Thibaut Mouthier

Biological and chemical pretreatment of grasses to overcome lignin's recalcitrance improving carbohydrate degradability

INVITATION

You are cordially invited to attend the public defence of my PhD thesis entitled

BIOLOGICAL AND CHEMICAL PRETREATMENT OF GRASSES TO OVERCOME LIGNIN'S RECALCITRANCE IMPROVING CARBOHYDRATE DEGRADABILITY

On **Tuesday 18 September 2018**
at **16.00 p.m.**
in the Aula of Wageningen University,
Generaal Foulkesweg 1A,
Wageningen

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PARANYMPHS

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Propositions

1. The degradation of lignin-carbohydrate complexes during ammonia treatment can be monitored through the analysis of 4-vinylguaiacol by py-GC-MS.
   (this thesis)

2. Severe pretreatment conditions prevent the use of lignin as building blocks for high value chemicals.
   (this thesis)

3. Laboratory simulation can replace laboratory classes.

4. The impact of retroviral genes from outside our planet on evolutionary genomic complexity in biological specimens on earth is underestimated.
   (Steele et al., Progress in Biophysics and Molecular Biology, 2018, 136: p. 3-23)

5. The mix of cultures is an undervalued ingredient in tasty recipes.

6. For lovers of French wine, the origin and age of the wine defines the taste more than the actual aroma.

Propositions belonging to the thesis, entitled

"Biological and chemical pretreatment of grasses to overcome lignin's recalcitrance improving carbohydrate degradability"

Thibaut M.B. Mouthier

Wageningen, 18th September 2018.

Biological and chemical pretreatment of grasses to overcome lignin's recalcitrance improving carbohydrate degradability

Thibaut M.B. Mouthier

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrotechnology, Nutrition and Health Sciences)

Biological and chemical pretreatment of grasses to overcome lignin's recalcitrance improving carbohydrate degradability

Thibaut M.B. Mouthier

Thesis

submitted in fulfilment of the requirements for the degree of doctor

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by the authority of the Rector Magnificus,

Prof. Dr A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

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at 4 p.m. in the Aula.

Thibaut M.B. Mouthier

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Table of Content

List of abbreviations

PCW	Plant cell wall	$P_{(1,2,3)}$	Phase 1, 2 or 3 of composting process
GAX	Glucuronoarabinoxylan	$wP_{(1,2,3)}$	Water unsoluble solids from $P_{(1,2,3)}$
LCC	Lignin carbohydrate complex		
Glc	Glucose	ADL	Acid detergent lignin
Ara	Arabinose	ADF	Acid detergent fibre
Xyl	Xylose	NDF	Neutral detergent fibre
GlcA	Glucuronic acid	pRDF	Potentially rumen degradable fibre
UA	Uronic acid	ASL	Acid soluble lignin
DS	Degree of substitution		
		H unit	Lignin *p*-hydroxyphenyl unit
CSF	Combined severity factor	G unit	Lignin guaiacyl unit
ARP	Ammonia recycle percolation	S unit	Lignin syringyl unit
SAA	Soaking in aqueous ammonia	Vinyl-H	4-vinylphenol
LAA	Low liquid ammonia	Vinyl-G	4-vinylguaiacol
LMAA	Low moisture anhydrous ammonia	Vinyl-S	4-vinylsyringol
AFEX	Ammonia fibre explosion	Unsub.	Unsubstituted lignin unit
		Cα-O	Oxidised carbon α
NH_3	Ammonia	Ph-C0-2	Lignin unit with non, 1 or 2 carbons on the side chain
H_2SO_4	Sulfuric acid	Ph-C-3	Lignin unit with 3 carbons on the side chain
HAc	Acetic acid		
NaOH	Sodium hydroxide	S/N	Signal to noise ratio
HMF	Hydroxymethylfurfural		
WUS	Water unsoluble solids		
WSS	Water soluble solids		

CHAPTER 1

General introduction

1.1 Relevance of this research

The valorisation of lignocellulosic grasses, such as wheat straw and corn stover, into renewable biochemicals and biofuels, is a promising alternative for fossil based products and contributes to sustainable 'whole crop use' approaches. Even, valorisation for food production (i.e. mushrooms) is a current use of lignocellulose. These feedstocks, being by-products, are not competing with food production, making them perfect candidates for valorisation. Hereto, it is aimed to convert the plant biomass into smaller building blocks like monosaccharides via enzymatic degradation. Lignocellulose, however, is not directly accessible for enzymes, hence, pretreatment of the material is required to open up the complex lignocellulose-matrix obtaining a commercially feasible process.

Lignocellulosic biomass is mainly composed of carbohydrates and lignin, which are interconnected within the plant cell wall (PCW). The complexity of the plant cell wall structures makes it hard-to-convert and lowers bioconversion of carbohydrates. The cellulose, which is the principal structure to enzymatically degrade into monosaccharides, is protected by xylan and lignin. In grasses, both xylan and lignin have complex structures; 1) xylan has a xylosyl backbone decorated with arabinosyl, glucuronic acid and acetyl groups preventing enzymatic degradation, 2) lignin is a phenolic polymer and the natural defence barrier preventing biological and chemical degradation. To be able to use the entire lignocellulosic material, the structures and their interconnections must be modified to have a maximal enzymatic conversion. Showing and proving that the interconnections between xylan and lignin play a key role in carbohydrate degradation will have a high impact for (chemical) treatment processes.

The research described in this PhD-thesis is conducted within a NWO-project in collaboration with two industrial partners, DSM and CNC, named "Cracking the recalcitrance of xylan in *hard-to-convert* biomass with novel enzymes and intrinsic chemical catalysts". The project aimed to identify new routes to release monosaccharides from hard-to-convert feedstocks by a detailed structural analysis of the recalcitrant carbohydrate and lignin released from grass feedstocks mildly treated with 1) intrinsic chemical catalysts (acetic acid and ammonium); 2) novel enzymes and; 3) combination of both. Loosening of the architecture of cell walls is known to enhance accessibility to enzymes. However, the residual lignin structures are not fully understood and will vary depending on the plant source and the targeted product (i.e. conversion to biochemicals, opening the structures for compost). Hence, this research focused on the variability and the lignin recalcitrant structures of 2 main grasses (corn stover, wheat straw) and how they can be affected by a biological process (rumen digestion or composting process), by an ammonia pretreatment, and by an acetic acid (enzymatically released or added to the treatment) or sulfuric acid pretreatment. The effects and the modifications of lignin were evaluated for to their influence on further enzymatic degradation of polysaccharides. Furthermore, the presence and the use of intrinsic catalysts within the biomass or within the process was investigated to increase the severity of pretreatment and its following degradability.

1.2 Grasses composition, plant cell wall structures and relevant carbohydrate degrading enzymes

Agro-industrial by-products (i.e. rice-, wheat-, and barley straws, corn stover, sugarcane bagasse) are available for large demand. Grass residues originating from the food industry are commonly used as livestock feed, but part of it could be used to produce biochemicals after a reorganisation of the feed supply chain [1]. In this thesis, the focus is on the by-products wheat straw and corn stover being agricultural residues and both belonging to the grasses. The composition of grasses varies, depending on the type, origin and year of harvest, although are largely comprised of lignin, hemicellulosic xylan and cellulose within their plant cell walls.

1.2.1 Composition and architecture of the plant cell wall

In general, plant cell walls (PCWs) are mainly composed of (hemi-) cellulose, pectin, protein and lignin. PCWs are assembled into three major layers: the middle lamella, the primary cell wall and the secondary cell wall [2]. A schematic representation of the PCW is shown in Figure 1.1. The primary and secondary cell walls mainly consist of interlinked (hemi-) cellulose, pectin and protein, while the middle lamella is the first synthetized layer containing pectin to allow high flexibility, cell expansion and plant cell adhesion. Lignin is majorly present in the secondary cell wall and is also interconnecting all the different layers rigidifying the plant cell wall [3].

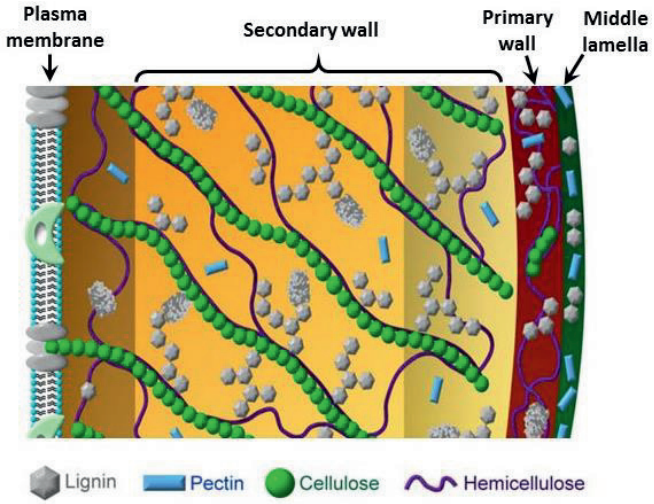


Figure 1.1: Plant cell wall model based on Achtyuthan et al. [3].

1.2.1.1 Primary and secondary cell wall

The primary cell wall is mainly composed of polysaccharides, while lignin is basically absent. Depending on the structure, primary cell wall can be classified into two different types: Type I and Type II.

Type I is mainly present in dicots and consists of a crystalline cellulosic microfibril network, which can be either embedded in a hemicellulosic xyloglucan or glucuronoarabinoxylan (GAX) network, together with pectic homogalacturonan and rhamnogalacturonan I structures [4, 5]. As an example, the primary cell wall is the main layer in the dry matter found in fruits or in vegetables [6]. Type II is mainly present in *Poaceae* (grasses) and in related monocots. It consists of cellulose, which can be embedded in network with GAX, glucomannan, or xyloglucans [7, 8].

In general, the secondary PCW mainly consists of cellulose, hemicellulose and lignin. Cellulose and hemicellulose form a carbohydrate complex [7], while lignin covers the carbohydrates strengthening the entire structure [9]. The secondary cell wall architecture with the main components is presented in Figure 1.2. For grasses and wood, the secondary PCW represents the main part of the dry matter, hence relevant in perspective of biorefinery approaches. The hemicellulosic fraction in the secondary PCW of grasses mainly consists of glucuronoarabinoxylan (see below), while in dicot walls it is mainly glucuronoxylan and (gluco)mannan, and in conifer walls it is mainly glucuronoarabinoxylan and (gluco)mannan [10].

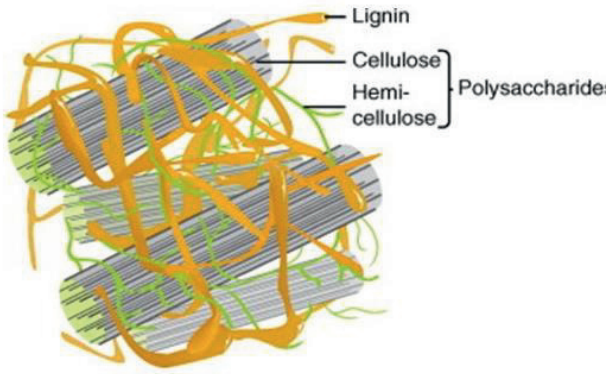


Figure 1.2: Interaction of the structural elements in the secondary cell wall as suggested by Vanholme et al. [11].

1.2.1.2 Main secondary cell wall polysaccharides structures in grasses

In grasses, cellulose, hemicellulosic GAX and lignin are the main components of the secondary PCWs. Although the amounts of these components vary widely between plant species [10], general structural characteristics are present and are discussed below. The variability and distribution of grasses are shown in Table 1.1. In grasses, cellulose ranges from 30 w/w % in corn stover up to 49 w/w % in

Miscanthus grass, while hemicellulosic GAX ranges from 16 w/w % for rice straw up to 28 w/w % in switch grass.

Table 1.1: Typical grasses based raw material, and their polysaccharides and cell wall composition.

Content w/w %	Wheat straw	Corn stover	Sugarcane bagasse	Rice straw	Miscanthus grass	Empty fruit bunch	Switch grass
Cellulose[a]	31	30	39	37	49	36	36
GAX[a,b]	23	26	27	16	25	19	28
Xylosyl[b]	20	19	21	16	22	15	24
Arabinosyl[b]	2.5	2.7	5.6	n.s.	1.8	n.s.	2.9
Uronic acid[b]	n.s.[c]	2.2	n.s.	n.s.	n.s.	n.s.	n.s.
Galactosyl	n.s.	1.0	n.s.	n.s.	0.4	n.s.	n.s.
Mannosyl	n.s.	0.5	n.s.	n.s.	0.3	3.6	1.3
Rhamnosyl	n.s.	1.1	n.s.	n.s.	n.s.	n.s.	n.s.
Lignin[d]	25	31	24	15	27[d]	35.4	26.4
Acetic acid[e]	1.7	2.4	n.s.	n.s.	n.s.	n.s.	n.s.
Protein (Nx6.25)	n.s.	4.2	n.s.	n.s.	n.s.	n.s.	n.s.
Ash	n.s.	n.s.	n.s.	14.5	n.s.	5.8	1.9
DS Ara[f]	12.5	14.2	26.7	n.s.	8.2	n.s.	12.1
DS UA[f]	n.s.	11.6	n.s.	n.s.	n.s.	n.s.	n.s.
Reference	[12]	[13]	[14]	[15]	[16]	[17]	[18]

[a] Carbohydrates contents (w/w %) represented as anhydro-residues

[b] Sum of xylosyl, arabinosyl and uronic acid residues = glucuronoarabinoxylan (GAX)

[c] n.s.= not specified

[d] Sum of acid insoluble lignin (including ash) and acid soluble lignin in w/w %

[e] Esterified acetic acid in w/w %

[f] DS Ara = mol of arabinosyl residues per 100 mol of xylosyl; DS UA = mol of uronic acid residues per 100 mol of xylosyl

1.2.1.3 Cellulose

Cellulose represents the major part of the carbohydrates in grasses (Table 1.1). Cellulose is a homogeneous linear polymer of β-(1→4)-linked glucan chains that can aggregate into microfibrils via hydrogen bonds and van der Waals forces [19, 20]. A simplified structure of the cellulose chain is shown in Figure 1.3. The microfibrils formed are highly crystalline and can form two different types of cellulose: cellulose Iα, which is mainly present in algae and bacteria, and Iβ present in plants [21-24]. The two forms of cellulose are present in different proportions in natural sources [25]. They differ from each other in the way they are packed together, but both cellulose form Iα and Iβ are formed from β-(1→4)-linked glucosyl units together forming a flat ribbon [22]. They are both assembled in a helical chain fixed by *O*3'-*O*5 hydrogen bonds and with *O*2-*O*6' hydrogen bonds between successive glucosyl residues [23].

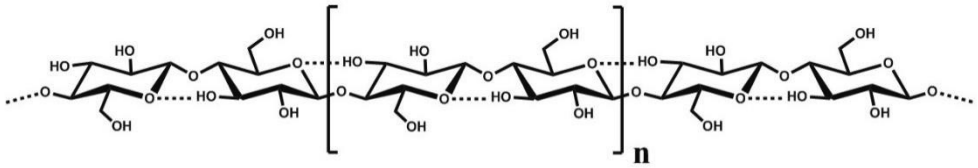
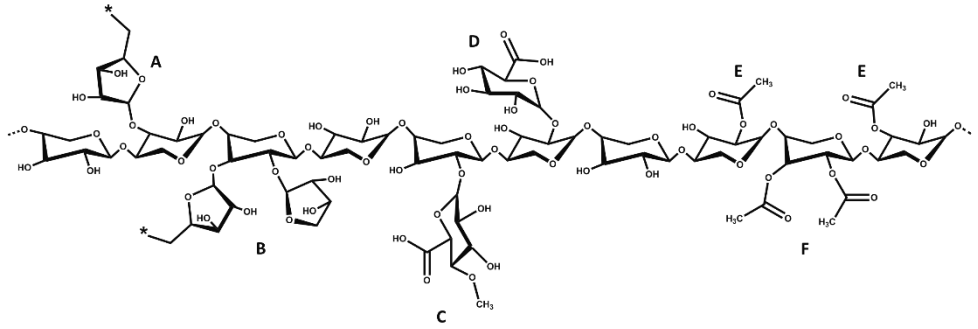


Figure 1.3: Structure of the cellulose chain consisting of β-(1→4)-linked glucosyl residues [22].

1.2.1.4 Glucuronoarabinoxylan (GAX)

As mentioned above, the major part of the hemicelluloses present in grasses is GAX. GAX is built with a backbone of β-(1→4)-linked xylosyl residues, with various types and degrees of ramifications. The latter depends on the type, origin and year of harvest of the grass material. A representation of GAX is shown in Figure 1.4.



Side chains attached to Xyl-β(1→4) backbone :

A. Ara*f*-α(1→3)Xyl-β(1→4)

B. Ara*f*-α(1→2)[Ara*f*-α(1→3)]Xyl-β(1→4)

C. (*O*-Me)GlcA-α(1→2)Xyl-β(1→4)

D. GlcA-α(1→2)Xyl-β(1→4)

E. *O*-2-acetyl-Xyl-β(1→4) and *O*-3-acetyl-Xyl-β(1→4)

F. *O*-3 and *O*-2-acetyl-Xyl-β(1→4)

* Indicates the position of the attachment of ferulic acid groups on the α-(1→3)-linked Ara*f* residues

Figure 1.4: Schematic representation of the glucuronoarabinoxylan structure found in grasses. Adapted from Faik [26].

The main ramifications of GAX in grasses are arabinosyl and (4-*O*-methyl-) glucuronosyl residues (Figure 1.4). Nevertheless, the degree of substitution of the xylosyl backbone with arabinosyl (DS Ara) and/or uronic acid (DS UA) residues varies between species (Table 1.1). As an example, the DS Ara of wheat straw, corn stover and switch grass is very similar, 12.5, 14.2 and 12.1, respectively, while it is almost doubled in sugarcane bagasse (26.7; Table 1.1). The (4-*O*-methyl-) glucuronosyl residues are α-(1→2)-linked to the xylan backbone and arabinofuranosyl residues can be linked to the xylosyl residues at either the *O*-3 or both *O*-2 and *O*-3 positions [27-29]. Via the *O*-5 position, the arabinofuranosyl residues can be further esterified to ferulic acid or diferulic acid groups [30]. In

addition, substitution with esterified acetic acid groups occur at the *O*-2 and/ or *O*-3 of the xylosyl residues [28]. Esters of *p*-coumaric acid have also been detected in GAX structures [29, 31].

1.2.1.5 Lignin and lignin-carbohydrate complexes (LCCs)

Lignin is an aromatic polymer representing 10 to 35 (w/w %) of the total dry matter content of grasses (see Table 1.1). For comparison it is good to note that in soft- and hardwoods lignin contents are higher than 30 (w/w %) of the dry matter [32, 33]. The lignin structures and the protection it provides to the plant cell makes it hard to degrade material. Thus, it is one of the most limiting structures in view of lignocellulosic biomass conversion [34]. Lignin is insoluble in most of the common solvents protecting the PCW from microbial attack and natural elements [35]. Together with hemicellulose, lignin protects the cellulose from degradation: linking PCW structures, lowering enzyme accessibility and adsorbing enzymes enabling biochemical reactions [36, 37].

Lignin is synthetized from the three monolignols *p*-coumaryl alcohol, coniferyl alcohol and synapyl alcohol (Figure 1.5). The monolignols are integrated within the lignin polymer forming the three main lignin building blocks units, *p*-hydroxyphenyl (H-unit), guaiacyl (G-unit) and syringyl (S-unit) [38] (Figure 1.5).

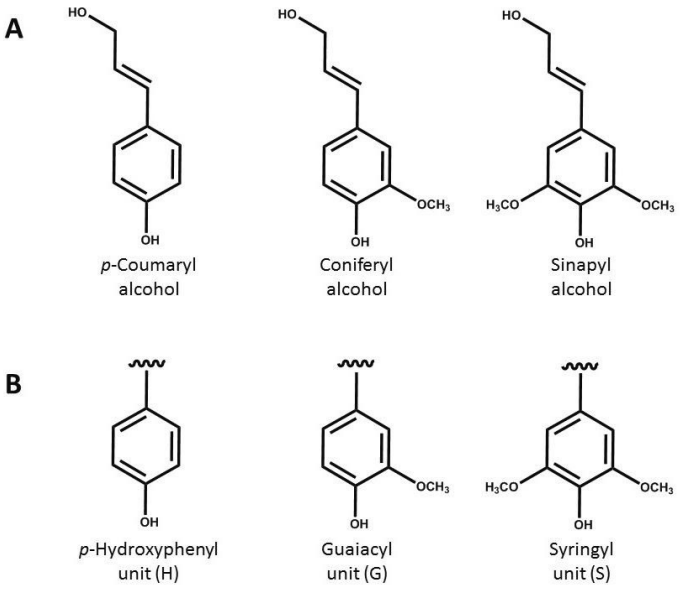


Figure 1.5: Primary lignin building blocks. **A** Monolignols. **B** Lignin units (H, G and S) derivating from the monolignols and integrated within the lignin polymer.

The composition of lignin varies within plant species resulting in different ratios of H-, G- and S-units. In grasses, lignin is composed of all three units in different ratios depending of the plant source. For

comparison, hardwood lignin is mainly composed of S and G units and softwood lignin is mainly composed of G units with a small amount of H units [39].

A schematic representation of the complex lignin network is shown in Figure 1.6. Lignin H-, G- and S-units are linked together through different types of C-C and C-O bonds of which the most abundant in grasses, soft and hardwood lignin is the β-*O*-4 aryl ether linkage (Figure 1.6). For example, for wheat straw it has been reported that the β-*O*-4 aryl ether linkage represents 75 %, while the second most abundant linkage (β-5' phenylcoumaran linkage; Figure 1.6) represents 11 % of the lignin linkages [33]. In hard wood, a third major lignin interunit linkage can be found (β- β' resinol linkage; Figure 1.6). In *Eucalyptus* wood, it has been reported that the β- β' resinol linkage represents 14 %, while the β-*O*-4 aryl ether linkage represents 80 % and the β-5' phenylcoumarans only 2 % of the lignin linkages [32]. Other linkages that can be found in grass lignin are: α,β-diaryl ethers, spirodienones , cinnamyl alcohol end-groups and cinnamyl aldehyde end groups [33].

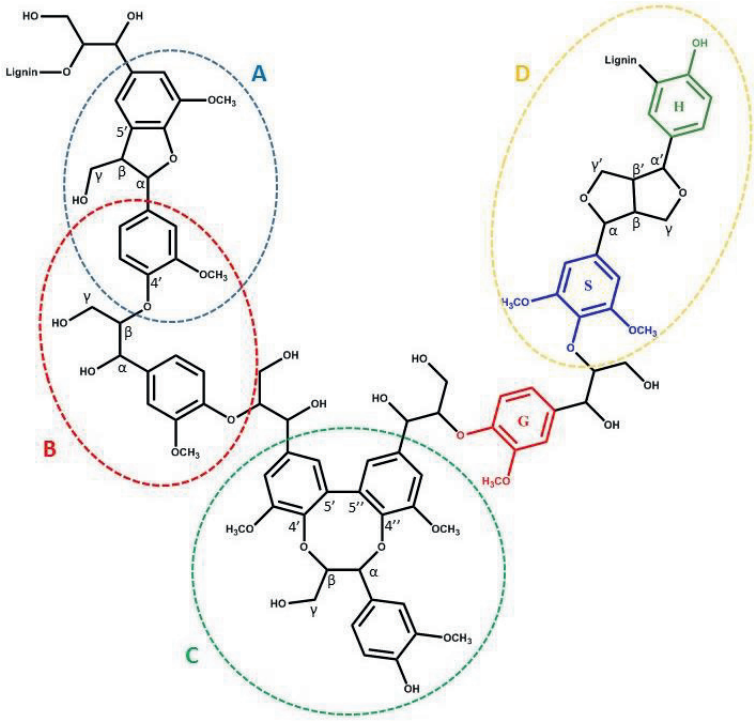


Figure 1.6: Schematic representation of lignin structures; H-unit (in green), G-unit (in red) and S-unit (in blue). The dotted circles represents the four main structures in lignin of grasses, being **(A)** β-5' phenylcoumaran, **(B)** β-O-4' aryl ether, **(C)** dibenzodioxocin and **(D)** β-β' resinol.

Furthermore, in grasses, lignin can be decorated with *p*-hydroxycinnamates (*p*-coumarates and ferulates) on the lignin side-chains: *p*-coumarates are mostly connecting to the γ–OH of the lignin side chains and are not found within lignin core, while ferulates and diferulates can be ester- or ether-

linked to lignin side chains or within the lignin core. Additionally, ferulates and diferulates can also be ester-linked to the cell wall carbohydrates crosslinking the two structures into a lignin-carbohydrate complex (LCC) [39].

In grasses, *p*-coumarates are mainly acylated towards the γ–OH of the side chains [33, 40, 41], but can also be acylated to the α-OH side chain in bamboo [40, 42]. For example, up to 10 % of the total lignin is acylated by cinnamyl aldehyde via the γ–OH of the side chains in wheat straw [33]. Additionally, in maize, up to 90 % of acylated *p*-coumarates are attached to a syringyl group [41]. Schematic *p*-coumarates linked to γ–OH and α-OH side chains are presented in Figure 1.7.

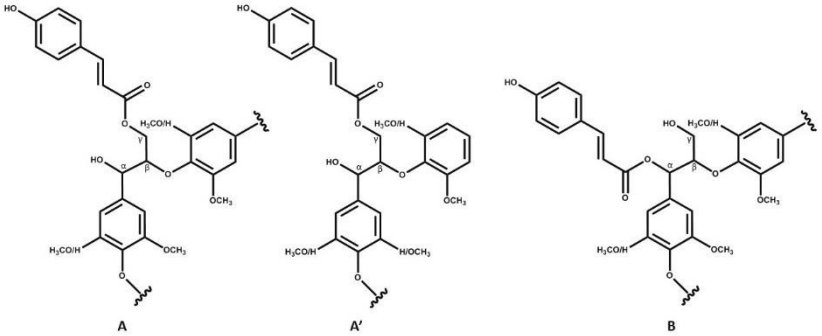


Figure 1.7: *p*-coumarates linked to γ–OH (**A** and **A'**) and α-OH (**B**) in lignin, forming end-groups of lignin.

Similar to *p*-coumarates, ferulates can also be linked to lignin side chains via ester or ether bonds, but can also be ester-linked to PCW polysaccharides (i.e. GAX). Ferulates and diferulates can be ester- or ether-linked to lignin on one side and be ester-linked to GAX on the other side forming a polysaccharides-lignin structures, named 'Lignin Carbohydrate Complexes (LCCs)' (Figure 1.8). Additionally, ferulates and diferulates can also be only ester-linked to GAX on one side or on both sides for diferulates, crosslink two GAX chains. The ferulates or diferulates are esterified to arabinosyl residues of xylan [30, 43-45]. The diferulates involved in cross-linking polysaccharides or polysaccharide and lignin follow different C-C and C-O bonds reducing carbohydrates availability and strengthening the plant cell wall. A schematic structure of cross-linked GAX-GAX and GAX-lignin chains is shown is Figure 1.8.

Diferulates involved in polysaccharides cross-linking increase cell wall rigidity and can also be incorporated within lignin. Many types of diferulates were identified in plant cell walls [30, 45, 47]. Figure 1.9 shows the different types of diferulates.

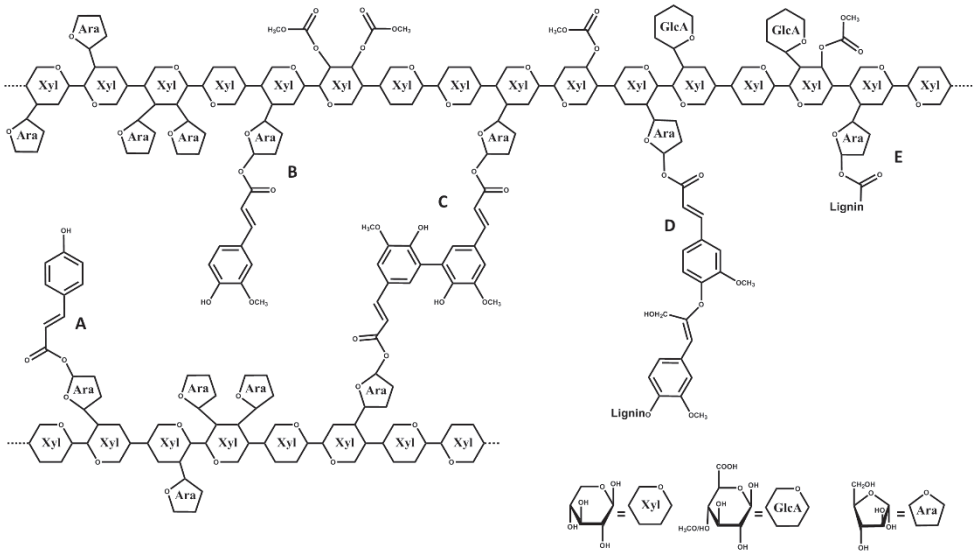


Figure 1.8: Schematic representation of *p*-coumarates, and of ferulates cross-linking glucuronoarabinoxylan in grass cell wall. Adapted from Hatfield et al. [31] and from Williamson et al. [46]. **A** Coumaric acid ester linked to an arabinosyl residue. **B** Ferulic acid ester-linked to an arabinosyl residue. **C** 5-5' diferulic acid cross-linking glucuronoarabinoxylan. **D** Ferulic acid ester-linked to an arabinosyl residue and ether linked to lignin. **E** Direct ester cross-linking between an arabinosyl residue and lignin.

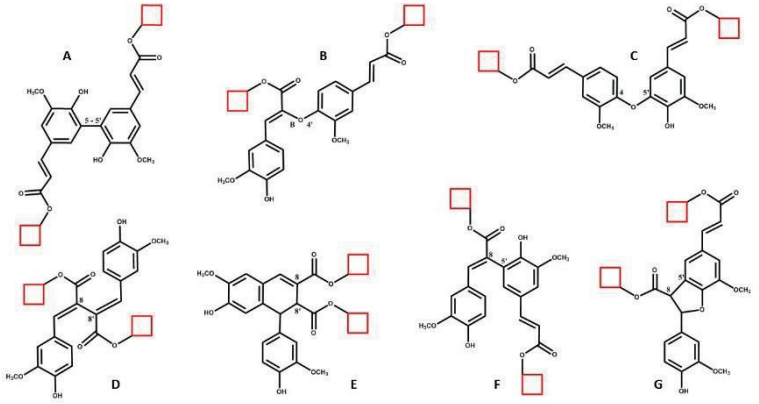


Figure 1.9: Diferulates found in grass cell. **A** 5-5' diferulate. **B** β-O-4' diferulate. **C** 4-O-5' diferulate. **D** 8-8 diferulate. **E** 8-8' diferulate. **F** 8-5' diferulate. **G** 8-5' diferulate. □ represents glucuronarabinoxylan or lignin.

1.2.2 Enzymes involved in cellulose and xylan degradation

The complexity of the plant cell wall requires a wide range of cell wall degrading enzymes to completely degrade all PCW structures. The continuous discovery and description of plant cell wall degrading enzymes, expressed by many different bacteria and fungi, resulted in the establishment of the CAZy database (CAZy.org [48]). This database classifies, in particular, carbohydrate active enzymes into families based on their amino acid sequence identity. Most enzymes known to act on cellulose or GAX are enzymes showing hydrolytic activity towards the glycosidic bonds of these polysaccharides. These hydrolases, classified in CAZy as glycoside hydrolase (GH) families, are discussed below.

1.2.2.1 Cellulose enzymatic digestibility

Cellulose is a water insoluble polysaccharide and consists of different regions having an organized crystalline structure and amorphous structure. To enzymatically degrade these structures, three different classes of cellulases can be used: endoglucanases, exoglucanases and β-1,4-glucosidases. Cellulose can be hydrolysed into cellobiose using endoglucanases (GH5, 7, 12 or 45) and exoglucanases (GH6 or 7) together (Figure 1.10) [49-52]. The resulting cellobiose can be converted into glucose by β-1,4-glucosidases (GH1 and GH3) [50], being also able to cleave small soluble oligo-saccharides (Figure 1.11). Other enzymes involved in the degradation of cellulose have recently been discribed, namely lytic polysaccharide monooxigenases (LPMOs), possessing a different catalytic mechanism compared to classical hydrolases and classified as Auxiliary Activities (AA). This novel mechanism involves oxidative cleavage of glycosidic linkages resulting in a substrate more susceptible to hydrolases [53].

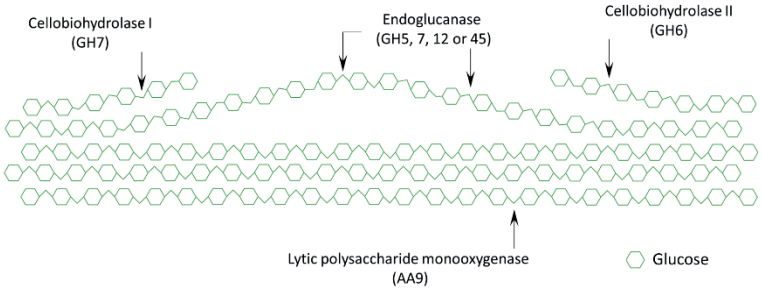


Figure 1.10: Cleavage sites of the main cellulose degrading enzymes.

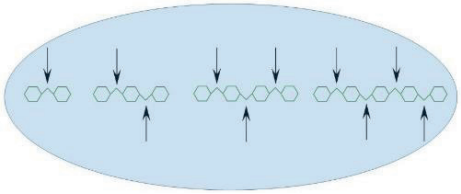


Figure 1.11: Cleavage sites (→) of the main soluble cello-oligosaccharides degrading enzymes

1.2.2.2 Xylan enzymatic digestibility

Xylan, unlike cellulose, is a highly heterogeneous structure as a whole, being substituted with glucuronic acids, arabinosyl and acetyl groups in grasses. Furthermore, cross-linkages with lignin makes its degradation by enzymes more difficult. Xylan complexity demands a variety of enzyme activities. An overview is shown in Figure 1.12.

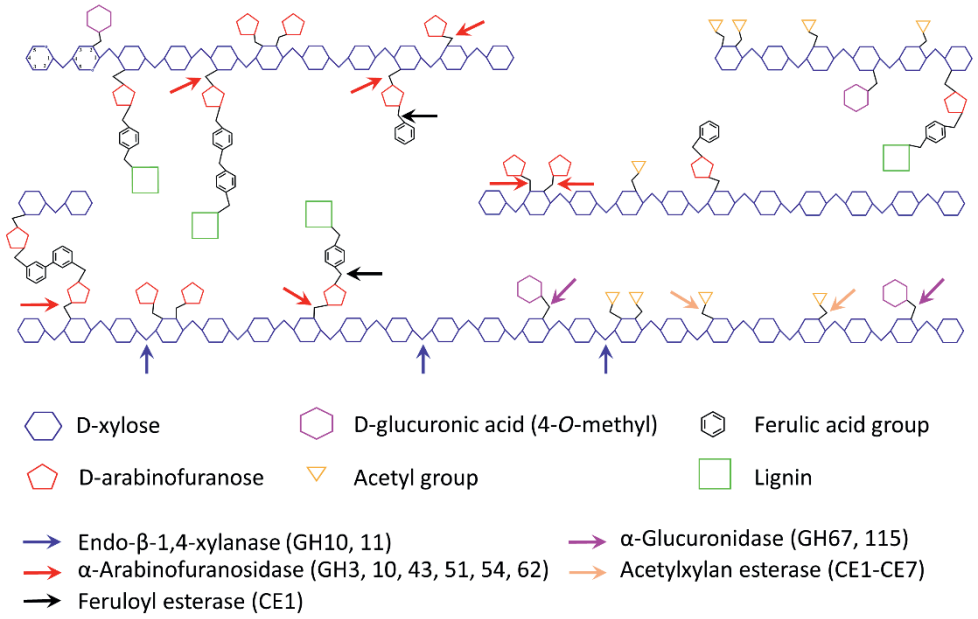


Figure 1.12: Cleavage sites of the major GAX degrading enzymes

The β-(1→4)-linked xylan backbone can be cleaved by endo-β-1,4-xylanases, mostly found in the CAZy families GH10 and 11, although other enzyme families can be involved. The activity needed will depend on the type of substitution linked to the xylan backbone. GH10 enzymes are more tolerant to degrade substituted xylan while GH11 are active towards low degree of substituted xylan. The released xylo-oligossaccharides can be further cleaved by β-xylosidases (GH3, 43 or 54) [50, 54]. The cleavage of xylan backbone side chains is performed by α-arabinofuranosidases (GH3, 10, 43, 51, 54, or 62) for arabinosyl residues and by α-glucuronidases (GH67 or 115) for (methylated-)glucuronic acid groups [54, 55]. Additionally, acetyl xylan esterases and feruloyl esterases, classified in the CAZy database in the carbohydrate esterase (CE) families, are needed to cleave acetyl groups (CE1 or CE7) and feruloyl groups (CE1), respectively [56].

1.3 Pretreatments of grasses and effects on individual structures present

Pretreatment of lignocellulosic biomass such as grass is a crucial step to achieve conversion in the subsequent enzymatic driven bioconversion step of the complex carbohydrate structures into the desired products.

Frequently described is the use of thermochemical pretreatments. These pretreatments are usually performed at temperatures ranging from 80 to 180 °C using different types of chemicals, varying from low to high pH. Other pretreatments comprise physical treatments to reduce the particle sizes, and biological treatment, such as composting (mix of chemicals and microrganisms) and fungal treatments. All pretreatments have as goal to lower the plant cell wall complexity and increase subsequent enzymatic conversion.

From the many different types of pretreatments described in literature [27, 57-59], acidic and ammonia pretreatments are used in the research of this thesis and, hence, will be discussed here.

1.3.1 Acidic pretreatment of corn stover and impact on structures

The common result of acidic thermo-pretreatment is solubilization of xylan and, hence, removal from the insoluble cellulose fibers (Figure 1.4, Tabel 1.2) enabling an increased accessibility for enzymes to the cellulose structures (Figure 1.3). The most commonly used acid during such pretreatments is dilute sulfuric acid (H_2SO_4) [12, 60, 61], while also hydrochloric acid, phosphoric acid or nitric acid have been studied [59]. In addition, weak acids such as acetic acid or formic acid have been applied for grasses pretreatments [62-64]. All acidic pretreatments studied are carried out at elevated temperatures; conditions range in temperature (120 – 240 °C), treatment times (5- 300 min), and acid concentration (1-1- w/w %) used [64-67]. The severity of such pretreatments differs and is determined by the chosen combinations for temperature, treatment time and final pH reached. Enabling comparison between pretreatment settings and resulting effects, such as enzymatic degradability, the Combined Severity Factor (CSF) has been introduced (Equation 1.1) [68].

Equation 1.1: $CSF = \log R_0 - pH = \log (t \cdot \exp[(T - 100)/14.75]) - pH$

where t is reaction time (minutes) and T is the treatment temperature (°C) and pH is the pH obtained after pretreatment.

Table 1.2: Effects, advantages and drawbacks of acidic pretreatment of grasses.

Effect of thermo-acidic pretreatment		Effects (CSF dependend)	References
Carbohydrate structures	Advantages	➢ High glucan recovery within the residues (80-95 %) ➢ Solubilisation of xylan (10-95 %) into mono- (5-25 %) or oligomeric xylose (30-50 %) ➢ High cellulose digestibility of the residues (20-90 %)	[12, 60, 61, 66, 69, 70]
	Drawbacks	➢ Xylan loss (up to 40%) ➢ Formation of carbohydrate degradation products inhibiting enzyme activity and fermentation (furfural, HMF, acetic acid)	[12, 64, 65, 67, 69, 70, 72, 73]
Lignin structures	Advantages	➢ Solubilisation of lignin and possible removal (10-30 %) in flowthrough pretreatment ➢ Decrease of β-*O*-4 lignin linkages (36 %)	[66, 76]
	Drawbacks	➢ Condensation and re-arrangement of lignin during cooling down of pretreatment ➢ Formation of lignin droplets on cellulose surface inhibiting enzyme activity ➢ No lignin removal (w/w %) in batch pretreatment	[66, 74-77]
Environment	Drawbacks	➢ Toxic, corrosive and hazardous and require reactors that are resistant to corrosion	[79]
Economics	Advantages	➢ Economically viable	[69, 78, 79]
	Drawbacks	➢ Acid must be recovered after hydrolysis ➢ Require reactors that are resistant to corrosion ➢ High salt content in hydrolysate	[78, 79]

Xylan is solubilised to different extents, depending on the CSF applied and type of grass used during the treatment, and solubilisation ranges from 10 to 50 % for CSFs < -0.5 for wheat staw [12] and CSF < 1.0 for corn stover [66, 69, 70], and from 50 to 95 % for higher CSFs for corn stover. For wheat straw, at CSF < -0.5, the solubilised xylan is present as xylo-fragments having high to low molecular weight (M_w): 50% of the xylo-fragments have a high M_w (degree of polymerization (DP)>25); 35% have a medium M_w (DP 9-25); and 15% have low M_w (DP<9). In comparison, at high CSF (>1.0) most xylo-fragments (>80% initially present) are converted further to xylose or low DP oligosaccharides [12, 71]. However, at high temperatures (>160 °C), xylose can be further converted to furfural and acetic acid, which are known inhibitors of the fermentation [12, 64, 65, 67, 69, 70, 72, 73]. Nevertheless, in corn stover, the removal of xylan further allows increased enzymatic cellulose digestibility (70-90 w/w %), in particular CSFs above 1.0 [66, 69, 70].

During thermo-acidic pretreatment lignin is also partly solubilized, however, lignin is reported to precipitate and recondensate during cooling of the pretreated biomass [66, 74-77]. This lignin

solubilization followed by recondensation, does not happen when operated in flowthrough pretreatment enabling 10-30 w/w % lignin removal at high CSF (3.0-4.5). Possibly due to rearrangements in the lignin structure during pretreatment or cooling, following acid pretreatment of switchgrass, the lignin β-*O*-4 linkages (Figure 1.6) reduced up to 36 % combined with a decrease of syringyl units (Figure 1.5) [76]. Furthermore, lignin droplets were shown to be formed at the surface of cellulose during acid pretreatment of maize stems decreasing enzymatic hydrolysis of cellulose [77].

Furthermore, it should be noted that acids are toxic, corrosive and hazardous and require reactors that are resistant to such effects. Preferably, the acid must be recovered after hydrolysis to make the process economically feasible [78, 79].

The effects of sulfuric acidic pretreatments on grasses are summarized in Table 1.2.

1.3.2 Ammonia pretreatment of wheat straw and impact on structures

Ammonia pretreatment of grasses is, like acid catalysed pretreatments, performed to increase enzymatic saccharification of the carbohydrate structures and aim to the same application as the acidic treatments. Nevertheless, the use of ammonia leads to different effects on the PCW structures (Table 1.3). The main effect of common ammonia pretreatments is lignin removal due to cleaved C-O-C bonds in the lignin (Figure 1.6). Second, lignin-carbohydrate complexes (LCCs) are disrupted due to cleaved ether and ester bonds within these LCCs (Figure 1.8 and Figure 1.9), and third, ammonia is responsible for swelling of the cellulose reducing crystallinity and increasing accessibility for enzymes [17, 80-86].

In terms of economics and environment, ammonia treatments have many practical advantages over acidic treatments, and is often used in different industries for the production of for example pharmaceuticals, chemicals and food [87]. Ammonia is non-toxic and not harmful for industrial equipment being non-corrosive for pipes and vessels, and is easily recoverable due to its high volatility. Furthermore, ammonia is used in aqueous form (25 % in water) and is less expensive compared to other chemicals used in biomass pretreatments [88]. As an example for comparison, on *alibaba.com*, 1 ton of H_2SO_4 industrial grade (purity 98%; used for treatment) can be bought for 200-400 $ while 1 ton aqueous ammonia industrial grade (25% in water, used for treatment) is 180-240 $.

The effects of various ammonia pretreatments on grasses are discussed below and a recapitulative summary is presented in Table 1.3.

1.3.2.1 Aqueous ammonia pretreatments

Ammonia recycle percolation (ARP)

In lignocellulosic biomass treatments, ammonia is used in aqueous form using ammonia recycle percolation (ARP) [81, 89]. ARP limits re-polymerization and re-precipitation of solubilized lignin, because in the process lignin is directly removed (70-85 %). Despite that (part of the) lignin is removed, ARP has as negative effect that it results in (partly) dissolved GAX (40-60 %), hence is removed with the soluble lignin.

Soaking in aqueous ammonia (SAA)

To solubilise and disrupt lignin without GAX removal, the lignocellulosic biomass is soaked in aqueous ammonia (SAA) at atmospheric pressure, at room to mild temperature (>80 °C), for hours to days [17, 82, 83, 85]. It has been shown [82, 90] that more than 80 % of hemicellulosic GAX and more than 95 % of cellulose could be retained after treatments ranging from 30 to 80 °C for 4 to 24h when corn stover was soaked in 15 to 30 % ammonia. Furthermore, corn stover treated in SAA setups has been successfully converted into bioethanol by simultaneous saccharification and co-fermentation (SSCF) [91], and shown to be effective in lignin removal (60–70%). The main drawbacks of SAA are the long treatment time and the high input of liquid (aqueous ammonia and water), which results in relatively high costs and needs of water recycling at industrial scale.

Low liquid ammonia (LLA)

To meet industrial scale requirements concerning economics and environment, low liquid ammonia (LLA) pretreatment has been developed [84]. Compared to ARP and SAA, the LLA process uses lower liquid inputs and lower energy spent, but has an extended treatment period (4-12 weeks). LAA is conducted using low amount of ammonia in a solid to liquid ratio of 1:0.2-5.0 w/w and at ambient temperature. This method resulted in 73 % ethanol yield based on initially present glucan and xylan in untreated grass w/w dm in a subsequent yeast fermentation step [84].

1.3.2.2 Anhydrous ammonia pretreatments

Low moisture anhydrous ammonia (LMAA)

Low moisture anhydrous ammonia (LMAA) pretreatments on corn stover have been investigated aiming at minimized water and ammonia used within the pretreatment process of lignocellulosic biomass. The difference with the aqueous ammonia pretreatments is that, in addition to reduced water inputs, ammonia is brought in gaseous phase instead of in the aqueous phase, limiting liquid-biomass reactions [86]. LMAA processes are constituted of three phases: 1) ammoniation at ambient temperature (gaseous ammonia being in contact with lignocellulosic biomass), 2) pretreatment at mild temperatures (40-150 °C) for 48-144 h, 3) excess ammonia recovery by evaporation. LMAA results, again, in increased enzymatic conversion, for example pretreated corn stover showed an ethanol yield of 89% based on total glucan and xylan untreated corn stover [86]. The main advantages of LMAA are: 1) the minimal use of water during the process including low water inputs for the pretreatment; 2) the absence of washing step (replaced by the evaporation phase); 3) the minimal

ammonia inputs; and 4) the beneficial outcome of ammoniated lignocellulosic biomass providing anti-microbial effects and nitrogen source for further microorganism use [86, 92].

Ammonia fiber explosion (AFEX)

AFEX pretreatment of lignocellulosic biomass is developed in the 80s using anhydrous liquid ammonia during a pressurized pretreatment with minimum water inputs where the biomass is moistured (1:0.5 < S:L< 1:1) [93]. The AFEX pretreatment conditions are commonly comprising mild temperatures (60-120 °C), short pretreatment times (5-15 min), and high pressures (250-300 psi). At the end of the pretreatment the pressure is rapidly released causing disruption of the cellulosic PCW-matrix and a decrease in cellulose crystallinity [59]. The AFEX pretreatment offers numerous other advantages regarding its effect on PCW structures, such as lignin removal, minimal removal of hemicellulosic GAX, and no washing step is needed after pretreatment [59, 94-96]. It has been reported that by using a commercial (hemi-) cellulase cocktail almost all cellulose has been converted to glucose and 80% of the xylan to xylose [96]. A subsequent ethanol formation in a fermentation step without washing, detoxification, and supplementation of nutrients for microorganisms, resulted in a yield of 60% based on total carbohydrates present at the start of the fermentation [97]. Nevertheless, AFEX has drawbacks such as that the high pressures involve high equipment costs and high energy consumption. Additionally, ethanol yields are lowered due to inhibition of the fermentation resulting from carbohydrate degradation compounds [59].

Table 1.3: Effects, advantages and drawbacks of the different ammonia pretreatment of grasses.

Effect of ammonia		Effects	Reference
Aqueous ammonia as chemical	General	➢ Cleavage of C-O-C bonds in lignin ➢ Cleavage of ether and ester bonds in LCCs ➢ Swelling reagent ➢ Removes lignin ➢ Easy to recover and reuse ➢ Non-polluting and non-corrosive chemical ➢ Cheap	[24, 59, 81, 98, 99]
ARP treatment	Advantages	➢ Lignin removal (70-85 w/w %) ➢ Glucan remained in the solid fraction (>90 w/w %) ➢ Removal of amorphous cellulose increasing crystallinity index	[81]
	Drawbacks	➢ Solubilisation and loss of hemicellulose (40-60 w/w %) ➢ Expensive reactor due to high pressure (2.3 MPa) and high temperature (170°C)	

SAA treatment	Advantages	➢ Lignin removal (40-75 w/w %) ➢ Coumaric acid removal (70 w/w %) ➢ Ferulic acid removal (70 w/w %) ➢ Glucan remained in the solid fraction (78-95 w/w %) ➢ Causes cellulose swelling ➢ Xylan remained in the solid fraction (> 80 w/w %) ➢ Ethanol maximum yield (65-83 % based on total glucan and xylan) ➢ Mild temperature (room temperature – 60 °C)	[17, 81-83, 85, 90, 91]
	Drawbacks	➢ Long treatment time (12 h – 60 days) ➢ High ammonia concentration (>20 w/w %)	
LLA treatment	Advantages	➢ Lignin removal (<20 w/w % for 0-5 w/w % NH_3; 20-55 w/w % for 5-50 w/w % NH_3) ➢ 45-85 w/w % glucan digestibility (5-50 w/w % NH_3) ➢ 15-70 w/w % xylan digestibility (5-50 w/w % NH_3) ➢ Ethanol maximum yield (75 % based on total glucan and xylan; 50 w/w % NH_3) ➢ Mild temperature (30 °C)	[84]
	Drawbacks	➢ High ammonia concentration (>5 w/w %) ➢ Long treatment time (28-84 days)	
LMAA treatment	Advantages	➢ Ethanol maximum yield (89 % based on total glucan and xylan) ➢ Moderate temperature (40-120 °C) ➢ Minimum water inputs - No washing step	[86]
	Drawbacks	➢ High cost due to use of gaseous ammonia (recompression of ammonia after treatment)	
AFEX treatment	Advantages	➢ Ethanol maximum yield (55-95 % based on total glucan and xylan) ➢ Glucan and xylan recovery (>95 w/w %) ➢ Cellulose swelling causing de-crystallisation ➢ Moderate temperature (60-120 °C)	[59, 93-96]
	Drawbacks	➢ Expensive reactor due to high pressure (250-300 psi) ➢ High cost due to use of gaseous ammonia (recompression of ammonia after treatment)	

1.3.1 Other pretreatments

1.3.1.1 Alkali pretreatment

Alkali pretreatments are usually based on sodium, potassium or calcium hydroxide catalysts [80]. Their main effects are similar to ammonia pretreatment such as solubilisation of lignin and disruption of LCCs (Figure 1.8 and Figure 1.9) increasing carbohydrate accessibility for enzymes [58, 100, 101]. Additionally, high carbohydrate recovery, based on total glucan and xylan, is reported for sodium hydroxide pretreatment (NaOH), as well as high monosaccharide yields (>78%) (based on total glucan and xylan) after subsequent enzymatic hydrolysis [102]. Alkali pretreatment is commonly performed at moderate temperatures (<140 °C). The main drawbacks are that recovery of the catalyst is expensive and alkali are corrosive for equipment [103].

1.3.1.2 Physico-chemical pretreatment

Steam explosion and hydrothermal pretreatment are common methods to pretreat lignocellulosic biomass having as main advantages that no catalyst is added [104]. The steam explosion method applies high pressure saturated steam for a short time (seconds to minutes) to lignocellulosic biomass degrading hemicellulose and lignin. Hydrothermal pretreatment are performed using only water within the reaction. Water is used as solvent and catalyst at elevated temperature (>200 °C) allowing xylan removal [59]. Nevertheless, such methods are performed at high temperature resulting in the formation of inhibitors, such as aromatic compounds, lowering further fermentation yields [105].

1.3.1.3 Physical pretreatment

Physical pretreatment can be used previously to another pretreatment in order to decrease the lignocellulosic biomass particle size. Chipping, grinding or milling is applied to increase particle size leading to higher enzymatic digestibility of the biomass [106]. However, at industrial scale it is preferred not to perform physical treatment due to energy costs.

1.3.1.4 Biological pretreatment

In biological pretreatments microorganisms are used to loosen the PCW-matrix in lignocellulose. Enabling the selective removal of lignin, leaving most of the carbohydrate fraction behind, white-rot fungi are the most widely investigated microorganisms. These fungi are well-known for their lignin-degrading machinery, mainly comprising oxidative enzymes. Those oxidative enzymes comprise laccases, lignin peroxidases, and manganese peroxidases [107, 108]. The most studied fungi producing lignin degrading enzymes are *Botrytis cinerea*, *Phanerochaete chrysosporium*, *Stropharia coronilla*, *Pleurotus ostreatus* and *Trametes versicolor* [108]. Other fungi such as brown-rot fungi are reported to degrade cellulose, leaving the lignin in the material, although modifying lignin linkages (Figure 1.6). Furthermore, soft-rot fungi have the ability to degrade lignin in angiosperm wood performing better in low lignin content biomass and containing higher humidity [108, 109].

Biological pretreatment has as advantage over the described chemical-thermo-pretreatments that only low amounts of or no energy and chemicals are required. So far, nevertheless, very long

treatment times (up to 6 or even 17 weeks) for sufficient fungal growth are reported, which might be a consideration for commercial applications [110].

1.3.1.5 Industrial scale biological pretreatment of grasses for mushroom production

Worldwide, white button mushrooms are grown on a for *Agaricus bisporus* selective and optimized substrate [111]. The substrate for fruiting of *Agaricus bisporus* is derived from wheat straw based compost mixed with horse and chicken manure [111] in 3 phases: i) biological composting at 80 °C (3-9 days), ii) further microbial conversion and conditioning (30-58 °C, 5 days), and iii) mycelium growth.

The first phase (Phase 1), biological composting at 80 °C, is essential to prepare the lignocellulosic material with respect to structure and composition for the following phases. Phase 1 is an aerobic fermentation, carried out in concrete tunnels, in which the temperature rises within 24 h to 80 °C due to microbial growth. At the same time ammonia is formed. The 80 °C and ammonia formation are maintained for 3 to 5 days. Both temperature and ammonia causes softening of the lignocellulose and is associated with the initial presence of mesophilic microbiota followed by their replacement by thermophilic microbiota [111]. It has been shown that the conditions in Phase 1 result in an increased enzymatic degradability of the carbohydrate fraction. Hence, this first phase (Phase 1) is actually a commercial biological pretreatment of the lignocellulosic biomass.

1.4 Aim and outline of the thesis

The background and aim of this project is described in **Chapter 1**. The cell wall architecture and structures are thoroughly described, which helps to understand the various structural effects of the pretreatments performed in previous and in the current research. Based on the information provided in Chapter I, two hypotheses for this PhD research are formulated: 1) mild acidic versus ammonia pretreatments show different effects on grass xylan and lignin structures and their interconnections, 2) the more xylan, lignin or LCCs disruption occurs during pretreatment of grasses, the higher the enzymatic degradability of residual material.

Chapter 2 describes the relationship between the chemical composition of maize stems and its *in vitro* degradation in the rumen of ruminants analysed as gas production. This chapter describes, moreover, the differences in effects on gas production of lignin content and composition found within different internodes from two maize cultivars. In **Chapter 3**, corn stover was treated with acetic, sulfuric acid, or a combination of both, to assess lignin composition and its consecutive modification to the treatment. The performances of the treatments were also evaluated for carbohydrate degradability of the residues. **Chapter 4** describes the modification of the composting process in absence of gypsum to evaluate its performance on the carbohydrate degradability of the residues of wheat straw for further mushroom (*Agaricus bisporus*) production. Additionally, lignin composition and content were determined to link to potential effect of the ammonia bio-process on lignin modifications and the

consequences on carbohydrate degradation. The effects of ammonia treatments on wheat straw were investigated in **Chapter 5.** Ammonia treatments were performed on wheat straw according to a statistical design of experiments (Taguchi design) to independently evaluate the effects of three parameters (temperature (°C), treatment time (hours) and the Solid:Liquid ratio (S:L)) on cellulose and xylan degradability of the residues, on carbohydrate and lignin composition, and on treatment mass balance. Finally, the relevance of this research and implication for future applications is discussed in **Chapter 6**.

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CHAPTER 2

Lignin composition is more important than content for maize stem cell wall degradation

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ABSTRACT

The relationship between the chemical and molecular properties, in particular the lignin (ADL) content and composition expressed as the ratio between syringyl and guaiacyl compounds (S:G ratio), of maize stems and *in vitro* gas production was studied in order to determine which is more important in the degradability of maize stem cell walls in the rumen of ruminants. Different internodes from two contrasting maize cultivars (Ambrosini and Aastar) were harvested during the growing season. The ADL content decreased with greater internode number within the stem, whereas the ADL content fluctuated during the season for both cultivars. The S:G ratio was lower in younger tissue (greater internode number or earlier harvest date) in both cultivars. For the gas produced between 3 and 20 hours, representing the fermentation of cell walls in rumen fluid, a stronger correlation (R^2=0.80) was found with the S:G ratio than with the ADL content (R^2=0.68). The relationship between ADL content or S:G ratio and 72-h gas production, representing total organic matter degradation, was weaker than that with gas produced between 3 and 20 hours. The S:G ratio plays a more dominant role than ADL content in maize stem cell wall degradation.

2.1 INTRODUCTION

Forage maize is an important forage for high yielding dairy cows in most areas of the world. A large part of the metabolisable energy in forage maize is derived from starch in the kernel. The fibre-rich remainder of the plant (the stover) also contains a significant amount of metabolisable energy and nutrients, despite its lower degradability. However, research over the past decades has mainly focused on improving yield and proportion of starch in forage maize in order to obtain a greater nutritional value and accommodate more to the nutritional needs of cattle [1]. This focus did not lead to greater and / or more rapid degradation of the non-starch fraction of the plant (mainly cell walls) [1]. Research into the causes of differences in degradability of the cell walls may result in better and faster degradability of cell walls of maize stems. Increased cell wall degradation of forage maize by harvesting at earlier stages of maturity will decrease starch content and increase enteric methane production [2].However, forage maize with a greater cell wall degradation at a similar growth stage will have a greater nutritional value, so lower costs, lower environmental emissions and a better performance of the animals.

The nutritive value of maize stover can vary widely and be affected by genotype, climate, maturity of the plant etc [3]. The relationship between the degradability of maize stem cell walls and the different properties of the plant is well documented. For example, there is a strong negative correlation between cell wall maturation and degradability, which generally is ascribed to the increasing amount of acid detergent lignin (ADL) compared with other cell wall compounds [4]. However, ADL content cannot fully explain the variation in cell wall degradability, with evidence that the ADL content may differ between cultivars without differences in cell wall degradability [5].

Lignin is an organic polymer made up of phenyl propane units organised in a three-dimensional structure. The precursors of these building blocks, coniferyl, sinapyl and *p*-coumaryl alcohols can be transformed into guaiacyl (G unit), syringyl (S unit) and *p*-hydroxyphenyl (H unit) units, respectively, through a complex dehydrogenative polymerisation process.[6]. Lignin content and composition change during plant maturation when more lignified primary and secondary cell walls of sclerenchyma and vascular tissues are developed [7]. Studies on forages of different physiological maturation indicate a shift towards a more S unit-type lignin with advancing maturity in some species [8-10]. As mentioned above, maturation with an increasing content of ADL reduces forage degradability. In view of the complexity of factors involved in cell wall development, insufficient information exists on which factors (ADL content or composition) precisely determine the degradability of maize stem cell walls.

The present study focused on the relationship between the chemical and molecular properties of maize stem cell walls and *in vitro* rumen fermentation, measured by an automated gas production technique. The goal was to provide further insights into the background of differences in the composition of cell walls among different samples, at a chemical and molecular level, and the relationship with degradability.

2.2 MATERIALS AND METHODS

2.2.1 Maize production and management

Seeds of the maize cultivars Ambrosini and Aastar (provided by Limagrain, Rilland, the Netherlands) were sown in the first week of May 2012 at the experimental fields of Unifarm in Wageningen, the Netherlands. The sowing density was 10 plants m^{-2}, with 13.3 cm between plants and 75 cm between the rows. The fields had a sandy soil with pH 5.5, 21 g kg^{-1} organic matter (OM) and adequate levels of macro- and micronutrients. The fields were fertilized with 40 kg ha^{-1} of cow manure, 150 kg ha^{-1} of calcium-ammonium-nitrate (CAN, 270 g kg^{-1} N), 160 kg ha^{-1} of potassium (K60, 600 g kg^{-1} K_2O) and 100 kg ha^{-1} phosphate (triple superphosphate, 450 g kg^{-1} P_2O_5).

2.2.2 Sample preparation

During 2012, the internodes were harvested excluding the lower and upper nodes. Only internode 7 (counted from the ground) was harvested on 28 and 14 days before anthesis (d -28 and d -14, respectively) and on 14, 28, 42 and 70 days after anthesis (d 14, d 28, d 42 and d 70, respectively). As the major changes occurred after anthesis and levelled off afterwards, the interval between the last two sampling dates was 4 weeks instead of 2 weeks. At anthesis (14 August), whole plants were harvested and internode 5, 7, 9, 11, 13 and 15 were collected. In all cases, internodes were collected from 12 plants (4 plots, 3 plants per plot) and randomly separated to be duplicated. All the internodes were stored at -20 °C directly after harvesting.

All the internodes were oven-dried at 70°C and ground to pass a 1 mm sieve using a Peppink 100 AN cross beater mill (Peppink, Deventer, The Netherlands) before chemical analysis, pyrolysis gas chromatography / mass spectrometry (Py-GC-MS) analysis, and *in vitro* fermentation.

2.2.3 Chemical analysis

Dry matter (DM) was determined gravimetrically after 4 hours at 103 °C and ash after 3 hours at 550 °C. Neutral detergent fibre (NDF) was determined by the method of Van Soest et al. [11] using a heat-resistant amylase and expressed exclusive of residual ash. Acid detergent fibre (ADF) and ADL were determined by the method of Van Soest and McQueen [12] and also expressed exclusive of residual ash. The difference between NDF and ADL was defined as potentially rumen degradable fibre (pRDF). Nitrogen (N) was determined by the Kjeldahl method, and crude protein (CP) was calculated as N × 6.25.

2.2.4 Py-GC-MS analysis

Pyrolysis of 100 µg, weighed on a Mettler-Toledo XP6 microbalance (Mettler-Toledo, Columbus, US) 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 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}). Species coming from lignin units and species coming from p-coumaric and ferulic acids were distinguished assuming more than 80-85 % of p-coumaric and ferulic acids was considered part of lignin and not xylan, also based on the ratios of esterified p-coumaric and ferulic acids to xylan reported by Van Dongen et al. [13]. The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and with those reported in literature [14]. The relative abundance of each identified compound was calculated based on the total relative area obtained from the pyrogram according to Jurak et al. [15]. All the compounds identified and their spectra were checked manually. As the method is time consuming, not all samples were analysed, but enough to see differences and trends. One sample from the duplicated samples of internodes 5, 9, 13 and 15 of both maize cultivars harvested at anthesis, and one sample from duplicated samples of internode 7 of both maize cultivars harvested on d -28, d 0, d 28 and d 70 were analysed using Py-GC-MS. The syringyl:guaiacyl (S:G) ratio was calculated by dividing the sum of the abundance of the syringyl compounds divided by the sum of the abundance of the guaiacyl compounds.

2.2.5 *In vitro* gas production

The fermentation kinetics were determined with the *in vitro* gas production technique.[16] Incubation of 0.5 g of OM was performed in 60 ml buffered rumen fluid (1 part of rumen fluid and 2 parts of buffer) in 250 ml bottles at 39 °C in a shaking water bath. Each sample was run in one bottle each time and each sample was run twice. Gas production was recorded for 72 h with an automated system [16]. Results were corrected for blank gas productions, i.e. buffered rumen fluid but without a substrate.

Rumen fluid was obtained from 2 non-lactating cows that were fed twice a day with hay, and with 1 kg of concentrate in the morning. Rumen fluid was collected 2 h after the morning feeding and was pooled, stored in a warm insulated flask filled with CO_2, filtered through cheesecloth, and mixed with an anaerobic buffer/mineral solution as described by Cone et al. [16]. All processing of rumen fluid took place under continuous flushing with CO_2.

The three phasic mathematical model for gas production as described by Cone et al. [16] and Groot et al. [17] was used to determine OM and cell wall degradation. The gas production curves are divided into three different sub-curves, each with an asymptote (A), a half-time value (B) and a shape

parameter (C) [17]. The asymptotes of sub-curve 1 (A1) correspond to the gas production between 0 and 3 h incubation and are caused by fermentation of the water-soluble components and that of sub-curve 2 (A2) to the gas production between 3 and 20 h incubation caused by fermentation of the non-soluble components [17]. The half-time value B is the incubation time (h) needed to reach half of the maximum gas production, representing a measure for the rate of degradation of the total OM.

2.2.6 Statistical analysis

Data were analysed using the GLM procedure of SAS/STAT® 9.3 (Statistical Analysis System, Cary, NC, USA) and the model included maize cultivar (Ambrosini and Aastar), maturity (different harvest dates or different internodes), and cultivar × maturity as fixed effects. Because Py-GC-MS analyses were performed on one sample of selected internodes and harvest dates only, no interaction effect was included in the model when analysing S:G ratio data. Differences among main effects were analysed using Tukey-Kramer's multiple comparison procedure in the LSMEANS statement of SAS. Significant effects were declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

2.3 RESULTS

2.3.1 Chemical composition of internode 7 at different harvest dates

The levels of ash, NDF, ADF, ADL, the ratio of lignin to pRDF (ADL:pRDF ratio) and the S:G ratio in internode 7, harvested at different dates during 2012, are shown in Table 2.1. Cultivar, harvest date and their interactions showed significant effects on the concentrations of ash, NDF, ADF and ADL and on the ADL:pRDF ratio. There was no clear trend for a greater level of NDF, ADF and ADL in more mature tissue. The ADL:pRDF ratio increased to the highest value on d 28 in Ambrosini and d 14 in Aastar and then decreased in both cultivars. The concentration of NDF, ADF, ADL and ADL:pRDF ratio were greater in Ambrosini than in Aastar except for the internode harvested on d 14. Cultivar and harvest date also showed significant effects on the S:G ratio, with a greater S:G ratio observed in Ambrosini than in Aastar and the lowest value of the S:G ratio observed in the youngest internode harvested on d -28 for both cultivars.

2.3.2 Chemical composition of several internodes harvested at anthesis

The levels of ash, NDF, ADF, ADL, ADL:pRDF ratio and S:G ratio in successive internodes within the stem harvested at anthesis for the two cultivars are shown in Table 2.2. Cultivar and internode significantly affected all these parameters and a significant cultivar × internode effect for ash, NDF, ADF and ADL:pRDF ratio was found. Again, no clear trend for a greater level of NDF in both cultivars and ADF in Ambrosini in older internodes (lower internode number) was observed.

Table 2.1: Ash content (g kg-1 DM±sd), NDF, ADF and ADL content (g kg-1 OM±sd), ADL:pRDF ratio, S:G ratio and *in vitro* gas production (ml g-1 OM±sd) parameters of fermentation of internode 7 of two maize cultivars (Ambrosini and Aastar) harvested at different dates (days before anthesis were expressed with "-") in 2012

Cultivar	Harvest date	Ash	NDF	ADF	ADL	ADL:pRDF ratio	S:G ratio	A1	A2	GP72	B
Ambrosini	d -28	71±0.9^{a}	715±6.4^{c*}	487±5.3^{c*}	51±0.1^{d*}	7.73±0.06^{b*}	0.62	43±1.2^{cd}	117±2.9^{a*}	249±6.3^{bc*}	12.9±0.5^{d}
	d -14	47±0.4^{d}	739±2.5^{b*}	515±8.2^{b}	69±4.4^{bc*}	10.25±0.80^{ab*}	ND	39±1.1^{d}	91±3.8^{cd*}	233±8.0^{cd*}	16.8±1.1^{b}
	Anthesis	40±0.2^{e*}	598±1.1^{d*}	421±0.6^{d*}	59±0.0^{cd*}	10.92±0.01^{a*}	0.72	66±3.4^{a*}	95±3.1^{c*}	258±12.4^{ab}	11.8±0.4^{de}
	d 14	41±0.1^{e*}	589±2.6^{d*}	414±0.2^{d*}	50±4.1^{d}	9.36±0.92^{ab}	ND	72±3.9^{a*}	106±2.1^{b*}	273±5.9^{a*}	10.4±0.3^{e*}
	d 28	51±0.6^{c*}	719±8.2^{c*}	515±9.1^{b*}	78±7.9^{ab*}	12.27±1.63^{a}	0.83	47±4.7^{bc*}	95±5.0^{c*}	245±11.9^{bcd*}	14.9±0.7^{c*}
	d 42	58±0.3^{b*}	796±1.4^{a*}	565±0.3^{a*}	86±1.7^{a*}	12.12±0.30^{a*}	ND	30±3.4^{e*}	85±5.6^{d*}	227±11.4^{d*}	19.8±0.5^{a*}
	d 70	58±0.0^{b*}	705±3.5^{c*}	489±1.9^{c*}	65±1.8^{bcd*}	10.16±0.28^{ab}	0.79	54±2.6^{b*}	86±3.7^{cd*}	246±5.0^{bcd*}	15.8±0.4^{bc*}
Aastar	d -28	69±0.5^{b}	672±3.7^{bc}	448±0.2^{b}	34±0.6^{c}	5.34±0.08^{c}	0.49	41±0.7^{e}	131±4.0^{a}	264±5.0^{bd}	13.4±0.1^{b}
	d -14	48±0.7^{d}	715±2.2^{a}	490±0.9^{a}	49±0.3^{b}	7.42±0.02^{bc}	ND	38±3.1^{e}	118±3.5^{ac}	267±8.9^{cde}	16.3±0.5^{a}
	Anthesis	41±0.0^{f}	554±0.0^{e}	376±2.8^{d}	42^{bc}	8.25^{ab}	0.61	77±3.6^{ab}	109±10.7^{cd}	284±23.2^{abc}	11.2±0.6^{cd}
	d 14	50±0.8^{c}	678±2.6^{b}	480±1.3^{a}	66±4.6^{a}	10.72±0.83^{a}	ND	54±5.0^{d}	102±2.5^{d}	258±7.7^{cd}	13.2±0.7^{b}
	d 28	44±0.4^{e}	552±7.9^{e}	375±1.3^{d}	42±0.7^{bc}	8.27±0.28^{b}	0.65	83±5.1^{a}	124±4.1^{ab}	310±11.6^{a}	10.4±0.4^{d}
	d 42	50±0.1^{c}	586±3.0^{d}	404±3.1^{c}	46±1.4^{bc}	8.44±0.24^{ab}	ND	73±1.7^{bc}	113±6.8^{bcd}	296±20.7^{abe}	12.6±0.9^{bc}
	d 70	71±0.2^{a}	660±4.1^{c}	441±10.4^{b}	44±5.3^{bc}	7.14±1.05^{bc}	0.65	65±3.4^{c}	114±4.3^{bcd}	282±7.2^{ad}	12.9±0.8^{b}
Significance (*P*)	***Cultivar (C)***	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	<0.001	<0.001
	Date (D)	<0.001	<0.001	<0.001	<0.001	<0.001	0.009	<0.001	<0.001	0.001	<0.001
	C × D	<0.001	<0.001	<0.001	<0.001	0.002	-	<0.001	<0.001	<0.001	<0.001

DM, dry matter; sd, standard deviation; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; OM, organic matter; ADL:pRDF ratio, (the ratio of ADL and potentially rumen degradable fibre (pRDF; calculated as the difference between NDF and ADL))×100; S:G ratio, the ratio of syringyl and guaiacyl compounds; A1 and A2, gas production within 3 h and between 3 and 20 h; B, time.

However, there was a clear trend for a lower concentration of ADL and a lower ADL:pRDF ratio and S:G ratio in younger tissues (higher internodes) for both cultivars. Aastar had significantly lower concentrations of NDF, ADF and ADL and a lower ADL:pRDF ratio and S:G ratio than Ambrosini.

2.3.3 *In vitro* fermentation of internode 7 at different harvest dates

The results of the kinetics of gas production of internode 7 harvested at different dates of the year are shown in Table 2.1. Cultivar, harvest date and the interactions showed significantly effects on A1, A2, total gas production after 72 h (GP72) and B. For A1, B and GP72 for both Ambrosini and Aastar, there was no clear increase or decrease at later harvest dates. The earliest harvested internodes had the highest A2, and there was a tendency for lower gas production as the internodes were harvested later.

2.3.4 *In vitro* fermentation of several different internodes harvested at anthesis

The results of the kinetics of gas production of internode 5, 7, 9, 11, 13 and 15 harvested at the same day for the two cultivars are shown in Table 2.2. Cultivar and internode showed significant effects on A1, A2, GP72 and B. The interactions between cultivar and internode only influenced A1 significantly. There was no clear trend for A1. A greater A2 was observed when the internode was younger (higher internode) in the plant for Aastar, while the lowest internode (internode 5) showed the lowest A2 and GP72 for both cultivars. The half-time value B tended to decrease from lower to higher internodes.

2.3.5 Relationship between ADL content or S:G ratio and *in vitro* fermentation

The relationships between ADL content and A2 and GP72 are shown in Figure 2.1. The relationship between ADL content and A2 (indicating cell wall degradation) was stronger (R^2=0.68) than the relationship between ADL content and GP72 (indicating OM degradation) (R^2=0.54).

The relationships between S:G ratio and A2 and GP72 are shown in Figure 2.2. The relationship between S:G ratio and A2 was stronger (R^2=0.80) than the relationship between S:G ratio and GP72 (R^2= 0.45). Upon comparison with the relationships between ADL content and A2, the results indicate that the S:G ratio plays a more dominant role in cell wall degradation than the ADL content; however, in comparison to ADL content, the S:G ratio appeared not to have a strong relationship with OM degradation.

Table 2.2: Ash content (g kg^{-1} DM±sd), NDF, ADF and ADL content (g kg^{-1} OM±sd), ADL:pRDF ratio, S:G ratio and *in vitro* gas production (ml g^{-1} OM±sd) parameters of fermentation of internodes (5, 7, 9, 11, 13 and 15) of two maize cultivars (Ambrosini and Aastar) harvested at anthesis in 2012.

Cultivar	Internode	Ash	NDF	ADF	ADL	ADL:pRDF ratio	S:G ratio	A1	A2	GP72	B
Ambrosini	5	76±0.1^{a*}	739±1.4^{a*}	521±0.8^{a*}	78±0.5^{a*}	11.88±0.06^{a*}	0.90	39±8.4^{b*}	97±6.3^{c*}	245±21.3^{b*}	17.1±1.4^{a*}
	7	44±0.4^{b}	646±0.6^{e*}	445±2.3^{b*}	63±2.2^{b*}	10.73±0.42^{b*}	ND	58±10.8^{ab}	111±4.6^{bc*}	282±16.0^{ab*}	14.6±1.2^{ab*}
	9	32±0.7^{e*}	686±2.5^{b*}	455±1.3^{b*}	60±0.5^{bc*}	9.58±0.13^{c*}	0.66	50±8.5^{ab*}	112±5.4^{bc*}	266±15.5^{ab*}	14.2±1.6^{ab*}
	11	35±0.6^{d}	664±0.7^{d*}	418±4.4^{c*}	57±0.0^{cd*}	9.41±0.01^{cd*}	ND	60±2.5^{a}	139±17.0^{a}	313±44.0^{a}	12.5±1.9^{b}
	13	37±0.1^{c}	671±0.0^{c*}	403±1.4^{d*}	51±1.0^{e*}	8.25±0.17^{e*}	0.47	60±8.2^{a}	132±9.9^{a}	299±26.4^{ab}	12.2±1.6^{b}
	15	36±0.4^{cd*}	674±2.1^{c}	407±4.2^{cd*}	53±1.6^{de*}	8.53±0.27^{de*}	0.53	51±3.0^{ab}	123±2.7^{ab*}	274±9.1^{ab}	13.0±0.1^{ab}
Aastar	5	60±0.3^{a}	599±1.3^{b}	421±4.0^{a}	57±0.1^{a}	10.51±0.01^{a}	0.73	60±3.3^{bc}	120±10.2^{b}	291±18.2	13.6±0.7^{a}
	7	45±0.1^{b}	562±2.8^{de}	388±0.1^{b}	43±1.2^{b}	8.38±0.20^{b}	ND	71±4.6^{ab}	133±6.7^{ab}	323±15.0	12.7±0.4^{ab}
	9	36±0.1^{e}	555±2.3^{e}	359±1.8^{c}	38±1.6^{c}	7.32±0.30^{c}	0.53	74±4.4^{a}	137±2.7^{ab}	327±8.0	11.7±0.4^{b}
	11	34±0.0^{f}	577±2.4^{cd}	356±2.3^{c}	38±0.0^{c}	6.89±0.10^{cd}	ND	67±5.9^{ac}	139±4.7^{ab}	316±16.6	11.7±0.3^{b}
	13	38±0.3^{d}	589±10.8^{bc}	350±7.1^{c}	33±0.8^{cd}	6.01±0.27^{de}	0.45	62±7.1^{ac}	142±10.4^{a}	310±25.8	11.5±0.9^{b}
	15	41±0.3^{c}	659±5.9^{a}	375±1.1^{b}	32±2.2^{d}	5.11±0.42^{e}	0.39	55±6.4^{c}	146±8.0^{a}	306±27.4	11.8±0.9^{b}
Significance (*P*)	**Cultivar (C)**	<0.001	<0.001	<0.001	<0.001	<0.001	0.039	<0.001	<0.001	<0.001	<0.001
	Internode (I)	<0.001	<0.001	<0.001	<0.001	<0.001	0.012	0.001	<0.001	0.010	<0.001
	C × I	<0.001	<0.001	<0.001	0.169	0.002	-	0.018	0.081	0.164	0.191

DM, dry matter; sd, standard deviation; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; OM, organic matter; ADL:pRDF ratio, (the ratio of ADL and potentially rumen degradable fibre (pRDF; calculated as the difference between NDF and ADL))×100; S:G ratio, the ratio of syringyl and guaiacyl compounds; A1 and A2, gas production within 3 h and between 3 and 20 h; B, time needed to reach half of GP72; GP72, gas production within 72 h. Values with different superscript letters (a, b, c, d, e) within cultivar are significantly different. Values with * are significantly ($P \leq 0.05$) different from corresponding harvest dates of Aastar.

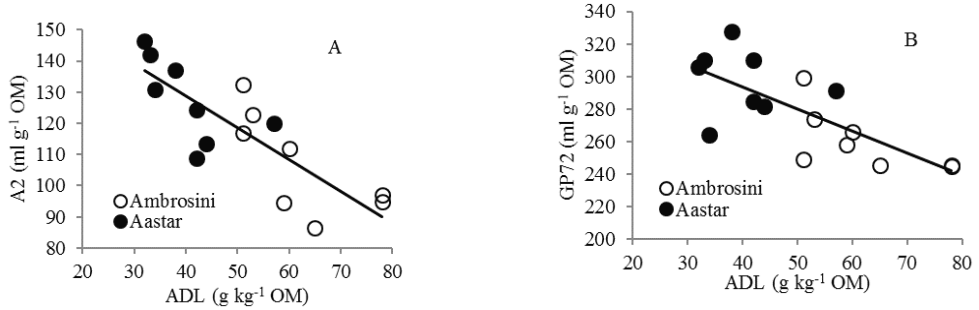

Figure 2.1: A: relationship between ADL content and *in vitro* gas production between 3 and 20 h incubation (parameter A2) of the internodes 5, 9, 13 and 15 harvested at anthesis and internode 7 harvested on 28 days before anthesis, at anthesis, and 28 and 70 days after anthesis from two maize cultivars (Ambrosini and Aastar): A2 = -1.01 ±0.19 × ADL + 169 ±9.9, (estimate ± SE), root mean square error = 10.54, R2=0.68; B: relationship between ADL content and *in vitro* gas production within 72 h incubation (GP72) of the internodes 5, 9, 13 and 15 harvested at anthesis and internode 7 harvested on 28 days before anthesis, at anthesis, and 28 and 70 days after anthesis from two maize cultivars (Ambrosini and Aastar): GP72 = -1.35 ±0.33 × ADL + 348 ±17.6, (estimate ± SE), root mean square error = 18.75, R2=0.54

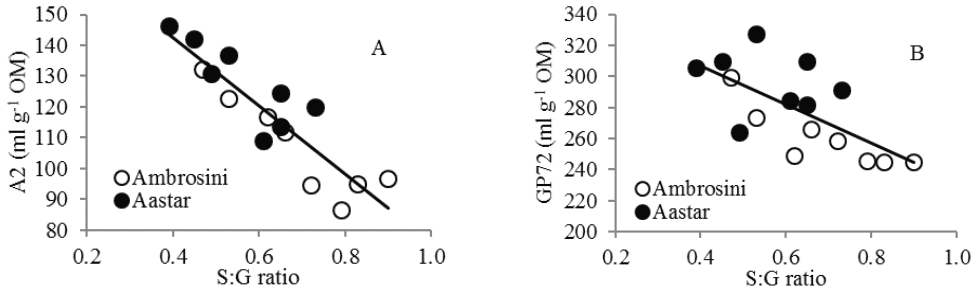

Figure 2.2: A: relationship between S:G ratio and *in vitro* gas production between 3 and 20 h incubation (parameter A2) of the internodes 5, 9, 13 and 15 harvested at anthesis and internode 7 harvested on 28 days before anthesis, at anthesis, and 28 and 70 days after anthesis from two maize cultivars (Ambrosini and Aastar), A2 = -110.99 ±14.85 × S:G ratio + 187 ±9.5, (estimate ± SE), root mean square error = 8.32, R2=0.80; B: relationship between S:G ratio and *in vitro* gas production within 72 h incubation (GP72) of the internodes 5, 9, 13 and 15 harvested at anthesis and internode 7 harvested on 28 days before anthesis, at anthesis, and 28 and 70 days after anthesis from two maize cultivars (Ambrosini and Aastar), GP72 = -124.27 ±36.67 × S:G ratio + 356 ±23.5, (estimate ± SE), root mean square error = 20.54, R2=0.45

2.4 DISCUSSION

2.4.1 Effect of maturity on chemical composition

The developing internodes of maize stem provide the opportunity to study how cell wall composition changes during maturation [18-20]. Different maturity stages can be represented by successive

internodes of the maize stem harvested at the same time [20] or by selecting a given internode and harvesting it at different dates [18] Within the stem, cell walls of the upper part (greater internode number) are physiologically younger and less lignified than those of the lower part. In the current study, internode 5, 7, 9, 11, 13 and 15 sampled at the same day and internode 7 sampled at different days during the growth were used to investigate how maturity influenced the chemical composition of cell walls, as well as cell wall degradation.

Cone and Engels [5] reported that NDF and ADF increased with increasing maturity, which was also demonstrated by Tolera and Sundstøl [21] However, NDF and ADF did not have the clear increasing trend with maturity in the present study which is in line with the results reported by Boon et al. [3] and Cone et al. [22]. In contrast with expected changes in the ADL content of the stems [5, 21] in the present study the ADL content did not increase when harvest date was used as an indicator of maturity. When different internodes sampled at anthesis were used to investigate the changes in the ADL content, as expected the ADL content increased from the upper internodes (internode 15, average 43 g kg^{-1} OM) to the lower internodes (internode 5, average 68 g kg^{-1} OM). The ADL:pRDF ratio, as an indication of potentially degradable OM, is expected to be greater in older internodes, which is demonstrated by different internodes harvested at the same date. However, when internode 7 was harvested at different days, an increasing ADL:pRDF ratio in early growth was observed followed by a trend to decrease in later growth.

The S:G ratio increased during the maturation, especially from the top to the bottom of the stem. This observation is in line with data from other studies [6, 8, 23] Deposition of G units continues throughout the lignification of cell walls while large amounts of S units are deposited mainly in the middle and late stages of lignification [6, 24]. Therefore, the composition of lignin shifts from lignin with primarily G units to lignin with mixed S-G units during cell wall development and the S:G ratio increases.

2.4.2 Effect of maturity on cell wall degradation

In this study, the *in vitro* gas production technique was used to assess the cell wall degradation. In the case of maize stems, cell wall degradability is represented by A2, being the gas production caused by fermentation of the non-soluble fraction while OM degradability is represented by GP72 (the gas produced after 72 h fermentation) as indicated by Cone et al. [16, 25] and Groot et al. [17].

The decline in cell wall degradability during maturation is well documented and it is generally accepted that the formation of more lignified plant cell walls during maturation is the major reason leading to lower cell wall degradation [24]. Jung and Casler [26] found that the degradability of cell walls in maize stems after both 24- and 96- h *in vitro* incubations with rumen fluid decreased as the stems were harvested later. Our results show that there was a clear trend that the cell wall degradability decreased only from the younger internode to the older internode within the stem. For internode 7,

sampled at different dates, there was rapid drop in cell wall degradability up to August 14, to remain fairly constant afterwards, which is in line with Cone and Engels [5], who reported that the cell wall degradability decreased significantly up to 15 August and remained fairly constant afterwards when the date of sowing was 25 April which is close to the date of sowing in our study.

2.4.3 Factors related to cell wall degradability

Lignin content of forages has long been reported to be negatively correlated with cell wall degradability [27] and this relationship which was also observed here is consistent with previous studies [28-32]. Boon et al. [3] found that the lignin content in the internode 7 of corn stem was significantly correlated with cell wall degradation assessed by *in vitro* gas production [29] The negative correlation between lignin content and cell wall degradation was also reported in both grass [28, 31, 32] and legumes [30, 31]. Even though these studies demonstrated that lignin content is an important factor that limits cell wall degradation in ruminants, the strength of the relationship varied among studies due to the methods that were used to evaluate cell wall degradation. However, comparing different corn genotypes, Cone and Engels [5] suggested that ADL content is not always a good indicator of degree of cell wall degradability, which is supported by Sommerfeldt et al., [33] who observed that degradability of cell walls was greater for bm3 maize than for a normal variety although no differences in ADL content were observed. However, both a reduced ADL content and a lower S:G ratio were found in bm3 maize in other studies [34, 35]. The relationship between ADL content or ADL composition and cell wall degradability is not fully understood even though this relationship has been investigated for many years. Compared with the S:G ratio, the ADL content has a weaker relationship with cell wall degradability (Panel A, Figures 1 and 2). This result is in accordance with what Vailhe et al. [36] and Sewalt et al. [37] observed for tobacco. Guo et al. [38] found that a greater S:G ratio in alfalfa resulted in a greater cell wall digestibility which is opposite to our results. The significant correlation between the S:G ratio and the cell wall degradability in maize stems was not found by Jung and Buxton [39] and Grabber et al. [40]. It should be noticed that only a single maturity stage was used in the research conducted by Jung and Buxton [39] and by Grabber et al., [40] which may be the reason of the discrepancy between the results of these studies. When forages were harvested across maturity stages, strong negative correlations between ADL content and cell wall degradability were shown, [32, 41] while only weak negative correlations existed between ADL content and cell wall degradability if forages of a single maturity stage were examined [39, 42].

The reason why a lower S:G ratio is related with greater degradability is difficult to explain. Jung and Deetz [24] hypothesised that syringyl-rich lignin would be more inhibitory to cell wall degradation, as syringyl monolignols have fewer potential polymerisation sites and should have a more linear polymer structure which could protect a larger area of the secondary cell walls from degradation than the more branched guaiacyl-rich lignin. However, Filley et al.[43] emphasized that the G-type lignin is more resistant to chemical and biological breakdown than the S-type lignin. In all likelihood, the influence of the S:G ratio on cell wall degradability should not be explained by the nature of the S or G unit itself

in view of the complex linkages between S or G units with other molecular structures that may be related to a reduction in degradability. One possible explanation is that during the formation of S units, mostly *p*-coumaric acid is connected with the S unit through ester bonds [44] and *p*-coumaric acid is thought to be more toxic to ruminal microorganisms than other phenolic acids and may limit cell wall degradation [45]. The latter is supported by Martínez et al. [46] who reported that removal of *p*-coumarate in sugar cane bagasse enhanced the enzyme degradation of cellulose and lead to a greater cell wall degradation.

2.5 CONCLUSIONS

The ADL content and the S:G ratio increased from the upper internode to the lower internode within the maize stem for both investigated cultivars (Aastar and Ambrosini) and the S:G ratio in internode 7 tended to increase during the growing season. However, the ADL content in internode 7 fluctuated during maturation. Cell wall degradability, as determined with the gas production technique, tended to decrease up to anthesis with no clear pattern after anthesis, whereas cell wall degradability increased with internode number (from bottom to top).

For maize stems, the S:G ratio (lignin composition) showed a better relationship with cell wall degradability than the ADL content. A lower S:G ratio was associated with a greater degradability.

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Additional files

Supplementary Table 2.1: Below is all data from pyrolysis gas chromatography / mass spectrometry analysis of the samples. The structures of the components are published by Jurak et al.[1] and by Martínez et al.[2]

	Compound	Origin[a]	Ambrosini				Aastar			
			17 Jul	14 Aug	11 Sep	23 Oct	17 Jul	14 Aug	11 Sep	23 Oct
1	Furfural	C	8.3	7.9	5.8	6.9	9.1	7.2	7.1	7.5
2	(5H)-furan-2-one	C	3.8	3.5	3.5	2.9	4.4	3.8	3.9	3.1
3	2-acetylfuran	C	1.0	1.0	0.8	0.8	1.3	1.0	0.9	1.0
4	4-hydroxy-5,6-dihydro-2H-pyran-2-one	C	0.7	1.2	0.5	0.6	0.8	1.5	1.2	0.5
5	1,4-anhydroarabinofuranose	C	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.0
6	5-(hydroxymethyl)dihydro-2(3H)-furanone	C	0.9	1.2	0.9	0.8	1.2	1.7	1.3	0.9
7	5-hydroxymethylfurfural	C	0.1	1.8	0.0	0.6	0.3	1.9	1.2	1.1
8	2-methylfuran	C	0.5	0.5	0.3	0.3	0.6	0.5	0.5	0.5
9	2,3-dihydro-5-methylfuran	C	13.7	11.8	10.0	9.1	15.6	10.4	10.5	9.9
10	1,6-anhydro-β-D-glucopyranose (levoglucosan)	C	0.4	1.4	0.1	0.5	0.4	0.7	0.8	0.4
11	Phenol	H	5.8	4.4	5.5	5.3	5.5	4.4	4.9	5.9
12	2-methylphenol (o-cresol)	H	0.8	0.7	0.7	0.6	0.8	0.7	0.7	0.8
13	4-methylphenol (p-cresol)	H	0.8	0.7	0.7	0.6	0.8	0.7	0.7	0.8
14	4-ethylphenol	H	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
15	4-vinylphenol	H/ PCA	38.7	41.8	42.7	42.9	39.1	42.9	43.2	42.7
16	Hydroquinone	H	0.7	0.7	0.5	0.7	0.9	0.9	0.8	0.9

	Compound	Origin[a]	Ambrosini				Aastar			
			17 Jul	14 Aug	17 Jul	14 Aug	17 Jul	14 Aug	17 Jul	14 Aug
17	Guaiacol	G	4.8	3.5	4.8	4.7	4.3	3.8	4.3	4.8
18	4-methylguaiacol	G	0.3	0.5	0.4	0.5	0.3	0.5	0.5	0.4
19	4-ethylguaiacol	G	0.5	0.7	0.6	0.6	0.5	0.6	0.7	0.7
20	4-vinylguaiacol	G/ FA	7.3	5.7	7.4	7.1	6.3	6.3	5.9	6.5
21	Eugenol	G	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.1
22	4-propylguaiacol	G	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
23	Vanillin	G	0.7	0.6	0.6	0.7	0.6	0.6	0.6	0.6
24	*cis*-isoeugenol	G	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.1
25	*trans*-isoeuganol	G	0.4	0.6	0.6	0.6	0.4	0.6	0.5	0.5
26	Acetovanillone	G	0.0	0.1	0.1	0.2	0.0	0.0	0.1	0.0
27	Guaiacylacetone	G	0.4	0.4	0.5	0.5	0.3	0.5	0.4	0.6
28	*trans*-coniferaldehyde	G	0.0	0.1	0.1	0.2	0.0	0.1	0.1	0.1
29	Syringol	S	5.3	5.0	6.9	6.4	3.7	4.4	5.0	5.4
30	4-methylsyringol	S	0.4	0.4	0.5	0.5	0.3	0.4	0.5	0.4
31	4-vinylsyringol	S	1.7	1.4	2.5	2.2	1.2	1.3	1.3	1.5
32	4-allyl-2,6-dimethoxyphenol	S	0.0	0.2	0.2	0.2	0.0	0.1	0.1	0.1
33	Syringaldehyde	S	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.3
34	*cis*-2,6-dimethoxy-4-propenylphenol	S	0.1	0.1	0.2	0.1	0.0	0.1	0.1	0.1
35	Homosyringaldehyde	S	0.0	0.0	0.2	0.2	0.0	0.1	0.1	0.1
36	*trans*-2,6-dimethoxy-4-propenylphenol	S	0.6	0.7	1.0	1.0	0.5	0.7	0.7	0.8
37	Acetosyringone	S	0.3	0.3	0.3	0.4	0.2	0.3	0.3	0.3

	Compound	Origin[a]	Ambrosini				Aastar			
			17 Jul	14 Aug	17 Jul	14 Aug	17 Jul	14 Aug	17 Jul	14 Aug
38	Syringylacetone	S	0.3	0.3	0.4	0.4	0.2	0.3	0.3	0.4
39	*trans*-sinapyl alcohol	S	0.1	0.1	0.1	0.2	0.0	0.1	0.1	0.1
	% Carbohydrates[b]		29.4	30.5	21.9	22.6	33.5	29.0	27.6	24.9
	% Lignin		70.6	69.5	78.1	77.4	66.5	71.0	72.4	75.1
	Ratio Lignin/Carbohydrate		2.40	2.28	3.56	3.42	1.98	2.45	2.62	3.02
	SUM H[c]		46.9	48.4	50.2	50.2	47.3	49.7	50.3	51.2
	SUM G[c]		14.6	12.3	15.2	15.2	12.8	13.2	13.4	14.5
	SUM S[c]		9.1	8.9	12.7	12.0	6.3	8.0	8.7	9.4
	Ratio Syringyl/Guaiacyl		0.62	0.72	0.83	0.79	0.49	0.61	0.65	0.65
	Ratio (Syringyl/Guaiacyl)$_{\text{except 4-vinylguaiacol}}$ [d]		1.01	1.13	1.30	1.21	0.79	0.97	1.00	0.99
Based on Pyrolysis GC-MS										
	% Cα-unsubstituted lignin		15.8	12.9	17.2	16.4	13.5	12.6	14.2	16.1
	% Cα-methylated lignin		2.2	2.2	2.3	2.2	2.3	2.3	2.3	2.3
	% Cα-vinyl lignin		47.7	48.8	52.5	52.1	46.6	50.5	50.4	50.8
	% Cα-oxidized lignin		2.0	2.0	2.2	2.5	1.6	1.9	1.9	2.2
	% Cα-oxidized G-units		1.2	1.1	1.2	1.3	0.9	1.1	1.1	1.3
	% Cα-oxidized S-units		0.9	0.9	1.0	1.2	0.6	0.8	0.9	0.9

[a] C, carbohydrate-derived compound; H, *p*-hydroxycinnamyl lignin-derived compounds; G, guaiacyl lignin-derived compounds; S, syringyl lignin-derived compounds; PCA, *p*-coumarates; FA, ferulates.

[b] Standard deviations of the % of carbohydrates were < 2.0

[c] Standard deviations of the sum of H, G and S compounds were < 1.

[d] All G and S derived peaks were used for the calculation of the S:G ratio except 4-vinylguaiacol which also can arise from ferulates.

	Compound	Origin[a]	Ambrosini Internodes				Aastar Internodes			
			5	9	13	15	5	9	13	15
1	Furfural	C	8.4	8.5	10.5	9.7	7.5	8.2	9.4	10.6
2	(5H)-furan-2-one	C	3.0	4.3	4.5	4.9	3.6	4.3	5.7	5.6
3	2-acetylfuran	C	0.8	0.7	1.0	1.0	0.7	0.8	1.0	1.1
4	4-hydroxy-5,6-dihydro-2H-pyran-2-one	C	2.4	1.7	2.0	1.8	2.6	2.6	2.0	1.8
5	1,4-anhydroarabinofuranose	C	0.2	0.1	0.3	0.2	0.1	0.1	0.2	0.2
6	5-(hydroxymethyl)dihydro-2(3H)furanone	C	1.0	1.3	1.3	1.5	1.2	1.9	2.1	1.8
7	5-hydroxymethylfurfural	C	1.3	0.6	0.7	0.3	2.4	1.5	0.7	0.4
8	2-methylfuran	C	0.5	0.5	0.5	0.3	0.5	0.6	0.5	0.6
9	2,3-dihydro-5-methylfuran	C	12.3	12.7	15.2	16.0	12.4	13.3	15.8	17.5
10	1,6-anhydro-β-D-glucopyranose (levoglucosan)	C	1.8	0.5	1.4	0.7	0.8	0.8	0.4	0.4
11	Phenol	H	3.9	3.6	3.8	3.7	3.6	3.5	3.9	4.0
12	2-methylphenol (o-cresol)	H	0.4	0.5	0.5	0.5	0.5	0.4	0.5	0.6
13	4-methylphenol (p-cresol)	H	0.4	0.5	0.5	0.5	0.5	0.4	0.5	0.6
14	4-ethylphenol	H	0.0	0.1	0.1	0.2	0.1	0.0	0.1	0.2
15	4-vinylphenol	H/PCA	42.9	37.7	32.9	34.3	42.1	38.3	32.6	30.1
16	Hydroquinone	H	0.5	0.6	0.8	0.6	0.4	0.6	0.8	0.8
17	Guaiacol	G	3.1	4.4	4.6	4.4	3.4	4.0	4.6	4.7
18	4-methylguaiacol	G	0.2	0.6	0.6	0.5	0.4	0.5	0.5	0.3
19	4-ethylguaiacol	G	0.4	0.5	0.6	0.5	0.4	0.6	0.6	0.5
20	4-vinylguaiacol	G/ FA	5.2	7.4	8.3	8.1	6.3	7.7	8.6	9.8

	Compound	Origin[a]	Ambrosini Internodes				Aastar Internodes			
			5	9	5	9	5	9	5	9
21	Eugenol	G	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0
22	4-propylguaiacol	G	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
23	Vanillin	G	0.6	0.7	0.8	0.7	0.5	0.7	0.7	0.7
24	*cis*-isoeugenol	G	0.0	0.1	0.0	0.1	0.1	0.1	0.0	0.0
25	*trans*-isoeuganol	G	0.4	0.9	0.6	0.4	0.4	0.6	0.6	0.4
26	Acetovanillone	G	0.2	0.1	0.1	0.1	0.0	0.1	0.0	0.0
27	Guaiacylacetone	G	0.3	0.5	0.4	0.5	0.4	0.3	0.5	0.5
28	*trans*-coniferaldehyde	G	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1
29	Syringol	S	5.1	5.1	3.7	4.1	4.7	4.1	3.7	3.5
30	4-methylsyringol	S	0.4	0.5	0.4	0.4	0.4	0.4	0.3	0.3
31	4-vinylsyringol	S	1.6	2.2	1.5	1.9	1.5	1.2	1.5	1.5
32	4-allyl-2,6-dimethoxyphenol	S	0.2	0.2	0.2	0.0	0.2	0.0	0.0	0.0
33	Syringaldehyde	S	0.4	0.3	0.2	0.2	0.4	0.3	0.2	0.2
34	*cis*-2,6-dimethoxy-4-propenylphenol	S	0.1	0.1	0.0	0.1	0.1	0.1	0.0	0.0
35	Homosyringaldehyde	S	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.1
36	*trans*-2,6-dimethoxy-4-propenylphenol	S	0.9	0.9	0.7	0.6	0.8	0.7	0.7	0.5
37	Acetosyringone	S	0.3	0.4	0.4	0.3	0.3	0.4	0.4	0.3
38	Syringylacetone	S	0.4	0.4	0.3	0.4	0.3	0.3	0.3	0.3
39	*trans*-sinapyl alcohol	S	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

	Compound	Ambrosini Internodes				Aastar Internodes			
		5	9	5	9	5	9	5	9
	% Carbohydrates[b]	31.6	30.9	37.3 ± 4.6	36.2 ± 2.2	31.9	34.1	37.8	40.0
	% Lignin	68.4	69.1	46.3	63.8	68.1	22.6	62.2	60.0
	Ratio Lignin/Carbohydrate	2.16	2.24	1.68	1.76	2.14	0.66	1.65	1.50
	SUM H[c]	48.2	43.1	38.6	39.8	47.1	43.2 ± 2.2	38.5	36.1
	SUM G[c]	10.6	15.7	16.3 ± 2.0	15.7	12.1	14.8	16.3	17.2
	SUM S[c]	9.5	10.3	7.7	8.3	8.9	7.8	7.4	6.7
	Ratio Syringyl/Guaiacyl	0.90	0.66	0.47	0.53	0.73	0.53	0.45	0.39
	Ratio (Syringyl/Guaiacyl)$_{\text{except 4-vinylguaiacol}}$[d]	1.47	0.99	0.76	0.85	1.28	0.93	0.77	0.71
Based on Pyrolysis GC-MS	% Cα-unsubstituted lignin	12.1	13.1	12.1	12.3	11.6	11.6	12.1	12.2
	% Cα-methylated lignin	1.5	2.1	1.9	1.9	1.6	1.8	1.9	1.7
	% Cα-vinyl lignin	49.7	47.3	42.8	44.3	49.9	47.2	42.8	41.4
	% Cα-oxidized lignin	2.0	2.4	2.2	2.3	1.9	2.0	2.1	2.0
	% Cα-oxidized G-units	1.0	1.4	1.3	1.4	1.0	1.1	1.2	1.2
	% Cα-oxidized S-units	1.0	1.1	0.9	0.9	1.0	1.0	0.9	0.8

[a] C, carbohydrate-derived compound; H, *p*-hydroxycinnamyl lignin-derived compounds; G, guaiacyl lignin-derived compounds; S, syringyl lignin-derived compounds; PCA, *p*-coumarates; FA, ferulates.

[b] Standard deviations of the % of carbohydrates were < 2.0 or indicated

[c] Standard deviations of the sum of H, G and S compounds were < 1.5 or indicated

[d] All G and S derived peaks were used for the calculation of the S:G ratio except 4-vinylguaiacol which also can arise from ferulates.

[1] Jurak E, Punt AM, Arts W, Kabel MA and Gruppen H, Fate of carbohydrates and lignin during composting and mycelium growth of *Agaricus bisporus* on wheat straw based compost. *PLoS One* **10.1371**:1-16 (2015). [2] Martínez PM, Punt AM, Kabel MA and Gruppen H, Deconstruction of lignin linked *p*-coumarates, ferulates and xylan by NaOH enhances the enzymatic conversion of glucan. *Bioresour Technol* **216**:44-51 (2016).

CHAPTER 3

Corn stover lignin is modified differently by acetic acid compared to sulfuric acid

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Abstract

In this study, two acid catalysts, acetic acid (HAc) and sulfuric acid (H_2SO_4), were compared in thermal pretreatments of corn stover, in particular to assess the less understood fate of lignin. HAc-insoluble lignin, analysed by pyrolysis GC-MS, showed decreasing levels (%) of Cα-oxidized (from 3.7±0.2 to 1.8±0.1), propenyl (from 2.5±0.1 to 1.0±0.1), vinyl-G (from 34.5±1.8 to 28.4±0.9), vinyl-S (from 4.2±0.2 to 3.7±0.1) and methylated (from 4.9±0.04 to 2.8±0.1) lignin units at increasing HAc amounts. Concurrently, unsubstituted and vinyl-H units increased (from 7.5±0.5 to 11.3±0.2 and from 40.5±1.9 to 49.9±0.9, respectively). Similar trends were seen for residual lignin in H_2SO_4 catalysed pretreatments, although the composition differed from that of residual HAc-lignin. In particular, H_2SO_4-lignin showed slightly lower values (%) for unsubstituted (9.9±0.2) and vinyl-H (45.7±4.1) units, while Cα-oxidized (3.4±0.4), propenyl (1.9±0.1), vinyl-G (28.5±0.9), vinyl-S (4.4±0.6) and methylated (4.6±0.2) lignin units remained higher compared to HAc-catalysis at similar pH values. Xylan yields and corresponding enzymatic conversions of the solids were similar regardless the type of acid. Our findings show that HAc in pretreatments decreased lignin complexity, possibly due to cleavage reactions, although subsequent recondensation reactions increased solid lignin yields, more than H_2SO_4, while removal of xylan and enzymatic conversion of solids were equal.

3.1 Introduction

Lignocellulosic biomass is considered as sustainable feedstock for the production of biochemicals or biofuels [1]. A majority of lignocellulosic by-products of the agro-industry are grass-like feedstocks, such as corn stover or wheat straw. These grasses are mainly constituted by the plant cell wall polysaccharides cellulose (30-50 w/w %) and xylan (20-40 w/w %), which together with the aromatic lignin polymer (5-25 w/w %) form a complex network [2]. Pretreatment of lignocellulosic biomass is required to open-up this complex network of polymers to give access to carbohydrate degrading enzymes to release monosaccharides that can be further used in biomass valorisation.

In commercial setups (i.e. POET-DSM in North America), majorly sulfuric acid is used as catalyst in thermal (above 160 °C) pretreatments of corn stover [1]. Such acid pretreatment largely allows to dissolve hemicellulosic xylan, while cellulose and most of the lignin remains in the solids [3, 4]. This residual carbohydrate fraction shows, as mentioned above, an improved enzymatic conversion [5, 6]. However, high pretreatment temperatures (above 160 °C) and residence time can lead to the formation of inhibitory by-products for further fermentation [7].

The amount of xylan dissolved has been reported to increase at decreased pH, which relates to an increased amount of xylan cleavages by acid generated H^+-ions in solution [1, 3]. In contrast, lignin becomes only partly soluble during acid catalysed thermal pretreatment and cellulose remains completely in the solid residue. In addition, during pretreatment dissolved lignin can again condensate and precipitate on the residual cellulosic fibres during cooling down [8]. The latter has been shown to considerably decrease carbohydrate degradability due to either physical hindrance of enzymes by lignin to reach the cellulose, or by enzyme-protein adsorption to lignin avoiding that the enzyme reaches the cellulose [9]. To overcome such lignin condensation and precipitation lignin should be degraded far enough to remain in the soluble, hence removable, fraction even upon cooling. Hereto, weak acids are reported to be more efficient than strong acids, although, so far only researched on dimeric lignin models. To be more specific, [10] have shown that weak acids, in particular formic and acetic acid, catalyse the cleavage of oxidized β-*O*-4 linked lignin dimers with a conversion yield of 61.2 w/w % into soluble low molecular-mass aromatics such as syringol, guaiacol and *p*-hydroxyphenol. In the same study, sulfuric acid was tested, but no cleavage of the oxidized dimer used was observed.

The lignin of grasses, like corn stover, is known to be rich in such β-*O*-4 linkages, which represents approximately 80% of the interunit linkages in grasses [11, 12]. Other linkages are, for example, β-5 phenyl coumaran, β-β' pinoresinol, 5-5' biphenyl and β-1 diaryl propane [13-15]. Moreover, in corn stover, acetic acid is intrinsically present as part of the xylan structure, hence, after it becomes released, it can possibly directly act on the lignin structures present [16].

Based on the above described literature findings, in our study, it was hypothesized that a weak acid such as acetic acid not only cleaves oxidized dimers, but also polymeric lignin via the cleavage of oxidized β-*O*-4 linkages. Hence, in this research, the effect of acetic and sulfuric acid was studied on the corn stover lignin structure during thermal pretreatments ranging in severity and pH. In addition, all dry matter and carbohydrate mass balances were evaluated, as well as water insoluble carbohydrate degradability using a (hemi-)cellulolytic enzyme cocktail.

3.2 Material and methods

3.2.1 Materials

Dried corn stover (98 w/w %) was provided by DSM (Delft, The Netherlands), and milled (<1 mm) (Retsch Mill MM 2000, Retsch, Haan, Germany). The corn stover was milled using consecutive 6, 4, 2 and 1 mm sieve. The corn stover was finally sieved through a 1 mm sieve.

3.2.2 Acetic acid and sulfuric acid pretreatment

Milled corn stover was subjected to acid catalysed hydrothermal pretreatment under defined conditions as shown in Table 3.1 using a 1L-Parr reactor (Moline, Illinois, USA). Each pretreatment was carried out with 37.5 g of corn stover to which 500 g of distilled water combined with acid was added. The reactor was stirred (100 rpm) during the total pretreatment time, including heating up and cooling down. The Parr reactor was heated with help of a heating mantle. Heating up time was 20 minutes till 160 °C was reached after which the treatment time was set (Table 3.1). At the end of the treatment time the Parr reactor was cooled within 40 minutes (10 min to reach 80 °C) with help of temperature controlled oil (set at -10 °C prior to the experiment), which was leaded through a metal spiral within the pretreated corn stover mixture. A typical heating up and cooling down profile is presented in Appendix A.

3.2.3 Separation of water soluble from water insoluble material

Pretreated corn stover samples were directly neutralized using 1M NaOH and centrifuged (10,000*g*, 15 min). Supernatants were collected and immediately frozen. Residues were washed 5 times with distilled water and each time centrifuged (10,000*g*, 15 min). Part of the washed residues was freeze dried for further analysis and part was kept as wet water unsoluble solids (WUS) for direct enzymatic hydrolysis (see 2.4). Freeze dried WUS was subjected to neutral sugar content and composition, uronic acid content, Klason lignin content and pyrolysis GC-MS.

3.2.4 Enzyme hydrolysis of wet water unsoluble solids pretreated corn stover

Wet WUS (not subjected to freeze drying (see 2.3)) (10 mg mL^{-1} dry matter) was suspended in 50 mM

citrate buffer (pH 4.5). Incubations were started by the addition of 2 w/w % DSM enzyme cocktail containing cellulases and hemicellulases (protein / dry matter (dm); protein concentration 52 mg mL^{-1}) (DSM, Delft, The Netherlands). Samples were incubated for 5, 24 and 48 h at 60 °C in a head over tail rotating device. Enzymes were inactivated by adding 50 µL of concentrated hydrochloric acid prior to centrifugation (10,000g, 5 min) of which was pretested to result in a pH lower than 2. The supernatants were collected and subjected to mono and oligo-saccharides analysis as discussed in 2.5.5. All enzyme hydrolyses were performed in duplicate.

Table 3.1. Acetic and sulfuric acid (HAc and H_2SO_4) pretreatment conditions of corn stover, including temperature (°C), treatment time (min), amount of acid added and resulting combined severity factor (CSF). Sample codes are given for each pretreated corn stover material. The pH prior to (soaked for 1 h at 20 °C) and after pretreatment are indicated as well as the amount of free acetic acid present after pretreatment (standard deviations are all lower than 0.01).

Sample code	Temperature (°C)	Treatment time (min)	HAc (w/w %)[a]	H_2SO_4 (w/w %)[a]	CSF[b]	pH prior	pH after	Free HAc after (% w/w)[c]
0-0	160	60	0	0	-0.63	5.85	4.17	1.9
5-0	160	60	5	0	-0.23	3.92	3.77	7.3
15-0	160	60	15	0	0.12	3.51	3.42	17.4
30-0	160	60	30	0	0.33	3.23	3.21	32.6
0-1.2	160	60	0	1.2	0.40	3.08	3.14	2.5
0-2	160	60	0	2	1.15	2.24	2.39	2.6
5-1.2	160	60	5	1.2	0.52	2.88	3.02	7.5
5-2	160	60	5	2	1.16	2.24	2.38	7.7

[a] Expressed per 100 g dry matter corn stover as g HAc added or g H_2SO_4 added or both.

[b] Combined severity factor (according to Abatzoglou et al., 1992). CSF = log R_0–pH = t . exp ((T-100)/14.75) - pH

[c] Expressed per 100 g dry matter corn stover as g free HAc present after pretreatment in the mixture.

3.2.5 Analytical methods

3.2.5.1 Neutral sugar content and composition

The neutral sugar content and composition was determined in duplicate according to [17], using inositol as an internal standard. Samples were treated with 72 w/w % H_2SO_4 (1 h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C and the constituent sugars released were analysed as their alditol acetates using gas chromatography (Focus-GC, ThermoScientific, Waltham, MA, USA). The column used was DB-225 (15 m × 0.53 mm id × 1 µm film thickness; Agilent Technologies, Santa Clara, CA, USA). The initial column temperature was 180 °C with 2 min holding time. The temperature was then increased to 210 °C with a ramp of 2 °C min^{-1}, followed by 5 min holding time. The injector and detector temperature was set at 220 °C. Helium was used as carrier gas with a constant pressure of

60 kPa. The analyses were performed in duplicate. Total carbohydrate content was calculated as the sum of neutral carbohydrates and uronic acids.

3.2.5.2 Uronic acid content

The uronic acid content was determined in duplicate as anhydro-uronic acid content by an automated *m*-hydroxydiphenyl assay [18] with addition of sodium tetraborate using an autoanalyser (Skalar Analytical BV, Breda, The Netherlands). Glucuronic acid (Fluka AG, Busch, Switzerland) was used as a reference (12.5–200 µg mL^{-1}).

3.2.5.3 Acid soluble and insoluble lignin content

To each freeze dried WUS sample of 300 mg dry matter, 3 mL of 72 w/w % H_2SO_4 was added and samples were pre-hydrolysed for 1 h at 30 °C. Distilled water (37 mL) was added to each sample and samples were put in a boiling water bath for 3 h and shaken every half hour. Next, the suspension was filtered over G4 glass filters (Duran Group, Wertheim/Main, Germany). The filtrate was measured for acid soluble lignin (ASL) spectrophotometrically at 205 nm. ASL was calculated according to the formula: ASL = (A*B*C)/(D*E), with A = absorption relative to 1M H_2SO_4, B = dilution factor, C = filtrate volume, D = extinction coefficient for lignin (110 g Lcm^{-1}), and E = weight of substrate (g).The residual part was washed until it was free of acid and dried overnight at 105 °C. Analysis was performed in duplicate.

3.2.5.4 Mono- and oligosaccharides analysis

High performance anion exchange chromatography (HPAEC) was performed on a Dionex ICS-5000 unit (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (2 mm x 250 mm ID) in combination with CarboPac guard column and PAD detection (Dionex). The system was controlled by Chromeleon software (Thermo Scientific, Sunnyvale, CA, USA). Elution and quantification of mono- and oligosaccharides (0.3 mL min^{-1}) was performed with a combination of 3 types of eluents, A: 0.1M NaOH; B: 1M NaOAc in 0.1M NaOH; C: H_2O.

The elution profile for the monosaccharides was as following: 0-30 min 15% A and 85% C, 30-35 min 100% B, 35-45 min 100% A, 45-60 min 15% A and 85% C. The elution profile for the oligosaccharides was as following: 0-35 min: 0-38% B in A, 35-38 min 100% B, 38-50 min 100% A. For quantification, glucose, xylose, xylo-oligosaccharides (XOS) with a degree of polymerization (DP) of 2 to 4 (Megazyme, Wicklow, Ireland) and glucuronic acid were used for calibration in at least 4 increasing concentrations between 5 and 30 µg mL^{-1}. Analysis was performed in duplicate.

3.2.5.5 Lignin analysis by pyrolysis GC-MS

Pyrolysis of 100 µg, weighed on a Mettler-Toledo XP6 microbalance (Mettler-Toledo, Columbus, US), was performed with an EGA/PY-3030D micro-furnace pyrolyzer (Frontier Laboratories, Fukushima, Japan), equipped with an AS-1020E Autoshot auto-sampler. The pyrolyzer was connected to a Thermo7820A gas chromatograph using a DB-1701 fused-silica capillary column (30 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 pyrolysis was performed at 500° C for 1 min, with an interface temperature of 300 °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}). Compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and with those reported in literature [19, 20]. Pyrograms were processed by AMDIS software according to [19]. Relative response factors (RRF) of the lignin-derived pyrolysis products were determined as described by [21], where AMDIS software was used for processing instead of Xcalibur software. Molar areas (A/M) were divided by the corresponding RRF and summed to give the total RRF corrected molar area. All lignin derived pyrolysis GC-MS compounds analysed and their structural features are shown in Appendix B.

3.3 Results and discussions

3.3.1 Distribution of solids and liquids of acetic or sulfuric acid pretreated corn stover

First, the composition of the corn stover prior to pretreatment was analysed. The corn stover contained 37.5±0.8 w/w % cellulose, 35.2±0.8 w/w % arabinoglucuronoxylan (29.7±0.5 w/w % xylosyl, 4.8±0.2 w/w % arabinosyl and 0.7±0.1 w/w % uronic acids), 1.8±0.1 w/w % other carbohydrates, 3.04±0.07 w/w % esterified HAc, 4.7±0.1 w/w % protein, 3.7±1.0 % acid soluble lignin and 14.5±0.2% acid insoluble lignin. As these data were in line with previous published composition of corn stover [16, 22], they were further used to calculate yields of components recovered after pretreatment in either the solids or the liquors discussed below.

To compare the effect of acetic versus sulfuric acid during thermal pretreatment, for two settings the dose of acid added was such that a similar pH was reached (pH 3.21 for 30 w/w % HAc and 3.14 for 1.2 w/w % H_2SO_4, Table 3.1). Further to study our hypothesis, various acid doses were tested (Table 3.1). Consecutively to HAc and H_2SO_4 pretreatments (160 °C for 60 min; Table 3.1) all the pretreated corn stover samples were analysed for dry matter, carbohydrates and lignin over the soluble (liquor) and insoluble fractions (WUS) as shown in Table 3.2.

Table 3.2: Distribution of soluble and insoluble material after acetic and sulfuric pretreatment of corn stover and the composition of the insoluble fractions (WUS) after pretreatments. Sample codes are explained in Table 3.1.

Sample code	0-0	5-0	15-0	30-0	0-1.2	0-2	5-1.2	5-2
CSF	-0.63	-0.23	0.12	0.33	0.40	1.15	0.52	1.16
WUS recovery (w/w % dm)	71.8	65.2	74.0	75.4	60.4	59.1	61.0	62.1
Carbohydrates recovery (w/w % dm)								
Carbohydrates in WUS	71.7	62.0	56.6	49.3	53.3	55.8	57.1	50.2
Carbohydrates in liquor	24.6	31.5	32.5	33.5	36.9	34.8	34.8	34.3
Losses	3.7	6.5	10.9	17.1	9.8	9.4	8.2	15.5
Lignin recovery in WUS (w/w % dm)	89.7	75.9	82.6	72.9	82.9	98.3	92.8	92.3
Composition of WUS (w/w % dm)								
Total carbohydrates	76.5	78.8	73.6	73.0	70.0	70.3	73.5	67.8
Glucan	53.3	61.6	61.6	64.5	60.3	63.3	64.4	61.8
Xylan	21.0	15.0	10.9	8.6	9.9	6.3	8.7	5.8
Lignin (Klason):								
Acid soluble lignin	1.6	1.2	1.1	1.0	1.1	1.0	0.5	0.5
Acid insoluble lignin	21.7	22.4	26.4	25.3	25.5	29.2	28.7	29.9
Xylan composition of WUS (mol %)								
Arabinosyl	6	7	8	8	7	10	9	9
Xylosyl	87	85	82	82	83	77	80	75
Galactosyl	5	7	9	10	9	13	10	15
Uronic acid	2	1	1	1	1	1	1	1
Degree of substitution (DS) WUS xylan								
DS_{Ara}[a]	7.4	8.4	9.6	9.3	8.8	12.2	11.0	13.0
DS_{GlcA}[a]	6.2	7.6	10.5	12.2	10.6	17.0	12.7	19.0

[a]Ratio mol/100mol; abbreviations: Ara, arabinosyl; GlcA, glucuronic acid

For all pretreatments, most of the dry matter was recovered in the WUS fractions (59-75%; Table 3.2), although the carbohydrate yields in the WUS decreased with increasing amounts of acid (49-71%; Table 3.2). As expected, this decrease was mainly due to xylan degradation and solubilisation. As shown in Figure 3.1, soluble xylan increased (from 40 to 59%) as acid concentration increased. However, increased acid concentration led to higher xylan losses (from 5 to 30%). Meanwhile, glucan yields in the WUS remained almost constant (86-99%; Figure 3.1). Previously, [3] showed comparable recoveries of both xylan and glucan for sulfuric acid treatment of wheat straw. Others also showed similar recoveries for corn stover pretreatments at similar CSFs. After a dilute sulfuric acid pretreatment, (CSF =1.2), [23] showed 56.7% solid recovery with glucan and xylan yields in the liquor of 7.5 and 58.1%, respectively. However, in our study, lignin recoveries where higher at similar CSF (98% compared to approximatively 80%) but starting with a higher initial lignin content (18 w/w % compared to 14 w/w %). At similar pH measured after HAc treatment (0.25 w/w %), [24] showed lower dm recovery (56.6% at pH 3.79 and 60.5 at pH 3.48)

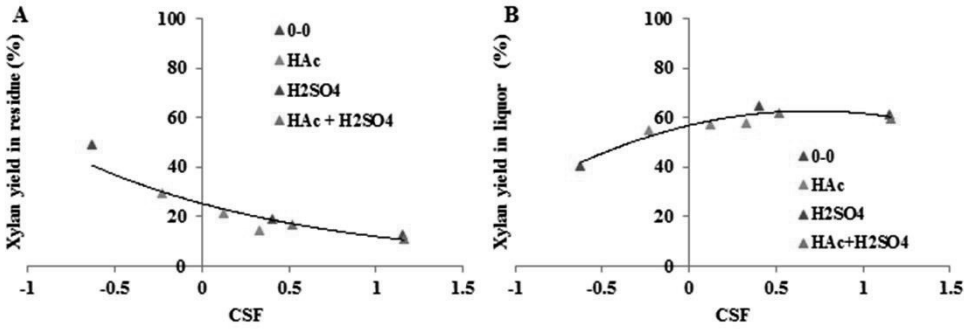

Figure 3.1: A: Xylan yield in the residue; B: Xylan yield in the liquor. Obtained after HAc- and H_2SO_4-catalyzed pretreatment, in % of the original amount in corn stover as the function of the CSF (see Table 3.1 for conditions at the various CSFs).

The pH reached in pretreatment, in combination with the temperature applied, is known to influence the amount of degraded xylan that becomes soluble [3, 25]. Corn stover pretreated without catalyst (0-0; Table 3.1) showed a pH of 5.85 and 4.17 prior to and after pretreatment, respectively. This decrease in pH most likely relates to the 1.1 w/w % HAc released from the corn stover xylan (Table 3.1) corresponding to 36.2% of the originally esterified HAc. The amount of released HAc was higher (2.5-2.6 w/w % based on corn stover dm) when H_2SO_4 was added during pretreatment (0-1.2, 0-2; Table 3.1). For the HAc catalysed pretreatment the released amount of HAc could not be determined due to interference with the added HAc. For both the HAc and H_2SO_4, the suspensions changed less than 0.15 units of pH during pretreatment (Table 3.1). Nevertheless, at similar final pH, remaining amounts of xylan in the WUS fractions were similar, independent of the type of acid used in pretreatment.

In addition to acetic ester substituents, corn stover xylan is known to be decorated with arabinosyl and (4-*O*-methyl-)glucuronic acids residues. In Table 3.2, the xylan composition and the degree of substitution (DS_{Ara} and DS_{GlcA}) is shown. Generally, the DS increased in the insoluble fraction after pretreatment upon increasing severity, for both HAc and H_2SO_4.

In contrast with the xylan yield, the yield of lignin in the solid fractions tended to increase at increasing acid concentration (Table 3.2); lignin yield was 63-72% at the higher acid dosages compared to 54-60% for the lower acid dosages. Even, at similar CSF, the HAc-catalysis showed a tendency in higher solid lignin yields compared to the H_2SO_4-catalysis. Hence, if HAc had another effect on the lignin than H_2SO_4, as suggested by our hypothesis, this did not result in an increased soluble lignin population due to cleavage. Nevertheless, the difference in the lignin fingerprint (see 3.2) after sulfuric and acetic acid pretreatment and after an increased concentration of acid pointed to a different reorganization (re-condensation) of the lignin after the pretreatments. Both recondensation and cleavage is reported to occur during acidic pretreatments of lignocellulose, reviewed by [8]. At increased acidity the

recondensation, preventing the lignin to solubilize, was reported to dominate [26], which is well in line with our observation of the higher lignin yields at higher acid dosages.

To summarize, corn stover pretreatments (160 °C for 60 min) lead to xylan solubilization, while more than 90% of the cellulose remained in the solids. A clear trend was obtained showing an increased amount of xylan solubilized at a decreased pH reached, which was independent of the type of acid (HAc or H_2SO_4) used. In contrast with xylan becoming soluble, lignin tended to increasingly remain in the solids at increasing acid concentrations, most likely due to recondensation reactions. Hence, the effect of HAc or H_2SO_4 on the insoluble lignin was further studied.

3.3.2 Lignin fingerprint analysed using pyrolysis GC-MS of HAc versus H_2SO_4 pretreated corn stover

In the introduction of this research, we have already mentioned that acidic pretreatment conditions at elevated temperatures in commercial set-up (> 160 °C) or in laboratory (> 130 °C) led to lignin fragmentation or lignin model fragmentation, respectively, by acidolysis of the aryl ether linkages (respectively, [8, 10]). We hypothesized that HAc catalysed pretreatments would lead to increased cleavage of the β-*O*-4 linkage in corn stover lignin, compared to H_2SO_4 catalysed pretreatments. Due to our results discussed above, which pointed at increased recondensation of lignin at higher acid dosages, our hypothesis was modified. The adapted hypothesis was that HAc catalysed pretreatments would lead to increased cleavage of the β-*O*-4 linkage in corn stover lignin with subsequently increased recondensation reactions, compared to H_2SO_4 catalysed pretreatments.

To analyse whether the lignin was modified differently by HAc compared to H_2SO_4, corn stover and all WUS-samples were subjected to analytical pyrolysis GC-MS. This method is shown to result in 'lignin-fingerprints' allowing a fair comparison of the starting material, the pretreated corn stover without addition of acids and the insoluble fractions after acidic pretreatments [21, 27, 28].

The results are presented in Table 3.3. The main lignin structures (ρ-hydroxyphenyl (H), guaiacyl (G) and syringyl (S)) were grouped based on the side-chain attached to the H, G or S unit (see Appendix 3.B).

Although the S:G ratio, commonly used to describe lignin structures, was not affected by the pretreatment conditions applied (0.3-0.4; Table 3.3), compositional changes of the lignin in the various solids were observed. At increasing severity (CSF) of the HAc catalysed pretreatments, the relative abundance of unsubstituted and vinyl-H lignin units obtained after py-GC-MS increased in the solids. For unsubstituted py-GC-MS residues even a linear increase was obtained (R^2 = 0.99; Figure 3.2), while for Vinyl-H the correlation with the CSF was less linear (R^2 = 0.72; Figure 3.2). The H_2SO_4 pretreatments also showed (Table 3.3) an increased level of unsubstituted (9.9±0.8 and 10.8±0.5) and vinyl-H (45.7±4.1 and 46.9±2.2) residues in the solids compared to the solids obtained without catalyst

(7.5±0.5 for unsubstituted and 40.5±1.9 for vinyl-H). But, no increasing trend with higher H_2SO_4 levels was obtained and the values obtained differed for the HAc pretreatments at similar pH (Table 3.3). For all residues analysed, the total level of H-units dominated, which was again dominated by the level of vinyl-H units (= 4-vinylphenol). The same was observed in previous studies for corn stover [29] and sugar cane bagasse [28], while in wheat straw lignin the level of H-units was about five times lower [11].

Table 3.3: Py-GC-MS relative abundance of structural features in the residues (WUS) after HAc and H_2SO_4 pretreatment of corn stover (codes in Table 3.1) on the basis of molar peak area corrected for relative response factors. H = ρ-hydroxyphenyl-units, G = guaiacyl-units, S = syringyl-units. Explanation of the structural features is shown in Appendix B. Average and standard deviation of triplicates. The Relative Standard Deviation (RSD) of the triplicates (total molar area divided by their weight) were calculated and kept below 10 %, otherwise re-analysed.

	WUS							
	0-0	**5-0**	**15-0**	**30-0**	**0-1.2**	**0-2**	**5-1.2**	**5-2**
H	44.9±1.9	54.2±2.1	56.7±1.2	55.2±0.9	51.2±4.2	53.3±2.2	54.5±3.8	54.0±0.6
G	42.2±1.8	34.8±1.1	32.8±1.5	34.7±0.9	36.1±1.0	36.0±1.3	34.5±2.0	35.1±0.9
S	12.9±0.3	11.0±0.2	10.5±0.4	10.2±0.2	12.7±0.8	10.7±0.5	11.0±0.7	10.9±0.3
S:G	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.3
Unsub.	7.5±0.5	9.1±0.3	10.4±0.2	11.3±0.2	9.9±0.8	10.8±0.5	9.8±0.7	11.4±0.4
Methyl	4.9±0.04	3.5±0.3	3.1±0.2	2.8±0.1	4.6±0.2	5.1±0.2	3.9±0.2	3.8±0.1
Ethyl	0.3±0.03	0.2±0.02	0.2±0.01	0.2±0.04	0.3±0.05	0.3±0.07	0.2±0.04	0.2±0.05
Vinyl	79.2±2.6	81.6±2.4	81.8±1.9	82.0±1.3	78.6±4.3	78.7±2.5	80.4±4.2	79.3±1.0
Vinyl-H	40.5±1.9	49.6±2.1	51.7±1.2	49.9±0.9	45.7±4.1	46.9±2.2	49.4±3.7	48.1±0.5
Vinyl-G	34.5±1.8	28.3±1.1	26.5±1.4	28.4±0.9	28.5±0.9	28.1±1.2	27.4±1.9	27.6±0.9
Vinyl-S	4.2±0.2	3.8±0.1	3.6±0.3	3.7±0.1	4.4±0.6	3.7±0.3	3.7±0.5	3.6±0.1
Propenyl	2.5±0.1	1.4±0.2	1.2±0.04	1.0±0.1	1.9±0.1	1.6±0.1	1.7±0.4	1.3±0.03
C_α-O[a]	3.7±0.2	2.6±0.1	2.2±0.2	1.8±0.1	3.4±0.4	3.0±0.2	2.8±0.2	2.6±0.2
C_β-O[b]	0.5±0.1	0.4±0.02	0.3±0.03	0.3±0.01	0.4±0.1	0.4±0.04	0.3±0.1	0.4±0.01
C_γ-O[c]	1.4±0.1	1.2±0.1	0.9±0.3	0.6±0.1	0.9±0.3	0.2±0.3	0.7±0.05	1.0±0.2

[a]C_α-oxygen, [b]C_β-oxygen, [c]C_γ-oxygen

It should be noted that py-GC-MS analysed vinyl-H and vinyl-G compounds solely comprised 4-vinylphenol and 4-vinylguaiacol, respectively. The predominance of 4-vinylphenol and 4-vinylguaiacol in the pyrograms of grasses is reported to be due to degradation during pyrolysis of *p*-coumarates and ferulates, respectively [11].

The shown increase was in favour of all other py-GC-MS residues analysed, which all showed decreasing trends (Table 3.3, Figure 3.2). Again, for the lignin remaining in the HAc-solids the trends showing these decreases, versus the CSF, was more linear compared to that of the H_2SO_4-solids.

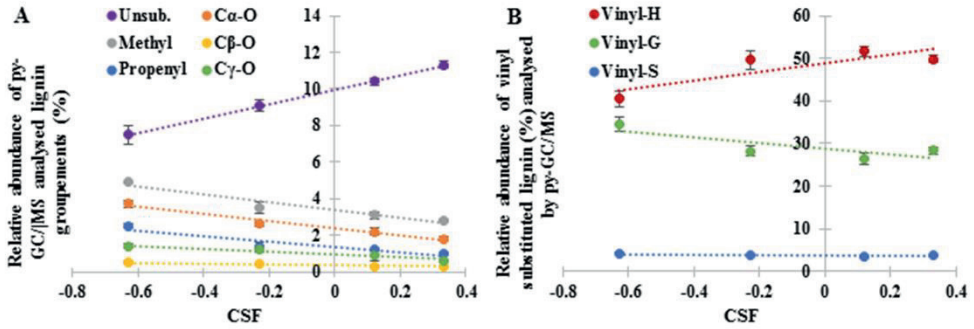

Figure 3.2: Relative abundance of lignin-GC-MS-units MS of corn stover residues obtained after pretreatment with acetic acid. A: Most abundant units excluding vinyl-units (see Appendix B for details). B: Vinyl-units.

Keeping in mind the increase in lignin content (w/w %) in the residues obtained (Table 3.2) at higher CSF, the abundance of two lignin structures (analysed by py-GC-MS) significantly increased: the sum of unsubstituted (py-GC-MS analysed) and 4-vinylphenol (vinyl-H) units (Figure 3.2B).

First, the increase in abundance of unsubstituted units can be speculated to result from the cleavage of β-aryl ether linkages (β-*O*-4), double β-*O*-4 linkage cleavage and oxidized β-*O*-4 linkage cleavage resulting in terminal phenolic groups and a lower abundance of oxygen within lignin (Figure 3.3). This speculation is based on previous studies as shown in Figure 3.3, which studied lignin reactions for lignin models or lignocellulosic biomass. It was reported that β-*O*-4 linkages can be cleaved in acid condition during a thioacidolisis reactions resulting in a terminal phenolic group [10, 30-32]. The same mechanism was shown by Miles-Barett and co-workers [33] on double β-*O*-4 linkage lignin-model. However, despite the cleavage of lignin β-*O*-4 linkages, repolymerization of cleaved lignin has been shown to occur during the pretreatment and its cooling down [30]. Similarly to thioacidolysis reactions, Li et al. [26] showed the cleavage of the β-*O*-4 linkage during autohydrolysis (3% acetic acid) of aspen wood and re-polymerization by acid-catalysed condensation. Oxidized β-*O*-4 linkages (lignin model compound) can also be cleaved into monomeric aromatics using formic acid as shown by Rahimi et al. [10]. Similar products were recovered using acetic acid but lower yields were achieved.

Second, the increase in abundance of py-GC-MS analysed 4-vinylphenol within the residues after acid pretreatment can be speculated to result from the loss of complexity in lignin as explained previously (Figure 3.3) and from the increased solubility of xylan and xylo-oligosaccharides linked coumaric acid (Table 3.2). Indeed, in previous studies, it was shown that mild acidic pretreatment showed increased solubility of xylan structures having coumaric acid and/ or ferulic acid esters [16, 34].

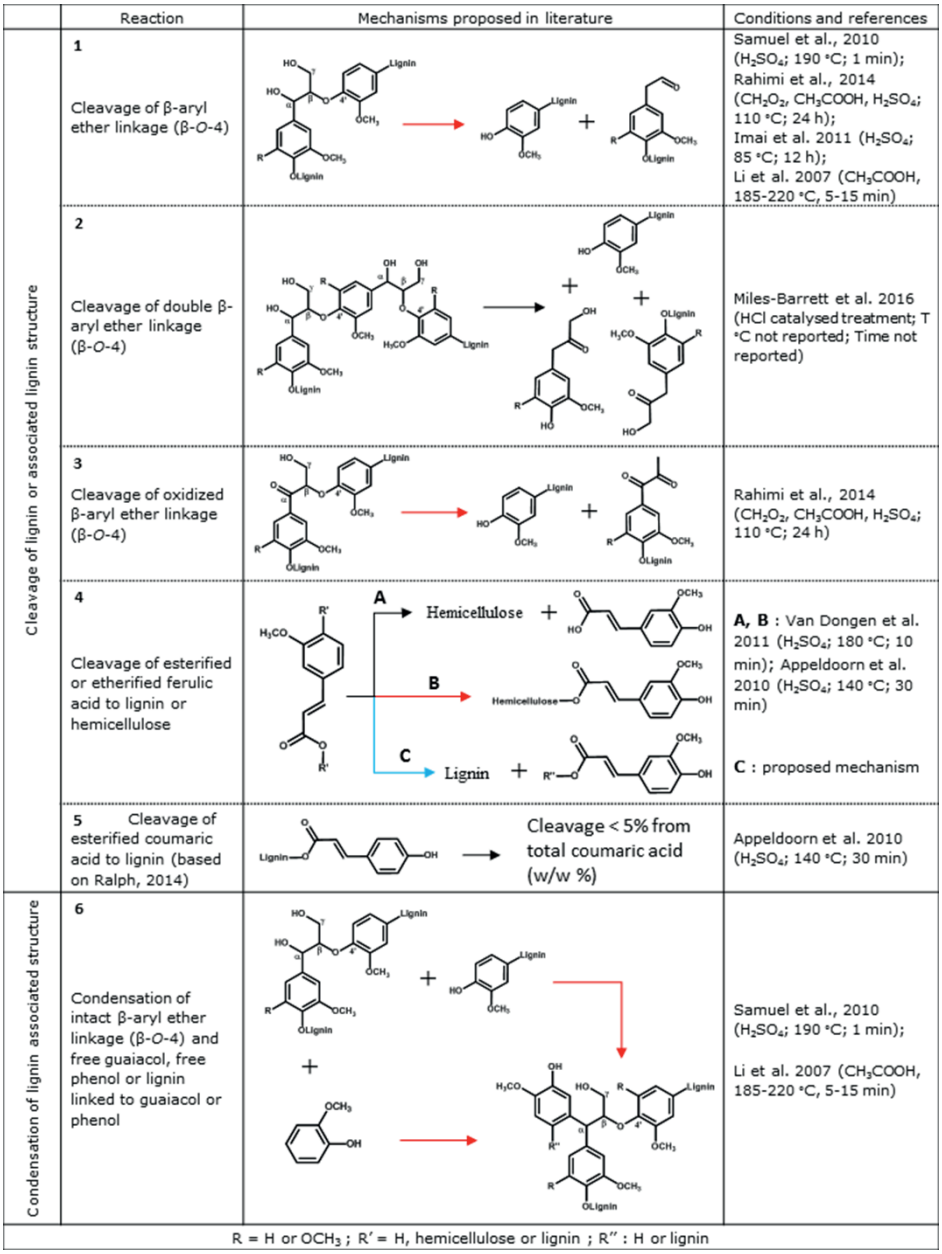

Figure 3.3: Proposed lignin modification mechanisms occurring during acid pretreatment of corn stover and during cooling down. (→) proposed mechanism in the literature; (→) proposed mechanism in the literature and according to our results; (→) proposed mechanism according to our results.

3.3.3 Enzymatic hydrolysis

The enzymatic degradability of the residues collected after pretreatment was studied. Hereto, an in xylanase-enriched cellulase cocktail was employed and released glucose and xylose was measured after 5, 24 and 48 hours of incubation (Table 3.4).

Table 3.4: Enzymatic conversion of glucan and xylan in the residues (WUS) after HAc and H_2SO_4 pretreatment of corn stover (codes in Table 3.1). Average and standard deviation of duplicates.

WUS	% glucose released from WUS total glucosyl			% xylose released from WUS total xylosyl		
	5 hours	*24 hours*	*48 hours*	*5 hours*	*24 hours*	*48 hours*
0-0	25.3±0.0	46.2±0.0	29.3±0.0	29.3±0.0	43.0±0.0	44.9±0.0
5-0	24.5±0.9	44.4±0.2	24.0±1.2	24.0±1.2	40.3±0.3	43.7±1.1
15-0	27.8±1.0	47.2±0.0	24.1±0.9	24.1±0.9	41.7±0.0	44.3±0.1
30-0	25.0±0.3	44.8±0.8	18.2±0.4	18.2±0.4	38.9±1.1	43.0±0.8
0-1.2	25.5±1.3	47.1±0.3	18.3±1.3	18.3±1.3	38.2±0.9	42.4±0.2
0-2	25.7±0.9	50.7±1.5	15.4±1.2	15.4±1.2	39.6±0.5	41.0±0.9
5-1.2	28.0±0.4	46.9±0.6	20.8±0.6	20.8±0.6	40.8±0.7	43.9±1.7
5-2	28.1±1.4	46±0.0	18.5±1.4	18.5±1.4	39.3±0.2	43.4±0.1

After 5 hours of incubation, it can be seen that the addition of acid (sulfuric or acetic acid) during the pretreatment did not increase glucan degradability into glucose, and is the range of 24.5 - 27.8%. It can be noticed that the combined addition of both acids led to a higher glucan degradability (28%). After 24 and 48 hours of incubation, glucan conversion increased to around 26% and 60%, respectively. No major difference were seen for the glucan degradability of residues obtained after pretreatments at various acid dosages of HAc or H_2SO_4. The latter conclusion was also valid for the xylan conversion (Table 3.4), which had already reached a conversion of around 39-43% after 24 hours and of 41-45% after 48 hours. A careful trend was observed showing that enzymatic xylan conversion decreased with increasing CSF, independent of the type of acid. Apparently, the xylan remaining in the residues at higher CSF was more difficult to degrade. An explanation can be the increased DS in these residues (Table 3.2), known to hinder a complete enzymatic degradation [35]. Alternatively, the increased level of (recondensed) lignin may have hindered the enzymes (Tables 3.2, 3.3; [36]).

3.4 Conclusions

All corn stover pretreatments (160 °C) resulted in increased solubility of xylan, while more than 90% of the cellulose remained in the solids. More xylan tended to solubilize at a decreased pH, which was independent of the type of acid (HAc or H_2SO_4) used. In contrast, lignin increasingly remained in the solids at decreased pH, for both HAc and H_2SO_4 catalysed pretreatments due to recondensation

reactions during pretreatment cooling. Still, different lignin compositions were obtained in the residues after HAc or H_2SO_4 catalysed pretreatments, even at similar pH. Overall, HAc decreased residual corn stover lignin complexity more than sulfuric acid.

Funding

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Additionnal files

Supplementary Table 3.1:Py-GC–MS compounds analyzed and structural features, including their CAS number, their molecular weight (Mw) and their relative response factor (Amdis-based).

Compound	CAS	Structural feature	M_w ^{12}C ($g\,mol^{-1}$)	RRF (−)[a]
phenol	108952	H, unsub.[b]	94	0.54
2-methylphenol	95487	H, methyl	108	0.57
4-methylphenol	106445	H, methyl	108	0.54
4-vinylphenol	2628173	H, vinyl	120	0.26
guaiacol	90051	G, unsub.[b]	124	0.49
4-methylguaiacol	93516	G, methyl	138	0.48
4-ethylguaiacol	2785899	G, ethyl	152	0.93
4-vinylguaiacol	7786610	G, vinyl	150	0.14
vanillin	121335	G, C_α-O	152	0.37
trans-isoeugenol	97541	G, propenyl	164	0.31
acetovanillone	498022	G, C_α-O	166	0.39
guaiacylacetone	2503460	G, C_β-O	180	0.52
syringol	91101	S, unsub.[b]	154	0.38
4-methylsyringol	6638057	S, methyl	168	0.46
4-vinylsyringol	28343228	S, vinyl	180	0.20
syringaldehyde	134963	S, C_α-O	182	0.18
trans-4-propenylsyringol	26624135	S, propenyl	194	0.30
acetosyringone	2478388	S, C_α-O	196	0.35
syringylacetone	19037582	S, C_β-O	210	0.35
trans-sinapyl-alcohol	537337	S, C_γ-O	210	0.03

[a] RRF based on van Erven et al. (2017).

[b] Unsub.=unsubstituted.

CHAPTER 4

Potential of a gypsum-free composting process of wheat straw for mushroom production

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Abstract

Wheat straw based composting generates a selective substrate for mushroom production. The first phase of this process requires 5 days, and a reduction in time is wished. Here, we aim at understanding the effect of gypsum on the duration of the first phase and the mechanism behind it. Hereto, the regular process with gypsum addition and the same process without gypsum were studied during a 5-day period. The compost quality was evaluated based on compost lignin composition analysed by py-GC-MS and its degradability by a commercial (hemi-)cellulolytic enzyme cocktail. The composting phase lead to the decrease of the pyrolysis products 4-vinylphenol and 4-vinylguaiacol that can be associated with *p*-coumarates and ferulates linking xylan and lignin. In the regular compost, the enzymatic conversion reached 32 and 39 % for cellulose, and 23 and 32 % for xylan after 3 and 5 days, respectively. In absence of gypsum similar values were reached after 2 and 4 days, respectively. Thus, our data show that in absence of gypsum the desired compost quality was reached 20 % earlier compared to the control process.

4.1 Introduction

The production of substrate used for the growth of the mushroom *Agaricus bisporus* is based on a composting process, which involves the bioconversion of a mixture of wheat straw with horse and chicken manure, water and gypsum [1]. The first phase of this biological process requires 5 days before the compost is considered ready for the second phase, and a time-reduction of the first phase would be desirable. In our research, we aim at understanding the effect of gypsum on the duration of the first phase, and plant cell wall structure modifications required for an optimal compost production.

Wheat straw is the second largest biomass feedstock in the world [2] and is a by-product of the agricultural industry. Nowadays, amongst others, it is used for animals as feed or bedding. Wheat straw is a lignocellulosic feedstock and mainly composed of cellulose (33-40 w/w %), hemicellulosic xylan (20-25 w/w %) and lignin 15-20 w/w %) as reviewed by Prasad and Singh et al. (2007) [3]. Wheat straw is one of the main ingredients of the composting process studied and serves as main carbon source for a wide range microorganisms and the mushroom *A. bisporus*. In addition to wheat straw, gypsum and water, also horse and chicken manure are added. Additionally, part of the wheat straw has already been mixed with horse manure originating from horse bedding and is considered as “not fresh”. The manure supplements the compost with microorganisms and ammonium [4] and is the main nitrogen source. Gypsum provides a calcium source for mushrooms and, in addition, is known to lower the pH [1, 4].

After mixing all ingredients, the composting process follows three phases [5]. The first phase is a thermo-biological treatment (Phase I), the second phase is used to condition the compost and to enable microorganisms other than A. bisporus to grow (Phase II). It is conducted for 5 to 9 days where the temperature is maintained at 56 °C for 24 h before cooling to room temperature by blowing indoor air. During the third phase (Phase III), mycelium growth of A. bisporus within the compost is achieved (Phase III). The mycelium spread within the compost and fully colonize it after 12 to 16 days. The latter two phases (Phase II and III) have been described previously. In particular the fate of compost content and composition over the three phases have already been studied [5] and will not be further discussed here. At industrial scale, Phase I is conducted in 180 tons compost capacity tunnels for 3 to 6 days in presence of oxygen. This thermo-biological phase aims to soften the wheat straw structure for the following phases through the action of ammonia [1]. Within the first 24 hours of phase I, microorganisms from the manure will grow releasing ammonia and generating heat up to 80 °C, consequently softening the mixture. As mesophilic microorganisms will grow from the initial starting temperature, the increase of temperature lead to their replacement by thermophilic microorganisms such as *Aspergillus fumigatus* and *Myceliophtora thermophila* [1, 6, 7]. The growth of microorganisms within the compost during phase I will enhance the degradation of the carbohydrate polymers and the decomposition of organic nitrogen releasing gaseous ammonia through ammonification and immobilisation increasing temperature and pH [1, 4, 8].

In the field of thermo-chemical conversion of lignocellulosic feedstocks into fermentable carbohydrates, meant for the production of biofuels or biochemicals, pretreatments with ammonia resulting in a pH above 8 were shown to increase enzymatic hydrolysis of (hemi)cellulose by (partial) delignification [9-11]. In these studies, pretreatment and enzymatic conversion aimed at a complete deconstruction of carbohydrates into monosaccharides and, therefore, required a more than five times higher ammonia loading than achieved in the Phase I composting process. Whether lignin structures become modified in compost during Phase I to improve carbohydrate accessibility is unknown. However, the latter has been studied during mushroom growth, such as for *A. bisporus* [12] and *Pleurotus eryngii* [13]. In these studies pyrolysis GC-MS was used to monitor lignin modifications.

In our research, it is hypothesized that higher ammonia loadings in Phase I would help to open up the structure, possibly by lignin modifications, to enable the development of the microorganisms in Phase II. In addition, it is hypothesized that a higher amount of ammonia is reached when pH is not controlled by gypsum. The amount of ammonia needed in order to achieve Phase I compost quality (5 composting days) is unknown.

The objective of this research is to assess the potential of biological ammonia pretreatment (Phase I) of a wheat straw based composting process by a gypsum free Phase I process. The gypsum free process was compared to the regular (control) process, in which gypsum was added. Lignin was fingerprinted with analytical pyrolysis GC-MS. The quality of the compost obtained at various time points during Phase I was evaluated based on its enzymatic degradation by using a commercial (hemi-)cellulolytic cocktail.

4.2 Material and methods

4.2.1 Sample of Phase I compost process in presence and absence of gypsum

Wheat straw and compost samples were obtained from CNC (CNC Grondstoffen B.V., Milsbeek, The Netherlands) in 2013 and in 2015. CNC is a worldwide supplier of substrate for mushroom growing.

The composting process of Phase I at CNC was conducted according to the same methodology as described by Jurak et al. (2014) [5]. Both in 2013 and 2015, composting tunnels were conducted in parallel. The ingredients, wheat straw, horse manure, chicken manure and water, were mixed and divided in two batches. One batch was mixed with gypsum and used to fill one tunnel (4 x 35 x 4.5 m). Samples taken from this tunnel were coded P-13 and P-15 for samples obtained in 2013 and 2015, respectively. The second batch was used to fill the second tunnel (absence of gypsum) and samples were coded as A-13 and A-15, respectively, for the 2013 and 2015 experiment. At the beginning of Phase I, the substrate of both tunnels had a 1:3 (w/w) solid-to-liquid ratio. The circulation of air inside both tunnels was performed in a closed cycle to avoid ammonia losses.

The temperature of the compost was measured 50 cm below the surface in hexaplicate using a thermometer (Testo 110, Testo Inc., Sparta Township, NJ, United States). Ammonium and pH were measured according to Kjeldahl (1883) [14]. The ammonia in the air was measured using a Dräger tube (CH31901) (Drägerwerk AG & Co. KGaA, Lübeck, Germany) in the closed-recycling air system from each tunnel.

In the 2013 experiment, Phase I samples were collected every day from both tunnels and were coded as follows: start of Phase I (P0-13 and A0-13), 1 day (P1-13 and A1-13), 2 days (P2-13 and A2-13), 3 days (P3-13 and A3-13), 4 days (P4-13 and A4-13), and 5 days (P5-13 and A5-13). Samples from tunnel P-15 and A-15 were collected at the beginning and at the end of Phase I (P0-15 or A0-15 and P5-15 or A5-15 for the tunnel in presence of gypsum and absence of gypsum, respectively). For every sample, 3 times 10 kg were collected from 50 cm below the surface (within the first 5 meters of the tunnels due to high temperature) and mixed thoroughly. The samples were subdivided to 1 kg amounts and frozen immediately. Samples were freeze dried and milled (<1 mm) (Retsch Mill MM 2000, Retsch, Haan, Germany).

4.2.2 Water extraction

Freeze dried and milled samples (10 g) were suspended in Millipore water (100 mL) overnight and were extracted at room temperature under continuous stirring. After centrifugation (10,000*g*; 15 min; 20 °C), the residues were washed three times with 100 mL Millipore water. The residues were recovered as water un-extractable solids (WUS) after freeze dried.

Freeze dried WUS-samples were coded as follow: samples from 2013, start of Phase I (wP0-13 and wA0-13), 1 day (wP1-13 and wA1-13), 2 days (wP2-13 and wA2-13), 3 days (wP3-13 and wA3-13), 4 days (wP4-13 and wA4-13), and 5 days (wP5-13 and wA5-13). Samples from 2015, start of Phase I (wP0-15 and wA0-15) and 5 days (wP5-15 and wA5-15).

4.2.3 Enzyme hydrolysis

WUS samples (25 mg) were suspended in 1 mL 50 mM sodium acetate buffer (pH 5.0). Samples were boiled for 10 min to stop remaining enzyme activity and to avoid growth of remaining microorganisms. Samples were then cooled to room temperature. Incubations were started by the addition of 2.7 w/w % CellicCtec2 ((protein / dry matter (dm); CellicCtec2 protein concentration 127 mg mL^{-1}) (Novozymes, Bagsværd, Denmark)) and 0.3 w/w % CellicHTec ((protein / dm); CellicHTec protein concentration 120 mg mL^{-1}) (Novozymes, Bagsværd, Denmark)) to the boiled and cooled sample. Samples were incubated for 24 h at 50°C in a head over tail rotator. Enzymes were inactivated (10 min at 100 °C) prior to centrifugation (10,000*g*, 5 min).The supernatants were collected and subjected to further analysis. Enzyme treatment was performed in duplicate and replicated for the beginning and end of Phase I.

For xylanase treatment, WUS samples (15 mg) were suspended in 1.5 mL mM sodium acetate buffer (pH 5.0) were incubated for 24 hours at 50 °C with a pure endo-(1,4)-β-D-xylanase 1 (EX1) (23 µL / 5 mg freeze dried WUS) from *Aspergillus awamori* (EC 3.2.18) (protein concentration 21.5 µg mL^{-1}) [14]. Prior to enzyme addition, samples were put at 100 °C for 10 min to inactivate remaining enzyme activity and cooled at room temperature. Samples were incubated for 24 h at 50°C in a head over tail rotator. Enzymes were inactivated (10 min at 100 °C) prior to centrifugation (10,000*g*, 5 min).The supernatants were collected and subjected to further analysis. Treatment were performed in duplicates.

4.2.4 Analytical methods

4.2.4.1 Neutral sugar content and composition

The neutral sugar content and composition was determined in duplicate according to Englyst and Cummings (1984) [15], using inositol as an internal standard. Samples were treated with 72 w/w % H_2SO_4 (1 h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C, uronic acids released were analysed (section below) and the constituent sugars released were analysed as their alditol acetates using gas chromatography (ThermoScientific, Waltham, MA, USA). Total carbohydrate content was calculated as the sum of neutral carbohydrates and uronic acids.

4.2.4.2 Uronic acid content

Uronic acid content was determined in duplicate as anhydro-uronic acid content by an automated m-hydroxydiphenyl assay [16] with addition of sodium tetraborate using an autoanalyser (Skalar Analytical BV, Breda, The Netherlands). Glucuronic acid (Fluka AG, Busch, Switzerland) was used as a reference (0-100 µg mL^{-1}).

4.2.4.3 Nitrogen and protein content

Samples were analysed in duplicate for nitrogen content in duplicate using the combustion (DUMAS) method on a Flash EA 1112 Nitrogen Analyzer (Thermo Scientific, Sunnyvale, CA, USA). Methionine (AcrosOrganics, Geel, Belgium) was used as a standard. Nitrogen to protein conversion factor of 6.25 was used [17].

4.2.4.4 Insoluble (Klason) lignin content

To each sample of 300 mg dm, 3 mL of 72 w/w% H_2SO_4 was added and samples were pre-hydrolysed for 1 h at 30 °C. Distilled water (37 mL) was added to each sample and samples were put in a boiling water bath for 3 h and shaken every half hour. Next, the suspension was filtered over G4 glass filters (Duran Group, Wertheim/Main, Germany). The residual part was washed until it was free of acid and dried overnight at 105 °C. Final residues were corrected for ash content and considered as a measure for the acid insoluble lignin content. Analysis was performed in duplicates.

4.2.4.5 Ash content

Freeze dried samples or lignin residues were first dried overnight at 105 °C in the oven and weighed and, subsequently, stored at 575 °C for 4 h and again weighed. The difference between the mass measured after incubation at 105 °C and after incubation at 575 °C was taken as ash content. Analysis was performed in duplicates.

4.2.4.6 High performance anion exchange chromatography

High performance anion exchange chromatography (HPAEC) was performed on a Dionex ICS-5000 unit (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (2 mm x 250 mm ID) in combination with CarboPac guard column and PAD detection (Dionex). The system was controlled by Chromelion software (Thermo Scientific, Sunnyvale, CA, USA). Elution and quantification of mono- and oligosaccharides was performed at 0.3 mL min^{-1} with a combination of 3 eluents, A: 0.1M NaOH; B: 1M NaOAc in 0.1M NaOH; C: H_2O. The elution profile for the monosaccharides was as following: 0-30 min 15 % A and 85 % C, 30-35 min 100 % B, 35-45 min 100 % A, 45-60 min 15 % A and 85 % C. The elution profile for the oligosaccharides was as following: 0-35 min: 0-38 % B mixed in A, 35-38 min 100 % B, 38-50 min 100 % A. For quantification, glucose, xylose, xylo-oligosaccharides (XOS) with a degree of polymerization (DP) of 2 to 4 (Megazyme, Wicklow, Ireland) and glucuronic acid were used for calibration in at least 4 increasing concentrations between 5 and 30 µg mL^{-1}. Analysis was performed in duplicate.

4.2.4.7 Analytical pyrolysis GC-MS analysis

Pyrolysis of WUS-samples (80-100 µg) were performed in triplicates, weighed on a Mettler-Toledo XP6 microbalance (Mettler-Toledo, Columbus, US) was performed with an EGA/PY-3030D micro-furnace pyrolyzer (Frontier Laboratories, Fukushima, Japan) mounted on to a Thermo7820A gas chromatograph equipped with a DB-1701 fused-silica capillary column (60 m × 0.25 mm internal diameter, 0.25 µm film thickness) and coupled to a DSQ-II thermo mass selective detector (EI at 70 eV) (Thermo Scientific, Waltham, MA, USA). The pyrolysis was performed at 500°C. The GC oven temperature was programmed as follows: 70°C (2 min hold) linearly increased to 230 °C at 5 °C per min, and to 270 °C at 2.5 °C per min. 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 [18]. The relative area per compound identified was calculated according to Jurak et al. (2015) [12]. For all compounds identified spectra were checked manually.

4.3 Results and discussion

4.3.1 Composting conditions in Phase I in presence or absence of gypsum

The composting process starts after mixing the ingredients (wheat straw, horse and chicken manure, water) and filling the Phase I-tunnel. In our research we compared a tunnel filled with mixed ingredients in absence of gypsum with a control tunnel filled with mixed ingredients and gypsum

during Phase I. The hypothesis was that the absence of gypsum will lead to higher amounts of gaseous ammonia, higher compost pH, and will help opening up the compost structure enabling growth of microorganisms in Phase II, compared to the control tunnel. Hence, the conditions reached in both tunnels, including pH and ammonia content, were measured and the data are presented in Figure 4.1. From Figure 4.1A, it can be seen that in both tunnels (P-13 and A-13) the temperature increased within 24 h to 80 °C and remained at that temperature till the end of Phase I. Such a temperature-profile is as expected for this phase and follows the observed pattern year round (CNC, The Netherlands, pers. comm.). The moisture content of compost from both tunnels (P-13 and A-13) was the same and remained constant during Phase I (73-75 w/w % (standard deviation < 0.1, Supplementary Table 4.1).

Gaseous ammonia (Figure 4.1B) and pH (Figure 4.1C) showed an increase for both tunnel (P-13 and A-13) within the first 24 h. In both tunnels, a similar trend was observed, but in the A-13 higher values for both pH and gaseous ammonia (pH 9 and 3.4 kg m^{-3}) were reached after 24 hours Phase I compared to the P-13 tunnel (pH 8.8 and 2.2 kg m^{-3}). Data from P-15 and A-15 confirmed the described trends of P-13 and A-13, showing a higher pH and higher gaseous ammonia in absence of gypsum. Previously, Gerrits (1988) [1] also showed that in absence of gypsum, pH and gaseous NH_3 was higher compared to composting in presence of gypsum. The measurements of NH_4^+ in compost (Figure 4.1D) did not show any major differences between P-13 and A-13 compost (ranging from 0.8 % to 0.6 % NH_4^+) although the A-13 samples gave a relatively high standard deviation during Phase I compared to the P-13 samples. Nevertheless, comparing the end of A-13 and A-15 to the end of P-13 and P-15 (0.4-0.5 % to 0.6 % NH_4^+, respectively), it can be suggested that the absence of gypsum led to a slightly lower NH_4^+ in the compost. Such a lower NH_4^+ content matched well with the measured higher pH and gaseous ammonia in absence of gypsum because the dissociation of NH_4^+ in NH_3 and H^+ is favoured at high pH. To summarize, the absence of gypsum in Phase I led to higher pH values and NH_3 gas concentration compared to composting with gypsum, confirming our hypothesis, and thus affected the “chemical” severity of the process. The other treatment conditions such as temperature (°C) and moisture content (w/w %) were found to be similar and followed the same trend during Phase I, regardless the presence or absence of gypsum.

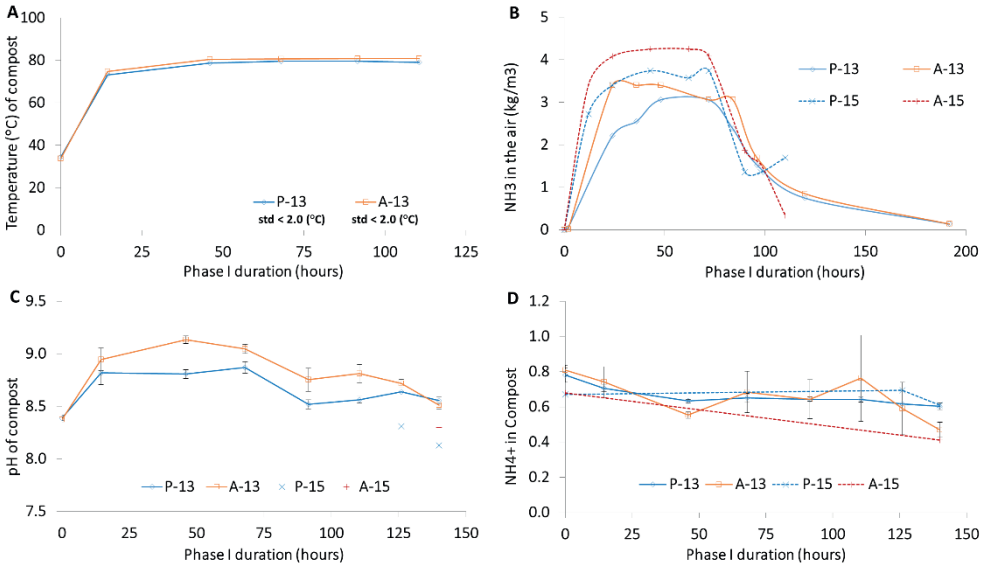

Figure 4.1: Conditions measured during the first 5-days of the composting process (Phase I) from a wheat straw-manure based mixture in presence (P) and in absence (A) of gypsum. A: Temperature (°C); B: Gaseous ammonia (NH_3) measured in the air (kg m^{-3}); C: pH of the compost; D: NH_4^+ content (%) in the compost, based on dry matter.

4.3.2 Enzymatic hydrolysis of Phase I-compost obtained in presence or absence of gypsum

As indicated in the previous section, the Phase I-samples obtained from the gypsum-free process were exposed to a higher pH and gaseous ammonia content compared to the Phase I-samples in presence of gypsum. To corroborate our main hypothesis that ammonia in Phase I helps to open up the structure to enable growth of the microorganisms in Phase II, the samples (either exposed to gypsum or not) were subjected to enzyme hydrolysis mimicking the microbial growth in Phase II. Only the enzymatic degradability of water unsoluble solids (WUS) was studied, because water soluble material is assumed to be well accessible for enzymes and microbes. The glucan to glucose and xylan to xylose conversions of enzymatic treated WUS are shown in Figure 4.2.

At the beginning of Phase I, as expected glucan (24.0 % ± 0.6) and xylan (12.2 % ± 0.3) conversions were similar for wP0-13 and wA0-13 (Figure 4.2). At the end of Phase I, glucan and xylan conversions were increased for wP5-13 (38.5 % (± 0.9) and 31.9 % (± 1.0), respectively). For wA5-13, conversions were even higher (44.3 % (± 1.3) and 39.6 % (± 0.8), respectively). The experiment and enzymatic hydrolysis was repeated in 2015. Glucan and xylan conversions of wP0-15 (19.0 % ± 0.4 and 10.6 % ± 0.0, respectively) were again similar to conversions of wA0-15 (21.4 % ± 1.9 and 11.1 % ± 0.6, respectively). At the end of Phase I, glucan and xylan conversions were found again to be higher for

wP5-15 (37.4 % ± 1.4 and 32.1 % ± 0.7, respectively), and again even higher for wA5-15 (39.1 % ± 2.2 and 38.1 % ± 1.2, respectively).

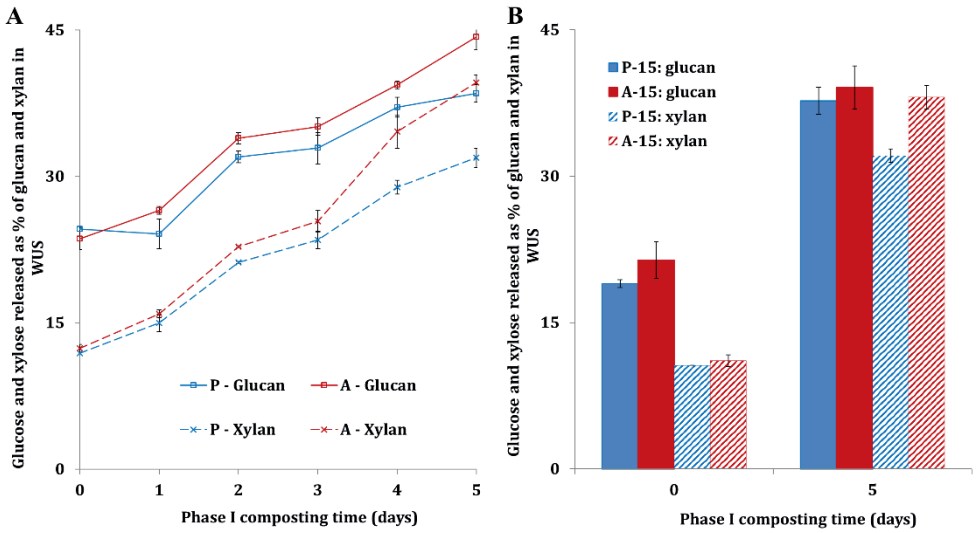

Figure 4.2: Enzymatic hydrolysis (CellicCTec2/ CellicHTec; 24 h) of WUS samples prepared from Phase I-compost expressed as glucose and xylose released (%) from total glucan and xylan in the corresponding WUS. Samples obtained from tunnels in presence (blue; wP-13 and wP-15) and in absence (red; wA-13 and wA-15) of gypsum. A: Phase I from 2013; B: Phase I from 2015.

Hence, it was concluded that compost of both tunnels (P-13 and A-13) became more enzyme degradable during Phase I, seen from the enzymatic glucan and xylan conversions of WUS (Figure 4.2) for both experiment performed in 2013 and 2015. Moreover, samples from the Phase I-tunnel in absence of gypsum resulted in WUS having higher glucan and xylan conversion compared to the WUS samples obtained in presence of gypsum, confirming our hypothesis.

Slight conversion differences for wP-15 and wA15 were observed compared to wP-13 and wA-13. These differences may be due to the initial difference in compost composition originating from straw, which is known to vary over the years [19, 20]. Carbohydrate composition will be discussed and shown in a lower section.

Usually, Phase I compost in presence of gypsum is known (CNC, pers. comm.) to be ready for Phase II after 5 days, although also a 3-day-Phase I is occasionally applied. Now, it was assumed that glucan and xylan conversion of compost-WUS obtained after 3 or 5 days mimics the microbial growth in Phase II or the 'readiness' of Phase-I compost. The same conversions of WUS obtained from Phase-I in presence of gypsum (32.0 and 38.5% for glucan, and 23.0 and 31.9 % for xylan conversion (wP3-13 and wP5-13, respectively)) were also reached one day earlier in absence of gypsum (33.9 and 39.4 for

glucan and 22.8 and 34.6 for xylan conversion, wA2-13 and wA4-13 respectively). This higher enzyme degradability of the composts obtained in the gypsum-free Phase I compared to those from the gypsum-rich Phase I, can be the result of higher pH (pH >8) and higher gaseous ammonia in the gypsum-free process. The latter conditions, in particular the higher pH, have been shown to induce both de-acetylation of xylan and hydrolysis of the carboxyl-ester linkages present between arabinosyl-substituents of xylan and ferulate or *p*-coumarate and, possibly, between xylan and lignin [21-23]. Therefore, it can be contemplated that the absence or a reduced amount of ester-linkages present between xylan and lignin resulted in a more open structure of the lignocellulosic network of the compost and a higher accessibility of xylan for enzymes, which favoured glucan and xylan conversions. Additionally, ammonia can be expected to partly degrade the lignin present, again allowing a better accessibility for enzymes, which has been reported frequently in previous research for lignocellulosic feedstocks such as corn stover and *Miscanthus* [22, 24]. It should be noted, however, that in the latter studies, aqueous ammonia is used at much higher concentration than reached in the current study. Hence, in our composting process, we expected a start of lignin deconstruction, which is discussed in more details in a lower section. Lignin deconstruction or partly degradation could lead to an increase in accessibility of xylans being linked to lignin via ferulates and *p*-coumarates [23].

To study whether the accessibility of xylan for enzymes in the compost samples of the gypsum-free Phase I was indeed higher compared to control-composts due to higher pH, both wP-13- and wA-13-samples were subjected to a pure endo-xylanase (*Aa*GH10) hydrolysis [14]. Xylose, xylobiose and total soluble xylooligomers were quantified after hydrolysis and presented as percentage of total xylosyl residues present in the corresponding WUS samples (Figure 4.3).

*Aa*GH10 hydrolysis of wP0-13 or wA0-13 (Figure 4.3), both prepared from compost obtained at the start of Phase I, showed a similar release of xylose (0.5 % ± 0.1), xylobiose (1.0 % ± 0.1) and total xylooligomers (3.5 % ± 0.2). For compost-WUS obtained at the end of Phase I, the percentage of xylose and xylobiose released from wP5-13 and for wA-13 (1.5 % ± 0.1 and 4.2 % ± 0.3, respectively) were almost similar, while the percentage total xylooligomers released for wA5-13 (13.9 % ± 0.0) was higher than for wP-13 (12.1 % ± 0.3).

These data suggest that, indeed, a higher pH and ammonia content in Phase I as obtained in absence of gypsum led to compost in which the xylan present was more accessible for the pure endo-xylanase used. This higher accessibility for enzymes did not influence the water binding capacity of the WUS samples; no difference in water binding was analysed for all samples of P-13 or wA-13 (from 1.7 to 1.5 water bound per total carbohydrate (w/w %), standard deviation < 0.1; Supplementary table 4.2).

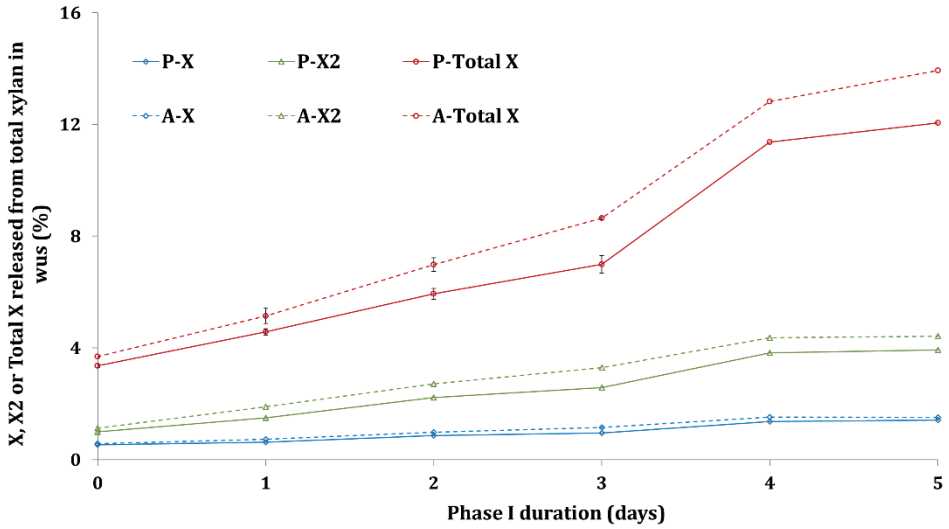

Figure 4.3: Endo-xylanase (*Aa*GH10) hydrolysis of WUS expressed as xylose (X1; in blue; standard deviation < 0.1%), xylobiose (X2; in green; standard deviation < 0.2%) and total xylosyl residues (Total X; in red) released from total xylan in WUS (%).

4.3.3 Composition and content of composts

The chemical compositions of all Phase I-compost samples obtained in presence or in absence of gypsum are shown in Table 4.1.

The total carbohydrate contents of the compost obtained at the start of Phase I, were 38 and 39 % (w/w), for P0-13 and A0-13 respectively, and 46 and 49 % (w/w) for P0-15 and A0-15. Previously, Jurak et al. (2014) [5] reported 34 % (w/w) carbohydrates to be present in compost WUS at the start of Phase I, while ash and lignin were reported to be both 25 w/w %. The observed differences of the initial compost compositions are, most likely, the result of year-to-year variations in ingredient or raw material composition [19, 20].

Table 4.1: Composition of Phase I-compost samples (2013 and 2015) of wheat straw compost obtained in presence (P) and in absence (A) of gypsum. Standard deviations for total carbohydrates content (w/w %) < 3.0 and for lignin < 2.0.

	PI-0[a]				PI-1[a]		PI-2[a]		PI-3[a]		PI-4[a]		PI-5[a]			
	P[b]		A[b]		P[b]	A[b]	P[b]	A[b]	P[b]	A[b]	P[b]	A[b]	P[b]		A[b]	
	P0-13[c]	P0-15[c]	A0-13[c]	A0-15[c]	P1-13[c]	A1-13[c]	P2-13[c]	A2-13[c]	P3-13[c]	A3-13[c]	P4-13[c]	A4-13[c]	P5-13[c]	P5-15[c]	A5-13[c]	A5-15[c]
Content (% w/w) dm[d]																
Lignin (Klason)[e,f]	20	22	17	23	19	19	20	20	19	18	21	20	19	21	19	18
Total carbohydrates	38	46	39	48	40	40	40	39	38	38	39	37	40	45	39	46
Ash	30	22	26	19	29	26	29	27	28	26	29	27	31	25	27	26
Total nitrogen	2	2	2	2	1	1	2	2	2	2	1	2	2	2	2	2
Protein[g]	10	8	10	8	9	9	10	9	10	10	9	10	10	7	10	7
Carbohydrate composition (mol %)																
Arabinosyl	5	5	5	5	5	5	5	5	6	5	6	5	6	5	5	6
Xylosyl	34	35	34	36	34	33	34	33	34	33	33	34	33	34	33	34
Mannosyl	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Galactosyl	2	1	1	1	2	1	2	1	2	1	2	1	2	1	2	2
Rhamnosyl	0	0	0	0	0	1	0	1	1	1	1	1	1	0	1	1
Glucosyl	57	53	54	52	56	55	56	55	56	55	57	54	57	54	55	52
Uronic acid	3	4	4	5	3	4	3	4	3	4	3	3	3	4	3	5
Degree of Substitution (DS)																
DS Ara[h]	15	14	14	13	16	15	16	16	17	16	17	16	18	16	17	17
DS GlcA[h]	10	12	11	13	10	11	10	11	10	11	10	10	10	13	9	13

[a]PI-0: Phase I initial mixture, PI-1, PI-2, PI-3, PI-4, PI-5 is phase I compost after 1, 2, 3, 4 and 5 days of composting time.
[b]P: Presence of gypsum within the composting process, A: Absence of gypsum within the composting process.
[c]13: Wheat straw based compost from 2013, 15: wheat straw based compost from 2015.
[d]Weight percentage is based on dry matter of composting samples and corrected for the amount of gypsum added.
[e]Corrected for ash.
[f]Calculated values as 100 minus total carbohydrates, ash, total nitrogen and protein for the "2015" samples.
[g]Nitrogen to protein conversion factor of 6.25 was used; protein content does not include N-containing salts.
[h]DS Ara = mol Ara/100 mol Xyl; DS GlcA = mol GlcA / 100 mol Xyl; abbreviations: Ara, arabinosyl; xyl, xylosyl; GlcA, glucuronic acid.

The total carbohydrate content was measured through Phase I for compost obtained from both P-13 and A-13, showing no change from day 0 to day 5 (from 38 to 40 w/w % for P-13 and from 39 to 39 w/w % for A-13). Likewise, no changes were found for P-15 and A-15 from day 0 to day 5 (from 46 to 45 w/w % for P-15 and from 48 to 46 w/w % for A-15). Previously, Jurak et al. (2014) [5] and Iiyama et al. (1994) [25] reported 38 and 40 % of total carbohydrates in compost obtained at the end of Phase I, which is similar to our data.

The carbohydrate compositions (mol %) were similar for all samples obtained during Phase I. For all samples, glucosyl (52–57 mol %) and xylosyl (33–36 mol %) residues were the main carbohydrate constituents of the compost originating from the wheat straw cellulose and xylan. Additionally, arabinosyl (5–6 mol %) and glucuronyl residues (3–5 mol %) were analysed in all composts, most likely mainly present as xylan substituents as expected based on previously reported xylan structures [5]. The degree of substitution of xylosyl residues were found to be similar in our study compared to Jurak et al. (2014) [5] for P0-13 and P1-13.

In short, for all samples obtained through the composting Phase I, no major difference in their chemical composition was found regardless presence or absence of gypsum. Contents of

carbohydrates, lignin, ash, total nitrogen and proteins were not affected during Phase I, matching with Jurak et al,. (2015) [12] data. Previously, Li and Kim (2011) [24] also showed that low ammonia concentrations within pretreatments of corn stover has no effect on the composition of the material obtained. Those results are in accordance to our data.

4.3.4 Dry matter and carbohydrate recoveries and composition of WUS

The dry matter recovery and composition of WUS was analysed for all compost samples collected during Phase I. The yield and composition of all WUS is presented in Table 4.2. Recoveries of dry matter after water extraction were similar for composts obtained at the start and at the end of Phase I (74-82 % for w0 (P-13, P-15, A-13 and A-15) and 73-81% for w5 (P-13, P-15, A-13 and A-15). Previously, recovery of dry matter was reported to be 82 % [5] for end of Phase I being similar to our data.

Cellulose, analysed as glucan, and xylan remained completely in the WUS for all samples analysed, which indicated that none of the carbohydrates became water soluble during Phase I composting (Table 4.2). Like for the composts, the main constituents of WUS-carbohydrates were glucosyl (51-56 mol %) and xylosyl (33-39 mol %) residues and the main xylan substituents were arabinosyl (DS 14-16) and glucuronyl (DS 9-13; UA) residues. The type and amount of xylan substituents, reflected in the degree of substitution (DS) (Table 4.2), were neither affected by presence or absence of gypsum nor by the duration of Phase I.

To conclude, the recoveries of insoluble matter and carbohydrates, compositions of WUS and type and amount of xylan substituents was similar for composts obtained at the start and end of both the gypsum-free Phase I and the Phase I control process in presence of gypsum. Hence, the higher enzyme degradability observed for the compost-WUS-samples obtained at the end of Phase I and of the gypsum-free Phase I is not related to the analysed carbohydrate content and composition of the WUS-samples.

Table 4.2: Recoveries and chemical composition of water unsoluble solids (WUS) of wheat straw based phase I composts that have been supplemented (P) or not (A) with gypsum, based on dry matter (DM). Standard deviation for total carbohydrates content (w/w %) dm < 3.0.

wPI-0[a]				wP1-1[a]		wPI-2[a]		wPI-3[a]		wPI-4[a]		wPI-5[a]			
wP[b]		wA[b]		wP[b]	wA[b]	wP[b]	wA[b]	wP[b]	wA[b]	wP[b]	wA[b]	wP[b]		wA[b]	
wP0-13[c]	wP0-15[c]	wA0-13[c]	wA0-15[c]	wP1-13[c]	wA1-13[c]	wP2-13[c]	wA2-13[c]	wP3-13[c]	wA3-13[c]	wP4-13[c]	wA4-13[c]	wP5-13[c]	wP5-15[c]	wA5-13[c]	wA5-15[c]
WUS recoveries (%)															
Total DM															
82	74	82	76	81	81	80	81	81	80	80	80	81	74	80	73
Total carbohydrates															
95	93	95	91	96	102	95	107	106	102	106	109	104	96	100	90
Glucan															
94	91	94	89	95	99	95	107	103	102	101	111	101	94	98	92
Xylan															
97	97	95	97	93	107	97	110	110	107	110	107	107	102	107	93
Content (% w/w) DM[d]															
Total carbohydrates															
45	58	44	58	47	51	47	51	50	49	51	51	51	58	49	57
Glucan															
26	32	26	32	28	29	28	30	29	29	30	30	30	33	29	33
Xylan															
13	18	13	20	14	15	14	15	15	15	15	15	15	18	15	18
Arabinan															
2.3	2.8	2.2	3.2	2.4	2.6	2.4	2.5	2.7	2.6	2.6	2.7	2.6	2.7	2.6	3.3
Carbohydrate composition (mol %)															
Arabinosyl															
5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	6
Xylosyl															
34	36	34	39	33	35	35	34	35	35	34	33	34	36	35	36
Mannosyl															
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Galactosyl															
1	2	1	1	1	1	1	1	1	1	1	1	1	2	1	1
Rhamnosyl															
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Glucosyl															
55	52	54	51	56	54	56	55	54	54	54	55	55	53	54	53
Uronic acid															
4	4	4	3	4	4	4	4	3	3	4	4	4	3	3	3
Degree of substitution (DS)															
DS Ara[e]															
15	13	15	14	15	15	15	14	16	15	15	16	15	13	15	16
DS GlcA[e]															
13	10	13	8	12	11	11	11	9	10	12	11	11	9	9	9

[a]wPI-0: water unsoluble solids Phase I initial mixture, wPI-1, wPI-2, wPI-3, wPI-4, wPI-5 is water unsoluble solids Phase I compost after 1, 2, 3, 4 and 5 days of composting time.

[b]P: Presence of gypsum within the composting process, A: Absence of gypsum within the composting process.

[c]13: Wheat straw based compost from 2013, 15: wheat straw based compost from 2015.

[d]Weight percentage is based on dry matter of composting phases and corrected for the amount of gypsum added.

[e] DS Ara = mol Ara/100 mol Xyl; DS GlcA = mol GlcA / 100 mol Xyl; abbreviations: Ara, arabinosyl; xyl, xylosyl; GlcA, glucuronic acid.

4.3.5 Lignin analysis of WUS compost by pyrolysis GC-MS

Compost samples in presence and in absence did not show differences in term of overall compositions and contents. A detailed lignin fingerprint obtained using pyrolysis GC-MS was used to investigate whether differences within the lignin component from the two experiments during Phase I were

obtained. Pyrolysis GC-MS was performed in triplicate on all WUS obtained during Phase I from compost in presence and in absence of gypsum (wP-13 and wA-13) from day 0 to day 5. The identities and relative abundances (average of triplicates) of the compounds formed upon pyrolysis are shown in Table 4.3. The pyrolysis of untreated wheat straw released similar compounds (derived from carbohydrates and lignin) as compost-WUS-samples, although in different proportions [26], and similarly as previously reported by Jurak et al. (2015) [12]. Interestingly, the sum of the relative abundances of the compounds derived from lignin decreased in WUS obtained from day 0 to day 5; 42.8 to 33.9 % for w0P-13 to w5P-13 and 46.9 to 33.1 % for w0A-13 to w5A-13. The main decrease was observed from day 0 to day 1 from 42.8 to 35.6 % and from 46.9 to 34.1 % (for wP-13 and wA-13, respectively) and then remained stable around the value of day 1. In a previous study, the composting process was evaluated for its mass balance showing minor losses of lignin content during composting Phase I [12]. The later research published a decrease in the total relative pyrogram area from 86 % ± 7 for Phase I-day 0 to 70 % ± 7 for end of Phase I. Those results are in accordance with the decrease of the relative abundance of lignin within our samples.

Table 4.3: Identity and relative abundance (average of triplicates) of the compounds obtained upon pyrolysis GC-MS of WUS wheat straw-based compost in presence (wP-13) and in absence (wA-13) of gypsum after 0, 1, 2, 3, 4 and 5 days of Phase I.

Based on Pyrolysis GC-MS	wP0-13	wP1-13	wP2-13	wP3-13	wP4-13	wP5-13	wA0-13	wA1-13	wA2-13	wA3-13	wA4-13	wA5-13
% Carbo-hydrates[a]	57.2 ±2.3	64.4 ±2.1	64.5	62.9	67.7	66.1	53.1	65.9	65.3	65.1 ±3.5	66.5 ±2.3	67.0 ±3.1
% Lignin	42.8	35.6	35.5	37.1	32.3	33.9	46.9	34.1	34.7	34.9	33.5	33.0
Ratio Lignin / Carbohydrate	0.7	0.6	0.6	0.6	0.5	0.5	0.9	0.5	0.5	0.6	0.5	0.5
SUM H[b]	33.9	30.8	31.7	29.8	30.6	31.7	28.0	32.8	34.0	31.5	30.7	30.8
SUM G[b]	47.7	49.2	49.1	48.8	48.4	47.1	48.3	50.0	46.3	48.5	48.6	48.7
SUM S[b]	18.5	20.0	19.2	21.4	21.0	21.2	23.7	17.2	19.7	20.0	20.7	20.5
Ratio Syringyl / Guaiacyl	0.39	0.41	0.39	0.44	0.43	0.45	0.49	0.34	0.43	0.41	0.42	0.42
Ratio Syringyl / Guaiacyl $_{except\ vinylguaiacol}$[c]	0.75	0.76	0.78	0.79	0.81	0.84	0.91	0.68	0.80	0.75	0.77	0.74
% Cα-unsubstituted lignin	37.9	33.7	39.4	37.0	44.3	46.2	43.2	40.1	42.2	43.4	44.2	44.6
% Cα-methylated lignin	7.7	7.5	8.2	7.9	7.8	8.7	6.3	7.3	7.8	8.1	7.7	8.2
% Cα-vinyl lignin	51.4	51.8	52.2	48.2	49.8	49.8	48.4	53.5	51.5	49.2	49.5	47.9
% Cα-oxidized lignin	7.3	8.7	8.5	9.8	9.1	8.9	7.2	7.9	9.1	9.3	9.4	9.8
% Cα-oxidized G-units	4.5	5.3	5.2	5.7	5.4	5.3	4.1	4.9	5.5	5.8	5.4	5.9
% Cα-oxidized S-units	2.9	3.4	3.3	4.1	3.7	3.6	3.1	2.9	3.6	3.5	3.9	3.9

[a] Standard deviation of the % of carbohydrates were < 1.5, except shown differently.
[b] Standard deviations of the sum of H, G and S compounds were < 1.5, except shown differently.
[c] All G and S derived peaks were used for the estimation of S:G ratio except 4-vinylguaiacol which also can arise from ferulates.

Lignin derived compound can be classified according the three main monolignols units: *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) [27, 28]. Furthermore, lignin-derived H, G or S units can be classified according to their aromatic-ring substituent such as Cα-unsubstituted lignin (e.g. guaiacol), Cα-methylated lignin (e.g. 4-methylguaiacol), Cα-vinyl lignin (e.g. 4-vinylguaiacol) or Cα-oxidized lignin (e.g. vanillin) [27, 28].

The composting process had different effects on lignin H, G and S units. The sum of H was similar after 5 days for wP5 and wA5 (31.7% and 30.8 %, respectively) even if the sum of H of wP0 was 33.9% and 28.0 for wA0. The sum of G remained stable from day 0 to day 5 for both tunnels, ranging from 47.7

to 47.1 % for wP and from 48.3 to 48.7 % for wA. Similarly to H units, the sum of S was similar after 5 days for wP5 and wA5 (21.2 % and 20.5 %, respectively) even if the sum of H of wP0 was 18.5 % and 23.7 % for wA0. Comparing both tunnels, no major differences could be found concerning the sum of H, G and S.

Unlike the type of lignin unit (H, G and S) abundance, the functional group attached to the aromatic units were affected over the process (Table 4.3). The percentages representing Cα-unsubstituted lignin decreased from day 0 (37.9 and 43.2 % for wP0 and wA0, respectively) to day 1 (33.7 and 40.1 % for wP1 and wA1, respectively) to increase again until day 5 (46.2 and 44.6 % for wP5 and wA5, respectively). Cα-methylated lignin and Cα-oxidized lignin increased from day 0 to day 5. The percentage of Cα-vinyl lignin, on the contrary, showed a minor decrease from day 0 to day 5. No major differences can be observed between wP-13 and wA-13 among lignin-substituents distribution.

Most pronounced was the decrease in 4-vinylphenol (compound 20) and 4-vinylguaiacol (compound 25), that can be derived from *p*-coumarates and ferulates [29, 30], respectively, from day 0 to day 5 (from 10.6 to 8.0 % and from 10.0 to 7.7 % for wP-13 and wA-13, respectively) for 4-vinylphenol and for 4-vinylguaiacol (from 9.9 to 7.5 % and from 10.4 to 6.9 % for wP-13 and wA-13, respectively) (supplementary Table 4.3). The later decrease may relate to a decrease in ester-linkages between xylan and lignin as previously described by Liu et al. (2013) [22] as a result of high ammonia concentration (>10 w/w %) treatment (temperature > 100 °C). The same was observed by Murciano et al. (2016) [31] for NaOH treated sugar cane bagasse showing a decrease of ester linked *p*-coumarates and ferulates , which positively affect enzymatic hydrolysis. Our data confirm our hypothesis that higher pH increased enzymatic hydrolysis, expectedly by opening the xylan-lignin structure by breaking ester-linked bounds.

4.4 Conclusions

In this study, a composting Phase I process was conducted in absence of gypsum and compared to the control process in presence of gypsum. Optimal compost properties were reached after 2 and 4 days in absence of gypsum compared to 3 and 5 days in the control. Glucan and xylan enzymatic degradation yields (34 and 23 %, respectively) were reached 1 day earlier in absence of gypsum. These results show the potential of a faster “gypsum-free” alternative for Phase I. Additionally, composting Phase I was confirmed to be crucial leading to a decrease of 4-vinylphenol and 4-vinylguaiacol that can be associated with *p*-coumarates, and ferulates linking lignin and xylan. Thus, authors recommend to introduce gypsum within the process by the end of Phase I accordingly to Phase II condition requirements.

Acknowledgements

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Additional files

Supplementary Table 4.1: Moisture content (w/w % dry matter based) measured during the first 5-days of the composting process (Phase I) from a wheat straw-manure based mixture. (STD < 0.1)

w/w %	PI-0		PI-1		PI-2		PI-3		PI-4		PI-5	
	P0-13	A0-13	P1-13	A1-13	P2-13	A2-13	P3-13	A3-13	P4-13	A4-13	P5-13	A5-13
Moisture per dry matter	74.09	75.18	73.53	74.30	72.20	73.35	73.63	74.07	73.18	73.60	72.76	73.65

Supplementary Table 4.2: Water binding capacity of WUS compost samples expressed as ratio of water bound per total carbohydrates (std < 0.1), per total glucans (std < 0.1) and per total xylan (STD< 0.2).

	PI-0		PI-1		PI-2		PI-3		PI-4		PI-5	
	wP0-13	wA0-13	wP1-13	wA1-13	wP2-13	wA2-13	wP3-13	wA3-13	wP4-13	wA4-13	wP5-13	wA5-13
Water bound per total carbohydrates	1.6	1.7	1.7	1.6	1.7	1.5	1.6	1.6	1.5	1.5	1.6	1.5
Water bound per total glucan	2.8	2.9	2.8	2.8	2.9	2.6	2.7	2.7	2.7	2.5	2.7	2.7
Water bound per total xylan	5.6	5.6	5.7	5.2	5.7	5.1	5.2	5.2	5.2	5.1	5.4	5.0

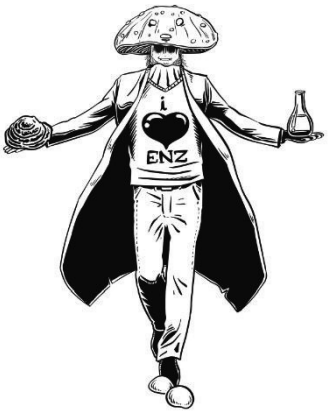
Supplementary Table 4.3: Identities and relative abundance (average of triplicates) of the compounds obtained and detected upon pyrolysis GC-MS of WUS wheat straw-based compost in presence (wP-13) and in absence (wA-13) of gypsum after 0, 1, 2, 3, 4 and 5 days of Phase I.

Label	compound	Origin[a]	wP0-13	wP1-13	wP2-13	wP3-13	wP4-13	wP5-13	wA0-13	wA1-13	wA2-13	wA3-13	wA4-13	wA5-13
1	2-methylfuran	C	0.4	0.2	0.2	0.2	0.2	0.2	0.3	0.0	0.4	0.1	0.2	0.1
2	Furfural	C	8.7	7.7	8.6	8.0	8.8	8.5	8.6	7.9	6.9	8.1	8.3	8.2
3	2-furanmethanol	C	1.2	0.7	0.9	0.8	0.8	1.0	1.7	0.8	0.7	0.8	0.8	0.9
4	2-acetylfuran	C	0.3	0.2	0.2	0.2	0.2	0.2	0.4	0.1	0.2	0.2	0.2	0.2
5	2,3-dihydro-5-methylfuran	C	7.5	4.2	6.1	5.8	5.5	6.5	11.6	5.4	5.6	6.2	5.3	5.9
6	2(5H)-furanone	C	5.1	2.3	3.2	2.9	3.0	3.1	7.1	3.1	3.6	3.1	3.5	3.4
7	5-ethyl-2-furaldehyde	C	0.3	0.2	0.3	0.3	0.3	0.3	0.2	0.2	0.3	0.3	0.3	0.3
8	5-(hydroxymethyl)dihydro-2(3H)-furanone	C	1.2	0.6	0.8	0.7	0.6	0.8	2.0	0.7	1.1	0.8	0.8	0.9
9	1,4-anhydroarabinofuranose	C	1.6	1.7	2.1	2.1	2.1	1.9	1.2	1.9	2.1	2.2	2.2	2.5
10	5-(hydroxymethyl)-2-Furancarboxaldehyde	C	1.7	2.5	2.3	2.6	2.3	2.4	1.1	2.2	2.4	2.3	2.5	2.6
11	1,6-anhydro-β-D-glucopyranose (levoglucosan)	C	8.4	15.3	14.3	13.9	15.2	15.3	5.0	14.8	16.8	13.7	13.9	14.6
12	2,4-dihydropyran-3-one	C	0.4	0.4	0.4	0.3	0.4	0.4	0.5	0.5	0.6	0.4	0.4	0.4
13	5-hydroxy-2-tetrahydrofuraldehyde-3-one	C	0.9	1.5	1.4	1.5	1.4	1.5	0.4	1.4	1.7	1.6	1.6	1.5
14	1,4-anhydroxylofuranose	C	1.6	2.9	2.6	2.5	2.9	2.6	1.2	2.6	2.5	2.4	2.6	2.8
15	4-hydroxy-5,6-dihydro-2H-pyran-2-one	C	17.9	24.1	21.1	21.2	24.0	21.2	11.8	24.2	20.3	22.7	24.0	22.7
16	Phenol	H	2.2	1.1	1.6	1.5	1.4	1.6	2.1	1.5	1.7	1.7	1.5	1.4
17	2-methylphenol (o-cresol)	H	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18	4-methylphenol (p-cresol)	H	1.3	0.6	0.9	0.8	0.7	0.9	0.8	0.7	0.9	0.8	0.7	0.8
19	4-ethylphenol	H	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
20	4-vinylphenol	H / PCA	10.6	9.0	8.5	8.5	7.6	8.0	10.0	8.7	9.0	8.3	7.9	7.7
21	Hydroquinone	H	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22	Guaiacol	G	3.1	1.9	2.2	2.3	2.1	2.4	4.4	2.1	2.3	2.4	2.3	2.2
23	4-methylguaiacol	G	1.3	1.3	1.3	1.4	1.2	1.3	1.4	1.2	1.2	1.4	1.3	1.3
24	4-ethylguaiacol	G	0.4	0.4	0.3	0.3	0.3	0.3	0.4	0.3	0.4	0.4	0.4	0.4
25	4-vinylguaiacol	G / FA	9.9	8.1	8.7	8.0	7.2	7.5	10.4	8.4	7.6	7.6	7.4	6.9
26	Eugenol	G	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.3	0.2	0.2
27	Vanillin	G	1.1	1.1	1.0	1.2	0.9	0.9	0.9	0.9	1.0	1.1	1.0	1.0
28	Acetovanillone	G	0.5	0.5	0.5	0.6	0.5	0.6	0.5	0.5	0.6	0.6	0.5	0.6
29	Guaiacylacetone	G	0.3	0.3	0.3	0.3	0.2	0.3	0.5	0.3	0.4	0.3	0.3	0.3
30	*cis*-isoeugenol	G	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.1
31	guaiacyl vinyl ketone	G	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1
32	*cis*-coniferyl-alcohol	G	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
33	*trans*-coniferyl-alcohol	G	2.7	2.8	2.0	2.8	2.1	1.8	3.1	2.3	1.6	2.0	2.2	2.2
34	*trans*-coniferaldehyde	G	0.3	0.4	0.4	0.4	0.4	0.3	0.3	0.4	0.4	0.4	0.4	0.4
35	Syringol	S	1.6	1.2	1.2	1.3	1.2	1.4	3.1	1.1	1.2	1.2	1.2	1.2
36	4-methylsyringol	S	0.7	0.7	0.7	0.7	0.6	0.7	0.8	0.5	0.6	0.7	0.6	0.6
37	4-ethylsyringol	S	0.2	0.1	0.2	0.2	0.1	0.2	0.3	0.1	0.1	0.1	0.1	0.2
38	4-vinylsyringol	S	1.5	1.3	1.3	1.5	1.3	1.4	2.3	1.1	1.3	1.3	1.3	1.2
39	4-allyl-2,6-dimethoxyphenol	S	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2
40	Syringaldehyde	S	0.4	0.5	0.4	0.6	0.5	0.5	0.5	0.4	0.5	0.5	0.6	0.5
41	Acetosyringone	S	0.5	0.5	0.5	0.6	0.5	0.5	0.6	0.4	0.5	0.5	0.5	0.5
42	Syringylacetone	S	0.3	0.2	0.2	0.3	0.2	0.2	0.4	0.2	0.2	0.2	0.2	0.2
43	*cis*-2,6-dimethoxy-4-propenylphenol	S	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1
44	1-(3,5-dimethoxy-4-hydroxyphenyl)propyne	S	0.1	0.2	0.1	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.1
45	*trans*-2,6-dimethoxy-4-propenylphenol	S	1.0	0.9	0.9	1.0	0.8	1.0	1.2	0.7	0.9	1.0	0.8	0.8
46	Homosyringaldehyde	S	0.2	0.2	0.2	0.1	0.3	0.1	0.2	0.2	0.3	0.2	0.2	0.2
47	*cis*-sinapyl-alcohol	S	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1
48	*trans*-sinapyl-alcohol	S	0.9	0.8	0.5	0.9	0.6	0.5	1.1	0.5	0.5	0.5	0.6	0.6
49	*trans*-sinapaldehyde	S	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

[a] C, carbohydrate-derived compound; H, *p*-hydroxycinnamyl lignin-derived compounds; G, guaiacyl lignin-derived compounds; S, syringyl lignin-derived compounds; PCA, *p*-coumarates; FA, ferulates

CHAPTER 5

Low liquid ammonia treatment of wheat straw increased carbohydrate degradability and decreased residual hydroxycinnamic acids



Mouthier, TMB, de Rink, B, van Erven, G, de Gijsel, P, Schols, HA, Kabel, MA, Low liquid ammonia treatment of wheat straw increased carbohydrate degradability and decreased residual hydroxycinnamic acids. S*ubmitted*

Abstract

Ammonia treatment of lignocellulose has frequently been studied resulting in improved degradability of the carbohydrate fraction. Mechanisms and effects during *low* ammonia dose treatment, however, are hardly considered. Even low ammonia dose treatment is expected to already loosen the lignocellulosic architecture and can possibly be applied during biomass storage. Hence, in this study the mechanisms involved during ammonia treatment of wheat straw were investigated, in particular at low doses of ammonia. In detail, a statistical design of experiments (Taguchi design) was performed to independently evaluate the effects of the three parameters (ammonia concentration, treatment time (hours) and the Solid:Liquid ratio (S:L)) on structure, composition and enzymatic degradability of the residual fractions. The results showed that low ammonia concentration (≤2 w/w % NH_3) resulted in a high carbohydrate recovery (> 80%) coupled to the solubilisation of 50% of total xylan and 40% of total glucan after enzymatic hydrolysis of the treated material using (hemi-)cellulase enzyme cocktail. This effect might result from the relative decrease in hydroxycinnamic acids analysed via pyrolyis-GC-MS. Of the sum of pyrolysis-GC-MS lignin related compounds, ferulic acid decreased by 10% and coumaric acid more than 50%, measured as 4-vinylphenol and 4-vinylguaiacol, respectively. Our findings show that the parameter effecting the residual fraction the most was the ammonia concentration even though treatment time was seen important for enzymatic degradability as well.

5.1 Introduction

Wheat straw is a main agricultural by-product worldwide [1]. This lignocellulosic biomass represents a great potential to be further used for production of biochemicals, biofuels and, also, for mushroom production [2, 3]. Hereto, first, a (thermo-) chemical treatment is a prerequisite to open up the material enabling further microbial or enzymatic treatment [3]. The composition of wheat straw has been well described, and include cellulose (33-40 w/w %), hemicellulosic xylan (20-25 w/w %) and lignin (15-20 w/w %) [4].

Microbial or enzymatic treatment, which focusses on conversion of the carbohydrate fraction, has been intensively studied and reviewed [3, 5-8]. Acid driven (thermo-) treatments, for example using sulphuric or acetic acid for catalysis, are well understood to increase enzymatic degradability. For these treatments the relationship between pH, temperature and treatment time has successfully been demonstrated to combine in a 'combined severity factor (CSF)' [9]. The CSF again was shown to correlate well with an increase in hemicellulose solubility, increasing the cellulose accessibility for enzymes [10, 11]. Nevertheless, such acid treatments have as drawbacks that they require corrosive acids and large amounts of water enabling separation of soluble from insoluble fractions. In this respect, ammonia catalysed (thermo-) treatments at relatively high solid to liquid ratios have an advantage. Ammonia is a cheap and widely used chemical in industries, (e.g. food-, pharmaceuticals- or chemicali) [12], and is recoverable for reuse [13]. Furthermore, such ammonia treatments have shown to lead, even at mild temperatures (120 °C) to: 1) the decrease of lignin rather than xylan in the residual fractions; 2) the increase of the enzymatic hydrolysis of residual carbohydrates [14, 15]. Additionally, lower temperatures (80 °C), low-liquid and low ammonia loading (1-2 w/w %) treatments in an industrial composting setting have also been shown to enhance subsequent microbial and enzymatic conversion, while lignin contents even remain similar [16]. Such industrial treatment of wheat straw is the first process phase resulting in a selective material to grow mushrooms on (Mouthier et al., 2017).

In an ammonia treatment of corn stover performed at high ammonia loadings (6:1 ammonia : biomass weight ratio), it was reported that approximatively 0.022 g ammonia per 100 g biomass (equals 2.2 w/w % ammonia loading) input could not be recycled due to reactions between ammonia and biomass, however, these reactions were not proven [14]. In the same study, it was shown that lignin ester-bonds and ether bonds (β-aryl ether bond) within lignin were cleaved resulting into delignification of biomass (35-45 % for 70-120 °C) increasing glucan conversion. In grass lignin, the β-aryl ether linkage is the most predominant grass lignin inter-linkage [17] (approx. 80% in isolated wheat straw lignin [18]), while alkaline labile ester-bonds link carbohydrates and lignin in so-called ester-linked lignin-carbohydrate complexes (LCCs) [19, 20]. In addition, grass LCCs are also reported to link xylan to lignin via ether bonds [21, 22]. Cleavage of ester bonds in both lignin and in LCCs is expected to open up the wheat straw structure considerably, increasing accessibility for enzymes.

Nevertheless, these reported effects of ammonia treatments on the lignin and LCC structure are not known for treatments at lower ammonia loadings in combination with high dry matter contents. To the best of our knowledge, the relationship between the mechanisms involved during low ammonia (thermo-) treatment and three main condition parameters being ammonia loading, treatment time and solid to liquid ratio to explain further enzymatic degradability is unknown.

Hence, in this study, the effect of three parameters (ammonia concentration, time, solid to liquid ratio) on the lignin structure and resulting enzymatic carbohydrate degradability was investigated. All experiments were conducted at 80 °C. The experiment was designed according to the Taguchi method, performing a set of 36 experiments, which allows to statistically define the parameter influencing the most the characteristics of the treated wheat straw. Results comprise carbohydrate composition, mass balance, enzymatic degradability, and lignin composition. Our study contributes to a further understanding of which parameter in low temperature ammonia treatment effects lignin and further enzymatic degradability of the carbohydrate fraction.

5.2 Material and methods

5.2.1 Material

Wheat straw was kindly provided by CNC (CNC Grondstoffen B.V., Milsbeek, The Netherlands). The wheat straw was milled with a Retsch ZM200 mill (Retsch, Haan, Germany), consecutively equipped with a 6, 4, 2 and 1 mm sieve. The straw was finally sieved through a 710 µm sieve. The wheat straw used contained 69.6±2.8 w/w % of total sugars (38.7±1.5 w/w % cellulose, 29.3 w/w % arabinoglucuronoxylan (23.3±0.9 w/w % xylosyl residues, 3.0±0.2 w/w % arabinosyl residues and 3.0±0.1 w/w % uronic acids), 1.6±0.1 w/w % other carbohydrates), in addition to 20.5±0.5 w/w % (corrected for ash) of Klason lignin, 1.1±0.1 w/w % protein and 3.6%±0.1 w/w % ash. Carbohydrates, proteins, ash and lignin were analysed as described previously [16].

5.2.2 Experimental design and low liquid ammonia (LLA) treatment

The effect of ammonia treatment conditions on wheat straw was evaluated by a minimum experimental set based on the Taguchi method [23]. The three parameters investigated were designated as follows: A: Ammonia loading w/w % (0, 0.2, 1, 2 and 20 %); B: treatment time (24, 48, 72, 96 and 120 hours); and C: Solid to Liquid ratio (S:L) (1:4 (L), 1:6 (M) and 1:10 (H)). The letter chose for S:L ratio (L, M and H) were given according to the amount of liquid added within the treatment. The parameters and levels are summarized in Table 5.1.

Table 5.1: Low liquid ammonia treatment parameters (A-C) designated for wheat straw treatments and their levels. Parameter A and B were performed using 5 levels while parameter was performed using only 3.

Parameter	Level				
	1	2	3	4	5
A: Ammonia loading (w/w %)[a]	0	0.2	1	2	20
B: Treatment time (hours)	24	48	72	96	120
C: Solid to Liquid ratio (S:L)[b]	1:4	1:6	1:10		

[a] Ammonia (w/w %) = g ammonia/ 100 g dry matter wheat straw.
[b] Solid to Liquid ratio = wheat straw dry mass (g) : (ammonia (g) + water (g)).

An L(25) orthogonal array was used to conduct the set of experimental conditions. To the 25 designed experiments, 11 additional conditions were added to cover lower ammonia concentration parameter (0.2 and 1 w/w % NH_3). Conditions chosen for all experiments are shown in Table 5.2. Each treatment was conducted in duplicate, and performed in 50 mL glass bottles tightly sealed with screwcaps and heated at 80 °C in a heating block (Dry bath, Profilab, Jena, Germany).

5.2.3 Separation of water soluble from water insoluble material

First, ammonia was removed of the treated wheat straw samples by blowing a stream of air for one hour. Subsequently, samples were washed 5 times with 50 mL water, centrifuged (10,000 *g*, 15 min) and supernatants were combined and immediately frozen. Washed residues were freeze dried (WUS) and subjected to enzymatic hydrolysis (see 5.2.4). Freeze dried WUS was analysed for neutral sugar content and composition, uronic acid content, and subjected to semi-quantitative to pyrolysis GC-MS for the estimation of lignin content and composition.

5.2.4 Enzyme hydrolysis

WUS samples (25 mg) were suspended in 1 mL 50 mM sodium acetate buffer (pH 5.0) containing 0.2 mg mL^{-1} sodium azide. Incubations were started by the addition of 2.7 w/w % CellicCTec2 ((protein per dry matter (dm); CellicCtec2 protein content 127 mg mL^{-1}) (Novozymes, Bagsværd, Denmark)) and 0.3 w/w % CellicHTec ((protein per dm); CellicHTec protein content 120 mg mL^{-1}) (Novozymes, Bagsværd, Denmark)). Samples were incubated for 24 h at 50°C in a head–over-tail rotating device. Enzymes were inactivated (10 min at 100 °C) prior to centrifugation (10,000 *g*, 5 min). The supernatants were collected and subjected to HPAEC for monosaccharide quantification. Enzyme treatment was performed in duplicate.

Table 5.2: Low liquid ammonia treatment conditions of wheat straw and experiment codes (ammonia (w/w %) / time (hours) / S:L ratio). Solid to Liquid ratio (S:L) is 1:4 (L), 1:6 (M) and 1:10 (H), according to a L(25) orthogonal array plus 11 extra conditions.

Code (NH_3 / Time / S:L ratio)	Treatment conditions		
	NH_3 w/w %	Treatment time (hours)	S:L ratio
0/0/L	0.0	0	1:04
0/24/L	0.0	24	1:04
0/120/L	0.0	120	1:04
0.2/24/L	0.2	24	1:04
0.2/48/L	0.2	48	1:04
0.2/72/L	0.2	72	1:04
0.2/96/L	0.2	96	1:04
1/24/L	1.0	24	1:04
1/72/L	1.0	72	1:04
1/96/L	1.0	96	1:04
1/120/L	1.0	120	1:04
2/96/L	2.0	96	1:04
20/24/L	20.0	24	1:04
20/72/L	20.0	72	1:04
20/96/L	20.0	96	1:04
20/120/L	20.0	120	1:04
0/0/M	0.0	0	1:06
0/48/M	0.0	48	1:06
0/96/M	0.0	96	1:06
0.2/72/M	0.2	72	1:06
1/48/M	1	48	1:06
1/96/M	1	96	1:06
2/120/M	2	120	1:06
20/24/M	20.0	24	1:06
20/72/M	20.0	72	1:06
20/96/M	20.0	96	1:06
0/0/H	0.0	0	1:10
0/72/H	0.0	72	1:10
0.2/96/H	0.2	96	1:10
0.2/120/H	0.2	120	1:10
1/120/H	1.0	120	1:10
2/24/H	2.0	24	1:10
2/48/H	2.0	48	1:10
2/72/H	2.0	72	1:10
20/48/H	20.0	48	1:10
20/120/H	20.0	120	1:10

5.2.5 Analytical methods

Analyses were carried out in duplicate for every duplicate treatment. The averages shown for carbohydrate analysis (content, composition and recovery) of one treatment is the average of analytical duplicates of the duplicate treatment with corresponding standard deviation ($std=\sqrt{(std_1^2+std_2^2)}$). The averages shown for the enzymatic hydrolysis from one treatment is the average of a duplicate hydrolysis performed for the duplicate treatment (4 hydrolysis per treatment setting) and the corresponding standard deviation ($std=[std_1+std_2+std_3+std_4]/4$). The averages shown for lignin composition of one treatment is the average of analytical triplicates of the duplicate treatment, including the standard deviation ($std=\sqrt{(std_1^2+std_2^2)}$).

5.2.5.1 Neutral sugar content and composition

The neutral sugar content and composition was determined in duplicate according to Englyst and Cummings (1984) [24], using inositol as an internal standard. Samples were treated with 72% (w/w) H_2SO_4 (1 h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C, uronic acids released were analysed. The constituent sugars released were analysed as their alditol acetates using gas chromatography (ThermoScientific, Waltham, MA, USA). Total carbohydrate content was calculated as the sum of neutral carbohydrates and uronic acids.

5.2.5.2 Uronic acid content

Uronic acid content was determined in duplicate as anhydro-uronic acid content by an automated m-hydroxydiphenyl assay [25] with addition of sodium tetraborate using an autoanalyser (Skalar Analytical BV, Breda, The Netherlands). Glucuronic acid (Fluka AG, Busch, Switzerland) was used as a reference (0-100 $\mu g\ mL^{-1}$).

5.2.5.3 High performance anion exchange chromatography

High performance anion exchange chromatography (HPAEC) was performed on a Dionex ICS-5000 unit (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (2 mm x 250 mm ID) in combination with CarboPac guard column and PAD detection (Dionex). The system was controlled by Chromelion software (Thermo Scientific, Sunnyvale, CA, USA). Elution and quantification of mono- and oligosaccharides was performed at 0.3 mL min^{-1} with a combination of 3 eluents, A: 0.1M NaOH; B: 1M NaOAc in 0.1M NaOH; C: H_2O. The elution profile for the monosaccharides was as follows: 0-30 min 15 % A and 85 % C, 30-35 min 100 % B, 35-45 min 100 % A, 45-60 min 15 % A and 85 % C. The elution profile for the oligosaccharides was as follows: 0-35 min: 0-38 % B mixed in A, 35-38 min 100 % B, 38-50 min 100 % A. For quantification, glucose, xylose, xylo-oligosaccharides (XOS) with a degree of polymerization (DP) of 2 to 4 (Megazyme, Wicklow, Ireland) and glucuronic acid were used for calibration in at least 4 increasing concentrations between 5 and 30 $\mu g\ mL^{-1}$.

5.2.5.4 Analytical pyrolysis GC-MS analysis

Pyrolysis of 100 µg WUS, weighed on a Mettler-Toledo XP6 microbalance (Mettler-Toledo, Columbus, US), was performed with an EGA/PY-3030D micro-furnace pyrolyzer (Frontier Laboratories, Fukushima, Japan), equipped with an AS-1020E Autoshot auto-sampler. The pyrolyzer was connected to a Thermo7820A gas chromatograph using a DB-1701 fused-silica capillary column (30 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 pyrolysis was performed at 500° C for 1 min, with an interface temperature of 300 °C. The GC 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}). Compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and with those reported in literature [26, 27]. Pyrograms were processed by AMDIS software according to [26]. Relative response factors (RRF) of the lignin-derived pyrolysis products were determined as described by [18], where AMDIS software was used for processing instead of Xcalibur software. Areas (A) were divided by the corresponding RRF and summed to give the total RRF corrected area. Relative abundance (mol %) of each compound was calculated by ($A_{RRF\ corrected\ compound}/A_{RRF\ corrected\ total}$*100). For estimating the lignin content, RRF corrected areas per compound were multiplied by the respective molecular weight and summed. The summed area was correlated to the total area of a wheat straw sample with known Klason lignin content (corrected for ash) (20.5 w/w %) [18]). Note that in these analyses the compounds trans/cis-coniferyl alcohol and trans/cis-sinapyl alcohol were not included, since their detection was severely affected by system performance as previously indicated by [27].

5.2.5.5 Calculation of Signal to Noise (S/N) according to the Taguchi method

All results collected from every duplicate treatment and analysis performed were subjected to calculation of the signal to noise (S/N) ratio based on the Taguchi method. Hence, for this S/N calculation, no averages of results were taken, but each single result was considered to include result deviations within the S/N calculation. The Taguchi method has originally been developed to optimize quality during goods manufacturing and is an appropriate method to evaluate experimental parameters within engineering and biotechnology [23, 28]. The S/N ratio is a measure of robustness, which is used to identify the control factor settings that minimize the effect of noise on the response. In our study, the S/N ratio was calculated according to the "nominal is best (II)" approach and calculated as Eq. (Equations 5.1, 5.2 and 5.3).The signal-to-noise (S/N) ratio is calculated for each factor level combination. The formula for the nominal-is-best (II) S/N ratio using base 10 log is:

Equation (5.1) Where,
$$SN_i = 10 \log \frac{\bar{\gamma}_i^2}{s_i^2}$$

Equation (5.2)
$$\bar{\gamma}_i = \frac{1}{N_i} \sum_{u=1}^{N_i} \gamma_{i,u}$$

Equation (5.3)

$$s_i^2 = \frac{1}{N_i - 1} \sum_{u=1}^{Ni} \left(\gamma_{i,u} - \bar{\gamma}_i\right)$$

With ***(i)*** being the number of experiments, ***(u)*** the trial number and ***(N_i)*** the number of trials for experiment ***i***

Finally, the average values of the SN ratio for each level of each of the parameter are calculated and differences of the maximum and minimum values is reported as the Delta. The higher the delta is, the greater the impact on the experiment.

To calculate which treatment-parameter had the largest impact, non-treated samples were not taken into account. SN ratios were calculated for every characteristic analysed: Mass balance recovery (%) (water unsoluble solids (WUS), water soluble solids (WSS), calculated losses) and carbohydrate recoveries (%) (glucan, xylan, arabinan, uronic acid), calculated lignin (lignin recovered WUS (%) and estimated lignin content w/w %), enzymatic hydrolysis (%) (glucans released from total glucans in WUS, xylan released from total xylans of WUS), WUS carbohydrate composition (mol%) (glucose, xylose, arabinose and uronic acid), lignin building block abundance (%) (*p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) building blocks), lignin groups abundance (%) (Cα-oxidised, vinyl, methyl + ethyl, and unsubstituted) and single lignin compound abundance (%) (4-vinylphenol and 4-vinylguaiacol).

5.3 Results and discussion

5.3.1 Effect of low liquid ammonia treatment (LLA) on dry matter and carbohydrate recovery

In this research, it was aimed to study which condition parameter of an ammonia treatment (at 80 °C) of wheat straw would affect the most the lignin structure and the enzymatic carbohydrate degradability. In particular low ammonia loadings (0–2 w/w %) were chosen to enable understanding and translating effects occurring within composting of wheat straw to produce a selective substrate for edible white button mushrooms [16].

First, the effect of the three main parameters on the recovery of dry matter (dm) and recovery of carbohydrates was analysed. Results are shown in Table 5.3. The recovery of dm in the solids (WUS) decreased at increasing ammonia concentration for all three different solid to liquid ratios (L, M and H) from around 85 % for non-treated (i.e. 0/0/L) or 24h treated samples (i.e. 0.2/24/L) to 75 % for samples treated at 20 w/w % ammonia (20/24/L, 20/120/L; Table 5.3). The decreased recovery of dm in the solids corresponded to an increase of dm in the liquid fractions for L, M and H ratios from 8% (0/0/L or 0.2/24/L) to 23 % (20/24/L). For all treatments of the lowest ammonia doses (0.2, 1 and 2

w/w % NH_3) the dm recoveries were comparable. Although less pronounced as for increasing doses of ammonia, longer treatment times also resulted in increased soluble dm recovery, for example from 20 % (20/24/M) to 24 % (20/96/M) or from 10 % (2/24/H) to 12% (2/72/H) (Table 5.3).

The carbohydrate recovery in the solids was, like for the dm, similar for 0.2, 1 and 2 w/w % NH_3 (i.e. glucan and xylan recoveries 80-90%) with no significant decreasing trend with increased dose of NH_3 concentration. Our data showed that at low ammonia doses the carbohydrates were recovered in the solids with overall losses of only 5% in the total mass balance. The trends in soluble versus insoluble glucan and xylan contents (w/w %) in our study, were comparable for a wheat straw composting process (80 °C; ammonia 1.2%) for 1 to 5 days [2, 16], as was aimed for. Other studies, for example evaluating the low-moisture anhydrous ammonia treatment (gaseous ammonia) of corn stover or aqueous-ammonia soaking (12-28 w/w % NH_3) of rice straw, also showed that there were no noticeable changes in carbohydrate contents (w/w %)[29, 30].

In particular for the treatments performed at 20 % w/w NH_3, glucan (from 90 to 70 %), xylan and arabinan (from 75 to 55 %) and glucuronic acid (70 to 60 %) recoveries in the solids decreased. Previous studies showed that glucan and xylan contents (w/w %) remain similar at increasing concentration of NH_3 (0.5-50 w/w %), treatment time and S:L ratio, while these studies did not focus on the recoveries [15, 31]. Ko et al. (2009) [29] found similar glucan recoveries in the solids (86-92 %) after 4-12 hours aqueous ammonia treatment (20 w/w %) of rice straw compared to the glucan recoveries after 24 h treatment of our study (88-91 % for 20/24/L, M and H).

In short, the dry matter and carbohydrate recoveries in the solids (WUS) after treatment were influenced mainly by an ammonia concentration > 2 % (in this study 20 w/w %), while both treatment time and the S:L ratio had no or little influence.

5.3.2 Effect of liquid ammonia treatment (LLA) on enzymatic glucan and xylan degradability of solids

Insoluble fractions (WUS; Solids) after low liquid ammonia treatment were subjected to enzymatic degradation by using a commercial (hemi-)cellulase cocktail. The enzymatic degradability in the solid fractions of either the cellulose or the xylan fraction is seen as a measure for the accessibility of the enzymes within the substrate and, hence, for the treatment efficiency.

For the treatments having a low S:L ratio (1:4), xylan degradability in the solids (Figure 5.1) increased from around 10 % for the treatment without addition of ammonia to around 20 % for the treatment performed at 1 w/w % ammonia (1/24/L, 1/72/L, 1/96/L, 1/120/L) and to more than 40 % for the treatment performed at 2 w/w % ammonia (2/96/L).

Table 5.3: Dry matter (dm) recovery in solids and liquids combined with calculated losses, and carbohydrate recovery in solids (WUS), of the 36 low liquid ammonia treatments of wheat straw. The treatment data were sorted in the following order; 1) from low (L) to high (H) Solid to Liquid ratio (S:L), 2) treatment time from 0 days up to 5 days, and 3) ammonia concentration from 0 up to 20 w/w % NH_3. Standard deviations combine the duplicate treatments and duplicate (carbohydrate) analyses.

code	Dry matter recovery %						Carbohydrate recovery in solids %[a]			
(NH_3/Time/ S:L ratio)	Solids		Liquids		Loss		Glucosyl	Xylosyl	Arabinosyl	Uronic acid
0/0/L	85.9	± 0.0	7.9	± 0.4	6.2	± 0.3	85.8 ± 1.2	85.0 ± 2.7	79.8 ± 1.6	78.7 ± 0.0
0/24/L	85.5	± 1.0	9.0	± 0.3	5.5	± 0.6	85.8 ± 1.2	80.1 ± 0.3	81.9 ± 1.1	87.4 ± 0.1
0/120/L	85.7	± 0.3	8.9	± 0.4	5.4	± 0.0	81.8 ± 0.6	81.6 ± 0.2	69.5 ± 2.4	79.1 ± 2.0
0.2/24/L	85.9	± 0.5	8.2	± 0.7	5.9	± 0.2	84.8 ± 3.4	82.5 ± 4.1	85.7 ± 7.6	86.2 ± 0.7
0.2/48/L	85.3	± 1.1	8.1	± 0.0	6.5	± 1.1	86.5 ± 0.1	80.8 ± 2.6	83.7 ± 2.1	85.4 ± 2.1
0.2/72/L	84.4	± 0.8	7.4	± 0.4	8.2	± 1.2	80.6 ± 5.5	82.1 ± 4.4	77.0 ± 4.1	75.0 ± 2.9
0.2/96/L	84.6	± 0.4	9.4	± 0.4	6.0	± 0.8	80.6 ± 2.0	81.5 ± 2.8	77.0 ± 5.5	84.9 ± 10.8
1/24/L	84.3	± 0.7	9.4	± 0.4	6.3	± 0.3	91.6 ± 0.8	89.8 ± 0.7	87.8 ± 0.7	85.2 ± 0.7
1/72/L	83.3	± 0.1	9.2	± 0.7	7.6	± 0.9	81.9 ± 1.4	83.5 ± 1.6	79.2 ± 2.5	73.2 ± 0.5
1/96/L	82.8	± 0.5	10.9	± 0.3	6.3	± 0.2	82.4 ± 1.5	79.0 ± 1.6	76.8 ± 1.0	101.6 ± 4.5
1/120/L	83.6	± 0.3	10.4	± 1.0	5.9	± 0.7	85.2 ± 7.0	82.3 ± 5.4	77.7 ± 4.0	76.6 ± 0.9
2/96/L	80.3	± 2.9	11.4	± 3.2	8.3	± 0.3	85.3 ± 2.9	78.3 ± 2.8	84.3 ± 3.3	76.9 ± 3.4
20/24/L	73.8	± 2.4	23.4	± 0.5	2.8	± 1.9	89.7 ± 8.5	69.4 ± 6.2	73.0 ± 3.8	70.8 ± 2.0
20/72/L	71.1	± 3.9	22.9	± 0.9	6.0	± 4.7	74.3 ± 3.2	60.2 ± 3.8	59.7 ± 2.2	63.4 ± 3.3
20/96/L	77.1	± 1.3	17.3	± 3.7	5.6	± 2.3	74.1 ± 3.4	57.2 ± 0.1	56.4 ± 1.8	67.4 ± 6.6
20/120/L	74.6	± 2.7	21.4	± 4.3	4.0	± 1.6	90.1 ± 3.4	76.3 ± 2.5	67.4 ± 1.3	78.7 ± 5.7
0/0/M	86.2	± 0.6	6.9	± 1.4	6.9	± 0.6	86.8 ± 0.8	82.9 ± 0.2	83.6 ± 1.1	86.4 ± 1.2
0/48/M	85.2	± 0.5	7.4	± 0.3	7.4	± 0.2	86.5 ± 5.3	82.7 ± 0.1	81.9 ± 2.7	83.2 ± 2.1
0/96/M	85.4	± 0.5	7.9	± 0.4	6.7	± 0.1	84.9 ± 2.3	85.7 ± 1.1	86.2 ± 1.6	86.1 ± 1.2
0.2/72/M	84.4	± 0.7	8.2	± 0.0	7.4	± 0.7	86.7 ± 2.1	89.7 ± 4.4	88.6 ± 6.3	84.3 ± 3.0
1/48/M	82.5	± 1.0	9.7	± 0.0	7.8	± 1.0	87.7 ± 0.8	85.1 ± 1.6	86.6 ± 0.9	82.8 ± 3.5
1/96/M	83.5	± 1.0	10.2	± 0.7	6.2	± 1.6	88.2 ± 2.6	85.5 ± 1.5	86.5 ± 3.4	81.6 ± 2.2
2/120/M	80.7	± 0.5	12.0	± 0.3	7.3	± 0.8	87.2 ± 2.8	85.0 ± 1.9	89.8 ± 0.3	79.8 ± 0.2
20/24/M	74.9	± 1.7	20.2	± 1.8	4.9	± 0.1	91.3 ± 4.1	70.5 ± 2.8	71.2 ± 3.0	68.0 ± 2.0
20/72/M	71.6	± 0.8	22.4	± 0.7	5.9	± 1.5	77.7 ± 5.0	57.8 ± 3.5	55.8 ± 4.2	56.6 ± 0.8
20/96/M	74.2	± 0.3	24.4	± 0.4	1.4	± 0.7	91.4 ± 9.8	72.0 ± 7.6	70.9 ± 6.7	64.1 ± 1.0
0/0/H	85.9	± 0.6	5.9	± 0.3	8.3	± 0.3	85.9 ± 4.1	85.9 ± 3.9	85.9 ± 6.6	85.9 ± 0.6
0/72/H	84.9	± 0.4	8.1	± 0.0	7.0	± 0.5	91.6 ± 0.1	86.7 ± 3.0	90.7 ± 5.0	85.3 ± 2.6
0.2/96/H	85.0	± 0.5	8.7	± 0.0	6.4	± 0.5	91.6 ± 0.8	90.6 ± 4.0	90.2 ± 6.4	85.1 ± 0.8
0.2/120/H	83.3	± 2.5	8.2	± 0.7	8.5	± 3.3	80.1 ± 2.4	75.4 ± 3.5	68.3 ± 0.4	84.9 ± 2.6
1/120/H	81.9	± 1.2	10.2	± n.a.[b]	8.1	± 0.8	82.1 ± 2.5	80.5 ± 0.9	76.0 ± 1.0	94.1 ± 3.3
2/24/H	82.0	± 0.5	10.4	± 0.4	7.5	± 0.1	88.3 ± 1.4	85.1 ± 2.7	92.1 ± 5.8	78.3 ± 1.8
2/48/H	80.8	± 2.0	11.9	± 0.4	7.3	± 1.6	86.6 ± 2.4	84.4 ± 3.9	87.2 ± 6.5	79.5 ± 3.0
2/72/H	79.7	± 0.2	12.5	± 0.3	7.8	± 0.5	85.0 ± 0.5	83.5 ± 0.3	89.8 ± 0.6	79.5 ± 4.2
20/48/H	70.7	± 1.4	21.6	± 0.3	7.7	± 1.7	72.3 ± 1.2	54.3 ± 0.7	53.2 ± 1.0	62.4 ± 2.2
20/120/H	76.3	± 0.3	22.3	± 0.2	1.4	± 0.5	88.2 ± 7.6	68.8 ± 5.5	70.8 ± 5.3	71.1 ± 0.2

[a] Carbohydrates presented as anhydro-sugars, recovered from originally present in untreated wheat straw

[b] N.a. = Not applicable, liquid duplicate treatment was lost.

Hence, even at such low ammonia concentrations (2 w/w %), an intriguing increase in xylan (and glucan) degradability was obtained, most likely to structural modifications of the network of lignin and carbohydrates present. Similarly, for the set M (S:L), xylan degradability increased from around 10 % (0/0/M, 0/48/M, 0/96/M) to more than 20 % for 1/48/M and 1/96/M, and to more than 40 % for 2/120/M. In the H set, similar trends were obtained compared to L and M. Therefore, it was concluded that the S:L ratio did not have an influence on xylan degradability in the solids, while ammonia concentration and treatment time did have an effect. Glucan degradability followed a similar trend as xylan degradability. Ammonia concentration and treatment time had an increasing effect on glucan degradability, while S:L ratio had no effect.

At highest ammonia concentration (20 w/w % NH_3), it can be seen from Figure 5.1 that glucan degradability is higher than 40 % (40-70 %) and xylan degradability higher than 70 % (70-90 %). The results confirmed that increasing ammonia concentration increased glucan and xylan degradability compared to lower ammonia concentration treatments, which has been concluded in previous researches as well [15].

Considering the enzymatic glucan and xylan degradability, and the fractions that get solubilised after the treatment and after enzymatic hydrolysis, the sum of glucan and xylan mass balances after both treatment and enzyme degradation is presented in Figure 5.2.

Keeping in mind that glucan and xylan recoveries in the solids were high (80-95%) at low NH_3 treatment (0-2 w/w %) and could be considered as similar, the treatments at ≤ 1 w/w % NH_3 doses were compared for their soluble matter after treatment and enzymatic hydrolysis. The addition of only 1 w/w % NH_3 during the treatment resulted to double the amount of soluble glucan and xylan after enzymatic hydrolysis (from 15 to 20 % and from 10 to 20 % for glucan and xylan, respectively) compared to samples without NH_3 addition (Figure 5.2) resulting of a total solubility after treatment and enzymatic hydrolysis up to 40 %. For treatments using 2 NH_3 w/w %, total glucan solubility after enzymatic hydrolysis even increased further to 50%, especially after 5 days treatment (2/120/M) (Figure 5.2B). The latter treatments resulted in a doubled or even tripled xylan solubility after enzymatic hydrolysis and this effect became larger at increasing treatment times. Possibly, the increased xylan degradability helped glucan degradability due to increased accessibility of the cellulose fibres as had already been suggested in previous research [11]. In contrast to treatment time, S:L ratio had no significant effect on increasing xylan and glucan degradability.

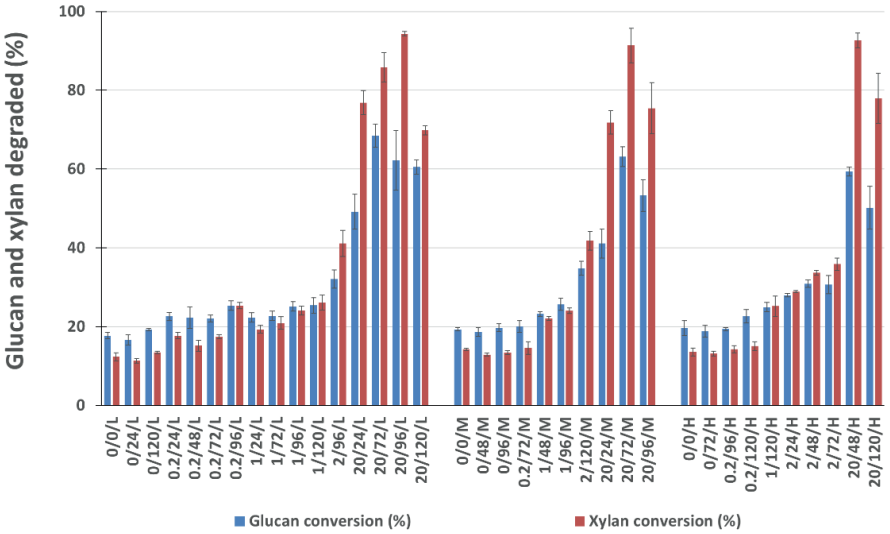


Figure 5.1: Enzymatic hydrolysis of water unsoluble solids (WUS) from ammonia treated wheat straw under varying conditions (NH_3 concentration, treatment and S:L ratio) using a (hemi-)cellulase cocktail. Conversions are based on glucose or xylose released and represented as maximum conversion of amounts of total glucosyl (cellulose) and xylosyl (xylan) residues, respectively. Treatment codes are explained in Table 5.2. Standard deviations combine the duplicate treatment and duplicate enzymatic hydrolysis.

For treatments performed at medium dose of ammonia (20 w/w %), the combined yields of xylan, which is the sum of soluble xylan due to treatment and xylose resulting from enzyme degradation of the solids, significantly increased compared to the low ammonia treatments performed and reached more than 80 % (Figure 5.2A). Furthermore, similarly, the combined yields of glucan in the soluble fraction after treatment and enzymatic hydrolysis reached 55 % (20/24/L) to 75 % (20/72/L) (Figure 5.2B). To summarize, the main effects on enzymatic degradability of the solids was due to ammonia dose with almost no effects of treatment time and S:L ratio parameters.

Although slightly higher ammonia were used (1-2 w/w %), the xylan and glucan degradability of WUS observed in the current study was similar to previous data for composting ammonia treatments performed on wheat straw or corn stover at low ammonia concentrations (0-5 w/w % NH_3). For example, in a composting process [16], xylan degradability of non-treated wheat straw was around 10 % and around 35 % after 5 days composting (0.2-1 % ammonia loading), and glucan degradability was around 20 % for non-treated wheat straw and between 35 and 40 % after 5 days composting. For treatments performed on corn stover, glucan digestibility was between 20 and 30 %, and xylan digestibility was between 10 and 20 % (< 2 w/w % NH_3) after 72 h hydrolysis [15]. These corresponding data will allow comparison of the mechanisms occurring at molecular level in the lignocellulose, during both the lab treatments and the commercial composting process mentioned above, to improve understanding of the degradability of the glucan and xylan fraction.

Figure 5.2: Xylan (A) and glucan (B) mass balances after ammonia treatment and enzymatic hydrolysis, represented as maximum conversion of amounts of total glucosyl (cellulose) and xylosyl (xylan) residues based on untreated wheat straw. Recalcitrant xylan or glucan = xylan or glucan remaining in solids. Treatment codes are explained in Table 5.2.

5.3.3 Effect of liquid ammonia treatment (LLA) on lignin

All solids (WUS) were subjected to pyrolysis (py)-GC-MS, without further sample treatment, to determine lignin composition and estimate their lignin contents. The detailed lignin fingerprints

obtained were used to express the relative abundances (%) of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units [17] and relative abundances of unsubstituted, methyl and ethyl, vinyl and Cα-oxidised lignin groups [18]. Results are shown in Table 5.4. It is good to note that all the py-GC-MS analysis of treated samples were performed using AMDIS software which is a software being more accurate for compound identification and relative abundance (mol %) rather than for compound quantification (w/w %). The estimated lignin content (w/w %) calculated from AMDIS presented in table 4 have to be taken carefully and were analysed regarding their trend rather than exact value.

Hence, looking at trend, based on py-GC-MS results, the lignin contents (w/w %) of the solids obtained at increasing concentrations of NH_3 decreased, reaching around 7-11 w/w % after a 20 w/w % ammonia treatment (Table 5.4). For comparison, the lignin content of untreated wheat straw was 20.5 w/w %. This decrease possibly related to degradation of lignin during the treatments performed, although results should be taken carefully as lignin was estimated and seen not correct for several solids analysed (i.e. treatments at lowest severity 0/0/L and 0/0/H both show only 12-14 w/w % lignin). Nevertheless, at low ammonia concentration, the lignin content of the solids did not clearly decrease.

Aside from the estimated decrease of lignin, also lignin modifications were observed based on the relative abundances of unsubstituted, methyl and ethyl, vinyl and Cα-oxidised lignin groups of py-GC-MS compounds analysed. The identities and the relative abundance of all individual, lignin-originating compounds for all solids analysed are shown in Supplementary 5.1.

The relative abundance of H units decreased in the solids obtained from increasing ammonia concentrations for the three different S:L ratio (L, M and H), from around 30 % (i.e. 0/0/LMH) to 20 % (i.e. 20/72/L, 20/72/L, 20/48/H) (Table 5.4). Correspondingly, at increasing ammonia concentration, the relative abundance of S units increased, while the abundance of G units was constant in a range of 52-58 %. The effect of treatment time on the distribution of lignin units (H, G and S) was inconclusive. For example, the relative abundance of H units either decreased upon increasing treatment times (i.e. 0.2/x/L or 2/x/H ; Table 5.4), remained constant (i.e. 1/x/L; Table 5.4), or even increased (i.e. 20/x/M from H%). Comparing the various lignin groups defined in Table 5.4 (unsubstituted, methyl and ethyl, vinyl, and Cα-oxidised lignin groups), based on grouping comparable pyrolysis compounds (Supplementary 5.1), the % vinyl groups were the most affected by the ammonia treatments. These vinyl lignin groups resulting from py-GC-MS are a measure for the amount of coumaric and ferulic acids present [17]. These groups decreased from more than 70 % in abundance of the totals of lignin measured to values lower than 60 % with increasing ammonia concentrations up to 20 w/w %. Possible mechanisms involved in the removal of these vinyl groups from the solids will be discussed below.

Table 5.4: Characteristics and relative abundance of lignin in the solids (WUS) of wheat straw samples treated with ammonia and their estimated lignin content (w/w % based on dry matter) as analysed by py-GC-MS. Treatment codes are explained in Table 5.2. Standard deviations combine analytical triplicates of the duplicate treated samples. The average was performed keeping RSD<10 %. If one sample out of 6 caused RSD>10 %, that sample was considered as outlier, otherwise the duplicate treatment was reanalysed. The identities and relative abundances of all lignin compounds analysed by py-GC-MS are presented in supplementary 5.1.

code (NH_3/Time/S:L ratio)	Analysed by pyrolysis GC-MS (composition %)							
	%H	%G	%S	%Unsubstituted[a]	% Methyl + % Ethyl[b]	%Vinyl[c]	%Cα-ox[d]	Estimated Lignin[e] (w/w %)
0/0/L	28.3 ± 1.3	56.5 ± 2.3	15.2 ± 0.4	11.3 ± 0.3	3.5 ± 0.1	71.2 ± 1.8	5.1 ± 0.2	14.3 ± 1.0[f]
0/24/L	28.7 ± 0.5	55.1 ± 1.2	16.2 ± 0.4	8.7 ± 0.2	2.9 ± 0.2	70.8 ± 1.1	5.1 ± 0.1	18.6 ± 0.1
0/120/L	25.6 ± 0.8	54.0 ± 1.9	20.4 ± 2.0	8.3 ± 0.3	3.4 ± 0.2	65.3 ± 1.9	6.5 ± 0.2	17.5 ± 1.1
0.2/24/L	29.4 ± 1.7	55.8 ± 2.9	14.8 ± 1.7	11.7 ± 1.3	3.7 ± 0.4	71.2 ± 2.5	4.5 ± 0.7	13.7 ± 0.8[f]
0.2/48/L	28.9 ± 1.5	55.5 ± 2.3	15.6 ± 0.5	10.4 ± 0.3	3.4 ± 0.3	69.9 ± 2.3	4.7 ± 0.2	14.0 ± 1.5[f]
0.2/72/L	27.2 ± 1.4	57.7 ± 3.2	15.1 ± 0.5	11.9 ± 0.4	3.6 ± 0.2	71.1 ± 3.2	4.5 ± 0.2	14.5 ± 1.2[f]
0.2/96/L	22.4 ± 6.1	56.2 ± 7.6	21.4 ± 1.8	8.0 ± 0.9	3.3 ± 0.3	67.1 ± 9.7	5.6 ± 0.4	19.4 ± 1.2
1/24/L	20.0 ± 1.8	58.1 ± 1.4	21.9 ± 0.4	8.8 ± 0.2	3.4 ± 0.2	65.0 ± 2.2	5.4 ± 0.2	19.7 ± 1.8
1/72/L	19.7 ± 0.3	57.4 ± 1.0	22.9 ± 0.4	8.7 ± 0.1	3.3 ± 0.1	64.9 ± 1.1	5.4 ± 0.2	18.9 ± 1.4
1/96/L	19.1 ± 0.8	57.2 ± 0.7	23.7 ± 1.2	8.6 ± 0.4	3.4 ± 0.2	63.9 ± 1.1	5.6 ± 0.2	19.7 ± 0.5
1/120/L	19.5 ± 0.5	56.6 ± 1.1	23.9 ± 0.9	8.3 ± 0.2	3.5 ± 0.2	64.0 ± 1.1	5.7 ± 0.2	19.9 ± 1.8
2/96/L	19.1 ± 0.4	55.8 ± 0.9	25.2 ± 0.7	9.9 ± 0.2	3.7 ± 0.2	61.7 ± 0.9	5.9 ± 0.1	16.0 ± 1.0[f]
20/24/L	23.7 ± 2.6	54.1 ± 2.9	22.2 ± 0.8	15.8 ± 0.8	5.7 ± 0.3	63.1 ± 3.9	6.5 ± 0.3	10.8 ± 1.5[g]
20/72/L	21.1 ± 1.4	57.4 ± 1.1	21.5 ± 0.9	16.1 ± 0.4	5.7 ± 0.2	62.8 ± 1.6	6.3 ± 0.3	11.8 ± 1.2[g]
20/96/L	24.0 ± 0.8	54.8 ± 1.1	21.2 ± 0.6	14.3 ± 0.5	5.0 ± 0.4	65.8 ± 1.2	7.3 ± 0.4	10.8 ± 1.2[g]
20/120/L	13.3 ± 0.5	52.7 ± 1.1	34.0 ± 1.6	10.2 ± 0.4	4.5 ± 0.5	49.8 ± 0.9	8.4 ± 0.2	11.3 ± 1.0[g]
0/0/M	31.9 ± 1.7	56.5 ± 2.0	11.6 ± 0.5	10.9 ± 0.5	3.5 ± 0.2	76.1 ± 2.6	5.3 ± 0.2	17.2 ± 1.1[f]
0/48/M	30.5 ± 1.6	54.1 ± 1.4	15.4 ± 0.6	10.2 ± 0.3	3.6 ± 0.2	69.8 ± 2.1	5.2 ± 0.2	16.1 ± 0.4[f]
0/96/M	28.6 ± 2.8	55.0 ± 2.0	16.4 ± 1.0	9.5 ± 0.4	3.7 ± 0.2	68.7 ± 3.2	5.5 ± 0.2	15.8 ± 1.6[f]
0.2/72/M	29.8 ± 2.1	55.0 ± 2.4	15.3 ± 0.5	11.1 ± 0.3	3.3 ± 0.3	72.6 ± 2.9	4.5 ± 0.1	13.7 ± 1.2[f]
1/48/M	18.7 ± 0.8	57.9 ± 1.3	23.4 ± 1.1	8.2 ± 0.3	3.1 ± 0.2	64.2 ± 1.4	5.5 ± 0.2	21.0 ± 0.2
1/96/M	22.1 ± 8.6	54.7 ± 6.9	23.2 ± 1.6	10.0 ± 1.0	4.0 ± 0.6	64.5 ± 11.0	5.3 ± 0.2	19.8 ± 1.3
2/120/M	19.5 ± 2.9	52.3 ± 1.6	28.1 ± 2.9	11.9 ± 0.7	4.5 ± 0.6	57.7 ± 3.2	6.6 ± 0.4	16.7 ± 1.9[f]
20/24/M	16.2 ± 2.0	52.2 ± 1.8	31.6 ± 1.1	10.5 ± 0.6	5.5 ± 0.5	52.5 ± 2.5	7.4 ± 0.3	12.2 ± 1.3[g]
20/72/M	19.8 ± 0.7	54.7 ± 1.4	25.5 ± 0.7	14.6 ± 0.3	5.0 ± 0.3	55.5 ± 1.5	7.0 ± 0.2	8.7 ± 1.0[g]
20/96/M	20.3 ± 0.4	58.4 ± 1.4	21.3 ± 0.6	16.7 ± 0.6	5.8 ± 0.2	61.9 ± 1.3	6.6 ± 0.3	7.0 ± 1.0[g]
0/0/H	31.6 ± 0.4	56.3 ± 0.8	12.1 ± 0.3	11.3 ± 0.1	3.4 ± 0.1	74.7 ± 0.9	6.0 ± 0.2	12.0 ± 1.2[f]
0/72/H	30.8 ± 3.2	54.2 ± 3.6	15.0 ± 0.5	9.7 ± 0.5	3.3 ± 0.2	71.3 ± 4.8	5.0 ± 0.3	16.3 ± 0.9[f]
0.2/96/H	20.5 ± 0.4	59.2 ± 1.5	20.3 ± 0.6	8.5 ± 0.2	3.2 ± 0.2	67.0 ± 1.5	5.2 ± 0.2	19.4 ± 0.9
0.2/120/	27.4 ± 1.0	56.8 ± 2.0	15.9 ± 0.5	10.5 ± 0.3	3.7 ± 0.2	70.6 ± 1.1	4.6 ± 0.2	16.1 ± 2.2[f]
1/120/H	19.6 ± 1.2	57.2 ± 1.6	23.2 ± 1.1	10.1 ± 0.6	3.8 ± 0.3	64.2 ± 2.0	5.0 ± 0.2	19.1 ± 1.7
2/24/H	30.1 ± 1.3	53.3 ± 2.9	16.6 ± 0.5	12.6 ± 0.4	3.8 ± 0.3	69.3 ± 2.5	4.1 ± 0.2	13.2 ± 1.9[f]
2/48/H	27.8 ± 1.4	55.3 ± 1.8	17.0 ± 0.6	13.6 ± 0.4	4.0 ± 0.2	67.3 ± 1.5	4.6 ± 0.2	13.0 ± 1.2[f]
2/72/H	18.0 ± 1.8	54.9 ± 2.9	27.0 ± 1.1	9.8 ± 0.6	4.1 ± 0.3	58.3 ± 3.4	6.0 ± 0.3	18.2 ± 1.2

20/48/H	21.5 ± 1.5	54.5 ± 1.6	23.9 ± 1.2	16.4 ± 0.7	5.7 ± 0.6	56.3 ± 2.0	5.8 ± 0.3	8.1 ± 0.6[g]
20/120/H	23.0 ± 1.0	58.4 ± 1.9	18.6 ± 0.5	15.1 ± 0.5	5.6 ± 0.2	65.3 ± 2.1	5.8 ± 0.3	10.1 ± 0.8[g]

[a] Unsubstituted lignin compounds correspond to the sum of the relative abundance (%) of phenol, guaiacol and syringol analysed by py-GC-MS.

[b] Methyl + ethyl lignin compounds correspond to the sum of the relative abundance (%) of methyl and ethylphenol, of methyl and ethylguaiacol and of methyl and ethylsyringol analysed by py-GC-MS.

[c] Vinyl lignin compounds correspond to the sum of the relative abundance (%) of 4-vinylphenol, 4-vinylguaiacol and 4-vinylsyringol which are the degradation products of coumaric, ferulic and syringic acid, respectively, under py-GC-MS analyses.

[d] Cα-ox lignin compounds correspond to the sum of the relative abundance (%) of hydroxybenzaldehyde, vanillin, guaiacyl vinyl ketone, syringaldehyde and acetosyringone analysed by py-GC-MS.

[e] The estimated lignin content values (w/w %) were calculated using AMDIS software and need to be taken carefully. Trends should be analysed rather than exact values. Samples performed with 0, 0.2, 1 and 2 w/w % NH3 should be analysed together and should show similar lignin content w/w %, while samples performed with 20 w/w % NH3 should show be analysed together.

[f] The low lignin content (<16 w/w %) for samples performed at 0, 0.2, 1 and 2 w/w % NH3 must be taken carefully and could be considered as underestimated knowing samples 0/0/L,M,H show 20.5 Klason lignin content w/w %. The calculation of lignin content using AMDIS can be underestimated depending of the amount of samples (µg) analysed.

[g] The lower values trend (despite differences as explained in f) found for the estimated lignin content for samples performed at 20 w/w % NH3 were trusted to be lower compared to the other samples performed at lower NH3 loading.

The treatment time and the S:L ratio did not show any clear effects on lignin structures. The other lignin groups (unsubstituted, methyl and ethyl) did not show a clear modification during the treatment. Finally, it is of interest to note that the abundance of Cα-oxidised lignin resulting from py-GC-MS slightly increased in the solids of low to high ammonia dose treatments. Cα-oxidised lignin could be cleaved in acidic conditions, but is rather stable at high pH [32]. The latter observation coincidences with the increase of Cα-oxidised lignin relative abundance after ammonia treatment (Table 5.4).

For all ammonia treatments, a relative decrease in vinyl groups in the solids was analysed. To further specify this decrease, the relative abundance to the total lignin area of the three different vinyl compounds measured (py-GC-MS) are plotted in Figure 5.3. At increasing concentrations of ammonia, a clear decrease of the relative abundance of the pyrolysis product 4-vinylphenol was seen (from 25-28 % (for 0/0M, 0/0/H, or 0.2/48/L) to values around 15 % (2/96/L, 20/72/M, 2/72/H)), while treatment time and the S:L ratio did not show a clear decrease of vinyl phenol abundance. As mentioned above, 4-vinylphenol in py-GC-MS mostly results from coumaric acids in the solids. In grasses, it has been shown that *p*-coumarates ester-link to xylan [33] and to lignin, partly as pendant units, representing up to 10 w/w % of the lignin [17]. Hence, the decrease observed is expected to result from the degradation of such ester bonds, decreasing the number of coumaric acids in the solids.

The relative abundance of 4-vinylguaiacol analysed also decreased in the solids, but only for the M and H (S:L) treatments at increasing ammonia loadings (i.e. from 40-43% for 0/0/M and 0/0/H to 30-35 % for 2/120/M). Within L (S:L) treatments, the % of 4-vinylguaiacol remained similar around 40%

(from 0/0/L to 20/96/L; Table 5.4). The py-GC-MS product 4-vinylguaiacol is majorly derived from ferulic acids in wheat straw [17]. Ferulates are known to partly link to xylan, via ester-linkage to arabinosyl substituents. In addition, ferulates can bridge xylan and lignin via ester or ether linkages [22, 34] forming cross-linkages in the so-called LCCs lowering enzyme accessibility [21, 22, 35]. Hence, the observed decrease in 4-vinylguaiacol in the solids for the M and H treatments at increasing ammonia doses pointed at the cleavage of these ferulate-linkages and subsequently releasing these ferulic acids in the soluble fractions.

Literature showed that the *p*-coumarate and ferulate ester linkages are cleaved in alkaline and ammonia treatments [14, 20, 36, 37] and that ammonia treatment leads to solubilisation and decrease of lignin content in the solids [14, 15, 29]. Our study pointed at the possible cleavage of such linkages already at low ammonia doses, which at the same time left the lignin relatively intact in contrast to these previous studied at higher alkaline conditions. Hence, low ammonia treatment (0.2-2 w/w %) is expected to result in the detachment of lignin from xylan without extensive degradation or modification of the lignin allowing further use of the residual material.

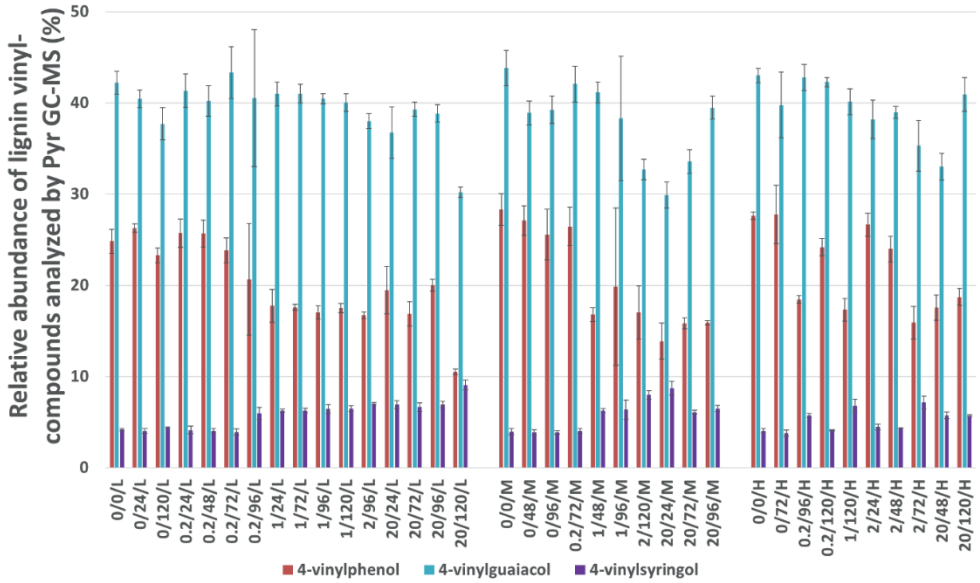


Figure 5.3: Relative abundance of 4-vinylphenol, 4-vinylguaiacol and 4-vinylsyringol (%) calculated from the total relative abundance of each individual lignin compound analysed by py-GC-MS.

5.3.4 Effect of the parameters analysed according Taguchi method and Signal to Noise ratio

The design of the experiments in this research was performed using the Taguchi method to determine which of the three defined parameters (i.e. ammonia dose, S:L ratio and treatment time) had the

largest impact on the outcome of the experiment (i.e. cellulose and xylan degradability of WUS, composition). For this determination the Signal to Noise (SN) ratio was calculated for every outcome of our study and is shown in Figure 5.4. The higher the SN ratio of the parameter the larger the effect on the outcomes.

First, the SN of the three different parameters were assessed versus the chemical compositions of the solids, which is shown in Figure 5.4A. From Figure 5.4A, we considered that SNs > 5 were having an effect on the treatment characteristics. Looking at the carbohydrate composition, xylan and, in particular, its decoration (arabinosyl and uronic acid residues) was the most affected by NH_3 concentration (SN = 10 and SN = 14, respectively), while treatment time affected xylosyl residues the most (SN = 11). These results confirmed our findings that the higher the ammonia dose and the longer the treatment time the more xylan was removed from the solids.

The analysis of the effect of the parameters on lignin composition (Figure 5.4A) confirmed that the relative abundance of H units was similarly affected by all three parameters (SNs > 12), while the relative abundance of G was mostly affected by NH_3 concentration and S:L ratio, and that the relative abundance of S was influenced by NH_3 concentration only. SN calculations also showed that the type of py-GC-MS analysed lignin groups (i.e. unsubstituted, methyl and ethyl and Cα-oxidised lignin groups) were not specifically targeted by the LLA treatment. This indicated that in combination with the measured relative abundances for individual compounds H, G and S, LLA treatments did not modify the core structure of lignin. The analysis of the effect of LLA parameters on the LLCs associated linkages (4-vinylguaiacol associated with ferulic acid upon pyrolysis), however, showed a clear effect of NH_3 concentration (SN = 12) and time (SN = 7). To a lower extent the relative abundance of 4-vinylphenol (degradation product of coumaric acid upon pyrolysis) was affected by treatment time (SN = 6). The Taguchi method confirmed that LLA treatment affected and decreased pendant *p*-coumarate and ferulate levels [17, 21, 22, 35], but not the core structures of lignin. The main parameters involved were NH_3 concentration and treatment time.

Second, the SN of the three different parameters were assessed versus recoveries and enzymatic degradability of the solids, shown in Figure 4B. The latter Figure clearly showed that the NH_3 concentration was the parameter with the highest effects, especially seen for carbohydrates (SN = 15 and SN = 12 for xylan and glucan recovery, respectively) and dm (SN = 11) recoveries, and for lignin contents (SN = 14). These findings matched with the data discussed above (Table 5.3, Figure 5.1 and 5.2). It should be noted that the strong effect of the NH_3 concentration on the recovery was also related to undesired losses observed (SN = 20).

The S:L ratio parameter had no major effects (SN < 5, except for liquid recovery (SN = 12)) confirming the above described results (Table 5.3, Figures 5.1 and 5.2). Although treatment time had no significant effect on the overall results, it equally affected enzymatic hydrolysis of the solids (WUS) compared to NH_3 concentration.

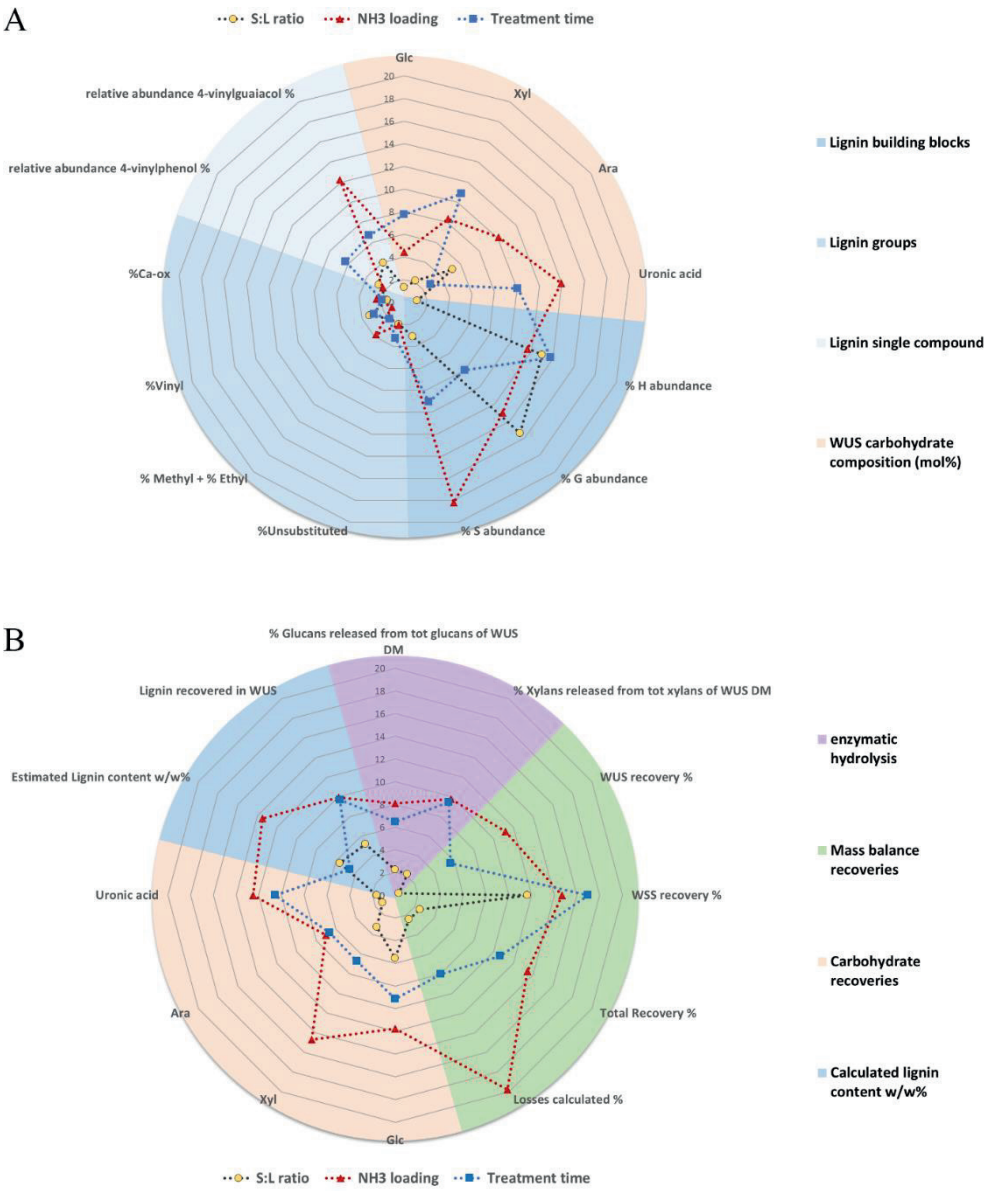


Figure 5.4: Representation of Signal to Noise ratio (SN) calculated for all results. High SN ratio represents high effect of the parameter on the result. **A**: SN calculated for compositional values of treated wheat straw. In blue, compositional relative abundance (%) of lignin, and orange the compositional values of carbohydrates (mol %). **B**: SN calculated for carbohydrate content (orange), lignin content w/w % and estimated recovery (blue), mass balance (green), and enzymatic hydrolysis of residues (purple).

5.4 Conclusions

LLA treatments, and especially at ≤ 2 w/w % ammonia doses, selectively reduced the ferulic (around 10% lower) and coumaric acids (> 50% lower) in the solids obtained, pointing at a looser structure, i.e. due to cleavages of LCC-linkages between xylan and lignin. The reduction of these hydroxycinnamic acids coincidence with increased enzymatic degradability of the material. The sum of ammonia treatment and enzyme degradation resulted in a total of 50 % of xylan and a total of 40% of glucan solubilization (2 w/w % NH_3). Additionally, low doses of NH_3 (≤ 2 w/w % NH_3) favoured maximum carbohydrate recovery (> 80 % for glycan and xylan) and minimal losses. The statistical evaluation (resulting from the Taguchi design of experiment) of this research confirmed that the ammonia loading was the parameter influencing the most carbohydrate and lignin recoveries and composition, while treatment time was as effective for enzymatic hydrolysis of the residues as the ammonia loading parameter. The environmental friendly nature (chemical and temperature) of low liquid ammonia treatment (≤ 2 w/w % NH_3) is a very promising primary step to make the resulting carbohydrates more susceptible for enzyme degradation without extensive lignin modification and is seen as relevant for further valorisation of lignocellulosic biomass.

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Supplementary 5.1: Identities and relative abundance (average of triplicates) of the compounds obtained and detected upon pyrolysis GC-MS of WUS wheat straw treated with ammonia.

	phenol	2-methylphenol	4-methylphenol	4-ethylphenol	4-vinylphenol	4-hydroxybenzaldehyde	guaiacol	4-methylguaiacol	4-ethylguaiacol	4-vinylguaiacol	eugenol	4-propylguaiacol	vanillin	homovanillin	acetovanillone	guaiacylacetone
CAS	108952	95487	106445	123079	2628173	123080	90051	93516	2785899	7786610	97530	2785877	121335	5703242	498022	2503460
MW	94	108	108	122	120	122	124	138	152	150	164	166	152	166	166	180
Origin	H	H	H	H	H	H	G	G	G	G	G	G	G	G	G	G
RRF[a]	0.54	0.57	0.55	1	0.26	0.408	0.49	0.48	0.93	0.14	0.38	0.345	0.37	0.37	0.39	0.52
Lignin group	Unsub.[b]	Methyl	Methyl	Ethyl	Vinyl	Cα-ox	Unsub.	Methyl	Ethyl	Vinyl	n.a.[c]	n.a.	Cα-ox	n.a.	Cα-ox	n.a.
0/0/L	2.1 ± 0.1	0.3 ± 0.0	0.7 ± 0.0	0.1 ± 0.1	24.8 ± 1.3	0.2 ± 0.0	5.8 ± 0.2	1.5 ± 0.1	0.3 ± 0.0	42.2 ± 1.2	0.2 ± 0.0	0.0 ± 0.0	2.1 ± 0.1	0.9 ± 0.1	0.7 ± 0.0	0.6 ± 0.0
0/24/L	1.5 ± 0.1	0.1 ± 0.1	0.5 ± 0.0	0.1 ± 0.0	26.3 ± 0.5	0.3 ± 0.0	4.3 ± 0.2	1.4 ± 0.1	0.2 ± 0.1	40.4 ± 1.0	0.2 ± 0.0	0.0 ± 0.0	2.0 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.4 ± 0.0
0/120/L	1.4 ± 0.1	0.1 ± 0.1	0.5 ± 0.0	0.0 ± 0.1	23.3 ± 0.8	0.3 ± 0.0	3.9 ± 0.2	1.6 ± 0.1	0.2 ± 0.1	37.7 ± 1.8	0.2 ± 0.0	0.1 ± 0.1	2.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.0
0.2/24/L	2.4 ± 0.6	0.2 ± 0.2	0.8 ± 0.0	0.1 ± 0.1	25.7 ± 1.6	0.2 ± 0.1	6.0 ± 1.1	1.5 ± 0.1	0.3 ± 0.2	41.3 ± 1.9	0.2 ± 0.1	0.0 ± 0.1	1.7 ± 0.5	1.0 ± 0.3	0.6 ± 0.1	0.5 ± 0.0
0.2/48/L	2.0 ± 0.1	0.2 ± 0.0	0.7 ± 0.1	0.0 ± 0.1	25.7 ± 1.5	0.2 ± 0.0	5.3 ± 0.3	1.4 ± 0.2	0.2 ± 0.1	40.2 ± 1.7	0.2 ± 0.0	0.0 ± 0.0	1.7 ± 0.1	1.0 ± 0.1	0.7 ± 0.0	0.5 ± 0.0
0.2/72/L	2.2 ± 0.1	0.3 ± 0.0	0.7 ± 0.1	0.0 ± 0.1	23.8 ± 1.4	0.2 ± 0.1	6.4 ± 0.3	1.5 ± 0.1	0.3 ± 0.2	43.3 ± 2.8	0.2 ± 0.0	0.0 ± 0.1	1.7 ± 0.1	1.0 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
0.2/96/L	1.2 ± 0.2	0.1 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	20.7 ± 6.1	0.1 ± 0.1	3.7 ± 0.7	1.4 ± 0.2	0.3 ± 0.1	40.5 ± 7.5	0.3 ± 0.0	0.1 ± 0.1	1.6 ± 0.2	1.2 ± 0.1	0.6 ± 0.0	0.5 ± 0.1
1/24/L	1.4 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	0.1 ± 0.0	17.7 ± 1.8	0.1 ± 0.0	4.2 ± 0.1	1.5 ± 0.1	0.3 ± 0.0	41.0 ± 1.3	0.3 ± 0.0	0.1 ± 0.1	1.5 ± 0.1	1.1 ± 0.1	0.6 ± 0.0	0.6 ± 0.0
1/72/L	1.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	17.6 ± 0.3	0.1 ± 0.0	4.1 ± 0.1	1.5 ± 0.0	0.2 ± 0.0	41.0 ± 1.0	0.2 ± 0.0	0.0 ± 0.1	1.4 ± 0.1	1.0 ± 0.0	0.7 ± 0.0	0.6 ± 0.0
1/96/L	1.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	17.0 ± 0.7	0.1 ± 0.0	4.0 ± 0.3	1.5 ± 0.1	0.3 ± 0.1	40.5 ± 0.6	0.2 ± 0.0	0.1 ± 0.1	1.5 ± 0.1	1.0 ± 0.1	0.7 ± 0.0	0.5 ± 0.0
1/120/L	1.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.1	17.5 ± 0.5	0.1 ± 0.0	3.8 ± 0.1	1.5 ± 0.2	0.3 ± 0.0	40.0 ± 1.0	0.3 ± 0.0	0.1 ± 0.1	1.5 ± 0.2	0.9 ± 0.1	0.7 ± 0.0	0.6 ± 0.0
2/96/L	1.6 ± 0.1	0.2 ± 0.0	0.5 ± 0.0	0.0 ± 0.1	16.7 ± 0.3	0.1 ± 0.1	4.5 ± 0.1	1.6 ± 0.1	0.3 ± 0.1	38.0 ± 0.8	0.3 ± 0.0	0.1 ± 0.1	1.5 ± 0.1	1.0 ± 0.1	0.7 ± 0.0	0.6 ± 0.0
20/24/L	2.6 ± 0.1	0.4 ± 0.0	0.9 ± 0.1	0.1 ± 0.1	19.5 ± 2.6	0.1 ± 0.1	7.1 ± 0.6	2.2 ± 0.3	0.4 ± 0.1	36.7 ± 2.8	0.4 ± 0.1	0.2 ± 0.1	1.7 ± 0.2	1.2 ± 0.5	0.8 ± 0.2	0.9 ± 0.1
20/72/L	2.8 ± 0.2	0.4 ± 0.0	0.8 ± 0.1	0.1 ± 0.1	16.9 ± 1.3	0.1 ± 0.1	7.6 ± 0.2	2.3 ± 0.1	0.5 ± 0.0	39.3 ± 0.8	0.4 ± 0.1	0.1 ± 0.2	1.5 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
20/96/L	2.7 ± 0.2	0.4 ± 0.1	0.7 ± 0.3	0.1 ± 0.1	20.0 ± 0.6	0.2 ± 0.0	6.4 ± 0.4	2.1 ± 0.1	0.3 ± 0.2	38.8 ± 0.9	0.3 ± 0.1	0.0 ± 0.0	1.9 ± 0.2	0.6 ± 0.3	1.1 ± 0.0	0.8 ± 0.0
20/120/L	2.1 ± 0.2	0.2 ± 0.0	0.3 ± 0.3	0.0 ± 0.1	10.5 ± 0.3	0.1 ± 0.1	4.3 ± 0.2	1.9 ± 0.2	0.3 ± 0.2	30.2 ± 0.6	0.4 ± 0.1	0.1 ± 0.2	1.9 ± 0.0	1.0 ± 0.2	1.1 ± 0.1	0.9 ± 0.0
0/0/M	2.2 ± 0.1	0.2 ± 0.1	0.8 ± 0.1	0.1 ± 0.0	28.3 ± 1.7	0.3 ± 0.0	5.4 ± 0.4	1.4 ± 0.1	0.3 ± 0.0	43.8 ± 1.9	0.1 ± 0.1	0.0 ± 0.1	2.1 ± 0.2	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.0
0/48/M	2.0 ± 0.1	0.2 ± 0.0	0.7 ± 0.1	0.1 ± 0.0	27.1 ± 1.6	0.3 ± 0.0	4.9 ± 0.3	1.4 ± 0.1	0.3 ± 0.0	38.9 ± 1.3	0.2 ± 0.0	0.0 ± 0.1	2.1 ± 0.1	0.5 ± 0.1	0.7 ± 0.0	0.5 ± 0.0
0/96/M	1.7 ± 0.2	0.2 ± 0.1	0.7 ± 0.1	0.1 ± 0.0	25.6 ± 2.8	0.3 ± 0.0	4.7 ± 0.3	1.6 ± 0.2	0.3 ± 0.1	39.2 ± 1.5	0.2 ± 0.0	0.0 ± 0.0	2.2 ± 0.1	0.6 ± 0.1	0.7 ± 0.0	0.5 ± 0.0
0.2/72/M	2.2 ± 0.2	0.2 ± 0.1	0.7 ± 0.1	0.0 ± 0.1	26.5 ± 2.1	0.2 ± 0.0	5.6 ± 0.2	1.4 ± 0.1	0.3 ± 0.1	42.1 ± 2.0	0.2 ± 0.0	0.0 ± 0.1	1.6 ± 0.0	0.9 ± 0.1	0.6 ± 0.0	0.5 ± 0.0
1/48/M	1.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.1	16.8 ± 0.8	0.1 ± 0.0	3.9 ± 0.3	1.4 ± 0.1	0.2 ± 0.1	41.1 ± 1.2	0.2 ± 0.0	0.1 ± 0.0	1.5 ± 0.1	1.1 ± 0.0	0.6 ± 0.0	0.5 ± 0.0
1/96/M	1.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	19.8 ± 8.6	0.1 ± 0.1	4.6 ± 0.9	1.6 ± 0.5	0.4 ± 0.2	38.3 ± 6.8	0.3 ± 0.1	0.1 ± 0.1	1.4 ± 0.2	1.0 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
2/120/M	1.9 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.1	17.0 ± 2.9	0.1 ± 0.1	5.2 ± 0.5	1.7 ± 0.3	0.5 ± 0.2	32.7 ± 1.1	0.4 ± 0.0	0.2 ± 0.1	1.5 ± 0.2	1.1 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
20/24/M	1.7 ± 0.1	0.2 ± 0.2	0.4 ± 0.1	0.1 ± 0.0	13.9 ± 2.0	0.0 ± 0.1	4.5 ± 0.6	2.3 ± 0.4	0.5 ± 0.2	29.9 ± 1.4	0.5 ± 0.1	0.1 ± 0.3	1.8 ± 0.0	0.9 ± 0.1	1.0 ± 0.1	0.8 ± 0.1
20/72/M	2.7 ± 0.2	0.2 ± 0.0	0.7 ± 0.2	0.1 ± 0.1	15.8 ± 0.6	0.2 ± 0.1	6.7 ± 0.2	2.2 ± 0.1	0.3 ± 0.2	33.6 ± 1.3	0.3 ± 0.0	0.1 ± 0.1	1.9 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
20/96/M	3.0 ± 0.3	0.5 ± 0.0	0.8 ± 0.1	0.1 ± 0.1	15.9 ± 0.2	0.0 ± 0.1	7.9 ± 0.3	2.4 ± 0.1	0.5 ± 0.0	39.5 ± 1.3	0.4 ± 0.0	0.1 ± 0.2	1.7 ± 0.1	1.4 ± 0.1	1.0 ± 0.1	0.9 ± 0.0
0/0/H	2.3 ± 0.1	0.3 ± 0.0	0.8 ± 0.0	0.2 ± 0.0	27.6 ± 0.4	0.4 ± 0.0	5.6 ± 0.1	1.3 ± 0.0	0.3 ± 0.0	43.0 ± 0.8	0.2 ± 0.0	0.0 ± 0.0	2.5 ± 0.2	0.9 ± 0.0	0.7 ± 0.0	0.5 ± 0.0
0/72/H	1.8 ± 0.2	0.2 ± 0.1	0.7 ± 0.1	0.1 ± 0.1	27.8 ± 3.2	0.3 ± 0.1	4.8 ± 0.3	1.4 ± 0.1	0.3 ± 0.0	39.8 ± 3.6	0.2 ± 0.0	0.0 ± 0.1	2.0 ± 0.2	0.5 ± 0.1	0.6 ± 0.0	0.5 ± 0.0
0.2/96/H	1.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	18.4 ± 0.4	0.1 ± 0.1	4.1 ± 0.2	1.5 ± 0.1	0.2 ± 0.1	42.8 ± 1.4	0.2 ± 0.0	0.0 ± 0.1	1.6 ± 0.0	1.0 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
0.2/120/H	2.0 ± 0.1	0.2 ± 0.1	0.7 ± 0.2	0.0 ± 0.1	24.2 ± 1.0	0.2 ± 0.0	5.4 ± 0.2	1.6 ± 0.1	0.3 ± 0.0	42.3 ± 0.5	0.2 ± 0.0	0.1 ± 0.1	1.7 ± 0.1	1.0 ± 0.1	0.7 ± 0.0	0.5 ± 0.0
1/120/H	1.5 ± 0.2	0.2 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	17.3 ± 1.2	0.1 ± 0.0	4.7 ± 0.3	1.6 ± 0.3	0.4 ± 0.1	40.1 ± 1.4	0.3 ± 0.0	0.2 ± 0.0	1.3 ± 0.0	1.0 ± 0.1	0.6 ± 0.1	0.6 ± 0.0
2/24/H	2.4 ± 0.1	0.2 ± 0.1	0.7 ± 0.2	0.1 ± 0.1	26.6 ± 1.3	0.1 ± 0.1	6.3 ± 0.3	1.6 ± 0.0	0.3 ± 0.1	38.2 ± 2.1	0.2 ± 0.0	0.0 ± 0.0	1.3 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
2/48/H	2.5 ± 0.2	0.3 ± 0.0	0.7 ± 0.1	0.0 ± 0.1	24.0 ± 1.4	0.2 ± 0.0	7.0 ± 0.3	1.6 ± 0.1	0.4 ± 0.0	39.0 ± 0.7	0.3 ± 0.0	0.1 ± 0.1	1.4 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.7 ± 0.0
2/72/H	1.5 ± 0.2	0.2 ± 0.0	0.3 ± 0.2	0.1 ± 0.0	15.9 ± 1.8	0.1 ± 0.1	4.4 ± 0.4	1.7 ± 0.1	0.4 ± 0.1	35.3 ± 2.8	0.3 ± 0.0	0.1 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	0.7 ± 0.0	0.7 ± 0.1
20/48/H	2.8 ± 0.1	0.4 ± 0.2	0.6 ± 0.4	0.1 ± 0.1	17.5 ± 1.4	0.1 ± 0.1	7.7 ± 0.4	2.4 ± 0.2	0.5 ± 0.1	33.0 ± 1.5	0.4 ± 0.0	0.1 ± 0.1	1.7 ± 0.1	0.5 ± 0.3	0.9 ± 0.1	0.8 ± 0.1
20/120/H	2.8 ± 0.2	0.4 ± 0.0	0.9 ± 0.1	0.2 ± 0.0	18.7 ± 0.9	0.1 ± 0.1	7.5 ± 0.3	2.4 ± 0.1	0.4 ± 0.2	40.9 ± 1.9	0.4 ± 0.1	0.1 ± 0.2	1.5 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	0.8 ± 0.1

	cis-isoeugenol	trans-isoeugenol	guaiacyl vinyl ketone	trans-Coniferaldehyde	syringol	4-methylsyringol	4-ethylsyringol	4-vinylsyringol	4-allyl-Syringol	syringaldehyde	acetosyringone	syringylacetone	cis-4-propenylsyringol	4-propynesyringol	4-allenesyringol	trans-4-propenylsyringol
CAS	cisIsoeugenol	transIsoeugenol	Unknown2	transConiferaldehyde	91101	6638057	14059928	28343228	6627889	134963	2478388	19037582	cis4propenylsyringol	R107	R107B	trans4propenylsyringol
MW	164	164	178	178	154	168	182	180	194	182	196	210	194	192	192	194
Origin	G	G	G	G	S	S	S	S	S	S	S	S	S	S	S	S
RRF[a]	0.31	0.31	0.52	0.046	0.38	0.46	0.965	0.2	0.3	0.18	0.35	0.35	0.3	0.3	0.3	0.3
Lignin group	n.a.[c]	n.a.	Cα-ox	n.a.	Unsub.[b]	Methyl	Ethyl	Vinyl	n.a.	Cα-ox	Cα-ox	n.a.	n.a.	n.a.	n.a.	n.a.
0/0/L	0.1± 0.1	1.1± 0.1	0.1± 0.0	0.9± 1.9	3.4± 0.2	0.6± 0.0	0.1± 0.0	4.2± 0.1	0.1± 0.1	0.9± 0.1	0.5± 0.0	0.0± 0.1	0.0± 0.1	0.0± 0.1	0.9± 0.1	3.2± 0.2
0/24/L	0.1 ± 0.0	1.0 ± 0.0	0.1 ± 0.0	3.9 ± 0.6	2.9 ± 0.1	0.7 ± 0.1	0.0 ± 0.0	4.0 ± 0.3	0.2 ± 0.1	0.9 ± 0.0	0.5 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	1.0 ± 0.1	4.3 ± 0.2
0/120/L	0.2 ± 0.0	1.3 ± 0.1	0.1 ± 0.0	4.5 ± 0.7	3.0 ± 0.1	1.0 ± 0.0	0.0 ± 0.0	4.4 ± 0.1	0.3 ± 0.1	1.2 ± 0.1	0.6 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	1.3 ± 0.1	6.5 ± 2.0
0.2/24/L	0.2 ± 0.0	1.3 ± 0.1	0.1 ± 0.1	0.9 ± 1.9	3.2 ± 0.3	0.7 ± 0.1	0.1 ± 0.1	4.1 ± 0.4	0.3 ± 0.4	0.8 ± 0.2	0.5 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	1.1 ± 0.1	2.7 ± 1.5
0.2/48/L	0.1 ± 0.1	1.2 ± 0.1	0.1 ± 0.1	2.8 ± 1.5	3.1 ± 0.1	0.7 ± 0.1	0.0 ± 0.0	4.0 ± 0.2	0.2 ± 0.1	0.9 ± 0.0	0.5 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	1.1 ± 0.1	3.5 ± 0.3
0.2/72/L	0.2 ± 0.1	1.3 ± 0.1	0.1 ± 0.1	0.5 ± 1.5	3.4 ± 0.3	0.7 ± 0.1	0.1 ± 0.0	3.9 ± 0.3	0.2 ± 0.2	0.9 ± 0.1	0.5 ± 0.0	0.0 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	1.0 ± 0.1	3.1 ± 0.2
0.2/96/L	0.2 ± 0.0	1.6 ± 0.2	0.1 ± 0.1	4.0 ± 0.9	3.2 ± 0.5	1.0 ± 0.1	0.1 ± 0.0	6.0 ± 0.6	0.4 ± 0.3	1.3 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.0 ± 0.0	1.8 ± 0.2	4.8 ± 1.5
1/24/L	0.2 ± 0.0	1.5 ± 0.2	0.1 ± 0.0	5.2 ± 0.2	3.3 ± 0.2	0.8 ± 0.1	0.1 ± 0.0	6.2 ± 0.2	0.3 ± 0.2	1.3 ± 0.1	0.6 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	1.6 ± 0.1	5.5 ± 0.2
1/72/L	0.2 ± 0.0	1.4 ± 0.0	0.1 ± 0.0	5.1 ± 0.1	3.4 ± 0.1	0.9 ± 0.1	0.1 ± 0.0	6.3 ± 0.2	0.3 ± 0.2	1.4 ± 0.1	0.6 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	1.7 ± 0.1	6.0 ± 0.2
1/96/L	0.2 ± 0.0	1.5 ± 0.2	0.1 ± 0.0	5.1 ± 0.3	3.3 ± 0.2	0.9 ± 0.1	0.1 ± 0.0	6.4 ± 0.5	0.4 ± 0.1	1.3 ± 0.0	0.6 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	1.8 ± 0.2	6.6 ± 1.0
1/120/L	0.2 ± 0.0	1.5 ± 0.4	0.1 ± 0.0	5.0 ± 0.1	3.3 ± 0.2	1.0 ± 0.1	0.1 ± 0.0	6.5 ± 0.3	0.4 ± 0.1	1.4 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	2.0 ± 0.3	6.4 ± 0.7
2/96/L	0.2 ± 0.0	1.6 ± 0.2	0.1 ± 0.0	5.2 ± 0.2	3.8 ± 0.2	1.0 ± 0.1	0.1 ± 0.0	7.0 ± 0.1	0.4 ± 0.1	1.5 ± 0.0	0.7 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	1.9 ± 0.1	6.4 ± 0.6
20/24/L	2.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	6.1 ± 0.5	1.4 ± 0.1	0.2 ± 0.0	6.9 ± 0.4	0.4 ± 0.1	1.5 ± 0.2	0.9 ± 0.1	0.3 ± 0.1	2.2 ± 0.3	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0
20/72/L	2.3 ± 0.7	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	5.7 ± 0.2	1.4 ± 0.1	0.2 ± 0.1	6.6 ± 0.5	0.5 ± 0.2	1.5 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	2.2 ± 0.6	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0
20/96/L	2.0 ± 0.1	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	5.3 ± 0.3	1.3 ± 0.1	0.1 ± 0.0	6.9 ± 0.4	0.4 ± 0.3	1.7 ± 0.1	0.8 ± 0.1	0.2 ± 0.1	2.0 ± 0.1	0.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0
20/120/L	0.4 ± 0.0	2.6 ± 0.2	0.1 ± 0.1	7.4 ± 0.8	3.9 ± 0.2	1.6 ± 0.2	0.2 ± 0.0	9.1 ± 0.6	0.6 ± 0.1	2.5 ± 0.1	1.1 ± 0.0	0.3 ± 0.2	0.1 ± 0.0	0.0 ± 0.0	2.9 ± 0.2	9.1 ± 1.4
0/0/M	1.0 ± 0.1	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	3.3 ± 0.2	0.6 ± 0.0	0.1 ± 0.0	4.0 ± 0.3	0.1 ± 0.1	0.9 ± 0.1	0.5 ± 0.0	0.0 ± 0.1	0.8 ± 0.1	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
0/48/M	0.2 ± 0.0	1.1 ± 0.1	0.1 ± 0.0	3.2 ± 0.4	3.2 ± 0.2	0.7 ± 0.0	0.1 ± 0.0	3.9 ± 0.3	0.2 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	1.0 ± 0.0	3.5 ± 0.5
0/96/M	0.2 ± 0.0	1.2 ± 0.2	0.1 ± 0.0	3.6 ± 1.2	3.0 ± 0.2	0.8 ± 0.1	0.1 ± 0.0	3.9 ± 0.2	0.2 ± 0.1	1.0 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	1.1 ± 0.1	4.2 ± 0.9
0.2/72/M	0.1 ± 0.1	1.1 ± 0.0	0.1 ± 0.1	0.5 ± 1.4	3.3 ± 0.2	0.7 ± 0.0	0.1 ± 0.0	4.0 ± 0.3	0.2 ± 0.0	0.9 ± 0.0	0.5 ± 0.0	0.0 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.9 ± 0.1	3.2 ± 0.3
1/48/M	0.2 ± 0.0	1.4 ± 0.1	0.1 ± 0.0	5.5 ± 0.5	3.2 ± 0.2	0.8 ± 0.1	0.1 ± 0.0	6.2 ± 0.2	0.4 ± 0.2	1.4 ± 0.1	0.6 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	1.7 ± 0.1	6.9 ± 1.1
1/96/M	0.3 ± 0.1	1.9 ± 0.5	0.0 ± 0.1	3.7 ± 0.7	4.0 ± 0.5	1.1 ± 0.3	0.1 ± 0.1	6.4 ± 1.1	0.5 ± 0.1	1.4 ± 0.2	0.6 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	2.1 ± 0.6	4.9 ± 0.8
2/120/M	0.4 ± 0.1	2.4 ± 0.4	0.0 ± 0.0	4.6 ± 0.8	4.9 ± 0.5	1.4 ± 0.3	0.2 ± 0.1	8.0 ± 0.5	0.6 ± 0.3	1.8 ± 0.3	1.0 ± 0.1	0.4 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	2.6 ± 0.4	4.9 ± 2.8
20/24/M	0.4 ± 0.1	2.9 ± 0.5	0.1 ± 0.0	6.4 ± 0.5	4.3 ± 0.1	1.8 ± 0.1	0.3 ± 0.0	8.7 ± 0.7	0.6 ± 0.3	2.1 ± 0.1	1.0 ± 0.1	0.4 ± 0.2	0.0 ± 0.1	0.0 ± 0.0	2.9 ± 0.1	7.0 ± 0.7
20/72/M	0.3 ± 0.0	2.0 ± 0.2	0.1 ± 0.0	4.6 ± 0.4	5.3 ± 0.1	1.3 ± 0.1	0.1 ± 0.0	6.1 ± 0.2	0.4 ± 0.1	1.8 ± 0.2	0.8 ± 0.1	0.2 ± 0.1	0.0 ± 0.1	0.2 ± 0.1	1.8 ± 0.2	5.4 ± 0.5
20/96/M	2.2 ± 0.2	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	5.7 ± 0.3	1.4 ± 0.0	0.1 ± 0.1	6.5 ± 0.3	0.5 ± 0.2	1.5 ± 0.1	0.9 ± 0.1	0.3 ± 0.1	2.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0
0/0/H	1.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	3.4 ± 0.1	0.6 ± 0.0	0.0 ± 0.0	4.1 ± 0.2	0.2 ± 0.0	0.9 ± 0.0	0.6 ± 0.0	0.0 ± 0.0	1.0 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
0/72/H	0.2 ± 0.0	1.0 ± 0.1	0.1 ± 0.0	2.9 ± 0.4	3.1 ± 0.3	0.7 ± 0.1	0.0 ± 0.0	3.8 ± 0.3	0.2 ± 0.2	0.9 ± 0.0	0.5 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	1.0 ± 0.1	3.4 ± 0.1
0.2/96/H	0.2 ± 0.0	1.3 ± 0.1	0.1 ± 0.0	4.9 ± 0.2	3.1 ± 0.1	0.8 ± 0.1	0.1 ± 0.0	5.7 ± 0.2	0.3 ± 0.2	1.1 ± 0.1	0.6 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	1.5 ± 0.1	5.1 ± 0.5
0.2/120/H	0.1 ± 0.1	1.4 ± 0.1	0.0 ± 0.1	1.3 ± 1.9	3.1 ± 0.1	0.7 ± 0.0	0.1 ± 0.0	4.1 ± 0.1	0.3 ± 0.1	0.9 ± 0.0	0.5 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	1.1 ± 0.1	3.6 ± 0.4
1/120/H	0.2 ± 0.0	1.6 ± 0.1	0.0 ± 0.1	4.7 ± 0.6	3.9 ± 0.4	1.0 ± 0.1	0.1 ± 0.0	6.8 ± 0.7	0.4 ± 0.2	1.2 ± 0.1	0.7 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	2.0 ± 0.1	5.1 ± 0.6
2/24/H	0.2 ± 0.0	1.4 ± 0.1	0.1 ± 0.0	1.6 ± 2.0	3.8 ± 0.3	0.8 ± 0.0	0.1 ± 0.0	4.5 ± 0.3	0.3 ± 0.0	0.8 ± 0.0	0.5 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	1.3 ± 0.1	3.0 ± 0.2
2/48/H	0.2 ± 0.0	1.5 ± 0.0	0.0 ± 0.0	1.3 ± 1.6	4.1 ± 0.1	0.8 ± 0.1	0.1 ± 0.0	4.3 ± 0.1	0.3 ± 0.1	1.0 ± 0.0	0.6 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	1.3 ± 0.1	2.9 ± 0.5
2/72/H	0.3 ± 0.1	2.2 ± 0.2	0.0 ± 0.1	6.1 ± 0.6	3.8 ± 0.4	1.3 ± 0.2	0.2 ± 0.0	7.1 ± 0.7	0.5 ± 0.2	1.6 ± 0.1	0.9 ± 0.1	0.3 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	2.2 ± 0.2	7.0 ± 0.6
20/48/H	0.3 ± 0.1	2.2 ± 0.2	0.1 ± 0.1	4.0 ± 0.3	5.9 ± 0.6	1.4 ± 0.2	0.2 ± 0.0	5.7 ± 0.4	0.4 ± 0.1	1.4 ± 0.1	0.7 ± 0.1	0.2 ± 0.1	0.1 ± 0.2	0.0 ± 0.1	1.9 ± 0.1	4.1 ± 0.9
20/120/H	2.2 ± 0.1	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	4.8 ± 0.3	1.3 ± 0.1	0.1 ± 0.1	5.7 ± 0.1	0.4 ± 0.3	1.3 ± 0.1	0.7 ± 0.0	0.2 ± 0.2	1.9 ± 0.2	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0

[a]RRF based on Van Erven et al., 2017. [b]Unsub. = unsubstituted. [c]n.a. = not assagnied. The compound are not categorised within any cited lignin groups.

CHAPTER 6

General discussion

6.1 Aim and hypothesis of the research

The core purpose of this research was to identify new routes to liberate monosaccharides from hard-to-convert grass feedstocks using mild treatments with intrinsic chemical catalysts, acetic acid and ammonium from manure, enzymes, and combination of both. As a results, two main hypothesis were drafted as: 1) mild acidic versus ammonia pretreatments show different effects on grass xylan and lignin structures, and their interconnections, 2) the more xylan, lignin or LCCs disruption occurs during pretreatment of grasses, the higher the enzymatic degradability of residual material. The first hypothesis was investigated performing acidic treatment of corn stover (**Chapter 3**) and ammonia treatment of wheat straw (**Chapter 5**) and the second hypothesis was studied and presented in the thesis for wheat straw (**Chapter 3** and **5**) and corn stover (**Chapter 2** and **4**). Of particular interest was the fate of lignin structures and enzymatic degradability of wheat straw within a Phase 1 composting process. In this Phase 1, ammonia is formed and temperature arises to 80 °C, which is the first step to form a good substrate for mushroom growth.

One of the main challenges in enzymatic degradation of treated biomass is the recalcitrant and protective structures of the plant itself. Lignin acts as a natural protective barrier against microorganisms or industrial biomass degradation [1, 2]. The effect of lignin removal or modification for the carbohydrate accessibility is, nevertheless, not fully understood. Hence, the detailed characterization of lignin structure and composition, and corresponding enzymatic degradation of xylan and cellulose, is expected to contribute to a better understanding of plant biomass degradability.

Within this research, the effect of lignin content and composition in relation to the enzymatic degradability of residual carbohydrates was investigated for mild pretreatments and two types of grasses, being corn stover and wheat straw. The main finding of **Chapter 2** was that, the S:G ratio in the maize stem lignin has a greater influence on carbohydrate degradability, representing ruminal digestion, than the acid detergent lignin (ADL) content, based on two cultivars and different internodes. In **Chapter 3**, it is shown that the lignin structures of corn stover resulting from acetic acid or sulfuric acid treatments was different, however, the enzymatic carbohydrate degradability was similar. **Chapter 4** shows that conducting a Phase 1 composting process at higher pH, without adding gypsum, led to higher enzymatic glucan and xylan degradability. **Chapter 5** was specifically designed to mimic a composting Phase 1 treatment in a lab-scale low ammonia treatment set up together with medium ammonia treatment for comparison. This design mainly showed that the ammonia dose affected more than treatment time the resulting enzymatic glucan and xylan degradability and lignin structure of wheat straw. In both **Chapter 4** and **5**, the increased carbohydrate degradability of treated wheat straw coincided with a decrease in py-GC-MS analysed hydroxycinnamic acids.

In this final **Chapter 6**, (bio-) ammonia and acid treatment are compared to discuss similarities and differences regarding the resulting enzymatic carbohydrate degradability and lignin structures. Limitations of current lignin analysis are discussed. Furthermore, treatment parameters specifically involved at low ammonia treatment (0, 0.2, 1 and 2 w/w % NH_3) influencing structures and enzymatic degradability are discussed. Hereto, the corresponding data shown in **Chapter 5** are re-evaluated via the Taguchi statistical analysis. Finally, recommendations will address possibilities of the (low ammonia) composting process beyond the mushroom industry and methodology for lignin analysis.

6.2 Limits of lignin quantification and composition analysis methods

Despite the use of multiple methods described in this thesis for analysis of lignin quantification and composition (i.e. Klason lignin", acid detergent Lignin (ADL) and py-GC-MS), lignin analysis remains one of the major bottlenecks in lignocellulose research. In this section the different lignin analysis methodology and their limits will be discussed.

The determination of lignin content is mostly performed gravimetrically, in which sulfuric acid is used to basically degrade and solubilize every component but lignin. Hereto, the two main methods used are "Klason lignin" [3] and Acid detergent Lignin (ADL) [4]. A major drawback of both methods is that due to the use of highly concentrated sulfuric acid the lignin remaining is altered in structure. Hence, subsequent structural characterisation is excluded. Moreover, the residues can still contain non-lignin compounds, such as proteineous residues or chitin in the case of samples subjected to microbial growth [5, 6]. Furthermore, since the procedures for Klason lignin versus ADL are slightly different, the two methods are known to result in different lignin contents, in particular for grasses [7-10]. As an example, lignin analysis of perennial ryegrass was reported to have 14.0 w/w % Klason lignin and 6.7 w/w % ADL [7], corn residues were reported to have 19.9 w/w % Klason lignin and 2.7 w/w % ADL (sequentially determined) [10] and switchgrass was reported to have 14.5 w/w% Klason lignin and 7.5 w/w % ADL [8]. In the literature, ADL is often used for the analysis of grasses in relation with digestibility of carbohydrates in rumen [11, 12], while Klason lignin is used to quantify lignin in grasses before or after a chemical treatment [13-16]. Unfortunately, due to the different outcomes of these methods, lignin contents from different research fields cannot be compared, and indicates the difficulty in specific lignin quantification.

Our research was both in the field of animal digestion and of lignocellulose valorisation. Therefore, in this thesis, ADL was used in **Chapter 2** to relate with gas production from carbohydrate degradability of maize stem in rumen conditions, while Klason lignin was used before and after composting treatments of wheat straw (**Chapter 4**) and acidic treatment of corn stover (**Chapter 3**). Nevertheless, in all chapters py-GC-MS lignin analysis was applied, but mainly for specific lignin characterisation rather than quantification. Recently, successful lignin contents were determined using py-GC-MS, by using a ^{13}C-lignin isolate from wheat straw as internal standard [6]. This new method has recently

been developed at the Laboratory of Food Chemistry of Wageningen University, however, was not yet available to analyse treated corn stover and wheat straw discussed in our project. Further, in our research AMDIS software was used, while for lignin quantification Xcalibur software resulted in more representative lignin contents [6]. AMDIS is a very powerful software for compound identification allowing to set expected molecular mass and retention time corresponding to a molecule from a library and identify if the match percentage is high enough. However, the integration of each compound consecutive to an analysis is performed using a mathematical model. Thus, a low intensity compound present within the analysis could be left out or underestimated if the matching percentage level is not high enough for AMDIS. Similarly, a high intensity compound is possibly overestimated since the molecular mass for that molecule could have a match percentage high within a wide range. Xcalibur on the other hand, is using the exact molecular mass and retention time of each known compound and allows to perform manual integration. Beyond lignin quantification, the method for lignin composition used for this thesis remains valid resulting in fast and qualitative lignin analysis during biomass treatments [5, 12, 17-20].

Although py-GC-MS showed to be powerful to study lignin composition (this thesis), no information is obtained about lignin linkages via this technique. Hereto, NMR spectroscopy is the main analytical technique giving information about both types of inter-unit linkages and functional group decoration of the lignin macromolecules [21]. However, NMR has to be performed on extracted lignin that is also performed in solvent (i.e. dioxane) that can react with lignin. In principal, solid-state NMR of (non-extracted) wheat straw lignin is performed and reported [22, 23], but lignin signals are highly disturbed by carbohydrate signals in the aliphatic region needed for linkage determinations. Also, NMR cannot be considered for lignin content analysis. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTI) can also be used to determine the chemical changes of the surface of a sample [14] and linked lignin surface modification during treatment using lignin indicators wavelengths representing C–H, O–H, or CH2 bending frequencies [24]. To finish, size exclusion chromatography can also be used to determine the weight-average mass (Mw) of lignin, however, also implying previous lignin extractions [14, 15]. The high number of samples produced during this thesis-research and the outcome of the lignin modification occurring during treatment to be analysed lead us to choose the py-GC-MS method. Py-GC-MS for lignin composition can be performed on dry treated sample without carbohydrate signal disturbance.

Overall, py-GC-MS, optimized with addition of ^{13}C-lignin as internal standard, seems a promising alternative for all research fields to both quantify and characterize specifically lignin in reasonably high-throughput with minimal sample handling.

6.3 Comparison of (bio-)ammonia and acid treatment of wheat straw and corn stover

6.3.1 Dry matter and carbohydrate recovery in soluble and solid fractions

In **Chapter 3, 4** and **5** grasses (corn stover and wheat straw) were pretreated at mild acidic or (bio-)ammonia conditions and assessed for enzymatic degradability. Here, the outcomes for dry matter and carbohydrate recoveries of these either acidic or (bio-)ammonia are compared.

To efficiently valorise grasses after pretreatment, the recoveries of both soluble and solid fractions need to be taken into account enabling conclusions regarding unwished losses. In addition, knowing whether the final target molecules (i.e. glucose, xylose, phenolic compounds from lignin) are in the soluble or solid fraction result in a greater understanding of the subsequent (enzymatic) degradation step. The latter is seen to contribute in the context of optimised production of biochemicals and biofuels, but also of optimised mushroom production. In **Chapter 3**, acidic treatment of grass dissolved xylan, and as expected, cellulose and lignin were retained in the solids, whereas in **Chapter 4** and **5**, xylan, cellulose and lignin were retained in the solids at mild (bio-)ammonia conditions. Despite the fact that acidic and (bio-)ammonia treatments were performed on different grass types, corn stover and wheat straw, respectively, the two treatments were compared. Mainly trends rather than a direct comparison was aimed at.

The results from the **Chapters 3, 4** and **5** were used to calculate dry matter and carbohydrate recoveries, for both soluble and solid fractions recovered after the various pretreatments selected, which are summarized in Table 6.1. The pretreatments, which were selected for comparison, are: acetic acid (sample code: (5-0) and (30-0)), sulfuric acid (sample code: (0-1.2) and (0-2)) treated corn stover (based on **Chapter 3**); ammonia (sample code 0.2/120/H, 1/120/L and 2/120/M) treated wheat straw (results from **Chapter 5**) and (bio-)ammonia treatment, which is actually Phase 1 composting (sample code: PI-P; **Chapter 4**) as described in Table 6.1.

In the literature, it is accepted that acid treatment solubilise xylan [13, 25-28] and that xylose and arabinose can be found in the soluble fraction as mono- or oligosaccharides [29, 30]. In comparison, ammonia treatment is known to retain xylan and cellulose in the solids while solubilising lignin [16, 31-33]. Similar to this previous research, for our experiments (Table 6.1), the total dry matter recovery in the solids was lower after acid treatment than after ammonia treatment (60-75 % compared to 80-91 %, respectively). This lower recovery was mainly due to a lower xylan recovery in the solids for the acid treatment. The latter is not expected to be an effect for only corn stover, since previous research showed similar dry matter recovering, or even lower, for wheat straw at similar CSFs [3, 6].

Table 6.1: Comparison of ammonia treated wheat straw and acid treated corn stover conditions and recoveries.

Sample code	(5-0)[a]	(30-0)[a]	(0-1.2)[a]	(0-2)[a]	0.2/120/H[b]	1/120/L[b]	2/120/M[b]	PI-P[c,d]
Grass type	Corn stover				Wheat straw			
Experiment	Chemical							Biological
Experimental conditions:								
NH_3 w/w %	n.a.[e]	n.a.[e]	n.a.[e]	n.a.[e]	0.2	1.0	2.0	0.2
HAc w/w %	5	30.0	n.a.[e]	n.a.[e]	n.a.[e]	n.a.[e]	n.a.[e]	n.a.[e]
H_2SO_4 w/w %	n.a.[e]	n.a.[e]	1.2	2.0	n.a.[e]	n.a.[e]	n.a.[e]	n.a.[e]
pH after treatment	3.8	3.2	3.1	2.4	n.a.[e]	n.a.[e]	n.a.[e]	8.6
Treatment time (hours)	1	1	1	1	120	120	120	120
Treatment temperature (°C)	160	160	160	160	80	80	80	80
S:L ratio	1:13	1:13	1:13	1:13	1:10	1:04	1:06	1:04
Recoveries:								
Total DM recovery %								
In solids	65.2	75.4	60.4	59.1	83.3	83.6	80.7	91.0
In solubles	34.8	24.6	39.6	40.9	8.2	10.4	12.0	n.m.[f]
Total carbohydrate recovery %[g]								
In solids	62.0	49.3	53.3	55.8	77.5	82.8	85.8	93.8
In solubles	31.5	33.5	36.9	34.8	n.m.[f]	n.m.[f]	n.m.[f]	n.m.[f]
Specific carbohydrate recovery %[g]								
In solids								
Glucosyl %	96.3	85.6	91.3	99.9	80.1	85.2	87.2	96.2
Xylosyl %	29.6	14.6	18.8	12.5	75.4	82.3	85.0	88.7
Arabinosyl %	9.7	4.3	6.1	4.1	68.3	77.7	89.8	102.3
Uronic acid %	18.3	13.5	16.5	15.9	84.9	76.6	79.8	94.6
In solubles[h]								
Glucosyl %	6.6	7.7	7.7	7.7	n.m.[f]	n.m.[f]	n.m.[f]	n.m.[f]
Xylosyl %	54.7	57.9	64.9	61.2	n.m.[f]	n.m.[f]	n.m.[f]	n.m.[f]
Arabinosyl %	48.9	50.2	58.9	54.5	n.m.[f]	n.m.[f]	n.m.[f]	n.m.[f]

[a] Acid treated samples (X-Y), where X: concentration of acetic acid w/w % dm; and Y: concentration of H_2SO_4 w/w % dm.
[b] Ammonia treated samples (X/Y/Z), where X: concentration of ammonia w/w % dm; Y: treatment time (hours); Z: solid: liquid ratio (L 1:4; M 1:6; H 1:10).
[c] PI-P: End-Phase I composting sample; 120 hours treatment at industrial scale.
[d] The recovery values were published by Jurak et al. (2015) [5].
[e] Not applicable.
[f] Not measured.
[g] Carbohydrates represented as anhydro-sugars, recovered from originally present in corresponding untreated corn stover or wheat straw.
[h] Uronic acid recovery in the solubles gave yields > 100 % for every treatments are not shown in the Table.

The chemical treatment using aqueous ammonia (0.2/120/H, 1/120/L and 2/120/M samples; codes are described in Table 6.1) and the bioprocess involving ammonia as intrinsic catalyst (PI-P sample; Table 6.1) led to similar complete carbohydrate recoveries in the solids of both xylan and glucan, also shown in literature [5]. In this respect, the industrial bioprocess (Phase 1 composting process) was well represented by the industrial simulation at lab-scale, which could help in future experiments to mimic and understand Phase 1 composting process since such processing phase is run at 180 tons of material.

It can also be noted that after acidic treatment of corn stover (**Chapter 3**), xylosyl and arabinosyl were not fully recovered in the solids nor in the solubles. It was reported in the literature that depending on treatment severity, the solubilisation of xylosyl and arabinosyl residues can further react and form inhibitors like furfural, [13, 26-28, 34, 35]. The formation of inhibitory products could explain the losses found in our experiments.

6.3.2 Residual hemicellulose structures in solid fractions recovered after treatment

In view of our hypothesis number 1, the effect of acid versus ammonia treatments on the residual grass xylan structures was compared as well. Hereto, the ratio of ramifications versus xylosyl residues is considered a relevant parameter (**Chapter 3** and **5**). Differences in residual hemicellulose structures has consequences for the type of enzymes needed in the subsequent enzymatic treatments. After mild acidic treatments (**Chapter 3**), the residual xylan structures in the solids showed a decrease in arabinosyl residues and an increase in uronic acid residues substituted to xylosyl residues. The (bio-) ammonia treatments (**Chapter 5**), however, showed almost similar degree of substitution of the xylosyl residues with arabinosyl and uronic acid residues compared to untreated material. As mentioned previously, the comparison between the acidic and ammonia solid fractions after treatment for residual hemicellulose structures were performed on different grass types and only trends were compared.

Unfortunately, in the literature, acidic and ammonia treatment of grasses usually focus on the description of the carbohydrate content and, less frequently, on carbohydrate recoveries in the solids. However, detailed carbohydrate compositions of water unsoluble solids are not shown [15, 25-28, 31, 33]. Additionally, comparisons of solids obtained for similar conditions is difficult. As an example, in Sousa et al. (2016) [16] the ammonia concentration used was equivalent to 600 w/w %, whereas it was lower than 20 w/w % in all our studies.

Our studies described carbohydrate composition resulting from mild acidic or ammonia treatment. The degree of substitution of the xylosyl residues in xylan with arabinosyl residues (DS_{Ara}) decreased after mild acidic treatment of corn stover (from 16.3 to 12.2 for untreated corn stover and sample (0-2), respectively), while it was almost similar after mild ammonia treatment of wheat straw (12.9 and

13.6 for untreated wheat straw and sample (2/120/M), respectively). A similar effect was observed in the composting process to the ammonia treatment, where the DS_{Ara} was 15 for P1-0 (0 days phase 1, **Chapter 4**) and 17 for P1-5 (5 days phase 1, **Chapter 4**). The degree of substitution of the xylosyl residues with uronic acid (DS_{UA}), however, increased in the solids after mild acidic treatment of corn stover (from 7.6 to 17 for untreated and treated (sample (0-2)) corn stover, respectively), while it was, again, found almost similar after mild ammonia treatment of wheat straw (9.5 to 8.9 for untreated wheat straw and sample (2/120/M), respectively) and for Phase 1 composting process (10 and 10 for P1-0 and P1-5, respectively).

As a consequence, enzymatic treatment of mild acid treated residues seem to ask for lower amounts of arabinofuranosidases and higher amounts of glucuronidases, whereas both enzyme activities will be needed after a mild ammonia treatment of grass. In addition, regardless the type of pretreatment, the full spectrum of cellulases (see Figures 1.10 and 1.11), endo-xylanases and beta-xylosidases are required.

6.3.3 Residual lignin structures in solid fractions recovered after treatment

In addition to the fate of the cellulose and xylan, the comparison of the residual fractions consecutive to mild acidic or ammonia treatment can be extended to the third main polymer of grasses, lignin. In **Chapter 2**, our main finding was that the S:G ratio (i.e. Syringyl (S) and Guaiacyl (G)) of maize stem lignin (analysed by py-GC-MS) was more important than lignin content for further carbohydrate degradability. Moreover the full composition of corn stover lignin (analysed by py-GC-MS) was presented being one of the first after acidic (**Chapter 3**) and ammonia (**Chapter 5**) treatments, without previous sample preparation or lignin extraction, and the effect of the treatment on lignin composition was assessed. Additionally, lignin resulting from a composting process phase 1 (modified and not-modified) was also described (analysed by py-GC-MS) (**Chapter 4**). The trend comparison for corn stover acid treated and wheat straw (bio-)ammonia treated discussed in the previous sections was also performed for the content and the composition of residual lignin in the solid fractions after treatment.

Lignin is a complex polymer of phenolic compounds that is under-considered for valorisation and is often combusted to generate energy in paper or pulp industry [36]. In the biochemical industry, lignocellulosic biomass is mostly seen as source of carbohydrates that could be used to produce valuable molecules such as bioethanol or lactic acid. The conversion of cellulose and hemicellulose is however hindered by lignin. The different treatments to valorise carbohydrates consider lignin as a limiting factor rather that a valuable source of phenolic compounds [37]. Nevertheless, recently, new biochemical routes are investigated for the valorisation of phenolic compounds from lignin that could be used in various industries such as healthcare, nylon, plastics, resins or lubricants after Kraft process, or used as biochemicals after oxidative depolymerization (i.e. muconic acid, adipic acid, styrene, gallic

acid, syringic acid, pyrogallol, catechol) [38]. Hereto, harsh lignin modification needs to be prevented and compositional analysis needs to be in place.

In this thesis, lignin composition was analysed by py-GC-MS. This technique can be directly performed on total dry samples resulting in the relative abundance of the pyrolysis compounds originating from lignin and representing a lignin fingerprint. Additionally, lignin contents were estimated using py-GC-MS as well (i.e. **Chapter 5**: sample 0.2/120/H, 1/120/L, 2/120/M; Table 6.2). However, this estimation was performed without internal standard addition and, hence, not considered representative. In general, the estimations underestimated lignin contents. For example the Klason lignin content corrected for ash of the wheat straw used for this experiment was 20.5 w/w % (WS, Table 6.2), whereas the estimation from py-GC-MS resulted into 14.3±1.0, 17.2±1.1 and 12.0±1.2 w/w % lignin for 0/0/L, 0/0M and 0/0/H, respectively (**Chapter 5**).

Table 6.2: Lignin content and recoveries of ammonia treated wheat straw and acid treated corn stover.

Sample code	CS	(5-0)	(30-0)	(0-1.2)	(0-2)	WS	0.2/120/H	1/120/L	2/120/M	PI-P	PI-A
Grass type	Corn stover					Wheat straw					
Experiment[a]	Chemical									biological	
Content w/w % and recoveries in solids %:											
Lignin content	18.2[b]	23.6[b]	26.3[b]	26.6[b]	30.2[b]	20.5[b]	18.1[c]	23.1[c]	17.9[c]	19.4[b]	19.0[b]
std	1.0	0.0	0.5	0.3	0.9	0.8	2.8	2.8	2.9	0.4	0.1

[a] Treatment conditions are shown in Table 6.1

[b] Calculated as Klason lignin corrected for ash.

[c] Estimated lignin content (w/w %) calculated from wheat straw standard (20.5 w/w % Klason lignin) based on py-GC-MS analysis

The effect of acidic and ammonia treatments were assessed to investigate if lignin structures were modified during their respective treatment (**Chapter 3** and **5**). Although lignin content estimation did not result in quantitative data, the identification and the relative abundance of the pyrolysis compounds originating from lignin did and these data were used throughout this thesis (**Chapter 2, 3, 4** and **5**). The composition of the lignin building blocks [21] presented as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units (see Figure 1.5) is shown in Figure 6.1.

First, the comparison of the two grasses, corn stover and wheat straw, show major differences in the distribution of the main lignin building blocks. The difference in lignin composition of corn stover and wheat straw was also found in the literature and corresponded to our data [21, 39, 40]. In corn stover (**Chapter 3,** CS, Figure 6.1), the relative abundance of S units was lower than 10 %, and the relative abundance of H and G were 50 and 40 %, respectively.

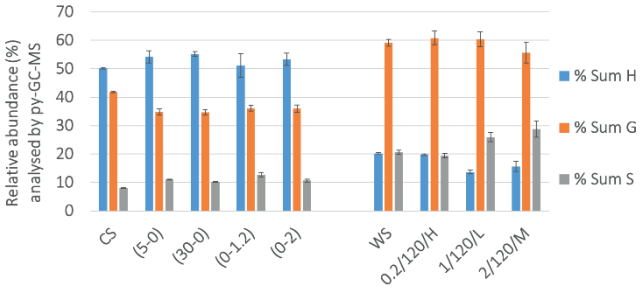

Figure 6.1: Lignin composition as analysed by py-GC-MS. Relative abundances (%) of the sum of *p*-hydroxyphenyl lignin py-GC-MS compounds (Sum H), sum of guaiacyl lignin py-GC-MS compounds (Sum G) and the sum of syringyl lignin py-GC-MS compounds (Sum S) in solids recovered from ammonia treated wheat straw and acid treated corn stover. Codes of treatments (x-axes) are explained in Table 6.1.

In **Chapter 2**, maize stem is described for 2 different cultivars and different internodes. The relative abundances matched between the different cultivars while slight differences were found through the internodes. In maize stem, H units are majorly present (60-70 %) followed by G units (15-20 %) and S units (9-15 %). Corn stover is a mix of maize stems, leaves and cobs (without the kernels) and harvested when the kernels are ripe, which may explain the different lignin composition (**Chapter 3**) with the younger and solely maize stems used in **Chapter 2**. Wheat straw, however, is mainly composed of G units representing 60 % of the total relative abundance, while both H and S building blocks both represent 20 % of the total relative abundance. The grass origin result in a very wide difference in composition of the building blocks. The valorisation of lignin for specific compounds must be chosen accordingly.

Previous studies showed that the treatment of grasses resulted in modification of lignin, however, the composition and the distribution of H, G and S units were not often described; an exception is the study of Murciano Martinez et al. (2016) [17]. In the latter study, sodium hydroxide treatment of sugar cane bagasse was reported to decrease the relative abundance of non-core lignin (i.e. *p*-coumarates and ferulates) while phenol, guaiacol and syringol accumulated in the residues, as analysed by py-GC-MS [17]. Further, it has been reported that acid treatments of switchgrass lead to the decrease of β-*O*-4 linkages by 36% as well as a minor decrease of β–β and β-5 linkages (See Figure 1.6) for a schematic lignin structure and linkages). Additionally, the S:G ratio decreased from 0.80 to 0.53 after acid treatment. It was also shown that guaiacyl phenolic OH group increased as well as condensed phenolic OH group, as analysed by ^{31}P-NMR. Size exclusion chromatography analysis showed a slight decrease in lignin molecular weights pointing to the condensation of lignin during dilute acid treatment [15]. Hydrothermal treatment of corn stover and wheat straw also showed a decrease in *Mw* of lignin after treatment [14]. Additionally, an increase in the surface lignin concentration after treatment was reported and was attributed to deposition of the lignin on the fibre surfaces [14], which was also shown after acid treatment of maize stem [41].

In our study, the composition of H, G, and S units in the solids obtained after the various acid treatments only varied slightly from untreated corn stover, with a slightly lower relative abundance of G corresponding to a slightly higher relative abundance of H and S units. Increasing the amount of acid (from (5-0) to (30-0) and from (0-1.2) to (0-2)) did not modify further the relative amounts of the main building blocks within the solids. Similarly, the ammonia treatment of wheat straw resulted with a slightly higher relative abundance S units in solids, now corresponding to a slightly lower H and, to a lesser extent, G units. Hence, acid or ammonia treatment did not modify to a high extent the relative abundance of the main building blocks initially present in grass, regardless the grass species used.

Although from the H, G and S composition no clear conclusion on specific modification was obtained, the analysis of treated grass lignin using py-GC-MS also allowed comparison of lignin structures in further detail. For example, py-GC-MS lignin compounds were grouped as vinyl, Cα-oxidised or unsubstituted compounds (see Figures 6.2 and 6.3B). Furthermore, relative abundances of targeted py-GC-MS compounds such as 4-vinylphenol and 4-vinylguaiacol, which are the pyrolysis products of coumaric acid and ferulic acid, were compared (Figure 6.3A) [6, 21].

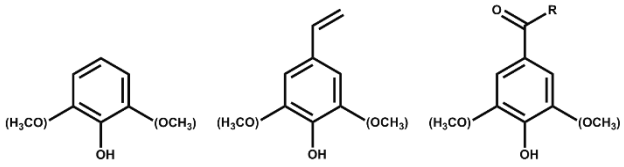

Figure 6.2: Representation of unsubstituted (A), vinyl (B) and Cα-oxidised lignin groups. R can be H or CH_3.

In our research, py-GC-MS for lignin characterisation was as one of the first used to analyse solids from acidic or ammonia treated grasses, without prior lignin extraction. This method has been applied in our laboratory already for lignin composition analysis in solids of hydrothermally treated sugar cane bagasse in presence of sodium hydroxide [17]. In the latter study, clearly a decrease of *p*-coumarates and ferulates enhancing enzymatic glucan conversion has been shown. Other groups also used py-GC-MS for lignin characterisation in grasses [42] and during fungal growth, in particular white rot fungal growth, allowing to describe specific modifications of lignin [12, 43, 44].

Recapitulative Figure 6.4 and Table 6.3 are presented at the end of this section to discuss and show observed effects of acid and ammonia treatments of grass on lignin structures and lignin structures involved in LCCs. Our py-GC-MS data are considered in view of literature py-GC-MS data, if present, and together with other lignin analysis data obtained from methods such as NMR, LC-MS.

In corn stover and wheat straw, 4-vinylphenol and 4-vinylguaiacol represent approximately 80 % and 70 % of the total lignin compounds, respectively (CS and WS, Figure 6.3B), which is in line with values reported in literature [6, 21, 42]. Hence, the relative abundance of these two vinyl-compounds is a

marker to assess structural modifications during pretreatments. Results mainly show a slight decrease of the relative abundance of 4-vinylguiaiacol linked to slight increases of 4-vinylphenol and 4-vinylsyringol for solids of acid treated corn stover (sample (5-0). (30-0), (0-1.2) and (0-2), Figure 6.3A and Table 6.1 for codes). The residues from mild ammonia treated wheat straw resulted in the decrease of both 4-vinylphenol and 4-vinylguaiacol at increasing concentration of ammonia (sample 0.2/120/H, 1/120/L, 2/120/M, Figure 6.3A), linked to an increase of 4-vinylsyringol. As the py-GC-MS products 4-vinylphenol and 4-vinylguaiacol are majorly derived from coumaric acid and ferulic acid, respectively, in wheat straw [21], we can speculate that the amount of (di-)ferulic (Figure 6.4, Table 6.3 (B, C, D and E)) and coumaric acid (Figure 6.4, Table 6.3 (A and H)) decreased in the solids after mild acid and ammonia treatment, respectively. The same has been shown, as mentioned above, for sodium hydroxide treatment of sugar cane bagasse [17]. Such (di-)ferulic acid in grasses are reported to link to xylan and even to link xylan to lignin in so-called carbohydrate complexes (LCCs; Figure 6.4 (D and E)) [45, 46]. Coumaric acids, in grasses, have been reported being pendant on lignin side chains (Figure 6.4 (H)) and representing up to 10 w/w % of the lignin (see Figure 1.7) [21]. The decrease in relative abundance of coumaric and ferulic acid could possibly result in opening of the grass cell wall during treatment by i.e. decreasing the LCC-linkages.

Although related, the ratio between vinyl compounds and other compound groups (Figure 6.3B) such as unsubstituted or Cα-ox gives us even a further insight in structural modifications. Furthermore, py-compounds can be either categorised into Ph-C0-2 lignin which represent compounds with none, 1 and 2 carbons in the side-chain or PhC-3 compounds having 3 carbons in the side chain. The latter represents more functionalised and larger lignin py-GC-MS products [21]. In addition, the increase in abundance of PhC-3 compared to Ph-C0-2 is described as an indicator for a more open lignin structure as shown for *L. edodes* treatment of wood chips increasing carbohydrate degradability [12].

In Figure 6.3B, it is shown that the ratio between vinyl- and Cα-ox compounds, and the ratio between Ph-C0-2/PhC-3 increased at increasing concentration of their respective acid (acetic or sulfuric acid). Simultaneously, the ratio between vinyl and unsubstituted compounds was slightly decreasing ((5-0). (30-0), (0-1.2) and (0-2), Figure 6.3B). These increased ratio for vinyl/Cα-ox and PhC0-2/PhC-3 can point out that either more lignin-like vinyl compounds were resulting from pyrolysis of solids, or less Cα-ox or PhC-3 compounds. The slight decrease in the ratio vinyl/unsubstituted favours the second option, possibly pointing at a decrease in lignin complexity (Figure 6.4 and Table 6.3 (K and L)) and increase in condensed structures for acid treated corn stover (Figure 6.4 and Table 6.3 (J)).

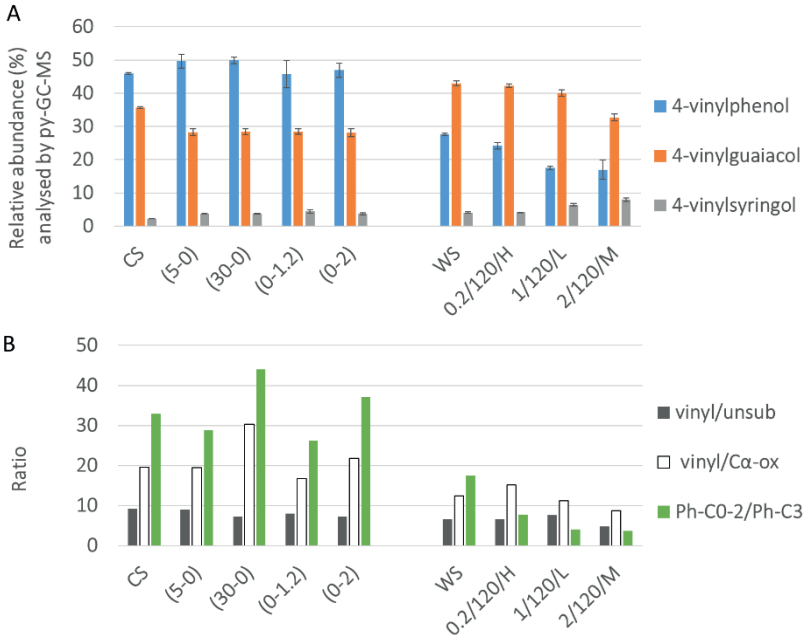

Figure 6.3: A. Relative abundance (%) of 4-vinylphenol, 4-vinylguaiacol and 4-vinylsyringol analysed by py-GC-MS within the solids of ammonia treated wheat straw and acid treated corn stover. B. Ratio between the sum of vinyl lignin compounds relative abundance to the sum of relative abundance of unsubstituted lignin compound (sum of phenol, guaiacol and syringol) and Cα-oxidised lignin compounds (sum of vanillin, syringaldehyde, acetovanillone and acetosyringone). Ratio between relative abundance of Ph-C0-2/PhC-3 is the ratio of the sum of py-GC-MS lignin-compounds with none, 1 and 2 carbons in the side-chain divided by that of py-GC-MS lignin-compounds with 3 carbons in the side chain [21]. For completeness, it should be noted that vinyl compounds belong to the Ph-C0-2 compounds.

Such a conclusion was also drawn in a recent study, which showed that oxidised inter-linkages (Figure 6.4 (K)) were prompt to be cleaved in acid conditions [47] explaining the decrease of Cα-ox within the residues. Additionally, it was also shown for aspen wood in a steam explosion setup that an increasing severity lead to a substantial cleavage of β-O-4 inter-linkages [48] (Figure 6.4 (I)). In addition, acid treatment has been reported to first solubilise lignin followed by its recondensation during cooling. This reactions result in lignin droplets deposition on cellulose [41]. We can speculate that during acid solubilisation, by oxidised and β-O-4 inter-linkages cleavage, and recondensation consecutive to an acid treatment, lignin loose part of its complexity in favour of unsubstituted lignin compound and results in a more condensed material. Such a conclusion is supported by our py-GC-MS data of the solids. It can be noted that as explained in **Chapter 3**, the residual lignin composition is not similar for acetic and sulfuric acid treatments, however followed a similar trend.

Table 6.3: Observed effects of acid and ammonia treatments of grass on specific and LCCs lignin structures. The coded crosslinks are shown in Figure 6.4.

Code Figure 6.4	Linkage type[a] or lignin group unit[b]	Structure shown in	Stability towards[c]		References
			Acid treatment	Ammonia treatment	
A	CA ester xylan	CF[d] [29], V[e][49], G[f][45, 46]	+ CS[n], CF[d]	- WS[h], SCB[l] (after NaOH)	Chapter 3 and 5, [17, 29, 50]
B	FA ester xylan	CF[d][29], M[g][51], G[f][45, 46]	+ CS[n], CF[d]	- WS[h], SCB[l] (after NaOH)	Chapter 3 and 5, [17, 29, 50, 52]
C	di-FA ester xylan	M[g][51], G[f][45, 46]	+ CS[n]	- WS[h], SCB[l] (after NaOH)	Chapter 3 and 5, [50]
D	di-FA ester lignin	M[g][51], G[f][45]	+ CS[n]	- WS[h], SCB[l] (after NaOH)	Chapter 3 and 5, [17, 50]
E	FA ether lignin	WS[h][6, 21], C[l][53], M[g][51], SG[j][54], RG[k][55]	-(→+) CS[n], SG[j]	- WS[h]	Chapter 3 and 5, [50, 52, 54]
F	Direct ether lignin-xylan	G[f][45]	n.d.[o]	n.d.[o]	
G	Direct ester UAme-lignin	G[f][45]	n.d.[o]	n.d.[o]	
H	CA ester lignin	V[e][49], M[g] [56], C[l][53], SCB[l][17],SG[j][54],WS[h] [21]	-(→+) CS[n], SG[j], M[g]	- WSh, SCBl (after NaOH)	Chapter 3 and 5, [17, 50, 54, 56]
I	β-*O*-4 lignin inter-linkage	WS[h][6, 21],V[e][49], SCB[l][6], BS[m][6], CS[n][6]	- CS[n], SG[j]	+ WS[h]	Chapter 3 and 5, [15]
J	Unsubstituted lignin units	WS[h][6, 21], SCB[l][6], BS[m][6], CS[n][6]	+ CS[n], SG[j]	+ WS[h]	Chapter 3 and 5, [15, 54]
K	Cα-oxidised lignin units	WS[h][6, 21], SCB[l][6], BS[m][6], CS[n][6]	- CS[n], SG[j]	+ WS[h]	Chapter 3 and 5, [15]
L	PhC-3 lignin units	WS[h][6, 21], SCB[l][6], BS[m][6], CS[n][6]	- CS[n], SG[j]	+ WS[h]	Chapter 3 and 5, [15, 54]

[a] FA= Ferulic acid; di-FA=di-ferulic acid, CA=p-coumaric acid; UAme=4*O*Me-uronic acid. [b] Lignin group units are presented as described in [6, 21].[c] Not stable (-) or stable (+) against acidic or ammonia treatment. [d] Corn fiber. [e] Various Herbaceous (sisal, kenaf, abaca and curaua). [f] Grasses (gramineous monocots). [g] Maize. [h] Wheat straw. [i] Corn stem .[j] Switch grass. [k] Rye grass. [l] Sugar cane bagasse. [m] Barley straw. [n] Corn stover. [o] Not described. (→+) Structure recondensation.

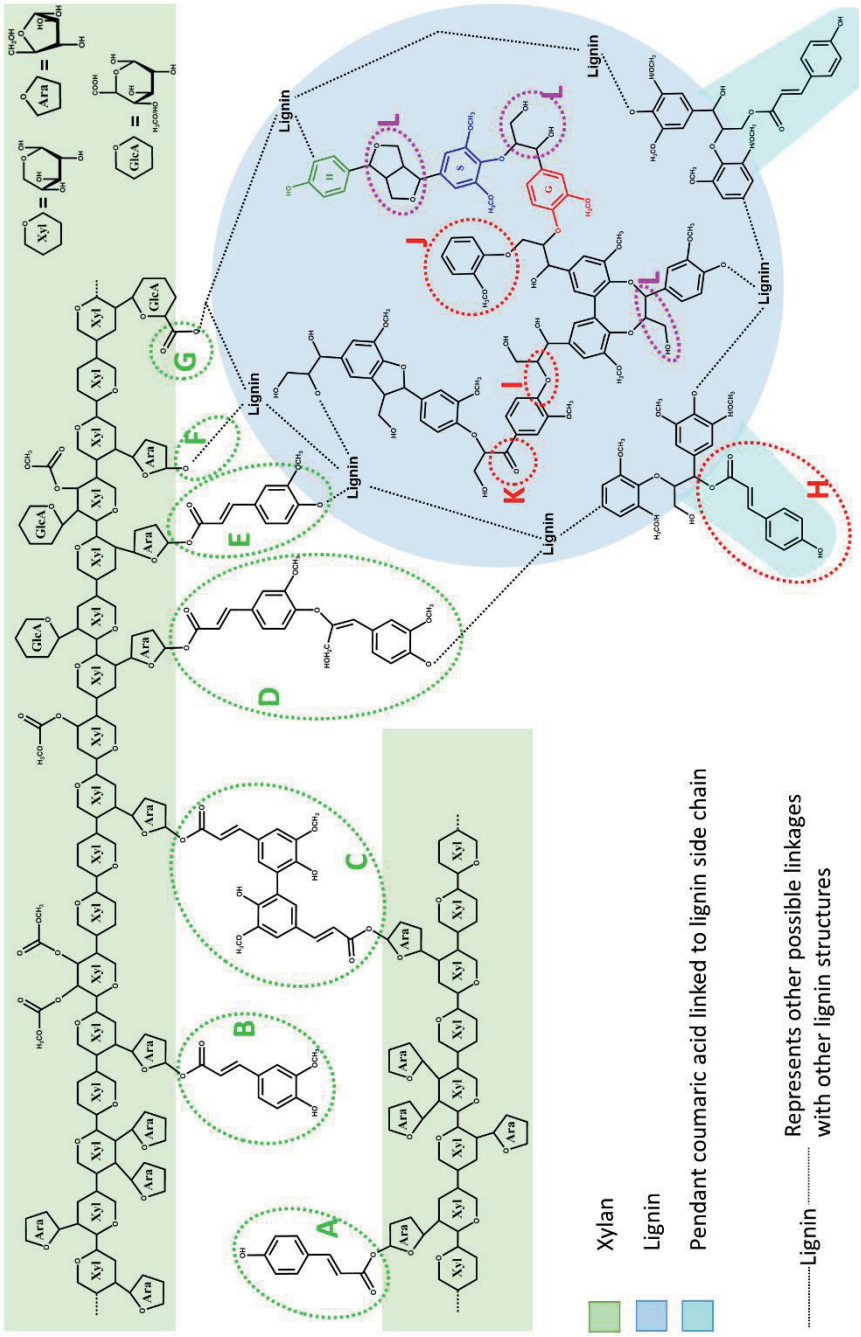

Figure 6.4: Hypothetical cross links with ferulic acid and coumaric, linking lignin and xylan, in grasses. Letters refer to linkages or lignin units groups presented in Table 6.3.

For the solids remaining after ammonia pretreatment of wheat straw, the above discussed ratios are also expressed in Figure 6.3, for 0.2/120/H, 1/120/L, 2/120/M (codes see Table 6.1). Based on the decrease in the specific structures 4-vinylphenol and 4-vinylguaiacol (Figure 6.3A) and the simultaneous decrease in vinyl/ Cα-ox and PhC0-2/PhC-3 ratio (Figure 6.3B), we can speculate that the relative abundance of Cα-ox and Ph-C3 increased within the residues possibly due to the fact that those structures were not targeted during ammonia treatment (Figure 6.4 and Table 6.3 (K and L)). Similarly, since the ratio vinyl/unsub was rather similar during the ammonia treatment it could indicate that the relative abundance of unsubstituted lignin were decreasing at a similar extent as 4-vinylphenol and 4-vinylguaiacol (Figure 6.3B). We can then speculate that the ammonia treatment did also targeted unsubstituted lignin by decreasing their relative abundance within treated residual lignin (Figure 6.4 and Table 6.3 (J)).

To finish, we could speculate that the first mechanism involving lignin solubilisation during an ammonia treatment is the cleavage of LCCs by the hydrolysis of ferulic acid linkages, and the cleavage of coumaric acid attached on lignin side chains.

6.3.4 Enzymatic degradability from pretreatment resulting solid fractions

Mild acid versus mild ammonia treatment

One of the aims of this study was to assess the carbohydrate degradability after a mild acidic or (bio) ammonia treatment of grass. In fact, our 2nd hypothesis was to aim whether the more xylan, lignin or LCCs disruption during pretreatment of grasses, resulted in a higher enzymatic degradability of residual material.

In view of this 2nd hypothesis, a main finding from **Chapter 3** was that the enzymatic glucan (47-50 %) and xylan (38-39 %) degradability was almost similar for the treatments performed with increasing doses of acid. In the same treatments, it was shown that xylan became soluble and that lignin was first disrupted, but subsequently re-condensed (6.2.1 and 6.2.3). In contrast, the bio-ammonia treatment reported in **Chapter 4**, showed that a slight increase in pH (from 8.6 to 8.8, equivalent to manure NH_3 input from 0.2 to 1 w/w %) during a composting Phase 1 process did increase enzymatic glucan (38 to 44 %) and xylan (32 to 40 %) degradability of the residues of 5 days treated wheat straw. Similarly, **Chapter 5** described an ammonia process and confirmed that at low concentration, a low increase in ammonia (from 0.2 to 1 w/w % NH_3), increased enzymatic glucan (22 to 26 %) and xylan (15 to 26 %) degradability of the treated residues. In the low dose ammonia treatments, xylan remained in the residues and indeed lignin or even LCC disruption occurred via a decrease in residual hydroxycinnamic acids (6.2.1 and 6.2.3). Figure 6.5 shows the comparison of glucan and xylan degradability after 24 h incubation using a (hemi-)cellulase cocktail to degrade the various treated residues. To be able to make a fair comparison of acid treated corn stover and ammonia treated wheat straw, only the trends of enzymatic degradability of both treatments were compared.

In literature, acidic processes are described to increase glucan degradability at increasing treatment severity via the solubilisation of xylan [13, 25, 26, 28]. The latter was not concluded from our study (**Chapter 3**), although solubilisation of xylan did occur to a similar extent as described in literature for similar conditions [13]. For example, the almost similar glucan degradability observed for the two doses of sulfuric acid treated solids (47 and 50 %, for 0-1.2 (CSF=0.40) and 0-2 (CSF =1.15), respectively, Figure 6.5) was unexpected and is shown to be lower as compared to previous research at similar treatment severities; glucan degradability was described to be around 50 % at CSF=-0.5 [13] and up to 80 % for CSF=1 [13, 26]. A reason for the lack of increase in degradability in our research with increased CSF treatments is unclear. Still, the enzymatic cocktail used was different compared to the one used in previous researches and we could speculate that our cocktail apparently was less powerful or did not contain the full spectrum of enzyme activities needed to degrade the remaining recalcitrant xylan in the solids to allow extensive cellulose degradation.

Ammonia is known to affect lignin structures and also known to result in improved enzymatic degradability of resulting solid fractions [16, 33, 57, 58]. Nevertheless, hereto, mainly medium and high ammonia concentrations (more than 5 w/w % and up to 600 w/w % NH_3) have been reported within the treatment of grasses indeed resulting in lignin removal [16, 31-33]. Our research is one of the first to compare enzymatic degradability of solids remaining after low ammonia concentration treatments (0.2/120/H, 1/120/L, 2/120/M, Figure 6.5 and **Chapter 5**) and remaining from a (bio-) ammonia setup (PI-P and PI-A, Figure 6.5 and **Chapter 4**).

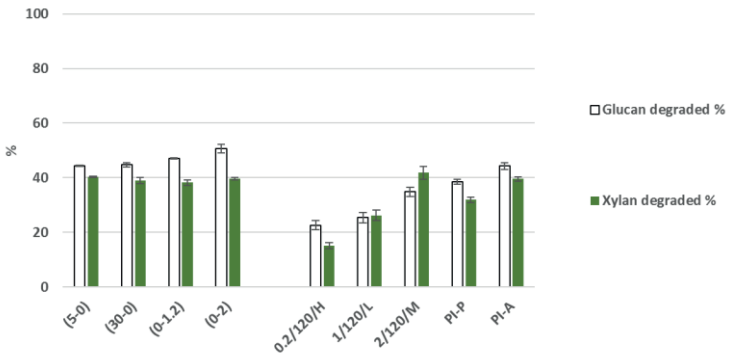

Figure 6.5: Enzymatic glucan (white bars) and xylan (green bars) degradability of the washed solids recovered after pretreatment (WUS). Conversions are based on glucose and xylose released, and represented as maximum conversion of amounts of total glucosyl (cellulose) and xylosyl (xylan) residues, respectively. (hemi-)cellulase cocktail were (see **Chapter 3**, **4** and **5** for details). Sample codes are as described in Table 6.1.

As described in literature for high dose ammonia treated solids, to a lesser extent also at low dose ammonia treatments a clear increase in enzymatic glucan (from 22 to 34 %; Figure 6.5) and xylan (from 15 to 42 %; Figure 6.5) degradation was observed. It should be noted that in **Chapter 4** and **5** another

enzyme cocktail was used compared to **Chapter 3**. Similar effects were observed in a composting process for increasing concentration of NH_3 input (PI-P 0.2 w/w % to PI-A 1 w/w %). Glucan degradability increased from 38 to 44 %, and xylan from 32 to 40 % (Figure 6.5).

In addition to dose, treatment temperature had an effect on the subsequent enzymatic degradability. A similar low ammonia dose treatment (0.5 w/w % NH_3) [33] of wheat straw, but at 30 °C and 28 days, instead of the 80 °C and 1-5 days used in our research, has been reported to lead to a lower enzymatic degradability: glucan degradability was 20-25 % and xylan degradability was 5-15 %. In that same study, glucan degradability equivalent to PI-A (1 w/w % NH_3) was only reached using 5 w/w % NH_3 addition, whereas equivalent xylan degradability to PI-A was reached using 10 w/w % NH_3 addition.

Overall, for the low dose ammonia treatments, enzymatic degradability improved, which matched with a decrease in hydroxycinnamic acids indicating lignin and/ or LCC degradation (Figure 6.3 and Table 6.3).

The comparison of the trends between our corn stover acidic (**Chapter 3)** and wheat straw (bio-)ammonia (**Chapter 4** and **5**) treatments is difficult since different enzyme cocktails were used. To our surprise and the fact that composting Phase 1 is applied at industrial scale, the composting process (Phase 1 setup) seem to be even more effective at increasing enzymatic carbohydrate degradability, especially xylan. Phase 1 composting process could be used as a storage phase for other commercial processes beyond making a selective substrate for the white button mushroom. Initial investments, obviously, are needed and include for example the construction of composting tunnels and air filtering facilities.

6.4 Phase 1 compost kinetics and severity

Understanding the different conditions and parameters affecting the composting process phase 1 helps to find ways to improve industrial yields of mushrooms or to be used in perspective of other industrial application as mentioned previously.

In **Chapter 4**, we showed that a composting phase 1 performed for 5 days increased enzymatic glucan and xylan degradability. In this section, we show that the carbohydrate degradability during composting process phase 1 (**Chapter 4**) and the laboratory ammonia treatment (**Chapter 5**) matched. Further, we show that the carbohydrate degradability increased gradually during the composting process phase 1 from 1 to 5 days, which has not been shown in previous research. Taking samples during phase 1 can be considered dangerous for human health (up to 4 kg/m^3 ammonia in the air and temperature of 80 °C, **Chapter 4**), however, taken as a challenge in our research.

The results of enzymatic degradation (24h) of glucan and xylan of solids obtained from phase I composting (**Chapter 4**) and from the low dose ammonia treatments at laboratory scale (**Chapter 5**), treated for 0 to 5 days, are shown in Figure 6.6. The laboratory scale experiment was designed according to the composting process conditions. The comparison of glucan degradability (Figure 6.6A) and xylan degradability (Figure 6.6B) showed that both treatments (lab scale and tunnel scale composting) followed a similar increasing trend. The composting process run at 0.2 (PI-P) and 1 w/w % NH_3 (PI-A) was higher in glucan degradability after 48 to 120 hours treatment compared to the treatment performed in laboratory at 2 w/w % NH_3. Xylan degradability, however, was higher for the 2 w/w % NH_3 treatment performed in laboratory compared to the composting process.

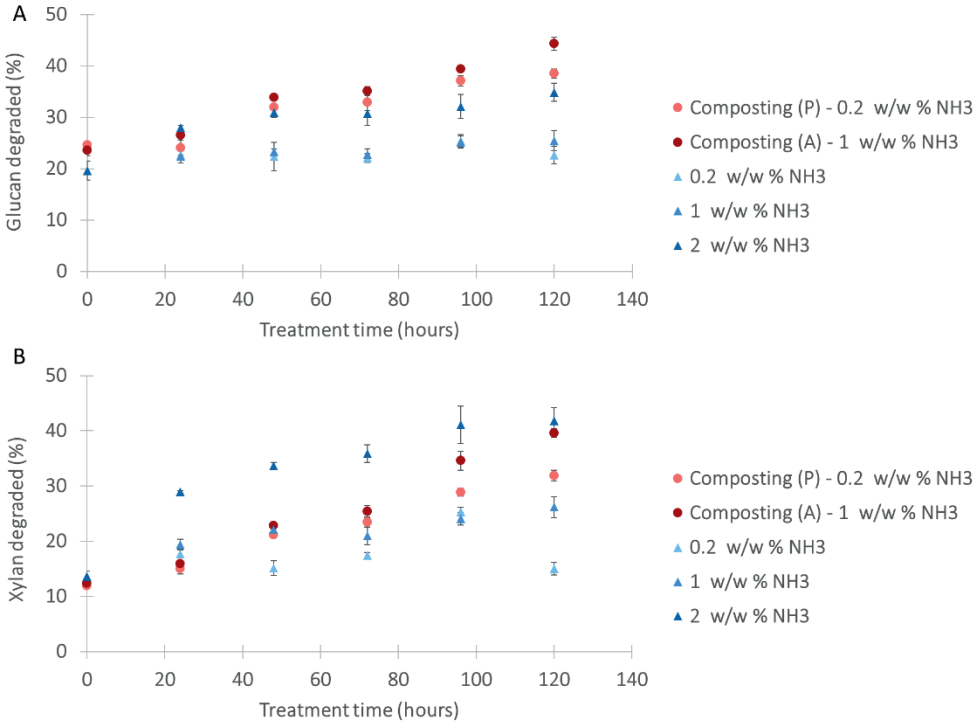

Figure 6.6: Enzymatic hydrolysis (24 hours) of washed solids (WUS) from ammonia treated wheat straw performed in a composting process and at laboratory scale using a commercial (hemi-)cellulase cocktail (see **Chapter 4** and **5** for details). Conversions are based on glucose or xylose released and represented as maximum conversion of amounts of total **(A)** glucosyl (cellulose) and **(B)** xylosyl (xylan) residues, respectively. Treatment times (x-axes) represent (ammonia-) pretreatment times.

This difference in glucan and xylan degradability could be explained that during composting process microorganisms are part of the treatment together with ammonia, increasing glucan degradability more than a treatment performed with a higher ammonia dosage (2 w/w % NH_3). Possibly, the microbial consortium had a more extensive 'glucan-opening' enzyme machinery at the conditions in

Phase 1 compared to 'xylan-opening' [59]. Studies showed that through the composting process the actinomycetal and fungal community composition evolves. After 9 days composting in Phase 1, the global composition of major thermophilic microorganisms was composed of both bacteria and fungi. *Corynebacterineae Corynebacterium* represented around 37 % of the total population [60] and were reported to have cellulase genes (*T. fusca* CelF) having synergistic effect with other cellulases [61]. The fungal population was mainly represented by *Sordariomycetes Miscroascus* (11%), *Sordariomycetes Acremonium* (11-22%), *Eurotiomycetes Thermomyces (11-38%)*, and *Eurotiomycetes Aspergillus* (11-15 %). Contrary to the bacteria found in compost, compost fungi did not have cellulase or xylanase genes activity [60]. Contrary to the glucan degradability, which is higher in the compost, the xylan degradability is higher at higher ammonia dosage (2 w/w % NH_3) than in a composting process confirming that ammonia alone improved xylan degradability. Most likely, during composting the more accessible xylan has been directly used by the microbial population present, which resulted in a lesser enzymatic accessible xylan population in the solids after composting.

Still, improvement of phase I degradability should be considered carefully, since improved degradability of phase 1 compost challenges the carbohydrate yields in the consecutive conditioning phase, still devoid of *A. bisporus,* and even may proliferate unwanted microorganisms that could hindered optimum mycelium growth during Phase 3.

6.5 Evaluation of effect treatment parameters for low ammonia dose treatments on structures and enzymatic degradability

The aim of **Chapter 5** was to independently evaluate the effects of three parameters (temperature (°C), treatment time (hours) and the Solid:Liquid ratio (S:L)) on enzymatic cellulose and xylan degradability of the residues, on carbohydrate and lignin composition, and on treatment mass balance. In **Chapter 5**, the condition of 20 w/w % ammonia (for 9 samples, **Chapter 5**) was included in the setup of the experiment to have a global overview of the effect of ammonia concentration from low to medium-high. In this section, a similar assessment, using the Taguchi statistical analysis, was now applied to visualize treatment condition parameters effects to assess only lower dose ammonia during treatments (0, 0.2, 1 and 2 w/w % NH_3). The choice to compare only lower ammonia doses is made to mimic Phase 1 composting conditions. Results are shown in Figure 6.7 for the lower ammonia doses. A rescaled Figure 5.4 from the results from **Chapter 5** is presented in Figure 6.8 to allow direct visual comparison.

The statistical analysis according to Taguchi methodology of the low ammonia doses (0, 0.2, 1 and 2 w/w % NH_3) (**this Chapter**) showed that both treatment time and ammonia concentration are the main parameters affecting wheat straw structures and composition, which was different within the

design when 20 w/w % NH_3 samples were included (**Chapter 5**). Hence, high ammonia doses (≥20 w/w %) treatment of wheat straw lowers the effects of treatment time.

In the literature, design of experiments allowing visualisation of parameters effects for treatment of biomass is often underestimated, although some researches show good examples of the value of experimental design [16, 62, 63]. In addition to the Taguchi-design used in our research, the Box-Behnken experimental design is also often applied. This Box-Behnken design is coupled to the use of Minitab software [16] and leads to surface response plots of the parameter effects on the investigated results (i.e. enzymatic degradability). The Taguchi design of experiment used in **Chapter 5** uses a similar matrix of parameters and levels, however, the visualisation of the effects of the parameters on the investigated results had to be performed manually. In our view, a Box-Behnken experimental design and surface response visualisation would have even increased the visual effects of the parameters investigated on every characteristics of low ammonia treatments. The obvious downside of Box-Behnken is that additional software needs to be acquired, which was not needed for our taken approach.

In view of ammonia treatments, either no or only Box-Behnken experimental designs were performed, but, as stated in the text above, ammonia concentration used in previous studies were ≥10 w/w % NH_3[16, 62, 63].

For only low dose ammonia treatments, the dry matter recovery in the solids (WUS) was affected more by treatment time (SN=9; Figure 6.7A) compared to ammonia dose (SN=5; Figure 6.7A). In **Chapter 5**, including the 20% ammonia dose, this effect was opposite (SN=12 for ammonia concentration and SN=6 for treatment time, as is again shown in Figure 6.8A). At low ammonia dose, basically all dry matter was recovered and this observed difference in SN has only a limited value. Interestingly, similarly to **Chapter 5** (again shown in Figure 6.8A), Figure 6.7A shows that the SN values related to dry matter losses are lower for treatment time parameter (SN=8) than for ammonia dose (SN=13). Apparently, losses relate primarily to ammonia dose and not treatment time both at medium-high and low ammonia dose treatments. In literature, Ko et al. (2009) [62] performed a treatment on rice straw at similar conditions compared to sample 20/24/L (**Chapter 5**) showing lower insoluble solid recovery (62.9 compared to 72.3 %) and similar glucan recovery (86.9 compared to 89.7 %), however, did not show losses. In **Chapter 5**, extensive losses were observed at 20 w/w % NH_3 doses. Other studies in the literature do not show complete mass balances of ammonia treatment of grasses and present carbohydrate content (w/w %) in the residues rather than recoveries. Performing low ammonia treatment (≤2 w/w % NH_3) of grass will minimise losses compare to higher ammonia concentrations (≥20 w/w % NH_3).

For specific carbohydrate recoveries, low dose ammonia treatment (Figure 6.7A) show most distinct effects on xylosyl, arabinosyl, uronic acid and glucosyl units. To be specific, treatment time had a strong effect on uronic acid recovery (SN=15), while its effects on the other mentioned carbohydrates

was present but lower (SN≤8). Ammonia concentration had a strong effect on xylosyl recoveries (SN=18), while having minor effects on the other mentioned carbohydrates (SN≤6) (Figure 6.7A). On the contrary, when the highest ammonia doses is included (**Chapter 5**, Figure 6.8A) the carbohydrate recovery is dictated by only the ammonia concentration parameter. The effect of treatment time only on uronic acid recovery may imply specific kinetics that could be investigated further. Interestingly, there was no difference in parameter effects, for low or high ammonia doses, for the enzymatic glucan and xylan degradability (Figure 6.7A and 6.8A).

In terms of lignin-composition of the solids, the effect of high ammonia concentration was more than time (SN>10 for relative abundance of H, G and S, Figure 6.8B). However, we can observe the increased effect of treatment time compared to ammonia concentration when only low ammonia doses are plotted (SN≥10 and SN≤10 for time and NH_3 concentration, respectively, for relative abundance of H, G and S, Figure 6.7B). Interestingly, ammonia concentration was not a parameter affecting the relative abundance of 4-vinylphenol and 4-vinylguaiacol at low ammonia doses, whereas treatment time had a strong effect (SN≥10, Figure 6.7B), which is opposite in **Chapter 5** (Figure 6.8B) where also 20 % NH_3 is included. Hence, most likely, for treatments within low ammonia dose ranges, treatment time affects more the cleavage of linkages with ferulic and coumaric acids than ammonia dose.

The study of low ammonia treatment of grasses through a design of experiments (Taguchi) show that low ammonia concentration minimised losses compared to higher NH_3 concentration and that the treatment time parameter had an increased effect on the treatment recoveries of grasses. Additionally, the ammonia concentration had a lower effect than time on lignin composition, while there was no clear trend for the enzymatic carbohydrate degradability.

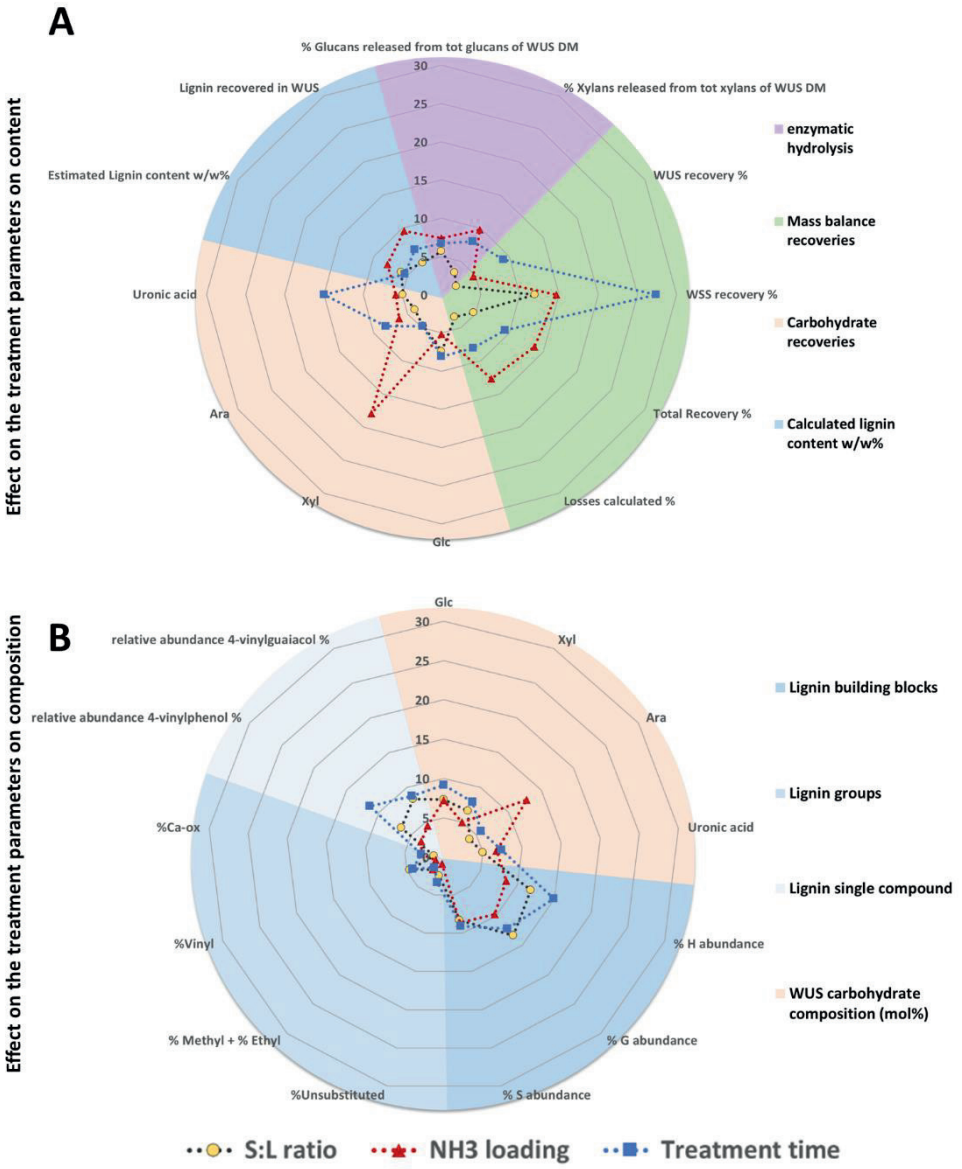

Figure 6.7: Representation of Signal to Noise ratio (SN) for only low ammonia treatment calculated from results from **Chapter 5** excluding treatment performed at 20 % w/w % NH_3. **A** SN calculated for carbohydrate (orange), lignin (blue) content w/w %, mass balance (green), and enzymatic hydrolysis of residues (purple). **B** SN calculated for lignin relative abundance (%) (orange), and the compositional values of carbohydrates (mol %) (blue).

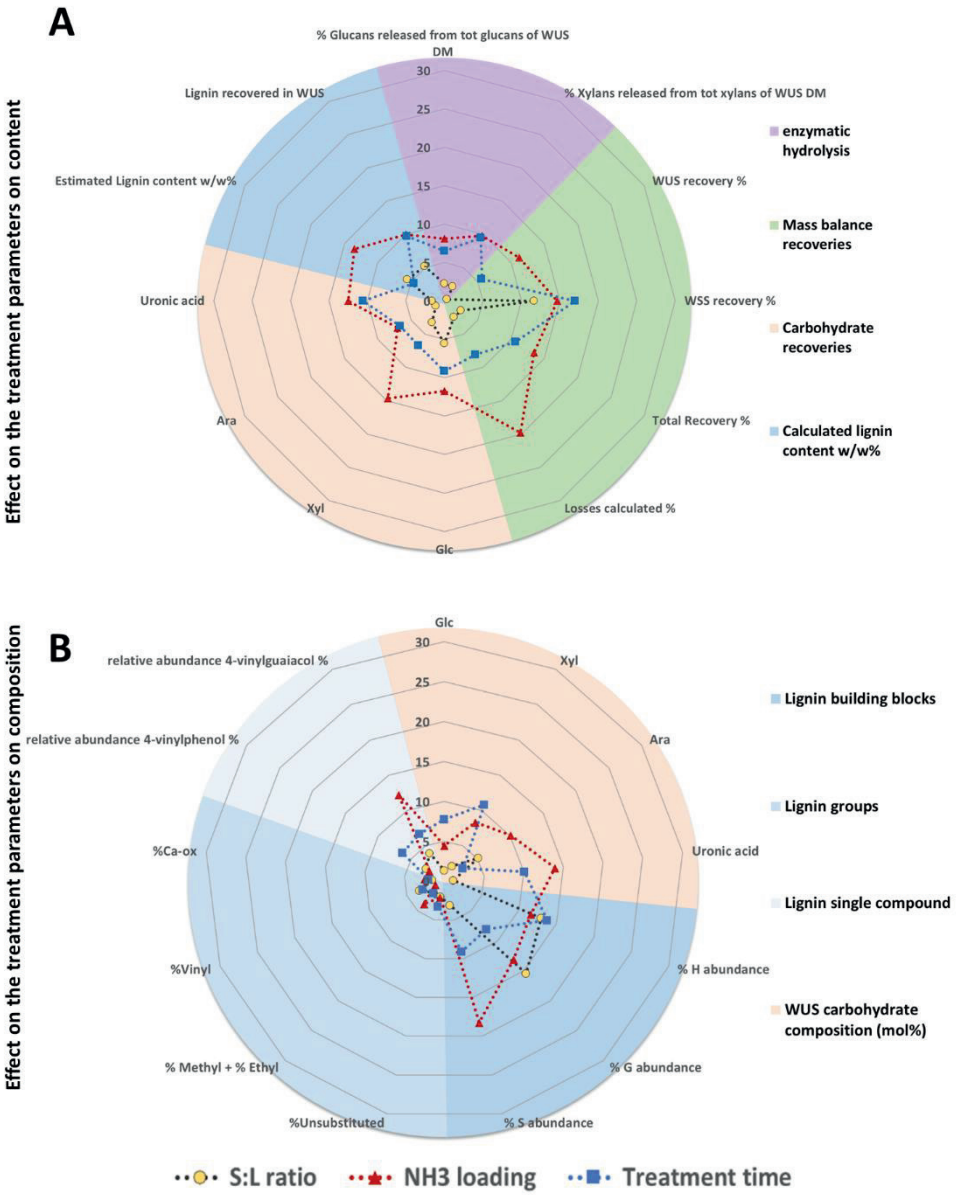

Figure 6.8: Rescaled representation of Signal to Noise ratio (SN) from results from **Chapter 5** (Figure 5.4) including 20 w/w % NH_3 samples. **A** SN calculated for carbohydrate (orange), lignin (blue) content w/w %, mass balance (green), and enzymatic hydrolysis of residues (purple). **B** SN calculated for lignin relative abundance (%) (orange), and the compositional values of carbohydrates (mol %) (blue).

6.6 Future perspectives

6.6.1 Low ammonia treatment for lignin valorisation

In this thesis, the analysis of lignin after a low ammonia treatment was investigated (**Chapter 5**). In **Chapter 5**, low ammonia treatment potentially lead to the cleavage of lignin carbohydrate complexes separating xylan from lignin and removing lignin side chains. Hence, low ammonia treatment of grasses has a great potential to be used prior to depolymerisation of lignin for lignin valorisation. It can, be expected that lignin caption is facilitated by the cleavage of the hydroxycinnamic acids.

Recently, valorisation of lignin has been proposed [36, 38] and the possibility to produce biochemicals from lignin through lignin depolymerisation was investigated extensively. Lignin structure recovery became the first step before oxidative depolymerisation [47, 64-69], depolymerisation via catalysts [66, 70-73] and also depolymerisation in combination with enzymes [69].

6.6.2 Lignin analysis

In this thesis, lignin analysis for content and composition was performed using various methods (**Chapter 2**, **3**, **4** and **5**). However lignin content (Klason lignin and ADL) and composition (NMR) analysis methods showed to have limitations. The use of py-GC-MS show many advantages for lignin content analysis compared to Klason lignin or ADL. An internal standard can be added within the sample for accurate quantification and system correction.

Overall, py-GC-MS, with addition of 13C-lignin as internal standard, seems a promising alternative for all research fields to both quantify and characterize specifically lignin in reasonably high-throughput with minimal sample handling.

6.6.3 Phase 1 composting phase integrated within bioethanol production process

Industrial composting (Phase 1; **Chapter 4**) was shown to result in increasing enzymatic xylan and glucan degradability. Currently, this composting is only used as pretreatment step to come to a selective substrate for white button mushroom production. It can be recommended to have a similar treatment, for example as storage phase, for other processes aiming at the valorisation of the carbohydrate fraction of lignocellulosic biomasses. An example of such a process is the 2nd generation bioethanol production process.

The production of bioethanol, currently performed via acidic hydrotreatment of grasses, implies large amount of feedstock storage to be processed for an extended period. Composting process phase 1 is a process that is conducted at industrial scale and would not need any optimisation nor development.

Despite the construction of the facilities to perform that storage phase, composting phase 1 could be very well integrated in the biorefinery cycle. The composting Phase 1 do not use the input of chemical as ammonia source, however, uses chicken and horse manure ammonium that will be transformed into gaseous ammonia during the aerobic fermentation.

Composting phase 1 showed real benefits towards both carbohydrate and lignin structures. First carbohydrate recoveries are very high and the treatment does not suffer from unwanted losses. Composting phase 1 increased carbohydrate degradability through the cleavage of ferulic and coumaric acid which link xylan and lignin opening the plant cell wall. Hence, if used prior to an acidic hydrothermal treatment, it can be envisaged that les acid and lower temperatures will result in similar carbohydrate conversion yields.

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Summary

The aim of this research was to identify new routes to liberate monosaccharides from hard-to-convert feedstocks by a detailed structural analysis of the recalcitrant carbohydrate and lignin released from grass feedstocks mildly treated with 1) intrinsic chemical catalysts (acetic acid and ammonium from manure); 2) novel enzymes and; 3) combination of both. The fate of the main polymers present, lignin, cellulose and xylan was studied, and also the fate of inter-linkages between carbohydrate and lignin was investigated using pyrolysis-GC-MS.

In **Chapter 1** an overview of the chemical composition of different grasses, in particular of corn stover and wheat straw, is presented. Also, the main biomass treatments currently studied are presented as well as the main enzyme activities involved in the hydrolysis of cellulose and xylan.

Chapter 2 shows the relationship between the lignin (ADL) content and composition resulting from pyrolysis-GC-MS of maize stem from different internodes and cultivars, and the *in vitro* gas production. The lignin content decreases with higher internode number within the stem, whereas the ADL content fluctuates during the season for both cultivars. The analysis of lignin composition performed using py-GC-MS shows that the S:G ratio is lower in younger tissue (higher internode number or earlier harvest date) in both cultivars. The gas produced within the first 20 hours of treatment strongly correlates with the S:G ratio, while ADL shows poor correlation. This correlation was not found after 20 hours treatment. It is concluded that the S:G ratio plays a more dominant role than ADL content in maize stem cell wall degradation associated with the first 20 hours treatment.

In **Chapter 3**, two acid catalysts, acetic acid (HAc) and sulfuric acid (H_2SO_4), is compared in thermal pretreatments of corn stover. The aim of this study is to assess the fate of the residual lignin consecutive to the acid pretreatment. The analysis of residual lignin composition from HAc and H_2SO_4 pretreatment performed using py-GC-MS shows similar trends, as the relative abundance (%) of Cα-oxidized, propenyl, vinyl-G, vinyl-S and methylated lignin units decrease, while unsubstituted vinyl-H units increase at increasing acid amounts. However, the composition itself differes. H_2SO_4-lignin shows slightly lower values (%) for unsubstituted and vinyl-H units, while the other lignin units remains higher compared to HAc-catalysis at similar pH values. The different composition of lignin resulting from both treatment, however does not result in a different carbohydrate degradability. It is concluded that HAc and H_2SO_4 treatment of corn stover lead to lignin recondensation after its solubilisation, while removal of xylan and enzymatic conversion of solids is similar.

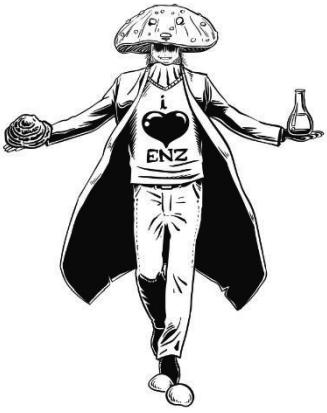
In **Chapter 4**, the research continues with wheat straw used within the context of commercial composting. The first composting phase involving a 5 days ammonia (bio-)treatment of wheat straw generates a selective substrate for mushroom (*A. bisporus*) production and its reduction in time is evaluated. The study focuses on understanding the effect of gypsum on the duration of the first phase and the mechanism behind it. The regular process with gypsum addition and the same process without gypsum is studied during a 5-day period. The enzymatic conversion of glucan and xylan is higher when the composting process is modified compared to regular compost and is reached 24

hours earlier. Additionally, composting Phase I decreases 4-vinylphenol and 4-vinylguaiacol, as analysed by py-GC-MS, that can be associated with *p*-coumarates, and ferulates linking lignin and xylan. We conclude that in absence of gypsum the desired compost quality is reached 20 % earlier compared to the control process.

The **Chapter 5** aims to understand the effect of ammonia treatment on wheat straw at similar condition as the composting process described in Chapter 4. Ammonia treatment of lignocellulose is known to improve degradability of the carbohydrate fraction. Low ammonia dose treatment is investigated to assess its potential for improved carbohydrate degradability. A statistical design of experiments (Taguchi design) is performed to independently evaluate the effects of three parameters, ammonia concentration, treatment time (hours) and the Solid:Liquid ratio (S:L), on structure, composition and enzymatic degradability of the residual fractions. The results shows that low ammonia concentration ($\leqslant$2 w/w % NH_3) resulted in a high carbohydrate recovery (> 80%) coupled to the solubilisation of 50% of total xylan and 40% of total glucan after treatment and enzymatic hydrolysis using an (hemi-)cellulase enzyme cocktail. This effect is linked to the decrease in the relative abundance of ferulic acid by 10% and of coumaric acid by more than 50%, as analysed by pyrolysis-GC-MS as 4-vinylphenol and 4-vinylguaiacol, respectively. Our findings show that the parameter effecting the most the treated wheat straw residues is the ammonia concentration even though treatment time is important for enzymatic degradability.

Finally, in **Chapter 6** the main findings of this thesis and future perspectives are discussed. Lignin compositional analysis by py-GC-MS is discussed, showing its great potential for treated grass lignin analysis compared to other methods. The effect of lignin content and composition in relation to the enzymatic degradability of residual carbohydrates are discussed and compared for different mild pretreatments and two types of grasses, being corn stover and wheat straw. As a results, mild acidic treatments solubilise xylan and retain cellulose and lignin, while mild ammonia treatment retain the three structures within the solids after treatments. Although, both treatment types retain lignin within the solid fraction, lignin is found to be modified in both cases, however, the modifications are different. Mild acidic treatments lead to the solubilisation followed by recondensation of lignin decreasing its complexity, and resulting in different lignin structures. Mild ammonia treatment, however, does not modify lignin complexity but results in the cleavage of ferulic and coumaric acid that are involved in LCCs and present on lignin side chains, respectively. The solubilisation of xylan during treatment results in an increased xylan and glucan degradability, while the cleavage of LCCs also results in increased xylan and glucan degradability using (hemi-)cellulase cocktails for both cases. The effect of only low ammonia treatment is found similar to medium-high ammonia treatment of wheat straw, however, resulting in lower losses after treatment, when analysed using the Taguchi design of experiment statistical analysis.

Acknowledgements



I will take this opportunity to express my gratitude to many of you who accompanied me during my PhD thesis project inside and outside Wageningen University over the past years. Although there is only one name stated on the cover page of this booklet it should be acknowledged that the final outcome of this thesis is a result of the contribution of many of you.

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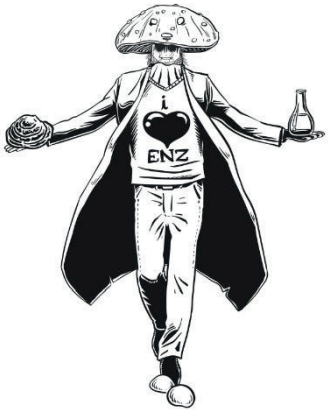
Cette thèse est dédiée à mon grand-père, Roger, qui nous a quitté cet été 2018.

Sincerely,

Thibaut

About the author



Thibaut Mouthier was born on September 4th 1986 in Paris, France. After obtaining his baccalauréat at the "Institut Charles Quentin" in Pierrefonds in 2005, he started studying biotechnology and engineering at Sup'Biotech in Paris. In 2011, he graduated as an Engineer in Biotechnology and started to work in Amsterdam, The Netherlands, in Avantium in the Research and development department as a Technologist. For 1 year and a half he worked on the development of Bio-based plastic. After this experience in the industry, he started a PhD in 2012 at the Laboratory of Food Chemistry in Wageningen University in collaboration with Royal DSM and CNC Grondstoffen BV on chemical and biological treatment of lignocellulosic biomass. The results of his PhD research are presented in this thesis.

Contact: thibaut.mouthier@gmail.com

List of Publications

He, Y*, **Mouthier, TMB***, Kabel, MA, Dijkstra, J, Hendriks, WH, Struik, C, Cone, JW, Lignin composition is more important than content for maize stem cell wall degradation. J Sci Food Agric. 2017;384-390.
** These authors contributed equally.*

Mouthier, TMB, Appeldoorn, MM, Pel, H, Schols, HA, Gruppen, H, Kabel, MA, Corn stover lignin is modified differently by acetic acid compared to sulfuric acid. Industrial Crops and Products. 2018; 121:160-168.

Mouthier, TMB, Kilic, B, Vervoort, P, Gruppen, H, Kabel, MA, Potential of a gypsum-free composting process of wheat straw for mushroom production. PlosOne. 2018; 1-15.

Mouthier, TMB, de Rink, B, van Erven, G, de Gijsel, P, Schols, HA, Kabel, MA, Low liquid ammonia treatment of wheat straw increased carbohydrate degradability and decreased residual hydroxycinnamic acids. *Submitted*

Overview of completed training activities

Disciplines and specific activities		
Courses		
Composting and growing	Mushroom office, Milsbeek	2013
Annual workshop on enzymatic hydrolysis of insoluble carbohydrates	University of Copenhagen, Holbaek	2013[a]
C4C scientific meeting	C4C, Milsbeek	2013[b]
Applied biocatalysis	VLAG, Wageningen	2014[a]
Advanced food analysis	VLAG, Wageningen	2015
Biorefinery for biomolecules	VLAG, Wageningen	2015[a]
Food and biorefinery Enzymology	VLAG, Wageningen	2015[a]
Conferences		
PolyRefNorth	Roskilde University and Copenhagen university, Copenhagen	2013[a]
EPNOE conference	Institute of Biopolymers and chemical fibers, Warsaw	2015[a]
General courses		
Techniques for writing and presenting a scientific paper	WGS, Wageningen	2014
Project and time management	WGS, Wageningen	2014
Career Orientation	WGS, Wageningen	2016
Scientific Writing	WGS, Wageningen	2016
Additional activities		
Preparation of research proposal	FCH, Wageningen	2012
Food chemistry PhD study trip	FCH, Germany, Denmark, Sweden, Finland	2014[a,b,c]
Food chemistry PhD study trip	FCH, Japan	2016[a,b]
PhD presentation and seminars	FCH, Wageningen	2012-2017
BSc and MSc Thesis Student Supervision, Presentation and Colloquiums	FCH, Wageningen	2013-2016

[a] Poster presentation, [b] Oral presentation, [c] Organizing committee

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