

**The role of accessory enzymes in enhancing the effective
hydrolysis of the cellulosic component of pretreated
biomass**

by

Jinguang Hu

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF**

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate and Postdoctoral Studies

(Forestry)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

March 2014

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Abstract

To achieve effective cellulose hydrolysis requires the synergistic cooperation of various cellulases and accessory enzymes/proteins. Most of previous synergism studies have used “model” cellulosic substrates, such as cotton or Avicel, and focused on the initial stages of hydrolysis. Previous studies have also demonstrated that the extent of synergism was influenced significantly by the composition and concentration of “cellulase” mixture and the nature of cellulosic substrate. To gain a better understanding of “cellulase synergism”, the actions of individual and combinations of cellulases, β -glucosidase and “accessory” enzymes (such as xylanases, xyloglucanases and AA9) were assessed on various pretreated lignocellulosic substrates at different enzyme loadings. The synergistic cooperation between cellulases and xylanases was found to enhance the extent of hydrolysis of steam pretreated corn stover and dramatically reduced the required cellulase dosage (about 7 times) needed to achieve reasonable cellulose hydrolysis (>70%). Xylanases appeared to act cooperatively with cellulases by solubilising the xylan and consequently increasing fibre swelling and cellulose accessibility. However, the observed synergism between the cellulase monocomponents and hemicellulases was highly substrate dependent. Those hemicellulases with broader substrate specificities, such as family 10 xylanase and family 5 xyloglucanase, promoted the greatest improvement in the hydrolytic performance of cellulases on a broader range of substrates. The “boosting effect” of AA9 on cellulase hydrolytic performance was highest on substrates showing a higher degree of accessible crystalline, rather than amorphous cellulose. The synergistic cooperation was probably, at least in part, due to AA9s oxidative cleavage, resulting in negatively charged sites on the cellulose. This likely increased the more rapid turn-over of processive enzyme CBHI. A greater degree of

synergism among cellulase components was demonstrated at lower enzyme concentrations and on pretreated substrates containing relatively accessible/disordered cellulose. Higher xylanase loadings were required to derive an “optimized mixture” when high solid loadings and substrates with a higher xylan content were used, while the addition of low amounts of AA9 (2mg/g cellulose) was beneficial in all cases. Determining the optimum enzyme composition for a particular substrate was shown to be a key strategy for reducing the protein loading required to achieve effective hydrolysis of pretreated biomass substrates.

Preface

List of publications

- Paper 1. Hu J, Arantes V, Saddler JN. 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? *Biotechnology for Biofuels*. 4:36.
- Paper 2. Hu J, Arantes V, Pribowo A & Saddler JN. 2013. The synergistic action of accessory enzymes enhances the hydrolytic potential of a "cellulase mixture" but is highly substrate specific. *Biotechnology for Biofuels* 6:112.
- Paper 3. Hu J, Arantes V, Pribowo A, Gourlay K, & Saddler JN. 2013. Hydrolytic and Oxidative Cellulose Cleavage Act Through a Synergistic Mechanism in Biomass Deconstruction. *In preparation*.
- Paper 4. Hu J, Arantes V, Pribowo A, Gourlay K, & Saddler JN. 2013. Synergistic cooperation between cellulase components during lignocellulose deconstruction. *In preparation*.
- Paper 5. Hu J, Arantes V, Pribowo A, Gourlay K, & Saddler JN. 2013. Can synergistic cooperation between cellulase and accessory enzymes enhance the high solid loading hydrolysis of pretreated lignocellulose?. *In preparation*.
- Paper 6. Shen F, Hu J, Zhong Y, Liu M, Saddler JN, Liu R. 2012. Ethanol production from steam-pretreated sweet sorghum bagasse with high substrate consistency enzymatic hydrolysis. *Biomass and Bioenergy*. 41:157-164.
- Paper 7. Gourlay K, Hu J, Arantes V, Andberg M, Saloheimo M, Penttila M, Saddler JN. 2013. Swollenin aids in the amorphogenesis step during the enzymatic hydrolysis of pretreated biomass. *Bioresource Technology*. 142:498-503.
- Paper 8. Pribowo A, Hu J, Arantes V, Saddler JN. 2013. The development and use of an ELISA-based method to follow the distribution of cellulase monocomponents during the hydrolysis of pretreated corn stover. 6:80.
- Paper 9. Shen F, Kumar L, Hu J, Saddler JN. 2011. Evaluation of hemicellulose removal by xylanase and delignification on SHF and SSF for bioethanol production with steam-pretreated substrates. 102:8945-51.

For paper 1 to 5, Jinguang Hu (JH), Jack Saddler (JS), and Valdeir Arantes (VA) contributed to the planning of the experimental work, interpretation of the results, and drafting of the manuscripts. JH carried out much of the laboratory work. For paper 6 to 9, JH helped develop the research and performed some of the experimental work.

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List of units and abbreviations

AA9	auxiliary activity family 9
AFEX	ammonia fibre expansion
AFM	atomic force microscopy
AIL	acid insoluble lignin
Ara	arabinan
ASL	acid soluble lignin
AX	arabinoxylan
BG	β -glucosidases
BMCC	bacterial microcrystalline cellulose
BSA	bovine serum albumin
CBH	exoglucanase
CBM	carbohydrate binding modules
CD	catalytic domain
CDH	cellobiodehydrogenase
CMC	carboxymethyl cellulose
CNC	cellulose nanocrystal
CrI	crystallinity
dA	de-acetylated
dl	delignified
DNS	3,5-Dinitrosalicylic acid
DP	degree of polymerization
DS	degree of synergism
DSP	dissolving pulp
DW	dry weight
EG	endoglucanase
ELISA	enzyme linked immunosorbent assay
EX	endo-xylanase
FPA	filter paper activity
FPLC	fast protein liquid chromatography
FQA	fibre quality analyzer

Gal	galactan
GH	glycoside hydrolase
Glu	glucan
GPC	gel permeation chromatography
GX	glucuronoxylans
HPLC	high performance liquid chromatography
IEA	international energy agency
LCC	lignin carbohydrate complexes
LPMOs	lytic polysaccharide monooxygenases
Man	mannan
M-DSP	mechanically modified dissolving pulp
MIX	reconstituted cellulase mixture
OPCS	organosolv pretreated corn stover
OPP	organosolv pretreated poplar
OPLP	organosolv pretreated lodgepole pine
PAGE	polyacrylamide gel electrophoresis
PASC	phosphoric acid-swollen cellulose
p-NPC	p-nitrophenyl- β -D-cellobioside
p-NPG	p-nitrophenyl- β -D-glucopyranoside
p-NPX	p-nitrophenyl- β -D-xylopyranoside
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SPCF	steam pretreatment corn fibre
SPCS	steam pretreatment corn stover
SPLP	steam pretreatment lodgepole pine
SPP	steam pretreatment poplar
SPSB	steam pretreatment sweet sorghum bagasse
SPSCB	steam pretreatment sugar cane bagasse
SPWS	steam pretreatment wheat straw
SS	Simons' stain
SSF	simultaneous saccharification and fermentation

TEA	triethanolamine
THF	tetrahydrofuran
XG	xyloglucanase
XRD	x-ray Diffraction
Xyl	xylan

Acknowledgements

I would like to express my sincere appreciation to my senior supervisor Dr. Jack Saddler for his mentorship, encouragement, support and guidance throughout my PhD studies. I would also like to thank my committee members Dr. Doug Kilburn and Dr. Paul McFarlane for their invaluable advice, guidance and support and contributions to the direction of this research.

Special thanks also go to Dr. Valdeir Arantes for selflessly helping me on this ride and for providing me with the tools for being the best scientist as I can be. I also wish to extend my thanks to all of the members in Forest Products Biotechnology/Bioenergy group for their friendship, support and encouragement.

I would also like to thank the Chinese Scholarship Council (CSC) for providing me the funding for this 4 years study.

1. Introduction

1.1 Background

The transition from the traditional “oil-refinery” to a renewable, biomass-based “bio-refinery” is crucial if we are to move to a more environmentally friendly economy (Ragauskas et al., 2006). However, commercially unattractive high enzyme loadings are still required to efficiently hydrolyse lignocellulosic materials into a sugar platform, which can subsequently be converted into various fuels and chemicals (Merino and Cherry, 2007; Stephen et al., 2012). One way to reduce the amount of enzyme usage is to improve the hydrolytic efficacy of “cellulase” mixtures. Although there has been a considerable amount of work done on improving individual cellulase properties such as binding affinity, catalytic activity, and thermostability (Zhang et al., 2006; He Jun et al., 2009; Wilson, 2009a), in most cases the extent of improvement achieved on simple, model cellulosic substrates does not directly translate into improved activity on the more complex, heterogeneous, lignocellulosic substrates (Zhang et al., 2006). In parallel, an alternative strategy has involved utilization of the synergistic cooperation among cellulase monocomponents and various accessory enzymes such as xylanases and AA9/GH61 (Wilson, 2009b; Harris et al., 2010; Hu et al., 2011). This approach has been shown to increase enzyme accessibility to the cellulose, and therefore the overall “specific activity” of the enzyme mixture.

Over the past few decades the mechanism of cellulose hydrolysis has been extensively researched since the “C1-Cx” hypothesis was first proposed by Reese et al. (1950). At that time, the mechanism of cellulose hydrolysis was suggested to be a result of an initial attack by a non-hydrolytic chain-separating factor “C1”, which was thought to

result in swelling of the cellulose, thereby facilitating subsequent attack by the hydrolytic enzyme complex “Cx”. Later, when it was shown that the reconstituted enzyme mixture, containing exoglucanases, endoglucanases and β -glucosidase, could effectively hydrolyze crystalline cellulose, the C1-Cx hypothesis was reinterpreted to suggest that, rather than C1 representing a “swelling factor”, the C1 and Cx components were in fact the exo- and endo-cellulase enzymes, respectively (Wood and McCrae, 1977; White and Brown, 1981). This suggested mechanism became the more widely-accepted model for cellulose breakdown which involves the synergistic cooperation between endo- and exo-types of cellulase enzymes (Henrissat et al., 1985; Woodward et al., 1988a). In the early nineties, multi-domain cellulase structures were identified, and the C1-Cx hypothesis was again revisited. In this iteration of the theory, it was suggested that the carbohydrate binding modules (CBMs) of cellulases could act as the C1 component, opening up the cellulose structure for subsequent attack by the Cx component, comprised of the catalytic domain (CD) of the cellulase (Din et al., 1991; 1994). The recently identified non-hydrolytic disruptive proteins, such as swollenin and expansin-like proteins, has once again revived the “C1-Cx” theory (Quiroz-Castaneda et al., 2011; Zhou et al., 2011; Gourlay et al., 2013). Although the “C1” concept is attractive, it is still hard to prove the existence of such a swelling factor. Alternatively, the recently identified lytic polysaccharide monooxygenases (LPMOs), such as AA9 (Auxiliary Activity family 9, previously known as GH61), are also expected to significantly increase the access of cellulases to cellulose by the oxidative cleavage/disruption of the crystalline cellulose region (Phillips et al., 2011; Quinlan et al., 2011). Thus, LPMOs seem to show some potential for improving the hydrolytic efficiency of cellulase enzymes.

In the case of lignocellulosic decomposition, a pretreatment process is first required to “open up” the complex biomass matrix, with thermochemical pretreatment being one example. High severity pretreatments can help create more easily digestible cellulose-enriched substrates. However, this is economically unattractive due to the low hemicellulose derived carbohydrate recovery and the high energy input required (Chandra et al., 2007; Yang and Wyman, 2008; Alvira et al., 2010). Thus, in many pretreatment strategies, such as steam explosion, mild severity conditions are often used to avoid or at least minimize sugar loss during pretreatment. Under these milder pretreatment conditions, some of the hemicellulose, mostly xylan in agricultural residues and hardwood, remains associated with the cellulosic-rich water insoluble fraction (Chandra et al., 2007). However, this residual hemicellulose component is known to exert a significant influence on the effectiveness of enzymatic hydrolysis of the cellulosic component (Bura et al., 2009; Kumar and Wyman, 2009b; Varnai et al., 2010). One of the general goals of the work described in this thesis was to investigate the reduction in the amount of cellulase enzymes that can be achieved by utilizing the synergistic cooperation between cellulases and hemicellulases, such as xylanases. An initial objective of this study was to assess how effective the xylanase-cellulase synergism would be on steam-pretreated corn stover. A review of the literature has indicated that previous studies in this area had used high amounts of xylanase supplementation that can be difficult to justify because of the increased enzyme costs (Berlin et al., 2006b; Kumar and Wyman, 2009b; Shen et al., 2011). The beneficial effect of supplementing xylanases into cellulase mixtures is thought to be a result of the improved cellulose accessibility resulting from xylan removal (Ohgren et al., 2007; Kumar and Wyman, 2009c; Morais et al., 2010). However, the fact that xylanase supplementation also has an impact on substrates with a very

low xylan content (Garcia-Aparicio et al., 2007; Kumar and Wyman, 2009c) indicates that xylanases might do more than just removing “blocking” xylan.

As will be discussed in detail within the main body of the thesis, when xylanases were used to partially replace the minimum amount of cellulase enzymes required to achieve 70% cellulose hydrolysis, a strong synergistic cooperation between cellulases and xylanases was apparent, which resulted in three times faster cellulose and xylan hydrolysis and a seven-fold decrease in cellulase loading (Hu et al., 2011). It was also apparent that the “blocking effect” of xylan was one of the major mechanisms that limited the accessibility of the cellulase enzymes to the cellulose. However, the synergistic interaction of the xylanase and cellulase enzymes was also shown to significantly improve cellulose accessibility through increased fiber swelling and fiber porosity (Hu et al., 2011).

One key component of the work was to better understand the synergistic interaction between cellulase monocomponents and various accessory enzymes during lignocellulose biodegradation and to formulate a more efficient and robust “enzyme cocktail” on a range of pretreated lignocellulosic substrates. Previous studies have demonstrated that xylanases from different glycoside hydrolase (GH) families had different specific activities on “model” xylanolytic substrates (Viikari et al., 1994; Pell et al., 2004; Ustinov et al., 2008) and also displayed different hydrolytic potentials on various lignocellulosic substrates (Beaugrand et al., 2004; Kim et al., 2004; Zhang et al., 2011a). In addition, xyloglucanases have been demonstrated to enhance the hydrolyzability of various lignocellulosic substrates when added to a cellulase mixture (Nishitani, 1995; Benko et al., 2008). Thus, one of the main objectives of this work was to increase our understanding of the synergistic cooperation of cellulases

and various hemicellulases on a range of substrates and the manner in which enzyme mixtures have to be adapted for different substrates.

Besides the heterogeneous nature of lignocellulose architecture, the highly organized, crystalline cellulose structure, present in low-severity pretreated lignocellulosic biomass, also restricts the hydrolytic performance of cellulase enzymes considerably (Chandra et al., 2007; Yang and Wyman, 2008). Recently, the accessory enzyme AA9/GH61 has attracted much attention as it can boost the hydrolytic potential of cellulases by oxidative cleavage of the highly ordered, crystalline cellulose regions (Phillips et al., 2011; Quinlan et al., 2011). In nature, brown-rot fungi, which are among the most effective microorganisms for cellulose degradation, have both cellulose hydrolytic oxidative cleavage systems (Kerem et al., 1999; Baldrian and Valaskova, 2008). This is similar to the two-type system that has also been identified for efficient biodegradation of chitin (Vaaje-Kolstad et al., 2010). Chitin is a cellulose derivative where the 2-hydroxy group is replaced with an acetyl amine group. Over the last few years, LPMOs were identified from many cellulolytic microorganisms (Harris et al., 2010), thus it is highly likely that an effective “cellulase cocktail” will also require the addition of a LPMO if we are to achieve fast and efficient cellulose hydrolysis with low enzyme loading.

As well as increasing the hydrolytic potential of enzyme mixtures by employing the synergism between cellulases and accessory enzymes, it is also important to obtain a better understanding of the synergistic interaction among cellulase monocomponents on lignocellulosic materials. Most previous studies have used “pure” cellulosic substrates such as Avicel, filter paper or cotton as the substrate to assess cellulase synergistic interactions (Chanzy and Henrissat, 1985; Converse et al., 1988; Jeoh et al., 2002; 2006). It has been

shown that the extent of cellulase synergism is very dependent on the enzyme concentrations used, as well as the substrate characteristics (Henrissat et al., 1985; Woodward et al., 1988a; 1991). Depending on the choice of pretreatment technique and the nature of the biomass substrate, several cellulose characteristics, e.g. cellulose accessibility, degree of polymerization (DP), crystallinity, and morphology can vary significantly among various pretreated substrates (Chandra et al., 2007; Yang and Wyman, 2008). Thus, assessing synergistic interaction of cellulases on a “library” of pretreated biomass substrates might provide us with invaluable information, not only expanding our fundamental knowledge of biomass degradation, but also contributing to the design of more efficient biomass degrading systems.

Previous studies have predominantly assessed enzyme synergism at low solid loadings, which is unlikely to be used on a commercial scale due to the low sugar concentrations that can be obtained, even at high levels of conversion (Hu et al., 2011; 2013; Harris et al., 2010; Jeoh et al., 2006). It was therefore an objective of this study to assess the impact of high-solid loadings on the synergistic interaction between cellulases and accessory enzymes. It was expected that the synergistic cooperation between cellulases and accessory enzymes would be intensified at high solid hydrolysis as some accessory enzymes, such as xylanases, might help the biomass liquefaction process by breaking down the hemicellulose-lignin matrix within the biomass. Therefore it would be useful if we could to optimize an appropriate enzyme mixture for high consistency hydrolysis (20%) by better utilizing the synergistic cooperation between cellulase enzymes and essential accessory enzymes, especially with the goal of reducing the amount of enzyme loading.

1.2 Biomass to sugar platform

Trends, such as the volatile and increasing oil price, global sustainability concerns such as climate change, and the economic malaise, have all influenced the ongoing and significant investment in what is generally termed as the “biorefinery” process (IEA, 2012). One of the essential strategies in a “biorefinery” is to hydrolyze the polysaccharides within the biomass into a sugar platform, which can be used as the feedstock for “bioconversion technologies” to produce sustainably derived products such as fuels and chemicals (Sims et al., 2010).

The development of biomass to sugar platforms in the past has been primarily based on sugar cane and starch crops such as corn, the two largest “sugar platform” industries in the world, with the former dominating in Brazil and the latter in the USA (Balat and Balat, 2009). The commercial success of these processes has increased interest in sustainable alternative approaches that convert lignocellulosic biomass into fermentable sugars. However, unlike sugar/starch-rich biomass, which is naturally designed as an energy-storage polymer in plants and is relatively easy to degrade into monomeric sugars, lignocellulosic biomass has evolved to withstand fast and effective microbial/enzymatic decomposition as it serves as structural materials in plants. Currently, the limited hydrolytic efficiency of “cellulase mixtures” for the deconstruction of lignocellulosic biomass is still one of the major techno-economic bottlenecks for achieving an economically feasible lignocellulose-derived sugar platform biorefinery (Stephen et al., 2012).

1.3 Recalcitrant characteristics of lignocellulosic biomass

Plants are composed of various cell types with a thick and complex cell wall structure to protect the plant from biological, chemical and physical attacks by the microbial and animal kingdoms, imparting a high level of biomass recalcitrance (Himmel et al., 2007). Biomass recalcitrance is a complex phenomenon that is governed by various physicochemical properties. Among them, several biomass properties, such as the heterogeneity of the cell wall constituents (e.g. the type, amount and distribution of hemicellulose and lignin) and the highly organized cellulose structure significantly restrict the accessibility of cellulolytic enzymes to cellulose (Himmel et al., 2007; Chundawat et al., 2011).

1.3.1 Cellulose structure and characteristics

Cellulose is the most abundant organic polysaccharide on earth and accounts for approximately 35% to 50% of the dry weight of plant biomass. Cellulose is also produced by a number of bacteria, algae, and tunicates (Lynd et al., 2002). Cellulose is a linear polymer of D-glucose units joined via β -1,4 glycosidic bonds, where the basic repetitive unit is cellobiose, a disaccharide with the glucose residues rotated 180° relative to each other along the main axis (Figure 1). Despite the simple chemical structure, the physical properties of cellulose, such as degree of polymerization (DP), crystallinity and allomorphic forms are highly variable (OSullivan, 1997).

The length of cellobiose is about 1.04 nm and a typical cellulose chain length (DP), depending on its source, varies from 100 to 10,000 (OSullivan, 1997). Although glucose is a

highly water soluble molecule, the solubility of cellooligomers decreases dramatically with an increase in DP. For example, cellohexaose (DP=6) is barely soluble in water. Every glucose molecule interacts through four intra-molecular hydrogen bonds with the adjacent two glucose molecules in the same glucan chain, and also through two inter-molecular hydrogen bonds between chains in the same layer (Figure 1) (Lynd et al., 2002; Saxena and Brown, 2005). The inter- and intra-molecular hydrogen bonds keep the chains straight and stacked in a sheet-like structure. Although no hydrogen bonds are found between the sheets, van der Waals forces appear to hold the sheets together (Saxena and Brown, 2005). Cellulose forms a highly organized, crystalline structure (interspersed by some disorganized amorphous or paracrystalline regions) under these strong hydrogen bonds and van der Waals forces (Cosgrove, 2005). This restricts the penetration of enzymes, or even small molecules, like water, into the structure entities (OSullivan, 1997; Arantes and Saddler, 2010). It has been proposed that the tightly packed crystalline regions are one of the major reasons for the low saccharification efficiency of cellulose, due to the limited accessibility of cellulase enzymes (Arantes and Saddler, 2010; Chundawat et al., 2011).

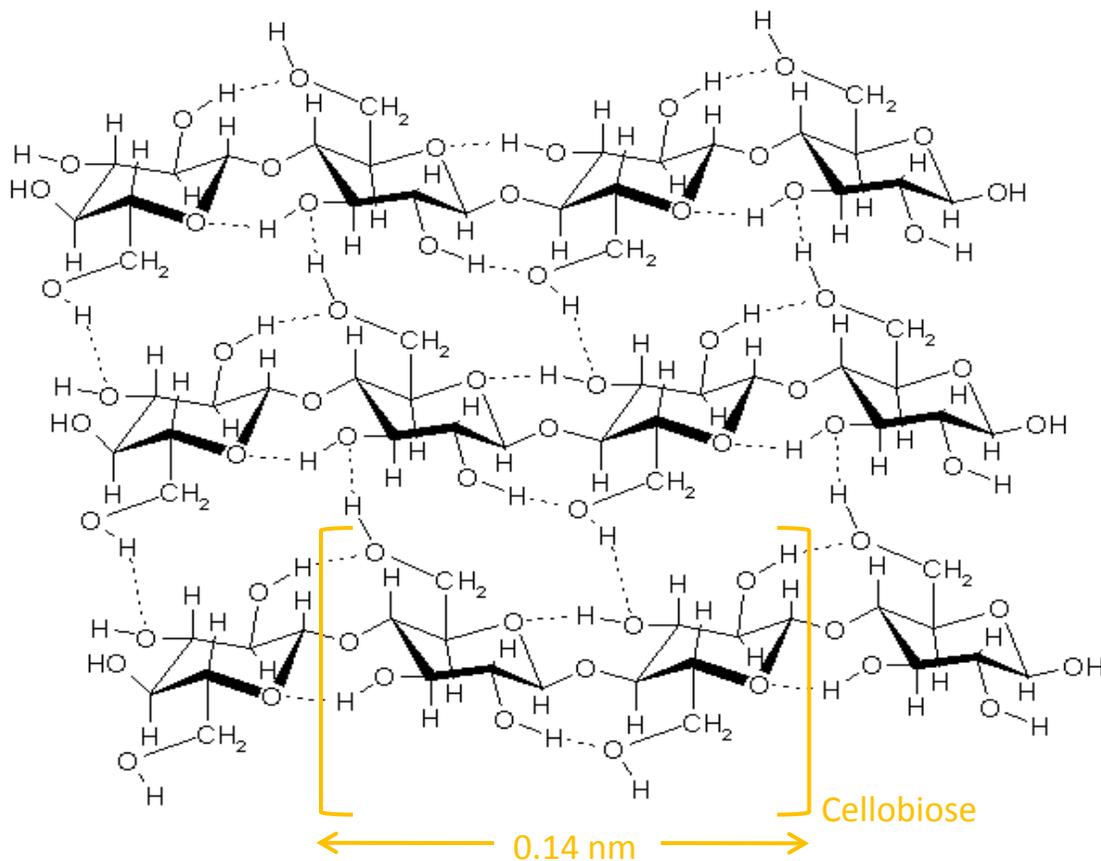


Figure 1. Cellulose structure

(http://www.doitpoms.ac.uk/tlplib/wood/structure_wood_pt1.php)

The most abundant cellulose allomorphic form in nature is defined as cellulose I, in which the triclinic cellulose I_{α} is dominant in bacterial and algal cellulose, while monoclinic cellulose I_{β} is dominant in higher plant cell walls (Atalla and Vanderhart, 1984). The cellulose I_{β} form is more stable than the I_{α} form, thus I_{β} cellulose is less susceptible to enzymatic hydrolysis (Lynd et al., 2002). Thermochemical treatments can transform cellulose I into other allomorphic forms, such as cellulose II by NaOH, cellulose III by ammonia, and cellulose IV by glycerol (Ishikawa et al., 1997). All these different cellulose allomorphs have been shown to influence the efficacy of downstream, enzymatic hydrolysis processes (Lynd et al., 2002).

1.3.2 Complex cell wall structure

In the plant cell wall, individual cellulose chains join together, shortly after biosynthesis, into elementary microfibrils, which consist of about 36 cellulose chains (3 to 5 nm in diameter). The elementary microfibrils are further packed into a hemicellulose-lignin matrix to form the rigid cell wall structure (Figure 2A) (Somerville et al., 2004; Cosgrove, 2005). These non-cellulosic components further increase the difficulty of the cellulose biodecomposition process by limiting the accessibility of cellulases to cellulose, causing unproductive adsorption of cellulase enzymes, and inhibiting the catalytic activity of cellulase enzymes (Lynd et al., 2002; Zhang and Lynd, 2004).

1.3.2.1 Hemicellulose

Hemicellulose is the second most abundant, renewable polysaccharide in nature after cellulose and accounts for about 20-35% of lignocellulosic biomass. Hemicellulose can be grouped into xylans (β -1,4-xylosyl backbone with arabinose, acetyl and uronic acid side chains), mannans (β -1,4-mannosyl backbone and/or glucosyl-mannosyl backbone with galactose side chains), xyloglucan (β -1,4-glucosyl backbone with xylose and other substituents), and β -glucan (β -1,3-1,4-glucosyl backbone). Unlike cellulose, hemicellulose composition differs between plant species and between cell tissues (Saha, 2003; Girio et al., 2010; Scheller and Ulvskov, 2010).

Xylan, a major hemicellulose component, has been found in large quantities in hardwoods (15-30% dry weight) and annual plants (<30%), but is less abundant in softwood (7-12%) (Wong et al., 1988; Viikari et al., 1994). Xylans are a diverse group of highly branched heteropolymers with a backbone of β -1,4-linked xylose residues, which are

substituted with glucuronosyl or 4-O-methyl glucuronosyl residues at the C-2 position, in glucuronoxylans (GX), or substituted with arabinofuranosyl residues at the C-3 position, in arabinoxylan and/or glucuronoarabinoxylans (AX) (Figure 2C) (Wong et al., 1988; Viikari et al., 1994; Saha, 2003). In addition, most xylans are acetylated to various degrees, normally at the C-3 position on xylopyranose (Wong et al., 1988; Viikari et al., 1994; Saha, 2003). The major xylan in hardwood (15-30% dry weight) is highly acetylated GX with a 10:7 ratio of xylose to acetyl. On the other hand, xylans in grasses (<30%) are mainly AX, with substitutions of various combinations of acetyl, arabinosyl, galactosyl, glucuronosyl, and xylosyl residues (Viikari et al., 1994). In softwood, a third of the hemicellulose consists of AX (7-12%), with a 4-O-methylglucuronic acid group found at the C-2 position at approximately every 5 xylose residues (Wong et al., 1988; Viikari et al., 1994). The structure and distribution of xylans vary among different plant species, and xylans interact with other plant carbohydrates through covalent (xylan-lignin, xylan-pectin, and xylan-protein) and non-covalent (xylan-cellulose, xylan-hemicellulose) associations, which contribute to fiber cohesion and cell wall integrity (Saha, 2003).

Mannan is the major hemicellulose in the plant cell walls of softwoods and consists of linear or branched polymers derived from mannose, glucose and galactose (Popper, 2008). Depending on the backbone and the galactose side chains, mannans can be grouped into mannans and galactomannans (backbones are entirely mannose), or glucomannan and galactoglucomannans (backbones containing mannose and glucose) (Moreira and Filho, 2008). In softwoods, the principal hemicellulose components are galactoglucomannan (mannose/glucose/galactose ratio 3:1:1) and glucomannan (mannose/glucose ratio 3:1), while a small portion of glucomannan (<5%) also exists in hardwoods with a mannose/glucose

ratio of 2:1 (Puls et al., 1993). Some of the mannosyl units in the mannan backbones are also substituted by O-acetyl groups (Moreira and Filho, 2008).

Xyloglucan, which is the most abundant hemicellulose in primary cell walls, has a cellulose-like backbone of β -1,4-linked D-glucopyranose residues with a D-xylopyranose substituted at the C-6 position in the majority of the glucopyranose residues (Scheller and Ulvskov, 2010). The xylose units can be further linked with other substituents and a special on-letter nomenclature system is used to denote these differences (Benko et al., 2008; Scheller and Ulvskov, 2010). The content of xyloglucan depends on the plant species, where it makes up about 20-25% of the dry weight of the primary cell wall in hardwood, 2-5% in grass, and 10% in softwood (Benko et al., 2008; Scheller and Ulvskov, 2010). Typically, there are three domains of xyloglucan found in plant cell walls. The first domain constitutes the cross-links between cellulose microfibrils as a tether; while the second domain binds to the surface of microfibrils; and the third domain is entrapped within the microfibrils (Pauly et al., 1999).

β -glucan is a mixed linkage glucan with cellootriosyl and cellotetrasyl (β -1,4-linked) segments linked by β -1,3-linkages, but longer β -1,4-linked units also exist. These β -(1,3; 1,4)-glucans are mostly found in grasses and they play an important role in cell expansion. So far, β -glucan has not been found in dicots, and the quantitative analysis of β -glucan is difficult as they are strongly growth-stage dependent (Burton et al., 2006; Scheller and Ulvskov, 2010).

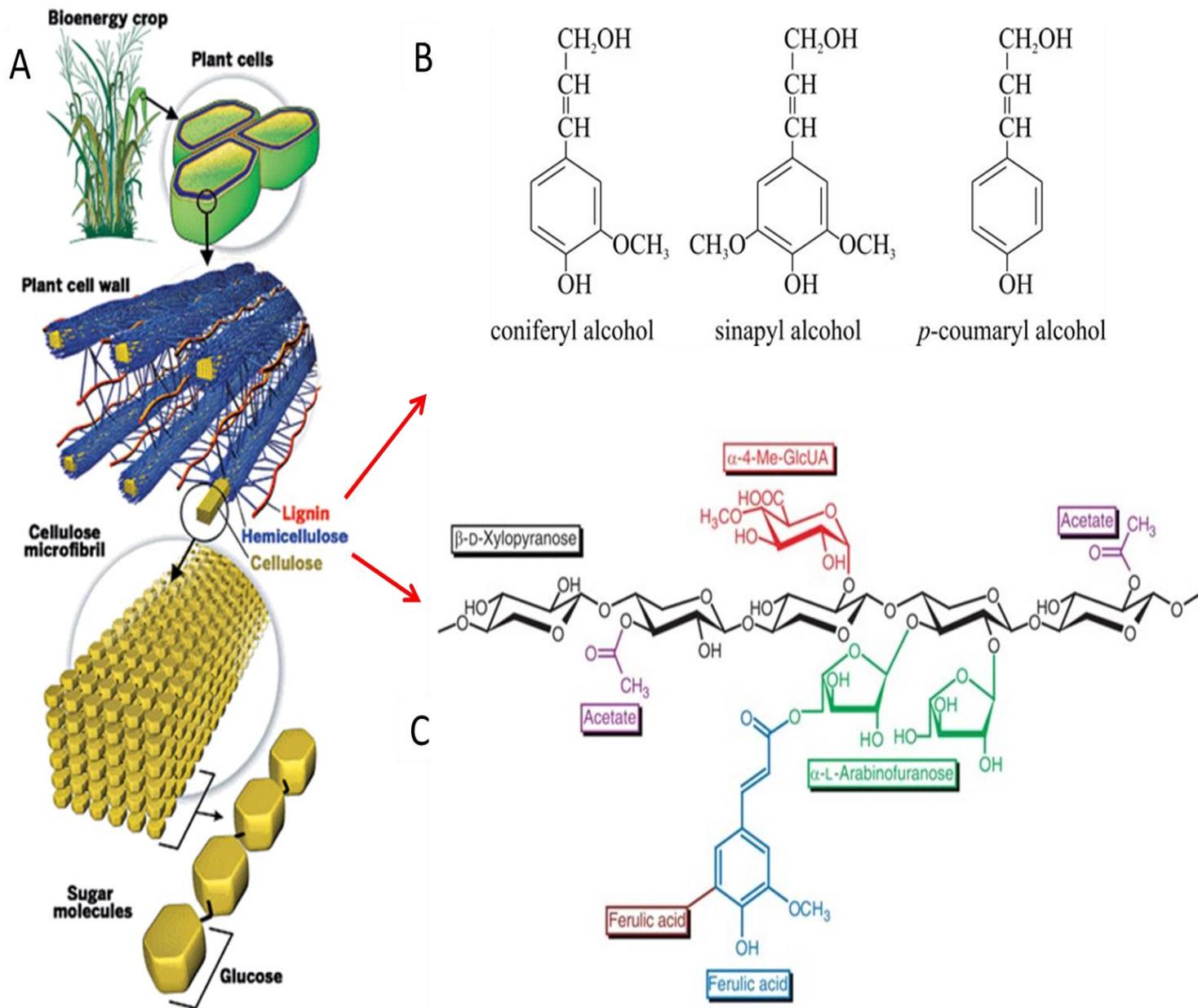


Figure 2. The complexity of plant cell wall structure. (A) Plant cell wall structure (Ritter, 2008); (B) Chemical structures of the phenylpropanoid alcohols used to construct the lignin polymers (Moore et al., 2011). (C) General structure showing the various linkages found in a variety of xylans isolated from plant cell walls. (Dodd and Cann, 2009)

1.3.2.2 Lignin

Lignin is a highly branched, three-dimensional polymer derived from three phenylpropane units (monolignols), namely guaiacyl (G, coniferyl alcohol), syringyl (S, sinapyl alcohol) and p-hydroxyphenyl (H, p-coumaryl alcohol) (Figure 2B). Lignin is synthesised by free-radical coupling of phenoxy radicals that are formed through enzymatic dehydrogenation of the monolignols (Humphreys and Chapple, 2002). Although the monolignols can be linked through a variety of carbon-carbon and carbon-oxygen bonds, lignin is dominated by β -O-4 linked aryl ether linkages (>50%) (Adler, 1977). The structure and composition of lignin is known to be very dependent on the source and type of lignocellulosic material. For example, softwood lignins (25-35% cell wall dry weight) are primarily composed of G units, whereas hardwood lignins (18-25%) are dominant in G and S units, and grass lignins (10-30%) contain all three monolignols (Kelley et al., 2004; Popper, 2008)

The main role of lignin is to provide mechanical strength and structural support in the cell wall to prevent compression and bending, and this structural support is mainly achieved through covalent bonding to the side chains of branched hemicelluloses (e.g. uronic acids and arabinose), known as lignin carbohydrate complexes (LCC) (Figure 2C) (Eriksson and Lindgren, 1977; Chabannes et al., 2001). LCCs are believed to form inclusion complexes that not only decrease the permeability of water across the cell wall, but also provide resistance against chemical- and/or enzyme-catalyzed deconstruction from microorganisms (Chundawat et al., 2011).

Even though the glycosidic bonds between monomer units in plant polysaccharides can be hydrolyzed under relatively mild conditions, the cleavage of ether linkages between

lignin monomers requires either high temperatures or oxidative reactions (Hammel et al., 1993; El Hage et al., 2010). Lignin can be softened and redistributed on the biomass surface above the glass transition temperature (T_g : 100-160 °C) (Selig et al., 2007). The amount and distribution of lignin cell walls have been shown to strongly influence cellulose hydrolysis by physically blocking access of cellulases to cellulose (steric hindrance) (Kumar et al., 2012), and/or by unproductive adsorption of enzymes via hydrophobic, ionic, and hydrogen bonding interactions (Eriksson et al., 2002a; Berlin et al., 2006b; Nakagame et al., 2011).

1.4 Biomass pretreatment

Due to the recalcitrant biomass characteristics discussed above, a pretreatment step is usually required to disrupt the highly organized cell wall structure before the bioconversion process. Ideally, the pretreatment should be performed at conditions that allow the various biomass components to be fractionated and recovered in an effective manner, while “opening up” the cellulosic fraction to enhance the rate and extent of enzymatic hydrolysis (Chandra et al., 2007; Yang and Wyman, 2008). Pretreatment strategies have generally been categorized into biological, physical and chemical processes, or a combination of these approaches (Table 1). Some of the advantages and disadvantages of different strategies are also listed in Table 1.

Biological pretreatment has been traditionally applied as a bio-pulping method in the pulp and paper industry (Chandra et al., 2007; Tian et al., 2012). In general, bio-based pretreatments have utilized wood degrading fungi such as soft rot, brown rot, and white rot fungi to modify the chemical composition of the lignocellulosic biomass. Soft and brown rot

fungi have been shown to primarily degrade the hemicellulose, while white rot fungi mainly attack the lignin (Hammel et al., 2002; Martinez et al., 2005). Even though this pretreatment approach consumes much less energy, is environmentally friendly and generates fewer inhibiting compounds than thermo/chemical pretreatment technologies, it requires careful growth conditions, large space for operating and a long pretreatment (incubation) period (at least 10-14 days) (Wyman et al., 2005; Tian et al., 2012). These limitations have made biological pretreatments commercially unattractive.

In contrast, physical pretreatments, such as milling and ultra-sonication, can break down feedstocks into smaller particles without changing their chemical composition (Mosier et al., 2005; Yunus et al., 2010). Physical pretreatments generally enhance enzymatic hydrolysis by disrupting the cellulose crystallinity and/or by increasing the surface area of the biomass (Mais et al., 2002; Yunus et al., 2010). Although physical pretreatments have been successfully applied to a variety of feedstocks, they are typically energetically demanding and do not remove or solubilize the hemicellulose and lignin, both of which generally restrict the accessibility of cellulases to cellulose (Wyman et al., 2005; Chandra et al., 2007). Physical pretreatments have yet to be shown to be economically viable on a commercial scale.

Chemical pretreatments (typically acid- or alkali-based) are usually initiated by chemical reactions with the main goal of enhancing enzyme accessibility to the cellulose by disrupting of the biomass structure and solubilizing the hemicellulose and lignin, and to a lesser degree by decreasing the DP and crystallinity of the cellulosic component (Chandra et al., 2007; Yang and Wyman, 2008). For example, organosolv pretreatments have been applied using a variety of solvents and this type of pretreatment has been shown to

significantly reduce the lignin content of the substrate (Pan et al., 2006). By combining it with swelling agents such as NaOH or sulfuric acid, organosolv pretreatments can also partially decrease cellulose crystallinity (Silverstein et al., 2007). Pretreatments that combine both chemical and physical processes are generally referred to as physicochemical processes and this type of pretreatment has received the most attention in recent years (Chandra et al., 2007; Yang and Wyman, 2008). For example, steam explosion, with the addition of a chemical catalyst, has shown significant advantages for generating easily hydrolysable substrates from most potential lignocellulosic biomass substrates (Chandra et al., 2007; Arantes and Saddler, 2011).

Unfortunately, as mentioned earlier, many pretreatment conditions that are optimized to result in easily-digestible cellulose (usually at very high severity) typically result in a significant loss of the hemicellulose component, which can account for 20-40% of the plant cell-wall polysaccharides (Chandra et al., 2007; Bura et al., 2009). The degradation of the hemicellulose not only reduces the sugar yield of the process, but also results in the formation of degradation products that can be inhibitory to the hydrolysis step, but particularly to the yeast used in the subsequent fermentation of the sugars to biofuels such as ethanol (Palmqvist et al., 1996; Ohgren et al., 2007). Lower severity pretreatments allow for the preservation of hemicellulose in a usable, insoluble form, resulting in an increase in overall sugar recovery. However, the subsequent enzymatic hydrolysis of the cellulosic component becomes more difficult due to the recalcitrant nature of the remaining, lightly-pretreated substrate (Chandra et al., 2007).

Table 1. Summary of various pretreatment technologies used for lignocellulosic biomass

Pretreatment technology	Examples	Major effects	Advantages	Disadvantages
Biological	white/brown-rot fungi	degradation of lignin and hemicellulose	low energy input; environmentally friendly	very slow
Physical	ball milling	reduce particle size; decrease cellulose crystallinity;	suitable for various substrates; no chemical input	large energy consumption
Chemical	dilute acid; alkaline; organosolv; ionic liquid	hemicellulose solubilisation; delignification; saponification of ester bonds	improve cellulose accessibility/digestibility	equipment corrosion; sugar degradation; toxic substrates; difficult to recover chemicals
Physico-chemical	steam explosion; AFEX	Disrupt LCC; hemicellulose degradation; decrease cellulose crystallinity	short time; cost effective; improve cellulose accessibility/digestibility;	sugar degradation; produce inhibitors for the following biological process

LCC: lignin-carbohydrate complex; AFEX: ammonia fibre expansion

In summary, an ideal pretreatment method should be cheap (both in terms of capital and operating costs), effective on a wide range of lignocellulosic substrates, require minimum preparation/handling steps prior to pretreatment, ensure recovery of all of the lignocellulosic components in a useable form and provide a cellulosic stream that can be efficiently hydrolyzed with low concentrations of enzymes. So far it is unlikely that one pretreatment process will be declared a “winner” for a range of biomass substrates as each method has its inherent advantages/disadvantages.

1.5 Major non-complexed enzymes for biomass decomposition

In nature, cellulolytic hydrolases (cellulases) can be produced by a wide variety of aerobic and anaerobic bacteria and fungi (Lynd et al., 2002; MacDonald et al., 2012; Wilson, 2008). Some anaerobic microorganisms have evolved to produce a large extracellular enzyme complex, known as a cellulosome, which contains a scaffolding protein and many bound enzymes (Doi and Kosugi, 2004), while aerobic fungi and bacteria typically secrete non-complexed, individual cellulase components and accessory enzymes that work synergistically in vitro to deconstruct cellulosic substrates (Lynd et al., 2002; Mahajan and Master, 2010). The non-complexed cellulase system from the filamentous fungus *Trichoderma viride* (also known as *Trichoderma reesei* or *Hypocrea jecorina*), was first identified by the U.S. Army Laboratory during World War II from the decay of canvas accoutrements in the South Pacific (Reese, 1976). It has been successfully and extensively studied and also currently dominates industrial cellulase applications (Nieves et al., 1998; Aslam et al., 2010).

1.5.1 Major cellulase components in the *Trichoderma* cellulase mixture

The traditional nomenclature of cellulases used to be based on either their substrate specificity (such as cellobiohydrolase and β -glucosidase) or on their molecular mechanism (such as endoglucanase and exoglucanase) (Wood and Mccrae, 1972; Woodward, 1991). However, it was recognised that this type of classification did not reflect the structural features of these enzymes (Henrissat and Bairoch, 1993). Thus, a new scheme was proposed and is now used that allocates the glycoside hydrolases into families based on their amino acid sequence similarities (Henrissat and Davies, 1997). In this system, glycoside hydrolases

(GH) have been grouped into 132 families as described in the Carbohydrate Active Enzymes Database (<http://www.cazy.org/CAZY>), in which cellulolytic enzymes belong to at least 14 families (Henrissat and Bairoch, 1993; Henrissat and Davies, 1997). Among them, *T. reesei* cellulases belong to at least 8 GH families. An “effective cellulase mixture” typically contains two exoglucanases (CBH I and CBH II), numerous endoglucanases (EG I-V) and two β -glucosidases (BG) (Markov et al., 2005; Zhou et al., 2008). Among them, CBHI, CBHII, EGI, EGII and BG have been identified as major cellulase components for the deconstruction of insoluble cellulose (Banerjee et al., 2010b; Gao et al., 2010).

The mechanism of enzymatic hydrolysis of the glycosidic bond takes place via an acid/base catalysis, which requires two critical carboxylate groups that play the role of a proton donor and a nucleophile/base (Koshland, 1953; Wilson et al., 1995). The hydrolytic cleavage mechanism can be divided into retaining or inverting mechanisms, where the configuration of the anomeric center can either be retained or inverted (Wilson et al., 1995). The nomenclature of sugar-binding subsites within the catalytic center of glycoside hydrolases is labeled from $-n$ (non-reducing end) to $+n$ (reducing end), with the hydrolytic cleavage taking place between -1 and $+1$ subsites (Davies et al., 1997).

1.5.1.1 CBHI (Cel7A)

Cel7A is a major component of the cellulase mixture, usually constituting 50-70% of the total protein expressed (Markov et al., 2005). Cel7A has a two-domain structure of a large catalytic domain (CD) that consists of a tunnel-like structure with 10 separate binding subsites (-7 to $+3$) around the active center (Divne et al., 1994), connected to a small family 1 cellulose-specific carbohydrate binding module (CBM) via a glycosylated linker peptide (Vantilbeurgh et al., 1986; Stahlberg et al., 1991). This special CD structure provides a large

number of interactions with a single cellulose chain and gives rise to a processive mode of action during hydrolytic events (Divne et al., 1994; Herner et al., 1999).

Cel7A has been shown to have retaining, exo-type activity and hydrolyzes β -1,4-glucosidic bonds from the reducing end, processively, liberating cellobiose as the main product (Teeri et al., 1992; Barr et al., 1996). Cel7A is arguably the most strongly adsorbed enzyme, and has been shown to adsorb to and preferentially hydrolyze crystalline regions of cellulose (Chanzy and Henrissat, 1985; Srisodsuk et al., 1998). Although most studies showed that Cel7A exhibits the typical exo-type activities, some work has indicated that Cel7A also displays endo-type activities (Henrissat et al., 1985; Barr et al., 1996).

1.5.1.2 CBHII (Cel6A)

Cel6A is another major exo-glucanase, which accounts for 10-15% of the total protein in the *T. reesei* cellulase system (Markov et al., 2005). Cel6A has a two domain structure, where the CD has a tunnel shape with four binding subsites (-2 to +2, about 20 Å long) in the active center (Koivula et al., 2002). The special feature of the Cel6A CD is that it has an extended binding site which can bind to the +4 position when working on crystalline substrates (Koivula et al., 1996; 2002). Cel6A hydrolyzes cellulose chains from the non-reducing ends, processively, via the inverting mechanism, with cellobiose as the main products, but the extent of processivity is significantly less than that of Cel7A (Tenkanen et al., 1999; Varrot et al., 2003). Although the synergistic cooperation between exoglucanases (Cel7A and Cel6A) and endoglucanase indicate that Cel6A behaves more like a typical exo-type enzyme (Henrissat et al., 1985), possible endo-type activities of Cel6A have also been reported (Stahlberg et al., 1993).

1.5.1.3 EGI (Cel7B)

Cel7B is the major endoglucanase in the cellulase system, which accounts for 6-10% of total protein (Markov et al., 2005). The physical structure of Cel7B is similar to Cel7A, but its CD has a more open active site cleft through the deletion of the loops that enclose the active site of Cel7A (Kleywegt et al., 1997). Cel7B has four kinetically significant binding subsites for cellulose, and carries out hydrolysis via a retaining mechanism (Kleywegt et al., 1997).

Cel7B dramatically decreases the DP of cellulose chains during hydrolysis, with the major hydrolysis products being cellotriose, cellobiose and glucose (Nakazawa et al., 2008). Cel7B has been identified as a non-specific endoglucanase, as it not only has activity on cellulosic substrates, but can also hydrolyze some hemicelluloses such as glucomannan, galactomannan, and xylan (Kleywegt et al., 1997). Interestingly, the core structure of EG I has been shown to exhibit similarities with family 11 xylanases (Biely et al., 1991; 1993).

1.5.1.4 EGII (Cel5A)

Cel5A makes up between 1% and 10% of the total protein mixture of *T. reesei* (Markov et al., 2005). Cel5A has a typical two-domain structure like most cellulase components and its CD has a cleft shape active center with an inverting catalytic mechanism (Nakazawa et al., 2008). Unlike Cel7B, Cel5A is a pure endoglucanase and only displays activity on cellulose and its derivatives (Nakazawa et al., 2008). In addition, Cel5A reduces the viscosity/DP of cellulosic substrates more efficiently compared to other endoglucanases, and the main products from hydrolysis of cellulose consist primarily of cellobiose, as well as small amounts of glucose and cellotriose (Medve et al., 1998).

1.5.1.5 β -glucosidase (BG)

β -glucosidases (BG), although not strictly “cellulases”, are known to be key components of an efficient cellulose hydrolysis system (Shewale, 1982; Korotkova et al., 2009). β -glucosidases can be grouped into glycoside hydrolase families 1 and 3 (GH1 and GH3) based on their amino acid sequence (Henrissat and Bairoch, 1993). In general, BG is known to have a pocket-like structure with hydrolytic activity towards cellodextrins, particularly cellobiose (Korotkova et al., 2009). The catalytic action of BG is similar to the retaining mechanisms of the exo-type enzymes, but instead of removing cellobiose from the chain ends, BG primarily removes glucose (Dan et al., 2000).

T. reesei typically produces low amounts of BG, which is not sufficient to hydrolyze the soluble cellooligosaccharides, particularly cellobiose, resulting from cellulose hydrolysis (Korotkova et al., 2009). Therefore, supplementation with BG from sources such as *Aspergillus niger* is considered necessary to achieve effective cellulose saccharification, and it has been shown to improve the efficiency of hydrolysis by several fold (Seidle et al., 2004), primarily by reducing cellobiose end-product inhibition.

The major BG from *A. niger* belongs to the GH 3 family, and its molecular weight is about 120 kDa based on SDS-PAGE (Seidle et al., 2004). This BG has been shown to be highly glycosylated, with D-mannose as the prevalent sugar, which makes up about 20-25 kDa of its molecular weight (Dan et al., 2000). Although different types of disaccharides and cellodextrins can be effectively hydrolyzed (Langston et al., 2006; Korotkova et al., 2009), glucose and gluconolactone have been shown to be strong competitive inhibitors to its activity (Nishimura and Ishihara, 2009).

1.5.2 Major accessory enzymes

Besides the cellulolytic enzymes, several other non-cellulolytic microbial enzymes, such as hemicellulases, lytic polysaccharide monooxygenases (LPMOs), ligninases and pectinases, have been shown to play a role during the lignocellulose biodegradation process (Alam et al., 2005; Wilson, 2008; 2009b). Among them, hemicellulases and LPMOs have received the most attention in the past number of years as they have been demonstrated to significantly improve the hydrolytic performance of cellulase enzymes on lignocellulosic biomass (Ohgren et al., 2007; Bura et al., 2009; Harris et al., 2010; Quinlan et al., 2011).

1.5.2.1 Hemicellulases

A range of hemicellulose-degrading enzymes are typically produced by microorganisms, including xylanases, mannanases, xyloglucanases, acetylxylan esterases, β -glucuronidases, arabinofuranosidases and galactosidases (Shallom and Shoham, 2003). Xylanases have, typically, been shown to be the most influential hemicellulase enzymes for the hydrolysis of pretreated lignocellulosic biomass by cellulases (Ohgren et al., 2007; Bura et al., 2009; Kumar and Wyman, 2009c). This is particularly evident during the hydrolysis of hardwoods and agricultural residues, where the hemicellulose left after pretreatment is typically composed of xylan, and blocks the access of cellulases to cellulose to a significant extent (Garcia-Aparicio et al., 2007; Bura et al., 2009).

Based on the CAZY classification system, most xylanases belong to glycoside hydrolase families 10 (GH10) and 11 (GH11) (Wong et al., 1988; Viikari et al., 1994). In general, GH11 endo-xylanases (GH11 EX), which shows similarity with the low molecular weight GH12 endo-glucanase, consist of a single catalytic domain (β -jelly roll structure) that

has a “right hand” shape (Pollet et al., 2010). Some of the GH10 endo-xylanases (GH10 EX), which are closely related to the GH5 endo-glucanases, have a two domain structure, and their catalytic domain (TIM-barrel structure) folds into a “salad bowl” shape (Pollet et al., 2010). Both families of these endo-xylanases follow the “retaining” mechanism during hydrolysis (Torrönen et al., 1994). GH10 EX is capable of attacking a glycosidic linkage close to a substituted xylose residue and requires only two unsubstituted xylose residues next to a substitution for activity, thus exhibiting greater catalytic versatility than GH11 EXs on branched substrates. On the other hand, GH11 EX requires at least three consecutive, unsubstituted xylose residues next to a branching point before cleavage can take place and it cannot cleave the linkage next to a branch point (Wong et al., 1988; Pollet et al., 2010). GH11 EX is characterized as “true” xylanases because of their exclusive activity on substrates with a D-xylose backbone (Ustinov et al., 2008).

1.5.2.2 GH61/AA9

GH61 enzymes were initially identified as endo-glucanases due to the specific, but weak, viscosity-reducing activity on some cellulose substrates such as CMC and β -glucan (Saloheimo et al., 1997; Karlsson et al., 2001). Therefore they did not attract much attention until about 2010 when Harris et al. (2010) showed the remarkable boosting effect of GH61 on hydrolysis of dilute-acid pretreated corn stover. Unlike the canonical cellulase enzymes, which have either channel or cleft-shaped active sites with conserved carboxylic acid residues for hydrolytic cleavage, GH61 enzymes have a planar active site surface, which contains a divalent metal ion (most likely copper) situated between three nitrogen atoms of the N-terminal histidine (Harris et al., 2010; Quinlan et al., 2011; Li et al., 2012). GH61 is a lytic polysaccharide monooxygenase (LPMOs), and similar to other LPMOs such as CBP21

and CelS2, it oxidatively cleaves substrates at highly organized regions (Vaaje-Kolstad et al., 2010; Forsberg et al., 2011; Quinlan et al., 2011). A possible mechanism for GH61 action was recently proposed (Phillips et al., 2011; Beeson et al., 2012), suggesting that oxygen is inserted into cellulose at C-H bonds at either C1 or C4 positions, thereby hydroxylating the polysaccharides to produce aldonic acid or 4-ketoaldose, respectively. However, the observed oxidation products at the C6 position (from C6-alcohol to C6-aldose) suggest a still unidentified mode of GH61 action (Quinlan et al., 2011). Recently, GH61, along with other LPMOs and the redox enzymes for lignin degradation, have been grouped into a novel category in the CAZy database, broadly termed “Auxiliary Activities” (AA). In this new classification system, GH61 belongs to the subfamily of AA9 (Levasseur et al., 2013).

In nature, many cellulose-degrading fungal species retain a heterogeneous collection of AA9-encoding genes (Harris et al., 2010). Besides the divalent metal ion buried in the active site, AA9 also requires a redox-active cofactor with metal reducing capacity to potentiate its activity. Compounds provided by cellobiodehydrogenase (CDH), synthetic small molecular reductants (e.g. gallate or ascorbate) and unidentified compounds in pretreated lignocellulosic biomass have been shown to act as AA9 cofactors (Langston et al., 2006; Dimarogona et al., 2012; Sigmund et al., 2012; Bey et al., 2013). However, the source of these cofactors within different types of pretreated substrates and their influence on cellulase-AA9 synergistic cooperation is still not fully resolved.

1.6 Synergism between cellulase components

The hydrolysis of insoluble cellulose requires a combination of cellulase components whose synergistic action is more efficient than the sum of the actions of the individual

enzymes (Henrissat et al., 1985; Merino and Cherry, 2007; Gao et al., 2010). Although synergism among these cellulases has been studied for more than sixty years and various hypotheses have been proposed to explain their synergistic interaction (e.g. exposing/creating of new hydrolysis sites, stereospecific enzymes and/or enzyme complexes, competitive adsorption, etc.), most of those synergism studies used relatively low enzyme loadings and “pure” cellulosic substrates, such as Avicel, filter paper and cotton, to assess their interactions (Chanzy and Henrissat, 1985; Converse et al., 1988; Jeoh et al., 2002; 2006). It has also been shown that the extent of synergism is very dependent on the enzyme concentrations and the nature of the substrates (Henrissat et al., 1985; Woodward et al., 1988a). Therefore, the exact mechanism of cellulase synergistic interaction, especially during hydrolysis of lignocellulosic biomass, is still not completely understood and the evidence is, in many cases, contradictory and inconclusive (Kostylev and Wilson, 2012; Andersen et al., 2008; Zhang et al., 2006; Wilson, 2008).

1.6.1 Synergistic interaction between endo- and exo- type cellulases

Most evidence indicates that hydrolysis of crystalline cellulose requires the combination of exoglucanases (CBHs), endoglucanases (EGs) and β -glucosidases (BG), instead of “C1-Cx” components. As BG is commonly believed to only hydrolyze cellobiose into glucoses, various models have been proposed to try to explain the mechanism of cellulose hydrolysis based on the synergistic interactions between CBHs and EGs. The early observation that not all EG components work synergistically with CBH was an unexpected one (Wood and McCrae, 1978; Lee and Woodward, 1988). Thus, the possibility of there being stereospecific EG components, which might attack the two different configurations of

the repeating β -1, 4-glycosidic bonds and generate the chain-ends specific for one type of CBH was proposed by Wood and McCrae (1986). Alternatively it was suggested that some EG components might form an enzyme complex with CBH, with the resulting complex having both endo and exo-type activities (Fagerstam and Pettersson, 1980; Wood et al., 1989). It was suggested that this CBH-EG complex could prevent the reformation of the covalent bond between two glucose residues separated by EGs, due to the rapid removal of cellobiose by CBHs after the EG cleavage. Rabinovich and Derjaguin (1988) have also suggested that the tightly adsorbed CBHs can bring about the dispersion of cellulose by hydrolytic (cellulose cleavage) and mechanical (penetration into interfibrillar space at micro-cracks in the surface; stratification) actions, which would open new sites for the action of weakly adsorbed CBHs and EGs. Similarly, Klyosov (1990) suggested that the existence of tightly adsorbed EG, which could produce glucose from transglycosylation, was crucial for hydrolysis of crystalline cellulose. In addition, some researchers have also mentioned that synergism between CBHs and EGs is highly dependent on the adsorption of the EG components, as a lack of synergistic cooperation between CBH and an EG, with low affinity for binding to cellulose, has been observed (Klyosov et al., 1986; Beldman et al., 1988).

So far, the model suggested by Wood and McCrae (1972), which was further supported by White and Brown (1981), using electron microscopy, has obtained the widest acceptance. This model suggested that the internal amorphous areas of the cellulose microfibrils are initially attacked by EG to split into new chain ends, which are subsequently attacked by the exo-acting CBH to cleave off cellobiose units in a processive manner. β -glucosidase further hydrolyses cellobiose to glucose units. Under this model, the synergistic interactions between CBH and EG can be readily explained. The hydrolysis of insoluble

cellulose by CBH alone will be negligible due to the limited chain ends. Alternatively, EG could expose chain ends on the surface of cellulose, but the internal bonds susceptible to EG cleavage would remain 'buried' within the substrate without the action of CBH to depolymerize the outer chains and expose these internal bonds (Woodward, 1991). From the pattern expected from exo/endo-type synergism, it has been postulated that only a small amount of endo-type activity would be needed for effective cooperation. The activities of CBH II is consistent with this hypothesis, but CBH I, which exhibits endo-type adsorption along the cellulose chain, has been shown to require additional endo-type activity to achieve an effective substrate conversion (Chanzy et al., 1984; Henrissat et al., 1985).

1.6.2 Synergistic interaction between exo- and endo-type cellulases

Initially, the observed synergistic effect between CBH I and CBH II was difficult to understand, as both of these enzymes were thought to hydrolyze cellulose chains from the non-reducing ends, implying that they would compete for the limited number of available sites, rather than working synergistically. As mentioned earlier, in order to explain the synergistic cooperation at the time, Wood and McCrae (1986) postulated that CBH I and CBH II are two stereospecific enzymes, which could hydrolyze the different configurations of the non-reducing groups in glucan chains. Tomme et al. (1990) also suggested that a loose enzyme complex was formed that showed a synergistic binding action to maximize hydrolysis.

Subsequently, CBH I and CBH II have been shown to hydrolyze cellulose chains from the reducing and non-reducing ends, respectively (Barr et al., 1996). This offered new insights into explaining the synergistic cooperation between these two enzymes. Barr et al.,

(1996) also suggested that the enzymes can expose new hydrolysis sites for each other, by depolymerizing the outer cellulose chains, thereby revealing previously hidden strand breaks buried within the fiber. This model was supported by Nidetzky et al. (1995), who observed that CBH I could produce a more readily hydrolysable substrate for CBH II and vice versa.

Several studies have shown that CBH II actually functions like an endo-type processive enzyme, and that the synergism between CBH I and CBH II was due to these endo-type CBH II actions during hydrolysis (Hoshino et al., 1997; Boisset et al., 2000). However, the broad substrate specificity of CBH I and its endo-type adsorption along the cellulose, with potential splitting ability, have also been suggested as the main reasons for the synergistic interactions between the two CBHs (Chanzy et al., 1983; 1984).

1.6.3 Synergistic interaction between endo- and endo-type cellulases

Synergism between EGs has not been widely demonstrated. Kraeva et al. (1986) observed the synergistic effect between tightly and weakly adsorbed endo-type enzymes, and this effect has also been shown during sequential hydrolysis. Therefore, it seems that these two kinds of enzymes attack different sites within the cellulose and can also expose new sites for each other. It should also be noted that some endo-type enzymes, such as EG I, have a broad substrate specificity (Kleywegt et al., 1997). For example, the hemicellulase activity of EG I could strongly influence the synergistic cooperation between it and other cellulases, especially on lignocellulosic substrates.

1.6.4 Intramolecular synergism (CBM & CD)

Carbohydrate binding modules (CBMs) are a common feature in enzymes that act on solid substrates. CBMs have been classified into 61 families based on the CAZy database (<http://www.cazy.org/CAZY>). The most common CBMs belong to families I, II and III. Among them, the family I CBMs, which come from fungi, are quite small (around 32-36 residues), while family II and III, which come from bacteria, are much larger (Carrard et al., 2000). In contrast to family I CBMs, family II and III CBMs have been reported to enhance the physical disruption of cellulose fibers and to release small particles from cotton fibers (Din et al., 1991; Tomme et al., 1995).

As mentioned earlier, most of the cellulase components from *T. reesei* possess a two domain structure: the catalytic domain (CD) and the cellulose-specific family I carbohydrate binding module (CBM). In summary, the amorphous cellulose is hydrolyzed to the same extent by a native cellulase and its CD, but the removal of the CBM makes the enzyme less active against insoluble crystalline cellulose (Vantilbeurgh et al., 1986; Igarashi et al., 2009). Din et al. (1994) observed intra-molecular synergism between the isolated CD and CBM from an endoglucanase during hydrolysis of cotton fiber, but not BMCC. This phenomenon has been explained by the differences in the physical structures, i.e. the cotton fiber is much more disordered than BMCC, so a CBM could easily bind to and penetrate into surface discontinuities, sloughing off cellulose fragments rather than just bringing the CD into close association with the substrate. However, negative effects can be observed with overloading of CBM (Schwarz, 2001). This has been explained by the competition for binding sites of the CBM with the CD on the surface where the crystalline structure has already been disrupted, thereby lowering the overall efficiency.

The functions of CBMs in lignocellulose hydrolysis are still unclear. Some researchers have indicated that these modules simply enhance the adsorption of the CD, with no additional active property during hydrolysis (Nidetzky et al., 1994), while others have suggested that they can disrupt the crystalline structure of cellulose and can play the role of the C1 non-hydrolytic, disruptive component (Jervis et al., 1997; Arantes and Saddler, 2010). Still others have claimed that CBMs have the function of specific adsorption (targeting), which brings the CD into close proximity with its target substrate (Arantes and Saddler, 2010). Last but not least, some researchers have shown that CBMs can diffuse along the cellulose chains, while actively directing the outer glucan chain into the CD. This activity was suggested to result in a caterpillar-like motion of the CBM and CD (Gerner et al., 2000).

1.6.5 Synergistic interactions during enzyme adsorption

As cellulase enzymes work on the solid-liquid interface during hydrolysis of insoluble cellulose, enzyme adsorption/desorption profiles are crucial for better understanding of the hydrolytic performance of cellulase components. Based on the commonly accepted cellulase synergism model, one may expect positive cooperation among cellulase components to increase the binding affinity of each other. Because cellulase components have binding sites of varying affinities, they can produce or expose more available sites for each other during hydrolysis (Tomme et al., 1990; Nidetzky et al., 1994).

However, competitive adsorption among cellulase components has been reported by many different research groups (Ryu et al., 1984; Medve et al., 1998; Andersen et al., 2008). For example, Medve et al. (1994; 1998) assessed the correlation between CBHI and CBHII/EGII adsorption and their synergistic cooperation on Avicel. Their results indicated

that fewer enzymes were adsorbed in the presence of other enzymes, and CBHI was more affected by other enzymes. They explained this phenomenon by proposing competitive enzyme adsorption on the common binding sites.

Alternatively, the cellulose “surface erosion model” was proposed by Valjamae et al. (1998; 1999) in the late nineties, in which he proposed that the processive CBHI is sterically hindered by the eroded cellulose surface during hydrolysis, and that other cellulase monocomponents, that diminish/modify the surface erosion pattern, can release those blocked CBHIs and facilitate or enhance its hydrolytic potential. In the following years, this model was supported by several studies using different techniques (e.g. FPLC, tritium labeled CBHI, etc.) to monitor the adsorption/desorption profile of CBHI during hydrolysis (Karlsson et al., 1999; Eriksson et al., 2002b; Valjamae et al., 2003). Recently, the “traffic jam” of CBHI was actually observed by microscopy technologies such as AFM (Igarashi et al., 2011), and the length of the obstacle-free path, available for a processive run of CBHI on the cellulose chain, has been described as the rate limiting step for cellulose hydrolysis (Kurasin and Valjamae, 2011).

1.6.6 Potential synergistic interaction between exo/endo-type cellulases and BG

As mentioned earlier, it is recognised that BG hydrolyzes cellobiose, a strong inhibitor of exo-type cellulases (Shewale, 1982; Langston et al., 2006). Some researchers have indicated that BG might be more engaged in hydrolysis than simply through its role in removing cellobiose, because it can also hydrolyze cellodextrins efficiently (Shewale, 1982; Seidle et al., 2004; Chauve et al., 2010). Several authors have even proposed that hydrolysis

may be mediated by exo/endo-type enzymes splitting cellulose and producing cellobiose and cellodextrins, followed by endo-enzymes and BG working together to further cleave these products into glucose (Shewale, 1982; Enari and Nikupaavola, 1987).

1.7 Synergism between cellulases and major accessory enzymes.

Although cellulase components work synergistically to hydrolyze insoluble cellulose, the extent of improvement of cellulose hydrolysis, achieved by optimizing their synergistic cooperation (e.g. ratio of major cellulase components), has been limited. Alternatively, we (Berlin et al., 2007; Bura et al., 2009; Shen et al., 2011) and other workers (Kumar and Wyman, 2009b; Harris et al., 2010; Quiroz-Castaneda et al., 2011; Zhou et al., 2011) have shown that some accessory enzymes and/or disrupting proteins, such as hemicellulases, LPMOs, and swollenin, can significantly enhance the hydrolytic performance of cellulase enzymes on a range of cellulosic substrates. Among them, xylanases, xyloglucanases, and AA9s have attracted much attention in the past few years since they have been shown to “boost” the enzymatic hydrolysis of pretreated lignocellulosic biomass by cellulase enzymes.

1.7.1 Potential synergism between cellulases and xylanases

Recently, some researchers found that supplementing xylanases into cellulase cocktails dramatically increased the hydrolytic performance of “cellulase mixtures” when added to some pretreated lignocellulosic substrates (Garcia-Aparicio et al., 2007; Selig et al., 2008; Kumar and Wyman, 2009b; Morais et al., 2010; Varnai et al., 2010). This phenomenon has been explained by suggesting that the beneficial effect of xylanase supplementation is a result of the improved cellulose accessibility caused by the removal of the xylan “coat”

(Selig et al., 2008; Kumar and Wyman, 2009b). Interestingly, it has also been shown that xylanases improve the hydrolysis of lignocellulose regardless of xylan content (Garcia-Aparicio et al., 2007). However, during these studies, the degree of improvement in substrate digestibility mediated by the xylanases varied widely. This is likely due to the fact that some of these studies evaluated the beneficial effect of xylanase supplementation at low glucan conversion yields (Murashima et al., 2003; Varnai et al., 2010), or made use of samples with very low xylan content (Garcia-Aparicio et al., 2007). Additionally, some workers have also used uneconomically high amounts of xylanase (Berlin et al., 2007). Therefore, the “real” interaction between cellulases and xylanases during hydrolysis of pretreated lignocellulose, at a reasonable enzyme loading capable of achieving effective substrate conversion, is still not completely understood.

A recent study has suggested that xylan may become ‘zipped in’ to elementary fibrils during crystallization directly after cellulose synthesis (Scheller and Ulvskov, 2010). Therefore, it would be expected that removal of this embedded xylan would allow cellulases to access and degrade cellulose embedded within the deeper cellulosic structure. In this manner, depolymerization of cellulose by cellulase enzymes in the deeper structure is also expected to expose the xylan chains, trapped within or between cellulose microfibrils, to the action of xylanases. This degradation model therefore predicts a synergistic effect between xylanases and cellulases.

1.7.2 Potential synergism between cellulases and xyloglucanases

Aside from endo-xylanases, another type of hemicellulase, xyloglucanase, has also been suggested to enhance the hydrolyzability of various lignocellulosic substrates by

cellulase enzymes (Nishitani, 1995; Benko et al., 2008). Xyloglucanase can hydrolyze xyloglucan, which is one of the major components in the primary cell wall of higher plants and has been suggested, like xylan, to limit the accessibility of cellulase enzymes to cellulose (Vincken et al., 1995; Chanliaud et al., 2004; Benko et al., 2008; Kaida et al., 2009; Powlowski, et al., 2009). The depolymerization and re-arrangement of the linkages in xyloglucan by hydrolases or transferases have been proven to be an essential step for plant cell wall expansion and deposition during cell growth (Vincken et al., 1995; Chanliaud et al., 2004). In a similar fashion, it has been suggested that the cleavage of glycosidic linkages in xyloglucan results in the swelling of cellulose microfibrils (Hayashi and Kaida, 2011), an effect that has been shown to increase cellulose accessibility and therefore cellulase performance. The cooperation between a xyloglucanase and cellulases has been assessed on some lignocellulosic substrates and shown to enhance cellulose hydrolysis in pretreated softwood (Benko et al., 2008), but the beneficial effects (if any) of xyloglucanase on the hydrolysis of various substrates still need to be assessed.

1.7.3 Potential synergism between cellulases and AA9

In nature, the cellulolytic mechanism of many microorganisms can be divided into two groups. As discussed earlier, the most extensively studied and exploited hydrolytic system consists of hydrolases that act cooperatively and synergistically (Zhang et al., 2006; Wilson, 2008). The second and less-explored mechanism reportedly breaks down cellulose to glucose by means of oxidative components acting cooperatively with hydrolases (Baldrian and Valaskova, 2008; Eastwood et al., 2011; Arantes et al., 2012). It has been shown that the efficient biodegradation of structural polysaccharides, such as cellulose and chitin, requires

the cooperative interactions between oxidative and hydrolytic systems (Eastwood et al., 2011; Arantes et al., 2012).

As also mentioned earlier, a new class of oxidative enzymes (recently classified as AA9) has been found in many cellulolytic microorganisms, including various species of industrial interest such as *T. reesei* (Harris et al., 2010). While purified AA9 enzymes exhibit negligible activity towards various polysaccharide substrates, they can synergistically cooperate with individual cellulase monocomponents and/or cellulase mixtures to hydrolyze both “pure” cellulolytic substrates (e.g. PASC, Avicel, and bacterial cellulose) and pretreated lignocellulosic substrates (Harris et al., 2010; Langston et al., 2011; Quinlan et al., 2011). While various AA9s have been identified, characterized and shown to act synergistically with hydrolases during enzymatic hydrolysis of a range of cellulosic substrates (Langston et al., 2011; Li et al., 2012; Bey et al., 2013), the extent of the observed synergistic cooperation between these two systems ranged from very low to high. Similar to the proposed function of the “C1”swelling factor, AA9 is also expected to significantly increase the access of cellulases to cellulose by the oxidative cleavage/disruption of the crystalline cellulose regions. Thus, it seems to hold huge potential for improving the hydrolytic efficiency of cellulase enzymes.

1.8 Synergism application

Over the last decade, biotechnology companies, such as Novozymes, Genencor International, etc., have reported a significant decrease in the cost of enzyme production, but it is generally recognised that a further 3-5 fold reduction is still needed in order for cellulosic ethanol to be economically feasible (Aden and Foust, 2009; Humbird et al., 2010).

However, the further cost reductions for enzyme production has been predicted to be challenging (Wilson, 2009a; Stephen et al., 2012). Alternatively, the strategy to make better “cellulase cocktails” by identifying the essential enzymes and optimizing their ratios has received considerable attention over the last few years.

Most of the initial studies that investigated the optimization of cellulose combinations focused on the hydrolysis of “model” cellulosic substrates such as cotton fiber, Avicel, and filter paper (Baker et al., 1998; Kim et al., 1998; Boisset et al., 2001; Gusakov et al., 2007). However, it has been realized that the optimized cellulase mixtures based on cellulosic substrates could not reflect their hydrolysis potential on “real-life” pretreated lignocellulosic biomass (Meyer et al., 2009; Gao et al., 2010). Thus, it appears that the enzyme mixture should be tailored for the particular lignocellulosic biomass and the specific pretreatment strategy (Berlin et al., 2007; Banerjee et al., 2010a).

1.8.1 Optimizing cellulase components on “model” cellulosic substrates

As the breakdown of cellulose into individual glucose monomers has been commonly believed to occur by the concerted action of cellobiohydrolases (CBHs), endoglucanases (EGs), and β -glucosidases (BG), these three types of cellulase enzymes from different fungal and bacterial sources were often chosen to formulate the cellulase mixture. However, even for the different cellulosic substrates, the essential cellulase components and their optimal ratios in a cellulase mixture are still uncertain, as summarized in Table 2. For example, the required portion of exoglucanase (CBHI) in the optimized mixture varied from 40% to about 70% during hydrolysis of Avicel and bacterial cellulose, respectively. Although 20% endoglucanase (Cel7B) was required for maximizing the hydrolysis of microcrystalline

cellulose, only 1.3% endoglucanase (EGV) was needed for efficient bacterial cellulose hydrolysis.

These observed differences might be the result of different experimental setups (e.g. hydrolysis time, temperature, solid loading, etc.), the different cellulosic substrates used in each study (e.g. Avicel, cotton, filter paper, etc.), and the different source of cellulases from various fungi and bacteria (e.g. *Thermobifida*, *Trichoderma*, *Hemicola insolens*, etc.). Generally, the reconstituted cellulase mixture containing two types of CBHs (working from different chain ends), at least one type of EG, and BG could efficiently hydrolyze insoluble cellulose (Table 2). Among them, CBHI is the major component (usually accounting for more than 50% of the protein content), followed by CBHII (around 20-30%) and EGs (around 10-20%). A certain amount of β -glucosidase was compulsory in all the optimized mixtures.

Table 2. Summary of enzyme optimization studies on “model” cellulosic substrates

Filter paper		Microcrystalline cellulose						Bacterial cellulose			Avicel	Cotton
<i>Thermobifidafusca</i> ; <i>Trichoderma reesei</i>		<i>Acidothermus cellulolyticus</i> ; <i>Trichoderma reesei</i> ; <i>Thermobifidafusca</i> ; <i>Thermotoganea politana</i>						<i>Humicola insolens</i>		<i>Chrysosporium lucknowense</i>		
CBHI (<i>T. reesei</i>)	43%	CBHI (<i>T. reesei</i>)	60%	40%	40%	60%	40%	Cel 7A	69%	CBH Ia	40%	40%
E1 (<i>T. fusca</i>)	16%	CBHII (<i>T. reesei</i>)	20%	20%				Cel 6A	30%	CBH Ib		
E2 (<i>T. fusca</i>)	7%	E3 (<i>T. fusca</i>)			20%	20%	40%	Cel 45A	1.25%	CBH IIb	40%	40%
E3 (<i>T. fusca</i>)	8%	EGI (<i>T. reesei</i>)	20%					BG	sufficient	EG II	16%	16%
E4 (<i>T. fusca</i>)	14%	EI (<i>A. cellulolyticus</i>)		40%	40%					EG V		
E5 (<i>T. fusca</i>)	12%	E5 (<i>T. fusca</i>)				20%				BG L	4%	4%
BG (<i>T. reesei</i>)	0.3U/ml	EndoA (<i>T. politana</i>)					20%			Xyl II		
		BG (<i>A. niger</i>)	sufficient									
Conversion	62%	Conversion	16%	16%	14%	11%	11%	Conversion	90%	Conversion	90%	84%
HC: 20 h, 50 °C, 0.85% C. EL: 0.07 µmol /g cellulose.		HC: 120 h, 50 °C, 5% C. EL: 0.4 mg/g cellulose.						HC: 24 h, 37 °C, 0.1% C. EL: 1.8 µmol /g cellulose.		HC: 72 h, 50 °C, 5%/2.5% Avicel/cotton. EL: 20/10 mg/g Avicel/cotton.		
<i>Kim et.al, 1998</i>		<i>Baker et.al, 1998</i>						<i>Boisset et.al, 2000</i>		<i>Gusakov et.al, 2007</i>		

HC: hydrolysis condition, EL: enzyme loading.

1.8.2 Optimizing cellulase components on pretreated biomass

Even though the reconstituted cellulase mixture, containing the major cellulase components, had better hydrolytic performance on certain cellulosic substrates, it did not perform well during hydrolysis of some pretreated lignocellulosic biomass (Banerjee et al., 2010a; Gao et al., 2010). Thus, it seems that the enzyme optimization should be based on the specific pretreated biomass, as both the cellulose characteristics (e.g. cellulose DP and crystallinity) and their association with hemicellulose and lignin will significantly influence the required essential enzymes and their ratio in the optimized “cellulase mixture” (Banerjee et al., 2010b; Gao et al., 2011).

Several groups have tried to optimize the “cellulase cocktail” on pretreated biomass (Rosgaard et al., 2007; Banerjee et al., 2010b; Gao et al., 2010). Although different optimization strategies and statistical design software were used in different studies, the common process was identical, as summarized in the flow chart (Figure 3). In short, the essential cellulase enzymes, namely “core cellulases”, were first identified by fractional factorial design, and then the required accessory enzymes were selected based on their ability to enhance the hydrolytic performance of the “core cellulases” on the specific pretreated substrates. After the “core cellulases” and the required accessory enzymes were identified, the optimized enzyme cocktail was formulated by ratio design of these enzyme components. Finally, the hydrolytic potential of the optimized enzyme cocktail was compared with commercially available cellulase mixtures on the pretreated biomass.

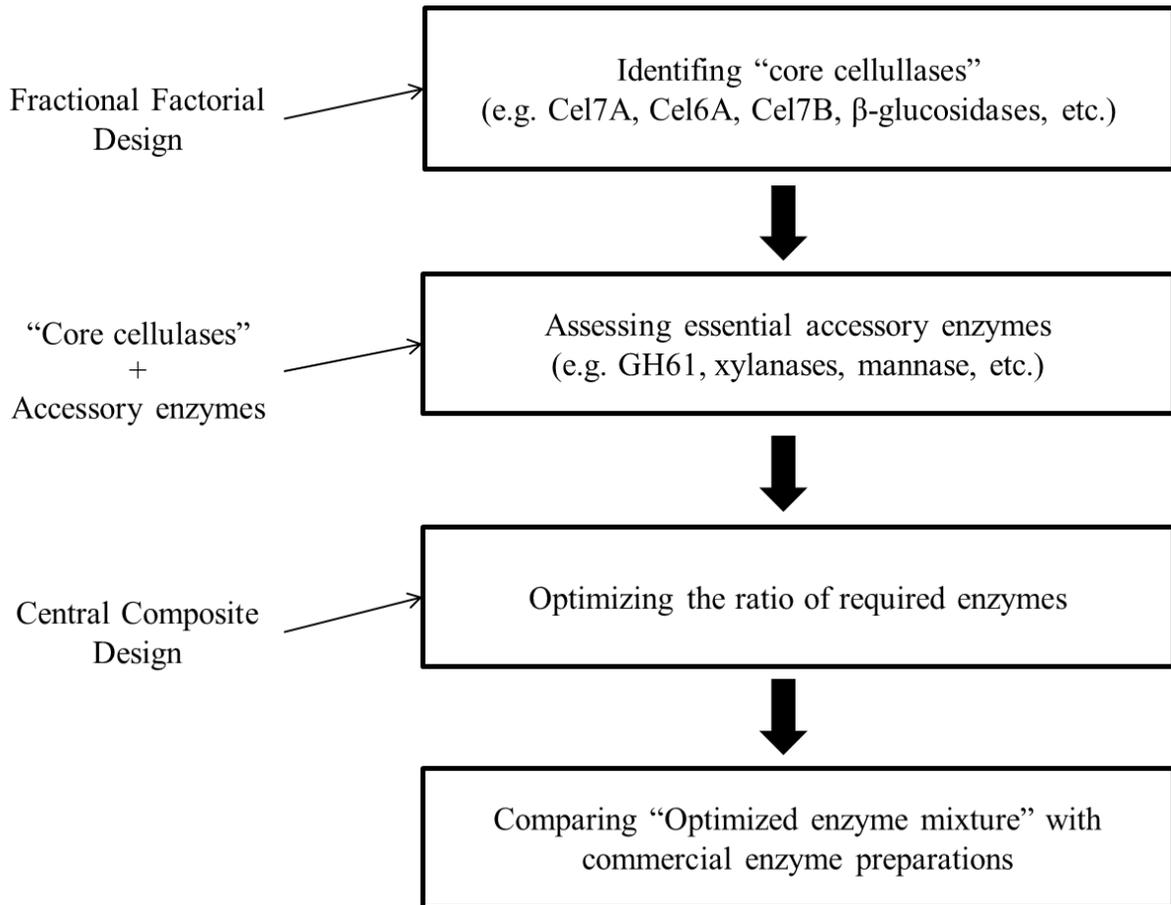


Figure 3. General process for optimizing an “enzyme cocktail” for lignocellulose deconstruction.

The reconstituted “optimized enzyme cocktails” for different pretreated biomass substrates are summarized in Table 3 and are based on reports in the literature. A large number of studies were carried out on ammonia fibre expansion (AFEX) pretreated corn stover by the Biomass Conversion Research Lab (BCRL) and the Great Lakes Bioenergy Research Center (GLBRC) in Michigan State University using a microplate technique (Banerjee et al., 2010b; Gao et al., 2010). The GENPLAT platform developed by GLBRC can analyze synthetic enzyme mixtures on pretreated biomass by robotic liquid handling and statistical experimental design. This system can automatically pipette substrate slurry, add

individual enzyme components, and determine the sugar products, thus providing a robust platform for identifying the essential enzymes and also for tailoring a more efficient and robust enzyme cocktail on various feedstocks.

For example, as shown in Table 3, the six core enzymes (CBHI, CBHII, EGI, BG, GH10 X, and BX) were first identified and optimized by GENPLAT on AFEX corn stover, but the hydrolytic efficiency of the optimized “core enzymes” was lower than the commercial enzyme preparations Accellerase 1000 plus Multifect xylanases. Therefore, 10 other potential accessory enzymes (GH11 X, Cel5A, arabinosidase B, Cel12A, Cel61A, Cel61B, glucuronidase, arabinosidase2, Cip1, and Cip2) were further assessed and a new “enzyme cocktail” was formulated. The new “enzyme cocktail” had a similar hydrolytic performance as the commercial enzymes (Accellerase and Spezyme), but only 50% of glucan and 33% of xylan were hydrolyzed. Therefore additional accessory enzymes, such as mannanase and amyloglucosidase, were further tested to improve the hydrolysis efficiency of the reconstituted “enzyme cocktail”. Although the GENPLAT system is very efficient for optimizing “enzyme cocktails”, one drawback is the very low substrate loading (usually 0.2%) used in the optimization, which may not be a true reflection of the optimized enzyme mixture’s performance in industrially relevant high-consistency hydrolysis (more than 10%).

In summary, optimizing “enzyme cocktails” by combining “core cellulases” and accessory enzymes provided valuable information to help better understand the essential enzymes required for efficient biodegradation of various pretreated biomass and it also significantly improve the efficiency of substrate hydrolysis. It appears that CBHI, CBHII and EGI are core enzymes for cellulose hydrolysis, while EGI, xylanase and BX are the essential ones for xylan hydrolysis. A sufficient amount of BG was an essential requirement in all the

optimized mixtures. Accessory enzymes such as AA9 and xylanases could significantly improve the hydrolytic efficiency of cellulases on a range of pretreated biomass substrates. Small amounts of other accessory enzymes such as arabinofuranosidase (Arb), glucuronidase (Gl), and mannanase, depending on the type of pretreated biomass, also contributed to cellulose hydrolysis. Selecting the “right” enzyme components is crucial to achieve a fast and efficient hydrolysis of pretreated biomass.

Table 3. Summary of enzyme optimization studies on pretreated lignocellulosic substrates

OPDF		SECS			a-SPBS	w-SPBS	AFEX CS (< 0.1mm)				AFEX CS (< 0.5mm)					
CBHIa	20%	CBHI	20%	CBHI	50%	45%	CBHI	30%	CBHII	25%	CBHII	5%	CBHII	4%	CBHII	4%
CBHIb	20%	CBHII	38%	CBHII	25%	17%	CBHII	19%	EGI	25%	EGI	22%	EGI	11%	EGI	19%
CBHIIb	40%	Cel 6B	5%	EGI	25%	38%	EGI	30%-35%	Xln GH10	13%	BG	15%	BG	4%	BG	8%
EGII	6%	EGI	18%	EGII	0%	0%	EX	15%	Xln GH11	13%	EX3	30%	EX3	12%	EX3	9%
EGV	8%	EGIII	15%	BG			BX	2%-6%	BG	" +2 mg"	BX	6%	BX	4%	BX	5%
BGL	4%	GH61A	2%				BG	" +10%"	BX	" +0.6 mg"			Cel61A	17%	Cel61A	18%
XyIII	2%	BG	3%						arabinofuranosidase	" +0.6 mg"			Cel61B		glucuronidase	
									glucuronidase	" +0.8 mg"			glucuronidase	1%	arabinosidase2	
													arabinosidase2	1%	Cip1	
													arabinosidaseB		Cip2	
													Cip 1		Cel5A	2%
													Cip 2		EX2	13%
													Cel 5A	5%	Cel12A	
													EX2	20%	mannase	
													Cel 12A		amyloglucosidase	
Conversion	88%G	73%G			56%G	38%G	80%G + 56%X		90%G + 70%X		40%G + 25%X		51%G + 33%X		52%G + 41%X	
HC: 72h, 50°C, 5% C EL: 12mg/g cellulose	HC: 72h, 50°C, 5% C EL: 57mg/g cellulose		HC: 48 h, 50 °C, 1% C. EL: 11 mg/g cellulose + BG			HC: 24 h, 50 °C, 0.2% C. EL: 33/22 mg/g cellulose				HC: 48 h, 50 °C, 0.2% C. EL: 15 mg/g cellulose						
<i>Gusakov et.al, 2007</i>	<i>Zhou et.al, 2009</i>		<i>Rosgaard et.al, 2007</i>			<i>Gao et.al, 2010,2011 (GLBRC, Michigan State University)</i>				<i>Banerjee, 2010, 2011 (GLBRC, Michigan State University)</i>						

HC: hydrolysis condition, EL: enzyme loading.

1.8.3 Case study: Novozymes cellulase preparations

Novozymes, the biggest enzyme production company in the world, produces “cellulase mixtures”. From the evolution of Novozymes’ cellulase products (Celluclast-2003; CTec 1-2009; CTec 2-2010; and CTec 3-2012), it can be observed that more and more enzymes/proteins have been added to these mixtures to improve their hydrolytic performance (Figure 4). Novozymes has claimed that their newest “cellulase mixture”, Cellic CTec 3, contains additional “cellulase components”, such as advanced AA9, improved β -glucosidases, and various hemicellulases, which enhances the hydrolytic efficiency of Cellic CTec3 to be 5 times better than the competing enzyme products on the market (Li et al., 2012).

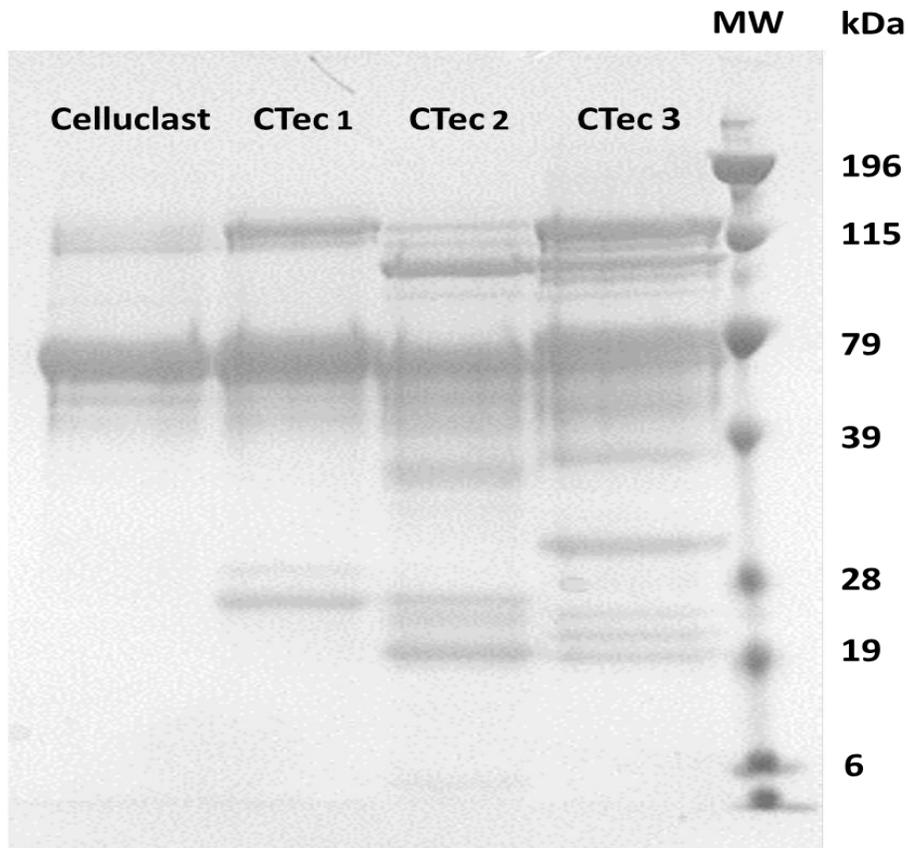


Figure 4. The evolution of Novozymes’ commercial cellulase preparations.

1.9 Existing challenges for synergism studies

As mentioned earlier, although synergism among cellulases has been studied for several decades, the exact mechanism of enzymatic cellulose biodegradation is still not completely understood and the evidence is, in many cases, contradictory and inconclusive (Zhang et al., 2006; Wilson, 2008; Kostylev and Wilson, 2012). This is likely due to the fact that synergism between cellulases involves multiple types of interactions in complicated cellulase mixtures, as discussed previously. In addition, there are also several different experimental factors, such as the method for assessment of enzyme synergism, the variety of substrates and cellulase components used in the studies, and different enzyme/substrate loadings, which can significantly affect the synergistic experimental data and their interpretation.

So far, most synergism studies have used glucose production as the way to evaluate the synergistic interaction among cellulase enzymes. However, as cellulose hydrolysis is such a complex process, where the multiple enzymes interact with insoluble substrates, other factors such as enzyme adsorption/desorption profiles and substrate physicochemical characteristics (e.g. cellulose accessibility, crystallinity, DP, etc.) should also be considered when assessing enzyme interactions (Woodward, 1991; Zhang and Lynd, 2004). Gaining a better understanding of the individual enzymes' interactions with other enzymes and/or with the substrate during hydrolysis has been challenging, primarily because of the lack of specific techniques that can assess/observe the individual enzymes and proteins in the "cellulase mixture" (Pribowo et al., 2013). For example, many model substrates used to evaluate the distribution of enzymes based on their activities, such as carboxymethyl

cellulose (CMC), filter paper, and various chromophoric substrates (e.g. p-nitrophenyl- based substrates) display overlapping specificities among different enzymes.

In addition, few, if any, current techniques are able to accurately assess the key substrate physicochemical properties during cellulose deconstruction. No single technique has the capacity to simultaneously quantify the effect occurring at multiple levels (from the microfibril up to the whole fibre) during the enzymatic hydrolysis process (Gourlay et al., 2012). Currently, various qualitative microscopic techniques, such as scanning electron microscopy (SEM) and atomic force microscopy (AFM), have been used to assess the changes in substrate physical properties. However, these techniques still have limitations in identifying some enzyme-substrate interactions, such as the changing of cellulose crystallinity and DP, and the amount of accessible cellulose. As for methods to assess cellulose crystallinity, a recent study (Park et al., 2010) pointed out that even the relatively more accurate methods such as XRD may not be capable of extracting exact information on crystal lattices within the cellulose structure.

1.10 High consistency hydrolysis

Regarding the overall commercial viability of the biomass-to-glucose process, it is crucial to produce a high sugar concentration from the enzymatic hydrolysis step. This would result in significant economic savings, not only in the operational cost for hydrolysis and fermentation, but also in minimizing energy consumption in the downstream process, such as distillation/evaporation (Jorgensen et al., 2007; Zhang et al., 2009). One way of obtaining a concentrated sugar solution is to use high substrate levels for the enzymatic hydrolysis process. For example, many authors claimed that at least 4-5% ethanol production is needed

to achieve an economically feasible bio-ethanol process (Varga et al., 2004; Jorgensen et al., 2007; Szijarto et al., 2011), which requires around 20% solid loading of the typical pretreated lignocellulosic substrates (50-60% cellulose content, dry weight DW) in the hydrolysis process to obtain enough glucose for the fermentation step. However, unlike the starch-based fuels hydrolysis, which can easily be carried out at 20-30% solid content (Bayrock and Ingledew, 2001), the high consistency hydrolysis of lignocellulosic biomass is more challenging due to inefficient mass transfer (e.g. rheological problems) and the increased levels of enzyme inhibitors from both sugars and various degradation products derived from other components (Cara et al., 2007; Zhang et al., 2009).

In order to overcome the challenges associated with the high solid hydrolysis, several strategies have been tried, such as using specially designed reactors, fed-batch systems, and simultaneous saccharification and fermentation (SSF) processes. Jorgensen et al., (2007) showed that up to 40% (DW) of pretreated wheat straw can be hydrolyzed by using a horizontally placed drum with a horizontal rotating shaft mounted with paddlers, while Zhang et al. (2009) reached 30% (DW) consistency hydrolysis of pretreated poplar by using the pulping equipment peg mixer. The combined fed-batch substrate loading and SSF processes also demonstrated the benefits of increasing the final ethanol concentration to around 50 g/L in several studies (Varga et al., 2004; Rudolf et al., 2005; Jorgensen et al., 2007).

Even though the solid loading can be increased to 30-40% by using the specially designed reactors, it seems that 20-25% (DW) consistency hydrolysis is more practical because a high glucose concentration can be achieved (>100g/L) without sacrificing hydrolysis yield (Cara et al., 2007; Zhang et al., 2009; Di Risio et al., 2011). By assessing the

rates and extent of enzymatic hydrolysis of pretreated corn stover with a range of solid loadings (2-32%, DW), Hodge et al. (2008) showed that the mass transfer limitations become apparent when solid concentrations approach 20%. Iogen's progress report for cellulosic ethanol production also indicated that 20% solid loading (DW) is the maximum that can be handled in pilot scale plants (Tolan, 2002). Similarly, in a practical fibre processing "pulp and paper" industry, a pulp consistency between 20-25% is typically encountered (Zhang et al., 2009).

So far, not many studies have assessed the influence of adding accessory enzymes and/or cellulase components during high consistency hydrolysis. Although Szijarto et al. (2011) suggested that Cel5A is a key enzyme in the "liquefaction" of biomass and that xylanases (GH11 EX) have virtually no effect on the viscosity of hydrothermally pretreated wheat straw, Di Risio et al. (2011) demonstrated that the rapid initial release of xylose in the high solid loading hydrolysis of steam pretreated poplar coincided with a significant reduction in slurry viscosity and particle size. Both studies observed increased sugar yields by supplementing xylanases to cellulase enzymes. Viamajala et al. (2009) claimed that the removal of hydrophilic polymers such as xylan can facilitate the liquefaction of high consistency slurry due to the increased availability of free water. In addition, it seems that there was always a recalcitrant cellulose fraction that remained inaccessible to cellulase enzymes (Cara et al., 2007), while accessory enzymes might increase the accessibility of cellulases to this inaccessible cellulose. Therefore, accessory enzymes, such as xylanases and AA9 hold the potential to synergistically cooperate with cellulase enzymes during high solid loading hydrolysis of pretreated lignocellulosic materials.

1.11 Thesis objectives

The work described in this thesis hoped to provide a better understanding of the roles and functions of cellulase components and several essential enzymes, such as xylanases, xyloglucanase, and lytic polysaccharide monooxygenase (AA9/GH61) during hydrolysis of “realistic” pretreated biomass substrates. By better understanding the interaction we hoped to improve the hydrolytic performance of an “enzyme cocktail” by generating high concentrations of biomass-derived sugar using low enzyme loading. The specific objectives of each chapter within the thesis is summarised below.

In Chapter 3.1 the interaction between a commercial cellulase preparation (Celluclast 1.5L) and xylanase preparation (Multifect xylanase) was assessed on steam pretreated corn stover (SPCS). The enzyme interactions were assessed at various cellulase:xylanase ratios. Different hydrolysis strategies were used such as adding the enzymes (cellulases and xylanase) separately, simultaneously, and sequentially. The changes in the gross fibre characteristics of SPCS was also monitored over the course of hydrolysis, with/without xylanase addition, to determine whether xylanases do more than just removing a xylan “coat” thus increasing accessibility to the cellulose.

In Chapter 3.2, to try to develop a better understanding of xylanase/cellulase synergism, more information on the interactions between individual cellulase components and xylanases and how these interactions vary on different pretreated biomass is required. Therefore, the major cellulase components (Cel7A, Cel6A, Cel7B, Cel5A) were purified from commercial cellulase preparations and their interaction with different family xylanases and xyloglucanases were assessed over a range of pretreated lignocellulosic substrates

(Chapter 3.2). By being able to assess the interaction between cellulase and various xylanases, we hoped to identify the best xylanase, if any, for enhancing the hydrolytic potential of cellulase enzymes. We also hoped to determine why different family xylanases performed differently on various “model” xylanolytic substrates and/or pretreated biomass.

Xylanases increased the accessibility of cellulase enzymes to cellulose and therefore significantly improved cellulose hydrolysis. Another barrier to cellulose hydrolysis comes from the highly organized crystalline cellulose structure, which limits the reactive sites of cellulose to cellulases. AA9 has been shown to be another promising accessory enzyme as they can attack the crystalline cellulose through an oxidative cleavage mechanism. Therefore, in Chapter 3.3 we assessed the interaction between cellulases and AA9 on various “model” cellulolytic and pretreated lignocellulosic substrates. The objective was to better understand the mechanism of the cooperation between cellulose hydrolytic and oxidative cleavage, but also to assess the potential of AA9 to enhance the hydrolytic performance of cellulase enzymes on various biomass substrates.

Previous cellulase synergism studies have mainly used “model” cellulosic substrates (e.g. cotton and Avicel) with low enzyme dosage and it has been shown that their synergism is highly dependent on enzyme concentration and substrate characteristics. Therefore we carried out a systematic cellulase synergism study (Chapter 3.4) with the expectation that we could further understand the synergistic cooperation of individual cellulase components, especially during hydrolysis of pretreated biomass.

Past work on assessing the beneficial effect of accessory enzymes was largely based on low solid loading (2-5%) hydrolysis, which is hard to justify because high solid loading

(~20%) hydrolysis is required for achieving economically feasible bio-fuel/chemical production. Therefore, we next assessed the influence of the essential accessory enzymes (xylanase and AA9) on the high solid loading hydrolysis of pretreated biomass by cellulases (Chapter 3.5). It was expected that the synergistic cooperation between cellulase and accessory enzymes would be intensified and more significant on high solid loading hydrolysis. Subsequently, we compared the hydrolysis ability of our customized enzyme cocktail (cellulases, xylanases, and AA9) with Novozymes' latest enzyme preparations on pretreated biomass to understand the extent of the beneficial effects that accessory enzymes have in the new commercial enzyme preparations.

The general goal of the thesis was to ascertain whether accessory enzymes would synergistically cooperate with cellulase enzymes during hydrolysis of pretreated biomass substrates. By identifying the essential enzymes and understanding the nature of their major interactions, we hoped to provide valuable information that is needed to formulate more efficient and robust enzyme cocktails that would be effective on a range of pretreated lignocellulosic substrates.

2. Materials and methods

2.1 Lignocellulosic/cellulosic substrates

A range of lignocellulosic substrates were used in this study, including three different agricultural residues (corn stover, sweet sorghum bagasse, and corn fibre), a hardwood substrate (poplar), and a softwood substrate (lodgepole pine). Cellulose nanocrystal (CNC), dissolving pulp (DSP) and cellulose III were kind gifts from the Forest Products Laboratory – USDA Forest Service and the MSU Biomass Conversion Research Laboratory, respectively. Avicel PH 101 was purchased from Sigma (MO, USA). PASC and Cellulose II were produced from Avicel according to Ishikawa et al. (1997).

2.2 Pretreatment

Steam pretreatment was conducted in a 2 L StakeTech III steam gun (Stake Technologies, Norvall, ON, Canada) in the Forest Products Biotechnology/Bioenergy Laboratory at the University of British Columbia. Briefly, corn stover, sweet sorghum bagasse, corn fibre, and wood chips from poplar and lodgepole pine (DW; dry weight of 300 g) were impregnated overnight with SO₂ (Table 4) in a sealable plastic bag. The bags were then opened and left for 60 min in a fumehood to release the unabsorbed SO₂. A batch of biomass (50 g) was loaded in the steam gun and the conditions used for pretreatment are listed in Table 4. Most of the pretreatment conditions were selected, based on previous work in this laboratory (Bura et al., 2003; Nakagame et al., 2010), to maximise overall sugar recovery (hemicellulose and cellulose) while providing a cellulosic component that could be readily hydrolysed with relatively low enzyme loadings. A poplar sample (SPP180) was also

pretreated at low severity in order to maintain a relatively high hemicellulose/xylan content in the water insoluble cellulosic fraction.

Organosolv pretreatment was carried out in a 2 L rotating digester (Aurora Products Ltd, Savona, BC, Canada) as described by Del Rio et al. (2010). Biomass (200 g DW) was incubated in a mixture containing water, ethanol and a chemical additive (H₂SO₄) as shown in Table 4. The vessels containing the biomass and the pretreatment liquor were heated up and maintained at the target temperature for a specified time. The vessels were then immediately cooled down to room temperature in a water bath, and the pretreated substrates were separated from the liquor using a nylon cloth.

Table 4. Pretreatment conditions for various biomass substrates used in this study (Steam pretreated corn stover at 190 °C, poplar at 200 °C, lodgepole pine, and organosolv pretreated corn stover, poplar, and lodgepole pine were kindly donated by Seiji Nakagame)

Pretreatment	Species	Temperature (°C)	Time (min)	SO ₂ (% w/w)	Ethanol (% v/v)	H ₂ SO ₄ (%)
Steam pretreatment	Corn stover	180-190	5	3	-	-
	Sweet sorghum bagasse	190	5	3	-	-
	Corn fibre	190	5	3	-	-
	Poplar	180-200	5	3	-	-
	Lodgepole pine	200	5	4	-	-
Organosolv pretreatment	Corn stover	200	60	-	75	1.0
	Poplar	200	60	-	60	1.25
	Lodgepole pine	170	50	-	60	1.0

After the steam/organosolv pretreatment, the solid fractions of the pretreated biomass were collected and washed with water and vacuum filtered to a final moisture content above 60%. A small fraction of this sample was kept at 4 °C for chemical composition analysis and daily experiments; and the rest was stored at -20 °C to prevent contamination.

2.3 Compositional analysis of pretreated substrates

The chemical composition of the substrates' water insoluble fraction after steam and organosolv pretreatment were determined using the modified Klason lignin method derived from the TAPPI standard method T222 om-88 as previously described by Bura et al. (2003). The carbohydrate composition of acid hydrolysate was determined by high performance anion exchange chromatography (Dionex DX-3000, Sunnyvale, CA). Acid soluble lignin was determined by reading the absorbance at 205 nm on a Cary 50 UV-Vis spectrometer. The acid insoluble lignin was determined gravimetrically by drying the insoluble lignin in an oven overnight.

2.4 Delignification and hemicellulose removal treatments

The complete delignification of the pretreated substrates was achieved using sodium chlorite according to the procedure in the Pulp and Paper Technical Association of Canada's (PAPTAC) Useful methods G10.U. Approximately 30 g (DW) of never-dried pulp substrates were resuspended in a 300 ml solution composed of 5% (w/v) NaClO₂ dissolved in 1% (v/v) acetic acid and incubated overnight in the dark at room temperature. The delignified substrates were then filtered using a funnel and washed thoroughly with water. The resulting

substrates (with no detectable lignin as characterised by the Klason method) were collected and kept in 4 °C.

The removal of residual hemicelluloses after delignification was executed as described in the TAPPI standard method T203 cm-99. Briefly, 15 g (DW) never-dried delignified substrates were resuspended in deionized water and 50% (w/v) NaOH to give a final volume of 750 ml and a final NaOH concentration of 17.5% (w/v). The pulp slurry was stirred for 30 min and then the deionized water was added to dilute the NaOH concentration to 9.5% (w/v). The diluted pulp slurry was stirred for a further 30 min and filtered under vacuum and rinsed 3 times with 9.5% (w/v) NaOH, followed by deionized water until the pH of the filtrate was neutral.

2.5 Enzymes

2.5.1 Commercial enzyme preparations

Commercial cellulase enzyme mixtures Celluclast 1.5L, CTec 1, CTec 2, and CTec 3 were from a genetically modified strain of *T. reesei* (Novozymes, Franklinton, NC). Novozyme 188 (protein content 233 mg/mL, Novozymes A/S, Bagsvaerd, Denmark) was the β -glucosidase preparation used in this study. Multifect xylanase (Genencor US Inc., Palo Alto, CA) and HTec 2 (Novozymes, Franklinton, NC) were the commercial xylanase preparations used.

2.5.2 Enzyme purification

The purification of the four major cellulase components of Celluclast 1.5L (*T. reesei*), namely Cel7A (CBHI), Cel6A (CBHII), Cel7B (EGI), and Cel5A (EGII), was based on a modified procedure from previously reported (Medve et al., 1998; Rosgaard et al., 2007; Zhou et al., 2008). The process is summarized in Figure 5. Briefly, proteins in Celluclast 1.5L were firstly separated by an anion exchange column (Hiload 16/10 Q Sepharose High Performance column) using a linear gradient change of buffer stock A (20 mM Triethanolamine (TEA), pH 7.0) to buffer stock B (20 mM TEA, 1 M NaCl, pH 7.0). This step resulted in four major peaks I, II, III, and IV, which were collected and used for further separation of the major cellulase components. CBHI was purified by rechromatographing the combined fractions from peak IV using a size exclusion column (Hiload 16/60 Superdex 75 prep grade column) with an isocratic flow of sodium acetate buffer (50 mM, pH 5.0). CBHII was purified from peak II by another high resolution anion exchange column (Bio-Rad UNO Q1 column) with a linear gradient change of buffer stock A (20 mM Tris-HCl, pH 8.8) to buffer stock B (20 mM Tris-HCl, 1 M NaCl, pH 8.0). The proteins eluting in peak III and peak IV were further purified by hydrophobic interaction chromatography (HiPrep 16/10 Phenyl FF high sub column), respectively, with a gradient change of start buffer (20 mM sodium phosphate buffer (SPB), 1 M $(\text{NH}_4)_2\text{SO}_4$, pH 7.0) to elution buffer (20 mM SPB, pH 7.0). After that, peak III was separated into another three peaks and one of the major peaks was collected and injected into UNO Q1 column with a linear gradient change of buffer stock A (20 mM Tris-HCl, pH 8.8) to buffer stock B (20 mM Tris-HCl, 1 M NaCl, pH 8.0), from which the purified EGI was obtained. Peak IV was also separated into three peaks and the

major peak was further purified by UNO Q1 column with a linear gradient change of the same buffer used for purification of EGI, then EGII was obtained.

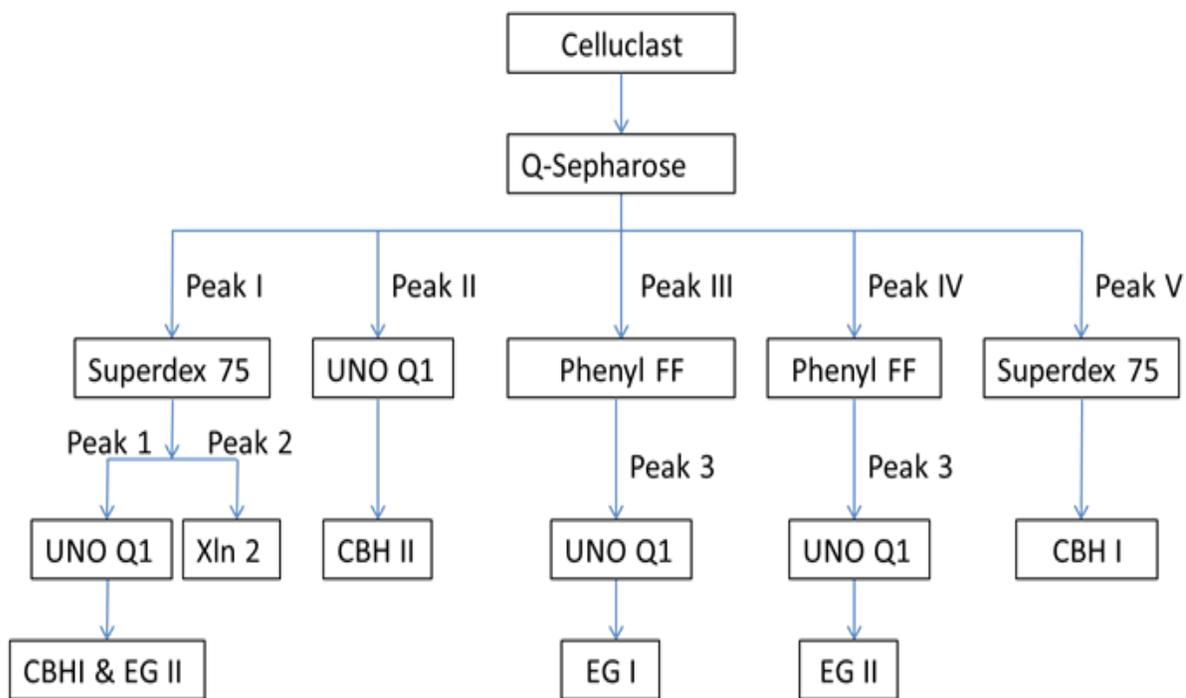


Figure 5. Purification of the major cellulase components from Celluclast 1.5L by FPLC. Q-Sepharose (anion exchange column); Superdex 75 (size exclusion column); Phenyl FF (hydrophobic interaction column); UNO Q1 (anion exchange column at high resolution).

Family 11 endo-xylanase (GH11 EX), family 10 endo-xylanase (GH10 EX), and family 3 β -glucosidase (GH3 BG) were purified from Multifect Xylanase, HTec 2, and Novozyme 188, respectively, by a two-step chromatography process (Gao et al., 2010). In brief, enzyme stocks were firstly injected into a size exclusion column (Hiload 16/60 Superdex 75 prep grade column) with an isocratic flow of TEA buffer (20 mM, pH 7.0), and the peaks containing major endo-xylanase and β -glucosidase activity were collected and further purified by ion exchange chromatography (UNO Q1 column) with a linear gradient

change of buffer stock A (20 mM TEA, pH 7.0) to buffer stock B (20 mM TEA, 1 M NaCl, pH 7.0).

The family 5 xyloglucanase (GH5 XG) derived from *Paenibacillus* sp. was purchased from Megazyme and desalted using a sodium acetate buffer (50 mM, pH 4.8). AA9/GH61 stock was kindly provided by Novozymes, and the pure AA9 enzymes could be separated by a one-step size exclusion chromatography process (Hiload 16/60 Superdex 75 prep grade column) with an isocratic flow of sodium acetate buffer (50 mM, pH 5.0).

All purification procedures were performed in an automated FPLC system (BioLogic Due-Flow). The buffers used for enzyme purification were prepared using nanopure water and filtered through a 0.22 μm membrane filter (Millipore) followed by sonication for at least 30 min. The purity of the enzymes and lack of contamination by other cellulases and hemicellulases was confirmed by SDS-PAGE and liquid chromatography–mass spectrometry/mass spectrometry (LCMS/MS) as described by Pribowo et al. (2012).

2.5.3 Enzyme activity assays

The filter paper activity of the commercial cellulase preparations was determined according to the method published by the International Union of Pure and Applied Chemistry (Ghose, 1987). Cellobiohydrolase, β -glucosidase and β -xylosidase activities were determined by using p-nitrophenyl- β -D-cellobioside (p-NPC), p-nitrophenyl- β -D-glucopyranoside (p-NPG), and p-nitrophenyl- β -D-xylopyranoside (p-NPX) as substrates, respectively, according to Saha and Bothast (1996).

Xylanase, xyloglucanase, and CMCase activities were determined by a method modified from Lin and Thomson (1991). Briefly, beechwood glucuronoxylan, birchwood glucuronoxylan, and oat spelt arabinoxylan were dissolved in 50 mM sodium acetate buffer (pH 5.0) by stirring overnight at room temperature, while Tamarind xyloglucan (Megazyme) and carboxymethylcellulose (CMC) were dissolved in the same buffer but only for 3 h. To assay the activities, 70 μ l substrates were added in microplates with 30 μ l of the appropriately diluted enzyme samples and mixed in an incubator at 400 rpm for various incubation times at 50 °C. The enzymatic reaction was stopped by adding 200 μ l of 3,5-dinitrosalicylic acid (DNS) reagent after exactly 5, 10, 20, and 30 min incubation. After that, the microplates were placed in an oven at 105 °C and boiled for 30 min, and the reducing sugar content of the samples were analyzed by measuring the absorbance at 540 nm. Xylose and glucose standards were used for calibration. The reducing sugar released (μ mol) at different hydrolysis times was plotted, and the enzyme activity (μ mol/min) was determined by the slope of the linear phase of the hyperbolic curve.

2.6 Enzymatic hydrolysis

The regular hydrolysis experiments (1 ml) were carried out at 2% (w/v) solids loading in sodium acetate buffer (50 mM, pH 4.8), 50 °C, 150 rpm in a benchtop hybridization incubator (combi - H12). The high consistency hydrolysis experiments were performed at 10% and 20% (w/v) solids content in the same buffer system, but the reaction mixtures were 20 ml and were incubated at 150 rpm, 50 °C, in a rotary shaker. Cellulases were used at a range of protein loadings (per gram of cellulose) with enough β -glucosidase supplementation to prevent inhibition from cellobiose accumulation.

The accessory enzymes were added using two strategies: i) supplementation – varying amounts of accessory enzymes were supplemented to the cellulase mixture; ii) cellulase replacement – varying amounts of the total cellulase loading was replaced with an amount of accessory enzymes to avoid an increase in the total protein loading. The restart hydrolysis experiments were carried out by incubating the substrates with cellulases or xylanases alone for 24 h. Thereafter, other enzymes or protein controls were added to the pre-hydrolyzed mixture and incubated for a further 48 h.

Samples were periodically taken during the course of hydrolysis. For the sugar analysis, the samples were heated at 100°C for 10 min to inactivate the enzymes. Supernatants were collected after centrifugation at 13000 rpm for 10 min and stored at -20°C. For protein analysis, the samples were collected without heating and kept at 4°C. The concentration of glucose and xylose in the supernatants were measured using HPLC (Dionex DX-3000, Sunnyvale, CA) as described elsewhere (Boussaid et al., 1999). Alternatively, the quantitative analysis of glucose concentration in the hydrolysate was also performed by a glucose oxidase assay (Berlin et al., 2006a). The hydrolysis yields (%) of the pretreated substrates were calculated from the cellulose and xylan content as a percentage of the theoretical cellulose and xylan available in the substrates. All hydrolysis experiments were performed in duplicate and mean values and standard deviations are presented. Substrate and enzyme blanks were run at the same time by incubating the substrates without enzymes and by incubating the enzymes without substrates.

2.7 Enzyme synergism

The following equation was used to calculate the degree of synergism between enzymes during hydrolysis (Woodward et al., 1988b; Kostylev and Wilson, 2012):

$$DS = \frac{GC_{\text{mixture}}}{\sum GC_{\text{individual}}}$$

where GC_{mixture} is the cellulose hydrolysis achieved with reconstituted enzyme mixture, while $\sum GC_{\text{individual}}$ is the sum of cellulose hydrolysis achieved with the individual enzymes.

2.8 Protein content assay

The protein concentration of enzyme mixtures and purified enzymes were measured using the ninhydrin assay and bovine serum albumin (BSA) as the protein standard (Starcher, 2001). Diluted samples (40 μl) were mixed with 1 N HCl (100 μl) in Eppendorf tubes (1.5 ml) and incubated in the oven (105 °C) overnight. The incubated tubes were cooled for 30 min and briefly centrifuged, after which 200 μl of 2% ninhydrin reagent (Sigma, MO, USA) was added. After mixing with a vortex mixer, the mixture was heated at 100°C for 20 min with subsequent cooling for 10 min at room temperature. Then 50% (v/v) of ethanol (1 ml) was added into the tube and absorption measured at 570 nm using a Bio50 UV-vis spectrophotometer.

The concentrations of Cel7A/Cel7B in the hydrolysis system were determined using an immunoassay to specifically quantify the amount of Cel7A and Cel7B in the supernatant (Pribowo et al., 2013). Briefly, a monoclonal antibody (MAb) specific for Cel7A/Cel7B was

used to distinguish this enzyme from the other enzymes present in the supernatant. A Cel7A/Cel7B polyclonal antibody (PAb) was then used to bind the captured Cel7A/Cel7B. The amount of Cel7A/Cel7B was indirectly quantified by measuring the bound PABs using a third antibody conjugated to alkaline phosphatase (AP, Biorad). The quantitation was achieved by adding p-nitrophenylphosphate (Bio-Rad), a substrate for alkaline phosphatase, and the reaction was incubated at room temperature for 30 min. The reaction was stopped by adding 100 mL glycine-NaOH (400 mM). The amount of Cel7A/Cel7B was then indirectly quantified by measuring the absorbance of p-nitrophenyl at 405 nm.

2.9 Fibre quality analysis

A fibre quality analyzer (FQA) (LDA02; OpTest Equipment, Inc., Hawkesbury, ON, Canada) was used to measure the gross fibre characteristics such as fibre length, width, and size distribution according to the procedure described by Robertson et al. (1999). Briefly, the settings on the FQA were adjusted to measure particles down to 0.07 mm. A dilute suspension of fibres with a fibre frequency of 25-40 events per second was transported through a sheath flow cell where the fibres are oriented and positioned. The images of the fibres were detected by a built-in CCD camera and the fibre length and width were calculated by circular polarized light. The weighted average fibre lengths and fibre width (L_w/W_w) were determined and the fibre size and distribution were plotted.

2.10 Cellulose accessibility

2.10.1 Simons' stain

Cellulose accessibility to cellulases was estimated by the Simons' stain technique according to the modified procedure by Chandra et al. (2008). Briefly, direct orange (DO-Pontamine Fast Orange 6RN) and direct blue (DB-Pontamine Fast Sky Blue 6BX) dyes were obtained from Pylam Products Co. Inc. (Garden City, NY, U.S.) and fractionated by an Amicon filtration system. For each type of substrate, approximately 100 mg (DW) of never-dried samples were weighed into each of six 15 ml polypropylene centrifuge tubes. Each tube received 1.0 ml of phosphate-buffered saline (PBS, pH 6) followed by the addition of DO and DB dyes solutions (10 mg/ml), in a series of increasing volumes (0.25, 0.50, 0.75, 1.0, 1.5, and 2.0 ml), to the six tubes with each set of tubes containing a 1:1 mixture of DO and DB dyes at increasing concentrations. Then nanopure water was added to make up the final volume of the samples to 10 mL in each tube. The tubes were incubated overnight at 70°C in an orbital shaker at 200 rpm. After the incubation, the tubes were centrifuged at 5,000 rpm for 10 min, and a sample of the supernatant was placed in a cuvette and the absorbance read on a Cary 50 UV-Vis spectrophotometer at 624 and 455 nm. The amount of dye adsorbed on the fibre was calculated using the difference in the concentration of the initially added dye and the concentration of the dye left in the supernatant according to the Beer-Lambert law. The extinction coefficients were calculated by preparing standard curves of DO and DB dyes and measuring the slope of their absorbance at 455 and 624 nm, respectively.

2.10.2 CBM assay

The relative amount of accessible crystalline cellulose and amorphous cellulose was assessed by two specific carbohydrate binding modules (CBM): CBM2a has a planar binding face and preferentially adsorbs to crystalline cellulose, while CBM44 has a cleft-shaped binding site that preferentially adsorbs to amorphous cellulose (Gourlay et al., 2012). Briefly, never-dried samples (containing about 4 mg cellulose) were mixed with 350 μg CBM and made up to a final volume of 1 ml with phosphate buffer (50 mM, pH 7.0). Samples were incubated at room temperature for 1 h followed by centrifugation at 18,000 $\times g$ for 10 min. The concentration of CBM2a in the supernatant was determined by measuring the absorbance of the solution at 280 nm (Cary 50 UN-Vis spectrophotometer). The extinction coefficients for CBM2a and CBM44 are 27,625 M^{-1} and 27,365 M^{-1} , respectively.

2.11 Cellulose crystallinity

Cellulose crystallinity (CrI) was measured by X-ray diffraction (XRD) as described in Nishiyama et al. (2002). Briefly, pulp samples were washed and filtered to prepare sheets with a relatively flat surface, and then mounted onto a zero-background plate. The data was collected with a Bruker D8-Advance powder X-ray diffractometer. Bruker TOPAS version 4.2 was used to model percent crystallinity and Nishiyama's cellulose 1 β was used to model cellulose. The percentage cellulose crystallinity was calculated as: 100 \times (crystalline area/total area), where the total area = crystalline area + amorphous area.

2.12 Cellulose degree of polymerization

Cellulose degree of polymerization (DP) was measured by gel permeation chromatography (GPC) as described in Del Rio et al. (2010). Briefly, the pulp substrates were carefully delignified and hemicellulose removed as described in section 2.4. Approximately 20 mg of oven-dried, delignified and hemicellulose-free samples were placed in glass tubes, where the samples were resuspended in 7 mL anhydrous pyridine followed by the addition of 3 mL phenylisocyanate. The tubes were then sealed with Teflon stoppers and incubated at 80°C with periodic vortexing for 48 h. The reaction was stopped by adding 1 mL of methanol to the mixture, and the tricarbonylated cellulose was recovered by precipitation in 4 volumes of methanol followed by centrifugation at 5,000 rpm for 20 min. The precipitated cellulose tricarbonylate was washed three times with methanol to remove all traces of pyridine. The washed samples were resuspended overnight in tetrahydrofuran (THF, final concentration 2 mg/mL) at 4°C and filtered through a 0.45 µm filter. The GPC analysis of the tricarbonylated samples was carried out in an Agilent 1100 HPLC system (Palo Alto, Ca) equipped with two styragel columns (HR5E and HR1 purchased from Waters, Milford, MA) in tandem. THF was used as the mobile phase and a GPC calibration curve was generated from the elution profile of polystyrene standards. The cellulose DP was calculated by dividing the molecular weight of the tricarbonylated polymer by the molecular weight of tricarbonylated anhydroglucose ($DP = MW/519$). The weight average (DP_w) was determined.

2.13 Acid group analysis

Incorporation of acid groups in the substrate was determined by conductometric titration as described by Katz et al. (1984). In brief, 0.05 g (dry weight base) substrate was

added to 15 ml of 0.10 N HCl. The mixture was incubated overnight. The pulp was then washed with nanopure water by adding 35 ml of water, mixing, and centrifuging it at 5000 xg for 10 min. The solution was carefully decanted and the pulp was further washed five times with 50 ml water. The washed pulp was re-suspended in 50 ml of NaCl (0.001 N) and 20 μ l HCl (0.50 N), stirred and conductometrically titrated with 0.05 N NaOH. The total acid groups were deduced by plots of the titration curve, namely volume of NaOH against conductivity (Del Rio et al., 2011).

2.14 Reducing capacity analysis

Substrate soluble components were prepared by incubating 2% of pretreated substrates with 50 mM acetate buffer in a rotating incubator overnight at 50°C. The soluble components were then separated from the solid substrates after centrifugation at 16000 x g for 30 min. Assays for reduction of Fe³⁺ ions by the soluble components were carried out in 1.5 ml eppendorf tubes containing 0.4 ml sodium acetate buffer at pH 5.0 (20 mM), 0.375 ml substrate soluble components, and 0.025 ml freshly prepared FeCl₃ (20 mM) at room temperature for 30 min. Then 0.2 ml ferrozine sodium salt 1% (w/v) was added to the tubes to make up a final volume of 1 ml and the absorption of Fe²⁺/ferrozine complexation was assessed on a UV/Visible spectrophotometer at 562 nm ($\epsilon_{562 \text{ nm}} = 27,900 \text{ M}^{-1} \text{ cm}^{-1}$) (Aguilar and Ferraz, 2007; Arantes and Milagres, 2007).

3. Results and discussion

3.1 The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect?

3.1.1 Background

As described earlier in Chapter 1 – 1.4, many pretreatment strategies such as steam explosion use mild severity conditions to avoid or at least minimize sugar loss during pretreatment (Chandra et al., 2007; Alvira et al., 2010). Under these milder pretreatment conditions, some of the hemicellulose, mostly xylan in agricultural residues and hardwood, remains associated with the cellulosic-rich water insoluble fraction (Chandra et al., 2007). However, this residual hemicellulose component is known to exert a significant influence on the effectiveness of enzymatic hydrolysis of its cellulosic component (Bura et al., 2009; Kumar and Wyman 2009b; Varnai et al., 2010).

Due to the fact that the hemicellulose-degrading enzyme activities detected in most of the commercially available cellulase preparations are low or insufficiently active to achieve significant conversion of the residual hemicellulose (Berlin et al., 2007; Ohgren et al., 2007), supplementation of the cellulase enzymes with the so-called “accessory enzymes”, such as xylanase-enriched preparations, has been the most common approach to increase the overall fermentable sugar yields from pretreated hardwoods (Bura et al., 2009; Kumar and Wyman 2009c) and agricultural residues (Garcia-Aparicio et al., 2007; Ohgren et al., 2007). Though accessory enzymes offer the potential to increase substrate digestibility, the high dosages of enzyme supplementation applied in many of the past studies can be difficult to justify

because of the increased enzyme costs that are incurred. It is generally acknowledged that, among the several factors that hamper our current ability to attain efficient lignocellulosic biomass conversion yields at low enzyme loadings, a major problem lies in our incomplete understanding of the cooperative action of the different enzymes acting on pretreated lignocellulosic substrates.

Hemicellulose, which is generally found in higher concentrations on the outer surface of cellulose fibres and is also diffused into the interfibrillar space through fibre pores (Oksanen et al., 1997; Kohnke et al., 2010), has been proposed to act as a physical barrier that limits the accessibility of the cellulase enzymes to the cellulose (Suurnakki et al., 1997; Bura et al., 2009; Selig et al., 2009). In recent work, we and other workers have also shown that the limited access of cellulase enzymes to the cellulose chains is a key factor which necessitates the use of relatively high enzyme dosages to attain effective cellulose hydrolysis (Arantes and Saddler, 2011). One of the main beneficial effects of cellulase supplementation with xylanase during biomass saccharification is thought to be the result of the improved cellulose accessibility as a result of xylan solubilisation (Ohgren et al., 2007; Kumar and Wyman, 2009b).

It has been suggested that xylan forms a sheath on each cellulose microfibril and that it is also “zipped into” the cellulose microfibrils during crystallization, shortly after cellulose synthesis (Pauly et al., 1999; Scheller and Ulvskov, 2010). Thus it might be anticipated that the depolymerization of cellulose by cellulases within the fibre would expose the xylan chains, naturally trapped within or between microfibrils, to the action of xylanase. If true, this degradation model might indicate the potential synergism between xylanase and cellulase enzymes during lignocellulose hydrolysis. Previous work looking at xylanase-aided

bleaching treatment of Kraft cellulosic pulps showed that the xylanase not only hydrolyzed the reprecipitated xylan on the fibre surface, but also increased pulp fibre porosity, resulting in a substantial increase in the permeability of the cellulosic pulp fibres (deJong et al., 1997; Suurnakki et al., 1997; Wong et al., 1997).

The synergistic action among the multiple forms of hemicellulose-degrading enzymes (e.g. enzymes acting on the xylan backbone and on the xylan side chains) and also among the cellulose-degrading enzymes (e.g. exoglucanases and endoglucanases) have been studied extensively (Woodward, 1991; Jeoh et al., 2006; Selig et al., 2008; Wilson, 2008). Although synergistic cooperation between cellulases and an endo-xylanase has been observed at low substrate conversion yields (Murashima et al., 2003; Selig et al., 2008; Morais et al., 2010), limited work has looked at the interaction between xylanase and cellulases at conditions relevant to the biofuels industry. This is when relatively low enzyme loadings are used to achieve fast and nearly complete hydrolysis of the cellulose. Similarly, the lack of relevant controls in this previous work has made it difficult to determine if the beneficial effect of the accessory enzyme addition was a result of a cooperative interaction (synergism) with cellulases or merely an additive effect, as the increased substrate hydrolyzability upon enzyme supplementation was typically associated with a corresponding and often substantial increase in protein loading.

In this section, the possible additive or synergistic interaction of xylanase with cellulases was determined during the hydrolysis of steam pretreated corn stover (SPCS) at the minimum enzyme loading required to achieve substantial (greater than 70%) cellulose hydrolysis. Initially, two strategies were evaluated; 1) xylanase supplementation of the minimum cellulase dose required for effective cellulose hydrolysis and, 2) replacement of a

portion of the minimum cellulase dose with xylanase, while keeping the total protein loading constant. The degree of synergism (DS) was calculated and compared at various xylanase:cellulase ratios. To further investigate the xylanase-cellulase interaction mechanisms, hydrolysis experiments were also carried out by adding the enzymes (cellulases and xylanase) separately, simultaneously, and sequentially. The changes in the gross fibre characteristics of SPCS were also monitored over the course of hydrolysis with or without xylanase addition. The beneficial hydrolysis-boosting effect of xylanase supplementation was then evaluated on a range of lignocellulosic substrates. Finally, the potential of further reducing xylanase loading in the “optimized” enzyme mixture was assessed, and the possibility of increasing cellulase enzyme recyclability by xylanase supplementation was also evaluated.

3.1.2 Results and discussion

3.1.2.1 The protein content and specific activities of the enzyme preparations

Initially the protein concentrations and specific activities of the three commercial glycoside hydrolase preparations used in this study were determined and compared (Table 5). As expected, all preparations demonstrated substantial differences in their protein content and specific activities towards model substrates. Novozyme 188, which is commonly used as the β -glucosidase (BG) supplementation, displayed the highest protein concentration and BG activity. The cellulase enzyme cocktail, Celluclast 1.5 L, displayed the highest activity towards all of the cellulosic substrates, while the other enzyme preparations had below detectable levels of filter paper activity and contained very low CMCase activity. The Celluclast 1.5L preparation contained low levels of endo-xylanase activity, indicating its low

hydrolytic capability towards xylan. In contrast, the Multifect xylanase showed very high endo-xylanase activity relative to the other enzyme preparations and very low activities towards the cellulosic substrates, indicating its overall low saccharification activity towards cellulose.

Table 5. Protein content (mg/ml), filter paper activity (FPU) and specific activities (U/ml) of the commercial enzyme preparations on model substrates

Enzyme preparation	Protein content	FPA	CMCase	β -glucosidase	CBH I	Xylanase	β -xylosidase
Celluclast 1.5L	129.2	50.3	474.7	17.0	158.0	438.8	37.8
Multifect Xylanase	37.1	N/A	9.0	12.7	2.7	2588.4	22.5
Novozyme 188	233.4	N/A	15.0	239.0	26.2	32.63	3.9

CMCase: carboxymethyl cellulase; CBH I: cellobiohydrolase I; FPA: filter paper activity; FPU: filter paper unit; n/a: negligible activity detected.

3.1.2.2 Effect of xylanase supplementation on the enzymatic hydrolysis of SPCS

In order to assess the influence of xylanase addition during the enzymatic hydrolysis of the SPCS substrate (Table 6), the hydrolysis experiments were carried out over a range of increasing dosages of cellulase activity (Celluclast 1.5L) in the absence and presence of fixed amounts of xylanase activity (Multifect xylanase) (Figure 6).

Table 6. Steam pretreatment conditions and chemical composition of pretreated biomass

Substrate	Pretreatment Conditions	Composition of pretreated feedstocks						Abbreviation
		<i>Ara</i>	<i>Gal</i>	<i>Glu</i>	<i>Xyl</i>	<i>Man</i>	<i>AIL</i>	
Corn stover	190 °C, 5 minutes, 3% SO ₂	1.0	0.7	56.1	7.0	1.1	27.0	SPCS
Sweet sorghum bagasse	190 °C, 5 minutes, 3% SO ₂	0.6	0.8	54.3	9.8	1.0	25.8	SPSB
Lodgepole pine	200 °C, 5 minutes, 4% SO ₂	bdl	bdl	46.4	bdl	bdl	45.0	SPLP

AIL: acid insoluble lignin; Ara: arabinan; bdl: below detectable level; Gal: Galactan; Glu: glucan; Man: mannan; Xyl: Xylan

In the absence of xylanase, the hydrolysis of the cellulose and xylan within the SPCS increased with increasing cellulase loading and reached a plateau at a cellulase protein loading of 35 mg/g cellulose (Figure 6). At this enzyme dosage, the cellulose and xylan hydrolysis were about 70 and 80%, respectively. The high xylan hydrolysis achieved by the Celluclast 1.5L might be caused by the xylanase activity within the Celluclast 1.5L as shown in Table 5. A further increase in cellulase loading beyond 35 mg/g cellulose resulted in only marginally improved hydrolysis yields. Alternatively, when the SPCS substrate was hydrolyzed with increasing cellulase loading in the presence of xylanase (60 mg/g cellulose), a significant increase in cellulose hydrolysis was observed, with more than 80% of the cellulose hydrolysed at a cellulase loading of 5 mg/g cellulose, compared to only about 45% in the absence of xylanase. The relatively high xylanase loading (60 mg/g cellulose) used here was chosen based on previous studies (Berlin et al., 2007; Ohgren et al., 2007).

As expected, xylan hydrolysis also increased significantly from 56% in the absence of xylanase to over 95% with xylanase supplementation. Further increasing the cellulase loading beyond 5 mg/g cellulose in the presence of xylanase did not significantly increase either cellulose or xylan hydrolysis. A good correlation ($R^2=0.98$) was observed between the extent of cellulose hydrolysis and xylan removal (Figure 6 inset). This good regression relationship indicated that the extent of cellulose hydrolysis could likely be used to predict the extent of xylan hydrolysis and vice versa.

It appears that the supplementation of cellulases with xylanase not only substantially increased the rate of hydrolysis of both the cellulose and xylan, it also increased the extent of hydrolysis. For example, the maximum degree of cellulose and xylan hydrolysis in the absence of xylanase were about 75% and 93%, respectively, at a cellulase loading of 95 mg/g cellulose, whereas cellulose and xylan hydrolysis were about 80 and 100%, respectively, when xylanase was added (60 mg/g cellulose), even at relatively low cellulase loadings of 5 mg/g cellulose. This increase suggested that the residual xylan present in the pretreated SPCS substrate played an important role in limiting the ease of cellulose hydrolysis.

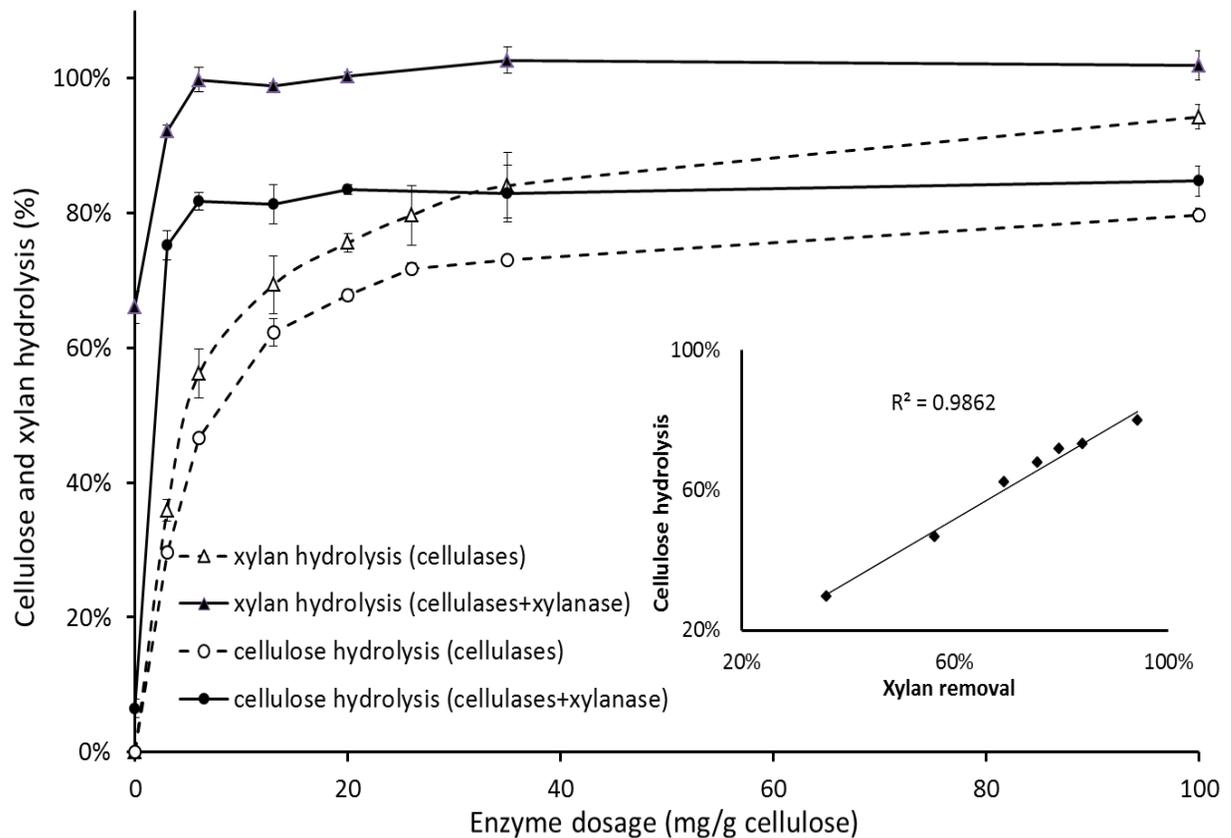


Figure 6. Conversion of SPCS at increasing cellulase doses with or without xylanase supplementation (60 mg/g cellulose) after 72 h hydrolysis. Relationship between xylan removal and cellulose conversion after 72 h hydrolysis at various enzyme doses (inset). Full line: hydrolysis in the presence of xylanase; dashed line: hydrolysis in the absence of xylanase. SPCS: steam pretreated corn stover.

The hydrolyzability of the xylan in the substrate by xylanases alone was further assessed by using a range of xylanase loadings to hydrolyze SPCS without cellulases supplementation, and also by a fed-batch strategy, adding xylanases to periodically refresh xylanase activity (Figure 7). Xylanase alone could only hydrolyze about 65% of the xylan even at very high enzyme loadings (100 mg/g cellulose) (Figure 7A), and adding further xylanases after a period of time could not further improve the extent of xylan removal (Figure 7B). Thus, it appears that there is a certain amount of xylan that is not accessible to

xylanase enzymes, and its access requires the action of cellulases as the addition of low amounts of cellulases (5 mg/g cellulose) significantly increased xylan hydrolysis (Figure 6). The observed simultaneous cellulose and xylan hydrolysis provided evidence for the potential synergistic cooperation between the cellulase enzymes and xylanase during hydrolysis.

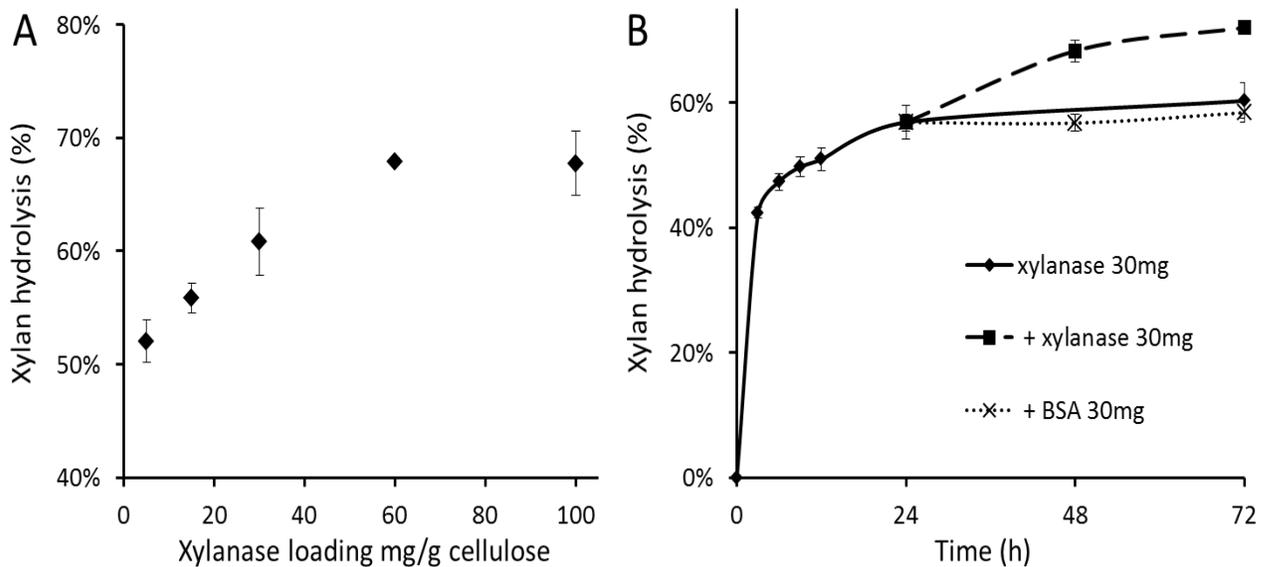


Figure 7. Xylan hydrolysis of SPCS. (A) Xylan hydrolysis with a range of xylanase loadings after 72 h. (B) Sequential addition of xylanases for xylan hydrolysis. SPCS: steam pretreated corn stover; protein control BSA: bovine serum albumin.

3.1.2.3 Cellulase replacement with xylanase vs. cellulase supplementation with xylanase

Although it was clear that xylanase supplementation improved both cellulose and xylan hydrolysis at various levels of cellulase loading (Figure 6), it appeared that a significant xylanase boosting effect was only achieved when low cellulase loadings were used. To further evaluate the interaction between the cellulase and xylanase enzymes during hydrolysis of SPCS, two sets of hydrolysis were carried out. In the xylanase supplementation

approach, varying amounts of xylanase (5–60 mg/g cellulose) were added to the minimum amount of cellulases (35 mg/g cellulose) which had previously been determined to be required for 70% of the cellulose to be hydrolysed (Figure 6). In the cellulase replacement approach, varying amounts of the cellulases were replaced (up to 86% cellulase replacement on a protein basis) with xylanase while the total amount of enzyme added, on a protein basis, was kept constant at 35 mg/g cellulose.

It was apparent that supplementing the cellulases with varying amounts of xylanase increased both the cellulose and xylan hydrolysis (Table 7). However, the degree of synergism (DS) between cellulases and xylanase was about 1, indicating that the observed improvement in SPCS hydrolyzability was more a product of increased enzyme loading. This could be termed an additive effect, as the specific activity of the unsupplemented cellulase mixture was 22.8 mg sugars/mg enzymes, while the specific activity of xylanase-supplemented cellulase mixture was 21.5 mg sugars/mg enzymes. Alternatively, when relatively small amounts of the cellulases were replaced with xylanase, a slight increase in the degree of synergism was observed, suggesting that synergistic cooperation was taking place between the cellulase and xylanase enzymes (Table 7). Although lower amounts of cellulase enzymes were present under these conditions (71% cellulase and 29% xylanase) as compared to hydrolysis runs with no cellulase replacement (100% cellulase, 35 mg/g), the same levels of SPCS hydrolysis were obtained (74% cellulose and 82% xylan hydrolysis, Table 7). As the percentage of cellulases replaced by xylanase was increased (up to 86%), the degree of synergism also increased. Under the conditions tested, the highest degree of synergism was observed at a cellulase and xylanase loading of 5 and 30 mg/g cellulose, respectively, which resulted in a substantial increase in both cellulose and xylan hydrolysis

(86% cellulose and 99% xylan hydrolysis, Table 7). It appears that with this enzyme ratio, the xylanase and cellulases worked synergistically together to hydrolyze the SPCS, resulting in an enzyme mixture with a relatively high specific activity (27.3 mg sugars/mg enzymes). Similar SPCS hydrolysis yields could be achieved by replacing cellulases with xylanase to a total final protein loading of 35 mg/g cellulose as compared to increasing the enzyme dosage by supplementing the 35 mg cellulases/g cellulose with increasing amounts of xylanase (Table 7). As the enzyme mixture containing 5 mg of cellulases/g cellulose and 30 mg xylanase/g cellulose displayed the highest degree of synergism (1.62) during the hydrolysis of the SPCS substrate, this mixture was used to determine the mechanism behind this observed synergism.

It was apparent that the type of interaction between xylanase and cellulase enzymes was dependent on the total enzyme loading and enzyme ratio, as has been observed previously (Woodward et al., 1988a; Jeoh et al., 2006). A strong synergistic effect was observed at low cellulase loadings and when a high xylanase to cellulase ratio was used. The highest degree of synergism (1.62) was observed at a xylanase to cellulase protein loading of 6:1, which resulted in a considerable reduction (7-fold) in the total amount of cellulase enzymes required for effective cellulose hydrolysis of the SPCS substrate. In related work, Bura et al. (2009) and Berlin et al. (2007) also found that a similar xylanase to cellulase ratio improved cellulose hydrolysis. However, in this earlier work it was more of an additive effect rather than a truly synergistic interaction.

Table 7. Effect of cellulase supplementation with xylanase and cellulase replacement with xylanase on cellulose and xylan hydrolysis, and on the degree of synergism during hydrolysis of SPCS after 72 h.

Hydrolysis strategy	Total protein (mg/g cellulose)	Enzyme mixture	Xylanase (% total protein)	DS	Cellulose hydrolysis (%)	Xylan hydrolysis (%)
Xylanase Supplementation	40	35mg C + 5mg X	13	0.99	73.9	80.1
	45	35mg C + 10mg X	22	0.96	74.1	83.6
	45	35mg C + 10mg B	0	–	72.9	81.3
	95	35mg C + 60mg X	63	1.02	87.1	100
Cellulase Replacement	35	35mg C	0	–	72.1	81.6
		25mg C + 10mg X	29	0.98	74.1	82.6
		15mg C + 20mg X	57	1.09	74.2	82.6
		10mg C + 25mg X	71	1.31	82.3	98.6
		5mg C + 30mg X	86	1.62	86.3	99.3
		5mg C + 30mg B	0	–	55.8	68.6
Separate Hydrolysis	5	5mg C	0	–	45.6	56.3
	30	30mg X	100	–	5.2	61.4

Separate hydrolysis used as control. B: bovine serum albumin; C: cellulases; DS: degree of synergism; SPCS: steam pretreated corn stover; X: xylanase; n/a: not applicable.

There are several possible explanations for the strong synergistic interaction observed between cellulases and xylanase. As mentioned earlier, xylanase have been suggested to remove the xylan “coat” on the surface of pulp fibre, improving cellulose accessibility to cellulase enzymes (Ohgren et al., 2007; Kumar and Wyman, 2009b). In a related work, Pauly et al. (1999) found that one third of the xyloglucan present in dicot plants was entrapped within the microfibril, and its removal required the action of both cellulase and xyloglucanase enzymes. Xylanases have also been suggested to increase the proportion of substrate available for productive cellulase interaction due to their unproductive binding to sites on lignin (Ryu and Kim, 1998; Kumar and Wyman, 2009a). The xylanase used in this work is categorised in the glycoside hydrolase family 11 (GH11), and this xylanase lacks a carbohydrate binding module (CBM) (Torrönen and Rouvinen, 1995). In most cellulase enzymes, the CBM has been shown to preferentially bind to lignin (Palonen et al., 2004). Previous work has also shown that lignin has very little influence on xylanase activity as compared to cellulases and β -glucosidase (Berlin et al., 2006b). Therefore, for the work reported here, an increase in cellulase availability due to xylanase binding to lignin is unlikely to be the major reason for the observed enhancement of cellulase activities in the presence of xylanase.

3.1.2.4 Simultaneous, separate, and sequential hydrolysis

Most of the previous work in this area has reported an increase in substrate digestibility upon xylanase supplementation, without further clarifying the likely mechanism behind the cooperative interaction between both types of enzymes. To further clarify the mechanisms behind the observed synergistic cooperation between the cellulases and xylanase,

various time course hydrolysis profiles were followed using simultaneous, separate, and sequential addition of cellulases and xylanase (Figure 8).

It seemed that, regardless of the enzyme addition strategy that was used, most of the xylan hydrolysis was accomplished during the first 3h (Figure 8B). The simultaneous (cellulases + xylanase) and the separate addition (cellulases) strategies showed similar trends of increased xylan removal occurred within 72 hours hydrolysis, resulting in about 100% and 50% xylan solubilisation, respectively. However, when xylanase was added alone (30 mg/g cellulose), xylan hydrolysis levelled off after 24 hours with only about 60% of the original xylan solubilised. It is likely that the readily accessible xylan was hydrolyzed in the first few hours of hydrolysis and that, in order to access the xylan that is more closely associated with the cellulose and “buried” within the fibre structure, the synergistic interaction with cellulases was required for more extensive xylan solubilisation (Figure 8B). The addition of xylanase alone left one third of the original xylan in the SPCS, even when very high xylanase loadings (100 mg/g cellulose) were used (Figure 7A). However, when a sequential cellulase addition approach was used (Figure 8B), the hydrolysis profile followed the same trend as when the cellulases and xylanase were added simultaneously at the beginning of the hydrolysis. However, the final xylan hydrolysis yields were lower (Figure 8B).

It was found that the addition of xylanase alone (30 mg/g cellulose) resulted in limited (5%) cellulose hydrolysis (Figure 8A). However, when cellulases (5 mg/g cellulose) were added to the xylanase pre-hydrolyzed SPCS (sequential hydrolysis, Figure 8A), cellulose hydrolysis increased sharply from 5% at 24 h of hydrolysis to about 53% at 48h, which was significantly higher than the cellulose hydrolysis achieved when adding cellulases only (36% at 48h hydrolysis). This cellulose hydrolysis yield was also higher than the

theoretical sum of cellulose hydrolysis obtained with cellulases and xylanase when used separately. It was apparent that pre-hydrolyzing SPCS with xylanase resulted in enhanced cellulose hydrolysis when cellulases were subsequently added.

When both types of enzymes were added simultaneously (simultaneous hydrolysis), the enzymatic digestibility of SPCS was substantially increased (Figure 8A). For example, when the enzymes were added together, about 50% of the cellulose was hydrolysed after 12 h hydrolysis, whereas in the absence of xylanase, only 20% of the cellulose was hydrolysed. After 72 h, approximately 45% of the cellulose was hydrolyzed when using cellulase enzymes (5 mg/g cellulose) alone, whereas similar hydrolysis yields could be achieved in just 10 h when xylanase was supplied together with cellulases at the beginning of hydrolysis. It was apparent that the combined addition of cellulases and xylanase not only resulted in higher hydrolysis rates, but also substantially increased the extent of SPCS hydrolysis. After 72 h, cellulose hydrolysis was almost 2-fold higher (87%) as compared to hydrolysis carried out in the absence of xylanase (45%). To ensure that there were no other mechanisms at play, such as a possible “detergent” effect of added protein, a protein control of bovine serum albumin (BSA) was shown to have almost no effect (Figure 8).

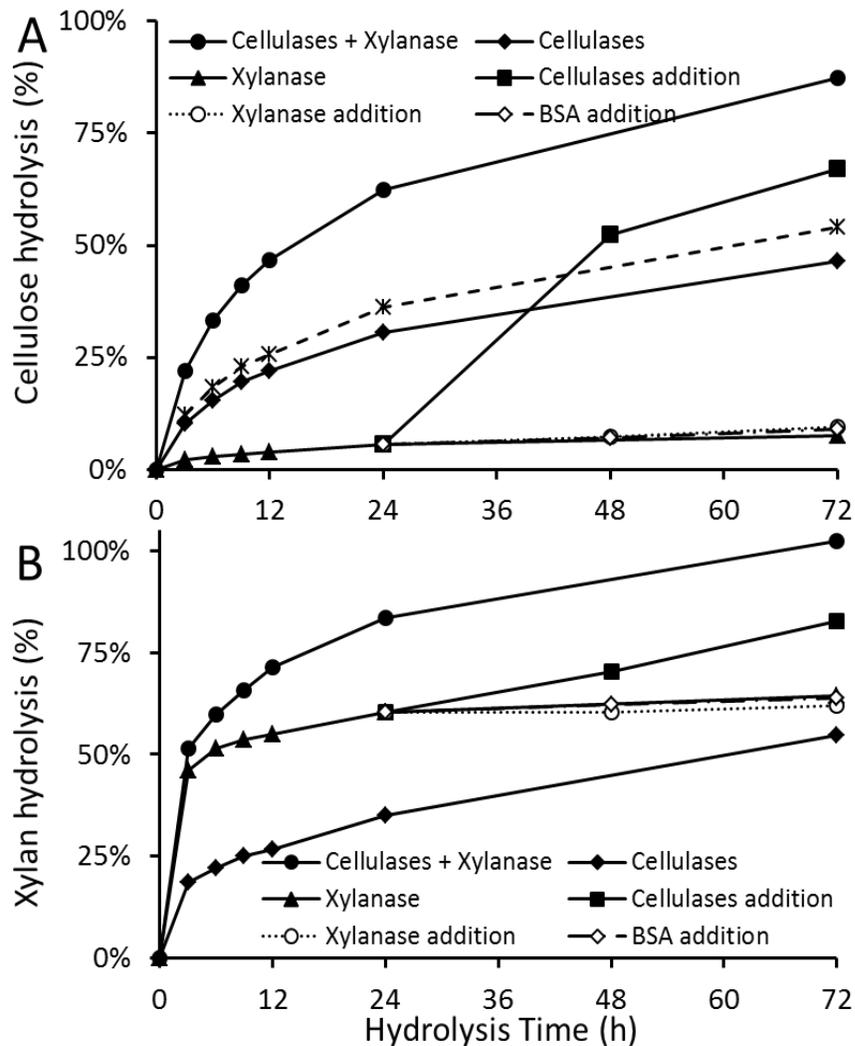


Figure 8. Time course of SPCS hydrolysis. Separate hydrolysis: (black rhombus) 5 mg cellulases or (black triangle) 30 mg xylanase; Simultaneous hydrolysis: (black circle) 5 mg cellulases and 30 mg xylanase; Sequential hydrolysis: addition of (black square) 5 mg cellulases, (clear circle) 5 mg xylanase and (clear rhombus) 5 mg BSA to pre-hydrolyzed SPCS with 30 mg xylanase for 24 h. Theoretical: (asterisk) sum of cellulose conversion after hydrolysis with 30 mg xylanase and 5 mg cellulases separately. (A) Cellulose hydrolysis. (B) Xylan hydrolysis. BSA: bovine serum albumin; SPCS: steam pretreated corn stover.

3.1.2.5 Pulp fibre properties

Hemicellulose is known to contribute to fibre strength and its solubilisation is known to influence pulp fibre properties. One method that has been successfully used by the pulp

and paper sector to evaluate changes at the fibre level is the use of a fibre quality analyzer (FQA). By using this equipment we hoped to determine any changes in the fibre dimensions and fibre size distribution of the unhydrolyzed and residual SPCS after simultaneous and separate hydrolysis with cellulase and xylanase addition over 24 h (Figure 9 A and B).

When compared to the unhydrolyzed SPCS control, xylanase addition alone did not result in any changes in the fibre length distribution (Figure 9A). In contrast, cellulase treatment resulted in a significant decrease in the population of fibres with lengths less than 0.15 mm and also a slight increase in the population of fibres with lengths within the range of 0.15 – 0.35 mm (Figure 9A). The latter group of fibres was likely due to the fragmentation of long fibres into shorter fibres as the average fibre length after 24 decreased from 0.555 mm to about 0.270 mm. Earlier work has shown that the smaller fibres were rapidly hydrolysed and solubilised (Saddler et al., 1999). When the xylanase and cellulases were added simultaneously, a different pattern was observed with a significant increase in the population of fibres in the length range of 0.09 – 0.23 mm (Figure 9A) and significantly more fibre fragmentation as the average fibre length was reduced from 0.55 mm to about 0.22 mm. The sizable amount of fibres in the length range of 0.09 – 0.23 mm was also likely due to the higher fragmentation of SPCS fibres, resulting in the higher amounts of shorter fibres.

We next measured changes in the fibre width of the residual SPCS after hydrolysis, as this value can provide a general indication of the degree of fibre swelling (Figure 9B). As was observed with the fibre length values, xylanase treatment alone promoted only a slight change in the average fibre width, whereas cellulase addition increased the fibre width by about 10%. A significant change in fibre width was observed when both the xylanase and cellulases were added simultaneously, with the fibre width increasing by about 30%, as

compared to the untreated fibres, and by 20%, as compared to the cellulase only-treated SPCS fibres. This increase in fibre width suggested a significant increase in fibre swelling as a result of the synergistic cooperation of the cellulases and xylanase.

To quantify any increases in accessibility of the SPCS fibres to the enzymes, we used the Simon's stain method, which had previously been shown to provide a good estimation of cellulose accessibility (Arantes and Saddler, 2011). An increase in the orange dye (DO) to blue dye (DB) ratio after xylanase treatment indicated that more of the cellulose was accessible, likely due to the removal of the xylan in the SPCS fibres (Figure 9C). The addition of cellulases alone resulted in a decrease in the DO:DB ratio primarily because of the decreasing amount of substrate that was available to measure as hydrolysis proceeds.

Xylanase-aided bleaching treatment of cellulosic pulps have also been demonstrated to promote physical changes in the pulp fibres, such as an increase in fibre porosity and fibre disintegration (deJong et al., 1997; Suurnakki et al., 1997; Wong et al., 1997). Similar changes during hydrolysis of lignocellulosic biomass are likely to increase the available specific surface area of the cellulose to cellulase enzymes and therefore, the effectiveness of the cellulases, a process termed amorphogenesis (Arantes and Saddler, 2010).

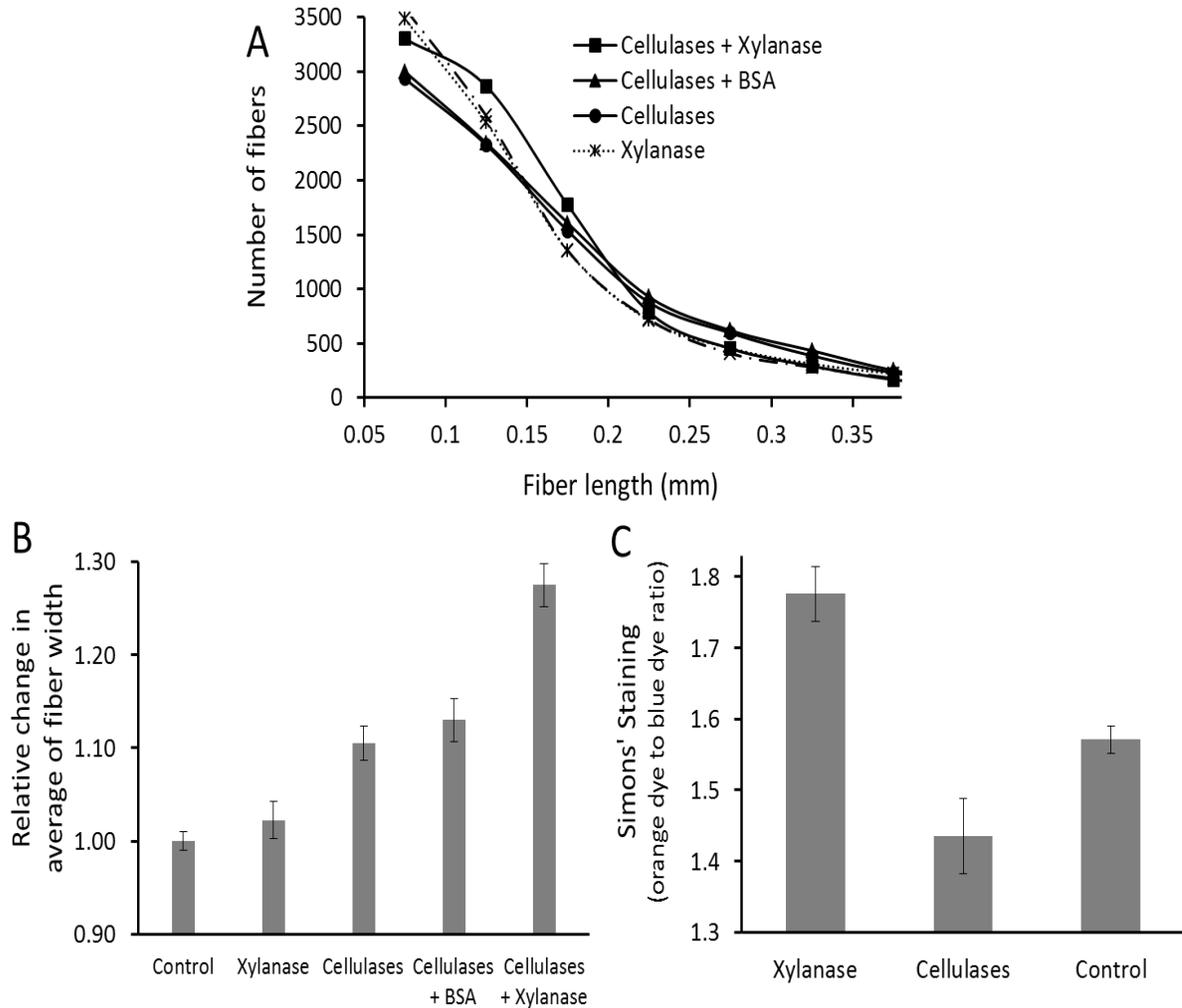


Figure 9. Change in SPCS fibre properties during separate and simultaneous hydrolysis with cellulases and xylanase after 24 h. (A) Fibre length distribution. (B) Average fibre width. (C) Fibre surface area (combination interior/exterior) determined by the Simons' stain technique. Enzyme loading (mg/g cellulose): cellulases (5), xylanase (30) and BSA (30). Control: substrates were incubated at the same condition without the addition of enzymes. BSA: bovine serum albumin; SPCS: steam pretreated corn stover.

3.1.2.6 The effect of xylanase supplementation on the enzymatic hydrolysis of other pretreated lignocellulosic substrates

To determine if the xylanase boosting effect, observed during hydrolysis of SPCS, could also be observed with other biomass substrates, steam pretreated sweet sorghum bagasse (SPSB) and Lodgepole pine (SPLP) were hydrolyzed with similar combinations of

cellulases and xylanase as was carried out with the SPCS substrate (Figure 10). The SPSB had a higher xylan content than did the SPCS substrate, whereas the xylan content of the SPLP was negligible (Table 6). The effectiveness of hydrolysis of both the SPSB and SPLP substrates was assessed using a total protein loading of 35 mg/g cellulose (5 mg cellulases + 30 mg xylanase per g cellulose and 5mg cellulases + 30 mg BSA per g cellulose). The simultaneous addition of xylanase and cellulases also significantly enhanced the cellulose hydrolysis of both the SPSB and SPLP substrates. Interestingly, xylanase supplementation boosted the cellulose hydrolysis of the SPLP substrate even though this substrate did not contain any xylan. This suggested that the mechanism behind the xylanase-boosting effect of cellulose hydrolysis in pretreated biomass was not solely due to increasing cellulose accessibility through xylan removal, and that it also involved contributions to changes in fibre morphology, as was shown with the FQA and Simons' stain values, obtained during the hydrolysis of the SPCS substrate. It is likely that this synergism occurs in a similar fashion to the amorphogenesis effect observed with co-factors such as GH61 or cellulose binding modules which have been suggested to enhance the effectiveness of cellulase enzymes (Arantes and Saddler, 2011).

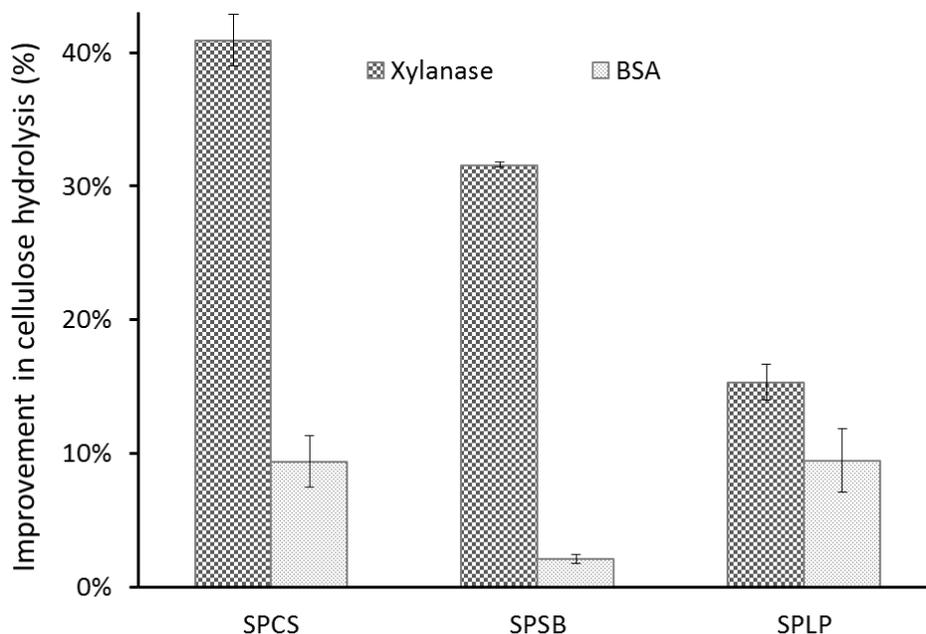


Figure 10. Improvement in cellulose hydrolysis yields in the presence of xylanase (30 mg/g cellulose) and cellulases (5 mg/g cellulose) as compared to hydrolysis yields in the presence of only cellulases (5 mg/g cellulose) after 72 h hydrolysis of various substrates. SPCS: steam pretreated corn stover; SPSB: steam pretreated sweet sorghum bagasse; SPLP: steam pretreated lodgepole pine.

3.1.2.7 The potential to further reduce xylanase loading in the enzyme mixture and xylanase thermostability.

Although the synergistic cooperation between cellulase and xylanase in the “optimized” enzyme mixture (5 mg C + 30 mg X, Table 7) significantly reduced the required cellulase loading (about 7 times) for an efficient hydrolysis of SPCS, the required amount of xylanase loading was still high. Thus, the potential of decreasing the xylanase dosage was assessed. The reduction of the xylanase dosage in the enzyme mixture resulted in a parallel decrease in both cellulose and xylan conversion after 72 h (Figure 11), while the further increase in the xylanase dosage to 60 mg/g cellulose did not improve the hydrolytic performance of the enzyme mixture (Figure 11). Again, the control of cellulase plus BSA

and xylanase alone, without cellulase, indicated that the improved cellulose and xylan hydrolysis by the cellulase/xylanase mixture came from their synergistic cooperation during hydrolysis.

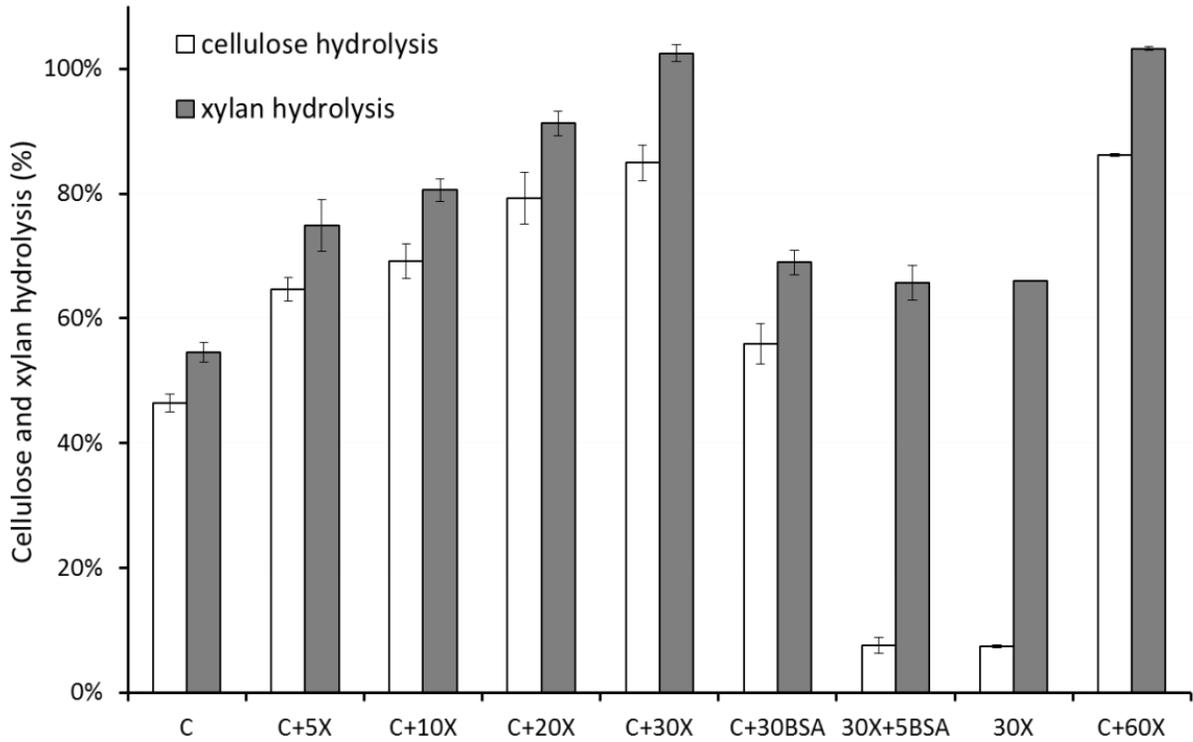


Figure 11. Effect of cellulases (5 mg/g cellulose) supplementation with xylanase (5-60 mg/g cellulose) on cellulose and xylan hydrolysis of SPCS after 72 h. C: cellulases, X: xylanases, BSA: bovine serum albumin, SPCS: steam pretreated corn stover.

One of the possible reasons for the required high xylanase loading might be the lack of thermostability of the xylanase preparation used in this study as reported in earlier studies (Shibuya et al., 2000; Sun et al., 2005). Therefore, the xylanase thermostability (reflected by the xylanase activity on birchwood xylan) was assessed at various temperatures and also during time course of incubation at the hydrolysis temperature 50°C (Figure 12). The xylanase activity decreased dramatically with the increased temperature (Figure 12A), with

less than 10% of original activity left at 50°C after 72 h incubation. The changes in the xylanase activity profile at 50°C showed that more than half of the xylanase activity was already lost in the first 3 h, followed by a gradual reduction in activity until 24 h incubation, where the majority of xylanase was deactivated (Figure 12B). Therefore, it seems that improving xylanase thermostability was a possible solution to further decrease the required xylanase loading in the enzyme mixture.

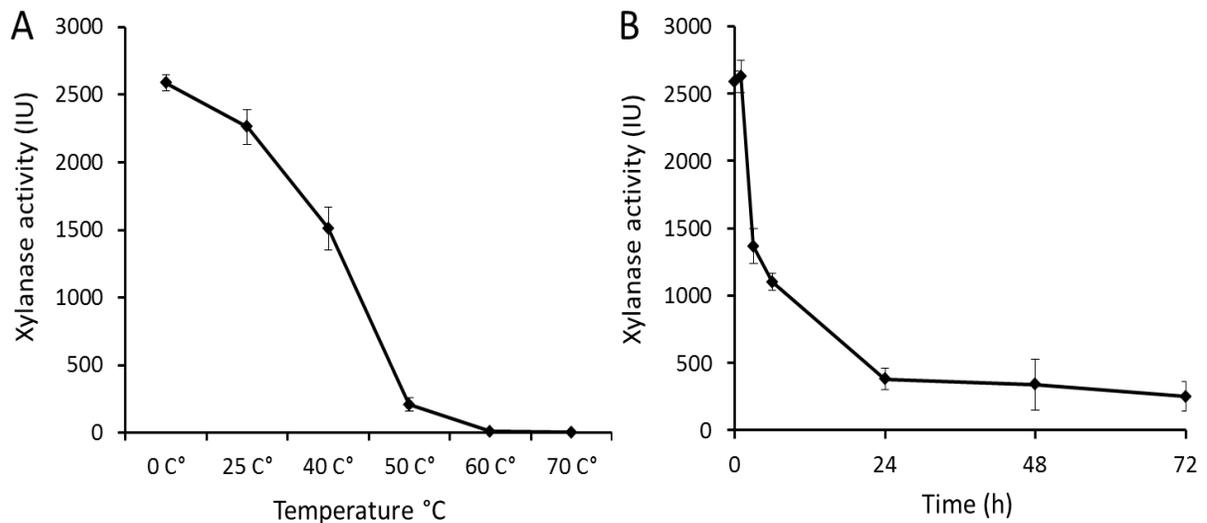
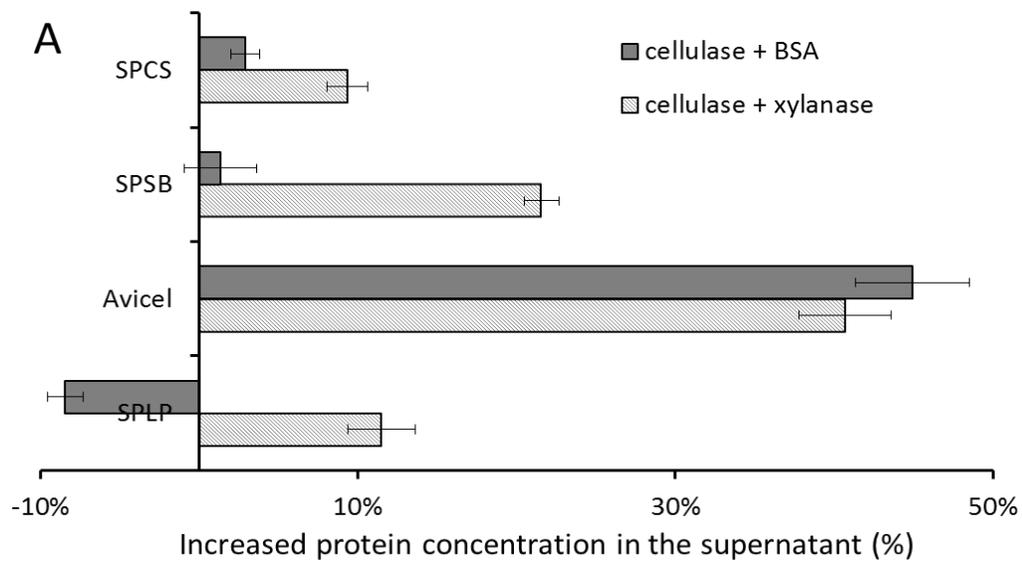


Figure 12. (A) Xylanase activity after incubating in buffer at different temperatures for 72 h. (B) The time course of xylanase activity at 50 °C. Xylanase activity was assessed using birchwood xylan as the substrate.

3.1.2.8 The potential for increasing cellulase recyclability by xylanase addition

The synergistic cooperation between cellulase and xylanase not only significantly increased cellulose hydrolysis, but also improved the rate and extent of xylan hydrolysis (Figure 8). Xylanase has also been suggested to solubilize the small lignin fractions from pretreated lignocellulosic biomass by breaking down the lignin-carbohydrate complex (deJong et al., 1997; Suurnakki et al., 1997). These factors might increase the potential of

recycling cellulases after hydrolysis as less xylan and lignin left in the solid part of the substrate may result in less cellulases that will be unproductively bound /stuck in hydrolysis residues. In order to test this hypothesis, the protein content and specific enzyme activities in the liquid phase were assessed after 72 h hydrolysis with/without xylanase addition. As expected, xylanases increased the total protein content in the hydrolysate of pretreated lignocellulosic substrates, but not the cellulosic substrate Avicel (Figure 13A). The increased specific activities of the enzymes in the supernatant indicated that these increased free enzymes were still reactive (recyclable) after hydrolysis (Figure 13B).



B Enzyme activity in the supernatant (IU)

	CMC	Xylan	p-NPC	p-NPG
5C + 30X	11.3	15.0	0.005	0.08
5C + 30BSA	9.7	10.7	0.003	0.05

Figure 13. Enzyme recycling potential by supplying xylanase to cellulase. (A) The increased protein concentration in the supernatant after 72 h hydrolysis of different substrates. (B) The enzyme specific activities in the supernatant after 72 h hydrolysis of SPCS. SPCS/SB/LP: steam pretreated corn stover/sweet sorghum bagasse/lodgepole pine.

3.1.3 Conclusion

It appears that the xylanase-boosting effect during hydrolysis of pretreated lignocellulosic biomass is a result of increased cellulose accessibility to cellulase enzymes due to xylan removal from the surface of pulp fibres. In addition, it seems that the synergistic action between xylanase and cellulase enzymes during lignocellulose hydrolysis may also increase cellulose accessibility through increased fibre swelling and fibre porosity, as illustrated in Figure 14. It is possible that at the macroscopic level, xylanase removes the surface xylan (“coat”) on the pulp fibres, which could significantly increase cellulose accessibility. At the microscopic level, xylanase can hydrolyze xylan connecting and covering the cellulose microfibrils, therefore, causing fibre swelling and further improving cellulose accessibility to cellulase. However, at this level, xylanases also require the cooperative action of cellulase enzymes to hydrolyze the cellulose which limit the access to the xylan chains embedded in the deeper cellulosic fibrillar structure. It is also possible that synergistic interaction between xylanase and cellulase can break down the lignin-carbohydrate complex, which could release lignin fractions during enzymatic hydrolysis, and therefore, increase substrate digestibility.

The overall protein loading required to achieve fast, nearly complete hydrolysis of a model cellulosic substrate (SPCS) could be significantly reduced by making use of the synergistic interaction that occurs between cellulases and xylanase. The ‘xylanase-boosting’ effect was observed on a range of pretreated lignocellulosic materials, regardless xylan content. So-called ‘accessory enzymes’ such as xylanase might offer considerable potential to increase the overall performance of cellulase enzyme mixtures, while reducing the protein loading required to achieve effective hydrolysis of pretreated lignocellulosic substrates.

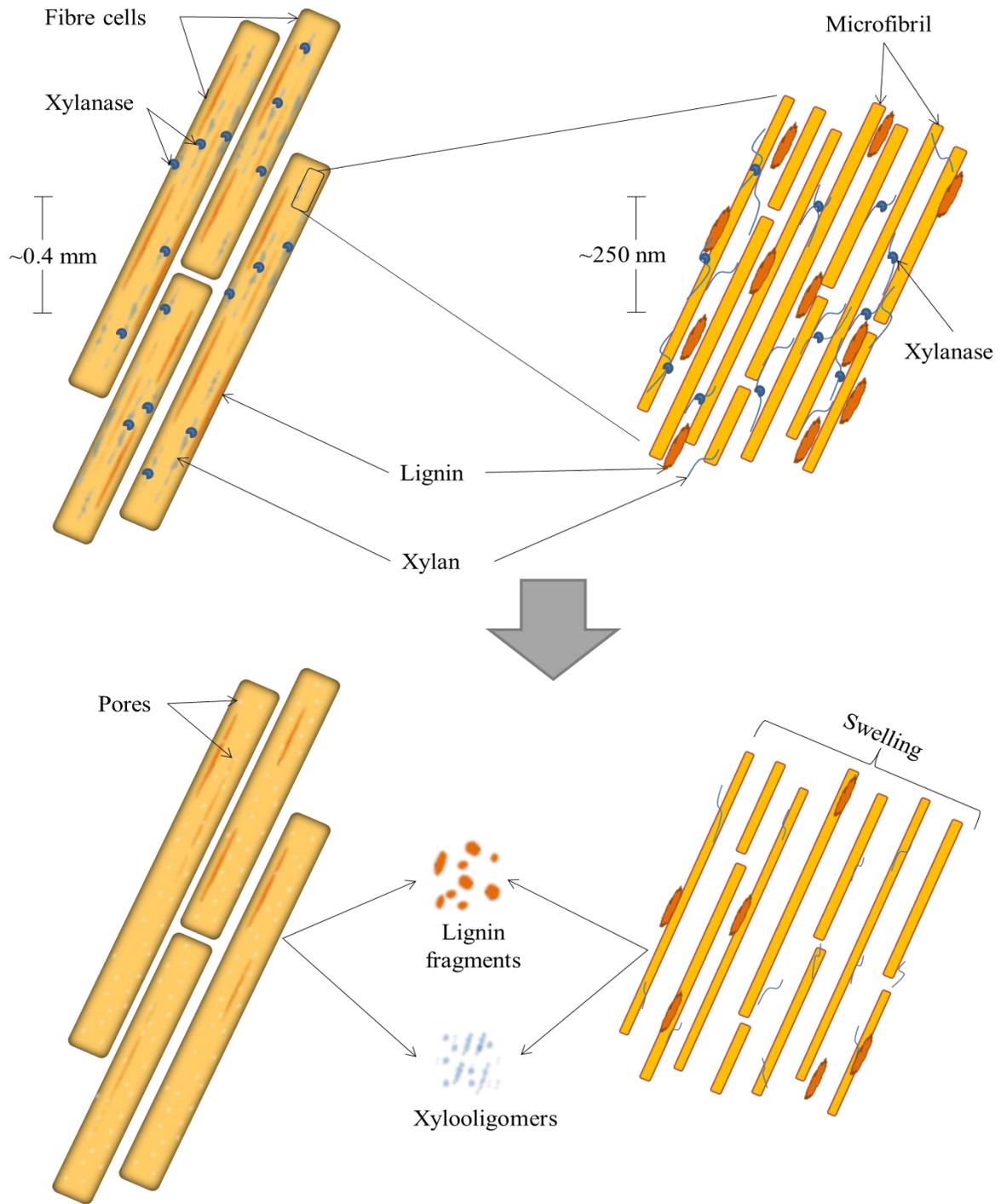


Figure 14. Schematic diagram showing the possible changes in lignocellulosic substrate in the presence of xylanase. At the macroscopic level, xylanase removes the xylan (shown as xylooligomers) and some of the lignin fragments on the outer surface of the pulp fibre, increasing fibre porosity and accessibility. At the microscopic level, xylanase and cellulase acting simultaneously remove xylan connecting and covering the cellulose microfibrils, releasing more xylooligomers and lignin fragments and promoting fibre swelling and increased cellulose accessibility.

3.2 The synergistic action of different family xylanases and xyloglucanase enhanced the hydrolytic potential of a “cellulase mixture”, but it was highly substrate specific

3.2.1 Background

Previous work (section 3.1) had shown that, when commercial xylanase preparations (Genencor Multifect Xylanase, enriched in family 11 endo-xylanases) were added to commercial cellulase preparations (Novozymes, Celluclast 1.5L), their synergistic cooperation not only substantially enhanced the hydrolysis extent of both the glucan and xylan present in steam pretreated corn stover, it also dramatically reduced the required cellulase dosage (about 7 times) needed to achieve reasonable cellulose hydrolysis yields (>70%). Even though the overall total protein loading did not change or was slightly lower, high amounts of xylanase were still required to achieve improved hydrolysis. In addition, as the commercial enzyme preparations used for the earlier work contained several enzymes/proteins, we were unable to assess the basis of the mechanisms involved in the observed synergism. Therefore, the interaction between purified cellulase components and different family xylanases and xyloglucanase were further assessed.

Based on their amino acid sequence similarities, most xylan hydrolyzing enzymes belong to either glycoside hydrolase family 10 (GH10) or family 11 (GH11) as described in detail in Chapter 1 (1.5.2.1). Although there is a considerable amount of information known about the differences between these two major families of endo-xylanases regarding their structure, catalytic mechanisms and specific activities on “model” xylanolytic substrates (Beaugrand et al., 2004; Kim et al., 2004; Pell et al., 2004), there has been little work carried out regarding their hydrolytic potential or their interactions with cellulases during hydrolysis

of industrially relevant pretreated lignocellulosic substrates (Beaugrand et al., 2004; Kim et al., 2004; Zhang et al., 2011a). However, previous work has shown that GH10 EX addition was more effective on corn fibre and hydrothermally pretreated wheat straw (Kim et al., 2004; Zhang et al., 2011a), while GH11 EX was more effective on destarched wheat bran arabinoxylan (Beaugrand et al., 2004). Although synergistic cooperation between GH10 EX and GH11 EX was observed during the hydrolysis of corn fibre (Morais et al., 2012), a similar level of synergism was not observed during hydrolysis of arabinoxylan obtained from wheat bran (Beaugrand et al., 2004). A related study (Gao et al., 2011) showed that the addition of both GH10 EX and GH11 EX, along with other enzymes, resulted in a more effective enzyme cocktail when hydrolysing the cellulosic component of ammonia fibre expansion (AFEX) pretreated corn stover. This earlier work suggested that GH10 EX had broad catalytic specificity and that this enzyme might prove to be a better candidate for enhancing the hydrolysis of more realistic biomass substrates.

As mentioned in Chapter 1 (1.7.2), xyloglucanase is another potential candidate to enhance the hydrolyzability of various lignocellulosic substrates when added to a cellulase mixture. Benko et al. (2008) showed that a xyloglucanase from *T. reesei* slightly enhanced the catalytic performance of a cellulase mixture during hydrolysis of some lignocellulosic substrates. Related work also showed that the depolymerization and re-arrangement of the linkages in xyloglucan by hydrolases or transferases was an essential step in plant cell wall expansion and deposition during cell growth (Hayashi and Kaida, 2011). This suggested that the cleavage of glycosidic linkages within the xyloglucan resulted in the swelling of the cellulose microfibrils (Vincken et al., 1995; Chanliaud et al., 2004), which has been shown to

increase cellulose accessibility and, consequently, effectiveness of enzyme hydrolysis (Hayashi and Kaida, 2011).

The work reported in this section investigated the potential synergistic interaction between cellulases and several hemicellulosic hydrolyzing enzymes (GH11 EX, GH10 EX), and a family 5 xyloglucanase (GH5 XG) during the enzymatic hydrolysis of various steam pretreated lignocellulosic substrates. As well as adjusting the “cocktails” at the beginning of hydrolysis we also tried to “re-start” hydrolysis by the addition of further enzymes when the rate of hydrolysis had levelled off. As is explained later, although GH11 EX exhibited higher activity on “model” xylanolytic substrates, GH10 EX addition was better able to boost the hydrolytic potential of the cellulase monocomponents during hydrolysis of pretreated lignocellulosic substrates. However, the observed improvements in hydrolysis yields were highly substrate dependent. In addition, the possible reasons why GH10 EX performed better than GH11 EX when acting synergistically with cellulase enzymes during hydrolysis of lignocellulosic substrates were also investigated through measuring xylan accessibility and enzyme thermostability.

3.2.2 Results and discussion

3.2.2.1 Enzyme purification and their activities on “model” substrates

The different family xylanases and the major cellulase components were purified from various commercial enzyme preparations as described in Chapter 2 (2.5.2). The major protein within the Multifect Xylanase was found to be GH11 EX, as shown by tandem mass spectrometry, with this protein constituting more than 80% of the total protein present in the mixture. As expected, it had a relatively low molecular weight of about 20 kDa (Figure 15).

The GH10 EX, which had a much larger molecular weight compared to the GH11 EX, was purified from HTec 2 (Figure 15). The major cellulase monocomponents within Celluclast 1.5L, on a protein weight basis, were *T. reesei* Cel7A, Cel6A, Cel7B, and Cel5A (Figure 15), which comprised about 56%, 12%, 5%, and 6% of the total protein, respectively. These values were similar to the proportion of protein concentrations reported earlier by other workers (Martinez et al., 2008; Zhou et al., 2008). The family 5 xyloglucanase (GH5 XG) and family 3 β -glucosidase (GH3 BG) were purified from Megazyme xyloglucanase stock and Novozymes 188, respectively.

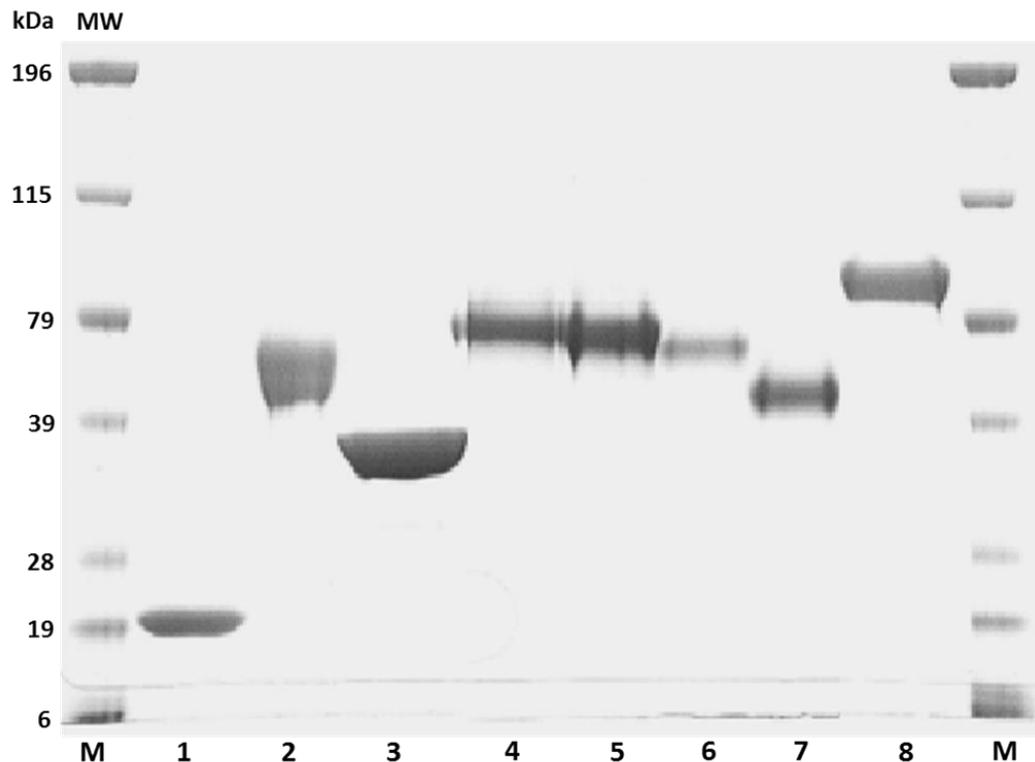


Figure 15. SDS-PAGE of purified enzymes: GH11 EX (lane 1), GH10 EX (lane 2), GH5 XG (lane 3), Cel7A (lane 4), Cel6A (lane 5), Cel7B (lane 6), Cel5A (lane 7), GH3 BG (lane 8) and marker (lane M). Proteins were identified by LC-MS/MS. Proteins are named according to their glycoside hydrolase family.

The xylanase, xyloglucanase, endo-glucanase, β -glucosidase and β -xylosidase activities of the purified enzymes were determined as detailed in the material and methods section (2.5.3) (Table 8). As expected, both GH10 and GH11 family endo-xylanases were able to effectively hydrolyze all of the xylan “model” substrates, while the other enzymes showed very low or undetectable levels of activity. The GH11 EX showed substantially higher hydrolytic activity (190-230 U/mg) on all of the xylan substrates compared with the GH10 EX (100-160 U/mg). This difference in activity was also observed previously (Zhang et al., 2011b) when using thermostable recombinant xylanases from *Nonomuraea flexuosa* and *Thermoascus aurantiacus*. When various *p*-nitrophenyl (pNP) substrates were used to assess any differences between the two xylanases, the GH10 EX showed detectable activities on pNPC, pNPG and pNPX, suggesting that GH10 EX had a broader catalytic versatility and may also have higher hydrolysis efficiency towards short xylooligomers (p-NPX activity) as compared with GH11 EX. In contrast, the family 5 xyloglucanase (GH5 XG) was the only enzyme that showed significant activity towards xyloglucan (146 U/mg) (Table 8). It also displayed noticeable hydrolytic activity towards CMC (3.7 U/mg). Of the various cellulase monocomponents that were assessed, only Cel7A showed any activity on the p-NPC substrate and it also showed detectable levels of activity on other “model” substrates such as CMC, xylan (birch wood, beech wood, oat spelts) and xyloglucan (Table 8). As expected, β -glucosidase was the only enzyme that displayed notable activity on the p-NPG substrate (0.4 U/mg).

Table 8. Specific activities (U/mg) of the purified enzymes assessed on “model” substrates.

Enzymes	Birch wood xylan	Beech wood xylan	Oat spelts xylan	Xylo-glucan	CMC	p-NPC	p-NPG	p-NPX
GH11 EX	193.2	191.5	229.3	n/a	n/a	n/a	n/a	n/a
GH10 EX	103.6	119.2	162.9	n/a	n/a	<0.2	<0.1	0.5
GH5 XG	n/a	n/a	n/a	145.6	3.7	n/a	n/a	n/a
Cel7A	<0.2	0.2	<0.2	1.24	<0.2	0.3	n/a	n/a
GH3 BG	n/a	0.2	0.35	n/a	n/a	<0.1	0.4	n/a

n/a - negligible activity was detected.

3.2.2.2 Interaction of GH11 EX with cellulase monocomponents

The possible synergistic interaction between the GH11 EX, the major xylanase component in Multifect Xylanase preparation, and the cellulase components from Celluclast 1.5L, Cel7A, Cel6A, and Cel5A was first assessed using both a “model” cellulosic substrate (dissolving pulp, DSP) and a pretreated biomass substrate (SPCS). As Cel7B had previously been shown to exhibit significant levels of endo-xylanase activity (Biely et al., 1991; Kleywegt et al., 1997), its interaction with GH11 EX was not assessed. When dissolving pulp was used as the substrate, the addition of GH11 EX enhanced the hydrolytic activity of both of the exo-type cellulases (Cel7A and Cel6A), but not the endo-type cellulase (Cel5A), as determined by the amount of glucose released (Table 9). When the enzyme interactions were assessed on the SPCS substrate, the addition of GH11 EX enhanced the hydrolytic potential

of all of the cellulase monocomponents (Cel7A, Cel6A, and Cel5A). In all cases, the highest enhancement of cellulolytic activity, promoted by GH11 EX, was observed with Cel7A (Table 9). Although the BSA protein control, used to substitute for GH11 EX, also improved the hydrolytic activity of the cellulase enzymes (Table 9), the slight increase in hydrolysis due to BSA addition was substantially lower than the benefit observed after GH11 EX addition. This beneficial action, combined with the previous observation that GH11 EX alone showed no hydrolytic activity towards the cellulose in either the DSP or SPCS substrates, indicated that the greater hydrolytic potential exhibited by the cellulase monocomponents after GH11 EX addition was the result of the synergistic interaction of the GH11 xylanase and cellulase monocomponents. As Cel7A displayed the highest degree of synergism with GH11 EX on both the DSP and SPCS substrates, this combination was used to elucidate the mechanisms behind the observed synergistic interaction between the enzymes.

Table 9. Cellulose hydrolysis of dissolving pulp (DSP) and steam pretreated corn stover (SPCS) by cellulase monocomponents with or without supplemental GH11 EX after 72 h.

	Cel7A	Cel7A + GH11EX	Cel7A + BSA	Cel6A	Cel6A + GH11EX	Cel6A + BSA	Cel5A	Cel5A + GH11EX	Cel5A + BSA
DSP	41.4%	51.3%	48.4%	10.8%	12.1%	11.7%	9.6%	8.8%	8.6%
SPCS	22.2%	30.1%	24.9%	3.4%	5.5%	3.9%	0.5%	1.5%	0.9%

Control: BSA.

3.2.2.3 Enhancement of the hydrolytic activity of Cel7A on various lignocellulosic substrates by the addition of accessory enzymes

The potential of the hemicellulose-hydrolyzing enzymes GH10 EX, GH11 EX, and GH5 XG to enhance the hydrolytic activity of Cel7A was next assessed on the dissolving pulp and the various pretreated lignocellulosic substrates (Table 10, Figure 16). It was apparent that both of the endo-xylanases (GH10 EX and GH11 EX) could effectively enhance the cellulolytic activity of Cel7A on all of the pretreated lignocellulosic substrates tested. Compared with GH11 EX, the addition of GH10 EX resulted in substantial increases in the hydrolytic action of Cel7A. The range of improvements varied from 10 to 100%, depending on the substrate used. A greater increase in hydrolysis due to GH10 addition was observed with the relatively higher xylan-containing substrates such as SPCS (7.0%), SPSB (9.8%) and SPP180 (6.6%), resulting in $\geq 80\%$ increase in the catalytic activity of the supplemented Cel7A (Figure 16). However, there was only a modest increase in the hydrolytic activity of Cel7A when each of the endo-xylanases was supplemented during the hydrolysis of the SPCF substrate, despite its high xylan (15%) content (Table 10). Endo-xylanases also significantly improved the hydrolytic activity of Cel7A when added to substrates containing very low or virtually no xylan, such as the DSP and SPLP substrates (Table 10). Although the addition of the BSA controls resulted in improvements in the range of 0.5-20% (Figure 16), these increases in the hydrolytic potential of Cel7A were substantially lower than those achieved after GH10 EX and GH11 EX supplementation.

We next used the two steam pretreated poplar samples with differing hemicellulose content (SPP180 (6.6% xylan) and SPP200 (3.7% xylan)) to assess the possible influence of the xylan on the hemicellulose-depolymerizing enzyme's ability to improve the cellulolytic

activity of Cel7A. The addition of the GH11 xylanase resulted in similar hydrolysis improvements (about 20%) on both substrates, despite the almost 2-fold higher xylan content in the SPP180 substrate as compared with the SPP200 substrate (Table 10). In contrast, GH10 EX addition improved the Cel7A catalytic activity 4-fold when applied to the SPP180 substrate (95%) compared with the hydrolysis yield observed with the SPP200 substrate (24%).

Table 10. Chemical composition of pretreated lignocellulosic substrates.

Substrate	Pretreatment Conditions	Composition of pretreated feedstocks						abbreviation
		<i>Ara</i>	<i>Gal</i>	<i>Glu</i>	<i>Xyl</i>	<i>Man</i>	<i>AIL</i>	
Corn fibre	190 °C, 5 min, 3% SO ₂	6.9	2.8	38.2	15.3	2.2	12.6	SPCF
Sweet sorghum bagasse	190 °C, 5 min, 3% SO ₂	0.6	0.8	54.3	9.8	1.0	25.8	SPSB
Corn stover	190 °C, 5 min, 3% SO ₂	1.0	0.7	56.1	7.0	1.1	27.0	SPCS
Poplar	180 °C, 5 min, 4% SO ₂	0.3	0.8	59.8	6.6	1.3	30.4	SPP180
Poplar	200 °C, 5 min, 4% SO ₂	0.3	0.8	59.3	3.7	1.2	33.9	SPP200
Lodgepole pine	200 °C, 5 min, 4% SO ₂	bdl	bdl	46.4	bdl	bdl	45.0	SPLP

Ara Arabinan, *Xyl* Xylan, *Glu* Glucan, *Gal* Galactan, *Man* Mannan, *AIL* Acid Insoluble Lignin, bdl below detectable level.

The trends observed in Figure 16 demonstrated that GH5 XG addition slightly improved Cel7A activity when acting on the SPCF and SPP180 substrates, whereas a significant improvement (about 20%) was observed on the SPLP substrate. It appears that the greatest improvements in Cel7A hydrolytic activity were observed when the GH5 XG was added to the hydrolysis of the pretreated wood substrates (SPP180, SPP200, and SPLP).

To assess the possible synergistic cooperation between cellulases and hemicellulases as a function of hemicellulase loading, the SPCS was hydrolyzed with cellulases supplemented with varying amounts of GH10 EX. The titration curve indicated that the cellulose hydrolysis boosting effect of adding xylanases decreased slightly (from ~90% to ~85%) when GH10 EX loading was decreased by half (5 mg/g glucan). Further decreasing the GH10 EX enzyme loading to 3 and 1 mg/g glucan could still improve cellulose hydrolysis by ~50% and ~18%, respectively.

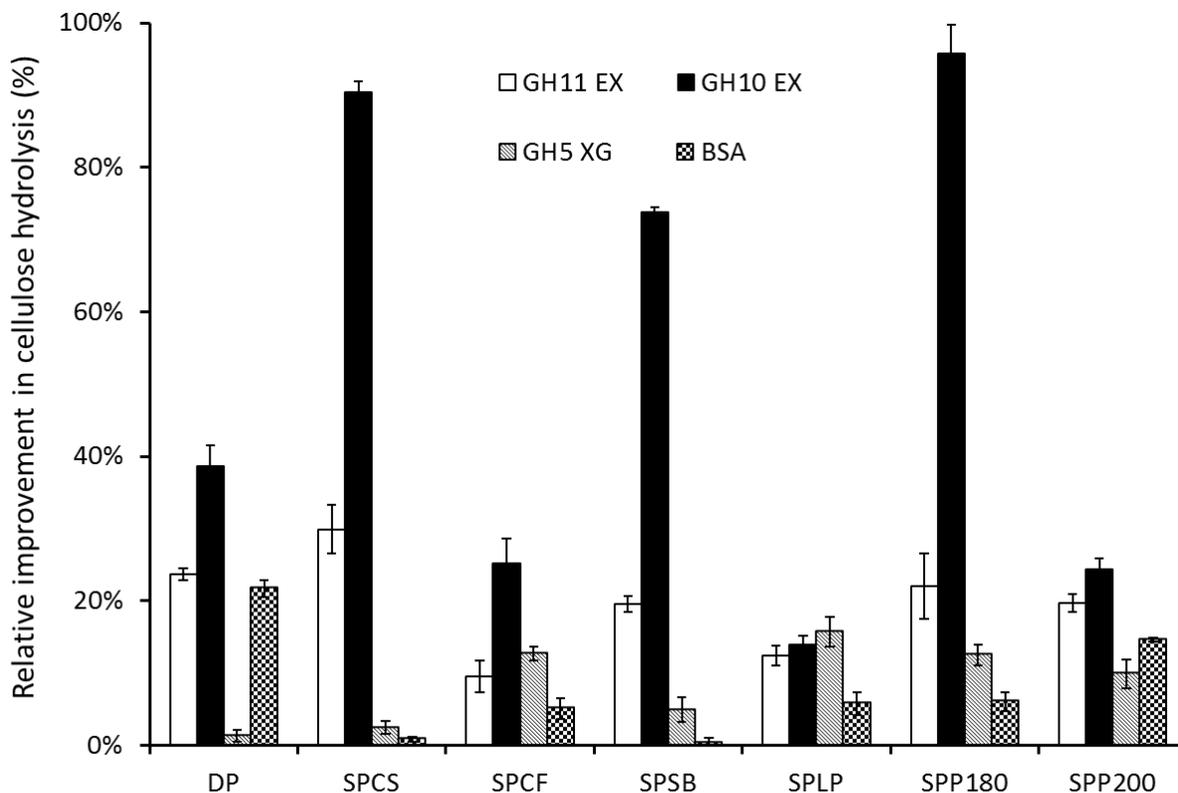


Figure 16. Relative improvement in cellulose hydrolysis by supplementation of accessory enzymes (GH11 EX, GH10 EX and GH5 XG) to Cel7A during hydrolysis of various pretreated lignocellulosic substrates (SPCS, SPCF, SPSB, SPLP, SPP180 and SPP200) after 72 h. Substrate control: dissolving pulp (DSP). Protein control: BSA.

Although all of the xylanases assessed in the current work acted synergistically with cellulases to enhance the hydrolysis of the cellulose present in a range of pretreated lignocellulosic substrates, the extent of improvement was highly dependent on the substrate used, as well as on the nature of the accessory enzyme added. In general, the endo-xylanases interacted synergistically with the cellulases in the hydrolysis of the steam pretreated agriculture residues (SPCS, SPSB) and hardwood (SPP180, SPP200). These substrates had relatively high xylan content. In contrast, as was previously observed by Benko et al. (2008), the greatest hydrolysis boosting effect obtained with xyloglucanase addition was observed during hydrolysis of steam pretreated softwood (SPLP) (Table 10). Interestingly, despite the

much higher xylan content of the SPCF substrate (15.3%) as compared to SPCS (7%) and SPSB (9.8%), no significant enhancement in cellulose hydrolysis was observed after endoxylanase addition (Figure 16). This could have been due to the highly branched xylan structure of the SPCF substrate which would be expected to restrict the accessibility of endoxylanase towards the glycosidic bonds within the xylan backbone (Bura et al., 2003; Appeldoorn et al., 2010).

3.2.2.4 Enhancement of the hydrolytic activity of Cel7A on various lignocellulosic substrates by the addition of binary mixtures of accessory enzymes

To assess the possible synergistic interaction between the accessory enzymes and Cel7A, binary mixtures of GH10 EX/GH11 EX, GH10 EX/GH5 XG, and GH11 EX/GH5 XG were formulated and compared with the hydrolysis obtained when Cel7A was supplemented with GH10 EX alone (Figure 17). With the exception of the binary mixture GH11 EX/GH5 XG, all of the other binary mixtures resulted in similar or increased hydrolysis as compared with Cel7A when supplemented with GH10 EX alone. The greatest improvement was observed with the binary mixture GH10 EX/GH5 XG, which enhanced glucose yields obtained from the SPCS, SPP200, DSP and SPLP substrates by 120, 60, 60 and 20%, respectively (Figure 17). As GH5 XG had previously exhibited detectable CMCase activity (Table 8), we next substituted GH5 XG in the binary mixture GH10 EX/GH5 XG with a purified endoglucanase (Cel5A) to assess the interaction of GH10 EX with a “true” endoglucanase enzyme. Although the addition of the GH10 EX/Cel5A mixture resulted in the improved hydrolytic activity of the Cel7A on the SPLP and SPP200 substrates as compared with the addition of GH10 EX alone, these improvements were similar (on SPLP) or lower (on DSP, SPCS and SPP200) to those observed with the GH10 EX/GH5 XG

mixture. This suggested that the detected endoglucanase activity of GH5 XG did not explain the strong synergistic interaction observed with the binary mixture GH10 EX/GH5 XG.

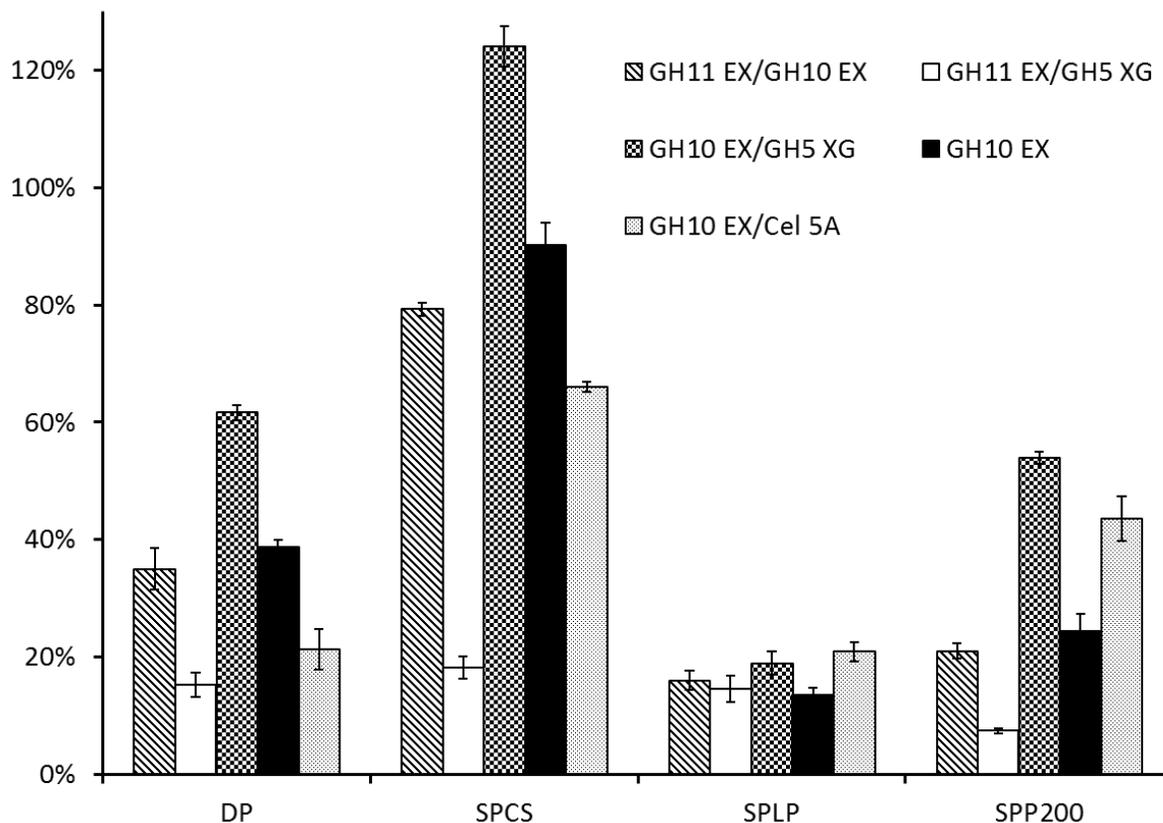


Figure 17. Relative improvement in cellulose hydrolysis by supplementation of binary mixtures of accessory enzymes (GH11EX/GH10EX, GH11EX/GH5XG and GH10EX/GH5XG) to Cel7A during the hydrolysis of various pretreated lignocellulosic substrates (SPCS, SPLP and SPP200) at 72 h. Substrate control: dissolving pulp (DSP). Enzyme control: GH10EX and GH10EX/Cel5A.

Although earlier work (Gao et al., 2011) had shown cooperative interaction between accessory enzymes such as GH11 EX and GH10 EX to enhance glucose release during lignocellulose hydrolysis, we found that the binary mixtures of these two family endo-xylanases mixture (GH11 EX/GH10 EX) resulted in equal or slightly lower improvements in cellulose hydrolysis yields than did the addition of only GH10 EX to the cellulases. However,

this earlier work (Gao et al., 2011) used AFEX pretreated corn stover, in contrast to the SO₂-catalyzed steam pretreated corn stover used in this study. It is highly likely that the AFEX treatment left a higher amount of residual hemicellulose in association with the cellulose, likely requiring the cooperation of both family endo-xylanases to achieve effective cellulose hydrolysis.

Although the GH11 EX/GH5 XG mixture did not appear to offer any advantages in enhancing the hydrolytic activity of Cel7A beyond the enhancement observed with the individual enzymes, a strong synergistic cooperation was observed with the GH10 EX/GH5 XG mixture. Although the exact mechanism behind this cooperation has yet to be fully resolved, it is likely that accessory enzymes with broader substrate specificities (such as GH10 EX and GH5 XG) resulted in stronger cooperative interactions with cellulase enzymes and therefore, the greater hydrolysis yields observed with the pretreated biomass substrates.

3.2.2.5 Subsequent GH10 EX addition releases bound Cel7A and restarts hydrolysis after glucose yields have levelled off

To determine the possible mechanisms involved in the observed improvements in the cellulolytic activity of Cel7A due to GH10 EX addition, hydrolysis experiments were carried out where the SPCS substrate was initially hydrolyzed with Cel7A followed by addition of GH10 EX (or other protein control) at a time when cellulose hydrolysis was observed to start levelling off. The hydrolysis of SPCS by Cel7A followed a typical hydrolysis profile (as shown in Figure 18) where, after an initial rapid rate of hydrolysis, the rate gradually decreased. The addition of more Cel7A, after 24 h, to the prehydrolyzed substrate resulted in an increase in hydrolysis, from 21 to 28% after 48 h, and from 28 to 32% after 72 h.

However, the addition of GH10 EX rather than the Cel7A to the 24 h-prehydrolyzed SPCS resulted in significantly better cellulose hydrolysis, with the yields increasing from 21 to 33% after 48 h and from 33 to 38% after 72 h (Figure 18). Controls where BSA and GH3 BG were each added, after 24 h, to the prehydrolyzed substrate did not promote a significant increase in cellulose hydrolysis.

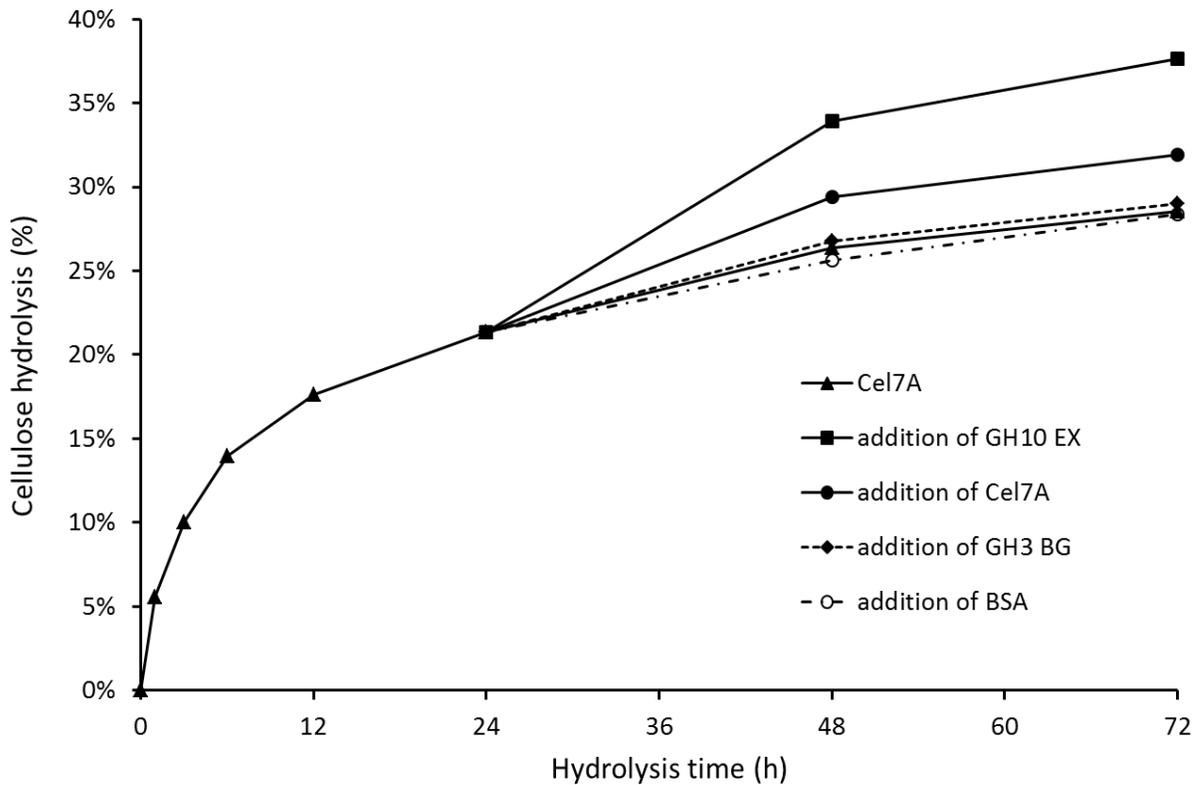


Figure 18. Time course of hydrolysis of steam pretreated corn stover (SPCS) by (▲) 15 mg Cel7A with the addition of (■) 10 mg GH10 EX, (●) 10 mg Cel7A, (◆) 10 mg GH3 BG and (○) 10 mg BSA at 24 h.

We next investigated the adsorption/desorption profile of Cel7A after 24 h hydrolysis in the presence and absence of GH10 EX or the protein control (BSA) (Figure 19). In general, when Cel7A alone was added, its adsorption profile did not change during the following 48 h,

other than a slight decrease in the amount of protein detected in the supernatant after this additional 48 h. However, the addition of GH10 EX resulted in a different Cel7A adsorption/desorption profile with almost 40% of the Cel7A desorbed within 60 seconds after the addition of GH10 EX. After a further 10 min, the amount of Cel7A in solution started to gradually decrease, indicating the re-adsorption of the Cel7A onto the SPCS substrate.

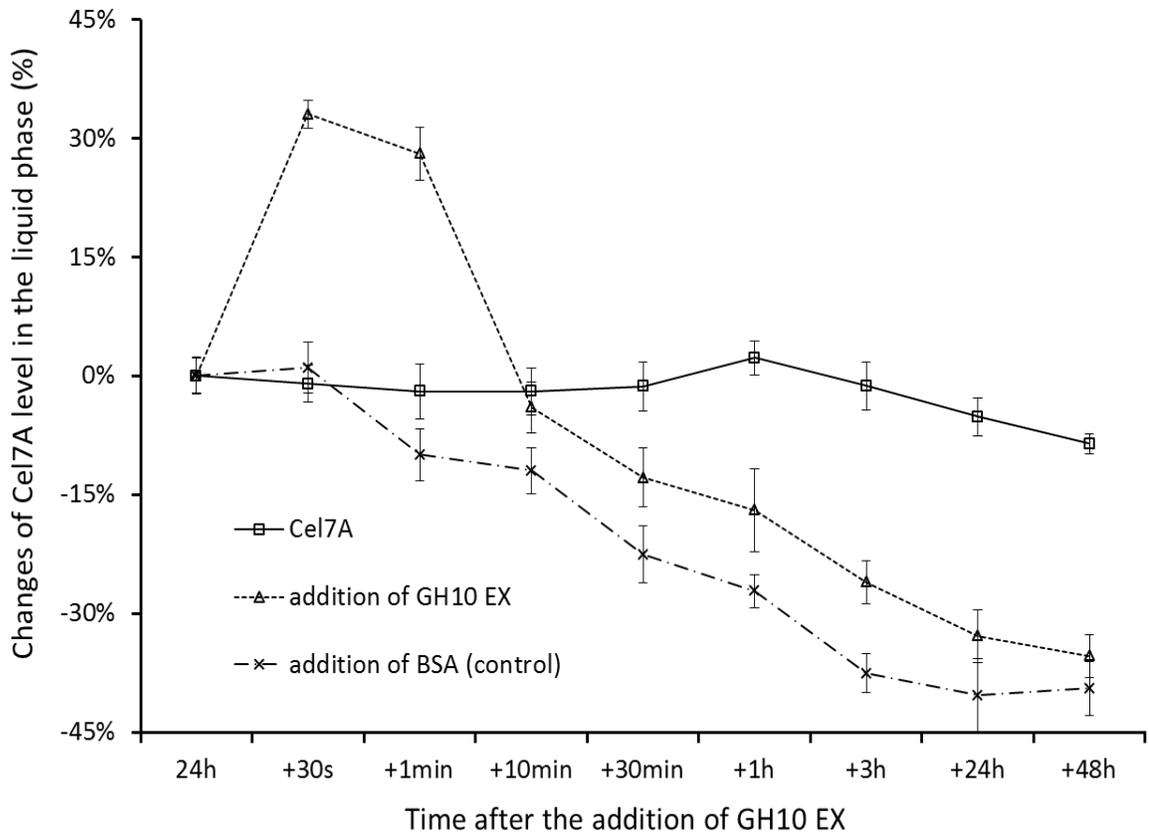


Figure 19. Changes in the amount of Cel7A in the liquid phase of steam pretreated corn stover (SPCS) after 24 h hydrolysis. (Δ) with and (\square) without addition of GH10 EX. Control: (*) addition of BSA.

One of the ways that accessory enzymes are thought to aid in achieving improved hydrolysis is by helping release or dislodge cellulases that seem to be stuck on the substrate. For example, Eriksson et al. (2002a) observed a change in the adsorption equilibrium of Cel7A upon addition of Cel7B when the hydrolysis of steam pretreated spruce had levelled off. They suggested this was due to competition between the two cellulases. It is likely that, in the work reported here, the observed change in the adsorption equilibrium of Cel7A upon addition of GH10 EX was also due to competitive adsorption. It is also worth noting that, like Cel7B, GH10 EX has a carbohydrate binding module with a high affinity for cellulose (Rabinovich et al., 2002; Collins et al., 2005). Previous work has shown that Cel7A gets stuck on cellulose microfibrils during enzymatic cellulose hydrolysis (Igarashi et al., 2011), leading to a substantial decrease in the rate of hydrolysis. It is possible that the GH10 EX hydrolyzed possible obstacles such as xylan that restricted the processive movement of the Cel7A.

One of the main challenges in achieving effective cellulose hydrolysis is to overcome the still-not-well-understood gradual decrease in hydrolysis rate in the latter stages of hydrolysis. Various mechanisms are thought to play a role, such as the inactivation of cellulase enzymes (denaturation, product inhibition, unproductive binding) and an increase in the recalcitrance of the residual substrate (Eriksson et al., 2002a; Yang et al., 2006). The results presented here indicate that two of the major roles that accessory enzymes, such as xylanases, might play in enhancing the hydrolytic potential of a “cellulase mixture” is to assist in the release of cellulases that are stuck on the substrate, while hydrolysing the non-cellulose components of the substrate that restrict access of the cellulases to the cellulose.

3.2.2.6 Assessing the influence of acetyl groups on xylanases' accessibility to xylan.

GH10 EX performed better than GH11 EX during hydrolysis of pretreated lignocellulosic substrates (Figure 16), even though GH11 EX had higher activity on “model” xylanolytic substrates (Table 8). This observation agrees with previous work (Pryor and Nahar, 2010), which suggested that “model” substrates used to detect enzyme activity do not necessarily predict their hydrolytic performance on heterogeneous lignocellulosic materials. The greater boosting effect of adding supplemental GH10 EX to cellulases was likely due to its ability to extensively cleave xylan into smaller chain products (Kolenova et al., 2006; Zhang et al., 2011b). It has recently been suggested that longer xylo-oligosaccharides inhibited cellulases to a greater extent (Qing et al., 2010; Qing and Wyman, 2011). In addition, GH10 EX has also been shown to have a higher affinity for the highly branched xylan backbone (Subramaniyan and Prema, 2002; Kolenova et al., 2006), and might be more thermostable than GH11 EX at the hydrolysis temperature (Sun et al., 2005; He Jun et al., 2009).

To understand why GH10 EX performed better than GH11 EX on pretreated biomass, a “model” cellulosic substrate with 10% xylan content was formulated by mixing birchwood xylan with the “pure” cellulosic substrate, dissolving pulp (DSP). In this “model” substrate, xylan was assumed to locate on the outside surface of the cellulose fibres. As expected, the additional xylan decreased the hydrolyzability of the DSP to cellulase enzyme (Figure 20), while the addition of xylanase to the cellulase enzymes recovered the cellulose hydrolysis after 72 h. Interestingly, the two different family xylanases had a similar boosting effect on cellulose hydrolysis. Therefore, it seems that both family xylanases had a similar affinity for the birchwood xylan relocated on the DSP surface.

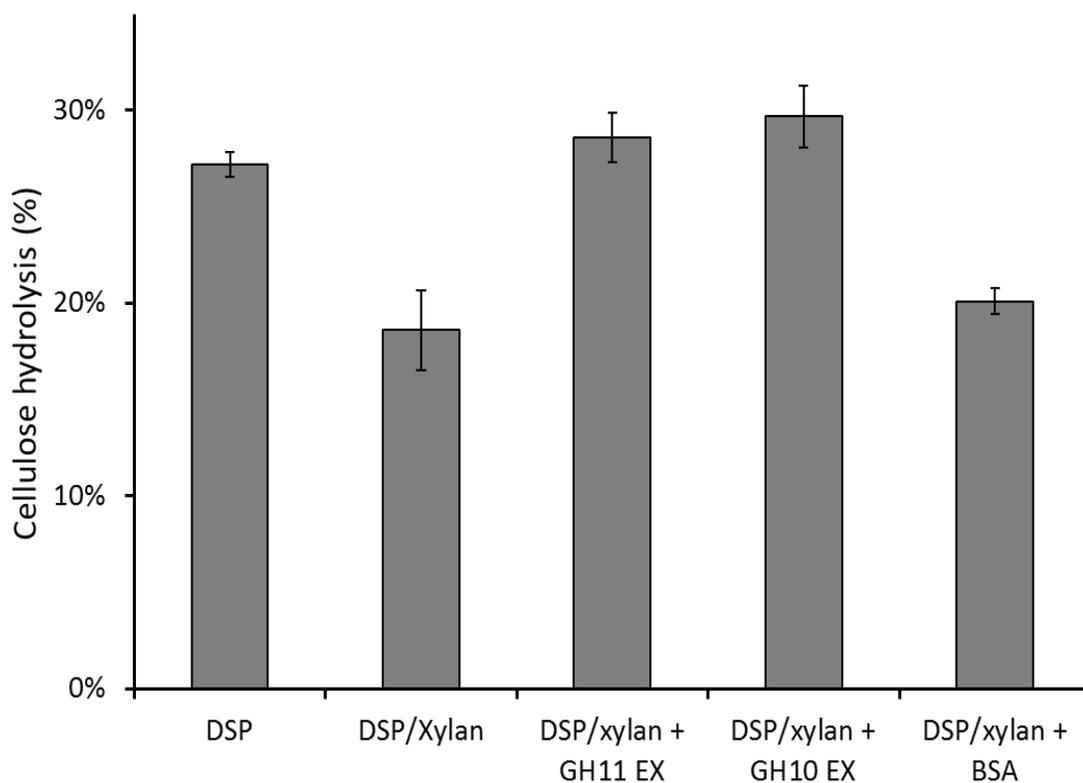


Figure 20. Cellulose hydrolysis of DSP (+ 10% birchwood xylan) by Cel7A with/without GH10/11 EX supplementation at a 2% (w/v) consistency. DSP: dissolving pulp.

Acetylation of the xylan backbone may block the access of xylanases to the xylan within the pretreated biomass. Therefore, the better performance of GH10 EX versus GH11 EX during hydrolysis of pretreated lignocellulosic substrates might be due to the GH10s higher affinity for substituted xylan, as discussed in more detail in Chapter 1 (1.5.2.1). To test this hypothesis, the two family xylanases were assessed on sugar cane bagasse pretreated using two different severities, resulting in a substrate with and a substrate without deacetylation. The SPSCB at higher severity (200°C, SPSCB 200) had less acetyl group content compared with the one pretreated at 190°C (SPSCB 190) as shown in Figure 21B. GH10 EX had a significantly better performance than GH11 EX during its synergistic cooperation with cellulase for cellulose hydrolysis on both SPSCB190 and SPSCB 200

(Figure 21A). However, the difference in cellulose hydrolysis boosting effects between family 10 and 11 xylanases was dramatically reduced on the deacetylated substrates dA-SPSCB 190 and dA-SPSCB 200 (Figure 21A). Thus, it seems that the acetyl group removal increased the beneficial effect of GH11 EX.

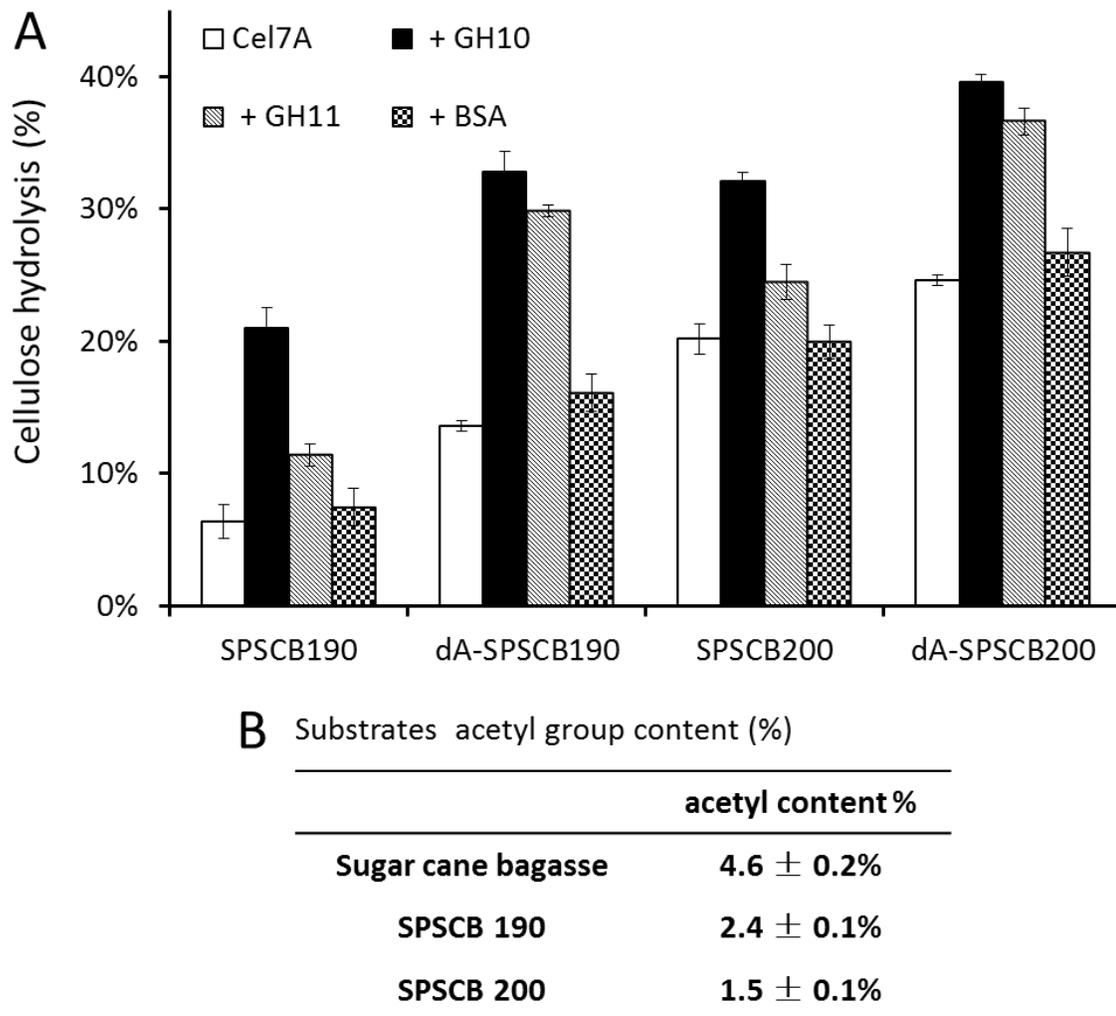


Figure 21. The influence of de-acetylation on cellulose hydrolysis by cellulase and different family xylanases. (A) Cellulose hydrolysis of de-acetylated (dA-) and original steam pretreated sugar cane bagasse (SPSCB) at two different temperatures (190 and 200°C) with/without GH10/11 EX after 72 h. (B) The acetyl content of original and steam pretreated sugar cane bagasse. GH10/11: glycoside hydrolase family 10/11 endo-xylanases.

We next assessed the adsorption profile of GH10/11 EX on various “model” xylanolytic substrates (birchwood xylan and oat spelt xylan), cellulosic substrates (Avicel and DSP), and lignin (isolated from softwood Douglas fir), and also on “real life” steam pretreated sugar cane bagasse (SPSCB190) and deacetylated SPSCB190 (dA-SPSCB190) (Table 11). It appeared that GH11 EX had a high adsorption affinity to both xylan substrates, but GH10 EX displayed greater absorption on the “model” cellulosic substrates. The higher adsorption of GH10 EX to cellulose could be a result of it having a cellulose binding module (CBM), which is not present in GH11 EX (Chapter 1 - 1.5.2.1). A greater amount of GH11 EX (~60%) than GH10 EX (~10%) adsorbed to isolated lignin at 4 °C, which indicated that a large amount of GH11 EX would unproductively bind to lignin during lignocellulose hydrolysis. Interestingly, the acetyl group removal significantly increased the affinity of GH11 EX to lignocellulosic substrates. Even though the same amount of GH10 EX was absorbed on SPSCB190 and dA-SPSCB190, the absorption of GH11 EX on deacetylated substrate was almost double compared with its absorption on SPSCB190 (Table 11). Combined with the previous result where the substrate deacetylation also increased the hydrolytic performance of GH11 EX (Figure 21), it was likely that the acetyl groups left in the pretreated biomass restricted the xylan accessibility more for GH11 EX than it did for GH10 EX.

Table 11. Xylanase adsorption on various substrates at 4°C.

	Beechwood xylan	Oat Spelts xylan	Avicel	DSP	Lignin	SPSCB190	dA- SPSCB190
GH11 EX	83.3%	76.8%	5.6%	24.8%	59.2%	47.3%	94.0%
GH10 EX	75.8%	74.3%	22.6%	38.6%	10.7%	53.1%	48.1%

GH10/11 EX: glycoside hydrolase family 10/11 endo-xylanases, DSP: dissolving pulp, SPSCB190: steam pretreated sugar cane bagasse at 190°C, dA-SPSCB190: de-acetylated SPSCB190

3.2.2.7 Comparing the thermostability of family 10 and 11 xylanases

As shown in the last section (3.1.2.7), Multifect Xylanase (enriched in GH11 EX) was thermo-unstable over prolonged hydrolysis, thus another possible reason for the better performance of family 10 xylanase might be higher thermostability than family 11 xylanase. In addition, the previous study used the whole xylanase mixture and only assessed its thermostability in the hydrolysis buffer. The enzyme might be more stable during the hydrolysis of lignocellulosic substrates as it can interact with the solid substrate. Therefore, the thermostability of the purified family 10 and 11 xylanase was assessed and compared in the hydrolysis buffer and in the realistic hydrolysis system containing both buffer and lignocellulosic substrates (Figure 22). In general, the GH10 EX was far more thermostable than the GH11 EX, with and without solid substrate (SPCS), while the presence of SPCS improved the thermostability of both family xylanases. Without SPCS, around 80% of the GH11 EX precipitated out in the first hour, while the solid SPCS in the system significantly decreased the extent of precipitation of the xylanase (Figure 22A). GH10 EX performed better than did the GH11 EX, with around 50% of the GH10 EX still active in the hydrolysis

buffer after 12 h incubation, while the GH11 EX had completely lost activity after 12 h (Figure 22A).

For the xylanase activity of both family xylanases, GH10 EX had a higher specific activity remaining in both hydrolysis buffer (Figure 22B) and hydrolysis buffer with SPCS than GH11 EX (Figure 22C). Thus, we concluded that the lower thermostability of GH11 EX was one of the main reasons for the lower observed hydrolytic efficacy of GH11 EX compared with GH10 EX when they were cooperating synergistically with cellulase in lignocellulose hydrolysis.

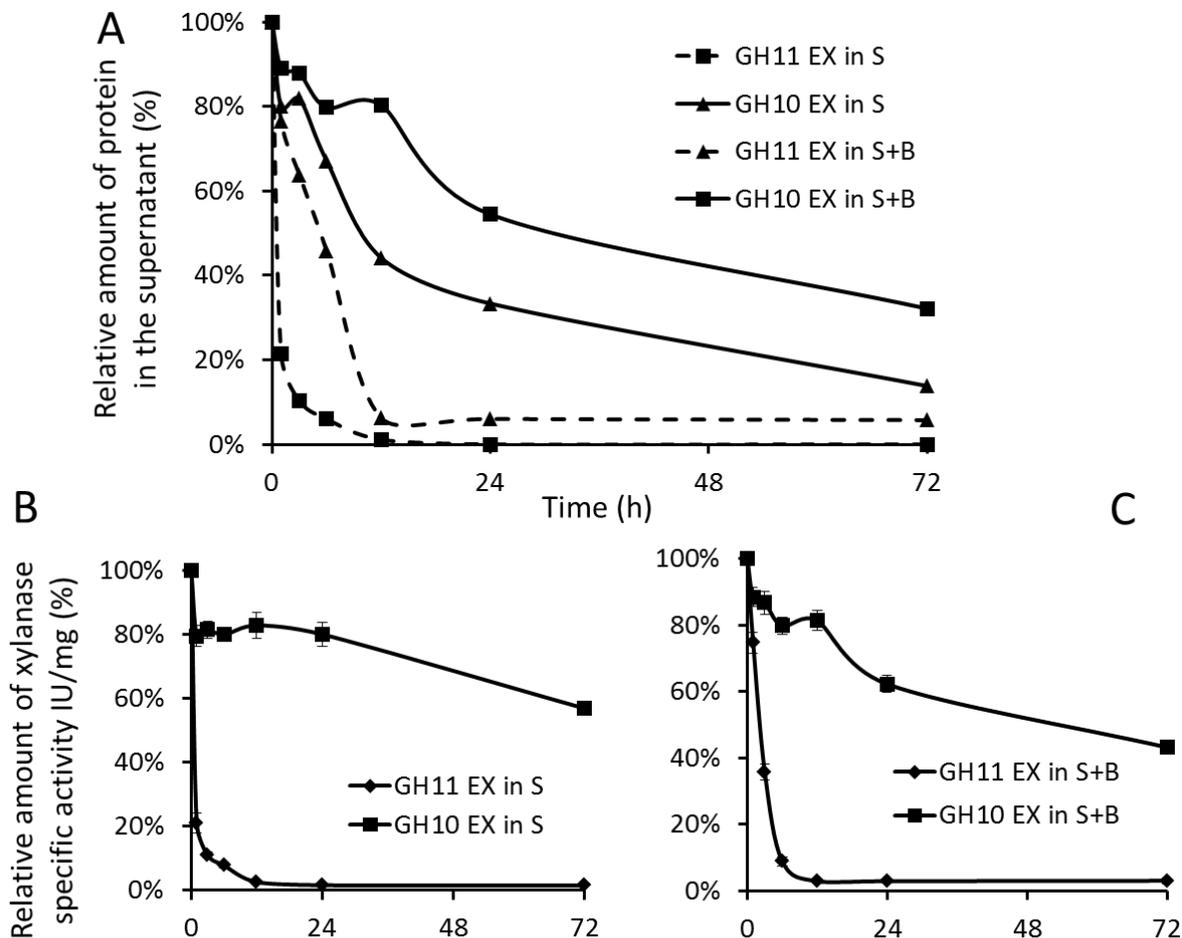


Figure 22. Xylanase thermostability at 50°C. (A) Relative amount of protein in the supernatant. Relative amount of xylanase specific activity (IU/mg) of different family xylanases in the hydrolysis system (B) and in the buffer only system (C). S: substrate SPCS, B: buffer.

3.2.3 Conclusion

It was apparent that the hydrolytic activity of cellulase monocomponents was enhanced by the addition of accessory enzymes, although the “boosting” effect was highly substrate specific. The GH10 EX and GH5 XG both exhibited broad substrate specificity and showed strong synergistic interaction with the cellulases when added individually. The GH10 EX was more effective on steam pretreated agricultural residues and hardwood substrates,

whereas GH5 XG addition was more effective on softwood substrates. The synergistic interaction between GH10 EX and GH5 XG, when added together, further enhanced the hydrolytic activity of the cellulase enzymes over a range of pretreated lignocellulosic substrates. GH10 EX addition could also stimulate further cellulose hydrolysis when added to the hydrolysis reactions after the rate of hydrolysis had levelled off. It appears that those accessory enzymes, such as GH10 EX and GH5 XG, with broader substrate specificities, promoted the greatest improvements in the hydrolytic performance of the cellulase mixture on all of the pretreated biomass substrates. In addition, family 10 xylanase performed better than family 11 xylanase due, in part, to the GH11 EX having lower accessibility than GH10 EX to the xylan backbone in the pretreated biomass with the acetyl group substitution. The GH11 EX was also demonstrated to be more thermo-unstable than GH10 EX in the hydrolysis system.

3.3 Hydrolytic and oxidative cellulose cleavage act synergistically to enhance biomass deconstruction

3.3.1 Background

In the previous two sections, we assessed the synergistic cooperation between cellulases and hemicellulases, (mainly xylanase), during the hydrolysis of pretreated lignocellulosic substrates. It was shown that xylanase could significantly improve the hydrolytic performance of cellulase enzymes by increasing the accessibility of cellulases to cellulose. After reaching cellulose, another barrier for cellulases is the highly organized crystalline cellulose structure which also restricts the accessibility of the cellulose to cellulase enzymes, as described in Chapter 1 (1.3.1). The existence and function of a cellulose swelling factor has been discussed for more than six decades since Reese proposed the “C1-Cx” hypothesis, where C1 was suggested to first swell the highly organized crystalline cellulose to facilitate the hydrolytic cleavage by cellulase enzymes Cx (Reese et al., 1950). Although several disrupting enzymes/proteins and cellulose binding domains (CBM) have been tested as the “C1” component that caused the so-called cellulose “amorphogenesis”, it is still difficult to prove the existence of such a swelling factor (Arantes and Saddler, 2010). Recently, the newly identified lytic polysaccharide monooxygenases, or AA9s, have attracted much attention as it can cleave cellulose crystalline regions oxidatively (Harris et al., 2010).

The most efficient wood biodegradation system in nature belongs to brown rot fungi, which dominates the decomposition in northern coniferous forest ecosystems, even though it only accounts 6% of wood-decay species (Baldrian and Valaskova, 2008; Eastwood et al., 2011). Brown rot decay has both an oxidative/radical based system, believed to initiate the attack by the hydroxyl radicals produced through Fenton reactions in incipient decay stages,

and an enzymatic based system that hydrolyzes the polysaccharides within the woody biomass using an incomplete cellulase suite (Eastwood et al., 2011; Arantes et al., 2012). In a similar pattern, this two-type system has also been found for efficient biodegradation of chitin (Vaaje-Kolstad et al., 2010), another major structural polymer existing in nature in the form of nanocrystallites. Thus, one can expect that synergistic cooperation between enzymatic hydrolysis and oxidative cleavage would be crucial for achieving an efficient biodegradation of cellulosic substrate. Developing a better understanding of cellulases-AA9 synergistic interactions might assist in designing a more efficient and robust “cellulase cocktail” for biomass deconstruction, but may also provide insights for improving the pretreatment strategy.

As described in more detail in Chapter 1 (1.5.2.2), AA9 cleaves cellulose chains through an oxidative mechanism at its planar active site surface which contains a divalent metal ion (most likely copper). The activation of AA9 enzymes also requires a redox-active cofactor. Even though several AA9 studies have been carried out in the last few years, most of this work has focused on the protein structure and the structure-function characteristics (Quinlan et al., 2011; Westereng et al., 2011; Beeson et al., 2012; Bey et al., 2013). The mechanism of the synergistic cooperation between cellulase enzymes (hydrolytic cellulose cleavage) and AA9 proteins (oxidative cellulose cleavage) has not been studied extensively. It is known that some non-cellulosic fractions from lignocellulosics can act as AA9 cofactors to potentiate its activity (Quinlan et al., 2011; Dimarogona et al., 2012), but the source of these cofactors within different types of pretreated substrates and the correlation between their reducing ability and the AA9s ability to boost cellulose hydrolysis by cellulases are still unclear. Since it has previously been shown that the synergism between

cellulases, as well as the overall hydrolysis extent, was highly affected by a variety of physicochemical substrate characteristics (e.g. cellulose accessibility, crystallinity, degree of polymerization, cellulose allomorphs, the type, amount and distribution of residual lignin and hemicellulose) (Woodward, 1991; Watson et al., 2002; Zhang and Lynd, 2004), it is not unrealistic to expect that these substrate characteristics will also impact the cellulases-AA9 interaction. Recent studies have shown that one of the rate limiting factors of cellulose hydrolysis is due to the unproductively bound/jammed processive enzymes (Igarashi et al., 2011; Ganner et al., 2012; Fox et al., 2013). Thus another beneficial function of AA9 enzymes might be the negative charge (aldonic acid) and the short chains produced on crystalline cellulose resulting from the oxidative cellulose cleavage and the consequential increased general turnover number of processive cellulases during hydrolysis.

In this part of the thesis we assessed the synergistic cooperation between *Thermoascus aurantiacus* AA9 and *T. reesei* cellulases during hydrolysis of a library of “industrially-relevant” pretreated lignocellulosic and “model” cellulosic substrates. We evaluated the influence of the physicochemical characteristics of these substrates on the ability of AA9 to enhance cellulose hydrolysis by cellulase enzymes. It was apparent that the non-cellulosic components in pretreated lignocellulosic substrates (i.e. soluble compounds, lignin fragments, and possibly xylan) possess high enough reducing capabilities to act as AA9 co-factors during hydrolysis. Out of the major substrate physicochemical characteristics studied, the relative amount of accessible crystalline cellulose appeared to be the key factor that influenced the extent of hydrolysis enhancement promoted by the synergistic cooperation between AA9 and cellulase enzymes. It was also apparent that AA9 treatment resulted in an increase in the amount of unbound processive cellulase enzyme (Cel7A) during

hydrolysis of semi-crystalline cellulosic substrates, possibly due to an increase in the overall negative charge of the cellulose fibres as a result of oxidative cellulose cleavage.

3.3.2 Results and discussion

3.3.2.1 Boosting effect of AA9 on various pretreated lignocellulosic biomass.

We next assess the synergistic cooperation between cellulases and AA9 on pretreated lignocellulosic substrates, corn stover, poplar and lodgepole pine. These substrates were chosen as representatives of agricultural residue, hardwood, and softwood substrates respectively and they were pretreated using steam explosion and/or organosolv pretreatment to create a range of biomass substrates with a variety of physicochemical properties (Table 12). In the presence of AA9, the hydrolysis yields of all pretreated substrates were significantly increased, but the extent of enhancement was highly substrate dependent (Figure 22A). The added AA9 displayed a higher boosting effect on organosolv pretreated substrates than on steam pretreated ones (e.g. 27% on OPCS vs. 14% on SPCS). For substrates pretreated by the same pretreatment technology, a higher AA9 boosting effect was observed on agricultural residues, followed by hardwood and to a lesser extent on softwood.

Table 12. Chemical composition of pretreated lignocellulosic biomass

Pretreatment	Samples	Major sugar compositions (%)			Lignin analysis (%)	
		Glu	Xyl	Man	AIL	ASL
Steam pretreatment	SPCS	50.7 ± 0.9	15.4 ± 0.1	0.7 ± 0.0	23.3 ± 0.3	3.4 ± 0.2
	SPP	58.2 ± 0.2	7.7 ± 0.1	2.0 ± 0.1	28.5 ± 0.2	2.8 ± 0.1
	SPLP	53.4 ± 1.0	0.2 ± 0.0	0.4 ± 0.1	42.8 ± 0.2	0.6 ± 0.0
Organosolv pretreatment	OPCS	60.3 ± 0.6	12.2 ± 0.5	1.0 ± 0.1	26.2 ± 0.7	1.6 ± 0.2
	OPP	78.2 ± 0.3	4.5 ± 0.0	2.2 ± 0.1	18.2 ± 0.5	1.0 ± 0.1
	OPLP	77.7 ± 0.7	1.5 ± 0.0	2.0 ± 0.0	25.4 ± 0.1	0.3 ± 0.0

Glu, glucose; Xyl, xylose; Man, mannose; AIL, acid insoluble lignin; ASL, acid soluble lignin; CS, corn stover; LPP, lodgepole pine.

Recently, it had been shown that the AA9 “boosting effect” during hydrolysis of “pure” cellulose by cellulases depended on the amount of exogenous reducing cofactor, such as gallate, that was added to the reaction mixture (Quinlan et al., 2011; Dimarogona et al., 2012). However, contrary to the hydrolysis of pure cellulosic substrates, the exogenous addition of a synthetic reducing agent did not further increase the hydrolysis yields of pretreated substrates by a cellulase/AA9 mixture (Figure 23A). When relatively pure cellulose (isolated from pretreated lignocellulosic substrates by extensive delignification and xylan removal) was hydrolyzed by cellulase/AA9, the AA9 boosting effect was only observed in the presence of a synthetic reducing agent (Figure 23B). This indicated that some components, other than cellulose, that were present in the pretreated lignocellulosic substrates were acting as AA9 reducing agents/cofactors. Thus, the different extents of enhancement in sugar yields by AA9 during hydrolysis of pretreated lignocellulosic

substrates by cellulases could be associated with the reducing agents within pretreated biomass.

To identify the source of AA9 cofactors in pretreated lignocellulosic substrates, different fractions/components (soluble substrate compounds, lignin, hemicellulose) were separately evaluated for their ability to act as an AA9 cofactor. In order to simplify the hydrolysis system, purified cellulase (Cel7A) and β -glucosidase (GH3 β G) were used. Soluble compounds derived from pretreated substrates have been suggested to potentiate AA9 activity due to their metal reducing capacity (Quinlan et al., 2011). The boosting effect of AA9 on a pure cellulosic substrate dissolving pulp (DSP) was found to correlate with the reducing capacity of the soluble substrate components added to the reaction (Figure 23B; Table 13). However, this trend did not hold for hydrolysis of the lignocellulosic substrates, where a correlation between the reducing capacity of a particular substrate's soluble components and the boosting effect of AA9 during hydrolysis of that substrate was not observed (Figure 23B; Table 13). For example, the soluble fractions of organosolv pretreated substrates exhibited significantly lower metal reducing ability compared to fractions derived from steam pretreated substrates. However, AA9 resulted in a greater boosting effect on the organosolv pretreated substrates.

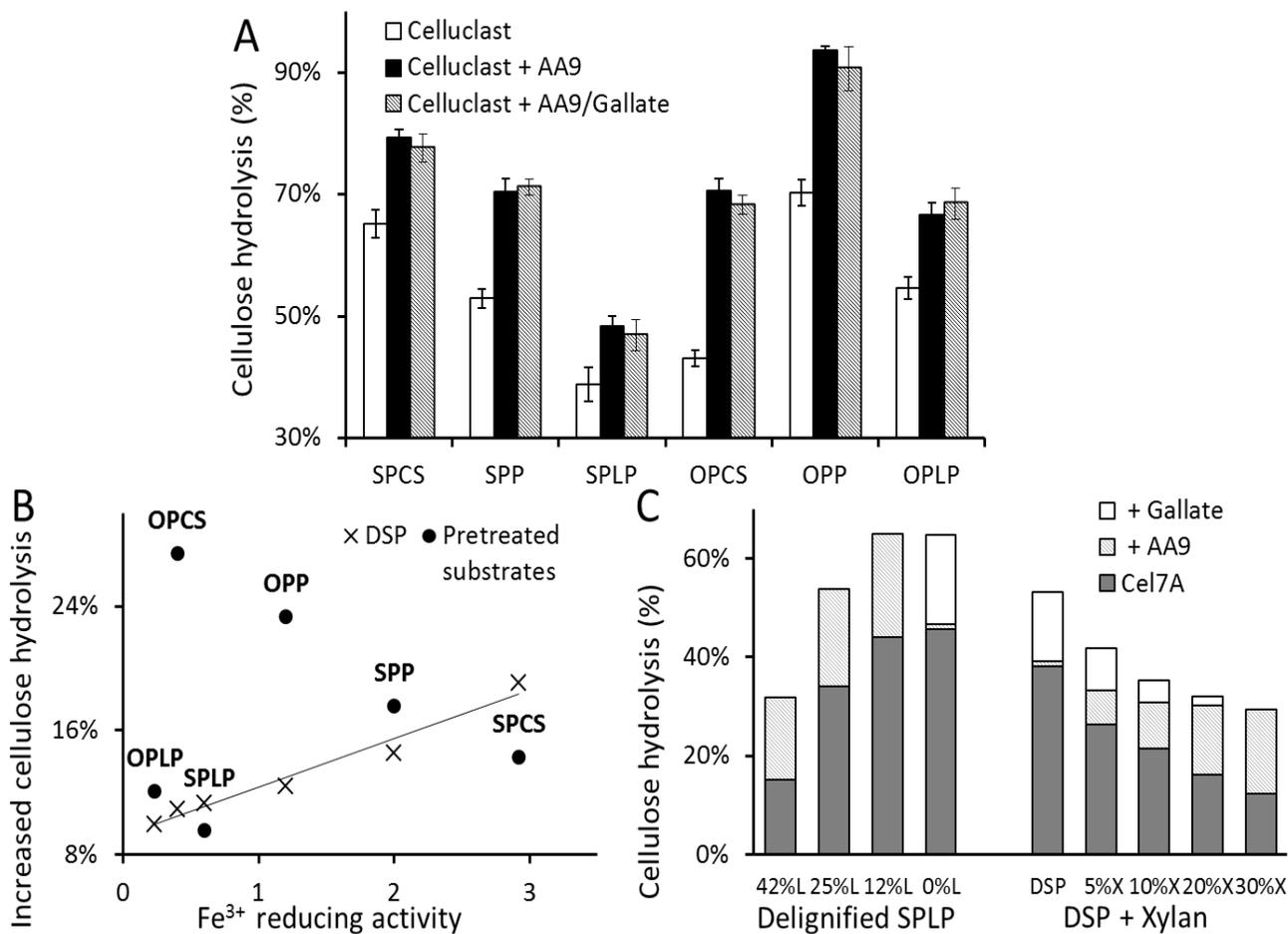


Figure 23. The boosting effects of AA9 on the hydrolysis of various pretreated lignocellulosic substrates and the influence of different non-cellulosic fractions on AA9 activity. (A) Cellulose hydrolysis of different substrates with/without AA9 after 48 h. (B) The relationship between the Fe³⁺ reducing activity of substrates' soluble compounds and AA9 increased cellulose hydrolysis on (●) pretreated substrates and (x) dissolving pulp (DSP). (C) The hydrolysis boosting effects of AA9 on SPLP with various lignin contents and on DSP with the supplementation of different portions of birchwood xylan after 48 h. Steam and organosolv pretreated corn stover, poplar and lodgepole pine: SP/OP- CS, P, and LP.

Table 13. The Fe³⁺ reducing activity of the soluble components from various pretreated substrates and the AA9 increased cellulose hydrolysis on pretreated substrates and on dissolving pulp (DSP) with the supplementation of these soluble components.

	reducing ability	increased DSP conversion	increased pulp conversion
SPCS	2.9	19.2%	14.3%
SPP	2.0	13.2%	18.1%
SPLP	0.4	11.0%	9.5%
OPCS	0.2	10.9%	27.4%
OPP	1.2	11.4%	23.3%
OPLP	0.2	9.9%	12.0%

Steam/organosolv pretreated corn stover, poplar, and lodgepole pine (SP/OP- CS, P, and LP).

The potential role of lignin as an AA9 cofactor was investigated by hydrolysing steam pretreated softwood (SPLP), delignified to various extents in the presence and absence of a synthetic reducing agent. The presence of the synthetic reducing agent was only required to potentiate AA9 activity on the completely delignified SPLP (Figure 23C, Figure 24B). Although no correlation was found between the amount of residual lignin and the extent of the AA9 boosting effect, it seems that when lignin was present, addition of a synthetic cofactor such as gallate was no longer required to potentiate the boosting effect of AA9 (Figure 23C).

The influence of hemicellulose was initially assessed after removing lignin and soluble components from pretreated substrates, resulting in substrates containing only cellulose or cellulose and hemicellulose (holocellulose). In the absence of a synthetic reducing agent, the AA9 boosting effect was only observed on substrates containing some residual hemicellulose, such as delignified hardwood and agricultural residues (Figure 24A).

These results suggested that the residual hemicellulose within holocellulose might also serve as an AA9 cofactor. To further test this hypothesis, birchwood xylan was added to dissolving pulp (DSP), a relatively “pure” cellulosic substrate (>97% cellulose), prior to hydrolysis. Again, the AA9 boosting effect was found to be dependent on exogenous xylan addition (Figure 23C). However, when the effect of varying amounts of residual hemicellulose in steam pretreated wheat straw (SPWS) was tested, no correlation was found between the extent of the AA9 boosting effect and the residual hemicellulose content (Figure 25). Interestingly, it seemed that the synergistic cooperation between the AA9 and GH10 EX also enhanced cellulose hydrolysis by the cellulases (Figure 25B). The degree of synergism between the AA9 enzymes, which have no activity towards hemicellulose (Harris et al., 2010), and xylanases in enhancing cellulose hydrolysis was found to be highly dependent on the xylan content of the substrate (Figure 25B). This suggested that xylanases might increase the accessibility of the cellulose to AA9 by solubilizing xylan and, in turn, allowing AA9 to then synergistically interact with cellulases and stimulate cellulose hydrolysis. Indeed, we (Bura et al., 2009; Hu et al., 2011) and others (Garcia-Aparicio et al., 2007; Selig et al., 2008; Kumar and Wyman, 2009b) have shown that the removal of residual hemicellulose in pretreated cellulosic substrates by xylanases can significantly increase cellulose accessibility to enzymes.

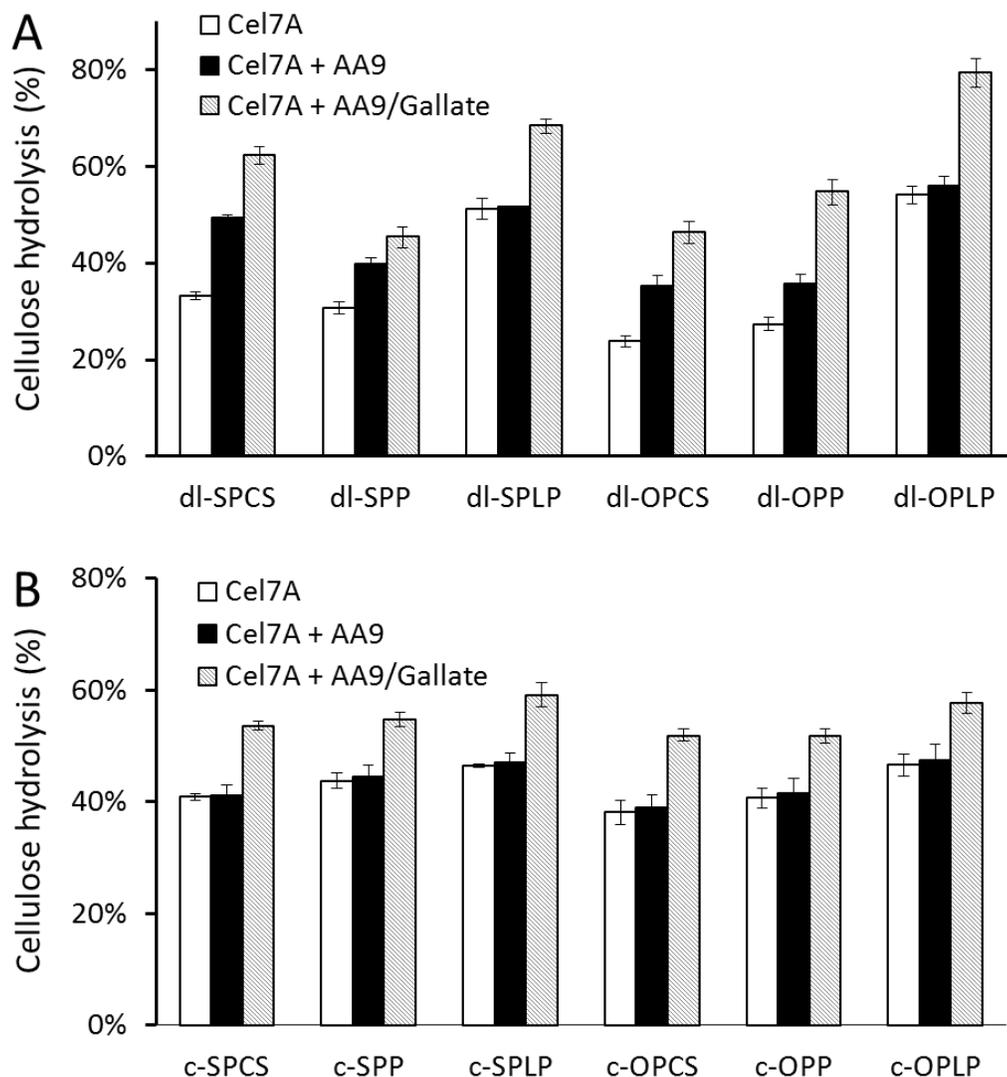


Figure 24. Cellulose hydrolysis of (A) holocellulose and (B) “pure” cellulose isolated from various pretreated lignocellulosic substrates with/without AA9 after 48 h.

Though it seemed that pretreated biomass-derived soluble compounds, lignin, and possibly residual xylan could potentially act as AA9 cofactors during hydrolysis of pretreated lignocellulosic substrates, their metal-reducing capability did not seem to be the determining factor for the observed substrate-dependent boosting effects of AA9. For example, even 12% residual lignin could provide enough reducing power to potentiate AA9 activity (Figure 23C).

Alternatively, the various extents of the AA9 boosting effect may be influenced by other substrate properties such as differing physical characteristics, as discussed below.

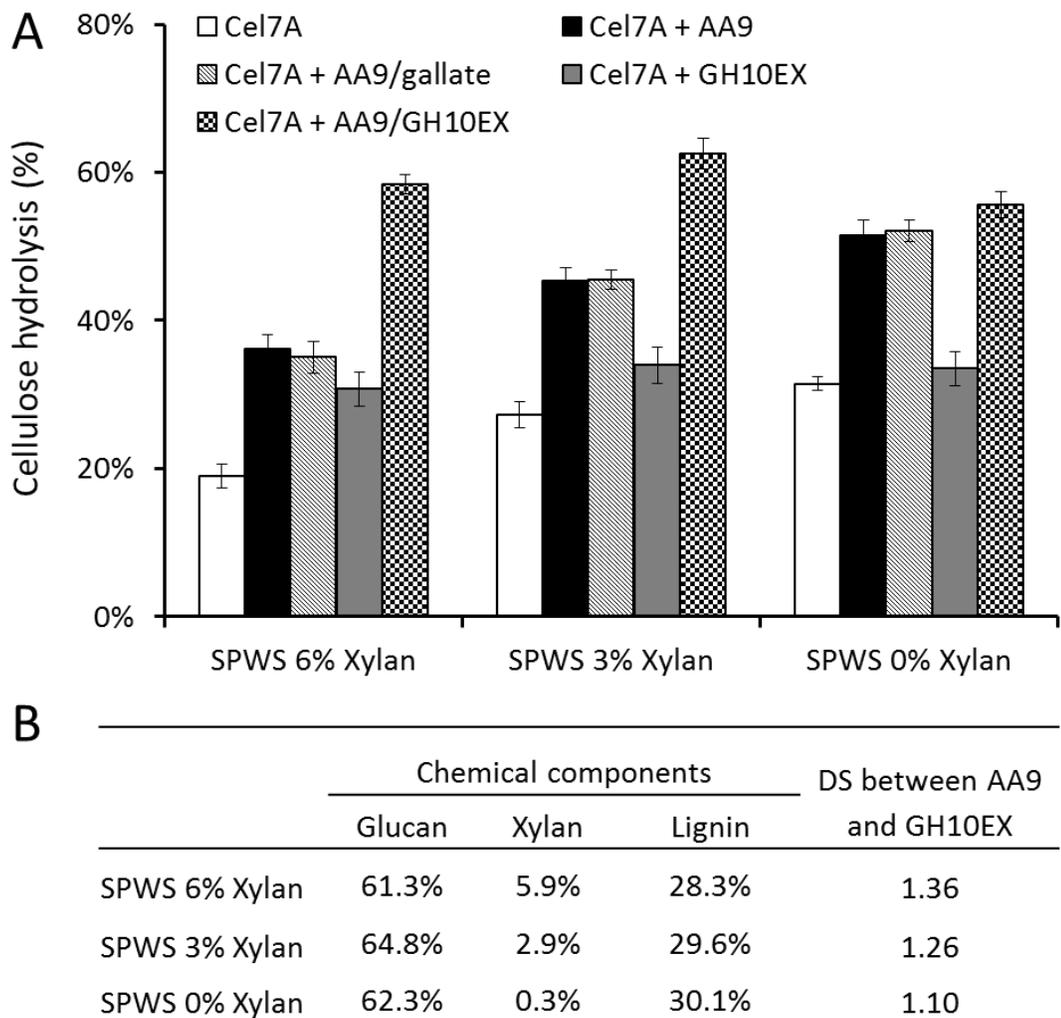


Figure 25. (A) The cellulose hydrolysis of steam pretreated wheat straw (SPWS) with different xylan content with/without AA9 and GH10 EX after 48 h. (B) The chemical composition of SPWS with different xylan content and the degree of synergism (DS) between AA9 and GH10 EX on the cellulose hydrolysis of SPWSs.

3.3.2.2 The influence of substrate physical characteristics on AA9 boosting effect

Several substrate properties such as gross fibre characteristics, cellulose accessibility, cellulose degree of polymerization (DP), and cellulose crystallinity (CrI), that have been suggested to influence the extent of cellulose hydrolysis. Thus changes in these characteristics were assessed for each of the lignocellulosic substrates (Figure 26A). These substrate characteristics were then related to the increase in hydrolysis yields in the presence of AA9. As AA9 has been shown to cleave cellulose chains within the crystalline cellulose regions, thereby increasing accessible binding sites to cellulase enzymes, it was anticipated that there might be a correlation between CrI and AA9 increased cellulose hydrolysis. However, when these physical characteristics were plotted against the extent of the AA9 boosting effect, a good relationship was only observed between cellulose accessibility, as determined by the Simons' Stain technique, and the AA9 boosting effect (Figure 26B).

The relationship between the AA9 boosting effect and cellulose accessibility indicated that, similarly to what has been shown for hydrolytic cellulases, oxidative AA9 enzymes also required access to cellulose. Indeed, the most recent mechanistic model of AA9 action suggests the direct enzyme-catalyzed oxidation of cellulose (Langston et al., 2011; Phillips et al., 2011; Beeson et al., 2012). This would require direct AA9 access to the cellulose, rather than the initially proposed generation and diffusion of reactive oxygen species (ROS) that randomly attacked the substrate (Karkehabadi et al., 2008; Rineau et al., 2012). The fact that the extent of AA9 stimulation of the hydrolytic cellulase activity seemed to correlate with the amount of cellulose surface area (as determined by the Simons stain values) supported a direct, AA9-catalyzed oxidation of cellulose. Further evidence to support

this mechanism comes from the observed synergistic cooperation between AA9 and xylanases that significantly enhanced cellulase hydrolysis of SPWS (Figure 25).

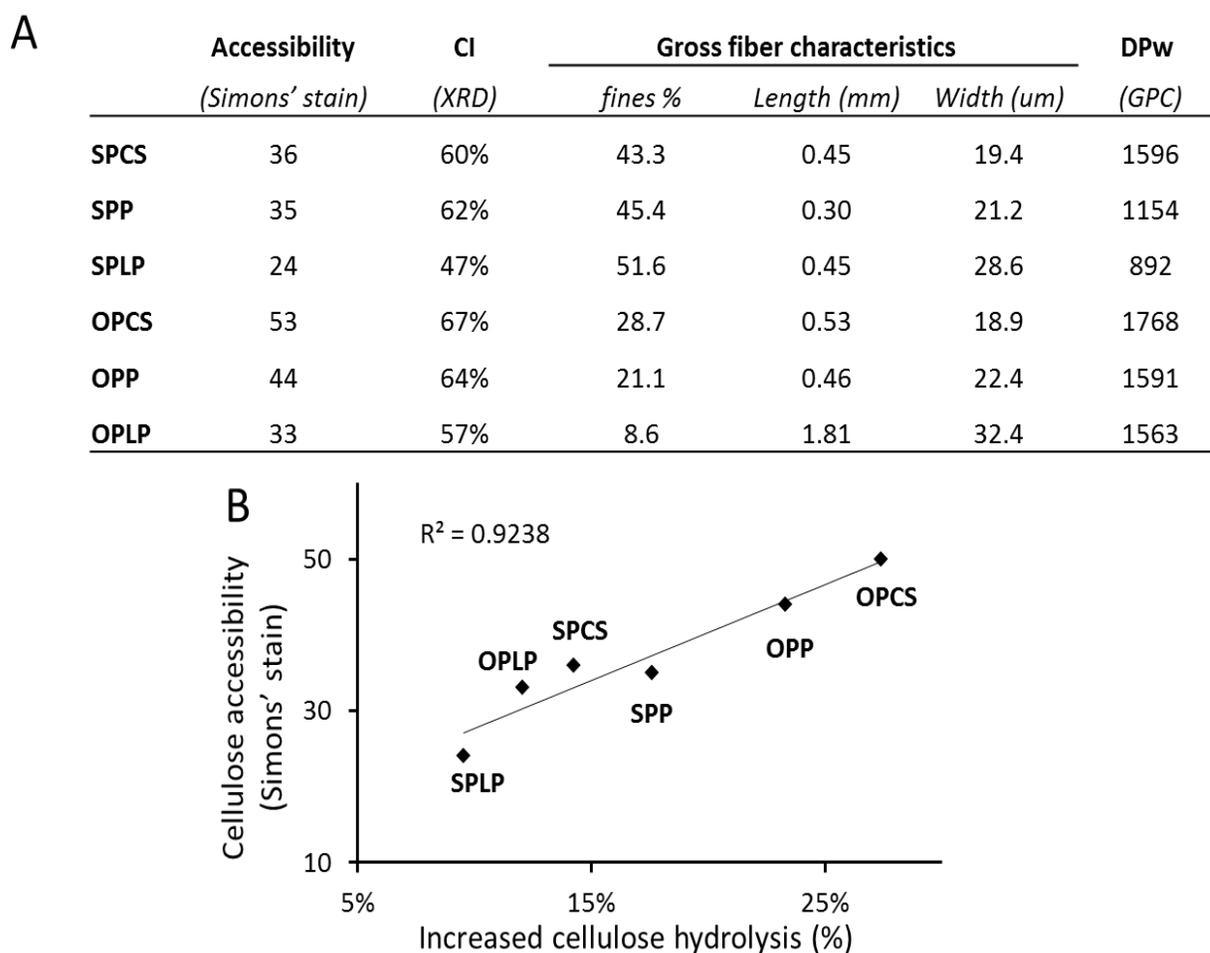


Figure 26. The influence of substrates' physical characteristics on AA9 boosting effect during enzymatic cellulose hydrolysis. (A) Substrate properties: cellulose accessibility, gross fibre characteristics: fibre length and width, cellulose degree of polymerization (DP), and cellulose crystallinity (CrI). (B) The correlation between cellulose accessibility and AA9 increased cellulose hydrolysis on various pretreated substrates.

3.3.2.3 The influence of cellulose allomorphs on AA9 boosting effect

The absence of a correlation between the crystallinity index (CrI) of pretreated lignocellulosic substrates and AA9-mediated enhancement of cellulose hydrolysis was

unexpected, as previous studies have reported AA9 enzymes to have endo-activity on crystalline cellulose (Langston et al., 2011), thereby synergizing with cellulase enzymes by creating new binding/initiation sites for cellulase activity. To further investigate the lack of correlation between CrI and the AA9 boosting effect on pretreated lignocellulosic substrates and also to better understand the influence of only the cellulose properties on cellulases-AA9 interactions, without interferences from other cell wall components, a range of model/pure cellulosic substrates with varying crystallinity and allomorphic forms were used to further assess the interaction between cellulases and AA9 (Figure 27A).

Generally, AA9 addition resulted in increased enzyme mediated cellulose hydrolysis on the highly crystalline cellulosic substrates such as cellulose nanocrystals (CNC) (Figure 27A). An increase in hydrolysis yield was also observed on those cellulosic substrates with a moderate level of crystallinity such as Avicel and DSP. No AA9 enhancement was observed on amorphous cellulosic substrates such as phosphoric acid swollen cellulose (PASC) (Figure 27A). When Avicel (Cellulose I) was mercerized to Cellulose II, the boosting effects of AA9 addition on cellulose hydrolysis was considerably reduced. This might be due to the expected decrease in cellulose crystallinity during the alkaline mercerization process to produce Cellulose II (Ishikawa et al., 1997). In addition, AA9 showed no enhancement in hydrolysis yields on Cellulose III (Figure 27A). Thus, it seems that AA9 enzymes can only stimulate cellulase activity on the crystalline regions of cellulose I. Thus, the cellulose CrI of pretreated lignocellulosic biomass, assessed by XRD, might not reflect the amount of crystalline cellulose that actually interacts with the cellulase-AA9 mixture.

Although the determination of cellulose CrI and its relationship with cellulose hydrolyzability has been investigated at length, it is still unclear which CrI determination

method is more accurate and whether or not the CrI measured by currently available methods can predict the digestibility of cellulose by cellulase enzymes (Zhang and Lynd, 2004; Park et al., 2010). A recent study (Park et al., 2010) compared the most commonly used methods for determining cellulose CrI (e.g. XRD, NMR, infrared (IR) and Raman spectroscopy) and emphasised that even the relatively more accurate methods such as XRD may not be capable of extracting exact information of crystal lattices within the cellulose structure. Therefore, other cellulose properties (e.g. accessibility, DP, particle sizes, etc.) should also be considered for predicting cellulose hydrolyzability. In addition, considering that the cellulose CrI of pretreated biomass represents the relative amount of crystalline material in the substrates (Nishiyama et al., 2002), CrI may not be able to predict the trend of cellulose crystallinity of different pretreated substrates as they contain different amounts of non-cellulosic components.

A study on the binding capacity of AA9 on model/pure cellulosic substrates showed that, as expected, the higher amount of AA9 adsorption to CNC (~35%) as compared with Avicel and DSP (~25%) is likely due to the CNC's higher crystallinity (Figure 27B). No AA9 adsorption was observed on Cellulose III, which suggests that the structure of Cellulose III may not be recognized by AA9 enzymes. Therefore, even though the Cellulose III structure is commonly regarded as more accessible to enzymes than Cellulose I (Gao et al., 2013), this lack of binding could explain the observed lack of AA9 boosting on Cellulose III hydrolysis (Figure 27A). Unexpectedly, a high amount of AA9 was adsorbed onto the amorphous cellulose preparations Cellulose II and PASC, despite a lack of AA9 boosting effects during hydrolysis of these two substrates (Figure 27A). This apparent paradox might be caused by the increased accessibility of small microcrystalline substructures, present

within the relatively amorphous bulk of these substrates (Gourlay et al., 2012), which might adsorb AA9. However, since these substrates were already highly accessible, the addition of AA9 had no beneficial effect on the hydrolyzability of cellulose by cellulase enzymes.

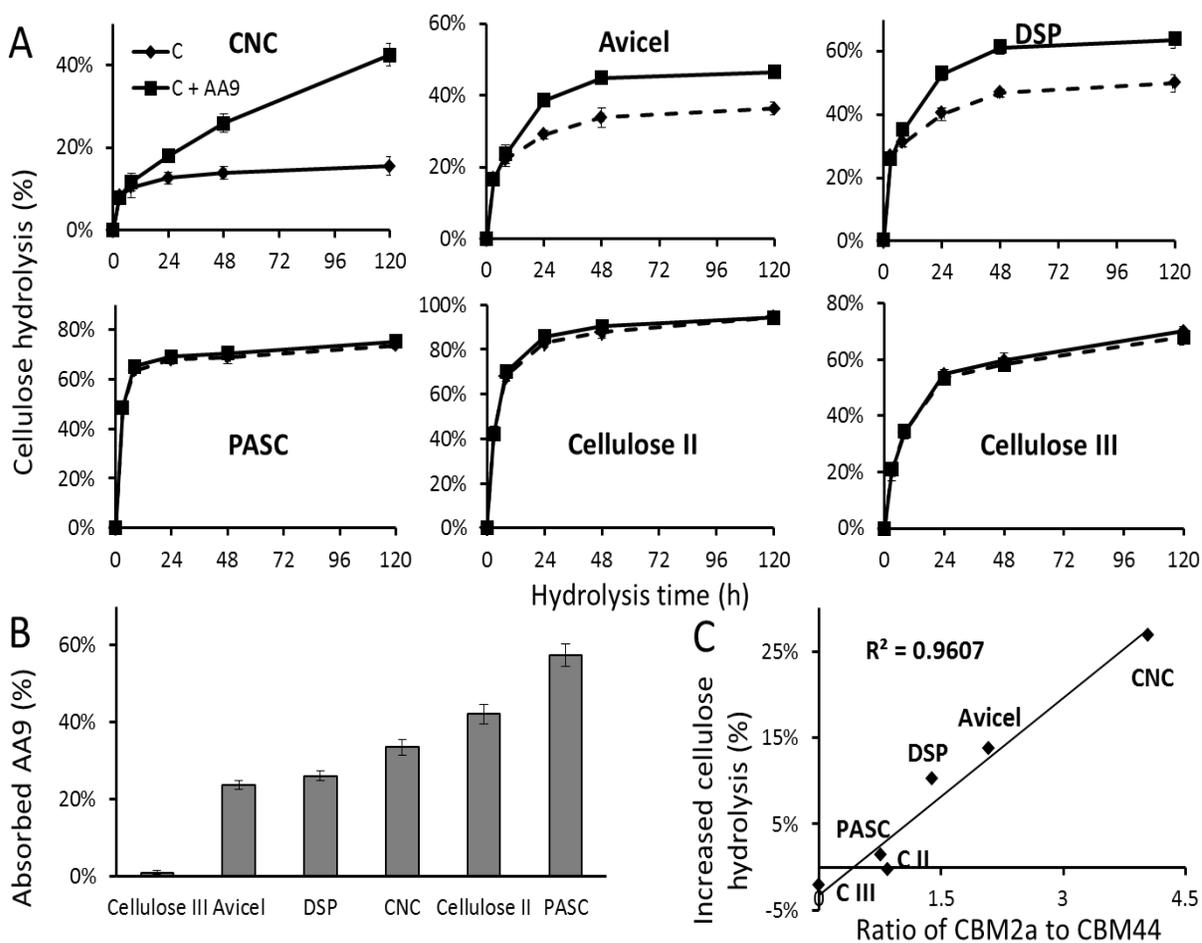


Figure 27. The effects of AA9 on cellulose hydrolysis of various “pure” cellulytic substrates. (A) The time course of hydrolysis of various cellulytic substrates with/without AA9. (B) The adsorption of AA9 on various cellulytic substrates. (C) The correlation between AA9 increased cellulose hydrolysis and the ratio of accessible crystalline cellulose (CBM2a) to accessible amorphous cellulose (CBM44). CNC: cellulose nanocrystals; DSP: dissolving pulp; PASC: phosphoric acid swollen cellulose; CBM: cellulose binding module.

In order to further elucidate the influence of the amount of accessible crystalline cellulose and amorphous cellulose on enzyme interaction, two cellulose binding modules (CBMs), one with a planar binding face that preferentially adsorbs to crystalline cellulose (CBM2a), and the other with a cleft-shaped binding site that preferentially adsorbs to amorphous cellulose (CBM44), were used to determine the accessible crystalline and amorphous cellulose within the cellulosic substrates (Figure 28). The adsorption of both CBMs exhibited a similar trend as the cellulose accessibility assessed by the Simons' Stain technique (Figure 28).

Interestingly, a significant amount of the crystalline cellulose-binding CBM2a and AA9 adsorbed onto amorphous cellulosic substrates such as PASC and Cellulose II (Figure 27B and Figure 28). As CBM2a and AA9 recognize only a few adjacent ordered cellulose chains as being "crystalline" (Bolam et al., 1998; Li et al., 2012), the observed high binding capacity of PASC and Cellulose II was likely due to the increased exposure of small microcrystalline substructures within the overall disorganized/amorphous cellulose (Gourlay et al., 2012). One possible explanation for the high adsorption, but weak hydrolytic boosting effect of AA9 on these amorphous substrates, could be that disruption of these microcrystalline regions by AA9 does not influence the hydrolytic performance of cellulase enzymes. This might be due to the large number of readily accessible, reactive sites that already exist within substrates such as PASC and Cellulose II. Therefore, even if reactive sites were created by AA9's oxidative cleavage, their impact on the hydrolytic performance of cellulases would be expected to be relatively low. The existence of microcrystalline substructures in the "amorphous" cellulose could also explain why, unlike the related bacterial CBM33 proteins (CBP21 and CelS2), that cleave only highly ordered/crystalline

substrates (Vaaje-Kolstad et al., 2010; Forsberg et al., 2011), AA9 has been reported to cleave amorphous cellulose (PASC), releasing various native and oxidized cello-oligosaccharide products (Quinlan et al., 2011; Westereng et al., 2011; Bey et al., 2013).

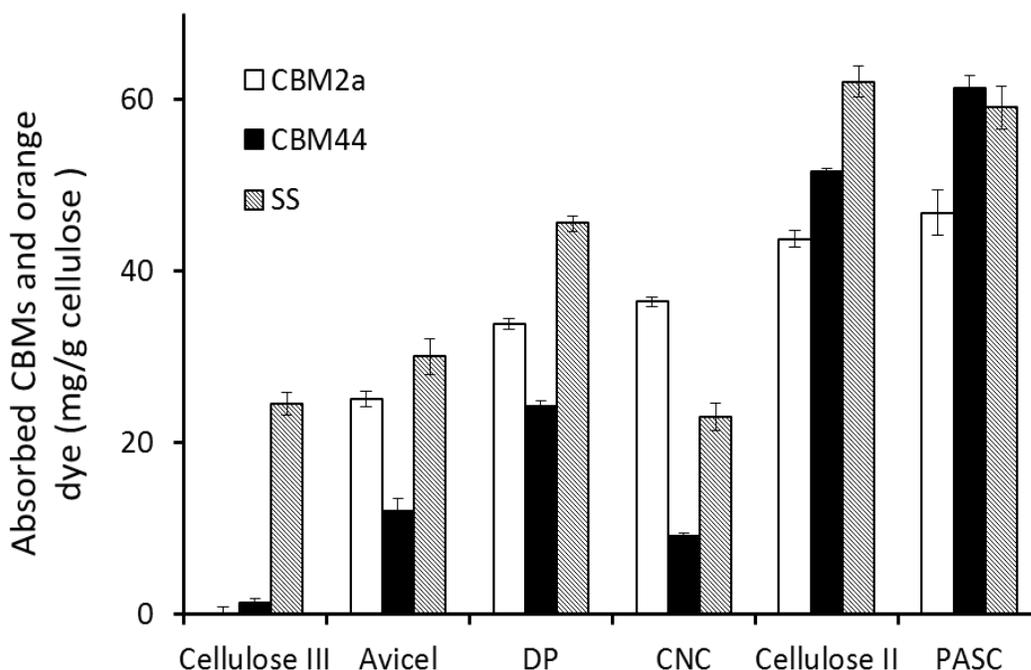


Figure 28. The adsorption of CBM2a (to crystalline cellulose), CBM44 (to amorphous cellulose) and orange dye on various “pure” cellulolytic substrates. CNC: cellulose nanocrystals; DSP: dissolving pulp; PASC: phosphoric acid swollen cellulose; SS: Simons’ Stain

The ratio between CBM2a and CBM44 provided a good indicator of the relative available crystalline cellulose to amorphous cellulose within the substrates. A linear correlation ($R^2 > 0.94$) was observed between the AA9 “boosting effects” on cellulose hydrolysis and the ratio of accessible crystalline to accessible amorphous cellulose (Figure 27C). We further applied this technique to the pretreated lignocellulosic substrates and a good relationship was also observed between CBM2a:CBM44 adsorption and the extent of

AA9 boosting (Figure 29). Thus, it could be concluded that the relative amount of accessible crystalline cellulose is the determining factor when maximising the synergistic cooperation between AA9 and the cellulase enzymes.

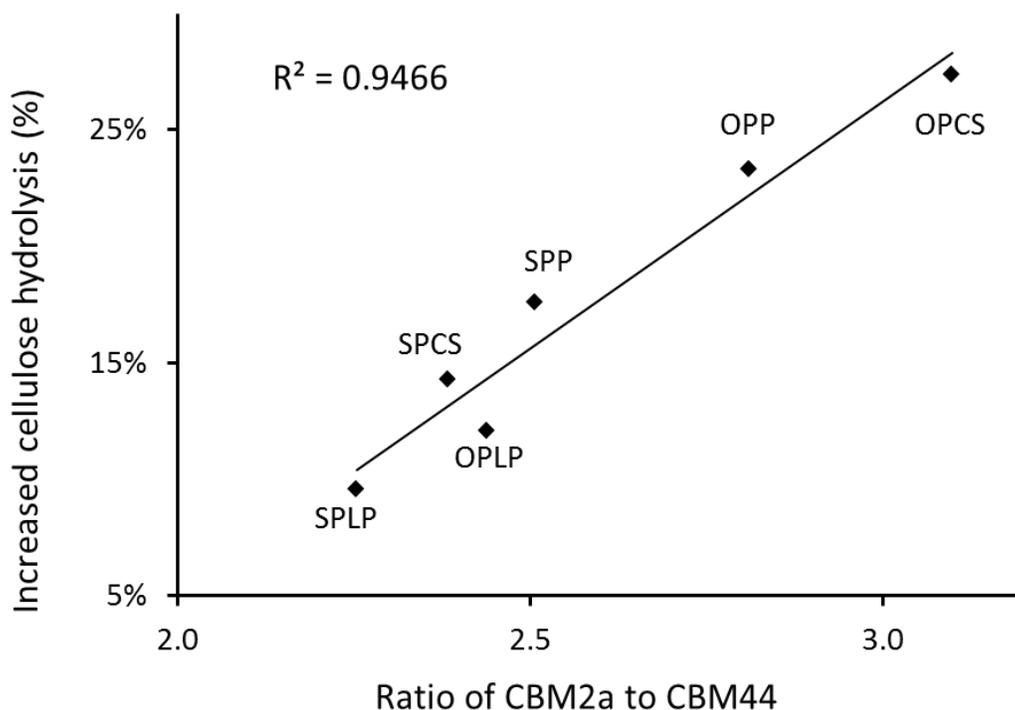


Figure 29. The correlation between AA9 increased cellulose hydrolysis and the ratio of accessible crystalline cellulose (CBM2a) to accessible amorphous cellulose (CBM44). Steam and organosolv pretreated corn stover, poplar and lodgepole pine: SP/OP- CS, P, and LP.

Contrary to the CrI techniques, the CBM assay only quantifies changes at the surface of the cellulose, rather than the bulk cellulose. The correlation between increases in the ratio of adsorbed CBM2a:CBM44 on the various celluloses with the increased boosting effect of AA9 therefore suggests that AA9 synergizes with cellulases by creating more reactive sites for cellulases on the originally recalcitrant crystalline regions. While this hypothesis can easily be used to explain the initial stages of hydrolysis (the CBMs were applied to

unhydrolyzed substrates), it does not explain how this initial CBM adsorption profile on the unhydrolyzed substrates is able to predict the boosting efficiency of AA9 over longer periods of hydrolysis. One possible explanation is that enzymatic hydrolysis may proceed in an “onion peeling” fashion, where layers at the cellulose surface (containing both amorphous and crystalline regions) are hydrolyzed together. This would suggest that, as hydrolysis proceeds, the removal of the outermost layers of the cellulose uncovers buried layers that have a similar surface morphology (relative amounts of crystalline and amorphous cellulose) as the previous layer. This model has previously been suggested (Chanzy and Henrissat, 1985; Resch et al., 2013), and the results presented here appear to support this hypothesis.

3.3.2.4 The influence of AA9 on enzyme adsorption profile

The adsorption/desorption profile of cellulase enzymes, with and without AA9, was also assessed during the time course of hydrolysis. Even though AA9 did not influence the adsorption profile of cellulases on highly crystalline and amorphous cellulosic substrates (CNC, cellulose II, cellulose III, and PASC), about 10% of the initially added total protein was released during hydrolysis of semi-crystalline cellulose substrates such as DSP and Avicel (Figure 30). Similarly, about 10% more enzymes in solution were observed during hydrolysis of pretreated lignocellulosic materials (SPCS and OPCS) (Figure 31).

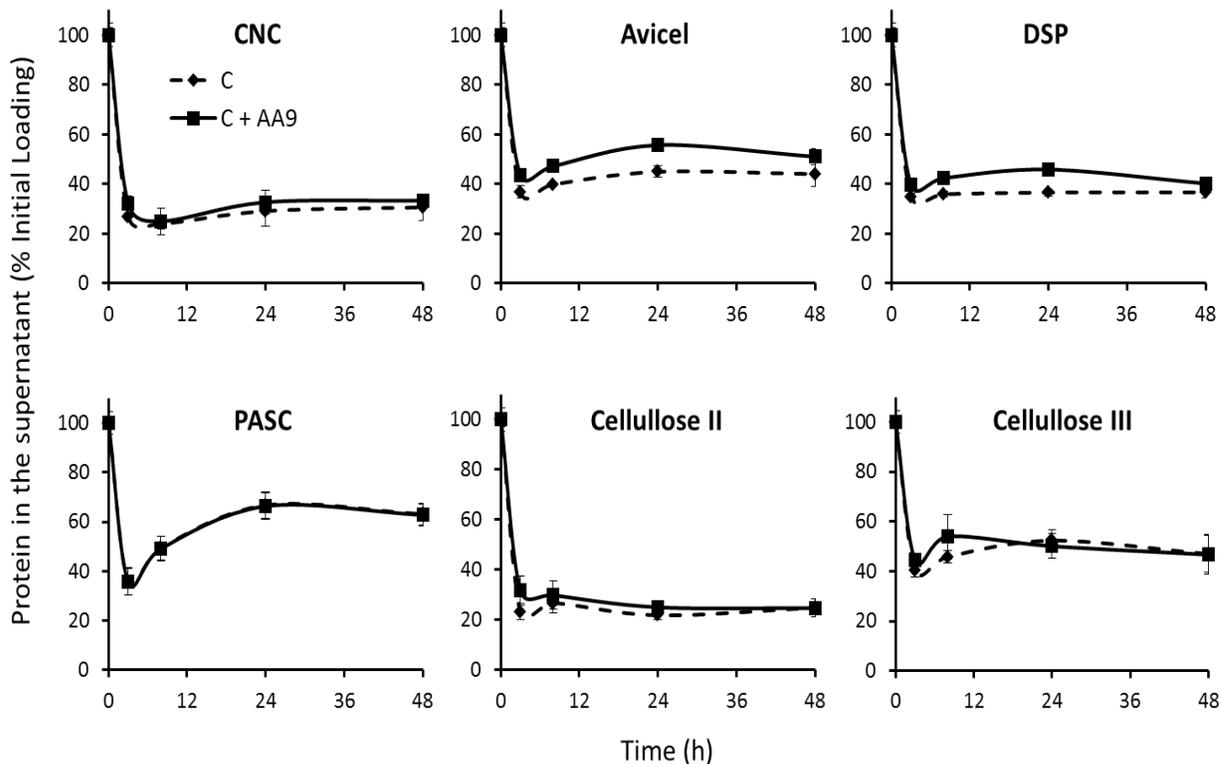


Figure 30. The adsorption/desorption profile of a "cellulase mixture" with and without AA9 during time course of hydrolysis of various "pure" cellulosic substrates. CNC: cellulose nanocrystals; DSP: dissolving pulp; PASC: phosphoric acid swollen cellulose.

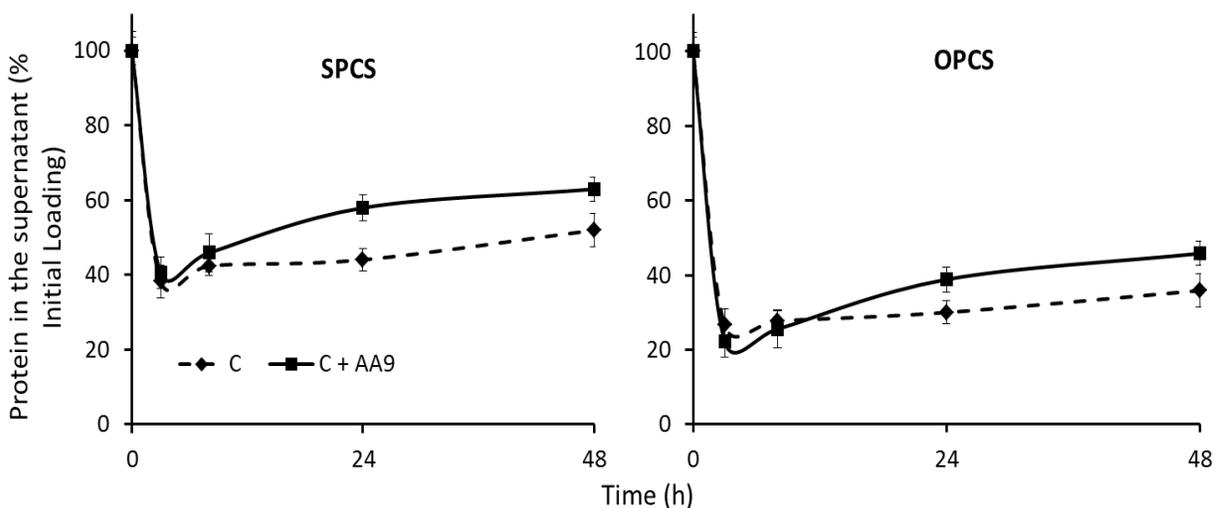


Figure 31. The adsorption/desorption profile of "cellulase mixture" with and without AA9 during time course of hydrolysis of pretreated lignocellulosic substrates (SPCS & OPCS). SPCS: steam pretreated corn stover; OPCS: organosolv pretreated corn stover. C: Celluclast 1.5L.

As the release of cellulase enzymes by the AA9-cellulase interaction was only observed on cellulosic substrates with semi-crystalline structure (e.g. DSP, Avicel and pretreated biomass) (Figure 30 and Figure 31), it was possible that the AA9 influenced the adsorption/desorption profile of the processive cellulase (Cel7A), which is commonly believed to get stuck/jammed by the disorganized cellulose surface during processive hydrolysis. We tried to test this hypothesis by using an immunoassay (ELISA) to specifically quantify the amount of Cel7A in the supernatant during Avicel hydrolysis in the presence and absence of AA9. An increase (~15%) in the concentration of Cel7A was observed in the liquid phase during hydrolysis with AA9 (Figure 32B). As a control, AA9 showed no influence on the adsorption profile of the major endoglucanase, Cel7B (Figure 32C).

One way in which AA9 may be changing the adsorption/desorption profile of Cel7A is by altering the surface charge of cellulose through its oxidative cleavage of glycosidic bonds. Compared to hydrolysis without AA9, the solid residues after Avicel hydrolysis with AA9, regardless of the enzyme loading, showed an increase in acid groups as quantified by conductometric titration (Figure 32A), which indicated an increase in negative charges on the cellulose. As an increase in enzyme desorption was also observed during hydrolysis of pretreated biomass with AA9, compared to hydrolysis without AA9 (Figure 31). The acid groups on the solid residues of one of the pretreated substrates (OPCS) after hydrolysis, with and without AA9, were quantified to determine if AA9 could also alter the surface charge of a lignocellulosic substrate. As was observed on Avicel, AA9 also caused an increase in acid groups on OPCS (Figure 33).

It appears that this increase in the amount of acid groups led to a decrease in the amount of the processive cellulase, Cel7A, adsorbed to the cellulose during hydrolysis of

semi-crystalline cellulosic substrates such as Avicel, DSP, and pretreated lignocellulosic substrates. Thus, this proposed “traffic jam” effect of processive Cel7A, where Cel7A gets stuck/jammed on the disorganized/eroded cellulose surface, and/or by other unproductively bound cellulases during hydrolysis (Igarashi et al., 2009; 2011; Cruys-Bagger et al., 2012) might increase the amount of enzymes unproductively bound onto the substrate. The release of unproductively bound/jammed Cel7A by other cellulase enzymes has been shown to significantly increase the hydrolytic efficiency of a “cellulase mixture” (Igarashi et al., 2011; Ganner et al., 2012). In a similar fashion, the data presented here suggests that an additional mechanism by which AA9 enzymes may stimulate cellulase hydrolytic activity may be by increasing the off-rate of processive Cel7A on the disorganized cellulose surface. In a similar manner, chemical post-treatment (sulphonation) of pretreated biomass prior to enzymatic hydrolysis has also been shown to increase the overall negative charge on the surface of pretreated cellulosic substrates, which led to a significant increase in the amount of unbound enzymes during hydrolysis and also significantly improved cellulose hydrolysis (Kumar et al., 2011).

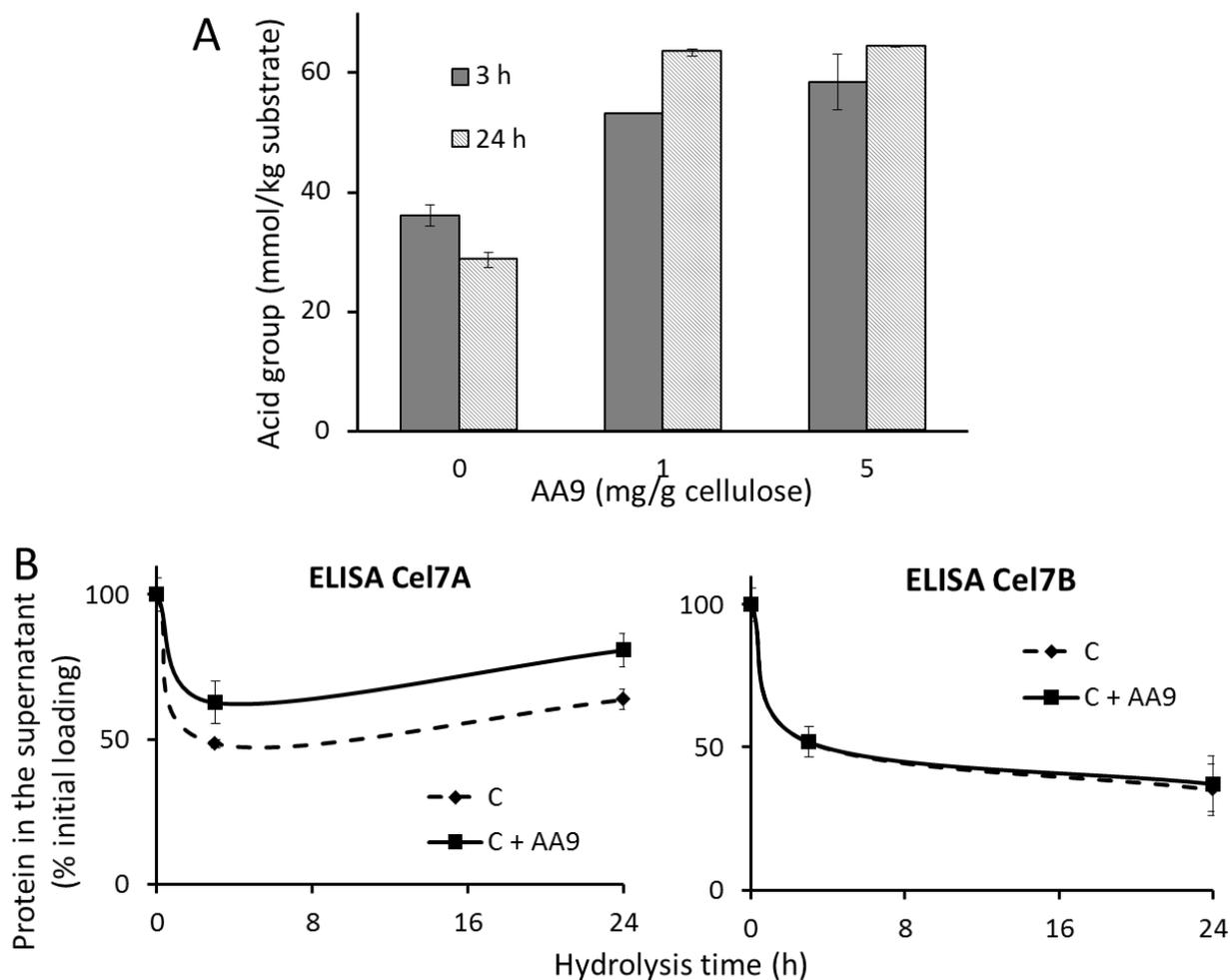


Figure 32. The influence of AA9 on processive exoglucanase Cel7A and endoglucanase Cel7B adsorption/desorption profile and on the acid group content of the residue after hydrolysis of Avicel. (A) The acid group content of the Avicel hydrolysis residue with different AA9 loadings after hydrolysis for 3 and 24 hours. (B) The adsorption profile of TrCel7A and TrCel7B in the cellulase mixture with and without AA9.

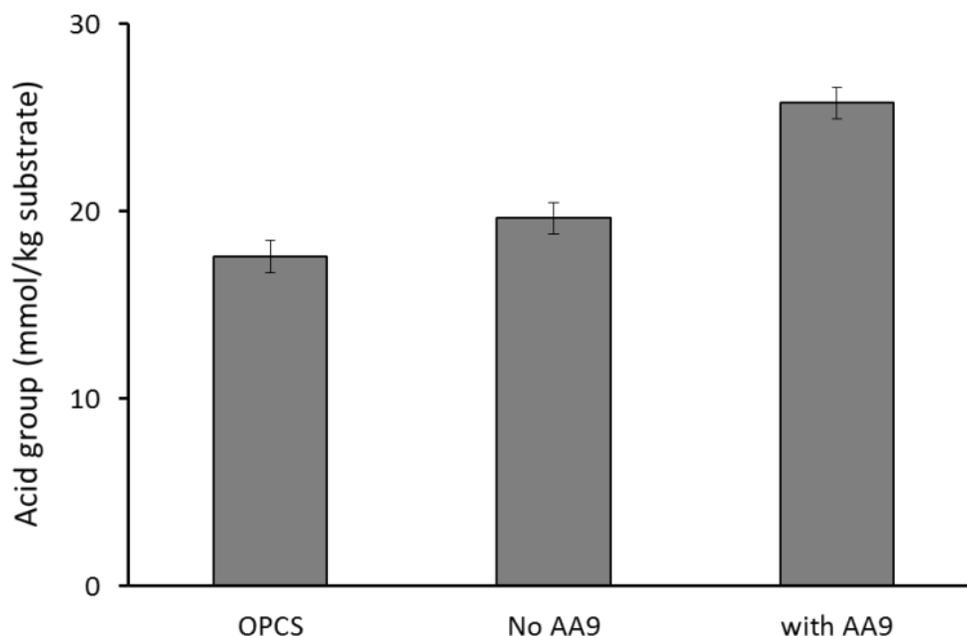


Figure 33. The acid group content of OPCS residue with and without AA9 after 24 hours hydrolysis. OPCS: organosolv pretreated corn stover.

3.3.2.5 The influence of AA9 dosage on cellulose hydrolysis

The required AA9 loading in the enzyme mixture was further assessed during Avicel hydrolysis (Figure 34). When cellulases were partially replaced by AA9 by up to 40% of protein loading (5 mg) it appeared that lower amounts of AA9 (1 mg) improved cellulose conversion during hydrolysis. Increasing the amount of AA9 up to 5 mg in the enzyme mixture was still beneficial for cellulose hydrolysis, but the final conversion was slightly decreased (Figure 34A). The decreased cellulose hydrolysis by the enzyme mixture could be as a result of the decreased amount of cellulase enzymes in the hydrolysis system. Thus, it seems that the hydrolytic potential of a cellulase mixture could be significantly boosted by the addition of low amounts of AA9.

The adsorption/desorption profile of the enzyme mixture with different amounts of AA9 indicated that there was, as shown earlier, about 10% more enzyme in the supernatant in the presence of AA9 enzyme (Figure 34B). However, the increased amount of AA9 had no influence on the amount of protein in the hydrolysate.

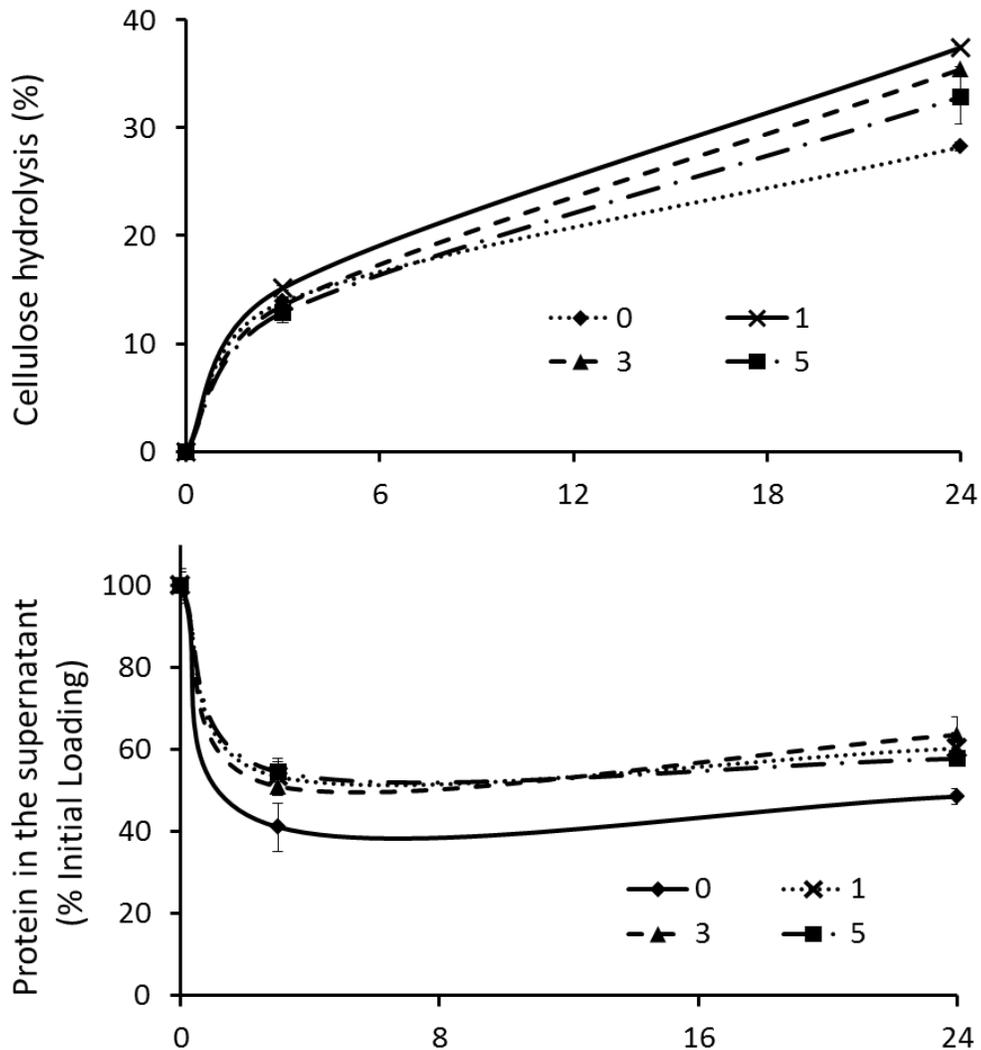


Figure 34. (A) The cellulose hydrolysis and (B) the relative amount of protein in the supernatant during time course of hydrolysis of Avicel by cellulases with/without AA9 (0 -5 mg/g cellulose).

3.3.3 Conclusions

Until recently, it was thought that the depolymerisation/cleavage of cellulose was mediated entirely through enzymes utilizing a hydrolytic catalytic mechanism. In the natural carbon cycle, oxidative cleavage significantly contributes to an efficient biodegradation process of structural polysaccharides such as cellulose and chitin. Over the past few years, it has become apparent that a previously unstudied family of enzymes (lytic polysaccharide monooxygenases: LPMOs), utilizing an oxidative rather than a hydrolytic mechanism, are also involved in structural polysaccharides degradation. We have shown that one of these LPMOs, AA9, synergistically cooperates with cellulose hydrolytic enzymes for a range of cellulosic and lignocellulosic substrates' decomposition. We showed how these enzymes take advantage of particular biomass components to potentiate their action. Additionally, we showed how these LPMOs act synergistically with the canonical hydrolases to facilitate the degradation of recalcitrant regions of cellulose that the hydrolases alone are incapable of efficiently degrading. Through a better understanding of the underlying mechanism behind the synergistic interaction between cellulose oxidative and hydrolytic cleavages, this work provided new insights into the mechanism of lignocellulosic deconstruction. This work should help with the development of more robust and efficient enzyme cocktails used for bioconversion.

3.4 Synergistic cooperation between canonical cellulase components during lignocellulose decomposition

3.4.1 Background

In previous sections, we assessed the synergistic interaction between canonical cellulases and the major accessory enzymes, such as xylanases and AA9, during deconstruction of a library of pretreated lignocellulosic substrates and “model” cellulosic substrates. It appeared that these accessory enzymes could significantly improve the hydrolytic performance of cellulase enzymes, but the extent of improvement was highly substrate dependent. The major contribution of xylanases was to increase the accessibility of cellulase enzymes to cellulose embedded into the hemicellulose-lignin matrix while AA9 addition could further increase the accessible/reactive sites of cellulose to cellulases by oxidative cleavage of the highly organized crystalline cellulose regions.

As described in Chapter 1 - 1.6, the hydrolysis of cellulose requires a suite of cellulase components that act cooperatively and synergistically at the ends (exo-glucanases) or randomly in the middle (endo-glucanases) of the cellulose chains to release soluble cellodextrins, mostly in the form of cellobiose. Cellodextrin is then further hydrolyzed to glucose by another hydrolase, β -glucosidase. Although the synergistic interaction among these canonical cellulases has been studied for a long time, most of the studies have used “model” cellulosic substrates with relatively low enzyme dosage and only looked at the initial stage of the hydrolysis (Chanzy and Henrissat, 1985; Converse et al., 1988; Irwin et al., 1993; Jeoh et al., 2002; 2006).

It has been shown that cellulase synergism is highly dependent on enzyme concentration and substrate characteristics (Henrissat et al., 1985; Woodward et al., 1988a; 1991). For example, although a strong synergism was displayed among cellulase components at a low enzyme dosage, the synergistic effect at a high enzyme loading was significantly reduced (Woodward et al., 1988a; 1988b). Usually, a higher level of cellulase synergism can be found during hydrolysis of cotton fibre, Avicel, and bacterial microcrystalline cellulose, while lower/no synergy was found when using acid/endoglucanase-treated bacterial cellulose and amorphous cellulose such as phosphoric acid swollen cellulose (PASC) to assess cellulase interaction (Samejima et al., 1997; Valjamae et al., 1998; Jeoh et al., 2006; Chen et al., 2007). However, opposite results have also been reported (Andersen et al., 2008). In addition, it is still difficult to explain why individual cellulase components can hydrolyze amorphous cellulose (e.g. PASC), while cooperation between cellulase components is required for the deconstruction of crystalline cellulose (e.g. cotton) (KlemanLeyer et al., 1996; Srisodsuk et al., 1998).

There are a number of studies that have assessed the synergistic cooperation between cellulase enzymes during hydrolysis of more realistic, pretreated lignocellulosic substrates, and most have focused on identifying the core enzymes and optimizing their ratios for achieving fast and efficient cellulose hydrolysis as described earlier. However, only a few of these studies assessed the mechanism of enzyme-enzyme and enzyme-substrate interactions during hydrolysis. For those synergism mechanism studies, Nutor and Converse (1991) showed that the cellulase hydrolysis rate and their degree of synergism was highly dependent on the substrate concentration during hydrolysis of pretreated poplar. Eriksson et al. (2002b) indicated that the synergism between CBH I and EG was mainly caused by EG facilitating

the processive hydrolytic movement of CBH I during hydrolysis of steam pretreated willow and spruce, and the ratio between CBH I and EG was not critical for their synergistic interaction. Jalak et al. (2012) claimed that the CBH-EG synergism on pretreated wheat straw at low enzyme loading was mainly through the recruitment of stalled processive CBH I during hydrolysis.

Depending on the choice of pretreatment strategies and the type of lignocellulosic material, several physicochemical characteristics of pretreated substrates (e.g. the residual non-cellulosic materials and their association with the cellulose, cellulose degree of polymerization (DP) and crystallinity, surface area of cellulose accessible to enzymes, etc.) can vary significantly (Chandra et al., 2007; Yang and Wyman, 2008). It is commonly acknowledged that one of the main factors that impede the efficacy of cellulases to hydrolyse lignocellulosic biomass, is the incomplete understanding of their cooperation when acting on pretreated lignocellulosic substrates. Thus, understanding cellulases' synergistic interaction on various pretreated lignocellulosic substrates will not only expand our fundamental knowledge of the mechanism of biomass degradation, but might also help us design more efficient biomass degrading systems.

In the work reported in this part of the thesis we assessed the synergistic cooperation among the major *T. reesei* cellulase monocomponents (Cel7A, Cel6A, Cel7B, and Cel5A) and *A. niger* β -glucosidase during hydrolysis of a library of realistic pretreated lignocellulosic substrates. We evaluated the enzyme concentration as well as the influence of the major physicochemical characteristics of the substrates on the extent of synergism among these cellulases. The results indicated that the reconstituted cellulase mixture of major cellulase components achieved a similar hydrolytic conversion as the commercial cellulase

preparation (Celluclast) when acting on substrates without any xylan content. It appeared that cellulase synergism intensified at unsaturated enzyme concentration. Under the reasonable/low enzyme loading required for reasonable cellulose hydrolysis of pretreated lignocellulose, synergism was observed among cellulase monocomponents (degree of synergism: 1.5-2.0). But the highest degree of synergism existed between cellulases and β -glucosidase (degree of synergism: 3-10). The cellulose hydrolysis was mainly achieved by Cel7A, the major cellulase component in the enzyme mixture, while other cellulase components seemed to synergistically enhance the hydrolytic efficiency of Cel7A during hydrolysis. Based on the major substrate physicochemical characteristics studied, the higher ratio of available amorphous to crystalline cellulose, which infers more disorganized/accessible cellulose morphology, led to the strongest synergistic cooperation among cellulase components. Finally, the synergism model based on the increased off-rate of processive Cel7A by other cellulase components was used to explain the observed substrate characteristics and enzyme loading-dependent cellulase synergy.

3.4.2 Results and discussion

3.4.2.1 The hydrolytic potential of reconstituted major cellulase components and their synergism on various pretreated lignocellulosic biomass.

To gain a better understanding of the synergistic cooperation among cellulase enzymes on pretreated lignocellulosic biomass, major cellulase monocomponents and β -glucosidase were purified from Celluclast 1.5L and Novozyme 188, respectively, and their synergistic interactions were assessed on six different substrates: steam and organosolv pretreated agricultural residue corn stover (SPCS/OPCS), a hardwood substrate poplar

(SPP/OPP), and a softwood substrate lodgepole pine (SPLP/OPLP). The major cellulase monocomponents obtained from Celluclast 1.5L, on a protein weight basis, were Cel7A, Cel6A, Cel7B, and Cel5A, which comprised about 56%, 12%, 5%, and 6% of the total protein in Celluclast 1.5L, respectively.

The hydrolytic potential of the reconstituted major cellulase components (MIX), based on their original ratio in Celluclast 1.5L, was firstly assessed on the various pretreated biomass substrates. The remaining ~20% of protein in the MIX was made up with BSA to maintain the same amount of protein in the hydrolysis system. Although the MIX exhibited a similar hydrolytic performance as compared with Celluclast 1.5L on SPLP, the MIX achieved lower cellulose conversion on other substrates (Figure 35). The chemical composition of these pretreated substrates showed that the major difference between SPLP and other substrates was the amount of xylan content, with SPLP containing negligible xylan content while the others had between 1.5-15.4% of xylan (Table 12). Thus, we hypothesized that the observed lower hydrolytic performance of the MIX might be due to the amount of xylanase activity present in the Celluclast 1.5L. When a glycoside hydrolase family 10 endo-xylanase (GH10 EX) was used to replace the BSA in the reconstituted enzyme mixture (MIX), the hydrolytic potential of the MIX was improved and reached the same level as Celluclast on all the substrates (Figure 35). Thus, it appeared that the major cellulase components plus xylanase could achieve similar hydrolysis ability as the commercial cellulase preparation Celluclast 1.5L on a range of pretreated biomass substrates.

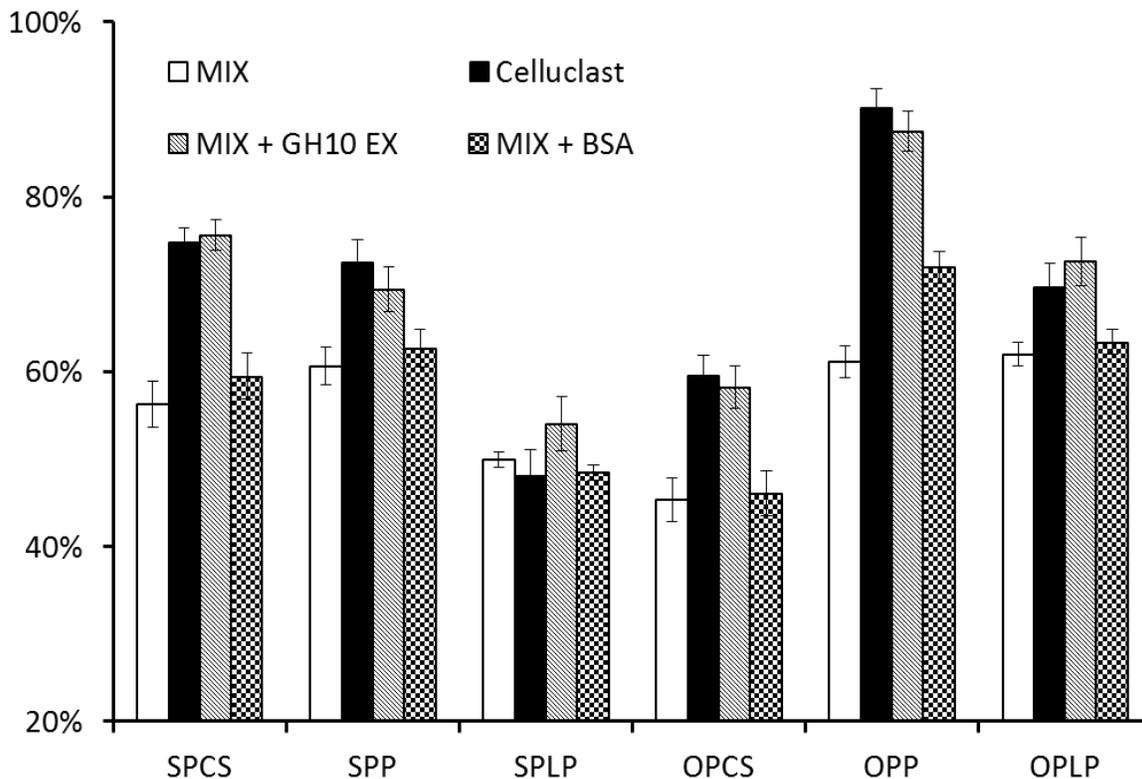


Figure 35. Cellulose hydrolysis of various pretreated lignocellulosic substrates after 72 h. MIX: reconstituted cellulases mixture by Cel7A, Cel6A, Cel7B, and Cel5A; GH10 EX: glycoside hydrolase family 10 endoxylanase; BSA: bovine serum albumin.

3.4.2.2 The dependence of cellulase synergism on enzyme concentration.

As cellulase synergism is highly dependent on enzyme concentration (Henrissat et al., 1985; Woodward et al., 1988a; 1991), the influence of cellulase loading on their extent of synergism was first assessed in a simplified hydrolysis system, where a “model” cellulosic substrate dissolving pulp (DSP) was hydrolyzed by the binary mixture between Cel7A and other cellulase components at unsaturated, saturated, and over-saturated Cel7A concentration. The maximum adsorption (saturation point) of Cel7A on DSP was assessed using a Scatchard plot of the equilibrium binding data, namely a plot of the amount of bound protein per gram of cellulose against the ratio of bound to free protein at equilibrium. The theoretical

maximum adsorption of protein (at saturation point) was obtained based on the horizontal axis intercept, which was about 40 mg Cel7A per gram of cellulose in DSP (Figure 36). The nonlinear relationship after reaching a certain enzyme loading was also observed by Woodward et al. (1988a) when assessing the cellulase adsorption on Avicel, which they concluded was due to the heterogeneous morphology of the cellulose.

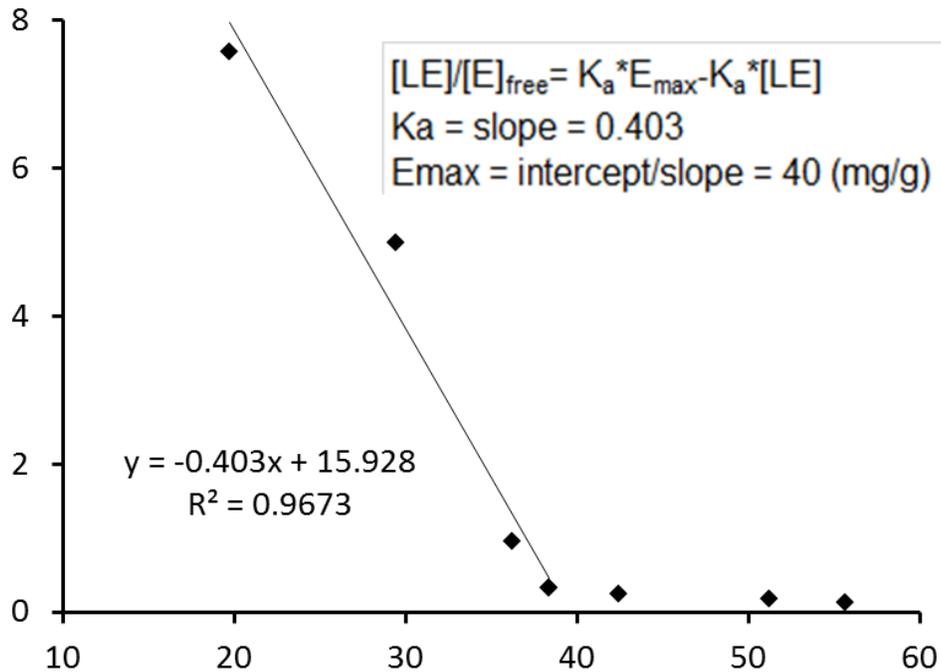


Figure 36. The maximum adsorption of Cel7A on DSP assessed by Scatchard plot of the equilibrium binding data. [LE]: concentration of bound enzyme per gram cellulose, [E]_{free}: concentration of free enzyme, E_{max}: enzyme maximum adsorption

When the interaction between Cel7A and Cel5A was assessed during hydrolysis of DSP at different Cel7A saturation status, synergistic cooperation was only observed under unsaturated Cel7A concentration (Figure 37). At unsaturated Cel7A loading, the cellulose hydrolysis plateaued after 24 h (Figure 37A), but the hydrolysis continued when using saturated and over-saturated Cel7A concentration, even though the hydrolysis rate decreased

dramatically (Figure 37B/C). When Cel5A appeared simultaneously with Cel7A in the hydrolysis system, the synergistic interaction between Cel7A and Cel5A was only observed at unsaturated Cel7A concentrations, where both the rate and the extent of cellulose hydrolysis were significantly improved (Figure 37B). The synergistic interactions between the binary mixtures of Cel7A-Cel6A and Cel7A-Cel7B displayed a similar pattern, where the extent of synergism intensified at unsaturated Cel7A concentration (Figure 38 and Figure 39). The control Cel5A, Cel6A and Cel7B alone only hydrolyzed very low amounts of cellulose (data not shown). Woodward et al. (1988a) also observed that the extent of cellulase synergism intensified at unsaturated enzyme loadings on a model cellulosic substrate (Avicel).

Among these cellulase components, Cel7B showed the greatest enhancement of cellulose hydrolysis by Cel7A at unsaturated Cel7A concentrations (Figure 39A), and a slight improvement was observed even under saturated Cel7A and over-saturated Cel7A loading. This might be caused by the lower amount of xylanase activity of the Cel7A enzyme, as a certain amount of xylan (about 4%) was present in the DSP substrate.

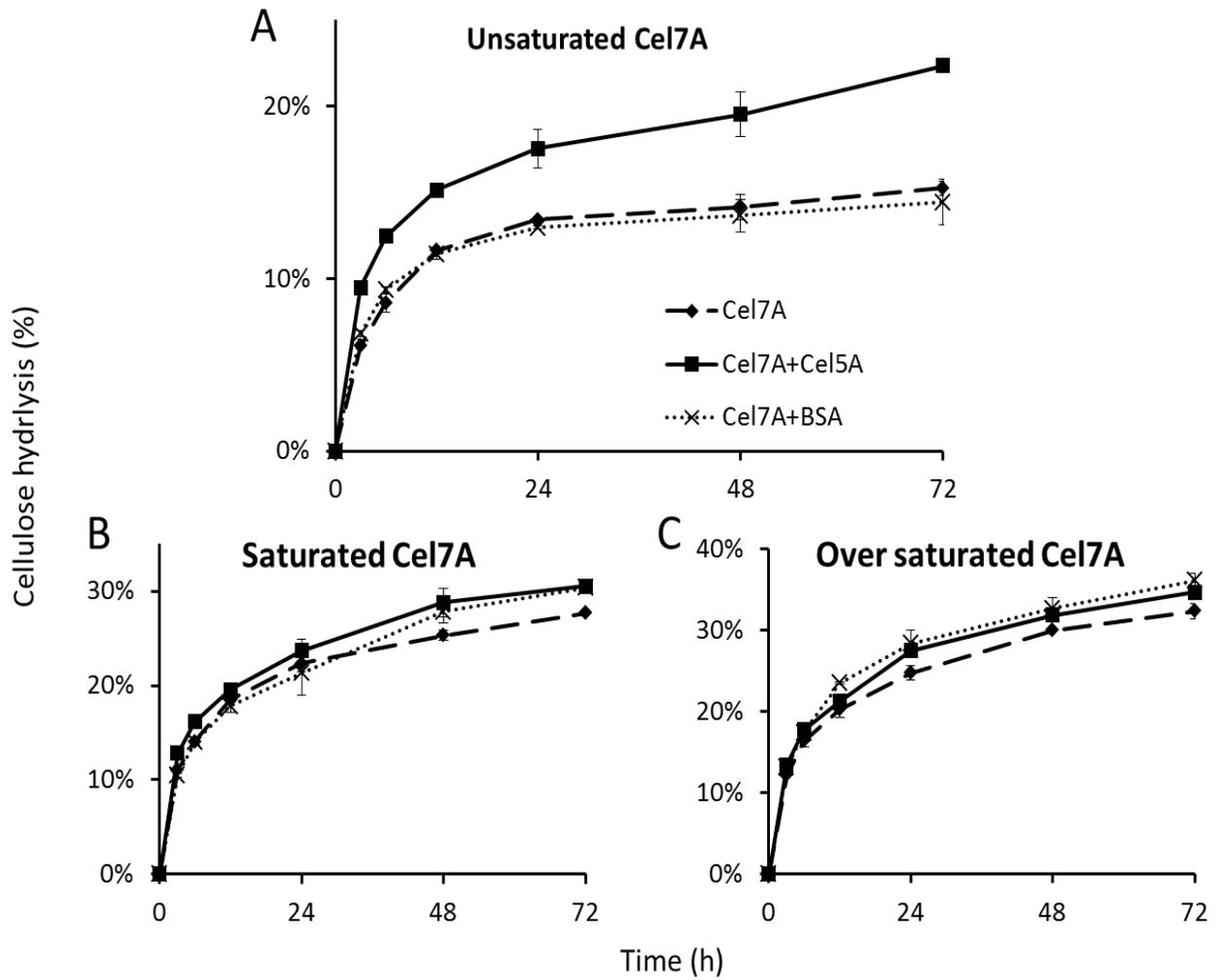


Figure 37. Cellulose hydrolysis of DSP by unsaturated (10mg/g cellulose), saturated (40 mg/g), and over saturated (70 mg/g) Cel7A with/without Cel5A.

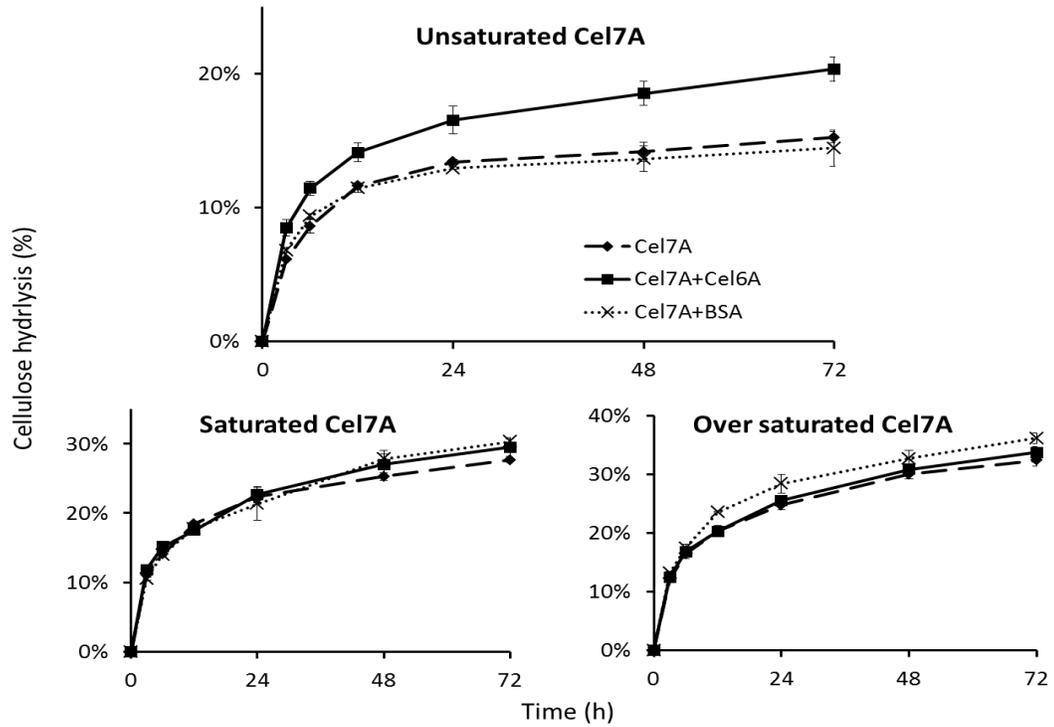


Figure 38. Cellulose hydrolysis of DSP by unsaturated (10mg/g cellulose), saturated (40 mg/g), and over saturated (70 mg/g) Cel7A with/without Cel6A.

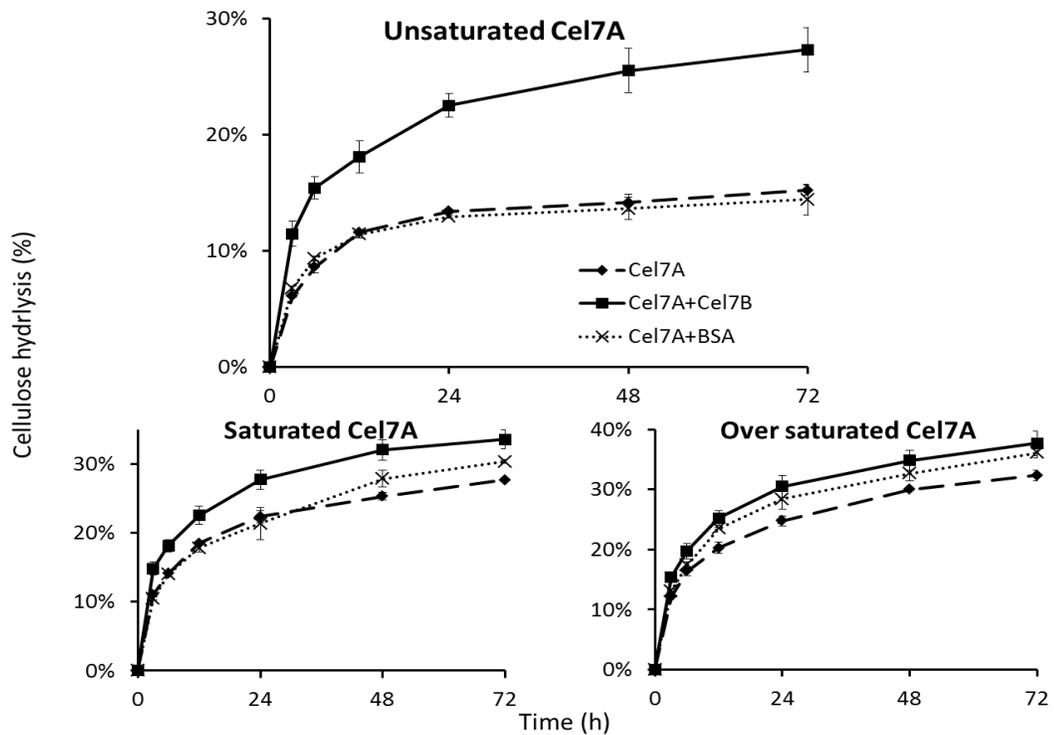


Figure 39. Cellulose hydrolysis of DSP by unsaturated (10mg/g cellulose), saturated (40 mg/g), and over saturated (70 mg/g) Cel7A with/without Cel7A.

3.4.2.3 The influence of substrate characteristics on cellulase synergy

As cellulase synergism was highly dependent on enzyme concentration and only occurred at unsaturated/low enzyme loading, their interactions during hydrolysis of pretreated lignocellulosic biomass were assessed at a more reasonable/low enzyme concentration required to achieve a realistic cellulose hydrolysis (around 50-60%). The hydrolysis was performed by individual cellulase components and their different combinations. And the ratio of individual cellulase components was kept consistent as they were in Celluclast 1.5L, because the purpose of this study was to gain a better understanding of their realistic interactions during hydrolysis, not to optimize their synergistic interaction.

Interestingly, it appears that Cel7A was the only cellulase component that could hydrolyze a certain amount of cellulose (20-32%) within the pretreated biomass substrates, while other enzymes displayed negligible cellulose conversion (<3%) (Table 14). Although the combined action of cellulase components did achieve greater cellulose hydrolysis than the sum of the action of individual cellulases (degree of synergism (DS): 1.5-2.0), the synergism between cellulases and β -glucosidase (DS: 3-10) was much higher (Table 15). The interaction of Cel7A-Cel6A (exo-exo), Cel7A-Cel7B (exo-endo), and Cel7B-Cel5A (endo-endo) were also assessed as their binary mixtures. Some synergism was observed in the exo-exo and the exo-endo combinations (DS: 1.1-1.7), but anti-synergism was observed between endo-type enzymes (Table 15). The synergistic cooperation between cellulases components indicated that the enzyme loading used here for lignocellulose deconstruction might be under unsaturated enzyme concentration, as a certain amount of enzymes was assumed to unproductively bind to lignin during hydrolysis.

Table 14. Cellulose hydrolysis by purified individual cellulase monocomponents and their different mixtures after 72 h.

	Cel7A	Cel6A	Cel7B	Cel5A	MIX	MIX (without BG)	Cel7A+C el6A	Cel7A+ Cel7B	Cel7B+ Cel5A
SPCS	23.9%	1.7%	2.6%	1.3%	56.3%	13.0%	37.6%	43.1%	3.2%
SPP	29.5%	2.6%	2.1%	1.2%	60.6%	6.1%	47.6%	43.8%	2.3%
SPLP	20.8%	3.1%	0.8%	0.3%	49.9%	6.4%	39.8%	24.3%	0.9%
OPCS	24.5%	2.4%	2.5%	0.9%	45.3%	15.1%	30.2%	36.8%	2.6%
OPP	32.0%	2.3%	1.8%	0.3%	61.1%	10.0%	38.2%	37.6%	2.2%
OPLP	29.5%	2.7%	1.4%	0.6%	62.0%	5.8%	37.8%	33.2%	1.2%

All hydrolysis experiments were supplemented with a sufficient amount of β -glucosidases (BG) (3mg/g cellulose), except the one labeled as MIX (without BG). MIX: the reconstituted cellulase mixture by Cel7A, Cel6A, Cel7B, and Cel5A

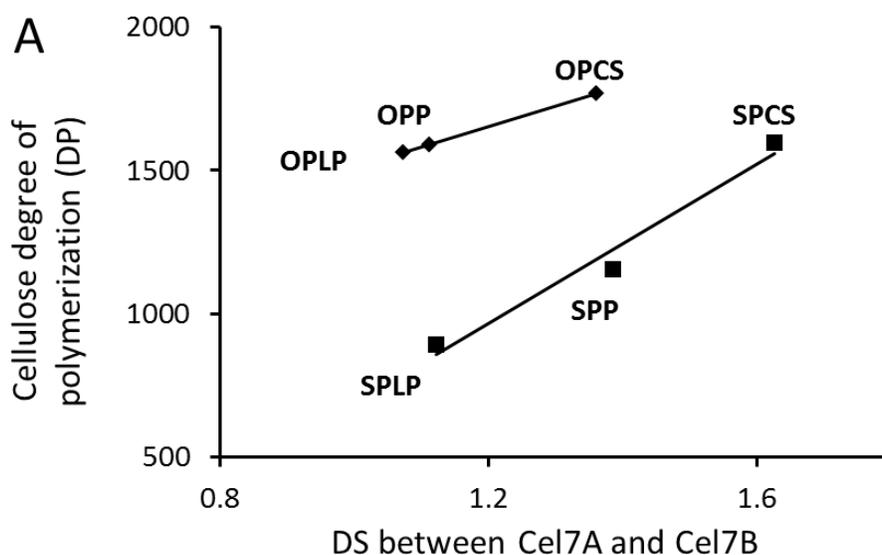
Table 15. Degree of synergism (DS) between cellulase monocomponents.

	DS MIX	DS MIX & BG	DS Cel7A/Cel6A	DS Cel7A/Cel7B	DS Cel7B/Cel5A
SPCS	1.9	4.3	1.5	1.6	0.8
SPP	1.7	10.0	1.5	1.4	0.7
SPLP	2.0	7.8	1.7	1.1	0.8
OPCS	1.5	3.0	1.1	1.4	0.8
OPP	1.7	6.1	1.1	1.1	1.0
OPLP	1.8	10.7	1.2	1.1	0.6

MIX: the reconstituted cellulase mixture by Cel7A, Cel6A, Cel7B, and Cel5A

3.4.2.4 The influence of substrate characteristics on cellulase synergy

Many of the substrate characteristics, that have been commonly believed to influence cellulose hydrolysis, were assessed (Figure 26A) and compared with the degree of synergism (DS) among the major cellulase components. When the gross fibre properties (fibre length, width, and the percentage of fines), cellulose accessibility, cellulose crystallinity (CrI), and cellulose degree of polymerization (DP) of the pretreated substrates were plotted against the degree of synergism (DS) among the mixture of cellulase components (MIX) and between the cellulase binary mixture (Table 15), a good relationship was only observed between the DP of the cellulose within the substrates pretreated in the same manner and the DS found with the binary mixture of exoglucanase (Cel7A) and endoglucanase (Cel7B) (Figure 40A). To confirm this observation, the extent of synergy between Cel7A and Cel7B was further assessed on the “pure” cellulolytic substrate, cotton fibre, with different fibre lengths (indicating different DP) to exclude the influence of hemicellulose and lignin within the pretreated biomass. Again, the highest DS of Cel7A-Cel7B was observed during hydrolysis of the longest cotton fibre (longer cellulose DP) (Figure 36B).



B

	Cel7A	Cel7B	Cel7A+Cel7B	Cel7A+BSA	DS
Long	6.1%	1.3%	16.7%	7.8%	2.3
Medium	10.3%	2.1%	20.4%	12.3%	1.6
Short	22.3%	3.6%	36.8%	24.6%	1.4

Figure 40. (A) The relationship between cellulose degree of polymerization (DP) and the degree of synergism (DS) of Cel7A/Cel7B, (B) DS of Cel7A and Cel7B on cotton fibre with different fibre lengths.

As these general substrate characteristics might not reflect the characteristics of the “accessible cellulose” that were actually interacting with cellulase enzymes during hydrolysis, we further assessed the amount of accessible crystalline and amorphous cellulose within various substrates by two specific carbohydrate binding domain (CBM): CBM2a specifically binds to crystalline cellulose, while CBM44 prefers to bind amorphous cellulose. As expected, the sum of both CBMs displayed a good correlation with cellulose accessibility assessed by the Simons’ Stain technique for the substrates pretreated with same strategy

(Figure 41A). When the ratio of the two CBMs (indicates the relative amount of accessible crystalline and/or amorphous cellulose) was calculated and correlated with the DS of cellulase components (Table 15), a strong relationship was observed between the ratio of CBM44:CBM2a and the DS among cellulase mixture (MIX) (Figure 41B).

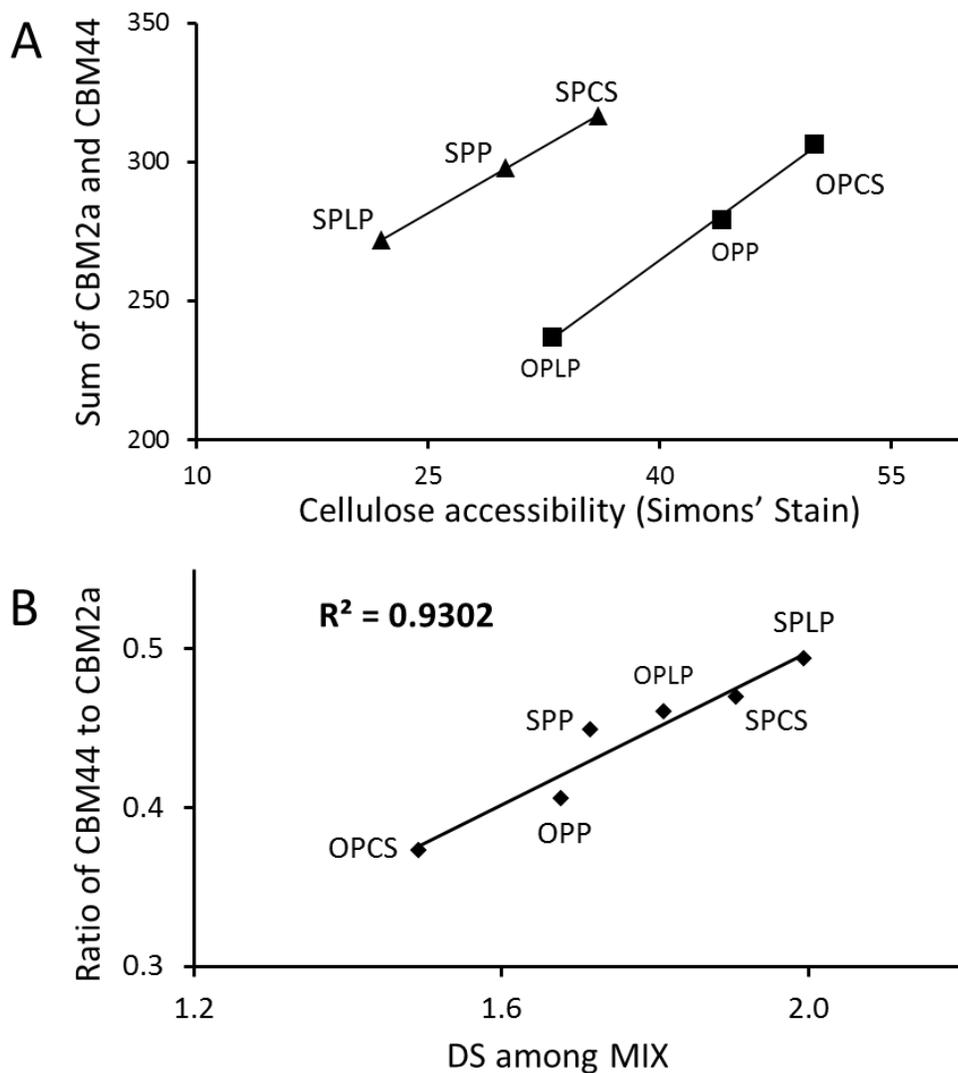
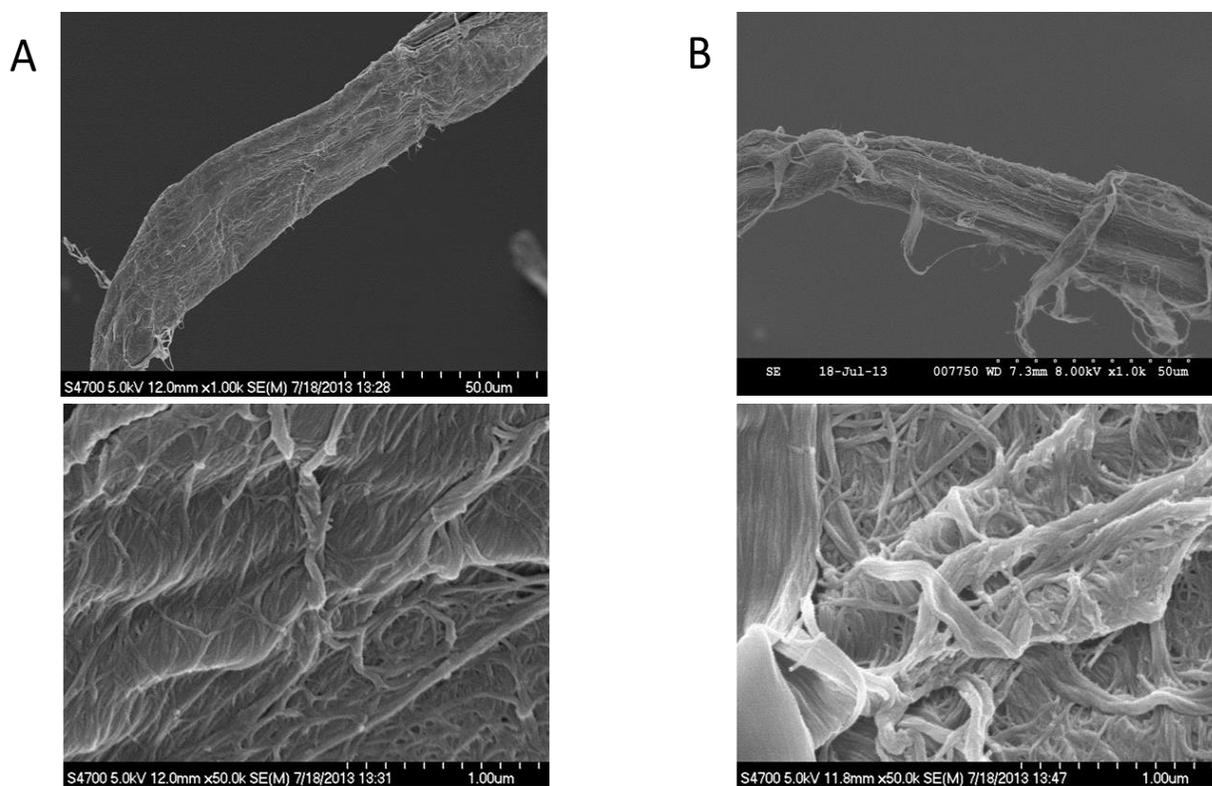


Figure 41. The correlation between (A) sum of CBM2a and CBM44 and cellulose accessibility, and between (B) the ratio of CBM44 to CBM2a and the degree of synergism (DS) among cellulase monocomponents (MIX). MIX: reconstituted cellulases mixture by Cel7A, Cel6A, Cel7B, and Cel5A.

With the same amount of accessible cellulose, it might be expected that the higher CBM44:CBM2a ratio indicates a more accessible/disorganized cellulose surface morphology, leading to the higher degree of synergism among the cellulase MIX during hydrolysis. In order to test this hypothesis, we assessed and compared the CBMs binding affinity on the “model” cellulosic substrate dissolving pulp (DSP) and on the mechanically modified dissolving pulp (M-DSP). M-DSP has a more accessible/disorganized cellulose surface morphology as shown under scanning electron microscopy (SEM) (Figure 42), which was expected to happen during the mechanical modification process. The higher ratio of CBM44:CBM2a was assessed on M-DSP (Figure 42), which supported the previous suggestion that, the higher the ratio of CBM44:CMB2a, the more accessible/disorganized the cellulose surface morphology.



	CBM44	CBM2a	CBM44/CBM2a
DSP	14.9	62.0	0.24
M-DSP	53.8	146.3	0.37

Figure 42. Cellulose allomorphs under scanning electron microscopy (SEM) at two different resolutions. (A) Dissolving pulp (DSP) and (B) mechanical modified DSP (M-DSP) under 50 and 1 μm scales.

Most cellulose hydrolysis was achieved by Cel7A enzymes alone (Table 14), with cellulase synergistic interaction increasing at unsaturated Cel7A concentrations (Figure 37, 38, and 39) and on the more disorganized cellulose (identified by using the CBM technique) (Figure 41). Therefore, it is possible that enzymatic hydrolysis is carried out in the same way an onion is peeled, where the processive Cel7A enzymes (usually accounting for more than 60% of the total protein in natural fungal cellulase systems) hydrolyses the cellulose from the reducing ends. However, Cel7A enzymes would become non-productively bound on the

cellulose through its cellulose binding module (CBM) and/or would therefore get stuck on the disordered cellulose surface morphology during the processive hydrolytic action. Therefore, the other cellulase monocomponents synergistically enhance the Cel7A hydrolytic potential by releasing the unproductively bound and/or trapped Cel7A enzymes. Some recent studies also showed a similar hydrolysis model. Ganner et al. (2012) have suggested that the role of the endoglucanase, Cel6A in particular, is to facilitate the processive performance of Cel7A during hydrolysis while Resch et al. (2013) showed that only the reducing end of the cellulose bundles were actually attacked and sharpened during hydrolysis.

This model might explain the observed synergistic cooperation between Cel7A and other cellulase components, which only happened at unsaturated Cel7A concentrations. Thus, the initial enzyme adsorption randomly distributes Cel7A enzymes on the accessible cellulose surface, where some have access the cellulose reducing ends to start the hydrolysis, processively, while others become unproductively bound to the cellulose surface. In addition, some of the productive Cel7A will be stuck/jammed by the eroded/disorganized cellulose surface and/or by the unproductively bound enzymes during the hydrolysis. Under conditions of low Cel7A concentrations, the average distance between trapped and/or unproductively bound Cel7A and other available chain ends is large, thus other cellulase components are required to reinitiate these Cel7A enzymes by releasing them to the liquid phase. This could be by competitive binding or by creating new reducing ends nearby which can be accessed by the trapped/unproductive bound Cel7A enzymes through lateral diffusion. However, at high enzyme concentrations the stuck Cel7A could be released by other Cel7A enzymes during their processive movement (Igarashi et al., 2011). The distance between the stuck Cel7A and other available reactive sites could also be significantly reduced due to the high

Cel7A density on the cellulose surface, thus the synergistic contribution of other cellulase components to Cel7A enzymes will be minimized during hydrolysis.

3.4.2.5 The influence of cellulase synergism on the adsorption profile of Cel7A.

To further assess the suggested synergistic interaction among cellulase components, the adsorption/desorption profile of Cel7A, with and without the addition of other cellulase monocomponents (Cel5A, Cel6A, Cel7B), were assessed by using an immunoassay (ELISA) during the course of hydrolysis of the SPCS. The synergistic interaction between Cel7A and other cellulase components increased the amount of Cel7A in the liquid phase (Figure 43). In general, the endo-type cellulases (Cel5A and Cel7B) released more Cel7A than the exo-type cellulase Cel6A, while the protein control BSA also caused a slight increase in Cel7A adsorption during hydrolysis.

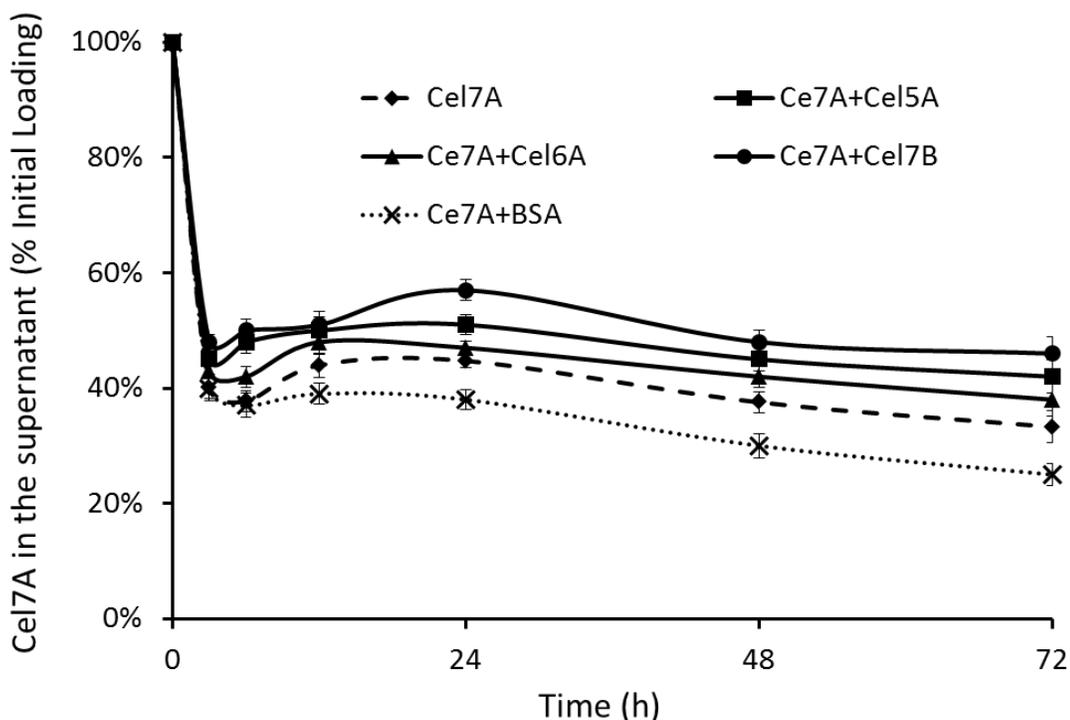


Figure 43. The adsorption/desorption profile of Cel7A with/without other cellulase components during time course of hydrolysis of SPCS. SPCS: steam pretreated corn stover.

The synergistic interaction between Cel7A and other cellulase components were further assessed by a sequential hydrolysis strategy, where the SPCS was initially hydrolyzed with Cel7A alone, followed by addition of other cellulase components when the cellulose hydrolysis levelled off. The addition of Cel7B, Cel5A, and Cel6A after 24 h boosted cellulose hydrolysis by about 50%, 30%, and 15%, respectively, as measured after 72 h (Figure 44). When controls were also included, where the same amount of BSA was added to Cel7A in place of another enzyme, or other cellulase components were added to the deactivated Cel7A, very low cellulose hydrolysis was detected (<2%) (data not shown). The final cellulose hydrolysis after 72 h with the sequential enzyme addition strategy was slightly lower compared to the simultaneous addition of the cellulase components at the beginning of

the hydrolysis of SPCS (Table 14), which indicated that the synergistic cooperation between cellulase components required the existence of all the individual cellulase components in the hydrolysis mixture.

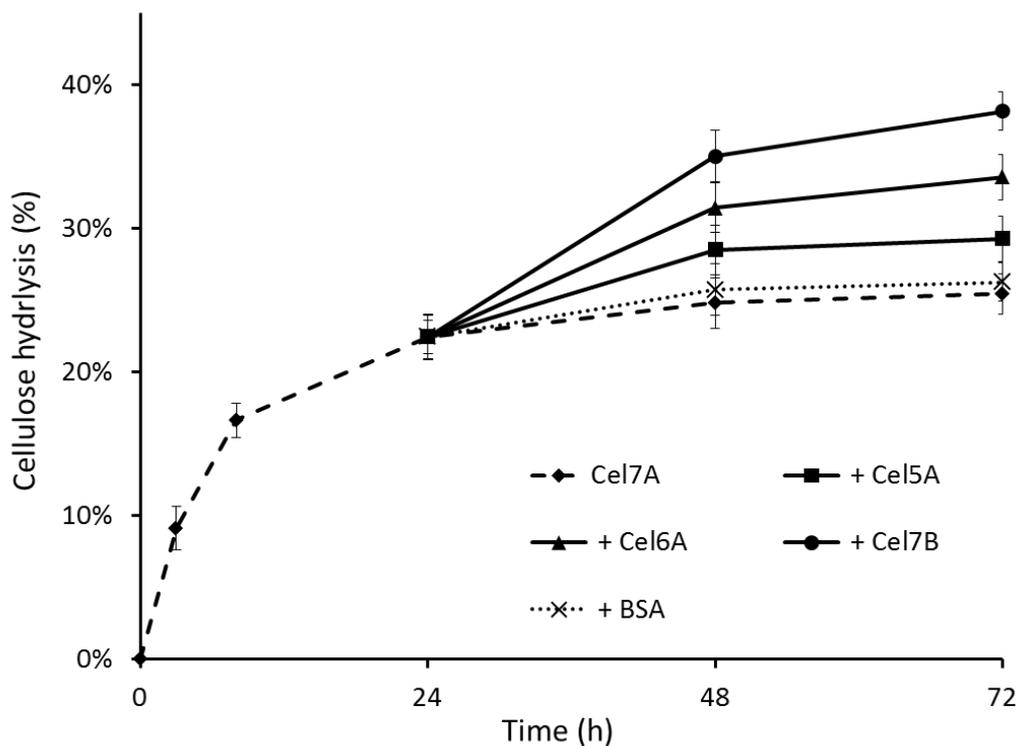


Figure 44. The time course hydrolysis of SPCS by the sequential addition of cellulase components. SPCS: steam pretreated corn stover.

When the adsorption/desorption profile of Cel7A was assessed after the addition of other cellulase enzymes, there was a quick release of Cel7A within a few minutes, followed by the re-adsorption of Cel7A on the SPCS substrate (Figure 45). After that, the Cel7A adsorption profile maintained a similar trend as the hydrolysis performed by adding Cel7A and other cellulase components at the beginning (Figure 43), where a certain amount of Cel7A was released in the liquid phase in the presence of other cellulase components. The

addition of Cel7B released the highest amount of Cel7A (around 20%) in the first 5 min, while the addition of Cel5A and Cel6A enzymes caused a release of ~10% Cel7A around the same time (Figure 45). On the other hand, the protein control increased the amount of absorbed Cel7A in the solid phase.

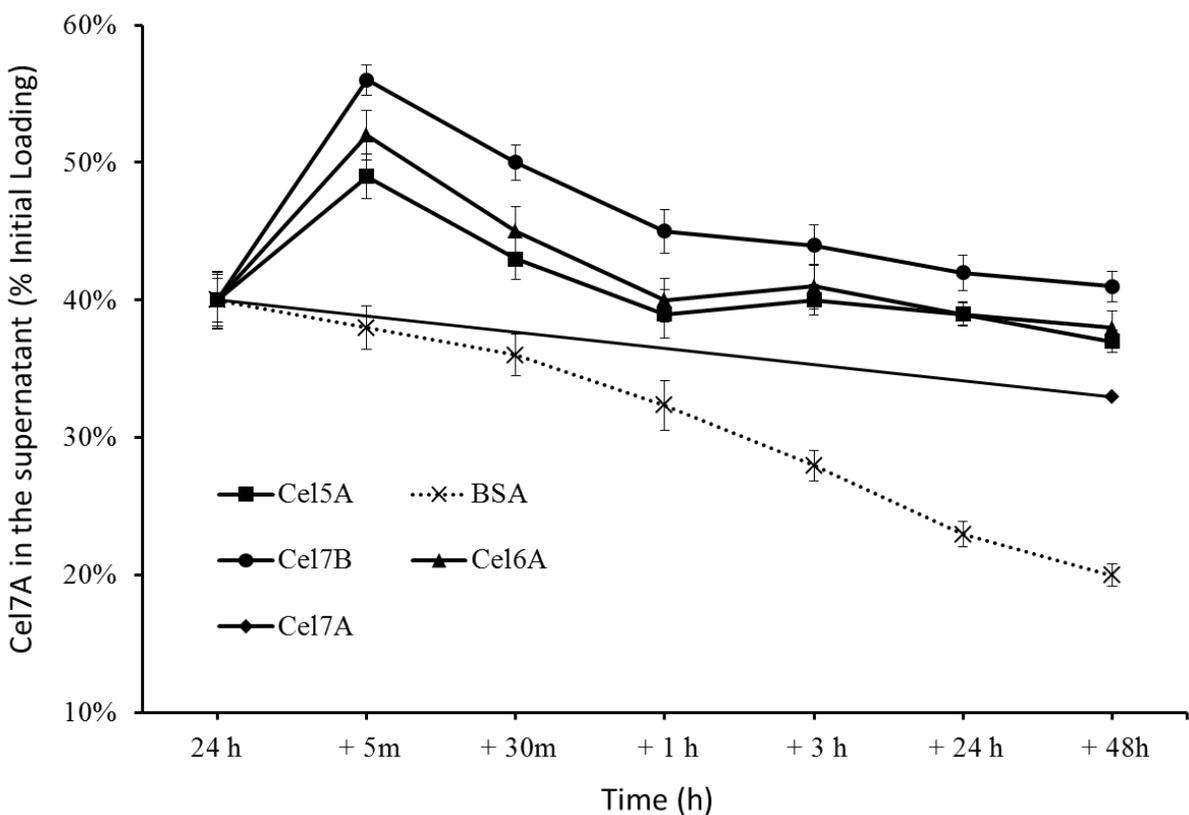


Figure 45. Change of Cel7A level in the liquid phase of SPCS after 24 h hydrolysis with the addition of Cel7B, Cel6A, and Cel5A. Protein control: BSA

The Cel7A adsorption/desorption profile during hydrolysis of SPCS with/without other cellulase components supported the proposed enzyme synergism model (Figure 43), where the synergistic cooperation between Cel7A and other cellulases should increase the

amount of free Cel7A enzymes in the liquid phase during hydrolysis. Further evidence came from restarting the levelled-off Cel7A hydrolysis by the addition of Cel6A, Cel7B, and Cel5A, which resulted in a release of Cel7A enzymes for a short period of time (Figure 45), and also improved the cellulose hydrolysis (Figure 44).

3.4.3 Conclusion

The synergistic cooperation between cellulase components is more apparent at low/unsaturated enzyme loadings and on the more accessible/disorganized cellulosic substrates. Cel7A, the major cellulase components in the cellulase mixture, appears to play the major role in hydrolyzing the cellulose within the pretreated lignocellulosic substrates, while other cellulase components seem to synergistically enhance the hydrolytic performance of Cel7A enzymes. Synergism among cellulase monocomponents was moderate (DS: 1.5-2.0), but high levels of synergism was observed between cellulases and B-glucosidase (DS: 3.0-10). The synergistic cooperation among cellulase components could be explained, at least in part, by the release of the “stuck” Cel7A during hydrolysis. It is possible that the processive Cel7A enzymes become unproductively bound and/or trapped on the cellulose surface during cellulose hydrolysis. Other cellulase components, which can release the Cel7A through competitive binding and/or by creating new reactive sites near the trapped Cel7A, can synergistically reinitiate the Cel7A hydrolysis.

3.5 Can synergistic cooperation between cellulase and accessory enzymes enhance the high solid loading hydrolysis of pretreated lignocellulose?

3.5.1 Background

As mentioned in Chapter 1 - 1.10, high solid loading hydrolysis is essential for achieving economically feasible bio-fuel production. But the increased rheological and inhibition problems can result in the low efficiency of the saccharification process. Several strategies have been tried to overcome these problems, such as using special customized reactors (Cara et al., 2007; Jorgensen et al., 2007; Zhang et al., 2009) and/or using a fed-batch approach to adding substrate (Rudolf et al., 2005; Zhang et al., 2009) to improve the biomass liquefaction process, as well as using simultaneous saccharification and fermentation (SSF) to decrease the inhibition by a build-up of sugar products (Varga et al., 2004; Rudolf et al., 2005). However, few studies have assessed the important role that accessory enzymes might play in improving the hydrolytic efficiency of high solid loading hydrolysis.

Though family 11 xylanases (GH11 EX) did not assist with the liquefaction process during hydrolysis of pretreated wheat straw, it synergistically improved the cellulose hydrolysis by cellulases (Szijarto et al., 2011). The beneficial effects of family 10 xylanase (GH10 EX), which is more efficient than GH11 EX during decomposition of lignocellulosic biomass (Chapter 3 - 3.2), have, to our knowledge, not been investigated previously. In previous work, a good correlation was observed between xylose release and the decrease in biomass viscosity and particle size during hydrolysis of steam pretreated poplar at high solid loading (Di Risio et al., 2011). The removal of hydrophilic xylan was also predicted to

facilitate the liquefaction step of high solid hydrolysis by increasing the amount of free water in the system (Viamajala et al., 2009).

In addition, the lytic polysaccharide monooxygenases (AA9) and β -glucosidase (BG) might also be important for high solid hydrolysis. As shown earlier, AA9 significantly improved the hydrolytic efficiency of cellulase enzymes on various cellulosic/lignocellulosic substrates by increasing the reactive sites on the highly organized cellulose and also releasing the unproductively bound processive enzyme during hydrolysis. Thus, these effects might be more beneficial for high solid loading hydrolysis as cellulase enzymes would have greater difficulty in accessing cellulose and might also get stuck more readily in the high solid: water environment. Although B-glucosidase showed the strongest synergism with cellulase enzymes during cellulose hydrolysis (Chapter 3 - 3.4), its activity will likely be strongly inhibited by the sugar production derived from hydrolysis. Therefore high solid hydrolysis might strongly hamper the performance of the B-glucosidase due to the high concentrations of sugar products.

As mentioned earlier, from the SDS-PAGE figure of the Novozymes' enzyme products (Figure 4), it appears that more enzymes/proteins were added to the new enzyme preparations. Novozymes also indicated that the new enzyme preparation, CTec 3, is more thermostable than previous enzyme preparations, which would allow for increased hydrolysis temperatures as another strategy for improving hydrolysis efficiency.

In this part of the thesis, we assessed the potential of using accessory enzymes to improve the hydrolytic efficiency of cellulase enzymes during high solid hydrolysis. First, the "optimized" enzyme cocktail was formulated by partially replacing a certain amount of

cellulase enzymes with GH10 EX and AA9 at 2%, 10%, and 20% solid loading of two pretreated lignocellulosic substrates. The hydrolytic performance of the “optimized” enzyme cocktail was compared with the leading Novozymes enzyme products, CTec 2 and CTec 3. Finally, the strategies for further improving the hydrolytic potential of CTec 2 and CTec 3 were investigated by using central composite design and altering the hydrolysis temperature, respectively. It appeared that synergistic cooperation between accessory enzymes and cellulases could significantly improve the hydrolytic potential of enzyme cocktails on high solid loading hydrolysis.

3.5.2 Results and discussion

3.5.2.1 “Optimizing” an enzyme cocktail for hydrolysis of pretreated lignocellulosic substrates at different solid loadings.

The enzyme optimization was first performed during hydrolysis of poplar, which was steam pretreated at 200°C (SPP 200), at three different solid loadings, namely 2%, 10%, and 20% (Figure 46). The required minimum cellulase loading for achieving reasonable cellulose hydrolysis was first assessed at the three solid loading. At 2% consistency, 10 mg cellulases per gram of cellulose were enough to convert about 70% of the cellulose after 72 h (Figure 46C). As expected, the required cellulase loading for 70% cellulose hydrolysis was significantly higher, requiring around 30 mg/g cellulose, when the initial solid loading was increased to 10% (Figure 46B). Using 20% solid loadings, the cellulases alone could not attain 70% cellulose hydrolysis, even with a high amount of enzyme loading (80 mg/g cellulose). Thus, we used an approximate final hydrolysis yield of 60% (instead of 70%) cellulose to quantify the minimum amount of cellulases required for 20% consistency

hydrolysis (Figure 46A). In the case of SPP 200 the required cellulase loading was 60 mg/g cellulose at a 20% consistency.

We then partially replaced some of the required cellulase enzymes with GH10 EX. This replacement strategy was chosen because our previous work had shown that it promoted the synergistic interaction between cellulases and accessory enzyme without increasing the total amount of protein loading. It appeared that 5% of GH10 EX was enough to boost the hydrolytic potential of the enzyme mixture for SPP 200 at 2% and 10% solid loading, while around 10% GH10 EX was required at a 20% solid loading (Figure 46). Further increasing the ratio of xylanases to cellulases slightly decreased the hydrolytic potential of the enzyme mixture. The required amount of xylanases in the enzyme mixture, although low, might be due to the low xylan content in the SPP 200 (~3.7%, Table 10). Interestingly, the extent of cellulose hydrolysis improvement achieved by GH10 EX was higher at relatively low solids (2% and 10%) than high solids (20%) loading (Figure 46).

After the required amount of GH10 EX in the enzyme mixture was identified, another major accessory enzyme, AA9, was used to further replace cellulase enzymes in the cellulase-xylanase mixture. Even low amounts of AA9 (0.5-1 mg/g cellulose) significantly improved cellulose hydrolysis at all different solid loadings, and the maximum cellulose conversion was achieved when 2 mg AA9 per gram of cellulose was used to replace an equal amount of cellulases in the “optimized” enzyme mixture (Figure 46). The most significant improvement was observed at 20% solid loading, where the existence of AA9 increased cellulose hydrolysis by about 20% after 72 h (Figure 46A).

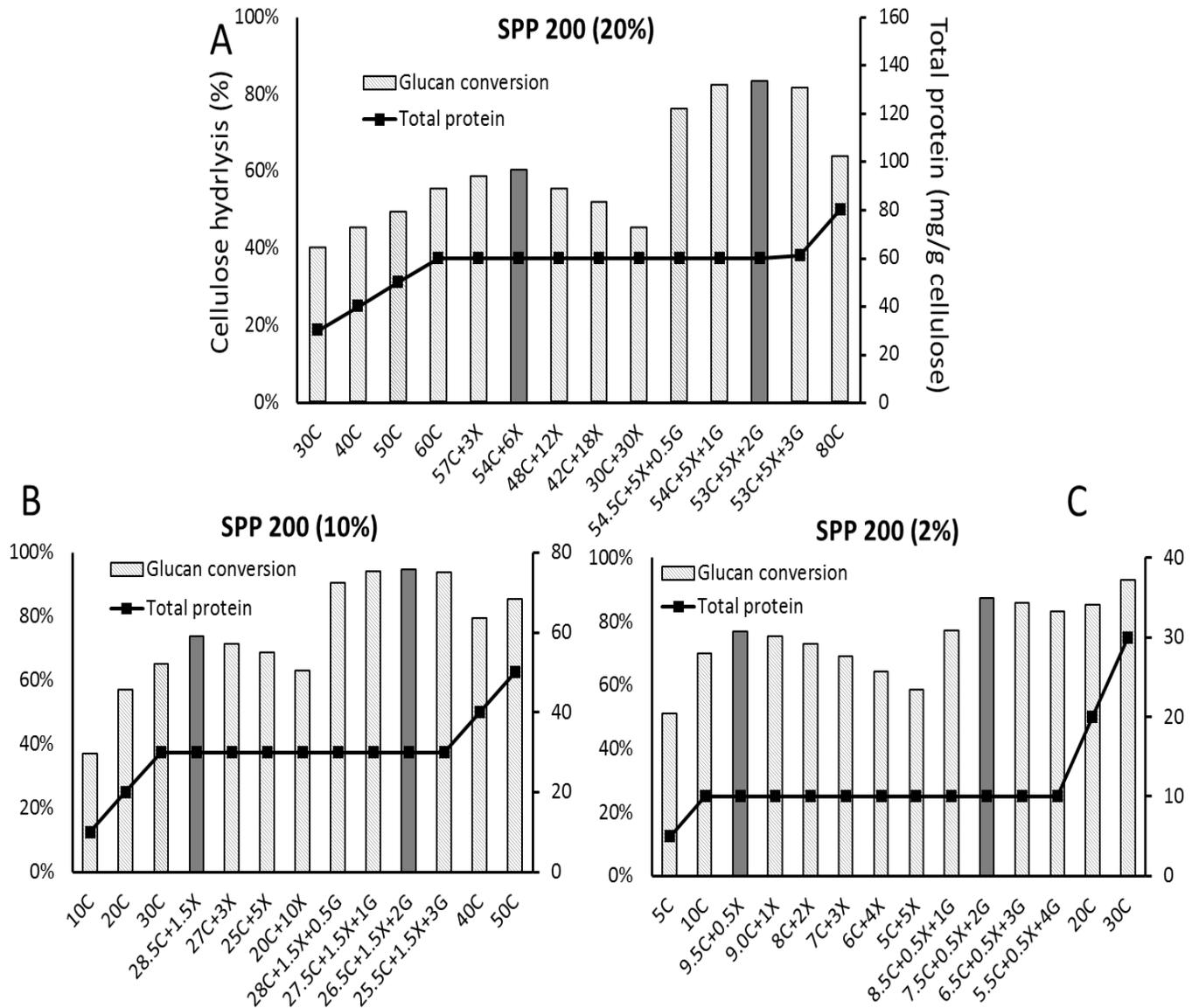


Figure 46. Cellulose hydrolysis of SPP 200 by partially replacing cellulases with accessory enzymes (GH10 EX and AA9) at (A) 20%, (B) 10%, and (C) 2% solid loading hydrolysis after 72 h. SPP 200: steam pretreated poplar at 200 °C.

The same strategy for “optimizing” enzyme mixtures was further applied on corn stover steam pretreated at 180°C (SPCS 180) (Figure 47). SPCS 180 is relatively harder to hydrolyze as compared with SPP 200 due to the lower severity (temperature) used in the pretreatment process, so almost double the amount of enzyme was required for a reasonable

cellulose hydrolysis. Similar to SPP 200, replacing cellulase enzymes with GH10 EX and AA9 significantly improved the hydrolytic potential of the enzyme mixture during hydrolysis of SPCS 180 at different solid loadings (Figure 47). However, unlike the hydrolysis of SPP 200, GH10 EX contributed more than AA9 in aiding in the hydrolysis of SPCS 180 hydrolysis. This was likely due to the higher xylan content (~20%) of the SPCS 180 substrate.

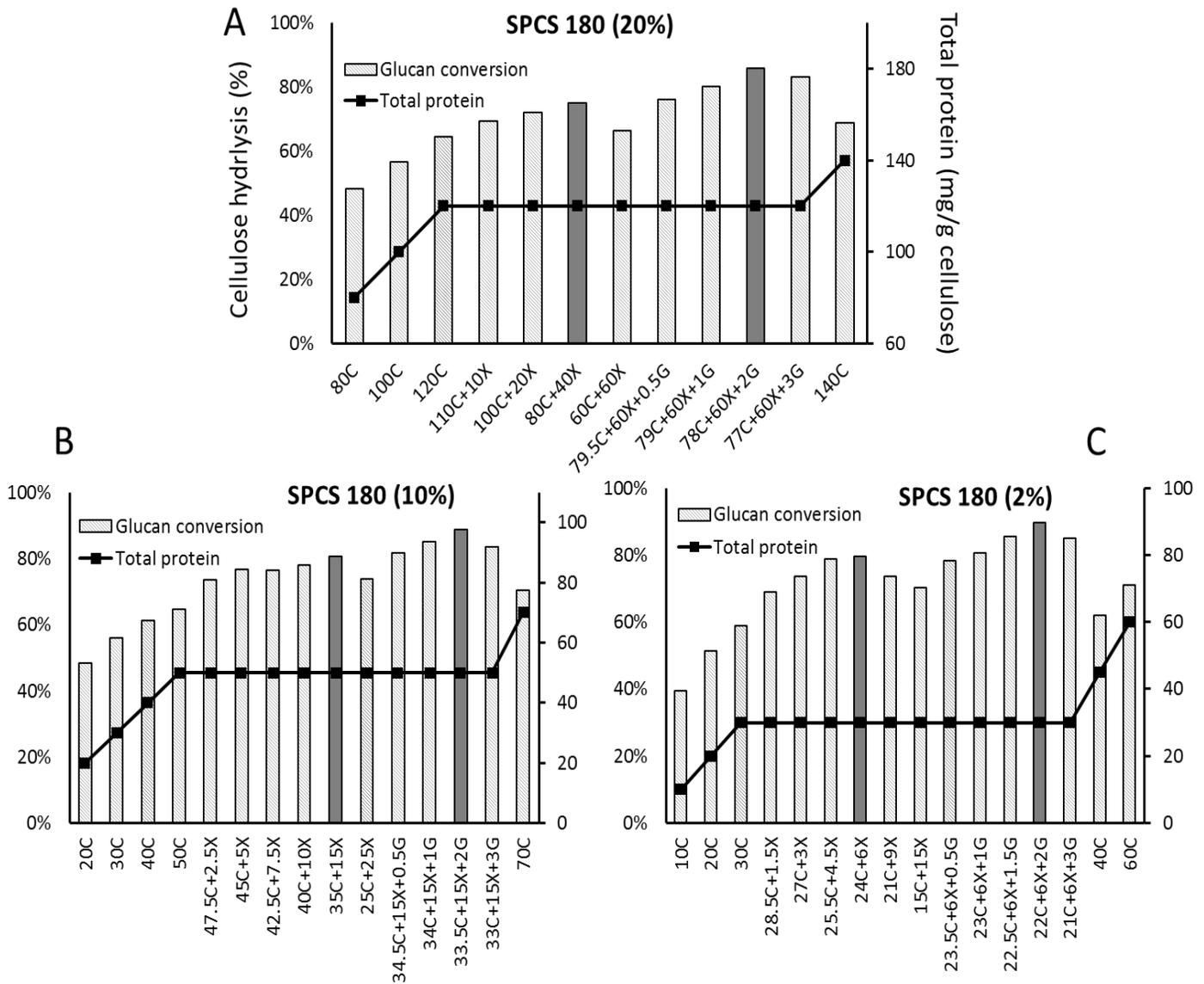


Figure 47. Cellulose hydrolysis of SPCS 180 by partially replacing cellulases with accessory enzymes (GH10 EX and AA9) at (A) 20%, (B) 10%, and (C) 2% solid loading hydrolysis after 72 h. SPCS 180: steam pretreated corn stover at 180°C.

The amount of required accessory enzymes in the “optimized” enzyme mixture was calculated and compared at different solid loadings. It appeared that the required amount of xylanases (GH10 EX) increased, while the required amount of AA9 decreased, with an increase in solid loading from 2% to 20% (Table 16). SPCS 180 required more GH10 EX in the “optimized” enzyme mixture for its hydrolysis than SPP 200 (Table 16). A small amount of AA9 (2 mg/g cellulose) was sufficient in all the “optimized” mixtures for both substrates and also for different consistency hydrolysis (Figure 46 and 47). By mixing and matching the cellulases with the essential accessory enzymes, we could significantly improve the final cellulose conversion and also reduce the amount of protein loading, especially at high solid loading hydrolysis. For example, 60 mg cellulase per gram of cellulose hydrolyzed about 55% of the cellulose in SPP 200 at 20% solid loading and further increasing the cellulase loading to 80 mg/g cellulose did not really improve the hydrolysis (Figure 47A), while the “optimized” enzyme mixture (60 mg/g cellulose) could achieve more than 80% cellulose hydrolysis.

Table 16. The enzyme composition in the “optimized mixture” during hydrolysis of SPP 200 and SPCS 180 at different solid loadings.

Substrates	Solid loading	Enzyme portions in the “optimized mixture”		
		Celluclast	GH10 EX	AA9
SPP 200	2%	75%	5%	20%
	10%	88%	5%	7%
	20%	87%	10%	3%
SPCS 180	2%	73%	20%	6.7%
	10%	66%	30%	4%
	20%	65%	33%	2%

SPP200: steam pretreated poplar at 200 °C, SPCS180: steam pretreated corn stover at 180 °C.

3.5.2.2 Comparing the hydrolytic ability of the “optimized” enzyme mixture and various Novozymes’ enzyme preparations

The saccharification performance efficiency of the “optimized” enzyme mixture was compared with a series of leading Novozymes’ products, CTec cellulase preparations, on SPP 200 as shown in Figure 48. CTec 2 and 3 performed better than CTec 1 for the cellulose hydrolysis of SPP 200, and this beneficial effect was amplified at high solid loading. CTec 1 achieved about 70% cellulose hydrolysis after 72 h at 2% solid loading, where the same amount of CTec 2 and CTec 3 hydrolyzed around 75% and 90% cellulose, respectively (Figure 48). When 20% initial solid loading was used, CTec 3 hydrolyzed about 40% more cellulose than CTec 1 under the same hydrolysis condition.

It is worth mentioning that our “optimized” enzyme mixture showed a similar hydrolytic ability as compared with the Novozymes’ CTec 3 enzyme preparation (Figure 48). It has been suggested that new enzyme products (such as CTec 3) contain enzymes with better catalytic efficiency and/or thermostability than the old enzyme preparations. By optimizing an enzyme mixture with essential accessory enzymes, our “optimized” enzyme mixture achieved a similar hydrolytic ability as CTec 3. Thus, it seems that identifying the “right” enzyme combination and optimizing the enzyme ratios is just as important as improving individual enzyme activities.

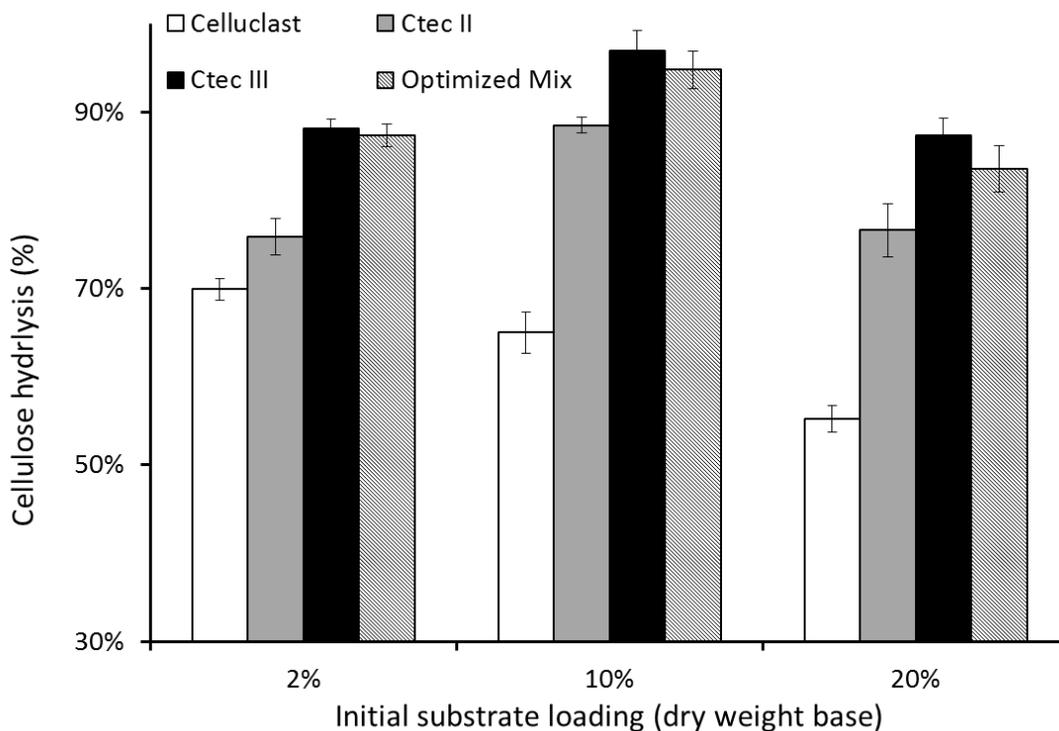


Figure 48. Cellulose hydrolysis of SPP 200 by various “cellulase cocktail” at different solid loading after 72 h. Optimized MIX: reconstituted cellulases mixture by cellulase enzymes (Celluclast, 1.5L), GH10 EX, and AA9.

3.5.2.3 Enhancing the hydrolytic potential of C Tec 2 by accessory enzymes

When the hydrolytic ability of Novozymes’ C Tec enzyme preparations and the “optimized” enzyme mixture was compared on SPP 200, C Tec 1 and C Tec 2 enzyme preparations showed a much lower efficiency than C Tec 3 and the “optimized” mixture (Figure 48). The C Tec 1 preparation, similar to Celluclast 1.5L that was used as the basic cellulase mixture for optimizing the enzyme cocktail, contains mostly cellulase activity. However, the other enzyme preparations might contain a considerable amount of accessory enzymes, such as hemicellulases and AA9. As the amount of AA9 required to supplement the cellulases was small (~2 mg/g cellulases), it is possible that the lower efficacy of C Tec 2 compared to C Tec 3 might be due to the relatively lower xylanolytic activity of this enzyme

mixture. In addition, CTec 3 has considerably higher β -glucosidase (BG) activity than does CTec2 (data not shown), and BG will have a significant contribution to high solid loading hydrolysis. Thus the lower hydrolytic efficiency of CTec 2 might also be caused by the lower BG activity in the enzyme preparation.

We further tried to enhance the hydrolytic potential of CTec 2 by mixing and matching it with xylanolytic enzymes, such as HTec 2, which contains mainly GH10 EX and β -xylosidase (BX), and BG, at relatively high solid loadings (10% to 20%). The central composite design was used in this study, instead of the enzyme replacement method used previously, in order to assess the beneficial effects of accessory enzymes. As shown in Table 17, five individual factors, namely CTec 2, HTec 2, BG, BX, and consistency (solid loading) were compared and the low and high value of each factor was based on previous experience. Full type design was chosen and one response variable, glucan conversion, was used, which led to 42 set-ups with 3 centre points (Table 18).

Table 17. Variables that were used for the central composite design to assess SPP hydrolysis

Central Composite Design			
Factors*	Units	low (-1) level	high (+1) level
CTec 2	mg/g Cellulose	30	70
HTec 2	mg/g Cellulose	0	10
BG	mg/g Cellulose	0	4
BX	mg/g Cellulose	0	4
Consistency	%	10	20

*CTec 2 (Cellulase mixture), HTec 2(Cellulase mixture), BG (β -Glucosidase), BX (β -Xylosidase); Full type design: Not center points 42; Center points 3; alpha = 1
Responses: Glucan conversion %

Table 18. Central composite design for SPP hydrolysis

		C-Tec (mg/g)	H-Tec (mg/g)	BG (mg/g)	BX (mg/g)	Consistency (%)	Glucan conversion (%)
1	Fact	30	0	0	0	10	74.2%
2	Fact	70	0	0	0	10	83.4%
3	Fact	30	10	0	0	10	80.9%
4	Fact	70	10	0	0	10	91.5%
5	Fact	30	0	4	0	10	77.8%
6	Fact	70	0	4	0	10	87.2%
7	Fact	30	10	4	0	10	85.2%
8	Fact	70	10	4	0	10	93.9%
9	Fact	30	0	0	4	10	76.9%
10	Fact	70	0	0	4	10	85.2%
11	Fact	30	10	0	4	10	81.1%
12	Fact	70	10	0	4	10	89.7%
13	Fact	30	0	4	4	10	77.8%
14	Fact	70	0	4	4	10	88.9%
15	Fact	30	10	4	4	10	84.8%
16	Fact	70	10	4	4	10	92.3%
17	Fact	30	0	0	0	20	56.2%
18	Fact	70	0	0	0	20	72.5%
19	Fact	30	10	0	0	20	68.2%
20	Fact	70	10	0	0	20	81.8%
21	Fact	30	0	4	0	20	59.5%
22	Fact	70	0	4	0	20	76.3%
23	Fact	30	10	4	0	20	73.6%
24	Fact	70	10	4	0	20	85.6%
25	Fact	30	0	0	4	20	58.7%
26	Fact	70	0	0	4	20	74.1%
27	Fact	30	10	0	4	20	69.2%
28	Fact	70	10	0	4	20	81.2%
29	Fact	30	0	4	4	20	60.5%
30	Fact	70	0	4	4	20	78.8%
31	Fact	30	10	4	4	20	73.9%
32	Fact	70	10	4	4	20	86.2%
33	Axial	30	5	2	2	15	68.6%
34	Axial	70	5	2	2	15	89.5%
35	Axial	50	0	2	2	15	75.4%
36	Axial	50	10	2	2	15	82.3%
37	Axial	50	5	0	2	15	78.3%
38	Axial	50	5	4	2	15	80.6%
39	Axial	50	5	2	0	15	79.2%
40	Axial	50	5	2	4	15	79.7%
41	Axial	50	5	2	2	10	78.6%

ANOVA for Response Surface Reduced 2FI Model, Response: Glucan conversion						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	3287.39	7	469.63	150.74	< 0.0001	significant
<i>A-C-Tec</i>	1309.44	1	1309.44	420.29	< 0.0001	
<i>B-H-Tec</i>	560.12	1	560.12	179.78	< 0.0001	
<i>C-BG</i>	105.18	1	105.18	33.76	< 0.0001	
<i>E-Consistency</i>	1203.68	1	1203.68	386.34	< 0.0001	
<i>AB11.88</i>	1	11.88	3.81	0.0584		
<i>AE58.59</i>	1	58.59	18.81	0.0001		
<i>BE38.50</i>	1	38.50	12.36	0.0012		
Residual	115.28	37	3.12			
<i>Lack of Fit</i>	114.63	35	3.28	10.13	0.0938	not significant

In the optimized mixture for 20% solid loading hydrolysis, with the goal of minimizing the amount of CTec 2 enzymes and maximizing glucan conversion, it was found that high amounts of BG loading (~4 mg/g cellulose) and HTec 2 loading (10 mg/g cellulose) were required. The addition of BX did not contribute to the cellulose hydrolysis (Figure 49). The highest cellulose hydrolysis yield in this scenario was about 80%, which required loadings of CTec 2, HTec 2, and BG at 55, 10, and 4 mg/g cellulose, respectively. The CTec 2 dosage could be significantly reduced by the supplementation of HTec 2 for hydrolysis of SPP 200. For example, more than 60 mg CTec 2 per gram of cellulose achieved about 73% of cellulose hydrolysis after 48 h, while the same extent of cellulose conversion could be reached with about 33 mg of CTec 2, when 10 mg of HTec 2 was supplied during hydrolysis (Figure 49).

Design-Expert® Software

Glucan conversion



X1 = A: C-Tec

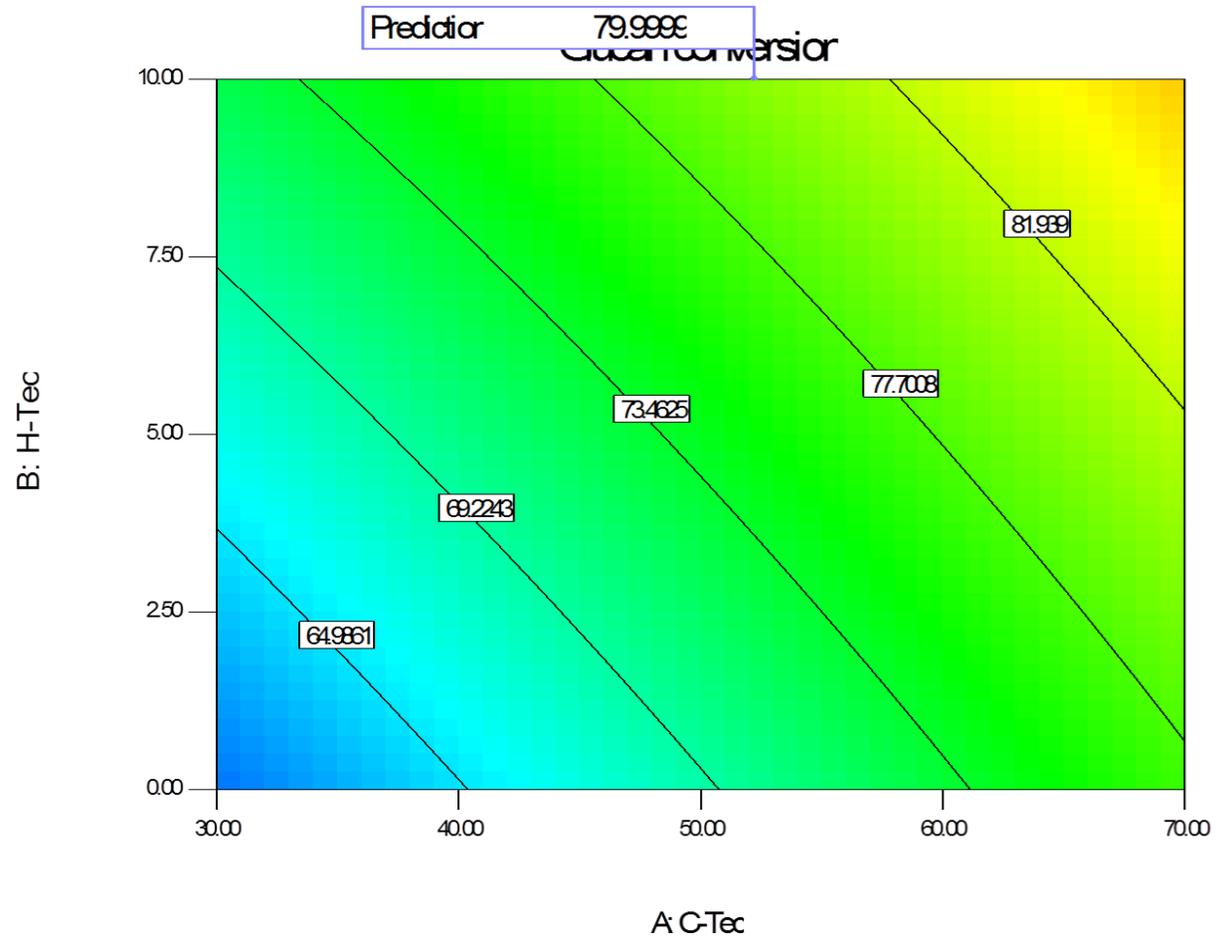
X2 = B: H-Tec

Actual Factors

C: BG = 3.89

D: BX = 0.00

E: Consistency = 20.00



$$\text{Glucan conversion} = + 86.78883 + 0.13779 * \text{C-Tec} + 0.45833 * \text{H-Tec} + 0.87941 * \text{BG} - 2.08594 * \text{Consistency} - 6.09375\text{E-}003 * \text{C-Tec} * \text{H-Tec} + 0.013531 * \text{C-Tec} * \text{Consistency} + 0.043875 * \text{H-Tec} * \text{Consistency}$$

Figure 49. Prediction of “optimized” enzyme cocktail by CTec2, HTec2, BG and BX for the hydrolysis of SPP at 20% solid loading.

3.5.2.4 The hydrolytic ability of CTec 3 at different temperatures.

The beneficial effect of addition of HTec 2 and/or BG to CTec 3 was also assessed during hydrolysis of SPP 200 at 20% solid loading at various temperatures. The addition of both enzymes improved cellulose conversion, but the extent of improvement was very small (Figure 50). However, the same amount of CTec 3 resulted in even better hydrolysis yield as compared with HTec 2 and BG supplementation (data not shown). Increasing the temperature from 45°C to 55°C further improved cellulose hydrolysis by about 10% (Figure 50), but further increasing the temperature to 60°C significantly reduced hydrolysis. The improved cellulose hydrolysis at 55°C might be a result of the higher enzyme catalytic efficiency at higher temperatures. Thus, improving enzyme thermostability is another possible approach to enhance the cellulase hydrolytic efficiency, especially at high solid loadings.

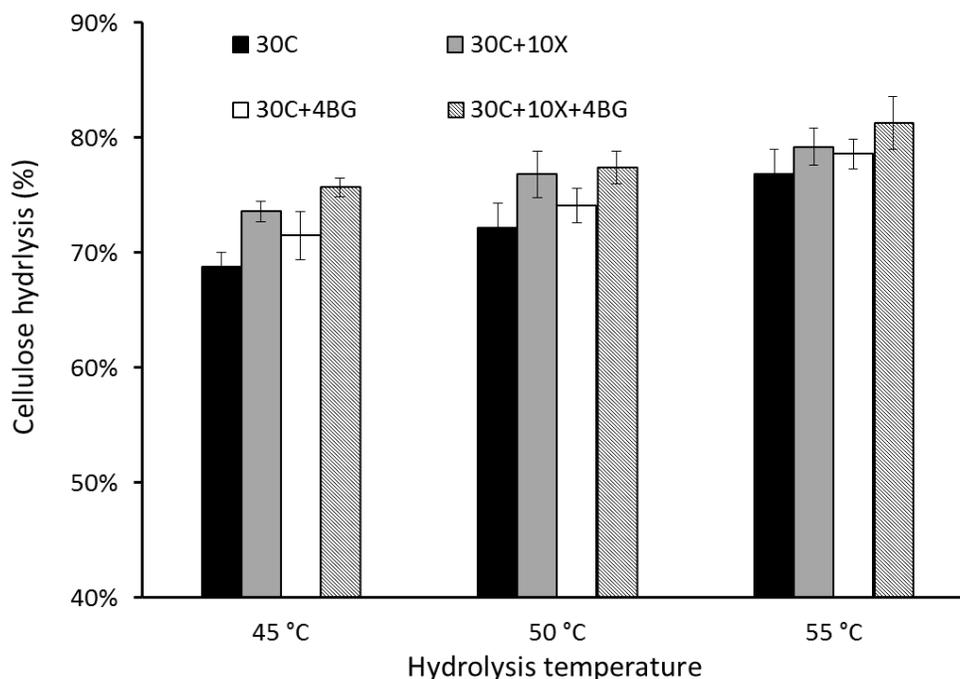


Figure 50. Cellulose hydrolysis of SPP 200 by CTec 3 with/without Htec 2 and BG supplementation at different temperatures. C: C-Tec 3; X: GH10 EX; BG: β -glucosidase

3.5.3 Conclusion

The synergistic cooperation between cellulases and the accessory enzymes, such as xylanases and AA9, significantly improved the hydrolytic potential of an enzyme mixture during hydrolysis of pretreated lignocellulosic substrates at high solid loadings. The “optimized” enzyme mixture required more xylanases when the substrates had a relatively high xylan content and when the hydrolysis was performed at comparatively high solid loading, while a low amount of AA9 (about 2mg/g cellulose) seemed to be sufficient in all the “optimized” enzyme cocktail, regardless of the substrates and solid loading. The “optimized” enzyme cocktail showed a similar hydrolytic potential as the leading Novozymes’s enzyme preparation CTec 3, which indicated that identifying and tailoring the “right” components in the enzyme cocktail is essential for achieving an efficient cellulose hydrolysis with low enzyme loading.

The hydrolytic potential of CTec 2 can be further improved by HTec 2 (mainly family 10 xylanase) and β -glucosidase (BG) supplementation, while the addition of β -xylosidase (BX) had no beneficial effects for CTec 2 hydrolysis ability. The synergistic cooperation among these enzyme preparations could reduce the amount of CTec 2 dose and also achieve better cellulose hydrolysis of pretreated biomass substrates..

4. Conclusions and future work

4.1 Conclusions

We first assessed the interaction between a commercial cellulase preparation (Celluclast 1.5L) and a xylanase preparation (Multifect Xylanase) and the potential to improve the hydrolysis of steam pretreated corn stover (SPCS) when added at low protein loadings. It was apparent that the type of interaction between xylanase and cellulase enzymes was dependent on the total enzyme loading and enzyme ratio. Their synergistic interaction resulted in three times faster cellulose and xylan hydrolysis, a seven-fold decrease in cellulase loading and a significant increase in the hydrolytic performance of the optimized enzyme mixture. This suggested that the 'blocking effect' of xylan was one of the major mechanisms that limited the accessibility of the cellulase enzymes to the cellulose. It was noticeable that the synergistic interaction of the xylanase and cellulase enzymes was also shown to significantly improve cellulose accessibility through increased fibre swelling and fibre porosity and these mechanisms also played a major role in enhancing enzyme accessibility.

Although the synergistic interaction between xylanase and cellulase enzymes significantly reduced the cellulase loading for efficient cellulose hydrolysis of SPCS, the required xylanase dose (mainly family 11 xylanase) was still high. In addition, as commercial enzyme preparations were used, we were not able to identify which cellulase components acted synergistically with xylanase. Thus, we subsequently investigated the potential synergistic interactions between purified cellulase monocomponents (Cel7A, Cel6A, Cel7B, Cel5A) and hemicellulases from family 10 and 11 endo-xylanases (GH10 EX and GH11 EX)

and family 5 xyloglucanase (GH5 XG), during hydrolysis of various steam pretreated lignocellulosic substrates. It was apparent that hemicellulases interacted synergistically with cellulase components (mainly Cel7A) to improve the hydrolysis of a range of pretreated lignocellulosic substrates. However, the extent of improved hydrolysis was highly substrate dependent. It appears that those accessory enzymes, such as GH10 EX and GH5 XG, with broader substrate specificities promoted the greatest improvements in the hydrolytic performance of the cellulase mixture on all of the pretreated biomass substrates. Besides the beneficial effects of xylanase described in earlier work, the results presented here indicated another role that xylanase might play in enhancing the hydrolytic potential of a “cellulase mixture”, namely to help release cellulases that are stuck on the substrate during hydrolysis.

Once cellulase enzymes access the cellulose within the pretreated biomass, another barrier that limits cellulase hydrolytic efficiency is the highly organized/crystalline cellulose structure. Thus, another type of accessory enzyme that might disrupt the crystalline cellulose would be beneficial if we want to achieve efficient cellulose hydrolysis with low enzyme loadings. Over the past few years, it has become apparent that a previously unstudied family of enzymes (lytic polysaccharide monooxygenases: PMOs), utilizing an oxidative rather than a hydrolytic mechanism to cleavage crystalline cellulose region, can significantly improve the cellulose hydrolysis by cellulases. We have shown that one of these PMOs, known as AA9, synergistically cooperates with cellulose hydrolytic enzymes on a range of cellulosic substrates. We also built on this more fundamental work by demonstrating how these enzymes take advantage of particular biomass components to potentiate their action. Additionally, we demonstrated how the AA9 enzymes act synergistically with the canonical hydrolases to facilitate the degradation of recalcitrant regions of cellulose that the hydrolases

alone are incapable of efficiently degrading. By promoting the synergistic cooperation between cellulase enzymes and AA9, we were able to improve the hydrolytic performance of the enzyme mixture on a range of pretreated biomass substrates at low enzyme loadings.

In addition to improving the hydrolytic potential of cellulase enzymes by their synergistic cooperation with the major accessory enzymes such as hemicellulases and AA9, it is also important to understand the synergistic interaction among cellulase components during hydrolysis of pretreated biomass substrates. Previous work has mainly used “model” cellulosic substrate to assess cellulase synergy, with very low enzyme loadings (<20% cellulose hydrolysis). The work presented here indicated that cellulase synergism intensified at low/unsaturated Cel7A concentration. When cellulase interaction was assessed over a range of pretreated lignocellulosic substrates using reasonable/low enzyme dosage (~60% cellulose hydrolysis), the highest synergism was observed between cellulase and β -glucosidase. It was apparent that Cel7A is the major enzyme involved in the hydrolysis of cellulose within the pretreated biomass, while other cellulase components synergistically enhanced its hydrolytic potential. The synergism among cellulase components is most apparent on the accessible/disorganized parts of the cellulose. It seems that cellulase synergism could be explained, at least in part, by the release of the trapped processive Cel7A enzymes during hydrolysis.

Previous work assessed enzyme interaction at low substrate concentrations. However, this is unlikely to be used at commercial scale as the low concentration of sugars obtained after hydrolysis limits the downstream bio-fuels/chemicals production. Therefore, the synergistic cooperation between cellulases and major accessory enzymes (GH10 EX and AA9) was further assessed during hydrolysis of pretreated lignocellulosic substrates at high

substrate concentrations. The “optimized” synergistic cooperation among cellulases, GH10 EX, and AA9 significantly enhanced the hydrolytic performance of the enzyme mixture during high solid loading. More GH10 EX was required for the hydrolysis of pretreated biomass substrate with a higher xylan content, while a low amount of AA9 was sufficient in all the substrates assessed in this study. It appears that identifying and deriving the optimum enzyme composition will help in enhancing the efficiency of cellulose hydrolysis when using with low enzyme loadings.

4.2 Future work

4.2.1 Further assessment of the role of xylanases

This study showed the strong synergistic cooperation between xylanase and cellulase enzymes during the deconstruction of various lignocellulosic substrates, even substrates with very low xylan content such as steam pretreated lodgepole pine. In addition, the work also indicated that the interaction between xylanases and cellulases also caused fibre swelling and increased fibre porosity. Thus, it would be interesting to investigate how xylanases assist in cellulose hydrolysis in substrates with a very low xylan content.

4.2.2 Improved understanding of the mechanism of cellulose enzymatic hydrolysis

In this study, we found that Cel7A is the major cellulase component for cellulose hydrolysis while other enzymes synergistically improved the hydrolytic potential of Cel7A. This work adds further evidence to the proposed “onion peeling” cellulose hydrolysis model.

Further assessing this proposed model by using microscopy and/or other techniques would be helpful in obtaining a better understanding of cellulose hydrolysis.

4.2.3 Improving the high solid loading hydrolysis

By “optimizing” the “cellulases mixture” by the addition of accessory enzymes we can significantly improve the high solid loading hydrolysis (up to 20%), but the total enzyme dosage is still not very commercially attractive. So further improving the efficiency of high solid loading by combining the use of accessory enzymes with other strategies such as fed-batch addition of substrates, SSF, and using a special reactor, would be interesting to pursue.

4.2.4 Using enzymes to improve the quality of cellulose products.

The production of high quality cellulose products such as dissolving pulp (DSP) and cellulose nanocrystalline cellulose (CNC) has received much attention in the past few years. As mentioned earlier, each accessory enzyme and individual cellulase component has special reactive sites and catalytic mechanism, which would likely influence the cellulose characteristics. Therefore, using an enzymology approach to improve the quality of these high value cellulose products is attractive. For example, xylanase and endo-glucanase can be used to improve the quality of DSP by reducing the xylan content and/or decreasing the degree of polymerization of cellulose, while AA9 might be a good candidate for enhancing the reactivity of CNC (increasing the acid groups) through oxidative cleavage of the crystalline cellulose surface.

4.2.5 Prebiotics (cello/xylo-oligosaccharides) production using tailored enzyme mixtures

Non-digestible oligosaccharides, such as cellooligosaccharides and xylooligosaccharides, serve as one of the major prebiotics and play an increasing role in feed, food and pharmaceutical industry. Acid-catalyzed cellulose hydrolysis has been performed to produce cellooligosaccharides at an industrial scale. However, this process results in a low yield of cellooligosaccharides and many by-products. Thus, tailoring cellulase-xylanase cocktails to produce oligosaccharides from lignocellulosic substrates might be interesting to pursue.

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