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Recent Developments in the Delignification and Exploitation of Grass Lignocellulosic Biomass

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Cite This: *ACS Sustainable Chem. Eng.* 2021, 9, 2412−2432

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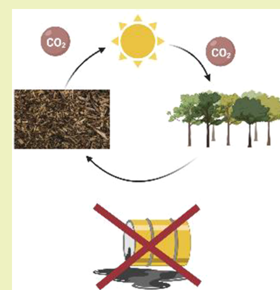
ABSTRACT: Biofuels could be defined as the fuels of the future, although much work is still required before they will replace fossil fuels. In this review, lignocellulosic biomass based on straw and related crops and wastes is described concerning its lignin contents, structure, and properties, and an overview on the means of current and predictable delignification protocols is presented. The discussion is focused on herbaceous monocot materials and their available pretreatments (physical, chemical, and enzymatic), with special emphasis on fungal ligninolytic enzymes and the most recent findings and developments in their current application, issues, and perspectives.



KEYWORDS: *Biofuels, Lignocellulosic biomass, Grass lignin, Delignification, Pretreatments, Ligninolytic enzymes*

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■ INTRODUCTION

Extensive use of fossil fuels is considered the main source of greenhouse gases and other polluting emissions. These phenomena have deep consequences on climate change and public health.[1] Conceivably, global energy demand will strongly rise in the next decade and beyond.[2,3] These issues have led to a growing interest in renewable energies such as biofuels.[4] Among these, bioethanol is used as a renewable substitute for gasoline. Bioethanol is obtained by fermentation of sugars (sugar cane, beet) or starch (maize). As it can be mixed in almost any ratio with gasoline, bioethanol is often used as an additive. It has been estimated that its use could potentially bring a reduction of 30% in our current oil consumption.[5] "First generation" bioethanol has been produced from food sources and is not sustainable because it competes with food crops. Instead, "second generation" bioethanol is obtained from nonedible materials, such as cellulose obtained from lignocellulosic biomass (LBM).[6−8] The economic assessment of lignocellulosic ethanol production has also been extensively studied.[9,10] LBM, earth's most abundant biopolymer-based material, is mainly composed of cellulose (40−50 wt %), hemicellulose (25−35 wt %), and lignin (15−30 wt %) biopolymers and a small percentage of minerals and extractives.[11] Cellulose, the main biopolymer present in lignocellulosic biomass, is a linear, regular, microcrystalline homopolymer $H(C_6H_{10}O_5)_nOH$, where n (degree of polymerization) is some thousands. Cellulose consists exclusively of glucose units linked by β-1,4 glycosidic bonds. Hemicelluloses are irregular, branched, amorphous heteropolymers consisting of different C_5 and C_6 monosaccharide units, such as xylose, mannose, galactose, arabinose, rhamnose, glucuronic acid, and galacturonic acid in various amounts and chemical linkage types, depending on biomass source.

Cellulose, hemicelluloses, and lignin are connected among themselves to form LBM (Figure 1). Cellulose is encapsulated in a cross-linked hemicellulose/pectin matrix through hydrogen bond linkages and van der Waals forces. Hemicellulose is presumably linked with lignin mainly via ester covalent bonds due to hydroxycinnamic acids present in lignin−carbohydrate complexes in the case of grasses.[12−14]

Bioethanol is only one among many products obtainable from cellulose processing. Indeed, according to the type of pretreatment, cellulose hydrolysis leads to a mixture of sugars (glucose, cellobiose, cellotriose, cellodextrins) and lower amounts of other compounds, such as 5-hydroxymethylfurfural (HMF), lactic acid, levulinic acid (LA), 1,2-ethylene glycol, sorbitol, 2,5-bis(hydroxymethyl)furan, formic acid, gluconic acid, and glycolic acid. Hemicellulose hydrolysis results in a mixture of xylose and other monosaccharides, such as arabinose, glucose, mannose, and uronic acids, depending on the particular source of the LBM. Lower amounts of furfural, LA, and γ-valerolactone (GVL) are also formed. Very recently, the topic of cellulose and hemicellulose enzymatic hydrolysis has been extensively reviewed.[15] Except for glucose and xylose, the other "platform compounds" can be valorized by synthesizing other value-added

Received: October 2, 2020
Revised: January 7, 2021
Published: February 4, 2021

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https://dx.doi.org/10.1021/acssuschemeng.0c07266
ACS Sustainable Chem. Eng. 2021, 9, 2412−2432

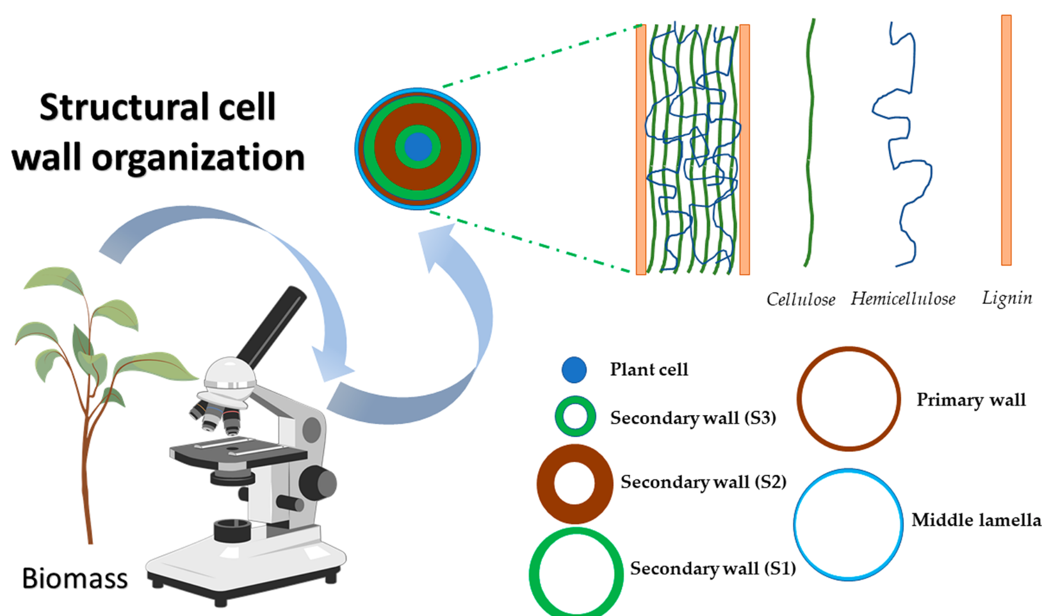

Figure 1. Lignocellulose biomass composition: cell wall composition and structure.

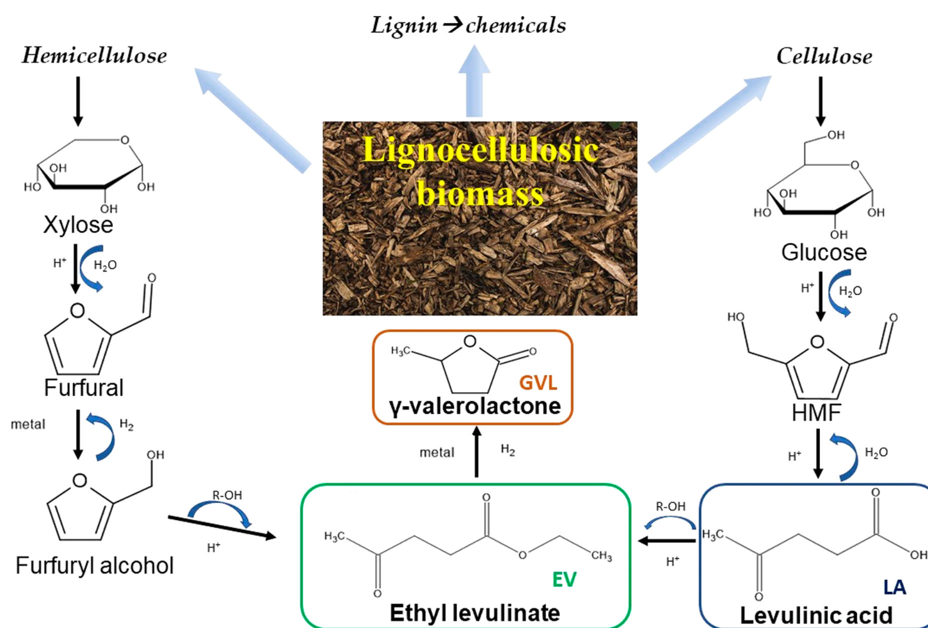

Figure 2. LA, EV, and GVL production from LBM.

chemicals or fuels. For example, LA is one of the most promising platform compounds obtainable from lignocellulose (Figure 2). LA can be used to produce fuel additives as GVL[16,17] or ethyl valerate (EV)[18,19] (Figure 2).

Among the various sources of LBM, grasses (in a general sense, but with particular emphasis on monocots, that is, essentially Poaceae, formerly Gramineae) occupy a special position for various reasons: (i) their lignin content is generally lower in comparison with woody LBM, and conversely their hydrolyzable and fermentable polysaccharide content is higher; (ii) herbaceous LBM usually derives as wastes from other crops or also can be purposely cultivated in marginal areas without hampering food production for humans and animals such as sheep or cattle; (iii) the lifecycle of these vegetables is often annual, and therefore they represent typical renewable resources. As a consequence, they have been and are nowadays the subject of many studies[20,21] to shed light on the particular structural and chemical properties of their lignin fraction and to find the best ways to exploit their high potential to obtain fermentable sugars, fine chemicals, and renewable biofuels.

The aim of the present review is to encompass the state of the art in the knowledge of these LBM materials and to critically examine the main issues and perspectives in their utilization.

■ AN OVERVIEW ON LIGNIN GENERAL FEATURES AND STRUCTURE

Lignin is completely different from the other biopolymers forming LBM, being a cross-linked molecular network based on phenylpropanoid units (monolignols) such as *p*-coumaryl, coniferyl, and syringyl alcohols (Figure 3) and minor amounts

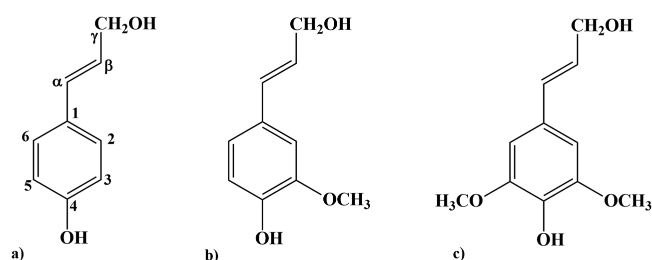

Figure 3. Three monolignols acting as the main comonomers in lignin biosynthesis: (a) *p*-coumaryl alcohol, becoming the *p*-hydroxyphenyl unit H in the polymer; (b) coniferyl alcohol, becoming the guaiacyl unit G in the polymer; (c) sinapyl alcohol, becoming the syringyl unit S in the polymer.

of other monomers.[22] To a certain extent, lignins show a different composition depending on the particular class of vegetables where they are found. Generally speaking, lignin structure forms a dense 3-D texture, based on *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, linked by a variety of C−O and C−C (most commonly, *β*-O−4, *β*−5, *β*−*β*, 5−5, 4-O−5, *β*−1 and *α*-O−4) bonds (Figure 4).[14,22] As noted above, the lignin content in LBM depends on the source: softwoods show the highest lignin content (28−32%), followed by hardwoods (20−25%) and grasses (17−24%).[24,25] Also, the relative amounts of the three main monomers differ significantly[23] among hardwood lignin (H/G/S = trace:50:50, GS-lignin), softwood lignin (H/G/S = 4:96:trace, G-lignin), and grass (monocot) lignin (H/G/S = 5:70:25, HGS-lignin). Important variations in the relative amounts of the three monolignols are often found within the same lignin group.[26,27] Moreover, the large uncertainty in the quantitative determinations of such percentages and ratios should be taken into due account, as the obtained values deeply depend both on the lignin isolation procedures and on the chosen analytical methods.[28]

As noted above, lignins of different botanical origin differ considerably in the proportions among the three main monolignols. This has important consequences in the 3-D structure of the resulting heteropolymers, because S units cannot participate in interunit linkages involving their 5-positions, which are occupied by methoxy substituents and indeed unavailable for participation in other bonds.[29] Consequently, S units cannot participate in 5−5, 4-O−5, and *β*−5 linkages. As a result, decidedly more cross-links are present in softwood lignin (nearly no S units) in comparison with hardwood lignin and, to a certain extent, in grass lignin (about 25% S units, although with high variations among different botanical species). Therefore, although present in substantially lower amounts, grass lignin forms a denser network with respect to the much more abundant hardwood lignin and is conceivably harder to remove.[29]

■ GRASS LIGNIN: RECALCITRANT THOUGH SCARCE

For the reasons underlined above, grasses (both crops and wastes coming from herbaceous crop processing) are of high interest as inexpensive and rich sources of polysaccharides and therefore fermentable sugars, as well as other sugar-derived chemicals. However, such potential is hindered by the recalcitrance of their lignin fraction,[30] which must be removed in whole or at least in part to fully exploit the polysaccharides. Accordingly, grass lignin structure has been and is nowadays the subject of much research to understand its peculiar structural motifs and to find suitable delignification treatments.

Grasses *stricto sensu* belong to the Poales,[31] and in particular to the family Poaceae, although also Cyperaceae include a vast variety of herbaceous species. On the other hand, many Bambusoideae have woody stems, though their "wood" shows the anatomical features, and the general chemical composition, typical of monocots,[32] and this is why they are considered in this description.

Since the pioneering studies, some points have been established about grass lignin general structure and properties, although with sometimes contradictory conclusions referring to, for example, the presence (if any) and abundance of *α*-O−4 ether linkages connecting the monolignol units.[33,34] Another special feature emphasized by early studies is the high relative abundance of H units in wheat straw lignin and therefore presumably also in taxonomically related straws. In the same study, the highly complex structure of Poaceae lignin in comparison to both hardwood and softwood lignin was confirmed.[35] This outstanding feature was also underlined in a general review[20] focused on the peculiarity of grass cell wall in comparison with dicots: in particular, a significant percentage of H units (∼4−15%) was claimed to be usually present in grass lignins, together with etherified or esterified ferulic and *p*-coumaric acids (Figure 5). However, the use of combined, different analytical techniques has shown that the relative abundance of H units is usually overestimated, because *p*-coumaryl residues are often and incorrectly ascribed to H units.[36] Also protein residues in cell walls contribute to such an overestimation.[37] Neither H units nor hydroxycinnamic acids are present in noticeable amounts in the large majority of dicot lignins. That of lignin acylation by hydroxycinnamic acid is a crucial feature in lignin of monocot grasses,[38] not only for their differentiation from nongrass lignins but because these hydroxycinnamoyl residues are the chemical bridges between lignin and cell wall polysaccharides. In particular, the *γ* position, predominantly or also exclusively of S units, is the acylation site, by acetyl, *p*-hydroxybenzoyl, and *p*-coumaroyl moieties. Also ferulic acid is present, though usually in smaller amounts than *p*-coumaric acid, as a constituent of grass lignins. Ferulic acid should be considered among the monomeric units of grass lignins, where it most probably acts as a polymerization initiator.[38] However, rather than forming esters at the *γ* positions of S units, ferulic acid tends to be linked preferentially to G units[39] as an *α*-alkyl-aryl ether (by means of its phenolic hydroxyl at the 4 (or *para*) position), whereas its carboxy function is esterified to hemicelluloses, mainly at the 5 positions of arabinose residues of arabinoxylans. Such ester bonds are alkali-labile, whereas the *α*-alkyl-aryl ether bonds are acid-labile. This observation has important practical consequences and should be taken into due account when planning delignification protocols for herbaceous crops (*vide infra*). Another peculiar feature of feruloyl residues in grass lignins is the definite tendency of these moieties to exist in part as various isomeric C−C and C−O dimers, arising from peroxidase action. Such dimers contribute to a close covalent bonding between lignin and cell wall polysaccharides. The very complicated topic of lignin−carbohydrate complexes (LCCs) has been recently reviewed[40] and has been and is still the objective of several studies, owing to its implications in delignification procedures (*vide infra*). In addition to the "indirect" mode of interaction (feruloyl moieties acting as bridges between lignin and carbohydrates), direct *α* ether linkages have been found, binding together lignin and arabinoxylans. In particular, the ether linkages involve usually the 5 position of the arabinose units. Moreover, a minor fraction

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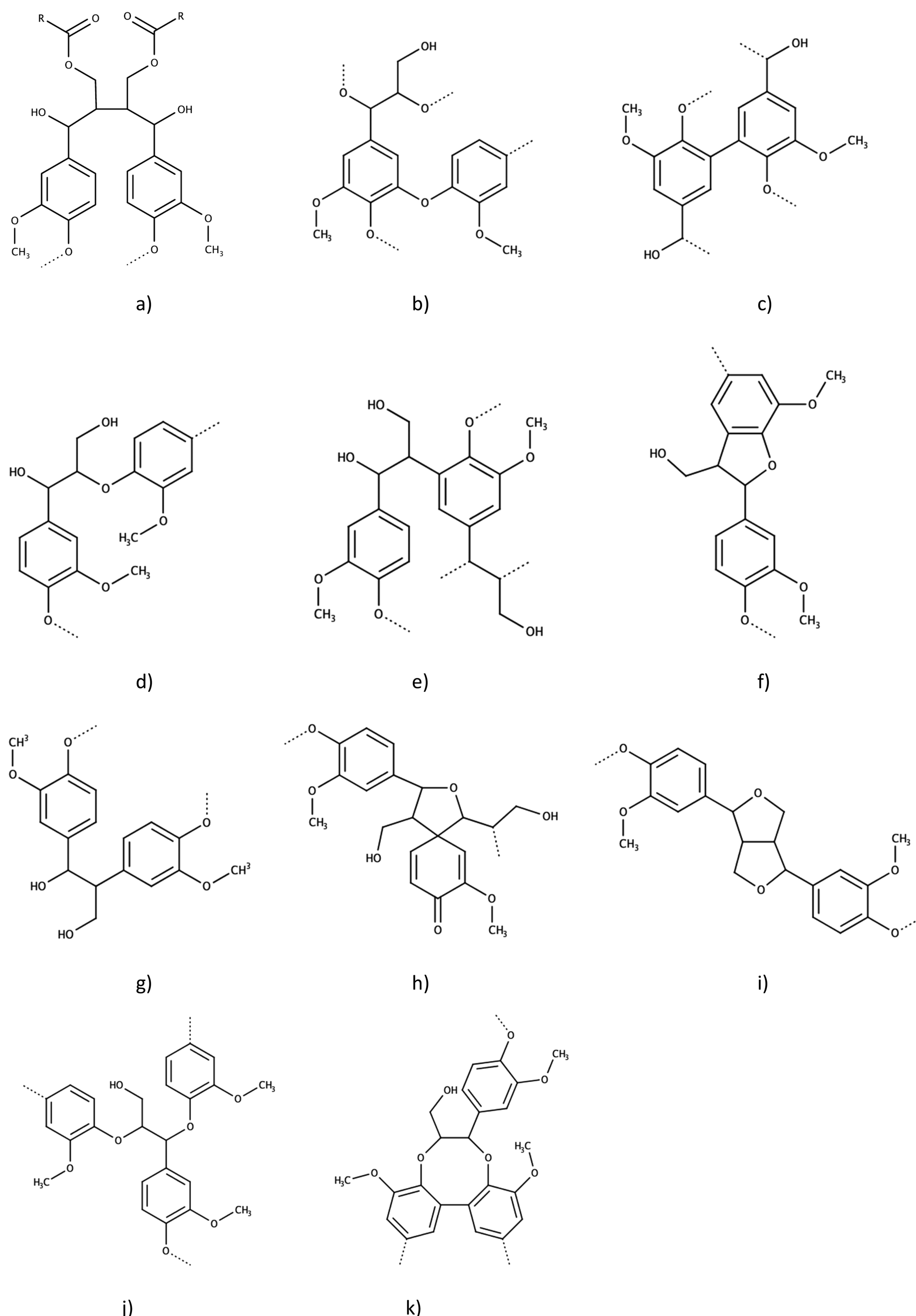

Figure 4. Most common chemical linkages interconnecting the monomeric units within the lignin 3-D polymer: (a) $\beta-\beta$; (b) 4-O−5; (c) 5−5; (d) β-O−4; (e) $\beta-5$; (f) phenylcoumaran, (α-O−4 and $\beta-5$); (g) $\beta-1$; (h) spirodienone (α-O−α and $\beta-1$); (i) resinol (α-O−γ, $\beta-\beta$, and γ-O−α); (j) α,β-diarylether (α-O−4 and β-O−4); (k) dibenzodioxocin (α-O−4, β-O−4, and 5−5).

of polysaccharides can form ester linkages involving glucuronic acid or 4-*O*-methyl-glucuronic acid moieties of the glucurono-arabinoxylans. The preferentially involved esterification sites are the γ positions of G units.[38,40]

The widely accepted structural models for cell walls in monocot grasses[20,41] suggest that the prevailing interbiopolymer covalent bonds mainly involve hemicelluloses. However, some indications of direct bonding between lignin and cellulose exist

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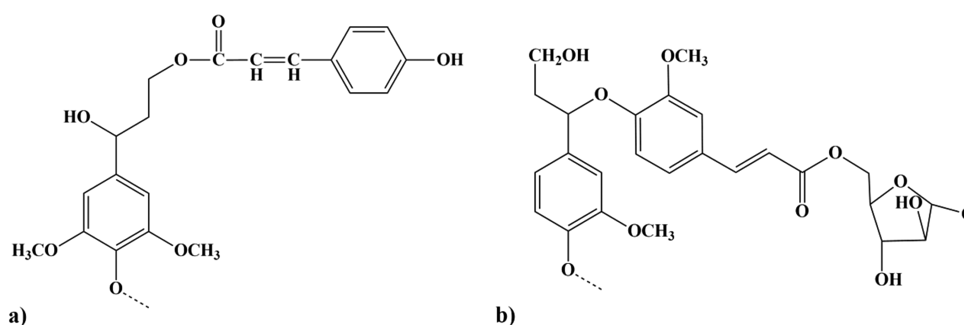

Figure 5. *p*-Coumaric (a) and ferulic (b) acids and their mode of bonding to grass lignin.

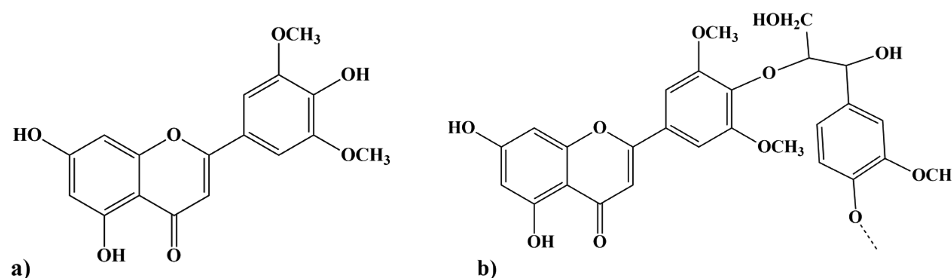

Figure 6. Flavone tricin (a) and its mode of bonding to the lignin (b).

from a long time ago and were recently confirmed with new analyses.[42] In particular, systematic NMR analysis of 80 species of the Poaceae has confirmed the wide presence of such bonds, involving essentially the 6-OH along the cellulose chains and the phenolic 4-OH of the monolignol units. In spite of the greater steric hindrance, also the 2- and 3-OH groups along the cellulose chains could be involved in such linkages, as well as the α- and β-hydroxyls of the phenylpropanoid moieties.

Another peculiar feature of the grass lignins is the significant presence of monomers, different from the "classical" monolignols,[22] that are sometimes found also in wood lignins, but as quite marginal components. Among those, the flavone tricin (Figure 6) occupies an outstanding position as a minor comonomer in grass lignins, where it participates in the formation of polymeric chains exclusively through 4′-*O*-β linkages. Therefore, it can occupy only the terminal positions in the lignin chains. A role for this flavonoid as a possible initiation molecule for lignification in monocots has been suggested.[43]

The large variety of monomers, additional compounds, and possible interunit linkages, together with the variety of grass LCCs, gives rise to a grass lignin family, where the main common features are counterbalanced by particular compositional and structural motifs, such as are briefly encompassed here below.

A comparative study[44] among wheat, barley, maize, alfa (*Stipa tenacissima*), *Miscanthus* straws, and oat husks has shown that the respective lignins are quite similar, with an almost identical S/G ratio (0.78 and 0.79, respectively) for barley and wheat straw lignins. Other Poaceae would be slightly different, with S/G ratios of 0.76 for maize and 0.81 for *Miscanthus* straws, respectively. These values have been determined by FT-IR analysis. In the same study, the condensation indices (CI, meaning the fraction of monolignol subunits, participating in additional C−C linkages or diphenyl ether linkages, besides those usually binding the monolignols) were measured. However, the claimed strict resemblance among cereal lignins has been questioned by other studies.[38,41] The H/G/S ratio is 4:35:61, 5:49:46, and 15:45:40 for maize stover, wheat straw, and rice straw, respectively. In particular, rice (*Oryza sativa*) straw contains less lignin in comparison with maize and wheat; wheat straw shows the almost unique property of containing a significant fraction ($\simeq$20%) of its total lignin content that is readily soluble in alkaline solutions. However, as a general observation, such data as well as other analytical measurements have to be considered with the greatest caution, owing to the significant structural alterations lignins can undergo as a consequence of even mild extraction and separation procedures.[45,46]

Bamboo[47] and giant bamboo[48] lignins have been investigated in detail with concern to their structural features, owing to the great importance of many Bambusoideae as potential pulp and paper sources as well as with reference to their application as starting raw materials in biorefineries. In brief, *Bambusa* lignin shows an unusual abundance of S units that sharply prevail over both G and H units (S/G ratio > 1.5). The monolignol units are bound by means of the usual linkages such as β-O−4 and so on, with the significant presence of α,β-diaryl ether motifs. The *Bambusa* lignin is noticeably heterogeneous, as a fraction with a lower S/G ratio could be separated by means of a milder extraction, whereas an alkaline extractant allows the dissolution of the high S/G ratio component. Also, significant amounts of *p*-coumaric and ferulic acids have been detected, covalently bound to the polymer. For *Dendrocalamus sinicus* (giant bamboo), the most interesting result is perhaps the high incidence of α-O−sugar linkages, maintaining covalently bound to both other lignins and hemicelluloses. The involved C atoms can be either terminal carbons (C_6 or C_5 depending on the hexose or pentose nature of the involved sugar) or secondary (C_2 or C_3) carbons. A detailed study on *Arundo donax* (common reed) lignins[49] revealed that, as found for other monocot grasses, some differences exist between stem and foliage lignins. On the whole, in spite of the apparent resemblance between reed and bamboo, the S/G ratio is decidedly lower in the former (<1), although with significant differences between stem and foliage

https://dx.doi.org/10.1021/acssuschemeng.0c07266
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lignins: the latter contains more H units and its S/G ratio is even lower. The β-O−4 linkages largely prevail in both lignins, although with higher frequency in stem; conversely, acylation at the γ positions is more present in the foliage lignin.

Alfa (*St. tenacissima*) is not to be confused with alfalfa (*Medicago sativa*), which belongs to Fabaceae and is therefore a dicot. Alfa lignin analyzed after a mild organosolv process,[26] revealed a "normal" composition of an HGS type polymer, where the main interunit linkage is, as usual, β-O−4 alkyl-aryl ether. Moreover, it contains a high amount of acetate, hydroxybenzoate, and hydroxycinnamate esters, with the acetyl residues mainly linked to the γ positions of the S units.

Sugar cane (*Saccharum* spp.) bagasse and straw are produced in large amounts in tropical countries as byproducts of the sugar industry. Therefore, they have been deeply studied as a potential source of fermentable sugars after saccharification. Consequently, their lignins have been the subject of a number of investigations to determine their structure and chemical properties.[50] In particular, bagasse lignin is a HGS-type polymer with a high S/G ratio, whereas the opposite is found in the straw, where G units sharply prevail. This finding is in full agreement with the consideration that bagasse arise mainly from mature stems, whereas straw contains also young leaves in addition to mature foliage. Moreover, the two types of lignin differ also in the proportions among the interunit linkages. In fact, bagasse lignin units are largely linked by β-O−4 bridges, whereas straw lignin contains comparatively higher proportions of other linking modes, such as phenylcoumaran (benzoxolane) moieties.

Pennisetum purpureum (elephant grass) is in top position for LBM productivity and is easily cultivated in tropical areas, and therefore its lignin is worth investigating in detail.[36] This lignin contains a low content of H units (~3%) and exhibits a high degree of γ-acylation by *p*-coumaric acid.

Switchgrass (*Panicum virgatum* L.), Poaceae, is a perennial monocot grass native of North America, forming vast prairies along the plains, in particular in marginal, arid, and poorly productive areas, although it has been introduced in Europe, owing to its high potential as an energy crop for ethanol production.[51] The switchgrass lignin, arising from the use of polysaccharide hydrolases, is characterized by a high prevalence of β-O−4 interunit linkage, followed by β−5−α-O−4 (phenylcoumaran) and β−β (resinol) linkages.[52] Inspection of lignin content and composition among different varieties of switchgrass showed very little variations, whereas lignin content and S/G ratio varied greatly when comparing internodes, nodes, and leaves: the content increases from internodes to nodes to leaves, whereas the S/G ratio decreases from about 0.7 for internodes to 0.6 for nodes to 0.46 for leaves.[53] A detailed comparative experimental study about the techno-economic feasibility of different delignification pretreatment procedures for switchgrass has been published.[54]

In conclusion, grass lignins are generally present in lesser amounts in straw in comparison to wood lignins, but their particular structure, typical for noticeable presence of etherified and esterified hydroxycinnamic acids, making close linkages with cell wall polysaccharides, requires careful (pre)treatments to fully exploit the fermentable sugar potential of the LBM.

DELIGNIFICATION MAIN STRATEGIES AND PROTOCOLS

As already noted, due to its highly cross-linked, three-dimensional structure, grass LBM is recalcitrant to degradation, making the overall lignocellulose conversion a challenge.[14] In order to separate cellulose and hemicelluloses from lignin, delignification pretreatments are needed.[55−57] During pretreatment, lignin is modified or partially separated and also dissolved and removed from cellulose and hemicelluloses making them more accessible to hydrolytic enzymes. With respect to the processes needed to obtain ethanol or the platform compounds from cellulose and hemicellulose, the delignification step is undoubtedly the most challenging and critical of the overall process. Different delignification strategies can be used:

- mechanical (microwave, ultrasound, mechanical extrusion);[58]
- physico-chemical (steam explosion, hot water, CO_2 explosion, NH_3 fiber explosion);[59,60]
- chemical (acid or alkali hydrolysis, organosolv, ionic liquids, reductive catalytic fractionation, selective oxidation);[61−67]
- biological (microorganisms, redox enzymes).[68]

Most of the listed pretreatments involve harsh conditions (high temperature and pressure, use of solvents, or extreme pH values) causing partial biomass degradation. As a consequence, wastes as well as enzyme inhibitors are produced, making such processes unsuitable from economic and environmental points of view.[6,69] Among pretreatments, the use of redox enzymes is very attractive for overcoming these drawbacks.[70] Moreover, they prevent the formation of furfural and phenolics, inhibitors of hydrolytic enzymes needed to obtain monosaccharides from cellulose and hemicelluloses, which are typically generated during mechanical, physicochemical, and chemical pretreatments.[71] For these reasons, the use of redox enzymes is becoming of increasing importance in the biorefinery field. In the last years, the role of enzymes for the production of biofuels has been widely investigated.[72]

The aim of the present section is to give an overview of the most recent developments in the field of industrial delignification, with particular focus on the use of redox enzymes.

A Brief Survey on the Nonenzymatic Delignification Methods for Straw. It is common knowledge that hemicelluloses, owing to their branched and irregular structure, are comparatively easier to remove than the other biopolymers forming LBM, and therefore relatively mild treatment such as extraction with hot water allows their removal. Conversely, lignin stubbornly resists such treatments, as does cellulose. As underlined in the previous section, grass lignins are engaged in a wide variety of covalent bonds with hemicelluloses and cellulose, and therefore any attempt at separation among the three main biopolymers usually produces raw fractions containing significant amounts of the other biopolymers, unless very harsh operative conditions (such as Kraft protocols) are adopted.[73] However, although the Kraft method is very suitable for preparing cellulose fibers (pulp and paper industries), it shows the drawback of completely dissolving the hemicelluloses, essentially (glucurono)arabinoxylans, which constitute a substantial source of fermentable sugars in straw.[74,75] Also for this reason, the Kraft process is not usually applied to straw delignification. The same could be said for the other widespread wood delignification process, which is based on soluble sulfites.[76] Therefore, some other protocols, not so destructive and above all more ecofriendly, have been developed, and some are currently used at industrial or semi-industrial scale. In particular, mild alkaline pretreatments could be resolutive for preliminary hemicellulose separation from lignin, owing to the

https://dx.doi.org/10.1021/acssuschemeng.0c07266
ACS Sustainable Chem. Eng. 2021, 9, 2412−2432

labile ester bonds joining the two polymers by means of ferulic acid bridges (*vide supra*). It is also worth noting that the fractionation protocols useful for research at laboratory scale are usually not suitable at all for the purpose of polysaccharide recovery. In fact, hemicelluloses are readily dissolved by comparatively smooth treatments, contrary to cellulose and lignin. Consequently, biorefinery-oriented procedures must include operative methods devoted to hemicellulose recovery and exploitation. Finally, the whole biorefinery process must be inexpensive enough to ensure a substantial profit when selling the obtained chemicals such as ethanol, xylose or xylitol, and so on.[77,78]

Physicochemical Treatments. These include the use of solvent systems that ideally do not alter significantly the covalent structures of the main LBM constituents. In practice, the dense 3-D lignin structure requires a partial molecular simplification to be brought into solution, unless hydrotropic solvents are used.[79] Generally speaking, there is growing interest in the field of lignin removal by organic aqueous solvents.[55] These should be inexpensive, easily recoverable and recyclable, and environmental friendly. Progress in this field has been recently reviewed.[80] Particularly promising appears a recent method based on lignin solubilization ("organosolv" process) with an ethanol–water mixture (60:40) under pressure at 160 °C. The dissolved lignin was then precipitated by proper dilution with moderately acidified water.[81] Ethanol (80% aqueous solution) was used similarly for solubilization of lignin from *Miscanthus*.[82] Another organosolv procedure on straw and other byproducts from cereals has been described, affording a highly efficient lignin extraction and recovery.[83] However, the whole fractionation process for the studied LBM was somewhat laborious. Other studies[84,85] have shown the efficient solubilizing action of hot imidazole over lignin, which could be isolated by simple dilution of the imidazole solution; this latter could be later recovered.

Ionic liquids (ILs) are organic salts that are nonvolatile liquids at room temperature (Figure 7). ILs have high solvating power and excellent chemical and thermal stabilities, and their solubilizing power toward lignin has been assessed by some studies.[86,87]

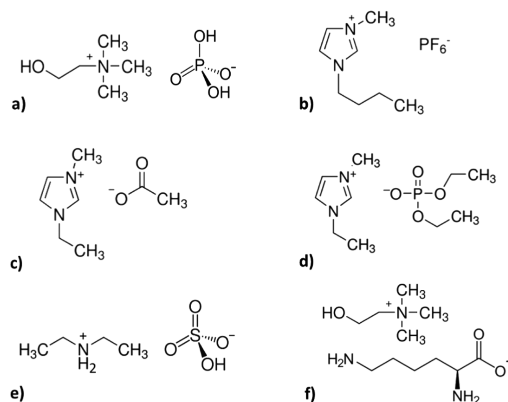

Figure 7. Common ILs used in delignification process: (a) Cholinium dihydrogen phosphate [Ch][H_2PO_4]; (b) 1-butyl-3-methylimidazolium hexafluorophosphate [Bmim][PF_6]; (c) 1-ethyl-3-methyl-imidazolium acetate [EMIM][OAc]; (d) 1-ethyl-3-methyl-imidazolium-diethyl phosphate [EMIM][DEP]; (e) diethylammonium hydrogensulfate ([DEA][HSO_4]); (f) cholinium L-lysinate [Ch][Lys].

Steam and Ammonia Explosion and Extrusion. Although known from about a century, steam explosion is still current and its use in LBM pretreatments is widespread. Several studies, even at the pilot or industrial scales, have been described related to various LBM materials from different plant sources and have been recently reviewed.[88,7] The main advantage of the technique is the absence of added chemicals, whereas the main limitation is the partial hydrolysis of the hemicelluloses and the formation of free acids such acetic and hydroxycinnamic. These cause dehydration and cyclization of xylose leading to the undesired 2-furaldehyde.

Ammonia explosion[89] is a related technique that among other effects prevents furaldehyde formation caused by free acidity. The technique, commonly referred to as AFEX (ammonia fiber expansion/explosion), requires the use of anhydrous ammonia,[90] that can be recovered at the end of the treatment owing to its high volatility. Excess ammonia could also be recovered by stripping with hot steam, resulting in a concentrated ammonia solution. On the whole such a pretreatment does not alter the LBM chemical composition too deeply, whereas it acts on ultrastructure,[91] making the material more accessible to further treatments such as those involving polysaccharide hydrolytic enzymes. This pretreatment has been recently reviewed together with the use of ammonia aqueous solutions.[90]

Extrusion[92] is to a certain extent similar to steam explosion. In this case, steam is substituted by a strong shear pressure, obtained by forcing LBM through a screw system, which also causes the LBM to heat at the same time.

Chemical Pretreatments. These could be subclassified into two main groups: (i) hydrolytic methods and (ii) oxidative methods.

In principle, hydrolysis could take place under either acidic or alkaline catalysis. Mild acidic hydrolysis is effective toward hemicelluloses, while lignin and cellulose remain untouched; as expected, the stronger the acidic conditions, the more efficient is the hydrolysis. However, excessive acidity could promote, besides other unwanted side reactions, partial hydrolysis of both cellulose and lignin. In particular, strong acids promote the formation of the undesired 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde, so milder conditions are generally adopted. On the other hand, alkaline hydrolysis removes a fraction of hemicellulose without forming 2-furaldehyde, but also a significant lignin fraction is removed and contaminates the obtained solubilized hemicellulose. A mild acidic pretreatment to remove and partly hydrolyze hemicellulose, followed by an alkaline one to remove a substantial lignin fraction, could be the optimal solution, although more costly and requiring more complex biorefineries. All these consideration have been extensively and recently reviewed.[93]

Some other pretreatments, in particular oxidative ones, find application in pulp and paper industry, as they leave a solid residue mainly formed of cellulose. In other words, hemicelluloses are removed (and substantially wasted) together with lignin. To prevent such waste, the above-mentioned mild acidic pretreatment[94] could allow for hemicellulose hydrolysate recovery prior to the delignification process.[93]

Lignin interunit linkages mainly arise from radical-based addition reactions, and consequently their hydrolysis requires harsh conditions (in the case of C–O–C diphenyl ethers or alkyl-aryl ethers) or is quite impossible (C–C bonds). Therefore, lignin solubilization, apart from the harsh pulping treatments such as Kraft, sulfite, or soda processes, is achieved by means of oxidative cleavage. Ozone treatment under suitable

https://dx.doi.org/10.1021/acssuschemeng.0c07266
ACS Sustainable Chem. Eng. 2021, 9, 2412–2432

operative conditions could be effective,[95,96] but its use is limited by high production costs, impossible storage, and the explosive and toxic nature of the reagent.

Chlorite pretreatment of LBM is a well-known process, affording substantial lignin removal without significant formation of highly recalcitrant chlorolignins. A deep study on the sodium chlorite/acetic acid pretreatment of hybrid *Pennisetum* straw, citing also recent developments with reference to biofuel production, has been recently published.[94] It is worth noting that such a pretreatment is rather selective toward lignin and increases substantially polysaccharide digestibility. A treatment of rice straw with chlorine dioxide has shown promising results[66] with respect to delignification of such a LBM. Alas, chlorine dioxide is an expensive, toxic, unstable, and explosive reagent, so these substantial limitations should be taken into due account.

A huge number of studies have been published concerning the use of hydrogen peroxide as a delignifying agent for LBM, usually under moderate alkaline conditions. Effectiveness of the process ranges from moderate to good, and its main advantages are economical (hydrogen peroxide is a comparatively inexpensive reagent and is manageable and safe enough for industrial applications) and environmental (the only stable end-products of its action are molecular oxygen and water). Generally speaking, although hydrogen peroxide is a stronger oxidant at low pH values, it is often more effective under moderately alkaline (pH 10−12) conditions, where its mode of action is at least in part based on the formation of superoxide and hydroxyl radicals and also the extremely reactive singlet oxygen. Important changes toward a more substantial, if not exclusively, radical character of H_2O_2 action are observed in the presence of certain redox-active transition metals such as Cu, Mn, and especially Fe (Fenton or Fenton-like reactions).

These general features of lignin oxidation by hydrogen peroxide have been recently reviewed.[97,98] Some outstanding examples are cited here, relative to straw LBM pretreatments involving H_2O_2. *Panicum virgatum* and *Zea mays* derived LBM were subjected to alkaline hydrogen peroxide pretreatment; in both materials, a substantial cleavage of alkyl aryl ether linkages was found, in particular of β-O−4 ones, leading to lignin solubilization and increase of polysaccharide hydrolysis.[99] Barley straw was investigated with relation to its response to optimized alkaline hydrogen peroxide pretreatment, and a substantial improvement of glucose yields through enzymatic hydrolysis of the solid residue was assessed.[100] Another promising study deals with hydrogen peroxide pretreatment prior to ammonia fiber expansion of corn stover; in detail, demethylation of G units took place, concomitant with cleavage of a significant fraction of β-O−4 linkages; conversely, resinol and phenylcoumaran moieties remained substantially unchanged. Some γ carbons were oxidized, and the primary alcohol functions were changed into carbamides. As a result, a substantial decrease in lignin MW was observed. Indeed, a sharp improvement in saccharification was obtained.[101]

Addition of hydrogen peroxide to acidic pretreating solutions affords the production of hydrolyzed hemicelluloses, where undesired furane aldehydes have been oxidized to carboxylic acids, thus preventing any inhibition phenomena of the microorganisms used to produce bioethanol. Various studies have been recently published[102−104] employing hydrogen peroxide in acetic or phosphoric acids; satisfactory extent of ligninolysis was observed besides a substantial hemicellulose hydrolytic removal. Performic acid (arising from the direct reaction between formic acid and H_2O_2) has a noticeable tendency toward heterolytic scission affording the virtual hydroxylium ion, which is a very effective electrophile and could efficiently break lignin down through a Baeyer−Villiger-like oxidation reaction cutting the Cα−Cβ linkages within the phenylpropanoid units.[105,106]

As noted above, H_2O_2 could also act as a source of hydroxyl radicals, under the conditions of the Fenton (and also Haber−Weiss) reaction, causing a noticeable improvement in straw LBM digestibility.[107−109]

Fungal Redox Enzymes for the Delignification of Straw LBM. In Nature some fungi, like white rot fungi (WRF), brown rot fungi (BRF), and soft rot fungi (SRF), live and grow on wood or on LBM, both natural or artificial. WRF and BRF belong to the group of basidiomycetes, while the SRF group consists mostly of ascomycetes.[110] It has been shown that basidiomycetous WRF are able to delignify lignocellulosic feedstock efficiently, unlike the BRF, which delignify biomass slowly and incompletely; SRF mainly attack polysaccharides, so their delignification efficiency is irrelevant.[111,112]

The use of fungi to pretreat biomass is called biological pretreatment. Due to its many advantages in the delignification process such as low energy supply, low environmental impact, and high substrate specificity, biological pretreatment is considered a cleaner and greener approach compared to physical and chemical pretreatments.[111,113,114] Nevertheless, this approach shows several drawbacks such as the loss of polysaccharides, which leads to low sugar and ethanol yields, and long reaction times (from 13 up to 50 days) to obtain high yields of delignification. Moreover, the process depends on many factors such as cultivation time, fungal strains, and culture conditions.[115] Therefore, biological pretreatments on industrial scale are still limited.[116] Fungal degradation of lignin is the result of the cooperative actions of several enzymes. The use of ligninolytic enzymes secreted by WRF involved in natural delignification processes could overcome these drawbacks.[117] Enzymatic delignification is attractive because it is faster than biological pretreatment and can operate in a wide range of pH (3−8) and temperature (25−80 °C). Moreover, it does not require supplementation of nutrients.

The most important ligninolytic enzymes (Figure 8) from WRF extracts[25] are laccases (phenol oxidases) and heme-peroxidases (lignin peroxidase, manganese peroxidase, versatile peroxidase). The delignification process could be enhanced by the cooperative action of several accessory enzymes, such as glyoxal oxidase, aryl alcohol oxidase, and veratryl alcohol oxidase able to generate hydrogen peroxide essential for peroxidase mechanism.[23,118] Initially somewhat surprisingly, early studies showed that purified ligninolytic enzymes are often most active toward oligomeric model compounds, whereas their activity tends to decrease when facing real lignin preparations. Moreover, it has been well-known for decades that sometimes the main effect of ligninolytic enzymes is further polymerization rather than depolymerization.[119,120] This depends mainly on the radical character of the reactions catalyzed by the ligninolytic enzymes: the arising radicals could spontaneously undergo cleavage, reaction with molecular oxygen, or disproportionation to quinones or can polymerize. *In vivo*, polymerization is usually prevented by the intervention of other enzymes, reducing reactive quinones to catechols,[121] which in turn undergo irreversible cleavage by dioxygenases. Obviously, the use of such complex mixtures of enzymes along industrial applications is only seldom feasible, and therefore other shrewdness must be adopted to overcome the problem.

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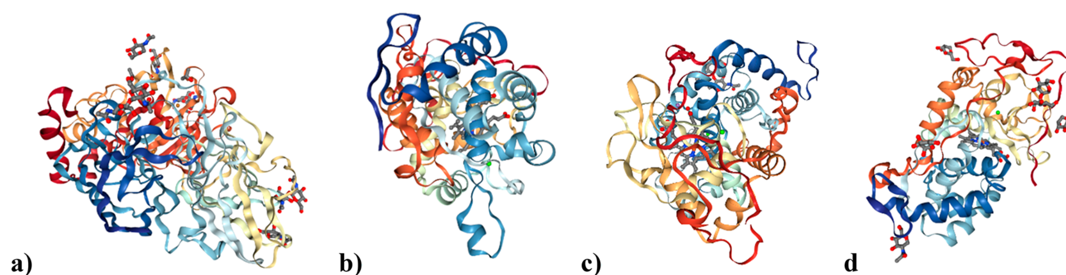

Figure 8. Structures of some ligninolytic enzymes: (a) laccase from *Trametes versicolor*, 1GYC; (b) lignin peroxidase from *Phanerochaete chrysosporium*, 1B85; (c) manganese peroxidase from *P. chrysosporium*, 1MNP, (d) Versatile peroxidase from *Pleurotus eryngii*, 3FJW.

Ligninolytic redox enzymes are described in the following paragraphs. The ligninolytic enzymes will be initially listed and briefly described, then the most promising strategies to improve their performance in terms of activity and stability will be described.[110]

Laccases. Laccases, LCs (EC 1.10.3.2, benzenediol:oxygen oxidoreductase), are extracellular N-glycosylated multicopper oxidases able to oxidize various phenolic and nonphenolic compounds by one electron transfer with the concomitant reduction of dioxygen to water.[122] They are widespread among higher plants, bacteria, insects and WRF.[123] Their physicochemical proprieties, such as isoelectric point, molecular size, stability, activity, etc. depend on the source. Indeed, due to their higher redox potential,[124] fungal laccases rather than bacterial laccases are widely used in biorefineries and bioremediation processes.[23] LCs typically comprise three domains and contain four copper ions arranged in mononuclear and trinuclear clusters. Substrate oxidation at the mononuclear site generates electrons that are transferred one by one to the trinuclear site where O_2 is reduced to water.[125] Their molecular mass commonly ranges between 50 and 140 kDa, but it has been reported to be from 34 to 383 kDa for laccases from *Pleurotus eryngii* and *Podospora anserina*.[116,122,126,127] Due to their low substrate specificity, LCs can degrade several compounds with a phenolic structure, including lignins.[128] For example, Sondhi et al. obtained a 28% reduction in kappa number (a measurement of total amount of material oxidizable by $KMnO_4$) by means of thermophilic LC from *Bacillus tequilensis*.[129]

To date, the number of microorganisms involved in the delignification process is still unknown;[25] however it is certain that the features of their secreted ligninolytic enzymes depend on their nature and ecology.[130] Due to their ability to produce ligninolytic enzymes showing different properties compared to terrestrial enzymes (such as high salt tolerance, pH extremes, and thermostability), interest in marine-derived fungal species is growing. Rodríguez-Couto investigated the ability of the marine fungus *Phlebia* sp. MG-60 to degrade sugar cane bagasse for 30 days. The treatment leads to about 52% decrease of lignin in the raw material.[131] D'Souza-Ticlo et al. extracted from fungus MTCC 5159 (*Cerrena unicolor*) a LC stable at pH 9 and able to retain >60% of its activity up to 180 min at 50 and 60 °C. The same enzyme was tested in the presence of Pb, Fe, Ni, Li, Co, and Cd ions at 1 mmol. The results showed no inhibition by salts.[132] Brenelli et al. obtained oxidative enzymes under saline and nonsaline conditions from the white-rot basidiomycete *Peniophora* sp. CBMAI 1063.[133] A LC form with thermal stability ranging from 30 to 50 °C (120 min) was extracted and characterized from the same fungus.[134]

The use of enzymes in their native form employed directly on industrial scales is often hampered by the industrial harsh reaction conditions (such as extreme values of pH and temperature), which could lead to a loss of activity.[135] Due to the need for obtaining enzymes able to work under extreme operative conditions, enzyme engineering is considered an attractive technology for making them suitable for industrial and biotechnological applications.[136] This technique implies the modification of the amino acid sequence, the main approaches reported in literature being rational design and directed evolution.[137] The engineering of ligninolytic enzymes could play a crucial role in the field of biomass delignification. Several works about this topic have been reported. The LC from *Pycnoporus cinnabarinus* is considered one of the most promising high-redox-potential enzymes for environmental biocatalysis; Camarero et al. expressed it in *Saccharomyces cerevisiae* obtaining a laccase total activity enhanced 8000-fold.[138] Kwiatos et al. described the engineering of *Fusarium oxysporum* LC expressed in *Saccharomyces cerevisiae* and engineered toward higher expression levels and higher reactivity toward 2,6-dimethoxyphenol, which could be used as a mediator for lignin modification.[139] It is known that laccases typically show their optimum activity below neutral pH values. It is supposed that the excess of OH^- ions inhibits electron transfer from T1 Cu to the trinuclear copper cluster. However, some important industrial applications require LCs active at pH ≥ 7, such as paper pulp bleaching and decolorization of industrial dyes.[140] LC engineering is considered a promising tool to overcome this bottleneck. Fang et al. expressed Lac15D, a bacterial LC screened from a marine metagenomic library, in *E. coli*, obtaining an enzyme with a high tolerance to halogen ions able to work under alkaline conditions.[141,142] Yin et al. engineered the LC from the basidiomycete *Coprinopsis cinerea*, which showed an optimum pH of 8.5 toward guaiacol and retained more than 70% of its activity at pH range of 7−9.[143]

However, due to their relatively low redox potential (0.5−0.8 V), LCs are not able to oxidize nonphenolic subunits with a high redox potential, which comprise more than 80% of lignin.[23,144] Moreover, because of their size, LCs are unable to penetrate the small pores of plant cell walls.[117] To overcome these issues, the use of mediators has been proposed.[145,146]

Laccase Mediators. Mediators are small, soluble molecules able to transfer electrons from the to-be-oxidized molecules to the LC active site.[117] In the case of LBM, they can shuttle between the surface of the solid substrate and the enzyme. Laccase mediator systems (LMSs) have a higher redox potential ($E°$ > 1.1 V) in comparison to LC in the Cu T1 site ($E°$ = 0.5−0.8 V) and are able to oxidize lignin to a good extent (Figure 9).[72,147,148]

Among them (Figure 10), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), 1-hydroxybenzotriazole (HBT), and 2,2,6,6-tetramethylpiperidine-1-oxyl

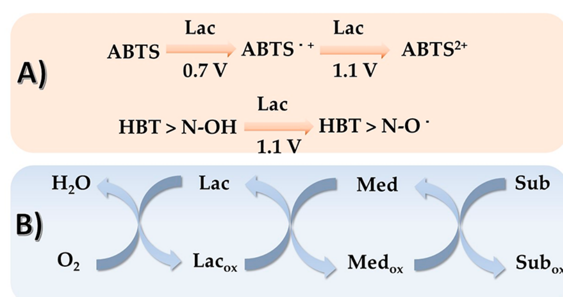

Figure 9. (A) Redox potentials of the oxidation reactions of ABTS and HBT by laccase and (B) LMS mechanism.

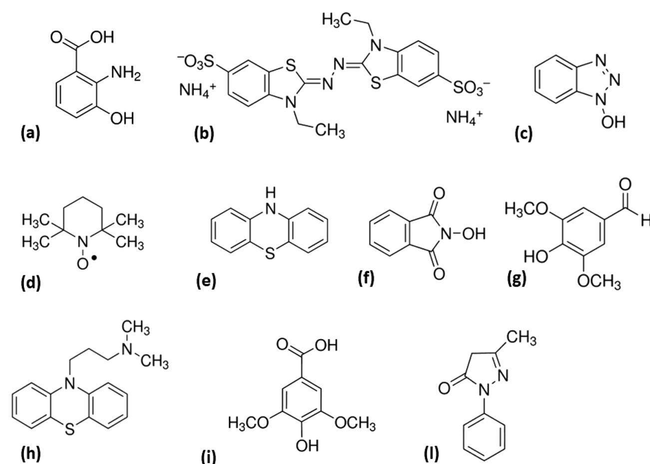

Figure 10. Most common laccase mediators: (a) 3-hydroxyanthranilic acid; (b) ABTS; (c) HBT; (d) TEMPO; (e) phenothiazine; (f) *N*-hydroxyphthalimide (HPI); (g) 3,5-dimethoxy-4-hydroxybenzaldehyde, 4-hydroxy-3,5-dimethoxybenzaldehyde (Syringaldehyde); (h) 10-(3-(dimethylamino)propyl)phenothiazine (Promazine); (i) methyl ester of 4-hydroxy-3,5-dimethoxy-benzoic acid (syringic acid); (l) 1-phenyl-3-methyl-5-pyrazolone.

(TEMPO) are likely the most used mediators.[117] However, a substantial substitution of 4-hydroxy-TEMPO for TEMPO in the near future could be reasonably forecast, as the former is substantially less expensive than the latter and shows comparable catalytic efficiency.[149]

It has been common knowledge for a long time that LC is deeply involved in delignification of LBM by WRF. However, fungal LCs alone are generally unable to efficiently promote delignification *in vitro*, most probably because of the concomitant presence of other necessary enzymes or natural redox mediators *in vivo*. The delignification degree in the presence of such mediators depends on reaction conditions, as well as on LBM type and enzyme source. For these reasons, the attainment of the highest delignification yield requires the systematic screening of enzymes from different sources, as well as of mediators and of operating conditions. Gutiérrez et al. reported the use of LC from *Trametes villosa*, with HBT as mediator and alkaline extraction, to delignify *Eucalyptus globulus* wood and *Pennisetum purpureum* nonwood with lignin removal of 48% and 32%, respectively.[150] Xie et al. achieved 35% solubilization of the insoluble Kraft lignin through the use of LC from *Trametes versicolor* in the presence of HBT.[151] The use of thermophilic enzymatic sources could allow overcoming of low thermal stabilities of conventional LCs. Navas et al. used a thermophilic LC from *Thermus* sp. 2.9 incubated with steam-exploded biomass (*Eucalyptus globulus*) at 60 °C for 24 h in the presence of HBT as mediator obtaining a decrease in the intensity of the IR bands associated with lignin aromatic backbone and lignin−hemicellulose linkages.[152] A very recent study sheds light on the intimate mechanism of straw delignification by a LC/HBT mediator system:[153] subtle analytical methods revealed Cα oxidation and Cα−Cβ and β-O−4 oxidative cleavage. In the same study, a significant reduction in phenylcoumaran and resinol linkages was found, showing that also the poorly reactive β−β and β−5 C−C links could be targets of the laccase/mediator system. The final result is a substantial delignification. Another study[154] on corn cob lignin and two fungal LCs (from *Cerrena unicolor* and *Trametes versicolor*) in the presence of some redox mediators has shown the importance of mediators in the delignification process. In another study[155] on sugar cane bagasse and straw, the LC from *Pycnoporus cinnabarinus* in the presence of HBT caused a noticeable delignification, which correlates with a substantial improvement of saccharification yields. Recombinant LC from *Pleurotus ostreatus* was shown to be able to degrade corn stover lignin in the presence of suitable mediators.[156] Also a bacterial LC from *Bacillus ligniniphilus*, alone or in the presence of mediators such as ABTS, has proven effective[157] in lignin (alkaline lignin and milled wood lignin) oxidative degradation, as it has been recently shown. In particular, a selective removal of S units, within the frame of general demethoxylation, was observed. Different from other fungal and bacterial LCs, this enzyme promoted only limited repolymerization of the hydroxy-aromatics it obtained from lignin. Overall, whereas being *per se* capable of degrading lignin, the enzyme worked better in the

Table 1. A Synopsis of Various LCs, Their Mediators (if Any), and Their Substrates

laccase source	mediator	solvent	lignocellulosic feedstock	lignin loss %	refs
Tramentes versicor	HBT	35 mM phosphate buffer (pH 7)		35%	151
Ascomycete Myceliophthora thermophila	Methyl syringate	50 mM sodium dihydrogen phosphate (pH 6.5)	*Eucalyptus globulus*	up to 50% (after alkaline peroxide extraction)	72
Trametes villosa	HBT	50 mM sodium tartrate buffer (pH 4)	*Eucalyptus globulus*	up to 48% (after alkaline peroxide extraction)	150
Thermus sp. 2.9			*Eucalyptus globulus*		152
Tramentes versicor	HBT	[C_2 mim][OAc]	seaweed biomass (a mix of *Chaetomorpha* and *Cladophora*)	up to 27%	162
Aquisalibacillus elongatus		[Bmim][PF_6]	sugar beet pulp	78.4%	163
Trametes sp.	HBT	[EMIM][DEP]	oil palm biomass	35.4%	158
Trametes versicolor IBL-04		50 mM sodium malonate buffer pH 4.5	sugar cane bagasse	78.3%	165
Trametes versicolor		[Bmim][PF_6]	lignocellosic biomass	kappa number 77.3%	166

presence of ABTS mediator. Table 1 presents some examples of laccases, alone or in the presence of suitable mediators, acting on the specified substrates.

Laccases in Ionic Liquids. Recent works have reported the possibility to obtain a quick conversion of lignocellulosic biomass to cellulose through the combined use of ionic liquids (ILs) and redox enzymes.[158,159]

These features allow such solvents to increase the enzyme accessible surface area of LBM, leading to a more efficient enzymatic delignification. Depending on the cation−anion pair, ILs can either activate or inhibit ligninolytic enzymes.[14,160] Galai et al. screened *Trametes versicolor* LC (TvL) activity in 56 different ILs and found that 13 of them improved laccase activity in comparison to buffer solutions. The most notable improvement was seen in 10 mM [Ch][H_2PO_4] (cholinium dihydrogen phosphate) in sodium acetate buffer (50 mM; pH 7.0), which increased TvL activity by 451% compared to the IL-free control acetate buffer.[161] Stevens et al. measured the enzyme activity of the LC from *Trametes versicolor* in different ILs. They found that [DEA][HSO_4] (diethylammonium hydrogen sulfate) allowed TvL to oxidize ABTS, while [EMIM][OAc] (1-ethyl-3-methylimidazolium acetate) and [Ch][Lys] (cholinium lysinate) inactivated the laccase even at very low concentrations.[160] Financie et al. obtained a significantly enhanced rate of enzymatic delignification of palm oil biomass (OPFB) by LC from *Trametes* sp. in [EMIM][DEP] (1-ethyl-3-methylimidazolium-diethyl phosphate). They obtained a final lignin content of about 8.5 wt %, compared to the initial 24.0 wt % of untreated OPFB.[158] Al-Zuhair et al. investigated the enzymatic delignification of seaweed biomass (a mix of *Chaetomorpha* and *Cladophora* species), carried out using TvL, HBT, and [EMIM][OAc] obtaining a delignification degree up to 27%.[162] Rezaei et al. reported the use of LC from the halophilic bacterium *Aquisalibacillus elongatus* in the presence of [BMIM]-[PF_6] (1-butyl-3-methylimidazolium hexafluorophosphate) to delignify sugar beet pulp, obtaining a delignification yield of 78.4% after 24 h.[163] The same enzyme was also tested in the same conditions in [BMIM][PF_6] to remove lignin from peanut shell, obtaining a lignin removal of 87% after 24 h.[164]

Deep Eutectic Solvents. Deep eutectic solvents (DESs) are a new class of green solvents composed of a hydrogen bond acceptor (HBA, usually a quaternary ammonium salt) and a hydrogen bond donor (HBD) such as glycerol, succinic acid, etc. (Figure 11). DESs are becoming an attractive alternative to ILs.[167]

Indeed, DESs show similar physical properties compared to traditional ILs, with the advantage of being less toxic, cheaper, and easier to prepare.[168] Some applications have been described where DESs are able to dissolve and remove lignin.[169,170] Moreover, DESs could avoid the loss of catalytic activity or enzyme denaturation that can occur in several organic solvents.[168,171] Toledo et al. screened laccase activity in 16 different DES/water mixtures.[172] The activity did not change in the presence of most DESs, whereas in some cases, such as with ChDHC/Xyl (Choline dihydrogen citrate in xylitol) (2:1) at 25 wt %, an increase of relative activity up to 200% was observed.[172] Khodaverdian et al.[174] studied the activity and stability of laccase from *Bacillus* HR03 in betaine-based natural DESs. These DESs are obtained from two or more compounds that are generally plant based primary metabolites, that is, organic acids, sugars, alcohols, amines, and amino acids.[173] The highest activity was obtained in 20% (v/v) glycerol/betaine (2:1).[174] Interest in DESs used in combination with mechanical[175,176] and chemical

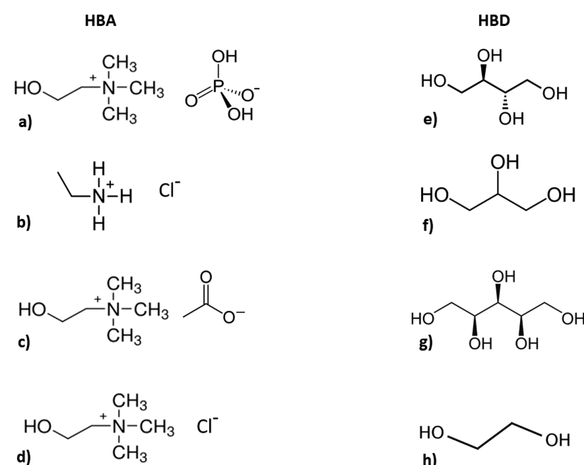

Figure 11. Some examples of acceptors (HBAs): (a) cholinium dihydrogen phosphate (ChH_2PO_4); (b) ethylammonium chloride (EACl); (c) cholinium acetate (ChAc); (d) Cholinium chloride (ChCl). Some examples of hydrogen bond donors (HBDs): (e) erythritol (Ery); (f) glycerol (Gly); (g) xylitol (Xyl); (h) ethylene glycol (EtG).

pretreatment[177,178] is growing. However, the use of DESs for enzymatic LBM delignification has still not been extensively studied. To date, very few works regarding laccases and DESs have been reported.[171,172,174] Hence, this topic is worthy of further investigation.

Peroxidases. Heme peroxidases are hemoproteins catalyzing the oxidation of various organic and inorganic substrates in the presence of hydrogen peroxide as the electron acceptor. They are widely distributed in nature: plants, animals, and microbes. Extracellular fungal peroxidases (lignin modifying peroxidases), such as lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP), are involved in lignin degradation.[179−181] In spite of looking like the best candidates for biodelignification, until now the ligninolytic peroxidases have been rather disappointing, owing to some important reasons: low productivity, unsuccessful attempts of heterologous expression and mutagenesis, and facile irreversible inactivation,[182] especially in the presence of even a slight excess of H_2O_2.[183] Therefore, the use of ligninolytic peroxidases could be considered as a blind alley. On the other hand, they are quite worth being studied, as at least some of the cited drawbacks could be conceivably removed in the near future. In fact, some promising studies have been carried out in the field of ligninolytic peroxidase engineering. For example, Pham et al. engineered the LiPH8 from *Phanerochaete chrysosporium*, which is generally unstable under acidic pH conditions, to make it able to work under acidic conditions.[184] One of the main issues that occur frequently during biomass pretreatment is the formation of inhibitory compounds such as 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde (furfural). Yee et al. reported that a recombinant manganese peroxidase (rMnP) produced from the yeast *Pichia pastoris* was able to degrade furfural and HMF.[185] Peroxidases are generally sensitive to H_2O_2 concentration. In the literature, several reports concern the engineering of peroxidases to avoid this issue and make them more suitable for delignification process.[186−188] Gonzalez-Perez et al. improved versatile peroxidase (VP) resistance to H_2O_2; an evolved version of this enzyme was subjected to a range of directed evolution and hybrid strategies in *Saccharomyces cerevisiae*. The result showed an increase of the half-life of the

protein from 3 (parental type) to 35 min in the presence of 3000 equiv of H_2O_2 and with a 6 °C upward shift in thermostability.[188]

Lignin Peroxidase. Lignin peroxidase (EC 1.11.1.14) with its various isoforms, was first discovered in the extracellular medium of white rot fungus *Phanerochaete chrysosporium* by Tien and Kirk.[189] The crystal structure of *P. chrysosporium* LiP is constituted by 343 amino acids. Two glycosylation sites, two Ca^{2+} binding sites, and four disulfide bridges stabilize the three-dimensional structure of the enzyme.[190] The molecular mass of LiPs ranges from 35 to 48 kDa, and p*I* values range from 3.1 to 4.7 depending on the enzymatic source. Due to their high redox potential (around 1.2 V at pH 3), LiPs are able to oxidize the aromatic rings of lignin (irrespective of their methoxylation degree, but with preference for nonphenolic units), dyes, and a variety of recalcitrant pollutants.[127,191,118] The LiP reaction cycle is comparable to other peroxidase cycle mechanisms (Figure 12).[23] First, the enzyme is oxidized by H_2O_2, leading to

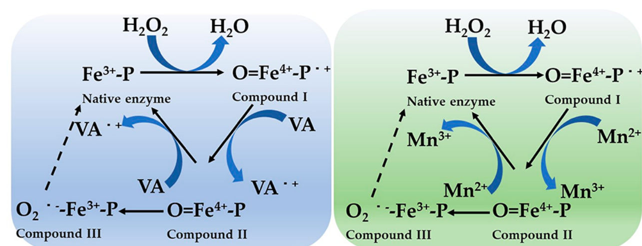

Figure 12. Catalytic cycle of LiP (left) and MnP (right).

the generation of the compound I that exists as a ferryl (iron(IV)-oxo complex) cation radical intermediate [$O{=}Fe^{IV}{-}P^{\bullet+}$]. Then, this undergoes two one-electron reduction steps by the electron donor substrate, such as lignin, lignin oligomers, or veratryl alcohol (VA), which acts as a mediator, leading to a transient formation of compound II [$O{=}Fe^{IV}{-}P$] and a very reactive radical cation ($VA^{\bullet+}$).[192] Compound II further oxidizes the second VA molecule, returning it to its native state before starting a new catalytic cycle of LiP.[23] The eventual excess of H_2O_2 at pH 3 in the absence of a substrate converts compound II to compound III [$Fe^{III}O_2^{\bullet-}$], which is rapidly and irreversibly bleached or returns to its native form by spontaneous autoxidation or oxidation through $VA^{\bullet+}$.[23,110,190]

Manganese Peroxidase. Manganese peroxidase (EC 1.11.1.13) and its isoforms were first detected in *P. chrysosporium* (the teleomorph of *Sporotrichum pruinosum*) by Glenn, Gold, et al.[179] It is an oxidoreductase with its heme group included between two α-helix domains, 350 amino acids, and a 43% sequence similarity with LiP.[190] It contains five disulfide bridges and two Ca^{2+} ions, which maintain the structure of the active enzyme. The Mn^{II}-binding site consists of two glutamate and one aspartate γ-carboxylic groups and is located close to the porphyrin macrocycle. Their molecular mass ranges from 38 to 62.5 kDa, and their p*I* values ranges from 2.9 to 7.1. The catalytic cycles of MnPs and LiPs are very similar, with the exception for the use of Mn^{II} as their reducing substrate, generating Mn^{III}, which diffuses from enzymes into the lignocellulose structure (Figure 8). To date, MnP has not been used for biomass delignification. Nevertheless, it is widely used for dye decolorization. For example, Zhang et al. reported the dye decolorization by a manganese peroxidase from *Cerrena unicolor* BBP6.[193] Siddeeg et al. immobilized MnP enzyme extracted from *Anthracophyllum discolor* on iron oxide/chitosan magnetic nanocomposite for decolorization of textile wastewater.[194]

Versatile Peroxidase. Versatile peroxidase (EC 1.11.1.16) was found for the first time in *Pleurotus eryngii* and *Bjerkandera adusta*.[195] It shows functional characteristics of both LiP (oxidation of substrates like VA and aromatic compounds) and MnP (oxidation of Mn^{II} to Mn^{III}) that make it able to oxidize high and medium redox potential compounds in the absence of mediators. VP shows a structure constituted by 11–12 helices, four disulfide bridges, two structural Ca^{2+} sites, a heme pocket, and a Mn^{2+}-binding site.[127] Its molecular mass ranges from 40 to 45 kDa with a p*I* range from 3.4 and 3.9. The basic catalytic cycle of VPs is similar to those of other peroxidases with the two intermediary compounds I and II.[25] One could expect that VP should be a very effective ligninolytic catalyst, combining the features of LiP and MnP. On the contrary, *Pleurotus eryngii*, a good VP producer, is slow and poorly efficient in ligninolysis.

Crude Enzyme Mixtures. The use of a single peroxidase for biomass pretreatment is ineffective to obtain a high delignification degree. Nevertheless, the use of crude enzyme mixtures (enzymatic cocktails composed mainly of LCs, LiP, MnP, and VP) constitutes a more effective strategy,[196] also when taking into account that LiP acts preferentially on nonphenolic units, whereas MnP shows sharp preference for the phenolic ones. There are several advantages in using crude enzyme mixtures. As reported by Asgher et al., they could contain accessory enzymes that enhance the degradation of lignin compounds. Moreover, some studies show that the extract could contain cellulases able to hydrolyze the delignified lignocellulosic biomass simultaneously. The delignification by means of crude enzyme mixtures is generally carried out under mild conditions.[115] Nonetheless, the efficiency of the process depends on several aspects, such as biomass features and operating conditions (pH, temperature, mediator(s), oxygen, and use of surfactants).[196] Asgher et al. reported the use of a crude enzyme mixture containing LiP, MnP, and LC extract from *Pleurotus ostreatus* IBL-02 to pretreat sugar cane bagasse, obtaining a 33.6% lignin loss.[197] Kong et al. reported the use of crude enzyme extract (MnP and LC) from white-rot fungus *Echinodontium taxodii* 2538 to delignify bamboo biomass. Their results showed a higher lignin degradation when enzymes were used simultaneously rather than singularly.[198]

Immobilized Enzymes. The use of free ligninolytic enzymes can often result in several limitations such as low operational stability (under extreme conditions of temperature and pH) and difficult recovery and reuse.[199] Most of these problems can be overcome through enzyme immobilization on solid supports such as xerogels, sand, clay, nanofibrous polymers, or nanoparticles.[147] Although immobilization usually worsens the kinetic parameters of the enzyme and therefore its overall catalytic efficiency, the facile recovery, in particular when using magnetic particles, and the improved stability largely counterbalance the drawback. In the case of LBM, the problem of the insolubility of lignin could be overcome in two ways: (a) the use of ILs or DESs at least in part solubilizes the lignin and therefore it can be efficiently oxidized by the enzyme; (b) the use of suitable mediators as molecular shuttles allows the effective flux of the electrons from lignin to LC. The two ways could well operate together for optimal performance of immobilized LCs. The choice of the support and the method involved in enzyme immobilization are important factors that affect the enzyme activity and stability. To the best of our knowledge, very few

https://dx.doi.org/10.1021/acssuschemeng.0c07266
ACS Sustainable Chem. Eng. 2021, 9, 2412−2432

Table 2. Immobilized Ligninolytic Enzymes at Work

enzyme	enzyme source	carrier	solvent	lignocellulosic feedstock	lignin loss %	refs
LC	*Trametes versicolor IBL-04*	alginate−chitosan	50 mM sodium malonate buffer (pH 4.5)	sugar cane bagasse	78.3%	165
LC	*Trametes versicolor*	Fe_3O_4@SiO_2@Kit-6 magnetite nanoparticles	[Bmim][PF_6]	olive pomace biowaste	kappa number 77.3%	166
LC	*Trametes versicolor*	ferrite (MNPs)	50 mM sodium citrate buffer (pH 4.8)	*Ipomoea carnea*	38.16	204
LC	*Trametes versicolor*	copper ferrite magnetic nanoparticles (CuMNPs)	50 mM sodium citrate buffer (pH 4.8)	*Ipomoea carnea*	43.3%	204
LiP, MnP, LC	*Ganoderma lucidum* IBL-05	alginate−chitosan beads	50 mM sodium malonate buffer (pH 4.5)		57.3%	200
LC	*Lentinus tigrinus*	SBA-15 mesoporous silica	50 mM citrate buffer (pH 4.5)	pistachio shell	91%	205
LC, cellulase, and β-glucosidase		co-immobilization in sodium alginate beds	100 mM citrate buffer (pH 4.8)	*Ipomoea carnea*	35.7	206
LC, cellulase, and β-glucosidase		co-immobilization in sodium alginate beds	100 mm citrate buffer (pH 4.8)	*Saccharum arundinaceum*	24.1%	206

works about the immobilization of crude enzyme mixtures have been reported. In contrast, LC immobilization has widely been investigated.[200−202] Bilal et al. reported the use of a ligninolytic enzyme cocktail from *Ganoderma lucidum* IBL-05 immobilized on alginate−chitosan beds to delignify sorghum stover, obtaining a delignification of 57.3% after 15 h. The reaction was carried out in 50 mM sodium malonate buffer (pH 4.5) and is relevant as delignification proceeds without the use of mediators.[200] Chang et al. studied the entrapment of laccase from the lacquer tree *Rhus vernicifera* in a cellulose ester membrane, finding that the immobilized LC had much higher levels of retained activity (20.34% after 2 days) compared with the free LC.[203] Amin et al. immobilized LC from *Trametes versicolor* by covalent attachment on modified Fe_3O_4@SiO_2@Kit-6 magnetite nanoparticles in the presence of IL for enhanced delignification of olive pomace biowaste.[166] They obtained up to 77.3% decrease in kappa number (after 6 h of incubation of the biomass with immobilized LC and [Bmim][PF_6] as ionic liquid without any mediator. Muthuvelu et al. immobilized LC from *Trametes versicolor* on ferrite (MNPs) and copper ferrite magnetic nanoparticles (CuMNPs).[204] They obtained higher activity recovery for the LC immobilized on CuMNPs (13.2 U/mL) than for that on MNPs (10.93 U/mL). Moreover, they investigated the delignification of *Ipomoea carnea* using both free and immobilized LC without mediator. After 20 h of incubation, they obtained lignin removal of 43% for the enzyme immobilized on CuMNPs, 40% for LC immobilized on MNPs, and 38% for the free enzyme.[204] Asgher et al. reported the use of LC from *Trametes versicolor* IBL-04 immobilized on alginate−chitosan beads.[165] A marked reduction in lignin content of all the studied LBM was obtained after 15 h, with the highest delignification (78%) for sugar cane bagasse, followed by wheat bran and maize stover. The experiments were carried out in 50 mM sodium malonate buffer (pH 4.5) without any mediator. Sadeghian-Abadi et al.[205] immobilized LC from *Lentinus tigrinus* by covalent attachment on SBA-15 mesoporous silica. The immobilized enzyme was active toward the delignification of pistachio shell, achieving an efficiency of 91% after 12 h of incubation without mediator in 50 mM citrate buffer (pH 4.5). Moreover, they screened a range of mediators (2,6-dimethoxyphenol, GA, HBT, TEMPO, and vanillin) at different concentration (1, 5, 10 mM) to test their effect on enzymatic delignification. Their results show that TEMPO was the most effective mediator at all concentrations with a maximum delignification degree of 68.77% after 8 h of reaction. This work showed that it is possible to obtain a good delignification degree with purified laccases without using mediators.[205] Muthuvelu et al. developed the coimmobilization of three enzymes (laccase, cellulase, and β-glucosidase) in sodium alginate beds for delignification of different biomasses such as *Typha angustifolia*, *Arundo donax*, *Saccharum arundinaceum*, and *Ipomoea carnea*.[206]

Table 2 encompasses some outstanding examples of ligninolytic enzymes (mainly LCs) working on the specified LBM.

■ CONCLUSIONS

Turning herbaceous LBM into platform chemicals and fuels is an important opportunity to produce sustainable economies. Nevertheless, their exploitation is affected by the need for pretreatments, enzymatic hydrolysis, and fermentation stages. One of the most critical steps is the pretreatment one. Among those reported in literature, enzymatic pretreatments are considered the most ecosustainable. However, due to high prices, possible low stability, and catalytic activity of the involved enzymes, this technology is still a challenge. Moreover, the delignification degree depends on the chosen enzymes, suitable supports for immobilization, use of redox mediators, reaction conditions, and solvents. Some of these issues have been analyzed in this review. In these last years, the interest to overcome these problems in order to increase the cost-effectiveness of biofuel production and to make the transition from the laboratory to the industrial/commercial scale is widely growing. The future goals to overcome these drawbacks could enhance the enzymatic stability and activity using new supports for enzyme immobilization, solvents as DESs, and redox mediators. To the best of our knowledge, there are not studies where white-rot fungi (WRF) ligninolytic peroxidases have been used on their own to delignify LBM, for the reasons depicted above. So the use of such promising but disappointing enzymes has not found any significant utilization in delignification processes, although these peroxidases are useful, for example, in decolorizing wastewaters from paint and textile factories. In contrast, fungal LCs fully keep what they promise, and therefore their use, under the operative conditions and tricks above-described, is actually booming. And a further expansion of their use could be easily and reasonably anticipated.

https://dx.doi.org/10.1021/acssuschemeng.0c07266
ACS Sustainable Chem. Eng. 2021, 9, 2412−2432

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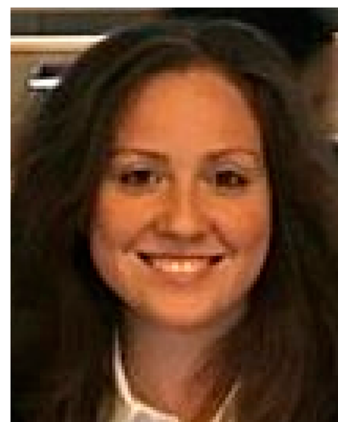

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https://dx.doi.org/10.1021/acssuschemeng.0c07266
ACS Sustainable Chem. Eng. 2021, 9, 2412−2432

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■ ACKNOWLEDGMENTS

D.T. thanks MIUR (PON RI 2014-2020, Azione I.1 "Dottorati Innovativi con Caratterizzazione industriale"; DOT1304455 2) for financing his Ph.D. scholarship. CC acknowledges MIUR (PON-AIM Azione I.2; DD no. 407-27.02.2018, AIM1890410-2). TOC image has been created at Biorender.com.

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https://dx.doi.org/10.1021/acssuschemeng.0c07266
ACS Sustainable Chem. Eng. 2021, 9, 2412−2432