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Research review paper

Biological valorization of low molecular weight lignin

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ABSTRACT

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 $\times$ 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, however, 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 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 (Sjöstrom, 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 Na_2S (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 hydrosulfide 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

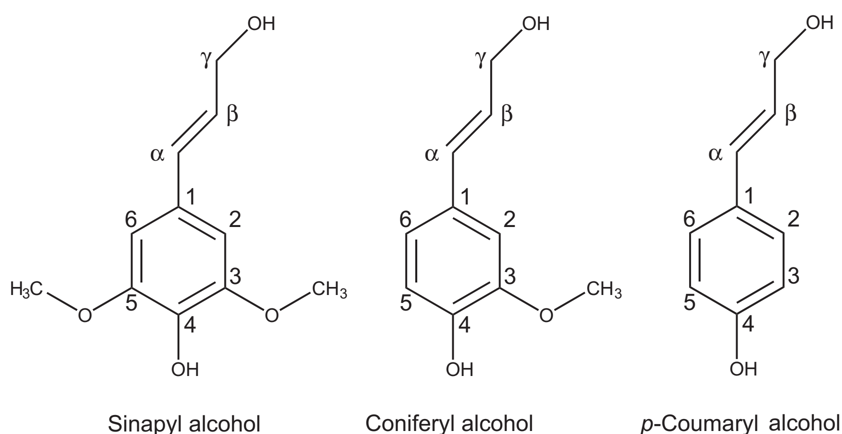

Fig. 1. The three primary monomeric building blocks of lignin (monolignols), showing also numbering of carbon atoms in the benzene ring and notation on the propylene side chain.

chemicals. The soda-anthraquinone (soda-AQ) process is based on soda (NaOH) and AQ, which catalyzes lignin depolymerization and retards the rate of alkaline peeling of carbohydrates. The soda has the same function as in the Kraft process, and AQ hence adds additional ether-bond breaking capability (Chung and Washburn, 2016).

The Alcell^{tm} and Organocell^{tm} processes are examples of organosolv processes, in which the removal of lignin takes place with an organic solvent. The former process uses an ethanol/water mixture with 50 wt% of each, and wood chips are cooked three times at 200 °C and 3.5 MPa. The same cooking conditions are used in the Organocell^{tm} process, but here a methanol/water mixture is used instead of the ethanol/water mixture. Furthermore, the product from the methanol/water cooking is 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 lignosulfonates, 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 sulfonate groups (Saake and Lehnen, 2007). The soda-AQ lignin has a relatively high availability, is low in price, and has high aliphatic and aromatic functionality (Calvo-Flores et al., 2015). A significant advantage 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 produced using organosolv processes (e.g. Alcel^{ltm} and Organocell^{tm}) (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 therefore 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 implementation. Lignosulfonates produced from the sulfite process is the dominating source of lignin for other applications than fuel, with a production of about 1 million tons per year (Strassberger et al., 2014). Lignosulfonates 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 (Werpy et al., 2004). In the lignin report, the production of a number of chemicals: fuels, macromolecules, syngas, hydrocarbons, 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).

Process	Availability	Price	Aliphatic/phenolic groups	Quality	Solubility in organic solvents	Sulfur content	Hemicellulose content
Kraft	High	Low	High	Varying	Insoluble	About 1 wt% (thiol groups)	High
Sulfite	High	Low	Low	Varying	Insoluble	5–6 wt% (sulfonate groups)	Some
Soda-Anthraquinone	High	Low	High	Varying	Partial	None	Some
Alcell™	Low	High	Etherified	Constant	High	None	None
Organocell™	Low	High	Etherified	Constant	High	None	None

Table 2
Examples of lignin use.

Principal use	Comments	References
Fuel	Fuel in recovery boiler in Kraft processes	Naqvi et al. (2012)
	Combined heat and power applications of lignin from 2nd generation ethanol. Could be a growing source of lignin in particular in US and Brazil	Hamelinck et al. (2005); Bugg and Rahmanpour (2015)
Concrete additive	Lignosulfonates have a plasticizing effect, and are used to give an increased pourability of concrete. Typical dosages are 0.1–0.3% by weight of cement.	Plank (2004)
Dispersant	For powder preparation in e.g. agriculture	
Animal feed additive	Lignosulfonates can be used in the production of feed pellets/blocks	Doherty et al. (2011)
Resins and adhesives	Lignin can be used to partly replace phenols in various types of adhesives and resins	Stewart (2008)
Vanillin production	Vanillin can be obtained through chemical processing of softwood lignin from sulfite pulping	Pacek et al. (2013)
Syngas	Gasification of lignin recovered from Kraft processing can give synthesis gas. This process has not gained commercial success yet.	Naqvi et al. (2012)

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 (Caballero et al., 1996; 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 H_2, CO, CO_2, CH_4, and minor amounts of compounds with two or more carbon atoms (C_2H_4, C_2H_6, C_3H_6, C_3H_8, and traces of C_4 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 compounds 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 as 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

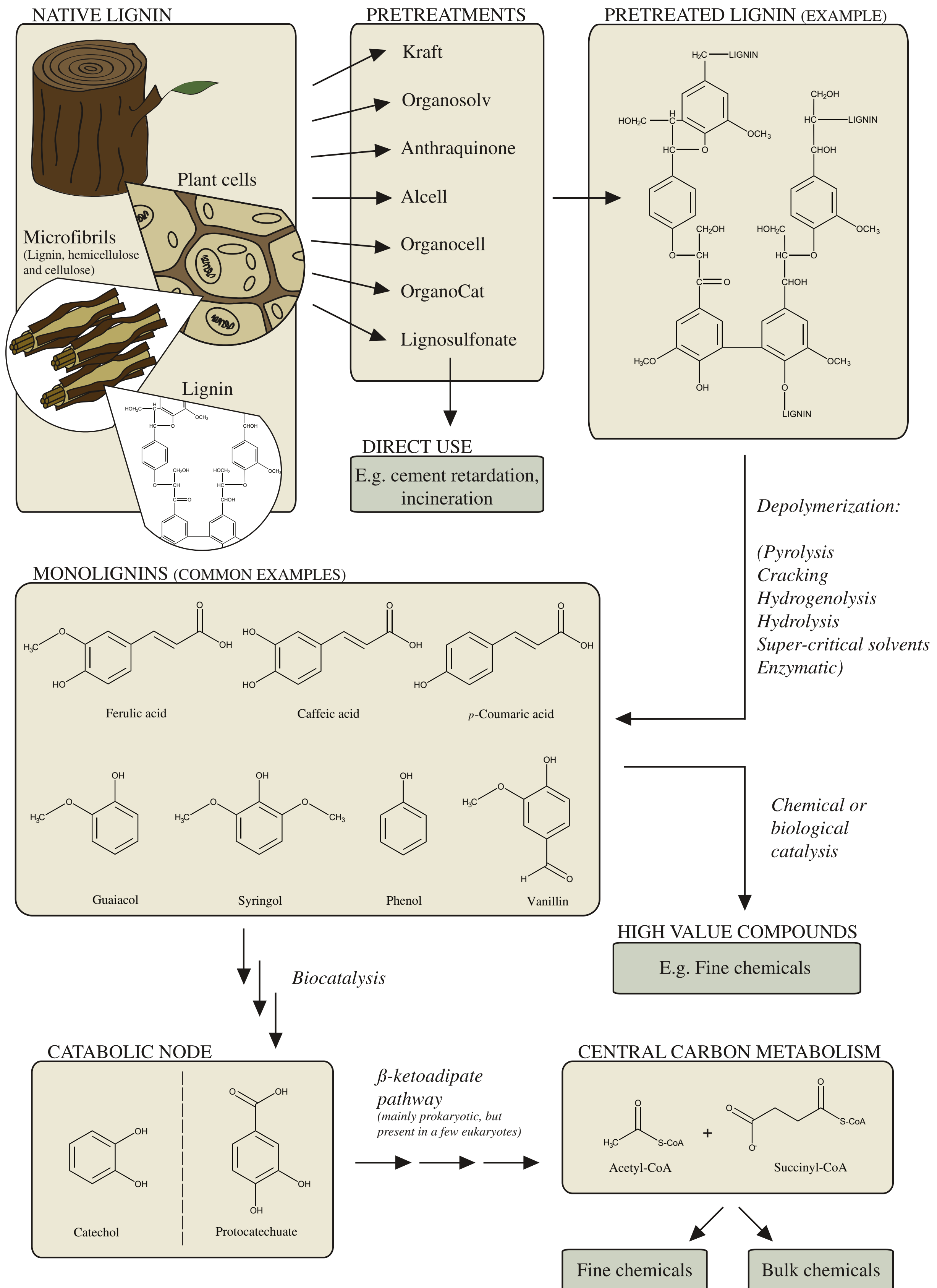

Fig. 2. Outline of the suggested process for valorization of lignin to chemicals.

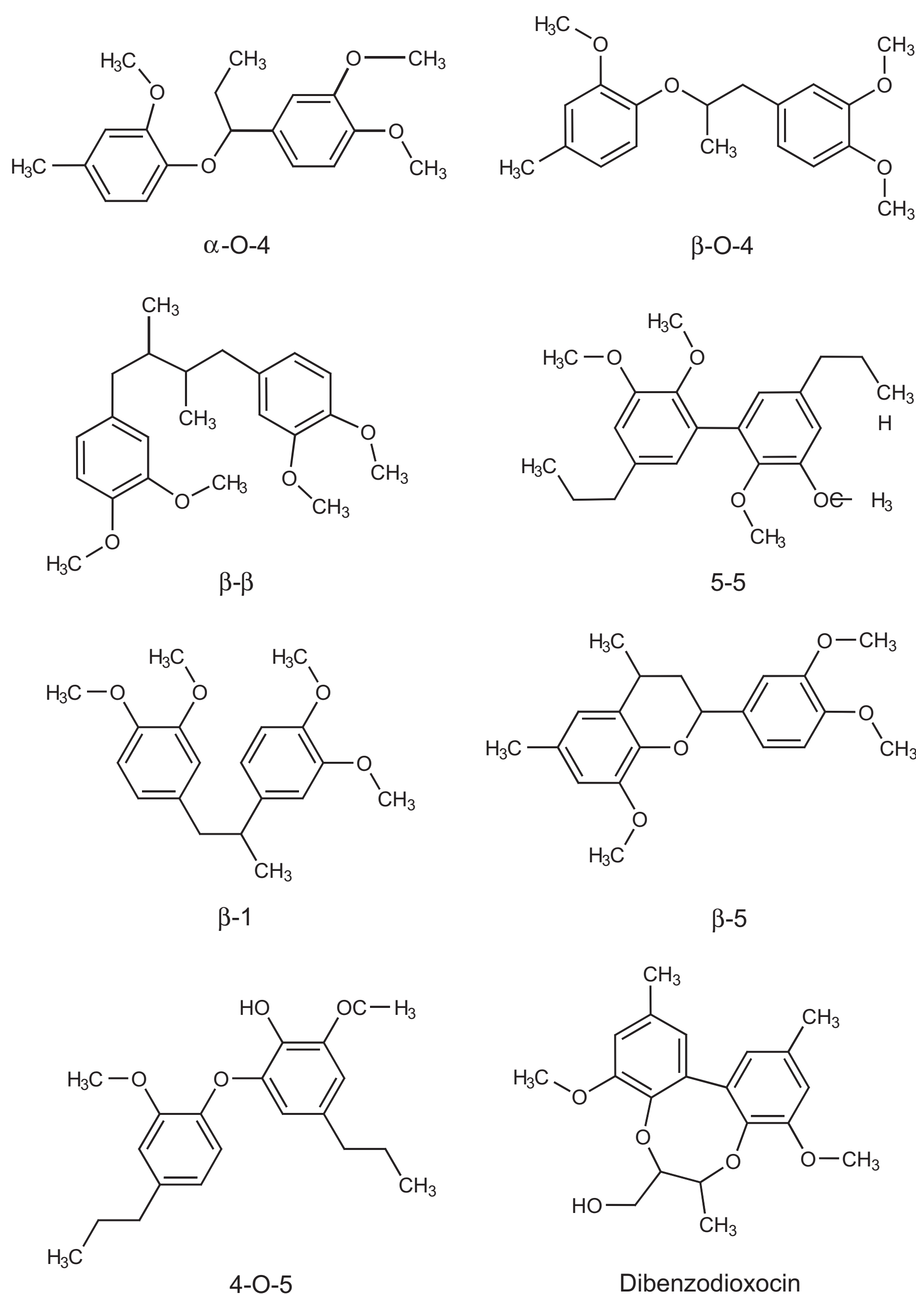

Fig. 3. Common linkages in lignin.

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 H_2SO_4), 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

Table 3
Common linkages and functional groups in lignin and their approximate proportions (Capanema et al., 2005; Chakar and Ragauskas, 2004; Sjöstrom, 1993; Zakzeski et al., 2010).

Linkage type	Share in softwood lignin (%)	Share in hardwood lignin (%)
β-O-4	45–50	60
5-5	10–27	3–9
β-5	9–12	6
α-O-4	2–8	7
β-β	2–6	3–12
β-1	7–10	1–7
4-O-5	4–8	7–9
Dibenzodioxocin	5–7	1–2
Functional group abundance per 100 C_9-units		
Aliphatic hydroxyl	115–120	88–166
Methoxyl	90–97	139–158
Phenolic hydroxyl	15–30	10–15
Carbonyl	10–20	17–24

conditions, i.e. high temperatures and pressures. The cleavage of functional groups may cause complete rearrangement of the carbon backbone 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 fragments 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; Oasmaa 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 Supathna, 1978; Şenol et al., 2007; Viljava et al., 2000; Yang et al., 2009; Zhao et al., 2009). These are usually a combination of Co, Mo, Pt, Ni, and W, which may be used on a support material (see e.g. Furimsky (2000)). Out of these, Co—Mo appears to be superior (Singh and Ekhe, 2015; Zakzeski et al., 2010) for producing monomeric phenols from the lignin.

Xu et al. investigated the depolymerization and hydrodeoxygenation of switch grass lignin using a formic acid hydrogen source, 20 wt% Pt/C catalyst, and ethanol solvent (Xu et al., 2012a). At a temperature of 350 °C, this combination yielded phenolic monomers and improved the O/C and H/C molar ratios. Furthermore, Cu-doped hydrotalcite-like precursor emerged to play a significant role in minimizing char formation and favoring the required hydrogenolysis, dehydration, and hydrogenation stages without reducing the aromatic rings, attaining catechols as major products from the complex lignin biopolymer (Barta et al., 2014). Jongerius et al. also proposed a two-step approach to the conversion of lignin to monoaromatic compounds of low oxygen content (Jongerius et al., 2013). The first step entails lignin depolymerization in a liquid phase reforming reaction using 1 wt% Pt/γ-Al_2O_3 catalyst at 225 °C in alkaline ethanol–water resulting in a decrease in lignin molecular weight. The lignin-oil isolated by extraction of the ethanol–water solution is further subjected to a hydrodeoxygenation reaction in the second conversion step, which is typically performed in dodecane at 300 °C under 5 MPa hydrogen pressure over $CoMo/Al_2O_3$ and carbon nanofiber-supported molybdenum carbide (Mo_2C/CNF) catalysts.

In an excellent contribution towards developing effective methods for lignin valorization, Song et al. revealed that nickel-based catalysts are highly active and selective in native lignin conversion towards producing various monomeric phenols (Song et al., 2013). Results indicated that lignin can be selectively cleaved into propylguaiacol and propylsyringol with total selectivity >90% at a lignin conversion of about 50%. This work contributes in understanding the behavior of the native lignin conversion and the monomeric phenolics formation through reductive depolymerization. In another example, Wang and Rinaldi demonstrated a novel route for the conversion of lignin to arenes (Wang and Rinaldi, 2013). The one-pot procedure was conducted in the presence of Raney Ni and β-zeolite using 2-propanol as H-donor in a molar ratio of 2-propanol to phenol ranging between 1 and 3. This lignin conversion, giving low boiling point arenes rather than high boiling phenols, facilitates lignin valorization by conventional petroleum refineries.

The efforts made on *homogeneous* catalysis in the hydrogenolysis of lignin are relatively small compared to the extensive research efforts on heterogeneous catalysis. The first studies with water soluble metal salts in biomass liquefaction was reported in 1931 by Lindblad (Lindblad, 1931), who used Ni, Co and Fe salts for the hydrotreatment of sawdust. Later salts of Ni and Mo have been reported as effective catalysts (Rogers et al., 1981). Oasmaa and Johansson managed to deoxygenate Kraft lignin to the degree of 98% by using a water soluble salt of Mo at 20 MPa (Oasmaa and Johansson, 1993). The most common approach appears to be the use of Rh-based complex with various organic anions such as 1,5-C_6H_{10}, triphenylphosphine (PPh_3), and C_6H_6 (Hu et al., 1997; Plasseraud and Süss-Fink, 1997; Suarez et al., 2006) or colloidal rhodium (Nasar et al., 1994; Zhao et al., 2007). An inorganic salt was, however, reported by Nagy et al. (Nagy et al., 2009). In most cases, the partial pressure of hydrogen has been relatively low (0.1–5 MPa), but there are exceptions with pressures of almost 10 MPa. The temperatures applied are low compared to the heterogeneous catalysis experiments, ranging from room temperature to 378 K.

During hydrogenolysis with gaseous hydrogen, depolymerization and char formation depends on the hydrogen partial pressure (Pandey and Kim, 2011). High partial pressure of hydrogen suppresses char formation and promotes depolymerization. During thermolysis of peat, large volume of gas (mainly CO_2) is evolved which dilutes the added hydrogen 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 hydrogen 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 latter will be dealt with in a later section.

Karagöz et al. used the carbonate salt of Rb and Cs for producing phenol by hydro-liquefaction of wood biomass (Karagöz et al., 2004). In a treatment at 553 K for 15 min, in which these salts were added at a concentration of 1 M, mainly phenolic and benzenediol derivatives were obtained. In the thermal process without the catalysts, the product distribution 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 depolymerization of lignin using NaOH and other basic media have shown to be an efficient 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 phenolic 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.,

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 biopolyols 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). Secondly, 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 organosolv lignins in a compressed fluid of carbon dioxide/acetone/water at 300 °C and 100 bar into 10–12% aromatic monomers through adding small amounts of formic acid as 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 $CuMgAlO_x$ 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. Catallo 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 alkane and alkene gases (C_2 to C_5). 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 viridosporus* 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 acetosyringone, 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 (Grelier and Koumbe, 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 Mn^{3+}, 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 *Thanatephorus 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

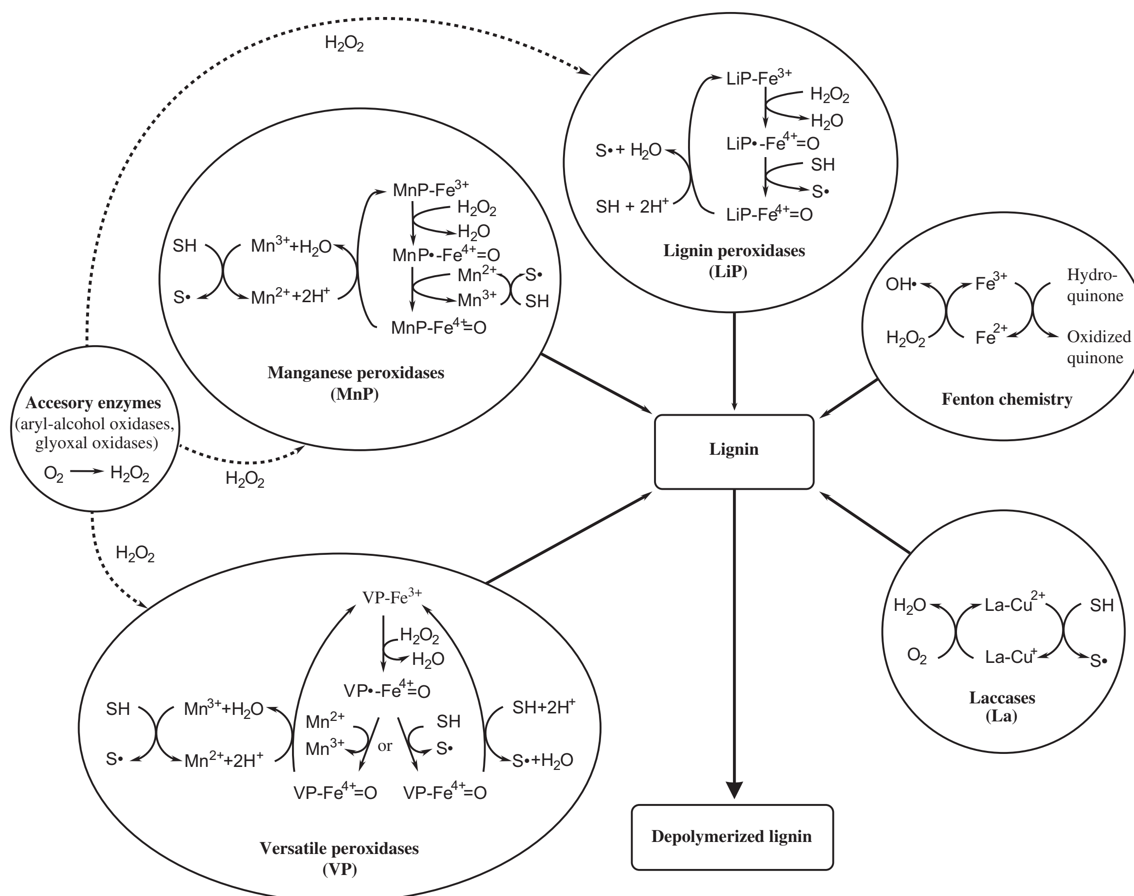

Fig. 4. Schematic representation of enzyme catalyzed lignin depolymerization. Aromatic radicals are generated through the oxidative action of laccases or peroxidases. Through reactions of these radicals lignin depolymerization will take place. Accessory enzymes generate hydrogen peroxide needed for the function of peroxidases. Attack on lignin in the initial stages, when the small size of pores in the still-intact cell wall prevents the penetration of ligninolytic enzymes, may take place by free hydroxyl radicals generated by Fenton chemistry. (Substrate is denoted by 'SH', which are phenolic lignin units in case of laccases, non-phenolic lignin units in case of lignin peroxidases, and non-phenolic/phenolic lignin units in case of manganese peroxidases and versatile peroxidases).

different C—C and C—O linkages in lignin, but it is crucial to enable an efficient biological upgrading of lignin. Only smaller fragments produced can subsequently be taken up by microbes and channeled into the intracellular catabolic routes. The two aspects of depolymerization reported here, chemical or enzymatic, pose different challenges. In the chemical depolymerization, controlling the char formation and preventing repolymerization of the produced monomers is crucial in order to obtain low molecular weight compounds. However, it is also important to avoid formation of compounds inhibiting microbial growth. The advantage of the chemical depolymerization is the high reaction rate, while the low reaction rate is a drawback with enzymatic depolymerization. Advantages of enzymatic depolymerization, e.g. high selectivity towards fragments which are more easily channeled into the microbial catabolic routes, may outweigh the lower reaction rate. The selection of depolymerization method needs to be based on the entire conversion process, i.e. not only the depolymerization itself but also the later biological conversion must be taken into account to avoid system sub optimization.

The small fragments from depolymerization may also be interesting compounds themselves – possibly after minor modifications. The further biocatalytic conversion of smaller lignin fractions, as well as monolignols, will be described in Section 3.

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).

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

Table 4
Summary of selected key publications in lignin depolymerization: 2005–2015.

Lignin substrate	Catalyst	Conditions	Major product(s)	Yield, wt%	References
Birch wood sawdust	Ru/C, Pd/C, Rh/C, Pt/C	200/250 °C, 40 bar	C_8–C_9 alkanes	42	Yan et al. (2008)
			C_{14}–C_{18} alkanes	10	
			Methanol	11	
Wood flours from Japanese beech (*Fagus crenata*)	1-ethyl-3-methylimidazolium chloride	90–120 °C	Sugars	Drop in mean MW	Miyafuji et al. (2009)
Organosolv lignin/model compounds	Various brønsted acid catalysts	180 °C, 69 bar	Guaiacol	11.6	Binder et al. (2009)
Kraft lignin	$H_3PMo_{12}O_{40}$ (phosphomolibdic acid)	170 °C, 10 bar	Vanillin	4.6	Voitl and Rohr (2009)
			Methyl vanillate	4.2	
Organosolv lignin	NaOH (2 wt%)	300 °C, 250 bar	Monomeric phenols and oligomers	85	Roberts et al. (2011)
Organosolv wheat straw	–	300–370 °C, 100 bar	Syringic acid	2	Gosselink et al. (2012)
Organosolv hardwood			Syringol	3.6	
Switchgrass lignin	20 wt% Pt/C, formic acid hydrogen source, ethanol solvent	350 °C	Monomeric guaiacol-type products	21	Xu et al. (2012b)
Aspen hardwood lignin/model compounds	4-acetamido-TEMPO in combination with HNO_3 and HCl (10 mol% each)	45 °C, 1 bar O_2	Ketones and acids	77–98	Rahimi et al. (2013)
Birch sawdust	Ni/C	200 °C, 1 bar Ar	Propenylguaiacol and propenylsyringol	54	Song et al. (2013)
Organosolv lignin (*Miscanthus giganteus*)	Vanadium catalysts	80 °C	Volatile monophenolic compounds	3	Chan et al. (2013)
Organosolv	Pt/Al_2O_3; $CoMo/Al_2O_3$ or Mo_2C/CNF	225 °C, 58 bar Ar; 300 °C, 55 bar H_2	Alkylated phenol, guaiacol, syringol-type products	11	Jongerius et al. (2013)
Kraft				9	
Sugarcane bagasse				5	
Organosolv lignin	Raney Ni and β-zeolite	2 h at160 °C; 2 h at 240 °C	Arenes	78	Wang and Rinaldi (2013)
			Alkanes	18	
			Phenols	4	
Organosolv lignin (*Aleurites moluccana*)	Cu-PMO	140–220 °C, 30–60 bar H_2	C_9 catechols and oligomers	60–93	Barta et al. (2014)
Soda lignin	$CuMgAlO_x$	300 °C, 10 bar	Aromatic monomers	23	Huang et al. (2014)
Organosolv and ball-milled lignin	Ni-HTC	270 °C	Alkyl-aromatic products	Decrease in MW	Sturgeon et al. (2014)
Organosolv lignin	NiAu	170 °C	Aromatic monomers	14	Zhang et al. (2014a)
Soda/Alcell™ Organosolv/Kraft	$CuMgAlO_x$	380 °C, 10 bar	Alkylated aromatics and cycloalka(e)nes	60–86	Huang et al. (2015)
Organosolv lignin	Mesostructured silica	200–350 °C, 1 bar	Syringol	23	Klamrassamee et al. (2015)
Birch sawdust (*Betula pendula*)	DDQ/tBuONO/O_2	80 °C	Phenolic monomers	5	Lancefield et al. (2015)
Kraft lignin	Cu/Mo loaded zeolite ZSM-5	220 °C	Alkyl phenols	22.5	Singh and Ekhe (2015)
Wood sawdust	$B(C_6F_5)_3$	Ambient T and P	Phenol derivatives	7–24	Feghali et al. (2015)

(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 propionic acid. This can in turn subsequently be converted to phenyl acetic acid. Aerobically, however, caffeic acid is typically oxidized to protocatechuic acid (Grbić-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 monomers 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 catabolic conversion of the lignin monomers. This will be central – regardless of end-product.

3.2. Biological 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 plethora of microbes present in the surroundings, or even endosymbiontic, 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; Zaldivar et al., 1999) constitutes a beneficial niche that may prove a competitive advantage of aromatic-degrading microbes over less tolerant ones, also to non-lignolytic 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 protocatechuate; 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 endpoint 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, good chemical analysis pipelines (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; Vinuselvi 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 lignocellulose biorefineries. As previously pointed out in Section 2, products from lignin depolymerization are very heterogeneous. This is true also for phenylpropenoid 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 hydroxycinammic 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 hydratases/lyases: After the initial activation, the propenoid 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.) (Masai 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-ketoadipate pathway (see Section 3.2.3) 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

Table 5
Enzymes in funneling pathways.

Enzyme type	Substrate	Product	Enzyme name	Microorganism	References
Acyl-CoA synthetase	Ferulate/*p*-coumarate/caffeate	Feruloyl-/p-coumaryl-/caffeoyl-CoA	HcaC	*Acinetobacter baylyi*, *Cupriavidus necator* JMP134	Fischer et al. (2008); Bleichrodt et al. (2010); Pérez-Pantoja et al. (2010)
	Ferulate/*p*-coumarate/caffeate/sinapate	Feruloyl-/p-coumaroyl-/caffeoyl-/sinapyl-CoA	FerA	*Sphingobium* sp. SYK-6	Masai et al. (2007)
	Ferulate	Feruloyl-CoA	Fcs	*Pseudomonas* sp. HR199, *P. putida* KT2440	Overhage et al. (1999); Jimenez et al. (2002)
Acyl-CoA hydratase/lyase	Feruloyl-/p-coumaroyl-/caffeoyl-CoA	Vanillin/p-hydroxybenzaldehyde/protocatechuic aldehyde	HcaA	*Acinetobacter baylyi*, *C. necator* JMP134	Fischer et al. (2008); Bleichrodt et al. (2010); Pérez-Pantoja et al. (2010)
			FerB, FerB2	*Sphingobium* sp. SYK-6	Masai et al. (2007)
			Ech	*P.* sp. HR199, *P. putida* KT2440, *P. fluorescens* AN103	Overhage et al. (1999); Jimenez et al. (2002)
Dehydrogenase	Vanillin/p-hydroxybenzaldehyde/ protocatechuic aldehyde	Vanillate/p-hydroxybenzoic acid/protocatechuate	HcaB	*A. baylyi*, *C. necator* JMP134	Fischer et al. (2008); Bleichrodt et al. (2010); Pérez-Pantoja et al. (2010)
	Vanillin	Vanillate	Vdh	*P.* sp. HR199, *P. putida* KT2440	Overhage et al. (1999); Jimenez et al. (2002)
	β-O-4 dimer, Cα alcohol type	β-O-4 dimer, Cα carbonyl type	LigD	*Sphingobium* sp. SYK-6	Masai et al. (2007)
	Coniferyl alcohol	Coniferyl aldehyde	CalA	*P.* sp. HR199	Overhage et al. (1999); Jimenez et al. (2002)
	Coniferyl aldehyde	Ferulate	CalB	*P.* sp. HR199, *P. putida* KT2440	
	Benzoate diol	Catechol	BenD	*P.* sp. PRS2000, *P. putida* KT2440, *C. necator* JMP134	Jimenez et al. (2002); Pérez-Pantoja et al. (2010)
Decarboxylases	Vanillate	Guaiacol	VdcB, C, D	*Streptomyces* sp. D7	Chow et al. (1999)
	5-carboxyvanillate	Vanillate	LigW	*Sphingobium* sp. SYK-6	Masai et al. (2007)
	Protocatechuate	Catechol	AroY	*Enterobacter cloacae* P241	Yoshida et al. (2010); Vardon et al. (2015)
O-Demethylase systems	Vanillate	Protocatechuate	VanA, B	*A. baylyi*, *P. putida* WCS358, *P. putida* KT2440	Fischer et al. (2008); Bleichrodt et al. (2010); Jimenez et al. (2002)
			LigM	*Sphingobium* sp. SYK-6	Masai et al. (2007)
	5–5′-dehydrodivanillate (Biphenyl dimer)	Demethylated biphenyl dimer	LigX		
	Methyl-THF	THF	LigH MetF		
	Syringate	3-*O*-methylgallate	DesA		
	Vanillate	Protocatechuate + Formaldehyde	VanA1B	*Bradyrhizobium japonicum*	Sudtachat et al. (2009)
	Corrinoid protein, primary methyl acceptor		Component A	*Acetobacterium dehalogenans*	Kaufmann et al. (1998)
	Vanillate/guaiacol/syringate/isovanillate	Protocatechuate/catechol/3-*O*-methylgallate/ gallate + methylated component A	Component B		
	Inactivated component A	Activated component A	Component C		
	Methylated component A + THF	Demethylated component A + CH_3-THF	Component D		

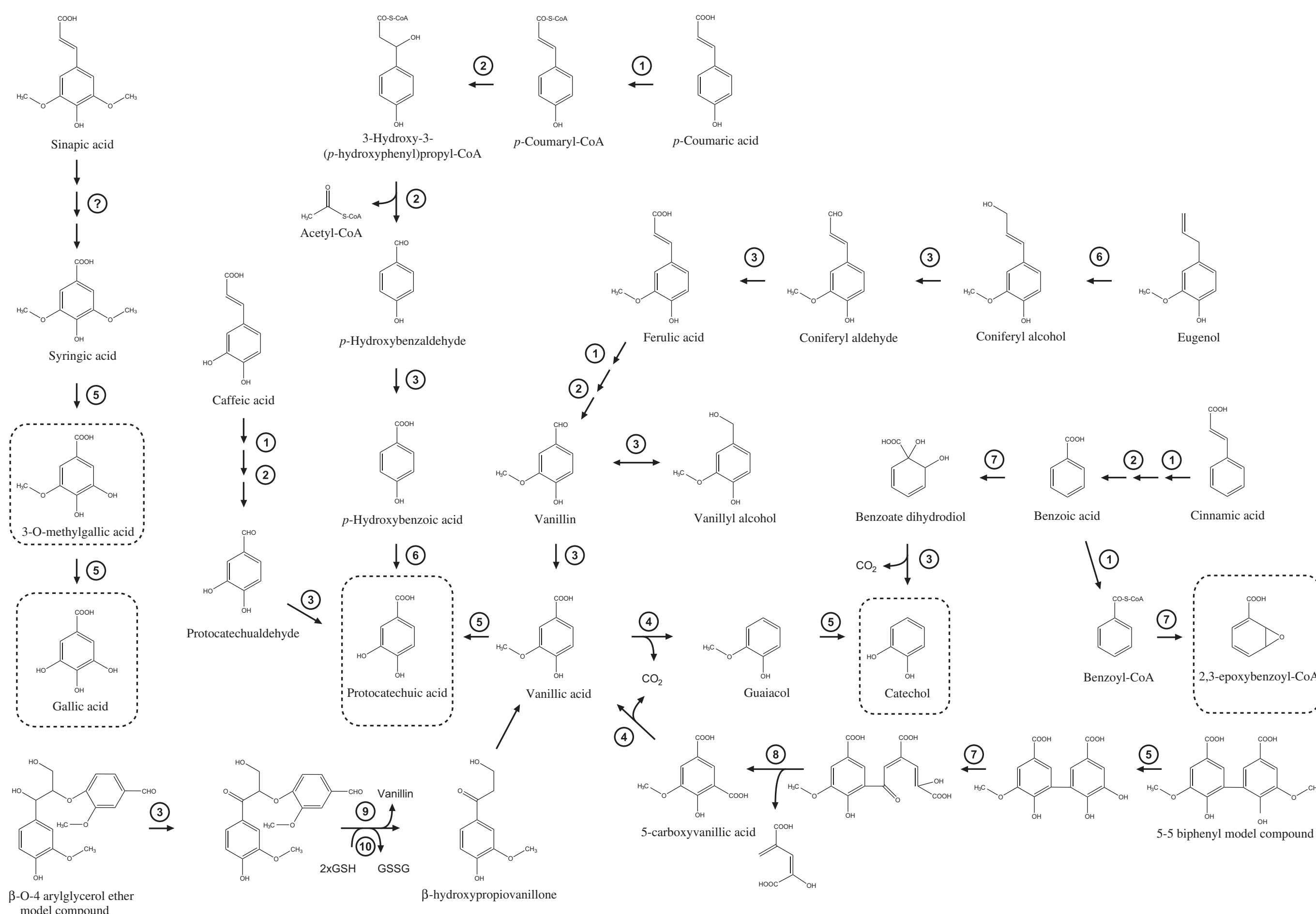

Fig. 5. Funneling pathways for the microbial catabolism of lignin-related aromatic compounds. Only several well-known examples are depicted, but different pathways can be observed in different microorganisms and environmental conditions. Most cofactors are omitted for the sake of simplicity. Enzyme type is indicated in each reaction step with the following code: **1**: Acyl-CoA synthase, **2**: Enoyl-CoA hydratase/lyase, **3**: Dehydrogenase, **4**: Decarboxylase, **5**: Demethylase system, **6**: Hydroxylase, **7**: (Di)oxygenase, **8**: Hydrolase, **9**: β-etherase, **10**: Glutathion-S-tranferase. Compounds enclosed in a dotted box can be subjected to enzymatic ring opening for further degradation.

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-ketoadipate pathway, as will be discussed in the following section. The most central node in lignin product metabolism is protocatechuic acid, followed by catechol, but there are also other aromatic molecules that can be subjected to ring cleavage, such as 3-*O*-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 phenolic 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

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

Organism	Common strains	Type and origin	Known pathways and enzymes	Genome sequenced?	Known carbon sources	References
Eukaryotes (fungi and yeast)						
Anthracophyllum discolour	Acevedo isolate	White rot fungi; forest	Mn peroxidase; laccase; lignin peroxidase	No	Three- and four-ring PAHs: phenanthrene, anthracene, fluoranthene, pyrene, benzo(*a*)pyrene	Acevedo et al. (2011)
Aspergillus nidulans	FGSC A4	Filamentous fungi	Eukaryotic β-ketoadipate pathway	Yes	Mono-chlorophenols and -chlorocatechols	Galagan et al. (2005); Martins et al. (2014); Martins et al. (2015)
Candida parapsilosis	CBS604	Yeast; pathogen	Variants of the eukaryotic β-ketoadipate and gentisate pathways	Yes	3-hydroxybenzoate, 4-hydroxybenzoate, gentisate, hydroquinone, protocatechuate, resorcinate, resorcinol	Middelhoven et al. (1992); Butler et al. (2009); Holesova et al. (2011)
Candida tropicalis	HP15; ATCC 20240 MYA-3404; JH8	Yeast; opportunistic pathogen	Eukaryotic β-ketoadipate pathway	Yes	phenol, resorcinol, quinol, hydroxyquinol, catechol; to a lesser extent 4-chloro-catechol, protocatechuate, *p*-cresol, *m*-chlorophenol, and *p*-chlorophenol; Kraft black liquor; Olive mill wastewater	Krug et al. (1985); Krug and Straube (1986); Potvin et al. (1988); Butler et al. (2009); Martinez-Garcia et al. (2009); Wang et al. (2011)
Fusarium oxysporum	MUCL 30736; Strain 4287	Filamentous fungi; soil	Eukaryotic β-ketoadipate pathway; extracellular peroxidases and laccases	Yes	Wheat straw lignin; ferulic acid, coumaric acid, vanillic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, cinnamic acid, catechol	Rodriguez et al. (1996); Ma et al. (2010); Michielse et al. (2012)
Phanerochaete chrysosporium	ATCC 34541; BKM-F-1767	White rot fungi	*Ortho*-cleavage pathway; phenanthrene metabolism	Yes	Biphenyl, biphenylene, dibenzofuran, dibenzo-*p*-dioxin, diphenyl ether, vanillin, phenol, hydroquinone, 4-chlorophenol, 4-nitrophenol; phenanthrene; cornstalk, cotton stalk, Olive pomace	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); Ichinose and Wariishi (2012); Zhao et al. (2012)
Rhodotorula graminis	WP1; Durham isolate	Yeast; soil	Eukaryotic β-ketoadipate pathway	Yes	Catechol, Protocatechuate, phenylalanine, mandelate, benzoate, *m*-hydroxybenzoate, salicylate	Durham et al. (1984); Firrincieli et al. (2015)
Rhodotorula mucilaginosa	CBS17	Yeast	Eukaryotic β-ketoadipate pathway	Yes	Caffeic acid, *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, protocatechuate, vanillic acid	Cook and Cain (1974); Sampaio (1999); Deligios et al. (2015)
Trametes versicolor	Strain 1	White rot fungi	Putative eukaryotic β-ketoadipate pathway; laccase	Yes	*p*-creosol, phenol	Cassland and Jönsson (1999); Alexieva et al. (2010); Floudas et al. (2012)
Trichoderma reesei	QM6a	Filamentous fungi	Laccase, peroxidase, glyoxal oxidase	Yes	Putative lignin degradation potential	Martinez et al. (2008); Adav et al. (2012)
Trichosporon cutaneum	–	Yeast, oleaginous	β-ketoadipate pathway	Yes	Catechol, gentisate, homoprotocatechuate, resorcinol, phenol, protocatechuate	Gaal and Neujahr (1979); Anderson and Dagley (1980)
Prokaryotes						
Acinetobacter baylyi	ADP1	γ-Proteobacterium; Gram negative,	β-ketoadipate pathway	No	Alkaline pretreated liquor from corn stover; *p*-coumaric acid, ferulic acid, 4-HBA, vanillic acid	Bleichrodt et al. (2010); Salvachúa et al. (2015)
Agrobacterium tumefaciens	B6;	α-Proteobacterium;	(Inducible) β-ketoadipate pathway	Yes	4-hydroxybenzoic acid	Parke and Ornston (1986);

(continued on next page)

Table 6 (*continued*)

Organism	Common strains	Type and origin	Known pathways and enzymes	Genome sequenced?	Known carbon sources	References
(Rhizobium radiobacter)	C58	Gram negative; soil				Parke (1997) Goodner et al. (2001)
Aneurinibacillus aneurinilyticus	AY856831	Bacillus; Gram positive; Paper mill sludge	oxidation of the sinapylic (G) and coniferylic (S) alcohol units in lignin	Yes	Kraft lignin	Chandra et al. (2007); Raj et al. (2007a)
Bacillus atrophaeus	B7	Bacillus; Gram positive; rain forest soil	Laccase	Yes	Kraft lignin; guaiacylglycerol-β-guaiacyl ether	Liu et al. (2012); Huang et al. (2013)
Cupriavidus necator	JMP134	β-Proteobacterium; Gram negative, soil	β-ketoadipate, (methyl)catechol *meta*-cleavage, gentisate, homogentisate, benzoyl-CoA, phenylacetyl-CoA, 2-aminobenzoyl-CoA, 2,3-dihydroxyphenylpropionate, (chloro)hydroxyquinol, (amino)hydroquinone, 3-hydroxyanthranilate pathways	Yes	60 aromatic compounds are known to serve as a sole carbon source in this organism	Pérez-Pantoja et al. (2008); Lykidis et al. (2010)
Corynebacterium glutamicum	ATCC13032	Actinobacterium; Gram positive; soil	β-ketoadipate, hydroxyquinol, mycothiol-dependent gentisate pathways	Yes	Catechol, protocatechuate, 4-cresol, gentisate, resorcinol	Kalinowski et al. (2003); Qi et al. (2007)
Enterobacter lignolyticus	SCF1	γ-Proteobacterium; Gram negative, facultative anaerobic; rain forest soil	4-hydroxyphenylacetate degradation pathway, glutathione biosynthesis and GST pathways, catalase/ peroxidaseHPI and DypB-type peroxidase	Yes	Alkali lignin (Sigma Aldrich)	DeAngelis et al. (2011); DeAngelis et al. (2013)
Klebsiella pneumoniae	M5a1	γ-Proteobacterium; Gram negative; soil	Gentisate pathway	Yes	Kraft lignin, Benzoic acid, p-Hydroxybenzoic acid, Protocatechuic acid	Deschamps et al. (1980); Jones and Cooper (1990); Pang et al. (2016)
Oceanimonas doudoroffii	JCM21046T	γ-Proteobacterium; Gram negative, marine	Lignolytic enzymes, aromatic fission pathways to yield acetyl-CoA with endpoint formation of polyhydroxyalkanoate (PHA)	No	Lignin; *p*-coumaric acid, caffeic acid, ferulic acid, sinapinic acid, 3-HBA, 4-HBA, 2,5-DHBA,3,4-DHBA, vanillic acid, syringic acid, and gallic acid	Numata and Morisaki (2015)
Pseudomonas putida	KT2440; mt-2	γ-Proteobacterium; Gram negative; soil	β-ketoadipate, phenylacetyl-CoA, homogentisate, gentisate, and homo-protocatechuate pathways	Yes	Catechol, protocatechuate, benzoate, *p*-hydroxybenzoate, benzylamine, phenylacetate, phenylalanine, tyrosine, phenylethylamine, phenylhexanoate, phenylheptanoate, phenyloctanoate, coniferyl alcohol, *p*-coumarate, ferulate, caffeate, vanillate, nicotinate and quinate	Nelson et al. (2002); Jimenez et al. (2002); Poblete-Castro et al. (2012)
Rhodococcus erythropolis	A5.1; TA421; 1CP; XP	Actinobacterium; Gram positive; Termite gut	Polychlorinated biphenyl (PCB) catabolic pathway, β-ketoadipate pathway	Yes	Biphenyl, PCB, *m*-cresol,*p*-cresol, vanillic acid, veratryl alcohol; (low molecular weight) Kraft lignin	Maeda et al. (1995); Eulberg et al. (1997); Tao et al. (2011) Taylor et al. (2012)
Rhodococcus jostii	RHA1	Actinobacterium; Gram positive; soil	E.g. the β-ketoadipate and gentisate pathways (in total 26 peripheral aromatic pathways); DypB lignin peroxidase	Yes	Lignin model compounds (β-aryl ether lignin dimers), Kraft lignin, polychlorinated biphenyls (PCB), vanillin	McLeod et al. (2006); Ahmad et al. (2011); Chen et al. (2012)
Rhodococcus opacus (*Nocardia* sp.)	DSM 1069; PD630	Actinobacterium; Gram positive; soil	β-ketoadipate pathway	Yes	Coniferyl alcohol, 4-Hydroxybenzoic acid, vanillic acid	Holder et al. (2011); Kosa and Ragauskas (2012)
Rhodopseudomonas palustris	CGA009	α-Proteobacterium; Gram negative	Anaerobic, photoheterotrophical growth on phenylpropenoid lignin monomers	Yes	Caffeate, cinnamate, coumarate, ferulate	Larimer et al. (2004); Salmon et al. (2013)
Sphingomonas paucimobilis (*Sphingobium* sp.)	SYK-6 (NBRC 103272)	α-Proteobacterium; Gram negative; soil	Protocatechuate 4,5-cleavage pathway, syringate degradation through multiple 3MGA degradation pathways	Yes	Lignin-derived biaryls and monoaryls, 5,5′-dehydrodivanillate (DDVA), β-aryl ether, phenylcoumarane, pinoresinol, diarylpropane, biphenyl	Masai et al. (1999a); Kasai et al. (2004); Peng et al. (2005); Masai et al. (2012)
Streptomyces viridosporus	T7A (ATCC 39115)	Actinobacterium; Gram positive; soil	β-ketoadipate pathway; extracellular peroxidase	Yes	Wheat straw, protocatechuate	Ramachandra et al. (1988); Davis and Sello (2010); Davis et al. (2013); Zeng et al. (2013)
Treponema primitia	ZAS-1; ZAS-2	Spirochete; Gram negative; termite gut	Catechol *meta*-cleavage pathway	Yes	Catechol, protocatechuic acid, hydrocaffeic acid and caffeic acid	Rosenthal et al. (2011); Lucey and Leadbetter (2014)

(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 Ornston, 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 microbial *ortho* and *meta* pathways converge on the tricarboxylic acid cycle (TCA) via their end-product formation (Bugg et al., 2011; Wells and Ragauskas, 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^+-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 cultivatable 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-oxoadipate pathway) 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 Ragauskas, 2012). Using *P. putida* as a model organism, the constituents and biochemistry of the different branches of the ß-ketoadipate pathway was elucidated in the mid-1960s by Ornston and Stainer (Ornston 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 Fujii, 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 lignin catabolism 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 bioremediation of pollutants and valorization of lignocellulosic waste streams (Wells and Ragauskas, 2012). The protocatechuate branch of *R. jostii* RHA1 has recently been reconstructed in vitro (Yamanashi 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).

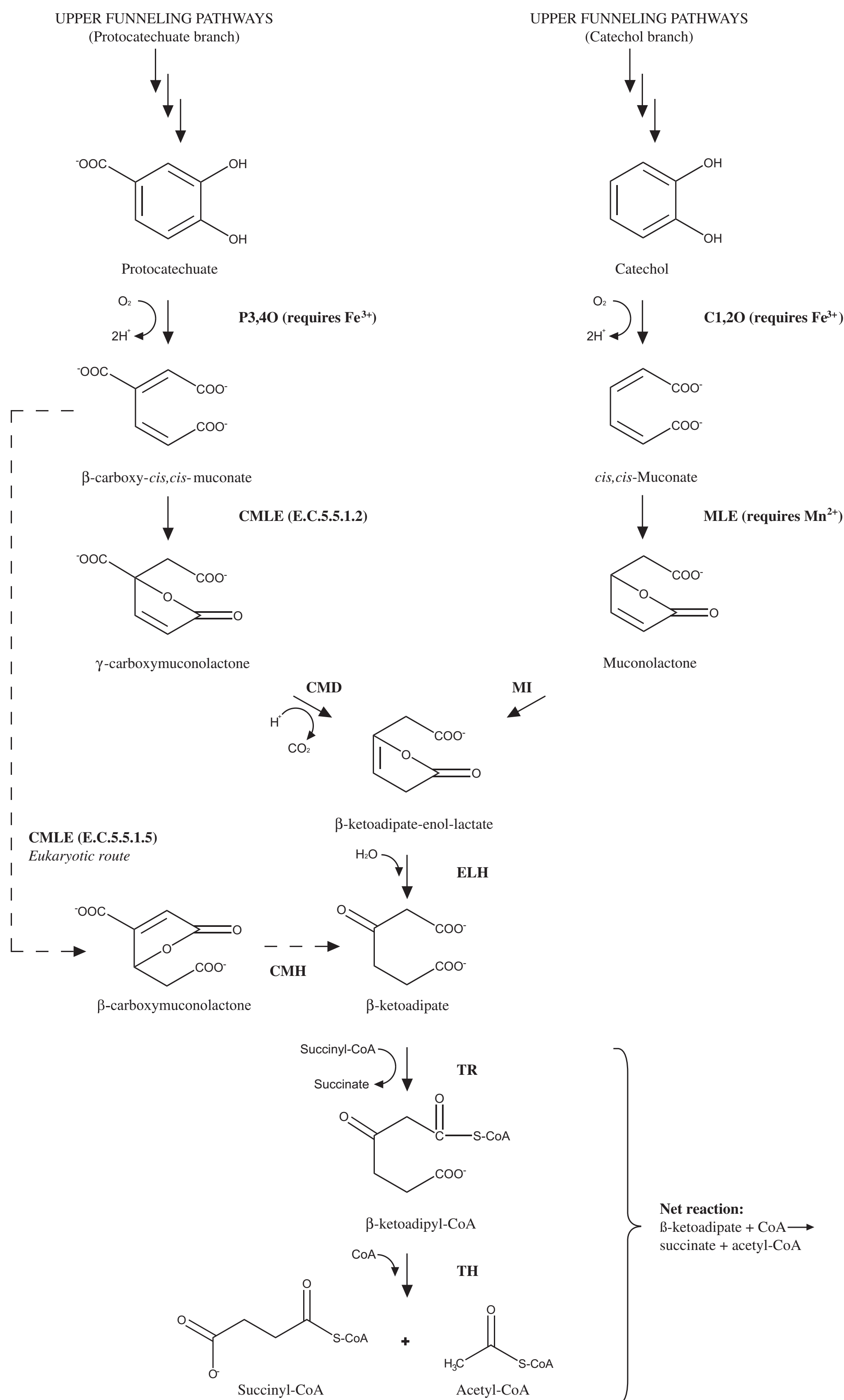

Fig. 6. The eukaryotic and prokaryotic β-ketoadipate pathways. Solid arrows indicate the reactions present in prokaryotes. The eukaryotic and prokaryotic pathways coincide except for the reactions in dashed arrows, which show the divergent route in the eukaryotic protocatechuate branch (Cook and Cain, 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 *Rhodococci* 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-etherases and glutathione lyases (from *Sphingobium* sp. SYK-6, *Novosphingobium* sp. PP1Y and *Thiobacillus denitrificans* ATCC 25259) as lignin depolymerization biocatalysts (Picart et al., 2015). The authors set up proof of concept one-pot processes combining the different β-etherases 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. 75iv2, *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 total lignin analysis is, however, not covered here. A comprehensive review was recently written by Lupoi 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 (Lupoi 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 7 summarizes 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 ionic liquids 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 (Sumerskiy 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 (Sumerskiy et al., 2015). In some of the studies, fractionations 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 pretreatment and detection techniques, have long been prevalently used in the analysis of lignin and lignin–carbohydrate complexes structures and linkages (del Río et al., 2012a; del Río et al., 2012b; Du et al., 2013; Du et al., 2014; Lupoi et al., 2015), the evaluation of lignin monomeric units (del Río 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; Lourenço et al., 2013). py-GC with a flame ionization detector (py-GC-FID) was proven to give comparable precision to that of the standard Klason 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 SiO_2 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^n) 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^n, 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; Savy 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 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 (Bogolitsyna et al., 2011; Dupont et al., 2007; Gebremeskel and Aldaeus, 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.,

Table 7
Chromatographic techniques applied to lignin samples.

Analytical purpose	Original sample type	Sample preparation	Chromatographic system	Mass spectrometric technique	References
Lignin-derived compounds	Wheat straw lignin	Ozone and soaking aqueous ammonia (OSAA) pre-treatment — pyrolysis	Py-GC–MS	EI-quadrupole	Azadfar et al. (2015)
	Non-woody lignin (agricultural waste including walnut shell, wheat straw, cotton stalk, rice husk, bamboo, rape straw, corncob and peanut shell)	Pyrolysis	Py-GC–MS	EI-quadrupole	Chen et al. (2015)
	Milled Cynara Lignin	Pyrolysis	Py-GC–MS	EI-quadrupole	Lourenço et al. (2015)
	Milled linden wood lignin, enzymatic hydrolysis corn stover l ignin, wheat straw alkali lignin, wheat straw sulfonate lignin	Pyrolysis	Py-GC–MS	EI-quadrupole	Lin et al. (2015)
	Kraft lignin, scots pine wood	Pyrolysis	Py-GC–MS	EI-ITMS	Ohra-aho and Linnekoski (2015)
	Alkaline lignin	Photocatalytic degradation – cyclic LLE	Py-GC–MS	EI-quadrupole	Fan et al. (2015)
	Kraft lignin	Hydrothermal liquefaction – LLE	GC–MS	EI-quadrupole	Zhou (2014)
	Kraft lignin	Pyrolysis	Py-GC–MS	EI-quadrupole	Shen et al. (2010)
	Wheat straw lignin	Pyrolysis	Py-GC–MS	EI-quadrupole	Yang et al. (2010)
	Flax shive lignin	Pyrolysis	Py-GC–MS	EI-quadrupole	Ross and Mazza (2010)
	Synthesized phenyl coumaran type lignin model compounds	–	–	TOF-SIMS-MS	Matsushita et al. (2012)
	Prairie cordgrass lignin, aspen trees hardwood lignin, synthetic Kraft lignin	Pyrolysis	Py-GC–MS	EI-quadrupole	Zhang et al. (2012)
	Three nearly pure lignin samples, one mixture 1:1 cellulose and lignin	Pyrolysis	Py-GC–MS	EI-quadrupole	Mullen and Boateng (2010)
	Bamboo wood lignin	Pyrolysis	Py-GC–MS	EI-quadrupole	Lou et al. (2010)
	Organosolv switchgrass lignin	Liquid-liquid extraction (LLE)	GC–MS	–	Xu et al. (2012b)
	Lignin-derived compounds	Water solution CE analysis	CE-UV	–	Rovio et al. (2010)
	Humic acid	CuO oxidation - LLE	CE-UV	–	Lima et al. (2007)
	Organosolv hardwood and wheat straw lignins	CO_2 pressure release and gas washing	GC–MS	–	Gosselink et al. (2012)
	Cornstover-derived organosolv lignin	Solvolysis in a micro reactor	Micro reactor-GC	–	Kim et al. (2014)
	Rice husk and peach pit	Pyrolysis - LLE	2D-GC-TOF-MS	–	Moraes et al. (2012)
	Pine wood	Pyrolysis	2D-GC-TOF-MS	–	Djokic et al. (2012)
	Red oak chips	LLE	HPLC-MS^n	ESI-LQIT-FT-ICR	Owen et al. (2012)
	E. Globulus lignin	Pyrolysis	Py-GC–MS	EI-quadrupole	Ibarra et al. (2007)
	Wood samples (*E. globulus, E. nitens, E. maidenii, E. grandis, and E. dunnii*)	Pyrolysis	Py-GC–MS	EI-quadrupole	Rencoret et al. (2007)
	Kraft lignin	LLE	GC–MS	EI-quadrupole	Raj et al. (2007b)
	E. Globulus wood chips	Alkaline extraction - Pyrolysis	Py-GC–MS	EI-quadrupole	Oudia et al. (2007)
	Palm fruit bunch and pine wood chips	Pyrolysis	2D-GC-TOF-MS	–	Tessarolo et al. (2013)
	Organosolv switchgrass lignin	LLE	HPLC-MS^n	ESI-LQIT-FT-ICR	Jarrell et al. (2014)
	Beech flakes	Flash pyrolysis- hydrodeoxygenation	2D–GC-TOF-MS	–	Marsman et al. (2008)
	Lolium and *Festuca* grasses	Pyrolysis	Py-GC–MS	EI-quadrupole	Fahmi et al. (2007)
	Poplar wood	–	–	LA-REMPI-TOF-MS	Mukarakate et al. (2011)
	Lignin from eucalypt pulps, milled wood lignin, Kraft lignin	Pyrolysis	Py-GC–MS	EI-quadrupole	Ibarra et al. (2005)
	Milled wood lignin, magnolia wood	–	–	TOF-SIMS	Saito et al. (2006)
	Lignin dimer model compounds	–	–	TOF-SIMS	Saito et al. (2005)
	Acacia, chestnut, cherry, mulberry and oak wood	SLE - SPE	GC–MS/MS	CI-ITMS	Flamini et al. (2007)
	Hardwood sulfite pulp bleaching effluent	Solid phase extraction (SPE); solid l iquid extraction (SLE)	CE-MS	ESI-ITMS	Bogolitsyna et al. (2011)
	Lignin from archaeological shipwrecks Lignin from spruce (soft wood) and birch (hard wood)	–	–	DE-ITMS	Modugno et al. (2008)

(continued on next page)

Table 7 (*continued*)

Analytical purpose	Original sample type	Sample preparation	Chromatographic system	Mass spectrometric technique	References
	Dissolved organic matter and sediments	LLE	HPLC-UV	–	Lobbes et al. (1999)
	Wheat straw	HS-SPME	HS-SPME/GC–MS	–	Kolb et al. (2013)
	Soluble lignin extracted from sugar cane	Freeze dry-ultrasonic SLE	UPLC-MS/MS	ESI-TQD	Kiyota et al. (2012)
	Pine sawdust	Depolymerization reaction-solvent evaporation; distillation	GC–MS	–	Galkin and Samec (2014)
	Dissolved organic matter, soil and sediments	CuO oxidation reaction-SPE clean-up-derivatisation	GC–MS	–	Kaiser and Benner (2012)
	Hemicellulose, cellulose and lignin standard mixture	Thermal and catalytic pyrolysis	2D-GC-TOF-MS	–	Michailof et al. (2014)
	Kraft lignin, soda lignin, organosolv lignin, pine wood pyrolytic lignin, lignin filtration residue from acid straw hydrolysis, lignin-enriched residues from anaerobic biomass fermentation	Off-line pyrolysis	2D-GC-TOF-MS	EI-TOF-MS	Windt et al. (2009)
	Beech lignin	LLE	GC–MS	–	Stärk et al. (2010)
	Aged paper	SLE	CE-MS	–	Dupont et al. (2012)
	Wheat Straw Lignin	CuO oxidation	UPLC-HRMS	–	Ouyang et al. (2014)
	Kraft mill residues	Pyrolysis - LLE	2D–GC-TOF-MS	–	Faccini et al. (2013)
	Standard mixture	None	HPLC-MS^n	ESI/APCI-LQIT	Haupert et al. (2012)
MW	Sulfonated lignin	Sulfonated lignin dissolve in water	IP-RPLC × IP-SEC	–	Brudin et al. (2008)
	Sulfonated lignin	Sulfonated lignin dissolve in water	IP-RPLC × THM-GC–MS	–	Brudin et al. (2010)
	Jack Pine Kraft lignin	Dissolve in either NaOH or deionized water	SEC-MALLS	–	Gidh et al. (2006b)
	Milled wood lignin and Kraft lignin	Acetobromination	Acetobromination-SEC	–	Asikkala et al. (2012)
	Softwood, Eucalyptus globulus and wheat straw lignin	Acetobromination	SEC-UV	–	Guerra et al. (2007)
	Mischantus and giant reed	Sulfuric acid hydrolysis and Alkaline oxidative hydrolysis for lignin extraction	SEC-UV	–	Savy and Piccolo (2014)
	Spent sulfite liquor samples, Kraft lignin, soda bagasse lignin, steam explosion lignin and lignosulfonate samples	Fractionation by ultrafiltration	SEC-multi-detection	–	Ringena et al. (2006)
	Kraft lignin, Soda bagasse lignin, Lignosulfonates, Steam explosion lignin and Alcell lignin	Acetylation	SEC-UV	–	Baumberger et al. (2007)
Structure and linkage	Synthetic lignin model compounds, lignin from xylem tissue	Grinding in liquid nitrogen, extraction - SLE – LLE	UPLC-MS^n	APCI-LQIT-FT-ICR; DI-APCI-ITMS	Morreel et al. (2010a)
	Synthetic lignin model compounds	–	–	APCI-ITMS	Morreel et al. (2010b)
	Eucalyptus grandis, Eucalyptus globulus	–	–	MALDI-LQIT-MS	Araújo et al. (2014)
	Miscanthus and Switchgrass Lignin	–	–	MALDI-TOF-MS	Richel et al. (2012)
	Wheat straw lignin	Extraction with CIMV procedure	–	APPI-QqTOF-MS	Banoub et al. (2007)
	Sulfonated lignin	Sulfonated lignin dissolve in water	IP-RPLC × THM-GC–MS	–	Brudin et al. (2010)
	Wheat straw lignin	Pyrolysis	Py-GC–MS	–	del Río et al. (2012b)
	Elephant grass	Pyrolysis	Py-GC–MS	–	del Río et al. (2012a)
	Lignin from jute fibers	Pyrolysis	Py-GC–MS	EI-quadrupole	del Río et al. (2009)
	Spruce wood LCC	Thioacidolysis; pyrolysis	Py-GC–MS	–	Du et al. (2013)
	Spruce wood LCC	Pyrolysis	Py-GC–MS	–	Du et al. (2014)
Lignin S/G ratio	*Eucalyptus globulus* wood	Pyrolysis	Py-GC–MS	–	del Río et al. (2005)
	Eucalyptus spp. wood	Nitrobenzene oxidation-LLE; pyrolysis	Py-GC–MS	–	Lima et al. (2008)
	Eucalyptus spp. wood lignin	Nitrobenzene oxidation-LLE; pyrolysis	Py-GC–MS	–	Nunes et al. (2010)
Lignin content	Pine, spruce, larch and compression wood	Pyrolysis	py-GC-FID	–	Alves et al. (2006) Alves et al. (2008) Alves et al. (2009)
	Kraft black liquor	Alkaline solvent dilution for CE analysis	CE-UV	–	Gebremeskel and Aldaeus (2013)
Characterization	Organocell lignin, dioxane lignin, Björkman lignin, and Klason lignins from both softwoods and hardwoods	Dissolve lignin sample in DMF/water mixture	RP-HPLC-DAD and Fluorometric detection	–	Gora et al. (2006)
	Sulfonated Kraft lignin and lignosulfonate from spruce sulfite process	Dissolve in Milli-Q water	HIC-UV	–	Ekeberg et al. (2006)
	Kraft lignin	Dissolve in either NaOH or deionized water	HPLC-MALLS	–	Gidh et al. (2006a)
Functional group	Kraft lignin	Chemical reduction in Head Space sample vial	HS-GC-TCD	–	Li et al. (2015)
	Lab lignin sample	Reaction in HS vial	HS-GC-FID	–	Li et al. (2012)

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 Aldaeus, 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; Windt et al., 2009). Quantitative studies of bio-oil composition were achieved by combining 2D–GC-FID 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 chromatography. The coupling of ion-pair reversed phase HPLC (IP-RPLC) with ion-pair SEC (IP-SEC) enabled the differentiation of “good quality” batches of sulfonated lignin from “bad quality” ones (Brudin et al., 2008). With the doubt 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, Windt 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 GCxGC-Time-of-Flight (TOF)-MS (Windt et al., 2009).

4.3.2. Analysis of oligomeric lignin-related compounds

In recent years, modern multiple stage tandem (MS^n) and high resolution mass spectrometry (HRMS) techniques like a linear quadrupole ion trap-Fourier transform ion cyclotron resonance hybrid mass spectrometer (LQIT-FT-ICR-MS) or a triple quadrupole mass spectrometer (TQD) were combined with liquid chromatography (LC) for structural lignin characterization with focus on lignin derived compounds with higher molecular weights (Jarrell et al., 2014; Kiyota et al., 2012; Morreel et al., 2010a; Owen et al., 2012). Jarrell et al. investigated lignin isolated from milled switch grass with an LQIT-FT-ICR-MS equipped with an electrospray ionization (ESI) source run in negative mode. Beside monomeric lignin derived compounds they focused their research also on dimeric lignin compounds, which they identified with the fragmentation pathways in MS^n experiments and the elemental compositions of the compounds obtained from exact mass measurements (Jarrell et al., 2014). With an LC × LC-LTQ-FT-ICR-MS method with negative ESI Morrell et al. investigated the fragmentation pathways in MS^2 experiments of synthesized lignin trimers, tetramers and pentamers bases on coniferyl alcohol (G) and sinapyl alcohol (S) monomeric subunits. Their developed analytical method was applied for detecting oligomeric lignin compounds on wild-type poplar xylem. 134 oligomeric lignin compounds were detected (Morreel et al., 2010a). Kiyota et al. investigated also synthesized lignin model compounds using a LC-TDQ method with negative ESI. The synthesized oligomeric lignin model compounds from dimers to tetramers were based on *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S) monomeric subunits. Their developed method was tested on soluble lignin extracted from sugarcane. Four monomeric, eight dimeric and three trimeric lignin compounds were identified (Kiyota et al., 2012). Bogolitsyna et al. coupled capillary electrophoresis (CE) to an ITMS using ESI in negative mode. They investigated a pulp bleaching effluent sample and detected lignin-derived monomeric compounds (Bogolitsyna et al., 2011).

Several groups have investigated isolated lignin samples or lignin model compounds with direct infusion MS (Banoub et al., 2007; Haupert et al., 2012; Morreel et al., 2010b). Haupert et al. compared the ionization properties of monomeric and dimeric lignin model compounds with atmospheric pressure chemical ionization (APCI) and ESI, both in negative and positive ionization mode (Haupert et al., 2012). Morreel et al. used an ITMS with APCI in negative mode and a FT-ICR-MS with ESI in negative mode to investigate the fragmentation pathways of four different bonding structures of self-synthesized dimeric lignin model compounds (Morreel et al., 2010b). Banoub et al. analyzed wheat straw lignin with a quadrupole-time-of-flight tandem MS (QTOF-MS/MS) with atmospheric pressure photoionization (APPI) in

both negative and positive ionization mode. They detected 63 new oligomeric compounds 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).

4.3.3. 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) (Araújo 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^n experiments of lignin-related oligomeric compounds higher than tetramers. 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
APPI 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^n 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^n multiple stage tandem mass spectrometry
PCA principal component analysis
py-GC pyrolysis-gas chromatography
qMS quadrupole mass spectrometer
QTOF quadrupole/Time-of-Flight 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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