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Ano 2015

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Melhoramento enzimático da pasta Kraft de Eucalipto na produção de pasta solúvel

Enzymatic upgrading of Eucalypt paper-grade kraft pulp within dissolving pulp production

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia – Ramo Industrial e Ambiental, realizado sob a orientação científica do Doutor Dmitry Victorovitch Evtyugin, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro e do Doutor Pedro Loureiro, Investigador Sénior no Departamento de Produtos Florestais da empresa Novozymes A/S, Dinamarca.

Dedico este trabalho aos meus pais e irmã.

o júri

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Agradecimentos

Em primeiro lugar gostaria de agradecer ao Dr. Pedro Loureiro, e ao Dr. Henrik Lund pela enriquecedora oportunidade que me proporcionaram de realizar a tese de mestrado na conceituada empresa Novozymes. Sem o vosso voto de confiança este sonho nunca se teria realizado.

Um muito obrigado aos meus orientadores, Dr. Pedro Loureiro e Dr. Prof. Dmitry pela amizade e apoio que deram, estando sempre disponíveis e prontos a ajudar. E um especial agradecimento ao Dr. Pedro Loureiro, não só pela paciência em explicar e esclarecer as vezes necessárias as dúvidas que foram surgindo ao longo do percurso, mas também pela simpatia durante a minha jornada na Dinamarca.

Aos meus pais e irmã pelo carinho e força que me deram, principalmente quanto estava longe de casa e as saudades apertavam. Amplio este obrigado, aos tios e primos que mesmo estando longe, estiveram sempre presentes.
Obrigado a todos os membros do Laboratório de Produtos Florestais, em especial à Jette e à Anja e por estarem sempre dispostas a ajudar no laboratório. Obrigada à Elenie, Betina, Lise, Ingrida, Pedro, Perrakis e Valentin pela indispensável amizade, ao Kim por me estar sempre alegrar com as suas cantorias, ao Pierre, Morten e Owik pelos sábios conselhos, às simpáticas Joan e Tania e a todos os colegas que não mencionei, que me fizeram crescer como pessoa.

Também gostaria de agradecer aos meus amigos de Gent, Louredo, e Aveiro em especial às colegas de Biotecnologia Inês Cardoso e Joana Couceiro pelo apoio e pela amizade.

Por último, mas igualmente importante, queria agradecer ao Dovydas Bacanskas, por interessar-se pelo que faço e por me apoiar incansavelmente.

Sonia Cadete
25 Novembro, 2015

palavras-chave Pasta solúvel, xilanases, cellulases, endoglucanases, pureza da pasta, celulose

Resumo

Pastas solúveis são normalmente usadas para a produção de derivativos de celulose e celulose regenerada. Alguns dos parâmetros que determinam a qualidade de uma pasta solúvel são: um elevado teor de celulose, baixo teor de material não-celulósico, elevada brancura, uma distribuição uniforme de pesos moleculares e elevada reactividade da celulose.

Na primeira parte deste trabalho, fez-se um estudo de optimização aplicando enzimas, previamente seleccionadas, em diferentes fases de um novo processo de purificação desenvolvido na Novozymes da pasta de eucalipto Kraft em celulose solúvel, como uma alternativa ao processo convencional de pré-hidrólise kraft.

Além da purificação, a aplicação de celulases (endoglucanase) no início da sequência possibilitou uma diminuição da viscosidade, enquanto que a aplicação de xilanases das famílias GH11 e GH10 contribuíram também para o aumento da brancura da pasta final.

A segunda parte deste trabalho teve como objectivo explorar várias actividades enzimáticas auxiliares conjuntamente com a melhor GH11 xilanase identifcada, de modo a promover a remoção das hemiceluloses mais recalciterantes de uma pasta Kraft de Eucalipto parcialmente branqueada. Todas as combinações das enzimas testadas resultaram numa fracção resistente de xilana residual (ca. 6% na pasta) que não foi possível hidrolisar.

A produção de uma pasta solúvel foi possível usando um estágio de extracção alkalino a frio (CCE) no fim de uma sequência composta pelos seguintes estágios: O-X-D-HCE-X-HCE-D-CCE. A aplicação de enzimas melhorou a eficiencia do processo. Com esta sequência,os principais requisitos para a produção de uma pasta solúvel (adequada para producao de viscose) foram cumpridos: 2,7% de xilana residual, 92,4% de brancura, uma viscosidade dentro dos valores de uma pasta solúvel comercial e elevada reactividade.

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keywords

Dissolving pulp, Xylanases, cellulases, endoglucanases, pulp purity, cellulose

abstract

Dissolving-grade pulps are commonly used for the production of cellulose derivatives and regenerated cellulose. High cellulose content, low content of non-cellulosic material, high brightness, a uniform molecular weight distribution and high cellulose reactivity are the key features that determine the quality of a dissolving pulp.

The first part of this work was an optimization study regarding the application of selected enzymes in different stages of a new purification process recently developed in Novozymes for purifying an eucalypt Kraft pulp into dissolving pulp, as an alternative to the pre-hydrolysis kraft (PHK) process. In addition, a viscosity reduction was achieved by cellulase (endoglucanase) treatment in the beginning of the sequence, while the GH11 and GH10 xylanases contributed to boost the brightness of the final pulp.

The second part of the work aimed at exploring different auxiliary enzyme activities together with a key xylanase towards further removal of recalcitrant hemicelluloses from a partially bleached Eucalypt Kraft pulp. The resistant fraction (ca. 6% xylan in pulp) was not hydrolysable by the different combinations of enzymes tested.

Production of a dissolving pulp was successful when using a cold caustic extraction (CCE) stage in the end of the sequence O-X-D-HCE-X-HCE-D-CCE.

The application of enzymes improved process efficiency.

The main requirements for the production of a dissolving pulp (suitable for viscose making) were fulfilled: 2,7% residual xylan, 92,4% of brightness, a viscosity within the values of a commercial dissolving pulp and increased reactivity.

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Abbreviations

-A-	Acidolysis stage
αGRN	α-glucuronosidase
AM	Amylase
BG	β-glucosidase or cellobiase
CAZy	Carbohydrate-Active enZYmes Database
CBM	Carbon binding module
CMC	Carboxymethyl cellulose
CBH	cellobiohydrolase or exoglucanase
CIP1	Cellulose induced protein-1
CIP2	Cellulose induced protein-2
R10, R18	Cellulosic residue in 10% and 18% NaOH
D-	Chlorine dioxide stage
CCE-	Cold caustic extraction
CUT	Cutinase
DP	Degree of Polymerization
DP	Dissolving Pulp
ECF	Elemental chlorine free
EG	Endoglucanase
EC	Enzyme class
EP	Enzyme protein
-X-	Enzymatic stage
E-	Esterase
EXP	Expansins

GE	Glucuronoyl esterase
GH	Glycoside Hydrolases
HexA	Acid Hexenuronic
HPAE	High-Performance Anion-Exchange Chromatography
HCE-	Hot caustic extraction
ISO	International Organization for Standardization
LCC	Lignin carbohydrate complexes
M	Mannanase
Tm	Melting temperature
MeGlcA	4-O-methyl-α-D-glucopyranosyl uronic acid
MN	Molecular weight number average
MWD	Molecular weight distribution
MW	Molecular weight
kDa	Molecular mass in kilo Dalton
NREL	National Renewable Energy Laboratory
O-	Oxygen stage
PHK	Pre-Hydrolysis Kraft
PDI	Polydispersity index
PAD	Pulsed Amperometric detection
SRS	Sugar Recovery Standards
SWO	Swollenins
TDP	Target to Dissolving pulp

1. Introduction

Until the end of the 19th century the main focus of the pulp and paper industry was solely the production of paper-grade pulps. The chemical conversion processes for the production of textile cellulosic fibers (e.g. viscose fibers) were first invented in 1892 and afterward cellulose acetate in 1904. Since then, the production of cellulose acetate led to the development of the present dissolving- grade pulp.[1]

Dissolving pulp is a pulp of high cellulose content, first produced merely from cotton linters. Nowadays there are two main processes for production of dissolving pulp: one starts with sulphite cooking and the other with pre-hydrolysis kraft cooking. The acidic conditions of the sulphite cooking removes part of hemicelluloses, which are impurities that interfere in the follow up processes for the manufacture of DP (conversion). But hemicelluloses cannot be solubilized under alkaline conditions (kraft cooking) effectively, thus an acid hydrolysis step was introduced prior to the alkaline pulping process, creating the pre-hydrolysis-kraft (PHK) process. [1]

The wood-derived celluloses made by the PHK and acid sulfite processes accounts for about 85–88% of the total dissolving pulp market and may comprise additional purification to achieve the required purity depending on the end-use product. [2] Typically, the sulphite process allows a pulp with 90-92% of α-cellulose (cellulose chains with highest degree of polymerization), whereas PHK process provides a pulp with 94-96% of α-cellulose. Purified cotton linters represent the dissolving pulp of highest cellulose purity particularly used for manufacturing acetate cellulose and high-viscosity cellulose ethers. [3] Viscose fibers and cellulose acetate are two of the main markets of a dissolving-grade pulp, being viscose by far the main segment.

The market of wood cellulosic products requires more developments in the processes in order to increase the purity level of the final product while decreasing the cost of dissolving pulp production.

The following dissertation has the purpose of describing a research work developed during the past nine months under the framework of an internship at the company Novozymes A/S.

Novozymes A/S is a global company headquartered in Bagsværd, Denmark. The main activity is to create new solutions for various industries, allowing the customers to achieve

more efficient use of raw materials, offer higher-quality products, reduce energy consumption and replace traditional chemicals with more sustainable alternatives, such as industrial enzymes, microorganisms, biopolymers and other proteins. The company, known as the world leader in bio-innovation, is focused on the production and commercialization of enzymes. The research work was developed particularly in the Forest Products R&D department.

The production of forest products is based on the specific knowledge of the wood composition with all its natural variability for the conversion into a variety of products with it own details and quality requirements. In order to make such products, a detailed knowledge about the starting material and the chemical conversion processes into the end products must constitute an integrated base to be used during the research work. Therefore, an overview in of the wood chemical composition, the chemistry and technology of the dissolving pulp production, the product quality requirements and a description of the relevant enzymes studied is given in the following sections.

1.1 Wood and pulp chemical composition

The type of wood is one of the most important variables in the pulping process in terms of the quality of the end product. It is normally divided into two kinds of wood: i) softwoods have a more simple macro-structure comprised of a limited number and uniform cell types; ii) hardwoods have a more complex and large number of different cell types. [4]

Softwoods used to be the main raw material for dissolving pulp. However, hardwood pulp obtained by sulphite process is even better than softwoods for the production ofe viscose.

Although the wood structure is very diverse, softwoods and hardwoods share the same three main components of the cell wall: cellulose 40-45%, hemicellulose 20-35%, and lignin 20-35%, together with small amounts of extractives, such as terpenoids, resin acids, fatty acids, pectin, proteins and inorganic matter.

Cellulose is a linear homopolysaccharide composed by D-glucose units joined by ß-1,4-glycosidic bonds (Figure 1). The strong tendency for cellulose to form both intra- and intermolecular hydrogen bonds leads to the formation of microfibrils, which in turn form either highly ordered (crystalline) or less ordered (amorphous) regions that can be packed tightly together to form even larger fibrils and finally cellulose fibers. [5]

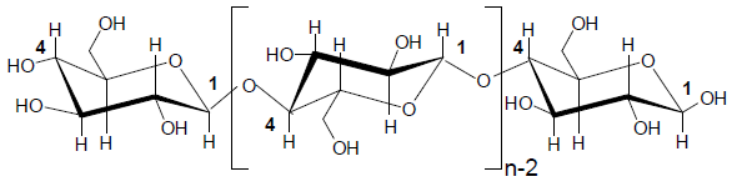


Figure 1 Structure of cellulose

Cellulose is a stiff molecule and difficult to dissolve in aqueous solutions due to the existence of huge quantities of inter- and intramolecular hydrogen bonds The native cellulose known as cellulose I, is composed of two crystalline phases: triclinic crystalline cellulose Iα and monoclinic Iβ. The ratio of Iα/Iβ in the composition varies depending on the source of the cellulose. In wood pulp, the cellulose Iβ dominates. [5]

Cellulose II also exists as a more complex network than the native molecule. The transformation of native cellulose I into cellulose II can occur upon a treatment with 10-18% of sodium hydroxide (NaOH) at room temperature by a process called mercerization. Cellulose II can also be formed in chemical conversion to produce regenerated fibers and cellulose derivatives.[6]

Hemicelluloses are the second abundant polyssacharides group composed by diverse sugar residues (D-xylose, L-arabinose, D-glucose, D-mannose, D-galactose, D-glucuronic acid, 4-*O*-methylglucuronic acid, D-galacturonic_acid) and other O-acetylated neutral sugars. These non-cellulosic polyssacharides provide a supporting function to the cell wall and are located in the matrix between cellulose fibrils and lignin. [7] Most hemicelluloses in wood also have a linear β-(1→4)-linked backbone of sugar residues. Unlike cellulose, hemicelluloses are mainly branched polymers of low molecular weight (DP 80-120) and can have substituted side chains which make them more soluble in water. [5]

Wood hemicelluloses consist of either xylan or glucomannan based backbone polymers. The structure of xylan and glucomannan in relation to hardwood and softwood is depicted in Figure 2.

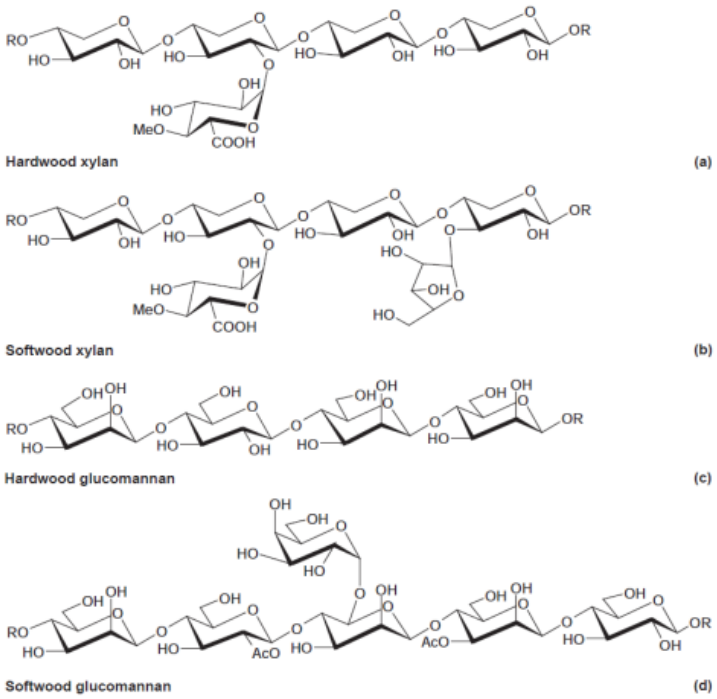


Figure 2 Molecular structure of xylan and mannan in hardwood and softwood; a) Harwood xylan; b) Softwood xylan; c) Hardwood glucomannan; d) Softwood glucomannan;

Hardwood and softwood xylans have different side group patterns. Hardwood xylan contains acetyl groups, while softwood contains-arabinose side groups. Xylan in softwood is less acidic than in hardwood due to the lower content of MeGlcA. [7] Hardwood xylan is *O*-acetyl-(4-*O*-methylglucurono) xylan, in which the backbone is substituted at random intervals with acetyl and 4-*O*-methylglucuronic acid side groups. The acetyl ester groups are present at the C2 and/or C3 positions, corresponding to approximately 4-7 acetyl per 10 xylopyranosyl residue. Softwood xylan is mainly arabino-(4-*O*-methylglucurono) xylan, in which L-arabinose units are α-1,3-linked to position C-3 of the xylan backbone. In softwood xylan there are no acetyl groups, unlike hardwood. [5]

Regard to mannans they are divided into two groups: galactoglucomannans and glucomannans. The first one are composed of β-1,4-linked glucose and mannose units, which are randomly distributed along the backbone and galactose side groups attached to glucose or mannose units through α-1,6-linkages. The second hardwood glucomannan contains glucose and mannose units in the ratio of 1:1-2 and neither galactose no acetyl groups are present. [5]

Besides cellulose and hemicelluloses, wood also contains starch and pectin. Starch is composed by linear amylose (glucose units linked by α-(1,4)-glycosidic bonds) linear and

branched amylopecin (α-1,6-glycosidic bonds). Pectin is composed by homogalacturan linear chains with highly branched galacturonic chains.

Lignin is a macromolecule with a more complex three dimentional structure.[8] It contains mainly phenolic hydroxyphenylpropane units (monolignols): coniferyl alcohol in softwood and coniferyl alcohol together with sinapyl alcohol in hardwood. In addition, both hardwood and softwood lignin often contain small amounts of p-coumaryl alcohol. [9]

There are covalent linkages between lignin and hemicelluloses in native wood. These structures are typically referred to as lignin-carbohydrate complexes or simply as LCCs. The formation of LCCs take place during lignification in growing plants and are present in native wood as ester, benzyl ethers and phenyl glycosidic linkages. [10] The lignin is covalently bound to the hemicelluloses which in turn, are bound to cellulose through extensive intermolecular hydrogen bonding.[10] The predominant bonds in lignin are: ether bonds (β-O-4 and α-O-4), which are easier to cleave; β-5, 4-O-5, β-1 bonds and β-β, 5-5 or C-C bonds, which are the hardest to cleave.

The extractive materials in wood are mainly volatile oils, resins, fats and waxes. In sofwood is mainly terpenes and tall oil (derived from fats and waxes). Fats are esters of fatty acids with glycerol, while waxes are esters of fatty acids with higher alcohols. The extractives of softwood also contain phenolic compounds derived from lignin biosynthesis. Hardwood contains a lower content of terpenes but also contains steroids which are esterified with fatty acids. Harwood also contains fats, waxes, fatty acids, alchool and low molecular weight phenols. The major group of extractives (phenolic compounds) in hardwood are the tanins and flavonoids. [11]

1.2 Dissolving-grade Pulp

Pulp represents the major raw material basis for two main applications:

(a) mechanical conversion to paper and board production, where the pulp fibers are modified to give a consistent sheet;

(b) chemical conversion to products such as regenerated fibers (as for viscose) or products of higher demands on purity called cellulose derivatives (such as acetates, ethers, nitrates).

The former is denoted as paper grade, the latter as dissolving grade pulp.

There are some differences on the pulping process of making regular paper pulp and dissolving pulp. Both processes use the same raw material: wood chips being cooked under pressure at a temperature of 130 to 180°C for several hours. The resulted sludge is put through a screening process to remove large fragments, dirt and other debris and what remains is called pulp. Afterwards, the pulp is washed and then bleached.

Besides lignin removal, in the manufacture of dissolving pulps, an important goal is to remove as much hemicelluloses as possible with minimum cellulose deterioration. As previously referred in the introduction, there are two main processes for the production of dissolving pulps: the acid sulfite process and the pre-hydrolysis kraft (or sulfate) process (also called PHK process). (Appendix 2, Figure 27 A)

1.2.1 Pre-hydrolysis Kraft Pulping

In the alkaline pulping process (e.g. Kraft process) both short-chain hemicellulose and cellulose are degraded and removed but xylan is more resistant and may precipitate on the cellulose microfibrils. For the manufacture of high-purity dissolving pulp, the pre-hydrolysis step is therefore applied before the actual Kraft process to solubilize xylan which is otherwise resistant under alkaline conditions.[12] The concept involves a pre-treatment at elevated temperatures (140-175°C) and high pressures (7-12 bar).

The principal reaction that occurs in the pre-hydrolysis step is the hydrolysis of acetyl groups either in xylan of hardwood or in galactoglucomannans of softwood, thus lowering the pH to 3 to 4. [13] The acids formed in this pre-hydrolysis step promote the acid hydrolysis of the wood components and a reduction of the wood mass. When a high intensity hydrolysis is applied, not only hemicelluloses will be depolymerized to achieve a higher purity in the final product, but also cellulose resulting in a lower overall yield of the process. In pre-hydrolysis, temperature, acidity and time are factors that determine the extent of the hydrolytic attack. A combination of these variables can be used to control various pulp properties, such as hemicellulose content, degree of polymerisation (DP) and crystallinity. Pre-hydrolysis affects lignin retention and structure in wood and may result in the formation of condensed lignin structures when high intensities are applied. [14]

Besides removing more hemicelluloses, this pre-hydrolysis can lead to the formation of highly reactive intermediates undergoing condensation products and pitch-like compounds are formed which may deposit on any surface available. Also, the two heating-up phases, prior to prehydrolysis and cooking require large amounts of steam, thus leading to a major

prolongation of the cook. [15] [16] Therefore, it would be an advantage if this step could be avoided in the process of making dissolving pulp.

The pre-hydrolysis step is followed by the actual Kraft cooking. During the kraft cooking, there are different phases with different selectivity towards dissolution of carbohydrates and lignin as shown in figure 3 and decribed bellow:

Initial phase: taking place at temperatures below 150°C, is characterized by losses in the carbohydrate fraction, which is more pronounced for hardwoods as compared to softwoods.[15] Physical dissolution and peeling reactions (depolymerization) start and hemicelluloses undergo deacetylation. Cellulose degradation by peeling is negligible in terms of yield loss. Reactive phenolic lignin units, such as β and α-O-4-ethers, are cleaved in the initial phase. [17]

Bulk phase: includes the heating period from 150°C to 170°C and a cooking treatment at 170°C. The main delignification occurs where phenolic and nonphenoplic β-O–4-ether bonds are cleaved. Typically, about 70% of the lignin is removed. The reactions of the carbohydrates are characterized by secondary peeling (i.e., alkaline cleavage of the glycosidic bonds) but also by stopping reactions, which are favored at elevated temperature (Figure 4). Methanol is liberated from 4-*O*-methylglucuronic acid side chains, and hexenuronic acid (HexA) units are formed.

Residual phase: is a very slow process which includes the final treatment at 170°C and finish at a delignification rate of about 90%. Delignification slows down considerably due to reduction of the amount of reactive lignin units. It is believed that the chemical nature of the residual lignin hampers further degradation reactions. A slow delignification is accompanied by the degradation of carbohydrates. [13] [14] [19]

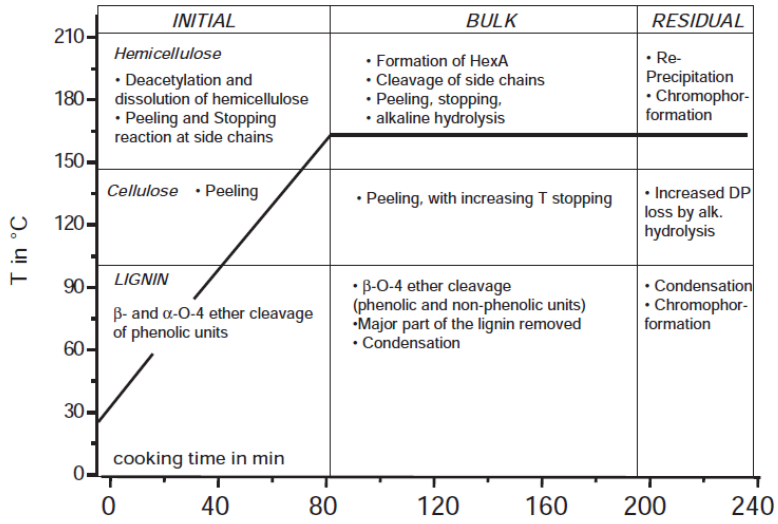


Figure 3 Phases of delignification and corresponding reactions of wood components[18]

Carbohydrate reactions

The degradation of carbohydrates in the alkaline conditions consists of mainly three reactions: A) peeling at the end of a carbohydrate chain (endwise or primary peeling); B) oxidative peeling randomly in the carbohydrate chain and C) alkaline hydrolysis (secondary peeling) occurring at higher temperatures (over 140°C). (Figure 4)

For the endwise peeling reactions, the reducing end groups in the cellulose or hemicellulose chain can undergo a series of keto-enol-transformations which will lead to the peeling off of one end unit at the end of the chain as one glucoisosaccharinic acid (for cellulose or glucomannans) or as xyloisosaccharinic acid (for xylan). This peeling effect can continue as long as a new reducing end group is formed but can be interrupted because of a competing "stopping'' reaction. The reaction pauses in more stable units when a substituent in position 2 or 3 of the xylan backbone is reached, for example a 4-*O*-methylglucuronic acid in position C2 of a side chain or arabinoxylan (softwood) in position C3, and will continue until a higher temperature is reached and the substituent is then cleaved. MeGlcA substituents are gradually converted to hexenuronic acid substituents through β-elimination of methoxyl groups in alkaline conditions. The mannose end unit in glucomannans is more stable to isomerisation than the anhydroglucose unit in cellulose; however, the extent of glucomannan degradation is more substantial than that of cellulose. [20]

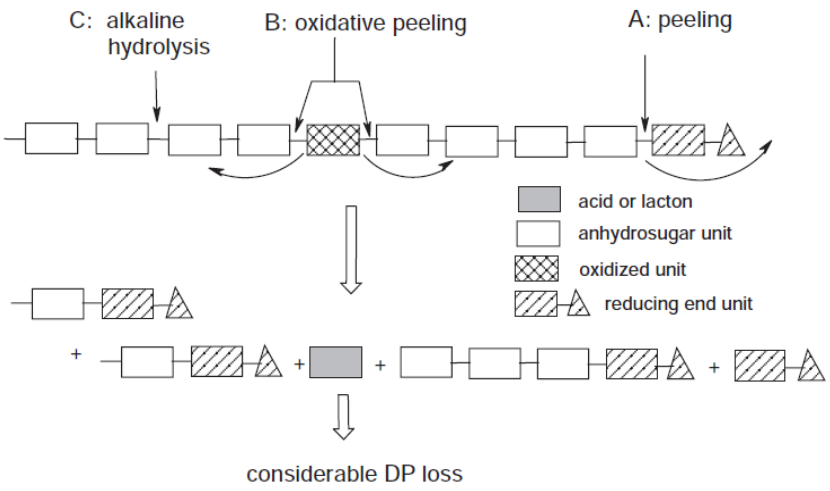


Figure 4 Schematic model of celulose/hemicellulose degradation under alkaline conditions

Formation of lignin carbohydrate (LC) bonds in kraft pulp

Lignin carbohydrate bonds can be generalized as either alkali-stable or alkali labile. [21, 22] The alkali-labile linkages, such as ester linkages, are readily cleaved under the harsh alkaline conditions of the kraft cook while the alkali-stable linkages, such as ether linkages, survive the kraft cook and have been suggested to be present in kraft pulps. These alkali-stable linkages may be enriched and contribute to the difficulty in removing lignin at the end of the cook. [23,24]

Besides natural LCCs, formation of LCCs occurs during the transition stage from the initial to the bulk phase of kraft cooking, where extensive lignin fragmentation takes place through cleavage of β-aryl ether linkages in non-phenolic units.

While the lignin fragment of the native LCCs is believed to be linked exclusively with hemicelluloses, the lignin fragment of artificial LCCs formed during pulping may be more frequently linked to cellulose. [25]

The role of Extractives

Regarding extractives, fatty acids and resin acid esters are saponified in alkaline pulping and can be recovered as tall oil soap. Wood terpenes undergo mainly condensation reactions during pulping, and can be collected as sulfate turpentine.

Extractives are responsible for pitch problems; they may also prevent delignification by covering parts of lignin with resinous material or simply reduce the penetrability of cooking chemicals into the wood. Part of extractives survives the cook more or less unchanged and this portion is referred to as the “unsaponifiable” fraction. [18]

1.2.2 Pulp Bleaching

The kraft pulping process does not achieve a more complete delignification in order to not negatively affect or harm yield and the quality of the pulp produced. The resulted pulp unbleached is composed of residual lignin and chromophoric compounds that gives a brownish color to the fibers. Therefore, for the production of bleached pulp, it is essential a further delignification and bleaching of the fibers by removing and modifying chromophoric groups which are assigned to residual lignin, carbohydrates, extractives and metal ions complexed with the pulp components. This is achieved by a series of oxidation and extraction steps leading to a lignin-free fiber by the use of appropriate chemicals and conditions in order to achieve the specific quality criteria of the pulp according to its final use. [26]

The bleaching sequence can include Oxygen (O) and chlorine dioxide (D) as pre-delignification agents in the beginning of the bleaching line, i.e. after the cooking process.[26] [27] Chlorine usage in pulp bleaching is harmful to the environment due to emissions of chlorinated organic compounds in the water effluents. Therefore, chlorine was substituted by chlorine dioxide, which originated the Elemental Chlorine Free (ECF) bleaching. Currently, ECF bleaching (e.g. DEDED, ODED and DEDP) is the dominant bleaching technology.

O_2 delignification

The oxygen delignification process is often applied as the first stage of a bleaching sequence. The oxygen molecule is unreactive unless is heated to elevated temperatures, thus this process has to be operated at high temperature (85-115°C) and pressure (4-8 bar). There are many possible reactions occuring during oxygen delignification: for lignin, degradation or condensation reactions take place while for carbohydrates it can be either random cleavage of the carbohydrate chain or the peeling reaction in the end of the carbohydrate chain.[28]

Chlorine Dioxide

Chlorine dioxide (ClO_2) is a powerful reactive oxidant, that reacts with lignin in pulp without significant degradation of cellulose or hemicelluloses. It breaks the lignin into small pieces by opening the aromatic rings and create structures that can be solubilized and removed under alkaline conditions. The Process variables are chemical dosage, end pH, temperature, time and pulp consistency. There are many possible reaction pathways during the oxidation of lignin with chlorine dioxide that are pH-dependent, thus the conditions have to be selected to favor a selective oxidation of lignin without degradading cellulose to preserve the fibers. [29] Another important aspect is the amount of chlorine dioxide applied and the best use of

chlorine dioxide is usually obtained when 20-30% of the total amount is applied in a second stage (D_1). For instance, the dosage in D_0 stage ranges from 10-30 Kg/t with typical end pH 3-4 and the dosage in D_1 range from 2-8 Kg/t with typical end pH 3-4. The brightness increases quickly with increasing charge of ClO_2 until a certain limit. One way to increase the brightness is the use of the alkaline extraction stage and a second chlorine dioxide stage after D_0. [30]

1.2.3 Pulp Purification

Unlike the paper-grade pulp, the production of dissolving-grade pulps comprises additional purification to remove non cellulosic material (e.g., extractives, lignin, hemicelluloses, proteins, waxes, pectins) and to change the molecular distribution to a narrow, type of distribution with a minimum amount of low molecular weight carbohydrates. The extent of purification should be adjusted to the need of the dissolving pulp process. [31]

Such alkaline purification can be carried out either as a cold or a hot stage. The cold stage is a physical process which solubilize low molecular weight carbohydrates during the swelling the primary wall, while the hot stage is a chemical process that removes the amorphous cellulose and hemicellulose chains of low molecular weight.

There are two ways of representing the dosage of sodium hydroxide applied in alkaline extraction: it can be calculated in the liquid phase (g NaOH/L) or based on the dry fiber (% odp ⇔ g NaOH/ g of odp).

A cold alkali extraction, also called Cold Caustic Extraction (CCE), usually takes place at a low temperatures, below 40ºC. The sodium hydroxide concentration in odp basis is 5-12% and the retention time short e.g. ca. 10 min. [32]

In a hot caustic extraction (HCE) stage the NaOH concentration in odp basis is below 2%, but the temperature is higher: usually between 80 and 120ºC. The retention time is in the range of 1 to 2 h. Under HCE conditions, alkaline peeling reactions of the polysaccharide are responsible for the removal of hemicelluloses. The same reactions, however, also cause significant cellulose yield loss. HCE also removes other pulp impurities such as lignin and extractives. The more alkali-resistant lignin, which might be associated with xylan, is gradually decreased with increasing NaOH charge.

The effect of CCE leads to a narrowing of the molar mass distribution (lower polydispersity index, PDI), where the main part of the short chain carbohydrates (from 2.5 to 12kDa) is

removed and the mid-molecular weight fraction (from 30 to 380kDa) increases. However, the CCE can also slightly damage the high molecular-weight fraction (>1000kDa) composed by long-chain carbohydrates. [31]

The polydispersity index (PDI) is the ratio of the weight average to the number average molecular weights (MW/MN). Normally, after a CCE treatment, the PDI and the low molecular-weight fractions decrease, while the high molecular-weight is not so affected.

In order to have a good quality dissolving pulp, not only the chemical composition is important. The physical properties, such as high level of brightness are also required. The data in Table 1 represents a simplified specification profile of the most important dissolving pulps, derived from hardwood, softwood and cotton linters and produced according to acid sulfite and PHK procedures.

Tabel 1Characterization of a selection of representative dissolving pulps [2]

		Preferred Application				
		Viscose	Ether (HV)	Viscose	Acetate	Acetate
Raw Material Cooking Process		Hardwood Sulfite	Softwood Sulfite	Hardwood PHK	Hardwood PHK	Cotton Linters
Brightness	% ISO	92.2	90.2	90.7	92.2	87.9
R_{18} content	%	93.4	95.2	97.9	98.2	99.0
R_{10} content	%	87.6	93.8	93.3	97.7	97.4
Xylan	%	3.6	3.1	1.5	0.9	0.2
Carbonyl	µmol g^{-1}	18.8	6.0	4.3	4.4	3.7
Carboxyl	µmol g^{-1}	35.6	59.8	32.0	15.0	12.4
DP_w		1790	4750	1400	2100	1250
DP_n		277	450	460	650	700
PDI		6.5	10.6	3.0	3.2	1.8
DP<100	wt%	9.0	0.5	2.5	2.0	0.3
DP>2000	wt%	26.8	61.0	19.9	35.0	15.5

Pulp purity can be determined by different analyses. One important method used in industry is the *Alkali solubility* (S18, S10) based on the degradation of cellulose and retention of hemicellulose. Pulp is extracted with a NaOH solution of 10%, 18% at 25ºC and dissolved carbohydrates determined by oxidation by potassium dichromate. The 18% caustic lye dissolves the shorter hemicellulose fraction whereas 10% caustic lye dissolves both hemicelluloses and shor chain celluloses. For dissolving pulp quality, it must be high R10, low S10-18 and low S18. (Figure 5)

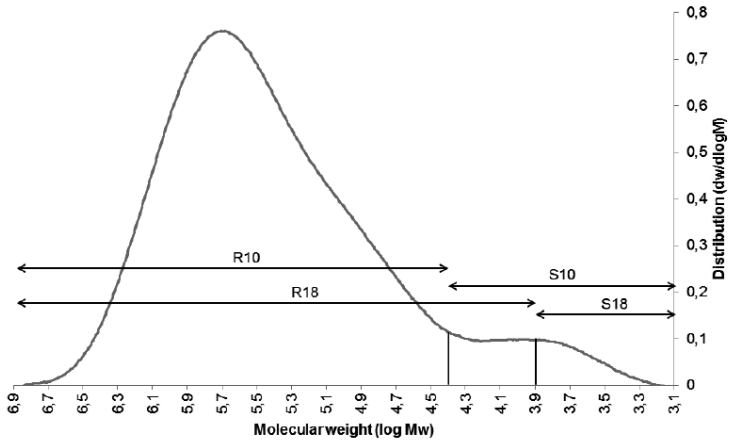


Figure 5 Molecular weight distribution curve and theoretical fractions of alkali resistance[75]

Another closely related and widely used method is the determination of α-, β- and ɣ-cellulose, whereas the α-cellulose indicates cellulose chains with high degree of polymerization (DP), β-cellulose indicates degraded cellulose with DP <200 and ɣ-cellulose consist in CBH with low DP below 10, such as hemicelluloses. For dissolving pulp, the content of β-cellulose and ɣ-cellulose must be as low as possible.

The reactivity of the pulp is another important parameter to consider in manufacture of a dissolving pulp, which determines the accessibility of chemicals to the hydroxyl groups of cellulose. Therefore, the structure and morphology of cellulose will affect the downstream conversion process and final product quality. The contamination from non-reacted cellulose particles is generally undesired, and the chemicals used for the production of regenerated cellulose are highly toxic, e.g. the CS_2 used in the viscose process. One of the most interesting approaches to increase the reactivity of DP, is treatment with cellulose degrading enzymes, cellulases.

1.3 The Conversion process and its applications

Dissolving pulp quality is linked to the conversion process into its end product: regenerated fibers (e.g., viscose, modal and lyocell) or cellulose derivatives (esters: acetates, nitrates and ethers:carboxymethyl, ethyl and methyl celluloses).

The conversion processes may involve steeping of the pulp in aqueous solutions of high NaOH concentration (18–25 wt.%) as in the viscose and carboxymethyl celullose (CMC) manufacture, followed by the addition of appropriate chemicals for subsequent derivatization (e.g., CS_2 for xanthation or alkylhalides for cellulose ethers). On the other hand, the acidic esterification processes can yield cellulose nitrate and cellulose acetate. The latter is a more important conversion process that involves a pretreatment with acetic acid prior to esterification to triacetate on the addition of acetic anhydride and a catalyst, usually sulfuric acid. The triacetate is hydrolyzed to the so called secondary acetate on dilution with water and precipitation of the flakes. These are then dissolved in acetone into a spinning dope from which fibers (e.g. textile filaments), lacquers or plastic are processed. These materials are subsequently used in a wide-range of production processes such as to produce rayon fabrics and tire coring and display screens; photografic films and additives in pharmaceutical and household products such as shampoos and toothpasts, as well as house paints, cigarrette fiters, food casings, and toothbrush fibers.(Appendix 2 – Figure 27 B)

1.4Enzymes applications in P&P

Many biological treatments (e.g., enzymatic and microbial treatments) have been developed for the pulp and paper industry, where the successful implementation of these treatments has become increasingly common. Of the different enzymes and their applications, cellulases has been used for deinking, xylanases have been implemented to improve the mechanical properties of pulps and the refining process, lipases have been used for pitch control, and ligninases, such as laccases or the laccase-mediator system, have been used in the bleaching process along with xylanases. [33] [34] [35]

Many studies have attempted to understand the mechanism of the interaction between the enzyme complex and the cellulose. However, due to the complex nature of the substrate, it is even still difficult to know which factors affect the speed of hydrolysis considering that all samples are different (non-uniform). It must be considered that the original cellulose structure might have been altered by different pretreatments and conditions, the proportions of ordered

and disordered regions vary among species and the different hierarchical levels (i.e., the fibrils and fibril aggregates) are modified during hydrolysis. In order to enhance delignification after kraft cooking, hemicellulases are used to modify the structure of xylan and glucomannan in the pulp fibers. This technology often named enzymatic bleach boosting can be combined with kraft pulping processes and different bleaching sequences and improves product quality. [36]

In order to produce a dissolving pulp from Kraft pulp, it is necessary to achieve several chemical requirements: reduce the content of hemicelluloses, decrease viscosity and increase the reactivity. The enzyme selection is very much based on the targeted substrate within the pulp suspension. The use of enzymes could be an alternative to various non-selective chemical treatments or improve already existed enzymatic processes for production of dissolving pulp.[21]

1.4.1 Cellulases

Cellulases are enzymes involved in the hydrolysis of β-1,4-glycosidic bonds of cellulose. They have been categorized based on their structural properties into three major classes: (a) **endoglucanase (EG)**, which attacks regions of low crystallinity in the cellulose fiber creating free chain ends; (b) **cellobiohydrolase, or exoglucanases (CBH)**, which are able to solubilize amorphous cellulose by hydrolysing cellulose from the free chain ends, producing cellobiose as an end product; and (c) β-**glucosidase or cellobiase (BG)**, which hydrolyzes cellobiose into glucose.

Although cellulose is relatively simple in terms of composition (anhydro-*Glc* units only) and morphology (mainly amorphous and monoclinic Iβ or triclinic Iα crystalline), there is a vast natural diversity of cellulases that act on the cellulose chain either alone or in concert. When they act together, synergy is often obtained towards an efficient degradation of the cellulose structure. Because enzymatic hydrolysis is a heterogeneous reaction and requires direct physical contact between enzyme and substrate, the diffusion of these enzymes towards the cellulosic substrate may be limited by physical barriers, such as lignin, until they adsorb on the substrate surface and then catalyze the hydrolysis. [30 37]

The performance of these enzymes with regard to the degradation of their substrates can be directly related to their size and structure. Their mode of action is in turn at the surface of the

substrate and thus is the main region where the modifications occur. It has been suggested that there are three primary parameters that affect the degree of enzymatic hydrolysis: the crystallinity, the specific surface area and the degree of polymerization of the cellulose [38]

Endoglucanase

The origin of endoglucanases (EGs) is usually distributed among various organisms. The catalytic core fits to several GH families, of which GH 5, 7, 9, 12, 45, and 48 are representative. Although all EGs act on the same substrate (amorphous cellulose), they have different mechanisms("inverting" for GH 6, 9, 45, and 48; "retaining" for GH 5, 7, and 12 (section). Such plurality found in Nature may relate to the different EGs' side-activities on hemicellulose, degrading complex lignocellulose.

The active sites of most EGs are cleft- or groove-shaped, inside which a cellodextrin or a cellulose segment may be bound before the catalytic hydrolysis by the endoglucanase. In addition to the catalytic core, EGs may have Cellulose binding domain (CBD) or other domains but these are not a pre-requisite for their action.

Glucosidase

Unlike CBHs and EGs, BGs, in general, are not modular (lacking distinct CBMs), and have pocket-shaped active sites to act on the non-reducing Glc unit from cellobiose or cellodextrin. [39] β-glucosidases (BG) belong to GH families 1, 3 and 9. Both of them are large families that show broad substrate specificities. Their molecular masses range from 35 to 640 kDa.

The GH family 3 β-D-glucosidases are broad specificity exo-hydrolases that remove single glucosyl residues from the nonreducing ends of oligo- and polysaccharides. The active site is largely independent of substrate conformation and will consequently accommodate a range of substrates. [40]

Non enzymatic proteins involved in cellulose hydrolysis

In addition to the enzymes listed above, novel biomolecules involved in cellulose hydrolysis have been recently identified. Swollenins (SWO) are documented to disrupt cellulosic fibres without showing any hydrolytic activity. Their role is analogous to expansins (EXP), which are thought to promote accessibility to cellulases that depolymerise cellulose fibrils.[40] These proteins are also called cell wall loosening proteins and they appear during the growing of young plant cells.

Swollenin and expansins are non-hydrolytic disruptive proteins. They can act synergistically with xylanases by the disruption of the hemicellulosic fraction of the biomass, enhancing xylan hydrolysis. The role of these proteins is to facilitate the action of the hydrolytic and oxidative enzymes by promoting amorphogenesis of the substrate, thereby opening up the plant cell wall and providing the catalytic enzymes with enhanced access to the glycosidic linkages within the sugar polymers. [41]

The structure of Swollenin includes an N-terminal cellulose-specific carbohydrate binding module (CBM) connected via a linker region to a C-terminal expansin-like domain. Swollenin has been shown to disrupt and swell α-cellulose without producing detectable amounts of reducing sugars. Swollenin acts by separating relatively intact (crystalline) regions of cellulose from each other (i.e. by separating microfibrils). [42] [43] [44]

Expansins are known to promote cell wall extension in plants, likely through a mechanism involving the disruption of hydrogen bonding between adjacent sugar chains. In this way, they are thought to allow the sliding of cellulose fibers and enlargement of the cell wall. Also it is known that the catalytic site of expansin domain 1 proteins share a number of conserved aminoacid residues with the GH45 family proteins. [40] [45]

CIP1 and CIP2 (cellulose induced protein-1 and -2, respectively) both contain a CBM and are co-regulated with cellulases. The function of CIP1 is unknown, though it is claimed that CIP1 has weak activity on p-nitrophenyl β-D-cellobioside and some synergistic activity with both GH61 and swollenin. Further investigation of both functions and the potential of these enzymes in industrial applications are needed. [47]

1.4.2 Hemicellulases

Unlike cellulose, hemicellulose has a more heterogeneous structure that comprises of a linear main chain of β-1,4 linked D-xylose backbone and short side chains of different sugar residues. Complete depolymerisation of hemicellulose fractions, into xylose and other monosaccharide sugars, requires a diverse range of enzymes with diverse modes of action and specificity.

Hemicellulases are enzymes that degrade hemicellulose by the hydrolytic action on glycosidic linkages. [36] Xylans and glucomannans are the most common hemicelluloses in

hardwood and softwood, respectively. Accordingly, **xylanases** and **mannanases** are the main **endoenzymes** needed in hemicellulose depolymerisation.

In addition, the released intermediary ptroducts can be further hydrolysed by **a set of exo-enzymes** (β-xylosidase and β-mannosidase) and **side group cleaving enzymes** (α-arabinosidase, α-galactosidase, α-glucuronidase and esterase), resulting in total polymeric hydrolysis into monomeric end products. [30]

The reaction mechanism seems to be dependent on the size of the enzyme, the accessible surface area of the pulp and topochemical substrate restriction. Furthermore the amount of carbohydrates dissolved by hemicellulases create internal structural changes in the pulp fibers and surface modifications. [48]

Xylanases

Endo-1, 4-β-xylanase enzymes do not cleave the xylan backbone randomly but cleave the glycosidic bonds in a selective manner depending on the chain length, degree of branching of substrate molecules and the presence of its constituents.

The enzymes can be divided into two categories: (i) enzymes degrading the polysaccharide main chain, which are endo-β-1,4-xylanase (EC 3.2.1.8) and β-**xylosidase** (EC 3.2.1.37) (ii) enzymes that release side chains, the main chain substituents, so called **accessory xylanolytic enzymes**, that include α-glucuronidase (EC 3.2.1.139), α-L-arabinofuranosidase (EC 3.2.1.55), acetylxylan esterase (EC 3.1.1.72) and feruloyl esterase. (3.1.1.73) (Figure 6). [49]

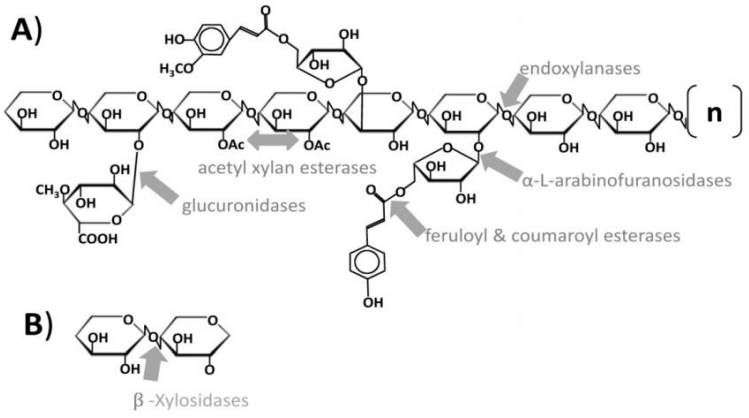


Figure 6 A) Action of Xylanolitic enzymes; B) Hydrolysis by β-xylosidase [50]

Due to the different physico-chemical properties, structure, mode of action and substrate specificity, xylanases have been classified in glycoside hydrolase families 5, 7, 8, 10, 11, 43

in the CAZy [Carbohydrate-Active enZYmes] database, but most of research has focused on families 11 and 10. [51]

In general, the GH10 xylanase family has a high MW (> 30 kDa) and low pI (isoelectric point) value (pH < 6.5), while GH 11 xylanases family have a low molecular mass (< 30 kDa) and high pI value (8.0‑10.0), even if some of the latter have low pI value of fungal origin. Family 11 has exclusive specificity for substrates containing D-xylose. In contrast, the family 5 xylanases possess carboxymethylcellulase activity, while the family 10 xylanases are active on cellulose of low molecular mass. [52] [16] [53]

Families 10 and 11 of endoxylanases essentially differ in their tertiary structure and substrate specificity.

With a molecular mass of approximately 35 kDa and structure of the catalytic domain with an eight -folded barrel, family GH10 xylanases are versatil due to the possibility of various accessory domains that can feature alternative catalytic domains . The substrate binds to the shallow groove on the bottom of the 'bowl'. The (α / β) barrel appears to be the structure of two other endoxylanases of family 10. (Figure 7A) The catalytic site is located at the narrower end, near the C-terminal of the barrel and there are five xylopyranose binding sites. The family GH10 xylanases have higher affinity for shorter linear β-1,4-xylo oligosaccharides comparing with family GH11, which has been proposed to be as a consequence of smaller substrate binding sites. This gives the family GH10 enzymes greater flexibility, but conversely restricts the ability of these enzymes to cleave branched substrates.[54]

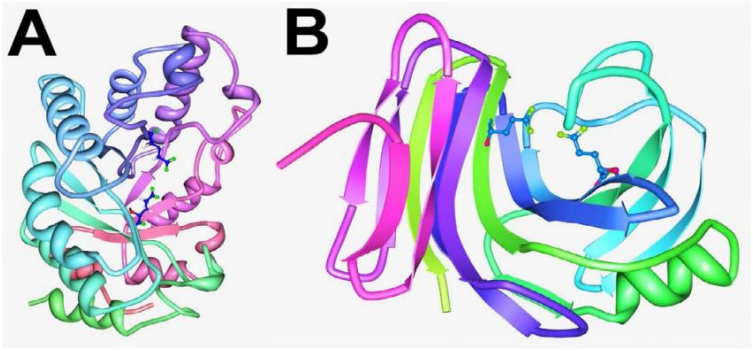


Figure 7 A) Structure of the family xylanase GH10 showing TIM-barrel fold with the catalytic glutamate residues projecting in to the active site cleft. B) Structure of the family xylanase GH11 showing β-jellyroll fold with the catalytic glutamate residues projecting in to the active site cleft [50]

Endoxylanases GH11 are the smallest xylanases with a core structure of 20-25 kDa molecular mass and a β-jelly-roll architecture. (Figure 7 B) Contrary to GH10, xylanases GH11 are highly specific and do not tolerate high substitutions on the xylan backbone. The family 11 endoxylanases are well packed molecules with molecular organisation mainly of β-pleated sheets. The catalytic domain folds into two β sheets (A and B) constituted mostly by antiparrellel β strands and one short α helix, which looks like a partly closed right hand. The catalytic groups present in the cleft accommodate a chain of five to seven xylopyranosyl residues. These xylanases possess 3-5 subsites for binding the xylopyranose rings close to the catalytic site. [55]

Regarding to catalytic properties, it is known that endoxylanases of Family10, in contrast to the members of Family 11, are capable of attacking the glycosidic linkages next to the branch points and towards the non-reducing end, leading to higher proportion of cleavage sites and releasing smaller size products. While endoxylanases of family 10 require two unsubstituted xylopyranosyl residues between the branches, endoxylanases of family 11 require three unsubstituted consecutive xylopyranosyl residues. [54 56]

The thermophilic nature and thermostability, important for pulp and paper applications, may be explained by several factors and structural parameters. For instance, it is believed that xylanases GH11 contain disulphide bridges that confer stability to enzymes.[53 57 49 50]

Xylanases are used in conventional bleaching of paper pulp to reduce the consumption of bleaching chemicals, in particular of chlorine dioxide in elemental chlorine free (ECF) bleaching sequences. In this pulp bleaching area, xylanases are also used to improve the otherwise lower final brightness of the pulp while maintaining savings in bleaching costs. They do not remove lignin-based chromophores directly, but instead degrade the xylan network that traps the residual lignin. [58 30 59]

Xylanases can be applied at different stages of the bleaching process, but most of the applications are within pre-bleaching, i.e. before or after the oxygen delignification. [60]

Mannanases

Endomannanases (M) catalyze the random hydrolysis of β-D-1,4 mannopyranosyl linkages within the main chain of mannans and of various polysaccharides consisting mainly of mannose, such as glucomannans (in hardwood), and galactoglucomannans (in softwood).

Degradation of (galacto)(gluco)mannans, (1→4)-D-mannosyl or manno/glucopyranosyl polymers with variable α(1→6) D-Gal side chain as well as O2 and/or O3 acetylation, can be carried out by mannanase, a group of widely distributed hydrolytic enzymes with catalytic cores belonging to GH5, 26 and 113 families. In addition to a catalytic core, mannanases may possess one or more CBMs (specific to mannan or cellulose) or other domains. Unlike kraft hardwood, softwood is abundant in (galacto)glucomannan. Mannanases in the pulp and paper industry can be used for increasing lignin extractability during the Kraft pulp bleaching in a similar way as with the application of xylanases [64]

1.4.3 Accessory xylanolitic enzymes

The accessory xylanolytic enzymes can be divided into two groups. Enzymes of the first group release side chains only from short branched oligosaccharides generated by endoxylananses. Enzymes of the second group attack both polymeric and oligomeric substrates.

Exo-1,4-β-D-xylosidase (BX) catalyses the hydrolysis of 1,4-β-D-xylo-oligosaccharides by removing successive D-xylose residues from the non-reducing ends of their substrates using the retaining Koshland double-displacement mechanism.

β-xylosidase GH3 are globular proteins with two-domains and are active on a wide range of substrates. The broad substrate specificity is likely caused by the fact that the glucosyl residue occupying binding subsite -1 is tightly locked into a relatively fixed position, whereas the position of the glucosyl residue at subsite +1 is rather flexible. The active site is therefore largely independent of substrate conformation and will consequently accommodate a wide range of substrates.

α-Glucuronidases are enzymes which are able to hydrolyse the α-1,2-linkage between 4-*O*-methylglucuronic/glucuronic acid and xylose. The hydrolysis of the stable α-(1,2)-linkage is often the bottleneck in the enzymatic hydrolysis of xylan, having the reported α-glucuronidases different substrate requirements towards this side-chain hydrolysis. [62]

The purified α-glucuronidases that have been studied are rather large proteins with molecular masses around 100 kDa. Most of the α-glucuronidases studied have been able to act only on 4-*O*-methylglucuronic acid-substituted xylooligosaccharides. Therefore, most α-

glucuronidases need to act synergistically with endo-xylanases in order to liberate substantial amounts of 4-*O*-methylglucuronic acid from xylans. [63]

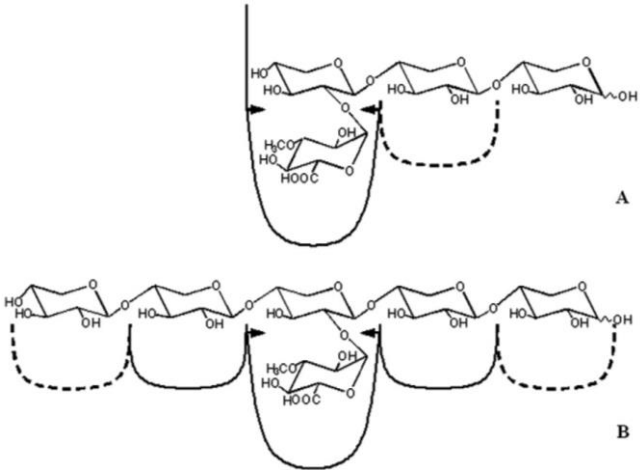


Figure 8 Schematic representation of the substrate binding sites of α-glucuronidase of (A) GH67 and (B) GH115 [61]

α-Glucuronidases assigned to glycoside hydrolase (GH) family GH67 belong to the first group of identified α-glucuronidases. They liberate only uronic acids linked to the non-reducing terminal xylosyl residues of xylooligosaccharides. The members of a recently established family of α-glucuronidases, GH115, differ from the α-glucuronidases of family GH67 by the ability to release MeGlcA linked not only to the non-reducing terminal of xylopyranosyl residues, but also those linked to the internal xylosyl residues. [61](Figure 2)

1.4.4 Esterases

Esterases are commonly used in P&P to break down pitch into smaller particles. However, they may also be important in the hydrolysis of ester-type LCCs. The most alkali labile chemical bonds are ester linkages. Ester linkages can be found between the free carboxy group of uronic acids or as acetyl side groups present in hemicellulose and in between hemicellulose chains. In lignin, ester linkages are present in benzyl groups. Many of these linkages in wood are easily broken by alkali and in particular during the kraft process.

Glucuronoyl esterases (GE) degrade ester LCC-type of bonds between glucuronic acids in xylans and lignin alcohols thereby potentially improving delignification of lignocellulosic biomass when applied in conjunction with other cellulases, hemicellulases and oxidoreductases. [10]

Among esterase families, it can also be found cutinases, which can be more non-specific in terms of substrate. Cutinases catalyse hydrolytic reactions of polyesters of the cuticle and of the suberin layers (protection of plant surfaces) in a wide range of substrates: short-chain soluble esters, water-insoluble medium and long-chain triacylglycerols, polyesters and waxes. [65]

1.4.5 Hydrolysis mechanism of GH enzyme families

Generally, the hydrolysis may result either in the retention or inversion of the anomeric centre of the reducing sugar monomer of the carbohydrate. [66]

Hydrolysis with retention of stereochemistry is achieved via 2 steps, double-displacement catalytic mechanism involving a covalent glycosyl-enzyme intermediate. (Figure 9A) Each step passes through a chemical (oxocarbenium ion-like) transition state. In the first step, glycosylation takes place, with an acid/base and nucleophilic assistance from two amino acid side chains, normally glutamate or aspartate. One catalytic residue plays the role of nucleophilic base by attacking the anomeric center, leading to the formation of a glycosyl enzyme intermediate and induces the departure of the leaving group. At the same time, the other catalytic residue acts as an acid and protonates the substrate (glycosidic oxygen) as the bond leaves.

In the second step, deglycosylation takes place, where the acid/base residue plays the role of a general base, extracts a proton from a water molecule which can then attack the anomeric carbon of the α-glycosyl enzyme intermediate. Then, a second substitution occurs, in which the anomeric carbon gives rise to a product with the β configuration, thus retaining the same initial configuration. [55]

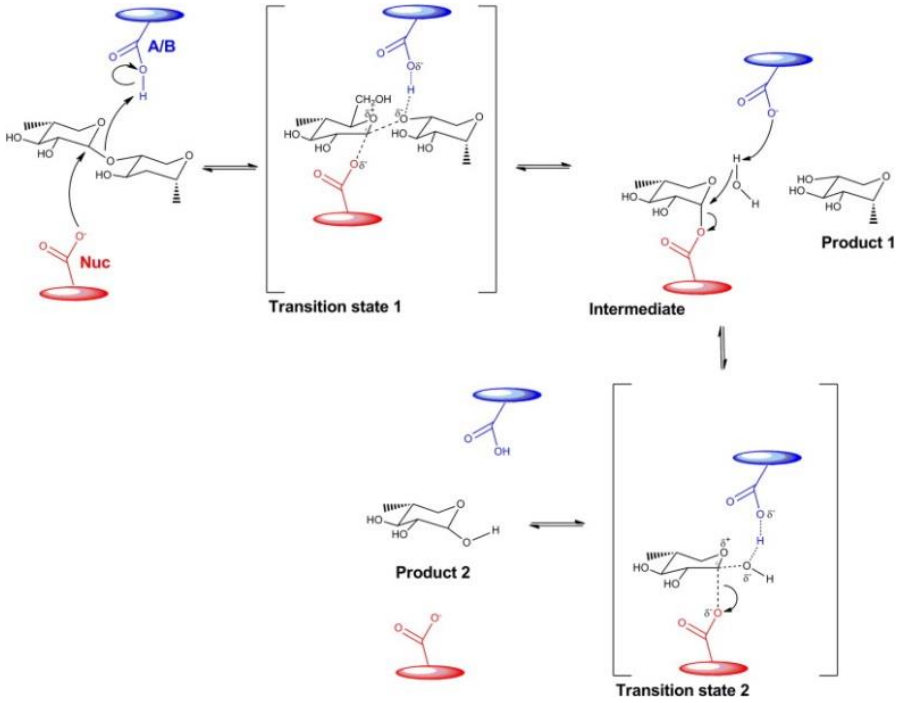


Figure 9 A Catalytic mechanism of GH 11Xylanase. **Nuc**: nucleophile catalytic residue; **A/B** acid/base catalytic residue; [55]

Hydrolysis of a glycoside with net inversion of anomeric configuration is generally achieved via a one step, single-displacement mechanism involving oxocarbenium ion-like transition states. (Figure 9 B) The reaction typically occurs with general acid and general base assistance from two amino acid side chains, normally glutamic or aspartic acids. [66]

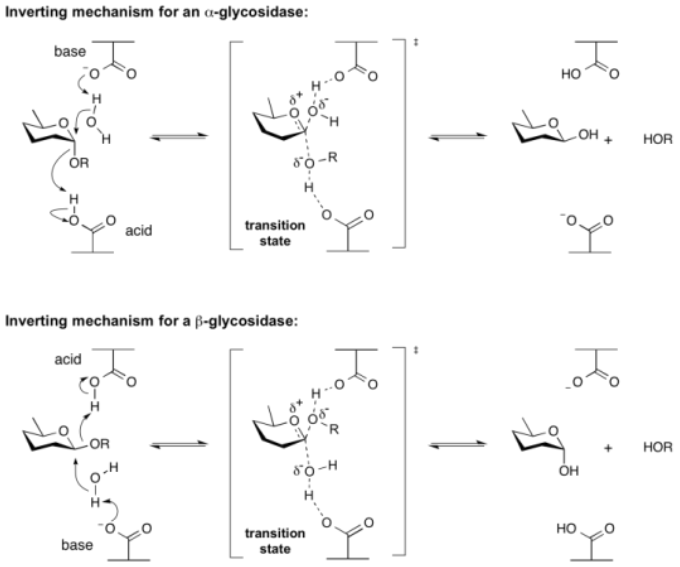


Figure 8 B Inverting mechanism of GH family

2. Motivation and objectives

The world dissolving pulp demand is increasing over the years and this encourages investments in new lines and paper grade producers to convert the existing lines in dissolving pulp.

The kraft cooking process is the first most important treatment used in paper-grade production, thus the viability of producing a dissolving kraft pulp without a pre-hydrolysis step and inclusion of enzymatic stages from conventional kraft cooking has to be studied.

In pulp and paper applications, it is required thermotolerant enzymes that survive in alkaline conditions, thus Tm and pH some of the important parameters for the selection of the candidates. The best xylanases GH11 and GH10 were previously screened in Novozymes Forest Products R&D, using bleached Eucalypt kraft pulp with regard to the amount of reducing and total sugars released.

This can be complemented with exploration of different enzyme auxiliary activities together with a key xylanase towards further removal of hemicelluloses from unbleached eucalypt kraft paper-grade pulp.

Novozymes A/S developed a new sequence based on the elemental chlorine free (ECF) bleaching sequence ($D_0ED_1ED_2$), where the extraction stages were substituted by hot caustic extraction (HCE) and enzymatic stage were added in pre-bleaching and in middle by replacing one chlorine dioxide stage, resulting the following sequence X-D_0-HCE-X-HCE-D_1.

The present dissertation aims at optimizing this bleaching and purification process developed by Novozymes, while converting the paper-grade pulp into a dissolving pulp, as an alternative to the PHK process.

Enzyme application in the pulp and paper industry has been subjected to exhaustive research because the conventional chemicals do not lead to such high selectivity on the substrate and environmental safety issues, as the enzymes. Hydrolytical enzymes, cellulases and xylanases, can enhance the fiber characteristics of both paper-grade and dissolving-grade pulp. However, little is known about the mechanism producing the effects of xylanase and cellulase interactions and the role that other essential enzymes may play thereby. There is a need to evaluate the effectiveness of accessory enzymes within the production of dissolving pulps.

3. Materials and Methods

3.1 Pulp and enzyme samples

A oxygen delignified eucalypt kraft pulp (51,4% ISO brightness; 11,0 kappa number and 18% xylan content) was provided by Forest Products R&D department of Novozymes and used in this study. (Figure 9)

Figure 9 Starting pulp - Oxygen delignified Eucalypt Kraft Pulp

A list of twenty seven samples of enzymes was provided by the Forest Products R&D department of Novozymes A/S. (Table) Two of the xylanases ("X" family GH 11 and X_A family GH 10) were selected within a previous screening made in Novozymes. The cellulosic products tested were a monocomponent product "EG_A" (purified GH45 endoglucanase) and a multicomponent product "EG_B" (a blend of cellulases).

For experiments in medium consistency only the previous enzymes referred (X, X_A, EG_A, EG_B) and the mannanase "M" were tested.

For low consistency experiments all enzymes were included.

Table 2 List of enzymes tested

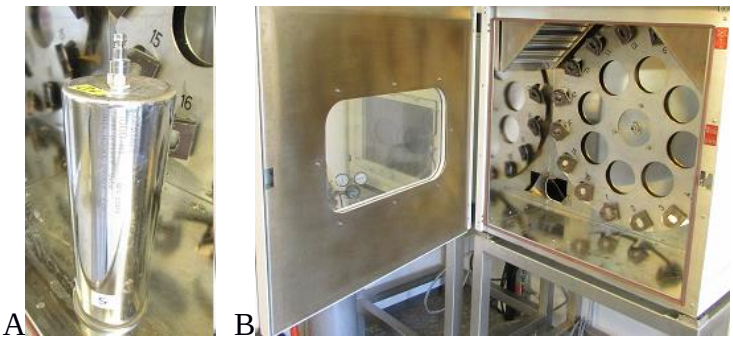
Class	Famiy	Enzyme	**Given ID**	Tm /Key properties/ features
Hemicellulases	GH11	Xylanase 11	**X**	
	GH10	Xylanase 10 A	**X $_{A}$**	89.0 At pH 5
	GH10	Xylanase 10 B	**X $_{B}$**	99.0 At pH 5
	GH10	Xylanase 10 C	**X $_{C}$**	109.0 At pH 5
	GH3	β-Xylosidase	**βX**	78.0 At pH 5
	GH115	α-Glucuronosidase	**α-GRN**	82.0
		Mannanase	**M**	
Cellulases	GH45	EndoGlucanase 45 (mono)	**EG A**	
	Mixture	EndoGlucanase (multi)	**EG B**	
	GH5	EndoGlucanase 5	**EG C**	89.0 At pH 5
	GH44	EndoGlucanase 44	**EG D**	
	GH3	β-Glucosidase	**β-Glc**	
Amylases		Amylase	**AM 1**	
		Amylase thm	**AM 2**	121.0 At pH 6.5
Esterases		Cutinase	**CUT**	87.0
		Esterase ht	**E-ht**	
		Esterase aromatic	**E-at**	70.0 At pH 5
		Esterase methyl gluc	**E-mt**	75.0 At pH 5
		Esterase stk	**E-st**	
		Esterase az 2X	**E-az**	75.6 At pH 7.0
Cell wall loosening proteins		swollenin 1	**Sw 1**	
		swollenin 2	**Sw 2**	
		expansin 1	**Ex 1**	
		expansin 2	**Ex 2**	
		Cip1 a	**Cip I A**	
		Cip1 b	**Cip I B**	
		Cip1 c	**Cip I C**	

Tm, melting temperature

3.2 Chemical treatments at medium consistency

Acidolysis stage (A)

Twenty grams of oxygen delignified eucalypt Kraft pulp (odp basis) were measured in plastic bags and mixed in acidified water at 10% consistency. The pulp was hand-mixed and the pH was adjusted with 2 M H_2SO_4 solution. Four different operating conditions: temperature 95,115°C; pH 2, 3; incubation time 90, 180 min; and pressure (atmospheric, 2 bar) were tested. (Table 2)



A B

Figure 10 Materials for A-stage: A) steel beaker; B) Labomat system

In the atmospheric A-stages, the bags were sealed and placed in water-bath at 95°C for 180 min. In the pressurized A-stages, the pulp was transfered into steel beakers, closed and pressurized with nitrogen (N_2) until 2,0 bar was reached (using a manometer to check the correct pressure). The beakers were introduced in the Labomat system (Werner Mathis AG) at 115°C for 90 min. (Figure 10) After the reaction time, the beakers were cooled and the pressure released. Afterwards, the pH of the filtrate was measured and all pulps were washed three times (3 x 1 L): 1x warm tap water, 1x deionized water and 1x0,001M $NaHCO_3$ for 15min. At the end, the pulp was kept wet in labelled plastic bags at ca. 5ºC in the fridge for the next step.

After the acidolysis stage, the dry matter was measured in order to divide approx. 15g of pulp for the next step (enzyme addition).

Chlorine dioxide (D) stage

The pulps were mixed with acidified water and pH adjusted to 2,5 with sulfuric acid before adding chlorine dioxide solution. The pulps were bleached with 1,10% ClO_2 (in odp basis). The solution was added cold ca. 5°C to avoid evaporation (decomposition) and treatment was performed in sealed plastic bags in water-bath at 80ºC during 1.5 h (10% consistency). After the incubation time, the mixture was washed three times (2x with hot water, 1x deionised

water) and filtered through 3 layers of filters (paper, thick textile, thin textile) in a Büchner funnel under vaccum.

Hot Caustic Extraction (HCE) stage

The pulps were subjected to a hot alkaline extraction at 95°C for 2 hours at a pulp consistency of 10%. Aiming at optimizing the process and facilitating the removal of low weight carbohydrates in the pulps upon purification, excessive charge of NaOH (6% and 12% on a dry pulp basis) was applied. The wet pulp was mixed with distilled water and pre-heated in water-bath before adding NaOH solution. When the temperature attained the set-point, NaOH was added, the pulp suspension was mixed and the sealed plastic bags kept under water for incubation. After the incubation time, the mixture was washed with hot water and filtered through 3 layers of filters (paper, thick textile, thin textile) in a Büchner funnel until the pH fall to neutral. The pulps treated with 12% NaOH required more washing due to the higher dosage of soda.

Cold Caustic Extraction (CCE) stage

The pulps were subjected to a cold alkaline extraction at 35°C for 30 min at a pulp consistency of 10%. The experiment was carried out in plastic bags and the amount of soda was calculated to have a final concentration of 40 g NaOH/L and 80 g NaOH/L in the liquid phase.

The wet pulp was mixed with distilled water and pre-heated in water-bath before adding NaOH solution. When the pulp temperature attains the set-point, NaOH was added, the pulp was mixed and the sealed bags were kept under water for incubation.

After the incubation time, the highly swollen pulp was washed with room temperature water and filtered through 3 layers of filters (paper, thick textil, thin textil) in a Büchner funnel. In the end the pulp was neutralized by adding room temperature water until ca. 5% consistency and pH adjusted with sulfuric acid until below 5.

3.3 *Enzymatic treatments at medium consistency*

Experimental xylanases X (11) and X A (10) were selected from a previous screening made in Novozymes of 68 xylanases. An experimental mannanase (M) and commercial endoglucanases (EG) were also tested along with xylanases, including a monocomponent EG_A (GH45) and multicomponent product EG_B (cellulase cocktail).

Figure 11 Pulp with enzymatic treatment

The enzymatic treatments were performed at pH 4.5 and pH 6.0 at 10% pulp consistency. The enzyme dosage was calculated in odp basis (mg EP/Kg odp) and in liquid phase (mg EP/mL).

Sodium acetate buffer (50 mM; pH 4.5) and phosphate buffer (50 mM, pH 6) were used. To carry out the treatment, the buffer was added to the pulp and pre-heated before adding the enzyme. After pre-heating the pulp with buffer, the enzyme was added into the liquid phase and mixed thoroughly in order to obtain a homogeneous mixture. The treatment was performed in sealed plastic bags, which were immersed in a water bath during 4 hours. To obtain a uniform distribution, the pulps were mixed after 2 hours in a paint-shaker for 2 min. The pulp was finally washed three times (2x with hot water, 1x deionised water) and filtered through 3 layers of filters (paper, thick textile, thin textile) in a Büchner funnel under vaccum.

3.4 Screening of Enzymes at low pulp consistency

The screening of enzymes was done at 1% consistency, using a partially bleached O-X_1-D_0-HCE Eucalypt kraft pulp (Figure 12), with ca. 10% residual xylan content and was performed in two steps: one enzymatic treatment, followed by an alkaline treatment at low dosage. It was supposed to be a hot caustic extraction at 6% dosage, but a mistake occurred in calculations of the sodium hydroxide to apply and the final dosage applied was more diluted (10 times) than it was expected.

The performance of enzymes on xylan removal was analysed at three different temperatures: 50, 75 and 90°C, in several combinations. The temperatures were selected according to the melting temperature (Tm), a common “rule of thumb” is to use a temperature at least -5°C below Tm.

For the experiments with cell wall loosening proteins (SWO,EXP and CIP1), the pulp was incubated overnight with these proteins.

Pre-heated acetate buffer (50 mM; pH 4.5), a steel ball and additional solutions were added to 0,50 g of pulp in 50 mL centrifuge tubes and placed into a thermomixer for pre-heating before adding the enzyme. To ensure a good performance of the enzymes Esterase ht, Esterase az and Esterase st, 1 mL of calcium (CaCO3 0,001 M) was added as co-factor.

The enzymes were previously diluted before addition to the pulp in the required dosage. After diluting the pulp fibers with buffer and after pre-heating, the enzyme was dosed into the liquid phase; the tubes were closed and hand-mixed to obtain a homogeneous mixture. The tubes were then introduced inside steel beakers and filled with hot water which was then placed in a Launder-Ometer® for an incubation time of 4 hours. After the incubation time, the tubes were placed in ice to stop the reaction and 1 mL of the supernatant was filtered (with lur-loc syringes coupled with 0.45 µm filter) and collected into Eppendorf's, which were then frozen for later analysis. The pulp was washed three times (2x with hot water, 1x deionized water) and filtered through 2 layers of filters (thin textile) in a system of suction funnels under vacuum. One sample of 0,25 g odp was washed pulp and taken for sugar analysis.

The remaining wet pulp (equivalent to 0,25 g odp) was subjected to an alkaline treatment at 85ºC for 1,5 hours, using the same instruments as the enzymatic screening procedure. Instead of buffer, pre-heated deionized water was added to dilute pulp fibers. The tubes with pulp and a steel ball were placed in thermomixer before adding sodium hydroxide. When the temperature reaches the set-point, 1 mL of NaOH solution was added to the target dosage. The tubes were capped tightly, hand-shaked a few times and placed in the Launder-Ometer® beakers for the incubation time pre-defined (90 min). After the incubation, the pulp was washed until reaching a neutral pH and collected for later sugar analysis.

3.5 Pulp characterization

Determination of carbonyl groups

Carbonyl groups, such as keto or aldehyde, in sugar units of polyssacharides increase the reactivity of the carbohydrate ring. Such groups can be generated upon enzymatic treatment. Thus aldehyde groups were quantified in order to observe the main differences between enzymatic treatments of the same family of enzymes, xylanases GH10.

The method is based on the ability of aldehyde groups to reduce 2,3,5 triphenyl-2H-tetrazoliym chloride (TTC) with formation of red colorant formazan that can be determined by spectrophotometry. The generation of formazan is quantified at 546 nm and is

proportional to the amount of aldehyde groups in the pulp, expressed as mmol COH per kg of odp (oven-dried pulp). The concentration of aldehydes groups in an unknown sample can be predicted from a standard curve constructed using glucose standards.

Structural carbohydrates composition

The analysis of monossacharides composition, requires their isolation from the pulp material in a pure state and its determination involves cleavage of glycosidic bonds and a subsequent analysis by a chromatographic technique. The glycosidic bonds are cleaved by hydrolysis and the liberated monossacharides are usually separated by gas chromatography (GC), high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE). [67]

In this work, the monosaccharide composition of the pulps was determined according to an analytical procedure of a national laboratory of the US (NREL) with a few adaptations. This procedure is based on a two-step acid hydrolysis to fractionate the polymeric carbohydrates into monomers to be quantified by HPLC. [68] The samples were previously dried in an oven at 45°C between 8-12 hours and then grinded. The moisture present in the oven dried samples (odp) was measured before weighing. The experiment was done using 30 mg of pulp and a commercial dissolving pulp was always used as a reference.

Thirty mg odp were transferred into glass screw-top tubes in duplicate. Six glass beads were added in each tube and 0,30 mL of 72% H_2SO_4 was then added. The tubes were kept in water-bath at 30°C for 1 hour and every 10 minutes the tubes were mixed using a vortex.

A set of sugar recovery standards (SRS) were included in the second hydrolysis in order to estimate the extent of monosaccharide degradation. These standards were prepared as follows: 3,0 mg/mL of D-(+)glucose, 1,0 mg/mL of D-(+)xylose, 0,3 mg/mL of D-(+)galactose, 0,3 mg/mL of L-(+)arabinose and 0,5 mg/mL of D-(+)mannose. For each analysis, 2 mL of the prepared SRS were transferred into glass screw-top tubes in duplicates. Then, 69,6 µL of 72% H_2SO_4 was added for the remaining hydrolysis. Likewise, for SRS without hydrolysis, 2 mL of SRS were diluted with 69,6 µL of MilliQ-water.

The hydrolyzed pulp samples were then diluted to 4% H_2SO_4 by adding 8,4 mL water and followed a second hydrolysis along with the prepared SRSs at 121°C during 1 hour in an autoclave. Afterwards, the hydrolysates were filtered through a 0.45 µm filter before separation and quantification.

A set of eight calibration standards (1,0; 1,5; 2,5; 5,0; 7,5; 10,0; 20,0; 30,0 mg/L) were prepared from a stock-solution (0,1 g/L) composed by L-(+)arabinose, D-(+)galactose, D-(+)glucose, D-(+)xylose and D-(+)mannose.

Calibration standards and hydrolyzates were quantified by HPAE-PAD (ICS-5000+) Dionex system, Thermo Fisher Scientific with a electromechanical detector and with a CarboPac SA 10-4 µm), under the following conditions: injection volume of 2,5 µL, at a flow rate of 0,380 mL/min, with pressure limits of 200-5000psi; column temperature 45°C; compartment (detector) temperature 25°C and elution carried out using 0.2 µm filtered water, with a run time of 9 minutes. The data was processed using Chromeleon software (version 7).

The quantification of monosaccharides was determined from the calibration curve in terms of the amount (mg/L) of each component present in the samples.

In order to calculate the corrected concentration of detected sugars for each hydrolyzed sample (Corrected concentration), it is necessary to take into account the average recovery of a specific SRS component. For this, the following equations (1-6) were used.

Equation 1 Recovery of SRS after acid hydrolysis

$$Sugar\ Recovery\ (\%) = \frac{SRS\ (mg/L)}{SRS\ without\ hydrolysis\ \ (mg/L)} \times 100$$

Equation 2 Corrected concentration with sugar recovery standards (SRS)

$$Corrected\ SC\ (mg/L) = \frac{DSC\ (\frac{mg}{L})}{Sugar\ Recovery\ (\%)} \times\ DF$$

DSC: Detected sugar concentration in hydrolysates from pulp samples

DF Dilution Factor

Equation 3 Calculate the polymeric sugar concentration

$$PSC(\frac{mg}{L}) = Corrected\ SC\ (\frac{mg}{L}) \times \frac{MW\ sugar - water}{MWsugar}$$

PSC=Polymeric Sugar Concentration

MW: Molecular weight

Equation 4Calculate the percentage of each sugar on ODP basis

$$Sugar\ = \frac{Polymeric\ sugar\ concentration(\frac{mg}{L})}{Sample\ weigh\ (mg)} \times Volume\ of\ hydrolysis(mL)$$

Equation 5 Volume of hydrolysis

$$mH2SO4\ at\ 72\%(g) = Volume\ added(mL) \times \rho\ H2SO4\ at\ 72\% \left(\frac{g}{mL}\right) = 0.30mL\ \times \frac{1{,}64g}{mL} =\ 0.49g$$

$$mH2O = Volume\ added\ (mL) \times \rho H2O \left(\frac{g}{mL}\right) = 8{,}4 \times 1{,}0000 = 8{,}4g$$

$$Volume\ of\ hydrolysis = m(H2SO4 + mH2O) \times \rho H2SO4\ at\ 4\% = (0{,}49 + 8{,}4) \times 1{,}025 = 8{,}673\ mL$$

Equation 6 Percentage of each sugar detected

$$Sugar\ or\ Xylan\ (\%) = \frac{Average\ of\ each\ sugar}{Sum\ of\ all\ sugars} \times 100$$

Viscosity measurements on pulp

Viscosity, which is an indirect measure of the cellulose chain length, was determined in accordance with TAPPI Standard T 230 om-94 "Viscosity of Pulp (capillary viscometer method)." The moisture content was determined for air-dried pulp and was used to weigh 0.2500 g odp. The weighed pulp was dissolved in distilled water, solvated with cupriethylenediamine and passed through a capillary viscometer at 25°C. The efflux time was measured twice for each sample and converted to viscosity as mPa.s or centipascal (cP).

Handsheets for Brightness measurements

The pulp handsheets were made based on the ISO standard 3688:1999 ("Pulps — Preparation of laboratory sheets for the measurement of diffuse blue reflectance factor (ISO brightness)").

Handsheets of 200 g/m^2 of basis weight were prepared: e.g. 4 g odp pulp was suspended with 1000 mL of de-ionized water and disintegrated with 10 000 rotations. The pH of the suspension was adjusted to 5,0 with solutions of 0,1 M of NaOH and 0,05 M of H2SO4. Then, the suspension was well mixed and divided into two equal portions.

The next step consisted in a vacuum filtration of the suspension in a Buchner funnel with around 150 mm of diameter and a thick filter paper. The sheet was removed from the funnel and placed in a blotter paper. Then the handsheets prepared were pressed during 80 sec. with a pressure around 300 kPa in the sheet press with the following sequence: metallic plate, 2

blotter paper, blotter paper with the sheet covered by the filter paper, 2 blotter paper, metallic plate and so on. The sequence was disassembled and the filter paper was detached and placed on top of the pulp handsheet in order to protect it from dust. The handsheets were placed on the metallic plate with metal rings and dried in a climate room for 20h, with controlled temperature and humidity (22°C and 50%), during at least 3-4 h until a moisture of 5-15% was reached. The sheets were pressed again for 50 sec, with a pressure between 300-500 kPa and dried for 1 h in the climate room (the total drying time not exceeded 24 h).

The ISO Brightness of the sheets was determined with the Technidyne Color Touch spectrophotometer using the built-in program "Brightness L*a*b Whiteness AVG". The procedure consisted of loading a pad of sheets into the machine, by gently pulling down the arm of the equipment and then releasing it to press the sample. Four readings were made on different points of the filter side of each pulp handsheet produced.

4. Results and discussion

This chapter is organized by the order of experiments performed: starting with quantification of aldehyde groups in pulps for the selection of enzymes, followed by a brief description of the pulp treatments along with its chemical analysis (monossacharides composition) and finally the physical properties measured: ISO brightness, viscosity and reactivity.

Quantification of aldehyde groups

The best xylanases GH11 and GH10 were selected from a previous screening made in Novozymes Forest Products R&D, with regard to the amount of reducing sugars released in filtrate.

Another parameter to take into account in the selection of enzymes is the carbonyl groups that can be generated in pulp upon enzymatic treatment. Thus, the aldehyde groups were quantified in pulp treated with each of the thirty four xylanases GH10 tested. The results were compared with the amount of reducing sugars previously measured in Novozymes. (Appendix 1) Enzymes of the same family respond differently in the treated pulps: some enzymes generated a high number of aldehyde groups (more linkages cleaved) and released more reducing sugars, others enzymes almost did not produce a significant effect in pulps. For the purpose of this study the enzyme 33 (corresponding to xylanase X_A in Table 1) was the selected one due to the highest values recorded of reducing and total sugars released. The same method was applied for the xylanases GH11.

4.1Pulp Bleaching and purification

Several experiments were tested using different enzymes and combinations of stages, either enzymatic or chemical for the optimization of the new sequence X-D_0-HCE-X-HCE-D_1 created by Novozymes.

The following scheme represents the experimental design for this optimization: the boxes contain a number of stages and a specific color representative of the enzymatic stage "-X-" applied. (Figure 123) The pulps were analyzed for monosaccharide composition in the last stage of each box and for each analysis performed the starting pulp (SP) and a commercial dissolving pulp (DP) were used to calculate the xylan removal or the target to dissolving pulp.

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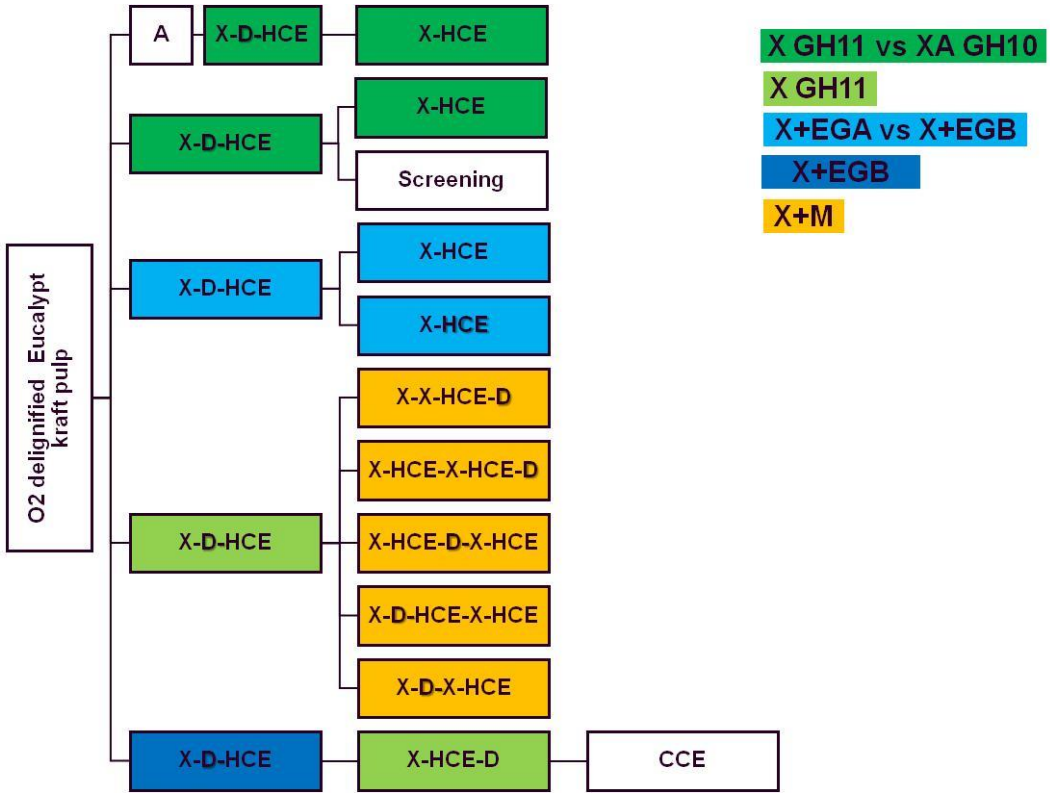


Figure 12 Schematic representation of the bleaching sequences tested

The first assumption was to introduce a hot acid stage (A) in pre-bleaching as an acidolysis in order to possibly cleave some of the more resistant ether linkages and make the lignin more accessible to chemical reactions during bleaching. Here, the best xylanases GH11 "X" *vs* GH10 "X_A" previously selected, were applied separately. (Figure 13, dark green boxes) The same sequence without hot acid stage was also tested. To achieve higher degree of purification of pulp and optimize the NaOH dosage, the hot caustic extraction (HCE) stages were tested at: 6% and 12% odp. The following experiments 6% NaOH was used.

The second assumption uses the combination of the xylanase GH11 with two cellulytic products (endoglucanases based) which are known to attack cellulose regions of low cristallinity. This selective action on the surface of the fibers was used as a hypothesis towards allowing the xylanase to act in deeper layers of the fiber wall and achieve higher xylan removal. Two different products were used: a mono-component endoglucanase GH45 "EG_A" and a multi-component product "EG_B", a blend of cellulases. Thus, the enzymatic combination of xylanase GH11 with endoglucanases "X+EG_A" and "X+EG_B" was tested within the sequence X_1-D_0-HCE-X_2-HCE. (Figure 13, light blue boxes)

The third assumption was to test three enzymatic treatments to reduce xylan. A partially bleached pulp (X_1-D_0-HCE with xylanase GH11 treatment) was subjected to five sequences with different order of enzymatic and chemical treatments, where xylanase GH11 and mannanase were equally combined in the second and third X-stage (X_2=X_3). (Figure 13, yellow boxes) The order of treatments changed but the conditions applied remained the same. The resulted pulps were analyzed for monosaccharide composition, viscosity and brightness at the end of sequence.

The next approach was to evaluate accessory enzymes with the key-xylanase GH11 through a screening experiment in X_2 at low consistency, in order to save resources and with the main goal of accessing the most resistant xylans. Though, xylanase was tested together with cellulases, amylases, esterases, cell wall loosening proteins and so-called accessory enzymes including β-D-xylosidases and α-glucuronidases. Several attempts were pursued to find an enzymatic cocktail that could boost the removal of xylan.

The last assumption was to access this resistant xylan through a cold caustic extraction (CCE) step, in post-bleaching. This CCE treatment mainly involves physical changes in the pulp substrate (preservation of pulp yield). Generally, higher alkali concentration and lower temperatures increase the degree of cellulose swelling and improve the extraction efficiency, which enhances the solubility of residual xylan in pulp. Thus for this experiment, a bleached pulp (X_1-D_0-HCE-X_2-HCE, where X_1="X+EG_B" a xylanase with endoglucanase treatment and X_2 ="X" xylanase GH11 treatment) was used. The CCE stage was applied at two dosages: 40 NaOH g /L and 80g/L in the liquid phase of the pulp suspension, resulting the following sequence: X_1-D_0-HCE-X_2-HCE-**D_1-CCE.** The resulted pulps were analyzed at the end of the sequence for monosaccharide composition, viscosity, brightness and reactivity.

The following sections of this chapter, discuss the results according to the effect of chemical and enzymatic treatments produced in pulps.

4.1.1Effect of a prior acidolysis treatment

The oxygen delignified kraft eucalypt pulp with 18% xylan content was submitted to a hot acid stage at four different conditions. (Table 3)

Table 3 Operating conditions of the A-stage

Operating Conditions	A1	A2	A3	A4
Temperature (°C)	95	95	115	115
pH	3	2	3	2
Incubation time (min)	180	180	90	90
Pressure (bar)	Atm	atm	2	2

To understand the effect of the A stage, it is important to discuss the analysis made on pulp in the middle of the sequence: A-X_1-D_0-HCE. When analyzing the results vertically in Table 4, it is clearly seen the effect of the A-stage: as more harsh conditions (from A1 to A4) of the A-stage applied, higher activation of lignin occours though hydrolysis of ether linkages, which then form new free phenolic hydroxyl groups to lower the residual xylan content. The highest decrease was observed on pulp controls (from 17,2% to 13,7% and from 16,6% to 13,3%) while the pulps treated with enzymes "X" and "X_A" show smaller variations, because the enzymes already removed part of the xylans linked to lignin.

However the A-stage is non selective which means that besides hemicellulose degradation, part of cellulose is also degraded, because the residual lignin tend to link to carbohydrate chains (cellulose or hemicellulose) by ether bonds that are labile to acid hydrolysis. It would be better to have a more selective approach, where the cellulose is not so affected. Thus, the acidolysis in pre-bleaching was an approach discarded for the optimization of the process.

4.1.2 Optimization of Alkali dosage

The hot caustic extraction (HCE) is a more selective way than acydolysis towards hemicellulose removal, thus when analyzing the results in Table 4 horizontally, it is possible to compare the differences among treatments at low 6% and high 12% dosage in HCE. Pulps treated with low dosage achieved 7% residual xylan. For more harsh and selective treatments at high dosage, the pulp afterwards became more difficult to be washed (achieve a neutral pH) and floor level of xylan also remained the same 7%. This means that for the purification

of pulps through HCE, the lower alkali dosage should be used for the next experiments to save in chemicals. However the treatment was not tested at lower charge than 6% and maybe it is possible to achieve the same levels of residual xylan, for example at 3%.

Table 4 Residual xylan content (%) on pulps analyzed in the middle and at the end of sequences: X_1-D_0-HCE*-X_2-HCE* and A-X_1-D_0-HCE*-X_2-HCE*. Starting pulp with 18% xylan content.

		(A)-X_1-D_0-HCE		X_2-HCE	
NaOH dosage in HCE (% odp)		6	12	6	12
No A	Control	**17,2**	**16,6**	**15,9**	**15,7**
	GH 11 X	10,2	9,5	7,1	6,7
	GH 10 X_A	11,6	11,2	8,7	8,5
A1	Control	15,8	17,0	13,6	8,8
	GH 11 X	11,4	11,8	8,6	6,5
	GH 10 X_A	12,8	12,5	10,2	6,6
A2	Control	15,2	15,2	12,7	6,6
	GH 11 X	10,9	11,0	7,4	7,1
	GH 10 X_A	11,8	11,8	8,4	6,0
A3	Control	15,1	15,4	13,0	**6,0**
	GH 11 X	11,1	10,1	7,7	6,1
	GH 10 X_A	12,4	11,9	8,7	9,5
A4	Control	**13,7**	**13,3**	**11,2**	6,3
	GH 11 X	10,4	10,2	7,0	6,6
	GH 10 X_A	11,2	10,8	8,0	6,5
Dissolving pulp		4,5			

4.1.3 Xylanases and endoglucanases in the bleaching sequence

The effectiveness of xylanases of different families, "X" GH11 and "X_A" GH10 was clearly observed. The results in figure 14 showed that the xylanase GH11 was more effective than GH10, either after one enzymatic stage or two enzymatic stages. There are many factors that affect the performance of enzymes, but here what matters are the differences between the structure and substrate specificity. Xylanase GH10 with affinity for shorter oligossacharides, they attack the glycosidic linkages next to the branch points and towards the non-reducing ends, leading to higher proportion of cleavage sites and releasing smaller size products. These are versatile enzymes because they can feature alternative catalytic domains. Contrary, xylanase GH11 are highly specific on the xylan backbone because the catalytic chain contains more subsites for binding the D-xylose. Besides that family GH11 has a lower MW (<30 kDa) which theoretically allows the enzyme to act on substrate localized in deeper layers of the fibers. After all, both enzymes were very efficient when comparing with the control pulps (where no enzyme was applied): after two enzymatic stages, xylanase GH11 "X" was able to decrease 55% of xylan content, while xylanase GH10 "X_A" reduced 45%.

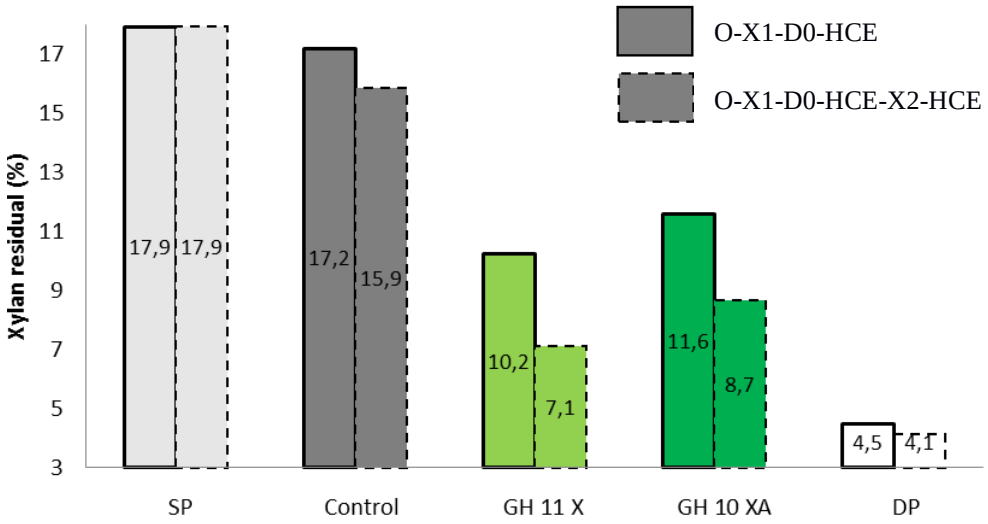


Figure 13 Residual xylan content (%) of pulps analysed in the middle and at the end of the sequence: X_1-D_0-HCE*-X_2-HCE*, with xylanases GH11 and GH10 treatment.

SP – Starting pulp (O2-delignifyed Eucalypt Kraft Pulp); DP – Dissolving pulp; HCE – Hot caustic extraction with 6% NaOH.

However, the target removal of xylan to achieve a commercial dissolving pulp should be higher than 68%. Thus the xylanase GH11 "X" was combined with endoglucanase to act the amorphous regions of cellulose and lead the xylanase to act on inaccessible linkages with xylan.

These EGs are cellulosic products not as tolerant to high temperatures and low pH as the xylanases, so the experiments were carried out at 60°C and pH 6. Besides that, these products are known as very effective and were used in lower dosages.

It must be pointed out that the same enzymatic treatment of Xylanase GH11 at lower temperature, lead to -1 residual xylan unit at end of the sequence, when compared with the previous experiment. (Figure 14) This observation permits to conclude that the "X" GH11 has a better performance at 75°C pH 4.5 than at 60°C pH 6.

The effect of endoglucanases action was not obvious at this point. After two enzymatic treatments with xylanase alone or combined with endoglucanases, the pulps still contain ca. 8% xylan. (Figure 14) The hypothetical "additive effect" of EGs in the degradation of xylan, didn´t have the expected decrease, therefore the minimal differences leads to conclude that the residual xylan is not entrapped between the amorphous cellulose.

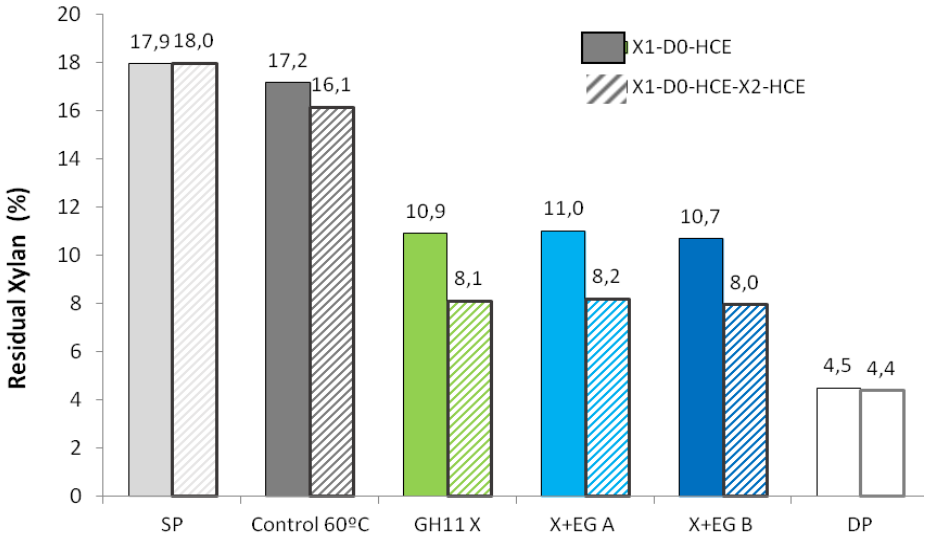


Figure 14 Residual xylan (%) of pulp in middle and end of the sequence X_1-D_0-HCE-X_2-HCE after HCE, using enzymatic combination of xylanase GH11 with endoglucanases "X+EG_A" and "X+EG_B". Endoglucanase monocomponent GH45 "EG_A"and multicomponent endoglucanase "EG_B".

4.1.4 Effect of three X-stages

The previous reference of a pulp treated with xylanase GH11 resulted in 7,1% residual xylan. (Figure 14) Having this value as a minimum achieved within a sequence O-X_1-D_0-HCE-X_2-HCE, the application of a third enzymatic stage was studied to pursue further improvement on the extent of xylan removal. A partially bleached pulp (X_1-D_0-HCE, where X1= Xylanase GH11 "X" at 60°C) with ca. 11% residual xylan and a control pulp (without enzyme) ca. 17% residual xylan, were subjected to five combinations of enzymatic and chemical treatments. The preceding monossacharides composition analysis resulted solely in glucose and xylose residues. Hardwood pulp contains very low amounts of mannan and mannose was not detected until now. It would make more sense to use mannanase for softwood pulp, however for these experiments mannanase combined with xylanase were also applied in the following stages.

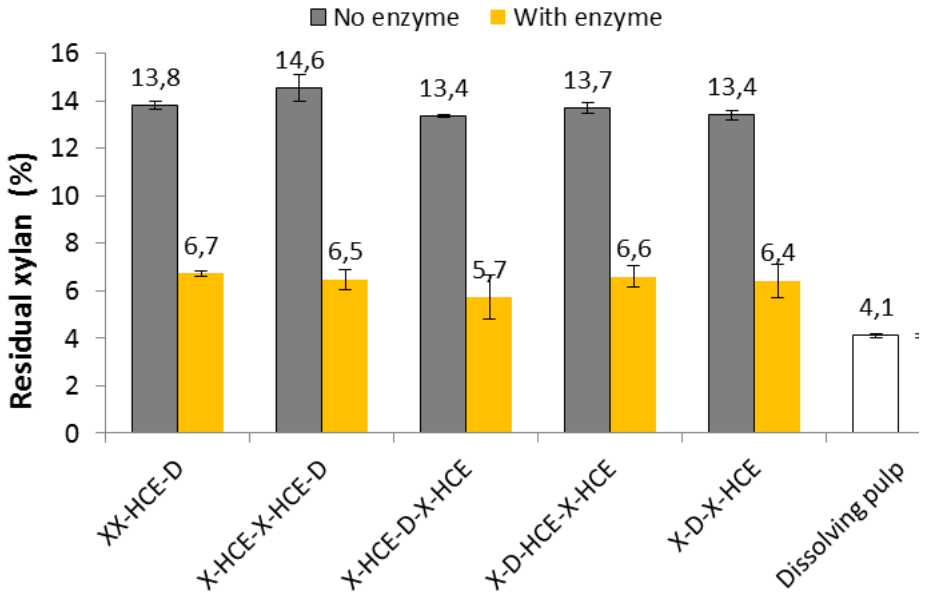


Figure 15 Residual xylan of pulp (%) resulted by combined bleaching sequences

Obviously these type of sequences are too long and not applicable in industry but the experiment led to understand that the order of treatments is important. From a chemistry point of view, HCE should be placed before any oxidative bleaching stage, as the efficiency of purification is reduced as soon as the aldehyde groups are oxidized to carboxyl groups.[31] The depicted figure 16 confirm that the order and number of treatments applied, slightly

influenced the residual xylan in pulp and the application of the 3rd enzymatic stage, only further reduce 1 unit, with 5,7% minimum achieved.

4.1.5 Screening of enzymes (low consistency)

It was not a matter of increasing the number of enzymatic stages, but it could be a matter of combining other enzymes to act differently on the substrate and make it more accessible.

The partially X_1-D-HCE bleached pulp studied in these screening experiments had already a xylanase treatment (X_1) that removed **55%** of xylan (corresponding to ca. 10% of residual xylan) and therefore the substrate used had a reduced amount of recalcitrant xylan.

The performance of auxiliary activities together with the best xylanase GH11 was studied in small scale experiments at 1% consistency aiming at selecting potential candidates able to boost the removal of the remaining xylan from the eucalypt kraft pulp. The screening experiments were divided in four parts comparing a range of temperatures (50-90°C) and dosages. (Table 5)

Table 5 Operating conditions of the screening experiments

Screening experiments	**Operating Conditions**		
	Temp (°C)	Dosage X_2-stage (mg EP/mL)	
1	75	0.002	
2	75	0.002	0,001
3	90	0.002	0,001
4	50	0.002	

The average amount of remaining xylan in the pulp (%) after the enzymatic stage (X_2) and after HCE in pulp is presented in Figures 17, 18, 19 and 20. The red line is the limit from which, any result bellow is considered an improvement.

In the experiment 1 and 2 the conditions were selected according to the enzymes tested, which of those were active at high temperatures. The aim of experiment 1 was to select one enzyme of each family tested. Regarding to amylases (AM1, AM2) tested at 75°C, only AM1 demonstrate an additional effect to Xylanase GH11 "X" but not to a significant extent. Regarding the α-glucuronidase GH115 (αGRN) enzymes, which are believed to act synergistically with xylanases in the full hydrolysis of xylan, releasing MeGlcA linked to the

non-reducing terminal and internal xylose residues, they did not show any improvements. This can be either linked to substrate specificity or poor availability of such groups on the surface of the fibers.

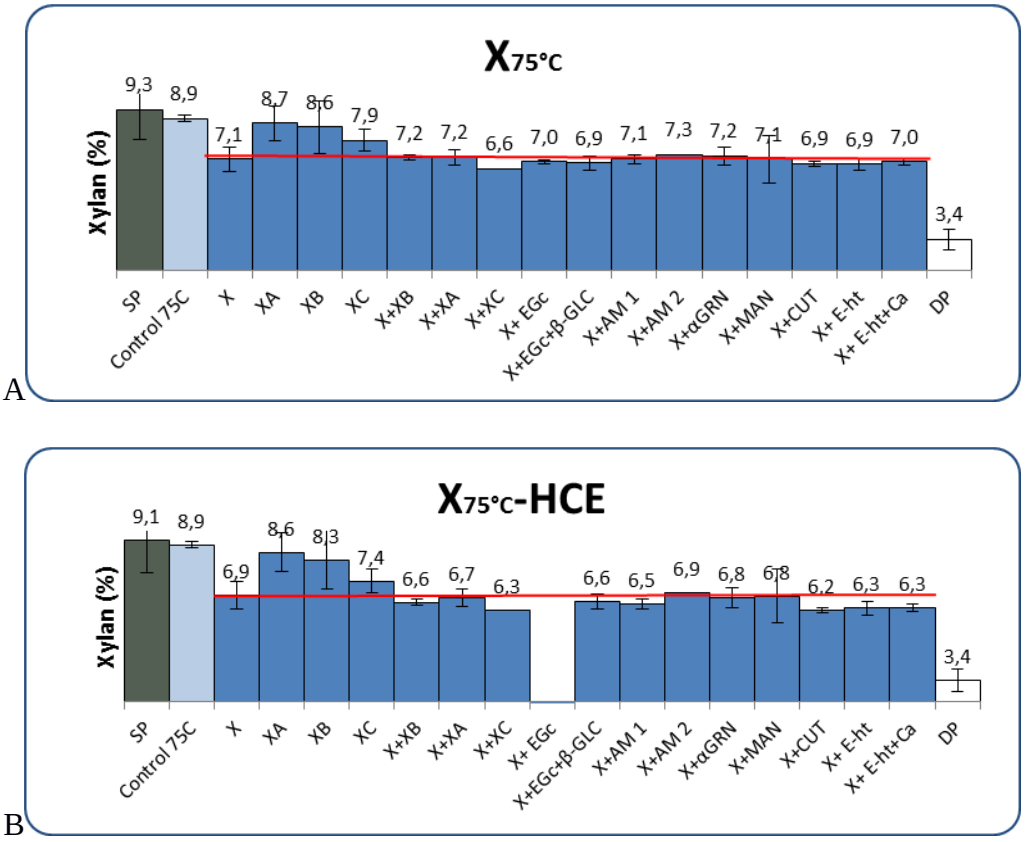
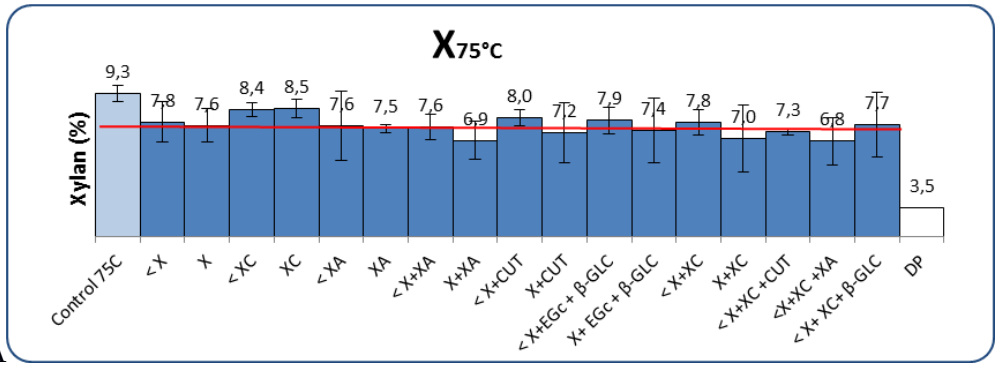


Figure 16 Experiment 1 at 75°C of various enzymes composed by X_2-stage at 75°C followed by HCE-stage at 6%NaOH; A) Sugar analysis after X_2-stage; B) Sugar analysis after HCE-stage;

SP: Starting pulp OX_1D-HCE-; **Control 75°C:** without Enzyme; **X:** Xylanase GH11; **X_A:** Xylanase A GH10; **X_B**: Xylanase B GH10; **X_C:** Xylanase C GH10; **EGc:** Endoglucanase GH5; **β-GLC:** β-Glucosidase GH3; **AM1:** Amylase; **AM2:** Amylase thm; **α-GLC:** α-Glucuronidase GH115; **MAN:** Mannanase; **CUT:** Cutinase; **E-ht:** Esterase-ht; **Eht+Ca:** Esterase ht+ CaCO3; **DP:** Dissolving pulp; **RED LINE**: result of Xylanase GH11 treatment alone;

Figure 18 (experiment 2) shows few combinations of enzymes (X+ XA; XC; XEGc+β-Glc; AM2; CUT; E-ht) that were selected from the previous experiment 1, to study the protein concentration effect. The purpose was to analyze if there is some synergy effect or cooperation between the enzymes. It was observed that the action of individual enzymes resulted in 7,8% xylan for "<X" and 8,4% for "<XC", but when combined in the same amount as in "<(X+XC)" resulted in lower xylan 7,0%, confirming the additive effect between xylanases GH11 and GH10. It was also observed that the action of enzymes at higher concentration always led to a pulp with slightly lower amount of residual xylan.

A



B

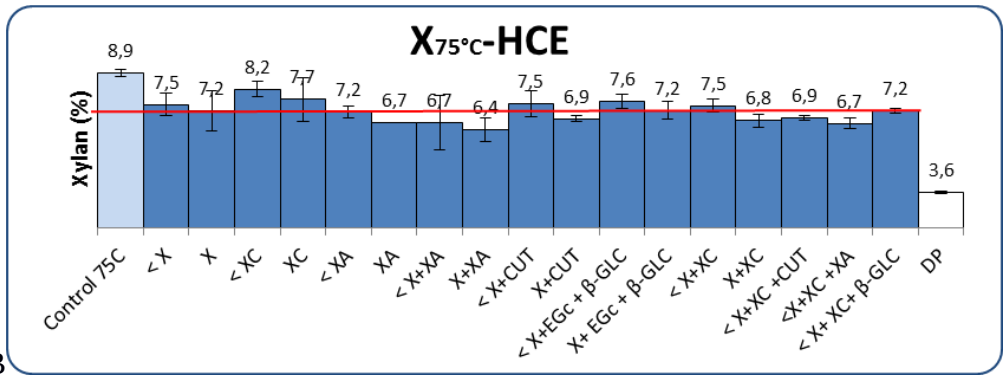


Figure 17 Experiment 2 Screening for the effect of protein concentration **A)** Sugar analysis after X_2-stage; **B)** Sugar analysis after HCE-stage; **SP:** Starting pulp OX1D-HCE-; **<X**: Half dosage; **X:** Xylanase GH11; **X_A:** Xylanase A GH10; **X_C:** Xylanase C GH10; **EGc:** Endoglucanase GH5; **β-GLC:** β-Glucosidase GH3; **CUT:** Cutinase; **DP:** Dissolving pulp; **RED LINE**: result of Xylanase GH11 treatment alone;

The Experiment 3 was done to evaluate the performance of xylanases at 90ºC. The singular enzymatic treatments with four xylanases: GH11 "X" and GH10 "X_A", "X_B", "X_C" in experiments 1 and 3 (Figure 18) shows that the selected xylanase GH11 "X" is again the preferred enzyme of all the xylanases tested. However, it is notable that the xylanase GH10 "X_C" seemed to be even more thermophilic than the tested xylanase GH11 as it had better performance at 90ºC than at 75ºC, despite of the high error bars that might compromise the conclusions. (Figure 18) One hypothesis to explain the difference might be due to the errors associated with long pulp treatments with enzyme followed by alkaline washing, which have several steps that might contribute to the error. The method of acid hydrolysis applied to quantify the monosaccharides composition possesses many steps, which can turn in associated errors, however the DP analysis made in all experiments always result in similar xylan content.

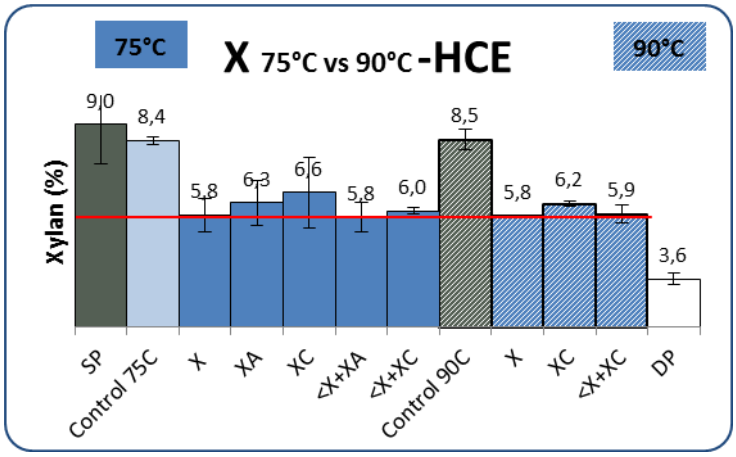
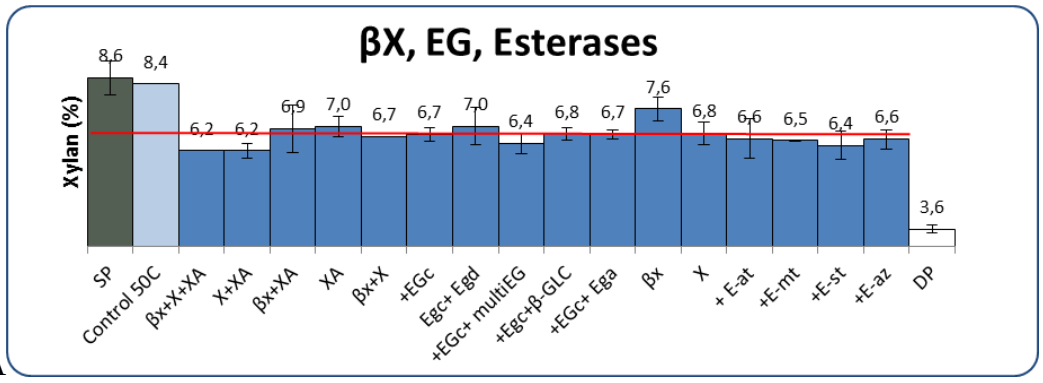


Figure 18 Experiment 3: Screening comparison at 75°C and 90°C (after Extraction stage) **SP:** Starting pulp OX1D-HCE-; **X:** Xylanase GH11; **X_A:** Xylanase A GH10; **X_C:** Xylanase C GH10; **DP:** Dissolving pulp; **<X:** Half dosage; **RED LINE**: result of Xylanase GH11 treatment alone;

A



B

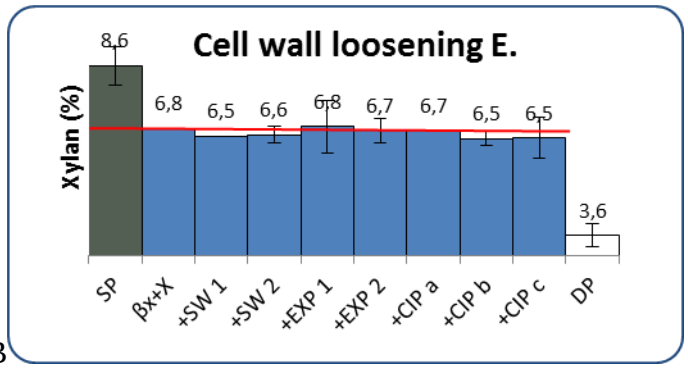


Figure 19 **Experiment 4 Screening of <u>various enzymes</u> composed by <u>X_2-stage at 50°C</u> followed by HCE-stage at 6%; A)** Sugar analysis after HCE-stage**; B)** Sugar analysis after HCE-stage; **SP:** Starting pulp OX1D-HCE-; **βX:** β-Xylosidase GH3; **X:** Xylanase GH11; **X_A:** Xylanase A GH10; **+(**β-Xylosidase,XylanaseGH11)**; EGc:** Endoglucanase GH5; **multiEG:** multicomponent Endoglucanases; **EG_D:** Endoglucanase GH44; **β-GLC:** β-Glucosidase GH3; **AM1:** Amylase; **AM2:** Amylase; **α-GLC:** α-Glucuronosidase GH115; **MAN:** Mannanase; **CUT:** Cutinase; **E-at:** Esterase aromatic; **E-mt:** Esterase methyl glucuronoyl; **E-st:** Esterase stk; **E-az:** Esterase Az 2X; **SW1**: Swollelin 1; **SW2:** Swollenin 2; **EXP1:** Expansin 1; **EXP2:** Expansin 2; **CIP1 a**: Cellulose Induced Protein 1 A; **CIP1 b**: Cellulose Induced Protein 1 B; **CIP1 c**: Cellulose Induced Protein 1 C; **DP:** Dissolving pulp; **RED LINE**: result of Xylanase GH11 treatment alone;

When it comes to combinations of xylanase GH11 "X+βX with cellulases (GH5 EGc, multi EG B, GH44 EG D , GH55 EG A) at 50°C, resulted in a pulp with lower yield.

Using a multi-component cellulolytic product (multi EG) at high dosage, is not advisable for this application because of the exo-activities leading to a more complete digestion of the fiber and thus resulting in severe loss of cellulose yield. In fact, using such an aggressive system, the pulp after such treatment completely lost its fibrous structure and properties with a concomitant high loss of solid substrate. (Figure 20) It is quite surprising that despite the severe degradation, the residual xylan remained in the analysis.

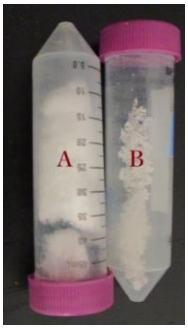


Figure 20 Resulted pulp in screening experiments. A) Pulp with xylanase treatment (normal content); B) Pulp with endoglucanase treatment (low content)

Regarding the combinations with esterases, (Figure 17,18) the cutinase (CUT) and esterase (E-ht) showed a decrease in residual xylan. As previously referred, esterases catalyze the hydrolysis of ester bonds in lipids while cutinases are specific for the hydrolysis of primary alcohol esters, e.g. contained in cutin. It seems that E-ht and CUT have promoted increased accessibility to the xylanase to hydrolyze more entrapped xylan by compounds with ester linkages. At 50°C the effect was not so notorious perhaps due to the fact that these esterases are more thermophilic. From the esterases tested (E-at, E-gmt, E-st, and E-2ax), E-st was the one that exhibited best results. The main difference between those enzymes is substrate specificity.

Regarding to cell wall loosening proteins, the role is to facilitate the action of the hydrolytic and oxidative enzymes by "opening up the plant cell". Even adding these proteins to the suspensions of pulp and left overnight, no additional effect was detected. (Figure 20)

A more extensive screening using others hydrolases could be tested, such as Pectinases and Exoglucosidases. In this context there are also several oxidoredutases such as LPMO's with oxidative ability to degrade crystalline cellulose that should be tested.

4.1.6 Effect of CCE stage

After several combinations of enzymeswithin a sequence X_1-D_0-HCE-X_2-HCE, the maximum amount of xylan removal was not improved beyond a remaining level of 6% recalcitrant residual xylan. The CCE stage was applied at the end of the sequence at 40 NaOH g /L and 80g/L in the liquid phase of the pulp suspension and the resulted pulps were analyzed for monosaccharide composition (*) at the end of sequence, after the second HCE and CCE stage: X_1-D_0-HCE-X_2-HCE*-**D_1-CCE*.**

The hemicelluloses content in the pulp, determined as xylan (%) below 5% indicate their suitability as a standard viscose-grade dissolving pulp. The results in **Erro! A origem da referência não foi encontrada.**2 A showed that the effect of a post-CCE_{80} treatment decreased the xylan content to a level of 2,7%, which is even lower than the commercial dissolving pulp used as reference.

In addition, the effect of the residual xylan content was studied as a function of NaOH concentration and it was obtained a linear correlation. (**Erro! A origem da referência não foi encontrada.** B) In order to theoretically remove all xylan, it would be necessary up to 100 g/L NaOH charge. However, such high amount of lye would lead to the change in supramolecular structure of cellulose, a transition of native cellulose I (native cellulose) into the Na-cellulose I (alkali cellulose). This gradual transformation of cellulose I to Na-cellulose I, as a result of the very high alkalinity at low temperature, results in the formation of a cellulose II structure upon washing. Because after this transformation, cellulose II is less crystalline thus the hydroxyl groups on C_6 and C_2 are more accessible to reagents. (e.g., CS_2 in the case of the viscose process) However, due to formation of inter- and intra-planar hydrogen bonds (hornification), the reactivity of cellulose II towards derivatization decreases upon thermal drying. [37]

The residual xylan content decreases with increasing NaOH concentration in the CCE-stage, but the application of enzymes provided higher purity (lower xylan content) and improved substantially the efficiency of the process. According to the correlation obtained, using a dosage of 80g/L NaOH in CCE stage, the enzymes were able to reduce the xylan content in pulp ca. 3 units lower than a pulp without enzyme. Besides that, enzymes lead to a substantial reduction in NaOH consumption of the CCE stage. For instance, to reach the amount of xylan

6% for pulp without enzyme treatment, it is necessary 80g/L NaOH, while for a pulp treated with enzyme, it is needed 30g/L of NaOH dosage.

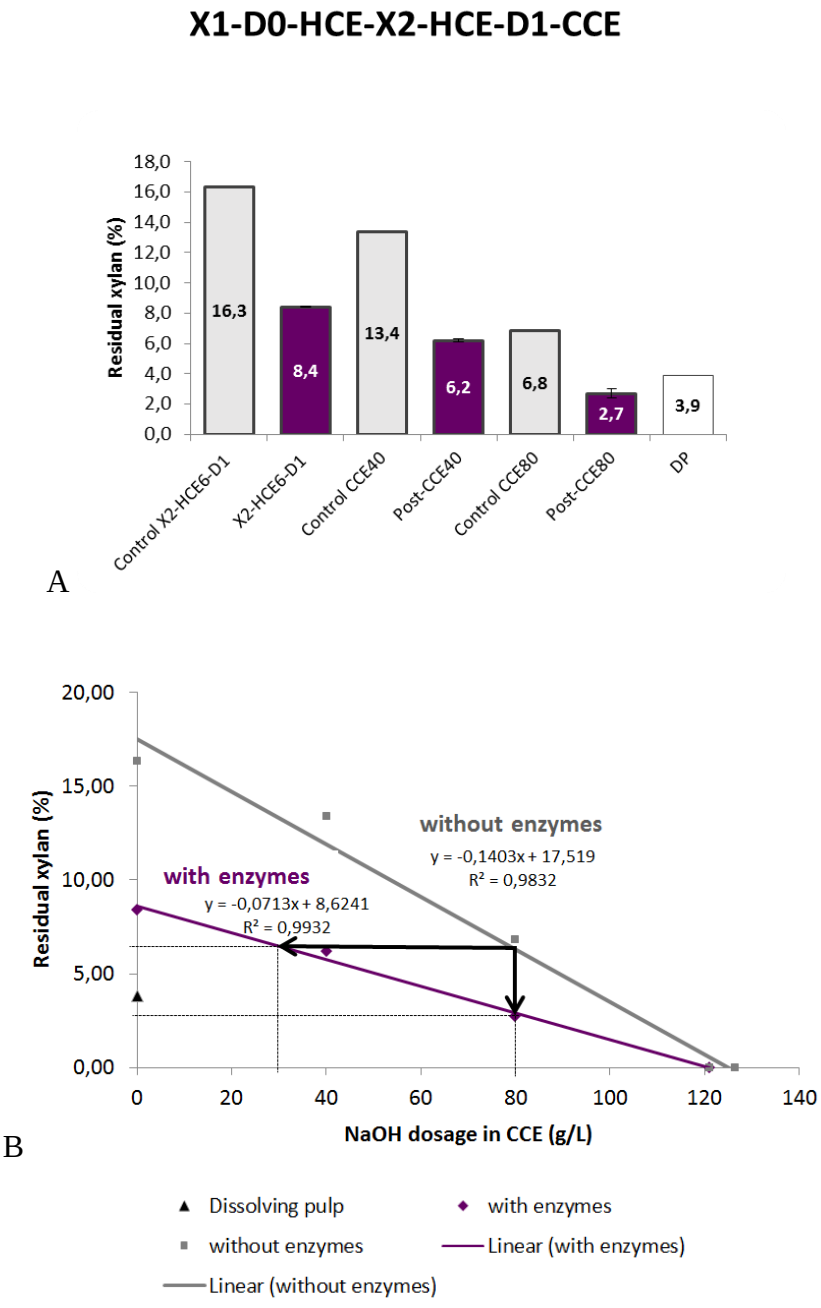


Figure 21 Effect of post-CCE stage

A) Residual xylan (%) of pulp after sequence: O-X1-D0-HCE-X2-HCE-D1-CCE.B) Correlation between the NaOH dosages applied in the CCE stage and residual xylan in pulp.

4.2 ISO Brightness

The demand for cellulose purity in dissolving pulps thus also requires a very low content of residual lignin and consequently the optical properties are also an important parameter for the quality. The residual lignin will affect negatively the downstream process of dissolving pulp conversion process (viscose).

In order to compare the brightness with the xylan content of all pulps, the data regarding the xylan content in pulp was normalized. Therefore, a dissolving pulp target (TDP) was calculated, as described in the following equation (1)

$$TDP: Target\ to\ Dissoving\ pulp\ = C - Ct$$

where C is the xylan content of fiber after treatment and Ct is the target xylan content of the commercial dissolving pulp (measured in sugar analysis 4.5%±0,5). (Figure 23)

It can be observed that without enzymes (grey bars) the brightness increased from an initial value of 51,3% to 75,8% in the partially bleached pulp (O-X_1-D_0-HCE) and finally reaching the maximum level of 91,8% after –X_2-HCE-D_1-CCE.

Regarding to the pulps treated with enzymes both tested xylanases GH11 and GH10, contributed to boost the brightness of the pulp. One hypothesis for the enzymatic mechanism in bleaching is that the disruption of the xylan chain by xylanase interrupts LCCs, improves the accessibility of the bleaching chemicals to the pulps and facilitates easier removal of solubilized lignin in bleaching.[69] The partially bleached pulps (O-X_1-D_0-HCE) treated with single xylanase GH 11 treatment, resulted in higher brightness (81,9% when treated at 75°C and 82,1% when treated at 60°C) than pulps treated with single xylanase GH 10 treatment (79,4%). Various factors such as substrate specificity and presence of CBD may explain the difference of these two particular enzymes tested; however it would not be scientifically correct to generalize a certain behavior for each GH family because, within the family, there are striking differences in terms of performance. [70] On the other hand, the pulps co-treated enzymatically with EGs (blue bars), resulted in a lower brightness 78,4% and 73,3%. It is noticed that the resulted brightness achieved within a treatment of EG_B lead to a lower value than in control. This observation indicates that an alkaline darkening effect might be caused by the HCE stage due to the release of carbohydrates by the previous action of the endoglucanases, which originated more degradation products of low molecular weight and increased the amount of reducing ends. These newly formed reducing ends in the cellulose

possibly led to the formation of new chromophores besides the peeling reactions under the harsh conditions of the HCE-stages. [71]

The pulp brightness of the final product also depends on the bleaching sequence used (pulp bleaching history). For the sequences with three enzymatic stages (Figure 23 Yellow bars), the pulp brightness (controls and enzyme-treated) decreases in the following order: X_2-HCE-D_2-X_3-HCE > X_2-D_2-HCE-X_3-HCE > X_2-D_2-X_3-HCE, which means that the variation is not a result of the enzymatic treatment. This behavior might be explained by the synergy between alkaline extraction and D stages.

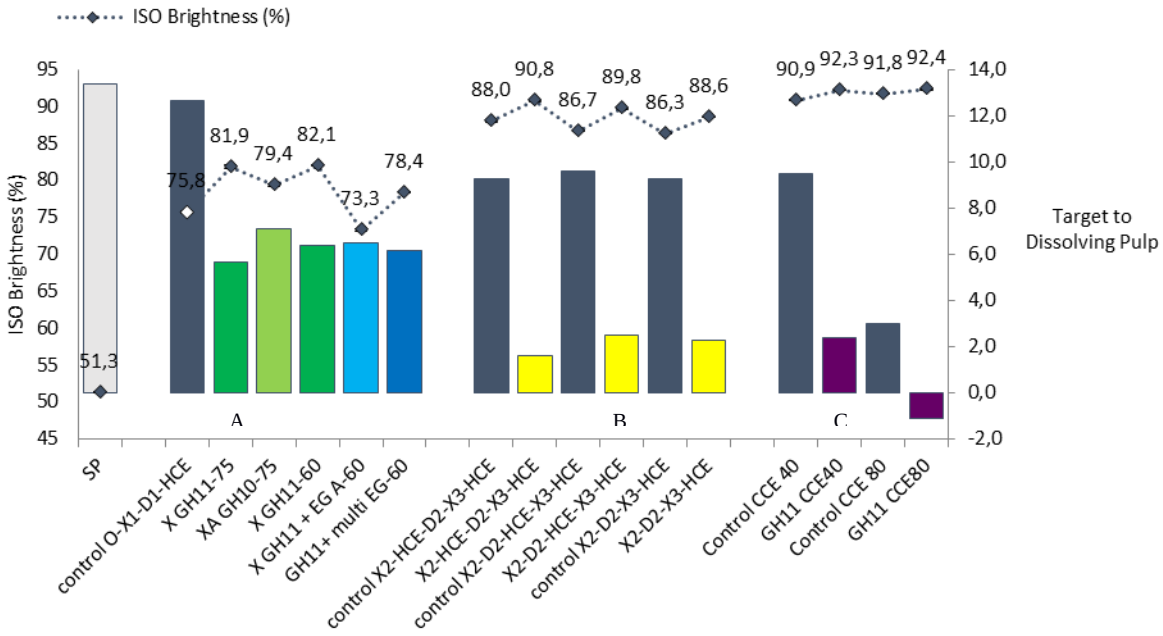


Figure 22 A) Brightness of pulp samples partilally bleached with sequence of treatments: O-X1-D0-HCE. Green and blue bars correspond to the type of enzyme applied in X1. B) Brightness of pulp samples within a sequence of alternate treatments O-X1-D0-HCE-(X2; X3; D1; HCE). Yellow bars correspond to enzyme treated pulps. The first X-stage (X1) with xylanase GH11 "X", second and third X-stage (X2=X3) with Xylanase+mannanase "X+M" treatment. C) Brightness of pulp samples within a sequence of treatments: X1-D0-HCE-X2-HCE-D1-CCE, with CCE-stage tested at 40g/L and 80g/L. Purple bars correspond to enzyme treated pulps. The first X-stage (X1) with "X+EG_B" and second X-stage (X2) with "X" treatment.

The maximum brightness reached was 92,4% and it was through the enzyme-based sequence including a post-CCE stage, which is more than needed for a standard viscose grade dissolving pulp.

4.3Viscosity of pulps

One of the main target parameters within dissolving pulp production is to adjust the average molecular weight of the polysaccharide fraction and the molecular weight distribution. The average MW of the pulp carbohydrates is commonly measured by the so-called pulp viscosity which is correlated with the average degree of polymerization of the cellulosic pulp.

It was previously studied that the viscosity of pulp may be enhanced by xylanase treatment because the removal of xylans, with lower DP than cellulose, will increase the average molecular weight. [72] The pulps with hemicellulases (xylanase GH11 and manannase) treatment (Figure 24 B yellow bars) confirm the expected slight increase in viscosity when compared to the control pulps (no enzyme). In the same manner, the removal of short-chain carbohydrates through HCE treatment, results in a slight increase in pulp viscosity because the stepwise degradation (end-wise peeling) has only a small effect on the molecular weight of long-chain cellulose. Regarding to the control pulps in Figure 24 B (grey bars - no enzyme), it is possible to observe how HCE stage has affected the viscosity. The two first sequences (from left to right) with three HCE treatments, resulted in viscosity slightly lower (ca. -1 unit mPa·s) than the third sequence which had only two HCE treatments.

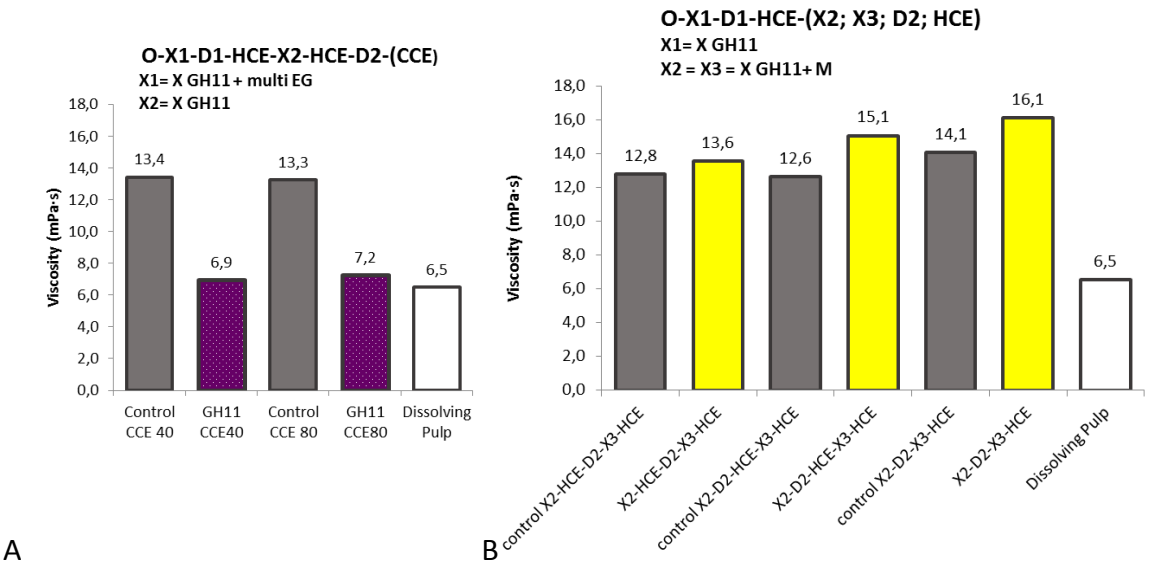


Figure 23 Viscosity of pulp upon the different sequences. Grey bars correspond to control pulps (no enzyme).

A) Viscosity of pulp samples within a sequence of treatments: X1-D0-HCE-X2-HCE-D1-CCE, with CCE-stage tested at 40g/L and 80g/L. Purple bars correspond to enzyme treated pulps. The first X-stage (X1) with "X+EG_B" and second X-stage (X2) with "X" treatment. B) Viscosity of pulp samples within a sequence of alternate treatments O-X1-D0-HCE-(X2; X3; D1; HCE). Yellow bars correspond to enzyme treated pulps. The first X-stage (X1) with xylanase GH11 "X", second and third X-stage (X2=X3) with Xylanase+mannanase "X+M" treatment.

As far as the sequences with CCE treatments are concerned (Figure 24 A purple bars), where the first enzymatic stage (X_1) had a multicomponent cellulase product applied in parallel with xylanase, the pulp exhibited a viscosity slightly higher but within an acceptable level of the commercial dissolving pulp used as reference. It is known that EGs are able to reduce the cellulose chain length and therefore they may efficiently provide a reduction in pulp viscosity. [73] In this case, it was used a multi-component cellulolytic product, where not only EGs are present, but also CBH cellulase, BG cellulase are included among others.

The dosage of enzyme applied is a crucial factor with regard to the degree of cellulose degradation. In this case, the cellulosic product was used in low dosage, thus the decrease of α-cellulose content is expected to be insignificant. However, when used at high dosage, it causes a deep degradation on the cellulose, as it was referred in the screening study.

The effect of the enzymatic action depends on the enzyme structure rather than other properties, so every EG enzyme should be tested for its ability to decrease pulp viscosity. Besides that, it would be interesting to test in the future the use of a monocomponent EGs, with and without a CBD, once that in several studies the role of CBD in EG applications was reported to contribute for the EG performance. [74]

Accordingly, the application of cellulases in the beginning of the process is a feasible way to control pulp viscosity or the cellulose average chain length.

It must be pointed out that this post-CCE stage might have an effect on viscosity decrease as well, nevertheless providing a nonselective reduction (compared to treatment with enzyme) in the degree of polymerization of cellulose. The determination of the average molecular weight is however not enough to predict specific product properties for the various possible applications. It is also necessary to measure the MWD and PDI. It is expected that the CCE-stage would lead to a low PDI and narrower molar mass distribution. [73]

4.4Reactivity measurements

The cellulose reactivity according to Fock's method was measured (as contracted service) to characterize the pulp quality for viscose making. The sample was stirred together with sodium hydroxide and carbon disulphide. Cellulose xanthate was formed and a certain amount of the xanthate was thereafter regenerated. Finally the regenerated cellulose yield was calculated.

The results of the reactivity measurements are interesting since it showed very significant differences between samples treated with enzymes and the controls. (Figure 24) The enzyme-treated samples showed a much higher reactivity. What is the mechanism behind the increase in reactivity? The product used in this experiment, "EG_B" is a blend of cellulases with high level of endoglucanases which preferably hydrolyse the amorphic cellulose and cleaves the cellulose randomly within the chain. Since less ordered or amorphous regions occur on the surface and between the microfibril endoglucanase treatment may lead to a swelling of the cell wall and thus increase the accessibility to solvents and reagents. [75]

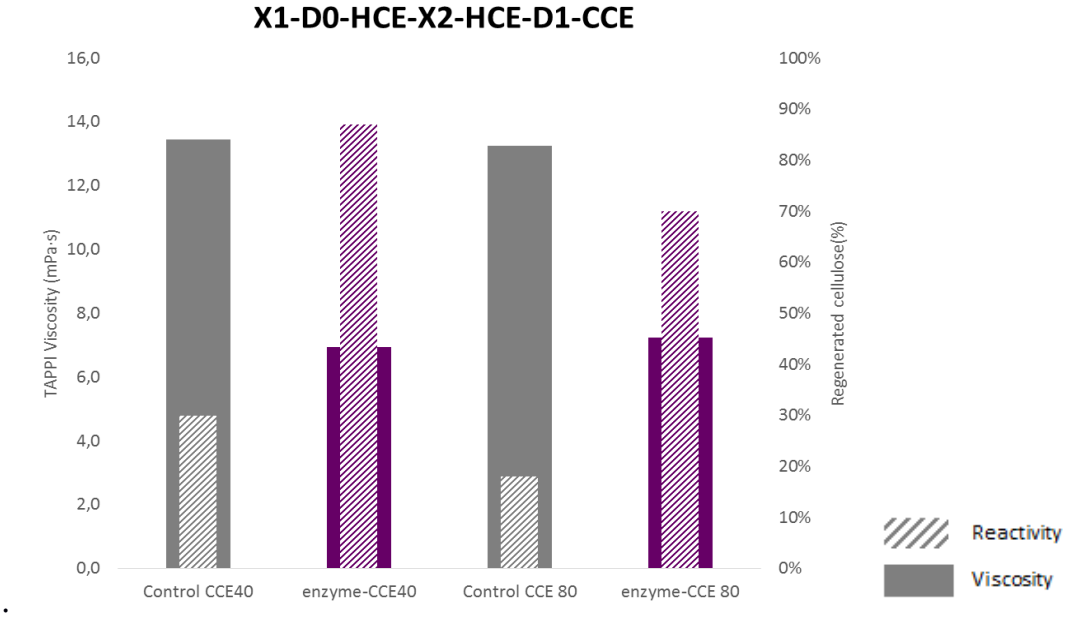


Figure 24 Reactivity according to Focks method versus viscosity of pulp samples within a sequence of treatments: X1-D0-HCE-X2-HCE-D1-CCE. Grey bars correspond to control pulps (no enzyme) and purple bars correspond to enzyme treated pulps. The first X-stage (X1) with "X+EG_B" and second X-stage (X2) with "X" treatment. The CCE-stage tested at 40g/L and 80g/L;

4.5Characterization of the treated pulps to be used as dissolving pulps

After evaluating the different treatments and conditions for the eucalypt kraft pulp to be converted into dissolving-grade pulps, the best treatment sequence and characteristics of the pulps were determined. A summary is presented in Table 6. The use of enzymes on this sequence of treatments allowed achieving a dissolving pulp without a pre-hydrolysis step and the requirements of a dissolving pulp suitable for viscose making. The possibilities for applying the method industrially seem promising.

Table 6 Sequence of treatments and characteristics of the treated eucalypt kraft pulp

Treatment sequence	X1-D0-HCE-X2-HCE-D1-CCE	
X1	Xylananse GH11 + Endoglucanase (multi)	
X2	Xylananse GH11	
HCE (% odp)	6%	
CCE (g/L)	80	
Characterists of resulted pulp:	Eucalypt kraft pulp	Commercial dissolving pulp
Xylan content(%)	2.7	4.5
Glucan content (%)	97.3	95.5
Pulp viscosity (mPa.s)	7.2	6.5
Cellulose reactivity by Fock (%)	70	-

Future Work

The optimization of this process can be further investigated. For instance, a lower alkali dosage must be tested in the hot caustic purification and besides that oxidoredutases should be tested within the same previous principle.

It was possible to obtain a dissolving pulp from a eucalypt kraft pulp without the pre-hydrolysis normally required in kraft cooking process. The next step is to test how this process can be implemented industrially. This concept provides the basis for the production of a new low cost technology for the manufacture of dissolving pulps of viscose pulps.

Depending on the mills, the investment to be made can be in order of producing a new line of a dissolving pulp or the conversion of paper-grade producers into dissolving pulp production. The second option requires a lower investment because the process created can be easily adapted with few modifications:

The first enzymatic stage application is not a difficult technology to apply, the only aspect to consider is to guarantee the right conditions, such as pH, after the oxygen delignification for the best performance of enzymes;

For the purification in HCE it just a matter of adjusting the alkaline conditions in the same tanks where extraction normally occours; the second enzymatic stage has to replace the second tower of chlorine stage and here the quantity of pollutants released is reduced, so as reduces the bleach chemical consumption. The main investment for the conversion is the application of the CCE technology. Of course the dosages applied experimentally won't be the same, because the NaOH can be recovered industrially. However this investment is much lower than adding a pre-hydrolysis technology for the kraft pulping process.

3. Conclusion

An enzyme-assisted method to produce dissolving pulp was studied in this work using oxygen delignified eucalypt kraft pulp from a paper-grade kraft pulping process.

Different sequences of treatments, which mainly consisted of enzymatic treatments using an endoglucanase and a xylanase (X-stage), hot caustic extraction (HCE-stage) and chlorine dioxide bleaching (D-stage), were evaluated. The sequence treatments conducted at medium pulp consistency revealed that: i) the acid treatment (A-stage) to the pulp prior to the enzymatic treatment does not support the reduction in the content of xylan; ii) the HCE was as effective at 6% as at 12% odp dosage, from the standpoint of xylan removal thus there is no need to increase NaOH dosage above 6%; and iii) the application of a 3rd enzymatic stage did not allow to reach a value below 5% residual xylan.

The evaluation of the quality of the final bleached and purified pulps revealed that when applying EGs in the beginning of the sequence, the pulp viscosity decreased quite effectively. As far as hemicellulases are concerned, the application of xylanases GH11 and GH10 at the very beginning of the sequence, contributed to boost the brightness of the final pulp at the middle and at the end of the sequence.

Enzyme screening studies using a partially bleached and purified pulp aided selecting relevant candidates from the point of view of xylan removal. A very resistant fraction (6% xylan in pulp) was not hydrolysable by the different combinations of enzymes tested. However, with regard to the results obtained, it seems that some candidates, e.g. cutinases, could be further explored as part of a future work aiming at increase the xylan accessibility by an enzymatic treatment.

It was also shown in this work that the production of a dissolving pulp from an O_2 delignified Eucalypt Kraft pulp was possible avoiding the pre-hydrolysis stage. The application of xylanase GH11 + EG in X_1 using a sequence O-X_1-D_0-HCE-X_2-HCE-D_1-CCE saves substantially high amounts of NaOH used in CCE stage. It was in addition possible to attain some requirements for the production of a dissolving pulp (suitable for viscose). The sequence used resulted in a pulp with 2,7% residual xylan, ISO brightness of 92,4%, a viscosity within the values of a commercial dissolving pulp and increased reactivity.

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Appendix 1

Quantification of Aldehyde groups in pulp

Several GH11 and GH10 xylanases were previously screened at 75°C and pH 4.5 in Novozymes Forest Products R&D, using bleached Eucalypt kraft pulp with regard to the amount of reducing sugars released. The pulps treated with GH10 xylanases were evaluated for the determination of aldehyde groups. The method is based on the ability of aldehyde groups to reduce 2, 3, 5 triphenyl-2H-tetrazolium chloride (TTC) with formation of red colorant *formazan* that can be determined by spectrophotometry.

The Figure 25 represent reducing sugars released in filtrate against the aldheyde groups in pulp. It is possible to confirm that enzymes from the same family, act in different way. The enzymes that released more reducing sugars more xylan has been removed. For the purpose of this study, as more xylan removed as better, thus the enzyme 33 was considered the best candidate.

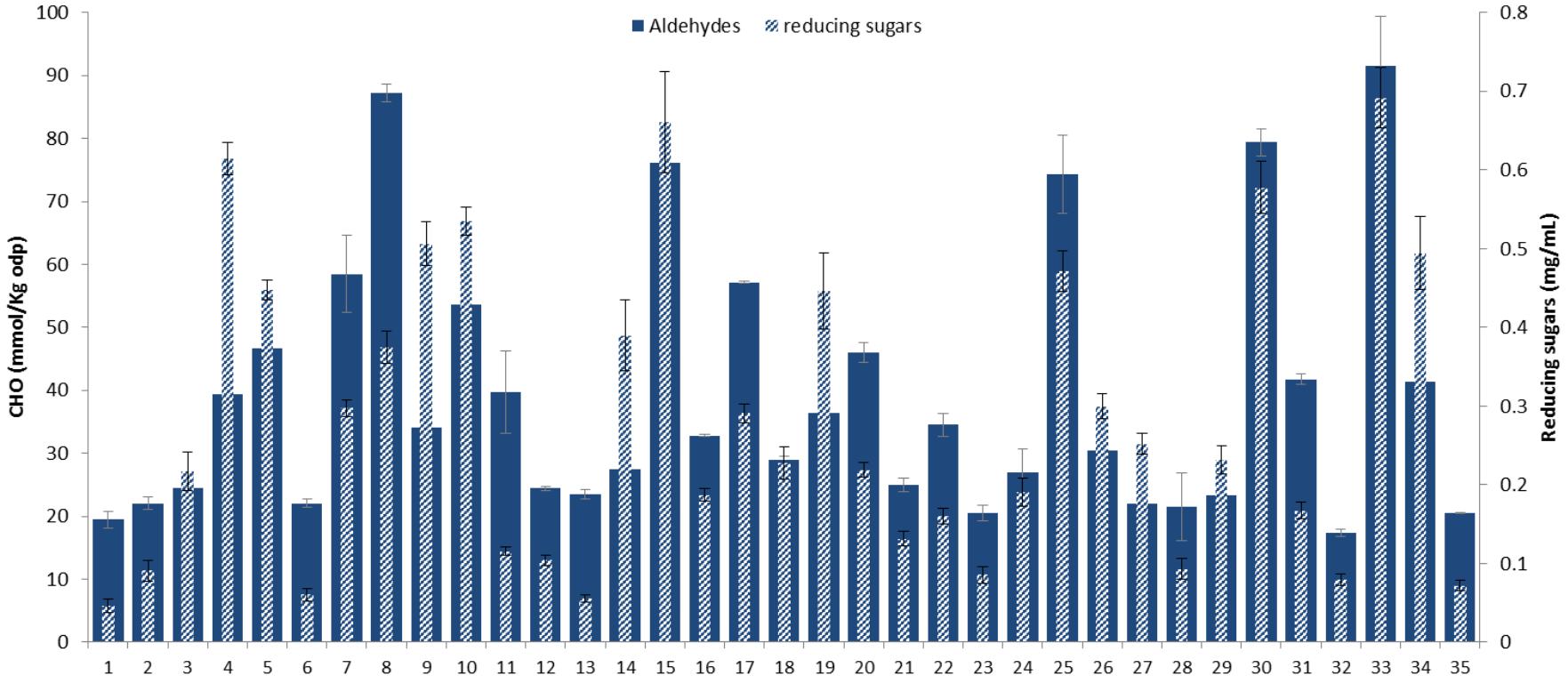

Figure 25 Screening of 34 GH10 xylanases with regard to the released reducing sugars (measured by Novozymes) and formed aldehyde groups in pulps; The pulp control is no. 1 (no enzyme)

Appendix 2

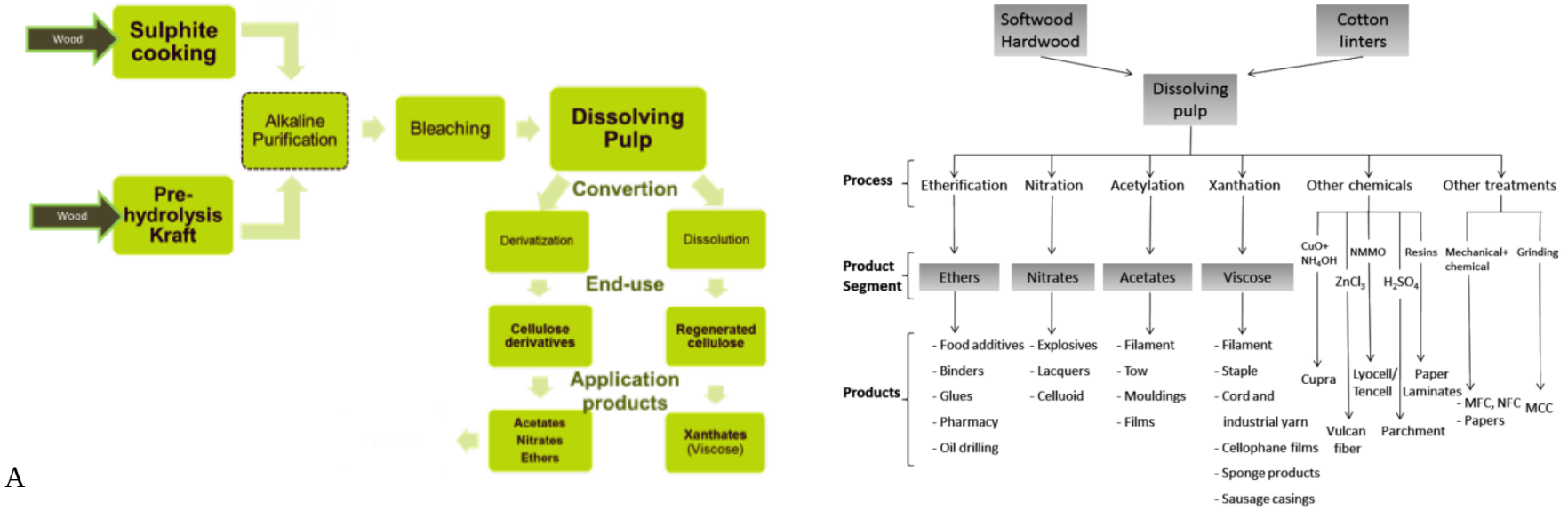

Figure 26 A) Production process of dissolving grade pulp B) Overview of some important products and processes originating from dissolving pulps[75]