
	Pseudochrobactrum glaciale	Pulp paper mill effluent	2012	(Chandra et al., 2012)
	Pantoea sp.	Pulp paper mill effluent	2012	(Chandra et al., 2012)
	Pseudomonas putida KT2440	Alkaline pretreated liquor	2014	(Linger et al., 2014a)
	Pseudomonas plecoglossicida ETLB-3	Black liquor	2015	(Paliwal et al., 2015)
	Pseudomonas putida A514	Alkali lignin	2016	(Lin et al., 2016)
	Pseudomonas strain	Alkaline insoluble lignin	2016	(Lin et al., 2016)
	Pseudomonas sp. Q18	Alkali lignin	2018	(Yang et al., 2018)

Microorganism	Strain	Lignin Source	Year	Ref.
	Pseudomonas putida NX-1	Kraft lignin	2018	(Xu et al., 2018)
	Pseudomonas strain Hu109A	Lignin	2023	(Nawaz et al., 2023)
	Pandoraea sp. B-6	Kraft lignin	2013	(Shi et al., 2013b)
	Enterobacter soil sp. nov.	Alkali lignin	2011	(Manter et al., 2011)
	Enterobacter lignolyticus SCF1	Alkali lignin	2013	(DeAngelis et al., 2013)
	Ochrobactrum pseudogrignonense	Nitrated lignin	2012	(Taylor et al., 2012)
	Ochrobactrum rhizosphaerae	Nitrated lignin	2012	(Taylor et al., 2012)
	Ochrobactrum tritici NX-1	Kraft lignin	2018	(Xu et al., 2018)
	Serratia marcescens (GU193982)	Black liquor	2011	(Chandra et al., 2011)
	Serratia liquefaciens	Pulp paper mill effluent	2012	(Chandra et al., 2012)
	Serratia liquefaciens LD-5	Pulp paper mill effluent	2016	(Haq et al., 2016)
Firmicutes	*Aneurinibacillus aneurinilyticus* (AY856831)	Kraft lignin	2007	(Chandra et al., 2007)
	Bacillus sp. (AY952465)	Kraft lignin	2007	(Chandra et al., 2007)
	Bacillus sp. (accession no. AY 952465)	Kraft lignin	2007	(Raj et al., 2007)
	Bacillus cereus (DQ002384)	Kraft lignin	2008	(Chandra et al., 2008)
	Bacillus atrophaeus LSSC3	Kraft lignin	2013	(Huang et al., 2013a)
	Bacillus pumilus CL29	Kraft lignin	2013	(Huang et al., 2013a)
	Bacillus sp. (CS-1 and CS-2)	Alkali lignin	2014	(Chang et al., 2014)
	Bacillus megaterium ETLB-1	Black liquor	2015	(Paliwal et al., 2015)

Microorganism	Strain	Lignin Source	Year	Ref.
	Bacillus ligniniphilus L1	Alkali lignin	2017	(Gücyeter et al., 2022)
	Bacillus endophyticus	Lignin	2016	(Ojha & Tiwari, 2016)
	Bacillus subtilis	Lignin	2016	(Ojha & Tiwari, 2016)
	Bacillus flexus RMWW II	Alkali lignin	2019	(Kumar et al., 2019)
	Bacillus altitudinis SL7	Purified synthetic alkali lignin	2021	(Khan et al., 2021)
	Paenibacillus sp. (AY952466)	Kraft lignin	2008	(Chandra et al., 2008)
	Paenibacillus glucanolyticus SLM1	Biochoice lignin	2016	(Mathews et al., 2016)
	Paenibacillus glucanolyticus 5162	Biochoice lignin	2016	(Mathews et al., 2016)
	Paenibacillus sp. strain LD1	Kraft lignin	2014	(Raj et al., 2014)
	Planococcus sp. TRC1	Lignin	2019	(Majumdar et al., 2019)
Extremophile bacteria	*Arthrobacter sp. C2*	Sodium lignin sulfonate	2022	(Jiang et al., 2022)

3 Enzymes for Lignin Depolymerization

Several successful examples of P. putida converting lignin-derived compounds into valuable products have been reported (Liu et al., 2024). However, despite these promising findings, achieving conversion starting from intact lignin remains challenging. Lignin degradation is a complex process that requires multiple enzymes and pathways. Many research efforts have been employed, trying to uncover the intricacies of these processes. These have yielded insights into fungal and bacterial enzymes with activity toward lignin degradation, with various bacterial enzymes putatively operating with higher specificity toward different lignin-specific linkages.

The initial and crucial step to effectively degrade lignin is to attack and depolymerize the complex lignin polymeric network into smaller phenoxy radical intermediates (Zhu

et al., 2022). This step can be facilitated by external oxidoreductases, including laccase (Lac, EC 1.10.3.2), lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), dye-decolorizing peroxidases (Dyp, EC 1.11.1.19), and versatile peroxidase (VP, EC 1.11.1.16) (Pollegioni et al., 2015). Typical enzymes capable of cleaving specific lignin linkages are summarized in Table 3. These enzymes have been extensively studied for their activity, however, almost exclusively on lignin model compounds (Lahive et al., 2020). They are known to target various linkages that occur within the lignin structure. These enzymes exhibit different substrate specificities and mechanisms of action; however, in most cases, their precise role in bioconversion of the lignin polymeric network remains elusive.

Table 3. Enzymes capable of cleaving specific lignin linkages. This table summarizes the types of enzymes involved in cleaving specific lignin linkages, their classification, their names, their source strains, the substrates utilized, and the relevant references. The enzymes listed are crucial for understanding the biochemical pathways of lignin degradation and highlight the diverse microbial sources capable of lignin bioconversion.

Linkage	Type of Enzyme	Name	Strains Source	Substrate Intracellular	Location	Refs.
β-O-4	*β*-Etherase	LigE; LigF	*Sphingobium sp.* SYK-6; *Novosphingobium sp. stra*in PP1Y	1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol	Intracellular	(Faix, 1991; Gall et al., 2014)
		Ds-GST1	*Dichomitus squalens* LYAD-421 SS1	dimeric lignin model compound contains β-O-4 aryl ether bond	Intracellular	(Marinović et al., 2018)
	laccase-like multicopper oxidases	CopA	*Pseudomonas putida* KT2440; *Pseudomonas fluorescen*s Pf-5	guaiacylglycerol-β-guaiacyl ether (GGE)	Secreted	(Granja-Travez & Bugg, 2018b)
	Dye-decolorizing peroxidase	Rh_DypB	*Rhodococcus jostii* RHA1	GGE	Secreted	(Vignali et al., 2018)
	heme-containing ligninolytic peroxidase	Versatile peroxidase	*Physisporinus vitr*eus	guaiacylglycerol β-guaiacyl ether (β-*O*-4 dimer)	Secreted	(Kong et al., 2017)
	heme-containing peroxidases	Manganese peroxidase	*Phanerochaete chrysospori*um	1-(3,5-dimethoxy-4-hydroxyphenyl)-2-[4-(hydroxymethyl)-2-methoxyphenoxy]-1,3-dihydro-xypropane	Secreted	(Wariishi et al., 1989)
	Laccases	Small laccase (SLAC)	*Streptomyces*	LM-OH (a phenolic *β-O*-4 lignin model compound)	Secreted	(Majumdar et al., 2014)

Linkage	Type of Enzyme	Name	Strains Source	Substrate Intracellular	Location	Refs.
		Laccase	*Bacillus ligniniphilus* L1	alkaline lignin and milled wood lignin	Intracellular	(Zhu et al., 2020)
5-5	C-C hydrolase	LigY	*Sphingomonas paucimobili*s SYK-6	2,2'-dihydroxy-3,3'-dimethoxy-5,5'-dicarboxybiphenyl (DDVA)	Intracellular	(Masai et al., 2007; Peng et al., 1998; Sonoki et al., 2000)
	heme-containing ligninolytic peroxidase	Versatile peroxidase	*Physisporinus vitreus*	dehydrodivanillic alcohol (5-5' dimer)	Secreted	(Kong et al., 2017)
	laccase-like multicopper oxidases	CopA	*Pseudomonas putida* KT2440; *Pseudomonas fluorescens* Pf-5	DDVA	Secreted	(Granja-Travez & Bugg, 2018b)
β-β	phenol-oxidizing enzymes		*Fusarium solani* M-13-1	l-syringaresinol	Secreted	(Kamaya et al., 1981)
Other bonds	oxygen oxidoreductase	Laccase	*Staphylococcus arlettae* S1-20		Secreted	(Chauhan et al., 2018)
		Lignin peroxidase	*Streptomyces viridosporus* T7A	GGE	Secreted	(Ramachandra et al., 1988)
		Lignin peroxidase	*Trametes versicolor* IBL-04	veratryl alcohol	Secreted	(Asgher et al., 2012)

3.1 The β-O-4 Bond

A number of enzymes are secreted by fungi and bacteria to degrade lignin or lignin-derived compounds (Janusz et al.); however, there is limited evidence regarding their ability to cleave specific linkages within the complex lignin structure. Reported evidence for linkage specificity mostly stems from studies with relatively simple model compounds for each of the lignin linkages Lahive et al., 2020). Among the various linkages present in lignin, 45–60% of the total linkages are β-O-4 aryl ether bonds (Adler, 1977). Cleaving this bond presents an essential step in the efficient use of lignin. Hence, enzymes that can cleave β-O-4 aryl-ether bonds are highly interesting for application in lignin valorization.

3.1.1 Fungal Lignin Depolymerization Enzymes

Lignin Peroxidases (LiPs)

Lignin peroxidase, a monomeric heme-containing enzyme, was the first enzyme found in *P. chrysosporium* that can degrade lignin (Glenn et al., 1983; Tien & Kirk, 1983). Its proficiency lies in the effective degradation of non-phenolic lignin units by catalyzing oxidative breakdown in the presence of H_2O_2 (Dashora et al., 2023). Therefore, it can catalyze the cleavage of *β-O-*4 ether bonds and Cα-C*β* linkages. LiPs are considered strong biocatalyst in the bioremediation of lignin and are represented in *Phanaerochaete chrysosporium*, *Trametes versicolor*, *Phanaerochaete sordida, and Phlebia radiata*.

Laccases

Laccases are widely found in plants, insects, fungi, and bacteria (Dwivedi et al., 2011; Giardina et al., 2010; Thurston, 1994). As a copper-containing enzyme of the polyphenol oxidases group, laccase catalyzes the oxidation of aromatic compounds, including phenols and phenolic derivatives during lignin degradation. Oxidation of these phenolic compounds leads to the formation of phenoxyl radicals, resulting in subsequent hydrolysis of C-C and β-aryl bonds in lignin's aromatic rings (Paul et al., 2023), yielding various products such as syringaldehyde, 1-(3,5-dimethoxy-4-ethoxyphenyl)-2-hydroxyethanone, 1-(3,5-dimethoxy-4-ethoxyphenyl)-2-hydroxypropanal, and 2,6-dimethoxy-*p*-benzoquinone. Laccases are present in various fungi species such as *Dichomitus squalens*, *Irpex lacteus*, *Lentinula edodes*, *Cerrena maxima*, *Trametes versicolor*, *Pleurotus ostreatus, and Phanaerochaete chrysosporium* (Dashora et al., 2023). Interestingly, *Peniophora lycii* LE-BIN 2142 lacks ligninolytic peroxidases, which are typically considered key enzymes in white-rot fungi. Instead, this species primarily relies on multiple laccase isozymes and unique FAD-binding proteins, suggesting an alternative oxidative strategy for lignin modification (Shabaev et al., 2022).

Versatile Peroxidases (VPs)

Versatile Peroxidase, a heme-containing ligninolytic peroxidase, was first found in white-rot fungi *Pleurotus eryngii* (Manavalan et al., 2015). VPs have been characterized to have catalytic functions of LiP, capable of oxidizing high redox potential substrates, combined with MnP, which oxidizes Mn^{2+} to Mn^{3+}, producing a diffusible oxidizing agent effective on low redox potential species. In the absence of mediators, they also oxidize azo-dyes and other non-phenolic compounds with high redox potentials (Civzele et al.,

2023). Different from MnPs and LiPs, VPs have a wider range of substrates. Evidence shows that VPs could catalyze *β-O*-4 lignin dimer to monomeric products (Zeng et al., 2017). Additionally, the VPs from *Physisporinus vitreus* oxidized the *β-O*-4 dimer, guaiacylglycerol *β*-guaiacyl ether, by depolymerization to a monomer or polymerization to a tetramer concurrently (Kong et al., 2017). VPs are found in *Pleurotus, Bjerkandera sp.*, *Panus*, *Calocybe*, *Trametes*, *Lepista*, *Dichomitous, and spongipelli* fungi species (Paul et al., 2023).

Manganese Peroxidases (MnPs)

Mangenese Peroxidase catalyzes the oxidation of a non-phenolic aromatic ring structure in lignin via oxidation of Mn^{2+} to Mn^{3+} as a redox mediator, leading to structural cleavage (Hofrichter, 2002). MnP from *Phanerochaete chrysosporium* was found able to cleave the *β-O*-4 of the phenolic lignin model dimer 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-[4-(hydroxymethyl)-2-methoxyphenoxy]-1,3-dihydro-xypropane (Tuor et al., 1992; Wariishi et al., 1989). MnP was first discovered in *P. chrysosporium* but was also later detected in other Basidiomycota species, including *Panus tigrinus*, *Lenzites betulinus*, *Agaricus bisporus*, *Bjerkandera* sp., and *Nematoloma frowardii* (Civzele et al., 2023).

Dye-decolorizing Peroxidases (DyPs)

Lastly, dye-decolorizing peroxidases (DyPs) are evolutionarily not related to the classical LME peroxidases (LiPs, MnPs, and VPs) but are a new class of heme-containing peroxidases found in bacteria and fungi (Paul et al., 2023). DyPs were first isolated in 1999 from the basidiomycetous fungus *Bjerkandera adusta* (Yoshida & Sugano, 2023). Some ligninolytic activity was found in *Termitomyces albuminosus*, *Auricularia auricula-judae*, and *Irpex lacteus.*

3.1.2 Bacterial Lignin Depolymerization Enzymes

In addition to harboring enzymes with characteristics comparable to fungal lignin-degrading enzymes, bacteria also have different lignin degradation mechanisms and enzymes. In the 1980s, *Pseudomonas acidovorans* had already been reported to degrade a *β*-aryl ether model compound (Vicuña et al.). Nevertheless, as of today, the actual number of functional bacterial enzymes well characterized in detail remains limited.

β-Etherase

β-Etherase, belonging to the protein superfamily of glutathione-S-transferase (GST; EC 2.5.1.18), is the first bacterial gene reported to function specifically in lignin degradation in *Sphingobium sp.* SYK-6 (Masai et al., 1991; Masai et al., 1989; Sheehan et al., 2001). *β*-Etherases exist especially in microorganisms that specialize in decomposing lignin (Voß et al., 2020). The *β-O-*4 aryl-ether bond degradation pathway in *Sphingobium sp.* SYK-6 needs three steps, involving three enzymes: an NAD^+-dependent Cα-dehydrogenase (LigD, LigL), a *β*-Etherase (LigE, LigF), and LigG, a glutathione-dependent lyase (LigG) (Reiter et al., 2013; Rosini et al., 2016). First, LigD/LigL oxidizes the Cα in model substrates, like 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenyl) propane-1,3-diol (GGE), under consumption of NAD^+. Only after this oxidation, LigE or LigF can cleave the C*β* ether bond, following the S_N2-type mechanism with high stereoselectivity. While LigE cleaves ether bonds in substrates with (R)-configured *β*-carbon, resulting in the corresponding (S)-configured glutathione adducts, LigF converts the corresponding (S)-substrate enantiomers (Helmich et al., 2016). Finally, LigG catalyzes the thioether cleavage of the chiral glutathione adducts to produce oxidized glutathione (GSSG) (Masai et al., 2003; Sato et al., 2009), as shown in Figure 2.

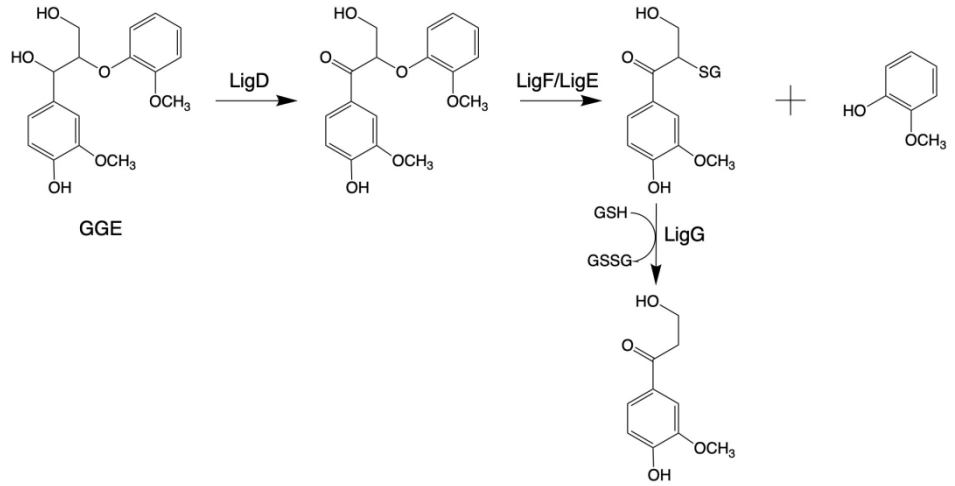

Figure 2. Pathways for the cleavage of *β-O-*4 bond by *β*-Etherase in *Sphingobium sp*. SYK-6 (adapted from (Bugg et al., 2011c)).

Dye-decolorizing Peroxidases (DyPs)

Dyps, heme-containing peroxidases are regarded as important enzymes involved

in lignin degradation, since they can specifically cleave and degrade a list of such lignin model dye compounds (Colpa et al., 2014). Generally, peroxidase can catalyze the degradation reaction of hydrogen peroxide, leading to the generation of reactive oxygen species, which in turn participate in lignin degradation. Additionally, DyP enzymes also catalyze the oxidation of -4 linkages, converting veratrylglycerol-*β*-guaiacyl ether into veratryl aldehyde and cleaving guaiacylglycerol-*β*-guaiacyl ether (Min et al., 2015; Rahmanpour et al., 2016). The DypB from *Rhodococcus jostii* RHA1 was the first bacterial lignin-degrading enzyme that has been characterized, which is capable of oxidizing polymeric lignin and lignin model compounds (Ahmad et al., 2011b). Novel research also found that Dyp1B from *Pseudomonas fluorescens* plays a significant role in lignin degradation (Ehibhatiomhan et al., 2023a).

Laccase-like multicopper oxidases (LMCOs)

Laccase-like multicopper oxidases (LMCOs) are a diverse group of oxidoreductases found in bacteria, fungi, and plants (Reiss et al., 2013). CopA is a member of LMCOs or pseudo-laccases (Rydén & Hunt, 1993; Solano et al., 2001). CopA enzymes from *P. putida* KT2440 and *P. fluorescens* Pf-5 catalyze the oxidization of the lignin model compound GGE (see above, Figure 2) and 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-dicarboxybiphenyl (DDVA, see blew, Figure 3), producing oxidized dimerized products (Granja-Travez & Bugg, 2018a; Xu et al., 2022).

Laccases

Laccase plays a crucial role in lignin biodepolymerization, but the reaction mechanism in bacteria remains incompletely elucidated.

Among bacterial laccases, small laccases (SLACs) are a type of laccase enzyme characterized by their smaller molecular size compared to traditional laccases (Machczynski et al., 2004). The SLAC from *Streptomyces* can degrade a phenolic *β-O-*4 lignin model compound (LM-OH) (Majumdar et al., 2014). Furthermore, SLAC variants have been functionally expressed in *Aspergillus niger* and are active in lignocellulose degradation (Sidar et al., 2024).

The laccase from *Bacillus ligniniphilus* L1 was found to promote lignin degradation by oxidizing phenolic and non-phenolic structures in lignin (Peng et al., 1998). In addition, this study highlights its potential role in cleaving key interunit linkages in lignin,

including β-O-4, β-5, β-β, 4-O-5, and 5-5.

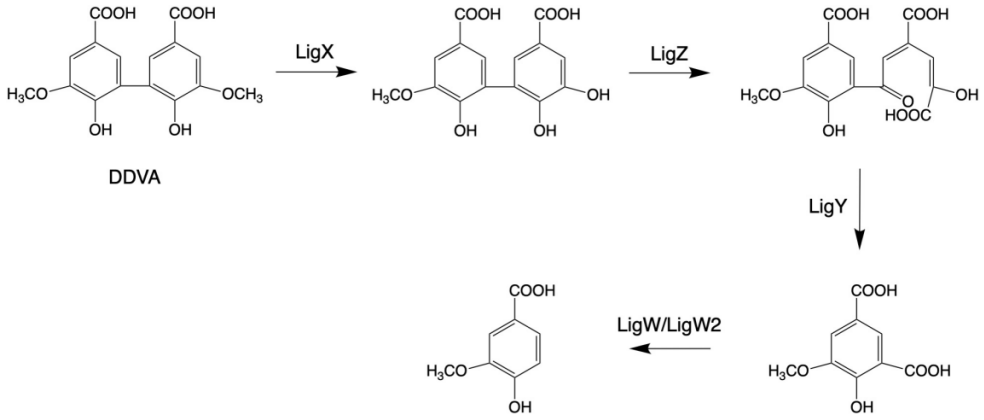

Figure 3. Pathways for the structural cleavage of biphenyl moieties in *Sphingobium sp*. SYK-6 (adapted from (Bugg et al., 2011c)).

3.1.3 5-5 Bond (Biphenyl Bond)

The proportion of 5-5 bonds in lignin is around 10% in softwood and 5% in hardwood (Pandey & Kim, 2011). Remarkably, it has been demonstrated that the cleavage of the biphenyl linkage plays a pivotal role in facilitating lignin degradation.

Amongst fungi, the versatile peroxidases (VPs) in *Physisporinus vitreus*, were also observed to cleave the 5-5 bond of dehydrodivanillic alcohol (5-5' dimer) in vitro for the first time (Kong et al., 2017).

The bacterial biphenyl degradation pathway was also found in *Sphingobium sp.* SYK-6 by growing on 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-dicarboxybiphenyl (DDVA) (Sonoki et al., 2009). In the mechanism of degrading DDVA, four enzymes are involved: LigX (a non-heme iron-dependent demethylase), LigZ (an extradiol dioxygenase), LigY (a C-C hydrolase), and LigW/LigW2 (decarboxylases) (Masai et al., 2007; Peng et al., 1998; Sonoki et al., 2000). In the catalytic progression of DDVA, the enzyme LigX catalyzes the elimination of a methoxy group, resulting in the formation of a hydroxyl group. Subsequently, the product generated by LigX serves as a substrate for oxidative meta-cleavage, facilitated by LigZ. Following this, LigY transforms the ring fission product into 4-carboxy-2-hydroxypentadienoic acid and 5-carboxyvanillic acid (5CVA). This sequence culminates with the participation of LigW and LigW2, which convert 5CVA into

the pivotal metabolic intermediate, vanillic acid or vanillate, essential for the synthesis of various bioproducts, as shown in Figure 3.

3.1.4 β-β Bond (Resinol Bond)

The breakdown of the pinoresinol lignin model compound has also been studied in *Fusarium solani* M-13-1 and *S. paucimobilis* SYK-6 (Kamaya et al., 1981; Masai et al., 2007). The catabolic pathways for both heterocyclic lignin components appear to involve alpha-hydroxylation as an initial step. However, enzymes that participate in the reactions have not been characterized clearly. Until 2018, the isolation of the highly efficient (+)-pinoresinol-mineralizing *Pseudomonas sp*. strain SG-MS2 and its catabolic pathway were reported, highlighting a significant advancement in understanding the catabolism of pinoresinol lignin dimers, as shown in Figure 4 (Shettigar et al., 2018).

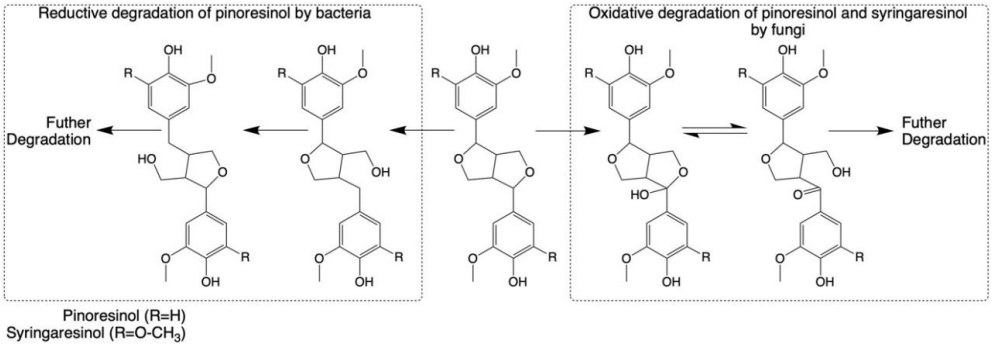

Figure 4. Pathways for the cleavage and subsequent degradation of pinoresinol and syringaresinol by bacteria and fungi, respectively (adapted from (Bugg et al., 2011c; Shettigar et al., 2018)).

3.1.5 Other C-C Bonds

Fungal MnP possesses the capability to not only break *β-O-4* linkages in phenolic structures but also disrupt C*α*-C*β* and *β*-aryl ether bonds in non-phenolic substances (Bao et al., 1994; Kapich et al., 1999). Studies indicate that laccases could cleave Cα–Cβ bonds or aryl–Cα bonds and catalyze the oxidation of Cα–OH to Cα=O of lignin model compounds (Kawai et al., 1988). The laccase degradation mechanism may vary depending on the substrate, pH, temperature, and other environmental conditions (Agustin et al., 2021). In addition, different types of laccases may have different substrate specificity and degradation efficiency. A deep understanding of these mechanisms is needed for developing effective biotechnological applications for lignin

degradation.

4 *Pseudomonas putida* as a Lignin-Degrading Cell Factory

Although the efficiency of lignin depolymerization by bacterial extracellular enzymes is less well studied than that of white-rot fungi, bacteria do provide a flexible platform for the heterologous expression of ligninolytic enzymes (Bugg et al., 2011b). We and others have investigated endogenous lignin degradation with strains of *Pseudomonas putida*. Moreover, strains of *P. putida* have been implicated in bioconversion and biosynthesis of valuable products from lignin-derived compounds (He et al., 2023; Johnson et al., 2016; Graf et al., 2014). The direct and grand challenge now is to directly access and utilize lignin as a source of valuable products. *P. putida* may prove a promising vehicle for the expression of various specific ligninolytic enzymes to construct cell factories, enabling the direct conversion of lignin into high-value products (see Figure 5).

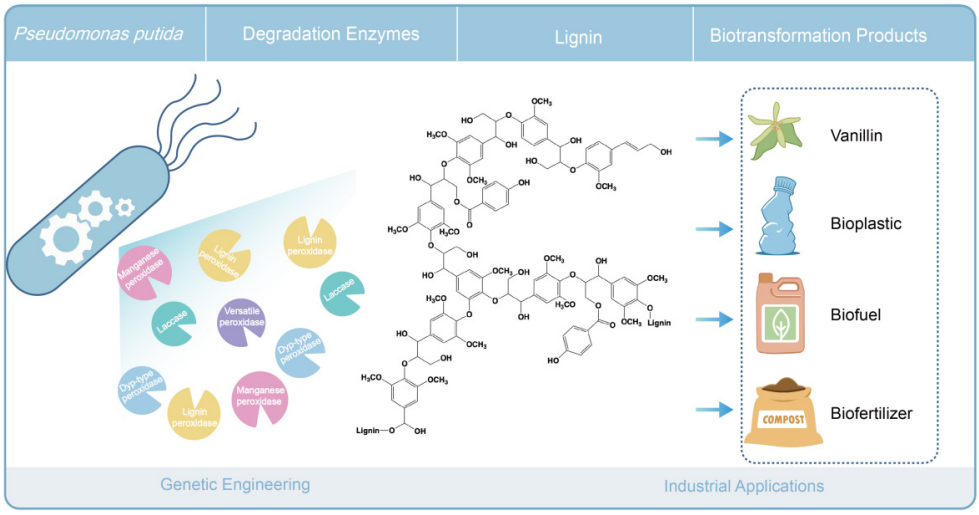

Figure 5. Conceptual representation of lignin biodegradation and engineering in *Pseudomonas putida*.

4.1 Natural Capabilities and Metabolic Pathways

P. putida strains like S12 and KT2440 are recognized as highly promising

industrial host strains (Ankenbauer et al., 2020). These strains can adapt to diverse physiochemical and nutritional niches and possess robust metabolic redox power, enabling them to survive under high oxidative stress. Moreover, *P. putida* has been found in natural environments degrading various organic compounds, including lignin-derived molecules. For example, *P. putida* KT2440 has been identified to possess the ability to degrade p-hydroxybenzoate, benzene, and xylene, which are components of lignin-derived aromatic hydrocarbons (Jiménez et al., 2002b). *P. putida* has a wide range of metabolic functions, combined with its extensive catabolic pathways, enabling it to utilize lignin-derived aromatic compounds as carbon sources.

In recent years, the depolymerization of lignin has become a research hotspot (Li et al., 2015; Zakzeski et al., 2010). Lignin-derived compounds like ferulic acid and vanillin have been given particular attention (Fache et al., 2016). *P. putida* KT2440 can metabolize vanillin by conversion into vanillate, at a rate of 4.87 mmol $(gCDW_{h})^{-1}$ (Ravi et al., 2017). Furthermore, *P. putida* KT2440 can degrade ferulic acid via a CoA-dependent non-β-oxidative pathway (Plaggenborg et al., 2003). Additionally, this strain can metabolize benzoate and catechol through the native β-ketoadipate pathway, further demonstrating its ability to process lignin-derived compounds (Jiménez et al., 2002b). The β-ketoadipate pathway is a chromosomally encoded aromatic compound degradation pathway that is widespread among soil bacteria and fungi (Harwood & Parales, 1996). Such pathways are essential for lignin degradation and valorization, turning complex aromatic polymers into economically valuable products.

P. putida demonstrates exceptional performance in the degradation of lignin derivatives and other aromatic compounds, showing the potential applications of biotechnology in lignin valorization. Therefore, *P. putida* strains are promising suitable platforms for the bioconversion of exogenous toxic chemical streams into valuable products that derive from lignin degradation.

4.2 Genetic Engineering of Pseudomonas putida

4.2.1 Genomic Tools

To develop efficient cell factories for lignin degradation, a specifically robust bacterial chassis is needed. *P. putida* is a robust platform with advanced metabolic

engineering applications (Nikel & de Lorenzo, 2018). The degradation capability of *P. putida* toward lignin can be further enhanced through genetic engineering. The introduction of genes encoding ligninolytic enzymes from other microorganisms can improve efficiency. Modifying the metabolic pathways of *P. putida* can convert the lignin degradation products into valuable compounds.

Over the years, dedicated genetic tools have been developed for the expression or deletion of genes in *P. putida* (Cook et al., 2018; Martin-Pascual et al., 2021); see Table 4. With the use of gene editing tools, it is highly possible to improve the lignin degradation of *P. putida*. For example, previous studies show that the expression of *Pseudomonas fluorescens* Dyp1B in *P. putida* KT2440 results in enhanced activity for the oxidation of 2,6-dichlorophenol (DCP) and polymeric lignin (Ehibhatiomhan et al., 2023b).

Table 4. Typical genomic tools for cloning, insertions, and deletions in *P. putida*.

Genomic Tools	Purpose	References
Tn5-based transposon system	Random insertion of genes	(Ahmad et al., 2011b)
Tn7-based transposon system	Insertions	(Zobel et al., 2015)
Inducible expression systems: XylS/Pm, LacIQ/Ptrc, Plac, Ptac	Expression of target genes	(Ahmad et al., 2011b; Bagdasarian et al., 1983; Calero et al., 2016)
pEMG	Scarless deletions and insertions	(Martínez-García et al., 2011)
pSNW	Scarless deletions and insertions	(Volke et al., 2020)
CRISPR-Cas9 systems	Precise genome editing, allowing for targeted gene knockouts and insertions	(Aparicio et al., 2018; Aparicio et al., 2019)
CRISPR-Cas3 systems	Precise genome editing, allowing for targeted gene knockouts and insertions	(Lammens et al., 2023)
phi15-based expression system	Expression of target genes	(Lammens et al., 2025)

4.2.2 Secretion System

Lignin, as a polymeric network, is obviously too large and complex to be transported into the cell and be degraded intracellularly (Bugg, 2024a). Hence, lignin-degrading enzymes for biotechnological applications should be extracellularly secreted with the microbial secretome. Several secretion systems have been studied in *P. putida*:

Outer membrane vesicles (OMVs) are secreted by the bacterium to deliver enzymes (Schwechheimer & Kuehn, 2015). Pioneering research has shown that OMVs in *P. putida* KT2440 can catabolize lignin-derived aromatic compounds (Salvachúa et al., 2020). This property can be exploited to deliver ligninolytic enzymes to lignin substrates, thereby enhancing the degradation process. A novel recombinant peroxidase secretion system has been constructed in *P. putida* KT-M2 (Lee et al., 2023). A flagellar type III secretion system was used for the dye decolorization peroxidase of *P. putida,* resulting in efficient oxidative activity of cell-free supernatants against a variety of chemicals, including the lignin model compound. Additionally, the periplasmic expression of peroxidase Dyp1B has been explored for lignin valorization in *P. putida* (Ehibhatiomhan et al., 2023b). The periplasmic expression strain shows higher lignin oxidation activity than the wide type.

These advancements in secretion systems, together with the genetic engineering tools, highlight *P. putida*'s potential as a powerful biotechnological platform for lignin degradation. By combining these approaches, it is possible to enhance the efficiency of lignin valorization processes, turning this complex and recalcitrant polymer into valuable bioproducts.

4.3 Biological Conversion and Reutilization

Biological funneling is a concept in bioconversion and metabolic engineering where a diverse array of complex molecules is funneled through a series of biological pathways to produce a single or a few specific valuable products (Becker & Wittmann, 2015; Weiland et al., 2022). Biological funnels can overcome the challenging heterogeneity of chemical mixtures as recently applied and demonstrated with low-molecular-weight lignin-derived aromatics (Abdelaziz et al., 2016; Kamimura et al., 2017). Indeed, *P. putida* may convert a mixture of lignin degradation products into useful compounds. These compounds can be further utilized, such as biofuels, chemical raw materials, and other bio-based products.

To obtain low-molecular-weight lignin-derived molecules, we may rely on a combination of chemical and biological treatment. Recently, conversion from lignin to medium chain-length polyhydroxyalkanoates was achieved in *P. putida* by combining microbial treatment with chemical pretreatment (Linger et al., 2014a). In a comparable effort, lignin conversion to β-ketoadipate was achieved with engineered *P. putida* (Werner

et al., 2023). In this work, genes encoding enzymes mediating 4-hydroxybenzoate hydroxylation and vanillate O-demethylation were overexpressed to improve the yield, and the gene that could cause intermediate accumulation was deleted. Additionally, through genetic engineering, Altenbuchner et al. successfully introduced key enzymes involved in the conversion of lignin-derived ferulic acid to vanillin in *P. putida* KT2440 (Graf & Altenbuchner, 2014). This research enhances vanillin production with up to 86% molar yields and few byproducts.

These studies further illustrate that engineering metabolic pathways in *P. putida* to funnel lignin breakdown products into desirable bioproducts can increase the economic value of lignin valorization processes.

5 Conclusions and Future Prospects

Lignin, the second most abundant terrestrial polymer found on Earth, constitutes an important part of plant fibers (Huang et al., 2020). Lignin is composed of a network of aromatic compounds and is highly resistant to decomposition. Currently, this rich aromatic compounds resource is mainly separated as a waste stream, where 98% is used as a heat source in factories (Yao et al., 2022). Only 2% is used in a chemical conversion to produce useful compounds like lignosulfonates (Demuner et al., 2019).

Importantly, we here make a case for lignin to be utilized much more effectively through biotechnological and chemical processes. Using those techniques, separately or in combination, lignin can be degraded into hundreds of valuable derivatives (Zhou et al., 2022). In nature, bacteria and fungi can degrade lignin, with some differences in the degradation mechanism, substrate specificity, and product generation. Only recently, research on the enzymatic processes involved in bacterial lignin degradation has led to the identification and documentation of specific enzymes dedicated to this purpose. This review provides a list of microorganisms reported to utilize lignin and potential enzymes involved in specific lignin depolymerization.

Fungi, especially white-rot fungi, produce a variety of enzymes (such as lignin peroxidase, manganese peroxidase, laccase, etc.) that can directly oxidize and degrade lignin. Fungi typically work by producing multiple enzymes that work together to break

down different lignin bonds and connections. The depolymerization of native lignin is facilitated by extracellular oxidative enzymes, including Lip, MnP, VP, and Lac, which have been extensively documented in fungi. The research on bacterial degradation of lignin is not as in-depth as that on fungi. It has only been a dozen years since the first bacterial enzyme that degrades lignin was discovered (Ahmad et al., 2011b). In bacteria, the lignin degradation enzyme systems are thought to be relatively simple and specific. Limited enzymes are involved, such as phenol oxidase. In bacteria like *Cupriavidus basilensis* B-8, Lac, and MnP activities were identified; however, no MnP or Lac genes were found (Shi et al., 2013a). Hence, bacteria are anticipated to possess distinctive lignin degradation mechanisms and novel types of peroxidases (Bugg et al., 2011b).

Typically, fungal lignin degradation spans 10–30 days, whereas in bacteria, it may be accomplished in as little as 2–7 days. From an industrial viewpoint, utilizing bacteria as a host strain to establish a lignin degradation and utilization cell factory would prove more cost-effective (Asina et al., 2016). In bioreactors, bacteria have advantages over fungi due to their rapid growth, simpler cultivation requirements, higher metabolic rates, easier genetic manipulation, and simpler product recovery (Cuebas-Irizarry & Grunden, 2024; Eng et al., 2021). While research on the enzymatic processes of lignin-degrading bacteria is currently relatively limited, using bacteria for lignin degradation still holds great prospects. This review demonstrates the great potential of *P. putida* as a microbial cell factory in lignin degradation and valorization, providing a sustainable approach to converting lignin into valuable bioproducts. The plasmid-free strain *P. putida* KT2440 is particularly regarded as a microbial host for biotechnological applications due to its biosafety status (Weimer et al., 2020) and is widely used in industrial production.

We believe that, through genetic engineering and process optimization, *P. putida* can be adapted to industrial needs and contribute to further developing the bioeconomy through sustainable industrial practices.