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Key Factors Affecting the Recalcitrance and Conversion Process of Biomass

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Abstract
Lignocellulosic biomass is one of the most abundant raw materials in the world, and it is mainly composed of carbohydrate polymers (cellulose and hemicellulose) and lignin. Its applications vary from the production of pulp and paper, to the most recent plant-based bioethanol production, which has challenge due to low hydrolysis conversion rates by the inherit recalcitrance of biomass. The biomass is naturally resistant due the high complexity in the component organization and interaction in the cell wall. The application of pretreatment technologies is one of the most used strategies to overcome biomass recalcitrance. These techniques often require a catalyst to modify the lignocellulosic structure which can be acids, alkaline compounds, ionic solutions, organic solvents, and even pressurized steam among others. The type of catalyst dictates the name of the pretreatment involved. This work presents an overview of these strategies, along with some recent contributions from the scientific community to improve biomass conversion technologies. The discussion is focused on the key factors related to the recalcitrance and conversion process, as well as the composition and physicochemical properties.

Keywords Biomass · Sugarcane · Hemicelluloses · Cellulose · Lignin · Pretreatment

Introduction

The study and use of biomass for biorefinery have increased significantly on the past few years due to the need to obtain new resources of energy that accomplish the demand for substitution of fossil fuels. These energy sources should be renewable with low environmental effects [1]. In this context, the lignocellulosic materials are promising alternative for replacing petroleum and its refined derivative forms for energy production, since they are available in huge amount and their conversion routes into industrial use products are well-known [2].

The global annual production of dry lignocellulosic material is around 10 to 50 billion tons, which represents approximately 50% of the income of biomass product on the planet [1]. The remarkable characteristic of lignocellulosic substrate is its high amount of carbohydrates (up to 75%). This carbohydrate-rich material is now an essential source of fermentable sugar for the production of biofuels, as well as a variety of chemical products and biodegradable materials [3]. The fermentable sugars available in the lignocellulosic biomasses have potential as feedstock for biorefineries. However, the compact and rigid structure of these biomasses results in the resistance to chemical and biological conversion due to their high degree of recalcitrance [1].

The biomass recalcitrance is closely associated with the physicochemical properties of the plant cell wall (PCW). The presence of lignin, hemicelluloses, pectin, ashes, etc., and their interconnections in the cell wall have built physical barriers that protect cellulose from enzyme or chemical deconstruction. The factors that affect the biomass cellulose from enzymatic hydrolysis include the rate of lignin, hemicelluloses and acetyl groups, cellulose crystallinity, polymerization

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degree, specific surface area, pores volume, and particle size [1, 4–7]. Although these factors have been exhaustively studied, the lignocellulosic biomass conversion routes remain economically unfeasible at industrial scale.

Several pretreatment methods have been developed on the last decades aiming to increase the material digestibility, such as acids [7–11], the alkaline [11], steam explosion [11–13], biological [14, 15], wet oxidation [16], organic-solvents [17–19], liquid hot water [20], ammonia water [21], ammonia fiber expansion (AFEX) [22], and ionic liquid [23]. All pretreatment approaches have the purpose of decreasing the recalcitrant barriers of biomass and increasing the enzymatic digestibility of cellulose. Thus, the understanding of how chemical compositions and physical structures influence the biomass recalcitrance and how they affect the lignocellulose enzymatic hydrolysis would greatly improve the current pretreatment technologies and to promote the development of new pretreatment processes [1].

The lignocellulosic material recalcitrance is originated mainly from the PCW structure, which consists of a matrix of reticulated polysaccharide nets, glycosylated proteins, and lignin 24. The vegetal biomass developed elaborated mechanisms to resist the attack of microorganisms (like bacteria and fungi), insects, and herbivores [24]. The biomass recalcitrance refers to the complex properties of PCW to protect its carbohydrates from degradation by microorganisms or deconstruction by enzymes or chemical products. This resistance is attributed to the aspects including (i) the epidermal tissue, cuticle, and the epicuticular waxes; (ii) the relative amount of sclerenchyma-like tissue (composed of thick-walled cells); (iv) the lignification degree; (v) the structural heterogeneity and the complexity of the cell wall microfibrils and matrix non-cellulosic polysaccharides; and (vi) the inhibitors of subsequent fermentations that naturally exist on cellular walls or that are generated during conversion processes [24].

The biomass heterogeneity of plant tissue and fractions, predominant type of cells, and lignin distribution also collaborates with material recalcitrance [4]. In addition, the crystallinity of the cellulose and the presence of inhibitors—such as the acetyl group, the proteins of the cell wall, and the uronic acid—contribute to the recalcitrance of the lignocellulosic biomass.

Structural changes are caused by the pretreatment processes applied to reduce the enzymatic digestibility. For instance, the high mechanic pressure from record feeders collapses the natural vascular structure; the acid pretreatments allow cellulose to repair, leading to cellulose being hornificated into microfibers; and some pretreatments solubilize and expand lignin onto the cellulose surfaces during the cooling off phase. In this article, we will discuss aspects of biomass heterogeneity, as well as its main structural components (cellulose, hemicellulose, and lignin). Also discussed are the pretreatments applied in them and how they can influence the modification of the biomass for its industrial application.

Biomass Model

Sugarcane: the Standard Biomass for Tropical Countries

The sugarcane is a perennial plant that stocks C4-kind sucrose (a reference to the way in which CO_2 is primarily organized in a compound containing four carbon atoms) belonging to the genus *Saccharum*, which was originated from Asia. The sugarcane is cultivated in tropical and subtropical countries all around the world, and the main producers are Brazil (739 million tons), India (341.2 million tons), China (125.5 million tons), and Thailand (100.1 million tons) [25].

The sugarcane plant is a clump forming cylindrical tall grass that grows erect reaching up to 5 to 6 m. The plant is divided in culms (called stems or stalks), leaves, and a system of roots. The unbranched aerial culms are differentiated into internodes and nodes with annular leaf scars (placed just below the bud, being a point of attachment of the leaf sheath to the stalk found at the node when the leaf drops off the plant) and loosely defined rows of adventitious root primordia in a several-tiered band that emerge just above each node. The bud is found at the root band of each node alternating between one side of the stalk to the other. The sprouting of the bud may give raise new plants. The sugarcane leaves are usually attached alternately to the nodes and are differentiated into leaf blade (comprising the lamina with a thickened midrib) and leaf sheath (which encloses the immature developing culm and young leaves) separated by a blade joint. In a mature plant, the number of green leaves per stalk is around 10. As characteristic of monocots, leaf venation runs parallel to each other from base to tip in both the blade and sheath. The belowground system of roots in sugarcane enables the intake of water and nutrients from the soil, which strongly influentiate the plant-water-soil relationship, and to anchor the plant to the soil. The volume of soil available for water and mineral uptake determines the depth of root system. Two types of downward-oriented buttress sugarcane root systems emerge from the nodes after planting a portion of sett (stem), the sett and shoot roots. The former are thin and much branched roots grow from the nodes of the sett in a young plant. The latter in comparison with sett roots are thicker, lesser white, succulent, and lesser branched and emerge from the base [26, 27].

In terms of morphological heterogeneity, it manly consists in a set of fibers and other elements, like vessels, parenchyma, and epithelial cells [28], being visually divided in culms, leaves, and a system of roots. The culm structure is heterogeneous, made by long and cylindric articulations (internode) interpolated by small nodes [29]. The external surface of the culm is covered by wax and impregnate with cutin, which is characteristic of the epidermis structure. The epidermis consists of a single coat with small and big alternating cells [29], resulting in a dense structure in which its content depends on

the type of plant [30]. The epidermis is organized in parallel walls covered by a thick impermeable coat called cuticle [29]. Moreover, from the recalcitrance and anatomy point of view, there is an elevated number of vascular bundles close to the epidermis. This region, defined as external fraction, is responsible to the recalcitrance of this region [4]. The external fraction is the most recalcitrant fraction of the sugarcane, followed by the node and internode [31], while the internal part of the internode is the less recalcitrant region [32].

According to Cerqueira et al. [33], a ton of sugarcane generates around 280 kg of bagasse. So, taking this prevision into account, approximately 1.7×10^8 tons of dry sugarcane will be produced in 2017/2018. In addition, a huge amount of this bagasse is used in the energy cogeneration. Also, there are biotechnological applications being studied with the aim to produce high-value molecules and biofuels, including second-generation ethanol (E2G), or cellulosic ethanol. In the past decades, the bagasse and straw were investigated as possible factors of environmental risks [34]. This thought was changed with its massive uses in the energy cogeneration. However, because of the importance of energy and building blocks replacing petroleum sources, the sugarcane bagasse and straw are agroindustrial residues with interest to be used in developing methods for the biofuel production and other high-value compounds that have economic advantages.

Corn: an Important Biomass for USA

Corn, the common name of the species *Zea mays*, is a tropical American plant that has been domesticated all over the world. It has Mexican-origin and is currently the third largest food-oriented crop in the world [35]. Corn is an annual C-4 plant with wide adaptation to different conditions of environments. Corn is used for green fodder, animal, and human feeding, since it is very energetic because starch is its main component, and also for the production of biofuels such as ethanol [36].

Like sugarcane, corn represents a source of abundant lignocellulosic material for biotechnological applications. According to data from the United States Department of Agriculture (USDA) [37], it was produced 1.037.9 million tons of maize worldwide in the 2016/2017. From this amount, the USA accounted for more than 37% of the production, totalizing 384.8 million tons. For comparison, the production in Brazil, Argentina, Paraguay, and Uruguay, the main producers in South America, reached about 124,265,000 tons, being Brazil the largest producer, with around 84,480,000 tons. Corn is for the USA just like sugarcane is for Brazil: a large production, which generates surpluses that could be used to generate other value-added products. For example, maize residues have huge potential for bioenergy production.

Corn plant has a single (in most cases) cylindrical culm that grows vertically upward from the ground, regularly reaching up 2 to 3 m at maturity (sometimes much taller). Near from the base of the main culm secondary stalks, called as tillers, may be produced. Typically, the plant is divided in culm (called stems or stalks), leaves, sheaths, cobs, and husks. The unbranched culm corresponds to approximately to 60% of the plant dry mass and is divided in internodes and ring-like nodes. From each node leaf forms, totalizing 16 to 22 alternately arranged leaves around the entirety of the culm. The leaf blades are linear to linear-lanceolate in shape with prominent whitened midrib. The leaf venation runs parallel to each other from base to tip in the blade. Two distinct sets of roots are found, the brace and fibrous roots. The brace root (adventitious) grows from the first node just above the soil while the fibrous roots develop from the lowest four nodes that are below ground. From the top of the stalk—above all leaves—emerges a single terminal many-ramified inflorescence named tassel, a male part the plant in which several small flowers are located. Many pollen grains, containing the male sex cells, are released from the flowers. The reproductive female part of the plant is axillary pistillate inflorescence called "ear," which grows at the tip of a shank, a small stalk-like structure that emerges from the leaf nodes located midway from the top to the bottom of the stalk. Several ears are found in the plant, and they are surrounded by the husk—a group of the green leaves attached to the shank—that plays function in the protection of the edible part of the plant, the kernels (or fruit). From the tip of the husk, the silk (long shiny fibers) grows [38–41].

The corn residues are basically composed of cobs, husks, stems, and leaves, which are currently used in limited quantities for animal bedding, furfural production, pulp production, and part of feeding rations [42]. Although, the straw that stays in the field provides erosion control, most of this residue is not removed from the field. As a by-product of maize production, corn cobs are produced in significant quantities in the USA, with an estimative of 68 tons generated each year. The process of harvesting and supplying corn bran for biorefineries is a first step towards energy generation from the material [43].

The chemical properties and physical characteristics of corn cobs make them a suitable raw material for various energy generation methods. Corn cobs contain about 32.3–45.6% of cellulose, 39.8% of hemicelluloses—mostly composed by pentoses—and 6.7–13.9% of lignin [44, 45]. Some studies have shown that, from corn residues, the corbs represent about 15–20% of this material [46].

As in sugarcane, the recalcitrance of the lignocellulosic material is a barrier to the conversion of the maize biomass. The composition of the corn stover may vary according to the harvest, location, climatic conditions, plant phenotype, etc. All these variables represent important determinants of cellulose, hemicellulose, and lignin determination [47].

Fractionating lignocellulosic material based on its compositional and anatomical differences might be useful in

separating the more recalcitrant fractions (i.e., cobs, stalk) from less recalcitrant portions (i.e., leaves, husk). It was found that the untreated leaf fraction gave the highest glucan conversion of 91%, whereas the untreated cobs and stalks had efficiencies of 63 and 33%, respectively. The trend is rather intuitive since the stalk is mostly composed of internodes rich in lignified xylem vessels with high degree of recalcitrance [48]. In the context of biorefinery and bioenergy production, this biomass is very important and may be a viable source of lignocellulosic material.

Heterogeneity of Biomass

PCW is an important characteristic of the plants (Fig. 1), which contributes to their survival, for being a physical barrier for pathogens and plays metabolic role for the maintenance of the plants. The PCW contains mainly cellulose, hemicellulose, and lignin. However, different crops have significant differences in the proportions of these biomolecules, as well as in the type of hemicellulose and/or the rate of monolignol monomers in lignin. Each type of plant presents distinct proportions of wall compositions in different parts of the plant. This biomass heterogeneity leads to great difficulty to its use as an industrial interest product [1].

The ligneous biomass is more abundant in cellulose and lignin molecules, while the grassy biomass has higher amounts of hemicellulose (mainly xylan) (Table 1). For biorefinery industry applications, cellulose, hemicellulose, and lignin are the main targets [1]. Cellulose (Fig. 1) is the most abundant renewable polysaccharide on earth. It is composed by a chain of unbranched homopolysaccharides made up of units of anhydroglucose linked by β-1,4-D-glucose. This gives rise to a crystalline structure due to extensive intra and intermolecular hydrogen linkages, which facilitate its assembly into fibrils [50]. The cellulose microfibrils have highly and poorly organized regions, defined as crystalline and amorphous regions, respectively. The native cellulose is a polymorphic structure, defined as cellulose I, which can be converted into polymorphs II, III, and IV through a variety of treatments. The native cellulose is found as two crystalline phases, known as I_α e I_β [51].

One unit of cellulose, known as elementary fibril, suffers a self-mounting to form microfibrils of several sizes, which is coated by hemicelluloses originating macrofibers, thus creating resistance to chemical and enzymatic degradation [52]. The degree of polymerization refers to the amount of glycose

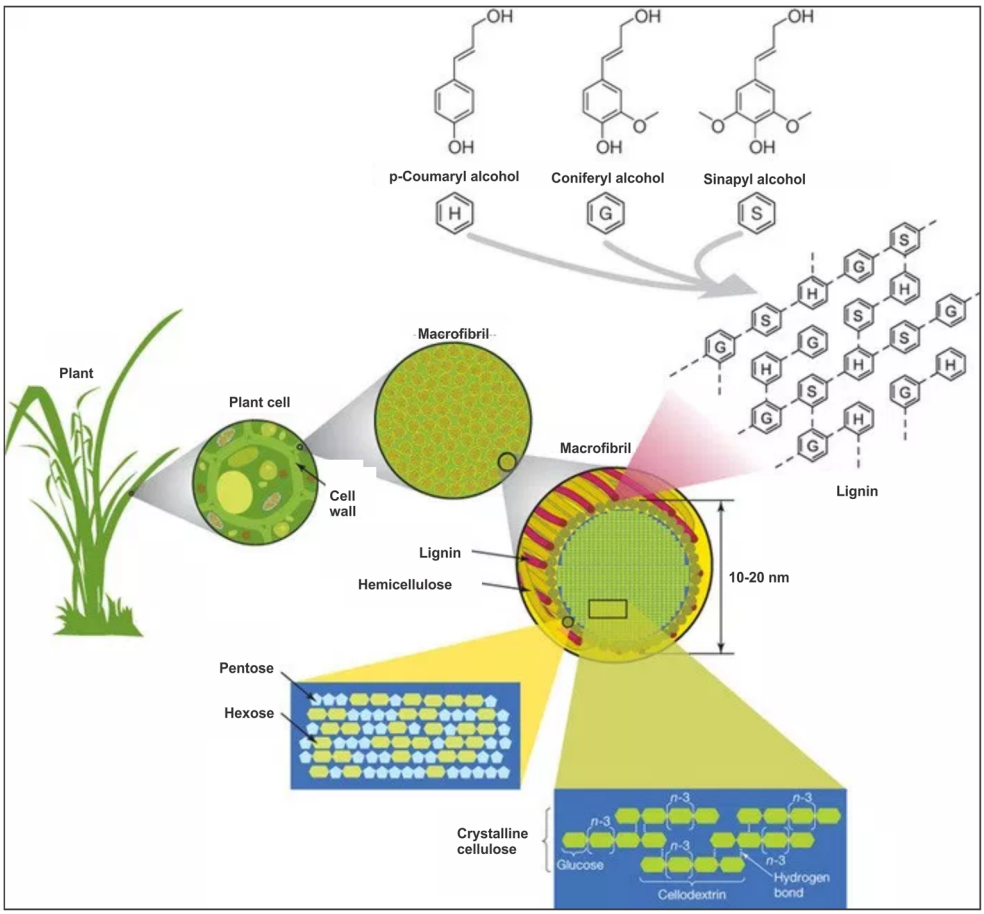

Fig. 1 Cell wall structure, detailing its subunits, like major components of cellulose, hemicellulose, and lignin [49]

Table 1 Main chemical components of several typical biomass feedstocks

Feedstock	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Nutshells	25–30	25–30	30–40	53
Hybrid poplar	48.6	15.7	21.8	1
Poplar	49.9	25.1	18.1	1
White oak	43.6	23.7	23.2	1
Red oak	43.4	23.5	25.8	1
Walnut	46.2	20.9	21.9	1
Maple	44.9	23	20.7	1
Corn cob	45	35	15	53
Corn stover	35.6	22.1	12.3	3
Sugarcane bagasse	44.7	28.8	19.5	54
Wheat straw	38.2	24.7	23.4	3
Rice straw	34.2	24.5	11.9	3
Switchgrass	45	31.4	12	53
Spruce	43.4	18	28.1	3
Pine	46.4	22.9	29.4	3
Birch	38.2	19.7	22.8	3
Willow	43	29.3	24.2	3

monomers present in the polymer in which the efficiency of the enzymatic hydrolysis is influenced. The natural cellulose structure is primarily resistant to the enzymatic hydrolysis as a result of its highly organized crystallinity and also its high polymerization degree, besides cellulose being water insoluble [53].

The hemicelluloses are recognized as the second most renewable polysaccharide in the world [30, 53]. However, the hemicelluloses are intimately associated to cellulose through physical connections and hydrogen links, meanwhile, lignins are linked to hemicellulose by covalent bonds (Fig. 2), mainly R (radical)-benzil and ether [53]. Generally, the hemicelluloses are branched polysaccharides made up by around 80 to 200 units of sugar residues with low molecular mass. The general chemical formula for the hemicelluloses is (C5H8O4)n or (C6H12O6)n, and they are classified as pentoses and hexoses, respectively [55]. The most abundant kind of hemicellulose in annual plants is arabinoxylan, which contains D-xilopiranosyl residues connected by glycosidic β-(1–4) connections. Sugarcane bagasse's hemicellulose is defined as L-arabino-(4-O-metyl-D-glucurone)-D-xylan [56, 57]. Connected to them, as simple unit chains, in positions C-2, C-3, or both, there are α-L-arabinofuranose and α-D-glucuronic acid (or its derived one, 4-O-metil) [58]. Xylan, which occurs naturally, contains O-acetyl groups located in some of the hydroxyl groups, in carbons 2 or 3. The most abundant kind of hemicellulose in annual plants is arabinoxylan, which contains D-xilopiranosyl residues connected by glycosidic β-(1–4) connections. Sugarcane bagasse's hemicellulose is defined as L-arabino-(4-O-metyl-D-glucurone)-D-xylan [56, 57]. Connected to them, as simple unit chains, in positions C-2, C-3, or both, there are α-L-arabinofuranose and α-D-glucuronic acid (or its derived one, 4-O-metil) [58]. Xylan, which occurs naturally, contains O-acetyl groups located in some of the hydroxyl groups, in carbons 2 or 3, in its main chain [58].

The ramification degree in the hemicellulose is given by the rate arabinose/xylose; therefore, the lower the rate, the higher the polymerization degree, and, likewise, the higher this rate, the smaller the polymer chain. The hemicellulose, in contrast to cellulose, is chemically heterogenous, and its composition shows a large proportion and content variation, as well as branches, resulting in an amorphous and hydrophilic structure. According to its composition and structural characteristics, hemicellulose is, therefore, more easily removed from PCW than cellulose, depending on the species, type of tissue, growth phase, and environmental and physiological conditions [30, 59, 60]. The xylose and the xylo-oligomers are frequently the main products obtained from hemicellulose pretreatment and enzymatic hydrolysis, allowing their use as fermentable sugars for ethanol generation, for instance [61]. Other products with aggregated value or intermediary compounds, such as xylitol, furfural, and levulinic acid, used for the production of chemicals and polymers, can also be generated from hemicellulose through appropriate catalytic approaches [62]. Other authors have suggested that the content, as well as the composition of the hemicellulose, may also affect the recalcitrance of the cell wall [63]. It is also believed that the interactions between the microfibers from cellulose and hemicellulose, as well as the lignin-carbohydrate connections, are capable of stopping the attack from the enzymes [64]. Transgenic Arabidopsis, with small content of methyl

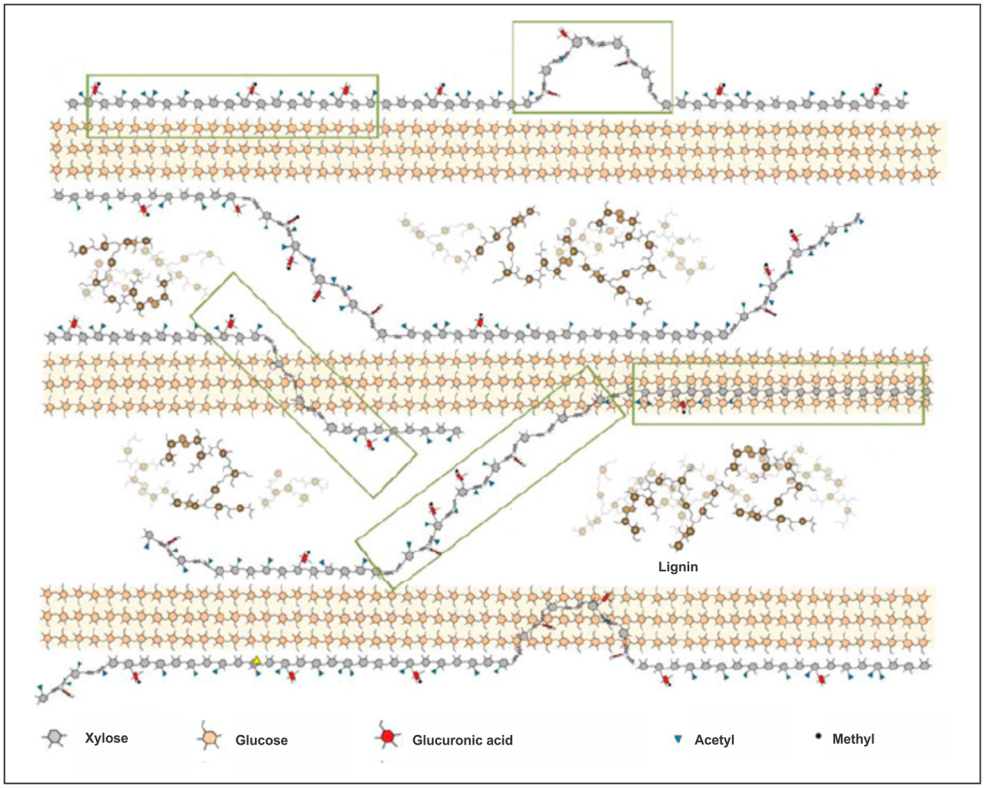

Fig. 2 Physical interaction among xylan, cellulose, and lignin in the plant cell wall [54]

groups in the side chains of glucuronoxylan, has released more xylose than the kind of wild control in less severe conditions after enzymatic hydrolysis [65]. Hemicellulose composition and proportion vary according to the biomass. Harwood and grasses usually are formed by pentosans (mainly xylans) and softwood by hexosans.

Lignin (Fig. 1) is known as the most abundant aromatic polymer in nature. It is a tridimensional phenolic biopolymer with amorphous structure. The lignin biosynthesis may be considered as a result of the polymerization of three main types of phenylpropane units, such as the monolignols: p alcohols—coumaryl, sinapyl, and coniferyl, which are responsible for its hard shape [42]. These monolignols may then be the origin of the p-hidroxyphenyl (H), syringyl (S), and guaiacyl lignin units [66], and these components vary according to the biomass, as well as hard and softwood grasses. Table 1 shows the relation between the amounts of celluloses, hemicelluloses, lignin, ashes, and extractives in some ligneous and grassy biomasses.

Lignin, Definition, and Function

Among the renewable resources available on earth, there is lignin, an amorphous macromolecule of high chemical and structural complexity which is widely available [67]. Lignin is composed basically of phenylpropane-derived units linked between themselves, creating a three-dimensional matrix. In 1838, Anselme Payen discovered a substance which would be later called lignin by Schulze in 1865, after a little bit less than 100 years later, the advances in analytical tools in biochemistry allowed the researchers to discover more about lignin [68]. In fact, lignin is a macromolecule of complex structure which varies according to the biomass of origin, and is basically constituted of different structures composed of aromatic rings, linked one to another by covalent bonds between carbon and carbon-oxygen [69]. This macromolecule plays a crucial role in plants, offering mechanical support due to the links of the fibers, while also decreasing the permeability of xylem cells, thus helping with the transport of water and nutrients through the plant, and offering protection to the cell against external enzyme attacks, and also increasing its structural strength [70, 71].

Although the challenge, there are many biotechnological applications for this macromolecule, like the production of biofuels, products of industrial interest like carbon fibers and many chemicals like aromatic compounds [72], cationic flocculants that have high dye removal rate [73], lignin can also be used in bio-based composites to modify its properties, such as crystallinity and hydrophobicity [74], and even in supercapacitors, which are used as a form of energy storage for wind turbines and electrical cars. A study showed that supercapacitors made of lignin derived activated carbon fibers had almost 400 F g^{-1} of capacitancy at 1 Volt of potential [75].

The precursors of the lignin-forming molecules are three, the para-coumaric acid, the para-coumaril-CoA, and the para-

coumaraldehyde [76]; these molecules are incorporated in the lignin macromolecule in the form of phenylpropanoid units, which are three: p-hydroxyphenyl, guaiacyl, and syringyl; these are derived respectively from the monolignols p-coumaryl, coniferyl, and synapyl alcohols [77]. The basic structure of the monolignols is separated into two, the phenolic fraction and the tri-carbonic chain, with the hydroxyl group being the only reactive structure of the molecule. Monolignols are composed of a C3 side chain coupled with an C6 aromatic ring [78]. When it comes to dry mass, most superior plants have between 20 and 25% of lignin [79].

As seen before, the lignin is comprised of three "monomeric" building blocks, the H, S, and G units; these units are not incorporated in the same ratio in all plants; there are variations between plant groups, in example, angiosperms have low amounts of H lignin incorporated and somewhat similar amounts of S and G lignin, while in gymnosperms, the amount of H lignin is still low but also the amount of S lignin, meaning that its cell wall is composed mostly of G-type phenylpropanoid units. In grasses and monocots, we have higher amounts of H lignin incorporated in the macromolecule, and a somewhat similar ratio of S to G lignin [81] (Fig. 3)

The synthesis of lignin in the cell wall occurs in an irregular mechanism; the phenylpropanoids are transported to the peripheric portion of the cell and incorporated in the cell wall through the action of oxidative enzymes, leading to the production of radicals that can create links between themselves, resulting the macromolecule of lignin. All genes related to monolignol synthesis have been described, and some of them have been modified or regulated to modify lignin production such as the ferulate-5-hydroxylase (F5H) and the caffeic acid O-methyltransferase (COMT); the regulators of lignin genes can also be targets of genetic studies [82]. Some research published by [83] showed that the suppression of the COMT gene could reduce the amount of lignin by 35% in *Brassica napus* in comparison with a wild specimen. There are however some cases where genetic modification of lignin-related genes results in problematic phenotypes such as dwarfism, sterility, and overall increase to environmental-related problems which can cause cell wall-related pathological conditions [83].

The lignin gives the lignocellulosic material a new recalcitrant characteristic, especially due to the binding capacity to the many kinds of molecules present in the cell wall [84], in a way that efforts are being made to obtain different varieties of genetically modified plants where the lignin content is inferior to the natural [85]. Although modifications in the amount of lignin can lead into an increasing in plant development problems, alterations in the composition of the lignin macromolecule seems to have no negative effect in plant growth [86], which can be explored to obtain new varieties with industrial favorable lignin chemical composition.

Other strategies are being developed to overcome those problems, and instead of focusing in modifying the expression of lignin biosynthesis genes, targeting the monomers can lead to better results, and the incorporation of some molecules into the lignin structure like coniferyl ferulate improves enzymatic hydrolysis glucose yield [88]. Another alternative to reduce lignin recalcitrance is to enhance the production of other lignin precursors of C6C1 structure; these compounds have lower polymerization capacity as they lack two carbons from the side chain, which correspond to one of the main reaction regions in common monolignols, known as the beta position [79]. Such strategy is efficient because it reduces lignin condensation in plant tissue making it easier to reach cellulose during the hydrolysis steps (Fig. 4).

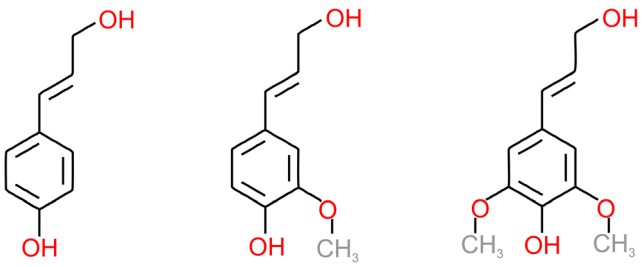

Fig. 3 Chemical structure of the three common monolignol components of the lignin [80]

Impact of Lignin in Biotechnological Processes

The use of lignocellulosic biomass as a material for ethanol production offers some challenges. The lignin, due to some of its characteristics such as complexity and structural and chemical resistance, shows itself as a problem for cellulose accessibility, mainly because of the enzymatic inhibition caused by the formation of inhibitory substances. Lignin works as a physical barrier protecting the cellulose from the action of hydrolytic enzymes like cellulases. The polymer is distributed within the empty spaces in the cell wall, interacting covalently with other molecules of that cell structure, granting the cell wall extra resistance. To overcome this problem, a series of different pretreatments have been developed to modify or remove lignin. It is also important to note that most of the pretreatment techniques also modify in some way the physicochemical properties of the biomass, leading to increased glucose yield [89].

The adsorption of the enzyme depends of the structure and composition of the lignin in the substrate; for this reason, it is important to study how to remove or modify lignin, in order to produce better results [1]. Lignin prevents access to cellulose by "capturing" cellulases as well, binding itself with the enzymes, sometimes irreversibly, causing a decrease in the reaction velocity by reducing the amount of available enzymes in the solution [90]. Lignin from grasses has lower unproductive adsorption rate in comparison to softwood lignin [91]. In *natura* lignin

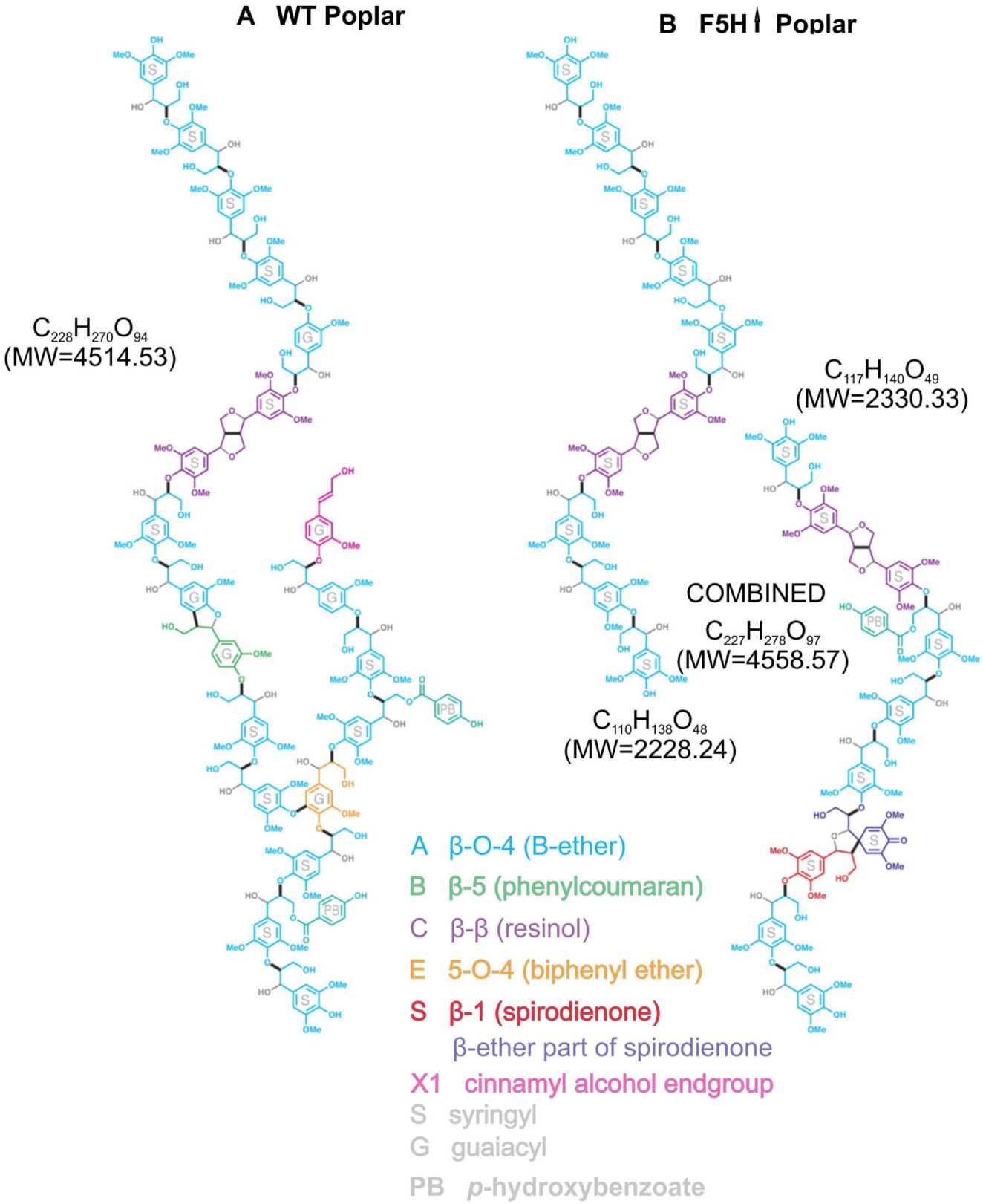

Fig. 4 Lignin models showing the main types and relative frequency of monomers in the lignin structure for wild-type (A) and the high Syringyl lignin (B). Reproduced with permission from [87]

can have lower unproductive adsorption rates in comparison with biomass pretreated by steam explosion, that happens mainly due to the lignin sub products generated [92]. The addition of non-ionic surfactants seems to reduce the enzyme-lignin

interaction by the use of surfactants such as Tween 20 in concentrations up to 0.05% resulted in higher rates of glucose conversion [93]. The article also shows other important results comparing the hydrolytic conversion rate using other surfactants based of poly-ethylene glycol (POG), and the POG-octylphenyl ether showed the highest score, improving the conversion rate up to two times when in comparison with the reaction without the surfactant, while Tween 20 improved the yield rates in about 50%. The surfactant addition increases the cellulose conversion rates, and the polyethylene glycol 4000 (PEG 4000) increased the conversion rate in about 80% [94]. Further, experiments conducted by Vaidya (2014) [95] demonstrated that the addition of PEG of up to 0.20 g of surfactant per gram of substrate increased the conversion rate of cellulose to glucose by more than 100%. In addition to that, he showed in his experiments that the usage of PEG in the enzymatic hydrolysis process increases enzyme activity, reducing incubation time and, possibly, enzyme loading.

Enzymes, such as albumin, could also be used to prevent lignin unproductive binding, making it so that instead of binding with cellulases, lignin ends up binding itself with the second enzyme, which has no effect in the hydrolysis reaction and therefore is disposable. Siqueira et al. (2017) [90] has observed a 136% increase in cellulose conversion of steam-pretreated sugarcane bagasse just by the addition of bovine serum albumin; the results of enzymatic hydrolysis using NaOH as reactant in the pretreatment also showed a 120% increase in glucose yield in comparison with the reaction without albumin (Fig. 3).

Modification of the lignin biosynthesis pathway can result in lower content increasing the enzymatic hydrolysis efficiency. Lignin structure and composition can be modified in order to decrease the severity of the conditions of the required pretreatments. The understanding of lignin biosynthesis can help redesign lignin, modifying its properties to make the processing easier and thus giving this molecule new economically viable industrial applications, all that without compromising plant development [96].

There are some correlations between genetic expression, lignin content, and enzymatic hydrolysis efficiency. In switchgrasses, the expression rate of one gene is related to lignin content (S/G ratio and overall lignin concentration) and to higher yields of glucose after hydrolysis, both with and without pretreatment, leading to an increase of up to 25 and 38% respectively, in comparison with the non-genetically modified specimen [97]. The syringyl/guaiacyl unit relation (S/G ratio) has been identified as a dominant factor in the cellulose accessibility, that happens mainly because it is easier to depolymerize S lignin in comparison with G lignin, as the ester bonds are easier to remove in the first case. In *Populus trichocarpa*, higher syringyl content resulted in higher yields of glucose after enzymatic hydrolysis [97].

Other studies suggest that S/G ratio has impact in cell wall disruption during chemical treatment and thus increasing cellulose conversion rates. The variance in this ratio has other impacts in the overall cell wall structure, influencing the cross-linkage between lignin and other constituents of the wall, increasing the number of micropores in the cell structure, consequently improving enzyme accessibility [98] (Fig. 5).

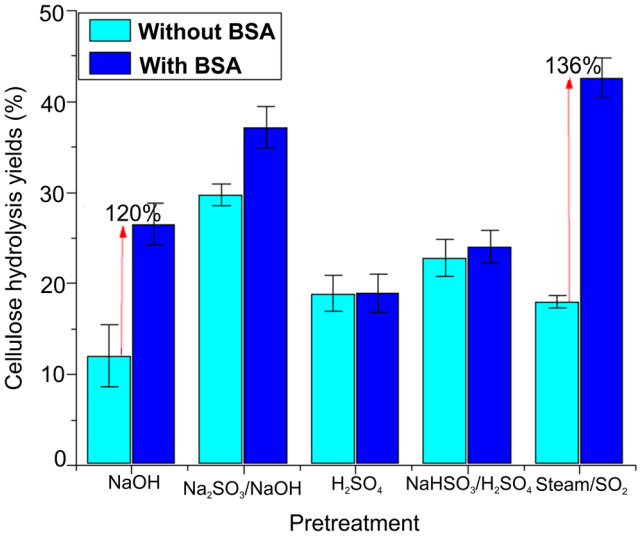

Fig. 5 Pretreated sugarcane bagasse cellulose hydrolysis yields after 72 h of hydrolysis and the effects of addition of bovine serum albumin [90]

Pretreatment Effects on Lignin

Enzymatic hydrolysis, as said previously, is the technique used to convert polymerized sugar, mostly in form of cellulose, to its monomeric form, glucose. For that conversion to happen, the enzymes responsible for breaking the chemical bonds between the sugars must have access to the polymer, and that is where lignin comes to turn things a little bit more complicated.

Pretreatment technology is the area of biomass processing that needs most improvements, and lignin is one of the molecules responsible for that, due to its presence and the ability to form covalent links with hemicellulose. To overcome those problems, pretreatment conditions must be severe, which make the process expensive, and sometimes too demanding of cellulose, converting part of it in degradation products. An efficient pretreatment must disrupt and remove the cross-links between lignin and hemicellulose, disrupt the hydrogen bonds formed between cellulose fibers, and increase the total surface area of cellulose for improved enzymatic hydrolysis results [99].

Some pretreatments can efficiently remove or modify lignin; sulfite soaking, for example, can delignify the material which, in turn, leads to an increase in cellulose accessibility; the main downside to the application of these techniques is the cost, which is too expensive; therefore, complete

delignification is not an option for the industry if they want to develop a competitive product [100].

The dilute acid pretreatment, used mainly to improve the digestibility of the lignocellulosic material, and attack the hemicellulose solubilizing it, making the cellulose more accessible [7, 101]. The conditions used vary, but it is commonly used in moderate temperatures and short reaction time [97]. The effect of this pretreatment method on lignin is the modification of its structure, leading to the formation of globes in the surface of the fibers as it gets solubilized and then repolymerized. On the other hand, the removal of lignin can be done with pretreatment technologies such as alkaline pretreatment and the oxidative pretreatment [4, 102].

Pretreatments with temperatures above 120 °C exceed the melting point of lignin, transforming it into a fluid, allowing the lignin to leave the cell wall matrix; and after the temperature cool down, the solubilized lignin will repolymerize upon the cell wall surface [103]. The lignin content increases during the enzymatic hydrolysis, meaning that as the reaction progresses and the accessible cellulose is hydrolyzed, the lignin droplets start to accumulate with other lignin residues in lower layers of the tissue, increasing the concentration of lignin deposited on the fiber [103]. This work showed that the lignin concentration started to increase up after 8 h of reaction, and with 144 h, the residual lignin had fully covered the substrate, preventing the action of the enzymes. Other work showed that the enzymatic hydrolysis slowdown is mainly provoked by enzyme and substrate factors [104]. Among the substrate factors, the lignin/cellulose ratio increases to 3.2, suggesting that the residual cellulose is protected by the lignin. During the enzymatic hydrolysis, the accessibility also decreases due the cellulose removal and lignin enrichment [104].

The acid pretreatment and steam explosion provoke increase in the percentage of insoluble lignin (Klason lignin) in comparison with the untreated biomass. The increase in the lignin content is because of hemicellulose removal, and mainly due to the polymerization of components to form a compound called pseudo-lignin [105, 106]. This material is not necessarily originated from lignin during the pretreatment. This compound is formed especially in severe conditions even using carbohydrates as substrates. The pretreatment of xylan and avicel resulted in the formation of pseudo-lignin leading to reduction of enzymatic hydrolysis glucose yield [107]. This work demonstrated that the color of the material changes according to the severity of the pretreatment (varying severity from 1.94 to 3.56), going from white, indicating the presence of cellulose and xylose (untreated), to black, indicating the presence of pseudo-lignin, when the pretreatment severity was the highest. The results indicate that the pseudo-lignin is formed from sugar degradation processes.

The steam explosion pretreatment is one of the most studied technologies for lignocellulose material conversion. In this method, the biomass is put in a reactor which is then heated to high temperatures and high pressure for a short period; after that, the reactor is suddenly depressurized, which "explodes" the lignocellulosic matrix, causing the rupture of the fibers, solubilizing part of the hemicellulose, offering similar results to the dilute acid pretreatment [108–110]. The steam penetrates the fibers and becomes condensed due to pressure; after the decompression of the system, the water formed inside the fibers evaporate quickly causing disturbance between the fibers and in the material. This pretreatment leads to the liberation of acetyl groups which will catalyze the hydrolysis of some polysaccharides [111].

The effects of this pretreatment on lignin depend on the biomass type; in hardwoods, for example, the break of ether type bonds leads to the formation of low molecular weight compounds and the increase in phenolic content; the increase in temperature also leads to the surging of condensed structures. Guaiacyl lignin shows the highest resistance to this method, and thus, the use of steam explosion pretreatment for softwoods leads to concentrations lower than 30% of soluble lignin [112]. To overcome this problem, it is necessary to pre-impregnate with SO_2, making the process more efficient for this type of biomass [113]. The percentage of insoluble lignin obtained through alkaline methods applied after the steam explosion pretreatment varies with the type of biomass, and softwoods like *Picea abies* (Norway spruce) and *Pinus sylvestris* (Scots pine) yield a maximum of 14 and 27% of insoluble lignin, respectively, while hardwoods like Birchwood and Aspen wood, the concentration of soluble lignin increases up to 86 and 48%, respectively. For sugarcane bagasse, the content of insoluble lignin after steam explosion followed by alkaline delignification reached 92% [55]. Even though the rates of solubilization were high, the combining of these two pretreatment technologies leads to a loss of about 31% of total cellulose, which is a considerable loss for the ethanol industry [55].

The content of lignin after the process increases according to the pretreatment conditions, and the higher is the severity, more is the residual lignin in the pretreated material [7]. This increase in lignin content can be justified by not only hemicellulose solubilization and condensation of lignin in the fiber surface, but also to the formation of pseudo-lignin due to sugar degradation processes [114].

There are other pretreatment technologies being developed to reduce lignin content and improve digestibility of cellulose. Nasirpour et al. (2014) [115] demonstrated that the combined usage of ionic liquids and surfactants such as Tween and PEG to pretreat sugarcane bagasse leads to a difference in digestibility of about 20% for tween, and 25% for PEG after 72 h of incubation.

Cellulose

The primary walls of plant cells contain cellulose assembled into long microfibrils a few nanometers in diameter [116].

Cellulose is so abundant in the globe that its annual biosynthesis reaches numbers varying between 10^{11} and 10^{12} tons [117]. It is composed of a non-branched linear chain, formed by D-glucose units linked by β (1 → 4) bonds. The cellulose macromolecules form a rigid network, resulting in a compact structure [118]. This network can be found inside the plant cell walls, where cellulose is organized in long and organized microfibrils, resulting in extensive fibers and producing a stiff structural reinforcement [119]. Cellulose is associated with other components such as hemicellulose, pectin, proteins, and lignin [120]. In a general view, the hemicellulose molecule structure, the cellulose fibers, and the lignin fill the spaces between these polysaccharides [121].

One of the most important properties of cellulose is the crystallinity. This property is observed in more organized regions of the cellulose chain, contrasting with its amorphous less organized regions. Crystalline regions are formed by microfibrils, which are paracrystalline structures of dozens of hydrogen-bonded (1,4) β-D-glucose chains, giving them a more organized cellulose chain than the other amorphous regions [122]. The hydrogen bonds (intra and interchain) in the crystalline regions give the materials higher recalcitrance, raising their resistance to enzymes and hindering cellulose digestibility and accessibility [1]. The x-ray diffraction method is commonly used to determine the crystallinity degree of cellulose; in the ligonocellulosic material, it is referred as biomass crystallinity index [1]. The diffraction intensities in the diffractograms allow to separate the crystalline regions from the other components of the cellulose (if pure) or the material in the case of lignocellulose [123].

Besides crystallinity, the degree of polymerization plays a large role in accessibility. Cellulose is a carbohydrate formed of glucose units whose number in the cellulose chain is what defines the degree of polymerization. A higher degree of polymerization hampers enzymatic activity as it produces a bigger and sturdier structure [1]. The degree of polymerization can be measured through the TAPPI T230 test method [124]. The degree of polymerization is positively correlated to enzymatic hydrolysis glucose yield for pure cellulose. However, no correlation is observed for cellulose degree of polymerization in lignocellulosic material [37].

Accessibility

Several factors influence the conversion of lignocellulosic biomass into biofuel and high-value molecules. Cellulose accessibility and the high cost of the technologies applied to solve these problems are among them. Among the factors related to substrate, the accessibility has been indicated as the most important since it directly correlates to the enzymatic hydrolysis glucose yield [125]. The accessibility is a reference of how much cellulose is exposed to enzyme action. Substrate properties on their own can influence the accessibility, due properties such as crystallinity, degree of polymerization, hemicellulose and lignin content/protection, and structural heterogeneity [126]. One of the biggest barriers to overcome is the limited access to a great portion of cellulose that is protected by a rigid and organized microfibril structure [127].

Evidence shows there is a positive relation between internal surface area and enzymatic hydrolysis effectiveness [128], considered one the most important factors hampering biomass hydrolysis [129]. In details, more than 90% of the enzymatic extract's digestibility comes from pore size distribution, much more than what the external surface area offers [130]. However, cellulases can reach internal surface by adequately size pores [131]. In fact, pores with diameters smaller than 5.1 nm are not big enough to allow enzyme entrance in the lignocellulosic material [132]. Furthermore, enzymatic hydrolysis studies use dried pretreated materials that diminishes digestibility due to pore size shrinkage [133].

A low degree of polymerization offers a larger number of binding sites for cellulases, promoting hydrolysis of the material. However, cellulose needs to be accessible to receive enzymatic action. Changes in the degree of polymerization are always accompanied by changes in porosity and crystallinity, as milling shortens the fibers and raises pore size distribution [1]. On the other hand, depolymerization is a process where polysaccharides are converted into monomers or a mixture of monomers [134]. The depolymerization of cellulose chains is part of the hydrolysis process. Cellulases are depolymerization agents as they break glucose into monomers [135]. The enzymes employed in the process and the substrate are worth noting as well. Enzyme diffusion through the pores, the binding sites used during hydrolysis, product inhibition, and the presence of other enzymes capable of attacking the plant cell wall are characteristics that should be considered to estimate cellulose accessibility [136].

Particle size can also be related to biomass digestibility since its influences the material surface [126]. Measuring particle size is a difficult task as it possesses irregular shapes and has a tendency to form agglomerates [137]. Sizes can be visually measured with the use of microscopy, image analysis, or by automated particle analyzers. However, these methods do not distinguish superficial topology and cracks on the surface that can raise the exposed surface area. Still, we can consider that smaller particles raise digestibility by offering a bigger exposed surface area for enzymatic hydrolysis [126]. Summarizing, not only hemicellulose and lignin removal impact biomass accessibility, but also the physical properties of the material.

The presence of lignin and hemicellulose interferes in the cellulose accessibility, altering pore distribution and the effectiveness of pretreatments and enzymatic components action [136]. Lignocellulosic materials are porous heterogeneous substrates with a superficial area divided into an external area

and an internal area, each with its own set of properties [126]. Looking at the internal surface, its accessibility can be measured by its superficial openings, rifts, and empty spaces created by the removal of non-cellulosic components like hemicellulose and lignin through pretreatments such as vapor explosion, diluted acid, alkaline, peroxide, and variants [137]. Hemicellulose removal can be considered a more impactful factor in cellulose accessibility than delignification [138]. Hemicelluloses act as physical barriers that hinder enzymatic hydrolysis due to their placement between and round the cellulose microfibrils inside the secondary cell walls [139]. Methods employing liquid phase analysis confirmed higher glucose yield during enzymatic hydrolysis on biomass that had its hemicelluloses removed [140].

Lignin naturally inhibits cellulolytic enzymes by creating a physical barrier and causes non-productive adsorption with consequent deactivation of adsorbed enzymes [100]. Adequate pretreatments may cause lignin removal and reduce or eliminate enzymatic hydrolysis inhibition [141]. Pretreatments that employ sulfite, ammonia, or even organosolv are capable of effectively delignify lignocellulosic materials, but complete lignin removal would be a far too expensive process [142]. A pretreatment condition with partial delignification increasing the accessibility could result in viable enzymatic hydrolysis yield with feasible scale up process.

Evaluating Accessibility

Several methods have been applied to measure and evaluate the accessibility of the lignocellulosic material, with certain advantages and disadvantages; they are correlated to enzymatic hydrolysis yield. Among the most used method are 3D electron tomogram, Simons' Stain, mercury intrusion, gas adsorption, and thermoporometry.

The Simons' stain method uses dyes to measure the microscopic damage caused on the fibers of lignocellulosic material as influence of the process [143]. The dye mixture used is composed of Direct Blue 1 and Direct Orange 15. First, Direct Blue molecules occupy the smaller pores in the fiber, while Direct Orange enters the bigger pores and covers the substrate's surface [126]. Tests showed that pore size expansion through physical or fungi action results in Direct Orange molecules gaining access to the larger pores and removing the Direct Blue molecules, a consequence of their higher affinity with the hydroxyl groups within the cellulose [144]. This method was efficient when comparing relative accessibility in pretreated cellulose samples [22]. Methods that employ only one dye such as Congo red are also effective. The dye covers the exposed cellulose surface area and has its supernatant quantified through spectrophotometry.

The mercury intrusion method can be used to measure the porosity of lignocellulosic materials [145], characterizing pores within the 3-nm and 100-μm size range. This method employs liquid mercury to analyze pores of solid and indefinite substrates. Liquid mercury is a non-stick substance, requiring pressure to penetrate into the pores. The required pressure is inversely proportional to the pore's radius, reaching 350 Mpa with 3-nm pores. This method offers information not only about the pores within the substrate, but also about the rifts between the particles inside the smaller portions of the material [146].

Gas adsorption is another method capable of measuring porosity where gases are enriched (adsorptive) in a solid substrate (adsorbent), allowing us to observe the volume of gas adsorbed (cm^3STP/g) [147]. Van der Walls interactions not only determine the physical adsorption controlling the intermolecular forces between adsorptive and adsorbent, but also the binary interactions between adsorptive molecules. The isotherm adsorption is obtained by measuring the adsorbed gas in the substrate while in crescent adsorptive pressure and constant temperature. Lowering the pressure allows evaluation of the desorption process. To avoid interferences within the adsorption process and release all adsorption spots available in the surface, a thorough purification of the samples must be done to remove any impurities. Degasification processes in high temperatures and low pressures are employed to purify the samples [146].

Thermoporometry is technique used to analyze wet porous materials, not requiring prior drying the material. The sample is washed with water that goes through temperatures ranging from freezing to melting extremes when confined in pores with diameters reaching submicrometers. This procedure allows to associate temperature variance with pore radius. Using an excess of liquid in the process produces two enthalpy peaks in freezing and melting thermograms. One of the peaks is related to an excess of free milligrams water on the sample surface, and the other, to low temperatures related to confined water or water bounded to the pores. This temperature variation corresponds to the positional difference between the confined/free water peaks and pore radius. Micropores with less than 2 nm cannot be analyzed because the superficial energy of the pores freezes the liquids [146].

The 3D electron tomography method allows to partition a tomogram in biomass and empty spaces, resulting in not only a volumetric map describing each type of biomass and rift, but also in a way to compute the biomass surface. It can be used to extract the nanoscale geometry of the cellulose microfibrils, reaching well-protected regions of the material [148]. The amount of exposed surface accessible to catalyzers of determined sizes in the biomass can be analyzed [146]. Furthermore, the generated images can be used to quantitatively measure pore size distribution [38] and have previously been used to verify the thickness of other solid objects [149].

Accessibility of the exposed surface area within the cellulose can be evaluated by hydrogen-deuterium exchange with

water. The heavy water is capable of labeling the accessible hydroxyl groups of cellulose exchanging hydrogen with water molecules [150]. This method was capable of offering information on the morphology and size of the cellulose fibril aggregates. In cotton cellulose, this method showed that the fibrils are arranged in a way that readily accessible surfaces are more abundant than slowly accessible ones, offering a different and more complex view of the material [151].

Pretreatments Affect the Accessibility

Several types of milling process can be used as step that precedes all pretreatments (Fig. 6), even though they can be considered one. Milling raises enzymatic hydrolysis effectiveness [152]. The material is milled to reduce particle size and raise the exposed surface area, diminishing the cohesiveness of the cellulose fibers [153]. The most utilized mills are the ball mill, which uses spheres of different sizes and materials to generate cuts and impacts to pulverize the biomass [154], and the discs mill, crushing the biomass with serrated plates [155]. Process involving milling is considered expensive and prohibited to large scale process. However, a combination of factors such as a properly biomass, partial milling associated to a chemical or physicochemical pretreatment could result feasibility. Furthermore, a milling step is necessary for size reduction prior pretreatment methods.

Acid pretreatment is recognized to solubilize hemicellulose with lower attachment to the cellulose. Acid pretreatments normally use sulfuric acid at 150 °C and 1 atm pressures [7]. The acid reaction can be divided into seven steps: (1) proton diffusion via wet lignocellulosic matrix, (2) protonation of the ether-oxygen bonds between the monomeric sugars, (3) breakage of the ether (glyosidic) bond and production of an intermediate carbocation, (4) solvation of the carbocation with water, (5) regeneration of protons and monomeric sugars (can be oligomers and polymers as well), (6) distribution of products in liquid phase, and (7) process returns to the second step [156]. Regarding the cellulose, acid pretreatments can cause losses up to 20% of the cellulose content [157]. The crystalline nature of the cellulose requires very low pH, high temperatures, and long reaction periods to hydrolyze significant portions of cellulose into glucose. These conditions favor acid hydrolysis of the cellulose, but degrade glucose into formic acid and levulinic acid [158]. Under mild conditions, the degree of polymerization suffers notable changes, rapidly decreasing in the initial stages and stabilizing after roughly 30 min of reaction [159]. The initial decrease in the degree of polymerization could be caused by the hydrolysis of the amorphous regions within the cellulose [41]. The ratio between amorphous and crystalline regions decreases after the pretreatment due to the partial removal of hemicellulose from the material [160].

As accessibility and enzymatic digestibility are severely affected by hemicellulose and lignin contents [161], their removal is a crucial part of the biomass conversion study. Hemicellulose has a branched structure, being much more susceptible to acid pretreatments than cellulose. Thus, hemicellulose can be completely removed without causing significant damage to the cellulose with the use of acid pretreatments [162]. The removal rate depends on the pretreatment conditions. Steam explosion using SO_2 2% at 200 °C for 2 min was capable of recovering 65% of xylan in corn straw, while pretreatments at 170 °C were able to recover only 18% [163]. The acidifying agent also influences hemicellulose removal. Sugarcane bagasse pretreated with H_2SO_4 resulted in complete hemicellulose removal while the steam explosion employing SO_2 was not able to remove as much. Despite removing all hemicellulose, the sulfuric acid pretreatment generated more degradation products than the sulfur dioxide

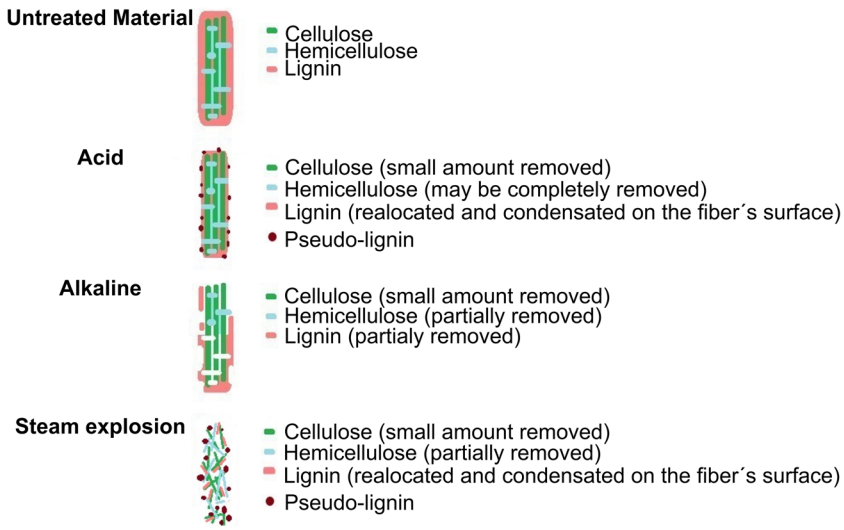

Fig. 6 Different pretreatment effects on the lignocellulosic biomass

method due to its aggressive behavior, attacking the broken sugars and generating furfural [164]. The sugars forming the hemicellulose are released in solution as oligomers or monomers, depending on temperature, reaction time, and acid concentration [48]. Raising the temperatures [12] and the acid concentration promotes hemicellulose removal [160].

Lignin removal by acid pretreatments is low regardless of biomass used and acidifying agent [146]. Generally, the increase of the severity of the acid pretreatment lowers the lignin solubilization, provoking lignin enrichment in the solid pretreated material [7]. Lignin removal is accompanied by the production of aromatic monomers in liquid phase, with phenolic types varying according to the type of biomass and pretreatment conditions [165]. Studies employing microscopy of the solid fraction revealed drastic changes in lignin morphology and distribution, showing spherical droplets in the cell wall with traces of lignin [61]. The morphology and location of these droplets on the biomass point to a cycle of breaking and condensation that would be responsible for the low removal and accumulation of the lignin [166].

Sodium, calcium, and potassium hydroxides can be used in alkaline pretreatments [167]. During this type of pretreatment, the first reactions that occur are solvation and saponification, causing swelling and raising accessibility to enzymes in the biomass. Severe pretreatment conditions cause dissolution, peeling of reducing ends, degradation, and decomposition of the dissolved polysaccharides [144]. Polysaccharide loss occurs mainly through hydrolytic reactions and peeling of the reducing ends present in the sugars [46]. The main purpose of alkaline pretreatments is the removal of lignin from lignocellulosic materials, reducing enzyme obstacles and improving polysaccharide reactivity. It is believed that this mechanism includes intermolecular saponification of the ester bonds between hemicelluloses and lignin, increasing porosity [165]. Peroxide in alkaline medium bursts the hemicellulose and lignin removal, resulting in a pretreated material with high enzymatic digestibility [102]. High yield of hemicellulose solubilization can be reached, around 90%, with low residual lignin [168].

Adding air to the reactive solution significantly increases delignification of materials rich in lignin [154]. The removal of acetyl groups from hemicelluloses through alkaline pretreatments exposes the cellulose, improving the enzymatic hydrolysis of both hemicellulose and cellulose [146]. In addition, alkaline pretreatments cause partial hemicellulose removal and cellulose swelling and decrease crystallinity [151]. This method provokes lower sugar degradation than the acid pretreatments. Lastly, the material is neutralized to adjust its pH and removes lignin, inhibitors, salts, and phenolic acids [154].

Recent studies used surfactants such as Tween 20 and Tween 80 combined with ethyl-3-methylimidazolium acetate as a pretreatment, soaking the material overnight to have the surfactants penetrate the pores. Cellulose fraction reached 65.6%, while hemicelluloses decreased to 17% and lignin to 14.7%. These results were also accompanied by a solid recovery of 83% at its best, denoting that material loss was caused mostly by hemicellulose and lignin removal. In addition, the material crystallinity was decreased and the microfibers within the cell wall were disrupted, increasing the exposed surface area. Porosity was also improved, as shown by the Simons' stain method. Similarly to other pretreatments, increased severity resulted in cellulose degradation [169].

Enzymatic Hydrolysis Influenced by Accessibility

Celullases are an enzymatic system of various components that depolymerize cellulose into glucose though breakage of glycosidic bonds. Cellulases are composed of at least three enzymes: endo-β-1,4-glucanases, exo-β-1,4-glucanase, and β-glucosidases. The cellulases act in synergic way, where the effect of the enzymes combined is bigger than the effect of them acting separately [157]. Among the cellulases, the endoglucanases are responsible for cleaving the cellulose through the interchain; the exo-β-1,4-glucanases are capable of acting on the free ends of the cellulose chain generated by the endo-β-1,4-glucanases, releasing water-soluble cellobiose; and the β-glucosidases ending the process by hydrolyzing cellobiose into glucose monomers [125]. The enzyme action is described as considering pure cellulose. However, in the lignocellulosic material are hemicellulose and lignin affecting the access of enzyme to cellulose chain. Probably, the endo-β-1,4-glucanase is the most affect enzyme in this process since this enzyme is recognized as initiate hydrolysis of the cellulose. The exposed cellulose, accessible to enzyme action, is directly correlated to the enzymatic hydrolysis glucose yield [7].

Cellulose chain hydrolysis can be schematically divided into five steps: (1) transference of the enzymes from a liquid medium to the cellulose particles surface; (2) enzyme adsorption and formation of the enzyme-substrate complex; (3) cellulose hydrolysis; (4) transference of the oligomers, glucose, and cellobiose from the particles surface to the liquid medium; and (5) hydrolysis of oligomers and cellobiose into glucose in liquid medium [56]. This process is influenced by many factors such as the presence of lignin and hemicellulose in the substrate, forming a chemical and physical barrier to the enzymes (Fig. 7) [170]. Moreover, during the enzymatic hydrolysis kinetics, the lignin content is increased, provoking slowdowns in the process. During enzymatic hydrolysis, the substrate changed, reduced the cellulose content, and increased the lignin physical barrier effect [148].

The delignification of substrates promotes better enzymatic conversion of cellulose, especially when lignin levels are lower than 10% [171]. Besides increasing hydrolysis efficiency,

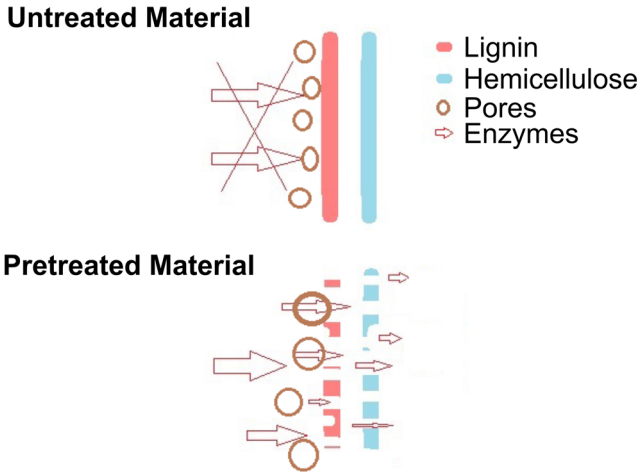

Fig. 7 Increased accessibility allows an efficient enzymatic hydrolysis

the amount of enzymes recovered after the process is also improved, probably due to reduced unproductive bind to lignin [69]. If not sufficiently removed, lignin can go through redeposition and occupy pores that were accessible before [172]. Moreover, cellulases adsorb unproductively into the lignin portion of the material, incapacitating the enzymatic hydrolysis [173]. In fact, lignin content in the raw biomass is indicated as a selection parameter for conversion process [33]. The pretreatment severity increases resulted in higher glucose yield since the accessibility increased [7].

Concluding Remarks

There are many contributions being made to improve our understanding of biomass composition, structure, and recalcitrance to enable better usage of this type of material. However, there are still plenty of questions that need to be answered as well as obstacles that need to be overcome. The cost of bioethanol conversion and other emerging technologies that require structural modification, solubilization, and purification of chemical components obtained from lignocellulosic biomass is still high, mainly due to the cost of the required reagents. In addition, the elevated cost is related to the low final concentration of the products obtained. Cellulose, hemicellulose, and lignin interact to create a matrix that offers structural rigidity and physical and chemical protection along with other properties that enable a plant to survive and dominate the environment in which it is grown. These three lignocellulosic macromolecules are composed of different monomers with different properties, making the lignocellulosic biomass a singular component. The differences in concentration, structure, and composition of each component dictate the physicochemical characteristics of biomass, and thus choosing a variety with a high cellulose content and a low syringyl/guaiacyl ratio along other well-studied properties will result in better conversion rates and reduced cost. If such varieties do not provide the expected results, pretreatments employing acid, steam, and alkaline agents can increase the pore size distribution and accessibility. However, based on the information gathered in this review, it is possible to conclude that the use of lignocellulosic biomass to produce biotechnological components of economic importance is here to stay, as it is the most widely available renewable resource worldwide. Its importance in reducing gas emissions and petroleum exploration is undeniable. Thus, improving existing technologies and developing new strategies to reduce the costs of lignocellulosic material conversion into bioethanol is an important step for popularization of this product.

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References

1. Zhao X, Zhang L, Liu D (2012) Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. Biofuels Bioprod Biorefin 6(4):465–482
2. Kamm B, & Kamm M. (2007). Biorefineries–multi product processes. In *White Biotechnology* (pp. 175-204). Springer Berlin Heidelberg
3. Jørgensen H, Kristensen JB, Felby C (2007) Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels Bioprod Biorefin 1(2):119–134
4. Brienzo M, Abud Y, Ferreira S, Corrales RC, Ferreira-Leitão VS, de Souza W, & Sant'Anna C (2016). Characterization of anatomy, lignin distribution, and response to pretreatments of sugarcane culm node and internode. Ind Crop Prod, 84, 305–313
5. Chang VS, & Holtzapple MT (2000). Fundamental factors affecting biomass enzymatic reactivity. In Twenty-first symposium on biotechnology for fuels and chemicals (pp. 5-37). Humana Press, Totowa
6. Zheng Y, Pan Z, Zhang R (2009) Overview of biomass pretreatment for cellulosic ethanol production. Int J Agric Biol Eng 2(3): 51–68
7. Brienzo M, Fikizolo S, Benjamin Y, Tyhoda L, Görgens J (2017) Influence of pretreatment severity on structural changes, lignin content and enzymatic hydrolysis of sugarcane bagasse samples. Renew Energy 104:271–280
8. Gámez S, González-Cabriales JJ, Ramírez JA, Garrote G, Vázquez M (2006) Study of the hydrolysis of sugar cane bagasse using phosphoric acid. J Food Eng 74(1):78–88
9. Rodrıguez-Chong A, Ramírez JA, Garrote G, Vázquez M (2004) Hydrolysis of sugar cane bagasse using nitric acid: a kinetic assessment. J Food Eng 61(2):143–152
10. Cheng KK, Cai BY, Zhang JA, Ling HZ, Zhou YJ, Ge JP, Xu JM (2008) Sugarcane bagasse hemicellulose hydrolysate for ethanol production by acid recovery process. Biochem Eng J 38(1):105–109
11. Hernández-Salas JM, Villa-Ramírez MS, Veloz-Rendón JS, Rivera-Hernández KN, González-César RA, Plascencia-

Espinosa MA, Trejo-Estrada SR (2009) Comparative hydrolysis and fermentation of sugarcane and agave bagasse. Bioresour Technol 100(3):1238–1245
12. Martín C, Galbe M, Nilvebrant NO, & Jönsson LJ (2002). Comparison of the fermentability of enzymatic hydrolyzates of sugarcane bagasse pretreated by steam explosion using different impregnating agents. In Biotechnology for Fuels and Chemicals (pp. 699-716). Humana Press, Totowa, NJ
13. Sendelius J (2005). Steam pretreatment optimisation for sugarcane bagasse in bioethanol production. Master of Science Thesis. Department of Chemical Engineering, Lund University, Sweden
14. Li X, Kondo R, & Sakai K (2002). In vivo and in vitro biobleaching of unbleached hardwood kraft pulp by a marine fungus, Phlebia sp. MG-60. *In Progress in Biotechnology* (Vol. 21, pp. 185-191). Elsevier
15. Camassola M, Dillon AJ (2009) Biological pretreatment of sugar cane bagasse for the production of cellulases and xylanases by Penicillium echinulatum. Ind Crop Prod 29(2–3):642–647
16. Martin C, Klinke HB, Thomsen AB (2007) Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzym Microb Technol 40(3):426–432
17. Pasquini D, Pimenta MT, Ferreira LH, Curvelo AA (2005) Sugar cane bagasse pulping using supercritical CO2 associated with co-solvent 1-butanol/water. J Supercrit Fluids 34(2):125–131
18. Pereira AA, Martins GF, Antunes PA, Conrrado R, Pasquini D, Job AE et al (2007) Lignin from sugar cane bagasse: extraction, fabrication of nanostructured films, and application. Langmuir 23(12):6652–6659
19. Tu Q, Fu S, Zhan H, Chai X, Lucia LA (2008) Kinetic modeling of formic acid pulping of bagasse. J Agric Food Chem 56(9):3097–3101
20. Laser M, Schulman D, Allen SG, Lichwa J, Antal MJ Jr, Lynd LR (2002) A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. Bioresour Technol 81(1):33–44
21. Kurakake M, Kisaka W, Ouchi K, Komaki T (2001) Pretreatment with ammonia water for enzymatic hydrolysis of corn husk, bagasse, and switchgrass. Appl Biochem Biotechnol 90(3):251–259
22. Li BZ, Balan V, Yuan YJ, Dale BE (2010) Process optimization to convert forage and sweet sorghum bagasse to ethanol based on ammonia fiber expansion (AFEX) pretreatment. Bioresour Technol 101(4):1285–1292
23. Shoda Y, Nakamoto A, Goto M, Tokuhara W, Noritake Y, Katahira S et al (2012) Short time ionic liquids pretreatment on lignocellulosic biomass to enhance enzymatic saccharification. Bioresour Technol 103(1):446–452
24. Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW & Foust TD (20/07). Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science, 315(5813), 804–807
25. K Sheth (2017). Top Sugarcane Producing Countries. http://www.worldatlas.com/articles/top-sugarcane-producing-countries.html. Accessed 20 December 2017;
26. Hussain, A., Khan, Z. I., Ghafoor, M. Y., & Ashraf, M. (2004). Review Sugarcane, Sugar Metabolism and Some Abiotic Stresses
27. Smith DM, Inman-Bamber NG, Thorburn PJ (2005) Growth and function of the sugarcane root system. Field Crop Res 92(2–3):169–183
28. Sanjuan R, Anzaldo J, Vargas J, Turrado J, Patt R (2001) Morphological and chemical composition of pith and fibers from Mexican sugarcane bagasse. Holz als Roh-und Werkstoff 59(6):447–450
29. Miller JD, Gilbert RA, & Odero DC (2009). Sugarcane botany: a brief view. University of Flórida, IFAS Extension
30. Sun RC, Fang JM, Tomkinson J, Geng ZC, Liu JC (2001) Fractional isolation, physico-chemical characterization and homogeneous esterification of hemicelluloses from fast-growing poplar wood. Carbohydr Polym 44(1):29–39
31. Brienzo M, Ferreira S, Vicentim MP, de Souza W, & Sant'Anna C (2014). Comparison study on the biomass recalcitrance of different tissue fractions of sugarcane culm. BioEnergy Res, 7(4), 1454–1465
32. Costa TH, Masarin F, Bonifácio TO, Milagres AM, Ferraz A (2013) The enzymatic recalcitrance of internodes of sugar cane hybrids with contrasting lignin contents. Ind Crop Prod 51:202–211
33. Cerqueira DA, Rodrigues Filho G, da Silva Meireles C (2007) Optimization of sugarcane bagasse cellulose acetylation. Carbohydr Polym 69(3):579–582
34. Adsul MG, Ghule JE, Singh R, Shaikh H, Bastawde KB, Gokhale DV, Varma AJ (2004) Polysaccharides from bagasse: applications in cellulase and xylanase production. Carbohydr Polym 57(1):67–72
35. Harris DR, & Hillman GC (Eds.). (2014). Foraging and farming: the evolution of plant exploitation (Vol. 31). Routledge
36. S Ullmann. Milho. (2001) http://www.ufrgs.br/afeira/materias-primas/cereais/millho. Accessed 20 December 2017
37. U. S. Department of Agriculture. USDA. (2017). https://www.usda.gov/. Accessed 20 December 2017
38. Bechoux N, Bernier G, Lejeune P (2000) Environmental effects on the early stages of tassel morphogenesis in maize (*Zea mays* L.). Plant, Cell Environ 23:91–98
39. Sridhar Viamajala; Michael J. Selig; Todd B. Vinant; Melvin P. Tucker; Michael E. Himmel; James D. McMillan; Stephen R. Decker Appl Biochem Biotechnol (2006) 130: 509
40. Hochholdinger, F. (2009). The maize root system: morphology, anatomy, and genetics. In Handbook of maize: its biology (pp. 145-160). Springer, New York, NY
41. Sylvester, A. W., & Smith, L. G. (2009). Cell biology of maize leaf development. In Handbook of Maize: its biology (pp. 179-203). Springer, New York, NY
42. Thompson JL, Tyner WE (2014) Corn stover for bioenergy production: cost estimates and farmer supply response. Biomass Bioenergy 62:166–173
43. Foley KM (1978). Chemical properties, physical properties, and uses of the Anderson'corncob products. The Andersons, Cob Division
44. Clark TF, & Lathrop EC (1953). Corncobs: their composition, availability, agricultural and industrial Uses. United States Department of Agriculture, Agricultural Research Administration, Bureau of Agricultural and Industrial Chemistry, Northern Regional Research Laboratory
45. Sokhansanj S, Turhollow A, Cushman J, Cundiff J (2002) Engineering aspects of collecting corn stover for bioenergy. Biomass Bioenergy 23(5):347–355
46. Chundawat SP, Venkatesh B, Dale BE (2007) Effect of particle size based separation of milled corn stover on AFEX pretreatment and enzymatic digestibility. Biotechnol Bioeng 96(2):219–231
47. Pu Y, Zhang D, Singh PM, Ragauskas AJ (2008) The new forestry biofuels sector. Biofuels Bioprod Biorefin 2(1):58–73
48. Atalla RH, Vanderhart DL (1984) Native cellulose: a composite of two distinct crystalline forms. Science 223(4633):283–285
49. Potters G, Van Goethem D, & Schutte F (2010). Promising biofuel resources: lignocellulose and algae. Nat Educ, 3(9), 14
50. Bezerra TL, Ragauskas AJ (2016) A review of sugarcane bagasse for second-generation bioethanol and biopower production. Biofuels Bioprod Biorefin 10(5):634–647
51. Sant'Anna C, De Souza W, & Brienzo M (2014). The influence of the heterogeneity, physicochemical and structural properties on the recalcitrance and conversion of sugarcane bagasse. Sugarcane: Production, Consumption and Agricultural Management Systems, 1–34

52. Peng F, Ren JL, Xu F, Bian J, Peng P, Sun RC (2009) Fractional study of alkali-soluble hemicelluloses obtained by graded ethanol precipitation from sugar cane bagasse. J Agric Food Chem 58(3): 1768–1776
53. Yao S, Nie S, Zhu H, Wang S, Song X, Qin C (2017) Extraction of hemicellulose by hot water to reduce adsorbable organic halogen formation in chlorine dioxide bleaching of bagasse pulp. Ind Crop Prod 96:178–185
54. Busse-Wicher M, Grantham NJ, Lyczakowski JJ, Nikolovski N, Dupree P (2016) Xylan decoration patterns and the plant secondary cell wall molecular architecture. Biochem Soc Trans 44(1):74–78
55. Chen H (2014). Chemical composition and structure of natural lignocellulose. In Biotechnology of lignocellulose (pp. 25-71). Springer, Dordrecht
56. Brienzo M, Siqueira AF, Milagres AMF (2009) Search for optimum conditions of sugarcane bagasse hemicellulose extraction. Biochem Eng J 46(2):199–204
57. Sun S, Cai Y, Liu H (2001) Biomass allocation of Scirpus mariqueter along an elevational gradient in a salt marsh of the Yangtse River estuary. Acta Bot Sin 43(2):178–185
58. Peng, J., Chen, P., Lou, H., & Zheng, X. (2009). Catalytic upgrading of bio-oil by HZSM-5 in
59. Fengel D, Wegener G (1984) Wood: chemistry, ultrastructure, reactions, vol 613. Walter Gruyter, pp 1960–1982
60. Brienzo M, Carvalho W, Milagres AM (2010) Xylooligosaccharides production from alkali-pretreated sugarcane bagasse using xylanases from Thermoascus aurantiacus. Appl Biochem Biotechnol 162(4):1195–1205
61. Zeng J, Helms GL, Gao X, Chen S (2013) Quantification of wheat straw lignin structure by comprehensive NMR analysis. J Agric Food Chem 61(46):10848–10857
62. Alonso DM, Bond JQ, Dumesic JA (2010) Catalytic conversion of biomass to biofuels. Green Chem 12(9):1493–1513
63. York WS, O'Neill MA (2008) Biochemical control of xylan biosynthesis—which end is up? Curr Opin Plant Biol 11(3): 258–265
64. Chundawat, S. P., Beckham, G. T., Himmel, M. E., & Dale, B. E. (2011). Deconstruction of lignocellulosic biomass to fuels and chemicals
65. Urbanowicz BR, Peña MJ, Ratnaparkhe S, Avci U, Backe J, Steet HF, Darvill AG (2012) 4-O-methylation of glucuronic acid in Arabidopsis glucuronoxylan is catalyzed by a domain of unknown function family 579 protein. Proc Natl Acad Sci 109(35):14253–14258
66. Moghaddam L, Zhang Z, Wellard RM, Bartley JP, O'Hara IM, Doherty WO (2014) Characterisation of lignins isolated from sugarcane bagasse pretreated with acidified ethylene glycol and ionic liquids. Biomass Bioenergy 70:498–512
67. Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83(1):1–11
68. Rocha GJM, Gonçalves AR, Nakanishi SC, Nascimento VM, Silva VFN (2015) Pilot scale steam explosion and diluted sulfuric acid pretreatments: comparative study aiming the sugarcane bagasse saccharification. Ind Crop Prod 74:810–816
69. Ghaffar SH, Fan M (2014) Lignin in straw and its applications as an adhesive. Int J Adhes Adhes 48:92–101
70. Holladay JE, White JF, Bozell JJ, & Johnson D (2007). Top value-added chemicals from biomass-volume II—results of screening for potential candidates from biorefinery lignin (no. PNNL-16983). Pacific *Northwest National Laboratory* (PNNL), Richland, WA (US)
71. Sjostrom E (2013). Wood chemistry: fundamentals and applications. Elsevier
72. Grisebach H (2016). Lignins I 5. Secondary Plant Products: a Comprehensive Treatise, 7, 457
73. Fang R, Cheng X, Xu X (2010) Synthesis of lignin-base cationic flocculant and its application in removing anionic azo-dyes from simulated wastewater. Bioresour Technol 101(19):7323–7329
74. Chung YL, Olsson JV, Li RJ, Frank CW, Waymouth RM, Billington SL, Sattely ES (2013) A renewable lignin–lactide copolymer and application in biobased composites. ACS Sustain Chem Eng 1(10):1231–1238
75. Hu S, Zhang S, Pan N, Hsieh YL (2014) High energy density supercapacitors from lignin derived submicron activated carbon fibers in aqueous electrolytes. J Power Sources 270:106–112
76. Labeeuw L, Martone PT, Boucher Y, Case RJ (2015) Ancient origin of the biosynthesis of lignin precursors. Biol Direct 10(1): 23
77. Boitte BC, Rivard M, Majira A, Beauhaire J, Allais F, Martens T et al (2015) Comparative electrochemical study on monolignols and dimers relevant for the comprehension of the lignification process. Phytochem Lett 13:280–285
78. Eudes A, George A, Mukerjee P, Kim JS, Pollet B, Benke PI et al (2012) Biosynthesis and incorporation of side-chain-truncated lignin monomers to reduce lignin polymerization and enhance saccharification. Plant Biotechnol J 10(5):609–620
79. Zeng Y, Zhao S, Yang S, Ding SY (2014) Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels. Curr Opin Biotechnol 27:38–45
80. Zakzeski J, Bruijnincx PC, Jongerius AL, Weckhuysen BM (2010) The catalytic valorization of lignin for the production of renewable chemicals. Chem Rev 110(6):3552–3599
81. Verma SR, Dwivedi UN (2014) Lignin genetic engineering for improvement of wood quality: applications in paper and textile industries, fodder and bioenergy production. S Afr J Bot 91: 107–125
82. Simmons BA, Loque D, Ralph J (2010) Advances in modifying lignin for enhanced biofuel production. Curr Opin Plant Biol 13(3):312–319
83. Oraby HF, Ramadan MF (2015) Impact of suppressing the caffeic acid O-methyltransferase (COMT) gene on lignin, fiber, and seed oil composition in Brassica napus transgenic plants. Eur Food Res Technol 240(5):931–938
84. Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W (2010) Lignin biosynthesis and structure. Plant Physiol 153(3):895–905
85. Carroll A, Somerville C (2009) Cellulosic biofuels. Annu Rev Plant Biol 60:165–182
86. Vanholme R, Morreel K, Darrah C, Oyarce P, Grabber JH, Ralph J, Boerjan W (2012) Metabolic engineering of novel lignin in biomass crops. New Phytol 196(4):978–1000
87. Stewart JJ, Akiyama T, Chapple C, Ralph J, Mansfield SD (2009) The effects on lignin structure of overexpression offerulate 5-hydroxylase in hybrid Poplar. Plant Physiology, 150, 621–635
88. Grabber JH, Hatfield RD, Lu F, Ralph J (2008) Coniferyl ferulate incorporation into lignin enhances the alkaline delignification and enzymatic degradation of cell walls. Biomacromolecules 9(9): 2510–2516
89. Brienzo M, Tyhoda L, Benjamin Y, Görgens J (2015) Relationship between physicochemical properties and enzymatic hydrolysis of sugarcane bagasse varieties for bioethanol production. New Biotechnol 32(2):253–262
90. Siqueira G, Arantes V, Saddler JN, Ferraz A, Milagres AM (2017) Limitation of cellulose accessibility and unproductive binding of cellulases by pretreated sugarcane bagasse lignin. Biotechnol Biofuels 10(1):176
91. Nakagame S, Chandra RP, Kadla JF, Saddler JN (2011) Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin. Biotechnol Bioeng 108(3):538–548
92. Rahikainen J, Mikander S, Marjamaa K, Tamminen T, Lappas A, Viikari L, Kruus K (2011) Inhibition of enzymatic hydrolysis by

residual lignins from softwood—study of enzyme binding and inactivation on lignin-rich surface. Biotechnol Bioeng 108(12): 2823–2834
93. Park JW, Takahata Y, Kajiuchi T, Akehata T (1992) Effects of nonionic surfactant on enzymatic hydrolysis of used newspaper. Biotechnol Bioeng 39(1):117–120
94. Börjesson J, Peterson R, Tjerneld F (2007) Enhanced enzymatic conversion of softwood lignocellulose by poly (ethylene glycol) addition. Enzym Microb Technol 40(4):754–762
95. Vaidya AA, Newman RH, Campion SH, Suckling ID (2014) Strength of adsorption of polyethylene glycol on pretreated Pinus radiata wood and consequences for enzymatic saccharification. Biomass Bioenergy 70:339–346
96. Fu C, Mielenz JR, Xiao X, Ge Y, Hamilton CY, Rodriguez M et al (2011) Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. Proc Natl Acad Sci 108(9):3803–3808
97. Studer MH, DeMartini JD, Davis MF, Sykes RW, Davison B, Keller M, Wyman CE (2011) Lignin content in natural Populus variants affects sugar release. Proc Natl Acad Sci 108(15):6300–6305
98. Papa G, Varanasi P, Sun L, Cheng G, Stavila V, Holmes B, Singh S (2012) Exploring the effect of different plant lignin content and composition on ionic liquid pretreatment efficiency and enzymatic saccharification of Eucalyptus globulus L. mutants. Bioresour Technol 117:352–359
99. Li C, Knierim B, Manisseri C, Arora R, Scheller HV, Auer M et al (2010) Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. Bioresour Technol 101(13):4900–4906
100. Yang Q, Pan X (2016) Correlation between lignin physicochemical properties and inhibition to enzymatic hydrolysis of cellulose. Biotechnol Bioeng 113(6):1213–1224
101. Hendriks ATWM, Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour Technol 100(1): 10–18
102. Selig MJ, Viamajala S, Decker SR, Tucker MP, Himmel ME, Vinzant TB (2007) Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose. Biotechnol Prog 23(6):1333–1339
103. Hansen MA, Kristensen JB, Felby C, Jørgensen H (2011) Pretreatment and enzymatic hydrolysis of wheat straw (Triticum aestivum L.)–the impact of lignin relocation and plant tissues on enzymatic accessibility. Bioresour Technol 102(3):2804–2811
104. Wallace J, Brienzo M, García-Aparicio MP, Görgens JF (2016) Lignin enrichment and enzyme deactivation as the root cause of enzymatic hydrolysis slowdown of steam pretreated sugarcane bagasse. New Biotechnol 33(3):361–371
105. Hu F, Jung S, Ragauskas A (2012) Pseudo-lignin formation and its impact on enzymatic hydrolysis. Bioresour Technol 117:7–12
106. Sannigrahi P, Kim DH, Jung S, Ragauskas A (2011) Pseudo-lignin and pretreatment chemistry. Energy Environ Sci 4(4):1306–1310
107. Kumar R, Hu F, Sannigrahi P, Jung S, Ragauskas AJ, Wyman CE (2013) Carbohydrate derived-pseudo-lignin can retard cellulose biological conversion. Biotechnol Bioeng 110(3):737–753
108. Alvira P, Tomás-Pejó E, Ballesteros MJ, Negro MJ (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour Technol 101(13):4851–4861
109. Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96(6):673–686
110. Oliveira FM, Pinheiro IO, Souto-Maior AM, Martin C, Gonçalves AR, Rocha GJ (2013) Industrial-scale steam explosion pretreatment of sugarcane straw for enzymatic hydrolysis of cellulose for production of second-generation ethanol and value-added products. Bioresour Technol 130:168–173
111. Shevchenko SM, Beatson RP, & Saddler JN (1999). The nature of lignin from steam explosion/enzymatic hydrolysis of softwood. In Twentieth Symposium on Biotechnology for Fuels and Chemicals (pp. 867-876). Humana Press, Totowa
112. Li J, Gellerstedt G, Toven K (2009) Steam explosion lignins; their extraction, structure and potential as feedstock for biodiesel and chemicals. Bioresour Technol 100(9):2556–2561
113. Rocha GDM, Gonçalves AR, Oliveira BR, Olivares EG, Rossell CEV (2012) Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production. Ind Crop Prod 35(1): 274–279
114. Nasirpour N, Mousavi SM, Shojaosadati SA (2014) A novel surfactant-assisted ionic liquid pretreatment of sugarcane bagasse for enhanced enzymatic hydrolysis. Bioresour Technol 169:33–37
115. Thomas LH, Forsyth VT, Šturcová A, Kennedy CJ, May RP, Altaner CM, Jarvis MC (2013) Structure of cellulose microfibrils in primary cell walls from collenchyma. Plant Physiol 161(1): 465–476
116. Kukrety A, Singh RK, Singh P, & Ray SS (2017). Comprehension on the synthesis of carboxymethylcellulose (CMC) utilizing various cellulose rich waste biomass resources. Waste Biomass Valorization, 1–9
117. French AD (2017) Glucose, not cellobiose, is the repeating unit of cellulose and why that is important. Cellulose 24(11):4605–4609
118. Bidhendi AJ, Geitmann A (2015) Relating the mechanics of the primary plant cell wall to morphogenesis. J Exp Bot 67(2):449–461
119. Davison BH, Parks J, Davis MF, & Donohoe BS (2013). Plant cell walls: basics of structure, chemistry, accessibility and the influence on conversion. Aqueous pretreatment of plant biomass for biological and chemical conversion to fuels and chemicals, 23–38
120. Wang S, Dai G, Yang H, Luo Z (2017) Lignocellulosic biomass pyrolysis mechanism: a state-of-the-art review. Prog Energy Combust Sci 62:33–86
121. Nakamura A, Watanabe H, Ishida T, Uchihashi T, Wada M, Ando T et al (2014) Trade-off between processivity and hydrolytic velocity of cellobiohydrolases at the surface of crystalline cellulose. J Am Chem Soc 136(12):4584–4592
122. Yuan X, Duan Y, He L, Singh S, Simmons B, Cheng G (2017) Characterization of white poplar and eucalyptus after ionic liquid pretreatment as a function of biomass loading using X-ray diffraction and small angle neutron scattering. Bioresour Technol 232: 113–118
123. Vena PF, García-Aparicio MP, Brienzo M, Görgens JF, Rypstra T (2013) Effect of alkaline hemicellulose extraction on kraft pulp fibers from Eucalyptus grandis. J Wood Chem Technol 33(3): 157–173
124. Crowe JD, Zarger RA, Hodge DB (2017) Relating nanoscale accessibility within plant cell walls to improved enzyme hydrolysis yields in corn stover subjected to diverse pretreatments. J Agric Food Chem 65(39):8652–8662
125. Chandra RP, Esteghlalian AR, & Saddler JN (2008). Assessing substrate accessibility to enzymatic hydrolysis by cellulases. Characterization of lignocellulosic materials, 60–80
126. Arantes V, Saddler JN (2010) Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnol Biofuels 3(1):4
127. Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol 6(11):850–861
128. Huang R, Su R, Qi W, He Z (2010) Understanding the key factors for enzymatic conversion of pretreated lignocellulose by partial least square analysis. Biotechnol Prog 26(2):384–392

129. Wang QQ, He Z, Zhu Z, Zhang YH, Ni Y, Luo XL, Zhu JY (2012) Evaluations of cellulose accessibilities of lignocelluloses by solute exclusion and protein adsorption techniques. Biotechnol Bioeng 109(2):381–389
130. Harmoko C, Sucipto KI, Retnoningtyas ES, Hartono SB (2016) Vinyl functionalized cubic mesoporous silica nanoparticles as supporting material to enhance cellulase enzyme stability. ARPN J Eng Appl Sci 11:2981–2992
131. Chandra RP, Bura R, Mabee WE, Berlin DA, Pan X, & Saddler JN (2007). Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics?. In Biofuels (pp. 67-93). *Springe*r Berlin Heidelberg
132. Luo X, Zhu JY (2011) Effects of drying-induced fiber hornification on enzymatic saccharification of lignocelluloses. Enzym Microb Technol 48(1):92–99
133. Goufo ED, & Mugisha S (2018). Complex harmonic poles in the evolution of macromolecules depolymerization. J Comput Anal Appl, 1490
134. Yücel Y, Göycıncık S (2015) Optimization and modelling of process conditions using response surface methodology (RSM) for enzymatic saccharification of spent tea waste (STW). Waste and Biomass Valorization 6(6):1077–1084
135. Zhao X, Li S, Wu R, & Liu D (2017). Organosolv fractionating pre-treatment of lignocellulosic biomass for efficient enzymatic saccharification: chemistry, kinetics, and substrate structures. Biofuels, Bioproducts and Biorefining
136. Karimi K, Taherzadeh MJ (2016) A critical review on analysis in pretreatment of lignocelluloses: degree of polymerization, adsorption/desorption, and accessibility. Bioresour Technol 203:348–356
137. Leu SY, Zhu JY (2013) Substrate-related factors affecting enzymatic saccharification of lignocelluloses: our recent understanding. Bioenergy Res 6(2):405–415
138. Zhu JY, Pan X, Zalesny RS (2010) Pretreatment of woody biomass for biofuel production: energy efficiency, technologies, and recalcitrance. Appl Microbiol Biotechnol 87(3):847–857
139. Chen JH, Wang K, Xu F, Sun RC (2015) Effect of hemicellulose removal on the structural and mechanical properties of regenerated fibers from bamboo. Cellulose 22(1):63–72
140. Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M (2011) Deactivation of cellulases by phenols. Enzym Microb Technol 48(1):54–60
141. Ek R, Alderborn G, & Nyström C (1994). Particle analysis of microcrystalline cellulose: differentiation between individual particles and their agglomerates. Int J Pharm, 111(1), 43–50
142. Simons FL (1950) A stain for use in the microscopy of beaten fibers. Tappi 33(7):312–314
143. Yu X, Minor JL, Atalla RH (1995) Mechanism of action of Simons' stain. TAPPI J 78(6):175–180
144. Berodier E, Bizzozero J, & Muller AC (2016). Mercury intrusion porosimetry. A Practical Guide to Microstructural Analysis of Cementitious Materials, 419
145. Orsolini P, Michen B, Huch A, Tingaut P, Caseri WR, Zimmermann T (2015) Characterization of pores in dense nanopapers and nanofibrillated cellulose membranes: a critical assessment of established methods. ACS Appl Mater Interfaces 7(46):25884–25897
146. Groen JC, Peffer LA, Pérez-Ramírez J (2003) Pore size determination in modified micro-and mesoporous materials. Pitfalls and limitations in gas adsorption data analysis. Microporous Mesoporous Mater 60(1–3):1–17
147. Ciesielski PN, Matthews JF, Tucker MP, Beckham GT, Crowley MF, Himmel ME, Donohoe BS (2013) 3D electron tomography of pretreated biomass informs atomic modeling of cellulose microfibrils. ACS Nano 7(9):8011–8019
148. Pu Y, Meng X, Yoo CG, Li M, & Ragauskas AJ (2016). Analytical methods for biomass characterization during pretreatment and bioconversion. Valorization of lignocellulosic biomass in a biorefinery: from logistics to environmental and performance impact New York: *Nova Science Publishers*, 37–78
149. McIntosh R, Nicastro D, Mastronarde D (2005) New views of cells in 3D: an introduction to electron tomography. Trends Cell Biol 15(1):43–51
150. Hildebrand T, Rüegsegger P (1997) A new method for the model-independent assessment of thickness in three-dimensional images. J Microsc 185(1):67–75
151. Reishofer D, & Spirk S (2015). Deuterium and cellulose: a comprehensive review. In Cellulose Chemistry and Properties: Fibers, Nanocelluloses and Advanced Materials (pp. 93-114*)*. Springer, Cham
152. Lindh EL, Salmén L (2017) Surface accessibility of cellulose fibrils studied by hydrogen–deuterium exchange with water. Cellulose 24(1):21–33
153. Licari A, Monlau F, Solhy A, Buche P, Barakat A (2016) Comparison of various milling modes combined to the enzymatic hydrolysis of lignocellulosic biomass for bioenergy production: glucose yield and energy efficiency. Energy 102:335–342
154. da Silva ASA, Teixeira RSS, de Oliveira Moutta R, Ferreira-Leitão VS, de Barros RDRO, Ferrara MA, & da Silva Bon EP (2013). Sugarcane and woody biomass pretreatments for ethanol production. In Sustainable degradation of lignocellulosic biomass-techniques, applications and commercialization. In*Tech*
155. Lin Z, Huang H, Zhang H, Zhang L, Yan L, Chen J (2010) Ball milling pretreatment of corn stover for enhancing the efficiency of enzymatic hydrolysis. Appl Biochem Biotechnol 162(7):1872–1880
156. Hideno A, Inoue H, Tsukahara K, Fujimoto S, Minowa T, Inoue S, Sawayama S (2009) Wet disk milling pretreatment without sulfuric acid for enzymatic hydrolysis of rice straw. Bioresour Technol 100(10):2706–2711
157. Herrera A, Téllez-Luis SJ, Ramırez JA, Vázquez M (2003) Production of xylose from sorghum straw using hydrochloric acid. J Cereal Sci 37(3):267–274
158. Trajano HL, & Wyman CE (2013). Fundamentals of biomass pretreatment at low pH. Aqueous pretreatment of plant biomass for biological and chemical conversion to fuels and chemicals, 103–128
159. Alonso DM, Gallo JMR, Mellmer MA, Wettstein SG, Dumesic JA (2013) Direct conversion of cellulose to levulinic acid and gamma-valerolactone using solid acid catalysts. Catal Sci Technol 3(4):927–931
160. Battista OA (1950) Hydrolysis and crystallization of cellulose. Ind Chemistry 42(3):502–507
161. Kumar R, Mago G, Balan V, Wyman CE (2009) Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. Bioresour Technol 100(17):3948–3962
162. Meng X, Pu Y, Yoo CG, Li M, Bali G, Park DY et al (2017) An in-depth understanding of biomass recalcitrance using natural poplar variants as the feedstock. ChemSusChem 10(1):139–150
163. Aguilar R, Ramırez JA, Garrote G, Vázquez M (2002) Kinetic study of the acid hydrolysis of sugar cane bagasse. J Food Eng 55(4):309–318
164. Öhgren K, Galbe M, Zacchi G (2005) Optimization of steam pretreatment of SO2-impregnated corn stover for fuel ethanol production. Appl Biochem Biotechnol 124(1–3):1055–1067
165. Excoffier G, Toussaint B, Vignon MR (1991) Saccharification of steam-exploded poplar wood. Biotechnol Bioeng 38(11):1308–1317
166. Du B, Sharma LN, Becker C, Chen SF, Mowery RA, van Walsum GP, & Chambliss CK (2010). Effect of varying feedstock–pretreatment chemistry combinations on the formation and accumulation of potentially inhibitory degradation products in biomass hydrolysates. Biotechnol Bioeng, 107(3), 430–440

167. Donohoe BS, Decker SR, Tucker MP, Himmel ME, Vinzant TB (2008) Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. Biotechnol Bioeng 101(5):913–925
168. Soto ML, Dominguez H, Nunez MJ, Lema JM (1994) Enzymatic saccharification of alkali-treated sunflower hulls. Bioresour Technol 49(1):53–59
169. Monte JR, Brienzo M, Milagres AMF (2011) Utilization of pineapple stem uice to enhance enzyme-hydrolytic efficiency for sugarcane bagasse after an optimized pre-treatment with alkaline peroxide. Appl Energy 88(1):403–408
170. Goshadrou A, Lefsrud M (2017) Synergistic surfactant-assisted [EMIM] OAc pretreatment of lignocellulosic waste for enhanced cellulose accessibility to cellulase. Carbohydr Polym 166:104–113
171. Walker LP, Wilson DB (1991) Enzymatic hydrolysis of cellulose: an overview. Bioresour Technol 36(1):3–14
172. Lu Y, Yang B, Gregg D, Saddler JN, Mansfield SD (2002) Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. Appl Biochem Biotechnol 98(1–9):641–654
173. Mooney CA, Mansfield SD, Touhy MG, Saddler JN (1998) The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods. Bioresour Technol 64(2):113–119