# Designed enzyme preparations for the hydrolysis of corn silage polysaccharides

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# Designed enzyme preparations for the hydrolysis of corn silage polysaccharides

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**Thesis**

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## Abstract

This thesis describes the design of hemicellulolytic enzyme preparations with high activity towards the rather recalcitrant xylan present in corn silage, a major biogas feedstock. Also, recalcitrance factors towards the enzymatic conversion of xylans, varying in type and level of substitution, are addressed. A $^{1}$H NMR based method was developed, allowing fast and robust quantification of the acetyl and phenoyl ester contents of plant polysaccharides. Lower enzymatic conversion of esterified xylan, embedded in the plant cell wall network, compared to extracted xylan was observed due to its recalcitrance through presence of acetyl and phenoyl esters and lower accessibility by the presence of lignin and cellulose. Combinations of enzyme preparations, showing increased substrate conversion, were recognized and provide a source for further optimization of hemicellulolytic mixtures. The data obtained show that enzyme activities that are underrepresented in enzyme preparations need to be adapted with proceeding hydrolysis in order to reach optimal conversion levels.

The approach of improving the enzymatic hydrolysis, as presented in this thesis, is shown to be an efficient strategy to obtain high conversion of polysaccharides. Industrial hemicellulolytic enzyme preparations were used for combinatorial screening and an individual, synergistic enzyme present in a “high activity” enzyme mixture was purified. A significant increase in the conversion of corn silage polysaccharides was obtained by supplementation of the acetyl xylan esterase 1 (*T. longibrachiatum*), classified as belonging to carbohydrate esterase (CE) family 5, to the *A. niger* / *T. emersonii* enzyme preparation. In addition, characterization of the deacetylation of partially acetylated xylo-oligosaccharides by acetyl esterases belonging to different CE families was performed. Acetyl esterases were categorized in three groups, with activity towards (i) 2-*O*, 3-*O* acetylated Xyl*p*, (ii) 2-*O*, 3-*O* and 2,3-di-*O* acetylated Xyl*p*, and (iii) 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p*, and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA at the non-reducing end. Positional preferences are shown towards 2,3-di-*O* acetylated Xyl*p* for the CE1, CE5 and CE6 classified esterases from *T. emersonii*, *A. niger* and *Orpinomyces sp.*, respecitively, and towards 3-*O* acetylated Xyl*p* for the CE4 classified esterase from *C. thermocellum*.

## List of abbreviations

| | |
|---|---|
| Ac | Acetyl residue |
| AcA | Acetic acid |
| AcXOS | Acetylated xylo-oligosaccharides |
| AcUXOS | Acidic acetylated xylo-oligosaccharides |
| AEC | Anion exchange chromatography |
| AFR | Anaerobic fermentation residue |
| An | *Aspergillus niger* |
| Ara | Arabinose |
| AX | Arabinoxylan |
| Axe | Acetyl xylan esterase |
| CAZy | Carbohydrate active enzyme |
| CBM | Carbohydrate binding module |
| CE | Carbohydrate esterase |
| CEC | Cation exchange chromatography |
| cWUS | Sodium chlorite treated water unextractable solids |
| cs soluble | Corn silage water soluble extract |
| Ct | *Clostridium thermocellum* |
| DM | Dry matter |
| DMSO | Dimethyl sulfoxide |
| DS | Degree of substitution |
| ErCS | Enzyme recalcitrant solids |
| EXH | Eucalyptus xylan hydrolysate |
| FA | Ferulic acid |
| Gal | Galactose |
| GH | Glycoside hydrolase |
| Glc | Glucose |
| GlcA | Glucuronic acid |
| H | Hexose |
| HIC | Hydrophobic interaction chromatography |
| HMBC | Heteronuclear multiple bond correlation |
| HPAEC | High performance anion exchange chromatography |
| HPLC | High performance liquid chromatography |
| HPSEC | High performance size exclusion chromatography |
| HSQC | Heteronuclear single quantum coherence spectroscopy |
| LC/MS | Liquid chromatography/mass spectrometry |
| MALDI TOF | Matrix assisted laser desorption/ionisation |
| Man | Mannose |
| Me | Methyl |
| MeGlcA | 4-*O*-methyl-glucopyranosyl uronic acid |
| MS | Mass spectrometry |
| n.d. | Not determined |
| NMR | Nuclear magnetic resonance |
| P | Pentose |
| pCA | p-Coumaric acid |
| p-NP-Ac | p-Nitrophenyl acetate |
| Rha | Rhamnose |
| RI | Refractive index |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SEC | Size exclusion chromatography |
| Te | *Talaromyces emersonii* |
| Tr | *Trichoderma reesei* |
| Os | *Orpinomyces sp.* PC-2 |
| UA | Uronic acid |
| WEX | Water extractable fraction from corn silage |
| WUS | Water unextractable solids |
| Xyl | Xylose |

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*CHAPTER 1*

# General introduction

## Preface

In order to improve the sustainable utilization of natural resources and to create high-added value products from biomass and agricultural raw material, the "LeanGreenFood" 7th Framework EU-program was initiated. For the globally fast growing industrial sector of biofuels, the efficient hydrolysis of plant polysaccharides is essential (Kircher 2014). For this reason, the development of efficient enzyme preparations for the hydrolysis of polysaccharides from corn silage was targeted within this thesis as part of the "LeanGreenFood" program.
One of the largest anthropogenic impacts on the environment is greenhouse gas emission by burning fossil fuels for electricity, transportation and heat. These sectors are responsible for 81 % of the total greenhouse gas emissions according to the United States Environmental Protection Agency (www.epa.gov). In order to significantly reduce the impact on climate change, greenhouse gas emissions must be lowered to more than half of their levels in 1990 (IPCC 2000), which is expected to boost the development of sustainable energy sources. One of such alternative resource to fossil fuels is biomass. Next to the use of lipids, monosaccharides released from plant polysaccharides can be fermented to biofuels. From a socio-economical point of view, agricultural, industrial and municipal carbohydrate-rich residues are sustainable and currently vastly unused energy feedstocks.

**Biofuels**

A main driver for the production of biofuels is the shift from fossil to biomass feedstocks (Kircher 2014). The global "energy from biomass" potential for sustainable use at competitive prices was estimated to be at 270 EJ/ yr (van Foreest 2012), which could cover 60 % of the current global energy demand (approximately 450 EJ/ yr; Faunce 2013).
Biodiesel, bioethanol and biogas are different examples of biofuels. Biodiesel is synthesized through transesterification of triacylglycerides (Bernal et al. 2012). Biodiesel is made from a diverse set of feedstocks including recycled cooking oil, soybean oil and animal fats. Biogas and bioethanol production are based on the anaerobic (biogas) and aerobic (bioethanol) fermentation of plant derived sugars. In order to be able to produce bioethanol and biogas, plant cell wall polysaccharides need to be converted into monosaccharides. For a sustainable, biochemical conversion of plant polysaccharides to fermentable sugars, efficient carbohydrate active enzymes are necessary. Process technologies for both bioethanol and biogas production are available and can be further optimized in order to efficiently utilize lignocellulosic feedstocks. However, the development of an economically feasible and sustainable, ideally carbon-neutral process is still an unresolved challenge. Current approaches to increase production in the bioethanol industry, involving harsh chemical and physical pretreatments, bear the disadvantages of high costs, energy needs (Blanch et al. 2011), environmental pollution and hinder the fermentation process of monosaccharides by the formation of

inhibitors (Horn et al. 2011). Therefore, a reduction of pretreatment severity is necessary regarding the development of a sustainable process. Furthermore, pretreatment is sometimes not applied due to economic and practical reasons as for example in the biogas industry.

*Biogas production process:* Biogas production involves a complex process, which can be divided into different phases, being hydrolysis (solubilization of particulates and conversion of polymers into oligomers and monomers), acidogenesis (conversion of monomers into volatile fatty acids), acetogenesis/dehydrogenation (conversion of volatile fatty acids into acetic acid, carbon dioxide, and hydrogen) and methanogenesis (conversion of acetates into methane and carbon dioxide, while hydrogen is consumed; Weiland 2010). Some acetate and hydrogen is directly produced by acidogenic fermentation of sugars and amino acids. However, acetate and hydrogen are primarily derived from the acetogenesis and dehydrogenation of higher volatile fatty acids (Miyamoto 1997). Figure 1 shows the stages of the methane formation process. Complex polymers are hydrolyzed and fermented by a consortium of mostly strictly anaerobic and some facultative anaerobic microorganisms. The higher volatile fatty acids are metabolized by obligate hydrogen-producing acetogenic bacteria. Methanogenic bacteria are strict anaerobes. Polysaccharides present in the plant biomass that are not converted into monosaccharides during the hydrolysis phase of the anaerobic fermentation process are recalcitrant towards the enzymes produced by the microorganisms present. Such recalcitrance of polysaccharides, as further described below, contributes to the current non-economic, partial conversion of the cellulose and hemicellulose (Himmel et al. 2007). Feedstock recalcitrance towards enzymatic processing can be diminished by the use of optimized enzyme preparations. Such enzyme preparations can be added during the hydrolysis stage of the methane formation process (Figure 1). Highly effective, optimized and inexpensive (hemi)cellulolytic enzyme preparations, may enable a more competitive utilization of non-food biomass for biomass conversion.

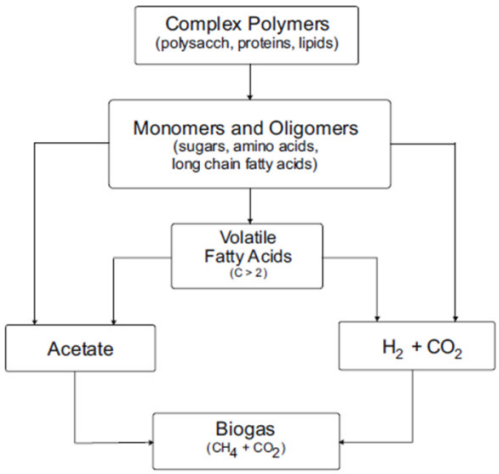


Figure 1: The stages of the methane formation process (Weiland 2010).

## The plant cell wall

The main components of plant cell walls of monocotyledons, such as corn, are hemicellulose, cellulose and lignin (Figure 2). The cell wall fulfills a range of physiological functions of the plant, such as stability, flexibility, water holding capacity or barrier against pathogens, as previously described (Carpita and Gibeaut 1993). Plant cell walls are often divided into two categories: (i) Primary walls, surrounding growing cells or cells capable of growth and (ii) secondary walls, containing lignin and surrounding specialized cells such as vessel elements. However, in reality primary and secondary walls are two extreme forms (Keegstra 2010), because specialized plant cells contain a spectrum of different cell wall characteristics.

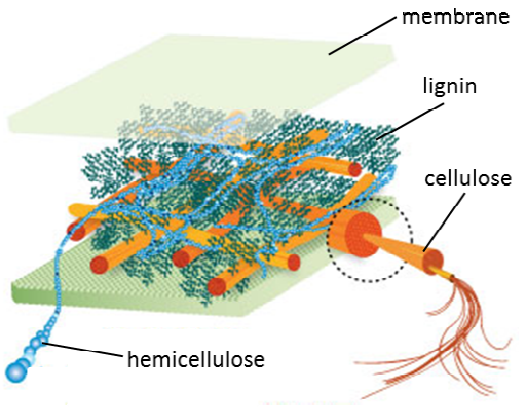


Figure 2: A simplified plant cell wall model containing cellulose microfibrils, hemicellulose and lignin (according to Ceres 2014).

### Cellulose

Cellulose is a linear insoluble polymer, consisting of β-(1,4)-linked glucose units. The structure of cellulose is shown in Figure 3. It is synthesized by plasma membrane-localized complexes containing structurally similar cellulose synthase subunits (Persson et al. 2005). The glucan chains can form interactions via hydrogen bonds resulting in water insoluble crystalline microfibrils (Somerville 2006). These crystalline regions widely persist hydrolysis by cellulases. Furthermore, hemicellulose coats cellulose through hydrogen bonding (MacAdam 2009).

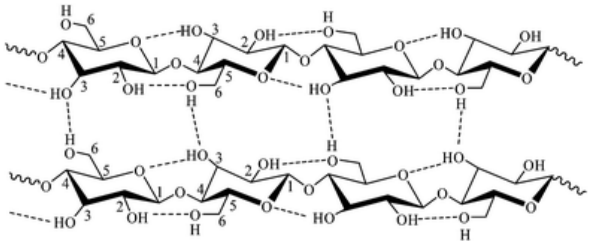

Figure 3: Structural model of cellulose (Lu et al. 2014).

**Hemicellulose**

Hemicellulose polysaccharides are divided into four general groups of structurally different polysaccharide types: mannans, xyloglucans, mixed-linkage β-glucans and xylans (Ebringerová 2006).

*Xylans can be divided in 5 different groups (Figure 4, Ebringerová 2006):*

(i) Homoxylans are polysaccharides composed of D-xylopyranosyl residues linked by β-(1->3)-linkages, β-(1->4)-linkages and/or mixed β-(1->3, 1->4)-linkages (Figure 4a). They are present for example in red algae (*Nemiales* and *Palmariales* sp.) and green algae (*Caulerpa* and *Brypsis* sp.) or seeds of *Plantago sp*. (Ebringerová 2006).

(ii) Glucuronoxylans (4-*O*-methyl-D-glucurono-D-xylan) consist of a β-(1->4)-D-xylopyranose backbone, substituted with single unit side chains of α-(1->2)-4-*O*-methyl-D-glucuronic acid and its non-methylated form (Figure 4b). Furthermore, the backbone is partially acetylated at the *O*-2 and *O*-3 position of Xyl*p*. They represent the main hemicellulose component of the secondary cell wall of woody tissues in dicots (Ebringerová 2006).

(iii) Glucuronoarabinoxylans [(D-glucurono)-L-arabino-D-xylan] consist of a partially substituted β-(1->4)-D-xylopyranose backbone. The xylopyranosyl units can be substituted with *O*-methyl-D-glucuronic acid at the *O*-3 position and L-arabinofuranosyl units at the *O*-3 or *O*-2 positions (Figure 4c). Dimeric arabinofuranosyl side groups might be present. These polysaccharides are present in lignified tissues of grasses and cereals.

(iv) Arabinoglucuronoxylans [(L-arabino)-4-O-methyl-D-glucurono-D-xylan] have the same structure as glucuronoxylan with additional single α-L-arabinofuranosyl residues at the *O*-3 position of the β-(1->4)-xylopyranan backbone (Figure 4d). They occur in softwoods and next to mannans as the minor hemicellulose in *Ginkgo biloba* (Ebringerová 2006).

(v) Arabinoxylans (L-arabino-D-xylan) are built of a β-(1->4)-D-xylopyranose backbone. The xylopyranosyl units are substituted at the *O*-2 and/or *O*-3 position with L-arabinofuranosyl units. Phenoyl and acetyl esters can also be present (Figure 4e). They are present in the cell wall of starchy endosperm, the outer layers of cereal grains and seeds of monocotyls (e.g. rye grass, bamboo shoots and pangola grass).

Very complex xylans were isolated for example from bran, seeds and gum exudates (Ebringerová 2006). These polysaccharides carry a variety of mono and oligosaccharide substituents on the β-(1->4)-D-xylopyranose backbone. Xylans present in corn have a high complexity (Figure 4f; Agger et al. 2010).

**Lignin**

Lignin is a complex aromatic heteropolymer composed of phenylpropanoid aryl-$C_3$ units which are linked via a variety of ether and C-C bonds (Bugg et al. 2011). Xylan can be covalently coupled to lignin via ferulates (Funk et al. 2007). Lignin is formed by radical polymerization of

guaiacyl units from precursor coniferyl alcohol, syringyl units from precursor sinapyl alcohol and p-hydrohyphenyl units from precursor p-coumaryl alcohol (Bugg et al. 2011). High recalcitrance of lignin towards enzymatic degradation has been described (Zeng et al. 2011).

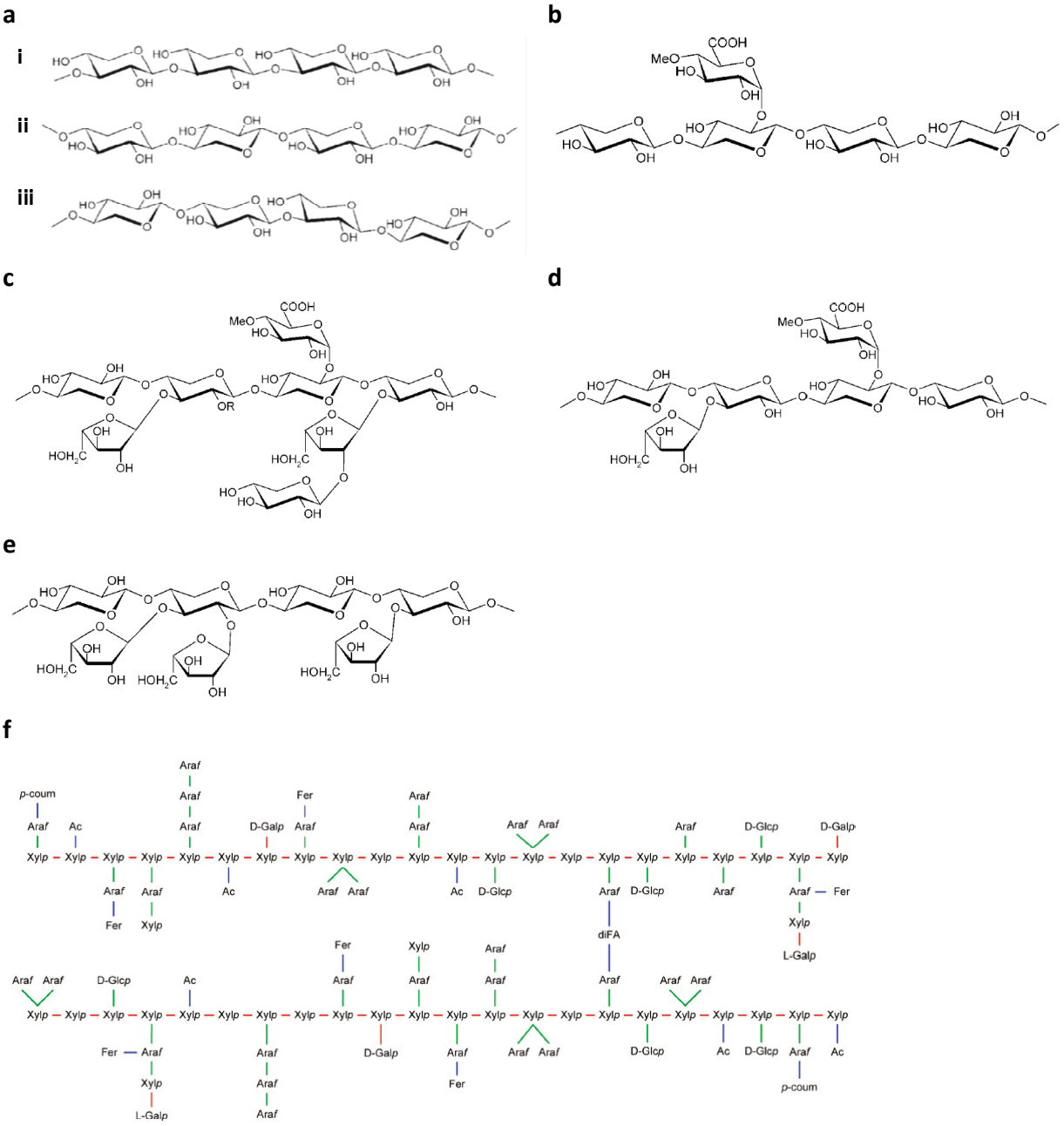


Figure 4: (a) homoxylan chains with i. β-(1->3)-linkages, ii. β-(1->4)-linkages and iii. mixed β-(1->3, 1->4)-linkages, (b) 4-*O*-methyl-D-glucurono-D-xylan, (c) (D-glucurono)-L-arabino-D-xylan, (d) (L-arabino)-4-*O*-methyl-D-glucurono-D-xylan, (e) L-arabino-D-xylan; modified from Ebringerová (2006), (f) a simplified sketch of corn bran xylan (Agger et al. 2010).

## Biogas feedstocks

Different types of biomass can be used for biogas production as long as they contain carbohydrates, proteins, fats, cellulose or hemicellulose as main components (Weiland 2010). Corn silage, as described below, and combinations of corn silage and manure are typically used for biogas production, but agricultural, forest and grass residues, urban waste streams and food waste are also examples of feedstocks that can be used for methane production. The cellulose and hemicellulose contents of a range of feedstocks are shown (Table 1). The amount of carbohydrates present determines the methane yield. Carbohydrates and proteins show fast conversion rates by anaerobic digestion. Fats provide higher gas yields than carbohydrates but have the disadvantage that they require long retention times. An increased utilization of feedstocks (Table 1) would be beneficial from a socio-economical point of view, as described above, and may co-exist next to the established biogas production from corn silage and manure. Furthermore, such feedstocks may be supplemented as co-substrates to corn silage, other energy crops or manure. However, the highest biogas potential results from energy crops (Weiland 2010), indicating that such crops are essential in order to produce biogas in an economically feasible way, while other feedstocks (Table 1) may be used as co-substrates.

Table 1: Cellulose and hemicellulose contents (w/ w %) of agricultural residues.

| **feedstocks / waste streams** | **lignocellulosic material** | **cellulose (%)** | **hemicellulose (%)** |
|---|---|---|---|
| agricultural residues | corn stover[a] | 30 | 26 |
| | corn fiber[a] | 14 | 39 |
| | corn cobs[a] | 33 | 34 |
| | wheat straw[b] | 30 | 50 |
| | cotton seed hairs[b] | 90 | 10 |
| manure | solid cattle manure[b] | 2-5 | 1-3 |
| urban waste streams | paper[b] | 85-99 | 0 |
| | sorted refuse[b] | 60 | 20 |
| | newspaper[b] | 40-55 | 25-40 |
| | waste paper from chemical pulps[b] | 65 | 10-20 |
| | primary wastewater solids[b] | 8-15 | |
| forest residues | hardwood stems[b] | 40-55 | 24-40 |
| | softwood stems[b] | 45-50 | 25-35 |
| | leaves[b] | 15-20 | 80-85 |
| grass feedstocks | Coastal bermudagrass[b] | 25 | 36 |
| | switchgrass[b] | 45 | 31 |
| | grasses[b] | 25-40 | 35-50 |

[a] Van Eylen et al. 2011.
[b] Kumar et al. (2009).

**Corn silage as a feedstock for biogas production**

Corn silage constitutes a major feedstock for biogas production. It is made from the aboveground part of the corn plant (ears, stalks and leaves; Bittman and Kowalenko 2004). Soluble plant sugars are fermented by anaerobic bacteria to organic acids, which reduce the pH of the plant material, preserving the silage and making it available the whole year round (Allen et al. 1995).

The composition of different plant parts derived from a corn crop varies (Table 2). Corn silage contains all the corn fractions as described in Table 2 [cobs, fiber (cellulose containing parts of grains; approximately 8 % w/w of the grain is fiber) and stover (stalks, leaves, husks and tassels; Pennington 2013)]. Also grain, containing bran (6 % of the grain), germ (11 % of the grain) and endosperm (83 % of the grain; White and Johnson 2003), which consists mainly of starch (88 %) and protein (8 %; Singletary and Below 1989), is present in corn silage.

The overall hemicellulose content as well as the ratio of individual hemicellulose building blocks (Xyl, Ara, Gal, UA and phenoyl and acetyl esters) show significant differences between corn fractions. While the cellulose and hemicellulose amounts present in corn cobs and corn stover do not differ significantly, corn fiber contains a much higher hemicellulose content than cellulose. Furthermore, the degree of substitution [DS (mol Ara, Gal, UA, ferulic acid, *p*-coumaric acid and acetic acid per mol Xyl)] is higher for corn fiber (DS = 1) compared to corn cobs and corn stover, which show a similar DS of 0.6 and 0.5, respectively. The protein and lignin contents vary between the different corn fractions. Regarding grain, starch constitutes the main part (Table 2). The heterogeneity of the polysaccharide composition present in different corn fractions reflects the high complexity of this feedstock. The xylan present in corn is complex as it is a heavily substituted polysaccharide, as discussed above and illustrated by the sketch of corn bran xylan (Figure 4f).

Table 2: Composition of different fractions from corn.

| | Composition (g $kg^{-1}$ dry matter) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | starch | cellulose | Ara | Xyl | Gal | Man + Rha | UA | ferulic acid | coumaric acid | acetic acid | protein | lignin |
| Cobs[a] | 2 | 33 | 2.4 | 28 | 0.8 | 0.8 | 1.8 | 1.1 | 1.7 | 4.3 | 1.0 | 17 |
| Fiber[a] | 16 | 14 | 12 | 18 | 3.3 | 2.0 | 3.7 | 3.1 | 0.3 | 3.2 | 10 | 5.7 |
| Stover[a] | 1 | 30 | 2.7 | 19 | 1.0 | 1.1 | 2.2 | 0.4 | 0.4 | 2.4 | 4.2 | 29 |
| Grain[b] | 73 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 9.1 | n.d. |
| Germ[b] | 8 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 18.4 | n.d. |
| Bran[c] | 14 | 40 | 20 | 30 | 5.6 | 3.9 | 2.2 | n.d. | n.d. | n.d. | 11.5 | n.d. |
| Endosperm[b] | 88 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 8.0 | n.d. |

n.d., not determined.

[a] Van Eylen et al. 2011.

[b] White and Johnson 2003.

[c] Ebringerová and Hromádková 2002.

## Utilization of enzymes in biogas production

The hydrolysis of biomass by microbial hemicellulolytic enzymes has been shown to be the rate limiting step in anaerobic digestion (Parawira 2011). Considering the currently available enzyme technology, industrial, *state-of-the-art* enzyme preparations provide a promising starting point for further improvements of the enzymatic hydrolysis process, especially because relatively cost efficient production of enzyme preparations by common microbial hosts is already established. So far, the current enzyme technology is not optimized for the conversion of plant polysaccharides. Commercial enzyme preparations are not yet routinely used for biogas production from bioenergy feedstocks, such as corn silage (Parawira 2014).
In this work the enzymatic hydrolysis of corn silage hemicellulose by hemicellulolytic enzyme preparations was studied. Recalcitrance characteristics of poly- and oligosaccharides towards the enzymatic conversion are targeted and strategies for an improved enzymatic hydrolysis are presented. A biochemical characterization of corn silage hemicellulose and its enzymatic hydrolysis enables insights for the design of improved, hemicellulolytic enzyme preparations. Optimized enzyme preparations are likely to be applied frequently for biogas production as they increase the profitability of biogas production.

## Plant cell wall polysaccharide degrading enzymes

In order to biochemically convert plant cell wall polysaccharides to monosaccharides, the action of (hemi)cellulolytic enzymes is required. Such carbohydrate active enzymes act on cellulose (cellulases) or on the xylan (xylan degrading enzymes). For the degradation of both cellulose and xylan, hydrolytic activity by endo-enzymes, acting upon inner bonds in polymeric substrates, and exo-enzymes, cleaving terminal residues of oligomers to monomers, are necessary. In order to efficiently degrade polysaccharide structures, specialized fungi and bacteria express a broad set of carbohydrate active enzymes (van den Brink and de Vries 2011). Synergy of such enzymes regarding plant cell wall saccharification has been reported for xylan backbone hydrolyzing endo-xylanases and accessory enzymes (de Vries et al. 2000).

### Classification of carbohydrate active enzymes

A comprehensive classification of carbohydrate active enzymes in families based on amino acid sequence similarities is available through the Carbohydrate Active Enzymes database (CAZy, www.cazy.org; Lombard et al. 2014). Enzymes are grouped under glycoside hydrolases, glycosyltransferases, polysaccharide lyases, carbohydrate esterases and auxiliary activities. Furthermore, protein fragments that do not have enzymatic activity, but target enzymes to the substrate can be classified under the carbohydrate-binding modules class (Cantarel et al. 2009, Table 3). Furthermore, enzymes, belonging to one enzyme class, are divided in families based

on significant amino acid sequence similarity with at least one biochemically characterized founding member (Lombard et al. 2014).

Table 3: Enzyme classes and their catalytic activity according to the Carbohydrate active enzymes database (www.cazy.org).

| Enzyme classes | Activity |
|---|---|
| Glycoside hydrolysases | Cleave glycosidic bond between carbohydrates or between a carbohydrate and a non-carbohydrate moiety by the addition of a water molecule (Davis and Henrissat 1995). |
| Glycosyltransferases | Catalyze the formation of glycosidic bonds from phospho-activated sugar donors (Cantarel et al. 2009). |
| Polysaccharide lyases | Cleave the glycosidic bond of uronic acid containing polysaccharides by the β-elimination mechanism (Yip and Withers 2006). |
| Carbohydrate esterases | Remove esters from oligo- and polysaccharides (Cantarel et al. 2009). |
| Auxiliary activities | Not strictly restricted to a single catalytic reaction mechanism; covers ligninolytic enzymes and lytic polysaccharide mono-oxygenases. |
| Carbohydrate-binding modules | Target enzymes to the substrate and may promote a longer interaction between enzymes and their substrate (Boraston et al. 2004). |

**Cellulases**

Mechanistically, cellulases form a family of different groups of enzymes, being endo-(1,4)-β-D-glucanases, exo-(1,4)-β-D-glucanases and β-glucosidases (Kuhad et al. 2011). Endo-glucanases hydrolyse “internal” glycosidic bonds and are mainly found in families GH5, GH9 and GH51 (Lopez-Casado et al. 2008). Exo-glucanases (Himmel et al. 2007) act on the ends of the glucan chain. In CAZy, entries of glycoside hydrolases under exo-glucanase appear within families GH3, GH6, GH7, GH 48 and GH 55. β-Glucosidases (GH1, GH3 or GH9) hydrolyse cellobiose.

**Xylan degrading enzymes**

Different enzymes are required to degrade the basic structural components which occur in hemicelluloses (Shallom and Shoham, 2003). The enzymes required for the hydrolysis of complex xylan as present in corn are described below. Figure 5 shows a simplified sketch for the degradation of xylan by fungal enzymes.

*(Endo-1,4*-β-*)-xylanases* catalyze the hydrolysis of 1,4-β-D-xylosidic linkages of xylan in an endo-fashion (Collins et al. 2005). Within the CAZy classification system xylanases are commonly found amongst families 10 and 11 (www.cazy.org). Catalytic domains with endo-1,4-β-xylanase activity can further be found in families 5, 7, 8 and 43 and bifunctional enzymes with a xylanase and a glycosidase domain are present amongst families 16, 52 and 62 (Collins et al. 2005). Endo-1,3-β-xylanases are present amongst family 26.

Xylanases constitute the main proportion of commercial xylan degrading enzyme preparations. 3420 patents have been listed to be introduced by the United States Patent and Trademark Office (www.uspto.gov) since 2001 to present with reference to xylanases.

β-*Xylosidases* are exo-acting enzymes that catalyze the cleavage of xylobiose and hydrolyze the non-reducing terminal end of D-xylan. According to CAZy, β-xylosidases are divided into families 3, 30, 39, 43, 52 and 54 (Knob et al. 2009).

α-*Arabinofuranosidases* cleave arabinosyl linkages. They are exo-acting enzymes, which hydrolyze terminal non-reducing residues from arabinose-containing polysaccharides (Saha 2000). They can hydrolyze (1->3)- and (1->5)-α-arabinosyl linkages within arabinan polymers and xylans substituted with arabinose (Bachmann and McCarthy 1991, Verbruggen et al. 1998). Subclasses of α-arabinofuranosidases are (i) enzymes with activity towards arabinose from singly and doubly substituted xylose and *p*-nitrophenyl α-L arabinofuranoside, (ii) enzymes that hydrolyze arabinose residues linked to the *O*-2 or *O*-3 position of xylose residues and do not hydrolyze *p*-nitrophenyl α-L-arabinofuranoside and (iii) enzymes that are able to release only *O*-3-linked arabinose residues from double- substituted xylose residues but do not hydrolyze *p*-nitrophenyl α-L-arabinofuranoside (Numan and Bhosle 2006). Entries under arabinofuranosidase are present in CAZy under families GH3, GH10, GH43, GH51, GH54, GH62 and GH 127 (www.cazy.org).

*α-Glucuronidases* hydrolyse the α-1,2-linkage between the (4-O-methyl)glucuronic acid and xylopyranosyl units. α-Glucuronidases from GH family 67 liberate GlcA or meGlcA only from fragments of glucuronoxylans in which the uronic acid is linked to the non-reducing terminal xylopyranosyl residue (Ryabova et al. 2009). Furthermore, the α-glucuronidase from *Schizophyllum commune* liberates meGlcA side chains from hardwood glucuronoxylan and the α-glucuronidase from the xylanolytic yeast *Pichia stipites* CBS 6054 liberates meGlcA residues linked to terminal or internal xylopyranosyl residues of glucuronoxylan and aldouric acids generated from polysaccharides by incubation with endoxylanases (Ryabova et al. 2009).

*Galactosidases:* α-Galactosidases release α-linked D-galactose residues from a wide range of substrates (van den Brink and de Vries, 2011) as for example xylan and galactomannans. They are present in CAZy under families GH4, GH27, GH36 and GH57. Presence of β-linked D-galactose in xylans and galactoglucomannans suggested that β-galactosidases (GH2 and GH35; van den Brink and de Vries, 2011) also play a role in the conversion of xylans (Sims et al. 1997, van den Brink and de Vries, 2011). Release of D-galactose residues by β-galactosidases was shown for wheat flour (de Vries et al. 2000).

*Carbohydrate Esterases* catalyze the deacetylation of substituted saccharides such as esters or amides in which sugars play the role of alcohol and amine. They are classified in 16

carbohydrate esterase (CE) families containing acetyl xylan esterases, acetyl esterases, chitin deacetylases, peptidoglycan deacetylases, feruloyl esterases, pectin acetyl esterases, pectin methylesterases, glucuronoyl esterases and enzymes catalyzing N-deacetylation of low molecular mass amino sugar derivatives (Biely 2012). Acetyl xylan esterases cleave the ester linkage between acetyl groups and xylose residues. This group of enzymes can be found in various CE families: CE1, 2, 3, 4, 5, 6, 7, 12 and 16 (www.cazy.org).

*Feruloyl esterases* are a heterogeneous group of enzymes that release ferulic acid from plant cell wall material as reported for sugar beet, spinach and true grasses (*Poaceae*; Kühnel 2012). These enzymes can be divided into four groups based on their activity towards synthetic substrates and dihydrodiferulic acids (Kühnel et al. 2011). There is a large diversity among feruloyl esterases, but their detailed classification in CAZy (now classified in CE family 1) is awaiting more biochemical, physiological and structural data (Ryabova et al. 2009).

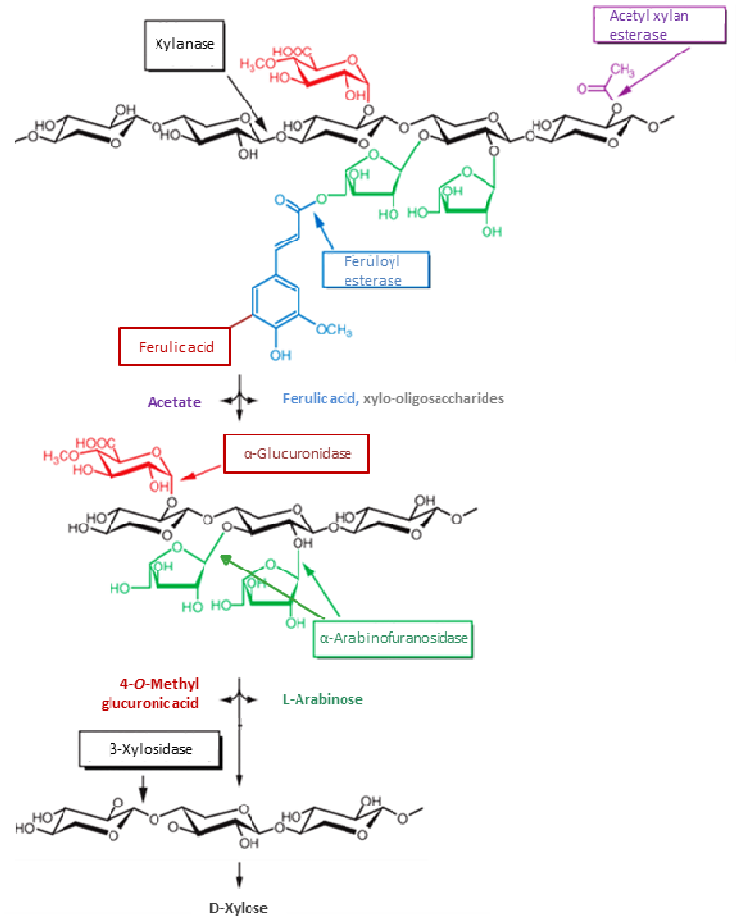


Figure 5: Simplified sketch of xylan degradation by fungal enzymes (modified from Dodd and Cann 2009).

## Rate limiting factors for biochemical feedstock conversion

### Feedstock recalcitrance

Bioconversion by (hemi)cellulolytic enzyme preparations is limited by the recalcitrance of feedstocks. In order to advance enzymatic conversion of lignocellulose it is of importance to understand the fundamental nature of biomass recalcitrance. Factors that contribute to recalcitrance of plant biomass include 1. the epidermal tissue of the plant body (cuticle and epicuticular waxes), 2. arrangement and density of the vascular bundles, 3. the amount of sclerenchymatous tissue, 4. the degree of lignification, 5. the complexity of cell wall regarding enzyme accessibility and 6. hindrance of the enzymatic hydrolysis by xylan substituents (Himmel et al. 2007).

Especially the hindrance of the enzymatic hydrolysis by the presence of substituents of the xylan backbone (Appeldoorn et al. 2010), crystallinity of the cellulose (Zhang and Lind 2004) and the presence of lignin as a factor affecting the enzymatic digestibility by physically impeding or non-specifically absorbing enzymes (Vanholme 2010) are important recalcitrance factors towards the enzymatic conversion. Another factor influencing the conversion of polysaccharides to monosaccharides is the particle size of the substrate. An increased surface availability affects conversion yields (Caulfield and Moore 1974) as the pore size of the substrate has been described as rate limiting (Thompson et al. 1992). Removal of the hemicellulose and lignin increases cellulose conversion by increasing the mean pore size of the substrate (Gregg and Saddler 1996, Grehtlein 1985, Palonen et al. 2004).

From the structural complexity of the xylan present in corn (Figure 4f) it can be anticipated that the complex pattern of xylan substituents is a major factor hindering the enzymatic hydrolysis. Regarding the conversion of lignocellulosic feedstocks for biofuels production, such as corn silage, it has been estimated that large cost-savings can be achieved by improving the enzymatic substrate conversion yield, reducing enzyme loading and by eliminating or reducing pretreatment (Lynd et al. 2008). All these aspects are linked to a necessity of gaining further insights in feedstock recalcitrance properties and the enzymatic hydrolysis process that decreases such recalcitrance.

### Conversion limiting enzyme properties

Regarding enzyme conversion limiting properties, irreversible or non-specific adsorption of the (hemi)cellulolytic enzymes (Zhang and Lynd 2004), inactivation of enzymes during the hydrolysis process (Eklund et al. 1990), product inhibition (Mansfield et al. 1999) and hindrance of the hydrolytic activity by xylan substituents play a crucial role. These rate limiting factors can by addressed by improvement of hemicellulolytic enzyme mixtures for polysaccharide conversion. Strategies to improve the enzymatic hydrolysis process are bioprospecting for efficient key enzymes, enzyme engineering (directed evolution, rational design and

multifunctional chimeras to increase the efficiency of enzymes) and the mining of plant pathogens for novel hydrolytic enzymes (Mohanram et al. 2013). By using *state-of-the-art* enzyme preparations, the hydrolytic activity towards recalcitrant polysaccharides may be further increased by the supplementation of "lacking" or synergistic key activities.

**Thermochemical pretreatment**

Thermochemical pretreatment processes either solubilize plant cell wall polysaccharides or render the recalcitrant cell wall architecture more accessible for enzymatic hydrolysis. Pretreatment technologies for lignocellulosic material have been reviewed previously (Kumar et al. 2009). Pretreatments include mechanical comminution, pyrolysis, steam explosion, ammonia fiber explosion, carbon dioxide explosion, ozonolysis, acid hydrolysis, alkaline hydrolysis, oxidative delignification, organosolv process treatment and biological pretreatment (lignin removal by rot fungi).

*Enzymatic conversion of non- (or mild) pretreated feedstocks:* As described above, hydrolytic enzymes convert polysaccharides to monosaccharides. Enzymatic conversion of polysaccharides can be done after or without pretreatment. Both, C5 and C6 monosaccharides released by the enzymatic hydrolysis can be further converted to biofuels by aerobic (bioethanol) or anaerobic (biogas) fermentation. In the case of processes without pretreatment, as for example the anaerobic conversion of corn silage to methane, the importance of an efficient enzymatic feedstock conversion process is pronounced. Regarding the use of corn silage for ethanol production, pretreatment is economically demanding (Kalač 2012) and therefore unfavorable. Feedstock conversion without the use of (harsh) pretreatment may be achieved by the development of an efficient enzymatic hydrolysis process of the plant cell wall polysaccharides.

**(Hemi)cellulolytic enzyme preparations**

(Hemi)cellulases are applied in various industrial segments, such as agriculture, food, pulp and paper, detergents, fermentation, or the textile industry (Kuhad et al. 2011, Henkelmann et al. 2012). (Hemi)cellulolytic enzyme preparations are commercially available and examples of such preparations are listed in Table 4.

Whereas a wide range of microorganisms produce (hemi)cellulases (Wilson 2011), only a limited number secrete these at levels appropriate for industrial use. Common microbial genera for industrial production are for example *Aspergillus, Bacillus, Cellulomonas, Myceliopthora, Clostridium, Humicola, Penicillium, Streptomyces, Talaromyces, Thermomonospora* or *Trichoderma*. *A. niger* is an example of a well characterized, commonly used microbial enzyme producer, secreting a range of (hemi)cellulases (Pel et al. 2007). Also other microbial hosts for industrial enzyme production have been extensively described in the literature (Kuhad et al. 2011, Waters et al. 2011, Petersson and Nevalainen 2012) and commercial enzyme preparations derived from these hosts are listed in Table 4 as well. As such industrial, microbial

production hosts are in use since a long time, they have undergone remarkable strain improvements towards high protein secretion and high (hemi)cellulolytic activity (Petersson and Nevalainen 2012). However, due to the large variety of enzyme producing microorganisms, many more hemicellulolytic fungi and bacteria provide interesting sources for the production of industrial complex xylan degrading enzymes (Kuhad et al. 2011). Examples of such (hemi)cellulolytic microbes are shown in Table 5. The characterization of enzymes secreted by these microorganisms and the further discovery of (hemi)cellulolytic fungi and bacteria is a current topic in biotechnological research.

Table 4: Examples of commercial (hemi)cellulolytic enzyme preparations from different production strains and their suppliers.

| **Strain** | **Enzyme preparation (supplier)** |
|---|---|
| *Aspergillus niger* | Amigase® Mega L (DSM), Amigase® TS (DSM), Cytolase® PL5 (DSM), Rapidase® Press (DSM), Rapidase® Smart Clear (DSM), Novozyme 188 (Novozymes), Pectinex®BE Colour (Novozymes), |
| *Aspergillus aculeatus* | Viscozyme® L (Novozymes) |
| *Aspergillus niger, Aspergillus aculeatus* | Pectinex® XXL (Novozymes) |
| *Aspergillus niger, Aspergillus oryzae* | Amigase® Plus (DSM) |
| *Bacillus* | Termamyl® SC DS (Novozymes), Validase® BG (DSM Valley Research) |
| *Bacillus licheniformis* | Termamyl® 120L (Novozymes), Spezyme® AA (Genencore), Mats® L (DSM) |
| *Bacillus amyloliquefaciens* | Filtrase® L (DSM) |
| *Bacillus amyloliquefaciens / Trichoderma longibrachiatum* | Filtrase® Deluxe (DSM) |
| *Humicola insolens* | Ultraflo® L (Novozymes) |
| *Myceliopthora thermophile C1* | CMAX® (Dyadic) |
| | SDS ALTERNAFUEL CMAX (Dyadic) |
| *Talaromyces emersonii / Trichoderma longibrachiatum* | Filtrase® NLC (DSM) |
| *Talaromyces emersonii / Disporotrichum dimorphosporum* | Filtrase® BR-X (DSM) |
| *Trichoderma reesei (teleomorph Hypocrea jecorina)* | Filtrase® Premium (DSM), MethaPlus® L100 (DSM), Laminex® BG2 (Genencor), Ultimase® BWL (Iogen), Celluclast® BG (Novozymes), Cellic® Ctec (Novozymes), |
| *Trichoderma reesei / Penicillium funiculosum* | Laminex® Super (Genencor) |
| *Trichoderma longibrachiatum* | Dyadic® Brewzyme LP (Dyadic), Dyadic® Cellulase PLUS (Dyadic), Dyadic® Xylanase PLUS (Dyadic), Laminex® BG (Genencor) |

Table 5: Examples of (hemi)cellulolytic microorganisms with application potential in industrial enzymes production (modified from Kuhad et al. 2011).

| | | | |
|---|---|---|---|
| **1. Fungi** | *Agaricus arvensis;*<br>*Aspergillus niger; A. nidulans; A. oryzae; A. terreus;*<br>*Chaetomium cellulyticum; C. thermophilum;*<br>*Coniophora puteana;*<br>*Fomitopsis sp.;*<br>*Fusarium solani; F. oxysporum;*<br>*Humicola insolens; H. grisea;*<br>*Lanzites trabeum;*<br>*Melanocarpus albomyces;*<br>*Mucor circinelloides;*<br>*Myceliopthora thermophile C1;*<br>*Neurospora crassa;*<br>*Penicillium brasilianum; P. occitanis; P. decumbans;*<br>*Phanerochaete chrysosporium;*<br>*Phlebia gigantea*<br>*Pleurotus ostreatus;*<br>*Poria placenta;*<br>*P. fumigosum;*<br>*P. janthinellum; Paecilomyces inflatus; P. echinulatum;*<br>*Sporotrichum thermophile;*<br>*Talaromyces emersonii;*<br>*Trichoderma reesei; T. longibrachiatum; T. harzianum;*<br>*Thermoascus aurantiacus;*<br>*Trichoderma atroviride;*<br>*Tyromyces palustris;*<br>*Trametes versicolor;* | **2. Bacteria** | *Acetivibrio cellulolyticus;*<br>*Acinetobacter junii; A. amitratus;*<br>*Acidothermus cellulolyticus;*<br>*Anoxybacillus sp.;*<br>*Bacillus subtilis; B. pumilus; B. amyloliquefaciens;*<br>*B. licheniformis; B. circulan; B. flexus;*<br>*Bacteriodes sp.;*<br>*Butyrivibrio fibrisolvens;*<br>*Cellulomonas biazotea;*<br>*Cellulomonas fimi; C. bioazotea; C. uda;*<br>*Cellvibrio gilvus;*<br>*Clostridium thermocellum; C. cellulolyticum; C. acetobutylium; C. papyrosolvens;*<br>*Eubacterium cellulosolvens;*<br>*Fibrobacter succinogenes;*<br>*Geobacillus sp.;*<br>*Microbispora bispora;*<br>*Paenibacillus curdlanolyticus;*<br>*Pseudomonas cellulosa;*<br>*Rhodothermus marinus;*<br>*Ruminococcus albus;*<br>*Salinivibrio sp.;*<br>*Streptomyces drozdowiczii; S. lividans;*<br>*Thermomonospora fusca; T. curvata* |

## The enzymatic toolbox of a common microbial host for industrial enzyme production - *Aspergillus niger*

Sequencing of the genome of *A. niger* has revealed the presence of a broad number of sequences of putative (hemi)cellulolytic enzymes (Pel et al. 2007). The numbers for genome sequences of putative carbohydrate active enzymes from different enzyme classes, as present in the CAZy database so far, are shown in Table 6. The high number of sequences annotated as belonging to different enzyme classes reflects the (hemi)cellulolytic potential by fungal enzyme producers, such as *A. niger*. The composition of the secretome of *A. niger* strongly depends on the carbon source used for growth. Different levels of individual enzymes are secreted depending on the substrate used for growth (Ferreira de Oliveira et al. 2011, Lu et al. 2010). For example, the secretion levels for hemicellulases were much lower with maltose and sorbitol as carbon sources compared to the use of xylose (Ferreira de Oliveira et al. 2011). Therefore, not only culture supernatants from different species, but also the culture supernatants obtained by cultivation of the same species under different growth conditions may result in secretomes that differ significantly in the enzyme activities present. There lies a significant, yet not fully used, hydrolytic potential within industrially used microbial enzyme producers. Certain enzymes may only be expressed under specific growth conditions and the different enzymes secreted by different hosts may be combined to more efficient hydrolytic mixtures.

Table 6: Genome sequences of putative carbohydrate active enzymes from *A. niger* as obtained from the CAZy database (status: May, 2014; www.cazy.org).

| Glycoside hydrolases | | Glycosyl Transferases | | Polysaccharide lyases | | Carbohydrate esterases | | Auxiliary activities | | Carbohydrate-binding modules | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| families | sequences | families | sequences | families | sequences | families | sequences | families | sequences | families | sequences |
| 55 | 245 | 30 | 117 | 2 | 8 | 7 | 20 | 5 | 34 | 13 | 44 |

## Aim and outline of the thesis

The aim of this thesis was the identification of rate limiting (recalcitrance) factors for the degradation of corn silage (xylan) and to increase the understanding of the enzymatic conversion process by hemicellulolytic enzyme preparations. As outlined in this chapter, enzyme preparations need to be further improved in order to obtain an efficient conversion of xylan.

**Chapter 2** describes the compositional characterization of xylan fractions obtained from corn silage water unextractable solids (WUS) by barium hydroxide extraction and fractionation by graded ethanol precipitation. The enzymatic conversion of the purified corn silage xylan fractions and the xylan conversion of corn silage water unextractable solids (WUS), chlorite treated WUS and a recalcitrant, industrial anaerobic fermentation residue is presented.

In **Chapter 3** a fast and robust method for the quantification of acetyl, feruloyl and *p*-coumaroyl ester contents of plant polysaccharides is described. In **chapter 4**, corn silage based feedstocks of different processing stages are studied towards their chemical composition. Different corn silage fractions were used as a model system in order to evaluate the hydrolytic potential of crude enzyme preparations. **Chapter 5** reports on the hydrolytic activities obtained by supplementation of sub-fractions of a crude *T. longibrachiatum* preparation to a (hemi)cellulolytic enzyme preparation. The deacetylation of corn and model substrates by an acetyl xylan esterase classified as belonging to CE family 5 from *T. longibrachiatum* was further investigated. In **chapter 6**, the positional preferences of acetyl esterases from different carbohydrate esterase families towards 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA are presented. The deacetylation efficiency and specificity for different acetylated xylo-oligosaccharides was studied.

The final chapter (**chapter 7**) discusses the major results obtained and their significance for future research in enzymatic feedstock conversion. In addition, the hydrolysis of corn silage polysaccharides by supplementation of the cloned acetyl xylan esterase classified as belonging to CE family 5 from *T. reesei* (a homologue of the acetyl xlyan esterase belonging to CE family 5 from *T. longibrachiatum*) to a hemicellulolytic enzyme preparation is shown.

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*CHAPTER 2*

# Characterization and enzymatic degradation of corn silage xylan populations


## Abstract

Xylan obtained from corn silage water unextractable solids (WUS) by barium hydroxide extraction was fractionated by graded ethanol precipitation. A population of xylan with a degree of substitution (DS) of 0.4 mol substituent (arabinose, galactose and uronic acid) per mol xylose, comprised 72 % of the xylan recovered by extraction and precipitation. In addition, low proportions of xylans with a DS ≤ 0.3 and ≥ 0.9 were present. Hydrolysis with an industrial *Aspergillus niger / Talaromyces emersonii* enzyme preparation revealed complete hydrolysis of the low substituted xylan (DS = 0.2). Only 11 % of the xylan present in the high substituted fraction (DS = 1.1) was hydrolyzed. Overall, 79 % of the xylan recovered by barium hydroxide extraction and graded ethanol precipitation was converted to Xyl. Recalcitrant uronic acid and galactose substituted (arabino-)xylo-oligosaccharides were present in the enzyme treated xylan fractions confirming that glycosidic substituents hinder complete hydrolysis of the xylopyranosyl units of the backbone. Xylan needs to be linearized by accessory enzymes for an efficient degradation. The conversion of the extracted xylan fractions was higher compared to the conversion levels obtained for the xylan present in the original WUS, delignified WUS and AFR, a recalcitrant, industrial residue from anaerobic fermentation, being 29 %, 69 % and 7 %, respectively. Presence of acetyl and phenoyl esters and physical inaccessibility of the polysaccharides present in WUS and AFR by the enzymes results in low conversion compared to extracted, de-esterified, xylans.



Neumüller K.G., Streekstra H., Schols H.A., Gruppen H., 2014. Characterization and enzymatic degradation of corn silage xylan populations.

## Introduction

Corn silage consists of the anaerobically processed aboveground part of corn (Bittman and Kowalenko 2004, Allen et al. 1995). Xylan from corn silage is a main source of fermentable C5 sugars for biofuels production (Weiland 2010). The backbone of corn xylan is formed by (1 → 4)-linked-β-D-xylopyranosyl units. α-(1→ 2)-Linked 4-(*O*-methyl-D)-glucopyranosyl uronic acid units and α-(1→ 3)-linked L-arabinofuranose residues, as well as 2-*O*-β-D-xylopyranosyl-α-L-arabinofuranose moieties constitute the substituents of the backbone (Ebringerová et al. 1992). Furthermore, complex side chains, such as α-L-galactopyranosyl-(1→2)-β-D-xylopyranosyl-(1→2)-5-*O*-trans-feruloyl-L-arabinofuranose attached to the *O*-3 position of Xyl*p*, were reported to be present in xylan from corn fiber (Appeldoorn et al. 2013). Acetyl and phenoyl esters are also present in xylan from corn silage (Neumüller et al. 2013).
In order to hydrolyze complex xylan, a mixture of hemicellulases is required. Accessory enzymes, such as arabinofuranosidases, α-glucuronidases, α-1,4-galactosidases, β-1,4-galactosidases, feruloyl/p-coumaroyl esterases and acetyl xylan esterases, linearize the xylan backbone in order to make it accessible for β-1,4-endo-xylanases and β-1,4-xylosidases. Enzymatic conversion of the xylan present in corn silage by a crude *A. niger / T. emersonii* enzyme mixture was comparable to other commercial enzyme preparations (Neumüller et al. 2014a). However, complete hydrolysis of the xylan present in corn silage has not yet been achieved with industrial enzyme preparations. The degree and type of backbone substitution (DS) and the distribution of substituents over the xylan backbone have a major influence on the degradability of xylans (Appeldoorn et al. 2010, Neumüller et al. 2014a). Investigation of the hydrolysis of different xylan populations present in corn silage by hemicellulolytic enzyme preparations would provide insights whether enzyme preparations contain the necessary accessory enzyme activities in order to degrade different populations of xylan present in corn silage. In order to determine the influence of the glycosidic substituents on the enzymatic hydrolysis, extracted and de-esterified xylans, differing in the DS, provide suitable model substrates. A comparison of the conversion of extracted (de-esterified) xylan populations to the xylans present in complex feedstocks, such as corn silage polysaccharides, can show to what extent esterification and physical inaccessibility (Zhao et al. 2012) of the xylans impede the conversion by hemicellulolytic enzyme preparations.
In this work, selectively extracted (Gruppen et al. 1991) and fractionated water unextractable xylan populations from corn silage WUS were characterized regarding their composition. The enzymatic conversion of the xylan fractions obtained by an industrial *A. niger / T. emersonii* enzyme preparation was investigated and the substrate conversion was correlated to the DS. The enzymatic conversion of the extracted xylan populations was compared to the conversion of the xylans as present in more complex feedstocks of the same source: Corn silage WUS (starting material), delignified WUS and an industrial anaerobic fermentation residue (AFR). Recalcitrant oligosaccharides of enzyme treated corn silage xylan fractions were identified by

mass spectrometry in order to obtain information on missing enzyme activities of the hemicellulolytic enzyme preparations.

## Materials and methods

### Materials

Isolation of corn silage water unextractable solids (WUS) and the industrial anaerobic fermentation residue (AFR) has been described previously (Neumüller et al. 2014a). Their composition is shown in Table 1. Chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). The *A. niger* and *T. emersonii* culture filtrates [Brewers Filtrase BXC (*T. emersonii*, DSM Heerlen, The Netherlands) and DSM-Arabanase (*A. niger*, DSM)] used have been described previously (Neumüller et al. 2014a).

### Extraction and fractionation of corn silage xylan

*Saturated barium hydroxide extraction:* The extraction of xylan from WUS with a saturated $Ba(OH)_2$ solution containing $NaBH_4$ was performed as described previously (Gruppen et al. 1991). The extracts were dialyzed extensively at 4 °C against sodium acetate buffer (200 mM, pH 5.0), then against running tap and finally against distilled water. After reduction of the volume by vacuum evaporation, the extract was fractionated by graded ethanol precipitation as described below.

*Fractionation by graded ethanol precipitation:* Graded ethanol fractionation of the xylan containing solution obtained after barium hydroxide extraction was obtained by an incremental increase of 20 % (v/v) in ethanol concentration ranging from 20 to 80 % (v/v). The mixture was stored at 4 °C for 16 h after each increase in ethanol concentration; each precipitate was collected by centrifugation (15000 g, 30 min, 4 °C). The pellet was dissolved in Milli-Q (MQ) water yielding fractions AX 20, AX 40, AX 60 and AX 80. The final supernatant was concentrated by vacuum evaporation (AX 80S). The five different fractions were freeze dried and stored at 4 °C.

### Chlorite treatment (delignification) of WUS

WUS was suspended in MQ water (20 g $L^{-1}$). The pH of the suspension was adjusted to 4.75 with glacial acetic acid. The suspension was heated to 70 °C. Sodium chlorite was added (4 g $L^{-1}$) followed by addition of glacial acetic acid (1 mL $L^{-1}$). The suspension was vortexed and incubated for 1 h. Again, sodium chlorite (4 g $L^{-1}$) and acetic acid (1 mL $L^{-1}$) were added and the suspension was vortexed and further incubated for 1 h. The delignified material was filtered (glass microfiber filters, 1.6 µm, GE Healthcare, Little Chalfont, UK) and washed with 800 mL MQ water. The filtered material was freeze dried.

**Compositional analysis**

*Constituent monosaccharide composition:* Extracted and fractionated xylan samples and chlorite treated WUS were pretreated in 72% (w/v) sulfuric acid for 1 h at 30 °C. Hydrolysis was done at 1 M sulfuric acid for 3 h at 100 °C. Sugars released were quantified as their alditol acetates by gas chromatography (Englyst and Cummings 1984). Inositol was used as internal standard. The UA content was determined as anhydro-uronic acid by a colorimetric m-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen 1973). A calibration curve was prepared with glucuronic acid.

*Acid insoluble material (acid insoluble lignin and ash):* Sulfuric acid hydrolysis of the xylan samples and chlorite treated WUS (100 mg) was performed according to the procedure described previously (Neumüller et al. 2014a). The suspension was filtered (Glass Fibre Filters, Whatman, Maidstone, UK). The residue was washed with MQ and dried at 100 °C for 2 h followed by cooling to RT in a desiccator overnight. The acid insoluble material was measured by weighing.

**Enzymatic hydrolysis**

The xylan fractions (AX 20 to AX 80S), WUS, delignified WUS and AFR were incubated with the *A. niger / T. emersonii* culture filtrates mixture (100 g protein $kg^{-1}$ substrate; 1:1 ratio based on protein content) at 2 mL scale at 50 °C, 700 rpm for 48 h in sodium acetate buffer (100 mM, pH 5.0). After incubation, the enzymes were heat inactivated (98 °C for 10 min), centrifuged (20000 g, 10 min, RT) and analyzed for the monosaccharides released by high performance anion-exchange chromatography (HPAEC) as previously described (Neumüller et al. 2014a).

**Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Maldi-Tof MS) of oligosaccharides**

Dowex AG 50W-X8 (Bio-Rad) was added to the enzyme treated xylan fractions to remove interfering salts. 2 µL 2,5-dihydroxybenzoic acid (10 g $L^{-1}$) and 1 µL of the enzyme treated xylan samples were transferred on a steel target plate (Bruker Daltonics, Bremen, Germany) followed by drying under a stream of hot air. Maldi-Tof MS was performed on an Ultraflextreme workstation (Bruker Daltonics) equipped with a 337 nm laser and controlled using FlexControl software. Calibration was done with a mixture of maltodextrins. The data analysis was done with the open source mass spectrometry tool mMass (Strohalm et al. 2008), retrieved from www.mmass.org.

## Results and discussion

### Characterization of corn silage xylan

*Extraction and fractionation of xylan populations from corn silage WUS.*

A selective extraction of xylans from corn silage WUS was performed using a saturated barium hydroxide solution. In order to fractionate the extracted xylans, graded ethanol precipitation of the extract was performed. 13 % (w/w) of WUS was recovered by extraction and graded ethanol precipitation, containing 23 % (w/w) of the total xylan present in WUS. This is in agreement with previously indicated xylan recoveries from corn endosperm after barium hydroxide extraction (13.8 % w/w, Van Laar et al. 2002). The relative amounts (% w/w) of the fractions obtained by graded ethanol precipitation of the xylans extracted from WUS are shown in Table 1. Fraction AX 60 constituted 55 % (w/w) of the extract, whereas fraction AX 20 represented 19 % (w/w) of the extracted material. Fractions AX 40 and AX 80 represented 5 % and 8 % (w/w), respectively. The remaining material (13 % w/w) of the barium extract could not be precipitated with the ethanol concentration of 80 % and was present in the supernatant (fraction AX 80S). Most of the proteins were either recovered in fraction AX 20, or remained in the supernatant (fraction AX 80S).

Table 1: Composition of xylan fractions (AX 20, 40, 60, 80 and 80S) of corn silage water unextractable solids (WUS), WUS, sodium chlorite treated WUS (chlorite WUS) and an anaerobic fermentation residue (AFR).

| | | **composition (g kg$^{-1}$ dry matter)** | | | | **molar composition of sugars[a]** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fraction | Yield | Sugars | Protein | Acid insoluble material | AX[b] | Xyl | Ara | UA | Gal | Rha | Man | Glc |
| WUS[c] | 40[d] | 61 | 4 | 20 | 32 | 73.0 | 12.2 | 6.8 | 2.5 | 0.5 | 2.5 | 2.5 |
| AX20 | 19[e] | 38 | 13 | 20 | 34 | 83.2 | 11.9 | 4.5 | 0.0 | 0.3 | 0.2 | 0.0 |
| AX40 | 5[e] | 55 | 7 | 5 | 57 | 78.9 | 14.0 | 3.9 | 1.4 | 0.2 | 0.1 | 1.4 |
| AX 60 | 55[e] | 69 | 2 | 3 | 74 | 68.2 | 20.1 | 5.0 | 3.3 | 0.1 | 0.0 | 3.3 |
| AX 80 | 8[e] | 64 | 5 | 4 | 69 | 51.1 | 32.7 | 6.4 | 4.6 | 0.3 | 0.3 | 4.6 |
| AX 80S | 13[e] | 7 | 19 | 43 | 7 | 39.8 | 26.5 | 9.9 | 10.8 | 0.0 | 2.2 | 10.8 |
| *AX total*[f] | 100 | 54 | 7 | 12 | 57 | 67.9 | 20.9 | 5.2 | 2.8 | 0.2 | 0.1 | 2.8 |
| chlorite WUS | 77[g] | 73 | n.d. | 2 | 34 | 73.8 | 14.8 | 6.6 | 2.4 | 0.0 | 0.0 | 2.4 |
| AFR[d] | n.d. | 23 | 31 | 48 | 13 | 58.7 | 14.7 | 11.0 | 6.0 | 1.3 | 2.4 | 6.0 |

[a]Expressed as percentage (mole per 100 mole).
[b] Xylan (AX) calculated as sum of Ara, Xyl, Gal and UA present.
[c] Neumüller et al. 2014a.
[d]Yield from corn silage.
[e] Yield from total xylan obtained by saturated barium hydroxide extraction and graded ethanol precipitation.
[f]Calculated based on the composition obtained for the fractions AX 20, 40, 60, 80 and 80S.
[g] Yield from WUS.
AX 20 to AX 80 S, xylan fractions obtained by graded ethanol precipitation of a barium hydroxide extract of WUS; n.d., not determined; UA, uronic acid.

*Compositional analysis of xylan fractions*

The xylan fractions obtained were analyzed for their carbohydrate composition. Ara and Xyl constituted the major part of the carbohydrates present (Table 1). Only a minor amount of Glc was extracted, mainly ending up in fraction AX 20. Figure 1 shows the proportion of carbohydrates recovered from the starting material (WUS) for each fraction obtained. 35 % of the Ara present in WUS was extracted by saturated barium hydroxide treatment and recovered by ethanol precipitation, while 21 % of all Xyl was recovered in the fractions. The extraction yields for UA and Gal were 18 % and 23 %, respectively. For all sugars, the main portion was present in fraction AX 60, representing in total 72 % of the extracted xylan recovered after the fractionation procedure. Acid insoluble material (acid insoluble lignin and ash) was observed mainly in fractions AX 20 and AX 80S (Table 1). Concomitantly, a lower relative xylan content was obtained for these fractions. The compositional data obtained show that fractions with a high xylan content were obtained by the barium hydroxide extraction and graded ethanol precipitation. The fractions vary significantly in their composition. Based on the results obtained the degree of substitution of the xylan fractions was calculated and is further discussed below.

*Compositional analysis of chlorite treated WUS*

In order to compare the enzymatic hydrolysis of the extracted corn silage xylan fractions to more complex feedstocks, corn silage WUS (starting material for the xylan extraction), delignified WUS, and a recalcitrant, corn silage based anaerobic fermentation residue (AFR), were included in this study. Table 1 illustrates the composition of WUS, AFR and the delignified WUS obtained by sodium chlorite treatment (chlorite WUS). The sodium chlorite treatment removes lignin and phenolic acids to a large extent (Bergmans et al. 1996). 77 % of the starting material was recovered following delignification. Only 2 % acid insoluble material was found to be present in chlorite WUS showing effective delignification of the starting material [20 % acid insoluble material containing acid insoluble lignin (17 %) and acid insoluble ash (3 %), Neumüller et al. 2014a]. In accordance with the low content of acid insoluble material, the sugar content was increased compared to WUS, confirming enrichment of polysaccharides in the delignified residue. A recovery of 92 % of the carbohydrates present in WUS was obtained for the residue after sodium chlorite treatment.

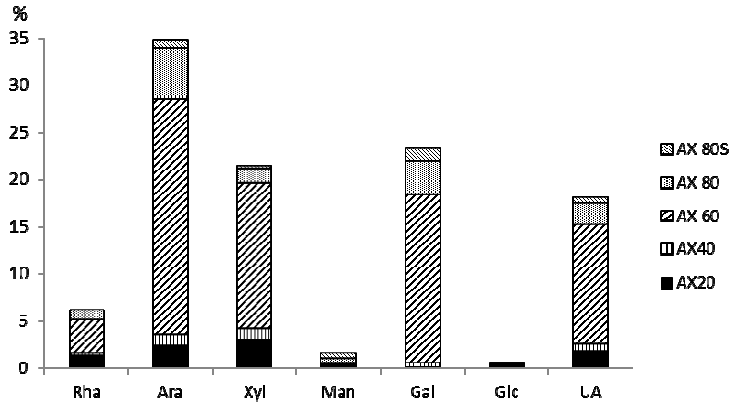


Figure 1: Yield (% w/w) of sugars obtained by barium hydroxide treatment of water unextractable solids from corn silage and fractionation by graded ethanol precipitation.

*Degree of substitution (DS)*

The DS of the glycosidic moieties which can occur in xylan side chains (Ara, Gal and UA; Agger et al. 2010, Ebringerová 2006, Appeldoorn et al. 2013) are displayed in Table 2 as mol substituent per mol Xyl present. Interestingly, the DS for Ara, UA and Gal of fraction AX 60 closely matched the DS of corn silage as determined previously ($DS_{Ara}$ = 0.23, $DS_{UA}$ = 0.11, $DS_{Gal}$ = 0.05, Neumüller et al. 2014a). Furthermore, small populations comprising 17 % (AX 20 plus AX 40) and 11 % (AX 80 plus AX 80S) of the xylan obtained by barium hydroxide extraction and graded ethanol precipitation, were present with a DS ≤ 0.26 and ≥ 0.90, respectively (Table 2). Ara was the main xylan backbone substituent for each fraction obtained. The DS of chlorite WUS regarding Ara, Gal and UA was similar to the DS of WUS.

The variability in the DS of the extracted and fractionated xylans illustrates different populations of xylan. These fractions provide a suitable model system to study the effect of glycosidic substitution towards the hydrolysis by hemicellulolytic enzyme preparations.

**Enzymatic hydrolysis of xylan**

*Enzymatic conversion of extracted and fractionated xylan*

In order to evaluate the enzymatic degradability of the extracted xylan fractions having varying DS of glycosidic substituents (Table 2), a hemicellulolytic *A. niger / T. emersonii* enzyme mixture was used for the hydrolysis. Table 3 shows the Ara and Xyl levels released as monomers after 48 h of incubation with the enzyme mixture as proportion of the Ara and Xyl present in the parental materials. The main population of the extracted xylan present (fraction AX 60) showed a xylan conversion of 76 %. It can be concluded that the enzyme mixture is efficiently, but not completely able to hydrolyze the major part of the extracted corn silage xylan. Complete xylan hydrolysis was obtained for fraction AX 20 ($DS_{total}$ = 0.22). A decrease in xylan conversion with increasing DS is evident (Tables 2 and 3). Fraction AX 80S ($DS_{total}$ = 1.08) showed high

recalcitrance towards enzymatic hydrolysis, resulting in xylan conversion of only 11 %. The presence of many substituents might result in a low accessibility and degradation regarding the β-1,4-endo-xylanases and β-1,4-xylosidases present in the *A. niger / T. emersonii* enzyme preparation. A high amount (43 %) of acid insoluble material was present in fraction AX 80S. Non-specific binding of enzymes to the acid insoluble material might also contribute to a non-efficient conversion. Adsorption of enzymes was for example shown for lignin (Rahikainen et al. 2011). However, presence of significant amounts of acid insoluble material (20 %) in fraction AX 20 did not impede complete xylan conversion.

In the case of extracted xylan, it is indicated that high substrate conversion was obtained when compared to WUS (29 % Xyl and 35 % Ara release; Table 3), due to the xylan purification from other cell wall material (cellulose, lignin, proteins) which results in optimal accessibility by hydrolytic enzymes. Furthermore, esters are removed by the alkaline extraction procedure, as described above.

Table 2: Degree of substitution (DS) of WUS, sodium chlorite treated WUS (chlorite WUS), the anaerobic fermentation residue (AFR) and xylan fractions obtained by graded ethanol precipitation of barium hydroxide extracted xylan from WUS (AX).

| Fraction | $DS_{Ara}$ [a] | $DS_{UA}$ [a] | $DS_{Gal}$ [a] | $DS_{total}$ [b] |
|---|---|---|---|---|
| WUS[c] | 0.18 | 0.08 | 0.04 | 0.30 (0.73[d]) |
| AX20 | 0.15 | 0.05 | 0.02 | 0.22 |
| AX40 | 0.18 | 0.06 | 0.02 | 0.26 |
| AX 60 | 0.29 | 0.09 | 0.05 | 0.43 |
| AX 80 | 0.63 | 0.15 | 0.12 | 0.90 |
| AX 80S | 0.72 | 0.19 | 0.17 | 1.08 |
| *AX total*[e] | 0.17 | 0.05 | 0.03 | 0.25 |
| chlorite WUS | 0.20 | 0.09 | 0.03 | 0.32 |
| AFR[c] | 0.21 | 0.14 | 0.07 | 0.42 (1.67[d]) |

[a] DS (mol Ara, UA and Gal/ mol Xyl). UA, uronic acid.
[b] Calculated as sum of $DS_{Ara}$, $DS_{UA}$ and $DS_{Gal}$.
[c] Neumüller et al. 2014a.
[d] DS including acetyl and phenoyl esters.
[e] AX total: calculated based on the composition obtained for the fractions AX 20, 40, 60, 80 and 80S.

Table 3: Levels of Ara and Xyl released from xylan fractions, water unextractable solids (WUS) from corn silage, sodium chlorite treated WUS (chlorite WUS) and the anaerobic fermentation residue (AFR) by enzymatic hydrolysis with the *A. niger / T. emersonii* enzyme preparation for 48 h.

| Fraction | **Ara** | **Xyl** |
|---|---|---|
| *AX total*[a] | 65 | 79 |
| AX20 | 93 | 100 |
| AX40 | 79 | 90 |
| AX 60 | 62 | 76 |
| AX 80 | 67 | 67 |
| AX 80S | 50 | 11 |
| chlorite WUS | 53 | 69 |
| WUS[b] | 35 | 29 |
| AFR | 9 | 7 |

[a] Calculated based on the Ara and Xyl conversion of fractions AX 20, 40, 60, 80 and 80S.
[b] WUS conversion by incubation with *A. niger / T. emersonii* preparation in a stirred bioreactor at 500 mL scale (10 g $L^{-1}$, Neumuller et al. 2014a).
Levels of Ara and Xyl are shown as proportion (%) of the Ara and Xyl present in the parental materials.

*Enzymatic conversion of xylan present in WUS, chlorite treated WUS and AFR*

The levels obtained for the degradation of WUS to monomers were 29 % (Xyl) and 35 % (Ara) after incubation of 48 h with the *A. niger / T. emersonii* enzyme preparation. The lower conversion of the xylan present in WUS compared to the extracted xylan (overall 79 % of the Xyl was released, Table 3) is most likely due to esterification and non-sufficient accessibility by hydrolytic enzymes (Zhao et al. 2012). Xylan degradation of sodium chlorite treated WUS (69 % Xyl and 53 % Ara) was higher compared to WUS after 48 h. Prolonged incubation of WUS resulted in higher Xyl conversion (60 %; Neumüller et al. 2014a), showing that the conversion rate of WUS is lower compared to chlorite WUS most likely due to low accessibility of enzymes to the xlyan present in the WUS. Chlorite treatment removes lignin and phenolic acids, which results in lower recalcitrance properties towards enzymatic degradation (Lacayo et al. 2013). Acetyl substituents at the *O*-2 or *O*-3 position of the xylopyranosyl units remain present after sodium chlorite treatment of corn xylan (Naran et al. 2009). The lower conversion of the xylan present in chlorite WUS (69 % Xyl and 53 % Ara) compared to barium hydroxide extracted xylans (79 % Xyl and 65 % Ara) can be explained by the presence of acetyl residues after sodium chlorite treatment. Degradability of corn silage fractions was increased by efficient deacetylation with a CE5 classified acetyl xylan esterase supplemented to the *A. niger / T. emersonii* enzyme preparation as observed previously (Neumüller et al. 2014b). Hydrolysis of AFR resulted in yields of 9 % (Ara) and 7 % (Xyl). The high DS of AFR ($DS_{total}$ = 1.67, Table 2) hinders hydrolysis by the *A. niger / T. emersonii* enzyme preparation, but especially the high acetylation ($DS_{Ac}$ = 1.17; Neumüller et al. 2014a) might significantly contribute to recalcitrance of this substrate. Substrates with a DS > 1 (fraction AX 80S and AFR) resist conversion into

monomers by the hemicellulolytic enzyme preparation. Different patterns of xylan backbone substitution were present for AFR and fraction AX 80S (mainly acetylation regarding AFR and arabinofuranosyl substituents regarding fraction AX 80S). The conversion of xylan by hemicellulolytic enzyme preparations might be more affected by the overall amount of substituents present than by the ratio between different xylan substituents.

*Correlation of xylan conversion with the degree of substitution (DS)*

As the hydrolysis yields obtained indicated a dependency of the substrate conversion to the DS, the correlation between the Xyl released and the amount of xylan backbone substituents present for each fraction, was determined. The results are shown in Figure 2. A negative correlation of $R^2$ = -0.87 was observed between the $DS_{total}$ and the Xyl released. This correlation was slightly higher ($R^2$ = -0.90) when exclusively extracted xylan populations were used for the calculation. Regarding extracted xylan fractions, recalcitrance is expected to be caused by backbone substitution only. The relative amount of individual substituents present differed amongst the extracted xylan fractions, WUS, chlorite WUS and AFR (Table 2). The data obtained indicate a lack of accessory enzymes, secreted by *A. niger* and *T. emersonii*, active towards glycosidic side chains or ester substituents that are necessary to linearize highly substituted xylan. Removal of xylan substituents is essential to render the xylan backbone vulnerable for enzymatic degradation. The type of substitution present in extracted, enzyme recalcitrant corn silage xylan fragments was further investigated by Maldi-Tof MS.

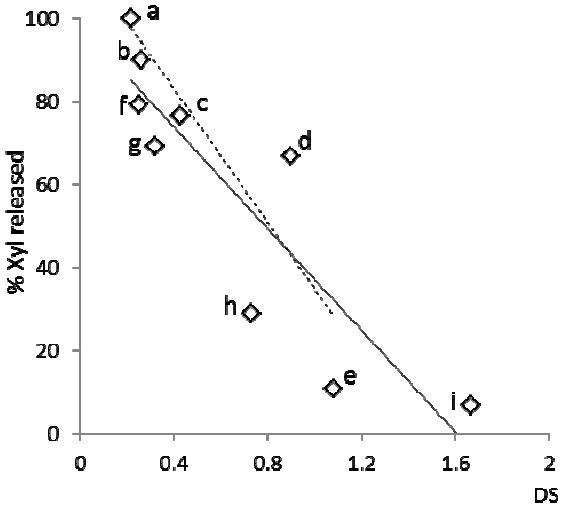


Figure 2: Correlation of the Xyl released (% of theoretical yield) of corn silage xylan fractions, WUS and AFR by incubation with the *A. niger / T. emersonii* enzyme preparation for 48 h with their total degree of substitution. (a) AX 20, (b) AX 40, (c) AX 60, (d) AX 80, (e) AX 80S, (f) AX total (theoretical value: calculated based on the results obtained for the fractions AX 20, 40, 60, 80 and 80S), (g) chlorite WUS, (h) WUS , (i) AFR. The solid line indicates the correlation between the Xyl released and the DS for all substrates (a to i). The punctuated line indicates the correlation between the Xyl released and the DS for the extracted xylan fractions only (a to f).

**Mass spectrometry of recalcitrant oligosaccharides**

In order to identify recalcitrant oligosaccharides present after enzymatic hydrolysis with respect to their building blocks [pentose (P), hexose (H), uronic acid (UA), *O*-methylated uronic acid (OmeUA) and acetyl groups (Ac)], mass spectrometry analysis of the enzyme treated xylan fractions (AX 20 to AX 80S) was performed. Table 4 illustrates the oligosaccharides corresponding to the masses of ions detected by Maldi-Tof MS.

Only few enzyme recalcitrant oligosaccharides ($P_2$, $P_1UA_1$ and $P_1UA_1H_1$) were present in fraction AX 20. $P_2$ was indicated to be present in abundance and was also observed in the corn stover fraction resistant to simultaneous saccharification and fermentation (Appeldoorn et al. 2010). Presence of few recalcitrant oligosaccharides in the enzyme treated fraction AX 20 is in agreement with the high conversion yield obtained for this fraction (Table 3). The oligosaccharides present in the enzyme treated fractions AX 40 to AX 80S are shown in Table 4. Ions corresponding to the masses of $P_2$, $P_4$, $P_5$ and $P_6$ are assumed to consist of a combination of Xyl and Ara as xylose oligomers would have been degraded by xylanases present. Other oligosaccharides detected ($P_xH_yUA_z$) most likely consist of Xyl carrying oligomeric side chains consisting of Ara, Gal and/or UA substituents (Table 4). Furthermore, presence of double substituted xylopyranosyl units with arabinofuranosyl moieties (Agger et al. 2010) is likely. The mass for ions correlating with meGlcA substituted $P_2$ was detected in fractions AX 40, AX 60 and AX 80. The presence of both GlcA and meGlcA in pretreated corn fiber oligosaccharides resistant to enzymatic saccharification has been reported previously (Appeldoorn et al. 2010). Two acetyl substituted oligosaccharides ($P_2Ac$ and $P_3Ac$) were detected (fractions AX 60, AX 80 and AX 80S), indicating that the alkaline xylan extraction did not completely deacetylate the xylan. However, the relative signal intensities for the acetylated oligosaccharides were relatively low.

The data obtained show that a range of recalcitrant oligosaccharides, consisting of pentoses, (methyl)GlcA, Ara or Gal residues, were present in fractions AX 40 to AX 80S. In order to obtain complete hydrolysis, additional accessory enzyme activities should be supplemented to the *A. niger / T. emersonii* secretome, targeting the resistant substituents (Table 4). Screening of different microbial sources for arabinofuranosidases, galactosidases and glucuronidases by use of recalcitrant, characterized substrates, such as the enzyme treated fractions AX 40 to AX 80S, will lead to the identification of additional, “lacking” accessory enzymes.

Table 4: Relative amounts (%) per oligosaccharide of the total oligosaccharides present for each enzyme treated xylan fraction (AX 20 to AX 80S) corresponding to the masses of ions detected by Maldi-Tof MS.

| oligosaccharides | AX 20 | AX 40 | AX 60 | AX 80 | AX 80S |
|---|---|---|---|---|---|
| $P_1H_1$ | | 7 | 1 | 3 | 5 |
| $P_1UA_1$ | 7 | 3 | | | 19 |
| $P_1UA_1H_1$ | 7 | | | | |
| $P_2$ | 86 | 56 | 64 | 82 | 67 |
| $P_2Ac_1$ | | | 1 | 3 | 3 |
| $P_2H_1$ | | 3 | | | |
| $P_2OmeUA_1$ | | 2 | 2 | 2 | |
| $P_3Ac_1$ | | | | | 3 |
| $P_3UA_1$ | | | | | 3 |
| $P_4$ | | 21 | 4 | 4 | |
| $P_4H_1$ | | 3 | 15 | 2 | |
| $P_4UA_1$ | | | 1 | | |
| $P_5$ | | 5 | 5 | | |
| $P_5H_1$ | | | 7 | 2 | |
| $P_6$ | | | | 2 | |

P, pentose; H, hexose; UA, uronic acid; OmeUA, *O*-methylated uronic acid; Ac, acetyl.

## Conclusions

Populations of xylan, obtained by saturated barium hydroxide extraction from WUS and graded ethanol precipitation, differed in their degree of glycosidic substitution ranging from low substituted (DS of 0.2) to heavily substituted (DS of 1.1) xylan (Table 2). Low substituted xylan is completely hydrolyzed by the hemicellulolytic enzyme preparation. The lower conversion obtained for esterified xylan embedded in plant cell wall structures (WUS, chlorite WUS and AFR) compared to extracted xylan (Table 3) indicate recalcitrance due to presence of acetyl and phenoyl esters and lower accessibility by the presence of lignin and cellulose. Xylan needs to be linearized by accessory enzymes for efficient conversion. It is indicated that efficient acetyl esterases need to be present in hemicellulolytic preparations in order to obtain high conversion of the esterified xylan as present in WUS. Enzyme recalcitrant structures of the extracted, de-esterified xylan were identified, being UA and Gal substituted (arabino-)xylo-oligosaccharides.

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## Supplementary information

## Characterization and enzymatic degradation of corn silage xylan populations.

Mass spectrometry (Maldi-Tof MS) was performed according to the procedure described in the original manuscript in order to characterize recalcitrant oligosaccharides towards the hydrolysis with a hemicellulolytic *A. niger / T. emersonii* enzyme preparation. Figure S1 shows the relative intensities of the masses of ions detected by Maldi-Tof MS corresponding to oligosaccharides.

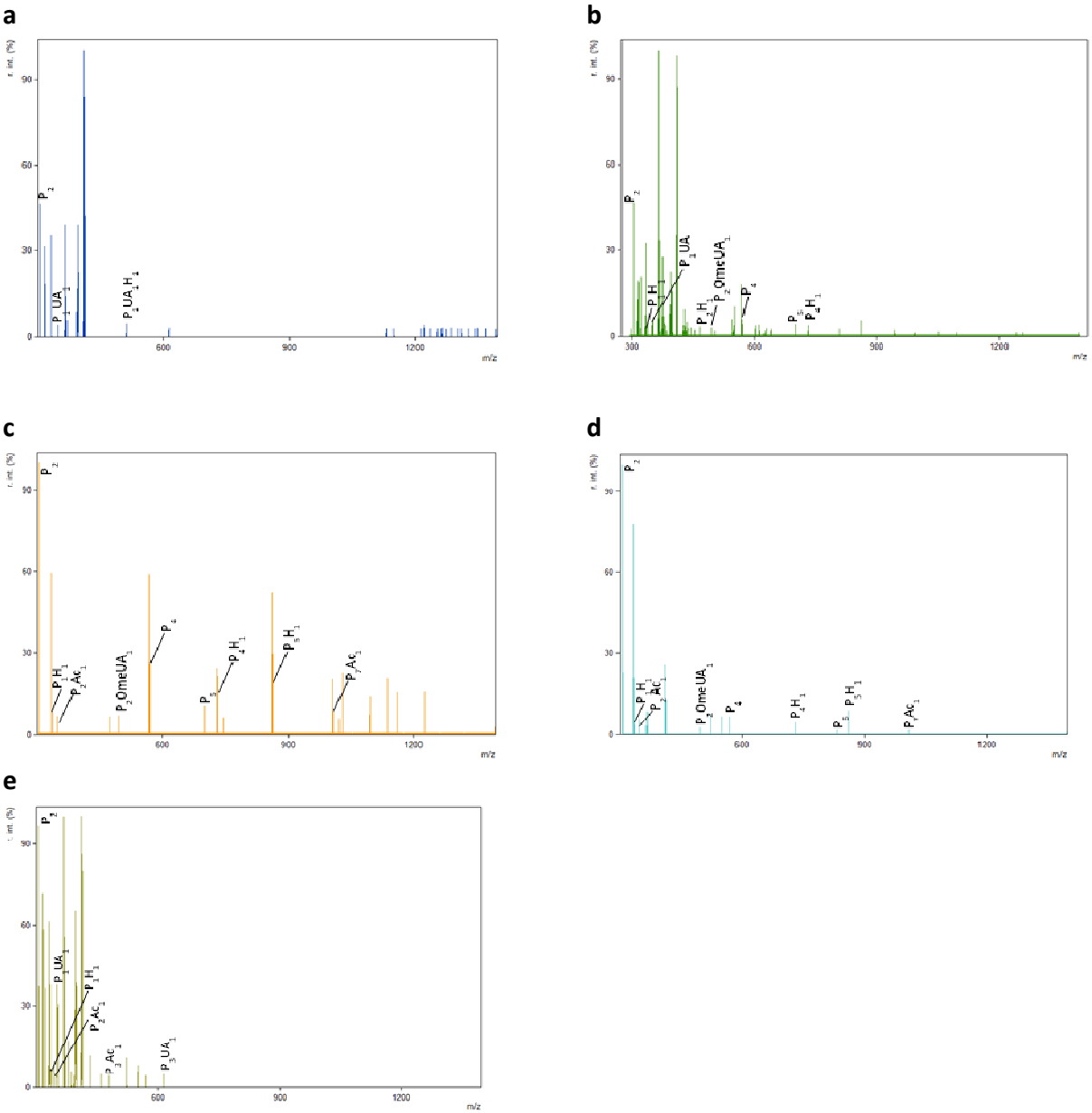


Figure S1: Maldi-Tof MS spectra of xylan fractions (a) AX 20, (b) AX 40, (c) AX 60, (d) AX 80 and (e) AX 80S hydrolyzed with the *A. niger / T. emersonii* enzyme preparation. P, pentose; H, hexose; UA, uronic acid; OmeUA, *O*-methylated uronic acid; Ac, acetyl

*CHAPTER 3*

# Fast and robust method to determine phenoyl and acetyl esters of polysaccharides by quantitative $^{1}$H NMR


## Abstract

The acetyl (AcE), feruloyl- (FE) and p-coumaroyl (pCE) ester contents of different cereal and grass polysaccharides were determined by a quantitative $^{1}$H NMR-based method. The repeatability and the robustness of the method were demonstrated by analyzing different plant polysaccharide preparations. Good sensitivity and selectivity for AcE, FE and pCE were observed. Moreover, an optimized and easy sample preparation allowed for simultaneous quantification of AcE, FE and pCE. The method is suitable for high throughput analysis and it is a good alternative for currently used analytical procedures. A comparison of the method presented to a conventional HPLC-based method showed that the results obtained are in good agreement, whereas the combination of the optimized sample preparation and analysis by the $^{1}$H NMR-based methodology results in significantly reduced analysis time.



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## Introduction

Analysis of plant cell wall polysaccharides from various feedstocks is necessary to characterize biomass conversion processes, to assist in the selection of suitable feedstocks, and for the development of optimized hydrolysis procedures (Foster 2010). Studies have shown that acetyl- (AcE), feruloyl- (FE) and p-coumaroyl (pCE) esters are xylan substituents present in cereal cell walls with their respective contents being dependent on the source of xylan (Badal 2003). Of particular relevance is the presence of AcE, FE, pCE and other substituents in oligosaccharides recalcitrant to enzymatic hydrolysis (Appeldoorn et al. 2010). A previous study demonstrated the presence of AcE, FE and pCE in recalcitrant polysaccharides at different levels, and enzymatic substrate conversion showed a dependency on the degree of xylan backbone substitution (Neumüller et al. 2012). In order to fully hydrolyse these polysaccharides, accessory enzymes, such as acetyl xylan esterases, and feruloyl esterases, are required. Their required dosage for efficient hydrolysis depends on the source of the feedstock, as feedstocks show significant differences in their composition (van Dyk et al. 2012). Although the presence of AcE, FE and pCE in plant cell wall polysaccharides has been known for a long time for various plant species (Buanafina 2009, Gille and Pauly 2012), little information is available for bioenergy feedstocks. Furthermore, different stages of maturation and growing conditions also have an influence on the composition of xylan substituents (Ellnain-Wojtaszek et al. 2001, Zheng and Wang 2001). Therefore, knowledge on the levels of substituents is important to assess the quality of a feedstock and its susceptibility to hydrolysis.

Analysis of AcE, FE and pCE contents by analyzing the acetic acid (AcA), feruloylic acid (FA) and p-coumaric acid (pCA) released upon alkaline treatment can be time-consuming due to the required sample preparation and chromatographic analysis. A variety of methods for HPLC analysis of phenolic acids has been reported (Robbins 2003), showing variations in solvent systems, elution, column choice, column temperature, flow rates and detection systems. No *single* standardized procedure for quantification has been generally adopted, indicating that the field is still in need of a robust technique for phenoyl and acetyl ester analysis. The use of different analytical procedures may lead to variations in the data and may cause difficulties in reproducing the data. Specific sample preparation and analysis procedures have been reported for the determination of AcA, FA and pCA separately by chromatographic analysis (Van Eylen et al. 2011). Separate analysis procedures may increase error rates compared to measurements by a single analysis procedure, and may be labor-intensive. Moreover, HPLC-based methods have rather long running times of 30 to 150 minutes (Robbins 2003), and they are dependent on the availability and inclusion of standards. A reduced sample analysis time (mobile phase sequence of 9.5 min and equilibration of 2.5 min) using an UPLC-MS/MS method has been reported (Gruz et al. 2008). In comparison, NMR allows for even shorter analysis times and has the advantage of being fully quantitative without the need of reference compounds for calibration curves

(Rizzo and Pinciroli 2005). This makes it a good alternative to chromatographic measurements (Wells et al. 2002). Several recent studies describe NMR as a useful technique regarding the characterization of plant extracts and polysaccharides (Charisiadis et al. 2012, Tizzotti et al. 2011, Qu et al. 2011, Pieri et al. 2011).
In the present study, a method allowing fast and robust determination of AcE, FE and pCE has been developed using quantitative $^{1}$H NMR as an analytical tool. The method allows quantification of AcA, FA and pCA by a single sample preparation procedure and a single recorded $^{1}$H-NMR spectrum. The results obtained are compared to results obtained by HPLC-based methods.

## Material and methods

### Feedstock materials

Dried, milled corn and grass silage were provided by DSM (Heerlen, The Netherlands). Water-unextractable solids (WUS) of corn cobs and corn stover were prepared as described elsewhere (Van Dongen et al. 2011). Corn fiber alcohol-insoluble solids (AIS) were prepared as described elsewhere (Kabel et al.2002). Commercially available potato starch (101252 starch) and maleic acid were from Merck (Darmstadt, Germany). Deuterated solvents were purchased from Cambridge Isotopes Laboratories (Andover, MA, USA).

### Sample preparation and analysis

*Sample preparation for NMR analysis:* Samples (10–30 mg) were accurately weighed in glass tubes. Hydrolysis was done in 1mL of 0.5 M NaOD in $D_2O$, under nitrogen atmosphere in the dark at room temperature (RT) with sonication with a Branson 5510 ultrasonic cleaner (Branson Ultrasonics, Danbury, CT, USA). Maleic acid (MA, 0.1 mL of a 5.277 g/L stock solution in $D_2O$) was added as internal standard.
The hydrolysis procedure involved sonication for 100 min followed by overnight incubation at RT and subsequent sonication for 100 min. Furthermore, shorter hydrolysis procedures with sonication times ranging from 0.5 h to 2 h and different time points of MA-addition (before or after hydrolysis with NaOD) were applied (Tables 1 and 2). $D_2O$ containing EDTA (0.1 g/ L; 2.5 mL) was added to the samples before analysis by NMR.

*Sample analysis by NMR:* A 700 MHz Bruker Avance III spectrometer, equipped with a 5 mm TCI cryoprobe and a SampleJet autosampler (Bruker, Billerica, MA, USA) suitable for 3x103.5 mm tubes was used for NMR analysis.
$^{1}$H NMR spectra were recorded at 300 K. For quantitative measurements a pulse program with water suppression was used (zgcppr) and water suppression power corresponding to 10 Hz suppression. A 90 degree pulse length was applied with a relaxation delay of 30 s. Number of

scans was 8. 65536 data points were collected and a spectrum width of 12ppm was used. The spectra were analysed with TopSpin 3.1 (Bruker).

*Quantification by NMR:* The acetic acid (AcA), ferulic acid (FA) and p-coumaric acid (pCA) concentrations were calculated from the $^{1}$H-NMR spectrum applying Equation 1. For pCA, the integrals corresponding to the aromatic proton peak at 7.44 ppm (doublet) and the integral area of the internal standard maleic acid proton resonance at 6.03 ppm (singlet) were used for this purpose. FA and AcA amounts were calculated using the peaks at 7.17 ppm and 1.95 ppm respectively, and the internal standard as mentioned above. The molecular weights used for pCA, FA and AcA were 164.16, 194.18 and 60.05, respectively.

$$P_x = \frac{A_x}{A_{st}} \times \frac{n_{st}}{n_x} \times \frac{MW_x}{MW_{st}} \times \frac{W_{st}}{W_x} \times P_{st} \qquad \text{Eq.1}$$

Ax = Area peak of product, Ast = Area peak of internal standard, Nst = number of protons corresponding to the internal standard peak, nx = number of protons corresponding to the product peak, MWx = molecular weight of product, MWst = molecular weight of the internal standard, Wst = Weight of the internal standard (g), Wx = Weight sample (g), Pst = purity internal standard (%)

*Sample preparation for HPLC analysis of FA, pCA, AcA:* FA and pCA were analyzed according to the procedure described by Appeldoorn et al. (2010) with some minor modifications: Each sample (10-20 mg) was dissolved in 200 µL methanol. 5 mL of 0.5 M KOH (flushed with N2) was added and samples were put under a $N_2$ atmosphere. The samples were kept in the dark for 16 h and sonicated 2 times for (100 min, 5510 Branson ultrasonic cleaner). After 16 h, the pH was adjusted to 2 by the addition of 0.75 mL of HCl (6 M). Extraction of FA and pCA was done by 4 mL ethyl acetate. The extraction was repeated once. The ethylacetate fraction was dried under $N_2$ at RT and the residue was dissolved in 1mL methanol.
*For AcA determination, e*ach sample (20-40 mg) was saponified with 1 mL of 0.4 N NaOH in isopropanol/H2O (1:1 mixture) for 1 h on ice and 2 h at room temperature under regular mixing.

*HPLC/UHPLC analysis of FA, pCA, AcA:* For phenolic acid analysis an Acella UHPLC system (Thermo Scientific, Rockford, IL, USA) equipped with a PDA detector and coupled to an LTQ XL mass detector equipped with an ESI source (Thermo Scientific) was used. Separation was performed on a Hypersyl GOLD column (1.9 x 150 mm; 1.9 µm; Thermo Scientific).
For the determination of FA and pCA the mobile phase was composed of (A) H2O + 1% (v/v) acetonitrile + 0.2% (v/v) acetic acid and (B) acetonitrile + 0.2% (v/v) acetic acid. Elution was done at 0.4 mL/min by the following profile: 5 min, isocratic 0% B; 5-23 min, linear from 0 to 50% B; 23-24 min, linear from 50 to 100% B; 24-27 min, isocratic at 100% B; 27-28 min, linear from 100 to 0% B, followed by reconditioning of the column for 7 min. Spectral data collection was done from 200 to 600 nm. Quantification was done at 320 nm on the basis of standards.

MS data were collected in the negative mode with an ion spray voltage of 3.5 kV, a capillary voltage of -20 V and a capillary temperature of 350 °C. Full MS scans were made within the range m/z 150-1500, and MS2 data of the most intense ions were obtained.
For acetic acid analysis, an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) equipped with a Shodex RI detector (Showa Denko Europe GmbH, Munich, Germany) and an Aminex HPX87H column (300mm x 7.8mm; Bio-Rad Laboratories, Hercules, CA, USA) were used for AcA analysis. For the determination of AcA by HPLC, elution was performed with H2SO4 (5 mM) at a flow rate of 0.6 mL/min and a column oven temperature of 40 °C.

## Results and discussion

### Determination of AcE, FE and pCE contents

*Optimization of experimental parameters*

Ferulic acid (FA), p-coumaric acid (pCA) and acetic acid (AcA) containing standards were analyzed by NMR. The quantified data obtained by NMR analysis were in agreement with the amounts weighed in [$R^2$ values >0.999; 0.2-0.02 mg/mL (FA, pCA); 20-0.0002 mg/mL (AcA)]. The signal-to-noise ratio was clearly above the quantification limit of 10:1 [International Conference on Harmonization (ICH), Chicago, 2005]. Linearity of the results was observed for the standard concentrations with signal-to-noise ratios > 10:1 [0.2-0.02 mg/mL (FA, pCA); 20-0.0002 mg/mL (AcA)]. This range covers the expected range of concentrations for AcA, FA and pCA in plant cell wall polysaccharides, using the sample preparation applied in this study. Whereas 0.02 mg/mL (FA, pCA) could be reliably quantified [signal-to-noise ratios of 19:1 (FA) and 32:1 (pCA)], quantification was not possible for FA and pCA below a concentration of 0.002 mg/mL and for AcA below a concentration of 0.0002 mg/mL with the measurement conditions applied (8 scans, analysis time: 4-5 min).
For the optimization of experimental parameters, plant polysaccharides were used. Figure 1 shows the recorded $^1$H-NMR spectrum after sample preparation of corn silage with the proton signals used for AcA, FA and pCA quantification (1.95, 7.17 and 7.44 ppm). The variance of the method (sample preparation and quantification) was assessed. The acetyl (AcE), feruloyl (FE) and p-coumaroyl (pCE) ester contents of corn silage by repeated measurements under the same or modified conditions (different saponification/sonication times, different amounts of sample, and addition of MA before and after hydrolysis) were calculated (% w/w) and are listed in Table 1. Standard deviations (stdv) within a series of repeated measurements were between 0.01 and 0.04 (Table 1) and ≤0.02 between the average values of three measurement series. Signal-to-noise ratios of >2000:1 for AcA, 45:1 for FA and 68:1 for pCA were observed for corn silage, being significantly above the detection and quantification limits. In order to investigate the suitability of the method for the determination of very low amounts of AcA, FA and pCA in polysaccharide-containing samples, corn silage/starch mixtures were analyzed [starch: no FE

and pCE present, and only minor amounts of acetic acid (0.015% w/w)]. Signal-to-noise ratios of 780:1, 45:1 and 50:1 for AcA, FA and pCA, respectively, allowed good quantification in a 1:10 corn silage/starch mixture. In a 1:100 corn silage/starch mixture, AcE could still be reliably quantified (Table 1) while reliable quantification of pCE and FE was not possible (signal-to-noise ratios <10:1). The quantification and detection limits for each sample can be easily assessed from each individually recorded NMR spectrum based on the signal-to-noise ratios.

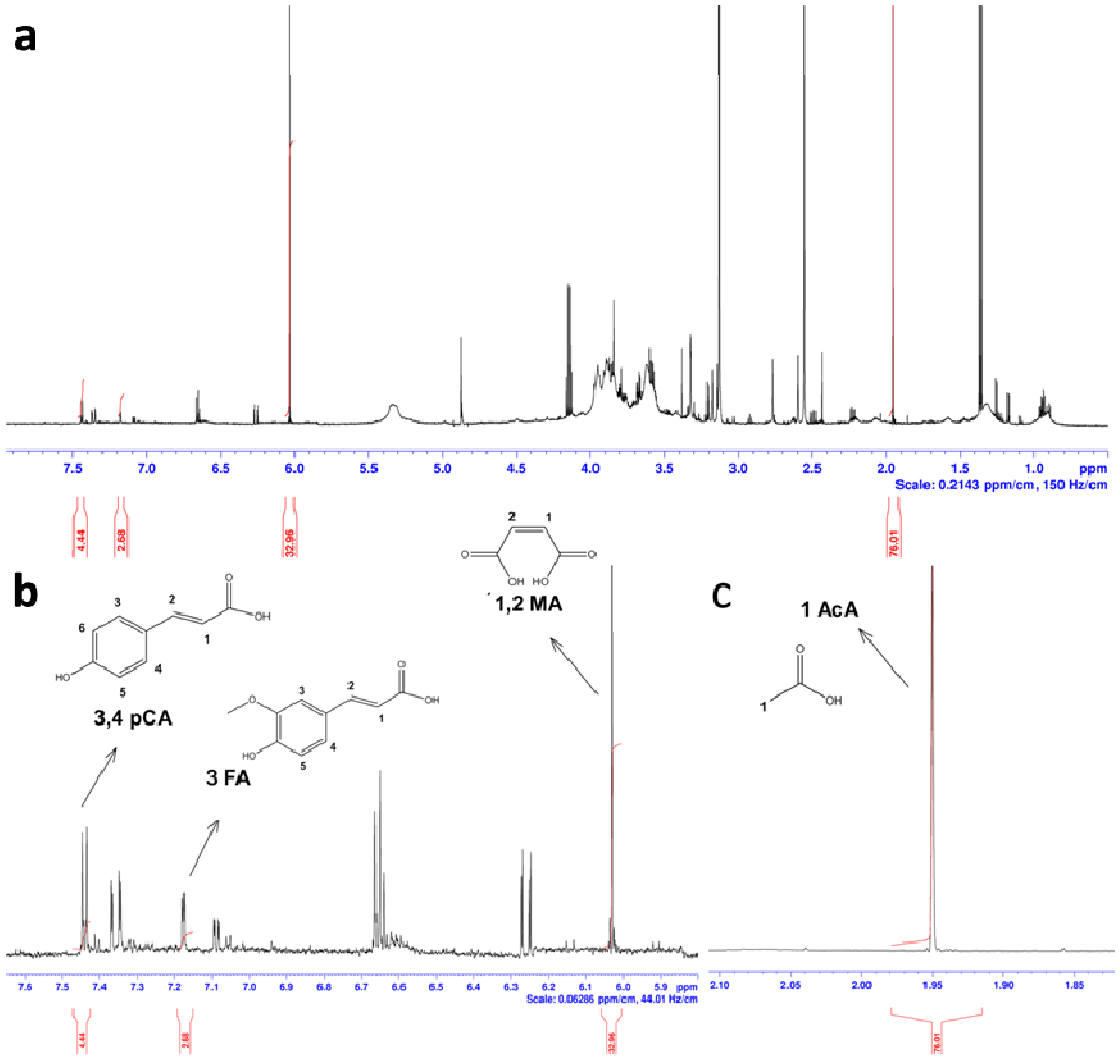


Figure 1: $^{1}$H NMR spectrum of saponified corn silage sample. (a) Region from 8.0 to 0.5 ppm, (b) 7.6 to 5.8ppm, (c) 2.1 to 1.85 ppm.

Table 1: AcE, FE and pCE contents (% w/w) determined by an NMR-based method of (i) corn silage (cs) with different saponification/sonication conditions and (ii) corn silage – starch mixtures (cs:starch).

| Sample | pCE (stdv) | FE (stdv) | AcE (stdv) | saponification/ | MA addition |
|---|---|---|---|---|---|
| $cs_{1-5}$ | 0.46 (0.01) | 0.44 (0.03) | 1.41 (0.03) | 1h | b.h.[a] |
| $cs_{6-7}$ | 0.45 (0.01) | 0.39 (0.02) | 1.39 (0.02) | 1h | a.h.[b] |
| $cs_{8-9}$ | 0.44 (0.03) | 0.43 (0.03) | 1.38 (0.01) | on[c] | b.h. |
| cs:starch (1:10) | 0.07 | 0.05 | 0.23 | 1h | b.h. |
| cs:starch (1:100) | 0.0 | 0.0 | 0.02 | 1h | b.h. |

[a] before hydrolysis (b.h).
[b] after hydrolysis (a.h.).
[c] 200 min sonication, incubation of 16 h.

*Optimization of pretreatment*

Saponification of the samples was done with NaOD (0.5 M) at room temperature. It has been reported that relatively mild NaOH extraction conditions (1 M or lower) at room temperature specifically release phenolic acid residues of non-lignin origin (Martens 2002, Provan et al. 1994), as the release of phenolic acids from lignin requires strongly oxidizing conditions (cupric oxide-NaOH, 175 °C; Chen 1992).

The saponification times ranged between 30 min and 16 h (Table 2), in agreement with reported saponification procedures (Robbins 2003). The differences observed when altering the saponification conditions are shown in Table 2 for four different corn-derived feedstocks (corn silage, corn cobs WUS, corn stover WUS, corn fiber AIS). In the case of corn silage, all saponification and sonication times tested resulted in comparable AcE levels, but short saponification/sonication times (0.5 h) gave lower pCE and slightly lower FE levels. Therefore, a saponification/sonication time of 1 h seems to be required to obtain full release of FE and pCE from corn silage. Prolonged saponification/sonication did not release additional FE or pCE.

For corn cobs and corn stover, an increase of pCE levels was observed with prolonged saponification/sonication time (2x 100 min sonication, overnight saponification). This indicates that the suitability of sample pretreatment conditions depends on the feedstock to be analyzed and its previous processing conditions. On the other hand, it is possible to optimize saponification/sonication times easily for routine analysis of a specific feedstock (as shown for corn silage), thereby reducing the sample preparation time. As pCE levels were higher with prolonged saponification/sonication for corn cobs, corn fiber and corn stover, overnight saponification was chosen as the standard sample pretreatment.

Table 2: Contents (% w/w) of pCE, FE and AcE in various corn samples (corn silage, corn cobs WUS, corn fiber AIS and corn stover WUS) following sample preparation with different saponification/sonication times.

| | **corn silage** | | | **corn cobs** | | | **corn fiber** | | | **corn stover** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| saponification | pCE | FE | AcE | pCE | FE | AcE | pCE | FE | AcE | pCE | FE | AcE |
| 0.5 | 0.49 | 0.36 | 1.40 | | | | | | | | | |
| 1 | 0.58 | 0.41 | 1.47 | 1.75 | 1.17 | 2.36 | 0.13 | 2.26 | 2.45 | 1.09 | 0.54 | 1.72 |
| 2 | 0.58 | 0.41 | 1.40 | | | | | | | | | |
| 16[a] | 0.56 | 0.43 | 1.45 | 2.33 | 1.09 | 2.36 | 0.19 | 2.06 | 2.47 | 1.87 | 0.46 | 1.85 |

[a]200 min sonication, incubation of 16 h.

**High-throughput determination of AcE, FE and pCE by NMR**

The analysis time of AcE, FE and pCE in plant feedstocks is significantly reduced by the method presented compared to conventional HPLC methods. NMR-analysis of a hydrolyzed sample took 4.52 min (equilibration time was set to 1 min). Reported HPLC methods for phenolic acid analysis vary significantly and range from 30 to 150 min, with some methods including equilibration, and others including it as a separate step (Robbins 2003). Analysis times by UPLC analysis are shorter (12 min, Gruz et al. 2008). Therefore, significant analysis time reductions of >80% and >50% were achieved by the NMR method compared to HPLC and UPLC methods, respectively.

Methods that include separate specific sample preparation and analysis procedures for AcE (Voragen et al. 1986), FE and pCE (Robbins 2003) are frequently used for compositional analysis of feedstocks (Appeldoorn et al. 2010, Van Dongen et al. 2011). However, separate analysis procedures may increase error rates and analysis time. Determination of AcE, FE and pCE from a single hydrolysate and a single recorded $^{1}$H-NMR spectrum significantly reduces labor input (a single sample preparation), analysis time (analysis by one recorded NMR spectrum) and may also lower error rates. The use of an autosampler for NMR analysis in this study allowed automated measurement of multiple samples, providing a platform for high throughput analysis with high precision by NMR quantification.

**Comparison of NMR- and HPLC-based methods**

AcE, FE and pCE content of five different substrates (grass and corn silage, corn cobs WUS, corn stover WUS and corn fiber AIS) were determined by NMR and the results were compared to those obtained by HPLC methods (Figure 2). A regression analysis of the AcE, pCE and FE contents independently determined by the method presented here and by a conventional HPLC-based method showed high correlations of 0.96, 0.94 and 0.93, respectively (Figure 2a). Therefore, the measured AcE, pCE and FE values are in good agreement and confirm the validity of the method presented. A comparison of the same hydrolysate prepared by a single sample preparation procedure (see *Sample preparation for NMR analysis*) of corn cobs WUS, corn

stover WUS and corn fiber AIS by NMR and HPLC analysis gave even higher correlations ($R^2$ = 0.99 for FA and pCA, Figure 2b). This observation indicates that small differences between the results obtained by the two methods are not due to the analytical technique used, but by errors that originate from the sample preparation procedure. Therefore, simplifying the sample preparation as well as analyzing a single hydrolysate, as described for the method presented here, lowers the error rate.

The results obtained by both the NMR- and the HPLC-based methods are in agreement with published data for corn cobs, corn stover and corn fiber (Van Eylen et al. 2011). Small differences between the values obtained and the published values are to be expected due to the preparation of the WUS (cobs, stover) fraction and the inhomogeneity of natural plant feedstocks regarding their chemical composition in general (Jahn et al. 2010, Liu 2011).

In conclusion, our data show that the NMR-based method presented is suitable for the determination of AcE, FE and pCE, occurring in plant cell wall polysaccharides. The method presented is a fast, robust and reliable alternative to conventional methods and it is therefore of relevance for the characterization of these substituents in cereal and grass cell wall polysaccharides.

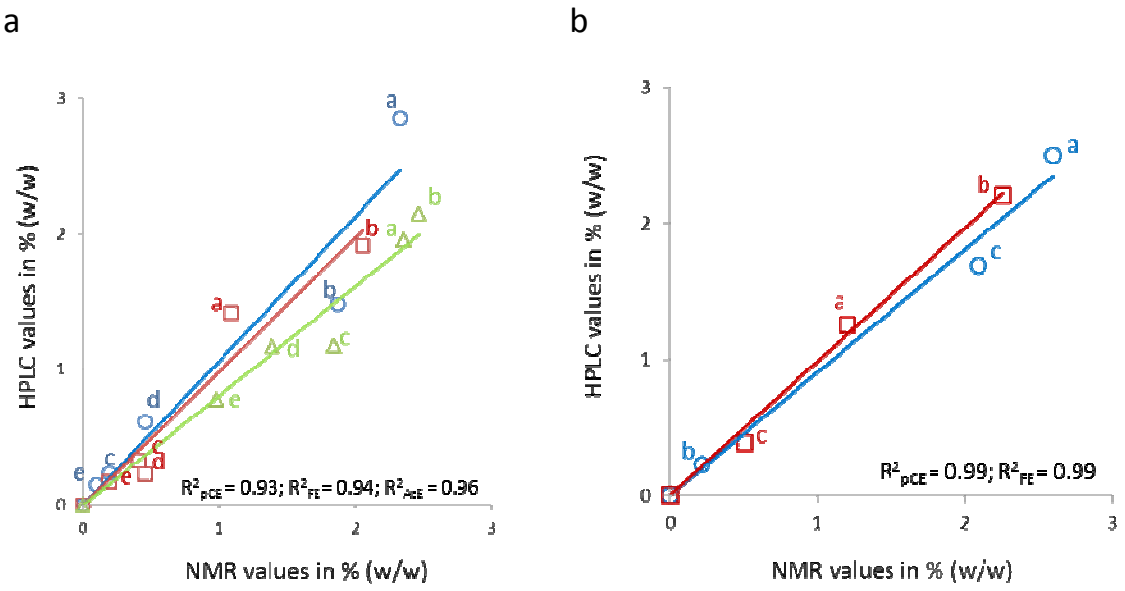


Figure 2: Correlations observed between the results obtained from independent NMR- and HPLC based analysis of different feedstocks. FE (red, squares), pCE (blue, circles), AcE (green, triangle), each data point represents the analysis of one sample. (a) Corn cobs, (b) corn fiber, (c) corn stover, (d) corn silage, (e) grass silage.
(a) analysis according to the NMR method presented and HPLC based methods
(feedstocks analyzed: grass silage, corn silage, corn cobs WUS, corn stover WUS, corn fiber AIS)
(b) FE and pCE levels of the same hydrolysate (feedstocks analyzed: corn cobs WUS, corn stover WUS, corn fiber AIS).

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*CHAPTER 4*

# Synergistic action of enzyme preparations towards recalcitrant corn silage polysaccharides


## Abstract

Corn silage, its water unextractable solids (WUS) and enzyme recalcitrant solids (ErCS) and an industrial corn silage-based anaerobic fermentation residue (AFR) represent corn substrates with different levels of recalcitrance. Compositional analysis reveals different levels of xylan substitution for WUS, ErCS and AFR, being most pronounced regarding acetic acid, glucuronic acid- and arabinose content. By screening for enzymatic degradation of WUS, ErCS and AFR, enzyme preparations exhibiting high conversion rates were identified. Furthermore, significant synergistic effects were detected by blending *Aspergillus niger / Talaromyces emersonii* culture filtrates with various enzymes. These findings clearly highlight a necessity for a combinatorial use of enzyme preparations towards substrates with high recalcitrance characteristics to reach high degrees of degradation. Enzyme blends were identified, outperforming the individual commercial preparations. These enzyme preparations provide a basis for new, designed enzyme mixtures for corn polysaccharide degradation as a source of necessary, accessory enzyme activities.



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## Introduction

Corn silage, a major biogas feedstock (Plöchl et al. 2009), provides a source of fermentable sugars. In order to enable efficient utilization of recalcitrant corn residues or wastes for biogas production, enzymatic preparations are necessary that can degrade these substrates.

Multiple enzyme classes are involved in the degradation of cellulose, including β-1,4-endoglucanases, exoglucanases/cellobiohydrolases and β-glucosidases. Hemicellulose requires even more enzyme activities for its complete hydrolysis. β-1,4-Endo-xylanases and β-1,4-xylosidases are necessary to degrade the xylan backbone. Important accessory activities are α-arabinofuranosidases, arabinoxylan arabinofuranohydrolases, α-glucuronidases, α-1,4-galactosidases, β-1,4- galactosidases, feruloyl/*p*-coumaroyl esterases and acetyl xylan esterases (Van den Brink and de Vries 2011). Furthermore, GH61 and CBM33 classified enzymes catalyze oxidative cleavage of polysaccharides (Vaaje-Kolstad et al. 2010). Other non-hydrolytic proteins such as expansins, expansin-like proteins (swollenin), carbohydrate-binding modules or low molecular weight peptides promote disruption of cellulosic material by amorphogenesis and deagglomeration (Arantes and Saddler 2010).

While a broad range of microorganisms express enzymes that are capable of hydrolyzing (hemi)cellulose, only a few strains secrete a complex of (hemi)cellulolytic enzymes with potential for practical application as in biofuels production (Sukumaran et al. 2005). Genera that are commonly commercially exploited for (hemi)cellulase production include *Aspergillus*, *Trichoderma*, *Humicola (*Fungi, Ascomycota*), Thermonospora (*Bacteria, Actinobacteria*) and Bacillus (*Bacteria, Firmicutes*)*. Bioconversion by (hemi)cellulolytic enzyme preparations is limited by the recalcitrance of feedstocks. Recalcitrant cell wall structures, like a high degree of backbone substitution (87%) of corn glucuronoarabinoxylan, has been reported (Huisman et al. 2000). Several substituents, like ferulic acid, diferulates, acetic acid, galactose, arabinose and uronic acid, make corn fiber resistant to enzymatic hydrolysis following mild acid pretreatment (Appeldoorn et al. 2010). Furthermore, biomass recalcitrance is derived from the architecture of cell walls, influencing the accessibility for enzymes (Arantes and Saddler 2010).

Different strategies to overcome plant cell wall recalcitrance have been applied. A frequently used strategy is the application of harsh physical and chemical pretreatment conditions, like steam explosion and acid hydrolysis. Such conditions typically cause the hydrolysis of the hemicellulose fraction, while rendering the cellulose fraction more susceptible to enzymatic degradation (Chen et al. 2012). Disadvantages of these methods are their high costs, energy needs (Blanch et al. 2011), their contribution to environmental pollution and the formation of inhibitors (Horn et al. 2011). Another promising approach is to diminish the necessity of intense pretreatment by improved enzymatic hydrolysis. The latter would allow better utilization of substrates in biogas production where usually no pretreatment is applied. Furthermore a better utilization of C-5 sugars without (intense) pretreatment would be of relevance for 2$^{nd}$

generation ethanol production as C-5 fermenting yeast has been reported as a powerful tool to ferment xylose and arabinose (Becker and Boles 2003, Shupe and Liu 2012).
Here we present fermentable sugar release by enzyme preparations screened on corn silage derived substrates: Water unextractable solids (WUS), enzyme recalcitrant solids (ErCS) and an anaerobic fermentation residue (AFR). WUS and ErCS (laboratory prepared corn silage model and -recalcitrant polysaccharides) and AFR (industrial residue) are substrates with increasing recalcitrance characteristics as determined by compositional analysis.
Substrate conversion by screening individual and blended enzyme preparations is assessed. For the latter a state-of-the-art *A.niger / T. emersonii* enzyme mixture is supplemented with a variety of commercial enzyme preparations resulting in increased conversion rates. Significant synergistic effects towards cellulose and hemicellulose degradation are identified for all substrates tested.

## Materials and methods

### Materials

*Preparation of water unextractable solids (WUS):* The preparation of water unextractable solids was based on the procedure described by Huisman et al. (2000). Defatting of milled corn silage [670 g; batch number: MS25032010 (harvested 2009 and ensilaged, Sperenberg, Brandenburg, Germany; dry matter (DM): 397 g $kg^{-1}$, pH 3.9, freeze dried January, 2010 and stored at 4 °C) provided by DSM Biopract (Berlin, Germany, 2009)] was performed overnight (16 h) by stirred extraction in *n*-h*exane* (6.7 L) at room temperature (RT). The suspension was filtered (Glass Fiber Filters 1.6µm, Millipore, Billerica, MA, USA) and the extracted lipids were quantified gravimetrically after evaporation of the organic solvent at RT. Destarching was done after gelatinization at 80 °C for 35 min and cooling to 60 °C in a stirred bioreactor by addition of α-amylase (BAN® 800, Novozymes, Bagsvaerd, Denmark) at 75 g $kg^{-1}$ DM (protein/substrate) for 2 h in Milli-Q water (pH 4.5). The suspension was centrifuged (25.000 × g, 15 min, RT).
The remaining destarched residue was suspended in 1 L SDS (0.052 mol $L^{-1}$) solution. The suspension was stirred at RT for 1 h, followed by centrifugation (25.000 × g, 15 min, RT). This deproteination procedure was repeated twice. The residue was washed thoroughly and repeatedly with destilled water to remove all remaining SDS. Subsequently, it was freeze-dried and denoted WUS.
*Preparation of enzyme recalcitrant solids (ErCS) from WUS:* Incubations with the *A. niger* / *T. emersonii* culture filtrates mixture [1:1 ratio, 100 g $kg^{-1}$ DM (protein/substrate)] were done, in Milli-Q water containing sodium azide (0.5 g $kg^{-1}$) at a substrate concentration of 10 g $kg^{-1}$ for a time period of 185 h in a stirred glass bioreactor at 50 °C. The protein content of the enzyme preparations was determined by the TCA biuret method. The optimal enzyme to substrate ratio [100 mg $g^{-1}$ DM (protein/substrate)] for a maximum degree of degradation was determined.

Higher enzyme to substrate ratios did not result in higher monosaccharide levels (data not shown). In order to produce an enzyme recalcitrant fraction from WUS, the latter was incubated with the *A. niger* / *T. emersonii* culture filtrates mixture at 500 mL volume. Free fermentable sugar release was monitored over time. After hydrolysis, the residue was centrifuged (17.000 × g, 15 min, RT). The pellet was re-suspended with 0.5 L SDS (0.052 mol $L^{-1}$) solution and stirred (52 rad $s^{-1}$) for 1 h at RT. The suspension was centrifuged (17.000 × g, 15 min, RT). This step was repeated once, followed by extensive washing with Milli-Q water, centrifugation (17.000 × g, 15 min, RT) and freeze drying. The final residue is referred to as ErCS.
*Anaerobic fermentation residue (AFR):* An anaerobic, industrial fermentation residue of a biogas production process was provided by DSM Biopract (Berlin, Germany, sampled January, 2011). The residue was derived from an incubation of corn silage ($w_{\text{corn silage}}$ = 710 g $kg^{-1}$), rye whole plant silage ($w_{\text{rye whole plant silage}}$ = 220 g $kg^{-1}$) and rye ($w_{\text{rye}}$ = 70 g $kg^{-1}$) without any pretreatment after a residence time of 63 days at a temperature of 39-40 °C. The fermentation residue was freeze dried, milled to homogeneity, sieved through a 355 µm sieve and stored at 4 °C.
*Particle size distribution (WUS, ErCS, AFR)*
The particle size distribution of WUS, ErCS and AFR in a water suspension (10 g $kg^{-1}$) was determined (Middlemas 2009) with an LS 13 320 Laser Diffraction Particle Size Analyzer (Beckman Coulter Inc., Fullerton, CA, USA) and is shown in Table 1.

Table 1: Particle size distribution of the corn fractions WUS, ErCS and AFR.

| Substrate | mean | 90% < |
|---|---|---|
| WUS | 410 µm | 840 µm |
| ErCS | 360 µm | 780 µm |
| AFR | 130 µm | 320 µm |

*Enzyme preparations:* Thirty-seven enzyme preparations [*Aspergillus niger* and *Talaromyces emersonii* enzyme preparations (DSM), a blend (1:1 mixture, based on protein content) of these preparations and thirty-four commercial preparations] were used for enzymatic hydrolysis. Further information (product name, supplier, production strain, main activities and known side activities) of the enzyme preparations can be found in Table S1. Protein contents of the *A. niger* and *T. emersonii* enzyme preparations were 70 and 90 g $L^{-1}$ respectively. These enzyme preparations can be obtained by ultrafiltration (10 kDa, 1h, 3200 g at 4°C) of Brewers Filtrase BXC (*T. emersonii*, DSM) and DSM-Arabanase (*A. niger*, DSM) followed by dilution of the retentate with a water glycerol (450 g $kg^{-1}$) mixture to a final concentration as stated above.

**Compositional analysis**
*Neutral sugar composition:* Corn silage, WUS, AFR and ErCS were pretreated in sulfuric acid (12 mol $L^{-1}$) for 1 h at 30 °C. Hydrolysis was done at 1 mol $L^{-1}$ sulfuric acid for 3 h at 100 °C. Sugars

released were quantified as their alditol acetates by gas chromatography (Englyst and Cummings 1984). Inositol was used as internal standard.

*Uronic acid (UA):* After hydrolysis with sulfuric acid (neutral sugar composition analysis) an aliquot was taken for the quantification of UA using an automated colorimetric *m*-hydroxydiphenyl assay (Thibault 1979). A calibration curve was prepared with galacturonic acid.

*Protein content (total nitrogen):* The protein content (N x 6.25) was determined with a Dumas-based nitrogen analyzer (Thermo Scientific, Waltham, MA, USA). Methionine was used for calibration.

*Starch:* The starch content was determined with the total starch assay (Megazyme International, Wicklow, Ireland).

*Dry matter (DM):* DM was determined by heating to 120 °C and measuring consistent weight for 120 seconds (120 s mode; LP16 dry matter analyzer, PM100 balance; Mettler Toledo, Columbus, OH, USA).

*Lignin (acid-soluble and -insoluble lignin):* Samples (100 mg) were treated with 1 mL sulfuric acid (12 mol $L^{-1}$) for 1 h at 30 °C. Hydrolysis was done for 3 h in a boiling water bath with 1 mol $L^{-1}$ sulfuric acid with regular vortex mixing every 30 minutes. The suspension was filtered (Glass Fibre Filters, Whatman, Maidstone, UK). Acid-soluble lignin in the filtrate was determined according to the procedure described by the National Renewable Energy Laboratory operated by the Midwest Research Institute (NREL/MRI, Golden, CO, USA; Sluiter et al., 2008). For the determination of acid-insoluble lignin, the residue was washed to remove residual acid and dried at 100 °C for 2 h followed by cooling to RT in a desiccator. Acid-insoluble lignin was measured by weighing and subtraction of the acid-insoluble ash content.

*Acid-insoluble ash:* The acid-insoluble residue of the lignin analysis was incinerated at 550 °C overnight and weighed. The residual material represents the acid-insoluble ash content.

*Ferulic acid (FA)-, p-coumaric acid (pCA) content, degree of acetylation (Ac):* FA, pCA and Ac of corn silage, WUS, ErCS and AFR were quantified by NMR (700 MHz, Bruker, Billerica, MA, USA) according to the procedure described previously (Neumüller et al. 2013). Maleic acid served as internal standard for quantification. Samples were hydrolyzed in 0.5 mol $L^{-1}$ NaOD under $N_2$, in the dark and exposed to ultrasonication (Branson 5510, Emerson, Ferguson, MO, USA) for 2 h at 35 °C.

**Analysis of mono- and oligosaccharides**

Mono- and oligosaccharides were analyzed by high-performance anion-exchange chromatography (HPAEC). An ICS 5000 system (Dionex Corporation, Sunnyvale, CA, USA), equipped with a CarboPac PA20 and an Amino Trap guard and Borate Trap pre-column was used with pulsed amperometry, gold electrode detection in triple mode waveform.

*Monosaccharide analysis:* After an equilibration step with NaOH (0.0005 mol $L^{-1}$, 15 min), elution was done according to the following procedure: Linear elution at 0.0005 mol $L^{-1}$ NaOH

(15 min, 0.5 mL $min^{-1}$), linear gradient from 0.0005 mol $L^{-1}$ to 0.375 mol $L^{-1}$ NaOH over 6 min (0.5 mL $min^{-1}$). The monosaccharides (Glc, Xyl, Ara) released were calculated as g $kg^{-1}$ DM (WUS, ErCS or AFR).

*Oligosaccharide analysis:* An equilibration step was done at 0.1 mol $L^{-1}$ NaOH (0.4 mL $min^{-1}$, 15 min). Elution was done according to the following HPLC procedure: 0.1 mol $L^{-1}$ NaOH and linear gradient elution from 0 mol $L^{-1}$ to 0.35 mol $L^{-1}$ sodium acetate (25 min, 0.5 mL $min^{-1}$), 0.1 mol $L^{-1}$ NaOH and linear gradient from 0.350 mol $L^{-1}$ to 1 mol $L^{-1}$ sodium acetate (2 min, 0.5 mL $min^{-1}$), followed by linear elution at 0.1 mol $L^{-1}$ NaOH and 1 mol $L^{-1}$ sodium acetate at a flow rate of 0.4 mL $min^{-1}$ (5 min).

**Screening of multi-component enzyme preparations**

Enzymatic incubations of WUS, ErCS and AFR were done on 1 mL scale in sealed 96 deep-well plates. Enzymes were added at a 2:1 ratio [(20 g $kg^{-1}$ enzyme preparation, 10 g $kg^{-1}$ substrate)], with or without the addition of the *A. niger / T. emersonii* culture filtrates mixture [1:1 protein ratio, 100 mg $g^{-1}$ DM (protein/substrate)]. Hydrolysis was done in MilliQ, containing sodium azide (0.5 g $kg^{-1}$), at 50 °C, shaking at 10.5 rad $s^{-1}$ for 48 h. When using AFR as substrate, Penicillin – Streptomycin 100X Solution GIBCO® (Invitrogen, Carlsbad, CA, USA) was added (1 g $kg^{-1}$). The pH of the suspension of WUS, ErCS and AFR (each 10 g $kg^{-1}$) was determined to be 5.6, 6.6 and 8.9 respectively. The pH of AFR decreased relatively fast after the start of hydrolysis to 8.3 within 1 h and 7.9 within 24 h. AFR is industrially processed at this pH (biogas formation process) and was therefore used to screen for enzymes active under industrially relevant conditions.

Enzyme controls (without substrate) were analyzed as well. Results were corrected for monosaccharides derived from the enzyme preparations.

After hydrolysis, the samples were centrifuged (1800 g, 15 min, RT), diluted in Milli-Q and heat-inactivated for 10 min at 95 °C. Analysis of the free fermentable sugars was done by HPAEC.

*Data visualization:* The percentage of the maximum theoretical yield of Glc, Xyl and Ara released was calculated based on measured levels of monosaccharides released and the sugar composition of the substrates. The calculated data-set was visualized in a heatmap. Heatmaps were built with the open source software Mayday (Battke et al. 2010). Enzymes were classified with respect to their hydrolytic activities in categories ranging from high (red) to medium (black) to low/no sugar release (green).

*Data-plots:* Data-plots in Figure 3 were built in Microsoft Excel.

Y(axis): g $kg^{-1}$ DM (Glc, Xyl, Ara released by CF in combination with E) – g $kg^{-1}$ DM (Glc, Xyl, Ara released by sole application of CF). X(axis): g $kg^{-1}$ DM (Glc, Xyl, Ara released by E).

CF = culture filtrates mixture (*A. niger / T. emersonii* enzyme mixture).

E = screened enzyme preparation.

## Results and Discussion

### Characterization of feedstock materials and recalcitrant residues

*Composition of corn silage and WUS*

Corn silage and its water unextractable solids (WUS) were analyzed for their compositions (Table 2). Regarding corn silage, the data for starch, xylan, lignin, protein and ash were in good agreement with previously published findings (Shao et al. 2010), while levels of cellulose, determined as non-starch Glc, were lower. Starch ($w_{starch}$ = 294 g $kg^{-1}$) represented the main originate of Glc. Xylose ($w_{Xyl}$ = 118 g $kg^{-1}$) constituted the main part of the hemicellulose [$w_{hemicellulose}$ = 203 g $kg^{-1}$ (Xyl, Ara, Gal, UA, FA, pCA, Ac)]. As preparation of corn silage involves harvesting before physiological maturity, the compositional data differ slightly from corn fiber-, cobs- and stover-composition (Van Eylen et al. 2011), which reflect mature stages of corn. Lignin was mainly present in an acid-insoluble form. Lipids were extracted and quantified ($w_{lipids}$ = 20 g $kg^{-1}$, minor amounts of lipids may still be present after extraction). Based on the amounts of xylan backbone substituents present (Ara, Gal, UA, FA, pCA, Ac, Table 2) the degree of substitution (DS) of the xylose backbone was calculated to be 0.85 (mol substituent per mol Xyl, Table 3).

As expected, compositional analysis of WUS revealed a similar hemicellulose composition as corn silage (Table 2). An increase of UA, FA, pCA and Ac was observed, consistent with an enrichment of cellulose and hemicellulose. The DS per Xyl (0.73, Table 3) was quite similar to corn silage. Hence, WUS represents a suitable model substrate for screening towards enzymatic degradation of corn silage polysaccharides.

*Preparation of ErCS (Hydrolysis of WUS by an A. niger / T. emersonii culture filtrates mixture)*

*A. niger* and *T. emersonii* both secrete a wide range of (hemi)cellulolytic enzymes (Sukumaran et al. 2005, Waters et al. 2011, Tuohy et al. 1993). A comparison of the hydrolysis by an *A. niger / T. emersonii* culture filtrates mixture to widely used *state-of-the-art* enzyme preparations (including industrially frequently used *Trichoderma* enzymes) revealed high levels of Ara and Xyl released by the *A. niger / T. emersonii* mixture [22 g $kg^{-1}$ DM (Ara) and 110 g $kg^{-1}$ DM (Xyl) after 48 h; incubations were done as described above: *Screening of multi-component enzyme preparations*]. The commercial enzyme preparations showed lower Ara and Xyl release [Laminex® super: 19 g $kg^{-1}$ DM (Ara) and 83 g $kg^{-1}$ DM (Xyl); Dyadic® Xylanase PLUS: 14 g $kg^{-1}$ DM (Ara) and 84 g $kg^{-1}$ DM (Xyl)]. As the *A. niger / T. emersonii* enzyme preparation showed high degrees of conversion, it was used to produce ErCS by the hydrolysis of WUS. Figure 1 shows Glc, Xyl and Ara released. The free monosaccharide-levels at the end of hydrolysis were 164 g $kg^{-1}$ DM (Glc), 136 g $kg^{-1}$ DM (Xyl) and 28 g $kg^{-1}$ DM (Ara).

Table 2: Compositional analysis of corn silage, WUS, ErCS and AFR [mass fraction (g $kg^{-1}$) of dry matter (DM)].

| | g $kg^{-1}$ | | | |
|---|---|---|---|---|
| | corn silage | WUS | ErCS | AFR |
| starch | 294 | 4 | 1 | 5 |
| non-starch carbohydrates: | | | | |
| Glc | 120 | 305 | 347 | 113 |
| Xyl | 118 | 236 | 227 | 75 |
| Ara | 27 | 43 | 29 | 16 |
| Gal | 8 | 12 | 9 | 6 |
| Man | 11 | 6 | 1 | 4 |
| Rha | 4 | 2 | 3 | 2 |
| UA | 18 | 28 | 17 | 15 |
| FA | 5 | 9 | 6 | not detected |
| pCA | 6 | 12 | 18 | 7 |
| acetic acid | 21 | 39 | 42 | 40 |
| protein | 75 | 42 | 33 | 305 |
| acid-insoluble lignin | 55 | 169 | 287 | 429 |
| acid-soluble lignin | 1 | <1 | <1 | <1 |
| acid-insoluble ash | 32 | 32 | 12 | 47 |
| lignin : non-starch | 0.2 | 0.3 | 0.5 | 1.9 |
| cellulose : hemicellulose | 0.6 | 0.8 | 1 | 0.7 |

Table 3: Degree of substitution (DS): mol substituent per mol of Xyl for corn silage, WUS, AFR and ErCS.

| | DS | | | |
|---|---|---|---|---|
| | corn silage | WUS | ErCS | AFR |
| $DS_{tot}$ [a] | 0.85 | 0.73 | 0.69 | 1.67 |
| $DS_{Ac}$ | 0.39 | 0.36 | 0.40 | 1.17 |
| $DS_{pCA}$ | 0.04 | 0.04 | 0.06 | 0.08 |
| $DS_{FA}$ | 0.03 | 0.03 | 0.02 | not detected |
| $DS_{UA}$ | 0.11 | 0.08 | 0.05 | 0.14 |
| $DS_{Ara}$ | 0.23 | 0.18 | 0.13 | 0.21 |
| $DS_{Gal}$ | 0.05 | 0.04 | 0.03 | 0.07 |

[a] total substituents: Ac, pCA, FA, UA, Ara, Gal

Monosaccharide release was significantly increased by blending the *A. niger* and *T. emersonii* preparations. Compared to hydrolysis with only the *A. niger* preparation (at the same total protein dosage, 100 mg $g^{-1}$ DM), blending lead to an increase in Glc release from 12 g $kg^{-1}$ DM to 164 g $kg^{-1}$ DM. Similar levels of released Xyl and Ara were obtained with the enzyme blend. The sole *T. emersonii* culture filtrate (protein dosage: 100 g $kg^{-1}$ DM), lead to a monosaccharide release of 90 g $kg^{-1}$ DM (Glc), 21 g $kg^{-1}$ DM (Xyl) and 2 g $kg^{-1}$ DM (Ara). The results described reveal a significant synergistic effect by the culture filtrates mixture. This is significant as enzyme optimization leading to higher conversion or reduced net protein requirements can

lower the costs of producing bulk enzyme mixtures for bioconversion. The mass fraction of oligosaccharides released from the total soluble sugars by hydrolysis with the *A. niger / T. emersonii* culture filtrates mixture was low (3.7 % after 72 h). This indicates the presence of enzymes being able to hydrolyze the majority of released oligosaccharides from WUS.
The *A. niger / T.emersonii* culture filtrates mixture is an efficient hemicellulolytic enzyme preparation leading to high degrees of conversion. Therefore treatment of corn silage-WUS with this mixture results in a residue that can be considered to be recalcitrant towards enzymatic hydrolysis ($w_{ErCS}$ = 465 g $kg^{-1}$, of the starting material WUS).

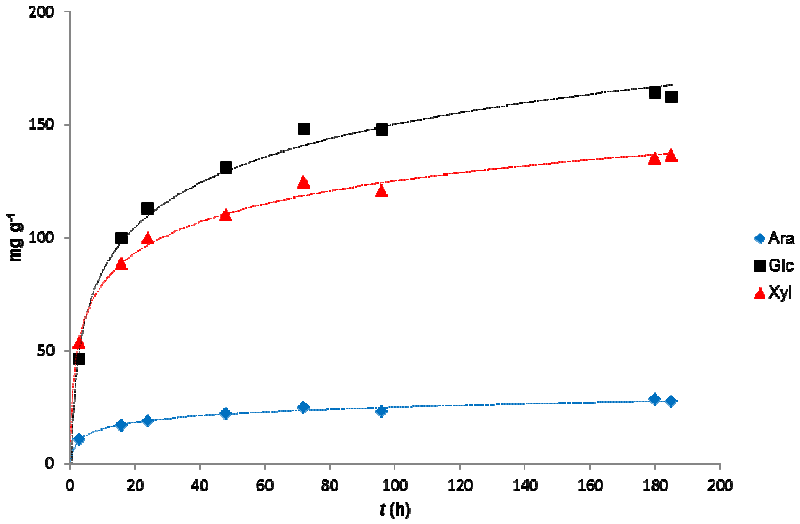


Figure 1: Glc, Xyl and Ara released (g $kg^{-1}$ DM) by hydrolysis of WUS with an *A. niger / T. emersonii* culture filtrates mixture.

*Composition of recalcitrant residues: ErCS and AFR*

The compositions of ErCS and AFR are shown in Table 2. Regarding ErCS, relatively high levels of non-starch carbohydrates were still present after enzymatic hydrolysis. As expected, lignin was clearly enriched ($w_{lignin}$ = 287 g $kg^{-1}$). It should be noted that lignin degrading enzymes such as laccases might have been inhibited by the antimicrobial growth agent present. Lignin may also contribute to feedstock recalcitrance (Rahikainen et al. 2011, Mauseth 1988). The DS was calculated based on the determined levels of xylan backbone substitution (Table 3). Regarding Ac and pCA, similar levels were obtained as for WUS. Other backbone substituents (Ara, Gal, FA, UA) were decreased by the *A. niger / T. emersonii* enzyme preparation. This indicates a high activity of the applied enzyme mixture towards major xylan backbone substituents.
Compositional analysis of AFR revealed a lower carbohydrate content than for ErCS (Table 2). A relatively high protein content was determined ($w_{protein}$ = 305 g $kg^{-1}$), most probably indicating the presence of bacterial protein originating from the anaerobic fermentation process. Based on the compositional data, a DS of 1.17, 0.21 and 0.14 (substituents per Xyl, Table 3) was

calculated for the three most dominant substituents Ac, Ara and UA, respectively. These levels were significantly higher than for ErCS (Table 3). AFR is an industrial residue and does not only contain corn derived- but also other (rye) residues. Corn xylan is a recalcitrant substrate due to complex xylan substitution (Appeldoorn et al. 2010) which is less present for rye (Pastell et al. 2009) and therefore may represent the major part of recalcitrant polysaccharides present. Regarding ErCS and AFR, substrate inaccessibility due to resistant plant cell wall architecture and the presence of hindering xylan substitution are envisaged to cause resistance to enzymatic hydrolysis. Xylan substitution, covalent linkage of the hemicellulose to lignin or the presence of proteins are relevant structural features determining recalcitrance properties of the substrate. The residual, recalcitrant polymers comprise a relevant amount of carbohydrates that could still be used for energy production. Therefore, they are a target for further enzyme screening.

**Screening of individual enzyme preparations**

(Hemi)cellulolytic enzyme preparations were assessed for the conversion of WUS, ErCS and AFR, representing substrates with increasing levels of recalcitrance. Enzyme preparations were screened at over-optimal and constant dosage (as described above, *Screening of multi-component enzyme preparations*). Application of a constant enzyme dosage is efficient for screening purposes (especially for large enzyme collections) and a suitable approach, as no direct relationship was observed between the amount of protein present and the enzyme activity, for a range of commercial enzyme preparations (Kabel et al. 2006). In addition, Kabel and colleagues reported in the same study that enzyme activity on natural substrates is not predicted by activities that were determined using standard assays. Application at over-optimal dosage has been described for various enzyme saccharification works (Selig et al. 2010, Wyman et al. 2005). Here we apply over-optimal dosage by screening different enzyme preparations on different substrates, which is intended to ensure that the enzyme activity of the respective preparation is maximal and not the limiting factor. Differences in substrate conversion are expected from different enzymes to be present in the preparations.

The heatmap in Figure 2 shows the released monosaccharide-levels as proportion of the theoretical yield. Under the screening conditions applied, a clear decrease in substrate conversion was observed from WUS to ErCS to AFR, in line with their expected recalcitrance. Some enzyme preparations were recognized to have high hydrolytic activity towards WUS (Figure 2, red) and also moderate to high activity towards the recalcitrant substrates ErCS (red, black) and AFR (black). Screened enzyme preparations with significant activity towards all three substrates were recognized (Optimase®, Dyadic® Xylanase PLUS, Ultraflo® L, Laminex® BG2, MethaPlus® L100). The ability to hydrolyze both, model polysaccharides (WUS) and recalcitrant substrates, indicates presence of enzymes for efficient hydrolysis of the carbohydrate backbone, while additional, necessary accessory activities are present.

Regarding AFR, a high structural complexity (Table 2) in addition to high pH values of the residue (which occur during the biogas formation process), presumably lead to low fermentable sugar release. This is relevant as enzymes with tolerance to high pH and at the same time activity towards xylan with a high degree of substitution, are advantageous for industrial processing applications of this residue. Enzyme preparations were recognized that are able to release additional Glc, Xyl and Ara from AFR (Figure 2a, AFR). The observed high Glc release from AFR (Figure 2a, enzyme 31) might be due to presence of particular GH61 classified proteins in this preparation (Pederson et al. 2011, Harris et al. 2010) by having an impact upon the number of entry sites for hydrolytic enzymes.

**Screening for synergy between an *A. niger / T. emersonii* mixture and commercial enzyme preparations**

Since the individual enzyme preparations do not contain all relevant enzymes at sufficient levels, a combinatorial use of enzyme preparations was assessed. The *A. niger / T. emersonii* culture filtrates mixture had shown high activity on WUS, comparable to conversion by industrially frequently used *state-of-the-art* enzyme preparations (Figure 2a). Its combinatorial application with commercial enzymes was assessed, to further increase substrate conversion. Combinatorial application allows recognition of enzyme preparations that contain important, accessory enzyme activities (usually not listed by the enzyme provider) which are active in synergy with the *A. niger / T. emersonii* culture filtrates mixture. This may result in increased degrees of degradation compared to hydrolysis by the individual enzyme preparations (Figure 2a) either by supplementing a limiting activity or by providing entirely new functionality.

Figure 2b shows hydrolysis levels obtained by blending the *A. niger / T. emersonii* culture filtrates mixture with the screened enzyme preparations. Clearly higher levels of Glc, Xyl and Ara released from WUS, ErCS and AFR were observed for several enzymes.

To evaluate the combinatorial use of the *A. niger / T. emersonii* mixture with the commercial preparations, values of released fermentable sugars were plotted, as described above, and are shown in Figure 3.

Enzyme preparations with synergistic key activities show high values on the Y-axis. These enzymes act in synergy with the *A. niger / T. emersonii* mixture.

Enzyme preparations showing high X-values and low Y-values do not contain enzymes that act in synergy with the *A. niger / T. emersonii* enzymes, in the sense that they do not show additional activity on top of the *A. niger / T. emersonii* culture filtrates mixture. These enzymes most likely target the same polysaccharide-fraction as the *A. niger / T. emersonii* mixture by the presence of overlapping enzyme activities.

Furthermore, enzyme preparations appear in the plots close to the 45° slope. These enzymes may show combinations of high synergy and overlapping individual enzyme activities with the

*A. niger / T. emersonii* mixture or hydrolyze parts that cannot be processed by the *A. niger / T. emersonii* enzyme preparation.
For each of the substrates, synergistically-acting enzymes were observed.

*Hydrolysis of WUS*
Regarding the hydrolysis of WUS, enzyme preparations can be identified which are likely to contain synergistic key activities (Figure 3). MethaPlus® L100 (enzyme 18) shows high levels of Xyl and Ara release when blended with the *A. niger / T. emersonii* culture filtrates mixture. Synergistic effects by accessory enzyme activities could lead to the observed increase in xylan backbone degradation. High levels for Xyl and Ara release along the 45° slope were found for Optimase® (enzyme 13).
As expected, the majority of the screened enzyme preparations show significant activities also without the *A. niger / T. emersonii* background, due to relatively low recalcitrance characteristics of WUS.
The highest monosaccharide levels were obtained with blends of the *A. niger / T. emersonii* culture filtrates mixture and [i] Metha Plus® L100 (enzyme 18) for Ara (31 g $kg^{-1}$ DM), [ii] Optimase® (enzyme 13) for Xyl (153 g $kg^{-1}$ DM) and [iii] Optimase® or Metha Plus® L100 for Glc released (223 g $kg^{-1}$ DM and 220 g $kg^{-1}$ DM respectively). The observed high conversion rates with *A. niger / T. emersonii* and Metha Plus® L100 or Optimase® demonstrate a strong activity of the identified blends for cellulose and xylan degradation. An efficient degradation of corn silage polysaccharides by a multi-component enzyme blend derived from *A. niger*, *T. emersonii* and *Trichoderma reesei* (teleomorph *Hypocrea jecorina*, Metha Plus® L100) can be recognized.

*Hydrolysis of ErCS*
Increased resistance towards enzymatic degradation was observed with ErCS. However, still considerably high amounts of monosaccharides were released by some enzyme preparations. Even though the *A. niger / T. emersonii* mixture itself showed only minor activity on ErCS (Figure 2, *A. niger / T. emersonii* culture filtrates mixture - background), synergy was observed with some screened enzymes. Dyadic® Cellulase PLUS (enzyme 30, Figure 3) showed synergy with the *A. niger / T. emersonii* preparation for Glc, Xyl as well as Ara release.
The majority of enzyme preparations that show activity on ErCS are found along the indicated 45° slope in Figure 3. These enzyme preparations can target structures which are not degraded by the *A. niger / T. emersonii* culture filtrates mixture, or represent combinations of high synergy and overlapping individual enzyme activities. Regarding the highest conversion levels of ErCS, different enzyme blends were identified for each sugar being: *A. niger / T. emersonii* culture filtrates mixture with [i] Ultraflo® L [enzyme 17; 17 g $kg^{-1}$ DM (Ara)], [ii] Dyadic® Xylanase PLUS [enzyme 1; 74 g $kg^{-1}$ DM (Xyl)] and [iii] Dyadic® Cellulase PLUS [enzyme 30; 285 g $kg^{-1}$ DM (Glc)].

Surprisingly, re-incubation of ErCS with the *A. niger / T. emersonii* mixture (which had also been used for the preparation of ErCS) resulted in additional fermentable sugar release [9 g $kg^{-1}$ DM (Glc), 26 g $kg^{-1}$ DM (Xyl),1 g $kg^{-1}$ DM (Ara)]. Plateau levels of Glc, Xyl and Ara had been reached with this enzyme mixture during ErCS preparation. An explanation for this observation could be an increased accessibility by physical changes of the substrate during freeze drying (Esteghlalian et al. 2001), or partial inactivation of the enzyme during hydrolysis and therefore incomplete degradation (Andric et al. 2010, Kristensen et al. 2009) [32, 33].

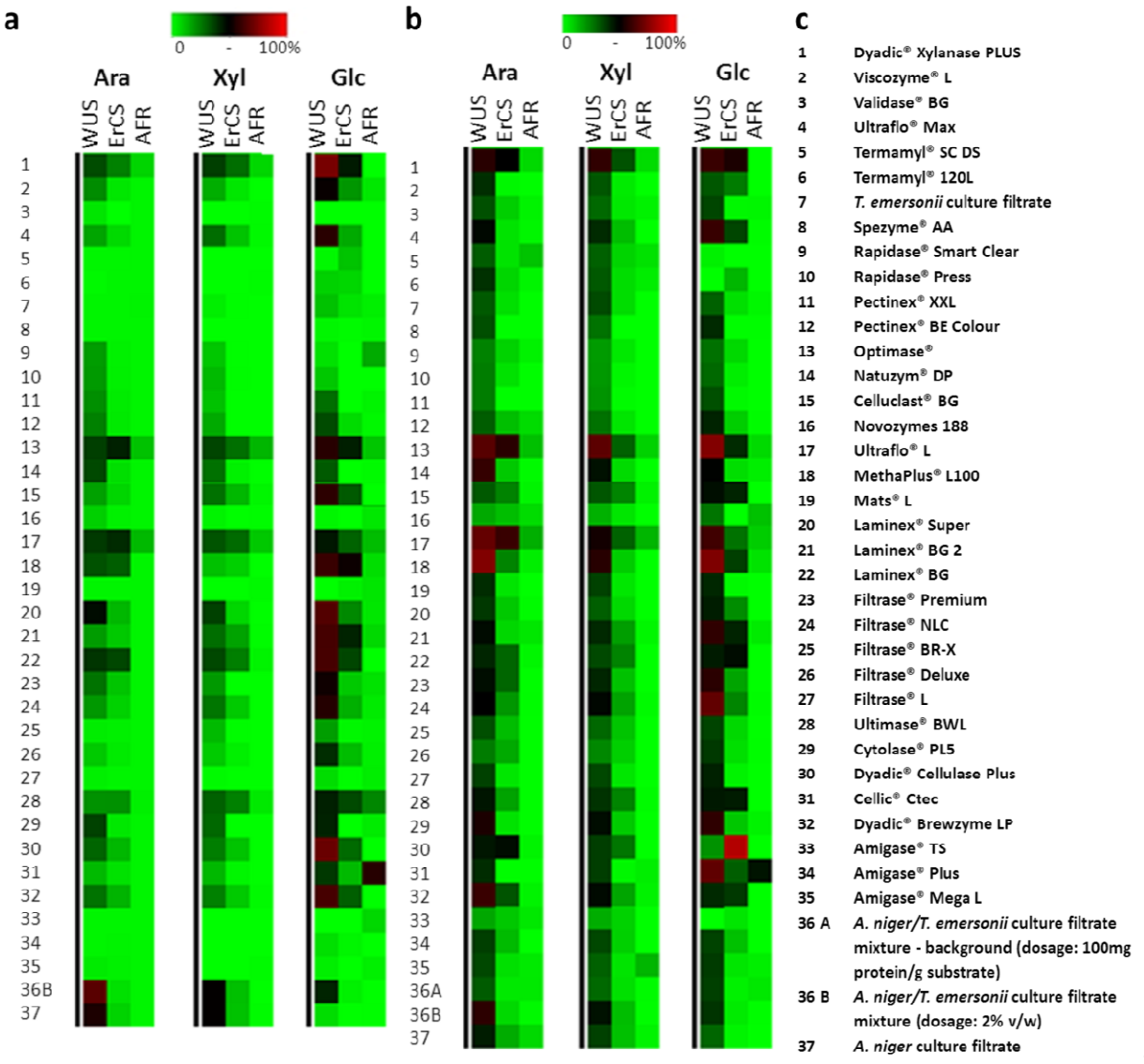


Figure 2: Heatmap of Ara, Xyl, Glc released (monosaccharide-levels as proportion of the theoretical yield based on compositional analysis) from WUS, ErCS and AFR by commercial and experimental enzyme preparations; (a) individual enzyme preparations; (b) individual enzyme preparations + *A. niger / T. emersonii* culture filtrates mixture [100 g $kg^{-1}$ (protein/substrate)]; (c) screened enzyme preparations.

*Hydrolysis of AFR*

In contrast to conversion of WUS and ErCS, the incubation of AFR resulted only in low to medium Glc, Xyl and Ara release. High resistance to enzymatic degradation was expected as discussed before. Therefore, the majority of enzymes showed no or only minor amounts of released fermentable sugars. However, we did observe individual and blended enzyme preparations, which were active towards this highly recalcitrant substrate. Synergy with the *A. niger / T. emersonii* mixture towards Ara and Xyl release was observed for Ultraflo® L (enzyme 17, Figure 3). This blend possessed enzymatic activity towards recalcitrant xylan and a required tolerance to high pH for further processing of this industrial residue.

For WUS, ErCS and AFR, enzyme blends were identified that outreach the individual commercial preparations and, therefore, provide a basis for the development of designed enzyme mixtures. These synergistic enzyme preparations have high activity on recalcitrant substrates and enable higher conversion of lignocellulosic, non-pretreated biomass. But even though hydrolysis was significantly increased, there is a need for additional activities to reach complete polysaccharide degradation as none of the blended enzyme preparations could completely degrade all cellulose and hemicellulose.

With increasing recalcitrance of the substrate less enzyme preparations showed presence of overlapping enzyme activities. Furthermore, less positive hits were found with increasing substrate recalcitrance. The highly substituted AFR showed only low substrate conversion by most screened enzymes. Therefore, a lack of specific enzymes towards highly substituted and recalcitrant polysaccharides is indicated, limiting efficient degradation of highly recalcitrant feedstocks.

Nevertheless, significant synergy of some enzyme preparations with the *A. niger / T. emersonii* culture filtrates mixture was found, resulting in increased conversion of WUS, ErCS and AFR. This suggests important enzymes to be present within the synergistically active enzyme preparations allowing increased hydrolysis of lignocellulosic, recalcitrant biomass.

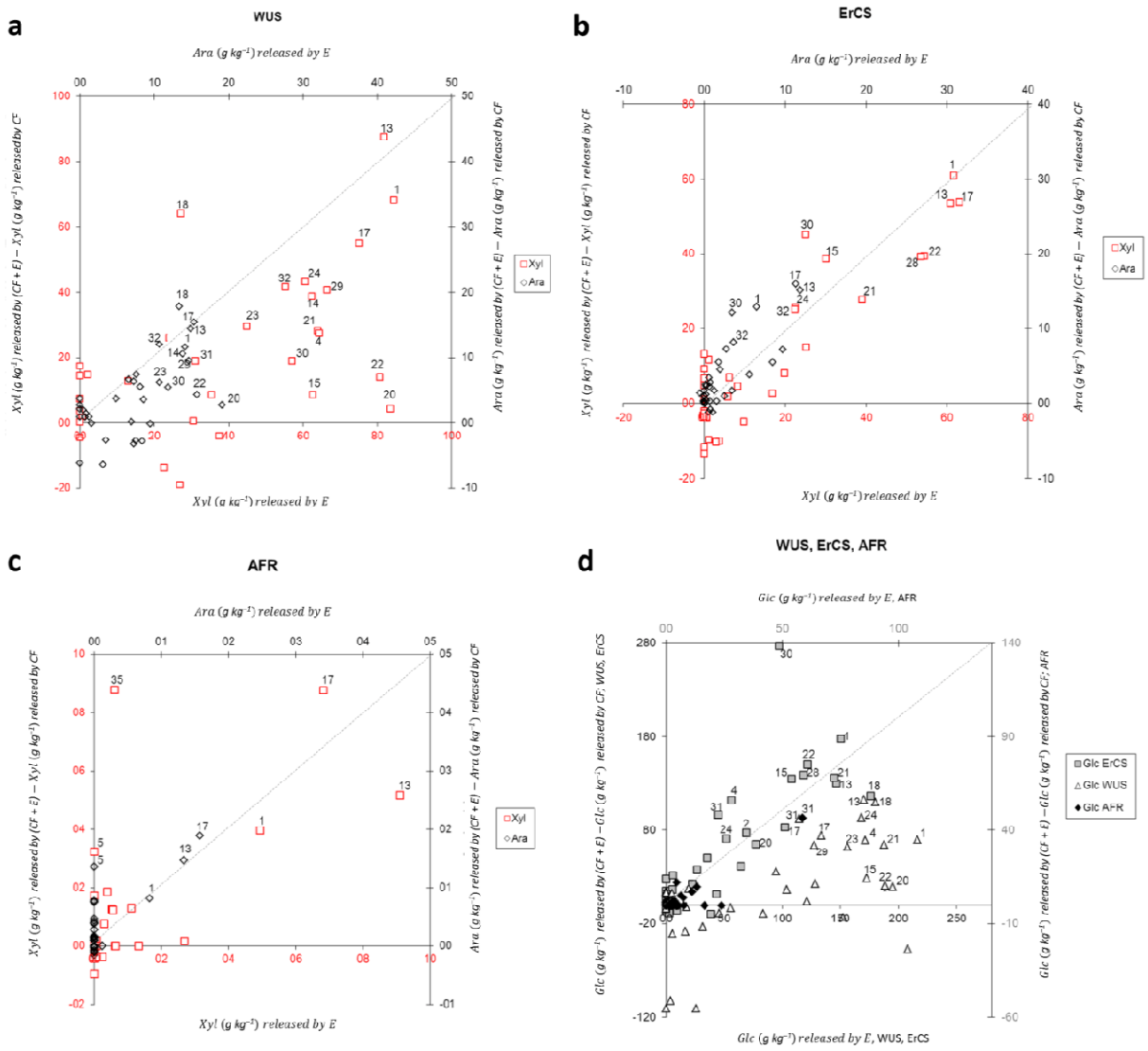


Figure 3: Data plots of fermentable sugars released (mg $g^{-1}$ DM): Y(axis): g $kg^{-1}$ DM (Glc, Xyl, Ara released by CF in combination with E) – g $kg^{-1}$ DM (Glc, Xyl, Ara released by sole application of CF); X(axis): g $kg^{-1}$ DM (Glc, Xyl, Ara released by E); CF = culture filtrates mixture (*A. niger / T. emersonii* enzyme mixture), E = screened enzyme preparation. The same number code was used for the enzyme preparations as in Figure 2. Different X, Y-axes were used for Ara and Xyl; (a) Ara, Xyl released (WUS); (b) Ara, Xyl released (ErCS); (c) Ara, Xyl released (AFR); (d) Glc released (WUS, ErCS, AFR).

## Conclusions

Increasing recalcitrance towards enzymatic degradation was observed for WUS, ErCS and AFR. Enrichment of certain substituents (Table 3) correlated with increasing recalcitrance characteristics. For AFR a high degree of acetylation (1.17), Ara (0.21) and UA (0.14)

substitution were determined. By enzyme screening towards degradation of WUS, ErCS and AFR, specific positive hits were identified for individual substrates only. Supplementation of an efficient (hemi)cellulolytic *A.niger / T.emersonii* mixture with certain commercial preparations resulted in increased conversion levels by enzyme synergy. The different positive hits obtained for substrates with increasing recalcitrance indicate that enzyme activities that may be underrepresented in the mixtures need to be adapted with proceeding hydrolysis to reach optimal conversion.

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# Supplementary information

## Synergistic action of enzyme preparations towards recalcitrant corn silage polysaccharides

Table S1: enzyme preparations (supplier, production strain, main activities). Side activities of several preparations are not available.

| enzyme preparation | supplier | strain | main activities |
|---|---|---|---|
| Natuzym® DP | Addfood | *fungal strain (unknown)* | unknown |
| Amigase® Mega L | DSM | *Aspergillus niger* | amyloglucosidase, side activities unknown |
| Amigase® Plus | DSM | *Aspergillus niger, Aspergillus oryzae* | amyloglucosidase, α-amylase, side activities unknown |
| Amigase® TS | DSM | *Aspergillus niger* | amyloglucosidase, side activities unknown |
| Arabanase | DSM | *Aspergillus niger* | cellulase, hemicellulase, side activities unkown |
| Brewers Filtrase® BXC | DSM | *Talaromyces emersonii* | cellulase, hemicellulase, side activities unkown |
| Filtrase® Deluxe | DSM | *Bacillus amyloliquefaciens / Trichoderma longibrachiatum* | β-glucanase, protease, side activities unknown |
| Filtrase® L | DSM | *Bacillus amyloliquefaciens* | β-glucanase, α-amylase, side activities unknown |
| Filtrase® NLC | DSM | *Talaromyces emersonii / Trichoderma longibrachiatum* | cellulase, xylanase, endoglucanase, side activities unknown |
| Filtrase® Premium | DSM | *Trichoderma reesei (teleomorph Hypocrea jecorina)* | β-glucanase, xylanase, side activities unknown |
| Mats® L | DSM | *Bacillus licheniformis* | β-glucanase, α-amylase, side activities unknown |
| MethaPlus® L100 | DSM | *Trichoderma reesei* | xylanase, β-glucanase, cellulase, side activities unknown |
| Rapidase® Press | DSM | *Aspergillus niger* | pectinase, β-glucanase, xylanase, side activities unknown |
| Rapidase® Smart Clear | DSM | *Aspergillus niger* | pectinase, side activities unknown |
| Validase® BG | DSM Valley Research | *Bacillus* | endo-glucanase,side activities unknown |
| Dyadic® Brewzyme LP | Dyadic | *Trichoderma longibrachiatum* | β-glucanase, (known side activities include: cellulase, xylanase, pectinase, mannanase, xyloglucanase, laminarase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, amylase, protease) |
| Dyadic® Cellulase PLUS | Dyadic | *Trichoderma longibrachiatum* | cellulase, (known side activities: β-glucanase, xylanase) |
| Dyadic® Xylanase PLUS | Dyadic | *Trichoderma longibrachiatum* | xylanase, (known side activities include: β-glucanase,cellulase, pectinase, mannanase, xyloglucanase, laminarase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, amylase, protease) |
| Laminex® BG | Genencor | *Trichoderma longibrachiatum* | β-glucanase, xylanase, side activities unknown |
| Laminex® BG2 | Genencor | *Trichoderma reesei* | β-glucanase, xylanase, side activities unknown |
| Laminex® Super | Genencor | *Trichoderma reesei / Penicillium funiculosum* | β-glucanase, xylanase, side activities unknown |
| Spezyme® AA | Genencor | *Bacillus licheniformis* | α-amylase, side activities unknown |
| Ultimase® BWL | Iogen | *Trichoderma reesei* | β-glucanase, xylanase, side activities unknown |
| Celluclast® BG | Novozymes | *Trichoderma reesei* | cellulase, side activities unknown |
| Cellic® Ctec | Novozymes | *Trichoderma reesei* | cellulase, side activities unknown |
| Novozyme 188 | Novozymes | *Aspergillus niger* | cellobiase, side activities unknown |
| Pectinex®BE Colour | Novozymes | *Aspergillus niger* | pectinase, side activities unknown |
| Pectinex® XXL | Novozymes | *Aspergillus niger, Aspergillus aculeatus* | pectinase, side activities unknown |
| Termamyl® 120L | Novozymes | *Bacillus licheniformis* | α-amylase, side activities unknown |
| Termamyl® SC DS | Novozymes | *Bacillus* | α-amylase, side activities unknown |
| Ultraflo® L | Novozymes | *Humicola insolens* | β-glucanase, xylanase, side activities unknown |
| Ultraflo® Max | Novozymes | *production strain unknown; produced by submerged fermentation GMOs* | β-glucanase, xylanase, side activities unknown |
| Viscozyme® L | Novozymes | *Aspergillus aculeatus* | cellulase, β-glucanase, hemicellulase, xylanase, arabanase, further side activities unknown |
| Optimase® | Schmack Viessman group | *Unknown* | cellulase, further side activities unknown |

Table S2: Ara, Xyl and Glc released (g $kg^{-1}$ DM) from WUS, ErCS and AFR by commercial and experimental enzyme preparations; (a) individual enzyme preparations; (b) individual enzyme preparations + *A. niger* / *T. emersonii* culture filtrates mixture.

**a**

**individual enzyme preparations**

monosaccharide yield (g $kg^{-1}$ DM)

| | **Ara** | | | **Xyl** | | | **Glc** | | |
|---|---|---|---|---|---|---|---|---|---|
| **enzyme preparations** | ***WUS*** | ***ErCS*** | ***AFR*** | ***WUS*** | ***ErCS*** | ***AFR*** | ***WUS*** | ***ErCS*** | ***AFR*** |
| Dyadic® Xylanase Plus | 13.9 | 6.5 | 0.8 | 84.4 | 61.7 | 4.9 | 216.1 | 150.1 | 0.0 |
| Viscozyme® L | 8.6 | 0.8 | 0.0 | 35.3 | 9.8 | 0.1 | 152.6 | 69.0 | 4.5 |
| Validase® BG | 1.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.6 | 0.0 | 0.0 | 0.0 |
| Ultraflo® Max | 6.6 | 1.5 | 0.0 | 64.4 | 25.1 | 0.6 | 171.4 | 55.9 | 0.0 |
| Termamyl® SC DS | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 3.8 | 38.5 | 0.0 |
| Termamyl® 120L | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 25.9 | 26.5 | 0.0 |
| *T. emersonii* culture filtrate | 0.0 | 0.0 | 0.1 | 6.8 | 0.7 | 0.0 | 35.3 | 17.4 | 3.1 |
| Spezyme® AA | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.3 | 2.7 | 1.7 |
| Rapidase® Smart Clear | 7.3 | 0.2 | 0.0 | 24.5 | 0.4 | 0.0 | 16.8 | 0.0 | 16.7 |
| Rapidase® Press | 7.5 | 0.3 | 0.0 | 30.4 | 1.1 | 0.0 | 31.8 | 0.0 | 0.0 |
| Pectinex® XXL | 8.4 | 0.5 | 0.0 | 26.8 | 0.0 | 0.0 | 83.2 | 0.0 | 2.2 |
| Pectinex® BE Colour | 9.5 | 0.6 | 0.0 | 37.4 | 0.0 | 0.0 | 103.8 | 22.8 | 1.4 |
| Optimase® | 14.9 | 11.9 | 1.3 | 81.9 | 60.8 | 9.1 | 170.2 | 146.7 | 11.3 |
| Natuzym® DP | 14.1 | 1.2 | 0.0 | 62.3 | 5.7 | 0.0 | 95.0 | 0.0 | 1.0 |
| Celluclast® BG | 6.9 | 1.8 | 0.0 | 62.6 | 30.1 | 1.1 | 172.9 | 108.2 | 0.0 |
| Novozymes 188 | 3.1 | 0.3 | 0.0 | 4.5 | 0.0 | 0.3 | 5.3 | 0.0 | 4.6 |
| Ultraflo® L | 15.4 | 11.3 | 1.6 | 75.2 | 63.0 | 6.8 | 133.8 | 102.2 | 13.3 |
| MethaPlus® L100 | 13.4 | 8.5 | 0.0 | 27.0 | 19.8 | 0.0 | 179.9 | 176.2 | 6.6 |
| Mats® L | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.6 | 0.0 | 9.4 | 4.9 |
| Laminex® Super | 19.1 | 3.4 | 0.0 | 83.5 | 16.8 | 0.0 | 195.2 | 77.4 | 4.7 |
| Laminex® BG2 | 7.3 | 2.5 | 0.0 | 63.9 | 39.0 | 0.3 | 187.6 | 144.7 | 7.7 |
| Laminex® BG | 15.7 | 9.7 | 0.0 | 80.6 | 54.3 | 1.3 | 188.2 | 121.6 | 0.0 |
| Filtrase® Premium | 10.7 | 2.7 | 0.0 | 44.8 | 0.0 | 0.0 | 156.1 | 35.5 | 5.5 |
| Filtrase® NLC | 7.6 | 2.0 | 0.0 | 60.5 | 22.5 | 0.1 | 168.2 | 51.9 | 4.0 |
| Filtrase® BR-X | 0.7 | 0.3 | 0.0 | 30.4 | 1.1 | 0.0 | 55.9 | 5.4 | 2.4 |
| Filtrase® Deluxe | 3.5 | 0.6 | 0.0 | 22.5 | 6.3 | 0.0 | 121.1 | 43.6 | 3.0 |
| Filtrase® L | 0.5 | 0.0 | 0.0 | 2.0 | 0.0 | 0.0 | 19.7 | 1.7 | 3.4 |
| Ultimase® BWL | 8.1 | 5.6 | 0.1 | 69.8 | 53.4 | 2.7 | 128.5 | 118.6 | 24.0 |
| Cytolase® PL5 | 14.7 | 0.7 | 0.0 | 66.4 | 0.0 | 0.0 | 127.6 | 0.0 | 3.8 |
| Dyadic® Cellulase Plus | 11.8 | 3.4 | 0.0 | 56.9 | 25.1 | 0.1 | 207.9 | 97.5 | 0.0 |
| CellicC® Tec | 4.9 | 1.1 | 0.0 | 31.1 | 3.7 | 0.0 | 114.7 | 44.9 | 58.8 |
| Dyadic® Brewzyme LP | 10.7 | 3.6 | 0.0 | 55.1 | 22.5 | 0.5 | 188.2 | 105.2 | 0.0 |
| Amigase® TS | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 7.8 |
| Amigase® Plus | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 17.6 | 6.0 | 0.0 |
| Amigase® Mega L | 0.9 | 0.2 | 0.0 | 0.0 | 0.0 | 0.6 | 9.1 | 0.0 | 3.0 |
| A. niger/T. emersonii mixture (2:1 v/w) | 27.5 | 1.1 | 0.0 | 114.3 | 26.6 | 0.0 | 126.1 | 9.0 | 0.1 |
| *A. niger* culture filtrate | 22.5 | 1.9 | 0.0 | 114.3 | 25.7 | 0.4 | 19.7 | 0.7 | 0.3 |

Table S2 (continued)

**b**

***A. niger/T. emersonii* mixture + enzyme preparations**

| | monosaccharide yield (g $kg^{-1}$ DM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Ara** | | | **Xyl** | | | **Glc** | | |
| **A. niger/T. emersonii mixture (background) + enzyme preparations** | ***WUS*** | ***ErCS*** | ***AFR*** | ***WUS*** | ***ErCS*** | ***AFR*** | ***WUS*** | ***ErCS*** | ***AFR*** |
| A. niger / T. emersonii mixture - background (100mg/g) | 13.1 | 1.1 | 0.5 | 66.2 | 13.3 | 0.6 | 110.0 | 9.0 | 4.6 |
| Dyadic® Xylanase Plus | 23.7 | 14.0 | 0.9 | 134.3 | 74.1 | 4.4 | 179.9 | 186.3 | 0.0 |
| Viscozyme® L | 16.7 | 0.3 | 0.0 | 74.7 | 8.5 | 0.0 | 97.0 | 86.1 | 0.0 |
| Validase® BG | 14.0 | 2.5 | 0.3 | 66.4 | 9.8 | 1.6 | 106.4 | 5.4 | 1.7 |
| Ultraflo® Max | 19.8 | 1.4 | 0.2 | 93.6 | 28.3 | 0.0 | 179.3 | 121.3 | 0.6 |
| Termamyl® SC DS | 13.0 | 1.4 | 1.5 | 69.8 | 18.3 | 3.6 | 7.9 | 0.0 | 0.0 |
| Termamyl® 120L | 16.4 | 2.2 | 0.2 | 73.4 | 14.4 | 2.2 | 0.0 | 46.9 | 0.0 |
| *T. emersonii* culture filtrate | 13.2 | 1.6 | 0.0 | 79.2 | 14.4 | 0.0 | 91.1 | 20.1 | 1.5 |
| Spezyme® AA | 14.1 | 0.0 | 0.0 | 61.7 | 0.0 | 0.0 | 122.9 | 1.0 | 0.3 |
| Rapidase® Smart Clear | 9.9 | 2.3 | 0.3 | 44.1 | 9.4 | 0.0 | 82.0 | 24.8 | 0.0 |
| Rapidase® Press | 10.4 | 1.5 | 0.0 | 44.8 | 3.7 | 0.0 | 86.4 | 11.4 | 0.0 |
| Pectinex® XXL | 10.3 | 0.0 | 0.0 | 47.3 | 1.7 | 0.0 | 101.1 | 4.0 | 0.0 |
| Pectinex® BE Colour | 13.0 | 3.4 | 0.9 | 62.1 | 10.7 | 0.0 | 127.0 | 31.8 | 0.5 |
| Optimase® | 27.6 | 16.3 | 1.6 | 153.5 | 66.9 | 5.6 | 222.9 | 139.0 | 7.4 |
| Natuzym® DP | 24.7 | 2.9 | 0.1 | 104.9 | 15.3 | 0.0 | 145.5 | 21.4 | 3.3 |
| Celluclast® BG | 13.3 | 6.7 | 0.2 | 74.5 | 51.9 | 1.7 | 138.8 | 144.1 | 0.0 |
| Novozymes 188 | 6.8 | 3.5 | 0.9 | 34.2 | 10.7 | 0.0 | 79.4 | 0.0 | 12.2 |
| Ultraflo® L | 28.5 | 17.1 | 2.0 | 121.3 | 67.1 | 9.2 | 184.6 | 92.5 | 10.0 |
| MethaPlus® L100 | 30.9 | 6.7 | 0.4 | 130.1 | 21.4 | 0.0 | 220.2 | 126.0 | 5.5 |
| Mats® L | 16.9 | 1.3 | 0.0 | 83.5 | 10.0 | 0.0 | 124.7 | 3.7 | 0.0 |
| Laminex® Super | 15.9 | 2.9 | 0.1 | 70.4 | 16.1 | 0.0 | 129.9 | 74.4 | 0.0 |
| Laminex® BG2 | 19.5 | 2.1 | 0.6 | 94.3 | 41.2 | 1.2 | 174.9 | 144.7 | 4.1 |
| Laminex® BG | 17.5 | 8.3 | 0.2 | 80.1 | 52.8 | 0.0 | 131.1 | 159.5 | 0.0 |
| Filtrase® Premium | 19.3 | 8.4 | 0.0 | 95.6 | 26.6 | 0.0 | 172.0 | 59.6 | 0.0 |
| Filtrase® NLC | 20.5 | 5.6 | 0.0 | 109.4 | 39.0 | 0.6 | 203.4 | 80.1 | 0.5 |
| Filtrase® BR-X | 14.1 | 3.5 | 0.0 | 66.6 | 25.1 | 0.0 | 107.6 | 25.8 | 0.0 |
| Filtrase® Deluxe | 10.5 | 4.6 | 0.0 | 52.2 | 20.3 | 0.0 | 114.1 | 21.1 | 0.3 |
| Filtrase® L | 15.2 | 1.2 | 0.0 | 80.8 | 10.0 | 0.0 | 127.9 | 8.0 | 0.0 |
| Ultimase® BWL | 18.7 | 5.0 | 0.0 | 89.8 | 52.5 | 0.5 | 132.9 | 148.7 | 0.0 |
| Cytolase® PL5 | 22.7 | 1.5 | 0.0 | 106.7 | 22.7 | 0.0 | 174.3 | 36.9 | 2.3 |
| Dyadic® Cellulase Plus | 18.6 | 13.3 | 0.2 | 85.1 | 58.4 | 0.0 | 62.9 | 285.8 | 0.0 |
| Cellic® CTec | 16.9 | 0.0 | 0.0 | 85.1 | 3.3 | 2.1 | 202.6 | 105.5 | 46.9 |
| Dyadic® Brewzyme LP | 25.2 | 9.3 | 0.3 | 107.8 | 38.4 | 1.7 | 123.2 | 130.0 | 0.0 |
| Amigase® TS | 6.9 | 2.9 | 0.5 | 36.0 | 19.0 | 0.1 | 0.0 | 16.1 | 0.0 |
| Amigase® Plus | 15.3 | 3.7 | 0.0 | 80.6 | 20.1 | 0.0 | 108.2 | 40.9 | 0.0 |
| Amigase® Mega L | 14.6 | 1.6 | 0.5 | 69.8 | 11.3 | 9.2 | 112.9 | 16.4 | 3.2 |
| *A. niger/T. emersonii* mixture (2:1 v/w) | 24.0 | 2.0 | 0.1 | 104.6 | 28.3 | 0.4 | 117.0 | 13.1 | 0.3 |
| *A. niger* culture filtrate | 18.2 | 4.5 | 0.9 | 83.9 | 27.0 | 1.5 | 88.2 | 7.0 | 0.2 |

# *CHAPTER 5*

# *Trichoderma longibrachiatum* acetyl xylan esterase 1 enhances hemicellulolytic preparations to degrade corn silage polysaccharides


## Abstract

Supplementation of a *Trichoderma longibrachiatum* preparation to an industrial *Aspergillus niger / Talaromyces emersonii* enzyme mixture demonstrated synergy for the saccharification of corn silage water-unextractable solids (WUS). Sub-fractions of the crude *T. longibrachiatum* preparation obtained after chromatography were analyzed regarding their hydrolytic activity. An acetyl xylan esterase 1 [Axe1, carbohydrate esterase (CE) family 5]-enriched sub-fraction closely mimicked the hydrolytic gain as obtained by supplementation of the complete, crude enzyme mixture (increase of 50, 62 and 29 % for Xyl, Ara and Glc, respectively). The acetic acid released from model polysaccharides (WUS) and oligosaccharides [neutral (AcXOS) and acidic (AcUXOS) xylo-oligosaccharides] by Axe1 was two and up to six times higher compared to the acetic acid released by acetyl xylan esterase A (AxeA, CE 1). Characterization of Axe1 treated AcXOS and AcUXOS revealed deacetylation of oligosaccharides that were not deacetylated by AxeA or the *A. niger / T. emersonii* preparation.



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## Introduction

Lignocellulosic feedstocks are a valuable renewable resource for biofuel production. The efficient utilization of lignocellulose is dependent on innovative processes, like enzymatic hydrolysis of polysaccharides. Both, C5 and C6 monosaccharides released are used for the production of 2$^{nd}$ generation biofuels by aerobic or anaerobic fermentation. Nonetheless, bioconversion is limited by feedstock recalcitrance. Corn fiber xylan has been reported to show resistance towards enzymatic hydrolysis following mild acid pretreatment due to presence of xylan substituents, like ferulic acid, diferulates, acetic acid, galactose, arabinose and uronic acid (Appeldoorn et al. 2010). These substituents differ among various xylan rich plant feedstocks and their relative amounts and distribution over the backbone have an impact on the recalcitrance towards enzymatic conversion (Neumüller et al. 2013).

Enzyme mixtures are required to remove xylan substituents from poly- and oligosaccharides (Kumar et al. 2008) enabling efficient degradation of the (1 → 4)-β-D-xylan backbone by β-1,4-endo-xylanases and β-1,4-xylosidases. Necessary accessory activities are α-arabinofuranosidases, arabinoxylan arabinofuranohydrolases, α-glucuronidases, α-1,4-galactosidases, β-1,4- galactosidases, feruloyl/p-coumaroyl esterases and acetyl xylan esterases. The latter are classified into sixteen structurally related carbohydrate esterase (CE) families (Carbohydrate-active enzymes database, www.cazy.org) and they are necessary enzymes for the degradation of acetylated polysaccharides. A high degree of acetylation (1 mol acetyl/ mol xylose) has been reported for an anaerobic corn silage residue that demonstrated high resistance against a range of commercial enzyme preparations (Neumüller et al. 2014). Thus, there may be a relation between the degree of acetylation and recalcitrance, which also suggests that available hemicellulolytic enzyme preparations are relatively deficient in such esterase activities. As the enzymatic hydrolysis process is still an impediment for achieving high yields from lignocellulosic material (Modenbach and Nokes 2013), the development of efficient enzyme preparations is crucial. Improved enzymatic hydrolysis of lignocellulosic biomass would reduce the negative impacts of harsh physical and chemical pretreatments (Blanch et al. 2011, Horn et al. 2011). Industrial microbial sources for hemicellulolytic preparations that produce appropriate levels of xylan degrading enzymes belong to genera such as *Aspergillus*, *Talaromyces* and *Trichoderma*. Nevertheless, many more sources are known (Adney et al. 1991, Dashtban et al. 2009, Das et al. 2007). Furthermore, the discovery of additional hemicellulolytic organisms is a topic of intensive research (Van Gool et al. 2011, 2012). Although common industrially used organisms secrete already a broad range of plant cell wall degrading enzymes (Waters et al. 2011, Ferreira de Oliveira 2011), no organism produces all the necessary enzymes in sufficient quantities (Kumar et al. 2008).

It has been reported that combinatorial application of industrially used enzyme preparations has synergistic potential (Neumüller et al. 2014). The same study showed significant hydrolytic

increase for the degradation of corn silage fractions by supplementation of a crude *T. longibrachiatum* enzyme preparation to an *A. niger / T. emersonii* enzyme mixture at saturated dosages.

In the present study the cause of the hydrolytic increase of this enzyme mixture for the hydrolysis of corn fractions is investigated by supplementation of purified fractions of the *T. longibrachiatum* enzyme preparation to the industrial *A. niger / T. emersonii* enzyme mixture. The synergistic potential of an acetyl xylan esterase 1 (Axe1, CE5)-rich fraction is presented. Deacetylation by Axe1 is demonstrated and compared to deacetylation by acetyl xylan esterase A (AxeA, CE1) from industrially used *A. niger*.

## Materials and methods

### Materials

*Substrates:* Corn silage water unextractable solids (WUS) and a corn silage based anaerobic fermentation residue (AFR) as used have been described previously (Neumüller et al. 2014, Table 1). A soluble extract of corn silage (cs soluble) was prepared by stirring corn silage in sodium citrate buffer (20 mM, pH 5, 200 g $L^{-1}$) at 700 rpm for 2 h at room temperature (RT). The suspension was centrifuged (3200 g, 30 min, RT) and the supernatant [cs soluble; dry matter (DM) 13 g $L^{-1}$] was collected. *Eucalyptus globulus* xylan hydrolysate (EXH) was kindly donated by Prof. Dr. J.C. Parajo of the University of Vigo-Ourense, Spain (Gullón et al., 2008). The production of neutral (AcXOS) and acidic (AcUXOS) fractions of eucalyptus oligosaccharides from EXH and their composition has been described previously (Koutaniemi et al. 2013, Table 1).

Table 1: Composition of corn fractions (WUS, AFR; Neumüller et al. 2014) and eucalyptus-derived xylo-oligosaccharides (AcXOS, AcUXOS, Koutaniemi et al. 2013).

| | Content[a] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| sample | starch | non-starch Glc | Xyl | Ara | Gal | Man | Rha | uronic acid | ferulic acid | *p*-coumaric acid | acetic acid | protein | acid-insoluble lignin | acid-soluble lignin | acid-insoluble ash | MeGlcA |
| WUS | 40 | 305 | 236 | 43 | 12 | 6 | 2 | 28 | 9 | 12 | 39 | 42 | 169 | <1 | 32 | n.d. |
| AFR | 5 | 113 | 75 | 16 | 6 | 4 | 2 | 15 | not detected | 7 | 40 | 305 | 429 | <1 | 47 | n.d. |
| AcXOS | n.d. | 93 | 803 | 18 | 75 | 62 | n.d. | n.d. | n.d. | n.d. | 117 | n.d. | n.d. | n.d. | n.d. | not detected |
| AcUXOS | n.d. | not detected | 765 | 0 | 42 | traces | n.d. | n.d. | n.d. | n.d. | 138 | n.d. | n.d. | n.d. | n.d. | 207 |

n.d., not determined.
[a] Results expressed as mg $g^{-1}$ dry matter (DM).

*Enzyme preparations:* Enzyme preparations from non-GMO production strains were used: *T. longibrachiatum* culture filtrate [Dyadic® Xylanase Plus (XP, Dyadic-NL, Wageningen, The Netherlands)], *A. niger* and *T. emersonii* culture filtrates [Brewers Filtrase BXC (*T. emersonii*, DSM Heerlen, The Netherlands) and DSM-Arabanase (*A. niger*, DSM)]. The *A. niger* and *T. emersonii* enzyme preparations were stored in a glycerol solution (450 g $kg^{-1}$; Neumüller et al. 2014). The *A. niger / T. emersonii* culture filtrates mixture results in comparable substrate

conversion of corn feedstocks as other, commercially available *state-of-the-art* enzyme preparations (Neumüller et al. 2014).

**Isolation of acetyl esterases**

*T. longibrachiatum*

*Anion exchange chromatography (AEC):* Fractionation of the culture filtrate was done at 4 °C. Each enzyme preparation was diluted 1:10 (v/v) in Tris-Hydrochloride (Tris-HCl, 100 mM, pH 8.0) followed by ultrafiltration [Amicon centrifugal filter units, 10 kDa molecular weight (MW) cut-off, 3200 g, 15-30 min, Merck-Millipore, Billerica, MA, USA]. The retentate was filled up to 10 mL (Tris-HCl, 100 mM, 5 mS $cm^{-1}$, pH 8.0) and applied onto an AEC column (Q-Sepharose XL 17-5072-01, GE Healthcare, Pittsburgh, PA, USA) connected to a liquid chromatography system (ÄKTA explorer, GE Healthcare). Equilibration of the column was done at a flow rate of 2 mL $min^{-1}$ with Tris-HCl (100 mM,pH 8.0). The elution of enzymes was performed by using a linear gradient from 0 to 0.5 M NaCl in the same buffer at 2 mL $min^{-1}$ and was monitored by measuring the adsorption at 280 nm. Fractions of 5 mL were collected and the pH was lowered immediately to pH 5.0 by addition of 5 mL of sodium acetate buffer (250 mM, pH 4.0). The fractions belonging to the same peaks on the basis of the chromatogram obtained, were pooled (XP1 to XP7, Fig. 2). After ultrafiltration of each fraction (10 kDa MW cut-off, 3200 g, 30 min, 4 °C), the retentate was filled to 0.5 mL with sodium acetate buffer (100 mM, pH 5.0). Aliquots were taken from each fraction obtained and protein contents were determined [Coomassie Plus (Bradford Assay) Kit, Sigma Aldrich, St. Louis, MO, USA].

*Size exclusion chromatography (SEC):* After ultrafiltration (10 kDa MW cut-off, 3200 g, 15-30 min, 4 °C) of the selected sample after AEC fractionation (XP1), the retentate was filled to 2 mL with 0.05 M sodium phosphate buffer containing 0.15 M NaCl (pH 5.0). The sample was applied onto a SEC column (Superdex 200 10/300 GL, GE Healthcare) which has been pre-equilibrated with 0.05 M sodium phosphate buffer containing 0.15 M NaCl (pH 5.0). SEC was performed at 4 °C. Elution of the enzymes was done at 0.4 mL $min^{-1}$ with the same buffer. Fractions of 1 mL were collected and the fractions (XP1-1 to XP1-9, Figure 2) were concentrated by ultrafiltration (10 kDa MW cut-off, 3200 g, 15 min, 4 °C). The protein contents were determined with the Coomassie Plus (Bradford Assay) Kit (Sigma Aldrich).

*A. niger*

AxeA was the only acetyl xylan esterase present in the crude *A. niger* culture filtrate as observed by mass spectrometry (data not shown) performed according to the procedure described below (*identification of proteins by liquid chromatography/mass spectrometry*). The crude *A. niger* preparation was fractionated by AEC and SEC according to the procedure described above. Presence of the purified acetyl xylan esterase was confirmed by the hydrolysis

of *p*-nitrophenyl acetate (90 IU). The calculated molecular mass of 33 kDa based on the protein sequence of AxeA matched with the protein band visible by the SDS-PAGE analysis.

**Enzyme assays**

*Synergy assay:* AEC and SEC purified enzyme fractions were applied at a total protein loading of (10 g protein $kg^{-1}$ substrate) in combination with the *A. niger / T. emersonii* culture filtrates mixture, which was applied at 100 g protein $kg^{-1}$ substrate, to incubate WUS. Higher concentrations of the *A. niger / T. emersonii* culture filtrates mixture did not result in increased monosaccharide release (data not shown). Incubations of WUS (10 g $L^{-1}$) were done at 1 mL scale using a Thermomixer comfort (Eppendorf, Hamburg, Germany) at 50 °C and 300 rpm for 48 h in sodium acetate buffer (100 mM, pH 5.0, 0.5 g $kg^{-1}$ sodium azide). After incubation, the samples were heat-inactivated (98 °C for 10 min), centrifuged (20.000 g, 10 min, RT) and analyzed for monosaccharides and oligosaccharides released.

*p-Nitrophenyl acetate assay:* Enzyme assays with *p*-nitrophenyl acetate, 4-nitrophenyl xylopyranoside and 4-nitrophenyl glucopyranoside (Sigma Aldrich) were carried out at a substrate concentration of 1 mM (sodium acetate buffer, 100 mM, pH 5.0) at 40 °C at a 300 µl scale in a 96 well plate. After addition of 0.1 mg enzyme the *p*-nitrophenol release was determined by continuous measurement for 15 min at 405 nm using an Infinite M1000 PRO microplate reader (Tecan, Männedorf, Switzerland). A calibration curve was prepared with *p*-nitrophenol standard solution (0.1 to 2 mM; Sigma Aldrich).

*Determination of acetic acid release:* WUS, AFR (10 g $L^{-1}$), acetylated eucalyptus oligosaccharides (5 g $L^{-1}$) and cs soluble (13 g $L^{-1}$) were incubated with the purified, acetyl xylan esterase rich enzyme fractions Axe1 (XP1-4) or AxeA (10 g protein $kg^{-1}$ substrate) at 50 °C for 24 h in sodium citrate buffer (50 mM, pH 5.0, containing 0.5 g $kg^{-1}$ sodium azide). The samples were heat-inactivated (98 °C for 10 min) and centrifuged (20.000 g, 10 min, RT). The acetic acid released was analyzed on an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) using an Aminex HPX 87H (Bio-Rad laboratories, Hercules, CA, USA) and AG 50W-X4 (Bio-Rad) guard column and RI detection. Elution was done with 0.005 M $H_2SO_4$ at a flow rate of 0.6 mL $min^{-1}$ at 40 °C.

*Adsorption of enzymes:* The insoluble substrates WUS and AFR (10 g $kg^{-1}$) were incubated with enzymes (Axe1 and AxeA) at 20 g enzyme $kg^{-1}$ substrate in sodium phosphate buffer (50 mM, pH 5.0, containing 0.5 g $kg^{-1}$ sodium azide) at 50 °C and 700 rpm for 48 h. Samples were taken at the start of the hydrolysis and after 48 h. The samples were centrifuged (20.000 g, 10 min, RT) and the unbound proteins remaining in the supernatant where analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Electrophoresis**

SDS-PAGE was performed by using a NuPAGE 10 % Bis-Tris gel (Life Technologies, Carlsbad, CA, USA) on a Power Ease 500 system (Life Technologies). Staining of protein bands was performed with the Instant Blue protein gel stain (Expedeon, San Diego, CA, USA). Mark12 and SeaBlue Plus2 (Life Technologies) were used as protein markers. Quantification of the protein bands obtained was performed with the software ImageJ, developed at the National Institute of Health (Bethesda, MD, USA).

**Analysis of mono- and oligosaccharides**

Monosaccharides and oligosaccharides released by enzymatic hydrolysis were analyzed by HPAEC as described elsewhere (Neumüller et al. 2014).The concentration of oligosaccharides released was quantified based on the response factor determined for a xylotetraose standard (Sigma-Aldrich; Van Gool et al. 2012).

**Identification of proteins by liquid chromatography/mass spectrometry (LC/MS)**

The sample (XP1-4) was heated to 95 °C for 10 min. After cooling the sample to 50 °C, ammonium bicarbonate was added (100 mM). The pH of the sample was 7.5. An aliquot was taken to perform an SDS-PAGE analysis. Dithiothreitol solution was added to the remaining sample to a final concentration of 12.5 mM, followed by an incubation step at 70 °C for 30 min. Iodoacetamide (5 mM) was added to the sample at RT as an alkylating agent followed by incubation for 30 min at 22 °C and 1000 rpm in the dark. Dithiothreitol solution was added again to the sample to a concentration of 12.5 mM, followed by incubation at RT for 15 min. Tryptic digestion (12.5 µg $mL^{-1}$) was done overnight at 37 °C. After addition of formic acid (2.5 % v/v), the sample was analyzed by LC/MS.

LC/MS analysis was done with an LTQ Orbitrap Velos Hybrid FT Mass Spectrometer coupled to an Accela system (Thermo Fisher Scientific, Waltham, MA, USA) at the DSM Analytical Department (Delft, The Netherlands) as principally described by Henne et al. (2012). The Swiss-Prot (http://www.expasy.org) protein databank was used for protein/peptide searches. The share of spectrum ID's (as displayed in Figure 3) was calculated with the ProteinProphet software (Nesvizhskii et al. 2003).

**Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Maldi-Tof MS) of oligosaccharides**

Dowex AG 50W-X8 (Bio-Rad) was added to the samples to remove interfering salts. 2 µL 2,5-dihydroxybenzoic acid (10 g $L^{-1}$) and 1 µL sample were transferred on a steel target plate (Bruker Daltonics, Bremen, Germany) and dried under hot air. Maldi-Tof MS was performed on a Ultraflextreme workstation (Bruker Daltonics) equipped with a 337 nm laser and controlled using FlexControl software according to the procedure described previously (Van Gool 2012).

Calibration was done with a mixture of maltodextrins (AVEBE, Veendam, The Netherlands; mass range 500–3500 Da).

## Results and Discussion

### Identification and characterization of a synergistic fraction of *T. longibrachiatum*

*Hydrolysis of WUS by A. niger / T. emersonii and T. longibrachiatum culture filtrates*

In order to investigate the potential for substrate conversion by combined application of a *T. longibrachiatum* with the *state-of-the-art*, industrial *A. niger / T. emersonii* enzyme preparation, WUS was incubated and the monosaccharides released were quantified (Figure 1, section a). The *A. niger / T. emersonii* and the *T. longibrachiatum* enzyme preparation were applied individually and in combination at saturated dosages [100 g $kg^{-1}$ (*A. niger / T. emersonii* preparation), 300 g $kg^{-1}$ (*T. longibrachiatum* preparation); higher concentrations of the enzyme preparations did not result in increased monosaccharide release, data not shown)], as previously described (Neumüller et al. 2014). The monosaccharide levels obtained by incubation with the individual enzyme preparations were lower than the monosaccharide level obtained by supplementation of a saturated dosage of the *T. longibrachiatum* to the *A. niger / T. emersonii* preparation (Figure 1 section a), confirming recently published findings (Neumüller et al. 2014). The total fermentable sugar level (sum of Glc, Xyl and Ara) observed by combined application was 304 mg $g^{-1}$ compared to 201 and 147 mg $g^{-1}$ by sole application of the *A. niger / T. emersonii* and the *T. longibrachiatum* preparation, respectively.

### Fractionation and characterization of a crude *T. longibrachiatum* preparation

In order to investigate if either individual fractions of the *T. longibrachiatum* preparation or all activities present in the commercial preparation are necessary to obtain optimal conversion levels, chromatographic fractionation of the enzyme preparation was performed.

*AEC fractionation*

Fractionation of the crude *T. longibrachiatum* preparation was obtained by anion exchange chromatography (AEC). Cation- exchange chromatography (CEC) and hydrophobic interaction chromatography (HIC) did not result in satisfactory fractionation of the crude preparation under conditions commonly used for protein separation (GE Healthcare Bio-Sciences AB 2010; data not shown). AEC was optimized in order to separate the enzyme mixture. A substantial part of the proteins present in the enzyme preparation showed no or only weak binding to the resin and eluted before the NaCl gradient (Figure 2a). Elution of the enzymes bound to the resin occurred up to 0.4 M NaCl. The fractions obtained were pooled in seven fractions (XP1 to XP7, Figure 2a). Protein composition, as analyzed by SDS-PAGE, differed between the fractions (Figure 2b). Following AEC separation, each fraction (10 g $kg^{-1}$ substrate) was supplemented to

the *A. niger / T. emersonii* preparation (100 g $kg^{-1}$ substrate) and incubated with WUS. The monosaccharides released were quantified (Figure 1, section b). Fraction XP1 demonstrated a significant increase in xylan conversion (increase of 34 mg $g^{-1}$ for Xyl) compared to the other AEC purified enzyme fractions (Figure 1, section b). The increased hydrolytic activity for the conversion of the xylan present in WUS by supplementation of fraction XP1 to the *A. niger / T. emersonii* preparation indicates enrichment of synergistically active enzymes in fraction XP1.

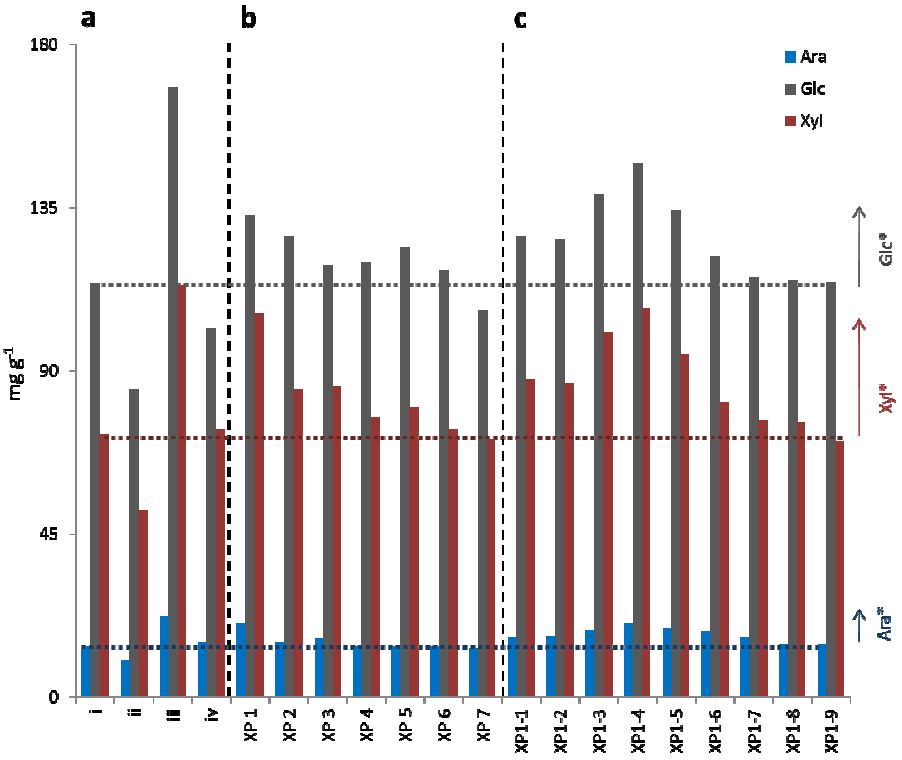


Figure 1: Monosaccharides (Glc, Xyl, Ara) released (mg $g^{-1}$, mean values of duplicates) by incubation with (a) crude enzyme preparations [i. *A. niger / T. emersonii* prep. (100 g $kg^{-1}$), ii. *T. longibrachiatum* prep. (300 g $kg^{-1}$), iii. *A. niger / T. emersonii* prep. (100 g $kg^{-1}$) plus *T. longibrachiatum* prep. (300 g $kg^{-1}$), iv. *A. niger / T. emersonii* prep. (100 g $kg^{-1}$) plus *T. longibrachiatum* prep. (10 g $kg^{-1}$)], (b) the *A. niger / T. emersonii* prep. (100 g $kg^{-1}$) plus AEC fractionated *T. longibrachiatum* prep. [fractions XP1 to XP7 (10 g $kg^{-1}$)], (c) the *A. niger / T. emersonii* prep. (100 g $kg^{-1}$) plus SEC fractionated XP1 [fractions XP1-1 to XP1-9 (10 g $kg^{-1}$)].

* hydrolytic gain for monosaccharides released by supplementation of enzyme fractions to the *A. niger / T. emersonii* preparation. The lines (blue, red, grey) mark the monosaccharide levels released by sole application of the *A. niger / T. emersonii* preparation.

### *SEC fractionation*

Further separation of the proteins present in XP1 was performed by SEC (Figure 2c). Figure 2d shows the SDS-PAGE analysis of the fractions obtained. SDS-PAGE analysis revealed a correlation of the hydrolytic activity obtained by supplementation of the SEC fractions to the

*A. niger / T. emersonii* preparation (Figure 1, section c) with a protein band at 34 kDa. This protein band was in abundance in fraction XP1-4. The monosaccharide levels obtained were also highest by supplementation of fraction XP1-4 to the enzyme mixture. Xylan degradation by this enzyme mixture was comparable to the degradation obtained by supplementation of the crude *T. longibrachiatum* preparation to the *A. niger / T. emersonii* enzyme cocktail, even though XP1-4 was applied at a much lower dosage [10 g $kg^{-1}$ substrate, compared to saturated dosage of the crude *T. longibrachiatum* preparation equal to 300 g $kg^{-1}$ substrate]. Incubation with the same dosage (10 g $kg^{-1}$) of the crude enzyme preparation did not show any increased hydrolytic activity regarding xylan degradation but comparable activity as obtained by sole application of the *A. niger / T. emersonii* mixture (Figure 1, section a). These findings demonstrate that certain individual enzymes have been enriched in fraction XP1-4 by the chromatographic separation that either provide entirely new functionality or supplement a limiting activity to the enzymes present in the *A. niger / T. emersonii* mixture.

**Identification of proteins present in high activity fraction (XP1-4)**

Identification of the proteins present in fraction XP1-4 was done by LC/MS. The proteins were assigned based on homology to known fungal proteins. By calculating the percent share of spectrum ID's value, a rough estimation of the relative protein abundance could be made (Figure 3). As the signal intensity of the MS analysis depends on the ionization of the peptides, only an indication of the relative abundance can be made by the calculation. The proteins identified by LC/MS were compared to protein bands obtained by the SDS-PAGE analysis (Figure 2d). The acetyl xylan esterase (EC.3.1.1.72, Axe1) of phylogenetically closely related *T. reesei* (teleomorph *H. jecorina*) was assigned. This enzyme was indicated to be present in high abundance (55 % share of spectrum ID's) in fraction XP1-4 (Figure 3). A protein band visible at 34 kDa by SDS-PAGE analysis (Figure 2d) matched with the reported molecular mass of Axe1 (*T. reesei*) as determined by Sundberg and Poutanen (1991). Cellobiohydrolase II (CBH II) was a second protein indicated to be present in high abundance by the LC/MS analysis, although with a clearly lower share of spectrum ID's value of 22 %. A protein band at approximately 44 kDa was observed by SDS-PAGE being close to the predicted MW of CBH II (49.6 kDa) based on its sequence (Teerii et al. 1987). The predicted molecular mass and values observed by SDS-PAGE analysis may differ due to post-translational modifications (Ahmad et al. 2005), errors due to varying charge / mass ratios (bound SDS) or protein mobility in the gel (Hjelmeland and Chrambach 1981). Although CBH II was present in noticeable levels in fraction XP1-4, its contribution to the increased substrate conversion is anticipated to be insignificant. Hydrolytic activity towards 1,4-β-D-glycosidic bonds was already present in abundance by application of the *A. niger / T. emersonii* preparation at a saturated dosage (accession number: An08g01760, An12g02220, *A. niger,* LC/MS results not shown and AAL78165.2, *T. emersonii,* Waters et al. 2011). A third protein band appeared in the gel at 23 kDa. Endo-beta-1,4-glucanase (4 % share

of spectrum ID's) and the 'hypothetical' protein AAO51653 (4 % share of spectrum ID's) were identified by LC/MS having predicted MWs of 25.2 and 25.8 kDa. Other proteins were not visible on the gel and were indicated at minor levels by LC/MS analysis (Figure 3).

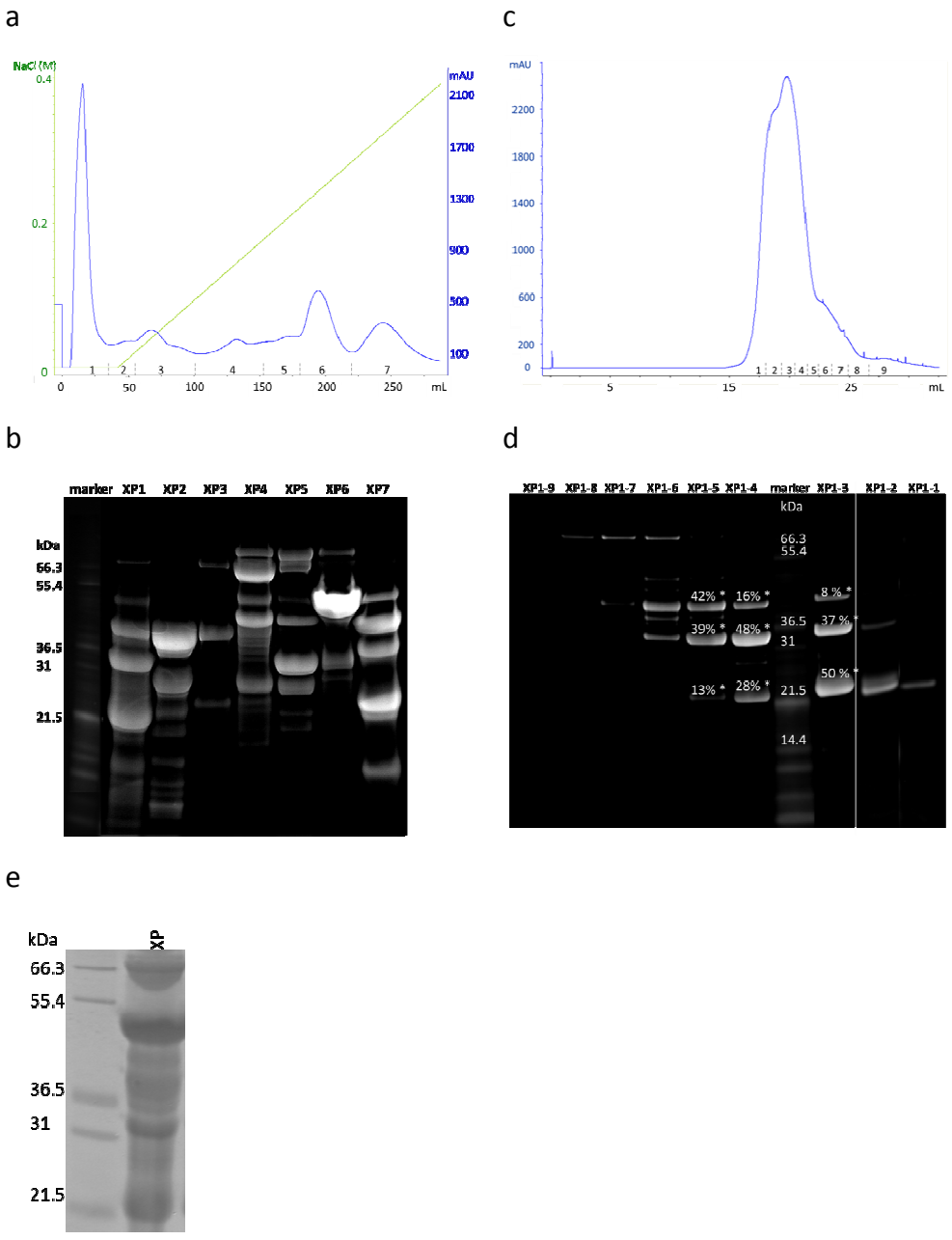


Figure 2: (a) Chromatogram (AEC) of the crude *T. longibrachiatum* preparation. The linear gradient by increasing concentration of NaCl from 0 to 0.4 M is shown in green. (b) SDS-PAGE of fractions obtained by AEC of the crude *T. longibrachiatum* preparation. (c) Chromatogram (SEC) of fraction XP-1. Adsorption was measured at 280 nm. The numbers 1-7 (a) and 1-9 (c) indicate the fractions that were tested by synergy assays. (d) SDS-PAGE of fractions obtained by SEC of fraction XP1. (e) SDS-PAGE of the crude *T. longibrachiatum* preparation (XP).
* percent of protein band of the total amount of proteins present obtained by Coomassie Brilliant Blue staining.

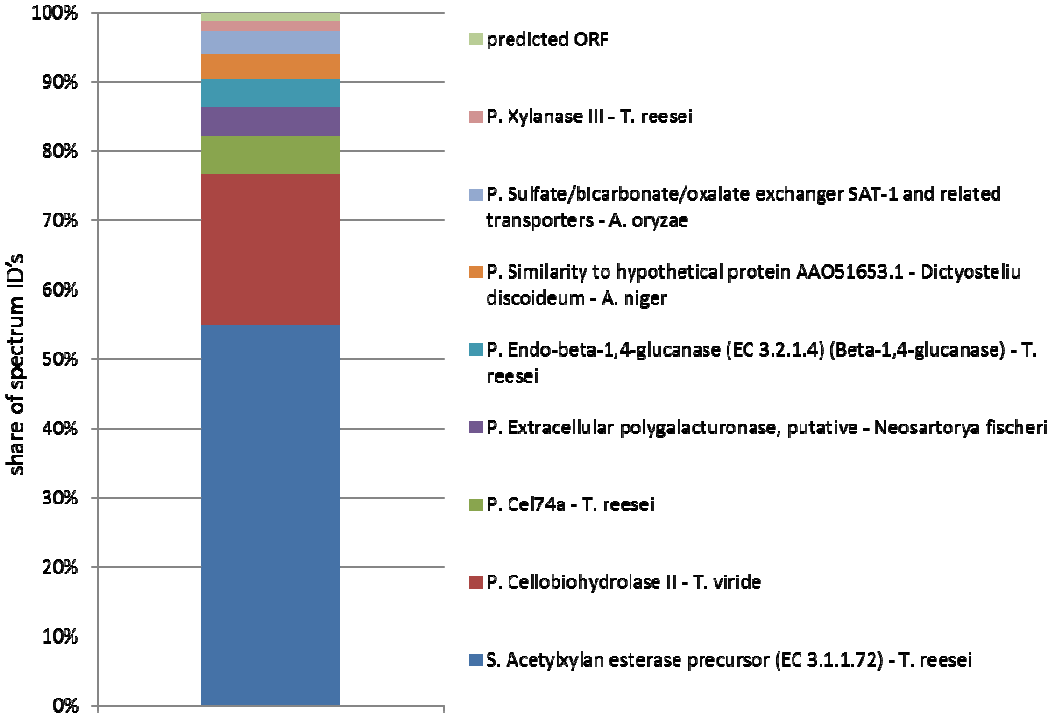


Figure 3: Protein identification by LC/MS analysis of fraction XP1-4 (purified from a *T. longibrachiatum* culture filtrate by anion exchange and size exclusion chromatography; the percent share of spectrum IDs for each protein present is shown).

*Presence of Axe1 in fraction XP1-4*

The hydrolysis of *p*-nitrophenyl-acetate showed a clear enrichment of the acetyl xylan esterase activity in fraction XP1-4 (495 IU, 15 fold increase in the hydrolysis of *p*-nitrophenyl-acetate) compared to the crude *T. longibrachiatum* preparation (33 IU). This is in agreement with the indicated high abundance of Axe1 in this fraction as described above. Only low activity was observed for fraction XP1-4 towards 4-nitrophenyl-xylopyranoside and 4-nitrophenyl-glucopyranoside compared to the crude *T. longibrachiatum* preparation [17 IU and 19 IU (XP1-4); 543 and 457 IU (*T. longibrachiatum* preparation) for the hydrolysis of 4-nitrophenyl-xylopyranoside and 4-nitrophenyl-glucopyronaside, respectively], indicating the removal of other enzymes during the Axe1 enrichment. The results confirm a selective enrichment of active Axe1 in fraction XP1-4.

Axe1 is classified as belonging the CE family 5 (Carbohydrate-active enzymes database; www.cazy.org). The acetyl xylan esterases found in the secretome of *A. niger* and *T. emersonii* are classified in CE1 (www.cazy.org, Waters et al. 2011, Ferreira de Oliviera et al. 2011). Acetyl xylan esterases belonging to different CE families are known to show a great diversity in structure and substrate specificity (Biely 2012). Furthermore, it should be noted that

differences in the hydrolytic activity and specificity may occur amongst enzymes within a CE family due to genetic variation. The data obtained [high abundance of Axe1 (belonging to CE family 5), enrichment in the activity towards *p*-nitrophenyl acetate and the observed high acetylation of the substrates used in this study (WUS: 39 g $kg^{-1}$ and AFR: 40 g $kg^{-1}$ acetic acid; equal to 0.36 and 1.17 acetyl groups per xylose residue, respectively)] suggest that the hydrolytic gain could indeed be induced by Axe1. Supplementation of the CE family 5 classified Axe1 may add missing deacetylation activities to the *A. niger / T. emersonii* enzyme cocktail.

**Synergistic potential of acetyl xylan esterase (Axe1, AxeA) rich fractions**

In order to investigate the synergistic potential for the saccharification of lignocellulosic feedstock by supplementation of Axe1 (XP1-4) to the *A. niger / T. emersonii* mixture, the mono- and oligosaccharides released from WUS and AFR were determined. Hydrolysis of these substrates was also performed by supplementation of additional CE family 1 classified AxeA, as already present in the *A. niger / T. emersonii* preparation, to the (hemi)cellulolytic preparation. This was done in order to analyze whether a high hydrolytic gain would occur specifically due to Axe1 (XP1-4, CE5) addition or also be obtained by application of additional quantities of CE1 acetyl xylan esterase.

*Saccharification by supplementation of Axe1 and AxeA to the (hemi)cullulolytic preparation*

The levels of monosaccharides released by supplementation of Axe1 or AxeA to the *A. niger / T. emersonii* preparation are shown in Figure 4a, 4b. Remarkably, application of 2.5 fold lower dosage of Axe1 compared to AxeA [Axe1 (XP1-4, 10 g $kg^{-1}$), AxeA (25 g $kg^{-1}$)] still resulted in a higher substrate conversion. The monosaccharide levels obtained for the hydrolysis of WUS were 147, 108 and 23 mg $g^{-1}$ (supplementation of Axe1) compared to 127, 93 and 19 mg $g^{-1}$ (supplementation of AxeA) for Glc, Xyl and Ara, respectively. The monosaccharide levels obtained by hydrolysis with the sole *A. niger / T. emersonii* preparation were clearly lower (114, 72 and 14 mg $g^{-1}$ for Glc, Xyl and Ara, respectively). With AFR, the relative increase in monosaccharides released by hydrolysis with the Axe1 supplemented enzyme mixture compared to the sole *A. niger / T. emersonii* preparation was even higher. The monosaccharide levels obtained for the hydrolysis of AFR were 10, 13 and 3 mg $g^{-1}$ (supplementation of Axe1) compared to 7, 9 and 2 mg $g^{-1}$ (supplementation of AxeA) and 6, 5 and 1 mg $g^{-1}$ by sole application of the *A. niger / T. emersonii* preparation for Glc, Xyl and Ara, respectively. As described above, AFR has a high acetyl/ Xyl ratio (1.17). The data demonstrate that deacetylation by acetyl xylan esterases enables additional release of monosaccharides from this recalcitrant residue by the (hemi)cellulolytic preparation. This suggests that Axe1 is able to increase substrate accessibility for other enzymes present in the *A. niger / T. emersonii* preparation and that xylanases depolymerize the xylan backbone more efficiently following

deacetylation. The increased Glc release is likely to be the consequence of opening up "resistant" cell wall structures by an increased degradation of hemicellulose.
The amount of oligosaccharides released by supplementation of Axe1 (XP1-4) or AxeA to the enzyme mixture is shown in Figure 4c and 4d. Oligosaccharide levels were also highest with the Axe1 (XP1-4) supplemented mixture. Especially low MW oligosaccharides with a retention time of 8 min (xylotetraose elutes at 7.9 min) and lower were present at elevated levels. The data obtained indicate a dependency of the oligosaccharide release on the deacetylation efficiency. Still, no complete degradation of the oligosaccharides released was observed with the (hemi)cellulolytic enzyme mixture.
Despite the fact that a relevant amount of acetyl xylan esterase activity was observed for the crude *A. niger / T. emersonii* preparation (245 IU, hydrolysis of *p*-nitrophenyl acetate), addition of Axe1 (495 IU) still increased the levels of mono- and oligosaccharides released. As described above, acetyl xylan esterases present in the *A. niger / T. emersonii* secretome belong to CE family 1 (Waters et al. 2011, Ferreira de Oliviera et al. 2011). The results demonstrate that certain acetyl xylan esterase activities (e.g. Axe1, CE5) are missing (or are underrepresented) in the industrial *A. niger* or *T. emersonii* preparations, and that these are necessary for the efficient conversion of recalcitrant corn substrates.

*Adsorption of Axe1 and AxeA*
Since the amount of enzyme bound to the substrate influences the hydrolysis rate, the adsorption of Axe1 and AxeA to WUS and AFR was analyzed (SDS-PAGE). High adsorption of Axe1 to the insoluble substrates was observed. 77 % of total Axe1 present was adsorbed to AFR and 98 % to WUS. Regarding AxeA, an adsorption of 51 % to AFR and 15 % to WUS was measured. The assigned sequence of Axe1 (*T. reesei*) has been reported to have a fungal type carbohydrate binding module (CBM) belonging to family 1 (Margolles-Clark et al. 1996). According to entries in the carbohydrate active enzymes database (www.cazy.org, 2013, Nov), CBM1 is not present in AxeA. Therefore, one might speculate that presence of CBM1 in Axe1 (*T. longibrachiatum*) may have a positive effect by increasing the concentration of the enzyme in close proximity to the substrate (Boraston et al. 2004). Carbohydrate binding by enzymes bearing only a single CBM, like Axe1, was described to be generally weak compared to the binding affinities observed for enzymes that have multiple carbohydrate binding sites (Boraston et al. 2004). Weak binding of enzymes might be advantageous for the hydrolytic processing of the substrate by enabling mobility of the enzyme. However, the exact role of CBMs present in hemicellulases needs to be further clarified.

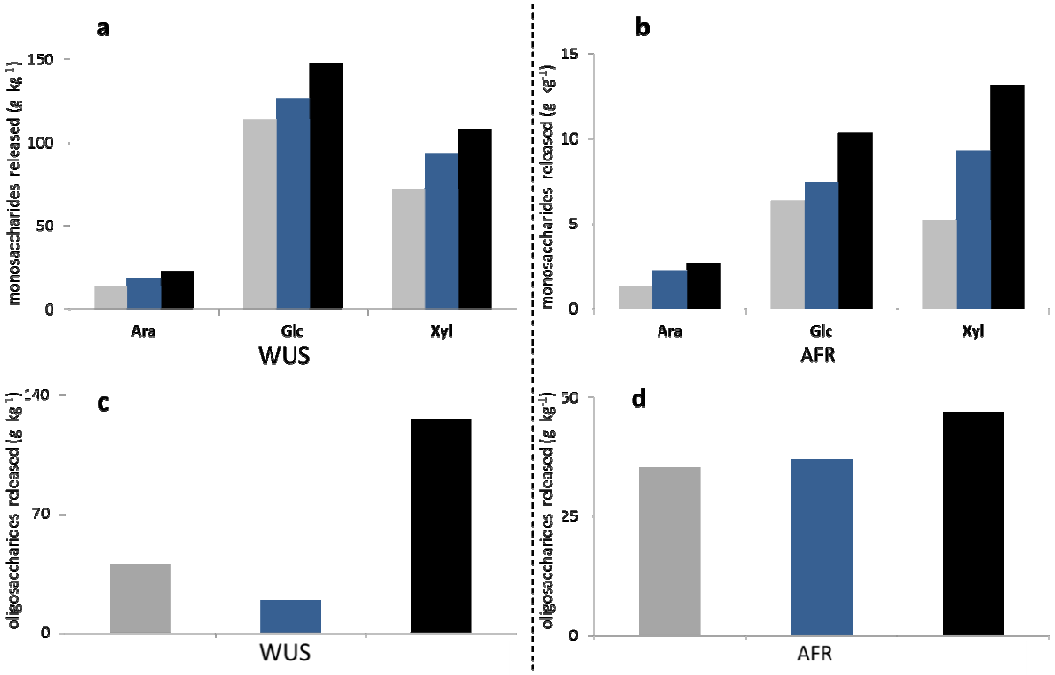


Figure 4: (a, b) Monosaccharides (g $kg^{-1}$) and (c, d) oligosaccharides (g $kg^{-1}$) released from corn silage water unextractable solids (WUS) and a recalcitrant, anaerobic fermentation residue (AFR) by incubation with the i. *A. niger / T. emersonii* prep. (100 g $kg^{-1}$; grey), ii. *A. niger / T. emersonii* prep. (100 g $kg^{-1}$) + AxeA (CE1, *A. niger*, 25 g $kg^{-1}$; blue), iii. *A. niger / T. emersonii* prep. (100 g $kg^{-1}$) + Axe1 (CE5, *T. longibrachiatum*, 10 g $kg^{-1}$; black).

**Deacetylation of poly- and oligosaccharides by Axe1**

*Deacetylation efficiency by acetyl xylan esterases Axe1 and AxeA*

The deacetylation of the corn- and eucalyptus-derived substrates, which show significant differences in xylan substitution, was determined by measuring the acetic acid release. Both, corn and hard-wood (eucalyptus) xylan contain 2-O, 3-O mono-acetylated xylan and [MeGlcA-α-(1 → 2)][3-*O*-Ac]-xylopyranosyl units (Naran et al. 2009). Furthermore, 2,3-di-*O* acetylated xylose was reported for hard-wood xylan (Naran et al. 2008, Evtuguin et al. 2002) and 2-*O* acetylated xylose next the an oligomeric side chain at the *O*-3 position was described for a corn fiber hydrolysate (Appeldoorn et al. 2013). Figure 5 shows the levels of acetic acid release for Axe1 (XP1-4), AxeA and a mixture of Axe1 (XP1-4) and AxeA (1:1 ratio based on protein content). All incubations were done at the same total protein loading (10 g protein $kg^{-1}$ substrate), as the acetyl xylan esterase containing fractions were not entirely pure after the chromatographic purification as described above. Application of Axe1 (XP1-4) exhibited a higher acetic acid release in all cases compared to AxeA, even though the purity for Axe1 was lower compared to AxeA. This observation confirmed that Axe1 is an interesting candidate for efficient deacetylation of insoluble substrates (WUS, AFR) as well as corn and eucalyptus derived oligosaccharides. For WUS, a combination of Axe1 (XP1-4) and AxeA resulted in highest yields.

Differences in deacetylation between Axe1 and AxeA could be a result of the affinity of the enzyme to the substrate or hindrance by xylan substituents. Furthermore, the positional preference of the enzyme might result in different overall deacetylation efficiencies. The positional preference of acetyl xylan esterases from *M. thermophile C1*, belonging to CE families 1 or 5, have been described. Both enzymes deacetylate the *O*-2 and *O*-3 position while showing a preference for *O*-2 (Pouvreau et al. 2011). Affinity of a CE1 classified acetyl xylan esterase from *S. commune* was reported to be higher towards 2,3-di-*O*acetylated than to monoacetylated xylopyranosyl units (Biely et al. 2013). Positional preferences might be conserved for acetyl xylan esterases belonging to the same CE family as these are classified on the basis of structurally related functional domains (www.cazy.org).
As described before, a high degree of acetylation contributes to the recalcitrance of AFR. Nevertheless, the data obtained show that Axe1, but also AxeA, effectively decrease its recalcitrance (Figure 4) and release nearly equally high amounts of acetic acid (Figure 5). Therefore, it is indicated that both enzymes, Axe1 and AxeA, are important processing enzymes for further utilization of cereal anaerobic fermentation residues. Regarding the deacetylation of oligosaccharides, Axe1 (XP1-4) showed higher efficiency with AcXOS, AcUXOS and the oligosaccharides present in cs-soluble compared to AxeA (Figure 5).

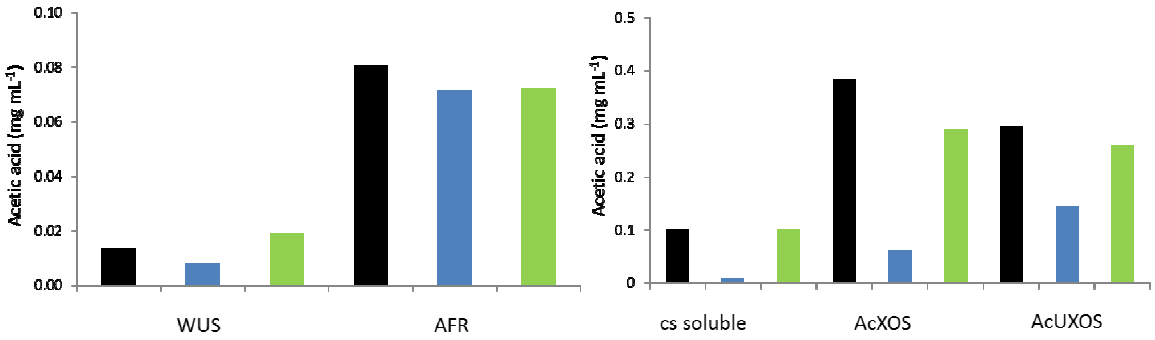


Figure 5: Acetic acid released (mg $mL^{-1}$) by incubation with Axe1 (CE5, black), AxeA (CE1, blue) and Axe1+AxeA (green) for 24 h. Corn silage water unextractable solids (WUS) and an anaerobic fermentation residue (AFR; left) and a soluble extract of corn silage (cs soluble), neutral (AcXOS) and acidic (AcUXOS) eucalyptus oligosaccharides (right) were used as substrates.

*Positional preference for the deacetylation of neutral (AcXOS) and acidic (AcUXOS) xylo-oligosaccharides*

In order to further characterize deacetylation of oligosaccharides by Axe1 (CE5) and AxeA (CE1), Maldi-Tof MS analysis of enzyme treated AcXOS and AcUXOS was performed. Eucalyptus xylo-oligosaccharides have been described as a suitable substrate to characterize acetyl xylan esterases (Koutaniemi et al. 2013, Pouvreau et al. 2011). AcXOS and AcUXOS show a high degree of acetylation (Table 1). Furthermore, MeGlcA is present in AcUXOS. Table 2 illustrates

the xylo-oligosaccharides corresponding to the masses of ions detected by Maldi-Tof MS before and after enzyme treatment.

*Deacetylation of AcXOS:* Hydrolysis with Axe1 resulted in low numbers of differently acetylated oligosaccharides (Table 2a) compared to AxeA. The accumulation of non- and mono-acetylated xylo-oligosaccharides indicates activity by Axe1 towards doubly acetylated β-D-xylopyranosyl units, which are present in *Eucalyptus globulus* xylan (Evtuguin et al. 2002). The remaining mono acetylated neutral oligosaccharides are resistant towards the acetyl xylan esterase. Acetyl migration (Mastihubová and Biely 2004) to the *O*-4 position (Biely 2012) could result in resistant oligosaccharides towards the acetyl xylan esterase applied. Only CE16 acetyl esterases have been described to deacetylate this position (Biely et al. 2012).

Hydrolysis with the Axe1 (XP1-4)-supplemented *A.niger / T.emersonii* preparation resulted in non-acetylated pentose oligosaccharides and mono- or diacetylated pentose oligosaccharides $P_3$ or $P_4$. The non-acetylated oligosaccharides were present in abundance demonstrating efficient deacetylation by the applied enzyme mixture. Hydrolysis with the *A. niger / T. emersonii* preparation containing additional AxeA, but no Axe1, showed presence of additional acetylated oligosaccharides ($P_2$ $Ac_3$, $P_4$ $Ac_2$ and $P_5$ $Ac_5$). The latter were present also in the sample hydrolyzed with AxeA only. This demonstrates the ability of Axe1 to deacetylate oligosaccharides that are not deacetylated by AxeA (CE1, *A. niger*) or the CE1 classified acetyl xylan esterase present in the *T. emersonii* preparation. Furthermore, the lower proportion of acetylated oligosaccharides present in the Axe1 (XP1-4) supplemented digest compared to the AxeA supplemented digest, shows a higher overall deacetylation level through Axe1 addition (Table 2a). This is in agreement with the high total acetic acid release by Axe1, as described above.

*Deacetylation of AcUXOS:* Recalcitrance towards enzymatic deacetylation was observed for AcUXOS (Table 2b). It has been reported that the MeGlcA moiety may hinder the hydrolysis of some acetyl groups (Van Gool et al. 2012). Still, several acetylated acidic oligosaccharides ($P_2$ MeGlcA Ac, $P_2$ MeGlcA $Ac_2$ and $P_3$ MeGlcA $Ac_3$) were hydrolyzed by Axe1 and AxeA. Axe1 also deacetylated $P_4$ MeGlcA $Ac_3$ and $P_5$ MeGlcA $Ac_2$. Combined hydrolysis by *A. niger / T. emersonii* plus Axe1 or AxeA resulted in the hydrolysis of additional [$P_x$MeGlcA $Ac_y$] oligosaccharides (Table 2b) compared to hydrolysis with Axe1 or AxeA only. All oligosaccharides besides $P_4$ MeGlcA Ac were deacetylated in the Axe1 (XP1-4) supplemented mixture and only a low proportion of acetylated $P_x$MeGlcA was observed after hydrolysis with Axe1 (XP1-4) only. The data indicate a certain tolerance of Axe1 towards MeGlcA substituents.

The results obtained suggest efficient deacetylation of eucalyptus *O*-acetyl-(4-*O*-methylglucurono)xylan by Axe1 and confirm a high degree of degradation by the Axe1 supplemented enzyme preparation.

Table 2: Maldi-Tof MS analysis of (A) neutral (AcXOS) and (B) acidic (AcUXOS) eucalyptus oligosaccharides. The oligosaccharides were incubated with purified enzyme fractions Axe1 (XP1-4) and AxeA, and with the *A. niger / T. emersonii* preparations supplemented with Axe1 (XP1-4) and AxeA. Data are displayed as relative amounts (%) per oligosaccharide of the total (acetylated and non-acetylated) pentose oligosaccharides P2 to P5 present per sample, based on peak intensities obtained by Maldi-Tof MS analysis.

| **A** | **oligosaccharides (AcXOS)** | **no enzyme** | **Axe1 (XP1-4)** | **AxeA** | ***A.niger/T.emersonii* + Axe1 (XP1-4)** | ***A.niger/T.emersonii* + AxeA** |
|---|---|---|---|---|---|---|
| | **$P_2$** | | 38 | 6 | | |
| | **$P_2Ac$** | 18 | 32 | 31 | | |
| | **$P_2Ac_2$** | | | 4 | | |
| | **$P_2Ac_3$** | | | 1 | | 4 |
| | **$P_3$** | | 10 | | | |
| | **$P_3Ac$** | 11 | 9 | 9 | 9 | 5 |
| | **$P_3Ac_2$** | | | 29 | 25 | 19 |
| | **$P_3Ac_3$** | 6 | | 6 | | |
| | **$P_3Ac_4$** | 3 | | | | |
| | **$P_4$** | | 4 | | 38 | 19 |
| | **$P_4Ac$** | 13 | 3 | 1 | 7 | 19 |
| | **$P_4Ac_2$** | 15 | | 3 | | 7 |
| | **$P_4Ac_3$** | 7 | | 4 | | |
| | **$P_4Ac_4$** | | | 2 | | |
| | **$P_5$** | | 4 | 2 | 21 | 21 |
| | **$P_5Ac$** | | | | | |
| | **$P_5Ac_2$** | 11 | | | | |
| | **$P_5Ac_3$** | 10 | | 1 | | |
| | **$P_5Ac_4$** | 6 | | | | |
| | **$P_5Ac_5$** | | | 1 | | 6 |

Table 2 (continued):

| B | oligosaccharides (AcUXOS) | no enzyme | Axe1 (XP1-4) | AxeA | *A.niger/T.emersonii* + Axe1 (XP1-4) | *A.niger/T.emersonii* + AxeA |
|---|---|---|---|---|---|---|
| | **$P_2$MeGlcA** | 7 | 61 | 4 | 37 | 9 |
| | **$P_2$MeGlcA Ac** | 5 | | | | |
| | **$P_2$MeGlcA $Ac_2$** | 4 | | | | |
| | **$P_3$MeGlcA** | | | | 30 | 16 |
| | **$P_3$MeGlcA Ac** | 10 | 3 | 39 | | 14 |
| | **$P_3$MeGlcA $Ac_2$** | 6 | 18 | 14 | | 8 |
| | **$P_3$MeGlcA $Ac_3$** | 3 | 1 | 3 | | |
| | **$P_4$MeGlcA** | | | | | |
| | **$P_4$MeGlcA Ac** | 15 | 2 | 9 | 33 | 33 |
| | **$P_4$MeGlcA $Ac_2$** | 7 | 2 | 16 | | 10 |
| | **$P_4$MeGlcA $Ac_3$** | 5 | | 5 | | |
| | **$P_4$MeGlcA $Ac_4$** | 17 | 9 | 4 | | 10 |
| | **$P_5$MeGlcA** | | | | | |
| | **$P_5$MeGlcA Ac** | | | | | |
| | **$P_5$MeGlcA $Ac_2$** | 8 | 1 | 2 | | |
| | **$P_5$MeGlcA $Ac_3$** | 8 | 2 | 3 | | |
| | **$P_5$MeGlcA $Ac_4$** | 5 | 1 | 1 | | |
| | **$P_5$MeGlcA $Ac_5$** | | | | | |

## Conclusions

A significant increase in the conversion of WUS (hydrolytic gain of 50, 62 and 29 % for Xyl, Ara and Glc, respectively) was obtained by supplementation of Axe1 (CE5) to the hemicellulolytic *A. niger / T. emersonii* preparation. The acetic acid released by purified Axe1 was two, six and two times higher compared to AxeA for WUS, AcXOS and AcUXOS respectively. Maldi-Tof MS demonstrated efficient deacetylation of *O*-acetyl-(4-*O*-methylglucurono)xylan by Axe1. Axe1 acts in synergy with the *A. niger / T. emersonii* preparation as accessibility for other carbohydrases present in the enzyme preparation is increased following efficient deacetylation of the substrate.

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## Supplementary information

**_Trichoderma longibrachiatum_ acetyl xylan esterase 1 enhances hemicellulolytic preparations to degrade corn silage polysaccharides.**

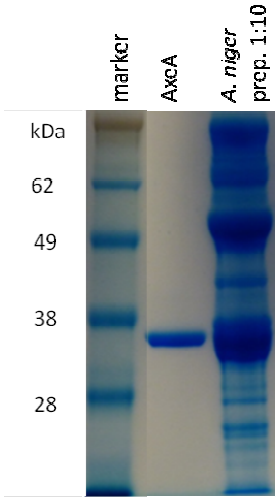


Figure S1: SDS-PAGE of purified AxeA (*A. niger*) and the crude *A. niger* culture filtrate.

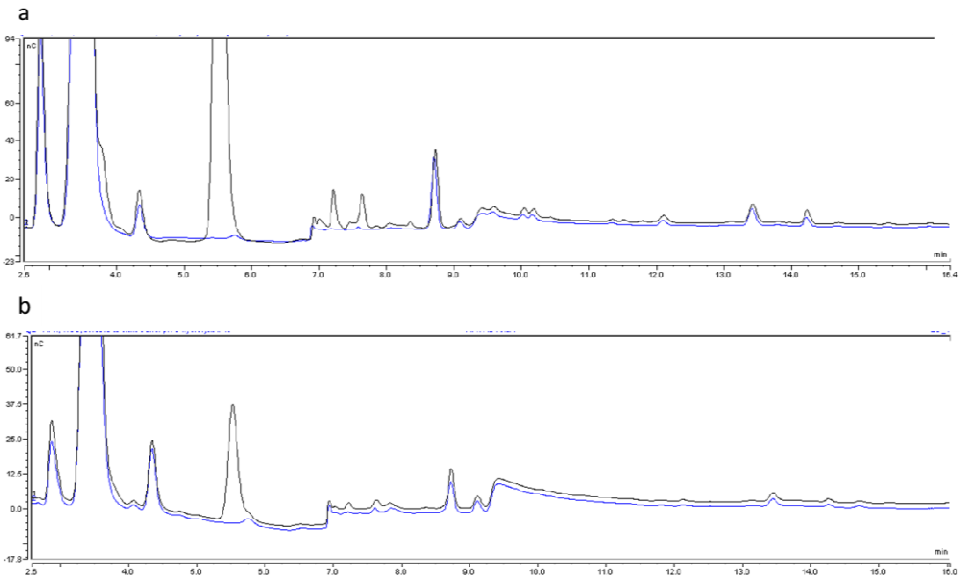


Figure S2: HPAEC analysis of oligosaccharides released from (a) corn silage water unextractable solids (WUS) and (b) an anaerobic fermentation residue (AFR) by incubation with the i. *A. niger / T. emersonii* prep. + AxeA (blue) and ii. *A. niger / T. emersonii* prep. + Axe1 (XP1-4; black). Oligosaccharides eluted after 3.8 min.

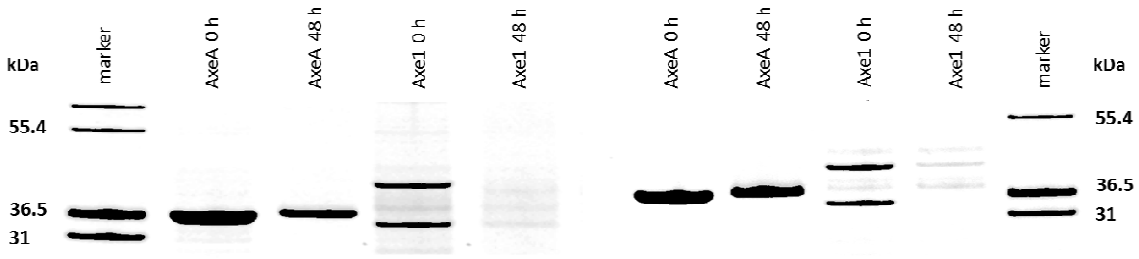


Figure S3: SDS-PAGE analysis of the supernatant (non-adsorbed enzyme fraction) from incubations of insoluble substrates [anaerobic fermentation residue (AFR, left) and water unextractable solids (WUS, right)] with Axe1 (XP1-4) and AxeA (0 h and 48 h).

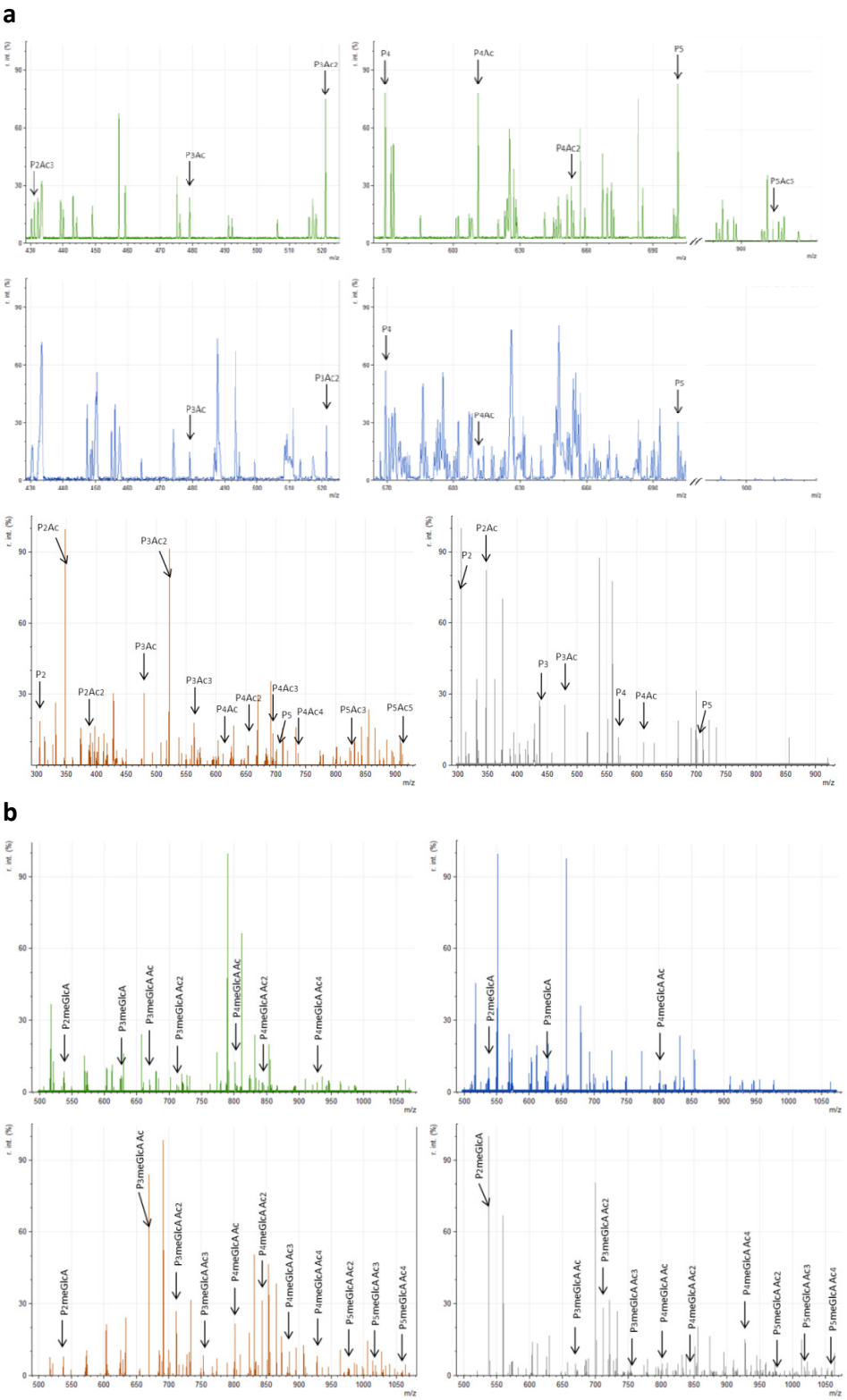


Figure S4: Maldi-Tof MS spectra of (a) neutral and (b) acidic eucalyptus oligosaccharides hydrolyzed with *A. niger / T. emersonii* preparation + AxeA (green), *A. niger / T. emersonii* preparation + Axe1 (XP1-4, blue), AxeA (brown) and Axe1 (XP1-4, grey). The relative intensities of oligosaccharides detected are shown in the graph.

*CHAPTER 6*

# Positional preferences of acetyl esterases from different CE families towards acetylated 4-*O*-methyl-glucuronic acid substituted xylo-oligosaccharides


## Abstract

The deacetylation efficiency and deacetylation rates for different acetylated Xyl*p* as present in 4-*O*-methyl-glucuronic acid (meGlcA)-substituted xylo-oligosaccharides (AcUXOS) derived from *Eucalyptus globulus* by acetyl esterases from seven different carbohydrate esterase (CE) families were monitored by $^{1}$H NMR. Differences were obtained regarding the hydrolysis of 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA. The acetyl esterases tested could be categorized in three groups having activities towards (i) 2-*O*, 3-*O* acetylated Xyl*p*, (ii) 2-*O*, 3-*O* and 2,3-di-*O* acetylated Xyl*p*, and (iii) 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p*, and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA at the non-reducing end of xylo-oligosaccharides. A high deacetylation efficiency of up to 83 % was observed for CE5 and CE1 classified acetyl esterases. The deacetylation rates for the different acetylated Xyl*p* differed between the acetyl esterases measured at pH 5.0 at 50 °C, and were highest for the CE5 classified acetyl esterase from *A. niger*, being 203, 276 and 274 µmol acetyl groups removed/ min/ mg enzyme for 2-*O*, 3-*O* acetylated and 2,3-di-*O* acetylated Xyl*p*, respectively. Positional preferences were obtained towards 2,3-di-*O* acetylated Xyl*p* (*Te*CE1, *An*CE5 and *Os*CE6) or 3-*O* acetylated Xyl*p* (*Ct*CE4).



Neumüller K.G., Carvalho de Souza A., Van Rijn J.H.J., Streekstra H., Gruppen H., Schols H.A., 2014. Positional preferences of acetyl esterases from different CE families towards acetylated 4-*O*-methyl-glucuronic acid substituted xylo-oligosaccharides. *Submitted for publication*.

## Introduction

Xylan is a valuable source of C-5 sugars for biorefinery. Acetylation of xylan restricts enzymatic degradation of the xylan backbone (Neumüller et al. 2014a). In order to increase the accessibility of β-1,4-endo-xylanases and β-1,4-xylosidases to the (1 → 4)-β-D-xylan backbone, enzymatic hydrolysis of glycosidic substituents and phenoyl and acetyl esters is required. Acetyl esterases are grouped in 16 carbohydrate esterase (CE) families based on the Carbohydrate-active enzymes database (CAZy) classification (www.cazy.org). Unfortunately, lack of knowledge in the sequence-to-specificity relationship in CAZyme families does not yet allow a reliable, automated substrate prediction (Lombard et al. 2014) for the esterases within a CE family.
Acetyl esterases with activity towards acetylated xylan were reported to fall into 8 different CE families (CE1, 2, 3, 4, 5, 6, 7 and 16; Biely 2012, Pawar et al. 2013). As reviewed previously (Biely 2012), the positional preference on synthetic substrates has been described for some acetyl xylan esterases. A characterization of the positional preference of acetyl esterases on partially acetylated xylo-oligosaccharides by NMR is only available for the CE4 (*Streptomyces lividans*) and CE6 (*Orpinomyces* sp.) classified acetyl xylan esterases (Uhliariková et al. 2013). Furthermore, only in few cases the specificity towards different acetylated Xyl*p* was investigated on partially acetylated plant oligosaccharides (Pouvreau et al. 2011, Koutaniemi et al. 2013). However, no deacetylation rates towards the differently acetylated Xyl*p* as present in plant xylo-oligosaccharides were reported by previous studies, which are necessary to determine the positional preference of the acetyl esterases at the start of hydrolysis.
Previous literature showed that CE1 and CE5 acetyl xylan esterases have a strong preference for the deacetylation of 2-*O* acetyl 4-nitrophenyl β-D-xylopyranoside (Biely et al. 2011). Esterases from the same families were reported to be active not only on 2-*O* but also on 3-*O* acetylated xylopyranosyl residues present in xylo-oligosaccharides (Pouvreau et al. 2011). Such differences between plant derived and generic substrates necessitate the investigation of the positional preference of acetyl esterases on natural substrates. A characterization of the deacetylation rates and the deacetylation efficiency after reaching the reaction endpoint by incubations with acetyl esterases from different CE families on differently acetylated, plant derived, Xyl*p* residues would provide insights into the mode of action of acetyl esterases. Especially the investigation of the hydrolytic properties of acetyl esterases from different CE families under hydrolysis conditions that are relevant for industrial biofuels production is important. It enables the design of efficient deacetylating hemicellulolytic enzyme preparations for application in the biofuels manufacturing process. Recently, supplementation of a CE5 classified acetyl xylan esterase from *Trichoderma longibrachiatum* to a hemicellulolytic enzyme preparation containing CE1 classified acetyl xylan esterases showed to increase substrate conversion to monomers (Neumüller et al. 2014b). Addition of acetyl esterases to hemicellulolytic enzyme preparations allows achieving efficient hydrolysis of hemicellulose while harsh alkaline

pretreatments (Agbor et al. 2011) are reduced. Acetylated 4-*O*-methyl glucuronic acid (meGlcA)-substituted xylo-oligosaccharides (AcUXOS) from *Eucalyptus globulus* are suitable substrates to characterize acetyl esterases (Koutaniemi et al. 2013, Pouvreau et al. 2011). 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and the 3-*O* acetylated Xyl*p* α-1,2-substituted with meGlcA are present in AcUXOS.

Here, we describe the deacetylation by cloned and commercially available acetyl esterases from different CE families on AcUXOS at pH 5 and 50 °C, which are common conditions for biofuels production (Sun 2010, Taherzadeh and Karimi 2007) and conditions under which the acetyl esterases are active. $^{1}$H and 2D NMR experiments were performed in order to characterize the deacetylation of 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and the 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA. The positional preference by the enzymes towards differently acetylated Xyl*p* is presented.

## Materials and methods

### Materials

*Substrates: Eucalyptus globulus* xylan hydrolysate was kindly donated by Prof. Dr. J.C. Parajo of the University of Vigo-Ourense, Spain (Gullón et al., 2008). The aceylated 4-*O*-methyl glucuronic acid (meGlcA)-substituted xylo-oligosaccharides (AcUXOS) fractionof the xylan hydrolysate was described previously (Koutaniemi et al. 2013). The Xyl, Ara, Gal and meGlcA contents of AcUXOS were 765, 0, 42 and 207 mg $g^{-1}$ dry matter (DM; Koutaniemi et al. 2013). The acetic acid was measured by $^{1}$H NMR (section 1.5.), being 210 mg $g^{-1}$ DM. Corn silage water unextractable solids (WUS) were used and have been described (Neumüllet et al. 2014a).

*Enzymes:* Cloning and expression of the acetyl xylan esterase 1 from *Trichoderma reesei* belonging to CE family 5 (*Tr*CE5, *T. reesei*, CAA93247.1) has been described previously (Neumüller et al. 2014c).

Acetyl xylan esterases from *Aspergillus niger* belonging to CE families 5 and 16 [*An*CE5 (CAK49022.1), *An*CE16 (CAK45102.1)] and the CE1 classified acetyl xylan esterase from *Talaromyces emersonii* [*Te*CE1 (ADX07526.1)] were obtained from DSM (Heerlen, The Netherlands). Expression of these genes in *A. niger* ISO527 was performed according to the procedure described previously (Neumüller et al. 2014c). Acetyl xylan esterases classified as belonging to CE families 2 [*Ct*CE2 (AAA23224)], 3 [*Ct*CE3 (ABN52033)] and 4 [*Ct*CE4 (ABN54169)] from *Clostridium thermocellum* were purchased from Prozomix (Haltwhistle, UK). The CE6 classified acetyl xylan esterase [*Os*CE6 (AAC14690.1)] from *Orpinomyces sp. PC-2* was purchased from Megazyme (Wicklow, Ireland).

**Electrophoresis**

SDS-PAGE was performed with a NuPAGE 10 % Bis-Tris gel (Life Technologies, Carlsbad, CA, USA) and a Power Ease 500 system (Life Technologies). The protein bands were stained with the Instant Blue protein gel stain (Expedeaon Inc. San Diego, CA, USA). The protein markers Mark 12 and SeaBlue Plus 2 (Life Technologies) were used. Quantification of the protein bands obtained by densitometry was performed with the software ImageJ, developed at the National Institute of Health (Bethesda, MD, USA)

*Calculation of molecular masses of proteins:* The protein sequences of the acetyl esterases [*Te*CE1, *An*CE5, *Tr*CE5 and *An*CE16] were retrieved from the National Center for Biotechnology Information (Bethesda, MD, USA, www.ncbi.nlm.nih.gov) and calculated with the Compute pI/Mw tool (Expasy Bioinformatics Resource Portal, www.expasy.org).

**Enzyme assays**

*p-Nitrophenyl acetate (p-NP-Ac) assay:* Enzyme assays with the acetyl esterases using *p*-NP-Ac were performed as described previously (Neumüller et al. 2014c). The enzymes were applied at a dosage of 0.3 mg total protein $mL^{-1}$ in sodium acetate buffer (10 mM, pH 5.0) at 40 °C. The *p*-nitrophenol release was determined by continuous measurement for 10 min at 405 nm.

*Monosaccharide releasing activities assay:* WUS (10 g $L^{-1}$) was incubated with acetyl xylan esterases (10 g protein $kg^{-1}$ substrate) in sodium citrate buffer (10 mM, pH 5.0, containing sodium azide 0.5 g $kg^{-1}$) at 50 °C and 700 rpm for 8h. The enzymes were heat inactivated (10 min, 98 °C) and analyzed for the presence of monosaccharides (mg monosaccharides released $g^{-1}$ dry matter) on an HPAEC system as described previously (Neumüller et al. 2014a).

**NMR**

*$^{1}$H and 2D NMR:* $^{1}$H-$^{13}$C HSQC spectra were recorded on an Avance III 700 MHz spectrometer (Bruker BioSpin, Billerica, MA, USA), equipped with a helium-cooled cryoprobe. The HSQC spectra were recorded using a correlation via double inept transfer pulse program using sensitivity improvement (hsqcetgpsi2), in 32 scans, 32 dummy scans, 512 increments, sweep width 130 ppm, relaxation delay of 1.2 s and acquisition time 0.36 s.

$^{1}$H-$^{13}$C HMBC spectra were recorded using heteronuclear zero and double quantum coherence with two-fold low-pass J-filter constant time version (shmbcctetgpl2nd), in 64 scans, 16 dummy scans, 1024 increments, sweep width 20 ppm, relaxation delay of 1.2 s and acquisition time 0.24 s.

*NMR enzyme assay:* AcUXOS was dissolved in deuterated sodium citrate buffer (10 mM, pH 5.0) at a concentration of 5 g $L^{-1}$, transferred to NMR tubes (3x103.5 mm glass tubes) and heated to 50 °C. A $^{1}$H NMR spectrum of each sample was measured before the addition of enzymes. A concentration of 5 g $kg^{-1}$ substrate for the acetyl esterases *Te*CE1, *Ct*CE2, *Ct*CE3, *Ct*CE4, *Tr*CE5, *An*CE5, *Os*CE6, *An*CE16 was determined as suitable for the purpose of measurement. *Ct*CE4 was

also dosed at 0.1 g $kg^{-1}$ substrate, as this enzyme showed a high hydrolytic rate (data not shown). The samples were vortex mixed after addition of the acetyl esterases. The incubations were done at 50 °C and monitored by continuous measurement of $^{1}$H NMR spectra for 30 min and a single measurement after 16 h on an Avance III 700 MHz spectrometer (Bruker).

*Calculation of deacetylation rates*

The concentration of acetic acid present in the hydrolysate was calculated from the $^{1}$H NMR spectrum as described previously (Neumüller et al. 2013) using the integral area of the internal standard 4,4-dimethyl-4-silapentane-1-sulfonic acid. Concentrations of 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* residues and 3-*O* acetylated Xyl*p* residues 2-*O* substituted with meGlcA were calculated accordingly based on the resonances obtained by $^{1}$H NMR analysis , being 2.17, 2.15, 2.11/2.10 and 2.22 ppm, respectively. The rates of the acetic acid release and the deacetylation rates for the different locations of acetyl within the Xyl*p* residues (2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and the 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA) were calculated as µmol acetic acid released/ min/ mg enzyme and µmol specific acetyl group removed/ min/ mg enzyme, respectively, after a measurement time of 5 min for relatively fast acetyl esterases (TeCE1, TrCE5, *An*CE5 and *Os*CE6), dosed at 5 g protein $kg^{-1}$ substrate, and after 30 min for the relatively slow acetyl esterases (*Ct*CE2, *Ct*CE3 and *An*CE16), dosed at 5 g protein $kg^{-1}$ substrate, and the *Ct*CE4, dosed at 0.1 g protein $kg^{-1}$ substrate. The purity of acetyl esterases present in the enzyme solutions was determined by quantitative densitometry of the gels obtained by the SDS-PAGE analysis, as described above. The hydrolytic rates were calculated per actual mg acetyl esterase present.

## Results and discussion

### Acetyl esterases

Selected acetyl esterases were analysed for their purity by SDS-PAGE and the results are shown in Figure 1. The protein band obtained of 34 kDa, 34 kDa, 26 kDa and 32 kDa for *Tr*CE5, *An*CE16, *An*CE5 and *Te*CE1, respectively, matched with the molecular mass reported previously (34 kDa, *Tr*CE5, *T. reesei*; Sundberg and Poutanen 1991) and the calculated molecular masses based on the protein sequences, being 33.8 kDa (*An*CE16), 25.8 kDa (*An*CE5) and 32.5 kDa (*Te*CE1). The protein band obtained by SDS-PAGE for *Ct*CE2, *Ct*CE3, *Ct*CE4 and *Os*CE6 (commercially available acetyl xylan esterases) was 37 kDa, 24kDa, 24kDa and 34kDa, respectively. The molecular masses obtained matched with the calculated molecular masses based on the protein sequences, being 36.4 (*Ct*CE2), 22.8 (*Ct*CE3), 22.8 (*Ct*CE4) and 34.9 (*Os*CE6). The purity of the acetyl esterases present in the enzyme solutions was determined by quantitative densitometry and is shown in Figure 1 as proportion (%) of the total proteins present as determined by SDS-PAGE analysis. The purities obtained reached from 15 % to 98 % (Figure 1). Also protein bands

different from the cloned acetyl esterases were present (Figure 1) in the supernatants obtained after expression by *A. niger* ISO527. Particularly for *An*CE5 a range of other protein bands were present, resulting in the lower purity (15 %) compared to the other acetyl esterase containing solutions. However, the production of recombinant enzymes was specific as secretion of other carbohydrate active enzymes is not induced by the expression and cultivation system used (no further data shown, Neumüller et al. 2014c). An assay to verify the absence of other monosaccharide releasing side activities was performed. No monosaccharide releasing activities were present by incubation of WUS (results not shown) with the acetyl esterase containing solutions (*Tr*CE1, *Ct*CE2, *Ct*CE3, *Ct*CE4, *Tr*CE5, *An*CE5, *Os*CE6 and *An*CE16). This confirms a selective secretion of the cloned esterases by *A. niger* ISO527 as already observed previously (Neumüller et al. 2014c). Absence of side activities and presence of significant amounts of each acetyl esterase enabled investigation of their modes of action.

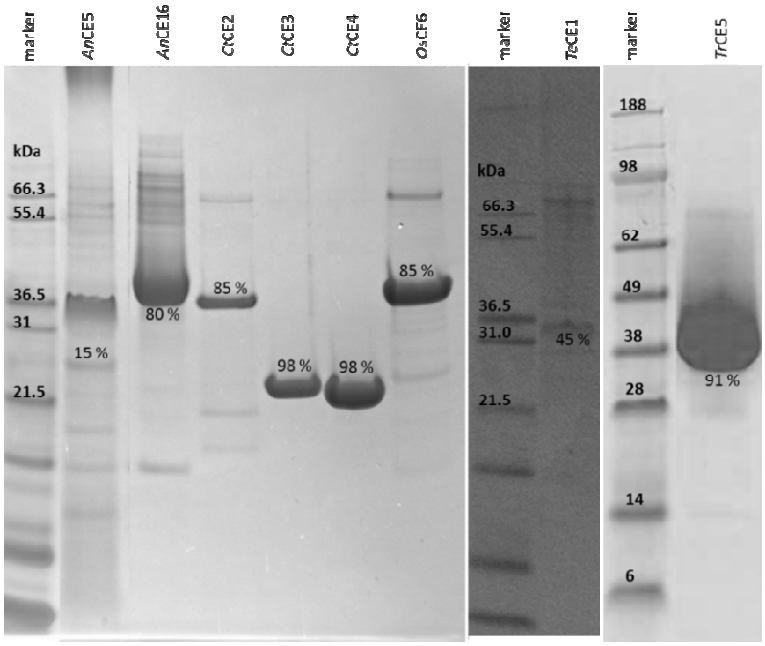


Figure 1: SDS-PAGE of acetyl esterases from *A. niger* (*An*CE5, *An*CE16), *C. thermocellum* (*Ct*CE2, *Ct*CE3, *Ct*CE4), *Orpinomyces sp.* (*Os*CE6), *T. emersonii* (*Te*CE1) and *T. reesei* (*Tr*CE5). The purity of the acetyl esterases present is shown as densimetric proportion (%) of the total proteins present as determined by SDS-PAGE analysis. The protein markers Mark 12 and SeaBlue Plus 2 were used.

**Activity of acetyl esterases**

*Activity on p-nitrophenyl acetate (p-NP-Ac)*

The generic esterase substrate *p*-NP-Ac was used to verify the presence of acetyl esterase activity. The *p*-nitrophenol released by hydrolysis of *p*-NP-Ac with the acetyl esterases is shown in Figure 2. The serine-type acetyl esterases, TeCE1, *Ct*CE2, *Ct*CE3, *An*CE5, *Tr*CE5, *Os*CE6 and *An*CE16, readily hydrolysed *p*-NP-Ac. The none serine-type, metal-dependent esterase *Ct*CE4

was not active on *p*-NP-Ac. The highest *p*-nitrophenol release was observed for the bacterial CE2 and CE3 classified acetyl xylan esterases from *C. thermocellum* (*Ct*CE2 and *Ct*CE3). The CE5 classified acetyl xylan esterases (*An*CE5 and *Tr*CE5) hydrolysed *p*-NP-Ac more efficiently than *Te*CE1, *Os*CE6 and *An*CE16. The hydrolysis of *p*-NP-Ac only gives an indication of the expected hydrolytic activity on acetylated oligosaccharides. Hence, characterization of the acetyl xylan esterases was continued with partially acetylated plant xylo-oligosaccharides.

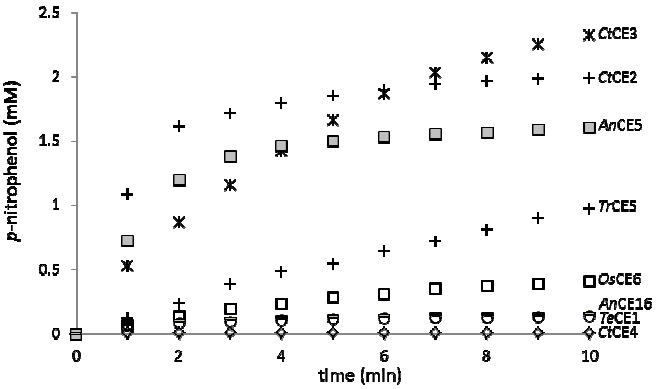


Figure 2: *p*-Nitrophenol released by incubation of *p*-nitrophenyl acetate with acetyl esterases (*Te*CE1, *Ct*CE2, *Ct*CE3, *Ct*CE4, *Tr*CE5, *An*CE5, *Os*CE6 and *An*CE16).

*Deacetylation efficiency of acetylated xylo-oligosaccharides*

The acetic acid released from AcUXOS after an incubation time of 16 h with acetyl esterases from different families as determined by $^{1}$H NMR is shown in Figure 3. All acetyl esterases showed deacetylation of AcUXOS. High levels of acetic acid released were obtained with the fungal *Te*CE1, *Tr*CE5, *An*CE5 and the bacterial *Ct*CE4 (Figure 3). The efficient deacetylation by the CE5 classified esterases is in agreement with the reported high deacetylation of poly- and oligosaccharides by Axe1 from *T. longibrachiatum* belonging to CE family 5 (Neumüller et al. 2014b). *Ct*CE4 efficiently deacetylated AcUXOS, in contrast to the absence of activity towards *p*-NP-Ac (Figure 2). This indicates that activities by acetyl esterases can differ significantly between acetylated oligosaccharides and generic substrates.

Moderate acetic acid release was obtained for *Ct*CE2, *Os*CE6 and *An*CE16 (Figure 3). Hydrolytic activity by *Ct*CE2 from *Neocallimastix patriciarum* towards acetylated glucuronoxylan has been reported before (Darlymple et al. 1997). *Os*CE6 was reported to remove acetyl groups from 2-*O*, 3-*O* and 2,3-di-*O* acetylated Xyl*p* (Uhliariková et al. 2013). The moderate deacetylation efficiency obtained in our study indicates that these positions were not completely deacetylated.

Although the position of all acetyl groups over the oligomeric substrate AcUXOS is not known, the number of acetyl groups removed by *An*CE16 (18 %) matches with the level of reducing end groups present as estimated from the mass spectrum obtained previously (Koutaniemi et al.

2013). The data obtained suggests exo-acting activity for the CE16 classified enzyme from *Aspergillus niger* as proposed previously for the CE16 classified acetyl esterase from *Trichoderma reesei* (Biely et al. 2014). So far, there is no structural information available for the CE16 family (Canteral et al. 2009), indicating a further need for characterization of this CE family. Only low deacetylation was obtained for *Ct*CE3, showing that this enzyme is not efficiently de-esterifying AcUXOS under the hydrolysis conditions applied.

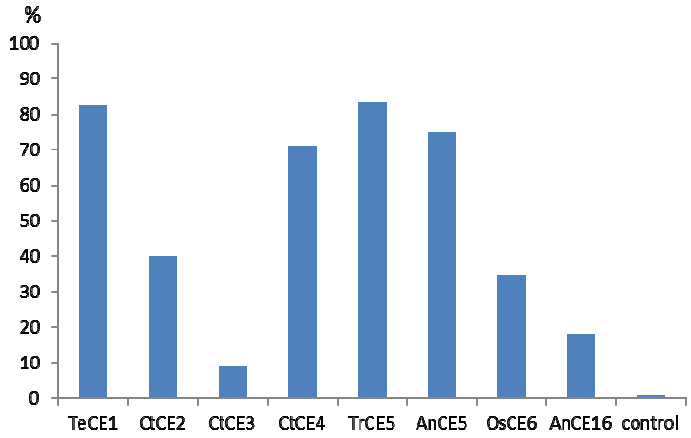


Figure 3: Acetic acid released as proportion (%) of the total amount of acetic acid present in acetylated 4-*O*-methyl glucuronic acid (meGlcA)-substituted xylo-oligosaccharide (AcUXOS) after an incubation time of 16h, pH 5.0 and 50 °C.

**Structural characterization of AcUXOS**

The AcUXOS fraction was further used to determine the positional specificity of acetyl esterases. In order to characterize the acetylation pattern of AcUXOS, $^{1}$H and 2D NMR measurements were performed. The relevant acetylated structural units of the eucalyptus xylo-oligosaccharides were assigned using literature data (Evtuguin et al. 2002) and confirmed by the reporter peaks observed for the AcUXOS fraction by $^{1}$H and $^{13}$C HMBC spectra (Table 1, Figure 4). Due to the focus of this study on monitoring the deacetylation of the structural units by various enzymes in time, a full assignment of all crosspeaks was not needed. Presence of the targeted structural units was confirmed by reporter peaks (Table 1), being in agreement with literature data (Evtuguin et al. 2002, Uhliariková et al. 2013). The acetyl positions and their corresponding chemical shifts for the observed 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and the 3-*O* acetylated α-1,2-meGlcA substituted Xyl*p* residues are shown in Table 1. The regions used for the integration of the different acetylated structural units ($^{1}$H NMR and $^{13}$C HMBC spectra), are shown in Figure 4. The NMR measurement showed that approximately 26 % of the acetyl groups present in AcUXOS are attached at *O*-2 and 46 % at *O*-3 of mono-acetylated Xyl*p*, 15 % represent di-acetylated Xyl*p* and 13 % are attached at *O*-3 of α-1,2-meGlcA substituted Xyl*p*. The $^{1}$H NMR spectra obtained (Figure 4), showed separated resonances (2.10/2.11, 2.15, 2.17 and 2.22 ppm) for the different, acetylated structural units (-Xyl 2,3-*O* Ac-, -Xyl 3-*O* Ac-, -Xyl 2-*O*

Ac- and –Xyl 3-*O* Ac 2-meGlcA-) present in AcUXOS (Table 1). By monitoring the changes in signal intensities for the region 2.1 to 2.22 ppm during incubation with acetyl esterases, the deacetylation efficiency and preference towards differently acetylated Xyl*p* can be investigated.

Table 1: Assignment of relevant chemical shifts of acetyl residues present in 4-*O*-methyl glucuronic acid (meGlcA)-substituted xylo-oligosaccharides (AcUXOS) from *E. globulus*.

| Structural unit | H2 | H3 | COC*H*$_3$ | C2 | C3 | *C*OCH$_3$ |
|---|---|---|---|---|---|---|
| -Xyl 2-*O* Ac- | 4.68 | - | 2.17 | 73.39 | - | 173.42 |
| -Xyl 3-*O* Ac- | - | 4.98 | 2.15 | - | 75.18 | 173.92 |
| -Xyl 2,3-*O* Ac- | 4.81 | 5.15 | 2.10/2.11 | 71.30 | 72.78 | 173.04/173.53 |
| -Xyl 3-*O* Ac 2-meGlcA- | - | 5.06 | 2.22 | - | 73.77 | 173.99 |

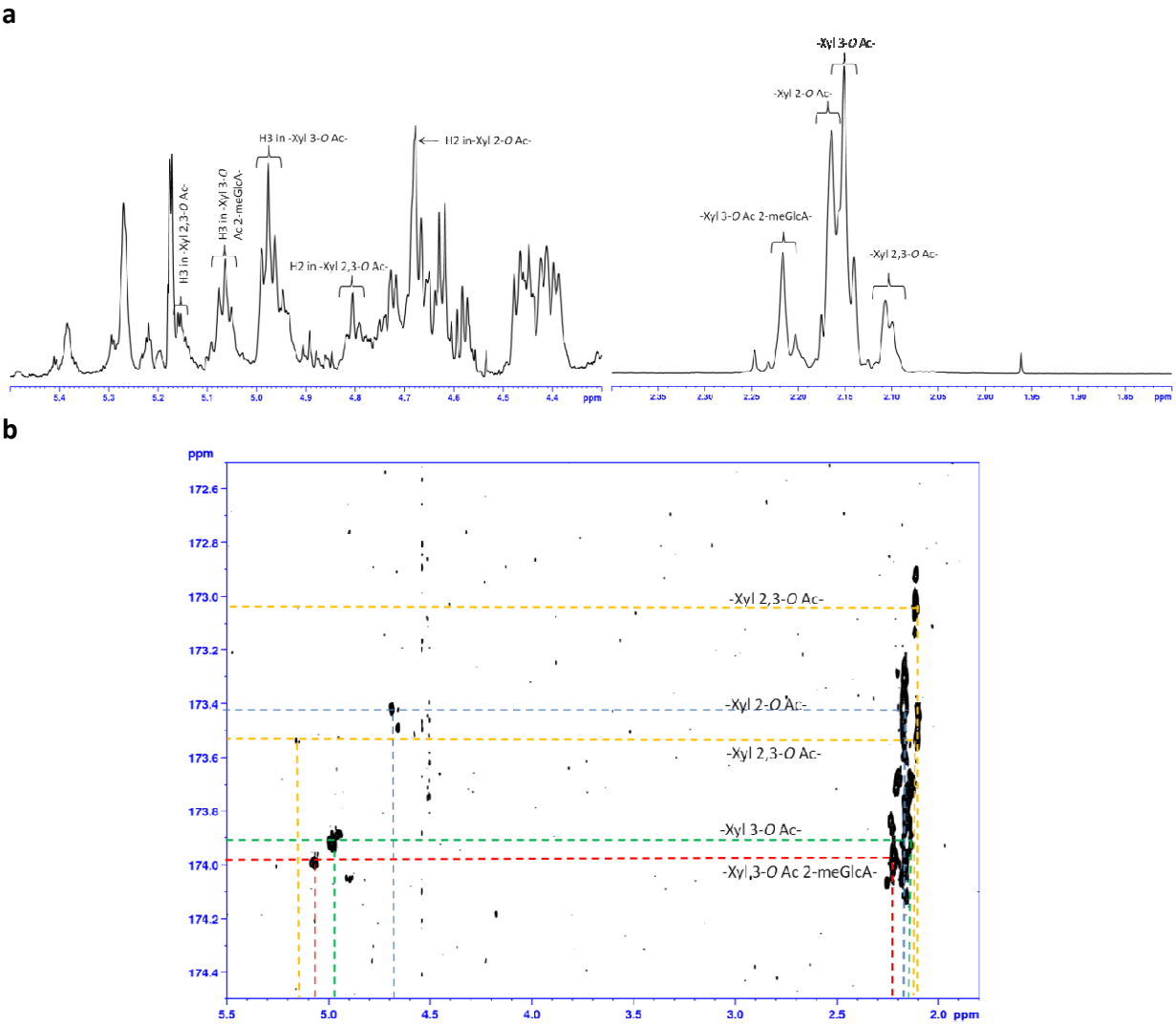


Figure 4: (a) $^{1}$H NMR and (b) $^{13}$C HMBC spectrum of acetylated 4-*O*-methyl glucuronic acid (meGlcA)-substituted xylo-oligosaccharides (AcUXOS) from *E. globulus*. Designations are the same as in Table 1.

**Positional specificity**

The distribution of acetyl moieties over 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-O substituted with meGlcA and the free acetic acid obtained after an incubation of 16 h (endpoint) of AcUXOS with acetyl esterases from different families by monitoring the changes in signal intensities for the region 2.1 to 2.22 ppm, corresponding to the structural units (Figure 4), are shown in Figure 5. The acetyl esterases can be divided in three groups with respect to their positional specificity. Activity of acetyl xylan esterases was observed either towards 2-*O*, 3-*O* acetylated Xyl*p* or 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* or activity towards 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA. Through deacetylation of 2,3-di-*O* acetylated Xyl*p*, 2-*O* and 3-*O* acetylated Xyl*p* moieties are generated. Even though only a relatively small fraction of 15 % of the total acetylation represents doubly acetylated Xyl*p*, the deacetylation efficiency determined must be interpreted in the context of small amounts of 2-*O* and 3-*O* acetylated Xyl*p* being generated if the enzyme is active on diacetylated Xyl*p*. Regarding *Te*CE1, *Tr*CE5 and *An*CE5, 2-*O*, 3-*O* and 2,3-di-*O* acetylated Xyl*p* were nearly completely deacetylated, as discussed below. Therefore the generation of 2-*O* and 3-*O* acetylated Xyl*p* from 2,3-di-*O* acetylated Xyl*p* is not relevant regarding the deacetylation efficiencies obtained for these enzymes. Regarding *Os*CE6 and *An*CE16, the deacetylation efficiency could be slightly higher for 2-*O* and 3-*O* acetylated Xyl*p* as small amounts of 2-*O* and 3-*O* acetylated Xyl*p* were generated. Monitoring of the initial 30 min of the hydrolysis, as discussed below, allowed a clear determination of the activity of acetyl esterases towards different acetylated Xyl*p*.

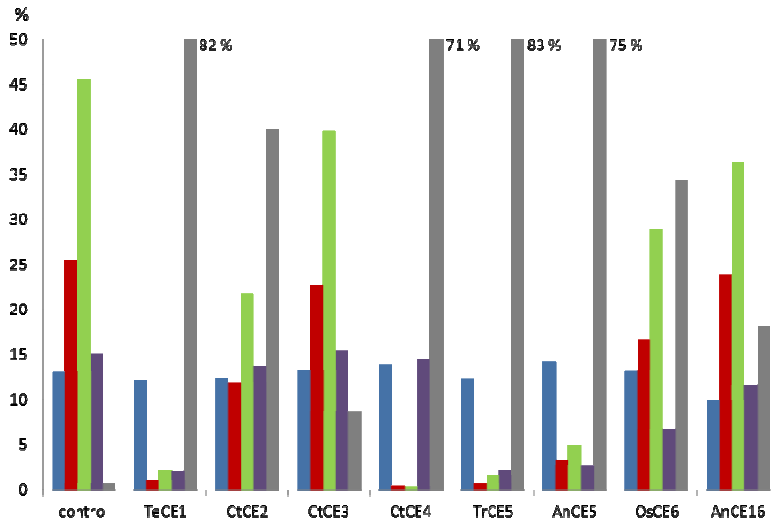


Figure 5: Distribution of acetyl moieties over 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* α-1,2-substituted with meGlcA present in acetylated 4-*O*-methyl glucuronic acid (meGlcA)-substituted xylo-oligosaccharides (AcUXOS) from *E. globulus* and free acetic acid as proportion (%) of the sum of the total acetyl groups present after incubation of AcUXOS with acetyl esterases from different CE families (incubation time: 16 h); structural units: (blue) –Xyl 3-*O* Ac 2-meGlcA-, (red) -Xyl 2-*O* Ac-, (green) –Xyl 3-*O* Ac-, (purple) –Xyl 2,3-*O* Ac-, (grey) acetic acid; the control represents AcUXOS without enzyme treatment.

*Activity exclusively towards 2-O and 3-O acetylated Xylp*

The bacterial acetyl xylan esterases from *C. thermocellum* (*Ct*CE2, *Ct*CE3 and *Ct*CE4) showed deacetylation of 2-*O* and 3-*O* acetylated Xyl*p*. No activity was observed towards 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA, until the end of hydrolysis. Complete deacetylation of 2-*O* and 3-*O* acetylated Xyl*p* was obtained for *Ct*CE4. Hydrolysis of the acetyl groups at these positions has also been described for the CE4 classified acetyl xylan esterase from *S. lividans* on aspen oligosaccharides (Uhliariková et al. 2013). As the $^{1}$H signals obtained show complete deacetylation of 2-*O* and 3-*O* acetylated Xyl*p*, activity of *Ct*CE4 towards terminal and internal acetylated xylopyranosyl units can be concluded. The presence of terminally acetylated xylo-oligosaccharides in AcUXOS was confirmed by mass spectrometry previously (Koutaniemi et al. 2013, Neumüller et al. 2014b). Furthermore, activity towards 2-*O* and 3-*O* acetylated Xyl*p* was not hindered by substituents (such as meGlcA) that could be present on neighbouring xylopyranosyl units. Moderate hydrolysis of these positions (52 % of both, the 2-*O* and 3-*O* acetylated Xyl*p*, were deacetylated) was observed for *Ct*CE2. Activity on synthetic substrates (4-*O*-acetyl 4-nitrophenyl β-D-xylopyranoside, methyl 3,4-*O*-diacetyl- and methyl 2,4-*O*-diacetyl- β-D-xylopyranoside) was reported for *Ct*CE2, suggesting a preference towards the artificial 4-*O* position which was observed for the CE4 esterase from *C. japonicus* (Topakas et al. 2010). In AcUXOS, the 4-*O* position is not acetylated. The data obtained show that in the absence of the 4-*O* acetyl group, a clear decrease of the 2-*O* and 3-*O* acetylated Xyl*p*, representing approximately 40 % of the total acetylation present in AcUXOS, was obtained with *Ct*CE2. Figure 3 shows that *Ct*CE3 was also able to hydrolyse both 2-*O* and 3-*O* acetylated Xyl*p*, although the deacetylation efficiency was low under the conditions applied. Approximately 9 % of the total acetylation present in AcUXOS was removed. Consistent to the activity by *Ct*CE3 towards acetylated xylo-oligosaccharides, activity by this enzyme was also reported towards birchwood xylan (Correia et al. 2008).

*Activity towards 2-O, 3-O and 2,3-di-O acetylated Xylp*

Clearly decreased $^{1}$H signals for 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* were obtained after incubation of AcUXOS with *Te*CE1, *Tr*CE5, *An*CE5 and *Os*CE6 in all cases, meaning that these enzymes are deacetylating 2-*O*, 3-*O* and 2,3-di-*O* acetylated Xyl*p*. Recalcitrance was observed towards the 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA. No decrease of the relative signal intensity as determined by $^{1}$H NMR was obtained for this position compared to the starting material. Nearly complete deacetylation of 2-*O*, 3-*O* and 2,3-di-*O* acetylated Xyl*p* was detected for *Te*CE1, *Tr*CE5 and *An*CE5. As described above, complete deacetylation indicates activity towards terminal and internal acetylated xylopyranosyl and tolerance towards meGlcA substituents that might be present on neighbouring xylopyranosyl units. These enzymes are efficiently deacetylating AcUXOS, releasing up to 83 % of the acetyl groups present in AcUXOS

as described above. The recalcitrant acetyl group (3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA) comprises 13 % of the total acetyl residues initially present, as described above.
Despite the lower deacetylation efficiency obtained for 2-*O*, 3-*O* and 2,3-di-*O* acetylated Xyl*p* by *Os*CE6 compared to *Tr*CE1 and the CE5 classified acetyl xylan esterases, a clear signal decrease (Figure 5) confirmed activity by *Os*CE6 towards the aforementioned positions.

*Activity towards 2-O, 3-O, 2,3-di-O acetylated Xylp and 3-O acetylated Xylp 2-O substituted with meGlcA*
For *An*CE16, moderate deacetylation efficiency was observed, as described above, for the acetylated positions present in AcUXOS. The signals corresponding to 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA decreased compared to the control (non-hydrolysed AcUXOS) while acetic acid was released. The decrease found for 2-*O* acetylated Xyl*p* after an incubation time of 16 h was low (13 %), indicating that *An*CE16 does not efficiently hydrolyse this position. A low deacetylation activity of 2-*O* acetylated Xyl*p* by the CE16 acetyl esterase from *T. reesei* was also observed on artificial substrate (Biely et al. 2011). Our data show that the signals obtained for 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA decreased more significantly (≥25 %). The highest signal decrease (30 %) was obtained for the 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA. This is relevant as previous studies using artificial substrates for the characterization of CE16 classified acetyl esterases do not include the determination of the activity towards acetylated Xyl*p* carrying a meGlcA substituent on the same residue (Biely et al. 2011). The proposed activity by *An*CE16 at the non-reducing end, as described above, explains the moderate deacetylation efficiency towards acetylated Xyl*p*. A higher deacetylation for the overnight incubation would have been expected if the enzyme would have activity towards “internal” acetylated Xyl*p* residues as well. Our data and previous observations (Biely et al. 2014) strengthen the hypothesized exo-acting activity by CE16 classified acetyl esterases from *A. niger* (this study) and *T. reesei* (Biely et al. 2014). The NMR data obtained showed no clear positional specificity towards the acetylated positions, while no obvious decrease was observed for the resonance corresponding to 2-*O* acetylated Xyl*p*.

*Deacetylation rates for different acetylated xylopyranosyl units*
Endpoint values for the deacetylation of AcUXOS indicate the ability of the acetyl esterases to catalyze the hydrolysis of acetyl groups at a specific location, but do not reflect the positional preferences at the start of hydrolysis of acetyl esterases. In order to determine the positional preferences on xylo-oligosaccharides, the deacetylation rate of acetyl esterases from different CE families was measured for 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA by $^{1}$H NMR spectroscopy. The effect of small amounts of 2-*O*, 3-*O* acetylated Xyl*p* being generated by acetyl esterases with activity towards 2,3-di-*O* acetylated Xyl*p* was discussed above. However, the initial deacetylation rates of the enzymes towards the

differently acetylated Xyl*p* could be determined as all acetylated Xyl*p* are present in access at the start of hydrolysis. Therefore, generation of small amounts of 2-*O* and 3-*O* acetylated Xyl*p* could be neglected for the calculation of the rates. A comparable relative decrease in signal intensities for 2-*O* and 3-*O* acetylated Xyl*p* was observed for the individual acetyl esterases with activity towards diacetylated Xyl*p* (data not shown), suggesting that both, 2-*O* and 3-*O* acetylated Xyl*p* could have been generated at comparable rates from 2,3-di-*O* acetylated Xyl*p*. Figure 6 shows an example of the hydrolysis curves obtained for the various acetyl positions by incubation with the CE5 classified acetyl xylan esterase (*An*CE5) from *A. niger* over 30 minutes. The hydrolysis curves obtained allowed investigation of the deacetylation rates on different acetylated Xyl*p* residues (acetyl groups removed/ min/ mg enzyme) by the acetyl esterases for each acetylated position based on the decrease of the resonance corresponding to 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xylp 2-*O* substituted with meGlcA. Enzyme recalcitrant acetyl groups were clearly identified by the presence of a constant signal over the time period, as observed for 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA, in the case of the *An*CE5 hydrolysed sample (Figure 6).

Table 2 shows the rates of hydrolysis for the deacetylation of 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA per mg acetyl esterase present. High deacetylation rates were observed for 2-*O* and 3-*O* acetylated Xyl*p* residues by *An*CE5, *Tr*CE5 and *Ct*CE4. *An*CE5 and *Tr*CE5 showed also high rates for the hydrolysis of the diacetylated xylopyranosyl units. Lower deacetylation rates were observed for *Te*CE1 and *Os*CE6 compared to *An*CE5 or *Tr*CE5. Furthermore, low rates were obtained for the bacterial *Ct*CE2 and *Ct*CE3. These enzymes were not active on diacetylated Xyl*p*. In the case of *Ct*CE3, activity on 2-*O* and 3-*O* acetylated Xyl*p* could only be concluded from the overnight incubation (Figure 5).

Regarding *An*CE16, a relatively low activity towards 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA was observed by monitoring the first 30 min of the hydrolysis (Table 2). Hydrolysis of these acetylated Xyl*p* residues was also clearly observed with the overnight incubation (Figure 5).

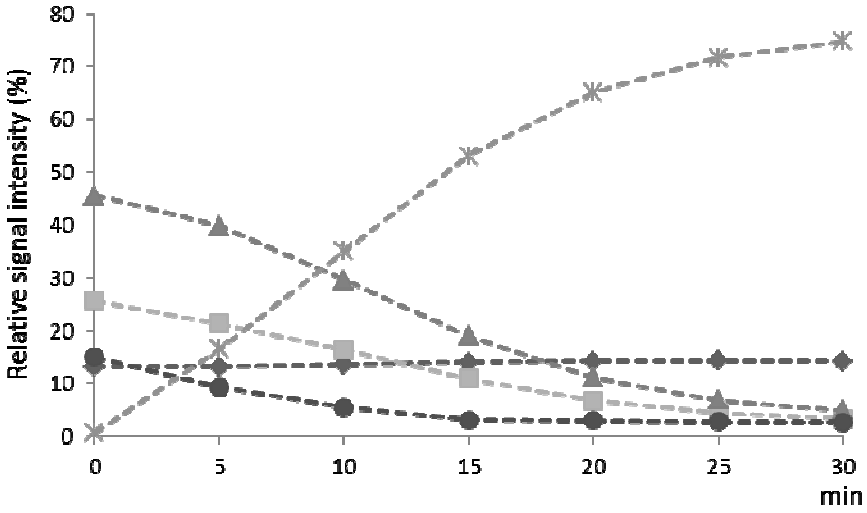


Figure 6: Relative signal intensities (% area of the total area of acetyl groups and acetic acid present) for acetyl xylan esterase CE 5 (*A. niger*) treated acetylated xylo-oligosaccharides. 3-*O* (triangle), 2-*O* (square), 2,3-di-*O* (circle) acetylated Xyl*p*, 3-*O* acetylated Xylp 2-*O* substituted with meGlcA (diamond) and acetic acid released (asterisk).

Table 2: Deacetylation rates (µmol acetyl groups removed/ min/ mg enzyme) by acetyl esterases for 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA from eucalyptus and rates for the total acetic acid released (µmol acetic acid released/ min/ mg enzyme).

| enzyme | -Xyl 3-*O* Ac 2-meGlcA- | -Xyl 2-*O* Ac- | -Xyl 3-*O* Ac- | -Xyl 2,3-*O* Ac- | acetic acid |
|---|---|---|---|---|---|
| *Te*CE1[a] | 0.0 | 12.7 | 25.5 | 44.0 | 74.6 |
| *Ct*CE2[b] | 0.0 | <0.01 | 6.5 | 0.0 | 5.7 |
| *Ct*CE3[b] | 0.0 | <0.01 | <0.01 | 0.0 | <0.01 |
| *Ct*CE4[b] | 0.0 | 88.1 | 356.6 | 0.0 | 365.7 |
| *An*CE5[a] | 0.0 | 202.7 | 276.2 | 274.4 | 754.7 |
| *Tr*CE5[a] | 0.0 | 171.8 | 289.2 | 92.5 | 540.9 |
| *Os*CE6[a] | 0.0 | 48.64 | 92.2 | 50.3 | 117.7 |
| *An*CE16[b] | 0.03 | 1.80 | 1.92 | 1.50 | 5.25 |

[a] µmol acetyl groups removed/ min/ mg enzyme and acetic acid released/ min/ mg enzyme after 5 min.
[b] µmol acetyl groups removed/ min/ mg enzyme and acetic acid released/ min/ mg enzyme after 30 min.

*Positional preference on acetylated xylo-oligosaccharides*

In order to evaluate whether acetyl esterases showed a positional preference towards certain positions, the ratios of the deacetylation rates obtained per relative amount of acetyl groups present for each position were calculated (Table 3). Determination of this ratio shows the preference of the enzyme towards each acetylated position. For some enzymes (*Ct*CE2, *Ct*CE3 and *An*CE16), the deacetylation activity was too low to determine clear positional preferences.

The effect by the generation of 2-*O* and 3-*O* acetylated Xyl*p* by deacetylation of 2,3-di-*O* acetylated Xyl*p*, has been discussed above.

The data obtained (Table 3) indicate a clear preference towards doubly acetylated Xylp for *Te*CE1. Preference towards diacetylated xylopyranosyl units has also been described for the CE1 classified acetyl xylan esterase from *S. commune* (Biely et al. 2013). For *Ct*CE4, a high affinity was obtained for the acetylated 3-*O* Xyl*p*, being more than twice as high as for 2-*O* Xyl*p*. This shows that *Ct*CE4 preferably attacks 3-*O* acetylated Xyl*p*. *An*CE5 and *Os*CE6 showed a higher preference towards 2,3-di-*O* acetylated Xyl*p* than for 2-*O* and 3-*O* acetylated Xyl*p*. No such positional preference was observed for *Tr*CE5, which deacetylated all positions (besides 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA) with similar preference. The results obtained for the CE5 classified esterases (*Tr*CE5 and *An*CE5) emphasize that differences can occur amongst acetyl xylan esterases from different species belonging to the same CE family regarding their positional preference. Therefore, it can be concluded that an automated, sequence based classification of esterases does not represent positional preferences of the enzymes. More representatives of one enzyme class need to be compared in order to conclude whether positional preferences are conserved for different CE families.

Table 3: Positional preference on acetylated xylo-oligosaccharides, calculated as the positional rate per relative amount of acetyl groups present (2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA) at the start of hydrolysis. The data were normalized.

| enzyme | -Xyl 3-*O* Ac 2-meGlcA- | -Xyl 2-*O* Ac- | -Xyl 3-*O* Ac- | -Xyl 2,3-*O* Ac- |
|---|---|---|---|---|
| *Te*CE1 | 0 | 12 | 14 | 74 |
| *Ct*CE2 | 0 | n.d. | n.d. | 0 |
| *Ct*CE3 | 0 | n.d. | n.d. | 0 |
| *Ct*CE4 | 0 | 30 | 70 | 0 |
| *An*CE5 | 0 | 24 | 19 | 57 |
| *Tr*CE5 | 0 | 35 | 33 | 32 |
| *Os*CE6 | 0 | 26 | 28 | 46 |
| *An*CE16 | n.d. | n.d. | n.d. | n.d. |

0, no activity; n.d., not determined.

## Conclusions

Depending on acetylation patterns of different substrates, suitable acetyl esterases can be selected from the data obtained (Tables 2 and 3). Knowledge on the deacetylation efficiency and the positional preference of acetyl esterases, as determined for common fungal and microbial esterases, allows to design optimal, deacetylating enzyme mixtures. The enzymes tested could be categorized in three groups of acetyl esterases with activities towards (i) 2-*O*,

3-*O* and (ii) 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and (iii) 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA. High deacetylation rates were obtained for *Ct*CE4, *Tr*CE5 and *An*CE5 (Table 2). Positional preferences were obtained towards 2,3-di-*O* acetylated Xyl*p* (*Te*CE1, *An*CE5 and *Os*CE6) or 3-*O* acetylated Xyl*p* (*Ct*CE4). For the deacetylation of acetylated 4-*O*-methyl glucuronic acid-substituted xylo-oligosaccharides a combination of the CE5 (*Tr*CE5 or *An*CE5) and the CE16 classified acetyl esterases (*An*CE16) is most efficient.

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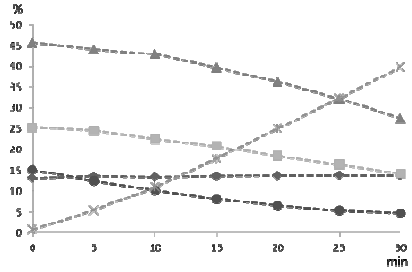
## Supplementary information

## Positional preferences of acetyl esterases from different CE families towards acetylated 4-*O*-methyl-glucuronic acid substituted xylo-oligosaccharides

*Te*CE1

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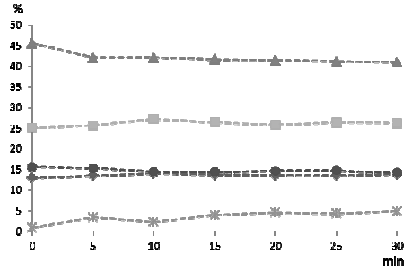


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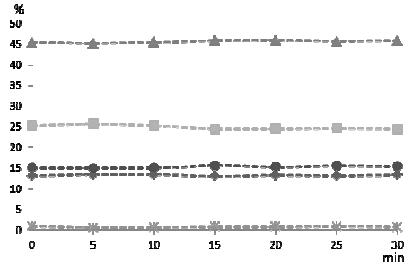


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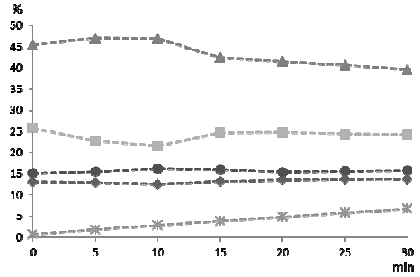
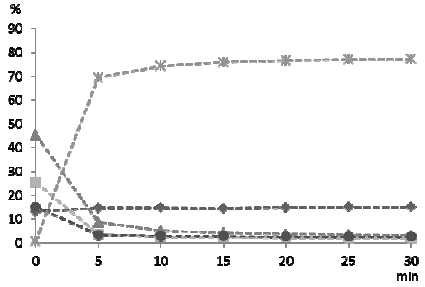
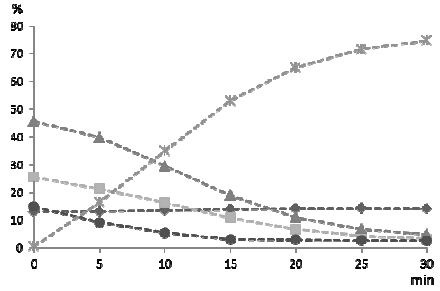


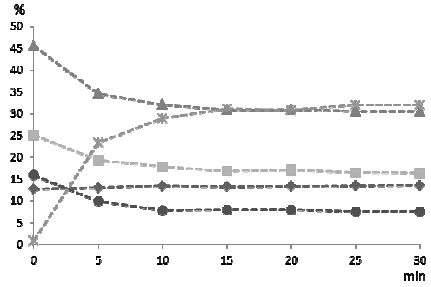

*Tr*CE5




*An*CE5




*Os*CE6




*An*CE16

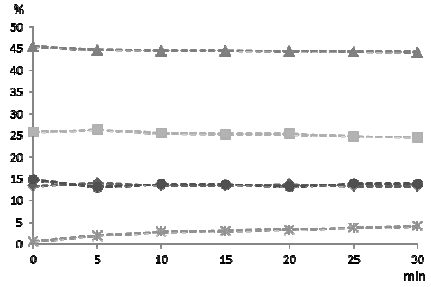



Figure S1: Relative signal intensities (% area of the total area of acetyl groups and acetic acid present) for enzyme treated acetylated xylo-oligosaccharides with acetyl esterase from different CE families. 3-*O* (triangle), 2-*O* (square), 2,3-di-*O* (circle) acetylated Xyl*p*, 3-*O* acetylated Xylp 2-*O* substituted with meGlcA (diamond) and acetic acid released (asterisk).

*CHAPTER 7*

# General discussion

## Motivation of the research

(Hemi)cellulolytic enzymes hydrolyze plant poly- and oligosaccharides to fermentable sugars. However, lignocellulosic feedstocks show significant recalcitrance towards commercially available enzyme preparations. A fundamental understanding of the nature of recalcitrance factors of lignocellulosic feedstocks on the one hand and the enzymatic hydrolysis process on the other hand are important to enhance biochemical feedstock conversion. An efficient enzymatic hydrolysis results in high conversion of poly- and oligosaccharides to monosaccharides and may require lower enzyme loadings or allow for a higher initial dry matter by an accelerated liquefaction. In the context of biogas from corn silage, these factors are crucial for the feasibility of the process. This thesis aimed for an improved understanding of the biochemical conversion regarding the rather recalcitrant xylan as present in corn silage. Therefore, recalcitrance factors towards the enzymatic conversion of corn silage xylans, varying in type and level of substitution, were addressed. A better understanding of the enzymatic hydrolysis of complex xylans enables the improvement of enzyme mixtures towards the rather recalcitrant hemicellulose.

## The role of glycosyl-, acetyl- and phenoyl-substitution in enzymatic hydrolysis

### Determination of acetyl and phenoyl ester contents of polysaccharides

For the determination of acetyl and phenoyl ester contents a $^{1}$H NMR based method was developed, as described in chapter 3. The method combines an optimized sample preparation procedure and high throughput analysis. This resulted in short analysis times and low errors as the method requires only a single and relatively easy sample preparation compared to other methods reported. The latter include separate and specific sample preparation and analysis procedures for acetyl (Voragen et al. 1986), feruloyl and *p*-coumaroyl esters (Robbins et al. 2003, Van Dongen et al. 2011). As NMR equipment is not always available, it should be noted that the optimized sample preparation procedure (saponification/sonication conditions) can also be transferred using (U)HPLC for quantification of acetyl and phenoyl ester contents by use of the appropriate standards (acetic, ferulic acid and *p*-coumaric acid).

### Acetylation

Acetylation is a major recalcitrance factor, especially for biogas production from corn silage. In corn silage, the degree of acetylation was approximately ten and thirteen times higher than the degree of *p*CA and FA substitution, respectively (Neumüller et al. 2014a).

The compositional analysis, as described in chapter 4, revealed high acetylation for corn silage water unextractable solids (WUS), representing all insoluble corn cell wall polysaccharides present, enzyme recalcitrant solids (ErCS) from WUS and an industrial corn silage based

anaerobic fermentation residue (AFR), being 0.36, 0.40 and 1.2 mol acetyl groups/ mol Xyl, respectively. The levels of individual glycosidic substitution were lower than the acetylation levels. The high acetylation of AFR shows that acetylated structures are recalcitrant towards utilization during the anaerobic fermentation in biogas plants. Furthermore, the high acetylation is likely to not only cause recalcitrance towards the anaerobic bacterial fermentation (biogas production process), but also towards the enzymatic hydrolysis by commercial, fungal enzyme preparations, as discussed below. The acetyl ester content of xylans is especially important for the enzymatic conversion of feedstocks, in case that no alkaline pretreatment causing removal of these esters (Agbor et al. 2011), is applied. Acetyl xylan esterases are necessary to render acetylated xylan accessible for glycosyl hydrolases.

In order to study the mode of action of acetyl xylan esterases, acetylated linear xylo-oligosaccharides (AcXOS) and 4-*O*-methyl glucuronic acid (meGlcA)-substituted xylo-oligosaccharides (AcUXOS) from *Eucalyptus globulus* (Table 1) are suitable model substrates, as described in chapters 5 and 6 (Neumüller et al. 2014b, 2014e). Differently acetylated Xyl*p*, being 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA, are present in eucalyptus xylan (Evtuguin et al. 2003). Characterization of the acetylated meGlcA substituted xylo-oligosaccharide fraction (AcUXOS) revealed a distribution of 26 % of the acetyl groups present being attached at *O*-2, 46 % at *O*-3 of mono-acetylated Xyl*p*, 15 % being present at 2,3-di-*O* acetylated Xyl*p* and 13 % being attached at *O*-3 of α-1,2-meGlcA substituted Xyl*p* (chapter 6). Presence of a second substituent (e.g. acetyl or meGlcA residue) on acetylated Xyl*p* renders the Xyl*p* recalcitrant towards enzymatic hydrolysis, as discussed below.

**Glycosidic substituents**

Next to acetylation, relatively high levels of glycosyl substitution, with Ara being the main glycosyl substituent, are present in xylan from corn silage fractions (Table 1). The xylan fractions obtained by saturated barium hydroxide extraction from WUS fractionated by graded ethanol precipitation (AX 20, AX 40 and AX 60; Table 1) show degrees of glycosidic substitution between 0.2 and 0.4. They were susceptible to enzymatic hydrolysis as incubation with the *A niger / T. emersonii* enzyme preparation resulted in Xyl release ≥ 76 %. With increasing DS, higher recalcitrance of the xylan towards enzymatic conversion is indicated. Especially the high substituted fraction present in corn silage (AX 80S, DS = 1.1) resists conversion, where Ara represents 67 % of the total substituents present. The industrial residue AFR (DS = 1.7) is highly recalcitrant as well, showing that xylan with a DS > 1 could not be hydrolyzed. Recalcitrant UA and Gal substituted (arabino-)xylo-oligosaccharides resist the enzyme treatment (*A. niger / T. emersonii* enzyme preparation) of the de-esterified barium hydroxide extracted xylan fractions (fractions AX 20 to AX 80S; Table 2). Such recalcitrant oligosaccharides (Table 2) provide a

suitable substrate for enzyme screening to explore microbes for the presence of accessory enzymes with activity towards recalcitrant structures present in corn.

Table 1: Composition of fractions from corn silage.

| | Composition[a] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fraction** | **starch** | **Rha** | **Ara** | **Xyl** | **Man** | **Gal** | **non-starch Glc** | **UA[b]** | **Ac** | **FA** | ***p*CA** | **protein** | **acid insoluble material** |
| Corn silage | 29 | 0.4 | 3 | 12 | 1 | 1 | 12 | 2 | 2.1 | 0.5 | 0.6 | 8 | 9 |
| WUS[c] | 0.4 | 0.2 | 4 | 24 | 1 | 1 | 31 | 3 | 3.9 | 0.9 | 1.2 | 4 | 20 |
| cWUS | 0.4 | <1 | 5 | 25 | <1 | 1 | 41 | 3 | n.d. | n.d. | n.d. | n.d. | 2 |
| ErCS | 0.1 | 0.3 | 3 | 23 | 0.1 | 1 | 35 | 2 | 4.2 | 0.6 | 1.8 | 3 | 30 |
| AFR[c] | 0.5 | 0.2 | 2 | 8 | 0.4 | 1 | 11 | 2 | 4.0 | not detected | 0.7 | 31 | 48 |
| WEX | n.d. | 0.4 | 1 | 1 | 5 | 1 | 12 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *AX total*[d] | n.d. | 0.1 | 12 | 39 | 0.1 | 2 | 1 | 4 | n.d. | n.d. | n.d. | 7 | 12 |
| AX 20 | n.d. | 0.1 | 4 | 28 | 0.1 | 0 | 6 | 2 | n.d. | n.d. | n.d. | 13 | 20 |
| AX 40 | n.d. | 0.1 | 8 | 45 | 0.1 | 1 | 1 | 3 | n.d. | n.d. | n.d. | 7 | 5 |
| AX 60 | n.d. | 0.1 | 15 | 51 | 0 | 3 | 0 | 5 | n.d. | n.d. | n.d. | 2 | 3 |
| AX 80 | n.d. | 0.2 | 23 | 36 | 0.3 | 4 | 1 | 6 | n.d. | n.d. | n.d. | 5 | 4 |
| AX 80S | n.d. | 0 | 2 | 3 | 0.2 | 1 | 1 | 1 | n.d. | n.d. | n.d. | 19 | 43 |
| AcXOS[e] | n.d. | n.d. | 2 | 80 | 6 | 8 | 9 | 0 | 11.7 | n.d. | n.d. | n.d. | n.d. |
| AcUXOS[e] | n.d. | n.d. | 0 | 77 | traces | 4 | 0 | 21[f] | 21.0[g] | n.d. | n.d. | n.d. | n.d. |

[a] expressed as weight percentage (DM).
[b] UA, uronic acid.
[c] Neumüller et al. 2014a.
[d] calculated from the compositional data obtained for fractions AX 20, 40, 60, 80 and 80S.
[e] Koutaniemi et al. 2013.
[f] present as meGlcA.
[g] determined by $^{1}$H NMR analysis (Neumüller et al., 2014e).
WUS, water unextractable solids from corn silage; cWUS, sodium chlorite treated WUS; ErCS, enzyme recalcitrant solids from WUS; AFR, anaerobic fermentation residue; WEX, water extractable fraction from corn silage; AX, xylan fractions obtained by saturated barium hydroxide extraction and graded ethanol precipitation from WUS; AcXOS linear xylo-oligosaccharides; AcUXOS, meGlcA substituted AcUXOS xylo-oligosaccharides; n.d., not dermined.

Table 2: Relative amounts (%) per oligosaccharide of the total oligosaccharides present for each enzyme treated xylan fraction (AX 20 to AX 80S) corresponding to the masses of ions detected by Maldi-Tof MS.

| oligosaccharides | AX 20 | AX 40 | AX 60 | AX 80 | AX 80S |
|---|---|---|---|---|---|
| $P_1H_1$ | | 7 | 1 | 3 | 5 |
| $P_1UA_1$ | 7 | 3 | | | 19 |
| $P_1UA_1H_1$ | 7 | | | | |
| $P_2$ | 86 | 56 | 64 | 82 | 67 |
| $P_2Ac_1$ | | | 1 | 3 | 3 |
| $P_2H_1$ | | 3 | | | |
| $P_2OmeUA_1$ | | 2 | 2 | 2 | |
| $P_3Ac_1$ | | | | | 3 |
| $P_3UA_1$ | | | | | 3 |
| $P_4$ | | 21 | 4 | 4 | |
| $P_4H_1$ | | 3 | 15 | 2 | |
| $P_4UA_1$ | | | 1 | | |
| $P_5$ | | 5 | 5 | | |
| $P_5H_1$ | | | 7 | 2 | |
| $P_6$ | | | | 2 | |

P, pentose; H, hexose; UA, uronic acid; OmeUA, *O*-methylated uronic acid; Ac, acetyl.

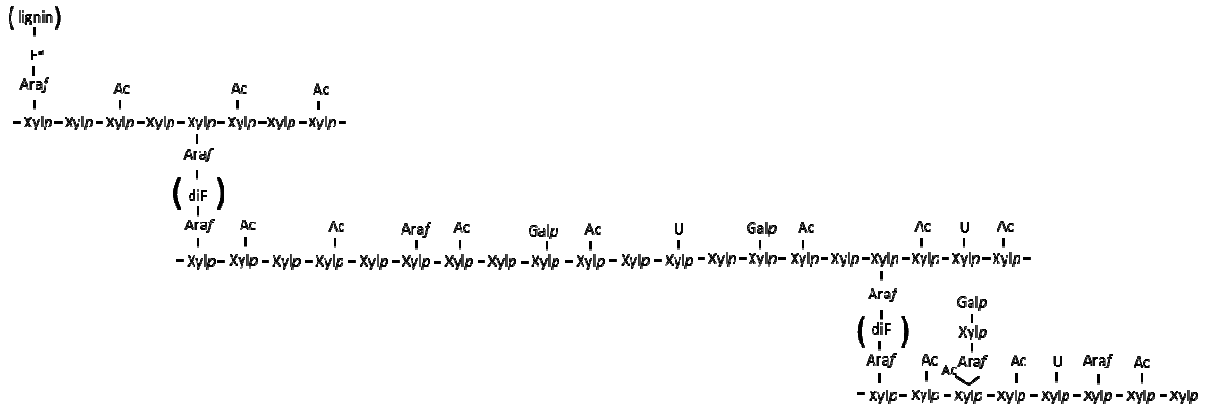
**Water unextrectable xylan present in corn silage**

Based on the compositional data obtained of different corn fractions (Table 1) and literature data (Agger et al. 2010, Ebringerová 2006, Appeldoorn et al. 2013), hypothetical models of the xylan as present in WUS and the recalcitrant AFR are proposed, as shown in Figure 1. Regarding WUS, the hypothetical model is based on the compositions of saturated barium hydroxide extracted xylan populations from WUS fractionated by graded ethanol precipitation (fractions AX 20 to AX 80S, Table 1) and the composition of the total xylan present (Table 1). The different xylan fractions (AX 20 to AX 80S) show a significant variation in DS (0.2 to 1.1) and these xylans might have been connected via diferulate crosslinks to form the complex xylan network in the parental material. A significant proportion (77 %) of xylan cannot be extracted from WUS by the saturated barium hydroxide treatment and may represent additional different (alkali unextrectable) xylan populations present in WUS. The acetyl and phenoyl ester contents of WUS were determined (Neumüller et al. 2014a), representing the esterification of the water unextractable xylan of corn silage (Table 1).

As illustrated in Figure 1a, the backbone of xylan as present in WUS is formed by xylopyranosyl units. Next to acetyl and phenoyl esters that decorate the xylan backbone (chapter 4), glycosidic substituents, such as α-(1→ 2)-linked 4-(*O*-methyl-D)-glucopyranosyl uronic acid units, α-(1→ 3)-linked L-arabinofuranose residues, 2-*O*-β-D-xylopyranosyl-α-L-arabinofuranose moieties (Ebringerová 2006) and complex side chains, such as α-L-galactopyranosyl-(1→2)-β-D-xylopyranosyl-(1→2)-5-*O*-trans-feruloyl-L-arabinofuranose attached to the *O*-3 position of Xyl*p*, are present (Appeldoorn et al. 2013). Ferulic acids can be esterified to arabinofuranosyl residues at position *O*-5 (Agger et al. 2010) and occur both on single arabinofuranosyl

substituents and arabinofuranosyl residues of heterogeneous side chains (Figure 1; Saulnier et al. 1995). Arabinofuranosyl residues can be linked to both *O*-2 and *O*-3 of doubly substituted xylopyranosyl units present in corn (Agger et al. 2010). Furthermore, ferulic acids have been isolated as dimers, trimers or tetramers from corn (Bunzel et al. 2006), whereas diferulates may cross-link xylans (Hatfield et al. 1999) or xylan and lignin (Lapierre et al. 2001).
A more heavily substituted xylan present in the corn silage based AFR (Figure 1b) indicates high recalcitrance towards enzymatic conversion.

**a**




**b**

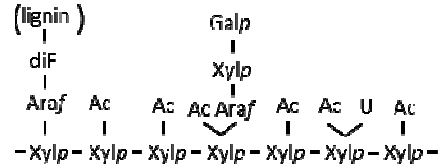



Figure 1: Simplified sketches of the (a) water unextractable xylan from corn silage and (b) xylan from a recalcitrant, corn silage based anaerobic fermentation residue. Xyl*p*, xylopyranosyl residue; Ara*f*, arabinofuranosyl residue; Gal*p*, galactopyranosyl residue; U, glucuronosyl residue; F, feruloyl residue; diF, diferulic acid; Ac, acetyl residue; possible linkage to lignin or xylan is shown in brackets.
[b] Can be present as diferulic acid and linked to lignin or xylan.

## Enzymatic hydrolysis of xylan

### Strategies for corn silage polysaccharide conversion by industrial enzyme preparations

An important conclusion of the data obtained is that combinatorial screening of crude enzyme preparations is suitable to identify enzyme combinations in order to improve the performance of hemicellulolytic mixtures. The efficient combinations of crude enzyme preparations and the *Aspergillus niger / Talaromyces emersonii* enzyme preparation towards the conversion of WUS, ErCS and AFR (chapter 4) provide a resource for the optimization of the enzymatic hydrolysis

towards corn silage polysaccharides and recalcitrant residues, using crude enzyme mixtures. Commercial enzyme preparations are not optimized for the hydrolysis of corn silage polysaccharides due to a lack of accessory activities present in the secretomes.

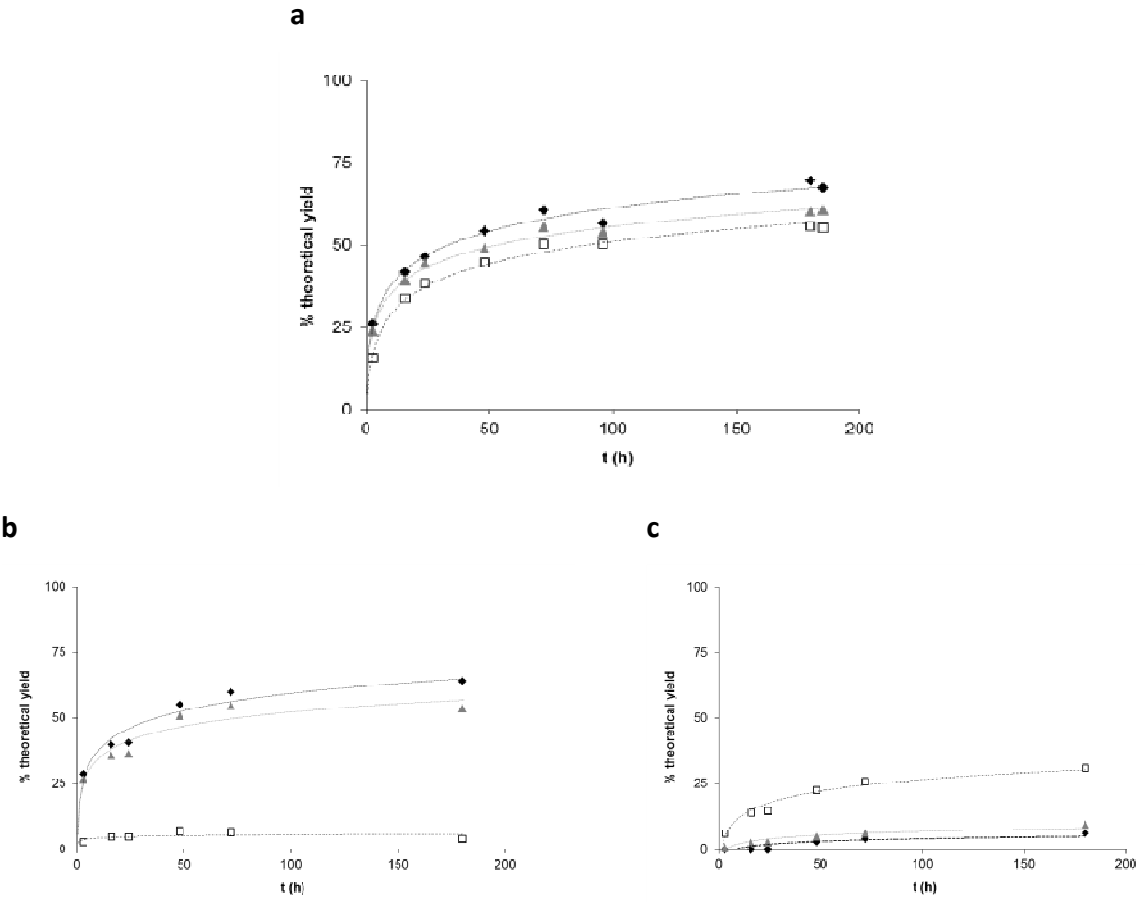



Figure 2: Proportion (%) of Xyl, Ara and Glc released from corn silage water unextractable solids by incubation with (a) an *A. niger / T. emersonii* culture filtrates mixture and culture filtrates from (b), *A. niger*, (c), *T. emersonii*, applied at a constant protein dosage (100 g protein $kg^{-1}$ substrate).

*A. niger / T. emersonii enzyme mixture*

An *A. niger / T. emersonii* culture filtrate mixture was recognized to be efficient towards the conversion of corn silage polysaccharides to monosaccharides (chapter 4). Combination of the individual culture filtrates (*A. niger* and *T. emersonii*) shows a significant increase in the Xyl, Ara and Glc release from cellulose and hemicellulose (Figure 2a) compared to the hydrolysis with individual culture filtrates (Figures 2b and 2c). The conversion of xylan and cellulose by the *A. niger / T. emersonii* enzyme mixture was comparable to the conversion by the most efficient commercial enzyme preparations (Neumüller et al. 2014a). This shows the potential of mixtures

of culture filtrates from different fungal species regarding the conversion of polysaccharides to monosaccharides and suggests a further search for combinations of crude culture filtrates in order to find highly efficient enzyme combinations. Despite the relatively high efficiency by the *A. niger / T. emersonii* enzyme mixture, a significant fraction of the xylan present in WUS (39 %) was still recalcitrant towards the conversion to monosaccharides (61 % of the Xyl was released, Figure 2a), indicating a further need for optimization in order to obtain complete conversion of xylan.

*Conversion of heavily glycosidic substituted xylan*

Low conversion of the extracted xylan fraction AX 80S was obtained with the *A. niger / T. emersonii* enzyme preparations. Considering the high DS of this fraction (1.1 mol substituent/ mol Xyl), it is clear that the *A. niger / T. emersonii* preparation lacks accessory activities towards heavily substituted xylan. A lack of accessory enzyme activities towards arabinofuranosyl residues or arabinofuranosyl residues present in complex side chains is indicated as fraction AX 80S showed a high $DS_{Ara}$ (0.7 mol Ara per mol Xyl), whereas only 50 % of this substituent (Ara) was released. Furthermore, the presence of recalcitrant Gal and UA substituted Ara and Xyl residues, after hydrolysis of the de-esterified, extracted xylan fractions (Table 2), confirms a lack of accessory enzymes targeting these structures. Hence, further optimization of the *A. niger / T. emersonii* preparation by addition of “lacking” accessory enzymes is required.

*Conversion of complex xylan embedded in plant cell wall structures*

The Xyl release by incubation with the *A. niger / T. emersonii* enzyme preparation as proportion from the xylan present in WUS (29 %), chlorite treated (delignified) WUS (69 %), and AFR (7 %) was significantly lower compared to Xyl released for extracted, de-esterified xylan fractions (a Xyl release of 79 % was calculated based on the Xyl released from fractions AX 20 to AX 80S; incubations were done at 50 °C, 48 h; chapter 2). This shows that enzymatic conversion of esterified xylan, embedded in other plant cell wall structures (cellulose, lignin and proteins) is not efficient. Recalcitrance factors, such as acetyl and phenoyl esterification, linkage of the hemicellulose to lignin (Vanholme 2010) or the interaction between cellulose and hemicellulose (Kabel et al., 2007) cause these lower conversion yields for WUS, chlorite WUS and AFR. Furthermore, the presence of acetyl groups and lignin is indicated to cause also a low rate of hydrolysis by limiting the access for glycosyl hydrolases. Regarding the recalcitrance caused by the presence of xylan substituents, it is indicated that especially acetylation is relevant, as the $DS_{Ac}$ was high for corn silage (0.39) and the highly recalcitrant AFR (1.2).

*Combinatorial screening of crude enzyme preparations*

Screening of combinations of the crude enzyme mixtures allowed a categorization of enzyme preparations in: (i) enzyme preparations containing synergistic key activities, (ii) enzyme

preparations that may hydrolyze parts that cannot be processed by the *A. niger / T. emersonii* mixture or (iii) enzyme preparations that do not show additional activity compared to application of the *A. niger / T. emersonii* mixture as they target the same polysaccharide-fraction (chapter 4). For the hydrolysis of WUS, representing corn silage polysaccharides, and ErCS, representing the enzyme recalcitrant fraction of corn silage model polysaccharides, the enzyme preparations were applied at a slightly acidic pH, optimal hydrolysis conditions of most hemicellulolytic fungal enzyme preparations and typical for the enzymatic hydrolysis step in biofuels production used by the industry (Sun 2010). The industrial biogas plant residue AFR was incubated at alkaline pH, as recalcitrance of this residue is, next to its structural complexity, also caused by the elevated alkaline processing conditions as occurring during the anaerobic fermentation process. Enzyme preparations with tolerance to high pH and at the same time activity towards xylan with a high degree of substitution, would be advantageous for industrial processing applications of this residue.

An efficient mixture of enzyme preparations towards WUS as well as the recalcitrant polysaccharides is a combination of culture filtrates from *A. niger*, *T. emersonii* and *Trichoderma longibrachiatum* (Figure 3). The high conversion as obtained by supplementation of the *T. longibrachiatum* enzyme preparation to the *A. niger / T. emersonii* preparation was further investigated and an individual synergistic enzyme was recognized, as discussed below (7.3.2.). Further examples of efficient enzyme combinations are shown in Figure 3 [(e.g. *A. niger / T. emersonii* together with *Humicola insolens* filtrate or with Optimase® (unknown fungal strain)]. Diverse sets of enzymes are present in industrial enzyme preparations that are not listed or are even not known to the enzyme provider. The hydrolytic gain by combinatorial application of culture supernatants is due to differences in the secretomes of different microbial species. Differences in the secretomes can even occur by the same species, when cultivated on distinct carbon sources (Ferreira de Oliveira et al. 2011). The positive hits obtained by the combinatorial screening approach (Figure 3) provide a profound source for the optimization of the industrial *A. niger / T. emersonii* enzyme preparation. Such screening approach towards efficient combinations of crude enzyme mixtures, embedding the current *state-of-the-art* enzyme technology, was shown to be efficient in increasing the conversion of recalcitrant substrates. However, in order to understand which enzymes present in such complex enzyme mixtures act in synergy with enzyme preparations, purification of individual enzymes and biochemical characterization is required, as discussed below for the mixture of the *T. longibrachiatum* enzyme preparation and the *A. niger / T. emersonii* enzyme preparation. Classical screening approaches towards the activity of enzymes on generic substrates are not suitable for a targeted identification of accessory enzymes that may act in synergy with crude enzyme preparations.

In order to further improve enzymatic conversion using available industrial enzyme preparations, a more dynamic process may be applied, where certain activities are

supplemented over time, adjusted to the changing recalcitrance state of the substrates with proceeding hydrolysis. An example is the supplementation of acetyl xylan esterase rich enzyme preparations with proceeding anaerobic fermentation of corn silage in order to obtain higher yields.

**a** **b**

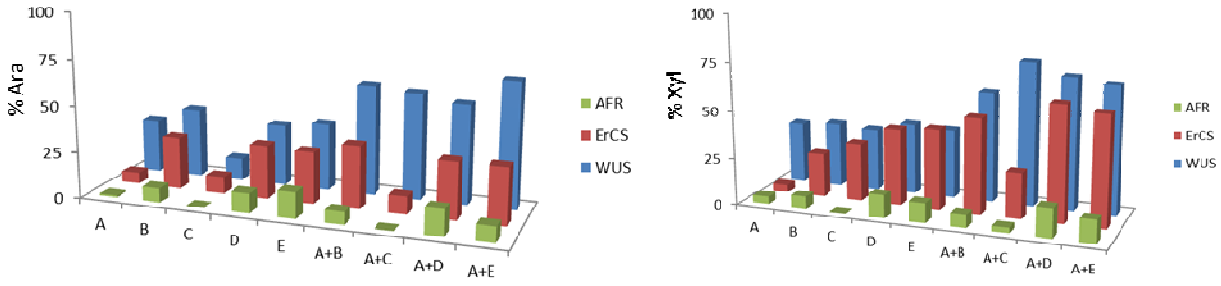



Figure 3: Proportion (%) of (a) Xyl and (b) Ara released from corn silage water unextractable solids (WUS), enzyme recalcitrante solids (ErCS) and an anaerobic fermentation residue (AFR) by incubation with crude enzyme preparations and combinations of crude enzyme preparations. A, *Aspergillus niger / Talaromyces emersonii* enzyme preparation; B, *Trichoderma longibrachiatum* enzyme preparation (Dyadic® Xylanase Plus); C, *Trichoderma reesei* enzyme preparation (MethaPlus®); D, *Humicola insolens* enzyme preparation (Ultraflo® L); E, *unknown fungal strain* (Optimase®).

**Recognition of synergistic individual enzymes from complex mixtures**

A major conclusion is that an efficient hemicellulolytic enzyme preparation towards corn silage polysaccharides consists of a culture filtrates mixture of *A. niger* and *T. emersonii* secretomes supplemented with an acetyl xylan esterase classified as belonging to CE family 5 from *T. longibrachiatum* or *Trichoderma reesei*. The acetyl xylan esterases belonging to CE family 5 (*T. reesei*, *T. longibrachiatum*) efficiently deacetylate xylan, rendering it more susceptible for the degradation by hemicellulolytic enzyme preparations.

The synergy observed by supplementation of the acetyl xylan esterase (CE5) rich fraction from *T. longibrachiatum* to the *A. niger / T. emersonii* enzyme preparation was confirmed by supplementation of a cloned, pure CE5 classified acetyl xylan esterase from *T. reesei* (Neumüller et al. 2014c), as described below (textbox 7.1.). This shows that the hydrolytic increase is due to supplementation of the CE5 acetyl xylan esterase to the CE1 esterase containing *A. niger / T. emersonii* enzyme preparations. The CE5 classified acetyl xylan esterase (*T. longibrachiatum*) resulted in the deacetylation of oligosaccharides, which were not hydrolyzed by the CE1 classified acetyl xylan esterases present in the *A. niger / T. emersonii* preparation as demonstrated by mass spectrometry (chapter 5). The differences observed for the deacetylation of acetylated xylo-oligosaccharides by the CE5 and CE1 classified acetyl xylan

esterases emphasize the importance for an analysis of the deacetylation efficiency on natural, plant substrates.

Textbox 7.1: *Acetyl xylan esterase 1 (T. reesei, carbohydrate esterase family 5) supplemented to a (hemi)cellulolytic preparation enhances degradation of recalcitrant corn silage polysaccharides (Neumüller et al. 2014c)*

The acetyl xylan esterase from *T. reesei* belonging to CE family 5 (*Tr*CE5), was cloned and expressed in *A. niger* ISO527 and supplemented to the *A. niger / T. emersonii* enzyme preparation. High homology between *Tr*CE5 and the CE 5 classified acetyl xylan esterase from *T. longibrachiatum* is indicated, as LC/MS analysis of the CE esterase enriched fraction from *T. longibrachiatum* matched with database entries for the protein sequence of *Tr*CE5 (Neumüller et al. 2014b). The conversion of corn silage water unextractable solids (WUS) by the hemicellulolytic enzyme preparation containing *Tr*CE5 was monitored.

Materials and methods

*Materials:* The corn silage water unextractable solids (WUS) used were described previously (Neumüller et al. 2014a). The composition of WUS is shown in Table 1. The *A. niger* ISO527 strain was from DSM (Heerlen, The Netherlands) and was used for expression of the cloned acetyl xylan esterase.

*Cloning and expression of TrCE5:* Cloning and expression of the codon pair optimized sequence of *Tr*CE5 for expression in *A. niger* ISO 527 was described previously (Neumüller et al. 2014c)

Codon pair optimized sequence, *Tr*CE5:

>jgi_trire1_32857 acetyl xylan esterase T. reesei (Hypocrea jecorina) – codon pair optimized for expression in *A. niger* ISO 527

aatgCCCTCCGTCAAGGAGACTCTGACCCTCCTCCTCTCCCAGGCTTTCCTGGCCACTGGCTCTCCTGTGGATGGCGAGACTGTTGTCAAGCGTCAGTGCCCCGCCATTCACGTTTTCGGTGCTCGCGAAACCACCGTCAGC
CAGGGCTACGGCAGCTCTGCCACTGTCGTCAACCTGGTCATTCAGGCTCACCCCGGCACCACCAGCGAAGCCATTGTCTACCCCGCCTGCGGTGGCCAGGCCTCTTGCGGTGGTATCTCCTACGCCAACTCCGTTGTCAA
CGGCACCAACGCCGCTGCTGCTGCCATCAACAACTTCCACAACTCCTGCCCCGACACCCAGCTTGTTCTTGTTGGATACAGCCAGGGTGCTCAGATCTTCGACAACGCCCTCTGCGGTGGTGGTGACCCCGGTGAGGGT
ATCACCAACACTGCTGTTCCTTTGACTGCTGGTGCTGTCTCTGCCGTCAAGGCCGCCATCTTCATGGGTGACCCTCGCAACATCCACGGTCTGCCCTACAACGTCGGCACCTGCACCACCCAGGGTTTCGATGCCCGTCCT
GCTGGCTTCGTCTGCCCCTCCGCCTCCAAGATCAAGTCCTACTGCGATGCTGCCGACCCCTACTGCTGCACTGGCAACGACCCCAACGTCCACCAGGGATACGGCCAGGAGTACGGCCAGCAGGCTCTTGCTTTCATCAA
CTCCCAGCTCTCCTCCGGTGGCAGCCAGCCTCCTGGTGGTGGTCCTACCTCGACCTCCCGCCCCACCTCGACCCGTACCGGAAGCTCTCCCGGTCCCACCCAGACCCACTGGGGCCAGTGCGGTGGTCAGGGCTGGACT
GGTCCCACTCAGTGCGAGTCCGGTACCACCTGCCAGGTCATCTCCCAGTGGTACTCTCAGTGCCTCtaaa

*Protein concentration:* The protein concentration was determined with the BioQuant protein reagent (Merck, Darmstadt, Germany; Neumüllet al. 2014c).

*Synergy assay:* The synergy assay for *Tr*CE5 and the *A. niger / T. emersonii* enzyme preparations was described previously (Neumüller et al. 2014c).

Results and discussion

*Cloning and expression of TrCE5:* High purity (91 %) was obtained for the *Tr*CE5 present in the culture filtrate from recombinant *A. niger* ISO527 (Neumüller et al. 2014c). The same study reported that no monosaccharide releasing side activities were observed by incubation of WUS with the *Tr*CE5 containing culture filtrate from *A. niger* ISO527, confirming selective expression of the cloned acetyl xylan esterase.

*Hydrolysis of WUS by supplementation of TrCE5 to an industrial (hemi)cellulolytic preparation:* The increase in monosaccharides released for the hydrolysis of WUS compared to hydrolysis with the sole *A. niger / T. emersonii* preparation was determined. An increase in the monosacchrides released of 23, 26 and 2 mg $g^{-1}$ for Xyl, Glc and Ara, respectively, was obtained for the hydrolysis of WUS by incubation with the *Tr*CE5 supplemented *A. niger / T. emersonii* preparation compared to levels obtained by sole application with the *A. niger / T. emersonii* preparation.

In conclusion the data obtained confirm the synergy by supplementation of CE5 classified acetyl xylan esterase (*T. longibrachiatum*, *T. reesei*) to the *A. niger / T. emersonii* culture filtrates mixture for the hydrolysis of corn polysaccharides.

**Design of efficient enzyme preparations**

An efficient approach to design hemicellulolytic enzyme preparations is the recognition and supplementation of “lacking” accessory activities to crude culture filtrates.

As described above, standardized enzyme preparations are not yet fully optimized to hydrolyze plant polysaccharides from corn. Recently, a platform for the optimization of enzyme mixtures has been developed using pure enzymes, covering endo- and exo xylanases and cellulases as well as accessory enzymes. However, the conversion of corn stover by mixtures of individual enzymes was not more efficient than by a crude, commercial enzyme preparation (Banerjee et al. 2010). The data obtained (chapters 4 and 5) suggest that an efficient crude preparation, or a mixture of crude enzyme preparations, is suitable to serve as a base to which accessory enzymes are supplemented in order to further optimize enzyme mixtures for a certain substrate or process. Such mixture (*A. niger*, *T. emersonii* culture filtrates and the CE5 acetyl xylan esterase from *T. reesei* or *T. longibrachiatum*) has been identified for the conversion of corn silage. The approach of identifying synergistic key enzymes from an efficient enzyme mixture is illustrated in Figure 4 and can also be applied for other mixes of efficient crude enzyme preparations (Figure 3) as for example *A. niger / T. emersonii* together with *H. insolens*. It is likely that additional individual synergistic enzyme activities will be identified. For future research, the identified combination of the *A. niger* and *T. emersonii* culture filtrates mixture containing additional acetyl xylan esterase from CE family 5 (*T. longibrachiatum* or *T. reesei*) would provide an ideal starting point to search for additional “lacking” enzyme activities towards the complete hydrolysis of corn polysaccharides. Synergistic, accessory enzymes can be supplemented to enzyme preparations or cloned in the production strain in order to already secrete an optimized hemicellulolytic enzyme mixture. “Lacking” activities of crude culture filtrates may easily be overlooked if standard assays are used to determine the hydrolytic efficiency of enzymes. Therefore, poly- or oligosaccharides resembling structural features as occur in nature are suitable substrates to study enzymatic hydrolysis for plant polysaccharide conversion.

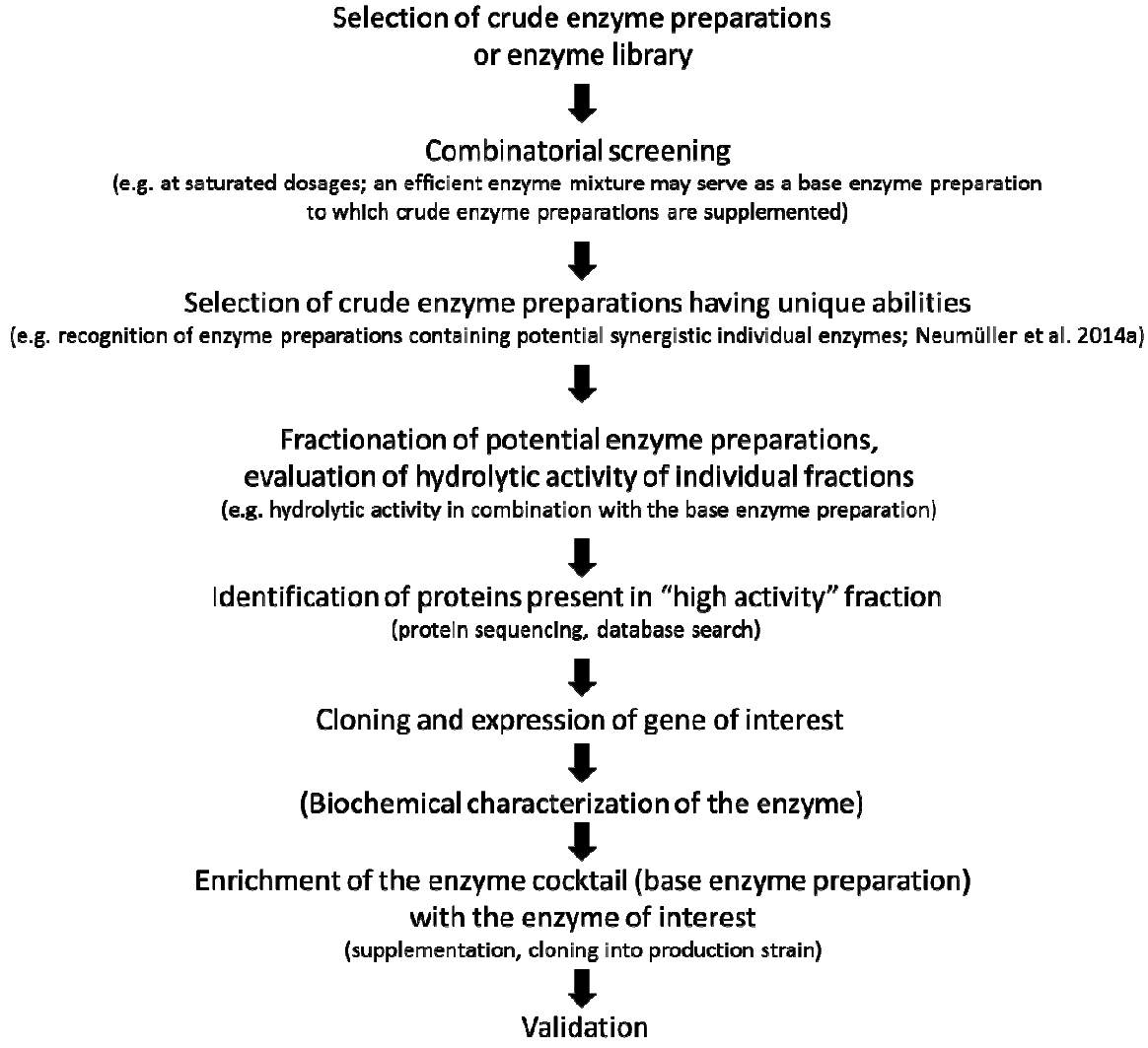



Figure 4: Flow chart for the optimization of enzyme preparations by identification and supplementation of necessary accessory enzymes from crude enzyme preparations.

## Comparing acetyl esterases from different CE families

The main conclusions obtained by comparing acetyl esterases from different CE families (chapter 6) were that acetyl esterases differ significantly in their positional preferences on partially acetylated xylo-oligosaccharides. Positional preferences were obtained towards 2,3-di-*O* acetylated Xyl*p* (*Te*CE1, *An*CE5 and *Os*CE6) or 3-*O* acetylated Xyl*p* (*Ct*CE4). Furthermore, high initial deacetylation rates towards different acetylated Xyl*p* were obtained for CE5 and CE4 classified acetyl xylan esterases. The results obtained regarding positional preferences and deacetylation efficiencies of acetyl esterases from different CE families enable the design of efficient deacetylating, hemicellulolytic enzyme preparations.

Since CE5 classified acetyl xylan esterases (*T. longibrachiatum* and *T. reesei*) showed to play a vital role in the conversion of plant biomass, the deacetylation of acetylated 4-*O*-methyl glucuronic acid (meGlcA)-substituted xylo-oligosaccharides (AcUXOS) by acetyl esterases from different carbohydrate esterase (CE) families was analysed by $^{1}$H NMR (chapter 6, Neumüller et al. 2014d). The enzymes tested can be categorized in three groups of acetyl esterases with activities towards acetylated (i) 2-*O*, 3-*O* and (ii) 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and (iii) activity towards 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA. The 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA was deacetylated by *An*CE16 only. CE16 classified esterases most likely act exclusively on terminal acetylated xylopyranosyl units as described for the CE16 classified acetyl esterases from *A. niger* (chapter 6) and *T. reesei* (Biely et al. 2014). This indicates that an enzyme needs to be found that can hydrolyse internal 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA. Screening, using the recalcitrant residue obtained by incubation of AcUXOS with CE5 and CE16 acetyl esterases from *A. niger*, resulting in exclusively "internal" 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA, may lead to the identification of such an acetyl xylan esterase. An enzyme that can target this position may also be of interest for the deacetylation of a range of other recalcitrant acetylated oligosaccharides, which carry complex side chains and acetyl residues on the same xylopyranosyl unit (Appeldoorn et al. 2013), as such enzyme must show a certain tolerance to substituents present next to the acetyl group. Acetyl esterases with different specificities are of interest for targeted modifications of partially acetylated xylans.

A combination of CE5 (*A. niger*, *T. reesei*) and *An*CE16 (*A. niger*) esterases results in high deacetylation of 4-*O*-methyl glucuronic acid (meGlcA)-substituted xylan. The CE5 classified esterases can remove 2-*O*, 3-*O* and 2,3-di-*O* acetylated Xyl*p* and *An*CE16 removes 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA from the non-reducing end. By addition of xylanases, removing unsubstituted xylpopyranosyl residues, "internal" 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA can also become accessible by *An*CE16. However, combination of acetyl esterases other than the CE5 and CE16 classified acetyl esterases or novel acetyl esterases that are not discovered yet might be necessary to obtain optimal deacetylation of other substrates having different patterns of acetylation. Deacetylating enzyme preparations for acetylated substrates can be designed based on the results obtained. Especially for biogas production, where pretreatment is usually not applied and where acetylation significantly restricts enzymatic hydrolysis, the characterization of the mode of action of acetyl esterases from different CE families is important. Application of a crude fungal culture filtrate with additional CE5 and CE16 classified acetyl esterases being present, is indicated to result in high conversion yields.

## Future Perspectives

The results obtained show that industrial enzyme preparations are not yet optimized for the degradation of corn silage polysaccharides. Their performance can be significantly improved by the supplementation of certain accessory enzymes as shown in this thesis. Supplementation of "lacking" accessory enzymes, such as acetyl and phenoyl esterases and glycosyl hydrolases, can result in a higher degree of conversion while keeping the enzyme loading low. An efficient enzyme mixture, as identified for the conversion of corn silage polysaccharides (chapter 5), may be even further improved by identifying additional accessory enzymes that target the residual recalcitrant poly- and oligosaccharides. Furthermore, other powerful combinations of crude enzyme preparations have been recognized (Figure 3) and provide a source for the identification of individual synergistic accessory enzymes. The enzyme resistant oligosaccharides as described in chapters 2 (Table 4), 4 and 6, may serve as a screening substrate in order to find additional necessary accessory activities. The developed approach to design powerful enzyme preparations (Figure 4) is important for future research and for the development of polysaccharide conversion technologies. It allows a fast optimization and adaptation of enzyme preparations towards different substrates and processes.

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## Summary

The monosaccharides released by enzymatic conversion of plant polysaccharides are utilized for the production of biofuels and chemicals. In order to reduce greenhouse gas emissions from fossil fuels, biogas production from lignocellulosic biomass will play a vital role in the future. In anaerobic digestion, the enzymatic hydrolysis of hemicellulose is a rate limiting factor. The enzymatic hydrolysis of the xylan present in corn silage, a major biogas feedstock, by efficient hemicellulolytic enzyme preparations is the focus of this thesis.

A general introduction to plant cell walls, recalcitrance of plant polysaccharides towards enzymatic conversion and carbohydrate active enzymes present in (hemi)cellulolytic enzyme preparations is presented in **chapter 1**. Populations of alkali extracted xylan from corn silage are described in **chapter 2**. Heavily substituted xylan is shown to be rather recalcitrant towards enzymatic conversion. Glucuronic acid and galactose substituted (arabino-)xylo-oligosaccharides are present in the enzyme treated xylan populations. The highly acetylated complex xylan, being part of a tight, insoluble cell wall network together with cellulose and lignin, was observed to be recalcitrant due to the presence of acetyl and phenoyl esters and a lower accessibility by enzymes to polysaccharides.

A fast and robust method to quantify acetyl and phenoyl esters of plant polysaccharides is described in **chapter 3**. Repeatability and robustness of the $^{1}$H NMR based method is shown. The method presented is suitable for high-throughput analysis and a suitable alternative to the more time-consuming, conventional (U)HPLC-based methods. The method described is of relevance for the characterization of acetyl and phenoyl ester substituents of the xylan backbone present in plant cell wall polysaccharides.

In **chapter 4**, compositions of corn silage fractions, being water unextractable solids (WUS), enzyme recalcitrant solids (ErCS) and an anaerobic fermentation residue (AFR) are described. Increased substitution of the xylan backbone was shown to correlate with increasing recalcitrance of corn silage fractions. Especially acetylation was high in the recalcitrant anaerobic fermentation residue. Evaluation of polysaccharide conversion by individual enzyme preparations or by combinations of commercial enzyme preparations with the *state-of-the-art Aspergillus niger / Talaromyces emersonii* enzyme preparation demonstrated a significant potential for the increase of conversion yields using combinations of crude enzyme mixtures. Via the data obtained by the screening it was found that commercial enzyme preparations are not optimized towards the conversion of corn polysaccharides due to a lack of accessory enzymes. Combinations of crude mixtures from different microbial hosts, secreting different sets of accessory enzymes, were recognized to efficiently hydrolyze corn polysaccharides, providing a source for the optimization of hemicellulolytic enzyme preparations.

In **chapter 5**, the optimization of an industrial hemicellulolytic enzyme preparation (*A. niger / T. emersonii* culture filtrates mixture) by supplementation of an acetyl xylan esterase from

*Trichoderma longibrachiatum* belonging to carbohydrate esterase (CE) family 5 is described. Analysis of the degradation products of enzyme treated partially acetylated xylo-oligosaccharides revealed that this acetyl xylan esterase deacetylates oligosaccharides which are not de-esterified by the CE1 classified acetyl xylan esterases present in the *A. niger / T. emersonii* preparation. Supplementation of the CE5 acetyl xylan esterase to the *A. niger / T. emersonii* enzyme preparation results in high conversion of xylan as accessibility for other carbohydrases present in the enzyme preparation is increased due to efficient deacetylation of the xylan backbone. A characterization of the mode of action of acetyl esterases from different carbohydrate esterase (CE) families towards, partially acetylated xylo-oligosaccharides is presented in **chapter 6**. The deacetylation efficiency and the positional preferences of acetyl esterases from different CE families towards 2-*O*, 3-*O*, 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA within the xylo-oligosaccharides are shown, allowing a categorization of the enzymes tested in three groups with activity towards (i) 2-*O*, 3-*O* acetylated Xyl*p* (ii) 2-*O*, 3-*O* and 2,3-di-*O* acetylated Xyl*p* and (iii) activity towards 2-*O*, 3-*O* and 2,3-di-*O* acetylated Xyl*p* and 3-*O* acetylated Xyl*p* 2-*O* substituted with meGlcA. High initial deacetylation rates are demonstrated for CE5 acetyl xylan esterases from *A. niger* and *Trichoderma reesei* and the CE4 classified acetyl xylan esterase from *Clostridium thermocellum* under the hydrolysis conditions applied (pH 5.0, 50 °C). Positional preferences are shown towards 2,3-di-*O* acetylated Xyl*p* for the CE1, CE5 and CE6 classified esterases from *T. emersonii*, *A. niger* and *Orpinomyces sp.*, respecitively, and towards 3-*O* acetylated Xyl*p* for the CE4 classified esterase from *C. thermocellum*.

**Chapter 7** presents a discussion of the different studies. Acetylation and presence of glycosidic substituents of the xylan present in corn are discussed. Differences amongst xylan populations present in corn silage are outlined and hypothetical model structures of water unextractable xylans present in corn silage are shown. A strategy for an efficient design of deacetylating, hemicellulolytic enzyme preparations and adaptation of hemicellulolytic enzyme mixtures towards different substrates or processes are addressed.

## Zusammenfassung

Die enzymatische Hydrolyse von Polysacchariden führt zur Freisetzung von Monosacchariden, welche für die Produktion von Biotreibstoffen vewendet werden. Die Produktion von Biogas aus lignocellulosehaltiger Biomasse wird zukünftig eine zentrale Rolle einnehmen, um die durch das Verbrennen fossiler Energieträger entstandenen Treibhausgasemissionen zu reduzieren. Die enzymatische Hydrolyse von Hemicellulose stellt einen limitierenden Faktor für die Abbaurate von Biomasse während der anaeroben Vergärung dar. Vor allem Maissilage ist ein wichtiger Rohstoff für die Biogaserzeugung. Die enzymatische Hydrolyse des in der Maissilage vorkommenden Xylans durch effiziente, hemicellulolytische Enzympräparate steht im Fokus dieser Dissertation.

Eine allgemeine Einführung zu Pflanzenzellwänden, der Resistenz von Polysacchariden gegenüber enzymatischer Hydrolyse und kohlenhydrataktiven Enzymen wird in **Kapitel 1** dargelegt. Populationen von alkalilöslichem Xylan aus Maissilage werden in **Kapitel 2** beschrieben. Ferner wird die Abbauresistenz von stark substituiertem Xylan gegenüber enzymatischer Hydrolyse dargestellt. Glucuronsäure und Galactose substitutierte (Arabino-)Xylo-Oligosaccharide wurden in enzymbehandelten Rückständen von Xylan-Populationen festgestellt. Insbesondere stark acetyliertes, komplexes Xylan, als Teil eines dichten, wasserunlöslichen Zellwand-Netzwerkes mit Cellulose und Lignin, wurde aufgrund der Präsenz von Acetyl- und Phenoylestern und der geringen Zugänglichkeit von hydrolytischen Enzymen zu Polysacchariden als abbauresistent charakterisiert.

In **Kapitel 3** wird eine schnelle und robuste Methode zur Quantifizierung von Acetyl- und Phenoylestern von Polysacchariden von Pflanzenzellwänden beschrieben. Die Reproduzierbarkeit und Robustheit dieser $^{1}$H NMR-basierten Methode wird dargelegt. Die vorgestellte Methode ermöglicht “high-throughput”-Analysen und ist eine geeignete Alternative zu den weitaus zeitaufwändigeren, konventionellen (U)HPLC-basierten Methoden. Die in Kapitel 3 beschriebene analytische Methode ist von Bedeutung für die Charakterisierung von Acetyl- und Phenoylestersubstituenten des Xylan-Rückgrats von Polysacchariden in Pflanzenzellwänden.

In **Kapitel 4** wird die Zusammensetzung von Maissilage und verschiedenen Fraktionen von Maissilage, wie nicht wasserextrahierbare Feststoffe (WUS), enzymresistente Feststoffe (ErCS) und eines Rückstandes einer anaeroben Vergärung dargelegt. Eine Korrelation eines steigenden Anteils von Xylansubstituenten in verschiedenen, aus Maissilage gewonnenen, Fraktionen mit steigender Abbauresistenz gegenüber enzymatischer Hydrolyse wurde festgestellt. Vor allem ein hoher Grad an Acetylierung des abbauresistenten, anaeroben Gärungsrückstandes wurde detektiert. Eine Evaluierung der Hydrolyse von Polysacchariden durch individuelle Enzympräparate oder Kombinationen von kommerziellen Enzympräparaten mit einem industriellen, dem ¨Stand der Technik¨ entsprechenden, Enzympräparat von *Aspergillus niger*

und *Talaromyces emersonii*, demonstrierte ein signifikantes Potential zur Steigerung der Substratumsetzung durch die Verwendung von Kombinationen aus Enzympräparaten. Durch die aus dem Screeningverfahren gewonnenen Daten ist ersichtlich, dass kommerzielle Enzympräparate nicht ausreichend optimiert sind für die Hydrolyse von Maispolysacchariden, da ein Mangel an "akzessorischen Enzymen" (accessory enzymes) vorliegt. Eine effiziente Hydrolyse von Maispolysacchariden durch Kombinationen aus Enzympräparaten von verschiedenen mikrobiellen Enzymproduzenten (mit Unterschieden bezüglich der Sekretion von kohlenhydrataktiven Enzymen) wurde beobachtet. Diese Daten stellen eine Quelle für die Optimierung von hemicellulolytischen Enzympräparaten dar.

**Kapitel 5** befasst sich mit der Optimierung einer industriellen hemicellulolytischen Enzymmischung (*A. niger / T. emersonii* Kulturfiltrat-Mischung) durch Beimengung einer Acetylxylan-Esterase von *Trichoderma longibrachiatum*, klassifiziert als Mitglied der Kohlenhydrat-Esterase [carbohydrate esterase (CE)] Familie 5. Eine Analyse der Abbauprodukte von enzymbehandelten, partiell acetylierten Xylo-Oligosacchariden zeigte, dass diese Acetylxylan-Esterase Oligosaccharide deacetyliert, welche Resistenz gegenüber CE1 klassifizierten Acetylxylan-Esterasen von *A. niger* und *T. emersonii* aufweisen. Beimengung der CE5 Acetylxylan-Esterase (*T. longibrachiatum*) zu dem *A. niger / T. emersonii* Enzympräparat resultierte in eine gesteigerte Hydrolyse von Xylan, da die Zugänglichkeit für andere kohlenhydrataktive Enzyme des Enzympräparates nach effizienter Deacetylierung des Xylan-Rückgrats erhöht wurde. Eine Charakterisierung der Wirkungsweise von Acetylxylan-Esterasen von verschiedenen CE Familien bezüglich der Deacetylierung von partiell acetylierten Xylo-Oligosacchariden wird in **Kapitel 6** beschrieben. Die Effizienz der Deacetylierung sowie die Positionspräferenz von Acetylxylan-Esterasen von verschiedenen CE Familien bezüglich der Hydrolyse von 2-*O*, 3-*O*, 2,3-di-*O* acetylierten Xylopyranosyl-Einheiten (Xyl*p*) und 3-*O* acetylierten Xyl*p*, welche an der *O*-2-Position mit 4-*O*-Methyl-Glucuronsäure (meGlcA) substituiert sind, werden dargelegt. Die erhaltenen Daten ermöglichten eine Kategorisierung der getesteten Acetylxylan-Esterasen in drei Gruppen mit Aktivität gegenüber (i) 2-*O*, 3-*O* acetylierten Xyl*p* (ii) 2-*O*, 3-*O* und 2,3-di-*O* acetylierten Xyl*p* und (iii) Aktivität gegenüber 2-*O*, 3-*O* und 2,3-di-*O* acetylierten Xyl*p* und 3-*O* acetylierten Xyl*p*, welche an der *O*-2-Position mit meGlcA substituiert sind. Hohe Anfangsgeschwindigkeiten (initial rate) für die Deacetylierung von partiell acetylierten Xylo-Oligosacchariden werden für die CE5 klassifizierten Acetylxylan-Esterasen von *A. niger* und *Trichoderma reesei* sowie für die CE4 klassifizierte Acetylxylan-Esterase von *Clostridium thermocellum* unter den angewandten Hydrolysebedingungen (pH 5.0, 50 °C) demonstriert. Positionspräferenzen gegenüber 2,3-di-*O* acetylierten Xyl*p* für die CE1, CE5 und CE6 klassifizierten Acetylxylan-Esterasen von *T. emersonii*, *A. niger* und *Orpinomyces sp.* und gegenüber 3-*O* acetylierten Xyl*p* für die CE4 klassifizierte Actylxylan-Esterase von *C. thermocellum* werden dargelegt.

**Kapitel 7** beschreibt eine Diskussion der verschiedenen Studien. Acetylierung und Präsenz von Substituenten des Xylan-Rückgrats werden diskutiert. Unterschiede zwischen Xylan-Populationen aus Maissilage werden beschrieben und hypothetische Modellstrukturen von nicht wasserextrahierbarem Xylan werden vorgestellt. Eine Strategie zum Design effizienter deacetylierender, hemicellulolytischer Enzympräparate und zur Anpassung von hemicellulolytischen Enzymmischungen an verschiedene Substrate und Prozesse wird diskutiert.

## Samenvatting

Enzymatische hydrolyse van polysachariden leidt tot het vrijkomen van monosachariden, die in de productie van biobrandstoffen een toepassing vinden. Productie van biogas uit lignocellulose-biomassa zal in de toekomst een belangrijke rol spelen om de uitstoot van broeikasgassen door het gebruik van fossiele brandstoffen te verminderen. Tijdens de anaërobe gisting is de enzymatische hydrolyse van hemicellulose een beperkende factor voor de snelheid van afbraak van biomassa. Kuilmais is een belangrijke grondstof voor de productie van biogas. De enzymatische hydrolyse van xylaan, aanwezig in de kuilmais, door efficiënte hemicellulolytische enzympreparaten, is de focus van dit proefschrift.

Een algemene inleiding tot de celwanden van planten, de weerstand van celwand-polysachariden tegen enzymatische hydrolyse en de polysacharide-actieve enzymen wordt gepresenteerd in **hoofdstuk 1**. Populaties van alkali-oplosbaar xylaan uit kuilmais worden beschreven in **hoofdstuk 2**. De weerstand van sterk-gesubstitueerd xylaan tegen enzymatische hydrolyse werd aangetoond. Glucuronzuur- en galactose-gesubstituteerde (arabino-)xylo-oligosachariden werden gedetecteerd in enzymatisch behandelde xylaanpopulaties. Bijzonder sterk geacetyleerd, complex xylaan, als onderdeel van een dicht, water-onoplosbaar celwandnetwerk, samen met cellulose en lignine, werd gekarakteriseerd als afbraak-resistent door de aanwezigheid van acetyl- en phenoylesters, die zorgen voor een slechte toegankelijkheid van de polysachariden voor hydrolytische enzymen.

In **hoofdstuk 3** is een snelle en robuuste methode voor de kwantificering van acetyl- en phenoylesters in polysachariden van plantaardige celwanden beschreven. De reproduceerbaarheid en de robuustheid van deze op $^{1}$H-NMR gebaseerde methodiek wordt gepresenteerd. De methode is een alternatief voor de veel tijdrovender, conventionele op (U)HPLC-gebaseerde methoden, en is geschikt voor "high-throughput" analyse.

In **hoofdstuk 4** is de samenstelling van kuilmais en verschillende fracties van kuilmais, zoals niet-water-extraheerbare vaste stoffen (WUS), enzym-resistente vaste stoffen (ErCS) en het residu na anaerobe vergisting, gepresenteerd. Een correlatie tussen een hoger gehalte aan xylaansubstituenten en een toenemende resistentie tegen enzymatische afbraak van verschillende kuilmaisfracties werd aangetoond. Met name bij het afbraak-resistente anaerobe fermentatieresidu werd een hoge mate van acetylering vastgesteld. Een evaluatie van de hydrolyse van polysachariden door afzonderlijke enzympreparaten en combinaties van commerciële enzympreparaten met industriële, *¨state-of-the-art¨* enzympreparaten van *Aspergillus niger* en *Talaromyces emersonii*, wees op een significant potentieel voor verbetering van de substraatomzetting door het gebruik van combinaties van enzympreparaten. Uit de screening bleek dat commerciële enzympreparaten onvoldoende geoptimaliseerd zijn voor de hydrolyse van maispolysachariden door een gebrek aan "accessoire enzymen" (accessory enzymes). Een efficiënte hydrolyse van maispolysachariden door combinaties van enzympreparaten afkomstig van diverse microbiële enzymproducenten (met verschillen in de

uitscheiding van koolhydraat-actieve enzymen) werd geconstateerd. Deze gegevens vormen een bron voor het optimaliseren van hemicellulolytische enzympreparaten.

**Hoofdstuk 5** behandelt de optimalisatie van een industrieel hemicellulolytisch enzymmengsel (*A. niger / T. emersonii* cultuurmix) door het bijmengen van een acetyl(xylaan)esterase van *Trichoderma longibrachiatum*, geclassificeerd als lid van de koolhydraatesterase [carbohydrate esterase (CE)] familie 5. Analyse van de afbraakproducten van enzym-behandelde, gedeeltelijk geacetyleerde, xylo-oligosacchariden toonde aan dat dit acetyl(xylaan)esterase (CE5) oligosacchariden deacetyleert die resistent zijn tegen de CE1-geclassificeerde acetyl(xylaan)esterasen aanwezig in het *A. niger* en *T. emersonii* mengsel. Toevoeging van acetyl(xylaan)esterase CE5 (*T. longibrachiatum*) aan het *A. niger / T. emersonii* enzympreparaat resulteerde in een verhoogde hydrolyse van xylaan, doordat de toegankelijkheid voor andere koolhydraat-actieve enzymen in het enzympreparaat is verhoogd door een efficiënte deacetylering van de xylaan-hoofdketen. Een karakterisering van de werking van acetyl(xylaan)esterases van verschillende CE families op gedeeltelijk geacetyleerde xylo-oligosacchariden is beschreven in **hoofdstuk 6**. De effectiviteit van de deacetylering en de voorkeurspositie van acetyl(xylaan)esterasen uit verschillende CE families qua hydrolyse van 2-*O*-, 3-*O*-, 2,3-di-*O*-geacetyleerde xylopyranosyl-eenheden (Xyl*p*) en 3-*O*-geacetyleerde Xyl*p* die een 4-*O*-methyl-glucuronzuur (meGlcA) unit op de *O*-2 positie heeft, zijn in dit hoofdstuk gepresenteerd. Met de verkregen gegevens is een indeling van de geteste acetyl(xylaan)esterasen in drie groepen gemaakt, met activiteit tegen (i) 2-*O*-, 3-*O*-geacetyleerde Xyl*p* (ii) 2-*O*-, 3-*O*- en 2,3-di-*O*-geacetyleerde Xyl*p* en (iii) activiteit tegen 2-*O*-, 3-*O*- en 2,3-di-*O*- en 3-*O*-geacetyleerde Xyl*p* die een meGlcA residu op de *O*-2 positie heeft. Een hoge initiële snelheid (initial rate) van de deacetylering van gedeeltelijk geacetyleerde xylo-oligosacchariden is aangetoond voor de in CE5 ingedeelde acetyl(xylaan)esterasen van *A. niger* en *Trichoderma reesei* en voor de in CE4 ingedeelde acetyl(xlyaan)esterase van *Clostridium thermocellum* onder de toegepaste hydrolyse-omstandigheden (pH 5.0, 50 °C ). Positievoorkeuren voor 2,3-di-*O*-*ge*acetyleerde Xyl*p* voor de in CE1, CE5 en CE6 ingedeelde acetyl(xylaan)esterasen van *T. emersonii*, *A. niger* en *Orpinomyces sp*. en voor 3-*O*-geacetyleerde Xyl*p* voor het in CE4 ingedeelde acetyl(xylaan)esterase van *C. thermocellum* zijn gevonden.

**Hoofdstuk 7** geeft een bespreking van de verschillende resultaten. Acetylering en de aanwezigheid van substituenten van xylaan worden besproken. Verschillen tussen xylaan populaties, die aanwezig zijn in kuilmais, worden beschreven en hypothetische modelstructuren van niet-water-extraheerbaar xylaan worden gepresenteerd. Een strategie voor het ontwerp van efficiënte deacetylerende, hemicellulolytische enzympreparaten en voor de aanpassing van hemicellulolytische enzymmengsels voor verschillende substraten en processen wordt besproken.

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# Klaus

## Curriculum vitae

Klaus Gerald Neumüller was born on the 30th of March, 1983 in Linz, Austria. After graduating from pre-university education at the grammar school “Europagymnasium Auhof” in 2002 and completing the 12 months civilian service of the Austrian government in the general hospital (Administrative department, Allgemeines Krankenhaus Linz) in 2003, he started his study of Genetics and Microbiology at the University of Vienna. As an undergraduate, he joined the research group of Dr. Jürgen Knoblich at the Institute of Molecular Biotechnology, Vienna, working as a Technical Research Assistant within a project on the genome-wide analysis of self-renewal in neuronal stem cells by transgenic RNAi. He did his master thesis (Diplomarbeit) under supervision of Dr. Katrin Heinze, focusing on the photounbinding of Calmodulin (Cam) from a family of Cam-binding peptides. During his undergraduate studies he wrote articles for publication in peer-reviewed journals as a first-author and co-author and graduated from the University of Vienna in September 2009.

Klaus started as an “Early Stage Researcher” at DSM (Delft, The Netherlands) and Wageningen University, funded by the EU Marie Curie LeanGreenFood programme in June 2010. His PhD project targeted the design of hemicellulolytic enzyme preparations for the degradation of recalcitrant corn silage polysaccharides, of which the results are discussed in this thesis.

## List of publications

Neumüller KG, Carvalho de Souza A, Van Rijn J, Streekstra H, Schols HA, Gruppen H, 2014. Positional preferences of acetyl esterases from different CE families towards acetylated 4-*O*-methyl-glucuronic acid substituted xylo-oligosaccharides. *Submitted for publication.*

Neumüller KG, Streekstra H, Schols HA, Gruppen H, 2014. *Trichoderma longibrachiatum* acetyl xylan esterase 1 enhances hemicellulolytic preparations to degrade polysaccharides. *Bioresource Technol*., 163, 64-73.

Neumüller KG, Streekstra H, Schols HA, Gruppen H, 2014. Acetyl xylan esterase Axe1 (*T. reesei*, Carbohydrate esterase family 5) supplemented to a hemicellulolytic preparation enhances degradation of recalcitrant corn silage polysaccharides. *Chem. Eng. Trans.,* 37, 277-282.

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Neumüller KG, Elsayad K, Reisecker JM, Waxham MN, Heinze KG, 2010. Photounbinding of Calmodulin from a family of CaM-binding peptides. *PloS One* 5: e14050.

## Overview of completed training activities

***Discipline specific activities***

Glycosciences Summer School[a], Wageningen, The Netherlands, 2010

Food and Bioresource Enzyme Technology, Wageningen, The Netherlands, 2010

Applied Enzyme Kinetics, Copenhagen, Denmark, 2010

Project Management, Copenhagen, Denmark, 2010

How to get Success in the Laboratory, Copenhagen, Denmark, 2010

Sustainability Analysis and Food Production, Wageningen, The Netherlands, 2011

Tools in Polysaccharide Engineering, Wageningen, The Netherlands, 2011

Science Ethics, Wageningen, The Netherlands, 2012

Global Food Production in a Changing World, Guangzhou, China, 2012

LeanGreenFood Work Package 5 meetings[b], Wageningen, The Netherlands, 2010-2013

***Excursions, study tours***

LeanGreenFood Excursion to Denmark, 2010

Food Chemistry PhD trip[a,b], Switzerland and Italy, 2010

LeanGreenFood Excursion to the Netherlands I (Gender Seminar, visit to industry, annual meeting[b]), 2011

LeanGreenFood Excursion to Greece (Visit to industry and annual meeting[b]), 2011

LeanGreenFood Excursion to China (Visit to industry and universities), 2012

LeanGreenFood Excursion to the Netherlands II (Science Ethics, annual meeting[b]), 2012

***Conferences***

EPNOE International Polysaccharide Conference[a], Wageningen, The Netherlands, 2011

International Carbohydrate Symposium[a], Madrid, Spain, 2012

Marie Curie LeanGreenFood conference: Enzymes in Sustainable Food Production[b], Halkidiki, Greece, 2013

International conference on biomass[b], Florence, Italy, 2014

[a] Poster presentation
[b] Oral presentation

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# Notes

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