Research review paper

Biological valorization of low molecular weight lignin

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Lignin is a major component of lignocellulosic biomass and as such, it is processed in enormous amounts in the pulp and paper industry worldwide. In such industry it mainly serves the purpose of a fuel to provide process steam and electricity, and to a minor extent to provide low grade heat for external purposes. Also from other biorefinery concepts, including 2nd generation ethanol, increasing amounts of lignin will be generated. Other uses for lignin – apart from fuel production – are of increasing interest not least in these new biorefinery concepts. These new uses can broadly be divided into application of the polymer as such, native or modified, or the use of lignin as a feedstock for the production of chemicals. The present review focuses on the latter and in particular the advances in the biological routes for chemicals production from lignin. Such a biological route will likely involve an initial depolymerization, which is followed by biological conversion of the obtained smaller lignin fragments. The conversion can be either a short catalytic conversion into desired chemicals, or a longer metabolic conversion. In this review, we give a brief summary of sources of lignin, methods of depolymerization, biological pathways for conversion of the lignin monomers and the analytical tools necessary for characterizing and evaluating key lignin attributes.

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1. Lignin – An introduction

1.1. Lignin in nature

Lignin is one of the main constituents of terrestrial plant biomass together with the carbohydrate polymers cellulose and hemicellulose. It is stated to be the second most abundant naturally occurring polymer on the planet, and it is by far the most important renewable source of aromatic compounds (Bozell et al., 2007). The amount of lignin formed annually in nature has been estimated to be in the range 5 to 36 × 10^8 tons (Gellerstedt and Henriksson, 2008). The lignin polymer is not found isolated in nature, but is strongly physically associated with hemicellulose and cellulose. The exact linkage structures in native biomass are not fully known. In technical processing of biomass, such as pulping, linkages are formed between the lignin and carbohydrates giving rise to a lignin carbohydrate complex (LCC) (Lawoko et al., 2005). The fraction of lignin varies widely between different types of biomass. The highest lignin fraction is typically found in softwood with a range of 25–32 wt% of dry matter, whereas the lignin content in hardwoods is slightly lower (18–25%) (Mutturi et al., 2014). The lignin content is even lower for straw and grasses, and lignin is almost completely missing in mosses and green algae (Vanholme et al., 2010). From an evolutionary perspective, the incorporation of lignin into the plant structure enabled the development of the tracheid cell type, and thereby a better transportation of water in the plant (Gellerstedt and Henriksson, 2008). This in turn made expansion of plants into dryer land possible. Lignin also has an important function as a protectant of the polysaccharides, since it is difficult to degrade.

Out of the three main polymers in plant, lignin has the most complex and heterogeneous composition and structure. The tridimensional polymer is built up from phenyl propanoid units which are substituted at various positions, linked by ether and C–C bonds. There are three basic building block structures abbreviated H (p-hydroxyphenyl), G (guaiacyl) and S (syringyl), differing in the number of methoxy groups on the aromatic ring; 0, 1 or 2, respectively. These structures can be represented in their alcohol form; namely as p-coumaryl, coniferyl, and sinapyl alcohols (Fig. 1). The primary lignin building blocks originate from the shikimate pathway (Higuchi, 1990). The initial step is the formation of cinnamic acid from phenylalanine, through the action of the enzyme phenylalanine lyase (PAL). Cinnamic acid is then further converted in a multistep process to the three basic structures. The polymer, in turn, is formed by radical coupling of the basic building blocks in a process involving enzymatically catalyzed oxidation. The initial step of this process is an oxidation of the phenol group of the monolignols, which gives rise to reactive radicals (Ralph et al., 2004). Since the monolignols are conjugated systems, mesomeric effects will give several ways of linking the building blocks together, gradually forming a lignin polymer. The relative proportions of the building blocks vary depending on the type of biomass. For instance, the coniferyl part (G) is completely dominant in softwoods (90–95%), whereas the proportion of sinapyl (S) (45–75%) is larger than the coniferyl part (25–50%) in hardwoods (Gellerstedt and Henriksson, 2008). Lignin in grasses contains significant amount of coumaryl (H) (5–35%), which is low in both softwoods and hardwoods. Lignin degradation in nature is slower than degradation of the carbohydrates, and lignin consequently constitutes a large part of the humic acid – the organic part of soil.

1.2. Technical lignin

Huge amounts of lignin are produced yearly in the pulp and paper industry as a co-product in the cooking process. Lignin is insoluble in water in its native state, and the purpose of the cooking process is to solubilize lignin and thereby separate it from the fiber fraction. The yearly amount of lignin produced in this manner can be estimated to be around 130 million tons (Rinaldi et al., 2016), most of which is directly used on-site. The Kraft cooking method is today by far the most common pulping method followed by sulfite-cooking (Sjöström, 1993). In particular for non-woody biomass, alkaline cooking with anthraquinone added, that is more selective towards lignin removal, is used to some extent (Hedjazi et al., 2009), and lignin may furthermore be removed using organic solvents, e.g. ethanol or methanol. A few different organosolv process concepts have been developed but these are of limited commercial significance for pulping at present (Viell et al., 2013), and the economic viability of the process needs to be clearly proven at pilot and demo scale (Michels and Wagemann, 2010).

The structure of the technical lignin is different from the native lignin and is furthermore dependent on the cooking method applied (Constant et al., 2016). As a result of the method used to obtain lignin, the abundances of different C–O and C–C linkages present in lignin will be substantially different from those existing for the native lignin (Abdelaziz and Hulteberg, 2016). This in turn affects the choices for further depolymerization. In the Kraft process, wood (normally softwood) is treated with an aqueous solution of NaOH and Na2S (white liquor) at a temperature range of 155–175 °C for several hours, giving OH– and HS– ions as active reactants. Aromatic ether bonds in the lignin structure are broken by the hydroxide and hydroxysulfide anions resulting in smaller water/alkali-soluble lignin fragments. These fragments, having a lower molecular mass, diffuse more rapidly into the cooking liquor – the black liquor. The mode of lignin removal is different in the sulfite-cooking process, which can take place under acidic, neutral, or even alkaline cooking conditions. Ether bonds are hydrolytically cleaved, after which sulfonations by the sulfite ions occur. The resulting lignosulfonates are highly water-soluble and dissolve in the cooking liquid (Adler, 1977). The sulfite process dominated the industry in the beginning of the 20th century, but has gradually been out-phased by the Kraft process, which gives stronger fibers and a more efficient recovery of
organosolv processes is currently very marginal, which means that the which are free of sulfur and hemicellulose, have an even quality, and are (will be rich in silicate. In many ways, the most attractive lignin is pro-
tage is that it is sulfur free. It is partially soluble in organic solvents,
be recooked in 20% NaOH (Hergert, 1998).
Some of the basic characteristics of lignins obtained from the
methods above are summarized in Table 1. The first two processes are large industrial processes, and large quantities of these lignins (i.e. Kraft lignin and lignosulfonates) are therefore potentially available. A drawback is that the lignins contain sulfur in both cases, and in addition some hemicellulose. The Kraft lignin has low water solubility, is high in phenolic contents and has a varying quality (Sjöstrom, 1993). The ligno-
sulfonates, on the other hand are polar and soluble in water, and their phenolic content is low. The molecular mass of lignosulfonates are higher than those of Kraft lignin, largely due to the incorporation of sul-
donate groups (Saake and Lehnen, 2007). The soda-AQ lignin has a rela-
tively high availability, is low in price, and has high aliphatic and aromatic functionality (Calvo-Flores et al., 2015). A significant advant-
age is that it is sulfur free. It is partially soluble in organic solvents, but contains hemicellulose. Its properties tend to vary somewhat, and since annual crops are the most common feedstocks, the resulting lignin will be rich in silicate. In many ways, the most attractive lignin is pro-
duced using organosolv processes (e.g. Alcell™ and Organocell™) (Abdelaziz et al., 2015; Nadif et al., 2002). These processes give lignins, which are free of sulfur and hemicellulose, have an even quality, and are soluble in organic solvents. However, industrial implementation of organosolv processes is currently very marginal, which means that the availability of these lignins is low and prices are high.

1.3. Lignin uses
Lignin has several different applications today (Table 2) and the market is slowly growing. However, the completely dominating use today is as fuel and only a few percent of the lignin produced in the industry is isolated for other purposes. The huge amounts of Kraft lignin produced yearly are used on-site for steam and electricity generation. When pulp is the primary product, the energy content in the plant will be higher than needed and surplus heat and electricity can there-
be used externally. However, this heat is instead needed for drying of pulp to paper if the plant is an integrated pulp and paper mill. Methods have been developed to gasify black liquor lignin into syngas, which can in turn be catalytically converted to e.g. methanol (Naqvi et al., 2012), but these have so far not reached commercial scale implement-
ation. Lignosulfonates produced from the sulfite process is the dominating source of lignin for other applications than fuel, with a produc-
tion of about 1 million tons per year (Strassberger et al., 2014). Lig-
osulfonates have an established market as additives in concrete, but are also used as binders, adhesive, or dispersants – in e.g. the agroindustry. In terms of production of chemical compounds, vanillin is a notable niche product. It is produced from softwood lignin, which is rich in guaiacyl units, in an oxidative alkaline chemical process (Pacek et al., 2013).
An extensive analysis on future potential lignin uses was made by researchers from the National Renewable Energy Laboratory (NREL) and the Pacific Northwest Laboratory (PNNL) resulting in the second volume of “Top-value added chemicals from biomass” (Bozell et al., 2007), where the first volume was the landmark study dealing with the carbohydrates (Wery et al., 2004). In the lignin report, the produc-
tion of a number of chemicals: fuels, macromolecules, syngas, hydrocar-
bons, phenols, and oxidized products are discussed. A central conclusion of the analysis of hurdles for production of smaller molecules, is the need for further development of technology to selectively break and make bonds, as well as better analysis and separation technology. These are topics to be discussed in this review, with a focus on lignin valorization using biological conversion, as outlined in Fig. 2.

Table 1
Comparison between lignin from different cooking processes (Calvo-Flores et al., 2015; Saake and Lehnen, 2007; Sjöstrom, 1993).

<table>
<thead>
<tr>
<th>Process</th>
<th>Availability</th>
<th>Price</th>
<th>Aliphatic/phenolic groups</th>
<th>Quality</th>
<th>Solubility in organic solvents</th>
<th>Sulfur content</th>
<th>Hemicellulose content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kraft</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>Varying</td>
<td>Insoluble</td>
<td>About 1 wt% (thiol groups)</td>
<td>High</td>
</tr>
<tr>
<td>Sulfité</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>Varying</td>
<td>Insoluble</td>
<td>5–6 wt% (sulfonate groups)</td>
<td>Some</td>
</tr>
<tr>
<td>Soda-Antraquinone</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Varying</td>
<td>Partial</td>
<td>None</td>
<td>Some</td>
</tr>
<tr>
<td>Alcell™</td>
<td>Low</td>
<td>High</td>
<td>Etherified</td>
<td>Constant</td>
<td>High</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Organocell™</td>
<td>Low</td>
<td>High</td>
<td>Etherified</td>
<td>Constant</td>
<td>High</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Fig. 1. The three primary monomeric building blocks of lignin (monolignols), showing also numbering of carbon atoms in the benzene ring and notation on the propylene side chain.
2. Depolymerization

A central process in the proposed strategy for lignin valorization (Fig. 2) is the breakdown of the lignin feedstock into compounds which can be further bioconverted by microorganisms. The depolymerization will need to yield low molecular weight lignins (mono- and oligomers), as only compounds of this size can serve as substrates for further cellular assimilation (see Section 3). Thorough methods for chemical analysis of the depolymerized lignin (see Section 4) will also be essential for a successful integration of the depolymerization and biocatalysis steps.

2.1. Bond breaking

The initial step allowing production of smaller molecules from lignin is depolymerization - a topic reviewed by e.g. (Amen-Chen et al., 2001; Brebu and Vasile, 2010; Huber et al., 2006; Ragauskas et al., 2014; Xu et al., 2014; Zakzeski et al., 2010). To obtain depolymerization of the lignin molecule, it is necessary to break the linkages between the phenyl propane units. The β-O-4 aryl glycerol ether bond is the dominant linkage type in native lignin. Typically, it constitutes > 50% of the bonding structures of a native lignin macromolecule in both softwood and hardwood lignins. Other major linkages comprise 5-5, β-5, α-O-4, β-β, β-1, 4-O-5, and dibenzodioxocin (Fig. 3). The functional groups in lignin, mainly methoxy and phenolic hydroxyl groups, have a significant effect on the molecule reactivity. Table 3 lists the different types of linkages as well as the functional groups common within a lignin macromolecule and their approximate proportions.

A classification of depolymerization methods is included in Fig. 2. In the following section, pyrolysis of lignin will be considered first, followed by catalytic cracking and hydrocracking. Thereafter the attention is turned to hydrogenolysis, with different types of catalysts, and hydrolysis of lignin – using subcritical or supercritical conditions. Finally, enzymatic depolymerization will be described.

2.2. Pyrolysis

Pyrolysis, the thermal degradation of an organic material at elevated temperature in inert environment, is a net endothermic reaction (He et al., 2006). There has been a strong interest in the pyrolysis of lignin, both for analytical purposes and for the production of fine chemicals and fuels. Lignin is a thermoplastic material and is rather recalcitrant towards thermal depolymerization (de Wild et al., 2012). Numerous studies have been performed for determining the temperature range and developing kinetic models for the decomposition of lignin (Cho et al., 2012; Montané et al., 2005; Sharma et al., 2004) and lignin model compounds (Chu et al., 2013). Kraft lignin, in particular, has been investigated within the field (Cahalilero et al., 1986; Fierro et al., 2005). Due to the natural variations in the lignin structure, the degradation of the various types is quite different. Ferdous et al. investigated the pyrolysis of Alcell and Kraft lignins in a fixed bed reactor and in a thermogravimetric analyzer using helium and nitrogen as carrier gas (Ferdous et al., 2002). The gaseous products obtained mainly consisted of H2, CO, CO2, CH4, and minor amounts of compounds with two or more carbon atoms (C2H4, C2H6, C3H6, C3H8, and traces of C4 compounds). Higher heating rates led to higher lignin conversion and larger syngas production for both Alcell and Kraft lignins. The kinetic parameters were highly dependent on the lignin substrate type, the lignin origin, and the equipment type adopted for pyrolysis reactions. In another example, Patwardhan et al. studied the pyrolysis of corn stover lignin using a micro-pyrolyzer coupled with a GC–MS/FID (Patwardhan et al., 2011). The pyrolysis resulted mainly in the formation of monomeric phenolic compounds, but the condensation of these vapors led further to other dimeric and oligomeric products. For example phenol, 4-vinyl phenol, 2-methoxy-4-vinyl phenol, and 2,6-dimethoxy phenol were reported as products. Zhang and coworkers employed Py-GC/MS and TGA/FTIR techniques in the non-catalytic/catalytic fast pyrolysis of several different types of lignin (Zhang et al., 2014b; Zhang et al., 2012). Approximately ten compounds were identified to account for almost 50% of the volatile products. Of the tested lignins, the Kraft lignin generated the least desirable pyrolysis products. The yields of volatiles were low, large amounts of char and in addition sulfur containing compounds were formed. In contrast, prairie cordgrass lignin potentially produced high quality of bio-oil and aspen lignin similarly gave high yields of bio-oil.

Toluene and p-Xylene were the most abundant hydrocarbons produced when micro-porous zeolite catalysts were used in the reaction. Catalytic fast pyrolysis of lignin is a promising approach where zeolite catalysts play a vital role in deoxygenating lignin-derived oxygenates (Yu et al., 2012).

Other recent reports on catalytic microwave-assisted pyrolysis over activated carbon state total phenolics yields of approximately 78% and hydrocarbon yields of 15% (Bu et al., 2014). The origin of phenolic oligomers during fast pyrolysis was investigated by Bai et al., and it was concluded that a significant fraction of phenolic oligomers available in bio-oil originates from repolymerization of smaller phenolic compounds formed during the pyrolytic depolymerization of lignin (Bai et al., 2014). In conclusion, pyrolysis may provide a route to obtain monomeric/oligomeric phenolic compounds, but further work on understanding the kinetics to enable process design to maximize yields of desired chemical products is needed. Clearly, the lignin origin plays a key role in the resultant pyrolysis products obtained.

2.3. Cracking

Cracking of hydrocarbons is a common unit operation in petroleum refineries, and contributes with somewhere between 20% and 50% of all gasoline produced. The cracking can be performed with hydrogen (hydrocracking) or without hydrogen. Cracking of lignin is differentiated in this context from pyrolysis of lignin by the presence of a heterogeneous catalyst. Corma and Huber discussed the catalytic cracking of lignin and stated that the conversion of this fuel is particularly challenging, as it contains stable aromatic structures (Huber and Corma, 2007). Indeed phenols, with similar structure to lignin produce large amounts of coke on the catalysts. Catalytic cracking with zeolite catalysts has been tested for lignin upgrading (Adjaye and Bakhshi, 1995). For example, Thring et al. performed cracking of lignin using the zeolite catalyst HZSM-5 (Thring et al., 2000), and obtained both liquid and light products.

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Table 2

<table>
<thead>
<tr>
<th>Principal use</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuel</td>
<td>Fuel in recovery boiler in Kraft processes</td>
<td>Naqvi et al. (2012)</td>
</tr>
<tr>
<td>Concrete additive</td>
<td>Lignosulfonates have a plasticizing effect, and are used to give an increased porosity of concrete. Typical dosages are 0.1–0.3% by weight of cement.</td>
<td>Plank (2004)</td>
</tr>
<tr>
<td>Dispersant</td>
<td>For powder preparation in e.g. agriculture</td>
<td>Doherty et al. (2011)</td>
</tr>
<tr>
<td>Animal feed additive</td>
<td>Lignosulfonates can be used in the production of feed pellets/blocks</td>
<td>Stewart (2008)</td>
</tr>
<tr>
<td>Resins and adhesives</td>
<td>Lignin can be used to partly replace phenols in various types of adhesives and resins</td>
<td>Pacek et al. (2013)</td>
</tr>
<tr>
<td>Vanillin production</td>
<td>Vanillin can be obtained through chemical processing of softwood lignin from sulfite pulping</td>
<td>Naqvi et al. (2012)</td>
</tr>
<tr>
<td>Syngas</td>
<td>Gasification of lignin recovered from Kraft processing can give synthesis gas. This process has not gained commercial success yet.</td>
<td>Naqvi et al. (2012)</td>
</tr>
</tbody>
</table>

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Fig. 2. Outline of the suggested process for valorization of lignin to chemicals.

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hydrocarbon gases directly in a fixed bed reactor in the temperature range 500–650 °C. The attained conversion values were high and ranged between 50% and 85%. More recently, Prado et al. suggested lignin as a good candidate for photocatalytic cracking because of the presence of hydroxyl groups (Prado et al., 2013a). In their study, the photocatalytic cracking reaction involved titanium oxide as a heterogeneous catalyst. High yields of lignin degradation compounds were obtained, with the main products syringaldehyde, pyrocatechol, and raspberry ketone. Furthermore, a two-step process incorporating depolymerization and catalytic cracking without the addition of hydrogen was conducted by Yoshikawa et al. in order to produce phenolic compounds from lignin over an iron oxide catalyst (Yoshikawa et al., 2014). It was concluded that the methoxyphenols and catechol in the lignin-derived slurry were selectively converted to phenols, cresols, and other alkyl phenols.

The major work in the field of hydrocracking of lignin has been done with the purpose of producing gasoline. To this end, the US NREL has developed and patented a process for converting lignin to motor fuel (Miller et al., 1999; Montague, 2003; Shabtai et al., 1999). The process includes a base-catalyzed lignin depolymerization using NaOH and methanol or ethanol as solvent at 593 K and 12 MPa. The liquid product is separated from the solids and neutralized (using H₂SO₄), after which the lignin is extracted using toluene. The toluene is further separated from the lignin and is hydrotreated in a two-reactor system, in which the first is a hydrodeoxygenation reaction (see Section 2.4) and the second is a hydrocracker.

2.4. Hydrogenolysis

Hydrogenolysis in general means decomposition in the presence of hydrogen, and in the specific context here it means a (reductive) depolymerization of the lignin molecule into smaller fragments, oligomers or monomers. Such hydrogenolysis of lignin is performed at severe conditions and is typically carried out using catalysts such as Pd or Pt. The process involves the breaking of the aromatic rings and the removal of oxygen-containing groups, resulting in a mixture of aliphatic and aromatic compounds. These compounds can then be further processed to produce fuels or other valuable chemicals.
about 50%. This work contributes in understanding the behavior of the
conditions, i.e., high temperatures and pressures. The cleavage of func-
tional groups may cause complete rearrangement of the carbon back-
bone leading to formation of 30–50 wt% char and release of volatile products (Brebu and Vasile, 2010). However, the presence of hydrogen can terminate the formed radicals in the carbon framework, suppress the char formation and promote the depolymerization into smaller frag-
ments and monomers.

The most investigated class of heterogeneous catalysts for lignin
hydrogenolysis is the hydrodeoxygenation catalysts, both for actual lignin (Harris et al., 1938; Meier et al., 1994; Domsa et al., 1993; Shabtai et al., 1999; Yan et al., 2008) and lignin model-compounds (Bredenberg et al., 1982; Bunch and Ozkan, 2002; De la Puente et al., 1999; Ferrari et al., 2002; Laurent and Delmon, 1994; Pepper and Furimsky (2000)) and lignin model-compounds (Furimsky (2000)). High partial pressure of hydrogen suppresses char for-
mation and promotes depolymerization. During thermoysis of peat, large volume of gas (mainly CO2) is evolved which dilutes the added hy-
drogen and increases the total pressure. This also probably applies to lignin, since a large gas volume is released during heating. It should be
mentioned that some of the hydrogen donor solutions are operating at super-critical conditions, but are sorted under this section due to the hy-
drogen donating nature of the reaction.

2.5. Hydrolysis

The hydrolysis reactions of lignin with water, often in the presence of a catalyst, have been investigated by several researchers. This
reaction may be performed using sub- or super-critical water, where the lat-
ter will be dealt with in a later section.

Karagoz et al. used the carbonate salt of Rb and Cs for producing phe-
nol by hydro-liquefaction of wood biomass (Karagoz et al., 2004). In a treatment at 553 K for 15 min, in which these salts were added at a con-
centration of 1 M, mainly phenolic and benzenoid derivatives were
obtained. In the thermal process without the catalysts, the product dis-
tribution markedly changed, giving as main products 4-methyl-phenol, 2-furan-carboxaldehyde and 2-methoxy-phenol. The two first products where not at all produced with the catalysts present.

Among the routes available for lignin conversion, the hydrolysis is
considered to be relatively mild, and the base-catalyzed depolymeriza-
tion of lignin using NaOH and other basic media have shown to be an ef-
cient depolymerization approach. A critical issue is to minimize the
formation of char and avoid lignin repolymerization, and capping agents
can be used for such purposes. Capping agents also enhance the yields of low molecular weight liquid products by stabilizing the present pheno-
lic compounds. Phenol was used as a capping agent by Yuan et al. in a study where high molecular weight alkaline lignin was hydrolyzed into small oligomers in a pressurized hot water-/ethanol mixture with NaOH as catalyst in the temperature range of 220–300 °C. At a reaction
temperature of 260 °C, and lignin/phenol ratio of 1:1 (w/w) an almost complete depolymerization with < 1% solid residue and only traces of gas products were obtained for a reaction time of 1 h (Yuan et al.,

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2010). Boric acid has also been shown to suppress addition and condensation reactions of initial products generated (Roberts et al., 2011). With a combination of base-catalyzed lignin hydrolysis and boric acid, a yield of low molecular weight products above 85% was obtained.

In a recent contribution, the depolymerization of Kraft lignin into polyols of moderately high hydroxyl number was successfully obtained by Mahmood et al. without using any organic solvent or capping agent but only direct hydrolysis using NaOH as a catalyst (Mahmood et al., 2013). The optimum operating conditions was found to be a temperature of 250 °C, a holding time of 1 h, and a NaOH/lignin mass ratio of 0.28 with 20 wt% substrate concentration. This resulted in <0.5% solid residues and 92% yield of depolymerized lignins suitable for bio-polylol production. Base-catalyzed depolymerization has been extended to lignin-rich residues after pretreatment and enzymatic hydrolysis, and significant yields of low molecular weight aromatics were obtained (Katahira et al., 2016).

2.6. Super-critical fluids

Super-critical water has several properties making it suitable as a solvent for lignin valorization. First of all, it is completely miscible with light gases, hydrocarbons, and aromatic compounds (Kanetake et al., 2007). Second, it has low viscosity, high diffusivity, and a dielectric constant similar to several organic solvents, with the added benefit of being thermally stable (Furusawa et al., 2007). Another advantage with the solvent is that separating the organic fractions from it is relatively simple. However, the high pressure and temperature required (above 647 K and 22 MPa) (Mörtstedt and Hellsten, 1999) are major drawbacks to the technology. This also makes it difficult to operate in a continuous mode, as feeding lignin during extraction is very difficult. Additionally, it is unclear whether it is possible to recuperate any of the energy needed in the creation of the super-critical water and if the materials issues can be solved.

A problem in supercritical liquid treatment is char formation, which has been observed when decomposing lignin in super-critical water and methanol (Yokoyama et al., 1998). Yokoyama et al. also established that the yield of char decreases and the yield of oil increases when the pressure is increased at constant temperature. Gosselink et al. depolymerized hardwood and wheat straw organosols lignins in a compressed fluid of carbon dioxide/acetone/air at 300 °C and 100 bar into 10~12% aromatic monomers through adding small amounts of formic acid as a hydrogen donor (Gosselink et al., 2012). However, a substantial amount of char was still formed indicating that further improvements are required in order to minimize losses as char. Recently, a one-step conversion of soda lignin was achieved in supercritical ethanol over Cu/Mg/AlOx catalyst resulting in high monomer yield (23 wt%) without the formation of char (Huang et al., 2014). Aromatics were the major products obtained. In addition, supercritical ethanol was found to be significantly more effective in producing monomers and avoiding char than supercritical methanol. The same research group further demonstrated the effectiveness of ethanol as a capping agent and formaldehyde as a scavenger, efficiently suppressing both depolymerization and char-formation reactions resulting in high-yield production of monomeric aromatics from lignin (Huang et al., 2015). Similarly, Güvenatam et al. used metal acetates, metal chlorides and metal trifluoromethanesulfonates as Lewis acid catalysts for the depolymerization of soda lignin at 400 °C in supercritical ethanol and water (Güvenatam et al., 2016). Char formation was also inhibited and high yields of low molecular weight organic products were produced. Catalo and Junk patented a process for converting wide ranges of biomass, including lignin, to hydrocarbon mixtures in near-critical or supercritical water (Catallo and Junk, 2001). The objective was to produce useful mixtures that are similar to a sweet crude petroleum, together with volatile and alkene gases (C2 to C5). It was claimed that such reactions may be carried out in continuous, batch, or semi-batch modes. However, only batch and stop-flow reactors were employed in the study. In another patent, Barta et al. developed a method using supercritical methanol. Lignin was extracted from biomass and subsequently depolymerized into mixtures of monomers using a porous metal oxide catalyst in the presence of the supercritical methanol (Barta et al., 2016).

2.7. Enzymatic depolymerization

A different option for lignin depolymerization is through enzyme catalysis, which is the main initial process in lignin degradation in nature. The lignin polymer is heterogeneous with many different bond types between the constituent ligno-monomers (cf. Fig. 3). As opposed to the targeted depolymerization of e.g. cellulose, the enzymatic degradation of lignin takes place with non-specific oxidative enzymes. Basidiomycetous fungi (primarily white rot fungi such as Phanerochaete chrysosporium) appear to have an important role for natural lignin degradation (recently reviewed by (Camarero et al., 2014)), but also bacteria such as Streptomyces viridusporus can degrade lignin by secreted enzymes (Dutta, 2015). White-rot fungi produce and secrete several kinds of oxidoreductases including laccases and heme peroxidases. These enzymes act indirectly in a cascade manner, which eventually results in an oxidative breakage of bonds in lignin. Laccases have a low redox potential, which only allows oxidation of the phenolic lignin units via the reduction of oxygen to water (Bugg et al., 2011; Sánchez, 2009). The substrate activity of laccases can be extended to non-phenolic lignin units if a mediator is available. The presence of mediator elements such as acetyl-syringone, syringaldehyde, vanillin, p-coumaric acid, 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), not only increases the oxidation capability and substrate specificity of laccases, but also prevents the polymerization of phenolic groups. These small redox molecules function as ‘electron shuttles’ across the enzyme and the lignin (Greier and Kombue, 2016; Huber et al., 2016; Jeon et al., 2012). Lignin peroxidases have a higher redox potential and attack non-phenolic lignin units by producing intermediate radicals, whereas manganese peroxidases generate Mn3+/4+, which acts on both phenolic and non-phenolic lignin units via lipid peroxidation reactions. Versatile peroxidases have in a sense combined the catalytic properties of both lignin peroxidase and manganese peroxidase. These enzymes have been found in relatively few organisms, e.g. fungal Pleurotus and Bjerkandera spp. (Ruiz-Dueñas et al., 2009). A different type of lignolytic peroxidase known as dye-decolorizing peroxidase (DYP) has been reported in the last decade for several organisms including Thanatophorus cucumeris Dec. 1 (fungus) (Sugano et al., 2007); Rhodococcus jostii (Ahmad et al., 2011) and Irpex lacteus (white rot fungi) (Salvachúa et al., 2013). These enzymes, named after their ability to degrade anthraquinone derived dyes, are structurally different from other peroxidases. Fungi also produce various additional accessory enzymes, which help the enzymatic degradation of lignin. Important examples are aryl-alcohol oxidases and glyoxal oxidases, which generate hydrogen peroxide needed for the function of peroxidases. Other examples of involved enzymes are aryl-alcohol dehydrogenases, copper radical oxidases, multicopper oxidases, cellobiose dehydrogenases, glucose-methanol-choline oxidoreductases and quinone reductases (Ayyachamy et al., 2013).

In addition to the enzymatic degradation of lignin, some fungi produce a hydroxyl radical via Fenton oxidation chemistry (Bugg et al., 2011). The reactive hydroxyl radical will oxidize compounds nearby, including lignin (Fig. 4). Through this chemical oxidation, the lignin structure will be more accessible for the lignin degrading enzymes.

2.8. Depolymerization for biological conversion

A compilation of depolymerization products reported by the methods above is given in Table 4. A range of different depolymerization products are formed – both due to differences in the original lignin source and depolymerization method employed. Depolymerization of lignin is challenging due to the distribution of strength of bonds in the

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previous work on lignin valorization during the past decades, a variety of methods and approaches have been developed for the extraction and utilization of lignin. However, the valorization process outlined in Fig. 2 continues with biocatalytical steps towards different specialized end-products (fine or bulk chemicals). Section 3.1 will address direct biocatalytic conversion of monomers in few steps, whereas Section 3.2 will outline strategies for microbial assimilation of said compounds into the central carbon metabolism (from where auxiliary pathways towards desired end-products can be attached by metabolic engineering). Finally, Section 3.3 will discuss approaches in which the depolymerization and bioconversion reactions are simultaneously combined (one-pot reactions and consolidated bioprocessing).

3.1. Direct biocatalytic conversion of specific monolignols

Successful depolymerization of lignin will yield a range of substituted phenols and propyl phenols, as well as oligomers of these (cf. Table 4). Direct conversion of these compounds into desired end-products – in one or few step process – is one approach for valorization. The market potential is somewhat difficult to assess, since many of the compounds are currently not available at a reasonable scale. However, there are many possible products which can in principle be derived from a depolymerized lignin mixture by reduction, oxidation or by shortening of the propylene-chain (see e.g. Bozell et al., 2007). Most fundamental work on biocatalytic conversion has focused on monolignols rather than compounds which are more likely to be obtained through lignin depolymerization. The biocatalytic conversion – as an alternative to the chemical conversion – to obtain the commercial product vanillin has been of considerable interest.

3. Biocatalysis

Following the depolymerization of natural and technical lignins aiming to yield low molecular weight lignins (monomers, oligomers), the valorization process outlined in Fig. 2 continues with biocatalytical steps towards different specialized end-products (fine or bulk chemicals). Section 3.1 will address direct biocatalytic conversion of monomers in few steps, whereas Section 3.2 will outline strategies for microbial assimilation of said compounds into the central carbon metabolism (from where auxiliary pathways towards desired end-products can be attached by metabolic engineering). Finally, Section 3.3 will discuss approaches in which the depolymerization and bioconversion reactions are simultaneously combined (one-pot reactions and consolidated bioprocessing).

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<table>
<thead>
<tr>
<th>Lignin substrate</th>
<th>Catalyst</th>
<th>Conditions</th>
<th>Major product(s)</th>
<th>Yield, wt%</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birch wood sawdust</td>
<td>Ru/C, Pd/C, Rh/C, Pt/C</td>
<td>200/250 °C, 40 bar</td>
<td>C₉-C₁₂ alkanes, C₁₄-C₁₈ alkanes, Methanol</td>
<td>42, 10, 11</td>
<td>Yan et al. (2008)</td>
</tr>
<tr>
<td>Wood flour from Japanese beech (Fagus crenata)</td>
<td>1-ethyl-3-methylimidazolium chloride</td>
<td>90–120 °C</td>
<td>Sugars, C₁₄–C₁₈ alkanes</td>
<td></td>
<td>Miyafuji et al. (2009)</td>
</tr>
<tr>
<td>Organosolv lignin/model compounds</td>
<td>Various brønsted acid catalysts</td>
<td>180 °C, 69 bar</td>
<td>Guaiacol</td>
<td>11.6</td>
<td>Binder et al. (2009)</td>
</tr>
<tr>
<td>Kraft lignin</td>
<td>H₃PMo₁₂O₴₀ (phosphomolibdic acid)</td>
<td>170 °C, 10 bar</td>
<td>Vanillin</td>
<td>4.6</td>
<td>Voit and Rohr (2009)</td>
</tr>
<tr>
<td>Organosolv lignin</td>
<td>NaOH (2 wt%)</td>
<td>300 °C, 250 bar</td>
<td>Methyl vanillate</td>
<td>4.2</td>
<td>Roberts et al. (2011)</td>
</tr>
<tr>
<td>Organosolv wheat straw</td>
<td>–</td>
<td>300–370 °C, 100 bar</td>
<td>Syringic acid</td>
<td>2</td>
<td>Geselin et al. (2012)</td>
</tr>
<tr>
<td>Organosolv hardwood</td>
<td>20 wt% Pt/C, formic acid hydrogen source, ethanol solvent</td>
<td>350 °C</td>
<td>Syringol</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Switchgrass lignin</td>
<td>4-acetamido-TEMPO in combination with HNO₃ and HCl (10 mol% each)</td>
<td>45 °C, 1 bar O₂</td>
<td>Monomeric guaiacol-type products</td>
<td>21</td>
<td>Xu et al. (2012b)</td>
</tr>
<tr>
<td>Aspen hardwood lignin/model compounds</td>
<td>Ni/C</td>
<td>200 °C, 1 bar Ar</td>
<td>Phenylglycaicol and propenyl-syringol</td>
<td>54</td>
<td>Song et al. (2013)</td>
</tr>
<tr>
<td>Birch sawdust</td>
<td>Vanadium catalysts</td>
<td>80 °C</td>
<td>Volatile monophenolic compounds</td>
<td>3</td>
<td>Chai et al. (2013)</td>
</tr>
<tr>
<td>Organosolv lignin (Miscanthus giganteus)</td>
<td>Pt/Al₂O₃, CoMo/Al₂O₃, or Mo₂C/CNF</td>
<td>225 °C, 58 bar Ar, 300 °C, 55 bar H₂</td>
<td>Alkylated phenol guaiacol,syringol-type products</td>
<td>11</td>
<td>Jongerius et al. (2013)</td>
</tr>
<tr>
<td>Kraft lignin</td>
<td>Raney Ni and 1/2-zeolite</td>
<td>2 h at 160 °C; 2 h at 240 °C</td>
<td>Aromes, Alkenes, Alkanes, Phenol</td>
<td>78, 18</td>
<td>Wang and Rinaldi (2013)</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>CuPMO</td>
<td>140–220 °C, 30–60 bar H₂</td>
<td>C₉ catechols and oligomers</td>
<td>60–93</td>
<td>Barata et al. (2014)</td>
</tr>
<tr>
<td>Organosolv lignin (Aleurites moluccana)</td>
<td>Cu/MgAlO₃</td>
<td>300 °C, 10 bar</td>
<td>Aromatic monomers</td>
<td>23</td>
<td>Huang et al. (2014)</td>
</tr>
<tr>
<td>Soda lignin</td>
<td>Ni-HF</td>
<td>270 °C</td>
<td>Alkyl-aromatic products</td>
<td>14</td>
<td>Zang et al. (2014)</td>
</tr>
<tr>
<td>Organosolv lignin</td>
<td>NaAu</td>
<td>170 °C</td>
<td>Aromatic monomers</td>
<td>14</td>
<td>Zang et al. (2014)</td>
</tr>
<tr>
<td>Soda/Akeel™ Organosolv/Kraft</td>
<td>CuMgAlO₃</td>
<td>380 °C, 10 bar</td>
<td>Alkylated aromatics and cycloalka(e)nes</td>
<td>60–86</td>
<td>Huang et al. (2015)</td>
</tr>
<tr>
<td>Birch sawdust (Betula pendula)</td>
<td>DDQ/TriBuONO₂O₂</td>
<td>80 °C</td>
<td>Phenolic monomers</td>
<td>5</td>
<td>Lancefield et al. (2015)</td>
</tr>
<tr>
<td>Kraft lignin</td>
<td>Cu/Mo loaded zeolite ZSM-5</td>
<td>220 °C</td>
<td>Alkyl phenols</td>
<td>22.5</td>
<td>Singh and Elise (2015)</td>
</tr>
<tr>
<td>Wood sawdust</td>
<td>H(C₆H₅)O₃</td>
<td>Ambient T and P</td>
<td>Phenol derivatives</td>
<td>7–24</td>
<td>Rghil et al. (2015)</td>
</tr>
</tbody>
</table>
(reviewed by e.g. (Priefert et al., 2001)). A potential starting point is ferulic acid, and a possible conversion is a two-step enzymatic conversion starting with a decarboxylation giving 4-vinylguaiacol, which is subsequently oxidized to vanillin. This can be achieved by whole cell catalysis with a suitable host (e.g. Escherichia coli) overexpressing the two enzymes (Furuya et al., 2015). Vanillin can in turn be reduced to vanillyl alcohol or oxidized to vanillic acid.

Rosazza et al. reviewed possible bioconversion routes of ferulic acid, including - apart from vanillin - also products such as caffeic acid, 4-vinylguaiacol, guaiacol, dihydroferulic acid, and polymers derived from ferulic acid (Rosazza et al., 1995). An initial demethylation of ferulic acid, a rather common microbial reaction, yields caffeic acid, which is a starting point for new product family. In the absence of oxygen, caffeic acid may be further converted through dehydroxylation to cinnamic acid, and then reduced to phenyl propanoid acid. This can in turn subsequently be converted to phenyl acetic acid. Aerobically, however, caffeic acid is typically oxidized to protocatechuic acid (Gribič-Galić, 1985).

The biocatalytic conversions of ferulic acid illustrate potential products. Since ferulic acid is one of the principal cinnamic acid derivatives, i.e. one of the lignin building blocks, one may believe that it can be easily obtained through depolymerization of lignin. However, ferulic acid is not a main product from depolymerization (cf. Table 4). Furthermore, purified ferulic acid may in fact be more valuable per se than e.g. vanillin, and the economics of such a process hinges on the availability of a low cost source of ferulic acid, even if there would be a premium price paid for “natural” vanillin as compared to chemically produced.

One main challenge when aiming for direct use of specific mono-mers from depolymerized lignin is no doubt separation. The depolymerized lignin will be a complex mixture – affected by both its biological origin and the fractionation and depolymerization methods applied. A different approach is therefore to work with the entire mixture as a carbon source in a complete biological conversion. In this process several of the compounds are to be metabolically converted and shunted to desired end-products as described by Linger et al. (Linger et al., 2014). This would – in a sense – be similar to the “sugar-platform” biorefinery concept, in which depolymerized carbohydrates are fermented into a wide range of desired end-products (e.g. alcohols, carboxylic acids, polyols) using genetically engineered microbes such as yeast (Nielsen et al., 2013). A wide range of products can be conceived also in a biological “lignin-platform” refinery, and several host organisms are possible. We will here focus on a common feature of these, which is the initial catalytic conversion of the lignin monomers. This will be central – regardless of end-product.

3.2. Biocatalytic conversion of depolymerized lignin

Wherever biodegradation of wood occurs in nature, there seems to be a symbiotic relationship between rot-type fungi and microbial species, where the former typically degrade macromolecules by secreted enzymes (as described in Section 2.7) giving smaller molecules which can be further catabolized by the secretors themselves or by the phlethora of microbes present in the surroundings, or even endosymbiotic, e.g. by termite gut microflora (Brune, 2014; Cragg et al., 2015; de Boer et al., 2005). From an evolutionary perspective, the toxic nature of many of these aromatic compounds (Schweigert et al., 2001; Zaldívar et al., 1999) constitutes a beneficial niche that may prove a competitive advantage of aromatic-degrading microbes over less tolerant ones, also to non- lignotyic species that have evolved both robustness to the (generally) toxic aromatic compounds and means of their catabolism (Davis and Sello, 2010; de Boer et al., 2005; dos Santos et al., 2004; Strachan et al., 2014).

There is a great diversity in the catabolism of biological degraders of lignin and lignin-derivatives. However, evidence across the prokaryotic kingdom (with few additional eukaryotic examples) indicates that a common catabolic node for aromatic breakdown is the formation of catechol or protocatechuic; this node is typically followed by aromatic ring fission and enzymatic conversion to acetyl-CoA and/or other constituents of the tricarboxylic acid (TCA) cycle (Bugg et al., 2011; Fuchs et al., 2011; Johnson and Beckham, 2015). As such, the major end-point of microbial aromatic catabolism - the central carbon metabolism - allows for many novel biotechnological prospects of propagating microorganisms solely on lignin-derived substrates for sustainable biovalorization of lignin waste streams.

Since most of the knowledge within the biological conversion field comes from fundamental microbiology, the examples given in this subsection (3.2) will focus on the known biochemistry of the lignin degradation that occurs in nature. This knowledge will be essential for future synthetic biology applications, e.g. for engineering of designer organisms for utilization of the specific mono- and oligomers resulting from depolymerized technical lignins. It is however very difficult to theoretically predict the exact chemical outcome of the different depolymerization methods (described in Section 2), and for this reason, chemical reaction pathways (Section 4) will be essential also for designing metabolic engineering strategies of the pathways described in the subsections below.

3.2.1. Catabolic funneling pathways

The enzymes responsible for the dissimilation of lignin-related compounds usually show less strict substrate specificity and are more tightly regulated than those catalyzing central metabolic pathways (Díaz et al., 2013). The expression of these enzymes are predominantly subjected to carbon catabolite repression, cross-regulation and vertical repression at different levels and by diverse mechanisms (Bleichrodt et al., 2010; Díaz et al., 2013; Vinsurveli et al., 2012), the complexity of which is not fully understood yet. This complex regulatory network has important repercussions in the biotechnological utilization of microorganisms to degrade lignin products in lignocellulosic biorefineries. As previously pointed out in Section 2, products from lignin depolymerization are very heterogeneous. This is true also for phenylpropanoid precursors involved in lignin biosynthesis (see Section 1.1), which are found naturally in soils and other environments after secretion by plants. Catabolism of these aromatic molecules involves more than ten different enzymatic activities (Table 5). The function of some of these can shortly be summarized as follows.

Acyl-CoA synthetases: This type of enzymes is responsible of the initial activation of hydroxycinnamic acids such as ferulic, p-coumaric, sinapic, caffeic or hydrocaffeic acids to acyl-CoA thioesters. Substrate specificity is typically low, and ATP is needed for this process (Pérez-Pantoja et al., 2010).

Acyl-CoA hydrolases/lases: After the initial activation, the propanoid aliphatic chain of the acyl-CoA product is hydrated to an aldehyde intermediate, and sequentially cleaved by this type of bifunctional enzyme, yielding acetyl-CoA and the corresponding aldehyde (vanillin, p-hydroxybenzaldehyde, syringaldehyde, etc.) (Masali et al., 2007; Pérez-Pantoja et al., 2010).

Dehydrogenases: These enzymes have an important role in funneling pathways, converting the different aldehydes generated into the corresponding carboxylic acid, which are less reactive and frequently less toxic to the host cell (Jimenez et al., 2002; Overhage et al., 1999; Pérez-Pantoja et al., 2010).

Decarboxylases: Non-oxidative decarboxylation of monoaromatic acids is carried out by microbial decarboxylase complexes. This process requires neither oxygen nor cofactors, and it is responsible for the conversion of 5-carboxyvanillic acid to vanillate and vanillate to guaiacol, the latter being a potential way to divert the protocatechuate branch of β-ketoacid pathway (see Section 3.2.1) towards the catechol branch (Chow et al., 1999; Yoshida et al., 2010).

O-Demethylases: This category comprises several types of enzymes that constitute demethylase systems, which are able to remove methyl...

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### Table 5: Enzymes in funneling pathways.

<table>
<thead>
<tr>
<th>Enzyme type</th>
<th>Substrate</th>
<th>Product</th>
<th>Enzyme name</th>
<th>Microorganism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl-CoA synthetase</td>
<td>Ferulate/p-coumarate/caffeate</td>
<td>Feruloyl-/p-coumaryl-/caffeoyl-CoA</td>
<td>HcaC</td>
<td>Acinetobacter baylyi, Cupriavidus necator JM134</td>
<td>Fischer et al. (2008); Bleichrodt et al. (2010); Pérez-Pantoja et al. (2010); Masai et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Ferulate/p-coumarate/caffeate/sinapate</td>
<td>Feruloyl-/p-coumaryl-/caffeoyl-/sinapyl-CoA</td>
<td>Fec</td>
<td>Pseudomonas sp. HR199, P. putida KT2440</td>
<td>Overhage et al. (1999); Jimenez et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Ferulate</td>
<td>Feruloyl-CoA</td>
<td>HcaA</td>
<td>Acinetobacter baylyi, C. necator JM134</td>
<td>Fischer et al. (2008); Bleichrodt et al. (2010); Pérez-Pantoja et al. (2010); Masai et al. (2007)</td>
</tr>
<tr>
<td>Acyl-CoA hydratase/lyase</td>
<td>Vanillin/p-hydroxybenzaldehyde/protocatechualdehyde</td>
<td>Vanillin Vanillate/p-hydroxybenzoic acid/protocatechualdehyde</td>
<td>HcaB</td>
<td>A. baylyi, C. necator JM134</td>
<td>Fischer et al. (2008); Bleichrodt et al. (2010); Pérez-Pantoja et al. (2010); Masai et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Vanillin</td>
<td>Vanillate</td>
<td>Vdh</td>
<td>P. sp. HR199, P. putida KT2440</td>
<td>Overhage et al. (1999); Jimenez et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>3-0-4 dimer, Cx alcohol type</td>
<td>Vanillate</td>
<td>UgD</td>
<td>Sphingobium sp. SYK-6</td>
<td>Masai et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Coniferyl alcohol</td>
<td>Vanillate</td>
<td>CalA</td>
<td>P. sp. HR199</td>
<td>Overhage et al. (1999); Jimenez et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Coniferyl aldehyde</td>
<td>Ferulate</td>
<td>CalB</td>
<td>P. sp. HR199, P. putida KT2440</td>
<td>Jimenez et al. (2002); Pérez-Pantoja et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>Benzoate diol</td>
<td>Catechol</td>
<td>BenD</td>
<td>P. sp. PR5200, P. putida KT2440, C. necator JM134</td>
<td>Masai et al. (2007)</td>
</tr>
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<td>Decarboxylases</td>
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<td>Guaiacol</td>
<td>VdrB, C, D</td>
<td>Streptomyces sp. D7</td>
<td>Chow et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>5-carboxylvanillate</td>
<td>Vanillate</td>
<td>UgW</td>
<td>Sphingobium sp. SYK-6</td>
<td>Masai et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Protocatechuate</td>
<td>Catechol</td>
<td>AorV</td>
<td>Enterobacter cloacae P241</td>
<td>Yoshida et al. (2010); Vardon et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>Vanillate</td>
<td>Protocatechuate</td>
<td>VanA, B</td>
<td>A. baylyi, P. putida WC3558, P. putida KT2440</td>
<td>Fischer et al. (2008); Bleichrodt et al. (2010); Jimenez et al. (2002)</td>
</tr>
<tr>
<td>O-Demethylation systems</td>
<td>5'-5'-dehydrovanillate (Biphenyl dimer)</td>
<td>Demethylated biphenyl dimer</td>
<td>Li6M</td>
<td>Sphingobium sp. SYK-6</td>
<td>Masai et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Methyl-THF</td>
<td>THF</td>
<td>Li6G</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Syringate</td>
<td>3'-0-3'-methyldigalate</td>
<td>DesA</td>
<td>Bradyrhizobium japonicum</td>
<td>Sudtachat et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>Vanillate</td>
<td>Protocatechuate + Formaldehyde</td>
<td>VanA1B</td>
<td>A. baylyi, C. necator JM134</td>
<td>A. baylyi, C. necator JM134</td>
</tr>
<tr>
<td></td>
<td>Corrinoid protein, primary methyl acceptor</td>
<td>Protocatechuate/caffeol/3'-0-3'-methyldigalate</td>
<td>Component A</td>
<td>Acetobacterium dehalogenans</td>
<td>Kaufmann et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Vanillate/syringate/isovanillate</td>
<td>Protocatechuate/caffeol/3'-0-3'-methyldigalate</td>
<td>Component B</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inactivated component A</td>
<td>Protocatechuate/caffeol/3'-0-3'-methyldigalate</td>
<td>Component C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methylated component A + THF</td>
<td>Demethylated component A + CH2-THF</td>
<td>Component D</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
moieties from methoxy-substituted aromatic molecules like vanillate, syringate or guaiacol by different mechanisms, with the help of cofactors (Kaufmann et al., 1998; Masai et al., 2007; Sudtachat et al., 2009). There are two main types of O-demethylase systems. The first type is preferred by aerobic microorganisms, and consists of two proteins: an IA type oxygenase and a reductase, both iron-sulfur enzymes containing [2Fe-2S] redox clusters (Masai et al., 2007). This system consumes oxygen and reducing power in the form of NADH, and as a result of demethylation generates formaldehyde, water and NAD^+ besides the demethylated substrate, mainly protocatechuate (Overhage et al., 1999).

The second type of demethylase system involves several enzymatic components and is always dependent on tetrahydrofolate (THF). One of the components is directly responsible for the removal of the methyl group from the main substrate, but the primary methyl acceptor for this group can be THF or a corrinoid protein, which will need to be regenerated by another methyl transferase that will transfer the methyl group to THF. Subsequently, THF also has to be regenerated by other enzymatic elements, yielding THF and formic acid, in order to recover this cofactor for further conversion of methoxylated substrates (Kaufmann et al., 1998; Masai et al., 2007).

This ensemble of funneling pathways converges in few metabolic nodes (Fig. 5), which lead further to intra- or extradiol ring opening by different types of specific dioxygenases, and will be further metabolized by the β-ketoapitate pathway, as will be discussed in the following section. The most central node in lignin product metabolism is protocatechuc acid, followed by catechol, but there are also aromatic molecules that can be subjected to ring cleavage, such as 3-0-methylgallate and gallic acid, as demonstrated in Sphingobium sp. SYK-6 (Kasai et al., 2004; Masai et al., 2007; Masai et al., 1999a). Furthermore, in this bacterial strain as well as strains from the order Burkholderiales, an alternative extradiol ring cleavage pathway has been described, which cleaves the aromatic ring between positions 4 and 5 of the phenolic ring (Kamimura and Masai, 2014; Masai et al., 1999b).

### 3.2.2. Microbial pathways for oxidative cleavage of aromatic rings

Cleavage of aromatic rings requires breaking strong bonds, and in nature this is not surprisingly predominantly an aerobic process. A few anaerobic pathways for dissimilation of aromatics have been identified (e.g. Benzoyl-CoA [Fuchs et al., 2011]), but these could rather be viewed as exceptions to the general rule. Oxidative ring cleavage of phenoic compounds, such as the ones found in low molecular weight lignin fractions, have classically been divided into three different categories depending on the relative positions of hydroxyl groups on the ring and the resulting fission (Harwood and Parales, 1996; Vaillancourt et al., 2006). Ortho-cleavage occurs in-between two adjacent OH-groups (intradiol) whereas meta-cleavage denotes fission adjacent to one of the OH-groups (extradiol) (Fuchs et al., 2011; Vaillancourt et al., 2006). Lastly, gentisate-cleavage can occur when two OH-groups are positioned in para position (Harpel and Lipscomb, 1990); however, due to the chemical structure, this type of ring dissimilation is not applicable for the catechol and protocatechuate node. It should also be noted that novel microbial strategies for aromatic catabolism that diverge from these classic pathways have recently been identified in nature.

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Table 6
A selection of known microbes with lignin- and/or aromatic degradation abilities.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Common strains</th>
<th>Type and origin</th>
<th>Known pathways and enzymes</th>
<th>Genome sequenced?</th>
<th>Known carbon sources</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eukaryotes (fungi and yeast)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthracophyllum discolor</td>
<td>Acevedo isolate</td>
<td>White rot fungi; forest</td>
<td>Mn peroxidase; laccase; lignin peroxidase</td>
<td>No</td>
<td>Three- and four-ring PAHs: phenanthrene, anthracene, fluoroanthene, pyrene, benzo(a)pyrene</td>
<td>Acevedo et al. (2011)</td>
</tr>
<tr>
<td>Aspergillus nidulans</td>
<td>FGSC A4</td>
<td>Filamentous fungi</td>
<td>Eukaryotic l-keto adipate pathway</td>
<td>Yes</td>
<td>Mono-chlorophenols and -chlorocatechols</td>
<td>Galagan et al. (2005); Martins et al. (2014); Martins et al. (2015)</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>CBS604</td>
<td>Yeast; pathogen</td>
<td>Variants of the eukaryotic l-keto adipate and gentisate pathways</td>
<td>Yes</td>
<td>3-hydroxybenzoate, 4-hydroxybenzoate, gentisate, hydroquinone, protocatechuate, resorcinol, resorcinol</td>
<td>Martins et al. (2014); Butler et al. (2009); Holesova et al. (2011)</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>HP15; ATCC 20290 MYA-3404; JH6</td>
<td>Yeast; opportunistic pathogen</td>
<td>Eukaryotic l-keto adipate pathway</td>
<td>Yes</td>
<td>phenol, resorcinol, quinol, hydroxyquinol, catehol; to a lesser extent 4-chloro-catehol, protocatechuate, p-cresol, m-chlorophenol, and p-chlorophenol; Kraft black liquor; Olive mill wastewater</td>
<td>Krug et al. (1985); Krug and Straube (1986); Povin et al. (1988); Butler et al. (2009); Martinez-Garcia et al. (2009); Wang et al. (2011); Rodriguez et al. (1996); Ma et al. (2010); Micheile et al. (2012); Martinez et al. (2004); Teramoto et al. (2004); Hirasuka et al. (2005); Shi et al. (2008); Haddadin et al. (2009); Ning et al. (2010); Ramirez et al. (2010); Khinode and Warishii (2012); Zhao et al. (2012); Durham et al. (1984); Furrance et al. (2015); Cook and Cain (1974); Sampaio (1999); Delgios et al. (2015); Cassland and Jonsson (1999); Alexieva et al. (2010); Floudas et al. (2012); Martinez et al. (2008); Adav et al. (2012); Gaal and Neujahr (1979); Anderon and Dagley (1980)</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>MUCL 30736; Strain 4287</td>
<td>Filamentous fungi; soil</td>
<td>Eukaryotic l-keto adipate pathway; extracellular peroxidases and laccases</td>
<td>Yes</td>
<td>Wheat straw lignin; ferulic acid, coumaric acid, vanillic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, cinamic acid, catehol (Biphenyl, biphenylene, dibenzofuran, dibenz-p-dioxin, diphenyl ether, vanillin, phenol, hydroquinone, 4-chlorophenol, 4-nitrophenol; phenanthrene; corn stalk, cotton stalk, Olive pomace</td>
<td>Wang et al. (2011); Rodriguez et al. (1996); Ma et al. (2010); Micheile et al. (2012); Martinez et al. (2004); Teramoto et al. (2004); Hirasuka et al. (2005); Shi et al. (2008); Haddadin et al. (2009); Ning et al. (2010); Ramirez et al. (2010); Khinode and Warishii (2012); Zhao et al. (2012); Durham et al. (1984); Furrance et al. (2015); Cook and Cain (1974); Sampaio (1999); Delgios et al. (2015); Cassland and Jonsson (1999); Alexieva et al. (2010); Floudas et al. (2012); Martinez et al. (2008); Adav et al. (2012); Gaal and Neujahr (1979); Anderon and Dagley (1980)</td>
</tr>
<tr>
<td>Phanerochaete chrysosporium</td>
<td>ATCC 34541; BKM-F-1767</td>
<td>White rot fungi</td>
<td>Ortho-cleavage pathway; phenanthrene metabolism</td>
<td>Yes</td>
<td>Catechol, Protocatechuate, phenylalanine, mandelate, benzoate, m-hydroxybenzoate, salicylate, Caffeic acid, p-coumaric acid, ferulic acid, p-hydroxybenzoic acid, m-hydroxybenzoic acid, protocatechuate, vanillic acid p-cresol, phenol</td>
<td>Martinez et al. (2004); Teramoto et al. (2004); Hiratsuka et al. (2005); Shi et al. (2008); Haddadin et al. (2009); Ning et al. (2010); Ramirez et al. (2010); Khinode and Warishii (2012); Zhao et al. (2012); Durham et al. (1984); Furrance et al. (2015); Cook and Cain (1974); Sampaio (1999); Delgios et al. (2015); Cassland and Jonsson (1999); Alexieva et al. (2010); Floudas et al. (2012); Martinez et al. (2008); Adav et al. (2012); Gaal and Neujahr (1979); Anderon and Dagley (1980)</td>
</tr>
<tr>
<td><strong>Prokaryotes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acinetobacter baylyi</td>
<td>ADP1</td>
<td>γ-Proteobacterium; Gram negative, α-Proteobacterium; Gram negative; soil</td>
<td>l-keto adipate pathway</td>
<td>No</td>
<td>Alkaline pretreated liquor from corn stover; p-coumaric acid, ferulic acid, 4-HBA, vanillic acid 4-hydroxybenzoic acid</td>
<td>Bleichrodt et al. (2010); Sabachúa et al. (2015); Park and Ormston (1986); Parke and Ormston (1986); Parke (1997); Goodner et al. (2001)</td>
</tr>
<tr>
<td>Agrobacterium tumefaciens (Rhizobium radiobacter)</td>
<td>B6; CS8</td>
<td>(Inducible) l-keto adipate pathway</td>
<td>(Inducible) l-keto adipate pathway</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus and species</td>
<td>Accession</td>
<td>Source</td>
<td>Carbon source</td>
<td>Lignin breakdown pathways</td>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-----------</td>
<td>--------</td>
<td>---------------</td>
<td>--------------------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>Anaeurinibacillus aneurinilyticus</td>
<td>AY856831</td>
<td>Bacillus; Gram positive; Paper mill sludge</td>
<td>Kraft lignin</td>
<td>oxidation of the sinapyl (G) and coniferyl (S) alcohol units in lignin</td>
<td>Chandra et al. (2007); Raj et al. (2007a)</td>
<td></td>
</tr>
<tr>
<td>Bacillus atrophaeus</td>
<td>B7</td>
<td>Bacillus; Gram positive; rain forest soil</td>
<td>Kraft lignin; guaiacylglycerol-δ-lignin ether</td>
<td>60 aromatic compounds are known to serve as a sole carbon source in this organism</td>
<td>Liu et al. (2012); Huang et al. (2013)</td>
<td></td>
</tr>
<tr>
<td>Cupriavidus necator</td>
<td>JMP134</td>
<td>β-Proteobacterium; Gram negative; Steelworks soil</td>
<td>Coniferyl alcohol, 4-Hydroxybenzoic acid, vanillic acid; fermentable substrate</td>
<td>Yes</td>
<td>Pérez-Pantoja et al. (2008); Lykidis et al. (2010)</td>
<td></td>
</tr>
<tr>
<td>Corynebacterium glutamicum</td>
<td>ATCC13032</td>
<td>Actinobacterium; Gram positive; facultative anaerobic; rain forest soil</td>
<td>Catechol, protocatechuic acid, 4-cresol, gentisate, resorcinol</td>
<td>Yes</td>
<td>Alkaline lignin (Sigma Aldrich)</td>
<td></td>
</tr>
<tr>
<td>Enterobacter lignolyticus</td>
<td>SCF1</td>
<td>β-Proteobacterium; Gram negative; facultative anaerobic; rain forest soil</td>
<td>Catechol, protocatechuic acid, benzoate, 4-hydroxyphenylacetate degradation pathway, glutathione biosynthesis and GST pathways, catalase and peroxidase HPI and DypB-type peroxidase</td>
<td>Yes</td>
<td>DeAngelis et al. (2013)</td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>M5a1</td>
<td>β-Proteobacterium; Gram negative; soil</td>
<td>Gentisate pathway</td>
<td>Yes</td>
<td>Kraft lignin, Benzonic acid, p-Hydroxybenzoic acid, Protocatechuic acid</td>
<td></td>
</tr>
<tr>
<td>Oceanimonas dougarii</td>
<td>JCM1046T</td>
<td>γ-Proteobacterium; Gram negative, marine</td>
<td>Lignolytic enzymes, aromatic fission pathways to yield aeryl-CoA with end point formation of polyhydroxyalkanoate (PHA)</td>
<td>No</td>
<td>Deschamps et al. (1980); Jones and Cooper (1990); Pang et al. (2016)</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>KT2440; mt-2</td>
<td>β-Proteobacterium; Gram negative; soil</td>
<td>Catechol, protocatechuic acid, benzoate, p-hydroxybenzoate, benzylation, phenylacetate, phenylalanine, tyrosine, phenylethylamine, phenylhexanoate, phenylheptanoate, phenyloctanoate, coniferyl alcohol, coumarate, ferulate, vanillate, nicotinate and quinate</td>
<td>Yes</td>
<td>Nelson et al. (2002); Jimenez et al. (2002); Poblete-Castro et al. (2012)</td>
<td></td>
</tr>
<tr>
<td>Rhodococcus erythropolis</td>
<td>A5.1; TAA21; TCP; XP</td>
<td>Actinobacterium; Gram positive; Termite gut</td>
<td>Polychlorinated biphenyl (PCB) catabolic pathway, β-ketoadipate pathway</td>
<td>Yes</td>
<td>Biphényl, PCB, m-cresol, p-cresol, vanilllic acid, veratryl alcohol; (low molecular weight) Kraft lignin</td>
<td></td>
</tr>
<tr>
<td>Rhodococcus jostii</td>
<td>RHA1</td>
<td>Actinobacterium; Gram positive; soil</td>
<td>E.g. the β-ketoadipate and gentisate pathways (in total 26 peripheral aromatic pathways); DypB lignin peroxidase</td>
<td>Yes</td>
<td>Kraft lignin model compounds (β-aryl ether lignin dimers), Kraft lignin, polychlorinated biphenyls (PCB), vanillinate, Catechol, protocatechuic acid, hydrocaffeic acid and caffeic acid</td>
<td></td>
</tr>
<tr>
<td>Rhodococcus opacus (Nocardia sp.)</td>
<td>DSM 1096; P6030</td>
<td>Actinobacterium; Gram positive; soil</td>
<td>Coniferyl alcohol, 4-Hydroxybenzoic acid, vanilllic acid; fermentable substrate</td>
<td>Yes</td>
<td>Meldau et al. (1995); Eiberg et al. (1997); Tao et al. (2011); Taylor et al. (2012)</td>
<td></td>
</tr>
<tr>
<td>Rhodoseudomonas palustris</td>
<td>CGA009</td>
<td>Actinobacterium; Gram positive; soil</td>
<td>Lignin degradation through multiple 3MGA pathways</td>
<td>Yes</td>
<td>Kosa and Ragauskas (2012)</td>
<td></td>
</tr>
<tr>
<td>Sphingomons paucimobilis (Sphingobium sp.)</td>
<td>SYK-6 (NBRC 103272)</td>
<td>Actinobacterium; Gram positive; soil</td>
<td>Caffeate, cinnamate, cumarate, ferulate</td>
<td>Yes</td>
<td>Larimer et al. (2004); Salmon et al. (2013)</td>
<td></td>
</tr>
<tr>
<td>Streptomyces viridochromogenes</td>
<td>T7A (ATCC 39115)</td>
<td>Actinomycetcales; Gram positive; soil</td>
<td>Catechol 4.5-cleavage pathway, syringate degradation through multiple 3MGA pathways</td>
<td>Yes</td>
<td>Major et al. (1999a,b)</td>
<td></td>
</tr>
<tr>
<td>Streptomyces viridochromogenes</td>
<td>T7A (ATCC 39115)</td>
<td>Actinomycetcales; Gram positive; soil</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Streptomyces viridochromogenes</td>
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<td>Actinomycetcales; Gram positive; soil</td>
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<td>Yes</td>
<td>Major et al. (1999a,b)</td>
<td></td>
</tr>
<tr>
<td>Streptomyces viridochromogenes</td>
<td>T7A (ATCC 39115)</td>
<td>Actinomycetcales; Gram positive; soil</td>
<td>Catechol 4.5-cleavage pathway, syringate degradation through multiple 3MGA pathways</td>
<td>Yes</td>
<td>Major et al. (1999a,b)</td>
<td></td>
</tr>
<tr>
<td>Streptomyces viridochromogenes</td>
<td>T7A (ATCC 39115)</td>
<td>Actinomycetcales; Gram positive; soil</td>
<td>Catechol 4.5-cleavage pathway, syringate degradation through multiple 3MGA pathways</td>
<td>Yes</td>
<td>Major et al. (1999a,b)</td>
<td></td>
</tr>
</tbody>
</table>
(Ismail and Gescher, 2012), which implies that this field is far from being fully elucidated. Based on their genetic makeup, microbes typically harbor dioxygenases specific for either ortho or meta cleavage, thus making the mode of phenol dissimilation species or even strain dependent (Stanier and Ornstorn, 1973). However, there are known exceptions of microorganisms that harbors both cleavage pathways or other substrate-specific redundancies (Pérez-Pantoja et al., 2010); in such cases the pathway co-regulation is governed by differential gene induction patterns, often through carbon catabolism repression; although still an emerging field, this phenomena has e.g. observed in Acinetobacter baylyi, Pseudomonas putida and Streptomyces spp. (Bleichrodt et al., 2010; Brzostowicz et al., 2003; Davis and Sello, 2010; Eaton and Ribbons, 1982; Morales et al., 2004). In addition to this, vertical regulation of the upper funneling pathways by presence of protocatechuate has also been observed in A. baylyi (Bleichrodt et al., 2010).

Following the individual routes of ring cleavage and catabolism, the micro-bial ortho and meta pathways converge on the tricarboxylic acid cycle (TCA) via their end-product formation (Bugg et al., 2011; Wells and Ragauskaus, 2012). Aromatic ortho-cleavage from the catechol/protocatechuate node - commonly referred to as the β-ketoadipate (β-KA) pathway in reference to its key metabolite - is cofactor independent and yields acetyl-CoA and succinate, whereas the meta-cleavage pathway starting this node is NAD⁺ dependend and yields acetyl-CoA and succinate, whereas the meta-cleavage pathway starting this node is NAD⁺-dependent with an end-point formation of pyruvate and acetyl-CoA (Inoue et al., 1995; Johnson and Beckham, 2015). Catechol is dissimilated with ortho or meta fission and with said outcome; however, due to the non-symmetric structure of protocatechuate (cf. Fig. 2), it can undergo meta-fission in two possible ways: either by NAD⁺-dependent meta cleavage as previously described (here occurring in the 2.3 position), or by NADP⁺-dependent 4,5-meta cleavage producing two molecules of pyruvate (Kamimura and Masai, 2014; Vaillancourt et al., 2006).

It is evident that the heterogeneous nature of lignin and the diversity in substrate specificity throughout the taxa of known lignin-degrading organisms limit the catabolic compatibility of these microbes with the type of lignin source, meaning that not all lignin-degrading organisms can utilize all types of lignin sources and their derivatives (Brown and Chang, 2014; Fernandez-Fueyo et al., 2012; Kasai et al., 2005). Catabolism of syringyl (S) lignin is for instance entirely dependent on the meta-cleavage pathway in the sense that the S-lignin degradation metabolites do not pass through the catechol/protocatechuate node but rather converge on the lower branch of the meta-pathway (Kasai et al., 2005). Derivatives of guaiacyl (G) lignin can on the contrary theoretically be catabolized by either ortho or meta-cleavage since the upper funneling pathways for such compounds converge on the catechol/protocatechuate node (Bugg et al., 2011).

The prevalence of ortho-cleavage pathways seems to be higher than meta-cleavage throughout the panel of currently known lignin-degrading prokaryotes (Bugg et al., 2011) (Table 6). Homologous enzymes for ortho fission of protocatechuate were predominantly found in actinobacteria and in a select set of proteobacteria, whilst meta-pathways were less prevalent and almost exclusive to proteobacteria (Bugg et al., 2011). Taken together with the fact that a high number of the cultivable lignin-degrading species belong to the actinobacteria phylum (Bugg et al., 2011; Tian et al., 2014; Větrovský et al., 2014), the ortho, or β-KA, pathway protrude as the more relevant of the two possible cleavage routes and will thus be highlighted in following section of this review.

3.2.3. Prevalence and prospects of the ortho-cleaving β-ketoadipate (β-KA) pathway

In nature, the β-KA pathway (also known as the 3-o xoacid path-
way) is a highly conserved metabolic route for ortho-cleaving ring fission that is yet very diverse when it comes to regulation and gene organization (Harwood and Parales, 1996). Although the form and redundancy of isoenzymes may differ both in and between species (Pérez-Pantoja et al., 2010), the pathway reactome is apparently consistent throughout the known hosts (Harwood and Parales, 1996; Wells and Ragauskaus, 2012), using P. putida as a model organism, the constituents and biochemistry of the different branches of the β-ketoadipate pathway was elucidated in the mid-1960s by Ornstorn and Stainer (Ornstorn and Stanier, 1966). Since then, this pathway has been discovered and described in numerous prokaryotes: predominantly in soil, forest and termite gut isolates (see Table 6) (Bugg et al., 2011; Tian et al., 2014). A few occurrences of the β-KA pathway have also been discovered in eukaryotes, including rot-type fungi such as Trametes versicolor (Alexieva et al., 2010), filamentous fungi (Martins et al., 2015; Michielse et al., 2012), as well as unicellular yeasts from the Rhodotorula (Cook and Cain, 1974; Jarboui et al., 2012; Katayama-Hirayama et al., 1992; Shimaya and Fuji, 2000) and Candida genera (Holesova et al., 2011; Krug et al., 1985; Wang et al., 2011). In a majority of the known aromatic degrading microbes, the upper funneling pathways are linked to the β-KA pathway by the protocatechuate/ catechol catabolic node (Harwood and Parales, 1996; Pérez-Pantoja et al., 2010). The β-KA pathway (extensively reviewed in (Harwood and Parales, 1996)) itself consists of nine enzymes allocated in two parallel upper branches (one from protocatechuate and one from catechol) that converge on a third branch. The latter has a starting point in the formation of the eponymous β-ketoadipate and an endpoint on acetyl-CoA formation (Fig. 6) and thus the β-KA pathway bridges the larger aromatic compounds (catabolized by the upper funneling pathways) with the TCA cycle.

According to present knowledge, not all microorganisms host both branches of the β-KA pathway; rather, many species seem have evolved to favor catabolism of either protocatechuate or catechol by regulation or absence of pathway genes (Harwood and Parales, 1996; Jimenez et al., 2002). For the eukaryotic β-KA pathway, there seems to be a preference towards the protocatechuate branch (Harwood and Parales, 1996), although species with the catechol or both branches have been discovered (Anderson and Dagley, 1980; Durham et al., 1984; Krug and Straube, 1986; Martins et al., 2015; Michielse et al., 2012; Santos and Linardi, 2004). Furthermore, the metabolic route of the eukaryotic version of this branch differs from that usually found in prokaryotes: here the lower part of the branch is bypassed as β-carboxy-cis,cis-muconate is converted to β-ketoadipate via β-carboxymuconolactone (Fig. 6) (Harwood and Parales, 1996).

Although a majority of the metabolic engineering approaches for improved production of aromatic compounds have focused on heterologous expression of extracellular lignolytic enzymes (laccases and peroxidases) in novel hosts (Bleve et al., 2008; Cassland and Jönsson, 1999; Gonzalez-Perez and Alcalde, 2014; Ryu et al., 2008; Wang and Wen, 2009), recent studies have focused on the catechol/protocatechuate node and its possibilities as a stepping stone for biological production of novel compounds from aromatic substrates. The biotechnological prospects of the β-KA pathways were recently comprehensively reviewed with a focus on bio-remediation of pollutants and valorization of lignocellulosic waste streams (Wells and Ragauskaus, 2012). The protocatechuate branch of R. jostii RHA1 has recently been reconstructed in vitro (Yamashita et al., 2015), which opens up for new knowledge on the characteristics of species- and/or strain-specific isoenzymes of the β-KA pathway. Other approaches have focused not on the acetyl-CoA end-point of this pathway, but rather on rerouting the carbon to other end compounds, e.g. adipic acid (Jung et al., 2015; Vardon et al., 2015). Furthermore, a recent study on P. putida demonstrated that the aromatic ring fission pathways are in fact interchangeable and are therefore promising targets for metabolic engineering. In fact, the authors conclude that the exogenous meta cleavage pathway from another strain of P. putida proved to be better than endogenous ortho in terms of pyruvate yield from lignin-derived substrates (Johnson and Beckham, 2015).
Fig. 6. The eukaryotic and prokaryotic β-ketoadipate pathways. Solid arrows indicate the reactions present in prokaryotes. The eukaryotic and prokaryotic pathways coincide except for the reactions in dashed arrows, which show the divergent route in the eukaryotic protocatechuate branch (Cook and Cain, 1974). Ligand requirements are according to (Bull and Ballou, 1981; Goldman et al., 1985). Enzyme abbreviations: P3,4O: Protocatechuate 3,4-dioxygenase; CMLE: Carboxy-cis-cis-muconate lactonizing enzyme; CMD: Carboxy-muconolactone decarboxylase;CMH: 3-carboxymuconolactone hydrolase; C1,2O: Catechol 1,2-dioxygenase; MLE: cis,cis-Muconate lactonizing enzyme; MI: Muconolactone isomerase; ELH: β-ketoadipate enol-lactone hydrolase; TR: β-ketoadipate succinyl CoA transferase; TH: β-ketoadipyl-CoA thiolase.
3.2.4. Microbial end-products from catabolized lignin

Through the natural variety in microbial metabolic products and the possibilities of metabolic engineering, there exist many routes to derive catabolized low molecular weight lignin to value-added end-products. Aromatic and phenolic bi-products (e.g. vanillin) (Masai et al., 2007) and central metabolites (e.g. pyruvate and lactate (Johnson and Beckham, 2015)) are obvious derivatives of the intracellular aromatic fluxes. Other enrichment options typically include rerouting of the flux from acetyl-CoA by endo- or exogenous pathways. Examples of studies on the latter include bioplastics from acetyl-CoA (Linger et al., 2014), biodiesel from lipids generated by oleaginous Rhodococcus from lignin model compounds (Kosa and Ragauskas, 2012) and polymers (Crawford et al., 1983; Michinobu et al., 2008; Otsuka et al., 2006; Trigo and Ball, 1994). Another feasible alternative is to engineer the catabolism upstream of the TCA cycle; this has for instance been demonstrated by a re-routing of the catechol branch of the β-KA pathway to form adipic acid instead of acetyl-CoA (Vardon et al., 2015). Strategies such as these are ultimately a crucial instrument in valorizing depolymerized lignin to products of biotechnical importance.

3.3. One-pot reactions and consolidated bioprocessing

Another possible biotechnical approach to valorization of lignin is to attempt to mimic the symbiotic system between fungi and microbes that occurs in nature, i.e. to combine the depolymerization and catabolic conversion of resulting smaller fragments into one step. This can potentially be implemented as a one-pot biocatalysis process, where multiple reaction steps are simultaneously performed in one reactor (e.g. by enzymatic or by engineered whole-cell catalysis) (Gasser et al., 2012), or through the means of consolidated bioprocessing (CBP), a process where degradation and metabolism are simultaneously performed by a microbial consortia in a single bioreactor, without the addition of external enzymes (Olson et al., 2012). While CBP was originally developed for ethanol production from lignocellulose (Olson et al., 2012), steps have recently been taken towards lignin CBP (Salvachúa et al., 2015).

The idea behind one-pot biocatalysis is to combine multiple enzymatic steps in one go in order to reduce the total unit operations. An example regarding lignin degradation was performed by Picart et al. that studied glutathione-dependent β-ethersases and glutathione lyases (from Sphingobium sp. SYK-6, Novosphingobium sp. PPIY and Thioacillus dentriticus ATCC 25259) as lignin depolymerization biocatalysts (Picart et al., 2015). The authors set up proof of concept one-pot processes combining the different β-ethersases and glutathione lyases and found that the reaction exhibited a good potential for selective cleaving of the ether bonds in the lignin macromolecule and for subsequent release of glutathione-free aromatic compounds (Picart et al., 2015).

Central to the consolidated bioprocessing approach is the design of the composition of the microbial consortia, i.e. to develop a working artificial niche; often aided by microbial metabolic engineering (Amore and Faraco, 2012; Olson et al., 2012). So far, lignin CBP studies have mostly focused on prokaryal co-cultures, and although fungal CBP is a relevant option, a drawback observed at least for lignocellulose CBP has been the low degradation rate and productivity (Panagiotou et al., 2005). For lignin CBP, Salvachúa and colleagues examined 14 bacterial species in order to identify the best biological catalysts for lignin depolymerization, secretion of lignolytic enzymes, consumption of aromatic compounds, and value-added chemicals production, using a lignin-enriched biorefinery stream as feedstock (Salvachúa et al., 2015). It was found that Acinetobacter sp. ADP1, Amycolatopsis sp. 75iw2, P. putida KT2440 and mt-2, and R. jostii RHA1 were able to depolymerize high molecular weight lignin complexes and catabolize appreciable portions of the low molecular weight aromatics. In a study by Wu and He, two sediment-free methanogenic microbial consortia were screened for lignin depolymerization under anaerobic conditions (Wu and He, 2013). This setup successfully resulted in biomethane production coupled lignin depolymerization, and also boosted the hydrolytic efficiency of the tested lignocellulosic materials (Wu and He, 2013). Other notable studies on fungal and prokaryal lignin degrading consortia include (Kausar et al., 2010; Rüttimann et al., 1991; van der Lelie et al., 2012; Wang et al., 2013).

4. Chemical analysis

A prerequisite for any valorization strategy is the ability to identify and quantify lignin and fraction products from lignin. This is in particular essential in order to be able to engineer tailor-made microorganisms (Section 3) that can utilize the specific low molecular weight lignins resulting from depolymerization of technical and natural lignins (Section 2), and as well to be able to assist in validation and troubleshooting of the metabolic engineering of said microorganisms.

This section deals with the chemical analysis of solid and liquid samples containing processed lignin, i.e. monolignols and oligolignols of different molecular weight distribution. Structural elucidation of intact lignin or its catabolism is, however, not covered here. A comprehensive review was recently written by Lupo et al., in which advances achieved in qualitative and quantitative analysis of lignin over the last ten years was evaluated based on their specific application fields (Lupo et al., 2015). There are also a few additional reviews describing chemical analysis of lignin-derived samples, see e.g. (Brudin and Schoenmakers, 2010; Vaz, 2014). Table 4 summarises the different analytical techniques used for lignin analysis.

Samples obtained from depolymerization reactions of lignin have different challenging characteristics. To start with, the samples are a mixture of many dissolved phenolic compounds, precipitated oligolignols and particles/agglomerates derived from the lignin. Furthermore, the pH might be extremely high or low, of which the former is a larger challenge than the latter in terms of compatibility of analytical equipment usually made of stainless steel and silica-based chromatographic columns. Moreover, the risk of re-polymerization reactions occurring after depolymerization prompts for rapid analysis in order to avoid transformation of the sample components.

4.1. Sample preparation

The first step of the analysis is to perform extraction and/or sample clean-up prior to separation and detection. Usually, conventional solvent extraction is used with solvents like diethyl ether (Llano et al., 2015; Mokochinski et al., 2015), ethyl acetate (Vigneault et al., 2007), n-hexane followed by ethyl acetate (Ribechini et al., 2015) or with ethanol and ethanol/water mixtures (Wang and Chen, 2013). A more academic study explored the use of liquid ions such as [Bmim][MeSO4] (Prado et al., 2013b), although such method is likely to be expensive as well as suffer from difficult separation of the lignin monomers from the ionic liquid. More rare is the use of pressurized hot (subcritical) water as extraction solvent (Sumersky et al., 2015). In the same study, XAD-7 resin was used for sample clean-up in order to remove carbohydrates from the hot water extract (Sumersky et al., 2015). In some of the studies, fractions have been done using a series of different solvents, such as ethanol containing different volume percentage of water (Wang and Chen, 2013).

4.2. Chromatography

Chromatography has been widely used in the analysis of lignin samples for various purposes. Major types of chromatographic techniques that are applied in lignin analysis are gas chromatography (GC), liquid chromatography (LC), size exclusion chromatography (SEC), capillary electrophoresis (CE) and two-dimensional (2D) chromatography. The combination of these techniques with advanced detectors and sample preparation procedures provides analytical tools with high separation capacity and resolution power. Remarkable progress has been made.
regarding the development of new chromatographic instrumentations and methods and the improvement of established methods in recent years (Lupoi et al., 2015). This section will focus on the introduction and assessment of latest research performed in each of the subcategories of chromatography.

4.2.1. Gas chromatography (GC)

Gas chromatography, combined with a series of sample pre-treatment and detection techniques, have long been prevalently used in the analysis of lignin and lignin–carbohydrate complexes structures and linkages (Del Rio et al., 2012a; del Rio et al., 2012b; Du et al., 2013; Du et al., 2014; Lupoi et al., 2015), the evaluation of lignin monomeric units (Del Rio et al., 2005; Kaiser and Benner, 2012; Lima et al., 2008; Nunes et al., 2010) and the determination of lignin depolymerization products (Galkin and Samec, 2014; Gosselink et al., 2012; Stärk et al., 2010; Xu et al., 2012b).

Standard wet chemistry procedures for lignin content determination requires vast time and labor input. A few studies have probed the possibility to utilize pyrolysis-gas chromatography (py-GC) as a substitute technique for lignin content determination of various hard- and softwoods (Alves et al., 2009; Alves et al., 2008; Alves et al., 2006; Loureno et al., 2003). py-GC with a flame ionization detector (py-GC-FID) was proven to give comparable precision to that of the standard Klonas analysis with advantages that no sulfuric acid pretreatment is needed and additional information on lignin structure can be obtained (Alves et al., 2006). The model was further evaluated with larch species and compression wood and principal component analysis (PCA) was applied to study species- and tissue-specific differences for classification of softwoods lignin (Alves et al., 2009; Alves et al., 2008).

Two research groups have investigated the analysis of lignin functional groups with headspace-gas chromatography (HS-GC). With this technique, accurate determination of methoxy groups in lignin was achieved as a substitute for the complicated and time-consuming traditional method (volumetric titration based on iodometry). The large uncertainty caused by sampling methyl iodide, which is highly volatile in the conventional GC method, was thus avoided (Li et al., 2012). Fast and efficient determination of lignin sample carbonyl group content was also enabled by using HS-GC. The authors found that carbonyl group reduction reactions could be significantly accelerated by the addition of SiO₂ powder (Li et al., 2015).

Phenolic monomers derived from lignin were analyzed by a headspace solid phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME/GC-MS). The method was optimized and applied on wheat straw. The speed and solvent-free feature of this technique is in accordance with the principles of green chemistry (Kolb et al., 2013). In the study of solvolysis of lignin in hydrogen donating solvents, the unfavorable time gap between solvolysis at given conditions and analysis was eliminated by using an online microreactor-gas chromatography system (Kim et al., 2014).

4.2.2. Liquid chromatography (LC)

The successful coupling of high-performance liquid chromatography and mass spectrometry (HPLC-MS) offers a powerful technique for the analysis of low molecular weight compounds in lignin samples with high selectivity and sensitivity. Mass spectrometry based analysis is discussed in more detail below (Section 4.3).

Negative-ion-mode electrospray ionization with NaOH dopant was proven to be able to ionize lignin degradation products very efficiently (Haupert et al., 2012). High-performance liquid chromatography–tandem mass spectrometry (HPLC-MS”) with this ionization technique was then utilized to separate and identify model compounds in complex degraded lignin product (Haupert et al., 2012; Owen et al., 2012). Lignin type monomeric and dimeric molecules in an organosolv lignin sample were thoroughly analyzed by HPLC-MS”, and this high-resolution approach elucidated the elemental compositions and structural information of the major compounds (Jarrell et al., 2014). Technology development in the packing material and instrumentation gave rise to the ultra-high-performance liquid chromatography (UHPLC). This advanced variant of traditional HPLC provides higher separation power in shorter analysis time. UHPLC coupled with an UV detector was applied in the analysis of mono-phenolic compounds from oxidative degradation of lignin (Ouyang et al., 2014). With the help of a self-constructed library of enzymatically synthesized monomers, dimers and oligomers, a fast and reliable analysis method of soluble lignin extracted from sugar cane utilizing UHPLC coupled with tandem mass spectrometry was successfully developed (Kiyota et al., 2012).

Besides the analysis of small lignin-derived compounds, HPLC has also been used in the characterization of processed lignin samples. 10-step gradients of N,N-dimethylformamide (DMF) in an aqueous mobile phase (buffered by phosphate) and a wide-pore octadecylsilica column was used to fractionate and characterize lignin samples of different origins, providing well-defined lignin peaks. Size exclusive effects were suppressed by the usage of a wide-pore reverse phase sorbent and surface interactions improved by good solvation power of DMF of lignin, which led to good reproducibility and robustness of the method (Gora et al., 2006).

Hydrophobic interaction chromatography (HIC) has also been used for fractionating processed lignin according to hydrophobicity differences (Ekeberg et al., 2006).

4.2.3. Size exclusion chromatography (SEC)

Despite the wide use of size exclusion chromatography for lignin molecular weight determination (Guerra et al., 2007; Ringena et al., 2006; Savvy and Piccolo, 2014), accurate and reproducible evaluation of lignin MW distribution with this technique is still elusive due to lignin’s degradation during isolation, large variance in polydispersity and solubility, associative behavior and detector limitations (Asikkala et al., 2012; Baumberger et al., 2007). For increasing the capability of SEC in lignin research, some efforts have been made and still need to be put into improving the reliability and reproducibility of SEC methods.

In sample preparation, a common method to minimize association interaction is acetylation using acetic anhydride in pyridine as a derivatization method, which suffers from a long reaction time (6 days). In contrast, acetobromination by using acetyl bromide in glacial acetic acid provided completely tetrahydrofuran-soluble lignin derivatives within 30 min (Asikkala et al., 2012). In a study towards standardization of SEC methods, the high molar-mass fraction of polydisperse lignins, in combination with different chromatographic conditions and data calculating strategies, were found to lead to large measurement variations of MW determination. The importance of recovery tests for unanalyzed lignin was also highlighted. In addition, two recommendations of chromatographic configurations were made for both aqueous and organic SEC (Baumberger et al., 2007). Multi-angle laser light scattering detector (MALLS) can be used for lignin molecular weight determination and has the ability to detect lignin aggregates (Contreras et al., 2008). Compared with traditional RI or UV detectors, MALLS coupled with size exclusion chromatography can enrich the results with more details, with the capability to effectively monitor the changes in lignin MW distribution, gyration radius and hydrodynamic radius (Gidh et al., 2006a; Gidh et al., 2006b). The interference of lignin fluorescence was confirmed and avoided by applying narrow band-pass filters.

4.2.4. Capillary electrophoresis (CE)

CE has in recent years proven to be a very useful technique for qualitative and quantitative determination of low molecular mass lignin degradation products as well as lignin content evaluation (Bogolysyna et al., 2011; Dupont et al., 2007; Gebremeskel and Aldea, 2013; Lima et al., 2007; Rovio et al., 2010). Compared with traditional GC and HPLC methods for the determination of lignin-derived compounds, CE offers shorter analysis time without the need for pre-derivatization. Coupled with UV detector, eight aromatic lignin derivatives in old book pages were separated and identified in 9 min (Dupont et al., 2014).

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Table 7
Chromatographic techniques applied to lignin samples.

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<td>Lignin from spruce (soft wood) and birch (hard wood)</td>
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<td>Lobbes et al. (1999)</td>
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<td></td>
<td>Dissolved organic matter and sediments</td>
<td>HPLC-UV</td>
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<td>Kob et al. (2012)</td>
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<td>Wheat straw</td>
<td>HS-SPME</td>
<td>HPLC-UV</td>
<td>–</td>
<td>HSPME,GC-MS</td>
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<tr>
<td>Analytical method</td>
<td>Soluble lignin extracted from sugar cane</td>
<td>Pine sawdust</td>
<td>Dissolved organic matter, soil and sediments</td>
<td>Hemicellulose, cellulose and lignin standard mixture</td>
<td>Kraft lignin, soda lignin, organosolv lignin, pine wood pyrolytic lignin, lignin filtration residue from acid straw hydrolysis, lignin-enriched residues from anaerobic biomass fermentation</td>
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<td>Freeze dry-ultrasonic SLE</td>
<td>UPLC-MS/MS</td>
<td>GC–MS</td>
<td>Odor plume evaporation; distillation</td>
<td>Odor plume evaporation; distillation</td>
<td>Heat and catalytic pyrolysis</td>
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<td>Depolymerization reaction-solvent-evaporation; distillation</td>
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<tr>
<td>UPLC–MS/MS</td>
<td>ESI-QTD</td>
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<td>Gallman and Samec (2014)</td>
<td>Kaisar and Benner (2012)</td>
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<td>Kiyota et al. (2012)</td>
<td>Galkin and Samec (2014)</td>
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<td>Windt et al. (2009)</td>
<td>Michailof et al. (2014)</td>
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2007). 11 lignin-derived monomers from alkaline CuO oxidation of humic acid were separated and quantified in 12 min, which was significantly faster than common HPLC and GC methods (Kaiser and Benner, 2012; Lobbes et al., 1999). The combination of CE with mass spectrometry detection (CE-MS) enabled the acquisition of structural information of the analytes. CE-MS was applied to the determination of lignin-derived phenolic model compounds in cellulose processing effluents and aged papers (Bogolitsyna et al., 2011). Besides the usage in analysis of low molecular mass lignin derivatives, the applicability of CE in the determination of lignin content was tested on black liquor samples. The results were in good agreement with those of the traditional sulfuric acid/UV method. Compared with the traditional absorption spectroscopic method where exact lignin absorption coefficient is very difficult to determine, the authors argued that the CE method holds the advantage that exact lignin absorption coefficient is not needed (Gebremeskel and Aldeaes, 2013).

4.2.5. Two-dimensional chromatography

The newly emerged multidimensional chromatography technique has drawn more and more attention in the field of lignin sample analysis. Compared with traditional one-dimensional chromatography, 2D chromatography can provide higher separation power and peak capacity, which allows the complex compositions of various lignin samples to be better investigated. Several research groups have performed the analysis of bio-oil samples and upgraded bio-oil samples with two-dimensional GC (2D-GC) in recent years. Many co-eluting compounds in conventional 1D-GC methods were clearly separated and identified (Faccini et al., 2013; Marsman et al., 2008; Moraes et al., 2012; Tessarolo et al., 2013; Winndt et al., 2009). Quantitative studies of bio-oil composition were achieved by combining 2D-GC-PID and 2D-GC-time of flight-mass spectrometry (2D-GC-TOF/MS). The great separation capability and resolution power provided by 2D-GC-MS can elucidate detailed differences between complex samples, which is beneficial for the study of pyrolysis mechanism and catalyst performance behind the bio-oil samples (Djokic et al., 2012; Michailof et al., 2014). sulfonated lignin dispersants in agrochemical formulations were fingerprinted by 2D-GC. The coupling of ion-pair reversed phase HPLC (IP-RPLC) with 2D-GC-PAC (IP-SEPAC) enabled the differentiation of “good quality” batches of sulfonated lignin from “bad quality” ones (Brudin et al., 2008). With the fact that size variations cannot fully account for the different behaviors of lignin in agrochemical formulations, the author developed a novel online ion-pair liquid chromatography-thermally assisted hydrolysis and methylation-gas chromatography-mass spectrometry system (IP-RPLC × THM-GC–MS). This hyphenation can determine not only the overall sulfonated lignin composition, but also the correlation between chemical compositions and sizes of lignin molecules (Brudin et al., 2010).

4.3. Mass spectrometry

Mass spectrometry (MS) is a commonly used analytical technique for structural characterization of isolated lignin. Commonly applied mass analyzer techniques are quadrupole, ion trap (ITMS) or Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometers. These techniques are described in more detail by Gross (Gross, 2011). There is an excellent review by Reale et al. in which MS applications related to structural characterization of isolated lignin are reported in detail (Reale et al., 2004). Hence, in this review the focus is bent on MS techniques used in lignin research in the last ten years since that review was published.

4.3.1. Analysis of monomeric lignin-related compounds

Pyrolysis combined with gas chromatography mass spectrometry (Py-GC–MS) is a widespread used technique for lignin degradation studies and the analysis of the monomeric lignin subunits. Due to its advantages for lignin degradation studies, because of short measurement times and the presence of GC–MS databases, Py-GC–MS is still the dominating analytical technique for analysis of lignin-derived compounds. In most studies the GC system is combined with a quadrupole mass spectrometer (qMS) and the focus is on the analysis of the monomeric lignin subunits. (Ibarra et al., 2007; Lourenço et al., 2015; Shen et al., 2010). Del Río et al. investigated lignin from jute fibers with a combination of Py-GC–qMS, 2D-NMR and thioacidolysis with a focus on structure elucidation. Besides monomers, they detected also dimeric lignin-derived molecules (del Río et al., 2009).

However, in a few lignin studies the GC system was coupled to a different mass spectrometer. For instance, Ohra-aho et al. used a Py-GC system coupled to an ion trap mass spectrometer (ITMS) in a lignin degradation study with different catalysts (Ohra-aho and Linnekoski, 2015). Flamini et al. used also a GC-ITMS system for the investigation of lignin-extracts from different trees in northern Italy (Flamini et al., 2007). Beside different kinds of lignins, Winndt et al. investigated Kraft lignin by offline Py and a subsequent analysis of the liquid and the gas phase by GC/MS/flame ionization detector (FID). The liquid phase was also analyzed with a combination of GCGC-Time-of-Flight (TOF)–MS (Winndt et al., 2009).
both negative and positive ionization mode. They detected 63 new oligomeric structures from dimers up to heptamers. The group proposed molecular structures of the oligomers and possible fragmentation pathways in tandem MS experiments (Banoub et al., 2007).

### 4.3.2. Analysis of solid lignin samples

With different MS techniques like TOF-secondary ion mass spectrometry (TOF-SIMS) (Matsushita et al., 2012; Saito et al., 2005; Saito et al., 2006), matrix-assisted laser desorption/ionization MS (MALDI-MS) (Aráoji et al., 2014; Richel et al., 2012), laser ablation resonance-enhanced multiphoton ionization TOF-MS (LA-REMPI-TOF-MS) (Mukarakate et al., 2011) or direct exposure MS (DE-MS) (Modugno et al., 2008) also solid lignin samples have been investigated. The usage of these MS techniques related to the investigation of lignin derived compound were recently described in detail in the review of Lupoi et al. (Lupoi et al., 2015).

### 5. Outlook

As a source of renewable carbon for chemicals, lignin is today the largest untapped terrestrial source. Use of whole lignin – either as a fuel or a polymer – has been the prevalent use until now. However, considering the richness of functional groups, other applications and alternatives for its conversion should be opted for. The challenges in using lignin as a raw material for low molecular weight chemicals originate from the fact that the polymer is heterogeneous - in many different ways. Lignin is firstly heterogeneous in the sense that different plants build their lignin with different proportions of the constitutive building blocks. Secondly, the cross-linking patterns are largely stochastically created, and the lignin is also connected to hemicellulose in the plant. There is thus heterogeneity for lignin even from the same plant, underlining the challenges in lignin valorization. Depolymerization requires the breaking of several types of bonds, which – if successful – will result in a complex mixture, which is highly influenced by the method of depolymerization used. The obtained mixture is difficult to chemically characterize and analytical methods need to be improved. For example, selective extraction methods, targeting low molecular weight aromatic compounds are needed, as well as suitable MS methods for the study of the fragmentation pathways in MS’ experiments of lignin-related oligomeric compounds higher than tetratramers. The lack of commercial standards or a database for lignin-related oligomers also complicates their analysis.

The use of individual compounds from a depolymerized mixture will be challenging from a separation technology point of view. A potential way forward is therefore to make use of the entire depolymerized mixture in a process towards targeted end-products. The converging pathways for catabolism of aromatic compounds found in many organisms in nature is a promising starting point, which could enable us to venture into the metabolic engineering route – this time not from the familiar starting point of polysaccharide derived sugars – but rather from lignin-derived aromatic compounds. Discoveries of novel isolates and enzymes acting on these compounds as well as a more detailed knowledge on the pathways – especially the upper funneling pathways – will be highly important. This exciting possibility will also require improved methods for lignin depolymerization, based on a better knowledge of lignin structure and depolymerization mechanism, as well as improved tools for efficient engineering of the aromatic catabolic pathways, based on an increased knowledge of these.

### List of abbreviations

- APCI: atmospheric pressure chemical ionization
- APP: atmospheric pressure photoionization
- β-KA: β-ketoadipate
- CBP: consolidated bioprocessing
- CE: capillary electrophoresis
- CE-MS: CE with mass spectrometry detection
- DMF: dimethylformamide
- DI: direct infusion
- DE-MS: direct exposure mass spectrometry
- EI: electron ionization
- ESI: electrospray ionization
- FID: flame ionization detector
- FT-ICR: MS Fourier transform-ion cyclotron resonance mass spectrometry
- GC: gas chromatography
- HS-GC: headspace-gas chromatography
- HS-SPME/GC–MS: headspace solid phase microextraction coupled with gas chromatography–mass spectrometry
- HPLC-MS: high-performance liquid chromatography and mass spectrometry
- HPLC-MS<sup>3</sup>: high-performance liquid chromatography–multiple stage tandem mass spectrometry
- HRMS: high resolution mass spectrometry
- HIC: hydrophobic interaction chromatography
- IP-RPLC: ion-pair reversed phase HPLC
- IP-SEC: ion-pair SEC
- IP-RPLC × THM-GC–MS: ion-pair liquid chromatography-thermally assisted hydrolysis and methylation–gas Chromatography–mass spectrometry system
- ITMS: ion trap mass spectrometer–LA-REMPI-TOF-MS: laser ablation–resonance-enhanced multiphoton ionization time-of-flight mass spectrometry
- LC: liquid chromatography
- LCC: lignin carbohydrate complex
- LLE: liquid–liquid-extraction
- LQIT: linear quadrupole ion trap
- MALDI-MS: matrix-assisted laser desorption/ionization mass spectrometry
- MW: molecular weight
- MALLS: multi-angle laser light scattering detector
- MS<sup>n</sup>: multiple stage tandem mass spectrometry
- PCA: principal component analysis
- py-GC: pyrolysis-gas chromatography
- qMS: quadrupole mass spectrometer
- QTOF: quadrupole/Time-Of-Fight hybrid mass spectrometer
- SEC: size exclusion chromatography
- SLE: solid-liquid extraction
- TCA: tricarboxylic acid
- TOF/MS: time of flight–mass spectrometry
- TOF-SIMS: time-of-Flight secondary ion mass spectrometry
- TQD: triple quadrupole mass spectrometer
- 2D–GC: two-dimensional GC
- UHPLC: ultra-high performance liquid chromatography

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