Enzyme-substrate interactions and their influence on enzyme recycling strategies as a way of reusing cellulases

by

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Abstract

Relatively high enzyme loadings are required for the bioconversion of lignocellulosic biomass, impeding the economical production of cellulosic sugars. The relative stability and robustness of these enzymes make enzyme recycling an attractive cost-reduction strategy. However, the efficiency of enzyme recycling has been limited by the complexity of enzyme-substrate interactions, which are influenced by enzyme, substrate, and physical factors. A lack of techniques to probe specific enzyme adsorption further limits our understanding of these interactions. Therefore, overcoming these challenges to better understand enzyme-substrate interactions is crucial if we are to improve the effectiveness of enzyme recycling strategies.

Initial work compared various ways to assess enzyme adsorption during hydrolysis of steam pretreated corn stover (SPCS) using a complete commercial cellulase mixture. While the distribution of six individual enzymes could be followed, the initial approach used was laborious, highlighting the limitations of techniques used to quantify individual enzyme adsorption profiles. A quicker, more sensitive double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was subsequently developed, to follow Cel7A, Cel6A, and Cel7B adsorption during hydrolysis, and shown to agree with earlier results.

As enzyme, substrate, and physical factors were known to affect enzyme recycling performance, their influence on individual enzyme adsorption was evaluated. Although the lignin present in the SPCS did not appear to influence enzyme adsorption (although Cel6A adsorbed more readily to the lignin-containing SPCS), cellulose allomorphs and crystallinity did appear to influence enzyme adsorption. The addition of Auxiliary Activity (AA) family 9, an oxidative enzyme, increased desorption of Cel7A, likely by increasing the substrate's negative charge. The AA9 itself remained primarily in the supernatant, which highlighted the importance of recovering enzymes from both the liquid and solid phases of the reaction.

The influence of glucose and ethanol on enzyme adsorption was evaluated, and a reduction in enzyme adsorption was observed at high glucose but not ethanol concentrations.

When the addition of fresh substrate was assessed as one way to recover enzymes, by combining enzyme recycling at low glucose concentrations with enzyme supplementation, good overall cellulose hydrolysis (~70%) over 5 rounds of enzyme recycle could be achieved with a 50% reduction in enzyme loading.

Preface

A version of section 3.1 was published as: Pribowo, A., Arantes, V., and Saddler, J.N. 2012. The adsorption and enzyme activity profiles of specific *Trichoderma reesei* cellulase/xylanase components when hydrolyzing steam pretreated corn stover. Enzyme Microb Technol. 50(3):195-203. I performed the experimental work and drafted the manuscript. Valdeir Arantes and Jack Saddler contributed to the planning, data interpretation and the writing of the manuscript.

A version of section 3.2 was published as: Pribowo, A., Hu, J., Arantes, V., and Saddler, J.N. 2013. The development and use of an ELISA-based method to follow the distribution of cellulase monocomponents during the hydrolysis of pretreated corn stover. Biotechnol Biofuels. 6:80-95. I performed the experimental work and drafted the manuscript. Jinguang Hu provided the purified enzymes used in the study. Valdeir Arantes and Jack Saddler contributed to the planning, data interpretation and writing of the manuscript.

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List of Abbreviations

AA9	auxiliary activity family 9
AFEX	ammonia-fiber explosion
AIL	acid-insoluble lignin
Ara	arabinan
ASL	acid-soluble lignin
Avi	avicel
BSA	bovine serum albumin
C II	cellulose II
C III	cellulose III
CBH	cellobiohydrolase
CBM	carbohydrate binding module
CD	catalytic domain
CMC	carboxymethyl cellulose
CNC	cellulose nanocrystals
DAS-ELISA	double-antibody sandwich enzyme-linked immunosorbent assay
DP	degree of polymerization
DsP	dissolving pulp
DSPCS	delignified steam pretreated corn stover
EG	endoglucanase
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
FPU	filter paper units

FTIR	Fourier-transform infrared
g	gram
Gal	galactan
GC	gas chromatography
GH61	glycoside hydrolase family 61
Glu	glucan
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
IU	international unit
kDa	kilo-Dalton
L	litre
LCC	lignin carbohydrate complex
m	meter
Man	mannan
mg	milligram
mL	millilitre
mmol	millimole
M	mole per litre
MAb	monoclonal antibody
MW	molecular weight
MUC	4-methylumbelliferryl β-D-cellobioside
MUL	4-methylumbelliferryl β-D-lactoside
MUG_3	4-methylumbelliferryl β-D-cellotrioside
NaOH	sodium hydroxide

NDn	not determined
nm	nanometer
Pab	polyclonal antibody
PASC	phosphoric acid swollen cellulose
PMO	polysaccharide mono-oxygenase
PNPC	*p*-nitrophenyl-β-D-cellobioside
PNPG	*p*-nitrophenyl-β-D-glucopyranoside
PNPX	*p*-nitrophenyl-β-D-xylopyranoside
s	second(s)
SS	Simon's Stain
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SO_2	sulphur dioxide
SPCS	steam pretreated corn stover
t	time
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
XPS	X-ray photoelectron spectroscopy
XSPCS	xylanase treated steam pretreated corn stover
Xyl	xylan
Xyn	xylanase

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Dedication

This thesis is dedicated to the FPB group. Thank you for a wonderful experience in the past 5 years.

1. Introduction

The transition from a fossil fuel-based economy to a sustainable bioeconomy requires a robust development of biorefineries where fuels and chemicals can be produced economically from renewable biomass resources such as lignocellulosic biomass (Bozell and Petersen, 2010; Ragauskas et al., 2006; Kamm and Kamm, 2004; Wijffels and Barbosa, 2010; Alonso, Bond and Dumesic, 2010). The use of lignocellulosic biomass such as agricultural and forest residues as feedstocks is particularly desirable because it adds value to waste materials and avoids the use of edible crops such as sugar, starch, vegetable oils, etc., for energy/ chemical production. Lignocellulosic biomass can be converted into power, heat, liquid fuels, and chemicals through either the thermochemical or biochemical routes (McKendry, 2002). Compared to the thermochemical route, the biochemical conversion of biomass to a sugar platform to produce renewable fuels and chemicals is preferred as it uses milder processes and can operate at a smaller economy of scale, allowing the use of local biomass residues compared to the thermochemical conversion which typically requires harsh conditions, such as high temperatures, pressures, or chemical concentrations, and a much larger amount of biomass to be economically feasible (Pu et al., 2008; Stephen, Mabee and Saddler, 2010). The ability to be economical at a small scale and to use local biomass resources is important for biorefineries as the low density of lignocellulosic biomass makes transporting large volume of biomass over long distance challenging and costly (Shabani, Akhtari and Sowlati, 2013).

The biochemical route from lignocellulosic biomass to sugars typically involves a pretreatment step to reduce biomass recalcitrance and an enzymatic saccharification step to hydrolyze cellulose and other sugar polymers to their monomeric sugars. The resulting simple sugars can subsequently be converted into a number of fuels and chemicals such as cellulosic ethanol via glucose fermentation to ethanol. Although extensive work has been done to improve the pretreatment technologies to break

open and increase the ease of enzymatic degradation of the biomass substrate, high enzyme loadings are still required for effective enzymatic hydrolysis of pretreated biomass (Arantes and Saddler, 2010; Klein-Marcuschamer et al., 2012). As a result, the enzymatic saccharification step remains the third most expensive cost component after capital and feedstock costs in the bioconversion of biomass to ethanol (Stephen, Mabee and Saddler, 2012; Isola, 2013; Pu et al., 2008; Aden and Foust, 2009).

Four ways of reducing the cost of enzymatic hydrolysis have so far been suggested. Improving the efficiency of pretreatment strategies to increase cellulose digestibility (Alvira et al., 2010; Mosier et al., 2005; Galbe and Zacchi, 2012); minimizing the cost of enzyme production (Merino and Cherry, 2007; Wilson, 2009); increasing the specific activity of the enzymes (Darias and Villalonga, 2001; Boer and Koivula, 2003); or recycling the enzymes for multiple rounds of hydrolysis (Tu et al., 2006; Tu, Chandra and Saddler, 2007). Significant research efforts over the last decade have reportedly lowered enzyme costs by 72-80% for a litre of ethanol (Isola, 2013). This reduction in enzyme cost has been achieved primarily by lowering the costs of enzyme/protein production and improving the efficiency of individual enzymes or enzyme mixtures (Banerjee, Scott-Craig and Walton, 2010). Following this significant cost reduction, the production costs of cellulase enzymes are estimated at around $10.14/kg of protein (Klein-Marcuschamer et al., 2012), which is similar to the production costs of amylase at around $10.40/kg of protein (de Castro et al., 2010).

Despite this similarity in production costs, the amount of protein required to achieve effective hydrolyses varies considerably! A recent analysis indicates that the cost contribution of enzymes in the production of ethanol from corn stover (biomass) is in the range of $0.13 - $0.38/ litre of ethanol, based on the amount of enzyme/protein required (Riisgaard, 2010; Klein-Marcuschamer et al., 2012). This is in stark contrast to the enzyme cost contributions to the production of corn ethanol which is estimated at $0.01/ litre of ethanol (McAloon et al., 2000). The 10 – 40x higher cost contribution of cellulase enzymes

compared to that of amylase enzymes is caused predominantly by the high enzyme loading needed to hydrolyze the highly recalcitrant lignocellulosic biomass compared to the easily hydrolyzed starch. It is estimated that 15 – 50 g of cellulase enzymes is needed for every kg of lignocellulosic biomass compared to 0.2 – 0.3 g of amylase enzymes/ kg of starch (Table 1). This high cost contributes to cellulosic ethanol being 40% more expensive than corn ethanol (Isola, 2013).

Table 1. Comparison of the use of enzymes for the production of cellulosic ethanol and corn ethanol

	Cellulosic Ethanol	**Corn Ethanol**
Ethanol production costs (US$/litre)	0.94[1]	0.67[1]
Enzyme costs (US$/litre ethanol)	0.13 – 0.38[2]	~0.01[3]
Enzyme contribution to ethanol production costs (%)	14 – 40[4]	~1.5[3]
Enzyme Quantity (g/kg cellulose or starch)	~10 – 50[5]	0.2 – 0.3[3]

Data obtained or adapted from (Isola, 2013)[1], (Klein-Marcuschamer et al., 2012)[2], and (McAloon et al., 2000)[3], (Stephen, Mabee and Saddler, 2012)[4], (Arantes and Saddler, 2011)[5]

It has been suggested that further reduction in enzyme production costs will be difficult to achieve. However, significant savings in the overall hydrolysis step could be gained if lower enzyme/protein loadings could be used to achieve efficient hydrolysis yields (Klein-Marcuschamer et al., 2012). Of the four enzyme cost reduction strategies mentioned earlier, a combination of improvements in pretreatment, increases in the hydrolytic potential of enzyme cocktails / specific enzyme activities, and the ability to re-use the enzymes may lead to better saccharification yields with reduced enzyme loading. Improving the activities of individual cellulase enzymes and the efficiency of enzyme cocktails is an area of ongoing research with companies such as Novozymes and Genencor continuing to develop and release their latest generation of “cellulase” enzyme mixtures (Lane, 2012).

As the hydrolytic potential of “cellulase enzyme mixtures” continues to improve (partly as a result of the inclusion of “accessory” enzymes and enzymes with higher activities and stability), recycling

these highly active and stable enzymes becomes an increasingly attractive enzyme-cost reduction strategy. Partly due to these reasons, a renewed interest in this area has recently developed, although research on cellulase enzyme recycling has been conducted for many years (Lee, Yu and Saddler, 1995; Deshpande and Eriksson, 1984; Gregg and Saddler, 1996). There have been several published studies on enzyme recycling which have explored various strategies, including enzyme immobilization (Gomez et al., 2010; Gomez et al., 2012), enzyme precipitation (Xu and Chen, 2009), membrane filtration (Qi et al., 2011; Yang et al., 2010a), enzyme desorption (Tu and Saddler, 2010; Ouyang et al., 2010; Rodrigues et al., 2012), recycling of the solid residues back to the subsequent round of hydrolysis (Weiss et al., 2013; Jin et al., 2012), and the addition of fresh substrates (Xue, Jameel and Park, 2012; Qi et al., 2011). Work has also been carried out to investigate the various enzyme characteristics that may influence their recoverability (Skovgaard and Jorgensen, 2013; Varnai, Siika-aho and Viikari, 2013; Du et al., 2012). The following section provides a summary of the various enzyme recycling strategies that have been assessed.

1.1 Enzyme Recycling Strategies

An effective enzyme recycling strategy should ideally achieve maximum recovery of the cellulase and auxiliary enzymes/ proteins with a minimum loss of sugars and enzyme activities and a minimum carry-over of residual substrate, sugars, ethanol, and hydrolysis inhibitors to the next round of hydrolysis. As cellulase enzymes are typically partitioned, to various extents, into the liquid phase or adsorbed onto the substrate (solid phase) during hydrolysis, maximizing enzyme recovery from these two phases will likely be key in the development of an effective enzyme recycle strategy. In the following sections, various enzyme recycle strategies are briefly reviewed and their effectiveness compared.

1.1.1 Enzyme Immobilization

Immobilization of cellulase enzymes on a solid support has been extensively studied with a primary objective of facilitating enzyme recovery and their reuse for cellulose hydrolysis (Gokhale and Lee, 2012). To date, most enzyme immobilization studies have focused on the enzyme β-glucosidase using various solid supports (Gomez et al., 2010; Gomez et al., 2012; Gomez, Romero and Fernandez, 2005). There are several reasons behind the efforts to immobilize this enzyme. First, unlike most of the other cellulase enzymes, β-glucosidase does not require adsorption to cellulose for its activity as it hydrolyzes the soluble substrate cellobiose into glucose in solution. Therefore, immobilization of the β-glucosidase enzyme has been shown not to impede enzyme activity and the diffusion of substrate into the enzyme's active site (Tu et al., 2006; Gomez et al., 2010). As this enzyme functions in the liquid phase, it is typically not recovered when enzymes are recycled by using readsorption to fresh substrates or by recycling the solid residues (Qi et al., 2011; Tu et al., 2009a).

Enzymes have typically been immobilized on various solid materials using three modes of attachment, namely direct physical adsorption on the surface of the solid materials, covalent binding, or encapsulation within the nanopores of the solid materials (Gokhale and Lee, 2012). A range of support materials have been used for enzyme immobilization, including nanoclay materials, gold nanoparticles, activated carbon, soil colloidal particles, liposomes, and latex colloidal particles (Gokhale and Lee, 2012). Direct physical adsorption is the simplest technique to immobilize enzymes on a solid carrier. Enzymes can adsorb on solid supports through hydrophobic interaction, hydrogen bonding, van der Waals forces, or electrostatic interaction (Gomez, Romero and Fernandez, 2005; Hanefeld, Gardossi and Magner, 2009). In addition to being relatively simple, direct physical adsorption of cellulase enzymes to a solid carrier may improve the enzyme's catalytic potential and facilitate enzyme recycling (Daoud, Kaddour and Sadoun, 2010; Yan et al., 2010). Direct adsorption of β-glucosidase to soil colloids, for example, has

been shown to improve enzyme activity due to the presence of metallic residues in the soil colloids and the formation of enzyme-metal complexes, which seemed to facilitate better substrate binding (Gokhale and Lee, 2012). Despite these advantages, this binding mechanism has its own drawbacks, including leaching of cellulase enzymes, for example due to cellulase enzymes' stronger affinity to cellulose than to the solid support, and loss of enzyme activity due to enzyme conformational changes upon adsorption (Yan et al., 2010; Gomez, Romero and Fernandez, 2005).

Enzymes can be covalently linked to a solid support in order to minimize enzyme leaching. However, covalently binding the enzyme requires the linkage of appropriate reactive groups within the enzyme structure with the support without obstruction of the active site. A decrease in enzyme activity has generally been observed upon covalent binding of enzymes to a solid support due to reasons such as long cross-linking time and reduction in enzyme mobility (Gomez et al., 2012).

Enzymes can also be encapsulated within an inorganic or polymeric matrix. By utilizing the size difference between the enzyme and the substrate or product molecule, some encapsulation media can be chosen to result in the free flow of substrate or product molecules from the bulk medium to the enzyme active site (Gokhale and Lee, 2012). A number of factors influence the efficiency of this method of enzyme immobilization, including pore size (Ortega, Busto and Perez-Mateos, 1998; Takimoto et al., 2008), the charge of the encapsulating matrix (Ortega, Busto and Perez-Mateos, 1998), shapes (Chang, Jang and Wu, 2011), and the functional groups present in the matrix (Hartono et al., 2009; Hartono et al., 2010). Immobilization of enzymes within the nanopores of a solid support similarly suffers from enzyme leaching as it relies more on a physical mode of attachment rather than covalent binding. It is worth noting that this method may not be suitable for enzymes that require direct adsorption on cellulose for their activity.

Cellulosomes, produced by anaerobic microorganisms, are nature's answer to immobilization of cellulase enzymes for efficient cellulose hydrolysis. The use of scaffoldin proteins as a support material for enzyme immobilization has recently gained support (Gokhale and Lee, 2012). This approach includes efforts to engineer "designer cellulosomes" to accommodate various cellulase and hemicellulase enzymes (Mingardon et al., 2007; Morais et al., 2011; Caspi et al., 2006), as well as attaching various catalytic domains (CDs) and carbohydrate binding modules (CBMs) on streptavidin or streptavidin-functionalized inorganic particles (Kim et al., 2011). Clustering of CDs and CBMs has been found to result in an increase in catalytic efficiency and enzyme-substrate interaction compared to the native free enzymes (Gokhale and Lee, 2012).

Enzyme immobilization as an enzyme recycling strategy may be appropriate for only a few enzymes, such as the β-glucosidase enzyme, that does not require adsorption on the lignocellulosic substrate for its activity. Hence, in order to facilitate the recovery of other cellulase enzymes, this strategy will likely need to be combined with other strategies such as membrane filtration. Furthermore, for this strategy to be commercially useful, more research is needed to improve the stability of immobilized enzyme activities and to assess and reduce the costs of enzyme immobilization.

1.1.2 Enzyme Precipitation

After hydrolysis, 40-60% of the original cellulase enzyme loading typically remains in the solution (Xu and Chen, 2009; Nakagame, Chandra and Saddler, 2010; Lu et al., 2002). Therefore, a strategy to recover enzymes from the liquid phase is important. Such a strategy was proposed by using tannin flocculation followed by PEG desorption (Xu and Chen, 2009). When using 5% (w/v) tannin and 1% (w/v) NaCl, these workers found that about 95% of the cellulase enzymes in the hydrolysate were precipitated into tannin-cellulase compounds. Cellulase enzymes were then recovered by desorption from these tannin-cellulase compounds using 1.5% (w/v) polyethyleneglycol (PEG) 6000. Although the

authors claimed that most of the cellulase enzymes in the liquid phase could be recovered by this strategy and that the use of PEG seemed to increase enzyme activities, enzyme recovery using enzyme precipitation has to be further explored to determine its practicality and efficiency.

1.1.3 Membrane Ultrafiltration

The use of membrane ultrafiltration is another strategy that has been widely explored to recover enzymes from the liquid phase. As most cellulase enzymes have molecular weights above 15 kDa (Vinzant et al., 2001), membranes with a molecular weight cut-off of 10 - 30 kDa are typically used to retain cellulase enzymes, while allowing sugars or ethanol to flow through (Yang et al., 2010a; Ramos and Saddler, 1994; Qi et al., 2012; Mores, Knutsen and Davis, 2001). The enzymes recovered in the retentate can then be reused for the next round of hydrolysis. Surfactants are usually added to the hydrolysis reaction, not only to improve hydrolysis efficiency, but also to increase the amount of cellulase enzymes present in the hydrolysate through desorption and prevention of enzyme unproductive adsorption to lignin. A filtration step to remove residual substrate from the hydrolysate can be carried out prior to a membrane ultrafiltration step (Steele et al., 2005). Similarly, the hydrolysis products in the filtrate, following the ultrafiltration step, can be further concentrated using nano-filtration (Qi et al., 2012).

The membrane ultrafiltration strategy is an effective strategy to recover most, if not all of the enzymes from the hydrolysate in their active form and to minimize the carry-over of hydrolysis or fermentation products. Incorporation of this strategy into a hydrolysis strategy using membrane bioreactors has also been shown to improve the hydrolysis rate and yield due to removal of sugars and other inhibitory compounds (Zhang et al., 2011). However, this strategy suffers from a number of drawbacks such as membrane fouling caused by solid lignocellulosic particles which would render the ultrafiltration inefficient (Lipnizki, 2010). As some enzymes may remain tightly bound to the solid

substrate, recovering enzymes from the solution alone may not significantly enhance the efficiency of hydrolysis (Knutsen and Davis, 2004). Also, the practical application and the cost efficiency of using membranes at a commercial scale have yet to be demonstrated.

1.1.4 Enzyme Desorption

Cellulase enzymes adsorbed to hydrolysis residues can be desorbed to facilitate enzyme recycling from the solid phase. Various desorption methods have been explored, including addition of buffer, high salt solutions, surfactants such as Tween, urea, polyhydric alcohols such glycerol and polyethylene glycol, and alkali (Tu and Saddler, 2010; Rodrigues et al., 2012; Du et al., 2012; Zhu, Sathitsuksanoh and Zhang, 2009; Tu et al., 2009b; Otter et al., 1989; Eriksson, Borjesson and Tjerneld, 2002b). Among these reagents, enzyme desorption in an alkaline solution at pH 13 and desorption using 72% (w/v) polyethylene glycol at neutral pH has been suggested to be among the most effective methods evaluated, although desorption at pH 13 resulted in cellulase deactivation (Zhu, Sathitsuksanoh and Zhang, 2009).

One study which tried to determine key factors influencing enzyme desorption found that pH, temperature, and surfactant concentrations were among the major factors which governed enzyme desorption from residual substrate (Tu et al., 2009a). Acidic pH (<4.8) has been shown to favor adsorption while desorption is favored at higher pH (Du et al., 2012). Enzyme denaturation at high pH is a major concern with using enzyme desorption as an enzyme recycling strategy. It was found that the effect of pH on enzyme activities and adsorption was temperature dependent. At high temperature and pH (for example 50^{o}C and pH 10), cellulase enzymes may lose their activities irreversibly, while at moderate temperature and pH (25^{o}C and pH 7, for example), almost 100% of cellulase activities could be restored upon adjusting the pH back to 4.8 (Du et al., 2012). Other workers also showed that more than 90% of the active cellulases could be recycled after hydrolysis of pretreated corn stover by washing at

pH 10 at 4^{o}C (Zhu, Sathitsuksanoh and Zhang, 2009). The desorption and reversibility of cellulase activities upon changes in pH were further investigated by other workers who used an alkaline wash treatment at pH 9 and 10 at room temperature for 2 hours to desorb Cel7A from lignin and showed that Cel7A underwent conformational changes at these high pHs which were reversible upon changing the pH back to 4.8 (Rodrigues et al., 2012). These reversible changes in protein conformation, or reversible enzyme denaturation, may explain the mechanism of enzyme desorption from lignocellulosic substrates and the reversibility of enzyme activities when the pH is reset to 4.8. These authors also suggested that it was more difficult to desorb enzymes from cellulose than from lignin using the alkaline elution method (Rodrigues et al., 2012). In contrast, Zhu *et al*. (2009) observed a higher enzyme desorption from Avicel compared to dilute-acid pretreated corn stover (Zhu, Sathitsuksanoh and Zhang, 2009). Therefore, more work is needed to better understand the mechanism of enzyme desorption from cellulose and lignocellulosic substrates, including the possibility of enzyme desorption due to changes in the surface charge of enzymes (and substrates) at a higher pH.

There are two primary advantages of the enzyme desorption method as an enzyme recycling strategy. First, enzyme desorption removes adsorbed cellulase enzymes from the solid phase back to the liquid phase. Therefore, following desorption, both the desorbed enzymes and liquid phase enzymes can be recovered from the liquid phase using methods such as membrane ultrafiltration or adsorption to fresh substrate (Qi et al., 2011; Xue, Jameel and Park, 2012). Second, following desorption, the enzyme-free hydrolysis residues can be removed from the recovery streams, and thus the accumulation of solid residues from the previous hydrolysis rounds can be minimized. The potential drawbacks of this enzyme desorption method include the irreversible loss of enzyme activities in harsh (high pH and high temperature) conditions or certain enzyme activities even at mild desorption conditions, the potential high costs associated with the use of desorbing agents, and in the case of alkaline elution, the high amount of salt that can be generated from constant adjustment of pH and its associated disposal costs.

As a further enzyme recovery strategy is still required to recover the desorbed enzymes from the liquid phase, the same drawbacks that currently limit the application of these enzyme recovery strategies will apply to the desorbed enzymes.

1.1.5 Recycling of Solid Residues

Recycling the recalcitrant, incompletely hydrolysed residue with adsorbed enzymes to the next round of hydrolysis is another way of recycling the cellulolytic enzymes. Previous work that looked into recycling increasing amount of solid residues after hydrolysis of dilute acid pretreated corn stover found that an increase in glucose production by ~15% of theoretical conversion yield was achieved when the amount of solid residues recycled was increased from 50% to 100% after washing (Weiss et al., 2013). By combining fresh enzyme supplementation with recycling of residual solids, these workers were able to decrease the enzyme loading by 30%, while achieving the same glucose yield (Weiss et al., 2013). Washing the solid residues prior to recycling was shown to also help improve the efficiency of hydrolysis, which was likely due to the removal of sugars and other soluble inhibitory compounds that may be trapped in the solid residue matrix (Weiss et al., 2013). When other researchers (Jin et al., 2012) recycled enzymes associated with the unhydrolyzed residues of AFEX^{TM} (ammonia fiber expansion)-treated corn stover, after separate hydrolysis and fermentation (SHF) and simultaneous saccharification and co-fermentation (SSCF) they managed to reduce enzyme loadings by ~38% and 28%, respectively. It appears that the enzymes which adsorbed to the lignin-rich hydrolysis residues maintained at least some of their activity. However, the retention of activities of enzymes adsorbed to lignin was likely influenced by the type of lignin and enzyme adsorption conditions. For instance, enzymes adsorbed to softwood lignin were shown to lose their activities at 45^{o}C but retained most of their activities at 4^{o}C. The thermal denaturation of enzymes upon adsorption to lignin was thought to be cause of this loss of enzyme activity (Rahikainen et al., 2011).

Simplicity and cost effectiveness are among the key strengths of this enzyme recycling strategy. The main limitation of this method is the increasing total solid content and reaction volume with increasing hydrolysis rounds which is likely to hamper hydrolysis efficiency. However, an increasing amount of lignin-rich residues derived from hydrolysis of dilute acid pretreated corn stover was shown not to have an inhibitory effect on the conversion efficiency at low solid hydrolysis conditions (Weiss et al., 2013). This is likely due to the properties of the lignin-rich residues used in this study. Previous work has shown that softwood-derived lignin has a more detrimental effect on hydrolysis yield compared to hardwood and agricultural residue-derived lignin (Nakagame, Chandra and Saddler, 2010). Therefore, the use of substrates with low lignin content and low inhibitory lignin is important for a successful implementation of this strategy. Another drawback of this method is the loss of enzymes in the liquid phase. As a result, a significant amount of fresh enzymes (~70% of the initial loading) typically needs to be added to each recycle round to maintain high conversion efficiency (Weiss et al., 2013; Jin et al., 2012).

1.1.6 Addition of Fresh Substrate

The addition of fresh substrate utilizes the cellulase enzymes' natural affinity to lignocellulosic substrates to readsorb and recover the enzymes in the liquid phase. Moreover, enzymes adsorbed to hydrolysis residues can be concurrently recovered to maximize enzyme recovery. Previous work which compared the efficiency of enzyme recovery from the liquid phase, (using readsorption to fresh substrate versus ultrafiltration, following hydrolysis of pretreated wheat straw), concluded that the hydrolysis efficiencies of the two strategies were comparable, provided that fresh β-glucosidase was added to complement enzymes recovered using readsorption to fresh substrate (Qi et al., 2011). This result highlights the efficiency of enzyme readsorption to fresh substrate in recovering most of the enzyme activities in the liquid phase. This work also showed that direct recycling of enzymes bound to

the solid residues resulted in better hydrolysis yields compared to a desorption method to recover the solid-phase enzymes (Qi et al., 2011). Previous studies done in our group to recycle enzymes used in hydrolysis of steam exploded hardwood (birch and eucalyptus) showed that recycling enzymes from both the unhydrolyzed residues and the liquid phase resulted in maximum hydrolysis efficiency (Lee, Yu and Saddler, 1995; Ramos, Breuil and Saddler, 1993). The efficiency of this enzyme recycling strategy was further enhanced when enzymes were recycled from partially delignified steam exploded birch substrate (Lee, Yu and Saddler, 1995). Related work emphasised the importance of a washing step after an enzyme readsorption step to enhance hydrolysis performance of recycled enzymes by reducing the amount of sugars carried over to the subsequent hydrolysis round (Xue, Jameel and Park, 2012). Overall, these studies demonstrated the advantage of enzyme recycling strategy using addition of fresh substrate in its ability to recover enzymes from both the liquid and the solid phase in one step. This simple recovery step can potentially minimize the costs needed to achieve maximum recovery of enzymes.

This strategy nonetheless suffers from several drawbacks such as an increase in the total solid content containing lignin-rich residues and reaction volumes (Lee, Yu and Saddler, 1995). Due to the low affinity of certain enzymes such as β-glucosidase, this strategy will also likely require fresh enzyme supplementation to maintain high hydrolysis yields, although at a reduced enzyme loading compared to recycling the residual solids alone. Therefore, to minimize these drawbacks, several factors seem to be important for the success of addition of fresh substrates as an enzyme recycling strategy. These include recovering the enzymes from both the solid and the liquid phases to ensure maximum enzyme recovery, achieving efficient hydrolysis in each hydrolysis round to minimize the amount of unhydrolyzed residues that are recycled to the next round, and having substrates with low lignin contents or low inhibitory lignin to minimize the amount of hydrolysis residues and to minimize the adverse effects of lignin on hydrolysis efficiency (Qi et al., 2011; Lee, Yu and Saddler, 1995).

1.1.7 Other Studies on Enzyme Recycling

To investigate the potential of enzyme recycling after distillation, some researchers explored the influence of ethanol and high temperature on the stability and activities of thermostable and mesophilic enzyme mixtures (Skovgaard and Jorgensen, 2013). It was found that ethanol had a destabilizing effect on enzyme activities when the enzyme mixtures reached their maximum temperature limit. Similar ethanol concentrations resulted in faster deactivation of enzymes at the upper temperature limit for enzyme activity compared to lower temperatures. Since the distillation temperature of ethanol is around 78^{o}C, enzyme recycling after distillation will only be possible with highly thermostable enzymes.

In other work that investigated the role of carbohydrate-binding modules (CBMs) in enzyme recycling following high solid hydrolysis, the authors (Varnai, Siika-aho and Viikari, 2013) suggested that CBMs may not be essential during high solid loading hydrolysis. This may be due to the proximity function of CBMs which may be less crucial in a low water environment such as during high solid hydrolysis compared to a high water environment (discussed in detail in Section 1.4.2.2) (Varnai, Siika-aho and Viikari, 2013). Thus the use of CBM-free cellulase enzymes was suggested to be advantageous for high solid hydrolysis as it was proposed to minimize unproductive binding of enzymes to the lignin-rich residue and facilitate enzyme recovery. However, the attainment of a high hydrolysis yield using CBM-free enzymes during high-solid loading hydrolysis is yet to be demonstrated.

1.2 Enzyme-Substrate Interaction and Enzyme Recycle Strategies

A central theme emerging from these previous enzyme recycle studies suggested that better understanding the dynamics of enzyme-substrate interactions and the distribution of enzymes in the liquid and solid phases during hydrolysis would be crucial in the development of an effective enzyme recycling strategy. Unfortunately, our efforts to better understand this fundamental interaction have been limited by two main challenges. First, the complexity of enzyme-substrate interactions, which

involve heterogeneous substrates, complex enzyme systems, and physical factors such as pH, temperature, and various soluble compounds all result in substantial challenges. Second, the lack of an effective methodology to specifically and quantitatively assay individual enzymes also makes work in this area challenging. As a result, this lack of detailed understanding on individual enzymes' interaction with the substrates has hampered the successful development of an efficient enzyme recycling strategy.

Due to these limitations, many previous enzyme adsorption studies have been carried out using purified enzymes and relatively simple, model cellulosic substrate. However, a comprehensive understanding of enzyme-substrate interaction will only be obtained by using a complete enzyme mixture on an industrially-relevant lignocellulosic substrate. In order to provide a perspective of the challenges in understanding individual enzyme-substrate interactions and to provide some insights to better develop improved enzyme recycling strategies, the next section of the thesis will review the various assays used in enzyme adsorption studies, along with their strengths and limitations. A brief review of the influence of substrate, enzyme, and physical factors in enzyme-substrate interaction is also added.

1.3 Assays for Determining Enzyme Adsorption Profiles

To be able to follow the distribution and activity profiles of individual enzymes during hydrolysis requires the identification of the enzymes of interest, their distribution within the solid or liquid phase, and the measurement of changes in their concentration and activity during hydrolysis. Throughout the history of cellulase research, a number of assays have been developed to both identify and measure the activity of the cellulase enzymes (Sharrock, 1988; Ghose, 1987). Despite the extensive number of assays available, there is a general agreement among researchers that one of the main challenges in cellulase research is the limited availability of simple, specific, and quantitative assays for individual cellulase enzymes. This section of the thesis has reviewed some of the assays that have been used in cellulase

research, including assays that have been recently developed or applied for cellulase enzyme adsorption studies. With respect to the use of these assays, they can be categorized into five groups of, general protein assays, protein labeling assays, antibody-based assays, chromogenic substrate-based assays, and other approaches for measuring cellulase activities.

1.3.1 General Protein Assays

Protein assays are grouped together because they can be used for protein quantification and identification, but not for enzyme activity measurement. These assays include chromatographic techniques to fractionate a mixture of proteins down to their individual protein components, gel electrophoresis to visualize proteins, mass spectrometry to identify proteins and various assays to measure protein concentrations.

Chromatographic techniques are normally employed to separate individual proteins from a mixture of proteins (Tenkanen, Puls and Poutanen, 1992; Yu, Lee and Saddler, 1993; Medve et al., 1998; Medve, Lee and Tjerneld, 1998; Medve, Stahlberg and Tjerneld, 1994). These techniques have been used to separate different enzyme components in a cellulase mixture after hydrolysis to determine the distribution of individual enzymes (Yu, Lee and Saddler, 1993). Proteins in isolated fractions can be subsequently identified using mass spectrometry or gel electrophoresis. Mass spectrometry is a powerful method to qualitatively identify specific proteins in a mixture of proteins (Vinzant et al., 2001).

Gel electrophoresis has been the standard assay to separate proteins based on their molecular weight (sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS-PAGE), electric charge (isoelectric focusing or IEF), or both (two-dimensional gel electrophoresis). Due to its simplicity, these assays are particularly useful in determining enzyme identity and purity when using purified enzymes. However, standard SDS-PAGE or IEF is typically not adequate to completely separate a cellulase mixture down to its individual enzyme components as most of the cellulase enzymes have similar molecular

weights and/ or isoelectric points (pI) and are present in multiple forms (Vinzant et al., 2001). Although concentrations of individual protein bands can be estimated based on their density, these techniques are only semi-quantitative.

Quantitative measurements of protein concentrations can be done by using techniques such as (ultraviolet) UV absorbance, Bradford, Lowry, bicinchoninic acid (BCA), ninhydrin, Kjeldahl, nitrogen absorption assays, and measurements with an elemental or CN analyzer (Haven and Jorgensen, 2013; Kumar and Wyman, 2008; Zhu, Sathitsuksanoh and Zhang, 2009). Some of these assays can only be used to measure proteins in a liquid sample, while others can be used for solid samples. The UV, Bradford, Lowry, BCA, and ninhydrin assays are routinely used to quantify the concentration of proteins in liquid solutions. Among these five assays, the ninhydrin assay is thought to exhibit the least interference from lignin, sugar and other materials that may be released during hydrolysis of lignocellulosic substrates and, therefore, has been used to quantify concentrations of cellulase enzymes during biomass hydrolysis (Zhu, Sathitsuksanoh and Zhang, 2009; Kumar and Wyman, 2008).

The Kjeldahl assay, nitrogen absorption assay, and a modified ninhydrin assay can be used to directly quantify the amount of proteins adsorbed to the solid surface. The Kjeldahl procedure is suitable to quantify proteins in solid or semi-solid samples. Therefore, it could potentially be applied to directly measure the amount of cellulases adsorbed to the substrates. This method, however, is tedious and time-consuming and involves the use of hazardous and corrosive chemicals (Wallace and Fox, 1998). A procedure based on nitrogen element analysis has recently been developed to measure protein adsorbed on a solid substrate (Kumar and Wyman, 2008). As well as a requirement for an expensive instrument, the presence of nitrogen-containing compounds in the substrate may obscure the results when using this method (Zhu, Sathitsuksanoh and Zhang, 2009). In response to these limitations other

workers have developed a method using the ninhydrin reagent to directly measure protein adsorption to the substrate (Zhu, Sathitsuksanoh and Zhang, 2009).

One recent study has suggested that the ninhydrin assay seems to be the preferred method in cellulase research to quantify the total concentration of proteins in the liquid and solid fractions (Haven and Jorgensen, 2013). While this technique is useful to measure the total amount of proteins in either fraction, it is not free from interference by sugars and other components that are found in a typical hydrolysate (Haven and Jorgensen, 2013; Schilling, Burchill and Clayton, 1963). In addition, more specific techniques must be employed to determine the adsorption profiles of individual enzyme components in the mixture.

1.3.2 Protein Labeling Assays

A number of researchers have explored the use of fluorescent or radioactive labeling to study enzyme adsorption and substrate accessibility (Hong, Ye and Zhang, 2007; Kawakubo et al., 2010; Wang, Wang and Ragauskas, 2010; Linder and Teeri, 1996; Nidetzky and Claeyssens, 1994; Nidetzky, Steiner and Claeyssens, 1994). Fluorescent proteins have been fused to cellulose binding modules (CBMs) to investigate cellulose accessibility to cellulase (Hong, Ye and Zhang, 2007), or to probe specific binding sites of different CBMs on pretreated substrates (Kawakubo et al., 2010). Constructing a fluorescent-labeled recombinant protein requires molecular biology techniques and is currently limited to small proteins as no work has been done to fuse an intact cellulase enzyme with a fluorescent protein. A recent publication reported a novel application of the fluorescence resonance energy transfer (FRET) method utilizing enzymes and carboxymethyl cellulose (CMC) labeled with fluorescent dyes to probe cellulase-cellulose interaction (Wang, Wang and Ragauskas, 2010). This method is discussed in detail later in the thesis.

Similarly, CBMs have also been labeled using radioactive isotopes to investigate their adsorption behaviour (Linder and Teeri, 1996). Radioactive labeling of an intact cellulase enzyme Cel6A with ^{125}I has also been performed to assess its adsorption. The non-productive adsorption of Cel6A to cellulose was observed although the activity of the labeled Cel6A was fully retained (Nidetzky, Steiner and Claeyssens, 1994).

In summary, protein labeling is an effective method to tag an enzyme and follow its interaction with the substrate during the course of hydrolysis. Depending on the labeling techniques used, protein labeling may not influence the enzymes activity. Therefore the activity of the labeled enzyme could further be measured using a standard cellulase activity assay to obtain the enzyme's activity profile during hydrolysis. However, protein labeling techniques have their disadvantages. Labeling with fluorescent proteins has been limited to the construction of recombinant proteins of small peptides or protein modules. Labeling of proteins using fluorescent dyes or radioactive isotopes may lead to a loss in enzyme activity. Because of the lack of specificity, labeling with radioactive isotopes generally requires a purification step to isolate the protein of interest prior to the labeling procedure. Therefore, protein labeling may not be an appropriate method that could be used to study the adsorption profiles of multiple enzymes.

1.3.3 Gel-based Assays

Due to the limitations of the standard SDS-PAGE or IEF method, a number of techniques are available to aid protein identification using gel electrophoresis, namely the Western Blot and zymograms (Aho et al., 1991; Beguin, 1983; Dan et al., 2000; Sun et al., 2008). Using the Western Blot technique, antibodies are applied to detect specific proteins of interest after gel electrophoresis. Due to their specificity, monoclonal antibodies (MAbs) have been applied to both identify and quantify (semi-quantitatively) the different cellulase components secreted by *Trichoderma reesei* (or *Hypocrea jecorina*)

(Aho et al., 1991). MAbs can also be used to detect the different domains of a cellulase enzyme (Aho et al., 1991). However, this technique is not suitable for detecting the activity of individual enzymes and the identification of enzymes depends on the availability and specificity of the antibodies.

The use of zymograms is another gel-related technique that can be used to identify and quantify (semi-quantitatively) the concentration of active cellulase enzymes. Zymograms help visualize enzyme activities after separation of the enzyme components under non-denaturing conditions using SDS-PAGE, IEF, or 2-dimensional SDS-PAGE (Beguin, 1983; Dan et al., 2000; Sun et al., 2008; SCHWARZ et al., 1987). This is done using the substrate in an overlaying gel and incubating it with the gel containing separated proteins. When CMC, xylan, or other polysaccharides are used as the substrate, the enzymatic activity can then be visualized as a clear zone against a dark background after the gel has been stained with Congo Red (Beguin, 1983; Dan et al., 2000; Sun et al., 2008; Schwarz et al., 1987). When a fluorescent substrate is used, the activity of enzymes for this substrate can be directly visualized under UV light (Sharrock, 1988). Therefore, this technique is useful for the identification of an enzyme based on its molecular weight and its activity on a particular substrate, as well as for the measurement of an enzyme's activity. However, this technique is highly dependent on the availability of a specific substrate for the enzyme of interest. Xylan, for example, can be hydrolysed by both endoglucanase (EG) I and xylanases (Lawoko et al., 2000). The multiplicity of an enzyme also limits the specificity of this technique.

1.3.4 Immunoassays

Antibodies raised against different cellulase components have been used to detect the presence and the concentrations of these enzymes in a reaction mixture (Riske, Eveleigh and Macmillan, 1990; Buhler, 1991; Kolbe and Kubicek, 1990). Two standard antibody-based techniques have been used in cellulase research, namely the Western blot technique and the enzyme-linked immunosorbent assay (ELISA). Western blot has been used to detect and quantify specific cellulase enzymes after gel

electrophoresis as described in the previous section (Aho et al., 1991). ELISA has also been used to identify and quantify the amount of a specific enzyme in culture filtrates and commercial enzyme preparations (Riske, Eveleigh and Macmillan, 1990; Den Haan et al., 2007). The ELISA method uses antibodies linked to a reporter enzyme to specifically recognize and bind a target compound in a mixture of compounds. This specific compound or protein can then be quantified by adding a substrate for the reporter enzyme and measuring the concentration of the product (Voller, Bartlett and Bidwell, 1978). Antibodies have also recently been used to visualize cellulase penetration of plant walls using an immuno-electron microscopy technique (Donohoe et al., 2009; Nieves et al., 1991).

Of these three techniques, the ELISA is the one that can give a true quantitative measurement of the level of a specific enzyme of interest. However, using antibodies alone will not give any information on the activity of the enzymes.

1.3.5 Substrate-dependent Assays

Various substrates are available to measure the total cellulase activity, the activity of a subset of enzymes in the cellulase mixture (for example the activity of the endoglucanases), or the activity of a specific enzyme component. Determination of the enzyme activity on these substrates can be done either by measuring the release of reducing sugars, release of chromogenic or fluorescent compounds, changes in viscosity, or in the case of dyed substrates, release of the dyed soluble fragments (Table 2).

The different methods and substrates commonly used in cellulase research have also been recently reviewed (Zhang, Himmel and Mielenz, 2006). As evident in Table 2 (adapted from (Zhang, Himmel and Mielenz, 2006)), the techniques that have been used to measure the activity of specific cellulase enzymes have been limited by the lack of specific substrates. There is currently no specific substrate for Cel7A, Cel6A, Cel12A, Cel61A, Cel45A and the hemicellulases. A recent *H. jecorina* enzyme characterization study lists some of the substrates commonly used for determination of various

hemicellulase activities (Sipos et al., 2010a). However, in most cases, multiple enzymes can act on these substrates. Xylanase activity, for example, is usually measured on xylan, but Cel7B is known to also display activity on xylan (Lawoko et al., 2000). Therefore, a new method is needed to overcome the limitation posed by the lack of substrates for specific enzymes.

Table 2. Methods of measuring cellulase activities (adapted from (Zhang, Himmel and Mielenz, 2006))

Enzyme	Substrate	Assay
Complete cellulase	Cotton	Solubilisation Estimation of cellulose in residue Release of reducing sugars Weight loss Loss in tensile strength
	Filter paper Hydrocellulose Avicel Solka Floc	Solubilisation Release of reducing sugars
	Dyed Avicel	Release of dyed soluble fragments
Cellobiohydrolase (CBH)	Avicel Hydrocellulose	Release of reducing sugars
	Dyed Avicel	Release of dyed cellobiose
	Amorphous cellulose	Solubilization: release of reducing sugars or decrease in turbidity
	Cellooligosaccharides	Increase in reducing sugars or analysis by High Performance Liquid Chromatography (HPLC)
Endoglucanase (EG)	Carboxymethylcellulose (CMC)	Release of reducing sugars
	Hydroxyethylcellulose	Decrease in viscosity
	Cellooligosaccharides	Increase in reducing sugars or analysis by HPLC
	Cotton	Swelling in alkali
	Amorphous cellulose	Solubilisation Release of reducing sugars Decrease in turbidity
H. jecorina Cel7A and Cel7B	*o*- or *p*-Nitrophenyl-β-D-cellobioside (with addition of gluconolactone to suppress β-glucosidases)(Deshpande, Eriksson and Pettersson, 1984)	Release of *o*- or *p*-nitrophenol
	4-methylumbelliferyl-β-D-lactoside (MUL) 4-methylumbelliferyl-β-D-cellobioside (MUC) (with addition of gluconolactone to suppress β-glucosidases) (Claeyssens and Aerts, 1992)	Release of fluorescent phenol

Enzyme	Substrate	Assay
H. jecorina Cel7B	MUL (with addition of cellobiose to suppress Cel7A) (Claeyssens and Aerts, 1992)	Release of fluorescent phenol
H. jecorina Cel5A	4-methylumbelliferyl-β-D-cellotrioside (MUG_3) (with addition of gluconolactone to suppress β-glucosidases) (Claeyssens and Aerts, 1992)	Release of fluorescent phenol
β-glucosidase	*o*- or *p*-Nitrophenyl-β-D-glucosides	Release of *o*- or *p*-nitrophenol
	Salicin Esculin	Release of glucose
	Cellobiose	Release of glucose
	Cellooligosaccharides	Release of reducing sugars

1.3.6 Other Approaches for Measurement of Cellulase Activity

As mentioned above, a lack of simple and quantitative assays to measure cellulase activities has been a major limitation in cellulase research. A number of studies have been done to expand the selection of techniques in our tool box for cellulase/ biomass research. One of the most recent methods uses a quartz crystal microbalance (QCM) piezoelectric-sensing technique to measure cellulase activity (Hu, Heitmann and Rojas, 2009). This method measures changes in frequency of a quartz crystal, which can be used to measure changes in the viscosity and density of a solution containing a cellulose substrate after enzymatic hydrolysis. The study shows that quantification of cellulase activity using QCM correlates with results obtained by measuring the actual reducing sugars (Hu, Heitmann and Rojas, 2009). A number of studies have also looked into improving standard cellulase assays through automation (Decker et al., 2003) or through the development of a miniaturized assay (King et al., 2009). These improvements have proven useful for high-throughput enzyme evaluations. An amperometric redox polymer-based biosensor which utilizes cellobiose dehydrogenase has also been used to determine the total concentration of soluble oligosaccharides. This method was claimed to be faster and

at least comparable with standard assays, such as the Somogyi-Nelson method, to measure reducing sugar concentrations (Hilden et al., 2001).

Another novel assay method utilized fluorescent microfibrils prepared using 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) as a grafting agent (Helbert et al., 2003). Although the concept of grafting dyes onto cellulose has been around for a while, the use of a fluorescent dye is thought to improve the assay sensitivity compared to the use of standard dyes such as Remazol Brilliant Blue, Reactive Orange, or Reactive Blue 19 (Zhang, Himmel and Mielenz, 2006; Helbert et al., 2003). The use of dyed cellulosic substrates allows for a comparison between the amount of released reducing sugar and released dyes from which one could differentiate between processive exo- and endocellulase activities (Helbert et al., 2003). Dyed substrates have been useful in determining cellulase activities in inhibition studies or when measurement of reducing sugars is difficult due to the presence of large amounts of existing hydrolysis products, such as cellobiose and glucose (Du et al., 2010). In terms of adsorption studies, dyed cellulosic substrates could potentially be used to assay the activity of re-adsorbed enzymes upon addition of dyed cellulosic substrates.

In addition to the five methods discussed in the review paper (Dashtban et al., 2010), an assay based on a novel application of fluorescence resonance energy transfer (FRET) has been recently developed to investigate cellulase-cellulose interaction (Wang, Wang and Ragauskas, 2010). In this method, CMC and the cellulase enzymes were labeled with different fluorescent dyes which respectively act as a FRET donor and acceptor. The FRET signal is generated only when the donor and acceptor dyes, bound to CMC and the cellulases respectively, come to within a certain distance of each other. This assay could serve as an indication of cellulase adsorption to the substrate. The investigators used this method to study the effect of temperature on cellulase binding to cellulose. While this work has been useful in developing a novel application of FRET for cellulase research more studies need to be done to

verify the potential of this method when assaying cellulase adsorption. One of the challenges includes the validity of using CMC, a soluble modified cellulosic substrate, for an adsorption study. It should also be noted that the authors did not specifically state which enzymes were labeled and they also observed a decrease in cellulase activity after labeling, which could make this technique inappropriate for following changes in enzymatic activity.

In the quest to expand the selection of techniques in our toolbox for cellulase/ biomass research, a recent study also explored the use of calorimetric assays to measure the rate of enzymatic hydrolysis of biomass. This work concluded that the heat flow measured by calorimetery could be correlated with the rate of hydrolysis and that it could provide a continuous picture of the hydrolytic rate (Murphy et al., 2010). As this technique measures the enthalpy change associated with enzymatic hydrolysis, it may also be used to determine the extent of hydrolysis. However, it might not be an appropriate method for following individual enzyme adsorption during the course of hydrolysis using a complete enzyme mixture as it would only be able to determine changes in the hydrolytic activity of the whole enzymes on the substrate.

1.4 Factors Influencing Enzyme Adsorption Behaviours

The limited availability of quantitative and specific techniques to monitor individual enzyme adsorption during hydrolysis has highlighted the complexity of cellulase enzyme interactions with lignocellulosic substrates. Three major factors influence this interaction, namely the properties of the substrates, the action of and interaction among individual enzyme components and the influence of physical factors during hydrolysis, such as pH and temperature, as well as the presence of soluble compounds, such as surfactants, sugars, and ethanol. The information summarised in Figure 1 describes the most recent model of enzymatic deconstruction of lignocellulosic biomass, highlighting the different factors that influence the enzyme-substrate interactions. The following section will provide a review of

the influence of substrate, enzyme, and physical factors on the adsorption of the major cellulase enzymes.

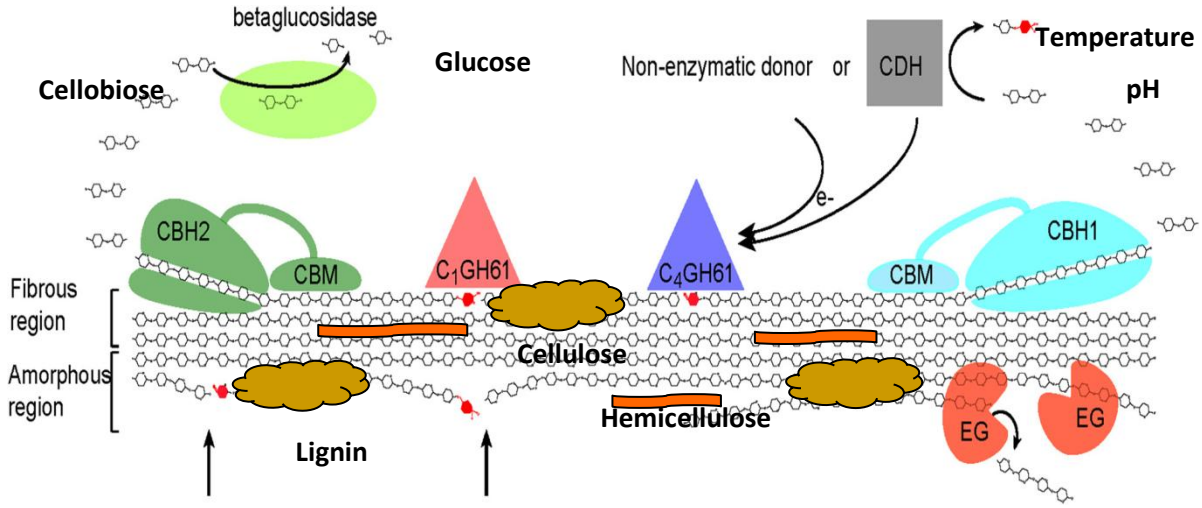

Figure 1. The complexity of the various factors involved in the hydrolysis of lignocellulosic biomass by canonical cellulase and accessory enzymes. Adapted from (Horn et al., 2012). CBM: cellulose binding module. CBH 1 and 2: cellobiohydrolase 1 and 2. C_1GH61 and C_4GH61: the two types of glycoside hydrolase family 61 (GH61 and now Auxiliary Activity family 9 (AA9)) enzyme, CDH: cellobiodehydrogenase enzyme, and EG: endoglucanase enzyme.

1.4.1 Enzyme Factors

The cellulase enzyme system involves a multitude of enzymes that have different affinities for and actions on lignocellulosic substrates (Quiocho, 1986; Yu et al., 1994) and, hence, should display different adsorption/desorption profiles. The actions of these enzymes may further influence the behaviour of other enzymes in the system (Eriksson, Karlsson and Tjerneld, 2002a). Enzyme-related factors include: (1) the molecular architecture of individual enzymes (such as the presence of a carbohydrate binding domain (CBM) and the structure of the catalytic domains (CDs); (2) their hydrolytic mechanism, such as processive or non-processive enzymes; (3) the source of enzymes; and (4) the nature of individual enzymes in the mixture. These enzymes have different affinities for lignocellulosic substrates (Quiocho, 1986; Yu et al., 1994) and hence, should display different adsorption/desorption

profiles. Due to the complexity of these enzyme mixtures, the roles of many of these enzymes, their interactions with one another, and their interactions with the substrates during hydrolysis are still poorly characterized. Many of these enzyme adsorption studies have been carried out using mono-component enzymes (Varnai et al., 2011; Gao et al., 2011; Eriksson, Karlsson and Tjerneld, 2002), Therefore, their adsorption behaviors may not represent their behaviors in a complete cellulase enzyme system.

1.4.1.1 Complexed Cellulase Systems (Cellulosomes)

In nature, various organisms, including bacteria, protozoa and fungi, produce a battery of enzymes to utilize lignocellulosic biomass as a nutrient source (Tanimura et al., 2013). These cellulase-producing organisms determine the molecular assembly of the cellulase enzymes produced. In turn, the molecular structures of cellulase enzymes highly influence the adsorption behavior of the enzyme.

Depending on their microbial source, the cellulase enzymes can either exist in a complexed cellulase system (cellulosomes) or in a non-complexed cellulase system (free enzymes) (Fontes and Gilbert, 2010). Cellulolytic aerobic fungi, such as *H. jecorina* and *Humicola insolens*, and aerobic actinomycete bacteria, such as *Cellulomonas* and *Thermobifida*, can penetrate cellulosic substrates through hyphal extension and secrete free cellulase enzymes into confined cavities within the penetrated cellulosic substrates (Lynd et al., 2002). In contrast, anaerobic bacteria, such as *Clostridia*, and anaerobic fungi synthesize cellulases and hemicellulases that are arranged in a large multienzyme complex called a cellulosome (Fontes and Gilbert, 2010; Lynd et al., 2002). It has been suggested that the energetic constraint of the anaerobic environment might have led to the construction of these cellulosomes (Fontes and Gilbert, 2010). The cellulosomes of certain microorganisms are tethered to the cell surface of the microorganisms (Beguin and Lemaire, 1996), enhancing the efficient uptake of the cellulose hydrolysis products (Bayer, Morag and Lamed, 1994; Schwarz, 2001). However, the importance

of the association of cellulosomes to the cell surface remains unclear as there is little evidence for membrane attachment in some microorganisms such as *Clostridium cellulolyticum* (Fontes and Gilbert, 2010).

A cellulosome is an enzyme complex that is typically bound to a bacterial cell wall and flexible enough to also bind tightly to microcrystalline cellulose. The assembly of these enzymes into a macromolecular complex may lead to a spatial enzyme proximity that allows for optimum synergistic interactions between the cellulosomal catalytic units (Fontes and Gilbert, 2010; Lynd et al., 2002). The architecture of cellulosomes is similar among various cellulosome-producing microorganisms, but the cellulosome composition differs depending on the species (Lynd et al., 2002). Cellulosomes were first identified in *Clostridium thermocellum*, and the cellulosome of *C. thermocellum* remains the paradigm for understanding the assembly and function of cellulosomes (Fontes and Gilbert, 2010). In *C. thermocellum*, cellulosomes are comprised of a large non-catalytic scaffoldin protein that is multimodular and typically contains cohesin domains, X (or hydrophilic) modules, and a CBM (Fontes and Gilbert, 2010). The scaffoldin is anchored to the cell wall via the type II cohesin domains. Catalytic modules with various activities, such as endo-glucanase, exo-glucanase, xylanase, and chitinase activities, have dockerin moieties that associate with the type I cohesin domains on the scaffoldin protein to form a cellulosome (Fontes and Gilbert, 2010).

While cellulosomes have been known to be very efficient at deconstructing plant biomass (Fontes and Gilbert, 2010), our understanding of the interaction between cellulosomes and lignocellulosic substrates during hydrolysis has yet to be fully resolved. During hydrolysis of cellulosic substrates, cellulosomes have been reported to physically separate individual cellulose microfibrils, increasing the surface area of cellulose and causing splayed end morphology on digested cellulose particles (Resch et al., 2013). This splaying of cellulose chain ends is in contrast to the well-known

narrowing/ tapering end effects of free cellulase enzymes (Resch et al., 2013; Chanzy and Henrissat, 1985). Due to its large molecular size, cellulosomes have been reported to be more prone to unproductive binding to lignin compared to free enzymes (Resch et al., 2013). As a result, cellulosomes have been shown to be less effective in hydrolyzing lignocellulosic substrates, but more effective on pure cellulosic substrates, compared with free enzymes on a mass basis (Resch et al., 2013).

1.4.1.2 Free Cellulase Systems

The individual enzymes in commercial enzyme mixtures have been mostly derived from free enzymes produced by the fungus *H. jecorina*. It is estimated that more than 200 different types of enzymes are present in these commercial cellulase mixtures (Merino and Cherry, 2007; Gomes et al., 2000). To date, several enzymes have been identified as important for efficient hydrolysis and, as a result, they have been the subject of considerable research. Key enzymes include two cellobiohydrolases (CBHs), Cel7A and Cel6A (previously known as CBH I and CBH II, respectively), several endoglucanases, primarily Cel7B, Cel5A, and Cel12A (previously known as EG I, EG II, and EG III, respectively), xylanases such as family 10 and 11 xylanases, β-glucosidase, and the lytic polysaccharide monooxygenases (LPMOs such as Auxiliary Activity family 9 (AA9), previously known as Glycoside Hydrolase family 61 (GH61) enzyme). In addition to these enzymes, *H. jecorina* is also known to produce a multitude of other enzymes that potentially contribute to lignocellulosic biomass degradation including endo- and exo-xylanases (Xyn I – IV), β-xylosidase, β-mannanase (Man5A), xyloglucanase (Cel74A), Cel5B, and acetyl xylan esterases (Axe 1 and 2). As additional types of enzymes or proteins with novel roles or improved properties continue to be identified (Kumar and Wyman, 2009; Harris et al., 2010), the compositions of today's commercial enzyme mixtures become increasingly complex with their inclusion.

Free cellulase enzymes are also modular in structure, consisting of CDs and CBMs that are connected via a linker domain (Bayer et al., 1998). The interaction of a cellulase enzyme with a

lignocellulosic substrate is determined by the presence/ absence of these domains, as well as the molecular structure of each domain. CBMs play an important role in promoting association of the enzyme with the substrate and are thought to impart three functions to the associated CD: (1) bringing the CD into close proximity with the substrate and increasing the enzyme concentration near the substrate (the proximity effect); (2) determining substrate specificity (the targeting effect); and (3) exerting a non-hydrolytic disruption of the cellulose chains that enhances hydrolysis efficiency (Boraston et al., 2004; Arantes and Saddler, 2010).

Based on their structures, CBMs are classified into three groups: type A, surface-binding CBMs; type B, glycan-chain binding CBMs; and type C, small-sugar binding CBMs (Boraston et al., 2004). The type A CBMs have a flat binding site containing aromatic amino acid residues. As a result, these CBMs promote enzyme binding to insoluble, crystalline cellulose and/or chitin, but show little or no affinity for soluble carbohydrates (Boraston et al., 2004). Type B CBMs have an extended binding site that is often described as grooves or clefts. While the presence of aromatic residues in the binding sites also plays a crucial role in ligand binding, type B CBMs are more effective in binding to individual glycan chains rather than crystalline surfaces. The binding affinity of these CBMs increases with an increase in the degree of polymerisation of the carbohydrate ligand. In contrast, type C CBMs lack the extended binding site grooves of type B CBMs, and therefore, bind optimally to mono-, di-, or tri-saccharides (Boraston et al., 2004).

At high solid consistency hydrolysis of lignocellulosic substrates, enzymes lacking CBMs were shown to outperform enzymes with CBMs, and it was suggested that the presence of a CBM might promote enzyme unproductive adsorption to lignin (Le Costaouec et al., 2013). The prevalence of aromatic residues on CBM binding sites may cause CBMs and their associated CDs to be susceptible to non-productive binding to lignin, which results from hydrophobic interaction between the aromatic

residues with the lignin. A recent study has suggested that the presence of CBMs in these free enzymes is influenced by the environment in which the host organisms live (Varnai, Siika-aho and Viikari, 2013). According to this hypothesis, cellulolytic organisms living in a low water environment tend to produce cellulase enzymes that lack CBMs, as the proximity function of CBMs is not really needed when enzymes do not have to diffuse far to associate with the substrate (Varnai, Siika-aho and Viikari, 2013). Therefore the use of CBM-lacking enzymes has been recommended when carrying out high-solid hydrolysis, to reduce non-productive adsorption to lignin and promote enzyme recycling (Varnai, Siika-aho and Viikari, 2013).

The source organisms also influence the structures and properties of the CDs of various glycoside hydrolases (GHs), which in turn determine their interaction with the substrate. For instance, a recent study has determined the crystal structure and properties of a novel endogenous GH family 7 produced by the marine wood boring animal *Limnoria quadripunctata* (Kern et al., 2013). This enzyme lacks a CBM, has a highly acidic surface charge compared to enzymes derived from terrestrial microbes and is highly salt tolerant, which makes it effective in hydrolyzing woody substrates in the marine environment at high solid loading in the animal's gut (Kern et al., 2013). To further illustrate the influence of enzyme structures on individual enzyme interaction with lignocellulosic substrate, the following section reviews previous enzyme adsorption studies of the major individual enzymes Cel7A, Cel6A, Cel7B, xylanases, β-glucosidase, and the AA9 enzyme.

1.4.1.2.1 Cel7A Adsorption Profiles

The cellobiohydrolase Cel7A is the major enzyme produced by *H. jecorina*, representing about 60% (on a mass basis) of the total cellulases produced by the fungus (Wood, 1992). It is also the major enzyme component in today's commercial cellulase enzyme mixtures (Horn et al., 2012; Rosgaard et al., 2007). The molecular architecture of the CD of Cel7A has been defined as having 4 surface loops that

form a tunnel with a length of 50 Å (DIVNE et al., 1994). The presence of this tunnel is responsible for the processive hydrolytic action of the enzyme, which cleaves cellulose chains from the reducing end of cellulose (Divne et al., 1994). The enzyme contains a CBM that is important for both its binding and processivity (Palonen, Tenkanen and Linder, 1999). Cellobiose is the main hydrolysis product and also the main inhibitor of Cel7A (Holtzapple et al., 1990).

The adsorption of Cel7A has been proposed to be reversible with rapid initial adsorption and a gradual desorption at the later stage of hydrolysis (Palonen, Tenkanen and Linder, 1999). It is generally agreed that Cel7A moves processively along the crystalline cellulose surface until it encounters an obstacle, resulting in the enzyme getting stuck and sometimes being released back into the solution. However, because Cel7A has a low dissociation rate constant, the release of Cel7A back to the solution is slow and has been proposed as the rate-limiting step for cellulose hydrolysis and one of the reasons behind the declining rate in hydrolysis (Eriksson, Karlsson and Tjerneld, 2002; Jalak and Vaeljamaee, 2010). The processive action of Cel7A seems to be influenced by the nature of the substrate, with the enzyme moving processively over a longer distance on a more crystalline cellulose surface (Kipper, Valjamae and Johansson, 2005). A study employing high-speed atomic force microscopy (AFM) to directly visualize Cel7A movement on cellulose surface seems to lend support to these observations (Igarashi et al., 2011). This study suggested that the roughness of the crystalline cellulose surface would lead to a traffic jam effect, where Cel7A enzymes would remain unproductively bound to the cellulose until the obstacle is removed or the enzyme is released back into the solution (Igarashi et al., 2011).

A related study visualized the movement of intact Cel7A, Cel7A without CBM, a catalytically inactive Cel7A, and a Cel7A lacking a tryptophan residue at the entrance of the active site tunnel (position 40) using high speed AFM (Igarashi et al., 2009). This study observed that CBM were required to promote the adsorption and the formation of enzyme-substrate complex. However, the movement of

Cel7A on cellulose did not depend on the CBM as both intact Cel7A and Cel7A lacking a CBM moved at the same speed on the cellulose surface. The Cel7A movement seemed to require the loading of a cellulose chain into the active site as no sliding was observed for the catalytically inactive Cel7A and the Cel7A lacking the tryptophan residue at the tunnel entrance. This tryptophan residue is thought to be important for the binding and loading of the cellulose chain into the CD of Cel7A (Igarashi et al., 2009).

1.4.1.2.2 Cel6A Adsorption Profiles

Cel6A is the second most abundant enzyme produced by *H. jecorina*, accounting for up to 20% of the total cellulase proteins produced on a mass basis (Wood, 1992). Cel6A also consists of a CD and a CBM (Abuja et al., 1988). The CD has 2 surface loops that give rise to a 20 Å tunnel (Lynd et al., 2002). Therefore, Cel6A is also a processive cellobiohydrolase that works from the non-reducing end of cellulose (Medve, Stahlberg and Tjerneld, 1994). Cellobiose is also the main product and inhibitor of Cel6A.

In contrast to Cel7A adsorption, Cel6A adsorption seems to be more irreversible (Palonen, Tenkanen and Linder, 1999). Cel6A has also been reported to have a low thermostability (Lantz et al., 2010). A study on Cel6A from *Humicola insolens* observed the ability of this enzyme to cut cellulose chains into shorter chains, indicating an endoglucanase-type activity and proposed that Cel6A can be classified as an endo-processive enzyme (Boisset et al., 2000).

1.4.1.2.3 Cel7B Adsorption Profiles

Cel7B is the most abundant endoglucanase produced by *H. jecorina*, constituting 5-10% of the total amount of cellulases produced (Kleywegt et al., 1997). It is a modular enzyme with a CBM connected to a CD and is structurally related to Cel7A, except that it has shorter loops that create an extended, open substrate-binding cleft instead of a tunnel (Kleywegt et al., 1997). Rapid adsorption of

Cel7B to the substrate has been generally observed, after which this enzyme becomes distributed in both the liquid and the solid phase (Eriksson, Karlsson and Tjerneld, 2002a; Karlsson et al., 2002). In the endo-exo synergism model, endoglucanases such as Cel7B are thought to cleave cellulose chains randomly with preference for the amorphous part of cellulose. This endoglucanase action creates new chain ends for exoglucanases to bind and start hydrolyzing the cellulose chains. More recently, an additional model of endo-exo synergism has been proposed upon an observation that addition of Cel7B caused an increased hydrolysis rate when hydrolysis by Cel7A has already levelled off (Eriksson, Karlsson and Tjerneld, 2002). A concurrent desorption of Cel7A was also observed upon addition of Cel7B. This study proposed that Cel7B also played a role in removing obstacles that block the movement of Cel7A along cellulose chains, releasing Cel7A from unproductive adsorption to cellulose (Eriksson, Karlsson and Tjerneld, 2002). In contrast, competitive adsorption between Cel7A and Cel7B was recently reported which showed that Cel7A had a much higher adsorption affinity to cellulose than Cel7B and that the two enzymes compete for surface sites (Maurer, Bedbrook and Radke, 2012). Due to the higher affinity of Cel7A, a higher proportion of Cel7B in the supernatant was required to achieve comparable surface Cel7B concentration. In another study, these authors also reported the synergistic interaction between Cel7A and Cel7B in which Cel7B was shown to enhance the activity of Cel7A by disrupting the hydrogen bonding structure of cellulose (Maurer et al., 2013).

1.4.1.2.4 β-glucosidase Adsorption Profiles

The enzyme β-glucosidase hydrolyzes cellobiose to glucose and therefore plays a crucial role in alleviating cellobiose product inhibition of other cellulase enzymes during cellulose hydrolysis (Berlin et al., 2007). The β-glucosidases from *H. jecorina* are prone to glucose inhibition, whereas those produced by *Aspergillus* species are more glucose tolerant (Lynd et al., 2002). The level of β-glucosidase secretion by *H. jecorina* has been shown to be insufficient for effective, in vitro, cellulose saccharification (Lynd et

al., 2002). Thus, β-glucosidase enzymes derived from *Aspergillus* have traditionally been used to complement cellulase enzymes from *H. jecorina*. However, due to advancement in enzyme cocktail formulation, β-glucosidase enzymes are usually already included in today's commercial enzyme mixtures such as Cellic CTec3 from Novozymes (Novozymes, 2012).

The adsorption profiles of the β-glucosidases can be easily determined by SDS-PAGE, as these enzymes typically have large molecular sizes of around 100 kDa, or by an activity assay due to the availability of a specific substrate *para*-nitrophenylglucopyranoside (PNPG) (Vinzant et al., 2001; Deshpande, Eriksson and Pettersson, 1984). A previous enzyme adsorption study has shown minimal adsorption of these enzymes to cellulose during hydrolysis (Yang and Wyman, 2006). In contrast, non-specific β-glucosidase adsorption to lignin has been observed (Rahikainen et al., 2011; Yang and Wyman, 2006). Due to minimum β-glucosidase adsorption to the solid substrate, enzyme recycle studies have typically supplemented recycled enzymes with fresh β-glucosidase enzymes (Qi et al., 2011; Tu et al., 2009a) or tried to immobilize the enzymes on a solid carrier to facilitate enzyme recycling (Tu et al., 2006; Gomez et al., 2010; Gomez, Romero and Fernandez, 2005). A recent study showed that β-glucosidases derived from different microorganisms exhibited different kinetics of cellobiose hydrolysis and glucose inhibition (Teugjas and Vaeljamaee, 2013). In turn, the adsorption of individual β-glucosidase enzymes to the substrates may be determined by both the characteristics of the substrate and the nature of the enzymes. The activity of oxidative enzymes such as AA9 enzymes during cellulose hydrolysis results in the production of aldonic acids, such as cellobionic or gluconic acids, which has been shown to be a stronger inhibitor of β-glucosidases than glucose itself (Cannella et al., 2012).

1.4.1.2.5 Xylanase Adsorption Profiles

Many microorganisms produce multiple xylanases with varied physicochemical properties, specific activities, and structures (Wong, Tan and Saddler, 1988). For instance, *Aspergillus niger* and

Trichoderma viride are reported to secrete 15 and 13 xylanases, respectively (Collins, Gerday and Feller, 2005; Biely, Markovic and Mislovicova, 1985). The diversity of xylosidic linkages has been proposed as the reason behind this diversity of xylanase enzymes (Wong, Tan and Saddler, 1988).

Enzymes with xylanase activities are found in GH families 5, 7, 8, 10, 11, and 43, although most research has been focused on xylanases belonging to family 10 and 11 (Collins, Gerday and Feller, 2005). The xylanases in GH family 10 are comprised of endo-1,4-β-xylanases and endo-1,3-β-xylanases. The substrate specificity of family 10 xylanases includes activity on xylan, but also low molecular weight cellulosic substrates (Collins, Gerday and Feller, 2005; Gilkes et al., 1991). The family 11 xylanases, on the other hand, are considered as true xylanases as they are specific for xylan-containing substrates (Collins, Gerday and Feller, 2005).

Due to the diversity of the xylanases, these enzymes may exhibit different interactions with lignocellulosic substrates. A study on an endo-1,4-β-xylanase derived from *Bacillus sp*. showed that the xylanase was irreversibly adsorbed to lignin and insoluble xylan at 4^{o}C (Zilliox and Debeire, 1998). Adsorption profiles of family 10 xylanases during hydrolysis are rather difficult to probe in the presence of other cellulases or hemicellulases because many cellulase enzymes have similar molecular weights to that of family 10 xylanase, and there is a lack of a specific substrate for family 10 xylanases. To overcome these difficulties, in section 3.1.8, we used a birchwood xylan zymogram assay to determine the adsorption profile of a family 10 xylanase. We observed xylanase activity at a molecular weight similar to that of the family 10 xylanases. The observed activity on zymogram was expected to be that of family 10 xylanase because this method preferably detected enzymes with higher activity towards the substrate rather than those with lower activities. The zymogram result showed that this enzyme was found in both the supernatant and adsorbed to the substrate. In contrast the adsorption profile of family 11 xylanase could be easily observed using SDS-PAGE as this enzyme has a molecular weight of 20 kDa, which is

lower than most cellulase enzymes. This family 11 xylanase has been shown to be distributed both in the supernatant and adsorbed to the substrate during the early stages of hydrolysis. After 6 hours, this enzyme could not be detected in either phases, presumably due to enzyme thermal deactivation (Janis et al., 2008).

The importance of xylanases in improving cellulose conversion by cellulases has been recently demonstrated in an experiment where substitution of a portion of the cellulases with xylanases resulted in an increase in cellulose conversion efficiency (Hu, Arantes and Saddler, 2011). The xylanases with broad activities showed a higher synergistic effect with cellulase enzymes than those with more specific activities (Hu et al., 2013).

1.4.1.2.6 AA9 enzyme Adsorption Profiles

The AA9 enzymes have been shown to significantly improve cellulose conversion efficiency by cellulases (Harris et al., 2010). As a result, these enzymes have been added to the latest commercial enzyme mixtures (Novozymes, 2012). Several studies have been carried out to determine the structures and mechanism of these enzymes (Harris et al., 2010; Bey et al., 2013; Langston et al., 2011; Quinlan et al., 2011). Unlike cellulase enzymes that have cleft or tunnel-type active sites, the AA9 enzyme has been shown to have a planar active site which contains a divalent metal ion (Harris et al., 2010; Quinlan et al., 2011). They also require soluble redox-active co-factors, such as reducing factors provided by cellobiodehydrogenase (CDH), or small molecular reductants, such as gallate or ascorbate (Langston et al., 2011; Quinlan et al., 2011). Studies on the catalytic activity of these enzymes have revealed that the AA9 enzymes improve cellulose hydrolysis by means of an oxidative cleavage of the crystalline cellulose through an insertion of an oxygen molecule into cellulose (Beeson et al., 2012). Therefore, these enzymes are thought to improve cellulose hydrolysis by increasing cellulose accessibility to cellulase enzymes. Despite extensive studies on this enzyme, little is known regarding the adsorption profiles of

this enzyme during hydrolysis. Thus, more work is needed to elucidate the adsorption of AA9 and its effects on the adsorption profiles of hydrolytic enzymes.

1.4.2 Substrate Factors

In addition to the characteristics of individual enzymes and their interactions with one another, enzyme-substrate interactions are also influenced by the properties of lignocellulosic biomass. Lignocellulosic biomass is composed of the three major polymers of cellulose, hemicellulose, and lignin. The amount and nature of these polymers vary in biomass from different sources. Their structures and distribution within a lignocellulosic substrate will also be further modified depending on the type of pretreatments used to break open the recalcitrant structure of this biomass. Lignin and hemicellulose cover cellulose microfibrils and they are known to impede the enzymes from accessing the cellulose (Himmel et al., 2007; Ding and Himmel, 2006). Therefore, their properties and distribution following pretreatment will strongly influence enzyme adsorption profiles and the efficiency of hydrolysis. Similarly, enzyme adsorption to cellulose is influenced by the structure and accessibility of cellulose following pretreatments (Nakagame, Chandra and Saddler, 2010; Bura, Chandra and Saddler, 2009). In addition, as hydrolysis proceeds, enzyme action on the substrate is likely to alter the structure of the substrates, which would further influence enzyme-substrate interactions.

It has been previously proposed that, during hydrolysis, the surface of cellulose becomes eroded and that these changes can present an obstacle and block the actions of processive enzymes (Yu, Lee and Saddler, 1993; Valjamae et al., 1998). In an effort to understand a possible cause of the decline in enzymatic hydrolysis rate over time, another study performed a restart hydrolysis experiment in which Avicel hydrolysis residues were recovered at different time points during hydrolysis (Yang, Willies and Wyman, 2006). After removing adsorbed cellulase enzymes through a proteinase treatment and excessive washing, fresh enzymes and buffer were added to these residues. The authors reported

similar initial enzymatic hydrolysis rates per adsorbed enzymes with these residues, suggesting that changes in substrate reactivity could not account for the decrease in enzymatic hydrolysis rate during hydrolysis but rather other mechanisms, such as the aforementioned enzymes getting unproductively adsorbed on cellulose, may play a role in the decline in hydrolysis rate (Yang, Willies and Wyman, 2006). As the amount of available cellulose decreases, the ratio between enzymes and available substrate increases. It has been suggested that, consequently, more enzymes try to access the same cellulose surface and these enzymes start to block each other as they compete for the same binding sites (Bommarius et al., 2008; Zhang et al., 2006). Therefore, as hydrolysis proceeds, the interplay between the enzymes and the substrate, and among the enzymes themselves, are likely to continue to change. Thus, these dynamic interactions add to the complexity of this enzyme-substrate interaction.

1.4.2.1 The Influence of Lignin on Enzyme Adsorption/Desorption

Lignin is a complex polymer composed of aromatic phenylpropane units (Boerjan, Ralph and Baucher, 2003). In plants, lignin acts as the glue that surrounds and binds microfibrils together, conferring mechanical support and structural support, decreasing water permeability across the cell wall, and protecting the cell wall from biodegradation (Sarkanen and Ludwig, 1971). In the bioconversion process, lignin is thought to affect enzymatic hydrolysis in two ways. First, it may act as a physical barrier which limits swelling of the substrate, while at the same time limiting the access of the enzymes to the cellulose. Secondly, it may non-productively bind the cellulase enzymes, reducing the amount of enzymes available for hydrolysis (Nakagame, Chandra and Saddler, 2010; Zhang et al., 2006; Chernoglazov, Ermolova and Klyosov, 1988; Palonen et al., 2004; Kumar et al., 2012).

The influence of lignin on enzyme adsorption is influenced by the characteristics of the lignin in the original biomass and after pretreatment, lignin content and lignin distribution within the substrate. The three major types of phenylpropane units that make up lignin in higher plants are guaiacyl (G),

syringyl (S), and p-hydroxyphenyl (H) units or monolignols. The composition of monolignols in lignin varies depending on the nature of the biomass substrate. For example, softwood lignin is primarily composed of G units (98%), hardwood lignin is composed primarily of both G and S units, and herbaceous lignin contains all three monolignols (Boerjan, Ralph and Baucher, 2003). The propensity of the G units in softwood lignin and the presence of a single methoxyl group on these G units result in a greater degree of cross linking among the lignin units as well as to hemicellulose (Boerjan, Ralph and Baucher, 2003). The lignin content in biomass also varies. In softwoods, lignin accounts for 25-35% (w/w) of the total dry matter while in hardwoods and herbaceous biomass, such as agricultural residues, lignin accounts for 18-25% (w/w) and 10-30%(w/w) of the total dry matter, respectively (Kelley et al., 2004). In general, softwoods are considered to be more recalcitrant compared to hardwood and herbaceous substrates (Nakagame, Chandra and Saddler, 2010), probably due to the higher amount of lignin and the higher degree of lignin cross-linking in this biomass.

A pretreatment method is typically required to increase biomass digestibility and enhance enzymatic hydrolysis. Due to lignin's major contribution to biomass recalcitrance, most pretreatment technologies seek to either reduce the lignin content in pretreated biomass or modify the physical and chemical properties of the lignin to reduce the inhibitory effects of lignin to enzymatic hydrolysis. Pretreatment methods, such as solvent extraction (organosolv pulping), alkaline (lime) pretreatments, and wet oxidation are intended to remove lignin (Alvira et al., 2010). Alternatively, ammonia fiber explosion (AFEX), dilute acid, and steam pretreatments tend to modify the properties and distribution of lignin, while leaving a large proportion of the lignin intact (Alvira et al., 2010). Due to the difficulty in pretreating softwood biomass to reduce its lignin content or lignin inhibitory effects, many researchers have explored various post-treatment methods to reduce the lignin content or further modify the lignin properties in pretreated softwood substrates (Galbe and Zacchi, 2012; Del Rio, Chandra and Saddler, 2011; Kumar, Chandra and Saddler, 2011; Yang et al., 2002).

The influence of lignin on enzyme-substrate interaction is determined both by its natural characteristics and the pretreatment/ post-treatment methods used (Nakagame, Chandra and Saddler, 2010; Sewalt, Glasser and Beauchemin, 1997). A previous study has compared the influence of lignin isolated from softwood, hardwood, and agricultural residues when added to hydrolysis of Avicel, and a decrease in hydrolysis efficiency was observed upon addition of softwood lignin but not lignin from agricultural residues (Nakagame, Chandra and Saddler, 2010). The modification of the physical and chemical properties of lignin during pretreatment or post-treatment also influences enzyme adsorption during hydrolysis. For example, organosolv pretreatment is effective in enhancing hydrolysis of softwood substrates due to its ability to remove most of the highly adsorptive lignin from the solid biomass, while it is reportedly detrimental to hydrolysis of agricultural residues (Sewalt, Glasser and Beauchemin, 1997). When the phenolic group contents in lignin derived from organosolv and steam pretreated agricultural residues were compared, the authors found a higher phenolic group content in organosolv lignin than in steam-pretreated lignin in the pretreated agricultural residues (Sewalt, Glasser and Beauchemin, 1997). This higher phenolic group content seems to be associated with the higher inhibitory effects of organosolv lignin, due to an increase in non-productive enzyme adsorption, as compared with steam-pretreated lignin in agricultural residues. For example, incorporation of sulfonic acid groups into the lignin structure has been shown to improve the efficiency of enzymatic hydrolysis by reducing lignin hydrophobicity and non-productive enzyme adsorption to lignin (Del Rio, Chandra and Saddler, 2011). High severity acid pretreatments have been reported to result in acid-catalyzed dehydration of carbohydrates, leading to the formation of pseudo-lignin which increases non-productive binding of enzymes (Sannigrahi et al., 2011).

While lignin removal is normally thought to improve cellulose hydrolysis performance, a recent publication reported a decrease in digestibility of acid-pretreated corn stover after complete delignification (Ishizawa et al., 2009). This decrease in digestibility after complete lignin removal was

found to be enhanced in substrates with low xylan contents. In these substrates, both xylan and lignin were thought to act as a spacer between cellulose microfibrils. Therefore, removal of lignin was suggested to result in cellulose aggregation, which subsequently decreased cellulose accessibility.

1.4.2.2 The Influence of Hemicellulose on Enzyme Adsorption/ Desorption

Hemicelluloses are heterogeneous, branched polymers composed of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, L-rhamnose, 4-O-methyl-D-glucuronic acid and galacturonic acid. Hemicelluloses have a significantly lower degree of polymerisation than cellulose and they can be acetylated (Kuhad, Singh and Eriksson, 1997; Selig et al., 2009). The hemicellulose content and type in lignocellulosic biomass differs between agricultural residues, hardwood, and softwood. The main hemicelluloses in softwood are galactoglucomannan containing a linear or branched chain of 1,4-linked glucose or mannose units, and arabinoxylans, representing approximately 20% and 5-10% of the total dry matter, respectively (Willfor et al., 2005a). The major hemicelluloses in hardwood and herbaceous biomass are 4-O-methylglucoronoxylans (15-30% of the total dry matter) and glucomannans (2-5% of the total dry matter) (Willfor et al., 2005b).

Hemicellulose in a substrate has been proposed to act as a barrier for enzyme access to the cellulase. Therefore, hemicellulose removal is thought to be important in increasing the effectiveness of cellulose hydrolysis (Alvira et al., 2010; Bura, Chandra and Saddler, 2009). Due to their low degree of polymerisation, hemicelluloses are particularly sensitive to the nature and severity of pretreatments. Alkaline pretreatments and AFEX, for example, modify and leave most of the hemicellulose intact in the solid fraction (Alvira et al., 2010). On the other hand, acid pretreatments, including acid-catalyzed steam pretreatments, remove hemicelluloses from the solid to the liquid fraction (Alvira et al., 2010).

Studies on the influence of hemicellulose on enzyme-substrate interactions are much more limited compared to studies on the influence of lignin. Removal of the hemicellulose content of the solid

substrate has been shown to increase cellulose hydrolyzability, presumably by increasing enzyme access to cellulose (Bura, Chandra and Saddler, 2009; Varnai, Siika-aho and Viikari, 2010). However, increases in severity of acid pretreatments also result in increased formation of degradation products such as furfural and hydroxymethyl furfural. These sugar degradation products have been shown to inhibit enzyme and yeast activities (Klinke, Thomsen and Ahring, 2004; Hodge et al., 2008). Therefore, researchers utilizing acid pretreatments have often opted for medium-severity pretreatment conditions that improve hydrolyzability of the solid fraction, preserve most of the hemicellulosic sugars, and minimize the formation of sugar degradation products. Hydrolysis of the resulting substrate can then be further improved by supplementing the cellulase enzymes with hemicellulases, such as xylanases, to enzymatically remove the remaining xylan (Bura, Chandra and Saddler, 2009; Selig et al., 2009). The acetyl groups, often found in xylan from agricultural residues, have been shown to restrict enzymatic hydrolysis, and hydrolysis can be improved by supplementation with accessory enzymes such as acetyl xylan esterase to remove the acetylated xylan (Selig et al., 2009; Christov and Prior, 1993). When Selig et al, (2009) investigated the effects of different degrees of lignin removal on the digestibility of alkaline-peroxide-pretreated corn stover substrates, they found that a decrease in lignin content did not significantly impact the extent of glucan conversion in the absence of xylan-degrading enzymes (Selig et al., 2009). Therefore, they concluded that the presence of lignin affected cellulose hydrolysis through its association with xylan. During pretreatment, xylan is degraded into its oligomeric or monomeric forms. Xylooligomers have been shown to be a potent inhibitor of cellulase enzymes (Qing, Yang and Wyman, 2010). Another study has shown that, by substituting a portion of a cellulase enzyme mixture with xylanase, an improvement in hydrolysis of steam pretreated corn stover could be achieved without increasing enzyme loading (Hu, Arantes and Saddler, 2011). The action of xylanases is thought to result in the removal of the hemicellulose barrier as well as causing changes in fiber structures, such as fiber swelling, which further improves enzyme access to cellulose (Hu, Arantes and Saddler, 2011). All these

studies show the importance of accessory hemicellulolytic enzymes to improve efficiency of hydrolysis of lignocellulosic substrates. The effect of hemicellulose on enzyme adsorption is, however, still not clearly defined.

1.4.2.2 The Influence of Cellulose on Enzyme Adsorption/ Desorption

Improving access to cellulose and therefore the hydrolysis efficiency of cellulose is one of the major goals of current bioconversion research. Cellulose is an unbranched homopolysaccharide composed of β-D-glucopyranose units linked by (1→4) glycosidic bonds. In nature, cellulose chains have a degree of polymerization (DP) of ~10,000 glucose units in woody biomass and 15,000 glucose units in native cotton cellulose. An AFM study on the structure of cellulose in plant cell walls has indicated that cellulose particles consist of cellulose elementary fibrils (CEF) with a dimension of 3 – 5 nm. These CEFs are an assembly of 36 cellulose chains. Groups of adjacent CEFs can subsequently be organized into macrofibrils. These macrofibrils eventually split at the end, get associated with other cell wall components such as hemicelluloses and lignin, and form parallel microfibrils (Ding and Himmel, 2006).

Various properties of cellulose, such as the type of allomorph, its crystallinity, its accessibility to enzymes, and its degree of polymerization (DP), have been extensively studied to determine their influence on enzymatic hydrolysis (Alvira et al., 2010). However, the extent to which these various cellulose properties influence enzyme adsorption has not been fully resolved.

There are four major types of cellulose allomorphs that can be interconverted including cellulose I, cellulose II, cellulose III, and cellulose IV. Cellulose I is the native form of cellulose found in nature. Two allomorphs of cellulose I have been identified based on their crystalline forms, cellulose Iα, found primarily in primitive organisms such as bacteria, and cellulose Iβ, the dominant form of cellulose in higher plants (OSullivan, 1997). These two forms of cellulose I have the same conformation but differ in their hydrogen bonding patterns (OSullivan, 1997). Cellulose II can be generated from cellulose I

through two processes, including regeneration, in which cellulose I is solubilized in a solvent followed by re-precipitation in water to give cellulose II and mercerization or swelling of cellulose I in concentrated NaOH. The conversion of cellulose I to cellulose II is irreversible. Cellulose III can be produced from cellulose I or cellulose II by treatment with liquid ammonia or some amines, followed by removal of the ammonia. Unlike cellulose II, the conversion of cellulose I to cellulose III is reversible. Finally, cellulose IV is produced by heating cellulose III to 206^{o}C in glycerol (OSullivan, 1997).

As cellulose I is the most abundant form of cellulose, most enzyme adsorption studies have been done on cellulose I although a number of enzyme adsorption studies has recently been carried out on cellulose III (Chundawat et al., 2011; Igarashi, Wada and Samejima, 2007). It was demonstrated that conversion of cellulose Iβ to cellulose III_1 using an ammonia-based pretreatment method, enhanced enzymatic hydrolysis by 5-fold, while reducing cellulase enzyme binding capacity. This seemingly anomalous observation is attributed to the alteration of the hydrogen bonding patterns in cellulose III_1. Compared to cellulose Iβ, cellulose III_1 has an increased number of intersheet hydrogen bonds, but a decrease in the number of intrasheet hydrogen bonds, resulting in a 50% increase in the number of glucan chain hydrogen bonds that are exposed to water. This increase in cellulose hydration was reported to facilitate easier glucan chain extraction by enzymes, enhancing enzymatic hydrolysis. The study also showed a reduction in the binding of Cel7A, Cel6A, and Cel7B (Chundawat et al., 2011). Ionic liquid pretreatments of cellulosic substrates may result in the production of cellulose II. While this conversion to cellulose II has been shown to dramatically increase enzyme hydrolysis efficiency (Dadi, Varanasi and Schall, 2006; Cheng et al., 2011), enzyme adsorption studies on cellulose II are still limited.

Cellulose accessibility to enzymes has been reported to be a critical parameter that determines the efficiency of enzymatic hydrolysis of cellulose (Arantes and Saddler, 2010a). Cellulose accessibility can be determined by using methods such as Simon's stain, water retention values, direct enzyme

adsorption, and cellulose binding module (CBM) adsorption (Chandra et al., 2009a; Wiman et al., 2012; Wang et al., 2012; Gourlay et al., 2013; Gourlay, Arantes and Saddler, 2012). An increase in cellulose accessibility is typically correlated with both an increase in enzyme adsorption and improved hydrolysis efficiency (Arantes and Saddler, 2010; Wiman et al., 2012; Wang et al., 2012; Piccolo et al., 2010).

Another important parameter of cellulose is cellulose crystallinity. Cellulose within biomass is typically composed of crystalline, paracrystalline, and amorphous regions. The influence of cellulose crystallinity on enzymatic hydrolysis has not been fully elucidated. A recent review paper highlighted the contradicting observations on the role of cellulose crystallinity on enzymatic hydrolysis rate (Yang et al., 2011). Although it has been suggested that crystallinity might increase during hydrolysis as the amorphous regions of cellulose would likely be degraded faster than the crystalline regions (Ooshima, Sakata and Harano, 1983; Paralikar and Betrabet, 1977), other studies have shown that the levels of cellulose crystallinity remained constant during enzymatic hydrolysis (Hall et al., 2010; Puls and Wood, 1991; Mansfield, Mooney and Saddler, 1999). Using a newly developed CBM adsorption method, our group demonstrated that initial enzymatic hydrolysis rates were correlated with the substrate's accessible crystalline and amorphous cellulose (Gourlay, Arantes and Saddler, 2012).

Cellulose crystallinity has been reported to influence enzyme adsorption (Kipper, Valjamae and Johansson, 2005; Hall et al., 2010; Jeoh et al., 2007; von Ossowski et al., 2003). Cel7A, for example, has been shown to display greater adsorption to amorphous cellulose than to crystalline cellulose (Jeoh et al., 2007). However, it is difficult to conclude if the increase in enzyme binding capacity observed with amorphous substrates was solely due to changes in cellulose crystallinity, as amorphous cellulose is expected to be more accessible than crystalline cellulose. Consequently, the increase in enzyme adsorption on amorphous cellulose could be due to an increase in cellulose accessibility rather than solely due to a decrease in cellulose crystallinity. Therefore, to conclusively determine the effect of

cellulose crystallinity on enzyme adsorption, there is a need for studies that isolate the effect of cellulose crystallinity from other cellulose properties. A recent study reported a rather different observation on the effect of cellulose crystallinity on enzyme adsorption (Hall et al., 2010). At low crystallinity index (CrI) values, a constant level of enzyme adsorption was observed on cellulosic substrates with increasing CrI values up to a certain level of crystallinity (35-45% CrI, depending on the enzyme loading). Above this CrI value, the amount of adsorbed enzymes decreased as the substrates became more crystalline. Consequently, while the initial rate of enzymatic hydrolysis was shown to be correlated to CrI, the initial rate was correlated to the extent of enzyme adsorption only up to a certain enzyme adsorption value. Above this value, increases in initial rate were observed even though the extent of enzyme adsorption remained constant (Hall et al., 2010). The reasons behind these observations are still unclear and warrant further investigation. A few studies have investigated the influence of cellulose crystallinity on enzyme processivity. In general, the processivity of Cel7A has been shown to be higher on crystalline cellulose than on amorphous cellulose or cellulose treated with endoglucanases (Kipper, Valjamae and Johansson, 2005; von Ossowski et al., 2003)

There is limited information in the literature on the influence of cellulose degree of polymerization (DP) on hydrolysis and enzyme adsorption. A recent review article has indicated that DP could play a role in enzyme synergy, with higher DP resulting in higher synergy between Cel7A and Cel7B (Yang et al., 2011). It could also influence enzyme processivity and adsorption, with a higher DP being important for an increase in enzyme processive action and enzyme adsorption Substrates with shorter DP, and consequently more reducing ends, are expected to result in improved hydrolysis efficiency by Cel7A (Yang et al., 2011). More studies are needed to further elucidate the importance of cellulose DP on enzymatic hydrolysis and individual enzyme adsorption.

1.4.3 Physical Factors

1.4.3.1 The Influence of Temperature on Enzyme Adsorption

Bioconversion of lignocellulosic biomass is typically done at 50^{o}C and a pH of 4.8 – 5.0. Changes in these parameters will not only affect enzyme activity, but also enzyme adsorption. In general, an increase in enzyme adsorption to cellulose and lignin has been observed when temperature is decreased (Kyriacou, Neufeld and Mackenzie, 1988;Kim, Yang and Jeong, 1988; Tu, Pan and Saddler, 2009). Adsorption of cellulase enzymes to lignin at high temperature has been suggested to cause a reduction in enzyme activities compared to adsorption at a lower temperature (Rahikainen et al., 2011).

1.4.3.2 Influence of pH on Enzyme Adsorption

The pH at which hydrolysis is conducted influences the charge of the enzymes and also of the lignocellulosic substrate components. At pH 4.8 – 5.0, the cellulose surface has a slightly negative charge. As most cellulase enzymes have pI values above 4.8 – 5.0, they will have positive charges at the typical hydrolysis pH (Nakagame et al., 2011). The enzyme Cel7A, on the other hand, has a pI around 3.6 – 3.9. Thus, at pH 4.8 – 5.0, this enzyme has a slight negative charge (Nakagame et al., 2011). An increase in pH would increase the negative charge on the surface of lignocellulosic substrate and shift the adsorption equilibrium towards enzyme desorption. Using an increase in pH to desorb cellulase enzymes has been explored as a potential enzyme recycling strategy (discussed in detail in section 1.1.4). A recent study has also shown that by increasing the hydrolysis pH to 5.5, enzyme non-productive adsorption to lignin was reduced due to an increase in the negative charge of lignin (Wang, Lan and Zhu, 2013).

1.4.3.3 Influence of Surfactants on Enzyme Adsorption

During cellulose bioconversion, the presence of soluble compounds such as sugars, ethanol, and surfactants may also influence enzyme binding to the substrate (Tu et al., 2009b; Eriksson, Borjesson and Tjerneld, 2002; Kristensen et al., 2007; Okino et al., 2013). Many studies have explored the influence of surfactants on enzyme adsorption to lignocellulosic substrates and enzyme activities (Tu et al., 2009b; Eriksson, Borjesson and Tjerneld, 2002; Kristensen et al., 2007; Okino et al., 2013). Addition of surfactants and other additives such as polyethylene glycol has been shown to improve conversion of lignocellulosic substrates, while at the same time reducing enzyme adsorption (Eriksson, Borjesson and Tjerneld, 2002b; Kristensen et al., 2007). The mechanism by which these additives improve hydrolysis has been attributed to a reduction in non-productive enzyme adsorption to lignin. Surfactants have been shown to decrease enzyme adsorption and increase cellulose conversion in lignin-containing substrates, while their effects on pure cellulosic or delignified substrates have been shown to be minimal (Eriksson, Borjesson and Tjerneld, 2002).

The types of surfactants, i.e. non-ionic, cationic, or anionic, also determine the influence of these surfactants on hydrolysis and enzyme adsorption (Eriksson, Borjesson and Tjerneld, 2002b). Non-ionic surfactants such as Tween have been shown to be the most effective in enhancing hydrolysis while a reduction in hydrolysis efficiency has been observed with charged surfactants (Eriksson, Borjesson and Tjerneld, 2002). Anionic surfactants have been shown to significantly reduce Cel7A adsorption, most likely by imparting a negative charge to the lignin which results in electrostatic repulsion with the negatively charged Cel7A at the hydrolysis pH (4.8) (Eriksson, Borjesson and Tjerneld, 2002). Although this would result in a reduction of unproductive binding to lignin, the presence of anionic surfactants may lead to enzyme denaturation. In contrast, the reduction in hydrolysis efficiency by cationic

surfactants may be caused by increased enzyme binding to positively charged lignin (Eriksson, Borjesson and Tjerneld, 2002b).

Past work has shown that the surfactant Tween 80 improved conversion of pure cellulose at high shaking speeds of 180 rpm but not at 0 – 100 rpm (Yang et al., 2011). This improvement in cellulose conversion was attributed to the protection effect conferred by Tween 80 on the adsorbed enzymes at high shaking speed.

1.4.3.4 Influence of Sugars and Ethanol on Enzyme Adsorption

Most studies on the effects of sugars and ethanol on enzymatic hydrolysis have evaluated their inhibitory effects on enzyme activities (Holtzapple et al., 1990; Kim et al., 2011), but not on enzyme-substrate interaction. Cellobiose, glucose, and ethanol are non-competitive inhibitors to cellulase enzymes, with cellobiose being the most powerful cellulase inhibitor, followed by glucose and ethanol (Holtzapple et al., 1990; Philippidis, Smith and Wyman, 1993; Gruno et al., 2004). The inhibition constants (K_I) of cellobiose have been reported to range from 3-6 g/L and those of glucose between 6-319 g/L (Andric et al., 2010).

The influence of these bioconversion products on enzyme-substrate interaction is only beginning to be elucidated through studies on high-solid hydrolysis. Increasing concentrations of glucose or xylose in a hydrolysis mixture has been reported to cause a decrease in water activity, which is a measure of the availability of water molecules, and this decrease was correlated to a decrease in conversion yields (Selig et al., 2012) In another study, a decrease in cellulase adsorption was observed as substrate loadings were increased and it has been suggested that the decline in enzyme binding capacity was responsible for the decreased sugar yield observed with high solids loading enzymatic hydrolysis (Wang et al., 2011). Similarly, another work reported that the decreased enzyme adsorption seemed to be associated with increasing concentrations of glucose and cellobiose in high-solid hydrolysis

(Kristensen, Felby and Jorgensen, 2009). A recent study has shown that at 20%(w/v) solid consistency the hydrolysis performance of intact enzymes was similar to that of enzymes lacking CBMs, suggesting that a loss in the intact enzyme adsorption capability may cause the observed decrease in hydrolysis performance at high solid consistency (Varnai, Siika-aho and Viikari, 2013). Therefore, based on these observations, the influence of co-solvent (sugars, ethanol) concentrations on enzyme adsorption, enzyme recycling, and hydrolysis efficiency needs to be clarified.

1.5 Thesis Overview and Objectives

1.5.1 Background

Primarily due to the recalcitrance of lignocellulosic biomass, high enzyme loadings are typically required to achieve effective hydrolysis of cellulosic substrates to their constituent sugars. However, high enzyme loadings increase the cost of the hydrolysis step, which has been identified as one of the major cost contributors to the overall bioconversion process (Klein-Marcuschamer et al., 2012; Stephen, Mabee and Saddler, 2012; Humbird et al., 2011).

To reduce the cost of enzymatic hydrolysis, achieving efficient conversion with reduced enzyme loadings has been proposed as a promising strategy (Klein-Marcuschamer et al., 2012). One way to achieve this is through recycling the enzymes for multiple rounds of hydrolysis. An ideal enzyme recycling strategy should recover most of the enzymes in their active form and be able to achieve consistently high conversion efficiency for a number of hydrolysis rounds. However, enzyme recovery requires a fundamental understanding of enzyme interaction with the substrates and enzyme distribution during hydrolysis. While many strategies have been explored as potential enzyme recycling strategies, there has been limited success in achieving efficient enzyme recovery and sustained conversion performance with recycled enzymes. Many of these strategies have recovered enzymes only from the liquid phase or the solid phase, which led to the loss of a large proportion of enzymes. Even

when enzymes have been recovered from both phases, only a few studies have attempted quantification and identification of enzyme distribution during hydrolysis and enzyme recycling. As a result, the identity and the quantity of specific enzyme activities that can be recovered from either phase are largely uncharacterized. One of the reasons for this lack of success in enzyme recycling is a lack of understanding the complex enzyme-substrate interactions that occur during hydrolysis, as well as a lack of available techniques to monitor enzyme distribution.

The primary objective of this thesis was to improve the efficiency of enzyme recycling in order to achieve efficient hydrolysis with reduced enzyme loadings. However, this has to be based on an improved understanding of enzyme-substrate interactions during hydrolysis at industrially relevant conditions before any improvement in enzyme recycling strategy is possible. Therefore this thesis has 3 sub-objectives:

1) to develop improved techniques to identify and quantify individual enzyme adsorption profiles during hydrolysis at industrially-relevant conditions;
2) to determine the influence of enzyme, substrate, and physical factors in individual enzyme distribution; and
3) to evaluate and improve an enzyme recycling strategy based on the addition of fresh substrates.

1.5.2 Research Approach

To achieve the 3 objectives outlined above, the work described in this thesis has been divided into five chapters. Due to a lack of specific techniques available to monitor individual cellulase enzyme adsorption during hydrolysis, most previous enzyme adsorption studies have used purified enzymes or model (cellulosic) substrates. However, the observed interactions in these simplified hydrolysis reactions

may not represent the actual enzyme-substrate interactions that would be used in an industrial setting, i.e. during hydrolysis of industrially-relevant lignocellulosic substrates using complete or commercial cellulase enzyme mixtures. Therefore, we wanted to determine individual enzyme adsorption profiles during an industrially-relevant hydrolysis reaction and evaluate the suitability of common enzyme adsorption techniques. To assess these individual enzyme adsorption profiles, section 3.1 attempted to follow individual enzyme adsorption profiles during hydrolysis of steam pretreated corn stover (SPCS), an industrially relevant lignocellulosic substrate, using a commercial enzyme mixture Accellerase 1000 (Genencor). The techniques evaluated included SDS-PAGE, zymograms, enzyme activity assays, ninhydrin, and mass spectrometry.

To try to overcome the limitations regarding the specificity of these techniques, section 3.2 describes the development of an ELISA technique to specifically and quantitatively determine individual enzyme adsorption profiles during cellulose hydrolysis using a complete enzyme mixture. A double-antibody sandwich ELISA method was developed for Cel7A, Cel6A, and Cel7B, utilizing a combination of monoclonal antibody (MAb) and a polyclonal antibody (PAb) to capture and quantify each of the target enzymes present in a mixture of enzymes. The applicability of this method to quantify specific enzyme adsorption profiles during hydrolysis was determined by comparing the ELISA method with other, less specific assays.

The development of the ELISA technique, when used in combination with other enzyme adsorption assays, resulted in the specific and quantitative determination of individual enzyme adsorption profiles during industrially-relevant hydrolysis reactions. Previous enzyme recycling studies had indicated that enzyme recycling performance was highly influenced by substrate properties, enzyme compositions, and hydrolysis conditions (Lee, Yu and Saddler, 1995; Qi et al., 2011; Jin et al., 2012; Ramos and Saddler, 1994). We therefore assessed the influence of these factors on the adsorption

profiles of individual enzymes during hydrolysis and quantified their recoverability. As this would provide invaluable information to help improve enzyme recycling performance, in the next three chapters, the influence of enzyme, substrate, and physical factors on individual enzyme distribution during hydrolysis was determined by using a combination of the ELISA technique and other protein assays. Specifically, in section 3.3, the influence of lignin on enzyme-substrate interaction was evaluated by hydrolyzing SPCS and delignified SPCS (DSPCS) using commercial enzyme mixtures. The influence of cellulose properties was also investigated using a range of pure cellulosic substrates including Avicel, dissolving pulp (DsP), cellulose II, phosphoric acid swollen cellulose (PASC), and cellulose III.

The results presented in section 3.3 showed that substrate, and especially cellulose properties, significantly influenced enzyme adsorption. As hydrolysis proceeds, the action of cellulase enzymes continues to alter the properties of the substrate (Igarashi et al., 2011; Valjamae et al., 1998; Yang, Willies and Wyman, 2006). It is likely that the action of cellulase enzymes on the cellulose influences the adsorption profiles of other enzymes, which in turn effects their distribution and recoverability. A significant boosting effect on cellulose hydrolysis has been observed by a recently re-classified family 9 Auxilliary Activity (AA9) oxidative enzyme. In contrast to the canonical hydrolytic cellulase enzymes, AA9 (GH61) has been shown to boost hydrolysis by oxidizing crystalline cellulose, creating new chain ends for the hydrolytic cellulase enzymes (Horn et al., 2012; Harris et al., 2010). Therefore, the oxidative action of this enzyme on cellulose may likely alter the cellulose properties and, in turn, the adsorption profiles of hydrolytic cellulase enzymes. Although the enzyme's mechanism of action has been investigated, little is known about its adsorption behaviour and, hence, its recoverability. Therefore, in section 3.4, we determine the recyclability of AA9 and its influence on the recoverability of the canonical cellulase enzymes. The adsorption profile of AA9 and its influence on adsorption profiles of hydrolytic enzymes were evaluated by hydrolyzing lignocellulosic substrates with a commercial enzyme mixture and substituting a portion of the enzyme loading with the AA9 enzyme. The adsorption of AA9 and other

enzymes were followed using the ELISA technique and other enzyme adsorption assays such as SDS-PAGE and enzyme activity assays.

In addition to substrate and enzyme properties, enzyme adsorption is also influenced by physical factors, such as temperature, pH, and concentrations of soluble compounds. Achieving high sugar and ethanol concentrations after a high consistency hydrolysis is needed to reduce costs. Therefore, an industrially relevant enzyme recycling strategy is likely to be carried out in the presence of high sugar or ethanol concentrations. Thus, understanding the influence of sugars and ethanol concentrations on enzyme adsorption and recoverability is becoming increasingly important. By better understanding the influence of sugars and ethanol this should also help determine the appropriate insertion point for enzyme recovery (for instance, after hydrolysis or after fermentation). In section 3.5, the effects of sugars and ethanol on enzyme adsorption and the efficiency of enzyme readsorption to fresh substrate as an enzyme recycling strategy were evaluated. Strategies to maximize enzyme recovery were evaluated by comparing enzyme recovery following hydrolysis and fermentation. Supplementation with fresh enzyme monocomponents to replenish lost enzyme activities was also evaluated as a strategy to improve conversion efficiency using recycled enzymes.

2. Methods and Materials

2.1 Preparation of Pretreated Biomass Feedstocks

The lignocellulosic substrate used in this study was SPCS that was pretreated by SO_2-catalyzed steam pretreatment at near optimal conditions that had previously been determined to provide maximum hemicellulose recovery while ensuring effective enzymatic hydrolysis of the cellulose component (Bura et al., 2003). After pretreatment, the cellulose rich water insoluble component was washed, filtered, and refrigerated for long-term storage. The resulting SPCS was further subjected to acid-chlorite delignification to produce delignified SPCS (DSPCS) by soaking SPCS in sodium chlorite-acetic acid solution for 24 hours at room temperature in the dark. The chemical loading used was 0.5 g of sodium chlorite dissolved in 10 ml of 1% acetic acid for every g dry-weight of SPCS. After incubation, the slurry was poured into 1 L of distilled water and filtered. The solid material was then washed thoroughly with 2 L of water. The whole procedure was repeated one more time until a visually white pulp was obtained. Xylanase-pretreated SPCS was produced by enzymatically hydrolyzing the xylan in SPCS at 2% (w/v) consistency with Multifect Xylanase (Novozymes) at a protein loading of 120 mg/g cellulose in 0.05 M Na-acetate buffer pH 4.8 at 50^{o}C for 24 hours. Following hydrolysis, the solid SPCS was recovered and further subjected to a protease treatment to remove xylanase enzymes bound to the solid. A protease solution was prepared by diluting proteinase from *Aspergillus melleus* (Sigma) to a concentration of 1 U/ml in 50 mM Na-phosphate buffer pH 7. Following an overnight incubation in the protease solution at 37^{o}C, the treated SPCS was heated at 90^{o}C for 2 hours to deactivate the protease. The solid substrate was then thoroughly washed (3x) with 50 mM Na-phosphate buffer, 1x with 1 M NaCl, and 3x with distilled water. Following the final wash, the resulting XSPCS was recovered using filtration.

In addition, 6 model cellulosic substrates were also used, including Avicel (Sigma), dissolving pulp (DsP), cellulose II, phosphoric acid swollen cellulose (PASC), cellulose nano crystal (CNC), and cellulose III derived from cotton linters. Cellulose III was a kind gift from National Renewable Energy Laboratory (NREL). Cellulose II was derived from Avicel according to a method described previously (Mittal et al., 2011). PASC was also derived from Avicel according to a method described previously (Zhang et al., 2006).

2.2 Substrate Characterization

The sugar and lignin compositions in the lignocellulosic and model cellulosic substrates were determined using the modified Tappi T-222 on-88 method, as previously described (Bura et al., 2003).

The lignin surface coverage of the SPCS, DSPCS, XSPCS and DsP was evaluated using the X-ray Photoelectron Spectroscopy (XPS) method. Pulp handsheets (60g/m^2) from the pretreated substrates were prepared according to TAPPI test method T-205 sp-06. Surface chemical characterization was carried out with a Leybold Max 200 X-ray photoelectron spectrometer (Cologne, Germany) with a monochromated Al-Ka X-ray source. The detector position was at an angle of 90^o relative to the sample surface. The samples for analysis (1 cm^2) were cut from the handsheets, attached to the sample holder and evacuated in a prechamber for 12 hours. The high resolution spectra were charge corrected using the C-C component of the C1s signal at 285 eV as an internal standard. A Gaussian curve fitting program (XPSPEAK 4.1) with the Shirley background was used to deconvolute the C1s signal. The following binding energies relative to the C-C position were used: 1.7 ± 0.2 eV for C-O, 3.1 ± 0.2 eV for C=O or O-C-O, and 4.2 ± 0.3 for O=C-O (Laine et al., 1994).

The theoretical surface lignin coverage (TSLC, Φ_{lig}) was calculated from the O/C ratios according to the following equation:

$$\Phi_{lig} = (O/C_{(sample)} - O/C_{(cellulose)}/(O/C_{(lignin)} - O/C_{(cellulose)})$$

Where $O/C_{(sample)}$ is the O/C ratio of the analyzed sample, and $O/C_{(cellulose)}$ and $O/C_{(lignin)}$ are the theoretical O/C ratios of pure cellulose and lignin (0.83 and 0.33) respectively (Laine et al., 1994).

Specific cellulose accessibility was determined by using the Simons' staining (SS) technique according to the modified procedure (Chandra et al., 2009). The relative amount of accessible crystalline cellulose and amorphous cellulose was also assessed using CBM2a and CBM44 adsorption, respectively (Gourlay, Arantes and Saddler, 2012).

2.3 Cellulase Enzymes and Comparison of Activities and Stabilities of Commercial Cellulase Mixtures

Commercial cellulase preparations Spezyme (Genencor), Accellerase™ 1000 (Genencor), Celluclast (Novozymes), Cellic CTec 2 (Novozymes), and Cellic CTec 3 (Novozymes) as well as β-glucosidase (Novozyme 188, Novozymes) were used in this study. Cellulase and β-glucosidase activities were expressed in terms of filter paper units (FPU) and international units (IUs), respectively. The cellulase monocomponents Cel7A, Cel6A, Cel7B, Cel5A, family 10 xylanase (Xyn10), and family 11 xylanase (Xyn11) were purified from Celluclast (Novozymes) using previously described methods (Medve et al., 1998; Rosgaard et al., 2007; Gama, Vilanova and Mota, 1998; Zhou et al., 2008).

In order to determine the cellulase mixture with the highest activity and stability, three commercial enzyme mixtures Celluclast, Spezyme and Accellerase™ 1000 were diluted 10-fold in Na-acetate buffer (0.05 M, pH 4.8), incubated at 40, 50, 60, 70, and 75^{o}C for 24 hours in a shaking incubator and assayed for protein content and enzymatic activities (section 2.10).

2.4 Enzymatic Hydrolysis

For the study on enzyme distribution during hydrolysis (section 3.1), enzymatic hydrolysis of SPCS was carried out in 15 ml tubes (Corning) in four replicates at 50^{o}C with a rotational mixing at 20 rpm in an incubator (Combi-D24). The SPCS was diluted to 2% solid loading (w/v) with Na-acetate buffer (0.05 M, pH 4.8) to a total volume of 10 ml. The antibiotics cycloheximide and tetracycline (Sigma) were added to the hydrolysis mixture at concentrations of 0.03 mg/ml and 0.04 mg/ml, respectively, to prevent microbial contamination. Accellerase^{TM} 1000 was added at 51 mg protein/g glucan or 20 FPU/g glucan. The reaction mixture was prepared on ice to limit hydrolysis. Samples were taken at different hydrolysis time over a period of 72 hours. After centrifugation, the proteins in the liquid phase were recovered by transferring the supernatant into new 15 ml tubes. One ml of the supernatant containing the liquid-fraction proteins was collected and boiled at 100^{o}C for 10 minutes for subsequent glucose measurement using the glucose oxidase assay (Berlin et al., 2006a). The remaining supernatant was stored at 4^{o}C for determination of enzyme activities, protein concentration, and gel electrophoresis and zymogram assays. Desorption of the proteins bound to the residual substrate (the solid phase) after each hydrolysis time was carried out according to previously described conditions (Tu et al., 2009a). Briefly, after collecting the supernatant, 5 ml of Na-acetate buffer (0.05 M, pH 5.3), containing 0.5% Tween 80 (v/v) (Sigma), was added to the solid SPCS hydrolysis residue. The tubes were incubated for 2 hours at 44^{o}C, after which they were centrifuged and the supernatant containing solid-phase proteins was collected. The solid-phase proteins were then stored at 4^{o}C for determination of enzyme activities, protein concentration, and gel electrophoresis and zymogram assays.

For determination of enzyme distribution using the double-antibody sandwich enzyme-linked immunosorbent assay (ELISA; section 3.2), SPCS hydrolysis was also carried out using Celluclast at 20 FPU/g glucan or 52 mg protein/g glucan supplemented with β-glucosidase at 1:2 FPU:IU. Concurrently,

SPCS was also incubated in Na-acetate buffer (0.05 M, pH 5.0), in the absence of enzymes, to serve as a substrate control (SPCS SC). During hydrolysis, samples were taken at different time points over a period of 72 hours. After centrifugation, the unbound proteins in the supernatant were recovered by transferring the supernatant into 15 ml tubes. One ml of the supernatant was collected and heated at 100^{o}C for 10 minutes for subsequent glucose measurement using the glucose oxidase assay. The remaining supernatant was stored at 4^{o}C for subsequent ELISA assay using the optimized conditions (described in section 2.13) to determine any changes in Cel7A, Cel6A, and Cel7B concentrations during hydrolysis.

2.5 The Influence of Substrate Characteristics on Enzymatic Hydrolysis and Enzyme Adsorption

SPCS, DSPCS, XSPCS, and DsP were hydrolyzed with Cellic CTec 2 (Novozymes) at 2% (w/v) substrate consistency and an enzyme loading of 15 mg/g cellulose in 0.05 M Na-acetate buffer pH 4.8 at 50^{o}C. No exogenous reducing factors were added to the hydrolysis reaction. The substrates were hydrolyzed for 72 hours, and samples were taken at different time points during hydrolysis. Supernatants were collected from these samples for sugar, protein, and enzyme activity analyses. For comparison purposes, a concurrent hydrolysis experiment was carried out on the four substrates using Celluclast at an enzyme loading of 20 mg/g glucan supplemented with 10 mg/g glucan of β-glucosidase. The 6 model cellulosic substrates were also hydrolyzed with Celluclast at an enzyme loading of 13 mg/g cellulose supplemented with 5 mg/g glucan of β-glucosidase (Novozym 188).

2.6 The Influence of AA9 Enzyme on Enzymatic Hydrolysis and Enzyme Adsorption

The influence of the AA9 enzyme on enzymatic hydrolysis and enzyme adsorption was determined by hydrolyzing steam pretreated corn stover (SPCS), delignified SPCS (DSPCS), and the 6 model cellulosic substrates using Celluclast and β-glucosidase at the same enzyme loadings and conditions used in section 2.5. The influence of AA9 enzyme was investigated by substituting a portion

of Celluclast with the same amount of AA9 enzyme to maintain a constant enzyme loading. The AA9 enzyme was derived from *Thermoascus auranticus* and kindly supplied by Novozymes. Samples were taken during hydrolysis, and supernatants were collected from the solid residues for sugar and protein assays.

Incorporation of acid groups in the substrate due to the action of AA9 enzyme was determined by conductometric titration as described previously (Del Rio, Chandra and Saddler, 2011). 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 with 50 ml water for 5 more times. The washed pulp was re-suspended in 50 ml of NaCl (0.001 N) and 20 ul 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, Chandra and Saddler, 2011).

2.7 The Influence of Soluble Compounds on Enzymatic Hydrolysis

The effects of glucose, xylose, and ethanol concentrations on protein adsorption were determined by adding a mixture of Celluclast and β-glucosidase to mixtures containing dissolving pulp (DsP) at 2% (w/w) solids loading and different concentrations of glucose, xylose, or ethanol. The enzyme loading added was ~50 mg/g glucan (37 mg Celluclast and 11 mg β-glucosidase/ g glucan). The enzyme, substrate, and sugar or ethanol mixtures were incubated on a rocking platform at 4^{o}C for 2 hours to minimize hydrolysis and to ensure that the adsorption equilibrium was achieved. Experiments were done in duplicate for each concentration. The concentrations of glucose and xylose added ranged from 0-250 g/L, and ethanol concentrations ranged from 2.5-20% (v/v). These concentrations correspond to

relevant concentrations that may be found during hydrolysis and fermentation, except for the xylose concentrations which typically do not exceed 100 g/L.

2.8 Enzyme Recycling with Addition of Fresh Substrates and Enzyme Supplementation

In Chapter 3.5, the efficiency of enzymatic hydrolysis combined with enzyme recycling was investigated by comparing hydrolysis performance after enzymatic hydrolysis (EH) and after hybrid hydrolysis and fermentation (HHF). During EH, DsP at 5% (w/w) consistency was hydrolyzed with a total cellulase enzyme loading of 25 mg/g glucan for 72 hours at 50^{o}C. The cellulase preparations used were either Cellic CTec 3 (at a loading of 25 mg/g glucan) or Celluclast at a loading of 20 mg/g glucan, supplemented with β-glucosidase at 4 mg/g glucan. Hydrolysis with Celluclast and β-glucosidase was also done with and without supplementation with 1 mg/g glucan of the AA9 enzyme. The HHF method started with a 24-hour hydrolysis after which a yeast pitch (*Saccharomyces cerevisiae* T2) was added to the whole mixture at a final OD_{600} of 6.5. The mixture then underwent simultaneous saccharification and fermentation (SSF) for 48 hours at 32^{o}C. Samples were taken at different time points during the first round of both EH and HHF to monitor glucan conversion and protein adsorption profiles.

After a 72-hour hydrolysis period (either EH or HHF), the same amount of fresh DsP was added to the hydrolysis mixture to recover proteins in the liquid and solid fraction. The mixtures were then incubated for 2 hours at 4^{o}C to allow the enzymes to re-adsorb to fresh substrate. After this re-adsorption period, the samples were centrifuged at 4696xg and 4^{o}C for 10 minutes to separate the solid (containing fresh DsP, residual solids, readsorbed enzymes, and residual sugars or ethanol) from the liquid fractions (containing unreadsorbed enzymes and sugars or ethanol). Fresh 0.05 M Na-acetate buffer pH 5 was then added to the solid fraction to a final solid loading of ~5% (w/w). The samples were then incubated for a second round of 24-hour hydrolysis. Samples were taken at the start and at the end of this 24-hour hydrolysis to determine the extent of glucan conversion attributed to the recovered

enzymes. The amount of proteins re-adsorbed was determined by measuring protein concentrations in the liquid fraction before and after the re-adsorption step.

Due to the superior enzyme recovery and hydrolysis performance observed with the HHF strategy, subsequent enzyme recycle experiments were carried out using the HHF method at the same solids loadings of 5% (w/w). To determine the influence of AA9 enzyme on hydrolysis and enzyme recycling, dissolving pulp (DsP) was hydrolyzed at 5% (w/w) solid loading using the HHF strategy, with and without 1 mg/g glucan of AA9 enzyme. The enzyme mixture containing AA9 enzyme was 20 mg/g glucan of Celluclast, 4 mg/g glucan of β-glucosidase, and 1 mg/g glucan of AA9 enzyme. The enzyme mixture without AA9 enzyme consisted of 21 mg/g glucan of Celluclast and 4 mg/g glucan of β-glucosidase.

Additionally, to better simulate the latest enzyme formulation, hydrolysis was done with Celluclast supplemented with the AA9 enzyme. The enzyme mixture in these subsequent experiments consisted of Celluclast, β-glucosidase, and AA9 enzyme at loadings of 20, 4, and 1 mg/g glucan, respectively. Parallel enzyme recycle experiments were also carried out using 25 mg/g glucan CTec 3 (Novozymes).

To determine if consistent hydrolysis performance could be achieved by supplementing recycled enzymes with fresh enzymes, the hydrolysis mixtures were supplemented with 40% of the original enzyme loading (either the mixture of Celluclast, β-glucosidase, and AA9 or CTec 3) following the enzyme recycle step. The hydrolysis was then carried out using the HHF strategy for a total of 5 rounds. Fresh substrate was added to the hydrolysis mixtures to recover the enzymes. Following centrifugation at 4696xg at 4^{o}C for 10 minutes to separate the liquid from the solid substrates, fresh buffer and 40% of the original enzyme loading were added to the solid substrates. These steps were repeated for 4 rounds of HHF and enzyme recycling. In the fifth round, the mixtures following enzyme recycle and

supplementation were hydrolyzed for a total of 96 hours. Time points were taken to monitor glucan conversion.

Although consistently high hydrolysis performance could be achieved in previous supplementation experiments where recycled enzymes were supplemented with 40% of the original enzyme composition, we wanted to determine if sugar production could be further improved by optimizing the composition of this enzyme supplement. In these optimization studies, DsP was initially hydrolyzed with a Celluclast, β-glucosidase, and AA9 enzyme mixture (25 mg/g glucan) using the HHF strategy for 48 hours as described before. Following addition of fresh substrate, recycled enzymes were similarly supplemented with 40% of the original amount of protein or 5 mg/g original glucan. While keeping the amount of protein constant, the ratio of the enzyme components in the fresh enzyme mixture was varied.

Initially, to determine the identity of important enzyme components needed for supplementation, the recycled enzymes were supplemented with 100% of Celluclast (5 mg/g original glucan), 60% of Celluclast and 40% of Xyn10, Xyn11, or β-glucosidase, or 80% of Celluclast and 20% of AA9. Because supplementation with the Celluclast + β-glucosidase mixture gave the highest increase in glucose production, the ratio of Celluclast and β-glucosidase in the mixture was further optimized by varying the amount of β-glucosidase in the mixture between 0-100%. Supplementation with the Celluclast + AA9 mixture also resulted in a slight increase in sugar production. Similarly, the ratio of the individual enzyme components in the mixture was optimized by adding 0-1.5 mg of AA9/g glucan to a mixture of 3 mg/g glucan of Celluclast and 2 mg/g glucan of β-glucosidase.

2.9 Measurements of Glucan Conversion and Ethanol Concentrations

Glucan conversion during hydrolysis was measured as glucose released during hydrolysis using the YSI glucose analyzer (YSI 2700 SELECT Biochemistry Analyzer, YSI Life Sciences) or the glucose

oxidase method. The extent of cellulose hydrolysis of the pretreated substrates was calculated from the glucan content as a percentage of the theoretical glucan available in the substrate. Ethanol concentrations after SSF were quantified by gas chromatography on a 5890 Series II chromatograph with a 6890 auto-injector with a splitless injector system and a flame ionisation detector (Hewlett Packard, Palo Alto, CA). Helium was used as the carrier gas (20 mL/min) for injection into a HP-Innowax analytical column (15m×0.53mm). In brief, the temperatures of the injection unit and flame ionization detector (FID) were set at 175°C and 250°C, respectively. The oven was heated to 45° for 2.5 minutes and the temperature was raised to 110°C at a rate of 20°C/minute and later held at 110°C for 2 minutes. Standards and samples were supplemented with 100% 1-butanol as the internal standard. Final glucan conversion for HHF was calculated by converting the amount of quantified ethanol to its glucose equivalent (Zhang and Bao, 2012).

2.10 Protein and Enzyme Activity Assays

The ninhydrin method (Pribowo, Arantes and Saddler, 2012) was used to quantify the protein content of the commercial cellulase mixtures and the amount of proteins recovered from the liquid fraction and desorbed from the solid fraction after hydrolysis in order to obtain protein distribution profiles during hydrolysis. Bovine serum albumin (BSA) was used as the protein standard.

In order to determine the distribution profiles of specific enzyme activities, the hydrolysates following hydrolysis were assayed using substrates, such as *p*-nitrophenyl-β-D-glucopyranoside (PNPG), *p*-nitrophenyl-β-D-cellobioside (PNPC), carboxymethyl cellulose (CMC), and birchwood xylan (Sigma) (Deshpande, Eriksson and Pettersson, 1984). PNPG is a specific substrate for β-glucosidase. Both Cel7A and β-glucosidase are known to hydrolyze PNPC (Wood and Bhat, 1988). Therefore, in order to obtain activity profile of Cel7A, β-glucosidase activity was specifically inhibited by adding 0.5 mg/ml of D-glucono-1,5-δ-lactone (Sigma) to the PNPC reaction (Deshpande, Eriksson and Pettersson, 1984). CMC

was used to determine the activity profiles of endoglucanases. PNPX and birchwood xylan were used to determine xylanase and endoglucanase activities.

2.11 SDS-PAGE and Zymograms

The SDS-PAGE assay of the proteins recovered during hydrolysis was performed in a 4-12% Bis-Tris Criterion XT polyacrylamide gel (Bio-Rad). In some cases, the proteins recovered from the liquid and solid phases had to be concentrated 10-fold by lyophilization. After staining with Coomassie Blue G-250 (Bio-Rad), the adsorption profiles of the different proteins on the gels were determined by measuring changes in band density using the densitometry function of Alpha Imager^{TM} 2200 (Alpha Innotech). Protein identification was done using mass spectrometry as described below.

In addition to SDS-PAGE, zymograms were used to visualize changes in enzymatic activities over time. The CMCase and xylanase activities were visualized by incorporating 0.1% (w/v) of CMC and birchwood xylan, respectively, into an 2% agarose overlay gel diluted in Na-acetate buffer (0.05 M, pH 4.8). The detection of CMCase activity on an overlay agarose gel was carried out as described previously (Sun et al., 2008) with some modifications. Briefly, prior to electrophoresis, the samples were first mixed with the XT sample buffer (Bio-Rad), which contains SDS, and heated at 60^{o}C for 45 minutes. Following electrophoresis, the polyacrylamide gel was washed twice with Na-acetate buffer (0.05 M, pH 4.8) containing 25% isopropanol (Sigma) for a total of 15 minutes to remove SDS. The gel was then washed two more times using Na-acetate buffer (0.05 M, pH 4.8) with no isopropanol for another 15 minutes. All washing steps were done at room temperature. This washing step renatures the proteins in the polyacrylamide gel. The polyacrylamide gel was then placed on top of a thin 2% agarose gel made with Na-acetate buffer containing 0.1% CMC, and the whole assembly was incubated at 50^{o}C for 60 minutes. The agarose gel was then stained with 0.1% Congo Red (Sigma) for 30 minutes. Excess Congo Red was poured off, and the agarose gel was washed with 1M NaCl for 30 minutes to remove excess stain. After

a final rinse in 5% acetic acid, the areas of hydrolysis appeared unstained against a dark blue background. The gel was then immediately photographed.

The xylanase activities were detected using a similar procedure, with an overlay agarose gel containing 0.1% birchwood xylan. The samples were not heated after the addition of XT sample buffer and the overlay agarose gel was incubated with the polyacrylamide gel for 1 hour instead of 30 minutes to increase contrast between hydrolysis zones and background staining.

Detection of cellobiohydrolase activities were carried out directly on the polyacrylamide gels using the synthetic substrate 4-methylumbeliferyl-β-D-cellobioside (MUC) (Sigma) (Sun et al., 2008). After addition of the XT sample buffer, the samples were heated for 45 minutes at 60^{o}C. After electrophoresis the polyacrylamide gel was rinsed according to the method described above for the CMCase zymogram. The gel was then directly soaked in a solution of Na-acetate buffer (0.05 M, pH 4.8) containing 0.1 mM MUC and incubated with shaking at 50^{o}C. After incubation for 1 hour, the gel was immediately visualized under UV illumination. To visualize the protein bands, the gel was also stained with Coomassie Blue G-250. The Cel7A, Cel7B and β-glucosidase have been shown to have activity on MUC. The Cel7A and β-glucosidase are easily distinguished based on their molecular weights (MW). However, this was not the case for Cel7A and Cel7B. In order to distinguish Cel7A activity from that of Cel7B, a concurrent zymogram assay was carried out using the same MUC concentration but with an addition of 1% cellobiose to specifically inhibit Cel7A activity (Du et al., 2010). The band intensities of the two zymogram gels were then measured and compared using the densitometry function of Alpha Imager^{TM} 2200.

2.12 Liquid Chromatography - Mass Spectrometry / Mass Spectrometry (LCMS/MS)

The bands of proteins on the SDS-PAGE gel were carefully excised, transferred to 1.5 ml centrifuge tubes, and digested with trypsin. The LC-MS/MS analysis of these bands was performed on an

LC Packings capillary LC system (Dionex) coupled to a tandem quadrupole time-of-flight mass spectrometer (QSTAR Pulsar, Applied Biosystems). Some peptide samples were purified by solid phase extraction. Purified peptides were analyzed using a linear-trapping quadrupole - Orbitrap mass spectrometer (LTQ-Orbitrap; ThermoFisher Scientific) on-line coupled to an Agilent 1100 Series nanoflow HPLC using a nanospray ionization source (ThermoFisher Scientific). Both analyses were carried out at the University of British Columbia (UBC) Michael Smith Laboratory / Centre for High Throughput Biology "CHiBi" Proteomics Core Facility. The mass accuracy: error of mass measurement is usually within 5 ppm and is not allowed to exceed 10 ppm.

The fragment spectra were extracted using DTASuper-Charge5 and searched using the Mascot algorithm against a database comprised of the protein sequences from the source organism using the following parameters: peptide mass accuracy 10 parts per million; fragment mass accuracy 0.6 Da; trypsin enzyme specificity, fixed modifications - carbamidomethyl, variable modifications - methionine oxidation and N-acetyl peptides, ESI-TRAP fragment characteristics. Only those peptides with IonScores exceeding the individually calculated 95% confidence limit (as opposed to the average limit for the whole experiment) were considered as accurately identified. This analysis was also carried out at the University of British Columbia (UBC) Michael Smith Laboratory / Centre for High Throughput Biology "CHiBi" Proteomics Core Facility.

2.13 Development of Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

2.13.1 Preparation of Antibodies for ELISA and Determination of Their Specificity

Monoclonal antibodies (MAbs) against Cel7A, Cel6A, and Cel7B as well as polyclonal antibodies (PAbs) against Cel7B were a kind gift from Dr. Larry Taylor of the National Renewable Energy Laboratory (NREL). PAbs against Cel7A and Cel6A were prepared commercially by Alpha Diagnostic International, Texas. Briefly, synthetic peptides containing amino acid sequence with high antigenicity from enzymes

Cel7A and Cel6A were identified and synthesized. The peptide sequence used to raise the Cel7A PAb was R-A-Q-S-A-C-T-L-Q-S-E-T-H-P-P-L-T-W-Q-K, and that for Cel6A PAb was C-D-T-L-D-K-T-P-L-M-E-Q-T-L-A-D-I-R. Following peptide conjugation, antibodies were raised by immunizing rabbits with these peptides. The antibody titers in the rabbit sera and its reactivity to the target peptide were tested using ELISA. Once the test results met the required criteria, the antibody was then purified from the sera by using affinity columns coated with the respective peptide.

The specificity of all MAbs and PAbs were first tested against purified enzymes and enzyme mixtures by using the Western Blot technique following a protocol described by the assay kit producer (Immun-Blot Assay Kit, Bio-Rad). The reactivity and specificity of MAbs against all 3 enzymes (Cel7A, Cel6A, and Cel7B) were tested against purified Cel7A from Celluclast and 3 commercial enzyme mixtures (30 µg each) Accellerase 1000, Celluclast, and Cellic CTec 2. PAbs against Cel7A and Cel6A were tested against purified Cel7A and Cel6A from Celluclast as well as the commercial cellulase mixtures Celluclast and Cellic CTec 2. The specificity and reactivity of PAb against Cel7B were similarly tested against purified Cel7A, Cel6A, and Cel7B from Celluclast as well as the 3 enzyme mixtures Accellerase 1000, Celluclast, and Cellic CTec 2.

Briefly, purified enzymes and enzyme mixtures were separated using SDS-PAGE on 4-12% (w/v) Bis-Tris Criterion XT polyacrylamide gels (Bio-Rad). Following electrophoresis, the polyacrylamide gel was equilibrated in the transfer buffer (Towbin buffer containing 25 mM Tris, 192 M glycine, and 20% (v/v) methanol) for 30 minutes. The proteins in the polyacrylamide gel were then transferred to a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) for 60 minutes at 15 V. After washing with Tris-buffered saline containing 0.05% (v/v) Tween 20 (TTBS), the membrane was immersed in Tris-buffered saline (TBS) containing 3% (w/v) gelatin to block any unoccupied sites on the membrane. Antibodies to be tested were then added at a

concentration of 5 µg/ml diluted in TTBS containing 1% (w/v) gelatin, and the membrane was incubated for 1 hour. Bound MAbs were detected by immersing the membrane in TTBS-1% (w/v) gelatin containing 1/3000 dilution of goat anti-mouse-IgG antibody conjugated to alkaline phosphatase (GAM-AP, Bio-Rad) for 1 hour whereas bound PAbs were detected by using goat anti-rabbit-IgG antibody conjugated to alkaline phosphatase (GAR-AP, Bio-Rad). After a final wash, the membrane was developed by incubation in the color development/substrate solution containing 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) and nitro-blue tetrazolium chloride (NBT) for 30 minutes. The reaction was stopped by immersing the membrane in nanopure water for 10 minutes.

2.13.2 Optimization of Sample Treatments for ELISA

A double-antibody sandwich ELISA was developed as it was previously shown (Buhler, 1991) to have improved specificity for a target cellulase enzyme present in a cellulase enzyme mixture. MAbs were used as the coating antibodies and PAbs as the detecting antibodies to minimize possible interference from other enzymes, sugars and other materials that may be present in the enzyme mixture. Unless otherwise stated, all reagents were added at a volume of 100 µl, and incubation was carried out at 37^{o}C. MaxiSorp plates (Nunc) were coated with MAb diluted in 1x phosphate-buffered saline (PBS) pH 7.5 at 4^{o}C overnight. The wells were then washed with PBS and blocked with 2% (w/v) BSA diluted in 1x PBS for 2 hours. After the wells were washed, enzyme standards and/or samples were added to the wells and incubated for 2 hours. As antibody-antigen interaction is optimum at pH > 7 (Riske, Eveleigh and Macmillan, 1990), the enzyme samples were added to the wells after dilution in PBS pH 7.5 to ensure that the enzyme samples were in a solution at greater that pH>7. Purified Cel7A, Cel6A, and Cel7B were serially diluted (concentrations 0 - 2.5 µg/ml) in PBS to develop standard curves. After incubation with each of the enzymes, the plate was washed, and the PAb, diluted in PBS with 1% (w/v) BSA, was added to each well. The plate was then incubated for 1 hour. Following another washing step,

the third antibody, a commercial GAR-AP (Bio-Rad) diluted in PBS with 1% (w/v) BSA, was added to the wells and incubated for another hour. After a final washing step, *p*-nitrophenylphosphate (Bio-Rad), a substrate for alkaline phosphatase (AP), was added to the wells and the plate was incubated at room temperature for 30 minutes or until sufficient colour had developed. Colour development was stopped by adding 400 mM glycine-NaOH. The amount of enzymes bound to the sandwich ELISA was quantified by measuring the absorbance of *p*-nitrophenyl at 405 nm.

The concentrations of the MAb, PAb, and GAR-AP were optimized for the Cel7A ELISA. Various concentrations of each antibody were tested against a series of concentrations of purified Cel7A. During each antibody optimization, the concentrations of the other two antibodies were kept constant. MAb's against Cel7A was tested at two different concentrations of 10 and 50 µg/ml. Once the concentration of the MAb was optimized, the PAb against Cel7A was assayed at concentrations of 1.75, 3.5, 7, and 14 µg/ml. Similarly, two different dilutions (1/500 and 1/1750) of the third antibody, (the GAR-AP conjugate) were assessed.

As heat treatment had previously been used successfully to improve the sensitivity of an ELISA technique for Cel7A (Riske, Eveleigh and Macmillan, 1990), we investigate the possible influence of heat treatment on the ELISA when 5 µg/ml of each of the purified enzymes were heated at 100^{o}C for 10 minutes. Each enzyme was heated in either Na-acetate buffer (0.05 M pH 5.0) or in PBS pH 7.5. After cooling the samples to room temperature, the enzymes that had been heated in Na-acetate buffer were first diluted with PBS and then added to the ELISA plate. Samples heated in PBS were directly added to the wells at the same final concentration. Unheated samples were added as controls.

2.13.3 Determination of the Specificity of the ELISA

The specificity of each ELISA was determined by comparing the ELISA signal of the target enzyme in the absence and presence of the three other cellulase enzymes (Cel7A, Cel6A, Cel7B, and Cel5A). The

reconstituted enzyme mixture consisted of 5 μg/ml of the target enzyme and 2.5 μg/ml of each of the other three cellulase enzymes in Na-acetate buffer (0.05M, pH 5.0). For Cel7A and Cel6A ELISA, the reconstituted enzyme mixture was heated at 100^{o}C for 10 minutes, serially diluted in PBS to make a standard curve, and then added to the well. Similarly, 5 μg/ml of the pure enzyme sample was subjected to the same treatment. The standard curve obtained from the purified enzyme sample was then compared with that obtained from the reconstituted enzyme mixture. The specificity of Cel7B ELISA was determined in a similar manner except that the enzyme samples were not heated but directly added to the wells after dilution in PBS. The specificity of ELISA was also tested using commercial enzyme mixtures to determine if a dilution of a commercial enzyme mixture can be used to construct a standard curve, obviating the need to use purified enzymes. Commercial enzyme mixtures were diluted in Na-acetate buffer (0.05M, pH 5.0), subjected to the heat treatment when required (i.e. for Cel7A and Cel6A ELISA), serially diluted in PBS, and then added to the wells.

2.13.4 Influence of Lignocellulosic-derived Components Present in the Hydrolysis Supernatants on the ELISA

Other than the enzymes, lignocellulosic hydrolysates can contain various materials derived from the biomass such as soluble phenolic compounds that may interfere with the ELISA technique. Therefore, to try to determine the possible influence of these substrate materials on the ELISA technique, lignocellulosic supernatants obtained from steam pretreated corn stover (SPCS), steam pretreated poplar (SPP), steam pretreated douglas fir (SPDF), and Avicel PH-101 (Sigma), a pure crystalline cellulose substrate, were incubated in 0.05 M Na-acetate buffer pH 5.0 for 24 hours at 50^{o}C with rotational mixing in an incubator (Combi-D24) in the absence of any enzymes. After centrifugation to remove the solid substrate, a known concentration of the target enzyme was added to these supernatants. The same enzyme concentration diluted in 0.05 M Na-acetate buffer pH 5.0 was used as a

control. These samples were subjected to heat treatment when required, diluted in PBS and then added to the well. The influence of sugar was not determined as previous work had shown that sugars did not interfere with the ELISA when a MAb was used as the first antibody (Riske, Eveleigh and Macmillan, 1990). As previous work had suggested that "diluting-out" these substrate-derived materials could minimize their interference of the ELISA (Conde et al., 2008) the supernatants were diluted 10 or 100 times with PBS.

2.13.5 The Development of Double-antibody Sandwich ELISA to Quantify Cel7A, Cel6A, and Cel7B Adsorption during SPCS Hydrolysis

A protocol for quantifying Cel7A, Cel6A, and Cel7B adsorption during hydrolysis was next developed. ELISA plates were incubated with 10 μg/ml of MAb in PBS at 4^{o}C overnight. The wells were then washed with PBS and blocked with 2% (w/v) BSA diluted in PBS for 2 hours. After the wells were washed, enzyme standards and/or samples were added to the wells and incubated for 2 hours. For the Cel7A and Cel6A ELISA's, before the addition of samples to the ELISA plate, the purified enzyme samples or the hydrolysate samples were first heated at 100^{o}C for 10 minutes. The heat treatment was always done in Na-acetate buffer (0.05 M pH 4.8). After cooling to room temperature, the samples were diluted in PBS and then added to the ELISA plate. Samples for Cel7B ELISA were not heated but directly diluted in PBS buffer. This dilution in PBS not only adjusted the pH of the added samples but also diluted any interfering materials that might be present in lignocellulosic supernatants. After incubation with the enzyme samples for 2 hours, the plate was washed with PBS. A PAb toward the enzyme of interest was added at a concentration of 14 μg/ml diluted in PBS containing 1% (w/v) BSA. The plate was then incubated for 1 hour. Following another washing step, the third antibody, a commercial GAR-AP (Bio-Rad) diluted 1/500 in PBS containing 1% (w/v) BSA, was added and incubated for another hour. After a final washing step, *p*-nitrophenylphosphate (Bio-Rad) was added, and the plate was incubated until

sufficient colour had developed. The colour development was stopped by adding 400 mM glycine-NaOH. The amount of enzymes bound to the sandwich ELISA was quantified by measuring the absorbance of *p*-nitrophenyl at 405 nm

By following this protocol, the amount of Cel7A, Cel6A, and Cel7B present in SPCS hydrolysates (unbound proteins) during 72-hour hydrolysis was quantified. Purified Cel7A, Cel6A, and Cel7B were used to make standard curves. In each of the ELISA assays, the SPCS SC were included and treated in the same way as the hydrolysate samples, to determine the possible influence of any materials in the hydrolysates. The initial enzyme in buffer without any substrate (enzyme control-EC) was also included, to determine the initial concentration of each enzyme. Protein samples for Cel7A ELISA were obtained from SPCS hydrolysis using 20 FPU/g cellulose of Accellerase 1000. Those for Cel6A and Cel7B ELISA were obtained from SPCS hydrolyzed by 20 FPU/ g Celluclast complemented with 40 CBU/ g cellulose of β-glucosidase.

3. Results and Discussion

3.1 The Adsorption and Enzyme Activity Profiles of Specific *Trichoderma reesei* Cellulase/ Xylanase Components when Hydrolyzing Steam Pretreated Corn Stover

3.1.1 Background

As mentioned in the introduction, cellulase enzyme recycling is a promising cost reduction strategy for enzymatic hydrolysis of lignocellulosic biomass for two main reasons. First, effective cellulose hydrolysis still requires relatively high enzyme loadings (Sun and Cheng, 2002; Himmel et al., 2007). Secondly, as the activity of many of the cellulase components has been shown to be relatively stable over extended hydrolysis times (Maheshwari, Bharadwaj and Bhat, 2000) there is an opportunity to perhaps re-use the enzymes for multiple rounds of hydrolysis. Previous work has shown that, during hydrolysis, the cellulase enzymes are distributed between the liquid phase or adsorbed onto the solid substrate (Yang et al., 2010; Tan et al., 1986; Ramos and Saddler, 1994; Lu et al., 2002; Knutsen and Davis, 2004; Steele et al., 2005). Several strategies have been pursued to recover cellulases from the liquid phase, including the use of ultrafiltration membranes; enzyme flocculation using tannins followed by the addition of polyethylene glycol (PEG) (Xu and Chen, 2009); and the use of techniques such as enzyme immobilization (Tu et al., 2006; Dourado et al., 2002). An alternative approach is to try to recover enzymes from the solid residue (the solid phase), where reutilization of bound enzymes to hydrolyze fresh substrates has shown some initially promising results (Yang et al., 2010; Knutsen and Davis, 2004; Lee, Yu and Saddler, 1995; Qi et al., 2011). An additional enzyme recycling strategy, namely enzyme readsorption to fresh substrates, could possibly provide a simple, cheap, and efficient way to recover enzymes from both the liquid and solid phases. However, enzyme readsorption strategies are known to be highly dependent on the nature of enzyme interaction with the substrate, resulting in

different recycling performance with different enzyme-substrate systems (Lu et al., 2002; Lee, Yu and Saddler, 1995; Qi et al., 2011; Tu, Chandra and Saddler, 2007).

The disparate enzyme recycling performance reported in the literature arises from our lack of understanding on the adsorption behavior of the different cellulase enzymes to the lignocellulosic substrates. Unfortunately, elucidating how individual cellulase enzymes interact with a lignocellulosic substrate during the course of hydrolysis has proven to be quite challenging. It is recognized that many factors influence this substrate-enzyme interaction, including hydrolysis conditions (e.g. temperatures, pH, protein loadings, and substrate concentrations) (Kumar, Singh and Singh, 2008; Zhang and Lynd, 2004); substrate factors (e.g. hemicellulose and lignin contents, cellulose structure and accessibility, changes in substrate characteristics during hydrolysis) (Arantes and Saddler, 2010; Bura, Chandra and Saddler, 2009; Nakagame, Chandra and Saddler, 2010; Bommarius et al., 2008; Valjamae et al., 1998; Quiocho, 1986); and enzyme related factors (e.g. source of enzymes, enzyme mixture composition and the proportions of individual enzymes in the mixture) (Palonen, Tenkanen and Linder, 1999; Palonen et al., 2004). To achieve effective hydrolysis, multiple types of enzymes are required in the mixture and these enzymes have been shown to have different roles and affinities to the various lignocellulosic substrates (Quiocho, 1986; Yu et al., 1994). However, the currently available assays used in enzyme adsorption studies, such as SDS-PAGE, zymograms, and enzyme activity assays lack the specificity to determine the adsorption profiles of these individual enzymes (Sharrock, 1988; Zhang, Himmel and Mielenz, 2006). As a result, enzyme-substrate interactions during hydrolysis remain complex and poorly understood.

To simplify this complex phenomenon, most previous enzyme adsorption studies have used model substrates and/ or determined the general changes in overall protein content or overall enzyme activities before and after hydrolysis (e.g. filter paper or carboxymethylcellulase (CMCase) activities)

(Boussaid and Saddler, 1999; Sipos et al., 2010). Only a few studies have tried to follow the adsorption behavior of individual enzyme components. However, these studies have either investigated changes in individual enzyme adsorption before and after hydrolysis, and not during hydrolysis (Sipos et al., 2010; Tu, Chandra and Saddler, 2007) or used purified enzyme components (Varnai et al., 2011). As the action of one enzyme is likely to affect the actions of other enzymes present in a mixture, the adsorption profiles of monocomponent enzymes in a reconstituted mixture are likely to be different than those present in a complete commercial cellulase mixture. Therefore, it would be desirable to obtain more detailed information on the adsorption and desorption profiles of individual enzyme components present in a whole cellulase mixture during hydrolysis.

The work reported here describes the adsorption and activity profiles of specific monocomponent enzymes present in a commercial cellulase mixture during hydrolysis of SO_2-catalyzed SPCS, a potentially important substrate for the production of lignocellulosic derived bioethanol. Commercially available cellulase cocktails were first screened for their activity and stability and the cellulase mixture with the highest activity was utilized for hydrolysis of SPCS. The various proteins were recovered from both the liquid and solid phases at different hydrolysis times. Using a combination of experimental techniques such as enzymatic activity assays, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), zymograms, and mass spectrometry, the adsorption and activity profiles of six specific enzymes during hydrolysis were followed. The activity and adsorption profiles of Cel7A, Cel7B, Cel5A, family 10 xylanase (Xyn10), family 11 xylanase (Xyn11), and β-glucosidase were compared and the factors influencing their distribution between the solid and liquid phases during cellulose hydrolysis as well as their effect on potential enzyme recycle strategies were then evaluated and discussed below.

3.1.2 Comparison of Activity and Stability of Commercial Cellulase Preparations

We first determined the thermal stability of the various commercial cellulase preparations as this was an important parameter in any enzyme recycling strategy. The three commercial cellulase preparations were incubated for 24 hours at different temperatures (40, 50, 60, 70, and 75^{o}C), after which the activities were measured using the standard substrates (PNPG for β-glucosidase activity, PNPC for Cel7A activity, and CMC for endoglucanase activities). All three enzyme preparations were able to retain most (more than 85%) of their activities on PNPG, PNPC, and CMC at incubation temperatures of up to 50^{o}C. At higher temperatures these activities decreased significantly and little or no activities were detected at temperatures of 70^{o}C and higher (data not shown).

Although the thermal activity profiles of all three preparations were similar at different temperatures, the loss of β-glucosidase activity in the Celluclast preparation was minimal, whereas a 40-50% reduction in β-glucosidase activity was observed with the Spezyme and Accellerase preparations (data not shown). When we compared the specific activities of the three cellulase preparations after 24-hour incubation at 50^{o}C, the Accellerase mixture showed the highest PNPCase and β-glucosidase activities, while the Spezyme preparation showed the highest CMCase activity (data not shown). Based on the relatively high specific activities of Accellerase, especially its high β-glucosidase activities, when compared to the other cellulase preparations, the Accellerase was chosen as the enzyme mixture used for further work.

3.1.3 Time Course of Enzymatic Hydrolysis

We next monitored the adsorption and activity profiles of various enzymes over time with the goal of achieving industrially-relevant cellulose hydrolysis yields. A cellulose hydrolysis yield of 70-80% was targeted as the goal for effective hydrolysis. Achieving this target conversion yield within a reasonable residence period was relevant, since techno-economic models of the overall biomass-to-

ethanol process had indicated that the generally long residence time needed to achieve complete cellulose hydrolysis adds significantly to operating costs (Aden and Foust, 2009; Yang et al., 2010). An enzyme loading of 20 FPU/g glucan, corresponding to 51 mg protein/g glucan, was found to be the minimum enzyme loading that resulted in 80% cellulose hydrolysis after 72 hours (data not shown). The time course of cellulose hydrolysis within the SPCS substrate appeared to be broken into 3 distinct phases where the fastest hydrolysis occurred in the first 3 hours of incubation in which ~35% of the initial cellulose was hydrolyzed. Phase two, which occurred from 3 to 24 hours, resulted in an increase in cellulose hydrolysis from 35% to around 75%. In the final phase, from 24 to 72 hours, very little further hydrolysis occurred (Figure 2), with most of the cellulose hydrolyzed (~75%) in the first 24 hours.

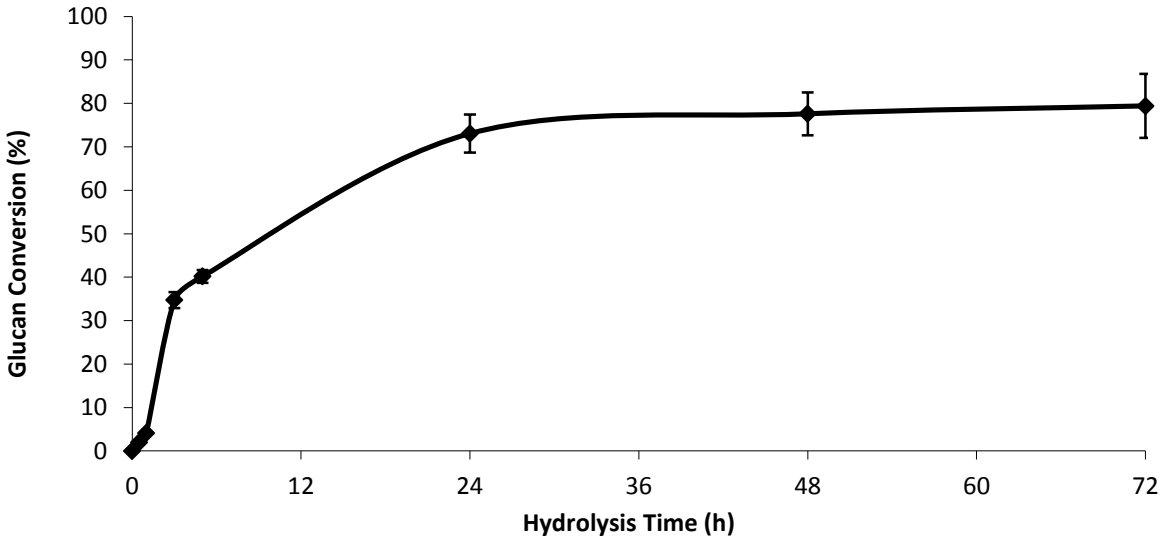

Figure 2. The time course of cellulose hydrolysis at 50^{o}C, 2% solid consistency (w/v), and an enzyme loading (Accellerase^{TM} 1000) of 51 mg/g glucan (20 FPU/g glucan)

3.1.4 Protein Distribution in the Liquid and Solid Phases

We then looked at the activity and distribution of the different enzymes present in the Accellerase mixture at various times during hydrolysis, in order to determine if this is linked to the

declining hydrolysis rate of a typical hydrolysis profile. The distribution of total proteins during the course of hydrolysis was determined by measuring the amount of proteins detected in either the liquid or solid phase (Figure 3). It was apparent that a significant amount of the added protein rapidly adsorbed to the SPCS substrate and that after thirty minutes, about 35% of the initial protein was adsorbed to the substrate (Figure 3). The amount of adsorbed protein remained relatively constant in the first two phases of hydrolysis (up to 24 hours; Figure 3 and Figure 4) when protein adsorption correlated with the corresponding hydrolysis rates. After 24 hours, when the hydrolysis was slowing down, some of the adsorbed proteins started to desorb back into the supernatant. At the end of the 72-hour hydrolysis, about a third of the initially adsorbed protein had been released back into the supernatant (Figure 3).

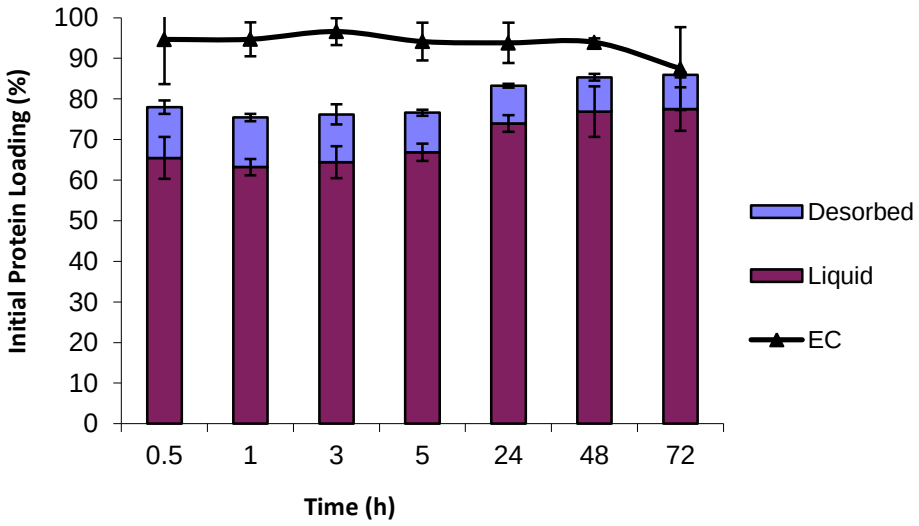

Figure 3. Time course of protein distribution in the liquid phase (Liquid) and desorbed from the solid substrate (Desorbed) during SPCS hydrolysis. EC: protein loading in the enzyme control samples**.**

Previous work, which looked at corn stover hydrolysis by Celluclast at 15 FPU/g cellulose and 10% substrate consistency (w/v), also found that 70% of the added proteins were present in the

supernatant after 24-hour hydrolysis (Yang et al., 2010). The low adsorptivity of cellulase enzymes onto the pretreated corn stover substrate might be due to the chemical composition and distribution of lignin on the substrates. Previous work has shown that cellulases had the lowest binding capacity to lignin isolated from corn stover, compared with lignin isolated from hardwood or softwood substrates (Nakagame, Chandra and Saddler, 2010), while related work showed that β-glucosidase was poorly adsorbed by lignin (Berlin et al., 2006). The relatively high β-glucosidase activity of the Accellerase preparation and low adsorption capacity of cellulases to corn stover lignin might partially explain the relatively high percentage of added protein that remained or desorbed back into the liquid phase during SPCS hydrolysis. About 86% of the initial protein added at the start of the reaction could be efficiently recovered in either the liquid or solid phases of SPCS hydrolysis. Thus, we next wanted to determine whether these proteins were active, as this would be important for any proposed enzyme recycling strategy.

3.1.5 Distribution and Activity Profiles of Specific Proteins in the Solid and Liquid Phases

To further investigate the adsorption profiles of specific enzyme components in the cellulase mixture, SDS-PAGE was carried out on proteins recovered from both the liquid and solid phases during hydrolysis of SPCS (Figure 4). At least eight protein bands could be identified within the control (enzyme alone) and possible protein identities were assigned to each band based on the MW of various known *H. jecorina* proteins listed in the CAZy database (Table 3). The polyacrylamide gels were analyzed using the densitometry function of the Alpha Imager™ 2200 (Alpha Innotech) and it was then possible to obtain qualitative information on the adsorption behavior of the protein(s) present in each band by plotting the density of bands 2, 3, 5, and 8 against the hydrolysis time (Figure 5). To confirm the identities of the proteins, each band was excised from the polyacrylamide gels and subsequently analyzed using mass spectrometry.

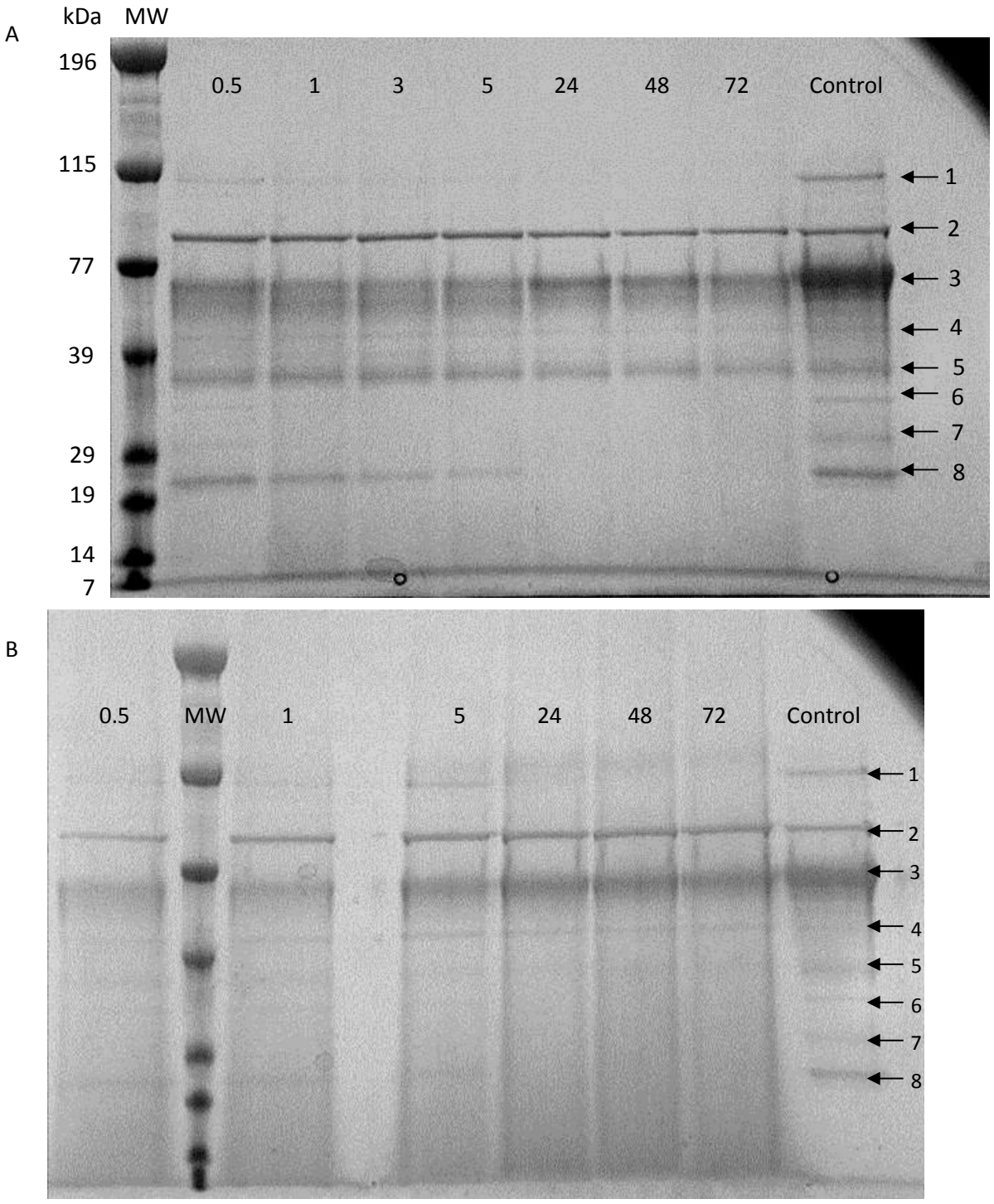

Figure 4. SDS-PAGE analysis of proteins during hydrolysis of SPCS. A: proteins in the liquid phase. B: proteins recovered from the solid phase. MW: molecular weight standards. The numbers above each lane indicate the time points during hydrolysis.

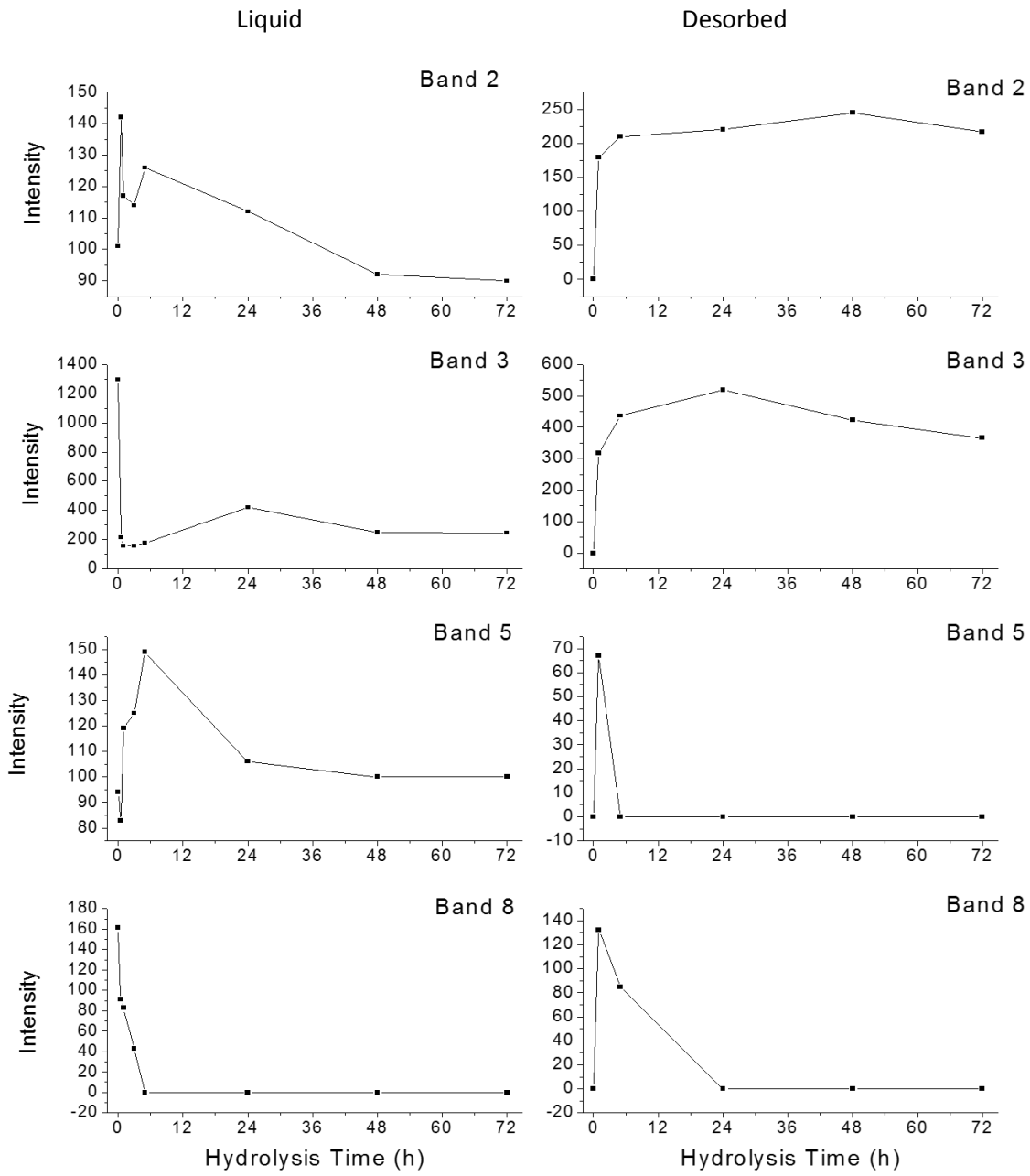

Figure 5. Densitometry analysis of bands 2, 3, 5, and 8 on SDS-PAGE gels of proteins in the liquid phase and desorbed from the solid substrate

Table 3. Summary of possible identities, mass spectrometry, and zymograms (CMC, MUC, Xylan)[a] results of the 8 bands identified on the SDS-PAGE gels

Band	MW (kDa)	Possible Identity based on MW [b]	Mass Spec	CMC		MUC		Xylan	
				L	S	L	S	L	S
1	114	Exo-β-D-glucosaminidase α-glucosidase β-galactosidase α-glucoronidase	Cel7A Cel6A Cel7B Cel5A α-glucoronidase	-	-	-	-	+	-
2	83	β-glucosidase β-xylosidase α-galactosidase II xyloglucanase	Cel7A Cel6A	-	-	-	-	-	-
3	56-62	Cel7A (CBH I) Cel6A (CBH II) Cel7B (EG I)	Cel7A Cel6A Cel7B	+++	++	+++	+++	+++	++
4	47	Cel5A (EG II) β-glucosidase 2 (Cel1A); Cel1B Man5A; Chitinase; α-galactosidase (Agl1) α-1,2-mannosidase (MDS1) α-L-arabinofuranosidase I (Abf1) 4-O-methylglucoronyl methylesterase	Cel7A Cel6A Cel7B Cel5A Cel61A	-	-	-	-	-	-
5	37	Cel61A (EG IV) Xylanase 10 (Xylanase III) α-L-arabinofuranosidase acetylesterase	Cel5A	++	+	-	-	+++	++
6	31	Endo-β-1,4-glucoronan lyase A; Axe 1; Axe 2	Cel7A						
7	23	Cel12A (EG III); Cel 45A (EG V) Xylanase 11 (Xylanase I);Cel 61B; DPM1	Cel7A Cel6A; Cel7B	++	+++	-	-	-	-
8	20	Xylanase 11 (Xylanase II)	Xyn 11	+	-	-	-	-	-

[a]Relative activities of enzymes recovered from the liquid phase (L) and the solid phase (S) are denoted as strong (+++), moderate (++), low (+), and none (-).

[b]Molecular weight of *H. jecorina* glycoside hydrolases were obtained from the CAZy website www.cazy.org.

3.1.6 Distribution and Activity Profiles of Cel7A (CBH I)

It was likely that Cel7A was present in band 3 (MW ~50-60 kDa) as it is known to have a MW of ~55 kDa (Figure 4). Mass spectrometry analysis of band 3 suggested that three major *H. jecorina* enzymes Cel7A, Cel6A, and Cel7B were present within this band (Table 3). This highlighted the challenge of following the adsorption profiles of individual enzymes in a complex enzyme mixture using SDS-PAGE. However, as the Cel7A normally accounts for up to 40-60% of the total protein produced by *H. jecorina* (Rosgaard et al., 2007) the densitometry analysis of this band should mostly follow the adsorption profile of Cel7A.

To further probe the adsorption profile of Cel7A and to correlate it with its corresponding activity, zymogram assays using 4-methylumbelliferyl-β-D-cellobioside (MUC), a substrate commonly used to detect cellobiohydrolase activity, were carried out. At least 3 bands were observed on the MUC zymogram gel with the brightest band located at MW ~55 kDa (Figure 6). The activity detected at this MW confirmed the presence of both Cel7A and Cel7B, but not Cel6A, as previous work has shown that Cel6A binds to but does not cleave MUC (Vantilbeurgh et al., 1985). In order to distinguish between the Cel7A and Cel7B activities, another zymogram assay was carried out using 0.1 mM MUC and 1% cellobiose (w/v) to specifically inhibit Cel7A activity (Du et al., 2010). Using the densitometry function of the Alpha Imager^{TM} 2200 to measure the resulting band densities, the addition of 1% cellobiose resulted in a ~70% decrease in activity of the enzymes recovered from both the liquid and the solid phase (data not shown). In both phases, the largest decrease in activity was observed for enzymes recovered after 72 hours, corresponding to about a 74% decrease for those enzymes recovered from the liquid phase and about an 85% decrease for the enzymes recovered from the solid phase (data not shown). These results indicated that most of the activity on MUC, represented by the band intensity at this MW, was due to Cel7A.

The slight changes in the activity profile observed using the MUC zymogram assay indicated that the adsorption of Cel7A to SPCS was reversible, with partial desorption occurring after 24 hours. This activity profile was in agreement with the adsorption profile observed on the SDS-PAGE gel. The reversibility of Cel7A adsorption is in agreement with previous work that suggested that both the binding of Cel7A and its cellulose binding module (CBM) to cellulose was reversible (Palonen, Tenkanen and Linder, 1999; Linder and Teeri, 1996).

The partial desorption of Cel7A after 24 hours observed on both SDS-PAGE and MUC zymogram gels occurred when the hydrolysis rate was starting to level off (Figure 5 and Figure 6C). Although the partial desorption of enzymes seems to occur whenever the hydrolysis rate starts to level off, this phenomenon has not been fully reported or explained (Sipos et al., 2010; Varnai et al., 2011). In related work, the partial desorption of proteins when the hydrolysis rate of steam pretreated spruce started to level off only occurred when polyethylene glycol (PEG) was added to the mixture (Sipos et al., 2010). Other workers found that the partial desorption of Cel7A only occurred during hydrolysis of catalytically delignified spruce substrates (Varnai et al., 2011). It is likely that lignin plays at least a partial role in this phenomenon, as the partial desorption of Cel7A observed in the work reported here suggested their low binding capacity to corn stover lignin.

As endo- and exo-glucanases, as well as β-glucosidase, are known to hydrolyze PNPC (Deshpande, Eriksson and Pettersson, 1984), changes in PNPC-ase activity were also measured for the proteins recovered from both phases throughout the course of hydrolysis. We added D-glucono-1,5-δ-lactone, a specific inhibitor for β-glucosidase (Deshpande, Eriksson and Pettersson, 1984), to inhibit the activity of this enzyme on PNPC. As previous work had shown that the endo-glucanases and Cel6A activity on PNPC was considerably lower than that of Cel7A (Deshpande, Eriksson and Pettersson, 1984), the PNPC-ase activity detected was likely due to the Cel7A component. The PNPC activity adsorption

profiles of proteins recovered from both the liquid and solid phases followed the same trend as was observed with the SDS-PAGE and MUC zymogram (Figure 7).

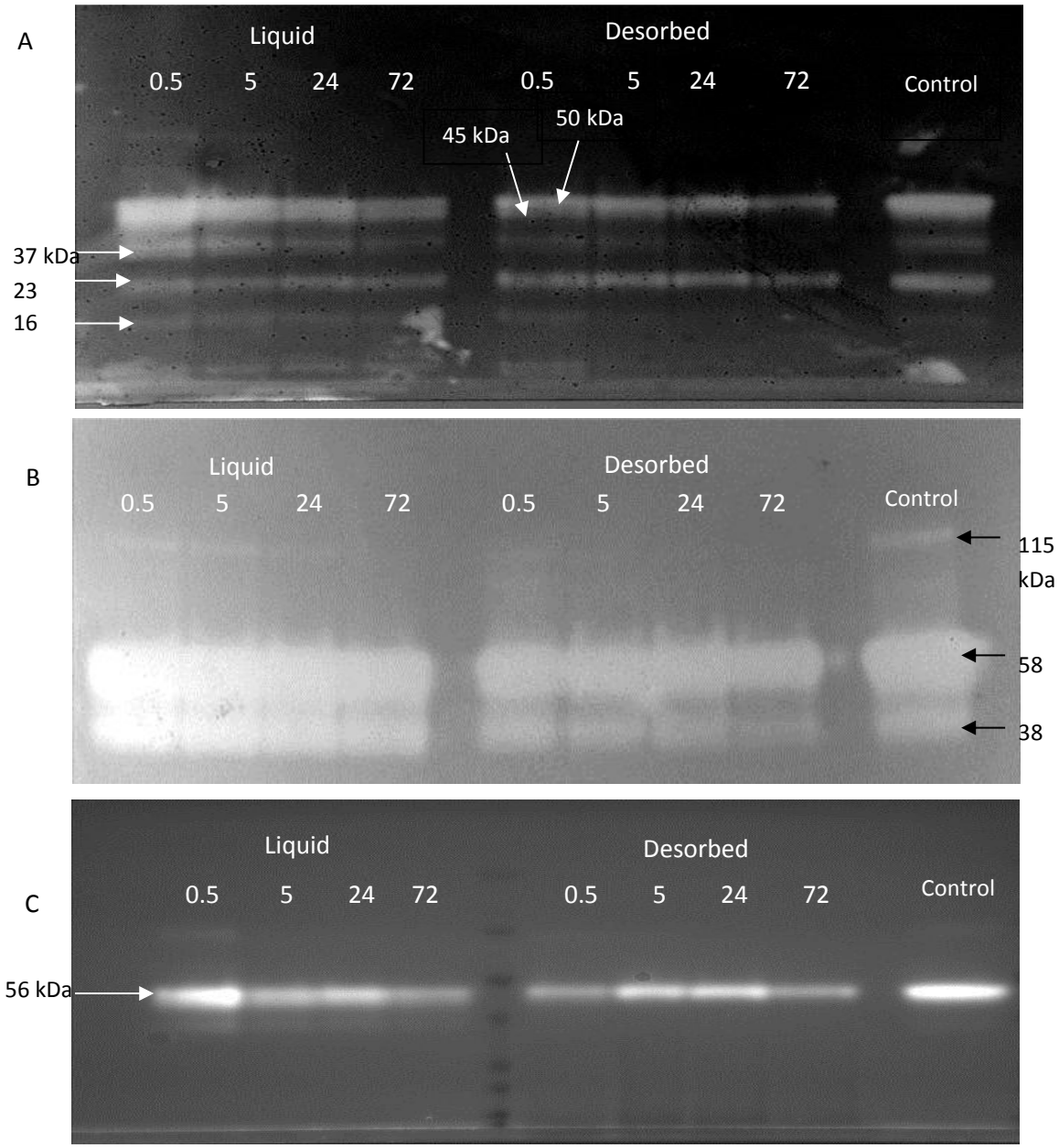

Figure 6. Activity and adsorption profiles using zymograms with 0.1% CMC (A), 0.1% birchwood xylan (B), and MUC (C) as substrates. Numbers above each lane indicate the different time points (hours) during hydrolysis**.**

3.1.7 Distribution and Activity Profiles of Cel7B (EG I)

When CMC was used as the substrate for the zymogram assay, a large zone of clearance (halo zone) was observed at a MW of 45-50 kDa (Figure 6A), corresponding to band 3 on the SDS-PAGE (Figure 4). As endoglucanases (EGs) are the major class of enzymes that are able to significantly hydrolyse CMC, as detected by Congo red staining (Gilkes et al., 1984), the halo zone detected at MW ~45-50 kDa was likely due to the action Cel7B. This assumption was further substantiated by the detection of a halo zone with a MW corresponding to band 3 on the xylanase zymogram gel (Figure 4). Of the three enzymes possibly present in band 3, only Cel7B would be expected to show activity on xylan (Lawoko et al., 2000). Both the CMC and xylan zymograms indicated that Cel7B was distributed in both the liquid and solid phases and that its activity decreased gradually in both phases during the course of hydrolysis (Figure 6A and B), presumably due to thermal deactivation.

3.1.8 Distribution and Activity Profiles of Cel5A (EG II), Xylanase III (Xyn 10) and Endo-xylanase II (Xyn 11)

The Cel5A was shown, by mass spectrometry (within 95% confidence limits), to be present in band 5 at a MW ~37 kDa (Figure 4), which was slightly smaller than the MW reported in the literature and on the CAZy website (~44 kDa). The halo zone at MW ~34 kDa on the CMC zymogram gel (Figure 6A) also corresponded to band 5 (MW ~37 kDa) (Figure 4), and hence was also indicative of Cel5A activity. Densitometry analysis of Cel5A on the SDS-PAGE gels indicated that the enzyme adsorbed to the substrate right after an initial mixing and then rapidly desorbed back to the liquid phase (Figure 5).

Xylanase activity was observed at 38 kDa (Figure 6B), which again corresponded to the MW of band 5 in figure 3. However, this xylanase activity was not likely to be caused by Cel5A (the only enzyme identified in band 5 using mass spectrometry), because Cel5A has been shown to have no xylanase activity (Lawoko et al., 2000). As *H .jecorina* Xyn 10 has a MW of 38 kDa, the observed xylanase activity

was presumably due to the activity of Xyn 10. Mass spectrometry did not identify Xyn 10 from the band, probably due to the presence of other proteins in the band that masked the presence of this enzyme. During the course of hydrolysis, after an initial spike in adsorption to the substrate, the xylanase remained primarily in the liquid phase and enzyme activity was lost over 72 hours, presumably due to thermal deactivation.

Mass spectrometry indicated that endo-1,4-β-xylanase II from GH family 11 (Xyn 11) was present in band 8 (MW ~20 kDa). On the SDS-PAGE gel, band 8 gradually disappeared from the liquid fraction within the first 5 hours of hydrolysis (Figure 4 and Figure 5). Similarly, the band intensity on the CMC zymogram gel decreased over time for enzyme activity detected in the liquid fraction (Figure 6A). Enhanced desorption using Tween 80 could only recover this protein from the solid phase in the early stages of hydrolysis (Figure 5), while no activity could be detected in the solid fraction, except at initial stages of hydrolysis (Figure 6A). After 24 hours, the enzyme was not detected in either the liquid or the solid phases (Figure 5). The rapid disappearance of Xyn 11 from the hydrolysate has been observed in previous work where the Xyn 11 could no longer be detected in the liquid phase after 6 hours of hydrolysis of steam pretreated spruce and Avicel (Varnai et al., 2011). As this enzyme is known to be thermally unstable and to form protein aggregates as it thermally denatures (Janis et al., 2008), the disappearance of band 8 from the liquid phase (Figure 4) after 24 hours was likely due to this protein aggregation, which may lead to protein precipitating from the solution.

3.1.9 Distribution and Activity Profiles of β-glucosidase

Based on its MW, band 2 (83 kDa) on the SDS-PAGE gels likely contained β-glucosidase (Figure 4). When the adsorption and activity profiles of this putative β-glucosidase were compared, the changes in PNPG activity in both the liquid and solid phases during the course of hydrolysis (Figure 7) indicated that this protein was distributed in both phases throughout hydrolysis. Approximately 60% of the initial

activity on PNPG was recovered from the liquid fraction, while the rest of the activity was recovered from the solid phase. Both the SDS-PAGE and PNPC data indicated a rapid initial enzyme adsorption to the substrate, which was immediately followed by enzyme desorption as the adsorption reached equilibrium (Figure 5 and Figure 7). The activity was also stable in the enzyme control samples, suggesting that the enzyme was stable both in the presence and absence of substrate (Figure 6).

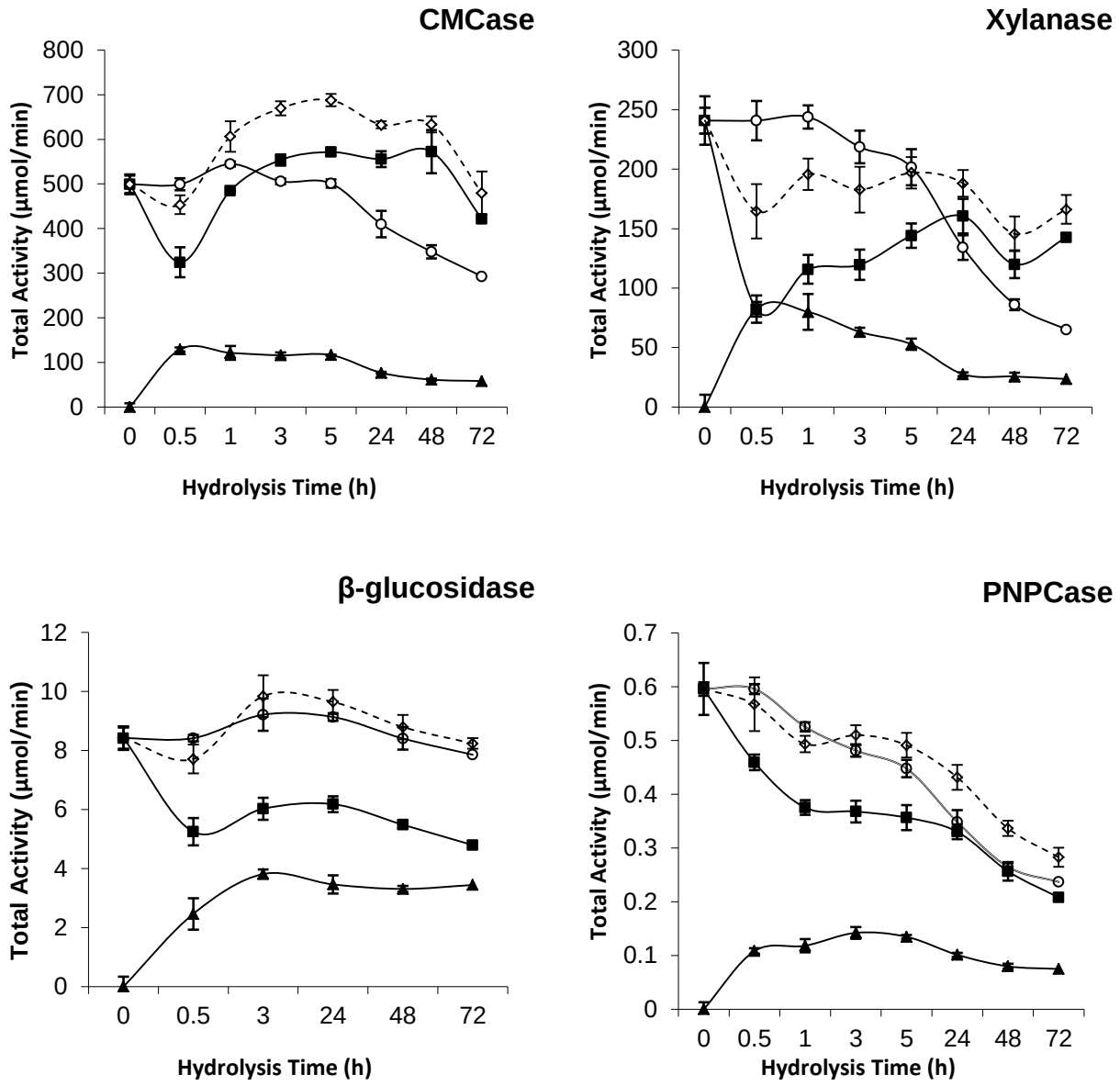

Figure 7. Activity data of liquid-phase enzymes (■) and solid-phase enzymes (▲) from SPCS hydrolysis and enzyme control samples (○). Dashed lines (◊) indicate the sum of activity from enzymes recovered from both phases.

3.1.10 Additional Observations

The enzymes that act on the CMC and xylan substrates seemed to be thermally deactivated over time, as was observed with the control samples. However, in the presence of substrate, this loss of activity was not as pronounced (Figure 7). The presence of the substrate was able to stabilize both the CMCase and xylanase activities over an extended period of hydrolysis. This was in agreement with earlier observations that the adsorption of cellulase enzymes to substrates was found to improve the stability of enzymes against repeated freeze-thaw and wet-dry events compared to non-adsorbed enzymes (Sinsabaugh and Linkins, 1989).

In the work reported here, Cel7A, Cel6A, Cel7B, and Cel5A were identified by mass spectrometry to be present in multiple bands (Table 3). The detection of enzymes in multiple bands has also been observed previously, even when the enzymes were fractionated using a 2-dimensional gel (Vinzant et al., 2001). The presence of multiple isozymes of a particular enzyme and the inability of the assay to fully resolve the protein mixture were proposed as an explanation for this observation (Vinzant et al., 2001). This highlights the need for better assays in order to resolve the different enzyme components present in a typical cellulase mixture.

3.1.11 Conclusions

Effective enzyme recycling of a cellulase mixture requires knowledge of individual enzyme-substrate interactions during the course of hydrolysis. Based on the data derived from the various techniques, it was apparent that the different constituents of the Accellerase cellulase enzyme mixture had different adsorption profiles on the SPCS substrate. All of the proteins appeared to be adsorbed, to some extent, within the first 30 minutes after the initial addition of the enzyme mixture to the substrate. This was evident in the lower activity detected for all classes of enzymes (β-glucosidase, CMCase, xylanase, and PNPCase) as compared to the enzyme control sample. After initial adsorption,

these enzymes either remained bound to the solid or desorbed back to the liquid phase to reach equilibrium. In general, Cel7A, Cel7B, Xyn 10, and β-glucosidase were all distributed in both the liquid and solid phases during the course of hydrolysis. Cel7A appeared to bind reversibly to the substrate with partial desorption occurring when the hydrolysis rate started to level off. However, Cel5A and Xyn 11 remained primarily in the liquid phase throughout the course of hydrolysis. In general, those enzymes that remained adsorbed to the solid retained their activity longer than those that remained mostly in the liquid phase.

With regards to enzyme recycling, these results indicate that maximum enzyme activity recovery could be obtained when enzymes were recovered from both the liquid and the solid phases after short-term rounds of hydrolysis (for example after 24 hours) to minimize the loss of enzyme activities. Although it was possible to determine the adsorption profiles of some individual enzymes using data derived using a combination of several techniques, the results were at best semi-quantitative, and the ability to determine enzyme identities were limited by the lack of specificity of the techniques. Therefore, a simple and quantitative technique with improved specificity would help to more accurately determine the distribution profiles of specific enzymes.

3.2 The Development and Use of an ELISA-based Method to Follow the Distribution of Cellulase Monocomponents during the Hydrolysis of Pretreated Corn Stover

3.2.1 Background

In section 3.1, the distribution of individual enzymes present in a commercial cellulase mixture (Accellerase 1000) was assessed during the hydrolysis of SPCS. A combination of methods, such as gel electrophoresis, zymograms, activity assays using chromophoric substrates, and mass spectrometry were used to determine the general distribution patterns of the major enzymes during SPCS hydrolysis. However, although enzyme distributions could be semi-quantitatively assessed by combining data derived from these techniques, the quantitative determination of the adsorption profiles of specific enzymes was not possible. A better understanding of the adsorption profiles of individual enzymes would be useful in aiding our efforts to improve enzyme recycling performance. For example by identifying and quantifying the types of enzymes that are lost during hydrolysis or enzyme recycling, an optimum mix of fresh enzymes could be created to replace these specific enzyme activities at a minimum loading. Therefore, a better technique that can specifically and quantitatively monitor enzyme adsorption profiles during hydrolysis would be invaluable.

It is well known that antibodies can bind to specific antigens and this ability has been used as the basis for many assays (Kolbe and Kubicek, 1990; Conde et al., 2008; Drow and Manning, 1980). This specific recognition and binding between antibodies and their antigens have been utilized in various techniques, including the enzyme-linked immunosorbent assay (ELISA). In cellulase enzyme research, the ELISA method, using monoclonal and/or polyclonal antibodies (MAbs and PAbs, respectively) raised against various cellulase enzymes, has been successfully used to quantify target enzymes both in culture filtrates and commercial enzyme preparations (Kolbe and Kubicek, 1990).

A double-antibody sandwich ELISA, which is an ELISA-based technique using a pair of antibodies to sandwich the target compound and specifically quantify it among other compounds in the mixture, has been successfully used to quantify the amount of Cel7A in a crude culture broth with minimal interference from other enzymes or other materials present in the broth (Riske, Eveleigh and Macmillan, 1990). Improved specificity of the assay was achieved when MAb was used as the coating antibody and PAb as the second, detecting antibody (Riske, Eveleigh and Macmillan, 1990). In related work, Buhler *et al.* (1991) optimized a double-antibody sandwich ELISA for Cel7B in a culture broth by also using MAb as the coating antibody and PAb as the detecting antibody and they were able to show that the assay was both sensitive and specific for Cel7B (Buhler, 1991). Although the specificity and quantitative nature of ELISA may allow for a specific determination of individual enzyme adsorption profiles during hydrolysis the feasibility of using ELISA to quantify specific proteins present in the supernatant after hydrolysis of a lignocellulosic substrate using a complete enzyme mixture has not yet been demonstrated.

In the work described here, a double-antibody sandwich ELISA was developed and used to specifically quantify the major cellulase enzymes Cel7A, Cel6A, and Cel7B present in the supernatant during hydrolysis of SPCS using a commercial enzyme mixture. The sensitivity of the assay was improved by subjecting the enzyme samples to a pH adjustment treatment and/or a heat treatment. While lignocellulosic substrate derived materials did interfere with the assay, this interference could be minimized by simple dilution.

3.2.2 Determination of the Specificity of the Different Monoclonal Antibodies (MAbs) and Polyclonal Antibodies (PAbs)

Our initial work investigated the specificity of the MAb and PAb for their target cellulase monocomponents. The specificity of Cel7A, Cel6A, and Cel7B MAbs were assessed using Western Blots against Cel7A, purified from a commercial Celluclast mixture, as well as against three commercial

enzyme mixtures (Celluclast, Accellerase, and CTec 2). The Cel7A MAb Western Blot showed a single band corresponding to the purified Cel7A and a major band with the three commercial enzyme mixtures at MW ~ 70 kDa, which indicated the binding of Cel7A MAb with the Cel7A monocomponent in the three commercial enzyme mixtures (Figure 8A). Although the Cel6A MAb also showed a band of protein at MW ~70 kDa with the three commercial enzyme mixtures (Figure 8B), this MAb did not react with the purified Cel7A. Similarly, the Western Blot that used the Cel7B MAb did not recognize the purified Cel7A, but recognized a protein band at MW ~60 kDa in all of the 3 commercial enzyme mixtures (Figure 8C). In the previous section, was shown that Cel7A, Cel6A, and Cel7B were all present within the same band on a SDS-PAGE gel (Figure 4 and Table 3) due to their similar MWs. Therefore, it appeared that all three of the MAbs were reactive and specific for their target enzymes.

The specificity and reactivity of Cel7A and Cel6A PAbs were also determined by Western Blots by using purified Cel7A and Cel6A from Celluclast as well as two commercial enzyme mixtures. The PAb against Cel7A was specific for its target enzyme as it only reacted with purified Cel7A and not with the purified Cel6A (Figure 9A). However, the PAb against Cel6A recognized both the purified Cel7A and Cel6A (Figure 9B). Possible contamination by Cel6A in the purified Cel7A fraction did not appear to be an issue as the Cel6A MAb did not react with the purified Cel7A preparation (Figure 8B). The reactivity and specificity of the Cel7B PAb was next determined using Western Blots against purified Cel7A, Cel6A, and Cel7B, as well as against three commercial cellulase mixtures. It was apparent that the Cel7B PAb recognized the purified Cel7B, but also cross-reacted with the purified Cel7A and Cel6A (Figure 9C). However, this cross-reactivity of the Cel6A and Cel7B PAbs was not expected to influence the specificity of the double-antibody sandwich ELISA, since both the Cel6A and Cel7B MAbs were shown to be specific to their respective target enzymes (Figure 8B and C).

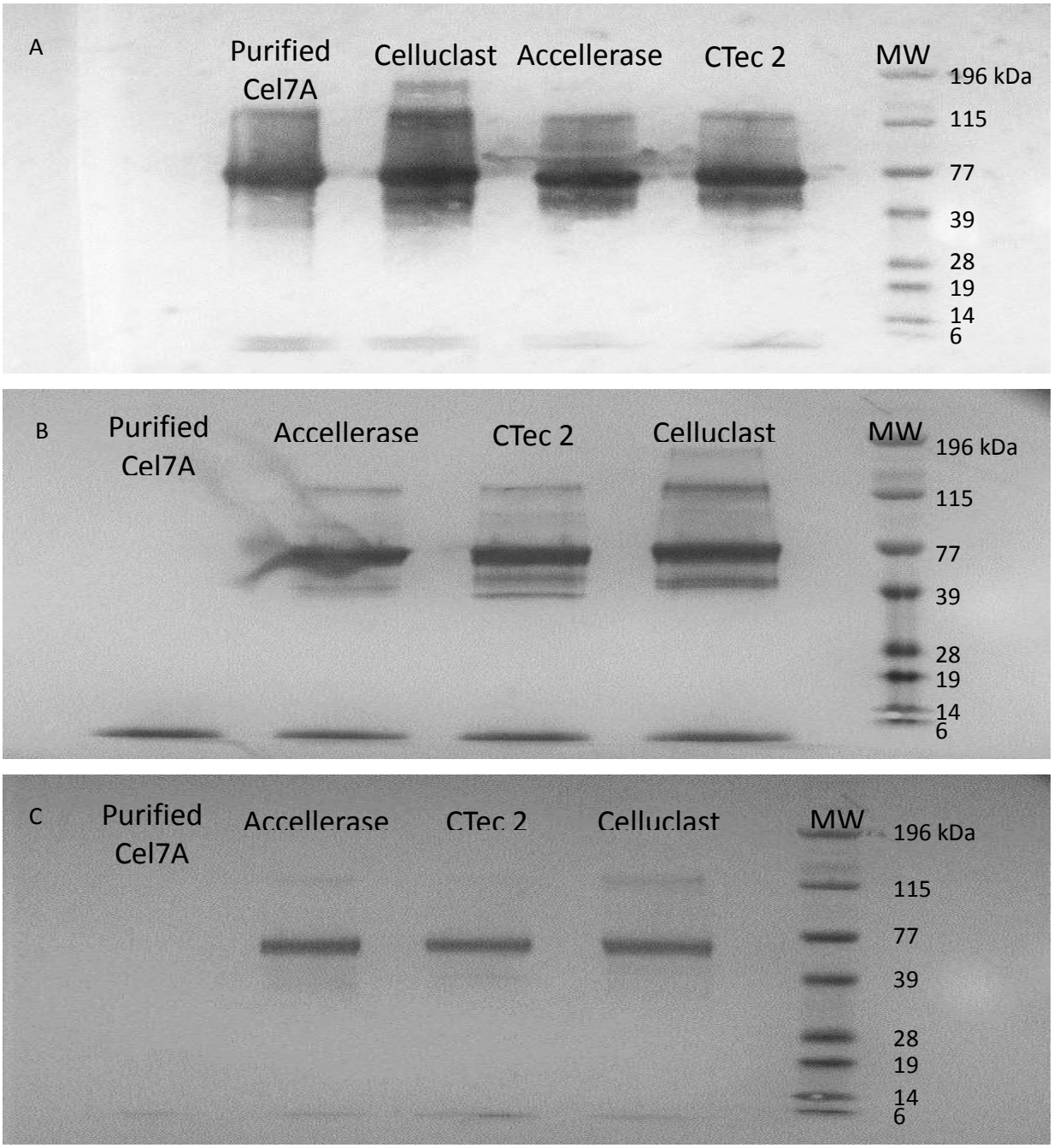

Figure 8. Reactivity and specificity of MAbs against Cel7A (A), Cel6A (B), and Cel7B (C) as determined using Western Blots.

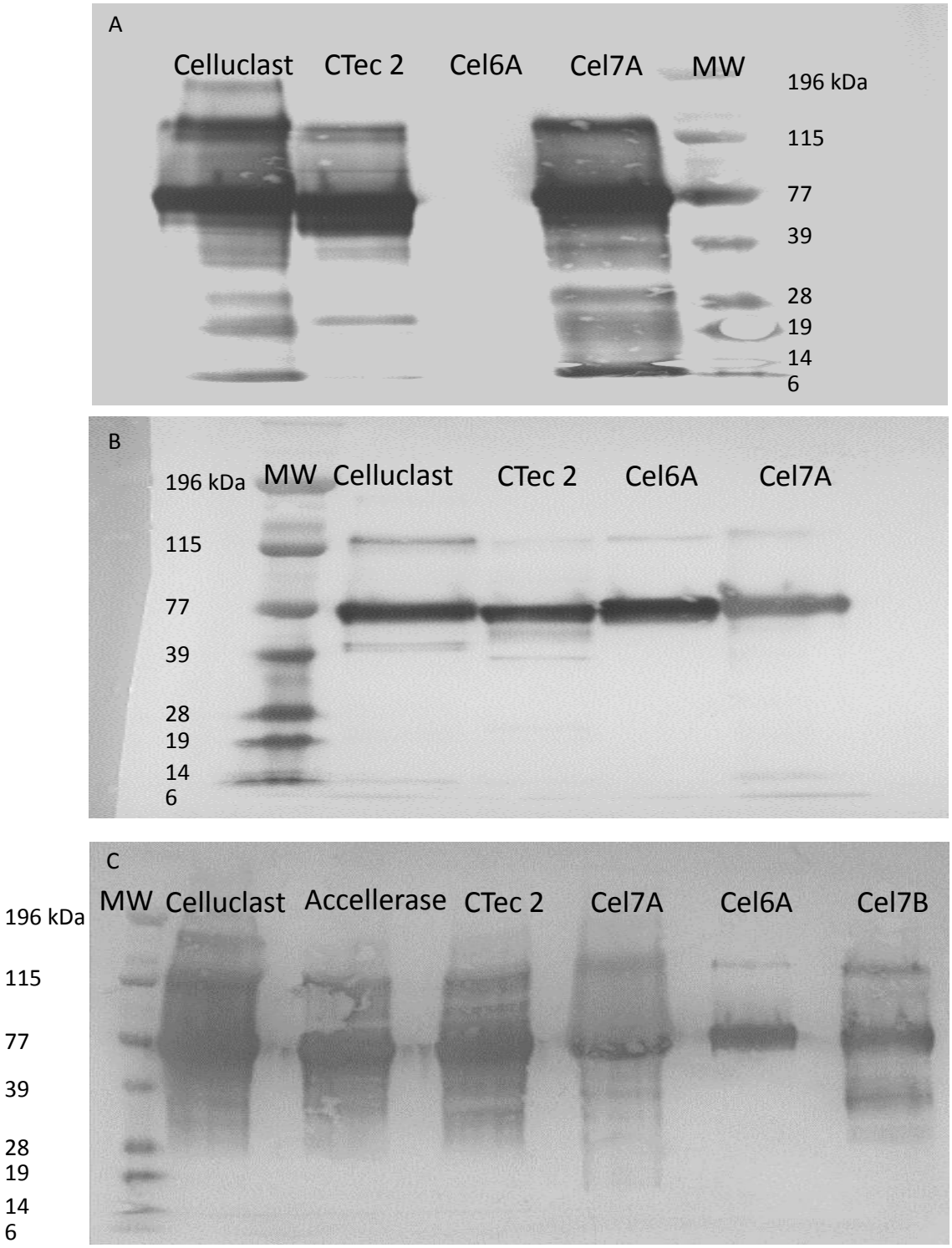

Figure 9. Reactivity and specificity of PAbs against Cel7A (A), Cel6A (B), and Cel7B (C) as determined using Western Blots.

3.2.3 Optimization of the Assay Protocols to Improve the Sensitivity of the Double-antibody Sandwich ELISA

Previous work had shown that a double-antibody sandwich ELISA, using a combination of a MAb and a PAb as the capture and detecting antibodies respectively, resulted in improved specificity compared to the normal ELISA or to a sandwich ELISA using PAb as the capture and MAb as the detecting antibody (Kolbe and Kubicek, 1990; Riske, Eveleigh and Macmillan, 1990). Thus, we next used a MAb as the capture antibody and a PAb as the detecting antibody to assay different concentrations of each of the three antibodies (MAb, PAb, and GAR-AP). In this way, we hoped to assess the sensitivity of the assay in detecting purified Cel7A at concentrations ranging from 0 - 2.5 µg/ml.

Two concentrations of Cel7A MAb (10 and 50 µg/ml diluted in 1x PBS) were initially assessed. However, as both concentrations gave similar absorbance values (Figure 10A), a MAb concentration of 10 µg/ml was used in subsequent work. Previous work had also determined that a concentration of 10 µg/ml was sufficient to coat the bottom surface of a well in a typical 96-well ELISA plate (Crowther, 2000). The concentrations of the PAb (detecting antibody) and GAR-AP, the tertiary antibody, were similarly optimized over the same range of Cel7A concentrations. A concentration of 0.14 µg/ml of PAb Cel7A and 1/500 dilution of GAR-AP were found to improve the sensitivity of the assay for all three enzymes (Figure 10B and C). These concentrations of antibodies were also used for the Cel6A and Cel7B based ELISA's.

Despite the increased sensitivity gained by optimizing the concentrations of all three antibodies, the improved signal was still quite low when compared to previously reported values (Riske, Eveleigh and Macmillan, 1990). Therefore, to further increase the sensitivity of the assay, the enzyme samples were subjected to pH adjustment and heat treatments prior to addition to the well. Although previous work had shown that the antigen-antibody interactions are typically optimum at pH > 7 (Buhler, 1991), fungal-derived enzymes are typically buffered and used at around pH < 5. We therefore brought the

enzyme samples up to pH 7.5, using PBS buffer, prior to their addition to the wells. Previously, Riske *et al.* (1990) had reported that a heat-sensitive fungal product caused a signal reduction with Cel7A ELISA and that this interference disappeared after the cellulase preparation was boiled, resulting in increased ELISA sensitivity (Riske, Eveleigh and Macmillan, 1990). Therefore, the enzyme monocomponents were also heated at 100°C for 10 minutes to determine if heat treatment could improve sensitivity.

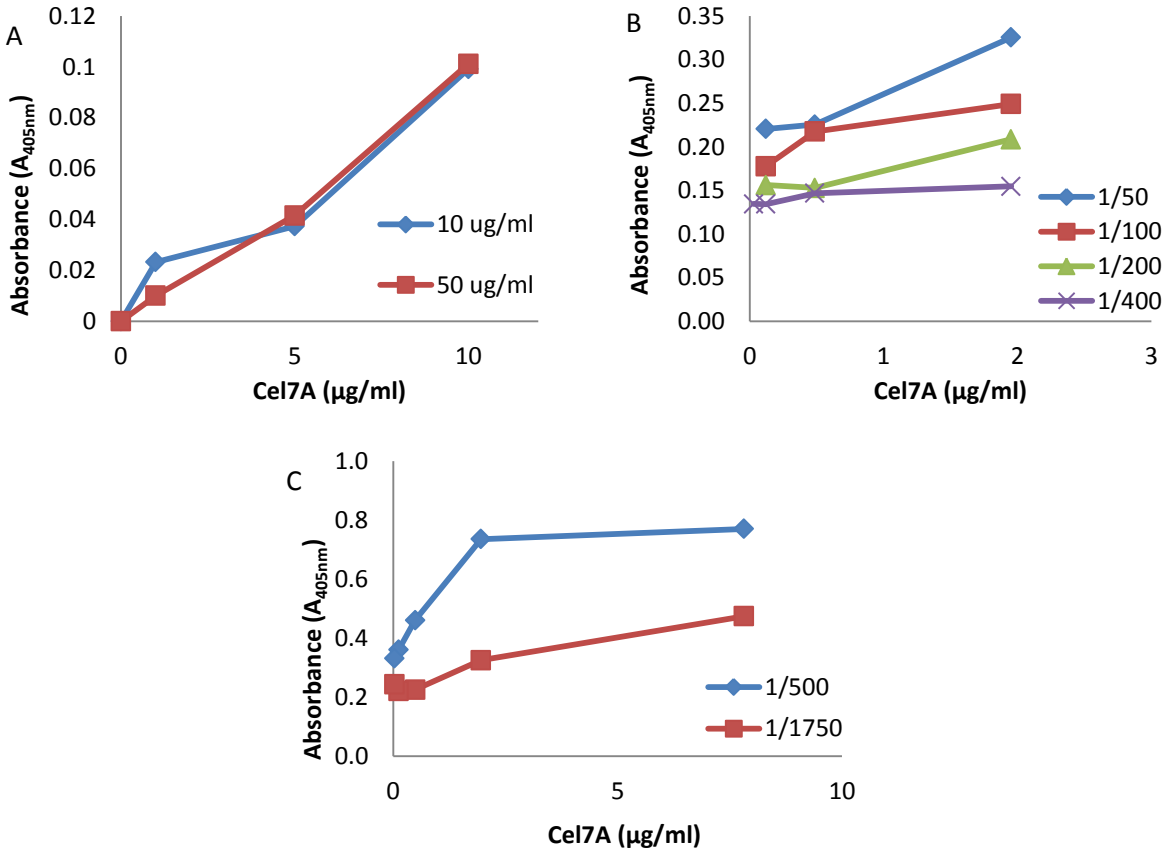

Figure 10. Optimization of the concentrations of MAb (A), PAb (B), and the third antibody, GAR-AP (C) over a range of concentration of purified Cel7A. (A). Two different concentrations of Cel7A MAb 10 µg/ml (◊) and 50 µg/ml (□) were added to the well. PAb and GAR-AP concentrations were kept constant at 1/400 and 1/1750 dilutions, respectively (B). Using 10 µg/ml Cel7A MAb, the Cel7A PAb was diluted to different degrees: 50x (◊), 100x (□), 200x (Δ), 400x (X). The GAR-AP was diluted 1750x. (C). The concentration of GAR-AP was varied by diluting it 500x (◊) or 1750x (□) in PBS. Cel7A MAb concentration was kept at 10 µg/ml, and Cel7A PAb was diluted 50x in PBS.

When the Cel7A and Cel6A were subjected to a heat treatment at 100°C for 10 minutes in a pH 5.0 buffer, followed by dilution in PBS buffer at pH 7.5, the sensitivity of ELISA increased by about 6x and 10x for Cel7A and Cel6A, respectively, when compared to the untreated samples at an enzyme concentration of 2.5 µg/ml (Figure 11A and B). However, heat treatment decreased the sensitivity of the Cel7B based ELISA (Figure 11C). Therefore, the enzyme samples for the Cel7B ELISA were not heated, but directly diluted in PBS buffer and then added to the wells.

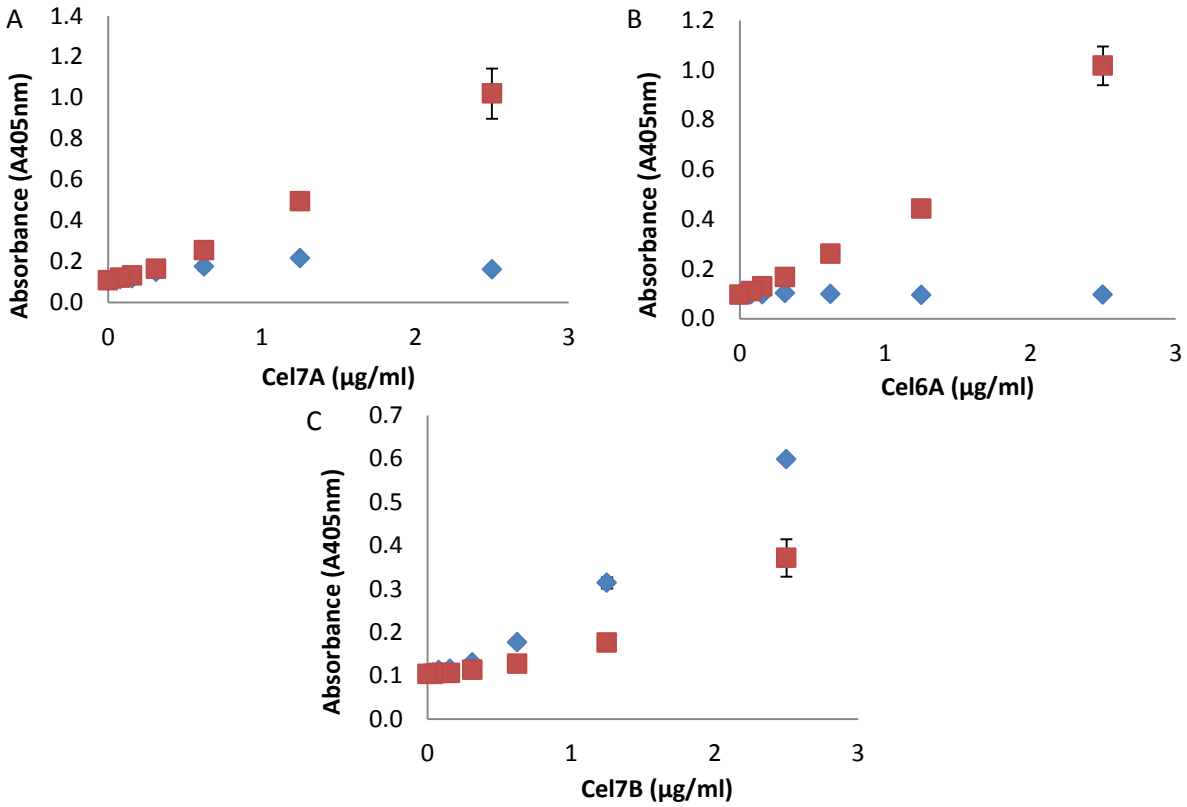

Figure 11. The effect of heat treatment on the sensitivity of ELISA for pure Cel7A (A) and Cel6A (B) and Cel7B (C). Heated enzyme samples were boiled in Na-acetate buffer pH 5.0 at 100°C for 10 minutes and then serially diluted in PBS (□). Non-heated samples were directly diluted in PBS (◊).

As mentioned earlier, the improved signal achieved by heating the enzymes used for the Cel7A and Cel6A based ELISA's could be caused by the removal of interfering heat-sensitive materials present in the samples (Riske, Eveleigh and Macmillan, 1990). The ineffectiveness of heating the Cel7B may

indicate that the interfering materials did not affect the Cel7B-based ELISA system. Enzyme denaturation after heating in Na-acetate buffer pH 5.0 did not seem to affect the ability of the antibody to bind to the antigen with the exception of Cel7B. This different response to the heat treatment highlighted the need to optimize the double-antibody sandwich ELISA for each specific enzyme-antibody system.

3.2.4 How Specific is the ELISA to the Enzyme of Interest?

The specificity of each ELISA was next determined by comparing the absorbance values of each enzyme when it was added as a single component and when it was added as a mixture of four purified enzymes (Cel7A, Cel6A, Cel7B, and Cel5A). For the entire enzyme based ELISAs (Cel7A, Cel6A, and Cel7B ELISA), the standard curves obtained with the purified enzymes were similar to those obtained with the reconstituted mixtures, especially when the target enzyme concentration was less than 1 µg/ml (Figure 12A, B, and C). It was apparent that the double-antibody sandwich ELISA assay was able to specifically quantify a target enzyme when it was present in a mixture with three other cellulase monocomponents.

We then investigated whether a whole commercial enzyme mixture could be used to make a standard curve, thus obviating the need for purified enzymes. A commercial enzyme mixture was diluted to 200 µg protein/ml in Na-acetate buffer (0.05 M, pH 5.0). When using the Cel7A and Cel6A based ELISAs, the commercial enzyme mixtures were heated, serially diluted 2-fold in PBS and then added to the wells. By sufficiently diluting the enzyme mixtures, a relatively linear standard curve could be obtained with whole enzyme mixtures when using the Cel7A and Cel7B based ELISAs (Figure 13A and C). A linear standard curve was also obtained with the Cel6A ELISA, although was only obtained with Celluclast and not with Accellerase or CTec 2 (Figure 13B).

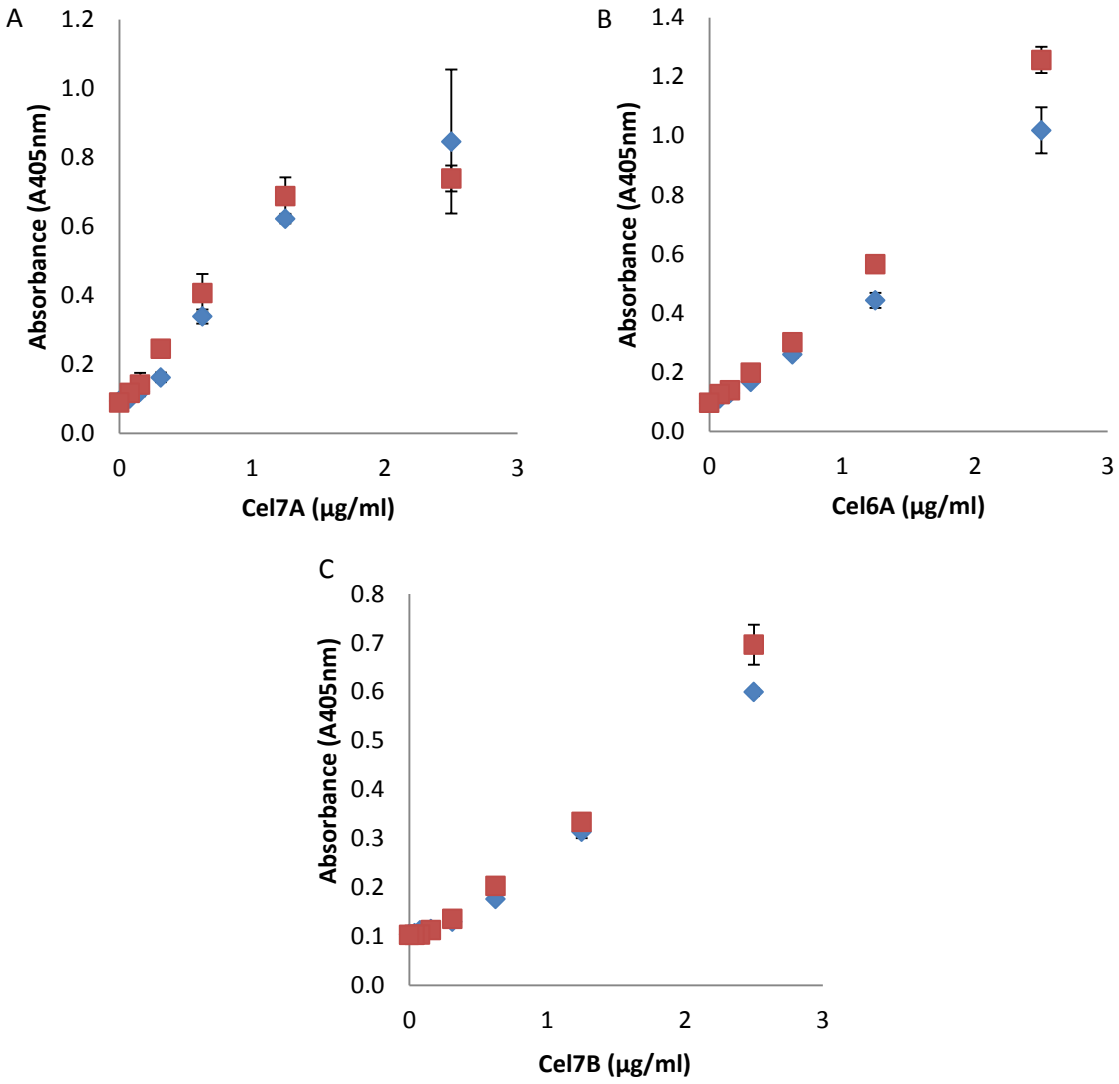

Figure 12. The specificity of ELISA for Cel7A (A), Cel6A (B), and Cel6A (C) as measured using pure enzymes (◊) and reconstituted mixtures of the 4 purified enzymes Cel7A, Cel6A, Cel7B, and Cel5A (□).

The linear standard curve obtained for all of the target enzymes highlighted the ability of the double-antibody sandwich ELISA to detect the target enzyme, even when present in complex enzyme mixtures. The high specificity of the MAbs could explain why Cel6A ELISA only worked with Celluclast and not with other commercial enzyme mixtures, as the Cel6A MAb was developed by colleagues at NREL to detect Cel6A in Celluclast. Although both the MAb and PAbs against Cel6A recognized the Cel6A present in Celluclast, Accellerase and CTec 2 (Figure 8B and Figure 9B), the lower ELISA signal observed

in the latter two commercial enzyme mixtures might be a result of a slight change in antigen recognition by the MAb. This may be caused by the inclusion of a different Cel6A in Accellerase and CTec 2 compared to Celluclast. Another possible reason was the presence of interfering compounds in Accellerase and CTec 2. When the concentration of the enzymes and antibodies were high, as in the case of the Western Blot studies (30 μg of enzyme samples and 250 μg MAb or PAb), there was likely enough interaction between the enzymes and antibodies, resulting in a significant band on the membrane. However, when the enzyme concentration was low (< 0.1 μg), as in the case with the ELISA, the lower binding affinity between the antibodies and Cel6A in Accellerase and CTec 2 resulted in a lower ELISA signal.

Therefore, it was apparent that a double-antibody sandwich ELISA was specific for target enzymes provided that appropriate MAbs and PAbs were available. Given the recent rapid development of enzyme cocktails to which new-and-improved enzymes have been introduced, (i.e. CTec 3), the highly specific nature of the antibody-antigen interaction shown in this assay will likely require the development of specific MAbs and PAbs that will recognize individual enzymes present in these new and improved enzyme mixtures.

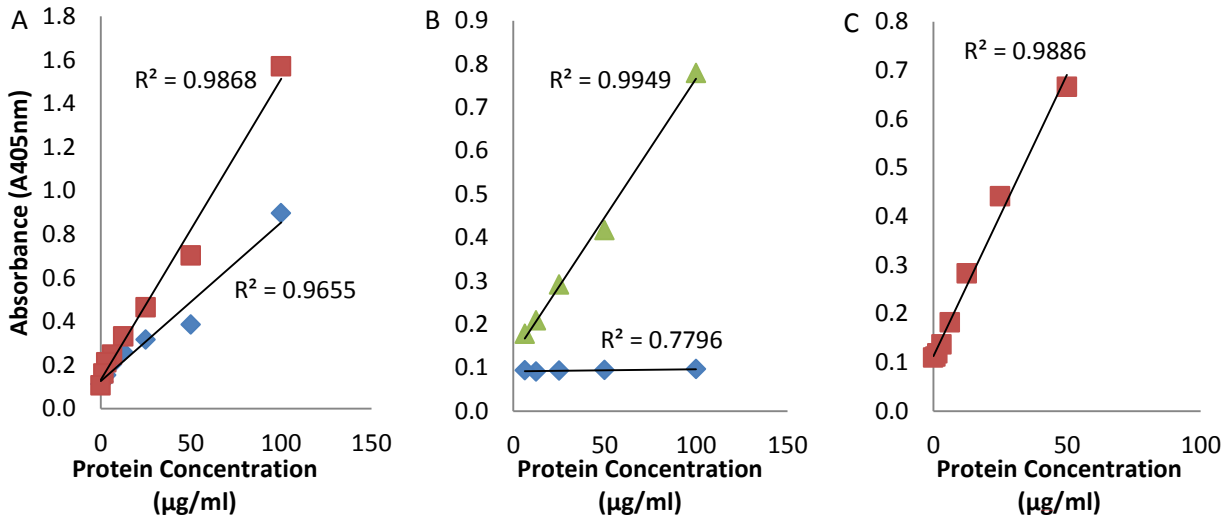

Figure 13. The construction of a standard curve for Cel7A ELISA (A), Cel6A ELISA (B), and Cel7B ELISA (C) using whole commercial enzyme mixtures Accellerase 1000 (◊), CTec 2 (□), and Celluclast 1.5L (Δ).

3.2.5 Determining the Possible Interference of Substrate-Derived Materials on the ELISA

Although various ELISA-based methods have been used to quantify cellulase enzymes, these assays have only been applied to commercial enzyme mixtures or to culture filtrates (Kolbe and Kubicek, 1990; Riske, Eveleigh and Macmillan, 1990; Buhler, 1991; Lynd and Zhang, 2002). The use of an ELISA to follow the distribution of cellulase enzymes during enzymatic hydrolysis of a realistic, lignocellulosic substrate has, so far, not been described in the literature. As a result, there is limited information on the possible influence of interfering materials that may be present when using ELISA under these conditions.

Previous work on the use of ELISAs to detect residual agrochemicals in soil samples had shown that humic substances in soil may result in an overestimation of the chemical concentrations (Conde et al., 2008; Toscano et al., 1998; Krotzky and Zeeh, 1995), and that sample dilution could be used to minimize interference (Conde et al., 2008). As a similar type of interference might occur with biomass-derived materials, such as soluble lignin fragments, supernatants derived from SPCS, SPP, SPDF, and

Avicel were assessed for their possible influence on the double-antibody ELISA. The supernatants were diluted in PBS to varying degrees to determine if a simple dilution could minimize the interference caused by these materials.

It was apparent that the undiluted biomass-derived supernatants resulted in considerable interference with all of the Cel7A, Cel6A, and Cel7B based ELISAs (Figure 14). The addition of the biomass-derived supernatants resulted in either over or under estimated the amount of enzyme with the Cel7A ELISA (Figure 14A) supernatants derived from the SPCS and SPP substrates resulting in an overestimation and the SPDF and Avicel supernatants resulted in an underestimation (Figure 14A). In contrast, only the SPP supernatants caused a signal overestimation with Cel6A ELISA, while the SPCS, SPDF, and Avicel supernatants gave a signal that was lower than the PBS control (Figure 14B). Interference with Cel7B based ELISA was only determined using the SPCS supernatant, which caused a slight overestimation (Figure 14C). To assess if a simple dilution could minimize interference caused by SPCS supernatant, this supernatant was diluted 10x or 100x in PBS. It was apparent that the interference caused by the addition of the undiluted SPCS supernatant could be minimized at both dilution levels (Figure 14A). This dilution strategy was also effective on both the Cel6A and Cel7B based ELISAs and a 100-fold dilution in PBS seemed to consistently give an ELISA signal similar to the PBS control for both Cel6A and Cel7B ELISA (Figure 14B and C).

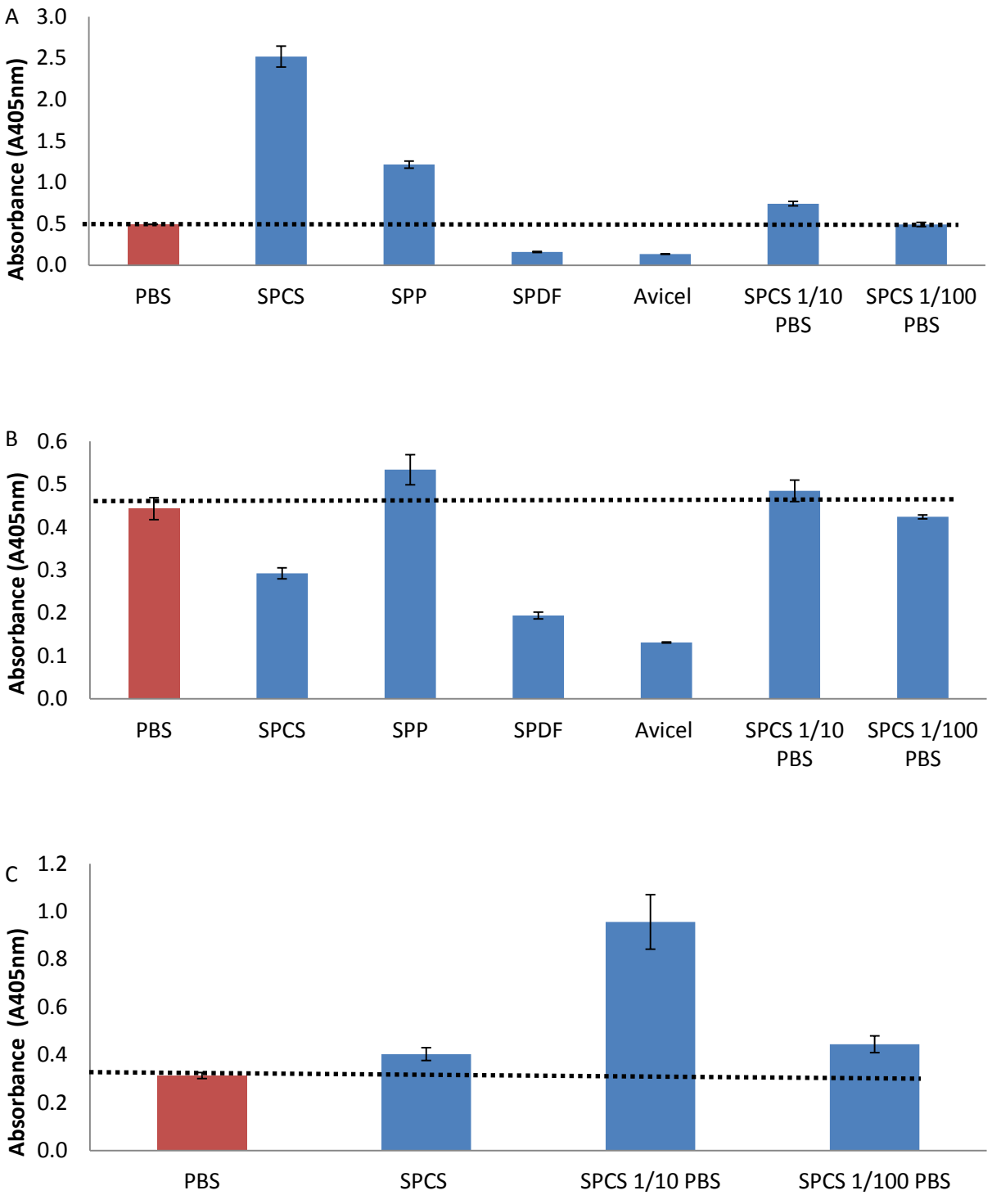

Figure 14. Effect of substrate supernatants on Cel7A ELISA (A), Cel6A ELISA (B) and Cel7B ELISA (C). Amount of purified enzymes added: 1.25 µg/ml.

3.2.6 Can the ELISA be used to Follow Enzyme Distribution during SPCS Hydrolysis?

We then assessed whether the double-antibody sandwich ELISA could be used to quantitatively monitor the time course of individual enzyme adsorption (Cel7A, Cel6A, and Cel7B) during the hydrolysis of SPCS. It was apparent that all three enzymes exhibited different adsorption profiles when incubated with SPCS (Figure 15A, B, and C). Most of Cel7A immediately adsorbed to the SPCS after mixing, with only about 30% of Cel7A remaining in the supernatant. After 3 hours of hydrolysis, Cel7A started to desorb back to the supernatant, with maximum desorption occurring after 6 hours of hydrolysis with about 65% of the initial Cel7A detected in the supernatant. Over prolonged hydrolysis, the concentration of Cel7A in the supernatant decreased progressively (Figure 15A). This partially reversible adsorption of Cel7A confirmed previous work in section 3.1 where a combination of techniques, such as zymogram, SDS-PAGE and enzyme activity assays, were used to semi-quantitatively determine specific Cel7A adsorption/desorption during SPCS hydrolysis.

In contrast, Cel6A directly adsorbed onto the SPCS within the first 3 hours and remained tightly bound throughout the course of hydrolysis (Figure 15B). The irreversible adsorption of Cel6A observed here was in a good agreement with previous work which looked at Cel6A adsorption using purified Cel6A (Palonen, Tenkanen and Linder, 1999).

Compared to Cel7A and Cel6A, the adsorption of Cel7B was more gradual with the amount of Cel7B detected in the supernatant continuously declining over the 72-hour hydrolysis period (Figure 15C). Prior to developing the ELISA method, we had tried to follow the specific adsorption profile of Cel7B by monitoring its profile, as determined by zymograms using CMC and xylan as substrates (section 3.1). The quantitative adsorption profiles obtained using the ELISA profile were in agreement with the qualitative results previously obtained using zymograms over 72-hour hydrolysis (Section 3.1.7).

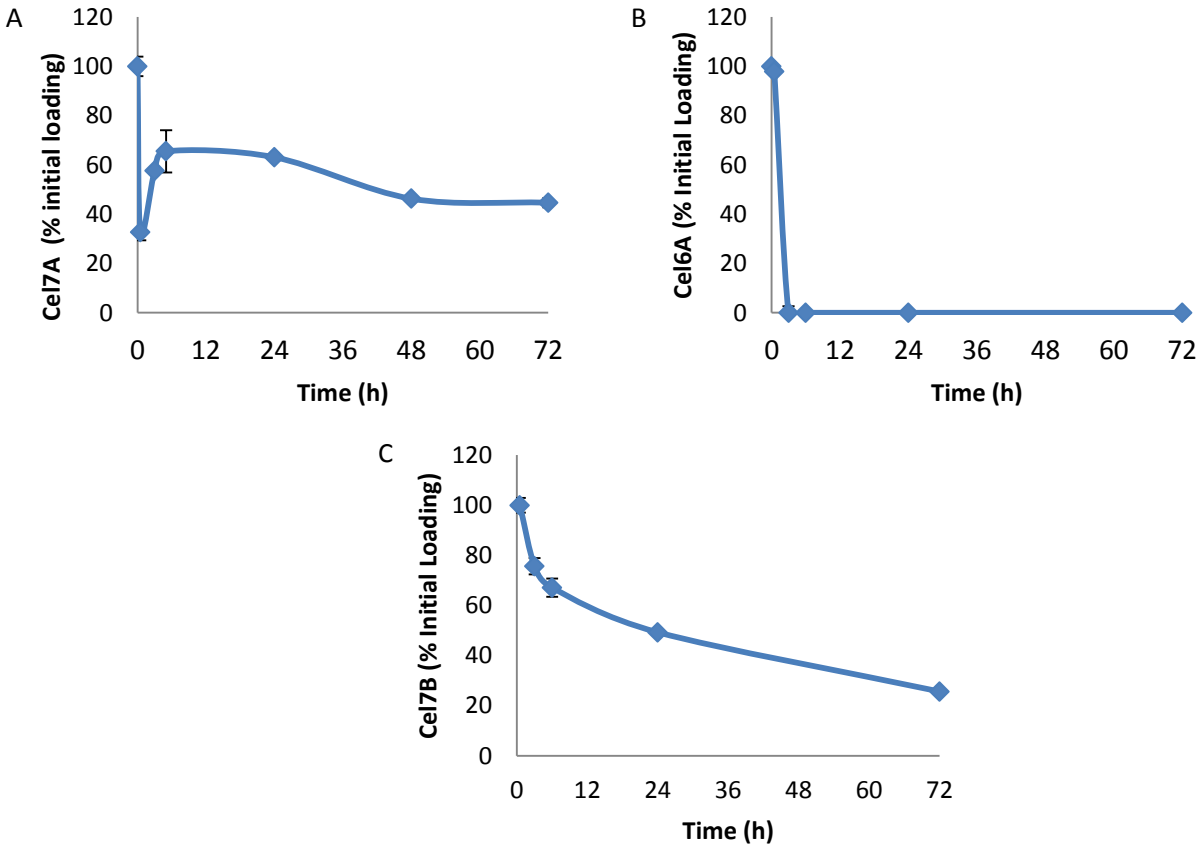

Figure 15. Adsorption profiles of Cel7A (A), Cel6A (B), and Cel7B (C) during hydrolysis of SPCS as determined by a double-antibody sandwich ELISA. Samples for Cel7A ELISA were obtained at different time points during hydrolysis of SPCS at 2% (w/v) substrate consistency in 0.05 M Na-acetate buffer pH 5.0 with 20 FPU/ g glucan Accellerase 1000. Samples for Cel6A and Cel7B ELISA were obtained from SPCS hydrolysis using Celluclast at 20 FPU/g glucan and β-glucosidase at 40 IU/g glucan.

3.2.7 Conclusions

A simple, high-throughput ELISA-based assay was developed to specifically quantify the adsorption of Cel7A, Cel6A, and Cel7B present in complex cellulase enzyme mixtures during hydrolysis of SPCS. A combination of MAb's and PAb's, as the respective coating and detecting antibodies, was used to develop a double-antibody sandwich ELISA. The method was able to detect and quantify individual enzymes present in cellulase mixtures. The assay was sensitive at relatively low enzyme concentrations (0 – 1 μg/ml), provided the samples were first pH adjusted and heat treated to increase the antigenicity of the target enzymes. Although lignocellulosic hydrolysates resulted in varying degrees of interference with the assay, the interference could be minimized by diluting the samples in PBS buffer. The immunoassay was employed to quantitatively monitor the adsorption of Cel7A, Cel6A, and Cel7B present in both Celluclast and Accellerase 1000, during the hydrolysis of SPCS. All three enzymes exhibited different individual adsorption profiles. The specific and quantitative adsorption profiles observed with the ELISA method were in good agreement with earlier work where more laborious enzyme assay techniques were used.

By using this ELISA technique, the distribution of specific enzymes during hydrolysis could be quantified, which would help determine the appropriate enzyme recycling strategy. By using the ELISA technique combined with other assays, the influence of substrate properties, enzyme actions, and physical factors on individual enzyme adsorption profiles could be assessed, helping us determine which substrate properties, enzyme composition and hydrolysis conditions were most influential in limiting enzyme recovery and enzyme recycling performance. As mentioned earlier, as the nature of the substrate had been shown to have a major influence on enzyme adsorption and related enzyme recycle strategies, we next assessed the influence of lignin and cellulose on adsorption/recycle.

3.3 The Influence of Lignin and Cellulose Properties on Enzymatic Hydrolysis and Enzyme Adsorption during Hydrolysis

3.3.1 Background

Due to the recalcitrance of lignocellulosic biomass, a pretreatment step is typically required to increase its enzymatic digestibility. These pretreatment strategies can be classified into the four major categories of physical, biological, and chemical pretreatments, and a combination of these pretreatment strategies. All of these pretreatment strategies share a common goal of both trying to maximise lignin and hemicellulose recovery but also in increasing cellulose accessibility to enzymes. However, they sometimes achieve these goals through different mechanisms such as by reducing the lignin content of the biomass, modifying lignin distribution, removing hemicellulose, and/or altering the structure of cellulose.

If a process such as steam pretreatment is used, in order to achieve high overall carbohydrate (hemicelluloses and cellulose) recovery while producing cellulose-rich substrates amenable to enzymatic hydrolysis, compromised pretreatment conditions at medium severity are used (Arantes and Saddler, 2011). This typically results in higher residual levels of hemicellulose and lignin in the pretreated substrates (Helbert et al., 2003; Schwarz, 2001). Depending on the pretreatment strategy and the characteristics of the biomass substrate, the properties of lignin, hemicellulose, and cellulose in the pretreated biomass will vary. The properties of these substrate constituents will have a significant effect on the hydrolysis efficiency and the enzyme-substrate interaction during hydrolysis. For example, following steam pretreatment, lignin depolymerisation and condensation on the cellulose fibers have been observed (Robert et al., 1988; Li, Henriksson and Gellerstedt, 2007). Cellulose itself may also exhibit different physical characteristics, such as different cellulose accessibility to enzymes, degree of polymerization (DP), crystallinity, and allomorphic forms. All of these substrate physicochemical

properties are known to significantly influence the efficiency of the cellulase enzymes (Ishizawa et al., 2009; Medve, Lee and Tjerneld, 1998) in breaking down cellulose to glucose. As previous enzyme recycling studies have indicated that substrate properties highly influence enzyme recycling performance (Lee, Yu and Saddler, 1995; Qi et al., 2011; Ramos and Saddler, 1994) a better understanding of how the properties of plant cell wall components, following pretreatments, influence enzymes' interaction with the substrate will help better develop an efficient enzyme recycling strategy.

To understand the influence of cellulose, hemicellulose, and lignin on enzyme adsorption and enzymatic hydrolysis, we followed cellulose hydrolysis efficiency and individual enzyme adsorption profiles during hydrolysis of the four different lignocellulosic substrates of SPCS, delignified SPCS (DSPCS), xylanase-treated SPCS (XSPCS), and dissolving pulp (DsP). To further assess the influence of cellulose properties, 6 cellulosic (or delignified lignocellulosic) substrates Avicel, DsP, cellulose II, phosphoric acid swollen cellulose (PASC), cellulose III, and cellulose nano-crystal (CNC) were hydrolyzed using a commercial enzyme mixture. The cellulose II and PASC in this study were derived from Avicel. The individual enzyme adsorption profiles were monitored during hydrolysis using the ELISA technique developed in section 3.2 and other techniques evaluated in section 3.1.

3.3.2 Substrate Compositions of SPCS, DSPCS, XSPCS, DsP, and the Six Cellulosic Substrates

The (ligno)cellulosic substrates were analyzed for their chemical compositions and accessibility.

Table 4 shows the chemical compositions of SPCS, DSPCS, XSPCS, and DsP. The acid-chlorite delignification method was effective in selectively removing the lignin in SPCS; the acid-insoluble lignin (AIL) content in DSPCS was 0.1% (w/w) of the total dry matter compared to 22.4% (w/w) in SPCS. The hemicellulose remained largely preserved following the delignification procedure as DSPCS still contained 8.0% (w/w) xylan compared to 8.4% (w/w) xylan in the SPCS. Due to the removal of most of the lignin, the cellulose (glucan) content in the DSPCS increased significantly to 91.1% (w/w) from 57.4%

(w/w) in the SPCS. The xylanase treatment of the SPCS, on the other hand, only reduced the xylan content by ~2% (w/w) from 8.4% (w/w) in the SPCS to 6.3% (w/w) in the XSPCS. The amount of AIL in XSPCS was comparable to that in SPCS. The gross characteristics of XSPCS were different from SPCS as the XSPCS was darker and felt stickier to the touch compared to SPCS. Due to the low xylan removal and possible changes in substrate properties other than hemicellulose properties alone, this substrate was not used further in this study. Comparing the almost pure cellulosic substrates DSPCS and DsP, both substrates had a cellulose content of above 90% (w/w) of the total dry matter. The DsP, on the other hand, had a much lower xylan content of 3.1% (w/w) compared to 8.0% in the DSPCS. The DsP also had slightly higher galactan and mannan contents as compared to the DSPCS (Table 4).

Other than the DsP, the chemical compositions of the six cellulosic substrates Avicel, cellulose II and PASC (derived from Avicel), cellulose III (derived from cotton linters), and CNC were not determined. For the purpose of determining enzyme loadings and conversion efficiency, the cellulose contents in all these six pure cellulosic substrates were regarded as 100%.

Table 4. Chemical compositions of steam pretreated corn stover (SPCS), delignified SPCS (DSPCS), xylanase-treated SPCS (XSPCS), and dissolving pulp (DsP).

Sample	Ara(%)	Gal(%)	Glu(%)	Xyl(%)	Man(%)	AIL(%)	ASL(%)	Ash(%)	Total(%)
SPCS	0.4	0.2	57.4	8.4	0.0	22.4	1.9	4.5	95.2
DSPCS	0.3	0.1	91.1	8.0	0.0	0.1	0.7	6.4	106.7
XSPCS	0.3	0.1	68.9	6.3	0.9	21.4	1.3	4.4	103.6
DsP	0.3	0.7	94.1	3.1	1.2	0.2	NDn	NDn	99.6

3.3.3 General Protein Adsorption Profiles during Hydrolysis of SPCS, DSPCS, and DsP

The protein adsorption profiles were determined using SDS-PAGE and the ninhydrin assay during hydrolysis of SPCS, DSPCS, and DsP using Cellic CTec2 without the addition of a reducing factor (Figure 16). In general, a rapid protein adsorption was observed upon addition of enzymes to all three substrates. These proteins then gradually desorbed back to the supernatant (Figure 16D). Interestingly,

relatively similar protein adsorption/ desorption profiles were observed for SPCS and DSPCS (Figure 16D). In contrast, the protein adsorption profile to DsP significantly differed from the adsorption profile to DSPCS despite both substrates having low lignin contents (Figure 16D). These results suggested that lignin removal from SPCS did not significantly influence protein adsorption, which may indicate that the lignin in SPCS did not contribute significantly to enzyme non-productive adsorption to lignin. As previous enzyme recycling studies reported improvements in enzyme recycling efficiency upon partial delignification of steam pretreated birch substrate (Ramos and Saddler, 1994; Lee, Yu and Saddler, 1995), the influence of lignin on enzyme adsorption and subsequently enzyme recycling might be dependent on the nature of the lignin. Indeed, subsequent work showed that lignin derived from corn stover had minimum inhibition on enzymatic hydrolysis compared to lignin isolated from hardwood and softwood substrates (Nakagame, Chandra and Saddler, 2010). However, the different protein adsorption profiles observed with DsP and DSPCS, despite both substrates having low amount of lignin, might indicate that cellulose properties may actually play a more important role in enzyme adsorption.

General protein adsorption profiles were further evaluated using SDS-PAGE. In general, the band density of the different proteins detected on SDS-PAGE confirmed the general trends observed with the ninhydrin assay (Figure 16D), showing similar protein adsorption profiles between SPCS and DSPCS and low level of protein adsorption to DsP (Figure 16A, B, and C). While it is difficult to quantify the level of protein adsorption to these three substrates using SDS-PAGE, the adsorption profiles of individual enzymes could be qualitatively determined. For example, high adsorption of proteins around MW of 50 kDa was observed on all three substrates (Figure 16A, B, and C). As discussed in Section 3.1, this protein band was likely to be indicative of the protein adsorption profile of the major cellulase enzyme Cel7A. A high adsorption of a protein band around MW 20 kDa, which likely indicated the adsorption profile of Xyn11, was observed on the DSPCS gel (Figure 16B), whereas the band's adsorption profile on SPCS was rather gradual (Figure 16A). A gradual adsorption of protein bands around MW of

150 kDa, likely indicating adsorption profiles of β-glucosidase, was observed on both the SPCS and DSPCS gels (Figure 16A and B). In contrast, no significant levels of adsorption were observed on the DsP gel for protein bands at MW 150 kDa and 20 kDa (Figure 16C). These observations again suggested that the substrate characteristics of DsP and DSPCS may be different and contribute to the different adsorption profiles observed on these substrates.

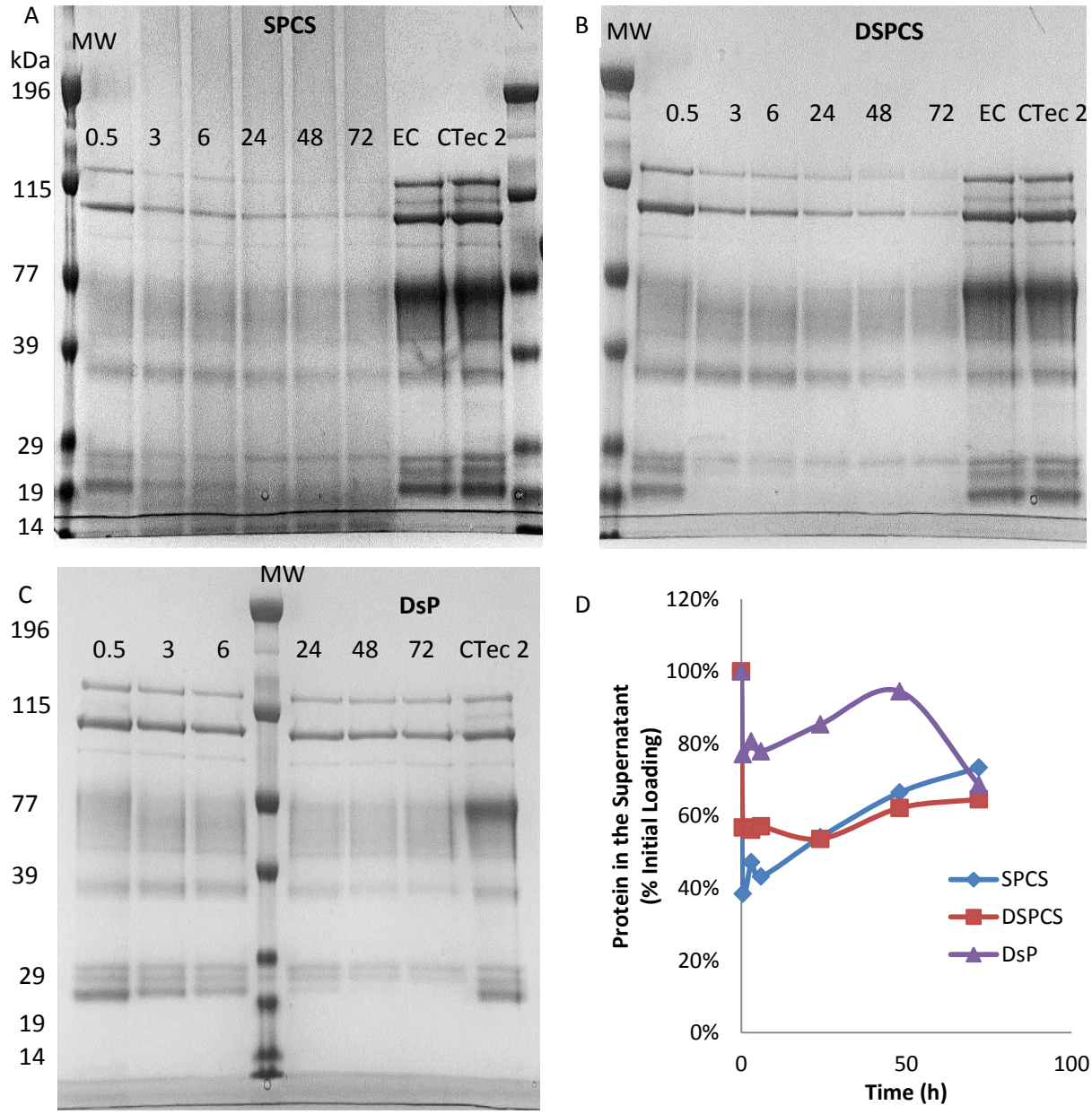

Figure 16. SDS-PAGE of proteins in the supernatants during hydrolysis of SPCS (A), DSPCS (B), and DsP (C), and protein adsorption profiles during hydrolysis of the 3 substrates as determined using the ninhydrin assay (D). Hydrolysis was conducted with Cellic CTec2 at enzyme loading of 15 mg/g glucan and 2% (w/v) solid consistency without the addition of any exogenous reducing factor.

3.3.4 Individual Enzyme Adsorption Profiles observed during Hydrolysis of SPCS, DSPCS, and DsP

The individual enzyme adsorption profiles were determined using enzyme activity assays and the ELISA assay developed in Section 3.2. The Cel7A adsorption profiles during hydrolysis of the three substrates with Cellic CTec 2 were determined based on Cel7A activity in the supernatant, as measured using PNPC. More than 80% of the initial Cel7A quickly adsorbed to SPCS and DSPCS upon the addition of enzymes. Alternatively, only about 50% of the initial Cel7A adsorbed onto the DsP, although the level of enzyme adsorption increased as hydrolysis proceeded. The Cel7A adsorption profiles onto SPCS and DSPCS were similar (Figure 17A). These observations confirmed the trends observed previously on SDS-PAGE and the ninhydrin assay as shown in Figure 16. Similar trends were also observed when the Cel7A adsorption profiles were determined using the ELISA method (data not shown). The similar Cel7A adsorption profiles on SPCS and DSPCS was unexpected as this suggested that lignin removal did not influence the level of Cel7A adsorption. As mentioned earlier, while enzymes have been shown to adsorb unproductively to lignin, certain types of lignin, such as lignin derived from agricultural residues, have been shown to have a minimum inhibition effect on hydrolysis yields and enzyme adsorption during hydrolysis (Nakagame, Chandra and Saddler, 2010). This low inhibitory lignin in SPCS may be the reason why similar Cel7A adsorption profiles were observed on this substrate, even after lignin removal.

When the adsorption profiles of β-glucosidase were determined using PNPG, a significant level of adsorption was observed on all of the substrates and the adsorption profiles were similar to those of Cel7A. The β-glucosidase enzyme also showed similar adsorption behaviors on SPCS and DSPCS and the least adsorption on DsP (Figure 17B). The adsorption profiles, as determined by PNPG, agreed well with the pattern observed on the SDS-PAGE gels for bands with a MW around 115 kDa, which showed minimal adsorption to DsP and a gradual adsorption to both SPCS and DSPCS (Figure 16A, B, and C). PNPX was used to determine the adsorption profiles of xylanase and endoglucanase enzymes. DSPCS

and DsP showed similar enzyme adsorption profiles as determined using PNPX. Alternatively, the PNPX-active enzymes adsorbed the least to SPCS (Figure 17C). More work is needed to try to further determine the possible substrate characteristics that contribute to the different adsorption profiles of these enzymes.

When the Cel6A adsorption profiles were determined by the ELISA technique, the Cel6A was shown to adsorb more rapidly to SPCS as compared to DSPCS and DsP. However, after 3 hours, all of the Cel6A was adsorbed to all of the substrates (Figure 17D). The Cel6A seemed to be more susceptible to adsorption to lignin even if SPCS lignin was not highly adsorptive to other enzymes such as Cel7A. The Cel6A also remained adsorbed to the solid for the remainder of hydrolysis. This irreversible adsorption of Cel6A agrees well with the Cel6A adsorption profile reported earlier (Palonen, Tenkanen and Linder, 1999).

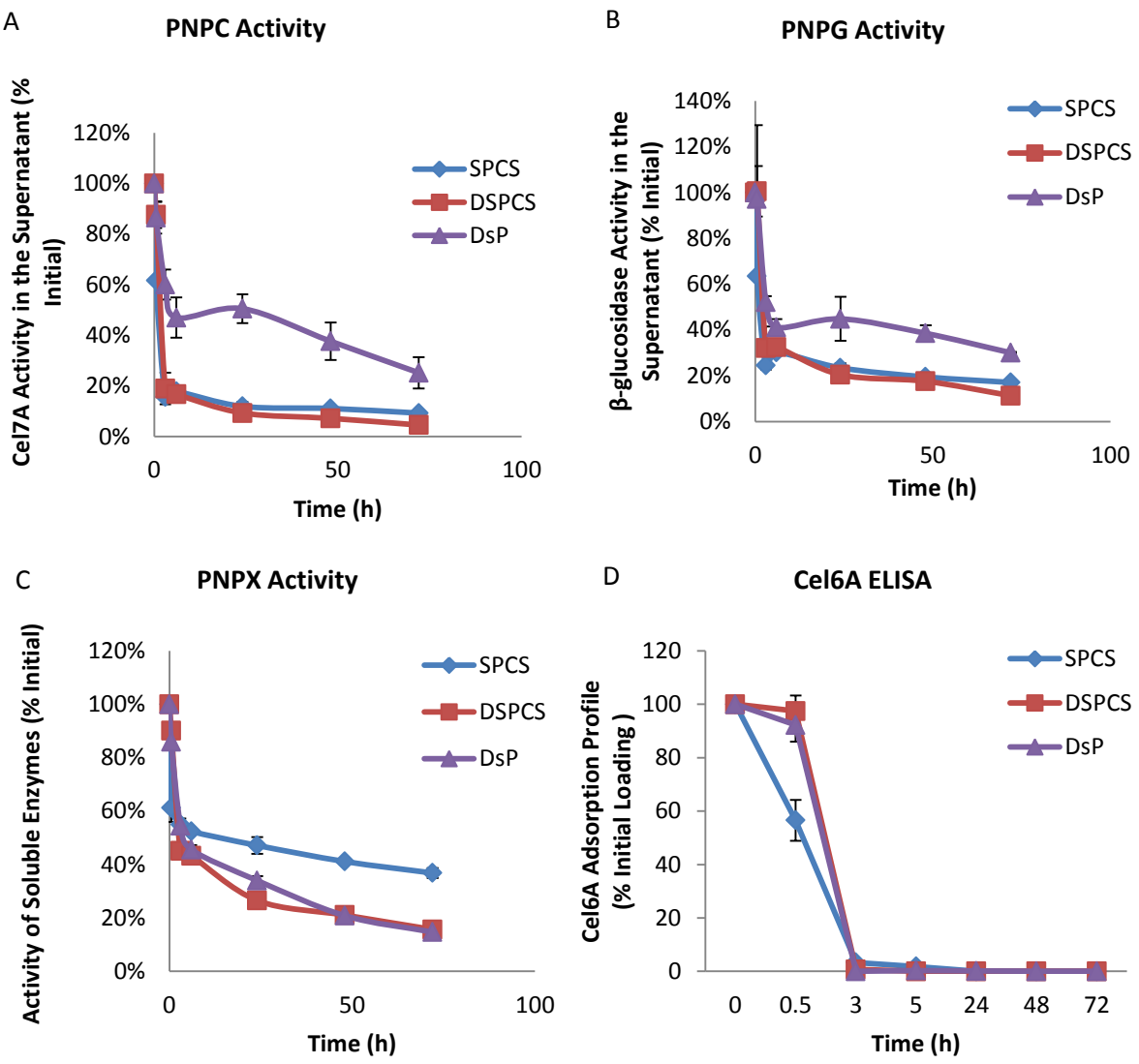

Figure 17. Individual enzyme adsorption profiles on SPCS, DSPCS, XSPCS, and DsP for Cel7A (A), β-glucosidase (B), xylanases and endoglucanases (C) as determined using PNPC, PNPG, and PNPX, respectively, and Cel6A as determined using DAS-ELISA.

3.3.5 General Protein Adsorption Profiles during Hydrolysis of Six Cellulosic Substrates

The general protein adsorption profiles during hydrolysis of the six pure cellulosic substrates were determined using the ninhydrin assay. Generally, upon addition of enzymes, a rapid enzyme adsorption was observed on all substrates. This rapid enzyme adsorption was followed by a partial enzyme desorption within 8 hours of hydrolysis (Figure 18). Although the general trend of enzyme

adsorption followed by desorption was similar among substrates, the levels of enzyme adsorption/ desorption differed with different substrates. Among the six substrates, cellulose II showed the highest level of enzyme adsorption (more than 80%), after which only a minimum (less than 10%) enzyme desorption was observed. On the other hand, similar levels of enzyme adsorption (~40% of initial protein loading) were observed at 3 hours with PASC, Avicel, and DsP. Following this adsorption, a high level of enzyme desorption was observed with PASC, followed by Avicel and DsP. After 24 hours, around 67% of the initial protein loading could be detected in the supernatant during PASC hydrolysis, while only ~45% and ~37% of the initial protein loading was detected during Avicel and DsP hydrolysis, respectively (Figure 18). Cellulose III was shown to adsorb the least enzymes and about 45% of the initial protein loading remained in the supernatant during hydrolysis. CNC was found to significantly adsorb the enzymes, and these adsorbed enzymes only showed a minimal desorption during the course of hydrolysis.

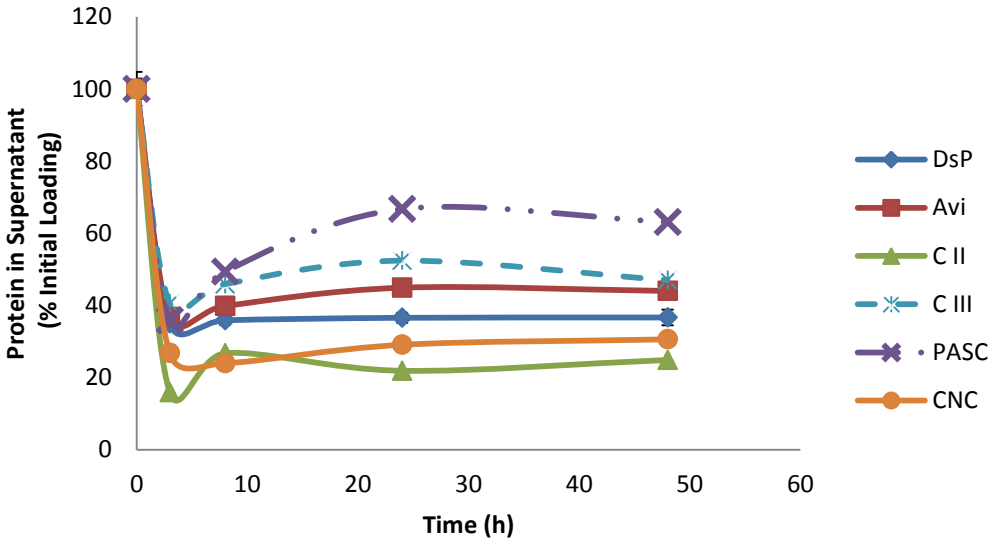

Figure 18. Protein adsorption profile during hydrolysis of 6 model cellulosic substrates: dissolving pulp (DsP), Avicel (Avi), cellulose II (C II), cellulose III (C III), phosphoric-acid swollen cellulose (PASC), and cellulose nano crystal (CNC). Hydrolysis was performed at 2% (w/v) substrate consistency using Celluclast and Novozym 188 at loadings of 13 and 5 mg/g cellulose, respectively.

3.3.6 Individual Enzyme Adsorption Profiles during Hydrolysis of Six Cellulosic Substrates

The adsorption profile of Cel7A on the six model cellulosic substrates was determined by measuring changes in Cel7A activities on PNPC in the supernatant during hydrolysis. Cel7A, being the most abundant enzyme in the cellulase enzyme mixture, displayed similar adsorption profiles to the adsorption profiles of the general proteins, as determined using the ninhydrin assay (Figure 18 and Figure 19A), across the six cellulosic substrates. Significant adsorption of Cel7A (more than 80% of initial activity) was observed on cellulose II, DsP, and PASC. Although the Cel7A remained adsorbed to cellulose II throughout hydrolysis, with little desorption occurring, desorption was observed on DsP and PASC (Figure 19A). Although around 70% of the added Cel7A was initially adsorbed to Avicel and CNC, desorption was observed with Avicel and no desorption was observed with CNC. The Cel7A showed the least (only around 60%) adsorption to cellulose III (Figure 19A).

A rather different enzyme activity adsorption profile was observed using PNPX. More than 80% of enzymes that were active on PNPX adsorbed to DsP, cellulose II, PASC, and CNC, with little desorption taking place during hydrolysis (Figure 19B). Interestingly, only around 60% of these enzymes were adsorbed to Avicel, while the least adsorption was observed on cellulose III. This high adsorption of endoglucanase and xylanase enzymes (enzymes that are expected to be active on PNPX) to pure cellulosic substrates was rather unexpected as these enzymes are typically shown to remain primarily in the liquid phase. More work is needed to assess possible reasons for this high adsorption to pure cellulosic substrates.

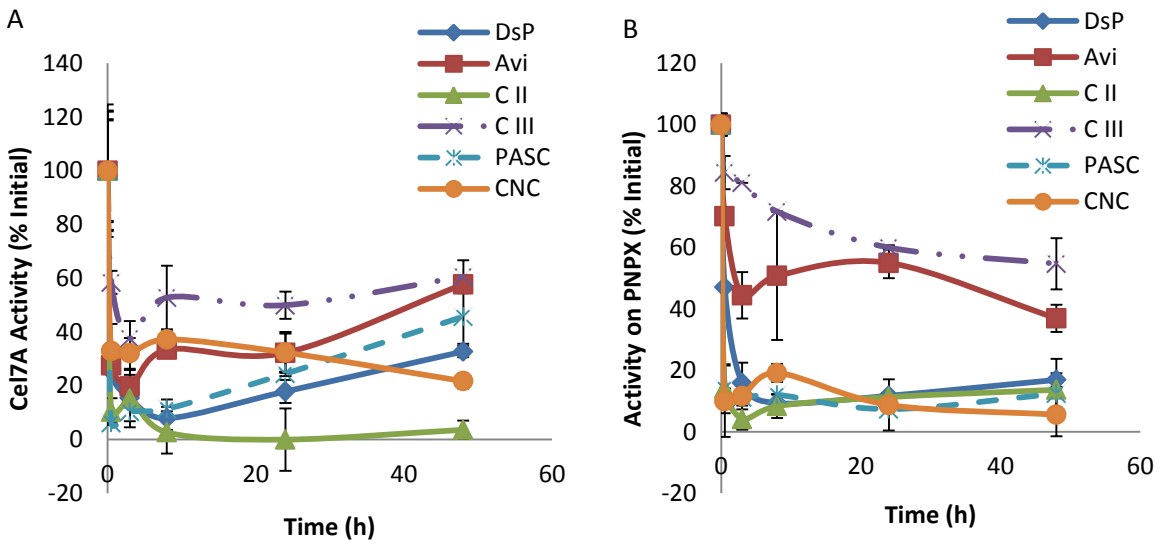

Figure 19. The adsorption profiles of Cel7A (A) and xylanase and endoglucanase enzymes (B) as determined by the PNPC and PNPX, respectively, during hydrolysis of 6 model cellulosic substrates using Celluclast and Novozym 188 at 2% (w/v) substrate consistency. The 6 cellulosic substrates included dissolving pulp (DsP), Avicel (Avi), cellulose II (C II), cellulose III (C III), phosphoric acid swollen cellulose (PASC), and cellulose nanocrystals (CNC).

3.3.7 Analyses of Substrate Properties

The substrate properties of the lignocellulosic and cellulosic substrates were next evaluated to try to determine which substrate properties might influence enzyme adsorption behavior. The accessibility of these substrates was determined by using the Simon's Stain method. After comparing SPCS, DSPCS, and DsP, the DSPCS was shown to have the most accessible surface area. The SPCS and DsP had comparable, highly accessible surface areas (Figure 20A) as lignin removal has previously been shown to increase cellulose accessibility (Kumar et al., 2012).

The (pure) cellulosic substrates were also analyzed using the Simon's Stain method to determine their accessible surface area. Based on the dye adsorption, CNC was determined to be the least accessible followed by the following substrates in the order of increasing accessibility, cellulose III, Avicel, DsP, cellulose II, and PASC (Figure 20B). In addition, the adsorption of CBM2a and CBM44, that

binds specifically to crystalline and amorphous cellulose, respectively (Gourlay, Arantes and Saddler, 2012), was determined on the six pure cellulosic substrates to assess the nature of the accessible cellulose (i.e. crystalline or amorphous). Earlier work had shown that the ratios between adsorbed CBM2a and CBM44 on a particular cellulosic substrate are good indicators of the crystallinity of the accessible cellulose (discussed in section 3.4). As expected, CNC had the highest CBM2a-to-CBM44 adsorption ratio, indicating the highly crystalline nature of the accessible cellulose in this substrate. Based on the CBM2a-to-CBM44 adsorption values, the crystallinity of the accessible cellulose in the six substrates were determined to be in the following order (from the most crystalline to the most amorphous) CNC, Avicel, DsP, cellulose II, and PASC (Figure 20B). No significant CBM adsorption was observed on cellulose III. This low adsorption of CBMs to cellulose III was expected as earlier work had reported the reduced enzyme adsorption to cellulose III due to increased cellulose hydration compared to cellulose I (Chundawat et al., 2011).

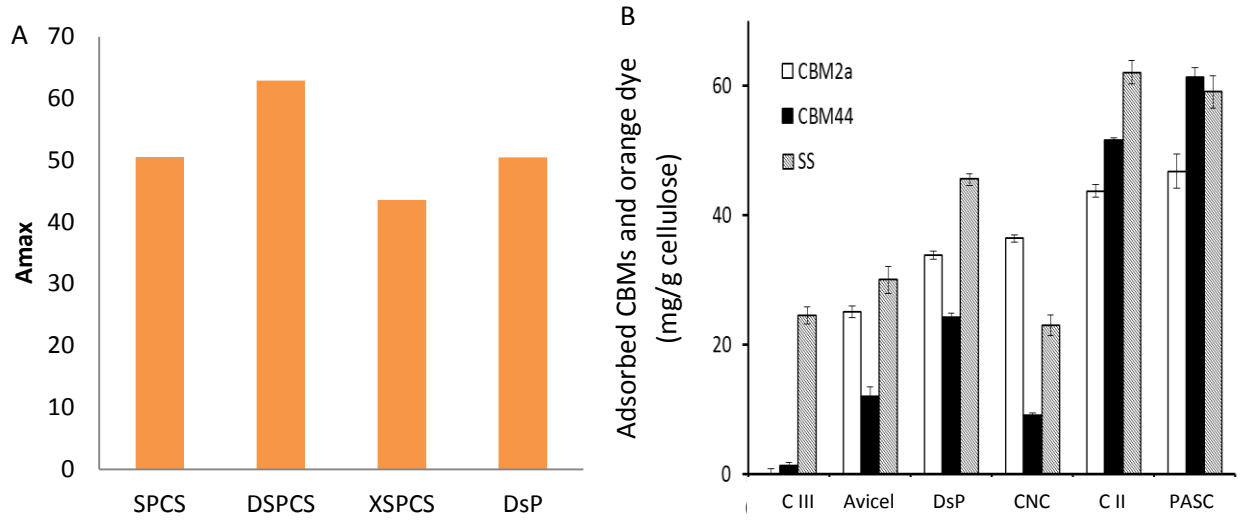

Figure 20. Substrate accessibility of steam pretreated corn stover (SPCS), delignified steam pretreated corn stover (DSPCS), xylanase-treated steam pretreated corn stover (XSPCS), and dissolving pulp (DsP) as determined using the Simon's Stain (SS) method (A) and the 6 model cellulosic substrates: cellulose III (C III), Avicel, dissolving pulp (DsP), cellulose nanocrystal (CNC), cellulose II (C II) and phosphoric acid swollen cellulose (PASC) were derived from Avicel (B). The cellulose accessibility of the 6 model cellulosic substrates was determined using the Simon's Stain (SS) method as well as cellulose binding module (CBM)2a with a preference for crystalline cellulose and CBM44 with a preference for amorphous cellulose.

While substrate accessibility has been shown to be a major factor influencing hydrolysis efficiency (Arantes and Saddler, 2010), its effects on enzyme adsorption are less understood. Surprisingly, the results presented here seem to indicate that cellulose accessibility may not be a major factor in determining enzyme adsorption profiles. The DsP and SPCS substrates were shown to have similar cellulose accessibility (Figure 20A), but the enzyme adsorption profiles to these two substrates were clearly different (Figure 16 and Figure 17). The enzyme showed similar adsorption profiles to SPCS and DSPCS (Figure 16 and Figure 17) despite DSPCS having a higher accessibility than SPCS (Figure 20A).

The accessibility of enzymes to the six cellulosic substrates again confirmed the earlier observation that, under the conditions studied here, cellulose accessibility did not seem to determine the nature of enzyme adsorption profile. For instance, PASC and cellulose II were shown to have similar

accessibility based on the Simon's Stain method and the CBM adsorption method. However, a higher level of enzyme adsorption was observed on cellulose II than on PASC (Figure 18). The enzymes seemed to bind irreversibly to cellulose II but reversibly to PASC (Figure 19A). These different enzyme adsorption profiles clearly showed the influence of different cellulose properties on enzyme adsorption. It is interesting to note that, although cellulose II and PASC were both considered to be swollen forms of cellulose following treatments with alkaline and phosphoric acid, respectively, differences seem to exist in the nature of these swollen cellulose particles, leading to different enzyme adsorption profiles.

Other than cellulose accessibility, other cellulose properties that may influence enzyme adsorption include cellulose allomorphs and crystallinity. When we compared cellulose I substrates Avicel and dissolving pulp (DsP) with cellulose II and cellulose III, the enzymes were shown to bind the most to cellulose II followed by the cellulose I substrates Avicel and DsP. Among the three cellulose allomorphs evaluated in this study, the enzymes seem to bind the least to cellulose III (Figure 18 andFigure 19A). This suggests that the type of cellulose allomorph may play an important role in determining the nature and extent of enzyme adsorption.

Cellulose crystallinity was also expected to influence enzyme adsorption. A recent study on the influence of cellulose crystallinity on enzyme adsorption reported a decreasing enzyme binding capacity above a certain cellulose crystallinity value as measured using the crystallinity index (CrI) (Hall et al., 2010). Another study has suggested that the processive action of Cel7A seems to be influenced by cellulose crystallinity, with the enzyme moving processively over a longer distance on a more crystalline cellulose surface (Kipper, Valjamae and Johansson, 2005). Therefore, it is possible that the differing crystalline natures of cellulose II and PASC may contribute to the different enzyme adsorption behaviors in these substrates. As shown by the increase in CBM44 adsorption to PASC compared to cellulose II (Figure 20B), PASC was a more amorphous substrate than was cellulose II. The higher amount of

accessible amorphous cellulose may explain the higher level of enzyme desorption observed with PASC as compared to cellulose II (Figure 18). The enzymes also seemed to adsorb irreversibly to the highly crystalline substrate CNC (Figure 19A).These results seemed to suggest that high cellulose crystallinity may contribute to the irreversible nature of enzyme adsorption.

Together, these results showed that cellulose accessibility may not be a major player in determining the nature of enzyme adsorption. Rather, cellulose properties such as the type of cellulose allomorph and cellulose crystallinity might have a more significant effect on enzyme adsorption/desorption.

3.3.8 Cellulose Hydrolysis Efficiency

Other than influencing enzyme recycling strategies, the different enzyme adsorption profiles on different substrates may also influence their conversion efficiency. Comparing the hydrolysis efficiency of SPCS, DSPCS, and DsP, the highest cellulose conversion was obtained during hydrolysis of the DSPCS followed by the SPCS and DsP (Figure 21A). Compared with the SPCS, lignin removal from the DSPCS seemed to improve hydrolysis efficiency by 20%. As DSPCS has been shown to have the most accessible surface area among the three substrates studied (Figure 20A), it was not surprising that this substrate was also the most efficiently hydrolyzed substrate. Interestingly, the SPCS was also more efficiently hydrolyzed compared to dissolving pulp (DsP) despite the low amount of lignin in the dissolving pulp. These two substrates have been shown to have a comparable degree of accessibility (Figure 20A), and thus the difference in accessibility could not account for this difference in hydrolysis efficiency. Rather, the increase in SPCS hydrolysis efficiency was more likely caused by the action of the AA9 enzyme in the Cellic CTec 2 enzyme mixture used to hydrolyze the substrates. This AA9 enzyme has been shown to require a reducing agent for its activity that would be present in the SPCS hydrolysate but absent in DsP hydrolysate (Quinlan et al., 2011).

The hydrolysis efficiency of the enriched cellulosic substrates was also compared and is shown in Figure 21B. Not surprisingly, those cellulosic substrates that were more accessible such as cellulose II and PASC were hydrolyzed more efficiently compared to substrates that were less accessible, with CNC being the least efficiently hydrolyzed. Cellulose III, on the other hand, was efficiently hydrolyzed although its accessible surface area was lower than Avicel as determined by the Simon Stain's method. This anomalous observation was likely due to the hydrophilic and low adsorptive nature of cellulose III, resulting in low Simon's Stain dye adsorption and therefore a seemingly low accessible surface area. Using a molecular dynamic technique, cellulose III has been shown to have increased the exposure of glucan chains to water and to allow easier extraction of glucan chains by enzymes (Gao et al., 2013). These two attributes of cellulose III allow for its efficient hydrolysis despite lower levels of enzyme adsorption (Gao et al., 2013; Gao et al., 2011). Unexpectedly, cellulose II showed higher conversion efficiency than PASC at 84% and 68% theoretical yield after 24 hours (Figure 21B) even though both PASC and cellulose II were shown to have similar accessibility (Figure 20B). The PASC seems to be more amorphous than cellulose II as determined using the CBM2a-to-CBM44 method (Figure 20B).Therefore, the increase in hydrolysis efficiency observed with cellulose II compared with PASC could not be explained solely based on substrate accessibility and the amorphous/crystalline nature of the two substrates. In contrast, a higher degree of enzyme adsorption with minimum desorption was observed on cellulose II than on PASC (Figure 18 and Figure 19). Arguably, given the same level of cellulose accessibility, a higher enzyme adsorption may be preferred as the prolonged enzyme adsorption may promote more efficient hydrolysis as observed with cellulose II and PASC. A fairly recent study has observed a similar high conversion efficiency with never dried cellulose II or cellulose II hydrate where almost 100% conversion was achieved within 12 hours (Wada, Ike and Tokuyasu, 2010). The authors argued that the high conversion efficiency of cellulose II hydrate compared to cellulose I could be ascribed to the following reasons. First, the authors determined the presence of two water molecules

intercalated between the molecular sheets of cellulose II hydrate, which caused cellulose II to have an inflated structure, and hence higher surface area, compared to cellulose I and dried cellulose II. Second, the structure of cellulose I was determined to consist of parallel chains forming hydrogen-bonded sheets that stack and are held together through hydrophobic van der Waals forces with no hydrogen bonding between the sheets (Nishiyama, Langan and Chanzy, 2002). In contrast, cellulose II consists of anti-parallel chains that are stacked and held together by both hydrophobic interaction and intermolecular hydrogen bonds (Langan, Nishiyama and Chanzy, 2001). The authors suggested that cellulose I would have a stronger hydrophobic interaction due to the smaller spacing between the cellulose sheets compared to cellulose II and that this hydrophobic interaction would act as a main factor to resist enzymatic hydrolysis, while hydrogen bonds could become unstable in water (Wada, Ike and Tokuyasu, 2010). Therefore, the authors argued that these weaker hydrophobic van der Waals forces in cellulose II compared to cellulose I would contribute to the enhanced hydrolysis efficiency of cellulose II (Wada, Ike and Tokuyasu, 2010).

The results described in this section of the thesis showed that understanding the influence of substrate properties, including cellulose properties, on enzyme adsorption profiles during hydrolysis can help us improve, not only enzyme recycling efficiency, but also hydrolysis efficiency.

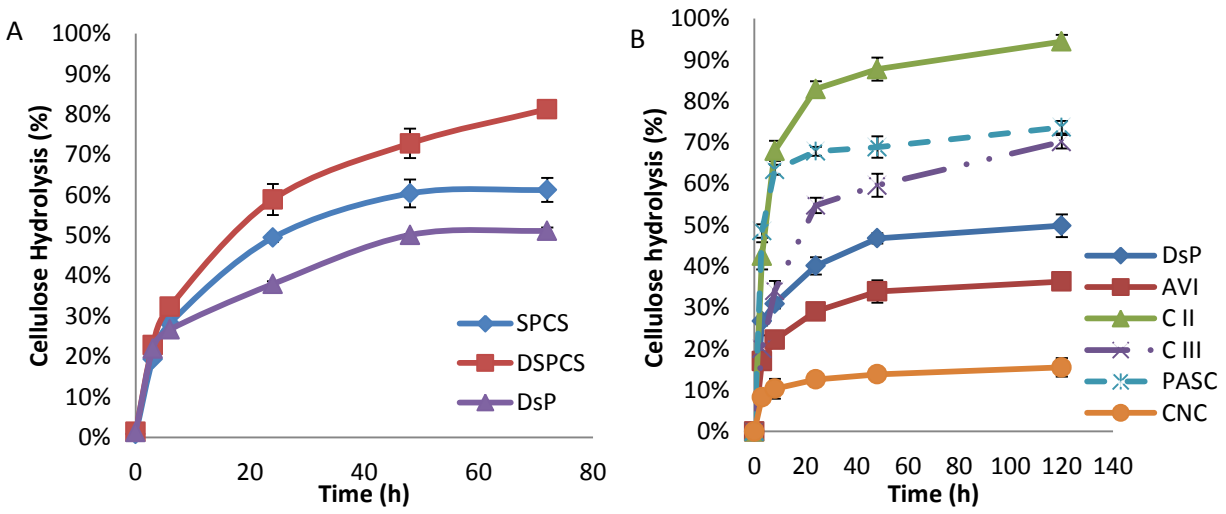

Figure 21. Cellulose hydrolysis of SPCS, DSPCS, and dissolving pulp (DsP) (A) and the six model cellulosic substrates DsP, Avicel (AVI), cellulose II (C II), cellulose III (C III), phosphoric acid swollen cellulose (PASC), and cellulose nanocrystals (CNC) (B). Hydrolysis of SPCS, DSPCS, and DsP was carried out using 15 mg/g cellulose of Cellic CTec 2 whereas hydrolysis of the 6 model cellulosic substrates was carried out using 13 mg/g cellulose of Celluclast and 5 mg/g cellulose of Novozym 188.

3.3.9 Conclusions

The work reported here showed that substrate characteristics influenced specific enzyme adsorption profiles and the efficiency of enzymatic hydrolysis. When comparing the SPCS and DSPCS substrates to determine the influence of lignin on enzyme adsorption, the removal of lignin did not significantly influence individual enzyme adsorption profiles with the exception of Cel6A which showed a faster binding to SPCS compared to DSPCS within the first half an hour of enzyme addition. Therefore, while partial delignification has previously been reported to improve enzyme recycling performance by reducing enzyme non-productive adsorption to lignin, the effect of lignin seems to be substrate-dependent. Comparing the hydrolysability of the substrates, lignin removal was shown to significantly increase the efficiency of cellulose hydrolysis in DSPCS compared to SPCS. The increase in cellulose hydrolysis efficiency was likely caused by an increase in substrate accessibility as determined using the

Simon's Stain method. Thus, while substrate accessibility was important for hydrolysis efficiency, this property did not seem to influence enzyme adsorption on the SPCS and DSPCS substrates.

In contrast, the nature of cellulose was shown to be an important factor in determining enzyme adsorption during hydrolysis and possibly hydrolysis efficiency. In particular, cellulose allomorphs and cellulose crystallinity seemed to influence the nature of enzyme-substrate interaction. When the three cellulose allomorphs were compared, the enzymes adsorbed the most to cellulose II and the least to cellulose III. The extent of cellulose crystallinity seemed to influence the nature of enzyme desorption during hydrolysis as an increase in the extent of enzyme desorption at a later stage of hydrolysis was observed with the more amorphous substrates such as PASC, DsP, and Avicel as compared to the highly crystalline substrate CNC. In contrast, cellulose accessibility alone did not seem to be a major factor in determining enzyme adsorption profiles during hydrolysis. For instance, while both the PASC and cellulose II were shown to have similar accessibility, an extensive enzyme desorption was observed during hydrolysis of PASC whereas a high degree of enzyme adsorption with little desorption was observed on cellulose II. Among the pure cellulosic substrates, cellulose II was the form of cellulose that was most efficiently hydrolyzed, followed by PASC, cellulose III, DsP, Avicel, and CNC. Although PASC and cellulose II were shown to have similar accessibilities, cellulose II was hydrolyzed more efficiently than PASC, indicating that factor(s) other than substrate accessibility may influence hydrolysis efficiency. As enzymes were observed to bind "more irreversibly" to cellulose II than to PASC, it would be interesting to further determine the possible influence of enzyme adsorption to hydrolysis efficiency.

With regards to enzyme recycling, these results confirm the importance of understanding the influence of substrate properties on enzyme adsorption. In addition to assessing the influence of lignin properties, cellulose properties also need to be considered when devising an enzyme recycling strategy. For example, enzyme recycling following hydrolysis of cellulose III substrates may need to emphasize

enzyme recovery from the liquid phase whereas following cellulose II hydrolysis, an efficient enzyme recovery from the solid phase will be important. Enzyme recycling after hydrolysis of a cellulose I substrate, on the other hand, will likely require enzyme recovery from both the liquid and the solid phases as enzymes have been observed to desorb during hydrolysis after an initial adsorption to cellulose I substrates. As cellulose I is the most common form of cellulose found in lignocellulosic biomass, even after pretreatments, enzyme recycling strategies that can recover enzymes from both phases will likely have to be developed. This part of the work showed that substrate properties greatly influence enzyme-substrate interaction and, possibly, cellulose hydrolysis efficiency.

However, as well as substrate properties the nature of the enzyme mixture used to hydrolyse the cellulosic component of the pretreated biomass substrate will also influence the enzyme recycle strategy that might be adopted. As mentioned earlier, as well as containing the more "traditional" cellulases in their commercial "cellulose deconstruction" enzyme cocktails the enzymes companies have shown how important it is to add so-called accessory enzyme such as xylanases and "oxidative" enzymes such as GH61/AA9 to the enzyme mixture. Thus in the next section of the thesis we wanted to determine the role that enzymes such as GH61/AA9 might play in developing an effective enzyme recycling strategy

3.4 Does Oxidative Cleavage of Cellulose by Auxiliary Activity Family 9 Enzymes Influence Enzyme Adsorption during Hydrolysis?

3.4.1 Background

Recently, several studies have shown that accessory enzymes and/or disrupting proteins, such as xylanases, lytic polysaccharide monooxygenases (PMOs, e.g. AA9), and swollenin, can considerably enhance the hydrolytic performance of cellulase enzymes on a range of lignocellulosic substrates (Harris et al., 2010; Zhou et al., 2011; Beeson et al., 2012). Among them, AA9 has attracted considerable attention in the past three years, since it has been shown to significantly boost the enzymatic hydrolysis of dilute-acid pretreated corn stover by cellulase enzymes (Harris et al., 2010; Zhou et al., 2011; Beeson et al., 2012). Over the last three years, several studies have tried to elucidate the mechanism by which the AA9 enzyme boosts hydrolysis (Phillips et al., 2011; Bey et al., 2013). Unlike the canonical cellulase enzymes, which cleave cellulose by a hydrolytic mechanism, AA9 appears to cleave the cellulose chains by an oxidative mechanism at its planar active site surface (Li et al., 2012; Quinlan et al., 2011; Beeson et al., 2012). The activation of AA9 enzyme has been reported to require a redox-active cofactor in the same way as cellobiodehydrogenase (CDH), needing either a synthetic small molecule reductants (eg. gallate or ascorbate), or still unidentified compounds in pretreated lignocellulosic biomass (Phillips et al., 2011; Bey et al., 2013; Langston et al., 2011; Cannella et al., 2012). The AA9 enzyme is thought to cleave the crystalline surface of cellulose and increase cellulose accessibility for the canonical cellulase enzymes (Horn et al., 2012). The oxidative cleavage of cellulose by the AA9 enzyme results in the production of soluble oxidized cellodextrins (aldonic acids) (Beeson et al., 2012a; Cannella et al., 2012). A recent study has shown that the aldonic acid products from the action of AA9 enzyme are stronger inhibitors of β-glucosidase activity than glucose (Cannella et al., 2012).

Despite this extensive body of work on the mechanisms of AA9 enzyme, not much research has been carried out to investigate the adsorption profile of AA9 and its influence on the adsorption behaviour of canonical cellulase enzymes. It is reasonable to expect that the oxidative action of the AA9 enzyme on the cellulose surface may create an oxidized chain end (Horn et al., 2012) that may influence enzyme adsorption behaviour. Due to its boosting effect on hydrolysis the AA9 enzyme is now included in the latest commercial "cellulase" enzyme mixtures (Novozymes, 2012). Therefore, understanding its adsorption and its influence on the adsorption profiles of other enzymes will be important in the development of enzyme recycling strategies to recover the enzymes after hydrolysis using today's commercial enzyme mixture(s).

We first investigated the adsorption profiles of the *Thermoascus auranticus* AA9 enzyme and the influence of its oxidative action on the adsorption behavior of canonical cellulase enzymes during hydrolysis of "model" pure cellulosic substrates and substrates derived from steam pretreated corn stover (SPCS). The AA9 enzyme was found to have minimum adsorption to the substrates and to remain primarily in the supernatant during hydrolysis. An increase in Cel7A desorption was observed during the course of hydrolysis when AA9 enzyme was present. The addition of AA9 to the enzyme cocktail also increased the negative charge of the hydrolysis residues as measured using an acid group quantification method. Thus, this increase in substrate negative charge may be a contributing factor to this increase in Cel7A desorption. The increase in desorption was observed even at a relatively low loadings of AA9 and further addition of AA9 did not have an increased effect. Increased desorption due to AA9 activity was observed with the processive Cel7A enzyme, but not with Cel7B.

3.4.2 The Adsorption Profile of AA9 Enzyme

Initially, the adsorption of AA9 was assessed on cellulosic substrates with different properties. The AA9 enzyme was shown to have minimal adsorption to cellulose III, moderate adsorption to Avicel,

DsP, CNC, and cellulose II and high adsorption to PASC (Figure 22). The low adsorption of AA9 onto the cellulosic substrates was expected as the AA9 has a flat active site (rather than a cleft or tunnel active site) and lacks a CBM (Horn et al., 2012). The presence of CBMs is thought to enhance enzyme adsorption to cellulose (Boraston, et al. 2004 ; Linder and Teeri 1997). While the flat binding site of CBM has been shown to promote binding to cellulose (Igarashi et al., 2011; Valjamae et al., 1998), its adsorption was reversible (Lehtio et al., 2003). For these reasons, the AA9 was expected to have insignificant or reversible adsorption to cellulose. Thus, the high adsorption of the AA9 enzyme on PASC was unexpected. The presence of high amount of accessible crystalline cellulose in PASC may explain the observed high level of adsorption of the AA9 on PASC.

In addition, the distribution of the AA9 enzyme during hydrolysis of SPCS and DSPCS was also determined using SDS-PAGE. This enzyme seemed to initially adsorb to the substrate followed by desorption as indicated by changes in band intensity (Figure 22B and C). The band intensity was significantly lower after 48 hours as compared to 24 hours. It was possible that, over the subsequent 24 hours, the enzyme was partially denatured or was adsorbed onto the solid substrate. Earlier work, described in Figure 4, showed that the decrease in the band intensity of some of the protein bands in the liquid phase corresponded to an increase in the band intensity of corresponding proteins desorbed from the solid phase. However, it was apparent in Figure 22B that AA9 did not adsorb significantly to the substrate or to SPCS lignin.

As mentioned earlier, a better understanding of the adsorption of AA9 during hydrolysis is important for enzyme recycling strategies. As the AA9 enzyme remained primarily in the supernatant with relatively pure cellulosic substrate, an effective enzyme recovery strategy from the liquid phase will be required to recycle this enzyme**.**

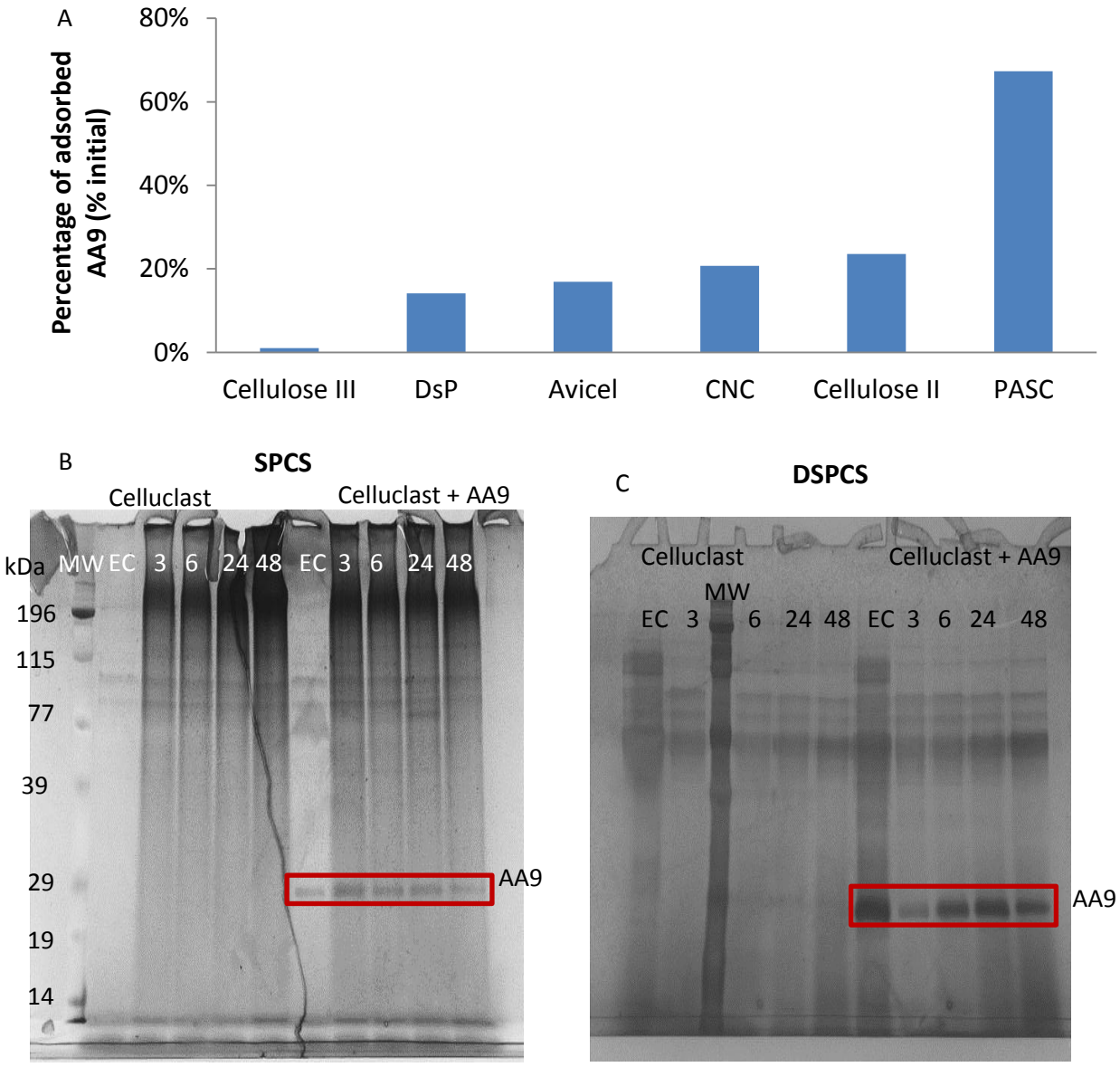

Figure 22. The adsorption of AA9 enzyme on various pure cellulosic substrates (A). Adsorption of AA9 enzyme during hydrolysis of SPCS (B) and DSPCS (C). EC: Enzyme control. The numbers indicate time points during hydrolysis.

3.4.3 The Influence of AA9 on Enzyme Adsorption Profiles

The influence of AA9 on the adsorption profiles of canonical cellulase enzymes was assessed during hydrolysis of six model cellulosic substrates. The properties of substrates used in this part of the thesis have been described previously in section 3.3. A slight increase in enzyme desorption was

observed, primarily on the semi-crystalline substrates Avicel and DsP at the early stages of hydrolysis (Figure 23). No apparent differences in enzyme adsorption/ desorption profiles were observed on the highly crystalline and amorphous cellulosic substrates (CNC, cellulose II, cellulose III, and PASC) (Figure 23). At the later stage of hydrolysis, desorption of enzymes from substrate hydrolysed without supplementation of the AA9 tended to catch up with the enzyme desorption observed in the presence of the AA9 enzyme.

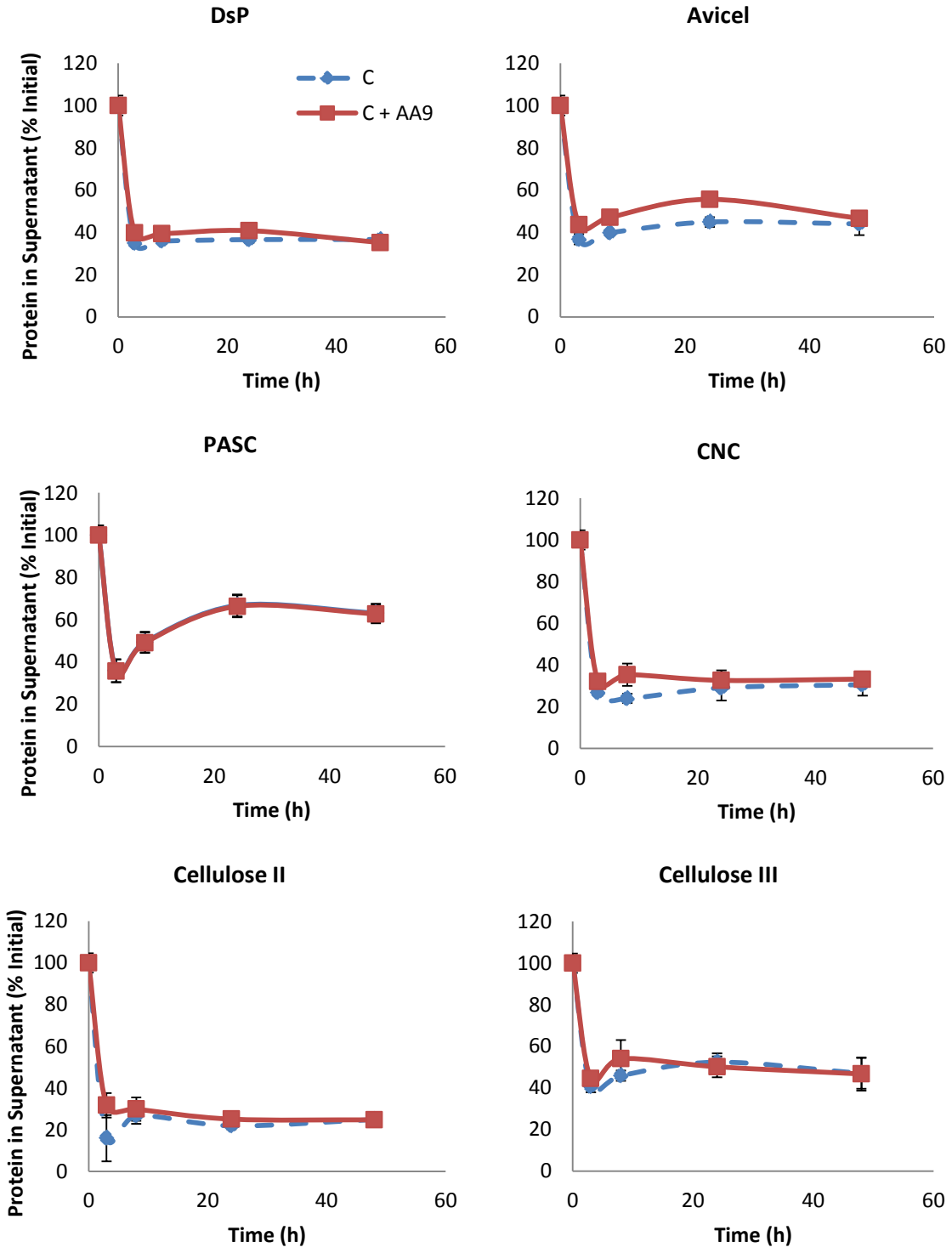

Figure 23. Protein adsorption profile during hydrolysis of pure cellulosic substrates using 13 mg/g glucan of Celluclast and 5 mg/g glucan of Novozym 188 with and without supplementation with 1 mg AA9/g glucan.

An increase in enzyme desorption was also observed when AA9 was added during hydrolysis of SPCS and DSPCS using Celluclast. A higher degree of enzyme desorption was observed during hydrolysis of DSPCS compared with SPCS (Figure 24A and B). After 48 hours of hydrolysis, about 61% of the initial protein loading was found in the hydrolysate of DSPCS with AA9 supplementation, compared with ~39% in the DSPCS hydrolysate without AA9 supplementation. A similar trend was observed when the activity of Cel7A in the supernatant during hydrolysis of SPCS and DSPCS was assessed using PNPC. An apparent higher desorption of Cel7A was observed with DSPCS compared with SPCS at 12% increase in Cel7A activity detected after 24 hours with DSPCS and 8% with SPCS (Figure 24C and D). More work is needed to determine if this difference was caused by the presence of lignin within the SPCS.

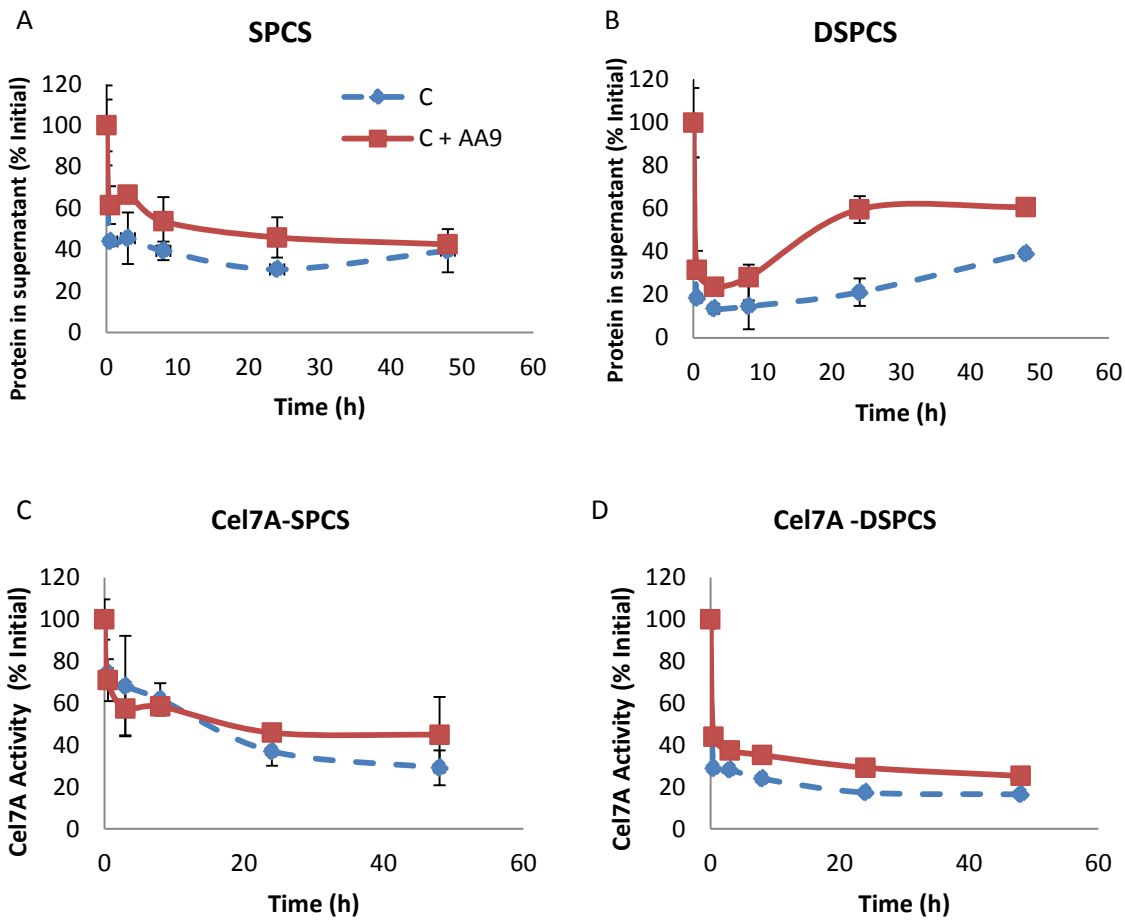

Figure 24. Protein adsorption profiles during hydrolysis of SPCS (A) and DSPCS (B) using 13 mg/g cellulose of Celluclast and 5 mg/g cellulose of Novozym 188 with or without supplementation with 1 mg AA9/g cellulose. Cel7A activity in the supernatant during hydrolysis of SPCS (C) and DSPCS (B) with or without supplementation with 1 mg AA9/g cellulose.

The results presented here provided an initial observation on the possible influence of substrate properties, especially cellulose properties, on the influence of AA9 on the adsorption profiles of canonical cellulase enzymes. As the release of cellulase enzymes by AA9 action was only observed on cellulosic substrates with semi-crystalline structure (e.g. DsP, Avicel and pretreated biomass), it was hypothesized that the AA9 may influence the adsorption/desorption profile of the processive cellulase Cel7A, which is has been shown to get stuck/jammed by the disorganized cellulose surface during processive hydrolysis (Igarashi et al., 2011; Valjamae et al., 1998). We tested 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 25B). As a control, AA9 showed no influence on the adsorption profile of the major endoglucanase Cel7B (Figure 25C).

One way by which the addition of 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 25A), which indicated an increase in negative charge of the cellulose. An increase in surface negative charge on lignocellulosic substrates, i.e. through a chemical post-treatment (sulfonation) of a pretreated biomass, has been shown previously to lead to reduction in enzyme adsorption (Del Rio, Chandra and Saddler, 2011). Thus, the action of the AA9 enzyme in releasing Cel7A, unproductively bound to cellulose, by altering the surface charge of cellulose or by removing other obstacles on the surface of cellulose may provide another mechanism by which the AA9 enzyme helps improve cellulose hydrolysis.

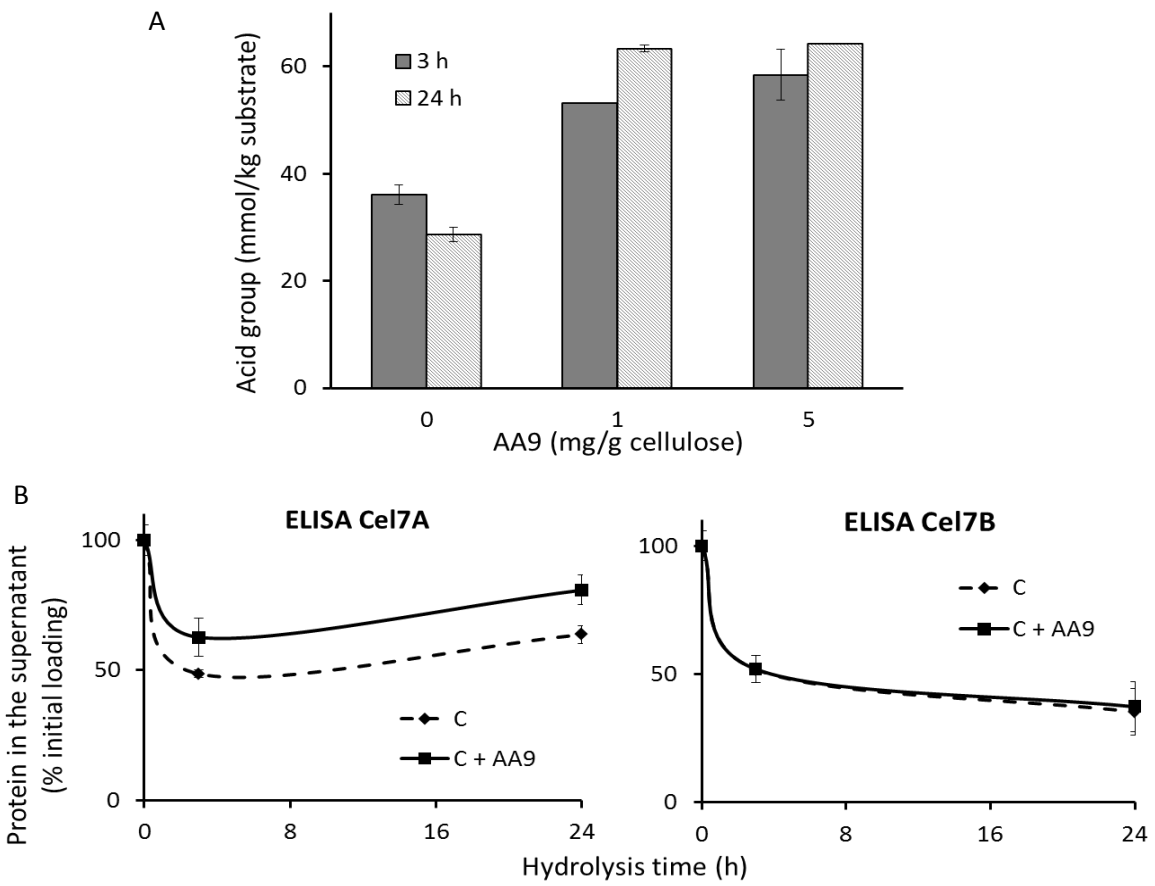

Figure 25. The influence of AA9 enzyme on the acid group concentration of the solid substrate after 3-hour and 24-hour hydrolysis of Avicel (A). The adsorption/ desorption profiles of Cel7A and Cel7B during hydrolysis of Avicel, with and without supplementation with 1 mg AA9/g glucan, as determined by a double antibody sandwich enzyme linked immunosorbent assay (ELISA) (B).

3.4.4 The Influence of the Loading of AA9 on Cellulase Enzyme Adsorption

We next determined the influence of the AA9 loading on the conversion and enzyme adsorption during Avicel hydrolysis. The AA9 loadings were varied and used to replace a corresponding amount of Celluclast enzyme to maintain a consistent enzyme loading. As expected, a ~10% increase in cellulose hydrolysis was observed with the replacement of 1 mg/g glucan of the Celluclast enzyme mixture with the AA9 enzyme. However, increasing the AA9 loadings above 1 mg/g of glucan, and hence decreasing corresponding amounts of the Celluclast enzyme mixture, led to a decrease in cellulose hydrolysis

(Figure 26A). Therefore, it could be concluded that only a small amount of the AA9 enzyme was needed to enhance cellulose hydrolysis. At the same enzyme loading, increasing the AA9 loading would lead to a decrease in cellulose hydrolysis, most likely due to the corresponding decrease in the loading of the canonical cellulase enzymes. An increase in enzyme desorption was also observed during hydrolysis of Avicel with AA9 addition, and changes in the AA9 loadings from 1 to 5 mg/g glucan did not seem to influence enzyme desorption (Figure 26B). This observation again indicates that only a small amount of the AA9 was needed for its effect on enzyme adsorption/ desorption during hydrolysis.

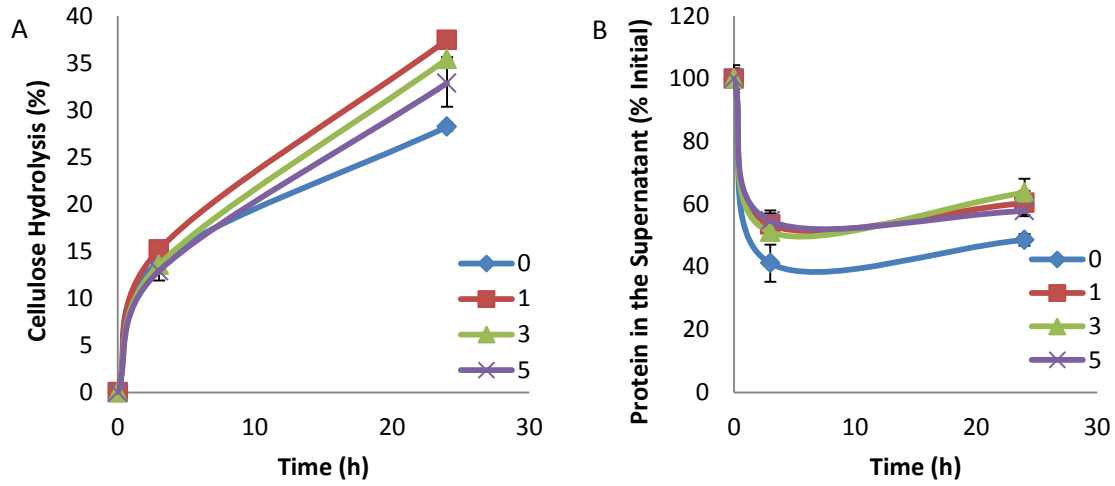

Figure 26. The effect of increasing loadings of AA9 enzyme on Avicel hydrolysis (A) and protein adsorption (B) at 2% (w/v) consistency. AA9 loadings: 0 (◊), 1 (■), 3 (▲), and 5 (x) mg/g glucan. The total protein loading (Celluclast, β-glucosidase, and AA9) was kept at 18mg/g glucan.

To determine if a similar boosting effect on cellulose hydrolysis and enzyme desorption could be obtained with lower AA9 enzyme loadings, the AA9 loadings were reduced to concentrations ranging from 0.1 to 1 mg/g glucan. After 24-hour hydrolysis, replacement of Celluclast with as little as 0.1 mg/g glucan of the AA9 was shown to boost cellulose hydrolysis by close to 10%. No additional enhancement in cellulose hydrolysis was observed with increasing loadings of the AA9 (Figure 27A). In contrast, increased enzyme desorption was observed with increased loadings of the AA9 enzyme up to a loading

of 0.7 mg/g glucan (Figure 27B). Thus, it was concluded that a small loading of the AA9 enzyme was able to enhance cellulose hydrolysis, while the enzyme's effect on enzyme desorption may be concentration dependent up to a certain AA9 enzyme loading.

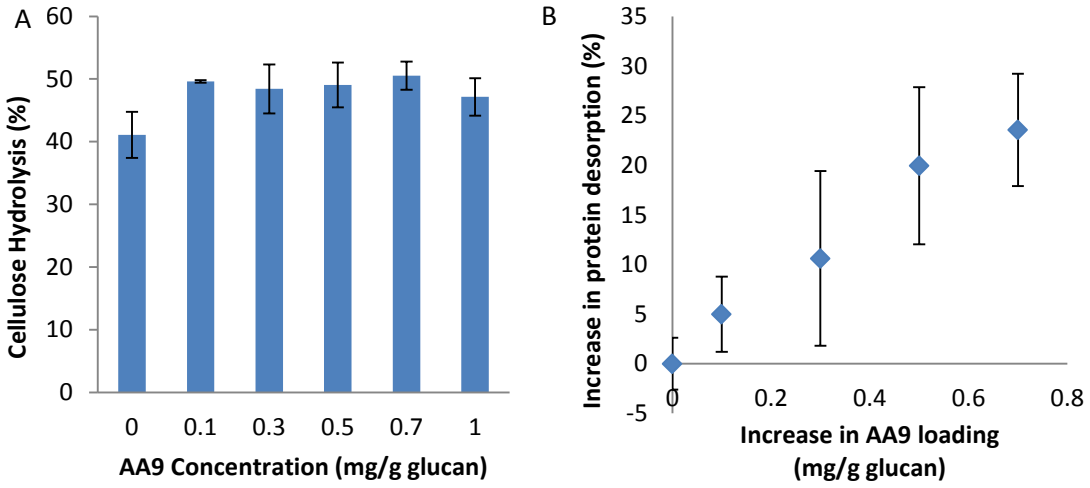

Figure 27. Increase in 24-hour cellulose hydrolysis yield (A) and protein desorption (B) during hydrolysis of Avicel with addition of small loadings of AA9 enzyme ranging from 0.1 to 1 mg/g glucan. Experiments were done in duplicates.

3.4.5 Boosting Effects of AA9 on Various Lignocellulosic Substrates

The increase in hydrolysis efficiency achieved by the addition of AA9 has been well established, and this increase in hydrolysis has been associated with oxidative cleavage of crystalline cellulose by the AA9, which opens up the substrate for other hydrolytic cellulase enzymes to hydrolyze (Harris et al., 2010; Horn et al., 2012). To determine if the observed increase in enzyme (Cel7A) desorption may indicate another potential synergistic interaction between the AA9 and Cel7A, namely by relieving Cel7A from unproductive adsorption to cellulose, hydrolysis was performed on SPCS, DSPCS, and the six pure cellulosic substrates, using Celluclast with or without supplementation of the AA9 enzyme. The chemical and physical characteristics of these substrates have been described in Table 4 and Figure 20.

The boosting effects of AA9 on conversion efficiency were shown to be highly substrate dependent. On the enriched cellulosic substrates, AA9 displayed a higher boosting effect on crystalline or semi-crystalline substrates, such as dissolving pulp, Avicel, and CNC, compared to swollen or amorphous cellulosic substrates, such as PASC and cellulose II. For example, the addition of AA9 increased CNC hydrolysis from 16% to 42% after 120 hours. The addition of AA9 to the dissolving pulp and Avicel had a slightly higher boosting effect on the dissolving pulp than the Avicel. The higher substrate accessibility of the dissolving pulp (Figure 20B) may contribute to this apparent higher boosting effect than on Avicel. No significant increase in cellulose hydrolysis was observed with the amorphous, highly accessible cellulosic substrates or cellulose III (Figure 28). With SPCS and DSPCS, the addition of AA9 boosted DSPCS hydrolysis the most, increasing the hydrolysis yield from ~56% to ~82%. The cellulose hydrolysis of SPCS was increased by ~10% (Figure 29).

As shown in Figure 23, an increase in enzyme desorption, especially at the early stage of hydrolysis (0 – 24 hours), was observed on the same substrates (dissolving pulp, Avicel and CNC) that exhibited a significant boosting in hydrolysis efficiency (Figure 28). Similarly, a higher increase in enzyme desorption was observed on DSPCS (Figure 24) which also showed a higher boosting effect upon the addition of AA9 (Figure 29) compared to SPCS.

Differences in substrate properties among the substrates evaluated in this study would definitely contribute to the observed differences in the boosting effects of AA9. We recently showed that the amount of accessible crystalline cellulose was a contributing factor to the boosting effect of the AA9 (manuscript in preparation), confirming the hypothesis that the main effect of AA9 addition to increasing cellulose hydrolysis was by opening up the crystalline regions of the cellulose to the canonical cellulase enzymes. Thus, this work has shown that the action of AA9 enzyme on crystalline cellulose led to both an increase in hydrolysis yield as well as in enzyme desorption. However, further work needs to

be done to determine if the increase in enzyme desorption would also contribute to the observed increase in hydrolysis efficiency.

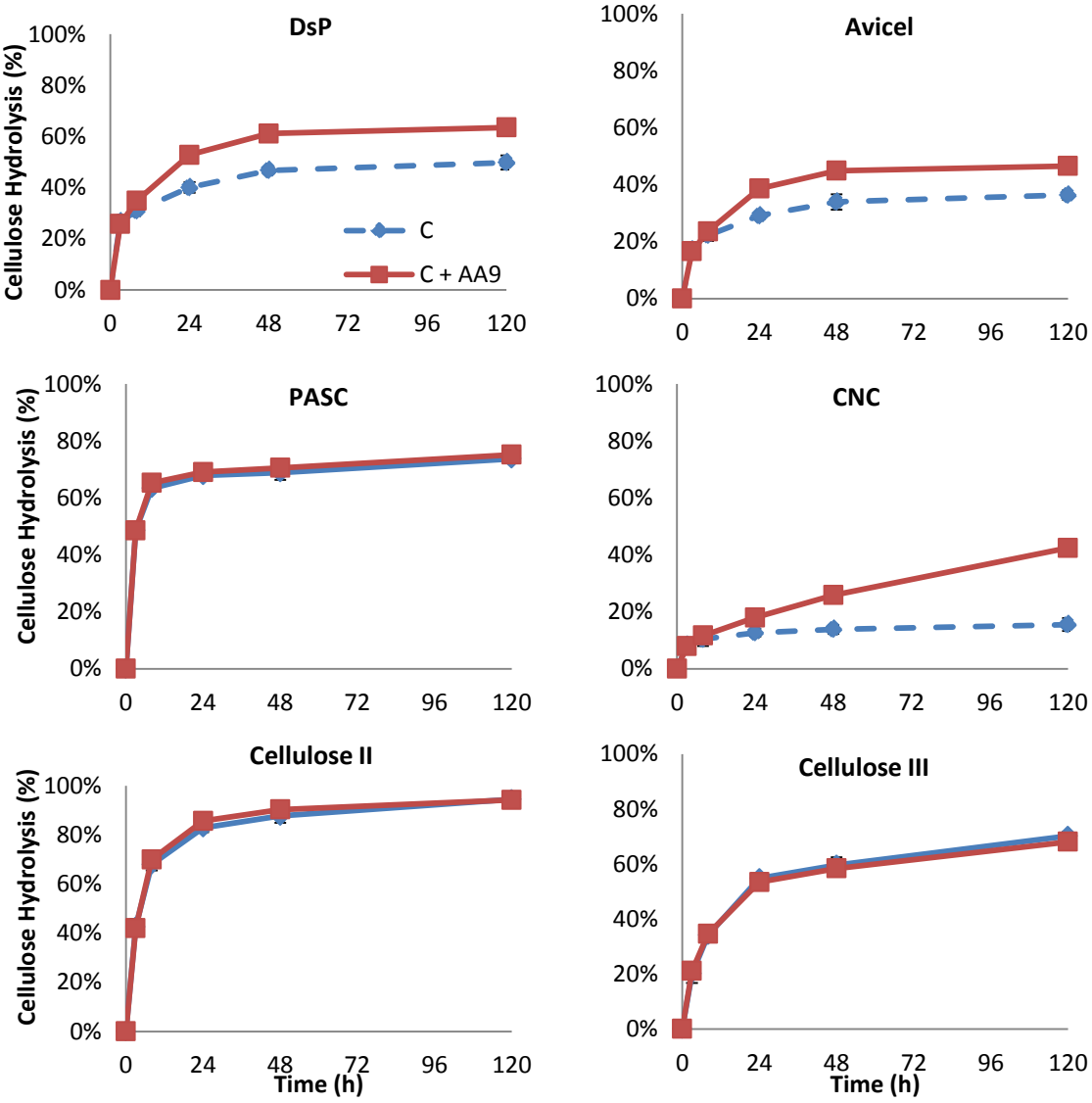

Figure 28. The boosting effect of AA9 on cellulose hydrolysis of pure cellulosic substrates: dissolving pulp (DsP), Avicel, phosphoric-acid swollen cellulose (PASC), cellulose nano crystal (CNC), cellulose II, and cellulose III. Cellulose II and PASC were derived from Avicel. The pure cellulosic substrates were hydrolyzed at 2% (w/v) consistency by Celluclast (13 or 12 mg/g glucan) and β-glucosidase (Novozyme 188 at 5 mg/g glucan), with or without supplementation of 1 mg of AA9/g glucan. The total protein loading was kept at 18 mg/g glucan.

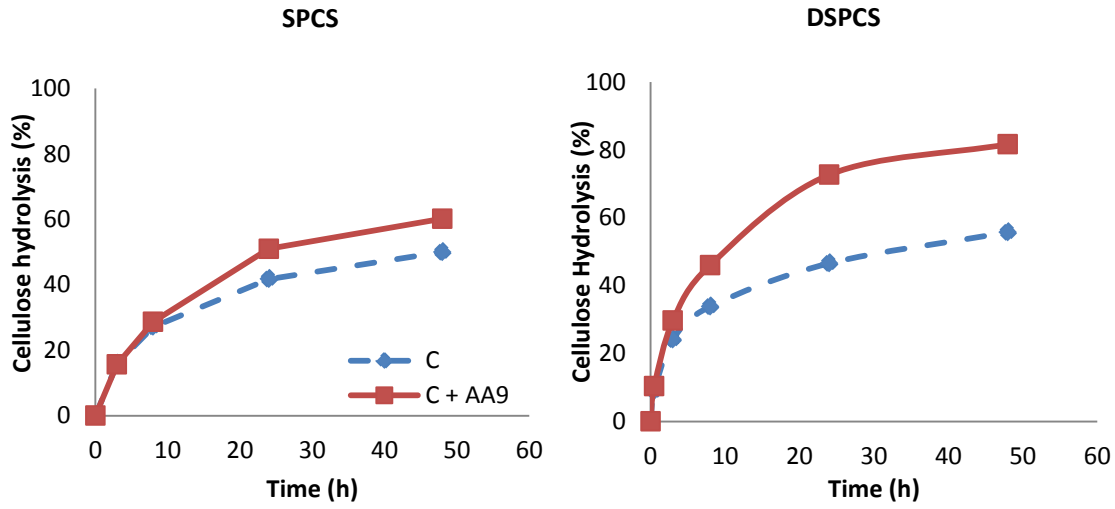

Figure 29. The boosting effect of AA9 on cellulose hydrolysis of steam-pretreated corn stover (SPCS), delignified SPCS (DSPCS), and xylanase-treated SPCS (XSPCS). The substrates were hydrolyzed at 2% (w/v) consistency with Celluclast (13 or 12 mg/g glucan) and β-glucosidase (Novozyme 188 at 5 mg/g glucan), with or without supplementation of 1 mg of AA9/g glucan. The total protein loading was kept at 18 mg/g glucan.

3.4.6 Conclusions

The *Thermoascus aurantiacus* derived AA9 enzyme was shown to not adsorb significantly to model cellulosic substrate or substrates derived from corn stover. We also helped better define the mode of AA9 action by demonstrating its influence on the adsorption profiles of canonical hydrolytic cellulase enzymes. The oxidative cleavage of crystalline cellulose by AA9 seemed to increase the negative charge of the substrate, likely contributing to the increased desorption of the processive enzyme Cel7A. However, the adsorption of Cel7B was shown to be unaffected. Additionally, we demonstrated how only a small amount of AA9 was needed to exert its influence on enzyme desorption. The increase in Cel7A desorption in the presence of AA9 may also indicate an additional mechanism by which the addition of AA9 helped boost enzymatic hydrolysis, namely by relieving unproductively adsorbed Cel7A. However, more work is needed to conclusively determine if the relieve of unproductively bound Cel7A would contribute to the boosting effect of AA9 addition.

With regards to enzyme recycling, the observed desorption of enzymes suggested that recovering enzymes from both the liquid and the solid phase, for example by using addition of fresh substrate, would be crucial to maximize recovery of these desorbed enzyme activities as well as to recovering the AA9 that remained primarily in the liquid phase. The low binding affinity of AA9 to the substrate may indicate poor enzyme recovery using addition of fresh substrate and, hence, the need to supplement subsequent rounds of hydrolysis with fresh AA9 enzyme.

In the last part of the thesis we then wanted to assess how all of the previous observations and results could be applied in a possible enzyme recycle scenario and how soluble components might influence enzyme adsorption and recycle.

3.5 High Performance Hydrolysis with Enzyme Recycling: The Role of Soluble Compounds and Mono-component Enzymes in Enzyme Adsorption and Recycling

3.5.1 Background

The efficiency of enzyme recycling strategies is highly influenced by the nature of the enzyme interaction with the substrates (Du et al., 2012; Zhu, Sathitsuksanoh and Zhang, 2009). In sections 3.3 and 3.4, we explored the influence of substrate and enzyme factors on enzyme-substrate interaction. It was apparent that the properties of cellulose in the lignocellulosic substrate significantly influenced enzyme adsorption profiles whereas lignin from corn stover was shown to have less of an effect on enzyme adsorption. In section 3.4, the addition of AA9 resulted in it remaining primarily in the liquid phase while its oxidative cleavage of cellulose increased the level of Cel7A desorption. In addition to enzyme and substrate factors, enzyme-substrate interaction during hydrolysis is also affected by the physical factors, such as temperature, pH, and concentrations of soluble compounds (Du et al., 2012; Kumar, Singh and Singh, 2008; Zhang and Lynd, 2004). Among these factors, the influence of soluble compounds, such as sugars and ethanol, on cellulase enzyme adsorption on insoluble lignocellulosic substrates has not been explored in detail.

Soluble compounds such as glycerol and sucrose have typically been used to prevent protein adsorption onto solid surface in fields as diverse as drug delivery, biofouling, and biocompatibility of implants (Evers et al., 2011). The soluble compounds present during bioconversion of lignocellulosic substrates, such as the released sugars or ethanol (in the case of simultaneous saccharification and fermentation or SSF) may likewise be expected to influence the adsorption behaviour of the cellulase enzymes. Selig *et al.* (2012) reported that increasing concentrations of glucose or xylose in a hydrolysis mixture caused a decrease in water activity, which was correlated to a decrease in conversion yields (Selig et al., 2012). Several studies have reported a decrease in cellulase adsorption with increasing

substrate loadings, and this decline in the binding capacity seemed to be associated with increasing concentrations of glucose and cellobiose in high-solid hydrolysis (Wang et al., 2011). Moreover, a recent study also showed that at 20% (w/v) solid consistency, intact enzymes achieved similar hydrolysis performance as enzymes lacking CBMs, suggesting a loss in enzyme binding capacity could play a role in the decrease in hydrolysis performance with increasing solid consistency (Varnai, Siika-aho and Viikari, 2013). Based on these observations, it seems that high concentrations of soluble compounds during hydrolysis may indeed reduce cellulose conversion by reducing enzyme adsorption.

Therefore, while the influence of sugars and ethanol on enzyme activities have been extensively investigated (Murphy et al., 2013; Holtzapple et al., 1990; Andric et al., 2010; Zhao et al., 2004), their influence on enzyme adsorption must not be overlooked as it seems to affect the efficiency of hydrolysis and possibly enzyme recycling. Moreover, as high concistency hydrolysis is increasingly desired to minimize downstream processing costs (Wang et al., 2011), an industrially-relevant enzyme recycling strategy will likely need to be done in the presence of high concentrations of these soluble compounds. The work described here seeks to evaluate the influence of soluble compounds, including glucose, xylose, and ethanol, on enzyme adsorption and enzyme recycling by readsorption to fresh substrate and, based on this understanding, to determine a potential strategy to improve the efficiency of this enzyme recycling strategy.

Enzyme recycling by addition of fresh substrate was chosen and evaluated in this study among the other enzyme recycling strategies due to several reasons. By taking advantage of the enzymes' natural affinity to the substrates, enzyme recycling by addition of fresh substrate has a number of advantages. This strategy is expected to be simpler and cheaper than others because it should not require any additional equipment, chemicals, or processing steps, such as a membrane for enzyme filtration or a solid carrier for enzyme immobilization. Moreover, by mixing the fresh substrate in the

hydrolysis mixture containing hydrolysates and residual solids, this strategy has the potential to recover enzymes from both the hydrolysates and the solid residues in a single recycling step. Results presented in previous sections have showed that recycling enzymes from both the solid and the liquid phase would be important to maximize enzyme recovery. In addition, as this strategy relies on enzyme natural readsorption to fresh substrate, any improvement in the efficiency of this strategy would depend on improved understanding of enzyme-substrate interaction.

To determine the influence of soluble compounds on enzyme readsorption to fresh substrate, we compared the efficiency of enzyme recovery following two hydrolysis strategies, an enzymatic hydrolysis (EH) and a hybrid hydrolysis and fermentation (HHF). These two hydrolysis strategies were chosen to compare the effect of glucose and ethanol on the efficiency of enzyme readsorption to fresh substrate. With EH, fresh substrate was added in the presence of high glucose concentrations following hydrolysis, whereas with HHF, fresh substrate was added in the presence of low glucose, but high ethanol concentrations following an SSF step. As some enzymes, such as Xyn 11, have been shown to quickly lose their activities after prolonged hydrolysis period (section 3.1), the HHF strategy, a combination of short-round hydrolysis (<24 hours) with SSF, was evaluated as a potential strategy to minimize the loss of enzyme activities.

We found that high glucose concentrations inhibited enzyme adsorption to the substrates more than did ethanol and xylose. Adding fresh substrate after SSF, which reduced glucose concentrations, resulted in both increased enzyme recovery and improved conversion in subsequent rounds of hydrolysis. Results in previous sections showed that certain enzyme activities were lost during hydrolysis and enzymes such as AA9 did not significantly bind to the substrate. Therefore, enzyme supplementation was evaluated as a strategy to further improve enzyme recycling performance. Overall, the results show that, to maximize the efficiency of enzyme recycling through addition of fresh

substrate, fresh substrate should be added after a sugar removal step, such as fermentation, to minimize the inhibitory effect of glucose on enzyme readsorption and an optimized ratio of key enzymes need to be supplemented to maintain high hydrolysis efficiency. Using this strategy, a consistently high hydrolysis performance was achieved for five recycling rounds with 50% reduction in enzyme loading.

3.5.2 The Influence of Sugars and Ethanol on Cellulase Enzyme Adsorption

The presence of soluble compounds such as surfactants, sugars, and alcohols is known to influence protein adsorption on a solid surface, including cellulase enzyme adsorption to lignocellulosic substrates (Kristensen, Felby and Jorgensen, 2009; Wendorf, Radke and Blanch, 2004; Eriksson, Borjesson and Tjerneld, 2002; Evers et al., 2011). Glucose and cellobiose, in particular, have been proposed to reduce enzyme adsorption to lignocellulosic substrates during high solid hydrolysis (Kristensen, Felby and Jorgensen, 2009). Xylose and ethanol are two other major soluble compounds that are typically present in the bioconversion of biomass to ethanol. While their effects on enzyme activities have been reported in many studies (Skovgaard and Jorgensen, 2013; Qing, Yang and Wyman, 2010; Podkaminer et al., 2012), little is known about their influence on cellulase enzyme adsorption, enzyme recycling, and hydrolysis performance.

In the work reported here, the adsorption of a commercial cellulase enzyme mixture (Celluclast and Novozym 188) to DsP, a model cellulosic substrate, was evaluated across a range of glucose, xylose, and ethanol concentrations. Control samples containing corresponding sugar or ethanol concentrations, but without proteins, were included to account for possible interference from the sugars and ethanol on the ninhydrin assay. The measured protein concentrations were then normalized to these control samples.

Glucose was shown to reduce enzyme adsorption at concentrations above 100 g/L. At a glucose concentration of 250 g/L, which could be found during high solids enzymatic hydrolysis, enzyme

adsorption was reduced by about 50%, compared with a sample with no added glucose (Figure 30A). Hydrolysis at high substrate loadings of around 20% (w/v) typically achieves final glucose concentrations in the range between 150 – 175 g/L (Zhang et al., 2009; Zhao et al., 2013; Yang et al., 2010). One study has reported a final glucose concentration of close to 250 g/L after hydrolysis using 30% (w/v) initial solid loading (Zhao et al., 2013). Therefore, enzyme recycling after a high solid loading hydrolysis could be expected to suffer from the inhibition effect of high glucose concentrations on enzyme adsorption.

Xylose, on the other hand, did not seem to significantly reduce enzyme adsorption, even at a very high concentration of 250 g/L (Figure 30A). Similarly, the level of enzyme adsorption remained constant at ethanol concentrations from 0-20% (v/v) (Figure 30B). Industrially relevant hydrolysis reactions are not expected to have this high concentration of xylose or ethanol. Therefore, xylose and ethanol were not expected to significantly influence enzyme adsorption and enzyme recycling. While glucose and xylose were found to significantly interfere with the ninhydrin assay, this interference could be accounted for by normalizing the absorbance of the protein samples to the absorbance of the sugar control samples. Therefore, while keeping in mind the limitation of this protein assay, it seems that glucose, and to a certain extent xylose, seemed to inhibit enzyme adsorption at concentrations above 100 g/L. Ethanol, on the other hand, seemed to have a minimum effect on enzyme adsorption, even at high concentrations.

While high glucose concentrations have been proposed to reduce enzyme binding capacity (Kristensen, Felby and Jorgensen, 2009), the mechanism behind this decrease in adsorption remains unclear. Previous studies on protein adsorption to solid surfaces may provide insights into possible mechanisms behind this decrease in enzyme adsorption. In the presence of non-ionic co-solvents such as glycerol and sucrose, a decrease in protein adsorption to both hydrophobic and hydrophilic solid surfaces has been observed (Wendorf, Radke and Blanch, 2004; Evers et al., 2011). These studies suggest that as these non-ionic co-solvents tend to be excluded from the protein surface, they are

thought to induce a preferential hydration of the protein, favoring the protein's native folded conformation to reduce the protein surface area. As protein adsorption usually includes an "opening up" of the protein conformation on the solid surface (Wendorf, Radke and Blanch, 2004), this protein hydration and the resulting shift in equilibrium towards the native folded state has been proposed as the mechanism behind this reduction in protein adsorption. These studies further argue that a reduction in protein adsorption is related to the molecular volume of the non-ionic co-solvents. Co-solvents with bigger molecular volumes and/or higher numbers of hydroxyl groups are thought to induce a greater reduction in protein adsorption (Wendorf, Radke and Blanch, 2004; Evers et al., 2011).

Glucose, xylose, and ethanol are expected to have different molecular sizes. To determine if the molecular size of non-ionic co-solvents might influence cellulase adsorption to (ligno)cellulosic substrate, cellulase adsorption to DsP was quantified in the presence of different concentrations of ethylene glycol with varying molecular sizes (ethlyne glycol (EG), polyethylene glycol (PEG) 200, and PEG 600). At concentrations up to 100 mmol/L (which corresponded to concentrations of 6.2, 20, and 60 g/L for EG, PEG 200, and PEG 600, respectively), no significant reduction in cellulase adsorption to DsP was observed for all of these non-ionic co-solvents. The level of cellulase adsorption was similar regardless of the molecular size of the co-solvents (Figure 30C), suggesting that the molecular size of non-ionic co-solvents may not play a crucial role in the reduction of cellulase adsorption to cellulose. These studies on protein adsorption on solid surfaces may offer insights to the possible mechanisms behind the reduction in cellulase enzyme adsorption in the presence of the high glucose concentrations observed in this study. However, the adsorption behaviours of cellulase enzymes to lignocellulosic substrates may be different from the adsorption behaviours of regular proteins to a solid surface as the presence of CBMs on some of these cellulase enzymes and the characteristics of the solid substrates may influence the nature of adsorption inhibition by these non-ionic compounds.

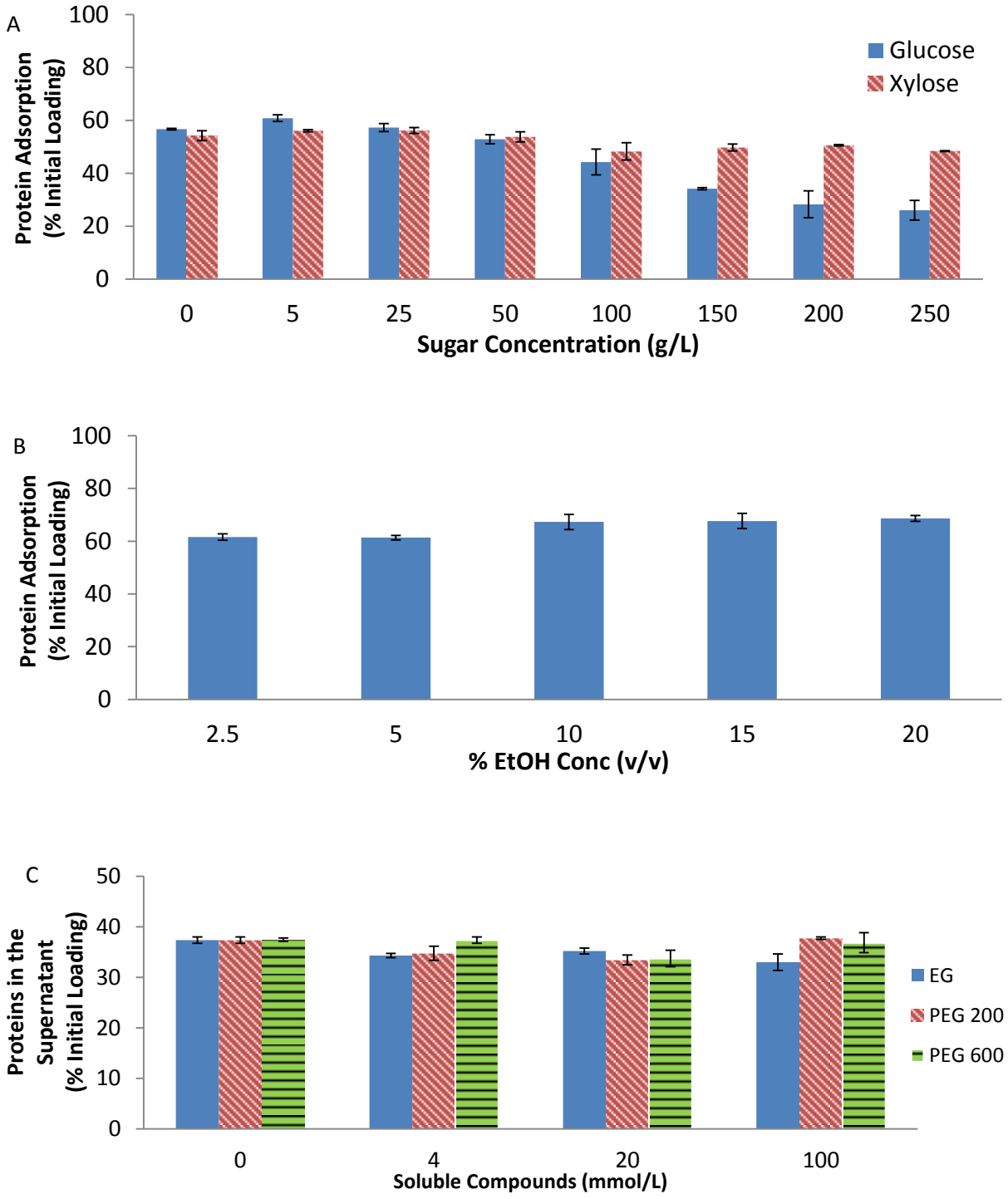

Figure 30. The effect of sugars (A) and ethanol (B) concentrations on enzyme adsorption at 50 mg/g glucan enzyme loading to DsP at 2% solid loading for 2 hours at 4°C. The influence of different concentrations of soluble compounds with varying molecular sizes on cellulase adsorption to DsP as measured using the ninhydrin assay. The soluble compounds used were ethylene glycol (EG), polyethylene glycol (PEG) 200, and PEG 600 (C).

Another possible mechanism for the reduction in cellulase adsorption at high sugar concentrations was formation of a layer of the glucose and xylose on the surface of the solid substrate. The decrease in water activity (or availability) at high sugar concentrations may indicate the displacement of water molecules from around the solid substrate by these sugar molecules (Selig et al., 2012). The presence of this sugar layer may prevent the hydrophobic interactions that are required for enzyme adsorption to cellulose. However, further work needs to be done to evaluate this hypothesis.

To determine if the reduced enzyme adsorption may be driven by inhibition of CBM adsorption at high sugar concentrations, the adsorption of CBM2a was evaluated in the presence of high concentrations of glucose, xylose, and ethanol. Glucose and xylose but not ethanol were shown to reduce adsorption of CBM2a (Figure 31A), suggesting that the inhibition in enzyme adsorption by high sugar concentrations may be driven by inhibition of CBM adsorption to cellulose. To further evaluate the influence of high sugar concentrations on the adsorption of other enzyme domains, the adsorption of CBM2a, Cel7A CD, and intact Cel7A to DsP was evaluated in the presence of glucose. A reduction in the adsorption of all enzyme domains was also observed at high glucose concentrations (Figure 31B). At glucose concentration of 250 g/L, adsorption of intact Cel7A was reduced from 37% to ~20% whereas CBM2a adsorption was reduced from 34% to 22%. About 7% of the initial Cel7A CD loading was adsorbed to DsP in the absence of glucose and no adsorption was observed at concentration of 250 g/L. Both the adsorption of CBM and CD of Cel7A on cellulose has been shown to be driven by a hydrophobic interaction between the enzyme domains and cellulose (Igarashi et al., 2009). Therefore, it seems that high sugar concentrations may prevent the hydrophobic interaction that drives the adsorption of enzymes to cellulose.

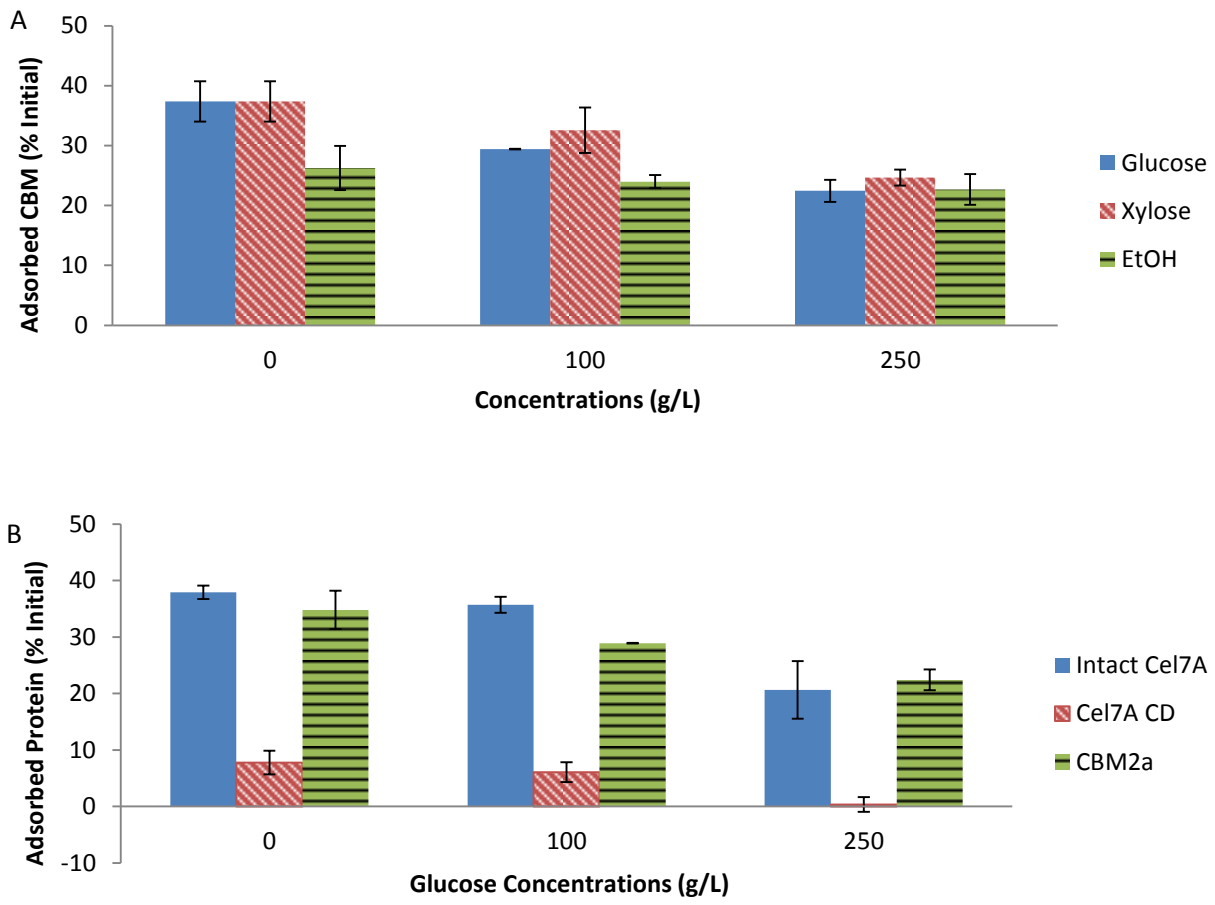

Figure 31. The effects of different concentrations of glucose, xylose, and ethanol (EtOH) on the adsorption of CBM2a on DsP (A) and the influence of different concentrations of glucose on the adsorption of intact Cel7A, Cel7A catalytic domain (CD), and CBM2a on DsP (B) as measured using the UV absorbance (A_{280})

Understanding the influence of sugars and ethanol on enzyme adsorption is important for both enzyme recycling and enzymatic hydrolysis. Their effects on enzyme adsorption is crucial for developing efficient enzyme recycling strategies, especially for those that depend on enzyme adsorption such as enzyme recycling through addition of fresh substrates. While the presence of high glucose concentrations may promote protein desorption back to the liquid phase and protein recovery for the next round of hydrolysis, the recovery of these proteins through re-adsorption to fresh substrate may likewise be limited in the presence of high glucose concentrations. Therefore, this particular enzyme

recycle strategy may benefit from minimizing glucose concentrations and/or converting it to a less inhibiting co-solvent such as ethanol.

The inhibition of enzyme adsorption by high sugar concentrations may also be crucial for our efforts to understand and improve hydrolysis efficiency. Desorption of enzymes from the solid substrate at the later stage of hydrolysis has been commonly reported in a number of studies and attributed to the decreasing availability of adsorption sites in the residual solid substrates (Tu et al., 2009; Berlin et al., 2005). Based on the observations reported here, high concentrations of glucose in the later stage of hydrolysis could also be expected to contribute to enzyme desorption. Aside from causing product inhibition, reduced enzyme adsorption caused by high glucose concentrations may also contribute to the decrease in hydrolysis efficiency. In a previous study, high glucose concentrations have been shown to contribute to the slowdown in hydrolysis rate at the later stage of hydrolysis or to the reduced conversion during high solids hydrolysis (Wang et al., 2011). To determine the importance of enzyme adsorption on hydrolysis efficiency, different ratios of intact Cel7A and Cel7A CD at a total protein loading of 50 mg/g glucan were used to hydrolyze Avicel. Glucose production was quantified after 72 hours hydrolysis. A gradual decrease in glucose production was observed when intact Cel7A was substituted with increasing amounts of Cel7A CD. About 85% reduction in glucose production was observed when 100% Cel7A CD was used compared to 100% intact Cel7A (Figure 32A). As a control, the activity of the different ratios of intact Cel7A/Cel7A CD was measured on PNPC, which showed a much less dramatic reduction in enzyme activity (about 15% reduction in activity with 100% Cel7A CD compared to 100% intact Cel7A, Figure 32A). Interestingly, the level of enzyme adsorption remained relatively stable when Cel7A proportion was more than 40% of the total enzyme loading. When the Cel7A proportion was less than 40% of total enzyme loading, no adsorption of enzyme was observed (Figure 32B). About a 50% reduction in Cel7A adsorption was observed at glucose concentrations of 250 g/L (Figure 31A). In contrast, Cel7A activity on PNPC only decreased by about 33% at glucose

concentration of 250 g/L (Figure 32C). Together, these results suggest that the reduction in enzyme adsorption to lignocellulosic substrate may significantly influence hydrolysis efficiency.

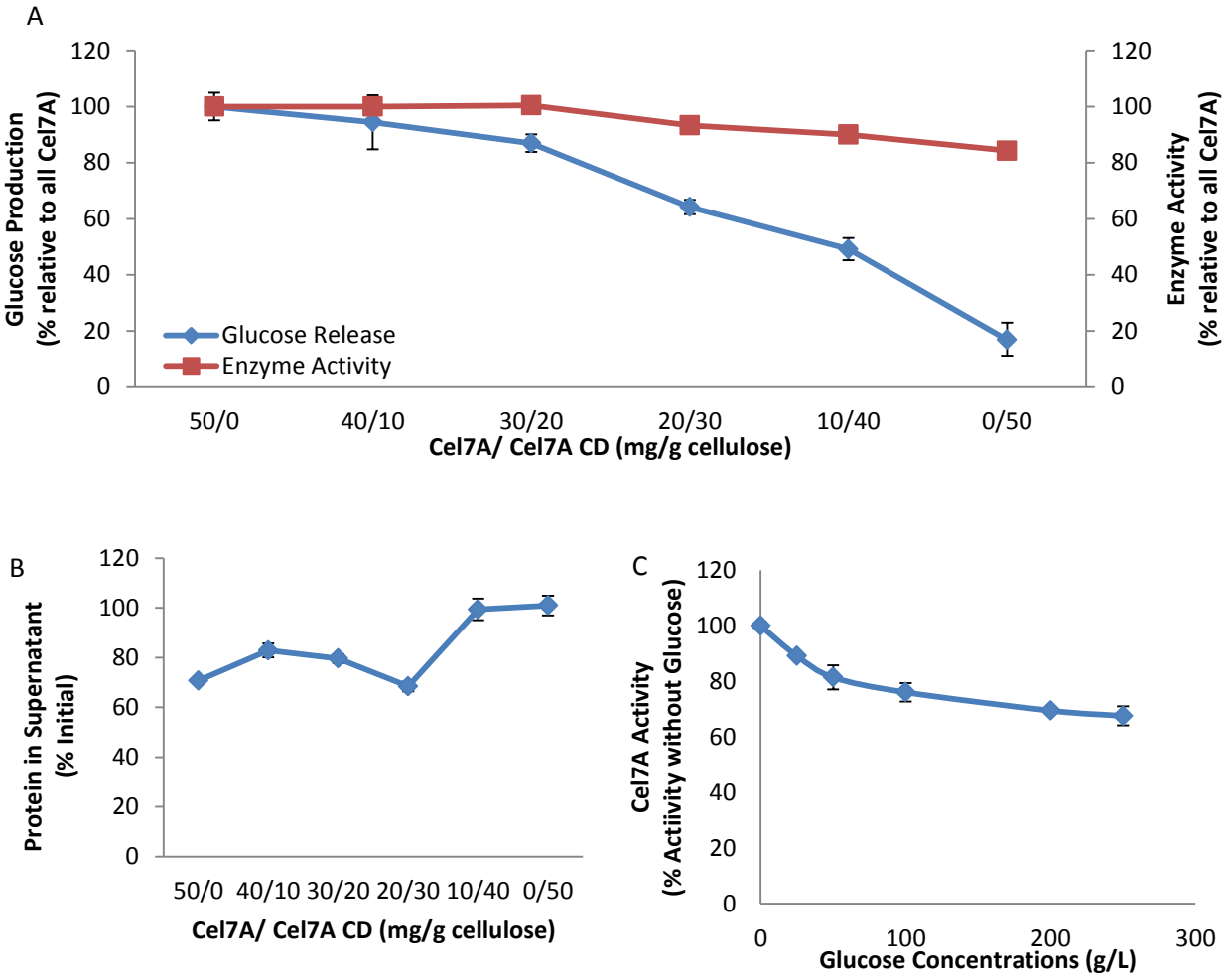

Figure 32. Reduction in hydrolysis efficiency of Avicel by Cel7A due to loss of enzyme adsorption capacity by substituting intact Cel7A with increasing proportion of Cel7A catalytic domain (Cel7A CD) (A). Protein adsorption to Avicel at different ratios of intact Cel7A and Cel7A CD (B). Cel7A activity as measured using PNPC at increasing glucose concentrations (C).

3.5.3 Comparison of Enzyme Recycling Strategies through the Addition of Fresh Substrates

In most of the previous enzyme recycling studies, the protein recovery step has been carried out after enzymatic hydrolysis (Tu et al., 2009; Weiss et al., 2013; Jin et al., 2012). However, given the higher

inhibitory effects of glucose on enzyme adsorption compared to ethanol (as shown in Figure 30A and B), enzyme recycling by addition of fresh substrate may be more efficient when done after fermentation compared to after hydrolysis. Glucose is also a more powerful inhibitor of enzyme activities compared to ethanol (Holtzapple et al., 1990). Therefore, to minimize the inhibition by glucose carried over to the next round of hydrolysis, a washing step was shown to be necessary to improve hydrolysis performance after enzyme recycling (Xue, Jameel and Park, 2012). By recovering the enzymes after fermentation rather than after hydrolysis, the glucose inhibition on enzyme activity was minimized and the efficiency of hydrolysis with enzyme recycling could potentially be improved without any additional washing steps.

Because fermenting glucose to ethanol would require the addition of yeast cells, we first assessed if the addition of yeast cells would affect protein adsorption by monitoring the concentration of proteins in the supernatant before and after addition of yeast. After taking into account the amount of proteins that may come from the yeast cells, the addition of yeast did not seem to influence the adsorption of the proteins in the reaction (data not shown).

We next compared two hydrolysis enzyme recycle strategies of either hybrid hydrolysis and fermentation (HHF) or straight enzymatic hydrolysis (EH) (Figure 33). For the HHF approach the substrate was prehydrolyzed for 24 hours at 50^{o}C and pH 4.8 and subsequently subjected to SSF for another 48 h at 32^{o}C and pH 4.8. For the EH approach, the substrate was hydrolyzed for 72 hours at 50^{o}C. Following either the HHF or EH approach, fresh substrate was added, and the reaction mixture was incubated at 4^{o}C for 2 hours to recover the enzymes in the liquid phase. Following the enzyme re-adsorption step, the liquid fraction was separated from the solid fraction and it was anticipated to contain the enzymes adsorbed to the residual substrate and the fresh substrate. Fresh buffer was then added to the solid fraction and the subsequent round of hydrolysis was initiated.

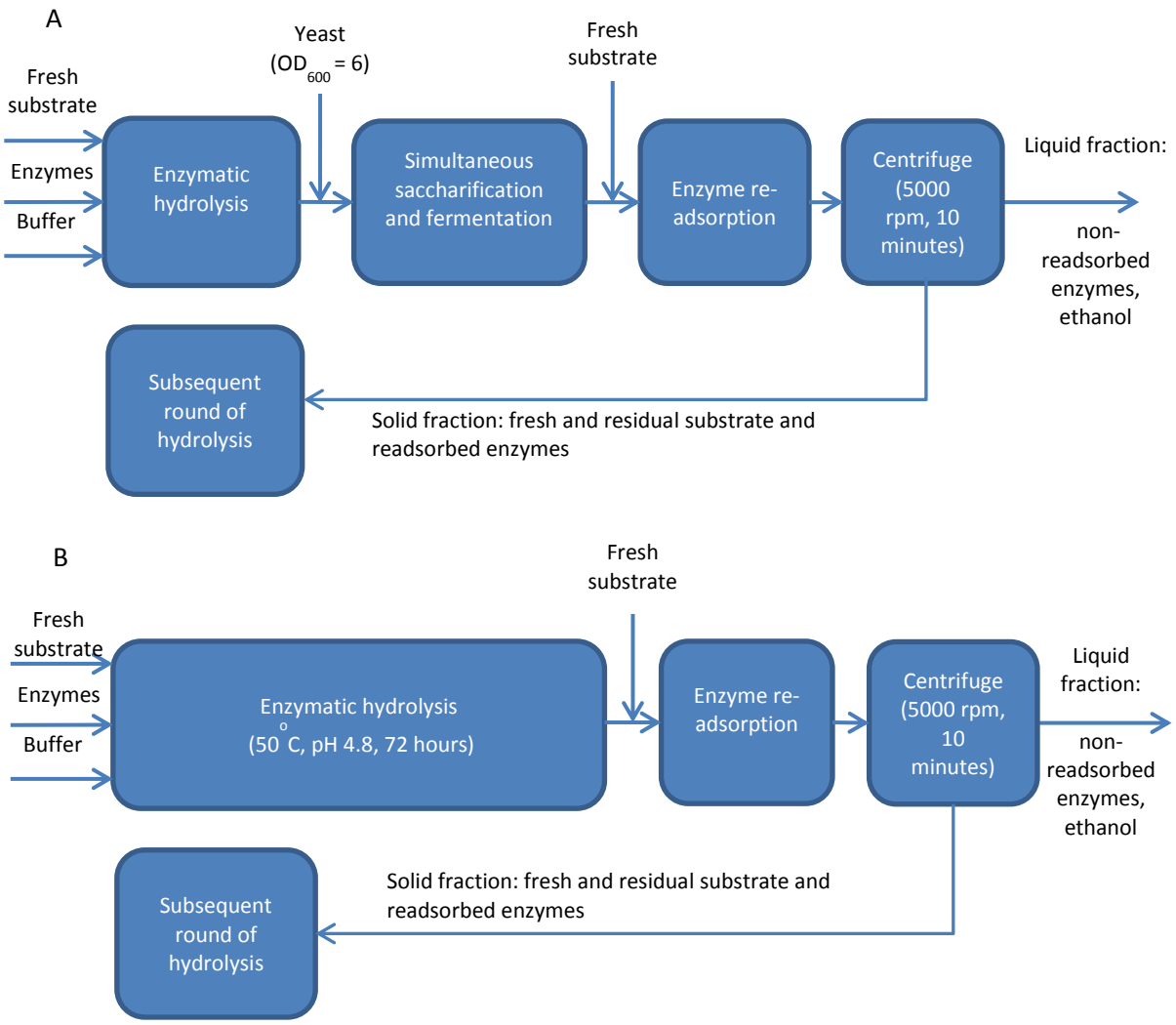

Figure 33. Schematic diagrams of the hybrid hydrolysis and fermentation (HHF) (A) and straight enzymatic hydrolysis (EH)(B)strategies with enzyme recycling through addition of fresh substrate.

Comparing the first round of 72-hour hydrolysis between the EH and HHF strategies, the HHF approach resulted in an about 10% higher glucan conversion compared to the EH approach (Figure 34A). This higher conversion with HHF was likely due to reduced product inhibition by glucose and reduced thermal denaturation of enzymes, due to lower temperature used with the HHF approach as compared

to the EH approach. By doing hydrolysis at 50°C in the first 24 hours, the HHF strategy was also expected to be superior compared to an SSF-only strategy in achieving fast initial conversion.

Following the first round of HHF and EH, fresh substrates were added to recover the enzymes. Two approaches to enzyme recycling were initially compared: the first, recovering proteins from the liquid fraction only, and the second, recovering from both the liquid fraction and the solid residue. Comparing cellulose hydrolysis after 24 hours of second round hydrolysis, recovering proteins from the liquid fraction only versus both the liquid fraction and solid residues resulted in comparable cellulose conversions (data not shown). Therefore, in subsequent experiments, fresh substrates were directly added to the hydrolysis mixture containing both the liquid fraction and the solid residues. Comparing cellulose hydrolysis after 24 hours using recycled enzymes, enzyme recovery after the HHF strategy improved cellulose conversion by 30% compared to recovering enzymes after the EH alone step (Figure 34B).

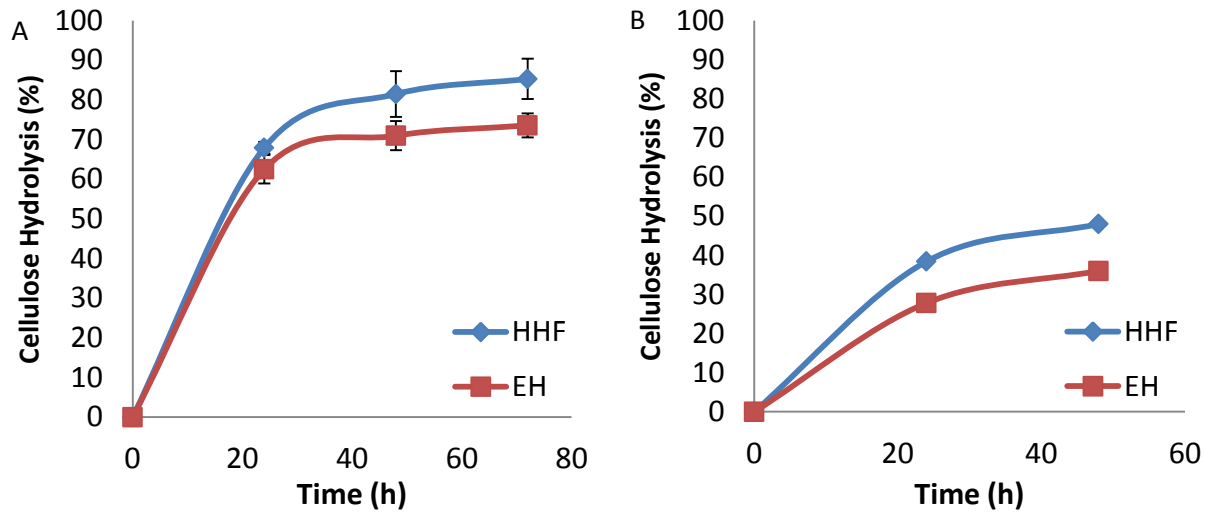

Figure 34. Cellulose hydrolysis of dissolving pulp (DsP) during 72 hours of the first round of hybrid hydrolysis and fermentation (HHF) and enzymatic hydrolysis (EH) using 21 mg/g glucan of Celluclast and 4 mg/g glucan of Novozyme 188 (A). Performance of recycled enzymes during the second-round of 48 hours of hydrolysis at 50°C following addition of fresh substrates to HHF and EH samples (B).

3.5.4 Enzyme-Substrate Interaction during Hybrid Hydrolysis and Fermentation (HHF) and Straight Enzymatic Hydrolysis (EH)

To determine if increased protein recovery after HHF compared to after EH may be the possible reason behind the increased conversion after the second round of hydrolysis, we compared the enzyme-substrate dynamics between the two strategies. After the first round, only ~60% of the initial protein loading was present in the supernatant after the HHF approach as compared to ~80% after the EH approach (Figure 35A). We expected this apparently lower protein concentration after HHF to be an experimental artifact due to normalization of the protein concentrations in the yeast control (YC) samples (samples containing a corresponding amount of yeast but with no enzymes). The concentration of yeast proteins in the YC sample was anticipated to be higher compared to that of the HHF samples due to the likely higher yeast mortality caused by a lack of nutrients and sugars in the control sample. As a result, the protein concentration in the HHF sample was likely to be underestimated after normalization to the yeast control sample.

To obtain better insights into the enzyme adsorption profiles and to minimize interference from yeast proteins, the specific enzyme activities during EH and HHF were compared. The adsorption profiles of Cel7A as determined from its activity on PNPC showed that the HHF approach resulted in a higher Cel7A activity in the supernatant (~84% initial activity) at 72 hours as compared to ~48% after EH (Figure 35B). A similar trend was observed for β-glucosidase activity where almost all of the initial β-glucosidase activity was detected after 72 hours of HHF compared to only around ~82% initial activity after the EH approach (Figure 35C). A slightly different adsorption profile was observed for xylanase activity as determined using PNPX. Xylanase activity quickly dropped after the first 24 hours and remained relatively constant at ~66% and ~54% throughout the 72-hour period of HHF and EH, respectively (Figure 35D).

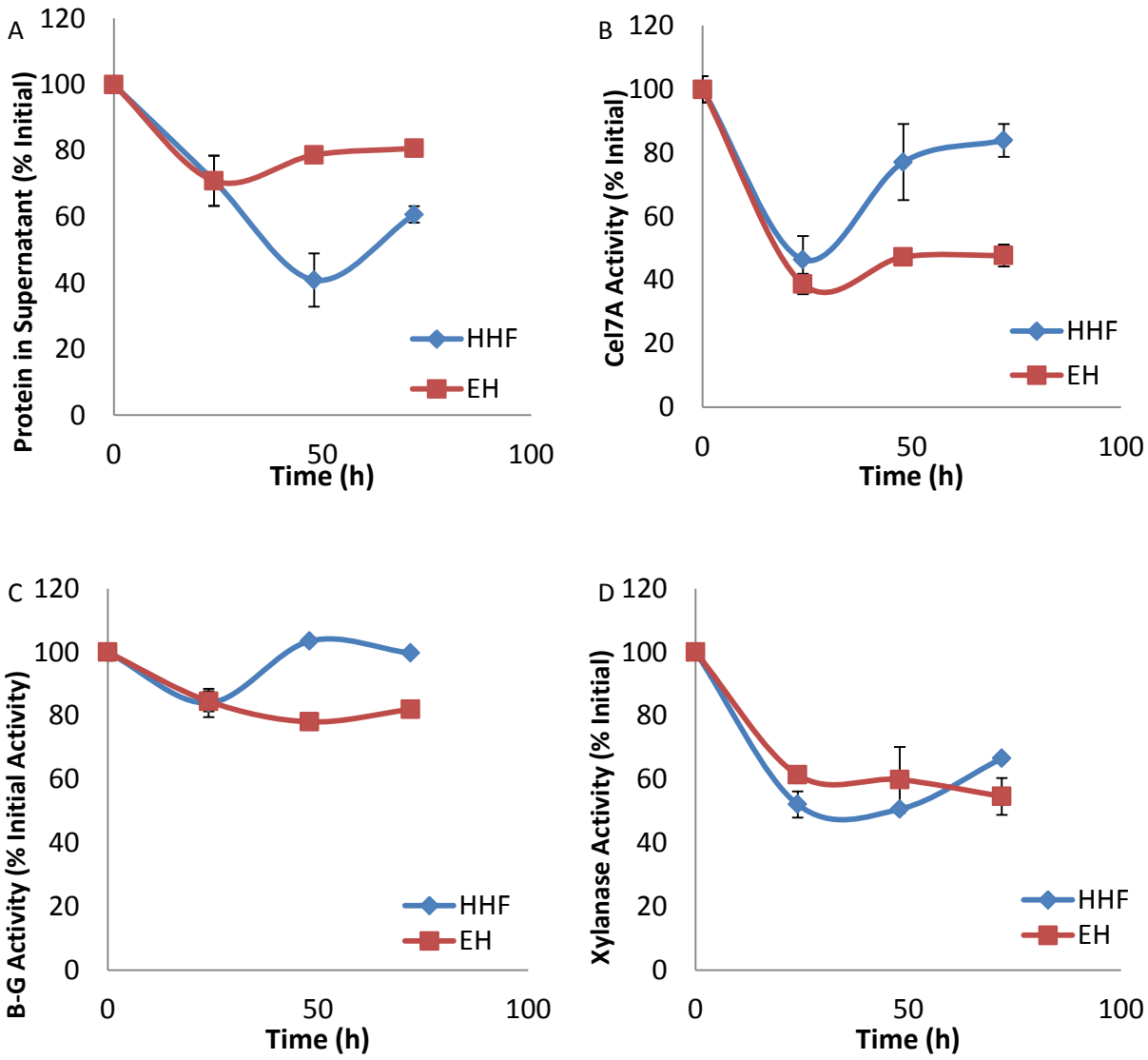

Figure 35. Profiles of protein (A), Cel7A (B), β-glucosidase (C), and xylanase enzyme (D) adsorption during the first round hydrolysis using the hybrid hydrolysis and fermentation (HHF) and enzymatic hydrolysis (EH) strategies as determined using the ninhydrin assay (A) and activity assays using PNPC (B), PNPG (C), and PNPX (D).

The higher enzyme activities observed after HHF for Cel7A and β-glucosidase compared to after EH could be attributed to two major reasons. First, the HHF approach resulted in a higher cellulose conversion as shown in Figure 34A and, hence, less residual solid compared to the EH approach. The enzymes have been shown to bind to solid residues after hydrolysis (Zhang and Lynd, 2004). Therefore,

minimizing residual solids would minimize the amount of enzyme binding sites and favour enzyme desorption back to the supernatant, resulting in a higher amount of protein in the liquid fraction. The second reason for the higher activities was likely due to the improved conservation of enzyme activities during HHF because of the lower temperatures used during the 48-hour SSF compared to the EH approach. The xylanase enzymes, especially the family 11 xylanase, have been shown to be thermally labile even after 24 hours of exposure to a typical hydrolysis temperature of 50^{o}C (section 3.1). This thermal instability may account for a dramatic loss of xylanase activities within the first 24 hours of hydrolysis, after which the lower temperature of SSF would not be beneficial in maintaining the activities of the enzymes. Due to the nature of these activity assays, one might expect that the presence of glucose in the EH samples might influence the measured activities of these enzymes. Although we could not fully rule out product inhibition by glucose, we expected its influence on the measured enzyme activities to be minimal, because the samples were diluted 4x for the activity assays. The expected final glucose concentration after dilution was less than 10 g/L, while glucose is reported to cause a 50% decrease in Cel7A activity at a concentration around 70 g/L (Murphy et al., 2013).

3.5.5 Enzyme Recovery by Re-adsorption to Fresh Substrate Following Hybrid Hydrolysis and Fermentation (HHF) and Straight Enzymatic Hydrolysis (EH)

While the HHF approach resulted in increased levels of enzyme activities in the supernatant after the first round of conversion, these increased enzyme activities would only be beneficial and contribute to the observed increased in the second round conversion if they were efficiently recovered. When comparing enzyme recycling after the HHF and EH approaches, the addition of fresh substrates after HHF resulted in the recovery of ~40% of the protein present in the supernatant compared to ~26% after the EH approach (Figure 36A). Based on the previous observation on the inhibitory effects of

glucose to enzyme adsorption (**Figure 30**A), this higher protein recovery with the HHF samples could be attributed to lower glucose inhibition to protein re-adsorption compared to EH samples.

To get a more detailed understanding of specific enzyme recovery, the activities of Cel7A, β-glucosidase, and xylanases in the supernatant, before and after addition of fresh substrate, were quantified to understand the efficiency of these enzymes in their re-adsorption to fresh substrates. These enzymes were specifically tracked because Cel7A is the dominant cellulase enzyme and both β-glucosidase and xylanases are the most studied accessory enzymes. Relatively specific activity assays are also available for these enzymes (Claeyssens and Aerts, 1992).

Our results showed that the addition of fresh substrate to the liquid fraction after the HHF approach recovered around 85% Cel7A, 23% β-glucosidase, and 63% xylanase activities, compared with 76% Cel7A, 25% β-glucosidase, and 75% xylanase activities after the EH approach (Figure 36B). To further confirm the different enzyme recycle efficiency after HHF and EH treatment, the activities of these enzymes in the supernatant were quantified after 24 hours of second round hydrolysis. The hydrolysates, containing enzymes recovered after HHF contained 7% of initial Cel7A activities, 32% of initial β-glucosidase activities and 13% of initial xylanase activities compared to 4%, 23%, and 9% of initial Cel7A, β-glucosidase, and xylanase activities, respectively, in hydrolysates with enzymes recovered after EH (Figure 36C). The higher Cel7A, β-glucosidase and xylanase activities, present after the second round hydrolysis following recovery from HHF samples, were expected given the higher amount of these activities present after HHF (Figure 35B and C) and their higher recovery rate through re-adsorption after HHF versus after EH.

The low activities of Cel7A after the second round of hydrolysis, despite relatively high recovery of these enzymes, could be attributed to the adsorption of these enzymes to the residual solids. In contrast, the relatively high activities of β-glucosidase, despite its low recovery efficiency, highlighted

the non-adsorptive nature of these enzymes to pure cellulosic substrate and also their thermal stability. Taken together, the addition of fresh substrate as an enzyme recycle strategy seemed to be advantageous when done after HHF compared to after EH, because of the reduced glucose inhibition to protein adsorption. By lowering glucose concentrations the enzymes would readsorb more readily upon addition of fresh substrates, resulting in an increased cellulose hydrolysis in the subsequent round of hydrolysis.

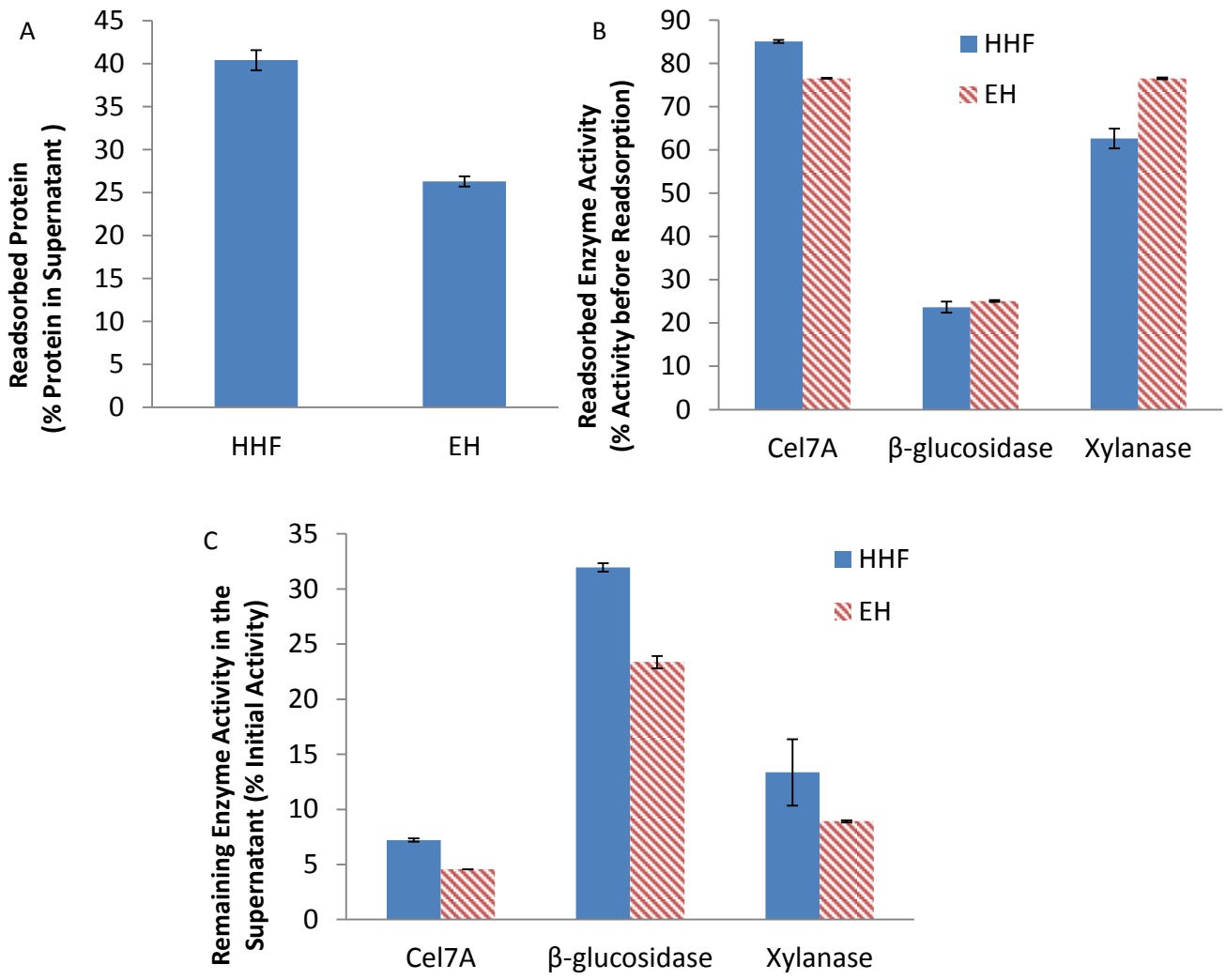

Figure 36. The amount of protein readsorption upon addition of fresh dissolving pulp (DsP), following hydrolysis with hybrid hydrolysis and fermentation (HHF) and enzymatic hydrolysis (EH) strategies (A). The readsorption of specific enzymes to fresh DsP following HHF and EH appraoches. The amount of enzyme activities (% of initial enzyme activity loading) of recycled enzymes after 24 hours of second round hydrolysis at 50°C (C).

To compare the efficiency of recycled enzymes after HHF with fresh enzymes in hydrolyzing cellulose, we constructed an enzyme efficiency curve by hydrolysing DsP at 5% solid loadings with three different enzyme loadings. By plotting the conversion yields (as a %) as a logarithmic function of the corresponding enzyme loading (in mg/g glucan), the resulting linear curve can predict the conversion yields that a given enzyme loading may achieve (manuscript in preparation). By using this method, 10

mg fresh enzymes/g cellulose, which corresponded to the 40% of the initial enzyme loading recovered after HHF, was predicted to result in ~39% conversion yield after 24 hours hydrolysis (Figure 37). In comparison, a similar conversion yield was achieved by the enzymes recycled after HHF after 24 hours of second round hydrolysis (Figure 34B). This indicated that the recycled enzymes did not suffer any major loss in efficiency following the recycling step after HHF. A similar result was reported in a related study which showed that, with a substrate containing a low amount of lignin, around 80-100% of original enzyme activity could be recovered from both the solid residue and the supernatant by addition of fresh substrate (Lee, Yu and Saddler, 1995). This conservation of enzyme activities following hydrolysis and a recycling step showed that devising an efficient enzyme recycle strategy is a worthwhile endeavor to reduce enzyme loading.

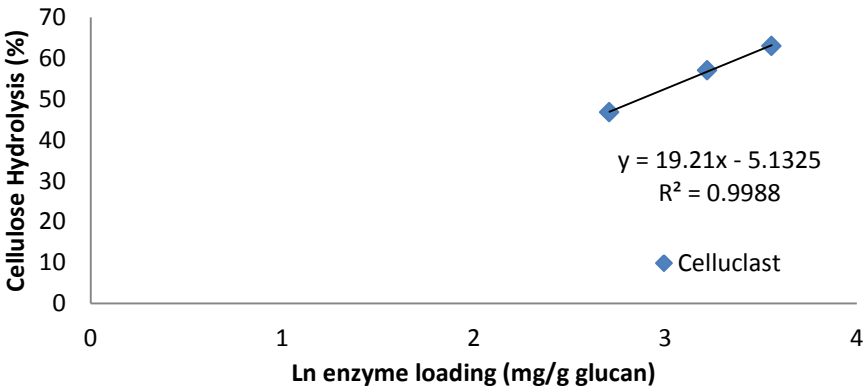

Figure 37. Enzyme efficiency curve as measured by using linearization of the natural log (ln) of enzyme loading and cellulose conversion (% theoretical) to predict hydrolysis efficiency at any given enzyme loadings.

3.5.6 The Effect of Family AA9 Enzyme on Hydrolysis and Enzyme Recycling

As mentioned earlier the role of the family AA9 enzymes in boosting hydrolysis has been documented. However, not much is known about its adsorption behavior during hydrolysis as well as its recyclability. Therefore, we conducted a study on the effect of a family AA9 enzyme on the performance

of enzymatic hydrolysis with enzyme recycling, using the HHF strategy whereby DsP was hydrolyzed for 24 hours at 50^{o}C, followed by 24 hours SSF at 32^{o}C. Hydrolysis was performed using 25 mg total enzyme loading/g glucan, with and without the substitution of 1 mg/g glucan of the enzyme loading with the AA9 enzyme. In addition to determining the effect of AA9 on cellulose hydrolysis, its effect on enzyme re-adsorption to fresh substrate was also evaluated.

As expected, the substitution of a portion of the enzyme loading with a corresponding amount of the AA9 enzyme resulted in an increase in cellulose hydrolysis by about 6-7% both after 24-hour hydrolysis and the subsequent 24-hour SSF (Figure 38A). However, this improvement in cellulose hydrolysis was not as pronounced in the second-round of HHF following a re-adsorption step to fresh substrate (Figure 38A).

To determine the adsorption behavior of the AA9 enzyme, the hydrolysates after three rounds of HHF with enzyme recycling were analyzed using SDS-PAGE. The AA9 remained in the supernatant throughout HHF and seemed to be re-adsorbed upon addition of fresh substrate and was then released back into the supernatant as the HHF proceeded (Figure 39). The fact that this enzyme did not boost hydrolysis following enzyme recycling suggests that the AA9 enzyme may have lost its activity, despite maintaining its presence in the hydrolysates.

The presence of the AA9 enzyme also resulted in a 5% increase in the concentrations of protein in the supernatant after 24 hours, compared to hydrolysis without the AA9 enzyme. However, addition of this enzyme did not increase enzyme desorption after the subsequent 24-hour SSF (Figure 38B). In a previous section, we had shown that the oxidative cleavage of cellulose chains by the AA9 enzyme resulted in an increase in acid groups in the solid substrate, which suggested an increase in negative charges (section 3.4.2).

This increase in negative charge may explain the increase in enzyme desorption (or decrease in unproductive enzyme adsorption) observed in this study (Del Rio, Chandra and Saddler, 2011). The presence of AA9 enzyme, on the other hand, did not seem to influence protein re-adsorption to fresh substrate (data not shown). The lack of AA9's influence on protein re-adsorption was expected as it is not likely that this enzyme will have enough time to significantly change the properties of the added fresh substrate during enzyme readsorption.

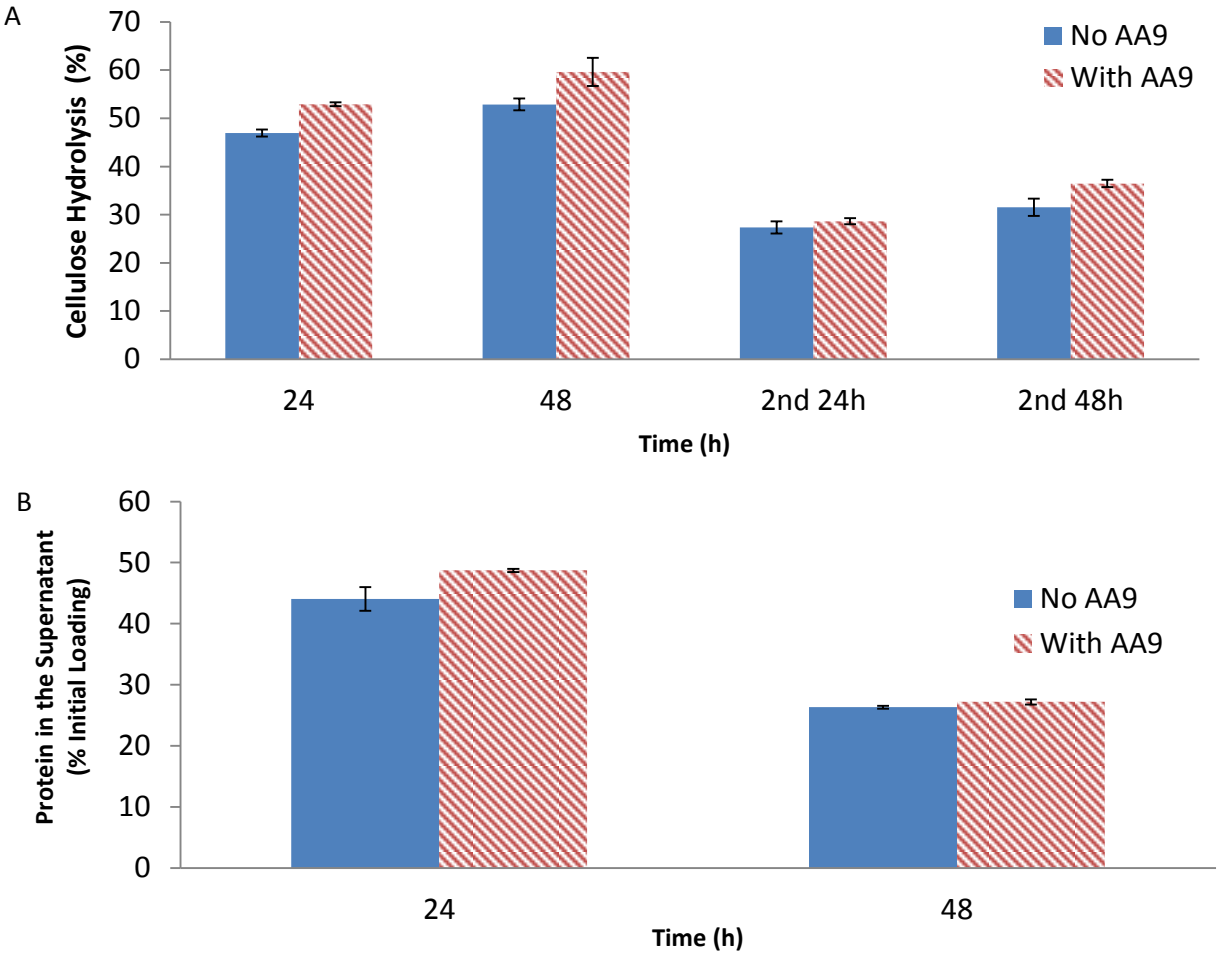

Figure 38. The influence of AA9 enzyme on the first round and second round of DsP hydrolysis at 5% (w/w) solid loading using the hybrid hydrolysis and fermentation (HHF) strategy (A). The amount of proteins in the supernatant during the first round of 48 hours of HHF –24 hours of hydrolysis at 50°C followed by 24 hours of simultaneous saccharification and hydrolysis (SSF) (B).

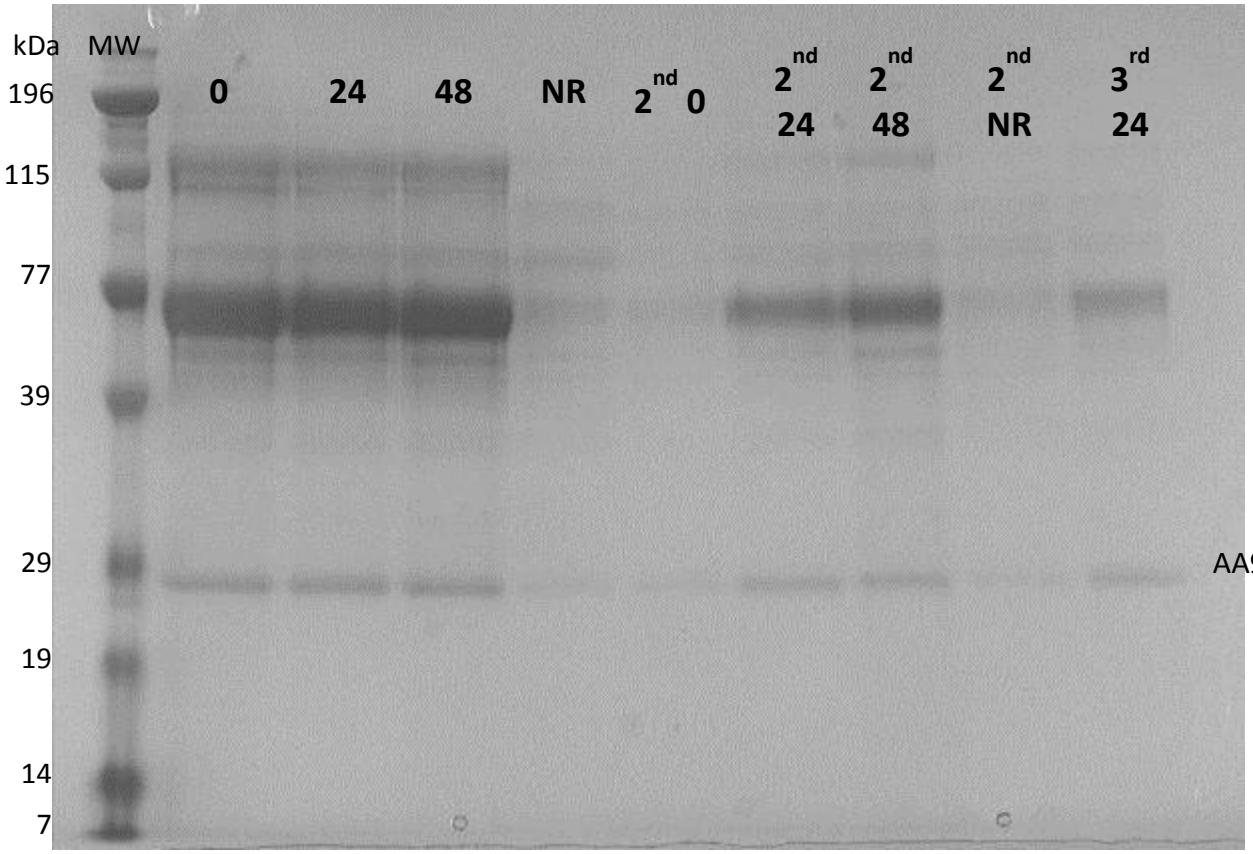

Figure 39. SDS-PAGE of proteins in the supernatant during three rounds of HHF with enzyme recycling by addition of fresh substrates. The numbers 0, 24, 48 indicate the time (hour) during hydrolysis at which samples were taken. The numbers 2nd and 3rd indicate the round of hydrolysis. NR indicates proteins that were not re-adsorbed after addition of fresh substrate.

3.5.7 Five Rounds of Enzyme Recycling by the Addition of Fresh Substrate and Enzyme Supplementation

Following enzyme recycling after HHF, hydrolysis using recycled enzymes achieved 48% conversion after 48 hours of the second round hydrolysis compared to 70% conversion in the first 48 hours of the first round of EH (Figure 34A and B). Depending on the nature of the substrates and enzyme recycling strategies, previous enzyme recycling studies have reported a second round

conversion yield between 40-90% of the original yield, and the higher conversion yields (above 50% of the first round yield) were typically obtained after supplementation with fresh β-glucosidase (Lee, Yu and Saddler, 1995; Tu et al., 2009; Weiss et al., 2013; Qi et al., 2011). Therefore, the ~68% of the first round yield obtained in this study by using only the recycled enzymes after HHF without addition of any fresh enzymes was among the highest second round conversion yields ever reported in the literature.

The need for fresh enzyme supplementation has been reported in previous studies (Lee, Yu and Saddler, 1995; Tu et al., 2009; Weiss et al., 2013; Qi et al., 2011). This is especially true when enzymes are recycled through re-adsorption to fresh substrate, because certain enzymes, such as β-glucosidase, do not efficiently readsorb to fresh substrate (Tu et al., 2009; Qi et al., 2011). Following addition of fresh substrate, around 50-60% of the protein in the supernatant was not recovered for the next round of conversion (Figure 36A). To determine if consistently high hydrolysis performance could be obtained by supplementing recycled enzymes with fresh enzymes, a study was carried out to supplement recycled enzymes with 40% of the original enzyme loading.

In this study, hydrolysis was performed with 25 mg/g glucan of CTec 3 or a mixture of Celluclast, β-glucosidase, and AA9 (20, 4, and 1 mg/g glucan, respectively) using the HHF strategy. Following addition of fresh substrate, 40% of the original enzyme composition was added to the hydrolysis mixture to replace lost enzyme activities. The process was repeated for a total of five rounds of hydrolysis. The cellulose conversions after 24 hours of hydrolysis at each round were compared to determine if enzyme supplementation would help maintain consistently high cellulose conversion.

Hydrolysis using CTec 3 achieved 10-15% higher cellulose conversion compared to hydrolysis with the Celluclast, β-glucosidase and the AA9 mixture (Figure 40). Fresh enzyme supplementation resulted in a consistently high hydrolysis performance, but also about 10% and 5% increase in cellulose conversion in the second round with CTec 3 and the Celluclast, β-glucosidase, and AA9 mixture,

respectively, compared to the first round, indicating that the amount of enzyme supplemented may have been higher than was required to replenish lost enzyme activities. This elevated hydrolysis performance remained relatively constant throughout all the subsequent rounds of hydrolysis (Figure 40). When the total enzyme use was calculated, combining enzyme recycling after HHF with fresh enzyme supplementation managed to achieve consistently high hydrolysis performance (60-70% with CTec 3 and ~50% cellulose conversion with Celluclast, β-glucosidase, and AA9 mixture) with a total of 13 mg enzyme/g cellulose or a ~50% saving compared to using 25 mg/g glucan when enzymes were not recycled. Recently, an enzyme recycling study using enzyme recycling from the solid residues and enzyme supplementation achieved a total enzyme saving of 38% and 28% following a separate hydrolysis and fermentation (SHF) and simultaneous saccharification and cofermentation (SSCF) processes, respectively (Jin et al., 2012). The higher enzyme savings achieved in the present study were likely due to a higher enzyme recovery when enzymes were recycled from both the supernatant and the solid residues through addition of fresh substrates in the presence of low glucose concentrations. By combining fast hydrolysis with fast SSF, a significant time saving was also achieved compared to doing a typical combination of 72-hour hydrolysis followed by 72-hour fermentation.

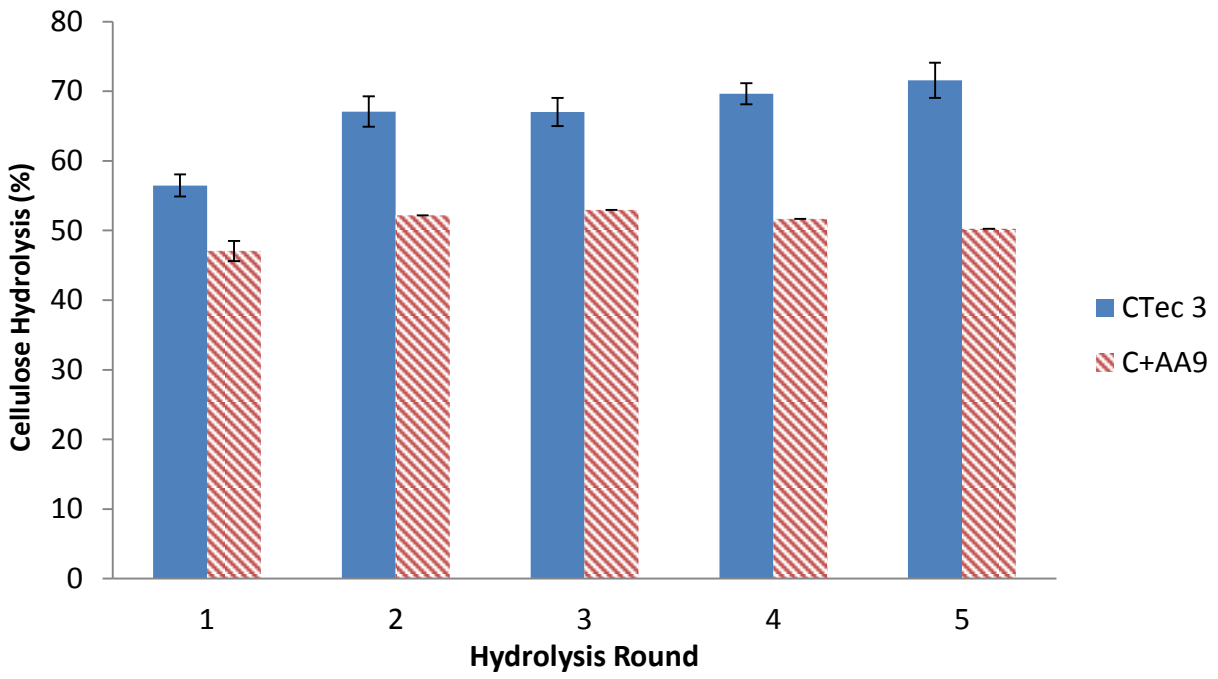

Figure 40. Consistently high hydrolysis performance for 5 rounds of dissolving pulp (DsP) hydrolysis using the hybrid hydrolysis and fermentation (HHF) strategy with 25 mg/g glucan of commercial enzyme mixtures CTec 3 and a mixture of Celluclast, β-glucosidase, and AA9 enzyme (C+AA9). Enzymes were recycled after each round by addition of fresh substrates and supplemented with 40% of the original enzyme loading.

3.5.8 Optimization of Mono-Component Enzymes for Enzyme Supplementation

Different enzymes seem to vary in their recovery efficiency upon addition of fresh substrate. In the work reported here, the addition of fresh DsP seemed to be more efficient in recovering Cel7A than xylanases, but not β-glucosidase (Figure 36B). Thus, in addition to losing the protein mass, certain key enzyme activities seemed to be more prone to being lost compared to others, which may result in lost enzyme synergy. In previous studies, enzyme supplementation has typically been carried out by adding β-glucosidase (Qi et al., 2011; Tu et al., 2009) or a certain fraction of the original enzyme loading (Weiss et al., 2013; Jin et al., 2012). Creating a custom enzyme cocktail for enzyme supplementation to

reconstitute the lost protein mass and to also selectively restore lost enzyme activities may provide an opportunity to further reduce enzyme loadings

To evaluate this hypothesis, the proteins recovered by re-adsorption to fresh substrate were supplemented with fresh enzymes corresponding to 20% of the original enzyme loading (or 5 mg/g glucan). To systematically determine possible key enzyme activities that may not be recovered, recycled enzymes were supplemented with a portion of the original cellulase mixture (Celluclast), along with various enzymes such as xylanase 10 (Xyn10), xylanase 11 (Xyn11), β-glucosidase and AA9. The complete protein-substrate mixture was then incubated at 50^{o}C for 24 hours.

Compared to the hydrolysis performance achieved without any enzyme supplementation, supplementing the hydrolysis with 20% of the original Celluclast loading resulted in an increase in sugar production by about 45% (Figure 41A). Substituting 40% (w/w) of this additional Celluclast loading with either Xyn10 or Xyn11 did not improve the sugar production significantly. The substitution of a portion of Celluclast with xylanase enzymes was done based on a previous observation which showed that xylanase enzymes, especially Xyn11, were prone to thermal denaturation (section 3.1). A lack of improvement might indicate that the amount of xylanase enzymes present in the Celluclast mixture may have been enough to restore lost xylanase activities. It is also possible that the xylanase enzymes were not key enzymes for DsP hydrolysis even though DsP contained around 3% xylan (Table 4. Chemical compositions of steam pretreated corn stover (SPCS), delignified SPCS (DSPCS), xylanase-treated SPCS (XSPCS), and dissolving pulp (DsP).It should be noted that a 20% (w/w) substitution of Celluclast with AA9 improved sugar production by around 60%, and substituting 40% of Celluclast with β-glucosidase resulted in an increase in sugar production by about 100% compared to non-supplemented hydrolysis (Figure 41A). These results agreed well with data presented in Figure 36B which shows that β-

glucosidase was the main enzyme that was not recovered by readsorption to fresh DsP and with the previous section which shows that AA9 lacked a significant adsorption to the substrate.

We further evaluated if adjusting the composition of these enzyme supplements would affect hydrolysis performance. By fixing the loading of the enzyme supplement to 5 mg/g glucan, we varied the amount of added Celluclast and β-glucosidase from 0 to 5 mg/g glucan. Supplementation with 60% and 40% (corresponding to 3 and 2 mg/g glucan) of Celluclast and β-glucosidase, respectively, resulting in the highest increase in cellulose conversion (45%) in the second round hydrolysis than any other ratios, when compared to cellulose conversion obtained without enzyme supplementation (Figure 41B). Further supplementing this enzyme loading combination with AA9 from 0.5 – 1.5 mg/g glucan further increased the conversion gradually by ~5-10% (Figure 41C). It was therefore concluded that the hydrolytic performance of recycled enzymes using re-adsorption to fresh substrates could be significantly improved by supplementing the recycled enzymes with an optimum ratio of selected enzyme activities to replace those lost during the recycling step.

In summary, high enzyme recovery could be achieved through readsorption to fresh substrate by reducing the glucose inhibition to enzyme adsorption. To compensate for enzymes lost during the hydrolysis and recycling steps, the recycled enzymes should be supplemented with an optimum ratio of key enzyme activities. This strategy achieved consistently high hydrolysis performance for five rounds of hydrolysis with a 50% saving in enzyme loading. This is the highest enzyme saving reported in the literature so far. While further research needs to be conducted to evaluate the efficiency of this particular enzyme recycle strategy with real lignocellulosic substrates, it is likely that the inhibition effect of glucose on enzyme adsorption, observed in this study is applicable to hydrolysis of all lignocellulosic substrates.

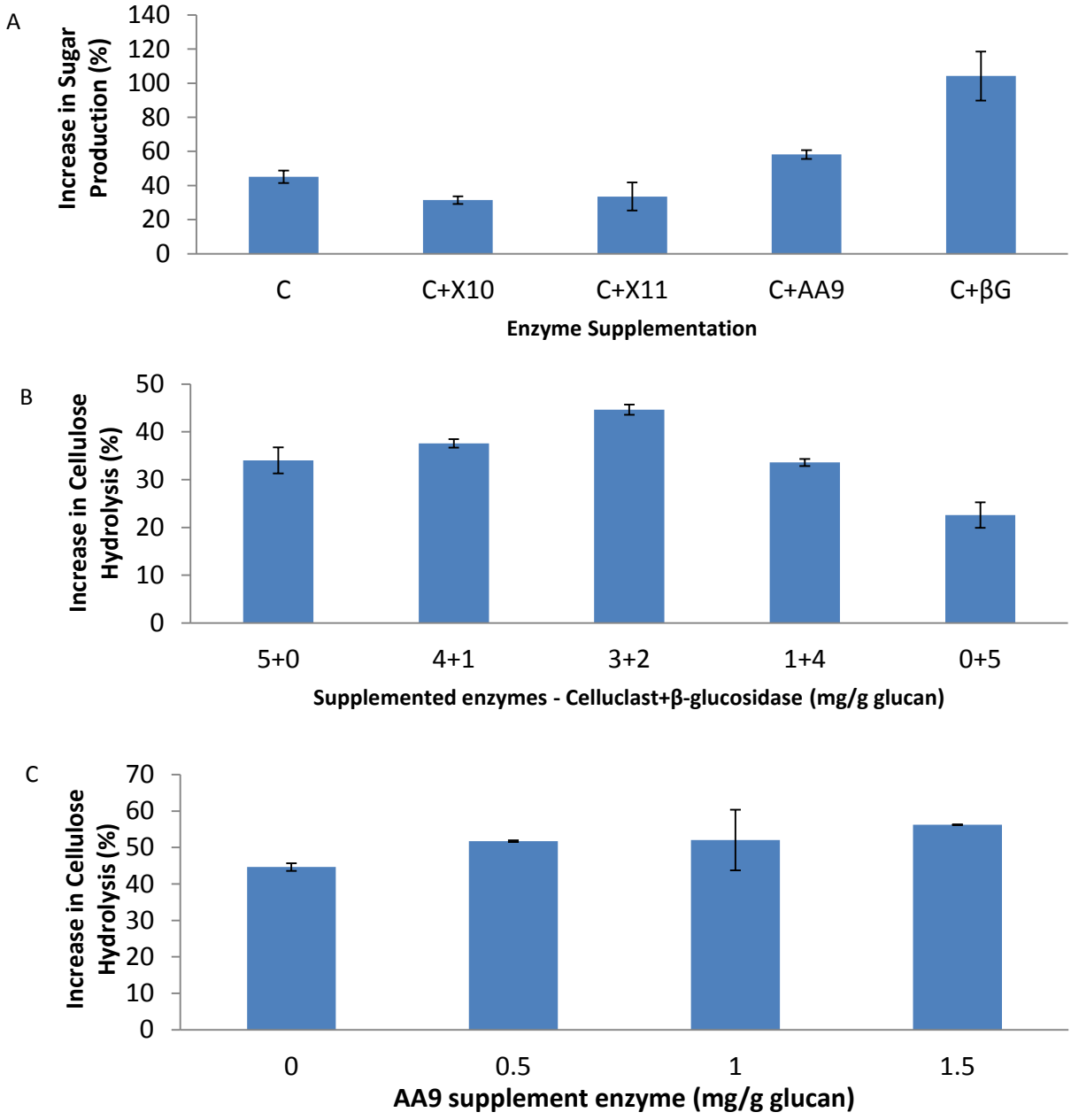

Figure 41. Identification of essential enzyme activities needed to restore lost activities after enzyme recycling by substituting a portion of Celluclast (C) mixture with enzyme monocomponents: family 10 xylanase (X10), family 11 xylanase (X11), AA9, and β-glucosidase (βG) and measuring increase in sugar production compared to hydrolysis using recycled enzymes without enzyme supplementation (A). Optimization of the ratio of Celluclast and β-glucosidase in the enzyme supplement (B). Optimization of AA9 enzyme dose to supplement Celluclast and β-glucosidase mixture (3 and 2 mg/g glucan, respectively) (C). Cellulose hydrolysis was measured after 24 hours of second-round hydrolysis at 50^{o}C with recycled enzyme and enzyme supplement.

3.5.9 Conclusions

Soluble compounds present during the bioconversion of lignocellulosic substrates play an important but often overlooked role in enzyme-substrate interaction. Glucose was shown to inhibit enzyme adsorption to cellulose at concentrations above 100 g/L. Xylose and ethanol, on the other hand, did not seem to significantly influence enzyme adsorption, even at concentrations as high as 250 g/L and 20% (v/v), respectively. The reduction in enzyme binding capacity caused by glucose influenced enzyme recycling efficiency, especially through readsorption to fresh substrates. Minimizing glucose concentrations during enzyme recycling by recovering the enzyme after HHF instead of EH, was shown to improve enzyme recovery and hydrolysis performance using recycled enzymes. These recycled enzymes (40% of the initial enzyme loading) were shown to be as efficient in hydrolysing the substrate as fresh enzymes at the same enzyme loading, indicating the stability of the enzyme activities. While the AA9 enzyme was shown to improve the overall hydrolysis efficiency in the first round, this enzyme did not increase hydrolysis after enzyme recycling, suggesting that this enzyme might not have been recovered or lost its activity.

As the results in the previous sections had shown that some enzyme activities were lost during hydrolysis or not recovered due to their low adsorption affinity, enzyme supplementation was evaluated as a potential strategy to improve the hydrolysis performance using recycled enzymes. Supplementing recycled enzymes with fresh enzymes (<40% of original enzyme loading) achieved consistently higher hydrolysis performance after five rounds of hydrolysis. By combining enzyme recovery after the HHF strategy with enzyme supplementation, an increase in the efficiency of hydrolysis was achieved for five rounds of hydrolysis resulting in a 50% reduction in enzyme loading, compared to hydrolysis without enzyme recycling. A further reduction in enzyme loading could be achieved by identifying the types of

enzymes that were lost during hydrolysis and enzyme recycling in order to create an optimum enzyme supplement mix.

Overall, the results presented in this thesis have shown that an improvement in enzyme recycling efficiency could be achieved by understanding the underlying enzyme-substrate interactions. By using specific techniques to identify individual enzyme adsorption profiles during hydrolysis, this thesis has identified the influence of substrate properties, especially cellulose properties, AA9 enzyme, and glucose concentrations on individual enzyme adsorption and enzyme recycling performance. Using these insights, an improvement in enzyme recycling performance by addition of fresh substrates was achieved by recovering enzymes from both phases in the presence of low glucose concentrations after HHF and supplementing recycled enzymes with key enzyme activities that were lost during hydrolysis and enzyme recycling.

4. Conclusions and Future Work

4.1 Conclusions

The overall objective of the work described in this thesis was to look at some of the enzyme-substrate interaction phenomena that would influence enzyme recycling efficiency, as one way to reduce enzyme loadings, while achieving/ maintaining overall high cellulose hydrolysis yields during hydrolysis of lignocellulosic substrates. By better understanding individual enzyme-substrate interactions during hydrolysis and the various factors that influenced these individual enzyme adsorption profiles, an improved enzyme recycling strategy was devised which maximized enzyme recovery and resulted in significant reductions in enzyme loadings. Due to the complexity of enzyme-substrate interactions and the limited number of techniques available to assess individual enzyme adsorption profiles, the overall goal had three major objectives:

(1) To assess and improve the techniques used to determine enzyme distribution during hydrolysis using commercial cellulase mixtures and industrially relevant substrates;

(2) To investigate the influence of the substrate, enzyme, and physical factors on individual enzyme adsorption; and

(3) To evaluate and improve the performance of an enzyme recycling strategy using addition of fresh substrates.

As most previous adsorption studies have typically used purified enzymes and model (pure) cellulosic substrates, the observed enzyme-substrate interactions may not represent the realistic interactions that occur during hydrolysis of lignocellulosic substrates by a complex enzyme mixture. Therefore, in the initial work, to try to obtain a realistic understanding of enzyme distribution during hydrolysis, the adsorption of individual enzymes during hydrolysis of steam pretreated corn stover

(SPCS) using a commercial enzyme mixture Accellerase 1000 were assessed. A variety of techniques, including SDS-PAGE, enzyme activity assays (PNPG, PNPC, PNPX, and CMC), mass spectrometry for protein identification, and zymograms, were used. By combining the data obtained from these assays, the distribution profiles of six individual enzymes (Cel7A, Cel7B, Cel5A, Xyn10, Xyn11, and β-glucosidase) in the liquid and solid phases during hydrolysis could be identified. However, this approach proved to be laborious as information on individual enzyme adsorption had to be derived from several techniques that lacked the specificity required to probe individual enzymes in a complex enzyme mixture and many of the techniques were qualitative or semi-quantitative in nature. This initial study highlighted the need for a better assay that could specifically and quantitatively monitor enzyme adsorption during hydrolysis using a complete commercial enzyme mixture. In general, this initial work showed that most of the enzymes were distributed in both the liquid and solid phases during hydrolysis with an increase in enzyme desorption to the liquid-phase occurring at the later stages of hydrolysis. The enzymes that remained adsorbed to the solid retained their activity longer than those that remained mostly in the liquid phase. Therefore, a fast hydrolysis combined with an enzyme recycling strategy that recovered the enzymes from both the solid and the liquid phase could potentially lead to a significant enzyme saving by minimizing the loss of enzyme activities during hydrolysis while maximizing enzyme recovery from both phases.

To address the limitation of the currently available protein assays used to determine individual enzyme adsorption profiles during hydrolysis of lignocellulosic substrates using commercial enzyme mixtures, we assessed the applicability of an ELISA technique for cellulase enzyme adsorption studies. A double-antibody sandwich ELISA (DAS-ELISA) method was developed and used to quantitatively determine specific enzyme adsorption profiles. By using a pair of monoclonal and polyclonal antibodies (MAbs and PAbs, respectively), a DAS-ELISA method was developed to specifically probe Cel7A, Cel6A, and Cel7B during SPCS hydrolysis using a complex cellulase enzyme mixture. By employing the

specificity and sensitivity of the interactions between MAbs and PAbs with the target enzyme, we were able to specifically and quantitatively determine the adsorption profiles of cellulase monocomponents Cel7A, Cel6A, and Cel7B present in both the Celluclast and Accellerase 1000 enzyme preparations during the hydrolysis of SPCS. The assay was shown to be sensitive at relatively low enzyme concentrations (0 – 1 μg/ml). All three enzymes exhibited different individual adsorption profiles. The adsorption profiles observed with the ELISA method were in agreement with the earlier, more laborious enzyme assay techniques, demonstrating that the ELISA technique could overcome the limitations with current techniques and obtain specific and quantitative enzyme adsorption profile data. Subsequent studies then investigated the effects of substrate properties, enzyme characteristics and hydrolysis conditions on specific enzyme adsorption profiles as these factors have been shown to significantly influence enzyme recycling performance.

The substrate properties evaluated in this study included the influence on lignin and cellulose properties on specific enzyme adsorption. The influence of lignin was assessed by removing lignin from SPCS using acid-chlorite delignification to produce delignified SPCS (DSPCS). Previous studies on the influence of lignin on enzyme adsorption have typically evaluated the influence of lignin on general protein adsorption and not on individual enzyme adsorption. An increase in enzyme recycling performance had previously been reported with partially delignified substrates. This increase in performance was associated with a decrease in enzyme non-productive adsorption to lignin. Surprisingly, when delignified SPCS (DSPCS) was studied, lignin removal did not seem to affect the adsorption profiles of Cel7A and β-glucosidase. In contrast, these enzymes showed different adsorption profiles to DSPCS and DsP even though both were relatively pure cellulosic substrates. These results suggested that, at least with these substrates, the cellulose properties were a major factor that influenced the enzyme adsorption profiles.

To further determine the influence of cellulose properties, individual enzyme adsorption was assessed by hydrolyzing the six (relatively) pure cellulosic substrates of Avicel, DsP, PASC, cellulose II, cellulose III, and CNC. Both the PASC and cellulose II were derived from Avicel. The nature of cellulose was shown to be an important factor in determining enzyme adsorption profiles during hydrolysis and cellulose hydrolysis yields. Individual enzymes showed different adsorption profiles to all six cellulosic substrates. Comparing different cellulose allomorphs, enzymes seemed to adsorb the most to cellulose II and the least to cellulose III. Cellulose crystallinity seemed to influence the extent of enzyme desorption at the later stage of hydrolysis. Minimal desorption was observed with the highly crystalline substrate CNC whereas an extensive desorption occurred with the highly amorphous PASC. Substrate accessibility, as measured by the Simon's Stain and CBM adsorption methods, seemed to correlate with the extent of initial enzyme adsorption. Although both cellulose II and PASC were shown to have similar accessibility, a high adsorption of Cel7A, with little desorption during hydrolysis, was observed with cellulose II, whereas with PASC, the initial adsorption was quickly followed by an extensive desorption. A similar phenomenon was observed with the SPCS and DSPCS substrates. Although DSPCS was shown to be more accessible than SPCS, both substrates exhibited similar enzyme adsorption profiles. Therefore, both studies using corn-stover derived substrates and pure cellulosic substrates showed that cellulose properties, especially the nature of the cellulose allomorphs and crystallinity, highly influenced the nature of enzyme adsorption and, consequently, the appropriate strategies for enzyme recycling. This study also confirmed previous observations that lignin in agricultural residues did not negatively affect enzyme adsorption, suggesting a possibility for recycling of the solid residues to recover enzymes adsorbed to the residues without a significant adverse effect on hydrolysis performance.

The adsorption profile of the accessory enzyme AA9 and its effects on individual enzyme adsorption during hydrolysis was subsequently investigated. The addition of AA9 has been shown to significantly improve hydrolysis efficiency of lignocellulosic substrates. Consequently, this enzyme has

been included in the most recent commercial cellulase enzyme mixtures for lignocellulosic bioconversion. While many studies have explored the mechanisms of oxidative cleavage of cellulose by the AA9 enzyme and the possible mechanism of its synergistic interactions with cellulose hydrolytic enzymes, no study has ever been done on its adsorption behaviour and its possible influence on the adsorption behavior of canonical cellulase enzymes. As cellulose properties have been shown to influence enzyme adsorption, the oxidation of cellulose by AA9 could be expected to influence the adsorption behavior of other enzymes. Therefore, understanding the recyclability of AA9 and its influence on other enzymes might be important for industrially-relevant enzyme recycling strategies. The AA9 enzyme was observed to remain primarily in the supernatant throughout hydrolysis. A slight increase in enzyme desorption was observed in the presence of AA9, primarily on substrates with higher accessible crystalline cellulose such as Avicel and CNC as well as with lignocellulosic substrates SPCS and DSPCS. This showed that, in addition to increasing hydrolysis efficiency, the addition of AA9 also influenced the adsorption profiles of canonical cellulase enzymes. An increase in the negative charge on the surface of cellulose, probably due to oxidation of cellulose, was shown to be a potential contributing factor behind this increase in enzyme desorption. By using ELISA, this increase in desorption was observed to occur primarily with Cel7A, a processive enzyme, but not with Cel7B. In general, an increase in protein desorption was shown to correlate with an increase in AA9 enzyme loading from 0.1 to 0.7 mg/g cellulose or 0.6% to 3.9% of total enzyme loading. The low adsorption of AA9 enzyme and the observed increase in Cel7A desorption in the presence of AA9 enzyme highlighted the importance of recovering enzymes from both the liquid and the solid phase to ensure maximum enzyme recovery. The observed release of Cel7A in the presence of AA9 enzyme may also suggest another mechanism for the synergistic interaction between AA9 enzyme and Cel7A, namely through reduction in Cel7A unproductive adsorption to cellulose.

In an industrial setting, in addition to using a complex enzyme mixture containing a range of enzymatic activities, enzymatic hydrolysis of lignocellulosic biomass needs to be carried out at high substrate consistency to increase sugar concentrations and minimize downstream costs. Soluble compounds such as glucose and ethanol that are present during the bioconversion of lignocellulosic substrates play an important, but often overlooked role in enzyme-substrate interaction. Understanding the influence of sugars and ethanol on enzyme adsorption is important to assess the appropriate enzyme recycling strategy, the optimum insertion point for enzyme recycling, and the influence of high consistency hydrolysis on enzyme recycling. Glucose was shown to inhibit enzyme adsorption to cellulose at concentrations above 100 g/L. In contrast, xylose and ethanol did not seem to significantly influence enzyme adsorption even at concentrations as high as 250 g/L and 20% (v/v), respectively. As some enzyme recycling strategies such as enzyme readsorption to fresh substrate or recycling of solid residues depend on the adsorption of enzymes to the substrate, the inhibitory effect of glucose to enzyme adsorption may influence the efficiency of these recycling strategies.

Based on these results, enzyme recycling strategies might be improved by performing short rounds of hydrolysis to minimize loss of enzyme activities, enzyme recovery from both the solid and the liquid phases to maximize enzyme recovery, enzyme recovery at low glucose concentrations to improve enzyme adsorption, and enzyme supplementation to replace loss enzyme activities. The efficiency of an enzyme recycling strategy using addition of fresh substrate was evaluated because of its simplicity and its ability to recover enzyme components from both the liquid and the solid phases. To minimize the loss of enzyme activity and improve enzyme recovery, enzyme recycling was done after an HHF strategy. In this strategy, substrates were hydrolyzed for 24 hours followed by SSF to minimize loss of enzyme activities. Enzyme recycling was performed after SSF. The reduction in glucose concentrations after SSF was shown to improve enzyme recovery and subsequent hydrolysis performance using recycled enzymes. As certain enzymes, such as AA9, Xyn 11, and β-glucosidase, were lost during hydrolysis or

enzyme recycling due to their low adsorption affinity, enzyme supplementation was evaluated as a potential strategy to improve the performance of recycled enzymes. By combining HHF followed by enzyme recycling and fresh enzyme supplementation, consistently high hydrolysis performance for five rounds of hydrolysis and a 50% reduction in enzyme loading were achieved compared to hydrolysis without enzyme recycling. Cellulose hydrolysis yields could be further improved by identifying key enzyme activities that were lost during hydrolysis or the recycling step to create an optimum mix of enzyme supplements.

Overall, this work has shown that, by better understanding the various factors that influence individual enzyme adsorption profiles, an improvement in an enzyme recycle strategy could be achieved, resulting in a significant reduction in enzyme loading. This improved understanding of enzyme adsorption profiles during hydrolysis of lignocellulosic substrates using commercial enzyme mixtures has been achieved by overcoming the lack of specific techniques for enzyme adsorption studies and by investigating the influence of various factors on individual enzyme adsorption during hydrolysis.

4.2 Future Work

4.2.1 Investigating Cellulose Properties that Determine Enzyme Adsorption

Despite cellulose II and PASC showing similar accessibility and cellulose hydrolysis rates, they displayed completely different enzyme adsorption profiles. There have been many studies on enzyme adsorption to cellulose III which show that, despite low levels of enzyme adsorption to the substrate, cellulose III can be efficiently hydrolyzed due to the increased hydration (and hence, hydrophilicity) of the substrate. Similarly, investigations into the structural differences between cellulose II and PASC, which may have led to the strikingly different enzyme adsorption profiles, may give further insight into cellulose structures that determine enzyme adsorption. This work might subsequently prove useful for

improving pretreatment strategies that result in easily digestible substrates with predictable enzyme adsorption profiles and enhancing enzyme recycling strategies.

4.2.2 Assessing the Influence of Other Accessory Enzymes/ Non-Hydrolytic Proteins such as Xylanases and Swollenin on Enzyme Adsorption

As well as AA9, a number of other accessory enzymes/ proteins, such as swollenin and xylanases, have been studied to assess their synergistic effects on hydrolysis of lignocellulosic substrates. The work reported in the thesis showed that AA9 likely plays a role in relieving unproductively adsorbed Cel7A, suggesting an additional mechanism by which this enzyme may boost cellulose hydrolysis. Thus, the influence of other accessory enzymes on enzyme-substrate interaction needs to be further investigated. In this way we would hope to obtain a better understanding of the dynamic interactions among enzymes and between enzymes and substrates during hydrolysis.

4.2.3 Determining the Influence of High Substrate Consistency on Individual Enzyme Adsorption Profiles

Several studies have suggested using high consistency hydrolysis to achieve high sugar or ethanol concentrations, consequently improving the commercial viability of the bioconversion process. Some of these studies have reported that enzyme adsorption tends to decrease as substrate consistency increased and that this decrease in enzyme binding capacity may be responsible for the lower hydrolysis rate and yield commonly observed at high consistency hydrolysis. Therefore, in order to further improve saccharification efficiency at high substrate consistency, it will be important to investigate the influence of increasing substrate consistency on the adsorption profiles of individual enzymes in order to gain a better understanding of the dynamics of enzyme-substrate interaction encountered at high substrate consistency.

4.2.4 Evaluating Enzyme Recycling using Real Lignocellulosic Substrates and High Consistency

The work in the thesis showed that the addition of fresh substrate was an effective enzyme recycling strategy due to its simplicity and efficiency in recovering enzymes from both the liquid and solid phases. A study evaluating the efficiency of this strategy to recycle enzymes after hydrolysis of real lignocellulosic substrate is necessary to determine the efficiency of this strategy with a "realistic" lignocellulosic biomass. As high sugar or ethanol concentrations will likely be needed to improve the economics of the bioconversion process, it would be of interest to evaluate hydrolysis and enzyme recycling strategies at high substrate consistency. These two approaches will help determine if enzyme recycling strategies can be used in realistic industrial conditions.

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