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# Agroindustrial Wastes as Substrates for Microbial Enzymes Production and Source of Sugar for Bioethanol Production

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## 1. Introduction

Environmental issues and concerns aimed at reducing the ambient pollution have boosted the search for “clean Technologies” to be used in the production of commodities of importance to chemical, energy and food industries. This practice makes use of alternative materials, requires less energy, and diminishes pollutants in industrial effluents, as well as being more economically advantageous due to its reduced costs. Considering this scenario, the use of residues from agroindustrial, forestry and urban sources in bioprocesses has aroused the interest of the scientific community lately. The utilization of such materials as substrates for microbial cultivation intended to produce cellular proteins, organic acids, mushrooms, biologically important secondary metabolites, enzymes, prebiotic oligosaccharides, and as sources of fermentable sugars in the second generation ethanol production has been reported (Sánchez, 2009). Notably, the microbial enzymes can be the products themselves as well as tools in these bioprocesses. Agroindustrial wastes are valuable sources of lignocellulosic materials. The lignocellulose is the main structural constituent of plants and represents the primary source of renewable organic matter on earth. It can be found at the cellular wall, and is composed of cellulose, hemicellulose and lignin, plus organic acids, salts and minerals (Pandey et al., 2000; Hamelinck et al., 2005). Therefore, such residues are superior substrates for the growth of filamentous fungi, which produce cellulolytic, hemicellulolytic and ligninolytic enzymes by solid state fermentation (SSF). These fungi are considered the better adapted organisms for SSF, since their hyphae can grow on the surface of particles and are also able to penetrate through the inter particle spaces, and then, to colonize it (Santos et al., 2004). Filamentous fungi are the most distinguished producers of enzymes involved in the degradation of lignocellulosic material, and the search for new strains displaying high potential of enzyme production is of great

biotechnological importance. Several agroindustrial wastes are commonly used for this purpose, such as sugarcane bagasse, wheat bran, corn cob and straw, rice straw and husk, soy bran, barley and coffee husk (Sánchez, 2009). Microbial cellulases, xylanases and ligninases are enzymes with potential application in several biotechnology processes. For decades, such enzymes have been used in the textile, detergent, pulp and paper, food for animals and humans (Bocchini et al., 2003; Kumar et al., 2004; Maicas & Mateo, 2005; Graminha et al., 2008; Hebeish et al., 2009). Recently, research has been focused on the potential use of these enzymes for the degradation of lignocellulosic materials, aiming at the releasing of fermentable sugars that can be converted to second generation ethanol by the action of fermentative microorganisms (Buaban et al., 2010; Talebnia et al., 2010). Among the enzymatically saccharified lignocellulosic wastes intended for the production of ethanol one can cite rice straw, wheat bran, wheat straw, sawdust, rice husk, corn straw and sugarcane bagasse, being the later greatly abundant in Brazil (Binod et al., 2010; Martín et al., 2007). Bearing this in mind, research has been focused on the development of new technologies capable of making the sugar available from bagasse, in order to supply the internal market and also to be exported (Cerqueira Leite et al., 2009). The intimate chemical and physical association between lignin and polysaccharides from the plant cell wall makes the enzymatic degradation of the carbohydrate portion difficult, and consequently the extraction of fermentable sugars, since this phenylpropanoid polymer is not easily degraded biologically. Furthermore, the crystalline structure of cellulose prevents the action of microbial enzymes (Gould, 1984). In order to facilitate the enzymes access to the polysaccharides, especially the cellulose, several pretreatments of the lignocellulosic materials have been proposed, with the intention of disorganizing the plant cell wall structure and lignin removal (Krishna, Chowdary, 2005). In this chapter, we will approach the application of lignocellulosic wastes as substrates for the growth of microorganisms able to produce enzymes such as cellulases, hemicellulases and ligninases, and as sources of fermentable sugars in the production of second generation ethanol, via enzymatic hydrolysis. It will be emphasized the composition of the main residues, the prominent microorganisms, their enzymes and mechanisms of action involved with lignocellulose degradation, SSF characteristics, pretreatment methods and enzymatic hydrolysis of lignocellulosic material, as well as the strategies that have been explored for second generation ethanol production.

## 2. Lignocellulose

*Lignocellulose* is the name given to the material present in the cell wall of higher terrestrial plants, made up of microfibriles of cellulose embebed in an amorphous matrix of hemicellulose and lignin (Fig. 1) (Martínez et al., 2009).

These three types of polymers are strongly bonded to one another and represent more than 90% of the vegetable cell's dry weight. The quantity of each polymer varies according to the species, harvest season and, also, throughout different parts of the same plant. In general, softwoods (gymnosperms such as pine and cedrus) have higher lignin content than hardwoods (angiosperm such as eucalyptus and oak). Hemicellulose content, however, is higher in gramineous plants. In average, lignocellulose consists of 45% of cellulose, 30% of hemicellulose and 25% of lignin (Glazer & Nikaido, 2007). Lignocellulosic materials also include agricultural residues (straws, stover, stalks, cobs, bagasses, shells), industrial

residues (sawdust and paper mill discards-, food industry residues), urban solid wastes e domestic wastes (garbage and sewage) (Mtui, 2009).

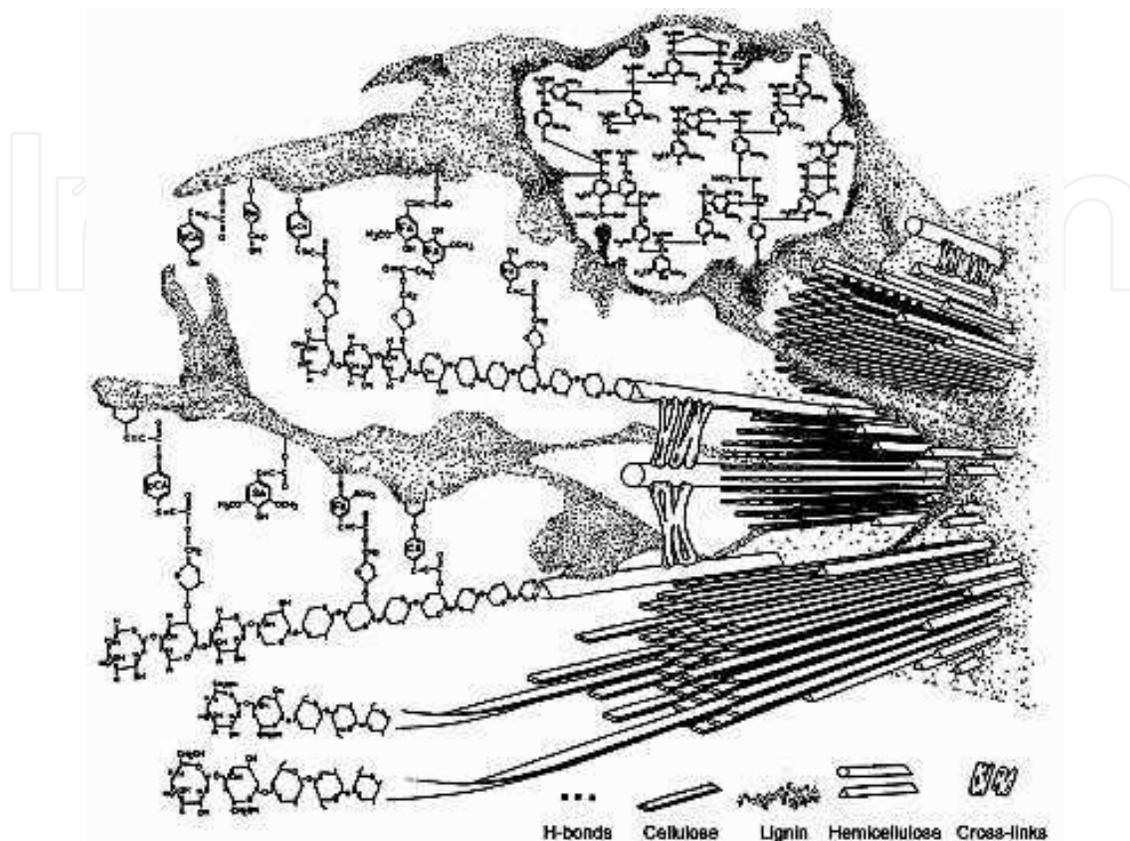


Fig. 1. Scheme of secondary plant cell wall. CA: *p*-coumaric acid; FA: ferulic acid; SA: sinapic acid. Source: Bidlack et al, 1992.

Lignocellulose is the world's main source of renewable organic matter and the chemical properties of its components make it a material of great biotechnological value. Therefore, a few years ago, the concept of lignocellulose biorefinery emerged, which has received growing attention due to the potential of conversion of this material into many high added value products such as chemical compounds, fermentation substrates, feedstock and biofuels (Ragauskas et al., 2006; Demirbas, 2008).

The accentuated growth of the world's consumption of energy originating from fossil resources has aggravated the problem of atmospheric pollution by the release of gases related to the greenhouse effect. For this reason, besides the high cost of petroleum and the eminent depletion of these resources in a few decades from now, the obtainment of fuels from renewable sources, such as lignocellulosic biomass, has aroused great interest in the last years. Currently, it is believed that ethanol, as the main form of bioenergy, is the best alternative to the use of fossil fuels (Wang et al., 2011).

Inside this context, new technologies have been developed for the efficient obtainment of fuels from lignocellulosic biomass and developed and developing Countries have been focusing efforts in researches aimed at obtaining the so called biofuels, such as bioethanol and biodiesel, as well as their introduction and prevalence in the market (Hamelinck et al., 2005; Prasad et al., 2007).

## 2.1 Cellulose

Cellulose is the most abundant organic compound on Earth and the main constituent of plant cell walls. It consists of linear chains of approximately 8,000 to 12,000 residues of D-glucose linked by  $\beta$ -1,4 bonds (Timell, 1967; Aro et al., 2005). Cellulose chains exhibit a flat structure, stabilized by internal hydrogen bonds (Fig. 2). All alternate glucose residues in the same cellulose chain are rotated 180°. One glucose residue is the monomeric unit of cellulose and the dimer, cellobiose, is the chain's repetitive structural unit (Brown Jr et al., 1996). Cellulose chain is polarized, once there is a nonreducing group at one of its end and, at the opposite end there is a reducing group. New glucose residues, originating from UDP-glucose, are added to the nonreducing end during polymer synthesis (Koyama et al., 1997). Parallel cellulose chains interact, through hydrogen bonds and van der Waals forces, resulting in microfibriles, which are very extensive and crystalline aggregates (Glazer & Nikaido, 2007; Somerville et al., 2004).

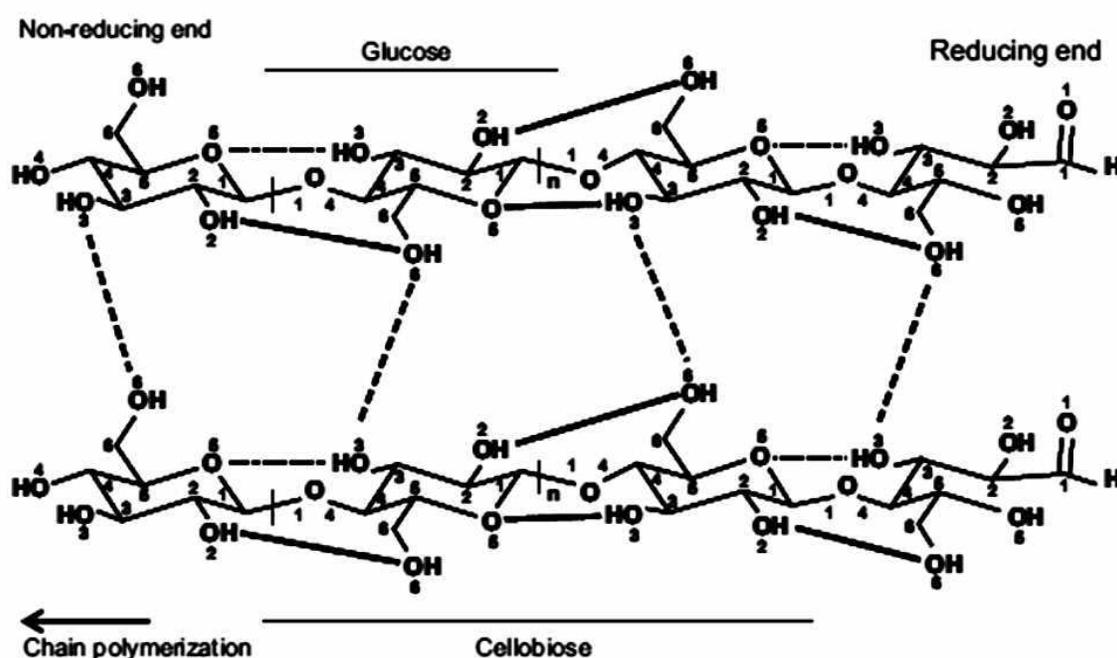


Fig. 2. Representative structure of cellulose chains. Dotted and solid lines: inter and intra chain hydrogen bonds, respectively. Source: Festucci-Buselli et al., 2007.

The microfibriles are made up of approximately 30-36 glucan chains, exhibit a 2-10 nm diameter and are cross-linked by other components of the cell wall, such as the xiloglucans. (Arantes & Saddler, 2010). The cellulose microfibriles networks are called macrofibrils, which are organized in lamellas to form the fibrous structure of the many layers of plant cell wall (Fig. 3) (Glazer & Nikaido, 2007).

In cellulose fibers, crystalline and amorphous regions alternate. The crystalline regions are very cohesive, with rigid structure, formed by the parallel configuration of linear chains, which results in the formation of intermolecular hydrogen bonds, contributing to cellulose's insolubility and low reactivity, at the same time making it more resistant to acid hydrolysis, making water entrance difficult and modifying fiber elasticity. The amorphous regions are formed by cellulose chains with weaker organization, being more accessible to enzymes and susceptible to hydrolysis (Bobbio & Bobbio, 2003; Nelson & Cox, 2006).

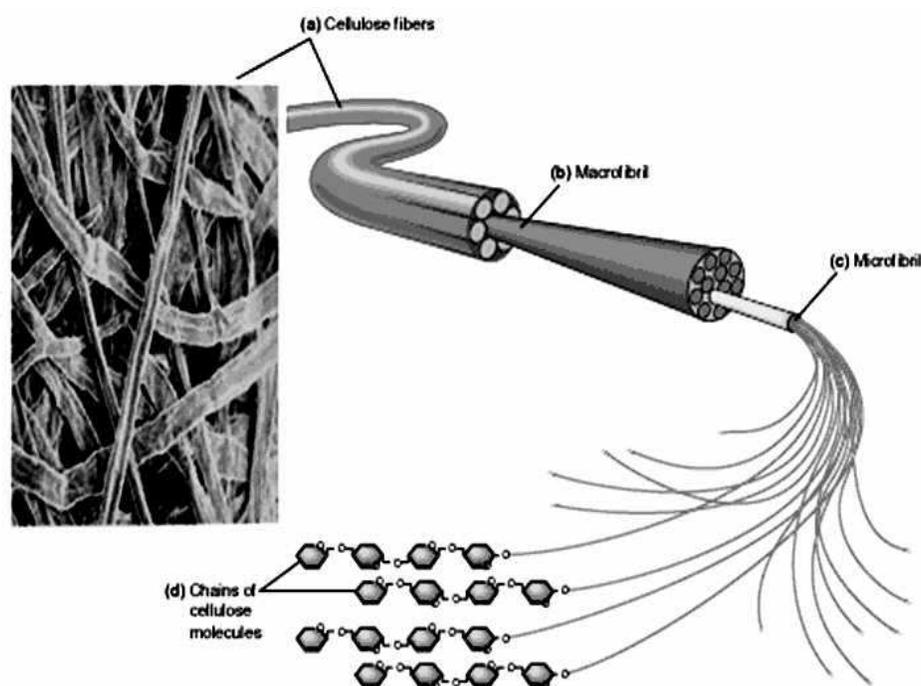


Fig. 3. Representative scheme of cellulose fiber Available from <http://nutrition.jbpub.com/resources/chemistryreview9.cfm>.

## 2.2 Hemicellulose

Hemicellulose is the second group of most abundant polysaccharide in plant cell wall and, differently from cellulose, it is made up of non crystalline heteropolysaccharides (Aspinall, 1959).

Schulze (1891) initially classified hemicellulose as the polysaccharide fraction of plant cell wall easily hydrolyzed, an imprecise classification which was used for a long time. The group also defined as polysaccharides present in plant cell wall and intercellularly (in the middle lamella), extracted from higher terrestrial plant tissues through alkaline treatment or, yet, as certain carbohydrates of cereal endosperms, which are non starch polysaccharides that are described as cereal gum or pentosans (Timell, 1965; Wilkie, 1979).

Afterwards, hemicellulose classification was redefined, based on the chemical properties of its components, including only cell wall polysaccharides non covalently bonded to cellulose made up by  $\beta$ -(1,4)-linked pyranosyl residues that have the O-4 in the equatorial position. Such characteristics result in a conformation that is very similar to cellulose (cellulose-like conformation) and cause a tendency to hydrogen-bond to cellulose chains (O'Neill & York, 2003).

In a general way, the hemicellulose fraction makes up 15 to 35% of plant biomass, representing a great renewable source of biopolymers which may contain pentoses ( $\beta$ -D-xylose,  $\alpha$ -L-arabinose), hexoses ( $\beta$ -D-mannose,  $\beta$ -D-glucose,  $\alpha$ -D-galactose) and/or uronic acids ( $\alpha$ -D-glucuronic,  $\alpha$ -D-4-O-methylgalacturonic and  $\alpha$ -D-galacturonic acids). Other sugars such as  $\alpha$ -L-rhamnose and  $\alpha$ -L-fucose may also be present in small amounts and the hydroxyl groups of sugars can be partially substituted with acetyl groups (Girio et al., 2010). Therefore, hemicellulose classification depends on the type of monomer constituent and these may be called xyloglucans, xylans (xyloglycans), mannans (mannoglycans) and  $\beta$ -(1 $\rightarrow$ 3,1 $\rightarrow$ 4)-glucans (mixed-linkage  $\beta$ -glucans). Galactans, arabinans and arabinogalactans are also many times

included in the hemicellulose group, however do not share the equatorial  $\beta$ -(1 $\rightarrow$ 4)-linked backbone structure (Scheller & Ulvskov, 2010). Its form and structure depend on where they are present, being in woods or fruits cell walls (Dey & Brinson, 1984).

Most of the hemicelluloses are relatively small molecules, containing 70 to 200 residues of monosaccharides, being hardwood hemicellulose the largest molecules with 150 to 200 units (Coughlan, 1992).

Hemicelluloses associate to cellulose by physical intermixing and hydrogen bonds, and to lignin e pectin, by covalent bonds (Freudenberg, 1965).

### 2.2.1 Xylans

Xylans, the most relevant components of hemicellulose, constitute the second group of polysaccharides most abundant in nature, contributing to approximately one third of all the renewable organic carbon on Earth (Prade, 1995). Xylans constitute about 20–30% of the biomass of hardwoods and herbaceous plants. In some tissues of grasses and cereals xylans can account up to 50% (Ebringerová et al., 2005).

Xylans are mainly situated in the secondary cell wall of plants (Timell, 1965), in close contact to cellulose through strong interactions established by hydrogen bonds and van der Waals forces. They may also be present in the primary cell wall, in particular in monocotyledonous (Wong et al., 1988). Their molecules are oriented parallel to cellulose chains, in the cell wall matrix, and are located between cellulose microfibrils (Northcote, 1972). Xylan, covalently bonded to lignin and non covalently bonded to cellulose, exhibits an important role in maintaining cellulose integrity in situ, protecting the fibers against the action of cellulases (Beg et al., 2001; Uffen, 1997).

Xylans of all higher plants are heteropolysaccharides with a backbone of  $\beta$ -(1 $\rightarrow$ 4) linked xylopyranose units, usually substituted with sugar units and O-acetyl groups (Stephen, 1983). Exception to this pattern have been isolated from the seeds of *Plantago* species (Sandhu et al., 1981; Samuelsen et al., 1999) The occurrence of homoxylans in higher plants is rather rare, being isolated from esparto grass (Chanda et al., 1950), tobacco caule (Eda et al., 1976), guar seeds (Montgomery et al., 1956) and from some marine algae (Barry & Dillon, 1940; Nunn et al., 1973).

Methylglucuronoxylans (MXG) (O-acetyl-4-O-methylglucuronoxylans) are dominating in the secondary walls of hardwoods (dicots such as eucalypto e carvalho) having single side chains of  $\alpha$ -D-glucuronic acid (GA) and/or its 4-O-methyl derivative (MeGA) attached at position 2 of the xylopyranose monomer units (Ebringerová & Heinze, 2005). The content of acetyl groups varies in the range 3–13% and is responsible for xylan's partial solubility in water (Sunna & Antranikian, 1997).

Arabinoglucuronoxylan (AGX) (arabino-4-O-methylglucuronoxylans) are the major components of non-woody materials (e.g., agricultural crops, grasses) and a minor component (5–10% of dry mass) for softwoods (ex gymnosperms such as pine and cedrus). They contain single side chains of 2-O-linked  $\alpha$ -D-glucopyranosyl uronic acid unit (GA) and/or its 4-O-methyl derivative (MeGA) and 3-linked  $\alpha$ -L-arabinofuranosyl unit (Timell, 1965; Sunna & Antranikian, 1997).

Into the cell walls polysaccharides of forage and grasses, ferulic acid residues are introduced via an ester linkage between their carboxylic acid group and the primary alcohol on the C5 carbon of the arabinose side chain of arabinoxylans (Hartley, 1973) (Fig. 4), but can also be covalently linked to lignin monomers via an ether linkage (Kondo et al., 1990). So, ferulic acid participates with lignin monomers in oxidative coupling pathways to generate ferulate-

polysaccharide–lignin complexes that cross-link the cell wall (Buanafina, 2009). Esters of *p*-coumaric acid are also abundant in grass cell walls, but it is not clear if they can be attached directly to the xylans, and they may be primarily associated with lignin (Hatfield et al., 2008).

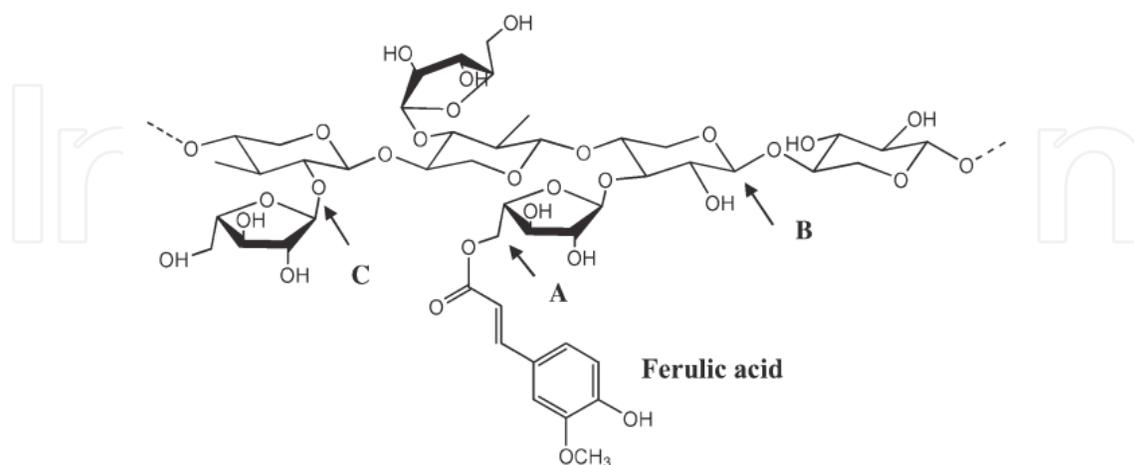


Fig. 4. Structure of ferulic acid esterified to arabinose units of arabinoxylan. A: ferulic acid linked to O-5 of arabinose chain of arabinoxylan; B:  $\beta$ -1,4-linked xylan backbone; C:  $\alpha$ -1,2-linked L-arabinose. Source: Buanafina, 2009. The occurrence and structural characteristics of other types of xylan have been reported in detail in previous review papers (Ebringerová & Heinze, 2005; Ebringerová et al., 2005).

### 2.3 Lignin

Lignin is the second most abundant polymer in the cell wall of vascular plants and in nature, after cellulose. Approximately 20% of the total carbon fixed by photosynthesis in land ecosystems is incorporated into lignin. It is a complex and recalcitrant aromatic polymer, without defined repetitive units (Hammel & Cullen, 2008), its precursors are three *p*-hydroxycinnamyl alcohols or monolignols (*p*-coumaryl, coniferyl and sinapyl) and their recently reported acylated forms (Ralph et al., 2004; Martínez et al., 2008) (Fig. 5).

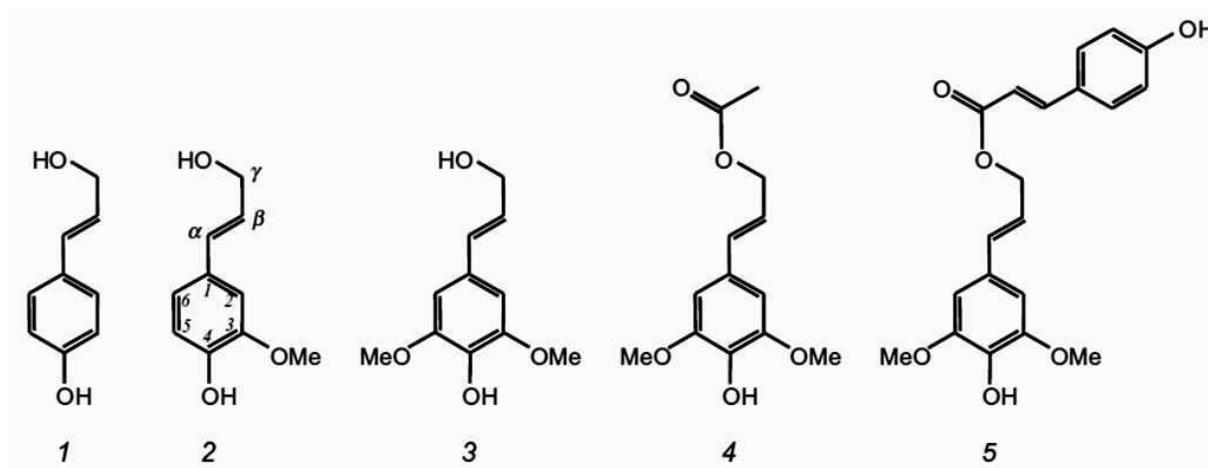


Fig. 5. Lignin precursors or monolignols. Classical: *p*-coumaryl (1), coniferyl (2), sinapyl (3). Acylated: derived from sinapyl alcohol  $\gamma$ -esterified with acetic (4) and *p*-coumaric acid (5). Source: Ruiz-Dueñas & Martínez, 2009 – partially reproduced

Although these precursors are phenolic compounds, the polymer is basically non-phenolic (Fig. 6), due to the high frequency of ether linkages between the phenolic position (C4) and a side-chain (or aromatic ring) carbon of the *p*-hydroxyphenylpropenoid precursors (Fig. 6, substructures A, B and D), strongly predominant in the growing polymer. Unlike cellulose and hemicelluloses, the lignin polymerization mechanism (based on resonant radical coupling) results in a complex three-dimensional network. During the polymer synthesis, a variety of ether and carbon-carbon inter-unit linkages are formed, resulting in many substructures, such as those shown in Figure 6 (Ruiz-Dueñas & Martínez, 2009).

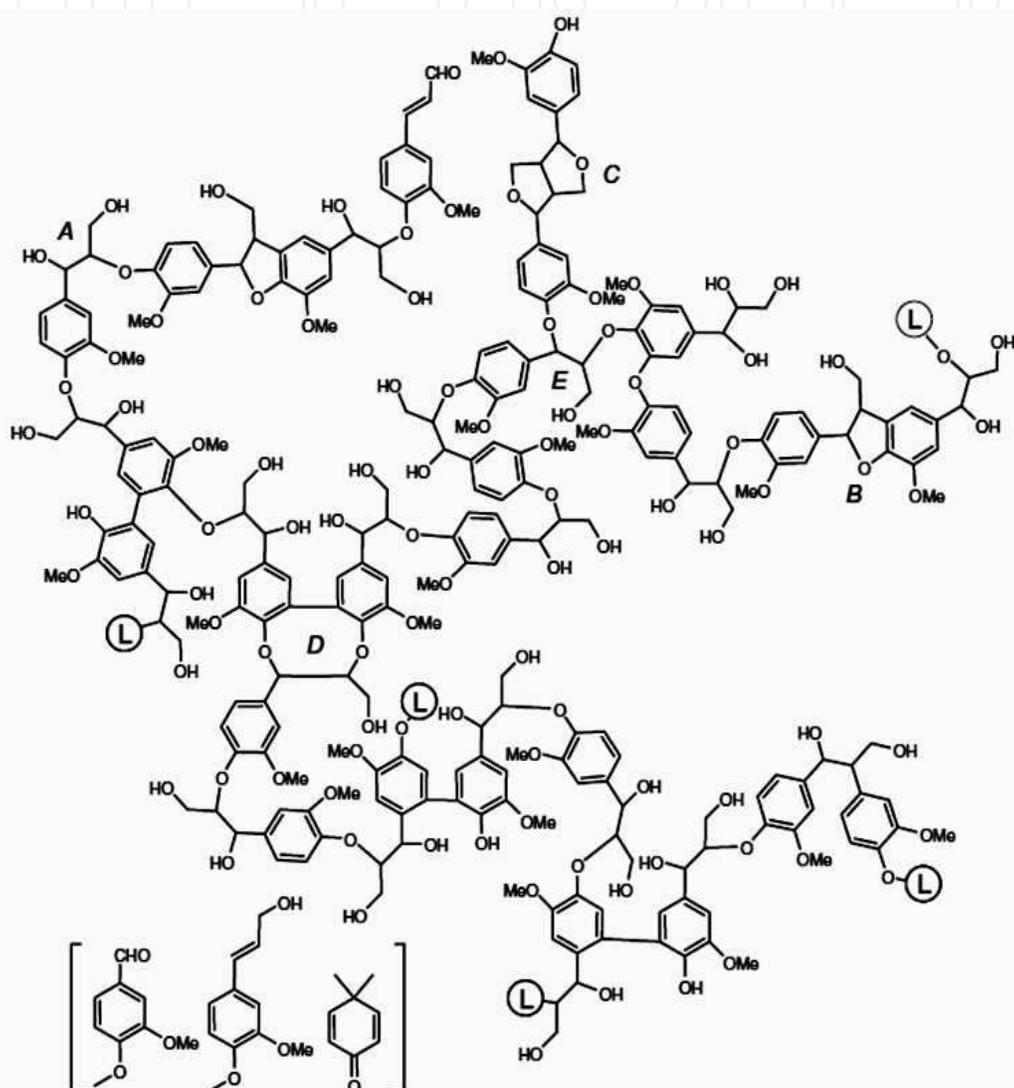


Fig. 6. Lignin structure. Substructures:  $\beta$ -O-4'(A); phenylcoumaran (B); pinoresinol (C) and dibenzodioxocin (D). L-containing circles indicate linkages to additional lignin chains. Brackets indicate other minor structures, such as vanillin, coniferyl alcohol and dimethylcyclohexadienone-type units, the latter in new spirodienone substructures. Source: Ruiz-Dueñas & Martínez, 2009 – partially reproduced.

The association of lignin and hemicelluloses occurs through covalent linkages (such as benzyl ester bonds with the carboxyl group of 4-O-methyl-d-glucuronic acid in xylan and more stable ether bonds between lignin and arabinose or galactose side groups in xyans

and mannans) (Kuhad et al., 1997) and also through noncovalent linkages. These interactions form a dense and organized network that surrounds the cellulose through extensive hydrogen bonding (Fig. 1) (Westbye et al., 2007). This network protects the cellulose and is one of the reasons for biomass recalcitrance. These lignin-carbohydrate complexes (LCC) represent an obstacle for lignocellulose bioconversion processes because lignin hinders enzyme-mediated hydrolysis of carbohydrates, since it acts as a physical barrier, restricting enzyme access to carbohydrates (Mooney et al., 1998). Lignin may also interact with enzymes possibly through hydrophobic interactions resulting in nonproductive binding (Sutcliffe & Saddler, 1986).

### 3. Lignocellulose-degradating enzymes

Lignocellulosic materials represent an important source of added-value chemicals, such as reducing sugars, furfural, ethanol and other products that can be obtained by enzymatic or chemical hydrolysis. Enzyme-catalyzed hydrolysis of lignocellulosic biomass provides better yields without the generation of side products (Demirbas, 2008).

Enzymatic degradation of vegetable biomass by microorganisms is carried out by a complex mixture of enzyme, amongst which cellulases and hemicellulases stand out, whose action results in free carbohydrates that may be hydrolyzed to soluble saccharides that can be further metabolized, besides ligninases, which promote lignin depolymerization. Most carbohydrate hydrolases are modular proteins that, besides the catalytic site, have a carbohydrate-binding module (CBM) (Jørgensen et al., 2007). CBMs were first discovered on cellulases but it is now evident that many carbohydrate hydrolases acting on insoluble but also soluble polysaccharides have CBMs, which function is to bring the catalytic site in close contact with the substrate and ensure correct orientation. Furthermore, for some CBMs a disruptive effect on the cellulose fibers has also been shown (Boraston et al., 2004).

#### 3.1 Cellulases

The production of cellulases by microorganisms occurs, mainly, by bacteria and filamentous fungi, with few reports of production by yeasts. Ascomycetes and imperfect fungi have great importance for degradating cellulose and decomposing soil vegetable residues, being known as brown rot fungi (Sandgren & Hiberg, 2005).

A cellulolytic system based on 'free' enzymes, that act synergistically to complete cellulose degradation, is typically produced by aerobic fungi and bacteria. This enzyme system include three types of cellulases (Fig. 7):

- i. *Endoglucanases* (EG, endo-1,4- $\beta$ -D-glucan 4-glucanohydrolase, EC 3.2.1.4): hydrolyses, at random,  $\beta$ -1,4 glucosidic bonds at internal amorphous sites in the cellulose chains, providing more ends for the cellobiohydrolases to act upon;
- ii. *Exoglucanases* or *cellobiohydrolases* (CBH, 1,4- $\beta$ -D-glucan cellobiodehydrolase, EC 3.2.1.91): act on the reducing (CBH I) or nonreducing (CBH II) ends of cellulose chains, liberating cellobiose;
- iii.  *$\beta$ -glucosidases* ( $\beta$ -glucoside glycosyl hydrolase or cellobiase, EC 3.2.1.21): hydrolyze cellobiose or cello-oligosaccharides to glucose and are also involved in transglycosylation reactions of  $\beta$ -glucosidic linkages of glucose conjugates (Coughlan & Ljungdahl, 1988).

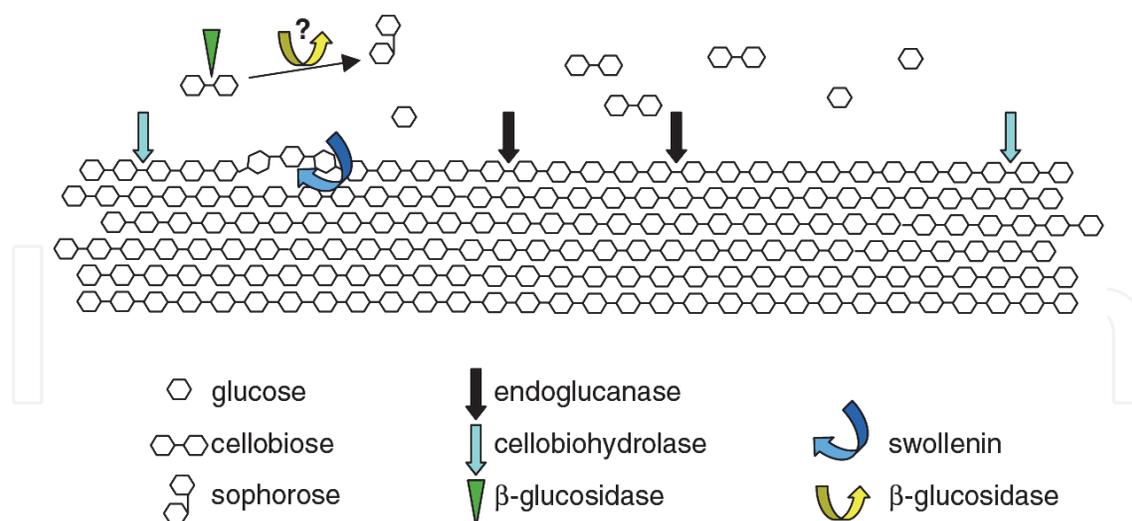


Fig. 7. Schematic representation of the cellulolytic system. Sites of intense cellulolytic enzyme activity are shown, besides an alternative path for the formation of sophorose by  $\beta$ -glucosidase's transglycosylating activity. Source: Aro et al, 2005.

In addition to the classical cellulases, Figure 7 also shows the action of swollenins, proteins with amino acid similarity to plant expansins that regulate cell wall enlargement in growing cells. Expansins were firstly isolated from *Trichoderma reesei* in 1992 and are thought to disrupt hydrogen bonding between cellulose microfibrils or between cellulose and other cell wall polysaccharides, without hydrolyzing them, causing sliding of cellulose fibers or expansion of the cell wall (Whitney et al., 2000). It has been reported that swollenin action helps enzymatic cellulose degradation since it causes a partial damage and loose structure on cellulose, similar to that caused by ultrasound treatment, without releasing reducing sugar (Saloheimo, et al. 2002)

The enzymes of the cellulolytic complex may be subject to catabolic repression by the final product of hydrolysis. For preventing accumulation of cellobiose,  $\beta$ -glucosidase is responsible for controlling the overall speed of the cellulolytic hydrolysis reaction, exhibiting a crucial effect on the polymer's enzymatic degradation (Leite et al., 2008).

Cellulases synthesized by anaerobes, particularly clostridia and rumen microorganisms, frequently assemble into a large multienzyme complex (molecular weight >3 MDa) termed cellulosome and first identified in 1983 from the thermophilic and spore-forming *Clostridium thermocellum* (Lamed et al., 1983). This bacterial cellulosome shows very high activity on crystalline cellulose ("true cellulase activity" or Avicelase) which is not commonly observed among fungal cellulases (Johnson et al., 1981).

In *C. thermocellum*, cellulolytic enzymes are typically distributed both in the liquid phase and on the surface of the cells. However, several anaerobic species that degrade cellulose do not release measurable amounts of extracellular cellulase, and instead have localized their complexed cellulases directly on the surface of the cell or the cell-glycocalyx matrix (Lynd et al., 2002).

Besides *Clostridium* and other anaerobic bacteria, evidences suggest the presence of cellulosome in at least one aerobic bacterium and a few anaerobic fungi such as *Neocallimastix*, *Piromyces* and *Orpinomyces* (Fanutti et al., 1995; Li et al., 1997).

In addition to cellulases, cellulosomes include xylanases, mannanases, arabionfuranosidases, lichenases, and pectin lyases (Bayer et al., 2004).

The structure and function of bacterial cellulosomes have been reviewed several times elsewhere (Bayer et al., 1998; Nordon et al., 2009). All cellulosome described share some characteristics (Fig. 8): their enzymes are linked to noncatalytic modules, called dockerins, by carbohydrate-binding modules (CBMs). Dockerins bind, by calcium-dependent interactions, to the cohesin modules, located in a large noncatalytic protein that acts as a scaffoldin. In general, the scaffoldin, a large and distinct protein, allows binding of the whole complex to the plant cell wall, via a cellulose-specific family 3 CBM (CBM3a), and to the bacterial cell via a C-terminal divergent dockerin (Fontes & Gilbert, 2010).

Since the recalcitrance and chemical complexity of some polymers represent an obstacle to the enzymatic degradation of lignocellulose, more efficient enzyme systems are required. Cellulosomes highlight as one of nature's most elaborate nanomachines and the arrangement of plant cell wall degrading enzymes into this complex has advantages over free enzyme systems. Less total protein may be required to solubilize cellulose, including crystalline cellulose, which suggests that specific activity of the cellulosome for such substrates is higher than that of free enzyme systems (Johnson et al., 1982; Boisset et al, 1999). We could say that cellulosome enzymes are "concentrated" and positioned in a suitable orientation both with respect to each other and to the cellulosic substrate, thereby facilitating stronger synergism among the catalytic units. Due to this optimum spacing of the components, working in a synergistic manner, non-productive adsorption is avoided. Since cellulosome is close to the microorganism cell surface, hydrolysis inhibitory products would not accumulate, but would be maintained at appropriate concentrations for most efficient use by the cell (Shoham et al, 1999).

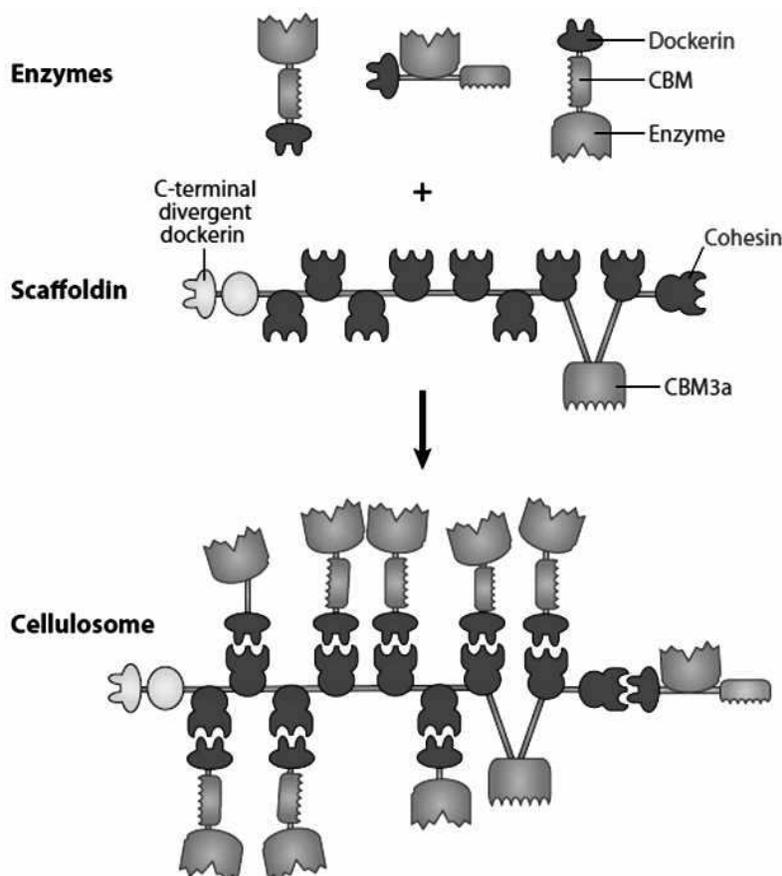


Fig. 8. Mechanism of cellulosome assembly. Source: Fontes & Gilbert, 2010.

### 3.2 Xylanases

Due to xylans heterogeneity and complexity, the complete hydrolysis of this polysaccharide requires the action of an enzyme system with different specificities and ways of action (Fig. 9). The microbial systems are made up by (Wong et al., 1988):

- i. *Endo-1,4- $\beta$ -D-xylanases* (EC 3.2.1.8): hydrolyze  $\beta$ -1,4 glycosidic linkages between xylose residues in the backbone of xylans;
- ii. *1,4- $\beta$ -D-xylosidases* (EC 3.2.1.37): release  $\beta$ -D-xylopyranosyl residues from the non-reducing terminus of xylobiose and some small 4- $\beta$ -D-xylooligosaccharides;
- iii.  *$\alpha$ -L-arabinofuranosidases* (EC 3.2.1.55): removes L-arabinose side chains from the xylose backbone of arabinoglucuronoxylan;
- iv.  *$\alpha$ -glucuronidases* (EC 3.2.1.1): hydrolyzes the  $\alpha$ -1,2 glycosidic bonds between the glucuronic acid residues and  $\beta$ -D-xylopyranosyl backbone units found in glucuronoxylan;
- v. *acetyl xylan esterases* (EC 3.1.1.72): removes the *O*-acetyl groups from positions 2 and/or 3 on the  $\beta$ -D-xylopyranosyl residues of acetyl xylan
- vi. **p*-coumaric and ferulic acid esterases* (EC 3.1.1.1): cleave ester bonds on xylan, between arabinose and erulic acid sidegroups and between arabinose and *p*-coumaric acid, respectively (Christov & Prior 1993).

The *feruloyl esterases* exhibit a key role providing an increase on hydrolytic enzyme's accesability on hemicellulose fibers due to the removal of ferulic acid from the side chains and cross links (Wong, 2006).

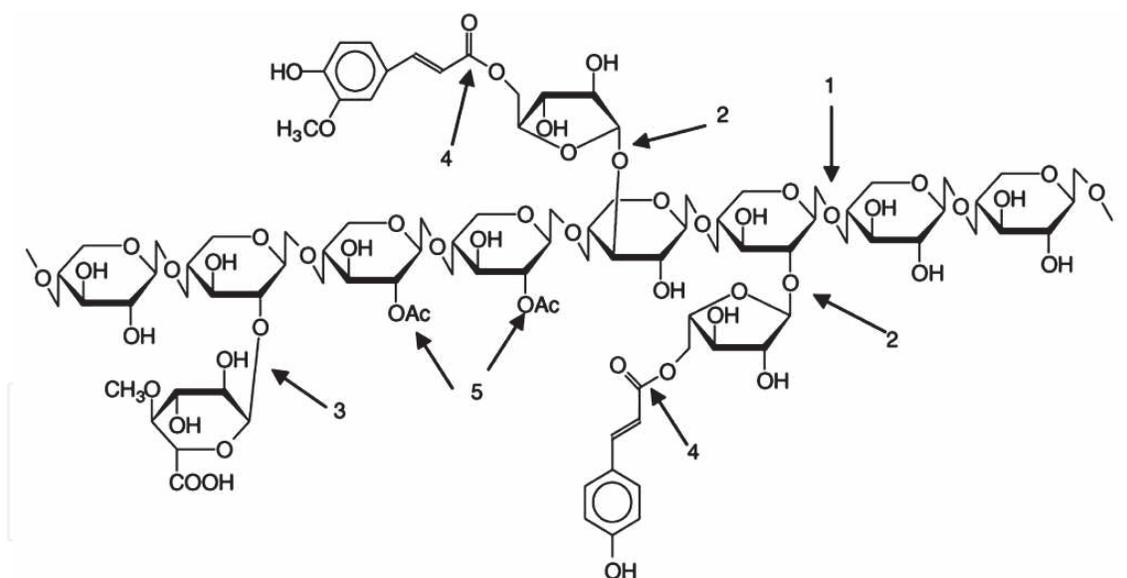


Fig. 9. Schematic representation of the action of the xylanolytic enzyme system. 1 - endoxyylanases; 2 -  $\alpha$ -L-arabinofuranosidases; 3 - glucuronidases; 4 - feruloyl and coumaroyl esterases; 5 - acetyl xylan esterases; 6 -  $\beta$ -xylosidases. Source: Chávez et al., 2006

All xylanolytic enzymes act in a cooperative way to convert xylan into its monomers (Wong et al., 1988). Such multifunctional xylanolytic system may be found in fungi and bacteria (Sunna & Antranikian, 1997), including the actinomycete (Elegir et al., 1995). Some of the most important microorganisms that produce xylanolytic enzymes belong to the genera

*Aspergillus*, *Trichoderma*, *Streptomyces*, *Phanerochaete*, *Clotridium* e *Bacillus* (Collins et al., 2005; Subramaniyan & Prema, 2002).

### 3.3 Ligninases

Lignin decomposition is indispensable for carbon recycling since it is the most abundant renewable source of aromatic polymer in nature. Due to its complex and heterogeneous structure, lignin is chemically recalcitrant to breakdown by most organisms. Only the basidiomycetous, called whiterot fungi, are able to degrade lignin efficiently by producing an array of extracellular oxidative enzymes that act synergistically. Among these enzymes, the major groups include lignin peroxidases, manganese peroxidases, and laccases (Wong, 2009).

#### 3.3.1 Laccases

Laccase (E.C. 1.10.3.2) is a multicopper protein belonging to the family of the blue oxidase enzymes. This enzyme generally contains four copper ions, grouped in three groups: T1, formed by one ion and is responsible for substrate oxidation and for electron transfer; T2, also formed by one ion and, together with the T3 group, which contains two ions, constitute the copper trinuclear center, involved in oxygen reduction and water release (Torres et al., 2003). Copper located at the T1 site is the one responsible for the strong absorption of the enzyme at the 600 nm range; however there has been reports of laccases with copper deficiency at T1, called white laccases, due to the absence of the characteristic absorbance at the blue range (Baldrian, 2006).

Laccase is classified as a phenol oxidase and catalyzes the oxidation of various aromatic and inorganic compounds (phenols in particular) and at the same time reduces oxygen to water. Phenolic dyes, phenols, chlorophenols, some diphenylmethanes and benzopirenes are amongst the substrates oxidized by this enzyme (Durán & Esposito, 2000). Laccase can degrade lignin even in the absence of other ligninases, such as manganese peroxidase and lignin peroxidase (Mayer & Staples, 2002).

The catalytic mechanism is based on the reduction of molecular oxygen forming water, at the expense of consecutive mono-electronic oxidation of the substrate.

Laccases may directly interact with phenolic substrates and oxidize them, however they are not capable of directly acting on aromatic non-phenolic molecules, thus requiring a mediator molecule for the degradation of such compounds. In this laccase-mediator mechanism, the mediators oxidize substrates of high molecular mass (Torres et al., 2003). Generally, the mediators are low molecular weight substances, secreted by the fungi itself, which when oxidized by laccases, are capable of oxidizing compounds that would not be the enzyme's direct target. The mediation phenomenon increases the range of substrates of these enzymes (Leonowicz et al., 1999)

#### 3.3.2 Lignin peroxidases (LiP)

Lignin peroxidase (LiP) (E. C. 1.11.1.14) is a glycoprotein that has a prosthetic group made up of iron protoporphyrin IX, with catalytic activity dependent on H<sub>2</sub>O<sub>2</sub> (Rodríguez & Durán, 1988). The H<sub>2</sub>O<sub>2</sub> required for LiP activity is originated from different biochemical pathways, expressed according to the nutritional factors and growth conditions of the microorganism (Pointing, 2001).

LiP is an enzyme capable of oxidizing various non-phenolic aromatic compounds such as benzyl alcohol, cleaving side chains of these compounds, catalyzing aromatic ring opening reactions, demethylation and oxidative dechlorination (Conessa et al., 2002).

Veratrilic alcohol may induce enzyme's action, protect it against activation by high levels of  $H_2O_2$ , besides acting as co-substrate, carrying out oxidation of the non-phenolic aromatic compounds (Mester et al., 1995; Pointing, 2001).

### 3.3.3 Manganese peroxidases (MnP)

Manganese peroxidase (MnP, E. C. 1.11.1.13) is a glycosylated extracellular enzyme that has an heme prosthetic group, found only in basidiomycetes. In general, it does not unleash direct transformations on its substrates. The electron transfers involving manganese happen in the presence of dicarboxylic radicals, such as oxalate, malate, fumarate and malonate (Leonowicz et al., 1999). These radicals are  $Mn^{3+}$  chelating agents. When complexed, apparently, they act as low molecular weight mediators which will effectively oxidize lignin's phenolic compounds (Higuchi, 2004). MnP's reaction mechanism is similar to that of other peroxidases, such as lignin peroxidase but, in this case, compounds I and II of MnP oxidize  $Mn^{2+}$  (Conessa et al., 2002).

## 4. Solid state fermentation (SSF) and its application on the cultivation of microorganisms that produce lignocellulolytic enzymes

SSF can be defined as the cultivation of microorganisms in a solid insoluble matrix, in the presence of enough water to only support microbial growth (held in the particles). The solid matrix may be used by the microorganism as substrate, or may just serve as an inert support for growth (in this last case, it should be soaked in a nutrient solution) (Pandey et al., 2000). The selection of an appropriate substrate is a key factor for the success of SSF. Besides having the adequate composition to induce the desired product, the particles size should also be considered, since this is a factor that greatly influences SSF. Small particles offer more contact surface, allowing more access of the microorganism to the nutrients; however, depending on the type of substrate and on the moisture level, it can get compacted, impairing aeration and oxygen availability, as well as heat dissipation, limiting microbial growth. Big particles, on the other hand, facilitate aeration, however may hinder microbial access, limiting substrate contact surface and making heat transfer difficult (PALMA, 2003). Other parameters should also be evaluated and optimized for higher process efficiency, such as initial moisture and pH, incubation temperature, aeration, inoculum size, nutrient supplementation, extraction and purification of the final product (Pandey, 2003).

Solid state fermentation (SSF) is an interesting process for lignocellulose-degrading enzymes production at low cost, due to the possibility of using agricultural and agroindustrial residues as substrate for microbial growth. Filamentous fungi are considered to be the most adapted microorganisms for SSF, since their hyphae may grow on the particles surface and penetrate the intra-particle spaces, and thus colonize solid substrates (Santos et al, 2004). In a general way, the demands of high water activity for the development of bacteria make them not adaptable to SSF processes. However, scientific reports have shown that some bacterial cultures may be adapted to this type of process (Nampoothiri & Pandey, 1996; Orozco et al., 2008).

Fig. 10 is a schematic representation of some biological and physical processes that take place during SSF. During a process of solid-state fermentation, fungi grow by forming a mycelial mat on the surface of the particles that comprise the solid substrate. Aerial hyphae protrude into the gaseous space and penetrative hyphae grow into liquid-filled pores. Unless the moisture is above the appropriate level, the empty spaces between the aerial

hyphae are filled with gas (g), whereas the empty spaces within the mycelial mat and within the substrate are filled with liquid (l). Near the substrate surface and within the pores are regions of most metabolic activities. However, the regions of aerial hyphae also show metabolism and there can be a transport of substances from the penetrative to the aerial hyphae. By producing hydrolytic enzymes that diffuse into the solid matrix, fungi can degrade macromolecules into smaller units which are taken up by mycelium to serve as nutrients. During fermentation, there is consumption of  $O_2$  and production of  $CO_2$ ,  $H_2O$ , other biochemical products and heat. Gradients develop within the biofilm that, for instance, force  $O_2$  to diffuse from the gaseous phase into deeper regions of the biofilm and  $CO_2$  to diffuse from these regions to the gaseous phase. Heat is removed from the substrate via conduction and also by evaporation, which is part of the complex balance of water in the system. The release of organic acids and ammonia might change the local pH (Hölker & Lenz, 2005).

The use of SSF for fungi cultivation presents many advantages, such as: simulation of their natural environment, which results in better adaptation to the medium and higher enzymatic productions; reduction of bacterial contamination, due to absence of free water; obtainment of more concentrated enzymes, once the enzymatic extracts may be extracted with small amounts of water (Bianchi et al, 2001). Other advantages of fungal enzyme production by SSF can also be mentioned when comparing it to submerged fermentation, such as use of lesser amount of water and consequently a decrease in effluent generation, lower requirement of space and energy, more stability of the obtained products, more biomass and enzyme production, less catabolic repression of enzymes and little protein degradation (Pandey et al., 2000; Viniegra-González et al., 2003).

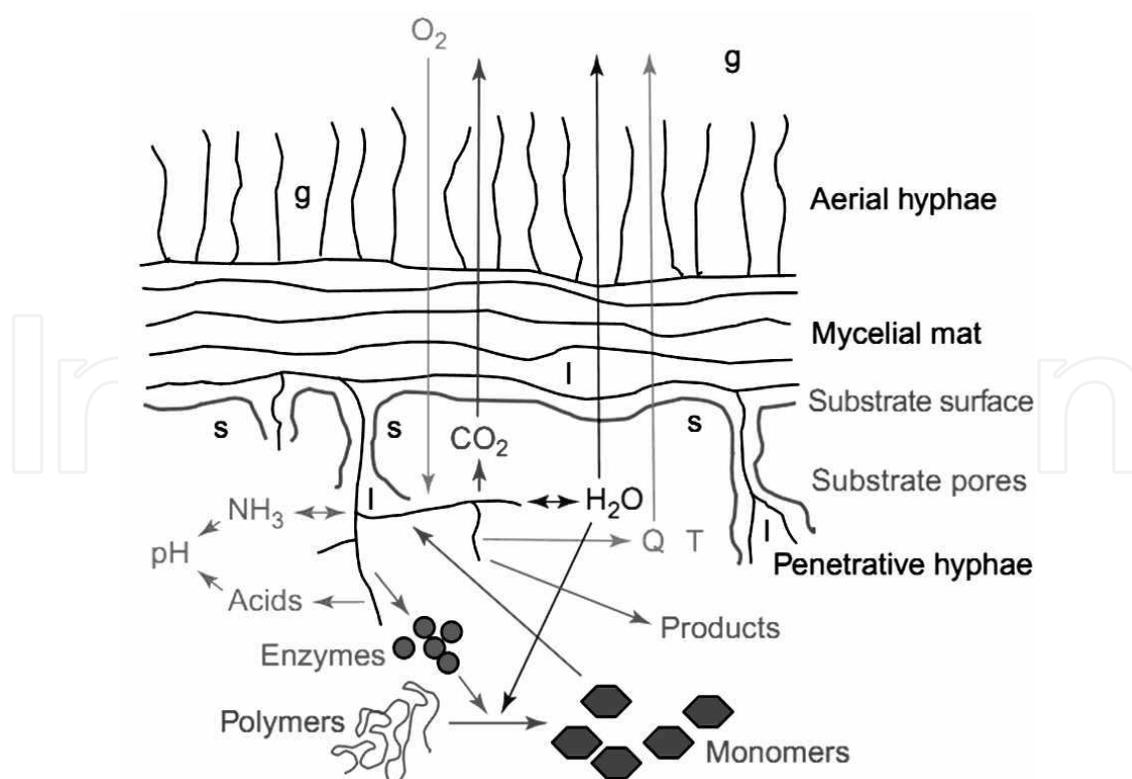


Fig. 10. A scheme of some microscale processes that occur during solid-state fermentation (SSF). Source: Holker & Lenz, 2005.

The main disadvantages of using SSF are the difficulty of heat dissipation. Temperature is directly related to water activity (*aw*) and aeration. The low thermal conductivity of the solid medium limits the removal of excess heat generated by microbial metabolism in SSF. In practice, SSF requires aeration mostly to dissipate heat rather than to provide oxygen (Viesturs et al., 1981). The temperature increase in bioreactors may cause protein denaturation, especially of the thermolabile ones (Santos et al., 2004). In this matter, thermophilic fungi encourage special interest for enzyme production processes by SSF. By exhibiting optimum growth at 45 and 55°C, such microorganisms better endure the possible temperature raise due to the difficulty of heat dissipation in SSF, especially in large-scale bioreactors, as well as secrete enzymes that are more active and stable at higher temperatures, which present many important properties from the industrial application point of view (Maheshwari et al., 2000).

Scale-up represents another obstacle to be overcome in SSF processes, once different gradients (moisture, temperature, substrate concentration and others) form along the bioreactor, which may negatively influence the process, especially the static bed ones. Heat dissipation, mass transfer and control of the fermentative parameters are the main challenges to be overcome in this sense (Hölker & Lenz, 2005; Khanahmadia et al., 2006). It is worth mentioning that, in the last decades, researches on design, modeling, operation and scaling up have brought progress to the knowledge of SSF processes involving bioreactors (Mitchell et al., 2000).

## **5. Lignocellulosic residues: Substrates for the production of microbial enzymes and source of fermentable sugars for second generation ethanol production**

Agricultural, industrial, forestry and urban solid residues are abundant sources of lignocellulose available worldwide that, in the past, were treated as waste in many countries (Dashtban et al., 2009). In some countries, these materials are still treated as wastes, disposed in the environment, many times without an adequate treatment, raising environmental damage. In other countries, they are also used to generate thermal energy by traditional (firing for cooking and heating) or modern uses (producing electricity and steam, and liquid biofuels) (Dawson & Boopathy, 2007; Kim & Dale, 2004).

With the increasing expansion of agro-industrial activities, large quantities of lignocellulosic residues are generated annually worldwide (Sánchez, 2009). In Brazil, according to the data cited by Ferreira-Leitão et al. (2010), the agroindustry of corn (13767400 ha), sugarcane (7080920 ha), rice (2890930 ha), cassava (1894460 ha), wheat (1853220 ha), citrus (930591 ha), coconut (283205 ha), and grass (140000 ha) collectively occupies an area of 28840726 ha and generates 597 million tons of residue per year.

Biotechnological innovations bring many significant and successful efforts to convert lignocellulosic residues into valuable products including enzymes for biotechnological and industrial applications (Leite et al., 2008; Tengerdy & Szakacs, 2003). However, the cost to produce these enzymes is still an obstacle that needs to be overcome. Therefore, the use of agroindustrial residues as carbon source for fungal growth and ligno/hemi/cellulolytic enzyme production through solid state fermentation (SSF) has been reported in many studies as a way of significantly reducing process cost. Many agricultural and agroindustrial by-products are used in SSF processes for the production of cellulases, xylanases and ligninases, among other enzymes (Table 1) (Gao et al., 2008; Kang et al., 2004).

As previously mentioned fungi are better adapted to SSF and are able to produce copious amounts of cellulases and hemicellulases which are secreted to the medium for easy extraction and purification. Their enzymes are often less complex than bacterial glycoside hydrolases and can therefore be more readily cloned and produced via recombination in a rapidly growing bacterial host, such as *Escherichia coli* (Maki et al., 2009). Besides, there are the white-rot fungi that significantly contribute to lignocellulose conversion since they are the best producers of the lignin degrading enzymes. Table 1 shows a few examples of lignocellulose-degrading enzymes produced by fungi in SSF.

Lignocellulosic residues can also be used to obtain biofuels like bioethanol, named second generation or cellulosic ethanol. New technologies promote the hydrolysis of the carbohydrate fraction of these residues into fermentable sugars that are converted into ethanol by fermenting microorganisms. This technology contrasts with the conventional first generation ethanol production, where carbohydrates of the edible portion (starch, sugar, oils, etc.) of food-based crops are used to be converted into ethanol (Balat et al., 2008).

Among lignocellulosic materials, crop residues are the major potential feedstock for second generation bioethanol production. Table 2 shows the composition of some crops and residues in terms of lignin and carbohydrates, besides the potential yield of second generation ethanol (Kim & Dale, 2004).

With the increase of world population and of the use of fossil fuels, together with constant climate changes, the search for sources of renewable energy has become more necessary, through the coordinated and sustainable actions, in environmental, social and economical aspects. So far, many countries have invested in research to introduce and ensure the prevalence of bioethanol on the market. In this context, Brazil stands out for having an energetic matrix with 46% of renewable sources, more than half of them represented by agroenergy, in a world where the use of these sources is of only approximately 15%. It is the first country to regulate the mandatory addition of a certain percentage of ethanol into motor fuel. In 1975, the Brazilian government implemented the *Proálcool* (Alcohol National Program) for the large scale substitution of petroleum originated vehicle fuels, due to the petroleum crises in 1973 and 1979. This program greatly contributed to the country's alcohol and sugar sector development. Currently, sugarcane and its by-products are the second major source of primary energy of the national energetic matrix and the use of ethanol is already superior to that of gasoline. This experience has won over the world and Brazil has become reference in this area (Statistical Yearbook AgriEnergy, 2009).

In Brazil, first generation ethanol is produced from sugarcane broth, a readily fermentable material, once the substrate (sucrose) is directly used by the fermentative agent, *Saccharomyces cerevisiae*. In the United States alcohol fuel is produced from corn starch, meanwhile in Europe (except for France, which uses beet sugar) wheat and barley starch are mainly used. In both cases, enzymatic hydrolysis of starch is employed, and these countries have, therefore, industrial experience in the use of enzymes for bioethanol production (Bon et al., 2008).

Presently, Brazil ranks No. 1 in using first generation ethanol, obtained from sugarcane broth, with the highest rate in the world (Wang et al., 2011). The country's interest in developing biofuels has driven researches and investments for the diversification of the national energetic matrix. Therefore, the use of sugarcane bagasse (the fibers which are left after milling), a lignocellulosic residue widely available in the country, stands out as a new technology to be explored for the production of second generation ethanol.

Enzymes	Fungi	Substrates	Reference
<b>Endoglucanase</b>	<i>Trichoderma reesei</i> QM9414	Rice bran	Rocky-Salimi & Hamidi-Esfahani, 2009
	<i>Aspergillus terreus</i> M11	Wheat bran and straw, corn cob, reed straw, sugarcane bagasse	Gao et. al, 2008
	<i>Myceliophthora</i> sp. IMI 387099	Rice straw	Badhan et. al, 2007
	<i>Fusarium oxisporum</i> F3 <i>Aspergillus Niger</i> 38	Corn Cob Wheat straw and bran	Panagiotou et al., 2003 Jecu, 2000
<b><math>\beta</math>-glucosidase</b>	<i>Aspergillus sydowii</i> BTMFS 55	Wheat bran	Madhu et al., 2009
	<i>Thermoascus aurantiacus</i> CBMAI 456 and <i>Aureobasidium pullulans</i> ER-16	Wheat bran, soy bran, soy peel, corncob and corn straw	Leite et al., 2008
<b>Xylanase</b>	<i>Neosartorya spinosa</i> D19	Wheat bran	Alves-Prado et al., 2010
	<i>Aspergillus terreus</i>	Palm waste	Lakshmia et al, 2009
	<i>Coprinellus disseminatus</i> <i>Aspergillus carneus</i> M34	Wheat bran Soybean Shell, Rice bran	Singh et al., 2009 Fang et al., 2009
	<i>Fomes sclerodemeus</i>	Wheat bran	Papinutti & Forchiassin, 2007
<b>Laccase</b>	<i>Coriolopsis byrsina</i>	Wheat bran	Gomes et al., 2009
	<i>Lentinus tigrinus</i> BAFC 197	Wheat straw	Lechner & Papinutti, 2006
	<i>Pleurotus</i> sp	Coconut coir	Bhattacharya et al., 2011
	<i>Pleurotus ostreatus</i> MTCC1804 and <i>Anacystis nidulans</i> IU625	Peanut shell	Mishra & Kumar, 2006
	<i>Trametes versicolor</i> <i>Trametes versicolor</i>	Horticultural wastes Sugarcane bagasse	Xin & Geng, 2001 Pal et al., 1995
<b>Manganese peroxidase</b>	<i>Lentinus</i> sp	Wheat bran	Gomes et al., 2009
	<i>Fomes sclerodemeus</i>		Papinutti & Forchiassin, 2007
	<i>Pycnoporus Cinnabarinus</i>	Wood	Alves et al., 2004
	<i>Ceriporiopsis subvermispota</i> <i>P. chrysosporium</i>	Wood waste Wheat straw	Ferraz et al., 2003 Fujian et al., 2001
<b>Lignin peroxidase</b>	<i>Lentinus</i> sp	Wheat bran	Gomes et al., 2009
	<i>Poliporus</i> sp, <i>Hexagonia</i> sp	Wheat bran	Abrahão et al., 2008
	<i>Pleurotus sajor-caju</i>	Banana waste	Reddy et al, 2003
	<i>Pleurotus ostreatus</i> , <i>Phanerochaete chrysosporium</i> <i>P. radiate</i>	sugarcane bagasse corn straw	Pradeep & Datta, 2002 Vares et al., 1995

Table 1. Production of enzymes related to the degradation of vegetable material by fungi cultivation in SSF, using agricultural residues as substrate.

Biomass	Residue/crop ratio	Dry matter <sup>1</sup>	Lignin <sup>1</sup>	Carbohydrates <sup>1</sup>	Ethanol yield <sup>2</sup>
Barley	1.2	88.7	2.90	67.10	0.41
Barley Straw		81.0	9.00	70.00	0.31
Corn	1.0	86.2	0.60	73.70	0.46
Corn stover		78.5	18.69	58.29	0.29
Oat	1.3	89.1	4.00	65.60	0.41
Oat straw		90.1	13.75	59.10	0.26
Rice	1.4	88.6		87.50	0.48
Rice Straw		88.0	7.13	49.33	0.28
Sorghum	1.3	89.0	1.40	71.60	0.44
Sorghum straw		88.0	15.0	61.00	0.27
Wheat	1.3	89.1		35.85	0.40
Wheat straw		90.1	16.0	54.00	0.29
Sugarcane		20.0		67.00	0.50
Sugarcane bagasse	0.6 <sup>3</sup>	71.0	14.50	67.15	0.28

<sup>1</sup>Data in percentage; <sup>2</sup>Data in L/ Kg of dry biomass; <sup>3</sup>Kg of bagasse/Kg of dry sugarcane. (Kim & Dale, 2004).

Table 2. Composition of some lignocellulosic biomass (based on dry biomass) and potential ethanol yield.

The Brazilian production of sugarcane for sugar and alcohol obtainment was of 563,638,524 milled tons in the 2008/2009 harvest (approximately 78 ton/ha). Ethanol yield per sugarcane ton in this harvest season was of approximately 49 L or 3,528 L/ha (Statistical Yearbook AgriEnergy, 2009).

In general, the processing of one sugarcane ton generates approximately 280 kg of bagasse. According to Macedo (2005), about 92% of sugarcane bagasse is used for heat process. If the remaining 8% were to be converted to ethanol, then one could expect an additional 2200 L of ethanol per hectare, bringing the ethanol yield per hectare to 5.728 L, reducing the land use requirements by 29% (Soccol et al., 2010).

Sugarcane bagasse represents the highest residue percentage of the Brazilian agroindustry, with an average of 75 million tons/year (Teixeira et al., 2007). Although a great part is used for thermal energy generation at the manufacturing plant, the excess bagasse represents a great opportunity for the national agro industry. Therefore, there is broadly recognized that the annual sugarcane bagasse production reaches considerable numbers and that the use of this residue is a national need, with wide space for the development of more noble activities than direct energy generation through combustion. According to a few authors, the use of the excess bagasse makes the investments needed to adapt the sugar and alcohol manufacturing plants economically feasible, solving the supplying problem of the sugar and alcohol industry, offering social and environmental advantages and increasing yield of the economical process (Gamez et al., 2006).

Sugarcane composition varies according to many factors, such as the extraction process, sugarcane variety and soil composition. Generally, it is made up of approximately 32-44% cellulose, 27-32% hemicellulose and 19-24% lignin (Malherbe & Cloete, 2002; Howard et al.,

2003). This residue presents small amounts of other compounds such as pectin, protein, minerals and low molecular weight compounds, besides having lower ash content (approximately 2.4%), which represents a great advantage for its bioconversion by microorganisms when compared to other residues such as rice and wheat straw, which contain approximately 17.5 and 11% ash, respectively. In addition, when compared to other agro industrial residues, bagasse can be considered a rich reservoir of energy due to its high yield (approximately 80 t/ha, when compared to 1, 2 and 20 t/ha for wheat, grasses and trees, respectively) and annual regeneration capacity (Pandey et al., 2000).

In Brazil, the South-Central region is responsible for approximately 90% of the sugarcane production and designated an area of 7.01 million hectares for its plantation in 2008. In the São Paulo State alone, 3.82 million hectares (54.5%) were occupied with this crop. The sugar and alcohol sector in Brazil has been keeping a continuous expansion along the past years, which can be noted by the enlargement of sugarcane planted area, construction of new manufacturing plants throughout the national territory and by the production of ethanol in the country (Teixeira et al., 2007). Often, this expansion raises a number of questions regarding its competitiveness with food crops and the possibility of destruction or damage of high biodiversity areas, deforestation and degradation or damaging of soils through the use of chemicals. Studies have demonstrated that such concerns have been grossly exaggerated (Cerqueira Leite et al., 2009; Goldemberg & Guardabassi, 2010). Goldenberg et al (2008) reported that, according Instituto de Economia Agrícola - IEA, a large portion of Brazil has conditions to economically support agricultural production while preserving vast forest areas with different biomass. Most expansion on existing sugarcane crops is taking place on degraded and pasture lands (Lora et al., 2006), areas are far from important biomes like Amazon Rain Forest, Cerrado, Atlantic Forest and Pantanal. Goldenberg et al. (2008) also presented in their paper that sugarcane growth does not seem to have an impact in food areas, since the area used for food crops has not decreased between 1990 and 2006, according data from IBGE and IEA. Besides, the amount of fertilizer used is also small compared to sugarcane crops in other countries (48% more is used in Australia). Authors also consider that there is an increase on ethanol production in Brazil, especially in Sao Paulo State, due to the growth of overall productivity related to the development of new species and to the improvement of agricultural practices.

## **6. Pretreatments and enzymatic saccharification of lignocellulosic biomass aiming ethanol production**

### **6.1 Types and effects of pretreatments**

One of the major obstacles towards saccharification of the lignocellulosic material is its recalcitrant nature. Heterogenous characteristic of biomass particles, surface area and the presence of hemicellulose-lignin complexes covering cellulose contribute to the resistance of lignocellulosic biomass towards hydrolysis (Chang & Holtzapple, 2000).

Structural and constitutive factors reflect the complexity of the lignocellulosic material. The variability of these characteristics contributes to the differences of recalcitrance among lignocellulose sources (Mosier et al., 2005). The removal of lignin, the reduction of cellulose cristalinity and the increase of fiber porosity as a means to facilitate enzyme access may significantly improve saccharification of the lignocellulosic material (McMillan, 1994). This way, the bioconversion of lignocellulosic material into ethanol involves many stages which include: a) pretreatment (physical, chemical and/or biological); b) lignin depolymerization

and carbohydrate hydrolysis; c) conversion of fermentable sugars liberated through the hydrolysis in ethanol and d) ethanol separation and purification.

The objective of any technology related to the pretreatment of lignocellulosic material is to minimize or remove the constraints of hydrolysis, improving enzymatic hydrolysis rate, thus increasing the yield of fermentative sugars. Generally, pretreatments alter the structure of lignocellulosic biomass, making it more accessible to the action of depolymerizing enzymes.

The success of pretreatments is based on a few factors such as: obtainment of fermentable sugars or of a facilitated subsequent obtainment of these sugars through enzymatic hydrolysis; no formation or low formation of products that may inhibit both enzymatic hydrolysis and the action of fermentative microorganisms; be a low cost technology (Sun & Cheng, 2002). Studies have shown that the pretreatment step defines at which extension and cost the carbohydrates will be converted into ethanol (Chandel et al., 2007). It should also be mentioned that lignin removal, during the pretreatment, is important due to the possibilities of its industrial use (Gopalakrishnan et al., 2010).

Table 3 shows a few examples of lignocellulosic biomass pretreatments. It should be mentioned that not all of these methods have been completely established in such a way as

	<b>Processes</b>	<b>Mechanism of changes on biomass</b>
<b>Physical</b>	Ball-milling; Two-roll milling; Hammer milling; Colloid milling; Vibro energy milling ; Hydrothermal; High pressure steaming; Extrusion; Expansion; Pyrolysis; Gamma-ray irradiation; Electron-beam irradiation; Microwave irradiation.	Increase in accessible surface area and pores size; decrease in cellulose crystallinity and polymerization degree; partial hydrolysis of hemicellulose and lignin depolymerization
<b>Physicochemical and chemical</b>	EXPLOSION: Steam explosion; Ammonia fiber explosion (AFEX); CO <sub>2</sub> explosion; SO <sub>2</sub> explosion. ALKALI: Sodium hydroxide; Ammonia; Ammonium sulfite. GAS: Chlorine dioxide; Nitrogen dioxide ACID: Sulfuric acid; Hydrochloric acid; Phosphoric acid; Sulfur dioxide. OXIDING AGENTS: Hydrogen peroxide; Wet oxidation; Ozone. CELLULOSE SOLVENTS: Cadoxen; CMCS; SOLVENT EXTRACTION OF LIGNIN: Ethanol-water extraction; Benzene- water extraction; Ethylene-water extraction; Butanol-water extraction; Swelling agents.	Delignification; decrease of cellulose crystallinity and polymerization degree; partial or complete hemicellulose hydrolysis
<b>Biological</b>	Actinomycetes and Fungi	Delignification; reduction of cellulose and hemicellulose polymerization degree

Source: Taherzadeh & Karimi, 2007.

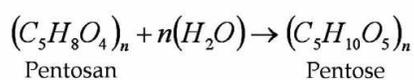
Table 3. Pretreatment methods of lignocellulosic materials for enzymatic hydrolysis.

to be technically and economically feasible for large scale processes. In some cases, a method is initially employed to increase the efficiency of another method to be used afterwards. Besides, it should be taken in consideration that the choice of pretreatment has to be compatible to the subsequent hydrolysis step. Hydrolysis with diluted acids (gentle acid treatments) is probably the most widely used amongst the chemical pretreatments, and it can be used for direct obtainment of fermentable sugars, originated from hemicellulose fraction or as precedent of enzymatic hydrolysis (Taherzadeh & Karimi, 2007). One of the main advantages of this type of pretreatment is the high xylose yield, from xylan degradation, representing an important economy in the ethanol production process as a whole, given that approximately 90% of sugars from hemicellulose are this way recovered (Sun & Cheng, 2002). However, *S. cerevisiae*, the most used microorganism for industrial ethanol fermentation processes, and the majority of bacteria and other yeasts do not utilize D-Xylose. Thus, there has been a great emphasis in the last two decades on developing an efficient organism for xylose fermentation through metabolic engineering (Jeffries, 2006; Martín et al., 2007a).

In the pretreatment with diluted acids, cellulose and lignin fractions remain unaltered. On the other hand, some disadvantages are the need of pH neutralization for the following enzymatic hydrolysis process, high maintenance cost due to corrosion problems and possible generation of cellulase activity inhibitors, furfural and other condensation products generated from the rapid xylose degradation, which should be removed through biomass washing prior to enzymatic hydrolysis (Sun & Cheng, 2002). Cellulose, on the other hand, needs more severe hydrolysis conditions, considering its crystalline nature. Acid-catalyzed cellulose hydrolysis is a complex and heterogeneous reaction, involving physical and chemical factors following the mechanism of  $\beta$ -1,4 glycosidic bond cleavage (Xiang et al., 2003).

Pretreatment with diluted sulphuric acid generally reaches high reaction rates and significantly provides cellulose hydrolysis (Esteghlalian et al., 1997). In a general way, acid hydrolysis reactions may be represented by:

1<sup>st</sup> hydrolyze step



Pentosan

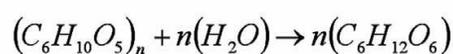
Pentose



Pentose

Furfural

2<sup>nd</sup> hydrolyze step



Pentose

Hexose

The explosion, with or without the presence of vapor, is one of the most cited pretreatment methods for lignocellulosic biomass. Vapor explosion removes most of the hemicellulose, promotes lignin depolymerization, facilitating the access of cellulases to their substrate (Moniruzzaman, 1996) decreasing the amount of lignin available for non specific and unproductive bonding to cellulases and hemicellulases. In this method, biomass is treated with saturated vapor (160-260 °C) for a few seconds to minutes, under high pressure (0.69-4.83 MPa), which is abruptly reduced to atmospheric pressure, making the material go through an explosive decompression (Varga et al., 2004). Adding H<sub>2</sub>SO<sub>4</sub> (or SO<sub>2</sub>) or CO<sub>2</sub> to the vapor explosion process may significantly improve enzymatic hydrolysis, decrease the formation of inhibiting compounds and promote a more complete hemicellulose removal (Morjanoff & Gray, 1987). The advantages of vapor explosion pretreatment include the low energetic requirement, when compared to mechanical fragmentation, and the absence of environmental or recycling costs. This type

of pretreatment is amongst the ones with lower cost for hardwood and agricultural residues (Clark & Mackie, 1987). Disadvantages of this method include incomplete dissociation of the lignin-carbohydrates matrix and formation of compounds that may have inhibitory effect on microorganisms used during the downstream processes (microbial growth, enzymatic hydrolysis and/or fermentation) (Mackie et al., 1985), which leads to the need of washing the pretreated biomass (McMillan, 1994). Washing decreases the overall yield of the saccharification process, once it removes part of the soluble sugars generated by hemicelluloses' hydrolysis (Mes-Hartree et al., 1988).

## 6.2 Enzymatic saccharification

The enzymatic hydrolysis of lignocellulosic biomass is advantageous when compared to the chemical conversion, once there is no substrate loss due to chemical modifications and moderate and non corrosive operational conditions may be used. In addition, it provides better yields since it does not generate collateral products. During lignocellulose saccharification, the use of auxiliary enzymes such as feruloyl esterases and ligninases is interesting, given that it facilitates the access of cellulases and hemicellulases to their substrates (Sandgren et al, 2005; Selig et al., 2008). Therefore, complex enzymatic preparations are more efficient for the degradation of lignocellulosic material than those that do not have the concerted action of these enzymes (Yu et al., 2005).

The different biomass pretreatment strategies and the optimization of the enzymes production processes have resulted in several-fold reduction of enzyme loading for lignocellulose hydrolysis (Merino & Cherry, 2007) but the efficient enzymatic conversion of cellulose and hemicellulose polymers is still the major bottleneck in the utilization of lignocellulosic residues for bioethanol production. Thus, the need of obtaining efficient enzyme mixtures, with the minimal number of key enzymatic activities, in an optimal combination is clear. Besides, the optimal reaction conditions and minimal dosage requirements must be known (Meyer et al., 2009).

With the cost reduction for enzyme production, caused by the use of alternative and inexpensive substrates such as the lignocellulosic residues or by the use of modern technologies for production optimization such as cloning and heterologous superexpression, enzymatic hydrolysis cost tends to become relatively low when compared to acid hydrolysis. Moreover, there is a possibility of recover and recycle of the enzymes bound to the residual substrate as well as the enzymes from reaction suspension.

One of the great disadvantages of the enzymatic hydrolysis of lignocellulosic material is related to the inhibition by the products of carbohydrate hydrolysis, especially when concerning enzymes from the cellulolytic complex, especially  $\beta$ -glucosidases, key enzymes for the complete saccharification of cellulose into glucose. The low activity of this enzyme or its inhibition by glucose ( $K_i$  of most  $\beta$ -glucosidases from typical cellulase-producing microorganisms is 1–14 mmol L<sup>-1</sup> glucose) (Yun et al., 2001; Decker et al., 2000) results in cellobiose accumulation, a potent inhibitor of cellobiohydrolases and endoglucanases, decreasing hydrolysis rates (Holtzapple et al., 1990; Kovács et al., 2009). Inhibition of the cellulases by hemicellulose-derived sugars has also been shown (Xiao et al., 2004).

The addition of a surplus of  $\beta$ -glucosidase activity is an alternative to overcome the competitive product inhibition of the  $\beta$ -glucosidases. However, the use of high concentrations of this enzyme to reduce inhibition problems does not seem advantageous, once it can significantly increase process cost. Another strategy is to screen for  $\beta$ -

glucosidases with high glucose tolerance.  $\beta$ -glucosidases with  $K_i$  up to 1400 mmol L<sup>-1</sup> have been reported (Decker et al., 2000) and these could be cloned into the cellulase-producing microorganisms to produce a more efficient enzyme mixture. The removal of sugars during hydrolysis by ultrafiltration or by employing the simultaneous saccharification and fermentation process (SSF), where the sugars produced during enzymatic hydrolysis are simultaneously fermented to ethanol, have also been reported as alternatives to overcome the problem of enzyme inhibition by the final products of carbohydrate degradation (Sun & Cheng, 2002; Jeffries & Jin, 2000).

It has been shown that ethanol also inhibits cellulases, although less intensely when compared to glucose (Holtzapple et al., 1990; Chen & Jin, 2006). Cellulases inhibition by ethanol follows a noncompetitive inhibition pattern for ethanol concentrations less than 4 M and, when the ethanol concentration is increased, the enzyme is denatured. Ethanol also interferes with enzyme (mainly cellobiohydrolases) adsorption to cellulose and modifies the cooperative effect between cellobiohydrolases and endoglucanases (Ooshima et al., 1985; Holtzapple et al., 1990).

During the pretreatment, the lignocellulose degradation products and hemicellulose-derived monomeric sugars formed and released into the liquid fraction (prehydrolysate) have also been shown to inhibit enzymes activities, since the remaining solid fraction (amorphous cellulose and lignin) is absorbed with this liquid up to 60–90% of its total weight. Among these degradation products we can mention organic acids (acetic acid, formic acid, and levulinic acid), sugar degradation products (furfural from xylose and 5-hydroxymethylfurfural - HMF - from hexoses at high temperature and pressure) and lignin degradation products (vanillin, syringaldehyde, and 4-hydroxybenzaldehyde) (Palmqvist et al., 1999a; Cantarella et al., 2004). However, the inhibition of enzymatic hydrolysis by these products has not been clearly elucidated (Jorgensen et al., 2007). It has been shown that washing the pretreated material results in faster conversion of cellulose due to removal of inhibitors (Tengborg et al., 2001). Ultrafiltration has also been used to remove sugars and other small compounds that may inhibit the action of the enzymes.

Another obstacle to enzymatic hydrolysis of lignocellulose carbohydrates is the possibility of unspecific adsorption of enzymes (both cellulases and hemicellulases) onto lignin particles or surfaces, mainly due to hydrophobic interaction and, possibly, due to ionic-type lignin-enzyme interaction. Actually, after almost complete hydrolysis of the cellulose fraction in lignocellulosic material, up to 60–70% of the total enzyme added can be bound to lignin (Lu et al., 2002). Therefore, cellulases with lower affinity for lignin could be explored in the development of new enzymatic complexes preparations (Berlin et al., 2005; Palonen et al., 2004).

The trouble of unspecific enzymes adsorption to lignin could be overcome by the addition of non-ionic surfactants like Tween 20 or Tween 80. It could also improve the hydrolysis rate so that the same degree of conversion can be obtained at lower enzyme loadings. Ethylene oxide polymers such as poly(ethylene glycol) (PEG) show a similar effect and it is advantageous due to its low cost (Kristensen et al., 2007). Besides the use of surfactants, other methods for desorbing enzymes have been developed, such as use of alkali, urea and buffers of varying pH (Otter et al., 1989).

As previously mentioned, recycling of the enzymes from the reaction suspension as well as from the residual substrates is an attractive way of reducing costs for enzymatic hydrolysis (Qi et al., 2011). The addition of fresh substrate could recover free cellulases in bulk solution

by adsorption, due to the high affinity of these enzymes for cellulose (Castanon & Wilke, 1980). The new material retaining up to 85% of the enzyme activity free solution could then be separated and hydrolyzed in fresh media eventually with supplementation of more enzyme (Tu et al., 2007). Since  $\beta$ -glucosidase does not typically bind to the cellulosic substrate it cannot be reused and supplementation with this enzyme is required at the beginning of each round of hydrolysis in order to avoid the buildup of cellobiose and the subsequent end-product inhibition of cellulase (Lee et al., 1995; Tu et al., 2007).

Ultrafiltration has been cited as viable process capable of recovering all of enzyme components (endoglucanase, exoglucanase and  $\beta$ -glucosidase) after complete hydrolysis of the cellulose (Mores et al., 2001; Qi et al., 2011). Depending on the lignin content of the substrate, only up to 50% of the cellulases can be recycled using this approach. The saving is therefore low, taking into account recovery costs (Singh et al., 1991; Lee et al., 1995).

The denaturation or loss of enzyme activity due to mechanical shear, proteolytic activity or low thermostability should also be considered as limiting factors for hydrolysis. Besides, due to cellobiohydrolases processivity and strong binding to cellulose chain (by the catalytic site) obstacles can make the enzymes halt and become unproductively bound. Summarizes the factors that limit efficient cellulose hydrolysis (Jorgensen et al., 2007).

The range of toxic compounds generated during some types of pretreatment and hydrolysis of lignocellulosic materials, mainly with high temperature and pressure, under acidic conditions, can limit the rapid and efficient fermentation of the hydrolysates by the fermenting microorganisms, such as *Saccharomyces cerevisiae*. The inhibiting compounds are divided in three main groups based on origin: weak acids, furan derivatives and phenolic compounds. As mentioned above, furfural and HMF are formed from xylose and hexoses respectively and when they are broken down, they generate formic acid. HMF degradation also yields levulinic acid. Besides, the partial lignin breakdown generates phenolic compounds (Palmqvist & Han-Hagerdal, 2000).

Undissociated weak acids inhibit cell growth since they are liposoluble when undissociated and can diffuse across the plasma membrane. In the cytosol, dissociation of the acid occurs due to the neutral intracellular pH, thus decreasing the cytosolic pH (Pampulha & Loureiro-Dias, 1989) and cell viability. According to Verduyn et al. (1990), when fermentation pH is low, cell proliferation and viability are inhibited also in the absence of weak acids, due to the increased proton gradient across the plasma membrane, resulting in an increase in the passive proton uptake rate.

Studies have been reported that furfural is metabolized by *S. cerevisiae* under aerobic, oxygen-limiting and anaerobic conditions (Taherzadeh et al., 1998; Navarro, 1994; Palmqvist et al., 1999b). Furfural is reduced to furfuryl alcohol during fermentation, with high yields, and the reduction increases with the increasing of inoculum size and of specific growth rate in chemostat (Fireoved & Mutharasan, 1986) and batch cultures (Taherzadeh et al., 1998). At high furfural concentrations (above 84 mmol.g<sup>-1</sup>) the reduction rate decreases in anaerobic batch fermentation, probably due to cell death (Palmqvist et al., 1999b). Aerobic growth is less sensible to inhibition by furfuryl alcohol in *S. cerevisiae* than in *Pichia stipitis* (Weigert et al., 1988; Palmqvist et al., 1999b). According to Palmqvist et al. (1999a) growth is more sensitive to furfural than is ethanol production. Indeed, at low concentrations of furfural (approximately 29 mmol/L) there is an invrease in ethanol yield. The authors reported that this probably occurs because the reduction of furfural to furfuryl alcohol, by NADH-dependent yeast dehydrogenases (which regenerates NAD<sup>+</sup>) has a higher priority than the

reduction of dihydroxyacetone phosphate to glycerol (which regenerates NADH). Thus the lower carbon consumption for glycerol production leads to an increase in ethanol yield.

Cell integrity is harmed by phenolic compounds, especially those of low molecular weight, since they affect the membrane ability to act as selective barrier and enzyme matrix (Heipieper et al., 1994). These compounds have a considerable inhibitory effect during the fermentation of lignocellulosic hydrolysates, by a not elucidated mechanism (Delgenes et al., 1996).

Another obstacle for the efficient enzymatic saccharification of lignocellulosic material is related to the cellulase recycling (turnover), since the absorption characteristics of these enzymes on lignocellulosic substrates have not yet been completely understood. The enzymatic degradation of cellulose is a complex process that occurs at the limit of solid/liquid phases, where the enzymes are the mobile components. When the cellulases act in vitro on the insoluble substrate, three processes occur simultaneously: (a) physical and chemical changes of cellulase at the solid phase (still not solubilized); (b) primary hydrolysis, involving the liberation of soluble intermediates from the surface of cellulose molecules that are in reaction and (c) secondary hydrolysis, involving the hydrolysis of soluble intermediates into others of low molecular weight and, finally, into glucose (MOISER; LADISCH; LADISCH, 2002).

In a general way, enzymatic hydrolysis rate of the lignocellulosic material rapidly decreases, with cellulose enzymatic degradation being characterized by a fast initial phase, followed by a slow secondary phase, which can last until all the substrate is degraded. This has been frequently explained by the rapid hydrolysis of the easily accessible cellulosic fraction, by strong enzyme inhibition, especially  $\beta$ -glucosidases, by the product and the low inactivation of absorbed enzyme molecules (Balat et al., 2008).

Cellulose is an insoluble substrate; the adsorption of the cellulases onto the cellulose surface is the first step in the initiation of hydrolysis. Therefore, the presence of CBMs is essential for fast and correct docking of the cellulases on the cellulose. Removal of CBMs significantly lowers the hydrolysis rate on cellulose (Suurnäkki et al., 2000).

## 7. Strategies for second generation ethanol production

Saccharification of lignocellulosic material and the conversion of sugars into ethanol may employ different strategies, carried out simultaneously or sequentially. In all cases, the pretreatment stage is of crucial importance to increase enzymatic conversion efficiency.

When enzymatic hydrolysis and alcoholic fermentation are carried out separately, the process is known as Separate (or Sequential) Hydrolysis and Fermentation (SHF). In this case, the enzymatic hydrolysis of the carbohydrates and the subsequent fermentation of hexoses and pentoses are carried out in distinct reactors and they can be performed under their optimum conditions, which is an advantage of this strategy. However, SHF leads to the accumulation of the glucose derived from the hydrolysis of cellulose that can inhibit cellulases, affecting the reaction rates and yields. Besides, part of glucose is adsorbed in the solid residual material, lowering the sugar conversion (Soccol et al., 2010; Olofsson et al., 2008).

Enzymatic hydrolysis and sugar fermentation can run together, in a same reactor, as Simultaneous Saccharification and Fermentation (SSF), is faster and presents a low cost process since only one reactor is necessary and the glucose formed is simultaneously

fermented to ethanol, which also avoid the problem of product inhibition associated with enzymes. The risk of contamination is lower due to the presence of ethanol, the anaerobic conditions and the continuous withdrawal of glucose. Pentoses fermentation can be performed in a separate reactor. One disadvantage of this strategy is relates to the different optimum temperature for enzymatic hydrolysis (45–50 °C) and alcoholic fermentation (28–35 °C) (Soccol et al., 2010).

The process called Simultaneous Saccharification and Co Fermentation SSCF, pentoses and hexoses conversion are carried out in the same reactor (Castro; Pereira Jr, 2010). Finally, in the Consolidated BioProcessing (CBP) a single microbial community produced all the required enzymes and converts sugars into ethanol in a single reactor (Lynd, 1996), lowering overall costs. Studies suggest that CBP may be feasible and the researches have focused on the development of new microorganisms adapted to this process, which has been a key challenge (Lynd et al., 2002).

## 8. Conclusions

The search for “clean technologies”, using alternative feedstocks, in order to obtain products of industrial interest, save energy and reduce effluent production is economically advantageous and has been encouraged by environmental issues during the last years. Researches dealing with the use of lignocellulosic wastes in bioprocesses, specially for microorganisms cultivation and cellulases, xylanases, ligninases and other enzymes production, stand out. These enzymes have potential for various biotechnological applications and in recent years special attention has been given to the destructuring, hydrolysis and saccharification of lignocellulosic material in order to obtain fermentable sugars that can be converted into second generation ethanol by fermenting microorganisms. However, for an efficient conversion of lignocellulosic materials into products of industrial interest, some bottlenecks must be overcome. The search for microbial strains suitable for cultivation in large scale, producing enzymes with characteristics appropriate to the biotechnological processes to which they are intended is of great importance.

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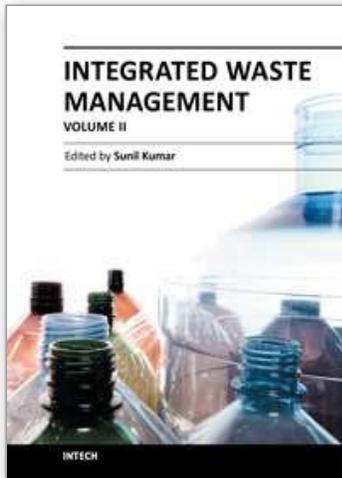
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