ENZYMATIC CONVERSION OF PRETREATED BIOMASS INTO FERMENTABLE SUGARS FOR BIOREFINERY OPERATION

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ABSTRACT

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Depleting petroleum reserves and potential climate change caused by fossil fuel consumption have attracted significant attention towards the use of alternative renewable resources for production of fuels and chemicals. Lignocellulosic biomass provides a plentiful resource for the sustainable production of biofuels and biochemicals and could serve as an important contributor to the world energy portfolio in the near future. Successful biological conversion of lignocellulosic biomass requires an efficient and economical pretreatment method, high glucose/xylose yields during enzymatic hydrolysis and fermentation of both hexose and pentose to ethanol. High enzyme loading is a major economic bottleneck for the commercial processing of pretreated lignocellulosic biomass to produce fermentable sugars.

Optimizing the enzyme cocktail for specific types of pretreated biomass allows for a significant reduction in enzyme loading without sacrificing hydrolysis yield. Core glycosyl hydrolases were isolated and purified from various sources to help rationally optimize an enzyme cocktail to digest ammonia fiber expansion (AFEX) treated corn stover. The four core cellulases were endoglucanase I (EG I), cellobiohydrolase I (CBH I), cellobiohydrolase II (CBH II) and β-Glucosidase (βG). The two core hemicellulases were an endoxylanase (EX) and a β-xylosidase (βX). A diverse set of accessory hemicellulases from bacterial sources was found necessary to enhance the synergistic action of cellulases hydrolysing AFEX pretreated corn stover. High glucose (around 80%) and xylose (around 70%) yields were achieved with a moderate enzyme

loading (~20 mg protein/g glucan) using an in-house developed enzyme cocktail and this cocktail was compared to commercial enzyme.

Studying the binding properties of cellulases to lignocellulosic substrates is critical to achieving a fundamental understanding of plant cell wall saccharification. Lignin auto-fluorescence and degradation products formed during pretreatment impede accurate quantification of individual glycosyl hydrolases (GH) binding to pretreated cell walls. A high-throughput Fast Protein Liquid Chromatography (HT-FPLC) based method has been developed to quantify CBH I, CBH II and EG I present in hydrolyzates of untreated, AFEX, and dilute-acid pretreated corn stover. This method can accurately quantify individual enzymes present in complex binary and ternary protein mixtures without interference from plant cell wall derived components.

The binding characteristics of CBH I, CBH II and EG I during 48 hours hydrolysis were studied on different cellulose allomorphs: microcrystalline cellulose Avicel (cellulose I_{β}), liquid ammonia treated cellulose (cellulose III), sodium hydroxide treated cellulose (cellulose II) and phosphoric acid swollen amorphous cellulose (AC). The digestibility ranking is AC>cellulose III>cellulose II>cellulose I. However, AC has the highest initial enzyme binding capacity while cellulose III had the lowest. CBH II is less stable during hydrolysis. Time course binding studies were also performed for pretreated biomass. Ammonia Fiber Expansion (AFEX) treated corn stover (CS), dilute acid (ACID) treated CS and ionic liquid (IL) pretreated CS were compared. The results indicate that presence of lignin is responsible for significant unproductive cellulase binding. These results are critical for improving our understanding of enzyme synergism, productive/unproductive enzyme binding and the role of pretreatment on enzyme accessibility to lignocellulosic plant cell walls. The results also assist in engineering novel low unproductive binding enzyme systems and developing economic enzyme recycle options.

This work is dedicated to my wife Haiyan Cen and my son Nicholas Jiatong Gao

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KEY TO SYMBOLS OR ABBREVIATIONS

acetonitrile (ACN)

dilute acid treated corn stover (ACID-CS)

ammonia fiber expansion (AFEX)

p-aminophenyl-1-thio-β-D-cellobioside (APTC)

ammonia recycle percolation (ARP),

β-glucosidase (βG)

β-xylosidase (βX)

carboxymethylcellulose (CMC)

consolidated bio-processing (CBP)

catalytic domain (CD)

cellobiohydrolases (CBHs)

carbohydrate binding domain (CBD)

Consortium for Applied Fundamentals and Innovation (CAFI)

3,5-dinitrosalicylic acid (DNS)

dissolved oxygen (DO)

degree of polymerization (DP)

degree of synergism (DS)

endo-1,4,-β-D-glucanases (EG)

endoxylanases (EX)

Fungal Genetics Stock Center (FGSC)

Fast Performance Liquid Chromatography (FPLC)

glycosyl hydrolases (GH)

ion exchange chromatography (IEX)

L-arabinofuranosidase (LArb)

α-glucuronidase (LαGl)

Liquid hot water (LHW)

bacterial endoxylanases (LXs)

para-nitrophenyl (*p*NP)

4-nitrophenyl-β-D-xylopyranoside (*p*NPX)

4-nitrophenyl-α-L-arabinofuranoside (*p*NPAf)

4-nitrophenyl-β-D-cellobioside (*p*NPC)

4-nitrophenyl-β-D-glucopyranoside (*p*NPG)

Recombinant endo-glucanase (REG)

trifluoroacetic acid (TFA)

yeast nitrogen base (YNB)

CHAPTER 1 INTRODUCTION

1.1 Background

Depleting crude oil reserves, environmental problems due to green house gas emissions using fossil fuel and long term energy security have attracted significant attention towards the use of alternative renewable resources for production of fuels and chemicals [1-3]. Among many types of biofuels, ethanol is the most employed liquid biofuel [4]. It can be directly used as fuel or mixed with gasoline at different ratios [5, 6]. Currently, 10% ethanol is mixed into gasoline as an oxygenate to boost octane number for combustion engines [7]. Slightly modified engines can run using 85% ethanol (E85) [6]. Almost 90% of Brazilian automotive fuel demand is now met using ethanol produced from sugar cane [5].

In the US, most of the ethanol is produced from corn starch [4]. Nowadays less than 10% of fuel demand is met using corn ethanol and there is a growing controversy of producing fuels out of food materials (about 30% of US corn is used for biofuels production) [8, 9]. Lignocellulosic biomass provides a renewable resource (approximately 1×10^{11} tons) for the sustainable production of biofuels/biochemicals, and could serve as an important contributor to the world energy portfolio in the near future [5]. The US has initiated several biofuels research and development programs that aim to make the cellulosic ethanol cost competitive by 2012 and replace 30% of the US's current fuel use by 2030 [10-12].

Although lignocellulosic biomass is considered as a renewable feedstock for biofuels production, it is quite challenging to convert it to biofuels in an industrial scale. Some of these challenges include: (i) the bulk density of biomass (agricultural residue) is low and has issues related to

logistics on the transportation and storage [13]; (ii) it is highly recalcitrant which makes it harder to digest by enzyme and requires pretreatment which could be an energy intensive process [1, 14]; (iii) high enzyme requirements add operating cost [1, 15, 16] and (iv) other issues related to the xylose utilization during fermentation [17, 18], inhibition of enzymes and microbes by degradation products formed during pretreatment [19-21].

1.2 Objectives

This research applies engineering strategies to optimize enzymatic hydrolysis process of pretreated biomass as well as to determine some important hydrolysis related parameters which are required to achieve fundamental understanding of the hydrolysis mechanism. The specific objectives are:

(1) Purify major biomass degrading enzymes including cellulases and hemicellulases using standard chromatography procedures.

(2) Optimize the ratio of major enzymes for digesting ammonia fiber expansion (AFEX) treated corn stover using statistical engineering design.

(3) Study the auxilliary enzymes (in low abundance but do help improve hydrolysis) performance on AFEX treated corn stover.

(4) Set up the experimental procedures to evaluate enzyme binding properties on biomass.

(5) Investigate the enzyme binding during hydrolysis and evaluate the unproductive binding of enzyme on lignin.

CHAPTER 2 LITERATURE REVIEW

2.1 Lignocellulosic biomass

A potential source for fermentable carbohydrates is lignocellulosic materials from plant cell walls which are composed of up to 75% carbohydrates. Normally, cellulosic biomass contains 40-50% of cellulose, 25-35%, of hemicelluloses, 15-20% of lignin and small amount of minerals, oils, sugar and other compounds[22]. The structure of the cell wall of plants (shown in Figure 2.1) is very complicated with cellulose microfibrils and hemicelluloses branches.

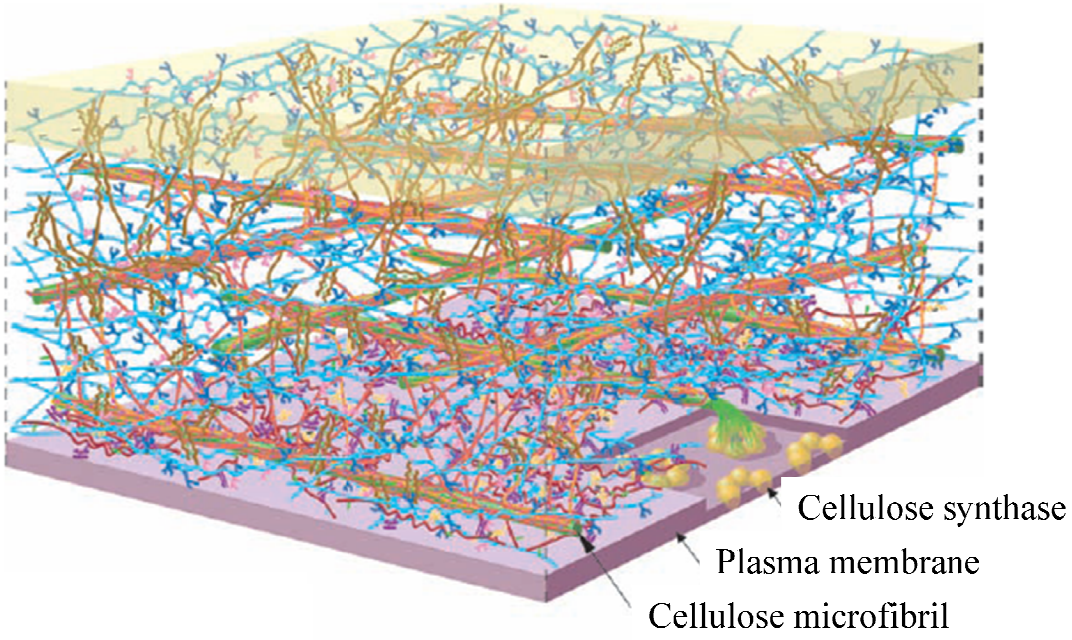


Figure 2.1 Scale model of the polysaccharides in an Arabidopsis leaf cell. Between microfibrils, the hemicelluloses cross-links (shown in orange) are extended[23].

"For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation."

2.1.1 Cellulose

Cellulose, characterized by Anselme Payen in 1838, is the main component in cell walls of higher plants. It is also formed by some algae, fungi, bacteria, etc. About 7.5×10^{10} tons of cellulose is formed and degraded each year, establishing it as the most abundant regenerated material on earth[24]. The structure of cellulose molecules (Figure 2.2) shows that cellulose is a linear polymer consisting of D-glucopyranose joined together by β-1,4 glycosidic bonds with a degree of polymerization (DP) up to 20,000. Anhydrocellobiose is the repeating unit of cellulose[24]. The cellulose molecule is very robust because of the hydrogen bonds and linear crystal structures. The half-times for spontaneous degradation of the glucosidic bond at 25 ºC is around 5 million years[25]. So enzyme-driven degradation of cellulose is very important to degrade the cellulose polymers.

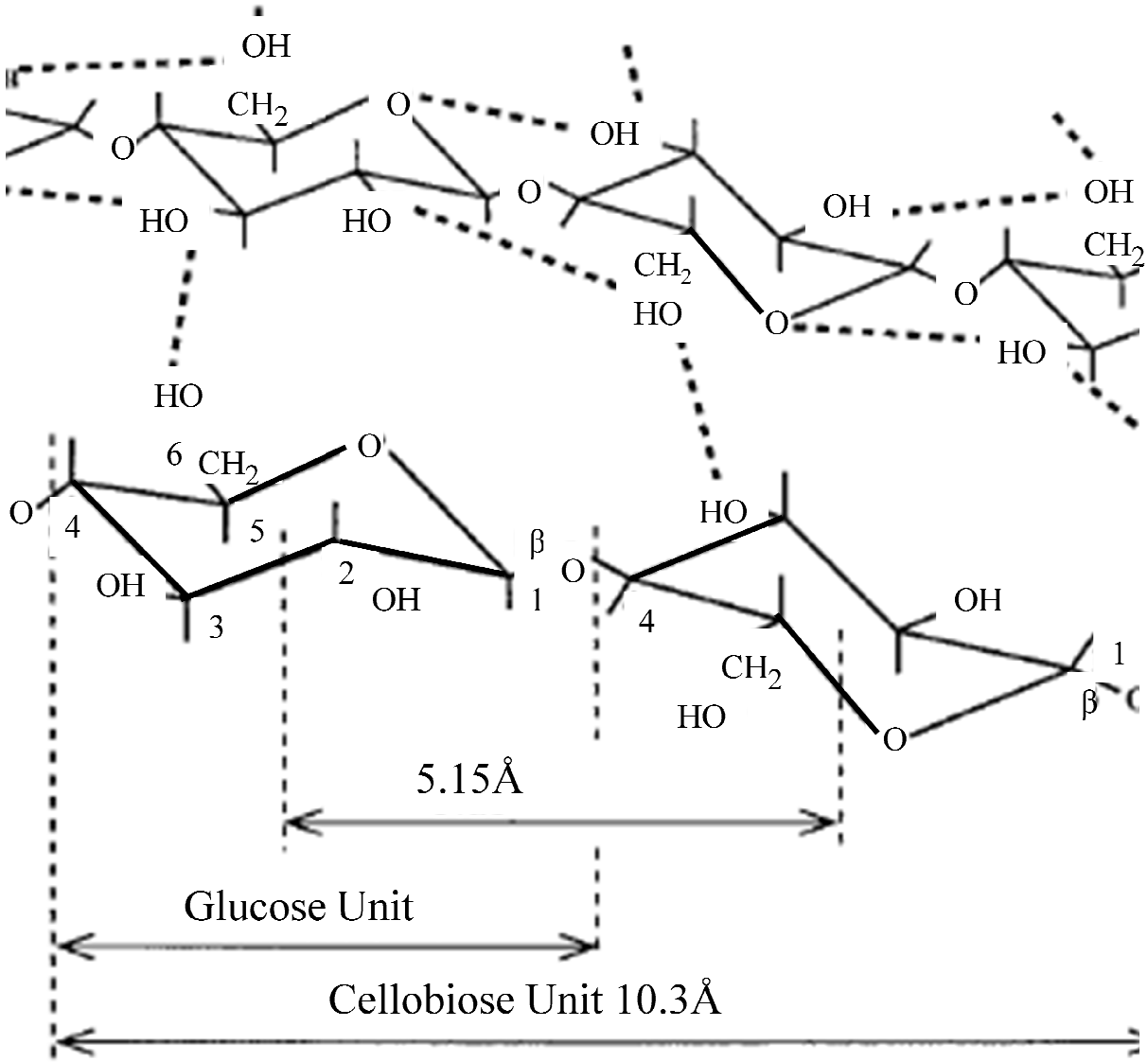


Figure 2.2 Cellulose structures with cellobiose unit linked through β-1,4-glycosic bond.**[26]**

2.1.2 Hemicellulose

Hemicellulose, the second most abundant polysaccharide in nature (about one-third of all renewable organic carbon on earth), is a major polysaccharide in plant cells[27]. It contains the following residues: D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, D-glucuronic acid and 4-O-methyl-D-glucuronic acid[28]. Xylan is the major constituent of hemicellulose. In addition to xylan, hemicellulose also contains other heteropolymers such as xyloglucan (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-glucose and D-mannose), galactoglucomannan (heteropolymer of D-galactose, D-glucose and D-mannose) and arabinogalactan (heteropolymer of D-galactose and arabinose)[29]. Hemicelluloses are connected through hydrogen bonds to cellulose microfibrils and also covalently attached to lignin to form a highly complex structure.

2.1.3 Lignin

Lignin is the main component of vascular plants. It is an amorphous, polyphenolic material composed of three phenylpropanoid monomers: (1) *p*-coumaryl alcohol, (2) coniferyl alcohol and (3) sinapyl alcohol[30].

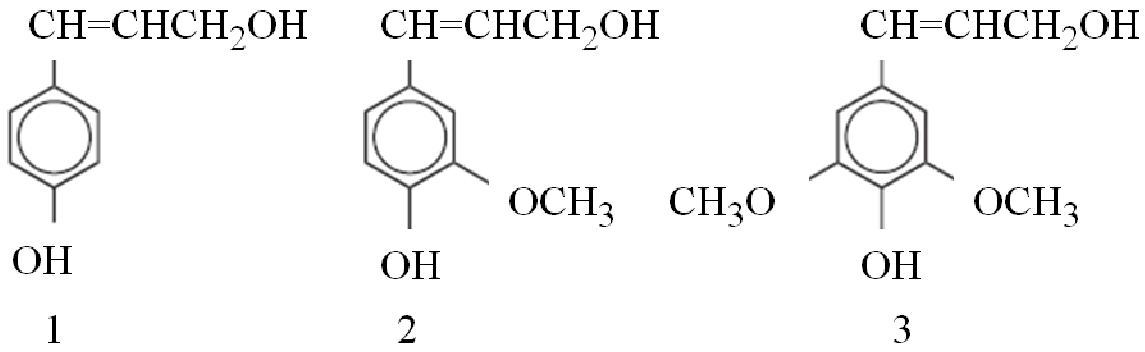


Figure 2.3 Lignin's three components.(1) *p*-coumaryl alcohol, (2) coniferyl alcohol and (3) sinapyl alcohol

Lignin embeds the cellulose and also forms covalent bonds to hemicelluloses to reduce microbial and chemical decomposition. Normally, herbaceous plants have a lower content of lignin, whereas softwoods have higher lignin level[28].

2.2 Pretreatment of biomass

Cellulose can be broken down to glucose either by enzyme hydrolysis or by acid hydrolysis. Generally, conversion of lignocellulosic substrates to ethanol includes four main steps: pretreatment, hydrolysis, fermentation and product separation/purification. Pretreatment is the crucial step which affects the overall biorefinery efficiency[22]. The resistance of lignocellulosic materials to conversion to fermentable sugar and the high cost of enzymes[31] are two major processing obstacles. According to the National Renewable Energy Laboratory's report[32], pretreatment is one of the most costly steps in cellulosic ethanol production, accounting for 33% of total processing costs. Hence, successful pretreatment methods not only can increase enzyme accessibility but also facilitate the downstream hydrolysis and fermentation process.

Although many physical, chemical or biological pretreatment techniques have been studied over many years, the total cost of pretreatment is still a major obstacle to make price competitive bio-fuels. So far, only a few pretreatment methods have been proved to be promising for increasing cellulases digestibility[33] and decreasing cost. Current leading biomass pretreatment technologies can be categorized into three groups: the acid based pretreatment such as dilute acid pretreatment and liquid hot water pretreatment, the alkaline based pretreatment including lime pretreatment, Ammonia recycle percolation (ARP), Ammonia fiber expansion (AFEX) and extraction based pretreatment such as ethanol organosolv pretreatment.

2.2.1 Dilute acid pretreatment

Dilute acid normally refers to 0.5-1.0% sulfuric acid. Although SO_2 can enhance the yield in a way similar to dilute sulfuric acid, it is not preferred from the economics consideration[34]. Acid or SO_2 catalyzed pretreatment can efficiently recover hemicelluloses-derived sugars and improve cellulosic biomass hydrolysis[35]. Despite little lignin being dissolved, results show that lignin is disrupted which increased cellulose susceptibility to enzyme. And it has also been shown that under severe conditions (high temperature, long residence time and high concentration of acid), nearly complete hydrolysis can be achieved. However, a high proportion of sugar was lost due to degradation. And degradation products are the main source of inhibitors of downstream hydrolysis and fermentation[36]. Detoxification techniques such as alkali treatment, sulfite treatment, anion exchange, enzymatic detoxification and other methods are available to decrease the inhibition of degradation sugar. However, the multiple steps involved inevitably increase the whole pretreatment cost. In addition, the corrosion of the equipment by acid solutions is also considered as an important cost factor.

2.2.2 Liquid hot water pretreatment

Liquid hot water (LHW) plays a role similar to dilute acid at hightemperatures. At 200 °C, water's pH is 5.6 compared with pH 7.0 when water is at 25 °C because the pKa of water is affected by temperature. By LHW pretreatment, acetic and uronic acids are formed by breaking hemiacetal linkages of hemicelluloses. Those acids catalyze the removal of oligosaccharides from hemicelluloses[37]. Compared with dilute acid pretreatment, some researchers[38, 39] believe the LHW pretreatment has the potential to increase cellulose digestibility, sugar yield, pentosan recovery[22] with the advantage of little or no inhibition of sugar fermentation[40] as

well as reducing the need for neutralization. Scale-up LHW treatment at a pilot plant has been done and hydrolysis cost is estimated equivalent to less than $0.84/gal of ethanol produced from the corn fiber[41].

2.2.3 Lime pretreatment

Lime (calcium hydroxide) is added to biomass as a pretreatment agent to enhance the enzymatic digestibility[42]. Approximately 10% of glucan is solublilized after treatment. During lime treatment, some lignin is removed, which is believed to be one of the reasons to increase biomass enzyme accessibility. Lime treatment method shows significantly enhanced enzymatic digestibility when dealing with low-lignin biomass materials, such as switchgrass, bagasse and wheat straw.

Compared with the NaOH (0.68$/kg)[43] pretreatment method, lime (0.06$/kg)[43] pretreatment is much cheaper. And the potential to recover lime[44] can also decrease the final cost. If the operation is employed under 100 °C, no pressure reactors will be required and cost efficiency will be improved consequently. In addition, lime has less health hazard concerns than some other pretreatment chemicals. However, the water loading required is more than 5 g H_2O/g biomass, which could be a problem either from the perspective of cost or industrial process burden. Also, some glucan dissolves in the water and results in the loss of substrate.

2.2.4 Ammonia recycle percolation

Ammonia has several advantages as a pretreatment agent. It is an effective swelling reagent for lignocellulosic biomass. It has high selectivity for reactions with lignin over with cellulose. And it is relatively easy to recover due to its highly volatile character. And as an industrially favored chemical, it is regarded as non-polluting and non-corrosive chemical with only one-fourth the

cost of sulfuric acid on molar basis. Kim *et al*.[45] studied the pretreatment effect on corn stover using aqueous ammonia in a flow-though column reactor. The corn stover feedstock was presoaked overnight in 15% NH_3, and then followed by the ammonia recycle percolation (APR) process at 170 °C and 15% of NH_3. The results show that the ARP process removed 70%-85% of the total lignin and that 70% of lignin was removed within 10 min of treatment. Approximately half of the hemicelluloses were also solubilized. ARP reduced the total solid mass and the solid remaining was in the range of 53.6%-61.4% of the starting material. Over 95% of the glucan and nearly 100% for xylan were preserved in the solid. The enzymatic digestibility at 60 and 10 FPU/g of glucan were near 100% and 92.5% respectively[45].

2.2.5 Ammonia Fiber Expansion

Ammonia fiber explosion (AFEX, now called ammonia fiber expansion) pretreatment was invented by Dale *et al [14, 15, 46, 47]*. In AFEX the lignocellulosic biomass was treated with liquid ammonia at temperatures (60-100 °C) and high pressure (250-300 psi) for 5-10 min., followed by rapidly releasing pressure. Both temperature and pressure decreased rapidly. The surface area available to microbial attack is increased, which was believed to be the primary mechanism by which AFEX treated biomass achieves the theoretical yields with lower enzyme loading (<5 FPU per gram of biomass)[48]. Lignin is cleaved and some is deposited on the surface of the biomass[49]. Compared with other pretreatment methods, AFEX has some unique characteristics. It can treat biomassthe samples without involving any aqueous solution[50] .The treatment is relatively rapid and consequently has the potential to get high efficiency treatment in industrial application. Most of the ammonia can be recovered and used for the next batch of treatment. Residual ammonia can be used as nitrogen source for downstream fermentation.

Because there is no wash stream required, cellulose and hemicelluloses are well preserved with little degradation product formation[51].

So far, AFEX has been applied to several cellulosic biomass materials such as alfalfa[52], *florigraze rhizoma* peanut[48], switchgrass[31], corn stover[53], reed canarygrass[54]. Various parameters affect AFEX treatment performance including, temperature, ammonia loading, retention time, particle size[49] and moisture content of biomass.

2.2.6 Ethanol Organosolv

This process extracts most of the lignin from lignocellulosic biomass using an ethanol and water mixture at around 200 °C and 400 psi[55]. The diluted spent liquor was processed by flashing and lignin was recovered as a precipitate. The cellulose and some residuals of lignin and hemicelluloses were retained in the pulp. Enzyme hydrolysis results showed that the lower the remaining lignin content, the higher cellulose to glucose conversion yield is achieved. The four independent operation conditions affecting the pretreatment include: temperature, time, catalyst dose and ethanol concentration[56]. Besides the cellulose substrate for fermentation obtained, many other co-products are also available after pretreatment. The multi-products refinery plant has the potential to offset the cost to produce ethanol by producing other materials or chemicals such as lignin, acetic acid, furfural and so on.

2.3 Enzyme systems

2.3.1 Cellulase

The half-times of the glucosidic bond at 25 ºC is around five million years [25]. So the enzyme-driven degradation of cellulose is very important to break the cellulose polymers. The exo-1, 4-β-D-glucanase or cellobiohydrolases (CBHs) move along the cellulose chain and release cellobiose

or glucose units from the end. CBH I breaks the cellulose from the reducing end [57] and for CBH II, it breaks from the non-reducing end [58]. However, CBH II is suspected to have some characteristics typical of endoglucanases [59, 60]. The endo-1,4,-β-D-glucanases (EG) hydrolyze internal glycosidic bonds randomly inside cellulose chains [61, 62]. 1,4-β-D-glucosidases, which mainly hydrolyze cellobiose to glucose, also cleave glucose units from cellodextrins with DP up to six [63].

2.3.2 Hemicellulase

Hemicellulases constitute two major classes of enzymes namely glycoside hydrolases and carbohydrate esterases. Xylanases (EC 3.2.1.8) break the xylan backbone at β-1,4 bonds [64-66]. Family 10 xylanases cleaves β-1,4 linkages at least one un-substituted xylopyranosyl residue adjacent to substituted xylopyranolsyl residues from reducing end. Family 11 xylanases only cleave from at least two un-substituted xylopyranosyl residue [67]. β-Xylosidases (EC 3.2.1.37) are exo-glycosidases that hydrolyze short xylooligomers into xylose units [64, 65, 68]. β-Mannanases hydrolyze mannan and release β-1.4-manno groups which can be further hydrolyzed to mannose by β-mannosidases (EC 3.2.1.25) [29]. The arabinofuranosidase (EC 3.2.1.55) removes arabinose and α-glucuronidase cleaves the α-1,2-glycosidic bond of the 4-O-methyl-D-glucuronic acid side chain from the xylan backbone [29, 69]. The major esterases are acetyl xylan esterases (EC 3.1.1.72) and ferulic acid esterases (EC 3.1.1.73), which hydrolyze acetyl groups on xylose moieties and the ester bond between the arabinose and ferulic acid respectively [29]. Characterization of enzymes by their substrates sometimes face limitations because some enzymes have multiple substrate activities. Some xylanases can hydrolyze cellulose [26] and EG I has xylan degradation activities [70, 71].

Table 2.1 Major enzymes and their functions on cell wall polymers.

Enzyme	Function	Ref.
Cellobiohydrolyase I (CBH I)	Break the cellulose chain from the reducing end and release cellobiose.	[57, 58]
Cellobiohydrolyase II (CBH II)	Break the cellulose chain from the non-reducing end and release cellobiose. Suspected endo-activities	[58, 59, 72]
Endo-glucanase (EG)	hydrolyze internal glulosidic bonds randomly inside cellulose chains. EG I also has significant xylanase and xyloglucanase activity	[61, 62, 70]
β-Glucosidase	Hydrolyze cellodextrins (DP 2-6, cellobiose, cellotriose, cellotetrose, cellopentose, cellohexose) into glucose	[63]
Endo-xylanase (GH 10)	cleaves β-1,4 linkages at least one unsustituted xylopyranosyl residue adjacent to substituted xylopyranolsyl residues from reducing end	[67]
Endo-xylanase (GH 11)	clearves from at least two unsubstituted xylopyranosyl residue	[67]
β-Xylosidase	hydrolyze short xylooligomers into xylose units	[64, 65, 68]
β-Mannanases	hydrolyze mannan and release β-1.4-manno oligomers	[29]
β-Mannosidases	Further hydrolyze manno oligomers into mannose	[29]
α-Arabinofuranosidases	remove arabinose from the xylan backbone	[29, 69]
α-D-Glucuronidases	cleave the α-1,2-glycosidic bond of the 4-O-methyl-D-glucuronic acid side chain from the xylan	[29, 69]
Acetyl xylan esterases	hydrolyze acetyl groups on xylose moieties	[29]
Ferulic acid esterases	hydrolyze the ester bond between the arabinose and ferulic acid	[29]
GH 61 enzymes	No major detectable activity. When adding with other enzyme, hydrolysis yield is increased	[73, 74]
Expansin protein	No detectable activity, disrupting the hydrogen bonds in cellulose, increase cellulase activity	[75, 76]

2.3.3 Other proteins

Some enzymes or proteins are identified to have no specific acidities toward cellulosic substrates, yet they increase the hydrolysis yield when combined with other cellulases and hemicelluloses. The newly discovered family GH 61 enzymes have little detectable activity on glucan. However, supplementation of GH 61 enzymes in cellulases mixture can decrease enzyme loading by 2 fold

[73]. The proper functioning of GH 61 enzyme requires a metal ion which is very different from other fungal enzymes [73]. Other expansin-like proteins have been found to increase the cellulase activity possibly by disrupting the hydrogen bonds in cellulose [75, 76]. Some properties of these enzymes are summarized in Table 2.1.

2.4 Enzyme synergism

The term synergy for enzyme hydrolysis is defined as the circumstance in which the amount of reducing sugar produced when two or more enzymes acting together is greater than the summation of the individual enzyme acting alone. Enzyme synergism is classified into two categories (i) one of the enzyme removes the major inhibitors of other enzymes. (e.g., β-glucosidase synergism involves the hydrolysis of cellobiose which is CBH I's inhibitor [16, 77]; endo-xylanases and xylosidases can break xylooligomers into xylose hence remove the inhibition caused to cellulose [78]). (ii) one of the enzyme can increase the accessibility of other enzymes on substrates (e.g., endo-exo synergism, a cellulose chain is attacked by endoglucanases by the random scission and generates more chain ends for cellobiohydrolases; exo-exo synergism, two exo acting in concert compared to individual activities [79]). Other possible synergies are, for example, whee hemicellulases remove hemicellulose wrapped around the cellulose micro fibrils and thereby increase the cellulases accessibility. The fundamental mechanism of synergisms is not well understood from a molecular basis due to lack of direct observation and debate regarding the specific action of individual cellulases. Some proposed mechanisms cannot explain other phenomena such as the lack of synergistic effect among cellulases from different microbial strains. [80].

2.5 Major challenges of enzymatic hydrolysis

Compared with easily hydrolysable carbohydrates (like corn grain starch), lignocellulosic biomass faces many challenges when used as feedstocks for industrial scale biofuel production. Compared to corn ethanol production in Table 2.2, the high cost of enzyme, long hydrolysis time, and low concentration of fermentable sugar in final hydrolyzates as well as the presence of unfermentable oligosaccharides all impede the industrial scale production of biofuels by using cellulosic feedstocks.

Table 2.2 Comparison of ethanol produced from corn kernel and cellulosic biomass

	Corn	Cellulosic biomass
Main Substrate	Amylose (α-1,4-linkage of glucose) and amylopectin (α-1,6-linkage of glucose)[81]	Cellulose (β-1,4-linkage of glucose) Hemicellulose, Lignin
Pretreatment	Mild	Intensive
Enzyme	α-Amylase Glucoamylase [4]	Cellulases Hemicellulases
Saccharification Temperature	90-110ºC, liquefy starch 60-70ºC, saccharification	50ºC
Enzyme dosage and cost	$0.02-0.05/gallon ethanol[82]	20-30 mg/g glucan $0.72/gallon ethanol[83]
Hydrolysis time	Hours to 1 day [81].	Up to several days
Hydrolysate	High concentration of fermentable sugars (glucose, maltose, maltotriose)[82]	Monomer glucose, xylose, arabinose at moderate concentration, significant amount of unfermentable oligosaccharides.[18] Inhibitory degradation product [19]. Insoluble lignin.
Expected ethanol concentration	Batch 80-100g/L[4]	Batch 40g/L [18] SSF 32-35g/L [84]

2.5.1 High cost of commercial enzymes

The most popular commercial enzymes currently available for the biomass hydrolysis are produced by the submerged fermentation of the fungus *T. reesei* [85]. Several mutants have been isolated and the cellulase productivity using the strain has been improved by more than 20 fold

over last several decades [86]. In addition, the fermentation conditions have also been optimized for growth medium including salts, nutrient, surfactant and inducer [87]. All those factors could affect the enzyme yield as well as the relative ratios of individual enzymes at different pH and temperature values. The submerged fermentation process requires higher capital cost, including higher demand for nutrient supplements as well as enzyme inducers [85, 88].

2.5.2 Low activity of enzymes

The Table 2.3 summarizes the specific activity for some enzymes when compared to commercial cellulases (from BRENDA database, http://www.brenda-enzymes.info/). Regardless of major difference in substrates and reaction conditions, commercial cellulases are still inefficient when compared to other enzymes used in various biological processes.

Table 2.3 Specific activity for different enzymes compared to commercial cellulases.

Enzyme	Specific activity*
H. sapiens Catalase	273,800
Aspergillus awamori glucoamylase	21,000
Bacillus sp. α-amylase	5,009
B. taurus DNase I (endo)	1,090
S. cerevisiae Hexokinase	120
H. sapiens DNA pol III	5.3
(Spezyme CP+ Novozyme 188) on Avicel	0.14

*1 μmol product/min/mg enzyme (specified conditions)

The primary reason for this apparent inefficiency is that cell walls are highly recalcitrant. For cellulose, intra and inter molecule hydrogen bonds lock the cellobiose unit in a tight packed structure [89]. Substantial energy is required either to peel the glucan chain or to cleave glycosic bond, thereby resulting in the low enzyme activity [90]. For cell walls, the existence of hemicellulose and lignin provides another steric hindrance and chemical shield against enzyme attachment to the cellulose. In other words, the structure of the cell wall is comparable to a

concrete structure where one can visualize the cellulose microfibrils as steel rods embedding in the mix of lignin and hemicellulose acting as cement [91, 92]. Such structures not only protect the cellulose from cellulases enzymes attack, they also provide the strength to biomass to resist tough environmental conditions.

2.5.3 Hydrolysis rate decreasing and lower sugar yield at high solid loading

The slowdown in the enzymatic hydrolysis rate with the increasing sugar conversion has been reported [93, 94]. The mechanism behind the phenomenon is still poorly understood [95]. It is widely believed that the substrate is becoming less reactive during hydrolysis. However the reactivity of substrates is difficult to measure [96]. Other proposed reasons include: (i) enzyme lose activity during hydrolysis [97, 98]; (ii) enzyme bind unproductively [95, 99] to lignin and (iii) inhibition by degradation product formed during pretreatment and end-product such as sugars and oligosaccharides [19-21, 100].

Yang and Wyman [101] have claimed that the decline of the hydrolysis rate for the pure cellulose is not due to decreased substrate activity. By restarting the hydrolysis at different conversion, almost same initial hydrolysis rate was observed. One possible explanation for such observations is that enzymes might be getting “stuck” during the hydrolysis process [101]. Similar results were found on dilute acid pretreated wood [96]. However, it is not clear whether the biomass is still same after extensive washing by salts and other reagents. Hodge et al found that the high sugar concentration is the major reason for the enzyme inhibition, while acetic acid (15 g/L) and phenolic compounds (9 g/L) and furans (8 g/L) have limited effect on reaction kinetics [21]. Kristensen reported that at a high solid loading, the binding efficiency of enzymes on biomass decreases and thereby results in a low sugar yield [93].

2.6 Current progress on enzymatic hydrolysis

2.6.1 Increase substrates digestibility by pretreatment

Different pretreatment technologies for decreasing the recalcitrance of cell walls [102, 103], increasing the cellulase accessibility and converting the highly crystalline cellulose to more amorphous structures [104-106] have been studied extensively [15, 35, 103, 107-109]. AFEX is an alkaline based “dry to dry” (i.e. no separate liquid fraction arises from the pretreatment) process and the composition of pretreated biomass is almost identical to that of untreated biomass [15]. In contrast, dilute acid pretreatment can remove a significant amount of hemicelluloses (60-80%) and some lignin (<5%) [14]. The degree of polymerization (DP) of cellulose is largely unchanged after AFEX pretreatment (~ 6000-7000 for corn stover), unlike dilute acid treatment that results in the decrease of the DP by 60-70% [110]. Both of these thermo chemical pretreatments are believed to increase the cellulose accessibility to cellulases through various ultra-structural and physicochemical changes that are not currently well understood [14, 19, 111]. The physiochemical properties of the pretreated biomass strongly influence the downstream hydrolysis and fermentation processes.

2.6.2 Hydrolysis of pretreated biomass using crude enzyme mixtures

Crude fungal enzymes from commercial sources normally contain many different types of proteins and enzymes. The available commercial enzymes cocktail for lignocellulosic biomass hydrolysis normally can be categorized into cellulase, such as Celluclast, Spezyme CP, Accellerase 1000, CTec, etc.; β-glucosidase, such as Novozyme 188, Accerllerase BG, etc.; hemicellulase such as Multifect xylanase, HTec, etc.; and pectinase such as Multifect Pectinase. Mostly, those commercial cocktails contain cross activities on cellulose, hemicellulose and other intermediate hydrolysis products such as cellobiose, xylobiose. The complexity of commercial

enzyme is seen from SDS-PAGE and proteomics analysis (As shown in Figure 2.4 and Table 2.4 [111]).

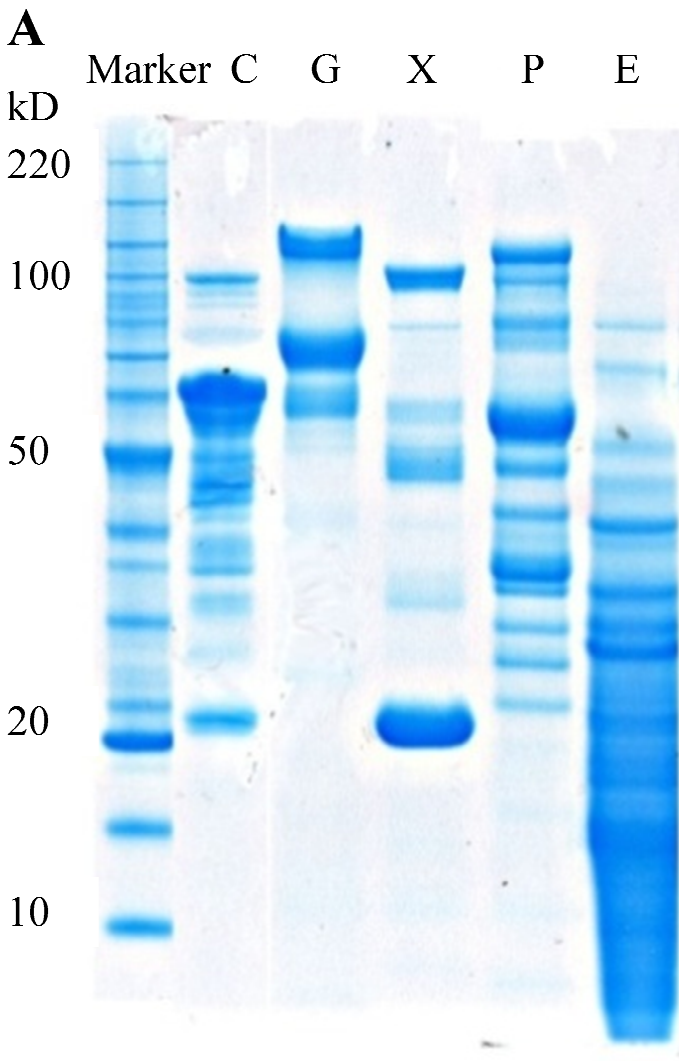


Figure 2.4 (A) SDS-PAGE of some commercial enzymes. C: Spezyme CP; G: Novozyme 188; X: Multifect Xylanase; P: Multifect Pectinase; E: Depol 670L

Table 2.4 Proteomics data for some commercial enzymes. The abundance of each protein is reflected by the peptide count number detected during analysis.

GH family	Enzyme Description	Spezyme CP	Novo 188	Multifect Xylanase	Multifect Pectinase	Depol 670L
	acetyl esterase	1.2		4.4		0.2
	acetyl xylan esterase I	0.7		1		
	acetyl xylan esterase II	0.2		0.4		
	Acetyl Xylan Esterases	2		5.8		0.2
	feruloyl esterase	-	0.2	-	1.2	-
	Feruloyl Esterases		0.2		1.2	
1	β-glucosidase					
3	β-glucosidase	4.1	7.7	2.9	4.6	2.2
	β-Glucosidases	4.1	7.7	2.9	4.6	2.2
3	β-xylosidase	3	1.1	6.5	4	-
	β-Xylosidases	3	1.1	6.5	4	
6a	exocellulase II	10.4	0.1	1	0.3	13
7a	exocellulase I	27.5	0.3	2	0.8	23
	Cellobiohydrolases	37.9	0.4	3	1	36.1
10	endoxylanase II	1.8	1.3	0.6	2.6	0.4
11	endoxylanase I (all family 11 included)	2.6		35.8	1.8	4.1
	Endo-xylanases	4.4	1.3	36.4	4.4	4.5
5	endoglucanase II	5.3	0.6	0.2	3.1	7.6
6	endoglucanase					0.4
7b	endoglucanase I	7.5		0.2		4.6
12	endoglucanase III	2.4	0.2	0.4	1.7	2.8
45	endoglucanase V					0.3
61a	endoglucanase IV	0.5		0.9	0.2	0.7
61b	endoglucanase VII	0.2		0.1		
	Endo-glucanases	15.8	0.8	1.9	5.1	16.5
43	arabinan arabinosidase		0.5		3.1	
51	α-arabinofuranosidase		0.9		3.6	
54	α-arabinofuranosidase	0.9	1.1	1.2	7.4	1.3
62	α-arabinofuranosidase	1		1.2	2.8	1.4
	Arabinofuranosidases	1.9	2.5	2.5	16.9	2.7
67	α-glucuronosidase	2.4		2.5	1.4	-
	α-Glucuronosidase	2.4		2.5	1.4	
74	xyloglucanase	11.3		21.3	0.7	12
	Xyloglucanases	11.3		21.3	0.7	12

Table 2.4 (cont'd)

GH family	Enzyme Description	Spezyme CP	Novo 188	Multifect Xylanase	Multifect Pectinase	Depol 670L
13	α-amylase	0.4	3.4		1.2	0.2
15	glucoamylase		40.2	0.2	3.3	2
	Amylases	0.4	43.6	0.2	4.4	2.2
	lyases (all PL families			-	4.2	0.5
	pectin methyl esterase				2.6	1.4
	rhamnogalacturonan acetyl				0.5	1.5
	α-rhamnosidase		0.2		1.5	
28	polygalacturonases (endo/	0.1			6.2	6.1
	Pectinases	0.1	0.2		15	9.5
	other noncellulolytic proteins	2.6	27.4	5.6	20	3
	cip protein 1	2.7		2.2		2.5
	cip protein 2	3		2.1		1.1
	swollenin	3.2				1.6
2	β-mannosidase		1.7		2	0.2
5	β-mannase	1.4		0.8	0.4	3.3
5	β-1,6-endogalactanase	0.7		1.5		0.4
16	GH 16 glycosyl hydrolase		1.4		0.6	
17	β-1,3-glucanase		1.3		0.7	
18	chitinase (endo)	0.2	0.1	0.3	0.2	
20	chitinase (exo)	0.3	1.1	0.3	0.3	
27	α-galactosidase	0.1	3.5	0.4	1.8	0.4
30	glucanase, glucosylamidase, glucuronoxylanase	0.2	0.4		0.1	
31	α-glucosidase		1		2.7	
32	β-fructofuranosidase		1.1		0.3	0.1
35	β-galactosidase	2.3	1.4	3.9	8.3	0.2
47	mannosidase (in oligomannosaccharides)				1.4	
53	arabinogalactan beta-galactosidase				1.2	1.2
55	β-1,3-glucanase (endo/exo)		0.8		1.3	
65	trehalase				0.4	
71	α-1,3-glucanase				0.1	
72	glycosyl transferase		0.3		0.6	
76	α-1,6-mannase				0.1	

Table 2.4 (cont'd)

GH family	Enzyme Description	Spezyme CP	Novo 188	Multifect Xylanase	Multifect Pectinase	Depol 670L
79	β-glucuronidase				0.8	
81	β-1,3-1,4 endoglucanase				0.1	
92	α-1,2-mannosidase		0.5		0.5	
	Other proteins	16.6	42.1	17.1	44.1	14.2

To measure those crude enzymes activities, artificial substrates or substrates which were subjected to significant chemical modifications have been used such as Avicel, xylan, carboxymethylcellulose (CMC), cellobiose, xylobiose and *para*-nitrophenol based glycosides [112, 113]. Activities based on the standard assay have limited ability to predict the real performance of those enzymes on the whole cell wall or the pretreated biomass [114]. Because hydrolysis normally operates in days whereas the activity assay normally is done in minutes/hours, enzymes stability during a long period of hydrolysis is also not reflected. In addition, the artificial substrates have many differences in physical and chemical structures when compared to the pretreated cell walls. Many effects such as unproductive binding of enzyme on lignin, inhibition of degradation products formed during pretreatment and heterogeneous structure of the cell walls cannot be mimiced using the artificial substrates only [115].

Many other researchers have focused on adding the commercial enzyme cocktail to evaluate biomass digestibility for a specific pretreatment. Kumar et al. compared the effect of xylanases supplements on the corn stover pretreated by different pretreatments and found the glucose yield increased linearly with the remove of xylan for all pretreatments [100]. Some reports compare different pretreatment technologies with different cellulosic biomass (corn stover, poplar,

switchgrass) using commercial crude enzymes, for example the reports by the Consortium for Applied Fundamentals and Innovation (CAFI) group [116-124].

2.6.3 Hydrolysis studies by using purified enzymes

Many researchers use artificial pure cellulose substrates, such as Avicel, CMC, filter paper, swollen cellulose. Irwin et al. measured the activity of the purified *T fusca* cellulases in the presence of *T reesei* enzymes (CBH I, CBH II). Addition of CBH I to *T. fusca* crude cellulase increased 1.7 fold itsactivity toward filter paper. Wood et al. found the optimal ratio of CBH I and CBH II was 1:1 and trace amount of endoglucanase activity enhanced CBHs mixture effective on hydrolyzing cotton [62]. Baker et al. compared the performance of 5 ternary enzyme cocktails of cellulase from different strains and found that the CBH I from *T. ressei*, an endoglucanases from *A.cellulolyticus*, and an exo cellulase delivered high hydrolysis rates over a wide range of mixture compositions on microcrystalline cellulose [125]. But the highest yield is less than 20% due to the low enzyme loading range selected by the authors. Similarly, Kit et al. optimized 6 cellulases mixtures on the filter paper and observed significant synergism among those cellulases [126]. The degree of synergism (DS), [127] which is defined as the activity exhibited by mixtures of enzyme components divided by the sum of the activities exhibited individually by the enzymes, is commonly used by many researchers. DS values larger than two are very common and in some cases, the DS was larger than five [128]. The DS is closely related to and affected by the dosage of the enzyme loading, characteristics of substrates, extent of hydrolysis, substrate concentration, etc [129, 130]. Hence it is difficult to compare DS from different researchers. Boiset et al. found the mixture of enzymes giving rise to the highest saccharification rate did not always correspond to mixtures with highest DS [131]. Therefore, from the engineering point of view, it is preferable to use the hydrolysis yield to evaluate the

hydrolysis. Hydrolysis yield not only reflects the extent of hydrolysis but also the available sugar for the downstream fermentation. In addition, the yield is an important factor for determining the capital and process cost in the large scale biofuel production.

The native *T. ressei* normally produces 40-60% CBH I, 12-20% CBH II, 5-10% EG I and 10-30% of other enzymes [132, 133]. This ratio indicates substrate-specific gene regulation and response in *Trichoderma* strain [132]. These enzyme cocktails may not work well for all pretreated biomass. Therefore, the ratio optimization of an individual enzyme for a specific pretreated biomass has an obvious engineering significance. The optimized cocktail can reduce the enzyme loading without sacrificing the hydrolysis yield or increasing the sugar yield in shorter incubation time. Eventually, it could help construct multi gene expression systems in fungi to produce optimized enzyme mixtures [134] or help more rationally blend crude enzyme mixtures from different strains. In addition, it helps to determine the critical enzyme therefore rationally design heterogeneous expression of the enzyme during consolidated bio-processing (CBP) [32, 135-137].

For the ratio optimization applied on pretreated biomass, Rosgaard et al. optimized CBH I, II, EG I, II on barley straw with different pretreatment methods[71]. The steam explosion and liquid hot water pretreated straw had the similar optimum ratio of CBH I, CBH II and EG I (0.43:0.20:0.37) while EG II was not required. If the biomass is first soaked with acid and then steam explosion pretreatment is performed, the EG I's ratio decreased to 0.27 from 0.37. The acid soaking hydrolyzes a part of the xylan and EG I has a significant activity on xylan hydrolysis [71]. Partial removal of xylan helps to reduce EG I and also facilitates the improved cellulose hydrolysis (20% yield increased compared with other two pretreatmented straw) [138]. Kim et al. optimized the five *Thermomonaspora fusca* cellulase with or without CBH I from *T*

reesei and found the enzyme cocktail with CBH I exhibited three times the yield when compared to non-CBH I contained cocktail. The optimized enzyme cocktail required 43.3% of CBH I in total enzymes [126]. Selig et al. found significant increases in the depolymerization of the liquid hot water pretreated corn stover by adding purified xylanase and esterases to CBH I. Xylanase and esterase work synergistically on the xylan hydrolysis and in turn improve the cellulose hydrolysis [139]. Zhou et al. optimized purified enzymes from *Trichoderma viride* on the steam-exploded corn stover and found the optimized relative ratio for CBH, EG and βG was 62.0%, 35.2% and 2.8% respectively which increased the glucose yield by 2.1 folds to 72.4% when compared to crude cellulase enzyme preparation [140]. Benko et al. reported that xyloglucanase (EG I, EG III and Cel74A xyloglucanase) can increase the hydrolysis yield on 11 different pretreated biomass with the combination of purified CBH I, II, EG II and βG [70].

2.6.4 Enzyme binding characteristics

Cellulase typically contains a catalytic domain (CD) and a carbohydrate binding domain (CBD) [141] joined by an extended interdomain linker peptide [142]. The extent of binding and processive hydrolytic action of CBHs on crystalline cellulose depends on both the CBD and CD [57, 142, 143]. CBDs are thought to enhance the CD hydrolysis efficiency on the insoluble substrates via increasing the local surface bound enzyme concentrations [141, 142, 144]. Binding of the cellulase enzymes precedes their hydrolytic actions. Therefore, it is critical to obtain a better understanding of the binding properties of individual cellulases on pretreated lignocellulosic biomass.

Currently, most cellulase binding studies fall into three categories: 1) quantification of the enzymes present in the supernatant using electrophoresis [145, 146]; 2) monitoring the modified enzymes with fluorescent tags [33, 147] or isotope labels [148, 149]; and 3) measuring the total

protein concentrations [150-153] or cellulase activity [154, 155] remaining in supernatant. The characteristic advantages and limitations of each technique are listed in Table 2.5.

Electrophoresis based quantification method is a simple technique with reproducibility problems which make it a semi-quantitative method for proteins that are able to be separated via electrophoresis. Fluorescent labeling methods are typically suitable for pure cellulosic substrates (e.g. Avicel, phosphoric acid swollen cotton, bacterial cellulose). Lignocellulosic biomass normally exhibits strong auto-fluorescence which can overwhelm the protein signal [33]. In addition, various labeling procedures can modify the native protein structures and thereby affect their binding properties. Protein radio-labeling, which requires specialized equipment, is labor intensive and hence is not widely used [148, 149]. Methods that measure total protein using bicinchoninic acid or other chemical reagents are unable to quantify individual enzymes in complex enzyme mixtures and are also prone to interference from reducing sugars, lignin phenolics and other non-hydrolytic proteins present within cell walls.

Table 2.5 Currently available cellulase adsorption measurement methodologies

Method	Main principle	Advantages	Limitation	References
SDS-PAGE	Enzyme concentration by relative band density on gel	Easy to operate, minimal interference by non-proteinaceous compounds	Semi-quantitative, Not applicable to proteins with similar molecular weight	[145, 146]
Fluorescent labeling	Label enzymes with fluorescent groups for quantification	Simulataneous quantification of multiple enzymes by labeling different emission wavelength fluorescent markers	Fluorescence bleaching, interference due to lignin/phenolic autofluorescence, risk modifying enzyme binding	[33, 147]
Isotope labeling	Culture strains on isotope labeled media and purify expressed enzymes	Quantify individual enzyme in mixture	Labor intensive, special equipment necessary	[148, 149]
Total protein	Measuring total nitrogen, or using Bradford or BCA reagents to quantify total protein concentration	Quick, simple, inexpensive measurement without need for sophisticated labware	Cannot differentiate between enzymes, interference by background sugars and phenolics	[150-153]
Enzyme activity	Measuring supernatant enzyme activities to correlate to enzyme concentration	Easy to assay, minimal interference by non-enzymatic proteins.	Lack of suitable substrates with enzyme specificity,	[154, 155]
FPLC	Using ion-exchange column to quantify individual enzymes with different *pI* by correlating to UV absorption peak area.	Quantify individual enzymes, accurate, minimal interference by background sugars and salts.	Low throughput and laborious, interference by UV absorbing compounds, applied to two enzyme based mixtures	[79, 156]

Hence, developing a robust analytical method that can quantify individual cellulases present within lignocellulosic hydrolyzates is critical to improve our understanding of the enzyme synergism, productive/unproductive enzyme binding and the role of pretreatment on the enzyme accessibility to lignocellulosic plant cell walls.

Fast Performance Liquid Chromatography (FPLC) based separation and quantification of proteins has been used previously by Medve et al. for CBH I/CBH II [156] and CBH I/EG II [79] mixtures hydrolyzing microcrystalline cellulose. Separation of these proteins is possible due to differences in their isoelectric points (*p*I). Enzymes bound on ion-exchange columns can be eluted separately by linear salt gradients. Pretreated cell walls have additional UV absorbing compounds (e.g. aldehydes, phenolics) that can interfere with the separation and quantification of enzymes [19].

2.6.5 Others

Other research to improve the hydrolysis efficiency include using genetic engineering tools to increase the yield of biomass or modify other characteristics of the biomass in favor of the fuel production [157], as well as modified lignin structures to the facilitate pretreatment [102, 158]. Protein engineering has been used to increase individual enzyme's stability and activity [159].

CHAPTER 3 GLYCOSYL HYDROLASES EXPRESSION AND PURIFICATION

3.1 Introduction

To our knowledge there have been few reports of the production of biomass hydrolyzing enzymes by culture of recombinant *Pichia pastoris* although several industrial enzymes have been produced from this yeast [160]. The recombinant cellulases produced by fermentation of *P. pastoris* were tested for their ability to act in synergism with purified commercial enzymes. An

advantage of using heterologous expression for protein isolation is to minimize the purification steps necessary to achieve high enzyme purity.

Fast-flow Protein Liquid Chromatography (FPLC) based methods have been used to purify cellulase enzyme components from *Trichoderma viride* [161], *Pennicillium brasilianum* [162], *Aspergillus sydowii* [163], and *Trichoderma reesei* [164]. It has been reported that CBH II from *Trichoderma reesei* is difficult to purify from enzyme mixtures due to the tendency of CBH II and EG's to form aggregates in solution[165]. To separate CBH from EG, *p*-aminophenyl β-cellobioside has been used as an effective affinity ligand to capture CBH[166]. However, this affinity resin is expensive, requires a hydrogenation reaction under platinum catalyst for synthesis and is not available commercially. Trace contamination of endoglucanases appearing in purified CBH's could result in confounded results during activity assays. Researchers have observed purified CBH's with very low CMCase activity, possibly due to endoglucanase contamination, resulting in conflicting reports [62, 164, 167]. Therefore, it is imperative to develop high-throughput purification methods that isolate high purity proteins for enzyme-synergy investigations. In this study, common high performance ion exchange and hydrophobic interaction based chromatographic columns were used to isolate electrophoretically pure cellulases and hemicellulases from commercial enzymes and culture broths of heterologously expressing recombinant yeasts.

3.2 Enzyme production

3.2.1 Organism

Recombinant *P. pastoris* strains containing cellulase and xylanase genes were obtained from the Fungal Genetics Stock Center (FGSC) at the University of Missouri (Kansas City, MO). The genes encoding cell wall degrading enzymes were isolated from *Aspergillus nidulans* and

integrated into the genome of *P. pastoris* X-33 by Bauer et al. (2006) [168]. The recombinant strains used were FGSC#10062 and FGSC#10077 expressing endo-glucanase (REG) and β-xylosidase (βX), respectively. The recombinant strains were maintained on YPD plates containing yeast extract (1 % w/v), peptone (2 % w/v), dextrose (2 % w/v) and agar (2 % w/v). Plate cultures were stored at 4 ^{O}C for routine use. Culture stocks in 40 % glycerol were stored at -80 ^{O}C for long term use.

3.2.2 Culture media

For preparation of seed culture BMGY medium was used. The BMGY medium contained (in 1L) 10 g of yeast extract, 20 g of peptone, 100 ml of yeast nitrogen base (YNB) (20.4 g of yeast nitrogen base without ammonium sulfate and amino acids and 60 g of ammonium sulfate in 600 ml water), 100 ml of 1M potassium phosphate buffer at pH 6.0, 100 ml of 10 % (v/v) glycerol and 2 ml of 0.02 % (w/v) biotin. YNB and biotin were filter sterilized and added to the sterile medium containing other components. For fermentations, rich media BMMY or synthetic basal salt medium were used. The BMMY medium was composed of yeast extract, peptone, yeast nitrogen base, phosphate buffer and biotin at concentrations similar to BMGY medium. Instead of glycerol, 5 ml of methanol was added as the carbon source. The basal salt medium contained phosphoric acid (26.7 ml), calcium sulfate (0.93 g), potassium sulfate (18.2 g), magnesium sulfate heptahydrate (14.9 g), potassium hydroxide (4.13 g), glycerol (40.0 g) and 1.4 ml of trace salts solution in a liter of distilled water. The trace salts solution was prepared by dissolving $CuSO_4$-$5H_2O$ (6 g), NaI (0.08 g), Mn SO_4-H_2O (3.0 g), $NaMoO_4$-$2H_2O$ (0.2 g), boric acid (0.02 g), $CoCl_2$ (0.5 g) ZnCl (20.0 g) $FeSO_4$-$7H_2O$ (65.0 g), biotin (0.2 g), H_2SO_4 (5.0 ml) in one liter of distilled water. The trace salts solution was filter sterilized using a 0.2 μm membrane.

3.2.3 Preparation of seed culture

A single colony from YPD plate was transferred to 5 ml YPD broth in test tubes and incubated at 28 ^{o}C for 24 h with agitation (225 rpm). One ml of the culture was then transferred into 500 ml baffled flasks containing 100 ml of BMGY medium and incubated at 28 ^{o}C for 24-36 h with agitation at 225 rpm. The culture broth was centrifuged at 3000 rpm at room temperature for 3 min and the supernatant was discarded. The cell pellet was re-suspended in fresh sterile medium and used to inoculate production medium for enzyme expression in methanol medium.

3.2.4 Expression of recombinant enzymes in shake flasks

The expression of recombinant enzymes was tested by cultivating *P. pastoris* in BMMY medium in shake flasks. Baffled Erlenmeyer flasks (1000 ml) with 200 ml of production medium were inoculated with seed culture to an initial OD_{600} of approximately 1.0 and incubated at 28^{o}C with agitation at 225 rpm. The cultures were supplemented with 0.6 % methanol at 24 h intervals for 120 h.

3.2.5 Fermentation

A lab scale 1L BIOSTAT B plus fermentor (Sartorius AG, Goettingen, Germany) was used for fed-batch fermentation. Cultivation of *P. pastoris* in batch phase is done for 24 h in glycerol medium. Then fed-batch fermentation was performed by adding methanol to induce the expression of recombinant protein. The batch fermentation phase was carried out using either rich BMMY medium or basal salt medium with 4% (w/v) glycerol. The bioreactor vessel was filled with 600 ml of the production medium and sterilized in an autoclave. The medium was inoculated with seed culture to an initial OD_{600} of about 1.0. The culture pH and temperature

were maintained at pH 6 and 30^{o}C. pH was adjusted using ammonium hydroxide (28%) which also served as nitrogen source during fermentation. The level of dissolved oxygen (DO) concentration was maintained over 20% throughout the fermentation with air and agitation controls. Anti-foaming agent was added at the start of batch culture to minimize foam formation. There was no foam formation during 120 h fermentation. The fed-batch fermentation was started after 24 h of fermentation. Methanol was added to the culture as an inducer for expression of recombinant enzyme. Addition of methanol was dependent on change in DO level, which was controlled by a multistage algorithm encoded into an Excel spreadsheet. Initially, the stirring rate was increased to maintain 20% DO. Once the stirring rate reached the maximum (900 rpm), oxygen was supplied to maintain the DO level. Methanol feeding was based on substrate depletion which was identified by a spike in the DO level. For basal salt medium based fermentation, the methanol was mixed with the trace element solution and used for fed batch additions. In the case of rich medium pure methanol was used in fed batch additions. The fed-batch fermentation was carried out for 120 h at pH 6 and 30^{o}C. During the fermentation culture, samples were removed at 24 h intervals and OD_{600} was measured. After removing the cells by centrifugation the supernatant was analyzed for expression of recombinant enzyme by activity assay and SDS-PAGE. At the end of fed-batch fermentation the cells were harvested and the wet weight of the cell pellet was measured.

3.2.6 Production of recombinant enzymes from P. pastoris

P. pastoris has been used for high level expression of heterologous proteins by employing a methanol inducible promoter [169]. Several recombinant enzymes and functional proteins such as lipase and human interferon have been successfully produced at high levels using the *P.*

pastoris system [160, 170]. However, the expression of hydrolases by *P. pastoris* for degradation of plant cell walls is not well known. The major advantage of using *P. pastoris* for glycosyl hydrolase expression is that individual enzymes of endo-, exo-acting and de-branching activity for degradation of complex biomass structure can be produced and purified relatively easily. In recombinant *P. pastoris* the target gene is placed under the control of methanol inducible alcohol oxidase 1 promoter (*AOX1*p) and integrated into its genome. Though a few commercial enzymes are available for biomass hydrolysis, the actual ratio of individual enzymes in the mixture required for efficient pretreated biomass hydrolysis has not yet been defined. This is partly due to inadequate knowledge of the precise composition of enzyme activities in crude commercial enzyme blends. Here, the *P. pastoris* expression system was used for production and purification of major glycosyl hydrolases (GH). The main biomass depolymerizing enzymes endo-glucanase, β-glucosidase and β-xylosidase were expressed using *P. pastoris* methanol induction system and purified. Recombinant *P. pastoris* strains containing the genes encoding the GH of *Aspergillus nidulans* were obtained from FGSC and used for fermentation. The recombinant enzymes were fused to 6xHis tag at N-terminus for simplified purification of recombinant enzymes [168].

All three enzymes were produced by fermentation using rich BMMY medium. After 24 h of batch fermentation in glycerol medium the OD_{600} of cultures reached about 50 which is 50 fold higher than the initial cell density. Analysis of the culture sample showed absence of recombinant enzyme expression. The onset of fed-batch culture with the addition of methanol caused expression of recombinant enzymes. The level of enzyme expression increased with fermentation time and the maximum level was obtained at 96 h (Figure 3.1). As methanol was used as the carbon source there was an increase in cell density during fed-batch phase. At the end of 120 h of fermentation the wet-weight of cells reached 100 to 150 g/L of culture medium. The

amount of extracellular protein produced reached about 0.7 – 1.0 g/L. As seen in the Fig. 1 the recombinant enzyme was the major protein. These results demonstrate the potential of the *P. patoris* system for high level expression of other accessory enzymes for efficient hydrolysis of biomass.

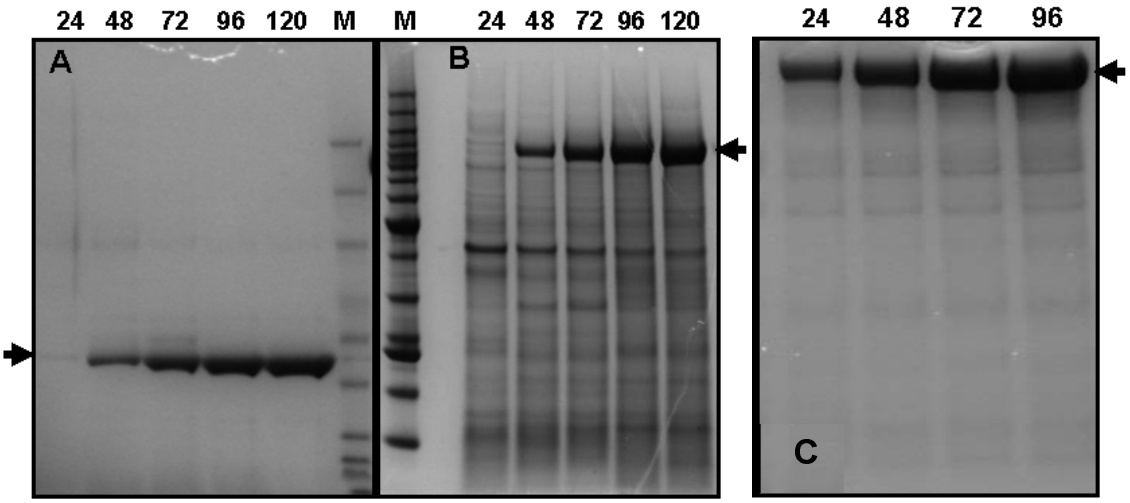


Figure 3.1 SDS-PAGE of expressed recombinant enzymes by *P. pastoris* during the course of fermentation. Each lane refers to culture supernatants of different fermentation times in hours. Each well was loaded with 10 µl of respective supernatant. Where; A - Engoglucanase, B - β-Glucosidase, C - β-Xylosidase. Arrows indicate respective enzymes of interest.

3.2.7 Purification of recombinant enzymes

Since the recombinant enzymes are fused with 6x His tag, a Ni-affinity column was used to purify enzymes. With low volume (4-5 ml) Ni-affinity column all the recombinant enzymes were electrophoretically purified. But large scale purification of enzymes from fermentation broth of cell culture using pre-packed FPLC based Ni-column was not successful. With the help of high throughput ion exchange column (Resource Q) we could rapidly purify all enzymes. These results demonstrated the use of *P. pastoris* for high level expression of individual biomass hydrolyzing enzymes and one step purification using fast flow ion exchange chromatography.

Recombinant endo-glucanase (REG) was produced by fermentation using basal salt medium to test the expression level and its effect on the purification method. The expression of endo-glucanase was at high level similar to rich medium and purification resulted in very pure enzyme. The fermentation strategy using synthetic medium has good potential to produce and then efficiently purify other biomass hydrolyzing enzymes. Compared to purification of enzymes from commercial enzyme source, the purification of recombinant enzymes was simple and efficient. Since purification of enzymes is a key limiting step for investigating enzyme synergy, these results are useful for purifying the spectrum of biomass hydrolyzing enzymes from the crude commercial enzymes and fermentation broth of recombinant *P. pastoris*. The purified β-glucosidase from recombinant fermentation was found to have no activity on cellobiose. The reasons are unknown.

3.3 Protein purification

Details of enzyme purification steps are shown in Table 3.1. Enzyme purification was performed using a FPLC system (GE Healthcare, Buckinghamshire, United Kingdom). The following FPLC columns were used: 51 ml HisPrep 26/10 desalting column (GE Healthcare, Lot # 17-5087-01), 6 ml Resource Q anion exchange column (GE Healthcare, Lot # 17-1179-01), 1.7 ml Mono S cation exchange column (GE Healthcare, Lot # 17-5180-01), 1.7 ml Mono Q anion exchange column (GE Healthcare, Lot # 17-5179-01) and 1 ml PHE hydrophobic interaction column (GE Healthcare, Lot # 17-1186-01). The crude enzyme samples were filtered (using 0.2 μm filter) and buffer exchanged to initial buffer (buffer A) using HisPrep 26/10 desalting column before injecting onto respective columns. CBH I and CBH II isolated from Spezyme CP (after steps 4.3 and 2.3) were further polished using APTC (p-aminophenyl-1-thio-β-D-cellobioside) based affinity chromatography to remove minor endoglucanase contaminants [166]. Protein samples

were concentrated using a tangential flow-filtration system (10 kDa Vivaflow membrane, Lot # 08VF5022, Sartorius, Bohemia, NY). Milli-Q water was used to prepare buffers. Elution buffers were filtered through 0.2 μm PES membrane and degassed prior to use.

Table 3.1 Chromatographic steps employed during FPLC based purification of various glycosyl hydrolases from crude enzyme mixtures.

Step	Sample	Column and Buffer	Gradient	Flow (ml/min)
1.0	Spezyme CP	6 ml Resource Q A: 20 mM Tris pH 7.3 B: A+1M NaCl	2 ml sample 15 CV 0-30% B	6
2.1	CBH II rich fraction in 1.0	1.7 ml Mono Q A: 20 mM Tris Buffer pH 7.5 B: A+1M NaCl	10 ml sample 10 CV 0-9% B	2
2.2	Major Peak in 2.1	1.7 ml Mono S A: 20 mM Citric Buffer pH 3.1 B: A+1M NaCl	11 ml sample 25 CV 0-15% B	2
2.3	Major Peak in 2.2	1 ml Resource PHE A: 20 mM Tris pH 7.5 B: 1M $(NH_4)_2SO_4$ +A	11 ml sample 40 CV 25%-0 B	4
3.1	EG I rich fraction in 1.0	1.7 ml Mono Q A: 20 mM Tris Buffer pH 7.5 B: A+1M NaCl	10 ml sample 20 CV 0-13% B	2
3.2	Major Peak in 3.1	1.7 ml Mono S A: 20 mM Citric Buffer pH 3.1 B: A+1M NaCl	10 ml sample 12 CV 0-8% B	2
3.3	Major Peak in 3.2	1 ml Resource PHE A: 20 mM Tris pH 7.5 B: 1M $(NH_4)_2SO_4$ +A	11 ml sample 40 CV 25%-0 B	4
4.1	CBH I rich fraction in 1.0	1.7 ml Mono Q A: 20 mM Tris Buffer pH 7.5 B: A+1M NaCl	5 ml sample 20 CV 20-30% B	2
4.2	Major Peak in 4.1	1.7 ml Mono S A: 20 mM Citric Buffer pH 3.1 B: A+1M NaCl	5 ml sample 10 CV 0-10% B	2

Table 3.1 (cont'd)

4.3	Major Peak in 4.2	1 ml Resource PHE A: 20 mM Tris pH 7.5 B: 1M $(NH_4)_2SO_4$ +A	5 ml sample 6 CV 55%-0 B	4
5.1	Novozyme 188	6 ml Resource Q A: 20 mM Tris pH 7.5 B: A+1M NaCl	2 ml sample 1 CV 20% B 4 CV 20%-44%B	6
5.2	βG rich fraction in 5.1	1.7 ml Mono S A: 20 mM Citric Buffer pH 3.1 B: A+1M NaCl	2 ml sample 10 CV 0-100% B	2
5.3	Major Peak in 5.2	1.7 ml Mono Q A: 20 mM Tris Buffer pH 7.5 B: A+1M NaCl	5 ml sample 20 CV 20-40% B	2
6.1	Multifect Xylanase	6 ml Resource Q A: 20 mM Piperazine pH 10.6 B: A+1M NaCl	2 ml sample 10 CV 0-50% B	6
6.2	EX rich fraction in 6.1	1.7 ml Mono S A: 20 mM Citric Buffer pH 3.1 B: A+1M NaCl	2 ml sample 15 CV 0-20% B	2
7	β-xylosidase fermentation broth from recombinant *Pichia pastoris* FGSC strain# 10077	6 ml Resource Q A: 20 mM Tris pH 7.5 B: A+1M NaCl	2 ml sample 15 CV 0-30% B	6

3.3.1 SDS-PAGE and protein purity quantification

SDS-PAGE was performed using a Novex® XCell *SureLock*™ Mini-Cell system (Invitrogen, Carlsbad, CA, USA) using pre-cast NuPAGE® Novex 4-12% Bis-Tris gels (Invitrogen, Lot # NP0321BOX). After electrophoresis the gels were fixed with 50% methanol and 7% acetic acid solution for 15 min and stained with GelCode Blue Stain Reagent (Thermo Fisher Scientific, Lot # KD131759, Rockford, IL, USA) to visualize the protein bands. The gel image was taken using the UVP BioDoc-It Imaging System (Upland, CA, USA). Protein purity was estimated by UN-SCAN-IT gel software (Version 6.1, Orem, Utah, USA).

3.3.2 Purification of glycosyl hydrolases from crude enzyme blends

The commercial enzyme blend, Spezyme CP, which has high activity on cellulose, was used as a source of EG and CBH. Purification of CBH I, CBH II and EG I was performed using suitable ion exchange columns. The crude enzyme sample was loaded onto the column, pre-equilibrated with 20 mM Tris buffer at pH 7.3. The bound proteins were eluted using a linear gradient of NaCl (0 to 1 M) in the same buffer. Four major protein peaks, I, II, III and IV were observed (Figure 3.2). The fractions corresponding to peaks I and IV had higher Avicelase activity, whereas Peak II had higher CMC activity (data not shown). Electrophoretically pure proteins were obtained by further polishing each fraction using higher resolution ion exchange (i.e. Mono Q and Mono S) and hydrophobic interaction chromatography (Resource PHE) based chromatography. CBH I and CBH II were further polished using APTC based affinity chromatography (after steps 4.3 and 2.3, respectively, as indicated in Table 3.1) to remove trace endoglucanase contaminants. The polished fractions showed single protein bands on SDS-PAGE

(Figure 3.5). The degree of purity was found to be >99 % based on quantification of the SDS-PAGE gel band intensity using UN-SCAN-IT gel^{TM} software.

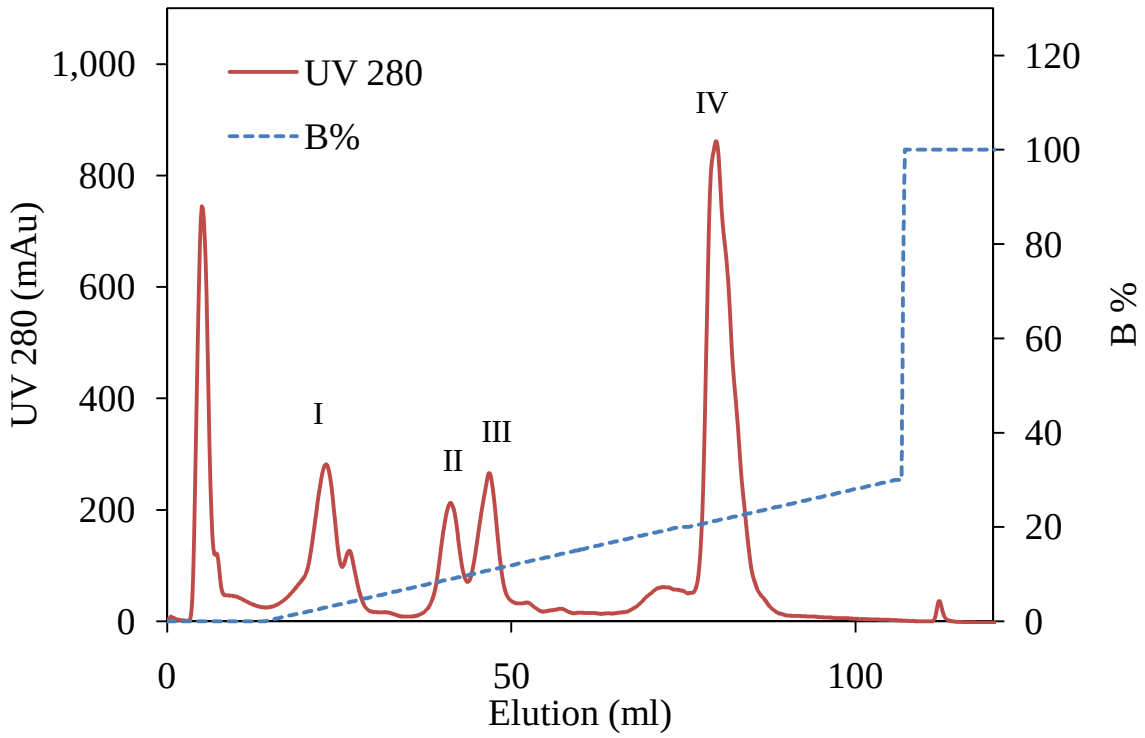


Figure 3.2 Separation of protein fractions (I-IV) from commercial enzyme blend (Spezyme CP) by anion exchange chromatography (Step 1.0) with respect to elution buffer gradient (% B).

Complete hydrolysis of cellulosic biomass necessitates avoiding inhibition of CBH's by cellobiose and other gluco-oligomers [171, 172]. Novozyme 188 was chosen as the source for purification of β-glucosidase. IEX chromatographic separation gave four major protein peaks (Figure 3.3), among which peak II gave the highest β-glucosidase activity (based on *p*NPG and cellobiose based activity assays). SDS-PAGE analysis revealed two major proteins in Peak II fraction (data not shown). This protein fraction was further purified (to 94% purity) using a

cation exchange column (Mono S). A third step of polishing with Mono Q further increased the purity to greater than 99% (Figure 3.5).

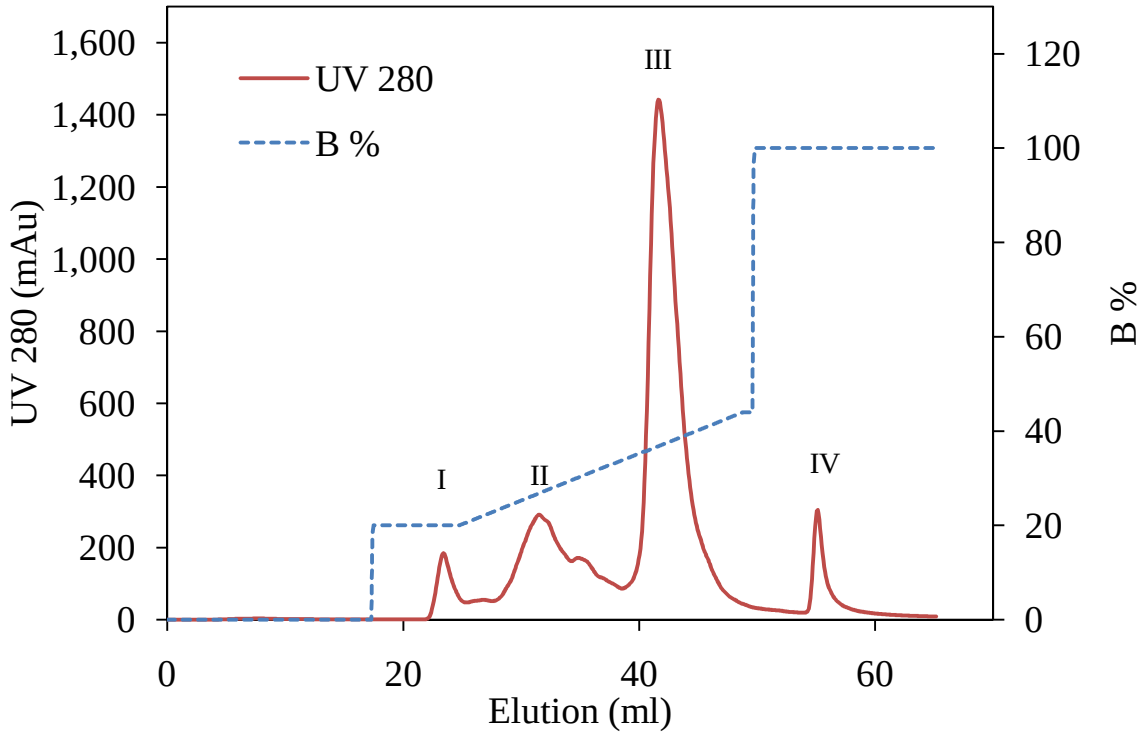


Figure 3.3 Separation of protein fractions (I-IV) from commercial enzyme blend (Novozyme 188) by anion exchange chromatography (Step 5.1) with respect to elution buffer gradient (% B).

Multifect® Xylanase was the source to isolate a suitable endo-xylanase. The separation was performed using the anion exchange column at pH 10.6 (20 mM Piperazine). Four major peaks (Figure 3.4) were obtained within which peak II gave the highest xylanase activity. Peak II fraction was further polished using a cation exchange (Mono S) column at pH 3.1, to obtain high purity (>99 %) endo-xylanase giving a single protein band on SDS-PAGE.

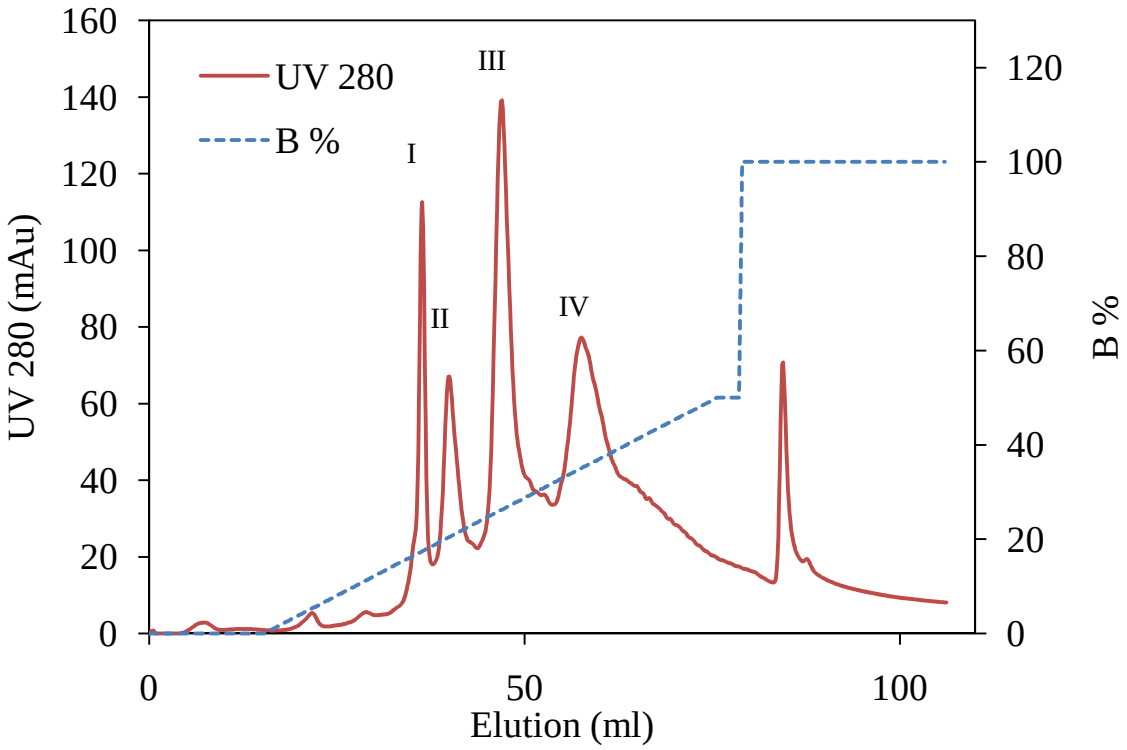


Figure 3.4 Separation of endo-xylanase from Multifect Xylanase (Step 6.1). Elution profile of protein UV adsorption (280 nm) with respect to elution buffer gradient.

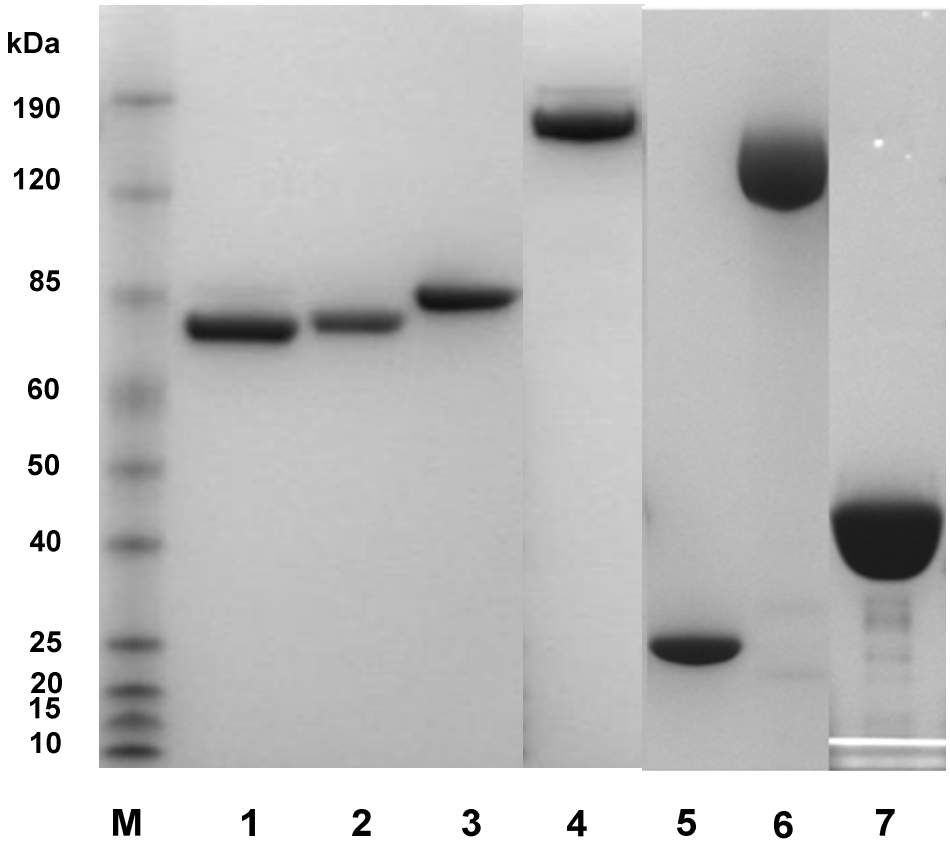


Figure 3.5 SDS-PAGE of purified EG I (lane 1), CBH II (lane 2), CBH I (lane 3), β-G (lane 4), EX (lane 5), β-X (lane 6), REG (lane 7) samples and marker ladder (lane M).

CHAPTER 4 MIXTURE OPTIMIZATION OF SIX CORE GLYCOSYL HYDROLASES FOR MAXIMIZING SACCHARIFICATION AFEX PRETREATED CORN STOVER

4.1 Introduction

From previous chapter, the most important cellulases and hemicellulases necessary to digest pretreated biomass have been purified. EG randomly hydrolyzes internal glycosidic bonds within cellulosic microfibrils [173], while CBH enzymes act processively along cellulosic chains cleaving off cellobiose units from either end (CBH I acts at reducing ends and CBH II acts at non-reducing ends) [174] with βG ultimately hydrolyzing cellodextrins to glucose [175]. EX cleaves the xylan backbone at internal β-1,4 xylosidic bonds, while βX hydrolyzes short xylooligomers to xylose [29]. All these enzymes are thought to work harmoniously, creating new accessible adsorption sites or active substrates for each other to act upon [16].

In this chapter, six core cellulases and hemicellulases were isolated using various purification and heterologous expression strategies. Various combinations of these enzymes were tested on AFEX treated corn stover to determine optimal combinations at three total protein loadings (8.25, 16.5 and 33 mg/g glucan) using a suitable design of experiments methodology. Synergistic interactions among different enzymes were then determined through various mixture optimization experiments. Optimum combinations were predicted from suitable statistical models which were able to further increase hydrolysis yields. These results demonstrate the potential to rationally design an enzyme mixture targeted towards a particular feedstock and pretreatment that can help maximize hydrolysis yields and minimize enzyme usage in a cellulosic biorefinery.

4.2 Materials and Methods

4.2.1 AFEX pretreatment

The detailed procedures of AFEX pretreatment have been described[176]. Pre-milled (passed through a 10 mm sieve) corn stover (Pioneer Hybrid seed variety (33A14) based stover, provided by NREL, was harvested in 2002 from the Kramer farm in Wray, CO) with 60% moisture (kg water/kg dry biomass), was transferred to a high-pressure Parr reactor. Heated liquid ammonia (1 kg of ammonia/kg of dry biomass) was charged to the reactor vessel resulting in immediate rise in temperature to 130 ^{o}C. The reactor was maintained at 130 ^{o}C for 15 min through an external heating mantle (within $\pm$ 10 °C). At the end of 15 min, the pressure was reduced to atmospheric level resulting in precipitous drop in temperature of the reactor contents. The very rapid pressure drop in the vessel caused the ammonia to vaporize, cooling the biomass to below 30 ^{o}C. The pretreated material was left under the hood overnight to ensure complete removal of residual ammonia. The AFEX treated stover was then milled to under 100 μm based on the methodology employed earlier [177] and kept under refrigeration until further use. The composition of the milled AFEX corn stover was found to be 34.4% glucan, 22.4% xylan, 4.2% arabinan, 0.6% mannan, 1.4% galactan, 3.8% uronyl, 11% lignin and 5.6% acetyl content.

4.2.2 Crude enzyme mixtures

Spezyme CP and Multifect Xylanase were a gift from Genencor (Danisco US Inc., Genencor Division, Rochester, NY), while Novo 188 (Sigma–Aldrich Corp., St. Louis, MO, Novozyme 188®, C6105) was procured from Sigma. The protein concentration was determined colorimetrically using the Pierce (Pierce Biotechnology, Rockford, USA) BCA (bicinchoninic acid) assay kit with bovine serum albumin (BSA) as the standard [178].

4.2.3 Heterologous enzyme expression

Detail information is described in 3.2.

4.2.4 Protein purification

Detail information is described in 3.3.

4.2.5 Proteomics analysis

A brief overview to the proteomics methodology is presented here while the detailed protocol is provided elsewhere [179]. Purified proteins of interest were denatured and reduced by adding urea and thiourea to a final concentration of 7 M and 2 M, respectively. Fresh dithiothreitol was added to a final concentration of 5mM, and samples were incubated at 60 °C for 30 min. Following incubation, the protein sample was diluted 10-fold with 100 mM NH_4HCO_3, pH 8.4, to reduce salt concentration. A volume of 1 M $CaCl_2$ was added to the diluted sample to a final concentration of 1 mM, and the sample was digested at 37 °C using sequencing grade trypsin (Promega, Madison,WI) at a ratio of 1 unit/50 units of protein (1 unit =< 1 μg of protein) for 4 h. Following incubation, digested samples were desalted using an appropriately sized C-18 SPE column (Supelco, St. Louis, MO) and vacuum manifold. Three column volumes of methanol were passed through the column followed by 2 column volumes of nanopure water. After passing the sample through the column, the column was washed with 4 volumes of a 95% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) solution. Peptides were eluted using one column volume of an 80% ACN, 0.1% TFA solution. The collected peptides were concentrated to a final volume of 150 μl and measured using the BCA assay (Pierce Biotechnology, Rockfort, IL) according to the manufacturer's instructions. Peptides from enzyme tryptic digests were analyzed using high resolution reversed-phase HPLC separation coupled to an ion trap mass

spectrometer (LTQ, Thermo Electron, San Jose CA) that was operated in a data-dependent MS/MS mode [179]. MS/MS spectra were analyzed using the SEQUEST algorithm in conjunction with a protein collection of all fungal entries from Uniprot (Swiss-Prot and TrEMBL). Preliminary filtering of identified peptides was performed using: a minimum cross-correlation cut-off (XCorr) of either 1.9, 3.0, or 3.2 for 1+, 2+, or 3+ charge states, respectively; partially and fully tryptic peptides (peptides that contained either an arginine or lysine at the site of cleavage); DelCn >= 0.1. Estimation of peptide False Discovery Rate (FDR) was calculated by decoy database searching techniques, resulting in values between 0.6% and 1.9%.

4.2.6 Enzyme activity assays

The enzyme activity assays were based on a high-throughput microplate based method as described in previous work [177]. A 2.2 ml deep-well microplate (Lot # 780271, Greiner, Monroe, NC) was used to add 250 µl of 1% (w/v) stock substrate (CMC, Avicel, oat spelt xylan, cellobiose, xylobiose), 50 µl of 0.5 M citrate buffer (pH 5.0) and 200 µl of appropriately diluted enzyme samples (20 ng to 100 µg/well). Carboxymethyl cellulose (CMC, Lot # 419273), cellobiose (Lot # C7252), oat spelt xylan (Lot # 9559) and Avicel (Lot # 11365) were procured from Sigma (Sigma-Aldrich, St. Louis, MO). The microplates were incubated at 50 ^{o}C with shaking at 200 rpm for 10 min (cellobiose), 60 min (CMC, xylan) or 300 min (Avicel). The amount of glucose released was estimated using an enzyme assay kit (R-Biopharm, Marshall, MI). One unit of cellobiase was defined as one micromole of glucose released per milligram enzyme per minute under the assay conditions. For CMC, Avicel and xylan based substrates the reducing sugars released were estimated using 3,5-dinitrosalicylic acid (DNS) based assay [180]. The hydrolyzate supernatants were filtered through 0.45 µm microplate filter (Lot # R6PN00144, Millipore, Ireland) and 50 µl of the supernatant was incubated with 100 µl of DNS reagent in

polypropylene microplate wells (Lot # 651201, Greiner, NC) at 100 °C for 30 min. After the plates cooled down to room temperature, 100 µl of the solution was transferred to a clear, flat-bottom microplate (Lot # 353072, Becton Dickinson Labware, NJ, USA) for measuring absorbance at 540 nm. Suitable reducing sugar standards (either glucose or xylose standards ranging from 0.1-2 g/l) were included for the DNS assay. One unit of CMCase, Avicelase and xylanase activity was defined as one micromole of reducing sugars (as glucose equivalents for Avicel/CMC and xylose equivalents for xylan) released per milligram enzyme per minute under the respective assay conditions.

The *para*-nitrophenyl (*p*NP) based chromogenic substrates used were 4-nitrophenyl-β-D-cellobioside (*p*NPC Lot # N5759), 4-nitrophenyl-β-D-glucopyranoside (*p*NPG Lot # N7006), 4-nitrophenyl-β-D-xylopyranoside (*p*NPX Lot # N2132) and 4-nitrophenyl-α-L-arabinofuranoside (*p*NPAf Lot # N3641)). All substrates were procured from Sigma (Sigma-Aldrich, St. Louis, MO). The assay mixtures containing 80 µl of 1 mM *p*NP substrate, 10 µl of 0.5 M citrate buffer (pH 5.0) and 10 µl of diluted enzymes (20 ng to 16 ug/well) in 350 µl micro plates were incubated at 50 ^{o}C with shaking at 200 rpm. After 15 min reaction time, 200 µl of 1M Na_2CO_3 was added to assay mixtures to arrest the hydrolytic reaction. The amount of *p*NP released was quantified by measuring absorbance (at 420 nm) of 4-nitrophenol (*p*NP, Lot # 1048) based standard curve (0.1 to 1 mM). One unit of enzyme activity was defined as one micromole of *p*-nitrophenol released per milligram enzyme per minute under the assay conditions.

4.2.7 Enzymatic hydrolysis of AFEX treated corn stover

All six core enzymes were used to hydrolyze AFEX treated corn stover in various relative amounts and total protein loadings to determine optimal enzyme combinations that maximize glucan and xylan digestibility. Minitab (Version 15.0, Minitab Inc, State College, PA) statistical

software was used to create a suitable mixture optimization design and analyze responses. In a mixture problem with q factors, it is common to define proportion variables x_i, for i = 1, 2, ..., q, where $x_i \geq 0$ represents the proportion of ingredient i in the mixture and $x_1+x_2+\ldots+x_i+\ldots+x_q=1$. The proportion variable allows one to consider a particular mixture experiment as a geometric point. In particular, the set of all points (x_1, x_2, ..., x_q) whose coordinates satisfy $x_i \geq 0$ and $x_1+x_2+\ldots+x_i+\ldots+x_q=1$ is called a q-dimensional simplex [181]. In this work, a fivecomponents simplex centroid mixture design was generated for CBH I, CBH II, EG I, EX and βX which were loaded at three different total protein loadings (7.5, 15 and 30 mg/g glucan). βG was loaded at 10% of the total enzyme loading (0.75, 1.5 and 3 mg/g glucan, respectively) to simplify dimensions of the experiment and this amount was later re-optimized. The hydrolysis data were analyzed by the software to generate a mixture regression statistical model and used to predict optimum mixture composition. The hydrolysis experiments were performed in 2.2 ml deep well microplates at 0.2% (w/w) total glucan loading along with 50 mM citrate buffer (pH 5.0) in a total volume of 500 µl per well [177]. The microplates were incubated at 50 ^{o}C with shaking at 200 rpm for 24 h. All experiments were carried out in quadruplicate; with mean values and standard deviations reported.

4.2.8 Glucose and xylose assays

Glucose and xylose released after hydrolysis were assayed using enzymatic kits purchased from R-Biopharm (Lot # 10716251035, Marshall, MI) and Megazyme (Lot # 80110-2, Bray, Ireland), respectively. The glucose assay was based on a two step enzymatic reactions, where D-Glucose was first phosphorylated to D-glucose-6-phosphate using ATP and hexokinase. The D-glucose-

6-phosphate was then reacted with $NADP^+$ by glucose-6-phosphate dehydrogenase to form D-gluconate-6-phosphate and NADPH. The reactions are stoichiometric to the amount of D-glucose and the corresponding increase in NADPH was measured at 340 nm to estimate glucose concentration. The xylose assay was based on an analogous two-step reaction method. α-D-xylose was first converted to isomeric β-D-xylose by xylose mutarotase. β-D-xylose was then reacted with NAD^+ to form D-xylonic acid and NADH. The corresponding increase of NADH was measured at 340 nm to estimate xylose concentration.

4.3 Results and Discussion

4.3.1 Activity of purified enzymes

Enzyme sources (in order to assign respective glycosyl hydrolase family to each purified protein) were determined through proteomics that helped identify the major tryptic peptides obtained for all six purified proteins (two major tryptic peptides for each enzyme is listed in Table 4.1**.**

Table 4.1 Identification of major peptides from six purified glycosyl hydrolases by LC-MS/MS.

Enzyme	Observation counts on LC-MS/MS		MaxXcorr	MaxDelCn	Peptides based on SEQUEST analysis		Similarity	Uniprot KB No.	Organism
	Total peptide	*Individual peptide*			*Sequence*	*Amino acid position*	*BLAST analysis*		
Purified CBH I	1602	475	6.6225	0.6159	K.KLTVVTQFETSGAINR.Y	18 AA (303 - 320)	100%	P62694	Trichoderma reesei
		400	4.0915	0.5665	K.YGTGYCDSQCPR.D	14 AA (183 - 196)	100%	P62694	Trichoderma reesei
Purified CBH II	428	63	3.6351	0.3634	K.YKNYIDTIR.Q	11 AA (218 - 228)	100%	P07987	Trichoderma reesei
		48	7.5152	0.5898	R.TLLVIEPDSLANLVTNLGTPK.C	23 AA (237 - 259)	100%	P07987	Trichoderma reesei
Purified EG I	166	21	6.4641	0.444	R.LYLLDSDGEYVMLK.L	16 AA (130 - 145)	100%	P07981	Trichoderma reesei
		18	6.0823	0.5831	K.TFTIITQFNTDNGSPSGNLVSITR.K	26 AA (269 - 294)	100%	P07981	Trichoderma reesei
Purified bG	2647	126	5.0463	0.5181	K.HYIAYEQEHFR.Q	13 AA (189 - 201)	100%	Q30BH9	Aspergillus niger
		126	6.9095	0.5488	R.DLANWNVETQDWEITSYPK.M	21 AA (820 - 840)	100%	Q30BH9	Aspergillus niger
Purified EX	397	121	5.5168	0.5299	K.LGEVTSDGSVYDIYR.T	17 AA (136 - 152)	100%	P36217	Trichoderma reesei
		48	7.8233	0.5948	R.NPLIEYYIVENFGTYNPSTGATK.L	25 AA (113 - 137)	100%	P36217	Trichoderma reesei
Purified bX	2811	330	6.3165	0.4447	R.SVMCSYNAVNGVPSCANK.F	20 AA (266 - 285)	100%	O42810	Aspergillus nidulans
		272	7.7185	0.4754	R.SVVVKFELKGEEAVILSWPEDTTSDFVSSIDGGLDR.K	38 AA (760 - 797)	100%	O42810	Aspergillus nidulans

The purified enzymes were evaluated for their hydrolytic activity on various substrates to determine activity and purity of isolated proteins. The activity of all enzymes with typical polymeric substrates (i.e. Avicel, oat spelt xylan, CMC) and *p*NP based chromogenic substrates was assessed. The activity assay results for all purified enzymes are shown in Table 4.1. The endo-acting enzyme EG I had high specific activity on CMC (6.69 U) and xylan (5.08 U); comparable to EX activity of xylan (8.24 U) as well. Interestingly, endoxylanase (and xylo-oligomerase) activity for EG I has been reported previously [182], suggesting that this enzyme couple play a dual role in hydrolyzing glucan and xylan in pretreated cell walls, unlike other endoglucanases. In addition, EG I showed significant activity on *p*NPC based chromogenic substrate.

Table 4.2 Purified enzymes activity assay on chromogenic p-nitrophenyl (*p*NP) glycosidic substrates, Avicel, CMC, oat spelt xylan, cellobiose and xylobiose.

Specific activity						
	CBH I	CBH II	EG I	βG	EX	βX
*p*NPC	0.0014	n.d.	0.0466	2.47	n.d.	n.d.
*p*NPG	n.d.	n.d.	n.d.	4.15	n.d.	0.0063
*p*NPX	n.d.	n.d.	n.d.	0.00952	n.d.	1.272
*p*NPAf	n.d.	n.d.	n.d.	n.d.	n.d.	0.142
Avicel	0.019	0.027	0.011	n.d.	n.d.	n.d.
Xylan	n.d.	n.d.	5.08	n.d.	8.24	n.d.
CMC	n.d.	n.d.	6.69	n.d.	n.d.	n.d.
Cellobiose	n.d.	n.d.	n.d.	124.9	n.d.	n.d.
Xylobiose	n.d.	n.d.	n.d.	n.d.	n.d.	52.2

One unit of specific activity was defined as one μmol *p*NP released per mg protein per minute. One unit of specific activity was defined as one μmol (as glucose equivalents) reducing sugars released based on DNS method (for Avicel and CMC) per mg protein per min. For oat spelt xylan, specific activity was defined based on xylose equivalents. For cellobiose and xylobiose, one unit of specific activity was denoted as one μmol of glucose or xylose released per mg protein per minute. Where; n.d. is not detectable.

Among the exo-acting enzymes, CBH I and CBH II had significant activity on Avicel (0.019 and 0.027 U, respectively), compared to the minor activity of EG I (0.011 U) seen on Avicel as well. This is not surprising considering Avicel has significant proportion of amorphous cellulose (nearly 20-30%) [183]. Also, if the CBH's are not extensively polished, they show significant CMC activity (1.3 U and 1.6 U for CBH I and CBH II, respectively). Hence, the polishing steps are crucial to remove endoglucanase contamination in cellobiohydrolases to obtain highly pure enzymes. Purified CBH I showed much lower *p*NPC activity (0.0014 U) than EG I, while CBH II had no detectable activity. βG and βX did not show appreciable activity on any of the polysaccharide based substrates. βG and βX had significant activity on cellobiose (124.9 U) and xylobiose (52.2 U), respectively. βG and βX also showed significant activity on *p*NPG (4.15 U) and *p*NPX (1.27 U), respectively. In addition, βG also had significant activity on *p*NPC (2.47 U) and trace activity on *p*NPX (0.0095 U). Similarly, βX has trace activity on *p*NPG (0.0063 U), but no detectable activity on cellobiose, while βX activity on *p*NPAf (0.142 U) would indicate α-arabinofuranosidase cross-activity. Similar cross-activity has been reported earlier for certain GH 3 β-xylosidases [184]. Other enzymes such as CBH II and EX had no detectable activities on any of the chromogenic *p*NP substrates.

4.3.2 Hydrolysis of AFEX treated corn stover by purified enzymes

Core enzyme mixtures were tested for their hydrolysis performance on AFEX treated corn stover. All enzyme loadings were based on equivalent bovine serum albumin BCA based measurement. To simplify the matrix of enzyme combinations to be tested, βG was loaded at 10% of the total cellulase/hemicellulase (CBH I + CBH II + EG + EX + βX) to prevent cellobiose inhibition. The optimum βG loading was later determined for the optimized five enzyme mixture.

From Table 4.3 (Experiments #1-32), the reproducibility of the hydrolysis experiment is satisfactory with low standard deviations (mostly < 2%) observed among quadruplicates. The trends for both glucose and xylose yields among different enzyme mixtures was dependent on the unique enzyme combinations. In terms of maximizing both glucose and xylose yield, the best experimental mixture tested contained CBH I, CBH II, EG I, EX and βX at equal protein loading (#31). Compared to Spezyme CP (at equal protein loading), glucose yield was 10-50% higher, whereas, xylan conversion was 40-225% higher, depending on total protein loading employed. Replacing the cellulase fraction with suitable hemicellulases (#29) helps increase xylan conversion by 500-1000% (with respect to #7) without causing significant change in glucan conversion. Selig et al. [185] reported 80-150% increase in xylan conversion to xylobiose due to supplementation of CBH I with an endoxylanase (from *Thermomyces lanuginosus*), with a corresponding increase of 15-20% in the glucan conversion to cellobiose during hydrolysis of hot water treated corn stover. However, in that experiment the protein loading for the endoxylanase supplementation was 5-15% higher, hence explaining the slight improvement in the overall glucan conversion as well. Individual enzymes (along with βG) generated very limited amount of glucose and xylose (#1 to #5). Interestingly, significant xylan conversion was obtained by only EG I (#3), which is not unexpected based on its significant oat spelt xylan activity. It may be possible that bi-functional enzymes like EG I have an advantage over endoglucanases from other GH families (e.g. GH 5, GH 12, GH 61) due to the fact that cellulose microfibrils are thought to be enclosed within hemicellulose rich sheaths [186] that would hinder the activity of mono-functional endoglucanases.

For binary enzyme combinations (#6 to #15), CBH I/EG I mixture (#7) gave the highest glucose yield (62.6% at 33 mg/g glucan) and the EX/βX mixture (#15) gave the highest xylose yield

(51.8% at 33 mg/g glucan) followed by EG I/βX (#14, 40.5% at 33 mg/g glucan) likely due to EG I's xylan cross-activity. Interestingly, it was possible to achieve close to 50% xylan conversion in the absence of any cellulases (< 5 % glucan conversion, #15). This suggests that the cell wall ultra-structure was significantly modified during AFEX pretreatment that allowed significant enzymatic accessibility to both glucan and xylan fractions, unlike untreated cell walls (data not shown) [187]. Interestingly, even though CBH II had significantly higher activity (42% higher) on crystalline cellulose than CBH I (Table 4.3), CBH I gave significantly higher conversions (45-50% higher) in binary/ternary/quartenary mixtures that had either one of the CBH's (Compare #7 vs. 10; 19 vs. 22; 29 vs. 30). This result reiterates the importance of optimizing enzyme cocktails on real pretreated lignocellulosic biomass and not on artificial cellulosic substrates like Avicel [114]

For ternary enzyme combinations (#16 to #25), CBH I/CBH II/EG I mixture (#16) gave the highest glucose yield but very low xylose yield. Binary mixtures of CBH I + EG I synergized together to give higher combined glucan and xylan conversion (1.5–2.5 fold, #7) compared to CBH I + EX, CBH II + EG I and CBH II + EX. However, for ternary systems that included CBH II the combined glucan and xylan conversions for CBH I + CBH II with either EG I or EX are remarkably similar. This suggests that presence of CBH II reduces the advantage of EG I over EX when synergizing with CBH I alone, possibly due to minor endo-activity typically seen for CBH II [156] In order to get significantly higher xylose yield, either the EG I-βX or EX-βX combination is necessary. For quarternary enzyme combinations (#26 to #30), without βX, xylan conversion dropped dramatically (#26) and without CBH I, glucan conversion decreased as well (#30).

From Table 4.3, it is not unexpected to find that CBH I, CBH II and EG I are critical for glucan hydrolysis; while EG I, EX and βX are important for xylan hydrolysis. By plotting glucose yield versus xylose yield at varying total enzyme loading (Figure 4.1), the glucose yield was found to be scattered from almost 0% to 75% while xylan conversion clustered into two sets. As long as EX or EG I and βX are present within the mixture (cluster 1), xylan conversion was found to be greater than 30% compared to cluster 2. Also, EX/βX mixture has higher xylose yield compared with EG I/βX containing mixtures. By comparing hydrolysis yields among different protein loadings, an interesting result comes to light. Reducing enzyme loading by 4 fold from 33 to 8.25 mg/g glucan resulted in quite different effects on overall glucose and xylose hydrolysis yields. For enzyme mixtures giving either higher or lower glucose yield, reduction of total enzyme loading resulted in a consistent drop of around 50% in glucose yield with no apparent correlation to initial glucose yield (33 mg/g glucan loading). However, xylan conversions dropped depending on the type of enzyme mixture. Enzyme mixtures (inclusive of EX/EG + βX, Cluster 1) that contributed to higher xylose yields saw a lower decrease in xylan conversions upon reducing enzyme loading by four-fold, compared to the low xylan hydrolyzing enzyme mixtures (Cluster 2). This suggests that it might be possible to further reduce hemicellulase loading without sacrificing xylan hydrolysis yields. Increasing enzyme loading helps increase glucose and xylose hydrolysis yields. However, the extent of improvement is quite different. Glucose yield increases by more than 20% while xylan conversion increases by less than 10% upon doubling the enzyme loading. The highest xylose yield was always below 60% conversion. This might be due to lack of other accessory enzyme activities such as α-arabinofuranosidase, feruloyl/acetyl xylan esterases, pectinases and α-glucuronidase.

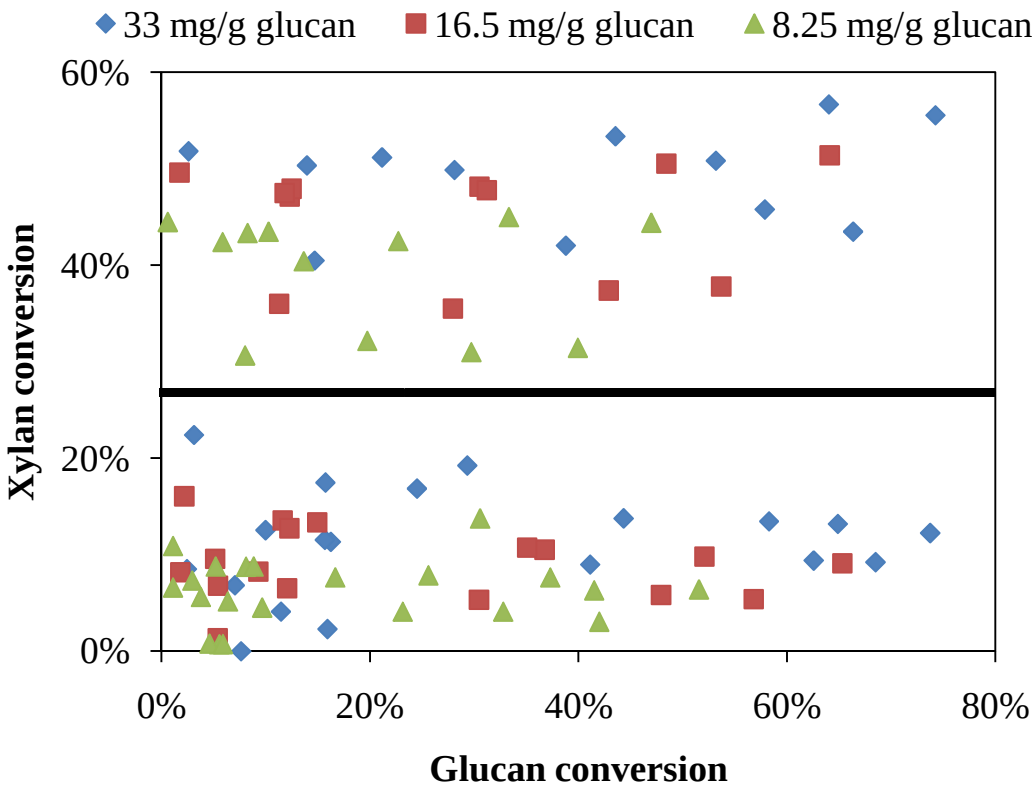


Figure 4.1 Twenty four hours glucose versus xylose yields for different total enzyme loading (33, 16.5 and 8.25 mg/g glucan) saccharifying AFEX treated corn stover. Above the bar, all enzyme mixtures contain either EG I/βX or EX/βX (Cluster 1) whereas below the bar (Cluster 2) no such combinations exist.

Table 4.3 24 hours hydrolysis yields of AFEX treated corn stover by various enzyme mixtures at three different total protein loadings. βG was loaded at 10% (mass loading) of all other proteins. An equivalent amount of Spezyme (equivalent mass basis 33, 16.5 and 8.25 mg/g glucan, respectively; with no βG supplementation) was included.

	Enzymes ratio					30 mg/g glucan plus 10% βG			
						Glucose yield		Xylose yield	
#	CBH I	CBH II	EG I	EX	βX	Avg	Stdev	Avg	Stdev
1	1	0	0	0	0	11.5%	1.0%	4.1%	0.5%
2	0	1	0	0	0	7.6%	0.2%	0.0%	1.6%
3	0	0	1	0	0	16.2%	0.8%	11.3%	2.4%
4	0	0	0	1	0	3.1%	0.5%	22.4%	2.0%
5	0	0	0	0	1	2.4%	0.4%	8.5%	2.3%
6	0.5	0.5	0	0	0	15.9%	0.8%	2.3%	0.5%
7	0.5	0	0.5	0	0	62.6%	0.9%	9.4%	0.8%
8	0.5	0	0	0.5	0	29.3%	1.3%	19.2%	0.7%
9	0.5	0	0	0	0.5	10.0%	1.4%	12.5%	0.8%
10	0	0.5	0.5	0	0	41.1%	0.8%	9.0%	1.4%
11	0	0.5	0	0.5	0	24.5%	1.1%	16.9%	1.4%
12	0	0.5	0	0	0.5	7.0%	0.5%	6.8%	1.5%
13	0	0	0.5	0.5	0	15.7%	0.9%	17.5%	2.0%
14	0	0	0.5	0	0.5	14.7%	1.2%	40.5%	1.8%
15	0	0	0	0.5	0.5	2.6%	1.1%	51.8%	2.6%
16	0.33	0.33	0.33	0	0	68.5%	1.7%	9.2%	0.5%
17	0.33	0.33	0	0.33	0	58.3%	1.7%	13.4%	1.2%
18	0.33	0.33	0	0	0.33	15.7%	0.7%	11.5%	1.2%
19	0.33	0	0.33	0.33	0	64.9%	1.8%	13.2%	1.4%
20	0.33	0	0.33	0	0.33	57.9%	1.1%	45.8%	1.0%
21	0.33	0	0	0.33	0.33	28.1%	2.2%	49.8%	2.3%
22	0	0.33	0.33	0.33	0	44.3%	0.8%	13.8%	1.2%
23	0	0.33	0.33	0	0.33	38.8%	1.4%	42.0%	0.5%
24	0	0.33	0	0.33	0.33	21.2%	0.8%	51.1%	1.5%
25	0	0	0.33	0.33	0.33	13.9%	0.6%	50.3%	1.4%
26	0.25	0.25	0.25	0.25	0	73.7%	1.4%	12.2%	1.0%
27	0.25	0.25	0.25	0	0.25	66.3%	1.6%	43.5%	1.0%
28	0.25	0.25	0	0.25	0.25	53.2%	0.9%	50.8%	0.6%
29	0.25	0	0.25	0.25	0.25	64.0%	1.5%	56.6%	2.9%
30	0	0.25	0.25	0.25	0.25	43.5%	0.5%	53.3%	1.6%
31	0.2	0.2	0.2	0.2	0.2	74.2%	1.5%	55.5%	1.6%
32	Spezyme CP					66.8%	1.3%	39.1%	1.5%

Table 4.3 (cont'd)

#	Enzymes ratio					15 mg/g glucan plus 10% βG			
						Glucose yield		Xylose yield	
	CBH I	CBH II	EG I	EX	βX	Avg	Stdev	Avg	Stdev
1	1	0	0	0	0	5.4%	0.7%	1.3%	1.0%
2	0	1	0	0	0	6.1%	1.0%	0.2%	1.0%
3	0	0	1	0	0	12.1%	0.5%	6.5%	1.7%
4	0	0	0	1	0	2.2%	0.2%	16.0%	0.9%
5	0	0	0	0	1	1.8%	0.2%	8.2%	0.3%
6	0.5	0.5	0	0	0	9.7%	0.9%	0.3%	0.7%
7	0.5	0	0.5	0	0	47.9%	0.9%	5.8%	0.6%
8	0.5	0	0	0.5	0	11.6%	1.0%	13.5%	1.0%
9	0.5	0	0	0	0.5	5.1%	0.2%	9.6%	0.6%
10	0	0.5	0.5	0	0	30.5%	1.1%	5.3%	0.6%
11	0	0.5	0	0.5	0	14.9%	1.0%	13.3%	0.7%
12	0	0.5	0	0	0.5	5.4%	0.4%	6.8%	0.7%
13	0	0	0.5	0.5	0	12.3%	0.7%	12.7%	0.8%
14	0	0	0.5	0	0.5	11.3%	0.7%	36.0%	1.5%
15	0	0	0	0.5	0.5	1.7%	1.1%	49.6%	1.3%
16	0.33	0.33	0.33	0	0	56.8%	2.2%	5.4%	0.7%
17	0.33	0.33	0	0.33	0	36.7%	1.6%	10.5%	1.2%
18	0.33	0.33	0	0	0.33	9.3%	0.7%	8.2%	0.3%
19	0.33	0	0.33	0.33	0	52.1%	1.0%	9.8%	1.3%
20	0.33	0	0.33	0	0.33	42.9%	0.5%	37.4%	0.7%
21	0.33	0	0	0.33	0.33	12.3%	2.5%	47.1%	1.1%
22	0	0.33	0.33	0.33	0	35.1%	0.7%	10.7%	0.8%
23	0	0.33	0.33	0	0.33	28.0%	1.1%	35.5%	1.9%
24	0	0.33	0	0.33	0.33	12.5%	0.9%	47.9%	1.9%
25	0	0	0.33	0.33	0.33	11.8%	0.6%	47.5%	0.3%
26	0.25	0.25	0.25	0.25	0	65.3%	0.8%	9.1%	1.1%
27	0.25	0.25	0.25	0	0.25	53.7%	1.2%	37.8%	1.4%
28	0.25	0.25	0	0.25	0.25	30.5%	1.3%	48.1%	1.0%
29	0.25	0	0.25	0.25	0.25	48.4%	1.2%	50.5%	0.9%
30	0	0.25	0.25	0.25	0.25	31.2%	0.9%	47.8%	0.4%
31	0.2	0.2	0.2	0.2	0.2	64.1%	1.4%	51.4%	1.6%
32	Spezyme CP					47.3%	1.4%	22.9%	2.8%

Table 4.3 (cont'd)

#	Enzymes ratio					7.5 mg/g glucan plus 10% βG			
						Glucose yield		Xylose yield	
	CBH I	CBH II	EG I	EX	βX	Avg	Stdev	Avg	Stdev
1	1	0	0	0	0	5.5%	3.1%	0.7%	0.3%
2	0	1	0	0	0	4.6%	0.5%	0.8%	0.6%
3	0	0	1	0	0	9.7%	0.5%	4.5%	0.5%
4	0	0	0	1	0	1.1%	0.8%	10.9%	0.3%
5	0	0	0	0	1	1.1%	0.9%	6.6%	0.9%
6	0.5	0.5	0	0	0	5.9%	0.2%	0.7%	0.3%
7	0.5	0	0.5	0	0	32.8%	1.6%	4.1%	0.5%
8	0.5	0	0	0.5	0	5.2%	1.2%	8.8%	1.9%
9	0.5	0	0	0	0.5	3.0%	0.4%	7.3%	0.9%
10	0	0.5	0.5	0	0	23.1%	1.2%	4.1%	0.7%
11	0	0.5	0	0.5	0	8.1%	0.5%	8.7%	0.5%
12	0	0.5	0	0	0.5	3.8%	0.4%	5.6%	0.6%
13	0	0	0.5	0.5	0	8.8%	0.9%	8.8%	0.2%
14	0	0	0.5	0	0.5	8.0%	0.3%	30.6%	0.6%
15	0	0	0	0.5	0.5	0.6%	0.2%	44.5%	1.0%
16	0.33	0.33	0.33	0	0	42.0%	1.1%	3.0%	0.5%
17	0.33	0.33	0	0.33	0	16.7%	1.6%	7.6%	1.0%
18	0.33	0.33	0	0	0.33	6.4%	1.3%	5.1%	1.9%
19	0.33	0	0.33	0.33	0	37.3%	1.4%	7.6%	0.7%
20	0.33	0	0.33	0	0.33	29.7%	0.6%	31.0%	1.4%
21	0.33	0	0	0.33	0.33	5.9%	1.1%	42.4%	1.2%
22	0	0.33	0.33	0.33	0	25.6%	0.4%	7.8%	0.5%
23	0	0.33	0.33	0	0.33	19.7%	0.3%	32.1%	3.4%
24	0	0.33	0	0.33	0.33	8.3%	0.8%	43.3%	0.8%
25	0	0	0.33	0.33	0.33	10.3%	1.1%	43.4%	1.2%
26	0.25	0.25	0.25	0.25	0	51.5%	1.3%	6.4%	0.7%
27	0.25	0.25	0.25	0	0.25	39.9%	1.1%	31.4%	0.9%
28	0.25	0.25	0	0.25	0.25	13.6%	0.4%	40.4%	0.8%
29	0.25	0	0.25	0.25	0.25	33.3%	0.4%	45.0%	0.9%
30	0	0.25	0.25	0.25	0.25	22.7%	0.5%	42.5%	1.5%
31	0.2	0.2	0.2	0.2	0.2	47.0%	0.7%	44.4%	1.1%
32	Spezyme CP					30.6%	0.8%	13.7%	1.7%

4.3.3 Optimal core enzyme mixture for hydrolyzing AFEX treated corn stover

Table 4.4 Statistical mixture model regression analysis for glucose hydrolysis yields at three total protein loadings for saccharifying AFEX treated corn stover.

	30 mg/g glucan		15 mg/g glucan		7.5 mg/g glucan	
Terms	Coefficient	P value	Coefficient	P value	Coefficient	P value
CBH I	0.2231	*	0.0965	*	0.0611	*
CBH II	0.0923	*	0.0737	*	0.0516	*
EG I	0.1699	*	0.1345	*	0.1065	*
EX	0.1192	*	0.055	*	0.0203	*
βX	0.0573	*	0.037	*	0.0221	*
CBH I*EG I	1.684	<0.001	1.4036	<0.001	0.9213	<0.001
CBH II*EG I	1.1135	<0.001	0.774	<0.001	0.5656	<0.001
CBH II*EX	0.6724	<0.001	0.3758	<0.001	0.1958	0.001
CBH I*CBH II*EG I	5.246	<0.001	6.1749	<0.001	5.4967	<0.001
CBH I*CBH II*EX	9.3207	<0.001	6.8796	<0.001	3.3075	<0.001
CBH I*EG I*EX	8.5795	<0.001	8.3711	<0.001	6.7795	<0.001
CBH I*EG I*βX	7.681	<0.001	5.7115	<0.001	3.8956	<0.001
CBH II*EG I*EX	3.3146	<0.001	4.2069	<0.001	3.9445	<0.001
CBH II*EG I*βX	4.8307	<0.001	3.2345	<0.001	2.1095	<0.001
EG I*EX*βX	2.4303	0.002	2.3416	<0.001	2.1053	<0.001
CBH I*CBH II*EG I*βX	20.7769	0.023	27.1232	<0.001	23.8943	<0.001
CBH I*CBH II*EX*βX	58.0654	<0.001	34.6422	<0.001	15.5443	<0.001
Regression R^2	96.48%		97.57%		97.15%	
R^2 (predicted)	93.48%		96.87%		96.15%	

Cellulose comprises the largest polysaccharide fraction in lignocellulosic cell walls and glucose is the preferred carbon substrate for many microorganisms. In order to further enhance the glucose yield, the enzyme mixture composition and glucan conversion data (Table 4.3) were analyzed by Minitab to develop a statistically based predictive model. Response surface models were generated for all three total protein loading experiments. Model terms, coefficients and P values are listed in Table 4.4. Only terms that have a significantly low P value (<0.05) have been included to develop statistically valid and highly predictive models. The model explains the

variance in the data very well with R^2 greater than 0.9. The optimum mixture composition (capable of giving the highest glucan conversion) from the response surface models was predicted using the response optimizer functionality available in Minitab. The optimized mixture composition and predicted responses are shown in Table 4.5 and Figure 4.4.

The coefficients of the model also provide insight into ranking the importance of each enzyme and synergistic interactions among various enzymes on the hydrolysis yields. The model clearly indicates that both EX and βX work synergistically with cellulases and can help significantly increase glucose yields. This might be due to the removal of hemicelluloses wrapped around glucan microfibrils to help increase cellulose accessibility [33] and also prevention of cellulase inhibition by hemicellulose oligomers [188]. Another interesting trend regarding relative importance of EG I based on total protein loading was noticed from the predicted coefficients of the model. This trend was harder to notice by simply glancing over the original dataset in Table 4.4. Ranking coefficients for single, binary, ternary and quaternary terms consistently highlighted EG I containing terms at the top of each list for the lowest total protein loading (0.1065 for EG I, 0.9213 for CBH I*EG I, 6.7795 for CBH I*EG I*EX and 23.8943 for CBH I*CBH II*EG I*βX). However, for the 30 mg/g glucan enzyme loading model most terms with the highest coefficient within each of the respective term families (i.e. single, ternary, quaternary) do not include EG I (0.2231 for CBH I, 9.3207 for CBH I*CBH II*EX and 58.0654 for CBH I*CBH II*EX*βX). This statistical result may have a phenomenological explanation based on substrate inhibition encountered at lower enzyme to substrate ratios [189]. At lower enzyme loadings, the CBH enzymes bound to the cellulosic substrate are further separated from potential reducing ends than at higher protein loadings (for identical relative enzyme ratios) due to relative surface density of the enzymes on the substrate. Therefore, in order to maximize glucan

digestibility at lower total protein loadings it would be necessary to increase the number of accessible reducing ends for CBH enzymes, possibly through increasing the relative EG I loading. This may help explain why the model predicts 15% higher EG I ratio (35% vs. 30%) in the lowest total protein loading compared to the 30 mg/g glucan enzyme loading optimum mixture (Table 4.5). However, in order to validate this hypothesis suitable adsorption experiments using purified cellulases on AFEX corn stover would need to be conducted at varying total protein loadings. The optimum mixtures for all three total enzyme loadings are similar, containing approximately 29-30% CBH I, 18-20% CBH II, 30-35% EG I, 14-15% EX and 2-6% βX based on total protein mass loading (excluding βG). These results also validate earlier findings that hemicellulase supplementation can help reduce cellulase loading while giving higher overall hydrolysis yields [185, 190].

The ternary plots (Figure 4.2) were generated by the regression model given in Table 4.4. The contour patterns of different protein loadings were quite congruous indicating that the optimum enzyme ratios were overlapping, though the three-dimensional shapes are quite different (due to variable slopes between low and high protein loading). By fixing EX and βX at their optimum ratio, the effect of varying CBH I, CBH II and EG I absolute ratios on glucose yield was determined. All three enzymes were found to be important. By increasing the total enzyme loading (A.1 to A.3), the glucose yield became less sensitive to relative ratios of CBH I, CBH II and EG I. Similar results are also seen in (B.1 to B.3). EX can increase hydrolysis yields and a small amount of βX (around 4%) was critical. Since xylan conversions are highly dependent on βX, including βX in the mixture was essential. The contour plots help visually demonstrate that the relative ratio of enzymes becomes less important at relatively higher total protein loadings, as long as there is a minimal amount of each individual component. This is an important finding, as

for a feasible cellulosic ethanol process, enzyme usage needs to be substantially minimized. This is possible via gravitating towards significantly lower protein loadings than what are currently utilized in most published research articles (e.g. reducing enzyme usage from 15 FPU/g glucan to say 5 FPU/g glucan; where 15 FPU typically corresponds to 25-35 mg enzyme/g glucan). This reduction in enzyme usage would be further facilitated by consumption of hydrolyzed sugars during the co-fermentation step (i.e. SSF).

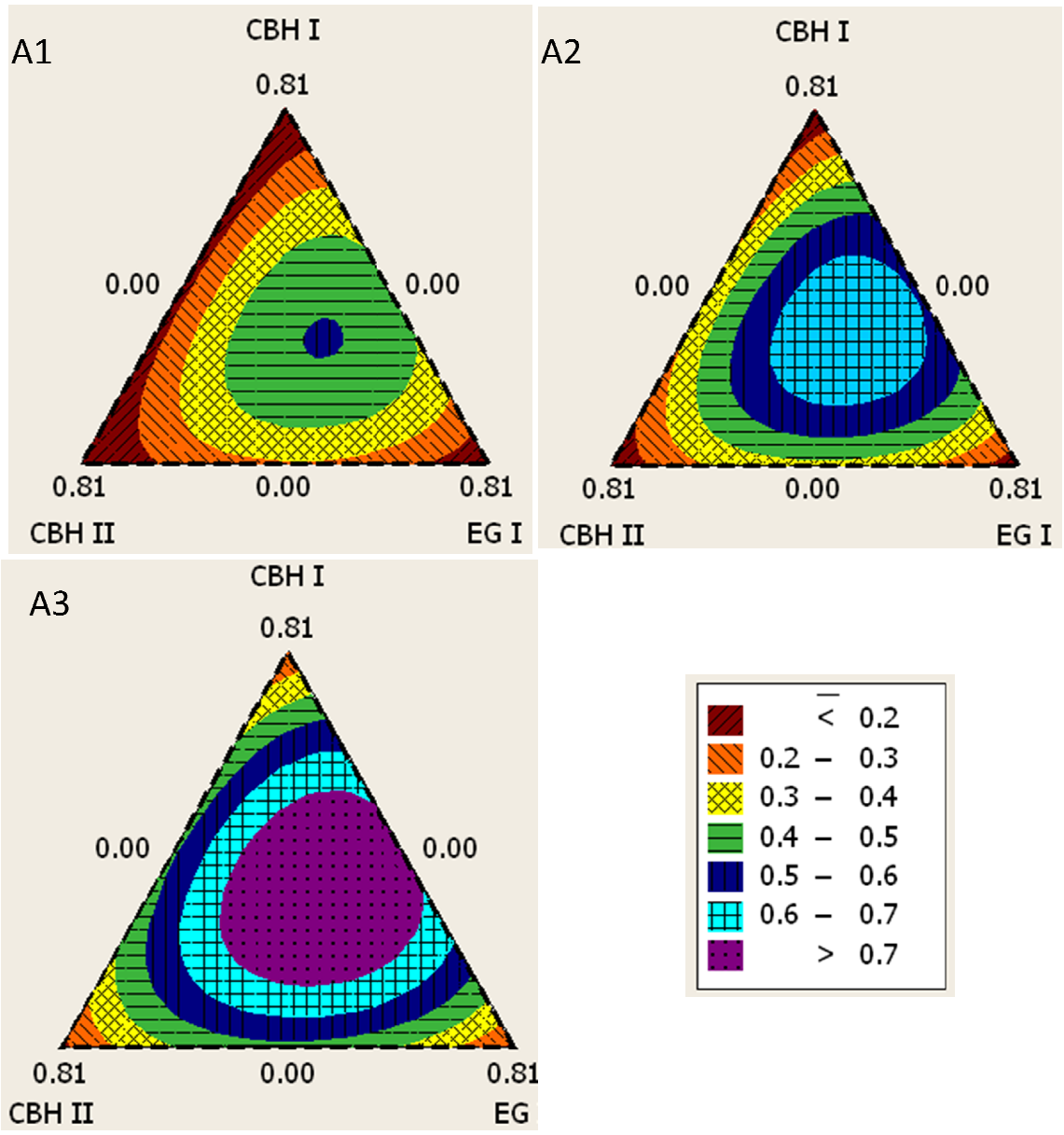
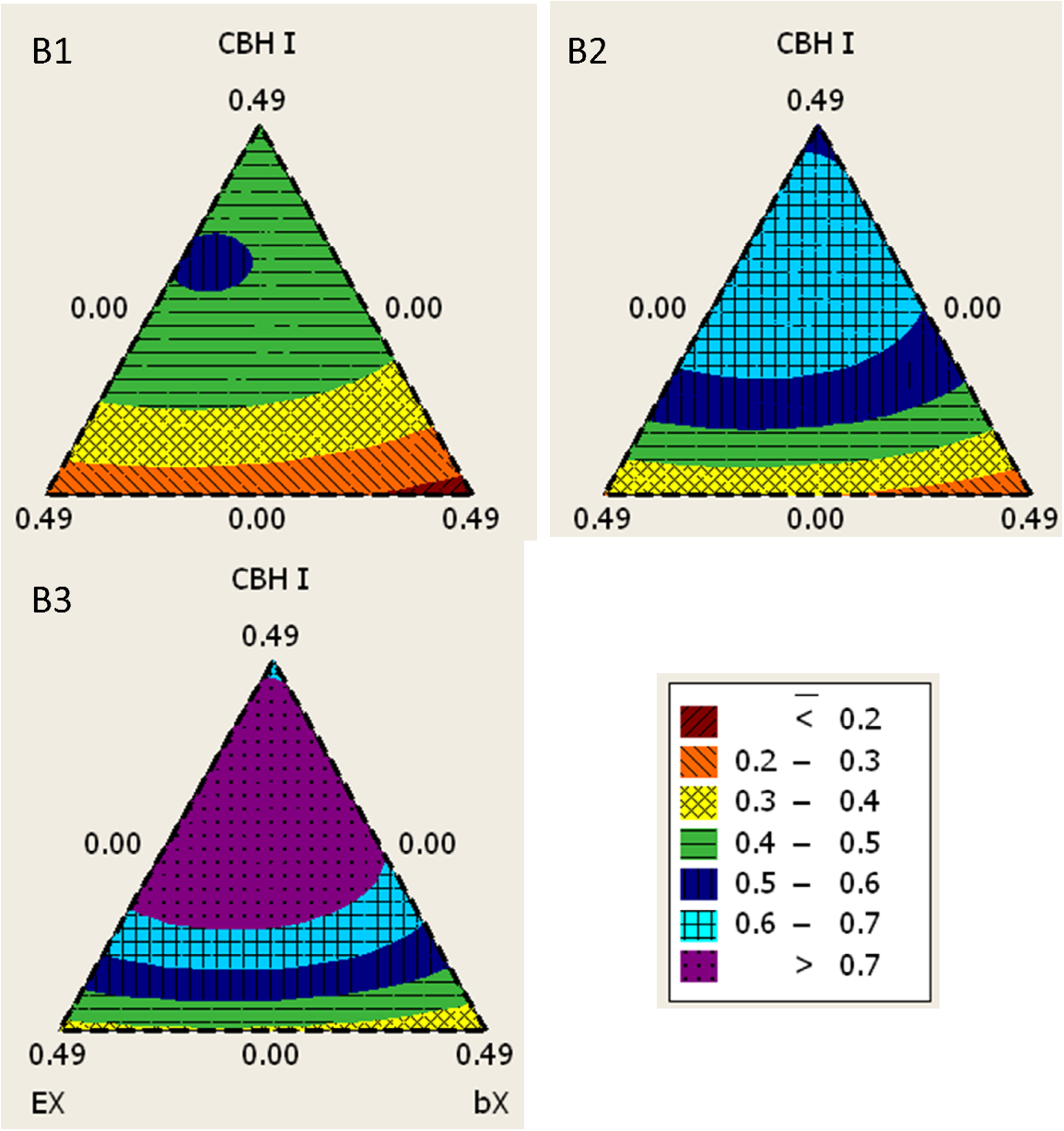


Figure 4.2 Ternary plots of models developed to predict glucose yields as a function of varying enzyme loadings upon hydrolysis of AFEX treated corn stover. Expected glucose yield ranges are denoted by the different colors/patterns in the legend. A.1, A.2 and A.3 are ternary plots of CBH I, CBH II and EG I, with EX and βX held constant at 0.15 and 0.04, respectively (as protein mass ratio where sum of all five protein ratios is unity). B.1, B.2 and B.3 are ternary plots of CBH I, EX and βX, with CBH II and EG I held constant at 0.19 and 0.32 ratio. A.1/B.1, A.2/B.2 and A.3/B.3 are plots of 7.5, 15 and 30 mg/g glucan enzyme loading, respectively. For all experiments, βG was loaded at 10% of the total enzyme (CBH I + CBH II + EG I + EX + βX) mass loading.

Figure 4.2 (cont'd)



In order to determine the minimal βG loading necessary, increasing amounts of βG were loaded to the optimal cocktail (predicted from previous experiments) for a total five enzyme (CBH I + CBH II + EG I + EX + βX) loading at 7.5, 15 and 30 mg/ g glucan. The results (shown in Figure 4.3) indicate that 1-5% of βG (i.e. 0.375, 0.75, 1.5 mg/g glucan, respectively, for all three total

protein loadings) is more than sufficient to prevent end-product inhibition. Xylose yield was not significantly affected by βG supplementation. The optimum mixtures for all three total enzyme loadings, inclusive of all six core enzymes, would probably contain 27-30% CBH I, 17-20% CBH II, 29-35% EG I, 14-15% EX, 2-6% βX and 1-5% βG based on total protein mass loading.

The statistical model predictions were tested for all three total protein loadings with βG supplementation at 10% of CBH I + CBH II + EG I + EX + βX (shown in Table 4.5). Since all optimally predicted combinations had around 15% EX and 4% βX loading, the xylan conversions were comparable to the data obtained in earlier designed experiments (Table 4.3). For 15 mg/g glucan loading, the model prediction closely matched the experimental data set. At 7.5 mg/g glucan, the result was 2% higher than the model expectation while at 30 mg/g glucan the result was 2% lower than model expectation. Compared with the best experimental combination **(**Table 4.3, #31), the glucose yield for optimized mixtures increased further (7.7%, 6.7% and 10.5% corresponding to 7.5, 15 and 30 mg/g glucan, respectively). Compared to Spezyme CP, where individual enzyme ratios have not been optimized for AFEX treated corn stover, the optimized cocktail gave much higher glucose and xylose yields. Also, when the enzyme loading was reduced by 4 fold from 33 to 8.25 mg/g glucan, the drop in the glucose and xylose yield was much lower for the optimized cocktail compared to Spezyme CP. For Spezyme CP, the glucose yield decreased from 66.8% to 30.6% and xylose yield decreased from 39.1% to 13.7%, whereas the optimized cocktail showed a decrease from 80% to 52.6% and 56.2% to 42.2% for glucose and xylose yields, respectively. This finding reconfirms the importance to tailor-make enzyme cocktails for pertinent pretreated substrates in order to minimize enzyme usage, maximize digestibility and hence reduce the overall cost of producing cellulosic ethanol.

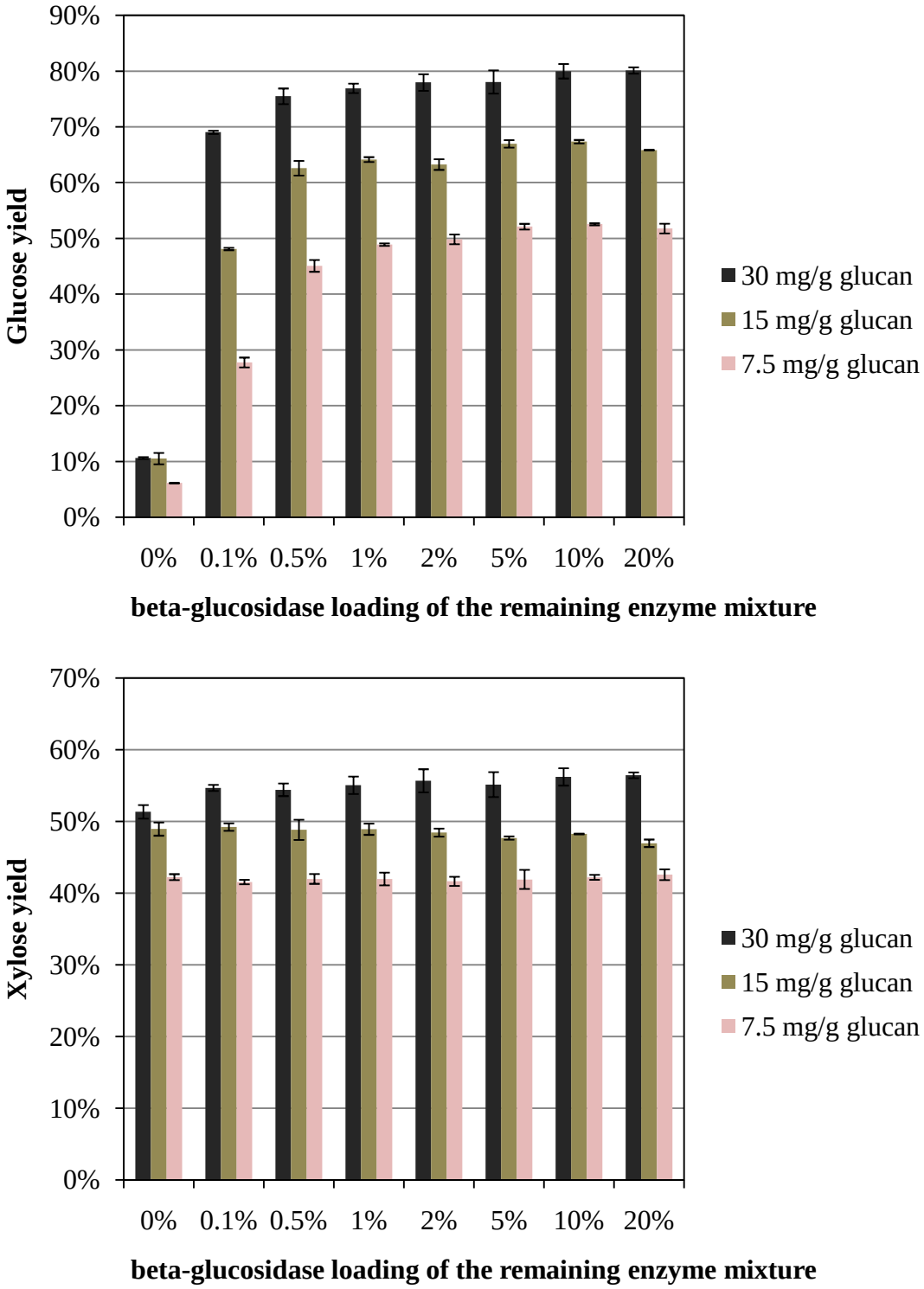


Figure 4.3 Twenty-four hours glucose (A) and xylose (B) yields for saccharified AFEX-treated corn stover for varying bG loadings, held at fixed respective optimum ratios of CBH I,CBH II, EG I, EX and bX for three different total enzyme loadings.

Table 4.5 24 hours glucose yield for AFEX treated corn stover saccharified at optimum enzyme loading. The model generated optimum mixture hydrolysis predictions were tested at varying total enzyme loadings.

5 core enzyme loading (mg/g glucan)	βG enzyme loading (mg/g glucan)	5 core enzyme mixture ratios (mass basis)					Glucose yield			Xylose yield	
		CBH I	CBH II	EG I	EX	βX	Model Expectation	Experimental Average	Standard Deviation	Experimental Average	Standard Deviation
30	3	0.305	0.182	0.300	0.152	0.061	82.0%	80.0%	1.3%	56.2%	1.2%
15	1.5	0.296	0.192	0.323	0.149	0.040	68.4%	67.4%	0.3%	48.3%	0.1%
7.5	0.75	0.293	0.193	0.352	0.141	0.021	50.6%	52.6%	0.2%	42.2%	0.4%

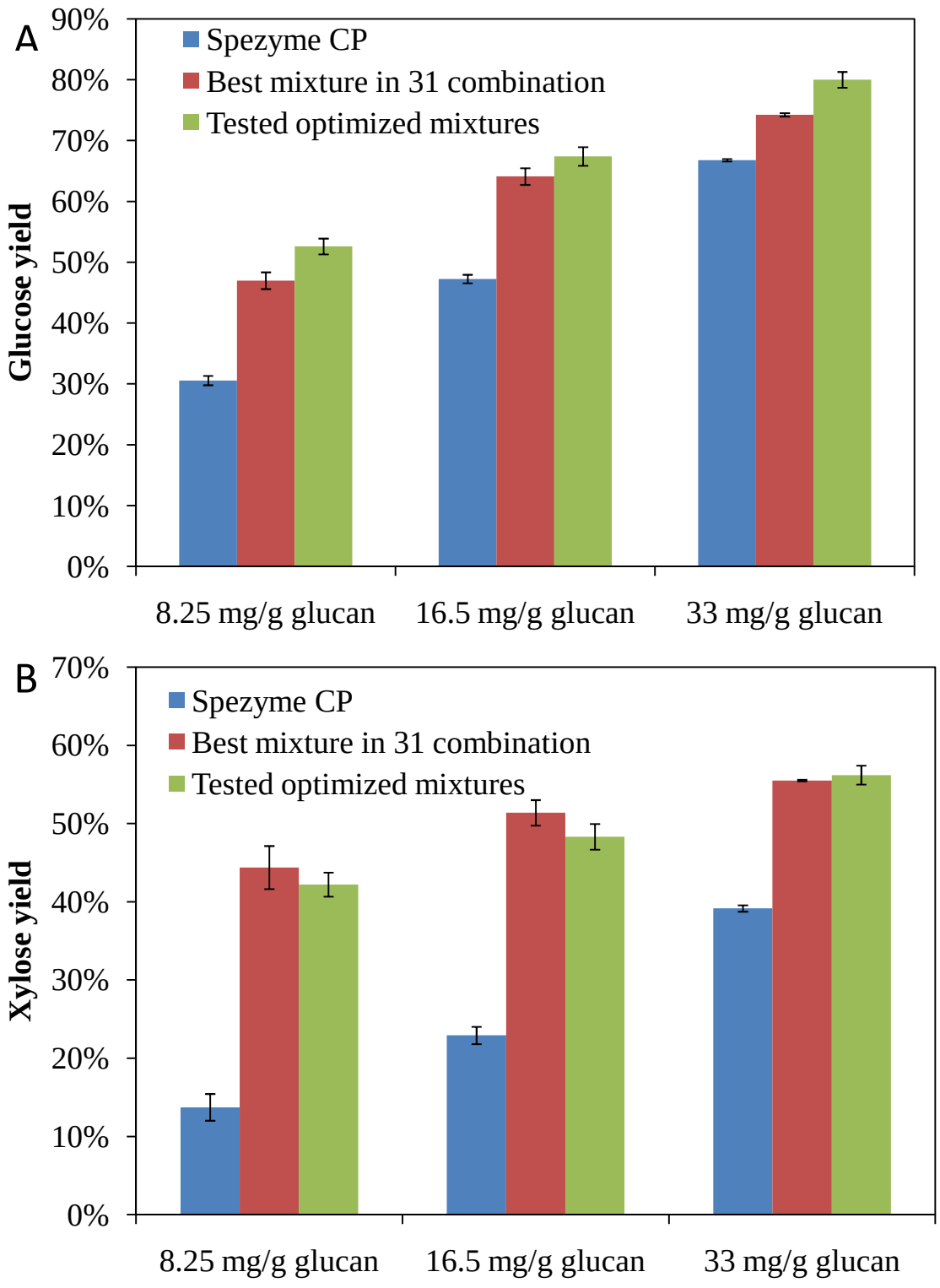


Figure 4.4 Glucose (A) and xylose (B) yield for different enzyme cocktails at 24 hours.

4.4 Conclusion

We have successfully isolated six core cellulases and hemicellulases through various purification and heterologous expression strategies. Thirty one unique combinations of purified fungal glycosyl hydrolase mixtures were tested on AFEX treated corn stover to determine glucose and xylose yields, at three total protein loadings (8.25, 16.5 and 33 mg/g glucan; inclusive of all six enzymes) using a suitable design of experiments methodology. The optimal enzyme ratios that maximized hydrolysis yields were found to be strongly dependent on the total enzyme loading employed, with endoglucanase I (EG I) playing a relatively more important role at lower total protein loadings. Reducing relative cellulase ratio by addition of suitable hemicellulases (endoxylanase, β-xylosidase) helped significantly enhance xylose yield with no decrease in glucose yield. The range of optimal ratios for the cocktail containing six core enzymes, maximizing glucan and xylan hydrolysis yields of AFEX treated corn stover, is comprised of 27-30% CBH I, 17-20% CBH II, 29-35% EG I, 14-15% EX, 2-6% βX and 1-5% βG based on total protein mass loading. These results demonstrate the potential to rationally design an enzyme mixture targeted towards a particular feedstock and pretreatment that can help maximize hydrolysis yields and minimize protein usage in a cellulosic biorefinery.

CHAPTER 5 STRATEGY FOR IDENTIFICATION OF NOVEL FUNGAL AND BACTERIAL GLYCOSYL HYDROLASE HYBRID MIXTURES

5.1 Introduction

Previous chapter demonstrate how fungal enzymes work synergistically. However, in nature, anaerobic bacterial enzymes are typically aggregated and assembled on a complex scaffold structure through various integrating modules known as cohesins and dockerins [191]. These enzyme complexes, known as cellulosomes, are attached to the surface of the bacterial cell walls [192-194]. Few studies have investigated the synergism among catalytic domains of various bacterial enzymes, and the synergistic interactions between bacterial and fungal hydrolases acting on pretreated lignocellulosic biomass. Some reports have shown exo/exo and exo/endo synergism between fungal and bacterial enzymes hydrolyzing crystalline cellulose [195, 196]. Recent publications have reported synergy between *Trichoderma* and *Serratia/Streptomyces* based on chitin degrading hydrolases completely hydrolyzing untreated crab shells [197]. But, very few reports are available on the nature of synergistic interactions between bacterial and fungal enzymes, especially bacterial hemicellulases hydrolyzing pretreated lignocellulosic biomass.

In this chapter, the enzymatic digestibility of Ammonia Fiber Expansion (AFEX) treated corn stover was evaluated by varying combinations of fungal and bacterial glycosyl hydrolases. Fungal enzymes (CBH I, CBH II and EG I) were purified from suitable commercial sources (Spezyme CP); while βG was purified from Novozyme 188. Two cellulases (LC1 and LC2), two xylanases (LX1 and LX2), one β-glucosidase (LβG) and one β-xylosidase (LβX) were obtained from various bacterial sources (e.g. *Clostridium, Geobacillus, Dictyoglomus*). This paper

presents a rational four-step strategy for designing an optimal enzyme cocktail, based on enzymes from multiple sources, to efficiently hydrolyze pretreated lignocellulosic biomass to help ultimately decrease the cost of cellulosic ethanol.

5.2 Materials and Methods

5.2.1 AFEX pretreatment

Detailed information is described in 4.2.1

5.2.2 Discovery and cloning of LX1, LX2, LβX and LβG

Detailed information is published by Gao et al[198]. Those discovery and cloning work have been done by Lucigen.

5.2.3 Discovery and cloning of LC1 and LC2

Detailed information is published by Gao et al[198]. The protein sequences and strain sources are listed in Table 5.1.

Table 5.1 Amino acid sequences and glycosyl hydrolase families for all six bacterial enzymes

Notation	Amino acid sequence	GH family	Uniprot No.	Source, name and predicted molecular weight
LC1	MNNLPIKRGINFGDALEAPYEGAWSGYIIKDEYFKIVKDAGFDHVRIPIKWSVYTQKEAPYSIEKRIFDRVDHLIEEGLKNNLHVIINIHHYEEIMEDPLGEKERFLAIWRQISEHYKDYPNNLYFELLNEPTQNLSSELWNQFLKEAIEVIRRTNPERKIIVGPDNWNSLYNLEKLIIPENDENIIITFHYYNPFPFTHQGAGWVKIDLPVGVKWLGTEEEKREIERELDMAVSWAEEHGNIPLYMGEFGAYSKADMESRVRWTDFVARSAEKRGIAWSYWEFYSGFGVFDPEKNEWRTPLLRALIPERNI*	5	B8DZM2	Dictyoglomus turgidum (Dtur_0670, 37 kDa)
LC2	MVSFKAGINLGGWISQYQVFSKEHFDTFITEKDIETIAEAGFDHVRLPFDYPIIESDDNVGEYKEDGLSYIDRCLEWCKKYNLGLVLDMHHAPGYRFQDFKTSTLFEDPNQQKRFVDIWRFLAKRYINEREHIAFELLNEVVEPDSTRWNKLMLEYIKAIREIDSTMWLYIGGNNYNSPDELKNLADIDDDYIVYNFHFYNPFFFTHQKAHWSESAMAYNRTVKYPGQYEGIEEFVKNNPKYSFMMELNNLKLNKELLRKDLKPAIEFREKKKCKLYCGEFGVIAIADLESRIKWHEDYISLLEEYDIGGAVWNYKKMDFEIYNEDRKPVSQELVNILARRKT*	5	P0C2S3	Clostridium thermocellum (CELC, 40.9 kDa)
LX 1	MAKTEQSYAKKPQISALHAPQLDQRYKDSFTIGAAVEPYQLLNEKDAQMLKRHFNSIVAENVMKPINIQPEEGKFNFAEADQIVRFAKKHHMDIRFHTLVWHSQVPQWFFLDKEGQPMVNETDPVKREQNKQLLLKRIETHIKTIVERYKDDIKYWDVVNEVVGDDGELRDSPWYQIAGIDYIKVAFQTARKYGGNKIKLYINDYNTEVEPKRSALYNLVKQLKEEGIPIDGIGHQSHIQIDWPSEEEIEKTIIMFADLGLDNQITELDVSMYGWPPRAYLSYDAIPEQKFLDQADRYDRLFKLYEKLSDKISNVTFWGIADNHTWLDSRADVYYDTDGNVIVDPKAPYTRVEKGNGKDAPFVFDPEYNVKPAYWAIIDHK*	10	B4BME8	Geobacillus sp. G11MC16 (47.4 kDa)
LX 2	MCSSIPSLREVFANDFRIGAAVNPVTLEAQQSLLIRHVNSLTAENHMKFEHLQPEEGRFTFDIAIKSSTSPFSSHGVRGHTLVWHNQTPSWVFQDSQGHFVGRDVLLERMKSHISTVVQRYKGKVYCWDVVNEAVADEGSEWLRSSTWRQIIGDDFIQQAFLYAHEADPEALLFYNDYNECFPEKREKIYTLVKSLRDKGIPIHGIGMQAHWSLTRPTLDEIRAAIERYASLGVILHITELDISMFEFDDHRKDLAAPTNEMVERQAERYEQIFSLFKEYRDVIQNVTFWGIADDHTWLDHFPVQGRKNWPLLFDEQHNPKPAFWRVVNI*	10	P45703	Geobacillus stearothermophilus (XynA, 38.5 kDa)

Table 5.1 (cont'd)

βX	MPTNVFFNAHHSPVGAFASFTLGFPGKSGGLDLELARPPRQNVFIGVESSHEPGL YHILPFAETAGEDESKRYDIENPDPNPQKPNILIPFAKERIEREFRVATDTWKAGD LTLTIYSPVKAVPDPETASEEELKLALVPAVIVEMTIDNTNGTRTRRAFFGFEGTD PYTSMRRIDDTCPQLRGVGQGRILGIASKDEGVRSALHFSMEDILTATLEENWTF GLGKVGALIADVPAGEKKTYQFAVCFYRGGYVTAGMDASYFYTRFFHNIEEVG LYALEQAEVLKEQAFCSNELIEKEWLSDDQKFMMAHAIRSYYGNTQLLEHEGK PIWVVNEGEYRMMNTFDLTVDQLFFELKMNPWTVKNVLDFYVERYSYEDRVR FPGDETEYPGGISFTHDMGVANTFSRPHYSSYELYGISGCFSHMTHEQLVNWVL CAAVYIEQTKDWAWRDRRLTILEQCLESMVRRDHPDPEKRNGVMGLDSTRTM GGAEITTYDSLDVSLGQARNNLYLAGKCWAAYVALEKLFRDVGKEELAALAGE QAEKCAATIVSHVTEDGYIPAVMGEGNDSKIIPAIEGLVFPYFTNCHEALKEDGR FGDYIRALRQHLQYVLREGICLFPDGGWKISSTSNNSWLSKIYLCQFIARHILGWE WDEQAKRADAAHVAWLTHPTLSIWSWSDQIIAGENYRSKYYPRGVTSILWLEE GE*	52	Q09LZ0	Geobacillus stearothermophilus (XYNB2, 79.8 kDa)
βG	MSKITFPKDFIWGSATAAYQIEGAYNEDGKGESIWDRFSHTPGNIADGHTGDVA CDHYHRYEEDIKIMKEIGIKSYRFSISWPRIFPEGTGKLNQKGLDFYKRLTNLLLE NGIMPAITLYHWDLPQKLQDKGGWKNRDTTDYFTEYSEVIFKNLGDIVPIWFTH NEPGVVSLLGHFLGIHAPGIKDLRTSLEVSHNLLLSHGKAVKLFREMNIDAQIGIA LNLSYHYPASEKAEDIEAAELSFSLAGRWYLDPVLKGRYPENALKLYKKKGIEL SFPEDDLKLISQPIDFIAFNNYSSEFIKYDPSSESGFSPANSILEKFEKTDMGWIIYPE GLYDLLMLLDRDYGKPNIVISENGAAFKDEIGSNGKIEDTKRIQYLKDYLTQAHR AIQDGVNLKAYYLWSLLDNFEWAYGYNKRFGIVHVNFDTLERKIKDSGYWYKE VIKNNGF*	1	P26208	Clostridium thermocellum (BGLA, 51.5 kDa)

5.2.4 Enzyme expression and purification

Detailed information is described in Chapter 3 and in a paper published by Gao et al[198].

5.2.5 Strategy for enzyme screening on realistic lignocellulosic substrates

A simple four-step strategy was applied for screening and comparing activities of novel glycosyl hydrolases to develop enzyme mixtures that can efficiently saccharify pretreated lignocellulosic biomass (Figure 5.1). A typical benchmark enzyme mixture could include fungal cellulases (CBH I + CBH II + EG I) along with a suitable β-glucosidase (βG). The goal was to compare the activity of novel enzymes with respect to a defined benchmark on realistic substrates like pretreated cellulosic biomass. The first step was to characterize the type of enzyme in order to assign it to a specific GH family (e.g. *p*NP-glycoside based activity assays and glycosyl hydrolase family determination based on amino acid sequence similarity). The second step was doping the new enzyme/s along with the benchmark mixture to determine the effect on digestibility of pretreated biomass. It may be necessary to swap the corresponding type of enzyme from the benchmark mixture before adding new enzymes to compare relative improvements. This iterative method allows one to determine the most efficient enzyme/enzyme mixtures that have high activity on pretreated lignocellulosic biomass and avoid the pitfall of screening individual enzymes on unrealistic substrates (e.g. CMC, *p*NP-glycosides) [114]. Once a minimal enzyme mixture has been defined it should be possible to further reduce enzyme dosage by optimizing the relative ratios of the enzymes in the mixture to maximize glucan and xylan digestibility [71].

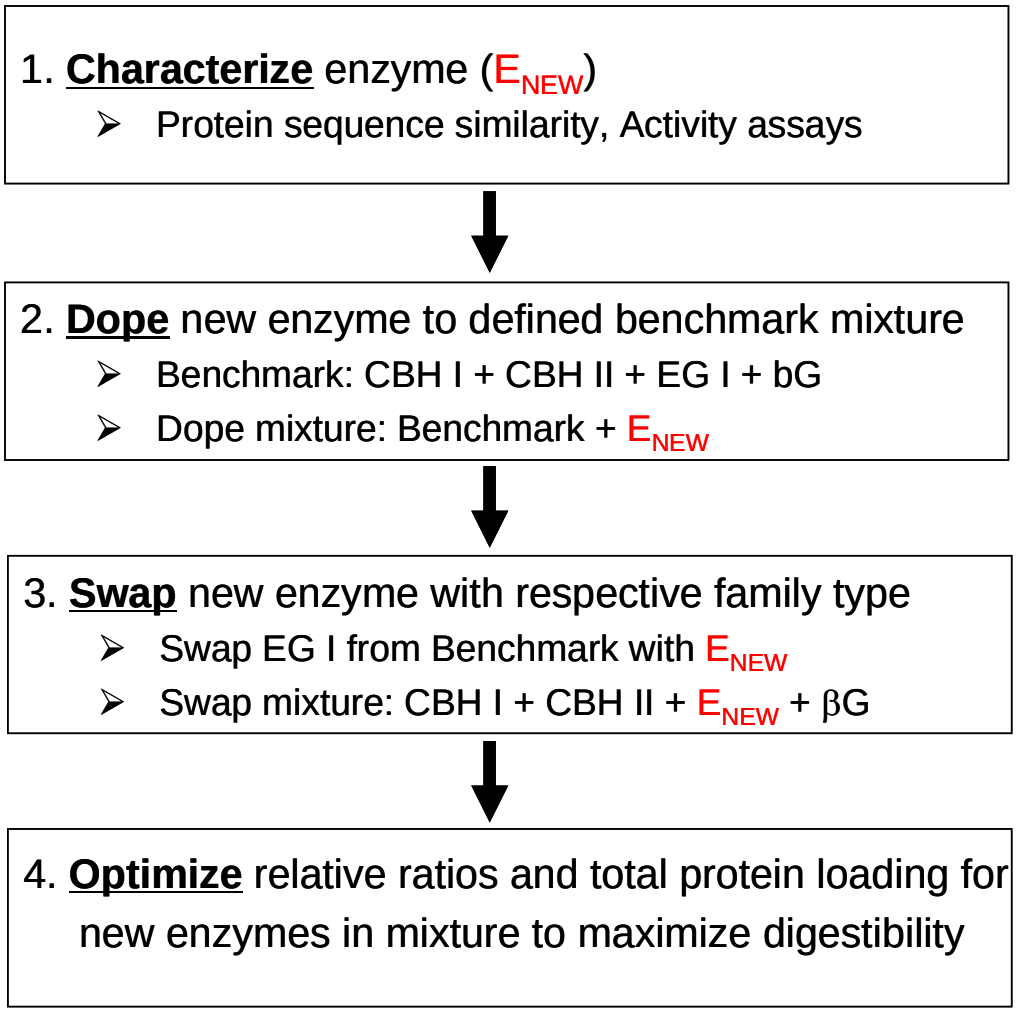


Figure 5.1 Four-step strategy for screening glycosyl hydrolases and developing novel enzyme mixtures to maximize digestibility of pretreated lignocellulose.

5.2.6 Enzyme activity assays

Detailed information is described in 4.2.6.

5.2.7 Enzymatic hydrolysis of pretreated biomass

Detailed information is described in 4.2.6.

5.2.8 Glucose and xylose assays

Detailed information is described in 4.2.8.

5.3 Results and discussions

5.3.1 Specific activities of bacterial and fungal enzymes

The enzymes were tested for their activity on different substrates at pH 5 (Table 5.2). LC1 and LC2 have significant *p*NP-cellobioside and CMC activity. Although both of the endocellulases were found to have significant activity on *p*NPC, their CMC activity was slightly lower or comparable to EG I. LX1 and LX2 were found to have much higher xylanase activity than EG I, though earlier work has reported another *Geobacillus* xylanase (with 88% similarity to LX1) to have lost 40-60% activity at pH 5 [66]. Recent work with a fungal endo-xylanase isolated from *Trichoderma* has shown that the *Geobacillus* enzyme has 3-5 fold higher activity on oat spelt xylan under identical conditions [71]. LβX was found to have high xylosidase activity but poor α-arabinofuranosidase activity, comparable to what has been reported earlier [199]. The bacterial β-glucosidase (LβG) had significantly lower cellobiose activity than its fungal counterpart (βG). No noticeable activity on Avicel was detected for any of the bacterial enzymes compared to the fungal cellulases.

Table 5.2 Activity assay data for bacterial and fungal enzymes.

Activity Units										
	LC1	LC2	LX1	LX2	LβX	LβG	CBH I	CBH II	EG I	βG
*p*NPC *	79.6	378.8	11.1	65.6	-	130.1	1.4	-	44.6	2470
*p*NPL *	207.7	353.1	-	8.3	-	143.8	7.4	-	19.3	-
*p*NPG *	-	-	-	-	1.5	320.3	-	-	-	4150
*p*NPAf *	-	-	-	-	9.4	-	-	-	-	-
*p*NPX *	-	-	0.9	4.2	1545	15.1	-	-	-	9.52
Avicel **	-	-	-	-	-	-	0.02	0.03	0.01	-
CMC **	6.6	1.1	-	-	-	-	-	-	6.7	-
Xylan **	-	-	19.3	24.4	-	-	-	-	5.1	-
Cellobiose **	-	-	-	-	7.8	31.1	-	-	-	125

One unit of activity on *p*NP based substrates (*) is equivalent to one nmol of *p*NP (*p*-nitrophenol) released/mg enzyme/min.

One unit of activity on Avicel/Xylan/CMC/Cellobiose (**) is equivalent to one μmol of glucose equivalent released/mg enzyme/min.

Where; "-" stands for no detectable activity; *p*NPC (*p*NP-β-D-cellobioside); *p*NPL (*p*NP-β-D-lactopyranoside); *p*NPG (*p*NP-β-D-glucopyranoside); *p*NPAf (*p*NP-α-L-arabinofuranoside); *p*NPX (*p*NP-β-D-xylopyranoside); CMC (Carboxymethyl cellulose).

Figure 5.2 depicts the hydrolysis yields on AFEX corn stover for all 6 bacterial enzymes added together as a mixture at varying pH and temperatures. The enzyme loading was 4 mg/g glucan each for LC1, LC2, LX1 and LX2; 2 mg/g glucan each for LβX and LβG. At pH 6.5 and 50 °C, the xylose yield was approximately 50%, suggesting the high hemicellulase activity for the enzymes. However, the glucan conversion was significantly lower (< 5%). The activity assays showed that none of the bacterial enzymes had any significant activity on Avicel (Table 5.2). Although the bacterial enzymes cloned belonged to thermophilic microbes, hydrolysis yields at 70 ^{o}C were lower compared to 50 ^{o}C. It is possible that the enzymes lost activity at high temperature during the prolonged incubation (24 hours). Since the bacterial and fungal enzymes have a different working pH ranges, a mixture of both enzymes was tested on pH 6.5 and pH 4.5. For fungal enzymes, the optimal pH was found to be at 4.5-5 (data not shown). When tested at pH 6.5, significant loss in activity was observed for the fungal enzymes. For an equimass mixture of CBH I, CBH II and EG I, the glucan hydrolysis yield decreased to 10% (pH 6.5) compared to 60% at pH 4.5 (24 hrs hydrolysis, data not shown).

Interestingly, the bacterial β-xylosidase has been reported earlier to retain about 40% activity at pH 5 vs. pH 6.5 [199]. We noticed only a 25% loss in activity based on the overall xylan conversions for AFEX corn stover (Figure 5.3). This would suggest that optimizing the bacterial

hemicellulases at pH 4.5-5 along with fungal cellulases would be possible considering the significant activity retained at acidic pH.

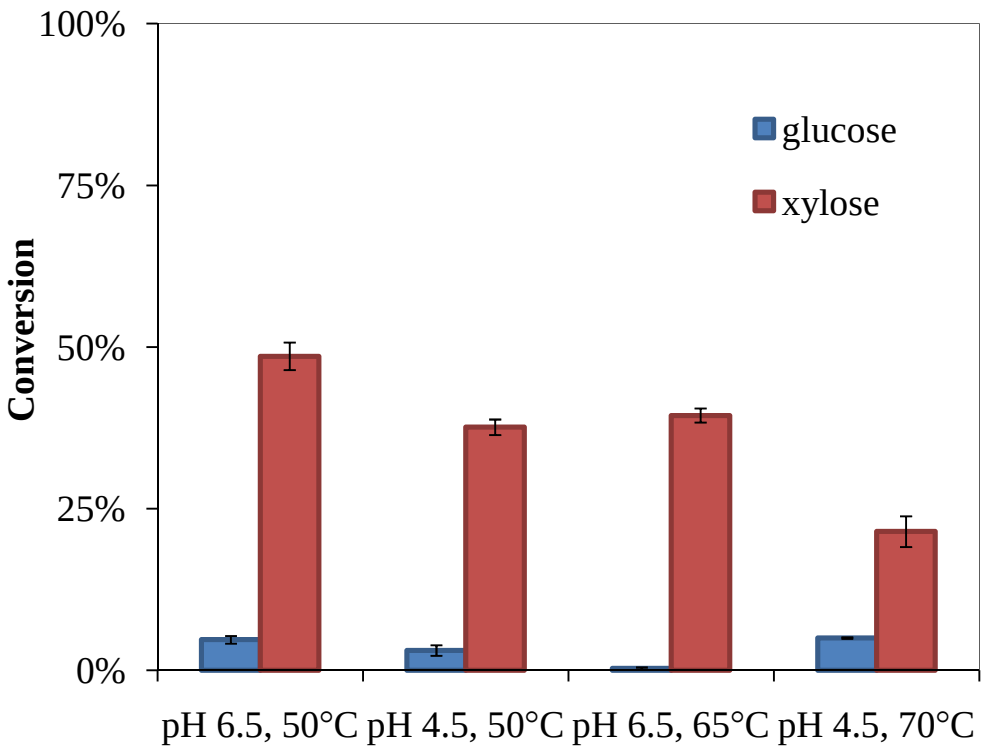


Figure 5.2 Percent glucan (blue bar) and xylan (red bar) conversion after 24 hours hydrolysis of AFEX treated corn stover. Six bacterial enzymes were added together as a mixture at varying pH and temperature. Each enzyme mixture contains both bacterial cellulases and hemicellulases (4 mg/g glucan each for LC1, LC2, LX1 and LX2; 2 mg/g glucan each for LβX and LβG).

5.3.2 Doping and swapping bacterial/fungal cellulases

The experimental design for the doping and swapping experiments is shown in Table 5.3, which was conducted under specific assay conditions (pH 4.5-5.0, 50 ^{o}C, 24 hrs) using AFEX treated corn stover. From Figure 5.3A, experiments #A-B show that swapping of LβG with βG does not significantly increase the glucose or xylose yield. From the results of experiment #G it can be

observed that LβG showed lower glucan conversion even at much higher enzyme loadings (Figure 5.3B and Table 5.3). Experiments #F-J also indicate that the bacterial β-glucosidase has much lower activity compared to its fungal counterpart. The current batch of bacterial enzymes does not possess substantial exo-cellulase activity to hydrolyze AFEX treated corn stover. Therefore, purified fungal cellulases (CBH I, CBH II, EG I) were doped into the enzyme mix in order to further enhance the glucose yield (Experiments #C-E). Doping any of the three fungal cellulases increased the glucan conversion to around 20%. Interestingly, #E has a higher xylose yield possibly due to cross-activity of EG I on xylan. Doping LC1, LC2 or both together into a fungal mixture did not significantly improve either the glucose or xylose hydrolysis yield (Experiments #K-M). The above results demonstrate that bacterial cellulases (LC1, LC2, LβG) do not significantly improve the digestibility of pretreated biomass compared to fungal cellulases (CBH I, CBH II, βG), despite the fact that the bacterial enzymes were found to have significant activity on artificial substrates (like CMC and *p*NP-glycosides). Wilson et al. have shown synergism between certain bacterial endo-glucanases and fungal exo-glucannases on filter paper, but the overall digestions were still quite low [130]. It is possible that due to lack of suitable exo-cellulase activity the bacterial cellulases (LC1 and LC2) are currently unable to completely hydrolyze the substrate. Previous results for *Clostridium thermocellum* β-glucosidase have also revealed that gene functions encoding for hydrolytic activity on MU-glucoside and/or cellobiose are associated closely on the chromosome [130].

Table 5.3 Mixtures of bacterial and fungal enzymes tested on AFEX treated corn stover. The experimental results for these enzyme combinations are shown in Figure 5.3.

			Individual enzyme loading (mg/g glucan)									
	#	Mix type	LC1	LC2	LX1	LX2	LβG	LβX	CBH I	CBH II	EG I	βG
A	A	Control	4	4	4	4	2	2				
	B	Swap	4	4	4	4		2				2
	C	Dope	4	4	4	4		2	4			2
	D	Dope	4	4	4	4		2		4		2
	E	Dope	4	4	4	4		2			4	2
B	F	Swap					2		4	4	4	
	G	Swap					10		4	4	4	
	H	Benchmark							4	4	4	2
	I	Swap/Dope					2	2	4	4	4	
	J	Dope						2	4	4	4	2
	K	Dope	4	4					4	4	4	2
	L	Dope	4						4	4	4	2
	M	Dope		4					4	4	4	2
C	O	Dope			4				4	4	4	2
	P	Dope				4			4	4	4	2
	Q	Dope			4	4			4	4	4	2
	R	Dope			4	4		2	4	4	4	2

5.3.3 Doping of bacterial hemicellulases to fungal benchmark mixture

Although bacterial cellulases (LC1, LC2 and LβG) did not work effectively on their own or work synergistically with fungal enzymes, the bacterial hemicellulases (LX1, LX2 and LβX) were found to have significant activity on pretreated corn stover (Experiments #O-R). As shown in Figure 5.3C (experiment design shown in Table 5.3), doping LX1 and LX2 to the fungal benchmark mixture helps increase the xylose and glucose yield. Hydrolyzing xylan enhances the accessibility of the cellulases to the residual cellulose microfibrils and results in higher glucan conversions. When LX1 and LX2 were doped together (Experiment #Q), glucan conversion increased to 70% while no noticeable increase was seen for xylose (Experiments #O-P). Addition of LβX (Experiment #R), helped increase the xylan conversion substantially (71% glucan and 76% xylan conversion). In order to enhance both glucan and xylan conversion, LβX is important to hydrolyze soluble xylan based oligosaccharides which are inhibitors of endoxylanases and cellulases. There have been reports on increased conversions on both xylan and glucan by supplementation of hemicellulases [139]. In order to completely digest the xylan fraction, β-xylosidase is indispensible to hydrolyze xylo-oligosaccharides, especially xylobiose to monomeric xylose [200]. Previous results have also shown that addition of bacterial hemicellulases to fungal cellulases, results in increasing both glucose and xylose yields [201].

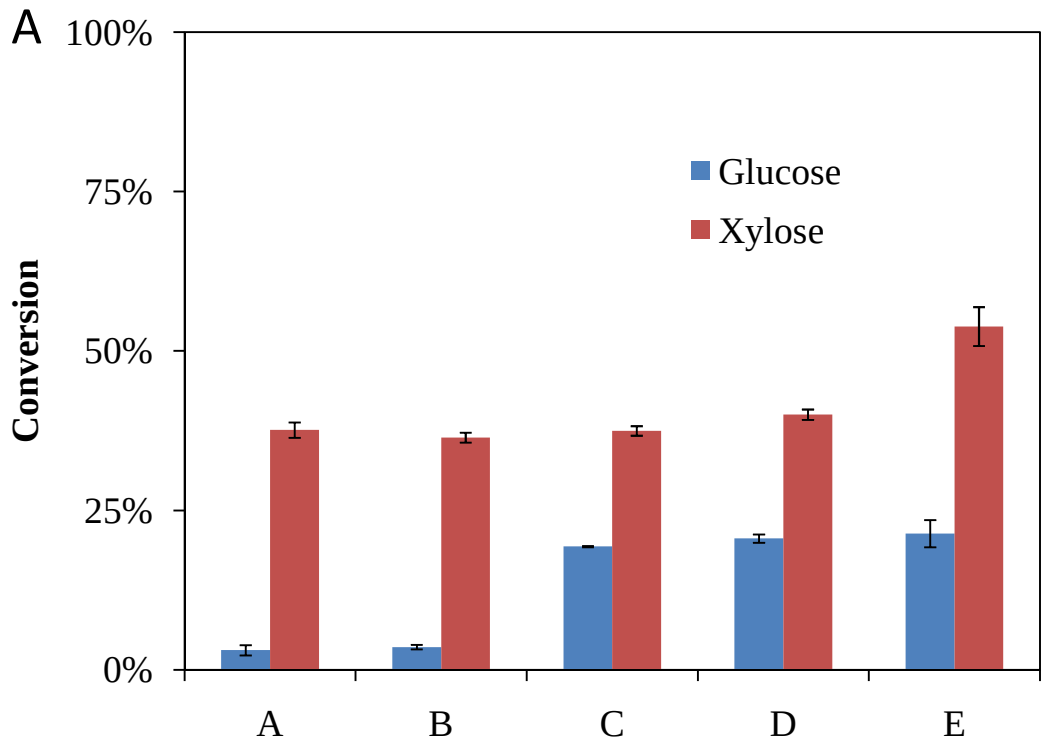
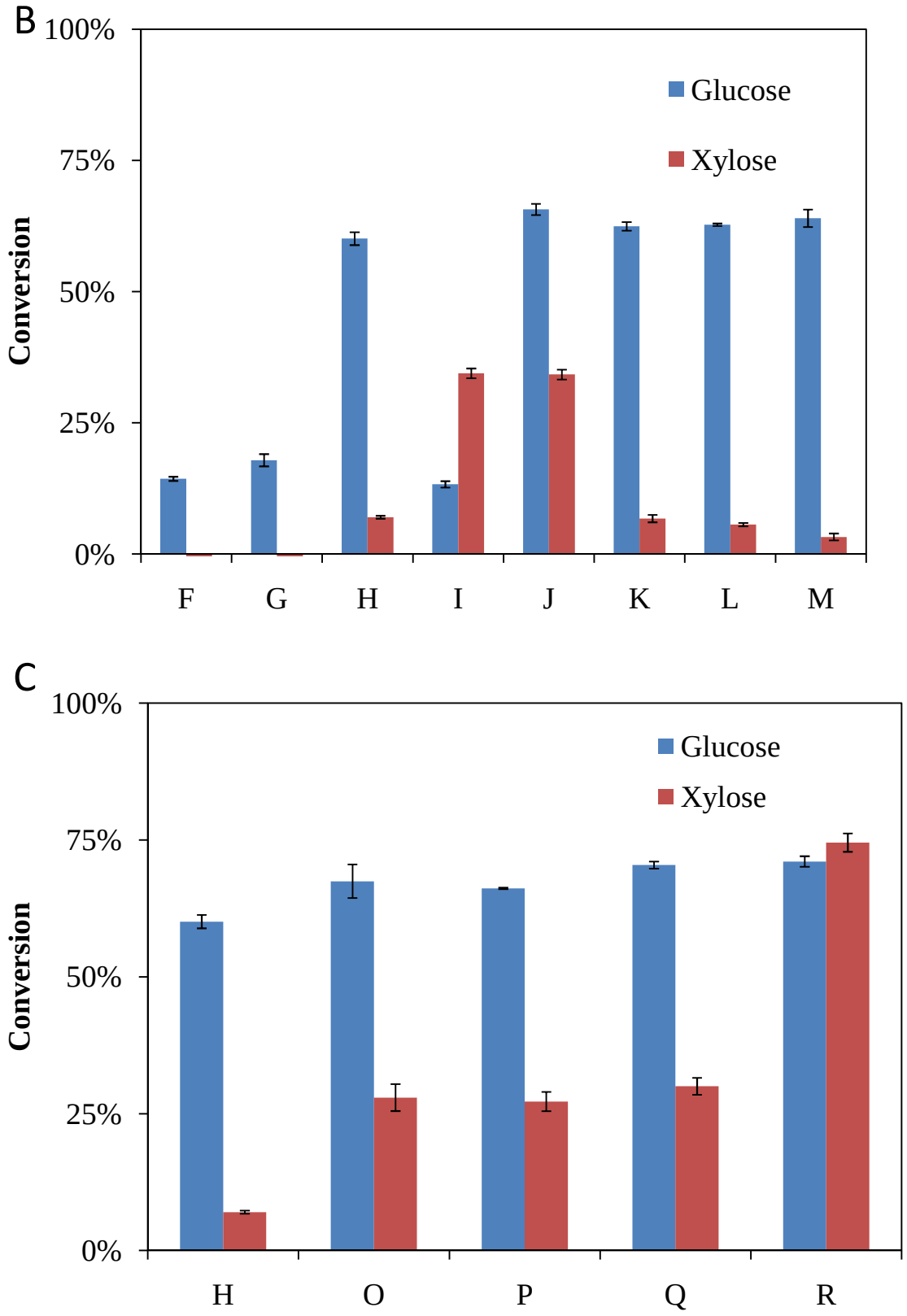


Figure 5.3 Percent glucan (blude bar) and xylan (red bar) conversions after 24 hours hydrolysis of AFEX treated corn stover. Enzyme mixtures used for experimental data sets in panel A (A to E), panel B (F to M) and panel C (H, O to R) are based on protein compositions listed in Table 5.3.

Figure 5.3 (cont'd)



5.3.4 Enzyme mixture optimization

Previous results have demonstrated the synergistic interactions between fungal cellulases (CBH I, CBH II, EG I and βG) and bacterial hemicellulases (LX1, LX2 and LβG). However, individual enzyme ratios need to be optimized to allow further increase in glucan and xylan conversions. In order to do this, 73 different enzyme combinations were tested in duplicates (standard deviations were less than 5% for the replicates) and the average hydrolysis yields for both glucan and xylan were determined for three different protein loadings (Table 5.4). βG was loaded at a 10% (mass ratio) of the total remaining enzymes to ensure complete hydrolysis of cellobiose [125]. CBH I and CBH II were added at more than 20% (total protein excluding βG) in all mixtures to ensure sufficient cellulase activity. EG I added was at least 10% of the mixture. Bacterial LX1 and LX2 together were always more than 5% of total enzyme loading while bacterial LβX was greater than 1%. All of the above constraints were based on the fact that cellobiohydrolase, endoglucanase, β-glucosidase, endoxylanase and β-xylosidase are indispensible for an efficient enzyme cocktail [71]. Deconstruction of crystalline cellulose is the major limiting step towards complete hydrolysis, and hence requires a significant amount of cellulase. It is clear that increasing the bacterial hemicellulases loading beyond 10 mg/g of glucan does not significant increase the xylan conversion. Using the current cocktail of enzymes the xylan conversion could not exceed 75% conversion even when glucan conversion was over 90%. It is possible that other hemicellulases (e.g. α-arabinofuranosidase, α-glucuronidase) are necessary to further increase the xylan conversion. Without suitable complementary hemicellulases, xylan conversion is a bottleneck and increasing the total enzyme loading alone would not result in 100% xylan conversion.

Table 5.4 24 hours glucan and xylan hydrolysis yields of AFEX treated corn stover by various enzyme mixtures at three different total protein loadings. βG was loaded at 10 (mass loading) of all other proteins.

	Enzymes ratio (%)						30 mg/g glucan	
#	CBH I	CBH II	EG I	LX1	LX2	LβX	Glucan Yield (%)	Xylan yield (%)
1	20.0	0.0	10.0	0.0	37.0	33.0	47.6	49.3
2	84.0	0.0	10.0	5.0	0.0	1.0	70.1	47.3
3	0.0	52.0	42.0	5.0	0.0	1.0	56.5	49.2
4	0.0	20.0	10.0	34.5	34.5	1.0	38.1	46.3
5	84.0	0.0	10.0	2.5	2.5	1.0	60.6	38.3
6	10.0	10.0	74.0	5.0	0.0	1.0	89.0	54.1
7	0.0	84.0	10.0	0.0	5.0	1.0	45.4	36.9
8	0.0	20.0	10.0	0.0	37.0	33.0	31.3	44.5
9	52.0	0.0	42.0	0.0	5.0	1.0	68.0	44.6
10	20.0	0.0	10.0	0.0	69.0	1.0	51.4	50.2
11	0.0	52.0	10.0	37.0	0.0	1.0	52.0	48.1
12	0.0	20.0	10.0	0.0	69.0	1.0	40.4	48.7
13	0.0	84.0	10.0	2.5	2.5	1.0	48.2	41.0
14	42.0	42.0	10.0	5.0	0.0	1.0	81.2	46.8
15	51.0	9.0	18.0	5.3	7.8	9.0	83.9	56.1
16	52.0	0.0	42.0	5.0	0.0	1.0	71.3	47.6
17	10.0	10.0	10.0	69.0	0.0	1.0	80.1	54.4
18	10.0	10.0	10.0	0.0	69.0	1.0	67.2	48.5
19	0.0	20.0	74.0	5.0	0.0	1.0	44.8	40.8
20	9.0	51.0	18.0	7.8	5.3	9.0	86.2	60.2
21	52.0	0.0	10.0	37.0	0.0	1.0	63.7	47.9
22	0.0	20.0	10.0	69.0	0.0	1.0	41.1	49.6
23	20.0	0.0	10.0	34.5	34.5	1.0	53.1	50.6
24	52.0	0.0	10.0	5.0	0.0	33.0	65.7	55.3
25	9.0	19.0	18.0	5.3	7.8	41.0	79.6	56.5
26	52.0	0.0	10.0	0.0	5.0	33.0	53.3	45.7
27	84.0	0.0	10.0	0.0	5.0	1.0	64.6	41.0
28	0.0	52.0	10.0	0.0	5.0	33.0	37.1	39.9
29	42.0	42.0	10.0	0.0	5.0	1.0	78.3	43.6
30	51.0	9.0	18.0	7.8	5.3	9.0	90.2	60.2
31	20.0	0.0	10.0	69.0	0.0	1.0	52.7	52.0
32	9.0	19.0	18.0	7.8	5.3	41.0	81.2	60.0
33	20.0	0.0	42.0	0.0	37.0	1.0	65.1	47.0
34	0.0	52.0	10.0	5.0	0.0	33.0	45.3	48.8

Table 5.4 (cont'd)

35	0.0	20.0	74.0	2.5	2.5	1.0	44.5	40.4
36	20.0	0.0	74.0	2.5	2.5	1.0	67.1	47.5
37	0.0	20.0	10.0	37.0	0.0	33.0	37.5	48.2
38	0.0	20.0	42.0	0.0	37.0	1.0	45.7	49.4
39	9.0	51.0	18.0	5.3	7.8	9.0	81.2	56.0
40	19.0	9.0	50.0	5.3	7.8	9.0	81.9	54.1
41	19.0	9.0	18.0	5.3	7.8	41.0	79.5	56.8
42	9.0	19.0	50.0	7.8	5.3	9.0	90.0	59.3
43	20.0	0.0	10.0	0.0	5.0	65.0	42.6	47.2
44	20.0	0.0	10.0	37.0	0.0	33.0	53.5	55.1
45	10.0	10.0	10.0	0.0	5.0	65.0	64.0	49.7
46	0.0	52.0	42.0	0.0	5.0	1.0	49.5	42.3
47	0.0	20.0	10.0	0.0	5.0	65.0	33.1	42.8
48	0.0	20.0	42.0	37.0	0.0	1.0	48.4	50.3
49	0.0	20.0	10.0	2.5	2.5	65.0	33.0	42.7
50	20.0	0.0	10.0	5.0	0.0	65.0	46.7	50.3
51	9.0	19.0	50.0	5.3	7.8	9.0	77.7	53.6
52	20.0	0.0	74.0	5.0	0.0	1.0	64.8	45.8
53	9.0	19.0	18.0	39.8	5.3	9.0	83.6	59.7
54	19.0	9.0	18.0	7.8	5.3	41.0	85.3	60.0
55	20.0	0.0	42.0	0.0	5.0	33.0	57.2	49.5
56	0.0	20.0	42.0	0.0	5.0	33.0	43.5	50.4
57	20.0	0.0	74.0	0.0	5.0	1.0	67.0	48.7
58	20.0	0.0	10.0	2.5	2.5	65.0	48.8	52.7
59	19.0	9.0	50.0	7.8	5.3	9.0	94.7	62.9
60	10.0	10.0	10.0	5.0	0.0	65.0	74.7	57.9
61	0.0	20.0	10.0	5.0	0.0	65.0	33.0	44.7
62	52.0	0.0	10.0	0.0	37.0	1.0	63.5	49.6
63	20.0	0.0	42.0	5.0	0.0	33.0	57.3	49.5
64	20.0	0.0	42.0	37.0	0.0	1.0	58.4	46.3
65	19.0	9.0	18.0	5.3	39.8	9.0	76.5	53.8
66	0.0	84.0	10.0	5.0	0.0	1.0	49.9	44.2
67	0.0	52.0	10.0	0.0	37.0	1.0	44.5	45.9
68	0.0	20.0	74.0	0.0	5.0	1.0	46.8	43.3
69	9.0	19.0	18.0	5.3	39.8	9.0	74.9	53.4
70	18.0	18.0	26.0	10.5	10.5	17.0	77.3	53.6
71	19.0	9.0	18.0	39.8	5.3	9.0	79.0	54.8
72	0.0	20.0	42.0	5.0	0.0	33.0	45.4	53.3
73	10.0	10.0	74.0	0.0	5.0	1.0	82.4	50.1

Table 5.4 (cont'd)

	Enzymes ratio (%)						15mg/g glucan	
#	CBH I	CBH II	EG I	LX1	LX2	LβX	Glucan Yield (%)	Xylan yield (%)
1	20.0	0.0	10.0	0.0	37.0	33.0	31.8	41.6
2	84.0	0.0	10.0	5.0	0.0	1.0	44.7	33.0
3	0.0	52.0	42.0	5.0	0.0	1.0	37.7	35.9
4	0.0	20.0	10.0	34.5	34.5	1.0	28.4	39.3
5	84.0	0.0	10.0	2.5	2.5	1.0	44.0	30.6
6	10.0	10.0	74.0	5.0	0.0	1.0	60.7	37.5
7	0.0	84.0	10.0	0.0	5.0	1.0	30.3	26.0
8	0.0	20.0	10.0	0.0	37.0	33.0	21.8	38.8
9	52.0	0.0	42.0	0.0	5.0	1.0	49.4	31.5
10	20.0	0.0	10.0	0.0	69.0	1.0	31.6	36.3
11	0.0	52.0	10.0	37.0	0.0	1.0	34.8	38.7
12	0.0	20.0	10.0	0.0	69.0	1.0	26.1	34.8
13	0.0	84.0	10.0	2.5	2.5	1.0	33.0	29.1
14	42.0	42.0	10.0	5.0	0.0	1.0	66.5	35.2
15	51.0	9.0	18.0	5.3	7.8	9.0	69.5	50.3
16	52.0	0.0	42.0	5.0	0.0	1.0	55.4	37.6
17	10.0	10.0	10.0	69.0	0.0	1.0	54.9	40.8
18	10.0	10.0	10.0	0.0	69.0	1.0	51.2	37.8
19	0.0	20.0	74.0	5.0	0.0	1.0	34.2	35.9
20	9.0	51.0	18.0	7.8	5.3	9.0	59.5	46.8
21	52.0	0.0	10.0	37.0	0.0	1.0	42.6	39.3
22	0.0	20.0	10.0	69.0	0.0	1.0	27.8	38.3
23	20.0	0.0	10.0	34.5	34.5	1.0	33.7	40.7
24	52.0	0.0	10.0	5.0	0.0	33.0	38.5	43.2
25	9.0	19.0	18.0	5.3	7.8	41.0	62.5	46.8
26	52.0	0.0	10.0	0.0	5.0	33.0	34.2	36.8
27	84.0	0.0	10.0	0.0	5.0	1.0	39.7	25.2
28	0.0	52.0	10.0	0.0	5.0	33.0	26.8	35.0
29	42.0	42.0	10.0	0.0	5.0	1.0	61.8	28.1
30	51.0	9.0	18.0	7.8	5.3	9.0	71.6	51.0
31	20.0	0.0	10.0	69.0	0.0	1.0	32.2	40.3
32	9.0	19.0	18.0	7.8	5.3	41.0	58.4	46.8
33	20.0	0.0	42.0	0.0	37.0	1.0	44.5	38.6
34	0.0	52.0	10.0	5.0	0.0	33.0	29.5	40.0

Table 5.4 (cont'd)

35	0.0	20.0	74.0	2.5	2.5	1.0	30.4	33.0
36	20.0	0.0	74.0	2.5	2.5	1.0	45.9	33.4
37	0.0	20.0	10.0	37.0	0.0	33.0	28.0	46.4
38	0.0	20.0	42.0	0.0	37.0	1.0	30.5	36.8
39	9.0	51.0	18.0	5.3	7.8	9.0	64.7	49.7
40	19.0	9.0	50.0	5.3	7.8	9.0	70.2	42.5
41	19.0	9.0	18.0	5.3	7.8	41.0	61.2	48.8
42	9.0	19.0	50.0	7.8	5.3	9.0	64.2	47.1
43	20.0	0.0	10.0	0.0	5.0	65.0	24.4	36.1
44	20.0	0.0	10.0	37.0	0.0	33.0	37.9	46.3
45	10.0	10.0	10.0	0.0	5.0	65.0	43.6	39.3
46	0.0	52.0	42.0	0.0	5.0	1.0	35.7	29.5
47	0.0	20.0	10.0	0.0	5.0	65.0	21.3	35.6
48	0.0	20.0	42.0	37.0	0.0	1.0	33.6	41.2
49	0.0	20.0	10.0	2.5	2.5	65.0	21.9	37.6
50	20.0	0.0	10.0	5.0	0.0	65.0	27.2	40.7
51	9.0	19.0	50.0	5.3	7.8	9.0	61.0	46.9
52	20.0	0.0	74.0	5.0	0.0	1.0	44.2	34.8
53	9.0	19.0	18.0	39.8	5.3	9.0	61.8	49.2
54	19.0	9.0	18.0	7.8	5.3	41.0	62.4	48.7
55	20.0	0.0	42.0	0.0	5.0	33.0	38.6	42.7
56	0.0	20.0	42.0	0.0	5.0	33.0	29.7	42.1
57	20.0	0.0	74.0	0.0	5.0	1.0	42.6	32.7
58	20.0	0.0	10.0	2.5	2.5	65.0	30.0	40.0
59	19.0	9.0	50.0	7.8	5.3	9.0	71.8	54.5
60	10.0	10.0	10.0	5.0	0.0	65.0	46.0	45.4
61	0.0	20.0	10.0	5.0	0.0	65.0	22.4	39.0
62	52.0	0.0	10.0	0.0	37.0	1.0	40.0	35.2
63	20.0	0.0	42.0	5.0	0.0	33.0	40.3	47.4
64	20.0	0.0	42.0	37.0	0.0	1.0	44.8	41.7
65	19.0	9.0	18.0	5.3	39.8	9.0	60.8	47.8
66	0.0	84.0	10.0	5.0	0.0	1.0	33.1	31.1
67	0.0	52.0	10.0	0.0	37.0	1.0	29.7	33.1
68	0.0	20.0	74.0	0.0	5.0	1.0	31.8	30.3
69	9.0	19.0	18.0	5.3	39.8	9.0	59.9	49.0
70	18.0	18.0	26.0	10.5	10.5	17.0	65.5	49.4
71	19.0	9.0	18.0	39.8	5.3	9.0	58.5	48.2
72	0.0	20.0	42.0	5.0	0.0	33.0	30.2	45.4
73	10.0	10.0	74.0	0.0	5.0	1.0	61.8	36.1

Table 5.4 (cont'd)

	Enzymes ratio (%)						10 mg/g glucan	
#	CBH I	CBH II	EG I	LX1	LX2	LβX	Glucan Yield (%)	Xylan yield (%)
1	20.0	0.0	10.0	0.0	37.0	33.0	26.2	43.9
2	84.0	0.0	10.0	5.0	0.0	1.0	34.8	26.3
3	0.0	52.0	42.0	5.0	0.0	1.0	31.9	27.2
4	0.0	20.0	10.0	34.5	34.5	1.0	23.1	35.5
5	84.0	0.0	10.0	2.5	2.5	1.0	34.7	21.5
6	10.0	10.0	74.0	5.0	0.0	1.0	51.4	29.8
7	0.0	84.0	10.0	0.0	5.0	1.0	25.0	20.1
8	0.0	20.0	10.0	0.0	37.0	33.0	19.5	40.2
9	52.0	0.0	42.0	0.0	5.0	1.0	37.5	24.5
10	20.0	0.0	10.0	0.0	69.0	1.0	20.1	26.1
11	0.0	52.0	10.0	37.0	0.0	1.0	28.6	31.2
12	0.0	20.0	10.0	0.0	69.0	1.0	20.9	29.5
13	0.0	84.0	10.0	2.5	2.5	1.0	25.7	20.6
14	42.0	42.0	10.0	5.0	0.0	1.0	61.3	28.6
15	51.0	9.0	18.0	5.3	7.8	9.0	55.9	43.0
16	52.0	0.0	42.0	5.0	0.0	1.0	45.0	30.0
17	10.0	10.0	10.0	69.0	0.0	1.0	45.2	34.3
18	10.0	10.0	10.0	0.0	69.0	1.0	41.9	30.3
19	0.0	20.0	74.0	5.0	0.0	1.0	23.7	26.1
20	9.0	51.0	18.0	7.8	5.3	9.0	53.4	44.1
21	52.0	0.0	10.0	37.0	0.0	1.0	37.4	34.6
22	0.0	20.0	10.0	69.0	0.0	1.0	21.7	33.8
23	20.0	0.0	10.0	34.5	34.5	1.0	25.6	33.3
24	52.0	0.0	10.0	5.0	0.0	33.0	30.0	39.2
25	9.0	19.0	18.0	5.3	7.8	41.0	53.3	48.3
26	52.0	0.0	10.0	0.0	5.0	33.0	30.6	36.3
27	84.0	0.0	10.0	0.0	5.0	1.0	30.0	20.9
28	0.0	52.0	10.0	0.0	5.0	33.0	20.6	29.9
29	42.0	42.0	10.0	0.0	5.0	1.0	52.4	20.3
30	51.0	9.0	18.0	7.8	5.3	9.0	58.8	47.9
31	20.0	0.0	10.0	69.0	0.0	1.0	25.3	34.0
32	9.0	19.0	18.0	7.8	5.3	41.0	51.7	50.5
33	20.0	0.0	42.0	0.0	37.0	1.0	34.3	30.8
34	0.0	52.0	10.0	5.0	0.0	33.0	24.9	39.7

Table 5.4 (cont'd)

35	0.0	20.0	74.0	2.5	2.5	1.0	24.8	25.7
36	20.0	0.0	74.0	2.5	2.5	1.0	35.4	25.6
37	0.0	20.0	10.0	37.0	0.0	33.0	23.1	46.5
38	0.0	20.0	42.0	0.0	37.0	1.0	24.0	27.5
39	9.0	51.0	18.0	5.3	7.8	9.0	61.0	43.4
40	19.0	9.0	50.0	5.3	7.8	9.0	63.3	52.2
41	19.0	9.0	18.0	5.3	7.8	41.0	46.9	39.2
42	9.0	19.0	50.0	7.8	5.3	9.0	56.9	37.3
43	20.0	0.0	10.0	0.0	5.0	65.0	18.2	34.2
44	20.0	0.0	10.0	37.0	0.0	33.0	30.2	45.3
45	10.0	10.0	10.0	0.0	5.0	65.0	32.9	35.9
46	0.0	52.0	42.0	0.0	5.0	1.0	32.8	26.4
47	0.0	20.0	10.0	0.0	5.0	65.0	16.9	33.8
48	0.0	20.0	42.0	37.0	0.0	1.0	28.7	35.6
49	0.0	20.0	10.0	2.5	2.5	65.0	16.5	33.2
50	20.0	0.0	10.0	5.0	0.0	65.0	20.4	35.7
51	9.0	19.0	50.0	5.3	7.8	9.0	60.1	48.8
52	20.0	0.0	74.0	5.0	0.0	1.0	37.1	31.2
53	9.0	19.0	18.0	39.8	5.3	9.0	51.6	48.3
54	19.0	9.0	18.0	7.8	5.3	41.0	54.8	44.9
55	20.0	0.0	42.0	0.0	5.0	33.0	29.7	42.2
56	0.0	20.0	42.0	0.0	5.0	33.0	22.6	38.7
57	20.0	0.0	74.0	0.0	5.0	1.0	31.6	25.7
58	20.0	0.0	10.0	2.5	2.5	65.0	24.5	39.6
59	19.0	9.0	50.0	7.8	5.3	9.0	63.1	52.3
60	10.0	10.0	10.0	5.0	0.0	65.0	35.4	43.5
61	0.0	20.0	10.0	5.0	0.0	65.0	18.2	38.6
62	52.0	0.0	10.0	0.0	37.0	1.0	34.1	28.9
63	20.0	0.0	42.0	5.0	0.0	33.0	29.6	40.1
64	20.0	0.0	42.0	37.0	0.0	1.0	37.1	36.6
65	19.0	9.0	18.0	5.3	39.8	9.0	52.8	47.9
66	0.0	84.0	10.0	5.0	0.0	1.0	28.4	25.6
67	0.0	52.0	10.0	0.0	37.0	1.0	24.5	25.8
68	0.0	20.0	74.0	0.0	5.0	1.0	26.0	24.1
69	9.0	19.0	18.0	5.3	39.8	9.0	47.4	42.6
70	18.0	18.0	26.0	10.5	10.5	17.0	58.6	49.9
71	19.0	9.0	18.0	39.8	5.3	9.0	52.2	49.3
72	0.0	20.0	42.0	5.0	0.0	33.0	24.9	40.5
73	10.0	10.0	74.0	0.0	5.0	1.0	43.7	25.1

Glucan and xylan hydrolysis results for all mixtures are shown in Figure 5.4 as a scatter plot. The relative ratio of the individual enzymes significantly affected the overall sugar yield. At 10 mg/g glucan loading, the highest glucan conversion is 63.1% while the lowest is 20.6%. The highest xylan conversion is 52.3% while the lowest is 20.1%, at the same protein loading. Similarly, major differences in overall conversions can be seen for other protein loadings as well. One of the best mixtures resulting in the highest glucan and xylan conversions contained 19% CBH I, 9% CBH II, 50% EG I, 7.8% LX1, 5.3% LX2 and 9.0% LβX.

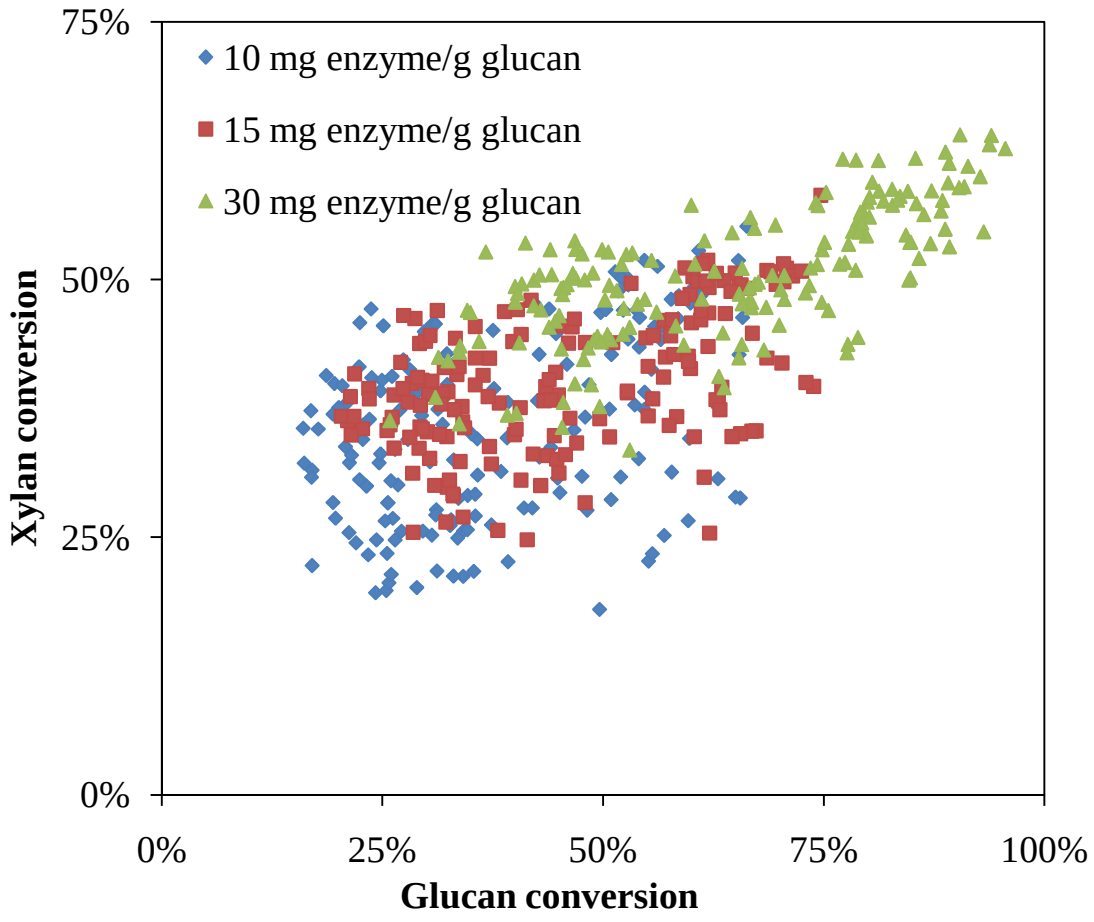


Figure 5.4 Glucan (X-axis) versus xylan (Y-axis) conversion after 24 hours hydrolysis of AFEX treated corn stover. Hydrolysis results from three different total enzyme loadings (inclusive of CBH I + CBH II + EG I + LX 1 + LX 2 + LβX, as listed in

Table 5.4) are depicted by green triangles (30 mg/g glucan), red squares (15 mg/g glucan) and blue diamonds (10 mg/g glucan). An additional loading of 3, 1.5 and 1 mg/g glucan of βG was supplemented in each case, respectively.

5.3.5 Relationship between glucan and xylan conversions

Glucan and xylan conversion for various combinations of enzymes at three different total enzyme loadings are shown in Figure 5.4. The three clusters for different enzyme loadings demonstrate that at higher enzyme loading, the glucan and xylan conversion is generally higher. Another interesting phenomenon observed is that at higher enzyme loadings, the shape of the data point cluster is narrower. At lower enzyme loadings, the data points are more scattered. By applying linear regression on xylan conversions vs. corresponding glucan conversion for various enzyme mixtures, a linear relationship between the two variables is confirmed (Table 5.5). The P values are close to 0, indicating that the linear relationship has statistical significance. When the total enzyme loading was increased (from 10 to 30 mg/g glucan) the R^2 (Coefficient of determination) value increases as well. This validates our visual interpretation of the shape of the data cluster at low vs. high enzyme loadings.

Table 5.5 Linear regression of xylan vs. glucan conversion at three different total enzyme loadings.

Xylan conversion = Constant + A*glucan conversion					
Enzyme loading (mg/g glucan)	Constant		A		R^2
	Coefficient	P	Coefficient	P	
10	0.24347	0.000	0.3061	<0.001	0.218
15	0.29395	0.000	0.23899	<0.001	0.262
30	0.34521	0.000	0.24774	<0.001	0.451

CBH I and CBH II are both indispensible for efficient hydrolysis. For all 3 varying enzyme loadings, if the mixture does not contain CBH I, the glucan conversions are quite low. When

both CBH I and CBH II are included, higher glucan conversions are possible (>90% glucan conversion at 30 mg/g glucan enzyme loading). Xylan conversion is not markedly affected by the presence of either CBH I or II. Xylan conversion tends to be slightly higher (5-10%) at higher enzyme loadings when both CBH's are present. LX1 has slightly higher specific activity compared to LX2 on AFEX treated corn stover.

Figure 5.5 is helpful in visually summarizing the optimal regions of enzyme ratios for maximizing both glucan and xylan digestibility. Different clusters of data (based Table 5.4) were separated based on the overall ratio of cellulases (I), xylanases (II) and βX (III). The three enzyme loadings were plotted as insets (a), (b) and (c) representing 10, 15 and 30 mg/g glucan enzyme loading, respectively. At high enzyme loadings, the higher glucan and xylan yielding data points aggregate closely compared to the lower enzyme loading. This suggests that glucan and xylan yields are more sensitive to individual enzyme ratios at lower enzyme loading. At high cellulase loading (94%), the hemicellulase loading is much lower and both glucan and xylan yields are relatively lower. This further confirms our previous assumption that in order to maximize glucan yield, higher xylan hydrolysis yields are desirable. On the other hand, for higher hemicellulase loading (>37%), xylan conversion is slightly lower while glucan conversions drop significantly. Similar trends for βX at around 9% loading of total protein mass ratio are seen as well. To achieve high conversions of glucan and xylan, 78% cellulases (CBH I, CBH II and EG I), 13% xylanase (LX1 and LX2) and 9% βX seems to be optimal. The optimal cellulase loading (total of 78%; total protein mass basis) for both CBH I and CBH II ranges from 9-51%; while EG I ranges from 10-50%.

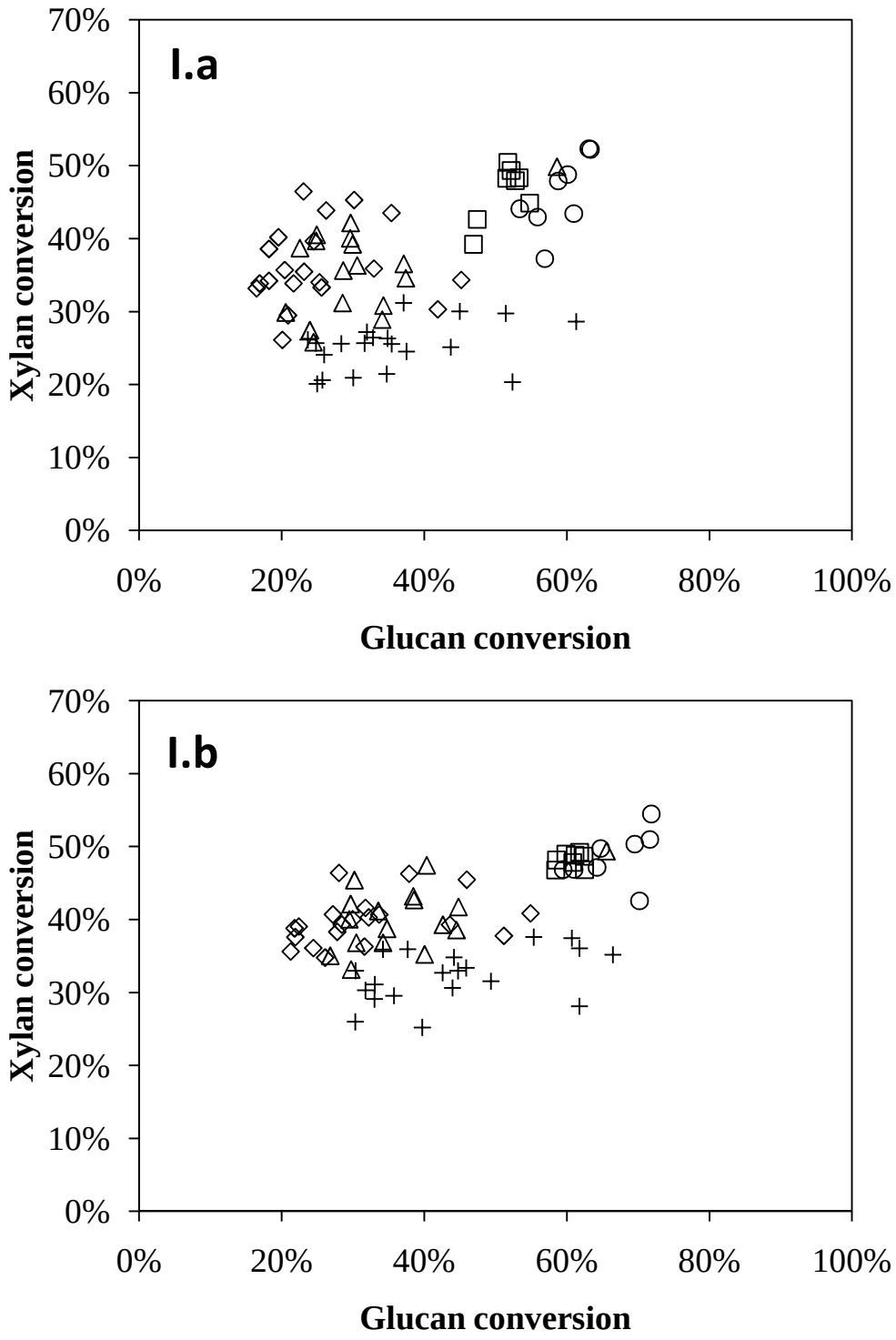
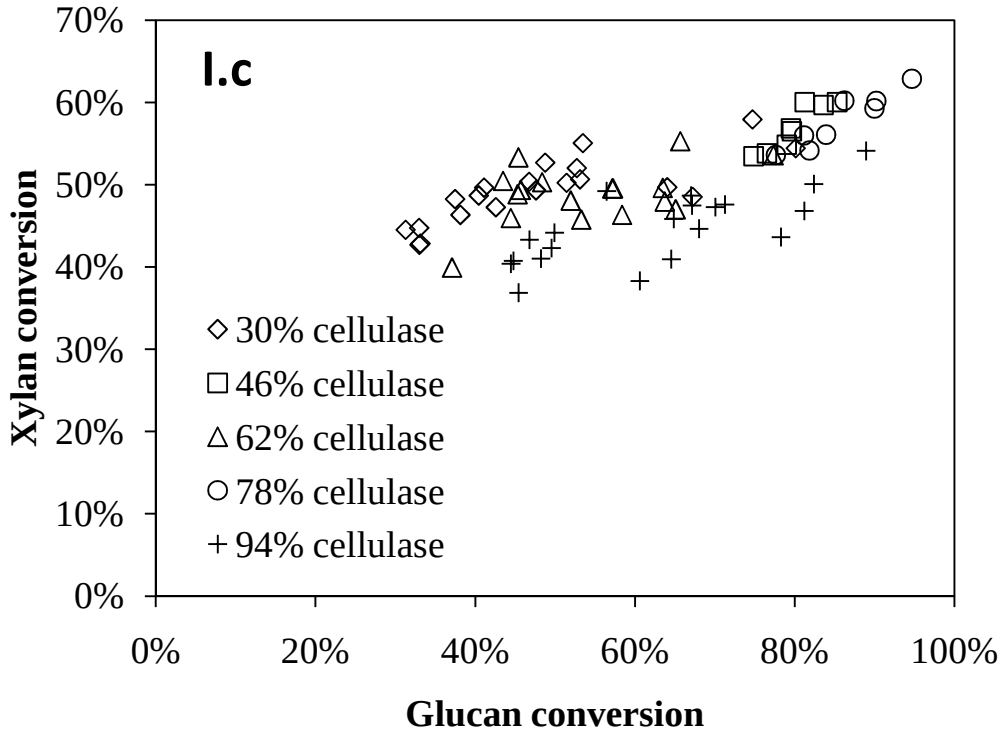


Figure 5.5 Glucan (X-axis) versus xylan (Y-axis) conversion after 24 hours hydrolysis of AFEX treated corn stover for varying relative ratios of cellulases (panel I), xylanases (panel II) and β-xylosidase (panel III) at three different total enzyme loadings (a, b and c correspond to 10, 15 and 30 mg/g glucan enzyme loading, respectively).

Figure 5.5 (cont'd)



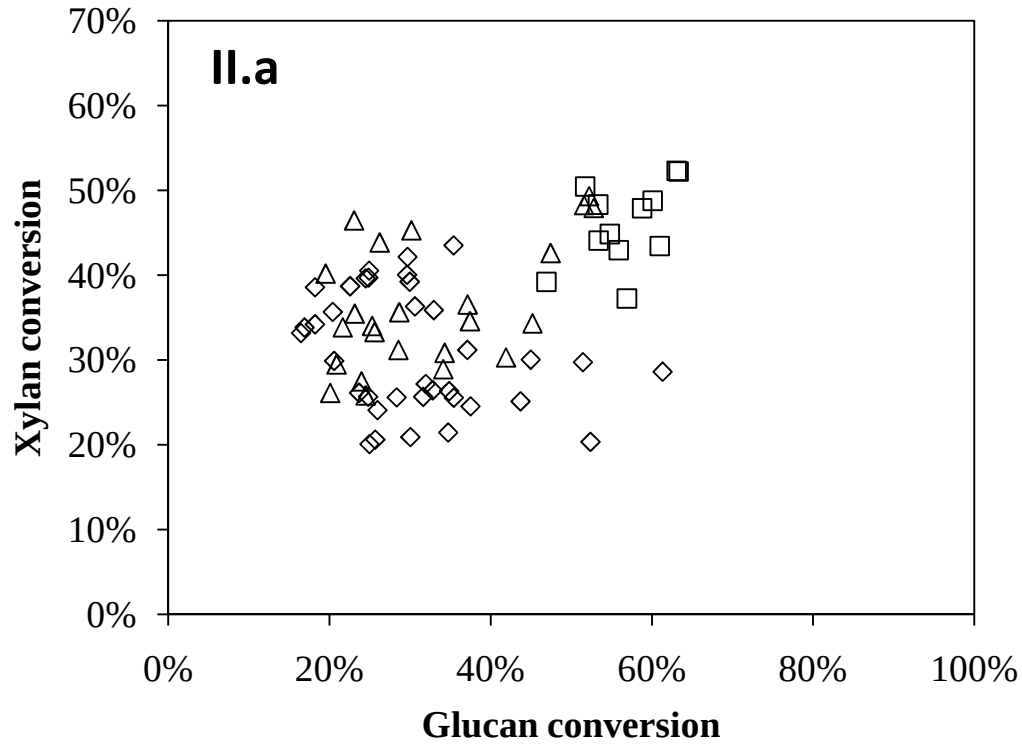
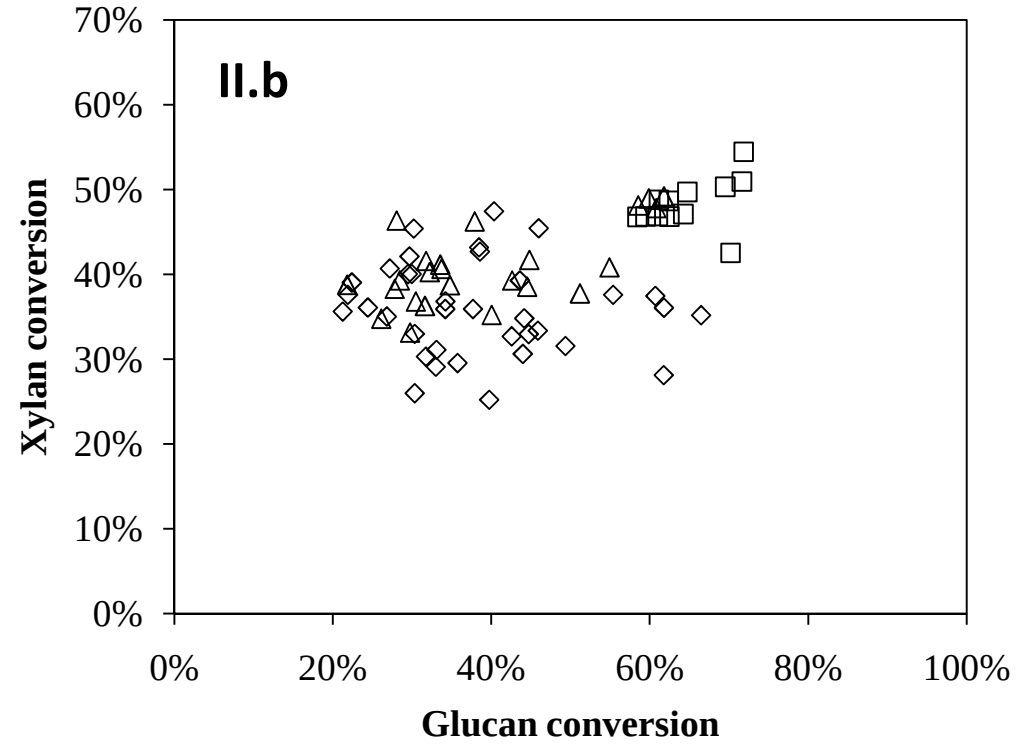
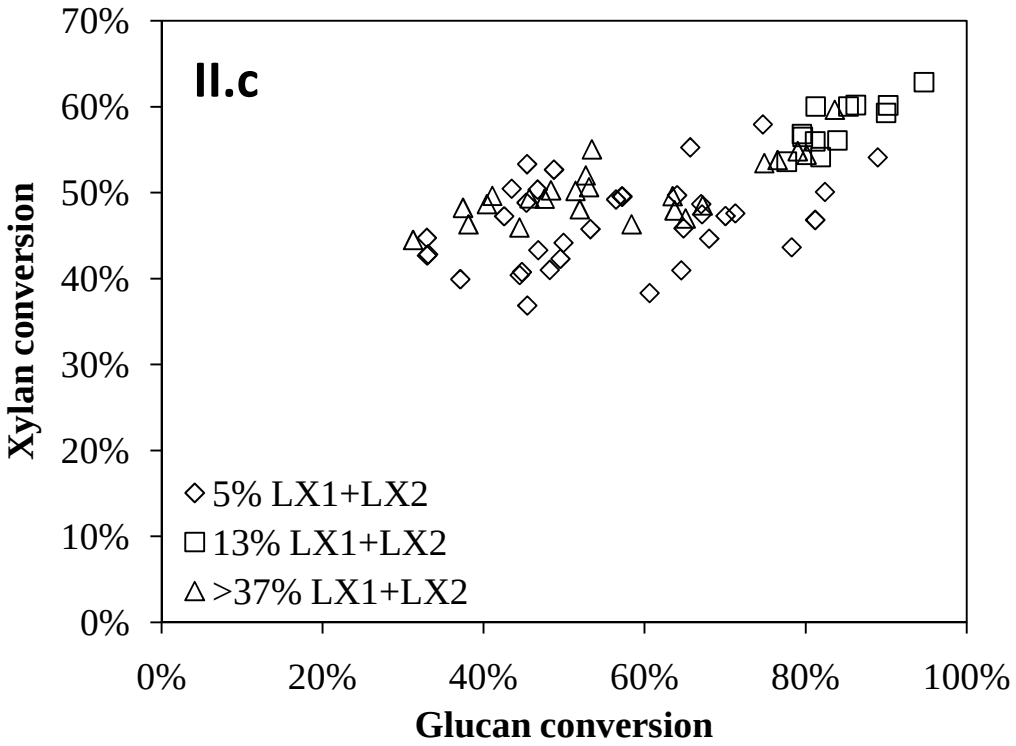


Figure 5.5 (cont'd)





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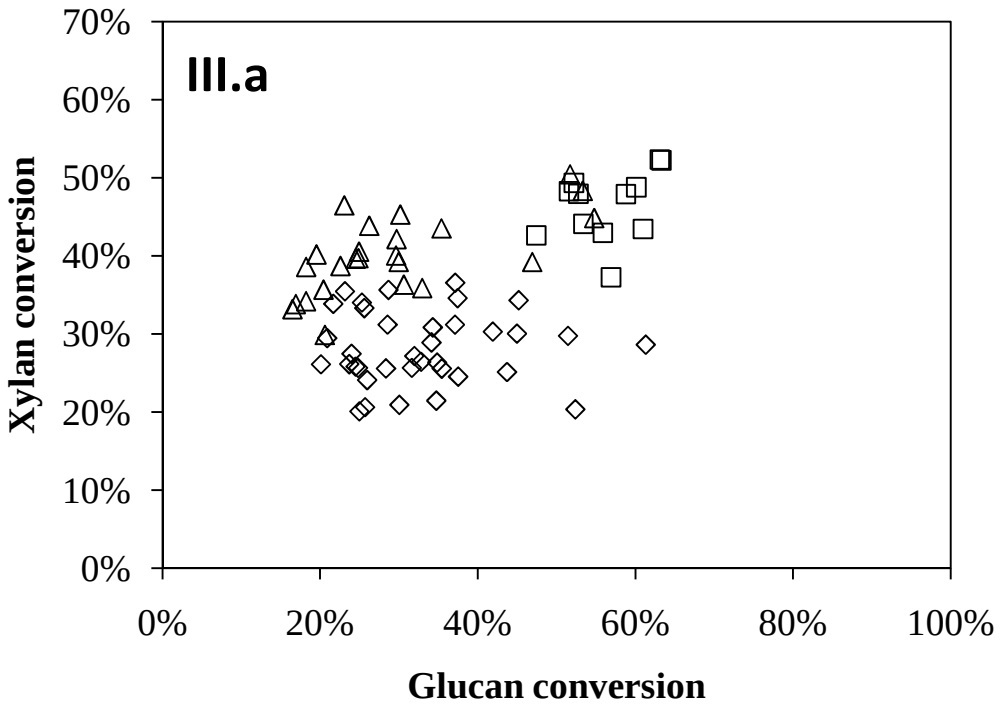

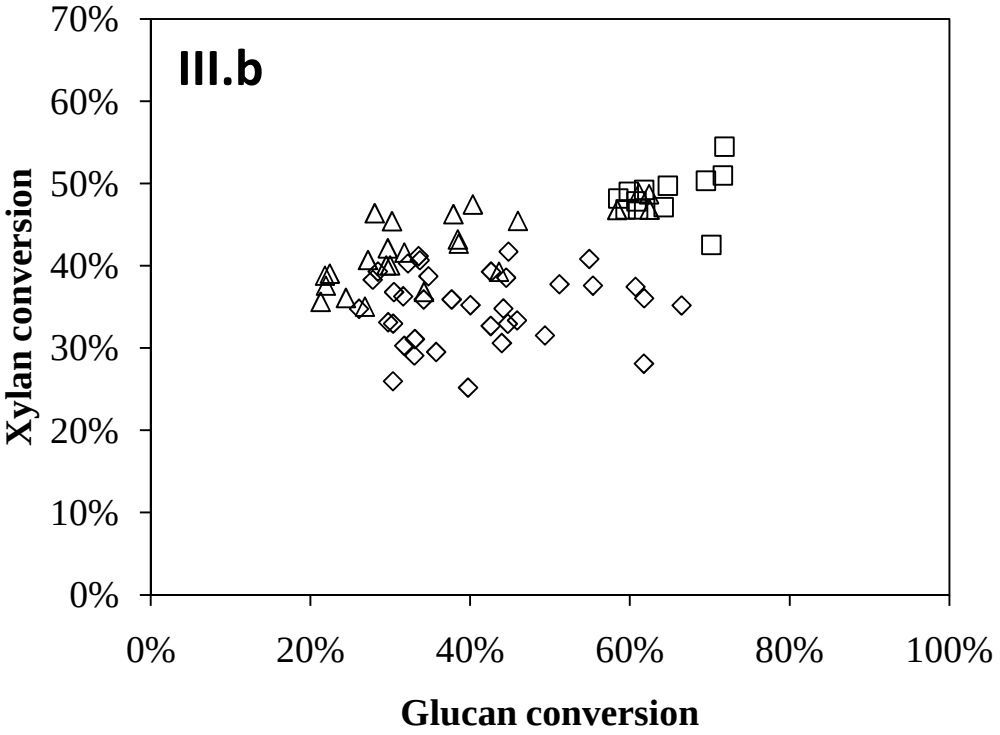

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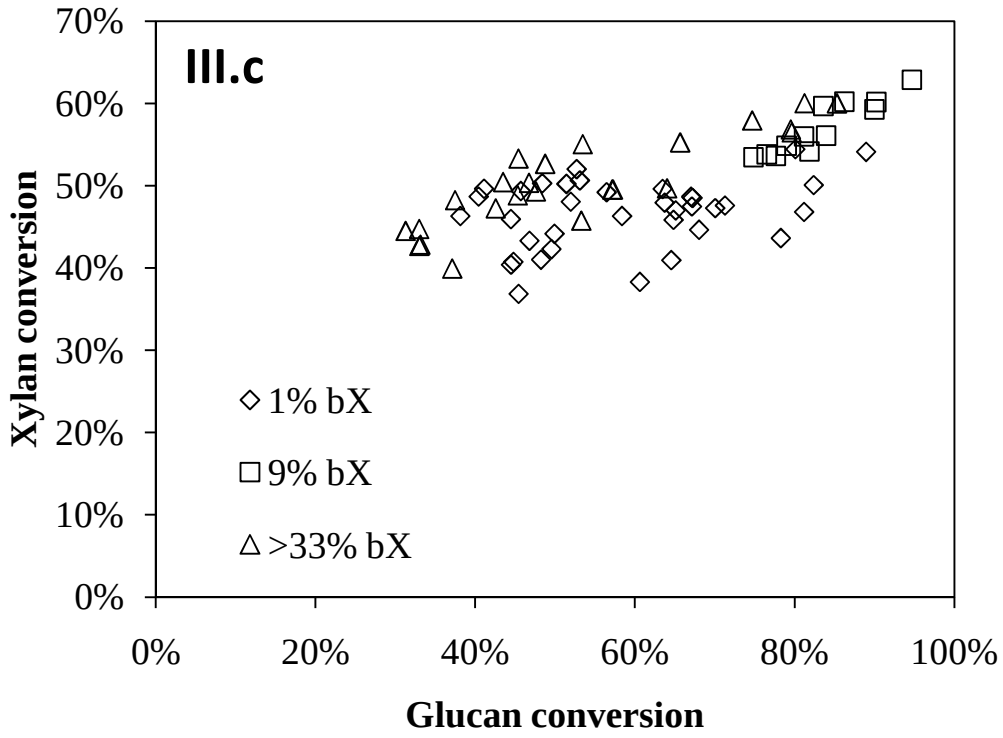


5.3.6 Discussion

It is interesting to note that the cellobiase activity for the bacterial enzyme (LβG) is significantly lower than its fungal counterpart (lower activity even at pH 6.5, data not shown). Previous work has reported anaerobic bacterial enzyme complexes to be easily inhibited by cellobiose [202], suggesting that cellulosomal activity on cellobiose is relatively poorer (compared to their fungal counterparts). It is also possible that due to preferred metabolism of cellobiose and the hydrolyzed oligomers after phosphorylation by the bacterial cell, the activity of β-glucosidase is relatively poor compared to their fungal counterparts [203].

One of the limitations of hydrolyzing the substrate at pH 5 is the relatively lower activity of these bacterial enzymes (40-50% of optimum at pH 6.5) reported by Bravman et al. [199]. This would suggest that in the presence of a suitable bacterial exo/endo-cellulase complex (that has an

optimum activity at pH 6.5), it would be possible to further lower the total enzyme loading (and maximize glucan/xylan conversion). There have also been several reports on the activity of GH family 43 β-xylosidase, with very few publications on their GH 52 counterparts [199]. This study is one of the first that reports the activity of GH 52 β-xylosidases on pretreated lignocellulosic biomass.

5.4 Conclusion

In summary, we have examined the activity of both fungal and bacterial based enzyme mixtures on a realistic lignocellulosic substrate (i.e. AFEX pretreated corn stover). The results indicate that certain fungal cellulases and bacterial hemicellulases work synergistically together to maximize glucan and xylan digestibility. Bacterial xylanases (LX1, LX2 and LβX) can increase both the glucan and xylan hydrolysis yield when added along with fungal cellulases. Optimized ratios for individual enzymes are obtained by examining 73 unique enzyme mixture combinations. Close to 90% glucan and 70% xylan conversion is achieved for the optimal enzyme combinations. There is a high linear correlation observed between glucose and xylose hydrolysis yield. Especially at high enzyme loading, this relationship is more obvious. This could be explained based on the cell wall structural organization and the effect of pretreatment on it. Within the untreated cell wall ultra-structure, cellulose fibrils are embedded among thin sheaths of hemicellulose [204]. After pretreatment, the cell wall structure is modified chemically and ultra-structurally, to substantially enhance enzyme accessibility [14, 33]. Unlike dilute acid pretreatment, AFEX does not hydrolyze and extract any hemicellulose from the cell wall. Therefore, hemicellulases are crucial not only to maximize hemicellulose hydrolysis but also help enhance glucan digestion. The current sets of bacterial hemicellulases are not sufficient to completely hydrolyze AFEX treated corn stover hemicellulose. In order to further increase xylan

conversion greater than 70%, other hemicellulases such as α-arabinofuranosidase and α-glucuronidases would be necessary.

CHAPTER 6 HEMICELLULASES AND AUXILIARY ENZYMES FOR IMPROVED CONVERSION OF LIGNOCELLULOSIC BIOMASS TO MONOSACCHARIDES

6.1 Introduction

Previous chapters have allowed us to define the optimum ratio of six core fungal enzymes for AFEX treated corn stover. The cocktail consisted of cellobiohydrolases I and II (CBH I and CBH II), endoglucanase I (EG I), β-glucosidase (βG), endoxylanase (EX) and β-xylosidase (βX). More than 80% of theoretical glucose yield could be achieved using this optimized cocktail. However, irrespective of the amount of EX and βX loaded, xylose yield never exceeded 56% [71], which suggests the inclusion of other hemicellulases and/or accessory enzymes is necessary in order to further increase xylose yields.

Glycosyl hydrolases from bacteria provide a plentiful source of enzymes which have the potential to be utilized in lignocellulose hydrolysis. Synergism between fungal cellulases and bacterial xylanases has been recently demonstrated in our previous studies [198]. In this work, we demonstrate that supplementing fungal cellulases and β-glucosidase (βG) with additional EXs and other hemicellulases can further increase the yields of glucose and xylose at reduced total enzyme loadings. Xylanases and two additional debranching hemicellulases from *Clostridium thermocellum, Geobacillus thermodenitrificans, G. stearothermophilus* and *Dictyoglomus turgidum* (cloned and expressed in *Escherichia coli*) along with fungal cellulases (CBH I, CBH II, EG I and βG purified from *Trichoderma reesei* and *Aspergillus niger* derived broths) were evaluated to test their hydrolytic efficacy on AFEX treated corn stover.

6.2 Materials and Methods

6.2.1 AFEX pretreatment

Detailed information is described in 4.2.1

6.2.2 Discovery and cloning of LX1, LX2, LβX and LβG

Detailed information is published by Gao et al [198].

6.2.3 Discovery and cloning of L-arabinofuranosidase (LArb)

6.2.4 Discovery and cloning of LX3, LX4, LX5, LX6 and LαGl

Detailed information is published by Gao et al[198, 205]. The protein sequences and strain sources are listed in Table 6.1.

Table 6.1 Amino acid sequences and glycosyl hydrolase families for all six bacterial enzymes.

Notation	Amino acid sequence	GH family	Uniprot No.	Source, name and predicted molecular weight
LX3	MSGNALRDYAEARGIKIGTCVNYPFYNNSDPTYNSILQREFS MVVCENEMKFDALQPRQNVFDFSKGDQLLAFAERNGMQM RGHTLIWHNQNPSWLTNGNWNRDSLLAVMKNHITTVMTHY KGKIVEWDVANECMDDSGNGLRSSIWRNVIGQDYLDYAFR YAREADPDALLFYNDYNIEDLGPKSNAVFNMIKSMKERGVPI DGVGFQCHFINGMSPEYLASIDQNIKRYAEIGVIVSFTEIDIRIP QSENPATAFQVQANNYKELMKICLANPNCNTFVMWGFTDK YTWIPGTFPGYGNPLIYDSNYNPKPAYNAIKEALMGYHHHH HH	**10**	P10478	Truncated *Clostridium thermocellum* (XynZ, 38.0 kDa) (corresponds to last 324 A.A. in protein of the mature enzyme)
LX4	MKMGKMYEVALVVEGYQSSGKADVTSMTITVGNAPSTSSP PGPTPEPTPRSAFSKIEAEEYNSLKSSTIQTIGTSDGGSGIGYIE SGDYLVFNKINFGNGANSFKARVASGADTPTNIQLRLGSPTG TLIGTLTVASTGGWNNYEEKSCSITNTTGQHDLYLVFSGPVN IDYFIFDSNGVNPTPTSQPQQGQVLGDLNGDKQVNSTDYTAL KRHLLNITRLSGTALANADLNGDGKVDSTDLMILHRYLLGII SSFPRSNPQPSSNPQPSSNPQPTINPNAKLVALTFDDGPDNVL TARVLDKLDKYNVKATFMVVGQRVNDSTAAIIRRMVNSGH EIGNHSWSYSGMANMSPDQIRKSIADTNAVIQKYAGTTPKFF RPPNLETSPTLFNNVDLVFVGGLTANDWIPSTTAEQRAAAVI NGVRDGTIILLHDVQPEPHPTPEALDIIIPTLKSRGYEFVTLTE LFTLKGVPIDPSVKRMYNSVP	**11**	O87119	Truncated *C. thermocellum* (XynA, 52.1 kDa) (corresponds to residues 199-683 of the mature enzyme)

Table 6.1 (cont'd)

LX5	KNKRVLAKITALVVLLGVFFVLPSNISQLYADYEVVHDTFEV NFDGWCNLGVDTYLTAVENEGNNGTRGMMVINRSSASDGA YSEKGFYLDGGVEYKYSVFVKHNGTGTETFKLSVSYLDSET EEENKEVIATKDVVAGEWTEISAKYKAPKTAVNITLSITTDST VDFIFDDVTITRKGMAEANTVYAANAVLKDMYANYFRVGS VLNSGTVNNSSIKALILREFNSITCENEMKPDATLVQSGSTNT NIRVSLNRAASILNFCAQNNIAVRGHTLVWHSQTPQWFFKD NFQDNGNWVSQSVMDQRLESYIKNMFAEIQRQYPSLNLYAY DVVNEAVSDDANRTRYYGGAREPGYGNGRSPWVQIYGDNK FIEKAFTYARKYAPANCKLYYNDYNEYWDHKRDCIASICAN LYNKGLLDGVGMQSHINADMNGFSGIQNYKAALQKYINIGC DVQITELDISTENGKFSLQQQADKYKAVFQAAVDINRTSSKG KVTAVCVWGPNDANTWLGSQNAPLLFNANNQPKPAYNAV ASIIPQSEWGDGNNPAGGGGGGKPEEPDANGYYYHDTFEGS VGQWTARGPAEVLLSGRTAYKGSESLLVRNRTAAWNGAQR ALNPRTFVPGNTYCFSVVASFIEGASSTTFCMKLQYVDGSGT QRYDTIDMKTVGPNQWVHLYNPQYRIPSDATDMYVYVETA DDTINFYIDEAIGAVAGTVIEGPAPQPTQPPVLLGDVNG	**10**	B4BME8	Truncated *C. thermocellum* (XynY, 81.4 kDa) (corresponds to residues 1-736 of mature enzyme)
LX6	MEIPSLKEVYKDYFPIGAAVSHLNIYTYEDLLKKHFNSLTPEN QMKWEVIHPKPYVYDFGPADEIVDFAMKNGMKVRGHTLV WHNQTPGWVYAGTKDEILARLKEHIYEVVGHYKGKVYAW DVVNEALSDNPNEFLRKAPWYDICGEEVIEKAFIWANEADP NAKLFYNDYNLEDPIKREKAYQLVKRLKEKGIPIHGVGIQGH WTLAWPTPKMLEDSIKRFSELGVEVQITEFDISIYYDRNENN NFKVPPDDRIEKQAQLYKQAFEILRKYRGVVTGVTFWGVAD DYTWLYFWPVRGREDYPLLFDKNHNPKKAFWEIVKFHHHH HH	**10**	B8E346	*Dictyoglomus turgidum* (Dtur_1647, 38.1 kDa)

Table 6.1 (cont'd)

LαGl	MAEVKPYNMCWLEYTDLSKYKNKYIKVFENVVVLGGNELN LPLKELKNFLTFSLNIKPKIFKNTLVKGRNYVLIGRLIEIKKIFK ESERFEKLLNDEGFIIKRIDIDGNKVLIITAKSYNGIVYGIFNLI ERLKRGEDIENIDIVSNPSLRFRMLNHWDNLDGSIERGYAGK SIFFRENKILINERTKDYARLLSSIGVNGVVINNVNVKKKEVE LITPSYLKKIGELSKIFSAYGIKIY LSINFASPIYLGGLNTADPLDKRVAVWWKAKVDEIYEYVPD FGGFLVKADSEFNPGPHMYGRTHADGANMLGEALESYGGF VIWRAFVYNCLQDWRDTNTDRAKAAYENFKPLDGKFSENVI VQIKYGPMDFQVREPVNPLFGGLEHTNQILELQITQEYTGQQI HLCYLGTLWKEVLDFDTYAKGEGSKVKEILKGNVFDLKNN GMAGVSNVGDDINWTGHDLAQANLYTFGALSWNPDERIEE VVKRWIELTFGDNEKVIKNISYMLLSSHKAYEKYTTPLGLG WMVNPGHHYGPNPEGYEYSKWGTYHRANYEAIGVDRSSRG TGYTLQYHSPWREIYDNIETCPEELLLFFHRVPYNYKLKSGK TLIQTYYDLHFEGVEEAEEIRKKWIELKGEIEDKIYERVLNRL DIQIEHAKEWRDVINTYFFRRTGIPDEKGRKIYPHHHHHH	**67**	B8E3 B2	*D. turgidum* (Dtur_1714, 79.4 kDa)

6.2.5 Enzyme expression and purification

Detailed information is described in Chapter 3 and in a paper published by Gao et al[198].

6.2.6 Enzymatic hydrolysis of pretreated biomass

Detailed information is described in 4.2.6.

6.2.7 Glucose and xylose assays

Detailed information is described in 4.2.8.

6.3 Results and discussion

6.3.1 Supplementing bacterial hemicellulases along with fungal cellulases

Previous work has shown that *Dictyoglomus* and *Clostridium* derived hemicellulases retain significant activity at pH 4.5-5, whereas *Trichoderma* derived cellulases lose considerable activity at pH greater than 5.0 [198]. Hence, all assays were conducted at pH 4.5-5 to maximize performance of both sets of enzymes. Overall strategies used to obtain an optimized cocktail and major findings from this study are highlighted in Figure 6.1. From step 2 onwards, each experimental step was designed and tested based on conclusions from preceding steps. For step 1, to test performance of the nine different hemicellases on AFEX treated corn stover, all six xylanases (4 mg/g glucan for each xylanase) and other three accessory enzymes (2 mg/g glucan for each of LβX, LαGl and LArb, respectively) were grouped and doped together along with the core cellulases mixture. This avoided the risk of missing any synergism among the endoxylanases (LXs) and also between LXs and accessory enzymes (LβX, LαGl and LArb). Core fungal enzymes consisted of CBH I, CBH II and EG I (4 mg/g glucan loading of each cellulase) plus 2 mg/g glucan βG loading. Previous results have shown the current βG loading is sufficient to prevent cellobiose build-up at the current glucan loading [71]. The protein mass

ratio of CBH I, CBH II and EG I was kept at 1:1:1 in this study since previous results have demonstrated that this ratio results in optimum activity [198]. The minimum loading of LβX, LαGl and LArb required was determined after screening for optimal endoxylanase combination.

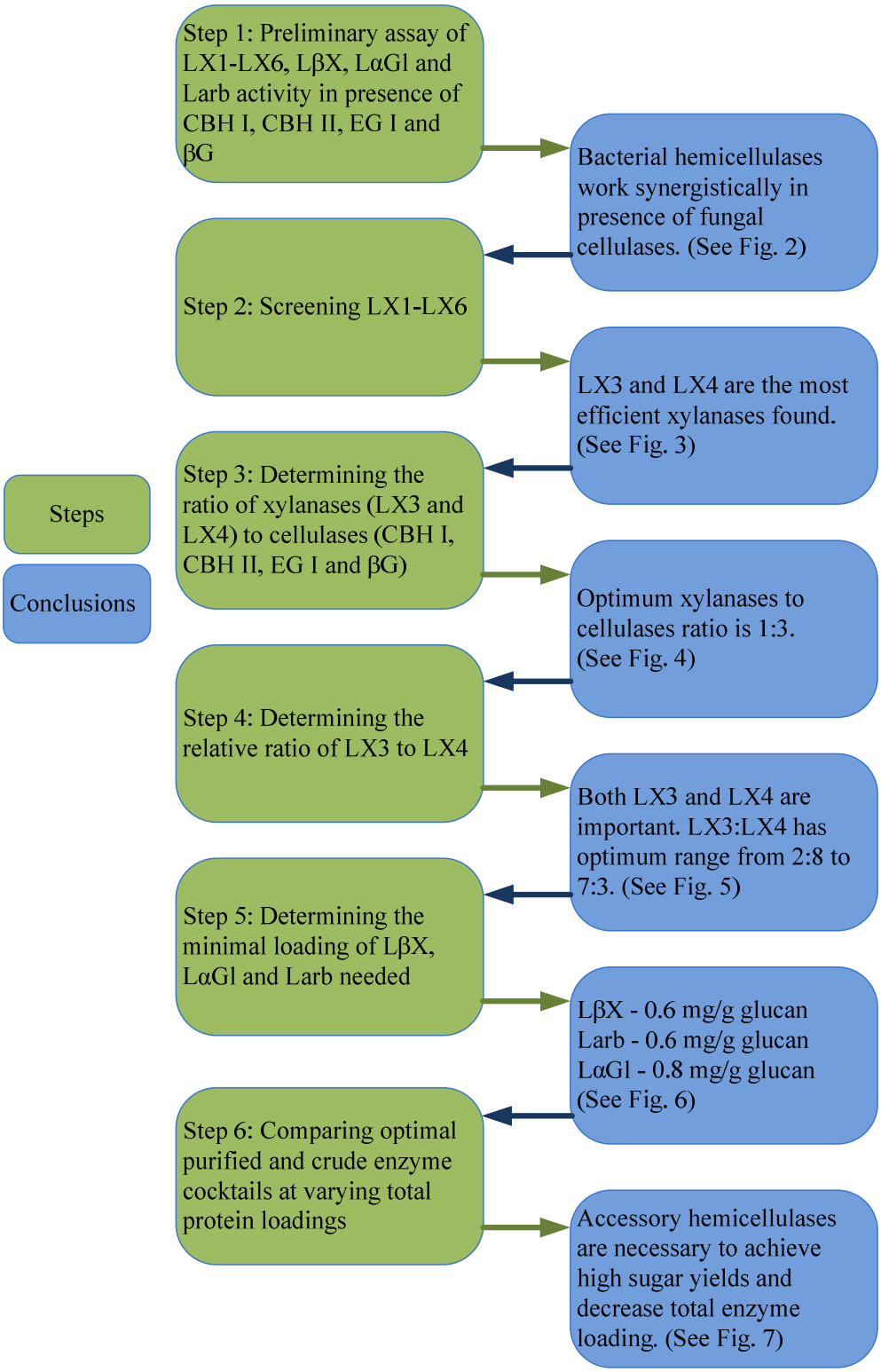


Figure 6.1 Overall conclusions drawn from each step of the process of hemicellulases optimization in presence of cellulases during hydrolysis of AFEX treated corn stover.

From Figure 6.2, core cellulases alone account for 56% glucose yield and minimal (3%) xylose yield within 24 h hydrolysis. Supplementing xylanases along with core cellulases increased glucose yield to as high as 83%. However, only 13% xylose yield was achieved. One possible reason for the improvement in glucan conversion is that xylanase supplementation helps remove xylan [200] sheathing cellulose fibrils and, hence, increases substrate accessibility [92]. LβX supplementation allows cleavage of xylo-oligomers to monomeric xylose and hence mixtures containing LβX have higher monomeric xylose yield. The α-arabinofuranosidase removes arabinose side-chains, while α-glucuronidase cleaves the α-1,2-glycosidic bond of the 4-O-methyl-D-glucuronic acid side chain from the xylan backbone [29, 69]. Adding LArb and LαGl along with cellulases and xylanases, further increased xylose yields by 12% and 7%, respectively. Adding both these enzymes, along with core cellulases and xylanases, allowed the xylose yield to reach as high as 74%. Hence, the removal of side chains which impede endoxylanase activity could enhance xylose yields significantly [67, 74, 206]. However, the total enzyme loading employed to obtain this conversion was 44 mg/g glucan, which is not viable for industrial processing. Therefore, systematically screening the critical endoxylanases and determining the minimum amounts of accessory enzymes is necessary to decrease the total enzymes loading without sacrificing hydrolysis performance.

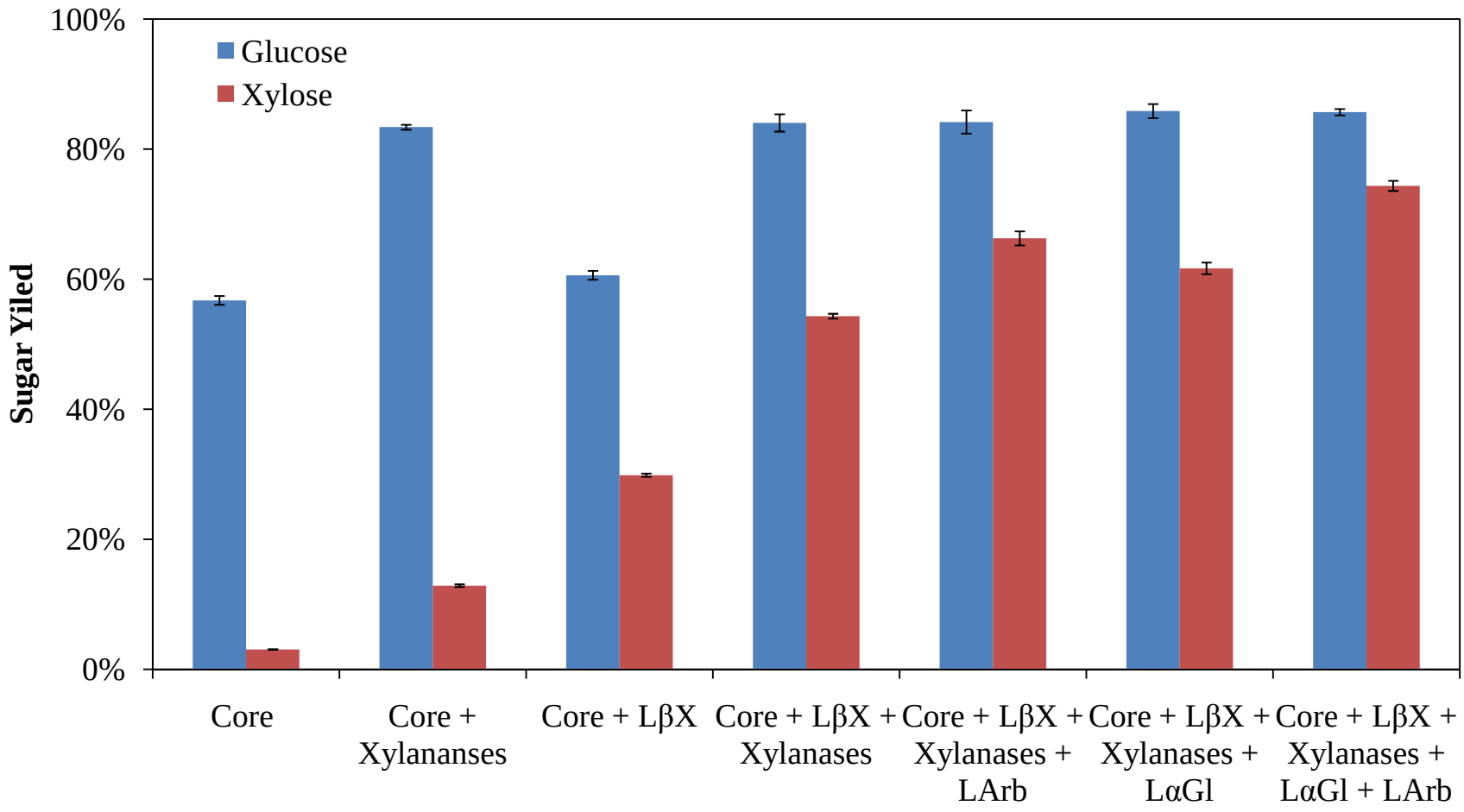


Figure 6.2 Glucose (blue bar) and xylose (red bar) yield after 24 h hydrolysis of AFEX-treated corn stover for different enzyme cocktails. Core enzymes only contain CBH I, CBH II, EG I (4 mg/g glucan each) and βG (2 mg/g glucan). Endoxylanases include LX1, LX2, LX3, LX4, LX5 and LX6 (4 mg/g glucan each). Accessory hemicellulases LArb, LαGl and LβX were loaded at 2 mg/g glucan each.

6.3.2 Screening endoxylanases

In Figure 6.2, all 6 xylanases were loaded together to evaluate their performance along with LβX, LαGl and LArb and also to prevent the risk of missing any synergism among the xylanases. However, not all xylanases may be necessary to bring about efficient xylan hydrolysis. In order to determine the most important xylanases or their combinations, a two level (0 and 4 mg/g glucan for low and high level of enzymes loading), six factor (LX 1 to LX 6) full factorial experiment was carried out by fixing the remaining enzyme loading (CBH I, CBH II and EG I at 4 mg/g glucan each; βG, LβX, LαGl and LArb at 2 mg/g glucan each).

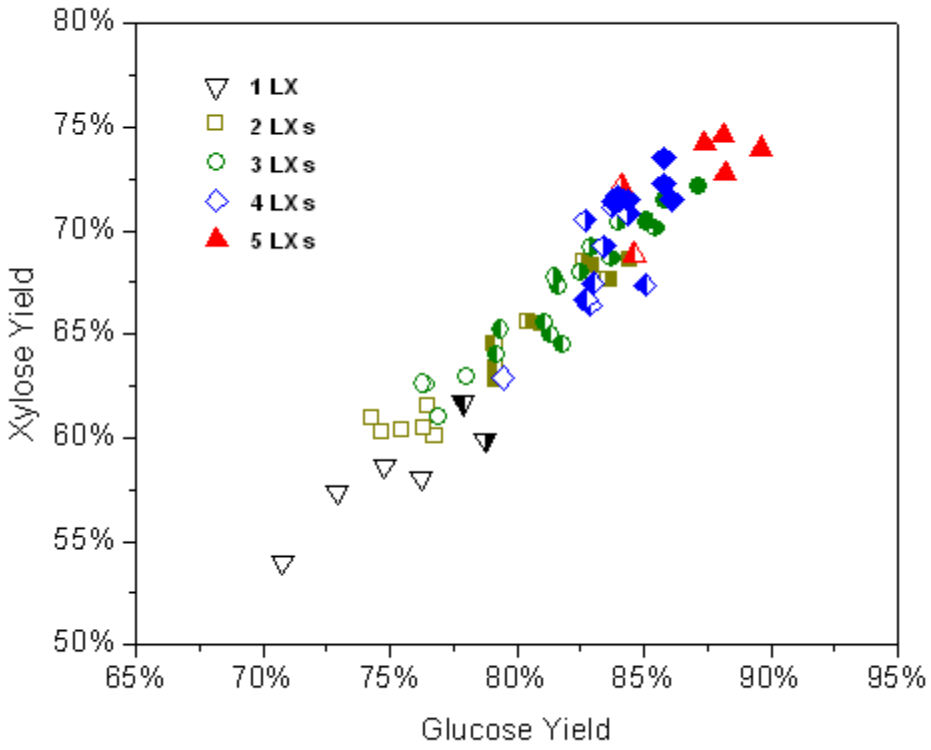


Figure 6.3 Glucose and xylose yield after 24 h hydrolysis of AFEX-treated corn stover for different xylanase loadings. LX1 to LX6 were loaded as different combinations (from single enzyme loading to 5 enzymes added simultaneously). Open symbol denotes no LX3 or LX4 were included in the mixture. Left half filled symbol denotes LX3 was included in the

mixture. Right half filled symbol denotes LX4 was included. Close symbol denotes both LX3 and LX4 were present in the mixture.

Results of the 24 h hydrolysis results for AFEX corn stover are shown in Figure 6.3 which depicts the glucose versus xylose yields. The xylose yield shows a strong linear relationship with the glucose yield. This reconfirmed previous findings that high glucose yields can be achieved at higher xylose yields for AFEX treated corn stover [198]. In this experiment, xylanase loading increased depending on the number of xylanases involved in the mixture. Experiments performed by adding individual xylanases showed that LX3 gave the highest conversion followed by LX4. For binary LX mixtures, the best mixture contained both LX3 and LX 4. Mixtures containing neither of these two enzymes resulted in 10% or more drop in both glucose and xylose yield. Combinations containing either LX3 or LX4 exhibited moderate conversions. For ternary mixtures, and other higher multiple LX loadings, LX3 and LX4 gave a similar trend which suggests that both LX3 and LX4 are superior compared to other xylanases.

6.3.3 Optimum cellulase to xylanase ratio

Based on previous results, both LX3 and LX4 were chosen as supplementary xylanases for further studies. As xylose and glucose yields are highly correlated, the ratio between cellulases and xylanases loaded was further optimized.

In Figure 6.4, varying ratios of xylanases (LX3 and LX 4 at equi-mass loading) were supplemented along with fungal core cellulases (CBH I, CBH II and EG I at equi-mass loading) for a fixed total protein loading of 20 mg/g glucan. A fixed amount (2 mg/g glucan) of other enzymes (βG, LβX, LaGl and LArb) were also loaded. Both the glucose and the xylose yields are shown in Figure 6.4. Without any xylanases, the glucose and xylose yields dropped to 63% and 43%, respectively. As EG I has some xylanase activity [70, 71], even in the absence of any other

xylanases, a significant xylose yield was obtained by the endocellulase in the presence of LβX. Supplementing the mixture with 5% xylanase increased both the glucose and xylose yields significantly. At 25% xylanase supplementation, the highest observed glucose and xylose yields were seen. When no cellulases were loaded, the glucose yield drops to less than 10%. Interestingly, xylanase loadings, ranging from 15% to 75% of total enzyme added, gave comparable glucose (>70%) and xylose yields (>60%).

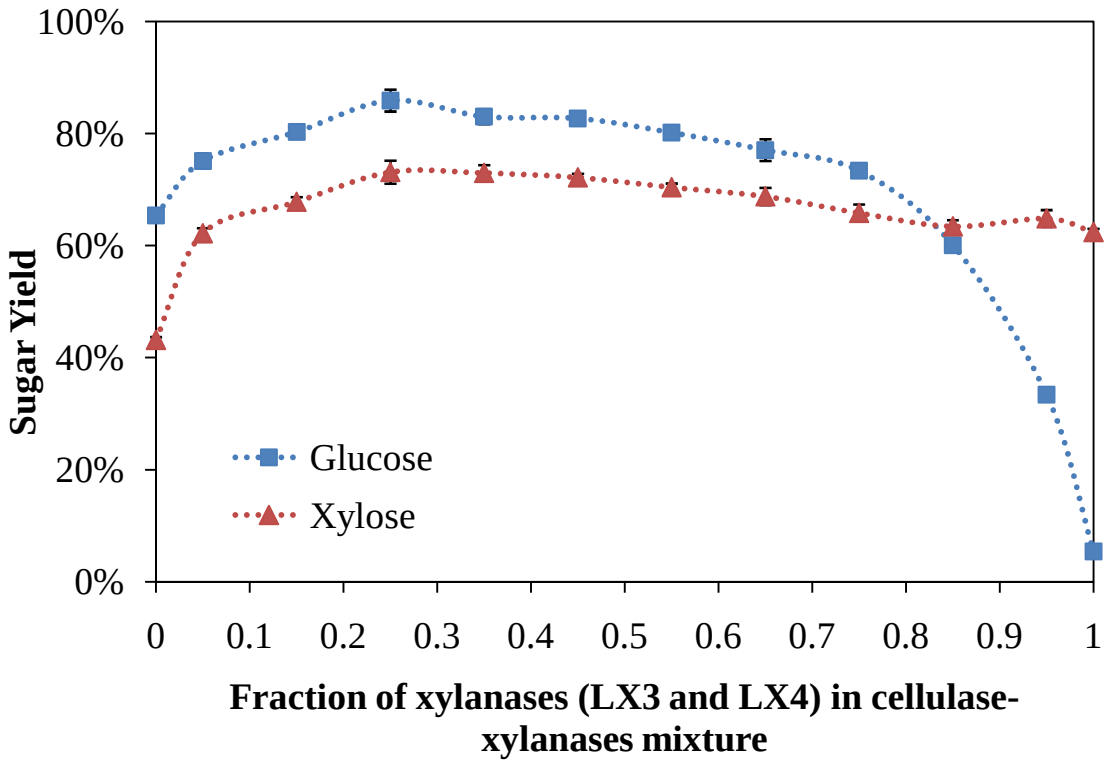


Figure 6.4 Glucose (blue) and xylose (red) yield after 24 h hydrolysis of AFEX-treated corn stover for different ratios of endoxylanases LX3 and LX4 in total enzymes (CBH I, CBH II, EG I, LX3 and LX4) loading of 20 mg/g glucan. All enzymes were loaded at equi-mass ratio, except βG, LβX, LαGl and LArb that were additionally supplemented at 2 mg/g glucan each.

6.3.4 Synergism between endoxylanases (LX3 and LX4)

The optimal endoxylanase loading is one-third of the amount of cellulases added for hydrolysis. It is interesting to compare the synergistic role of each xylanase in the mixture. Based on previous results, cellulases (CBH I, CBH II and EG I) were loaded at 5 mg/g glucan each, xylanases (LX3 and LX4) were loaded at 5 mg/g glucan total and accessory enzymes (βG, LβX, LαGl and LArb) were loaded at 2 mg/g glucan each. Several reports highlight the synergism between exo-glucanases and also between different families of hemicellulases [139, 207, 208]. It is interesting to determine if there is similar synergism between the endoxylanases. Figure 6.5 shows that supplementing only LX4 or LX3 along with core cellulases and accessory enzymes results in xylose yields of 60% and 65%, respectively. However, synergistic combinations of LX3 and LX4 resulted in a greater than 70% xylose yield. In addition, the glucose yield also benefit from including both LX3 and LX4. Table 6.1 shows that LX3 and LX4 belong to GH family 10 and 11, respectively. These results suggest that both family 10 and 11 glycosyl hydrolases are necessary to achieve complete xylan conversion for AFEX treated corn stover. It is normally believed that family 10 xylanases are more versatile and efficient than family 11 xylanases [67, 207]. Family 10 xylanases cleave β-1,4 linkages from at least one unsubstituted xylopyranosyl residue adjacent to substituted xylopyranolsyl residues towards the reducing end. However, family 11 xylanases only cleave linkages adjacent to at least two unsubstituted xylopyranosyl residues [67]. Our results are also consistent with Banerjee *et al*., who found that a combination of GH family 10 and 11 fungal xylanases help increase both glucose and xylose yields [74]. Ratios of LX3/(LX3+LX4) between 0.4-0.7 yielded comparable total monosaccharides yields. These results reconfirm previous observations that both LX3 and LX4 are required in order to achieve higher monosaccharides yields.

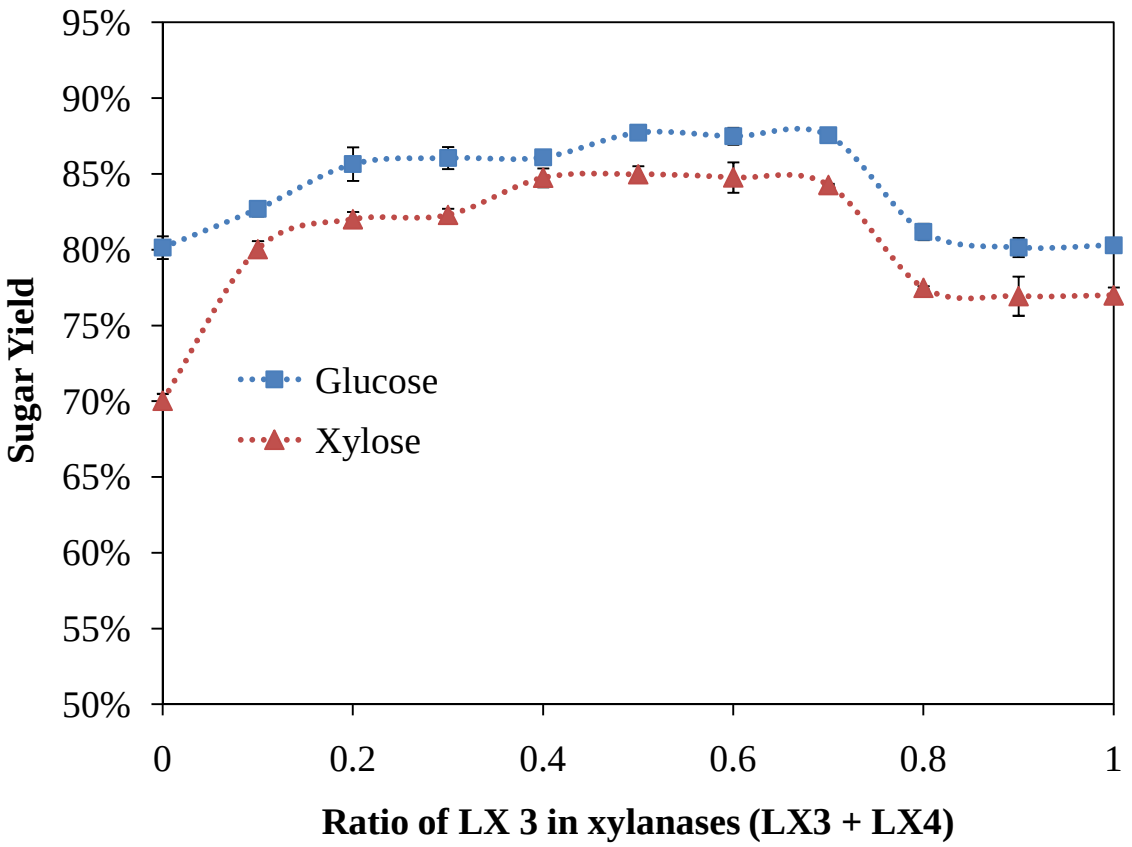


Figure 6.5 Glucose (blue) and xylose (red) yield after 24 h hydrolysis of AFEX-treated corn stover for different ratios of LX3 and LX4. 5 mg/glucan of each CBH I, CBH II, EG I and (LX3 +LX4) were added to the hydrolysis. Other enzymes (βG, LβX, LαGl, and LArb) were each added at 2 mg/g glucan.

6.3.5 Minimizing loading of accessory enzymes (LβX, LαGl and LArb)

In all of the above studies, LβX, LαGl and LArb loading was in excess. In order to determine the minimum loading of each enzyme, different amounts of each were loaded while the other two were kept in excess. Glucose yields for all experiments were comparable (data not shown) which suggests that these enzymes do not influence glucan hydrolysis. However, there was a significant change in the xylose yields as shown in Figure 6.6. The minimum loading for LβX, LαGl and LArb was 0.6, 0.8 and 0.6 mg/g glucan, respectively. Note: LβX plays a very important role on xylose yield. Without LβX, the xylose yield is very low, even when LαGl and LArb are loaded.

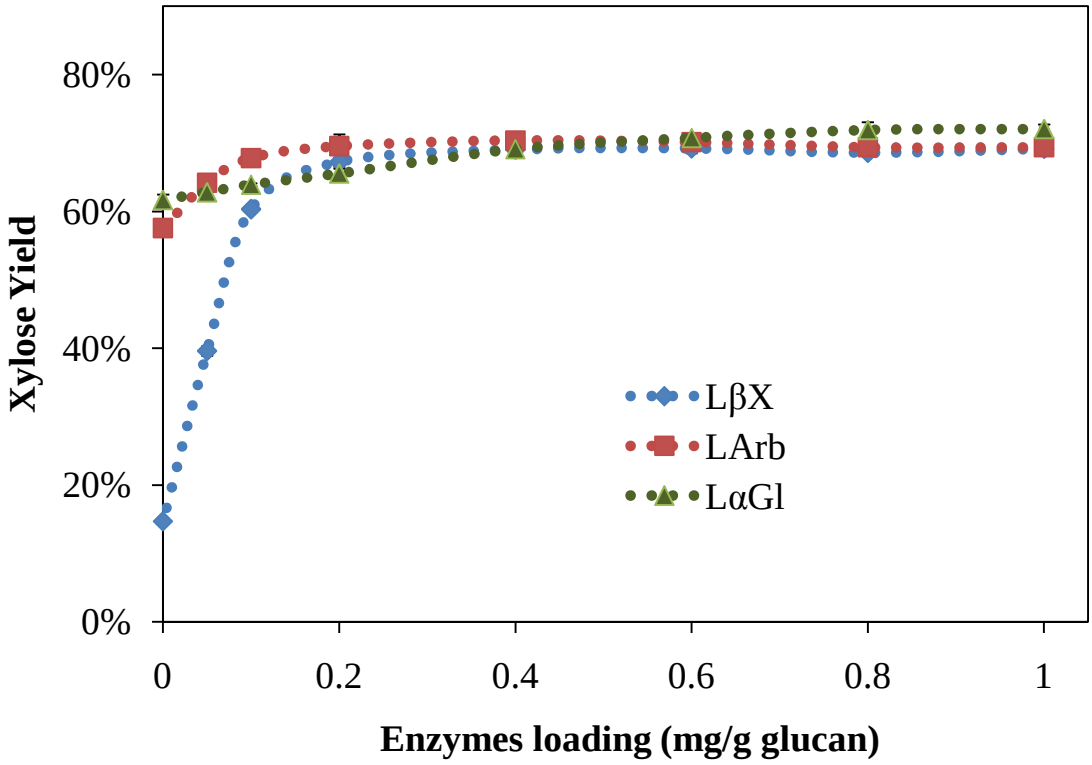


Figure 6.6 Xylose yield after 24 h hydrolysis of AFEX-treated corn stover in presence of accessory enzymes. LβX, LArb and LαGl were loaded individually at different protein loadings, in presence of an excess of the other two enzymes (1 mg/g glucan each). Major cellulases (CBH I, CBH II and EG I), hemicellulases (LX3 and LX4) were loaded at 5 and 2.5 mg/g glucan each, respectively. Additional βG (2 mg/g glucan) was added to prevent cellobiose build-up.

6.3.6 Comparison of monosaccharides yield for optimized and crude commercial enzyme preparations

The previous experiments helped define the optimal cellulase and hemicellulase loadings necessary to maximize monomeric glucose and xylose yields from AFEX treated corn stover. It was interesting to compare the hydrolytic activity of optimized cellulase-hemicellulase cocktail with purified core cellulases and crude commercially available enzymes on pretreated biomass with respect to the maximum monosaccharide yield achieved. in Figure 6.7.

From Figure 6.7, at a fixed total protein loading (30 mg/g of glucan), it is clear that the commercial enzyme (Accellerase 1000) has limited hydrolytic activity on both glucan (70%) and xylan (20%) fractions for AFEX treated corn stover due to the lack of suitable hemicellulase activity. The core cellulase mixture (CBH I, CBH II and EG I) had slightly better glucan conversions but lower xylan yields (<5%) compared to the commercial preparations. Interestingly, the inclusion of xylanases and xylosidases enhanced both glucose and xylose yields significantly. When small amounts of accessory hemicellulases (LαGl and LArb) were included in the core cellulase-xylanase mixtures, glucose yields were unchanged while xylose yields increased to >70%. For the total monosaccharide yield (Figure 6.7C), Accellerase 1000 at 30 mg/g glucan loading has a similar yield (50%) compared to the optimized cocktail at around 7 mg/g glucan.

In order to achieve monosaccharides yields greater than 80%, which is necessary for an economical industrial process, the optimized cocktail concentration needed to be around 22 mg/g glucan while the non-optimal cellulase-xylanase mixtures required more than 33 mg/g glucan. These results demonstrate how trace amounts of important accessory hemicellulases can further enhance the overall polysaccharide hydrolysis yields for AFEX pretreated corn stover. About 1.2 mg/g glucan total loading of LArb and LαGl can decrease the overall enzyme loading by over 33%. Furthermore, if the cocktail does not contain specific activities, such as β-xylosidase, α-arabinofuranosidase and α-glucuronidase, no matter how much of the other enzymes are loaded, it is impossible to achieve high xylose hydrolysis yields.

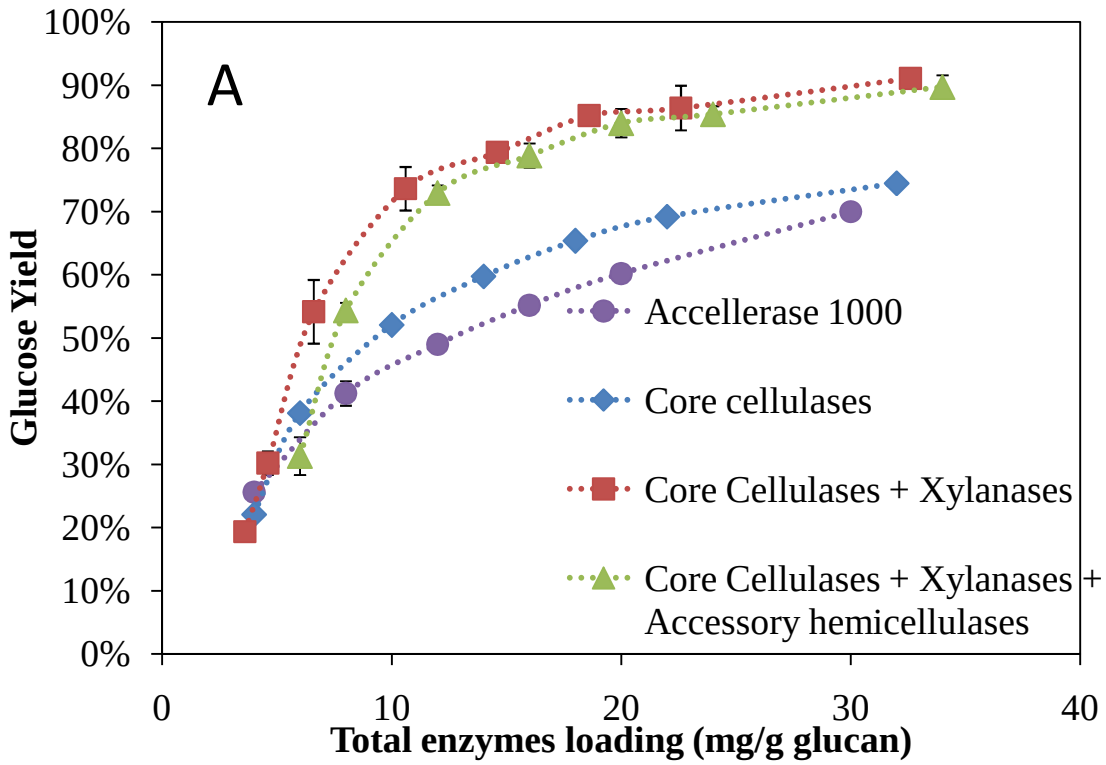
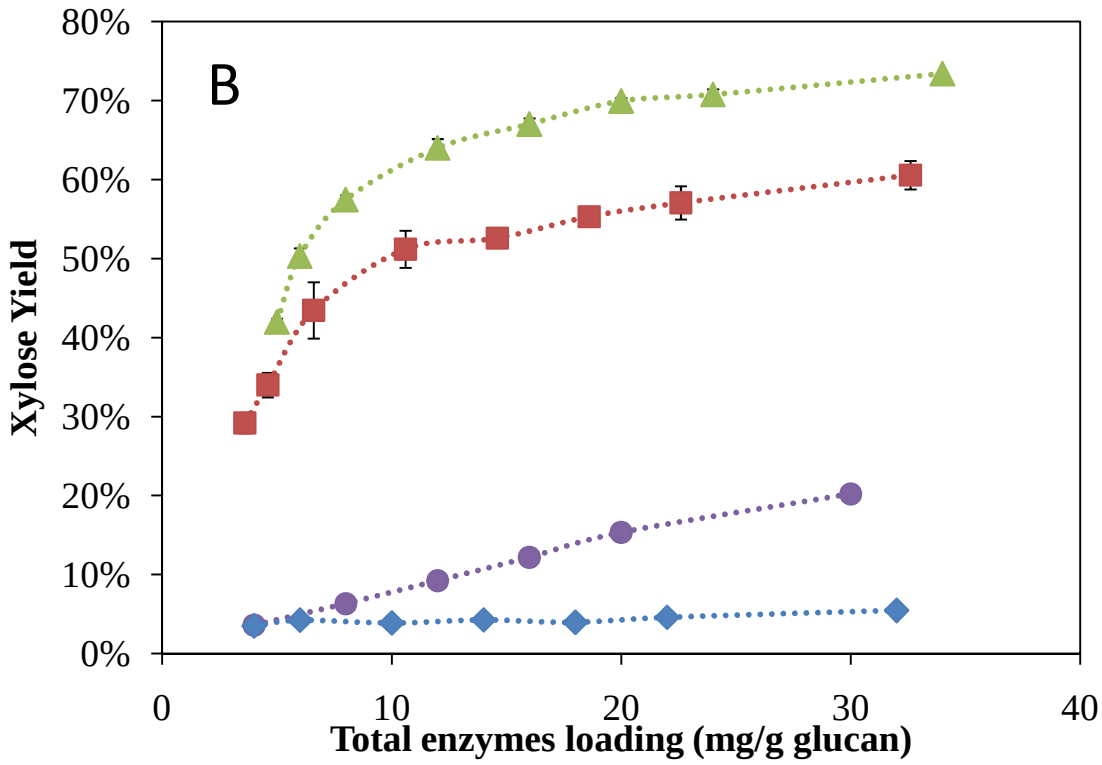
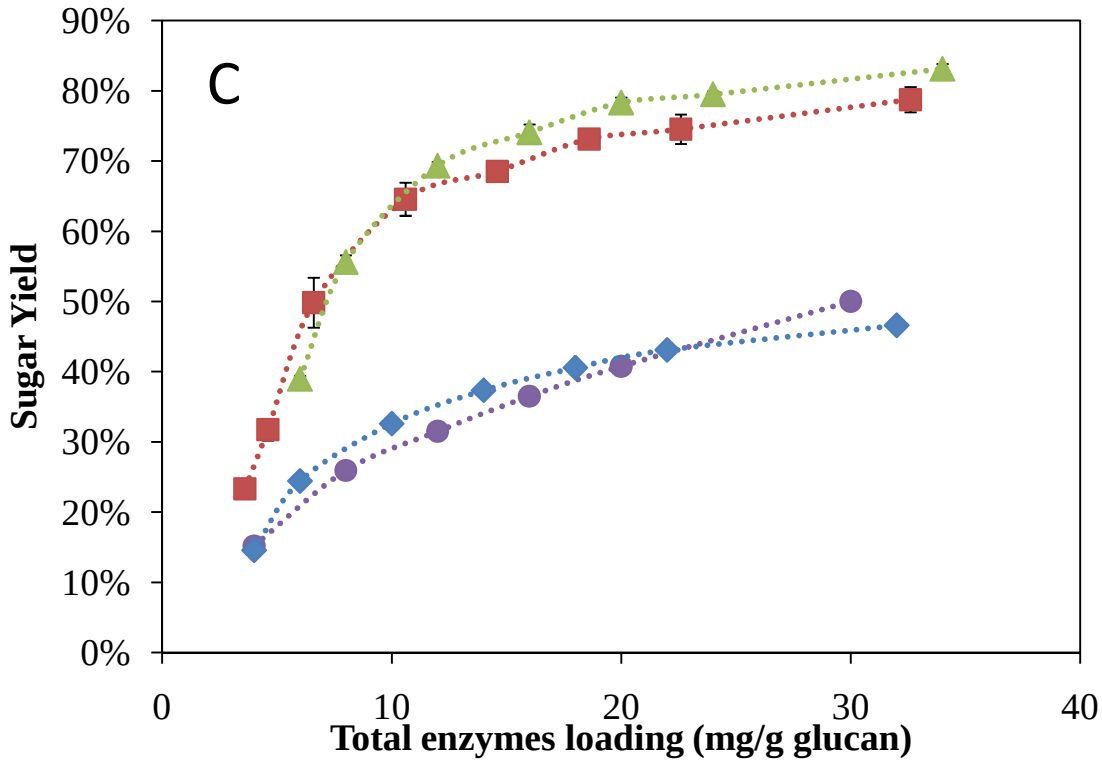


Figure 6.7 Glucose (A), xylose (B) and total monosaccharide (C) yields after 24 h hydrolysis of AFEX-treated corn stover for various enzyme cocktails. Accellerase 1000 is a commercial preparation. Core cellulases contain CBH I, CBH II and EG I at equi-mass loading along with 2 mg/g glucan βG. Xylanases (LX3 and LX4 at equi-mass ratio) to cellulases (CBH I, CBH II and EG I) ratio is fixed at 1:3. LβX, LαGl and LArb were loaded at 0.6, 0.8 and 0.6 mg/g glucan, respectively).

Figure Figure 6.7 (cont'd)





6.4 Conclusion

In this work, an optimized cocktail of xylanases and accessory enzymes was identified for AFEX treated corn stover. This cocktail included both fungal cellulases (CBH I, CBH II, EG I and βG) and bacterial hemicellulases (LX3, LX4, LArb, LαGl and LβX). The optimized cocktail can hydrolyze AFEX treated corn stover, resulting in glucose and xylose hydrolysis yields greater than 80% and 70%, respectively, at a reasonable protein loading (~20 mg/g glucan). Adding endoxylanases increases both xylose and glucose yields significantly (along with a suitable β-xylosidase). Supplementation of accessory α-arabinofuranosidase (LArb) and α-glucuronidase (LαGl) further increased xylose yields by 20 percentage points. This study clearly demonstrates that for biomass pretreated by a specific technology (for example, AFEX or similar alkaline pretreatments), it is both possible, and important, to tailor-make specific enzyme cocktails with optimal individual enzyme ratios to achieve higher monosaccharide yields.

This study was carried out at low solids loading (0.2% glucan or 0.58% solids basis, respectively) due to mass-transfer limitations typical for microplate based assays [177]. Therefore, the monosaccharide yield and enzyme loadings discussed here are valid for the best case scenario and there were no significant end-product inhibition or mass transfer limitations typically encountered during high solid loading (15-30% solids loading)-based saccharification. These results should mimic a simultaneous saccharification and fermentation process where the monosaccharides are directly fermented to ethanol without allowing a significant build of sugars. For an industrial high solid loading hydrolysis, more βG and βX might be necessary in order to overcome the build-up of sugar oligomers due to the inhibition of enzymes at high concentrations of monosaccharides and potential unproductive binding to lignin.

AFEX pretreated biomass, which contains nearly all of the original xylan content from untreated biomass, is very different from acid pretreated biomass in terms of its physicochemical composition and enzymatic digestibility [19, 209, 210]. The efficacy of any pretreatment is normally evaluated by enzymatic hydrolysis at specific conditions (for example, enzyme loading, hydrolysis time, solid loading). In order to achieve an unbiased comparison between pretreated samples, enzymes used for evaluation should be able to reflect the true efficacy of the pretreatment. When comparing AFEX versus dilute acid pretreated biomass, one should include necessary hemicellulase and accessory enzyme activities in the enzymatic cocktail. Otherwise the lack of specific activities could result in underestimating the true digestibility of AFEX pretreated biomass. In addition, for other low severity pretreatments (such as alkaline peroxide) which do not remove most of the hemicellulose fraction, the results in this paper could also be useful for the development of more balanced enzyme cocktails in order to evaluate the pretreatment effectiveness.

CHAPTER 7 INTERACTION OF TRICHODERMA REESEI CELLULASES WITH UNTREATED, AFEX, AND DILUTE-ACID PRETREATED BIOMASS

7.1 Introduction

From previous chapters, enzyme cocktail can be optimized to improve hydrolysis yield. Among those optimized cocktail, CBH I, CBH II and EG I are the three major enzymes contribute to >60% of total enzyme loading. If one can improve their efficiency by understand the mechanism of these enzymes during hdyrolsysi, a more efficient hydrolysis process can be expected.

All of the three enzymes have CBD. It has been suggested that almost all of the hydrolysis parameters, such as temperature [156], pH [211], ionic strength [149], solids loading [93], enzyme type [212], pretreatment and substrate ultrastructure [213] can influence cellulase binding. The limitation of current enzyme binding studies are shown in Table 2.5.

Among those techniques, Fast Performance Liquid Chromatography (FPLC) based separation and quantification of proteins is able to quantify protein based on the differences in their isoelectric points (*p*I). Pretreated cell walls have additional UV-absorbing compounds (e.g., aldehydes, furans, phenolics) that can interfere with the separation and quantification of enzymes using IEX (Figure 7.1) [19]. In order to overcome this problem and study the binding behavior of purified cellulase (i.e., CBH I, CBH II and EG I) mixtures on untreated (UN-CS), dilute acid (ACID-CS) and ammonia fiber expansion pretreated corn stover (AFEX-CS), we report here a modified, semi-automated HT-FPLC method. This method utilizes gel filtration prior to ion exchange based cellulase separation and quantification using a 96-well microplate assay format that is compatible with a high-throughput hydrolytic assay method recently reported by our group [71, 177]. Using this method we are able to accurately quantify unbound CBH I, CBH II

and EG I within untreated and pretreated corn stover hydrolyzates. This method was then used to study multiple enzyme and pretreated biomass combinations to illustrate its utility. Our results reveal the importance of how different pretreatments (i.e., AFEX and dilute-acid) can influence accessibility and total available binding sites for individual cellulases as well as how these enzymes can compete and/or cooperate with each other for those binding sites.

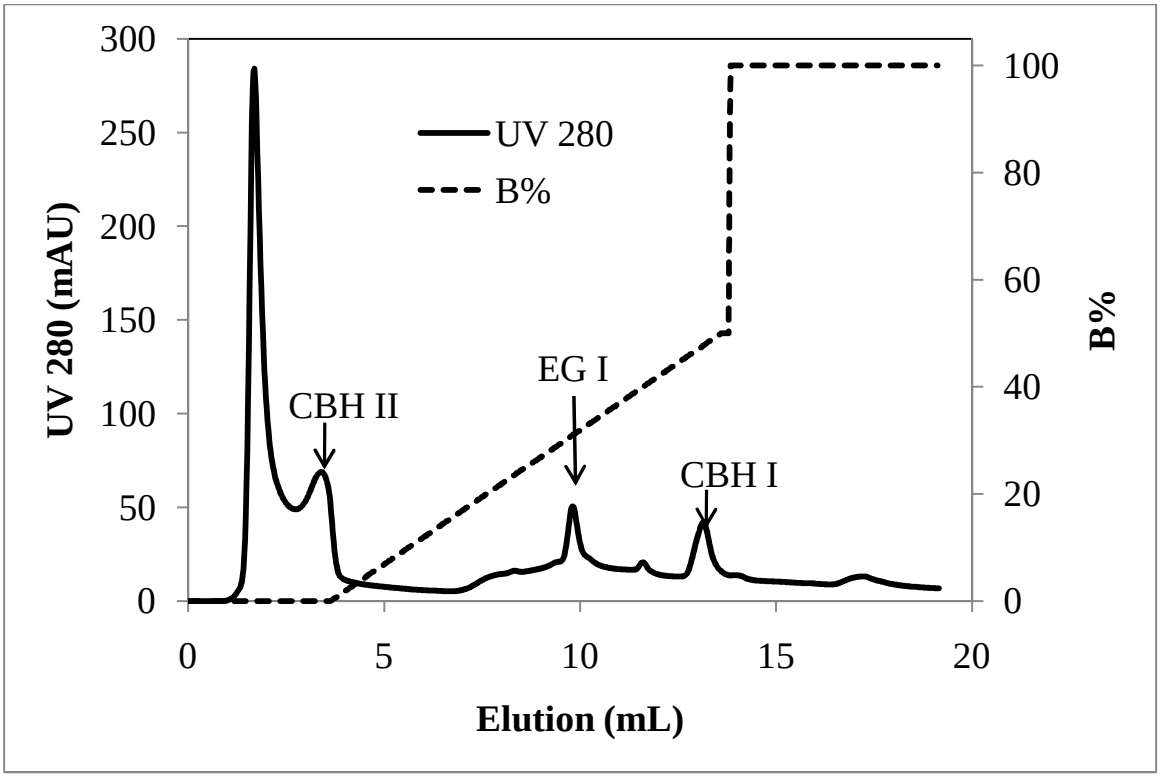


Figure 7.1 Separation of individual cellulases by anion exchange chromatography after 20-fold dilution of AFEX corn stover hydrolyzate supernatant in pH 7.5, 20 mM Tris buffer without removal of lignocellulose derived UV-absorbing decomposition products. Enzymes were eluted by applying a linear gradient of 1M NaCl to the column (dotted line; shown as %B on secondary Y-axis).

7.2 Materials and Methods

7.2.1 AFEX pretreatment

Detailed information is described in 4.2.1

7.2.2 Dilute acid pretreated corn stover

The dilute acid pretreatment was performed with a 1.0 L Parr reactor made of Hastelloy C (Parr Instruments, Moline, IL, USA). CS was presoaked in 1.0% w/v dilute sulfuric acid solution at 5.0% solids (w/w) overnight. The total weight of the pretreatment mixture was 800 g. The presoaked slurry was transferred into the reactor, which was then sealed and fitted to the impeller driver motor which was set at 150 rpm. The vessel was lowered into a hot sand bath and heated rapidly (within 2 min) to an internal temperature of 140 ± 2°C and maintained at 140 ± 2°C in the fluidized heating sand bath for 40 min. At the end of the reaction time, the reactor was cooled to below 50°C in a water bath. The dilute acid pretreated CS slurry was filtered through Whatman no. 1 filter paper. Details of the apparatus, experimental procedure and combined severity calculation are described elsewhere [214, 215]. After pretreatment, the dilute acid treated corn stover (ACID-CS) solids were washed to neutral pH, dried at room temperature in a fume hood, milled using a 0.1 mm screen (as described previously) and stored at 4^{o}C. The composition of the ACID-CS solids was approximately 60.6% glucan, 3.3% xylan, and 32.9% lignin.

7.2.3 Enzyme purification

Detailed information is given in Chapter 3 and in a paper published by Gao et al[71, 198].

7.2.4 Cellulase Binding and Quantitation

For enzyme quantification study, the total enzyme loaded was assumed to be equal to the enzyme bound to the solid biomass plus the free enzyme left in the supernatant. Thus, by assaying the amount of free enzyme in the hydrolyzate, one can determine the amount of enzyme bound to the

biomass. An illustrative schematic highlighting the cellulase adsorption and HT-FPLC based quantification methodology is shown in Figure 7.2. A 2.2 ml deep-well microplate (Lot # 780271, Greiner, Monroe, NC) was used to load 200 μl of 1.45% (w/v) AFEX-CS/UN-CS or 200 ul of 0.825% (w/v) ACID-CS along with 25 μl 1M citrate buffer (pH 4.8) and 275 μl of enzyme mixtures using epMotion 5070 automatic pipetting work station (Eppendorf, Hamburg, Germany) as described previously [177]. Each well contained 50 mM citrate buffer and 1 mg glucan equivalent solids in a total reaction volume of 0.5 ml. After incubation for 2 hours at 4^{o}C at 250 rpm, the supernatant was separated from the insoluble solids by filtering through a 0.45 μm low protein binding hydrophilic microplate based filter (Lot # R6PN00144, Millipore, Ireland). The FPLC system (ÄKTA purifier) and autosampler (A905) used for enzyme filtrate separation and quantification were from GE Healthcare (Buckinghamshire, United Kingdom). The enzyme filtrate was loaded into the FPLC autosampler for gel filtration chromatography to isolate the cellulases from the UV-absorbing background (Figure 7.2). The gel filtration column was packed with 28 mL of Superdex 200 (Lot #S6782, Sigma-Aldrich, St. Louis, MO) media in a XK 16/20 column (Lot #18-8773-01, GE Healthcare). While during eluting out 2 column volumes (CV) of 20 mM Tris buffer (pH 7.5), the enzyme rich fractions were collected as 6 mL aliquots. Part of the aliquots (0.5 mL) were then injected into a Mono Q column (Lot #17-5179-01, GE Healthcare) followed by 6 CV linear gradient elution with 1M NaCl in 20 mM pH 7.5 Tris buffer. The concentration of individual enzymes was correlated to the elution peak area detected at UV 280 nm and calculated using the Unicorn 5.11 software. The binding studies are performed at 4 ^{o}C at different individual cellulase loading ranging from 25 to 450 mg/g glucan. CBH I, CBH II and EG I bindings were studied individually as single enzyme systems. For binary enzyme mixtures, pairs of CBH I/CBH II, CBH I/EG I and CBH II/EG I were loaded at equal mass

loading (e.g., 25 mg/g cellulase loading binary mixture experiment comprised of 25 mg/g glucan loading of enzyme A and B each, respectively. Hence the total cellulase loading was actually 50 mg/g glucan for this binary mixture experiment.), and each enzyme's binding characteristics were studied in the presence of the other enzyme present in the pair. Similarly, for ternary enzymes system, equal mass ratios of all three enzymes were loaded and each individual enzyme's binding characteristics were studied.

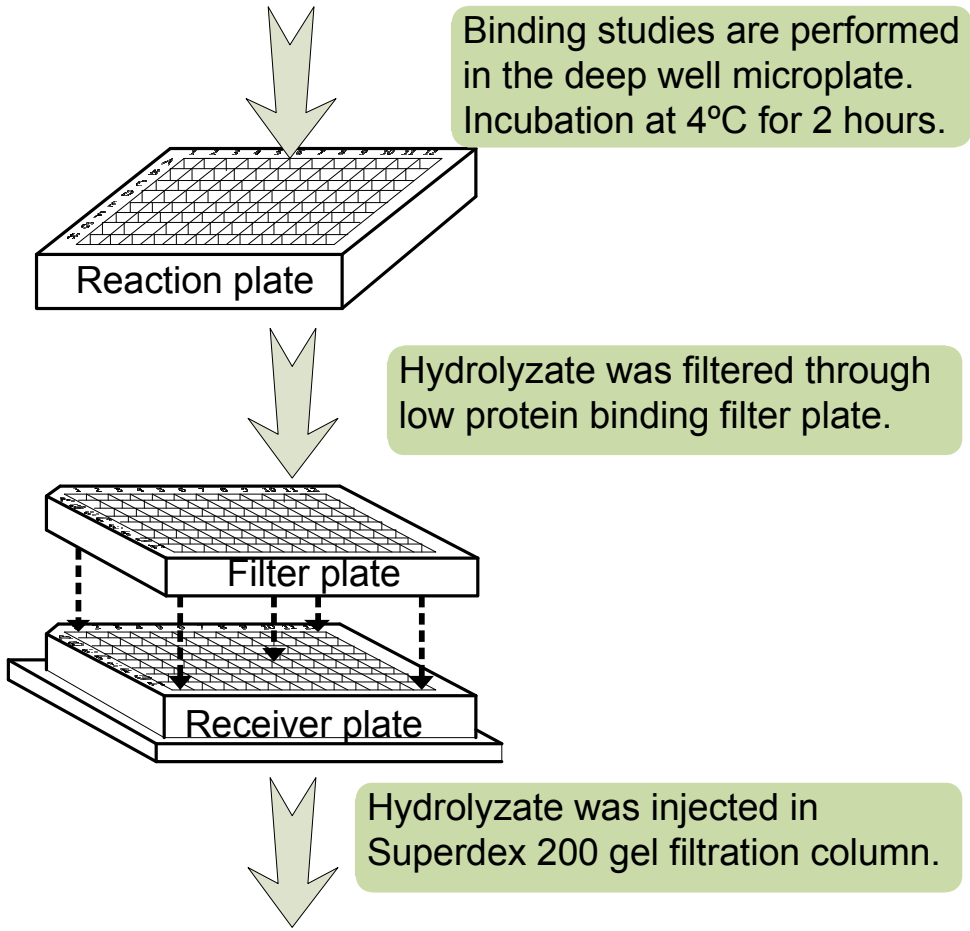
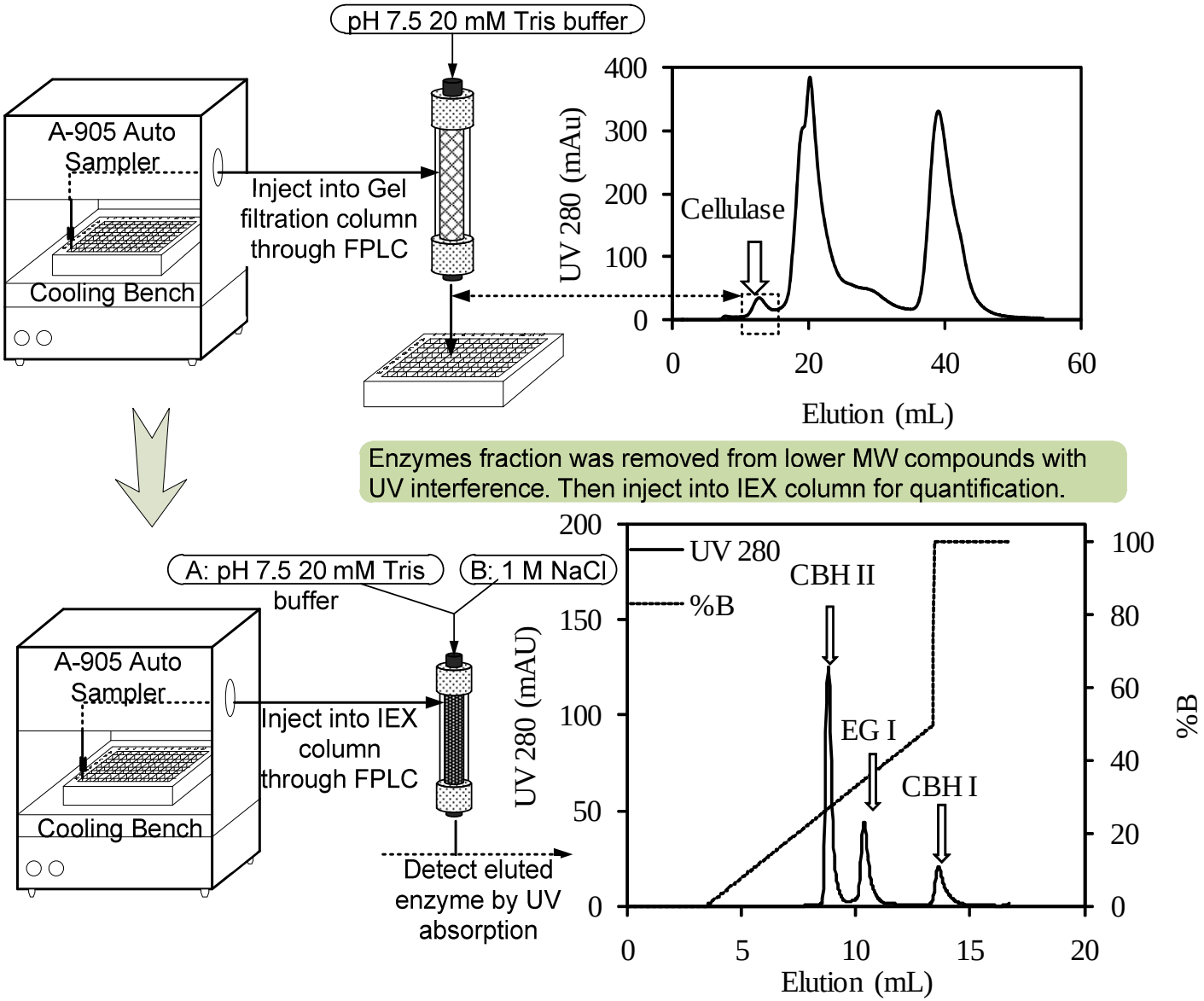


Figure 7.2 Two-step high-throughput HT-FPLC method for separation and quantification of complex cellulase mixtures present in lignocellulosic biomass derived hydrolyzates. High molecular weight (>50 kDa) cellulases are separated from AFEX pretreated corn stover hydrolyzate derived UV-absorbing components (e.g., phenolics) by Superdex 75 gel filtration and then quantified by anion exchange chromatography (IEX). Enzymes were eluted applying a linear gradient of 1M NaCl (dotted line; shown as %B on secondary Y axis).

Figure 7.2(cont'd)



7.2.5 Langmuir Model Fitting

The binding isotherms were fit to a Langmuir isotherm model using Matlab 7.0 (MathWorks Inc., Natick, MA). The curve fitting toolbox in Matlab was set using the Trust-Region algorithm to fit the isotherm data. The Langmuir model is described using the following equation:

$$E_b = \frac{E_{bm} K_a E_f}{1 + K_a E_f}$$

Where E_b is the bound enzyme (mg/g glucan); E_{bm} is the total available binding sites (mg/g glucan); E_f is the concentration of free enzyme present in liquid phase (mg/L) and K_a is the association constant (L/mg).

7.3 Results and Discussion

7.3.1 FPLC Quantification

However, for real lignocellulosic biomass, directly injecting the lignocellulosic hydrolyzate (even after 20 fold dilution) into the ion-exchange column results in poor protein binding to the column and causes severe base line fluctuation during separation (Figure 7.1). CBH II cannot bind to the column effectively and the baseline fluctuates considerably during the separation, making it difficult to achieve reliable peak area quantification. Hence, desalting and buffer exchange of the hydrolyzate using an appropriate buffer is necessary prior to IEX.

Thus, a preliminary gel filtration chromatography step was used to separate the high molecular weight cellulases from the lower molecular weight background components. Large molecules, such as enzymes, elute from the gel filtration column earlier than low molecular weight

compounds. The gel filtration step separated cellulases (CBH I, CBH II and EG I) from other cell wall derived compounds that have significant UV absorption (Figure 7.2). In addition, gel filtration acted as a buffer exchange step which is also necessary for subsequent ion-exchange chromatographic separation (Note: enzymatic hydrolysis is done typically at pH 4.5-5.0 while, the optimal ion-exchange separation takes place at pH 7.5 for *T. reesei* cellulases.) This pH adjustment during gel-filtration depends on the exact protein pI and ion-exchange method employed and can be optimized for other non-trichoderma enzymes as well.). After gel filtration, the enzyme fractions were injected into an anion exchange column (Mono Q), and the bound enzymes (CBH I, CBH II and EG I) were eluted using a linear gradient of NaCl (Figure 7.2). Based on the differences in *p*I, CBH II (*p*I ~ 6.8) eluted out first, followed by EG I (*p*I ~ 4.5) and CBH I (*p*I ~ 4.2). CBH I, CBH II and EG I were separated with good baseline resolution. Calibration curves based on peak areas were used to determine the concentration of individual enzymes. Cellulase mixtures of known concentration for individual enzymes that could be reliably quantified by this method lies within the range of 50 μg/ml to 0.9 mg/ml (this corresponds to 25 to 450 mg/g glucan loading). Linear regression standard curves with satisfactory linearity (R^2>0.999) were obtained to determine unknown protein concentrations. All standard enzymes mixtures of known concentrations had also undergone the exact same sample preparation procedure as the enzymes in the biomass hydrolyzates to minimize the potential error associated (e.g., non-specific binding of enzymes to microplate walls, enzyme loss during microplate filtration and FPLC based gel filtration) with the absolute quantification. The UV-900 multi-wavelength UV sensor in the current FPLC system is incorporated with a xenon flash lamp which supplies a higher sensitivity and higher signal to noise ratio compared to regular mercury lamps. To evaluate how UV signal drifts during long run time measurements

affects the reproducibility of this method, 98 samples of hydrolyzate containing around 0.3 mg/ml each of three cellulases were tested continuously for 3 days. The coefficients of variation (based on detected protein peak areas) for CBH I, CBH II and EG I are 0.9%, 1.5% and 1.6% respectively and there is no obvious increasing or decreasing trend of UV signal strength during measurement. These results demonstrate that the current FPLC methodology is both robust and reproducible.

One could speculate that inclusion of an enzyme blank (e.g., hot water extract from pretreated biomass alone) to re-calibrate the standard curve could overcome the lignin interference to UV absorbance. However, this could be challenging because during the course of enzymatic hydrolysis increasing amounts of soluble lignin are expected to be released [216]. Hence, the standard curve would need to be adjusted for each time point sampled. Incorporating a gel filtration step to separate the lignin (and other UV absorbing cell wall components) from the enzymes in varying time-course hydrolyzates prevented such difficulty with satisfactory reliability (data not shown). Another solution to this problem was illustrated by a recent study that used a dual-wavelength spectral correction method to minimize lignin interference on UV-Vis spectrophotometric measurements to quantify cellulase adsorption to lignocellulosic biomass [217]. Compared to other reported methodologies (Table 2.5) [79, 156], the current FPLC method has certain advantages. First, the method gives reproducible and accurate results due to semi-automation of the protocol (e.g., via use of a FPLC microplate based autosampler). Second, by removing 280 nm UV-light absorbing background compounds using a gel filtration column this method can be used as a tool to probe the binding properties of cellulases on real lignocellulosic biomass (unlike previous binding studies that used purified cellulosic materials devoid of these background compounds). Third, all major cellulases involved in hydrolysis

(CBH I, CBH II and EG I make up nearly 80% of total enzyme present in crude cellulase broths) [71, 210] can be simultaneously monitored, allowing us to study protein-protein interactions for real lignocellulosic biomass.

7.3.2 Binding isotherms for single, binary and ternary cellulase mixtures

Compared to UN-CS, both AFEX-CS and ACID-CS have much higher cellulase binding capacities (Figure 7.3, Figure 7.4 and Figure 7.5). For example, when CBH I was added alone at high enzyme loading (450 mg/g glucan), only 60 mg/g glucan CBH I bound to UN-CS whereas 190 and 147 mg/g glucan CBH I was bound to AFEX-CS and ACID-CS, respectively. This suggests that both pretreatments significantly increase cellulase accessibility for corn stover. These results agree with earlier studies by Jeoh et al. [33] and Kumar et al. [153, 218] showing that pretreated corn stover has higher CBH I binding capacity. We also found similar results for CBH II and EG I.

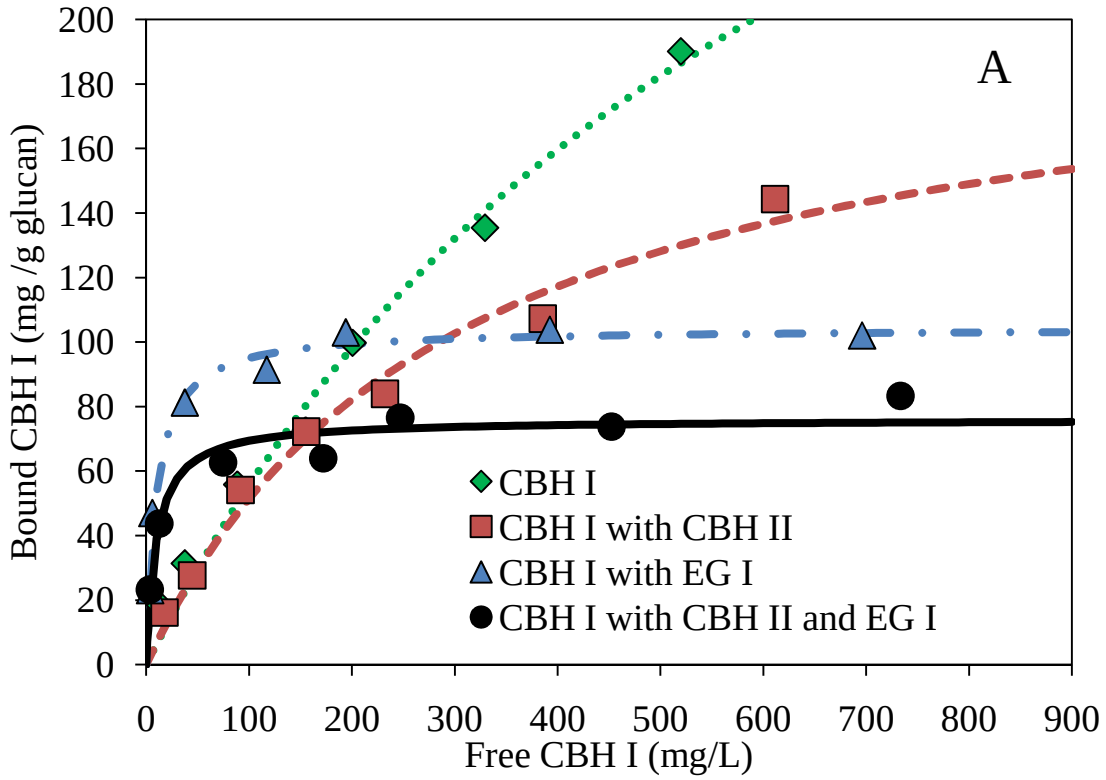
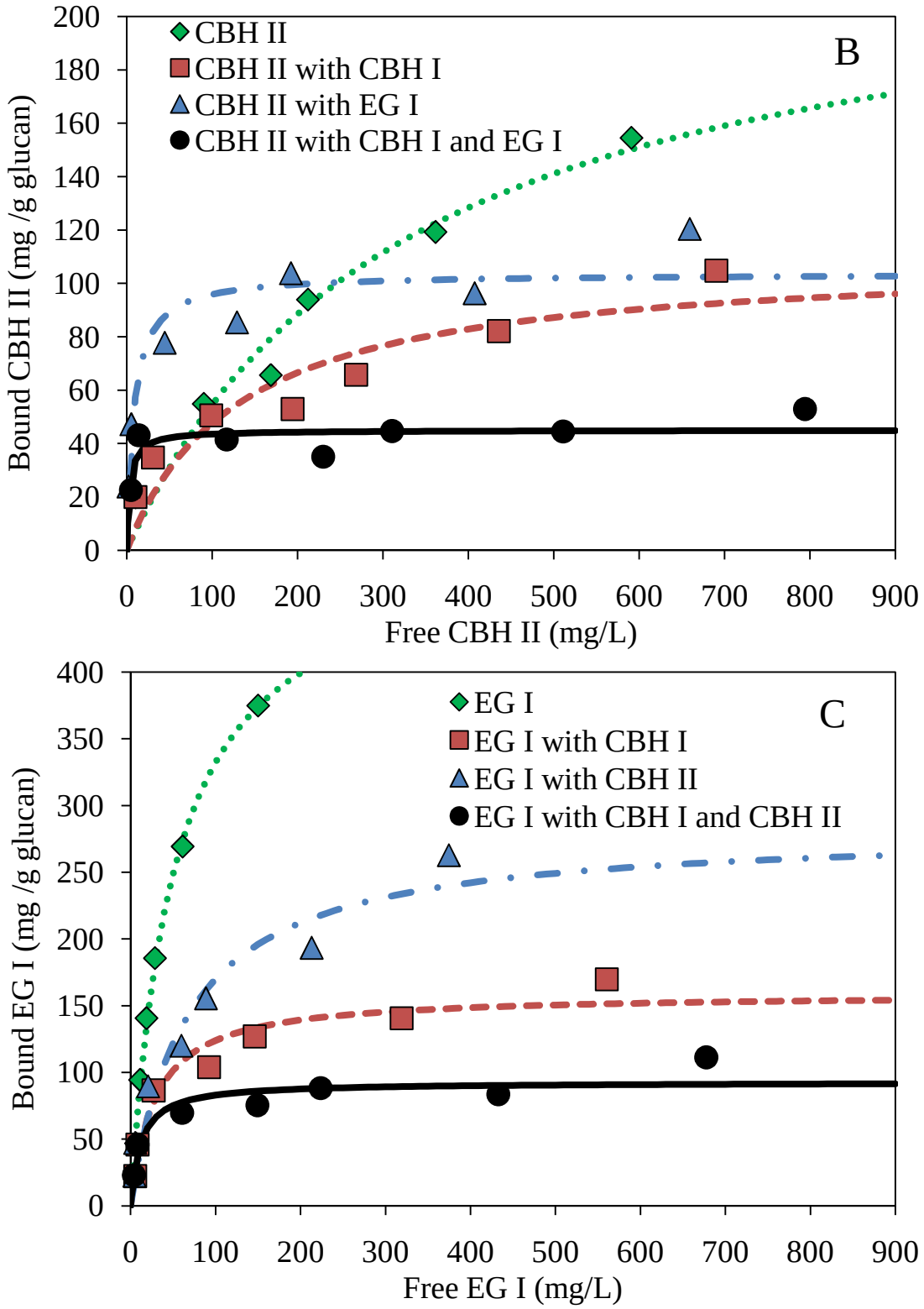


Figure 7.3 Binding isotherms for CBH I (A), CBH II (B) and EG I (C) on AFEX pretreated corn stover. The extent of binding for individual enzymes was measured for single, binary and ternary cellulase mixtures.

Figure 7.3 (cont'd)



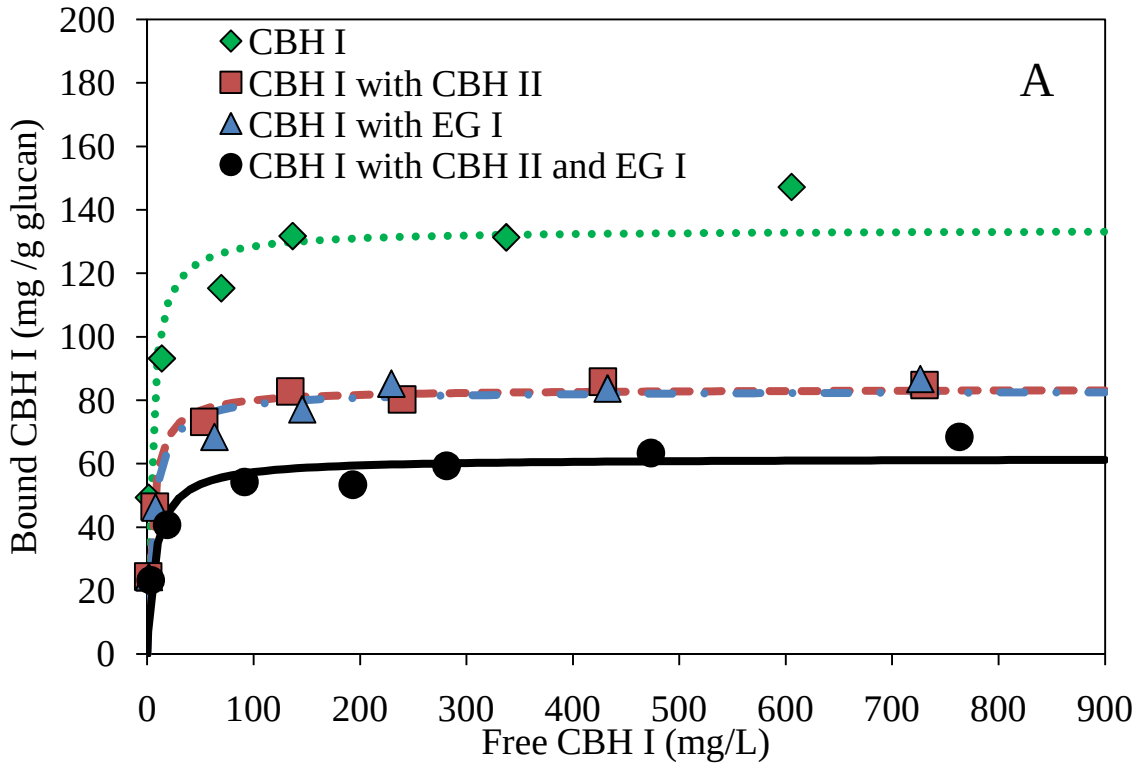
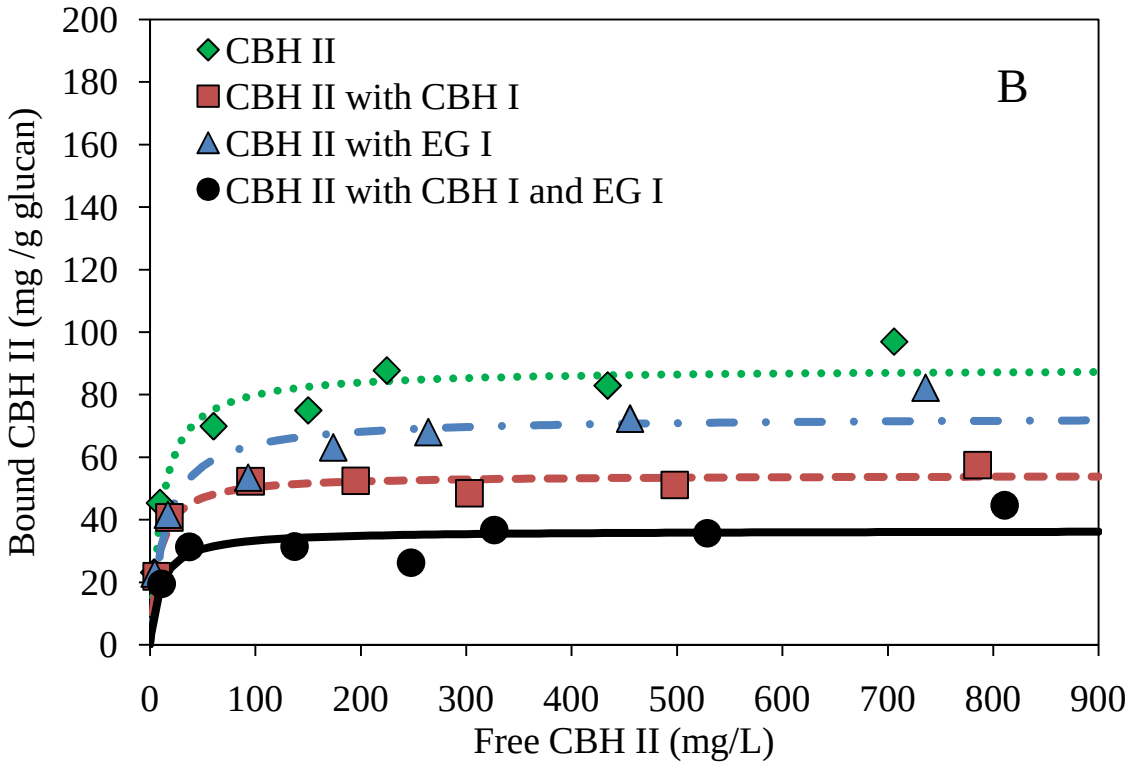
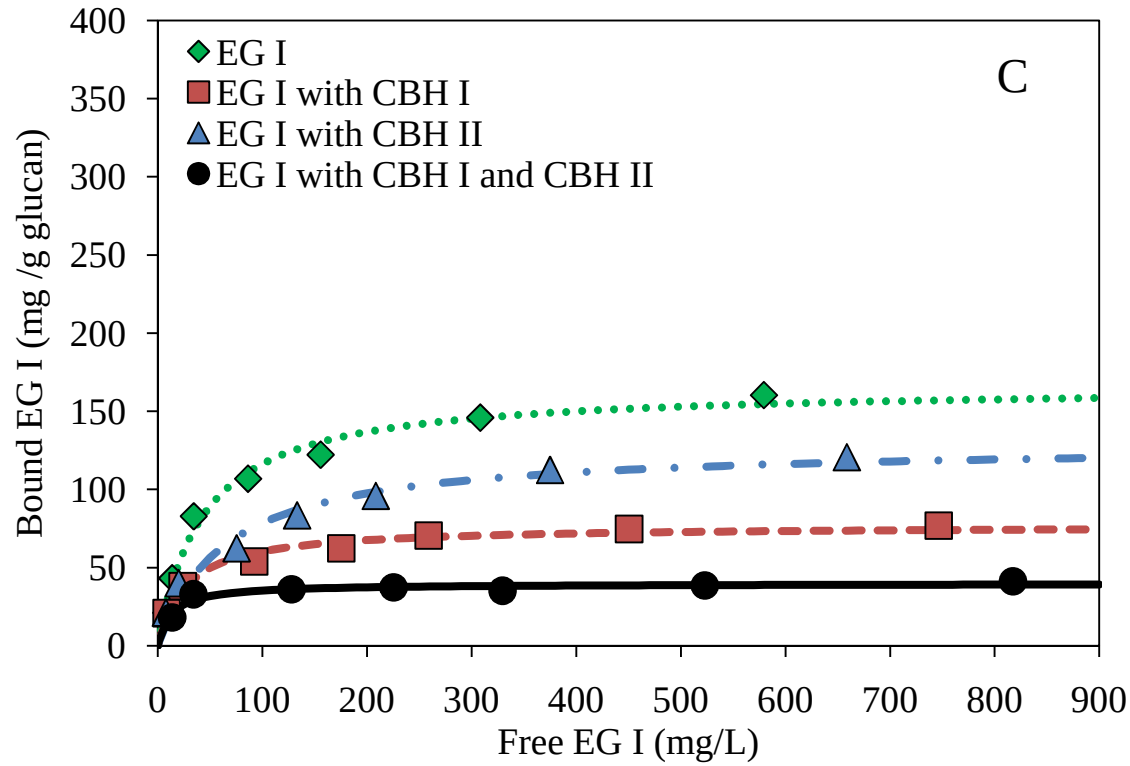


Figure 7.4 Binding isotherms for CBH I (A), CBH II (B) and EG I (C) on dilute acid pretreated corn stover. The extent of binding for individual enzymes was measured for single, binary and ternary cellulase mixtures.

Figure 7.4 (cont'd)





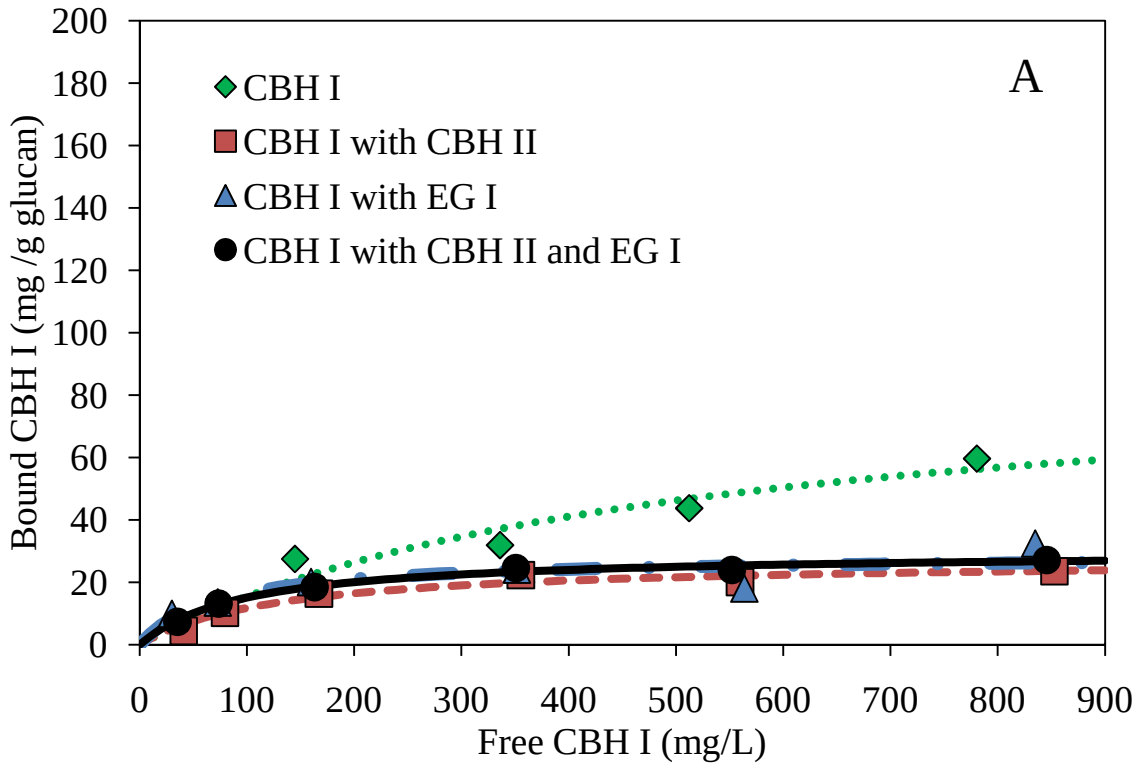
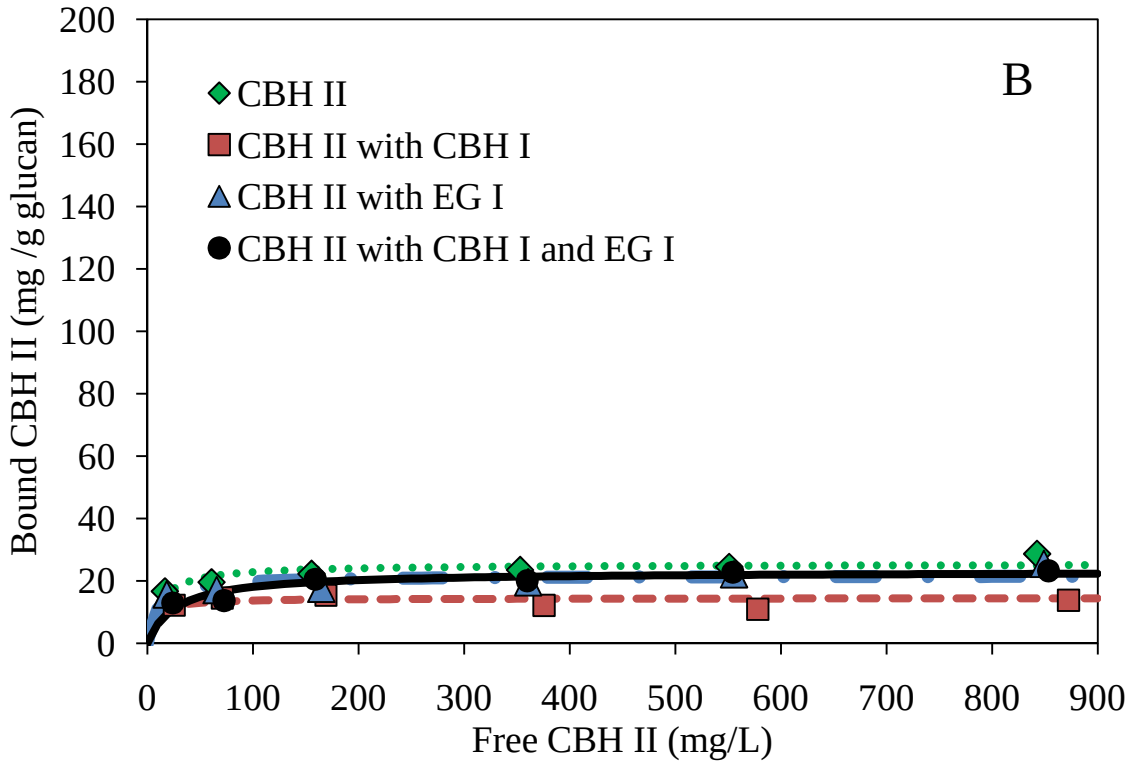
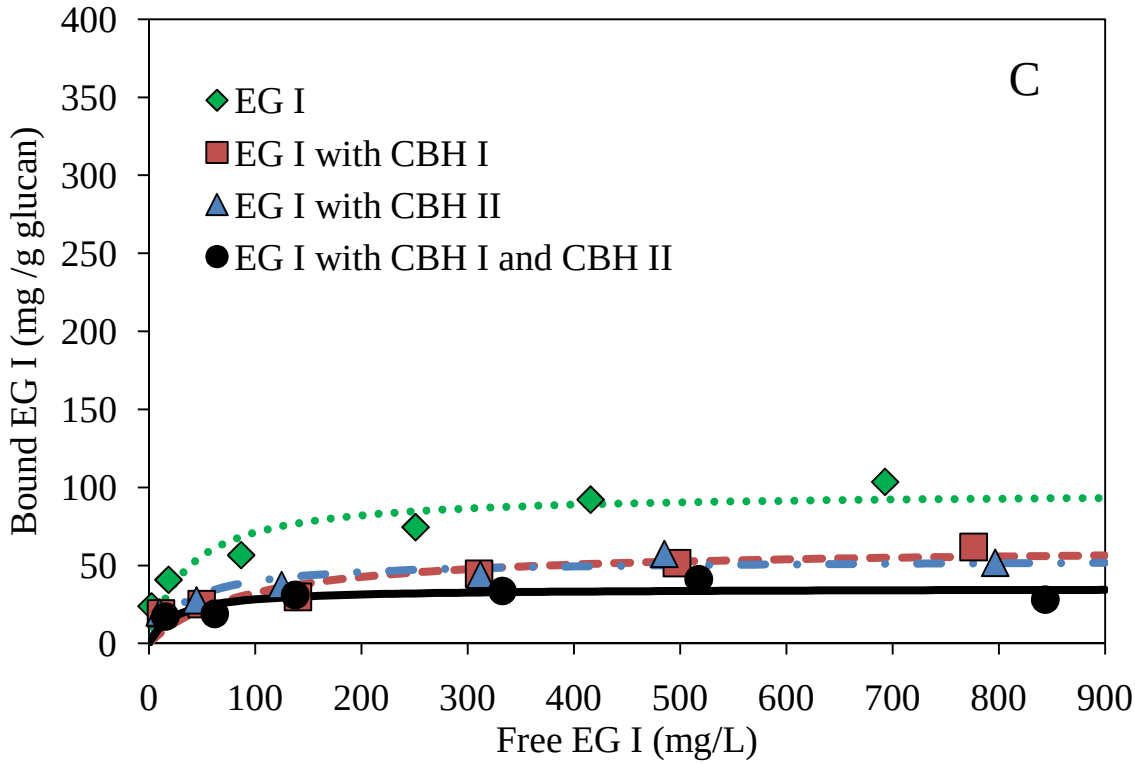


Figure 7.5 Binding isotherms for CBH I (A), CBH II (B) and EG I (C) on untreated corn stover. The extent of binding for individual enzymes was measured for single, binary and ternary cellulase mixtures.

Figure 7.5 (cont'd)





Although the binding isotherms and overall binding capacities for individual enzymes are quite different for AFEX-CS, ACID-CS and UN-CS, the relative order of binding capacities among the samples is the same for each protein. At 450 mg/g glucan protein loading EG I added alone had the highest binding capacity (374, 160 and 103 mg/g glucan for AFEX-CS, ACID-CS and UN-CS, respectively), followed by CBH I (190, 147 and 60 mg/g glucan for AFEX-CS, ACID-CS and UN-CS, respectively) and CBH II (154, 97 and 29 mg/g glucan for AFEX-CS, ACID-CS and UN-CS, respectively).

We studied the binding isotherms for binary mixtures of enzymes in which one enzyme's binding properties were investigated in the presence of another enzyme at equivalent protein mass loading. Overall, these isotherms demonstrate that the relative extent of binding depends on both the type of cellulase and the substrate. For AFEX-CS, two different types of binding phenomena were observed; one was cooperative binding for CBHs in the presence of EG I, the other was competitive binding between CBH I and CBH II. For CBH I-CBH II binary mixtures, the presence of either exocellulase prevented the other enzyme from binding and resulted in reduced levels of bound enzymes. For CBH I-EG I and CBH II-EG I mixtures, presence of EG I significantly enhanced CBH I and CBH II binding to AFEX-CS. At 100 mg/g glucan enzyme loading for each component, EG I increased CBH I and CBH II binding to AFEX-CS by 71% and 42%, respectively, compared to the case in which CBH I or CBH II were added alone. This enhancement in exocellulase binding due to EG I was not observed when individual enzyme loadings exceeded 200 mg/g glucan. Under those conditions, the binding capacities for CBH I or CBH II (each in the presence of EG I) were significantly lower compared to each exocellulase added alone. Previous saccharification experiments for AFEX-CS have shown significant synergism between CBH I-EG I and CBH II-EG I mixtures hydrolyzing both the glucan and

xylan fractions of the substrate compared to CBH I-CBH II combinations [71]. This hydrolytic synergism between exo- and endo- cellulases on AFEX-CS is consistent with both CBH II and CBH I exhibiting cooperative binding in presence of EG I. On the other hand, no cooperative binding occurred between any of the binary mixtures for UN-CS; only competitive binding was observed. For ACID-CS also, only competitive binding was observed. Introduction of another enzyme to the binary mixture was found to always decrease the binding capacity of the other enzyme in the pair.

In addition, CBH I was found to compete more strongly than CBH II for available binding sites in the presence of EG I. Since though both cellulases have highly homologous CBD domains their binding affinity is considerably different, suggesting the CD would also play an important role of directing exo-cellulase binding to crystalline cellulose as suggested elsewhere as well [210]. CBH I is a processive enzyme that moves along the cellulose chain during hydrolysis [57]. For complete biomass hydrolysis, it is necessary for EG I, which creates endo-cuts on the cellulose chain, to return to the liquid phase when competing with CBH and hence bind on another cellulose chain to create additional endo-cuts. Other binding properties of binary enzyme mixtures have been reported on isolated crystalline cellulose. Jeoh et al. found cooperative binding for Cel5A and Cel6B from *T. fusca* on bacterial microcrystalline cellulose at 50 °C while competitive binding was observed at 5°C [147]. Medve et al. found no cooperative binding between CBH I and EG II on Avicel at 4 °C [79]. However, others have shown competition during binding at 4°C on Avicel for CBH I and CBH II [156, 219].

For ternary enzyme mixtures with AFEX-CS as substrate, CBH I binding reflects both cooperative and competitive effects depending on the total enzyme loading employed. EG I increased the binding of both exocellulases at lower enzyme loading (<150 mg/g glucan for CBH

I and <100 mg/g glucan for CBH II). On the other hand, CBH I and CBH II compete with each other and gave lower maximum binding capacities than previous single and binary component mixtures. EG I was unable to bind cooperatively with either CBH I or CBH II, reducing the extent of bound EG I dramatically. As the enzyme loading increased, these trends become more obvious. At lower protein loadings, the available AFEX-CS substrate binding sites are sufficiently numerous to allow cooperative binding between enzymes. But as the protein loadings increase, fewer binding sites are available for all enzymes resulting in increased competitive binding. CBH I had the highest binding affinity followed by EG I and CBH II for the ternary system. Similar results were found for ACID-CS, with CBH I having the highest binding capacity, followed by EG I and CBH II.

To assist the reader in understanding these complex binding patterns, we have summarized these results in a tabular form. In Table 7.1, the cooperative and competitive binding in binary enzyme mixture for different substrates was shown to vary with the total enzyme loading employed. It is also interesting to note, for some enzymes mixtures such as CBH I and CBH II in AFEX-CS, neither cooperative nor competitive binding was observed at low enzyme loading, which suggests independent binding of the enzyme pair on biomass.

Table 7.1 Cooperative and competitive binding among various binary cellulase mixtures for AFEX pretreated (AFEX-CS), dilute-acid pretreated (ACID-CS) and untreated (UN-CS) corn stover. Binding types (cooperative versus competitive) are generalized in the table and the respective enzyme loading ranges within which that particular binding behavior is seen are shown in brackets.

		Binding type and range (mg/g glucan)					
	Bound Enzyme	CBH I		CBH II		EG I	
	In presence of	CBH II	EG I	CBH I	EG I	CBH I	CBH II
Substrates	AFEX-CS	0 (25~150) - (150~450)	+ (25~200) - (200~450)	0 (25~50) -(150~450)	+ (25~200) - (200~450)	0 (25~50) - (50~450)	0 (25~50) - (50~450)
	ACID-CS	0 (25~50) - (50~450)	0 (25~50) - (50~450)	0 (25) - (25~450)	0 (25) - (25~450)	0 (25) - (25~450)	0 (25~50) - (50~450)
	UN-CS	0 (25~50) - (50~450)	0 (25~50) - (50~450)	- (25~450)	0 (25~50) - (50~450)	- (25~450)	- (50~450)

+ : Significant cooperative binding observed.

-: Significant competitive binding observed.

0: No significant influence on binding in presence of background enzyme.

Table 7.2 Langmuir type (single-site model) binding isotherm parameters for individual cellulase enzymes binding to untreated (UN-CS), ammonia fiber expansion (AFEX-CS) and dilute acid treated (ACID-CS) corn stover for various single, binary and ternary enzyme mixtures.

		AFEX-CS				ACID-CS				UN-CS			
		$K_a x10^3$	E_{bm} mg/g			$K_a x10^3$	E_{bm} mg/g			$K_a x10^3$	E_{bm} mg/g		
Enzyme	Mixture	L/mg	glucan	biomass	R^2	L/mg	glucan	biomass	R^2	L/mg	glucan	biomass	R^2
	Alone	1.5	426.6	146.8	0.99	246.5	133.7	81.0	0.94	2	92.1	31.7	0.95
	+ CBH II	3.4	204.4	70.3	0.98	227.4	83.5	50.6	0.96	7.6	27.4	9.4	0.95
	+ EG I	103.5*	104.2	35.8	0.98	184.4	83	50.3	0.95	14	28.5	9.8	0.7
CBH I	+ CBH II + EG I	104.4*	76	26.1	0.93	130.9	61.7	37.4	0.91	10.3	29.9	10.3	0.99
	Alone	3.1	233.5	80.3	0.95	95.9	88.3	53.5	0.94	91.4	25.4	8.7	0.74
	+ CBH I	7.7	110	37.8	0.85	129.1	54.3	32.9	0.94	175.8	14.5	5.0	0.67
	+ EG I	123.9*	103.6	35.6	0.9	72.3	72.9	44.2	0.89	102.8	21.7	7.5	0.5
CBH II	+ CBH I + EG I	292.9*	45	15.5	0.59	104.1	36.5	22.1	0.57	37.2	23	7.9	0.82
	Alone	19.8	500.4	172.1	0.99	23.4	166	100.6	0.99	27.5	97	33.4	0.84
	+ CBH I	35.3	159	54.7	0.94	36.9	76.7	46.5	0.97	10.9	62.2	21.4	0.8
	+ CBH II	15.1	282.1	97.0	0.94	15.6	128.7	78.0	0.97	29	53.6	18.4	0.85
EG I	+ CBH I + CBH II	85.7	92.6	31.9	0.87	80.4	39.7	24.1	0.89	39.7	35.3	12.1	0.64

*Cooperative binding was observed. K_a increased significantly in the presence of EG I for AFEX-CS.

#This value was calculated based on the fitted isotherm E_{bm} (mg/g glucan) value and original glucan content (dry weight basis) for each substrate.

7.3.3 Modeling Enzyme Binding Properties

Langmuir single-site adsorption model was used to describe cellulase binding to untreated and pretreated corn stover under different enzyme loading conditions (single, binary or ternary mixtures). The K_a, E_{bm} and coefficient of determination (R^2) values are shown in Table 7.2. The E_{bm} values based on both glucan and total biomass basis are shown for comparison. The model fits for pretreated biomass, both AFEX-CS and ACID-CS, are much better (R^2>0.9) than those for untreated corn stover, UN-CS. The E_{bm} for AFEX-CS and ACID-CS are much higher compared to UN-CS, which is consistent with increased cellulose accessibility after pretreatment. Hong et al. also found a linear relationship between increased substrate digestibility and available binding sites for CBM on different cellulosic substrates [220]. Interestingly, E_{bm} for AFEX-CS is typically higher than ACID-CS for these enzymes. Since the total biomass loading for AFEX-CS is higher than ACID-CS (all experiments are based on equivalent glucan loading, thus AFEX-CS has much higher xylan content than ACID-CS), one would expect the higher xylan content for AFEX-CS could result in greater cellulase binding than ACID-CS. The effect of individual cell wall components (e.g., xylan, lignin) on non-productive cellulase binding is not clear based on these experiments and requires further investigation.

In addition, comparing the E_{bm} for CBH I, CBH II and EG I among different substrates suggest that binding strengths for each enzyme depend on the substrate and cellulase type. For AFEX-CS treated corn stover, EG I had the highest E_{bm} (500.4 mg/g glucan) followed by CBH I (426.6 mg/g glucan). For UN-CS, EG I and CBH I have a comparable E_{bm} (~95 mg/g glucan).

Interestingly, CBH II had the lowest E_{bm} (25.4 mg/g glucan) for untreated corn stover compared to other cellulases. However, after pretreatment the E_{bm} for CBH II increased dramatically to 233.5 (AFEX-CS) and 88.3 (ACID-CS) mg/g glucan, respectively. These results suggest that the preferred binding sites for CBH II, possibly inclusive of cellulose non-reducing ends [221], are exposed and become more readily accessible after thermochemical pretreatments. As explained previously, adding EG I increased CBH I and CBH II binding for AFEX-CS. This incremental increase in binding was also reflected in the K_a values. The K_a value reflects the initial slope of the isotherm, and hence corresponds to enzyme affinity for the substrate when the substrate is present in excess. During cooperative binding of cellulases to AFEX-CS, the K_a values for CBH increased dramatically for binary mixtures incorporating EG I compared to single exocellulases which suggests that the CBH binding affinity to AFEX-CS increased significantly in the presence of EG I. For ternary mixtures, K_a was also significantly higher than for single enzyme experiments. For ACID-CS, K_a values (i.e., only for exocellulases in absence of EG I) are much higher than AFEX-CS which suggests that removal of xylan can significantly modify the substrate and hence alter its cellulase binding properties. In subsequent work, we hope to explore these trends in the presence of xylanases, which may significantly alter the observed binding patterns by exposing additional cellulose, perhaps in ACID-CS, but certainly in AFEX-CS. In this work, the binding of enzymes onto the whole biomass was observed. However, it is difficult to evaluate the individual contribution to binding for each cell wall component (e.g., crystalline cellulose, xylan, lignin, amorphous cellulose). Yang and Wyman added bovine serum albumin as a “lignin blocker” to minimize unproductive binding during enzymatic hydrolysis of

dilute acid and AFEX treated corn stover [213]. They found that this approach benefitted hydrolysis of AFEX-CS much less than it did for ACID-CS. This would lead one to speculate that the lignin exposed during AFEX has lesser binding capacity for cellulases, possibly due to lower lignin hydrophobicity as suggested recently [209], than lignin exposed during dilute acid treatment [213]. Preliminary unpublished data from our lab also validates this finding. Though binding of individual cellulase enzymes to lignin and hemicellulose fractions was not measured in this study, it will be explored in future work.

7.3.4 Impact of AFEX pretreatment on cooperative cellulase binding

AFEX is a "dry to dry" (i.e., no separate liquid fraction arises from pretreatment) process and the composition of pretreated biomass is almost identical to that of untreated biomass [15]. In contrast, dilute acid pretreatment can remove a significant amount of hemicellulose and some lignin [14]. Cellulose degree of polymerization (DP) is largely unchanged by AFEX (~ 6000-7000 for corn stover), unlike dilute acid treatment that results in decreasing the DP by 60-70% [110]. Both of these thermochemical pretreatments are believed to increase cellulase accessibility to cellulose (and hemicellulose, lignin) through various ultra-structural and physicochemical changes that are being unraveled in recent years [14, 19, 111, 209].

One possible reason for cooperative binding between CBH-EG I binary mixtures for AFEX-CS might be due to the ultrastructure of AFEX-CS compared to UN-CS and ACID-CS. AFEX has been recently shown to create internal porosity within the cell walls by physically delocalizing some of the hemicellulose and lignin to the outer wall surfaces. However, there is likely a significant amount of xylan strongly associated with cellulose microfibrils after pretreatment [209]. In contrast, acidic treatments hydrolyze and strip out most of the hemicellulose and some of the lignin into a separate liquid stream during pretreatment [110, 210]. EG I is known to have

significant xylanase and xyloglucanase activity [70, 71], and hence could have prevented non-productive binding of exo-cellulases to the hemicellulose fraction by binding to it instead. Although EG I's hydrolytic activity is mostly suppressed at 4ºC, it had marginal activity on xylan tightly associated to cellulose especially at higher enzyme loadings (Note: EG I loading at 100 mg/g glucan or more resulted in ~2% xylan hydrolysis to soluble sugars at 4ºC). Removal of this xylan sheath by EG I could have exposed the underlying cellulose surface, hence enhancing exocellulase binding to cellulose. This also explains why at high total enzyme loading CBH enzyme binding is much less when it is part of binary mixtures than when it is added as a single enzyme. Since there are only trace amounts of hemicellulose left behind in the cell wall after dilute acid treatment this cooperative binding phenomena is not readily observed for ACID-CS (at least in presence of EG I). On the other hand, this phenomenon is also not observed for UN-CS due to limited enzyme accessibility to the embedded cellulose sheathed by tightly cross-linked lignin and hemicellulose [33, 209].

7.4 Conclusions

Both AFEX and dilute acid pretreatment were found to increase cellulase binding to embedded cellulose microfibrils within cell walls. Presence of EG I enhanced exo-cellulase cooperative binding to AFEX pretreated cell walls likely due to presence of residual hemicellulose that sheathed cellulose fibrils unlike what was seen for dilute acid treated cell walls. Competitive binding among enzymes was also observed for certain substrates, cellulase combinations and protein loadings employed. Though it was not possible to determine extent of non-productive binding of cellulases from the current set of experiments, future studies with isolated lignin and hemicellulose fractions will help gain a better understanding of this phenomenon. These studies could assist in enzyme engineering efforts to minimize unproductive binding and help design

improved pretreatments that facilitate productive binding hence lowering the enzyme dosage necessary for cost-effective enzymatic hydrolysis of lignocellulosic biomass.

CHAPTER 8 CELLULOSE CRYSTALLINITY AND LIGNIN: THEIR IMPACT ON EFFICIENT CELLULASE ACTION

8.1 Introduction

In the previous chapter, the binding studies were done at 4 ºC on pretreated/untreated biomass. Biomass is heterogeneous and 4 ºC inhibit enzyme activities significantly. To have a better understand of how enzyme work, time course hydrolysis studies with tracking of individual enzyme's distribution between liquid phase and solid phase are indispensible. Both pure cellulosic and lignocellulosic substrates are used in this chapter.

The extent of cellulase binding to crystalline cellulose has been generally found to be directly correlated to the enzymatic hydrolysis rate [222-224]. However, the role of productive and non-productive binding is difficult to discern from most experiments. Also, the role of cellulose allomorph type on cellulase binding has not been studied before. Cellulose allomorph type (or the lack of crystallinity) has been suggested recently to determine how difficult it is for fungal cellulases to abstract individual glucan strands from the crystal surface prior to hydrolysis of the glycosidic bond [210]. This suggests that different allomorph types may result in different binding behavior for cellulases.

Lignin is thought to impede efficient enzymatic hydrolysis possibly due to unproductive binding of cellulases to it and/or through steric hindrance facilitated by the lignin-carbohydrate complex that decrease cellulose accessibility [33]. In addition, lignin derived degradation products produced during pretreatment could further inhibit enzyme activity [20, 209]. Recalcitrance towards enzymatic digestion is believed to be directly proportional to lignin content [102]. Delignification of biomass during or after pretreatment can enhance hydrolysis rate and overall

sugar yield [225]. Several studies have reported the beneficial effect of surfactants and other proteins that can prevent unproductive binding of cellulases to lignin to some extent [145, 213, 226]. However, the affinity of lignin towards individual cellulases is still far from clear due to the lack of reliable techniques to track individual enzyme binding present within complex protein mixtures during saccharification. Doping extracted lignin to pure cellulosic substrates to mimic pretreated biomass is one way to quantify the inhibitory role of lignin [115, 227]. However, the major limitation of this approach is that lignin structure can be chemically or physically modified during extraction and hence its affinity to enzymes could change. On the other hand, the pretreated cell wall microstructure (e.g., porosity, lignin redistribution due to pretreatment) and chemical linkages between different components (i.e., cellulose, hemicellulose and lignin) cannot be simulated by simply recombining the respective purified components. Tracking enzyme binding during hydrolysis has been carried out by measuring total crude protein concentration and/or activities of unbound enzymes in the hydrolyzate [146, 153]. However, these methods have limitations because it is difficult to differentiate individual enzyme binding from these experiments.

To achieve a comprehensive understanding of cellulase binding to cellulosic biomass during course of hydrolysis, we have applied a novel FPLC based methodology to quantify CBH I, CBH II and EG I concentration in complex hydrolyzate mixtures [228]. Pure cellulose with different crystal structures were included as well: microcrystalline cellulose I, cellulose II, cellulose III, and regenerated amorphous cellulose. Lignocellulosic biomass materials included in this study were: pretreated (ammonia fiber expansion or AFEX, dilute acid and ionic liquid pretreated) corn stover to compare cellulase adsorption characteristics and overall digestibility as a function of pretreatment type. These studies have revealed several interesting phenomena that

were previously unknown. These include; (i) relative cellulase binding affinity is different for cellulose allomorphs and lignin-rich pretreated biomass, (ii) extent of unproductive cellulase binding to lignin depends on pretreatment type and was found to negatively correlate with hydrolysis yields, (iii) increased cellulase affinity for crystalline cellulose does not always correlate with enhanced digestibility, and (iv) the relative thermostability of *Trichoderma reesei* CBH II during course of saccharification is lower. We believe these findings will aid in engineering low unproductive binding cellulases for novel pretreatments and lead to development of economic enzyme recycling options.

8.2 Materials and Methods

8.2.1 Cellulosic substrates

Avicel (PH 101, Sigma-Aldrich, St Louis) was the pure cellulose source used to prepare all other allomorphs. Cellulose III was prepared by soaking Avicel in anhydrous liquid ammonia at 100 ^{o}C for 30 min. The samples were dried under nitrogen and purged overnight to remove residual ammonia. Cellulose II was prepared by adding 25% NaOH to Avicel at 4 ^{o}C for 60 min. The slurry was then centrifuged, filtered and washed with water till neutral pH. Regenerated amorphous cellulose (AC) from Avicel was prepared using 83% phosphoric acid at 4 ^{o}C for 60 min based on published protocol [229] Cellulose II and AC were lyophilized to dryness prior to storage at 4 ^{o}C for future experiments. Cellulose crystallinity index was estimated using XRD amorphous subtraction method [230].

8.2.2 AFEX pretreated corn stover

Detailed information is described in 4.2.1

8.2.3 Dilute acid pretreated corn stover

Detailed information is described in 7.2.2

8.2.4 Ionic liquid pretreated corn stover

Ionic liquid pretreated corn stover was a generous gift from JBEI. Details on ionic liquid pretreatment methodology are provided elsewhere [105].

8.2.5 Cellulase Purification

Detailed information is described in Chapter 3.

8.2.6 Enzymatic Hydrolysis

All hydrolysis experiments were performed in a 2.2 ml deep-well microplate (Lot # 780271, Greiner, Monroe, NC) at 1% (w/w) glucan loading along with 50 mM pH 4.5 citrate buffer in total reaction volume of 500 µl. 15 mg/g glucan (corresponding to 0.15 mg/mL) each of CBH I, CBH II and EG I are loaded along with 2 mg/g glucan of βG (C Cocktail). This enzyme loading allowed maximum digestion to be achieved for all cellulosic allomorphs within 48 h. For pretreated biomass, the C Cocktail included an additional xylanase (5 mg/g glucan) and βX (2mg/g glucan) (CX cocktail) to achieve near-theoretical glucan conversions within 48 h. The microplates were incubated at 50 ºC with shaking at 250 rpm for 48 h. Sampling was conducted at 1, 4, 12, 24 and 48 h. The supernatant was then separated from the insoluble solids by filtering through a 0.45 µm low protein binding hydrophilic microplate based filter (Lot # R6PN00144, Millipore, Ireland) for protein and sugar analysis. All experiments were carried out in triplicates with mean values and standard deviations reported in the figures.

8.2.7 Sugar analysis

Glucose and xylose concentration within the hydrolyzate was analyzed by HPLC with details provided elsewhere [209].

8.2.8 Quantitation of unbound CBH I, CBH II and EG I

8.2.9 Thermostability of CBH I, CBH II and EG I

0.15 mg/mL of purified CBH I, CBH II and EG I were incubated at 50 ºC. Samples from 0, 1, 4, 12, 24 and 48 hours incubation are evaluated for their specific activities on various substrates (CBH I and CBH II were tested on Avicel with incubation at 50 ºC for 24 hours; EG I was tested on carboxymethyl cellulose with incubation at 50 ºC for 1 hour). The reducing sugars were measured using the 3,5-dinitrosalicylic acid (DNS) based assay [71] . Relative activities are reported based on samples from 0 hour incubation.

8.3 Results and Discussion

8.3.1 Cellulase hydrolytic activity on different cellulosic substrates

The relative enzymatic digestibilities of various cellulose allomorphs and amorphous cellulose were very different (Figure 8.1). All substrates were hydrolyzed using a defined equi-mass cellulase cocktail comprising of CBH I, CBH II and EG I (at 45 mg total cellulase loading/g glucan). The digestibility of cellulose, during the first 4 hours of digestion, was found to rank in the following order: Amorphous cellulose or AC (90%) > cellulose III (58%) > cellulose II (54%) > cellulose I (43%). Figure 8.1E depicts the average hydrolysis rate at different time points. Near-theoretical glucan conversions were achieved for cellulose I, cellulose II and III, and amorphous cellulose at 48 h, 24 h, and 12 h respectively. Cellulose I saw the most significant decrease in its hydrolysis rate (within the first 4h of hydrolysis) among all allomorphs. Cellulose

II maintained a similar hydrolysis rate that was marginally higher than Cellulose III and AC at 4h. These results demonstrate how the recalcitrance of cellulose is closely related to its allomorph.

8.3.2 Cellulase binding to different cellulosic substrates during course of hydrolysis

The extent of unbound cellulases (CBH I, CBH II and EG I) for the various cellulose allomorphs and amorphous cellulose during saccharification was found to vary considerably. The level of unbound (or free) CBH I, CBH II and EG I for various cellulosic substrates was estimated using the depletion method and are shown in Figure 8.1A-D. The initial bound enzyme concentration (after 1 hour incubation) was calculated by the depletion method, i.e., subtracting free enzyme in supernatant from total enzyme loaded (Figure 8.2). AC had the highest binding capacity for CBH I, CBH II and EG I (more than 85% of added enzymes were bound after 1 hour). Cellulose II's binding capacity for CBH I and CBH II was greater than 70%, while 60% of EG I was bound after 1 hour. A greater percentage of the originally added cellulases were bound to AC and Cellulose II compared to Cellulose I (33-44% of cellulases are bound and EG I bind 10% more than CBH). On the contrary for cellulose III, only 17% CBH I, 12% CBH II and 6.6% EG I was bound to the substrate after 1 h of saccharification. This is striking considering that the digestion rate for Cellulose III is lower only compared to amorphous cellulose. Comparing the levels of unbound cellulases for amorphous cellulose after 1 h of saccharification and cellulose allomorphs after 4 h of saccharification is appropriate since the extent of glucan conversions is comparable. However, the trends noted after 1 h of hydrolysis were also seen for various cellulose allomorphs (I, II and III) after 4 h of hydrolysis.

For later stages of hydrolysis (24-48 hours), most of the cellulases are present in their unbound state (>90% CBH I and EG I are present as free enzymes at 48 h) in the supernatant as the cellulose is depleted (>95% glucan conversion for all substrates). These results are consistent

with previous work that reported industrial-scale cellulase production using pure cellulose (e.g., milled cotton, sulfite pulp and Solka Floc) to induce enzymes and protein recovery after complete substrate solubilization [85]. However, after 12 hours of saccharification the level of unbound CBH II in the supernatant keeps decreasing for all substrates. It's likely that CBH II has lower thermostability compared to the other cellulases that causes it to unfold and alter its structural properties (e.g., pI, molecular weight, amino acid composition) that influences its detection using the ion-exchange chromatography method. Our method to quantify cellulases is based on separation of enzymes from background UV-absorbing lower molecular components based on their molecular weight by gel filtration chromatography followed by individual cellulase separation and quantification via ion exchange chromatography [228]. If a certain fraction of CBH II is excluded from the chromatographic steps due to change in its molecular/structural properties then the total quantifies CBH II will be lower than theoretical available unbound protein in the supernatant.

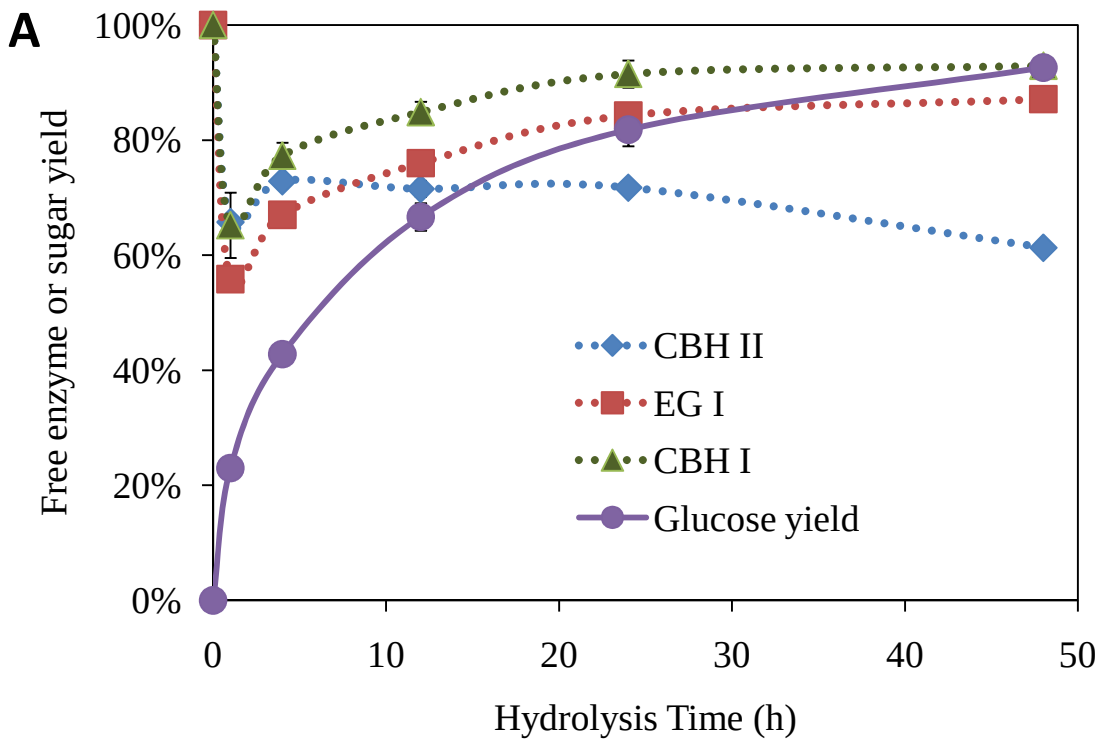
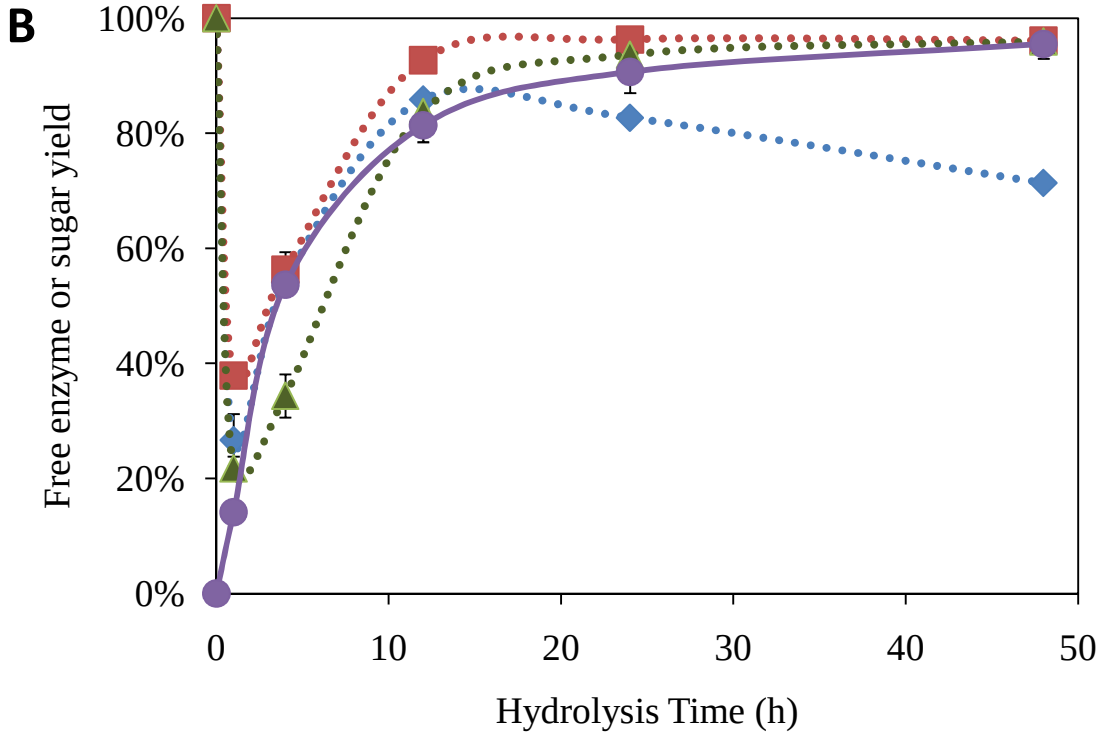


Figure 8.1 Sugar yield and the percentage of enzymes in supernatant for Cellulose I (A), Cellulose II (B), Cellulose III (C) and amorphous cellulose (D) during 48 hour hydrolysis at 50 °C and comparison of average hydrolysis rate for different cellulose allomorphs (E).

Figure 8.1 (cont'd)



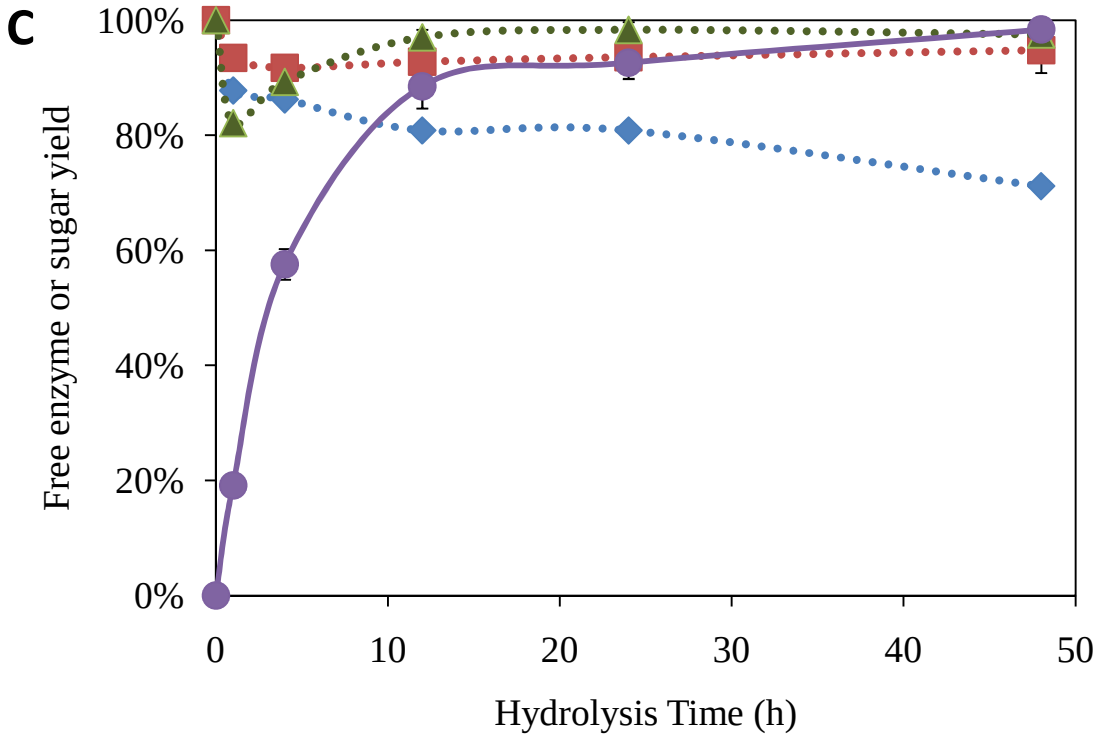
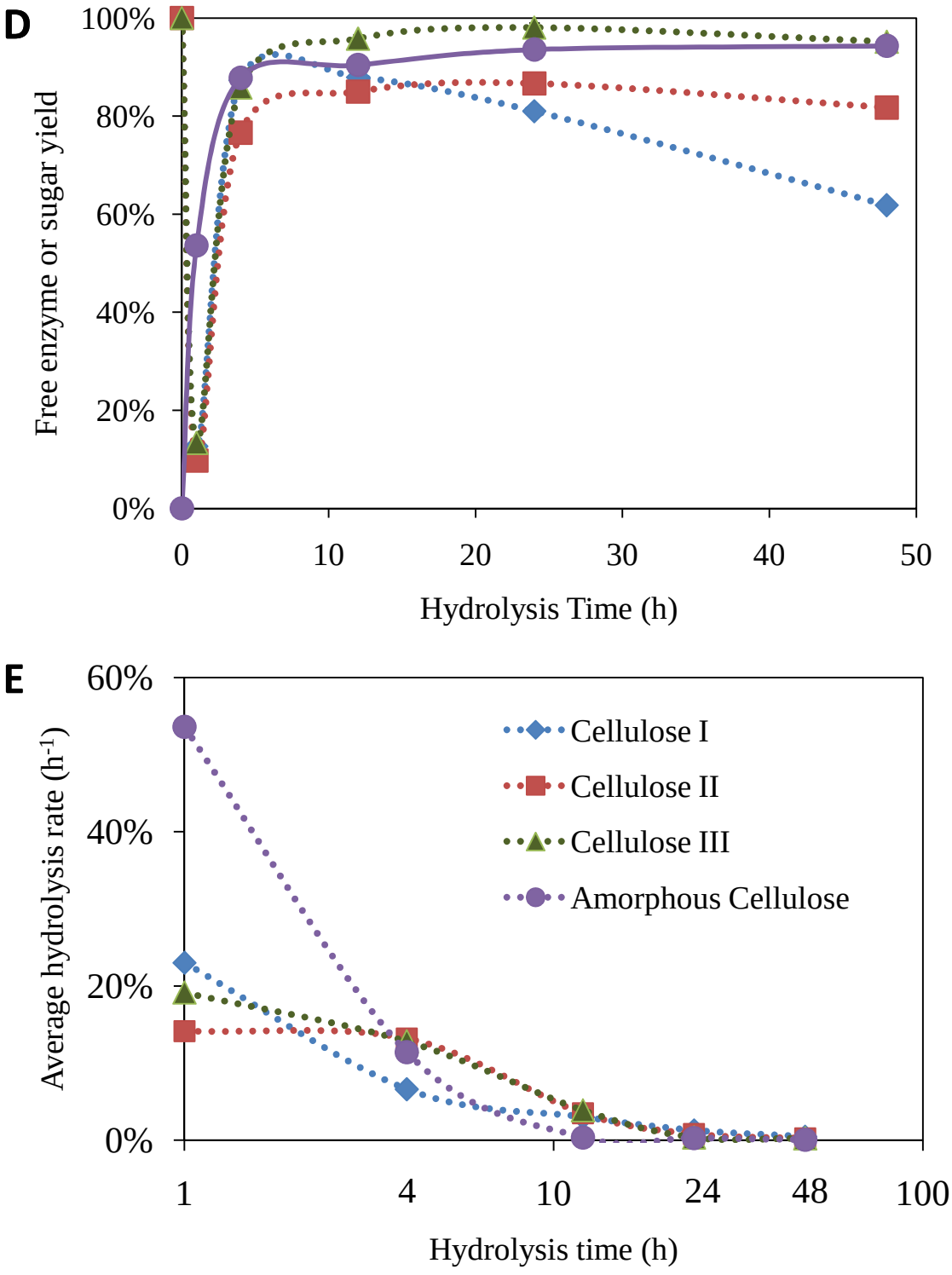


Figure 8.1 (cont'd)



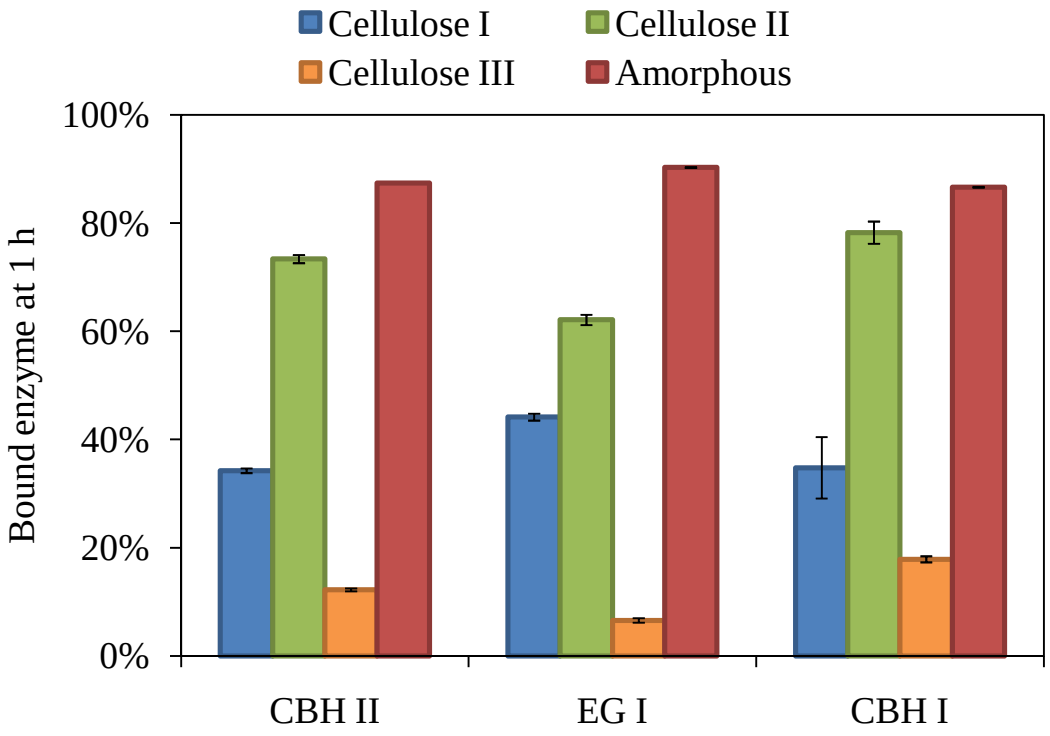


Figure 8.2 The level of bound CBH I, CBH II and EG I for different cellulose allomorphs at 1 hour.

8.3.3 Trichoderma cellulases thermostability

In order to confirm previous hypothesis that CBH II tends to easily lose activity during hydrolysis at 50ºC, purified cellulases were incubated at 50ºC for different time periods and assayed to check their specific activity. From Figure 3, we can clearly see that ~60% of CBH II's original activity was preserved after incubation at 50ºC for 48 hours; whereas, CBH I and EG I retain more than 90% of their original activity. In the case of Cellulose III, 70% of unbound CBH II is found in the supernatant after 48 hours, while only 60% of the enzyme is found for the other substrates (Figure 1). These results suggest that thermal denaturation of CBH II, as indicated by the loss in enzymatic activity, is responsible for altering the protein structure and hence impacting protein quantification using the ion-exchange chromatography method. A noticeable drop in cellulase activity is seen after 12 hour of incubation at 50ºC which is

consistent with the corresponding decrease in predicted concentration of unbound CBH II seen in Fig 1A-D. In the current set of thermostability experiments no substrates was added, however, it is reasonable to suspect that cellulase activity preserved would be different in a heterogeneous environment in the presence of cellulose. Binding to cellulose has been reported to stabilize cellulase activities and prevent thermally-induced denaturation [231, 232]. However, since it is difficult to directly measure individual bound enzyme's activity during hydrolysis, these results offer evidence of extent of activity loss during incubation.Pretreated corn stover hydrolysis

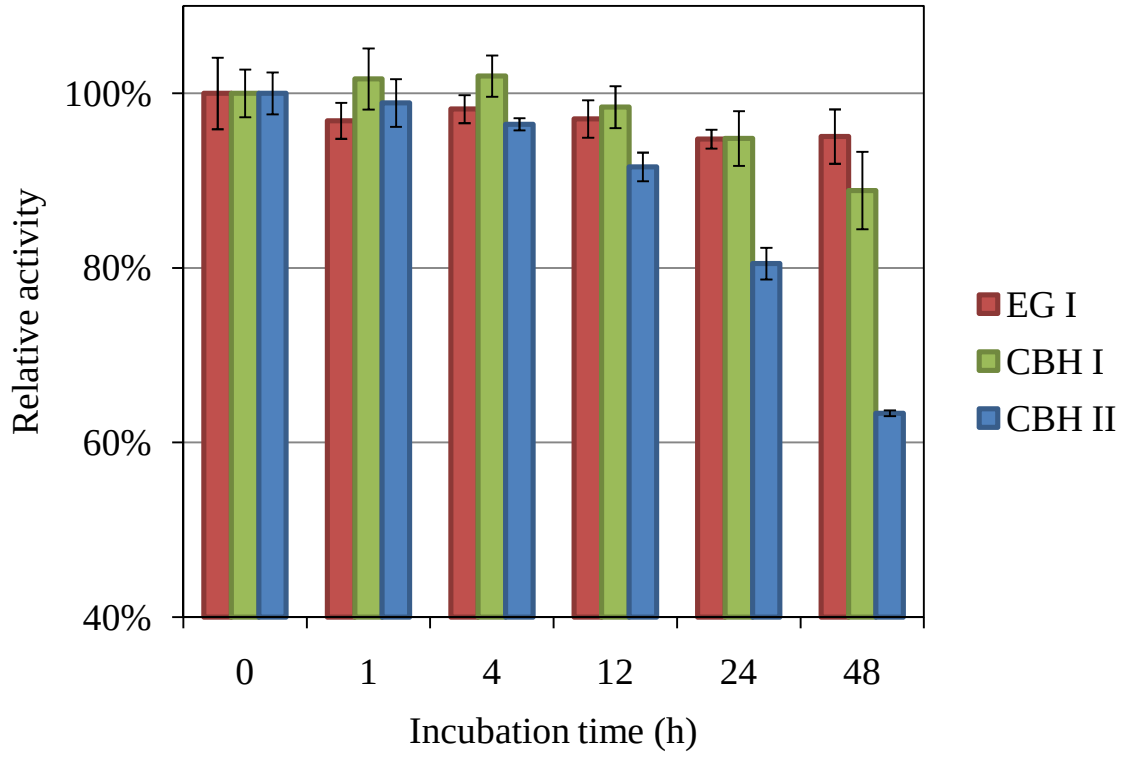


Figure 8.3 Relative activity for CBH I, CBH II and EG I after certain time of incubation at 50 ºC.

8.3.4 Cellulase binding and hydrolytic activity on pretreated lignocellulosic biomass

Commercialized large scale hydrolysis for biofuels or chemicals production has to be considered by operating with pretreated biomass rather than pure cellulose. After pretreatment, biomass is

modified physically and chemically to decrease the recalcitrance which facilitated quick sugar releasing during saccharification. To avoid plant species different, corn stover is chosen as the single feedstock. AFEX is a "dry to dry" process which does not remove any liquid stream during pretreatment hence preserves almost all of the composition [103, 209]. Dilute acid pretreatment, which remove most of the hemicelluloses from liquid stream and part of lignin. The removal of most hemicellulose modifies the substrate composition. Both cellulose and lignin are enriched compare to untreated biomass [103]. Ionic liquid pretreatment, a novel pretreatment technology, swells the cell wall and remove significant amount of lignin from biomass hence the glucan and xylan component are enriched. These three pretreatments are representatives for many other alkaline, acid and extraction based pretreatment such as lime, steam explosion and organosolv. The composition of pretreated corn stover is shown in Table 8.1. AFEX CS has almost identical composition with untreated one. Dilute acid remove most of the xylan and consequently has the highest glucan and lignin content. IL pretreatment removes most of the lignin and preserved most xylan. All of the experiments are performed at 1% standard glucan loading so their theoretical glucose yields are same. Theoretical xylose and lignin concentration therefore are quite different from each other.

CBH I, CBH II and EG I are the "work horse" enzymes for biomass saccharification. For AFEX CS, optimized cocktail need around 70%-80% of those enzymes [71, 74, 198]. Endo-xylanase, β-xylosidase and other auxiliary hemicellulases are necessary for hemicellulose hydrolysis to achieve high xylose yield [205]. To evaluate how xylan component affect cellulases binding, all pretreated CS experiments are performed in two set of enzymes cocktail. One is only with CBH I, CBH II, EG I and βG (cellulases, abbreviation of C Cocktail). The other is C Cocktail with addition of EX and βX (cellulases with xylanases, abbreviation of CX Cocktail).

Table 8.1 Composition and theoretical concentration of cellulose allomorphs (cellulose I, II, III and amorphous cellulose) and pretreated corn stover.

	Composition*			Theoretical concentration (g/L)		
Substrates	Glucan	Xylan	Lignin**	Glucose	Xylose	Lignin
Cellulose allomorphs	~100%***	0	0	11.11	0.00	0.00
Untreated/AFEX CS	34.6%	19.6%	11.0%	11.11	5.66	3.18
IL CS	46.9%	29.8%	2.7%	11.11	7.22	0.58
ACID	60.6%	3.3%	32.9%	11.11	0.62	5.43

*Based on dry weight

**Insoluble Klason lignin

***Non-cellulose component is neglectable

For AFEX CS, as show in Figure 8.4, with the C Cocktail, the glucose yield can get around 80% after 48 hours while xylose yield is very low (<10%). This is consistent with our previous experiment results [71, 205]. Addition of EX and βX can enhance the xylose yield to around 54% and most of the glucan are hydrolyzed into monomeric glucose. The ratios of enzyme in supernatant are quite different from that in pure cellulose. At 1 hour, most of cellulases are bound onto biomass. With the saccharification moving forward, only limited amount of enzymes return to supernatant. CBH I has the highest recovery with 47% and 60% for C and CX cocktail respectively. For CBH II, much less amount of enzyme can be desorbed from biomass. CX cocktail can help to increase 8% more CBH II to final 37% in supernatant. For EG I, even in CX cocktail, only 28% is detected in the supernatant. The xylanases help remove xylan wrapped around glucan chain hence increase cellulose digestion. Just EX and βX are sufficient to solubilized xylan with some of xylose in brach substituted oligomeric sugar form. So for CX cocktail digested AFEX CS, the most of the remaining insoluble biomass is lignin residual. For AFEX CS, the EG I has the highest unproductive binding level onto lignin and CBH I has the lowest. But still, quite amount of CBH I (40%) cannot be recovered. Regarding previous research focused on mixture optimization of CX cocktail on AFEX CS, EG I has the highest

ratio counting 31% of the total enzyme loading [71]. The EG I has the highest unproductive binding level could explain why the optimum cocktail requires so much EG I.

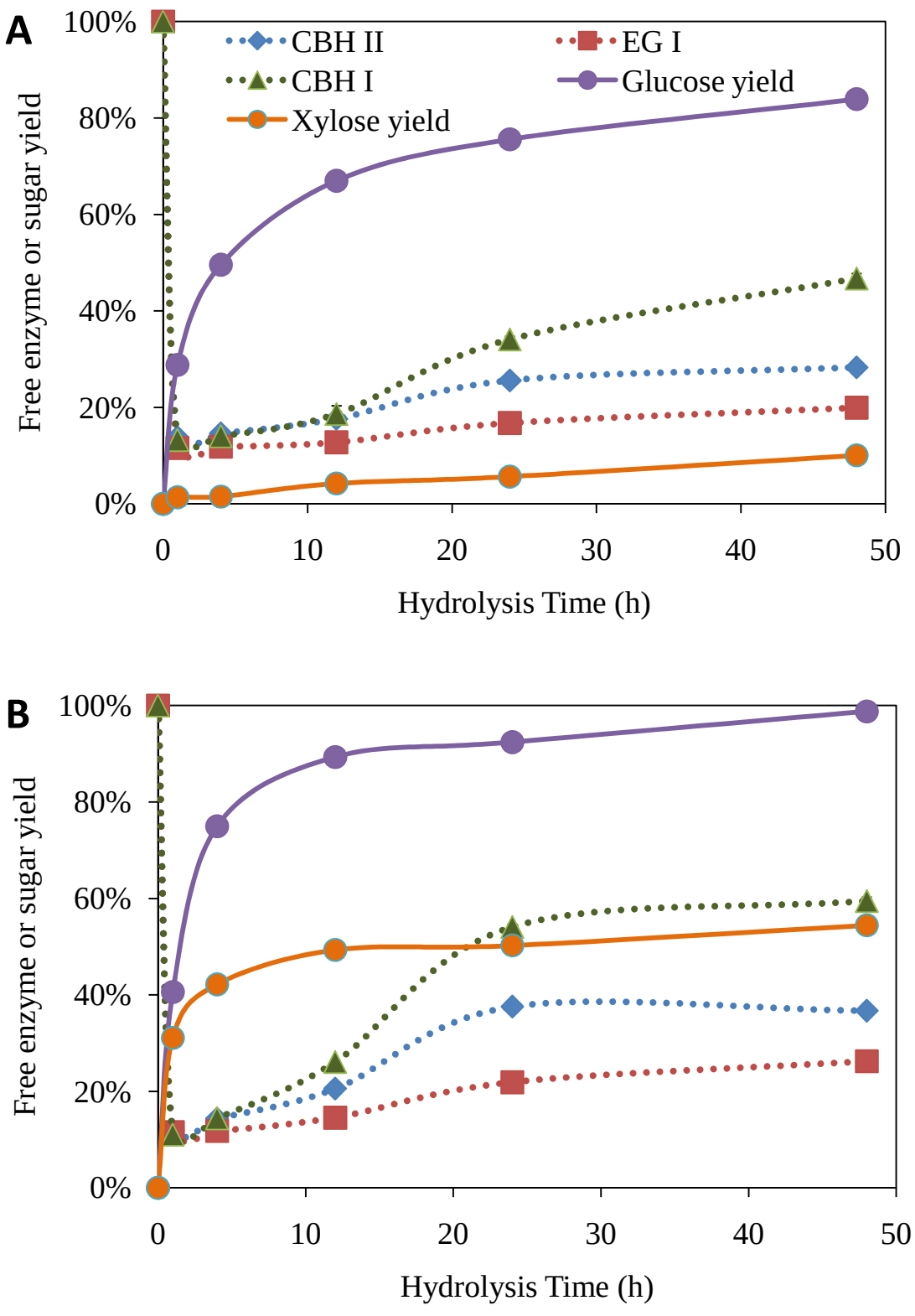


Figure 8.4 Sugar yield and the percentage of enzymes in supernatant for AFEX CS with (B) and without (A) endoxylanase and β-xylosidase during 48 hour hydrolysis at 50 °C.

For ACID CS (Figure 8.5), C cocktail is sufficient to get almost 90% glucose yield. The CX cocktail slightly improves glucose yield. However, for both cocktail, most of cellulases bound unproductively after 48 hours hydrolysis. Only around 30% CBH I can be recovered, which is around half of AFEX CS. Around 20% of EG I and 10% of CBH II remain in the supernatant. These results suggest the high lignin content in AICD CS contribute less enzyme recovery.

IL CS only contains less than 3% of lignin and the time course hydrolysis and free enzyme assay results are shown in Figure 8.6. With the C cocktail, more than 80% of glucose yield is obtained after 48 hours, while the CX cocktail can release around 90% of glucose. It takes 24 hours to release most the xylose, which is much slower compared to AFEX CS. Most cellulases bound onto biomass at 1 hour. With the conversion increasing, enzymes return to supernatant with different extent. CBH II is the highest recoverable enzymes. 60% can be recovered. Regarding the CBH II lost activity during hydrolysis for pure cellulose allomorphs. It is likely that most of CBH II can be recovered after hydrolysis. The recovery of EG I in IL CS is only around 30% but is still higher than AFEX CS and ACID CS. The interesting result is for CBH I. Less than 50% can be recovered in IL CS, which is less than AFEX CS although the lignin content of AFEX CS is much higher (Figure 8.7).

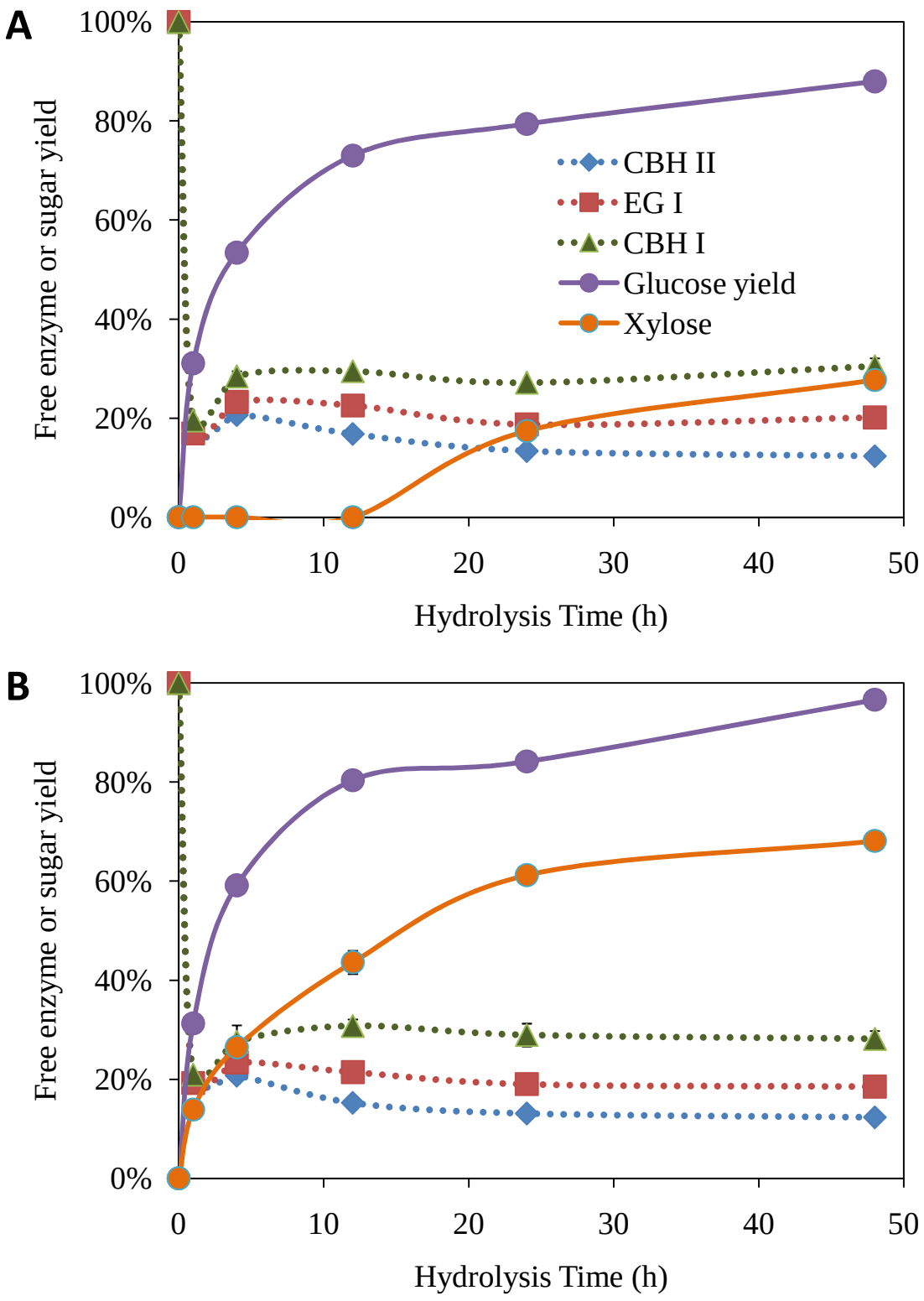


Figure 8.5 Sugar yield and the percentage of enzymes in supernatant for ACID CS with (B) and without (A) endoxylanase and β-xylosidase during 48 hour hydrolysis at 50 ºC.

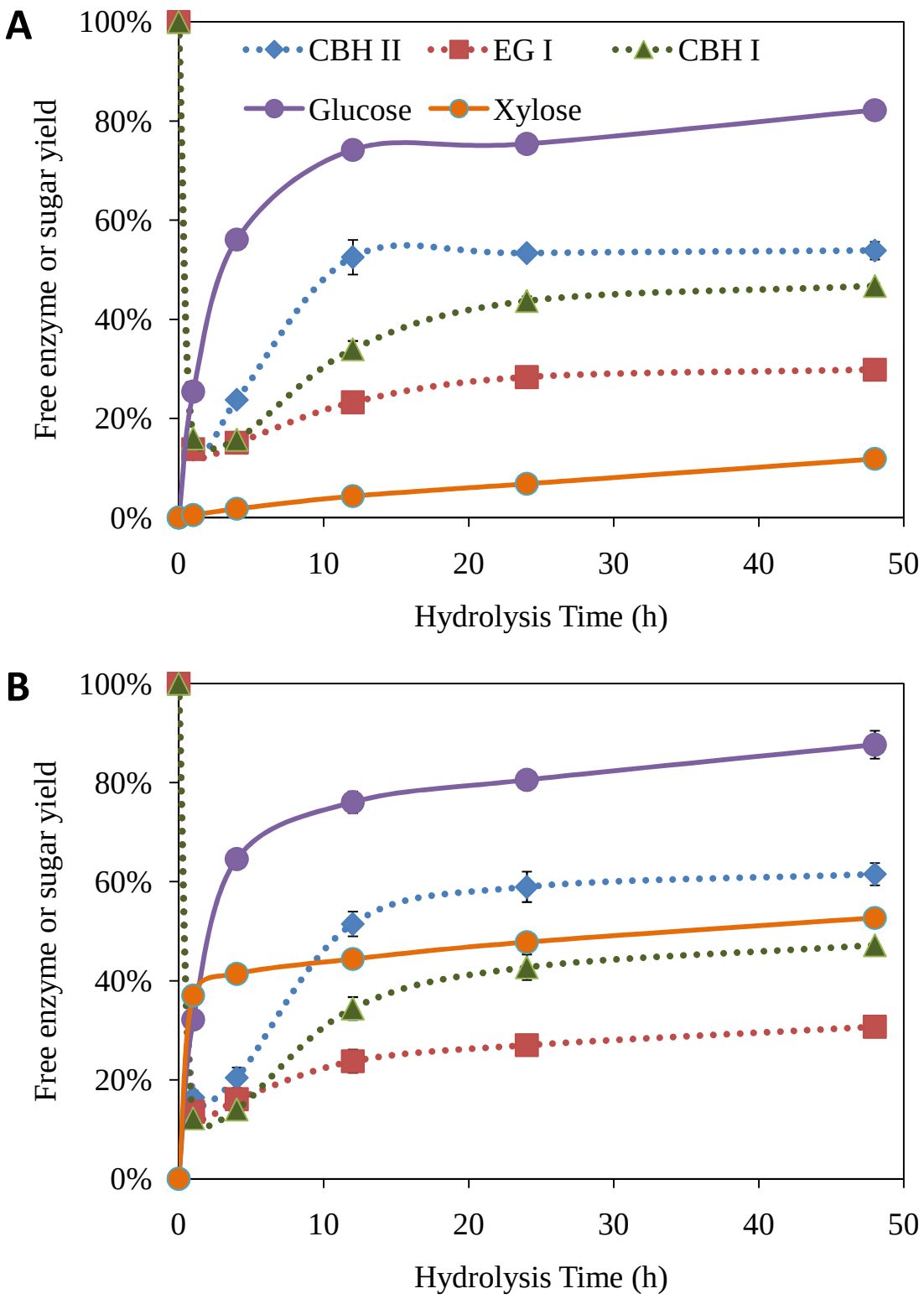


Figure 8.6 Sugar yield and the percentage of enzymes in supernatant for IL CS with (B) and without (A) endoxylanase and β-xylosidase during 48 hour hydrolysis at 50 ºC.

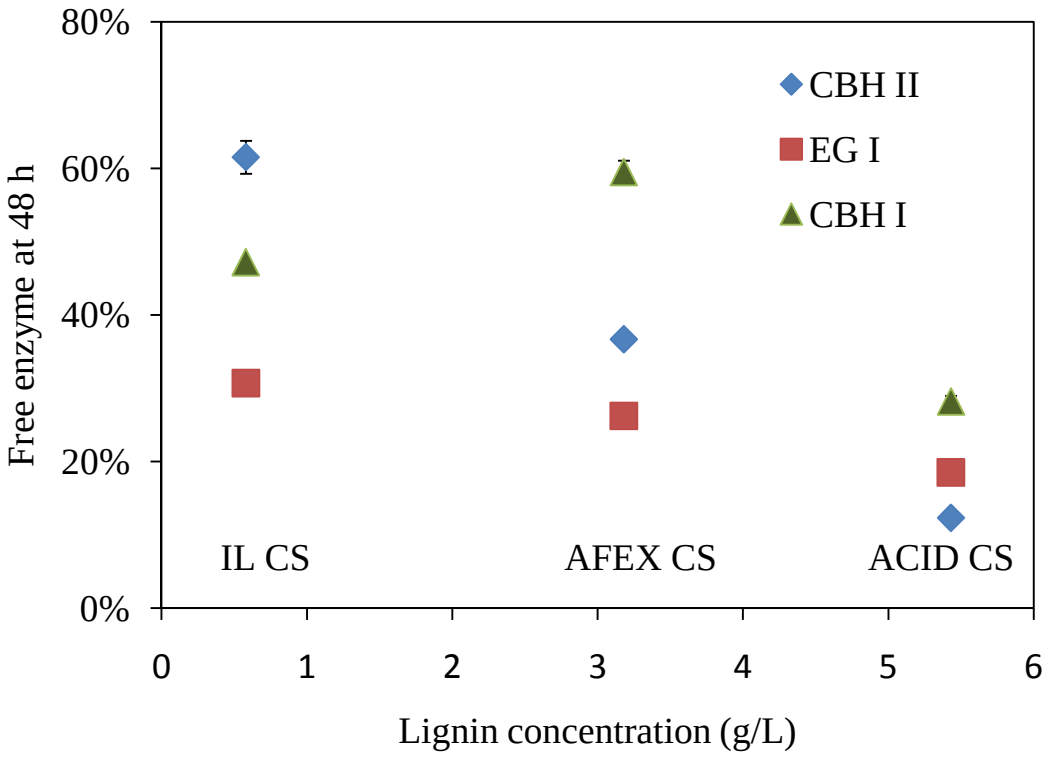


Figure 8.7 Percentage of enzyme in supernatant after 48 hours hydrolysis of AFEX CS, ACID CS and IL CS.

How inhibitory lignin is always bewilder researchers to fully understand the hydrolysis process. Many efforts have been done to understand the lignin's role. However, most of those researches are limited from two major drawbacks: (1) they lack the tools to monitor individual enzyme. Only the total protein concentration is reported [153, 228]; (2) By extract lignin from biomass, the structure of lignin might be modified significantly hence their inhibitory effect could also has been changed and this is why some results show little or no inhibitory effect of extracted lignin [115, 227]. These experiments reveal meaningful results for enzymatic hydrolysis and cellulases binding. High unproductive binding level of enzymes onto lignin is confirmed. Generally, high lignin content correlated to high level of unproductive binding. The binding of cellulases is both governed by substrate composition and crystal structure. Among cellulose allomorphs, different

binding levels are observed due to the variation of surface area, surface hydrophobicity, etc. The stability of each cellulase is different. CBH II is prior to lose activity at 50 ºC whereas CBH I and EG I still maintain most of their activities after 48 hours incubation.

All these findings and hypothesis shed a light to the future researches which are focused on understanding hydrolysis mechanism and engineering high efficient hydrolysis systems. For pretreatment studies, with this techniques applied, rational design and evaluation of a novel pretreatment are possible by focusing on minimizing unproductive binding of enzyme onto biomass. Hence the enzymes could be recovered for the following batch of hydrolysis. From the protein engineering point of view, with above tools, evaluating the mutant enzymes to make them have less unproductive binding level (for EG I) as well as increase their thermostability (for CBH II) could help to develop a superior cocktail.

8.4 Conclusion

In this research, we have performed experiments to track CBH I, CBH II and EG I during enzymatic hydrolysis. Cellulose allomorphs and pretreated corn stover are chosen as substrates for continuous 48 hours saccharification. The digestibility from highest to lowest for cellulose allomorphs is amorphous cellulose > cellulose III > cellulose II > cellulose I. The binding capacity rank from highest to lowest is amorphous cellulose > cellulose II > cellulose I > cellulose III. For pretreated biomass, AICD CS has high unproductive binding due to its high lignin content. EG I is much hard to recover compared to CBH I. Removal of lignin by IL facilitate enzymes recovery especially for CBH II, but less favorable for CBH I compared to AFEX CS.

CONCLUSION AND SUGGESTIONS FOR FUTURE RESEARCH

The fate of cellulosic ethanol production is largely decided by how large quantity of cheap fermentable sugar can be obtained. The work in this thesis focused on enzymatic hdyrolsys of pretreated biomass. The major conclusion is

1) Optimizing the enzyme cocktail for specific types of pretreated biomass allows for a significant reduction in enzyme loading without sacrificing hydrolysis yield.
2) A diverse set of accessory hemicellulases from bacterial sources was found necessary to enhance the synergistic action of cellulases hydrolysing AFEX pretreated corn stover.
3) A high-throughput Fast Protein Liquid Chromatography (HT-FPLC) based method has been developed to quantify CBH I, CBH II and EG I present in hydrolyzates of untreated, AFEX, and dilute-acid pretreated corn stover. This method can accurately quantify individual enzymes present in complex binary and ternary protein mixtures without interference from plant cell wall derived components.
4) The binding studies on pure cellulose allomorphs reveal the binding level is not alway positive correlate to substrate digestibility. CBH II is less stable during hydrolysis. Presence of lignin is responsible for significant unproductive cellulase binding.

All these results in this work shed a light to the future research which is focused on engineering high efficient hydrolysis systems and understanding lignocellulosic biomass hydrolysis mechanism. The following suggestions and ideas could be helpful for future work.

1) Screening and evaluating novel enzymes for AFEX pretreated biomass. Esterase, GH 61 enzymes and many other possible cocktail candidates are possible to work synergistically with current cocktail and hence decreasing total enzyme loading.

2) Evaluating enzyme cocktail at different pretreatment severity. Pretreatment is normally evaluated by using commercial enzymes at fixed conditions. Pretreated biomass at different pretreatment severity may require different combination of enzymes at different loading dosage. So far, few studies have been focused on this area which is also important to evaluate pretreatment and hydrolysis economic analysis as an integrated system.
3) Studing the binding properties of other enzymes. In this work, CBH I, CBH II and EG I's binding properties are studied. Other enzymes, such as xylanases, endo-glucanases are also binding on biomass and it will be interesting to study their binding behaviors. GH 61 enzymes have no detectable activities and have no effect on pure cellulose/xylan but they can decrease total enzyme loading significantly on pretreated biomass. Is that possible that GH 61 enzymes can reduce unproductive binding level of other work horse enzymes? Is any protein-protein interaction between those enzymes?
4) Understanding the effect of hydrolysis end products and degradation products. During hydrolysis, sugars (monomeric and oligomeric format) and degradation compounds formed during pretreatment are released into the liquid phase. At high solid loading hydrolysis experiment, these compounds are in much higher concentration hence could inhibit enzyme or affect their binding properties. Studing how these compounds affect cocktail, enzyme binding could help to improve hydrolysis at high solid loading.
5) Establishing a comprehensive hydrolysis model. With the tools developed in this work, purified enzymes and their binding level on substrates can be used to build a model which is integrated by individual enzyme's parameters. Rather than many other

simplified models, this comprehensive model could reveal more important information during hydrolysis and help to design a more efficient hydrolysis process.

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