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REVIEW ARTICLE

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# Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview

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**Abstract** In nature, cellulose, lignocellulose and lignin are major sources of plant biomass; therefore, their recycling is indispensable for the carbon cycle. Each polymer is degraded by a variety of microorganisms which produce a battery of enzymes that work synergically. In the near future, processes that use lignocellulolytic enzymes or are based on microorganisms could lead to new, environmentally friendly technologies. This study reviews recent advances in the various biological treatments that can turn these three lignicellulose biopolymers into alternative fuels. In addition, biotechnological innovations based on natural delignification and applied to pulp and paper manufacture are also outlined.

**Keywords** Cellulose · Hemicellulose · Lignin · Biodegradation

## Introduction

Lignocellulose, the major component of biomass, makes up about half of the matter produced by photosynthesis. It consists of three types of polymers – cellulose, hemicellulose, and lignin – that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages. A great variety of fungi and bacteria can fragment these macromolecules by using a battery of hydrolytic or oxidative enzymes. In native substrates, binding of the polymers hinders their biodegradation. Molecular genetics of cellulose-, hemicellulose- and lignin-degrading systems advanced considerably during the 1990s. Most of the enzymes have been cloned, sequenced, and expressed both in homologous and in heterologous hosts. Much is known about the structure, genomic organization, and regulation of the genes encoding these proteins. It is beyond the scope of this review, however, summarize these findings. Our purpose is to provide an overview of the degradation of cellulose, hemicellulose, and lignin and the enzymatic systems involved. For a better understanding of enzymology and degradation, we will first briefly describe the structure of the cell wall and its components.

Lignocelluloses in nature derive from wood, grass, agricultural residues, forestry wastes and municipal solid wastes. Several biological methods for lignocellulose recycling, based on the enzymology of cellulose-, hemicellulose- and lignin degradation, have been suggested. Among them, compostage and their use as raw material for the production of ethanol as an alternative combustible seem to be the most economically feasible. Moreover, the general use of alternative, environmentally friendly technologies that introduce lignocellulose enzymes at different stages of pulp and paper manufacture as a pretreatment to pulping (biopulping), bleaching (biobleaching), or wastewater treatment has allowed considerable electrical power savings and a reduction of pollutants in the waste water from these industries. In addition, pretreatment of agricultural wastes with ligninolytic fungi enables their use as raw material for paper manufacturing. The use of microorganisms or their enzymes to enhance the de-inking of recycled fibers and the release of toners from office wastes is another promising field that is under research.

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## Composition of lignocellulose materials and structure of the wood wall

The major component of lignocellulose materials is cellulose, along with lignin and hemicellulose. Cellulose

and hemicellulose are macromolecules from different sugars; whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors. The composition and percentages of these polymers vary from one plant species to another. Moreover, the composition within a single plant varies with age, stage of growth, and other conditions [21]. Long cells enveloped by a characteristic cellular wall form wood. This wall is a complex structure that acts at the same time as plant skin and backbone (Fig. 1).

Cellulose makes up about 45% of the dry weight of wood. This lineal polymer is composed of D-glucose subunits linked by $\beta$-1,4 glycosidic bonds forming cellobiose molecules. These form long chains (called elemental fibrils) linked together by hydrogen bonds and van der Waals forces. Hemicellulose and lignin cover microfibrils (which are formed by elemental fibrils). The orientation of microfibrils is different in the different wall levels. Microfibrils group together to constitute the cellulose fiber. Cellulose can appear in crystalline form, called crystalline cellulose. In addition, there is a small percentage of non-organized cellulose chains, which form amorphous cellulose. In this conformation, cellulose is more susceptible to enzymatic degradation [5]. Cellulose appears in nature associated with other plant substances and this association may affect its biodegradation.

Hemicellulose is a complex carbohydrate polymer and makes up 25–30% of total wood dry weight. It is a polysaccharide with a lower molecular weight than cellulose. It consists of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids. Sugars are linked together by $\beta$-1,4- and occasionally $\beta$-1,3-glycosidic bonds. The principal component of hardwood hemicellulose is glucuronoxylan (Fig. 2A), whereas glucomannan is predominant in softwood (Fig. 2B). Structures of hemicelluloses are described in several reviews [see

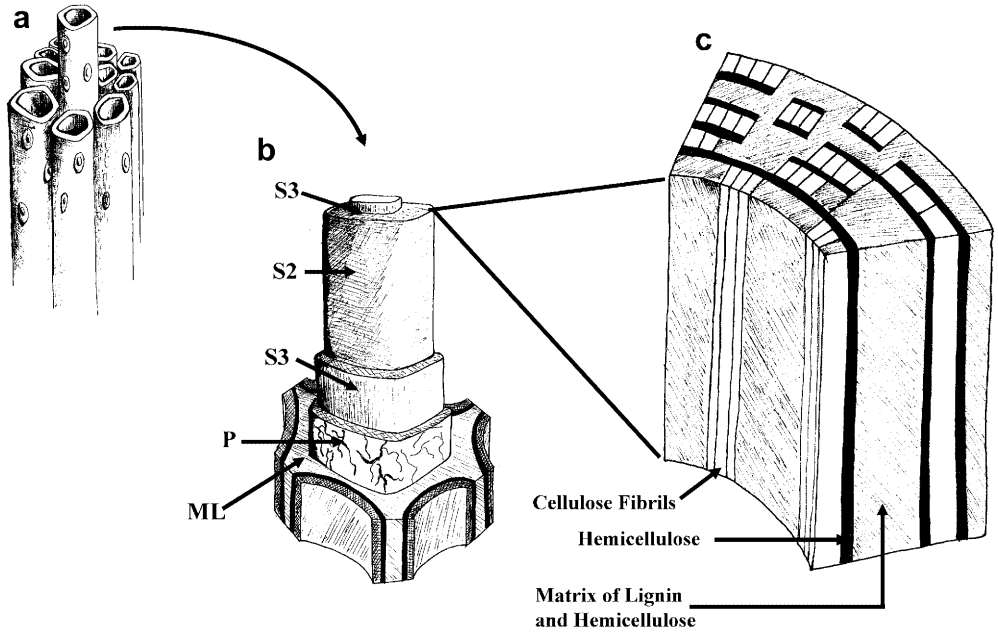



**Fig. 1A–C.** Configuration of wood tissues. **A** Adjacent cells, **B** cell wall layers. *S1, S2, S3* Secondary cell wall layers, *P* primary wall, *ML* middle lamella. **C** Distribution of lignin, hemicellulose and cellulose in the secondary wall. (Adapted from Kirk and Cullen [24])

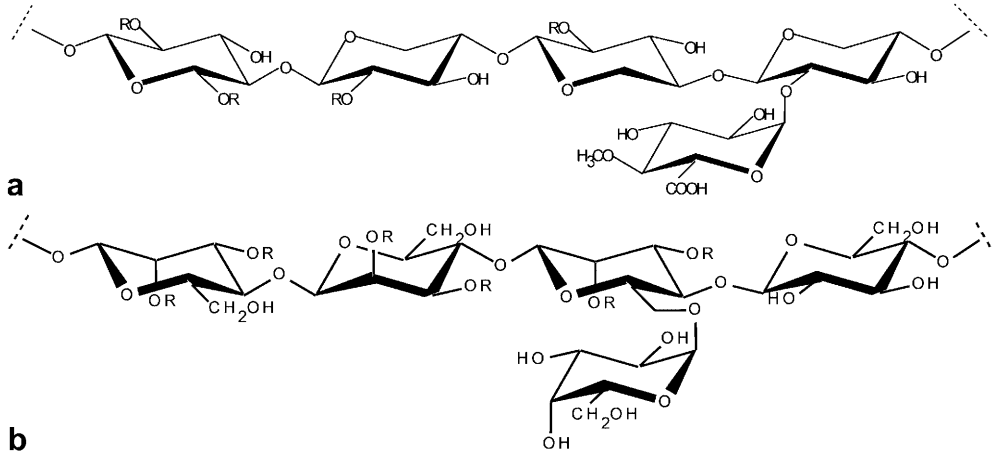



**Fig. 2.** **A** O-acetyl-4-O-methylglucuronoxylan from angiosperms. *R* H or acetyl group. Gymnosperm hemicellulose has the same basic structure but lacks acetyl groups and $\alpha$-1,3-l-arabinose residues. **B** O-acetyl-galactoglucomannan from gymnosperms. *R* H or acetyl group. Angiosperms contain lesser amounts of glucomannan, which always lacks $\alpha$-D-galactose

reference 21]. The main difference with cellulose is that hemicellulose has branches with short lateral chains consisting of different sugars. In contrast to cellulose, they are easily hydrolyzable polymers. They do not form aggregates, even when they are co-crystallized with cellulose chains.

Lignin (along with cellulose) is the most abundant polymer in nature. It is present in the cellular cell wall, conferring structural support, impermeability, and resistance against microbial attack and oxidative stress. Structurally, lignin is an amorphous heteropolymer, non-water soluble and optically inactive; it consists of phenylpropane units joined together by different types of linkages. The polymer is synthesized by the generation of free radicals, which are released in the peroxidase-mediated dehydrogenation of three phenyl propionic alcohols: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (*p*-hydroxyphenyl propanol), and sinapyl alcohol (syringyl propanol). Coniferyl alcohol is the principal component of softwood lignins, whereas guaiacyl and syringyl alcohols are the main constituents of hardwood lignins. The final result of this polymerization is a heterogeneous structure whose basic units are linked by C-C and aryl-ether linkages, with aryl-glycerol $\beta$-aryl ether being the predominant structure (Fig. 3).

The biological degradation of cellulose, hemicellulose, and lignin has attracted the interest of microbiologists and biotechnologists for many years. The diversity of cellulosic and lignicellulosic substrates has contributed to the difficulties found in enzymatic studies. Fungi are the best-known microorganisms capable of degrading these three polymers. Because the substrates are insoluble, both bacterial and fungal degradation have to occur exocellularly, either in association with the outer cell envelope layer or extracellularly. Microorganisms have two types of extracellular enzymatic systems: the hydrolytic system, which produces hydrolases and is responsible for cellulose and hemicellulose degradation; and a unique oxidative and extracellular ligninolytic system, which depolymerizes lignin. A discussion of the biochemistry and genetics of the enzymes involved in cellulosic and lignocellulosic materials is beyond the scope of this review.

## Cellulose biodegradation

Most of the cellulolytic microorganisms belong to eubacteria and fungi, even though some anaerobic protozoa and slime molds able to degrade cellulose have also been described. Cellulolytic microorganisms can establish synergistic relationships with non-cellulolytic species in cellulosic wastes. The interactions between both populations lead to complete degradation of cellulose, releasing carbon dioxide and water under aerobic conditions, and carbon dioxide, methane and water under anaerobic conditions [5,30].

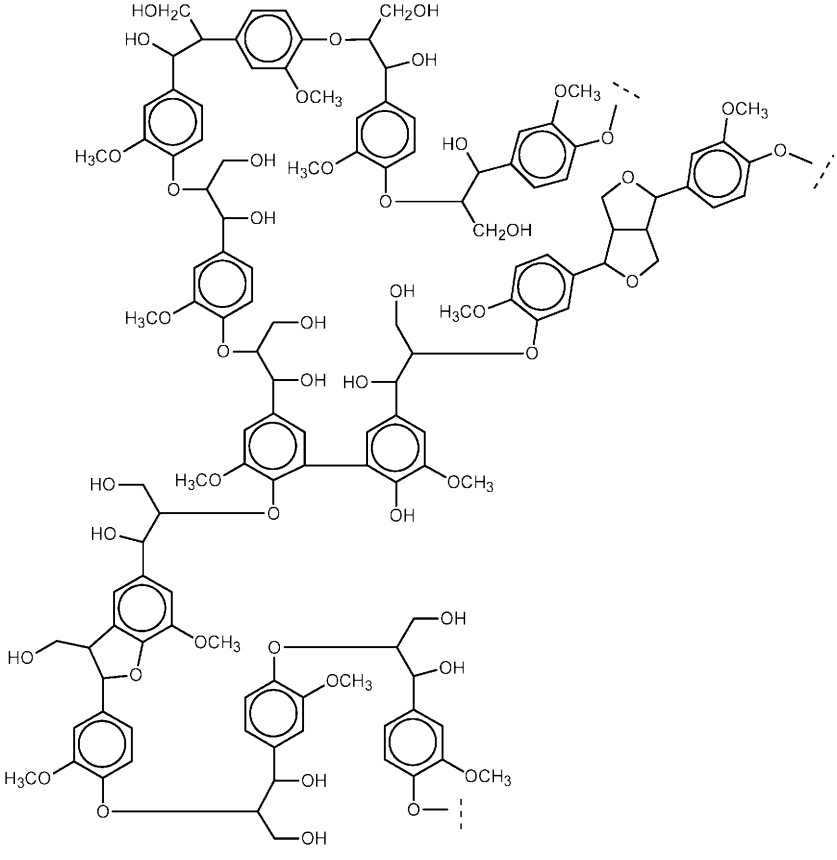


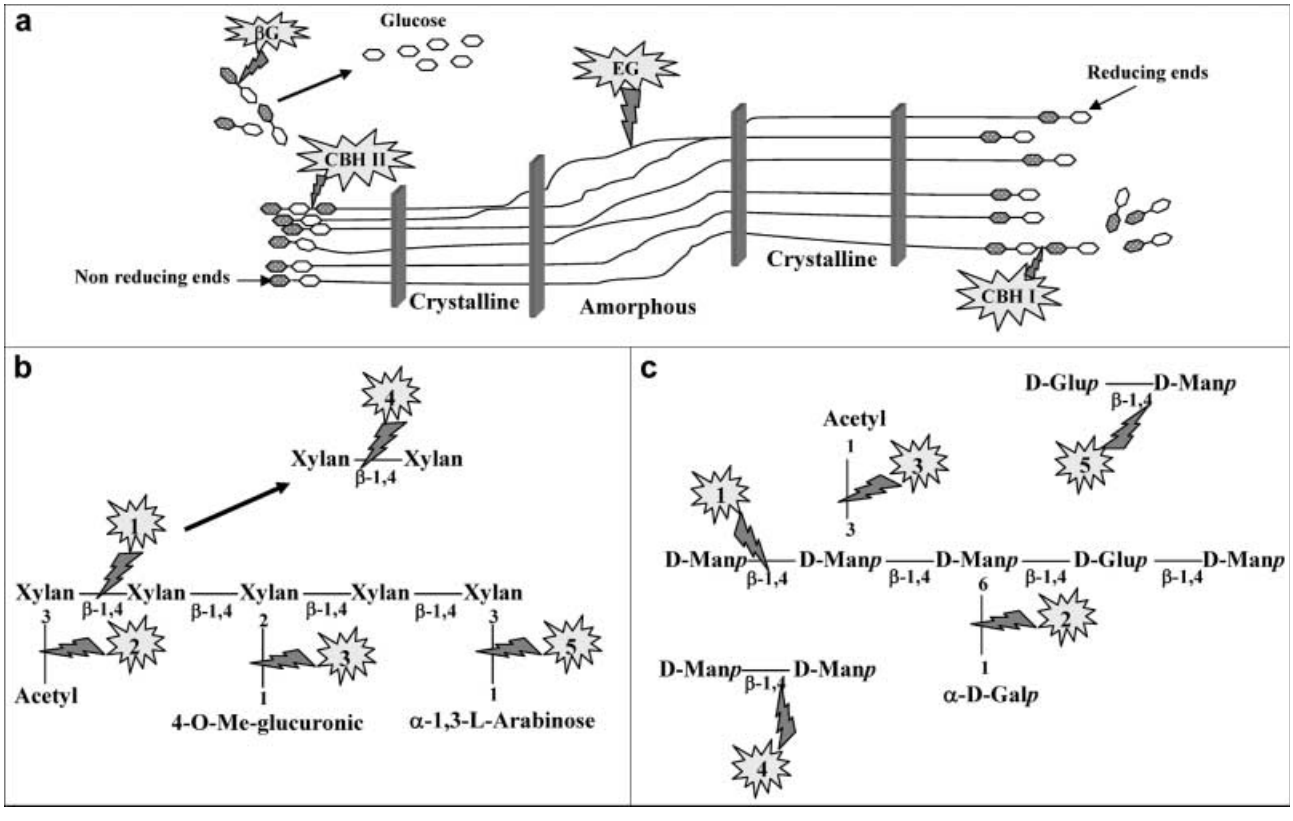

**Fig. 3.** Lignin from gymnosperms showing the different linkages between the phenylpropane units. Angiosperm lignin is very similar, but phenylpropane units contains two methoxyl groups in ortho position to oxygen

Microorganisms capable of degrading cellulose produce a battery of enzymes with different specificities, working together. Cellulases hydrolyze the $\beta$-1,4-glycosidic linkages of cellulose. Traditionally, they are divided into two classes referred to as endoglucanases and cellobiohydrolases. Endoglucanases (endo-1,4-$\beta$-glucanases, EGs) can hydrolyze internal bonds (preferably in cellulose amorphous regions) releasing new terminal ends. Cellobiohydrolases (exo-1,4-$\beta$-glucanases, CBHs) act on the existing or endoglucanase-generated chain ends. Both enzymes can degrade amorphous cellulose but, with some exceptions, CBHs are the only enzymes that efficiently degrade crystalline cellulose. CBHs and EGs release cellobiose molecules. An effective hydrolysis of cellulose also requires $\beta$-glucosidases, which break down cellobiose releasing two glucose molecules (Fig. 4A). Products of cellulose hydrolysis are available as carbon and energy sources for cellulolytic microorganisms or other microbes living in the environment where cellulose is being degraded. In fact, this release of sugars from cellulose is the main basis of microbial interactions occurring in such environments [30]. To function correctly, endoglucanases, exoglucanases and $\beta$-glycosidases must be stable in the exocellular environment and may form a ternary complex with the substrate.

The cellulase systems of the mesophilic fungi *Trichoderma reesei* and *Phanerochaete chrysosporium* are the most thoroughly studied [review in 24]. The EGs of these consortiums have two structural domains: the catalytic domain and the union domain. Their molecular masses range from 25 to 50 kDa, and they have optimum activities at acidic pH. CBHs act synergistically with EGs to solubilize high-molecular-weight cellulose molecules. They are also glycosylated and present an optima activity at acidic pH. *P. chrysosporium* has several $\beta$-glucosidases, whereas only one isoenzyme has been described in *T. reesei*. All of them have a high molecular masses ranging from 165 to 182 kDa.

Several thermophilic fungi can degrade cellulose faster than *T. reesei*. The EGs of thermophilic fungi are thermostable and their molecular masses range from 30 to 100 kDa. They show optimal activity between 55 and 80 °C at pH 5.0–5.5. Exoglucanases (40–70 kDa) are optimally active at 50–75 °C. The molecular characteristics of $\beta$-glucosidases are variable; their molecular masses range from 45 to 250 kDa, optimal pH from 4.1 to 8.1, and optimal temperature from 35 ° to 71 °C [32]. Among aerobic cellulolytic bacteria, species from the genera *Cellulomonas*, *Pseudomonas*, and *Streptomyces* are the best studied [5].

**Fig. 4A–C.** Enzymatic degradation of cellulose to glucose. *CBHI* Cellobiohydrolase I acts on the reducing ends; *CBHII* cellobiohydrolase II acts on the non-reducing ends; *EG* endoglucanases hydrolyze internal bonds. *$\beta$-G* $\beta$-Glucosidase cleaves the cellobiose disaccharide to glucose. **B** Enzymatic degradation of glucuronoxylans. *1* Endoxylanase, *2* acetylxylan-esterase, *3* $\alpha$-glucuronidase. *4* $\beta$-xylosidase, *5* $\alpha$-arabinose (required only to hydrolyze glucuronoxylan from gymnosperms (see Fig. 2A). **C** Enzymatic hydrolysis of glucomannan. *1* Endomannase, *2* $\alpha$-galactosidase (for gymnosperm hemicellulases, see Fig. 2B), *3* acetylglucomannan-esterase, *4* $\beta$-mannosidase, *5* $\beta$-glucosidase



About 5–10% of cellulose is degraded in nature under anaerobic conditions. The cellulose system of anaerobic microorganisms is clearly different from that of aerobic fungi and bacteria. The best characterized is that of *Clostridium thermocellum*, a strict anaerobic gram-positive, sporulated bacterium. In this system, enzymes are organized into large functional entities termed cellulosomes (see below). The organization of enzymes into cellulosome concentrates and positions them in such a manner that promotes synergism among catalytic units. The advantages of these arrangements of cellulolytic enzymes are that, since the cellulosome is attached to the cell surface, the enzymes are located at the interface between the cell and the insoluble substrate. The products of cellulolysis (such as cellobiose) can pass inside the bacterium via extended fibrous materials that are present between the cell and cellulose. However, these microorganisms require high temperatures for growth and cellulose degradation, for this reason they probably play a minor role in cellulose biodegradation in nature.

Several mesophilic cellulolytic anaerobes have been isolated from common environments including soil and sediments, compost, sewage, sludge, and anaerobic digestors [30]. Other well-known anaerobic cellulolytic microorganisms are rumen bacteria, fungi and protozoa, which degrade vast amounts of cellulose [30].

## Hemicellulose biodegradation

Hemicelluloses are biodegraded to monomeric sugars and acetic acid. Hemicellulases are frequently classified according to their action on distinct substrates. Xylan is the main carbohydrate found in hemicellulose. Its complete degradation requires the cooperative action of a variety of hydrolytic enzymes. An important distinction should be made between endo-1,4-$\beta$-xylanase and xylan 1,4-$\beta$-xylosidase. The former generates oligosaccharides from the cleavage of xylan; the latter works on xylan oligosaccharides, producing xylose [21]. In addition, hemicellulose biodegradation needs accessory enzymes such as xylan esterases, ferulic and *p*-coumaric esterases, $\alpha$-l-arabinofuranosidases, and $\alpha$-4-O-methyl glucuronosidases acting synergistically to efficiently hydrolyze wood xylans and mannans [for a review about hydrolysis of other hemicellulases see 24].

In the case of O-acetyl-4-O-methylglucuronxylan, one of the most common hemicelluloses, four different enzymes are required degradation: endo-1,4-$\beta$-xylanase (endoxylanase), acetyl esterase, $\alpha$-glucuronidase and $\beta$-xylosidase (Fig. 4B). The degradation of O-acetylgalactoglucomanann starts with rupture of the polymer by endomannases. Acetylglucomannan esterases remove acetyl groups, and $\alpha$-galactosidases eliminate galactose residues. Finally, $\beta$-mannosidase and $\beta$-glycosidase break down the endomannases-generated oligomers $\beta$-1,4 bonds (Fig. 4C).

Xylanases, the major component of hemicellulases, have been isolated from many ecological niches where plant material is present. Due to the important biotechnological exploitations of xylanases, especially in biopulping and bleaching, many publications have appeared in recent years [27]. The white-rot fungus *Phanerochaete chrysosporium* has been shown to produce multiple endoxylanases [24]. Also, bacterial xylanases have been described in several aerobic species and some ruminal genera [7,27].

Hydrolysis of $\beta$-glycosidic linkages is carried out by acid catalytic reactions common to all glycanases. Many microorganisms contain multiple loci encoding overlapping xylanolytic functions. Xylanases, like many other cellulolytic and hemicellulolytic enzymes, are highly modular in structure. They consist of either a single domain or a number of different domains, classified as catalytic and non-catalytic domains. Based on the homology of the conserved amino acids, xylanases can be grouped into two different families: family 10 (F), with relatively high molecular weight, and family 11 (G), with lower molecular weight. The catalytic domains for the two families differ in their molecular masses, net charge and isoelectric points [27] and may play a major role in determining specificity and reactivity. Biochemically and structurally, the two families are unrelated. The release of reducing sugars from purified xylan is highly dependent on the xylanase pI. Isoelectric points for endoxylanases from various microorganisms vary from 3 to 10. Optimum temperature for xylanases from bacterial and fungal origin ranges from 40 to 60 °C. Fungal xylanases are generally less thermostable than bacterial xylanases.

Researchers have paid special attention to thermostable hemicellulases because of their biotechnological applications (see below). Thermophilic xylanases have been described in actinobacteria (formerly actinomycetes) such as *Thermomonospora* and A*ctinomadura* [14]. Also, a very thermostable xylanase has been isolated from the hyperthermophilic primitive bacterium *Thermotoga* [45]. Xylanases of thermophilic fungi are also receiving considerable attention. As in mesophilic fungi, a multiplicity of xylanases differing in stability, catalytic efficiency, and activity on substrates has been observed [32]. The optimal temperatures vary from 60 to 80 °C and the pI ranges from 3.7 to 9.0. This diversity of xylanase isoenzymes of different molecular masses might be to allow their diffusion into the plant cell walls.

The use of xylanases in bleaching pulps has stimulated the search for enzymes with alkaline pH optima. Most xylanases from fungi have pH optima between 4.5 and 5.5. Xylanases from actinobacteria are active at pH 6.0–7.0 [21]. However, xylanases active at alkaline pH have been described from *Bacillus* sp. or S*treptomyces viridosporus* [7]. Genes encoding several xylanases have been cloned in homologous and heterologous hosts in order to overproduce the enzyme with the goal of altering its properties so that it can be used for commercial applications [27].

β-Xylosidases are less common than endoxylanases. Most of them are cell-bound and larger than xylanases. They have been described in several fungi such as *T. reesei* and *P. chrysosporium* with molecular masses ranging from 90 to 122 kDa, and most have acidic pH optima and pIs [24]. β-Xylosidases have also been described in *B. stearothermophilus* and the ruminal bacterium *Butyrivibrio fibrisolvens* [20]. Glucomannan-degrading enzymes have been described less frequently than xylanases. Several microbial mannanases from gram-positive and gram-negative bacteria have been studied [41]. Endomananases have also been described in white-rot fungi and in ascomycetes [24]. The most thoroughly studied α-galactosidases and acetylglucomanane esterases are those from the genus *Aspergillus*, while the best-studied β-mannonidases are from the fungi *Polyporus sulfureus* and *A. niger* [24]. To our knowledge, no α-galactosidases or β-manosidases have been isolated to date from white-rot fungi.

## Lignin biodegradation

The structural complexity of lignin, its high molecular weight and its insolubility make its degradation very difficult. Extracellular, oxidative, and unspecific enzymes that can liberate highly unstable products which further undergo many different oxidative reactions catalyze the initial steps of lignin depolymerization. This non-specific oxidation of lignin has been referred to as "enzymatic combustion" [25]. White-rot fungi are the microorganisms that most efficiently degrade lignin from wood. Of these, *Phanerochaete chrysosporium* is the most extensively. For recent reviews on lignin biodegradation by white-rot fungi and advances in the molecular genetic of ligninolytic fungi, see [10,50].

Two major families of enzymes are involved in ligninolysis by white-rot fungi: peroxidases and laccases. Apparently, these enzymes act using low-molecular-weight mediators to carry out lignin degradation. Several classifications of fungi have been proposed based on their ligninolytic enzymes. Some of them produce all of the major enzymes, others only two of them, or even only one. In addition, reductive enzymes including cellobiose oxidizing enzymes, aryl alcohol oxidases, and aryl alcohol dehydrogenases seem to play major roles in ligninolysis [10].

Two groups of peroxidases, lignin peroxidases (LiPs) and manganese-dependent peroxidases (MnPs), have been well-characterized. LiP has been isolated from several white-rot fungi. The catalytic, oxidative cycle of LiP has been well-established and is similar to those of other peroxidases. In most fungi, LiP is present as a series of isoenzymes encoded by different genes. LiP is a glycoprotein with a heme group in its active center. Its molecular mass ranges from 38 to 43 kDa and its pI from 3.3 to 4.7. So far, it is the most effective peroxidase and can oxidize phenolic and non-phenolic compounds, amines, aromatic ethers, and polycyclic aromatics with appropriate ionization potential [24]. Since LiP is too large to enter the plant cell, it degradation is carried out only in exposed regions of lumen. This kind of degradation is found in simultaneous wood decays. However, microscopic studies of selective lignin biodegradation reveal that white-rot fungi remove the polymer from inside the cell wall. An indirect oxidation by LiP of low-molecular-weight diffusible compounds capable of penetrating the cell wall and oxidizing the polymer has been suggested. However, this theory lacks evidence since low-molecular-weight intermediates such as veratryl alcohol cation radical are too short-lived to act as mediators [23]. MnPs are molecularly very similar to LiPs and are also glycosylated proteins, but they have slightly higher molecular masses, ranging from 45 to 60 kDa. MnPs oxidize Mn(II) to Mn(III). They have a conventional peroxidase catalytic cycle, but with Mn(II) as substrate. This Mn(II) must be chelated by organic acid chelators, which stabilize the product Mn(III) [40]. Mn(III) is a strong oxidant that can leave the active center and oxidize phenolic compounds, but it cannot attack non-phenolic units of lignin. MnP generates phenoxy-radicals which in turn undergo a variety of reactions, resulting in depolymerization [16]. In addition, MnP oxidizes non-phenolic lignin model compounds in the presence of Mn(II) via peroxidation of unsaturated lipids [24]. A novel versatile peroxidase (VP), which has both manganese peroxidase and lignin peroxidase activities and which is involved in the natural degradation of lignin has been described [8]. VP can oxidize hydroquinone in the absence of exogenous $H_2O_2$ when Mn(II) is present in the reaction. It has been suggested that chemical oxidation of hydroquinones promoted by Mn(II) could be important during the initial steps of wood biodegradations because ligninolytic enzymes are too large to penetrate into non-modified wood cell walls [17].

Laccases are blue-copper phenoloxidases that catalyze the one-electron oxidation mainly of phenolic compounds and non-phenolics in the presence of mediators [15]. The phenolic nucleus is oxidized by removal of one electron, generating phenoxy-free-radical products, which can lead to polymer cleavage. Wood-rotting fungi are the main producers of laccases but this oxidase has been isolated from many fungi including *Aspergillus* and the thermophilic fungi *Myceliophora thermophila* and *Chaemotium thermophilium* [29]. Recently, bacterial laccases-like proteins have been found [3]. These enzymes polymerized a low-molecular-weight, water-soluble organic matter fraction isolated from compost into high-molecular-weight products, suggesting the involvement of laccase in humification during composting [32]. The role of laccases in lignin biodegradation has been discussed recently [29].

The potential biotechnological applications of white-rot fungi or their ligninolytic enzymes are many. The most promising applications may be biopulping and bleaching of chemical pulps (see below). White-rot fungi can degrade/mineralize a wide variety of toxic

xenobiotics including polycyclic aromatic hydrocarbons, chlorophenols, nitrotoluenes, dyes, and polychlorinated and biphenyls [for a review see 42]. An obvious application is in situ bioremediation of contaminated soils [39]. Other fields under research are the use of these fungi for biocatalysis in the production of fine chemicals and natural flavors (e.g. vanillin), and the biotreatment of several waste waters such as bleach plant effluents (see below) or other waste water containing lignin-like polymers, as is the cases of dye-industry effluents and olive-oil-mill waste-waters [33].

Degradation of lignin and lignin-degrading enzymes hasalso been reported for actinobacteria from the *Streptomyces* genus [6]. Even though lignin biodegradation is accepted as an aerobic process, some authors have reported that anaerobic microorganisms in the rumen may alter, if not partially degrade, portions of lignified plant cells [2].

## Biological treatments of cellulosic and lignocellulosic materials

### Biotransformation of lignocellulosic biomass into alternative fuels

Obtaining ethanol as an alternative fuel using cellulose and lignocellulosic residues as a raw material has been strongly considered, especially after the fossil fuel crisis. World-wide, the conversion of starch from corn and other crops into ethanol is one of the largest-scale applications of biotechnology. Ethanol blended with gasoline (10:90) reduces carbon monoxide emissions. Lignocellulosic biomass offers a feedstock lower in price than starch. Over the last 20 years much progress has been made in the enzymatic conversion of lignocelluloses into ethanol, and the price of this product has dropped so much that nowadays ethanol can compete with gasoline. The transformation of lignocellulose into ethanol is completed in two steps: (1) hydrolysis of the polymer, delignification to liberate cellulose and hemicellulose from their complex with lignin, and depolymerization of carbohydrate polymer to produce free sugars; and (b) fermentation to ethanol using pentoses and hexoses liberated in the first step. In conventional processes, lignins present in the raw materials and releasing fermentable sugars are eliminated by chemical and/or thermic pretreatment followed by enzymatic/acidic hydrolysis. However, biological treatments have been proposed either to replace the physicochemical treatment or for detoxification or specific removal of inhibitors prior to fermentation. The aforementioned white-rot fungi or other ligninolytic microorganisms including *Streptomyces* can achieve delignification. The main advantages of biological delignification include mild reaction conditions, higher product yields, fewer side reactions and less energy demand [28]. An attractive alternative to the two-step bioconversion is simultaneous saccharification and fermentation [9], in which hydrolytic enzymes (e.g. *T. reesei* cellulases) and fermentative microorganism(s) are present in the reactor.

When the substrate is lignocellulose, the use of xylan is essential for biological transformation. Microbial xylanases catalyze xylan hydrolysis to xylose in a mild reaction with negligible substrate loss. However the enzymatic treatment is expensive and is one of the factors limiting enzymatic hydrolysis.

Xylose and other hemicellulose sugars are easily liberated by acid hydrolysis. Even thought they make up only 15% of total wood sugars, they comprise 45% of hydrolysis-recoverable sugars. For many years, the conversion of sugars into ethanol was limited to hexoses because xylose is a hardly fermentable sugar. Xylose can be fermented into ethanol or xylitol (which is used as a sweetener). In most yeast, the metabolism of xylose requires aerobic conditions to stimulate ethanol production and even then the resulting ethanol levels are very low. A recent breakthrough in this respect is the development of improved strains of fermentative microorganisms capable of fermenting pentose and hexose sugars into ethanol [22].

During the 1990s, many laboratories in the world put their efforts into obtaining the production of ethanol directly from lignocellulosic materials using anaerobic thermophilic microorganisms, *Clostridium thermocellum* being the best-studied. The great advantage of using these bacteria is that they hydrolyze cellulose and, at the same time, also ferment the resulting sugars directly into ethanol. The fact that *C. thermocellum* is a thermophilic bacterium offers several additional advantages, such as higher growth rates, higher metabolic activities, and increased enzyme stability. In addition, recovery of the products is easier. The cellulosome of *C. thermocellum* (Fig. 5) contains different types of glycosyl hydrolases, including cellulases, and hemicellulases, all of which are bound to a major polypeptide called scaffoldin or CipA (for cellulosome-integrating protein). Scaffoldin contains several functional modules. One of them is a single cellulose-binding domain (CBD), and there are nine repeating domains, called cohesins, which interact with cellulosomal enzymes. In addition, scaffoldin has a domain that allows it to attach to the cell surface [44]. However, wild-type strains have a limited ethanol tolerance. Genetic engineering will help to alleviate this problem [44].

Anaerobic digestion to convert lignocellulosic biomass into methane has been assayed; however, further advances in this technology, such as enhancing the enzyme levels for the breakdown of cellulose into fermentable sugars, and improved organisms that tolerate wider pH differences, are required to achieve economic potential [51].

### Compostage of lignocellulosic materials

Compostage is a dynamic procedure in which the synergistic action of a variety of microorganisms is needed for

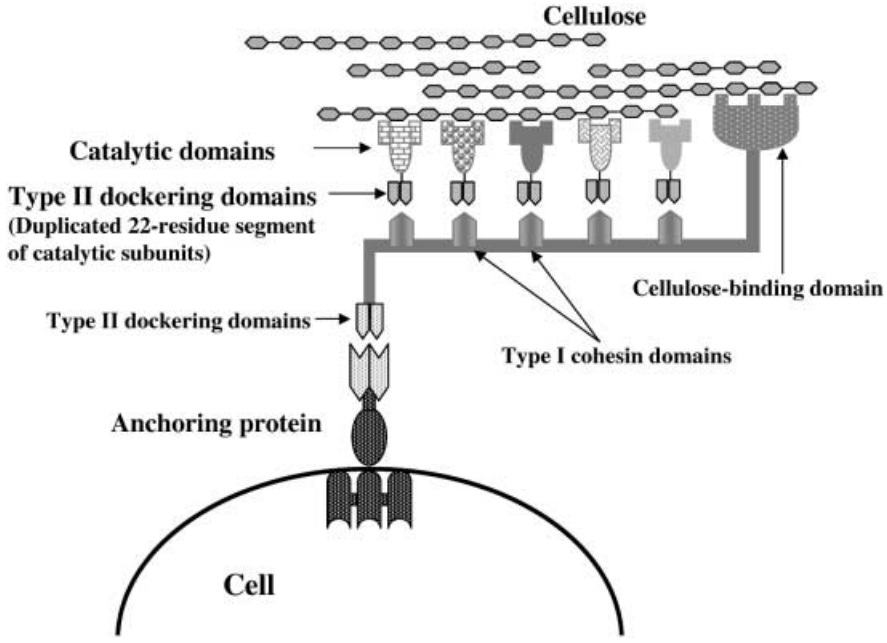



**Fig. 5.** Interaction between the cellulosome and cellulose. The association with the bacterial cell (*Clostridium thermocellum*) takes place through an anchoring protein

recycling lignocellulosic materials. The capacities of microorganisms to assimilate complex carbohydrates, such as cellulose, hemicellulose and lignin, depend on the ability to produce the enzymes described above. The lignocellulosic compost microbiota have been recently reviewed by Tuomela et al.[47]. Populations growing in compost piles consist mainly of bacteria (including actinobacteria) and fungi. Polymers such as hemicellulose, cellulose, and lignin are only degraded once the more easily degradable compounds have been consumed. Afterwards, the lignocellulosic materials are partly transformed into humus.

According to Tuomela et al. [47], at the beginning of the composting mesophilic bacteria are predominant. Once the temperature has reached more than 40 °C, thermophilic bacteria and fungi appear in the compost piles. When the temperatures reaches more than 60 °C, the microbial activity decrease, and as the compost pile cools, mesophilic bacteria appear again. Except for 1% of anaerobic bacteria in the compost process, which carry out much of the cellulolytic activity and so play a major role in the biodegradation of lignocellulosic materials, most biodegradation is carried out under aerobic conditions.

Among the aerobic bacteria that have been isolated from compost, actinobacteria probably play a major role in the degradation of lignocelluloses. These bacteria can degrade cellulose and solubilize lignin. However, it seems that thermophilic and thermotolerant fungi, which are known to have cellulolytic and ligninolitic activity, are critical to the degradation of lignocellulosic materials in compost. In compostage processes, white-rot fungi have been isolated only during the cooling and maturation phases. Some authors believe that, even if these fungi are very important in the degradation of lignin in nature, they must not be involved in degrading of this polymer in compost environments.

## Biotreatment of pulp and paper waste waters

In the last few years environmental protection agencies have become more restrictive regarding air and water discharges from chemical pulp and paper mills. The reductions of contaminants affects all stages of the paper industry, including pulping, bleaching, and papermaking. Pulping processes release colored compounds such as residual lignins, carbohydrate degradation products, and extractives (lipophilic compounds) into the effluent streams. The removal of this residual lignin can significantly reduce the volume of the chemicals used for bleaching, thus lowering the amount of hazardous compounds formed in bleach-plant waste waters. Residual lignins in pulps are the cause of their typical dark brownish color, which must be removed by multistage bleaching. The use of elemental chlorine for bleaching leads to environmental problems including release of toxic and recalcitrant chlorinated aromatics such as dioxins. The number of paper industries using chlorine dioxide in elemental chlorine-free (ECF) bleaching is decreasing, especially in Europe. Totally chlorine-free (TCF) output is estimated as 15% of total production. TCF pulp production is growing slowly. Alternative chemicals for bleaching have been used, e.g. oxygen, hydrogen peroxide, and ozone. These compounds have some disadvantages, and the paper loses quality during the bleaching. Alternatively, environmentally friendly technologies, based on the capacity of microorganisms to degrade cellulose, hemicellulose, or lignin, have been developed.

Present-day treatments of pulp and paper-mill effluents were reviewed by Ali and Sreekrishnan [4] and by Thompson et al. [46]. All pulp and paper mills have facilities to assure compliance with the regulations established by environmental agencies in their own coun-

try. Even so, the residual waters impose coloration and toxicity problems on the receiving waters, causing environmental hazards. Currently, most paper industries use the classical primary and secondary biological treatments. Almost half the paper industries use the aerobic activated-sludge method for the secondary treatment. Some industries apply physical and chemical tertiary treatments, especially to remove the residual dark color; but these treatments are expensive and ineffective.

The dark color of these effluents is mainly due to lignin and its derivatives, released during the different stages of pulp and paper-making processes. Lignin is converted into thio- and alkali-lignin during the kraft process, and to lignosulfonates in the sulfite process. Chlorolignins are the main byproducts from chemical wood-pulp bleaching. These compounds of high molecular weight are not degraded by any of the above-described biological treatments, and their final fates are unknown. The discharge of these colored wastes is not only a problem of aesthetics, but it has also been proven that chlorolignins are toxic and mutagenic [4]. Physical and chemical processes to remove the dark color are expensive and do not solve the problem because the lignins persist, although in different form. An alternative treatment is the use of ligninolytic microorganisms [13]. Several species of bacteria have been studied for their decolorization abilities. While some of them are able to decolorize, only a few strains metabolize lignin derivatives [37]. Color removal was efficiently achieved by employing the FPL/NCSU Mycor method, which uses *Phanerochaete chrysosporium* in rotating biological contactors [12]. A later modification of this method, termed MYCOPOR, was developed by Messner et al. [34]. Biological decolorization of paper mill wastes using either fungal mycelia, pellets, or immobilized cells was achieved with different strains [4,33]. Also, soluble or immobilized ligninolytic enzymes can be employed for effluent decolorization [36]. In addition to white-rot fungi, other strains evaluated for the decolorization and decontamination of kraft effluents include ascomycetes and *Thermoascus aurantiacus* [31,43].

## Fungal and enzymatic treatments of wood chips and pulps: biopulping and biobleaching

Biopulping is defined as the appropriate treatment of wood chips with lignin-degrading fungi prior to pulping. This biotreatment not only reduces energy consumption in the process of pulping, but also improves paper strength and removes wood extractives, leading to additional benefits such as fewer pitch problems and lower effluent toxicity [1]. The pulp and paper industry uses mechanical or chemical pulping processes or a combination of both to produce pulps. Mechanical pulping involves mechanical force to separate wood fibers. With this method, the yield is high, and the paper produced is of good quality. In chemical pulping processes the yield is low, but the pulp produced has a higher strength.

Pretreatments of wood chips for mechanical and chemical pulping with several white-rot fungi have been developed in several laboratories [1]. Biomechanical pulping using these microorganism has proven to be feasible from both the engineering and the economic point of view. Global concerns for the preservation of forests and the elimination of environmental pollution from pulp and paper industries have focused research on alternative fibrous resources for papermaking, such as non-woody plants or agricultural residues. Biopulping of non-woody plants with *C. subvermispora*, *Pleurotus* and other basidiomycetes reduces the amount of electrical power used for the refining stage by as much as 30%and improves paper properties [6,11].

Fungal pretreatment removes some and/or modifies; these changes make it easier to remove macromolecules, probably because they improve chemical penetration and so minimize the use of chemicals. Biopulping also benefits other pulping techniques such as sulfite, organosolv and dissolving pulp production. Fungal treatment prior to kraft pulping have received little attention. However, several studies indicate that biokraft pulping with white-rot fungi increases pulp yield and strength and reduces the cooking time during kraft pulping [1,35]. Taken together, the data suggest that biopulping could be an environmental friendly and cost-effective alternative to current pulping methods.

Bleaching of pulps using enzymes or ligninolytic fungi is known as biobleaching. The use of ligninolytic enzymes and hemicellulases aids in pulp bleaching and decreases the amount of chemical bleach required to obtain a desirable brightness of pulps. Vikarii et al. [48] were the first to show that treating kraft pulps with fungal hemicellulases reduces subsequent chlorine bleaching requirements. Other authors have confirmed these studies. Xylanase treatments decrease chlorine demand for kraft pulp by 6–15% [26]. In recent years at least 15 patents dealing with enzymatic treatments to enhance kraft pulps have been released. There are different hypotheses to explain the mechanism of hemicellulose prebleaching. One of them suggests that the hydrolysis of relocated and reprecipitated xylan on the surface of pulp fibers renders the pulp more permeable, thereby enhancing residual lignin extraction. The second model suggests that lignin or chromophores generated during kraft cooking react with carbohydrates moieties. Hemicellulases liberate lignin by releasing xylan-chromophore fragments and lignin extraction increases. Mannanases interact synergistically with xylanases and improve biobleaching, especially in softwood. Although promising results were obtained initially, mannanases seem to be less effective than xylanases [49]. Several xylanases from different microorganisms have proven to be effective for biobleaching of various kinds of pulps. On a laboratory scale, xylanases from fungi and bacteria have been assayed [27]. Most commercial preparations contain xylanases from different *Trichoderma* species.

Ligninolytic fungi have also been evaluated for biobleaching. The ligninolytic system seems to be involved in the process, but low-molecular-weight cofactors such as veratryl alcohol for LiP, manganese and organic acids for MnP, and *n*-substituted aromatic for laccase have to be added. Several fungi are effective in biobleaching; among these, very good results have been reported with *Trametes versicolor* [37], *Bjerkandera* sp., *Phlebia radiata* and *Lentinus tigrinus* [36]. Laccase and MnP seem to play a primary role in biobleaching, while the role of LiP remains unclear because this enzyme has not been detected during the process. Also, lignocellulosic enzymes, such as endoglucanases from *Paenibacillus* sp., have been applied to biorefining as an alternative to mechanical refining [38].

### Enzymatic deinking and pitch control

Paper and paperboard are the most important recyclable materials in Europe. About 40% of the total production of both materials is actually recycled and the proportion is growing. In Spain, 81% of the total raw material for papermaking is recycled paper. Printing and writing-grade papers amount to about 20 million metric tons per year in the United States [26]. Waste papers contain toner and other inks. These are synthetic polymers that do not disperse during conventional repulping. Conventional deinking uses surfactants to float the toner followed by high temperatures to aggregate it, and then vigorous dispersion for size reduction. Those processes are expensive and energy-consuming. Cellulases and xylanases release toner particles facilitating flotation and subsequent steps. This enzymatic method has been scaled up and has proven to be economically feasible [19].

The term "pitch" is applied to both wood extractives and the deposits that these extractives cause during pulp and paper manufacture. The toxicity of pitch constituents is well-established and its extractable fraction is currently the source of a major environmental problem in pulp and paper waste waters. Several ligninolytic fungi have been assayed for biotechnological control of pitch. From the point of view of industrial applicability, the most promising ones are *Phlebia radiata* and *Poria subvermispora* [18].

## Conclusions

This literature survey has shown that a variety of microorganisms can degrade the three major natural biopolymers that make up lignocellulosic materials: cellulose, hemicellulose, and lignin. Many laboratories around the world are involved in research on the different aspects of natural biodegradation of theses compounds. Our current knowledge of the enzymology, physiology, biochemistry, and molecular biology of these enzymes and of the producer fungi and bacteria is considerable. Consequently, processes that use enzymes and microorganisms are being developed to explore the potential for their biotechnological applications. Production of ethanol and other alternative fuels from lignocellulosic biomass can reduce urban air pollution, decrease the release of carbon dioxide in the atmosphere, and provide new markets for agricultural wastes. Biopulping and biobleaching are leading to cleaner and more efficient pulp and paper manufacture. Despite the progress achieved, more effort is needed for lignocellulosic enzymes and/or microorganisms to have significant industrial impact.

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