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Ciências

Effect of physical and physical-chemical pretreatments on the sugar release of lignocellulosic materials through an enzymatic cocktail

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Dedication

To my parents, who taught me the meaning of the word courage.

Acknowledgements

Getting so close to another goal in my life, I cannot help looking back and realizing I was never alone throughout this journey: there were people without whom it would not be possible for me to carry on. For this reason, I would like to express my deep gratitude for all of you, regarding particularly the following people:

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To all of you, my most sincere thanks!

"There's a tingling in the spine, a catch in the voice, a faint sensation as of a distant memory of falling from a great height… We know we're approaching the grandest of mysteries."

Carl Sagan, Cosmos.

Abstract

This work intended to begin an investigation course which aims to deepen the mechanisms that determine the sugar release from lignocellulosic materials, using adequate enzymatic complexes. In this study, it was used an enzymatic complex, kindly provided by Novozymes, which includes a diverse set of enzymes, designed for the hydrolysis of lignocellulosic materials. Provided with this tool, the work began by evaluating the response of two model cellulosic fibrous materials: a chemical bleached pulp (with lignin-free fibers and high specific area) and a mechanical pulp (lignin-rich and also with high specific area). These choices provide high and comparable specific areas, allowing the isolation of the effect of material's composition. While the chemical bleached pulp is mate exclusively by polysaccharides, the mechanical pulp contains all the lignin from its original wood, apart from the polysaccharides, preserving the wood's original ultra-structure. In order to distinguish extreme cases, the pulps were also subjected to a beating process in a PFI mill, which additionally increased the material's specific area, and were afterwards subjected to an enzymatic cocktail. The obtained results revealed completely different answers from both pulps, beaten and unbeaten. The lignin-free pulp has released practically all its carbohydrates, while the mechanical pulp released only about 20% of its potential. The pulp beating had a limited effect on the mechanical pulp and increased the sugar release rate and slightly increased its extension in the chemical bleached pulp. In conclusion, the behavior differences are not due to specific area, but to the chemical composition and/or to the differences in the ultra-structure of both fibrous materials studied. The performed studies point to a combined effect of both factors, which are difficult to isolate due to the fact that the lignin extraction process also induces modifications on the organizational structure of the polymers. The second stage of this work involved non-previously processed prime-matters, namely, pine and eucalyptus wood chips and also broom wood. In these cases, it is essential to submit the material to a pretreatment prior to subject it to enzymatic hydrolysis, aiming its sugars release. In this study, we chose to explore the sodium bisulfite potential, at different pH levels, taking into account the few published studies with this treatment, and the team's experience on wood coking processes. Based on the literature, the operating conditions of the sulfite stage were chosen, maintaining the enzymatic hydrolysis conditions. After the pretreatment, the material was subjected to a controlled disintegration treatment. The sugars and their byproducts from the pretreatment hydrolysate were analyzed by HPLC and the solid residue was afterwards subjected to enzymatic hydrolysis. The increase on acid charge (H_2SO_4), for a fixed sulfite level, translated in the increase of sugar release, particularly xylose, and in an increase of byproducts, potentially inhibitors of subsequent bioethanol production stages, and in a darker solid residue with more condensed lignin and

higher tendency to fragment in the disintegration step. An attempt of global mass balance was undertaken with consistent results, although they might require adjustments from further investigations. Generally, all the solid residues exhibited a very positive answer on the enzymatic hydrolysis, achieving polysaccharide conversions in the range of 65 to 98%. The sugar release rate proved to be fast in the beginning, gradually decreasing with contact time, until it is annulled. In some cases, a decrease in sugar concentration in the reaction medium takes place, in around 72 hours of enzymatic hydrolysis. Since this decrease is not expectable, it can be related with the existence of microorganisms detected when hydrolyzed samples were observed in an optic microscope. The microscopic observation of samples subjected to different times of enzymatic hydrolysis revealed the enzymes' ability of fragmenting the fibers; at the end of two days, the fibers were mostly converted to fine elements.

Key-words

Bioethanol; enzymatic hydrolysis; lignocellulosic materials; sulfite pretreatment; ultra-strucutre

Resumo

Com o presente trabalho pretendeu-se iniciar uma linha de investigação que visa aprofundar os mecanismos determinantes da libertação de açúcares de materiais lenhocelulósicos utilizando complexos enzimáticos adequados. No presente estudo utilizou-se um complexo enzimático amavelmente cedido pela Novozymes, que inclui um conjunto diverso de enzimas desenhadas para a hidrólise de materiais lenhocelulósicos. Munidos desta ferramenta, começou por avaliar-se a resposta de dois materiais fibrosos celulósicos modelo: uma pasta química branqueada (fibras livres de lenhina e com elevada área específica) e uma pasta mecânica (rica em lenhina e também com elevada área específica). Estas escolhas proporcionam áreas específicas elevadas e comparáveis, permitindo isolar o efeito da composição do material. Enquanto que a pasta química branqueada é constituída exclusivamente por polissacarídeos, a pasta mecânica contém toda a lenhina da madeira que lhe deu origem, para além dos polissacarídeos, preservando a ultra-estrutura original da madeira. Para extremar condições, as pastas foram ainda submetidas ao processo de refinação em moinho PFI, que aumentou ainda mais a área específica do material posteriormente submetido à acção do cocktail enzimático. Os resultados obtidos revelaram respostas completamente diferentes das duas pastas, refinadas ou não, com a pasta isenta de lenhina a libertar praticamente todos os seus hidratos de carbono, enquanto que a pasta mecânica libertou apenas cerca de 20% do seu potencial. A refinação teve um efeito limitado na pasta mecânica, e aumentou a velocidade de libertação dos açúcares e, marginalmente, a sua extensão na pasta química branqueada. Em conclusão, as diferenças de comportamento não se ficam a dever à área específica, mas sim à composição química e/ou às diferenças na ultra-estrutura dos dois materiais fibrosos objecto de estudo. Os estudos realizados apontam para um efeito conjunto dos dois factores, difíceis de separar em virtude de o processo de extracção da lenhina também induzir modificações ao nível da estrutura organizacional dos polímeros.

Numa segunda fase do trabalho passou a trabalhar-se com matérias-primas não previamente processadas, isto é, partiu-se de aparas de madeira de pinho e eucalipto e ainda de giesta. Nestes caso, é essencial submeter o material a um pré-tratamento antes de o submeter à hidrólise enzimática com vista à libertação dos açúcares. No presente estudo, optou-se por explorar o potencial do sulfito de sódio, a diferentes níveis de pH, tendo em conta os poucos trabalhos publicados com este tratamento e a experiência da equipa nos processos de cozimento de madeira. Com base na literatura, escolheram-se as condições de operação no estágio do sulfito, tendo-se mantido constantes as condições da hidrólise enzimática. Após o pré-tratamento, o material foi sujeito a um tratamento de desintegração controlado. No hidrolisado determinaram-se, por HPLC, os açúcares e os seus produtos de degradação, e recolheu-se o resíduo sólido, que posteriormente foi submetido à hidrólise enzimática.

O aumento da carga de ácido (H_2SO_4), para um dado nível de sulfito, traduz-se no aumento da libertação de açúcares, particularmente xilose, e num acréscimo dos produtos de degradação inibidores de algumas etapas seguintes no processo de produção de etanol, bem como na produção de um resíduo sólido com lenhina mais condensada (mais escuro), e uma maior tendência para a fragmentação na etapa de desintegração. Levou-se a cabo uma tentativa de balanço global de massa, tendo-se obtido resultados consistentes, mas que requerem afinação em trabalhos futuros. De uma maneira geral, todos os resíduos sólidos exibiram uma resposta muito positiva na hidrólise enzimática, tendo-se atingido conversões dos polissacarídeos na gama de 65 a 98%. A velocidade de libertação dos açúcares é rápida no início, decrescendo gradualmente com a passagem do tempo de contacto, até se anular. Em alguns casos assiste-se a uma diminuição da concentração de açúcares no meio reaccional para tempos de hidrólise da ordem das 72 horas. Esta diminuição não expectável pode estar relacionada com a existência de microrganismos detectados na observação microscópica das amostras hidrolisadas. A observação microscópica das amostras sujeitas a diferentes tempos de hidrólise enzimática revelou ainda a capacidade das enzimas para fragmentar as fibras; ao cabo de 2 dias as fibras estão maioritariamente convertidas em elementos finos.

Palavras-chave

Bioetanol; hidrólise enzimátic;, materiais lenhocellulósicos; pretratamento ao sulfito; ultra-estrutura.

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Acronyms list

AFEX	Ammonia fiber explosion
AGU	Amyloglucosidase Unit
CBU	Cellobiose unit
CED	Cupriethylenediamine
DNS	3,5-Dinitrosalicylic acid
DP	Degree of polymerization
ECF	Elemental chlorine free
EGU	Endo-Glucanase Unit
EU	European Union
FBG	Fungal Beta-Glucanase Unit
FGU	PolyGalacturonase Unit
FPU	Filter paper unit
FXU-S	Fungal Xylanase Unit
HPLC	High performance liquid chromatography
ICE	Internal combustion engines
IR	Infrared
IUPAC	International Union of Pure and Applied Chemistry
K-No	Kappa number
LAP	Laboratory analytical procedure
NREL	National renewable energy laboratory
PEG	Polyethylene glycol
PGWP	Pressurized ground wood pulp
RI	Refraction index
SEM	Scanning electron microscopy
SHF	Separate enzymatic hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
TS	Total solids
UV	Ultraviolet
WRV	Water retention value

Chapter I - Introduction

In the last few years, the increasing and continuous consumption of energy due to the world population growth and the rising number of industrialized countries, as well as the growing carbon dioxide emission issues and the aspiration of low fossil fuels dependence, have brought into focus the need to develop sustainable green technologies for many of our most basic manufacturing and energy needs (Sun e Cheng, 2002; Jørgensen *et al.*, 2007; Rass-Hansen *et al.*, 2007; Pu *et al.*, 2008; Alvira *et al.*, 2010).

Biomass is the only renewable source of energy that can provide short-term alternative fuels, such as bioethanol or biodiesel, for the transportation sector (Rass-Hansen *et al.*, 2007; Alvira *et al.*, 2010). Many countries, namely Brazil and USA, frequently use ethanol as a gasoline addictive and have invested significant resources on the utilization of ethanol/gasoline blends as fuel for internal combustion engines (Rass-Hansen *et al.*, 2007). Adding ethanol to the gasoline increases the octane number and reduces the carbon dioxide emissions (Hamelinck *et al.*, 2005).

Bioethanol can be produced from different types of feedstock: crops of starchy vegetables such as maize grains (1st generation bioethanol), and lignocellulosic materials such as wood, agriculture residues and waste paper, for instance (2nd generation bioethanol) (Sivakumar *et al.*, 2010; Balat, 2011). Although most of the global bioethanol supply comes from starchy materials, lignocellulosic materials are the most abundant feedstock for bioethanol production (Sivakumar *et al.*, 2010).

Lignocellulosic materials are fundamentally made out of carbohydrates polymers (cellulose and hemicelluloses), lignin and a smaller amount of other compounds, such as extractives and organic acids. Cellulose and hemicelluloses, which constitute about two thirds of the dry weight, are hydrolysable polysaccharides that generate sugars which, in turn, can be fermented into ethanol. Since lignin cannot be used for ethanol production, it can be harnessed for heat production by combustion, among other uses (Hamelinck *et al.*, 2005).

In order to take advantage of lignocellulosic residues as a source of renewable energy, it is important to convert them in the largest possible amount of fermentable sugars, which means that both the glucose obtained from the cellulose and hemicellulose fraction must be taken into account, contributing to the economic viability of the process (Cara *et al.*, 2008).

Obtaining ethanol from lignocellulosic materials takes essentially four steps: pretreatment, which breaks the lignin matrix in order to improve the fibers accessibility; hydrolysis, where the substrates are converted into sugars; fermentation, which transforms the sugars in

ethanol; and the distillation, where the previously produced ethanol is purified according to fuel specifications (Balat *et al.*, 2008; Margeot *et al.*, 2009; Mészáros *et al.*, 2009).

The experiments undertaken and described in this dissertation aimed to study the effect of some physical and physical-chemical pretreatments. Two approaches were followed:

(1) Using two model lignocellulosic materials (a lignin-free bleached pulp and a lignin-rich mechanical pulp), the effect of some physical pretreatments were studied, namely beating and drying;

(2) Wood chips of three different woody species (*Eucalyptus globulus, Pinus pinaster* and *Cytisus striatus*) were pretreated with different sulfite charges at different pH levels and the effect on the sugar and fermentation inhibitors release in the hydrolysates were studied. In addition, the solid residue was submitted to the effect of an enzymatic cocktail from Novozymes, in order to investigate the sugar release.

This dissertation is divided in 5 chapters, being this introduction the first of them. Chapter II consists of a literature review about the production of bioethanol from lignocellulosic materials, their main pretreatments, types of hydrolysis and fermentation of released sugars. Chapter III describes the reagents, materials and analytical methods used in the experiments, as well as the experimental procedures undertaken. Chapter IV shows the main results obtained and discusses their meaning and significance. Chapter V resumes the main conclusions and final considerations.

Chapter II – Literature Review

II - 1 Vegetable Biomass

Vegetable biomass is created in a process called photosynthesis, where the reactions between the water, sunlight and the atmospheric CO_2 originate the carbon hydrates, which are the main foundations of all vegetable materials. In this process, solar energy is stored in the chemical bonds of the structural components of biomass as chemical energy. Thus, if the biomass is efficiently processed, extracting the stored energy, the carbon is oxidised, producing CO_2 and water. The whole process is a closed cycle, as represented in the figure 1, once the CO_2 liberated is availed to produce new biomass: hence the energy obtained from these materials is designated renewable. While the ordinary vegetable biomass can be used as a renewable source of energy, fossilized biomass, like coal or petroleum, takes millions of years until it can be used as fuel. This is the reason why fossil fuels are considered nonrenewable sources of energy. Because burning these fuels exhausts a nonrenewable resource and contributes for the greenhouse effect, by spending "old" biomass and liberating "new" CO_2, this process is not a cycle (McKendry, 2002).

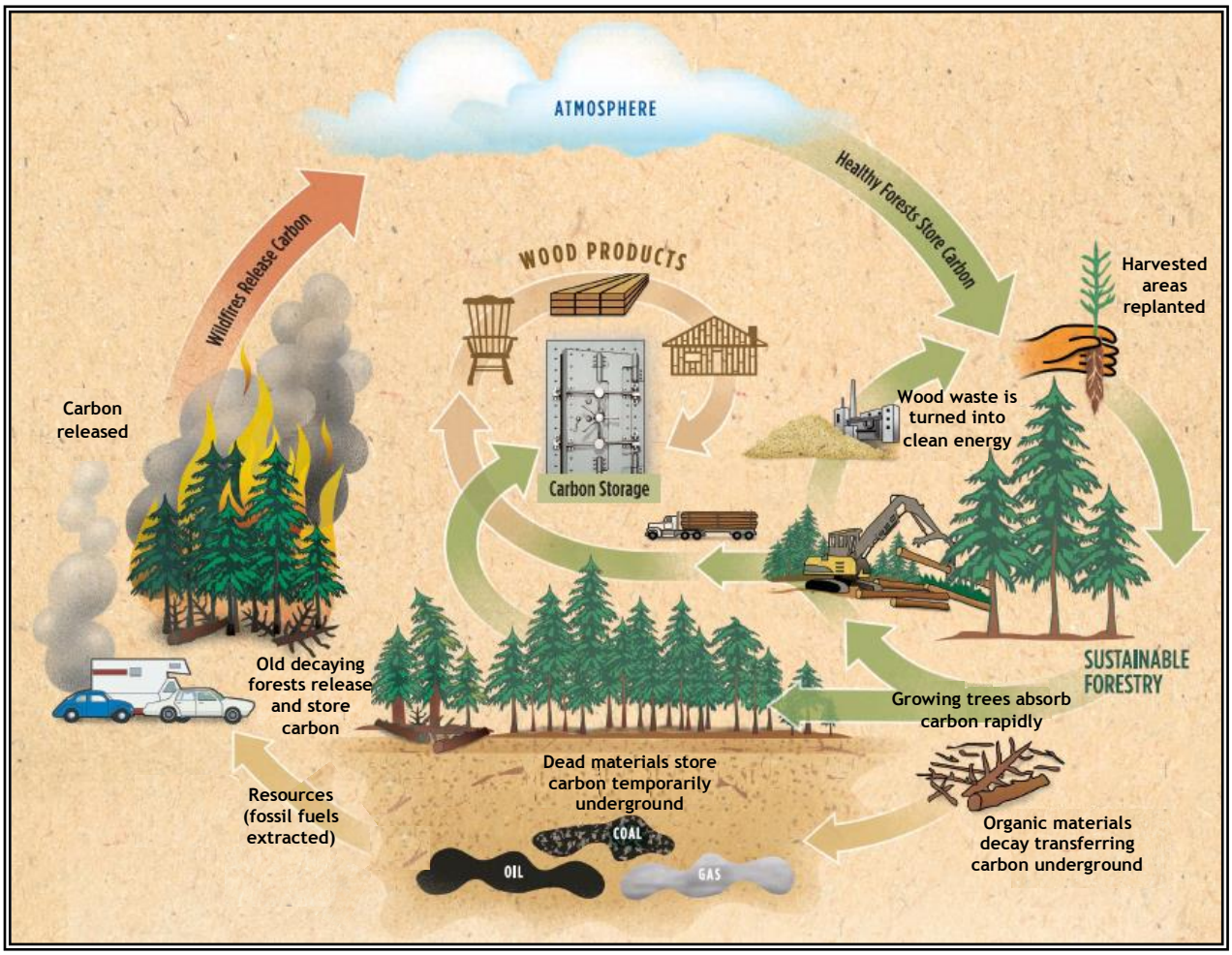


Figure 1 - Representation of the carbon cycle (Adapted from The Forest Foundation, 2013).

II - 1.1 Biomass as a source of energy

Biomass is generally designated as any biological material, most often referring to plants or plant-derived matter which can be transformed into diverse and appropriate types of bioenergy. In this context, examples of biomass can be wood, sawdust, agricultural residues, vegetable coal and biogas originated in organic waste decomposition. In bioengineering, much attention has been given to the production of liquid fuels from biomass (generally designated biofuels), mainly to try to ensure the transportation sector needs. Actually, until today, there haven´t been found any alternative renewable sources of fuel, apart from the biofuels, that are economically reliable and technologically mature (Zhang e Smith, 2007).

Bioethanol can be produced from different kinds of agricultural raw materials, which can be divided in three categories: simple sugars, starch and cellulose. Cellulosic materials can be supplied from a variety of inexpensive resources (Demirbaş, 2005).

Cellulosic or lignocellulosic materials are generic terms that designate the main constituents of the majority of vegetables (Ogeda and Petri, 2010).

Although the structural and chemical composition of lignocellulosic materials varies greatly with environmental and genetic issues (Ballat, 2011), the average composition of various types of cellulosic biomass materials is given in Table 1.

Table 1 - Composition in percentage of dry weight of various types of cellulosic biomass materials (Adapted from Demirbaş, 2005).

Material	Cellulose	Hemicelluloses	Lignin	Ash	Extractives
Green Algae	20-40	20-50	—	—	—
Cotton	80-95	5-20	—	—	—
Grasses	25-40	25-50	10-30	—	—
Hardwoods	45±2	30±2	20±4	0.6±0.2	5±3
Hardwoods Barks	22-40	20-38	30-55	0.8±0.2	6±2
Softwoods	42±2	27±2	28±3	0.5±0.1	3±2
Softwood Barks	18-38	15-33	30-60	0.8±0.2	4±2
Cornstalks	39-47	26-31	3-5	12-16	1-3
Wheat straw	37-41	27-32	13-15	11-14	7±2
Newspapers	40-55	25-40	18-30	—	—
Chemical pulps	60-80	20-30	2-10	—	—

II - 1.2 Importance of renewable energy sources

Nowadays, world's economy is highly dependent on various fossil energy sources, such as oil, coal, natural gas, among others (Sarkar *et al.*, 2012).

The transportation sector is worldwide considered to play a large role in the consumption of these energy sources, being almost entirely dependent on petroleum-based fuels. In addition, this sector requires liquid fuels. It accounts for more than 70% of global carbon monoxide (CO) emissions and 19% of global CO_2 emissions (Ogeda e Petri, 2010; Balat, 2011).

The increasing number of cars on the road, registered in the last few years, its predicted growth and consequent increase of emissions, will affect the stability of ecosystems, exhaust global oil reserves and give rise to global climate effects like the increase of greenhouse gases, which contributes to global warming (Ogeda e Petri, 2010; Balat, 2011).

In addition, because the reserves of fossil fuel are limited, annual global oil production will, inevitably, begin to decline within near future. Thus, renewable sources of energy might constitute a light at the end of the tunnel, being an alternative to petroleum-based fuels (Sarkar *et al.*, 2012).

II - 1.3 Bioethanol as an alternative fuel

Ethanol is a colourless flammable liquid, which has a boiling point of 78.4ºC, a melting point of -114.3 ªC and a density of 0.79 g/cm^3. Due to its heat potential, ethanol has been largely used as a source of heat, light and, as referred before, a fuel in the transportation sector, most specifically in internal combustion engines (Quilhó, 2011).

Basically, any of the members of the alcohol family can be used as a fuel, being ethanol (C_2H_5OH), methanol (CH_3OH), propanol (C_3H_7OH) and butanol (C_4H_9OH) the most suitable for motorfuels. However, only methanol and ethanol are technically and economically appropriate to be used as fuel in internal combustion engines (ICE) (Balat, 2011; Quilhó, 2011).

Unlike gasoline, ethanol is an oxygenated fuel that contains 35% oxygen, which reduces particulate and NOx emissions from combustion. Ethanol has a higher octane number (108), larger flammability limits and both higher flame velocity and vaporization heat. These features enable a higher compression rate and a lower combustion time, resulting in a greater efficiency (Balat, 2011; Quilhó, 2011).

Bioethanol has been applied directly as a gasoline improver (most commonly blended in concentrations of 10% bioethanol and 90% gasoline, known as E10) or substituent and in bioethanol-diesel blends with the particular purpose to reduce the emissions of exhaust gases (Balat, 2011).

Bioethanol can be used as a 5% blend with petrol under the European Union (EU) quality standard EN 228. This blend requires no engine modification and is covered by vehicle warranties. With engine modification, bioethanol can be used at higher levels, for example, E85 (Balat, 2011).

II - 1.4 Production of bioethanol from lignocellulosic materials

While renewable energy sources such as, for instance, the sun, the wind, the water and geothermal heat can substitute the petroleum-based fuels in energy industry, in a near future, fuel production and chemical industry may depend on biomass as an energy supply (Sarkar *et al.*, 2012).

Regarding bioethanol, there are, nowadays, two classifications of this fuel, depending on the feedstock from which it's produced: 1st generation bioethanol, essentially produced from crops of starchy feedstock such as maize grains and sugarcane, and 2nd generation bioethanol, produced from lignocellulosic based biomass, which includes agriculture and wood residues, as well as waste paper. An important part of the world's supply of bioethanol nowadays is constituted by 1st generation bioethanol. However, lignocellulosic biomass has some important advantages over starch crops in bioethanol production: lignocellulosic material represents a much more abundant feedstock and, unlike the crops, it does not compete for use as food. Thus, 2nd generation bioethanol is becoming widely accepted as superior to 1st generation one (Sivakumar *et al.*, 2010).

Bioethanol has been produced from the hydrolysation and fermentation of lignocellulosic material since the end of the 20st century, but the perspective of using bioethanol to supply the fuel market is relatively recent (Macedo *et al.*, 2008).

The biorefining of biomass has attracted much interest over the last few decades and, particularly in recent years, more extensive attention has been paid to the production of biofuels and biochemicals from biomass (Zhao *et al.*, 2011).

II - 2 Basic components of lignocellulosic materials

Lignocellulosic materials are a greatly attractive feedstock in industrial terms because they can be used to produce, apart from glucose and bioethanol, a large variety of other products. For instance, when lignin is degraded, the lower molar mass fractions obtained can be used in the fabrication of polyurethane foams and phenolic resins, among others (Ogeda e Petri, 2010). The different fractions of these materials must be selectively separated according to its characteristics and requirements of the final products (Pereira Jr. *et al., 2008)*.

Lignocellulosic materials include wood, herbaceous crops, agricultural and forestry residues, waste paper and paper products, pulp and paper mill waste, and municipal solid waste (Gong *et al.*, 1999).

II – 2.1 Hardwoods and softwoods

Botanically speaking, softwoods are those woods that come from gymnosperms (mostly conifers), and hardwoods are woods that come from angiosperms (flowering plants). Softwoods are generally needle-leaved evergreen trees such as pine (*Pinus*) and spruce (*Picea*), whereas hardwoods are typically broadleaf, deciduous trees such as maple (*Acer*) and birch (*Betula*) (Wiedenhoeft and Miller, 2005).

Not only do softwoods and hardwoods differ in terms of the types of trees from which they are derived, but they also differ in terms of their component cells. The single most important distinction between the two general kinds of wood is that hardwoods have a characteristic type of cell called a vessel element, whereas softwoods lack these. An important cellular similarity between softwoods and hardwoods is that in both kinds of wood, most of the cells are dead at maturity even in the sapwood. The cells that are alive at maturity are known as parenchyma cells, and can be found in both softwoods and hardwoods. Additionally, despite what one might conclude based on the names, not all softwoods have soft, lightweight wood, nor do all hardwoods have hard, heavy wood (Wiedenhoeft and Miller, 2005).

II – 2.2 Anatomical and morphological characterization of vegetable biomass

When a tree stem is cut transversely, as it is illustrated in figure 2, a portion of “heartwood” can be seen frequently as a dark-colored zone near the center of the stem. This portion is surrounded by a lightcolored peripheral zone called “sapwood” (Fujita and Harada, 2001).

Each year, tree species growing in temperate climates add one growth increment or ring to their diameter in the sapwood. For most species, this ring shows two distinct periods of growth and therefore two bands, called earlywood (springwood) and latewood (summerwood). Latewood is denser, harder, smoother, and darker than earlywood, and its cells have thicker walls and smaller cavities (Williams, 2005).

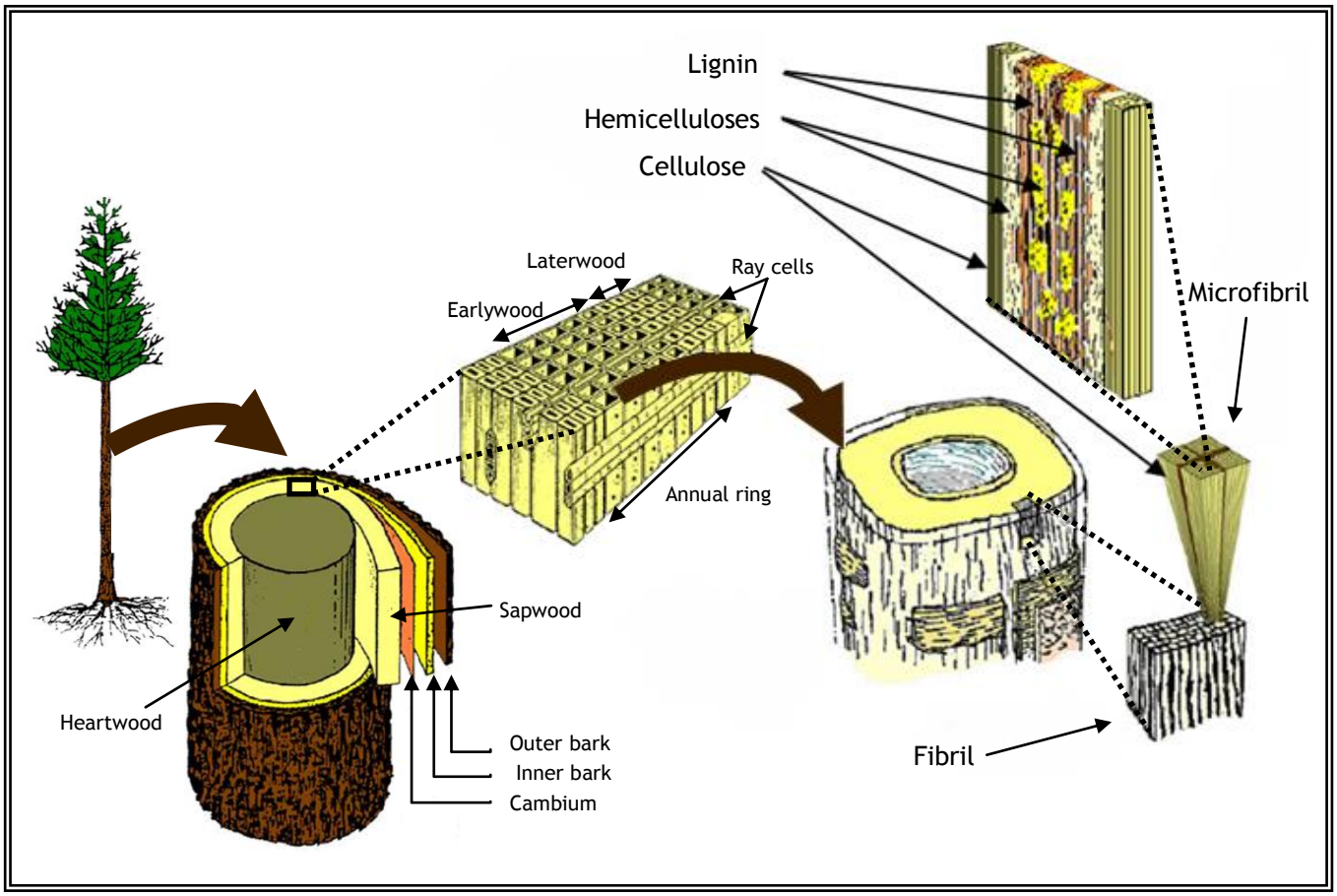


Figure 2 - Illustration of wood/plants, cell and cell wall components (Adapted from Novozymes, 2012).

The sapwood conducts water and mineral nutrients through the tissue, from the roots into the wood. In addition, the sapwood has living parenchyma tissue, which often plays some physiological role such as the storage of starch. From this point of view, the sapwood is considered an active xylem tissue (Fujita and Harada, 2001).

In contrast to sapwood, heartwood is dead xylem. Thus, heartwood does not participate in water conduction. As the tree matures, all parenchyma cells of the sapwood die, and other types of cells become occluded with pigment composed of polyphenols and flavanoids supplied mainly from the ray parenchyma (Fujita and Harada, 2001).

Although the conducting and physiological functions are lost in heartwood, the durability of wood against rot or insect decay is remarkably improved due to an addition of such pigments (Fujita and Harada, 2001).

The outer bark, not only provides mechanical protection to the softer inner bark, but also helps to limit evaporative water loss. Inner bark (or phloem) is the tissue through which sugars produced by photosynthesis are translocated from the leaves to the roots or growing portions of the tree. The vascular cambium is the layer between the bark and the wood that is responsible for producing both of these tissues just mentioned (Wiedenhoeft and Miller, 2005).

Digging deep into the woody biomass, the cell walls are made out of fibrils which themselves are built of microfibrils (figure 2). At a fundamental level, microfibrils are chains of cellulose covered by hemicelluloses, which, in their turn, *are enclosed by lignin* (Ogeda e Petri, 2010).

The most common organization of the vegetable fiber components is represented in the figure 3. Each fiber is made out of complex layers: a thin primary wall surrounds the secondary wall which is, in its turn, divided in three layers (S1, S2, S3), where the intermediate layer, S2, determines the mechanical properties of the fiber. This layer consists in a series of microfibrils made of long chains of helicoidally organized cellulose disposed along the fiber. Each microfibril is, on average, 10 to 30 nm long and is built by 30 to 100 chains of cellulose packed together (Silva *et al.*, 2009).

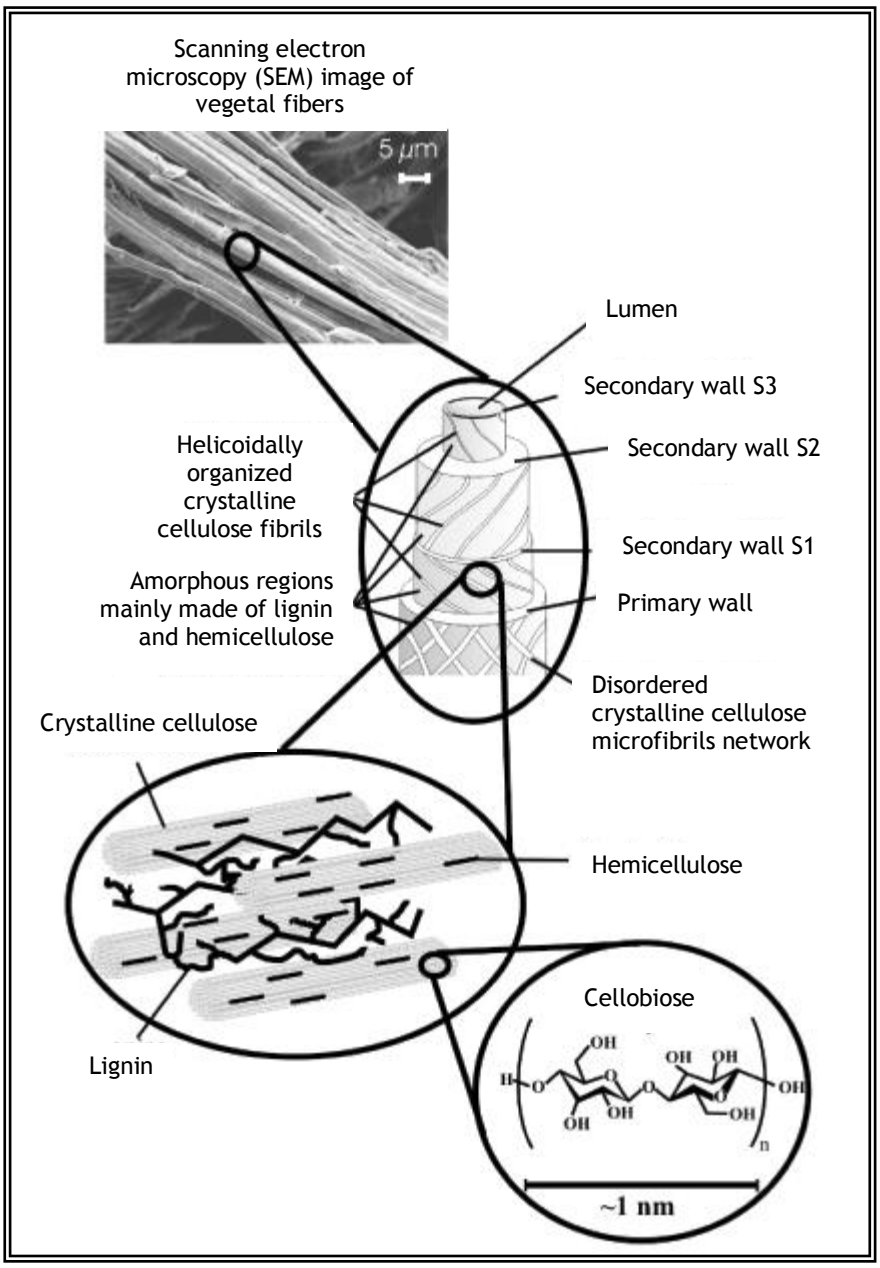


Figure 3 - Representation of vegetable fibers structure. The SEM image refers to eucalyptus fibers (Adapted from Silva *et al.*, 2009).

The proportion of the wood constituents varies between species, age as well as growing stage, and also between hardwoods and softwoods. Softwoods, like the pine, for instance, have a more significant percentage of lignin than that of the hardwoods. On the other hand, hardwoods, as the eucalyptus tree, present a higher content in cellulose and hemicellulose than softwoods (Pereira Jr. *et al., 2008;* Sivakumar *et al.*, 2010).

II – 2.3 Chemical structure

Woody biomass is mainly composed of structural substances (cellulose, hemicellulose and lignin), but other polymeric constituents present in lesser and often varying quantities are pectin, starch and proteins. In addition to these macromolecular components, various nonstructural and mostly low-molecular-mass compounds (extractives, acids, salts and minerals) also are present in small quantities. The relative mass proportions of structural carbohydrates and lignin can vary widely, depending on the morphological region and age of the wood (Chen, 2011).

II - 2.3.1 Cellulose

Cellulose is located mostly on the secondary cell wall. It is an organic polymer with a high degree of polymerization (1000 to 15000 sugar units), built with D-glucose monomers connected through ß-1,4-glycosdic bonds (Pereira Jr. *et al., 2008)*. The cellulosic chains are linked parallelly to one another by hydrogen bonds, as shown in figure 4. Although the strength of these bonds is relatively weak, their great number and their organization throughout the glucose chain is responsible for the resilience of the cellulose (Jørgensen *et al.*, 2007; Ogeda e Petri, 2010; Quilhó, 2011).

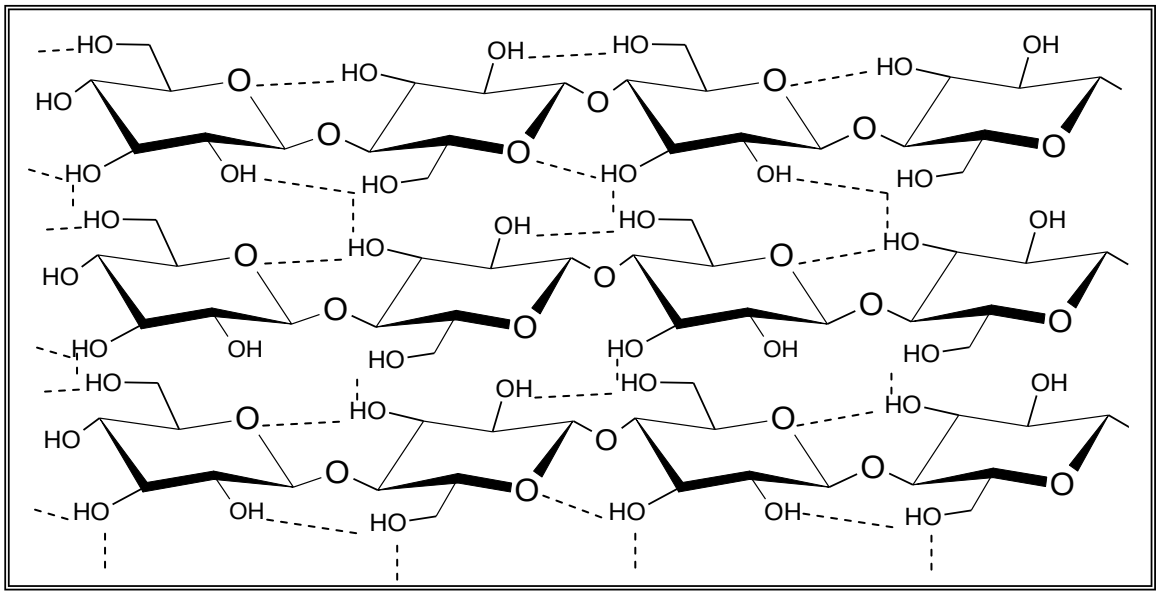


Figure 4 - Illustration of cellulose structure. The dashed lines represent the inter- and intra-chain hydrogen bonding pattern (Adapted from Festucci-Busell *et al.*, 2007).

Cellulose is insoluble in a large amount of solvents. The more crystalline is its structure, the less accessible it is to acid and enzymatic hydrolysis, where the polysaccharide is broken down to free sugar molecules by the addition of water (Quilhó, 2011; Batat, 2011).

The crystalline nature of cellulose in wood has been demonstrated by studies with X-ray diffractometry and polarization microscopy. This crystalline nature was also confirmed by the electron diffraction patterns of the secondary walls of wood cells in selected areas (Fujita and Harada, 2001). Biomass can also contain a small fraction of amorphous cellulose, more susceptible of undergoing hydrolysis (Pu *et al.*, 2008; Quilhó, 2011).

II - 2.3.2 Hemicelluloses

Hemicelluloses are ramified polymer that helps keep the cellulose microfibrils together. They are made out of pentose sugars (as xylose and arabinose), hexose sugars (like mannose, galactose and glucose) and uronic acids (glucuronic and mannuronic acids) (Quilhó, 2011; Batat, 2011). Figure 5 exemplifies the structure of a xylan hemicellulose.

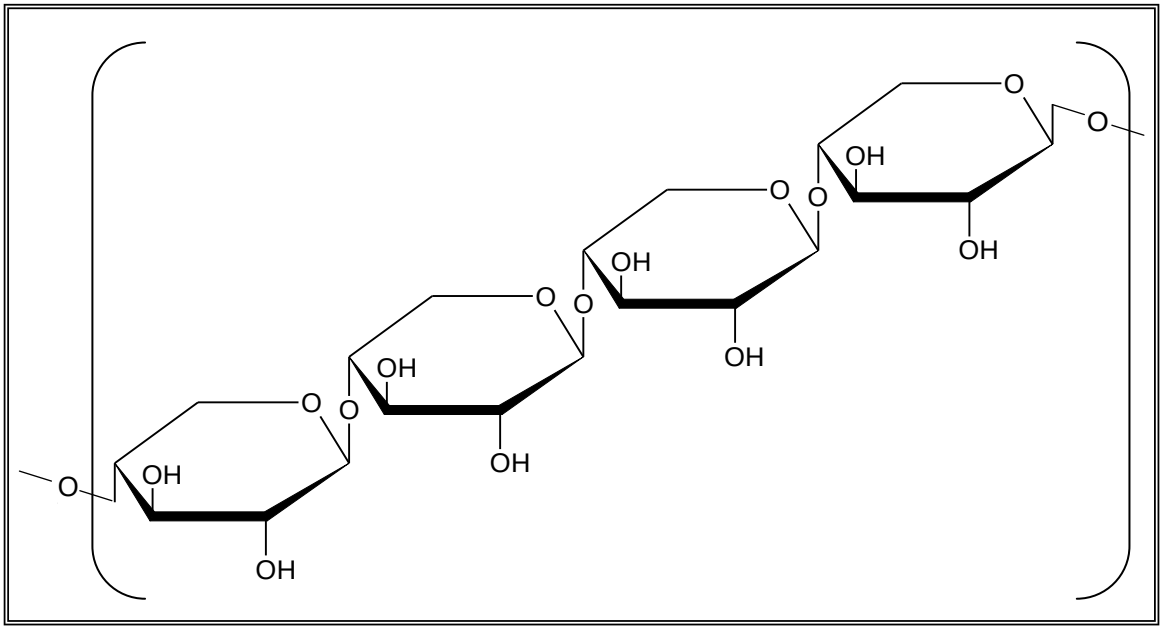


Figure 5 – ß-(1-4)-D-xylopyranosyl units from a xylan hemicellulose polymer (Adapted from Sigma-Aldrich, 2013).

Contrary to cellulose, hemicelluloses are relatively easy to hydrolyse, due to its amorphous nature and low degree of polymerization (60 to 300 sugar units), and is very much soluble in alkaline solutions (Sun e Cheng, 2005; Kumar *et al.*, 2009).

Commonly, the combination of cellulose and the hemicelluloses is designated as holocellulose (Williams, 2005).

II - 2.3.3 Lignin

Lignin is the non-polysaccharide fraction of the lignocellulosic materials. It is a natural aromatic macromolecule that has its origin in the polymerization of coumaric, coniferyl and sinapyl alcohols (illustrated in figure 6 (a)), organized in a complex net (Jørgensen *et al.*, 2007; Silva, 2010). The structural building blocks of lignin are joined together by ether linkages and carbon-carbon bonds (figure 6 (b)). Functional groups, including phenolic hydroxyl, aliphatic hydroxyl, methoxyl and carbonyl, may be introduced into the lignin polymer during its synthesis. They impart some polarity to the lignin macromolecule (Jørgensen *et al.*, 2007; Silva, 2010; Chen, 2011).

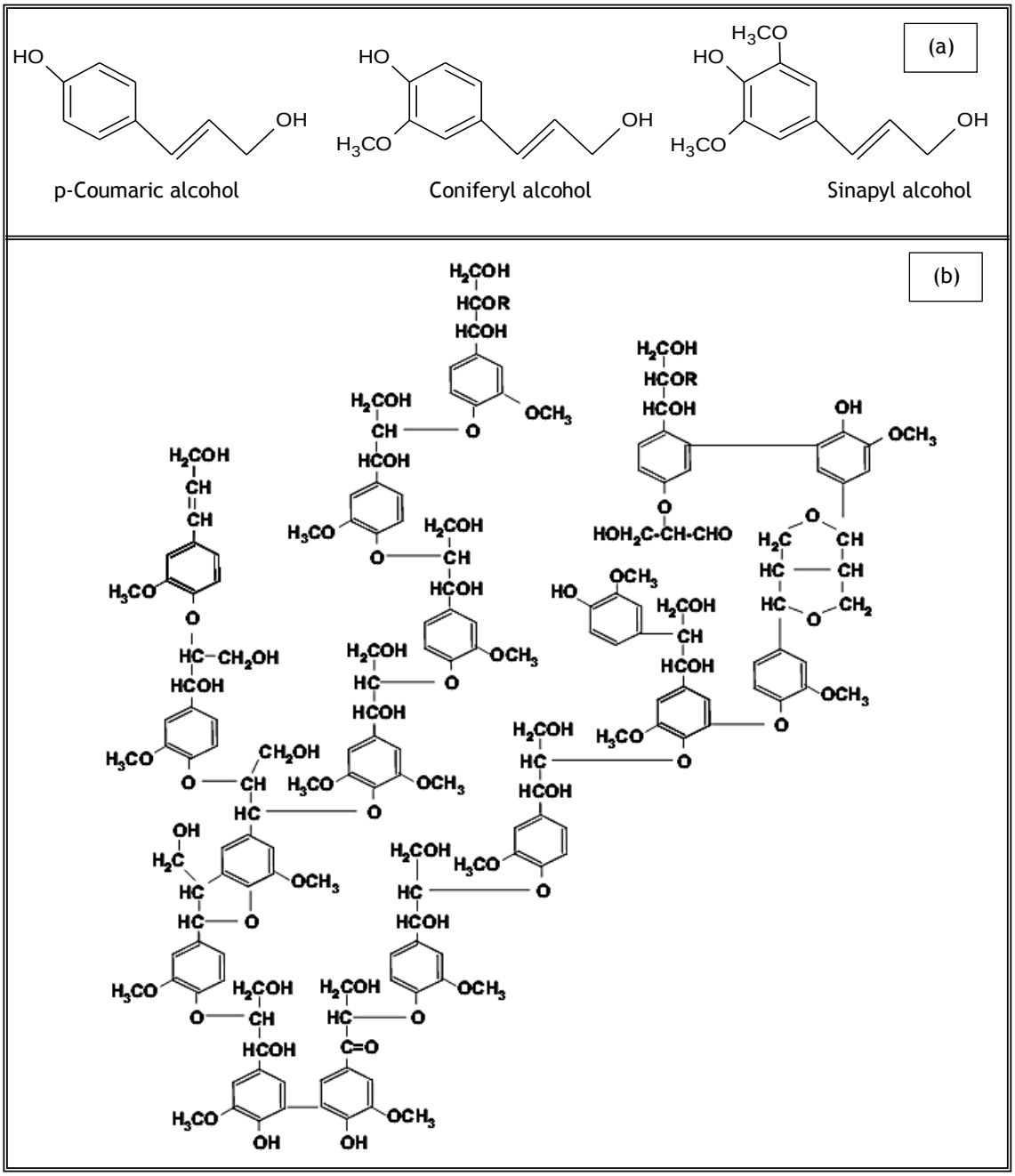


Figure 6 – Illustration of lignin precursors (a) (Adapted from Bierman, 1996); typical structure of a softwood lignin (b) (Adapted from Faravelli *et al.*, 2010).

Lignin provides toughness, impermeability and resistance against microorganism attacks. It is also the most recalcitrant constituent of vegetable cell walls. The higher its portion, the higher is the resistance of the material to hydrolysis. Consequently, lignins mechanical and chemical resilience is the main reason why it is so hard to access the cellulose and hemicelluloses (Taherzadeh e Karimi, 2007; Quilhó, 2011).

II - 2.3.4 Extractives

Generally speaking, extractives are chemicals in the wood that can be extracted using solvents, being frequently classified according to the solvent used to extract them: for instance, water-soluble, toluene-ethanol–soluble or ether-soluble extractives (Rowell *et al.*, 2005).

Although present in small fractions, extractives comprise an extraordinarily large number of lipophilic or hydrophilic substances such as fats, fatty acids, fatty alcohols, phenols, terpenes, steroids, resin acids, rosin, waxes, and many other minor organic compounds. These chemicals exist as monomers, dimers, and polymers (Rowell *et al.*, 2005).

Extractives not only impart color, odor and taste to wood, but they also protect woods against microbial damage or insect attacks. The amount of extractives and their composition vary with respect to the botanical families, wood species, growth regions and tissues, as well as the solvent used for the extraction. In general, softwoods have higher extractives content than hardwoods. (Rowell *et al.*, 2005; Chen, 2011).

Extractives are formed by parenchyma cells at the heartwood-sapwood boundary and are then exuded through pits into adjacent cells. In this way it is possible for dead cells to become occluded or infiltrated with extractives despite the fact that these cells lack the ability to synthesize or accumulate these compounds on their own (Wiedenhoeft and Miller, 2005).

Reactions of extractives during pretreatments are complex and are strongly dependent on pretreatment chemicals (Rowell *et al.*, 2005).

II - 3 Lignocellulosic materials pretreatments

In plants, the cell wall usually acts like a physical protection or barrier against pathogenic microorganisms, which can segregate specific hydrolytic enzymes and promote the fibres destruction. The issues involving the enzymatic digestibility are related, at a macro scale, not only with the size of the particle of biomass, but also with the overall porosity of the cell wall. At a micro scale, these issues are directly connected with the cellulose crystallinity, its polymerization degree, with the ramifications of the hemicellulose chain and with the composition of the lignin. Thus, it is important to modify these either chemical or physical properties of the cell wall in order to magnify the biologic conversion of the cellulose into sugars, in a process known as saccharification (Balat *et al.*, 2008; Sousa *et al.*, 2009).

The pretreatment stage promotes disruption of the lignocellulosic matrix in order to facilitate enzyme-catalyzed hydrolysis (Chen, 2011).

Feedstock pretreatment is one of the most critical steps in biochemical conversion of lignocellulose for commercial production of biofuel and bioproducts (Wang *et al.*, 2009). Figure 7 illustrates a comparison between a Scanning Electron Microscopy (SEM) of corn stover subjected only to enzymatic hydrolysis (a) and the same feedstock subjected to pretreatment prior to enzymatic hydrolysis (b). The results show that pretreatment changes lignocellulosic-structure susceptibility to be attacked by enzymes.

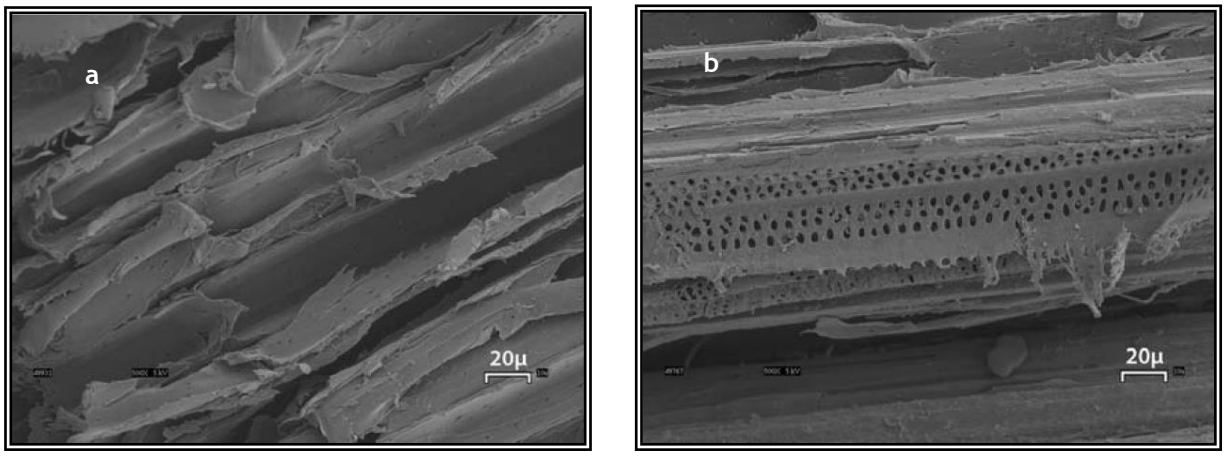


Figure 7 – Scanning Electron Microscopy comparing pretreated and not pretreated corn stover subjected to enzymatic hydrolysis: (a) A corn stover particle shows a smooth surface with a few micron-sized pores after only enzymatic hydrolysis. 11% of cellulose has been converted to glucose in 3 hours; (b) Corn stover particle with many more pores. It was pretreated in water at 190°C for 15 min and hydrolyzed by enzymes at 50°C for 3 hours, resulting in 40% cellulose conversion to glucose (Adapted from Houghton *et al.*, 2006).

The main objectives of every lignocellulosic material pretreatment are, therefore, to modify or remove any structural obstruction, breaking the lignin barrier and opening the crystalline regions of the cellulose, in order to improve the fermentable sugars yield, remove acetyl group for eliminating its interference with enzyme recognition to cellulose, reducing particle size or increasing the porosity of the substrates for facilitating the penetration of hydrolysis agents (Gong *et al.*, 1999; Balat *et al.*, 2008; Zhao, 2010). Figure 8 represents the role of the pretreatment in the conversion of lignocellulosic materials to fuel.

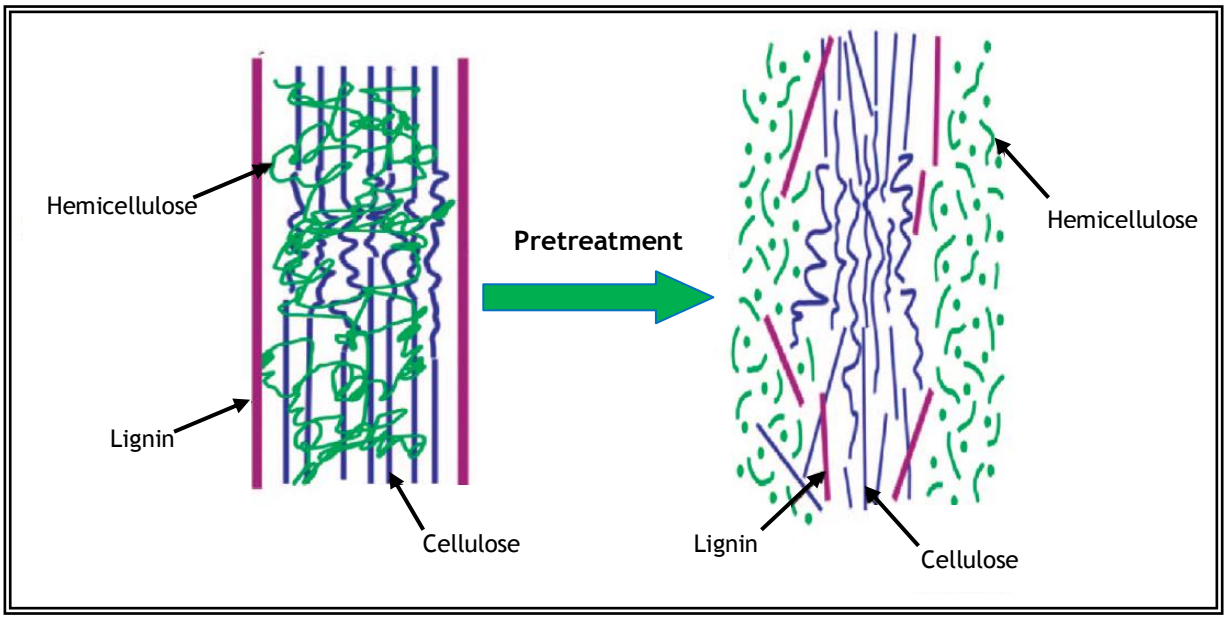


Figure 8 - Representation of the role of the pretreatment in the conversion of lignocellulosic materials to fuel (Adapted from Kumar *et al.*, 2009).

The pretreatment has to be efficient enough so that the resultant material can easily be accessed and hydrolyzed, and it cannot be so severe that it originates byproducts that can likely inhibit the microbial metabolism of the sugars into bioethanol (Kumar *et al.*, 2009; Balat, 2011).

Although the pretreatment is necessary to enhance the obtainment of fermentable sugars from lignocellulosic material, it is considered as one of the most expensive processing steps. The major goal, concerning bioethanol production, is to develop pretreatment technologies that comprise an acceptable overall cost-benefit relation, since the efficiency of this stage will have an economical impact on every step of the lignocellulosic biomass refinery for the production of either fuels or chemical compounds (Balat *et al.*, 2008; Kumar *et al.*, 2009; Sousa *et al.*, 2009).

Ultimately, each feedstock and pretreatment combination should be evaluated on an individual basis to determine the best process configuration to enable the industry implementation (Novozymes, 2012).

II - 3.1 Key factors for an effective pretreatment of lignocellulosic materials

For the pretreatment to be considered effective, the following requirements must be taken into account:

- High yields for multiple crops and harvesting times.

  It has been shown that various pretreatments are more suitable for specific feedstocks, depending on their type (from hardwood or softwood to agriculture residues), their origin (crops, agricultural and wood residues, etc.) and harvesting time, among others (Chandra *et al.*, 2007; Pan and Zhu, 2011).

- Highly digestible pretreated biomass.

  Cellulose from pretreatment should be readily digestible with yields higher than 90% in less than five or, preferably, less than three days, with an enzyme loading lower than 10 FPU/g cellulose (Yang and Wyman, 2008; Pan and Zhu, 2011).

- No significant sugars degradation.

  Through the pretreatment step, high yields of fermentable sugars should me achievable in the hydrolyzation stage, with no sugar loss by degradation: in other words, maximization of overall sugar recovery (Oliva *et al.*, 2003; Yang and Wyman, 2008, Kumar *et al.*, 2009; Pan and Zhu, 2011).

- Minimum amount of toxic compounds/ fermentation inhibitors.

  Harsh conditions during pretreatment can lead to partial hemicellulose degradation and also to the generation of toxic compounds resultant of the sugar decomposition, which can influence the proceeding hydrolysis and fermentation steps, mainly by inhibition. The liquid hydrolyzate from pretreatment must be fermentable following a low-cost, high yield conditioning step (Oliva *et al.*, 2003; Pan and Zhu, 2011).

- Effective biomass size reduction.

  The biomass fragmentation should be carried out in low cost reactors through minimizing their volume, employing appropriate materials of construction for highly corrosive chemical environments, and keeping operating pressures reasonable (Yang and Wyman, 2008).

↳ Non-production of solid-waste residues.

The chemicals formed during the pretreatment step should not represent a disposal issue (Alvira *et al.*, 2010).

↳ Obtainment of high sugar concentration.

The sugars concentration obtained from the coupled operations of pretreatment and enzymatic hydrolysis should be above 10% in order to ensure an adequate ethanol concentration at the end of the fermentation stage and to keep recovery and other downstream cost manageable (Alvira *et al.*, 2010).

↳ Fermentation compatibility.

The distribution of sugar recovery between pretreatment and subsequent enzymatic hydrolysis should be compatible with the choice of an organism able to ferment pentoses in hemicellulose (Yang and Wyman, 2008).

↳ Lignin recovery.

Lignin should be recovered with the purpose of simplifying downstream processes or for conversion into valuable coproducts (Yang and Wyman, 2008).

↳ Minimum heat and power requirements.

Heat and power demands for pretreatment should be low and/or compatible with the thermally integrated processes (Yang and Wyman, 2008; Kumar *et al.*, 2009; Alvira *et al.*, 2010).

II - 3.2 Inhibition issues

Lignocellulosic hydrolysates are complex mixtures of hexose and pentose sugars together with other compounds some of which can act as fermentation inhibitors (Weber *et al.*, 2010). Different pretreatments deliver different inhibitor profiles. Inhibitors of enzymes are not necessarily the same as for microorganisms (Novozymes, 2012).

During the pretreatment, aliphatic acids such as acetic and formic acids might originate from wood extractives, lignin degradation and sugar degradation (Chen, 2011).

Based on their origin, inhibition compounds are divided in three main groups: weak acids (formic, levulinic and acetic acids), furan derivatives (furfural and 5-hydroxy-2-methyllfurfural (HMF), from pentose and hexose sugars respectively), and phenolic compounds (lignin degradation products) (Araque *et al.*, 2008; Novozymes, 2012). It also was indicated that the lignin-degradation products have a more inhibitory effect on the fermentation microorganisms than the sugar-derived products (Chen, 2011).

On the other hand, sugar degradation products appear to be the strongest cellulase inhibitors, followed by lignin degradation products, whereas organic acids, salts, and oligomers are weaker enzyme inhibitors (Novozymes, 2012).

Adaptation of bacteria and yeast to the potentially toxic substances to increase their tolerance is another method to overcome inhibition without involvement of expensive and complicated procedures (Chen, 2011), but ultimately, optimization of pretreatment conditions is the best approach to minimize the formation of inhibitors that are not intrinsically present in the biomass feedstock and to provide a cost-conscious balance between substrate accessibility and inhibitor formation. (Novozymes, 2011).

II - 3.3 Physical pretreatments

According to what has been said previously, bioconversion of lignocellulosic materials to bioethanol begins with the pretreatment, which can be divided into three categories: physical, physical-chemical and chemical (Balat, 2011).

Usually, the lignocellulosic materials processing starts with physical pretreatments that involve the breakdown of the biomass feedstock into smaller particles, increasing the specific surface area and disruption of the cellulose crystallinity, so that the fibres accessibility can be improved in the subsequent stages (Chandra *et al.*, 2007; Balat *et al.*, 2008). However, the physical pretreatments do not result in lignin removal, which has been shown to restrict the efficiency of the succeeding processing stages (Chandra *et al.*, 2007).

There has been recently suggested another physical pretreatment which consist in irradiating cellulose with gamma rays, cleaving the *β*-1,4-glycosidic bonds, thus giving a larger surface area and a lower crystallinity. Despite being applicable at a small scale, this method is far too expensive to be used in a full-scale process. Furthermore, it is also doubtful that it can be used in combination with technologies supposed to be environmentally friendly (Galbe and Zacchi, 2007).

II - 3.3.1 Mechanical fragmentation

Although the mechanical fragmentation alone is not enough to increase the sugar conversion, the lignocellulosic materials processing requires a particle maximum size reduction, so that they can be effective (Sousa *et al.* 2009). The size reduction of the lignocellulosic material can be achieved by a combination of chipping, grinding and milling, in order to reduce cellulose crystallinity or its polymerization degree (Kumar *et al.*, 2009; Sousa *et al.* 2009; Balat, 2011).

Power requirements of mechanical fragmentation depend on the final particle size and the biomass characteristics. It increases rapidly with decreasing particle size. Besides being energy intensive, fragmentation techniques are also time-consuming and expensive (Balat, 2011). Besides, it is important to perceive that when a very small particle size is reached, a huge amount of energy has been consumed, and the method becomes economically unworkable (Sousa *et al*. 2009).

II - 3.3.2 Pyrolysis

Pyrolysis has been used as a pretreatment for lignocellulosic biomass, since it can be used as substrate for a fast pyrolysis for thermal conversion of cellulose and hemicelluloses into fermentable sugars with reasonable yields. When cellulosic materials are subjected to high temperatures (above 300ºC), cellulose rapidly decomposes to produce gaseous products and residual char (Sun, 2008; Balat, 2011). At lower temperatures, the decomposition is much slower, and the products formed are less volatile. The pyrolysis process is enhanced when carried out in the presence of oxygen (Kumar *et al.*, 2009).

II – 3.4 Physical-chemical pretreatments

This category on pretreatments includes mixtures of purely physical and chemical methods, and methods in between those two (Galbe and Zacchi, 2007).

II – 3.4.1 Steam explosion

Steam pretreatment, also known as steam explosion, is one of the most widely used methods for pretreatment of lignocellulosic materials (Galbe and Zacchi, 2007; Balat, 2011).

According to this method, the raw material is treated with high-pressure saturated steam at a temperature typically between 160 and 240ºC (corresponding to a pressure between 6 and 34 bar), which is maintained for several seconds to a few minutes, after which a swift cooling and an adiabatic expansion takes place, making the materials undergo an explosive decomposition, hence the designation of the pretreatment (Emmel *et al.*, 2003; Galbe and Zacchi, 2007; Sousa *et al.*, 2009).

During the steam explosion pretreatment, the overall crystallinity of the cellulose is increased by the crystallization of its amorphous portions. Hemicelluloses are thought to be hydrolyzed by acetic and other acids released during the pretreatment and found in the liquid phase as oligomeric and monomeric sugars and there is evidence that steam explosion promotes delignification to a limited extent, since lignin is redistributed on the fiber surfaces occurs as a result of melting and depolymerization/repolymerization reactions.

Depending on the severity of the pretreatment, some degradation of the cellulose to glucose can take place (Galbe and Zacchi, 2007; Kumar *et al.*, 2009; Balat, 2011).

Addition of H_2SO_4 or CO_2, usually between 0.3 to 3% (w/w), can substantially decrease time and temperature needed, effectively improve hydrolysis, decrease inhibitory compounds production and completely remove hemicellulose. Actually, for the pretreatment of softwoods, adding an acid as a catalyst is a prerequisite to increase the accessibility of the substrate (Kumar *et al.*, 2009).

Since it's difficult to find conditions that originate high yields of both hexose and pentose sugars, and at the same time also create a cellulose fraction which is easy to hydrolyze to glucose, a two-stage steam explosion pretreatment may be useful: it has been suggested that two-stage pretreatment using H2SO4 impregnation in the first stage under mild conditions, followed by SO2 impregnation in the second stage under harsher conditions, could be advantageous due to the difference in optimal pretreatment conditions between cellulose and hemicelluloses, thus obtaining high yields of both sugars and avoiding the release of fermentation inhibitors (Galbe and Zacchi, 2007; Chen, 2011). Thereby, it's relatively easy to optimize the pretreatment conditions so that it is effective for a large variety of vegetal biomass (Emmel *et al.*, 2003).

Although it has some attractive advantages, such as high ethanol yield, better utilization of raw materials and lower enzyme consumption, two-stage steam explosion pretreatment requires economic analysis to determine whether these advantages outweigh the extra cost involved (Chen, 2011).

The major issues that can influence this pretreatment are residence time, temperature, particle size and catalyst addiction, which can be sulfuric acid or sulfur dioxide (Sassner *et al.*, 2008). However, experiments made without addiction of catalysts report conversions in xylose between 45 and 65% (Hamelinck *et al.*, 2005).

Among the advantages of steam explosion pretreatment, it is important to emphasize the low energy requirement compared to mechanical fragmentation (about 70% less, to achieve the same size particle reduction) (Kumar *et al.*, 2009).

Nevertheless, using steam explosion to pretreat woody biomass produces a substrate containing a significant amount of lignin. Alkali post-treatment has been applied to steam exploded biomass to remove residual lignin (Chen, 2011).

II – 3.4.2 Ammonia fiber explosion

Ammonia fiber explosion (AFEX) is a method which, similarly to the steam pretreatment process, operates at high pressures. The biomass is treated with liquid ammonia (up to 2 kg per kg of dry biomass) for about 10 to 60 minutes at temperatures below 100 ºC and pressure above 3MPa). The ammonia is recycled after pretreatment by reducing the pressure, as ammonia is very volatile at atmospheric pressure. During pretreatment only a small amount of the solid material is solubilized which means that almost no hemicellulose or lignin is removed. However, the materials structure is changed resulting in an increasing of the water holding capacity, decrystallization of cellulose, partial depolymerization of hemicellulose, deacetylation of acetyl groups and a higher enzymatic digestibility. Shao *et al.* (2010) concluded that AFEX-pretreated starchy substrates had a 1.5 to 3 times higher enzymatic hydrolysis yield compared with untreated substrates, which means that AFEX effects on lignocellulosic biomass can be an important factor that influences the yield on enzymatic hydrolysis and microbial fermentation (Chundawat *et al.*, 2006; Galbe and Zacchi, 2007; Kumar *et al.*, 2009; Shao *et al.*, 2010).

Despite performing best on agricultural waste and have attractive economics compared to several leading pretreatment technologies, AFEX has not proven to be efficient on wood due to its higher lignin content. Nevertheless, this process does not produce inhibitors that may affect downstream biological processes (Chundawat *et al.*, 2006; Galbe and Zacchi, 2007).

II – 3.4.3 Liquid hot-water

Liquid hot-water pretreatment, also designated hydrothermolysis, uses water as media to pretreat biomass under pressure, maintaining it in the liquid state at elevated temperatures (160–240 ºC). It converts approximately 40–60% of the total biomass with 4–22% of the cellulose and nearly all of the hemicellulose to originate liquid soluble oligosaccharides. Although lignin is partially depolymerized and solubilized, complete delignification is not possible using hot water alone, since water cannot solubilize a large amount of lignin fragments. Condensation and redeposition of dissolved lignin onto fiber surface can also happen during hot-water pre-treatment, especially at high temperatures. It is also likely that structural and chemical changes occur to the lignin during hot-water pretreatment as well, but this phenomenon is yet to be confirmed by more effective analytical methods (Monsier et al., 2005a; Zhao, 2012). It has been proven though, that liquid hot-water pretreatment decreases cellulose crystallinity, increases its depolymerization and hydrolyzes almost all of the hemicellulose, which contributes to enhance cellulose accessibility (Zhao, 2012).

In order to avoid the formation of inhibitors during the hot-water pretreatment, the pH should be kept between 4 and 7 because, at this pH level, hemicellulosic sugars are retained in oligomeric form and monomers formation is minimized. Therefore the formation of degradation products is also lower (Mosier *et al.*, 2005).

It is believed that, throughout the hot-water pretreatment, cell wall disruption occurs, associated with a rupture of glycosidic bonds in hemicelluloses and amorphous regions of cellulose, essentially due to hydrolytic mechanisms. Moreover, cleavage of O-C-O bonds and methoxyl groups in lignin, and depolymerization of lignin down to relatively small subunits take place (Zhao, 2012).

Liquid hot-water has been shown to remove up to 80% of the hemicellulose and to enhance the enzymatic digestibility of pretreated material in herbaceous feedstocks, such as corn, sugarcane bagasse and wheat straw (Alvira *et al.*, 2010).

Two-step pretreatments have been studied to optimize hemicellulosic sugars recovery and to enhance enzymatic hydrolysis yields. Yu *et al.* (2010) have developed a two-step liquid hot-water pretreatment with the objective of achieving complete saccharification of both hemicellulose and cellulose of *Eucalyptus grandis*. In the first step, the highest yield of total xylose was achieved after 20 minutes at 180ºC. The optimum conditions, with minimal sugar degradation, for the second step were at 200ºC for 20 minutes, since it was found that the conversion into sugars is more sensitive to temperature than it is to time. The total sugar recovery from *E. grandis* with the optimized pretreatment and 72 hours of enzymatic hydrolysis, reached 96.63%, which is superior to the recovery from a single-step pretreatment with liquid hot-water or dilute acid (Yu *et al.*, 2010).

In general, liquid hot-water pretreatments are attractive from an economic point of view, since they require no catalyst and involve low-cost reactors, because there is a low corrosion potential. In comparison to steam explosion, lower formation of inhibitors is obtained. However, water demanding in the process and energetic requirements are higher. Furthermore, this pretreatment is not yet developed at commercial scale (Alvira *et al.*, 2010).

II - 3.5 Chemical pretreatments

In the following section, the major chemical pretreatment technologies are presented. They diverge essentially in the kind of chemical compounds used and mechanisms responsible for the structural disruptions and chemical modifications (Sousa *et al.*, 2009).

II - 3.5.1 Ozonolysis

Ozonolysis can be used as a pretreatment to degrade lignin (selectively reacting with the carbon-carbon bonds) and hemicellulose in lignocellulosic materials, keeping cellulose hardly affected (Sun and Cheng, 2008; Sugimoto, *et al*. 2009).

According to investigations undertaken by Sugimoto *et al*. (2009), the enzymatic saccharification sugar yield of Japanese cedar sawdust increased linearly with the progress of lignin degradation by the ozone pretreatment. The pretreatment was also effective with Hinoki cypress sawdust, lumber and board wastes (Sugimoto, *et al*. 2009).

This pretreatment method has many advantages like removing lignin effectively, not producing toxic residues for downstream processes and, thus, absence of enzymatic and fermentation inhibitors (except in extensive treatment) and the possibility of being carried out at room temperature. However, ozonolysis is a rather expensive process due to the large amount of ozone required for it to be effective (Sun and Cheng, 2008; Sugimoto, *et al*. 2009).

II - 3.5.2 Alkaline pretreatment

Alkaline pretreatments use alkaline compounds, such as NaOH or $Ca(OH)_2$, to pretreat lignocellulosic materials and their effect depends mainly on the lignin content. All lignin as well as part of the hemicelluloses are removed, and the reactivity of cellulose for later hydrolysis is increased (Hamelinck *et al.*, 2005).

The treatment of lignocellulosic materials with diluted sodium hydroxide causes the materials to swell, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Sun and Cheng, 2002; Balat *et al*., 2008; Sánchez and Cardona 2008) and providing more accessibility for enzymes and bacteria (Hendriks and Zeeman, 2009). In addition, this pretreatment removes acetyl and the various uronic acid substitutions on hemicelluloses that lower the accessibility of the enzyme to the hemicelluloses and cellulose surface. (Balat *et al*., 2008).

In alkaline treatment, the saponifiables (triglycerides, fatty acids, resin acids and steryl esters) are saponified to sodium soaps, glycerol and sterols. Above a certain concentration, the dissolved fatty and resin acid soaps may form micelles or resin/fatty acid mixed micelles, which may solubilize other nonsaponifiable compounds (Chen, 2011).

Alkaline treatment can also cause the solubilization, redistribution and condensation of lignin. These issues must be taken into account, since they can nullify or neutralize the effects of the lignin removal and cellulose swelling (Hendriks and Zeeman, 2009).

An important feature of this pretreatment is that the biomass alone is responsible for the consumption of some alkalinity of the solution, due to the conversion of alkali into irrecoverable salts or incorporation as salts into the biomass. Therefore, the remaining solution is called residual alkaline solution (Balat *et al.*, 2008; Hendriks and Zeeman, 2009).

It is also important to emphasize that the alkaline pretreatment implies the changing of the cellulose structure into a denser and a thermodynamically more stable form than that of the native cellulose (Hendriks and Zeeman, 2009).

Considering economic and environmental aspects, dilute NaOH treatment would be much more suitable than the concentrated NaOH pretreatment. Combination of dilute NaOH treatment and other treatments seems to be more efficient (Balat *et al.*, 2008).

II - 3.5.3 Acid pretreatment

There are many acid pretreatment technologies available and, despite being chemically alike, they work differently, though. While diluted acid pretreatment uses a strong acid as a catalyst, we can also think of liquid hotwater pretreatment as an acid pretreatment, since water acts like an acid at high temperatures and also due to the release of acetic acid, originated by the hemicelluloses degradation during the process (Sousa *et al.*, 2009).

Strong acids such as sulfuric and hydrochloric acids have been frequently used to treat lignocellulosic materials, but acid pretreatment can also involve nitric or acetic acids, depending on the feedstock. Despite being powerful agents, strong acids are toxic, corrosive and hazardous and require corrosion resistant reactors (Sun and Cheng, 2002; Herrera *et al.*, 2004; Silverstein *et al.*, 2007).

The acid pretreatment can be done with dilute or concentrated acids (Hendriks and Zeeman, 2009).

The main reaction that occurs during acid pretreatment is the chemical hydrolysis of hemicellulose, especially xylan, exposing cellulose to enzymatic digestion (Hendriks and Zeeman, 2009; Balat, 2011). Solubilized hemicelluloses (oligomers) can be subjected to hydrolytic reactions producing monomers, furfural, hydroxymethylfurfural and other volatile products in acidic environments. Besides, lignin quickly condensates and precipitates during the process. Hemicellulose solubilization and precipitation of solubilized lignin are, however, more pronounced during concentrated acid pretreatment when compared to dilute acid pretreatment (Hendriks and Zeeman, 2009). However, the concentrated acid must be recovered after hydrolysis in order to make the process economically feasible (Sun and Cheng, 2002).

There are mainly two types of dilute acid pretreatment processes:

- Continuous-flow process with low solids loading (5–10% [w/w]) at high temperature (above 160ºC);
- Batch process with high solids loading (10–40% [w/w]) and lower temperature (below 160ºC).

In general, higher pretreatment temperatures and shorter residence times result in higher soluble xylose recovery yields and enzymatic cellulose digestibility (Sun and Cheng, 2002; Balat, 2011).

Although dilute acid pretreatment gives high reaction rates and improves the hydrolysis significantly, its cost is usually higher than some physic-chemical pretreatment methods as AFEX or steam explosion. Furthermore, a neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes (Sun and Cheng, 2002; Balat, 2011).

Acid pretreatment comprise also some disadvantages, such as the following:

- The need to use expensive, corrosion resistant construction materials;
- The requirement of previous particle size reduction;
- Formation of degradation products and release of natural biomass fermentation inhibitors (Balat, 2011).

II - 3.5.4 Sulfite pretreatment

Existing alkaline, acid, liquid hot-water, steam explosion, AFEX, and organosolv pretreatment technologies have achieved satisfactory levels of success. Nevertheless, some critical issues associated with woody biomass bioconversion remained unresolved:

- Most existing pretreatment processes have low cellulose conversion of softwood, except for organosolv pretreatment;
- These pretreatments commonly require energy intensive size reduction from wood chips to particles of millimeters or less (fiber or powder) prior to chemical pretreatment to achieve satisfactory cellulose conversion efficiencies (typical size reduction energy consumptions for fiberization wood chips are about 200–600 Wh electricity/kg oven dry wood);
- Limited removal of feedstock recalcitrance, leads to a slow enzymatic hydrolysis rate and affects the process efficiency and, therefore, the economics of cellulosic ethanol production.
- Most of the existing pretreatment technologies have poor scalability (Zhu *et al.*, 2009).

Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose (SPORL) is a new method recently developed for cellulosic ethanol production from lignocellulosic materials (Zhu *et al.*, 2009).

SPORL is a two-step pretreatment: a chemical treatment with sulfite, followed by a mechanical size reduction (fiberization). With moderate amount of sulfuric acid and sulfite dosages, SPORL pretreatment works effectively with softwoods and hardwoods (Zhang *et al.*, 2013). The terms sulfite and bisulfite are used interchangeably in SPORL because active reagents in the pretreatment liquor can be sulfite (SO_3^{-2}), bisulfite (HSO_3^{-1}), or the combination of both with an acid medium (Wang *et al.*, 2009).

Hardwoods contain significant amounts of acetyl groups (3–5%). The formation of acetic acid from acetyl groups during pretreatment can maintain the pH value required for effective SPORL pretreatment without the application of additional acid (Wang *et al.*, 2009).

Unlike traditional sulfite pulping, whose goal is delignification while preserving cellulose for strong pulp, SPORL is different from traditional sulfite pulping in terms of objectives and process operating conditions (pH, temperature and chemical dosage). During the SPORL pretreatment, most of hemicelluloses are removed in the form of fermentable sugars with limited formation of fermentation inhibitors (such as furfural and hydroxymethylfurfural), cellulose is depolymerized and lignin condensates (excessive lignin condensation is prevented through proper operating conditions control). The removal of hemicelluloses and lignin makes the pretreated substrate readily digestible by enzymes. The dissolved hemicellulosic sugars (a mixture of hexoses and pentoses) in the pretreatment liquor are also fermentable because of limited formation of fermentation inhibitors during the pretreatment. In addition, the energy consumption for size reduction after the chemical pretreatment is significantly reduced (Wang *et al.*, 2009; Pan and Zhu, 2011; Zhang *et al.*, 2013).

The development of the SPORL process is based on the following fundamental understandings of sulfite pulping:

- A considerable amount of hemicellulose degradation and removal takes place during sulfite pulping, as evidenced by the predominant xylose content in pulping spent liquor;
- The degrees of polymerization of xylan and cellulose are reduced;
- Sulfonation of lignin increases the hydrophilicity of lignin (and therefore reduced hydrophobic interaction with enzymes;
- The hemicelluloses degree of dissolution, degradation of cellulose, and sulfonation and condensation of lignin increase with increasing reaction time and temperature, and with decrease of pH (Zhu *et al.*, 2009; Pan and Zhu, 2011).

Studies report that SPORL was very effective even when directly applied to chip-size (approximately 2×3×0,5 cm^3) woody biomass and without prior chip impregnation and mechanical fragmentation, resulting in lower energy consumption, and therefore, significantly reduced pretreatment cost and excellent substrate digestibility (Wang *et al.*, 2009; Pan and Zhu, 2011).

Furthermore, the SPORL pretreatment has a great scalability because the process can adapt to existing infrastructure and equipment in pulp industry and offers many advantages over existing processes for commercialization, with low environmental and technological barriers and risks. (Wang *et al.*, 2009; Zhu *et al.*, 2009; Zhang *et al.*, 2013).

Zhu *et al.* (2009) submitted spruce wood chips to SPORL with 8-10% bisulfite and 1.8 – 3.7% sulfuric acid at 180ºC for 30 minutes. More than 90% of cellulose conversion was achieved with an enzyme loading of about 14.6 filter paper units (FPU) of cellulase plus 22.5 cellobiose unit (CBU) ß-glucosidase per gram of oven dry substrate after 48 hours of hydrolysis. Electric energy consumption for size reduction was reduced to about 19 Wh/kg of oven dried untreated wood and the amounts of fermentation inhibitors (furfural and HMF) produced were low (1 and 5 mg/g of untreated oven dried wood, respectively). Similar results were achieved when the SPORL was applied to red pine (Zhu *et al.*, 2009).

Table 2 summarizes and compares mass balance results from spruce pretreated with SPORL and Dilute Acid methods.

Table 2 - Mass balance of SPORL and Dilute Acid pretreated Spruce (Adapted from Pan and Zhu, 2011).

	SPORL	**Dilute acid**
Unpretreated spruce (g)	100	100
Glucose	46.7	46.7
Galactose	2.6	2.6
Mannose	10.8	10.8
Arabinose	1.2	1.2
Xylose	5.5	5.5
Substrate (g)	60.5	64.1
Glucose	40.3	33.3
Mannose	7.1	_
Pretreatment Líquor (g)		
Glucose	2.9	3.0
Galactose	1.3	0.4
Mannose	4.5	0.9
Arabinose	0.4	0.1
Xylose	2.2	0.2
Inhibitor in liquor (g/L)		
Acid-soluble lignin	16.6	4.8
Formic acid	1.9	7.4
Acetic acid	2.7	5.3
Furfural	1.3	2.9
Hydroxymethylfurfural	2.0	4.7
Levulinic acid	3.2	11.4

Note: Pretreatment conditions were sulfuric acid 5% on wood, sulfite 9% (or 0% for dilute acid) on wood, 180ºC for 30 minutes at 5/1 liquid to wood ratio.

The results listed in table 2 clearly indicate that under the same acid loading, temperature and reaction time, SPORL is superior to dilute acid for the recovery of total sugars (hexoses and pentoses). Besides, fewer inhibitors were formed from degradation of saccharides during the SPORL pretreatment than the dilute acid pretreatment, due to the addition of sulfite in the SPORL pretreatment, which increased the pH value of the pretreatment liquor, limiting extensive degradation of saccharides (Pan and Zhu, 2011).

II - 3.5.5 Organosolv

Organosolv pulping is a method to extract lignin from lignocellulosic materials with organic solvents aqueous solutions. Organosolv pretreatment is, in many ways, similar to organosolv pulping, except in the degree of delignification, which is not demanded to be as high as that of pulping (Zhao, *et al.*, 2009).

This process is used to break the structural linkages between lignin and hemicellulose. The organic solvents commonly used for such purpose are methanol, ethanol, acetone, ethylene glycol, among others (Sun and Cheng, 2002; Mamman *et al.*, 2008). Alcohols, especially the lower molecular weight aliphatic alcohols, are the most frequently used solvents in organosolv pretreatment. An advantage of employing low boiling point alcohol (mainly methanol and ethanol) is of their low boiling point, ease of recovery by simple distillation with concomitant low energy requirement for their recovery. They are of low cost and also fully miscible with water (Zhao, *et al.*, 2009).

A considerable part of the lignin is separated from the pulp, mainly cellulose, which can be easily enzymatically hydrolyzed by endo, exo-glucanases and ß-glucosidases (Araque *et a.*, 2008).

Usually a high yield of xylose and higher delignification can be obtained at high temperatures (above 185ºC) and in an acidic environment (Sun and Cheng, 2002; Mamman *et al.*, 2008; Sousa *et al.*, 2009), although there might be no need for acid addition as a catalyst, as it is believed that organic acids released from the biomass during the pretreatment act as catalysts for the rupture of the lignin-carbohydrate complex (Zhao, *et al.*, 2009).

Organosolv pretreatment presents some advantages; organic solvents are usually easy to recover by distillation and recycled. Actually, recovery of solvent is of supreme importance to make this process more cost effective: solvents used in the process need to be drained from the reactor, evaporated, condensed and recycled to reduce the cost (Sun and Cheng, 2002; Mamman *et al.*, 2008; Zhao, *et al.*, 2009; Kupiainen *et al.*, 2012); in addition, organosolv processes can isolate lignin as a solid material and carbohydrates as syrup, both of which show promise as chemical feedstocks (Zhao, *et al.*, 2009).

On the other hand, there are inherent drawbacks to the organosolv pretreatment: the pretreated solids always need to be water washed in order to avoid the reprecipitation of dissolved lignin and avoid inhibition of enzymatic hydrolysis and fermentation; furthermore, organosolv pretreatment must be performed under extremely tight and efficient control due to the volatility of the organic and due to fire and explosion hazard (Sun and Cheng, 2002; Zhao, *et al.*, 2009).

II - 3.6 Biological pretreatments

Biological pretreatment, also called biodelignification, is the biological degradation of lignin by microorganisms (Balat *et al.*, 2008). It is a safe and environmental friendly method for lignin removal from lignocellulosic materials which is attracting extensive interests (Yu *et al.*, 2009). It is also energy saving as it is performed at low temperature and needs no use of chemicals (Hamelinck, 2005; Galbe and Zacchi, 2007).

These pretreatments typically use wood degrading fungi (soft, brown and white-rot) to modify the chemical composition of the lignocellulosic feedstock (Chandra *et al.*, 2007; Galbe and Zacchi, 2007).

Generally, soft and brown-rot fungi primarily degrade the hemicellulose while imparting minor modifications to lignin. White-rot fungi are the most promising microorganisms used for biological pretreatment because of their abilities to selectively degradation of lignin (Chandra *et al.*, 2007; Yu *et al.*, 2009).

As advantageous as it might be, this pretreatment has some drawbacks: the rate of biological pretreatment processes is far too low for industrial use, hence long residence times are needed (10 to 14 days) (Sun and Cheng, 2002; Chandra *et al.*, 2007; Galbe and Zacchi, 2007); biological pretreatment also requires careful control of growth conditions and large amounts of space (Chandra *et al.*, 2007; Galbe and Zacchi, 2007). Besides, some material is lost as the microorganisms used tend to consume hemicellulose and cellulose, or lignin, depending on the extension of the pretreatment (Galbe and Zacchi, 2007).

II – 4 Hydrolysis of lignocellulosic materials

The carbohydrate polymers in lignocellulosic materials need to be converted to simple sugars before fermentation, through a process called hydrolysis (Ballat, 2011). Cellulose hydrolysis is considered the major hydrolysis step (Hamelinck *et al.*, 2005).

Various methods for the hydrolysis of lignocellulosic materials have recently been described. The most commonly applied methods can be classified in two groups: chemical hydrolysis (dilute and concentrated acid hydrolysis) and enzymatic hydrolysis (Hamelinck *et al.*, 2005; Ballat, 2011). There are, however, some other hydrolysis methods in which no chemicals or enzymes are applied. For instance, lignocellulosic materials may be hydrolyzed by gamma-ray, electron-beam or microwave irradiation. However, those processes are considered commercially unimportant (Ballat, 2011).

II – 4.1 Chemical hydrolysis

Chemical hydrolysis comprises acid catalyzed hydrolysis – concentrated and dilute – and has been employed for yielding free sugars from the highly complex lignocellulosic biomass (El-Zawawy *et al.*, 2011).

II – 4.1.1 Acid hydrolysis

Acid hydrolysis is probably the most commonly applied method among the chemical hydrolysis methods. As has been said before, it can be used either as a pretreatment preceding enzymatic hydrolysis, or as the actual method of hydrolyzing lignocellulose to sugars. One of the main advantages of dilute acid hydrolysis is achieving high xylan to xylose conversion yields, leading to a high recovery of hemicellulose sugars. On the other hand, it may lead to corrosion issues and generation of fermentation inhibitory compounds (Taherzadeh and Karimi, 2007).

Dilute acid hydrolysis (0.7–3.0%) requires high operating temperatures (200–240 ◦C). Concentrated acid hydrolysis requires high amounts of acid and hence becomes uneconomical; acid recycling also entails considerable costs (El-Zawawy *et al.*, 2011).

II – 4.2 Enzymatic hydrolysis

As has been said previously, lignocellulosic biomass is a complex structure with crystalline cellulose, hemicelluloses, and lignin as major components (Houghton *et al.*, 2006). Enzymatic hydrolysis of cellulose in plant and wood cell walls is expected to be affected by its chemical composition as well as structural and morphological features (Agarwal *et al.*, 2011).

Enzymatic hydrolysis of lignocellulosic materials is carried out by highly specific enzymes (Sun and Cheng, 2002).

Utility cost of enzymatic hydrolysis is low compared to other hydrolysis methods because enzymatic hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45 to 50ºC) and corrosion issued do not represent a problem (Sun and Cheng, 2002).

Since every enzyme is specific to a certain substrate, the use of enzymatic cocktails for efficiently catalyzing the conversion of many lignocellulosic components has been suggested. To date, the best enzyme cocktails proposed for saccharification of this material are synergistic mixtures of enzymes with defined activities, primarily those that degrade cellulose (Houghton *et al.*, 2006).

Both bacteria and fungi can produce enzymes for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic (Sun and Cheng, 2002).

II – 4.2.1 Cellulases and hemicellulases

Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process (Sun and Cheng, 2002):

- Endoglucanase (EG or endo-1,4-D-glucanohydrolase) which attacks regions of low crystallinity on the cellulose fiber, creating free chain-ends;
- Exoglucanase or cellobiohydrolase (CBH or ß-D-glucan cellobiohydrolase) which degrades the molecule further by removing cellobiose units from the free chain-ends;
- ß-glucosidase which hydrolyzes cellobiose to produce glucose (Sun and Cheng, 2002).

During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol (Sun and Cheng, 2002).

In addition to the cellulases, there are also a number of auxiliary enzymes that attack hemicelluloses, such as glucuronidase, acetylesterase, xylanase, ß-xylosidase, galactomannanase and glucomannanase (Sun and Cheng, 2002).

One of the major limitations of this process is the consistently high cost of the enzymes involved in the conversion of the cellulose component into fermentable sugars. This is primarily due to the comparatively high (compared with amylase loadings required for starch hydrolysis) protein loadings commonly required to overcome the substrate features and enzyme-related factors limiting effective cellulose hydrolysis (Arantes and Saddler, 2011).

One question remains: how will structural and chemical details of enzyme substrate–binding sites affect enzyme adsorption and reaction rates? From cellulase kinetics alone, that question would never be answered (Houghton *et al.*, 2006).

II – 4.2.2 Enzymatic hydrolysis of cellulose

The rate-limiting step in hydrolysis is not catalytic cleavage but disruption of a single substrate chain from its native matrix, thereby rendering it accessible to the catalytically active cellulase site. Thus, the processes and interactions that facilitate this disruption of insoluble celulose must be analyzed and understood. To approach this problem, a detailed understanding of the structure of both the crystalline and noncrystalline portions of cellulose fibrils is first necessary:

- Hydrolysis of crystalline cellulose is the rate-limiting step in biomass conversion to ethanol because aqueous enzyme solutions have difficulty acting on this insoluble, highly ordered structure. Cellulose molecules in their crystalline form are packed so tightly that enzymes and even small molecules, such as water, are unable to permeate this structure (Houghton *et al.*, 2006).
- Cellulases and hemicellulases are secreted from cells as free enzymes or as extracellular cellulosomes. The collective activity of enzyme systems is believed to be much more efficient than the individual activity of any isolated enzyme (synergic effect); therefore, to truly understand how enzymes function, they must be studied as systems rather than individually or a few at a time. Besides, systems eventually must be analyzed under laboratory conditions more representative of real-world environments. For instance, laboratories often use purified cellulose as the substrate for enzyme analysis rather than more heterogeneous, natural lignocellulosic materials. This can provide erroneous conclusions about natural enzyme activity (Houghton *et al.*, 2006).

II – 4.2.3 Enzymatic cocktail

Although the reduction of pretreatment severity is sometimes required to reduce costs, low severity factors results in less sugar-release and consequently higher amount and different types of enzymes will be required to achieve high sugar yields from both cellulose and hemicelluloses fraction. In this context, development of hemicellulases and other accessory enzymes needed for complete degradation of lignocellulose components has become an important issue. Recent studies show the importance of new balanced enzymatic complexes or cocktails containing optimal combinations to effectively modify the complex structure of lignocellulosic materials (García-Aparicio *et al.*, 2007; Merino and Cherry, 2007).

However, achieving rapid and complete enzymatic hydrolysis of lignocellulosic biomass at low enzyme loadings is a major technical challenge in the commercialization of cellulose-based processes converting biomass to ethanol (Arantes and Saddler, 2011).

In a typical batch enzyme-based process, cellulose conversion-time experiments are characterized by a three-phase curve (Figure 9). This usually starts with the rapid adsorption of the cellulases onto the readily accessible cellulose, followed by an initial, fast rate of hydrolysis. Nonetheless, the reaction quickly reaches an intermediate phase, characterized by a moderate hydrolysis reaction rate when about 50-70% of the original substrate has been hydrolyzed. Thereafter, a very slow phase is characterized by a steady decrease of the reaction rate, which results in only a slight increase in the conversion of the remaining (the so-called “inaccessible” or recalcitrant) cellulose (Arantes and Saddler, 2011).

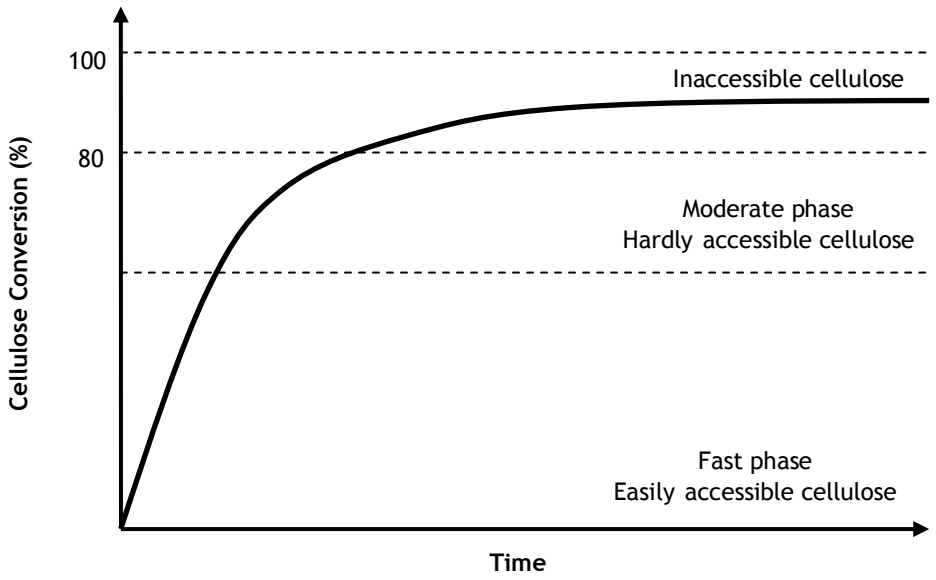


Figure 9 - Typical time course of the enzymatic hydrolysis of cellulose (adapted from Arantes and Saddler, 2011).

Typically, extended hydrolysis times or high protein loadings are required to achieve a near-complete conversion of cellulose. In some cases, depending on the nature of the substrate and the pretreatment method used, even at very high protein loadings of the commercially available cellulase mixtures and extensive hydrolysis times, complete cellulose hydrolysis cannot be achieved (Arantes and Saddler, 2011).

II – 4.2.4 Limiting factors of enzymatic hydrolysis

The enzymatic hydrolysis of biomass cellulose is a complicated process involving the adsorption of cellulases onto cellulose surface, synergic effects of cellulase components to hydrolyze cellulose fiber, followed by release and transfer of products into bulk liquid. Many factors have been found to affect the enzymatic hydrolysis of lignocellulose, mainly related to lignocellulose structural features (including chemical composition and physical structure) and related to mechanisms and interactions of the cellulolytic enzymes (Zhao, 2011).

II – 4.2.4.1 Structural limitations

Several structural factors have been found to affect the enzymatic digestibility of biomass, which include contents of lignin, hemicelluloses, acetyl group and cell wall proteins (structural barriers), cellulose crystallinity, degree of polymerization, pore volume, accessible surface, particle size, cell wall thickness. (Zhao, 2011).

Agarwal *et al.* (2011) studied the roles of cellulose crystallinity, cell wall particle size, and lignin removal on enzymatic hydrolysis and found out that the factors that modify the ultrastructure of the cell wall are more important than the cellulose crystallinity. They suggested that factors like lignin removal indirectly influence the hydrolysis because it causes physicochemical modification of the cell wall structure that, in turn, makes enzymatic access to cellulose easier (Agarwal *et al.*, 2011).

Figure 10 illustrates the main groups of structural limitations of enzymatic hydrolysis of lignocellulosic materials. The accumulation of these limitations leads to compounded effects.

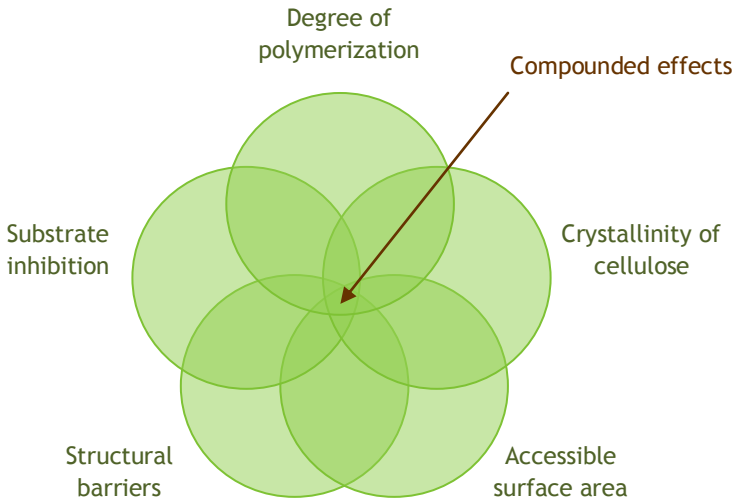


Figure 10 - Representation of the main structural limitations of enzymatic hydrolysis of lignocellulosic materials (Adapted from Novozymes, 2012).

II – 4.2.4.2 Enzyme-related and substrate-related limitations

The choice of pretreatment technology for a particular raw material depends on several factors, some of them directly related to the enzymatic hydrolysis step such as sugar-release patterns and enzymes employed. Thus, the combination of the composition of the substrate, type of pretreatment, dosage and efficiency of the enzymes used for the hydrolysis have a great influence on biomass digestibility, although the individual impacts of these factors on the enzymatic hydrolysis are still unclear (García-Aparicio *et al.*, 2007; Merino and Cherry, 2007).

The main factors that influence the enzymatic hydrolysis of cellulose in lignocellulosic feedstocks can be divided in two groups: enzyme-related and substrate-related factors, though many of them are interrelated during the hydrolysis process (García-Aparicio *et al.*, 2007; Merino and Cherry, 2007).

Substrate-related factors limiting enzymatic hydrolysis are directly connected to the pretreatment employed. Even though the effect of these factors is normally interrelate, they are described separately (García-Aparicio *et al.*, 2007; Merino and Cherry, 2007):

↳ Avaiable surface area

It has been suggested that the accessibility of the substrate to the cellulolytic enzymes is one of the most important factors influencing the rate and extent of enzymatic hydrolysis of lignocellulosic substrates. This is not surprising, as the enzymatic hydrolysis of cellulose is a surface-dominated phenomenon, and direct physical contact between the cellulase enzymes and substrate must occur (Arantes and Saddler, 2011). Thus, one of the main objectives of the pretreatment is to increase the available surface area for the enzymatic attack (Alvira *et al.*, 2010).

↳ Crystallinity index

Cristallinity index has been considered an important factor in hydrolysis rates of relatively refined cellulosic substrates (Chang and Holtzapple, 2000). It measures the relative fraction of crystalline cellulose in the total solid and it is impacted by the presence of lignin and hemicellulose, which are both amorphous. The presence of these compounds can increase the crystallinity index of the pretreated solid (Zhao, 2011).

Although the majority of pretreatments that affect the cristallinity index of cellulose do it in such a way that this parameter is reduced, it has been observed that, in some cases, pretreatment of lignocellulosic materials increases the cristallinity index of the cellulose fraction. This fact has been suggested to be due to the removal or reduction of more easily available amorphous cellulose after pretreatments such as steam explosion. In contrast, high pH pretreatments have been shown to have less effect and gave even reduced biomass crystallinity in some instances (Alvira *et al.*, 2010).

According to Zhu *et al.* (2008), at short hydrolysis periods, lignin content is not important to digestibility when crystallinity is low, and similarly, at long hydrolysis periods, crystallinity is not important to digestibility when lignin content is low.

↳ Degree of polymerization

The cellulose degree of polymerization translates into the number of glycosyl residues per cellulose chain. This parameter is essentially related to other substrate characteristics, such as cristallinity (Alvira *et al.*, 2010).

Depolymerization depends on the nature of cellulosic substrate. As mentioned before, in the enzymatic hydrolysis, endoglucanases cut at internal sites of the cellulose chains, preferentially less ordered, being primarily responsible for decreasing the degree of polymerization of cellulosic substrates. Studies on the effect of different pretreatments on cellulose chain length have suggested that xylan removal have a more severe impact on cellulose chain length than lignin removal (Kumar and Wyman, 2009; Alvira *et al.*, 2010).

↳ Porosity

The porosity or pore volume of the substrate is directly related to its available surface area (Alvira *et al.*, 2010). The pore size of the substrate in relation to the size of the enzymes is the main limiting factor in the enzymatic hydrolysis of lignocellulosic biomass (Hendriks and Zeeman, 2009).

It has been shown that lignin removal increases the porosity of the biomass and the increase in median pore width corresponds to the average molecular weight of the lignin molecules removed (Chandra *et al.*, 2007; Zhao, 2011).

Further research showed that the process of drying fibers causes a significant loss of large pores and a reduction of surface area, as it stiffens the fibers structure in a process known as *hornification* (Diniz *et al.*, 2004).

When wood pulp fibers are dried, the internal fiber volume shrinks, because of structural changes in wood pulp fibers. If fibers are resuspended in water, the original water-swollen state is not regained. This effect is dependent on the cell wall physical and chemical structure of the never-dried material, the susceptibility of the never-dried materials to drying, the drying method, and drying duration. The concept of hornification was first introduced by G. Jayme in 1944. Jayme introduced the water retention value (WRV) measurement by centrifugation and defined irreversible hornification as a decrease in WRV, expressed in percentage of the original value of the same never-dried sample (Diniz *et al.*, 2004; Luo and Zhu, 2011).

↳ Lignin barrier

As has been said before, lignin and hemicelluloses difficult the access of cellulose enzymes to cellulose, thus reducing the efficiency of the enzymatic hydrolysis. Lignin limits the rate of enzymatic hydrolysis by acting as a physical barrier, preventing the digestible parts of the substrate to be hydrolyzed (Chang and Holtzapple, 2000). Besides, lignin appears to reduce cellulose hydrolysis by adsorption of cellulases on its surface (Alvira *et al.*, 2010).

↳ Hemicellulose content

Removal of hemicellulose increases the average pore size of the substrate and therefore increases the accessibility and the probability of the cellulose to become hydrolyzed (Chandra *et al.*, 2007).

↳ Feedstock particle size

There is some evidence to support that reduction of particle size increases specific surface area and subsequently the accessibility of cellulose to the enzymes (Sun and Cheng, 2002).

↳ Cell wall thickness (coarseness)

The waxy barrier comprising grass cuticle and tree bark impedes penetration of enzymes; even milled, plant stems and woody_tissues limit liquid penetration by their nature (Alvira *et al.*, 2010).

II – 5 Fermentation

Yeasts are generally robust organisms, but they can be susceptible to environmental stress just like other microbes. High sugar concentrations encountered immediately after hydrolysis exert osmotic stress on yeast. A high concentration of solute outside the cell will cause water to leave the cell to reach equilibrium. This results in a longer lag phase at the beginning of fermentation. This can be overcome by performing a fed-batch or continuous fermentation so that sugar is added at approximately the rate at which the yeast consumes it (Novozymes, 2012).

Another osmotic stressor, and thus a yeast inhibitor, is high salt concentration, which is a result of pretreatment and neutralization of the biomass prior to hydrolysis and fermentation. To combat this, an alternative pretreatment that does not require significant pH adjustment might be utilized. A second route is choosing a fermenting organism that is salt tolerant (Novozymes, 2012).

Lastly, ethanol is inhibitory to yeast at high concentration by disrupting the integrity of the cell membrane. Some yeast strains are more ethanol tolerant than others, and for hydrolyzed, acid-pretreated corn stover, the incoming sugar concentrations are not high enough to generate an inhibitory ethanol concentration. This could be an issue for other feedstock or if an evaporation step is used prior to fermentation to concentrate the sugars (Novozymes, 2012).

II – 5.1 Hydrolysis and fermentation strategies

There are several viable fermentation options available, each with benefits and drawbacks. The most economically viable process options reach for configurations that maximize enzyme performance (Novozymes, 2012). The most common strategies are the following.

II – 5.1.1 Separate enzymatic hydrolysis and fermentation

In separate enzymatic hydrolysis and fermentation (SHF), pretreated lignocellulosic material is hydrolyzed to glucose and subsequently fermented to ethanol in separate units. The major advantage of this method is that it is possible to carry out the cellulose hydrolysis and fermentation at their own optimal conditions, since the optimal temperature for cellulase is usually between 45 and 50 ºC, and for the fermentation microorganisms it varies between 30 and 37ºC. The main drawback of SHF is the possible inhibition of due to released sugars in the hydrolysis stage (Taherzadeh and Karimi, 2007).

With respect to separate fermentation, there are batch and fed-batch processes; Batch fermentation of mixed sugar streams typically takes two to three days depending on the yeast pitch used because the fermenting organism will preferentially consume the sugar on which it can grow faster (typically glucose), then switch gears metabolically to utilize the other sugars; Fed-batch fermentation limits the effective concentration of the preferentially consumed sugar.

This process can force the fermenting organism to utilize both sugars concurrently, which can dramatically decrease fermentation time (Novozymes, 2012).

II – 5.1.2 Simultaneous saccharification and fermentation

Issues related to inhibition by sugar concentration can be minimized using simultaneous saccharification and fermentation (SSF). In this process, the glucose produced by the hydrolyzing enzymes is consumed immediately by the fermenting microorganisms present in the culture, keeping a low concentration of sugars in the media. SSF gives a higher reported ethanol yields from cellulose than SHF and requires lower amounts of enzyme. However, it is important to have the optimum conditions for the enzymatic hydrolysis and fermentation as close as possible, particularly with respect to pH and temperature (Taherzadeh and Karimi, 2007).

In advanced biofuel production, cellulase enzyme cocktails typically operate at temperatures exceeding those at which the fermenting organism (typically mesophilic yeast) can survive. Thus, hydrolysis of cellulose and fermentation of glucose occur concurrently. From the fermenting organism's perspective, this prevents stress on the yeast cell from high initial concentrations of sugar, and also allows the organism to operate at its optimal temperature and pH (Novozymes, 2012).

The inhibition effect of ethanol can also represent a disadvantage in SSF (Taherzadeh and Karimi, 2007).

II – 5.1.3 Simultaneous saccharification and cofermentation

Another mode of operation is simultaneous saccharification and cofermentation (SSCF), in which cofermentation refers to fermentation of both five-carbon and six-carbon sugars to ethanol. The hydrolyzed hemicelluloses during pretreatment and the solid cellulose are not separated after pretreatment, allowing the hemicellulose sugars to be converted to ethanol together with simultaneous fermentation of the cellulose (Teixeira *et al.*, 2010).

Unlike SSF, where only hexoses are converted to ethanol and pentoses can be fermented in another bioreactor with a different microorganism, SSCF requires only a single fermentation step to process hydrolyzed and solid fractions of pretreated lignocellulose, since it is suggested to ferment both hexoses and pentoses in a single bioreactor with a single microorganism (Novozymes, 2012).

Chapter III – Experimental part

This chapter describes the raw materials, equipments and reagents used, as well as the experimental proceedings followed.

III – 1 Materials and Reagents

III - 1.1 Biomass

In a first approach, industrial samples of *Pinus pinaster* bleached kraft pulp and Pressurized Ground Wood Pulp (PGWP) were used to determine the activity of the cellulases and to study the influence of pulp beating on the sugar release during the enzymatic hydrolysis.

Later, for the sulfite pretreatment, *Eucalyptus globulus* and *Pinus pinaster* chips were used (figure 11 (a) and (b)). *Cytisus striatus* (broom) wood branches were also tested (figure 11 (c)). After bark removal, the branches were milled in a grinding mill (Retsch Mühle – West Germany) (figure 11 (d) and figure 12) and subjected to sulfite pretreatment.

Figure 11 - Chips of *Eucalyptus globulus* (a), *Pinus pinaster* (b) and branches of *Cytisus striatus* before (c) and after bark removal and milling (d).

Figure 12 - Grinding mill.

III - 1.2 Labware

During the experimental proceedings, diverse labware was used, namely glass material (such as pipettes, beakers, cups, test-tubes, among others), plastic material (falcon tubes), as well as electro-mechanical material (such as a vortex mixer, a laboratory blender and so on). All the apparatus used specifically for each stage will be described in the respective section of the experimental proceedings.

III - 1.3 Reagents

All the reagents used in the experiments were of analytical grade (their properties are described in table A1 of the Annex A).

III - 1.4 Enzymatic cocktail

For the enzymatic hydrolysis it was used a Novozymes' cellulosic ethanol enzyme kit (figure 13), which is an enzymatic cocktail appropriate for the hydrolysis of lignocellulosic materials with the objective of producing bioethanol. The enzymes, their classification and the recommended usage conditions were provided by Novozymes and are described in table 3.

Figure 13 - Novozymes cellulosic ethanol enzyme kit.

Table 3 - Enzyme classification, and recommended usage conditions.

NS number	Enzyme type	Activity[1]	Density[2] (g/ml)	Range of pH	Rage of temperature (ºC)	Dosage (% w/w (TS))[3]
NS22086	Cellulase complex	1000 BHU(2)/g	1.15	5.0 – 5.5	45 – 50	1 – 5
NS22083	Xylanase	2500 FXU-S/g	1.09	4.5 – 6.0	35 – 55	0.05 – 0.25
NS22118	ß-glucosidase	250 CBU/g	1.2	2.5 – 6.5	45 – 70	0.2 – 0.6
NS22119	Enzyme complex	100FBG/g (≈13700PGU/g)	1.19	4.5 – 6.0	25 – 55	0.05 – 0.4
NS22002	Hemicellulase	45 FBG/g (≈470 FXU/g)	1.20	5.0 – 6.5	40 – 60	0.4 – 2
NS22035	Glucoamylase	750 AGU/g	1.15	4.5 – 5.5	60 - 70	0.01 – 0.06

Notes: 1) BHU(2) = Biomass Hydrolysis Unit; CBU = Cellobiose Unit; FBG = Fungal Beta-Glucanase Unit; PGU = PolyGalacturonase Unit; FXU-S = Fungal Xylanase Unit; AGU = AmyloGlucosidase Unit.
2) Density values are approximate.
3) TS = Total solids.

III - 1.4.1 Enzymatic cocktail preparation

In order to minimize dilution inaccuracies due to the small quantities of the enzymes required for the hydrolysis and due to their high viscosity, enzymatic solutions were prepared prior to adding them to the falcon tubes, where the enzymatic hydrolysis took place.

The enzymatic solutions were made so that one milliliter of each solution had the maximum recommended dosage of enzyme, regarding the total solids in each falcon tube. Thus, the enzymatic cocktail was performed by adding to the falcon tubes one milliliter of each enzyme solution.

III – 2 Analytical methods

The analytical methods used are described below. Every equipment used and adjustments made are properly described.

III – 2.1 Dry matter content determination

The dry matter content of the wood and pulp samples was carried out following the guidelines of the standard ISO 638:08. The dry matter content is defined as the ratio of the mass of a test piece, after drying to constant mass at a temperature of 105 °C ± 2 °C under specified conditions, to its mass before drying. Hence, it is usually expressed as a percentage mass fraction.

For some samples, the determination of the dry matter content was performed using an infrared (IR) balance, which simulates the proceeding referred above.

III – 2.2 Kappa Number determination

In order to determine the efficiency of the pretreatment and compare the degree of delignification obtained for wood samples subjected to different treatment conditions, the Kappa Number (K-No) of the pretreated materials was determined. Although this procedure is usually performed with the purpose of measuring the residual lignin of a pulp, it was also applied to the uncooked material (although in some cases full disintegration couldn't be reached), because it was important to have estimatives for the residual lignin content in the treated materials and for the delignificion obtained with the pretreatment.

The fundament on which the K-No determination is based, is related to the susceptibility of lignin to undergo oxidation (particularly in its aromatic rings) in acidic conditions, providing a measurement of the residual lignin. However, K-No measures the total amount of material in the pulp that is oxidizable with $KMnO_4$; the method does not distinguish between oxidizable material in residual lignin and in other structures, such as double bonds in hexenuronic acids, extractives and carbonyl groups in the pulp. Therefore, the presence of this compound might interfere with the method (Costa and Colodette, 2007).

For the determinations of K-No of the pretreated unbleached pulp and uncooked material, the procedures described in the Portuguese standard NP-3186/95 was followed. K-No is defined as the consumed volume of a 0.1 N $KMnO_4$ solution, in acidic environment conditions, per gram of *od* pulp, during 10 minutes at controlled temperature. The remaining $KMnO_4$ is determined by iodometric analysis (excess KI is added to the suspension and the formed iodine is titrated with a 0.2 N $H_2S_2O_3$ solution). Two separate titrations were performed on each sample.

III - 2.3 Water retention value determination

The water retention value (WRV) is an empirical measure of the capacity of a test pad of fibers to hold water. This value usually increases with increasing beating because of internal fibrillation and widening of internal pores.

The determination of WRV followed the proceeding described in the standard SCAN-C 62:00, in which a test pad consisting of pulp fibers is formed by dewatering a pulp suspension on a test-pad former.

The test-pad is centrifuged under a specific centrifugal force (3000 g) for a specified time (15 minutes), weighted, dried and weighted again. The WRV is then calculated from the wet mass of the test pad after centrifugation and the dry mass of the same pad. Each pulp was tested in triplicate.

Although this method is not applicable to mechanical pulps, it was applied to the PGWP in order to be able to confirm the increase of water holding capacity with beating and compare it with that of the kraft pulp.

III - 2.4 Limiting viscosity number determination

Aiming to follow the enzymatic hydrolysis effect on pulps, the intrinsic pulp viscosity was determined. The viscosity of cellulose solutions is highly dependent on its concentration. Therefore, it must be determined in conditions in which the interaction between the molecular chains can be despised, reaching what's called the limiting viscosity number (a known as intrinsic viscosity).

As has been mentioned in chapter II, due to the high molecular mass and degree of crystallization of the cellulose, among other reasons, this polymer is insoluble in the majority of the solvents. Ideally, the cellulose dissolution should originate complete disintegration in individual molecular chains, without modification of their length. Among the solvents used for that purpose, cupriethylenediamine (CED) is the most commonly used, even in viscosity control at an industrial level (Carvalho, 2000).

The limiting viscosity number determinations followed the procedure described in the standard SCAN-CM 15:99, which consists on comparing the outflow time of a certain cellulosic pulp solution volume through a viscometer, with that of the solvent: in this case, a CED solution.

III - 2.5 HPLC

HPLC was the analytical method selected to monitor the sugars release, as well as their degradation products, during the pretreatment and the enzymatic stages. The determination of sugar content in the hydrolysates was performed by High Performance Liquid Chromatography (HPLC) with an Aminex® HPX-87H (300 × 7.8 mm) column (Bio-Rad). The column is packed with a polymer-based matrix (polystyrene divinylbenzene) and separates compounds using the ion-moderated partition chromatography technique (Bio-Rad, 2013). The compounds are afterwards perceived by a refraction index (RI) detector and an ultraviolet (UV) detector, generating the respective chromatograms. Figure 14 represents a scheme of the HPLC apparatus.

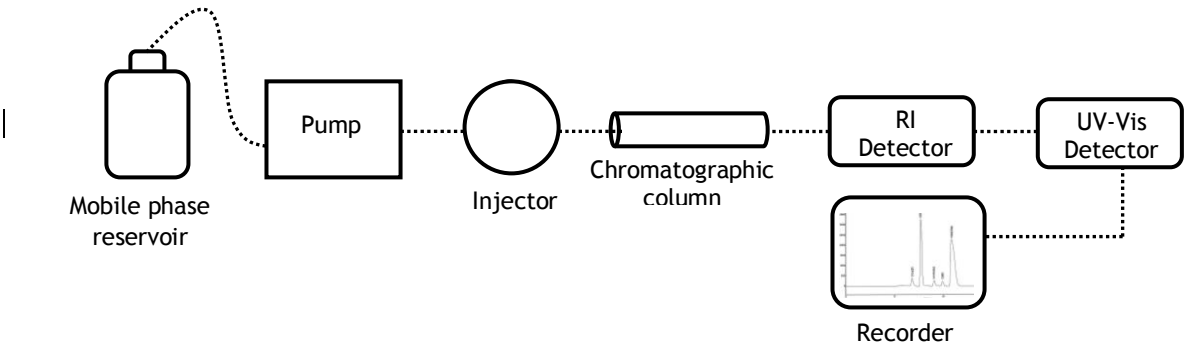


Figure 14 - Schematic representation of the HPLC apparatus.

Standard solutions of glucose (1, 2, 4 e 10 mg/mL), xylose (1, 2 e 4 mg/mL), cellobiose (1, 2 e 4 mg/mL), and acetic acid (1, 2 e 4 mg/mL) were prepared and analyzed by HPLC in duplicate. The average retention times and peak areas were obtained from the respective chromatograms. The peak areas were related to the compound concentrations in calibration curves, which are displayed in figure 15, along with the respective equations and linear correlation coefficients. Apart from the sugar calibration curves, the acetic acid calibration was also performed with the intent of monitoring byproducts generation in the enzymatic hydrolysis stage. For furfural and HMF, calibration curves available in the lab were used. Sugars and organic acids are detected by the HPLC system's RI detector, while furfural and HMF are monitored by the UV-Vis detector at a wavelength of 280 nm.

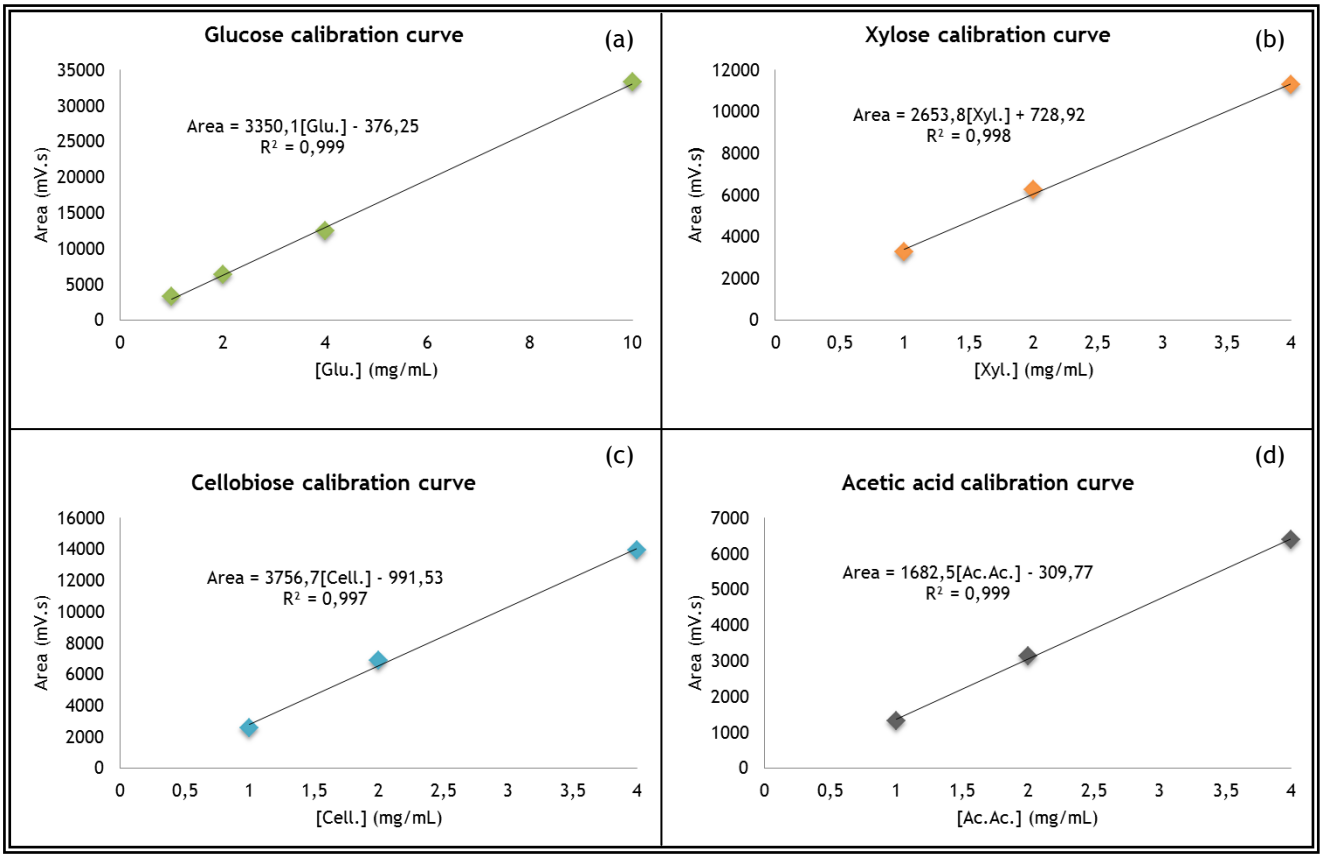


Figure 15- HPLC calibration curves obtained for glucose (a), xylose (b), cellobiose (c) and acetic acid (d).

III - 2.5.1 Determination of sugar content by HPLC

Every sample was filtered with 0.22 µm pore membranes prior to HPLC analysis. They were eluted in isocratic system with 5 mM H_2SO_4 at 60ºC. The injected volume into the column was of 50 µL (loop volume) and the sugars were perceived by the IR detector; degradation products, such as furfural and HMF, were monitored with a UV-Vis detector. The calibration curve equations were used to determine the glucose, xylose, cellobiose, acetic acid, furfural and hydroximethyl-furfural content in every sample.

Hydration correction factors were introduced in the sample's glucose and xylose content calculation: the glucose and xylose readings were multiplied by 0.9 and 0.88, respectively, as a correction for the water molecules added upon hydrolysis of cellulose and xylose.

III - 2.6 Morphological analysis

The following procedure intended to study the effect of the enzymatic hydrolysis on the fiber morphology. For this purpose, fine element quantification was monitored throughout the enzymatic hydrolysis, along with sample imagining by optic microscopy.

III - 2.6.1 Fine elements quantification

The fine elements quantification was performed by a Techpap MorFi LB-01 fiber analyzer (figure 16). The MorFi apparatus measures many different fiber indices, namely fibers' dimensions and statistical distribution, as well as other features, including fine elements. By default, MorFi classifies as fine elements any objects present in the pulp whose dimensions are too small for it to be considered as a fiber (a length less than 200 µm and/or a width less than 5 µm) (Techpap).

Figure 16 - Techpap MorFi LB-01 fiber analyzer.

In the case of the samples which didn't undergo enzymatic hydrolysis, the aliquot consisted in 0.4 g of *od* biomass suspended in water. To determine fine elements content in the enzymatic hydrolisate throughout the enzymatic hydrolysis, a full 10 mL syringe of each sample (2, 48 and 144 hours) was analyzed in the MorFI.

III - 2.6.2 Optic microscopy

In order to monitor the modifications of the fibers along the course of the enzymatic hydrolysis, samples were taken at 0, 2, 48 and 144 hours. Temporary microscopy slide preparations were performed, according to the wet-mount technique: a drop of each sample was placed in a clean flat slide and a cover slip was placed above it, forming an angle, with one edge touching the slide, and then gently lowed. The observations were made using a dark field condenser and pictures were taken.

III – 3 Experimental procedures

III – 3.1 Measurement of cellulases activity

According to International Union of Pure and Applied Chemistry (IUPAC) guidelines, the value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes, at the enzyme's optimum temperature and pH, has been designated as the intercept for calculating filter paper cellulase units (FPU) (Adney and Baker, 2008).

Based on this definition, and since the literature refers the preferable loading of cellulase as being less than 10 FPU/g of cellulose (Yang and Wyman, 2008), the activity of the enzyme NS22086 was checked following the general guidelines of the Laboratory Analytical Procedure (LAP) for Measurement of Cellulase Activities from the National Renewable Energy Laboratory (NREL) (Adney and Baker, 2008).

In 50 mL falcon tubes, 50 mg strips (*od*) of filter paper were placed. 50 mM citrate buffer with pH 6.5 and the enzyme NS22086 (in concentrations of 0.25; 0.5; 1; 2; 2.5 and 20 µL/mL) were added, assessing the tubes to a total volume of 40 mL. Note that the term "concentration" is used to represent the proportion of the original enzyme solution present in the dilution added to the assay mixture. Since the original enzyme solution has glucose in its composition, blanks for every concentration were prepared, proceeding as mentioned above but without adding any filter paper. Triplicates were prepared for each sample.

All the tubes were let to digest in a 50ºC water bath for 60 minutes and then transferred to an ice bath in order to stop the reaction.

Although NREL'S LAP states that the glucose content should be determined by adding DNS reagent and letting color develop by boiling, glucose content of all samples was determined by HPLC, as has been described in section III - 2.5.2, due to issues related to the presence of glucose in the original enzyme solution and its intense color (which might interfere in absorbance measurement). The glucose content of the blanks was subtracted to that of the test samples.

III – 3.2 Effect of pulp beating on the sugar release in the enzymatic hydrolysis

For this study, two industrial pulps were used: Pine bleached kraft pulp and PGWP (figure 17). The dry matter content of the pulps was determined using an IR balance and 30 g (*od*) of each pulp were weighted, roughly torn by hand and let to soak in warm water for half an hour. Afterwards, pulp suspensions were made in a laboratory defragmenter.

Figure 17 - *Pinus pinaster* kraft pulp and PGWP (left to right).

III – 3.2.1 Pulp beating

The pulps were collected in a test-pad former and then beaten in a PFI mill (figure 18) at 3000 and 6000 revolutions with a beating intensity of 1.5 N/m. The WRV's of the beaten, as well as unbeaten pulps were determined as described in section III – 2.3, with the purpose of estimating their water holding capacity and relating it to the overall porosity of the fibres.

Figure 18 - PFI mill.

III – 3.2.2 Enzymatic hydrolysis

In order to study the effect of pulp beating on the sugar release in the enzymatic hydrolysis, the pulps were treated with an enzymatic cocktail as follows.

Both beaten and unbeaten pulps were placed in 50 mL falcon tubes at a solid content of 1% (each falcon tube had 0.4 g (*od*) of pulp. A citrate 50 mM buffer solution with pH of 5.5 was added to the tubes until a total volume of 40 mL per tube. The enzymatic cocktail, prepared according to the procedure described in section III – 1.4.1, was added to the tubes. A spoonful of 0.3 mm glass spheres was placed in each tube in an intent of improving agitation. All tubes were homogenized in a vortex and inserted into a thermostated water bath (figure 19) with continuous agitation at 50ºC. The pulps were subjected to enzymatic hydrolysis for 2, 7, 15, 24, 48, 72, 96, 120 and 144 hours and the sugar content was determined by HPLC. Each sample was made in triplicate.

Figure 19 - Thermostated water bath with continuous agitation.

III – 3.3 Effect of sulfite pretreatment and bleaching on the sugar release in the enzymatic hydrolysis

The aim of this experiment was to study the effect of sulfite pretreatment and pulp bleaching on the sugar release in the enzymatic hydrolysis stage. For that purpose, eucalyptus wood chips were subjected to sulfite pretreatment and part of the resultant pulp was bleached. Both pretreated pulp and uncooked material, as well as bleached pulp were subjected to hydrolysis with an enzymatic cocktail.

III - 3.3.1 Biomass preparation

Chips of eucalyptus wood were roughly cut with cutting pliers and the dry matter content was determined using an IR balance.

III - 3.3.2 Sulfite pretreatment

1 kg (*od*) of eucalyptus chips were placed in a forced circulation digester (Figure 20) with a ratio of pretreatment liquor to wood chip of 5/1 (v/W). The composition of the pretreatment liquor was 9% (w/w) $NaHSO_3$ and 0.9% (w/w) H_2SO_4, based on wood, corresponding to a pH of 1.88. The temperature was increased gradually until 90ºc, during 60 minutes. Thereafter, the temperature remained constant for 120 minutes, in order to enable complete impregnation of the wood chips. Immediately after impregnation, the temperature was raised to 165ºC in about 60 minutes and maintained for an additional 120 minutes.

Figure 20 - Forced circulation digester.

The resulting solid was disintegrated and washed in a laboratory strainer (figure 21). The pulp and the uncooked material were collected. The uncooked material was transferred to a laboratory blender and was grinded for 1 minute in high speed mode. Part of the uncooked material was grinded for an additional minute (over grinded material). Both pulp and uncooked material were kept at 4ºC, being careful not to let them dry completely. The Kappa Number (K-No) of the pulp and uncooked material was determined as described in section III – 2.2 and 100 g (*od*) of the pulp obtained was bleached as described below.

Figure 21 - Laboratory strainer.

III – 3.3.3 Bleaching

The pulp bleaching was carried out using an Elemental Chlorine Free (ECF) sequence of 4 stages: alternate ClO_2 (D_n) stages followed by alkaline extractions with NaOH (E_n). The reagents charges and operating conditions are explained in table 4.

Table 4 - Operating conditions of the sulphite pulp bleaching [1].

Stage	Reagent	Reagent charge (%) [2]	Temperature (ºC)	Time (min.)	Consistency (%)
D_0	ClO_2	11,52	50	30	10
E_0	NaOH	5,91	65	90	10
D_1	ClO_2	2,0	75	60	10
D_2	ClO_2	0,5	75	30	10

Notes: [1] ClO_2 charge = 100 × reagent mass/ *od* pulp mass; Consistency = 100 × od pulp mass/ suspension's total mass.

[2] ClO_2 charge as active chlorine; NaOH charge = ClO_2 loading/2 + 0.15

The pulp was placed in plastic bags, closed and put in a thermostated water bath at the referred temperatures. The bags were occasionally and externally manipulated in order to ensure an adequate homogenization of both reagents and pulp fibers.

Every bleaching stage was followed by an intermediate stage, which consisted on washing the pulp with water and filtering it with a fritted funnel in vacuum. Once the residual water reached neutral pH, the following stage was performed. Between stage D_1 and D_2, NaOH was added to the pulp until pH reached 11, and the bag was manipulated for a couple of minutes before washing with water.

III – 3.3.4 Enzymatic hydrolysis

The dry matter content of all bleached pulp, unbleached pulp and uncooked material was determined with an IR balance and were subjected to enzymatic hydrolysis with the enzymatic cocktail, following the procedure described in section III – 3.2.2. Duplicates were performed, except for the samples corresponding to 2 and 7 hours of enzymatic hydrolysis: 6 tubes of each were prepared in order to be able to collect enough pulp to determine the limiting viscosity. The sugar content of the samples was determined by HPLC and the fine elements quantification throughout the enzymatic hydrolysis was performed as described in the sections III – 2.5.2 and III – 2.6.1, respectively.

The pulp from the tubes that underwent 2 hours and 7 hours of enzymatic hydrolysis was collected with a steel mesh and the their dry matter content was determined as described in section III – 2.1. The limiting viscosity of these pulps, as well as the limiting viscosity of the original bleached pulp was determined as described in section III – 2.4, in order to study the effect of the enzymatic hydrolysis on the cellulose depolymerization / degradation.

III - 4 Influence of pulp drying on the sugar release in the enzymatic hydrolysis

With the objective of studying the influence of pulp drying (relating it to the fiber hornification phenomena), part of the bleached and unbleached pulp prepared in sections III – 3.3.2 and III – 3.3.3 was let to dry at 60ºC overnight in a laboratory oven. Due to fiber aggregation and stiffening, the pulps had to be resuspended in water in a laboratory defragmenter. They were collected on a test-pad former and their dry matter content was determined in an IR balance. The enzymatic hydrolysis procedure was repeated for these pulps. The sugar content of the samples was determined by HPLC as described in section III – 2.5.2.

III - 5 Effect of sulfite pretreatment on different woody species

For each assay, 30 g (*od*) of eucalyptus, pine and broom were placed in 200 mL stainless steel reactors (Figure 22 (b)) with a ratio of pretreatment liquor to wood chip of 5/1 (v/W). The reactors were locked and placed in a thermostated polyethylene glycol (PEG) bath with continuous agitation (Figure 22 (a)).

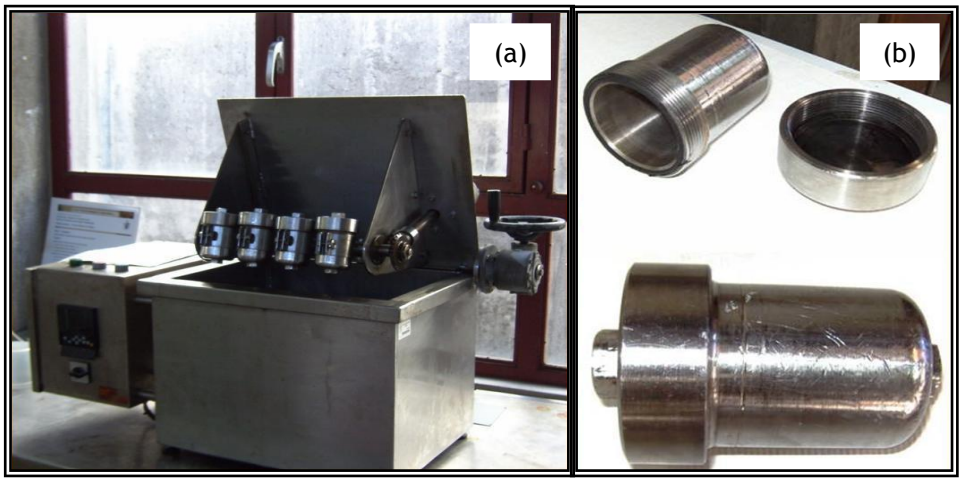


Figure 22 - Thermostated PEG bath (a) and stainless steel reactors (b).

The composition of the pretreatment liquors as well as the operating conditions are is represented in table 5.

Table 5 - Pretreatment liquor composition and operating conditions of the sulfite pretreatments.

Biomass	**Pretreatment liquor**			**Operating conditions**	
	Bissulfite (% (w/w))	H_2SO_4 (% (w/w))	pH	Pretreatment temperature (ºC)	Pretreatment time (minutes)
Eucalyptus	5	0	2.21	170	30
		0.9	1.75		
	9	0	3.35		
	15		2.61		
		2	2.13		
		5	1.70		
Broom	5	0.9	1.80	170	30
					90
	9	0	2.81		30
		0.9	1.88		
					90
	15	0	2.61		20
		2	2.13		
		5	1.70		
Pine	5	0.9	2.26	180	30
	10	0	4.41		20
		2	2.22		
		5	1.57		
	15	0	2.61		
		2	3.13		
		5	1.70		

The wood was first impregnated with pretreatment liquor at 90ºC for 30 to 60 minutes. The temperature was then raised to the cooking temperature designated in table 5 in about 60 to 90 minutes and maintained for the amount of time also mentioned in table 5.

The reactors were subsequently cooled down with tap running water and opened. Samples of the hydrolysates were collected, their pH was measured with a pH sensor, and they were searched for sugars (with the RI detector) and possible fermentation inhibitors (with the UV-Vis detector) by HPLC as described in section III – 2.5.2, performing 1:10 dilutions for quantification of furfural and HMF.

The pretreated wood from each reactor was grinded in a laboratory blender for 1 minute at its higher speed and washed in vacuum with water in a Büchner funnel covered with a filter paper, until the wash water turned clear. The resultant solids and filter papers were weighted and the dry matter content was determined as described in section III – 2.1. The solids residue of the pretreatment was determined for each reactor with the following formula:

$$Solid\ residue\ (\%) = \frac{(Wet\ weight - Filter\ paper\ weight) \times Dry\ matter\ content}{Initial\ wood\ dry\ weight} \quad (1)$$

The K-No was determined for every pretreated biomass as described in section III – 2.2 with the objective of elucidating about their lignin content. Based on the K-No and the solid residue, samples were chosen to undergo enzymatic hydrolysis, following the procedure depicted in section III – 3.2.2. The enzymatic hydrolysates were searched for sugars by HPLC according to what has been described in section III – 2.5.2.

The fine elements of every pretreated solid during enzymatic hydrolysis (at 0, 2, 48 and 144 hours) were quantified as described in section III – 2.6.1.

Samples of the three biomasses pretreated with 15% bisulfite/ 0% acid, and 15% bisulfite/ 5%acid were observed at an optic microscope throughout the enzymatic hydrolysis (at 0, 2, 48 and 144 hours) as described in section III – 2.6.2.

Chapter IV – Results and discussion

This chapter presents the results obtained for the effect of several physical and physical chemical pretreatments on the sugar release using a complex of enzymes, as well as their discussion.

IV – 1 Measurements of cellulases activity

As has previously been mentioned, 1 FPU corresponds to the amount of enzyme that, in the specified conditions, converts 4% of the cellulose into glucose.

As expected, the sugar yield did not behave as a linear function of the quantity of enzyme in the assay mixture in all the enzyme concentrations range used (figure B1 of Annex B). However, the three lowest enzyme concentrations (0.25, 0.5 and 1 µL/mL) had an approximately linear correlation with the glucose yield (figure 23). A linear regression equation of these values was used to interpolate the cellulose concentration correspondent to a sugar yield of 4% (1FPU).

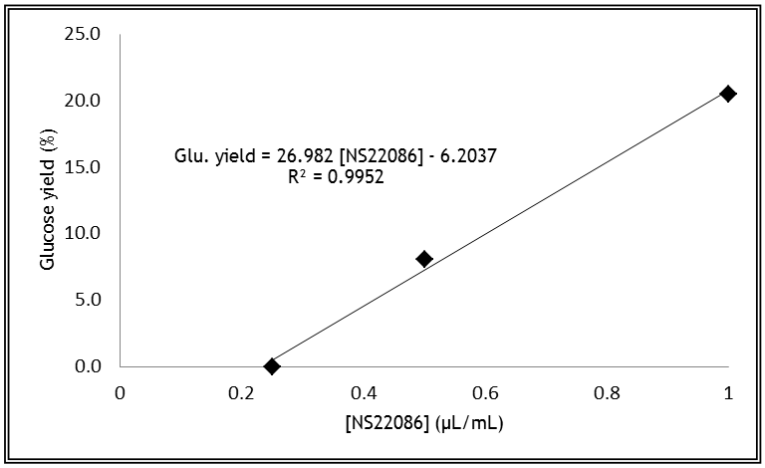


Figure 23 - Variation of glucose yield with cellulase concentration.

According to the linear regression equation obtained, 4% of glucose from 50 mg of filter paper corresponds to an enzyme concentration of 0.378 µL/mL. Since this assay was performed in a total volume of 40 mL, 1 FPU = 15.12 µL of the undiluted cellulase solution. In other words, the original enzyme solution's activity is 66.14 FPU/mL.

The cellulases dosage used in the enzymatic cocktail throughout the enzymatic hydrolysis experiments, as recommended by Novozymes, was of 5% (w/w (TS)), corresponding to 17.4 µL for 0.4 g of solid biomass used in each falcon tube. This value is equivalent to 2.877 FPU/g of cellulose (17.4 µL/15.12 µL /0.4g). Since the literature reports that, for the enzymatic hydrolysis to be viable, the cellulase loading should be less than 10 FPU/g of cellulose (Yang and Wyman, 2008), the applied value is consistent with that of literature.

IV - 2 Effect of pulp beating on the sugar release in the enzymatic hydrolysis

Pulp beating is a unit operation that significantly increases the specific surface area of the pulp fibers. Since enzyme accessibility depends on surface area, it is expected that pulp beating increases sugar release in enzymatic hydrolysis. A lignin free pulp (bleached kraft pulp) and a lignin rich pulp (PGWP) were selected for this study.

IV - 2.1 Enzymatic hydrolysis

The concentration profiles of cellobiose, glucose and xylose released throughout the enzymatic hydrolysis with the enzymatic cocktail (see sections III – 1.4 and III – 1.4.1) for the unbeaten PGWP and bleached kraft pulps are represented in figure 24.

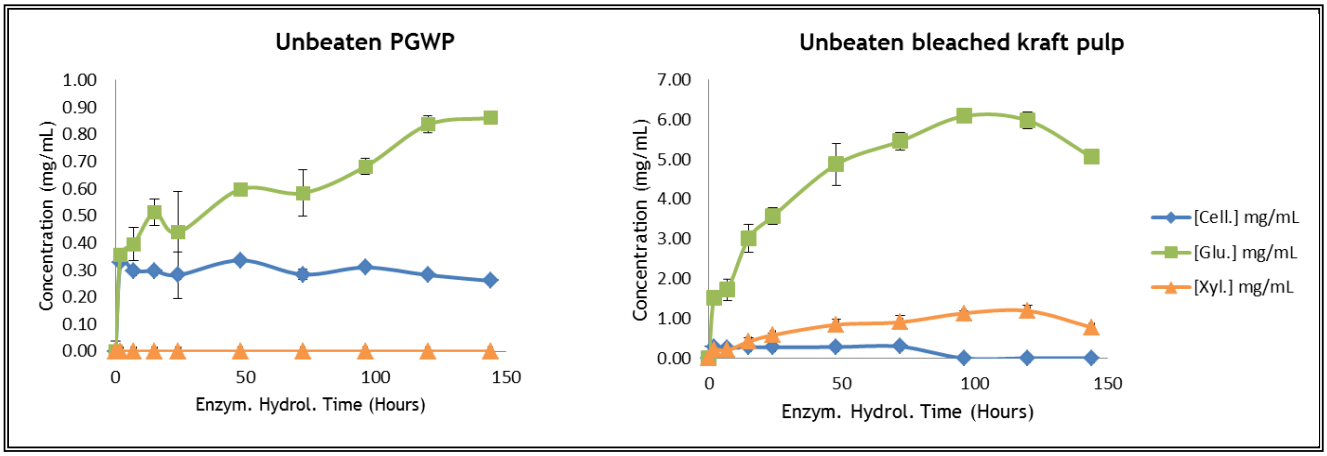


Figure 24 - Concentration profiles of cellobiose, glucose and xylose achieved in the enzymatic hydrolysis of unbeated PGWP and bleached kraft pulp.

Figure 24 shows that, for the bleached kraft pulp, much higher concentrations of cellobiose, glucose and xylose were achieved: the glucose concentration reached about 0.9 mg/mL at 144 hours of enzymatic hydrolysis for the PGWP, while the kraft pulp released around 6 mg/mL of glucose at 96 hours.

Since the standard deviation was very low ($\sigma \leq 0.52$), and consequently the average coefficient of variation of the sugar concentrations from the samples (from three tubes of the same time series and conditions) were also low, the sugar concentrations profiles throughout the enzymatic hydrolysis will be displayed without error bars from now on.

Figure 25 plots the concentrations of the above referred sugars during the course of enzymatic hydrolysis, this time for beaten PGWP and bleached kraft pulp, both beaten at 3000 revolutions in the PFI mill.

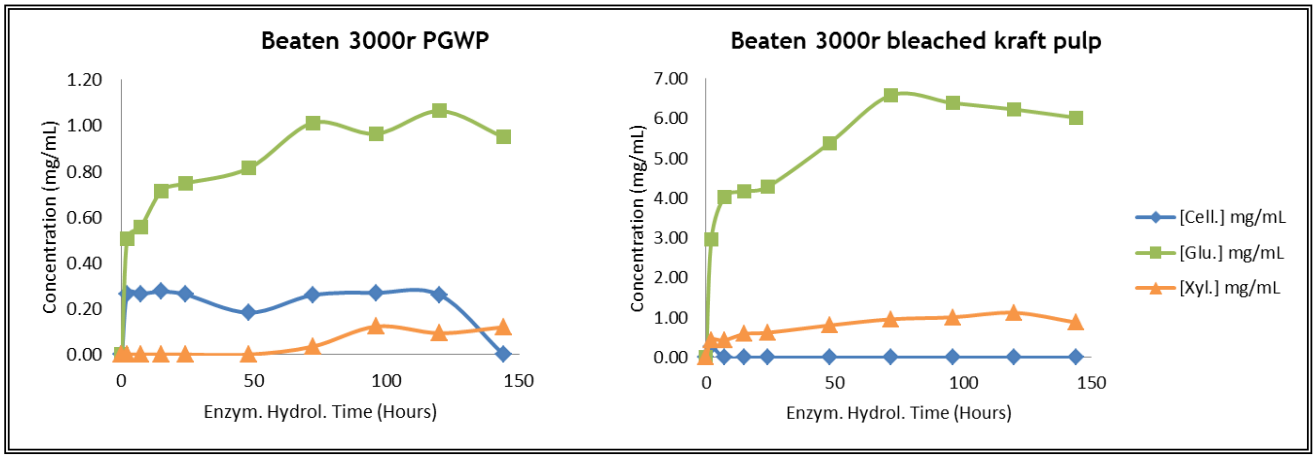


Figure 25 - Concentration profiles of cellobiose, glucose and xylose achieved in the enzymatic hydrolysis of PGWP and bleached kraft pulp beaten at 3000 revolutions and a beating intensity of 1.5 N/m.

For the pulps beaten at 3000 revolutions, maximum glucose concentration reached 6.6 mg/mL at 72 hours for the bleached kraft pulp, and 1 mg/mL at 120 hours for PGWP (figure 25).

When beating extent increased to 6000 revolutions, glucose concentrations increased to 7.8 mg/mL at 96 hours and 1.3 mg/mL at 144 hours for the bleached kraft pulp and PGWP, respectively, as it can be seen in figure 26.

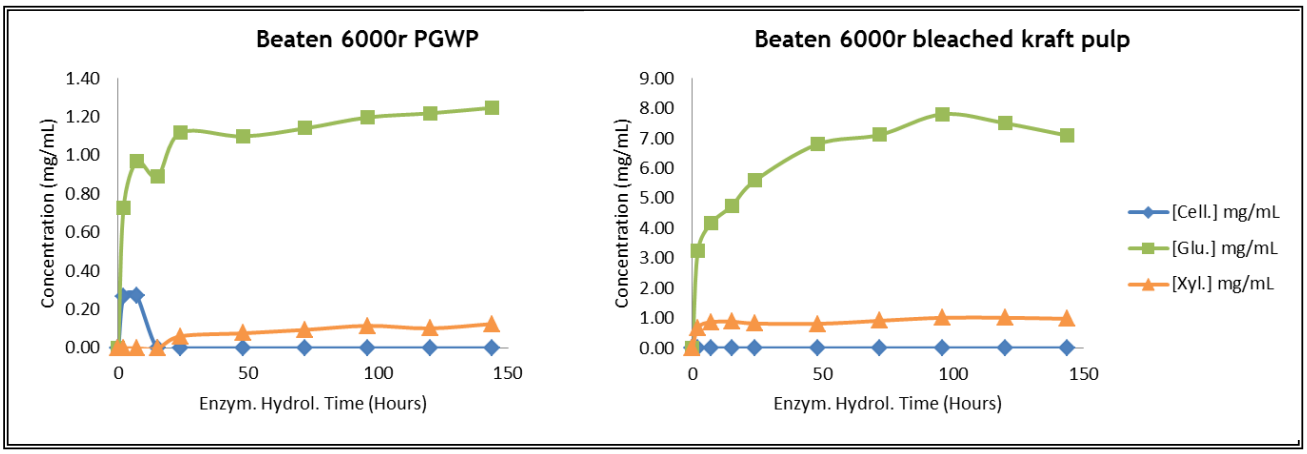


Figure 26 - Concentration profiles of cellobiose, glucose and xylose achieved in the enzymatic hydrolysis of PGWP and bleached kraft pulp beaten at 6000 revolutions and a beating intensity of 1.5 N/m.

Summarizing, the obtained results have shown that the sugar concentrations, along the enzymatic hydrolysis, increased with increasing beating extent for both bleached kraft and PGWP.

Figure 27 shows the overall carbohydrate yield variation during the enzymatic hydrolysis. The PGWP carbohydrate yield was calculated based on an estimated carbohydrates availability of 70% (considering 27% of lignin and 3% of extractives), while bleached kraft pulp was considered to have 100% of total carbohydrate availability.

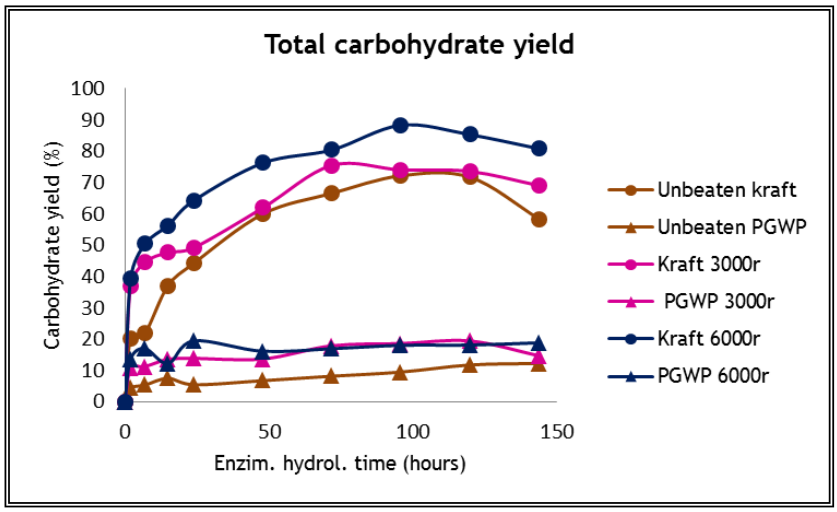


Figure 27 - Overall carbohydrate yield for the different beating extents of PGWP and bleached kraft pulp.

From figure 27 it can be observed that the chemical and physical characteristics of the raw material impart a drastic effect on the enzymatic cocktail performance; the maximum carbohydrate yield increases from less than 20% to close to 90%, when the raw material changed from an only mechanical processed wood (PGWP) to an extremely chemically processed wood (bleached kraft pulp).

It is also noteworthy that the specific surface area of the PGWP is not lower than the corresponding area for the chemical pulp. Therefore, the different behavior of the two raw materials can certainly be ascribed to the chemical and ultra-structural differences.

The results suggest that the beating increases the initial reaction rate and somewhat the carbohydrates yield, but it is incapable of substantially increasing the availability of the polysaccharides of the mechanical pulp (PGWP).

IV - 2.2 WRV determination

In order to estimate how the beating process affects the internal porosity of the fibers, the WRV of the pulps was determined.

The WRV obtained for the bleached kraft pulp and PGWP, subjected to the different beating extents, are recorded in table 6.

Table 6 - WRV obtained for the bleached kraft pulp and PGWP, subjected to different beating extents.

	WRV	
Beating extents	**Bleached kraft**	**PGWP**
0 rev	0.809	0.771
3000 rev	1.262	0.964
6000 rev	1.451	1.391

Note: WRV = (wet mass – dry mass)/ dry mass

As it can be observed in table 6, the increase of beating extent has led to an increase in the WRV for both pulps. The WRV has increased from 0.809 and 0.771 for unbeaten bleached kraft pulp and PGWP, to 1.451 and 1.391 for the respective pulps, beaten at 6000 revolutions. Since this feature is related to the overall porosity of the fibers, the results obtained for the WRV are consistent with those obtained for the sugar release in the enzymatic hydrolysis stage, leading to the assumption that pulp beating effectively increases the fibers accessibility to enzymes by increasing their overall porosity.

IV - 3 Effect of sulfite pretreatment and bleaching on the sugar release in the enzymatic hydrolysis

IV - 3.1 Chemical pretreatments

The figure 28 shows the evolution of the biomass throughout the sulfite pretreatment and bleaching processes, from pretreated wood to bleached pulp.

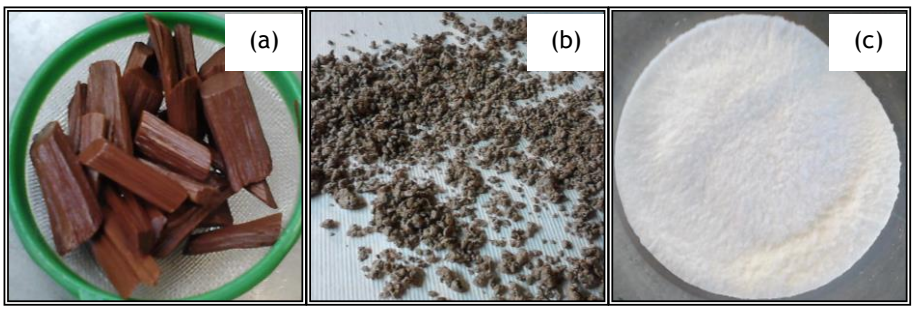


Figure 28 - Evolution of the biomass appearance: (a) sulfite pretreated wood; (b) sulfite pretreated unbleached pulp; (d) bleached pulp after bleaching stage D_2.

These pretreatments aimed to provide biomass samples with marked differences in their chemical and physical properties, in order to evaluate their behavior in the enzymatic hydrolysis.

IV – 3.2 Enzymatic hydrolysis

The concentration profiles of cellobiose, glucose and xylose released throughout the enzymatic hydrolysis of the sulfite bleached (figure 29 (a)) and unbleached (figure 29 (b)) pulps, as well as the grinded uncooked material (figure 29 (c)), are depicted in figure 29.

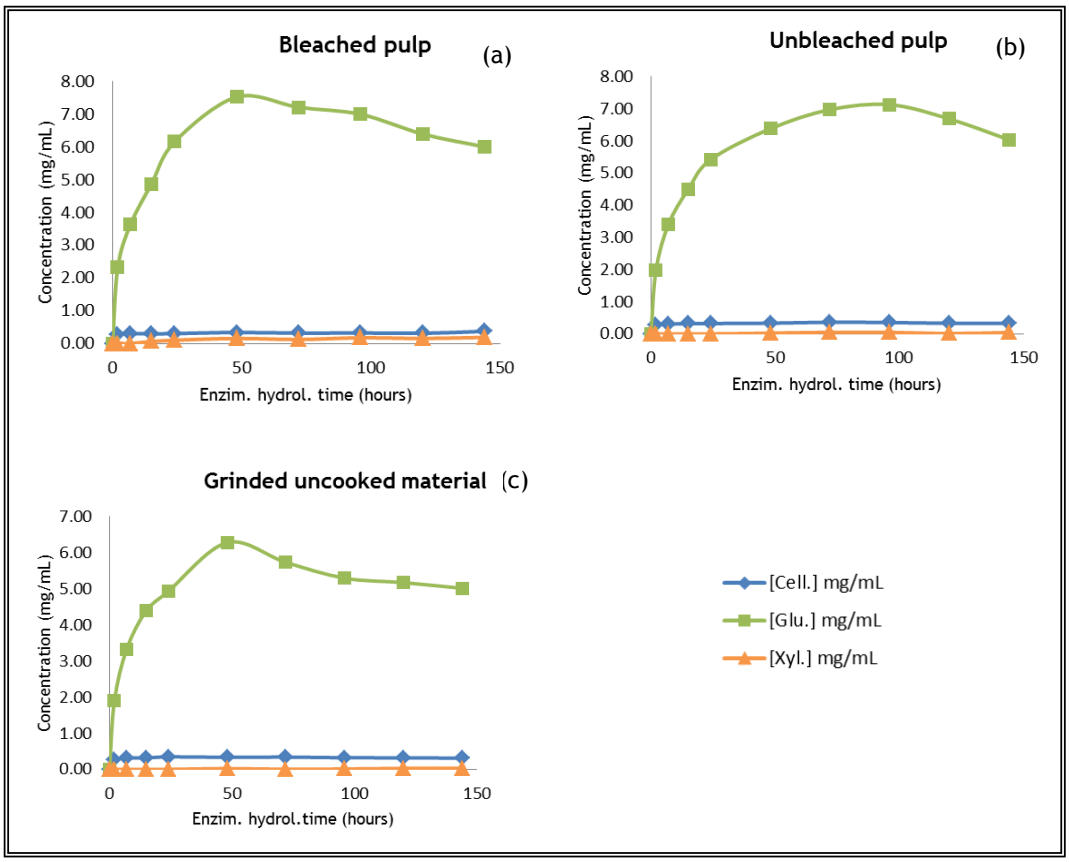


Figure 29 - Concentration profiles of cellobiose, glucose and xylose achieved in the enzymatic hydrolysis of the sulfite bleached pulp (a), unbleached pulp (b) and grinded uncooked material (c).

Analyzing the sugar concentrations plotted in the figure 29, it is possible to see that the cellobiose concentration remained extremely low and approximately constant throughout the enzymatic hydrolysis. This residual level is probably required to maintain the cellobiase (ß-glucosidase) activity.

The glucose concentration increased faster for the bleached pulp and uncooked materials when compared to that of the unbleached pulp. Glucose concentration reached 7.53 mg/mL and 6.28 mg/mL in 48 hours, for the bleached pulp and uncooked materials, respectively, while the unbleached pulp released 7.13 mg/mL of glucose at 96 hours of enzymatic hydrolysis.

The K-No determined for the sulfite pretreated unbleached pulp and uncooked material were 72 and 274, respectively. These values, despite their associated error, were used to estimate the lignin content in the materials (% lignin = K-No × 0.12). The complementary values were considered as the amount of carbohydrates available for the enzymatic hydrolysis and taken into account to calculate the carbohydrate yield for every assay. The bleached pulp was considered to have no lignin content; in other words, its total carbohydrate availability was assumed to be 100%.

Figure 30 shows the evolution of the total carbohydrates yield of the sulfite pretreated bleached, unbleached and uncooked materials. As has been described in section III – 3.3.2, a second set of assays was performed about a month later, to check the repeatability of the results. Moreover, the firstly grinded uncooked materials were additionally grinded for one more minute (over grinded uncooked material) and were also subjected to enzymatic hydrolysis, in order to understand the effect of the particle size reduction on the sugar release during enzymatic hydrolysis with the enzymatic cocktail.

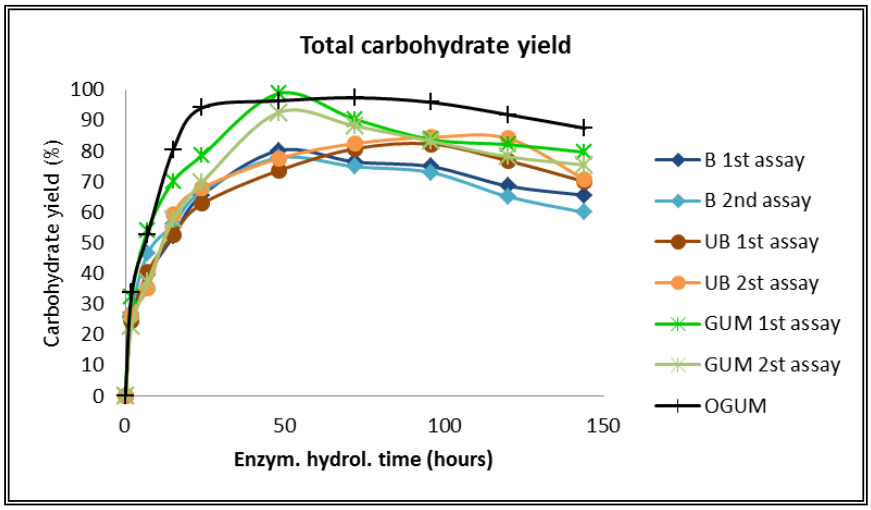


Figure 30 - Total carbohydrates yield of sulfite pretreated pulps (B = bleached pulp; UB = unbleached pulp; GUM = grinded uncooked material; OGUM = over grinded uncooked material).

As it is shown in figure 30 the enzymatic hydrolysis had a good repeatability, since the carbohydrate yield achieved during enzymatic hydrolysis for the first and second set of assays, concerning all three biomasses, was very close.

Contrarily to what was expected, the uncooked material reached a higher carbohydrate yield when compared to the bleached and unbleached pulp, attaining 92.6 to 98.6% in 48 hours. The reason why the enzymatic hydrolysis of the uncooked material resulted in a higher carbohydrate yield than the bleached pulp is probably related to an overestimation of lignin content (and consequent underestimation of available carbohydrates) from the kappa number determination procedure.

Concerning the particle size reduction effect study for the uncooked material, this feature proved to be important for the enzymatic hydrolysis reaction rate, which was increased with the increase of superficial area. However, the carbohydrate yield of the grinded uncooked material is similar to that of the over grinded uncooked material.

It can be observed, however, that for several pretreated solids, the sugar concentrations, and consequently, the carbohydrate yields, increase throughout the enzymatic hydrolysis but then tend to decrease afterwards. These results were thought to be due to adsorption phenomena. To test this hypothesis, sulfite pretreated bleached pulp was introduced in 50 mL falcon tubes with a solid content of 1% and a glucose concentration of 10 mg/mL, and were placed in a water bath with continuous agitation, at 50 ºC (the same conditions used for the enzymatic hydrolysis). Samples were taken at 2, 48 and 96 hours and were searched for sugars by HPLC. The concentration of glucose remained constant, leading to no conclusions regarding adsorption phenomena of sugars on the pulp fibers. This occurrence remains an issue which needs further investigation.

IV – 3.3 Viscosity determination

To characterize the effect of the enzymatic hydrolysis, the limiting viscosity of the sulfite bleached pulp was monitored. The pulp's initial average viscosity decreased from 475 cm^3/g (corresponding to a degree of polymerization (DP) of 383) to 220 cm^3/g (DP = 177) after 7 hours of enzymatic hydrolysis. Thus, the results obtained indicate a degradation of cellulose chains, translated in a decrease of their limiting viscosity (and consequently, of its DP).

IV – 4 Influence of pulp drying on the sugar release in the enzymatic hydrolysis

Figure 31 compares the total carbohydrates yield of both never-dried sulfite bleached and unbleached pulps (from the first assay of III – 3.3) with the same pulps dried, rewetted and resuspended prior to subjecting them to enzymatic hydrolysis with the enzymatic cocktail.

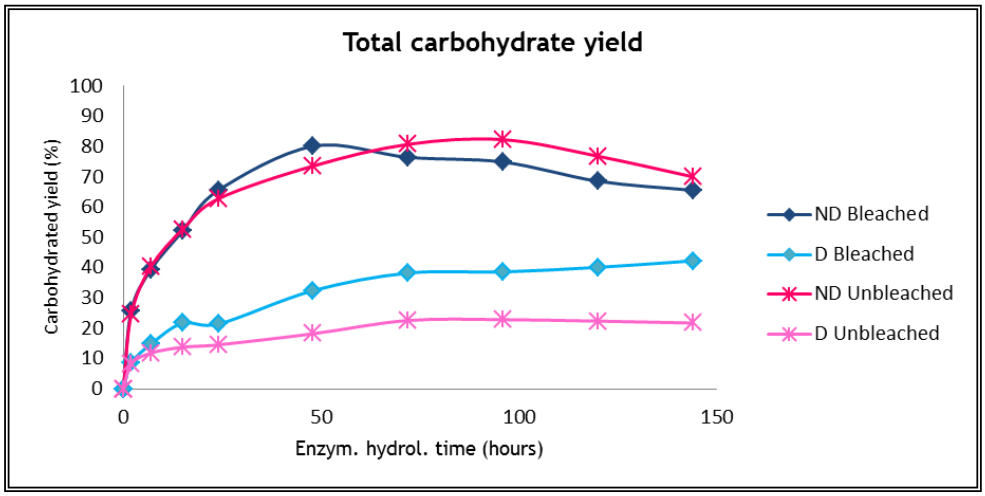


Figure 31 - Comparison between the total carbohydrate yields of never-dried and dried bleached and unbleached pulps, throughout the enzymatic hydrolysis (B = bleached pulp; UB = unbleached pulp; UM = uncooked material; GUM = grinded uncooked material).

Based on the obtained results, it was possible to verify that drying the pulps caused a drastic decrease on the total carbohydrates yield: the bleached pulp carbohydrate yield decreased from 80.2% at 48 hours to 40.3% at 144 hours of enzymatic hydrolysis. The same phenomena happened with the unbleached pulp: there was a decrease in the carbohydrate yield from 82.4 to 22.9 % at 96 hours of enzymatic hydrolysis.

The decrease of the carbohydrates yield in the enzymatic hydrolysis due to pulp drying is probably related to fiber hornification, which is responsible for the fiber's pore collapse and consequent decrease in enzyme accessibility.

The results obtained are consistent with the ones achieved by Luo and Zhu (2011), where several substrates with different degrees of drying-induced hornification were prepared and subjected to enzymatic hydrolysis. Due to fiber hornification, substrate enzymatic digestibility decreased about 90%, in 20 minutes of drying, for a SPORL pretreated lodgepole pine solid substrate, and slightly below 80% for a commercial bleached eucalyptus pulp, in 5 hours of drying, both at 150ºC (Luo and Zhu, 2011).

IV – 5 Effect of sulfite pretreatment on different woody species

IV – 5.1 Pretreatment analysis

Table 7 shows the pH of the pretreatment hydrolysates, as well as the K-No of the resultant solids and recovered solid residues.

Table 7 - Final pH of the hydrolysates, K-No and solid residue of the resultant solids for the different biomasses sulfite pretreatments.

Biomass	**Pretreatment conditions**			**Hydrolysates and solid residues**			Subjected to enzymatic hydrolysis and morphological analysis
	Bisulfite (% (w/w))	H_2SO_4 (% (w/w))	Pretreat. time (minutes)	Final pH	K-No	Solid residue (%)	
Eucalyptus	5	0	30	1.93	149	65.5	☒
		0.9		1.80	144	62.4	☑
	9	0		2.35	102	71.6	☑
	15			2.62	78	62.5	☑
		2		1.64	83	57.0	☑
		5		1.34	123	52.3	☑
Broom	5	0.9		2.56	132	71,5	☑
			90	2.63	155	72.4	☒
	9	0	30	3.61	109	64.0	☑
		0.9		2.66	114	55.7	☒
			90	2.76	134	51.3	☒
	15	0	30	3.38	80	63.4	☑
		2		2.17	100	56.4	☑
		5		1.61	116	54.5	☑
Pine	5	0.9		1.81	125	71.3	☑
	10	0		3.20	136	71.4	☑
		2		1.81	154	67.7	☒
		5		1.42	173	64.7	☒
	15	0		3.05	68	60.3	☑
		2		1.89	101	57.0	☑
		5		1.40	188	52.0	☑

Note: Only the pretreated solids marked with ☑ were subjected to enzymatic hydrolysis and morphological analysis.

As it can be deduced by table 7, acidic components were formed during the pretreatment, which leads to an overall decrease of pretreatment hydrolysates pH. Generally, the eucalyptus hydrolysate is more acidic than that of the pine (probably due to the release of acetic acid from eucalyptus xylan degradation). It is also possible to realize that, for the same pretreatment conditions, the broom's hydrolysate is less acidic than the corresponding for eucalyptus and pine.

It can also be seen that, in general, the increase of H_2SO_4 in the pretreatment liquor causes the resultant solids to have a higher K-No. This increase of the K-No is probably due to a redeposition of lignin on the fibers, which is consistent with the color presented by the pretreated solids: the higher the concentration of H_2SO_4, the darker and brownish the solids would appear (for the same concentration of sulfite). This phenomenon is illustrated in figure 32.

Figure 32 - Sulfite pretreated pine. The pretreatment liquors compositions (w/w) were, from left to right, 15% bisulfite and 0% H_2SO_4; 15% bisulfite and 2% H_2SO_4; 15% bisulfite and 5% H_2SO_4.

The inverse phenomenon happened with increasing the bisulfite concentration in pretreatment liquor (figure 33), which caused a clearing in the samples, due to sulfonation and removal of lignin.

Figure 33 - Sulfite pretreated eucalyptus. The pretreatment liquors compositions (w/w) were, from left to right, 9% bisulfite and 0% H_2SO_4; 5% bisulfite and 0% H_2SO_4.

Along with the darkening, the solids pretreated with a higher charge of H_2SO_4 presented a much more fragmented structure: the fibers were visibly more broken.

It is also important to emphasize the difficulty of filtering the pretreated solids in a Büchner covered with a qualitative filter paper, after grinding them in the laboratory blender. Although the use of wood chips in the sulfite pretreatment, without the need of prior particle size reduction, may be an advantage in the process, the difficulty of filtering the pretreated solids may also represent a drawback at an industrial scale.

With the objective of having an overall perspective of the pretreatments, a mass balance was attempted and the results were recorded in table 8. Glucose, xylose, furfural and HMF concentrations in the pretreatment hydrolysates were determined by HPLC and the respective weight percentages removal were calculated based on the initial biomass dry weigh and liquor to biomass ratio. The solid residue was also calculated based on the dry weight of the recovered solids and that of the initial biomass.

Despite some uncertainty in lignin estimation in the solid residues, the global mass balance is in the range of 90—110%, which is acceptable. The balance can be improved by measuring the lignin content of the hydrolysates and solid residues, using an appropriate method, such as Klason lignin. In addition, the wood chemical composition can be determined, in order to provide more exact estimations of total available carbohydrates and lignin.

Table 8 - Mass balance of the sulfite pretreatment for eucalyptus, broom and pine.

		Pretreatment hydrolysates						Sugars + Byproducts	Others * (%)	Sugars+ byproducts+others	Solid residue (%)	Total (%)
		Sugars (%)			Byproducts (%)							
Biomass	Liquor composition	Glu.	Xyl.	Glu. + Xyl.	Fur.	HMF	Fur.+HMF					
Eucalyptus	E_9s_0ac	0.73	8.71	9.44	0.60	0.25	0.84	10.29	16.24	26.53	71.6	98.08
	E_5s_0.9ac	2.55	17.20	19.75	3.19	0.05	3.23	22.98	14.22	37.20	62.4	99.61
	E_15s_0ac	0.74	5.99	6.73	0.69	0.17	0.86	7.58	19.15	26.73	62.5	89.23
	E_15s_2ac	1.27	12.80	14.07	2.22	0.28	2.49	16.56	19.32	35.88	57.0	92.89
	E_15s_5ac	2.52	12.84	15.35	3.19	0.39	3.58	18.94	17.28	36.22	52.3	88.51
Broom	E_9s_0ac	0.74	2.35	3.09	1.27	0.13	1.40	4.49	24.02	28.51	64.0	92.55
	E_5s_0.9ac	2.24	8.31	10.55	2.27	0.43	2.71	13.26	22.62	35.88	71.5	107.38
	E_15s_0ac	1.12	0.79	1.90	0.09	0.02	0.11	2.02	26.31	28.33	63.4	91.74
	E_15s_2ac	1.81	8.01	9.81	0.70	0.09	0.79	10.61	25.63	36.24	56.4	92.67
	E_15s_5ac	2.89	12.88	15.78	2.07	0.18	2.24	18.02	24.81	42.83	54.5	97.33
Pine	E_10s_0ac	0.47	3.79	4.26	0.75	0.52	1.27	5.53	19.35	24.87	71.4	96.28
	E_5s_0.9ac	2.25	11.78	14.03	2.07	1.36	3.44	17.47	20.31	37.77	71.3	109.06
	E_15s_0ac	0.75	6.48	7.24	0.80	0.67	1.46	8.70	26.08	34.78	60.3	95.12
	E_15s_2ac	2.48	10.82	13.30	1.87	1.12	2.99	16.29	24.10	40.39	57.0	97.34
	E_15s_5ac	7.54	14.31	21.86	2.89	1.71	4.60	26.46	19.36	45.82	51.6	97.41

Note: * Others = (Soluble lignin + insoluble lignin + extractives) - (K-No*0.12*solid residue/100) for eucalyptus and broom
or (Soluble lignin + insoluble lignin + extractives) - (K-No*0.15*solid residue/100) for pine

Assuming that Furfural and HMF derive from pentoses and hexoses, the total carbohydrates release should include both sugars and byproducts. As it can be observed from table 8, a higher sugar removal (column "Sugars + Byproducts") from eucalyptus is registered, mainly at low acid charges, compared to the other raw materials. This is an expectable result considering the release of acetic acid from xylan, which increases the acid concentration in the reaction medium.

Figure 34 illustrates the evolution of sugars and byproducts found in the hydrolysates with the increase of acid charge, concerning the highlighted areas of table 8.

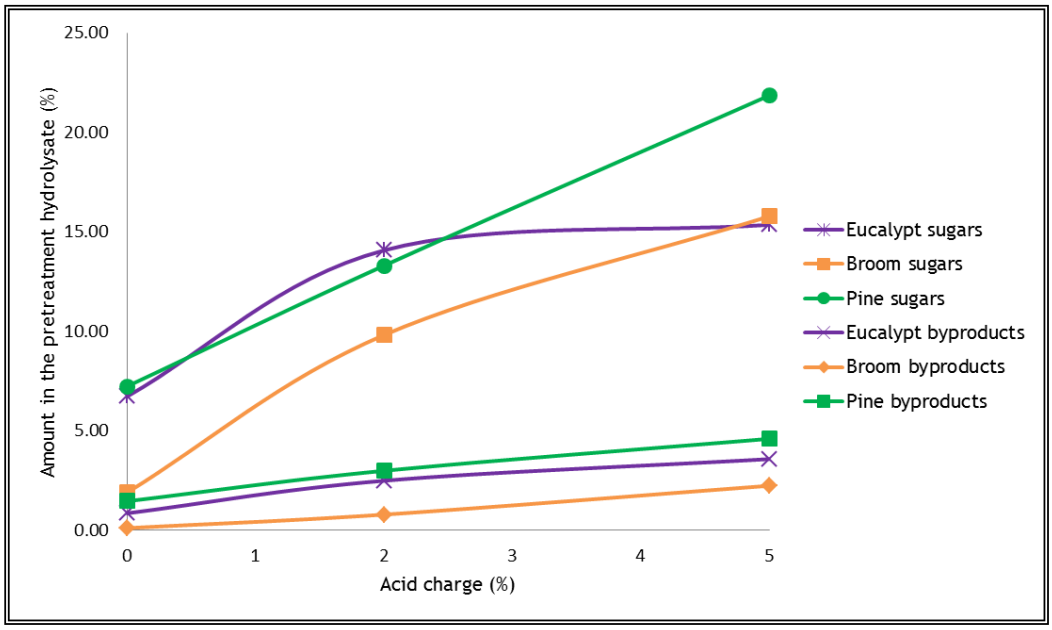


Figure 34 - Variations of the amounts of sugars and byproducts found in the hydrolysates with the acid charge of the pretreatment liquors.

The results illustrated in figure 34 suggest that, for all the raw materials, and with the same bisulfite charge (15%), the increase in the acid charge translates into an increase of the amount of removed sugars (table 8, highlighted areas), due to the more acidic pH. In addition, the amounts of sugars and byproducts found in the pine hydrolysates are generally higher, compared to those of the eucalyptus and broom, probably due to the higher temperature of the pretreatment (table 5, 180ºC *vs* 170ºC).

Figure 35 shows the amounts of glucose and xylose (a) as well as the amounts of furfural and HMF (b) found in the hydrolysates for the several materials, with increasing acid charge and 15% bisulfite.

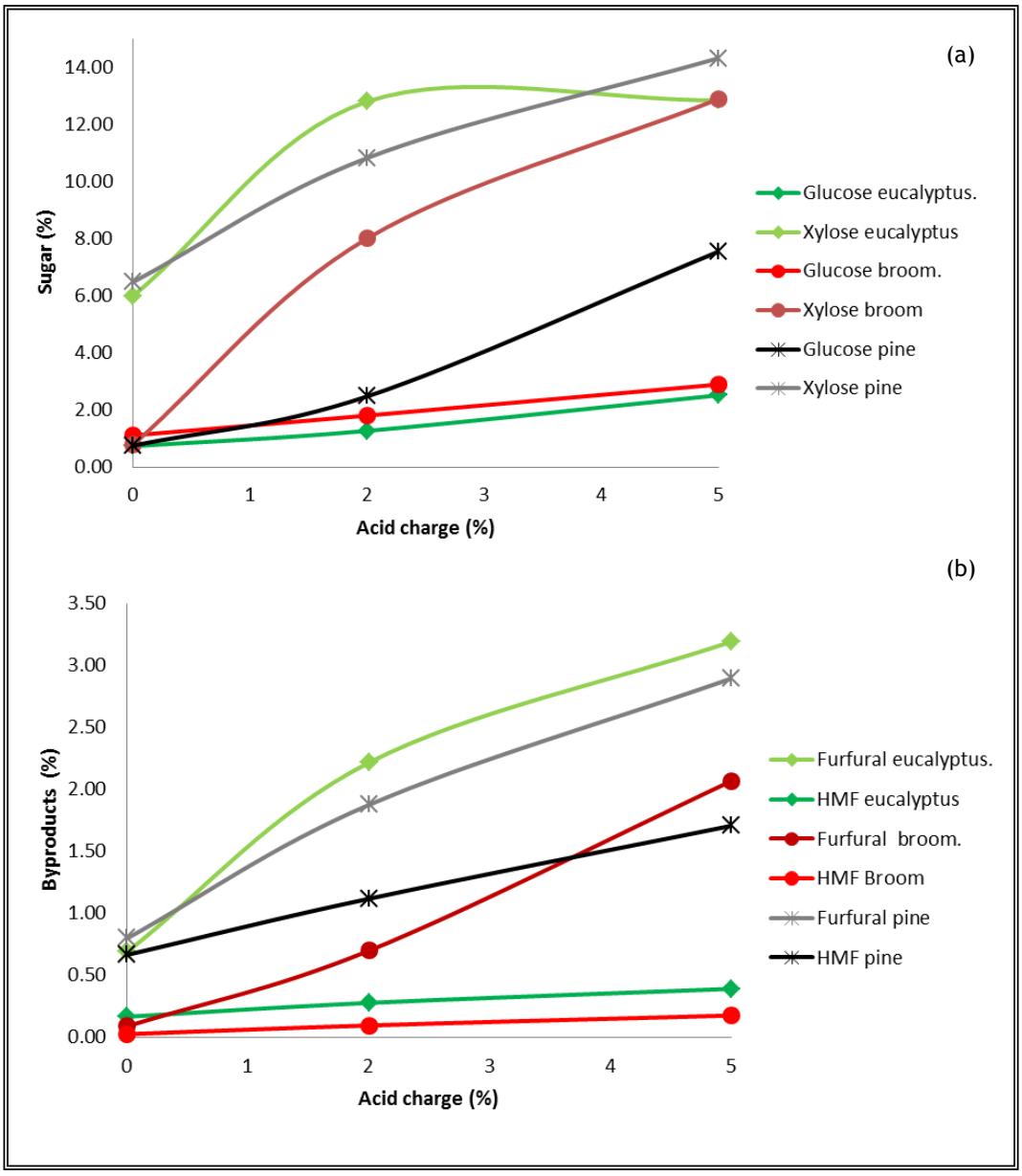


Figure 35 - Variations of the amounts of glucose and xylose (a), furfural and HMF (b) found in the hydrolysates, with the acid charge of the pretreatment liquors.

As can be observed in figure 35 (a), higher amounts of xylose are released into the hydrolysates (consequence of the xylan degradation), compared with the amounts of glucose. Since furfural and HMF derive from pentoses and hexoses, respectively (Larsson *et al.*, 1999), it is expected that the amounts of furfural found in the hydrolysates are higher than those of the HMF (figure 35 (b)), which agree with the results achieved.

IV – 5.2 Enzymatic hydrolysis of the sulfite pretreated material

Total available carbohydrates of broom wood (67.6%) were collected from chemical composition studies performed by Gil *et al.* (2012). For pine and eucalyptus, the correspondent values were estimated from table 1, in chapter II, (adding the cellulose and hemicelluloses dry weight percentage), being 66 and 75%, respectively. Total available carbohydrates estimates were used to calculate the total carbohydrate yield of the untreated material throughout the enzymatic hydrolysis.

For the pretreated material, the lignin content, in percentage, was estimated as being 0.12 × K-No for eucalyptus and broom, and 0.15 × K-No for pine. The total carbohydrates yield calculations were based on the lignin free material.

Figures 36, 37 and 38 show the total carbohydrates yield throughout the enzymatic hydrolysis for the several pretreated solids. The following notation for the pretreatment liquor composition was adopted: *X*s_*Y*ac, where *X* is the percentage in weight of bisulfite and *Y* is the percentage in weight of H_2SO_4.

Grinded eucalyptus, pine and broom were subjected to enzymatic hydrolysis without any chemical pretreatment, as a control group.

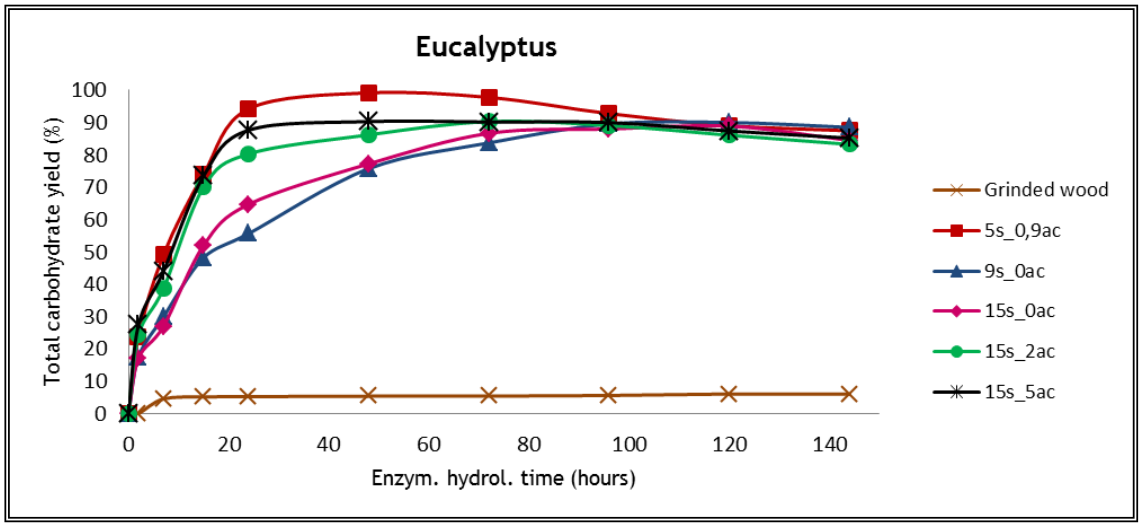


Figure 36 - Total carbohydrate yield throughout enzymatic hydrolysis of pretreated eucalyptus wood.

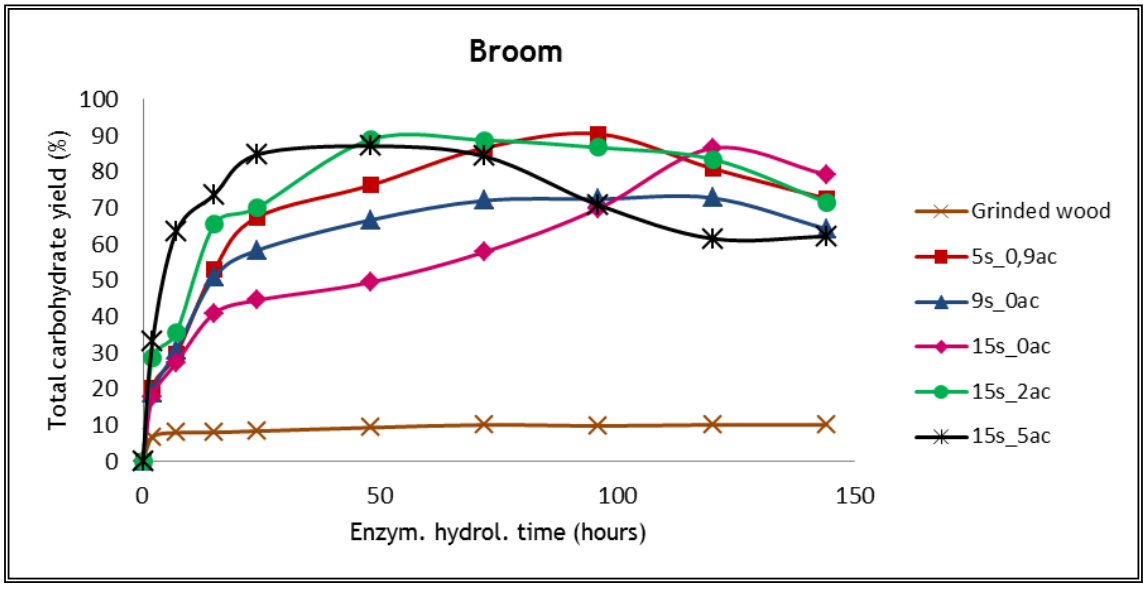


Figure 35 - Total carbohydrate yield throughout enzymatic hydrolysis of pretreated broom wood.

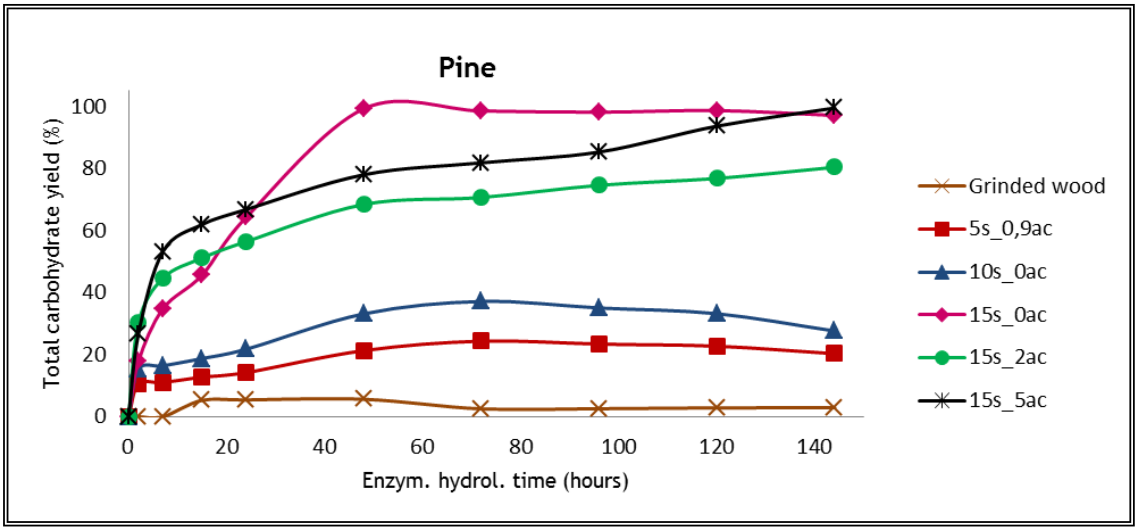


Figure 37 - Total carbohydrate yield throughout enzymatic hydrolysis of pretreated pine wood.

Analyzing the figures 36, 37 and 38, it can be observed that, for the three studied species, the total carbohydrate yield for grinded wood was extremely low comparatively to sulfite pretreated substrates. Despite the experimental uncertainty, the experimental data suggests that the sugar release is lowest for pine and highest for broom.

Regarding the pretreatments, and despite the higher lignin content estimated with K-No determination, the solids treated with 15% sulfite and 5% acid (w/w) exhibited a faster initial hydrolysis rate, probably due to their higher initial surface area, measured by morphological analysis. These results suggest that the sulfonated lignin does not hinder the action of the enzymes.

IV – 5.3 Effect of enzymatic hydrolysis on fiber morphology

Table 9 shows the fine elements quantification of the samples subjected to enzymatic hydrolysis. The results are expressed in percentage of area.

Table 9 - Fine element quantification of sulfite pretreated biomasses throughout the enzymatic hydrolysis.

		Fine elements (%) area				
Biomass	**Enzym. hydrol. time (hours)**	**5s_0.9ac**	**9(10)s_0ac**	**15s_0ac**	**15s_2ac**	**15s_5ac**
Eucalyptus	0	17.8	13.3	9.6	11.0	16.4
	2	50.6	36.2	32.6	30.5	45.5
	48	50.1	53.6	34.3	32.5	48.6
	144	48.6	45.2	40.4	45.3	49.9
Broom	0	22.6	20.4	15.5	19.6	22.6
	2	42.9	29.8	24.3	33.7	49.4
	48	50.3	48.7	36.1	42.8	50.3
	144	59.6	49.4	40.2	43.9	54.6
Pine	0	25.5	16.7	7.3	13.4	40.9
	2	25.7	19.9	16.0	35.1	41.1
	48	31.6	35.0	34.1	39.1	47.3
	144	41.6	50.8	32.0	39.6	55.6

As has been said previously, increasing acid content of pretreatment liquor caused the resultant solids to be visibly more broken, particularly for the solid pretreated with 15% bisulfite and 5% acid. This phenomenon is consistent with the results obtained for fine element quantification, registered in table 9, where it can be observed that solids pretreated with 5% bisulfite and 0.9% acid, and 15% bisulfite and 5% acid show a higher initial content of fine elements.

Table 9 also shows that, for all biomasses studied, fine elements increase throughout enzymatic hydrolysis, particularly in the first two hours. These results agree with what was expected, since the fibers go through physical modifications, getting smaller as the enzymes break their structure during hydrolysis.

Figures 38, 39 and 40 depicture some samples of pretreated solids from eucalyptus, broom and pine, and their evolution during enzymatic hydrolysis, observed with an optic microscope under dark field, at 160×.

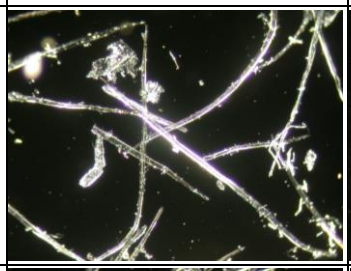
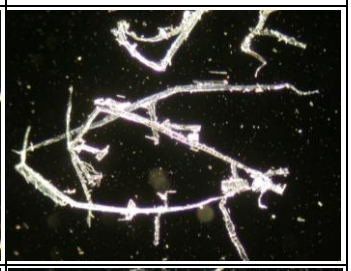
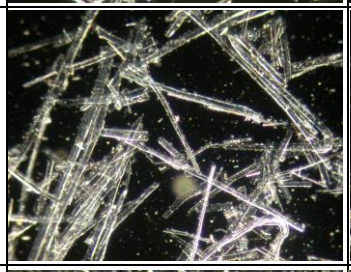
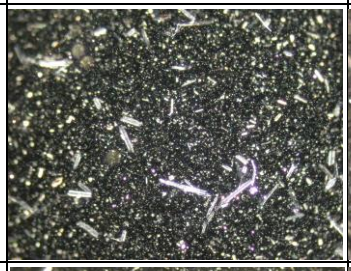
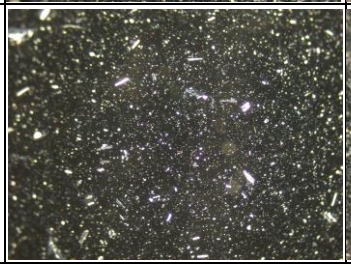
Eucalyptus		
Enzym. hydrol. time (hours)	**Pretreatment liquor composition**	
	15s_0ac	**15s_5ac**
0		
2		
48		
144		

Figure 38 - Samples of sulfite pretreated eucalyptus during enzymatic hydrolysis, observed with an optic microscope at 160×.

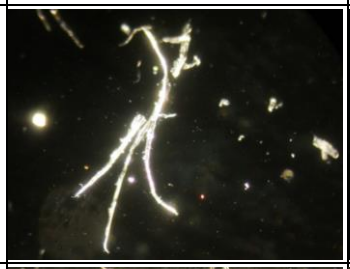
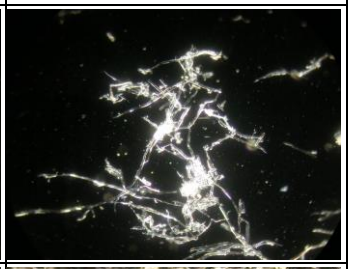
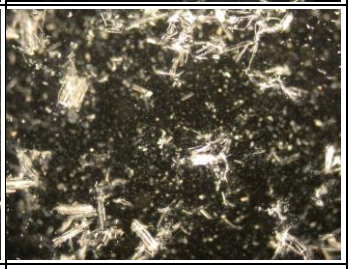
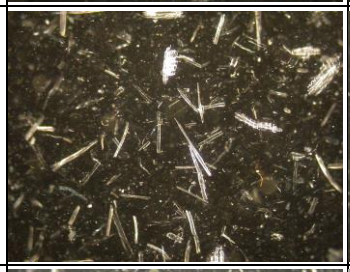
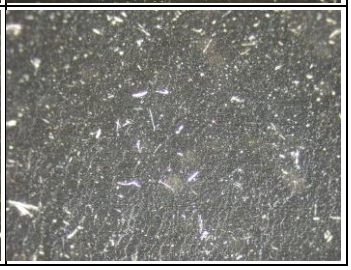
Broom		
Enzym. hydrol. time (hours)	Pretreatment liquor composition	
	15s_0ac	15s_5ac
0		
2		
48		
144		

Figure 39 - Samples of sulfite pretreated broom during enzymatic hydrolysis, observed with an optic microscope at 160×.

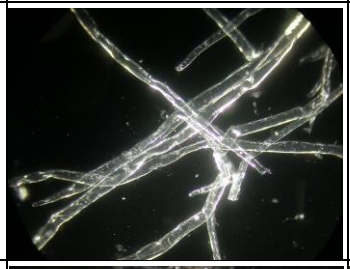
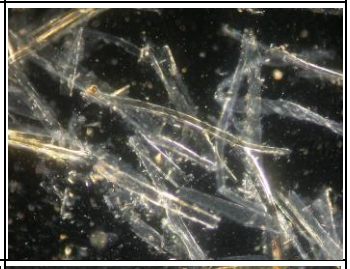
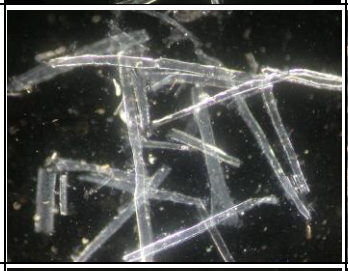
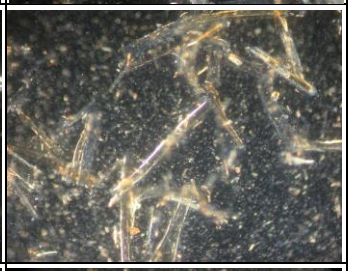
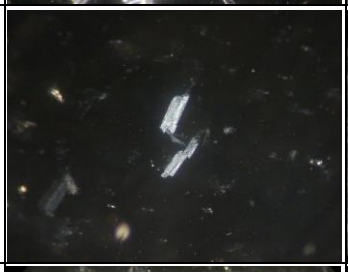
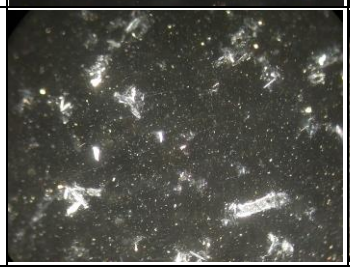
Pine		
Enzym. hydrol. time (hours)	Pretreatment liquor composition	
	15s_0ac	15s_5ac
0		
2		
48		
144		

Figure 40 - Samples of sulfite pretreated pine during enzymatic hydrolysis, observed with an optic microscope at 160×.

As it can be observed for the three biomasses, there is a decrease in the size of the fibers throughout the enzymatic hydrolysis, along with an increase of the amount of fine elements (figures 38, 39 and 40), which agrees with what has previously been confirmed in table 9.

Despite the visible fiber degradation, observations under a microscope also surprisingly revealed the presence of several microorganisms, predominantly in the samples that underwent a longer time of enzymatic hydrolysis. Because these microorganisms can contribute to either fiber degradation or consumption of the released sugars, the introduction of an antibiotic in the samples might be plausible.

Chapter V – Conclusions and final considerations

The main conclusions of this work are the following:

- The obtained results with model fibrous materials suggest that the presence of native lignin (not modified) and the polymers ultra-structure of the wood are two determinant features of the enzymatic hydrolysis, even when a specially designed enzymatic cocktail is used for hydrolyzing lignocellulosic materials. The structural organization determinant role was emphasized when a chemical bleached pulp (lignin-free) drastically lost its potential to be enzymatically hydrolyzed after being subjected to smooth drying process (60ºC, during 12 hours).

- The sulfite pretreatment of eucalyptus and pine woodchips and also broom wood, and the enzymatic hydrolysis of the correspondent solid residues, has confirmed the potential previously reported by other authors for this method.

- The temperature and pH level of the medium are the variables that favor the most the sugar release, but also their conversion into furfural and HMF, which are compounds with known inhibitory effects on the microorganisms that convert the sugars into ethanol though alcoholic fermentation.

- The solid residues from the pretreatment with sodium sulfite and sulfuric acid exhibited conversions in the range of 65 to 98%, compared to its theoretical values.

- Despite high residual lignin content of the solid residues from the sulfite pretreatment, the enzymatic hydrolysis extension was quite high, contrarily to what has occurred for the mechanical pulp, in which the lignin was in its original form.

- The solid residues of eucalyptus had an overall higher sugar conversion during the enzymatic hydrolysis than pine and broom.

- In future investigations, another method to determine the lignin content of the pretreated materials should be used, apart from kappa number determination, in order to decrease the uncertainty associated to this method, or optimize the correlation between the kappa number and the lignin content.

- The connection between the sugars concentration decrease after around 72 hours of enzymatic hydrolysis might be related with the presence of microorganism in the medium, which can consume the sugars released. Thus, the insertion of an antibiotic during the enzymatic hydrolysis might be a plausible option to avoid this issue.

- The optimization of sugar release during enzymatic hydrolysis could be improved by adjusting the enzymatic cocktail composition, adapting it to the type or composition of the biomass subjected to it.

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Paper, board and pulps; *Determination of dry matter content - Oven-drying method*; ISO 638:2000

Annexes

Annex A

Table A1 - Reagents used in the experimental procedures.

Reagent	Molecular formula	Properties
Citric acid monohydrate	$C_6H_8O_7.H_2O$	Molecular weight: 210.14 g/mol Purity: 99.5% Label: Panreac CAS number: 5949-29-1
Tri-Sodium acetate 2-hydrate	$C_6H_5Na_3O_7.2H_2O$	Molecular weight: 294.10 g/mol Purity: 99.0% Label: Panreac CAS number: 6132-04-3
Sulfuric acid	H_2SO_4	Molecular weight: 98.08 g/mol Purity:96% Label: Panreac CAS number: 7664-93-9
Sodium bissulfite	$NaHSO_3$	Molecular weight: 104.061 g/mol Purity: 99% Label: Sigma Aldrich CAS number: 7631-90-5
Anhydrous D-glucose	$C_6H_{12}O_6$	Molecular weight: 108.16 g/mol Purity: 99.5% Label: Pronolab CAS number: 50-99-7
Cellobiose	$C_{11}H_{22}O_{11}$	Molecular weight: 342.30 g/mol Purity: 99.5% Label: Merk CAS number: 528-50-7
D-xylose	$C_5H_{10}O_5$	Molecular weight: 150.13 g/mol Purity: 99.5% Label: Merck CAS number: 58-86-6

Table A1 (continuation) - Reagents used in the experimental procedures.

Reagent	Molecular formula	Properties
Sodium hydroxide	NaOH	Molecular weight: 40.0 g/mol Purity: ≥ 98% Label: Pronolab CAS number: 1310-73-2
Acetic acid glacial	CH_3COOH	Molecular weight: 60.5 g/mol Purity:99.7% Label: Panreac CAS number: 64-19-7
Sodium thiosulphate pentahydrate	$Na_2S_2O_3.5H_2O$	Molecular weight: 248.18 g/mol Purity: ≥ 99.5% Label: Merck CAS number: 7772-98-7
Potassium iodide	KI	Molecular weight: 166.0028 g/mol Purity: 99.0% Label: LaborSpirit CAS number: 7681-11-0
Potassium permanganate	$KMnO_4$	Molecular weight: 158.03 g/mol Purity: ≥ 99% Label: BDH Laboratory Supplies CAS number: 7722-64-7
Cupriethylenediamine solution	$Cu(NH_2CH_2CH_2NH_2)_2(OH)_2$	Molecular weight: — Purity: — Label: Carlo Erba Reagents CAS number: 14552-35-3

Annex B

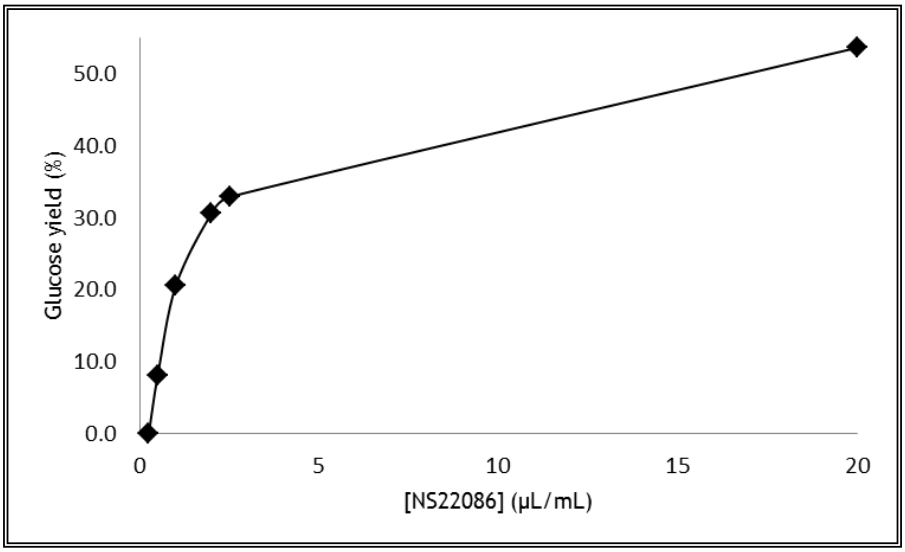

Figure B1 - Variation of glucose yield with cellulase concentration for the determination of cellulases activity.