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# **SYNTHETIC BIOLOGY TOWARDS LIGNIN UTILIZATION**

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# TIIVISTELMÄ

Olivia Kohl: Synteettinen biologia ligniinin hyödyntämisessä  
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Ligniini, kasvisolujen soluseinän komponentti, on maailman runsain aromaattisten polymeerien uusiutuva resurssi. Ligniinin aromaattinen rakenne mahdollistaa sen hyödyntämisen soveluksissa, joiden valmistamiseen perinteisesti käytetään fossiilisia raaka-aineita. Paperi- ja selluteollisuus tuottavat runsaasti ligniinipitoisia sivuvirtoja. Ligniiniä hyödynnetään yleisesti paperi- ja selluteollisuuden energian- ja lämmöntuotannossa. Tällöin ligniinin taloudellinen arvo jää murtoosaan verrattuna siihen, että ligniinistä valmistettaisiin korkeamman jalostusasteen tuotteita kuten materiaaleja, polttoaineita tai kemikaaleja.

Kirjallisuuskatsauksessa selvitetään ligniinin prosessoinnin ketju ligniinin hyödyntämiseksi. Tässä työssä keskitytään biologisiin menetelmiin sekä tutkitaan synteettisen biologian vaikutusta ligniinin hyödyntämisen tehokkuuteen.

Ligniinillä on heterogeeninen, haaroittunut ja ristosilloitettu rakenne, mikä tuottaa haasteita sen hyödyntämisessä. Muita haasteita ovat ligniinin liukenemattomuus veteen, myrkyllisyys mikroorganismille ja huono kemiallinen reaktiokyky. Ligniinin ominaisuudet vaihtelevat myös niiden lähteen mukaan: teollisten sivuvirtojen ligniinit saattavat tarvita kemiallisia esikäsittelyjä niiden ominaisuuksien parantamiseksi.

Tästä huolimatta ligniinin hajottamiseen monomeereiksi on useita eri termokemiallisia ja biologisia menetelmiä. Tätä hajotusprosessia kutsutaan depolymerisaatioksi. Sienet ovat luonnossa tehokkaita depolymerisoimaan ligniiniä. Toisaalta bakteeritkin kykenevät ligniinin hajottamiseen. Niiden etuna on nopea kasvu ja helppo geneettinen muokattavuus.

Depolymerisoinnin jälkeen ligniinistä muodostuu sekoitus erilaisia aromaattisia monomeerejä. Näiden erottelun ja jatkojalostuksen tehokkain menetelmä on biologinen konversio. Mikrobit hyödyntävät hajotustuotteita hiilen lähteenä ja muokkaavat niistä enää muutamia aineenvaihdunnan välituotteita kuten esimerkiksi katekolia ja protokatekuaattia (protocatechuate). Tämän jälkeen mikrobit muodostavat keskeisen aineenvaihduntareitin kautta esimerkiksi cis,cis-mukonihappoa.

Tästä eteenpäin jatkojalostamisen menetelmät riippuvat sovelluskohteesta. Nailonia ja polyeteenitereftalaattia (PET) voidaan muodostaa cis,cis-mukonihaposta. Ligniiniä voidaan myös hyödyntää materiaalina 3D-tulostamiseen, täyteaineena epokseihin ja liimoihin sekä lääketieteessä lääkeaineina tai lääkkeen kuljetukseen.

Synteettistä biologiaa käytetään biologisten menetelmien tehostamiseksi. Esimerkiksi biologia voidaan muokata siten, että se ilmentää samanaikaisesti useampaa ligniiniä hajottavaa entsyymiä. Ligniinin tehokas biologinen hyödyntäminen vaatii syvällistä ymmärrystä mikrobien ligniinin aineenvaihdunnasta. Lisäksi täytyy hallita synteettisen biologian työkalujen käyttö, jotta mikrobeille saadaan muokattua haluttuja ominaisuuksia. Tutkimus ligniinin hyödyntämiseksi on lisääntynyt viime vuosina ja uusia mahdollisuuksia löytyy jatkuvasti. Synteettinen biologia tehostaa ligniinin hyödyntämisen prosesseja, mikä lopulta voi mahdollistaa ligniinin hyödyntämisen teollisella tasolla.

Avainsanat: ligniinin depolymerisointi, biologinen konversio, geneettinen muokkaaminen, *Pseudomonas putida*, jalostus

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# ABSTRACT

Olivia Kohl: Synthetic Biology towards Lignin Utilization  
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Lignin, a component of plant cell walls, is the world's most abundant sustainable resource of aromatic polymers. The aromatic structure of lignin allows it to be used in applications that traditionally rely on fossil resources. The paper and pulp industry produce large lignin-rich side streams. Lignin is commonly used in these industries for energy and heat production. This leaves the value of lignin at a fraction of what it would be if it were used to produce higher value-added products such as materials, fuels or chemicals.

This literature review will explore the whole process chain that enables lignin utilization. The focus is on biological methods and the impact of synthetic biology on the efficiency of lignin utilization is explored as well.

Lignin has a heterogeneous, branched and cross-linked structure, which raises challenges in its utilization. Other challenges of lignin utilization include its insolubility in water, toxicity to microorganisms and poor chemical reactivity. The properties of lignins also vary depending on their source: lignins from industrial side streams may need chemical pretreatments to improve their characteristics.

Nevertheless, there are several different thermochemical and biological methods to break down lignin into monomers, which is called depolymerization. Fungi are efficient biological depolymerizers in nature. On the other hand, bacteria are also capable of breaking down lignin and they have the advantage of rapid growth rates and ease of genetic modifications.

After depolymerization, the lignin-derived products are a mixture of different aromatic monomers. The most efficient method for their separation and further processing is biological conversion. Microbes use the degradation products as a source of carbon and convert them towards some intermediates such as catechol and protocatechuate. The microbes can form e.g. *cis,cis*-muconic acid through a central metabolic pathway.

From here on, the downstream processing methods depend on the application. Nylon and polyethylene terephthalate (PET) can be formed from *cis,cis*-muconic acid. Lignin can also be used as a material for 3D printing, as a filler for epoxies and adhesives, and in medicine for drug delivery or as pharmaceuticals.

Synthetic biology is used to enhance biological processes. For example, a microbe can be engineered to express several lignin-degrading enzymes simultaneously. Effective biological utilization of lignin can only be achieved through a deep understanding of microbial lignin metabolism. In addition, the use of synthetic biology tools should be mastered in order to achieve the desired properties for microbes. Research into lignin utilization has increased in recent years and new opportunities are constantly being discovered. Synthetic biology enhances lignin utilization processes, which may eventually allow lignin to be utilized on an industrial scale.

Key words: lignin depolymerization, biological conversion, genetic manipulation, *Pseudo-*monas putida**, refinement

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# ARTIFICIAL INTELLIGENCE TOOLS

The AI tools used in my thesis and the purpose of their use has been described below:

ChatGPT 3.5

*Drafting the structure of the text, formulating research questions, improving writing and translations.*

DeepL Translator

*Assistance in translating the Finnish abstract into English.*

I am aware that I am totally responsible for the entire content of the thesis, including the parts generated by AI, and accept the responsibility for any violations of the ethical standards of publications.

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# 1. INTRODUCTION

Lignin, a component of plant cell walls, is the largest renewable aromatic polymer source on earth (Li et al. 2022; Yoo and Ragauskas 2021). In the global biosphere, approximately 300 billion tons of lignin is available and the amount increases by about 20 billion tons annually making lignin an abundant renewable resource (Becker and Wittmann 2019).

The pulp and paper industry produces roughly 50 million tons of lignin in a year as a major side stream that is mostly burned for the production of heat and electricity to power the industrial processes (Hämäläinen et al. 2018; Ragauskas et al. 2014). This use of lignin only realizes its energetic value (Becker and Wittmann 2019) although lignin holds significant potential to serve as an alternative source for aromatic compounds e.g. chemicals, fuels and materials that are typically obtained from petroleum (Sun et al. 2018; Yoo and Ragauskas 2021). The utilization of lignin follows the principles of circular economy which is the objective that today's economy is gradually shifting towards.

The current total market value of lignin-derived products is at \$3,3 billion of which 89 % is covered by energy. Other current markets include vanillin production and cement additives. (Lux 2014 as per Hämäläinen et al. 2018) With possible high-value applications like carbon fibers and phenols, lignin is currently utilized in its lowest value application as fuel with an energy value of about 22 MJ/kg. If lignin-based phenols and carbon fiber captured the largest market share, could the potential market value of lignin-based products rise to \$13,9 billion. (Hämäläinen et al. 2018; Smith et al. 2016)

Lignin is a complex molecule with strong bonds between its building blocks and its structure is heterogeneous (Becker and Wittmann 2019; Yoo and Ragauskas 2021). These features are only the beginning of challenges concerning the valorization of lignin i.e. the conversion of lignin into value-added products. Nevertheless, numerous approaches have been developed for the depolymerization i.e. breaking down of lignin and also towards the conversion of degraded lignin into value-added products. This thesis especially concentrates on the biological pathways towards lignin utilization with a focus on synthetic biology as a method for improving the efficiencies of lignin valorization processes.

The European Commission defines synthetic biology (SynBio) as follows: “SynBio is the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in living organisms.” (European Commission, Scientific Committee of Health and Environmental Risks 2014, p. 5) Practically, synthetic biology aims to optimize metabolic pathways by redesigning natural pathways or by designing and building synthetic pathways in microorganisms (Zhang et al. 2019). For example, molecular biology tools can be utilized in synthetic biology (Becker and Wittmann 2019) to produce changes in gene expression that could facilitate a better efficiency in lignin utilization.

This literature review aims to demonstrate lignin utilization from start to finish. A variety of lignin sources, depolymerization methods and value-added products along with their advantages and issues are presented to enable a thorough understanding of this field. The focus on biological processes is chosen due to its major potential in lignin valorization. This thesis tries to find answers for how lignin utilization is conducted currently and how synthetic biology can overcome prevailing challenges to enhance lignin utilization. First is considered, how lignin is degraded and which micro-organism is most efficient for this purpose. Next is explored, how these degradation products are turned into products of value and what kinds of products can be produced.

The theory of lignin structure and composition is explained in chapter 2, which makes understanding the upcoming chapters easier. In chapter 3 the major industrial processes that produce lignin as a side stream are reviewed and compared. Chapter 4 introduces challenges that need to be realized for lignin valorization. Some of the different chemical, thermal and biological methods for lignin depolymerization are presented in chapter 5. Chapter 6 concentrates on the biological conversion of lignin into value-added products and mentions several current applications of lignin-derived products. Some necessary requirements for the upscaling of lignin valorization processes are discussed in chapter 7 and chapter 8 compiles the conclusions of this thesis.

## 2. LIGNIN STRUCTURE AND COMPOSITION

Lignin is a biopolymer that provides structural integrity in wood and plants (Calvo-Flores et al. 2015). In nature, lignin only exists as part of lignocellulose alongside cellulose and hemicellulose (Chio et al. 2019). Inside plants, lignin functions as a water channel, structural stabilizer and it provides protection against microbial decay (Becker and Wittmann 2019).

Natural lignin mainly consists of three phenolic units, called monolignols, and a few carbohydrate components (Calvo-Flores et al. 2015). The lignin that is produced as a by-product in the cellulose-centered biorefinery and paper industry differs from native lignin because the process conditions alter the structure and composition of lignin (Yoo and Ragauskas 2021). Therefore, modified lignin is referred to as technical lignin. A single definition for the structure of lignin has not been established due to its intrinsic molecular complexity and diverse structural composition – this applies to both native and technical lignin (Calvo-Flores et al. 2015). This chapter describes how the structure of lignin is formed, why it varies so much and how the structures of softwood and hardwood differ from each other.

The structure of lignin is built from different combinations of monomers that are called monolignols. The main monolignols are hydroxycinnamyl alcohols that share the phenylpropane unit (Calvo-Flores et al. 2015) but differ in the degree of substitution by methoxyl groups on the aromatic ring (Duval and Lawoko 2014). These phenolic monolignols are commonly known as coniferyl, p-coumaryl and sinapyl alcohols. Monolignols are incorporated in the polymer as the following phenylpropanoid constituents: p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), as presented in figure 1. The structural diversity of lignin mainly stems from the different combinations of these three building units (Calvo-Flores et al. 2015).

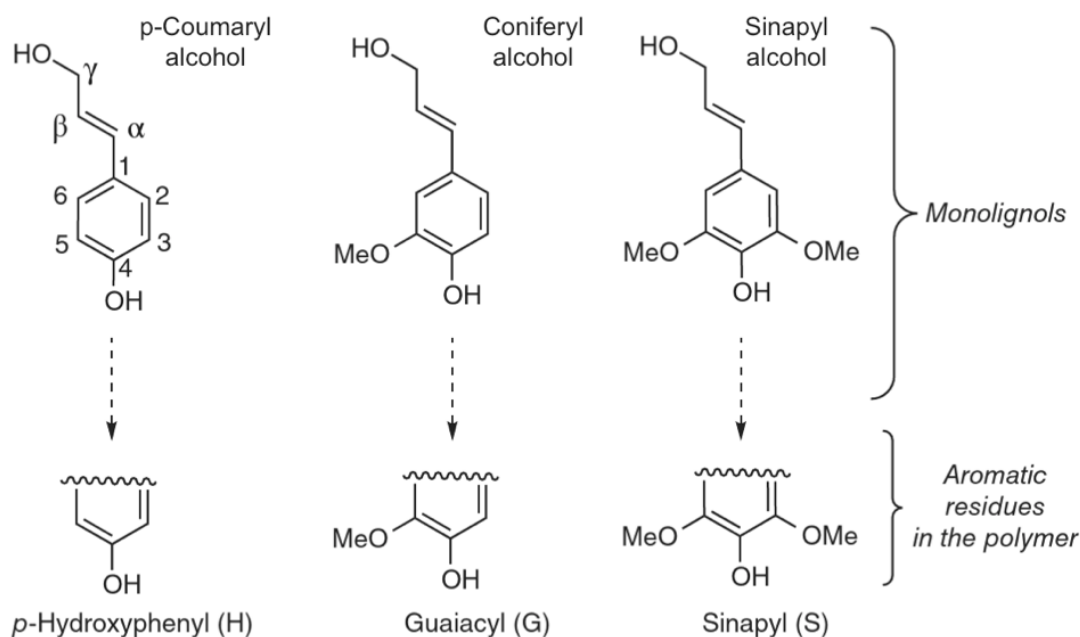


Figure 1: Chemical formulas and atom numbering of monolignols as well as their residues in the polymer. Modified from (Calvo-Flores et al. 2015).

Lignin polymers are synthesized through reactive radical intermediates which are generated by laccase and peroxidase enzymes (Becker and Wittmann 2019; Duval and Lawoko 2014). The delocalized electrons of these radicals facilitate radical coupling reactions that result in various types of inter-unit linkages at several positions between monolignols (Van den Bosch et al. 2018). One ether linkage is formed when the phenolic hydroxyl of one unit and the  $\beta$  carbon of another unit bond into the so called  $\beta$ -O-4 linkage (Duval and Lawoko 2014). The  $\beta$ -O-4 linkage is by far the most abundant inter-unit linkage in lignin, accounting for approximately 50–80% of all inter-unit linkages (Van den Bosch et al. 2018). Other linkages bonding the lignin subunits together are carbon-carbon bonds and ether bonds such as  $\beta$ -5 linkages, 5-5 linkages,  $\alpha$ -O-4 linkages and bi-phenyl or diaryl ether structures (Becker and Wittmann 2019). During processing, the  $\beta$ -O-4 linkage is readily cleaved. This coupled with the abundance of this linkage, the  $\beta$ -O-4 linkage is the most investigated inter-unit linkage in lignin research (Van den Bosch et al. 2018).

The formation of phenylpropanoid macromolecules is called lignification. In plants, lignification includes the biosynthesis of monolignols, their transport to the cell wall and finally the polymerization into the final macromolecule. The relatively simple constituents end up in a complex structure through the variety of possible linkages and arrangements. The heterogeneity and structural diversity of lignin is one factor that complicates lignin utilization. (Calvo-Flores et al. 2015)

The origin of lignin, i.e. the plant species, affects the structure, composition and ratio of lignin. For example, lignin comprises about 30% of the total mass in softwood but only 20–25% in hardwood and even less in herbaceous species. (Calvo-Flores et al. 2015) Softwoods are composed of mainly coniferyl units (type G). In comparison, hardwoods like birch contain less lignin and are composed of both coniferyl and sinapyl alcohols (types G and S). Herbaceous crops and grasses are composed of all three major monolignols (types H, G and S) with a majority of G units. (Becker and Wittmann 2019; Calvo-Flores et al. 2015; Salmela 2020; Van den Bosch et al. 2018)

The different ratios of H, G and S units and their distribution throughout the lignin polymer determine the types and amounts of inter-unit linkages and with it the branching and reactivity of lignin. In the G and S monolignols, methoxy groups occupy one or two of the ortho-positions relative to the phenolic group on the aromatic ring, respectively. The methoxy groups block the formation of the resilient 5-5 and  $\beta$ -5 carbon-carbon bonds. (Becker and Wittmann 2019; Duval and Lawoko 2014; Van den Bosch et al. 2018) This implies that G-type lignin contains a lower fraction of resistant carbon-carbon bonds than H-type lignin and S-type lignin contains the lowest fraction of these bonds (Van den Bosch et al. 2018).

Consequently, hardwood lignin with a high content of S-units contains less carbon-carbon bonds than G-rich softwood and is more susceptible towards depolymerization (Becker and Wittmann 2019; Calvo-Flores et al. 2015; Van den Bosch et al. 2018). Softwood lignins are also generally more branched than hardwood lignins because softwood lignins lack S units (Ragauskas et al. 2014) On the other hand G- and H-type lignins are better for biological conversion of lignin because their degradation products can be directly converted into metabolites like catechol. Though with enough thermochemical pre-treatments, S-type lignin can also be turned into catechol. (Kohlstedt et al. 2018)

## 3. LIGNIN SOURCES

In the pulp and paper industry, the traditional processes are designed for the efficient isolation and purification of the fibrous cellulose stream. Lignin has been perceived as an uninteresting component and therefore lignocellulose is processed under harsh conditions when separating the carbohydrates from lignin. This causes irreversible alterations in the chemical structure of lignin. (Van den Bosch et al. 2018) Essentially, different pulping and extraction processes result in different molecular compositions and molecular weights for lignin (Chio et al. 2019). The following steps in lignin valorization, which are depolymerization and synthesizing new products, may thus require different approaches for different types of lignin. Kraft lignin, liginosulfonates, soda lignin and organosolv lignin are related to the most important pulping processes (Becker and Wittmann 2019). These pulping processes, the extraction of lignin and the impact on the chemical structure of lignin are considered next. Table 1 summarizes and compares the important characteristics of the mentioned types of lignin.

The structure of native lignin is more prone to depolymerization because it has a higher content of ether bonds and a lower degree of carbon-carbon crosslinking. The structures of technical lignins often experience condensation as a result of the harsh industrial processes. Also, the presence of impurities such as alkali metals, biomass degradation products and sulfur impact the depolymerization efficiency. This means that technical lignins are less reactive substrates for further conversion into chemicals compared to native lignins. The reactivity of isolated lignin is also affected by the process severity and the lignocellulosic feedstock. Therefore, isolating lignins from industrial processes, whose structure resembles native lignin the most, is a good strategy for further chemical conversions. Especially the preservation of  $\beta$ -O-4 ether bonds is desired since it is the most abundant linkage in lignin structure. (Van den Bosch et al. 2018)

### 3.1 Kraft Lignin

Kraft process is currently the dominant method for producing pulp: kraft processing is used to produce 75 % of the total amount of pulp annually. This corresponds to 170 million tons of kraft pulp. In this context, above 70 million tons of kraft lignin, that could be extracted, is produced as a side stream. (Argyropoulos et al. 2023)

In kraft processing, lignin is separated from lignocellulose by white liquor, which mainly consists of sodium hydroxide (NaOH) and sodium sulfide (Na<sub>2</sub>S). The process is executed at 160°C or higher temperatures and the environment causes delignification, which means the separation of cellulose and hemicellulose from lignin. The kraft pulping process ends up with kraft pulp and black liquor. Black liquor contains the dissolved lignin among with water, salts, dissolved organic material and various sulfur species. (Argyropoulos et al. 2023; Van den Bosch et al. 2018)

Kraft process produces the largest lignin stream in industry, but black liquor is mainly used for the generation of electricity and heat as well as the regeneration of sodium sulfide. Presently, only less than 2% of the lignin stream is isolated as kraft lignin. (Van den Bosch et al. 2018)

Nevertheless, lignin can be isolated from black liquor by precipitation (Argyropoulos et al. 2023). Two similar methods to industrially isolate lignin from black liquor are LignoBoost (Zhu et al. 2014) and LignoForce (Kouisni et al. 2016). Another way for lignin extraction is referred to as the sequential liquid-lignin recovery and purification (SLRP) method (Lake and Blackburn 2014). All mentioned precipitation methods use acidification of black liquor to lower the solubility of lignin. LignoBoost and LignoForce use filtration to finally collect the lignin. In SLRP lignin is collected via phase separation in liquid form. In certain applications, lignin in crude black liquor may be directly utilized, like in gasification. (Argyropoulos et al. 2023)

Strong nucleophilic ions present in the kraft process initiate the cleavage of  $\beta$ -O-4 linkages. As sulfur is incorporated, thiol groups are formed in lignin. The alkaline conditions of the kraft process promote a network of complex repolymerization reactions. Ultimately, kraft processing results in a water-insoluble, condensed and recalcitrant lignin product that has lost the majority of native  $\beta$ -O-4 ether linkages. Kraft lignin contains a relatively low sulfur content ranging from 1–3% but this is enough to act as catalyst poison. Therefore, kraft lignin is considered a challenging source for catalytic conversions to chemicals and fuels. (Van den Bosch et al. 2018)

### 3.2 Lignosulfonates

Sulfite pulping was the prevalent pulping method until 1950s, but today it only represents less than 10% of the chemical pulping industry (Van den Bosch et al. 2018). Sulfite pulping breaks down wood at high temperature and pressure with an aqueous solution of sulfur dioxide combined with sodium, magnesium, calcium or ammonium sulfite or bisulfite salts (Calvo-Flores et al. 2015; Mboowa 2024).

Sulfite pulping is only possible for softwood species and the produced pulps can be of different types and qualities. This is because the sulfite cooking liquor can be engineered within the entire pH range through different chemical combinations. Advantages of sulfite pulping over kraft pulping include a higher yield, brightness and pulp is easily refined and bleached. On the contrary, chemical recovery is nearly impossible in sulfite pulping which makes the process environmentally harmful. (Mboowa 2024)

The waste liquid that forms during the separation of cellulose is rich with lignins. These lignins are called lignosulfonates because of the sulfonate groups present in the structure (Calvo-Flores et al. 2015). The lignosulfonate still needs to be isolated from the spent sulfite liquor. There are many ways to achieve extraction: sugar removal by chemical destruction or ultrafiltration, alcoholic fermentation of the sugars and precipitation. An issue with extracting lignosulfonates is the formation of an emulsion and foam. This can be avoided by proper selection of alkyl alcohol and amine in the extraction procedure. (Calvo-Flores et al. 2015)

Lignosulfonates have a higher sulfur content compared to kraft lignin: roughly 4–8 wt% (Calvo-Flores et al. 2015; Van den Bosch et al. 2018). Though sulfite pulping is pH-dependent, the lignin is sulfonated upon a nucleophilic attack of the (bi)sulfite anion. This results in the cleavage of  $\beta$ -O-4 bonds. (Van den Bosch et al. 2018) The produced lignosulfonates possess an improved solubility in water (Becker and Wittmann 2019; Van den Bosch et al. 2018) which has enabled their utilization as macromolecular components in multiple applications such as adhesives or dispersants. For these reasons, lignosulfonates represent a large source of lignin, despite the smaller production of the sulfite process. (Van den Bosch et al. 2018)

### 3.3 Soda Lignin

Soda pulping is a third important pulping method. It is similar to kraft pulping but uses only an aqueous solution of sodium hydroxide (NaOH) without sodium sulfide ( $\text{Na}_2\text{S}$ ) at a temperature of 160°C or less (Calvo-Flores et al. 2015; Van den Bosch et al. 2018). The absence of a strong nucleophile makes this process less efficient. Thus, herbaceous fibers are used for soda pulping because of their more accessible structure, lower lignin content and higher amount of unstable ester linkages. (Van den Bosch et al. 2018) Raw materials like sugarcane bagasse, straw and flax are still notable sources for paper in many countries (Calvo-Flores et al. 2015).

Lignin extraction is performed by lowering the pH until precipitation is possible. The formed lignin is called soda lignin and it is finally isolated by filtration or decantation.

(Calvo-Flores et al. 2015) The absence of a strong nucleophile also leads to a less efficient cleavage of  $\beta$ -O-4 bonds and causes similar repolymerization reactions as in the formation of kraft lignin. The advantage of soda pulping is the production of a more valuable sulfur-free lignin which avoids catalyst poisoning and reduces bad odors in downstream processing of lignin. (Van den Bosch et al. 2018)

### 3.4 Organosolv Lignin

Organosolv pulping uses organic solvents for the delignification of wood (Calvo-Flores et al. 2015). Generally, biomass is treated with a mixture of water, organic solvents and a catalyst at 140°C to 200°C (Ragauskas et al. 2014). A wide range of organic solvents such as alcohols, cyclic ethers, organic acids and ketones are used in combination with water or acids in the organosolv treatment (Van den Bosch et al. 2018). After this, the organosolv lignin may be precipitated and recovered from the concentrated liquor. (Ragauskas et al. 2014)

There are many different methods for organosolv pulping including co-solvent enhanced lignocellulosic fractionation (CELf) pretreatment (Yoo and Ragauskas 2021), Alcell, Acetosolv, Batelle and Organocell (Calvo-Flores et al. 2015). One popular type of organosolv lignin is ethanol organosolv lignin, the characteristics of which can be found in table 1 (Yoo and Ragauskas 2021).

Organosolv treatment for delignification is a great strategy for increasing the solubility of lignin in the processing liquor. This minimizes the redeposition of lignin onto cellulose fibers. (Van den Bosch et al. 2018) The preservation of the original functionalities like the  $\beta$ -O-4 bonds depend on the used solvents and processes. Usually, the final lignin structure is either extensively degraded or partially preserved. (Van den Bosch et al. 2018)

Organosolv lignins typically possess a low molecular weight, high purity and recovery yield (Van den Bosch et al. 2018; Yoo and Ragauskas 2021). In addition, these lignins have limited carbohydrate contamination, are sulfur-free and rich in functionality (Ragauskas et al. 2014). Organic solvents limit the presence of condensation reactions which makes the structure of this lignin more similar to native lignin (Duval and Lawoko 2014). Therefore, this high-quality lignin has generated interest for higher value applications such as polymers (Calvo-Flores et al. 2015; Duval and Lawoko 2014). On the other hand, organic solvents are expensive and the produced pulp possesses the lowest quality compared to kraft and soda pulps (Duval and Lawoko 2014).

Table 1 summarizes the characteristics of mentioned lignin types. Lignin recovery rates are high from all the discussed sources. The purity of lignin can be measured by investigating the amounts of other substances such as inorganic salts, metals or sulfur compared to the amount of lignin. Lignosulfonates are not as pure as the other lignin types. Lignosulfonates also exhibit the largest range of molecular weights of extracted lignin. Generally, the amounts of preserved  $\beta$ -O-4 linkages is relatively low in all lignin types.

**Table 1:** Lignin recovery, purity and molecular weight range for different types of lignin. Based on (Gabriel et al. 2017; Hemmilä et al. 2020; Patil et al. 2020; Yoo and Ragauskas 2021).

Lignin type	Lignin recovery	Purity	Molecular weight range (g/mol)	Amount of $\beta$ -O-4 linkages
Kraft Lignin	High	High (>90%)	2 000 – 7 000	Low
Lignosulfonates	High	Low – Medium	1 000 – 150 000	Low
Soda Lignin	High	High (~93%)	1 000 – 3 000	Low
Ethanol Organosolv Lignin	High	High	1 000 – 4 000	Low – Medium

Lignin is also available through other processes. For example, biorefineries produce substantial amounts of residual lignin while converting cellulosic biomass into liquid transportation fuels (Radhika et al. 2022; Ragauskas et al. 2014). Most biorefineries contain the following processes: pretreatments, enzymatic hydrolysis and sugar fermentation into fuels. In biorefineries, it is important to consider whether lignin is extracted at the end of processing or before enzymatic hydrolysis and fermentation. Lignin last means that the plant carbohydrates are extracted first leaving most of the lignin in solid residue. This lignin can be used for low-value markets. In order to produce higher value products, it needs additional purification because of the presence of enzymes and fermentation components. In the lignin first approach, pretreatments are applied to extract the lignin, before extracting the carbohydrates. This can modify structural features of lignin, but sometimes this is an advantage. Prerecovery of lignin can be more expensive compared to the lignin last approach but higher value products are more feasible through this approach. (Ragauskas et al. 2014)

## 4. CHALLENGES IN LIGNIN UTILIZATION

The utilization of lignin is not straightforward. Instead, there are multiple challenges that need to be considered in order to convert lignin into value-added products successfully and efficiently. The main challenges are the following: lignin reactivity, poor solubility in water, repolymerization after depolymerization and toxicity against microorganisms. The diverse and heterogenous structure of lignin is a general challenge. Different treatments prior to further processing of lignin can improve lignin characteristics related to the mentioned challenges. The use of e.g. ionic liquids, water or acid as pretreatments modify and produce other lignin characteristics that are necessary for further processing and applications (Rath et al. 2024). Some challenges can be addressed by modifying microorganisms through synthetic biology. This chapter discusses prevailing challenges in lignin utilization and presents solutions for some of the challenges.

The chemical reactivity of lignin is essential because further applications involve chemical processes. The  $\beta$ -O-4 linkages are relatively stable. Chemical reactivity of  $\beta$ -O-4 bonds is affected by the position and number of functional groups in the aromatic rings and side chains. (Yoo and Ragauskas 2021) In isolated lignin, a reduced content of  $\beta$ -O-4 bonds is linked to a weakened lignin reactivity. This stems from the condensed structure of lignin, when it is deficient in  $\beta$ -O-4 bonds and carbon-carbon bonds are retained (Salvachúa et al. 2016; Van den Bosch et al. 2018). As seen in table 1, the major industrial fractionation methods cause cleavage of  $\beta$ -O-4 bonds, which reduces lignin reactivity. Therefore, a lower phenolic monomer yield upon depolymerization might be expected if no pretreatments are applied. (Van den Bosch et al. 2018)

Lignin is often considered as a by-product from cellulose-centered biorefinery processes. Therefore, the fractionated lignin stream contains process solvents and chemicals as well as other decomposed contents like extractives and inorganic components. (Yoo and Ragauskas 2021) Such substances, e.g. sulfur, acetate and sodium hydroxide, are toxic for microorganisms. Additionally, recovered and pretreated lignin streams contain crude mixtures of different aromatics of which many also exhibit toxicity in microorganisms when these are to be used as carbon substrates. There are two options for solving this issue: either technical lignin is engineered to be non-toxic for microorganisms or the stress tolerance of microorganisms is tailored to endure lignins. (Becker and Wittmann 2019)

The solubility of lignin is a major issue in further processing. The high molecular weight and branched structure of lignin make it poorly soluble in process media (Hämäläinen et al. 2018; Yoo and Ragauskas 2021). The heterogeneity of lignin streams enhances this issue. For example, isolated kraft lignin appears to contain a high molecular weight fraction which is insoluble in acetone as well as a lower molecular weight fraction that is soluble in acetone (Argyropoulos et al. 2023). The content of hydroxyl groups to some extent determines the hydrophilicity of lignin. Modifying lignin in a manner that increases hydroxyl groups improves the solubility of lignin in aqueous solutions. (Yoo and Ragauskas 2021)

Another factor to consider in lignin solubility is the pH of the solution. Laccases are fungal enzymes that are utilized for the depolymerization of lignin. Laccases mostly function in acidic pH aqueous solutions in which technical lignins are hardly soluble. (Hämäläinen et al. 2018) Most other enzymes also function in moderate or acidic environments (Becker and Wittmann 2019). On the other hand, lignin can be efficiently solubilized in water with alkaline pH. As a solution, organisms can be metabolically engineered through synthetic biology to extend the pH range of enzymes to function in more alkaline conditions. (Hämäläinen et al. 2018)

A further challenge is that most oxidative enzymes employed for lignin depolymerization also have the capability to polymerize aromatic compounds (Salvachúa et al. 2016). This means that depolymerized degradation products may be repolymerized which counters the purpose of depolymerization (Hämäläinen et al. 2018). A solution is to integrate different lignin-degrading enzymes into a synergistic system that prevents the repolymerization of degraded lignin monomers (Li et al. 2022).

There are still other challenges to be solved in lignin valorization. Many of those can be solved through synthetic biology. Examples of synthetic biology modifications are mentioned in the upcoming sections. Novel issues are faced when considering the upscaling of lignin utilization processes. Maintaining a decent efficiency while scaling up the production is discussed in chapter 7.

## 5. LIGNIN DEPOLYMERIZATION METHODS

In nature, lignin depolymerization is a complex process that typically involves many microorganisms including yeasts, fungi and bacteria. These organisms provide various enzymes that contribute to different metabolic pathways for degrading lignin. (Nguyen et al. 2021) The depolymerization of lignin is a critical element for upgrading lignin (Becker and Wittmann 2019).

So far, microbial depolymerization processes demonstrate insufficient productivities and yields than those required by the industry. Thermal and chemical processing for lignin depolymerization can be beneficial due to rapid processing times and high yields. (Nguyen et al. 2021) However, catalyst selectivity is a major challenge in these depolymerization methods (Becker and Wittmann 2019). Also, thermochemical methods require severe reaction conditions and a large amount of energy (Chio et al. 2019). Lignin depolymerization using microbes and enzymes occurs more selectively, under milder conditions, in a more environmentally friendly manner and with less energy intensity, making the biological approach attractive (Becker and Wittmann 2019; Hämäläinen et al. 2018; Liu et al. 2024). Engineering microbes through synthetic biology might increase the efficiency of biological depolymerization.

The target of lignin depolymerization is to cleave inter-unit linkages in a manner that the heterogeneous lignin molecules are converted into relatively uniform monomers (Liu et al. 2024). These low-molecular-weight mono- or oligolignols function as bioavailable carbon for microbial hosts in further production. Different thermal, chemical and biological methods can produce phenolic monomers, including vanillin, guaiacol and catechol, and these may be utilized for a variety of applications. (Becker and Wittmann 2019)

### 5.1 Thermal and Chemical Depolymerization

Thermochemical treatments are applied for the depolymerization of lignin since increasing the temperature is a common and effective method for cleaving chemical bonds. Lignin can be thermally treated with or without catalysts. (Chio et al. 2019) The following methods and the depolymerized products are reviewed: pyrolysis, gasification, microwave-assisted depolymerization, oxidative depolymerization and reductive depolymerization.

Pyrolysis is a common thermochemical method for depolymerizing lignin. The advantages of this method are its low cost and the simplicity as well as the robustness of

the process. Disadvantages of pyrolysis include low product selectivity and separation efficiency. Pyrolysis is often carried out in a range of 400–800°C (Nguyen et al. 2021) because  $\beta$ -O-4 and  $\alpha$ -O-4 bonds break at around 200–400°C and carbon-carbon bonds break in temperatures higher than 400°C (Chettri et al. 2023). Pyrolysis takes place in limited amounts or in the absence of oxygen (Chettri et al. 2023; Nguyen et al. 2021). In these conditions, lignin is converted mainly into vanillin, syringol, 4-methylguaiacol, coniferyl alcohol and some unsaturated alkyls. Char is a dominant product in pyrolysis but its amount can be suppressed by high heating rates and higher temperatures of around 700°C (Patwardhan et al. 2011).

Gasification aims at producing synthesis gas (syngas) from carbon-containing material at high temperatures of 600–800°C in the presence of oxidizing agents like steam, oxygen or carbon dioxide (Argyropoulos et al. 2023; Nguyen et al. 2021). Syngas can be upgraded into varying fuels and platform chemicals using commercial petrochemical processes (Argyropoulos et al. 2023). A major challenge in lignin gasification is the condensation of aromatic compounds which leads to char products like coke and tar and this results in poor syngas yields (Argyropoulos et al. 2023; Nguyen et al. 2021). As lignin gasification methods are being developed, hydrogen, hydrocarbons, methane and methanol are among the expected products (Argyropoulos et al. 2023).

Lignin depolymerization can be assisted by microwave irradiation. The electromagnetic radiation causes rotatory movement of polar molecules. Eventually, an immense amount of heat is generated, and this breaks lignin polymers into monomers and oligomers. The products depend on the exposure time and intensity of the microwaves. (Chettri et al. 2023) Using microwaves is economical, energy-efficient and it reduces reaction times (Chio et al. 2019; Roy et al. 2021). However, microwave heating is non-uniform and it has a low diffusion rate in bulk, which restrict commercial utilization (Roy et al. 2021). Microwaves may also be utilized for heating in pyrolysis or other thermochemical depolymerization methods. Char formation in pyrolysis may be suppressed using microwaves as a pretreatment (Duan et al. 2018). Microwave-assisted pyrolysis of lignin can produce e.g. the following valuable compounds: syringol, phenol, guaiacol and catechol (Roy et al. 2021).

There are many approaches for oxidative depolymerization of lignin. Since oxidative methods are already widely used in the pulp and paper industry for pulp bleaching, can oxidative lignin depolymerization become an important and economical technology. (Sun et al. 2018) In oxidative lignin depolymerization, an oxidant is employed to transform the lignin polymer into oxygenated compounds of low molecular weight. Oxidants enable the

selective cleavage of both ether and carbon-carbon bonds. (Van den Bosch et al. 2018) For example, oxygen, hydrogen peroxide, peroxyacids and metal oxides are employed as oxidants for lignin depolymerization (Roy et al. 2021; Sun et al. 2018; Van den Bosch et al. 2018). Ideally, oxidative techniques aim for efficient depolymerization under mild conditions, directly converting lignin into fine chemicals containing aldehyde, alcohol, ketone or carboxylic acid components (Sun et al. 2018; Van den Bosch et al. 2018).

The production of vanillin, a flavoring component, is a well-known application of oxidative lignin depolymerization. The Norwegian company Borregaard produces vanillin at a commercial level via oxidation of liginosulfonates from sulfite liquor. (Roy et al. 2021; Van den Bosch et al. 2018) Also novel strategies for oxidative lignin depolymerization have been developed, including photocatalysis (Chen et al. 2021), electrochemistry (Luo and Liu 2023) and the implementation of ionic liquids (Szalaty et al. 2020) or heterogenous catalysts (Behling et al. 2016).

Reductive approaches are also capable of lignin depolymerization. Reductive depolymerization is generally performed using hydrogen gas or a hydrogen-donating compound in the presence of a redox catalyst (Roy et al. 2021; Sun et al. 2018). For example, noble metals and base metals can act as redox catalysts. Also acid and base catalysts are utilized in both reductive and oxidative lignin depolymerization. (Van den Bosch et al. 2018) Hydrogenation and hydrodeoxygenation are the generally occurring reductive reactions and these can provide clean mixtures of alkanes targeted for the production of bio-oils and fuels (Sun et al. 2018). Depolymerization is achieved mainly through the cleavage of ether bonds, but carbon-carbon bonds are not cleaved like in oxidative depolymerization (Van den Bosch et al. 2018). However, the redox catalyst prevents any further repolymerization by stabilizing reactive sites. Products from reductive depolymerization also include benzene, xylene, toluene and phenol compounds. (Roy et al. 2021)

The mentioned thermochemical depolymerization methods are only a few of the existing depolymerization methods. Other much researched depolymerization methods include electrochemical depolymerization, acid or base catalyzed depolymerization, photocatalytic depolymerization and hydro- or solvothermal liquefaction (Chettri et al. 2023; Roy et al. 2021; Van den Bosch et al. 2018). Research is aiming towards improving these methods but also novel thermochemical depolymerization methods are being developed.

## 5.2 Biological Depolymerization

The natural decomposition of lignin is mainly carried out by fungi and some bacteria. White-rot fungi depolymerize lignin the most efficiently in nature. (Li et al. 2022; Yaguchi

et al. 2021) These fungi possess two types of enzymes that enable the breakage of different inter-unit linkages connecting the G, H and S lignin monomers: laccases and peroxidases (Liu et al. 2024; Roy et al. 2021; Yaguchi et al. 2021). In addition, various other types of enzymes contribute to lignin depolymerization (Li et al. 2021).

A hypothesis on how lignin degradation works in nature is that white rot fungi and bacteria secrete extracellular oxidative enzymes that produce a pool of available aromatic carbon. Other microbes have evolved pathways to utilize these compounds as energy and carbon sources. This chain of events could prevent the repolymerization of depolymerized low-molecular weight aromatic compounds. It remains somewhat unclear, how these enzymes work together to depolymerize lignin. This uncertainty acts as another reason why the employment of lignin-degrading enzymes for industrial lignin depolymerization is still limited. (Salvachúa et al. 2016)

Lignin-degrading enzymes are a group of biocatalysts that comprise lignin-degrading modifying enzymes (LDMEs) and lignin-degrading auxiliary enzymes (LDAEs). (Li et al. 2021; Liu et al. 2024) Laccases and peroxidases are LDMEs and they directly depolymerize lignin by oxidizing the phenolic group in lignin. This generates free radicals that cleave various linkages and finally cause lignin depolymerization. (Li et al. 2021; Roy et al. 2021) Laccases use molecular oxygen ( $O_2$ ) as electron acceptors and produce water as the only by-product (Hämäläinen et al. 2018; Roy et al. 2021). Mediators are small compounds e.g. vanillin or p-coumarate and they enable laccases to also degrade non-phenolic compounds (Becker and Wittmann 2019; Chio et al. 2019; Roy et al. 2021). Peroxidases require hydrogen peroxide ( $H_2O_2$ ) as electron acceptors (Roy et al. 2021).

LDAEs cannot directly depolymerize lignin but they are essential for microorganisms to complete lignin degradation. These enzymes participate in lignin degradation by oxidizing small molecular compounds and generating hydrogen peroxide ( $H_2O_2$ ). As mentioned, hydrogen peroxide is a necessary cosubstrate for peroxidases to oxidize lignin. Also, hydrogen peroxide may be reduced by ferrous iron to generate strong hydroxyl radicals that trigger lignin depolymerization. Essentially, lignin depolymerization is more efficient when LDMEs and LDAEs are coordinately expressed because the reaction requires sequential electron transferring. (Li et al. 2022) Table 2 compiles the most important enzymes, their type and functions in lignin depolymerization.

**Table 2:** Major lignin-degrading enzymes, their type and functions in lignin depolymerization. Modified from (Liu et al. 2024).

Enzymes	Type	Functions in lignin degradation
Lignin peroxidase (LiP)	LDME	Oxidation of phenolic and non-phenolic structures in lignin with presence of H <sub>2</sub> O <sub>2</sub> .
Manganese peroxidase (MnP)	LDME	Requires manganese for lignin degradation in the form of H <sub>2</sub> O <sub>2</sub> Mn(II).
Versatile peroxidase (VP)	LDME	Works through typical characteristics of both LiP and MnP.
Dye-decoloring peroxidase (DyP)	LDME	Requires H <sub>2</sub> O <sub>2</sub> for lignin degradation.
Laccase	LDME	Uses oxygen as final electron acceptor. Mediators are required for oxidizing non-phenolic compounds.
Glyoxal oxidase (GLOX)	LDAE	Provides H <sub>2</sub> O <sub>2</sub> for peroxidases.
Aryl alcohol oxidase (AAO)	LDAE	Provides H <sub>2</sub> O <sub>2</sub> for peroxidases.

As mentioned in chapter 4, recovered technical lignin is a mixture of different aromatics of which many are toxic. In addition, technical lignins may exhibit extreme pH values and contain inhibiting substances such as sulfite. Consequently, the microbial depolymerization of technical lignins likely requires stress tolerance engineering, extending of the substrate spectrums and pathway regulation e.g. of aromatic catabolism. (Becker and Wittmann 2019)

Metabolic engineering is applied to prime microbes for lignin valorization. Synthetic biology has a major role in executing modifications that improve lignin utilization. Gene expression may be engineered with the help of e.g. libraries of ribosomal binding sites and synthetic promoters. Genetic modifications are faster executed through genome editing e.g. DNA synthesis and assembly. Lastly, an important approach is the implementation of synthetic pathways. This entails the design and engineering of novel enzymes with desired features. For example, hybrid enzyme variants may be engineered to have preferred substrate and cofactor specificity as well as an improved tolerance. These enzymes may be highly efficient compared to unmodified enzymes. (Becker and Wittmann 2019)

Choosing an appropriate host for lignin degradation is vital. The ideal host should not only be genetically accessible and easily modifiable, but it should also inherently possess the necessary metabolic traits for substrate utilization and product synthesis. It is beneficial that the host is known to perform at industrial scale. Since lignin is an aromatic compound, it is essential that the host has catabolic pathways for aromatic degradation.

As discussed earlier, extracted lignin is often a toxic substrate. Therefore, a natural robustness towards toxic substrates is an important trait. (Becker and Wittmann 2019)

Bacteria generally degrade lignin less effectively than fungi and one reason for this is that there are fewer lignin degrading species in bacteria (Chio et al. 2019). On the other hand, bacterial systems are easier to genetically engineer (Yaguchi et al. 2021). An aromatic catabolism is ideal for lignin depolymerization and the widely used *Escherichia coli* does not possess it. Although, lignin utilization by *E. coli* could work through elaborate genetic engineering. (Becker and Wittmann 2019) For example, genetically engineering *E. coli* by combining *in vitro* maturation and chaperones has resulted in active bacterially expressed manganese peroxidase (MnP) (Alfi et al. 2019).

*Pseudomonas putida* and *Corynebacterium glutamicum* have highly desirable features for lignin degradation: versatile aromatic pathways, suitability for industrial-scale applications, high tolerance and robustness, fast growth and genetic accessibility. These bacteria are most suitable for applications that require a large volumetric production. In contrast, other natural aromatic degraders, such as *Sphingomonas*, *Rhodococcus* and *Amycolatopsis*, show promise in their tolerance and their ability to utilize substrates. But these might be recalcitrant towards genetic manipulation and cannot be applied for industrial-scale production. On the other hand, highly priced fine and specialty chemicals like flavors and fragrances may be produced by unconventional species instead of typical microbes. (Becker and Wittmann 2019) For example, fatty acid methyl esters (FAMEs) are naturally produced by *Amycolatopsis* and *Rhodococcus* and this could make an application for lignin utilization e.g. as biodiesel (Kosa and Ragauskas 2012; Salvachúa et al. 2015).

The expression of fungal enzymes in bacteria poses difficulties because bacteria lack necessary post-translational modifications, chaperones and cofactors for successful folding of proteins. Also, insufficient secretion mechanisms are an issue with bacteria. (Yaguchi et al. 2021)

Yeast is a unicellular fungus and it can serve as a better platform for lignin degradation compared to bacteria, as the aforementioned issues do not apply to yeast. Yeast has been used for engineering laccases and peroxidases to improve enzyme kinetics, protein performance and concentration in different reaction conditions. As disadvantages, yeast-derived enzymes have lower catalytic efficiencies than native fungal enzymes and lignin-degrading enzymes are particularly challenging to recombinantly express in yeast. (Yaguchi et al. 2021) Still, utilization of yeast has been determined to be suitable for the biological conversion of lignin into valuable chemical compounds (Putra et al. 2022).

*Pichia pastoris* is an example of yeast that has been engineered towards lignin utilization (Song et al. 2020).

Especially white rot fungi have higher depolymerization rates and conversion efficiencies compared to bacteria due to their versatile and efficient production of various extracellular oxidases. (Chen and Wan 2017; Chio et al. 2019; Yaguchi et al. 2021) However, fungi grow slowly, it is difficult to genetically modify fungi and many produce harmful secondary metabolites that complicate processes (Yaguchi et al. 2021; Yoo and Ragauskas 2021).

Nevertheless, fungi are studied and engineered towards lignin utilization. For example, the white-rot fungus *Pleurotus ostreatus* has removed roughly 71 % of lignin from black liquor (Wu et al. 2005) and another white-rot fungus *Phanerochaete chrysosporium* removed up to 99,2 % of lignin from bagasse effluent (Sharari et al. 2011). Certain fungi have the ability to simultaneously break down lignin and utilize it as a carbon source for synthesizing fatty acids (Chen and Wan 2017). The native activities and catalytic efficiencies of fungi, which exceed those of many bacterial and yeast systems, suggest a serious consideration in enhancing synthetic biology methods to tailor fungi for lignin utilization (Yaguchi et al. 2021). Table 3 summarizes all mentioned depolymerization methods including their advantages and disadvantages.

**Table 3:** A summary of mentioned lignin depolymerization methods, their products, advantages and disadvantages. The information is collected from several references. (Becker and Wittmann 2019; Chettri et al. 2023; Chio et al. 2019)

Method	Approach	Examples of products	Advantages	Disadvantages
Pyrolysis	Heating at 400–800 °C in the absence of O <sub>2</sub>	Vanillin, syringol, 4-methylguaiacol, coniferyl alcohol, some unsaturated alkyls	Low cost, rapid, easy to operate	Low selectivity, severe reaction conditions, char formation
Gasification	Production of syngas at temperatures of 600–800 °C	Hydrogen, hydrocarbons, methane and methanol	Syngas can be turned into fuels and chemicals	Condensation of aromatic compounds, char formation
Microwave-assisted	Heat is generated via electromagnetic radiation	Syringol, phenol, guaiacol, catechol	Precise control, avoids surface heating	High energy consumption, non-uniform, hard to upscale
Oxidative	An oxidant transforms lignin into oxidated low molecular weight compounds	Vanillin, chemicals that contain aldehyde, alcohol, ketone or carboxylic acid components	Relatively mild conditions, widely used in pulp and paper industry	High cost, not environmentally friendly, low selectivity
Reductive	Depolymerization in the presence of a hydrogen-donating compound and a redox catalyst	Alkanes targeted for the production of bio-oils and fuels, benzene, xylene, toluene and phenol compounds	Redox catalyst prevents repolymerization of degraded lignin	Carbon-carbon bonds are not cleaved
Biological depolymerization	Microbes secrete enzymes that are capable of lignin depolymerization	Ferulate, p-coumarate, guaiacol, vanillin, syringate, gallate, benzoate, phenol, cresol, caffeate	Environmentally friendly, ability to genetically modify for desired characteristics	Low efficiency, long-lasting, multiple organism may be required for complete degradation

The initial microbial degradation of lignin produces a diverse spectrum of varying monolignols such as phenol, vanillin, ferulate, guaiacol, p-coumarate and cresols as seen in table 3. Microbes and their enzymes can be utilized to further refine these products into ones of even higher value. Utilizing microbes for initial degradation of lignin increases its bioavailability for subsequent bioconversions. (Becker and Wittmann 2019) The biological conversion of degraded lignin products will be discussed in the next chapter.

## 6. BIOLOGICAL CONVERSION INTO VALUE-ADDED PRODUCTS

Whether thermochemical or biological methods are applied for the depolymerization of lignin, they all produce a mixture of various aromatic compounds. The separation and purification of desired products is challenging but important considering the commercialization of lignin-derived products. (Nguyen et al. 2021) Different methods, such as precipitation, crystallization and extraction, should be sequentially combined to successfully separate the compounds. This is complicated and expensive, which does not support the industrial application of lignin utilization. (Roy et al. 2021) Instead, the biological conversion of degradation products should be considered because of the efficiency of microbes that funnel various substrates toward a single product and adapt to toxic environmental conditions (Borchert et al. 2022; Nguyen et al. 2021). Biological conversion can be applied for degradation products from thermal, chemical and biological treatments.

Lignin depolymerization is executed by relatively specialized bacteria and fungi in order to break down the complex structure of lignin. After depolymerization, the structurally simple aromatics are released and various other microbes continue the biological conversion into value-added products. (Becker and Wittmann 2019) First, the heterogeneous stream of compounds is funneled towards a single product; this process is called biological funneling (Borchert et al. 2022) and its principle is graphically presented below in figure 2. The utilized microbes possess a network of catabolic pathways that funnel the mixture of aromatic compounds towards so-called central intermediates. (Becker and Wittmann 2019) These biological pathways are referred to as upper pathways (Li et al. 2022).

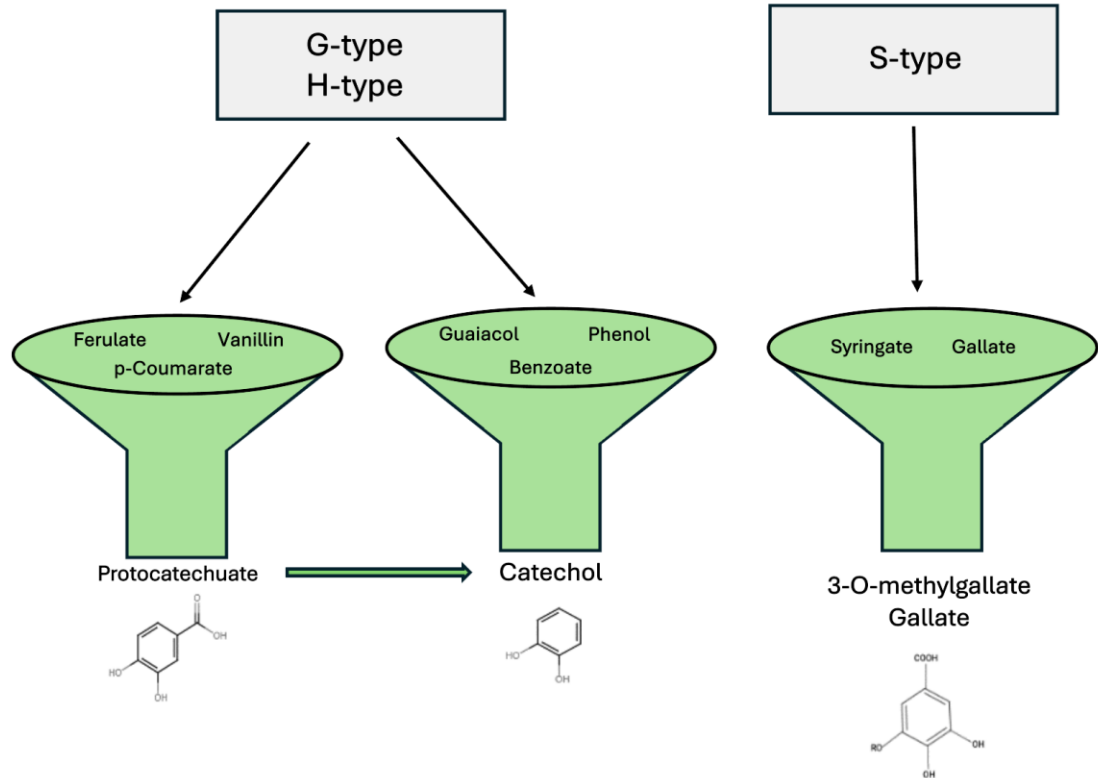


Figure 2: A schematic figure of biological funneling of degraded lignin. S-type lignin is funneled through a separate metabolic route. The green arrow indicates that protocatechuate can be turned into catechol through synthetic biology. Inspired from (Becker and Wittmann 2019).

Compounds derived from G- and H-type lignins include e.g. guaiacol, vanillin, ferulate, p-coumarate and benzoate. The aromatic compounds, that are derived from H-type and G-type lignins, are funneled into two central intermediates: catechol and protocatechuate, as seen in figure 2. (Becker and Wittmann 2019; Salvachúa et al. 2016) Depolymerized S-type lignin, generally obtained from hardwood, mainly converts into syringate. However, the funneling of syringate occurs separately via degradation into gallate and 3-O-methylgallate. (Becker and Wittmann 2019; Nguyen et al. 2021)

Some of the enzymes involved in degrading aromatics into catechol and protocatechuate are promiscuous i.e. responsible for the breakdown of many different aromatics. For example, in *Pseudomonas*, the enzymes acyl-CoA synthetase, aldehyde dehydrogenase and enoyl-CoA hydratase are shared for the degradation routes of caffeate, coumarate and ferulate. In addition, several microbes are capable of converting protocatechuate into catechol via the activity of protocatechuate decarboxylase, as indicated by the green arrow in figure 2. This useful feature can be transferred into other microbes through synthetic biology. Therefore, catechol is a central compound in the biological funneling of lignin-derived compounds. (Becker and Wittmann 2019)

Catechol and protocatechuate are further degraded by cleavage of their aromatic rings. The aromatic rings can be cleaved at ortho- or meta-positions and the different ring-cleavage procedures produce different metabolites. The  $\beta$ -keto adipate pathway is a major catabolic route and it relies on ortho-cleaving dioxygenases, while some microbes execute meta-cleaving in a different catabolic route. The  $\beta$ -keto adipate pathway produces a significant product from catechol, *cis,cis*-muconic acid (MA). (Becker and Wittmann 2019; Kohlstedt et al. 2018; Zhang et al. 2019) The relevance of MA in biotechnological applications is explained below.

Gallate and 3-O-methylgallate also experience ring-cleavage in their further catabolism. The central intermediate that forms from lignin-derived S-type compounds is 4-oxalomesaconate. (Becker and Wittmann 2019) The central intermediates that have formed from ring-cleavage processes then enter the central metabolism through different pathways in lignin-degrading microbes (Becker and Wittmann 2019; Zhang et al. 2019). The central metabolism produces compounds of biotechnological relevance. Central metabolites include pyruvate, acetyl CoA and oxaloacetate. (Becker and Wittmann 2019)

Aromatic compound metabolism can also be engineered through synthetic biology. For example, enzyme variants, alternative pathways and other novel findings are being developed in current research that aim towards a more efficient utilization of lignin. (Becker and Wittmann 2019) A variety of these are discussed in the next chapter.

MA is especially recognized for its industrial value. MA is a precursor of adipic and terephthalic acids, which act as important building blocks for commercial plastics such as nylon or polyethylene terephthalate (PET) polymers. (Kohlstedt et al. 2018; Zhang et al. 2019) As described above, MA is synthesized of so-called upper catabolic pathways that funnel into catechol as a central intermediate, which then undergoes ortho-cleavage into MA (Kohlstedt et al. 2018). *Pseudomonas putida* was earlier determined to be suitable for lignin depolymerization. This species also holds potential for the production of MA through fermentation (Kohlstedt et al. 2018, 2022).

Catechol is a highly toxic substance for microbes e.g. causing protein damage. Aromatic mixtures can also contain compounds that are toxic. Kohlstedt et al. have researched the impact of improving catechol tolerance, conversion efficiency and enhanced substrate spectrum in *P. putida* through metabolic engineering. The produced MA is applied for the production of nylon and PET in cascaded biochemical and chemical processes. (Kohlstedt et al. 2018, 2022)

Kohlstedt et al. were able to polymerize nylon from lignin for the first time. Nylon is produced from MA by hydrogenating to adipic acid and its methylated derivative. With production of nylon 6,6 in mind, *P. putida* KT2440 was engineered to contain a synthetic pathway that consists of both native catechol 1,2-dioxygenases under the same promoter. This modification provides a powerful engine for catechol conversion: catechol tolerance, catechol 1,2-dioxygenase levels and catechol conversion rates were increased. *P. putida* KT2440 was also engineered to express phenol hydroxylase genes from *P. putida* CF600. This alteration extends the substrate spectrum to cresols and enables the simultaneous conversion of catechol and phenol to MA. Production was scaled-up to produce kilograms of MA and it was successful with a purity of 97.9 %. Hydrothermally depolymerized softwood lignin was converted to MA with a yield of 13 g/L. This yield makes the engineered *P. putida* MA-9 strain a feasible host for technical scale production of nylon 6,6. (Kohlstedt et al. 2018)

Kohlstedt et al. are also responsible for producing the first PET out of lignin. This is significant because PET is currently the most commonly produced synthetic polyester. PET is chemically synthesized through five steps that produce the monomer terephthalic acid and comonomer diethyl glycol terephthalic acid. This study aimed to solve the challenge of a toxicity mechanism that enhances NADPH supply as a stress reaction and consequently sacrifices ATP generation. Thereby, the efficiency of MA production is hindered. (Kohlstedt et al. 2022)

*P. putida* EM42, which is a genome-reduced version of *P. putida* KT2440, is used as a template for this application. EM42 was engineered by deleting the genes of two isomerase enzymes. A similar modification to the previously mentioned study was performed: two native catechol 1,2-dioxygenases were placed under the same promoter. The modifications were successful, since the genome-reduced strains MA-10 and MA-11 enabled a more efficient pathway use under stress. A higher tolerance for a range of aromatics including catechol, cinnamates and phenolics, was exhibited as well. A striking titer of 74 g/L with a purity of 98,5 % was produced from MA-11. This yield is over five times greater than that of the MA-9 strain from the previous study. Another great advantage in MA-11 is the production of MA at a 30 % reduced glucose requirement compared to strain MA-6, which provides a remarkable economic advantage. Generally, the production of PET out of lignin is an attractive application but further catalytic optimization will make it even more suitable for industrial production. (Kohlstedt et al. 2022)

Direct bioconversion of lignin-derived aromatics is a promising approach for valorizing lignin. In a study, *P. putida* was engineered to directly convert funneled degradation products into  $\beta$ -ketoadipate since it has a native  $\beta$ -ketoadipate pathway.  $\beta$ -ketoadipate can

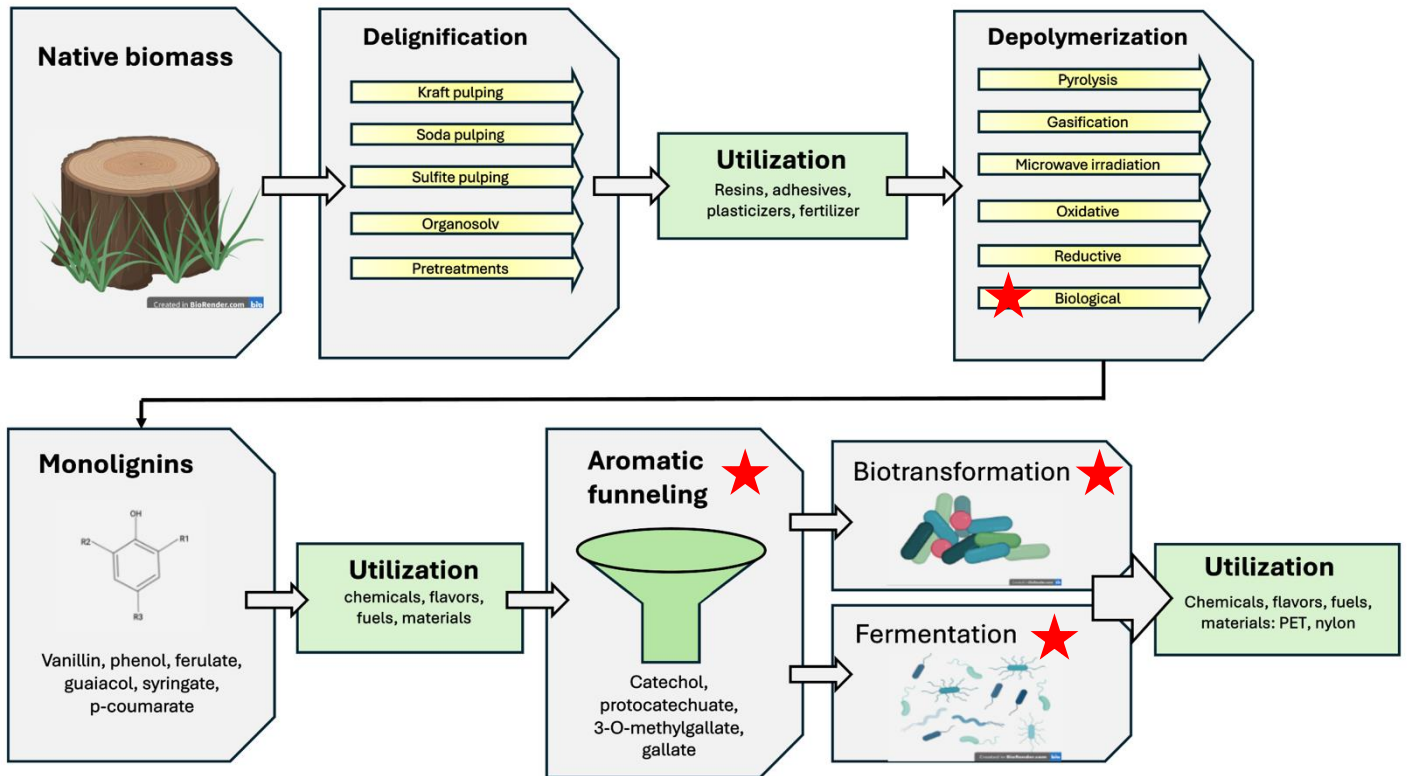
be polymerized into a nylon analog with a reduced water permeability compared to nylon from fossil resources. This lignin-based nylon analog is thus a performance-advantaged bioproduct. (Werner et al. 2023)

Another product that is obtained similarly via the  $\beta$ -ketoadipate pathway and direct bio-conversion is polyhydroxyalkanoate (PHA), which is formed from the intermediate acetyl-CoA (Salmela 2020). This PHA has features such as excellent biocompatibility and biodegradability (Becker and Wittmann 2019; Nguyen et al. 2021). Therefore, lignin-derived PHAs are suitable for many applications including packaging and tissue engineering (Becker and Wittmann 2019).

A wide selection of lignin-based biocomposites have already been developed. Composites comprise a polymer matrix and a reinforcing material that can be a synthetic or natural fiber. Generally, lignin-based biocomposites are eco-friendly, cost-effective and applicable in various industries. (Rath et al. 2024)

Lignin-based composites that have shown promise include fertilizers, plasticizers, varnishes, flame retardants (Wu et al. 2021), resins (Zhen et al. 2021) and adhesives (Gouveia et al. 2020) (Rath et al. 2024). These applications do not necessarily require thorough depolymerization of lignin. Instead, lignin is obtained after various delignification processes and a pretreatment suitable for the application is applied. (Becker and Wittmann 2019) For example, a lignin-epoxy composite resin with 50–60 % kraft lignin was produced. This environmentally friendly composite also had an improved thermal stability and uses little water and energy during manufacture. (Martin et al. 2022)

Lignin based composites may also be utilized as 3D printing materials (Sethupathy et al. 2022). 3D printing uses thermoplastics that are extruded through a hot nozzle into complex shapes with precise dimensions. A thermoplastic lignin composite outperformed common thermoplastics, like high-impact polystyrene (HIPS), in mechanical performance and 3D printability. In this study, 40–60 wt% of unmodified kraft and organosolv lignin were combined with nitrile-butadiene or nylon. Lignin-modified nylon exhibited enhanced tensile strength and stiffness in room temperature as well as a reduced viscosity in the melt – desirable features for efficient 3D printing. Thus, lignin based composites for 3D printing have superior mechanical and printing characteristics, are a greener alternative and generate additional revenue from waste streams. (Nguyen et al. 2018) AR-BOFORM® and Xylomer™ are lignin-based thermoplastics that already are commercially available (Sethupathy et al. 2022).



*Figure 3: Graphical summary of the full chain of events for lignin utilization. The red stars mark the processes that can be enhanced through synthetic biology. Inspired from (Becker and Wittmann 2019).*

The versatility of lignin-derived products does not end there. Lignin nanocomposites can have antibacterial and antioxidative properties and therefore they can be applied in food packaging or as a coating for materials (Sethupathy et al. 2022). Lignin can also be utilized in the medical sector in drug delivery, as wound dressings and they can even be turned into pharmaceuticals (Sun et al. 2018; Yu and Kim 2020). The enormous quantity of applications for lignin in different industries support the benefits of lignin valorization. Figure 3 summarizes the full chain of events of lignin utilization through biological conversion. It also mentions some applications and the points of the process chain where they can be realized. Red stars indicate the processes where synthetic biology may be utilized in order to increase lignin utilization efficiencies.

## 7. PROCESS EFFICIENCY AND UPSCALING

The feasibility of lignin valorization has been proven by many studies. However, industrial application requires a high efficiency and yield which still need to be addressed in lignin utilization. (Chen and Wan 2017) The entire valorization chain needs to be optimized for upscaling, starting with lignin extraction from biomass. Next, the depolymerization methods need to be refined for a better and more efficient yield. The biological conversion into value-added products should also be enhanced and this is mostly executed through synthetic biology.

Firstly, lignin should be efficiently extracted. Here it is important to consider that the extracted lignin should also be suitable for the further processing steps. If lignin is extracted from industrial processes e.g. kraft process, it is likely that pretreatments need to be applied since the characteristics of kraft lignin might be challenging in further chemical processes, as discussed in chapters 3 and 4. The application of pretreatments can be time-consuming and expensive but on the other hand, they allow the utilization of large industrial side streams. It is also important to consider, that increased process severities increase lignin yields but also decrease lignin reactivity – a balance between these two factors should be established (Van den Bosch et al. 2018).

Biorefineries as a source for lignin could be a more efficient option since their processing readily include a sort of pretreatment (Ragauskas et al. 2014). In the lignin first approach, the pretreatment might improve the structure of lignin and additionally no fermentation remains are included. Svensson et al. aimed to create a scalable process to purify lignin meanwhile producing ethanol at a bioethanol plant, using the lignin last approach. Lignin was separated by hydrolysis from fermented poplar wood rests. The wet fiber residue was turned into concentrated sugar solution by enzymatic hydrolysis and its fermentability was confirmed. The upscaling produced 128.6 kg of dry lignin. While the upscaling was successful, the yield was only at 36 % – processes still need to be improved before proper industrial scale production. (Svensson et al. 2020)

The extraction of lignin can also be enhanced by manipulation of plant genes to reduce cross-linking to other cell wall polymers. For example, genetically engineering plants to incorporate unnatural monomers with shortened side chains has reduced the degree of polymerization. (Ragauskas et al. 2014) Another way to engineer plants towards easier lignin extraction is to incorporate a gene that adds readily cleavable bonds into the backbone of the lignin polymer. These added ester linkages are called ‘zips’ and the lignin

that contains these is called 'zip-lignin'. (Ralph et al. 2019) Modifications to plant genes facilitate biomass processing and the depolymerization of lignin since the interunit linkages are more easily cleavable. These and other modifications to lignin monomer compositions can also reduce recalcitrance and facilitate a lignin structure that is easier to convert into value-added products. (Ragauskas et al. 2014) Overall, applying synthetic biology to manipulate plant genes can enhance lignin valorization significantly, but can be expensive and more research is still required.

Thermal and chemical depolymerization methods struggle with similar lignin condensation and repolymerization issues as biological methods. The following strategies are mentioned to alleviate these issues: isolation of native-like lignin with preserved reactivity for depolymerization or instantly converting lignin into stable monomers during lignocellulose processing. (Van den Bosch et al. 2018)

Synthetic biology is necessary for enhancing biological depolymerization. But first, a suitable host should be found. A few common hosts have been mentioned in chapter 5, but new lignin degraders are also identified for lignin valorization. Lignin-degrading enzymes might be discovered in research as well. It is essential to sequence microbes that possess lignin-degrading genes and to create libraries out of them. The information of new and already known lignin utilization genes can be utilized in synthetic biology e.g. while creating recombinant strains that are capable of several complicated metabolic tasks. (Chen and Wan 2017)

Avoiding the repolymerization during enzymatic depolymerization could be achieved for example by having a synergy between  $H_2O_2$ -generating enzymes and peroxidases. The enzymatic reaction efficiency is improved by a slow release of  $H_2O_2$  that keeps the peroxidases active. (Sutor et al. 2020) Another method for partially avoiding lignin depolymerization combined the expression of enzymes LiP and quinone reductase. This led to an increased yield of phenolic compounds from lignin degradation. (Chen and Wan 2017) The strategy of coordinating lignin depolymerization with downstream aromatic catabolism can improve the degradation efficiency by avoiding repolymerization since the processes occur in succession. This was demonstrated by an aromatic-degrading bacteria, *Rhodococcus opacus*, that was engineered to express laccase. In this study, lignin utilization for lipid production was significantly increased. (Levy-Booth et al. 2019)

The efficiency of biological depolymerization can be enhanced by expressing multiple lignin-degrading enzymes simultaneously. This was successfully exhibited for a com-

bined treatment of laccase and peroxidase (Lubbers et al. 2019). Also a combined expression of MnP and LiP significantly increased the depolymerization of lignin compared to MnP or LiP alone (Shin et al. 2019).

Preventing toxicity of the substrate can be achieved by creating inducible promoters that are activated by toxic substrates. This in turn activates a synthetic pathway that turns the toxic substrate into less toxic compounds. These kinds of inducible promoters provide flexibility in operating conditions and thereby increase the efficiency of lignin degradation. (Becker and Wittmann 2019)

Biological conversion into value-added products can also be enhanced through synthetic biology. A common bottleneck is the biological funneling capacity. Advanced synthetic biology should be applied to construct a super strain that integrates key pathways and enzymes from various microbes. (Li et al. 2022)

Avoiding the further degradation of already produced valuable products like vanillin or 4-hydroxybenzoic acid can be achieved by blocking the ring opening reaction (Chen and Wan 2017; Li et al. 2022). For example, the vanillin dehydrogenase gene was deleted in *R. jostii* and this resulted in an accumulation of vanillin (Sainsbury et al. 2013). A reduced energy consumption and a reduced carbon loss are additional benefits of this efficient approach (Li et al. 2022). Removing the complexity of aromatic pathway regulation is another promising strategy for using aromatics as substrates (Becker and Wittmann 2019).

Converting protocatechuate into catechol enhances MA production because MA is only produced from catechol. Since protocatechuate decarboxylase can convert protocatechuate into catechol, its gene can be expressed in organisms that do not naturally express it to enhance MA production. (Li et al. 2022)

It is known that the aforementioned *P. putida* KT2440 possesses considerable tolerance towards many substrates and inhibitors (Becker and Wittmann 2019). Its tolerance towards catechol was even further increased by expression of a synthetic gene. This was likely achieved through the accelerated degradation of catechol that made the cultivation environment detoxified. (Kohlstedt et al. 2018)

Adaptive evolution may also be utilized to achieve desired tolerance or other improvements. The benefit of adaptive evolution is that it mimics natural selection by fortifying genetic diversification in response to selective pressure. (Becker and Wittmann 2019) For example, overcoming the toxicity of ferulate, a lignin-derived compound, was achieved by using adaptive evolution to create a highly ferulate-tolerant strain of *Acineto-*

*bacter baylyi* ADP1. When a synthesis pathway was also added to this strain, *Acinetobacter baylyi* ADP1 was able to use ferulate as its sole carbon source for cell growth and product synthesis. (Luo et al. 2019)

An understudied approach that could potentially lead to industrial level production is the use of microbial consortia. This refers to communities of microorganisms that coexist and interact with each other, often in collaborative processes. Microbial consortia could have benefits like mutual interaction of different metabolic systems, broader metabolic flexibility and a better resistance towards environmental changes. Wang et al. demonstrated a successful microbial consortia that degraded aromatics (Wang et al. 2013).

It is important to remember that manipulation of catabolic pathways may also disable normal functions, which decreases lignin utilization efficiency (Chen and Wan 2017). For example, in *P. putida* KT2440 slow growth and inefficient use of substrate were shown in an engineered strain. The probable reason is the lack of downstream intermediates that would induce the regulation of upper pathways. (Johnson and Beckham 2015)

Biological functions and regulatory mechanisms of ligninolytic enzymes are still poorly understood which makes conducting research challenging. However, it is known that no single organism can utilize all lignin-derived aromatics efficiently, fungal manipulation is difficult and in biological conversion, the upper pathways of lignin utilization is the rate-limiting step. An in-depth comprehension of ligninolytic enzymes, including their regulatory mechanisms, enzymes, synergy, and interaction among different enzyme systems, is essential to guarantee extensive applications for lignin valorization. (Li et al. 2022) In the future, an industrial scale processing of lignin could benefit from a continuous process setup with reusable enzymes (Hämäläinen et al. 2018).

## 8. CONCLUSION

Lignin utilization includes the following parts: lignin source selection, lignin extraction from biomass, lignin depolymerization and the conversion into value-added products. This literature review has given insights on important features of all mentioned parts and a comprehension within the trending field of lignin utilization through the perspective of synthetic biology.

The aromatic structure of lignin is the key element that enables its utilization for higher-value applications such as chemicals, materials and pharmaceuticals. Yet, it is the structure of lignin that also complicates its utilization. The branched, heterogenous and cross-linked structure of lignin is difficult to break down due to the strength of abundant ether and carbon-carbon bonds.

Nevertheless, a plethora of thermal, chemical and biological methods have been developed towards the depolymerization of lignin. These need to be optimized since lignin reactivity, repolymerization, solubility and toxicity are still prevailing challenges. Fungi are naturally efficient lignin-degraders but are difficult to genetically modify which is required for increasing production. On the other hand, bacteria may lack features required for efficient lignin degradation but can be easily modified with desired features. This ability to engineer bacteria is the reason why they are often chosen for lignin depolymerization.

Biological conversion of degraded lignin products benefits from its ability to utilize mixtures of lignin-derived aromatics. Biological conversion usually involves biological funneling and the fermentation or biotransformation of funneled products. Bacteria are commonly used for biological conversion for the same reason as above, the ease of genetic modifications. As an example, nylon and PET were successfully produced in a bacterial strain of *P. putida*, that is also broadly utilized for lignin depolymerization. The yield of PET was impressive but insufficient for industrial scale production – unlike its purity of 98,5 %.

Synthetic biology is harnessed to overcome challenges and to enhance the yields of microbial production. For example, blocking ring-opening reactions can accumulate valuable compounds that do not require further degradation. A goal for lignin utilization, that requires advanced synthetic biology, is to develop a super strain capable of multiple complex metabolic pathways and that contains enzymes from different organisms.

The efficient biological valorization of lignin can only be realized through a deep understanding of microbial lignin metabolism and of the synthetic biology tools that are able to improve desired characteristics. Since lignin valorization is recently a common field of interest, great progress has been made towards lignin utilization. However, most studies are still in the proof-of-concept stage. An industrial scale production might require the integration of biological depolymerization and conversion processes. Nevertheless, ongoing research on biological degradation and conversion of lignin have unveiled exciting opportunities and a bright future towards the utilization of lignin.

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