

# Pretreatment Strategies for Delignification of Sugarcane Bagasse: A Review

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## ABSTRACT

*The valorization of agro-residues by biological routes is a key technology that contributes to the development of sustainable processes and the generation of value-added products. Sugarcane bagasse is an agro-residue generated by the sugar and alcohol industry in Brazil (186 million tons per year), composed essentially of cellulose (32-44%), hemicellulose (27-32%) and lignin (19-24%). The conversion of sugarcane bagasse into fermentable sugars requires essentially two steps: pretreatment and hydrolysis. The aim of the pretreatment is to separate the lignin and break the structure of lignocellulose, and it is one of the most critical steps in the process of converting biomass to fermentable sugars. The aim of this review is to describe different pretreatment strategies to promote the delignification of the sugarcane bagasse by thermo-chemical and biological processes.*

**Key words:** Sugarcane Bagasse, Lignocellulose, Pretreatment, Delignification

## INTRODUCTION

The valorization of agro-residues by biological routes is a key technology that contributes to the development of sustainable processes and the generation of value-added products. Brazilian economy is one of the most important agricultural-based economies in the world, being the first ranked worldwide in production and exportation of coffee, sugarcane, tropical fruits, beans and meat; second ranked in the production of ethanol, soybean and cassava and the third ranked in the production of corn. The residues generated by this intense agricultural activity represent potential feedstock that could be inserted in diverse production chains instead of discarding them (Soccol and Vandenberghe 2003; Singhania et al. 2009).

Agro-industrial wastes are interesting substrates for fermentative processes since they are easily available, rich in carbon and often represent a problem of disposal (Gassara et al. 2010). There are several publications describing bioprocesses to use the wastes such as hulls and bagasse as raw materials to produce ethanol, single-cell protein, mushrooms, enzymes, organic acids, amino acids, biologically active secondary metabolites, among other products (Soccol and Vandenberghe 2003). An important example is the production of second-generation biofuels, i.e., biofuels produced from non-food resources such as sugarcane bagasse. Sugarcane bagasse is a lignocellulosic agro-residue generated in high amount by the sugar and alcohol industry in Brazil. One of the main challenges in the utilization of lignocellulosic biomass in fermentative processes is the

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transformation of the complex polysaccharides into simple sugars that can be assimilated by the microorganisms. This can be achieved by chemical or enzymatic hydrolysis, preceded by appropriate pretreatments that enhance the efficiency of hydrolysis. The aim of the pretreatment is to separate lignin and break the structure of lignocellulose, and it is one of the most expensive steps in the process of converting biomass to fermentable sugars (Binod et al. 2012). The aim of this review is to describe different pretreatment strategies to promote delignification of the sugarcane bagasse by thermo-chemical and biological processes.

### CHARACTERISTICS OF THE SUGARCANE BAGASSE

Sugarcane bagasse is an agro-residue generated in high amount (186 million tons / year) by the sugar

and alcohol industry in Brazil. It is a porous residue of cane stalks left over after the crushing and extraction of the juice from sugarcane (Soccol et al. 2011a). Sugar mills generate approximately 270-280 kg of bagasse (50% moisture) per metric ton of sugarcane (Rodrigues et al. 2003). Considering that about 92% of the sugarcane bagasse is burned in the industry for process heat generation, if the remaining 8% could be used for second generation ethanol production, an additional ethanol yield of 2,200 L would be produced per hectare of sugarcane. The production of ethanol from sugarcane bagasse, through hydrolysis and fermentation, could yield about 280-330 L per ton of dry bagasse considering a cellulose content of 40% in the bagasse (Leite et al. 2009). Table 1 presents the chemical composition of sugarcane bagasse reported by different authors.

**Table 1** - Chemical composition of sugarcane bagasse.

Component	Soccol et al. (2011a)	Rocha et al. (2011)	Bertoti et al. (2009)
Cellulose	32-44%	45.5%	47.5-51.1%
Hemicellulose	27-32%	27%	26.7-28.5%
Lignin	19-24%	21.1%	20.2-20.8%
Extractives	-	4.6%	0.8-3% other
Ashes	4.5-9%	2.2%	compounds

Lignin is present in the cellular wall and confers structural support, impermeability and resistance against microbial attack and oxidative stress, and among the components of lignocellulose, it is the most recalcitrant to biodegradation. Lignin is formed from three precursor alcohols: *p*-hydroxycinnamyl (coumaryl) alcohol, which forms *p*-hydroxyphenyl units in the polymer; 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, the guaiacyl units; and 3,5-dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol, the syringyl units. Free radical copolymerization of these alcohols produces the heterogeneous, optically inactive, cross-linked and highly polydisperse polymer (Lee 1997; Soccol et al. 2011b).

Cellulose is a linear polymer composed of D-glucose subunits linked by  $\beta$ -1,4 glycosidic bonds forming the dimer cellobiose. These form long chains (or elemental fibrils) linked together by hydrogen bonds and intra- and inter-molecular van der Waals forces. Cellulose is usually present as a crystalline form and a small amount of non-organized cellulose chains forms amorphous

cellulose. In the latter conformation, cellulose is more susceptible to enzymatic degradation (Pérez et al. 2002).

Hemicellulose is a polysaccharide with a lower molecular weight than cellulose. It is formed from D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids, depending on the hemicellulose source. Sugars are linked together by  $\beta$ -1,4- and sometimes by  $\beta$ -1,3-glycosidic bonds (Soccol et al. 2011b).

The conversion of sugarcane bagasse into fermentable sugars requires essentially two steps: pretreatment and hydrolysis. Hydrolysis is conducted in the presence of enzymes (exo-glucanases, endo-glucanases and cellobiases) or mineral acids and releases glucose units from the cellulose molecules (Hernández-Salas et al. 2009). Cellulose is the main targeted polysaccharide because it is composed only by D-glucose units, which is a fermentable sugar for most microorganisms. Pentoses released from

hemicellulose, however, are not fermented by most of the yeasts used for ethanol production.

The enzymatic hydrolysis is a multi-step reaction that takes place in a heterogeneous system, in which insoluble cellulose is initially broken down at the solid-liquid interface via the synergistic action of endoglucanases (EC 3.2.1.4) and exoglucanases / cellobiohydrolases (EC 3.2.1.91). Subsequently, a liquid phase hydrolysis of soluble intermediate products takes place, i.e., short cellulo-oligosaccharides and cellobiose that are catalytically cleaved to produce glucose by the action of  $\beta$ -glucosidase (EC 3.2.1.21) (Andric et al. 2010). The cost of utilities for enzymatic hydrolysis is low compared to acid, or alkaline hydrolysis because it is usually conducted at mild conditions and does not cause corrosion problems (Duff and Murray 1996). However, the cost of enzymes can limit their application.

## PRETREATMENT STRATEGIES FOR DELIGNIFICATION OF SUGARCANE BAGASSE

Pretreatment is a very important step in the conversion of lignocellulose to fermentable sugars because the crystallinity of cellulose, degree of polymerization, moisture content, available surface area and lignin content are factors that hinder the action of the hydrolysis agents. The aim of the pretreatment is to separate the lignin and break the structure of lignocellulose, and it is usually performed by thermo-chemical processes. It is one of the most expensive and least technologically mature steps in the process of converting biomass to fermentable sugars. In order to be effective, pretreatment should avoid the need for reducing the size of biomass particles, preserve the pentose (hemicellulose) fractions, limit the formation of degradation products, minimize energy demands and the pretreatment agent should have low cost and be capable of recycling inexpensively (Binod et al. 2012).

Many methods have been used for pretreating the lignocellulosic materials. These are steam explosion, alkali washing, dilute acid hydrolysis, ammonia fiber expansion, liquid hot water and wet oxidation, etc. (Glasser and Wright 1997; Ramos et al. 1992, 2000; Martín et al. 2007; Zhang et al. 2007; Balat et al. 2008; Hernández-Salas et al. 2009; Hendriks and Zeeman 2009).

### Steam Explosion

Steam explosion is one of the most common methods for the pretreatment of lignocellulosic biomass (Soccol et al. 2011b) and can be performed in the presence, or absence of a catalyst (alkali, or acid). The grinded biomass is treated with high-pressure saturated steam at temperatures varying from 160 to 260°C and pressures of 0.69 to 4.83 MPa, and then the pressure is quickly reduced, which makes the material undergo an explosive decompression. The process causes the disrupting the structure of the material, degradation of hemicellulose and lignin transformation due to the high temperature, thus facilitating the subsequent hydrolysis of cellulose (Öhgren et al. 2007).

Rocha et al. (2012) reported the results of a steam explosion treatment of the sugarcane bagasse containing approximately 50% of moisture. Steam was injected in the reactor up to a pressure of almost 1.3 MPa equivalent to 190°C and was maintained for 15 min. After this period, the reactor was suddenly depressurized. The treatment solubilized an average of  $82.7 \pm 4.3\%$  of the hemicelluloses. Cellulose was hydrolyzed at the ratio of  $11.8 \pm 3.7\%$ , which probably corresponded to the polymer amorphous region. Lignin was solubilized at the proportion of  $7.9 \pm 9.1\%$ .

A study developed by Kaar et al. (1998) varying the temperature and residence time in the steam explosion treatment of sugarcane bagasse showed that the furfural content that represented the degradation of sugars, reported as a percentage of original xylose, was higher for higher severity and/or low temperature samples. The observed relationship with respect to the temperature at constant severity could be due to the thermal decomposition/polymerization of the furfural in the reactor during the runs.

### Alkaline Pretreatment

Alkaline pretreatment has received a lot of attention lately because it can remove the lignin from the biomass, thus improving the reactivity of the remaining polysaccharides and removing acetyl groups and various uronic acid substitutions on hemicellulose (Chen et al. 2011). The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and other components, for example, lignin and other hemicelluloses. Dilute NaOH treatment of lignocellulosic material causes swelling, leading to

an increase of internal surface area, a decrease in the degree of polymerization, a decrease in the crystallinity, separation of structural linkages between lignin and carbohydrates and disruption of the lignin structure (Fan et al. 1987; Soccol et al. 2011b). In the process of wood delignification, alkaline treatment results in a residue, called black liquor that must be disposed of.

In the delignification process developed by Rocha et al. (2012), the steam explosion pretreated bagasse was reacted with a 1.0% NaOH solution (w/v), using a solid-liquid ratio of 1:10 (w/v). The operation was carried out at 100°C for 1 h and there was an excellent removal of lignin from the biomass (92.7±3.9%).

The process hydrolyzed 31.1±3.5% of the cellulose and the percentage of hemicellulose hydrolysis was 94.7±0.9%.

The chemical pretreatment for sugarcane bagasse developed by Aguiar et al. (2010) to produce exoglucanases and endoglucanases resulted in better breakage of the fibers when using 2% H<sub>2</sub>O<sub>2</sub> together with 1.5% NaOH at 121°C for 15 min. This treatment increased the cellulose level up to 1.2 times and decreased the hemicellulose content 8.5 times, promoting a better accessibility of the fungi to the fibers.

### Acid Pretreatment

Dilute acid pre-hydrolysis can be an effective pretreatment process for sugarcane bagasse. Sulfuric acid is the most commonly used acid in the pretreatment of sugarcane bagasse (Lavarack and Griffin 2002) but other reagents such as hydrochloric, nitric and phosphoric acids can also be used (Rodríguez-Chong et al. 2004; Gámez et al. 2006). The use of acetic acid and hydrogen peroxide has been reported by Tan et al. (2010) as a method for removing lignin prior to bagasse enzymatic hydrolysis.

According to Chen et al. (2011), the pretreatment using dilute sulfuric acid has been considered as one of the most cost-effective methods. The mixture of biomass and dilute acid solution is usually controlled at a moderate temperature by means of conventional heating, or microwave-assisted heating, which is another effective route to pretreat the biomass. The electromagnetic field used in microwaves may create non-thermal effects that also accelerate the destruction of crystal structures. The process developed by Binod et al. (2012) using microwave-alkali (1% NaOH), followed by acid pretreatment (1% H<sub>2</sub>SO<sub>4</sub>) and

enzymatic hydrolysis gave an overall reducing sugar yield of 0.83 g/g dry sugarcane bagasse.

During the hot acid pretreatment, some of the polysaccharides are hydrolyzed, mostly hemicelluloses. The resulting free sugars can degrade to furfural (from pentoses) and to 5-hydroxy-methyl-furfural, or HMF (from hexoses). These compounds are inhibitory for the microorganisms, and their production means loss of fermentable sugars. Organic acids such as maleic and fumaric have been suggested as alternatives to avoid HMF formation (Kootstra et al. 2009).

### Ammonia Fiber Expansion

Ammonia fiber expansion (AFEX) is a process in which liquid ammonia is added to the biomass under moderate pressure (100 to 400 psi) and temperature (70 to 200°C) before rapidly releasing the pressure (Bals et al. 2010). This process decrystallizes the cellulose, hydrolyses hemicellulose, removes and depolymerises lignin and increases the size and number of micropores in the cell wall, thereby significantly increasing the rate of enzymatic hydrolysis (Mosier et al. 2005). As reported by Krishnan et al. (2010), the AFEX pretreatment improved the accessibility of cellulose and hemicelluloses in sugarcane bagasse during enzymatic hydrolysis by breaking down the ester linkages and other lignin carbohydrate complex bonds. The maximum glucan conversion of the AFEX pretreated bagasse and cane leaf residue by cellulases was approximately 85%, and the supplementation with hemicellulases during enzymatic hydrolysis improved the xylan conversion to 95-98%.

### Organosolv

The treatment with organosolvents involves the use of an organic liquid and water, with or without the addition of a catalyst (acid, or alkali). Organosolv pretreatments efficiently remove lignin from lignocellulosic materials through the partial hydrolysis of lignin bonds, resulting in a pulp enriched in cellulose. The addition of a catalyst can enhance the selectivity of the solvent with respect to lignin. Most of the hemicellulose sugars are also solubilized by this process (Sun and Cheng 2002; Mesa et al. 2011). This technique presents advantages when compared with aqueous based processes. In particular, the recovery of lignins and polyoses from the liquor is easily performed by distillation with the simultaneous

recycling of solvents (Novo et al. 2011). Mesa et al. (2011) demonstrated that the combination of a dilute-acid pretreatment followed by the organosolv pretreatment with NaOH under optimized conditions (60 min, 195°C, 30% v/v ethanol) was efficient for the fractionation of sugarcane bagasse for subsequent enzymatic hydrolysis, yielding a residual solid material containing 67.3% (w/w) glucose, which was easily recovered by the enzymatic hydrolysis. Novo et al. (2011) developed a process using glycerol- water mixtures and obtained a pulp with a residual lignin amount lower than 8%; the delignification was close to 80% and residual cellulose content was higher than 80%.

### Liquid Hot Water

Liquid hot water is a hydrothermal pretreatment, where pressure is applied to maintain the water in the liquid state at elevated temperature. Temperatures in the range of 170-230°C and pressure higher than 5 MPa are commonly used (Talebnia et al. 2010). This subcritical fluid presents particular properties in relation to water at ambient conditions, i.e., dielectric strength and ionic product and these properties can be easily controlled as the functions of pressure and temperature (Schacht et al. 2008).

The treatment presents high yields and low generation of undesired products (Hamelinck et al. 2005). Allen et al. (1996) described a process to fractionate the sugarcane bagasse and leaves using liquid hot water at 190-230°C by rapid immersed percolation (45 s to 4 min). Over 50% of the biomass could be solubilized; all of the hemicellulose together with more than 60% of the acid-insoluble lignin was solubilized, while less than 10% of the cellulose entered the liquid phase. The recovery of the hemicellulose as monomeric sugars after a mild post-hydrolysis exceeded 80% and less than 5% of the hemicellulose was converted to furfural.

### Wet Oxidation

Wet oxidation is a hydrothermal treatment, the process of treating material with water and air, or oxygen at temperatures above 120°C (McGinnis et al. 1983). Two types of reactions occur during wet oxidation: a low-temperature hydrolytic reaction and a high-temperature oxidative reaction (Martín et al. 2007).

Martín et al. (2007) demonstrated that alkaline wet oxidation at 195°C during 15 min yielded a solid

material with nearly 70% of cellulose, with a solubilization of approximately 93% of hemicelluloses and 50% of lignin, and an enzymatic cellulose convertibility of around 75%. Acid wet oxidation at 195°C for 15 min gave a good fractionation of bagasse, but a significant part of the polysaccharides was lost, and the enzymatic convertibility of the pretreated material was poor.

### Biological Treatments

Delignification can be performed not only by thermo-chemical processes but also by the biological route, using enzymes, or microorganisms. An example is the bleaching process of the wood pulp with ligninolytic enzymes that can provide mild and clean strategies for pretreatment (Kuhad et al. 1997). The advantages of biological delignification over the previous methods may include mild reaction conditions, higher product yields and fewer side reactions, less energy demand and less reactor resistance to pressure and corrosion (Lee 1997). Moreover, biological treatments are usually considered environmentally-friendly technologies. Lignin decomposition in nature is primarily attributed to the metabolism of microorganisms. Among all other organisms, white-rot basidiomycetes degrade lignin more rapidly and extensively than other groups (Falcón et al. 1995). These microorganisms produce several ligninolytic enzymes (laccases, manganese peroxidases and lignin peroxidases) that catalyze one-electron oxidation of lignin units, producing aromatic radicals (Giardina et al. 2000). Lignin degradation is mainly attributed to the secondary metabolism, or to restricted availability of nitrogen, carbon, or sulphur, and it is normally not degraded as sole carbon and energy sources, requiring additional co-substrates such as cellulose, hemicellulose or glucose (Silva et al. 2010). Some white-rot fungi preferentially attack lignin more readily than hemicellulose and cellulose. *Ceriporiopsis subvermispora*, *Phellinus pini*, *Phlebia* spp. and *Pleurotus* spp. belong to this group. Many white-rot fungi, however, exhibit a pattern of simultaneous decay characterized by degradation of all cell wall components. Examples of this group include *Trametes versicolor*, *Heterobasidium annosum* and *Irpex lacteus* (Wong 2009).

Solid-state fermentation is an interesting process to perform biological delignification because it

mimics the natural environment of lignin-degrading fungi. The advantages of the solid-state fermentation process over the submerged fermentation include: smaller fermenter volume; lower sterilization energy costs; easier aeration; reduced or eliminated costs for stirring and effluent treatment; lower costs for product recovery and drying; less favorable environment for many bacteria, lowering the risk of contamination (Lee 1997). The guiding principles to design the solid-state fermenters for biological delignification should be, first, to provide optimum conditions for the activity of the fungus through effective mixing, heat removal and oxygen supply and, second, to keep the equipment as simple and inexpensive as possible (Reid 1989). One of the main disadvantages of the biological delignification by fermentation is the long incubation time. The process developed by Pellinen et al. (1989) to delignify the kraft pulp and chemithermo-mechanical pulp (CTMP) using *Phanerochaete chrysosporium* presented delignification times of around two weeks, the kappa number being reduced from 33 to less than 10 for the kraft pulp and the lignin content decreasing from 26.5 to 21.3% for the CTMP. Gupta et al. (2011) achieved 6-10% of delignification of *Prosopis juliflora* and *Lantana camara* after 15 days of solid-state fermentation with *Pycnoporus cinnabarinus*. Reid (1989) estimated a cost of C\$ 720 per batch for a hypothetical process to delignify 10 tons of aspen wood. The duration of the batch was considered as eight weeks and the highest cost was attributed to the wood (C\$ 56 per ton).

In comparison with the solid-state fermentation, enzymatic delignification processes demand less incubation time. Kuila et al. (2011) reported a maximum delignification of *Bambusa bambos* of 84% using laccase from *Pleurotus* sp. at 400 IU/mL after 8h of incubation. Highest reducing sugar yield from the enzyme-pretreated biomass was 818.01 mg/g dry substrate after 8 h of incubation time using the following enzyme loading: endoglucanase 1.63 IU/mL; beta-glucosidase 1.28 IU/mL; exoglucanase 0.08 IU/mL and xylanase 47.93 IU/mL.

Recently, Moniruzzaman and Ono (2012) described an enhanced method to promote the enzymatic delignification of wood chips, using ionic liquid swollen biomass in ionic liquid aqueous systems with the aim of overcoming the difficulties with accessibility of the enzyme and

solubility of substrates and products. Enzymatic delignification in ionic liquid aqueous media containing 5% ionic liquid resulted in around 50% delignification of wood biomass after 24 h in the presence of laccase, whereas only 10% delignification was obtained when original wood chips (without ionic liquid pretreatment) were used.

## LIGNINOLYTIC ENZYMES

There are four major groups of ligninolytic enzymes produced by the white-rot fungi: lignin peroxidase (LiP; 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol:hydrogen-peroxide oxidoreductase; EC 1.11.1.14), manganese dependent peroxidase (MnP; Mn(II):hydrogen-peroxide oxidoreductase, or manganese peroxidase; EC 1.11.1.13), versatile peroxidase (VP; EC 1.11.1.16) and laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2). However, the process of lignin biodegradation can be further enhanced by the action of other enzymes such as glyoxal oxidase (EC 1.2.3.5), aryl alcohol oxidase (veratryl alcohol oxidase; EC 1.1.3.7), pyranose 2-oxidase (glucose 1-oxidase; EC 1.1.3.4), cellobiose/quinone oxidoreductase (EC 1.1.5.1) and cellobiose dehydrogenase (EC 1.1.99.18) (Wong 2009).

Both LiP and MnP belong to the class of peroxidases that oxidize their substrates by two consecutive one-electron oxidation steps with intermediate cation radical formation. Due to its high redox potential, the preferred substrates for LiP are nonphenolic methoxyl-substituted lignin subunits and the oxidation occurs in the presence of H<sub>2</sub>O<sub>2</sub> (Tuor et al. 1995; Wong 2009) whereas MnP acts exclusively as a phenoloxidase on phenolic substrates using Mn<sup>2+</sup>/Mn<sup>3+</sup> as an intermediate redox couple (Tuor et al. 1995). Versatile peroxidases are a group of enzymes, primarily recognized as manganese peroxidases, which exhibit activities on the aromatic substrates similar to that of LiP. These enzymes are not only specific for Mn (II), but also oxidize phenolic and non-phenolic substrates that are typical for LiP, including veratryl alcohol, methoxybenzenes and lignin model compounds in the absence of manganese (Wong 2009).

Laccases are blue multicopper oxidases able to oxidize a variety of phenolic compounds, including polyphenols, methoxy-substituted

phenols, diamines and a considerable range of other compounds, with concomitant reduction of molecular oxygen to water (Autore et al. 2009; Dwivedi et al. 2011). They oxidize phenols and phenolic lignin substructures by one-electron abstraction with formation of radicals that can repolymerize, or lead to depolymerization (Higuchi 1989). These enzymes have been found to oxidize also non-phenolic compounds in the presence of a mediator (e.g., 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonate or ABTS) (Wong 2009). Laccases are more readily available and easier to manipulate than LiP and MnP. These enzymes find many industrial applications in the areas of food products, pulp and paper, textiles, nanobiotechnology, soil bioremediation, synthetic chemistry and cosmetics (Couto and Herrera 2006).

The overall reaction catalyzed by these phenoloxidases is  $4 \text{ benzenediol} + \text{O}_2 \leftrightarrow 4 \text{ benzoquinone} + 2\text{H}_2\text{O}$  (Wong 2009). The laccase molecule is a dimeric, or tetrameric glycoprotein, which usually contains four copper atoms per monomer distributed in three redox sites named T1, T2 and T3. In the resting enzymes, all four coppers are in the  $2^+$  oxidation state (Couto and Herrera 2006; Wong 2009). Intracellular and extracellular isoenzymes may be produced from a single organism. The monomeric proteins have a molecular mass ranging from 50 to 110 kDa (Thurston 1994).

As described by Wong (2009), the first step of catalysis is an electron transfer between the reducing substrate and copper ( $\text{Cu}^{2+}$  to  $\text{Cu}^+$ ) at the T1 site, which is the primary electron acceptor. The electrons extracted from the reducing substrate are transferred to the T2/T3 site and the enzyme changes from its fully oxidized form to a fully reduced state. A successive four-electron oxidation, from four substrate molecules, is required to fully reduce the enzyme. The rate-limiting step in the catalytic cycle is the intramolecular electron transfer from T1 to the T2/T3 trinuclear copper site. The reduction of oxygen occurs at the T2/T3 site and the diffusion of oxygen to the trinuclear site is also a rate-limiting step.

The highest amounts of laccases are produced by white-rot fungi, which are the only organisms able to mineralize all components of lignin to carbon dioxide and water. Fungal laccases are secreted into the medium by the mycelium of filamentous fungi (Couto and Toca-Herrera 2007). Examples

of microorganisms that produce laccase with high activity are *Trametes pubescens* (740,000 U/L) (Galhaup et al. 2002), *Coriolus hirsutus* (83,830 U/L) (Koroleva et al. 2002), *Trametes hirsuta* (19,400 U/L) (Rodríguez-Couto et al. 2006), *T. versicolor* (16,000 U/L) (Font et al. 2003), *Pycnoporus cinnabarinus* (10,000 U/L) (Meza et al. 2006), *Neurospora crassa* (10,000 U/L) (Luke and Burton 2001), *Pleurotus ostreatus* (3,500 U/L) (Lenz and Hölker 2004). *P. ostreatus* belongs to a class of white-rot fungi that produces laccases, manganese peroxidases but not lignin peroxidases (Giardina et al. 2000).

Laccases are synthesized in multiple isoforms depending on the fungal species and the environmental conditions and this variety is related to the diversity of their roles: lignin degradation/synthesis, fruiting bodies development, pigment production, cell detoxification (Piscitelli et al. 2011). The biochemical diversity of laccase isoenzymes appears to be due to the multiplicity of laccase genes; however, regulation of their expression can be substantially diverse between the fungal species (Palmieri et al. 2003). The sequencing and annotation of the *P. ostreatus* PC 15 genome version 2.0 (Joint Genome Institute 2011) indicates the presence of at least twelve genes of multicopper oxidases. Some of the corresponding enzymes have been purified and characterized; these include POXA1b (Giardina et al. 1999), POXA1w and POXA2 (Palmieri et al. 1997), POXA3 (Palmieri et al. 2003) and POXC, previously named POX2 (Giardina et al. 1996) where POX means phenol oxidase. Other isoenzymes whose sequences have been determined are POX1 (Giardina et al. 1995), POX3 and POX4 (Joint Genome Institute 2011). POXC is the most abundantly produced under all growth conditions examined according to Giardina et al. (1999).

Laccases can be industrially employed in the degradation of phenolic compounds, promoting their oxidation. Enzymatic oxidation is advantageous in relation to chemical because of its specificity, biodegradability and requirement of mild conditions. In the food and beverages industry, laccases can be applied to remove undesired phenolics responsible for browning, haze formation and turbidity. In the pulp and paper industry, the biopulping and biobleaching using enzymes are alternatives to the conventional chemical processes. In the textile industry, the

decolourization of dyes is the main application of laccases. Laccases are also used in the fields of nanotechnology, bioremediation and synthetic chemistry (Couto and Herrera 2006). All these features indicate that the production of ligninolytic enzymes, especially laccases, could be considered

as a process to integrate the biological delignification within the concept of a lignocellulose biorefinery. Table 2 summarizes the different methods used for delignification and their results.

**Table 2** – Methods and results of delignification technologies reported by different authors.

Method	Conditions	Results	Reference
Steam explosion	1.3 MPa, 190°C, 15 min	7.9±9.1% lignin solubilization from sugarcane bagasse	Rocha et al. (2012)
Alkaline pretreatment	NaOH 1.0% (w/v), solid-liquid ratio 1:10 (w/v), 100°C, 1 h	92.7±3.9% lignin solubilization from sugarcane bagasse after steam explosion	Rocha et al. (2012)
Alkaline pretreatment	2% H <sub>2</sub> O <sub>2</sub> with 1.5% NaOH, 121°C, 15 min	Increased cellulose level 1.2 times and decreased hemicellulose content 8.5 times in sugarcane bagasse	Aguiar et al. (2010)
Acid pretreatment	1% H <sub>2</sub> SO <sub>4</sub> and enzymatic hydrolysis after microwave-alkali treatment (1% NaOH)	0.83 g reducing sugars / g dry sugarcane bagasse	Binod et al. (2012)
Ammonia fiber expansion	2 kg ammonia + 1.5 kg water / kg dry bagasse, 140°C, 30 min	85% glucan conversion by cellulases and 95-98% xylan conversion by hemicellulases in bagasse and cane leaf residue	Krishnan et al. (2010)
Organosolv	30% (v/v) ethanol with NaOH, 60 min, 195°C, preceded by dilute-acid pretreatment	Residual solid material from sugarcane bagasse containing 67.3% (w/w) glucose	Mesa et al. (2011)
Organosolv	Glycerol 80% in water, 150 min, 198.3°C	Pulp with 7.75% residual lignin (81.4% delignification)	Novo et al. (2011)
Liquid hot water	190-230°C, rapid immersed percolation (45 s to 4 min)	50% solubilization of sugarcane bagasse and leaves (all of the hemicellulose, 60% of the acid-insoluble lignin and less than 10% of the cellulose)	Allen et al. (1996)
Wet oxidation	Alkaline, 195°C, 15 min	Solid material with 70% cellulose, with solubilization of 93% hemicelluloses and 50% lignin	Martín et al. (2007)
Solid state fermentation	<i>Phanerochaete chrysosporium</i> , two weeks	Reduction of lignin content from 26.5% to 21.3% in chemithermo-mechanical pulp	Pellinen et al. (1989)
Solid state fermentation	<i>Pycnoporus cinnabarinus</i> , two weeks	6-10% delignification of <i>Prosopis juliflora</i> and <i>Lantana camara</i>	Gupta et al. (2011)
Enzymatic delignification	Laccase from <i>Pleurotus</i> sp. at 400 IU/mL, 8h	84% delignification of <i>Bambusa bambos</i>	Kuila et al. (2011)
Enzymatic delignification	Laccase delignification in ionic liquid aqueous media containing 5% ionic liquid	50% delignification of wood biomass after 24 h	Moniruzzaman and Ono (2012)



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Received: January 03, 2012;  
Accepted: June 12, 2013.

