Targeted discovery and functional characterisation of complex-xylan degrading enzymes

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Abstract

This thesis describes the development of a screening method to discover efficient hemicellulase producers in a wide range of fungi. The method is based on the potential of soil fungi to degrade soluble and insoluble xylan-rich substrates, by assigning various individual enzyme activities. Released mono- and oligosaccharides were monitored by high performance anion exchange chromatography and matrix assisted laser desorption/ionisation time-of-flight mass spectrometry. In addition, the released amounts of acetic acid were measured. Fungal strains, grown on wheat straw, with an efficient production of hemicellulolytic enzymes were *Aspergillus lentulus*, *Penicllium pinophilum*, *and Trichoderma harzianum*.

Next, two glycoside hydrolase (GH) family 10 endo-xylanases and two GH11 endo-xylanases of *Myceliophthora thermophila* C1 (C1) were subjected to a detailed characterisation. The degradation products from xylan-rich substrates, varying in type and degree of substituents, displayed that both intra- and interfamily differences occurred for these endo-xylanases. The different degradation profiles were correlated with a difference in the predicted secondary protein structure of the GH10 xylanases and with a minor difference in the primary protein structure of the GH11 xylanases. An additional cellulose binding domain attached to the endo-xylanases did not enhance the degradation of insoluble substrates.

In addition, synergy studies were performed on acetyl (xylan) esterases with glucuronidases. A concerted action of acetyl (xylan) esterases from different carbohydrate esterase families as well as synergy with glucuronidases of different GH families was observed.

List of abbreviations

Ac	Acetyl residue
AcXOS	Acetylated xylooligosaccharides
AcUXOS	Acidic acetylated xylooligosaccharides
AE	Acetyl esterase
AGU	Glucuronidase
An	*Aspergillus nig*er
Ao	*Aspergillus oryzae*
Ara	Arabinose
AXE	Acetyl xylan esterase
AXHm	Arabinoxylan arabinofuranohydrolase-mono
AXHd3	Arabinoxylan arabinofuranohydrolase-double-position 3
BeWX	Beech wood xylan
BiWX	Birch wood xylan
BSA	Bovine serum albumin
C1	*Myceliophthora thermophila* C1
CAZy	Carbohydrate-Active iinzyme
CBM	Carbohydrate binding module
CE	Carbohydrate esterase
CF AIS	Corn fiber alcohol insoluble solids
Cg	*Chaetomium globosum*
Ct	*Chaetomium thermophilum*
CV	Column volume
DMSO	Dimethyl sulfoxide
DNS	Dinitrosalicyl acid
DP	Degree of polymerisation
EC	Enzyme Commission
ESI	Electro spray ionisation
EXH	Eucalyptus xylan hydrolysate
Gal	Galactose
GH	Glycoside hydrolase
Glc	Glucose
GlcA	Glucuronic acid
Glur	Glucuronidase
H	Hexose
HPAEC	High performance anion exchange chromatography
HPLC	High performance liquid chromatography
HPSEC	High performance size exclusion chromatography
LC-strain	Low-cellulase C1 strain
MALDI TOF	Matrix assited laser desorption/ionisation
Man	Mannose
Me	Methyl
MeGlcA	4-*O*-methyl-glucopyranosyl uronic acid
m-HDP	m-hydroxy diphenyl
MS	Mass spectrometry
n.a.	Not analysed
OSX	Oat spelt xylan
P	Pentose
PAD	Pulsed amperometric detection
PAHBAH	p-Hydroxy benzoic acid hydrazide
Pc	*Penicillium chrysogenum*
PDA	Potato-dextrose-agar
PDB	Protein data bank
Pf	*Penicillium funiculosum*
PL	Polysaccharide lyase
PNP	*p*-nitrophenyl
Ps	*Penicillium simplicissimum*
Rha	Rhamnose
RI	Refractive index
Sc	*Schizophyllum commune*
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
Ta	*Thermoascus aurantiacus*
Tr	*Trichoderma reesei*
Trp	Tryptophan
Tyr	Tyrosine
U	Uronic acid (in MALDI-TOF mass spectra)
UA	Uronic acid
Val	Valine
WAX	Wheat arabinoxylan
WS KOHmix	Alkali-treated wheat straw
WS KOHres	Wheat straw alkaline residue
WS KOHss	Wheat straw alkaline soluble solids
WS WUS	Wheat straw water unextractable solids
Xyl_n	Xylooligomer of n units
XOS	Xylooligosaccharides
Xyl	Xylose
Xyl*p*	Xylopyranosyl

Table of contents

Chapter 1

General introduction

1

1.1 Project outline

Due to rapid growth in industrialisation there is an excessive consumption of fossil fuels. Concomitant with the excessive consumption, the depletion in fossil fuels leads to an increased interest in alternative fuels. An attractive alternative to fossil fuels is the production of bioethanol from lignocellulosic biomass. These lignocellulosic feedstocks are renewable and abundant. In terms of treatment of lignocellulosic biomass, the conversion of mildly pretreated biomass to bioethanol via enzymatic hydrolysis seems to be the most environmental sustainable technology. However, until now most commercial enzyme preparations are lacking one or more of the enzymes needed for complete hydrolysis of biomass.

The FP7 EU-project 'DISCO' ('Targeted DISCOvery of novel cellulases and hemicellulases and their reaction mechanisms for hydrolysis of lignocellulosic biomass') is aiming at the development of efficient and cost-effective enzyme tools to produce bioethanol from lignocellulosic biomass, and to understand how these enzymes work. Hemicellulases are crucial in achieving optimal hydrolysis of lignocellulosic feedstock, since hemicelluloses are interlinked within the cellulose matrix and thereby prevent cellulases from complete degradation of the cellulosic material. Even more, the monomeric degradation products of hemicelluloses can add up to the ethanol yield. As part of the DISCO-project, the presented PhD research focused on the activity-based screening for hemicellulases in various strain collections and soil samples. Furthermore, the enzymatic reaction mechanisms of endo-xylanases from *Myceliophthora thermophila* C1 are elucidated on a molecular level. Their functionality is combined with structural features of the protein. In addition, synergistic studies were performed with accessory enzymes on natural substrates.

1.2 Renewable energy resource

The increased use of fossil fuels contributes to the high level of CO_2 in the atmosphere, which is directly related to the global warming observed in the last decades (Sánchez

and Cardona, 2008; Vogt *et al.*, 2008). Liquid biofuels, produced from renewable resources, could provide one of the solutions to replace fossil fuel and in addition mitigate CO_2 emission (Rubin, 2008). The 'first generation' biofuels are made of sugar, starch or vegetable oils and appear unsustainable, due to the stress it puts on food commodities. In contrast, 'second generation' biofuels of feedstocks with no food use, could be part of the solution to fulfil energy requirements in an environmentally friendly way (Olsson *et al.*, 2007). Bioethanol is by far the most widely used biofuel for transportation world-wide. Lignocellulosic materials serve as cheap and abundant feedstock for bioethanol production (Balat *et al.*, 2008). Possible renewable feedstocks for this are: agricultural residues like straw (rice, barley, wheat), bagasse and corn stalks or -stover; forest wastes like saw dust or thinned wood; or can be municipal or industrial wastes (Zaldivar *et al.*, 2001; Pauly and Keegstra, 2010). Two main routes are available for the production of ethanol from lignocellulosic biomass. The first one is via thermochemical processing and the other one is through biochemical processing. In thermochemical processing the conversion of biomass is carried out through the combination of thermal decay and chemical treatment. This treatment will either solubilize the main components present in the cell wall structure, or just open up the cell wall structure. Both results will lead to more susceptible polymers for the enzyme treatment (Roberto *et al.*, 2003; Wyman *et al.*, 2005). The biochemical process to produce bioethanol from lignocellulosic biomass is related to this research. It may involve a mild pretreatment in combination with the conversion of biomass into monosaccharides, by use of enzymes. These monosaccharides subsequently can be fermented to produce ethanol (Gomez *et al.*, 2008).

1.3 Lignocellulosic biomass: Plant cell walls

Lignocellulosic biomass refers to plants and plant-derived organic material, which contain hemicellulose, cellulose and lignin as major components (De Wild, 2011). Different plant species and different developmental stages of the plant, tissues and cells vary in the structure and composition of the plant cell wall. A differentiation in

the plant cell walls is made in the primary wall layer, a flexible layer which is formed as the cell is still capable of growing (Keegstra *et al.*, 1973); the secondary wall layer, which is formed after the cell is fully grown; and the middle lamella gluing the plant cells (Buchanan *et al.*, 2000), as visualized in Fig. 1.1. The plant cell wall is highly organized and contains networks of polysaccharides, proteins and aromatic substances (Carpita and Gibeaut, 1993). The cell walls form the exterior of the plasma membrane and serve multiple purposes. They prevent membrane rupture, are involved in cell size and shape determination and control the rate and direction of cell growth. By providing rigidity to the plant cells, the walls function as the skeleton of the plant (Cosgrove, 2001).

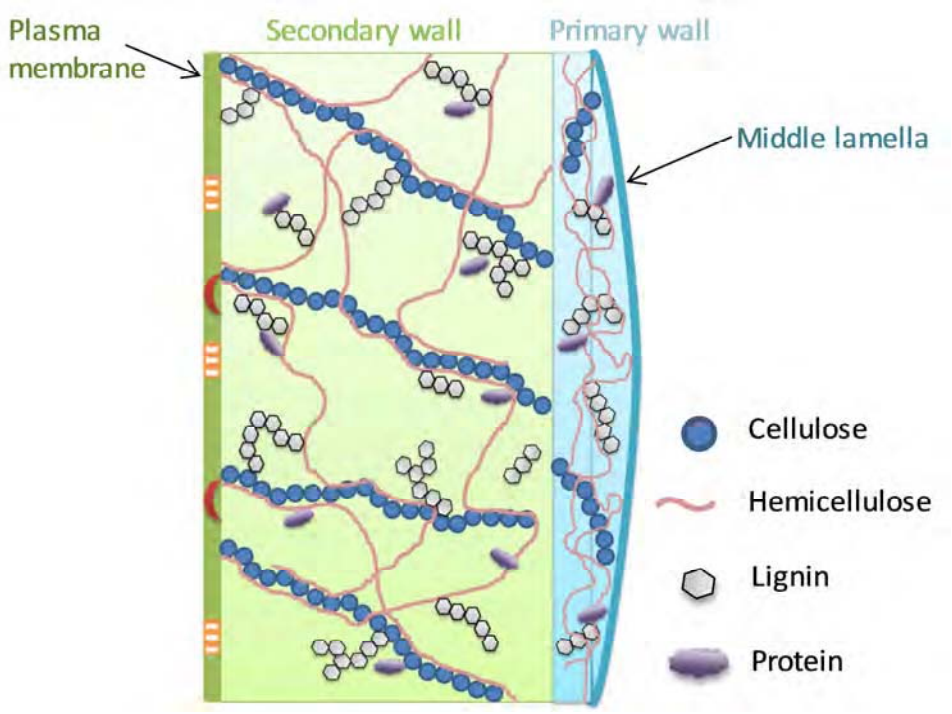

Fig. 1.1: Simplified model of the plant cell wall, including the specific localization of the various cell wall components, such as cellulose, hemicellulose, lignin and protein (adapted from Achyuthan et al. (2010)).

1.4 Cell wall composition

The lignocellulosic feedstocks used for bioethanol production consist for the major part of secondary cell walls (Gomez *et al.*, 2008). The secondary cell wall is composed of the carbohydrate polymers cellulose and hemicellulose, lignin and some minor components, like proteins and minerals (Fig. 1.1). Lignocellulosic biomass typically contains 55-75% (w/w) of polysaccharides, built-up of various pentose and hexose units. These polysaccharides are important for bioethanol production (Himmel *et al.*, 1999).

1.4.1 Cellulose

Cellulose is a linear insoluble homogeneous polymer of D-glucose residues, connected via β-(1→4) glycosidic linkages (Himmel *et al.*, 1999). It represents in general 40-80% of secondary cell walls (Salisbury and Ross, 1992). The hydroxyl groups present in the cellulose macromolecule can be involved in intra- and inter-molecular hydrogen bonds, resulting in bundels of cellulose molecules that aggregate to microfibrils. The microfibrils form a highly ordered crystalline arrangement, or a less ordered amorphous region (Kolpak and Blackwell, 1976).

1.4.2 Hemicellulose

Hemicelluloses are a heterogeneous class of polymers representing, in general, 10–40% of plant biomass (Salisbury and Ross, 1992). They may contain pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α-D-galactose) and/or uronic acids (α-D-glucuronic and α-4-*O*-methyl-D-glucuronic acids). Other sugars, such as α-L-rhamnose and α-L-fucose, may also be present in small amounts. The hydroxyl groups of sugars can be partially substituted with acetyl groups (de Vries and Visser, 2001; Ebringerová *et al.*, 2005). The hemicelluloses may coat cellulose microfibrils through hydrogen bonding. They are long enough to span between two microfibrils and link them together to form a network (MacAdam, 2009; Fig 1.1). The

structural and compositional heterogeneity of hemicellulose varies for different plant families, cell types, and even cell wall subsections. Different plant families have different hemicelluloses, in which herbaceous plants contain mainly arabinoxylans, while hardwood xylans are predominantly glucuronoxylans. Softwoods mainly consist of glucomannan, galactomannan, and galactoglucomannan. In addition, other hemicelluloses are present in many plant cell walls, including xyloglucan and β-glucans (only 1→3 or 1→4 and mixed 1→3, 1→4 linkages) (Decker *et al.*, 2009).

1.4.3 Lignin

The main component following cellulose and hemicellulose in lignocellulosic biomass is lignin, comprising 5-25% of the biomass feedstock (Saha *et al.*, 1998). The lignin is hydrophobic in nature and is thought to be bound covalently to hemicellulose and cellulose (Pérez *et al.*, 2002; Decker *et al.*, 2009). It is an aromatic polymer consisting of three different phenylpropane units (*p*-coumaryl, coniferyl and sinapyl alcohol) (Pérez *et al.*, 2002). The main purpose of lignin is to give the plant structural support, impermeability and is acting as shield against microbial attack (Hendriks and Zeeman, 2009). Lignin is attributed to be the most recalcitrant component in terms of breakdown of plant cell walls (Sánchez, 2009).

1.5 Hemicelluloses in more detail: Complex xylans

The most abundant hemicelluloses in nature are xylans. Xylans are the main hemicellulose components of secondary cell walls constituting about 10–30% of the biomass of hardwoods and herbaceous plants. In some tissues of grasses and cereals xylans can constitute up to 50% of the biomass (Stephen, 1983). Xylans comprise a β-(1→4)-D-xylopyranose backbone, which can be substituted with short carbohydrate chains based on L-arabinose, 4-*O*-methyl-D-glucuronic acid, D- or L-galactose or D-glucuronic acid (Buchanan *et al.*, 2003). The botanical source strongly determines the specific features with respect to type, amount, position and distribution of substituents over the xylan backbone (Kabel *et al.*, 2007). Xylans containing few

substituents tend to self-associate, forming aggregates with a low solubility (Andrewartha *et al.*, 1979; Linder *et al.*, 2003).

A frequently used classification is based on the types of side groups (Ebringerová, 2006):

a) Homoxylans: linear 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. They are common in seaweeds.

b) Glucuronoxylans: the β-(1→4)-D-xylopyranose backbone is substituted with α-(1→2)-4-*O*-methyl-D-glucuronic acid, while the non-methylated form may also be present. In addition, the backbone residues can be partially acetylated. Glucuronoxylans are commonly found in dicotyledons and in hardwoods.

c) Arabinoxylans: the β-(1→4)-D-xylopyranose backbone is substituted at position 2 and/or 3 of the xylose moiety with L-arabinofuranosyl units, which can be partly esterified with phenolic acids. Arabinoxylans are typical hemicellulose components from the cell wall of starchy endosperm, in outer layers of cereal grains as well as in seeds from other monocotyls, such as grasses.

d) Glucuronoarabinoxylans: next to α-(1→2)-4-*O*-methyl-D-glucuronic acid, substitution with α-(1→3) or α-(1→2)-L-arabinofuranosyl units is present on the β-(1→4)-D-xylopyranose backbone. In some glucuronoarabinoxylans also dimeric arabinosyl side chains can be present. The glucuronoarabinoxylans are often found in lignified tissue of grasses and cereals.

e) Heteroxylans: the β-(1→4)-D-xylopyranose backbone is heavily substituted with a variety of mono- and oligosaccharide side chains. They are mainly present in cereal bran. Corn fiber, for example, consists of a highly substituted xylan, having substituents at both positions *O*-2 and *O*-3 of the backbone xylopyranosyl units. Monomeric substituents or oligomeric side-chains can contain D-xylopyranosyl, L-arabinofuranosyl, (4-*O*-methyl)-D-glucuronic acid and galactose, mainly at the terminal position of an oligomeric side chain. Additionally, ferulic acid moieties can be

ester linked to the *O*-5 position of the arabinofuranosyl residues (Appeldoorn *et al.*, 2010).

A structural model of each of the xylan classes is presented in Fig. 1.2.

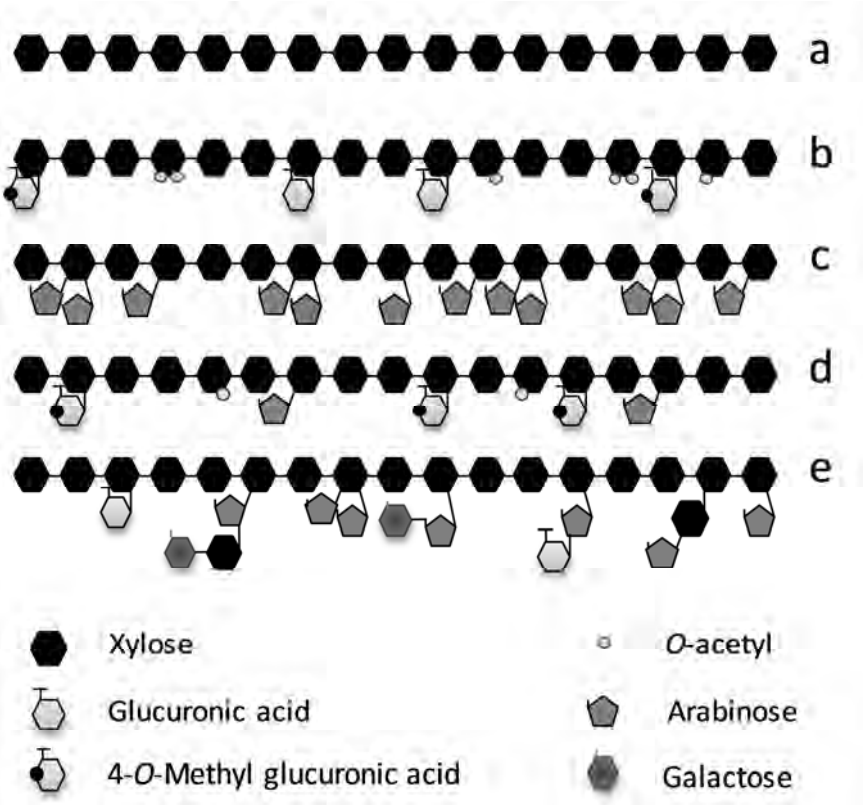

Fig. 1.2: Structural models of xylans. a) homoxylan; b) glucuronoxylan; c) arabinoxylan; d) glucuronoarabinoxylan; e) heteroxylan (based on Ebringerová et al. (2005)).

1.5.1 Xylan composition of common feedstocks for bioethanol production

The two major feedstocks currently used for biofuel ethanol production are sugarcane and corn grain. As ethanol demand grows, these feedstocks will eventually reach limits for the amounts that can be sustainably used for this purpose (Somerville *et al.*, 2010). Rice straw, wheat straw, corn stover, sugarcane bagasse, switch grass and poplar wood are the major agricultural alternatives available for bioethanol production (Kim and Dale, 2004; Wyman *et al.*, 2005). These feedstocks differ in

monosaccharide composition. Even though their major constituent sugar is cellulose, they can vary greatly in the hemicellulose composition. Even within a crop variation occurs e.g for the different parts of the corn plant (Table 1.1).

Table 1.1: Hemicellulose composition (w/w% of dry material) of major agricultural products available for bioethanol production (adapted from Gírio et al. (2010)).

	Xyl	**Ara**	**Man**	**Gal**	**Rha**	**UA**	**Ac**
Softwoods							
Pine	5-11	2-4	6-13	2-4	-	3-6	1-2
Spruce	5-10	1	9-15	2-4	-	2-6	1-2
Hardwoods							
Birch	19-25	0-1	2-3	1	1	4-6	4
Eucalyptus	14-19	1	1-2	1-2	0-1	2	3-4
Poplar	18-21	1	3-4	1	-	2-4	1-4
Willow	12-17	2	2-3	2	-	-	-
Agricultural and agro-industrial materials							
Barley straw[a]	15-22	3-4	-	1	-	-	-
Brewerey's spent grain[b]	15-16	8	1	1	-	2	1
Corn cobs[c]	28-35	3-8	-	1	1	2-3	2-4
Corn fiber[d]	18-22	11-12	0-2	3-4	0-2	4	3
Corn stalks	26	4	0-3	0-3	-	-	-
Corn stover[c,f]	15-26	2-6	0-2	1-3	0-1	2	2
Rice straw	15-23	3-5	2	-	-	-	-
Sugarcane bagasse	21-26	2-6	1	2	-	-	-
Switch grass[e]	20-24	3-4	0-1	1-2			
Wheat straw[f]	19-21	2-4	0-1	1-2	-	-	-

Xyl: xylose, Ara: arabinose, Man: mannose, Gal: galactose, Rha: rhamnose, UA: uronic acid, Ac: acetyl esters, -: not reported
[a] Combined with data Persson *et al.* (2009)
[b] Combined with data Beldman *et al.* (1987)
[c] Combined with data Van Dongen *et al.* (2011)
[d] Combined with data Van Eylen *et al.* (2011)
[e] From Lee *et al.* (2007)
[f] Combined with data Lee *et al.* (2007)

1.6 Pretreatment of lignocelluloses prior to enzymatic hydrolysis

One of the crucial steps in the biochemical production of bioethanol is the hydrolysis of the lignocellulosic carbohydrates to monosaccharides by use of enzymes. Given the

complexity of the carbohydrates, it is not surprising that on a molecular level they are recalcitrant to hydrolysis. In addition, enzymatic hydrolysis of the lignocellulosic material without pretreatment is usually not effective (Olsson *et al.*, 2007; Taherzadeh and Karimi, 2008). An effective and economical pretreatment should meet the following requirements (Taherzadeh and Karimi, 2008): (a) produce enzyme-accessible carbohydrates, (b) avoid destruction of the cellulose and hemicellulose constituting monosaccharides, (c) avoid formation of undesired inhibitors for the hydrolytic enzymes and fermenting microorganisms, (d) minimize energy demand, (e) reduce amount of residue, (f) minimize the use of chemicals, (g) reduce the overall production costs.

Methods that have been introduced for the pretreatment of lignocellulosics comprise physical methods, like milling, irradiation or high-temperature/high-pressure; (physico-)chemical methods, like explosion with alkali/acid-, gas- or oxidizing agents, or solvent extraction of lignin; and biological methods using fungi and Actinomycetes (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008). However, when lignocellulosic material is pre-treated, a hydrolysate rich in substances inhibitory to yeast and enzymes is formed. The amounts and nature of the inhibitory compounds is dependent on the pretreatment method and conditions used, but in general they can be put into three major groups: furaldehydes, weak acids and phenolics (Parawira and Tekere, 2011).

The dominating strategy for all pretreatments until now, is that they have been designed and optimized to make cellulose digestible, without considering hemicellulose digestion as a factor (Gírio *et al.*, 2010). The efficient utilization of the hemicelluloses is an opportunity to reduce the cost of ethanol production (Taherzadeh and Karimi, 2008), since the hemicellulose can represent 20-50% of the carbohydrates in the feedstock (Stephen, 1983). To preserve the hemicellulose, the most effective pretreatment at the moment seems to be the (compressed) hot water extraction. It is a mild pretreatment by which the hemicellulose is released as oligomers and not much inhibitors are formed (Dien *et al.*, 2006; Gírio *et al.*, 2010). To maintain the advantages of a mild pretreatment while increasing conversion rates and

levels, hemicellulases are becoming prominent in biomass conversion technology (Decker *et al.*, 2009).

1.7 Cell wall degrading enzymes

The degradation of cell wall polysaccharides requires two types of enzymes: Endo-enzymes and exo-enzymes. Endo-enzymes will act upon inner chemical bonds in a chain of molecules. The exo-enzymes will only cleave the terminal residue(s) from a polymer and hydrolyse oligomers to monomers. The action of endo-enzymes will lead to a rapid decrease of the molecular mass of a substrate and thereby produce new substrates for the exo-acting enzymes (Henrissat and Davies, 1997). It is not fully known how many different enzymes are involved in cell wall deconstruction, but the complete degradation of cell wall polysaccharides requires the synergistic action of a spectrum of enzymes. The use of both cellulases and hemicellulases seems to be the solution for complete degradation of the cell wall polysaccharides (Himmel *et al.*, 1997; Kristensen *et al.*, 2008).

1.7.1 Hydrolytic conversion process

The hydrolytic conversion by which complex polysaccharides are converted into simple monomers leads to the technical barriers for commercialising lignocellulosic bioethanol. The process is too slow and the variability in contents of lignin and hemicellulose composition makes that there is no generic way to treat different feedstocks (Sánchez and Cardona, 2008). Two main routes for the enzymatic hydrolysis for bioethanol production are used: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The two-step process of SHF makes use of optimal conditions for separate hydrolysis and fermentation. However, end-product inhibition by the resulting monosaccharides will limit enzyme activity (Wingren *et al.*, 2003). SSF is a process option, in which the enzymatic hydrolysis is performed together with the fermentation. This reduces

product inhibition of hydrolytic enzymes and reduces investment costs. However, one has to compromise on temperature (Olofsson *et al.*, 2008).

1.7.2 Classification of cell wall degrading enzymes

In practice, the 'exo' versus 'endo' distinction of enzymes seems difficult to measure, with many enzymes displaying properties that appear to be intermediate between the two types (Henrissat and Davies, 1997). Another classification has been developed by the International Commission on Enzymes of the International Union of Biochemistry. It was based on enzyme division by the type of reaction they catalysed: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Each enzyme was assigned an Enzyme Commission (EC) number in the respective category. The list of enzymes was subsequently updated, the most recent being 'Enzyme Nomenclature' in 1992 (Webb, 1992). As the EC numbers did not cover all functions characterized experimentally, an even more extended system was developed, which also describes the families of structurally-related catalytic and carbohydrate-binding modules of enzymes that degrade, modify, or create glycosidic bonds (Henrissat, 1991; Coutinho and Henrissat, 1999; Cantarel *et al.*, 2009). The classification contains cell wall modifying enzymes or modules in the following classes: Glycoside Hydrolases (GHs; EC.3.2.1.x), Polysaccharide Lyases (PLs; EC.4.2.2.x), Carbohydrate Esterases (CEs; EC.3.1.1.x) and Carbohydrate Binding Modules (CBMs) (Cantarel *et al.*, 2009), which are all collected in the carbohydrate-active enzyme database (CAZy). GHs will cleave the glycosidic bond between two sugar residues by the addition of a water molecule (Davies and Henrissat, 1995). PLs cleave the glycosidic bond of uronic acid-containing polysaccharides by a β-elimination mechanism (Yip and Withers, 2006). The CEs remove ester-linked components present in mono-, oligo- and polysaccharides, thereby facilitating the action of GHs on complex carbohydrates (Lombard *et al.*, 2010). The CBMs promote the interaction of the enzyme with the substrate, thereby increasing the hydrolysis rate of the enzyme (Boraston *et al.*, 2004).

The CAZy database presents an overview of all enzymes and classes and is updated continuously with novel enzymes and enzyme families (Cantarel *et al.*, 2009). It should be taken into account that GH families and classes might change in time due to new insights.

1.7.3 Cellulose degrading enzymes

For fermentation of lignocellulosic materials, the cellulose fraction should be degraded into glucose. Four classes of enzymes are involved in the biodegradation of cellulose and require synergistic action (Himmel *et al.*, 2007). The endo-glucanases (EC 3.2.1.4) hydrolyse cellulose to glucooligosaccharides and are mainly found in GH5 and GH9, but also in GH51 (Lopez-Casado *et al.*, 2008). Cellobiohydrolases (GH6, GH7 or GH48; EC 3.2.1.91) release cellobiose from crystalline cellulose (Duan and Feng, 2010). β-Glucosidases (GH1, GH3 or GH9; EC 3.2.1.21) degrade the oligosaccharides to glucose (Saibi and Gargouri, 2011). The exo-glucanases release glucose from cellulose and glucooligosaccharides (Himmel *et al.*, 1997). Primary hydrolysis occurs on the surface of solid substrates. Soluble oligosaccharides with a degree of polymerization (DP) up to six are released into the liquid phase upon hydrolysis by endo-glucanases and exo-glucanases. Secondary hydrolysis occurs in the liquid phase and involves the degradation of the glucooligosaccharides and hydrolysis of cellobiose to glucose by β-glucosidases (Zhang *et al.*, 2006).

1.7.4 Hemicellulose degrading enzymes

The term 'hemicellulase' is referring to a mix of enzyme activities that act upon the hemicellulose fraction. The enzymes involved in hemicellulose degradation can be divided into two major categories: depolymerizing and debranching/linearizing (Decker *et al.*, 2009).

The hemicelluloses xyloglucan and mannan require (xylo)glucanases (GH5, GH12, GH74; EC. 3.2.1. 151) (Master *et al.*, 2008), β-glucosidases, endo-mannanases (GH5, GH26; EC. 3.2.1.78) and β-mannosidases (GH1, GH2, GH5; EC. 3.2.1.25) for backbone

depolymerisation (Dhawan and Kaur, 2007). α-Xylosidases (GH31; EC. 3.2.1.177) (Lopez-Casado *et al.*, 2008), α-galactosidases (GH4, GH27, GH36, GH57; EC. 3.2.1.22), α-fucosidases (EC. 3.2.1.51), α-arabinofuranosidases (GH3, GH43, GH51, GH54, GH62; EC. 3.2.1.55) and acetyl esterases (CE16; EC. 3.1.1.6) are needed for debranching/linearizing the xyloglucan- or mannan backbones (de Vries and Visser, 2001; Shallom and Shoham, 2003).

Total biodegradation of xylans, being the most abundant hemicellulose, requires synergistic action of many different enzymes, due to the heterogenous character of the polymer. Xylan degrading enzymes comprise endo-β-1,4-xylanases (GH5, GH8, GH10, GH11, GH43; EC. 3.2.1.8) and β-xylosidases (GH3, GH30, GH39, GH43, GH52, GH54, GH116, GH120; EC. 3.2.1.37) for depolymerisation (Shallom and Shoham, 2003). Accessory enzymes, such as α-arabinofuranosidases, α-glucuronidases (GH67, GH115; EC. 3.2.1.139) (Kolenová *et al.*, 2010), acetyl xylan esterases (CE1-7 and CE12; EC. 3.1.1.72), ferulic acid esterases (CE1; EC. 3.1.1.73), and *p*-coumaric acid esterases (CE1; No EC code) are necessary for debranching/linearizing the xylans (Saha, 2003; Shallom and Shoham, 2003). Many xylanases do not cleave glycosidic bonds between substituted xylose units. The substituents must be removed in order to obtain complete xylan backbone degradation. On the other hand, several accessory enzymes are only active on xylooligosaccharides. These enzymes require endo-xylanases to hydrolyse the backbone partially before the substituents can be removed from the soluble oligomeric fragments (Biely, 1985; Poutanen *et al.*, 1987). Rapid debranching of polymers without backbone depolymerisation results in intermolecular aggregation of the backbone polymers, causing precipitation and makes the molecule even more difficult for depolymerising enzymes to access. Therefore, hemicellulose hydrolysis is optimal when all enzymes act simultaneously (Decker *et al.*, 2009). A schematic overview of the main enzymes involved in complex-xylan hydrolysis is displayed in Fig. 1.3.

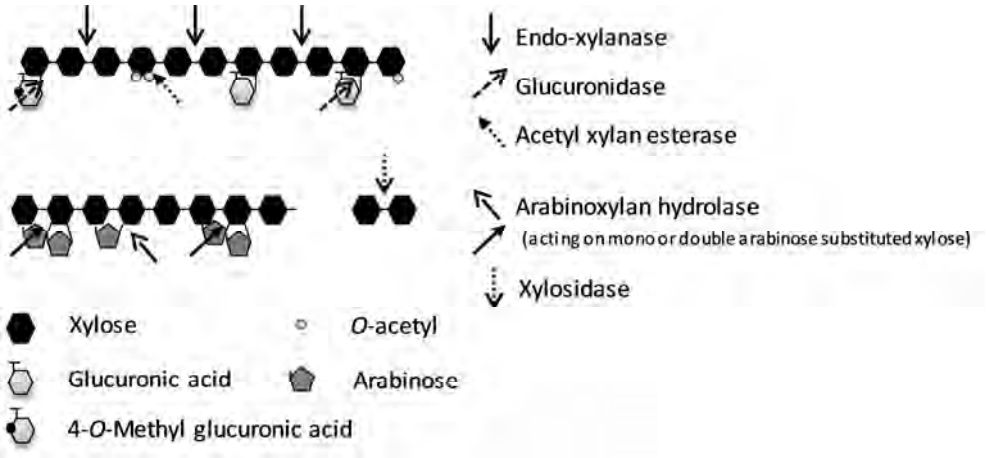

Fig. 1.3: Schematic overview of points of attack of main hemicellulases involved in complex-xylan degradation (Shallom and Shoham, 2003).

Endo-xylanases

Endo-xylanases (EC.3.2.1.8) are endo-acting enzymes, hydrolysing β-1,4- or β-1,3 linkage in xylan. At the moment of writing they are found in GH families 5, 8, 10, 11 and 43 (www.cazy.org) of which the GH10 and GH11 families are predominant (Moers *et al.*, 2003). The GH family 10 members have average molecular masses of approximately 40 kDa (Beaugrand *et al.*, 2004). Regarding their molecular structures, the GH10 family members display an 8-fold (α/β)-barrel resulting in a 'salad bowl' shape (Biely *et al.*, 1997; Collins *et al.*, 2005). An example of the protein fold of a GH10 endo-xylanase is displayed in Fig. 1.4A. GH10 xylanases are highly active towards short xylooligomers. GH10 xylanases exhibit lower substrate specificity than GH11 xylanases (Collins *et al.*, 2005). They tolerate substitution on xylose residues nearby the cleavage site, resulting in shorter xylooligomers than those produced by GH11 xylanases. Family 11 endo-xylanases are highly specific and do not tolerate many decorations on the xylan backbone (Biely *et al.*, 1997). These GH11 endo-xylanases are composed of three antiparallel β-sheets and one α-helix. The three-dimensional structure of these GH11 endo-xylanases has been described as a "partly closed right hand" of which an example is displayed in Fig.1.4B. This "hand" contains a thumb-like structure, which is the most flexible region of the xylanase. The movement of this

thumb could be essential for the function of the enzyme (Torronen and Rouvinen, 1997; Collins *et al.*, 2005).

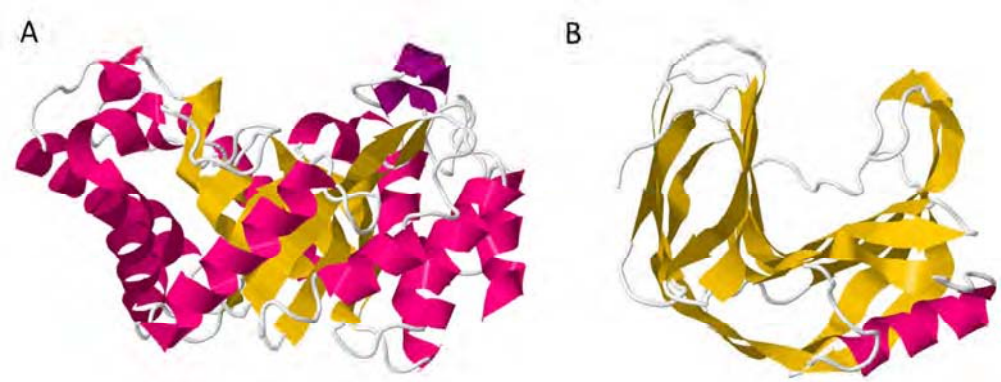

Fig. 1.4: Example of the three dimensional structure of a family 10 and a family 11 endo-xylanase. A: GH10 endo-xylanase of Penicillium simplicissimum (Schmidt et al., 1999), B: GH11 endo-xylanase of Hypocrea jecorina RUT-C30 (Torronen and Rouvinen, 1997).

β-Xylosidases

β-Xylosidases (EC. 3.2.1.37) are exo-enzymes, hydrolysing xylose units from the non-reducing terminus of D-xylan chains (Ward *et al.*, 1989). They are found in families GH3, GH30, GH39, which display the same fold as GH10 endo-xylanases (Harvey *et al.*, 2000; Yang *et al.*, 2004; St John *et al.*, 2010); and in GH43 displaying a five-bladed-β-propeller. The propeller is based upon a 5-fold repeat of blades composed of four-stranded β-sheets (Nurizzo *et al.*, 2002b). Xylosidases are also found in families GH52, GH54. GH116 and GH120, however, their 3D structure is unknown until now (www.cazy.org).

α-Arabinofuranosidases

Arabinofuranosidases (EC. 3.2.1.55) are enzymes that can hydrolyse arabinosyl linkages from various substrates, including hemicelluloses (Saha, 2000). As stated before, arabinoxylans consist of linear chains of β-D-(1,4)-linked xylopyranosyl residues, which can be substituted with α-L-arabinofuranosyl residue at the *O*-2 and/or *O*-3 position (fig. 1.2C; Ebringerová *et al.* (2000)). The arabinoxylan

arabinofuranohydrolases act specifically against the α-1,2- or α-1,3-linkage of the arabinose residues of arabinoxylan (Verbruggen *et al.*, 1998). Arabinoxylan hydrolases able to release arabinose only from position *O*-3 of the double substituted xylopyranosyl unit are quite unique. Only three were reported so far (Van Laere *et al.*, 1997; Sørensen *et al.*, 2006; Pouvreau *et al.*, 2011).

Arabinofuranosidases can be found in families GH3, GH43, GH51, GH54 and GH62. The folds of only families 43 and 51 are known: 8-fold (α/β)-barrel and the 5-fold propeller blades, respectively (Flipphi *et al.*, 1993; Hovel *et al.*, 2003).

α-Glucuronidases

Glucuronidases (EC. 3.2.1.139) are enzymes that are able to hydrolyse the α-1,2-linkage between glucuronic acid or 4-*O*-methyl glucuronic acid and xylose (Fig. 1.2B). Glucuronidase activity has been detected in the culture filtrates of both fungi and bacteria (Tenkanen and Siika-Aho, 2000). Most often the α-glucuronidases are found in family GH67 or GH115. GH67 enzymes display the 8-fold (α/β)-barrel (Nurizzo *et al.*, 2002a; Golan *et al.*, 2004). In contrast to GH67 enzymes which only cleave glucuronosyl linkages at the non-reducing ends of xylooligosaccharides, GH115 enzymes remove glucuronic acid from both the terminal and internal regions of xylooligosaccharides and xylans (Ryabova *et al.*, 2009).

Esterases

The esterases hydrolyse the ester linkages between xylose units and non-glycoside substituents. Acetyl xylan esterases (EC. 3.1.1.72) are able to hydrolyse the ester linkage between acetyl and xylose residues in xylans. They are distributed over families CE1-7, CE12 and CE16, displaying various folds (www.cazy.org). Feruloyl esterases (CE1; EC. 3.1.1.73) have many different specificities and can also hydrolyse the ester linkage between arabinose side chain residues and ferulic acid (Saha and Bothast, 1997; Kühnel *et al.*, 2012).

1.7.5 Rate determining factors in enzymatic hydrolysis of cell wall material

The rate determining factors of enzymatic hydrolysis of lignocellulosic material are either related to the structure of the substrate or are related to the enzymes. The factors related to the substrate include the partially crystalline nature of cellulose, accessible surface area, moisture content, degree of polymerization, particle size and lignin distribution (Sun and Cheng, 2002; Laureano-Perez *et al.*, 2005; Hendriks and Zeeman, 2009). Crystalline cellulose is known to be hard to be degraded by enzymes (Zhang and Lynd, 2004). The reduction in particle size leads to an increase of available specific surface and a reduction of the degree of polymerization of the cellulose (Hendriks and Zeeman, 2009). Lignin limits both the rate and extent of (enzymatic) hydrolysis by hindering the accessibility of the digestible parts of the substrate (Fig. 1.1; Chang *et al.,* (2000)). Interactions between cellulose and hemicellulose may also hinder efficient degradation of both these polymers (Kabel *et al.*, 2007).

The rate determining factors related to enzymes include end-product inhibition (Mansfield *et al.*, 1999), inactivation of the enzymes during hydrolysis (Eklund *et al.*, 1990) and irreversible or non-specific binding of enzymes (Zhang and Lynd, 2004). On the one hand, cellulases can adsorb on the substrate which can be an essential mechanism in cellulose hydrolysis (Ooshima *et al.*, 1990), on the other hand a carbohydrate binding module attached to an enzyme improves binding of the enzyme thereby facilitating activity of the catalytic domain on the surface of a solid substrate (Linder and Teeri, 1997). However, in addition to productive binding, cellulases and hemicellulases can bind in a non-productive way to lignin, which hinders efficient hydrolysis (Palonen *et al.*, 2004; Berlin *et al.*, 2006). Furthermore, a carbohydrate binding module also seemed to participate in the unspecific binding to lignin (Palonen *et al.*, 2004), thereby decreasing catalytic activity of the enzyme.

1.8 Sources of carbohydrate active enzymes

In nature, plant cell wall degradation is performed by a wide range of enzymes. These enzymes are produced by fungi, bacteria, yeast, marine algae, snails, insects, etc., but the principal commercial source of enzymes is filamentous fungi (Polizeli *et al.*, 2005).

1.8.1 Fungi

Most fungi secrete extracellular enzymes, which together act on the plant cell wall material to liberate monosaccharides as a directly assimilable end product allowing the organisms to grow heterotrophically (Kulkarni *et al.*, 1999). Many fungi have been isolated and were identified as efficient lignocellulolytic-enzyme producers (Sánchez, 2009).

The degradation of lignocellulose by filamentous fungi has been studied in a range of Basidiomycetes and Ascomycetes. It is known that the species of lignocellulolytic Basidiomycetes are extremely abundant in all forest types and that they are the major wood decomposers in most ecosystems. Among those wood-decaying Basidiomycetes, the white-rot fungi *Phanerochaete chrysosporium* contains many genes encoding oxidases, peroxidases and hydrolytic enzymes that cooperate in wood decay (Martinez *et al.*, 2004). The Ascomycete brown-rot fungus *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is used extensively in industry due to its capacity to secrete high levels of cellulases and hemicellulases (Martinez *et al.*, 2008). Many other *Trichoderma* species produce cellulolytic enzymes (Esposito and Silva, 1998). Furthermore, several *Aspergilli* are known for their efficient degradation of plant cell wall polysaccharides (de Vries and Visser, 2001).

The production of cellulases and hemicellulases by various *Penicillium* species and by *Myceliophthora thermophila* C1 has also been well studied (Aro *et al.*, 2005; Jørgensen *et al.*, 2005; Chávez *et al.*, 2006; Hinz *et al.*, 2009).

Though many of these fungi produce efficient lignocellulosic enzymes, the development of cost-effective enzymes for utilization of lignocellulosic biomass will require continuous research, as there is currently no powerful and effective enzyme

mixture to degrade the complex biomass efficiently. There are thousands of organisms involved in the natural decomposition of plant material in our biosphere, and only a fraction of those have been isolated or investigated (Merino and Cherry, 2007). It is very well possible that a very efficient lignocellulose-degrading organism exists, but it is not yet identified as such. This is partly due to the lack of effective screening methods. The development of a useful and predictive screening is particularly difficult because of the heterogeneous nature of solid substrates, like plant cell walls (Zhang *et al.*, 2006).

1.8.2 The enzymatic toolbox of *Myceliophthora thermophila* C1[1]

The filamentous fungus *Myceliophthora thermophila* C1 (C1), previously denoted as *Chrysosporium lucknowense* C1, was found to express a broad range of cellulases and hemicellulases that degrade plant cell walls. The fungus was first isolated from a soil sample collected in Lucknow, India by the Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences (Garg, 1966; Bukhtojarov *et al.*, 2004). After improvement of the original isolate, a vector was designed that enables the construction of complex libraries in the fungus, thereby facilitating a production strain as host for rapid screening (Verdoes *et al.*, 2007; Visser *et al.*, 2011). In the genome of the fungus, 200 genes were putatively encoding carbohydrate-active enzymes. This indicated that it is a potent strain to degrade plant cell wall material (Hinz *et al.*, 2009). As the C1 strain allows high overexpression levels of the enzymes with low background expression of other proteins (Verdoes *et al.*, 2007), it is an interesting alternative for the production of cellulolytic and hemicellulolytic enzymes. Among these putative carbohydrate active enzymes, 102 are potentially active in cellulose and hemicellulose degradation (Table 1.2). Compared to the common strains of *Aspergillus*, *Trichoderma* and *Penicillium*, C1 contains many putative

[1] During the present PhD study, molecular phylogenetic studies revealed that C1, previously classified as *Chrysosporium lucknowense* based on morphological characteristics, is actually a *Myceliophthora thermophila* isolate (Visser *et al.*, 2011). For clarity reasons it was decided to use only the new annotation within this thesis.

hemicellulolytic enzymes (Table 1.2). This makes it a highly interesting alternative to the common strains for the production of hemicellulolytic enzymes. However, research is needed to prove the potential of the putative enzymes.

Table 1.2: Putative hemicellulolytic enzymes present in the genome of Myceliophthora thermophila C1 (C1), Aspergillus niger (An), Trichoderma reesei (Tr), Aspergillus oryzae (Ao) and Penicillium chrysogenum (Pc).

Annotated enzymes	CAZy family	Number of enzymes*				
		C1[a]	*An*[b]	*Tr*[c]	*Ao*[b]	*Pc*[b]
β-Glucosidases/β-xylosidases	GH3	11	17	12	23	17
Endo-glucanases/cellobiohydrolases	GH5/GH6/GH7	19	14	7	18	16
Xylanases	GH10/GH11	11	5	5	8	4
Endo-glucanases	GH12/GH45/ GH61	28	11	10	12	7
Arabinases/arabinofuranosidases/ β-xylosidases	GH43	12	10	2	20	14
Arabinofuranosidases	GH51/GH62	4	5	1	5	3
α-Glucuronidase	GH67	1	1	1	1	1
Acetyl xylan esterases/ ferulic acid esterases	CE1/CE4/CE5	16	12	2	12	10
Total		**102**	**75**	**40**	**99**	**72**

* Total values per enzyme activity, corresponding to the theoretical number of enzymes within the CAZy families
[a] Personal communication S.W.A. Hinz, Dyadic Netherlands
[b] From the CAZy database (Henrissat *et al.*, 2012)
[c] From Martinez *et al.* (2008) and the JGI database (Grigoriev *et al.*, 2011)

1.9 Aim and outline of the thesis

The aim of this PhD study was to develop an effective screening method to discover efficient hemicellulolytic enzyme producing fungi for the hydrolysis of lignocellulosic biomass to fermentable monomers. Furthermore, the potential of enzymes from the promising *Myceliophthora thermophila* C1 strain was tested by analysing their ability to degrade various xylans and synergy of some accessory enzymes was studied.
An effective screening method for the detection of various hemicellulolytic enzymes in crude fermentation supernatants of fungi collected from various parts of the world is described in chapter 2.

Next, in chapter 3, the screening method was extended by the use of insoluble and complex substrates. By use of the insoluble and complex substrates efficient strains for the degradation of lignocellulosic biomass could be detected.

The strain *Myceliophthora thermophila* C1 (C1) produced many endo-xylanases, of which two GH family 10 endo-xylanases with and without carbohydrate binding domain are characterized in chapter 4. Their performances were correlated to the predicted protein structure of the enzymes.

In chapter 5 two novel C1 endo-xylanases of GH family 11 were subjected to purification and characterization. The formation of different degradation products by the enzymes was correlated to a single amino acid substitution in the predicted protein structures. Their performances were also compared with that of the C1 GH10 endo-xylanases. Based on these results the current classification system of carbohydrate active enzymes was criticized.

The combined action of various acetyl (xylan) esterases and their synergy with two different glucuronidases towards *Eucalyptus globulus* xylan is described in chapter 6.

The final chapter (chapter 7) discusses the major results found and the relevance of this study for future research in the search and characterization of novel and efficient enzymes.

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Chapter 2

Screening for distinct xylan degrading enzymes in complex shake flask fermentation supernatants

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Abstract

The efficient degradation of complex xylans needs collaboration of many xylan degrading enzymes. Assays for xylan degrading activities based on reducing sugars or PNP substrates are not indicative for the presence of enzymes able to degrade complex xylans: They do not provide insight into the possible presence of xylanase-accessory enzymes within enzyme mixtures. A new screening method is described, by which specific xylan modifying enzymes can be detected.

Fermentation supernatants of 78 different fungal soil isolates grown on wheat straw were analysed by high performance liquid chromatography and mass spectrometry. This strategy is powerful in recognising xylanases, arabinoxylan hydrolases, acetyl xylan esterases and glucuronidases.

No fungus produced all enzymes necessary to totally degrade the substrates tested. Some fungi produce high levels of xylanase active against linear xylan, but are unable to degrade complex xylans. Other fungi producing relative low levels of xylanase secrete many useful accessory enzyme component(s).

2.1 Introduction

In order to convert lignocellulosic biomass to ethanol, a whole array of enzymes is needed to degrade such a complex structure to monomers (Carvalheiro *et al.*, 2008). An important part of cereal and wood hemicellulose is the class of xylans, comprising a backbone of β-(1→4)-xylopyranosyl units containing various side groups like, amongst others, 4-*O*-methyl-α-D-glucopyranosyl uronic acid, ferulic acid, arabinofuranose and *O*-acetyl groups (Ebringerová and Heinze, 2000). The composition of the lignocellulose determines the enzymes required for complete degradation of the substrate to monomers.

Wheat arabinoxylan (WAX) and *Eucalyptus* xylan hydrolysate (EXH) have been used before to identify and characterize an array of xylan degrading enzymes (Christov *et al.*, 2000; Tenkanen and Siika-Aho, 2000; Pouvreau *et al.*, 2011), since these substrates contain the most abundant substituents present in cereal lignocellulosic xylans (Gírio *et al.*, 2010), except for ferulic acid (Vidmantiene *et al.*, 2006). WAX contains mono- and/or double α-L-arabinosylated xylopyranosyl units through *O*-2 and/or *O*-3 (Ebringerová and Heinze, 2000). The degradation of WAX requires, next to the presence of endo-xylanases (EC 3.2.1.8), the activity of AXH-m (releasing mono substituted arabinose: EC 3.2.1.55), AXH-d3 (releasing double substituted arabinose from position *O*-3: EC 3.2.1.55) or β-xylosidase (EC 3.2.1.37) within the fermentation supernatants (Van Laere *et al.*, 1997; Gírio *et al.*, 2010). The soluble EXH consists of *O*-acetyl-(4-*O*-methylglucurono)xylooligosaccharides (Christov *et al.*, 2000). The acetyl substituents are closely associated with the 4-*O*-methylglucuronic acid (Evtuguin *et al.*, 2003). The 4-*O*-methylglucuronic acid substituent may be substituted at *O*-2 with α-D-galactose (Shatalov *et al.*, 1999). The degradation of EXH is only successful in the presence of acetyl xylan esterases (EC 3.1.1.72), endo- and exo-xylanases and α-glucuronidases (EC 3.2.1.131) (Christov *et al.*, 2000; Tenkanen and Siika-Aho, 2000). α-Glucuronidases are enzymes that are able to hydrolyse the α-1,2-linkage between 4-*O*-methylglucuronic/glucuronic acid and xylose.

In the search for novel enzymes, in which the enzyme activity is often monitored using dyed substrates or via the formation of reducing end groups, no distinction can be made between different enzymes (Biely and Puchart, 2006; Ghatora *et al.*, 2006). Next to these assays, proteomic approaches making use of genomic libraries will often result in the annotation of known enzymes instead of identifying real novel or desired enzymes (Wang *et al.*, 2010).

Filamentous fungi are a good source of xylan degrading enzymes and their levels of enzyme excretion in the fermentation media are high, which makes them interesting for screening (Handelsman *et al.*, 1998; Polizeli *et al.*, 2005; Gírio *et al.*, 2010). However, more precise assays are necessary to include a wide range of enzymes in such screening (Biely and Puchart, 2006).

In this paper a screening method is presented in which a range of xylan degrading enzymes in fungal fermentation liquids are identified. Analysis of the xylan digests was done by using HPAEC, HPLC-organic acids and MALDI-TOF MS.

2.2 Materials and methods

2.2.1 Fungi

Shake flask fermentation supernatants (78) of mesophilic lignocellulolytic fungi were obtained from the Budapest University of Technology and Economics (BUTE), Hungary. These fungi have been isolated from soil samples and decaying plant materials collected worldwide. A few taxonomically identified fungi were also introduced into the study. They were obtained from known culture collections.

2.2.2 Enzymes

As reference enzyme endo-(1,4)-β -D-xylanase-I of *Aspergillus awamori* (GH10) was used. The purification and mode of action of this enzyme is described elsewhere (Kormelink *et al.*, 1993b). In addition, an acetyl xylan esterase (AXE: CE5) and an α-

glucuronidase (AGU1: GH67) of *Myceliophthora thermophila* C1 (Hinz *et al.*, 2009) were used as reference enzymes.

2.2.3 Chemicals and substrates

All chemicals used were, if not mentioned otherwise, of analytical grade.
The substrates used were Wheat arabinoxylan (WAX), medium viscosity (Megazyme, Wicklow, Ireland) and an *Eucalyptus* xylan hydrolysate (EXH) as produced by hydrothermal treatment (Garrote *et al.*, 1999), kindly provided by Prof. Dr. J.C. Parajo of the University of Vigo-Ourense, Spain.

2.2.4 Sugar composition of substrates

In order to determine the sugar composition, the substrates were hydrolysed using 72% (w/w) sulphuric acid at 30 °C for one hour followed by hydrolysis with 1M sulphuric acid at 100 °C for three hours. The neutral monosaccharides were analysed as their alditol acetates, using inositol as internal standard. A Focus gas chromatograph (Thermo Scientific, Waltham MA, USA) equipped with a Supelco SP 2380 column was used.
A part of the hydrolysate was used for the determination of the uronic acid content by the colorimetric m-HDP assay according to Ahmed and Labavitch (1978) using an auto-analyser (Skalar Analytical, Breda, The Netherlands) and using a galacturonic acid (0-100 µg/mL) calibration curve.

2.2.5 Isolation of lignocellulolytic fungi

Two types of agar media were used to isolate the cellulase and hemicellulase producing fungi. The medium contained either 30 g/L cellulose powder (Sigmacell Type 20, Sigma-Aldrich, St. Louis, MO, USA) or 30 g/L finely milled wheat straw (<0.3 mm) as carbon source. The other components were similar for both media (in g/L); $NaNO_3$, 3; $(NH_4)_2SO_4$, 1; KH_2PO_4, 1; $(NH_4)_2HPO_4$, 0.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; Difco yeast extract, 0.3; Bacto agar, 20; and (in mg/L) $FeSO_4.7H_2O$, 5; $MnSO_4$, 1.6;

$CoCl_2.6H_2O$, 2; $ZnSO_4.7H_2O$, 3.45. The pH (before sterilization) was set to 6.5 using sulphuric acid. Media were autoclaved routinely (30 min, 121 °C). The isolation agar media were supplemented with 100 µg/mL doxycycline hyclate (Sigma-Aldrich) to suppress bacterial growth. After incubation at 30 °C primary fungal colonies were inoculated in Petri plates by streaking twice on potato-dextrose-agar (PDA) supplemented with 1 g/L Triton X100 in order to obtain single colonies. The fungal isolates were freeze-dried for long term storage.

2.2.6 Enzyme production by shake flask fermentation

The lyophilized fungi were revitalized on PDA medium at 30 °C and the properly sporulated Petri plate cultures were used for inoculation. Two types of shake flask cultivation media (LC-3 and LC-4) were used. Medium LC-3 contained (in g/L): finely milled wheat straw (<0.3 mm), 20; KH_2PO_4, 1.5; $(NH_4)_2HPO_4$, 2; defatted soybean meal, 1; corn steep liquor, 50 % DM (SIGMA), 1; NaCl, 0.5; $CaCO_3$, 1; urea, 0.3; $MgSO_4.7H_2O$, 0.3; $CaCl_2$, 0.3; and (in mg/L) $FeSO_4.7H_2O$, 2.5; $MnSO_4$, 0.8; $CoCl_2.6H_2O$, 1; $ZnSO_4.7H_2O$, 1.7. Medium LC-4 contained (in g/L): finely milled wheat straw (<0.3 mm), 20; KH_2PO_4, 1; $(NH_4)_2HPO_4$, 1; $(NH_4)_2SO_4$, 1; defatted soybean meal, 1; corn steep liquor, 50 % DM (Sigma-Aldrich), 1; NaCl, 0.5; $CaCO_3$, 0.5; and (in mg/L) $FeSO_4.7H_2O$, 2.5; $MnSO_4$, 0.8; $CoCl_2.6H_2O$, 1; $ZnSO_4.7H_2O$, 1.7. For both media the pH (before sterilization) was set to pH 5.0 using sulphuric acid.

Of both media 150 mL was sterilized in 750 mL cotton-plugged Erlenmeyer flasks. They were inoculated with 3 loopful of spores per flask. Fermentation was carried out on a rotary shaker at 30 °C and 220 rpm for 5 days. Supernatants were stored at -18 °C for further analysis.

2.2.7 Enzyme screening

Endo-xylanase activity was determined using the colorimetric dinitrosalicylicacid (DNS) assay at pH5.0 using birch glucuronoxylan as substrate (Bailey *et al.*, 1992). Xylanase activity was expressed as IU/ml.

The 1,4-β-D-xylosidase activity was assayed as described by Herr *et al*. (1978). The liberated 4-nitrophenol was measured at 400 nm.
α-L-arabinofuranosidase activity was assayed according to Poutanen *et al*. (1987). 4-Nitrophenol was used for the standard curve.
For novel screening approaches WAX and EXH (both 5 mg/mL) were dissolved in 0.05 M sodium acetate buffer, pH 5.0. Next, shake flask fermentation supernatant was added to a final concentration of 1% (v/v). For determination of the degree of acetylation, the sodium acetate buffer was replaced by 0.05 M sodium citrate buffer (pH 5.0). Incubation took 24 h at 500 rpm and 35 °C. Enzymes were inactivated by boiling the digests for 10 min.

2.2.8 Quantification and characterisation of monomers and oligomers using HPAEC

The digests were 100x diluted with Millipore water and analysed by high performance anion exchange chromatography (HPAEC) using an ICS3000 HPLC system (Dionex, Sunnyvale, CA), equipped with a CarboPac PA-1 column (2 mm ID x 250 mm; Dionex) in combination with a CarboPac PA guard column (2 mm ID x 25 mm) and a ISC3000 ED PAD-detector (Dionex). A flow rate of 0.3 mL/min was used with the following gradient of 0.1 M sodium hydroxide (NaOH) and 1 M sodium acetate (NaOAc) in 0.1 M NaOH: 0-45 min, 0-500mM NaOAc in 0.1 M NaOH; 45-48 min washing step with 1 M NaOAc in 0.1 M NaOH; 48-60 min, equilibration with 0.1 M NaOH. Twenty µl of sample was injected each time. Quantification is based on the response factor of the standard xylose solutions of xylose to xylotetraose (Xyl_1 Sigma Aldrich, Steinheim, Germany, Xyl_{2-4} Megazyme, Wicklow, Ireland), D-arabinose and D-glucuronic acid (Sigma Aldrich).

2.2.9 Characterisation of oligomers by use of MALDI-TOF MS

For matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analysis an Ultraflex workstation (Bruker Daltonics, Bremen,

Germany) equipped with a 337 nm laser was used. The mass spectrometer was operated in the positive mode and calibrated with a mixture of maltodextrins (AVEBE, Veendam, The Netherlands; mass range 500-3500 Da). After a delayed extraction time of 120 ns, the ions were accelerated with a 25 kV voltage and subsequently detected using the reflector mode. One µL of desalted sample solution (AG 50 W-X8 resin; Bio-Rad, Hercules CA, USA) was mixed with 1 µL of matrix and dried under a stream of warm air. The matrix solution was prepared by dissolving 10 mg 2,5-dihydroxybenzoic acid (Bruker Daltonics) in a mixture of 700 µL water and 300 µL acetonitrile.

2.2.10 Determination of acetic acid

To determine the total acetyl content present in the substrate, the EXH blank was saponified by adding 50 µL 8 M NaOH to 0.45 mL of an EXH solution (5 mg/mL). The sample was left one hour on ice and 2 h at room temperature. This saponified EXH solution and all samples were analysed for acetic acid using an Ultimate 3000 system (Dionex) equipped with an Shodex RI detector and an Aminex HPX 87H column (300 mm x 7.8 mm) (BioRad, Hercules, CA). Twenty microliter of sample was injected. Elution was performed with 0.005 M H_2SO_4 at a flow rate of 0.6 mL/min and a column oven temperature of 40 °C. The amount of acetic acid released was calculated as percentage of the total amount of acetic acid present in the sample.

2.3 Results and discussion

The level of complexity of the xylans is illustrated by the ratio of the various substituents to xylose (Table 2.1). WAX is mainly substituted with arabinose. In EXH the xylan backbone is substituted with 4-*O*-methylglucuronic acid and acetic acid. This data is consistent with literature findings (Kormelink *et al.*, 1993a; Christov *et al.*, 2000). The lower sugar content of the EXH compared to the WAX is explained by the presence of reaction products of the hydrothermal treatment (Garrote *et al.*, 1999).

Table 2.1: Constituent monosaccharide composition and total sugar content (w/w%) of wheat arabinoxylan (WAX) and Eucalyptus xylan hydrolysate (EXH).

Sample name	Sugar composition (w/w%)[a]								Total sugar content (w/w%)	Ara/ Xyl[b]	UA/ Xyl[b]	Ac/ Xyl[b]
	Rha	Ara	Xyl	Man	Gal	Glc	UA	Ac				
WAX	0	32	58	---	---	---	1	---	91	0.6	0	---
EXH	2	2	39	2	5	2	6	8	59	0.1	0.1	0.8

[a] Neutral sugars, uronic acids or acetic acid expressed as weight percentage: Rha: rhamnose, Ara: arabinose, Xyl: xylose, Man: mannose, Gal: galactose, Glc: glucose, UA: uronic acid, Ac: acetyl esters
[b] Ratio mol/mol

2.3.1 Production and selection of the fermentation liquids

The initial screening of the culture collection and selected filamentous fungal samples was based on the ability of the micro-organisms to grow on cellulose or wheat straw as a sole carbon source. The extracellular production of xylanase, 1,4-β-D-xylosidase and α-L-arabinofuranosidase in the fermentation liquids for fungi selected by the HPLC and MS screening (*vide infra*) is summarized in Table 2.2.

The randomly selected lignocellulolytic fungi express in a wide range all enzyme activities tested. Strains ATCC 10864 and ATCC 14916 (two black *Aspergilli*) and three *Trichoderma* strains (F-1647, F-1702 and F-2380) are relatively good producers of endo-xylanase, β-xylosidase and arabinose releasing enzymes. These two black *Aspergilli* were previously found to be good xylan decomposers (Bailey and Poutanen, 1989; Linden *et al.*, 1994). Also other, non-identified fungi exhibit similar good secretion of xylan degrading enzymes. However, traditional colorimetric assays do not allow us to draw conclusions concerning the presence of individual enzymes involved in the degradation of complex xylans. Also weak producers of xylanases may excrete relevant hemicellulolytic enzymes needed for total degradation. Therefore, the application and combination of other methods (HPAEC, HPLC and MALDI-TOF MS) for screening of the same supernatants may reveal new details.

Table 2.2: Sample information of selected fermentation liquids including sample number, strain, and fermentation medium used, the origin of the strain and endo-xylanase, β-xylosidase and α-arabinofuranosidase activity in each sample.

Sample number	Strain	Shake flask medium	Isolation origin of strain	Colorimetric methods		
				Xylanase (IU/ml)	β-xylosidase (IU/ml)	α-arabino-furanosidase (IU/ml)
B1	*Trichoderma sp.* TUB F-1702	LC3	Soil, Ho Chi Minh City (Saigon), Vietnam	17.9	1.05	50.4
B4	Non-identified fungus TUB F-2292	LC4	Soil, Sibelius park, Helsinki, Finland	0.5	0.05	3.0
B7	Non-identified fungus TUB F-2346	LC3	Decaying forest litter, Camlidere, Turkey	2.3	0.13	11.7
B8	Non-identified fungus TUB F-2346	LC4	Decaying forest litter, Camlidere, Turkey	3.3	0.11	12.8
B12	Non-identified fungus TUB F-2342	LC4	Soil, Horsetooth Mt., Ft.Collins, Colorado, USA	31.2	0.15	6.0
B14	Non-identified fungus TUB F-2353	LC4	Soil, Horsetooth Mt., Ft.Collins, Colorado, USA	28.6	0.15	6.4
B27	Non-identified fungus TUB F-2348	LC4	Soil under thuya tree, city park, Budapest, Hungary	1.4	0.02	7.2
B29	*Trichoderma sp.* TUB F-2350	LC4	Meadow soil, 1180 m elevation, mountain near Yalta, Crimera, Ukraine	1.7	0.07	2.7
B30	Non-identified fungus TUB F-2352	LC4	Decaying forest litter, Camlidere, Turkey	10.7	1.02	25.5
B34	Non-identified fungus TUB F-2385	LC4	Soil, near Dead Sea, Jordan	1.6	0.04	3.0
B36	Non-identified fungus TUB F-2388	LC4	Soil in oasis, Syrian desert, Palmira, Syria	0.3	0.04	2.5
B38	Non-identified fungus TUB F-2390	LC4	Soil, Queensland, Australia	5.9	0.52	11.8
B40	*Aspergillus oryzae* NRRL 3485	LC4	NRRL 3485 (=ATCC 46244)	22.8	0.40	5.9
B41	*Aspergillus niger* ATCC 10864	LC4	ATCC 10864	24.9	1.09	14.1
B42	*Aspergillus foetidus* ATCC 14916	LC4	ATCC 14916	33.8	0.55	12.1
B43	Non-identified fungus TUB F-2386	LC4	Soil, near Dead Sea, Jordan	2.2	0.07	2.9

Continued on next page

Sample number	Strain	Shake flask medium	Isolation origin of strain	Colorimetric methods		
				Xylanase (IU/ml)	β-xylosidase (IU/ml)	α-arabino-furanosidase (IU/ml)
B45	*Trichoderma sp.* TUB F-1647	LC4	Dead bark of an unidentified tree with lichen, Embudu Village Island, South-Male Atoll, Maldives	11.0	0.14	11.5
B49	Non-identified fungus TUB F-2372	LC4	Soil under Parrotia persica (Persian ironwood), Arboretum, Szarvas, Hungary	6.5	0.22	19.2
B52	*Aspergillus terreus* OKI 16/5 (=ATCC 62406)	LC4	Cellulose pulp, Hungary	3.5	0.22	5.6
B53	Non-identified fungus TUB F-2394	LC4	Soil, New South Wales, Australia	1.8	0.03	3.2
B54	Non-identified fungus TUB F-2361	LC4	Garden soil under thorn-bush, Budapest, Hungary	2.1	0.04	4.3
B62	*Chaetomium globosum* OKI 270	LC4	OKI 270	0.7	0.02	3.1
B66	Non-identified fungus TUB F-2378	LC4	Soil under Parrotia persica (Persian ironwood), Arboretum, Szarvas, Hungary	9.7	0.56	8.2
B68	*Paecilomyces bacillisporus* IFO 9387	LC4	IFO 9387	1.0	0.03	5.8
B72	*Myrothecium verrucaria* NRRL 2003	LC4	NRRL 2003 (=ATCC 9095)	1.2	0.02	4.9
B73	Non-identified fungus TUB F-2341	LC4	Soil, Horsetooth Mt., Ft.Collins, Colorado, USA	2.7	0.12	5.6
B74	*Trichoderma sp.* TUB F-2380	LC4	Soil under Gynerium argenteum (pampas grass), Arboretum, Szarvas, Hungary	8.4	0.36	25.5
B78	*Paecilomyces varioti* IFO 4855	LC4	IFO 4855	1.1	0.14	2.9

ATCC: American Type Culture Collection, Manassas, Virginia, USA; IFO: Institute for Fermentation, Osaka, Japan; NRRL: Northern Regional Research Center, USDA, Peoria, Illinois, USA; OKI: National Institute for Public Health,Budapest, Hungary; TUB: Technical University of Budapest, microbial culture collection.

2.3.2 Enzyme recognition by WAX degradation

The digestion of WAX by the fermentation liquids resulted in various degradation products, which were visualized by use of HPAEC (Fig. 1).

The elution patterns differed for the different fermentation liquids. Some of the fermentation liquids released quite some monosaccharides, whereas others yielded substituted oligomers only. The hydrolysis products and HPAEC pattern of WAX degraded by an endo-xylanase I from *A. awamori* have been extensively described by Gruppen *et al*. (1992) and Kormelink *et al*. (1993c). The endo-xylanase I digest was used as reference for identification of the arabinoxylan oligosaccharides in the digests obtained.

Within the elution profiles of the 78 digests, four types of patterns could be distinguished, which are displayed in Fig. 2.1:

Pattern 1: Arabinose and xylose are present next to linear xylooligomers and (single, double and multiple) substituted arabinoxylooligomers.

Pattern 2: Arabinose, xylose, linear xylooligomers and double substituted arabinoxylooligomers are present. Multiple and single substituted arabinoxylooligomers are not detected.

Pattern 3: Arabinose, xylose and linear xylooligomers are present. No substituted (single, double and multiple) arabinoxylooligomers are detected.

Pattern 4: Arabinose, xylose, double and multiple substituted arabinoxylooligomers are present. Linear xylooligomers and single substituted arabinoxylooligomers are not detected.

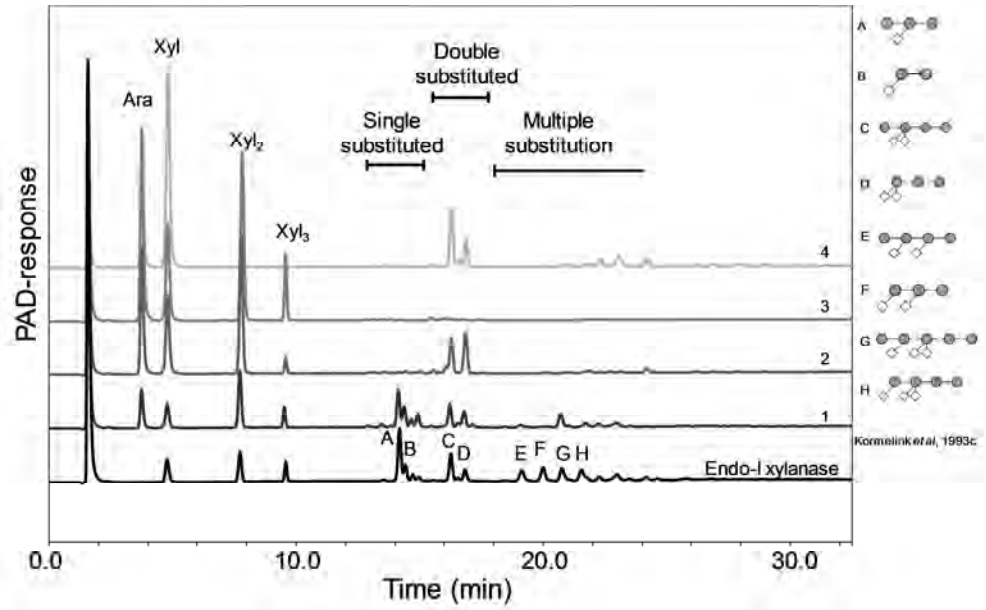

Fig. 2.1: Endo-xylanase I digest pattern and four typical high performance anion exchange chromatography (HPAEC) patterns as found for digests of fermentation liquid on wheat arabinoxylan (WAX) (line 1: degradation pattern of WAX after hydrolysis with sample B6, line 2: WAX after hydrolysis with sample B13, line 3: WAX after hydrolysis with sample B7, line 4: WAX after hydrolysis with sample B42).

Group 1

The oligomers released by the fermentation liquids displaying pattern 1 on HPAEC are almost similar to the oligomers released by WAX hydrolysed with endo-xylanase I (GH10), requiring two unsubstituted xylopyranosyl residues between the branches to be able to split (Kormelink *et al.*, 1993a; Biely *et al.*, 1997). The fermentation liquids contain mainly endo-xylanases. Some small differences compared to the endo-xylanase I pattern are observed for group 1, for example the presence of small amounts of arabinose (Fig. 2.1, line 1). Forty-two fermentation liquids displayed degradation pattern 1, indicating that the majority of strains tested only expressed xylanases when grown on wheat straw.

Group 2

Group number 2 represents 22 fermentation liquids displaying pattern 2. These fermentation liquids released quite some arabinose from the single substituted xylose residues, but the linear xylooligomers and double substituted arabinoxylooligomers are not hydrolysed. This indicates that the enzyme mixture contains, in addition of an endo-xylanase, also an arabinoxylan hydrolase acting on single substituted xyloses. No arabinoxylan hydrolase acting on the double substituted xylopyranosyl units is present. Arabinoxylan hydrolases able to release arabinose from the double substituted xylopyranosyl unit are quite unique. Only three were reported so far (Van Laere *et al.*, 1997; Sørensen *et al.*, 2006; Pouvreau *et al.*, 2011). Since linear xylooligomers are still present, no β-xylosidase activity is expected in the fermentation liquids of group 2.

Group 3

The digests of the fermentation liquids in group number 3 (8 in total) contain only monomers and linear xylooligomers. Both single and double arabinoxylooligomers have been degraded. This indicates that the fermentation liquids contain, next to endo-xylanases, arabinoxylan hydrolases active on both mono and double substituted xylopyranosyl units. High levels of linear xylooligomers are predominantly present, indicating the lack of a β-xylosidase.

Group 4

Group number 4 includes 5 fermentation liquids releasing high quantities of xylose monomer, indicating that a powerful β-xylosidase is present. Next to this β-xylosidase, an arabinoxylan hydrolase active on single substituted arabinoxylooligomers is present. This is illustrated by the absence of mono substituted oligomers and the presence of arabinose, while the double substituted arabinoxylooligomers are still present.

One of the HPAEC patterns, displaying the hydrolysis products of fermentation liquid number B63 after WAX digestion, could not be placed in one of the four groups. This

fermentation liquid was able to release arabinose from double substituted arabinoxylooligomers, but not from the single substituted arabinoxylooligomers.

Within the HPAEC-patterns of the fermentation liquids, the intensity of the different peaks can be used to quantify the xylan degrading activity. However, one should keep in mind that fixed amounts of the crude fermentation liquids are added, so no conclusions can be drawn on the specific activity or efficiency of the enzymes. Since the total content of each monosaccharide constituent in the initial substrate is known, the relative amount of released material (w/w%) was calculated. Fermentation liquids able to release a minimum of 50% product (arabinose, linear xylooligomer and total WAX) are shown in Table 2.3. The criterion for xylose release was 25%, since xylose release was limited for these digests. This was expected as β-xylosidase production in fungal fermentation broths is limited (Basaran and Ozcan, 2008). The quantitative data of the hydrolysis products formed by all fermentation liquids can be found in Supplementary Table A1.

It is shown that 10 out of 78 fermentation liquids were able to release >50% of the arabinose present in the initial substrate as monomer. Three fermentation liquids released >50% of the total xylose to xylooligomers. Seven out of 78 fermentation liquids were able to hydrolyse >50% of the total WAX to monomers or oligomers. For β-xylosidase activity, it can be seen that only 5 out of 78 fermentation liquids had the ability to hydrolyse >25% of the total xylose present to monomer.

Table 2.3: Fermentation liquids with distinct xylan degrading activity resulting from screening on wheat arabinoxylan (WAX) and Eucalyptus xylan hydrolysate (EXH), based on high performance anion exchange chromatography (HPAEC), matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography for organic acids (HPLC).

		Hydrolysis products formed of WAX				**Hydrolysis products formed of EXH**					
Sample number	**Group[a]**	**Arabinose[b]**	**Xylose[b]**	**Xylooligomers[c] (DP2-4)**	**Total WAX[d]**	**4-*O*-Methyl Glucuronic Acid[b]**	**Acetyl[e]**	**Xylose[b]**	**Xylooligomers[c] (DP2-4)**	**Galactose[f]**	**Total EXH[d]**
**		0	4	9	8	39	n.a.	2	10	-	15
B1	**4**	**35**	**27**	**10**	**36**	12	48	9	2	-	11
B4	1	12	1	12	12	**15**	**54**	**1**	**9**	**+**	**11**
B7	**3**	**74**	**17**	**42**	**63**	6	68	12	0	-	11
B8	**3**	**51**	**17**	**23**	**43**	5	58	13	0	-	12
B12	2	36	10	25	34	**47**	**63**	**5**	**7**	**-**	**17**
B14	2	43	12	26	40	**47**	**66**	**5**	**6**	**-**	**16**
B27	1	38	7	28	35	**17**	**80**	**3**	**10**	**-**	**14**
B29	1	13	4	8	12	**14**	**19**	**3**	**6**	**+**	**10**
B30	**4**	**37**	**34**	**4**	**36**	3	68	11	0	-	10
B34	1	42	6	32	39	**47**	**54**	**2**	**8**	**-**	**15**
B36	1	8	1	7	8	**12**	**34**	**1**	**7**	**+**	**9**
B38	**3**	**87**	**23**	**44**	**73**	7	60	11	2	-	11
B40	**2 or 4**	**54**	**25**	**22**	**49**	**19**	**79**	**10**	**1**	**-**	**13**
B41	**4**	**32**	**31**	**2**	**32**	**17**	**84**	**12**	**0**	**-**	**12**
B42	**4**	**33**	**35**	**1**	**34**	**23**	**75**	**11**	**0**	**-**	**13**
B43	1	4	4	8	9	**22**	**47**	**3**	**7**	**+**	**12**
B45	**3**	**70**	**16**	**52**	**64**	31	57	10	3	-	15
B49	**3**	**72**	**19**	**49**	**64**	27	60	10	3	-	15
B52	**3**	**68**	**8**	**55**	**60**	3	58	3	8	-	10
B53	1	25	2	25	25	**3**	**55**	**3**	**10**	**+**	**11**
B54	1	22	4	23	24	**46**	**76**	**3**	**8**	**-**	**16**
B62	1	26	1	21	22	**66**	**41**	**1**	**1**	**+**	**11**
B66	**3**	**69**	**19**	**46**	**62**	4	56	5	8	-	12
B68	1	20	4	20	21	**16**	**42**	**2**	**11**	**+**	**13**
B72	1	20	5	20	21	**28**	**79**	**3**	**7**	**-**	**12**
B73	**2**	**54**	**7**	**44**	**48**	28	62	3	9	-	13
B74	**3**	**77**	**22**	**51**	**69**	7	56	4	7	-	11
B78	1	13	4	10	13	**20**	**18**	**6**	**4**	**+**	**11**

The best performing fermentation liquids per substrate are in bold font. n.a.: Not analysed. ** Endo-xylanase I (WAX) or AGU1, Endo-xylanase I, AXE (EXH); + = Galactose released, - = No galactose released; [a] Based on degradation pattern on HPAEC after hydrolysis of WAX; [b] Amount as percentage of total amount of that component present in initial substrate (w/w%); [c] Amount of xylooligomers as percentage of xylose present in initial substrate (w/w%); [d] Monomers and linear xylooligomer products as percentage of total amount of these components present in initial substrate (w/w%); [e] Based on combined HPLC and MALDI-TOF MS results, expressed as percentage of total amount of that component present in the initial substrate; [f] Based on MALDI-TOF MS results, qualitative result.

2.3.3 Enzyme recognition by EXH degradation

Since WAX does not cover all substituents that can be present in a complex lignocellulose, EXH was used as additional substrate.

Oligosaccharides released

Enzymatic degradation of the EXH by the different fermentation liquids resulted in various HPAEC patterns of which a typical example is shown in Fig. 2.2, line 2.

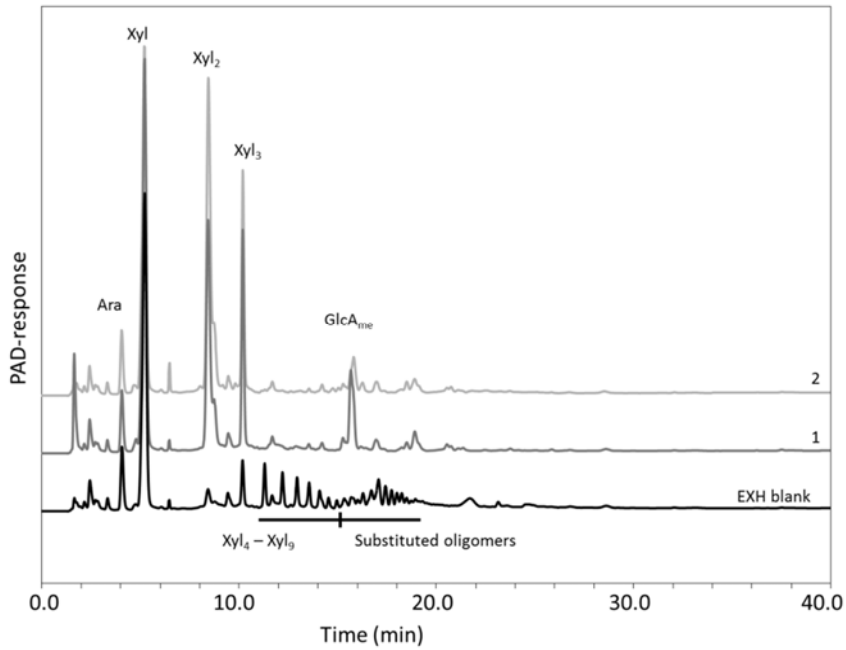

Fig. 2.2: High performance anion exchange chromatography (HPAEC) patterns derived from Eucalyptus xylan hydrolysate blank, line 1: Pattern of the digest of Eucalyptus xylan hydrolysate with the reference enzyme mixture (Endo-xylanase, Glucuronidase and acetyl xylan esterase) and line 2: Pattern of the digest of Eucalyptus xylan hydrolysate with fermentation liquid B35.

The HPAEC pattern of the blank EXH (Fig. 2.2), shows that already quite some monomeric xylose, monomeric arabinose, linear xylooligomers and substituted oligomers are present. The sample treated with the reference enzymes endo-xylanase I, glucuronidase and acetyl xylan esterase (Fig. 2.2, line 1) contains 4-*O*-

methylglucuronic acid, xylose, xylobiose and xylotriose. The substituted oligomers are partly degraded. The fermentation liquid B35 (Fig. 2.2, line 2), just as an example, has the ability to release arabinose, xylobiose, xylotriose and some 4-*O*-methylglucuronic acid. The HPAEC elution patterns of the hydrolysis products of EXH after digestion by all fermentation liquids were quite different. Therefore, it is hard to recognize specific activities responsible for similar patterns. Hence, the results were quantified for xylose, xylooligomers (DP2-DP4) and 4-*O*-methylglucuronic acid. These results are displayed in Table A1 for all fermentation liquids and for selected fermentation liquids in Table 2.3. These data ignore any galactosidase activity as galactose co-elutes with xylose under the conditions used. Furthermore, due to the highly alkaline conditions on HPAEC, acetyl xylan esterase activity could not be analysed using this technique.

Oligosaccharide profile using MALDI-TOF MS

To recognize acetyl xylan esterase activity, glucuronidase activity and to confirm the ability of a fermentation liquid to release the galactose unit from the *O*-2 position of the 4-*O*-methylglucuronic acid, MALDI-TOF MS was performed. In each spectrum the main peaks were annotated (Fig. 2.3). Hypothetical structures could be drawn based on the m/z value and the calculated putative structures of the main oligosaccharides, which gave insight in the degree of substitution. The positions of the substituents are still hypothetical. The blank EXH sample mainly contained acetylated glucuronoxylooligomers. The saponified sample only contains non-acetylated oligomers. Quite some fermentation liquids were able to release the acetyl substituents. The example of fermentation liquid B41 shows that it typically releases the acetyl groups and degrades the oligomers to smaller molecules (Fig. 2.3). The spectra of the blank sample and of fermentation liquid B41 are significantly different from each other and show that the release of side groups is necessary to allow xylanases to degrade the substrate to smaller molecules.

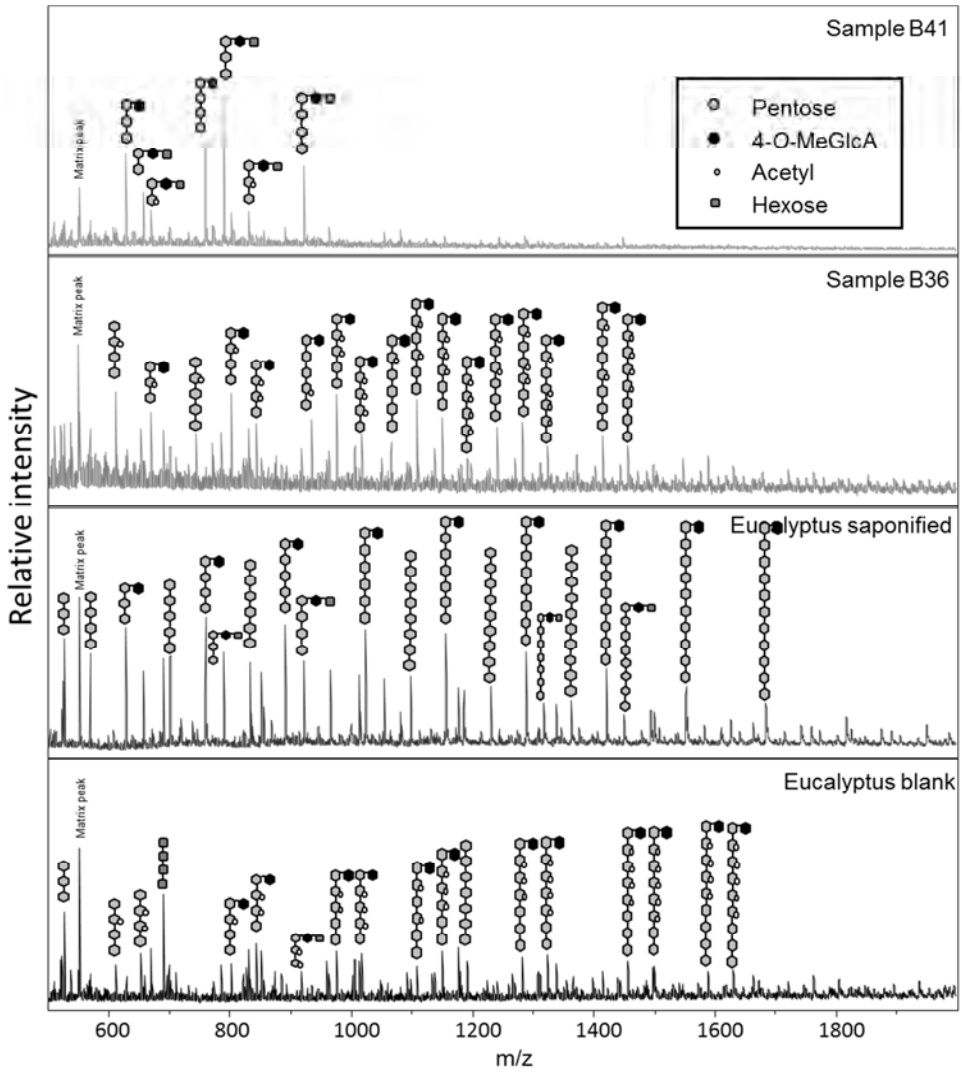

Fig. 2.3: Matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectra with hypothetical structures of Eucalyptus xylan hydrolysate before and after saponification and two fermentation liquids: example of digest strong in hexose release (B36), example of digest strong in acetyl and xylose release (B41).

Using HPLC it was shown that although most fermentation liquids had acetyl esterase activity, only 5% of the fermentation liquids could remove more than 75% of the acetyl substituents of the backbone (Table 2.3, Table A1). Information on the

positional specificity of the acetyl xylan esterases could only be obtained when using synthetical Ac-NPh-Xyl (Biely and Puchart, 2006).

Only 8 out of the 78 fermentation liquids contain galactosidase activity (Table 2.3, Table A1). Fermentation liquid B36 is an example of a fermentation liquid able to hydrolyze the galactose unit from the 4-*O*-methylglucuronic acid.

All MALDI-TOF mass spectra revealed typical resistant structures based on the five main peaks in each spectrum. The positions of the substituents are only hypothetically, based on the sugar composition of the EXH (Table 2.1) and literature (Garrote *et al.*, 1999; Christov *et al.*, 2000; Kabel *et al.*, 2002). Eighty percent of the fermentation liquids degraded higher oligomers and accumulated $X^{2a}XU^{M2,4m2}$ (code according to (Fauré *et al.*, 2009)) representing 2-*O*-Acetyl-β-D-$(Xylp)_2$-α-D-Galp-(1→2)-4-*O*-Methyl-α-D-GlcpA-(1→2). This component was present as one of the five main peaks in 80% of the spectra. Next to this component, in 87% of the fermentation liquids accumulation of $X^{2a}XU^{4m2}$ was seen, representing 2-*O*-Acetyl-β-D-$(Xylp)_2$- 4-*O*-Methyl-α-D-GlcpA-(1→2).

For each of the hydrolysis products formed, a top five was selected based on the amount of product present after digestion (Table 2.3). For 4-*O*-methylglucuronic acid release the top five ranged from 46-66% of the total amount of 4-*O*-methylglucuronic acid present in the initial substrate. Acetic acid release top five ranged from 75-84% of all acetic acid present. Xylose and xylooligomers (DP2-4) were released in much lower amounts, 1-13% and 0-11% respectively. Furthermore, the qualitative results on galactosidase activity are given, based on the five main structures present in the MALDI-TOF mass spectra lacking any galactose moiety. The selection in Table 2.3 shows that 17 fermentation liquids were active towards EXH. However, none of the fermentation liquids was able to hydrolyse more than 17% of the total substrate to monomers and xylooligomers (Sample B12, Table 2.3).

Table 2.4: Top three fermentation liquids per enzyme activity, based on the results of the classical colorimetric screening method and the presented screening method.

Sample number	Top 3 per activity determined by classical colorimetric methods			Top 3 per activity determined by current screening method				
	Endo-xylanase[a]	β-xylosidase[b]	α-arabino furanosidase[b]	Endo-xylanase[c]	β-xylosidase[d]	α-arabino furanosidase[e]	Glucuronidase[f]	Acetyl xylan esterase[g]
B1		X	X					
B7						X		
B8								
B12	X						X	
B14	X							
B27								X
B30		X	X		X			
B34							X	
B38						X		
B41		X			X			X
B42	X				X			
B45				X				
B52				X				
B62							X	
B72								X
B74			X	X		X		

a Based on reducing sugar release from birch glucuronoxylan.
b Based on liberated 4-nitrophenol from the respective PNP substrates.
c Based on the release of xylooligomers (DP2-4) from WAX.
d Based on the release of xylose from WAX/EXH.
e Based on the release of arabinose from WAX (either at *O*-2 or *O*-3 position).
f Based on the release of 4-*O*-methylglucuronic acid from EXH.
g Based on the acetic acid release from EXH.

Table 2.4 presents the three best enzyme producing soil fungi per activity and/or method. Surprisingly, only a few fungi were selected as best producer for more than one enzyme activity. As example samples B12 and B14 are indicated as best endo-xylanase producers. Nevertheless, both samples were not able to degrade WAX substrate, indicating that the endo-xylanases are hindered by substitution of the xylan

backbone. In contrast sample B30, B41 and B74 are able to produce quite some different accessory enzymes, but are not selected for their endo-xylanase expression.

2.4 Conclusions

The new screening method yields valuable information concerning the enzyme activities present in 78 fungal fermentation liquids. An overview of the three most active fermentation liquids per enzyme activity shows that enzyme selection via the classical screening method does not result in the best enzyme cocktail to degrade real substrates. Our method combines different analytical tools able to distinguish various specific enzyme activities in crude fermentation liquids using only two model substrates. It is a precise method for detailed screening of complex enzyme mixtures.

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Supplementary data

Table A1: Xylan degrading activity of all fermentation supernatants resulting from screening on wheat arabinoxylan (WAX) and Eucalyptus xylan hydrolysate (EXH), based on high performance anion exchange chromatography (HPAEC), matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography for organic acids (HPLC).

		Hydrolysis products formed of WAX				Hydrolysis products formed of EXH					
Sample number	**Group[a]**	**Arabinose[b]**	**Xylose[b]**	**Xylooligomers[c] (DP2-4)**	**Total WAX[d]**	**4-*O*-Methyl Glucuronic Acid[b]**	**Acetyl[e]**	**Xylose[b]**	**Xylooligomers[c] (DP2-4)**	**Galactose[f]**	**Total EXH[d]**
**		0	4	9	8	39	n.a.	2	10	-	15
B1	4	35	27	10	36	12	48	9	2	-	11
B2	2	36	18	19	36	18	55	4	2	-	8
B3	1	25	3	24	26	22	64	3	9	-	14
B4	1	12	1	12	12	15	54	1	9	+	11
B5	1	17	5	13	18	12	71	3	12	-	14
B6	1	14	4	14	16	8	56	2	13	-	14
B7	3	74	17	42	63	6	68	12	0	-	11
B8	3	51	17	23	43	5	58	13	0	-	12
B9	1	20	6	14	19	5	53	4	8	-	11
B10	1	27	8	19	27	3	57	3	8	-	10
B11	2	47	12	28	42	25	62	7	5	-	14
B12	2	36	10	25	34	47	63	5	7	-	17
B13	2	48	13	28	43	24	65	5	9	-	15
B14	2	43	12	26	40	47	66	5	6	-	16
B15	2	33	11	23	34	9	58	5	6	-	11
B16	2	32	9	24	32	10	58	4	7	-	11
B17	1	21	6	16	22	27	47	5	6	-	13
B18	1	28	7	22	28	21	42	4	6	-	11
B19	2	43	17	22	40	9	68	10	1	-	11
B20	2	31	9	22	31	7	64	4	7	-	10
B21	2	33	13	21	33	6	53	6	5	-	11
B22	2	34	13	22	34	8	66	5	5	-	10
B23	1	29	11	20	30	8	41	4	8	-	11
B24	1	40	8	24	34	25	45	6	6	-	13
B25	1	22	8	12	21	8	24	6	5	-	10

		Hydrolysis products formed of WAX				Hydrolysis products formed of EXH					
Sample number	**Group[a]**	**Arabinose[b]**	**Xylose[b]**	**Xylooligomers[c] (DP2-4)**	**Total WAX[d]**	**4-*O*-Methyl Glucuronic Acid[b]**	**Acetyl[e]**	**Xylose[b]**	**Xylooligomers[c] (DP2-4)**	**Galactose[f]**	**Total EXH[d]**
B26	2	35	10	25	34	8	51	4	7	-	11
B27	1	38	7	28	35	17	80	3	10	-	14
B28	1	14	4	13	16	13	55	4	8	-	12
B29	1	13	4	8	12	14	19	3	6	+	10
B30	4	37	34	4	36	3	68	11	0	-	10
B31	1	51	12	28	44	13	64	7	6	-	13
B32	1	33	5	23	29	12	58	4	9	-	13
B33	1	22	6	15	21	12	56	4	9	-	13
B34	1	42	6	32	39	47	54	2	8	-	15
B35	1	30	3	26	29	19	58	1	13	-	14
B36	1	8	1	7	8	12	34	1	7	+	9
B37	1	21	7	17	23	13	55	3	7	-	10
B38	3	87	23	44	73	7	60	11	2	-	11
B39	2	63	23	28	54	20	63	11	3	-	14
B40	2 or 4	54	25	22	49	19	79	10	1	-	13
B41	4	32	31	2	32	17	84	12	0	-	12
B42	4	33	35	1	34	23	75	11	0	-	13
B43	1	4	4	8	9	22	47	3	7	+	12
B44	1	36	3	31	32	31	69	2	8	-	13
B45	3	70	16	52	64	31	57	10	3	-	15
B46	2	26	8	24	28	20	56	6	8	-	14
B47	2	27	10	24	29	4	39	6	6	-	11
B48	2	29	2	30	29	3	59	3	10	-	11
B49	3	72	19	49	64	27	60	10	3	-	15
B50	2	34	6	33	34	23	40	5	8	-	14
B51	1	22	8	19	23	38	56	6	5	-	15
B52	3	68	8	55	60	3	58	3	8	-	10
B53	1	25	2	25	25	3	55	3	10	+	11
B54	1	22	4	23	24	46	76	3	8	-	16
B55	1	12	8	9	14	14	55	5	7	-	12
B56	2	30	13	25	33	23	47	5	7	-	13

		Hydrolysis products formed of WAX				Hydrolysis products formed of EXH					
Sample number	**Group[a]**	**Arabinose[b]**	**Xylose[b]**	**Xylooligomers[c] (DP2-4)**	**Total WAX[d]**	**4-*O*-Methyl Glucuronic Acid[b]**	**Acetyl[e]**	**Xylose[b]**	**Xylooligomers[c] (DP2-4)**	**Galactose[f]**	**Total EXH[d]**
B57	2	46	9	33	40	13	62	3	8	-	11
B58	1	27	3	22	24	13	71	3	11	-	14
B59	1	25	5	23	25	7	66	6	9	-	14
B60	1	24	7	22	25	38	60	4	8	-	16
B61	1	28	6	27	29	8	66	5	8	-	13
B62	1	26	1	21	22	66	41	1	1	+	11
B63	x	47	9	32	40	29	66	3	8	-	14
B64	1	24	5	24	25	18	70	3	9	-	13
B65	1	40	4	35	36	6	51	3	6	-	9
B66	3	69	19	46	62	4	56	5	8	-	12
B67	1	25	8	23	27	4	44	4	9	-	12
B68	1	20	4	20	21	16	42	2	11	+	13
B69	2	27	11	22	29	22	62	5	8	-	14
B70	1	31	5	25	28	18	61	2	10	-	13
B71	1	10	6	14	15	29	64	3	9	-	14
B72	1	20	5	20	21	28	79	3	7	-	12
B73	2	54	7	44	48	15	62	4	9	-	13
B74	3	77	22	51	69	7	56	4	7	-	11
B75	1	48	10	32	41	21	72	4	8	-	13
B76	1	16	2	15	15	13	72	3	11	-	14
B77	1	22	6	22	24	25	61	6	4	-	12
B78	1	13	4	10	13	20	18	6	4	+	11

** Endo-xylanase-I (WAX) / AGU1, endo-xylanase-I, AXE (EXH); + = Galactose released, - = No galactose released; [a] Based on degradation pattern on HPAEC after hydrolysis of WAX; [b] Amount as percentage of total amount of that component present in initial substrate (w/w %); [c] Amount of xylooligomers as percentage of xylose present in initial substrate (w/w %); [d] Monomers and linear xylooligomer products as percentage of total amount of these components present in initial substrate (w/w %); [e] Based on combined HPLC & MALDI-TOF MS results, expressed as percentage of total amount of that component present in the initial substrate; [f] Based on MALDI-TOF MS results, qualitative result. X: Could not be placed in one of the four groups

Chapter 3

Performance of hemicellulolytic enzymes in culture supernatants from a wide range of fungi on insoluble wheat straw and corn fiber fractions

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Abstract

Filamentous fungi are a good source of hemicellulolytic enzymes for biomass degradation. Enzyme preparations were obtained as culture supernatants from 78 fungal isolates grown on wheat straw as carbon source. These enzyme preparations were utilised in the hydrolysis of insoluble wheat straw and corn fiber xylan rich fractions. Up to 14% of the carbohydrates in wheat straw and 34% of those in corn fiber were hydrolysed. The degree of hydrolysis by the enzymes depended on the origin of the fungal isolate and on the complexity of the substrate to be degraded. *Penicillium*, *Trichoderma* or *Aspergillus* species, and some non-identified fungi proved to be the best producers of hemicellulolytic enzymes for degradation of xylan rich materials. This study proves that the choice for an enzyme preparation to efficiently degrade a natural xylan-rich substrate is dependent on the xylan characteristics and could not be estimated by using model substrates.

3.1 Introduction

Filamentous fungi are a good source of hemicellulolytic enzymes for biomass degradation. Their levels of enzyme excretion in the fermentation media are high, although strongly dependent on growth conditions (Gírio *et al.*, 2010; Van Gool *et al.*, 2011). By degradation of soluble complex substrates it was found that hemicellulolytic enzymes are produced by soil microbes. Between different types of soil microbes, a huge variation in production of specific hemicellulolytic enzymes exists, next to various levels of expression of those enzymes (Van Gool *et al.*, 2011). For the selection of powerful cellulases it has been stated before that instead of using dyed substrates, the use of real-life insoluble substrates is a better selection tool (Kabel *et al.*, 2006; Zhang *et al.*, 2006). Furthermore, Berrin and Juge (2008) reported that solubility of a substrate is one of the factors affecting xylanase functionality. Therefore, different insoluble substrates, varying from a relatively simple to a complex xylan-cellulose network like wheat straw and corn fiber, may be useful to reveal the potential ability of the xylan degrading enzyme preparations to degrade these substrates.

The composition of wheat straw and corn fibers is described in literature (Remond-Zilliox *et al.*, 1997; Appeldoorn *et al.*, 2010; Van Eylen *et al.*, 2011). Next to cellulases to degrade the cellulose, hydrolysis of xylans requires a variety of enzymes. Hydrolysis of the xylan backbone is done by endo-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37). The endo-xylanases will cleave the xylan backbone into oligosaccharides, which are degraded to xylose by β-xylosidases (EC 3.2.1.37). Arabinose residues can be removed by arabinofuranosidases (EC 3.2.1.55). Glucuronic acid residues and their 4-*O*-methyl ethers can be removed from the xylan backbone by α-glucuronidases (EC 3.2.1.131). Acetyl xylan esterases (EC 3.1.1.72) release acetyl residues from the backbone of cell wall polysaccharides (Van Gool *et al.*, 2011). Finally, feruloyl and *p*-coumaroyl esterases (EC 3.1.1.73) can remove the ferulic- and coumaric acid residues (Appeldoorn *et al.*, 2010).

The current research focused on the screening of 78 fungal culture supernatants for their ability to digest insoluble wheat straw and corn fiber fractions. Results will be discussed and compared with the results of previously tested wheat arabinoxylan (WAX) and *Eucalyptus* xylan hydrolysate (EXH) (Van Gool *et al.*, 2011).

3.2 Materials and methods

3.2.1 Fungi

Culture supernatants of 78 mesophilic lignocellulolytic fungi grown on wheat straw as carbon source, were obtained as described previously (Van Gool et al., 2011). The taxonomy the fungi is described in Supplementary Table A1.

3.2.2 Chemicals and substrates

All chemicals were, if not mentioned otherwise, of analytical grade. Wheat straw water unextractable solids (WS WUS) and corn fiber alcohol insoluble solids (CF AIS) were used as substrates.

The WS WUS were obtained by mixing of 60 ± 0,1 g of wheat straw (Pärnu Jaagupi, Estonia - harvested in winter 2009, kindly provided by Andres Käsper, Biogold Estonia) with an excess of Millipore water (70 °C) in a shaking incubator for one hour at 60 °C and 150 rpm. Next, the samples were filtered (110 mm Ø, Schleicher & Schuell, Dassel, Germany), the residue was collected, again mixed with water of 70 °C under the same conditions. After filtration the residues were collected and freeze-dried.

The CF AIS was produced as described by Kabel et al. (Kabel *et al.*, 2002). The first step included the removal of starch using an α-amylase and amyloglucosidase after which all low molecular weight material was removed by washing with 70% ethanol. The final alcohol insoluble solids were washed with acetone and dried in the air. The corn fiber originated from wet milling process (Archer Daniels Midland, Decatur, IL, USA).

3.2.3 Sugar composition of substrates

The sugar composition of the substrates was determined as described previously (Van Gool *et al.*, 2011); the neutral monosaccharides were analysed as their alditol acetates on a gas chromatograph after intensive hydrolysis with 72% (w/w) sulfuric acid. A part of the hydrolysate was used for the determination of the uronic acid content by the colorimetric m-HDP assay.

3.2.4 Enzyme screening

Wheat straw WUS (10 mg/mL) and corn fiber AIS (10 mg/mL) were suspended in 50 mM sodium acetate buffer, pH 5 to which 1% (v/v) shake flask fermentation supernatant was added. Incubation was accomplished for 24 h in a head-over-tail rotator at 35 °C. Enzymes were inactivated by boiling the samples for 10 min. Prior to analysis, the samples were centrifuged (5 min, 1500g, ambient temperature).
Based on data analysis a top five of culture supernatants per released component was selected. In some cases only two of the best supernatants were selected if the positions 3-5 were not discriminative enough.

3.2.5 Molecular weight distribution measurement by HPSEC

To determine the molecular weight distribution of polysaccharides and large oligosaccharides, high performance size exclusion chromatography (HPSEC) was performed on an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA, USA) equipped with three TSKgel SuperAW columns (6.0 mm ID x 15 cm per column; 4 µm) in series (SuperAW4000, SuperAW3000, SuperAW2500; Tosohaas Bioscience, Stuttgart, Germany), in combination with a guard column (Tosohaas Bioscience). Elution took place at 55 °C with 0.2 M sodium nitrate at 0.6 mL/min. The eluate was monitored with a Shodex RI-101 (Kawasaki, Japan) refractive index (RI) detector. The system was calibrated with a pullulan series (Sigma-Aldrich, St Louis, MO, USA).

3.2.6 Characterisation of oligomers by HPAEC

After treatment with the culture supernatants, the solubilized fragments were analysed by high performance anion exchange chromatography (HPAEC) as described by Van Gool et al. (2011). Quantification was based on the response factor of the standard solutions of xylose to xylotetraose (Xyl_1 Sigma Aldrich, Steinheim, Germany, Xyl_{2-4} Megazyme, Wicklow, Ireland), D-arabinose and D-glucuronic acid (Sigma Aldrich).

3.2.7 Characterisation of monomers by HPAEC

After incubation with the culture supernatants, the solubilized fragments upon enzymatic incubation were analysed by HPAEC using a dedicated gradient for monomer analysis. The flow rate was 0.3 mL/min and the mobile phase consisted of H2O, 0.1 M NaOH and 1 M NaOAc in 0.1 M NaOH. The elution profile was: 20 min isocratic at 16 mM NaOH; 20.1- 22.0 min isocratic at 0.1 M NaOH; 22.1-26 min, isocratic at 1M NaOAc in 0.1 M NaOH; 26.1-30 min isocratic at 0.1 M NaOH; followed by reconditioning of the column for 15 min with the starting gradient.

3.3 Results and discussion

3.3.1 Substrate composition

Table 3.1 displays that the WS WUS mainly contained xylose and glucose. The relative simple structure of WS WUS hemicellulose can be displayed by the degree of substitution using the molar ratio of arabinose:xylose and UA:xylose, which are only 1:8 and 1:16, respectively. Furthermore, the low abundance of other monomers in the substrate suggests that the hemicellulose is composed of a linear xylan backbone with minor amounts of substituents. This illustrates the presence of a predominantly ‘linear’ hemicellulose and a significant amount of cellulose. The level of acetylation of WS WUS was 1.8% (w/w), indicating a molar ratio of Ac:xylose of 1:4. The

composition of this substrate is in agreement with that provided in the literature (Miron and Ben-Ghedalia, 1982; Kabel *et al.*, 2007a).

The CF AIS was more complex as can be concluded from the various monosaccharides present. Xylose, arabinose and glucose were the main sugars and the molar ratios of arabinose:xylose and UA:xylose were 1:2 and 1:9 respectively. Furthermore, the substrate was highly acetylated, 1:2 for Ac:xylose (mol based). The ratios of substitution illustrate a highly decorated xylan backbone. These findings are in line with the complexity of corn fiber xylan as described by others (Appeldoorn *et al.*, 2010; Van Eylen *et al.*, 2011). Compared to WS WUS, a lower amount of cellulose was present in CF AIS, indicated by the lower amount of glucose. Together with the complex xylan structure, this lower cellulose content contributes to a different architecture of insoluble CF AIS compared to WS WUS. Both substrates had a total sugar content of ~70% (w/w), which could be explained by the presence of other components like lignin, proteins and/or salts.

Table 3.1: Constituent monosaccharide composition and total sugar content (w/w%) of the xylan rich substrates wheat straw water unextractable solids (WS WUS) and corn fiber alcohol insoluble solids (CF AIS).

Sample name	Sugar composition (w/w%)[a]								Total sugar content (w/w%)
	Rha	Ara	Xyl	Man	Gal	Glc	UA	Ac	
WS WUS	2	3	24	0	1	36	2	2	68
CF AIS	1	16	27	1	5	20	4	4	74

[a] Neutral sugars, uronic acids or acetic acid expressed as weight percentage: Rha: rhamnose, Ara: arabinose, Xyl: xylose, Man: mannose, Gal: galactose, Glc: glucose, UA: uronic acid, Ac: acetyl esters

3.3.2 Enzyme screening on insoluble WS WUS

Fig. 3.1 shows examples of HPAEC elution patterns of untreated WS WUS (blank) and WS WUS after incubation with three culture supernatants (#13, 36 and 57).

HPSEC results showed that no polymeric soluble material was present in any of the samples (data not shown). The blank sample confirms that the substrate only contains insoluble material. Culture supernatant #36, together with 21 other culture

supernatants, hardly released any monomers or oligomers, indicating that those culture supernatants could not hydrolyse the insoluble substrate. Fifty-six out of 78 hydrolysed >5% of the substrate to monomers or oligomers. The wheat straw WUS digest of culture supernatant #13 showed release of glucuronic acid, a high release of arabinose and other monomers. Culture supernatant #57 released also relative large amounts of xylobiose and xylotriose, but was lacking a glucuronic acid releasing enzyme.

To define the peak of co-eluted xylose, glucose and/or galactose, a more specific gradient was applied to HPAEC. Using this gradient, the different monomers could be separated and quantified as displayed in Table 3.2 for selected culture supernatants. A complete overview of the quantitative data of all culture supernatants can be found in the Supplementary Table A1.

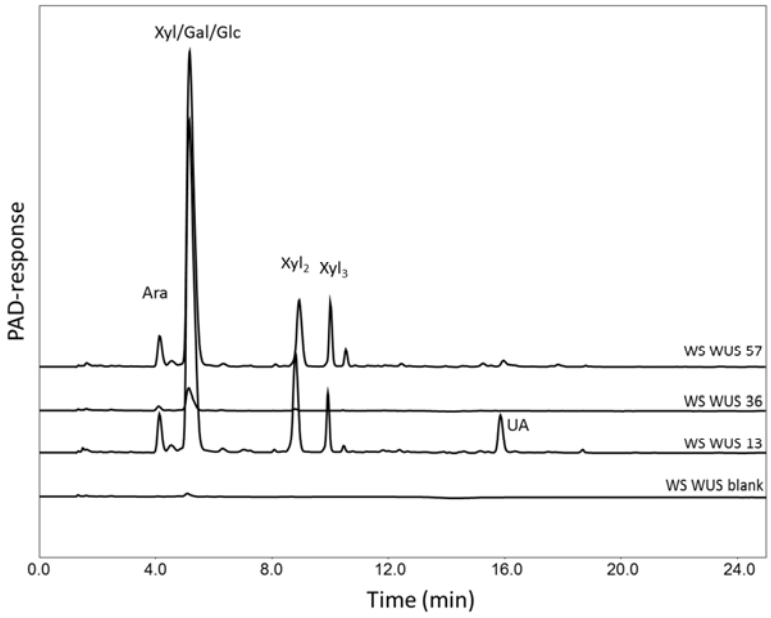

Fig. 3.1: Examples of high performance anion exchange chromatography (HPAEC) patterns derived from incubation of wheat straw water unextractable solids (WS WUS) with the culture supernatants for 24h at 35°C pH 5. Wheat straw blank and the culture supernatants #13, 36 and 57 are shown. Ara: arabinose; Xyl/Gal/Glc: xylose, galactose, glucose; $Xyl_{2,3}$: xylobiose, xylotriose; UA: glucuronic acid.

Tables 3.2 and A1 show that 13 out of 78 culture supernatants could solubilize more than 10% of the total insoluble substrate WS WUS. Only 3 samples were able to solubilize 14% of the WS WUS. Typical values for total cellulose hydrolysis in WS WUS by various cellulase preparations are around 10-20% (Koullas *et al.*, 1992; Lamsal *et al.*, 2011). Pretreatment enhanced total hydrolysis by opening up the cell wall structure (Pedersen and Meyer, 2009) and values up to 63% solubilisation after pretreatment of wheat straw have been reported (Lamsal *et al.*, 2011).

The enzymes in the culture supernatants releasing high amounts of monosaccharides are able to hydrolyse part of the complex hemicellulose and cellulose network. The maximum amount of glucose and xylose released from the WS WUS was reached by sample #38 (21 and 22% respectively). In general, both cellulases and xylanases are necessary for cell wall deconstruction (Wang, 2010) and these enzymes enhance the release of monosaccharides synergistically (Ghose and Bisaria, 1979). The release of other substituents e.g. arabinose, or galactose by accessory enzymes is of importance for complete hydrolysis of the xylan backbone. Nevertheless, these substituents are only present in small quantities and, therefore, do not play a pivotal role within the degradation of WS WUS.

It is remarkable that only two culture supernatants (samples #7 and 38) of the top five best-performing strains in hydrolysis of WAX (Van Gool *et al.*, 2011) and WS WUS are the same. As expected from the sugar composition, xylanases and cellulases are necessary for main degradation of WS WUS. WAX digestion already indicated that active xylanases were present in many of the culture supernatants (Van Gool *et al.*, 2011). However, these were not effective in the degradation of WS WUS. This is visualized by culture supernatants #36 and #13. Culture supernatant #36 was selected previously for enzyme activities which were not tested by using WS WUS. Culture supernatant #13 was already active towards WAX, but not selected as a top five best-performing sample (Van Gool *et al.*, 2011). However, this culture supernatant seems to have better performance in WS WUS degradation which indicates that the substrate used in a screening procedure is of importance to predict the efficiency of enzymes towards that particular substrate.

The differences between the culture supernatants hydrolysing the WS WUS could be caused by both substrate and enzyme characteristics. One of the reasons could be enzyme adsorption onto lignin and/or cellulose, causing the hydrolysis rate towards xylan to decrease (Lee *et al.*, 1995; Yang and Wyman, 2006). Other reasons could be product inhibition, substrate crystallinity or accessibility (Bansal *et al.*, 2009; Van Eylen *et al.*, 2011). Furthermore, it has been mentioned that xylanase activity may depend on the presence and type of a substrate binding domain attached to the xylanase (Sun *et al.*, 1998; Ustinov *et al.*, 2008; Cuyvers *et al.*, 2011). One should also note that, although the same conditions were used (pH 5.0, 35°C) to screen for efficient degradation of soluble and insoluble substrates, these conditions could be optimized for each individual enzyme preparation to obtain even more enhanced degradation (Polizeli *et al.*, 2005).

Table 3.2: Selected culture supernatants (bold font) with high enzyme activity towards wheat straw water unextractable solids (WS WUS) and corn fiber alcohol insoluble solids (CF AIS), based on high performance anion exchange chromatography (HPAEC) results.

	Hydrolysis products formed of WS WUS (w/w %)							**Hydrolysis products formed of CF AIS (w/w %)**						
Sample number	**Arabinose [a]**	**Galactose [a]**	**Glucose [a]**	**Xylose [a]**	**Glucuronic acid (+/- 4OMethyl) [a]**	**Xylooligomers (DP2-4) [b]**	**Total WS WUS [d]**	**Arabinose [a]**	**Galactose [a]**	**Glucose [a]**	**Xylose [a]**	**Glucuronic acid (+/- 4OMethyl) [a]**	**Other oligomers [c]**	**Total CF AIS [d]**
B1	35	12	15	**20**	3	1	11	14	5	16	6	1	10	18
B7	**38**	**47**	**19**	**23**	1	3	**14**	14	**12**	14	8	1	8	15
B8	26	**37**	14	**16**	1	1	10	11	**12**	11	5	1	7	13
B11	21	15	10	10	3	8	8	26	**11**	20	8	**6**	15	26
B12	32	17	17	13	3	**11**	13	24	10	18	6	3	16	26
B13	**41**	**22**	17	15	**5**	**16**	**14**	24	10	17	6	**9**	19	**29**
B14	24	15	14	11	2	8	10	**29**	10	19	7	**7**	**20**	**31**
B19	**38**	10	**19**	**21**	**5**	1	13	**29**	9	20	**11**	**6**	13	26
B20	34	5	**19**	11	2	8	12	15	1	14	3	1	15	21
B31	23	18	10	11	2	1	7	25	8	18	**10**	**7**	14	25
B38	**37**	**31**	**21**	**22**	1	1	**14**	22	**13**	21	**10**	1	8	19
B44	22	4	11	4	0	4	7	23	3	12	1	1	**21**	**28**
B45	33	21	**20**	14	1	7	13	26	10	**24**	8	0	14	26
B49	31	**22**	**19**	15	2	2	12	**28**	**11**	**22**	**11**	1	14	26
B57	**38**	19	18	12	3	**14**	13	20	5	16	3	0	13	20
B63	29	14	17	10	3	**11**	12	19	5	17	3	5	16	23
B66	30	17	18	7	1	6	11	**28**	8	**23**	6	2	15	26
B70	19	6	12	6	1	7	8	17	2	8	1	1	**20**	25
B73	32	20	16	8	2	3	10	**32**	8	**23**	5	2	**23**	**34**
B74	29	13	11	9	1	**12**	10	**35**	6	**23**	8	0	**21**	**34**
B75	33	16	18	11	3	**11**	13	25	5	21	4	1	17	27

[a] Amount as percentage of total amount of that component(s) present in initial substrate (w/w%)
[b] Amount of xylooligomers as percentage of xylose present in initial substrate (w/w%)
[c] Unidentified oligomers as percentage of total amount of sugars in initial substrate (w/w%)
[d] Monomers and oligomer products as percentage of total amount of these components present in initial substrate (w/w%)

3.3.3 Enzyme screening on insoluble CF AIS

The results obtained using WS WUS gave insight in the crude culture supernatants able to degrade a relatively simple xylan rich substrate. The hydrolysis of CF AIS provided insight towards the ability of the enzymes in the culture supernatants to degrade a complex insoluble substrate. HPSEC analysis of the CF AIS samples did not display any high molecular weight material being solubilized by the enzymes present in any of the culture supernatants (data not shown). The relative amount of each constituent monosaccharide in the substrate was calculated and is displayed in the Supplementary Table A1. Table 3.2 displays the top enzyme producers hydrolysing CF AIS as expressed per component released. The blank sample contained a small amount of glucose (Table A1), which originated from starch removal (Van Eylen *et al.*, 2011). Fifteen culture supernatants were selected as containing the best enzymes for hydrolysing CF AIS. Seven of these 15 supernatants were not selected for their performance on WS WUS and, therefore, seem to be efficient specifically for CF AIS hydrolysis.

Table 3.2 also shows that three out of 78 culture supernatants were able to solubilize 30-34% of the total CF AIS. Compared to WS WUS, a significant larger part of the cell wall polysaccharides in CF AIS was solubilized. Since CF AIS xylan is reported to be extremely complex (§3.3.1; Appeldoorn *et al.*, 2010; Van Eylen *et al.*, 2011), this is likely to be caused by the architecture of the substrate. It has been reported that more substituted xylans, like corn fiber xylan, absorb much less cellulose than a more linear xylan, like wheat straw xylan. This may lead to a tighter and denser structure of WS WUS, thereby making it less accessible for enzymes (Kabel *et al.*, 2007b).

The main building blocks of CF AIS are xylose and glucose substituted with arabinose. It can be seen that the β-1→4 linked xylan backbone of this substrate must have been less available for the enzymes since only up to 10% of the xylose available in the initial substrate was hydrolysed to monomer, compared to up to 20% for WS WUS. Sample #7 did not release much xylose from CF AIS, although it demonstrated to contain endo-xylanases and β-xylosidases active on insoluble WS WUS by the release of

xylose. This confirms that the β-1→4 linked xylan backbone in the substrate was less available for the enzymes present in this culture supernatant.

Table 3.2 also shows that six out of 78 culture supernatants were able to release ~30% of arabinose from the insoluble CF AIS. The release of arabinose substituents is necessary to allow endo-xylanases to hydrolyse the backbone (Kormelink *et al.*, 1991). However, in all cases no synergy between arabinofuranosidases and xylanases was observed, since an increased level of released arabinose was not always correlated to an increased level of xylose (-oligomers) released, e.g. samples #14, 73 and 74. Since the digests had a high content of 'other oligomers' indicating the presence of an endo-xylanase, they may have lacked an efficient β-xylosidase hydrolysing the oligomers to monomers, or the β-xylosidase was hindered by substitution of the oligomers (Kormelink *et al.*, 1993). The hydrolysis of WS WUS by these culture supernatants did also result in higher amounts of released xylooligomers, but not in high xylose release which makes the assumption of a lack in efficient β-xylosidase activity likely.

The glucuronic acid seems to have been difficult to release. The maximum amount of glucuronic acid released was 9%. The lack of enzymes able to release this glucuronic acid group from the backbone of xylooligomers in corn fiber was described by others as well (Appeldoorn *et al.*, 2010).

The best-performing culture supernatants on CF AIS were #14, 73 and 74, which were not selected for their performance on WS WUS. Again this confirms the importance of selection of the substrate used in a screening procedure.

3.3.4 Importance of the choice of substrate in screening for enzyme activity

In total 21 samples were selected as top hydrolysers of either WS WUS or CF AIS (Table 2). Out of these 21 samples, fourteen were selected for their performance on WS WUS, as well as 15 for their performance on CF AIS. Eight of the samples were selected for efficient solubilisation of both substrates. Five samples originated from the *Trichoderma* genus, of which especially the *Trichoderma reesei* is known for efficient production of cellulases and hemicellulases (Banerjee *et al.*, 2010; Gusakov,

2011). Furthermore, four *Penicillium* strains were selected as good producers of enzymes degrading insoluble xylan-rich material. The efficient production of cellulases and hemicellulases by various *Penicillium* species was reported by others (Jørgensen *et al.*, 2005; Gusakov, 2011). Sample #70 originated from *Phanerochaete chrysosporium*. This fungus is one of the best-studied fungi for bioconversion of plant biomass and is known to produce hemicellulolytic enzymes (Hori *et al.*, 2011). Eleven selected strains remained unidentified.

Based on the results for soluble substrates (Van Gool *et al.*, 2011) and insoluble substrates in this study, the five best fungi can be selected for each substrate. The selection was based on the total amount of main building blocks released. Table 3.3 shows that for each substrate, different culture supernatants were selected as having the top five enzyme contents.

Table 3.3: Top enzyme producers for the different substrates, based on the release of the main building blocks in the substrates: wheat arabinoxylan (WAX); Eucalyptus xylan hydrolysate (EXH); wheat straw water unextractable solids (WS WUS); corn fiber alcohol insoluble solids (CF AIS).

Rank	Top five producers on different substrates			
	WAX[a] *	EXH[b] *	WS WUS[c]	CF AIS[d]
1	38 (*Penicillium sp.*)	41 (*Aspergillus sp.*)	13 (non-identified)	73 (non-identified)
2	74 (*Trichoderma sp.*)	40 (*Aspergillus sp.*)	7 (*Penicillium sp.*)	74 (*Trichoderma sp.*)
3	49 (*Penicillium sp.*)	42 (*Aspergillus sp.*)	57 (non-identified)	14 (non-identified)
4	45 (*Trichoderma sp.*)	27 (*Bionectria sp.*)	38 (*Penicillium sp.*)	13 (non-identified)
5	7 (*Penicillium sp.*)	7 (*Penicillium sp.*)	12 (non-identified)	44 (non-identified)

[a] Main building blocks: arabinose, xylose, xylooligomers DP2-4
[b] Main building blocks: xylose, xylooligomers DP2-4, acetyl
[c] Main building blocks: glucose, xylose, xylooligomers DP2-4
[d] Main building blocks: arabinose, xylose/glucose, various oligomers
* Van Gool *et al.* (2011)

Only supernatant #7 was selected for three out of four substrates, indicating the presence of highly active and/or efficient enzymes in this sample. The supernatant was derived from a *Penicillium* species previously described as a good producer of cellulases and hemicellulases (Jørgensen *et al.*, 2005). Also *Trichoderma* and *Aspergillus* species were selected. Sample #74 and #13 were selected twice for

different substrates. Sample #74 was derived from a *Trichoderma* species; the fungus producing sample #13 remains unidentified. The selection of different culture supernatants for each substrate proves the need of using different substrates for identification of efficient enzymes within crude culture supernatants. This study demonstrates even more that the choice of a substrate to be degraded is of importance to screen for specific enzyme activity.

The novelty of the enzymes present in the culture supernatants will be established by identification of the strains, purification and characterisation of the enzymes as well as using optimized conditions for each enzyme.

3.4 Conclusions

The screening for specific enzyme activity within crude lignocellulolytic enzyme mixtures is only effective when using the correct substrate. Solubility, polymer chemical fine structure and the cell wall architecture of the substrates used in screenings will influence enzyme performance and thus the outcome of the screening. Especially *Penicillium, Aspergillus* and *Trichoderma* species produced high hemicellulolytic activity. The use of targeted substrates allows recognition of several efficient enzymes within crude culture supernatants.

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Supplementary data

Table A1: Sample information and results of all fermentation supernatants after hydrolysis of insoluble wheat straw water unextractable solids (WS WUS) and corn fiber alcohol insoluble solids (CF AIS), based on high performance anion exchange chromatography results

Fungi			Hydrolysis products formed of WS WUS (w/w %)							Hydrolysis products formed of CF AIS (w/w %)						
Sample number	Strain	Taxonomical positsion	Arabinose [a]	Galactose [a]	Glucose [a]	Xylose [a]	Glucuronic acid (+/- 4OMethyl) [a]	Xylooligomers (DP2-4) [b]	Total WS WUS [d]	Arabinose [a]	Galactose [a]	Glucose [a]	Xylose [a]	Glucuronic acid (+/- 4OMethyl) [a]	Other oligomers [c]	Total CF AIS [d]
Blank sample			0	0	0	0	0	0	0	0	0	2	0	0	0	0
1	TUB F-1702	*Trichoderma sp.*	35	12	15	20	3	1	11	14	5	16	6	1	10	18
2	TUB F-1702	*Trichoderma sp.*	27	7	14	15	1	1	9	9	2	11	3	0	8	13
3	TUB F-2292	Non-identified fungus	6	4	3	1	0	1	2	5	2	4	1	0	12	14
4	TUB F-2292	Non-identified fungus	6	2	3	1	0	1	2	3	1	3	0	0	7	8
5	TUB F-2293	*Coniochaeta ligniaria*	6	3	4	3	1	3	3	4	2	5	1	3	12	14
6	TUB F-2293	*Coniochaeta ligniaria*	8	7	9	2	1	3	5	5	2	8	1	3	10	13
7	TUB F-2346	*Penicillium sp.*	38	47	19	23	1	3	14	14	12	14	8	1	8	15
8	TUB F-2346	*Penicillium sp.*	26	37	14	16	1	1	10	11	12	11	5	1	7	13
9	QM6a	*Trichoderma reesei*	22	10	11	8	2	3	7	8	2	11	2	1	8	13
10	QM6a	*Trichoderma reesei*	20	5	15	8	1	6	9	8	2	12	1	1	8	12
11	TUB F-2342	Non-identified fungus	21	15	10	10	3	8	8	26	11	20	8	6	15	26
12	TUB F-2342	Non-identified fungus	32	17	17	13	3	11	13	24	10	18	6	3	16	26
13	TUB F-2353	Non-identified fungus	41	22	17	15	5	16	14	24	10	17	6	9	19	29
14	TUB F-2353	Non-identified fungus	24	15	14	11	2	8	10	29	10	19	7	7	20	31
15	TUB F-2358	*Trichoderma harzianum*	21	7	8	7	1	7	7	15	4	19	4	0	13	20
16	TUB F-2358	*Trichoderma harzianum*	21	4	13	7	0	4	8	10	1	15	2	0	8	13
17	TUB F-2360	*Trichoderma sp.*	16	7	6	7	1	1	4	6	1	9	3	0	8	11
18	TUB F-2360	*Trichoderma sp.*	17	4	7	7	1	2	5	8	2	13	2	0	7	11
19	RUT C30	*Trichoderma reesei*	38	10	19	21	5	1	13	29	9	20	11	6	13	26
20	RUT C30	*Trichoderma reesei*	34	5	19	11	2	8	12	15	1	14	3	1	15	21
21	TUB F-1598	*Trichoderma harzianum*	26	12	17	15	1	1	11	12	4	17	4	0	8	14
22	TUB F-2294	*Trichoderma sp.*	30	10	18	13	1	2	11	9	2	15	3	0	7	12
23	TUB F-2295	*Trichoderma sp.*	20	5	10	5	1	2	6	7	2	12	1	0	5	9
24	TUB F-2335	*Trichoderma sp.*	20	6	7	9	1	2	6	20	3	13	6	2	4	12
25	TUB F-2343	*Trichoderma sp.*	18	5	7	7	1	1	5	7	1	9	2	1	10	13
26	TUB F-2344	*Trichoderma sp.*	27	6	18	10	0	5	11	10	2	17	2	0	8	14
27	TUB F-2348	*Bionectria ochroleuca*	23	10	9	6	2	5	7	18	8	13	3	4	19	26
28	TUB F-2349	Non-identified fungus	6	3	3	1	0	0	2	7	2	4	1	0	5	7
29	TUB F-2350	*Trichoderma sp.*	11	8	3	4	0	0	2	4	1	4	1	0	3	5
30	TUB F-2352	Non-identified fungus	14	10	4	5	1	0	3	9	0	12	3	0	4	8
31	TUB F-2356	Non-identified fungus	23	18	10	11	2	1	7	25	8	18	10	7	14	25

Fungi			Hydrolysis products formed of WS WUS (w/w %)							Hydrolysis products formed of CF AIS (w/w %)						
Sample number	Strain	Taxonomical posistion	Arabinose [a]	Galactose [a]	Glucose [a]	Xylose [a]	Glucuronic acid (+/- 4OMethyl) [a]	Xylooligomers (DP2-4) [b]	Total WS WUS [d]	Arabinose [a]	Galactose [a]	Glucose [a]	Xylose [a]	Glucuronic acid (+/- 4OMethyl) [a]	Other oligomers [c]	Total CF AIS [d]
33	QM6a	*Trichoderma reesei*	27	9	11	7	0	4	7	12	2	16	2	0	10	15
34	TUB F-2385	*Corynascus verrucosus*	17	14	2	5	1	3	3	10	5	4	1	1	8	11
35	TUB F-2387	Non-identified fungus	17	6	5	3	1	3	4	12	3	11	1	1	7	12
36	TUB F-2388	Non-identified fungus	6	3	1	1	0	0	1	8	2	5	1	0	5	7
37	TUB F-2389	Non-identified fungus	7	4	3	2	0	0	2	6	2	4	1	0	4	6
38	TUB F-2390	*Penicillium sp.*	37	31	21	22	1	1	14	22	13	21	10	1	8	19
39	NRRL1808	*Aspergillus oryzae*	18	17	10	10	2	1	7	11	5	14	6	3	5	11
40	NRRL3485	*Aspergillus oryzae*	19	9	7	13	2	1	6	10	4	11	6	5	4	10
41	ATCC10864	*Aspergillus niger*	18	8	9	9	1	0	6	9	5	14	5	4	5	11
42	ATCC14916	*Aspergillus foetidus*	16	13	8	7	1	0	5	9	4	15	5	2	5	11
43	TUB F-2386	Non-identified fungus	5	6	4	2	0	0	2	1	1	3	0	0	3	4
44	TUB F-2382	Non-identified fungus	22	4	11	4	0	4	7	23	3	12	1	1	21	28
45	TUB F-1647	*Trichoderma sp.*	33	21	20	14	1	7	13	26	10	24	8	0	14	26
46	TUB F-2369	*Trichoderma sp.*	27	8	15	9	1	6	10	13	2	17	3	0	9	16
47	TUB F-2370	Non-identified fungus	30	10	8	12	1	2	7	9	5	11	3	1	7	11
48	TUB F-2371	Non-identified fungus	9	3	3	1	0	1	2	13	5	5	1	0	8	12
49	TUB F-2372	*Penicillium ochrochloron*	31	22	19	15	2	2	12	28	11	22	11	1	14	26
50	TUB F-2374	Non-identified fungus	21	11	11	5	1	2	6	15	6	15	4	0	12	19
51	QM 6a	*Trichoderma reesei*	26	11	13	8	1	4	8	13	2	18	3	1	11	17
52	OKI 16/5	*Aspergillus terreus*	32	4	17	10	0	6	11	26	2	19	4	0	15	24
53	TUB F-2394	*Humicola grisea/Humicola nigrescens*	8	7	9	1	1	1	4	15	2	9	1	0	17	22
54	TUB F-2361	*Bionectria ochroleuca*	16	5	7	3	1	3	5	13	3	10	1	0	16	21
55	TUB F-2375	*Trichoderma sp.*	14	4	8	6	1	7	6	10	2	13	2	0	15	20
56	TUB F-2376	*Trichoderma sp.*	27	11	15	9	1	6	10	15	3	20	3	0	12	20
57	TUB F-2377	Non-identified fungus	38	19	18	12	3	14	13	20	5	16	3	0	13	20
58	QM 7721	*Paecilomyces terricola, Synonym: Acremonium implicatum ; Fusidium terricola*	19	9	9	4	1	4	6	15	3	8	1	1	15	20
59	ATCC 11282	*Cladosporium herbarum*	8	5	2	3	1	1	2	11	4	6	2	0	10	13
60	RUT C30	*Trichoderma reesei*	22	3	14	6	1	5	9	11	1	10	1	0	15	20
61	BSUM	*Epicoccum nigrum*	20	9	6	3	1	1	4	15	4	8	3	0	9	14

Sample number	Fungi: Strain	Fungi: Taxonomical posistion	Hydrolysis products formed of WS WUS (w/w %): Arabinose [a]	Galactose [a]	Glucose [a]	Xylose [a]	Glucuronic acid (+/- 4OMethyl) [a]	Xylooligomers (DP2-4) [b]	Total WS WUS [d]	Hydrolysis products formed of CF AIS (w/w %): Arabinose [a]	Galactose [a]	Glucose [a]	Xylose [a]	Glucuronic acid (+/- 4OMethyl) [a]	Other oligomers [c]	Total CF AIS [d]
62	OKI 270	*Chaetomium globosum*	8	5	4	0	1	0	2	9	1	3	0	0	4	6
63	TUB F-2291	Non-identified fungus	29	14	17	10	3	11	12	19	5	17	3	5	16	23
64	TUB F-2303	Non-identified fungus	27	7	12	6	1	5	8	17	5	11	1	0	16	21
65	TUB F-2305	Non-identified fungus	22	6	13	6	1	4	8	12	1	11	1	0	13	18
66	TUB F-2378	Non-identified fungus	30	17	18	7	1	6	11	28	8	23	6	2	15	26
67	TUB F-2379	*Trichoderma sp.*	18	6	10	4	1	4	6	11	1	16	1	0	8	14
68	IFO 9387	*Paecilomyces bacillisporus, Synonym: Acremonium bacillisporum*	14	7	8	2	1	3	5	14	4	9	1	0	13	18
69	RUT C30	*Trichoderma reesei*	32	9	16	9	1	9	11	16	3	20	2	1	13	21
70	NCAIM F-00740	*Phanerochaete chrysosporium*	19	6	12	6	1	7	8	17	2	8	1	1	20	25
71	VKM F-1767	*Phanerochaete chrysosporium*	14	7	15	6	1	6	9	4	3	8	1	1	11	14
72	NRRL 2003	*Myrothecium verrucaria*	13	6	9	4	1	3	5	15	3	10	2	2	14	20
73	TUB F-2341	Non-identified fungus	32	20	16	8	2	3	10	32	8	23	5	2	23	34
74	TUB F-2380	*Hypocrea lixii (Trichoderma harzianum)*	29	13	11	9	1	12	10	35	6	23	8	0	21	34
75	TUB F-2381	Non-identified fungus	33	16	18	11	3	11	13	25	5	21	4	1	17	27
76	TUB F-2383	Non-identified fungus	14	3	4	2	0	4	3	14	1	6	1	0	10	13
77	TUB F-2384	Non-identified fungus	4	2	2	2	0	0	1	6	2	6	2	1	3	6
78	IFO 4855	*Paecilomyces varioti*	15	6	3	4	1	0	3	13	3	7	3	1	5	9

[a] Amount as percentage of total amount of that component(s) present in initial substrate (w/w%)

[b] Amount of xylooligomers as percentage of xylose present in initial substrate (w/w%)

[c] Unidentified oligomers as percentage of total amount of sugars in initial substrate

[d] Monomers and linear xylooligomer products as percentage of total amount of these components present in initial substrate (w/w%)

Origin of strains: ATCC: American Type Culture Collection, Manassas, Virginia, USA; BSUM: Botany School, University of Melbourne, Parkville, Australia; IFO: Institute for Fermentation, Osaka, Japan; NRRL: Northern Regional Research Center, USDA, Peoria, Illinois, USA; OKI: National Institute for Public Health, Budapest, Hungary; QM: Quartermaster Research and Development Center, U.S. Army, Natick, Massachusetts; RUT: Rutgers University, New Brunswick, New Jersey, USA; TUB F-...: Technical University of Budapest, culture collection of fungi; VKM: Russian Collection of Microorganisms, Moscow, Russia

Chapter 4

Two GH10 endo-xylanases from Myceliophthora thermophila C1 with and without cellulose binding module act differently towards soluble and insoluble substrates

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Abstract

Xylanases are mostly classified as belonging to glycoside hydrolase (GH) family 10 and 11, which differ in catalytic properties and structures. However, within one family, differences may also be present. The influence of solubility and molecular structure of substrates towards the efficiency of two GH10 xylanases from *Myceliophthora thermophila* C1 was investigated. The xylanases differed in degradation of high and low substituted substrate and the substitution pattern was an important factor influencing their efficiency. Alkali-labile interactions, as well as the presence of cellulose within the complex cell wall structure hindered efficient hydrolysis for both xylanases. The presence of a carbohydrate binding module did not enhance the degradation of the substrates. The differences in degradation could be related to the protein structure of the two xylanases. The study shows that the classification of enzymes does not predict their performance towards various substrates.

4.1 Introduction

Enzyme classification is based on amino acid sequence similarities and structural features. Currently, 130 different families of glycoside hydrolases have been identified (http://www.cazy.org). Most xylanases belong to glycoside hydrolase (GH) families 10 and 11, although some other xylanases are classified in families 5, 8, and 30 (St John *et al.*, 2010; Paës *et al.*, 2012; http://www.cazy.org). The xylanases in the various families differ both in catalytic properties and structure.

GH family 10 members have an average molecular mass of approximately 40 kDa (Beaugrand *et al.*, 2004) and display an 8-fold (α/β)-barrel resulting in a 'salad bowl' shape (Biely *et al.*, 1997; Collins *et al.*, 2005). According to Collins et al. (2005), GH10 xylanases are highly active towards short xylooligomers, thereby indicating a small substrate binding site. GH10 xylanases exhibit a lower substrate specificity than GH11 xylanases (Collins *et al.*, 2005). They tolerate an arabinose-decorated xylose residue, resulting in shorter xylooligomers than those produced by GH11 xylanases. Both enzymes exhibit endo-activity and thus show extended active site clefts which are commonly composed of three, five or more subsites (Beaugrand *et al.*, 2004). Although GH10 xylanases display the same folding, the topology of their substrate binding clefts is not conserved (Charnock *et al.*, 1998).

In the genome of the Ascomycetous fungus *Myceliophthora thermophila* C1 (previously known as *Chrysosporium lucknowense* C1) about 250 genes encode carbohydrate-active enzymes of which 180 are potential glycoside hydrolases. Out of these 180 genes, thirteen encode xylanases (Hinz *et al.*, 2009; Berka *et al.*, 2011). Six of the xylanases have been purified from the culture supernatant of C1 and preliminary laboratory tests demonstrated high efficiencies of some of the C1 xylanases (Ustinov *et al.*, 2008). Two of the xylanases from GH family 10, Xyl1 and Xyl3, which were overexpressed in a C1 strain dedicated to hemicellulase production, possess a family 1 carbohydrate-binding module (CBM) at the N- or C-terminus, respectively (Ustinov *et*

al., 2008). This CBM has a cellulose-binding function and is almost exclusively found in enzymes of fungal origin (http://www.cazy.org; Guillén *et al.*, 2009).

In many xylanase characterisation studies, for example those of Pollet *et al*. (2010) and Ustinov *et al*. (2008), the preference of enzymes to degrade substrates with different chain lengths, degrees of substitution and substitution patterns were determined; however, solubility as well as complexity of the cell wall structure is often not taken into account in these mechanistic studies. This omission hinders a concise overview of the enzyme characteristics, resulting in the suggestion that GH10 family xylanases perform similarly (Biely *et al.*, 1997; Beaugrand *et al.*, 2004; Ustinov *et al.*, 2008).

This study focused on two endo-xylanases from GH family 10; Xyl1 and Xyl3 from *Myceliophthora thermophila* C1, both with and without CBM, towards soluble and insoluble xylan-rich material. Their functionality was correlated to structural features of the protein.

4.2 Materials and methods

4.2.1 Enzyme production

Two different xylanases with and without carbohydrate binding module; Xyl1 (representing the enzyme with CBM), Xyl1dCBM (representing the enzyme without CBM), Xyl3 and Xyl3dCBM from *Myceliophthora thermophila* C1 were overproduced in a specially designed C1-expression host (LC strain) (Visser *et al.*, 2011). The strains, containing extra copies of the *xyl*1*, xyl*1*dcbm, xyl*3 and *xyl*3*dcbm* gene were grown aerobically in 2-L fermentors in mineral medium, containing glucose as carbon source, ammonium sulphate as nitrogen source and trace elements for the essential salts (Verdoes *et al.*, 2010). Growth was performed as described elsewhere (Visser *et al.*, 2011).

4.2.2 Enzyme purification

Xyl1 was purified using a BioGelP4 column (Biorad, Hercules, CA) with 0.01 M Bis-Tris/HCl buffer pH 6.9, followed by ion exchange chromatography using a Source15Q column (GE Healthcare, Uppsala, Sweden) with 0.01 M Bis-Tris/HCl buffer pH 6.9. The last step was hydrophobic interaction chromatography using a Source15ISO column (GE Healthcare) with 1.7 M $(NH_4)_2SO_4$ pH 5.0 as buffer. Crude Xyl1dCBM was desalted using a PD10 desalting column (GE Healthcare) with 10 mM sodium acetate buffer pH 5.0. The desalted enzyme was purified using a Source30S column (GE Healthcare) with 10mM sodium acetate buffer pH 5.0 in combination with a 1 M NaCl solution in the same buffer using a gradient of five column volumes from 0-0.5 M NaCl. Subsequently, fractions containing the xylanase were pooled and further purified on a Source30Q column (GE Healthcare) using the same buffers as described for the Source30S column with a gradient of two column volumes from 0-1 M NaCl. Xyl3 was purified by ion exchange chromatography using a Source15Q column with 0.01 M Bis-Tris/HCl buffer pH 6.8. Subsequently, fractions containing xylanase were pooled and further purified with hydrophobic interaction chromatography using a Source15ISO column with 1.7 M $(NH_4)_2SO_4$ pH 5.0 as buffer. Xyl3dCBM was purified according to the same protocol as used for Xyl3. After purification, all enzyme solutions were concentrated by ultrafiltration and freeze dried. All enzymes were dissolved in 10mM sodium acetate buffer pH 5.0. Table 4.1 provides an overview of the characteristics per enzyme.

Table 4.1: Overview of the characteristics of GH10 xylanases with and without carbohydrate binding module (dCBM) from Myceliophthora thermophila C1 in this study.

Sample	Code used in Ustinov *et al*, (2008)	Activity (U/mg protein)*	Molecular mass (kDa)**	pI	pH optimum	Temperature optimum (°C)
Xyl1dCBM	Xyn10A (lm)	33.7	31[a]	8.9 [a]	6[a]	65-70 [a]
Xyl1	Xyn10A (hm)	88.6	42[a]	7.8 [a]	5.5-6[a]	65-70 [a]
Xyl3dCBM	Xyn10B (lm)	68.3	46[a]	4.3 [a]	6-6.5[a]	80-85 [a]
Xyl3	Xyn10B (hm)	61.0	57[a]	4.4 [a]	6-6.5[a]	80-85 [a]

* Using wheat arabinoxylan as substrate, 30 min incubation, reducing sugar assay
** Measured by SDS PAGE
[a] As determined by Ustinov *et al*. (2008), and verified in this study

4.2.3 Chemicals & substrates

All chemicals were, if not mentioned otherwise, of analytical grade. The substrates used were: wheat arabinoxylan (WAX), medium viscosity (Megazyme, Wicklow, Ireland), beech wood xylan (BeWX) (Sigma-Aldrich, St. Louis, MO, USA), birch wood xylan (BiWX) (Sigma-Aldrich) and oat spelt xylan (OSX) (Sigma-Aldrich). The commercial substrates contain different type and amount of substituents, resulting in different solubility in watery environment (Table 4.2). Since BiWX and BeWX were obtained through alkali extraction, acetate groups are not present as substituents. Wheat Straw Water Unextractable Solids (WS WUS) (wheat straw, Groningen, The Netherlands, May 2003), WS KOHss and WS KOHres were prepared as described in Kabel et al. (2006). WS KOHmix contained a mixture of the WS KOHss and WS KOHres in the same weight yields as obtained during extraction, taking into account the loss of low molecular weight material during dialysis after the extraction. The constituent monosaccharide composition of the substrates is displayed in Table 4.3.

Table 4.2: Type of substituents present in wheat arabinoxylan (WAX), beech wood xylan (BeWX), birch wood xylan (BiWX) and oat spelt xylan (OSX).

Substrate	Backbone	Substituents	Solubility in water	References
WAX	β-(1→4)-D-Xyl	α-(1→2) Ara α-(1→3) Ara	++	(Van Gool *et al.*, 2011)
BeWX (random distribution)/ BiWX (blockwise distribution)	β-(1→4)-D-Xyl	α-(1→2) 4-*O*-Me-α-D-GlcA *O*-2 Ac *O*-3 Ac	+/-	(Ebringerová *et al.*, 2005; Hromádková *et al.*, 2005; Ren and Sun, 2010)
OSX	β-(1→4)-D-Xyl	α-(1→2) 4-*O*-Me-α-D-GlcA α-(1→3) Ara	-	(Saake *et al.*, 2001)

Xyl: xylose, Ara: arabinose, Ac: acetic acid, Me: methyl, GlcA: glucuronic acid

Table 4.3: Constituent monosaccharide composition (w/w%) of the substrates used for xylanase characterisation.

Sample name[a]	Sugar composition (w/w%)						Total sugar content (w/w%)
	Ara	Xyl	Man	Gal	Glc	UA	
WAX	32	58	0	0	0	1	91
BeWX	1	68	0	0	1	9	79
BiWX	0	69	0	0	1	9	81
OSX	7	61	0	2	5	2	76
WS WUS[b]	2	23	3	1	40	1	70
WS KOHss	5	46	1	1	3	1	56
WS KOHres	1	6	7	0	54	0	69
WS KOHmix	1	20	5	0	36	0	64

[a]Wheat arabinoxylan (WAX), beech wood xylan (BeWX), birch wood xylan (BiWX), oat spelt xylan (OSX), wheat straw water unextractable solids (WS WUS), wheat straw alkaline soluble solids (WS KOHss), wheat straw alkaline residue (WS KOHres), wheat straw after alkaline treatment (WS KOHmix)
Ara: arabinose, Xyl: xylose, Man: mannose, Gal: galactose, Glc: glucose, UA: uronic acid
[b] Only WS WUS contains 2% (w/w) acetic acid

4.2.4 Xylanase activity

Xylanase activity was determined by using the PAHBAH assay for reducing sugars (Kühnel *et al.*, 2010). The method is based on the colouring reaction of reducing sugar with PAHBAH in aqueous alkali. Substrate concentration: 2 mg/mL WAX (in 50 mM sodium acetate buffer, pH 5.0). The concentration of released reducing ends was determined using a D-xylose calibration curve.

4.2.5 Protein content determination

Protein concentrations were measured using the Bradford method (Bradford, 1976). Bovine serum albumin (BSA, Sigma-Aldrich Inc., St. Louis, MO, USA) was used as standard.

4.2.6 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using precast 8-16% gradient gels (Lonza, Basel, Switzerland) in running buffer

containing 25 mM Tris/HCl, 192 mM glycine and 0.1 % SDS (pH 8.3) at 125 V. The samples were denatured before being loaded on to the gel at 95 °C for 5 min in loading buffer containing 0.35 M Tris/HCl, 10.3 % (w/v) SDS, 36 % (v/v) glycerol, 5 % (v/v) 2-mercapto-ethanol and 0.012% (w/v) bromo-phenol-blue (pH 6.8). The gels were stained using Coomassie Brilliant Blue.

4.2.7 Enzyme degradation of the substrates

WAX, BeWX, BiWX and OSX were used next to insoluble WS WUS and WS KOHres, and partly soluble WS KOHss, and WS KOHmix. The substrate concentration was 5 mg/mL in 50 mM sodium acetate buffer, pH 5.0; allowing comparison with the enzyme characteristics published by Ustinov et al. (2008). WS KOHss was the only substrate that was also dissolved in 50mM sodium acetate buffer, pH 5.0 containing 20% (v/v) dimethylsulfoxide (DMSO).

Sufficient enzyme was added to theoretically degrade all xylosidic linkages of wheat arabinoxylan within 6 h (0.063 units/mL; one unit of enzyme activity is defined as the amount which liberates 1 µmol reducing end group min^{-1} at 50 °C). The samples were incubated at 50 °C. Aliquots were taken at 30 min, and at 1, 2, 4, 8 and, 24 h. An equal dose of fresh enzyme was added at 24 h and the reaction was stopped after 48 h. For the insoluble substrates WS WUS, WS KOHmix and WS KOHres 10x the amount of enzyme was added, compared to the amount used for the other commercial and partly soluble substrates. Aliquots were taken at 8 and 24 h. Substrate solutions without enzyme addition were used as blanks.

4.2.8 Characterisation of oligomers

Degradation was monitored by high performance anion exchange chromatography (HPAEC) as described before (Van Gool *et al.*, 2011). Quantification of the results was based on the response factor of xylotetraose. Relative amounts of monomers and oligomers were calculated either as percentage of the particular sugar present in the initial substrate, or as percentage of the total amount of carbohydrates in the initial

substrate. Results and discussion are based on samples incubated for 30 min, 2, 8 and 24 h for the commercial substrate digests. Although the results for the different time points were fully in line with each other, only the 24 h samples are discussed for the various WS WUS fractions.

4.2.9 Sequence analysis

The DNA sequence data are available at the GenBank database under the accession numbers, *xyl*1 JF508853 and *xyl*3 JF508854. The alignment of the amino acid sequences was made with the ClustalW multiple-alignment-program (Thompson *et al.*, 1994).
The CPHmodels 3.0 program (available at www.cbs.dtu.dk/services/CPHmodels/; Nielsen et al. (2010)) was used for homology modelling.

4.3 Results and discussion

4.3.1 HPAEC profiles of commercial xylans

The degradation products of the various substrates produced by the C1 endo-xylanases were visualised by high performance anion exchange chromatography (HPAEC). The HPAEC patterns display the composition in monomers and oligomers of the substrates after endo-xylanase digestion (Fig. 4.1). Xyl3dCBM was chosen to be displayed in the chromatograms.

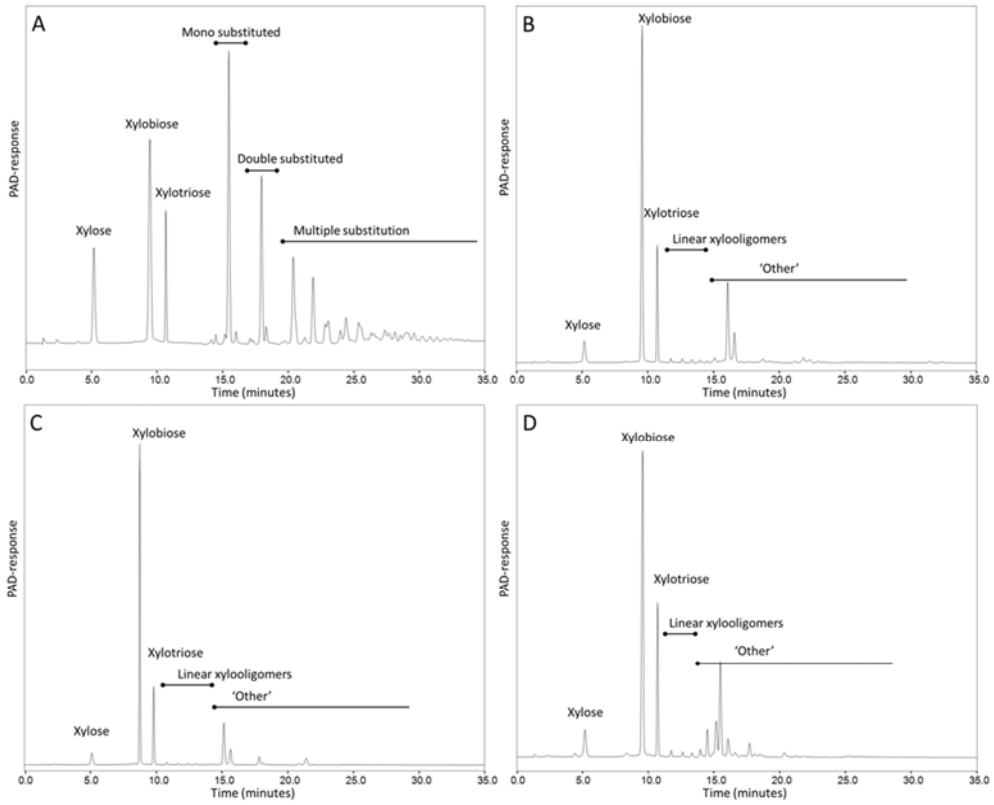

Fig. 4.1: Typical high performance anion exchange chromatography patterns for wheat arabinoxylan (WAX; A), beech wood xylan (BeWX; B), birch wood xylan (BiWX; C) and oat spelt xylan (OSX; D), digested with the C1 endo-xylanase 3 without carbohydrate binding module (Xyl3dCBM).

The pattern of WAX (Fig. 4.1A) displays many arabinose-substituted oligomers next to some monomers and linear xylooligomers. The abundant presence of substituted oligomers reflects the high level of arabinose substitution of WAX. The chromatograms of the BeWX, BiWX and OSX digests mainly display xylose and short linear xylooligomers, next to substituted oligosaccharides indicated with 'other' (Fig. 4.1B, C and D). The HPAEC patterns of BeWX and BiWX are comparable (Fig. 4.1B and C). This can be explained by their similar sugar composition (Table 4.3).

Though endo-xylanases will hydrolyse the xylan backbone to some extent, the degree of substitution and solubility of a particular xylan are important factors influencing enzyme efficiency (Paës *et al.*, 2012).

Overview of WAX hydrolysis products after endo-xylanase treatment

For each enzyme and each substrate amounts of mono- and oligosaccharides were quantified and plotted as percentage of xylose present in the substrate; or as percentage of the total substrate for unknown oligomers. Fig. 4.2 provides an overview of WAX hydrolysis products for each enzyme. The supplementary Fig. A1-A3 show the products of BeWX, BiWX and OSX released by the different xylanases.

Fig. 4.2 shows that the amount of the products released from WAX varied for the different endo-xylanases. Furthermore, the ratio of the released components is different for each individual enzyme hydrolysing WAX. Xyl1 (with and without CBM) hydrolysis led to more mono- and multiple substituted oligomers than both variants of Xyl3. The enzymes without binding module released more xylose than the enzymes with CBM. The release of xylobiose was lower for Xyl1. Despite the same amount of units dosed, Xyl1dCBM degraded WAX faster than Xyl1, whereas Xyl3 was faster than Xyl3dCBM.

In some cases a decrease is seen in the total amount of degradation products, for instance for WAX degradation by Xyl1 after 500 min. It could be that only after 500 min the preferred substrate for the xylanase became limiting.

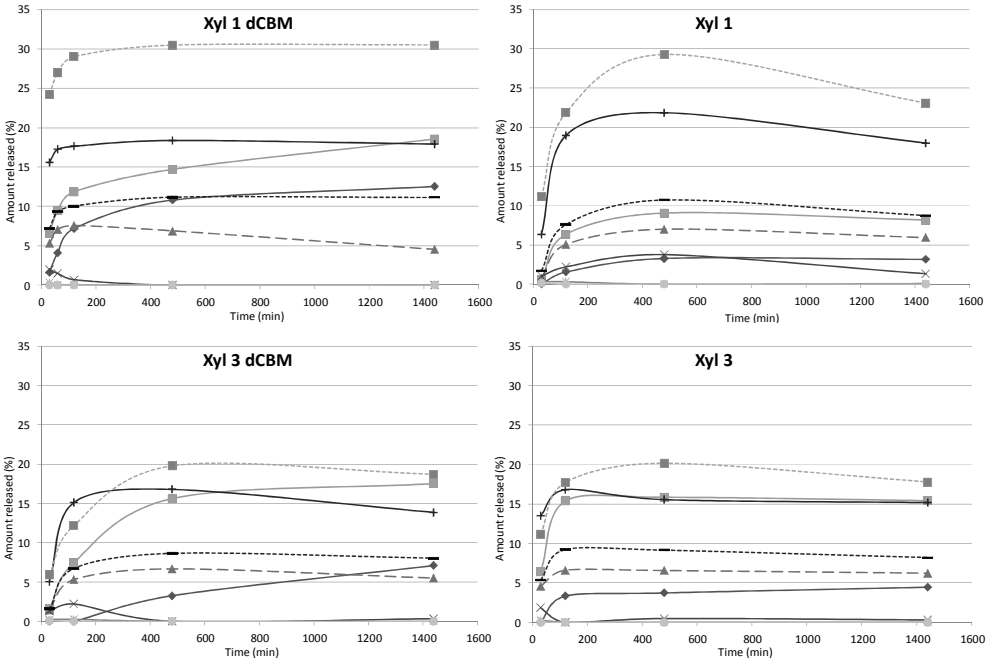

Fig. 4.2: Release of xylose and substituted oligomers after wheat arabinoxylan (WAX) digestion by different C1 GH10 xylanases. The amount of xylose components is displayed as weight percentage of the total amount of xylose. The amount of substituted oligomers is displayed as weight percentage of the total amount of carbohydrates in WAX. Xylose; Xylobiose; Xylotriose; Xylotetraose; Xylopentaose; Xylohexaose; Mono substituted oligomers; Double substituted oligomers; Multiple substituted oligomers.

Comparison of the hydrolysing capacity of the xylanases towards commercial xylans

The total hydrolysing capacity after 24 h per enzyme per substrate is displayed in Fig. 4.3 and is defined as the percentage (w/w) of the total substrate that is degraded by the enzymes to monomers and oligomers. It shows that Xyl1dCBM was the most efficient enzyme in the hydrolysis of the commercial substrates. Xyl1dCBM released ~65-80% of the carbohydrates present in the initial substrates as mono- and oligomers. Xyl3dCBM was quite efficient in BeWX and OSX hydrolysis (~70% degraded to monomer/oligomers). Xyl1 and Xyl3 were both less efficient on BeWX, BiWX and OSX compared to their counterpart without a binding domain. Especially

OSX hydrolysis was difficult for these variants of the C1 xylanases (~30-40% degraded to monomer/oligomers).

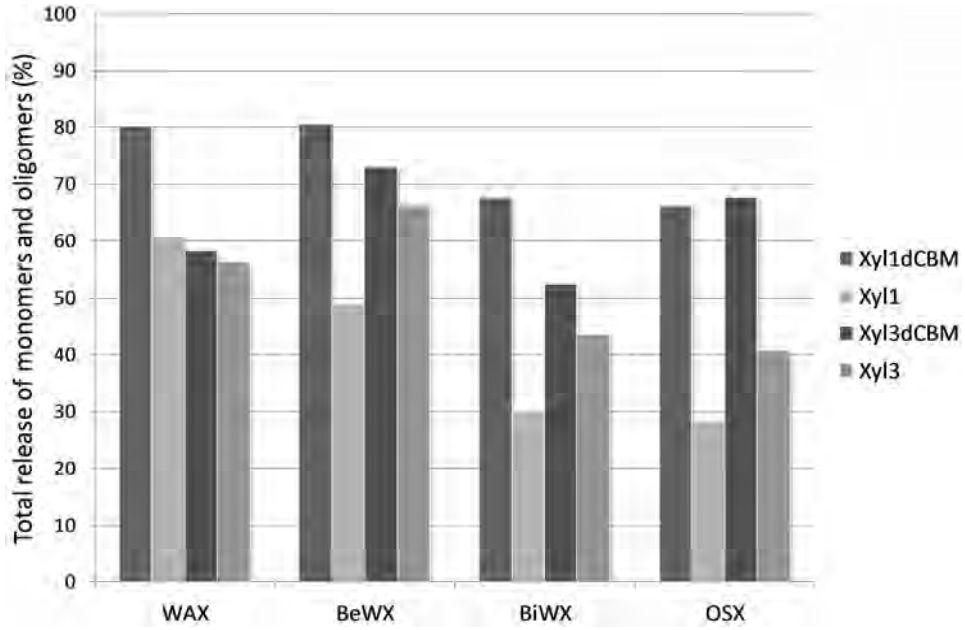

Fig. 4.3: Release (w/w%) of monomers and oligomers from the carbohydrate fraction of wheat arabinoxylan (WAX), beech wood xylan (BeWX), birch wood xylan (BiWX) and oat spelt xylan (OSX) by the C1 endo-xylanases with and without carbohydrate binding module (dCBM) after 24h of incubation, based on high performance anion exchange chromatography results.

Compared to BiWX, BeWX was more accessible for all the enzymes. In contrast to the other enzymes (Supplementary data Fig. A1-A2) Xyl1 could not degrade xylotetraose, xylopentaose and even xylohexaose in the BiWX digest, although this is assumed to be a suitable substrate for an endo-xylanase of GH family 10 (Pollet *et al.*, 2009). The addition of fresh enzyme after 24 h did affect the linear xylooligomers which were then degraded to xylotetraose or smaller oligomers (data not shown). This finding indicates that the enzyme has a low affinity for linear xylooligomers. Inactivation of the enzyme seems unlikely, since the enzyme showed activity on the other substrates after 24h under the same conditions. Ustinov et al. (2008) concluded that no changes occurred after addition of fresh enzyme.

In general, Xyl1dCBM released more xylose than the other enzymes. Furthermore, Xyl1dCBM, Xyl3 and Xyl3dCBM released high amounts of xylobiose. This can be attributed to a low binding affinity at the -1 and +1 subsites, where the sum of the binding affinities is negative (Pollet *et al.*, 2010). The total hydrolysing capacity of each enzyme was comparable to the results found by Ustinov et al. (2008) who also found higher activity for Xyl1 compared to Xyl3; however, their reaction products remained unspecified. They attributed the lower activity of Xyl3 to a longer and more glycosylated peptide linker compared to that of Xyl1, which is supposed to have a negative effect on the catalytic activity against polymeric xylans. Xyl1 has six possible glycosylation sites (based on serine), Xyl3 contains ten possible sites. In addition, Xyl3 does not contain any glycine residues, which usually add flexibility to the linker peptide (Yan and Sun, 1997), whereas Xyl1 contains nine glycine residues (not shown).

Influence of CBM on hydrolysis

The enzymes without binding module were more efficient in hydrolysis of the substrates than the enzymes with binding module (Fig. 4.3). The difference increased with decreasing degree of substitution, and decreasing solubility of the substrate. The type of substituents may also cause differences in hydrolysis efficiency.

It is supposed that the CBM hinders efficient docking of substrates and subsequently lowers the release of xylobiose. This might be caused either by the different conformation of the protein due to the presence of the CBM or by the fact that the CBM causes the enzyme to interact with the substrate in such a way that xylobiose could not be released. The observation that the enzymes without CBM are more efficient towards soluble substrate hydrolysis has been made before (Sun *et al.*, 1998). Both binding modules are classified as cellulose binding modules of family 1. An alignment was made of several CBMs from GH10 xylanases, GH11 xylanases and GH7 endo-glucanases/cellobiohydrolases which displayed around 70% homology with the cellulose binding modules of the C1 endo-xylanases (data not shown). It is not known whether this binding module is also able to bind to xylan. In literature (Black et al.,

1995) an alternative type of binding module is described that does bind xylan; however this alternative type of binding module is not yet recognized in C1 enzymes. This alternative type of CBM is an internal CBM homologue that exhibits 65 % sequence identity with the C-terminal CBM. The presence of such an internal CBM homologue affects the affinity for a xylan (Black et al., 1995). For a *Clostridium stercorarium* xylanase containing two CBMs of family IV at the C-terminal, binding of one of the CBMs to OSX has been reported (Sun et al., 1998); however, the C1 xylanases only contain one CBM of family 1. Therefore, this seems an unlikely explanation for the differences observed in C1 xylanases.

The differences found for the hydrolysing capacity of the two types of C1 xylanases on commercial substrate was previously attributed to the lower enzyme dose, based on protein content (Ustinov *et al.*, 2008). This seems unlikely, since the enzymes were highly overdosed in this study.

From the results obtained it cannot be decided which of the options described in the literature is occurring in C1 xylanases.

4.3.2 Enzyme performance using fractions of insoluble xylan-rich substrate

Fig. 4.4 shows the total release of monosaccharides and oligosaccharides from the partly insoluble substrates by the four enzymes tested. Hydrolysis of wheat straw is difficult for all xylanases, since only around 4% of the total substrate is hydrolysed to monomers and oligomers, representing around 17% of all xylose present in the WS WUS. It seems that a binding module is not helpful for degrading this cell wall material.

When alkali-labile interactions in WS WUS are broken by alkaline treatment and the hemicellulose-cellulose network is repacked upon neutralization, it is observed that the substrate was more available for the enzymes (Fig. 4.4, WS KOHmix). The total hydrolysis capacity of the enzymes increased from 4% on WS WUS to 20-30% on WS KOHmix, indicating complete hydrolysis of the xylan present (Table 4.3). The biggest effect of the alkaline treatment is observed for the enzymes without binding module.

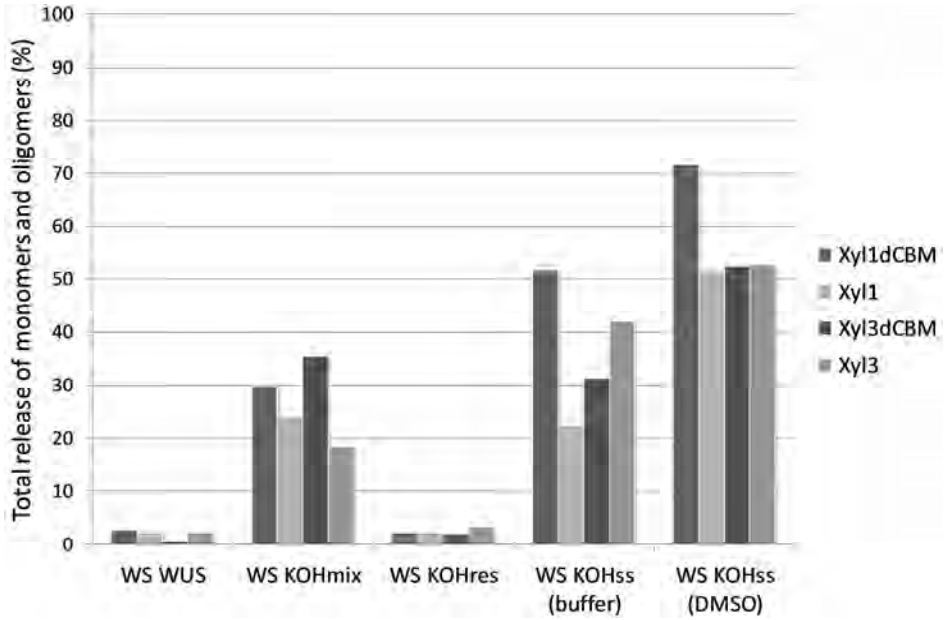

Fig. 4.4: Release (w/w%) of monomers and oligomers from the carbohydrate fraction of wheat straw water unextractable solids (WS WUS), wheat straw after alkaline treatment (WS KOHmix), wheat straw alkaline residue (WS KOHres), wheat straw alkaline soluble solids (WS KOHss) suspended in buffer and WS KOHss suspended in 20% DMSO by the different endo-xylanases after 24h of incubation; based on high performance anion exchange chromatography results.

Although cellulose was present, the enzymes containing a CBM were not more efficient than the enzymes without binding module. However, the differences are smaller than those seen on commercial substrates. In the literature two mechanisms are proposed by which a CBM could be beneficial for the enzyme: 1) bringing the enzyme into close vicinity with its substrate, thereby increasing the local concentration of enzyme and thus enhancing hydrolysis; or 2) binding of the CBM containing xylanases to crystalline cellulose, leading to disruption of the ordered cellulose network and enhancement of cellulose availability (Guillén *et al.*, 2009). The better performance of the xylanases without CBM could also indicate irreversible adsorption of the CBM to the target substrate, cellulose as has been found for several other CBM families (Linder and Teeri, 1997), but not for family 1 CBMs (Tomme *et al.*, 1998). Another possibility could be that the cellulose-hemicellulose network is not repacked as it was originally present in WS WUS. The enzymes containing a CBM will

bind to the cellulose, but in that case might not reach the vicinity of the target substrate, hemicellulose.

WS KOHres contains hardly any substrate available for the enzymes. Four percent of the carbohydrates in WS KOHres were solubilized by the enzymes as xylose and oligosaccharides, which is about 60% of the xylan present in this fraction. This indicates that the residual xylan in this fraction is accessible to the enzymes.

The xylan extracted from WS WUS is partly soluble in buffer and seems to be a good substrate for the enzymes (Fig. 4.4; WS KOHss). Xyl1dCBM was the most efficient enzyme, releasing 50% of all carbohydrates in this fraction as monomer and oligomers. The incomplete hydrolysis of linear xylan polymers in WS KOHss can be explained by xylan self-association. Therefore, the fraction was also dissolved in 20% (v/v) DMSO in buffer to prevent this behaviour (Ebringerová *et al.*, 1994) and to make the polymers more readily available for the enzymes. The results with the xylan dissolved in 20% (v/v) DMSO show that especially Xyl1 (both with and without binding module) was influenced by self-association of xylan, releasing up to 70% of the carbohydrates to monomers and oligomers. Xyl3 was less influenced by the self-association, but total degradation was still significantly higher than when the reaction was performed in buffer (50% vs. 30-40% of all carbohydrates). Remarkably, there was no difference in efficiency between the Xyl3 enzymes with and without CBM. The importance of hydrogen bond formation in CBM-ligand binding could be the reason why no differences were observed when using 20% (v/v) DMSO (Pell *et al.*, 2003).

Since the degree of substitution of the xylan in WS KOHmix and WS KOHss is equal, any difference in hydrolysis efficiency of the xylanases in a buffer system cannot be attributed to enzyme hindering caused by substituents. As there still were differences visible between Xyl1 and Xyl3, these should be derived from interaction between (partly) insoluble substrate and enzyme, or from differences in the conformation of the enzymes. It was found that differences in enzyme functionality occur for various GH11 endo-xylanases degrading soluble or insoluble arabinoxylans (Moers *et al.*, 2005). Only a GH10 endo-xylanase from *Thermoascus aurantiacus* showed similar degradation products compared to those of other GH10 endo-xylanases from both

soluble and insoluble xylans. No differences were reported in the degradation of soluble or insoluble xylans by GH10 xylanases (Zhang *et al.*, 2011). A GH8 xylanase of *Pseudoalteromonas haloplanktis* exhibited higher activity towards insoluble substrate than towards soluble substrate, by which the activity and solubilisation of insoluble substrates was significantly favoured by the presence of a secondary substrate binding site in the xylanase (Cuyvers *et al.*, 2011). However, such a secondary substrate binding site was not found in the xylanases studied. To understand the exact mechanisms underlying endo-xylanase activity towards the soluble and insoluble substrates, the protein structure of the xylanases was studied.

4.3.3 Differences in performance correlate with a difference in protein structure

The differences between both xylanases can be attributed to the protein structure of the enzymes. Intrafamily differences have been observed before in xylanases and are caused by variations in amino acid sequence of the enzymes (Moers *et al.*, 2005; Bonnin *et al.*, 2006; Pollet *et al.*, 2009).

Fig. 4.5 indicates that Xyl1 and Xyl3 have homology (~50% average) with heterologous GH10 xylanases from *Penicillium funiculosum, Aspergillus niger, Hypocrea jecorina, Chaetomiun globosum, Penicillium simplicissium* and *Thermoascus aurantiacus*. Both catalytic residues are conserved, as well as the residues involved in substrate binding (Schmidt *et al.*, 1998; Lo Leggio *et al.*, 2001). Schmidt et al. (1998) described two types of GH10 xylanases: one type contains two extra loops in the structure compared to the other type. These loops form a lid above the catalytic site, suggesting a role in the binding of substrates. These conserved amino acid sequences were also found in the C1 Xyl3, as indicated by boxes in Fig. 4.5. Xyl1 does not contain these two loops. Based on homology, model structures of Xyl1 and Xyl3 were constructed to show the presence of the two loops.

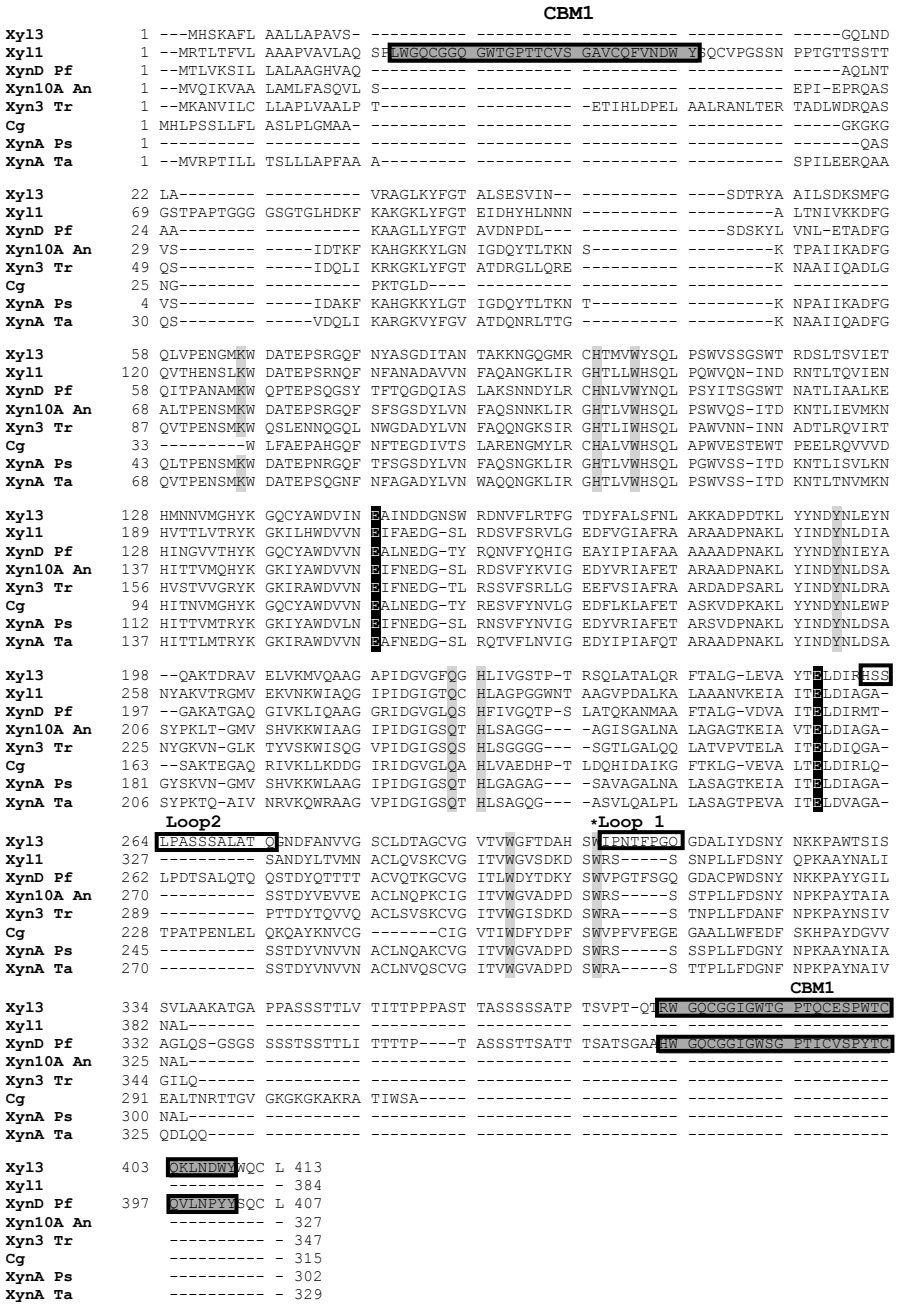

Fig. 4.5: Alignment of C1 GH10 xylanases with different heterologous GH10 xylanases. (continued on next page)

*Fig. 4.5 (continued):The black shaded residues are the catalytic residues, the grey shaded residues are involved in binding of the substrate, the residues in the boxes are two loops in the model structure of the C1 enzyme Xyl3 (based on Schmidt et al. (1998) and Lo Leggio et al. (2001)) and the residues in the grey coloured boxes are the residues from CBM1. XynD Pf: Xylanase XynD from Penicillium funiculosum, Genbank accession nr. CAG25554; Xyn10A An: Xylanase Xyn10A from Aspergillus niger, Genbank accession nr. CAK38067; Xyn3 Tr: Xylanase Xyn3 from Hypocrea jecorina, Genbank accession nr. BAA89465; Cg: Putative xylanase from Chaetomium globosum CBS 148.51, Genbank accession nr. EAQ91745; XynA Ps: Xylanase XynA from Penicillium simplicissium, Genbank accession nr. AAC23574; XynA Ta: Xylanase XynA from Thermoascus aurantiacus, Genbank accession nr.CAB65468, * : Trp305 in Xyl3 and Trp358 in Xyl1.*

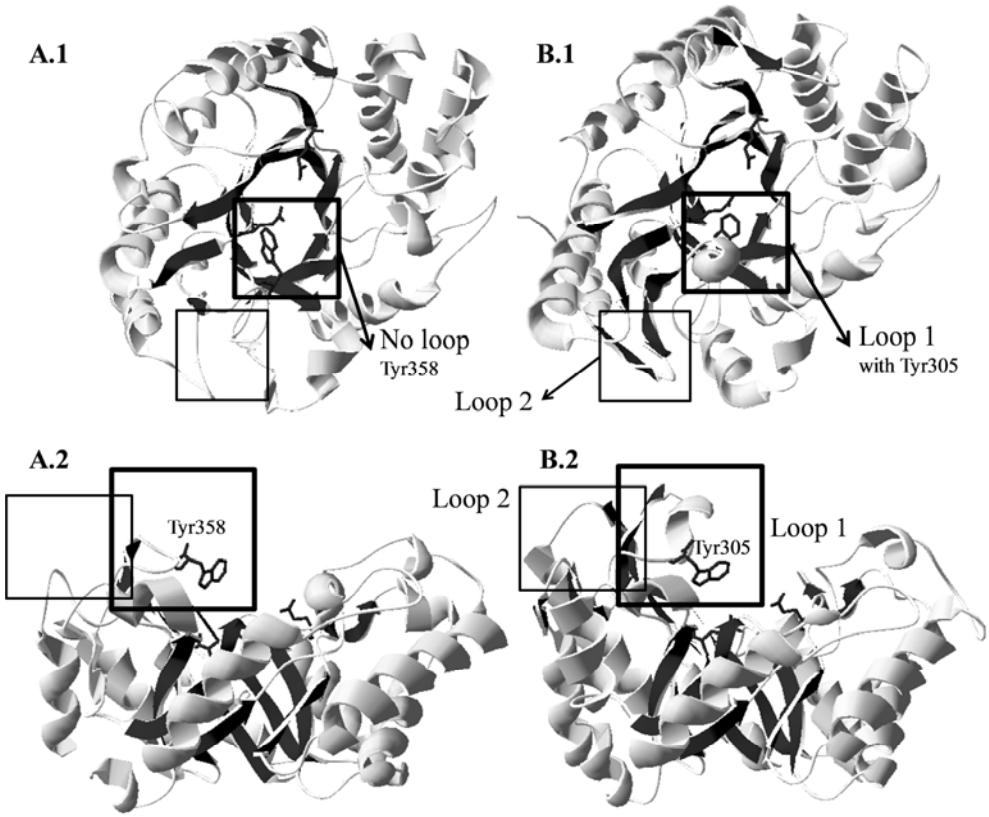

Fig. 4.6: Two views of the model structure of Xyl1 (A) and Xyl3 (B). The top view (1) and the side view (2) of the catalytic domains are shown. The model of Xyl1 is obtained using xylanase XynA from Penicillium simplicissium (Genbank accession nr. AAC23574) as template. The model of Xyl3 is obtained using xylanase Xyn10A from Streptomyces olivaceoviridis E-86 (PDB nr. 1V6Y) as template. The catalytic residues are indicated in black and the residue Tyr305 (Xyl3) and Tyr358 (Xyl1) are indicated in black as well (based on Schmidt et al. (1998) and Lo Leggio et al. (2001)).

Fig. 4.6 shows that these loops are indeed present for Xyl3 and not for Xyl1. Due to the presence of loop 1, amino acid Trp305 in Xyl3 is forming a lid above the catalytic cleft and therewith shields the active site. The position of the homologous Trp358 is such that in Xyl1 a more open structure is observed. Schmidt et al. (1998) suggested that xylanases with a more open cleft (without the two loops) hydrolyse branched xylooligosaccharides and larger oligosaccharides to a higher extent. Xylanases with the more closed cleft (with the two loops) are suggested to act more on linear xylans and can act on smaller oligosaccharides. This is in line with our findings for Xyl1 and Xyl3. Xyl1 was more active towards WAX, whereas Xyl3 was most efficient in OSX hydrolysis. Furthermore, the suggestion concerning the size of the catalytic cleft confirms the observation of low affinity of Xyl1 towards xylotetraose.

To prove the exact mechanisms underlying the endo-xylanase loops, engineering xylanases with and without loops could be interesting, in which their properties are tested using the methods described in the present study.

4.3.4 Substrate-protein interaction influences enzyme performance

A schematic representation of the factors influencing enzyme behaviour is displayed in Table 4.4. Although all enzymes belong to GH family 10, it can be seen that they reacted differently under various circumstances. Their functionality towards the various substrates was not characteristic of GH family 10 enzymes.

Table 4.4 shows that Xyl1dCBM was not much influenced by the degree of substitution of the substrate. Xyl1 degraded the less substituted substrate to a much lesser extent. This can be attributed to the CBM. In contrast, the Xyl3 enzymes degraded the less substituted xylan more efficiently, likely due to the structural features of the Xyl3 enzymes, in which loop 1 hinders efficient substrate docking.

Table 4.4: Performance of the C1 GH10 xylanases Xyl1, Xyl1 without carbohydrate binding module (Xyl1dCBM), Xyl3 and Xyl3 without carbohydrate binding module (Xyl3dCBM) as influenced by various substrate characteristics.

Enzyme	Lower degree of substitution	Randomisation of glucuronic acid substituents	Alkaline treatment of wheat straw	Removal of cellulose	Prevention of xylan self-association
Xyl1dCBM	0	+	++	++	++
Xyl1	-	+	++	-	++
Xyl3dCBM	++	+	++	-	++
Xyl3	+	+	++	++	+

-: Low efficiency
0: No effect
+: High efficiency
++: Very high efficiency

All xylanases had higher efficiency towards substrate with a random distribution of glucuronic acid substituents compared to a blockwise distribution. This can be attributed to the self-association of the xylan, which has significant influence on enzyme performance. All enzymes were hindered by the presence of alkali-labile interactions in the cell wall. The accessibility of xylan after alkaline treatment was increased. Also the minimal amount of xylan in the residue after alkaline extraction was more accessible.

Some of the xylanases are hindered by cellulose in the cell wall; however this could not be correlated to the presence or absence of a CBM.

4.4 Conclusions

The classification of enzymes within a certain family does not predict their performance towards various substrates. Use of different xylan-rich substrates revealed differences in enzyme efficiency of GH family 10 xylanases. The presence of a CBM was not beneficial for the GH10 xylanases. The different performance towards the substrates is explained by the structural conformation of the enzymes. The study

shows the importance of detailed enzyme characterisation for drawing the correct conclusions towards their efficiency.

Acknowledgements

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Supplementary data

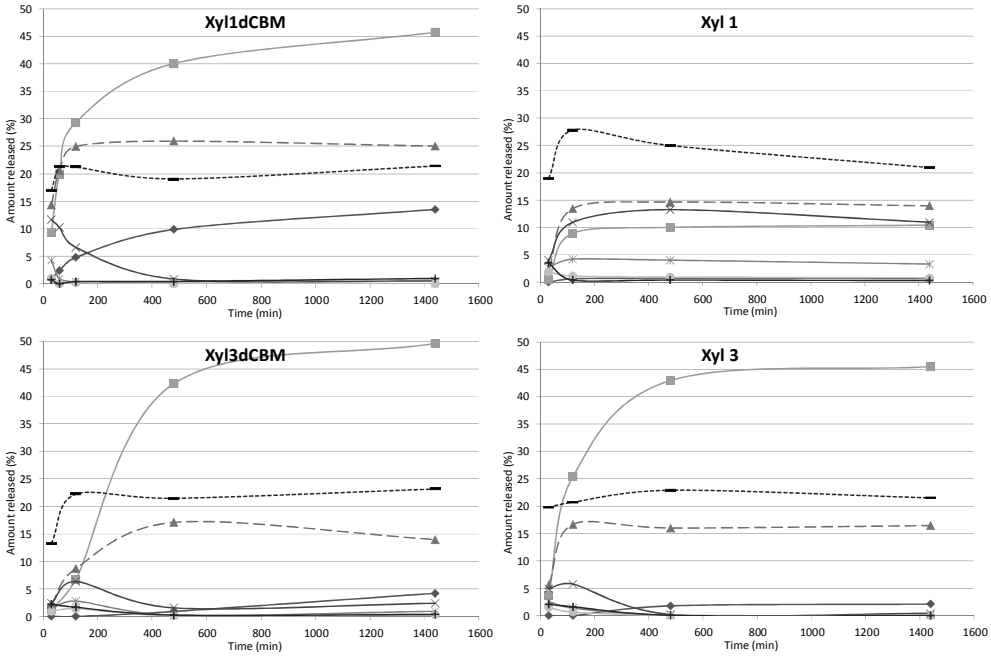

Fig. A1: Release of xylose and oligomers after beech wood xylan (BeWX) digestion by different C1 GH10 xylanases. The amount of xylose components is displayed as weight percentage of the total amount of xylose. The amount of substituted oligomers is displayed as weight percentage of the total amount of carbohydrates in BeWX. Xylose; Xylobiose; Xylotriose; Xylotetraose; Xylopentaose; Xylohexaose; Linear xylooligomers DP>6; Other.

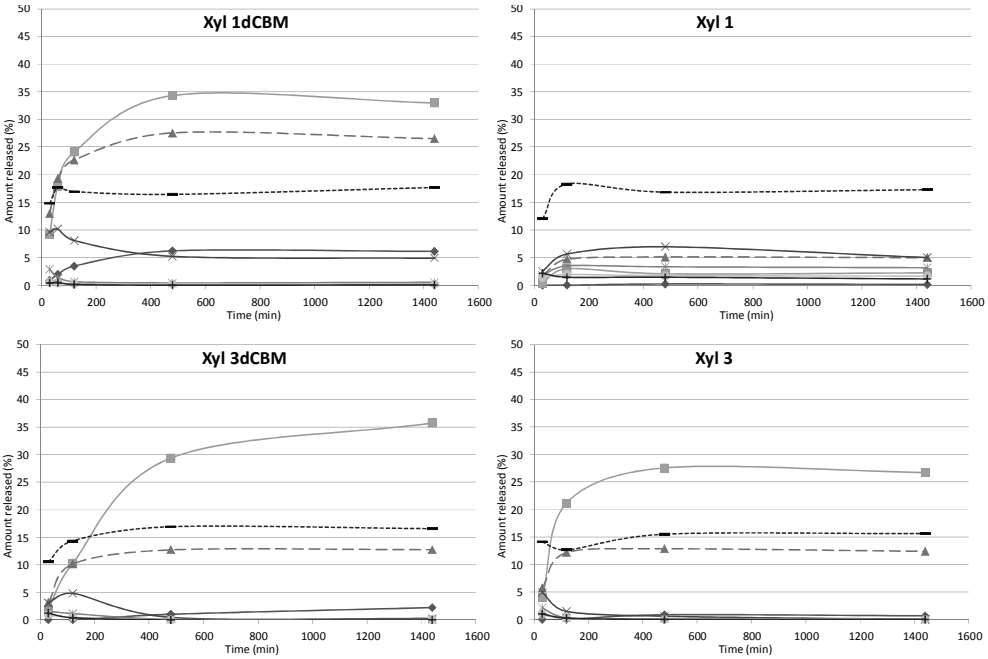

Fig. A2: Release of xylose and oligomers after birch wood xylan (BiWX) digestion by different C1 GH10 xylanases. The amount of xylose components is displayed as weight percentage of the total amount of xylose. The amount of substituted oligomers is displayed as weight percentage of the total amount of carbohydrates in BiWX. Xylose; Xylobiose; Xylotriose; Xylotetraose; Xylopentaose; Xylohexaose; Linear xylooligomers DP>6; Other.

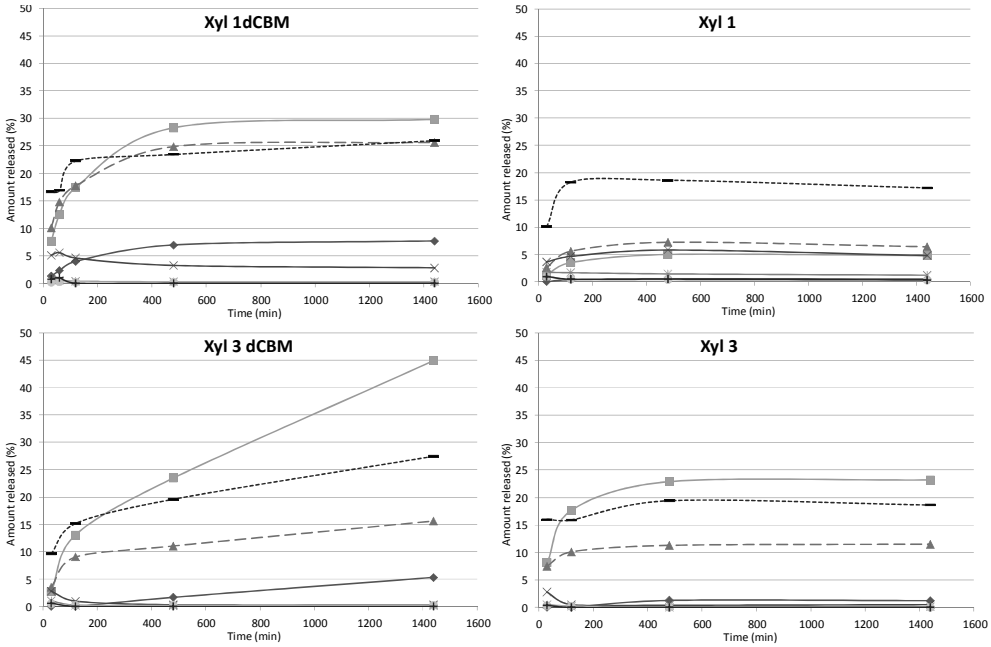

Fig. A3: Release of xylose and oligomers after oat spelt xylan (OSX) digestion by different C1 GH10 xylanases. The amount of xylose components is displayed as weight percentage of the total amount of xylose. The amount of substituted oligomers is displayed as weight percentage of the total amount of carbohydrates in OSX. Xylose; Xylobiose; Xylotriose; Xylotetraose; Xylopentaose; Xylohexaose; Linear xylooligomers DP>6; Other.

Chapter 5

Differences between two novel GH11 endo-xylanases from Myceliophthora thermophila C1 towards soluble and insoluble xylans

Van Gool, M.P., Van Muiswinkel, G.C.J., Hinz, S.W.A., Schols, H.A., Sinitsyn, A.P., Gruppen, H. (2012) Submitted for publication

Abstract

Several studies showed that glycoside hydrolase (GH) family classification only cannot predict enzyme performance. Therefore, two novel GH11 endo-xylanases from *Myceliophthora thermophila* C1 (C1) were purified and the influence of solubility and molecular structure of various substrates on their efficiency was investigated. Both endo-xylanases were more hindered by a higher degree of substitution than by a decreased solubility of the xylan. The different product formation from the various xylans could be correlated to the tyrosine at position 163 in the amino acid sequence of the enzymes, which is replaced in some xylanases by a valine. This replacement in GH11 xylanases results in a degradation product composition closer to GH10 xylanases.

The GH11 xylanases were more efficient on low substituted xylan compared to C1 GH10 endo-xylanases and from those substrates they released more small oligomers, which is contrary to the general assumption that GH10 xylanases degrade xylans to a higher degree.

5.1 Introduction

Xylans represent the main hemicellulose component of secondary plant cell walls. They comprise an (1,4)-β-D xylopyranose backbone, which can be substituted with arabinose, xylose, galactose, glucuronic acid or its 4-*O*-methyl ether and may also contain acetyl, feruloyl or *p*-coumaroyl groups (Saha, 2003). The biodegradation of xylans requires endo-1,4-β-D-xylanases (EC 3.2.1.8), β-D-xylosidases (EC 3.2.1.37) and several accessory enzymes, such as α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase (Collins *et al.*, 2005; Gírio *et al.*, 2010).

The principal commercial sources for these enzymes are filamentous fungi, of which strains of *Aspergillus* and *Trichoderma* are the most common ones used (McKelvey and Murphy, 2011). Also, the genome of the Ascomycetous fungus *Myceliophthora thermophila* C1 (C1) has been shown to contain many genes encoding carbohydrate-active enzymes, including several xylanases or xylan degrading activities (Hinz *et al.*, 2009; Van Gool *et al.*, 2012).

Most endo-xylanases belong to glycoside hydrolase (GH) family 10 or 11, based on amino acid similarities and structural features (Henrissat and Bairoch, 1993; Paës *et al.*, 2012). GH10 endo-xylanases are highly active towards short xylooligomers. They exhibit less substrate specificity than GH11 endo-xylanases and can hydrolyse different types of decorated xylans (Collins *et al.*, 2005). GH11 endo-xylanases are highly specific and do not tolerate many decorations on the xylan backbone (Biely *et al.*, 1997). These GH11 endo-xylanases are composed of three antiparallel β-sheets and one α-helix. The three-dimensional structure of these endo-xylanases has been described as a “partly closed right hand”. This “hand” contains a thumb-like structure, which is the most flexible region of the xylanase. The movement of this thumb could be essential for the function of the enzyme, as it determines the width of the cleft and by accompanying binding of substrates in the catalytic cleft (Paës *et al.*, 2007).

Although classification of the glycoside hydrolase enzymes is aimed at providing a convenient tool to derive mechanistic information, it has been shown that the GH family classification only cannot predict enzyme breakdown products and enzyme efficiency towards soluble and insoluble substrates (Van Gool *et al*. (2012) *and references therein*). Furthermore, differences in amino acid sequence and conformation of the enzymes can partly explain the formation of different breakdown products by the enzymes (Van Gool *et al.*, 2012).

Only three C1 GH11 xylanases have been subjected to preliminary xylanase characterisation tests before (Ustinov *et al.*, 2008). In this study, two novel C1 GH11 endo-xylanases, Xyl7 and Xyl8, were subjected to detailed analysis using the same effective approach as described previously for C1 GH10 endo-xylanases (Van Gool *et al.*, 2012). They were purified and characterised biochemically in order to determine their action pattern towards different xylans. Their functionality was combined with predicted structural features of the protein. Furthermore, their performance was compared with the results obtained for GH10 endo-xylanases of *M. thermophila* C1 as described previously (Van Gool *et al.*, 2012).

5.2 Materials and methods

5.2.1 Enzyme production

Xyl7 and Xyl8 from *Myceliophthora thermophila* C1 (C1) were overproduced in a specially designed C1-expression host (LC strain) (Visser *et al.*, 2011). In addition, a construct of the *xyl7* gene, which lacked the carbohydrate binding module (CBM) was used to transform the LC strain. These three strains were made as described previously (Visser *et al.*, 2011) and grown aerobically in 2-L fermentors in mineral medium, containing glucose, ammonium sulphate and trace elements for the essential salts (Verdoes *et al.*, 2010). After biomass formation, the enzyme was produced in a fed-batch process under glucose limitation at pH 6.0 and 32 °C. The production resulted in: Xyl7 (representing a xylanase with CBM), Xyl7dCBM (representing a

xylanase without CBM) and Xyl8 (representing a xylanase, naturally present without CBM).

5.2.2 Enzyme purification

The first two purification steps of Xyl7dCBM included ion exchange chromatography using a Source30S column (GE Healthcare, Uppsala, Sweden) with 15 mM potassium phosphate buffer, pH 6.0 and a gradient of 0-0.5 M potassium chloride (KCl) in five column volumes (CV). The active fraction was loaded onto a Source30Q column (GE Healthcare) with the same buffer with a gradient of 0-0.5 M KCl in six CV. Next, a phenyl sepharose 6 HS FF column (GE Healthcare) with 10 mM potassium phosphate buffer, pH 6.0 and a gradient of 1-0 M ammonium sulphate in eight CV was used. A Superdex 75 column (GE Healthcare) with 10 mM sodium acetate buffer, pH 5.0 as eluent was used for final purification.

The purification of Xyl7 started with ion exchange chromatography using a Source30S column with 10 mM sodium acetate buffer, pH 5.0 and a gradient of 0-0.5 M sodium chloride (NaCl) in five CV. Secondly, a Source30Q column was used with the same buffer with a gradient 0-0.5 NaCl in five CV. Finally, the active fractions were applied to a Superdex 75 column using a 10 mM sodium acetate buffer, pH 5.0.

The overexpressed Xyl8 was purified in three steps. The first step included gel-filtration using a BioGel P4 column (Biorad, Hercules, CA, USA) with a 10 mM Bis-Tris/HCl buffer, pH 6.8. Secondly anion exchange chromatography was applied using a Source30Q column (GE Healthcare). Elution was done by 10 mM Bis-Tris/HCl buffer of pH 6.8 with a gradient of 0-0.5 M NaCl in six CV. Finally, separation on a SourceISO column (GE Healthcare) was carried out in 50 mM sodium acetate buffer with 1.7 M ammonium sulphate, pH 5.0 with a gradient of 1.7-0 M ammonium sulphate in six CV. A Millipore Pellicon XL system was used for final desalting and concentrating the Xyl8 fraction.

After ultrafiltration and freeze drying, fractions were characterised by SDS-PAGE (as described below) and tested for xylan degrading activity towards wheat arabinoxylan

(WAX, medium viscosity, Megazyme) with the PAHBAH reducing sugar assay (Kühnel *et al.*, 2010).
The protein bands of SDS-PAGE corresponding to Xyl7 were isolated from the gel and sent to The Scripps Research Institute (Florida, USA) for ESI-MS/MS analysis.

5.2.3 Temperature and pH optimum

The temperature optima were determined within the range of 20 to 90 °C using 1% (w/v) birch wood xylan (Sigma) with 0.01% (w/w on protein basis) enzyme in 0.2 M sodium acetate buffer (pH 5.0). The reaction was stopped after 10 min. by boiling the digests.
The pH optima of the endo-xylanases were determined at the temperature optimum of each enzyme, using 1% (w/v) birch wood xylan with 0.01% (w/w on protein basis) enzyme. McIlvaine buffers were prepared by mixing 0.1 M citric acid and 0.2 M disodium hydrogen phosphate to obtain buffers in a pH range of 3.0–8.0. Activity was measured using the PAHBAH assay (Kühnel *et al.*, 2010).

5.2.4 Substrates

Wheat arabinoxylan (WAX), medium viscosity was obtained from Megazyme (Wicklow, Ireland). Beech wood xylan (BeWX), birch wood xylan (BiWX) and oat spelt xylan (OSX) were from Sigma-Aldrich (St. Louis, MO, USA). Wheat straw water unextractable solids (WS WUS) (wheat straw, Groningen, The Netherlands, May 2003), wheat straw alkaline soluble solids (WS KOHss), wheat straw alkaline residue (WS KOHres) and a mixture of the WS KOHss and WS KOHres in the same weight ratios as obtained during extraction (WS KOHmix) were prepared in our laboratory as described previously (Van Gool *et al.*, 2012). The constituent monosaccharide composition of the substrates is added as Supplementary table (Table A1).

5.2.5 Protein analysis

For protein characterisation SDS-PAGE was performed by using precast 8-16% gradient gels (Lonza, Basel, Switzerland) in running buffer containing 25 mM Tris/HCl, 192 mM glycine and 0.1 % (w/v) SDS (pH 8.3) at 125 V. The samples were denatured before being loaded on to the gel at 95°C for 5 min in loading buffer containing 0.35 M Tris/HCl, 10.3 % (w/v) SDS, 36 % (v/v) glycerol, 5 % (v/v) 2-mercapto-ethanol and 0.012% (w/v) bromo-phenol-blue (pH 6.8). The gels were stained using Coomassie Brilliant Blue. Protein concentration was measured using the Bradford method (Bradford, 1976).

5.2.6 Enzyme degradation of the substrates

Substrates were suspended in 50 mM sodium acetate buffer, pH 5.0 (5 mg/mL). WS KOHss was the only substrate that was also dissolved in 50 mM sodium acetate buffer, pH 5.0 containing 20% (v/v) dimethylsulfoxide (DMSO). Enzyme was added (0.063 units/mL; one unit of enzyme activity is defined as the amount which liberates 1 µmol reducing end group min^{-1} at 50 °C) and the mixture was incubated at 50 °C for 30 min, 1, 2, 4, 8 and, 24 h. An equal dose of fresh enzyme was added at 24 h and the reaction was stopped by boiling the samples after 48 h. Results and discussion are based on samples of 30 min, 2, 8 and 24 h (using the response factor of xylotetraose), as the 1 and 4 h samples were fully in line with the other samples and as there were no significant differences observed after 48h. For the insoluble substrates WS WUS, WS KOHmix and WS KOHres 10x the amount of enzyme was added. Aliquots were taken at 8 and 24 h. Substrate solutions without enzyme addition were used as blanks. Although the results for the different time points were fully in line with each other, only the 24 h samples are discussed.

Substrate degradation was monitored by high performance anion exchange chromatography (HPAEC) as described before (Van Gool *et al.*, 2011). Quantification of the results was based on the response factor of xylotetraose. Relative amounts of monomers and oligomers were calculated either as percentage of the particular sugar

present in the initial substrate, or as percentage of the total amount of carbohydrates in the initial substrate.

5.2.7 Sequence analysis

The DNA sequence data are available at the GenBank database under the accession numbers, *xyl*7 JF508855 and *xyl*8 JF508856. The alignment of the amino acid sequences was made with the ClustalW multiple-alignment-program (Thompson *et al.*, 1994).
The CPHmodels 3.0 program (available at www.cbs.dtu.dk/services/CPHmodels/; Nielsen et al. (2010)) was used for homology modelling.

5.3 Results and discussion

5.3.1 GH11 endo-xylanases from *Myceliophthora thermophila* C1

The genes *xyl7* and *xyl8* that have been found in the *M. thermophila* C1 genome were encoding glycoside hydrolase family 11 (GH11) endo-xylanases. Xyl7 naturally occurs with a family 1 carbohydrate binding domain (CBM) at the C-terminus of the protein.

Purification of the endo-xylanases

The purification of Xyl7 yielded in total 3 fractions containing active forms of Xyl7. ESI-MS/MS analysis on the fraction with the highest molecular mass confirmed that this fraction contained the full length Xyl7 (30 kDa) with the intact CBM. The second fraction contained another active form of Xyl7, which was named Xyl7qc. The molecular mass of the fraction Xyl7qc (25 kDa, determined by SDS-PAGE) was lower than that of Xyl7, but higher than the Xyl7dCBM (22 kDa), which was overproduced without the CBM in a separate fermentation (see below). This suggested that Xyl7 was modified in the CBM, which was confirmed by ESI-MS/MS analysis. Partial proteolysis has been observed before for a GH10 xylanase of *Clostridium josui* (Feng *et al.*, 2000).

The third active fraction contained both the full length Xyl7 as well as the processed enzyme Xyl7qc. The study was only continued with the pure fractions Xyl7 and Xyl7qc. After four sequential purification steps, the pool of Xyl7dCBM was still not completely pure. Therefore, the extra proteins found in the SDS-PAGE gel were analysed by ESI-MS/MS. The bands were identified as proteins not involved in plant cell wall degradation. It was decided to continue with this not completely pure preparation.

Xyl8 naturally occurs in C1 as a xylanase without CBM. After the three purification steps, the pool of Xyl8 contained, similarly to Xyl7dCBM, some proteins that were not involved in cell wall degradation. Also for Xyl8 it was decided to continue with this not completely pure preparation. Table 5.1 gives an overview of the characteristics per enzyme.

Table 5.1: Overview of the characteristics of the GH11 endo-xylanases from Myceliophthora thermophila C1 in this study.

Sample	Activity (U/mg protein)[a]	Molecular mass (kDa)[b]	pI[c]	pH optimum	Temperature optimum (˚C)
Xyl7dCBM	19.5	22	7.3	5.5-6.5	50-60 °C
Xyl7qc	42.2	25	--	5.5-6.5	50-60 °C
Xyl7	27.2	30	7.6	5.5-6.5	50-60 °C
Xyl8	170.6	22	6.2	5.5-6.0	50-65°C

[a] Using wheat arabinoxylan as substrate, 30 min incubation, reducing sugar assay
[b] Measured by SDS PAGE
[c] Calculated value
-- Not available

Temperature and pH optima

The temperature optima for all enzymes were between 50 and 60 °C. All enzymes retained ≥50% of their maximum activity between 20 and 65 °C. Xyl8 even had 80% of its maximum activity at 20 °C. The pH optima showed that the enzymes were mostly active between pH 5.0 and 6.5, exhibiting at least 80% of their maximum activity. The maximum activity was found at pH 6.0 for Xyl7dCBM, Xyl7qc and Xyl7 and at pH 5.5 for Xyl8 (no further data shown).

5.3.2 Enzyme performance on commercial xylans

Degradation products

The degradation of wheat arabinoxylan (WAX), beech wood xylan (BeWX), birch wood xylan (BiWX) and oat spelt xylan (OSX) was followed by high performance anion exchange chromatography (HPAEC). An example (Xyl7) of each chromatogram with peak identification (Van Gool *et al.*, 2012) is added as supplementary Fig. A1. For all substrates the amounts of monomers and oligomers were plotted against time. Fig.5.1 shows the data for BiWX. The results of the WAX, BeWX and OSX degradation products can be found in the Fig. A2-A4.

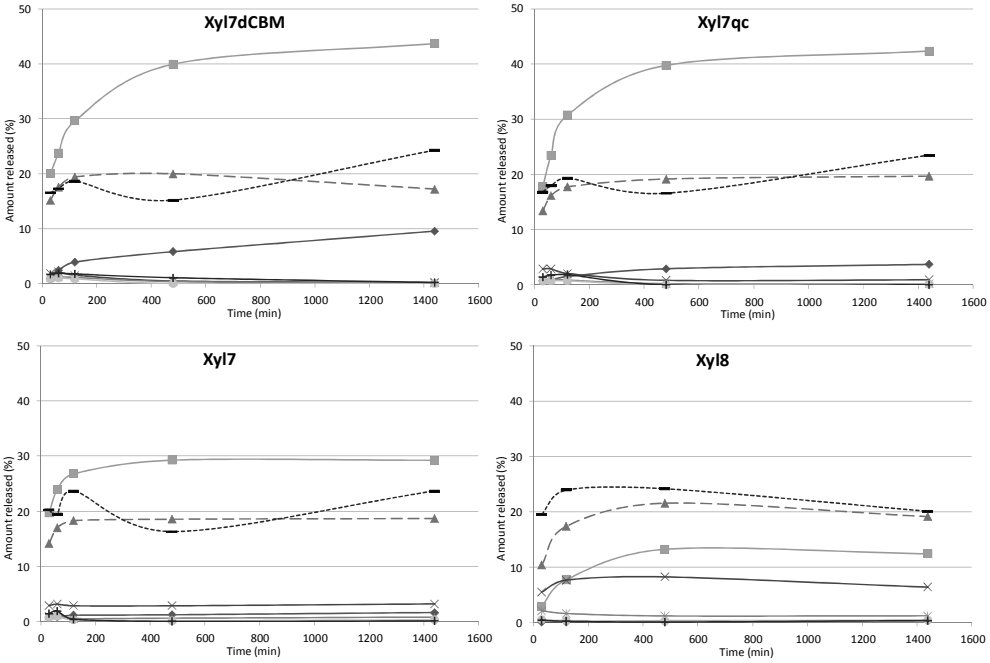

Fig. 5.1: Release of xylose and oligomers after birch wood xylan (BiWX) digestion by different C1 GH11 endo-xylanases. The amount of xylose components is displayed as weight percentage of the total amount of xylose. The amount of substituted oligomers is displayed as weight percentage of the total amount of carbohydrates in BiWX. Xylose; Xylobiose; Xylotriose; Xylotetraose; Xylopentaose; Xylohexaose; Linear xylooligomers DP>6; Other.

As WAX is often used to determine substrate selectivity of endo-xylanases (Moers *et al.*, 2003), we will start discussing the WAX results. The main components released from WAX by the endo-xylanases were multiple arabinose substituted xylooligomers, followed by single arabinose substituted oligomers. The ratio of the different products released from WAX was comparable for all the endo-xylanases (Fig. A2). In our study only small differences between the enzymes were observed in the amount of xylose and xylobiose released.

On BeWX, BiWX and OSX all variants of the Xyl7 endo-xylanase released xylobiose, substituted oligomers indicated with 'other' (mainly glucuronic acid substituted oligomers; Fig. A1) and xylotriose (Fig. 1 and Fig. A3-4). In all cases Xyl7dCBM and Xyl7qc behaved similarly, the only exception being the release of more xylose by Xyl7dCBM, indicating a high binding affinity at the subsites (-1) and (+1). The majority of GH11 endo-xylanases contain five subsites from (-2) to (+3) (Paës *et al.*, 2012) and the release of xylose is not often observed for endo-xylanases belonging to GH11 (Ustinov *et al.*, 2008; Pollet *et al.*, 2010). Therefore, this seems to be characteristic for the C1 xylanase Xyl7dCBM. Xyl7qc also released xylose, though to a lower extent than Xyl7dCBM.

Xyl8 released mainly substituted oligomers and xylotriose, and to a lesser extent xylobiose, xylotetraose and xylopentaose. Xyl8 displayed on BiWX, BeWX and OSX a completely different release of components compared to the Xyl7 endo-xylanases. Especially more of the larger oligosaccharides and less of the small oligosaccharides were released by Xyl8. The total amount of products released from these substrates is lower for Xyl8 compared to all Xyl7 variants.

Hydrolysing capacity of the endo-xylanases on commercial xylans

The total hydrolysing capacity (the percentage (w/w) of the total substrate that is degraded by the enzymes to monomers and oligomers) after 24 h per enzyme per substrate is displayed in Fig. 5.2A.

In general, the performance of the GH11 endo-xylanases of C1 increased with decreasing degree of substitution, despite the fact that a less substituted substrate

shows lower solubility. The former corresponds with the performance of GH11 endo-xylanases of *Fusarium graminearum* (Pollet *et al.*, 2009).

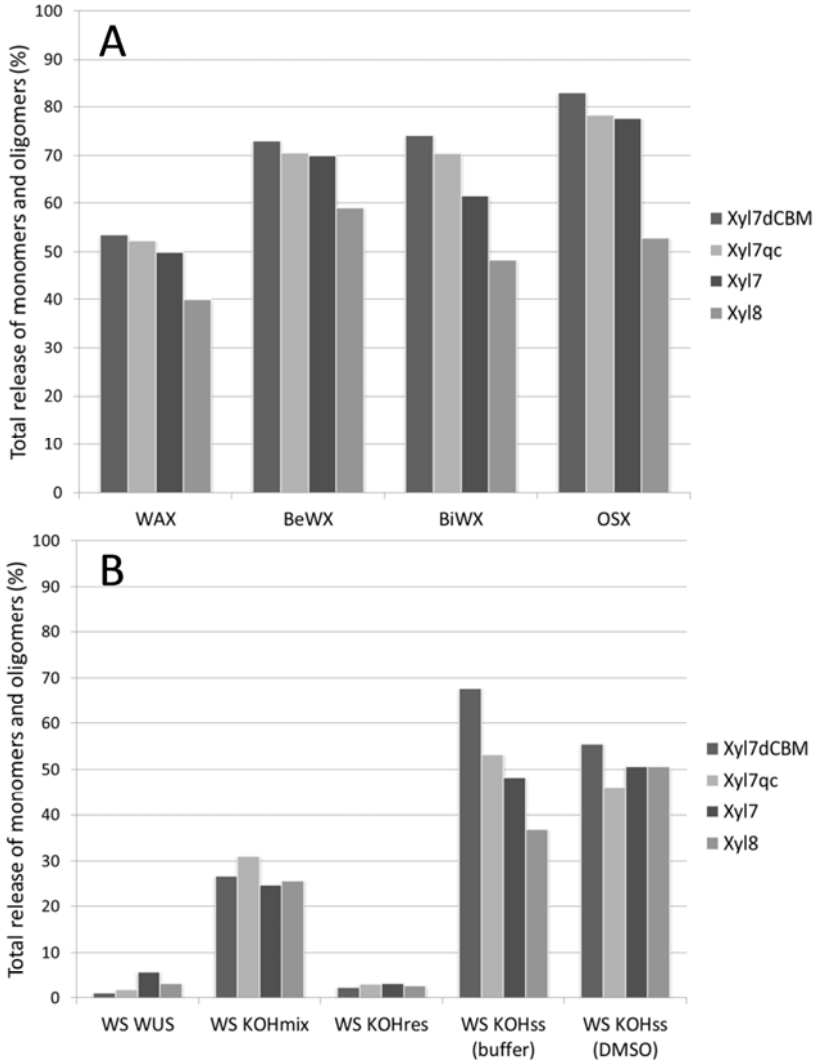

Fig. 5.2: Release (w/w%) of monomers and oligomers from the carbohydrate fraction of wheat arabinoxylan (WAX), beech wood xylan (BeWX), birch wood xylan (BiWX) and oat spelt xylan (OSX) (A) and from the carbohydrate fraction of wheat straw water unextractable solids (WS WUS), wheat straw after alkaline treatment (WS KOHmix), wheat straw alkaline residue (WS KOHres), wheat straw alkaline soluble solids (WS KOHss) suspended in buffer and WS KOHss suspended in 20% DMSO (B), by the C1 endo-xylanases with and without carbohydrate binding module (dCBM) after 24h of incubation based on high performance anion exchange chromatography results.

The total hydrolysing capacity on BeWX and BiWX was higher than that on WAX. The lower degrees of substitution of BeWX and BiWX in comparison to WAX might cause the better performance of the enzymes on these substrates. Also, the presence of negatively charged substituents may enhance the performance of GH11 endo-xylanases, especially when very positively charged areas are found on the surface of a xylanase, which has been suggested for the endo-xylanases of *Trichoderma harzianum* and *Bacillus circulans* (Torronen and Rouvinen, 1997). This could not be confirmed for the C1 xylanases, since Xyl7 contains around 7% positively charged amino acids; Xyl8 around 6%. Furthermore, it was observed visually in the structure model of both enzymes that these residues do not display large charged areas on the surface.

The difference between BeWX and BiWX is the distribution of the glucuronic acid residues. In BeWX they are randomly distributed; in BiWX they are blockwise distributed. This blockwise distribution causes the unsubstituted regions to self-associate, resulting in an aggregated substrate and partial insolubility of the substrate (Hromádková *et al.*, 2005; Ren and Sun, 2010). Xyl7 (with CBM) and Xyl8 were negatively influenced by the blockwise substitution of the glucuronic acid residues in BiWX compared to BeWX, while Xyl7dCBM and Xyl7qc did not exhibit significant differences in performance. OSX, a very linear xylan, also self-associates (Ren and Sun, 2010). Nevertheless, Xyl7 (with CBM) performed very well on OSX. This indicates that Xyl7 is not hindered by self-association of xylan. Therefore, the lower hydrolysing capacity of Xyl7 on BiWX was likely caused by the blockwise distributed glucuronic acid sequences in BiWX. In contrast, Xyl8 performed equally well on OSX and BiWX. This suggests that Xyl8 indeed was influenced by self-association of xylan.

Xyl7dCBM was most efficient in total hydrolysing capacity of all substrates, followed by Xyl7qc, Xyl7 and Xyl8. Xyl8 clearly performed different from the Xyl7 variants, even though they are GH11 endo-xylanases of the same origin. Xyl7dCBM performed similar as the C1 GH11 endo-xylanase Xyl5 (Xyn11C) reported previously (Ustinov *et al.*, 2008). None of the xylanases reported by these authors was comparable to the characteristics found for Xyl8.

5.3.3 Enzyme performance on xylan rich wheat straw fractions

Fig. 5.2B shows that the solubilisation of WS WUS was highest for Xyl7 (6% mono- and oligomers released). Xyl8 released 3% of the total substrate in monomers and oligomers, even though it does not contain a CBM. WS WUS consists of the water unextractable solids of wheat straw and the carbohydrates are still interlinked within the complex cell wall matrix.

The alkaline treatment of the WS WUS increased the amount of monomers and oligomers released by the enzymes up to 20-30% (WS KOHmix). After separation of the xylan fraction, the residue still contained 6% (w/w) of xylose, of which 60% could be solubilized by the GH11 endo-xylanases.

The xylans extracted from WS WUS were partly soluble in buffer and were very well degraded by all Xyl7 variants, where Xyl7dCBM was most efficient and Xyl7 least efficient as indicated by 68% and 48% mono- and oligomers released, respectively. Xyl8 degraded 37% of this extracted xylan fraction to mono- and oligomers. The behaviour of the GH11 endo-xylanases on WS KOHss in buffer resembled that of OSX, which can be ascribed to self-association of the wheat straw xylan and OSX.

By use of DMSO the possible effect of self-association of xylan was ruled out by the exclusion of hydrogen bond formation between the xylan polymers (Ebringerová *et al.*, 1994). Hence, in this solvent the only factors determining the enzyme performance were the degree and type of substitution of the xylan. The enzymes were not influenced by the more a-polar environment, as WAX in 20% (v/v) DMSO incubated with all GH11 xylanases showed similar degradation patterns compared to the buffer condition alone (no further data shown).

The effect of WS KOHss solubilisation in 20% (v/v) DMSO decreased the amount of released monomers and oligomers for Xyl7dCBM and Xyl7qc (decrease of ~10%). Xyl7 performed equally well in the more a-polar environment, whereas Xyl8 benefits from xylan solubilisation in DMSO.

From the results on all substrates, it was concluded that Xyl7 (with CBM) was not hindered by self-association of xylans and is only hindered by the type and degree of

substitution of a xylan. Xyl7dCBM was very efficient in the degradation of self-associated xylan. Xyl8 displayed a limited activity when xylans were self-associated and formed insoluble aggregates. In addition it was concluded that the CBM was not beneficial for the activity of Xyl7 on insoluble substrates.

5.3.4 Differences in enzyme behaviour correlate with protein structure

Alignment with heterologous GH11 endo-xylanases

In order to relate the differences in enzyme performance of the C1 GH11 endo-xylanases with their protein structures, the alignments of Xyl7 and Xyl8 with heterologous GH11 endo-xylanases are indicated in Fig. 5.3. These alignments show the conserved catalytic residues (black residues) and the residues involved in binding of the substrate (grey residues). Based on these alignments, Xyl7 and Xyl8 showed homology (40-60%) to heterologous GH11 endo-xylanases from *Hypocrea jecorina, Aspergillus niger, Penicillium funicolosum and Chaetomium thermophilum*, with respect to residues involved in substrate binding and the structure of the catalytic cleft (Hakulinen *et al.*, 2003). Compared to the other xylanases, Xyl8 had additional amino acids at the N-terminal part of the protein sequence, but these residues did not seem to be involved in catalysis neither were they recognized as a CBM (indicated with x in Fig. 5.3).

Construction of model structures of both C1 GH11 endo-xylanases showed that these enzymes were very similar (Fig. 5.4), which corresponds with the observation that many residues and residue clusters are conserved in GH11 endo-xylanases (Paës *et al.*, 2012).

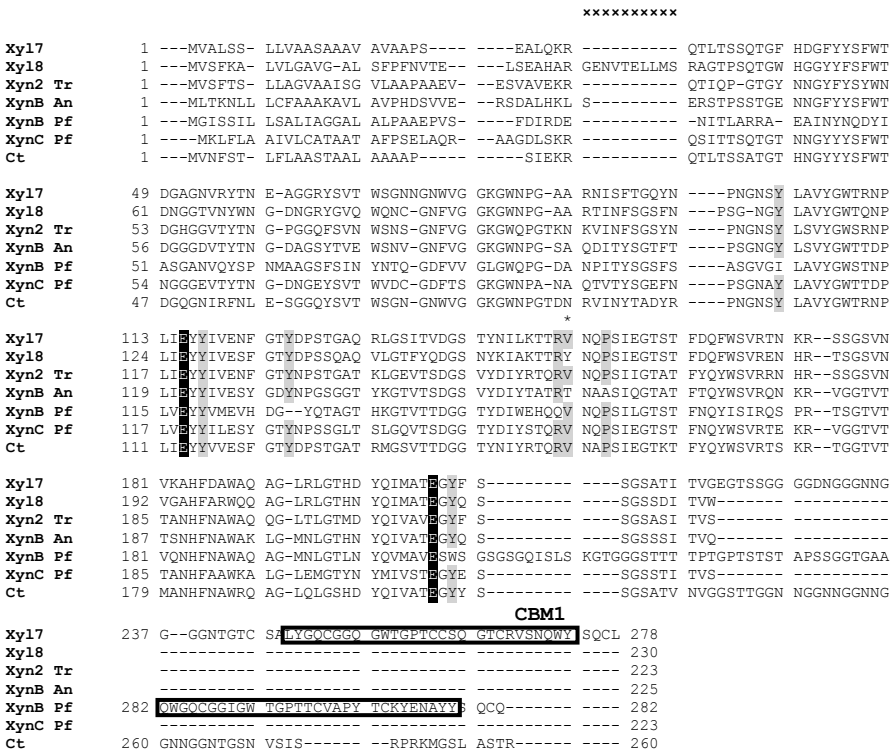

*Fig. 5.3: Alignment of C1 GH11 endo-xylanases with different heterologous GH11 endo-xylanases. The black shaded residues are the catalytic residues, the grey shaded residues are involved in binding of the substrate and the residues in the box are the CBM1residues in the enzymes (based on Hakulinen et al (2003). Xyn2 Tr: Xylanase Xyn2 from Hypocrea jecorina, Genbank accession nr. AAB29346; XynB An: Xylanase XynB from Aspergillus niger, Genbank accession nr. AAM95167; XynB Pf: Xylanase XynB from Penicillium funicolosum, Genbank accession nr. CAD33900; XynC Pf: Xylanase XynC from Penicillium funicolosum, Genbank accession nr. CAC15487; Ct: xylanase Xyn11A from Chaetomium thermophilum, Genbank accession nr. CAD48749; × extra amino acids in N-terminal part of Xyl8, * Tyr163 in Xyl8 and Val152 in Xyl7.*

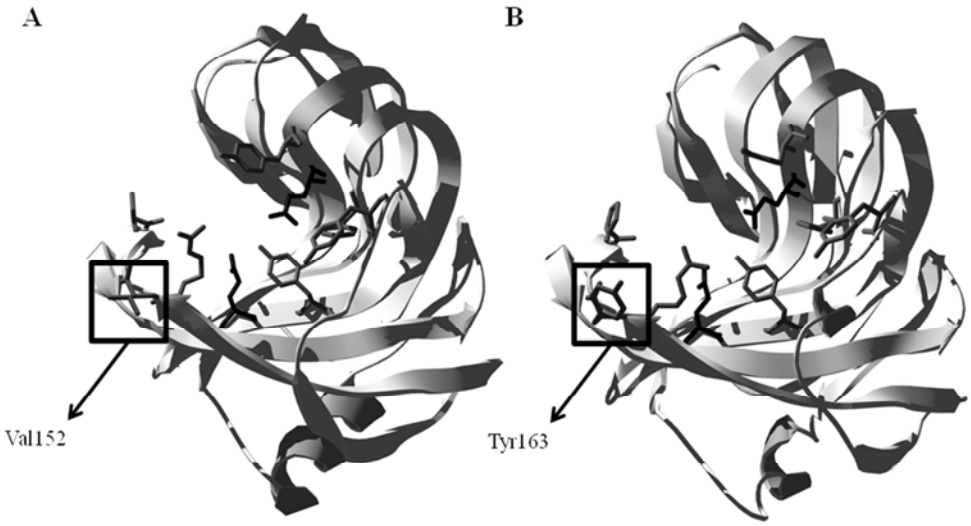

Fig. 5.4: Model structure of the catalytic domains of Xyl7 (A) and Xyl8 (B). The model of Xyl7 is obtained using xylanase Xyn11A from Chaetomium thermophilum (Genbank accession nr. CAD48749) as template. The model of Xyl8 is obtained using xylanase XynC from Penicillium funicolosum (Genbank accession nr. CAC15487) as template. The catalytic residues are indicated in black and the residues involved with binding are indicated in grey (based on Hakulinen et al. (2003)).

Difference in one amino acid responsible for different enzyme behaviour?

One of the differences between the amino acid sequence of Xyl7 and Xyl8, was residue Tyr163 in Xyl8, which was a valine in Xyl7 (Val152), indicated with * in the alignment in Fig. 5.3. It has recently been reported that the Tyr163 is variable in 68% of the GH11 endo-xylanases and that it is involved in the stacking interaction at the non-reducing subsite position (-2) (Paës *et al.*, 2012). It was hypothesized that the difference in subsite (-2) caused flexibility of this region, thereby becoming more tolerant towards substituents on the xylan backbone nearby the cleavage site of the enzyme; or by a change in relative binding affinity at the other subsites due to a larger distance to this (-2) subsite. This flexibility in the catalytic cleft can also explain the behaviour found for other GH11 xylanases reported previously (Ustinov *et al.*, 2008; Cuyvers *et al.*, 2011a). The presence of a valine instead of tyrosine is also found for Xyl5 (Xyn11C) which displayed, after glucuronoxylan and arabinoxylan digestion, a product composition closer to the GH10 family enzymes (Ustinov *et al.*, 2008). The

other two C1 GH11 xylanases reported by these authors, did not display GH10-like behaviour and it turned out that both contain a tyrosine at position 163 (Ustinov *et al.*, 2008). For GH11 endo-xylanases of *Trichoderma longibrachiatum*, *Aspergillus niger* and *Trichoderma viride* release of xylose (Cuyvers *et al.*, 2011a) can be linked to the valine on this position, whereas the lack of xylose release for *Bacillus subtilis* (Cuyvers *et al.*, 2011a) was correlated to the tyrosine at this position. The only exception in this case is the high pI-endo-xylanase of *Trichoderma longibrachiatum*, which released only minor amounts of xylose (Cuyvers *et al.*, 2011a), but does contain a valine. Endo-xylanases of *Penicillium funiculosum* both contain a valine (* in Fig. 3), and showed also xylose release (Berrin *et al.*, 2007). However, the different performance of this xylanase compared to other GH11 endo-xylanases has been attributed to different amino acid residues in the loop forming "thumb" (André-Leroux *et al.*, 2008) and was not correlated to the valine.

The structural difference and sequence variation of the tyrosine at position 163 seems to be an explanation for the different hydrolytic performance towards various xylans and seems to be especially related to xylose release by the GH11 endo-xylanases.

5.3.5 Interfamily differences for GH10 and GH11 endo-xylanases from *Myceliophthora thermophila* C1

Different action on soluble substrates

Both GH11 (this study) and GH10 (Van Gool *et al.*, 2012) endo-xylanases of *M. thermophila* C1 display intrafamily differences. Next to these intrafamily differences, interfamily differences were evaluated. Table 5.2 displays a schematical representation of the factors that were studied. It shows that the family division is clear when comparing the performance of the enzymes towards random versus blockwise distributed 4-*O*-methyl glucuronic acid substituted xylan. Furthermore, limited performance due to xylan self-association is only seen for GH10 enzymes.

Table 5.2: Performance of the GH10 and GH11 C1 endo-xylanases as influenced by various substrate characteristics. GH10 xylanases: Xyl1, Xyl1 without carbohydrate binding module (Xyl1dCBM), Xyl3 and Xyl3 without carbohydrate binding module (Xyl3dCBM). GH11 xylanases: Xyl7, a processed variant of Xyl7 (Xyl7qc), Xyl7 without carbohydrate binding module (Xyl7dCBM) and Xyl8, naturally without binding module.

Family	Enzyme	Lower degree of substitution	Randomisation of glucuronic acid substituents	Alkaline treatment of wheat straw	Removal of cellulose	Prevention of xylan self-association
GH10	Xyl1dCBM	**0**	**+**	**++**	**++**	**++**
	Xyl1	**-**	**+**	**++**	**-**	**++**
	Xyl3dCBM	**++**	**+**	**++**	**-**	**++**
	Xyl3	**+**	**+**	**++**	**++**	**+**
GH11	Xyl7dCBM	**++**	**0**	**++**	**++**	**-**
	Xyl7qc	**++**	**0**	**++**	**++**	**-**
	Xyl7	**+**	**-**	**++**	**++**	**0**
	Xyl8	**+**	**-**	**++**	**+**	**+**

-: Low efficiency
0: No effect
+: High efficiency
++: Very high efficiency

The amount of small degradation products from the various substrates by all C1 GH10 and GH11 xylanases was taken from the HPAEC results and plotted for each substrate (supplementary Fig. A5). The GH10 xylanases produced more small degradation products from WAX, such as xylose, xylobiose and xylotriose than the GH11 endo-xylanases. Towards glucuronic acid substituted xylan (BeWX and BiWX), the GH11 endo-xylanases produced more small degradation products (Fig. A5B-C). Towards more linear xylan, the GH11 endo-xylanases produced more small degradation products than the GH10 endo-xylanases, including xylose up to xylotetraose and substituted oligomers. Especially Xyl7dCBM (GH11) was very efficient in the release of small products, including xylose, which was in some cases even higher than the release of xylose by the GH10 enzymes.

The general assumption that GH10 endo-xylanases hydrolyse xylans to a higher degree than GH11 endo-xylanases (Biely *et al.*, 1997; Ustinov *et al.*, 2008; Dumon *et al.*, 2012), is in this study only valid for the degradation products from WAX.

Different action on insoluble substrates

Towards the various insoluble fractions of wheat straw, the GH11 endo-xylanases were as efficient as the GH10 endo-xylanases (Supplementary Fig. A6). In general, the better performance of GH11 endo-xylanases on insoluble substrate was assumed to be caused by the size of the xylanases: they are smaller compared to GH10 endo-xylanases, causing easier penetration into the cell wall (Torronen and Rouvinen, 1997; Beaugrand *et al.*, 2004).

The degree of substitution was more limiting for the C1 GH11 xylanases than the solubility of the substrate was. In contrast, the performance of C1 GH10 enzymes was negatively influenced by self-association of the xylan and the degree of substitution played a minor role (Table 5.2).

For the endo-xylanases of *M. thermophila* C1 studied, the CBM1 did not enhance the degradation of soluble or insoluble material (this study; Van Gool *et al.* (2012)). The CBM1 did enhance the solubilisation of insoluble material, i.e. xylan embedded in cell wall matrix for a GH10 xylanase of *Aspergillus aculeatus* (Cuyvers *et al.*, 2011a). However, it was reported that other endo-xylanases of GH10 with CBM were low in the solubilisation of insoluble substrate (Cuyvers *et al.*, 2011b). No correlation was found for the performance of the xylanases and the N- or C-terminal position of their CBM.

Relation between protein structure and the different performance of the C1 xylanases

Minor differences in the predicted protein structure of the GH11 xylanases already resulted in major differences in enzyme performance towards the various substrates, even though their 'hand-shaped' architecture, topology and catalytic cleft residues were conserved (Paës *et al.*, 2012). For C1 GH10 xylanases major differences in

predicted protein structure and folding resulted in differences in enzyme performance towards various soluble and insoluble substrates (Van Gool *et al.*, 2012).

5.4 Conclusions

Type and degree of substitution of xylan as well as self-association of linear xylans displayed intrafamily differences within two GH11 endo-xylanases of *Myceliophthora thermophila* C1. In the amino acid sequence of the enzymes a valine instead of tyrosine at position 163, seemed an explanation for the differences in xylanase performance found, especially concerning xylose release. A carbohydrate binding module did not enhance xylan degradation. It can be concluded that the family division for endo-xylanases does not provide information on degradation products and that a detailed characterisation and protein structure elucidation is necessary to display intra- and interfamily differences.

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Supplementary data

Table A1: Constituent monosaccharide composition (w/w%) of the substrates used for xylanase characterisation.

Sample name[a]	Sugar composition (w/w%)						Total sugar content (w/w%)
	Ara	Xyl	Man	Gal	Glc	UA	
WAX	32	58	0	0	0	1	91
BeWX	1	68	0	0	1	9	79
BiWX	0	69	0	0	1	9	81
OSX	7	61	0	2	5	2	76
WS WUS[b]	2	23	3	1	40	1	70
WS KOHss	5	46	1	1	3	1	56
WS KOHres	1	6	7	0	54	0	69
WS KOHmix	1	20	5	0	36	0	64

[a] Wheat arabinoxylan (WAX), beech wood xylan (BeWX), birch wood xylan (BiWX), oat spelt xylan (OSX), wheat straw water unextractable solids (WS WUS), wheat straw alkaline soluble solids (WS KOHss), wheat straw alkaline residue (WS KOHres), wheat straw after alkaline treatment (WS KOHmix)
Ara: arabinose, Xyl: xylose, Man: mannose, Gal: galactose, Glc: glucose, UA: uronic acid
[b] Only WS WUS contains 2% (w/w) Ac

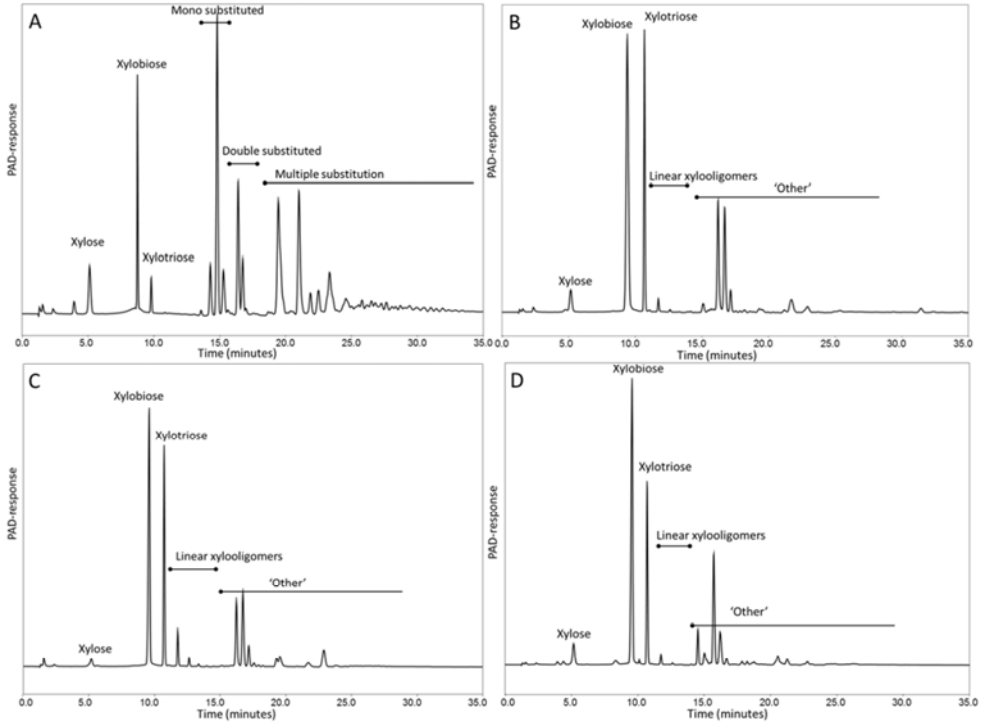

Fig. A1: Typical high performance anion exchange chromatography (HPAEC) patterns for wheat arabinoxylan (WAX; A), beech wood xylan (BeWX; B), birch wood xylan (BiWX; C) and oat spelt xylan (OSX; D), digested with the endo-xylanase 7 (Xyl7).

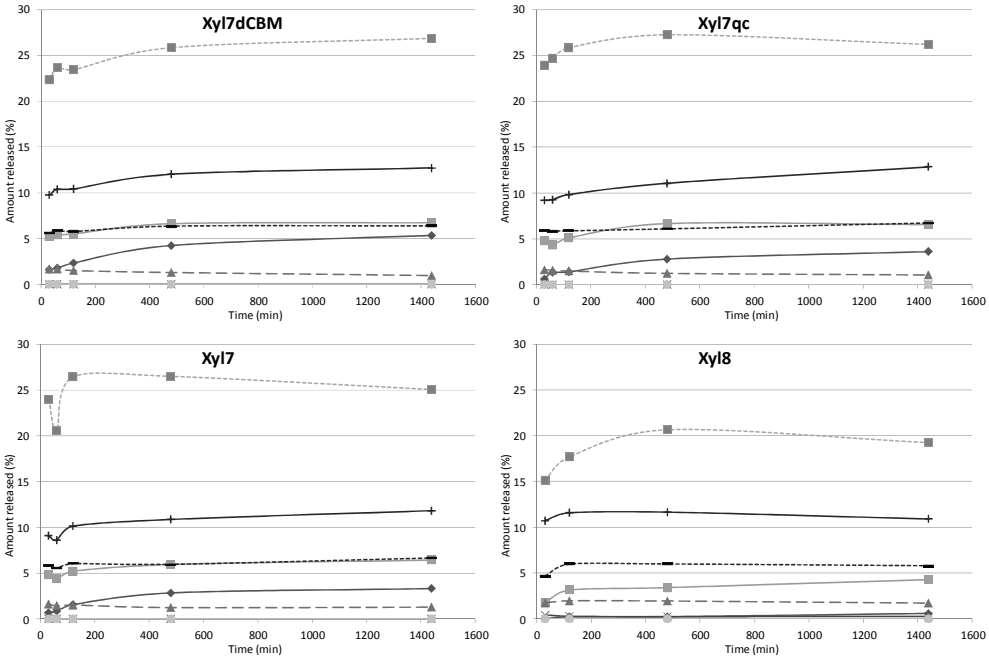

Fig. A2: Release of xylose and substituted oligomers after wheat arabinoxylan (WAX) digestion by different C1 GH10 xylanases. The amount of xylose components is displayed as weight percentage of the total amount of xylose. The amount of substituted oligomers is displayed as weight percentage of the total amount of carbohydrates in WAX. Xylose; Xylobiose; Xylotriose; Xylotetraose; Xylopentaose; Xylohexaose; Mono substituted oligomers; Double substituted oligomers; Multiple substituted oligomers.

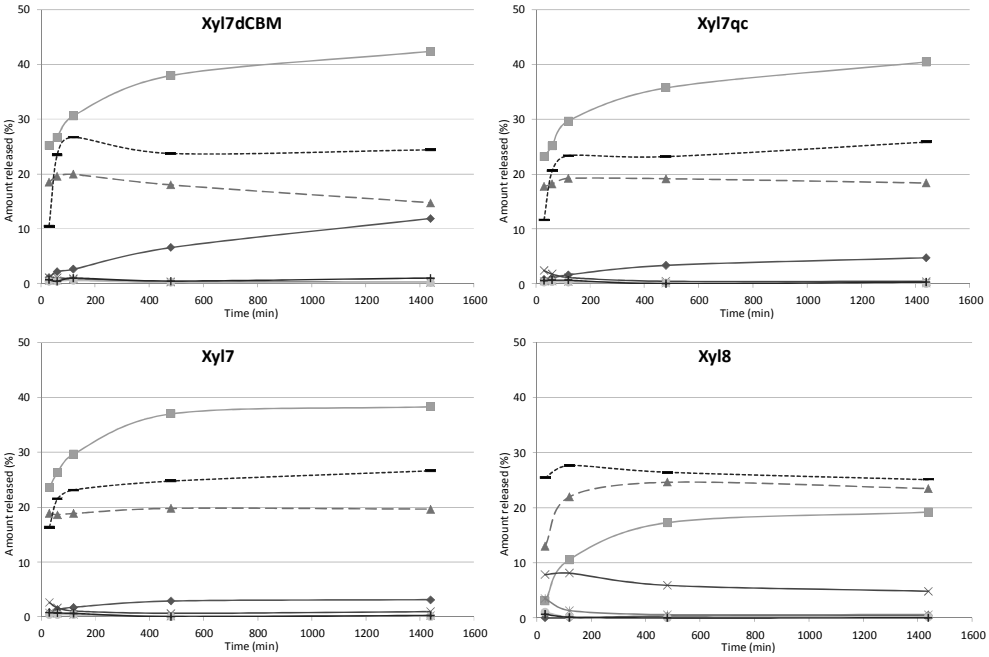

Fig. A3: Release of xylose and oligomers after beech wood xylan (BeWX) digestion by different C1 GH11 endo-xylanases. The amount of xylose components is displayed as weight percentage of the total amount of xylose. The amount of substituted oligomers is displayed as weight percentage of the total amount of carbohydrates in BeWX. Xylose; Xylobiose; Xylotriose; Xylotetraose; Xylopentaose; Xylohexaose; Linear xylooligomers DP>6; Other.

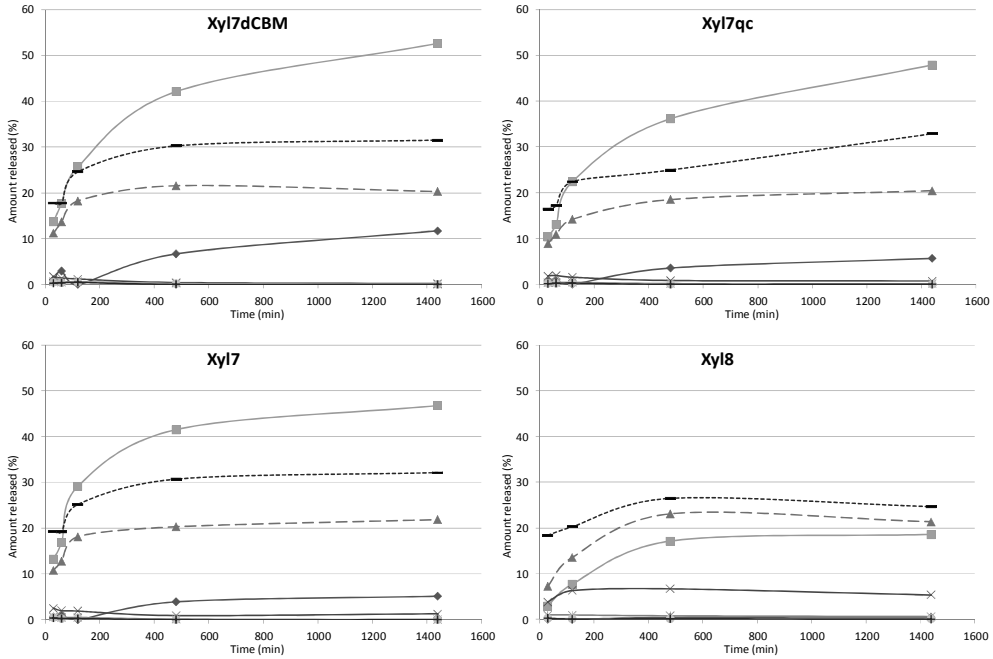

Fig. A4: Release of xylose and oligomers after oat spelt xylan (OSX) digestion by different C1 GH11 endo-xylanases. The amount of xylose components is displayed as weight percentage of the total amount of xylose. The amount of substituted oligomers is displayed as weight percentage of the total amount of carbohydrates in OSX. Xylose; Xylobiose; Xylotriose; Xylotetraose; Xylopentaose; Xylohexaose; Linear xylooligomers DP>6; Other.

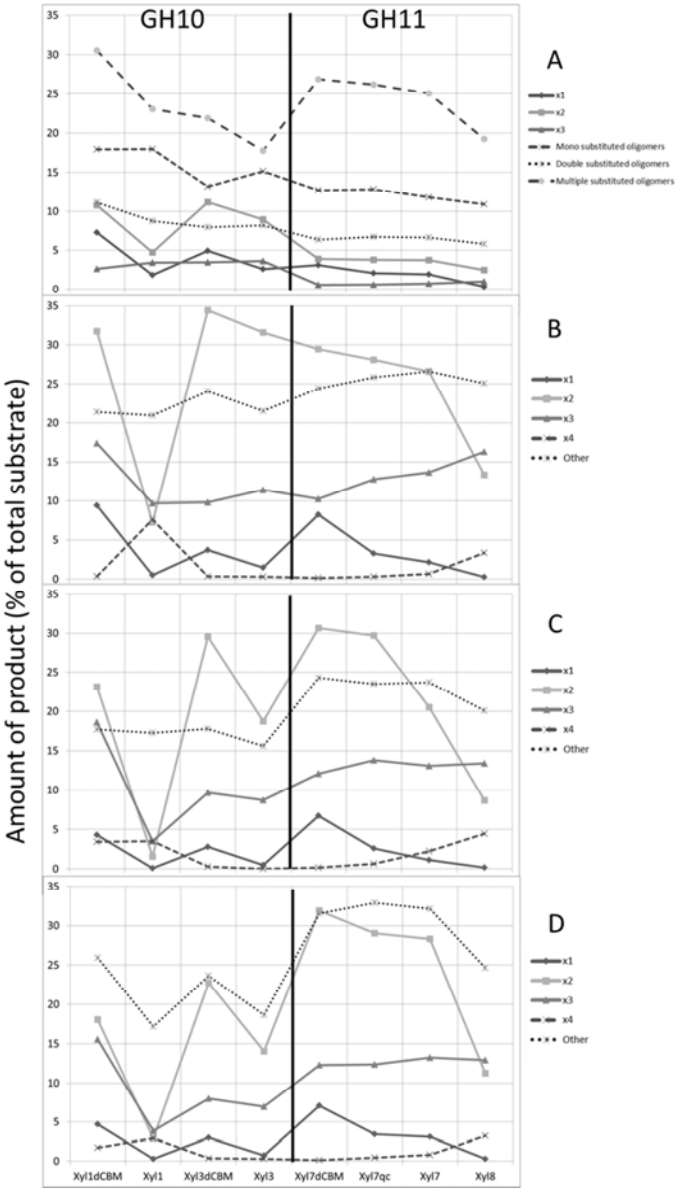

Fig. A5: Small degradation products released by the endo-xylanases relative to the total amount of substrate A: wheat arabinoxylan (WAX), B: beech wood xylan (BeWX), C: birch wood xylan (BiWX) and D: oat spelt xylan (OSX). X1: Xylose, X2: Xylobiose, X3: Xylotriose, X4: Xylotetraose. For WAX different arabinose substituted oligomers are indicated (mono, double and multiple substitution according to (Van Gool et al. 2011)). For BeWX, BiWX and OSX the substituted oligomers are indicated with 'Other'. The graph displays the GH10 endo-xylanases on the left and the GH11 endo-xylanases on the right side.

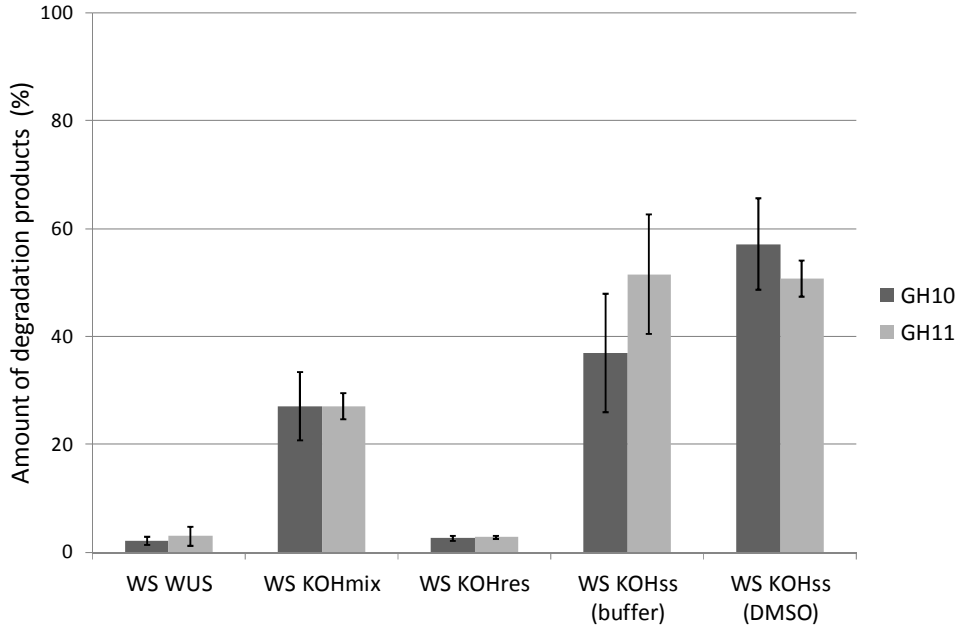

Fig. A6: Total amount of degradation products released (mean value ± standard deviation) by C1 GH10 endo-xylanases or C1 GH11 endo-xylanases from the various wheat straw fractions: water unextractable solids (WS WUS), wheat straw after alkaline treatment (WS KOHmix), residue after alkaline treatment (WS KOHres), alkaline soluble solids suspended in buffer (WS KOHss buffer), alkaline soluble solids suspended in 20% DMSO in buffer (WS KOHss DMSO).

Chapter 6

Concerted action of acetyl (xylan) esterases and α–glucuronidases on Eucalyptus xylan oligosaccharides

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Part of this chapter to be submitted for publication

Abstract

The enzymatic removal of both acetyl- (Ac) and 4-*O*-methyl glucuronic acid (MeGlcA) substituents from *Eucalyptus* xylan oligosaccharides requires the action of acetyl xylan esterases (AXEs; specific in deacetylating xylose residues), acetyl esterases (AEs; non-specific deacetylation) and glucuronidases. They are distributed over different carbohydrate esterase (CE) or glycoside hydrolase (GH) families, where each family contains a specific action towards acetylated substrate. So far, the family dependent specificity was mainly studied with artificial substrates. In addition, the complementary action of esterases from different families and the synergistic action with glucuronidases towards natural substrates is largely unknown.

In this study the action of acetyl (xylan) esterases from family CE1, 5 and CE16 either individually or AXEs in combination with AE was tested using a neutral and an acidic fraction of acetylated xylooligosaccharides from *Eucalyptus*. Furthermore, their synergistic action with a GH115 and GH67 glucuronidase was studied using the acidic fraction.

The individual esterases could deacetylate 35-90% of neutral acetylated xylooligosaccharides, with CE1 AXEs being most efficient. The combined action of CE16 AE with the CE1 and CE5 AXEs resulted in all cases in 80-90% deacetylation. Deacetylation of the acidic fraction (30-60%) was hindered by MeGlcA substituents on the xylooligosaccharides. Synergy was observed when the AXEs were combined with the GH115 glucuronidase. The action of the GH67 glucuronidase was restricted by Ac substitution, presumably on the same xylose residue as the MeGlcA. The double MeGlcA substituted oligosaccharide was also accumulating in the GH67 digests.

Complete deacetylation was difficult to achieve by the concerted action of the enzymes. The study showed that testing the action of acetyl (xylan) esterases from different families towards artificial substrates is not sufficient to predict their action towards natural substrate. Analysis of released acetic acid in combination with MALDI-TOF mass spectrometry was a powerful combination to reveal the action of the enzymes.

6.1 Introduction

Hard wood xylans, like *Eucalyptus* xylans, are highly substituted with acetyl residues (Ac) and α-(1→2)-linked D-glucopyranosyl uronic acid and/or its 4-*O*-methyl ether (MeGlcA) (Togashi *et al.*, 2009; Pouvreau *et al.*, 2011). In addition, the MeGlcA substituent may be substituted at *O*-2 with α-D-galactose (Shatalov *et al.*, 1999). Acetylation of xylose residues restricts the action of endo-xylanases, resulting in long oligosaccharides and decreased turnover to monosaccharides (Biely *et al.*, 1986; Kormelink *et al.*, 1993; Pouvreau *et al.*, 2011). Enzymatic removal of the acetyl residues from xylan can be obtained by esterases which cleave the ester bonds between the acetyl and the xylopyranosyl (Xyl*p*) residue. Two types of these esterases have been described: acetyl esterases (AE; E.C. 3.1.1.6) are non-specific and in the case of xylan remove Ac groups preferably from the non-reducing end of short xylooligosaccharides (XOS) (Poutanen *et al.*, 1990; Mukhopadhyay *et al.*, 1997), and acetyl xylan esterases (AXE; E.C. 3.1.1.72) specifically deacetylate xylose residues in both polymeric xylan and XOS (Poutanen *et al.*, 1990; Pouvreau *et al.*, 2011). The xylan deacetylating enzymes are found in carbohydrate esterase (CE) families 1-7, 12 and 16 (Henrissat *et al.*, 2012). AXE2 (CE5) and AXE3 (CE1) of *Myceliophthora thermophila* C1, previously denoted as *Chrysosporium lucknowense* C1 (Visser *et al.*, 2011), are assumed to remove acetyl groups from both positions *O*-2 and *O*-3 (Pouvreau *et al.*, 2011). AXEs from *Trichoderma. reesei* (TrAXE; CE5)*, Schizophyllum commune* (CE1)*, Streptomyces lividans* (CE4) and *Penicillium purpurogenum* (CE1 and CE5) strongly prefer deacetylation of the *O*-2 position. The AE of *Trichoderma reesei* (TrAE; CE16) prefers removal of acetyl groups from the *O*-3 or *O*-4 position (Li *et al.*, 2008; Biely *et al.*, 2011). This suggests a complementary action of the different esterases.

In addition, removal of the MeGlcA substituents is necessary to obtain a complete degradation of hard wood xylans. α-Glucuronidases (EC 3.2.1.139) hydrolyse the α-1,2-linkage between MeGlcA and xylose. Currently known, the α-glucuronidases are found in glycoside hydrolase (GH) families 67 or 115 (Henrissat *et al.*, 2012). GH67

enzymes only cleave glucuronosyl linkages from the Xyl*p* unit present at the non-reducing end of xylooligosaccharides; GH115 enzymes remove glucuronic acid also from polymeric glucuronoxylan from both the non-reducing terminal Xyl*p* as well as from internal residues (Tenkanen and Siika-Aho, 2000; Ryabova *et al.*, 2009; Kolenová *et al.*, 2010).

Specificity of AXEs and AEs have often been studied with model substrates, such as acetylated 4-*O*-nitrophenyl-β-D-xylosides or methyl-β-D-xylosides, or α-naphtyl acetate or 4-methyl umbelliferyl acetate (Sundberg and Poutanen, 1991; Biely *et al.*, 1996; Biely, 2012), although some studies used natural substrates (Tenkanen, 1998; Pouvreau *et al.*, 2011). In this study we tested the role and specificity of acetyl (xylan) esterases and α-glucuronidases in the enzymatic hydrolysis of both the neutral and acetylated acidic xylooligosaccharides from *Eucalyptus globulus* wood. The combined action of the various polymer acting AXEs of CE1 and CE5 with oligomer-acting AE of CE16 was tested. In addition, their synergistic action with a GH115 α-glucuronidase from *Schizophyllum commune* and a GH67 α-glucuronidase from *Myceliophthora thermophila* C1 was studied.

6.2 Materials and methods

6.2.1 Acetylated xylooligosaccharides

Eucalyptus globulus xylan hydrolysate obtained from hydrothermally treated *Eucalyptus* wood, of which the major part consists of acetylated (acidic) xylooligosaccharides, was kindly donated by Prof. Dr. J.C. Parajo of the University of Vigo-Ourense, Spain (Gullón *et al.*, 2008). The carbohydrate composition of the substrate was determined by methanolysis (Sundberg *et al.*, 1996; Table 6.1). The acetic acid content was determined after saponification: sodium hydroxide (NaOH) was added to a final concentration of 0.1 M, samples were incubated overnight and neutralised to pH 5-7 with hydrochloric acid (HCl) before analysis using the K-ACET kit (Megazyme, Wicklow, Ireland).

6.2.2 Production of neutral and acidic fraction of xylooligosaccharides

The production of oligosaccharides from the *Eucalyptus* hydrolysate was achieved with Shearzyme 500L (GH10 xylanase; 10,000 nkat/g XOS) (Novozymes, Bagsvaerd, Denmark) in 50 mM sodium citrate buffer pH 5.0 at 40 °C for 24 h. The enzymes were inactivated by boiling the samples for 10 min. The sample was fractionated using a Dowex 1x2 anion exchange column (2.5 x 16 cm, 100-200 mesh; Sigma-Aldrich, St Louis, MO, USA) regenerated with 2 M ammonium formate pH 6.0 and equilibrated with water. The unbound linear acetylated XOS (AcXOS) were eluted with water and concentrated by evaporation. The 4-*O*-methyl glucuronic acid substituted acetylated XOS (AcUXOS) were eluted with 300 mM ammonium formate pH 6.0. Oligosaccharide-containing fractions were repeatedly washed with water and evaporated to dryness. Finally, the oligosaccharides were dissolved in water.

Table 6.1: Constituent monosaccharide composition (w/w%) and acetic acid content (w/w%) of Eucalyptus xylan hydrolysate.

	Sugar composition (w/w%)[a]								Total sugar content (w/w%)
	Rha	Ara	Xyl	Man	Gal	Glc	MeGlcA	Ac	
Eucalyptus hydrolysate	n.d.	<2	63	<2	4	4	4	13	76

[a] Neutral sugars: arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glc); 4-*O*-methyl glucuronic acid (MeGlcA) and acetic acid (Ac) expressed as weight percentage
n.d. not detected; <2 traces, too low to be quantified

6.2.3 Deacetylating and deglucuronosylating enzymes

T. reesei AE (used at 4 mg/g XOS) and AXE (4 mg/g XOS), *M. thermophila* AXE2 and AXE3 (4 mg/g XOS), *S. commune* AGU (3 mg/g XOS) and *M. thermophila* AGU (3 mg/g XOS) were used in the study. Their isolation and purification is described in the references displayed in Table 6.2. Furthermore, their classification and used abbreviations are given (Table 6.2).

Table 6.2: Characteristics of the enzymes used in this study, including abbreviation, CAZy family and references.

Activity	*Abbreviation*	*Origin*	*CAZy family*	*Reference*
Acetyl esterase (AE)	TrAE	*Trichoderma reesei*	CE16	(Poutanen and Sundberg, 1988; Poutanen *et al.*, 1990)
Acetyl xylan esterase (AXE)	TrAXE	*Trichoderma reesei*	CE5	(Sundberg and Poutanen, 1991)
	C1 AXE2	*Myceliophthora thermophila* C1	CE5	(Pouvreau *et al.*, 2011)
	C1 AXE3	*Myceliophthora thermophila* C1	CE1	(Pouvreau *et al.*, 2011)
	AnAXE	*Aspergillus niger*	CE1	(Kormelink *et al.*, 1993)
α-Glucuronidase	ScGLUR	*Schizophyllum commune*	GH115	(Tenkanen and Siika-Aho, 2000)
	C1 AGU1	*Myceliophthora thermophila* C1	GH67	(Hinz *et al.*, 2009)

6.2.4 Enzyme incubations

Different combinations of the enzymes were added to 2 mg/mL AcXOS or AcUXOS in 35 mM sodium citrate buffer pH 5.5. Incubation was performed at 40 °C for 24 h. The enzymes were inactivated by boiling the samples for 3 min. Released acetic acid was measured with the K-ACET kit (Megazyme, Wicklow, Ireland).

6.2.5 MALDI-TOF MS analysis

Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed as described previously (Van Gool *et al.*, 2011) for both the untreated AcXOS and AcUXOS as well as all digests.

6.3 Results and discussion

6.3.1 Characterisation of the substrates

Endo-xylanase hydrolysis of *Eucalyptus* acetylated xylooligosaccharides (XOS) and subsequent separation with anion exchange chromatography produced two fractions: Linear acetylated xylooligosaccharides (AcXOS) and acetylated 4-*O*-methyl glucuronic acid (MeGlcA)-substituted xylooligosaccharides (AcUXOS). MALDI-TOF MS showed that next to acetylated pentose oligosaccharides also small amounts of acetylated hexose oligosaccharides were present in AcXOS (data not shown). Based on the constituent monosaccharide composition of the starting material it is clear that the pentose series reflect xylooligosaccharides. The hexose series are assumed to reflect glucose oligosaccharides potentially derived from glucomannan, as the galactose is known to be a substituent of the MeGlcA (Shatalov *et al.*, 1999) and only traces of other hexoses were found after methanolysis (Table 6.1). Acetylated glucose units have been found in pine wood glucomannan, and only contain acetyl groups at position *O*-3 (Tenkanen *et al.*, 1993). The xylooligosaccharides consisted of oligomers with a degree of polymerization (DP) ranging from 1-8 carrying 0 to 7 acetyl groups. The main oligosaccharides in AcUXOS fraction had a DP 2-8, substituted with one or two MeGlcA and 0 to 7 Ac groups. The total acetic acid content in AcXOS was 11.4% and in AcUXOS 19.8%.

6.3.2 Enzymatic deacetylation of neutral acetylated xylooligosaccharides

Acetic acid released

After incubation of the neutral acetylated xylooligosaccharides with the acetyl (xylan) esterases, the efficiency of the enzymes was established by measuring the liberated acetic acid. Fig. 6.1 shows the release of acetic acid from the linear AcXOS by the acetyl (xylan) esterases alone, and by a combination of the *Trichderma reesei* acetyl esterase (TrAE) with each of the acetyl xylan esterases from *Myceliophthora thermophila* C1 (C1 AXE2 and C1 AXE3), *Trichoderma reesei* (TrAXE) and *Aspergillus niger* (AnAXE).

TrAE alone is able to release 60% of the acetic acid from the AcXOS. The various acetyl xylan esterases differ in the amounts of released acetic acid from linear AcXOS. TrAXE displayed a very efficient removal of acetyl groups by almost 80%. The C1 AXE3 and AnAXE also released 80-90% of the acetic acid. The latter ones belong to CE family 1, which are reported to be fast in deacetylation and they are able to release acetyl groups from both position *O*-2 and *O*-3 (Altaner *et al.*, 2003). TrAXE belongs to CE family 5, which is known to release acetic acid only from position *O*-2 (Altaner *et al.*, 2003). C1 AXE2 released only 40% of the total acetic acid present in AcXOS, In this study we used pH 5.5 to enable comparison of all enzymes, however a higher activity at the pH optimum has been reported, being at pH 7.0 (Pouvreau *et al.*, 2011). The performance of C1 AXE2 was strongly enhanced by the addition of TrAE. TrAE is known to remove Ac only from position *O*-3 or *O*-4 of the xylopyranosyl residue of artificial substrates (Biely *et al.*, 2011), indicating that AXE2 especially removed the acetyl group from position *O*-2 under the conditions used. The enzyme belongs to CE5, reported previously for being specific in the release of acetic acid only from position *O*-2 (Altaner *et al.*, 2003). However, previous research reported deacetylation of xylooligosaccharides by AXE2 of both position *O*-2 and *O*-3 (Pouvreau *et al.*, 2011). TrAXE of CE5 is also known to prefer deacetylation of the *O*-2 position, but displays high release of acetic acid (Biely *et al.*, 2011). This indicates that the restricted action of C1 AXE2 is not caused by the position of the acetyl groups. It is assumed that migration has occurred, which is known to take place upon long incubation of acetylated samples (Mitchell *et al.*, 1990; Mastihubová and Biely, 2004; Biely *et al.*, 2011). In all cases the combination of TrAE and AXE resulted in 80-90% acetic acid released. The lower the individual AXE performance, the greater was the improvement in combination with TrAE.

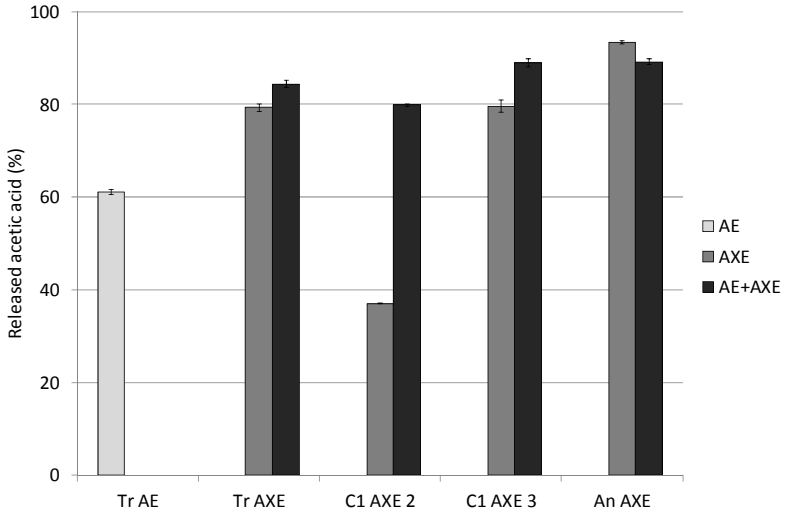

Fig. 6.1: Acetic acid release from AcXOS after treatment with the acetyl esterase (AE), acetyl xylan esterases (AXE) or a combination of the two (AE + AXE). TrAE: Trichoderma reesei acetyl esterase, TrAXE: Trichoderma reesei acetyl xylan esterase, C1 AXE 2: Myceliophthora thermophila C1 acetyl xylan esterase 2, C1 AXE 3: Myceliophthora thermophila C1 acetyl xylan esterase 3, An AXE: Aspergillus niger acetyl xylan esterase.

Neutral xylooligosaccharides resistant to enzymatic deacetylation

The composition of oligosaccharides remaining after deacetylation by the various esterases was analysed by MALDI-TOF MS. Fig. 6.2 displays as example the mass spectrum of AcXOS treated with TrAE. It displays a series of acetylated pentose oligosaccharides in the range of DP3-6 and hexose oligosaccharides of DP2-5. They contained up to four acetyl groups per oligosaccharide. Xylotriose (P_3), mono acetylated xylotriose (P_3Ac) and double acetylated xylotetraose (P_4Ac_2) were present predominantly. Simple monomers and dimers are not visible using MALDI-TOF MS, due to the detection limit of 300 Da.

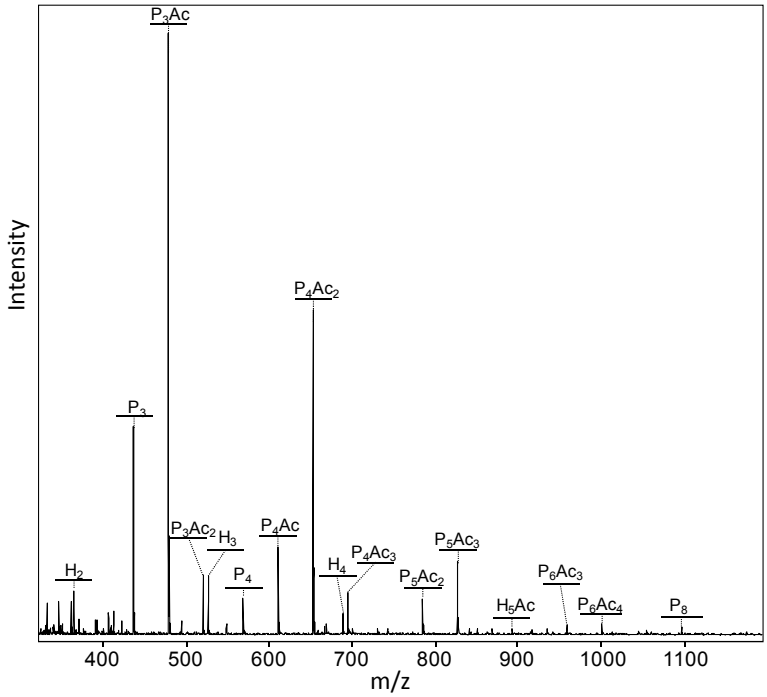

Fig. 6.2: Matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrum of the end-point products from neutral acetylated xylooligosaccharides from Eucalyptus treated with carbohydrate esterase family 16 acetyl esterase of Trichoderma reesei. H: hexose, P: pentose, Ac: acetyl group.

For each of the individual and combined acetyl (xylan) esterase digests as well as the untreated sample, the mass spectrum was used to determine the oligosaccharide composition. The total amount of oligosaccharides present of each DP was set as 100%. Fig. 6.3, shows the relative amount of xylooligosaccharides varying in degree of polymerization and amount of acetyl groups attached to these oligosaccharides for untreated AcXOS (blank) and after incubation with the acetyl (xylan) esterases. As mentioned earlier the AcXOS fraction also contained low amounts of acetylated hexoses. Combining the data of the (de)acetylated pentoses and hexoses would become too complex. Therefore, only acetyl substitution of the major xylooligosaccharide fraction is plotted.

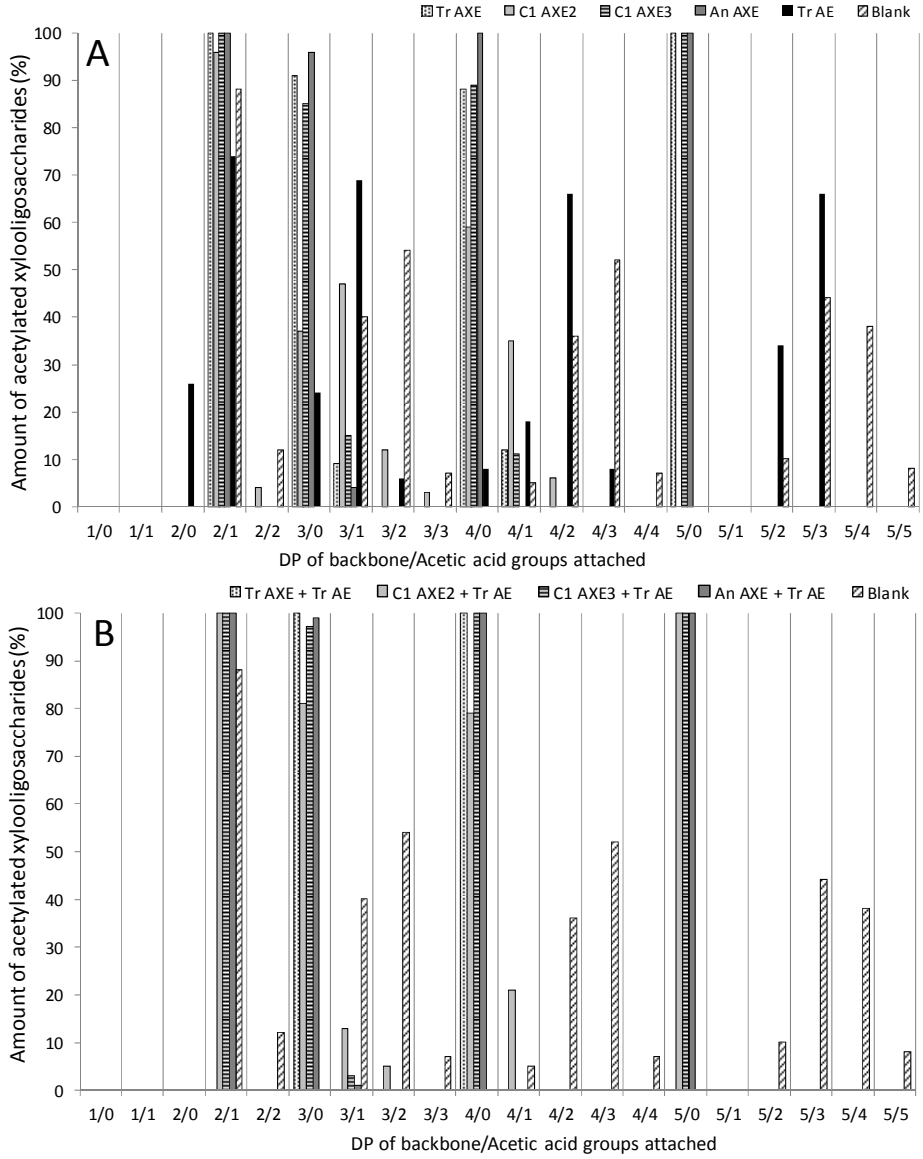

Fig. 6.3: Relative amount of acetylated xylooligosaccharides present in the AcXOS blank and AcXOS after treatment with acetyl (xylan) esterases individually (A) or after treatment of acetyl esterase in combination with each of the acetyl xylan esterases (B). The relative amount is based on the peak-intensities in the MALDI-TOF mass spectra. The total amount of oligosaccharides present for each degree of polymerization (DP) was set at 100%. TrAE: Trichoderma reesei acetyl esterase, TrAXE: Trichoderma reesei acetyl xylan esterase, C1 AXE 2: Myceliophthora thermophila C1 acetyl xylan esterase 2, C1 AXE 3: Myceliophthora thermophila C1 acetyl xylan esterase 3, An AXE: Aspergillus niger acetyl xylan esterase, Blank: untreated sample.

Fig. 6.3A shows oligosaccharides in the range of DP2-5. The untreated AcXOS (blank) contains 1-5 acetyl groups per oligosaccharide, where the long oligosaccharides carrying more acetyl groups than the short oligosaccharides. After TrAE treatment the oligosaccharides contained 0-3 acetyl groups per oligosaccharide, of which especially oligosaccharides of DP2-4 are present in the total deacetylated form. This is conform the action of TrAE reported in literature, stating that it especially removes acetyl groups from small oligosaccharides (Poutanen and Sundberg, 1988).

AnAXE was the most efficient acetyl xylan esterase: both DP4 and DP5 oligosaccharides were completely deacetylated, while for DP3 some oligosaccharides with only one acetyl group attached were remaining. DP2 was present with one acetyl group attached. This is in line with the amount of acetic acid released, showing that AnAXE released 90% acetic acid (Fig. 6.1). The other acetyl xylan esterases left mainly one or two acetyl groups attached to the oligosaccharides, of which C1 AXE2 displayed oligosaccharides containing the most acetyl groups. This also confirms the lower level of acetic acid released by this enzyme (Fig. 6.1). The more efficient deacetylation through tolerance for both long and short oligosaccharides of the AXEs, compared to AE, is in line with literature (Mitchell *et al.*, 1990; Poutanen *et al.*, 1990; Kormelink *et al.*, 1993; Pouvreau *et al.*, 2011). Acetylation remained on xylobiose for all samples, suggesting that the AXEs cannot act on acetylated xylobiose. The position of this remaining acetyl group can be either on the reducing end residue or on the non-reducing end residue, depending on the enzyme specificity. For C1 AXE2 it would remain at the non-reducing end as was found previously (Pouvreau *et al.*, 2011). It is also possible that migration of the acetyl group to an unnatural *O*-1 or *O*-4 position occurred, like has been described for partially acetylated plant carbohydrates in aqueous media (Mitchell *et al.*, 1990; Mastihubová and Biely, 2004; Biely *et al.*, 2011). This could also explain the complete deacetylation of part of the xylobiose by TrAE, which can remove acetyl groups also from position *O*-4 (Biely *et al.*, 2011) in contrast to the other acetyl xylan esterases.

TrAE was also capable of removing acetyl groups from the hexose oligosaccharides which were present in small quantities, whereas the AXEs could not deacetylate the

hexoses (data not shown). The activity of TrAE towards acetylated glucooligosaccharides has already been described (Poutanen and Sundberg, 1988; Tenkanen *et al.*, 1993).

The composition of the remaining oligosaccharides after the combined action of TrAE with each of the AXEs is displayed in Fig. 6.3B. AcXOS of DP4-5 were completely deacetylated by the combined action of TrAE with each of the AXEs. The combination of C1 AXE2 with TrAE displayed the lowest efficiency in deacetylation of the oligosaccharides. Xylobiose containing one acetyl group was present in all samples treated with both TrAE and AXE, displaying a recalcitrant structure in the oligosaccharides. The position of this remaining acetyl group could not be determined and is dependent on each of the enzyme specificities. However, acetyl migration to position *O*-4 on the non-reducing end was unlikely since the TrAE could have removed the acetyl group from this position.

6.3.3 Enzymatic deacetylation of acetylated acidic xylooligosaccharides

Similar to the neutral acetylated oligosaccharides, also the acetylated acidic oligosaccharides were treated with the acetyl (xylan) esterases. The released acetic acid was measured and the composition of the remaining oligosaccharides was determined by MALDI-TOF MS.

Acetic acid released

Fig. 6.4 shows the release of acetic acid from AcUXOS by the acetyl (xylan) esterases alone, and by a combination of the acetyl esterase with each of the acetyl xylan esterases.

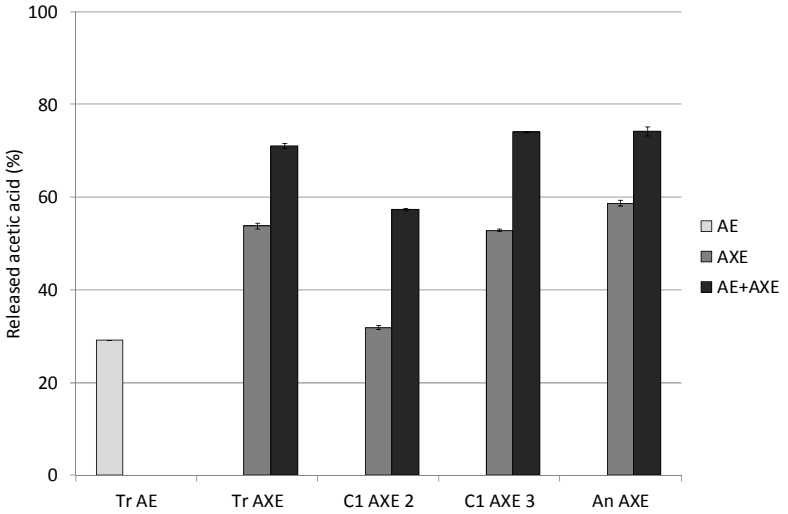

Fig. 6.4: Acetic acid release from AcUXOS after treatment with the acetyl esterase (AE), acetyl xylan esterases (AXE) or a combination of the two (AE + AXE). TrAE: Trichoderma reesei acetyl esterase, TrAXE: Trichoderma reesei acetyl xylan esterase, C1 AXE 2: Myceliophthora thermophila C1 acetyl xylan esterase 2, C1 AXE 3: Myceliophthora thermophila C1 acetyl xylan esterase 3, An AXE: Aspergillus niger acetyl xylan esterase.

The limited acetic acid release of the AcUXOS compared to the release from AcXOS shows that the MeGlcA substituents restrict the action of all esterases individually, but also the combination of TrAE and AXEs. All individual acetyl (xylan) esterases released 30% less acetic acid from the xylooligosaccharides if a MeGlcA substituent is present, although C1 AXE2 released 30-40% acetic acid from both the neutral as acidic fraction (Fig. 6.1 and 6.4). The combined action of TrAE with each of the AXEs resulted in 15-30% more acetic acid released than found for the individual AXEs. However, still 10-20% less acetic acid is released from this acidic oligosaccharide fraction compared to the neutral AcXOS. Literature describes that practically each xylopyranosyl unit carrying a MeGlcA at position *O*-2 is also carrying an acetyl group at position *O*-3 (Teleman *et al.*, 2000; Evtuguin *et al.*, 2003). Less acetic acid released from the acidic fraction compared to the neutral XOS was therefore assumed to be caused by steric hindrance through the MeGlcA at position *O*-2. This hindrance was not observed in previous studies, where the specificity of acetyl xylan esterases was only studied with model substrates (Sundberg and Poutanen, 1991; Biely *et al.*, 1996).

Acidic xylooligosaccharides resistant to enzymatic deacetylation

Fig. 6.5 displays as example the mass spectrum of AcUXOS treated with TrAXE. Acidic oligosaccharides in the range of DP2-9 with 0-2 acetyl groups were present.

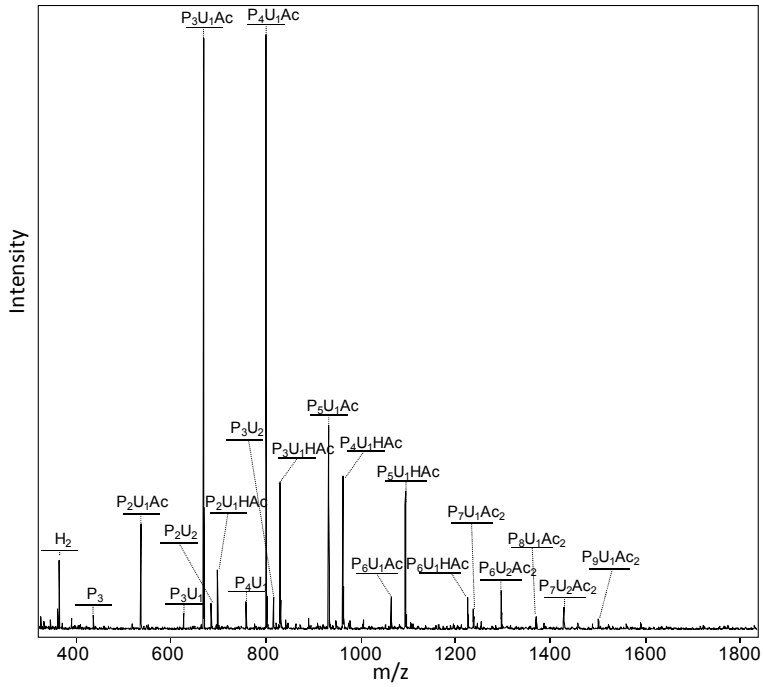

Fig. 6.5: Matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrum of acetylated acidic xylooligosaccharides from Eucalyptus treated with acetyl xylan esterase of Trichoderma reesei. H: hexose, P: pentose, U: 4-O-methyl glucuronic acid, Ac: acetyl group.

The MALDI-TOF mass spectra of acetylated acidic xylooligosaccharides after acetyl (xylan) esterase treatment displayed acidic xylooligosaccharides in the range of DP2-9 (MeGlcA not included). The oligosaccharides were substituted with 0-6 acetyl groups per oligosaccharide with the long oligosaccharides carrying more acetyl groups, 1 or 2 MeGlcA (double MeGlcA only for xylooligosaccharides of DP>5) and in some cases an additional hexose was present, assumed to be the galactose substitution at the MeGlcA (Shatalov *et al.*, 1999). Similar to the neutral acetylated xylooligosaccharides, the spectra of all samples were analysed for their degree of polymerization of the xylooligosaccharides and amount of acetyl groups attached. The total amount of

xylooligosaccharides present for each DP was set as 100%. It was decided to display only the acetylated acidic oligosaccharides in the range of DP4-7 as this gives a good overview of the capacities of the esterases (Fig. 6.6). Displaying all oligosaccharides would result in a too complex figure comprising the same conclusion, and our results pointed out that the same conversions were obtained for DP<4 or DP>7 oligosaccharides (not shown).

After incubation of AcUXOS with the individual acetyl (xylan) esterases, it is seen that the TrAE is not efficient in removal of acetyl groups from the acidic xylooligosaccharides (Fig. 6.6A). The low activity of TrAE could be a consequence of the position of the MeGlcA substituent in AcUXOS. The oligosaccharides are produced by GH10 endoxylanase hydrolysis. It was reported that the smallest oligosaccharides produced by GH10 endo-xylanases contained the MeGlcA substituent at the non-reducing Xyl*p* residue if no acetyl groups are present (Biely *et al.*, 1997). This is also the favoured Xyl*p* residue for the TrAE to remove an acetyl group from position *O*-3 or *O*-4 (Biely *et al.*, 2011). The MeGlcA at position *O*-2 is therefore assumed to cause steric hindrance for TrAE if it is indeed on the terminal xylose residue. This MeGlcA restricts the action of TrAE towards the acidic xylooligosaccharides. This would not have been detected when enzyme specificity was determined only towards artificial substrates.

AnAXE was most efficient in deacetylating AcUXOS as was displayed by the high amount of acetic acid released (Fig 6.4). The MALDI-TOF MS results showed that it was able to release acetic acid leaving 1-2 acetyl groups per oligosaccharide (DP>7). Some of the oligosaccharides of DP<6 were even completely deacetylated. C1 AXE3 was also very efficient in deacetylation of the acidic oligosaccharides, although this enzyme has more difficulties with the deacetylation of smaller oligosaccharides than AnAXE. C1 AXE2 showed to be less active towards the oligosaccharides with a DP<7, leaving 1-3 acetyl groups attached to the oligosaccharide, which explains the low amount of acetic acid released (Fig. 6.4).

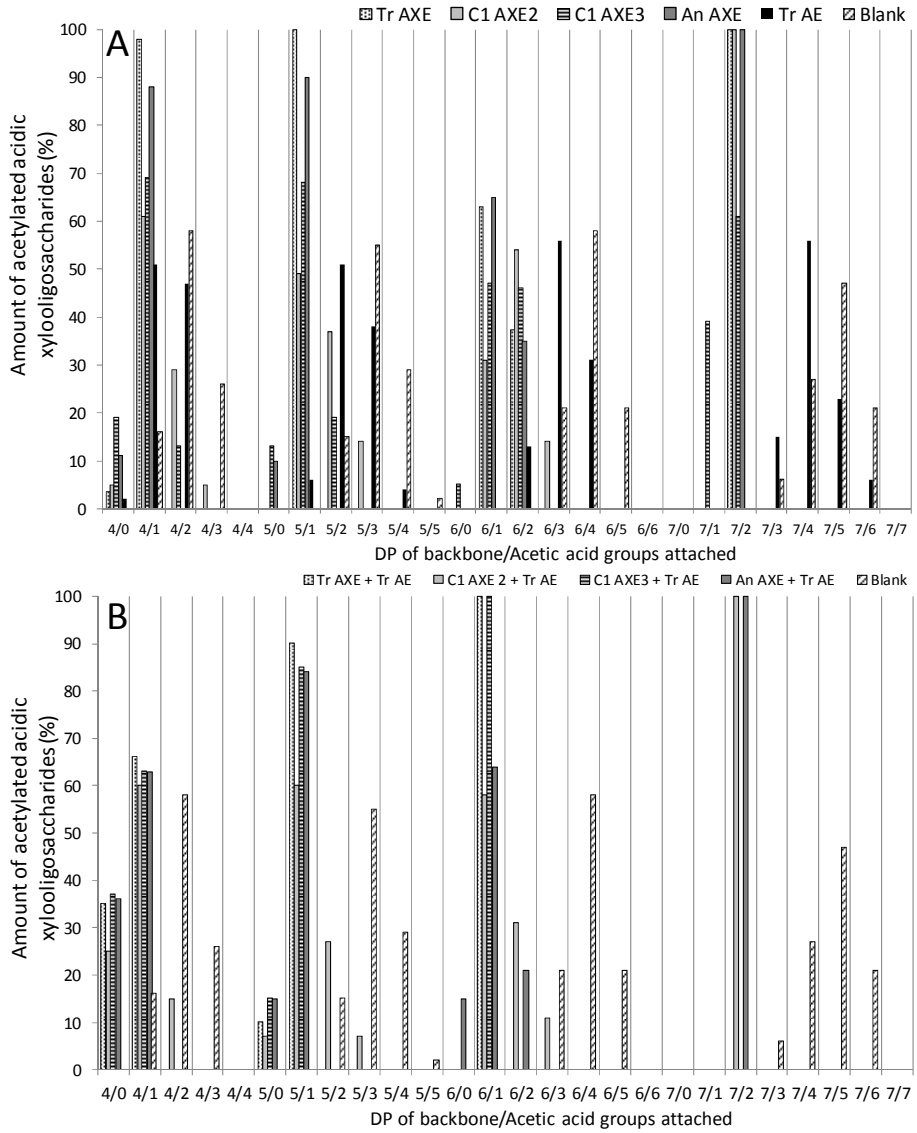

Fig. 6.6: Relative amount of acetylated acidic xylooligosaccharides of DP4-7 present in the AcUXOS blank and AcUXOS after treatment with acetyl (xylan) esterases individually (A) or in acetyl esterase (AE) in combination with each of the acetyl xylan esterases (AXE) (B). The relative amount is based on the peak-intensities in the MALDI-TOF mass spectra. The total amount of xylooligosaccharides present for each degree of polymerization (DP) was set at 100%. TrAE: Trichoderma reesei acetyl esterase, TrAXE: Trichoderma reesei acetyl xylan esterase, C1 AXE 2: Myceliophthora thermophila C1 acetyl xylan esterase 2, C1 AXE 3: Myceliophthora thermophila C1 acetyl xylan esterase 3, An AXE: Aspergillus niger acetyl xylan esterase, Blank: untreated sample DP value n=Xyl$_n$.

Similar to AcXOS, the concerted action of the acetyl esterase in combination with each of the acetyl xylan esterases on AcUXOS resulted in less remaining acetyl groups attached to the oligosaccharides compared to the action of the acetyl (xylan) esterases individually (Fig. 6.6B). Again C1 AXE2 had difficulties in the deacetylation of the oligosaccharides smaller than DP7, since Ac substitution was still present with three acetyl groups per oligosaccharide. The complementary action of C1 AXE2 and TrAE was not as strong for the acidic fraction as found for the neutral acetylated oligosaccharides. This could indeed confirm the earlier mentioned assumption that C1 AXE2 prefers to de-esterify the *O*-2 linked acetyl group, whereas the TrAE prefers the acetyl group on the *O*-3 position. As the de-esterification of the *O*-3 linked acetyl group is hindered by the MeGlcA on the neighboring *O*-2 position, this will lead to less acetic acid released. The other acetyl xylan esterases in combination with TrAE were able to remove acetyl groups present in the oligosaccharides leaving maximally one acetyl group attached per oligosaccharide. This indicates that the MeGlcA substituent hinders de-esterification: in the AcUXOS digests there was always one acetyl group more attached to the oligosaccharides than in the AcXOS digests.

Acetyl migration has less effect on the MeGlcA-substituted AcUXOS since the α-(1→2) linked MeGlcA prevents the migration involving *O*-2, although migration from *O*-3 to *O*-4 on the non-reducing end Xyl*p* may take place.

6.3.4 Synergistic action of acetyl xylan esterases and glucuronidases

To confirm the restricted action of the acetyl xylan esterases by the MeGlcA substituent, the AcUXOS fraction was also treated with two glucuronidases individually and in combination with each of the acetyl xylan esterases. Due to insufficient amounts of enzyme, TrAE could not be included in this experiment.

As expected, the glucuronidases alone did not show any acetyl esterase side-activity (data not shown). Galactose substitution of the MeGlcA moiety hindered the removal of the MeGlcA from the backbone by the glucuronidases. The acetic acid released by

the acetyl xylan esterases in combination with each of the glucuronidases is displayed in Fig. 6.7.

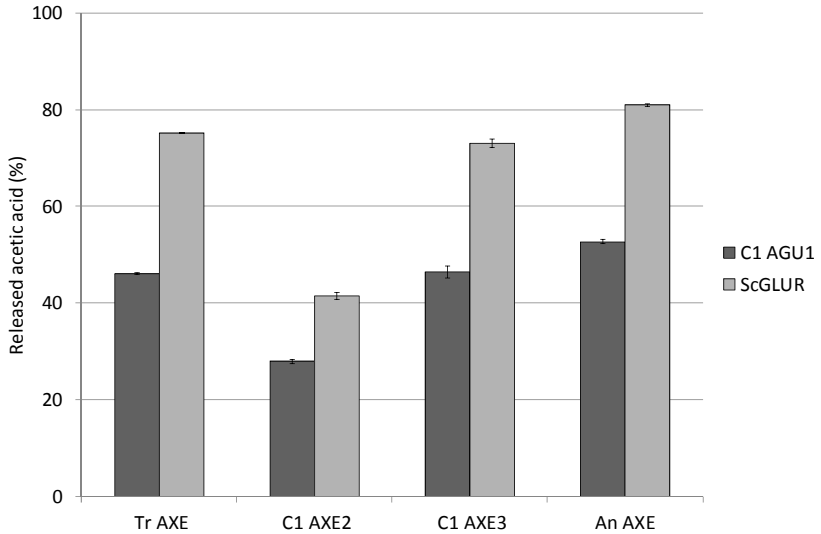

Fig. 6.7: Acetic acid release from meGlcA-substituted AcUXOS after treatment with different acetyl xylan esterases: TrAXE: Trichoderma reesei, C1 AXE 2/3: Myceliophthora thermophila C1 , An AXE: Aspergillus niger and glucuronidases: C1 AGU1: Myceliophthora thermophila C1, ScGLUR: Schizophyllum commune.

When compared to Fig 6.4, Fig. 6.7 shows that the C1 glucuronidase (C1 AGU1) does not enhance the acetyl xylan esterase activity, as no higher amounts of acetic acid were released by their combined action. The results obtained from MALDI-TOF MS (data not shown) showed that the C1 AGU1 in combination with an acetyl xylan esterase could deacetylate and deglucuronosylate high DP oligosaccharides. In general up to two acetyl groups per oligosaccharide were remaining. C1 AGU1 belongs to glycoside hydrolase (GH) family 67 and is known to only remove the MeGlcA substituent from the non-reducing end of xylooligosaccharides (Hinz *et al.*, 2009). As already mentioned, the oligosaccharides formed by the action of a GH10 endo-xylanase contain a MeGlcA at the non-reducing end of non-acetylated oligosaccharides (Biely *et al.*, 1997). This indicates that the glucuronidase is hindered by the neighbouring acetyl substituent(s) present on the same Xyl*p* moiety or on a Xyl*p*

moiety close by, which was already suggested previously for this enzyme (Hinz *et al.*, 2009). Oligosaccharides with two MeGlcA groups were accumulating in the digests after glucuronidase treatment, meaning that the glucuronidase could not hydrolyze these and is presumed to be caused by steric hindrance.

Polymer active GH115 glucuronidases enhances AXE activity. Up to 80% deacetylation is seen after combined AXE and ScGLUR treatment. The family GH115 glucuronidases act typically on polymeric xylan, but can also remove both internal MeGlcA and non-reducing end linked MeGlcA from AcUXOS (Tenkanen and Siika-Aho, 2000; Ryabova *et al.*, 2009; Kolenová *et al.*, 2010). MALDI-TOF MS displayed that the glucuronidase of *S. commune* is able to hydrolyze the 4-*O*-methyl glucuronic acid from acetylated oligosaccharides up to DP8 (data not shown). This is remarkable, as the glucuronidase has preference for polymeric xylan (Tenkanen and Siika-Aho, 2000; Ryabova *et al.*, 2009; Kolenová *et al.*, 2010). It is assumed that the large amount of acetyl groups per oligosaccharide (Fig. 6.6) restrict the action of the glucuronidase on large oligosaccharides. The oligosaccharides after treatment with ScGLUR were composed of acetylated oligosaccharides both without MeGlcA substitution or substituted with one MeGlcA. Acetyl substitution restricts the action of ScGLUR, but still a low action towards acetylated glucuronoxylan has been detected (Tenkanen and Siika-Aho, 2000). When acetyl xylan esterases are added in combination with the ScGLUR hardly any double MeGlcA substituted oligosaccharide is present. This indicates that ScGLUR is able to degrade this complex oligosaccharide. Only up to two acetyl groups per oligosaccharide were present after the synergistic action of the acetyl xylan esterases in combination with ScGLUR (data not shown), which is in accordance to the results of the AXEs alone (Fig.6.3A), assuming that the oligosaccharides carrying two acetyl groups are also MeGlcA substituted. By the synergistic action with ScGLUR, all acetyl xylan esterases released 10-25% more acetic acid compared to the release obtained by the acetyl xylan esterases alone (Fig. 6.4 and 6.7).

6.4 Conclusions

Analysis of acetic acid release and MALDI-TOF mass spectrometry was a powerful strategy to reveal the action of acetyl (xylan) esterases towards neutral and acidic acetylated *Eucalyptus* xylooligosaccharides. Acetyl xylan esterases of CE1 were most efficient in the deacetylation of both fractions xylooligosaccharides, followed by CE5 acetyl xylan esterases. CE16 acetyl esterase could not deacetylate the xylooligosaccharides, but could enhance the action of the CE5 esterases. One acetyl group on low DP oligosaccharides was remaining in most cases. MeGlcA substituents restricted the action of the acetyl (xylan) esterases. Complete deacetylation was difficult to achieve and only a part of the AcUXOS of low DP could be completely deacetylated. Often one or two acetyl groups were remaining on the acidic oligosaccharides. Synergy towards acetylated acidic xylooligosaccharides of the acetyl xylan esterases in combination with GH67 and GH115 glucuronidases was observed, in which the combination of CE1 AXE with GH115 glucuronidase was most efficient. A maximum of two acetyl groups per oligosaccharide were remaining after their combined action.

The results showed that testing the enzyme specificity on artificial substrates only was insufficient to understand their action towards natural substrates.

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Chapter 7

General discussion

7.1 Motivation of the research

Hemicellulases are crucial in achieving optimal hydrolysis of lignocellulosic feedstock. Several fungal strains have been established for industrial production of cell wall degrading enzymes. Nevertheless, most commercial enzyme preparations are lacking one or more of the enzymes needed for complete hydrolysis of biomass.

Carried out within the EU-project 'Targeted discovery of novel cellulases and hemicellulases and their reaction mechanisms for hydrolysis of lignocellulosic biomass', acronym DISCO, the aim of this PhD research was to focus on the efficient utilisation of the hemicellulolytic part of biomass for the production of bioethanol. Fungal strains have been fermented and were studied for their production of cell wall degrading enzymes. Some fungal endo-xylanases have been purified and characterised in this study. In addition, synergistic studies were performed to understand the role of accessory enzymes. The DISCO-project integrated the findings of the discovery of cellulases and hemicellulases and the investigation of their reaction mechanisms to completely degrade pretreated lignocellulosic biomass.

7.2 Searching for efficient hemicellulolytic enzymes

7.2.1 Substrate choice in screening

In chapter 2 a screening method is described, which is based on both the quantitative and qualitative analysis of individual degradation products from wheat arabinoxylan (WAX) and *Eucalyptus* xylan hydrolysate (EXH) (Van Gool *et al.*, 2011). Both these soluble xylans contain many of the substituents that can be present in complex xylans. The degradation products of WAX reflected the possible presence and activity of endo-xylanases (EC 3.2.1.8), of AXH-m (releasing mono substituted arabinose: EC 3.2.1.55), of AXH-d3 (releasing double substituted arabinose from position *O*-3: EC 3.2.1.55) or β-xylosidase (EC 3.2.1.37) within the crude fermentation supernatants. In addition, degradation of EXH provided information concerning the presence and activity of

acetyl xylan esterases (EC 3.1.1.72), endo- and exo-xylanases and α-glucuronidases (EC 3.2.1.131). Different enzyme activities within the crude fermentation supernatants could be recognized individually by the high performance anion exchange chromatography (HPAEC) degradation profiles of the digests and by qualitative determination of the composition of remaining oligomers by matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Using these substrates and methods, powerful enzyme producers were assigned. However, as described in chapter 3, the complexity and insolubility of agro-industrial substrates will also influence enzyme performance. The screening was extended using insoluble wheat straw (WS WUS) and corn fiber (CF AIS) fractions. This resulted in other powerful and dedicated enzyme producers than those selected for their performance on WAX and EXH (Chapters 2 and 3; Van Gool *et al.* (2011; 2012a)). Compared to the traditional screening methods using artificial substrates or reducing-end assays with a colouring reaction, the method gives much more information in a single analysis concerning the presence of enzymes in complex mixtures. Screening for efficient enzymes using model substrates already resulted in many interesting enzyme sources. Inclusion of agro-industrial substrates is considered to be essential to also predict enzyme performance towards more complex and insoluble substrates.

7.2.2 Fungi excreting high hemicellulolytic activities

The screening as described in chapters 2 and 3, resulted in several strains active towards the various xylan-rich substrates. The top enzyme producers for the different soluble and insoluble substrates, being WAX, EXH, WS WUS and CF AIS, were selected on the basis of the release of monomers and oligomers from the substrates. For EXH also acetic acid release was evaluated. Some of the 'unknown' strains mentioned previously (Chapters 2 and 3; Van Gool *et al.* (2011; 2012a)) have been identified in the meantime. The genomic DNA was isolated from the strains and was sent to the CBS (Fungal Biodiversity Centre, Utrecht, The Netherlands). This resulted in an

updated table of top enzyme-producers displayed in table 7.1. It shows that the main fungal strains originate from *Trichoderma, Penicillium* and *Aspergillus* species.

Aspergillus lentulus was selected four times, being efficient in degradation of the hemicelluloses from the insoluble wheat straw and corn fiber. It displayed relative high arabinose and xylooligomer release towards these complex and insoluble substrates. On EXH *A. lentulus* displayed high acetyl xylan esterase activity, this could also play a pivotal role in the degradation of WS WUS and CF AIS. However, the release of acetic acid was not quantified for those substrates. Using assays based on colour reactions, the strain only displayed high endo-xylanase activity and moderate arabinofuranosidase activity (Chapter 2; Van Gool *et al.* (2011)). Towards WAX the strain displayed the release of arabinose and xylooligomers, though not significantly high compared to other strains tested (Van Gool *et al.*, 2011). The strain would thus not have been selected if only these substrates were used in the screening. Many *Aspergillus* species are known for the production of cellulases and hemicellulases (Bailey and Poutanen, 1989; Gusakov, 2011), but the potential of cellulase and hemicellulase production by the species *A*. *lentulus* was not yet described in literature. *A. lentulus* was only discovered in 2005 and seems closely related to *A. fumigatus* (Balajee *et al.*, 2005). The latter is known for cellulase and hemicellulase production (Stewart *et al.*, 1984). The only application so far for the opportunistic pathogenic fungal isolate *A. lentulus* is the removal of textile dyes by the ligninolytic enzymes secreted by the species (Varga, 2006; Kaushik and Malik, 2010). The results of the screening show that *A. lentulus* is an interesting source for the production of efficient lignocellulose degrading enzymes.

Table 7.1: Top enzyme producers degrading the different complex xylans, based on the release of the main building blocks from wheat arabinoxylan (WAX); eucalyptus xylan hydrolysate (EXH); wheat straw water unextractable solids (WS WUS) and corn fiber alcohol insoluble solids (CF AIS) (Chapters 2 and 3; Van Gool et al. (2011; 2012a)). Sample numbers refer to numbers attributed previously (Chapter 2; Van Gool et al. (2011)).

Rank	Top five producers on different substrates			
	WAX[a]	EXH[b]	WS WUS[c]	CF AIS[d]
1	38 (*Penicillium pinophilum*)	41 (*Aspergillus niger*)	13 (*Aspergillus lentulus*)	73 (non-identified)
2	74 (*Trichoderma harzianum*)	40 (*Aspergillus oryzae*)	7 (*Penicillium pinophilum*)	74 (*Trichoderma harzianum*)
3	49 (*Penicillium ochrochloron*)	42 (*Aspergillus foetidus*)	57 (non-identified)	14 (*Aspergillus lentulus*)
4	45 (*Trichoderma sp.*)	27 (*Bionectria ochroleuca)*	38 (*Penicillium pinophilum*)	13 (*Aspergillus lentulus*)
5	7 (*Penicillium pinophilum*)	7 (*Penicillium pinophilum*)	12 (*Aspergillus lentulus*)	44 (non-identified)

[a] Main building blocks: arabinose, xylose, xylooligomers DP2-4
[b] Main building blocks: xylose, xylooligomers DP2-4, acetic acid
[c] Main building blocks: glucose, xylose, xylooligomers DP2-4
[d] Main building blocks: arabinose, xylose/glucose, various oligomers

Penicillium pinophilum was selected for the excellent performance in degradation of WAX, EXH and WS WUS. It displayed an active arabinofuranosidase. The arabinose of mono-, double and multiple arabinose substituted xylopyranosyl units in WAX could be released (Chapter 2; Van Gool *et al.* (2011)). Although the high arabinofuranosidase activity was also found using *p*-nitrophenyl arabinofuranoside (Van Gool *et al.*, 2011), the positional specificity as determined towards WAX by HPAEC could not be elucidated with the colorimetric method. Also endo-xylanase and β-xylosidase activity were recognized, which were not distinguished using colorimetric methods (Van Gool *et al.*, 2011). In literature this microorganism has already been ascribed a good producer of cellulases (Wood and McCrae, 1986; Clayssens *et al.*, 1989) and also of xylan degrading enzymes, but only when it was grown on xylan as carbon source during fermentation (Jørgensen *et al.*, 2005; Gusakov, 2011).

Trichoderma harzianum was selected for efficient degradation of WAX and CF AIS. In both cases arabinose was the major component being released, next to some xylooligomers (Chapter 3; Van Gool *et al.* (2012a)). The arabinose could be released from both positions *O*-2 and *O*-3 of the xylopyranosyl units in WAX. The strain did not perform extremely well in the degradation of EXH. However, acetic acid released from EXH by this strain was significant, though not high enough to be selected as one of the best strains (Chapter 2; Van Gool *et al.* (2011)). One of the most common *Trichoderma* species is *Trichoderma reesei*, which is known because of its efficient production of cellulases and hemicellulases (Banerjee *et al.*, 2010; Gusakov, 2011). *T. harzianum* is the most common *Trichoderma* species in nature, as it is the most frequently found *Trichoderma* species in samples world-wide (Druzhinina *et al.*, 2010). However, the *T. harzianum* species is very diverse; many sub-groups (sub-clades) exist (Druzhinina *et al.*, 2010). The expression of cellulose degrading enzymes by *T. harzianum* has been described previously (Ahmed *et al.*, 2009). Recently, the potential of hemicellulolytic activity by the *T. harzianum* species was published (Delabona *et al.*, 2012). These authors found high xylanase and cellulase activity for the strain when grown on sugar cane bagasse, by measuring reducing sugar release of beech wood xylan and hydroxyethyl cellulose respectively. The carbohydrate composition of sugar cane

bagasse and wheat straw is comparable (Lee *et al.*, 2007; Chandel *et al.*, 2012). Grown on these comparable substrates as carbon source during fermentation, it is expected that the strain excretes the same enzymes and thus confirms our findings. However, much more detailed information on the type and activity of the hemicellulolytic enzymes was found when using our approach.

7.2.3 Effect of the carbon source in growth medium[1]

All fungi tested in the screening method of chapters 2 and 3 were grown on wheat straw as carbon source during fermentation. It is thought that fungi produce enzymes during fermentation, to efficiently degrade the carbon source present in the fermentation medium (Jørgensen and Olsson, 2006; Sørensen *et al.*, 2011). For *Aspergillus* species it is known that they are not able to import large carbohydrate molecules, thus monosaccharides and small oligosaccharides are the actual inducers of the regulatory systems involved in the activation of expression of the genes encoding plant cell wall polysaccharide-degrading enzymes (De Vries, 2003). A series of various signals in the fungus leads to the production of enzymes able to degrade the polymers (Gielkens, 1999).

In order to vary the available carbohydrate molecules in the carbon source compared to those in wheat straw, all fungi were also grown on *Eucalyptus globulus* wood and corn fiber, of which their sugar composition has been described previously (Christov *et al.*, 2000; Van Eylen *et al.*, 2011). With these strains the same effective screening procedure was followed as for the wheat straw-grown samples (Chapters 2 and 3; Van Gool *et al.* (2011; 2012a)).

[1] The work described in §7.2.3-7.2.5 was carried out in collaboration with the Department of Applied Biotechnology and Food Science of Budapest University of Technology and Economics, Budapest, Hungary.

Materials and methods

Isolation and production of the fungi was carried out the same as described previously (2011; 2012a), only the wheat straw as carbon source in the fermentation medium was replaced in these experiments by either finely ground corn fiber (<0.3 mm, Hungrana Kft, 2432 Szabadegyhaza, Hungary) or finely ground *Eucalyptus* wood (<0.3 mm, kindly provided by Prof. dr. J.C. Parajo of the University of Vigo-Ourense, Spain). Enzyme activity was demonstrated by HPAEC analysis of the degradation products after incubation on wheat arabinoxylan (WAX), *Eucalyptus* xylan hydrolysate (EXH), wheat straw water unextractable solids (WS WUS) and corn fiber alcohol insoluble solids (CF AIS).

The results obtained in the study on the influence of the carbon source are summarized in table 7.2. The top-five enzyme producers to degrade the soluble WAX and EXH were comparable to the strains selected when grown on wheat straw as carbon source (Chapter 2; Van Gool *et al.* (2011)). They include *Penicillium pinophilum, Trichoderma harzianum, Aspergillus oryzae* and *Bionectria ochroleuca* (§7.2.2; Van Gool *et al.* (2011; 2012a)). This indicates that the fungus initiates which xylan degrading enzymes are produced with the ability to degrade the soluble substrates. The production of xylan degrading enzymes will be independent of the carbon source in the fermentation medium. However, when grown on corn fiber instead of wheat straw, the top-five degraders of the insoluble xylans were other strains (with the exception of *Penicillium pinophilum*). Unfortunately, the identity of many strains efficiently degrading the insoluble xylans remains unknown. Nevertheless, it points out that there are specific strains able to efficiently degrade the complex corn fiber fraction, when they were grown on corn fiber as carbon source during fermentation. It is hypothesized that these fungi produce more efficient enzymes by e.g. having a weak affinity for lignin (Berlin *et al.*, 2006); or a strong affinity for or interaction with the xylan-cellulose network (Guillén *et al.*, 2009).

The strains grown on *Eucalyptus* wood displayed six *Trichoderma species* out of ten selected strains. The reason for this may be assigned to the origin of *Trichoderma*, as the variety of the enzymes produced differs between fungi and often corresponds to the requirements of its habitat (Howell, 2003; Van Den Brink and De Vries, 2011).

Table 7.2: Selected fungi grown on corn fiber or Eucalyptus wood for the production of efficient enzymes to degrade wheat arabinoxylan (WAX) Eucalyptus xylan hydrolysate (EXH), wheat straw water unextractable solids (WS WUS) and corn fiber alcohol insoluble solids (CF AIS), based on the release of the main building blocks in the substrates. Sample numbers refer to numbers attributed previously (Chapters 2 and 3; Van Gool *et al. (2011; 2012a)).*

Carbon source	*Rank*	*Top five producers*			
		WAX[a]	EXH[b]	WS WUS[c]	CF AIS[d]
Corn fiber	1	7/8 (*Penicillium pinophilum*)	71 (*Phanerochaete chrysosporium*)	7/8 (*Penicillium pinophilum*)	63 (non-identified)
	2	31 (non-identified)	60/69 (*Trichoderma reesei*)	66 (non-identified)	66 (non-identified)
	3	11/12 (*Aspergillus lentulus*)	11/12 (*Aspergillus lentulus*)	75 (non-identified)	31 (non-identified)
	4	59 (*Cladosporium herbarum*)	38 (*Penicillium pinophilum)*	63 (non-identified)	15/16 (*Trichoderma sp.*)
	5	15/16 (*Trichoderma sp.*)	72 (*Myrothecium verrucaria*)	38 (*Penicillium pinophilum)*	57 (non-identified)
Eucalyptus wood	1	74 (*Trichoderma harzianum*)	40 (*Aspergillus oryzae*)	49 (*Penicillium ochrochloron*)	66 (non-identified)
	2	15/16 (*Trichoderma sp.*)	27 (*Bionectria ochroleuca*)	56 (*Trichoderma sp.*)	56 (*Trichoderma sp.*)
	3	47 (non-identified)	60/69 (*Trichoderma reesei*)	26 (*Trichoderma sp.*)	74 (*Trichoderma harzianum*)
	4	26 (*Trichoderma sp.*)	34 (*Corynascus verrucosus*)	15/16 (*Trichoderma sp.*)	52 (*Aspergillus terreus*)
	5	1/2 (*Trichoderma sp.*)	11/12 (*Aspergillus lentulus*)	66 (non-identified)	26 (*Trichoderma sp.*)

[a] Main building blocks: arabinose, xylose, xylooligomers DP2-4
[b] Main building blocks: xylose, xylooligomers DP2-4, acetic acid
[c] Main building blocks: glucose, xylose, xylooligomers DP2-4
[d] Main building blocks: arabinose, xylose/glucose, various oligomers

Interestingly, an overall comparison of the fungal strains grown on corn fiber and *Eucalyptus* wood as carbon sources, led to the conclusion that the most efficient enzyme mixtures were produced by fungi grown on complex corn fiber. These enzymes produced by fungi grown on corn fiber released on average twice as much monomers and oligomers from the substrates compared to the enzyme mixtures produced by the top-5 enzyme producing fungi grown on *Eucalyptus* wood (data not shown). This led to the suggestion that the corn fiber should contain, next to the complex xylan, an easily fermentable carbohydrate which can boost fungal growth in the initial phase of fermentation.

7.2.4 Does residual starch in corn fiber influence the expression of highly active enzymes?

The most efficient enzymes described in the previous paragraph were expressed by fungi grown on complex corn fiber. It was hypothesized that this was caused by the presence of 16 w/w% remaining starch, which is present next to the 53 w/w% of non-starch polysaccharides in corn fiber (Van Eylen *et al.*, 2011). Fermentation supernatants of eight strains were taken after 1, 3 and 5 days of fermentation to be able to detect which enzyme activities are present. These supernatants were tested for their starch degrading activity and arabinoxylan degrading activity.

Materials and methods

Eight strains (table 7.3) were fermented on corn fiber. Samples of the fermentation supernatants were collected after 1, 3 and 5 days incubation. Enzyme activity was demonstrated by high performance anion exchange chromatography (HPAEC) analysis of the degradation products after incubation on wheat arabinoxylan (WAX) (Van Gool *et al.*, 2011). In addition 5 mg/mL soluble potato starch (Sigma-Aldrich, St. Louis, MO, USA) in 50mM sodium acetate buffer pH 5.0 was incubated with 1% v/v fermentation supernatant for 1 h and 24 h at 37°C. The samples were boiled for 10 min to inactivate the enzymes. Glucose release was measured by HPAEC. The 1,4-β-D-xylosidase activity was assayed as described by Herr *et al.* (1978). The liberated 4-nitrophenol was measured at 400 nm.

Table 7.3: Strains used in the time-course fermentation on corn fiber.

*Sample number**	*Collection number***	*Origin*
B41	ATCC 10864	*Aspergillus niger*
B42	ATCC 14916	*Aspergillus foetidus*
B1/2	TUB F-1702	*Trichoderma sp.*
B40	NRRL 3485	*Aspergillus oryzae*
B78	IFO 4855	*Paecilomyces variotii*
(new)	NRRL 2129	*Penicillium aculeatum*
B52	OKI 16/5	*Aspergillus terreus*
B62	OKI 270	*Chaetomium globosum*

* Sample number as used in Van Gool *et al.* (2011; 2012a)
**ATCC: American Type Culture Collection, Manassas, VI, USA
IFO: Institute for Fermentation, Osaka, Japan (more recently: NBRC Culture Collection, NITE Biological Resources Center, Chiba, Japan)
NRRL: Northern Regional Research Centre, USDA, Peoria, IL, USA
OKI: Institute for Public Health, Budapest, Hungary
TUB: Technical University of Budapest culture collection, Budapest, Hungary (F-...: collection of fungi)

Eight strains were chosen to include several different fungal sources with proven hemicellulolytic activity (Chapters 2 and 3; Van Gool *et al.* (2011; 2012a)). The results of the time-course samples after starch and WAX degradation are summarised in Fig. 7.1. The figure shows for each of the strains: 1) the amount of xylose and xylooligomers (DP2-4) released from WAX as percentage of the total xylose in WAX; 2) arabinose released from WAX as percentage of the total arabinose present in WAX; and 3) glucose released after 24h incubation with soluble starch, which is assumed to display the end-point of the starch degradation. The glucose released after 1h incubation was used to determine starch degrading activity. In addition, β-xylosidase activity is plotted, which is determined using PNP-xylose.

Fig. 7.1 displays that β-xylosidase activity towards PNP substrate is rarely present in the strains tested, except for TUB F-1702 (Fig. 7.1C). Starch degrading activity is already present in the first fermentation samples (1 day), except for TUB F-1702 (Fig. 7.1C) and NRRL 3485 (Fig. 7.1D). After one day of fermentation both the *Aspergillus* strains ATCC 10864 (Fig. 7.1A) and ATCC 14916 (Fig. 7.1B) displayed a significant release of the mono- and oligosaccharides from WAX. The amount of mono- and oligosaccharides released from WAX did not change for the longer fermented samples (3 and 5 days). Only a slight increase in β-xylosidase activity was observed towards PNP-xylose and WAX. This indicates that all enzyme activity was constant over the whole fermentation period and that the initial production of hemicellulolytic enzymes

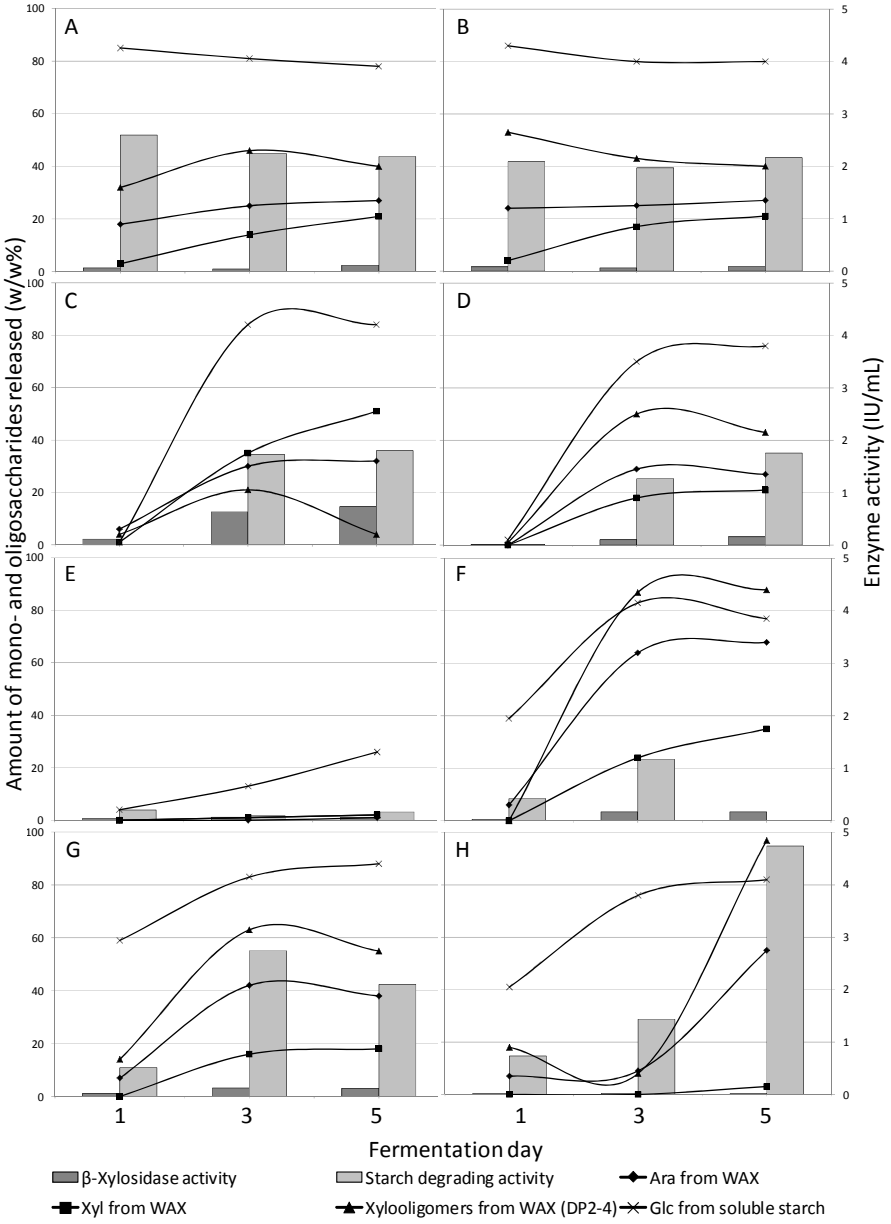

Fig. 7.1: β-Xylosidase and starch degrading activity (IU/mL), xylose and xylooligomers release from WAX (w/w% of total xylose present in WAX), arabinose released from WAX (w/w% of total arabinose present in WAX) and glucose released from soluble starch (w/w% of total glucose present in soluble starch) after 24h incubation. Each plot displays a strain grown on corn fiber for 1, 3 and 5 days. A: ATCC 10864, B: ATCC 14916, C: TUB F-1702, D: NRRL 3485, E: IFO 4855, F: NRRL 2129, G: OKI 16/5, H: OKI 270.

was not 'postponed' by the presence of starch in the fermentation medium. The two other *Aspergillus* strains (NRRL 3485 and OKI 16/5, Fig. 7.1D and G, respectively) and the *Trichoderma* strain (TUB F-1702, Fig. 7.1C) displayed an increased product release and enzyme activities upon longer fermentation. This indicates that these strains produce the various enzymes simultaneously. The main differences between the strains are that strain OKI 16/5 displayed starch degrading activity already after one day of fermentation, whereas this was not present for both other strains. Furthermore, TUB F-1702 produces a more efficient β-xylosidase than the other strains.

Fig. 7.1H displays the enzyme activity and products released by OKI 270. This strain released significant amounts of xylooligomers and arabinose, but only after five days of fermentation. The strain produces starch degrading activity after one day of fermentation, which is increasing rapidly upon fermentation, but it does not produce β-xylosidase. This indicates that the strain is slowly growing, first on starch and later on the hemicellulose of corn fiber. The *Penicillium* strain NRRL 2129 (Fig. 7.1F) seems to prefer the degradation of the hemicellulose in the carbon source of the fermentation medium. It does not produce starch degrading activity, but after 3-5 days of fermentation high amounts of arabinose and xylooligomers are released. Strain IFO 4855 (Fig. 7.1E) was hardly active towards WAX and starch, since not much mono- and oligosaccharides were released from these substrates. The conditions used upon fermentation were probably not optimal for this fungus to grow, since it is known that the fungus can produce hemicellulolytic enzymes when grown on wheat straw or *Eucalyptus* wood (Almeida e Silva *et al.*, 1995; Van Gool *et al.*, 2011).

The results display that starch is degraded by the starch degrading enzymes simultaneous with the xylan being degraded by xylan degrading enzymes for most of the fungi. This indicates that during fermentation, starch is utilised at the same time as the hemicellulose from corn fiber. The production of efficient hemicellulolytic enzymes is increasing upon fermentation time. Starch degrading activity is in most cases increasing or constantly present. Two strains have significant hemicellulolytic activity produced immediately. It has been reported previously that during

development the fungus will initially use the easily available carbon sources and after limitation of these sources the fungus starts to produce enzymes necessary to degrade the more complex polysaccharides (Maheshwari *et al.*, 2000). Our results show that this is only true for some of the strains tested.

A suggestion for future research would be to add an easily available carbon source to fermentation media having complex carbohydrates as target carbon source. The easily available carbon source should be added in such an amount that the fungus will survive the first days, if it will initially use the easily available carbohydrate source. After limitation of this easily available substrate the fungus can produce enzymes necessary to degrade the more complex carbon source. Nevertheless, it should be mentioned that probably not all fungi are growing on the complex carbon source, like strain IFO 4855.

7.2.5 Does cellulose induces production of hemicellulases?

Another suggestion for enzyme secretion by fungi is that cellulose functions as a trigger for the production of xylanolytic enzymes, which has been described for *Aspergillus species* (Hrmova *et al.*, 1989). Likewise, cellulolytic enzymes are produced in the presence of xylan as xylanolytic enzymes are expressed when fungi are grown on arabinose (de Vries and Visser, 2001). A trigger function for the production of enzymes through the carbon source has been suggested for *Aspergillus* and *Trichoderma species* producing plant cell wall degrading enzymes (Hrmova *et al.*, 1989; Margolles-clark *et al.*, 1997; De Vries, 2003). This was tested by growing the eight strains displayed in table 7.3 for 5 days on Avicel (microcrystalline cellulose). It was expected that hemicellulolytic enzymes were produced by the trigger function of cellulose. However, the sugar composition of Avicel (92 w/w% carbohydrates) displayed that the major part is composed of glucose (88 w/w%), but still some xylose (2 w/w%) was present (no further data shown). After WAX incubation with corn fiber grown supernatants or Avicel grown supernatants, the degradation products showed that the Avicel-grown strains contained three times less xylanolytic activity, compared

to the fungal strains grown on corn fiber. However, xylanolytic activity was observed. As the Avicel was not composed of pure cellulose and contained minor amounts of xylose we cannot conclude if the xylan degrading enzymes were expressed due to the presence of xylose or that another mechanism caused xylan degrading enzymes to be formed. It has been reported that *Aspergilli* did not produce xylanolytic enzymes when grown on substrates other than xylose-containing substrates, although literature is contradictory on this (Hrmova *et al.*, 1989; de Vries and Visser, 2001). To confirm the hypothesis of the induction of hemicellulolytic enzyme production by fungi grown on cellulose, the xylan-free bacterial cellulose produced by *Acetobacter xylinum* can provide a way to test this (Masaoka *et al.*, 1993).

7.2.6 Consequences of our research for future screening approach

The methods used in the screening for specific enzymes resulted in several interesting fungi efficiently producing xylan degrading enzymes (Table 7.1). In advance of starting a screening, one should consider (a) the application of the enzymes to screen for, i.e. the target substrate to be degraded, either by the selection of a representative carbon source used upon fermentation or by the substrate to be used to detect specific enzyme activity; (b) the fungal source, as it was shown that not all fungi are good in the expression of specific efficient enzymes; (c) the hydrolysis conditions, which should be adapted to the conditions used for the application, in terms of temperature, pH and residence time.

As an alternative of the activity based screening, genome-mining is a way to discover enzyme activities. The genomic data of micro-organisms can give a quick insight in the presence of certain potential enzymes in the genome (de Vries *et al.*, 2005; Margulies *et al.*, 2005). However, the functional annotation of a gene needs quite some time and expertise. Furthermore, one will only search for genes encoding 'known' enzymes (Jacobsen *et al.*, 2005) Hence, novel enzymes will not easily be detected.

Analytical tools

Next to these considerations in advance of a screening, novel techniques can be beneficial for an efficient screening in future. The screening method presented was very effective, but made use of non-high throughput analytical tools.

By use of HPAEC, one can easily distinguish endo-xylanase activity, arabinofuranosidase activity and glucuronidase activity. To prevent co-elution of glucose, galactose and xylose, an HPAEC method able to distinguish all monomers as well as the oligomers in a single run could be beneficial. The approach can be extended by use of tandem mass spectrometry (MS^n) or MALDI-TOF/TOF to distinguish the monosaccharide moiety that carries the acetyl group(s) and to specifiy the position of the acetyl group on the xylopyranosyl moiety. However, fragmentation of acetylated xylooligosaccharides is difficult due to the acetyl substituents present in the oligomer. A new development on MALDI-TOF MS is on-target labelling of the oligomers with 3-aminoquinoline to obtain information on oligosaccharide sequence, linkage, and branching (Rohmer *et al.*, 2010). Another commonly used technique is nuclear magnetic resonance (NMR) spectroscopy, by which the position of the acetyl groups could be determined (Kabel *et al.*, 2003). To encompass all information in a single method, hydrophilic interaction chromatography coupled with UV, evaporative light scattering and mass spectrometry detection seems a promising technique to separate, identify and quantify acetylated oligomers (Leijdekkers *et al.*, 2011; Remoroza *et al.*, 2012).

Using HPAEC for monomer & oligomer composition, HPLC analysis of organic acids for the degree of acetylation and MALDI-TOF MS to identify the remaining structures seems to be the best approach possible at the moment.

Impact of fungi selected

In general, the organisms selected for the efficient degradation of the complex xylans, often contained high activities of the accessory enzymes, e.g. arabinofuranosidase and acetyl xylan esterase. This indicates that especially the presence of highly active accessory enzymes is important for the selection of an efficient enzyme producer to

degrade complex xylans. Fungal sources known for their highly efficient hemicellulolytic activity were also selected in this study for the efficient degradation of complex xylans. These fungal sources were *Aspergillus, Penicillium* and *Trichoderma*. However, the selection includes different species than the common ones, displaying that the common industrial strains are possibly not the most effective ones for the degradation of complex xylans. An option towards even more enzyme activity produced could be obtained by co-cultivation of various strains, thereby improving enzyme production of the fungi by the induction of specific enzymes (Hu *et al.*, 2011). These results open doors for industry to produce more effective enzymes for the degradation of complex xylans by selection of new strains. In addition, the selected strains in this study could be very suitable for on-site production of hemicellulolytic enzymes in various biotechnological applications, since they were extracted from a soil sample, were grown on cheap agro-industrial waste products and displayed good enzyme performance towards agro-industrial substrates.

7.3 Detailed characterisation of hemicellulolytic enzymes

7.3.1 *Myceliophthora thermophila* C1 endo-xylanases from glycoside hydrolase families 10 and 11

Endo-xylanases are endo-acting enzymes hydrolysing the β-1,4 or β-1,3 linkage in xylans. They are mainly found in glycoside hydrolase (GH) families 10 and 11, while some xylanases are classified in families 5, 8, and 30 (Henrissat *et al.*, 2012). Chapter 4 describes the performance of two GH family 10 endo-xylanases from *Myceliophthora thermophila* C1 (C1) towards various soluble and insoluble xylans. By analysis of the degradation products from various xylan-rich substrates, varying in type and degree of substituents, it was found that the two C1 GH10 endo-xylanases formed different products in different amounts. The impact of substrate-related factors on enzyme efficiency of the C1 endo-xylanases is summarized in Table 7.4. It shows that the C1

GH10 endo-xylanases are hindered by self-association of xylan. An alkaline treatment of wheat straw increased the performance of the endo-xylanases.

Chapter 5 describes the differences found for two GH11 endo-xylanases from *M. thermophila* C1. Different type and degree of substitution of a xylan as well as self-association of linear xylans resulted in intrafamily differences for the two GH11 endo-xylanases, summarized in Table 7.4. The C1 GH11 xylanases are more efficient on low substituted xylan compared to C1 GH10 endo-xylanases as from those substrates they release more small oligomers. This is contradictory to the general assumption that GH10 xylanases degrade xylans to a higher degree (Biely *et al.*, 1997; Ustinov *et al.*, 2008; Dumon *et al.*, 2012).

The differences found for the C1 GH10 endo-xylanases could be correlated to the predicted 3D-structure of the enzymes. An additional loop shielded the catalytic cleft of Xyl3, thereby hindering efficient substrate docking (Chapter 4; Van Gool *et al.* (2012c)). Also for the C1 GH11 endo-xylanases the differences could be related to a different predicted protein structure. In contrast to the GH10 xylanases, minor differences in the predicted structure of the GH11 xylanases already caused the formation of different degradation products. Especially the variation of the amino acid moiety on position 163 of the enzymes seems to cause the different degradation product profile. A valine instead of a tyrosine on this position resulted in a degradation product profile closer to GH10 xylanases, presumably caused by a different substrate-enzyme interaction (Chapter 5; Van Gool *et al.* (2012b)).

The efficient production of cellulases and hemicellulases in *Myceliophthora thermophila* has been reported previously, however it is not completely representative for the C1 strain used in this study (Oberson *et al.*, 1992; Chadha *et al.*, 2004; Berka *et al.*, 2011).

Table 7.4: Performance of the GH10 and GH11 C1 endo-xylanases as influenced by various substrate characteristics. GH10 xylanases: Xyl1, Xyl1 without carbohydrate binding module (Xyl1dCBM), Xyl3 and Xyl3 without carbohydrate binding module (Xyl3dCBM). GH11 xylanases: Xyl7, a processed variant of Xyl7 (Xyl7qc), Xyl7 without carbohydrate binding module (Xyl7dCBM) and Xyl8, naturally without binding module.

Family	Enzyme	Lower degree of substitution	Randomisation of glucuronic acid substituents	Alkaline treatment of wheat straw	Removal of cellulose	Prevention of xylan self-association
GH10	Xyl1dCBM	0	+	++	++	++
	Xyl1	-	+	++	-	++
	Xyl3dCBM	++	+	++	-	++
	Xyl3	+	+	++	++	+
GH11	Xyl7dCBM	++	0	++	++	-
	Xyl7qc	++	0	++	++	-
	Xyl7	+	-	++	++	0
	Xyl8	+	-	++	+	+

-: Low efficiency
0: No effect
+: High efficiency
++: Very high efficiency

For all endo-xylanases it was found that the carbohydrate binding domain (CBM), being a contiguous amino acid sequence within a carbohydrate-active enzyme with a discreet fold having cellulose-binding activity in these enzymes, did not increase enzyme activity towards insoluble material (Chapters 4 and 5; Van Gool *et al.* (2012c; 2012b)). This result was independent of the position of the CBM, being either at the N- or C-terminal end of the enzyme.

7.3.2 Carbohydrate active enzyme classification system

From the results of chapters 4 and 5, it can clearly be concluded that GH family classification is not indicative for the degradation products formed by these carbohydrate active enzymes. In future research one should, therefore, always be aware of the differences that can be present within a family before making assumptions on the hydrolysing properties of the xylanase.

The carbohydrate active enzymes (CAZy) classification was developed following the enzyme nomenclature of the International Commission on Enzymes. The latter system did not cover all functions characterised experimentally anymore (Henrissat, 1991; Coutinho and Henrissat, 1999; Cantarel *et al.*, 2009). The CAZy classification includes the families of structurally-related catalytic modules and is based on sequence similarities. The classification system promised to reflect the structural features of the enzymes better than their sole substrate specificity and should provide a convenient framework to understand and predict mechanistic properties (Henrissat, 1991; Coutinho and Henrissat, 1999; Cantarel *et al.*, 2009). However, our results showed that even the combination of sequence similarities, protein model structures and substrate specificity is also not completely covering the variations within enzyme families. It has been described that the 3D structure is more conserved than sequence, indicating that sequence similarities are not enough to distinguish 3D structures. There are many examples of domains adopting highly similar 3D structures despite no apparent similarity in sequence and vice versa (Ponting and Russell, 2002). In addition, substitution of only one single amino acid causing differences in substrate specificity will not be distinguished in a comparative 3D approach as has been described in several occasions (Rutten *et al.*, 2009; Paës *et al.*, 2012; Van Gool *et al.*, 2012b).

The research presented in chapters 4 and 5 displayed differences within the CAZy families, thereby indicating that also this system is not able to make a proper distinction between all different enzymes and activities. This aspect is certainly recognised in the CAZy classification system, as illustrated by the ever expanding invention of subfamilies, new families and change of families described for various enzymes (Lombard *et al.*, 2010; St John *et al.*, 2010; Liu *et al.*, 2012; Sakurama *et al.*, 2012). Interestingly, not only the classification of carbohydrate active enzymes is difficult. Recently, a new classification system has been proposed for esterases/lipases, based on various criteria of physico-chemical, chemical, anatomical, or cellular nature (Ali *et al.*, 2012).

A solution for the classification of carbohydrate active enzymes could be to extend the CAZy classification system with subclasses displaying substrate specificity of each enzyme, which is related to (minor) differences in protein structure (Sakurama *et al.*, 2012; Van Gool *et al.*, 2012c; Van Gool *et al.*, 2012b). Nevertheless, this will be an enormous operation to fulfil for all enzymes already classified in the CAZy system.

7.3.3 Protein engineering to reveal structure-function relationship of enzymes

The differences in enzyme performance of the C1 GH10 and GH11 xylanases could be explained by combination of the degradation profile of the various substrates with the predicted protein structure of the enzymes. An alternative route to understand the importance of enzyme primary and secondary structure on performance can be via protein engineering.

Many examples of enzyme-protein engineering could be given in the context of the study presented. Just to illustrate the possibilities, two evident issues are discussed: 1) to confirm that the tyrosine or valine on position 163 in the C1 GH11 xylanases is responsible for the different degradation profile, site-directed mutagenesis on this position followed by the same detailed characterisation should be suitable. By use of crystallographic analysis of the xylanase structure, the exact mechanism of the amino acid on this position interacting with the substrate can be unravelled (Pollet *et al.*, 2009; Paës *et al.*, 2012). 2) The influence of the terminal position of the CBM in hindering efficient substrate docking. This could be examined by replacing the CBM from one terminal end of the enzyme to the other via protein engineering, followed by the same approach as used previously (Chapters 4 and 5; Van Gool *et al.* (2012c; 2012b)).

7.3.4 Accessory enzymes and other proteins involved in cell wall modification

Chapter 6 describes the concerted action of various acetyl (xylan) esterases and glucuronidases. Literature showed that acetylated xylooligomers with 4-*O*-methylglucuronic acid at position *O*-2 of the xylopyranosyl moiety are resistant to

further enzymatic degradation (Chapter 2; Van Gool *et al.* (2011)). Chapter 6 describes the need of synergy of enzymes to degrade such recalcitrant structures as the accessory enzymes are often hindered by neighbouring substituents. In addition, it showed that no effective acetyl (xylan) esterase or glucuronidase family exists that is able to remove all substituents from these oligosaccharides. It could be useful to add other analytical techniques in this study to be able to define positional specificity of the acetyl (xylan) esterases towards the used substrate and to define the lacking enzyme activity necessary to remove all substituents from these oligosaccharides. The analytical tools are not easily available or applicable as was discussed in §7.2.6.

Other proteins involved in cell wall modification, thereby enhancing the effect of cell wall degrading enzymes are expansins, swollenins and loosinins are non-enzymatic plant, fungal or bacterial proteins, respectively. They interact with and modify cell walls and/or cell wall components by an unknown action. They do not contain hydrolysing activity, but can alter the cellulose fiber structure, thereby enhancing the breakdown by cellulases. Further studies will be required in order to determine the mechanism by which these molecules increase lignocellulose conversion and allow industrial exploitation of this class of proteins (Sweeney and Xu, 2012). Nevertheless, they provide a promising tool to increase the enzymatic conversion of lignocelluloses in future.

7.4 Perspectives of biomass degradation

7.4.1 Applications of hemicellulolytic enzymes

The enzymes described in this PhD thesis will contribute to the valorisation of biomass, by conversion of the polysaccharides to monosaccharides. Next to the contribution of the enzymes in the production of biofuels or chemicals, they might also be applied in the food industry. They can be used for clarifying fruit juices, wine and beer and for extracting coffee (Hongpattarakere, 2002). They can also be applied in the bakery industry, where they solubilise the arabinoxylan fraction of the dough,

resulting in increased bread volume and an improved quality of the dough (de Vries and Visser, 2001). Furthermore, hemicellulases are used in non-food industry, e.g. to enhance chemical delignification in pulps for paper making (Suurnäkki *et al.*, 1997). Other applications include the increased nutritional properties of agricultural silage in the feed industry (Fodge and Anderson, 1997).

7.4.2 Applications of biomass degradation products

The conversion of biomass degradation products to high-value co-products seems an attractive opportunity to valorise agro-industrial wastes. The valorisation can be achieved by performing the overall processes with a minimum input of energy and mass and a maximal overall value of the production chain. It will improve the economy of lignocellulose degradation, minimise the waste discharge and reduce the dependence of petroleum-based products (Menon *et al.*, 2012). Examples of chemicals from hemicelluloses are, next to ethanol, xylitol, butanol, ferulic acid, lactic acid, furfural, vanillin and vanillic acid (Menon *et al.*, 2012). Oligosaccharides produced from biomass can be used as functional food additives or alternative sweeteners with beneficial properties (de Vries and Visser, 2001). Similarly, chemicals can be produced from the degradation products of the cellulose and lignin fraction of biomass, e.g. levulinic acid, lactic acid, or syngas products as ammonia and methanol (Menon *et al.*, 2012).

The degradation of biomass deals with the complex and recalcitrant structure of biomass, therefore, it requires a pretreatment to be applicable in industry.

7.4.3 Suitable biomass and pretreatment

Characterisation, understanding and overcoming the barriers for enzymatic hydrolysis of different raw materials is essential for the development of economically competitive processes based on enzymatic treatments (Arantes and Saddler, 2010). Availability and sustainability of feedstocks are major criteria to be addressed. Various feedstocks can be a potential source for bioethanol production. The

complexity of the feedstock determines the need for pretreatments and/or dedicated enzyme mixtures. Until now, a pretreatment is necessary for a feasible production of bioethanol from lignocellulosic biomass, as it was shown that untreated biomass is solubilised to oligo- and monosaccharides only up to 14-34 w/w% by various crude fungal fermentation supernatants (Chapter 3; Van Gool *et al.* (2012a)). The pretreatment can also be seen as a sterilisation step of the feedstock to prevent unwanted microbial growth. However, pretreatments have drawbacks, like the production of undesired products from mono- or oligosaccharides or lignin (Parawira and Tekere, 2011), resulting in loss of saccharides and inhibition of the bioconversion process. Therefore, the pretreatment must be appropriate to achieve the complete potential of lignocellulosic ethanol.

To screen for effective enzymes to be used in lignocellulose saccharification of the pretreated biomass an option could be to screen in the presence of high concentrations lignin or any other inhibiting components. A mild pretreatment of lignocellulosic biomass would be the most favourable option. An optimal balance should be found between pretreatment and enzyme hydrolysis to obtain a profitable process. In case of a mild pretreatment in combination with a variety of enzymes, simultaneous saccharification and fermentation seems to be the most suitable option. No end-product inhibition by the resulting monosaccharides, lower temperature, higher yields of both cellulose- and hemicellulose-derived monosaccharides and shorter residence time are main advantages leading to this conclusion (Wingren *et al.*, 2003).

7.5 Concluding remarks

In conclusion, this thesis shows that the discovery of new complex-xylan degrading enzyme producers is achieved. During the selection procedure of enzyme producing fungi, important factors to take into account are: the fermentation conditions, the screening method, the target substrate and the analytical tools to be used. Many new fungal strains were found, which produce efficient complex-xylan degrading enzymes.

Both the screening approach and the new strains provide opportunities for the enzyme producing industries by discovering enzymes with improved properties for the degradation of complex biomass.

The functional characterisation of endo-xylanases in this study displays the limitations of the current CAZy classification system, as it was found that quite some differences exist within two carbohydrate active enzyme-families studied. The substrate specificity of xylanases from different families is important for defining their suitability for various applications. In addition, the synergistic action of enzymes demonstrated that a mixture of enzymes is needed to obtain full degradation of recalcitrant substrate.

The results obtained in this study can contribute to the economic feasibility of biomass degradation in industry.

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Summary

The degradation products from enzymatically treated lignocellulosic biomass can be valorised by many promising applications, among which the production of bioethanol. By utilisation of the hemicellulolytic part of the biomass, next to the cellulose, additional 10-40% monosaccharides can be obtained.

In the framework of an EU-project, the development of efficient cellulosic and hemicellulosic enzymes for the enhanced hydrolysis of pretreated lignocellulosic biomass, in simultaneous saccharification and fermentation conditions to produce bioethanol was investigated. Within this project, the aim of the presented PhD study was to develop a screening method to detect hemicellulolytic activity in fungal complex fermentation supernatants, to study the performance of purified endo-xylanases and to investigate the function and synergy of accessory xylan degrading enzymes.

Chapter 1 provides a general introduction to biomass and the composition of the plant cell walls. The cell wall degrading enzymes necessary for the degradation of complex biomass are introduced and a brief overview of the production of bioethanol from these feedstocks is given.

As fungi are the principal source for cell wall degrading enzymes, **chapter 2** describes a new screening method enabling the discovery of many individual enzyme activities in complex fungal fermentation supernatants. The screening method uses soluble wheat arabinoxylan and *Eucalyptus* xylan hydrolysate. Through the analysis of the degradation products by high performance anion exchange chromatography and matrix assisted laser desorption/ionization time-of-flight mass spectrometry, combined with the measurement of released acetic acid, different enzyme activities in the fermentation supernatants were identified and quantified. This resulted in the selection of interesting strains for the production of effective hemicellulolytic enzymes.

Next, the screening method was extended with insoluble xylan-rich substrates, which is described in **chapter 3**. The addition of wheat straw and corn fiber to the screening method results in the selection of other strains than those selected on soluble substrates. This indicates that indeed screening towards soluble substrates only is not

sufficient to find active enzymes towards insoluble substrates. The main fungi selected for their production of efficient hemicellulolytic enzymes are *Aspergillus lentulus*, *Penicillium pinophilum* and *Trichoderma harzianum*.

Chapter 4 describes the detailed characterisation of two glycoside hydrolase (GH) family 10 endo-xylanases from *Myceliophthora thermophila* C1 (C1). The endo-xylanases perform differently towards substrates varying in degree and type of substitution. They are more efficient in the degradation of highly substituted xylans compared to low substituted xylans. This is attributed to the self-association of the low substituted xylans. Their different performances and products released could be related to their different predicted secondary protein structure. When the enzymes contain an additional carbohydrate binding module, they do not display enhanced enzyme activity towards the xylan-rich substrates compared to their counterpart without this binding module.

In **chapter 5** the detailed characterisation of two novel GH11 endo-xylanases of C1 is described. They differ in their actions towards various xylans and are very efficient in the degradation of low substituted xylan. They are not hindered by self-association of these low substituted xylans. Both GH11 endo-xylanases show the typical GH11 fold. Hence, their different degradation profiles could not be related to their predicted secondary protein structures. Interestingly, in the alignment a difference in the amino acid at position 163 is found, which seems to be responsible for their rather different behaviour. Also for the GH11 endo-xylanases studied an additional binding module is not beneficial for the degradation of insoluble substrate. The studies in both chapters 4 and 5 show that the general assumption towards the action of GH10 and GH11 enzymes, i.e. GH10 enzymes produce smaller oligomers compared to GH11 enzymes, is not valid for the C1 enzymes tested.

The action of endo-xylanases cannot be maximally profitable without the action of accessory enzymes. In **chapter 6** the concerted action of acetyl (xylan) esterases and glucuronidases towards two *Eucalyptus* wood hydrolysate fractions is described. It is shown that there is a complementary action for the acetyl (xylan) esterases belonging to different carbohydrate esterase (CE) families. The action of *Trichoderma reesei*

acetyl esterase of CE16 is most efficiently deacetylating xylooligomers from *Eucalyptus* in combination with acetyl xylan esterases of CE1. The presence of 4-*O*-methyl glucuronic acid in the acetylated xylooligomers restricts the action of the acetyl (xylan) esterases. The addition of GH115 glucuronidase results in higher amounts of deacetylation and deglucuronidation of the substrates.

In **chapter 7** the achievements of the previous chapters are summarised and discussed in the context of cell wall degrading enzymes. Fungal strains selected for the effective production of hemicellulolytic enzymes are discussed. Furthermore, the influence of the composition of the carbon source in the fermentation medium is addressed as well as the approach and analytical tools used in the screening. The action of the endo-xylanases is discussed and a critical view on the CAZy classification system is raised. Finally, the application of the biomass degradation products is discussed and more options for future industrial applications are given.

Samenvatting

De afbraakproducten van enzymatisch behandelde biomassa bieden vele mogelijke waardevolle toepassingen, waaronder de productie van bio-ethanol. Door het gebruik van de hemicellulose fractie uit biomassa, kan naast de glucose opbrengst uit cellulose, 10-40% extra monosacchariden worden verkregen.

In het kader van een EU-project, werd gezocht naar efficiënte cellulose- en hemicellulose afbrekende enzymen voor de productie van bio-ethanol. Doelstelling hierbij was om een toename in hydrolyse van voorbehandelde lignocellulose-bevattende biomassa te behalen onder gelijktijdige versuikering- en fermentatiecondities.

Binnen dit project richtte het hier beschreven promotieonderzoek zich op de ontwikkeling van een screeningsmethode die hemicellulose afbrekende enzymactiviteit kan detecteren in complexe fermentatievloeistoffen van schimmels, op de prestaties van gezuiverde endo-xylanases en op de functie en synergie van ondersteunende enzymen voor xylaan afbraak.

Hoofdstuk 1 bevat een algemene introductie over biomassa en plantencelwanden. De enzymen die nodig zijn voor volledige afbraak van de celwand worden geïntroduceerd. Daarnaast is een overzicht van bio-ethanolproductie uit verscheidene grondstoffen kort beschreven.

Schimmels zijn de belangrijkste bron voor celwand-afbrekende enzymen. **Hoofdstuk 2** beschrijft een nieuwe screeningsmethode die het mogelijk maakt om diverse enzymactiviteiten te herkennen in complexe fermentatievloeistoffen van schimmels. De methode maakt gebruik van tarwe arabinoxylaan en *Eucalyptus* xylaan. Door middel van analyse met 'high performance anion exchange chromatography' en 'matrix assisted laser desorption/ionization time-of-flight mass spectrometry' in combinatie met meting van vrijgemaakt azijnzuur, kunnen verschillende enzymactiviteiten in de fermentatievloeistoffen worden geïdentificeerd en gekwantificeerd. Dit resulteert in een selectie van schimmels die zeer effectieve hemicellulose-afbrekende enzymen produceren.

Vervolgens is de screeningsmethode uitgebreid door toevoeging van tarwe stro en mais vezel als substraat, zoals is beschreven in **hoofdstuk 3**. Deze uitbreinding op de

screeningsmethode resulteert in andere geselecteerde stammen, dan degene geselecteerd op oplosbare substraten. Dit impliceert dat het gebruik van alleen modelsubstraat in een screening niet afdoende is om actieve enzymen op onoplosbaar xylaan-rijke grondstoffen te vinden. De belangrijkste schimmels, geselecteerd voor de productie van efficiënte hemicellulose-afbrekende enzymen, zijn *Aspergillus lentulus*, *Penicillium pinophilum* en *Trichoderma harzianum*.

Hoofdstuk 4 beschrijft een gedetailleerde karakterisering van twee GH familie 10 endo-xylanases van *Myceliophthora thermophila* C1 (C1). De endo-xylanases vertonen verschillend gedrag op substraten die variëren in type- en mate van substitutie. De enzymen zijn efficiënter in de afbraak van hoog vertakt xylaan dan in de afbraak van laag vertakt xylaan. Dit is toegewezen aan de zelf-associatie van de laag vertakte xylanen. Het verschillende gedrag van de enzymen evenals de gevormde producten kan worden gerelateerd aan een verschil in de hypothetische secundaire eiwit structuur. Een extra koolhydraat-bindingsmodule aan de enzymen was niet van nut voor de afbraak van de xylaan-rijke substraten.

In **hoofdstuk 5** wordt de gedetailleerde karakterisering van twee nieuwe GH familie 11 endo-xylanases van C1 beschreven. Ze verschillen in gedrag ten opzichte van verschillende xylanen en zijn vooral erg efficiënt in de afbraak van laag vertakt xylaan. De GH11 C1 endo-xylanases worden niet gehinderd door zelf-associatie van de laag vertakte xylanen. Beide GH11 endo-xylanases vertonen een typische GH11-vouwing. De verschillende afbraakprofielen kunnen dus niet worden gerelateerd aan de hypothetische secundaire eiwitstructuur. Opvallend genoeg is er een verschil in het aminozuur op positie 163 gevonden, welke verantwoordelijk lijkt te zijn voor het verschillende gedrag van de twee enzymen. Ook voor de GH11 endo-xylanases is een extra bindingsmodule niet van nut voor de afbraak van de xylaan-rijke substraten. De resultaten beschreven in zowel hoofdstuk 4 als 5 laten zien dat de algemene aanname betreffende GH10 en GH11 enzymen, te weten GH10 enzymen produceren kleinere oligomeren in verhouding tot GH11 enzymen, niet geldig is voor de onderzochte C1 enzymen.

Endo-xylanases kunnen vaak niet optimaal functioneren, zonder de actie van ondersteunende enzymen. **Hoofdstuk 6** beschrijft de resultaten van acetyl (xylaan) esterases en glucuronidases die zijn getest op twee *Eucalyptus* hout hydrolysaat fracties. Er is aangetoond dat de acetyl(xylaan)esterases van verschillende koolhydraat-esterase (CE) families elkaar versterken. De CE16 acetylesterase van *Trichoderma reesei* is het meest efficiënt in het verwijderen van acetylgroepen van *Eucalyptus* xylan, in combinatie met de acetylxylaanesterases van CE1. De aanwezigheid van 4-*O*-methyl glucuronzuur in de geacetyleerde xylaanoligomeren hindert de werking van de acetyl(xylaan)esterases. Door toevoeging van een GH115 glucuronidase wordt hogere deacetylering en deglucuronosylering van de substraten behaald.

In **hoofdstuk 7** zijn de behaalde resultaten uit de voorgaande hoofdstukken samengevat en bediscussieerd. De geselecteerde schimmels voor de efficiënte productie van hemicellulases worden besproken en de invloed van de samenstelling van de koolstofbron in het fermentatiemedium wordt behandeld. Tevens worden de aanpak en de analytische technieken van de screeningsmethode geëvalueerd. De endo-xylanases worden bediscussieerd en een kritische blik wordt geworpen op het CAZy classificatie systeem. Tenslotte wordt de toepassing van de afbraakproducten uit biomassa besproken.

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Martine

About the author

Curriculum vitae

List of publications

Overview of completed training activities

Curriculum vitae

Martine Paula van Gool was born on the 18th of March 1982 in Leiden. After graduating from pre-university education (VWO) at the Sint Adelbert College in Wassenaar in 2000, she started her study in Food Technology at Wageningen University, specialising in Product Functionality. Her minor thesis was performed at the Department of Product Design and Quality Management. Her major thesis was performed at the Laboratory of Food Chemistry, focusing on the function and controlled degradation of soy saponins. Martine spent the last 5 months of her study in Montpellier, France, where she studied the influence of carbonation and iron fortification on the behaviour of milk caseins at the Polytech'Montpellier institute of the Université de Montpellier II.

After graduating in September 2006, she was working as product developer at Koninklijke Peijnenburg for one year. Afterwards, she spent 10 months at the Laboratory of Food Chemistry of Wageningen University, working on the proof of principle of a project entitled 'Advanced pretreatment for common biomass feedstocks'.

In August 2008 she started her PhD, of which the results are discussed in this thesis. She is now temporarily employed as post-doc researcher at the Laboratory of Food Chemistry of Wageningen University, while looking for a new job.

List of publications

Van Gool, M. P., Van Muiswinkel, G. C. J., Hinz, S. W. A., Schols, H. A., Sinitsyn, A. P. and Gruppen, H. (2012). Intra- and interfamily differences of two novel GH11 endo-xylanases from *Myceliophthora thermophila* C1 towards soluble and insoluble xylans, *submitted for publication*.

Toth, K., **Van Gool, M.P.**, Schols, H.A., Samuels, G.J., Gruppen, H., Szakacs, G. (2012). Diversity in production of xylan degrading enzymes among species belonging to the *Trichoderma* section Longibrachiatum, *submitted for publication.*

Makaravicius, T., Basinskiene, L., Juodeikiene, G., **Van Gool, M. P.** and Schols, H. A. (2012). Production of oligosaccharides from extruded wheat and rye biomass using enzymatic treatment, Catalysis Today, *in press*.

Van Gool, M. P., Van Muiswinkel, G. C. J., Hinz, S. W. A., Schols, H. A., Sinitsyn, A. P. and Gruppen, H. (2012). Two GH10 endo-xylanases from *Myceliophthora thermophila* C1 with and without cellulose binding module act differently towards soluble and insoluble xylans, Bioresource Technology 119: 123-132.

Van Gool, M. P., Toth, K., Schols, H. A., Szakacs, G. and Gruppen, H. (2012). Performance of hemicellulolytic enzymes in culture supernatants from a wide range of fungi on insoluble wheat straw and corn fiber fractions, Bioresource Technology 114: 523-528.

Van Gool, M. P., Vancsó, I., Schols, H. A., Toth, K., Szakacs, G. and Gruppen, H. (2011). Screening for distinct xylan degrading enzymes in complex shake flask fermentation supernatants, Bioresource Technology 102 (10): 6039-6047.

Gullón, P., González-Muñoz, M. J., **Van Gool, M. P.**, Schols, H. A., Hirsch, J., Ebringerová, A. and Parajó, J. C. (2011). Structural features and properties of soluble products derived from *Eucalyptus globulus* hemicelluloses, Food Chemistry 127 (4): 1798-1807.

Gullón, P., González-Muñoz, M. J., **Van Gool, M. P.**, Schols, H. A., Hirsch, J., Ebringerová, A. and Parajó, J. C. (2010). Production, refining, structural characterization and fermentability of rice husk xylooligosaccharides, Journal of Agricultural and Food Chemistry 58 (6): 3632-3641.

Overview of completed training activities

Discipline specific activities

- Advanced Food Enzymology, Wageningen, The Netherlands, 2008
- Summer Course Glycosciences, Wageningen, The Netherlands, 2008
- EPNOE-Utrecht meeting‡, Utrecht, The Netherlands, 2009
- Polysaccharides as a source of advanced materials†, Turku, Finland, 2009
- 61. Starch convention, Detmold, Germany, 2010
- 6. Bioethanol Technology meeting‡, Detmold, Germany, 2010
- Advanced Food analysis†, Wageningen, The Netherlands 2010
- Plant and Seaweed Polysaccharides workshop‡, Nantes, France, 2012

General courses

- PhD introduction week, Bilthoven, The Netherlands, 2008
- Philosophy and Ethics of Food Science & Technology, Wageningen, The Netherlands, 2008
- Techniques for writing and presenting a scientific paper, Wageningen, The Netherlands, 2009
- Project and Time management, Wageningen, The Netherlands, 2009
- Information Literacy for PhD-students, Wageningen, The Netherlands, 2010
- Kwantificeren en rapporteren in Xcalibur software, Wageningen, The Netherlands, 2008
- Career assessment, Wageningen, The Netherlands, 2012

Optionals

- Preparing PhD research proposal, Wageningen, The Netherlands, 2008
- Food Chemistry PhD trip, Beijing and Shanghai area, China, 2008
- Food Chemistry study trip‡, Ghent, Belgium, 2009
- Organization PhD trip Switzerland/Italy, Wageningen, The Netherlands, 2009/2010
- Food Chemistry PhD trip‡, Switzerland and Italy, 2010
- Food Chemistry Seminars, Wageningen, The Netherlands, 2008-2012
- Food Chemistry Colloquia, Wageningen, The Netherlands, 2008-2012
- DISCO EU-project meetings‡, Europe 2008-2012

† Poster presentation
‡ Oral presentation

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Notes

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