

Alkaline pretreatments of lignin-rich by-products and their implications for enzymatic degradation

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Thesis

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Chapter 1

General Introduction

THE INCREASING INTEREST IN PLANT BIOMASS BASED BIOFUELS AND CHEMICALS arouses mainly from the increased awareness of a possible finiteness of fuels. The current main challenge to produce such biofuels and biochemicals is economic efficiency, but also knowledge concerning type and effectiveness of both thermally assisted chemical and enzymatic treatments, needed to generate fermentable sugars, is lacking. The subject of this thesis is to gain understanding of the effect of both sulphuric acid and NaOH catalysed pretreatments of sugar cane bagasse and oil palm empty fruit bunches on subsequent enzymatic saccharification, with a focus on the NaOH catalysed pretreatments. The fate of the main polymers present, lignin, cellulose and xylan, was studied and also single-activity xylan degrading enzymes, all from *Rasamsonia emersonii*, were studied for their mode-of-action.

1.1 Current challenges for plant biomass conversion into monosaccharides

Agricultural by-products (e.g. sugar cane bagasse) useable for the production of biochemicals and biofuels are mainly composed of cellulose, hemicellulose and lignin. Agricultural by-products are often referred to as lignocellulose. The conversion process of such lignocellulose into monosaccharides for the production of biochemicals or biofuels can be achieved by three major steps (Figure 1): pretreatment, enzymatic saccharification and sugar fermentation.

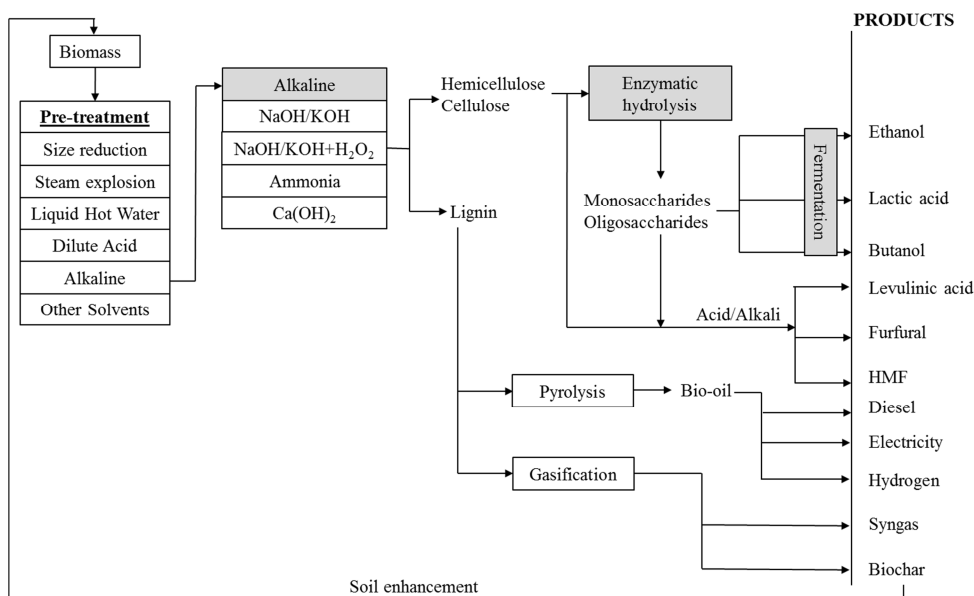


Figure 1. Scheme of a potential second generation biorefinery plant, to convert lignocellulose into products (adapted from Hayes, 2009).

Pretreatment technologies have been developed in order to increase enzyme accessibility to the (hemi-)cellulose (Mosier et al., 2005). The main currently studied pretreatments are shown in Figure 1, and the most used, alkaline, acid and oxidative, will be further described later on. After pretreatment, the lignocellulose is generally separated and the (hemi-)cellulose fraction is subjected to enzymatic saccharification. The residual lignin fraction is normally converted into energy or fuels. The valorization of lignin is beyond the scope of this thesis and it will not be further discussed.

The main technical challenges of each process are: i) finding the optimal pretreatment severity to open up the compact lignocellulosic cell wall architecture with minimal production of fermentation inhibitors (Palmqvist & Hahn-Hägerdal,

2000), ii) finding the optimal combination of enzyme activities for the complete degradation of the lignocellulose cell walls, and iii) to match process conditions, like pH and temperature, for co-saccharification and fermentation (Olofsson et al., 2008).

Table 1. Typical compositional data values based on dry matter of various grass type feedstocks.

Content ^a							
	Barley straw ^d	Sugarcane bagasse ^d	Empty fruit bunch ^d	Corn stover ^e	Wheat straw ^f	Miscanthus grass ^g	Switch grass ^h
Cellulose	35	37	26	30	31	31	39
Hemicellulose (total)	33	26	27	26	27	30	37
Arabinose	3	2	2	2.7	3	n.s. ⁱ	4
Xylose	22	22	18	19	22	n.s.	30
Galactose	1	0	1	1	1	n.s.	2
Mannose+Rhamnose	4	0	1	1.1	1	n.s.	1
Uronic acids	3	1	5	2.2	2	n.s.	n.s.
Lignin	24	28	33	29	25	11	25
Acetic acid	2	1	3	2.4	1.7	n.s.	n.s.
Protein ^b	2	1	3	4.2	n.s.	n.s.	n.s.
Ash content	1	5	5	n.s.	n.s.	2.8	2
Degree of substitution ^c							
Ara/Xyl	13	8	13	14	14	n.s.	n.s.
GlcA/Xyl	8	5	18	8	7	n.s.	n.s.
Ac/Xyl	17	7	27	34	32	n.s.	n.s.

^a% (w/w) based on dry matter

^b%Nx6.25

^cNumber of substituent residues per 100 xylosyl residues. Ara=arabinose, Xyl=xylose, GlcA=glucuronic acid, Ac=acetyl ester

^dMurciano Martínez et al., 2015

^eVan Eylen et al., 2011

^fJurak et al., 2015; Kabel et al., 2007

^gHodgson et al., 2011; Si et al., 2015

^hYan et al., 2010

ⁱn.s.=not specified

1.2 Lignocellulosic feedstocks and their cell wall architecture

Lignocellulosic by-products are very abundant worldwide, which makes them easily accessible at industrial amounts (Reddy & Yang, 2005).

Composition of grasses as agricultural lignocellulosic by-products

The three main components of many agricultural lignocellulosic by-products are cellulose, hemicellulose and lignin. The amount in which each component is present in the feedstock varies depending on the family, species and part of the plant of which the by-product originate from (Iqbal et al., 2013). In Table 1 the composition of seven agricultural by-products is shown. The contents of the main three polymers composing the cell wall, are quite similar among the seven grasses in the table; between 26-39% for cellulose, 26-37% for hemicellulose and 11-33% for lignin. The main differences are found when looking at the detailed carbohydrate composition of each by-product, although also the compositions are largely the same. Differences in xylan structures can be seen (Table 1), but these will be discussed further on in the text.

Plant cell wall architecture

The composition of lignocellulose shown in Table 1 indicates the amounts of the various components present. In the plant cell wall, these components are present in a complex network, in which cross-linked polysaccharides form a network with lignin and, to a minor extent, with cell wall proteins. The plant cell wall is constituted by the middle lamella, primary cell wall, secondary cell wall and plasma membrane, of which a model is shown in Figure 2. Primary cell walls are formed during early cell growth and are normally lignified to a low extent. Secondary cell walls are deposited when cell growth has ceased and they are often highly lignified (Figure 2).

Lignocellulosic agricultural by-products, and their corresponding plant materials, are mainly composed of secondary cell walls. So, they are generally comprised of long cellulose microfibrils interconnected by hemicelluloses (Ebringerová, Hromádková & Heinze, 2005). Additional polymers, such as lignin, fill the spaces in structures (Cosgrove, 2005).

Cellulose

Cellulose is an unbranched 1,4-linked β -D-glucan. Many parallel glucan chains form a crystalline microfibril that is mechanically strong and highly resistant to enzymatic attack. These long, crystalline microfibrils are 3 to 5 nm wide. In growing cells, they are aligned with each other, which gives further structural support to the cell wall (Cosgrove, 2005).

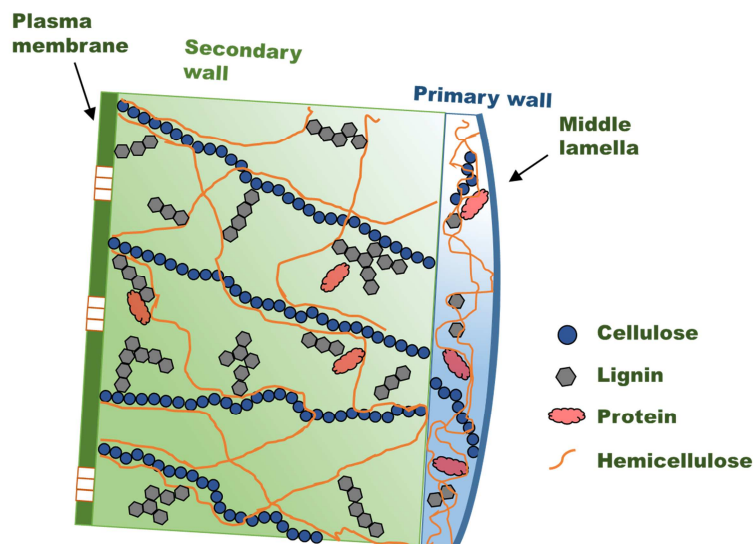


Figure 2. Plant cell wall model of grasses (adapted from Achyuthan et al., 2010).

Hemicellulose - xylan

Hemicellulose is a heterogeneous group of polysaccharides closely associated with cellulose in the plant cell walls (Figure 2). Depending on their origin hemicelluloses are characterized by having β -(1 \rightarrow 4)-linked backbones of glucosyl, mannosyl or xylosyl residues (Scheller & Ulvskov, 2010). Also other carbohydrates can be part of the hemicellulose structure as substituents, such as α -L-arabinosyl, β -D-glucosyl, α -D-galactosyl, α -D-glucuronic, α -D-4-O-methylglucuronic acid residues.

The agricultural by-products studied in this thesis, sugar cane bagasse, oil palm empty fruit bunch and barley straw, all belong to the grass type or gramineae family. In these grasses, the major hemicellulosic population is composed of xylans, which can represent, as seen from Table 1 based on the xylosyl residues content, 18 to 30% of the dry matter biomass (Gírio et al., 2010). But, in grasses and cereals, xylans can account up to 50% of the dry matter (Ebringerová et al., 2005).

Xylans are considered as the second abundant polysaccharide in plants next to cellulose (Ebringerová & Heinze, 2000). The oldest plants, as green algae and red seaweeds, are rich in homoxylans (β -1 \rightarrow 3, β -1 \rightarrow 4) (Ebringerová et al., 2005). However, homoxylans are rarely present in higher plants. Apparently, higher plants developed a more complex heteroxylan structure increasing the resistance to

enzymatic attack. Heteroxylans are composed of a β -(1 \rightarrow 4)-D-xylopyranosyl linked backbone, commonly substituted by various carbohydrate units and/ or *O*-acetyl groups. The structure of heteroxylans varies a lot depending on the source, which is discussed in more detail below.

Heteroxylans from monocots

In monocot plants, which includes grasses and some related species, xylan usually contains arabinosyl residues attached to the backbone and is known as arabinoxylan (AX). The arabinosyl group can be attached to the *O*-2 and/ or *O*-3 position of a xylosyl residue. When the xylan contains glucuronic acids as well it is denoted as glucuronoarabinoxylan (GAX) (Scheller & Ulvskov, 2010). In addition, for various GAX, for example present in corn stover (CS) and oil palm empty fruit bunch (EFB), it is reported that the xylan present is acetylated (Table 1, Figure 4). Another important characteristic of grass type xylans is the presence of ferulic and *p*-coumaric acid esters (Wende & Fry, 1997). These esters are normally attached to the *O*-5 position of an arabinosyl substituent. Ferulic and *p*-coumaric acid esters can also be crosslinked to lignin and, hereby, are assumed to form covalent linkages between xylan and lignin. The presence of such esters is a characteristic of grasses, which is not found in other types of xylan-rich feedstocks, such as hardwood (Faik, 2010).

The amount and type of xylan substitution, in general, varies and depends on the origin of the xylan (Table 1). A schematic structure of five grass-type xylans is shown in Figure 4. Ferulic and *p*-coumaric acid is not represented in this figure. When comparing the degree of substitution of the xylans in the different grass types (Table 1), it can be observed that the main difference is the abundance rather than the types of substituents present. For example, when comparing xylan from sugarcane bagasse (SCB) and EFB, EFB xylan contains three times more acetyl substituents and two times more glucuronic acids than SCB xylan (Table 1). These differences influence the solubility of the xylan. A higher content of acetyl groups leads to higher solubility of xylans (Van Gool, 2012). In order to completely enzymatically degrade the xylan into its monosaccharides, it is important to know the type of substituent remaining after pretreatment.

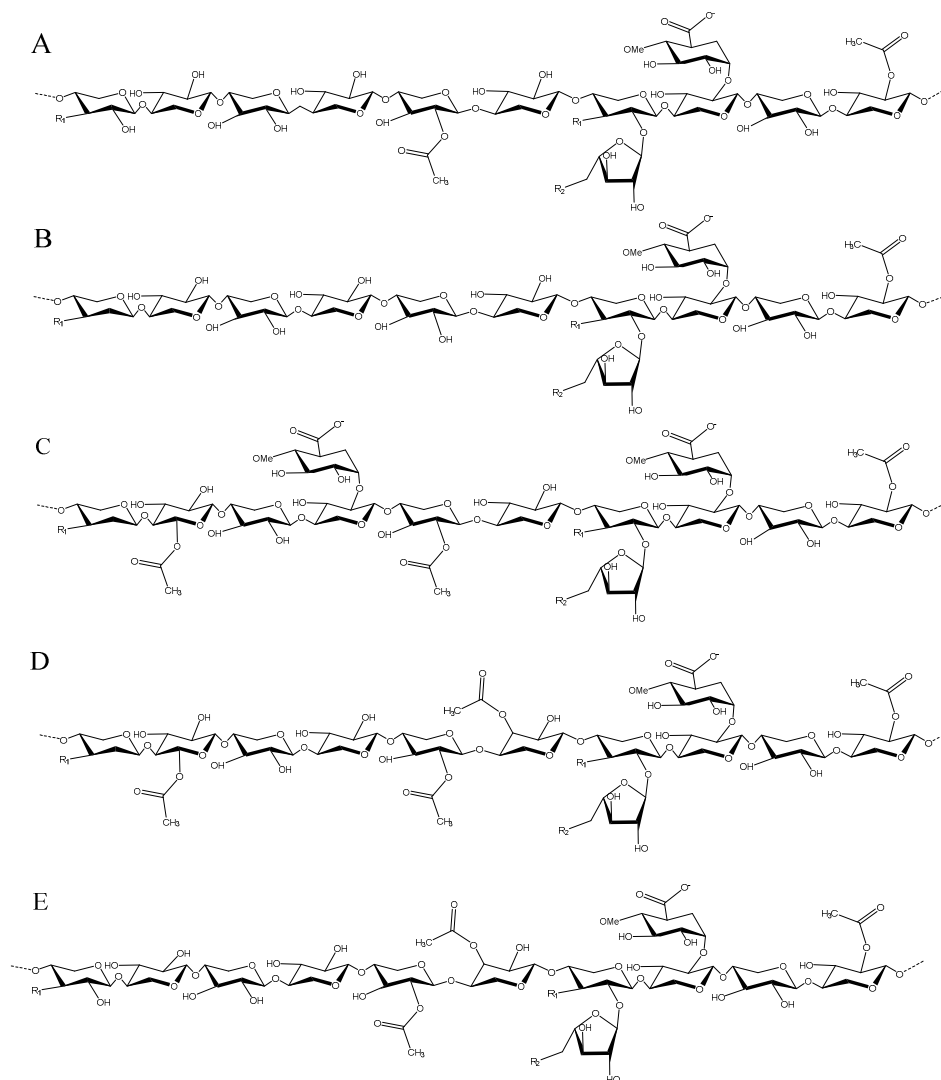


Figure 4. Schematic presentation of xylans from different grasses. The type and number of substituents related to the number of xylosyl residues is based on Murciano Martinez, et al. (2015) for barley straw (A), sugarcane bagasse (B), and oil palm empty fruit bunch (C), on Van Eylen, et al. (2011) for corn stover (D), and on Jurak, et al. (2015) for wheat straw (E).

Heteroxylans from dicots

Not only in monocots xylans are present, but also in certain dicots, like hardwood, the main hemicellulose is xylan. It constitutes about 20-30% of the biomass in hardwood. Heteroxylans from hardwoods, such as beech, birch, *Eucalyptus* and aspen wood, are mainly composed of a β -(1 \rightarrow 4)-linked β -D-xylosyl backbone substituted by 4-*O*-methylglucuronic acids (Ebringerová, 2006), named glucuronoxylan (GX). These GXs are normally highly acetylated. The percentage of acetylation is between 8-17% (w/w) (Gírio et al., 2010).

Lignin

Lignin is composed of a polyphenolic network, in which different repeating units can be identified. Lignin is synthesized mainly from three hydroxycinnamyl alcohols that differ in their degree of methoxylation: *p*-coumaryl, coniferyl, and sinapyl alcohol (Rencoret et al., 2011). Each of these monolignols gives rise to a different type of lignin unit called *p*-hydroxy-phenyl (H), guaiacyl (G), and syringyl (S) units, when incorporated into the polymer (Figure 5). The occurrence of each individual unit varies with the type of cell wall, tissue or plant family.

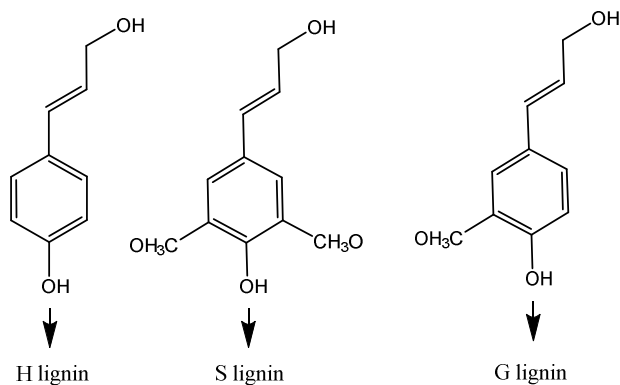


Figure 5. The three lignin precursors, *p*-coumaryl (H), coniferyl (G) and sinapyl (S) alcohol.

Hardwood lignin is generally rich in S and G units, with low levels of H units (Nimz, Nemr, Robert & Faix, 1981). Lignin in gymnosperms (softwood) is mainly composed of G units with low levels of S and H units (Boerjan et al., 2003). For grass type feedstocks the abundant presence of H units is a common factor. However, there is no agreement in literature about how H units should be classified. A high proportion

of H units is reported to be esterified to lignin units and not further linked, as is the case for *p*-coumarates in grasses (Del Río, Gutiérrez, Rodríguez, Ibarra & Martínez, 2007). In the latter case, such H units are seen as non core lignin units. The same is true for G units, which can partly be present as ferulates in grasses. In grasses, the major inter unit linkage is the aryl glycerol- β -aryl ether (β -O-4) linkage (García et al., 2009). Also ester linkages are present between lignin compounds (S, G, H) and hydroxycinnamates, such as ferulate, *p*-coumarate or *p*-hydroxybenzoate (Buranov & Mazza, 2008; Rencoret, Ralph, Marques, Gutiérrez, Martínez & Del Río, 2013; Ralph, 2010).

1.3 Pretreatment of lignocellulosic agricultural by-products

Several pretreatment technologies are available, and indicated in Figure 1. They can be classified into three categories based on the nature of the cell wall disruption: physical, chemical pretreatments and solvent fractionation (Da Costa Sousa et al., 2009). All attempt to break the recalcitrant lignocellulosic network in order to improve the access for microbial enzymes degrading the carbohydrates polymers (Zhao, et al., 2012).

The recalcitrant plant network, as explained in section 1.2, can be divided in four categories: 1) densely substituted hemicellulose structures shielding the access to cellulose and resisting breakdown to their constituent monosaccharides, 2) lignin networks embedding cellulose and hemicellulose and thus restricting enzyme access, 3) covalent hemicellulose-lignin junctions tying the two polymers together and hampering their removal, and 4) highly crystalline cellulose microfibrils.

Physical pretreatment

Physical pretreatments include treatments to reduce the particle sizes of the material and heat treatments, usually combined with chemicals. The latter will be discussed in the next section (chemical pretreatments). Particle size reduction treatments are carried out by applying mechanical stress (Zhu & Pan, 2010). The aim is to increase the surface area to improve the subsequent enzyme hydrolysis and, in some cases, by reducing the degree of polymerization (DP) of xylan and cellulose, and crystallinity of cellulose (Alvira, Tomás-Pejó, Ballesteros & Negro, 2010). Physical pretreatments, however, are in most of the cases not enough to modify the molecular size of the main biomass components and to make them more accessible for enzymes (Karimi, Shafiei & Kumar, 2013). Nonetheless, most other pretreatments require a maximal particle size in order to be effective, especially to overcome mass and heat

transport problems. It is also important to notice that below a certain particle size the treatment becomes economically unfeasible (Hendriks & Zeeman, 2009).

Strong acid or base pretreatments: H_2SO_4 or $NaOH$ catalysed

Chemical pretreatments are commonly applied in order to remove lignin, hemicellulose, or both, from the cellulose surface. The concentration of the chemical catalyst, time and temperature are kept the lowest possible in order to minimise economical costs and formation of fermentation inhibitors. Mainly, two types of chemical pretreatments are described in literature: acid and alkali catalysed treatments.

One of the most common acids used for the degradation of plant secondary walls is sulphuric acid. Many studies report the use of diluted sulphuric acid (1-10%) at high temperatures over 120°C (e.g. Canilha et al., 2011; Ferrer, Requejo, Rodríguez & Jiménez, 2013; Hernández, Pérez-Pimienta, Messina & Saldaña Durán, 2012; Lloyd & Wyman, 2005; Martin, Alriksson, Sjöde, Nilvebrant & Jönsson, 2007; Van Eylen, van Dongen, Kabel & de Bont, 2011). However, the severity of the acid pretreatments is difficult to compare, because the concentration of acid is not always well specified. The percentage of acid can reflect the volume of acid used per volume of pretreated material, or it can reflect the grams of acid based on grams of dry matter of pretreated feedstock. The aim of diluted sulphuric acid pretreatment is the solubilisation of the hemicelluloses, like xylan, as indicated in Figure 6. Thereby, it provides an improved access for enzymes to the cellulose fibres. Depending on the severity of the pretreatment (concentration of acid (pH), temperature and time applied), the production of carbohydrate degradation products such as furfural, HMF and organic acids varies (Palmqvist & Hahn-Hägerdal, 2000). These products may cause inhibition of the fermentation of monosaccharides into fermentation products like ethanol or lactic acid. Van Eylen et al. (2011), studied the pretreatment of corn cobs, stover and fibres using H_2SO_4 as a catalyst. They reported that at $\log R'_0$ severity factors of 0.12, 0.28 and 0.83, the amounts of furfural produced were 0, 0.1 and 0.25 g L⁻¹. Xylan solubilization at the mentioned $\log R'_0$ were around 70, 75 and 80%.

Alkaline pretreatments are commonly based on the use of sodium, potassium or calcium hydroxide (Carvalho et al., 2008). The major effect of such alkaline pretreatments is the solubilization of lignin, as shown in Figure 6, by the rupture of ester and hydrogen bonds cross-linking lignin and xylan (Sun & Cheng, 2002; Jacquet, Pollet, Lapierre, Mhamdi & Rolando, 1995). The solubilization of lignin enhances the accessibility of the residual cellulose and hemicellulose for enzymes.

Alkaline treatment also causes swelling of the treated biomass, which increases the surface area of (hemi-)celluloses for enzymes (Ibrahim et al., 2011).

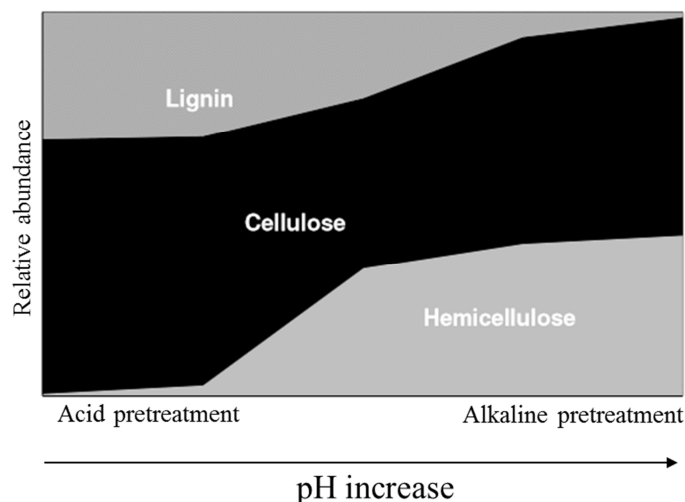


Figure 6. Relative abundance of lignin, cellulose and hemicellulose in the solids remaining after acid or alkaline pretreatment of lignocellulosics (adapted from Carvalho et al., 2008).

In addition, alkaline pretreatments provoke partial decrystallization of the cellulose (Brodeur et al., 2011). With loadings of 0.05–0.3 g of alkali/g of biomass, microwave pretreatment resulted in higher sugar yields than conventional heating, with the highest yield (90% of maximum potential sugars) being achieved at an alkali loading of 0.1 g/g (Kumar, Barrett, Delwiche & Stroeve, 2009).

Alkaline pretreatments are often carried out in presence of oxidative agents, such as H_2O_2 , O_2 , or peracetic acid. For example, Kim et al. (2006) reported that the use of O_2 in combination with $\text{Ca}(\text{OH})_2$ resulted in an extensive delignification of corn stover and a subsequent complete enzymatic hydrolysis of the residual cellulose into glucose. Despite the fact that oxidative treatments are mostly performed in alkaline conditions, also, oxidative treatments in absence of alkali have shown to result in complete enzymatic conversions of the residual cellulose. For example, about 50% of the lignin and most of the hemicellulose were solubilized by 2% (w/w) H_2O_2 at 30 °C within 8 h, and 95% cellulose conversion to glucose was achieved in the subsequent enzymatic saccharification (Azzam, 1989).

Other pretreatments

Solvent fractionation aims to disrupt the linkages between cellulose, hemicelluloses and lignin, again to increase the accessibility of the cellulose surface for enzymes. Reduction of the degree of crystallization of the cellulose is also aimed at (Sathitsuksanoh et al., 2010). Solvent fractionation includes the use of weak bases or

acids, and other organic solvents like ionic liquids. Three examples of solvent fractionations are i) soaking in aqueous ammonia (SAA) (Kim et al., 2008); ii) organic solvent based lignocelluloses fractionation (COSLIF) (Sathitsuksanoh et al., 2010) and iii) dissolution of lignocellulose constituents in ionic liquids such 1-allyl-3-methylimidazolium chlorides (Mäki-Arvela et al., 2010). Explanation of the different solvent fractionations described in literature is out of the scopus of this thesis and, therefore, not further discussed.

1.4 Enzyme hydrolysis of pretreated lignocellulosics

As mentioned above, one of the challenges in the production of monosaccharides from lignocellulosic material is the enzyme hydrolysis step. Recently, a survey concluded that in lignocellulosic ethanol production the three largest cost contributors are capital costs (41%), feedstock costs (27%) and enzyme costs (16%) (Bloomberg, 2013). The high enzyme costs are due to the high loadings in the saccharification. Typically, this step is carried out with 20 kg of enzyme per ton of biomass for about 3 days to achieve a 90% yield of glucose (NREL, 2011). This is in sharp contrast to the similar step in the first generation corn ethanol production where hydrolysis of starch is performed with about 2 kg of enzyme per ton of substrate for about 5 hours to afford 99% yield of glucose (Kwiatkowski, et al., 2006). As a result, the current production price for 2nd generation lignocellulosic ethanol is about €0.68/L, while the price for 1st generation corn ethanol is significantly lower at approximately €0.49/L. In order to make 2nd generation lignocellulosic ethanol a success, better utilization of the feedstock is a prerequisite.

As explained above, the physicochemical process of the pretreatment influences the recalcitrance of the material obtained and, thereby, the enzyme hydrolysis of the (hemi-) cellulosic feedstock. Both cellulose and xylan degrading enzymes, and also accessory xylan substituent removing enzymes are required. All carbohydrate degrading enzymes are classified in the Carbohydrate Active enZyme database (CAZy, www.cazy.org). The CAZy database classifies carbohydrate active enzymes based on their amino acid sequence similarities with at least one biochemically characterized member per family (Lombard et al., 2014). The enzymes mentioned in the next sections are discussed mainly based on their CAZy classification.

Cellulose degrading enzymes

In order to fully hydrolyze cellulose into glucose several enzymes are required, preferably acting synergistically. Industrial cellulases are almost all

produced by aerobic fermentations of cellulolytic fungi such as *Trichoderma reesei* or *Humicola insolens* (Wilson, 2009). Most cellulases belong to the glycoside hydrolase (GH) families 5, 6, 7, 12 and 45. The classical action of cellulases comprises the combined activity of endoglucanases, cellobiohydrolases and β -glucosidases (Goyal, Ghosh & Eveleigh, 1991). Endoglucanases attack the cellulose chains in a first step, followed by the combined action of both endoglucanases and cellobiohydrolases. Finally, the smaller oligosaccharides formed are hydrolysed to glucose by the action of the β -glucosidases. In addition, recently, oxidative enzymes classified as auxiliary activities (AAs) have been demonstrated to remarkably improve the degradation of cellulose.

Xylan degrading enzymes

In the last years, extensive research has been performed in order to reveal the structure and biochemical characteristics of many xylan degrading enzymes (Manju & Singh Chadha, 2011). Xylanases, like cellulases, have a catalytic domain and, often, other functional modules such as carbohydrate-binding modules (CBMs). CBMs facilitate the recognition of the substrate for the enzyme and improve the binding to insoluble substrates (Van Gool et al., 2012). Xylan degrading enzymes are classified as GH or as carbohydrate esterases (CE). Taking into consideration the composition of GAX, the following enzymes are necessary to degrade the polymer into monosaccharides: endoxylanases, β -xylosidases, α -L-arabinofuranosidases, α -D-glucuronidases and carbohydrate esterases.

Endoxylanases

Endo- β -1,4-xylanases (systematic name 1,4- β -D-xylan xylanohydrolase, (EC 3.2.1.8)) cleave the β -xylosidic bond between two β -(1 \rightarrow 4)-linked xylopyranosyl residues. Endoxylanases have been classified within the glycoside hydrolase families GH5, 7, 8, 10, 11, and 43.

Endo-xylanase belonging to GH10 and GH11 are the most extensively characterized and described in literature (Shallom & Shoham, 2003). Enzymes from family GH10 can tolerate the presence of substituents, such as arabinosyl residues, better in comparison with GH11 endo-xylanases (Beaugrand et al., 2004). An example of the mode of action of two endoxylanases, belonging to families GH10 and GH11, from *Aspergillus awamori* towards an arabinoxylan model described by Kormelink et al. (1993) is depicted in Figure 6.

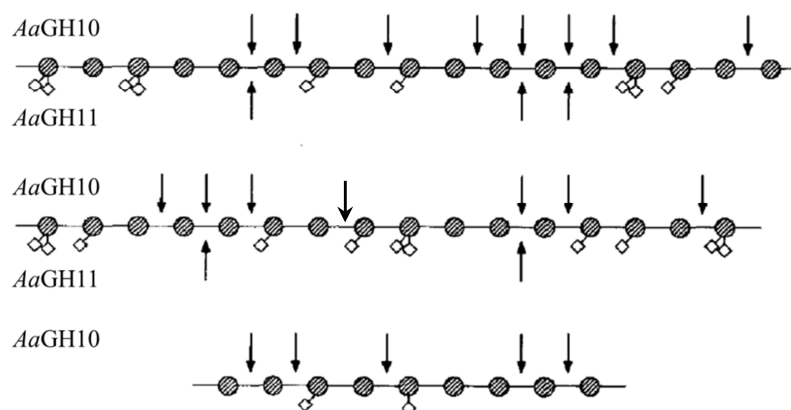


Figure 6. Cleavage sites (↓) of two endoxylanases GH10 and GH11 from *Aspergillus awamori* (Aa) towards an arabinoxylan model, adapted from Kormelink et al. (1993). ● = xylosyl residue ◐ = xylosyl residue substituted by an arabinosyl residue.

The endoxylanase belonging to family GH10 (AaGH10) is able to cleave the xylan backbone next to a xylosyl residue substituted with one or two arabinosyl residues. The xylanase from family GH11 (AaGH11) is more hindered by arabinosyl substituents and cleaves the xylan backbone only next to an unsubstituted xylosyl residue.

***β*-Xylosidases**

β-Xylosidases hydrolyse xylobiose and xylo-oligosaccharides (XOS) to xylose acting from the non-reducing end. According to the CAZy-database, *β*-xylosidases belong to GH3, 30, 39, 43, 52 and 54. (Cantarel et al., 2009). Filamentous fungal *β*-xylosidases have been described only for families 3, 43 and 54 (Knob et al., 2010). GH30 and GH52 *β*-xylosidases are found in bacteria and GH39 *β*-xylosidases in mammals.

***α*-L-Arabinofuranosidases**

α-L-Arabinofuranosidases (EC 3.2.1.55) are exo-enzymes degrading terminal arabinoses present in polysaccharides (Saha, 2000). These enzymes are found in the GH families 3, 43, 51, 54 and 62. AX specific arabinofuranosidases cleave arabinosyl residues linked to the *O*-3 or *O*-2 position of xylosyl residues in the xylan-backbone, as indicated in Figure 7. Some arabinofuranosidases are known to be very specific towards the position of the arabinose in the xylosyl residue. Considering substrate

specificity, arabinofuranosidases are classified into three subclasses (Ferré et al., 2000; Van Laere et al., 1997; Van Laere et al., 1999). Subclass 1 includes enzymes able to release arabinose from singly and doubly substituted AX and is able to hydrolyze *p*-nitrophenyl- α -L-arabinofuranoside at a rate similar to that for oligosaccharides (Numan & Bhosle, 2006). Subclass 2 includes enzymes able to release arabinose linked at the *O*-2 or *O*-3 position of a single substituted xylosyl residue. Subclass 3 includes enzymes releasing only arabinose linked at the *O*-3 position from doubly substituted xylosyl residues.

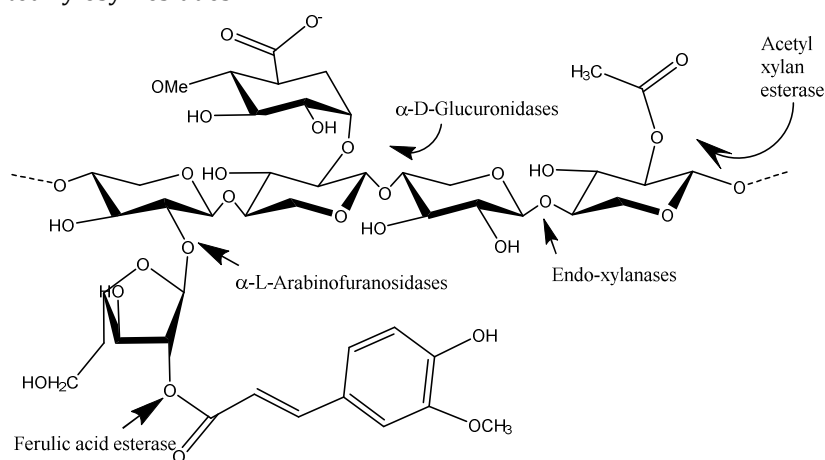


Figure 7. Sites of attack of xylanolytic enzymes on xylan (adapted from Shallom & Shoham, 2003).

α -D-Glucuronidases

α -D-glucuronidases are classified into the GH families 67 and 115. GH67 α -D-glucuronidases are extensively described in literature. These enzymes are able to act only on xylo-oligosaccharides (XOS) substituted with 4-*O*-methyl glucuronic acid (UA_{me}) at the non-reducing end (Golan et al., 2004; Nurizzo et al., 2002). On the contrary, GH115 α -D-glucuronidases are poorly described. Only six articles have been published describing the isolation or characterization of GH115 enzymes, of which three cover a biochemical characterisation (Chong et al., 2011; Fujimoto et al., 2011; Kolenová et al., 2010; Ryabova et al., 2009; Tenkanen & Siika-aho, 2000). These three GH115 α -D-glucuronidases are reported to release 4-*O*-methylglucuronic acid from polymeric glucuronoxylan and from glucurono-XOS.

Carbohydrate esterases

The CAZy database covers 16 different carbohydrate esterase (CE) families (Cantarel et al., 2009). Within these families acetylxyylan esterases, acetyl esterases, chitin deacetylases, peptidoglycan deacetylases, feruloyl esterases, pectin acetyl esterases, pectin methylesterases and glucuronoyl esterases are present (Biely, 2012). For the purpose of this research, only acetyl xylan esterases and feruloyl esterases are described. These esterases belong to the families CE1, 2, 3, 4, 5, 6, 7 and 16.

Acetyl xylan esterases (AXEs) cleave the ester bonds between the acetyl residue and the xylopyranosyl residue, as shown in Figure 7. Various acetyl xylan esterases are shown to be selective towards certain xylan structures. For example, the AXE from *Trichoderma reesei* (CE5) is more active towards acetylated polymeric xylan compared to acetylated XOS. It also has a preference towards cleaving the acetylated *O*-2 position of the xylosyl residue (Poutanen et al., 1990). Another AXE, also from *Trichoderma reesei* (CE 16), shows a preference to cleave off the acetyl residues linked to the *O*-3 or to the occasionally acetylated *O*-4 position of a xylosyl residue (Biely et al., 2011).

Feruloyl esterases (FAEs; EC 3.1.1.73) belong to a subclass of carboxylic esterases (EC 3.1.1). These esterases cleave the ester bonds between hydroxycinnamic acids esterified to arabinoxylans (Figure 7) and to certain pectins present in plant cell walls (Wong, 2006). A more elaborate classification based on substrate utilization and supported by primary sequence identity has been proposed to consist of four subclasses: types A, B, C and D. Type A is active on methyl ferulate (MFA), methyl *p*-coumarate (MpCA) and methyl sinapate (MSA). FAEs of type B are specific for MFA, MpCA and methyl caffeate (MCA), but not against MSA. These enzymes do not release diferulic acid and show sequence similarities to carboxylic esterase family 1 (CE1). Types C and D act on all four hydroxycinnamic acid methyl esters. Type C enzymes do not release diferulic acids from synthesized models like MpCA or MSA and complex substrates (arabinoxylans) (Crepin et al., 2004), whereas type D enzymes are able to release diferulic acids from arabinoxylan oligomers (Bartolomé et al., 1997).

1.5 Aim and outline of the thesis

The aim of this thesis is to characterise recalcitrant structures of mainly sugar cane bagasse and of empty fruit bunches remaining after pretreatments and to understand underlying reactions occurring. To this end, also pretreatment of barley straw is studied. In addition, it is aimed to study the mode-of-action of single-activity xylan degrading enzymes, all from *Rasamsonia emersonii*.

Chapter 2 describes the characterization of xylan structures from oil palm empty fruit bunch. The performance of oxidative delignification is compared to the conventional alkaline extraction of xylan in order to characterize the xylan by enzymatic fingerprinting. In **Chapter 3**, sugarcane bagasse, barley straw and oil palm empty fruit bunch, are pretreated using different concentrations of H_2SO_4 and NaOH . Carbohydrate mass balances and the enzymatic saccharifications of the residual glucan and xylan are shown. The effectiveness of the two types of pretreatments performed is assessed according to the yields of glucose and xylan obtained after enzymatic hydrolysis. **Chapter 4** describes the influence of NaOH pretreatments on the enzymatic saccharification of glucan and xylan from sugarcane bagasse. Various NaOH dosages are tested at different temperatures and residence times. NaOH residues are analysed for glucan, xylan and lignin contents. The data obtained are fitted into an empirical model. Of the same set of samples, the residual lignin structures are investigated in **Chapter 5**. Lignin units are analysed in all residues, and changes in their relative abundance of the main units are correlated with the enzymatic conversion of glucan. Amounts of residual xylan are also correlated with the enzymatic conversion of residual glucan. In **Chapter 6** the purification and characterization of two fungal α -glucuronidases produced by *Rasamsonia emersonii* is described. The results obtained in the above mentioned chapters are discussed in **Chapter 7**.

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Chapter 2

Delignification outperforms alkaline extraction as pretreatment for enzymatic fingerprinting of xylan from oil palm empty fruit bunch

ABSTRACT

Enzyme hydrolysed (hemi-) celluloses from oil palm empty fruit bunches (EFBs) are a source for production of bio-fuels or chemicals. In this study, EFB hemicellulose structures were characterized after either delignification or alkaline extraction. Endo-xylanase hydrolysis helped this structural characterisation.

Delignification of EFB facilitated the hydrolysis of EFB-xylan by a pure endo- β -1,4-xylanase, because it pushed the enzymatic hydrolysis of non-extracted xylan up to 91% (w/w) compared to less than 4% (w/w) prior to delignification. From the xylan present in the alcohol insoluble fraction (AIS) of EFB, after delignification and endo-xylanase hydrolysis, 21% was annotated by HPAEC as (acetylated) xylo-oligosaccharides. Without delignification this percentage was only around 5% (w/w). Removal of acetyl groups by alkali provoked xylan aggregation, hindering its enzymatic hydrolysis. From the analysis of the oligosaccharide-fingerprint of EFB xylan, the structure was defined as acetylated 4-*O*-methylglucuronoxylan. It is concluded that delignification alone outperforms alkaline extraction as pretreatment for enzymatic fingerprinting of EFB xyans.

P. Murciano Martínez, M. A. Kabel, H. Gruppen.

2.1 Introduction

Plant residues from agriculture, forestry and industry are accounted as a source of monosaccharides, to be released from the plant polysaccharides, for production of biofuels and chemicals (Cherubini, 2010). Oil palm empty fruit bunches (EFBs), which are the empty bunches remaining after removal of the oil-rich berries, are an example of such plant residues. EFB represents most of the residues from oil palms, and about 15.8 MT is produced per year worldwide (Sumathi et al., 2008). The oil palm (*Elaeis guineensis*) belongs to the *Arecaceae* family, which is part of the monocot class or true grasses. EFBs contain about 35% (w/w) cellulose, 25% (w/w) hemicellulose and 25% (w/w) lignin (Escarnot et al., 2011). Structures of EFB hemicellulose have not been described in detail. Nevertheless, based on its published carbohydrate composition, it can be assumed that the hemicellulose component is mainly composed of xylans (Sun et al., 1999). The latter is in agreement with the fact that hemicelluloses in grasses predominantly consist of glucuronoarabinoxylans (GAX). In grasses, the backbone of GAX consists of β -D-(1-4)-linked xylopyranosyl units that can be substituted at the O2, O3 or both positions with α -L-arabinofuranosyl residues or acetyl residues and/or at the O2 position with (4-O-methyl)- α -D-glucuronyl acid residues (Ebringerová et al., 2005).

Sun et al. (1999) reported on the characterisation of EFB hemicelluloses at the level of total sugar composition of the different fractions obtained after alkaline extraction. In that study, the alkaline extraction, which is generally performed to extract hemicelluloses, only yielded 50% (w/w) of the total xylans present in EFBs. As a comparison, for other grass-like feedstocks, like wheat straw, the extracted xylans studied represented 70-90% of all xylans present in the feedstock (Fang et al., 1999; Escarnot et al., 2011; Sun et al., 2013). In addition, the alkaline extraction used does not allow analysis of acetyl substituents due to saponification reactions provoked by the alkaline conditions. Hence, in our research, it is aimed at finding a good pretreatment to perform a representative enzymatic fingerprinting of the acetylated EFB xylan structure.

Various phenomena may underlie extraction efficiencies. First, the type and distribution of substituents along the xylan backbone influence the solubility of xylan, which will influence the extraction yield. Second, the amount and type of lignin differs per feedstock and may influence the extractability of xylans. Lignin is known to covalently crosslink the plant cell wall polysaccharides (cellulose and xylan) together, thereby forming a compact network (Du et al., 2014).

In the present study, EFB xylans are analysed including their acetyl substituents. Hereto, the EFB was delignified with peracetic acid prior to enzymatic fingerprinting of the EFB xylan, using a pure endo- β -1,4-xylanase. The delignification

method is compared to the conventional alkaline extraction of xylans for enzymatic fingerprinting.

2.2 Materials and methods

Materials used

Oil palm empty fruit bunches (EFBs) were kindly provided by Sime Darby (Kuala Lumpur, Malaysia). From a continuous palm oil crusher mill, during one hour five EFB-batches of 200 g each were collected (one per 10 minutes). The batches were dried at 40°C for 24 hours. Dried EFB-batches were milled (<1mm) in a 50 mL ball mill cuvette (Retsch, Haan, Germany) and equal amounts of all five batches were mixed to obtain the EFB-sample used. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) if not otherwise specified.

Fractionation of EFB

The EFB-sample (EFB dried milled fibres) was fractionated according to the scheme shown in Figure 1. First, EFB (35 g) was defatted by petroleum ether using a Soxhlet-unit (Gerhardt, Bonn, Germany). The defatted EFB was dried at 20°C overnight. Next, the material (33 g) was suspended in 70% (v/v) aqueous ethanol (1 L) and stirred at 20°C for 1 hour. After centrifugation (10000×g; 15min; 20°C), the supernatant was discarded and the residue was again suspended in 70% (v/v) ethanol (1 L), stirred at 20°C for 1 hour and centrifuged. Again, the supernatant was discarded. The final residue was dried at 20°C overnight, freeze dried and encoded AIS. Part of the AIS was delignified using peracetic acid (3 g peracetic acid/g dry solids; 25°C; 24h) as described by Kumar et al. (2013). Both the delignified fraction (DAIS) and the non-delignified AIS were sequentially extracted with 1 and 4 M NaOH, each containing 1% (w/w) NaBH₄, at 4°C (24h per extraction). After extraction, the supernatants obtained, denoted 1Mss and 4Mss, were separated from the insoluble residues (1M and 4MRes) by centrifugation (10000×g; 15min; 20°C). After centrifugation, all fractions were neutralised (pH 5.0) using glacial acetic acid, dialysed against demineralised water and freeze dried. Fractions encoded as D1Mss, D4Mss and D4MRes were prepared from DAIS.

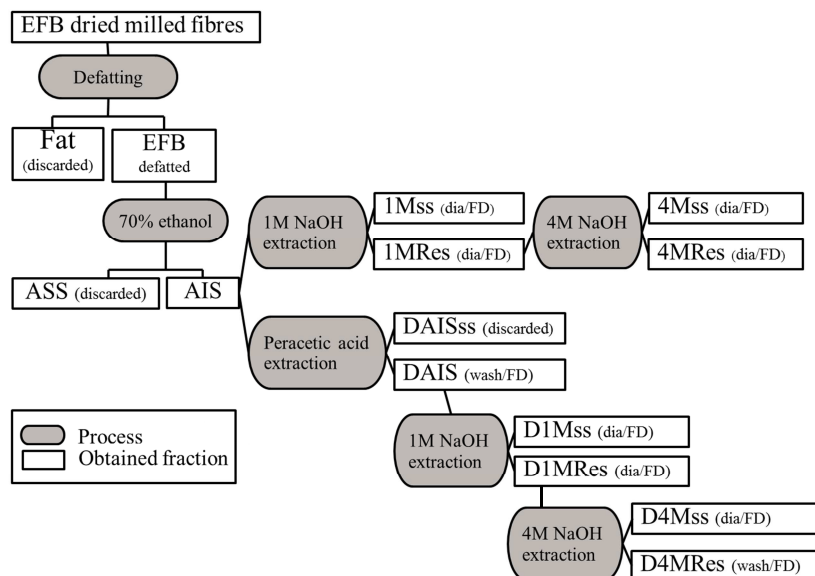


Figure 1. Schematic overview of xylan fractionation from empty fruit bunch (dia= dialyzed, FD= freeze dried).

Enzyme purification

An endo-xylanase from *Rasamsonia emersonii* (ReGH10), expressed and produced in *A. niger* ISO527 as described previously (Neumüller et al., 2015), was kindly provided by DSM Biotechnology Center (Delft, The Netherlands) and purified. Hereto, the enzyme was fractionated using a Superdex 200 26/60 column (GE Healthcare, Uppsala, Sweden) eluted with 20mM Tris HCl buffer pH 7.0 containing 0.15M NaCl at 20° C, over 5 column volumes at 6mL min⁻¹. Fractions (3 mL) collected were concentrated using 10 kDa Amicon filters (Merck-Millipore, Billerica, MA, USA). ReGH10 containing fractions were selected based on activity towards beechwood xylan (see *Enzyme hydrolysis*) and SDS-page analysis (selection based on the expected molecular mass of 55kDa. The fractions containing ReGH10 were pooled and again fractionated with the same column and gradient.

Enzyme hydrolysis

EFB fractions, beechwood xylan and birchwood xylan were incubated in duplicate with the purified ReGH10 (or with fractions of ReGH10 during purification) in 10mM NaOAc buffer, pH 4.5 (1 mL, 10 mg substrate dry matter) at 55°C during 24 hours. The (purified) ReGH10 was dosed at 0.2% (w/w) protein per weight of substrate added. After incubation, the samples were heated at 100°C for 10 minutes

and centrifuged (10000×*g*; 10 minutes; 10°C) prior to analysis. The supernatants obtained were analysed for dry matter, carbohydrate and acetyl ester contents. Oligosaccharide profiles were obtained by HPAEC and MALDI-TOF MS.

Protein content

To analyse protein contents, the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) was used with bovine serum albumin (BSA) as calibration.

SDS-PAGE

The protein purity was analysed by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Therefore, proteins were reduced with β-mercaptoethanol, heated for 10min and loaded on 12% polyacrylamide gels (Mini-PROTEAN TGX Gels, Bio-Rad Laboratories, Hempel Hempstead, UK). In addition, a protein marker (Protein All Blue Standards, Bio-Rad Laboratories) was loaded for mass calibration. Gels were stained with the EZBlue Gel Staining Reagent (Sigma Aldrich, Steinheim, Germany).

Carbohydrate content and composition, including acetyl ester and lignin contents

The neutral carbohydrate content and composition, including uronic acid content, and the contents of acetyl esters and lignin (acid soluble and acid insoluble (Klason) lignin) were determined as previously described by Murciano Martinez et al. (2015).

High-performance anion exchange chromatography (HPAEC)

Monosaccharides (xylose, glucose, arabinose, galactose, mannose) present in the supernatants after enzymatic incubation were analysed directly and after subsequent trifluoroacetic acid hydrolysis (TFA) (100°C, 1h) by HPAEC as described by Murciano Martinez et al. (2015).

Oligosaccharides present in the supernatants were analysed by HPAEC performed on an ICS-5000 unit (Dionex, Sunnyvale, CA, USA) using a CarboPac PA1 column (2 × 250 mm) with a guard column (Dionex) and PAD detection. Samples (25 µL) were eluted using the following elution profile: 1-30 min, water; 30-45 min, 0.1 M NaOH-0.4 M NaOAc in 0.1 M NaOH; 45-50 min, 1 M NaOAc in 0.1 M NaOH; 50-58 min, 0.1 M NaOH; 58-73 min (equilibration). Elution was performed at 0.3 mL min⁻¹ at 25°C. All analyses were carried out in duplicate. For quantification, xylose, xylo-oligomers (XOS) with a degree of polymerization (DP) of 2 to 6 (Megazyme, Wicklow,

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Ireland) and glucuronic acid were used as standards in at least 5 increasing concentrations between 10 and 50 $\mu\text{g mL}^{-1}$.

Analytical pyrolysis GC-MS

Pyrolysis of EFB extracted fractions (100 μg) was performed with an EGA/PY-3030D micro-furnace pyrolyzer (Frontier Laboratories, Fukushima, Japan) connected to a 7820A (Thermo Scientific, Waltham, MA, USA) gas chromatograph using a DB-1701 fused-silica capillary column (60 m \times 0.25 mm internal diameter, 0.25 μm film thickness) and a Thermomass selective detector (EI at 70 eV). The pyrolysis was performed at 500°C. The oven temperature was programmed from 45°C (0-4 min) to 280°C (5-60 min) at 4°C min⁻¹. Helium was the carrier gas (1 mL min⁻¹). The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and those reported in literature (Ralph, 1991). Peak molar areas were calculated for the lignin-degradation products, the summed areas were normalized. Data of each peak from three repetitive analyses were averaged and expressed as percentages. The relative standard deviation for the pyrolysis data was less than 5%.

Matrix-Assisted Laser Desorption/Ionisation Time Of Flight (MALDI-TOF) mass spectrometry

MALDI-TOF mass spectrometry was performed as previously described by Murciano Martínez et al. (2015).

Calculations of xylan blocks for fingerprinting of EFB-xylans

All EFB-fractions (prior to enzyme incubation) were hydrolysed using H_2SO_4 and analysed for the contents of total xylosyl residues ($[\text{X}_{\text{tot}}^{\text{F}}]$) and 4-*O*-methylglucuronic acids ($[\text{UA}_{\text{me}}^{\text{F}}]$). The content of acetyl esters ($[\text{Ac}^{\text{F}}]$) was also determined. The methods used are mentioned in the carbohydrates content and composition section.

The supernatants (SN) obtained after incubation with ReGH10 and subsequent centrifugation were analysed by HPAEC. Xylose was not present in any of the SNs. Xylo-oligosaccharides (XOS) with a DP of 2 to 6 ($[(\text{X}_2^{\text{SN}} + \text{X}_3^{\text{SN}} + \text{X}_4^{\text{SN}} + \text{X}_5^{\text{SN}} + \text{X}_6^{\text{SN}})]$) were quantified (mol L^{-1}). In the SN of ReGH10 hydrolysed DAIS all X_2 to X_6 were acetylated. Therefore, the contents of X_2 to X_6 analysed in this particular SN were presented as $[\text{X}_2\text{Ac}^{\text{SN}} + \text{X}_3\text{Ac}^{\text{SN}} + \text{X}_4\text{Ac}^{\text{SN}} + \text{X}_5\text{Ac}^{\text{SN}} + \text{X}_6\text{Ac}^{\text{SN}}]$. In addition, all SNs were hydrolysed with TFA and subsequently analysed by HPAEC (see HPAEC section) for contents of total xylosyl residues ($[\text{X}_{\text{tot}}^{\text{SN}}]$) and 4-*O*-methylglucuronic acids ($[\text{UA}_{\text{me}}^{\text{SN}}]$). The SNs

were analysed for acetyl ester content as well ($[Ac^{SN}]$). For the Res, the contents of total xylosyl residues ($[X_{tot}^{Res}]$), 4-*O*-methyl glucuronic acids ($[UA_{me}^{Res}]$) and acetyl esters ($[Ac^{Res}]$), was calculated as the difference of the amount present in the EFB-fraction and the amounts present in the respective SNs (after enzymes).

The data obtained were grouped on the basis of main substituent present and the latter were used to define xylan blocks. The following five blocks were defined in the SN and Res of enzyme treated fractions: linear xylan blocks larger than DP 6 ($\sum_{n=6}^{+\infty} X_n$), acetylated xylan blocks larger than DP 6 ($\sum_{n=6}^{+\infty} X_n Ac$), xylosyl residues linked to 4-*O*-methyl glucuronic acid (XUA_{me}), acetylated xylo-oligosaccharides from DP 2 to 6 ($\sum_{n=2}^6 X_n Ac$) and linear xylo-oligosaccharides from DP 2 to 6 ($\sum_{n=2}^6 X_n$). It should be noted that $\sum_{n=2}^6 X_n Ac$ and $\sum_{n=6}^{+\infty} X_n Ac$ was only applied to AIS and DAIS, as explained above, and that to all other EFB-fractions $\sum_{n=2}^6 X_n$ was applied.

The xylan blocks were now calculated based on the analysed values according to the equations 1 to 5 presented below. For the XUA_{me}^{SN} and XAc molar ratio of 1 to 1 of glucuronic acids or acetyl esters to xylosyl residues, respectively, was assumed. All concentrations are in mol L⁻¹. The distribution of the different xylan blocks over the SN and Res was calculated as percentage based on the total xylosyl residues analysed $[X_{tot}^F]$.

1. $\sum_{n=6}^{+\infty} X_n = [X_{tot}^{SN \text{ or } Res}] - \sum_{n=6}^{+\infty} X_n Ac - (\sum_{n=2}^6 X_n) - (\sum_{n=2}^6 X_n Ac) - XUA_{me}^{SN \text{ or } Res}$
2. $\sum_{n=6}^{+\infty} X_n Ac = XAc^{SN \text{ or } Res} - (\sum_{n=2}^6 X_n Ac)$
3. $XUA_{me}^{SN} = [UA_{me}^{SN}]$; $XUA_{me}^{Res} = [UA_{me}^{Res}]$
4. $\sum_{n=2}^6 X_n Ac = [X_2 Ac^{SN}] + [X_3 Ac^{SN}] + [X_4 Ac^{SN}] + [X_5 Ac^{SN}] + [X_6 Ac^{SN}]$
5. $\sum_{n=2}^6 X_n = [X_2^{SN}] + [X_3^{SN}] + [X_4^{SN}] + [X_5^{SN}] + [X_6^{SN}]$

Using:

6. $[X_{tot}^{Res}] = [X_{tot}^F] - [X_{tot}^{SN}]$; $[UA_{me}^{Res}] = [UA_{me}^F] - [UA_{me}^{SN}]$; $[Ac^{Res}] = [Ac^F] - [Ac^{SN}]$
7. $XAc^{SN} = [Ac^{SN}]$; $XAc^{Res} = [Ac^{Res}]$

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2.3 Results and discussion

General composition of EFB

The composition of the EFB milled fibres is presented in Table 1. EFB contains 30 (± 2) % w/w glucans and 33 (± 0) % w/w glucuronoarabinoxylans (GAX), which is defined as the sum of the xylosyl, arabinosyl and uronic acids residues. Acetyl esters (7 \pm 0.1 % w/w) are known be part of GAX in grasses, but can also be attached to the lignin (Martínez et al., 2008) and, therefore, not included in the GAX content. The amount of lignin, being the sum of acid soluble and acid insoluble lignin, present in EFB was 24 (\pm 0.1) % (w/w).

Table 1. Composition of empty fruit bunches (EFB), expressed as grams of compound per 100 grams of dry matter.

Component	Content (% w/w) ^a	Component	Content (% w/w) ^a
Glucan	30 \pm 2	Acid insoluble lignin ^d	23 \pm 0.1
GAX ^b	26	Syringyl units (S) ^e	9 \pm 0.5
Uronic acids ^{b,c}	4 \pm 0.1	Guaiacyl units (G) ^e	5 \pm 0.8
Arabinose ^{b,c}	2 \pm 0.1	Hydroxyphenyl units (H) ^e	9 \pm 1
Xylose ^{b,c}	20 \pm 0.1	Acid soluble lignin	1 \pm 0.1
Acetyl esters	7 \pm 0.1	Protein (%Nx6.25)	3 \pm 0.1
Other carbohydrates	2	Lipids	5 \pm 0.1
Mannose ^c	1 \pm 0.1	Ash	5 \pm 0.1
Galactose ^c	1 \pm 0.1		

^aExpressed on dry matter basis.

^bGAX= Glucuronoarabinoxylan; sum of xylosyl, arabinosyl and uronic acid residues.

^cCarbohydrates are presented as polymers.

^dKlason lignin, corrected for ash content.

^e% calculated from the % of molar area (py-GC/MS) based on the acid insoluble lignin content as 100%.

These results correspond with previous data (Hamzah et al., 2011; 2013; Sun et al., 1999). The content of arabinosyl residues (2% (w/w)) was similar to those of other grass feedstocks, like barley straw and sugarcane bagasse (Murciano Martínez et al., 2015). On the other hand, the contents of uronyl and acetyl substituents, 4 and 7% (w/w) respectively, were up to two times higher than those found for corn stover, sugarcane bagasse or barley straw (Appeldoorn et al., 2010; Murciano Martínez et al., 2015; Van Eylenet al., 2011).

The content of lignin present in EFB was similar to those in barley straw and sugarcane bagasse (Murciano Martínez et al., 2015). However, comparing the contents

of the lignin units in EFB lignin, as determined by pyrolysis-GC/MS (Table 1), the proportion of syringyl units in the EFB lignin (around 40%) is up to two times higher than those in sugarcane bagasse and barley straw (Murciano Matinez et al., 2015). S-units are composed of a highly substituted monolignol ring, providing a more open structure in the polymeric lignin when compared to guaiacyl units (Davison et al., 2006). The same proportion of hydroxyphenyl as of S- units was present in EFB lignin. However, hydroxyphenyl units are commonly found in grasses as product of the decarboxylation of *p*-coumaric acid during pyrolysis (Del Río et al., 2015). The *p*-coumaric acid is normally attached via ester linkages to S-units. They are considered as pendant units, and not as part of the core lignin.

EFB delignification and characterization of extracted hemicelluloses

First, EFB was defatted and extracted with ethanol resulting in AIS. Part of the AIS was delignified, resulting in DAIS. Subsequently, sequential extraction of both EFB AIS and DAIS (Figure 1) was performed. The dry matter yield and carbohydrate contents of all fractions are presented in Table 2.

Table 2. Dry matter yields of fractions, carbohydrate content and the degree of substitution (DS) of xylan of EFB and associated fractions.

	Dry matter yield w/w %		Carbohydrate content (w/w %) ^a							DS ^b		
	relative to EFB	relative to AIS	Ara ^c	Xyl ^c	Man ^c	Gal ^c	Glc ^c	UA ^c	Total	Ara	UA	Ac
EFB	100	-	1±0	20±0	1±0	1±0	29±2	3±0	57±1	10	15	25
EFB _(defatted)	96	-	2±0	19±0	1±0	1±0	28±2	4±0	55±2	10	15	25
AIS	76	100	2±0	23±0	1±0	1±0	33±0	4±0	64±0	7	12	25
DAIS	58	77	1±0	23±1	1±0	1±0	40±0	4±0	70±1	5	11	20
D1Mss	24	31	3±0	50±0	0±0	1±0	1±0	6±0	61±1	6	9	-
D4Mss	8	11	1±0	29±0	4±0	1±0	14±1	3±0	52±4	3	7	-
D4MRes	33	43	n.d. ^d	2±0	n.d. ^d	n.d. ^d	79±2	2±0	83±2	0	67	-
1Mss	17	22	4±0	37±1	0±0	1±0	1±0	6±0	49±1	10	11	-
4Mss	8	10	2±0	22±1	5±1	1±0	18±4	3±0	51±6	9	11	-
4MRes	50	66	1±0	15±0	n.d. ^c	n.d. ^c	47±1	2±0	65±1	7	7	-

^aExpressed as %(w/w) based on dry matter; rhamnose was not present in the samples.

^bDegree of substitution (DS) expressed as mol substituent per 100 mol of xylosyl residues.

^cAra=arabinose, Xyl=xylose, Man=mannose, Gal=galactose, Glc=glucose, UA=uronic acids. Ac= acetyl esters.

^dn.d.= not detected; under detection limits.

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From this table, it can be seen that the dry matter (DM) yields of EFB_(defatted), AIS and DAIS were, 96%, 76% and 58% (w/w), respectively. The carbohydrate contents were 55%, 64% and 70% (w/w) for EFB_(defatted), AIS and DAIS, respectively. The sequential extraction procedure aimed at extracting GAX from AIS or DAIS. Hence, the weight percentages of GAX (as sum of arabinosyl, xylosyl and glucuronic acid residues) based on DM were calculated from the numbers in Table 2.

These GAX contents were 28, 59, 33 and 4%, based on DM, in the fractions DAIS, D1Mss, D4Mss and D4MRes, respectively. These GAX contents are 29, 47, 27 and 18% in the DAIS, D1Mss, D4Mss and D4MRes fraction. Glucan contents are 1%, 14% and 79% (w/w) for D1Mss, D4Mss and D4MRes, respectively. The glucan content was higher in D4MRes, compared to that 4MRes, which was 47%. From the dry matter yield of the fractions and the carbohydrate content analysed, the distributions of GAX, glucan and total carbohydrates over the fractions were calculated (Figure 2).

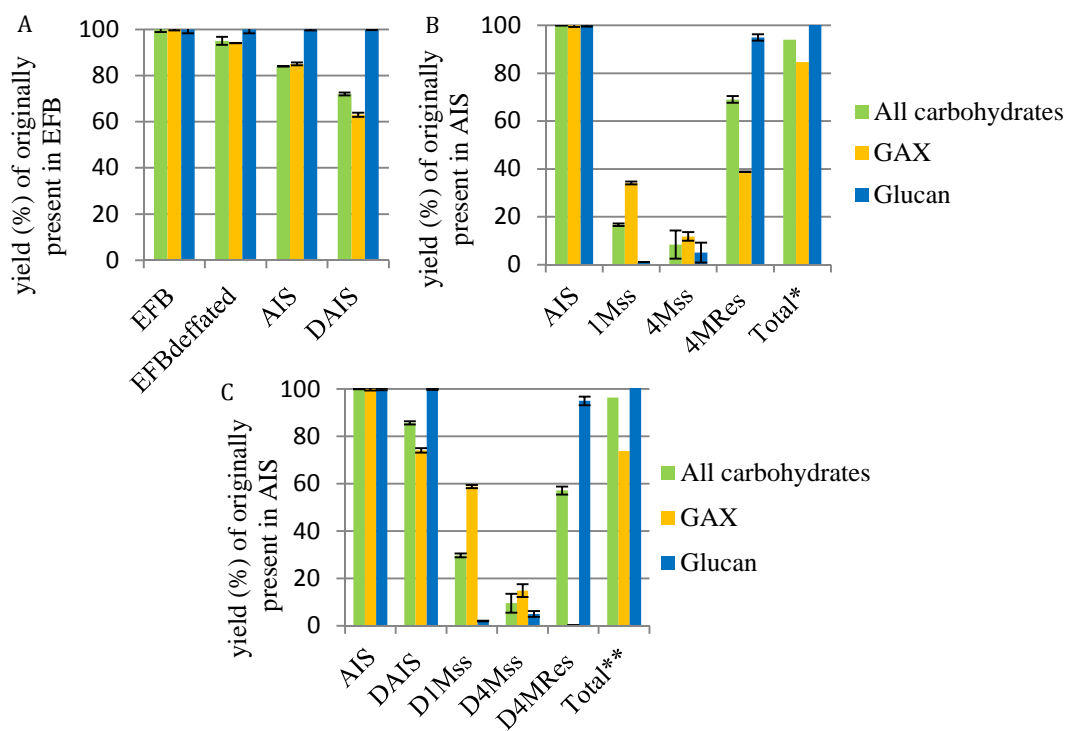


Figure 2. All carbohydrates, glucuronoarabinoxylan (GAX as sum of of arabinosyl, xylosyl and glucuronic acid residues) and glucan yields in fractions from originally present in EFB (A), or from originally present in AIS, with (B) and without delignification (C). *Sum of: 1Mss, 4Mss and 4MRes. **Sum of: D1Mss, D4Mss and D4MRes.

As shown in Figure 2, after defatting and ethanol extraction of EFB, 85% (w/w) of all carbohydrates and of the three monosaccharides from which GAX is constituted remained in AIS. For DAIS, these percentages were 72% and 63%, respectively. Hence, in DAIS, 85% of all carbohydrates and 74% of GAX constituting monosaccharides present in AIS were recovered (Figure 2). So, it was concluded that 15% (w/w) of GAX constituting monosaccharides from originally present in EFB solubilised during the defatting plus ethanol extraction and, in addition, 22% during the treatment with peracetic acid, which was not recovered in DAIS. During ethanol extraction, only small sugars are extracted and not polymeric xylan. Therefore, in Figures 2B and 2C total carbohydrates, GAX and glucan yields are expressed on the basis of AIS. In order to judge the effectiveness of the lignin removal, DAIS was subjected to analytical py-GC/MS analysis that showed no lignin degradation products (no further data shown). Hence, it was concluded that DAIS was devoid of lignin.

AIS and DAIS were sequentially extracted with 1 and 4M NaOH containing 1% (w/v) NaBH₄. From AIS, 37% (w/w) and 13% (w/w) of GAX was extracted in 1Mss and 4Mss, respectively, while 40% (w/w) remained un-extracted in 4MRes (Figure 2). The lacking 10% (w/w) was assumed to be lost due to sample handling during the extraction. From the delignified material, 59% (w/w) and 16% (w/w) of GAX present in the parental AIS was recovered in D1Mss and D4Mss, respectively (Figure 2). The lacking xylan was extracted in the delignification step (around 22% (w/w)) or ended up in extraction losses (around 3% (w/w)). Glucan was mainly present as cellulose, seen from the fact that around 95% of glucan present in AIS was recovered in either 4MRes or D4MRes (Figure 2).

Delignification of AIS resulted in an increase of GAX-yield from 50 to 75% (w/w based on AIS) when extracted sequentially with 1M and 4M NaOH. Furthermore, the carbohydrate composition of D1Mss and D4Mss differed from the composition of the non delignified fractions, 1Mss and 4Mss, (Table 2). These differences were the most reflected in the degree of substitution (DS). Specifically, the total DS (defined as the sum of UA_{me}, Ara and Ac) of AIS (44) was higher than that of DAIS (36). For 1Mss and 4Mss, the total DS were 21 and 20, while for D1Mss and D4Mss the DS were 15 and 10, respectively. Most likely, not only more xylan (Figure 2), but in particular, more linear xylan was extracted after delignification in D1Mss and D4Mss compared to the non delignified fractions.

Further characterisation of the xylans in the various EFB fractions was carried out by enzymatic fingerprinting by using endo- β -1,4-xylanase hydrolysis.

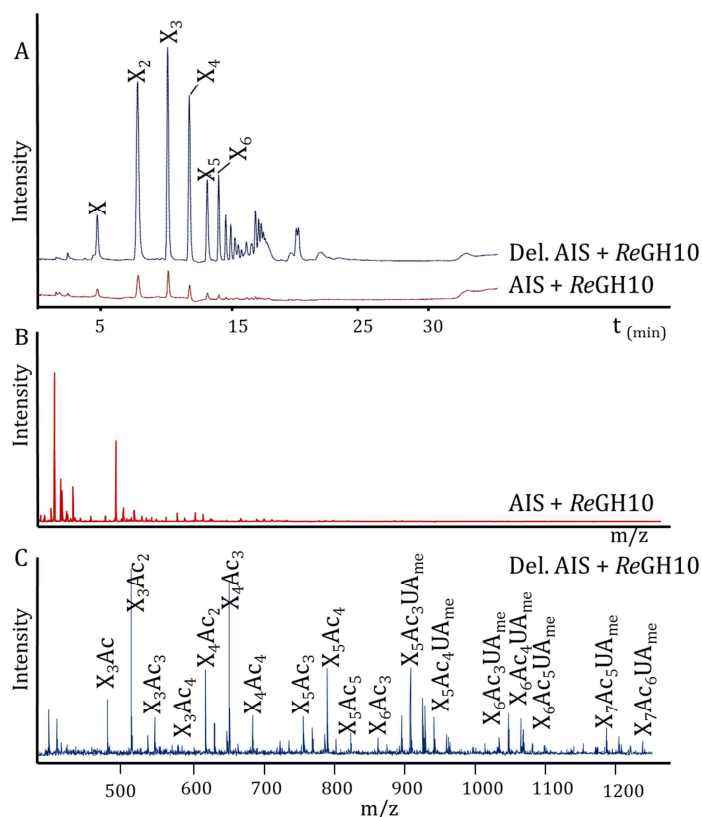


Figure 3. HPAEC chromatograms (A) and MALDI-TOF mass spectra (B, C) of AIS supernatants (SN) from incubated with *ReGH10* (A, B) and DAIS incubated with *ReGH10* (A, C). X=xylose, Ac=acetyl esters, UA_{me}= 4-*O*-methyl uronic acid.

EFB fractions hydrolysed by ReGH10

Figure 3 shows that DAIS hydrolysed by *ReGH10* yielded a remarkable higher content of xylo-oligosaccharides (XOS) compared to the XOS content released by *ReGH10* from AIS. In all *ReGH10* digests the total contents of xylosyl residues ($[X_{\text{tot}}^{\text{SN}}]$), of uronic acids ($[UA_{\text{me}}^{\text{SN}}]$) and acetyl esters ($[Ac^{\text{SN}}]$) present in the SN fractions were determined. The distributions of different xylan blocks over SN and Res were calculated following the equations explained in the materials and methods, and related to the parental fraction (Table 3).

Delignification outperforms alkaline extraction of xylan from EFB

Table 3. Distribution (%) of X_{tot} , X_{2-6} , UA_{me} and Ac in supernatant (SN) and residue (Res) of *ReGH10* hydrolysed EFB fractions.

Fraction	Distribution (%) over SN & Res							
	X_{tot}^a		$\sum_{n=2}^6 X_n^{\text{SN a,b}}$		$UA_{\text{me}}^{\text{a,b}}$		$Ac^{\text{a,b}}$	
	$X_{\text{tot}}^{\text{SN}}$	$X_{\text{tot}}^{\text{Res}}$	$\sum_{n=2}^6 X_n^{\text{SN}}$	$\sum_{n=2}^6 X_n^{\text{Res}}$	$UA_{\text{me}}^{\text{SN}}$	$UA_{\text{me}}^{\text{Res}}$	Ac^{SN}	Ac^{Res}
AIS	4 (1)	96 (22)	100	0 ^c	1(0.1)	99 (3.9)	n.d. ^c	100 (5)
1Mss	43 (16)	57 (21)	100	0 ^c	4(0.2)	96 (5.8)	0	0
4Mss	36 (8)	64 (14)	100	0 ^c	12(0.4)	88(2.6)	0	0
DAIS	91 (21)	9 (2)	100	0 ^c	55(2.2)	45(1.8)	87 (4)	13 (1)
D1Mss	54 (27)	46 (23)	100	0 ^c	23(1)	77 (5)	0	0
D4Mss	50 (15)	50 (15)	100	0 ^c	11(0.3)	89 (2.7)	0	0

^aXylosyl residues (presented as polymers); Numbers between brackets represent the content of xylan per 100g dry matter of Res or SN.

^b X = xylose, UA_{me} = 4-*O*-methyl glucuronic acid, Ac = Acetyl ester.

^cn.d. =under detection limits.

For DAIS, the xylan present in DAIS became almost completely solubilised (91%) upon *ReGH10* hydrolysis (Table 3) compared to only 4% solubilization of AIS. The xylan in the other EFB fractions became solubilized for 36 to 54% by *ReGH10* hydrolysis in a range of 36 to 54% (Table 3). So, delignification with peracetic acid had a strong positive influence on xylan solubilisation by *ReGH10*. Therefore, DAIS was considered as the most representative fraction for enzymatic fingerprinting the EFB xylan by *ReGH10*. In the enzymatic hydrolysate of DAIS, the major soluble xylo-oligomers present were all highly acetylated as seen from the MALDI-TOF mass spectrum (Figure 3C). Non-acetylated xylo dimers to xylohexamers were absent. Therefore, xylo-oligomers quantified by HPAEC (DP 2-6) in the SN from DAIS were all considered to be acetylated.

Next, from the values presented in Table 3 and the equations given in the materials and methods, the distribution of five xylan blocks over SN and Res after *ReGH10* hydrolysis were calculated and shown in Figure 4.

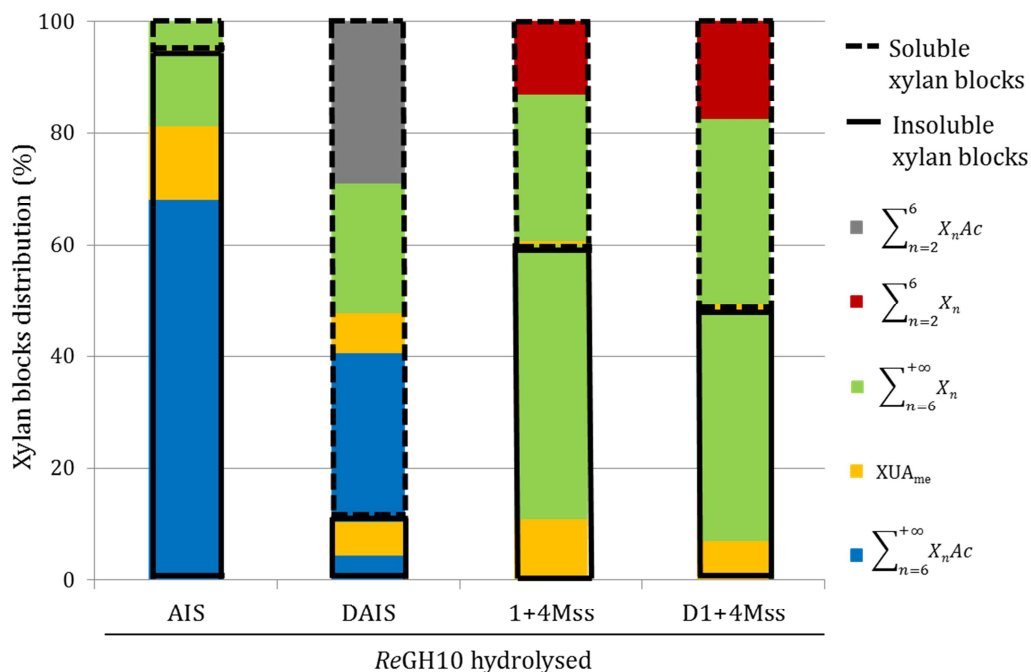


Figure 4. The distribution of xylan blocks of *ReGH10* hydrolysed EFB fractions over supernatants and residues.

The five xylan blocks defined are: linear xylan blocks larger than DP 6 ($\sum_{n=6}^{+\infty} X_n$), acetylated xylan blocks larger than DP 6 ($\sum_{n=6}^{+\infty} X_nAc$), xylosyl residues linked to 4-*O*-methyl glucuronic acid (XUA_{me}^{SN} and XUA_{me}^{Res}), acetylated xylo-oligosaccharides from DP 2 to 6 ($\sum_{n=2}^6 X_nAc$) and linear xylo-oligosaccharides from DP 2 to 6 ($\sum_{n=2}^6 X_n$).

In Figure 4 it can be seen that the 91% of xylan material solubilised from DAIS, as previously defined in the text, was mainly present as acetylated xylo-oligomers (DP 2 to 6) ($\sum_{n=2}^6 X_nAc$; 30%), linear xylan blocks larger than DP 6 ($\sum_{n=6}^{+\infty} X_n$; 24%), acetylated xylan blocks larger than DP 6 ($\sum_{n=6}^{+\infty} X_nAc$; 30%) and 7% xylosyl residues substituted with 4-*O*-methyl glucuronic acids (XUA_{me}). The acetylated xylo-oligomers (DP 2 to 6) were analysed by HPAEC, while the other blocks were based on both analysis and calculations as provided in the materials and methods. These HPAEC analysed xylo-oligosaccharides represented 30% (w/w) of xylan present in DAIS (Figure 4), which corresponded (Figure 2) to 21% (w/w) of xylan present in AIS. The 10% residual xylan contained 3% acetylated blocks ($\sum_{n=2}^6 X_nAc$), 6% xylosyl residues substituted with 4-*O*-methylglucuronic acids (XUA_{me}) (Figure 4).

Interestingly, the fingerprint of *ReGH10* treated DAIS allowed the characterisation of the xylan including almost all (80% (w/w) from present in AIS) acetyl ester substituents.

Following the conventional combination of alkaline extraction and enzyme hydrolysis, *ReGH10* dissolved only 40% of the xylan present in the sum of both extracts (1+4Mss; Figure 4), partitioned as 13% annotated by HPAEC as XOS (DP 2 to 6) and the rest as longer xylo-oligosaccharides. The annotated fraction represented around 5% of xylan present in AIS (data not shown). This low annotated proportion might be related with the fact that acetyl substituents were removed by the alkali used resulting in aggregation of linear xylan-blocks, as has been observed previously (Ebringerova et al., 1994). Aggregation will limit the endo-xylanase degradation. Delignification and conservation of acetyl groups not only enhanced solubility and degradability of the AIS fraction, but it also provided a more realistic picture of the GAX structure of EFB.

2.4 Conclusion

Successful characterization of hemicelluloses from EFB was achieved after delignification with peracetic acid and *ReGH10* fingerprinting. Specifically, the composition of the DAIS xylan after *ReGH10* hydrolysis was: 23% of linear xylan blocks, 64% of acetylated xylan blocks and 13% of 4-*O*-methyl glucuronic acid substituted xylan blocks. The removal of lignin outperformed the conventional alkaline extraction for enzymatic fingerprinting of GAX from EFB, now defined as acetyl 4-*O*-methylglucuronic acid.

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Chapter 3

Importance of acid or alkali concentration on the removal of xylan and lignin for enzymatic cellulose hydrolysis

ABSTRACT

The effect of hemicellulose and lignin solubilisation by H_2SO_4 and NaOH catalysed pretreatments was correlated to the extent of subsequent enzymatic cellulose hydrolysis. Three different grass-type feedstocks, palm empty fruit bunch, sugarcane bagasse and barley straw, were investigated. Soluble fractions after catalysis were characterised for mono- and oligosaccharides contents, while the residues were analysed for constituent monosaccharides composition.

Alkali pretreatment resulted into extensive lignin removal. This removal resulted in up to 90% (w/w) conversion of glucan into glucose by enzymes. But, the alkaline conditions also provoked up to 50% unwanted xylan losses. Acid pretreatment resulted into solubilisation (70-80% (w/w)) of xylan with almost no losses, while lignin remained. Although moderate xylan solubilisation increased enzymatic cellulose hydrolysis of residual glucan, extensive removal of xylan decreased it. Therefore, under the treatment conditions, the alkali treatments were the most efficient in terms of enzymatic release of xylose and glucose from the insoluble residues.

Based on: P. Murciano Martínez, R. Bakker, P. Harmsen, H. Gruppen, M.A. Kabel, 2015. Importance of acid or alkali concentration on the removal of xylan and lignin for enzymatic cellulose hydrolysis. *Industrial Crops and Products*, 64, 88-96.

3.1 Introduction

Plant biomass based fuels and chemicals are gaining interest due to the rapidly decreasing amounts in fossil fuel resources. Their production comprises four parts. Harvest/storage of lignocellulosic biomass, pretreatment, enzymatic saccharification and fermentation/modification (Himmel et al., 2007). The mechanisms behind various pretreatments and enzymatic saccharification are not fully understood yet. Interestingly, recent microscopic observations have shown (Ding et al., 2012) that extensive hemicellulose removal resulted in a decreased enzymatic cellulose degradability. In addition, lignin removal was suggested to increase this. Nonetheless, quantitative biochemical evidence of the degradation behaviour of lignin or hemicellulose depleted lignocellulosic residues are limited, although they are expected to be complementary to microscopic observations.

Sugarcane (*Saccharum officinarum*) and barley (*Hordeum vulgare*) belong to the Poaceae family, while oil palm (*Elaeis guineensis*) belongs to the Arecaceae family. All three are monocots or true grasses and are typical lignocellulosic materials composed of around $\pm 25\%$ (w/w) hemicellulose, $\pm 35\%$ (w/w) cellulose and $\pm 25\%$ (w/w) lignin (Escarnot et al., 2011). The hemicelluloses in monocots mainly consist of substituted glucuronoarabinoxylans (GAX). The backbone of GAX consists of β -D-(1-4)- linked xylopyranosyl units that can be substituted with α -L-arabinofuranosyl residues, acetyl residues and/or (4-O-methyl)- α -D-glucuronic acids (Ebringerová et al., 2005). Cellulose consists of β -D-(1-4)- linked glucose units, forming a crystalline polymer. Lignin is an aromatic polymer synthesised from phenylpropanoid precursors.

Chemical pretreatment processes described, resulting in an increased subsequent enzymatic hydrolysis of plant biomass, comprise acid or alkaline based processes. Acid pretreatment aims at solubilisation of hemicellulose leaving the cellulose accessible for enzymatic saccharification (Lloyd & Wyman, 2005). In these pretreatments, most of the lignin remains in the insoluble cellulosic residue. Alkali pretreatments have been proven to solubilise lignin, while hemicellulose remains in the cellulosic residue. Alkali breaks ester-linkages and may cause swelling of the lignocellulosic material, which increases accessibility for enzymes and thereby increases the accessibility for enzymatic hydrolysis (Park & Kim, 2012; Sun & Cheng, 2002). For both acid and alkali treatments, there is a remarkable effect on the composition and structure of the insoluble material. Clearly, the concentration of the acid or alkali is an important factor. At present, comparison of the effect of diluted alkali or acid on enzymatic hydrolysis has been reported (Wilkinson et al., 2014). However, a very limited number of conditions are applied (Giese et al., 2013; Kataria et al., 2013). Or in case of many conditions applied, no detailed quantitative

information is given with respect to carbohydrates present in the different fractions obtained.

The present study evaluates for BS, SCB and EFB, the effect of acid and alkali catalysis at elevated temperatures on their enzymatic hydrolysis. Various concentrations of acid (0-6% (w/w) based on dry matter) and alkali (0-12% (w/w) based on dry matter) are applied. Carbohydrate and lignin mass distributions over insoluble residues and soluble hydrolysates after catalysis are determined. In addition, enzymatic saccharification of the insoluble residues is correlated to the ratio of hemicellulose/lignin/cellulose present in these samples.

3.2. Materials and methods

Materials used

Sugarcane bagasse (95% (w/w) DM), barley straw (92% (w/w) DM) and empty fruit bunch (37% (w/w) DM) were supplied by Purac Thailand (Banchang, Thailand), Unifarm Wageningen UR (Wageningen, The Netherlands) and Sime Darby (Kuala Lumpur, Malaysia), respectively. Enzyme cocktails CellicCTec2 and CellicHTec were provided by Novozymes (Bagsvaerd, Denmark) and stored at 4°C.

Acid & alkali treatments

Stainless steel non stirred reactors (0.1 L) were loaded with a fixed solid:liquid ratio of 1:10 (w/w), and suspensions were just steerable. Residence time for acid and alkali treatments at 140°C and 120°C, were 30 and 60 minutes, respectively. All reactors were equipped with a controlled thermocouple (Pico Technology, Saint Neots, UK). The reactors were introduced into silicon oil preheated at the desired pretreatment temperature. Reactors removed from the oil after pretreatment were directly submerged in ice-water. The maximum heating and cooling times were 22 and 15 min, respectively. After cooling down to ambient temperature, the pretreated mixture was transferred into 0.25 L polypropylene tubes and centrifuged (10000 g, 15 min, ambient temperature). The supernatant was decanted and the residue was recovered and washed 3-4 times with water, until the pH of the suspension was neutral. For each raw material, the first supernatant and 3 wash supernatants were combined and freeze dried (denoted as "soluble fraction"). The remaining washed residues were denoted as "residues". All samples obtained, including their pretreatment conditions, are shown in Table 1.

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Table 1. Catalysis conditions, including acid or alkali load, temperature, residence time, inhibitors formed and residual xylan and glucan.

	H ₂ SO ₄ (w/w%) DM	NaOH (w/w%) DM	T (°C)	t (min)	pH before	pH after	Inhibitors ^b (g L ⁻¹)	Xylan content in residue (w/w%) DM ^a	Glucan content in residue (w/w%) DM ^a	Glucan degraded to glucose by CTec2 from residue(w/w%) DM	Xylan degraded to xylose by CTec2 from residue(w/w%) DM	Lignin content in residue (w/w%)DM ^a
Empty fruit bunch	0	-	140	30	6.6	5.3	n.a. ^c	23.9	35.5	-	-	46.6
	1	-			4.2	4.5	n.a.	23.1	35.0	9.8 ± (3.5)	11.9 ± (1.8)	n.a.
	2	-			2.9	3.6	n.a.	19.6	35.2	18.1 ± (2.4)	18.0 ± (1.1)	44.3
	3	-			2.3	2.9	n.a.	18.7	35.5	13.7 ± (0.9)	19.2 ± (0.8)	n.a.
	4	-			2.1	2.3	n.a.	17.4	34.3	20.8 ± (0.8)	25.1 ± (0.4)	34.0
	5	-			2.1	2.1	n.a.	13.4	29.7	37.0 ± (4.9)	30.3 ± (2.7)	n.a.
	6	-			1.8	2.0	n.a.	11.9	34.9	24.0 ± (1.9)	26.0 ± (1.3)	43.1
	-	0	120	60	7.3	5.9	n.a.	21.4	32.2	8.3 ± (1.3)	11.6 ± (0.5)	25.7
	-	2			12.2	7.2	n.a.	21.8	34.7	8.8 ± (1.0)	12.0 ± (0.5)	24.2
	-	4			12.6	7.7	n.a.	22.8	35.3	16.8 ± (0.5)	19.3 ± (0.5)	n.a.
	-	6			12.9	9.5	n.a.	20.9	30.0	47.6 ± (2.6)	38.5 ± (1.9)	17.8
	-	8			13.0	11.7	n.a.	24.9	33.2	62.3 ± (5.4)	38.1 ± (3.6)	n.a.
	-	10			13.0	12.4	n.a.	23.3	36.8	60.6 ± (2.0)	40.6 ± (1.1)	17.0
	-	12			13.0	12.5	n.a.	23.5	31.8	69.5 ± (18.2)	48.5 ± (8.1)	30.4

Importance of acid and alkali concentrations for the conversion of cellulose

Continuation Table 1. Catalysis conditions, including acid or alkali load, temperature, residence time, inhibitors formed and residual xylan and glucan

	H ₂ SO ₄ (w/w%) DM	NaOH (w/w%) DM	T (°C)	t (min)	pH before	pH after	Inhibitors ^b (g L ⁻¹)	Xylan content in residue (w/w%) DM ^a	Glucan content in residue (w/w%) DM ^a	Glucan degraded to glucose by CTec2 from residue(w/w%)DM	Xylan degraded to xylose by CTec2 from residue(w/w%) DM	Lignin content in residue (w/w%)DM ^a
Sugarcane bagasse	0	-	140	30	3.2	3.4	0.7	16.9	38.6	24.0 ± (1.6)	32.6 ± (1.0)	46.7
	1	-			2.2	2.4	1.5	11.1	44.2	25.1 ± (0.4)	39.2 ± (0.2)	n.a.
	2	-			2.1	2.2	2.0	8.4	39.8	34.5 ± (1.7)	46.4 ± (0.6)	20.0
	3	-			2.1	2.1	2.3	6.6	44.7	26.1 ± (1.4)	40.8 ± (1.4)	n.a.
	4	-			2.0	1.7	2.7	6.5	43.2	29.0 ± (1.6)	45.2 ± (2.1)	27.4
	5	-			1.9	1.6	2.7	5.4	43.2	23.2 ± (0.6)	42.4 ± (1.1)	n.a.
	6	-			1.8	1.5	3.3	5.0	48.3	20.3 ± (1.3)	38.2 ± (0.5)	25.3
	-	0	120	60	3.2	3.3	0.4	17.3	39.2	22.8 ± (1.4)	32.1 ± (0.8)	25.1
	-	2			12.4	6.0	0.9	18.0	36.3	28.2 ± (0.3)	33.9 ± (0.1)	21.2
	-	4			12.8	7.1	1.5	17.3	39.8	29.1 ± (9.0)	35.3 ± (6.6)	n.a.
	-	6			13.0	8.6	1.9	15.7	44.2	30.8 ± (1.3)	40.3 ± (0.8)	17.8
	-	8			13.1	10.3	2.0	13.5	37.9	60.6 ± (1.2)	63.1 ± (0.4)	n.a.
	-	10			13.1	11.5	2.2	14.9	41.1	46.5 ± (0.4)	50.1 ± (0.7)	n.a.
	-	12			13.1	12.5	2.3	14.8	42.7	44.3 ± (11.2)	52.3 ± (5.8)	15.0

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Continuation Table 1. Catalysis conditions, including acid or alkali load, temperature, residence time, inhibitors formed and residual xylan and glucan.

	H ₂ SO ₄ (w/w%) DM	NaOH (w/w%) DM	T (°C)	t (min)	pH before	pH after	Inhibitors ^b (g L ⁻¹)	Xylan content in residue (w/w%) DM ^a	Glucan content in residue (w/w%) DM ^a	Glucan degraded to glucose by CTec2 from residue(w/w%)D M	Xylan degraded to xylose by CTec2 from residue(w/w%) DM	Lignin content in residue (w/w%)DM ^a
Barley straw	0	-	140	30	6.7	5.1	0.9	24.0	33.0	15.1 ± (2.3)	9.0 ± (0.9)	20.8
	1	-			2.3	2.5	0.9	20.0	31.8	39.2 ± (2.1)	31.4 ± (1.2)	n.a.
	2	-			2.1	2.2	0.9	17.0	41.0	28.3 ± (2.1)	17.5 ± (0.6)	21.4
	3	-			2.1	1.9	2.4	11.0	44.0	21.3 ± (2.6)	32.5 ± (2.5)	n.a.
	4	-			1.9	1.7	4.2	10.0	44.0	22.9 ± (0.9)	32.3 ± (0.6)	23.3
	5	-			1.9	1.5	5.6	9.0	50.0	20.1 ± (0.1)	25.9 ± (0.6)	n.a.
	6	-			1.8	1.5	5.6	9.0	54.0	18.4 ± (1.1)	29.7 ± (0.5)	20.4
	-	0	120	60	6.7	5.4	0.7	22.0	40.0	9.4 ± (0.4)	6.1 ± (0.2)	n.a.
	-	2			12.5	7.6	2.3	23.0	41.3	18.2 ± (2.4)	13.5 ± (1.2)	17.7
	-	4			13.1	9.9	2.9	21.0	33.0	63.8 ± (4.8)	50.3 ± (3.4)	n.a.
	-	6			13.3	11.9	3.1	22.0	45.0	57.2 ± (1.5)	62.7 ± (0.4)	17.5
	-	8			13.2	13.0	3.4	21.0	47.0	75.0 ± (3.7)	63.6 ± (3.0)	n.a.
	-	10			13.2	12.9	3.0	21.0	47.0	77.0 ± (3.7)	68.2 ± (0.8)	10.0
	-	12			13.2	13.0	2.6	22.0	55.0	51.9 ± (3.3)	50.0 ± (6.8)	n.a.

Enzymatic hydrolysis

Incubations were performed with CellicCTec2 and with a combination of CellicCTec2 plus CellicHTec (ratio 10:1 (protein (N% \times 6.25) basis). The activities of the enzyme cocktails were 1000 Biomass Hydrolysis Units (BHU)/g and 2500 Fungal Xylanase Units (FXU)/g, respectively. All residues were milled (<1 mm) (MM 2000, Retsch, Haan, Germany) and incubated in 50mM sodium acetate buffer pH 5.5. The milled substrate load was 1% (w/w) based on dry matter. The enzyme incubations were performed in duplicate. Residues from selected samples (0, 2, 4, 6 % (w/w) sulphuric acid based on dry matter and 0, 4, 8, 12% (w/w) sodium hydroxide based on dry matter (Table 1) were incubated at 55°C, and rotated head-over-tail for 72h. Incubation conditions were chosen considering optimum temperature and pH of both enzyme preparations. Incubation time 72h was considered as end-point incubation. Enzyme dosage was in total 3% (w/w) of protein based on dry matter of the amount of residue/sample loaded. The protein concentration (N% \times 6.25) of CellicCTec2 and CellicHTec was 127 mg/ml and 120 mg/ml, respectively. Sodium azide 0.01% (w/v) was used to prevent bacterial growth. After incubation, the digests were heated at 100°C for 5 min, centrifuged (10000g, 5 min, ambient temperature), and analysed for their total carbohydrate content and constituent monosaccharide composition.

Analytical methods

Neutral constituent monosaccharides content and composition

Neutral carbohydrate content and composition was determined using inositol as an internal standard. Samples were pretreated with 72% (w/w) H₂SO₄ (1h, 30°C) followed by hydrolysis with 1M H₂SO₄ for 3h at 100°C. The constituent sugars released were derivatised and analysed as their alditol acetates using gas chromatography (Englyst & Cummings, 1984).

Uronic acid content

Uronic acid content was determined as anhydro-uronic acid by an automated m-hydroxydiphenyl assay (Thibault, 1979), including 0.3% (w/w) tetraborate in the sulphuric acid, with an autoanalyser (Skalar Analytical BV, Breda, The Netherlands). Glucuronic acid was used as reference.

Esterified acetic acid content

Samples (20 mg) were saponified with 1 mL of 0.4M NaOH in isopropanol/H₂O (1:1 v/v) for 3 h at room temperature. The level of acetic acid

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substituents was corrected for the free acetic acid present in non-saponified samples. The acetic acid content was determined with an Ultimate system (Thermo Scientific, Sunnyvale, CA, USA) equipped with a Shodex RI detector and an Aminex HPX 87H column (300 mm x 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA) plus pre-column. Elution was performed by using 5 mM H₂SO₄ at a flow rate of 0.6 mL min⁻¹ at 40°C.

Lignin content

Selected residues were analysed for acid insoluble (Klason) lignin. To each sample of 300 mg (dry matter) 3 ml of 72% (w/w) H₂SO₄ was added and samples were pre-hydrolysed for 1 h at 30°C. After this pre-hydrolysis, 37 ml of distilled water was added and samples were put in a boiling water bath for 3 h and shaken every half hour. Next, suspension was filtered over G4 glass filters (Duran, Mainz, Germany). The residual part was washed until it was free of acid and dried overnight at 105°C. The weight of the dried residual part was taken as a measure of the acid insoluble lignin content, after correction for the amount of ash in this fraction (2.4.6.).

Protein content

Nitrogen content (N%*6.25) was analysed of all raw feedstocks using the combustion (DUMAS) method on a Flash EA 1112 Nitrogen Analyser (Thermo Scientific, Rockford, IL, USA). Methionine (Acros Organics, New Jersey, USA) was used as a standard.

Ash content

Samples (0.5 g) were dried in an oven overnight (105°C), weighed and put in an oven at 504°C overnight. Next day, samples were weighed and the difference between the mass at 105°C and 504°C was taken as ash content.

High-performance anion exchange chromatography (HPAEC)

Monosaccharides (xylose, glucose, arabinose, galactose, mannose, fructose) were analyzed by high-performance anion exchange chromatography (HPAEC). Postcolumn addition was performed on an ICS-3000 unit using a CarboPac PA1 column (2 × 250 mm) (Dionex, Sunnyvale, CA, USA). Samples (25 µL) were eluted isocratically in 30 min with Millipore water. Afterwards, the following elution profile was applied: 30–45 min, 0.1 M NaOH–0.4 M NaOAc in 0.1 M NaOH; 45–50 min, 1 M NaOAc in 0.1 M NaOH; 50–58 min, 0.1 M NaOH; 58–73 min, Millipore water (equilibration) in order to clean and equilibrate the column. A flow of 0.1 mL/ min 0.5

M NaOH was added post column allowing pulsed amperimetric detection. Analysis were performed in duplicate.

MALDI-TOF mass spectrometry

The masses of soluble oligosaccharides present after pretreatment were characterised by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionisation Time Of Flight) mass spectrometry. The analysis was performed using an Ultraextreme instrument (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam II laser of 355 nm and operated in the positive mode. After a delayed extraction time of 350 ns, the ions were accelerated to a kinetic energy of 22 kV and detected using a FlashDetector. The data were collected from averaging 200 laser shots, with the lowest laser energy needed to obtain sufficient spectra. External calibration was performed using maltodextrins (Paselli MD-20, AVEBE, Veendam, The Netherlands). Samples were desalted prior to analysis using AG 50W-X8 Resin (BioRad Laboratories, Hercules, CA, USA). 1 μ L desalted sample was mixed with 1 μ L matrix solution of 10 mg/mL 2,5-dihydroxy-benzoic acid (Bruker Daltonics) in 50% (v/v) acetonitrile and dried under a stream of air.

High performance liquid chromatography (HPLC)

Furfural, HMF, acetic acid, levulinic acid and succinic acid were analysed by using high-performance liquid chromatography. HPLC instrument was equipped with a 717 Plus auto sampler, an isocratic pump 1515 (Waters) and a UV detector 486 (Waters, Hertfordshire, UK). Shodex RSPAK KC-811 ion exchange column (length 300mm-I.D. 8mm) (Shodex) with precolumn. Isocratic elution (30°C, 1 ml min⁻¹) was performed with water added with 0.021% (w/v) H₃PO₄ in water. Analysis were performed in duplicate.

3.3. Results and discussion

Composition of the feedstocks

The compositions of EFB, SCB and BS are shown in Table 2. The cellulose content addressed as total of glucosyl units was similar for BS and SCB and was slightly lower for EFB. Hemicellulose structures present in these grass materials are expected to be mainly glucoronoarabinoxylans (Ebringerová et al., 2005). Therefore, the glucoronoarabinoxylan (GAX) content was calculated as the sum of xylosyl, arabinosyl, and glucuronic acid residues. The amount of GAX for all the three materials is around 30% (w/w), which corresponds to literature values (Canilha et al., 2011; Hamzah et al., 2011; Kim & Day, 2011; Sun et al., 2011). Lignin contents of BS,

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Table 2. Compositional data of sugarcane bagasse, barley straw, and oil palm empty fruit bunch (%w/w; based on dry matter).

Component	Barley straw	Sugarcane bagasse	Empty fruit bunch
Cellulose	35	37	26
Hemicellulose (total)	33	26	27
Arabinose ^a	3	2	2
Xylose ^a	22	22	18
Galactose ^a	1	0	1
Mannose+Rhamnose ^a	4	1	1
Glucuronic acid ^a	3	1	5
Lignin ^b	24	28	33
Acetic acid (esters)	2	1	3
Protein (Nx6.25)	2	1	3
Ash content	1	5	5

^aPresented as polymers; part of hemicellulose content.

^bCorrected for ash content.

SCB and EFB were quite similar, 24-28 % (w/w), (Table 2) and comparable to other lignocellulosic feedstocks (Haque et al., 2012; Van Eylen et al., 2011). The acetic acid ester contents were around 2% (w/w), and the protein contents were lower than 5% (w/w). The degree of substitution of the xylan backbone was calculated and expressed as moles of arabinosyl, acetyl and glucuronic acid residues per 100 mols of xylosyl residues (Table 3). Using this parameter, double substitution and chains longer than 1 substituent are not taken into account.

Table 3. Moles of esterified acetic acid, arabinosyl and glucuronic acid residues per 100 moles.

Feedstock	mol/100mol xylosyl residues				
	Acetyl ester	Arabinose	Glucuronic acid	Total substituted ^a	Unsubstituted ^a
SCB	7	8	5	20	80
BS	17	13	8	38	62
EFB	27	13	18	57	43

^aTotal of substituted or unsubstituted xylosyl residues assuming that substituted sugars are single substituted and they are not longer than one sugar residue.

The resulting degree of substitution of the xylan backbone differed considerably among the three feedstocks (Table 3). In EFB almost 60% of xylosyl residues were substituted. For BS and SCB these values were 20% and 38% (w/w), respectively.

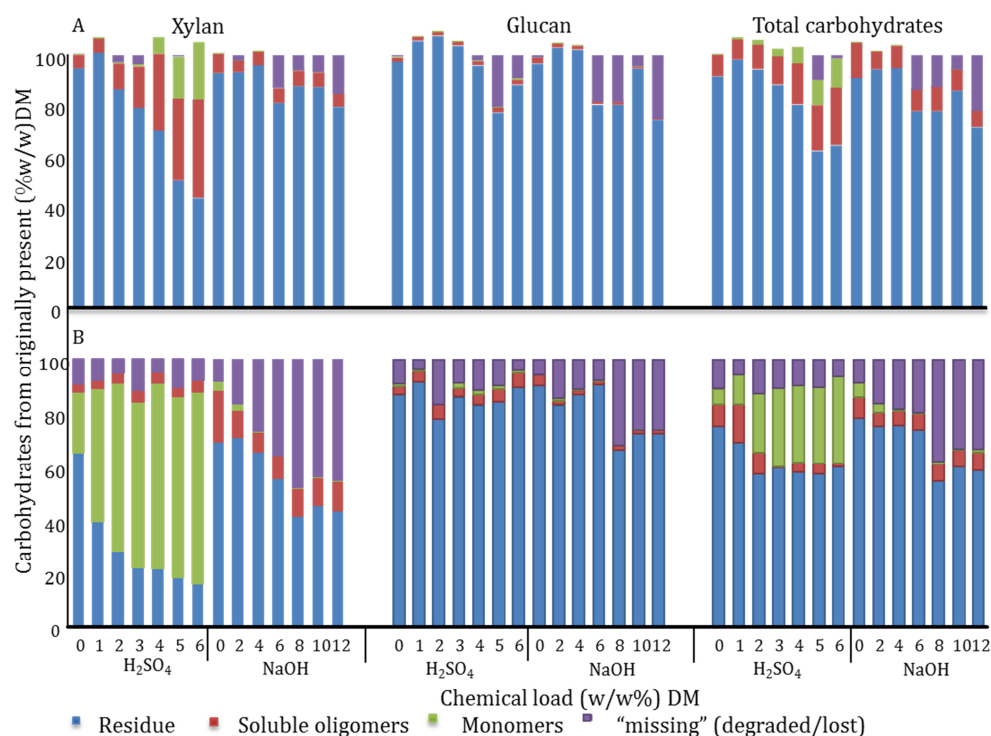


Figure 1. Distribution of xylose, glucose and total carbohydrates after acid and alkali pretreatment of empty fruit bunch (EFB) (A) and sugarcane bagasse (SCB) (B). Blue bar represents insoluble carbohydrates, red bar represents soluble oligosaccharides/polysaccharides in hydrolysates, green bar represents soluble monosaccharides in the hydrolysates and purple bar represents “missing” carbohydrates (degraded/lost in pretreatment)

Carbohydrate distribution over hydrolysates and residues after pretreatment.

Various concentrations of diluted H_2SO_4 and NaOH were applied during pretreatment of EFB SCB and BS to obtain different amounts of residual xylan and lignin (Table 1). The different structures of the hemicelluloses (Tables 2&3) provoked variations in the amount of hemicelluloses solubilised from EFB, BS and SCB. The resulting carbohydrate distributions over insoluble residues and water soluble hydrolysates are shown in figure 1 for SCB and EFB. BS behaved similar to SCB the pretreatment performed and is, therefore, not included in the figure. These data are shown in supplementary figure1.

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Alkali treatment solubilised lignin. But, it solubilised very little amounts of xylan, less than 10% (w/w) based on xylan originally present in the non-treated sample. Acid treatment solubilised high amounts of hemicelluloses when applying the highest doses of acid.

Acid treated samples showed xylan solubilisation reaching a maximum (80% (w/w)) in 6% (w/w) H₂SO₄ for SCB and BS, mainly as xylose monosaccharides. Corresponding glucan solubilisation was lower than 15% (w/w). It can be noted that for EFB similar conditions resulted in a much lower release of xylan as monosaccharides plus oligosaccharides compared to SCB and BS. The amount of lignin solubilised in the hydrolysate is for acid treated samples in the range of 0-15% (w/w) based on the original amount of lignin present (Supplementary Figure 2 and Table 1). Part of the xylan released in the hydrolysates of the acid pretreatments was present as oligosaccharides. These oligosaccharides were most abundant in the hydrolysates from EFB (Figure 1).

Next, MALDI-TOF MS was applied. In the hydrolysate of EFB not only acetylated, but also 4-*O*-methyluronylated oligosaccharides were determined (Figure 2). Although being a relatively small part of the SCB and BS hydrolysates, series of oligosaccharides composed of acetylated pentoses were found. The presence of the substituents corresponded to the carbohydrate composition analysis (Table 3). The substituents in these oligosaccharides may hinder complete enzyme hydrolysis into monosaccharides (Chang & Holtzaple, 2000; Leu & Zhu, 2012; Wang et al., 2012).

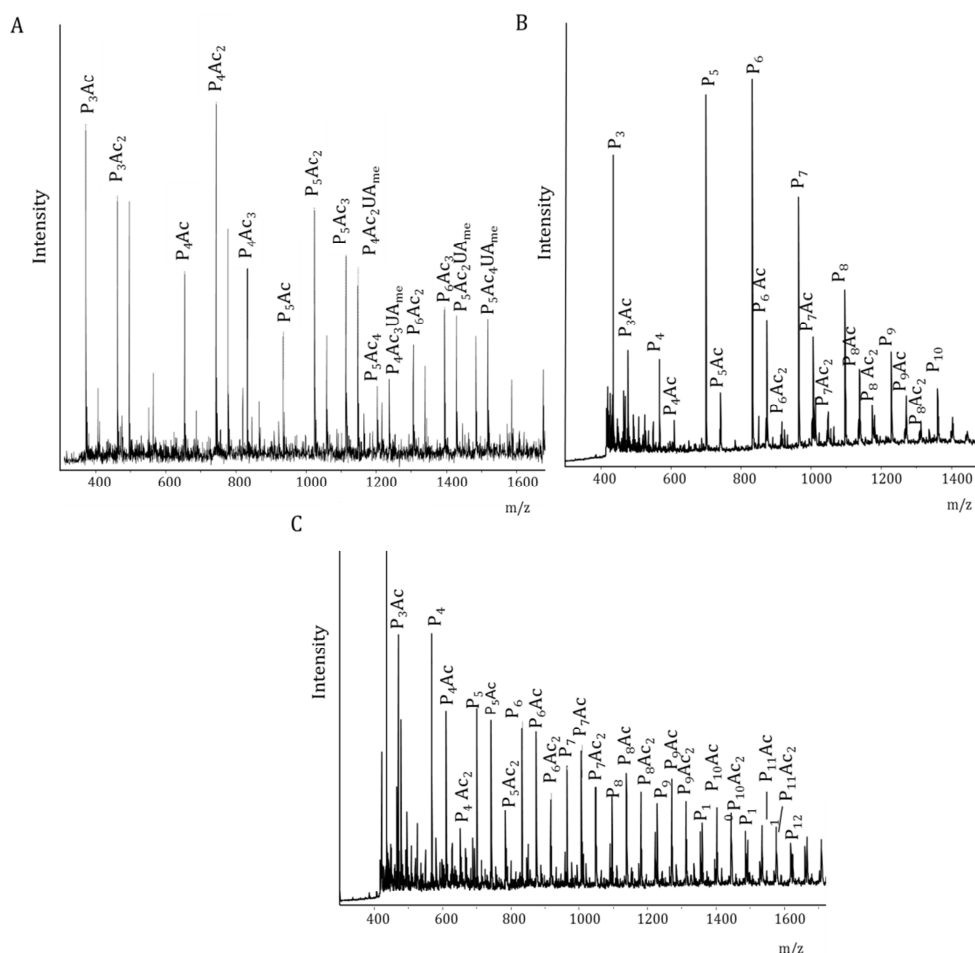


Figure 2. MALDI-TOF mass spectra of empty fruit bunch (EFB) 2% (w/w) acid pretreated (A), Sugarcane bagasse (SCB) 2% (w/w) Acid pretreated (B) and BS 2% (w/w) acid pretreated (C) hydrolysates. Peaks were tentatively annotated (P= pentose; Ac= acetyl group; UAme= 4-O-methyluronic acid).

After alkaline pretreatment swelling of the material was visually observed, but it resulted in almost no solubilisation of carbohydrates (less than 20% (w/w)) (Figure 1). Moreover, unexpectedly, the amount of calculated missing carbohydrates (up to 40% (w/w)) was remarkable. This missing fraction was calculated taking the total amount of xylan in the feedstock prior to pretreatment, minus the amount of xylan analyzed in residue and corresponding hydrolysate. It was hypothesized, that part of the xylan present was converted into non-carbohydrate degradation products during alkaline pretreatment, for example, caused by peeling (Danielsson &

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Lindström, 2005). The total inhibitors concentration found in the hydrolysate increased upon increased alkali concentration. However, the amount does not correspond to the missing xylan fraction (Table 1). Recently, Li et al., (2012) reported significant hemicellulose losses during alkaline pretreatment under similar conditions. During alkaline pretreatment up to 50 (w/w%) of lignin originally present in the feedstocks was solubilized (data not further shown). Carbohydrate and lignin removal by the pretreatments used, resulted in residues with different contents of xylan and lignin next to cellulose (Table 1).

Enzymatic degradability of residues resulting from the various pretreatments

To study the enzymatic degradability of the residues described above, they were incubated by using 2 different enzyme cocktails: CellicCTec2 (CCT) and a combination of CCT with CellicHTec (CHT). CCT is, principally, a cellulose degrading enzyme cocktail, while CHT is enriched in xylan degrading enzymes. Degradation of glucan and xylan into corresponding glucose and xylose is shown in Figures 3 and 4.

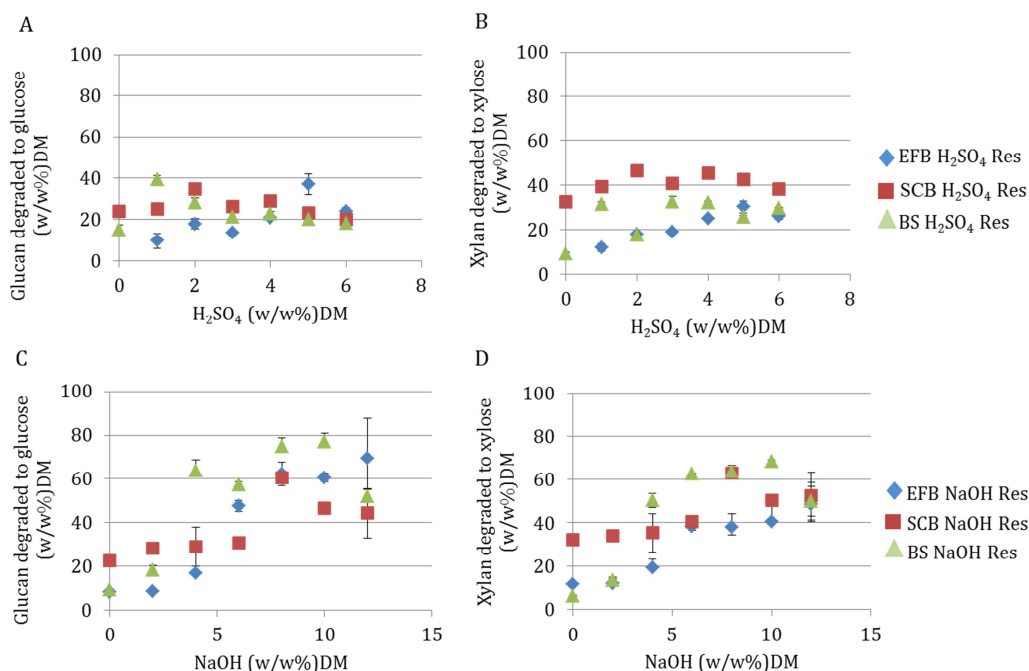


Figure 3. Proportion (%) of glucose from glucan after enzyme treatment (CellicCTec2) of acid treated residues (A) alkali treated residues (C), corresponding percentages of xylose from xylan acid pretreated residues (B) and alkali pretreated residues (D) for empty fruit bunch (EFB), sugarcane bagasse (SCB), barley straw (BS).

Degradation of glucan and xylan of residues from the acid pretreatments incubated with CCT reached a maximum of 40-45% (w/w). For the alkaline pretreatments a maximum of $\pm 80\%$ (w/w) for glucan and xylan conversion was observed. The combination of enzymes (figure 4) showed the highest yields for both pretreatments. This effect was most pronounced on residues from acid pretreatment, where glucose yield increased from 30 to 80% (w/w) upon addition of CHT to CCT. It should be noted that enzyme studies were performed with freeze dried material to allow good comparison between samples and to minimize inhomogeneity issues. Drying may negatively affect enzyme hydrolysis (Wang et al., 2012). Nonetheless, the results are expected to reflect the comparisons made.

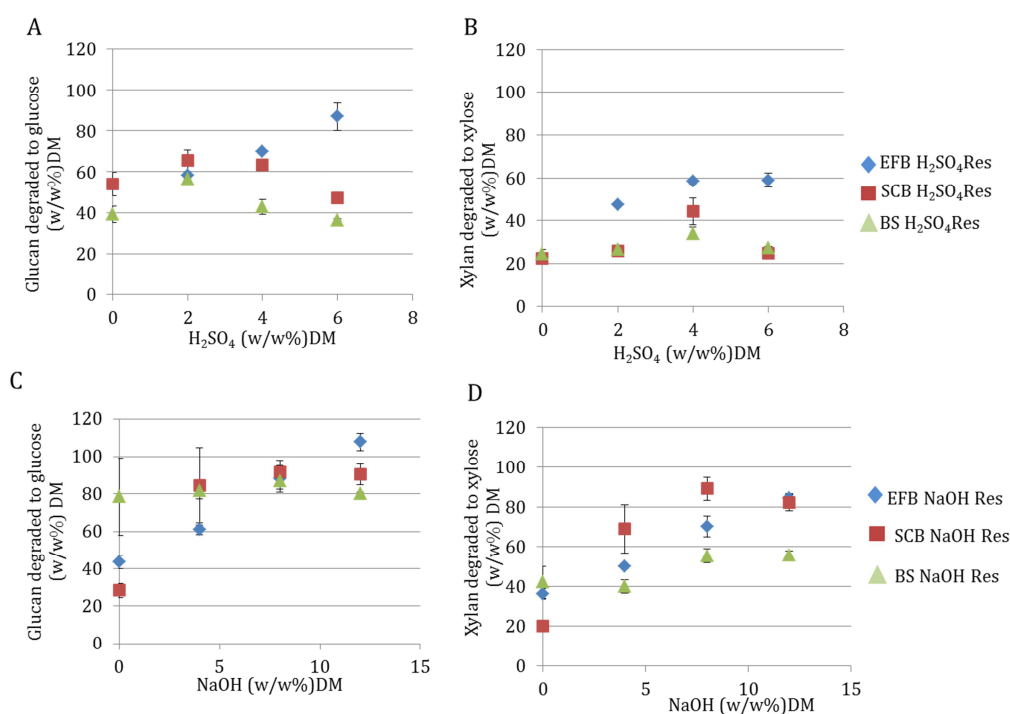


Figure 4. Proportion (%) of glucose from glucan after enzyme treatment (CellicCTec2+CellicHTec) of acid treated residues (A), alkali treated residues (C) and corresponding percentages of xylose from xylan in acid pretreated residues (B) and alkali pretreated residues (D) for empty fruit bunch (EFB), sugarcane bagasse (SCB), barley straw (BS).

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Hemicellulose solubilisation in acid pretreatment affecting residual glucan conversion

As during the acid pretreatments mainly xylan was removed and not lignin, there was interest to correlate the xylan/glucan (x/g) ratios of the residues with the enzymatic conversion (Figure 5). Acid pretreatments of SCB/BS resulted in residues with lower x/g ratios compared to the EFB residues obtained under similar conditions. Nevertheless, the trend observed in Figures 5 A and B was similar for SCB, BS and EFB. Residues obtained after acid pretreatment were better degraded by CCT at x/g ratios lower than 0.4. Ratios higher than 0,4 showed glucan conversion yields lower than 20% (w/w).

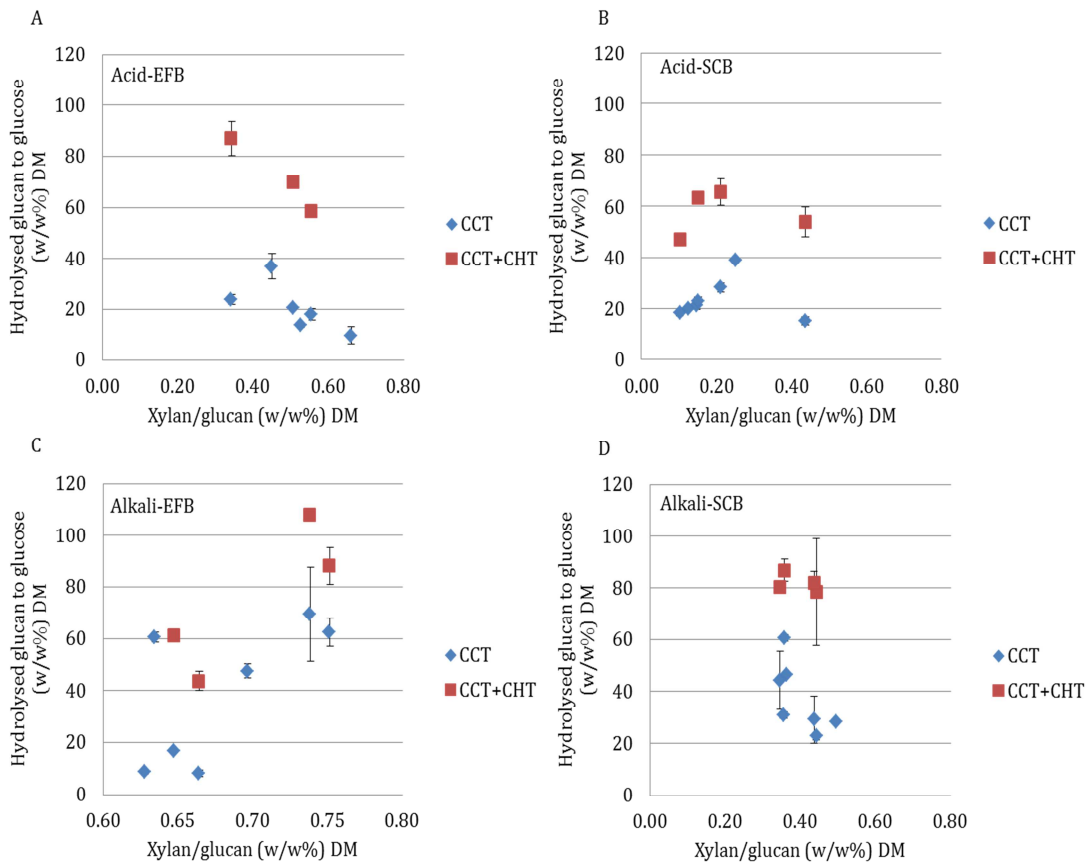


Figure 5. Proportion (%) of glucose from glucan (CellicCTec2 and CellicHighTec) vs residual xylan/glucan ratio after pretreatment expressed as percentage of weigh based on dry matter of EFB acid (A) and alkali (C) pretreated residues and SCB acid (B) and alkali (D) pretreated residues respectively.

In literature the removal of hemicelluloses was also suggested to enhance cellulose conversion into glucose by enzymes (Ding et al., 2012; Kabel et al., 2006; Palonen et al., 2004). Interestingly, at relatively low x/g ratios, the enzyme degradability of the residues after acid pretreatment decreased. For EFB, this point was reached at x/g ratios lower than 0.4, while for SCB and BS this was lower than 0.2. The decreasing trend was more pronounced for SCB and BS than for EFB. Hydrolysis by CCT+CHT showed for SCB and BS acid pretreated residues again that the yield of monosaccharides decreased at lowest x/g ratios (Figure 5A and B). On the contrary, for the EFB acid residues the hydrolysis yield by CCT+CHT increased at low x/g ratios (Figure 5). For SCB/BS acid treated residues the xylan hydrolysis yield decreased together with the glucan hydrolysis yield by CCT+CHT at low x/g ratios. To understand these phenomena, the remaining composition of the residues should be considered. The residues of the acid pretreatment were high in glucan content as well as in lignin content (Table 1). Both were hardly solubilized during pretreatment, while xylan was progressively removed upon higher acid concentrations (lower x/g ratio). Presence of lignin and of xylan has been shown to inhibit enzymatic glucan conversion by physically hindering the enzymes to access the cellulose fibers.

This physical hindrance of xylan was partly overcome by addition of endoxylanase activity to the cellulase cocktails, especially for the relatively xylan-rich EFB residues (Figures 4B and 5A). Physical hindrance by lignin is hardly described in literature. More important, it should be considered that lignin structures changes upon acid pretreatment, which may have resulted in re-condensation of lignin on to the cellulose fibers (Pu et al., 2013). Re-condensed, structurally altered lignin may physically block enzyme hydrolysis of cellulose, which could explain the decrease in hydrolysis observed for SCB and BS at lowest x/g ratios (Figure 5). As this was less observed for the EFB acid treated residues, it may implicate that relatively high amounts of residual xylan present prevented re-condensation of lignin present on cellulose fibers. In these residues the presence of xylan was the main physical barrier for glucan hydrolysis by cellulase enzyme preparations with low endoxylanase activity. To summarize, excessive removal of xylan from glucan fibers before addition of cellulases and xylanases, in the presence of lignin, negatively influenced glucan conversion into glucose. This confirms recent microscopy studies by (Ding et al, 2012).

Lignin solubilisation in alkaline pretreatments affecting residual glucan conversion

During alkaline pretreatment mainly lignin was removed for EFB, SCB and BS. To compare the enzymatic digestion of the residues with the acid treated residues,

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their enzymatic conversion was also plotted versus the x/g ratios (Figures 5 C and D). The enzymatic cellulose to glucose conversion yields differed after the various alkaline pretreatments within the same feedstock of the residues obtained. As x/g ratios of alkaline treated residues were rather similar within one feedstock, it was concluded that enzymatic glucan degradation did not relate to x/g ratios for alkaline residues. Apparently, another factor than x/g ratio influenced the enzymatic degradation of glucan. The residues contained different levels of lignin (Table 1). Therefore, in Figure 6, next to x/g ratios, also the lignin content was taken into account and plotted versus enzymatic degradation of glucan. In this figure, for the alkaline pretreated residues of EFB, an increase in hydrolysis by CCT of glucan was observed with decreasing lignin/glucan ratios. The same trend was observed for BS and SCB, albeit for the latter less pronounced. Apparently, lignin solubilization together with the, visually observed, swelling of the feedstocks during treatment facilitated the hydrolysis of the residual glucan up to 60% by CCT, while x/g ratios remained the same.

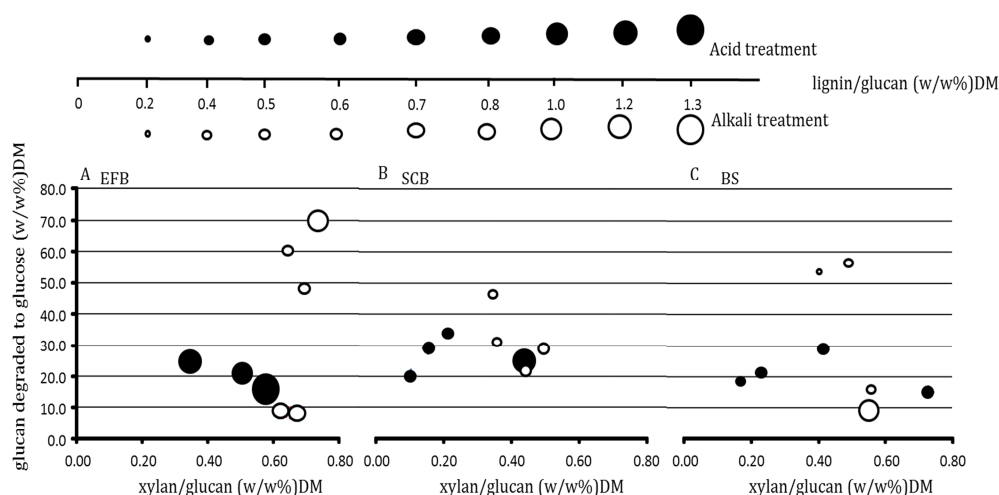


Figure 6. Proportion (%) of glucose from glucan (CellicCTec2) vs xylan/glucan ratios after catalysis expressed as w/w% based on dry matter of empty fruit bunch (EFB) acid (●) and alkali (○) catalysed residues (A), sugarcane bagasse (SCB) acid and alkali catalysed residues (B), barley straw (BS) acid and alkali catalysed residues (C). The size of the points represents lignin/glucan ratios.

The addition of CHT (CCT+CHT) increased the glucan hydrolysis even further, as well as the xylan hydrolysis (Figure 5 C and D), for alkaline treated residues.

Understanding the influence of lignin content on glucose yields after saccharification asks for more specific techniques, than the Klason lignin analysis applied, for example pyrolysis GC-MS (Chen & Dixon, 2007; Studer et al., 2011; Sykes et al., 2009), which we consider for future research.

3.4 Conclusion

Acid pretreatment solubilized large amounts xylan and increased cellulose (CCT) hydrolysis of residual glucan. But, upon extensive removal of xylan with lignin remaining, the glucan hydrolysis became less efficient. On the contrary, solubilization of lignin in alkaline pretreatments resulted in a rather high residual glucan hydrolysis. This showed that, under the conditions applied, alkali pretreatment resulted in the highest glucose from cellulose yield.

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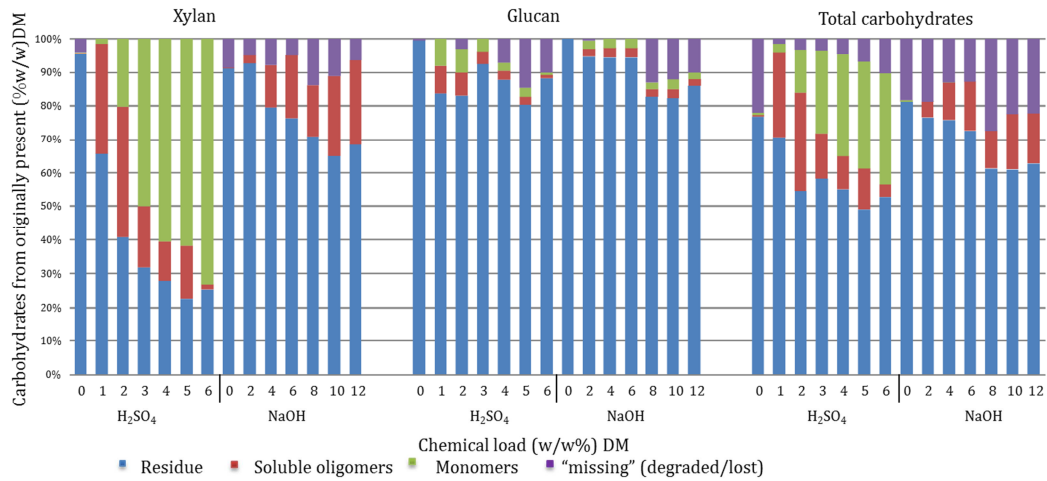
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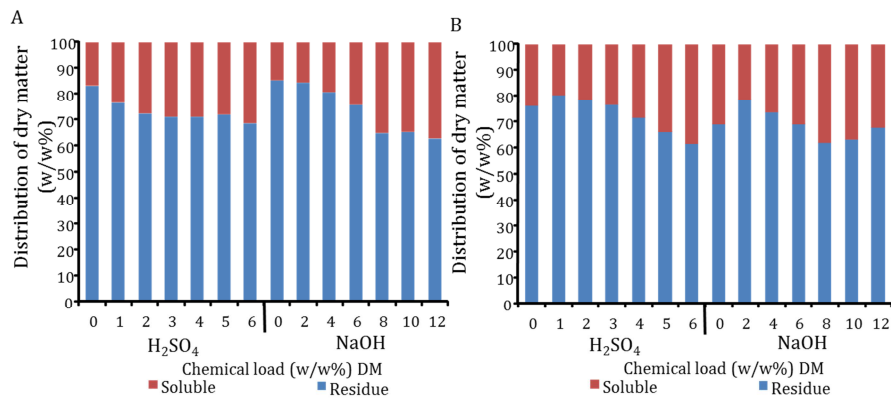
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Supplementary data



Supplementary figure 1. Distribution of xylose, glucose and total carbohydrates after acid and alkali pretreatment of Barley straw (BS). Blue bar represents insoluble carbohydrates, red bar represents soluble oligosaccharides/polysaccharides in hydrolysates, green bar represents soluble monosaccharides in the hydrolysates and purple bar represents "missing" carbohydrates (degraded/lost in pretreatment).



Supplementary figure 2. Distribution of dry matter after acid and alkali pretreatment of empty fruit bunch (EFB) (A) and sugarcane bagasse (SCB) (B). Blue bar represents insoluble dry matter and red bar represents soluble dry matter in hydrolysates.

Chapter 4

Alkaline pretreatment and enzymatic hydrolysis of sugar cane bagasse: An empirical model based on experimental data

ABSTRACT

The conversion of sugar cane bagasse (SCB) using alkaline pretreatment and enzymatic hydrolysis is an option to produce fermentable sugars. Many authors have reported the effect of alkaline pretreatment on the enzymatic hydrolysis rate, but no quantitative model has been formulated. In this research, pretreatment experiments were performed at different sodium hydroxide concentrations, temperatures and pretreatment times. The composition of SCB was analysed prior to pretreatment and, after pretreatment, the distribution of cellulose and hemicellulosic xylan over the soluble and insoluble phases were determined. In addition, lignin was analysed in the remaining solid fractions. The pretreated samples obtained were hydrolysed using cellulolytic and hemicellulolytic enzymes. Glucose and xylose released was measured at various time points during the enzymatic hydrolysis. The sodium hydroxide concentration was the variable with the largest effect on the enzyme hydrolysis. At a sodium hydroxide concentration of 9 (w/w)% a maximum hydrolysis of 81% of glucan and xylan present after pretreatment was found, while also the highest lignin solubilization was reached. These pretreatment conditions, however, also resulted in a significant loss of xylan. The experimental data were fitted in a hyperbolic empirical model. The constants of the empirical model were related to the pretreatment conditions with linear multivariable relations. A rate equation of the enzymatic hydrolysis was obtained. It describes the influence of the alkaline pretreatment conditions on the enzyme hydrolysis and can be used to design unit operations to convert SCB into fermentable sugars.

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4.1. Introduction

The increase in world population and in living standards comes along with an increased demand in fuel, energy and chemicals. These products are largely derived from the depleting sources of fossil fuels (Bo, 2011). Hence, many approaches aim to find good alternatives. An example is the successful production of bioethanol from sugar cane (Goldemberg, 2007), which leaves sugar cane bagasse (SCB) as a by-product constituting about 26 wt% of the total mass of the sugar cane. SCB is composed of the polysaccharides cellulose and hemicellulosic xylan, next to lignin (Kim and Day, 2011). The polysaccharides can potentially be converted to monosaccharides, which can be fermented to biofuels and other added value chemicals (Jørgensen et al., 2007).

Cellulose consists of β -(1 \rightarrow 4)-linked glucosyl chains aligned in a crystalline form. Xylan is composed of a backbone of β -(1 \rightarrow 4)-linked xylosyl residues that, in SCB, is mainly substituted with acetyl residues (Murciano Martinez et al., 2015). In the plant cell walls of SCB, cellulose is covered by xylan and kept together by lignin. Lignin is an aromatic polymer synthesized from phenylpropanoid precursors, forming a hard to degrade matrix (Bianfang et al., 2013). Enzymatic hydrolysis using cellulases and xylanases, is one of the most accepted and efficient technologies to hydrolyze the cellulose and xylan in SCB to glucose and xylose, respectively (Kumar et al., 2009). The compact structure of SCB, however, limits the access of these enzymes to their substrates. In addition, Nakagame et al. (2010) and Ximenes et al. (2011) have mentioned that lignin can inhibit and absorb the cellulolytic enzymes. Therefore, the lignin, xylan and cellulose have to be separated from each other such that the enzymes have free access and can release the fermentable sugars.

Several pretreatment technologies are described to separate the lignin from the polysaccharides. All involve the exposure of the lignocellulosic biomass to high temperatures, pressure, extreme pH and/ or solvents (Mosier et al., 2005). The severity factor describes how the pretreatment conditions affect the biomass during pretreatment (Chandra et al., 2007). Nevertheless, little is known about the influence of alkaline pretreatment conditions on the enzymatic hydrolysis reaction rates. Some models use linear empirical relations between the alkaline pretreatment conditions and the glucose release after enzymatic hydrolysis (e.g. Fuentes et al., 2011). Chen et al. (2013) and Rabelo et al. (2014) propose statistical models that describe the effect of temperature and alkali loading during pretreatment on the sugar yield of enzymatic hydrolysis. Gupta et al. (2012) propose a model that describes the cellulose hydrolysis rate depending on the percentage of pretreated biomass loading. However, none of these proposed model can be used to optimize the alkaline pretreatment conditions and operation modes.

This paper focuses on alkaline pretreatment of SCB, combined with enzymatic hydrolysis. A range of sodium hydroxide concentrations is applied at 40, 80 and 120 °C to

calculate the parameters of a proposed reaction rate model. The model can be used in mass balances of enzymatic hydrolysis equipment, to enable economical optimization of the overall process.

4.2. Materials and methods

Feedstocks

Sugar cane bagasse (SCB) was supplied by Corbion-Purac Thailand (Banchang, Thailand). It was obtained as air dried shredded fibers. The fibers were ball milled to a <1mm particle size using a Retsch ball mill. (Retsch, Haan, Germany). The SCB was composed (based on % (w/w) dry matter) of cellulose (37), hemicellulosic xylan (26), lignin (28), ash (5), esterified acetic acid (1), and protein (N% \times 6.25; 1) (Murciano Martinez et al., 2015). All chemicals and carbohydrate standards were provided by Sigma-Aldrich (St. Louis, MO, USA), unless stated differently.

Pretreatment

Pretreatment experiments were performed in a 100 mL vessel containing 7.7 g of SCB. Different pretreatment conditions were used as shown in Table 1. The dry biomass loading was fixed at a solid:liquid ratio of 1:10 (w/w) including alkali, if present. The pH of the reaction mixture was measured prior to and after the pretreatment. After pretreatment, the samples were neutralized using acetic acid, centrifuged (10000 xg, 15 min, 4 °C) and separated into a wet residue and supernatant.

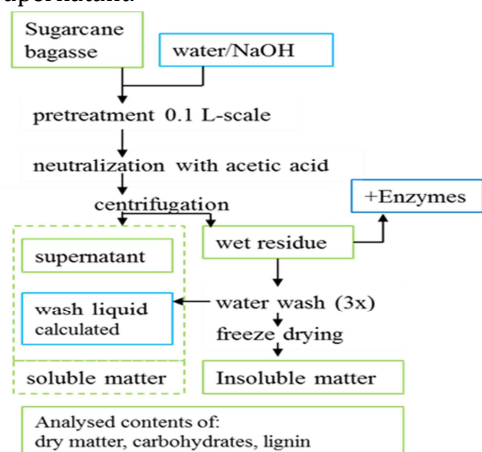


Figure 1. Schematic overview of the pretreatment of sugar cane bagasse and fractions obtained.

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The supernatant was weighed, analysed for dry matter and analysed for carbohydrate content and composition. The wet solid residue was weighed and analysed for dry matter. Afterwards, it was washed 3 times with deionized water, freeze dried and analysed for total carbohydrate content, composition and lignin content. Figure 1 shows the steps taken during the whole experimental procedure. To calculate the distribution of dry matter after pretreatment over wet solid residue and SN, a correction for the losses obtained due to sample handling during pretreatment was performed as follows. The total dry matter (g) (TDM) subjected to pretreatment (SCB+alkali) was divided by the total recovered dry matter (RDM) analysed in wet residue and supernatant (g) (ratio TDM/RDM). This ratio was higher than 0.9 for all pretreatments. Subsequently, the ratio TDM/RDM was multiplied by either the recovered dry matter in the residue or in the supernatant. To calculate the insoluble matter after washing, the now calculated wet residue was corrected for the analysed dry matter washed out.

Enzymatic hydrolysis

The wet neutralized residues (200 mg dry matter) were subjected to enzymatic hydrolysis in 10 mL NaOAc buffer pH 5.5, in duplicate, by using a combination of two enzyme-cocktails, kindly supplied by Novozymes, CellicCTec2 and CellicHTec (ratio 10:1 (w/w)). The total enzyme loading was 3% (w/w; enzyme protein/dry matter substrate). The protein content was 127 and 120 g L⁻¹ for CellicCTec 2 and Cellic HTec, respectively. The incubations were performed at 50°C, rotated head-over-tail for 72 h. Sodium azide was added as bacteriostatic agent (0.02 %w/v). The enzyme cocktail has cellulase and xylanase activities.

Analytical methods

Glucose and xylose content during enzymatic hydrolysis

The enzyme digests (72h, duplicates) were analysed for glucose and xylose contents using high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). Hereto, a Dionex ICS-5000 unit (Dionex, Sunnyvale, CA, USA) was equipped with a CarboPac PA-1 column (2 mm x 250 mm ID) in combination with a CarboPac guard column (2 mm x 50 mm ID) and PAD detection. The system was controlled by Chromelion software (Thermo Scientific, Sunnyvale, CA, USA). Elution and quantification (0.3 mL min⁻¹) was performed with a combination of isocratic elution and linear gradients from three types of eluents, A: 0.1 mol L⁻¹ NaOH, B: 1 mol L⁻¹ NaOAc in 0.1 mol L⁻¹ NaOH, C: Millipore water. The elution profile was: 0-

18 min: 18% A in 82% D, 18-30 min 0-100% B in 100-0% A, a cleaning step of 5 min 100% B, an equilibration step 5 min 100% A and 15 min at starting conditions.

The glucose oxidase/peroxidase assay (GOPOD; Megazyme, Wicklow, Ireland) was performed, according to the assay's protocol, to analyse the glucose content during enzyme hydrolysis at various hydrolysis times. In brief, 3 mL of GOPOD reagent was added to 0.1 mL sample (in duplicate), incubated at 50 °C for 20 min, analysed at 510 nm with a UV1240 mini spectrophotometer (Shimadzu, Kyoto, Japan).

Lignin and neutral carbohydrates after pretreatment

Lignin and carbohydrate contents and composition were analysed in duplicate, as described elsewhere (Murciano Martinez et al., 2015). Neutral carbohydrates refers to the cellulose and xylan that is present in SCB after pretreatment.

4.3. Results

Pretreatment

The exposure of the SCB-biomass to the pretreatment conditions (Table 1) caused solubilization in the supernatant of xylan (0-13%), as can be seen in Table 2, and lignin was partially removed from the wet residue (up to 39%; Table 2). In addition, in Table 2, the proportions of xylan and glucan that remained in the insoluble matter are shown.

It was clear that the more severe the pretreatment conditions, the less xylan and the less lignin remained in the residues after pretreatment. Seen from Tables 1 and 2, the sodium hydroxide concentration had the most effect on residual xylan yield, as has already been found by Karp et al. (2014) for sodium hydroxide pretreatment of corn stover. For example, at a sodium hydroxide loading of 9 wt% (samples 2, 4, 7, 9, 11, 14, 17), up to 44% of xylan was removed or degraded from the wet residues.

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Table 1. Conditions and amounts of sugar cane bagasse (SCB) used during pretreatments, measured pH and recovery of dry matter after pretreatment.

Experiment	Pretreatment conditions			SCB ² (g)	Total Initial wet weight ³ (g)	pH before ⁴	pH after ⁴	Recovery (%) ⁵
	Time (min)	Temperature (°C)	NaOH (w/w%) ¹					
1	30	40	4	7.3	77.3	11.9	11.6	85
2	30	40	9	7.6	77.6	13.1	13.0	100
3	30	80	4	7.3	77.3	12.1	10.7	103
4	30	80	9	7.6	77.6	13.1	12.7	88
5	30	120	0	7.0	77.0	3.9	4.0	96
6	30	120	4	7.3	77.3	12.1	8.9	78
7	30	120	9	7.6	77.6	13.1	10.8	81
8	60	40	4	7.3	77.3	12.1	11.7	88
9	60	40	9	7.6	77.6	13.1	13.0	99
10	60	80	4	7.3	77.3	12.2	9.9	103
11	60	80	9	7.6	77.6	13.1	12.7	111
12	60	120	0	7.0	77.0	3.9	4.0	95
13	60	120	4	7.3	77.3	12.0	8.6	90
14	60	120	9	7.6	77.6	13.0	10.2	96
15	180	40	0	7.0	77.0	4.0	4.0	90
16	180	40	4	7.3	77.3	12.0	11.7	86
17	180	40	9	7.6	77.6	13.1	12.9	91

¹Based on dry matter (dm) SCB (g NaOH/ 100g dm SCB).

²Dry matter used in pretreatment.

³Totals of grams of water, NaOH and SCB used for the pretreatment.

⁴Before pretreatment, SCB, NaOH and water were mixed and after 1h the pH was measured (pH before). After pretreatment the pH was measured again (pH after).

⁵Recovery of dry matter after pretreatment from originally present in SCB (w/w%).

Murciano Martinez et al. (2015) obtained similar results. After pretreatment the pH was lower for most samples compared to the pH before pretreatments (see Table 1), especially in the samples with 0 or 4% sodium hydroxide loading (samples 1, 3, 5, 6, 8, 10, 12, 13, 15, 16) observed from Table 1. Not only polysaccharide hydrolysis reactions occurred during pretreatment. Table 2 shows that not all polysaccharides removed from the residue were analyzed to be present in the supernatant, meaning that they were further converted into compounds that were not recognized or quantified by the carbohydrate analysis performed. Li et al. (2012) also found such a loss xylan after pretreatment. The part of the xylan and to a much lower extent the part of cellulose that were removed were, most likely, partially converted to unknown compounds upon prolonged reactions in alkali, which could include small organic acids (Aspinall et al., 1961).

Alkaline pretreatment of sugarcane bagasse. An empirical model

Table 2 Distribution of total dry matter (dm), total carbohydrates (tc), glucan (Glc) and xylan (Xyl) after pretreatment over supernatant and wet residue. Lignin (Lig) yield in the wet residues is included. Standard deviations are lower than 1% unless specified differently.

¹Distribution over supernatant and wet residue analysed and based on originally present in SCB.

Exp	Supernatant (%) ¹				Wet residue (%) ¹					Insoluble matter (%) ²				Total (%) ³		
	dm	tc	Glc	Xyl	dm	tc	Glc	Xyl	Lig	dm	tc	Glc	Xyl	tc	Glc	Xyl
1	11	6	2	12	89	97	98	93	68	75	92	96	83	100	100	100
2	14	6	2	11	86	86	87	81	51	61	80	85	70	92	89	92
3	10	5	2	8	90	88	91	77	68	71	84±3	90±2	70±6	92	93	85
4	17	3	1	5	83	80	86	66	56	59	77	85±2	61	83	87	72
5	8	7	2	13	92	98	100	90	82	83	93±2	98±2	81	100	100	100
6	12	3	1	5	88	94	98	82	67	74	91	97	78±2	97	100	87
7	20	3	1	5	80	79	89	56	61	60	77	89±2	53	82	90	61
8	11	6	2	11	89	90	91	85	84	73	86	90±2	76	96	94	96
9	14	6	2	11	86	83	85	76	70	62	78±2	83±3	66	89	87	87
10	8	4	2	8	92	84	89	76	82	72	78	87	66	88	91	84
11	14	3	1	5	86	72	79	62	66	61	69	78	57	75	80	67
12	7	7	2	13	93	100	102	93	92	86	95±2	100±4	83	100	100	100
13	10	2	1	3	90	93	99	78	75	75	91	99	75	95	100	81
14	17	1	0	3	83	86	99	59	66	62	85	98	57	87	99	62
15	8	0	0	0	92	91	101	76	97	86	91	101	76	91	100	76
16	13	0	0	0	87	85	96	71	90	78	85	96	71	85	96	71
17	15	6	2	11	85	89	98	76	76	69	83±2	96	65±2	95	100	87

¹Distribution over supernatant and wet residue

²Insoluble part only of the wet residue.

³Total = supernatant (%) + wet residue (%).

Enzymatic hydrolysis

Table 3 presents the enzymatic hydrolysis yields at 72h of hydrolysis of glucan to glucose and xylan to xylose of most of the pretreated samples. At the highest sodium hydroxide loadings used, at 80 or 120°C, the glucose hydrolysis yields were highest; 55, 79, 87 and 68% for experiments 4, 7, 11 and 14, respectively (table 3). The glucose hydrolysis yields was lowest (35%) if no sodium hydroxide was supplied and a temperature of 40 °C or 120 °C was used (experiment 12 and 15; table 3). Hence, a clear trend was observed that the more severe pretreatment conditions led to a higher yield of monosaccharides in enzymatic hydrolysis. This trend can be attributed to the fact that at in particular high sodium hydroxide concentration at elevated temperatures (> 80°C), the lowest amounts of residual lignin and xylan were recovered, which facilitated the access of the enzymes to the residual celluloses (Jin et al., 2013).

Table 3. Enzymatic hydrolysis of selected wet residues obtained after pretreatment; xylan to xylose and glucan to glucose conversion analysed by HPAEC after 72 hours of enzymatic hydrolysis.

Experiment	Pretreatment conditions		Time (min)	Enzymatic hydrolysis	
	NaOH (w/w%)	Temperature (°C)		Xylan to xylose (%)	Glucan to glucose (%)
4	9	80	30	48±1	55±2
5	0	120	30	33±7	40±7
6	4	120	30	39±0	52±0
7	9	120	30	69±13	79±12
8	4	40	60	34±2	35±12
9	9	40	60	55±5	54±4
10	4	80	60	41±4	59±5
11	9	80	60	75±0	87±0
12	0	120	60	31±1	35±1
13	4	120	60	31±1	47±2
14	9	120	60	63±2	68±3
15	0	40	180	33±4	35±2
16	4	40	180	38±1	49±2

Figure 2 shows the enzymatic release of glucose in time, for five selected pretreated samples. These samples represent mild, medium and severe pretreatment

conditions. It can be observed from figure 2, that if no sodium hydroxide is supplied during pretreatment, the enzymatic release of glucose was around half compared to sample prepared at 9 (w/w)% DM sodium hydroxide (6.14 g L⁻¹ versus 3.35 g L⁻¹ after 72 h). In addition to sodium hydroxide loading, it can also be seen that at a pretreatment temperature of 120 °C and a pretreatment time of 60 min, the production of glucose is around 18% higher than when the temperature is 80 °C and the pretreatment time 30 min.

Modelling the influence of the pretreatment conditions on enzymatic hydrolysis

The concentration of glucose measured at different time points during enzymatic hydrolysis for the differently pretreated samples (Figure 2) was used to obtain a mathematical relation that describes the influence of the pretreatment conditions on the glucose production rate. In the experimental data it can be seen that the enzymatic hydrolysis reaction although not specified (Table 3) tends to a maximum yield.

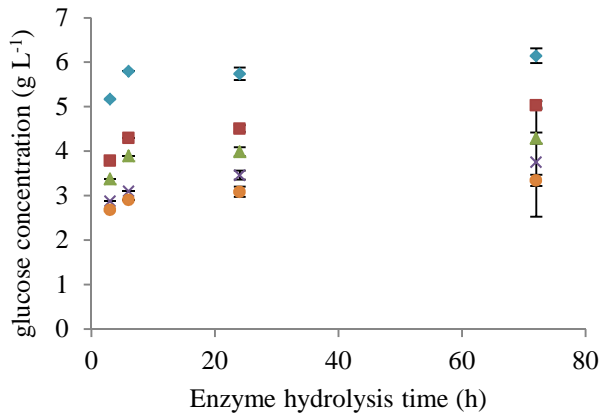


Figure 2 Concentration of glucose during enzymatic hydrolysis of pretreated samples. Pretreatment conditions: ♦ 9 dry mass wt% NaOH, 120°C and 60 min; ■ 9 dry mass wt% NaOH, 80°C and 30 min; ▲ 4 dry mass wt% NaOH, 120°C and 30 min; × 4 dry mass wt% NaOH, 40°C and 60 min; ● 0 dry mass wt% NaOH, 120°C and 60 min.

The reaction also starts fast and the slope decreases with time (Figure 2), which showed a hyperbolic nature and, therefore, a hyperbolic function of the following type was proposed:

$$C_{glc} = \frac{k_{1g} t}{k_{2g} + t} \quad (1)$$

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Where C_{glc} is the glucose concentration, k_{1g} and k_{2g} are empirical constants while t is the enzymatic hydrolysis time. The concentration of glucose can be directly related to the mass of cellulose (C_{cell}) if the initial amount of cellulose (C_{cell0}) is known:

$$C_{cell} = C_{cell0} - C_{glc} * \frac{Mw_{Cell}}{Mw_{glc}} \quad (2)$$

Where Mw_{cell} and Mw_{glc} are the molecular masses of anhydro-glucose (162 Da) and glucose (180 Da), respectively. The concentration of residual cellulose can be empirically modelled against time with equation 3.

$$C_{cell} = \frac{k_1 t}{k_2 + t} + C_{cell0} \quad (3)$$

Equation 3 was used to describe the degradation of cellulose. k_1 and k_2 are empirical constants. Figure 3 shows how the model parameters were adjusted to fit the transformed experimental data of figure 2.

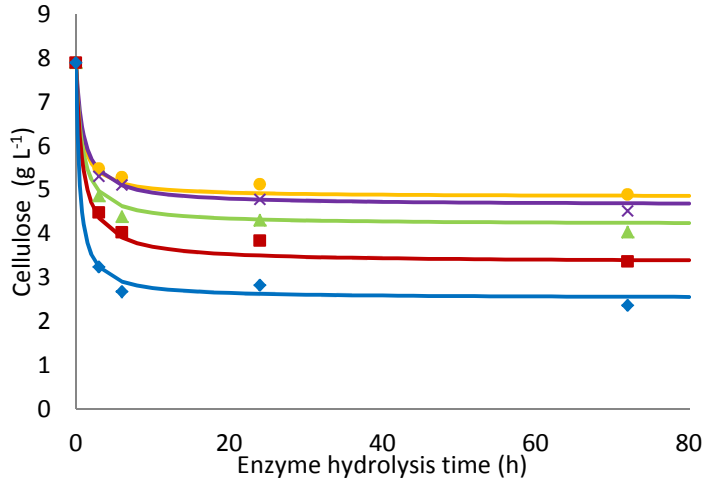


Figure 3 Empirical hyperbolic model fitted to experimental data points of pretreated and enzyme hydrolysed SCB. The lines represent the model and the shapes the experimental data. Pretreatment conditions: ♦(blue) 9 dry mass wt% NaOH, 120°C and 60 min; ■(red) 9 dry mass wt% NaOH, 80°C and 30 min; ▲(green) 4 dry mass wt% NaOH, 120°C and 30 min; ×(purple) 4 dry mass wt% NaOH, 120°C and 60 min; ●(orange) 0 dry mass wt% NaOH, 120°C and 60 min.

Each differently pretreated sample has its own empirical constants. In order to relate the pretreatment conditions to the extent of enzymatic hydrolysis, empirical linear relations were used that relate the pretreatment temperature, sodium hydroxide loading and pretreatment time to the enzymatic hydrolysis reaction rate constants (equations 4 and 5).

$$k_1 = A_1 + B_1 T + C_1 t_{pt} + D_1 C_{NaOH} \quad (4)$$

$$k_2 = A_2 + B_2 T + C_2 t_{pt} + D_2 C_{NaOH} \quad (5)$$

Where A_1, A_2, B_1, B_2, C_1 and C_2 are empirical parameters, T is temperature in °C, t_{pt} is the pretreatment time in minutes and C_{NaOH} is the pretreatment sodium hydroxide loading in wt% dry biomass.

Taking the derivative of equation 3 gives the rate (r_{cell}) of conversion of cellulose during enzymatic hydrolysis of pretreated SCB, using a batch material balance:

$$r_{cell} = \frac{dC_{cell}}{dt} = \frac{k_1 k_2}{(k_2 + t)^2} \quad (6)$$

To obtain a relation between the conversion of cellulose during enzymatic treatment and the concentration of cellulose, equation 7, derived from equation 3, is used to obtain time as explicit function of cellulose concentration. By doing this, the resulting reaction rate model can be used to describe continuous unit operations.

$$t = \frac{k_2(C_{cell} - C_{cello})}{k_1 - C_{cell} + C_{cello}} \quad (7)$$

Substituting equation 7 in the rate equation 6 gives:

$$r_{cell} = \frac{dC_{cell}}{dt} = \frac{(k_1 - C_{cell} - C_{cello})^2}{k_1 k_2} \quad (8)$$

In this rate equation k_1 and k_2 can be substituted by the empirical equations 4 and 5:

$$r_{cell} = \frac{((A_1 + B_1 T + C_1 t_{pt} + D_1 C_{NaOH}) - C_{cell} - C_{cello})^2}{(A_1 + B_1 T + C_1 t_{pt} + D_1 C_{NaOH})(A_2 + B_2 T + C_2 t_{pt} + D_2 C_{NaOH})} \quad (9)$$

Equation 9 is a quadratic equation that describes the solubilization rate of cellulose as a function of pretreatment conditions. Figure 4 shows the reaction rate against concentration of cellulose for the conditions from the experimental data.

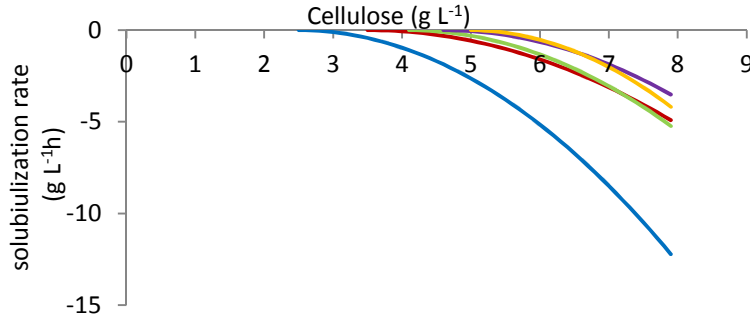


Figure 4: Enzymatic hydrolysis reaction (solubilization) rate against concentration of cellulose of pretreated samples. Pretreatment conditions: (blue) 9 dry mass wt% NaOH, 120°C and 60 min; (red) 9 dry mass wt% NaOH, 80°C and 30 min; (green) 4 dry mass wt% NaOH, 120°C and 30 min; (purple) dry mass wt% NaOH, 40°C and 60 min; (orange) 0 dry mass wt% NaOH, 120°C and 60 min.

There is a cellulose conversion rate with a maximum yield (maximum point of the parabolas). The process conditions included in the model defined the maximum yield. After the maximum yield has been achieved, the reaction stops, which can happen before all cellulose has been converted and depends on the accessibility of the enzyme to the substrate (Iberahim et al., 2013). The cellulose concentrations at the maximum points of the parabolas are the same concentration as the respective asymptotes of the models in figure 4.

The cellulose concentration at the maximum points of the parabolas, where the maximum hydrolysis yield is found and the reaction rate is zero is obtained when the derivative of equation 8 equals zero (equation 10).

$$C_{cell}(0) = k_1 + C_{cello} \quad (10)$$

The parameters of the model were adjusted to the experimental data. Table 4 shows the results.

Table 4 Empirical parameters (A, B, C and D) that relate to the pretreatment conditions with the enzymatic hydrolysis rate model.

k_1^1		k_2^1	
Parameter	Value	Parameter	Value
A_1 [g L ⁻¹]	-1.014	A_2 [h]	1.656
B_1 [g L ⁻¹ °C ⁻¹]	-0.011	B_2 [h °C ⁻¹]	-0.005
C_1 [g L ⁻¹ min ⁻¹]	-0.013	C_2 [h min ⁻¹]	-0.007
D_1 [g L ⁻¹ wt% ⁻¹]	-0.257	D_2 [h wt% ⁻¹]	-0.023

¹ k_1 and k_2 are enzymatic hydrolysis rate constants

4.4. Discussion

The experimental results obtained show that there is a strong influence of the pretreatment conditions on the performance of enzymatic hydrolysis (Table 3). This conclusion was also drawn by Zhao et al. (2007) for spruce and NaOH pretreatment, by Fuentes et al. (2011) for SCB and lime pretreatment, by McIntosh et al. (2011) for wheat straw and NaOH pretreatment and by Rabelo et al. (2014) for SCB with alkaline hydrogen peroxide pretreatment. Strong pretreatment conditions, such as 9 wt% sodium hydroxide and 120 °C, show a faster enzymatic hydrolysis reaction and a higher yield reaching up to 87% conversion of cellulose to glucose and 75% of conversion of xylan to xylose. This can be attributed mainly to the opening of the SCB structure and the solubilization of lignin, which is removed at larger quantities if the pretreatment conditions are stronger.

Lignin has inhibition and adsorption effects on the cellulolytic enzymes, reducing their activity (Rahikainen, 2013; Meng and Ragauskas, 2014). Therefore, the solubilization of lignin yields a higher enzymatic hydrolysis of the low-lignin content residues. Figure 5 shows the relation between the k_1 parameter, which describes the maximum yield during cellulose enzymatic hydrolysis and the percentage of lignin removed during pretreatment.

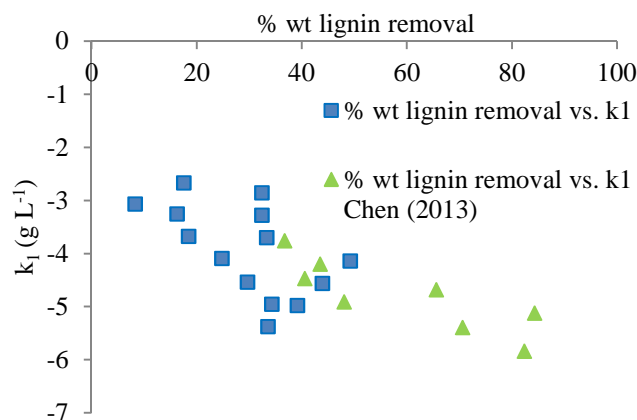


Figure 5 Enzymatic hydrolysis parameter k_1 plotted against the removal (%) of lignin from SCB by the pretreatments performed; ■ (blue) this study, ▲ (green) based on Chen et al. (2013).

It can be seen that the more lignin removed, the lower the k_1 will be, which possibly means that cellulose hydrolysis yield will be higher. k_1 constants were also calculated for the pretreatment conditions by Chen et al. (2013) and plotted against their lignin solubilization results. A similar trend is observed, where the more lignin removed, the higher the cellulose hydrolysis reactivity. However, Chen et al. (2013) used longer pretreatment times, which explains the higher lignin solubilization.

In addition to lignin removal from the residues, also hemicellulose removal had an influence on the enzymatic hydrolysis yield of the residues. The more severe the pretreatment conditions, in particular at higher sodium hydroxide loadings (9%) during pretreatment, the more xylan was removed. Figure 6 shows that the less xylan remains in a wet residue that was used for the enzymatic hydrolysis, the higher the overall enzyme conversion was for both glucan and xylan present in that residue. The reason for this can be that removing hemicellulose allows a better mass transfer between the substrate and the enzyme by opening the structure of SCB (Meng and Ragauskas, 2014).

During pretreatment, a decrease in pH was observed, which can be caused by phenolic acid groups that may be liberated by lignin degradations and turn into phenolate groups. Also, carboxylic groups formed by alkaline carbohydrate degradation can cause pH decrease (Palmqvist et al., 2000). To determine the optimal pretreatment conditions and the size of the enzymatic hydrolysis reactors a model is needed, that clearly describes the enzymatic hydrolysis reaction rate depending on the pretreatment conditions.

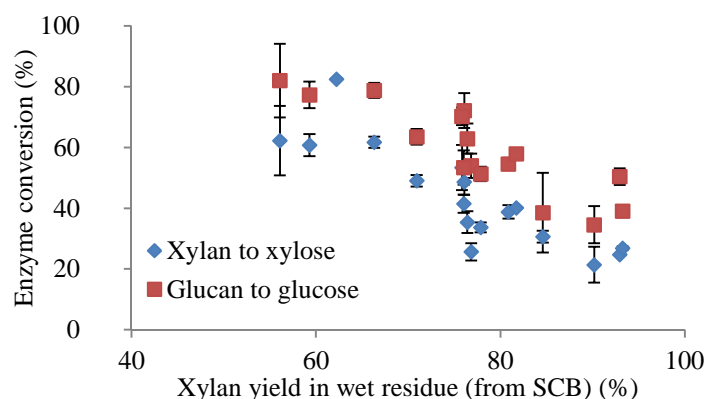


Figure 6: Enzymatic conversion (72 h) of the 'wet residue' obtained after pretreatment of SCB related to the xylan recovery in the 'Wet residue' (%) (calculated as originally present in SCB).

The obtained model can be substituted in the reaction rate term of a continuous or batch mass balance and can be optimized together with other models of unit operations of the whole process. The model has a good fit to the data as the R^2 of the model was calculated taking into account each experimental point in Figure 3 and was found to be 0.9644. This means that it simulated properly the cellulose enzymatic hydrolysis reaction for given pretreatment conditions. This is due to the large numbers of fitting parameters compared to the number of experimental data points. Consequently, the parameter values should be considered with precaution and the model should preferably be used to design pretreatment and enzymatic hydrolysis processes and unit operations within the range of studied pretreatment conditions. A similar model can be created for xylan hydrolysis if more time data points are available for xylose.

4.5. Conclusion

Alkaline pretreatment removed lignin, xylan and small amounts of cellulose from SCB. The more severe the conditions, the more xylan and lignin was solubilized. This resulted in increased enzymatic hydrolysis yield of xylan and glucan. The model presented here describes the enzymatic hydrolysis rate of cellulose in SCB depending on the sodium hydroxide pretreatment conditions. It can be used to design continuous pretreatment and enzymatic hydrolysis unit operations and optimize the whole SCB to fermentable sugar conversion process.

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Chapter 5

Deconstruction of lignin linked *p*-coumarates, ferulates and xylan by NaOH enhances the enzymatic conversion of glucan

ABSTRACT

Thermo-assisted NaOH pretreatment to deconstruct xylan and lignin in sugar cane bagasse (SCB) thereby, affecting its enzymatic degradability into fermentable sugars, is poorly understood. In this research, SCB milled fibres were pretreated using different dosages of NaOH (0, 4, 9% w/w per SCB dry matter) at different temperatures and residence times. At 0 and 4% w/w NaOH, between 17% and 34% of SCB xylan was not recovered in the residues, while at 9% NaOH both xylan and lignin were dissolved up to 47% and 48%, respectively. About 63% of the lignin compounds in SCB were *p*-coumarates and ferulates, analysed by pyrolysis-GC/MS as 4-vinyl phenol and 4-vinyl guaiacol, and designated as non-core lignin (NCL) compounds. The lower the relative abundance of NCL, the lower the recovery of xylan in the residues. At 4%NaOH mostly *p*-coumarates were removed, while at 9% NaOH both *p*-coumarates and ferulates were removed. Core lignin compounds, analysed as phenol, guaiacol and syringol, accumulated in the residues. Enzymatic glucan hydrolysis correlated positively with the simultaneous decrease in residual xylan and NCL.

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5.1 Introduction

There is an increasing interest to use sugarcane bagasse (SCB) as a source of monosaccharides, to be released from the polysaccharides present, for the production of biofuels and chemicals. SCB is composed of cellulose (37-45 %w/w), hemicellulosic xylan (26-32 %w/w) and lignin (11-28 %w/w). The contents vary due to, for example, seasonal crop variations or differences in conditions used to extract sucrose from the sugar cane (Canilha et al., 2011; Kim & Day, 2011; Murciano Martínez et al., 2015). A combination of physical pretreatment followed by a thermo-assisted chemical pretreatment is commonly performed in order to disrupt the structure of the lignocellulosic complex architecture (Mosier et al., 2005). A subsequent enzymatic hydrolysis step is required to convert the residual (hemi-) cellulosic structures into monosaccharides. Two of the most promising thermo-assisted chemical pretreatments used are dilute acid catalysed and dilute alkali catalysed treatments and both enhance the subsequent enzyme hydrolysis (Kumar et al., 2009). Thermo-assisted dilute acid pretreatments are reported to depolymerise and dissolve hemicellulose and minor amounts of lignin (Alvira et al., 2010). This may lead to formation of fermentation inhibitors, such as furans or organic acids, and require an additional detoxification step. Thermo-assisted NaOH pretreatments are known to dissolve lignin and hemicelluloses, and only low amounts of inhibitors remain in the (hemi-)cellulosic residues (Palmqvist & Hahn-Hägerdal, 2000; van der Pol et al., 2015). How NaOH interacts with lignin, however, is poorly understood. That may be partly due to difficulties encountered in the analysis of residual lignin. Also, to which extent the remaining lignin or hemicellulose in the cellulose-rich residues obtained after alkaline pretreatments affects the enzymatic hydrolysis of these residues is unknown.

In general, lignin is composed of 4-phenylpropanoid units, categorized based on the degree of methoxylation of the benzene ring as *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S) (Adler, 1977). The proportion of the three monolignol-derived units in lignin varies depending on the type of plant biomass (Ralph et al., 2004). For example, the main lignin unit present in softwood is generally the S unit, while grass type biomasses, like SCB, are rich in H units. In addition, a fraction of the H units in grasses is suggested to be interlinked with xylan. Therefore, such units are pending from the lignin bulk (Del Río et al., 2012b; Rencoret et al., 2013) and considered as non-core lignin (NCL). The complex formed when lignin is interlinked with xylan is called lignin-carbohydrate complex (LCC) (Fengel & Wegener, 1984). Within lignin, the units are inter-linked and thereby form a hydrophobic polymer. The most common linkages described are β -O-4, β -5, β - β , 5-5 and 5-O-4, with β -O-4 as the predominant one (Boerjan et al., 2003; Lewis &

Yamamoto, 1990). Recently, Del Río et al. (2015) published an extensive characterization of SCB lignin in which β -O-4 and β -5 are described as the main linkages, representing 83% and 6% of the total linkages present, respectively. Lapierre et al. (1989) showed that during 1M NaOH extraction of wheat straw, the main cleavages within the lignin bulk were in the aryl ether linkages, like β -O-4. In addition, peroxide treatments of grasses are reported to cleave alkyl-aryl ether linkages at pH values around 11 (Li et al., 2012). Nonetheless, when the aryl ether linkages are part of LCCs they appear to be stable in mild alkaline conditions (Kosikova et al., 1979). Ester linkages, for example existing between lignin units and carbohydrates, are the most labile under alkaline conditions (Buranov & Mazza, 2008). Nonetheless, no information on which lignin linkages are cleaved at different conditions of thermal pretreatments, ranging from a pH of 4 to 11, of particularly SCB is described in literature.

As has already been shown in chapter 3 and 4, the removal of xylan and lignin from the residues improves the enzymatic saccharification of glucan and xylan, into glucose and xylose. A removal of around 30% of xylan from SCB residues improved the enzymatic conversion of glucan into glucose (Chapters 3 and 4). But, removal of lignin was not quantified in that studies. In this study, it is hypothesized that only if both lignin and xylan are removed, for at least 30% each, from the residual cellulose, the enzymatic (hemi-)cellulose hydrolysis enhances as based on preliminary data from Chapter 3. It is also hypothesized that ester linkages between H units and xylan are cleaved at 4% of NaOH (w/w per substrate dry matter), reaching a pH above 8.6 (Chapter 4), thereby provoking removal of xylan, but not of lignin. At 9% NaOH pretreatments the pH is above 10 (Chapter 4), which is hypothesized to cleave β -O-4 linkages resulting in a release of NCL phenolics, such as ferulates and coumarates. As a consequence, xylan and lignin removal from residual cellulose should be observed. Therefore, the chemical composition of residual lignin remaining after alkaline pretreatment of SCB is determined by analytical pyrolysis-GC/MS and residual xylan recoveries are analysed. Finally, the extent of enzymatic glucan hydrolysis of the residues obtained after the pretreatments is correlated to the lignin and xylan recovery and type of lignin present in the residues.

5.2 Materials and methods

Materials used

Sugarcane bagasse (95% (w/w) dry matter (DM)), was kindly supplied by Corbion Thailand (Banchang, Thailand). The enzyme cocktails CellicCTec2 and

CellicHTec were kindly provided by Novozymes (Bagsvaerd, Denmark) and stored at 4°C. All chemicals used were purchased at Sigma-Aldrich (St. Louis, MO, USA), unless stated differently.

Alkaline pretreatments of SCB

Alkaline pretreatment of SCB fibres was conducted as explained in Chapter 4. The conditions used are shown in Table 1. The pretreated SCB samples were centrifuged (10000xg; 15min; 4°C) and the residues obtained were washed three times with water prior to freeze-drying (Chapter 4).

Table 1: Dry matter yield, Klason lignin and xylan contents in the residues after alkaline pretreatment of sugarcane bagasse (SCB) prior to enzyme hydrolysis (adapted from Chapter 4).

Sample code	Pretreatment conditions			Yield of DM in residue(%) ^b	Xylan in residue % (w/w) DM ^c	Klason lignin in residue % (w/w) DM ^c
	NaOH ^a (% w/w)	Temperature (°C)	Time (min)			
1	4	80	30	73.7	16.7±6	26.2±1
2	9	80	30	64.8	16.7±1	24.7±2
3	0	120	30	83.0	17.3±1	28.4±1
4	4	120	30	76.6	17.9±2	24.9±0
5	9	120	30	65.6	14.3±0	26.5±0
6	4	40	60	76.1	17.8±1	31.4±3
7	9	40	60	67.8	17.3±1	29.6±5
8	4	80	60	75.2	15.5±1	31.0±2
9	9	80	60	66.3	15.1±0	28.3±4
10	0	120	60	85.5	17.3±0	30.6±1
11	4	120	60	78.2	17.1±0	27.5±3
12	9	120	60	67.7	15.0±0	28.0±5

^a NaOH (g) per 100 g SCB dry matter

^bBased on dry matter present in SCB

^cDM= dry matter based

Enzymatic incubation of the residues obtained after alkaline pretreatment of SCB

The residues, obtained after alkaline pretreatment of SCB, were incubated with a combination of CellicCTec2 and CellicHTec (ratio 9:1) as described in Chapter

4. The analysis and quantification of monosaccharides released by the enzymes was performed by using HPAEC of which the details are provided in Chapter 4.

Analytical pyrolysis GC-MS

Pyrolysis of 100 µg, weighed on a Mettler-Toledo XP6 microbalance (Mettler-Toledo, Columbus, OH, USA), of all SCB residues was performed with an EGA/PY-3030D micro-furnace pyrolyzer (Frontier Laboratories, Fukushima, Japan) connected to a Thermo7820A gas chromatograph using a DB-1701 fused-silica capillary column (60 m × 0.25 mm internal diameter, 0.25 µm film thickness) coupled to a DSQ-II thermo mass selective detector (EI at 70 eV) (Thermo Scientific, Waltham, MA, USA). The same residues were submitted to pyrolysis-GC/MS (py-GC/MS) after tetramethylammonium hydroxide (TMAH) treatment performed as described elsewhere (Kuroda et al., 2001). The pyrolysis was performed at 500°C for 1min using a split flow of 1:133 after pyrolysis. The oven temperature was programmed from 45°C (0-4 min) to 280°C (5-60 min) at 4°C min⁻¹. Helium was the carrier gas (1 mL min⁻¹). The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and with those reported in literature (Ralph, 1991). Peak molar areas were calculated for all py-GC/MS products analysed and converted into relative abundance (RA) by dividing the peak area by the total area. Peak molar areas divided by the weight of dry matter subjected to pyrolysis were calculated and denoted as area (A_x). The abundance of a certain compound in the residues (A_x) is compared to the relative abundance of the same compound in the residue obtained after the mildest pretreatment (Sample code 3; Table 1; A_3) as relative area (A_x/A_3).

As a measure for the normalised lignin compound yields (NA_x ; equation 1), A_x was multiplied with the amount of dry matter recovered (RDM) after pretreatment in the residue (Chapter 4, Table 1). A_x was related to A_3 to the normalise the lignin yield related to the mildest pretreatment as $NY^{PL}_{x/3}$ (equation 2). Measurements were performed in triplicate and samples treated with tetramethylammonium hydroxide (TMAH) were analysed in duplicate.

$$(1) \quad NA_x = A_x \times RDM_x \text{ (g)}$$

RDM = recovered dry matter from SCB in the residue after pretreatment (g)

$$(2) \quad NY^{PL}_{x/3} \text{ (\%)} = \frac{NA_x}{A_3} \times 100$$

5. 3 Results and discussion

Enzymatic hydrolysis versus xylan and lignin yield of alkaline pretreated SCB

SCB milled fibres were treated with different concentrations of NaOH and at various temperatures and residence times (Table 1). Such pretreatment conditions have been described to increase the surface area of (hemi-)celluloses by swelling of the material and removal of lignin (Park & Kim, 2012). It was hypothesized, based on data from Chapters 3 and 4, that at least 30% (w/w) of lignin and xylan needs to be separated from the insoluble residues remaining after pretreatment to obtain an increase in the enzymatic conversion of residual (hemi-)cellulose. The yield of xylan in the Residues obtained after pretreatments is shown in Table 1. In Figure 1A, it is shown that the residual xylan recovery correlated well with the enzymatic conversion of glucan to glucose. In Chapter 4, it is shown that the conversion of residual xylan to xylose followed the same trend. Next, it was studied whether this correlation was due to xylan removal alone, or also partly due to lignin removal. Hence, lignin and xylan yields in the residues were analysed and plotted as shown in Figure 1B. The lignin yields of the mildest pretreatment performed ($NY^{PL_{x/3}}$; Table 1) was set as 100%. It was assumed that in the residue obtained after the mildest pretreatment all lignin present from SCB was recovered. Calculations are given in the materials and methods.

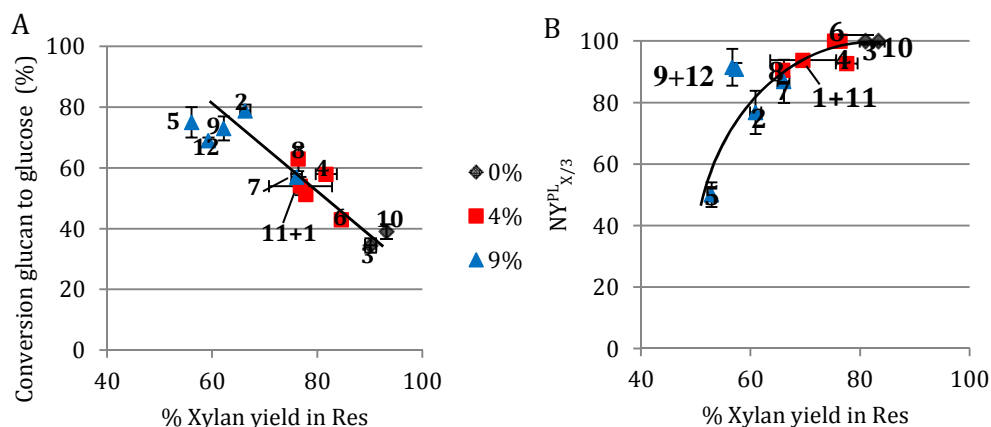


Figure 1. Xylan yield in the residues (Res) (% w/w) versus conversion of glucan in the residues to glucose (A, adapted from Chapter 4) and xylan yield versus normalised lignin yields obtained in the residues (Res) based on pyrolysis GC/MS ($NY^{PL_{x/3}}$), after alkaline pretreatment at 0% (blue), 4% (red) and 9% (green) NaOH dosages (B). Lines (black curves) represent only trends. Numbers refer to the sample codes described in Table 1.

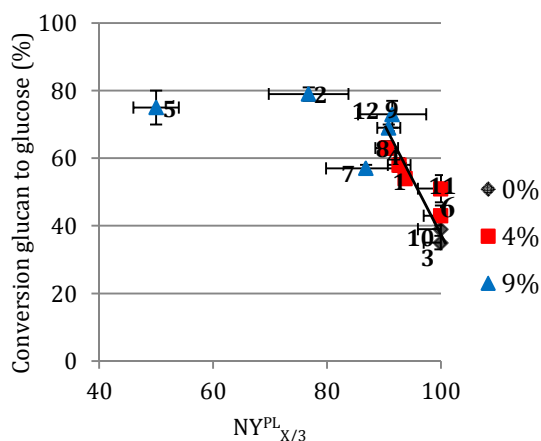


Figure 2. Normalised lignin yields obtained in the residues (Res) based on pyrolysis GC/MS ($NY^{PL}_{x/3}$), after alkaline pretreatment at 0% (blue), 4% (red) and 9% (green) NaOH dosages, plotted versus the enzymatic conversion of glucan to glucose. Lines (black curves) represent only trends. Numbers refer to the sample codes described in Table 1.

Analytical pyrolysis-GC/MS (py-GC/MS) was used to determine the normalised residual lignin yield instead of the traditionally used Klason lignin method (Hatfield et al., 2005). The aspecific character of the latter method was the main reason to choose for py-GC/MS. It can be seen from Figure 1 that at 4% NaOH pretreatment, however, the xylan yield (65 to 80%) was decreased more than the $NY^{PL}_{x/3}$ (90 to 100%), while for the pretreatments at 9% NaOH, the $NY^{PL}_{x/3}$ (50 to 90%) decreased more than the xylan yield (55 to 65%). Hence, the lower the xylan yield the lower the normalised lignin yield ($NY^{PL}_{x/3}$), but not following a linear trend. In order to determine the influence of lignin removal on the enzymatic conversion of glucan into glucose, $NY^{PL}_{x/3}$ is plotted against enzymatic conversion of glucan in Figure 2. The figure shows that the higher the normalised yield of lignin ($NY^{PL}_{x/3}$), the lower the enzymatic conversion of glucan into glucose in the residues with the exception of residue 5. This trend was most pronounced for the residues obtained after the 9% NaOH pretreatments. However, the correlation is not as linear as shown in Figure 1A for xylan yield in residues versus enzymatic conversion of glucan. The latter indicates that xylan removal had a more pronounced influence on the enzymatic conversion of glucan than lignin removal. A recent study of Yu et al. (2015) showed a complete enzymatic conversion of glucan into glucose after pretreatment of SCB by using alkaline peroxide, which provoked a release of the lignin and, to a lower extent, of the xylan from SCB in the remaining residues.

To sum up, a decrease in xylan yield of 30% gave an increased enzymatic glucan conversion, but more than 70% of lignin remained. Hence, the hypothesis proposing that a solubilisation of at least 30% of both xylan and lignin lead to an increase in enzymatic conversion of residual (hemi-)celluloses was corroborated for the xylan yield, but rejected for the lignin yield. Nevertheless, a trend showing an increase in enzyme hydrolysis with lower normalised lignin yields was observed.

Characterization of alkaline treated SCB residues by pyrolysis-GC/MS and TMAH-pyrolysis-GC/MS

The second hypothesis proposes that β -O-4 linkages are cleaved at 9% NaOH, as proposed in literature (Lapierre et al., 1989), which should result in a release of non-core lignin phenolics, such as ferulates and coumarates. In order to corroborate this hypothesis, the residual lignin compounds were analysed by py-GC/MS, with and without TMAH. Py-GC/MS with TMAH is used to discriminate non-core from core lignin units (Del Río et al., 2007). The pyrograms of the residues with the highest normalized lignin yield (sample 3 (Res₃); Figure 1) and the lowest lignin yield (sample 5 (Res₅); Figure 1) are shown in Figure 3. The identities of the compounds analysed and their relative abundances are given in Table 2. Pyrolysis derived compounds originating from carbohydrates were also present and indicated in Figure 3, but not further taken into account in this research. The most abundant lignin units in untreated SCB were 4-vinyl phenol (52%), 4-vinyl guaiacol (11%), phenol (5%), guaiacol (4.6%) and syringol (3.6%) (Table 2).

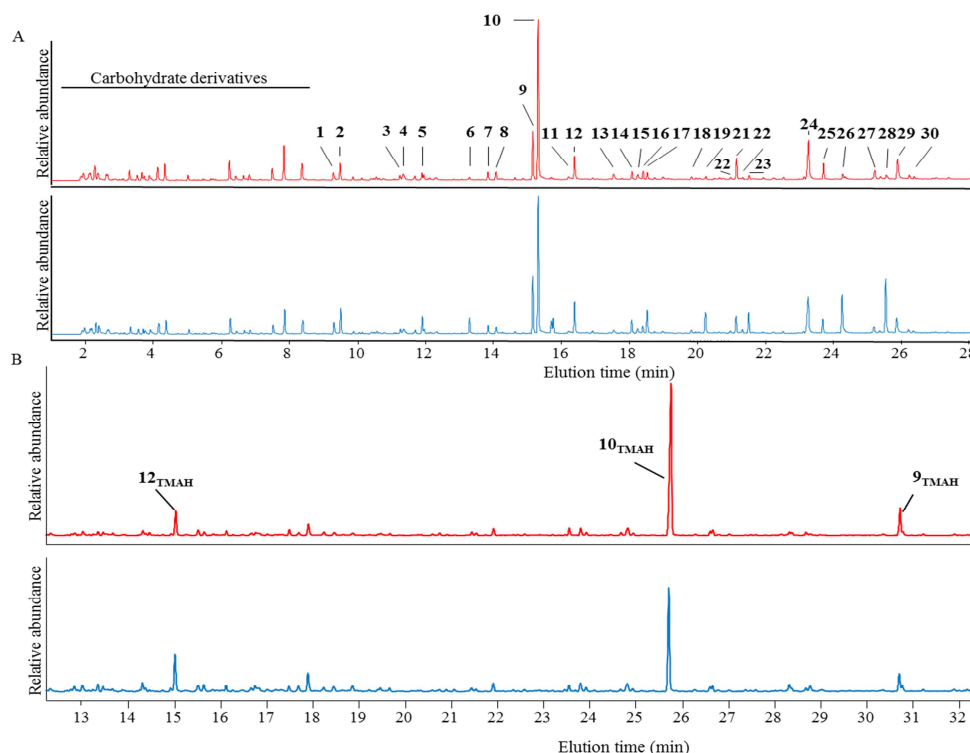


Figure 3. Pyrolysis GC/MS pyrograms of SCB residues 3 (red) and 5 (blue), in absence (A) and in presence of TMAH (B). Numbers (N) refer to the compounds in Table 2.

The sum of these compounds account for 76% of all lignin units present in SCB and were also annotated as the main lignin units for untreated SCB in literature (Lopes et al., 2011; Del Río et al., 2015).

Not only in untreated SCB, but in all residues analysed, 4-vinyl phenol, 4-vinyl guaiacol, guaiacol, syringol and phenol were the most abundant lignin units and covered at least 70% of the total lignin peaks analysed, with 4-vinyl phenol as most dominant (Table 2). The relative abundance (RA), however, of these main SCB lignin compounds varied (Table 2), with Res₃ and Res₅ being the two extremes. Seen from Table 2, the RA of 4-vinyl phenol (peak 10) the RA was 56 and 38%, for Res₃ and Res₅, respectively. Of 4-vinyl guaiacol (peak 9) the RA was 11% for both. Of phenol (peak 1) the RA was 3.7 and 6.8%, for Res₃ and Res₅, respectively. Of guaiacol (peak 2) the RA was 4 and 9%, for Res₃ and Res₅, respectively and of syringol (peak 12), 3 and 6.8%, for Res₃ and Res₅, respectively.

CHAPTER 5

Table 2. Relative abundance of Py-GC/MS analysed lignin compounds (numbered as N 1 to 30). Standard deviations are lower than ± 0.5 unless presented differently. Chemical structures of the lignin compounds are represented in Supplementary Figure 1.

N ¹	Compound name	Residues ²												
		SCB	1	2	3	4	5	6	7	8	9	10	11	12
1	Phenol	5.0	6.1	5.7	3.7	4.8	6.8	4.7	5.4	4.5	6.4	3.5	4.6	15 \pm 0.6
2	Guaiacol	4.6	6.3	6.9	3.6	5.1	8.6	5	6.3 \pm 0.5	4.7	7.3 \pm 0.7	3.4	4.9	15
3	4-Methylphenol	2.0	1.6	1.6	1.8	1.6	1.8	1.5	1.5	1.6	1.8	1.7	1.5	2.3
5	4-Methyl guaiacol	2.2	1.5	1.8	2	1.6	2.2	1.5	1.4	1.9	1.8	2	1.6	1.3
6	4-Ethylphenol	1.4	1.6	1.7	1.1	1.3	1.7	1.2	2.1	1.4	1.6	1.1	1.3	3.3
9	4-Vinylguaiacol	11 \pm 0.5	10	11 \pm 0.8	11 \pm 0.6	10	11.2	9.8	9.5	11 \pm 0.6	11.1	10.7	10.3	11 \pm 0.5
10	4-Vinylphenol	52 \pm 1.7	49 \pm 1.1	46 \pm 0.8	56 \pm 0.7	52 \pm 1.4	38 \pm 0.8	54 \pm 0.7	48 \pm 2.1	52 \pm 1.1	43 \pm 2.2	57 \pm 0.6	52 \pm 0.9	27.4
12	Syringol	3.6	5.2	5.7	3	4.6	6.8	4.4	7 \pm 0.8	4.2	6.2	3	4.7	8.5
14	Trans- Isoeugenol	1.6	1.4	1.7	1.4	1.4	1.9	1.3	1.4	1.5	1.6	1.4	1.4	1.4
16	4-Methyl syringol	1.8	1.3	1.6	1.8	1.6	1.7	1.5	1.7	1.7	1.5	1.8	1.6	0.9
17	Vanillin	2	1.2	1.3	1.6	1.4	1.6	1.4	1.1	1.4	1.3	1.4	1.4	1.5
18	Homovanillyl	0.7	0.6	0.7	0.9	0.7	0.7	0.6	0.5	0.7	0.7	0.9	0.7	0.4
19	Acetovanillone	0.7	0.6	0.8	0.7	0.7	0.9	0.7	0.6	0.7	0.8	0.7	0.7	1.1

Deconstruction of *p*-coumarates and ferulates enhances glucan conversion

Continuation Table 2. Relative abundance of Py-GC/MS analysed lignin compounds (numbered as N 1 to 30).

N ¹	Compound name	Residues ²												
		SCB	1	2	3	4	5	6	7	8	9	10	11	12
20	4-Hydroxy-benzaldehyde	0.7	0.5	0.5	0.8	0.6	0.3	0.6	0.5	0.5	0.4	0.7	0.6	0.7
21	4-Vinylsyringol	2.7	4.5	4.5	2.7	3.8	5.1	3.6	4.5	3.4	4.8	2.6	3.7	3.5
22	Guaiacylacetone	0.6	0.7	0.7	0.7	0.6	1.1	0.5	0.6	0.6	0.8	0.5	0.6	1.2
23	4-Allyl-2,6-di methoxyphenol	0.5	0.4	0.6	0.5	0.4	0.6	0.4	0.5	0.5	0.5	0.5	0.5	0.3
25	4-Propenyl phenol	2.2	2.1	2.5	2.3	2.1	2.4	2.1	2.3	2.2	2.4	2.3	2.1	1.4
26	Syringaldehyde	0.7	0.7	0.7	0.8	0.8	0.9	0.7	0.7	0.8	0.8	0.9	0.8	0.5
28	Acetosyringone	0.5	0.6	0.8	0.6	0.6	1	0.6	0.7	0.7	0.8	0.6	0.6	0.9
29	Trans-coniferyl-alcohol	1	1.5	1.4	1.2	1.8	1.8	1.8	1.6	1.6	1.7±0.5	1.2	2	0.1
30	Syringylacetone	0.5	0.6	0.6	0.6	0.6	0.8	0.6	0.7	0.6	0.8	0.6	0.6	0.8
	Sum	98	96	95	96	96	95	96	95	96	95	96	96	97

¹Peak annotation numbers used in Figure 2

²Standard deviations are lower than ±0.5 unless presented differently.

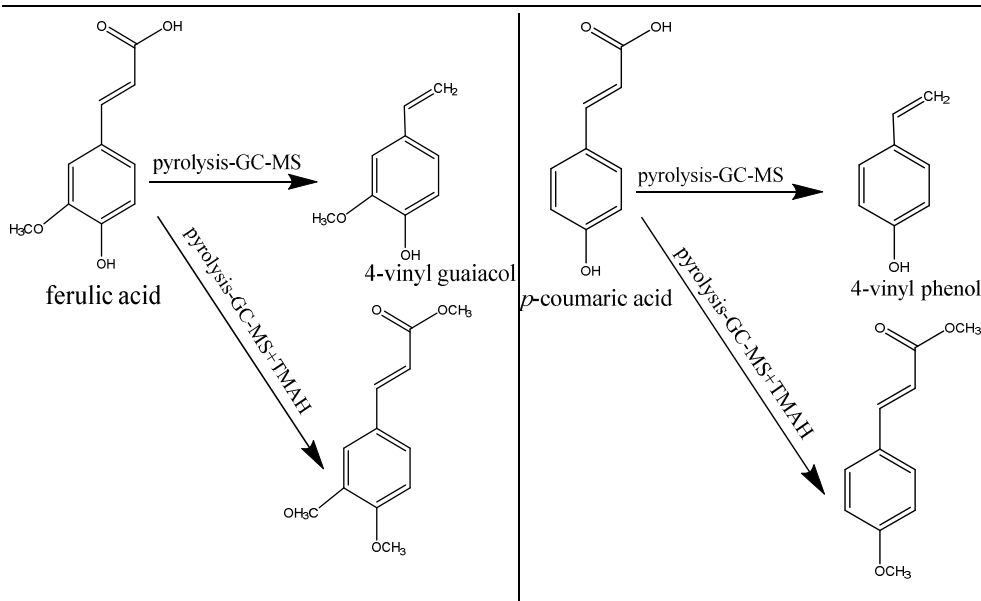


Figure 4. Schematic representation of TMAH derivatization and corresponding expected compounds in py-GC/MS analysis of *p*-coumaric acid (A) and ferulic acid (B).

Previous studies report that the predominance of 4-vinyl phenol and 4-vinyl guaiacol in the pyrograms of grasses is due to degradation during pyrolysis of *p*-coumarates and ferulates, respectively (Del Río et al., 2012a, b). To find out if the 4-vinyl phenol and 4-vinyl guaiacol analysed in SCB residues originated from *p*-coumaric acid and ferulic acid, the residues were subjected to methylation by TMAH followed by subsequent py-GC/MS. The use of TMAH in py-GC/MS avoids decarboxylation of *p*-coumarates and ferulates, as shown in Figure 4. TMAH also induces cleavage of alkyl-aryl ether bonds in lignin releasing methylated derivatives, such as 4-vinyl phenol and 4-vinyl guaiacol. This method allowed the discrimination between 4-vinyl phenol/4-vinyl guaiacol present as such from being py-GC/MS products of decarboxylated *p*-coumarates and ferulates, respectively (Del Río et al., 2007). TMAH py-GC/MS (Figure 2B) of Res₃ and Res₅ showed three main peaks coded as 9_{TMAH}, 10_{TMAH}, 12_{TMAH}, of which the structures are shown in Supplementary Figure 1. No methylated derivatives from 4-vinyl phenol and 4-vinyl guaiacol were detected in the TMAH pyrograms. Therefore, it was concluded that most of the 4-vinyl phenol and 4-vinyl guaiacol analysed by py-GC/MS (without TMAH) of the residues indeed originated from *p*-coumarates and ferulates, respectively (Del Río et al., 2007). The third major annotated compound was 1,2,3-trimethoxybenzene (12_{TMAH}), which has a syringol like ring-structure. In grasses, S units like syringol, are reported to be linked to *p*-coumarates mainly via ester linkages (Del Río et al., 2007; Del Río et al., 2008; Grabber et al., 1996). The cleavage of a syringol-*p*-coumarate linkage and methylation

of both units is possible in TMAH py-GC/MS as this method induces the cleavage of esters or alkyl-aryl ether bonds (Filley et al., 1999; Kuroda & Nakagawa-izumi, 2006). The 1,2,3-trimethoxybenzene could also be derived from triclin after the alkyl cleavage of the structure by TMAH pyrolysis. The presence of triclin, which is an *O*-methylated flavone, is recently reported to be a generic characteristic of grass lignin (Lan et al., 2015), and it is also reported to be present in SCB lignin (Del Río et al., 2015).

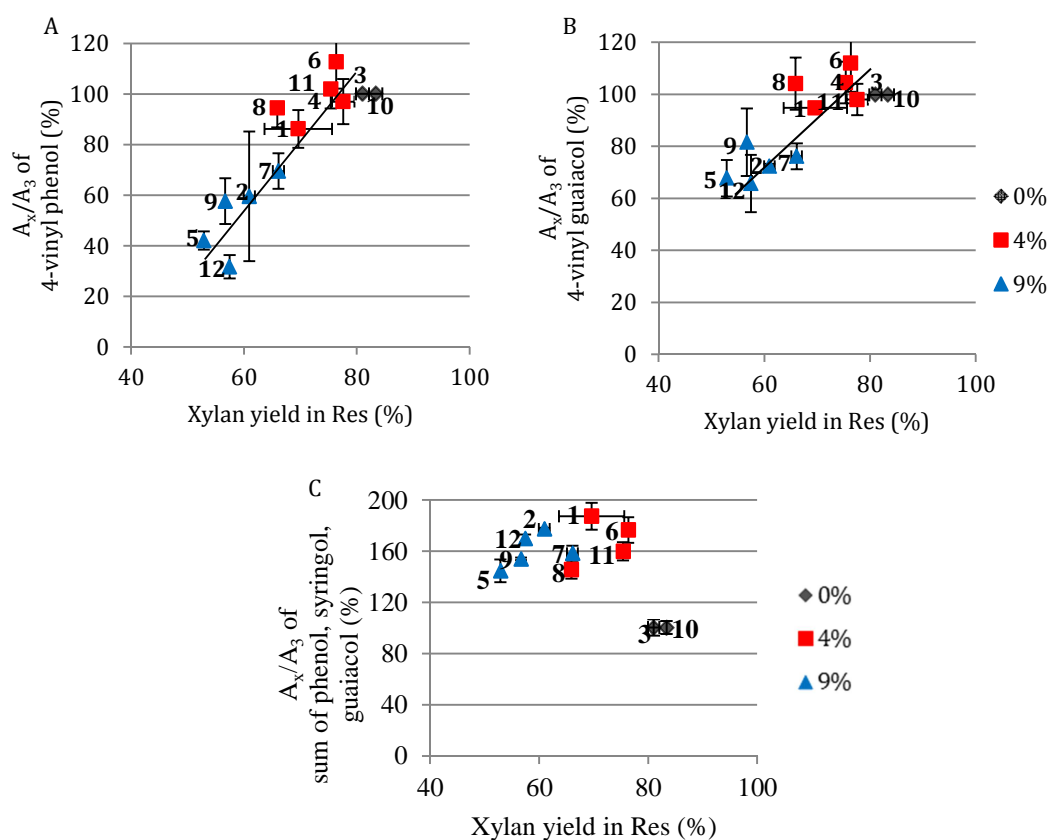


Figure 5. The relative area, related to Res₃ (A_x/A_3), of 4-vinyl phenol (A), 4-vinyl guaiacol (B) and the sum of phenol, guaiacol and syringol (C) versus the xylan yield in the residues obtained after NaOH pretreatments of SCB. Black lines represent only trends.

As mentioned above, the xylan yield in the residues correlated with the enzymatic hydrolysis of glucan to glucose, and is also shown in Chapter 4. Therefore, it is proposed to use the residual xylan yield as a measure of the severity of the pretreatment performed. In Figure 5, the relative area of the most pronounced lignin compounds analysed in the residues, related to Res₃ (A_x/A_3), is plotted versus the

residual xylan yields and shown in Figure 5 as 4-vinyl phenol (Figure 4A), 4-vinyl guaiacol (Figure 5B) and the sum of phenol, syringol and guaiacol (Figure 5C). It should be mentioned that the trend of the individual compounds phenol, syringol and guaiacol was similar to their sum (Table 2). Interestingly, an almost linear correlation was found between the residual xylan yield and the RA_x/RA_3 of 4-vinyl phenol, originating from *p*-coumarates (Figure 5A). The same trend was visible for the A_x/A_3 of 4-vinyl guaiacol, originating from ferulates, versus the xylan yield. The A_x/A_3 -sum of phenol, syringol and guaiacol (Figure 5C) versus the xylan yield, did not show any trend. The correlation possibly indicated that xylan and lignin are linked via *p*-coumarates and/or ferulates in sugarcane bagasse and dissolved together by the NaOH pretreatments performed. The latter is supported by the fact that ferulates are described in grasses to be attached mainly to carbohydrates via ester linkages and ether (β -O-4) linked to lignin. These ferulates can participate as bridges in LCCs and carbohydrate-carbohydrate complexes. For completeness, it should be noted that also a fraction of ferulates is described to be only linked to lignin via ether-linkages in wheat straw (Sun & Cheng, 2002).

In figure 6, a summary of the possible linkages in which ferulates participate in grasses is given and also possible linkages in which *p*-coumarates participate. Like ferulates, *p*-coumarates are described to be attached via ester bonds, mainly to lignin units and specifically to S units or to tricin. In addition, *p*-coumarates can be linked via ether-linkages to lignin. Nonetheless, the hydroxyl group of the phenolic ring is mainly reported to not be further linked (Del Río et al., 2008; Ralph, 2010; Ralph et al., 1994).

In SCB 4% NaOH residues, ester linkages are considered to be cleaved, because of the high pH values (pH>8.6, 0.1M NaOH). Hence, the decrease in xylan and relative area of 4-vinyl phenol in the residues after 4% NaOH pretreatments may be the result of cleavage of the ester linkages between ferulates and xylan and between *p*-coumarates and S/tricin (Figure 6). At 4% NaOH, ferulates analysed as 4-vinyl guaiacol, are less extracted compared to *p*-coumarates, analysed as 4-vinyl phenol (Figure 5). This indicates that ferulates are mostly still linked to lignin in the residues via the in literature reported β -O-4 linkages (Figure 6). In the residues obtained after the SCB pretreatments at 9% NaOH, both *p*-coumarates and ferulates levels, analysed as 4-vinyl phenol and 4-vinyl guaiacol, respectively, decreased significantly (Figure 5). This may indicate cleavage of both ester and β -O-4-linkages (Figure 6). Hence, the non-core lignin (NCL) units, analysed by py-GC/MS as 4-vinyl phenol and 4-vinyl guaiacol were more affected by the alkaline pretreatments performed compared to the core lignin (CL) units, analysed by py-GC/MS as guaiacol, syringol and phenol. CL compounds are reported to be linked via alkaline recalcitrant linkages, such as β -5 or 5-5 linkages, which are not easily degraded under the alkaline conditions used in this research (Yang et al., 2012).

Deconstruction of *p*-coumarates and ferulates enhances glucan conversion

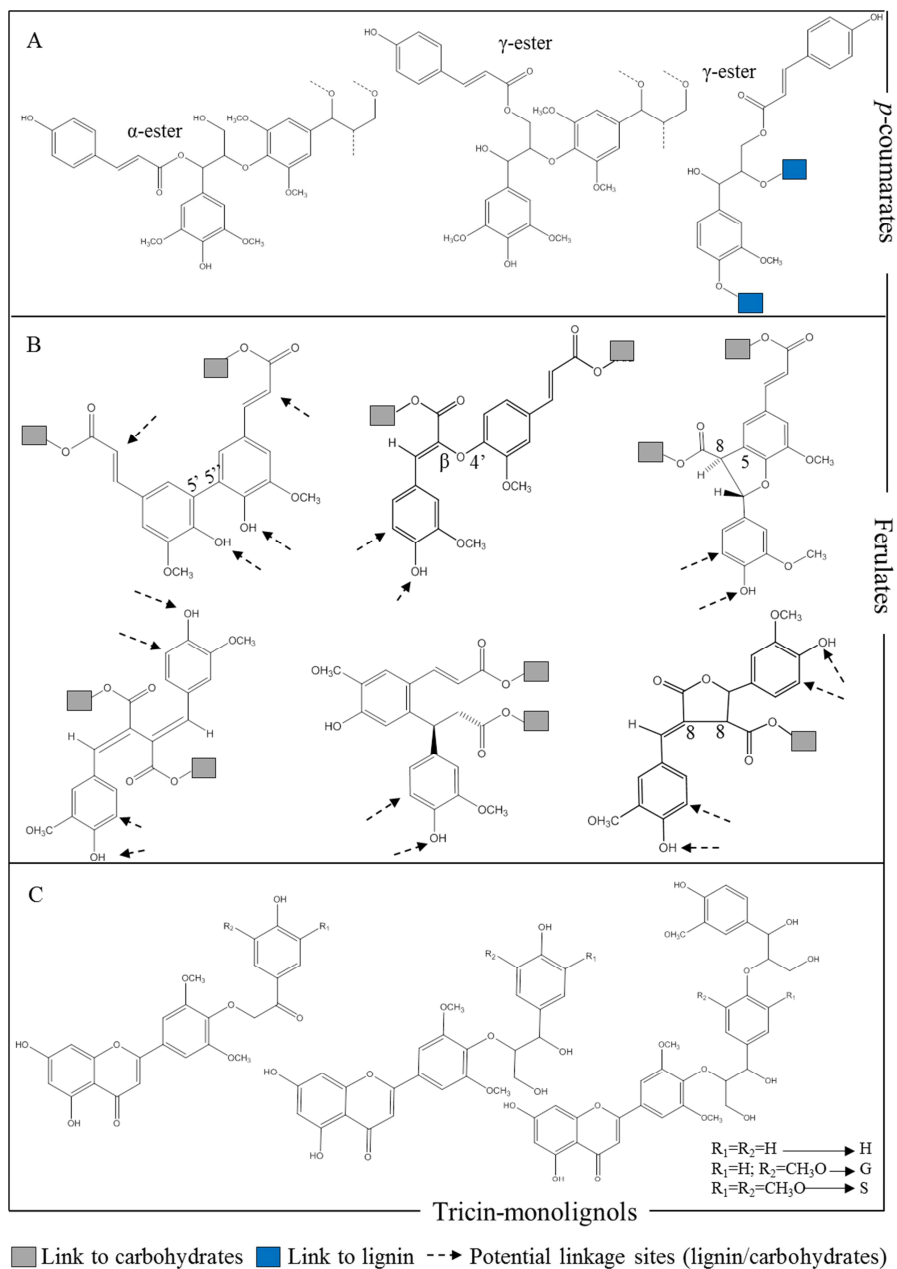


Figure 6. *p*-Coumarates (A) and ferulates (B) in lignin-xylan or xylan-xylan complexes reported to occur in grass (adapted from Crestini et al. (1997), Grabber et al. (1996, 2000) and Del Río et al. (2015)). Tricin-monolignol synthetic oligomers (C) are adapted from Lan et al. (2015)).

Now, we come back at our hypothesis that ester linkages between H units and xylan are cleaved at 4% of NaOH provoking removal of xylan, but not removing lignin. Indeed, at 4% NaOH xylan yields were lower than 70% and ester linkages are known to be cleaved ($\text{pH} > 8.6$). Hence, the hypothesis was mostly corroborated with the remark that *p*-coumarate levels had already decreased in the residues obtained after 4% NaOH pretreatments. At 9% NaOH pretreatments both *p*-coumarates and ferulates levels were lower than at 0 or 4% NaOH, in the residues obtained. The latter observation corroborates with our hypothesis based on literature knowledge, that in the 9% NaOH pretreatments of SCB both ester and β -O-4 linkages are cleaved, resulting in a release of non-core lignin phenolics, such as ferulates and coumarates. Obviously, in addition to these linkage cleavages, the release of xylan is also due to other degradation mechanisms like alkaline peeling of the xylan (Murciano Martínez et al., 2015) and alkaline disruption of hydrogen bonds between xylan and between cellulose and xylan (Xiao et al., 2001).

Extraction of different lignin populations and relation to enzymatic degradation of glucan

Figure 7 shows the plots of the relative area (A_x/A_3) or the yield ($\text{NY}^{\text{PL}}_{x/3}$) versus the enzymatic conversion of the glucan to glucose of the most abundant residual lignin compounds. Both the A_x/A_3 and the normalized lignin yield ($\text{NY}^{\text{PL}}_{x/3}$) of the sum of guaiacol, syringol and phenol, increased or remained constant upon increasing conversion of glucan into glucose.

The relative area of 4-vinyl phenol and 4-vinyl guaiacol, (Figures 7C and E, respectively), was constant and around 100%, compared to Res_3 , after 4% NaOH treatments. Nevertheless, in two of the residues obtained after the pretreatments at 4% NaOH (Res_1 and Res_4) the $\text{NY}^{\text{PL}}_{x/3}$ of 4-vinyl phenol and 4-vinyl guaiacol was decreased. Therefore, it was concluded that py-GC/MS analysed 4-vinyl phenol and 4-vinyl guaiacol were extracted from Res_1 and Res_4 and to a higher extent than the CL compounds, analysed as phenol, syringol and guaiacol. The glucan conversion was higher for the residues obtained after 4% NaOH pretreatments compared to 0% NaOH. As explained above and shown in Figure 1, the increased enzymatic conversion of glucan may be related to the xylan removal, rather than the removal of NCLs. In the residues obtained after the 9% NaOH pretreatments, a significant lower RA_x/RA_3 was shown of both 4-vinyl phenol and 4-vinyl guaiacol. The corresponding enzymatic glucan conversion was also highest, ranging from 60 to 80% (Figure 7).

Deconstruction of *p*-coumarates and ferulates enhances glucan conversion

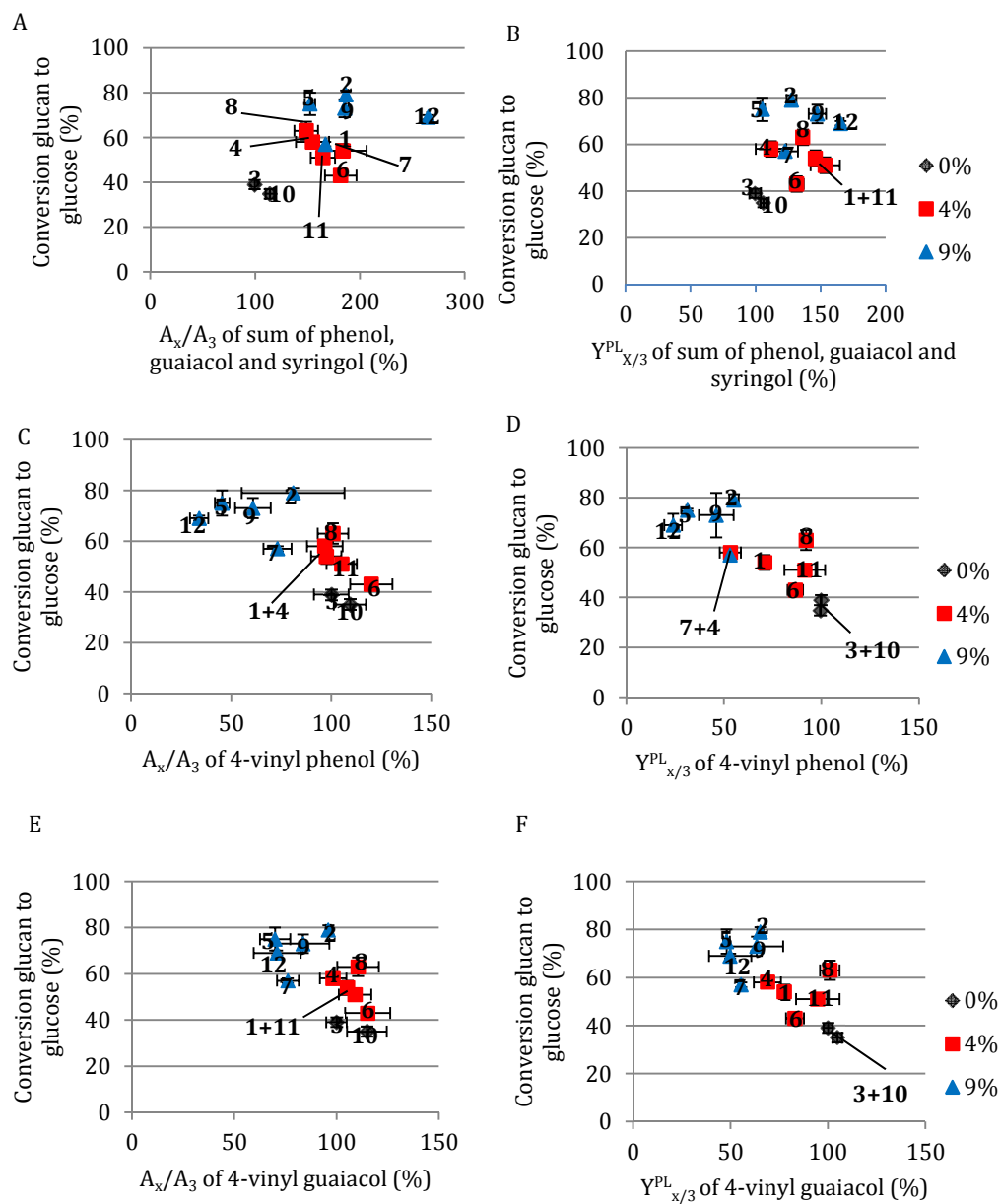


Figure 7. Conversion of residual glucan to glucose compared to the relative area (A_x/A_3) and yield ($Y^{PL}_{x/3}$) of the major residual lignin compounds analysed in the residues by py GC/MS: sum of guaiacol, syringol and phenol (A,B), 4-vinyl phenol (C,D) and 4-vinyl guaiacol (E,F).

During all SCB pretreatments at 9% NaOH, py-GC/MS analysed 4-vinyl phenol and 4-vinyl guaiacol were extracted to the highest extent, as $Y^{PL}_{x/3}$ were lowest (Figure 7). The lowest $Y^{PL}_{x/3}$ of both units was observed for Res₅ for which the enzymatic conversion of glucan into glucose was among the highest obtained (79%). These observations strengthen the conclusions given above, that ester linkages between H units and xylan are cleaved at 4% and β -O-4 link at 9% of NaOH and not only resulting in lower levels (A_x/A_3), but also in lower yields ($Y^{PL}_{x/3}$) of *p*-coumarates and ferulates.

5.4 Conclusion

In conclusion, the two initial hypothesis proposed were answered. The solubilisation of 30% of xylan increased the enzymatic hydrolysis of glucan to 65% as hypothesized. The percentage of lignin solubilised to reach 65% glucan conversion was around 10%, which was lower than hypothesized (30%). However, the maximum conversion of glucan obtained (79%) was achieved when 40% of xylan and 25% of lignin was solubilised. The selective solubilisation of ester linked *p*-coumarates at 4% NaOH and of both *p*-coumarates and ferulates at 9% NaOH correlated with the decrease in residual xylan and an increase in enzymatic hydrolysis of residual glucan.

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CHAPTER 5

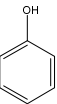
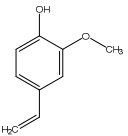
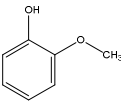
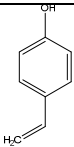
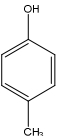
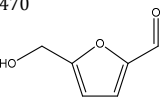
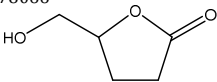
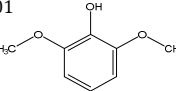
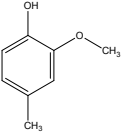
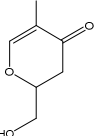
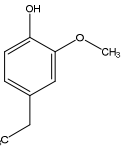
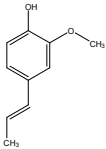
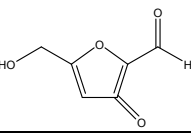
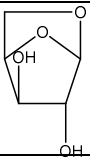
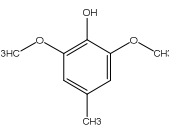
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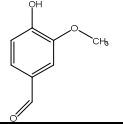
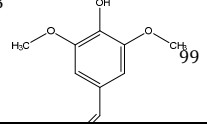
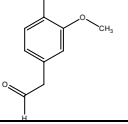
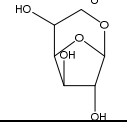
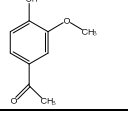
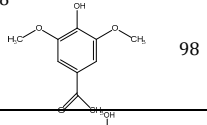
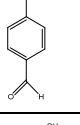
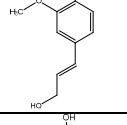
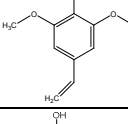
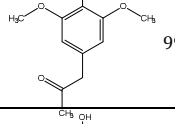
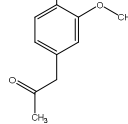
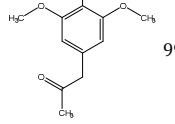
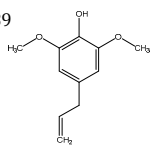
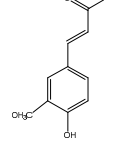
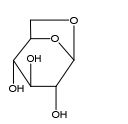
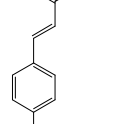
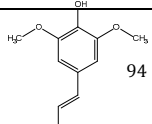
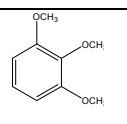
CHAPTER 5

Supplementary data

Supplementary Figure 1. Identities of lignin-derived structures annotated with py-GC/MS and relative molar area higher than 1% in SCB.

N.	Compound	CAS number	Chemical structure	Reverse search (in untreated SCB)	N.	Compound	CAS number	Chemical structure	Reverse search (in untreated SCB)
1	Phenol ^a	108952		99	9	4-vinylguaiacol ^a	7786610		100
2	Guaiacol ^a	90051		99	10	4-vinylphenol ^a	2628173		100
3	<i>p</i> -cresol ^a	106445		99	11	5-hydroxy-methylfurfural ^a	67470		88
4	5-(Hydroxy methyl) dihydro-2(3H)-furanone ^b	3278066		90	12	Syringol ^a	91101		100
5	4-methyl guaiacol ^a	93516		96	13	1,4-dideoxy-D-glycerohex-1-enopyranos-3-ulose ^b	-		98
6	4-ethyl guaiacol ^a	2785899		98	14	<i>Trans</i> -isoeugenol ^a	97541		99
7	5-hydroxy-2-tetrahydrofural dehyde-3-one ^b	Unknown		99	15	1,4-anhydroxylo-furanose ^b	-		99
8	Unknown (dihydroxy pyran-1-one) ^b	Unknown		100	16	4-methyl syringol ^a	6638057		99

Deconstruction of *p*-coumarates and ferulates enhances glucan conversion

N.	Compound	CAS number	Chemical structure	Reverse search value	N.	Compound	CAS number	Chemical structure	Reverse search value
17	Vanillin ^a	121335		100	26	Syringaldehyde ^a	134963		99
18	Homovanillyl ^b	26303902		100	27	1,6-anhydro-β-D-glucofuranose ^b	-		-
19	Acetovanillone ^a	498022		98	28	Acetosyringone ^a	2478388		98
20	4-hydroxy-benzaldehyde ^b	123080		100	29	<i>Trans</i> -coniferyl-alcohol ^a	458355		99
21	4-vinylsyringol ^b	28343228		100	30	Syringylacetone ^a	19037582		99
22	Guaiacylacetone ^a	2503460		98	30	Syringylacetone ^a	19037582		99
23	4-allyl-2,6-dimethoxyphenol ^a	6627889		97	9	3-(3-(4,6-dimethoxyphenyl)-2-propenoic acid methyl ester			99
24	Levoglucosan ^a	498077		94	10	3-(4-methoxyphenyl)-2-propenoic acid methyl ester			99
25	<i>Trans</i> -2,6-dimethoxy-4-propenylphenol ^b	26624135		94	12	1,2,3-trimethoxybenzene			99

^aInterpretation based on pure compounds

^bInterpretation based on Ralph (1991)

Chapter 6

The two *Rasamsonia emersonii* α -glucuronidases, ReGH67 and ReGH115, show a different mode-of-action towards glucuronoxylan and glucuronoxylo-oligosaccharides

ABSTRACT

Partly substituted xylan-structures remain after physical/chemical pretreatment of grass type plant biomass, in particular the ones substituted with (4-*O*-methyl-)glucuronic acids (UA_{me}). Hence, α -glucuronidases play an important role in the degradation of UA_{me}xylan structures, thereby facilitating the complete utilization of plant biomass. Here, the mode-of-action of two α -glucuronidases was demonstrated, both obtained from the fungus *Rasamsonia emersonii*; one belonging to the glycoside hydrolase (GH) family 67 (ReGH67) and the other to GH115 (ReGH115). Both enzymes functioned optimal at around pH 4 and 70°C. ReGH67 was able to release UA_{me} from with UA_{me} substituted xylo-oligosaccharides (UA_{me}XOS), but only the UA_{me} linked to the non-reducing end xylosyl residue was cleaved. In particular, in a mixture of oligosaccharides, UA_{me}XOS having a degree of polymerization (DP) of 2 were hydrolysed to a further extent than longer UA_{me}XOS (DP 3-4). On the contrary, ReGH115 was able to release UA_{me} from both polymeric UA_{me}xylan and UA_{me}XOS. ReGH115 removed UA_{me} from both internal and non-reducing end xylosyl residues, with the exception of UA_{me} attached to the non-reducing end of a xylotriose oligosaccharide.

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6.1 Introduction

For the production of biofuels and chemicals from plant biomass, a complete utilisation of the cellulose and hemicellulose present is desired. The degradation of these polymers is commonly approached via a physical and/ or thermo-assisted chemical pretreatment, followed by enzymatic hydrolysis. In grass type feedstocks, glucuronoarabinoxylan (GAX) is the major hemicellulose. It is constituted of a β -(1-4) linked xylopyranosyl backbone, substituted by side groups, like *O*-acetyl groups, arabinofuranosyl residues, 4-*O*-methyl- α -D-glucopyranosyl uronic acids and ferulic acids. The occurrence of substituents in the xylan backbone is highly dependent on the feedstock used. In addition, the abundance and distribution of substituents can be affected by the type and severity of the pretreatment performed (Murciano Matinez et al., 2015). For example, *O*-acetyl groups and arabinosyl residues are released during hydrothermal pretreatments catalysed by alkali or acids (Kootstra et al., 2009; Pietrobon et al., 2011; Samanta et al., 2012). However, glucuronic acid (UA) and its 4-*O*-methyl etherified derivative (UA_{me}) are hardly removed from the xylan backbone during such treatments (Murciano Martínez et al., 2015). Therefore, in commercial enzyme cocktails α -glucuronidases are crucial in addition to endo-xylanases and β -xylosidases, to achieve a complete hydrolysis to monosaccharides.

Such commercial enzyme cocktails mostly contain enzymes produced by ascomycetes, like *Aspergillus* species and *Trichoderma* species. In addition, the ascomycete *Rasamsonia emersonii* is a candidate for the production of (hemi-) cellulolytic enzymes. In this research, α -glucuronidases from *Rasamsonia emersonii* were studied.

In fungi, α -glucuronidases are classified based on the Carbohydrate-Active enZymes Database (CAZy.org; Cantarel et al., 2009) in two glycoside hydrolase (GH) families, which are GH67 and GH115. In the genome of 38 different basidiomycetes, only genes encoding GH115 are described and zero encoding GH67, which indicates that GH115 is preferred over to GH67 in basidiomycetes (Rytioja et al., 2014). For ascomycetes, like for various *Aspergillus* strains, such a distinct choice between GH115 or GH67 is not present. Depending on the strain, genes are present encoding only GH67 or both GH67 and GH115 (Rytioja et al., 2014). In addition to genome annotation, mainly based on putative functions, it is even more valuable to characterize the mode-of-action of the enzyme proteins corresponding to the annotated genes. The mode-of-action of GH67 α -glucuronidases is well-known, because many GH67 α -glucuronidases have been characterised and all are able to release UA_{me} linked to the non-reducing xylosyl end in xylo-oligosaccharides (UA_{me}XOS) (Gottschalk et al., 1996; Rosa et al., 2013; Siika-aho et al., 1994). GH67 α -glucuronidases are not able to release UA_{me} from polymeric glucuronoxyylan (UA_{me}xylan) (Rosa et al., 2013; Siika-aho et al., 1994). In contrast with the GH67 α -

glucuronidases, the mode-of-action of only a limited number of GH115 α -glucuronidases has been described. Only four α -glucuronidases have been biochemically characterised so far, one isolated from the basidiomycete *Schizophyllum commune*, one from the ascomycete *Pichia stipitis* and two from the bacteria *Streptomyces pristinaespiralis* and *Bacteroides ovatus*. All four are able to release UA_{me} from UA_{me}xylan (Fujimoto et al., 2011; Rogowski et al., 2014; Ryabova et al., 2009; Tenkanen & Siika-aho, 2000). In addition, all four GH115 α -glucuronidases are able to release UA_{me} from UA_{me}XOS, linked to either internal or to the non-reducing end xylosyl residues (Chong et al., 2011; Ryabova et al., 2009).

In this research, for the first time, two purified α -glucuronidases, both from the ascomycete *Rasamsonia emersonii*, belonging to either GH67 or GH115, are extensively characterised for their mode-of-action towards UA_{me}xylan and UA_{me}XOS. It is hypothesized that the two enzymes studied show a different mode-of-action towards the substrates studied at 65 °C and pH 4.5.

6.2 Materials and methods

Materials used

The alduronic acids mixture (AAC) containing xylo-oligosaccharides of a degree of polymerization (DP) of 2 to 5, having one UA_{me} substituent, was supplied by Megazyme (Wicklow, Ireland). Beechwood xylan (BeWX), birchwood xylan (BiWX) and all chemicals used were purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise specified. The carbohydrate composition of BeWX and BiWX was 68 and 69% (w/w) xylan, respectively, and 9% (w/w) UA_{me} for both substrates as determined by Van Gool, et al. (2012).

Enzymes expression, production and purification

The two α -glucuronidases from *Rasamsonia emersonii* (CBS 393.64), ReGH67 and ReGH115, were expressed and produced in *Aspergillus niger* as described previously (Neumüller et al., 2015). Purification of both ReGH67 and ReGH115 was carried out with a multiple-step chromatographic separation approach, as described in detail below, using an AKTA-Explorer preparative chromatography system (GE Healthcare, Uppsala, Sweden). As a first step, the crude enzyme mixture (around 2 mg mL⁻¹ protein based) was subjected to a self-packed Superdex 200 26/60 column (GE Healthcare), pre-equilibrated in 20 mM Tris HCl buffer (pH 7.0). After protein application, the column was eluted with 3 column volumes of buffer. Elution was performed at 6 mL min⁻¹. The eluate was monitored at 214 and 280 nm. Fractions (4 mL) were collected and immediately stored on ice. Peak fractions were pooled and concentrated by ultrafiltration (Amicon Ultra, 10 kDa, Merck Millipore, Cork, Ireland).

at 4°C. The concentrated pools were subjected to SDS-PAGE and analysis of xylanase activity. Fractions close to the expected molecular mass (*ReGH67*= 92kDa; *ReGH115*=111kDa) were re-submitted to Superdex 200 26/60 fractionation (2nd step). Now, the *ReGH67*-containing pool was devoid of xylanase activity. The *ReGH115*-containing pool was subjected to further purification (3rd step), and loaded onto a Resource S column (30 mm x 16 mm i.d., GE Healthcare) pre equilibrated with 20 mM sodium acetate buffer (pH 4.0). After protein application, the column was washed with 20 column volumes of starting buffer. Elution at 6 mL min⁻¹ was performed with a linear gradient of 0-1 M NaCl in 20 mM sodium acetate buffer (pH 4) over 20 column volumes. Elution was monitored at 214 and 280nm. Fractions (4 mL) were immediately stored on ice. Peak fractions were pooled, concentrated by ultrafiltration and subjected to SDS-PAGE as described above. Fractions close to the expected molecular weight (111kDa) were pooled to obtain *ReGH115*.

Enzymatic hydrolysis

BeWX and BiWX were incubated with the purified α -glucuronidases (*ReGH67* and *ReGH115*) in 10mM NaOAc buffer, pH 4.0 (1 mL, 10 mg substrate dry matter) at 70°C for 24 hours. The enzymes were dosed at 0.05% (w/w) protein per substrate added. Next, 2 μ L of 4M HCl was added and the sample was centrifuged (10000 $\times g$, 10 min, 10°C) prior to analysis.

The pH and temperature optima were tested by incubating *ReGH67* and *ReGH115* with AAc and BeWX in a range of pH 2 to 7 using 200mM NaOAc, and temperatures ranging from 40 to 90°C. Incubation time, enzyme dose and stopping the reaction was performed as described above.

The AAc was used as a substrate to determine the preferential substrate cleavage site of the enzymes over time. Hereto, 200 μ L of sample was collected at various hydrolysis times: 0, 5, 10, 15, 20, 60 minutes, and 2, 8 and 24 hours. All samples collected were submitted to HPAEC and, after 2-AA labelling, to RP-UHPLC-MS. The incubation was performed under the same conditions, protein and substrate concentrations as described above for the incubation with BeWX.

Protein analysis

SDS-PAGE was performed by using precast 8-16% bis-acrylamide gradient gels (Bio-rad, Hercules, CA, USA) in running buffer containing 25 mM Tris HCl, 10.3% (w/v) SDS at 100V. The samples were denatured prior to loading to the gel at 95°C for 5 min in loading buffer containing 0.35M TrisHCl, 10.3% (w/v) SDS, 36% (v/v) glycerol, 5% (v/v) 2-mercapto-ethanol and 0.012% (w/v) bromo-phenol-blue (pH 6.8). The gels were stained using coomassie brilliant blue. Protein content was determined according to Bradford (1976).

High performance size exclusion chromatography (HPSEC)

HPSEC of BiWX and BeWX before and after incubation with *ReGH115* was performed on an Ultimate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA) equipped with a set of three TSK-gel columns (6.0 mm x 15.0 cm per column) in series (SuperAW4000, SuperAW3000, SuperAW25000, Tosoh Bioscience, Stuttgart, Germany) in combination with a PWX-guard column (Tosoh Bioscience, Stuttgart, Germany). HPSEC was controlled by the Chromeleon software (Thermo Scientific, Sunnyvale, CA, USA). Elution took place at 40°C with 0.2M sodium nitrate at a flow rate of 0.6 mL min⁻¹. The eluate was monitored using a refractive index (RI) detector (Shodex RI-101, Kawasaki, Japan). Calibration was made by using pullulan series (Polymer Laboratories, Union, NY, USA) with a molecular weight in the range of 0.18-788 kDa.

High performance anion exchange chromatography (HPAEC)

Oligosaccharides and 4-*O*-methylglucuronic acids released after enzymatic incubation of AAc and BeWX with *ReGH67* or *ReGH115* were analysed by HPAEC as described elsewhere (Murciano Martínez et al. 2015). Quantification of 4-*O*-methylglucuronic acid was based on a calibration curve of glucuronic acid.

Reverse phase ultra high performance liquid chromatography-mass spectrometry (RP-UHPLC-MS)

A mixture of AAc, xylose, xylobiose, xylotriose and xylotetraose were labelled with anthranilic acid (2-AA) as described by Ruhaak et al. (2010) with some modifications: 50 μ L of sample (2 mg mL⁻¹) was dried under vacuum and mixed with 50 μ L of a freshly prepared mixture (1:4) of 2-AA (192 mg mL⁻¹) and 2-picoline borane (143 mg mL⁻¹) in DMSO containing 30% (v/v) of glacial acetic acid.

Also, AAc incubated with *ReGH67* or by *ReGH115* were labelled following the above described procedure. All 2-AA labelled samples were submitted to RP-UHPLC UV-MS analysis. Labelled oligomers were separated on a UHPLC Shield C18 BEH column (2.1 x 150mm, 1.7 μ m particle size; Waters, Milford MA, USA) using an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with pump, degasser, auto sampler, photo diode array (PDA) detector and coupled *in-line* to an LTQ-Velos double ion trap mass spectrometer equipped with a heated ESI probe (Thermo Scientific). The eluents were: 0.1% (v/v) formic acid in demineralised water (A), 0.1% (v/v) formic acid in acetonitrile (B) and 50% (v/v) acetonitrile in demineralised water (C). The flow rate was 300 μ L min⁻¹ and the sample injection volume was 10 μ L. The elution program was started at 95% (v/v) A, 8% (v/v) B for 25 min and followed by: 25-35 min linear gradient to 80% (v/v) A, 20% (v/v) B; 35-36 min linear gradient to 50% (v/v) A, 50% (v/v) B; 36-41 min 50% (v/v) A, 50% (v/v) B; 41-42 min linear

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gradient to 92% (v/v) A, 8% (v/v) B; 42-50 min 92% (v/v) A, 8% (v/v) B. Re-equilibration was performed for 17 min at starting condition. The cleaning step (250 μ L/min). The eluate was measured at 254 nm (Maslen et al., 2007). The compounds eluted were detected by MS in negative mode. Source heater temperature was set at 225 °C and the capillary temperature was 350 °C. Ion source voltage was set at -4.5 kV. The detected mass range was 300-2000 Da. MS² was performed on the most intense ion detected, with normalized collision energy of 30 (arbitrary units). Single reaction monitoring (SRM) was acquired by using the above mentioned MS settings. The main MS² fragments of each individual compound present in AAc were monitored together with the parent mass and retention time of the compound and given in Table 1.

Table 1. Single reaction monitoring (SRM) settings of AAc analysed by UHPLC-MS.

Segment 1 (0-16 min)					Segment 2 (16-50 min)				
Scan event name	Parent mass (m/z)	Main MS ² fragment (F)		Rt ^a (min)	Scan event name	Parent mass (m/z)	Main MS ² fragment (F)		Rt ^a (min)
		F1 (m/z)	F2 (m/z)				F1 (m/z)	F2 (m/z)	
TIC	50-1000	n.a. ^b	n.a. ^b	n.a. ^b	TIC	50-1000	n.a. ^b	n.a. ^b	n.a. ^b
SRM 1	856	666	780	14	SRM 1	460	270	384	16.2
SRM 2	724	533	648	14.7	SRM 2	724	534	648	17.5
SRM 2	724	533	648	15.3	SRM 3	592	402	516	17.5
SRM 3	n.a. ^b	n.a. ^b	n.a. ^b	n.a. ^b	SRM 3	592	402	516	21.2

^aRt = retention time in minutes

^bn.a.= not applicable.

The most abundant fragments from each RP18-separated single mass were determined (Table 1). In a separate analysis, these most abundant fragments, which are considered to be the fingerprint of the structure, were selectively monitored. From the latter analysis, the sum of the areas of the two main fragments was used for quantification of the structures. In addition, increasing concentrations of 2-AA labelled standards (xylose, xylobiose and xylotriose) were analysed. Samples were assumed to be labelled equally efficient as the standards, which showed a linear correlation ($R^2 = 0.99$) of their UV response area (340nm) upon increasing concentrations, as shown in Figure 1.

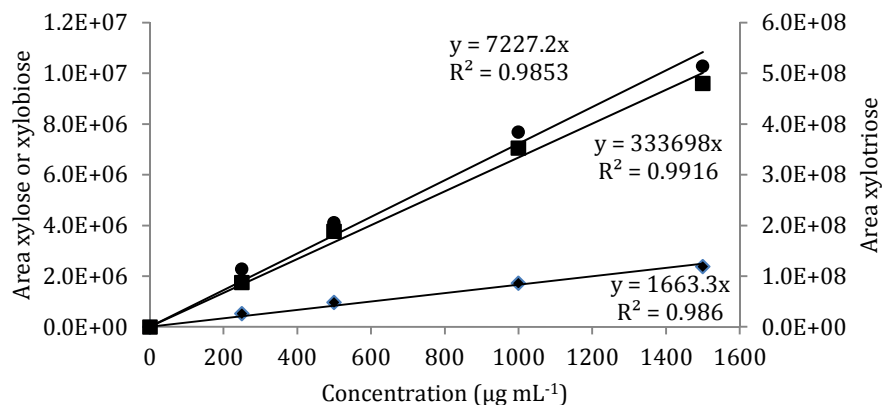


Figure 1. RP-UHPLC-UV (340nm) response area of various concentrations of 2-AA labelled xylose♦, xylobiose● and xylotriose■. The concentrations correspond to the amount subjected to labelling.

6.3 Results and discussion

Purification of the α -glucuronidases ReGH67 and ReGH115

The crude enzyme extracts from *Aspergillus niger* after overexpression of ReGH67 or ReGH115 were purified by a 2-step size exclusion chromatography; a subsequent cation exchange step was applied to the SEC 2-fraction of ReGH115 (data not shown).

SDS-PAGE of the purified ReGH67 and ReGH115 showed bands of a molecular mass (Mw) of 100 kDa and 150 kDa, for ReGH67 and ReGH115, respectively, compared to the marker (data not shown). These masses differed with the predicted masses of the enzymes based on their amino acids sequences, which were 91 and 111kDa for ReGH67 and ReGH115, respectively.

In addition, it was shown that no xylanase activity was present in the purified ReGH67 and ReGH115; neither towards 4-O-methyl glucuronoxylan (Figure 2), nor towards linear xylo-oligosaccharides (XOS) (Supplementary figure 2).

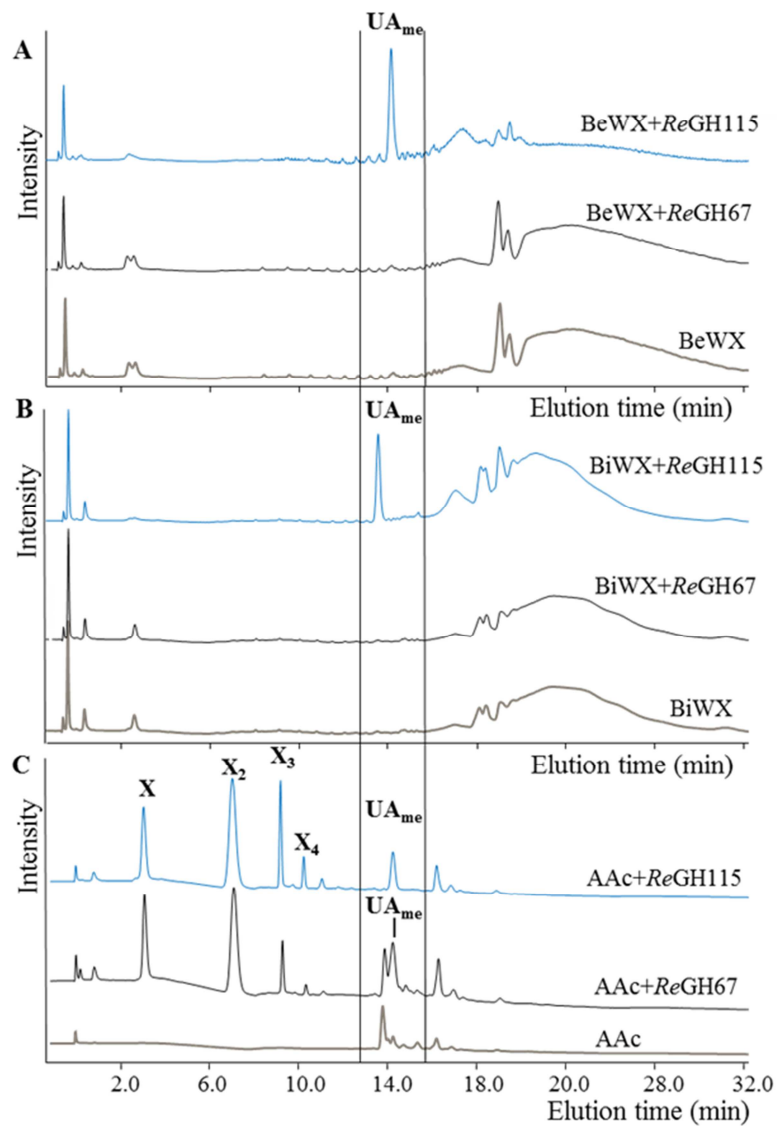


Figure 2. High performance anion exchange chromatogram of beechwood xylan (BeWX) (A), birchwood xylan (BiWX) (B) and the aldouronic acids mixture (AAc) (C) before and after incubation with *ReGH115* and *ReGH67*. X= xylose, X_2 = xylobiose, X_3 = xylotriose, UA_{me} = 4-*O*-methylglucuronic acid.

Optimum temperature and pH

ReGH67 showed a maximum release of UA_{me} from AAC at a pH-range from 4 to 6 and at a temperature range from 50 to 70°C (Supplementary Figure 1). In case of *ReGH115*, the optimum release of UA_{me} from BeWX was observed at pH 4 and at 65 to 70°C. Considering these optima for further hydrolysis experiments a pH of 4.0 at 70°C was taken for both enzymes to allow best comparison.

Activity of ReGH115 and ReGH67 towards 4-O-methylglucuronoxylan.

To date, only four α -glucuronidases belonging to GH115 have been characterised, of which only one originates from an ascomycete, while many GH67 α -glucuronidases have been studied. Therefore, we are in particular interested in the mode-of-action of *ReGH115*, although *ReGH67* is also of interest as this enzyme originates from the same ascomycete *R. emersonii*.

The activity of the two purified α -glucuronidases was first tested towards BeWX and BiWX, which both are polymeric xylans constituted of β -(1-4) linked xylosyl residues substituted with α -(1-2) linked 4-*O*-methylglucuronic acids (UA_{me}). The UA_{me} substitution pattern of BeWX is more random compared to BiWX, which is more blockwise (Van Gool et al., 2012). A blockwise distribution of UA_{me} leaves larger blocks of unsubstituted xylosyl residues, allowing self-association of these linear xylan blocks and, hereby, decreasing its solubility (Hromádková et al., 2006; Ren & Sun, 2010).

BiWX and BeWX were incubated with both enzymes for 24 h. *ReGH67* was not able to remove UA_{me} from the two UA_{me}xylans (Figure 2), as is also reported in previous studies with other GH67 α -glucuronidases (Nurizzo et al., 2002; Siika-aho et al., 1994). In contrast, *ReGH115* was able to release UA_{me} from both BiWX and BeWX. The release of UA_{me} from the xylan-backbone led to aggregation. The latter was seen from the increase in higher molecular mass material (around 1128 kDa) and from the decrease of lower molecular weight material (around 55kDa) seen by HPSEC analysis of BiWX (Figure 3). As mentioned earlier in the text, in literature only four GH115 α -glucuronidases are described for their mode-of-action. All four were able to release UA_{me} from UA_{me}xylan, as was observed in our study for *ReGH115*.

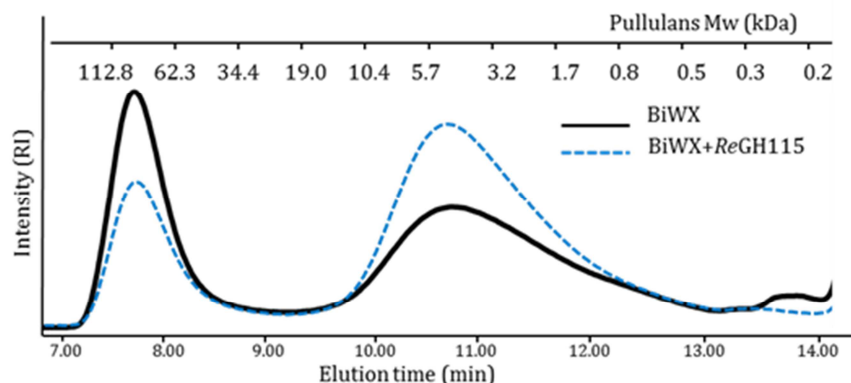


Figure 3. High performance size exclusion chromatograms of birchwood xylan before (solid line in black) and after (dotted line in blue) hydrolysis with *ReGH115*.

Mode-of-action of ReGH115 and ReGH67 towards AAC

AAC is a commercially available mixture of various xylo-oligosaccharides (XOS) with one UA_{me} linked per xylo-oligomer. The position of the UA_{me} in each oligomer is, however, not specified by the supplier. To enable the analysis of the mode-of-action of *ReGH67* and *ReGH115* towards characterized UA_{me}XOS, first the exact structures of the UA_{me}XOS present in AAC were determined. Hereto, AAC was labelled under reducing conditions with 2-AA and submitted to RP-UHPLC-MS analysis. The RP-UHPLC-UV absorbance at 254 and 340nm showed the presence of 6 peaks. The number of peaks detected by total ion current (TIC) was 8. The RP-separated 2-AA labelled UA_{me}XOS MS and MS² fragmentation patterns were recorded. Seven out of the 8 peaks detected by TIC were identified based on the MS² spectra and, the exact position of the UA_{me} for each of the xylo-oligosaccharides present in AAC was determined (Figure 4).

In a separate analysis, the elution of the TIC analyzed masses in 2-AA labelled AAC was detected as single reaction monitoring (SRM) to overcome the problem of co-elution of certain structures, as shown in Figure 4A. Assisted by the separation, the detected parent mass, and the mass fragmentation pattern, seven structures were confirmed in AAC.

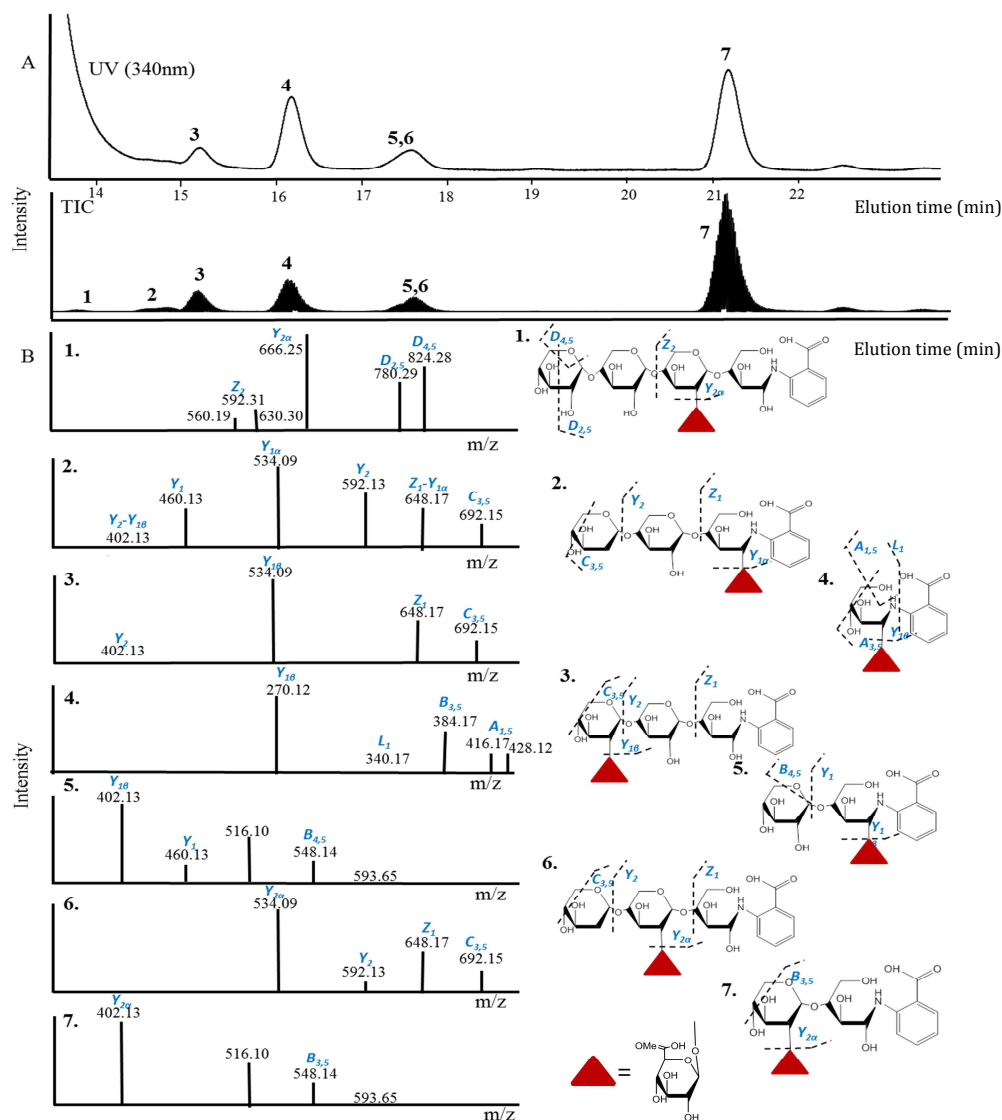


Figure 4. RP-UHPLC-UV-MS profiles (UV (340nm) and total ion current (TIC)) of 2-AA labelled AAc (A) and MS² fragmentation spectra (B) of the in A annotated peaks: 1=856 m/z, 2,3,6=724 m/z, 4= 460 m/z, 5,7=592 m/z,.

These seven structures are depicted in Figure 4, including their fragmentation patterns and masses. From Figure 4 it is clear that UA_{me}XOS are present having the UA_{me} linked to either the non-reducing, the internal or reducing end xylosyl residues. According to the nomenclature for UA_{me}XOS structures (Fauré et al., 2009), which uses 'X' for xylosyl residues and 'U^{4m2}' for xylosyl residues attached via their O2 to a 4-O-

methylglucuronic acid, the structures were named as 1=XXU^{4m2}X, 2=XXU^{4m2}, 3=U^{4m2}XX, 4=U^{4m2}, 5=XU^{4m2}, 6=XU^{4m2}X and 7=U^{4m2}X. The numbers correspond to the numbers shown next to the structures in Figure 4.

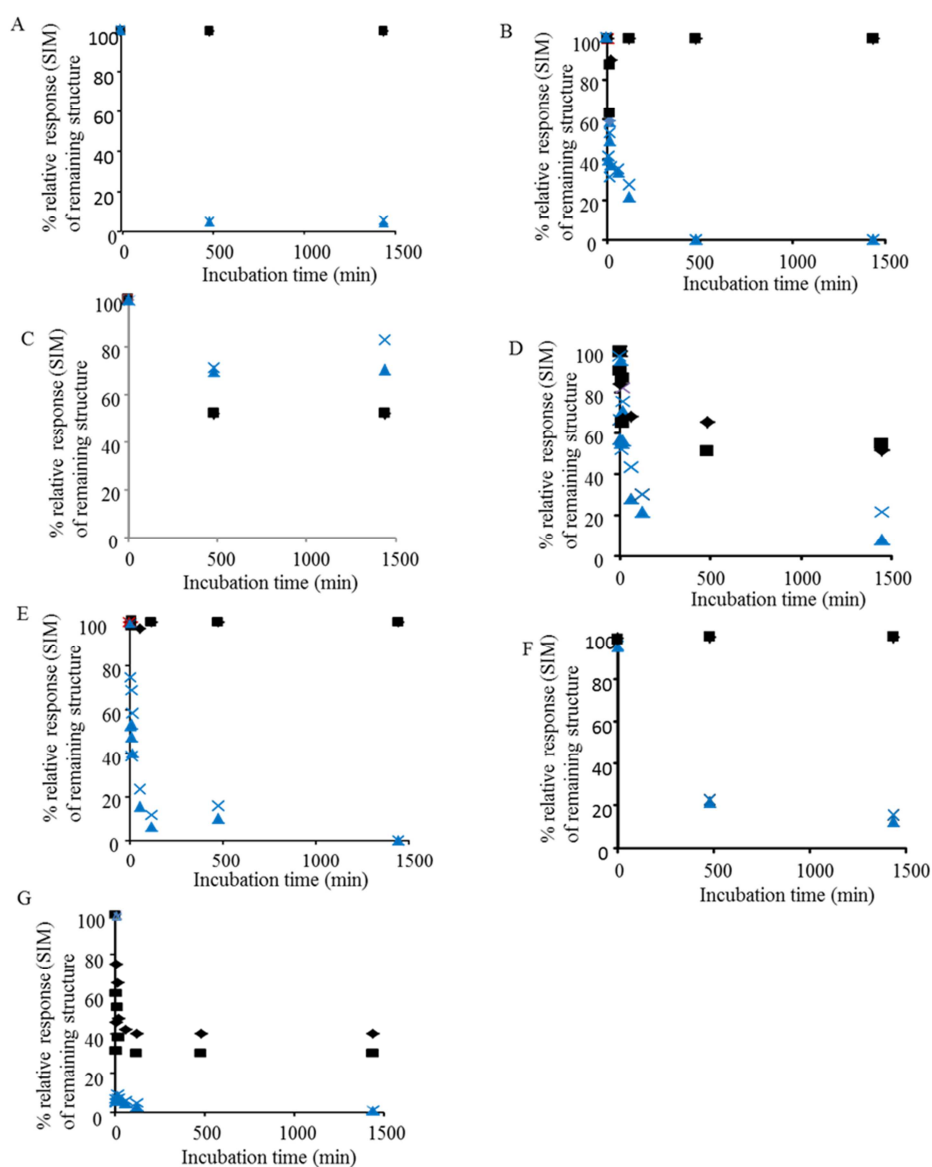


Figure 5. SRM relative abundance of AAc characterized structures during incubation with *ReGH67* (■ and ♦) or *ReGH115* (x and ▲): 1=XXU^{4m2}X (A), 2=XXU^{4m2} (B), 3=U^{4m2}XX (C), 4=U^{4m2} (D), 5=XU^{4m2} (E), 6=XU^{4m2}X (F) and 7=U^{4m2}X (G). Experimental duplicates are displayed individually.

Quantification of 2-AA labelled UA_{me}XOS in the *ReGH67* and *ReGH115* digests was achieved by using single ion monitoring (SRM). It allowed the quantification of selected fragments as described in the materials and methods (Table 1).

Figure 5 shows that *ReGH67* only cleaved UA_{me} from the structures 3 (U^{4m2}XX), 4 (U^{4m2}) and 7 (U^{4m2}X), which are substituted at the non-reducing xylosyl residue. Hence, the mode-of-action of *ReGH67* was comparable to those of many GH67 α -glucuronidases described previously (Gottschalk et al., 1996; Rosa et al., 2013; Siika-aho et al., 1994).

The activity of *ReGH115* towards AAc was of particular interest, because its mode-of-action is expected to be a valuable contribution to the so far poorly described α -glucuronidases from the GH115 family. *ReGH115* was able to release UA_{me} from *all* AAc structures, although, U^{4m2}XX mostly remained (Figure 5). Therefore, it was concluded that *ReGH115* did not show a distinct preference for a cleavage site in UA_{me}XOS. UA_{me} is removed from xylose moieties at the reducing side, internal and non-reducing-end side positions. So, under the conditions applied, clearly, *ReGH115* degraded AAc to a further extent than *ReGH67*.

In terms of oligosaccharides, a summary of the mode-of-action of the four α -glucuronidases from family GH115 described in literature and of our *ReGH115* is shown in Table 2. In contrast to the absence of preference of *ReGH115*, the *S. commune* GH115 α -glucuronidase prefers to cleave UA_{me} from internal xylosyl residues of UA_{me}XOS with a DP of 4 to 6 (Chong et al., 2011). The *B. ovatus* GH115 α -glucuronidase prefers to hydrolyze UA_{me} from either internal or non-reducing xylosyl residues in UA_{me}XOS (DP 2 to 4) (Rogowski et al., 2014). The *P. stipites* GH115 α -glucuronidase cleaved the UA_{me} from UA_{me}XOS in a DP range from 3 to 6 (Kolenová et al., 2010). However, the preference for internal or outer units remains undefined in that research. The *S. pristinaespiralis* GH115 α -glucuronidase was only tested towards one UA_{me}XOS having a DP of 5. To summarize, in comparison with the to date published activities of GH115 α -glucuronidases (Table 2), our *ReGH115* showed activity towards UA_{me}Xylan and UA_{me}XOS and showed no preference for the position of the UA_{me} in the oligosaccharides tested.

6.3 Conclusion

Both enzymes showed highest activity around pH 4 and at a temperature between 65 and 70°C. *ReGH67* released only UA_{me} attached to the xylosyl residue located at the non-reducing end of UA_{me}XOS. *ReGH115* was able to release UA_{me} from both UA_{me}Xylan and UA_{me}XOS. It showed no preference for the position of the UA_{me} in the UA_{me}XOS. This analysed mode-of-action is a valuable contribution to the so far poorly described α -glucuronidases from the GH115 family. Also, the knowledge presented is helpful to improve current enzyme cocktails for biorefinery applications.

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Table 2. Performance of GH115 α -glucuronidases described in literature towards polymeric and oligomeric xylan substrates. Active towards substrate (+), preferential degradation (++).

α -glucuronidases	ScAgu115 ^a	PsAgu115 ^a	SpAgu115 ^a	BoAgu115 ^a	ReGH115
Organism	<i>Schizophillum commune</i>	<i>Pichia stipitis</i>	<i>Streptomyces pristinaespiralis</i>	<i>Bacteroides ovatus</i>	<i>Rasamsonia emersonii</i>
Division	Basidiomycete	Ascomycete	Actinobacterium	Bacteroidete	Ascomycete
Mw (Kda)	125	120	n.t. ^b	199	150
pH optimum	n.t. ^b	4.4	n.t. ^b	n.t. ^b	4.0
T optimum (°C)	n.t. ^b	60	n.t. ^b	n.t. ^b	65-70
3D-structure	No	No	No	Yes	No
Activity on xylan ^c :					
BeWX	n.t. ^b	+	+	n.t. ^b	+
BiWX	n.t. ^b	n.t. ^b	+	n.t. ^b	+
SpW	+	n.t. ^b	n.t. ^b	n.t. ^b	n.t. ^b
WWX	n.t. ^b	n.t. ^b	n.t. ^b	+	n.t. ^b
Activity on U ^{4m2} X _n ^d :					
U ^{4m2}	n.t. ^b	n.t. ^b	n.t. ^b	+	+
U ^{4m2} X	n.t. ^b	n.t. ^b	n.t. ^b	+	+
XU ^{4m2}	+	+	n.t. ^b	n.t. ^b	+
U ^{4m2} XX	+	+	n.t. ^b	+	+
XU ^{4m2} X	++	+	n.t. ^b	++	+
XXU ^{4m2}	n.t. ^b	n.t. ^b	n.t. ^b	n.t. ^b	+
U ^{4m2} XXX	n.t. ^b	n.t. ^b	n.t. ^b	++	n.t. ^b
XU ^{4m2} XX	++	+	n.t. ^b	n.t. ^b	n.t. ^b
XXU ^{4m2} X	n.t. ^b	n.t. ^b	+	n.t. ^b	+
XXU ^{4m2} XX	++	+	n.t. ^b	n.t. ^b	n.t. ^b

^aScAgu115=Chong et al. (2011); Tenkanen & Siika-aho, (2000); PsAgu115=Ryabova et al. (2009); Kolenová et al. (2010); SpAgu115=Fujimoto et al. (2011); BoAgu115=Rogowski et al. (2014).

^bn.t.=Not tested.

^cBeWX= Beech wood xylan, BiWX= Birch wood xylan, SpW= Spruce wood, WWX=Willow wood xylan.

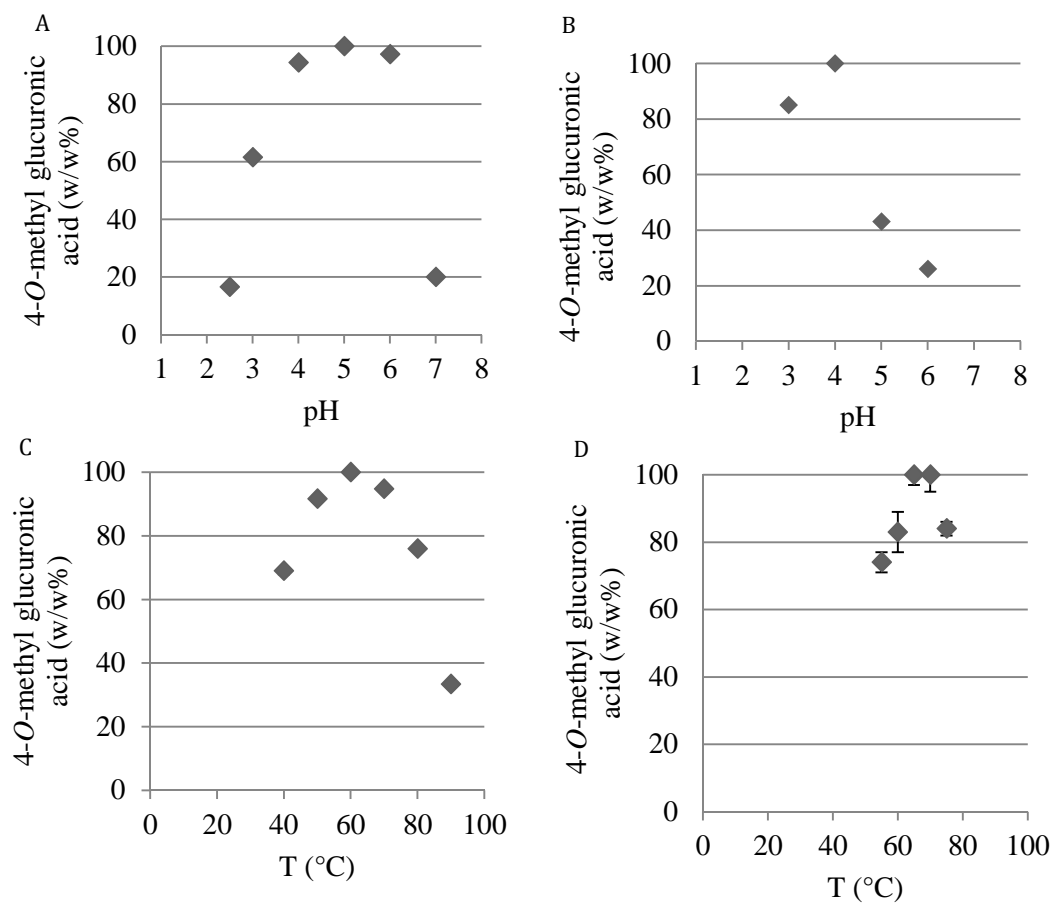
^dNomenclature used is according to Faure, et al. (2009).

References

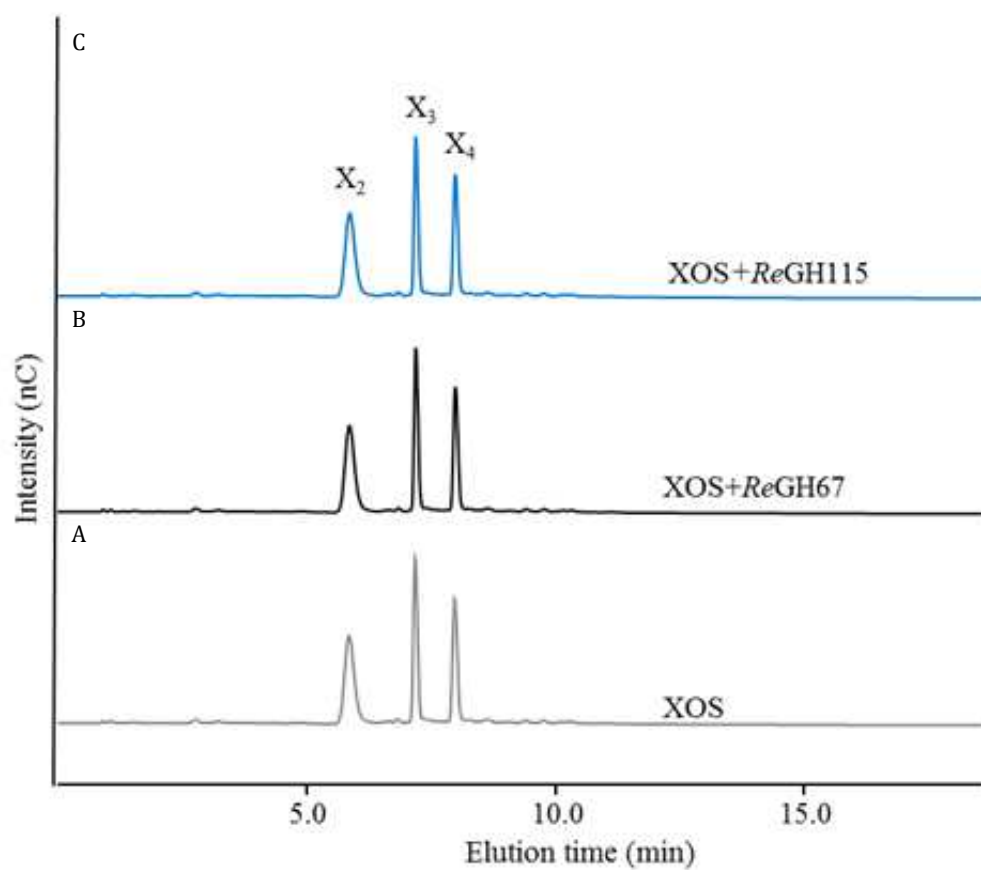
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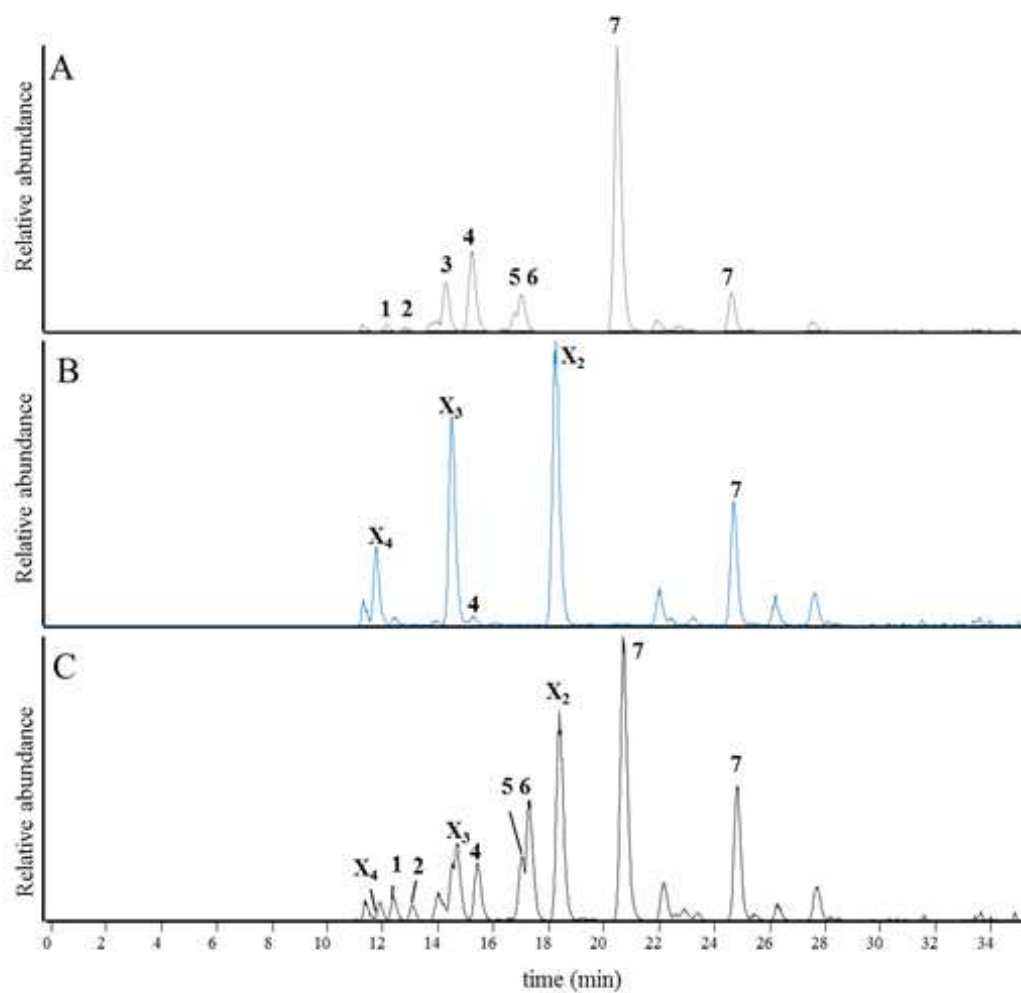
Supplementary data



Supplementary figure 1. pH (A) and temperature profiles (C) of the incubated *ReGH67* with the aldouronic acids mixture (AAc) and the pH (B) and temperature profiles (D) of the incubated *ReGH115* with beechwood xylan.



Supplementary figure 2. HPAEC chromatograms of xylo-oligosaccharides (XOS) before (A) and after 24h incubation with *ReGH67* (B) and *ReGH115* (C). XOS=xylo-oligosaccharides; X₂=xylobiose; X₃=xylotriose; X₄=xylotetraose.



Supplementary figure 3. RP-UHPLC-MS profile (total ion current (TIC)) of AAc before (A) and after incubation with *ReGH115* (B) and *ReGH67* (C). X₂= xylobiose, X₃= xylotriose, X₄= xylo-tetraose.

Chapter 7

General discussion

7.1 Aim & hypothesis of the research

The main aim of this research was to understand the enzymatic degradation of pretreated lignin-rich agricultural by-products, into their constituent monosaccharides, xylose and glucose. Mostly, the lignin-rich by-products sugarcane bagasse (SCB) and oil palm empty fruit bunch (EFB) were studied (Chapters 2, 3, 4 and 5), whereas barley straw was part of the research in Chapter 3 as well.

One of the main challenges in enzymatic plant biomass degradation is to improve the accessibility for enzymes to the complex and recalcitrant hemicellulosic xylan-cellulose-lignin network present in lignin-rich plant materials. Another important challenge is to find the most suitable accessory enzymes, in particular to degrade branched xylans. Once found, these can be added to enzyme cellulase-cocktails improving the enzymatic degradation of both xylans and celluloses.

Within this research, the effects of heat-assisted H_2SO_4 - (Chapter 3) and NaOH- (Chapters 2, 3, 4 and 5) catalysed pretreatments on the accessibility of residual (hemi-) cellulose for enzymes were studied. The focus was on the heat-assisted NaOH-catalysed pretreatments. Such NaOH-catalysed pretreatments are known to cause the solubilization of lignin, which is subsequently washed out in the liquid fraction and separated from the residual celluloses remaining in the solid fraction. In addition, xylans are not completely recovered in the residual fraction, partly due to their solubilization and partly due to their decomposition, e.g. peeling, under the alkaline conditions performed. Considering these premises, it was hypothesized that a yield of lignin and xylan in the cellulosic residues of around 70% each, based on the amounts originally present in the feedstock, would substantially improve the conversion of residual xylan and cellulose into xylose and glucose, respectively, by a commercial enzyme cocktail (Chapters 3 and 5). Residual and original xylan structures were also studied to corroborate the second main hypothesis: characterization of xylan structures predicts which accessory enzymes improve the complete conversion of the residual polysaccharides (Chapters 2 and 7).

7.2 Plant cell wall modifications by alkaline pretreatments

Extraction of xylan by alkaline solutions as a tool to characterise the xylan structure

The main conclusion of Chapter 2 is that delignification by the oxidative agent peracetic acid outperforms alkaline extraction of xylan from EFB with the aim to characterise the xylan structures present. It was shown in this chapter that by applying an endoxylanase, 91% of the xylan present in the delignified EFB, was released as soluble xylan structures. Without delignification, this percentage was below 5%. Furthermore, delignification improved the alkaline extraction yield of

xylan from 50%, in absence of delignification, to 75% after delignification. In the latter extraction procedure, a sequential extraction with 1M and 4 M NaOH was applied at room temperature and to the alkaline solutions a reducing agent (NaBH_4) was added. Under such conditions (temperature and reducing agent) the extracted xylan is protected against destructive reactions like peeling, which will be discussed later in the text.

Alkaline extraction is the most common extraction method for hemicellulose of grass type biomass when its structural characterisation is aimed at. NaOH, KOH, LiOH and $\text{Ca}(\text{OH})_2$ are extensively used. Such extractions have shown high yields of xylan, mainly when extracted from substrates with low lignin contents, as shown in Table 1. Extractions performed on the lignin-poor substrates corn bran, wheat flour and wheat bran (Table 1) showed xylan yields higher than 80% using a single step extraction. In case of substrates containing higher amounts of lignin, however, like the EFB or SCB studied in this thesis, one step extraction at high alkaline concentration does normally result in low xylan yields (Table 1).

A sequential extraction with increasing concentrations of alkali is reported to achieve higher yields than a single step extraction (Shi et al., 2011). Nonetheless, when comparing the yields of lignin rich substrates, in a one step or sequential extraction (32-73% of xylan extracted), with the yields of lignin poor substrates (83-87%), it seems obvious that the lignin hinders the extraction. This conclusion is supported by the fact that when a lignin degrading oxidative agent is used, in the presence or absence of alkali, the xylan extraction yields of lignin rich substrates are higher (75-95%) compared to the use of only alkali (Table 1). A difficulty in comparing xylan yields from alkaline extractions with yields from oxidative extractions is that many studies did not report if reducing agents, such as NaBH_4 , were added to the alkaline solutions. Therefore, low yields cannot always be addressed to the extraction method only, because of the fact that losses of xylan by peeling reactions can occur in absence of a reducing agent. The latter is, for example, expected to be the case for the low yields reported for alkali extracted rapeseed straw and wheat straw in Table 1. Others also report that delignification by oxidative agents, such as peroxide, resulted in higher xylan extraction yields of lignin rich materials (Brienzo et al., 2009). Oxidative agents have the advantage of degrading the xylan less in comparison with strong alkali.

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Table 1. Grass type feedstocks hemicelluloses. Isolation conditions and characteristics.

	FS ^a	Extraction method	Yield %	Lignin content % (w/w)	Oxidant	T (°C)	t (h)	Xylan type ^d	Reducing agent	Ref ^e
Lignin-poor ^b	CB	0.8M KOH	87	n.s. ^f	No	85	2	AX	No	1
	WF	Saturated Ba(OH) ₂	80	n.s. ^f	No	20	16	AX	NaBH ₄	2
	WF	1M NaOH	83	n.s. ^f	No	20	16	AX	NaBH ₄	3
Lignin-rich no oxidant added ^c	EFB	1M NaOH, 4MNaOH	50	33	No	25	24	GX	NaBH ₄	4
	CS	1M KOH, 4MKOH	65	24	No	n.s.	24	GAX	NaBH ₄	5
	RSS	0.5M NaOH	32	15	No	140	0.3	GAX	No	6
	RS	1M NaOH	73	n.s. ^f	No	30	18	GAX	No	7
	WS	2M NaOH	34	n.s. ^f	No	30	24	AX	No	8
Lignin-rich oxidant added ^c	WS	4MNaOH	92	n.s. ^f	H ₂ O ₂	80	16	AX	No	9
	SCB	NaOH to pH11.6	95	n.s. ^f	H ₂ O ₂	40	10	GAX	No	10
	EFB	1MNaOH 4MNaOH	75	33	C ₂ H ₄ O ₃ ^g	25	24	GX	No	11

^aFS=feedstock; CB=Corn bran; WF=Wheat flour; WB=Wheat bran; EFB=oil palm empty fruit bunch; CS=Corn stover; RSS=Rapeseed straw; RS=Rye straw; WS=Wheat straw; SCB=Sugarcane bagasse

^bLignin contents ranging from 1-5% (w/w based on dry matter)

^cLignin contents ranging from 10-33% (w/w based on dry matter)

^dAX=arabinoxylan; GAX= glucuronoarabinoxylan; GX=glucuronoxylan

^eReferences: 1=Chanliaud et al. (1995); 2,3=Gruppen et al. (1991); 4, 11=Chapter 2; 5=Van Dongen et al. (2011); 6=Svård et al. (2015); 7= Xiao, (2001); 8=Sun et al. (1996); 9= Fang et al. (1999); 10=Brienzo et al. (2009).

^fn.s.=not specified

^gC₂H₄O₃= Peracetic acid

Palamae et al. (2014) report that a peracetic acid pretreatment (60% (v/v) peracetic acid, in a ratio 1:20 solid:liquid based on EFB dry matter) for 9h, keeps EFB xylan for 90% (w/w) intact. In contrast, strong alkaline conditions are known to

provoke the peeling of the xylan backbone, decreasing the extraction yields considerably. This issue will be further discussed in the next section. Hence, it is concluded that oxidative treatments, by using for example H_2O_2 or peracetic acid, are a potential tool for high extraction yields and for extensive characterization of native xylan from lignin rich grass-type biomasses.

Xylan degradation reactions by alkaline pretreatments

NaOH catalysed pretreatments not only solubilized lignin, but also part of the xylan, which enhanced the access of enzymes to the cellulose surface seen from an increase in enzymatic degradability (Chapter 3). Due to the alkaline conditions (0.05-0.3M), however, the solubilized xylan reacted further to non-carbohydrate compounds considered as undesired carbohydrate losses (Chapters 3 and 4). In these NaOH-catalysed pretreatments no reducing agents were added. Interestingly, in Chapter 3, we show that glucuronoxylans (as present in EFB; Chapter 2) were less affected by the alkaline conditions applied compared to arabinoxylans, acetylated or non-substituted xylans (as present in SCB and BS). For the acetylated and majorly linear SCB xylan, up to 40% was lost during the pretreatment at 12% NaOH (w/w), 120 °C; 60 min. This percentage was lower than the 20% analysed in case of EFB xylan at comparable conditions. Expected reactions occurring in the alkaline conditions applied are alkaline hydrolysis and alkaline peeling or β -elimination reactions of hemicelluloses (Aspinall et al., 1961). Although the mechanisms of such reactions have been reported, the protective effect of (4-*O*-methyl-)glucuronic acid (UA_{me}) substituents on the xylan, observed in Chapter 3, is poorly described. In Figure 1, the proposed β -elimination (or peeling) and stopping reaction mechanisms occurring in alkaline conditions of glucurono- and arabinoxylans are shown. In glucuronoxylans, the (4-*O*-methyl-)glucuronic acid substituents are linked to the xylan backbone via a strong covalent linkage, which is resistant to the thermo-assisted alkaline conditions applied (Chapter 3). The stabilization of the glucuronic acid by the formation of 2-*O*-(4-*O*-methyl- α -D-glucopyranosyluronic acid)-3-deoxy-pent-2-enose under alkaline conditions (Johansson & Samuelson, 1977) functions as a stop for the peeling reactions as proposed in Figure 1A. On the contrary, the linkages between the xylan backbone and the arabinosyl substituents in arabinoxylans are more labile under the alkaline pretreatments applied and susceptible to alkaline hydrolysis (Chapter 3). After removal of the arabinosyl substituent from the backbone, β -elimination of the reducing end xylosyl residue of the xylan backbone can easily occur. Subsequently, the open xylosyl ring leads to the formation of isosaccharinic acids that can react further into a wide variety of organic acids, like levulinic acid and formic acid (Knill & Kennedy, 2002), as proposed in Figure 1B. Obviously, the latter cascade of reactions also occur for non-substituted xylan populations. Acetyl group substituents are readily released from both glucurono- and arabinoxylans, during alkaline

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pretreatments. Therefore, they are not expected to have an influence on the susceptibility of the xylan to peeling.

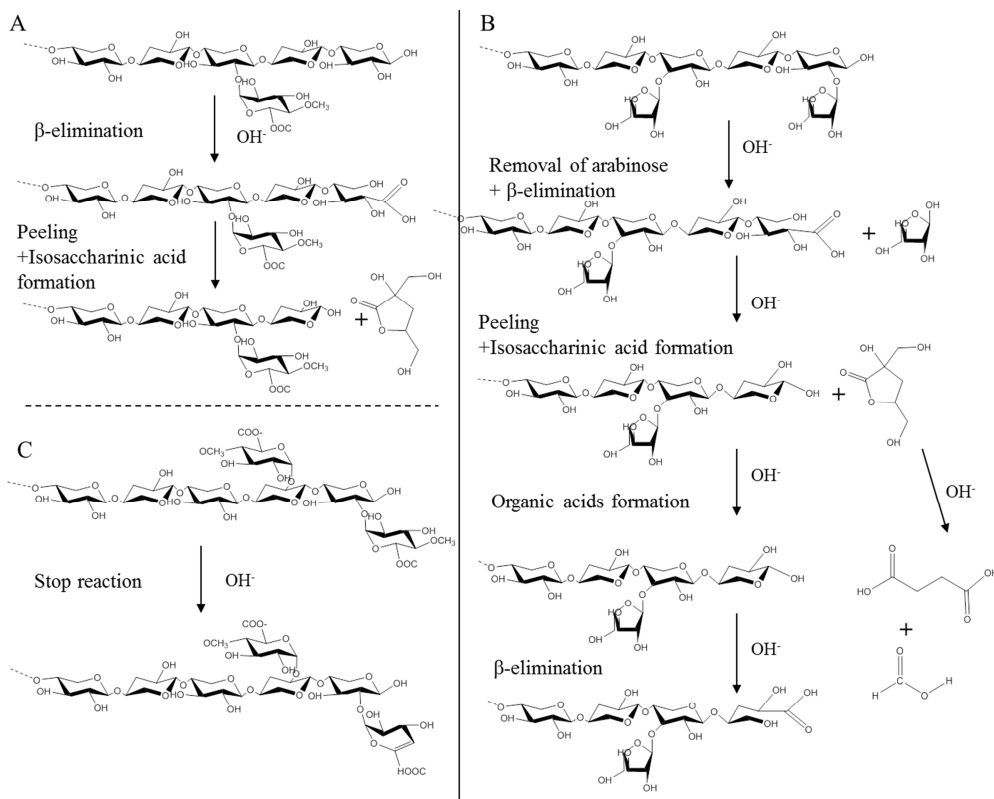


Figure 1. Proposed peeling and stop reactions occurring during alkaline pretreatment of glucuronoxylan (A, C) and arabinoxylan (B).

The NaOH pretreatments performed in Chapters 3 and 4 at 4% NaOH had a pH>8.6 throughout the complete pretreatment, which resulted in a complete deacetylation of the xylans. The xylan losses experienced can be prevented by protecting the reducing end with reducing agents, like sodium borohydride (NaBH_4) (Wigell et al., 2007). Indeed, in an additional experiment, alkaline pretreatment of SCB in the presence of NaBH_4 reduced the xylan losses at the highest dosage of NaOH applied (12% NaOH; Chapter 3) from around 40% to 20% (no further data shown). NaBH_4 is commonly used in the process of hemicelluloses extraction and characterization as previously shown in Table 1 and in Chapter 2. Alternatives are for example polysulfide (PS) and anthraquinone (AQ), but these chemicals are not as effective as NaBH_4 (Wang et al., 2015).

Lignin degradation in alkaline (NaOH) conditions

In addition to the above described degradation of xylans during the NaOH treatments performed, also, lignin solubilization and partial degradation occurred. That is demonstrated in Chapter 5, in which it was hypothesized that the removal of lignin from SCB, for optimal enzymatic degradability of the residual (hemi-) cellulose, required the cleavage of ester and ether bonds in lignin. Such ether bonds occur mainly as β -O-4 and α -O-4 linkages between *p*-hydroxycinnamic acids attached to lignin, to xylan or to both. When a covalent bond is established between a lignin unit and a carbohydrate (mainly xylan), the complex formed is called lignin carbohydrate complex (LCC). From the data presented in Chapter 5, indeed, it can be considered that ferulic acid and *p*-coumaric acid is not only linked to lignin or to xylan alone, but are also present in LCCs. With increasing severity of alkaline catalysed pretreatments a linear trend was seen between the extraction yield of these *p*-hydroxycinnamic acids and that of xylan. At both 4 and 9% NaOH pretreatments *p*-coumaric acid solubilised, while ferulic acid mainly solubilised at 9% NaOH (0.2M). Apparently, at 9% NaOH both ether and ester linkages, also the ones present in the LCCs, were cleaved releasing both *p*-hydroxycinnamic acids as well as xylan. In addition, the residues with the lowest contents of *p*-hydroxycinnamic acids and xylan also showed the highest enzymatic conversion of residual glucan and xylan into glucose and xylose, respectively.

The hydrolysis of the LCCs is important for the enzymatic hydrolysis of glucan and xylan (Buranov & Mazza, 2008). Several linkages occurring in LCCs have been proposed, mainly analysed in isolated LCCs from hardwoods, such as beech, eucalyptus, spruce and pine wood (Choi et al., 2007; Miyagawa et al., 2012; Takahashi & Koshijima, 1988a; Takahashi & Koshijima, 1988b).

The characterization of LCCs from grasses is still a challenge. Currently, the most common isolation and characterization method, also for grasses, is the so-called milled wood lignin procedure (MWL), followed by NMR analysis (Balakshin et al., 2014). LCCs in grasses have become of interest since 1990 (Hatfield et al., 2005). Grasses seem more abundant in such LCC studies compared to hardwood or soft wood. This is probably due to the high abundance of hydroxycinnamic acids (H) in grasses. Such H units are often considered as non-core lignin units as they are pendant from the lignin bulk (shown in Chapter 5). The majority of these non-core lignin (NCL) units are *p*-hydroxycinnamates, like ferulic acid, coumaric acid and sinapic acid, or *p*-hydroxybenzoates (Grabber et al., 2004). As mentioned, these NCL units can cross-link lignin and xylan via ester and ether (β -O-4, α -O-4) linkages. Figure 2 shows a schematic representation of LCC linkages summarized from literature describing LCCs for grass-type biomasses. *P*-hydroxycinnamates, like *p*-coumaric acid when they are linked to an S unit via an ester and not further linked leaving a phenyl hydroxy group free, should also be considered as NCL. Nonetheless, the difficulties in terms of

isolation of LCCs for NMR analysis are still an issue. Therefore, the exact location position of lignin to the carbohydrates is still unclear. Based on different literature studies we propose in Figure 2 the linkages present in LCCs between xylan and lignin as present in cell walls from grasses (Crestini & Argyropoulos, 1997; Helm & Ralph, 1993; Ralph et al., 1994; Liyama et al., 1994).

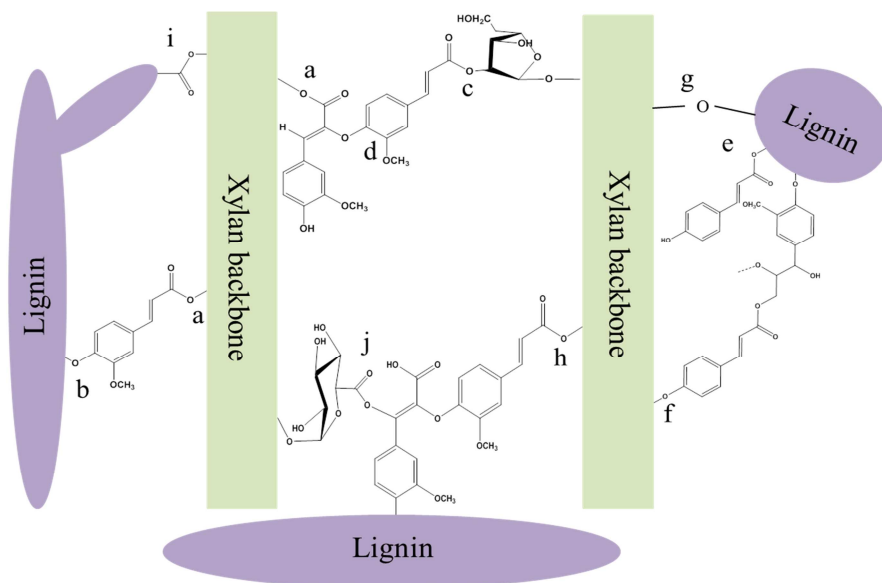


Figure 2. Hypothetical cross links with ferulic acid and coumaric acid, linking lignin and xylan, in grasses. Letters refer to linkages presented in Table 2.

Figure 2 includes ester and ether linkages between lignin moieties, which are the most abundant linkages in NCLs from grasses. Also, lignin linkages to the xylan backbone and to the different xylan substituents, such as arabinose and glucuronic acids, are represented. The stability of an LCC linkage depends on the type of pretreatment used, which will affect the separation and solubilization of lignin and xylan and the subsequent enzymatic saccharification. Depending on which of the two proposed linkages (ether or ester) is most abundant, alkaline, acid or oxidative pretreatment will have different effects on the extent of lignin degradation. Ester linkages proposed in Figure 3 (a, c, e, h, i and j) are cleaved during mild (<0.2M) alkaline pretreatments, as shown in Table 2. Ether linkages (b, d, f and g) are cleaved by oxidative treatments, using for example peroxide or peracetic acid, especially affecting β -O-4 linkages (Sun et al., 2000; Li et al., 2012). The cleavage of such ethers (Figure 2, linkage b) reduces the content of dimeric di-ferulic acids cross-linking xylan and lignin. Also during severe alkaline pretreatments at high NaOH dosages ($\geq 9\%$ NaOH dosage, or $\geq 0.2\text{M}$ at 100g SCB/L) (Chapter 5) ether linkages are proposed to be

cleaved. Acid pretreatments are reported to cleave alkyl-aryl ether linkages, however, condensation reactions between monolignols are reported to occur after the pretreatment (Glasser et al, 1983).

Table 2. Hypothetical cross-links between xylan and lignin present in grasses and their stability during alkaline, acid and oxidative pretreatments. The coded cross-links are shown in Figure 3.

Linkage code	linkage type ^a	Stability towards ^b			Reference
		OH ⁻ (8<pH<12)	H ⁺ (1<pH<5)	Ox	
a	FA ester xylan	-	+	+	MacAdam & Grabber, 2002
b	FA ether lignin	+	-	-	Sun & Cheng, 2002
c	diFA ester Arabinose	-	-	+	Del Río et al., 2008
d	β -O-4	+	+	-	Del Río et al., 2008
e	CA ester lignin	-	+	+	Sun et al., 2000; Sun & Cheng, 2002
f	CA ether xylan	+	-	-	Similar to b
g	direct ether lignin-xylan	+	-	-	Similar to b
h	diFA ester xylan	-	+	+	Helm & Ralph, 1993; Sun et al., 2000
i	ester lignin-xylan	-	-	+	Similar to a
j	ester diFA UAmc	-	+	+	Similar to a

^aFA=Ferulic acid; diFA=di-ferulic acid; CA= *p*-coumaric acid.

^bNot stable (-) or stable (+) against alkaline (OH⁻), acid (H⁺) and oxidative treatment (Ox).

In the case of SCB, the proposed linkages based on the occurrence of *p*-coumarates and ferulates are described in chapter 5. The linkage lability, proposed in Table 2, influences the solubilisation behaviour of lignin towards different concentrations of alkali (Chapter 5). Solubilisation of *p*-hydroxycinnamates (*p*-coumarates and ferulates) influences the corresponding enzymatic degradation of the glucan and xylan present in the NaOH residues (Chapters 4 and 5). Considering the above, we propose a schematic model of changes occurring in the SCB cell wall during alkaline pretreatments as depicted in Figure 4.

When no catalyst (0% NaOH) is used and applying a temperature of 120°C for 30-60 min (pH remained around 4), the plant cell wall is only slightly disrupted as seen from the low enzymatic conversion of glucan into glucose (35% (w/w); Chapter 4). About 83% (w/w) of xylan and all lignin originally present in SCB remained in the residues obtained.

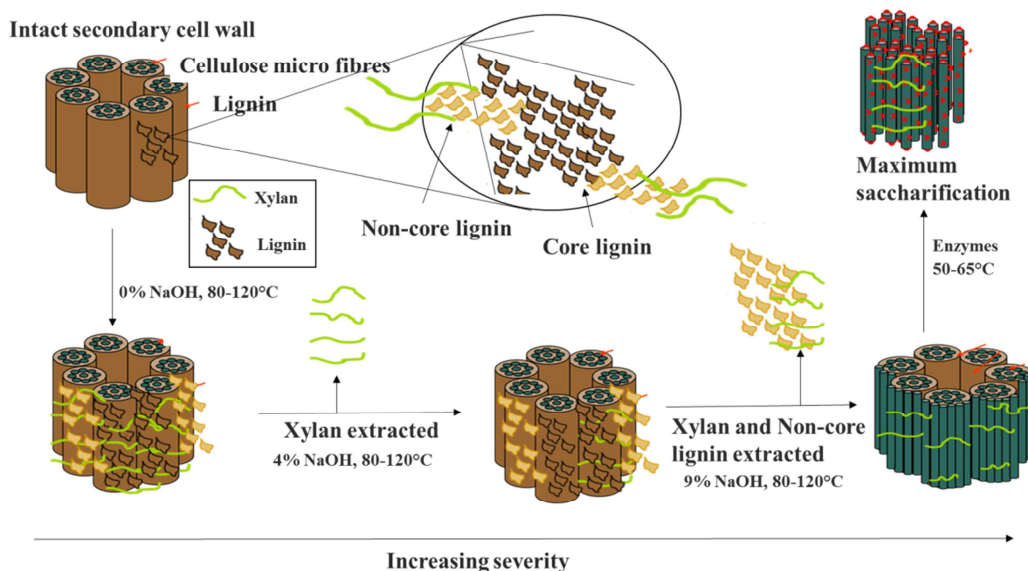


Figure 3. Hypothetical SCB secondary cell wall modifications during alkaline pretreatments. Adapted from Prihardi, 2013.

At 4% NaOH dosage based on dry matter substrate (0.1M) during the thermo-assisted pretreatments performed (Chapters 4 and 5), the pH ranged between 8.6 to 11.7. The latter condition enhanced the cleavage of ester linkages provoking the disruption of cross links between xylan and lignin via ferulates and *p*-coumarates (Chapter 5 and Figure 2). Hence, a subsequent removal of xylan from the residue was achieved. SCB had a high buffering capacity; at 4% NaOH dosage the initial pH was around 12. The pH already decreased from pH 13, which is the pH of 4% NaOH at 100g SCB/L. At the end of the pretreatment the pH was decreased up to 8.6 in some cases. The formation of organic acids from xylan, as discussed earlier in the text, may have contributed to this pH decrease. At these treatment conditions the enzymatic saccharification of glucan into glucose was not complete, being 40 to 58% (w/w). When the concentration of NaOH was increased to 9% NaOH (w/w; 0.2M) in the thermo-assisted pretreatments, the yield of xylan, *p*-coumarates and ferulates in the residues was lowest; all around 50% (w/w) of the amounts originally present in SCB (Chapter 5). The low yield of the NCLs coumarates and ferulates is expected to be due to the cleavage of ether linkages (β -O-4 or α -O-4) by alkali (Dimmel & Schuller, 1986). The residues after 9% NaOH treatment showed the highest enzymatic conversions of glucan and xylan (58-82% for glucan and 53-67% for xylan; Chapters 4 and 5). Other abundant lignin units, such as guaiacol, phenol and syringol, were not extracted in the same proportion as NCL units from the residue. The former units are considered as

core lignin units (CL; Chapter 5). Furthermore, such units might undergo condensation reactions promoted by alkali, which hinders the access of enzymes to the cellulose surface (Chakar & Ragauskas, 2004).

7.3 Hemicellulolytic enzymes from *Rasamsonia emersonii*

The second hypothesis postulated in this thesis was that the characterization of xylan structures predicts which xylan-degrading enzymes improve the complete conversion of the residual polysaccharides. Therefore, the remaining xylan structures after alkaline treatment (Chapter 3) and, more in detail, the xylan structures remaining after oxidative delignification (Chapter 2) were characterised. Xylan remaining after oxidative delignification of EFB showed a degree of substitution of 5 mol of arabinosyl, 11 mol of UA_{me} and 20 mol of acetyl residues per 100 mol of xylosyl residues. The xylan remaining in the residues after alkaline pretreatment of EFB and SCB was shown in Chapter 3. The yield of residual xylan, based on the original amount of xylan present, varied from 80-90% in EFB and from 10 to 50% in SCB. The residual xylan of EFB was composed of 11-15 mol of UA_{me} and of 5-13 mol arabinosyl per 100 mol of xylosyl residues. In SCB NaOH residues the xylan constituents were 7 mol of arabinosyl and 7 mol of glucuronic acid per 100 mol of xylosyl residues. Different from xylan from delignified EFB, xylans from NaOH treated EFB and SCB lacked acetyl substituents due to saponification reactions occurring during the alkaline pretreatments. Also, celluloses were mainly remaining in the NaOH or peracetic acid pretreated residues as seen from the Chapters 2, 3 and 4. Therefore, a combined addition of cellulases and xylanases, including accessory enzymes for the release of xylan substituents, is necessary for a maximum glucan and xylan conversion into their constituent monosaccharides.

***Rasamsonia emersonii* xylan-degrading enzymes for the degradation of xylan and cellulose remaining after alkaline pretreatments of SCB**

Degradation of xylan was not only beneficial for the increased yield of monosaccharides produced from xylan, but it also enhanced the conversion of glucan as seen in the Chapters 4 and 5. Hence, as an additional experiment, NaOH pretreated residues studied in Chapters 4 and 5 were incubated with an *Rasamsonia emersonii* (*Re*) cellulolytic cocktail, and with the same *Re* cocktail enriched with *Re*-xylan degrading enzymes. The exact types of xylan degrading enzymes was not specified, but include endo-xylanases and (4-*O*-methyl-)glucuronic acid releasing enzymes, as mostly glucuronoxylan-like structures were remaining in the residues described. Hereto, as an additional experiment, SCB NaOH residues (Chapter 4) were selected from mildest (coded 6 and 11) to harshest (5 and 9) pretreatment severities and were

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incubated with a cellulolytic cocktail (CA) (Figure 5 A&C) and with a cellulolytic cocktail enriched in xylan degrading enzymes (CB) (Figure 5 B&D).

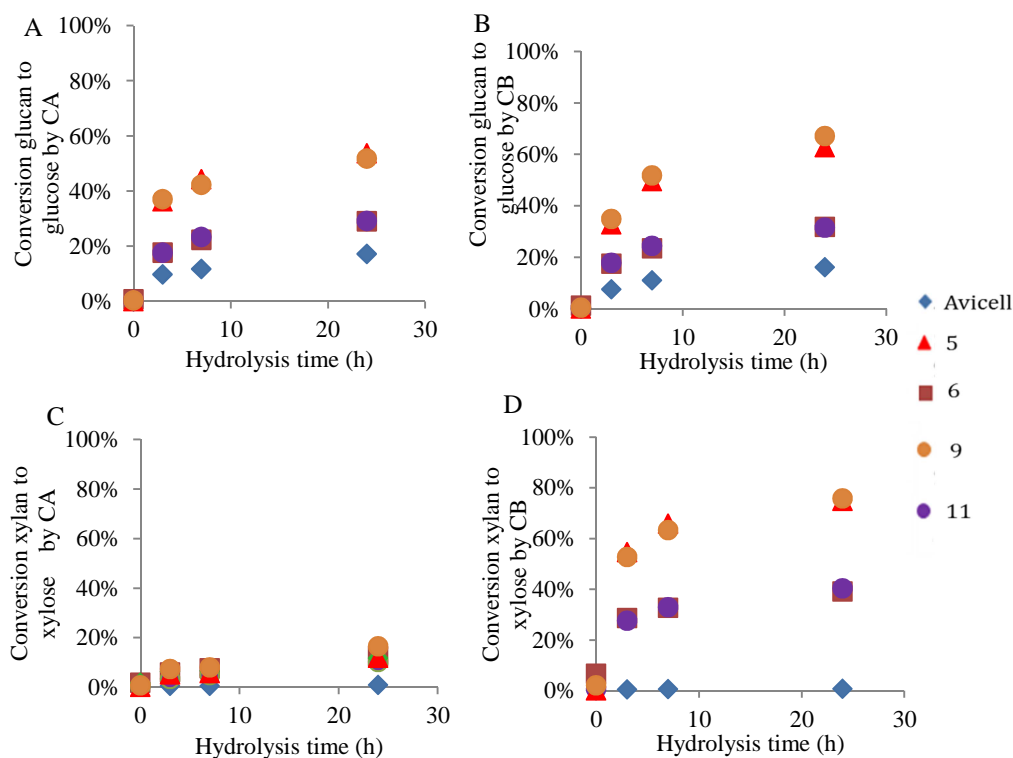


Figure 4. Enzymatic conversion of residues obtained after NaOH-pretreatments of SCB by the cellulolytic enzyme cocktail CA (A and C) and the cellulolytic enzyme cocktail CB enriched in xylan degrading enzymes (B and D). SCB NaOH residues (Chapter 4) were selected from mildest (residue codes 6 and 11) to harshest (5 and 9) pretreatment severities. Avicell was incubated as a control.

Materials and methods: 10mg of a selection of residues (Chapter 4&5) and avicell (control) were incubated together with CA or CB enzyme cocktail provided by DSM (Biotechnology Center, Delft, The Netherlands). Incubation was performed in 1mL citrate buffer, pH of 4.5, at 62°C, and during 24h. The enzyme dose was 2% (w/w) based on substrate dry matter of CA or CB. After 24h incubation the reaction was stopped by boiling the mixtures at 100°C for 5 minutes. Mixtures were centrifuged (10000xg; 25°C; 5min) and supernatants were separated and submitted to HPAEC analysis of glucose and xylose released as described in Chapter 4.

Avicell was also incubated with the same CA and CB cocktails as a control.

It was found that after incubation with CA, the residues treated with 9% NaOH showed the highest glucan and xylan conversion into glucose (55%) and xylose

(18%), respectively. The SCB residues incubated with CB (Figure 4 B&D) showed not only a higher xylan conversion (80% for residues 5 and 9) in comparison to CA (18% for residue 9), but also a higher glucan degradation (55 and 70% for residue 9 degraded by CA and CB, respectively).

Re is a good (hemi-) cellulase producer. It produces a wide range of xylan degrading enzymes activities, based on their annotation in the genome. Five of these enzymes are shown in Table 3: endoxylanase (*ReGH10*), arabinofuranosidase (*ReGH51*), two α -glucuronidases (*ReGH67* and *ReGH115*) and acetyl xylan esterase (*CE1*).

Table 3. Biochemical characteristics of five single-activity enzymes from *Rasamsonia emersonii*.

Enzyme code	Enzyme family CAZy ^a	Mw (kDa)	Predicted activity ^b	Tested Activity	Reference
<i>ReGH10</i>	GH10	44	Xylanase	Endo-1,4- β -xylanase	Chapter 2
<i>ReGH51</i>	GH51	69	Arabino-furanosidase	Arabinofuranosidase, O-3 position	Chapter 7
<i>ReGH67</i>	GH67	91	α -Glucuronidase	α -Glucuronidase UA _{me} -XOS	Chapter 6
<i>ReGH115</i>	GH115	111	α -Glucuronidase	α -Glucuronidase UA _{me} -polymer	Chapter 6
<i>ReCE1</i>	CE1	33	Acetyl xylan esterase	Acetyl xylan esterase	(Neumüller et al., 2015)

^aCAZy=carbohydrate active enzymes database (CAZy.org)

^bPredicted activity based on genome annotation.

Although the type of activity of the single activity enzymes was predicted by their gene annotation (Table 3), the real mode-of-action can only be confirmed by testing the enzymes towards various substrates. Hence, in our research, these single-activity enzymes, as shown in Table 3, were characterised prior to their use for the degradation of xylans remaining after delignification of EFB or after alkaline pretreatments of SCB.

Re grows well at temperatures around 45-50°C and the genome is sequenced and available (Houbraken et al., 2012); Uniprot: CBS393.64). The choice for *Re* was determined by the project partner DSM. DSM produces enzyme cocktails which are built around enzymes derived from *Re* (Pel, 2015). But, more important for the selection of *Re* is the thermostability of its enzymes produced. Although thermostability experiments were only performed with *ReGH10*, it is assumed that as all the other activities above mentioned belong to the same organism, they are expected to show a similar thermostability as *ReGH10*. The thermostability of *ReGH10*

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was studied in comparison with a GH10 endoxylanase from *Aspergillus awamori* (*AaGH10*; Kormelink et al., 1993) and the results are shown in Figure 5.

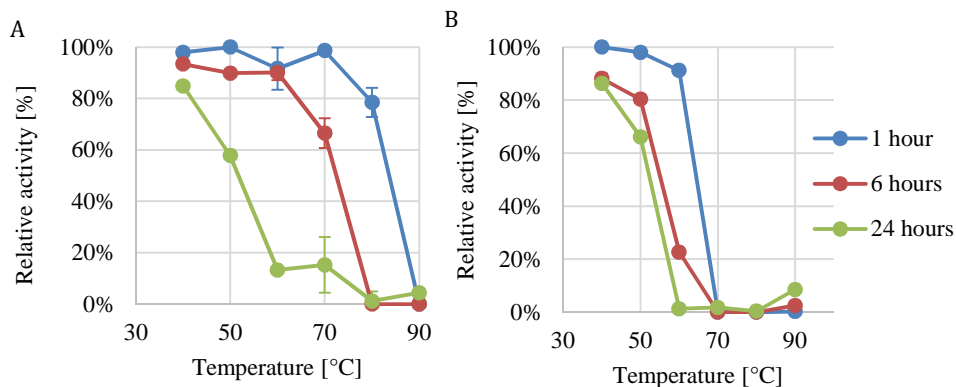


Figure 5. Residual xylanase activity of *ReGH10* (A) compared to *AaGH10* (B) on BeWX at optimum enzyme conditions after incubation of enzymes in absence of substrate at 40, 60, 70, 80 and 90°C for 1h, 6h and 24h.

Materials and methods: *ReGH10* and *AaGH10* were incubated in 1mL 50mM NaOAc buffer, pH 4.5 at 40, 60, 70, 80 and 90°C for 1h, 6h and 24h. To these enzyme incubated solutions, the substrate was added (5mg of BeWX dry matter) and a 40 min incubation was performed at 50°C at pH 4.5. After that, the mixture was acidified to pH 2 by 4M HCl and centrifuged (10000xg; 10min; 25°C). The supernatants obtained were analysed for the amount of reducing ends present by the PAHBAH reducing assay in duplicate (Lever, 1972).

ReGH10 kept 80% of its activity towards BeWX after storage at 80°C for 1 hour. The same activity was remained after 6h storage between 60 and 65 °C. In contrast, *AaGH10* kept 80% of its activity only at, for example, 1h and 60°C or 6h at 50°C. Hence, it is concluded that *ReGH10* showed a better thermostability than *AaGH10*.

ReGH10

ReGH10 was described as “xylanases” based on the genome annotation. The enzyme showed to be active towards EFB xylan (Chapter 2) and BeWX (results not shown). From BeWX, *ReGH10* released xylobiose (X₂), linear xylo-oligosaccharides (XOS) and xylo-oligosaccharides substituted by 4-*O*-methylglucuronic acid (UA_{me}-XOS). Hence, *ReGH10* can be classified as an endo-1,4-β-xylanase.

ReGH51

The single-activity arabinofuranosidase from *R. emersonii*, *ReGH51* (Table 3) was tested towards WAX in combination with the previously characterised *AaGH10* (Kormelink, 1993). *AaGH10* released a series of XOS substituted by arabinosyl groups at the *O*-3 or at the *O*-2 and *O*-3 positions as shown in Figure 5.

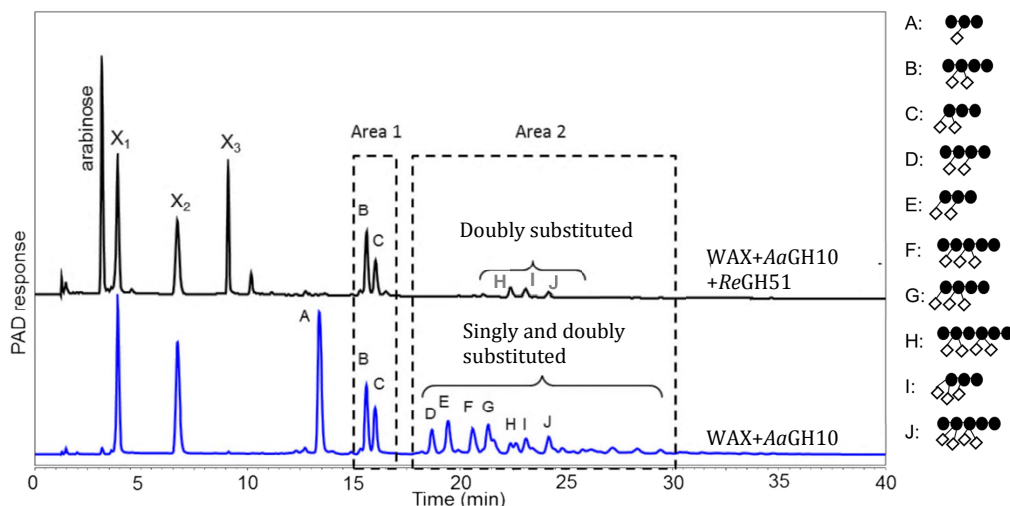


Figure 6: HPAEC patterns of WAX digested by *AaGH10* or by a combination of *AaGH10* and *ReGH51*. Digest products of WAX degraded by *AaGH10* were previously described by Kormelink et al. (1993) and shown on the right side of the figure. Black dots represent xylosyl residues and rhombuses represent arabinosyl residues.

Comparing the two chromatograms in Figure 6, the combination of *AaGH10* with *ReGH51* released mainly X, X₂ and X₃, and no single substituted XOS remained. *ReGH51* was apparently able to degrade the WAX structures substituted by a single arabinosyl residue at the *O*-3 position. This was concluded as HPAEC chromatogram peaks representing structures A, D, E, F, and G from *AaGH10* digested WAX disappeared after incubation with *ReGH10*. Nevertheless, *ReGH51* was not able to degrade those xylo-oligosaccharides substituted by two arabinosyl residues (at the *O*-3 and *O*-2 positions), seen from the remaining structures H, I and J. Based on the results obtained, *ReGH51* was confirmed to be an arabinofuranosidase specifically cleaving *O*3-substituted arabinosyl residues from xylan-structures.

ReGH67 and ReGH115

R. emersonii produces two α -glucuronidases (*ReGH67* and *ReGH115*), which were characterised in Chapter 6. *ReGH115* released UA_{me} from polymeric xylan and xylo-oligomers independently from the position of the UA_{me}. In contrast, *ReGH67* was

only active towards xylo-oligosaccharides substituted by UA_{me} at the non-reducing end.

ReCE1

Biochemical characteristics of CE1 were previously published by Neumüller, et al. (2015). CE1 showed to be active towards di-acetylated xylosyl residues in xylo-oligosaccharides substituted at the *O*-2 and *O*-3 position.

In conclusion, alkaline pretreatments of lignin rich grasses showed to mainly solubilise lignins hydroxycinnamic acids and minor amounts of xylan. Nonetheless, alkaline conditions provoke xylan degradations by peeling reactions, which are dependent on the amount and type of substituents present in xylan. Prevention of peeling reactions can be achieved by the use of reducing agents, such as NaBH₄. However, this is not feasible at industrial scale due to costs and safety issues. An alternative is the introduction of an oxidative agent to a mild alkaline solution as a pretreatment. Alkaline conditions are still relevant as they are responsible for the release of esterified hydroxycinnamic acids, which represent a large amount of the lignin present in grasses. An extensive release of lignin and a less extensive release of xylan during alkaline pretreatment enhances the enzymatic conversion of glucan and xylan into its constituent monosaccharides. Furthermore, the characterisation of xylan structures remaining in the residues after pretreatments is important to select the most effective enzymes. These enzymes should include endoxylanases plus accessory enzymes, such as α -glucuronidases as (4-*O*-methyl)glucuronic acids showed to be resistant towards acid, alkali or oxidative pretreatments. It was shown that the addition of the accessory enzymes optimised the conversion of xylan into xylose, and that their addition to a cellulase based cocktail also increased the glucan conversion.

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Summary

Summary

The aim of this thesis was to understand the effect of both H_2SO_4 and NaOH catalysed pretreatments of sugar cane bagasse, barley straw and oil palm empty fruit bunches on the subsequent enzymatic saccharification, with a focus on the NaOH catalysed pretreatments. The fate of the main polymers present, lignin, cellulose and xylan was studied, and also single-activity xylan degrading enzymes, all from *Rasamsonia emersonii*, were studied for their modes of action.

In **Chapter 1** an overview of the chemical composition of different grass type biomasses is presented. Also, the main biomass pretreatments currently studied are presented as well as the main enzyme activities involved in the saccharification of xylan and cellulose.

Chapter 2 shows the enzymatic fingerprint of xylan from oil palm empty fruit bunches (EFB) after either sequential extraction with 1 and 4M NaOH or delignification with peracetic acid as an oxidative agent. Delignification of EFB resulted in an almost complete hydrolysis, while after sequential extraction without delignification only 40% of the extract was enzymatically hydrolysed. Removal of acetyl groups by alkali provoked xylan aggregation, hindering its enzyme hydrolysis. From the analysis of the oligosaccharide-fingerprint of enzyme hydrolysed delignified EFB xylan, the structure was defined as acetylated 4-*O*-methylglucuronoxylan. It was concluded that delignification alone outperforms alkaline extraction as pretreatment for enzymatic fingerprinting of EFB xyans.

In **Chapter 3**, the extent of hemicellulose and lignin solubilisation by H_2SO_4 and NaOH catalysed pretreatments was correlated to the extent of subsequent enzymatic cellulose hydrolysis. Oil palm empty fruit bunch, sugarcane bagasse and barley straw were investigated. Alkali pretreatment resulted into extensive lignin removal. This removal resulted in up to 90% (w/w) conversion of glucan into glucose by enzymes. But, the alkaline conditions also provoked unwanted xylan losses. Acid pretreatment resulted into solubilisation of xylan with almost no losses, while lignin remained. Although moderate xylan solubilisation increased enzymatic cellulose hydrolysis of residual glucan, extensive removal of xylan decreased it. Therefore, from the pretreatments performed in Chapter 3, the NaOH pretreatments were the most efficient for the subsequent enzymatic release of xylose and glucose from the insoluble residues.

In **Chapter 4**, a broad range of alkaline concentrations, time and temperatures were applied to sugarcane bagasse. The pretreated samples obtained were hydrolysed using cellulolytic and hemicellulolytic enzymes. The NaOH concentration was the variable with the largest effect on the enzyme hydrolysis. High NaOH dosages, however, also resulted in a significant loss of xylan. The experimental data were fitted in a hyperbolic empirical model. A rate equation of the enzymatic hydrolysis was obtained, which described the influence of the alkaline pretreatment conditions on the enzyme hydrolysis and can be used to design unit operations to convert sugarcane bagasse into fermentable sugars.

In **Chapter 5**, modifications in lignin of sugarcane bagasse alkaline resulting from alkaline pretreatments (Chapter 4) were studied. The modifications were correlated to the enzymatic saccharification of glucan. Sugarcane bagasse lignin showed to be rich in hydroxycinnamates, mainly *p*-coumarates and ferulates, which were considered as non-core lignin units. The *p*-coumarates were extensively removed by alkali. Ferulates were also removed but, to a lesser extent than the *p*-coumarates. The lower the relative abundance of non-core lignin, the lower the xylan recovery was in the residues. Core lignin compounds, analysed as phenol, guaiacol and syringol, accumulated in the residues. Enzyme hydrolysis correlated positively with the simultaneous decrease in residual xylan and non-core lignin.

A common factor of NaOH and H₂SO₄ pretreated residues was the presence of (4-*O*-methyl)glucuronic acids (UA_{me}), which were not released during pretreatment. Hence, the release of (4-*O*-methyl)glucuronic acids from the xylan backbone is needed for the complete enzymatic conversion of xylan into xylose. Therefore, in **Chapter 6**, the mode-of-action of two α -glucuronidases was characterised. Both enzymes were obtained from the fungus *Rasamsonia emersonii*; one belonging to the glycoside hydrolase (GH) family 67 (*ReGH67*) and the other to GH115 (*ReGH115*). Both enzymes functioned optimal at around pH 4 and 70°C. *ReGH67* showed limited substrate specificity towards only xylo-oligosaccharides substituted with a 4-*O*-methylglucuronic acid group (UA_{me}XOS), cleaving UA_{me} only when attached to the non-reducing end xylosyl residue. *ReGH115* was much less substrate specific compared to *ReGH67*, because UA_{me} was released from both polymeric UA_{me}xylan and UA_{me}XOS, from both internal and non-reducing end xylosyl residues.

Finally, in **Chapter 7** the main findings of this thesis were discussed. As a conclusion, alkaline pretreatments of lignin rich grasses showed to mainly solubilise lignin and minor amounts of xylan. Nonetheless, alkaline conditions provoked xylan degradations by peeling reactions, which are very much dependent on the amount and type of substituents present in xylan. Prevention of peeling reactions can be achieved by the use of reducing agents such as NaBH₄, however, not feasible at industrial scale due to costs and safety issues. An alternative is the introduction of an oxidative agent to a mild alkaline solution as a pretreatment. Alkaline conditions, also showed to release esterified hydroxycinnamic acids, which in grasses represent a large amount of the lignin. Furthermore, the characterisation of xylan structures remaining in the residues after pretreatments is important to select the most effective enzymes. These enzymes should include endoxylanases, plus accessory enzymes, such as α -glucuronidases. It has been shown that the addition of the accessory enzymes optimised the conversion of xylan into xylose. Their addition to a cellulase based cocktail, also, increased the glucan conversion.

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“Paso de buey,
ojo de lince
diente de lobo y...
hacerse el bobo.”

Patricia.

Curriculum vitae

Patricia Murciano Martínez was born on June 5th 1987 in Teruel, Spain. She finished her secondary education at the I.E.S Vega del Turia in 2005. In September 2005 she started Human nutrition and health studies at the University of Valencia. She obtained her B.Sc. diploma after completing her thesis on nutrition of patients with kidney condition at Obispo Ploanco Hospital. In 2008, Patricia started the M.Sc. program Food Technology at the Polytechnic University of Valencia. During this period she worked as an intern at an artisan cheese maker: Queso artesano de Albarracín. She worked in the development of a new cheese product matured in wine. In 2011 she obtained her M.Sc. diploma after completing her thesis at the Institute of Food Research (Norwich, UK) where she participated in the development of a MATLAB model of carbohydrate characterization of wheat straw, based on FT-IR spectra. In September 2011, she started as a PhD student at the Laboratory of Food Chemistry, Wageningen University, within the BE-Basic project entitled: "C3-acids production from lignocellulosic feedstocks". The results of her work are described in this thesis. Patricia continues to work at the Laboratory of Food Chemistry as a researcher.



Contact: patricia.murciano@gmail.com

List of publications

P. Murciano Martínez, P. Harmsen, R. Bakker, H. Gruppen, M. A. Kabel. Importance of acid and alkali concentration on the removal of xylan and lignin for enzymatic hydrolysis. *Industrial Crops and Products*, 2015. Vol. 64, Pages 1-270.

P. Murciano Martínez, M.A. Kabel, H. Gruppen. Delignification outperforms alkaline extraction for enzymatic fingerprinting of xylan from oil palm empty fruit bunch, 2015. Submitted.

P. Murciano Martínez, M.A. Kabel, H. Gruppen. The two *Rasamsonia emersonii* α -glucuronidases, *ReGH67* and *ReGH115*, show a different mode-of-action towards glucuronoxylan and glucuronoxyloligosaccharides. 2015. Submitted.

P. Murciano Martínez, A. M. Punt, , M. A. Kabel, H. Gruppen. Deconstruction of lignin linked *p*-coumarates, ferulates and xylan by NaOH enhances the enzymatic conversion of glucan, 2015. Submitted.

Overview of completed training activities

Discipline specific activities

BE-Basic Symposium	2012-2015 ¹
Food & Biorefinery Enzymology (4th edition)	2013
Advanced Food Analysis	2013 ¹
Cell Wall Meeting	2013 ¹
Summer Course Glycosciences	2014 ¹
Lignin Conference	2014 ¹

General courses/activities

Interpersonal Communication Skills For PhD Students	2011
Scientific Publishing	2011
VLAG PhD Week	2012
Project And Time Management	2013
Scientific Writing	2013
Career Perspectives	2015

Additional activities

Preparation Of Research Proposal	2011
PhD Presentations Food Chemistry	2011-2015
B.Sc./M.Sc. Thesis Student Presentation And Colloquia	2011-2015
Food Chemistry PhD-trip Singapore and Malaysia	2012/2014 ^{1,2}
Food Chemistry PhD-trip Germany, Denmark, Sweden and Finland	2012/2014 ^{1,2}

¹Poster presentation

²Oral presentation

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Patricia Murciano Martínez, 2016.