UNIVERSITA' DEGLI STUDI DELL'INSUBRIA

DOTTORATO DI RICERCA IN BIOTECNOLOGIE, BIOSCIENZE E TECNOLOGIE CHIRURGICHE

Curriculum Biotecnologie Molecolari e Alimentari

XXIX CICLO

Development of an enzymatic tool-box for lignin oxidation/degradation

Sviluppo di un tool-box enzimatico per l'ossidazione e la degradazione della lignina

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Anno accademico 2015-2016

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1. Introduction

1.1. Lignin

Lignin was first identified by the Swiss botanist Anselme Payen de Candolle in 1813, who described it as *le materiel incrustant*, a separate component of wood, a fibrous and tasteless material, insoluble in water and alcohol but soluble in alkaline solutions (Figure 1). He named it "*lignine*", which derived from the Latin word *lignum*, meaning wood [1].

Since that time, about 15000 scientific papers, patents, and hundreds of books, have been published about this important natural polymer.

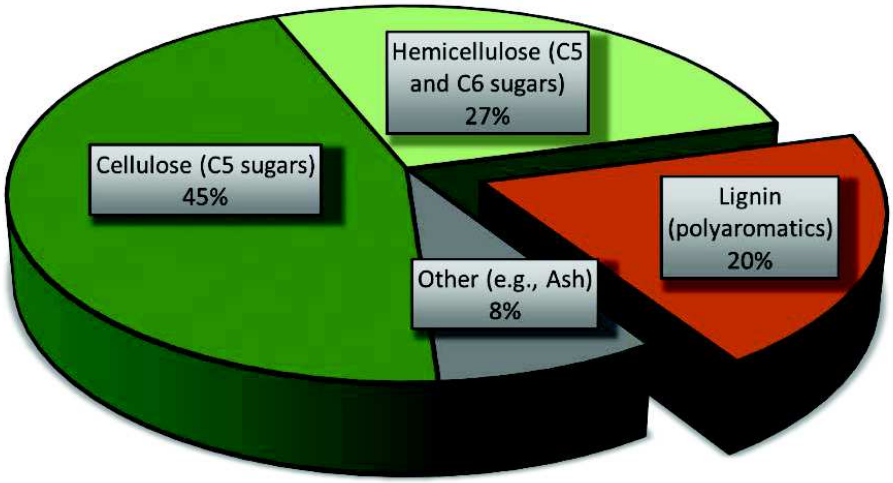


Figure 1: lignin is the second most abundant natural substance on earth after cellulose; it represent the 20% of total biomass.

Nowadays, the term lignin is referred to a mix of biopolymers, chemically very different one another, composed of phenolic and aliphatic substances. Lignin has different physiological functions: together with cellulose fibers, it provides strength to the stem and the bole of higher plants, it protects sugars from microorganisms and insects attack and it has a role in the nutrient transport [2-5] (Figure 2).

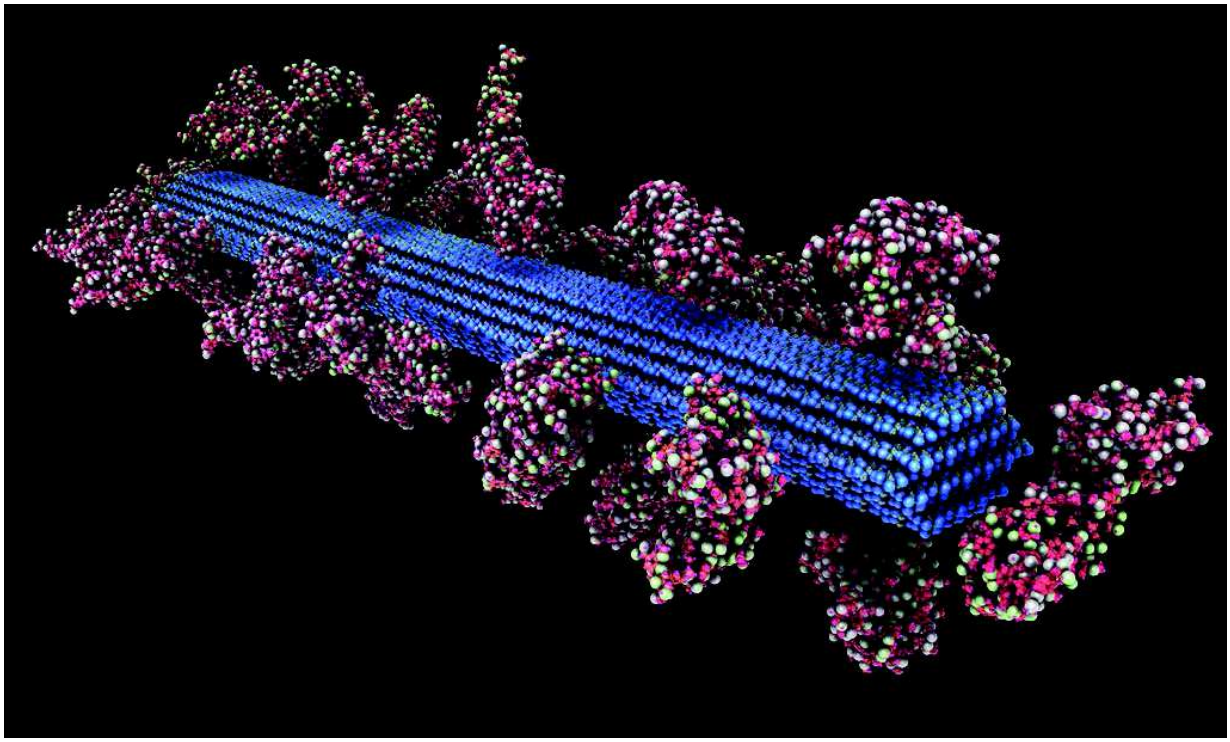


Figure 2: an atomistic model of cellulose (blue) surrounded by lignin molecules (red) comprising of a total of 3.3 million atoms. Water molecules that are present in the model are not shown. Simulation: Jeremy Smith, University of Tennessee and ORNL.

Thanks to its peculiar structure and properties, lignin can be useful as a starting material for valuable products. About 50 million tons of lignin are produced every year worldwide, but only 1% is currently valorised [6]. Despite the natural abundance of this compound as byproduct of the manufacturing processes for paper and pulp, up to now there is not a lignin-valorisation technology that could be enviromentally and economically suitable. One of the biorefinery-level challenge for lignin is the implementation of an effective separation/isolation process.

1.1.1. Chemical structure

Lignin possesses a three-dimensional amorphous structure constituted by methoxylated phenylpropane units [7] (Figure 3). In plant cell walls, lignin is intercalated between cellulose and hemicellulose, holding the lignocellulose matrix together [2]. The cross-linking with carbohydrates can give to the structure a higher strength [8]. In order to obtain a detailed structural and functional characterization, a number of studies where carried out. The composition, molecular weight, and amount of lignin differ from plant to plant, with lignin abundance generally decreasing from softwoods to hardwoods and eventually grasses [9].

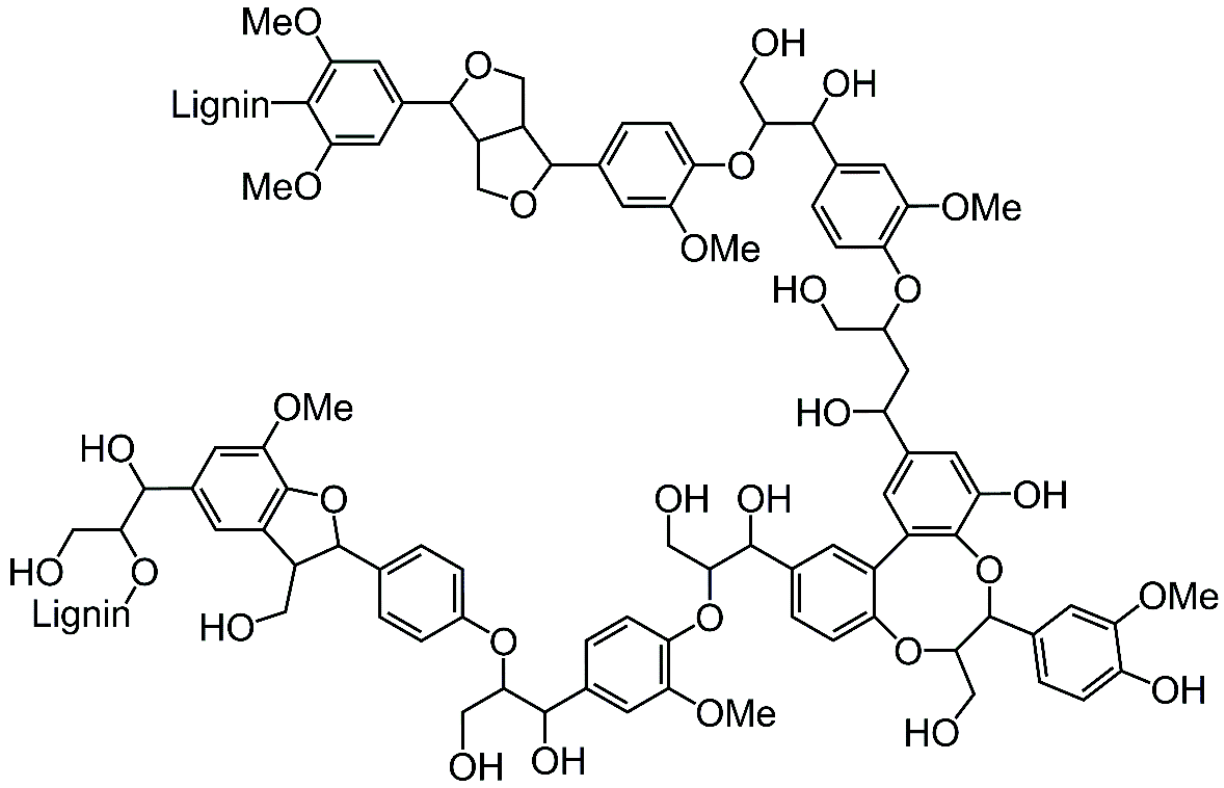


Figure 3: a small segment of softwood lignin polymer

As general rule, the most abundant linkage in both softwood and hardwood is the β-O-4 linkage, representing approximately from 50% of spruce linkages up to 60% of birch and eucalyptus linkages. Because the lignin molecule is very complex, the characterization and quantification of the various sub-structures is challenging even with advanced NMR techniques [10]. The relative abundance of *para*-coumaryl, coniferyl, and sinapyl units is different between plant species [11]. Softwood lignin is composed predominantly by coniferyl alcohols units, while an equal proportion of coniferyl alcohol and sinapyl alcohol appears in hardwood lignin [12]. The additional methyl ether groups on the aromatic rings prevent formation of 5-5 or dibenzodioxocin linkages, bringing to a more linear structure [13]. The relative abundance of the various linkages in softwoods and hardwoods are summarised in Table 1.

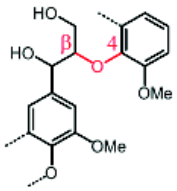
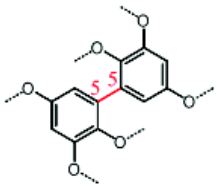
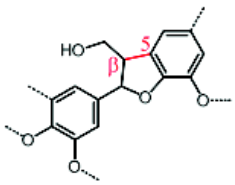
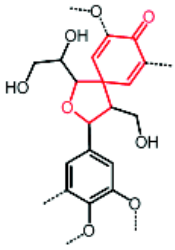
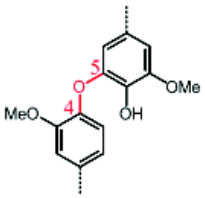
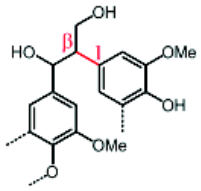
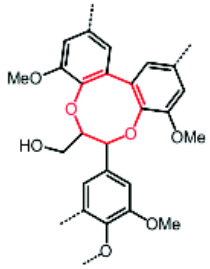
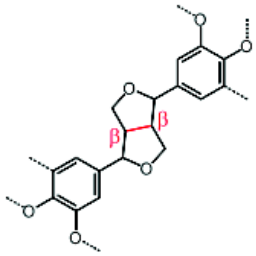
1.2. Biosynthesis

Lignin is a polymer of aromatic subunits usually derived from phenylalanine [19, 20].

Although different studies have been done, there is not a complete picture of the enzymes that play a role in lignin biosynthesis and of the order in which reactions take

place. The biosynthesis of lignin can be divided in two parts: the production of lignin monomers (monolignols) and the polymerization processes, through which the building-blocks are polymerized.

Table 1: common linkages (and approximated abundance per C_9-units) connecting the phenyl-propane units in softwood and hardwood [13]; [a] etherified: 19, phenolic: 5-8; nd: not determined.

	Linkage	β-O-4 	5-5 	β-5 	Spirodienone 
Softwood	Spruce [8, 14]	45-50	19-22	9-12	nd
	Spruce [15]	nd	22	12	nd
	Spruce [16]	45	24-27[a]	9	nd
	Spruce [11]	45	24-27	9	2
Hardwood	Birch [8, 14]	60	9	6	nd
	E. grandis [17]	61	6	3	nd
	E. grandis [10]	61	3	3	5
	P. fortune [18]	62	nd	11	3
	Linkage	4-O-5 	β-1 	Dibenzodioxacin 	β-β 
Softwood	Spruce [8, 14]	4-7	7-9	nd	2-4
	Spruce [15]	nd	2	5	3
	Spruce [16]	nd	1	7	2
	Spruce [11]	nd	1	7	6
Hardwood	Birch [8, 14]	7	7	nd	3
	E. grandis [17]	9	1	<1	3
	E. grandis [10]	9	2	nd	3
	P. fortune [18]	nd	1	2	12

Three monolignols, differing only in the substitution pattern on the aromatic ring, can be polymerized into lignin (Figure 4). The relative abundance of the different monolignol residues in lignin varies between species and within species, as does the total lignin content in wood.

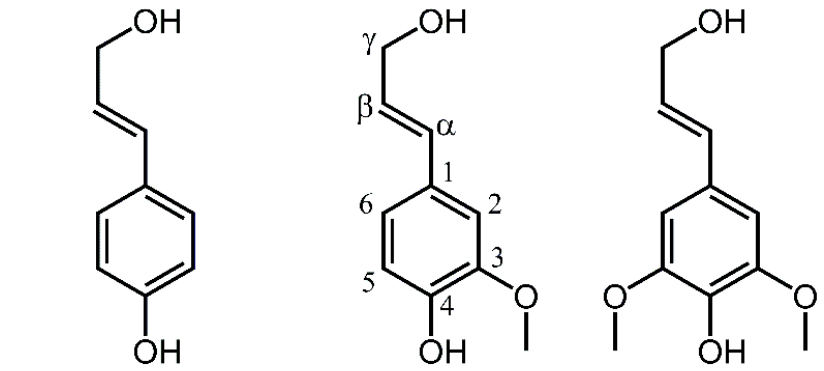


Figure 4: stuctures of the three monolignols

Plants obtain coniferyl alcohol, *para*-coumaryl alcohol and sinapyl alcohol from phenylalanine by a multistep process: the first step consists in phenylalanine deamination by phenylalanine ammonia-lyase (PAL) [21, 22]. This reaction yields cinnamate, that is hydroxylated by cinnamate 4-hydroxylase to *para*-coumarate. Subsequently, this acid molecule is reduced to alcohol form by a 3-enzymes cascade: firstly, *para*-coumarate is conjugated to coenzyme A by 4-coumarate:CoA ligase (forming an activated thioester precursor); subsequently, the CoA thioester is reduced to aldehyde by cinnamoyl-COA reductase and finally to alcohol by cinnamyl alcohol dehydrogenase, giving the first monolignol. The other substituted monolignols are produced starting from *para*-coumarate or its CoA-activated form by a sequential hydroxylation and O-methylation (catalysed by different hydroxylase and O-methyltrasferase, respectively). The final molecules are generated following the same reduction pathway described for *para*-coumaryl alcohol (Figure 5).

Overall, approximately 15 enzymes are implied in the synthesis of the three monolignols. The monolignes are relatively toxic and unstable: for these reasons many other enzymes are implied into their transport, storage in vacuole and glycosylation [19]. Regarding this process, the production of monolignols-glucosides by glucosyl trasferases is essential to stabilize the phenolic compounds and to lower their toxicity

in cytosol. Once they reach the outside of the cell, de-glycosylation can occur, catalysed by specific β-glucosidases produced by many species of plants [23].

The mechanisms of polymerization of monolignols into lignin have not been clearly defined yet. The monolignols are polymerized into lignin by a free radical mechanism [24] that starts with the oxidation of monolignols by two different classes of enzymes, peroxidases and laccases, that are both able to oxidise monolignols to free radicals [25-28].

In particular, the phenol group in position 4 can be oxidised to the radical form. Thank to the electron-conjugated chemical-structure of monolignols, the free radical can be delocalized on the benzyl ring, in position 3 and/or 5, or on the allylic chain, in β-position.

At this point, the radical coupling between two radical-activated monolignol can occour: the coupling of different monolignols with different activated-positions can lead to the formation of all the bound observed in lignin.

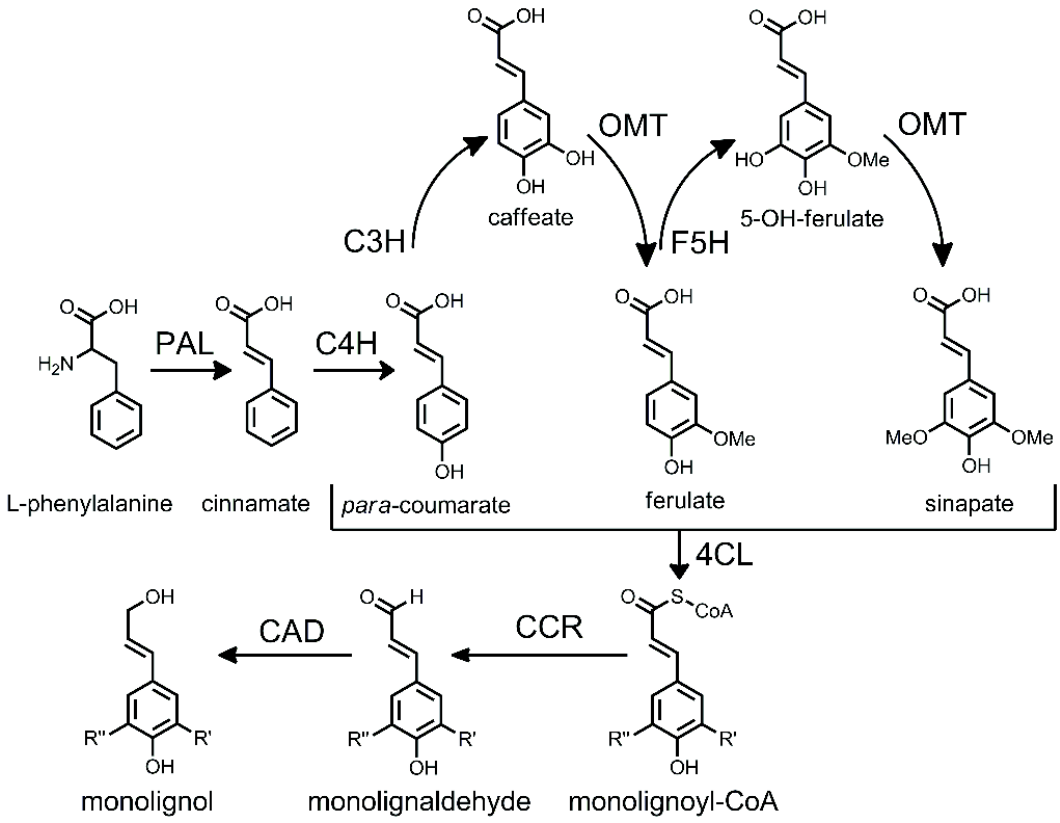


Figure 5: biosynthesis of monolignols; PAL: phenylalanine ammonia-lyase; C4H: cinnamate-4-hydroxylase; C3H: 4-coumarate-3-hydroxylase; F5H: ferulate-5-hydroxylase; OMT: O-methyltransferase; 4CL: 4-coumarate:CoA ligase; CCR: cinnamoyl-CoA reductase; CAD: cinnamyl alcohol reductase.

For instance, the β-O-4 linkage derives by the coupling of two monolignols present in one free radical in position 4 (phenolic –OH) and one in the β-allylic position (Figure 6). Despite the apparent random mechanism of polymerization, analysis of lignin structure shows that the ratio of lignin linkages can be modulated by the concentration and the type of monolignols. For example, sinapyl alcohol, because of the occupation of positions 3 and 5 by methylether groups, can carry the free-radical only in positions 4 and β-allylic. This leads to the formation of a lower numbers of possible dimerization products.

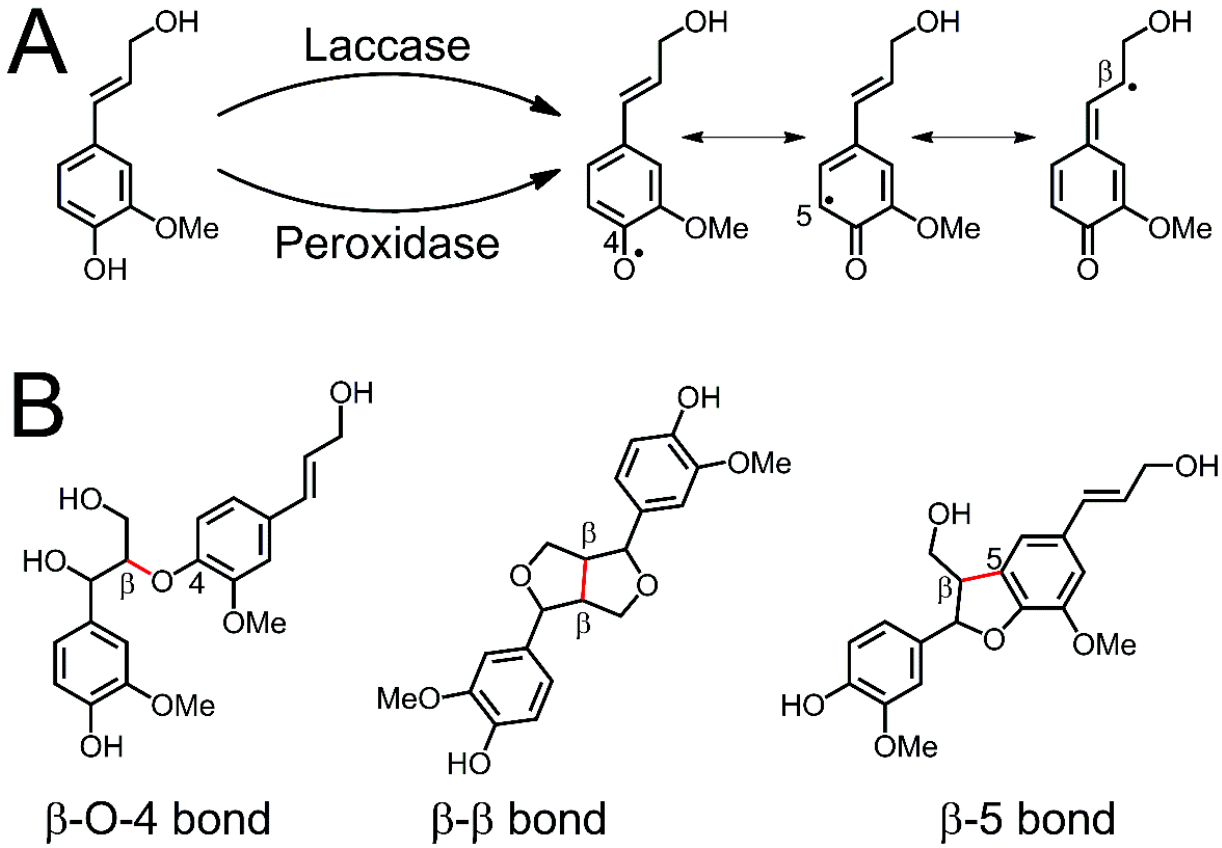


Figure 6: A) laccases and peroxidases start the polymerization process activating the monolignols by a radical oxidation; the formed radicals can be present in different resonance forms: covalent bonds are formed when two radicals meet. B) chemical structures resulting from the possible bonding between two monomers; the red line indicates the bonds generated by couplings of the β position and the 4, β, or 5 position [29].

Using the same mechanism, further free radical monolignol blocks can couple to the previous dimer and so on.

Various studies about the coupling and polymerization of monolignols using oxidative enzymes have proved that different enzymes produce different oligolignols, suggesting that free radical polymerization does not necessary imply the formation of a random

structure [30-33]. In the same way, a stereoselective synthesis of dilignols was performed using crude enzymatic plant extracts: it represents another evidence that polymerization of monolignols, although it seems to be a random free radical coupling process, can give a reasonably ordered structure [34].

1.3. Challenges for lignin use within the biorefinery

There are numbers of technical challenges associated with the lignocellulosic biorefinery operation: every biorefinery will use local biomass feedstocks and the heterogeneity of the starting material can influence the process: the target product and the biomass processing schemes can be different depending on many factors. This results in a high degree of variability in recovered lignin from different refineries [13, 35]. Lignin has a poly-phenolic structure that does not react as carbohydrates or oils because of its structural and chemical complexity. In biorefineries carbohydrates and oils fraction can be separated from the wood. These two streams are then directed to the production of biofuels via fermentation or chemical modification (esterification).

Lignin resulting from the biorefinery recovery process presents different features in terms of complexity, polydispersivity and molecular weight. This variability comes from the different chemical composition, which in turn depends on the kind of feedstock used. Nowadays, the extraction method used in a local biorefinery is manly chosen to optimize the carbohydrate stream and depends on, for example, biomass sources and flagship products [35]. Accordingly, it is easy to understand how the obtained lignins can have several different properties, including molecular weight distribution, isoelectric point, solubility, reactivity, number of free phenolic, hydroxyl and carboxyl groups, percent aromaticity, the types of aromatic substitutions (proton, hydroxyl, methoxy and alklyl), ramification and amount of residual bound carbohydrate fragments [13, 35].

One of the main long-term objective of modern biorefineries is the discovery and development of economical processes for the exploitation of lignin as material or as chemical. The effectiveness of such processes for the processing of biomass from diverse sources must be produced, otherwise lignins utility will be limited. One concept that may be of value is to separate lignin, used for production of chemicals, early from the biomass in biorefinery operations, using mild methods to preserve its

structure. The development and validation of the technology for this approach represents another challenge.

1.4. Lignin pretreatment

Lignin pretreatment is a foundamental step in the initial phase of biorefinery operations. In order to obtain different streams of biomass, the components (cellulosic and lignin) must be separated. Efficient biomass fractionation is currently one of the major challenges in biorefinery: the complex structure of the plant cell wall and the high crystallinity of cellulose make the feedstock recalcitrant to separate into its components. The various pretreatment technologies can be clustered into four categories: physical pretreatment (i.e., ball milling), solvent fractionation (i.e. organosolv process or the use of ionic liquids), chemical pretreatment (acidic, alkaline, or oxidative), and biological treatment (mainly using fungi) [36].

The structure of the isolated lignin material depends on the isolation method employed. For this reason, pretreatment methods can generate lignin material of different quality and purity.

In addition, the combination of the kind of biomass and the pretreatment used, is a key-point for lignin valorization in a biorefinery. Every different pretreatment method has both advantages and disadvantages, which will be discussed below.

1.1.1. Kraft lignin

Kraft pulping is the mainly spread pulping process worldwide. The process consists in boiling lignin at temperatures in the range of 150-180 ºC for few hours in the presence of aqueous sulfide, sulfhydryl and polysulfide, at alkaline pH [35]. The chemistry of this kind of extraction has been the subject of several studies. The solubilized lignin is isolated from the pulping liquor (also called “black liquor”) by acidic precipitation and it can be used as fuel to supply the energy for the bio-refinery and for paper and pulp manufacturers. This process can be performed with CO_2 obtained from the previous reactors. Precipitated kraft lignin may be recovered also by filtering and washing, resulting into a low sulfur content kraft lignin (less than 1-2% of –SH linkages) [13, 35].

The sulfonate functionalities are linked into aliphatic side chains or on the aromatic rings, depending on the treatment temperatures (100 or >150 ºC, respectively). Sulfonylation at aliphatic, benzylic or aromatic sites confer solubility and surfactant properties to the lignin (Figure 7).

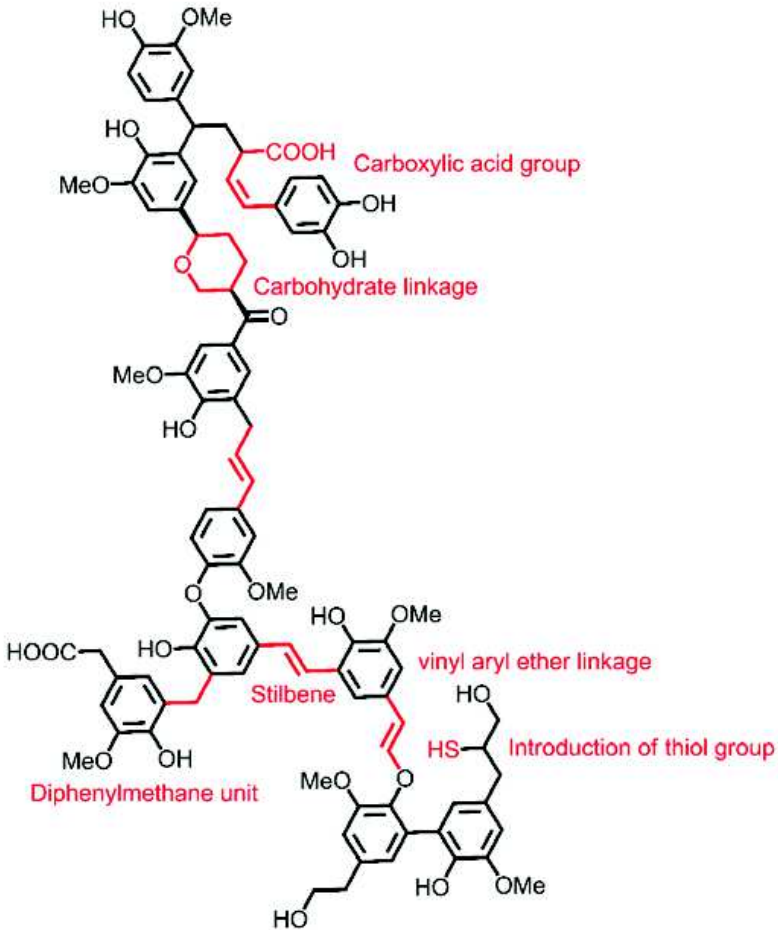


Figure 7: model depicting structural features characteristic of kraft lignin. In red are highlighted the peculiar chemical bound present in this kind of lignin.

The molecular weight of kraft lignin is generally between 1000 and 3000 Da [37, 38].

Nowadays, MeadWestvaco (Charleston, South Carolina, US) is the main producer of kraft lignin. Approximately 70-75% of their isolated lignin is chemically sulfonated using these kind of processes and about 692 x 10^3 tons of kraft lignin are produced every year by this company.

1.4.1. Sulfite lignin (Lignosulfonates)

The sulfite pulping process is a promising method for processing of forest lignin. Differently from kraft pulping, sulfite pulping generally yields a chemically useful lignin-rich black liquor stream. Because of the sulfite process methods, the isolated

lignin contains high quantity of sulfur as sulfonate groups linked to the side aliphatic chains (Figure 8) [39]. Lignosulfonates can be produced at different pH values (2-12), depending on the cationic composition of the reaction mixtures. Acidic sulfite processes use calcium and magnesium bivalent cations as counterions, meanwhile alkaline processes use sodium or ammonium counterions [13, 35]. Since sulfite lignin is generally soluble over the entire pH range it cannot be easily isolated by acid precipitation: the recovery is commonly performed through precipitation of calcium lignosulfonate with lime (Howard process). Lignosulfonates have a higher average molecular weight compared to the kraft lignin (commonly 5,000-20,000 Da). Lignosulfonates are soluble in water, highly polar organics solvents and amines. Sulfite lignins show several disadvantages: they are generally impure materials, contaminated by the couterions. Commercial lignosulfonates also exhibit a wide molecular weight outline and different degrees of sulfonation. Sulfur incorporation into final products represents a problem, and its removal would add expenses and/or environmental difficulties. For all these reasons, sulfite pulping accounts for only 2% of the global pulp production.

Lignotech (US) produces about 120 x 10^3 tons/yr of sulphite lignin by purchasing sulfite liquors from nearby mills. Worldwide production of lignosulfonates accounts 1060 x 10^3 tons/yr.

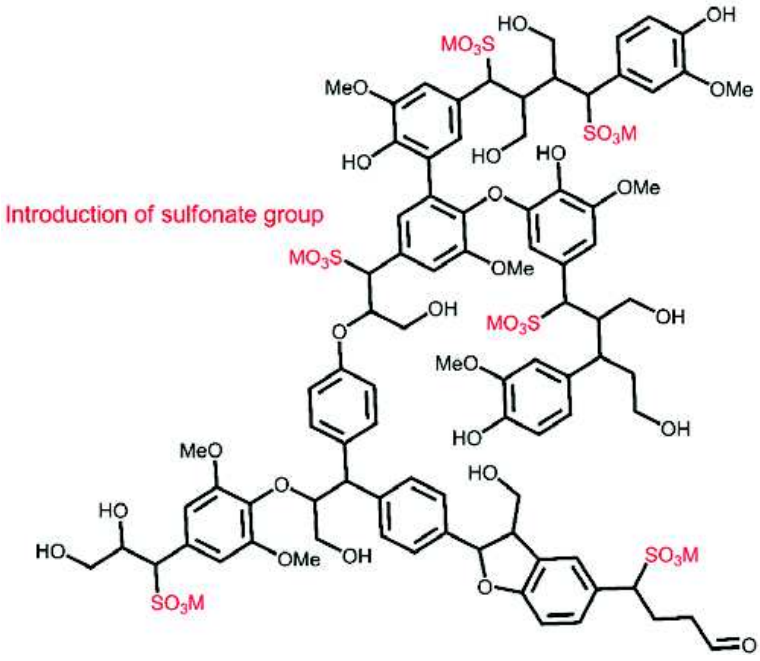


Figure 8: model depicting structural features characteristic of lignosulfonate lignin

1.4.2. Organosolv

Organosolv pulping is a group of treatments for the separation of wood components using organic solvents extraction [13, 35]. Using this operation cellulose, hemicellulose and lignin can be separated in 3 different working streams. During a typical organosolv procedure, lignin extraction is performed heating the wood at high temperatures in aqueous dioxane. Lignin obtained using this method conserves the major part of its original structure in particular the (β-O-4 linkages). Several solvents and combinations are used for organosolv pulping. The addition of acids or alkali is used to enhance pulping rates and yields. Organosolv lignin can be isolated by removing the solvents, distillation or precipitation with water [40]. Organosolv processes can be considered more environmentally friendly than sulfite or kraft pulping. Since it maintains its original structure, organosolv lignin is insoluble in water but dissolves in mild alkali enviroments and in many organic solvents [41].

The molecular weights of organosolv lignin are typically around 1000 Da. This peculiar feature, within the low sulfur content, makes organosolv lignin attractive as a low-molecular weight source (phenols or aromatics).

Organosolv processes have some disadvantages related to the handling and recovery of the organic solvent. Nowadays, no commercial source of organosolv lignin is available.

Lignol Innovations Corporation (Vancouver, Canada) is starting to apply organosolv pulping using ethanol or ethanol-water as solvents (Alcell's technology)

1.4.3. Ionic liquid lignins

Ionic liquids can be described as organic salts present in liquid form at relatively low temperature. According to their peculiar chemical features, ionic liquids have been proposed as "green solvents" for biorefineries [42-44]. In fact, their ability to extract lignin from biomasses is interesting for a number of reasons: it guarantees a high quality of lignin (similar to the organosolv one), high yields of extraction (93% using alkylbenzenesulfonate) [45], easy recovery by precipitation after addition of water and recyclability of ionic liquids.

Nowadays, lignin obtained with ionic liquids is not yet available on an industrial scale and it cannot be fully regarded as a technical lignin [46].

1.4.4. Other processes

Lignin pyrolytic processes (thermal decompositions occurring in the absence of oxygen) produce a lignin stream potentially available for biorefinery use. The pyrolysis process involves high temperatures (>400 °C). Reported MW values are around 300-600 Da.

The main disadvantage is the high level of carbohydrate consumption required by the process. In comparison to other processes, pyrolysed lignin shows different structural characteristics: as a double-bladed knife, these features represent either problems (different reactivity than "classical" ones) or advantages (unique opportunities to make specific aromatic hydrocarbons not available via other processes) [35].

The steam explosion is performed impregnating wood with steam under pressure (1.38−3.45 MPa and >180 °C, 1−20 min), then the pressure is released rapidly. During this process the lignocellulosic components are separated within the breaking of lignin structure [47, 48]. The obtained lignin has a molecular weight distribution similar to the organosolv one. This process does not use sulfur and this represents its main advantage. StakeTech Technology (Norval, Ontario, Canada) offers a commercial process and proprietary machinery for steam explosion pulping.

Ammonia fiber explosion (AFEX) process has been tried out for lignin but does not offer a well-defined lignin stream [49]. Dilute acid lignin process is also available but the low yields and the reactor corrosion make it an unsuitable method for biorefinery [35]. Pulping could be performed via alkaline oxidation using O_2 or H_2O_2. Obtained lignin is then easily recovered with acid precipitation. The principal drawback of this process is the slow delignification rates. These treatments provide lignin monomers with similar weight distributions as the organosolv lignin.

1.5. Lignin derived products

The commercial use of lignin derived products is limited nowadays: most of the lignin obtained from biorefineries and pulp and paper industries is addressed to combustion for the production of energy and steam. There are several uses of lignin based on its

polymeric and polyelectrolyte properties. Dispersants, emulsifiers, binders, and sequestrants could be obtained from lignin [50-53]. These products represent nearly the 75% of commercial lignin products. Other, less diffused, applications that can be found in the market include adhesives and fillers.

Examples of opportunities from macromolecules include: carbon fibers, polymer modifiers, adhesives and resins.

1.5.1. Carbon fibers

Lignin can be used as a cheap source of carbon suitable for the synthesis of synthetic polymers. Hypothetically, if only the 10% of the lignin would been used to produce carbon fibers, this would halve the consumption of steel, and decrease the petroleum use and the environmental impacts [35]. Unfortunately, the bottleneck of the production of carbon fibers from lignin is the purification and the molecular homogeneity of lignin raw material. For this reason the main goal is to make lignin from different sources an acceptable raw material for high-rate melt spinning and simultaneously, for delivering high-carbon weight yields when the melt spun lignin fiber is thermally converted to carbon fiber [35].

1.5.2. Polymer modifiers

Lignin can be simply used as a low-cost filler [54] for various polymer to enhance physical and chemical properties. The use of lignins as filler was tested in a panel of high-value products: applications may include high-strength engineering plastics, heat-resistant polymers, antibacterial surfaces and light- and ultraviolet-resistant polymers. The technical challenges that underlying this polymers production are focused on the economical cost and the applicability of lignins from different sources. Control of lignin color represents another challenge for some of these applications [35].

1.5.3. Resins/Adhesives/Binders

Others options for lignin based materials are resins and adhesives, especially in formaldehyde-free applications. Also in this case, to develop lignin based resins, adhesive and binders, a suitable molecular weight homogeneity is requested. Viscosity and amount of functional groups must be controlled for further enhancement of the material properties by chemical modification (i.e. carbonylation, carboxylation,

amination, epoxidation, demethylation). These modifications could improve oxidative and thermal stability and provide consistent mechanical processing properties [35].

1.6. Aromatic chemicals

Lignin is the sole renewable source of aromatic molecules, an important and high-volume class of compounds. For this reason the conversion of lignin to distinct low-molecular weight aromatic molecules is an attractive goal. In addition, another driving force in this direction is the lowering of petroleum availability and the increasing of prices [35].

1.6.1. BTX chemicals

In the petroleum refining and petrochemical industries, BTX refers to mixtures of benzene, toluene, and the three xylene isomers, all of which are aromatic hydrocarbons. Considering the structure of lignin, it is clear how two classes of compounds could be obtained from its depolymerisation. The first one can derive from an aggressive depolymerisation that results to C-C and C-O linkage break: BTX aromatics, phenols and some short chain aliphatics can be obtained. These products can be easily and directly used in conventional petrochemical processes. Low-molecular weight aliphatics can also be applied to syngas (fuel gas mixture consisting primarily of hydrogen, carbon monoxide, and very often some carbon dioxide) or fuel production. Indeed, low-molecular weight olefins could be obtained from the dehydrogenation of these compounds. The last option is represented by the use of low molecular weight compounds for power or heat production with the final aim to provide the energy for lignin conversion processes [35].

1.6.2. Monomeric lignin molecules

In order to obtain high-value products as starter for synthetic-chemistry, antioxidants, molecule of pharmaceutical interest that are difficult to make by conventional petrochemical routes a selective and mild depolymerizations should be preferred. These compounds are similar to the basic building blocks of lignin and, could be of economical relevance if produced in a commercial quantity. However, for a selective and efficient depolymerisation of lignin, three major problems must be solved: i) a selective depolymerisation technology needs to be developed; ii) markets and

applications for monomeric lignin building blocks need to be developed; iii) separation techniques for aromatic lignin monomers have to be improved [35].

To this aim, enzymatic hydrolysis of lignin represents a promising technology to obtain a selective depolymerisation of lignin using a green chemistry approach. The benefits and challenges in this field will be discussed in Chapter 9.

1.6.3. Fermentation products

Another option for the use of lignin is represented by the fermentation of the raw material by "white-rot" fungi. Nowadays, few fermentation routes that use lignin as carbon source are available. Fermentation products could be obtained (e.g. 2-ketoadipic acid) but unfortunately, this technology represents a higher-risk area of research [35].

1.7. Lignin model compounds

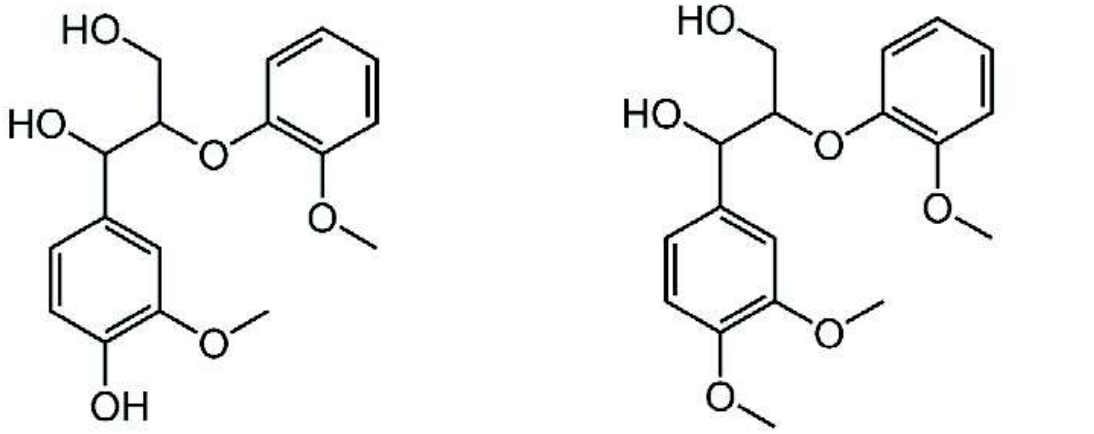
The complexity and variability of lignin has prompted the use of several simpler, lower molecular weight model compounds for the study of lignin valorization. Lignin model compounds present linkages that resemble those found in the lignin polymer. Molecules used as model compounds can be found in lignin degradation streams after a depolymerisation process: their valorization them to high-value chemicals is therefore of almost importance. Lignin model compounds can also simplify the analytical challenges relative to lignin analysis since they often contain only few types of linkage, simplifying the reaction paths. Lignin model compounds can be clustered relying based on the type of linkage that resemble. Many of reported compounds differ only by the number and type of functional groups (i.e., an additional methoxy group on an aromatic ring or one replacing a hydroxyl group) [13].

1.7.1. β-O-4 linkage model compounds

As previously said, the most abundant linkage in lignin is the β-O-4 [7, 10]. During alkaline pulping, the ether bond cleavage is the main pathway of lignin is depolymerisation which lead to the production of water-soluble compounds containing phenolic hydroxyl groups. Byproducts, e.g. 3-hydroxypropaldehyde and arenes, can be formed during the fragmentation. Several transformations in which the β-O-4 bond is not broken have been reported, such as the oxidation of the alcohol group in α and γ

positions to the corresponding carbonyl groups. Quinone species were also identified following oxidation of the model compound [13].

The most commonly used lignin model compound is 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (GGE) and its methoxy-substituted derivative 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (Me-GGE) (Figure 9).



1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (GGE)

1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (Me-GGE)

Figure 9: chemical structure of two lignin model compounds containing β-O-4 linkage

Although these two molecules share the major part of their structure, the reactivity can be very different because of the availability of the phenolic OH- group in GGE that can be oxidised.

1.7.2. Other linkage model compounds

The carbon-carbon bonds in lignin represent some of the most difficult bonds to break; accordingly many of these linkages tend to survive the pulping process [7]. The development of catalysts able to perform these disruptions (particularly the aryl-aryl linkages) is an important challenge that has not yet been addressed [13]. The structure of model compounds containing the 5-5, β-1 and β-5 linkages are illustrated in Figure 10.

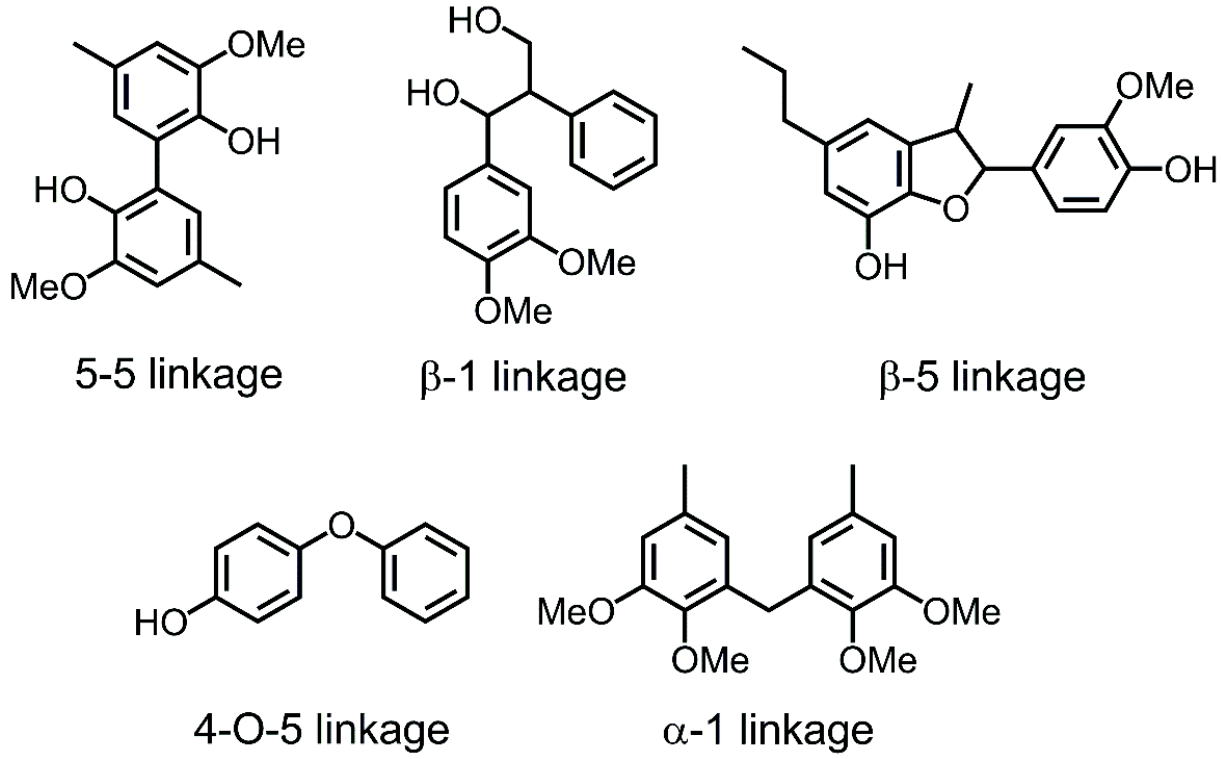


Figure 10: example of lignin linkage model compound not containing the β-O-4 linkage.

The 4-O-5 is an another further linkage present in lignin: different studies on model compounds containly this linkage have been carried out since this kind of linkage mainly results from oligomer-oligomer couplings and leads to the branching of the polymer. Model compounds that resemble the α-1 linkage have also been reported: although it is not naturally present in lignin, this linkage has been observed in kraft lignin [55].

1.8. Lignin depolymerisation

Lignin depolymerisation is one of the main challenges of modern biorefinery. The idea to generate value-added products from lignin raw materials is very interesting from the economical and ambiental point of view. The primary aim of lignin depolymerisation is to obtain small molecules that can be used as fuels or as backbone chemicals for further synthesis [56].

As described before, the thermal treatment (300 to 600 °C) of lignin in anaerobic condition (pyrolysis) can lead to the cleavage of OH groups linked to aliphatic side chain, to the break of alkyl side chain and linkage between aromatic rings: a mixture of phenol, guaiacol, syringol, and catechols can be obtained [57]. Unfortunately, this

process is affected by the feedstock type, the heating rate, and the reaction temperature [58].

The same problem has been identified in the gasification process: lignocellulosic material is converted into CO_2, CO, and H_2 at temperature >700 °C. The obtained syngas is the only useful product from this process [57]. Biochemical methods, for example lignin depolymerisation using fungi, were also employed, but the low efficiency and the slow growing rate of fungi make them unsuitable in biorefineries. Compared to pyrolysis, chemical treatment and biochemical depolymerisation offer the possibility to control the reaction and a great potential in lignin conversion [59, 60].

There are five categories of lignin chemical depolymerisation: base-catalysed, acid-catalysed, metallic catalysed, ionic liquids-assisted, and supercritical fluids-assisted.

1.8.1. Base-catalysed lignin depolymerisation

From the treatment of lignin with sodium hydroxide (or other bases) at high temperature it is possible to obtain phenols and phenol derivatives. Base-catalysed lignin depolymerisations are performed at above 300 °C and at high pressure. In this process, the cleavage of the aryl-alkyl (β-O-4 bond, in particular) occurs, also helped by sodium cation adducts [61]. The efficiency of this process depends on the concentration of base in relation with the lignin ratio.

1.8.2. Acid-catalysed lignin depolymerisation

The cleavage of β-O-4 bond of the lignin can also be performed by acid-catalysed depolymerisation [62]. For example, formic acid or other acids can act as hydrogen sources in the hydrolysis with the purpose of forming H_3O^+ on the β-O-4 bond or the cationic aromatic rings. As in the case of the base-catalysed process, the acid-catalysed depolymerisation requires harsh reaction conditions (T > 300 °C, 2-4 hours of reaction) [56]. Further problem is the deterioration of reactors due to the high concentration of acids used.

1.8.3. Metallic catalysed lignin depolymerisation

In order to increase the selectivity and efficiency of the depolymerisation process, the addition of metallic catalysts during the process was taken into account. The introduction of metallic catalysts can decrease the activation energy of the

depolymerisation reaction: for this reason, the process can be carried out in mild reaction conditions. Metallic mediated catalysis targets the cleavage of C–O and C–C bonds of lignin in presence of hydrogen sources like ethanol or water. Nickel, iron, palladium, platinum, zirconium or other solid catalysts can be used to obtain a selective depolymerisation. The main drawback of this technology are related to the use of catalysts themselves since heavy-metals are expensive and environmentally toxic [56].

1.8.4. Ionic liquid-assisted lignin depolymerisation

In the previous chapter, the ability of ionic liquids for lignin extraction was discussed [63]: ionic liquid can be employed in the depolymerisation of lignin. Under these conditions the break of β-O-4 bond under mild conditions (below 250 °C) can be obtained. The mechanism of depolymerisation is not currently elucidated but is believed that the acids associated with ionic liquid, such as Brønsted acid, can act as catalysts and hydrogen sources. The use of ionic liquid in lignin depolymerisation is limited by the high cost of ionic liquids that must be recycled to lower the cost [64]. Furthermore, there is a difficulty in separation of ionic liquid with lignin-derived molecules because of the π-π interaction between ionic liquid and aromatic moieties.

1.8.5. Supercritical fluid-assisted lignin depolymerisation

Supercritical fluids can be chosen as media for lignin depolymerisation [56]: similar to ionic liquid, supercritical fluids were employed as the solvent. Also in this case, the hydrogen sources were provided from acids and alcohols. The stumbling block in the development and use of this technology is the high cost of this process.

1.9. Lignin-degrading enzymes

Lignin degradation and valorization could be obtained exploining microbiologic and enzymatic approaches. Although many microorganisms are able to degrade the sugar-based biomass, only few groups of filamentous fungi (white-rot) possess the ability to metabolize lignin [65]. The lignin degradative process is an oxidative process where phenol oxidases (laccases, manganese peroxidases and lignin peroxidases) represent the key players [66-68].

The importance of these enzymes in lignin degradation process was established in different studies [69-71]: however, an efficient *in vitro* depolymerisation of lignin catalysed by these enzymes has not been yet reported [72-74].

It is clear that fugal intracellular compartmentalisation and others recently discovered enzymes (aryl-alcohol oxidase [75], glyoxal oxidase [76], aryl-alcohol dehydrogenases [77] and quinone reductases [78]) are involved in lignin degradation.

A recent published work reports that different soil bacteria are able to break down lignin. The enzymology of bacterial lignin breakdown is currently not well understood, but extracellular peroxidase and laccase enzymes appear to be involved [79]. In addition to oxidative enzymes, non-radical ligninolytic enzymes represent a promising alternative for lignin cleavage. For example, a system of 5 enzymes named Lig System was reported to be able to catalyse the cleavage of β-aryl ether bonds of a β-O-4 linkage model compound [80, 81].

Enzymatic lignin degradation offers different peculiar advantages:

- The production of valuable, industrially useful, low-molecular-mass aromatic compounds that can be used as important building blocks for preparative organic chemistry;
- Unlike methods based on the exploitation of microorganism, enzymatic depolymerisation can be more controllable and stable. In addition, different enzymes can be selected according to the type of technical lignin used;
- Enzymatic methods are energetically and environmentally “green”.

In order to have an overview of the large landscape of lignin degrading enzymes, the review “Lignin-degrading enzymes” is reported in the next pages.