How can we better predict the hydrolytic performance of commercial cellulase enzyme preparations on a range of biomass substrates?

by

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Abstract

Predicting the hydrolytic potential of a "cellulase enzyme cocktail" on lignocellulosic substrates is an ongoing challenge, particularly as enzyme companies try to reduce the required enzyme loading to achieve rapid and complete cellulose hydrolysis. The filter paper assay (FPA) is still widely used to assess the hydrolytic potential of cellulase enzyme preparations, as the method is clearly documented and Whatman No.1 paper is a universally available and consistent substrate. However, characteristics of filter paper such as its high cellulose content and dried nature, as well as the short (1 hour) duration of the assay, etc., all compromise the ability of the FPA to predict how enzyme cocktails will hydrolyze lignocellulosic substrates. To assess the influence of factors such as drying and the presence of lignin and hemicellulose on the FPA, "model/paper sheet" substrates were prepared and substituted for filter paper. When a paper sheet prepared from never-dried pulp was used in the assay, it was apparent that drying was a major, negative influence. Using sheets prepared from pretreated substrates containing lignin and hemicellulose resulted in a 53% decrease in the measured filter paper activity, illustrating the detrimental effects of lignin and hemicellulose on cellulase activity. Therefore, several realistic substrates were prepared that were rich in either xylan, mannan and/or lignin. These substrates were used to assess the beneficial effects of substituting cellulases with accessory enzymes (e.g. xylanases, mannanases). In many cases up to 50% of the cellulases in the enzyme cocktail could be substituted with accessory enzymes to achieve hydrolysis yields >70%. However, when the substituted enzyme cocktail was assessed via the filter paper assay, the resulting activity decreased by up to 58%. It was apparent that the predictability of the FPA was highly dependent on the composition/characteristics of both the substrate and the enzyme cocktail. The results indicated a potentially more useful method to predict the effectiveness of a cellulase mixture on a given

substrate may be to perform a longer-time hydrolysis of the actual biomass substrate while using the filter paper assay to provide an estimate of the initial protein/activity loading.

Lay Summary

In a proposed biorefinery, it is likely that lignocellulosic substrates will be used as feedstock. Currently, the filter paper assay (FPA) is used to predict cellulase performance when breaking down a range of substrates to their monomeric units. The FPA quantifies the amount of cellulase enzymes required to result in a 3.6% cellulose conversion in 60 minutes. However, the FPA has shortcomings such as, limitations with the Whatman No.1 filter paper used in the assay being dried and is almost pure cellulose, yet realistic biomass substrates will likely never be dried and will usually contain cellulose, lignin and hemicellulose. In addition, lignin and hemicellulose degrading enzymes have been shown to have no hydrolytic activity on cellulose. Thus, the main goal of the thesis work was to assess how representative the FPA is of the hydrolytic potential of a complex cellulase cocktail when breaking down complexed substrate.

Preface

I, Drake Mboowa affiliated to the Forest Products Biotechnology/Bioenergy Research Laboratory at the University of British Columbia, Point Grey campus, conducted the work presented in this thesis.

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List of Acronyms and Units

µL	Microliters
β-G	B-Glucosidases
0.5% acid	0.5% Acid Pretreated Organosolv Maple
^{0}C	Degree Celsius
1% acid	1% Acid Pretreated Organosolv Maple
AFEX	Ammonia Fibre Expansion
AIL	Acid Insoluble Lignin
Ara	Arabinan
ASL	Acid Insoluble Lignin
Bdl	Below Detectable Levels
BSA	Bovine Serum Albumin
C_5	Pentoses
C_6	Hexoses
CAZy	Carbohydrate-Active Enzymes Database
CBH	B-1,4-Glucan Cellobiohydrolases
CBMs	Carbohydrate-Binding Modules
CD	Catalytic Domain
CMC	Carboxymethyl Cellulose
CMCase	Carboxymethyl Cellulose Enzymes
DACS	De-Acetylated Corn Stover
DNS	3,5-Dinitrosalicylic Acid
DO	Direct Orange
DP	Degree of Polymerization
DS	Degree of Synergy
EG	Endoglucanases
Endo	Endoglucanase
Exo	Exoglucanase

FP	Filter Paper
FPA	Filter Paper Assay
FPB/B	Forest Products Biotechnology/Bioenergy
FPU	Filter Paper Units
g	Gram
Gal	Galactose
Glu	Glucan
HBKP	Hardwood Bleached Kraft Pulp
HCl	Hydrochloric Acid
HMW	High Molecular Weight
HPLC	High-Performance Liquid Chromatography
HS	High Severity
IU	International Units
IUPAC	International Union Of Pure And Applied Chemistry
L	Litres
LMW	Low Molecular Weight
LPMOs	Lytic Polysaccharide Monooxygenases
LS	Low Severity
M	Molar
Man	Mannan
mg	Milligram
Min	Minutes
ml	Millilitres
mM	Milli Molar
NA-	Not Analysed
$NaBH_4$	Sodium Borohydride
NaOH	Sodium Hydroxide
nm	Nanometres

NR	Not Replaced
ODW	Oven Dry Weight
PAPTAC	Pulp and Paper Technical Association of Canada
PASC	Phosphoric Acid Swollen Cellulose
PBS	Phosphate-Buffered Saline
PD-TMP	Partially Delignified TMP
PHK	Pre-Hydrolysed Kraft Pulp
Rpm	Revolutions per Minute
SBKP	Softwood Bleached Kraft Pulp
SO_2	Sulphur dioxide
SPCS	Steam Pretreated Corn Stover
SPS	Steam Pretreated Softwood
TAPPI	Technical Association of the Pulp and Paper Industry
TMP	Thermomechanical Pulp
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight
WRV	Water Retention Value
Xyl	Xylan
YSI	Yellow Springs Instrument

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I am grateful for the all the previous and current members of the Forest Products Biotechnology/Bioenergy Research group for their support, ideas, friendship and help with some laboratory techniques, which has made me become so experienced and knowledgeable in the bio-conversion/biorefinery research field.

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1. Introduction

1.1. Background

Many microorganisms, predominantly fungi, have been shown to degrade cellulose and other plant cell wall components (Sánchez, 2009). However, historically, it was thought enzyme mediated cellulose saccharification was primarily mediated by a cellulase enzyme mixture which involved three major types of enzymes, endoglucanases, exoglucanase/cellobiohydrolases, and β-glucosidases (Dashtban et al., 2009). Owing to the complexity of realistic lignocellulosic substrates, their recalcitrance and the desire to reduce protein loading, traditional types of cellulase preparations (Celluclast and spezyme) have more recently been supplemented with so-called accessory enzymes. The accessory enzymes, which include hemicellulases, Lytic polysaccharide monooxygenases (LPMOs), expansins etc., when added to traditional cellulase enzymes form an enzyme complex which is more efficient at deconstructing more realistic lignocellulosic substrates (Hu, 2014; Kim et al., 2014; Obeng et al., 2017). These accessory enzymes cooperate synergistically with the cellulase enzymes and have been shown to significantly improve saccharification yields when lignocellulosic substrates are hydrolyzed (Hu et al., 2014).

However, it has proven to be challenging to predict how effectively a complex cellulase enzyme preparation might hydrolyze a given substrate. Historically the hydrolytic efficiency of cellulase enzymes was assessed by the standardized filter paper assay (FPA) (Ghose, 1987). Although the FPA predominantly used filter paper as the "standard" substrate, other substrates such as solka floc, Avicel and cotton have also been used to assess the potential hydrolytic efficacy of a cellulase enzyme preparation (Ghose, 1987; Mandels et al., 1976). The standardized FPA is a volumetric based assay, which determines the dilution of the original cellulase enzyme stock required to catalyze a 3.6% cellulose conversion (2 mg glucose) of a 50

mg Whatman No. 1 filter paper in 60 minutes. The filter paper activity is then defined as the amount of enzyme, which releases one (1) μmol of glucose-equivalents per millilitre per minute from 50 mg of filter paper and is measured in terms of filter paper units (FPU's).

However, the FPA, although widely used, has several shortcomings that may limit its ability to predict the hydrolytic performance of cellulase enzyme mixtures when hydrolyzing more "realistic" cellulosic substrates such as pretreated lignocellulosic materials. For instance, even the cellulose component itself can differ in its physical characteristics such as the degree of crystallinity, particle size, pore volume, the degree of polymerization (DP), etc. Hence, the ability to predict the hydrolytic potential of an enzyme preparation with the FPA using Whatman No.1 filter paper may exhibit limitations when assaying cellulase enzyme activity on a range of more realistic cellulosic substrates. Whatman No.1 filter paper is an almost pure cellulosic substrate. However, substrates that are more realistic typically contain lignin, hemicellulose and cellulose. Furthermore, there are also an infinite number of combinations of pretreatments and conditions, which can make lignocellulosic substrates even more heterogeneous. Hence, predicting how well cellulase enzyme preparations might perform on realistic substrates has proven to be a challenge when the FPA is used.

As one example, Whatman No.1 filter paper is typically dried. However, more realistic, potential bioenergy substrates will usually have never been dried. During papermaking, the cell wall fibres are pressed between flat plates to ensure uniform thickness and then dried. Pressing aggregates the fibers, while drying shrinks and collapses the pores of cellulose fibers by removing the inter-fibril water resulting in an increased crosslinking between the adjacent fibers (Esteghlalian et al., 2001). Drying leads to "hornification" of fibers and is not completely reversible upon rewetting of the cellulose fibers (H. Chen et al., 2012). Hornification renders the

cellulosic fibers less accessible to the cellulase enzymes and reduces the rate and extent of enzymatic hydrolysis (Esteghlalian et al., 2001). This implies that assaying cellulase enzyme activity on a dried and pressed filter paper substrate might require a high enzyme loading for effective hydrolysis. Therefore, it is likely that using a dried Whatman No.1 filter paper in the FPA may not be representative of how any cellulase mixture will hydrolyze a never-dried substrate.

Another goal of the thesis work was to determine if the FPA could detect an enhancement or reduction in hydrolysis yields when cellulase enzymes were supplemented with accessory enzymes such as hemicellulases. Previous saccharification studies using realistic lignocellulosic substrates have shown that accessory enzymes can be very beneficial in enhancing hydrolysis (Hu et al., 2014, 2011; Sun et al., 2015). Accessory enzymes are thought to predominantly act on the hemicellulose and lignin components to swell/disrupt the substrate (Gourlay et al., 2013; Hu, 2014; Kim et al., 2014; Obeng et al., 2017). However, it has been shown that some accessory enzymes such as hemicellulases and ligninases don't show specific hydrolytic activity on pure cellulosic substrates such as Whatman No.1 filter paper (Hu, 2014; Pryor and Nahar, 2010; Sun et al., 2015). Therefore, the utilization of the FPA to accurately determine the total cellulase activity of a complexed cellulase enzyme cocktails which degrade lignocellulose is likely not representative.

1.2. What constitutes a complexed cellulase mixture?

The C_1-C_X hypothesis that was originally proposed by Reese et al. (1950) to facilitate cellulose hydrolysis was theorized to take place due to an initial swelling and chain separation of the cellulose by the C_x non-hydrolytic enzyme. This enhanced subsequent attack by the C_1 hydrolytic enzyme that would then act to release glucose as the main product. Subsequently, the hypothesis was modified to suggest that the C_x component carried out swelling and chain

separation while the C_1 was the hydrolytic component. Later, the C_x and C_1 components were reclassified as endoglucanases and exoglucanases (cellobiohydrolases) respectively (Reese et al., 1950; Wood, 1969, 1968; Wood and McCrae, 1972).

Initially, although the C_1-C_X model between the endoglucanase-exoglucanase cellulase enzymes was the accepted hypothesis for bioconversion of cellulose to soluble sugars (Henrissat et al., 1985; Wood, 1969), it was later observed that, as enzymatic hydrolysis proceeded, there was a significant build-up of cellobiose which inhibited C_1 activity. This led to the suggestion that β-glucosidase, which breaks down the cellobiose to glucose, should be added to the cocktail to maximize glucose production (Wood, 1969; Wood and McCrae, 1972). Consequently, it was suggested that a complete enzyme mixture was needed to efficiently hydrolyze cellulose and this mixture needed the synergistic cooperation of endoglucanase, exoglucanase and β-glucosidase enzymes to be effective

As mentioned earlier, as well as cellulose, lignocellulosic substrates contain lignin and hemicellulose. The nature, amount and distribution of these biomass components depends on the substrate type and pretreatment used. Briefly, lignin makes the plant cell wall rigid and recalcitrant to enzymatic degradation and also unproductively binds the cellulase enzymes (Mansfield et al., 1999). A predominant role of the hemicelluloses is to form a sheath around cellulose chains, thus restricting cellulase accessibility to the cellulose (Kumar and Wyman, 2013). Thus, to completely break down lignocellulosic substrates, lignin and hemicellulose degrading (accessory) enzymes are required to cooperate synergistically with the cellulase enzymes. Although the accessory enzymes do not hydrolyze the cellulose directly, earlier work has shown that accessory enzymes act in a variety of ways to improve cellulose accessibility when supplemented to “traditional” cellulase enzyme cocktails (Gao et al., 2011; Hu et al., 2011; Sun et

al., 2015; Várnai et al., 2011). Thus, the more recent "cellulase cocktails" that have been formulated to deconstruct more realistic lignocellulosic substrates contain "traditional" cellulases (exoglucanase, endoglucanase and β-glucosidase) as well as a range of accessory enzymes such as hemicellulases, lignin degrading enzymes and other swelling proteins.

1.2.1. Brief description of the traditional "cellulase" enzymes

1.2.1.1. Exoglucanase (C_1) enzymes

Exoglucanase (C_1) enzymes, also known as β-1,4-glucan cellobiohydrolases (CBH), are defined by the Carbohydrate-Active Enzymes database (CAZy) as glycosyl hydrolase EC 3.2.1.91. (Henrissat, 1991; Henrissat and Bairoch, 1993). They have been shown to catalyze the breakdown of cellulose to, glucose, cellobiose or cellotriose, from both the reducing and non-reducing ends, in a processive manner (Henrissat et al., 1988). The CBH enzymes are substrate specific and their activity decreases with a decrease in length of the oligosaccharide chain (Chen, 2014).

The CBH enzymes are often subdivided into CBH I and CBH II (currently referred to as Cel7A and Cel6A respectively). The CBH I and CBH II enzymes act from the reducing and non-reducing ends of the cellulose chain respectively during enzymatic hydrolysis (Boisset et al., 2000; Lynd et al., 2002). Earlier workers have reported CBH I and CBH II enzymes account for 50-70% and 10-20% respectively of the total protein produced by *Trichoderma reesei* fungus (Markov et al., 2005; Ramos et al., 1992). It has been shown that, to be effective, the CBH I and CBH II enzymes require endoglucanases since they tend to be very slow when reducing the DP of cellulose chains (Lynd et al., 2002).

1.2.1.2. Endoglucanase (C_x) enzymes

According to the Carbohydrate-Active Enzymes database (CAZy), endoglucanases (EG) are 1,4-β-D-glucan-4-glucan hydrolases, with a classification of glycosyl hydrolase EC 3.2.1.4 (Henrissat, 1991). Historically, the EG enzymes account for 20-30% of the total protein in a cellulase enzyme preparations (Chen, 2014). The EG enzymes primarily cut at random positions in the amorphous regions of β-1,4-glycosidic bonds of cellulose chains (Boisset et al., 2000). The EG enzymes are sometime referred to as the carboxymethyl cellulose enzymes (CMCase) since the CMC substrate is often used to measure its activity. The EG's from *Trichoderma reesei* (EGI, EGII, EGIII, EGV, and EGVI) have all been cloned (Schülein, 1997). However EGI and EGII (currently referred to as Cel7B and Cel5A respectively) have traditionally been the most predominant species used when formulating cellulase cocktails (Poeta et al., 2018).

The EGI and EGII enzymes account for 10-20% of the protein secreted from *Trichoderma reesei* on a mass basis (Markov et al., 2005). The EGI enzyme is the main endoglucanase and accounts for 6-10% of the total protein excreted by *Trichoderma reesei* (Markov et al., 2005). The EGI reduces the DP of cellulose chain, producing cellotriose, cellobiose, and glucose. Unlike EGII, EG1 is not substrate specific and has been reported to hydrolyze hemicellulose (Kleywegt et al., 1997; Nakazawa et al., 2008). The EGII accounts for 1-10% of the total protein from *Trichoderma reesei* and decreases the DP of cellulose chains more efficiently as compared to the EGI, producing cellobiose with small amounts of glucose and cellotriose (Zhiwei et al., 2012). Additionally, supplementary cellobiose is produced by the exo-endo synergism. Consequently, inhibition can only be alleviated by supplementing the β-glucosidase enzyme in the cocktail (Mosier et al., 1999).

1.2.1.3. β-glucosidases

As discussed earlier, β-glucosidases (β-G) hydrolyzes cellobiose to glucose and the removal of accumulated cellobiose during enzymatic hydrolysis is a vital step as it helps to overcome the inhibitory effects of cellobiose to CBH enzymes (minimizing end-product inhibition). The β-G enzyme has a low substrate specificity and its activity increases with a decrease in substrate concentration (Chen, 2014). Apart from its role in hydrolysing cellobiose to glucose, β-G plays an important role in trans-glycosylation, ensuring that glucose maintains its β-conformation (Chen, 2014; Watt et al., 1998). As stated earlier, when hydrolyzing realistic substrates, the major cellulases (endoglucanase, exoglucanase and β-glucosidase) are often supplemented with accessory enzymes.

1.2.2. Brief description of the major "accessory enzymes"

1.2.2.1. Hemicellulose-degrading enzymes

As stated earlier, hemicellulose forms a sheath around cellulose fibrils resulting in reduced cellulose accessibility to cellulases. Hemicellulose is a complex polymer with different branched and linear monosaccharides and non-carbohydrate subunits that bind to cellulose through hydrogen bonding. Hemicelluloses are comprised of C_6 or hexose (glucose, mannose, and galactose) and C_5 or pentose (xylose and arabinose) sugars as well as other acidic sugars (Hendriks and Zeeman, 2009). Due to the heterogeneity of hemicelluloses, its de-polymerization requires a concerted action of a variety of enzymes. Generally, hemicellulases can be divided into those enzymes which breakdown the main backbone (core hemicellulases), and those that remove branched groups attached to the main backbone (Bhattacharya et al., 2015). A detailed description of some of the microbial hemicellulose degrading enzymes (hemicellulases) which have proven to boost enzymatic hydrolysis of hemicellulose has been published by Shallom and Shoham (2003).

Hemicellulases belong to the glycoside hydrolases classification EC 3.2.1 according to the Carbohydrate-Active Enzymes (CAZy) server (http://afmb.cnrs-mrs.fr/CAZy). They include endo-β-1,4-xylanase (EC 3.2.1.8) which hydrolyzes β-1,4-xylan yielding short oligomers (β-1,4-xyloligomers). The β-1,4-xyloligomers are then solubilized by exo-β-1,4-xylosidase (EC 3.2.1.3). The α-l-Arabinofuranosidase (EC 3.2.1.55) and endo-α-1,5-arabinanase (EC 3.2.1.99) de-branch arabinose groups that are attached to the xylan backbone. The α-Glucuronidases (EC 3.2.1.139) cleave glucuronic acid side chains from xylan whereas α-galactosidases (EC 3.2.1.22) and endo-galactanases (EC 3.2.1.89) remove galactose and β-1,4-galactan side chains within galactoglucomannans respectively. Endo-β-1,4-mannanase (EC 3.2.1.78) de-polymerize β-1,4-mannan, liberating short β-1,4-manno-oligomer which are subsequently hydrolyzed by exo-β-1,4-mannosidases (EC 3.2.1.25). The acetyl groups linked to the xylan, glucomannan, and sugar acid groups can be hydrolyzed by acetyl xylan esterase (EC 3.1.1.72), acetyl mannan esterase (EC 3.1.1.6), and ferulic & *p*-cumaric acid esterases (EC 3.1.1.73).

It should be noted that cellulases, especially those from families 5 and 7, possess hemicellulolytic activities towards mannan and xylan (Vlasenko et al., 2010). The hydrolytic action of hemicellulases are similar to those of cellulase enzyme because they both work to cleave the β-1,4- glycosidic bonds within the backbone of the hemicellulose and cellulose respectively (Chang et al., 2011). Most hemicellulase enzymes possess a catalytic domain (CD) which hydrolyzes the β-1,4- glycosidic bonds and a carbohydrate-binding modules (CBMs) that enable the binding of the enzymes on the insoluble substrate (Schwarz, 2001; Shallom and Shoham, 2003).

Earlier workers that supplemented cellulase enzymes with hemicellulases showed that these additions boosted the hydrolysis yields of lignocellulosic substrates (Berlin et al., 2005a;

Gao et al., 2011; Hu et al., 2014, 2013, 2011; Sun et al., 2015; Várnai et al., 2011). For example, Gao et al. (2011) used a cellulase-hemicellulose enzyme cocktail to resulted in a 2-fold increase in the hydrolysis of ammonia fibre expansion (AFEX)-treated corn-stover. The most influential hemicellulose degrading enzymes are the xylanases and mannanases (Várnai et al., 2011). Both groups of enzymes play a vital role in the hydrolysis of hardwoods, softwoods and agricultural residues. This is why, in the work reported in section 3.2, xylanase and mannanase were used to study their possible synergism with cellulase when hydrolyzing lignocellulose and how their addition might influence the FPA.

1.2.2.2. Lignin-degrading/modifying enzymes

As stated earlier, lignin forms a barrier around the cellulose making lignocellulosic substrates more recalcitrant to enzymatic degradation. Lignin also unproductively binds cellulase enzymes. Although some pretreatments can significantly decrease the recalcitrance of lignocellulosic substrates through the partial removal of lignin, residual lignin can still play a role in inhibiting cellulase enzyme activity (Siqueira et al., 2017). Unlike cellulose and hemicellulose, lignin does not contain hydrolyzable β-1,4 bonds. Thus, lignin is predominantly degraded via oxidative mechanisms used by lignin degrading enzymes.

The most studied lignin degrading enzymes are laccases (EC 1.10.3.2), lignin peroxidases (EC 1.11.1.14) and manganese peroxidases (EC 1.11.1.13). Laccases are copper-containing polyphenol oxidase enzyme which oxidize the phenolic groups within lignin to phenoxyl radicals (Thurston, 1994). Lignin and manganese peroxidase are extracellular heme-glycoproteins which utilize peroxide to oxidize and degrade lignin (Chen, 2014). In this thesis work, lignin-degrading enzymes were not used but rather bovine serum albumin (BSA) was used to overcome unproductive binding of cellulases to lignin. BSA has a specificity for lignin and

works in a way that it blocks lignin unproductive binding sites thereby allowing better access of cellulases to the cellulose (Kumar et al., 2012; Siqueira et al., 2017). This is why in work reported in section 3.2, BSA was supplemented to cellulase when hydrolyzing the lignin rich steam pretreated softwood.

1.3. Synergism of a complexed cellulase mixture

To better quantify cellulase performance and to better anticipate cellulase activity on a range of realistic substrates, we need to determine how different cellulase mono components and accessory enzymes might cooperate when degrading cellulose. Earlier studies have shown that cellulase enzyme mixtures had a higher cooperative activity than the sum of individual cellulase mono-enzyme activities. This phenomenon has been termed synergism (Henrissat et al., 1985; Hu et al., 2011; Teeri, 1997). Synergism among cellulase enzymes and accessory enzymes has been quantified using the "degree of synergism" (DS) formulation, defined as the ratio of enzyme activity showed by a mixture of cellulase mono-components acting together to the sum of cellulase mono-component activities acting individually (Andersen et al., 2008; Hu et al., 2011). The degree of synergism is often calculated based on reducing sugar formation and rate of hydrolysis (Andersen et al., 2008; Jeoh et al., 2006).

Synergism between cellulase mono components has been extensively investigated and reported to occur in four ways (Jeoh et al., 2006, 2002; Lynd et al., 2002; Medve et al., 1998; Teeri, 1997; Woodward et al., 1988; Yang et al., 2016). They include: (i) exo-exo synergism, (ii) endo-endo synergism, (iii) exo-endo synergism and, (iv) intra-molecular synergy between CDs and CBMs. However, the quantification of synergism between cellulase mono components has predominantly used pure cellulosic substrates such as Avicel, filter paper, solkafloc and cotton and may not represent the actual synergism achieved on more realistic lignocellulosic substrates.

Synergism of cellulase cocktails when hydrolysing lignocellulosic substrates often involves the cooperation between cellulases and accessory enzymes (specifically hemicellulases, ligninases and swelling/disrupting enzymes) (Howard et al., 2003; Malherbe and Cloete, 2002; Menon and Rao, 2012).

1.3.1. Synergism among "traditional" cellulase enzymes

1.3.1.1. Synergism between CBH1 and CBH2 exoglucanase

The synergism between CBH I and CBH II, also known as exo-exo synergism was first reported as early as 1980 by Fagerstam and Patterson (1980). In this study, avicel and filter paper were hydrolyzed using CBH I and CBH II (also referred to as Cel6A and Cel7A respectively). High hydrolysis yields were achieved compared to when the CBH I and CBH II were used individually. This observed synergism has been confirmed by other studies carried out on a range of pure cellulosic substrates (Barr et al., 1996; Hoshino et al., 1997; Nidetzky et al., 1994a; Wood and McCrae, 1986). The exo-exo synergism has been described as resulting from the processive action of CBH II, which splits cellulose fibrils, providing new ends for the CBH I to deconstruct (Boisset et al., 2000; Duedu and French, 2016; Henrissat et al., 1985).

1.3.1.2. Synergism between endoglucanases and exoglucanases

Endo-Exo synergism is the major synergistic cooperation of cellulase enzymes that has been reported in the literature. As noted earlier, endo-exo synergism was first suggested by Reese et al. (1950) when they recognized that it required more than one enzyme to hydrolyze insoluble cellulose (the C_1-C_X hypothesis). Subsequently, Bhat and Hazlewood (2001) and Wood and McCrae (1972) described endo-exo synergism as a sequential unidirectional interaction between these two enzymes. This involved an initial attack on the cellulose chains by the endoglucanase to create new ends upon which the CBH would cleave off cellobiose in a processive manner. This has been a widely accepted model for endo-exo synergism because the synergistic

actions of endoglucanases and CBHs could be more easily explained (Wood and McCrae, 1972). Wood and McCrae (1978) later showed that not all endoglucanases work synergistically with CBHs to hydrolyze insoluble cellulose. As a consequence, they (Wood and McCrae, 1986) suggested that endoglucanase components are stereospecific and conduct a non-processively attack on two different configurations of non-reducing ends to generate new chain-ends for CBH.

1.3.1.3. Synergism between EG1 and EG2 endoglucanases

There is limited literature on the potential synergism between endoglucanase enzymes, especially for EG1 and EG2. Unlike the exo-exo synergism described earlier, it is challenging to visualize the action of one endoglucanase working with another endoglucanase. When Tuka et al. (1992) studied possible synergism between endoglucanases, when hydrolyzing avicel and filter paper, a DS above 1 was obtained. This indicated synergism between these two enzymes. Various studies have attributed this observation to the fact that some endoglucanases possess some type of exoglucanase activity (Boisset et al., 2000; Gilad et al., 2003; Riedel et al., 2006; Wilson, 2004). However, other studies have reported a DS of less than 1, indicating a more competitive interaction between endo-type cellulases. It has also be suggested that some endoglucanases are very substrate specific (Baker et al., 1996; Irwin et al., 1993). Collectively, these findings suggest that the detailed mechanisms of endo-endo synergism have still to be fully elucidated.

1.3.1.4. The intra-molecular synergism between catalytic domain and carbohydrate binding module

Most of the enzyme components of *Trichoderma reesei,* such as the Cel7A, Cel6A, and Cel7B enzymes, possess a modular structure consisting of a catalytic domain CD and a carbohydrate binding module (CBM) (Kleywegt et al., 1997; Teeri, 1997). According to the CAZy database server, most CBMs can be classified into 61 families (http://afmb.cnrs-mrs.fr/CAZy).

The most commonly researched CBMs belong to families I, II, and III and have been reported to boost disruption of cellulose fibers (Din et al., 1991). Families II and III originate from bacteria while family I originates from fungus. It has been suggested that the CD polishes the surface of the fiber associated with the release of reducing sugars (Din et al., 1991).

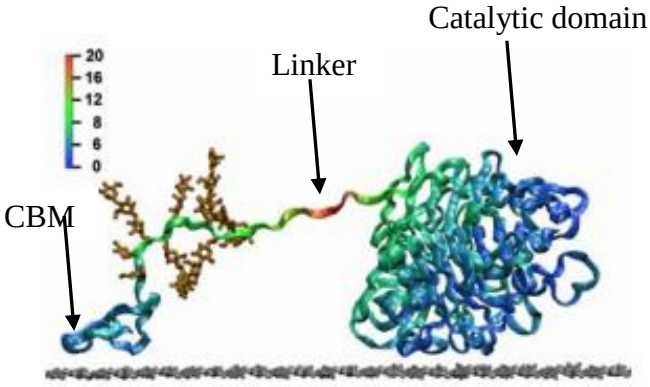

Figure 1.1: The modular structure of CBH Cel7A from Trichoderma reesei showing a CBM, linker and catalytic domain Source: (Zhong et al., 2008)

The CBM is connected to the catalytic domain (Figure 1.1) through a flexible O-glycosylated linker peptide (Zhong et al., 2008). The specific role of CBM's is still unclear, though some researchers have suggested that they initiate the binding of the cellulase enzymes to the cellulose (Várnai et al., 2013). Gilkes et al. (1992) demonstrated the key role that CBMs play in binding as the removal of CBMs from some enzymes decreases their activity by 50-80%. CBMs are reported to initiate binding through hydrophobic interactions, thereby enhancing cellulase adsorption to the insoluble substrate (Lehtiö et al., 2003; Ståhlberg et al., 1991). The O-glycosylated linker peptide connects the CBM and CD and also functions to maintain a suitable distance between the CBM and CD (Beckham et al., 2010). The linker has also been reported to contribute to the binding of the enzyme to the cellulose surface (Arola and Linder, 2016; Jalak and

Väljamäe, 2014; Nakamura et al., 2016). Finally, the CD possesses the active site which is in charge of conducting the hydrolytic cleavage of the β-0-4 bond (Divne et al., 1994).

1.3.2. Possible synergism between cellulases and major accessory enzymes

1.3.2.1. Synergism between cellulases and hemicellulases

Synergistic cooperation between canonical cellulases and hemicellulases has been shown to increase the hydrolysis yield of pretreated lignocellulosic substrates (Berlin et al., 2005a; Ji et al., 2014; Kumar and Wyman, 2009; Várnai et al., 2011). This boost in hydrolysis yields has been attributed to the fact that hemicellulase enzymes remove the hemicellulose from the cell wall, thereby increasing cellulose accessibility (Kumar and Wyman, 2009; Van Dyk and Pletschke, 2012). As described earlier, hemicellulose is quite complex, and its complete hydrolysis requires a variety of enzymes. Synergistic studies between cellulases and hemicellulases have mostly focused on enzymes which degrade the xylan and mannan backbones (Gübitz et al., 1996; Hu et al., 2011). Xylan is the main hemicellulose in hardwoods and agricultural residues, while mannan is the main hemicellulose in softwoods.

In earlier work, Hu et al. (2011) demonstrated the important role that xylanases play when added to cellulase cocktails. This work reported a 3-times increase in cellulose and xylose hydrolysis when xylanases were added to the commercial Celluclast enzyme preparation (Hu et al., 2011). In subsequent work, Hu et al. (2013) showed how xylanases and xyloglucanase improved the hydrolytic performance of commercial Celluclast on a range of pre-treated lignocellulosic substrates. In related work (Várnai et al., 2011), showed the synergistic action of both xylanase and mannanase with cellulases components from *Trichoderma reesei* on a range of lignocellulosic substrates. This work reported significantly high sugar yields during the hydrolysis of softwoods (Várnai et al., 2011). These workers concluded that, when xylanases and mannanases

were supplemented to the cellulases, this resulted in the peeling off the cellulose and hemicelluloses, leading to exposure of new substrate components which become more readily available for hydrolysis (Várnai et al., 2011).

It has been suggested that, when cellulases are supplemented with hemicellulases, the enzymatic hydrolysis of celluloses and hemicelluloses is increased (Selig et al., 2008). This provided part of the motivation for the research described within this thesis while, at the same time, assessing if any enhancement in enzymatic hydrolysis is reflected in the standardized filter paper assay (FPA).

1.3.2.2. Synergism between cellulase and ligninases/lignin modifying enzymes

Another interesting type of synergism between cellulases and accessory enzymes is that which takes place between cellulases and lignin degrading enzymes (ligninases). Although several pretreatment methods have been developed and have proven to remove lignin from biomass, complete removal of lignin is difficult or expensive to achieve (Chandra et al., 2007; Singh et al., 2017). A reduction in the lignin content of a biomass substrate is desirable, not only to increase cellulose accessibility, but also to decrease the inhibitory effects of lignin on the "cellulase cocktail" enzymes (Grabber et al., 2009). Residual lignin has been shown to inhibit the hydrolytic activity of cellulases, xylanases, β-glucosidase and other enzymes (Berlin et al., 2006a; Nakagame et al., 2011b; Siqueira et al., 2017).

Although during the thesis work there was not enough time available to assess the possible synergistic action of cellulases with ligninases, BSA treatment of pre-treated lignocellulosic substrates was used as a surrogate to determine the possible role that enzyme binding to lignin might play in inhibiting the enzymatic hydrolysis of cellulose. As noted earlier BSA has a high affinity for lignin binding sites (Kumar et al., 2012; Siqueira et al., 2017) and it

was used to block unproductive binding of the enzymes to the lignin. BSA does not disrupt the lignocellulose matrix but rather adsorbs to lignin, resulting in limited availability of unspecific binding sites on the lignin surface (Eriksson et al., 2002). Haynes and Norde (1994), showed that BSA possess hydrophobic sites and readily adsorbs to the hydrophobic surfaces of lignin. Adding BSA to substrates prior to enzymatic hydrolysis has been shown to enhance cellulase performance on lignin rich substrates (Siqueira et al., 2017). Thus, it was of interest to assess how the FPA reflects the improvement in cellulase performance on more realistic biomass substrates when BSA replaced a portion of cellulase enzymes.

1.4. Synergism Case studies

Cellulase enzymes companies like Novozymes, DSM and others have assessed synergism among various cellulase mono-components and accessory enzymes. In parallel, they have been able to improve protein expression thereby reducing on the protein loading required to achieve complete hydrolysis (Aden and Foust, 2009; Li et al., 2012). Novozymes has been one of the best case-studies that has exploited synergism to improve its cellulase enzyme preparations. Novozymes released CTec 1 and CTec 2 in 2009 and 2010 respectively with CTec 2 having more enzyme/protein bands added to it and proving to be more efficient than CTec 1. In 2012, Novozymes again launched a new enzyme called CTec 3, which was suitable for agricultural wastes and residues. The CTec 3 enzyme has been cited in many studies to be 1.5 times better than CTec 2 and requires 5 times less enzyme loading compared to enzymes from competing companies ("https://www.novozymes.com/en/news/news-archive/2012/02/advanced-biofuels-becoming-reality-with-novozymes-new-enzyme-technology," 2012).

Unlike CTec 1 and CTec 2, CTec 3 contains higher β-glucosidase activity, oxidative LPMOs (AA9), xylanase and is more thermo-stable and resistant to inhibitory effect of lignin

("https://www.novozymes.com/en/news/news-archive/2012/02/advanced-biofuels-becoming-reality-with-novozymes-new-enzyme-technology," 2012). Cellic CTec3 was designed for a wide range of pretreated agricultural feedstock and has proven effective on corn stover, corncob, corn fiber, wheat straw, sugarcane bagasse, wood pulp, and municipal solid waste (http://www.synbioproject.org/cpi/applications/cellic-ctec/). Although the newly developed enzymes (CTec series) have been helpful in lowering the protein loading required to achieve complete hydrolysis, a 3-5 fold reduction is still needed for bioethanol production to be economically possible (Hu et al., 2012; Humbird et al., 2010). To further improve the cellulase cocktail, more studies need to be conducted to better understand synergism among enzymes and how they relate to the available cellulase activity assays.

1.5. Cellulase activity assays

Measurement of cellulase activity is quite complex since it involves a reaction between an enzyme and insoluble heterogeneous cellulose. Unlike starch, which is soluble and typically hydrolyzed by one type of enzyme (amylases), enzyme activity on cellulosic substrates cannot be predicted using the classical Michealis-Menten's curve. For instance, different workers (Bezerra and Dias, 2005, 2004; Corazza et al., 2005; Sousa et al., 2011; Wald et al., 1984) have showed that the full applicability of the Michaelis-Menten model to cellulose hydrolysis requires various modifications to take into account the substrate concentration variables in addition to the substrate structural state concentration and multiple enzymes. On the other hand, Long et al. (2015) investigated the rate of starch hydrolysis by glucoamylase using the Michaelis-Menten kinetics and reported that the Michaelis-Menten model provides a satisfactory description of how amylases breakdown starch.

Cellulosic substrates are recalcitrant to enzymatic degradation and hence their enzymatic hydrolysis requires a concerted effort of many enzymes, which constitute a cellulase complex. Initially the evaluation of the hydrolytic potential of cellulase enzymes was based on individual cellulase enzyme activity assays for endoglucanases, exoglucanases, and β-glucosidases (Sharrock, 1988; Zhang et al., 2006). However, assessing the contribution of each cellulase mono components to enzymatic hydrolysis was often challenging because the differences in substrate specificity of each enzyme was relative, not absolute (Sharrock, 1988). In addition, most of the individual cellulase enzymes attack the β-l,4-glucosidic bond and their cleavage efficiency differs depending on the chemical surrounding environment of the bond (Sharrock, 1988). Such differences in the hydrolytic efficacy of individual cellulases were very difficult to quantify.

Furthermore, the synergistic cooperation of the different cellulase enzymes when breaking down cellulose complicates their individual assessment (Mandels et al., 1976). For instance, certain cellulase mono components are very important during enzymatic hydrolysis of cellulose when acting with other enzymes than in isolation. Due to the complexity of measuring individual cellulase enzyme assays, the measurement of total cellulase activity was developed (Sharrock, 1988; Zhang et al., 2006). The measure of total cellulase activity is based on the filter paper assay (FPA) which uses a filter paper substrate whose degradation requires the action of all the cellulase enzymes.

1.5.1. Brief description of Individual and total cellulase activity assays

1.5.1.1. Endoglucanase (EG) activity

As discussed earlier, the EG enzymes cleave internal β-1,4-glucosidic linkages randomly creating new active sites for the CBH to act on. EG activity is assayed using soluble cellulose substrates such as carboxymethyl cellulose (CMC) which have a high DP (Dashtban et

al., 2010; Zhang et al., 2006). The reducing ends of CMC are carboxymethylated. Carboxymethylation prohibits the cellulose chains from shortening and thus their degradation can only be done by an enzyme that can cleavage internal bonds (Zhang et al., 2006). Several methods can be used to measure the endoglucanase activity. The first assesses the decrease in viscosity of CMC and the consequent increase in the number of reducing ends measured using the reducing sugar assays (3,5-Dinitrosalicylic Acid (DNS) assay, Nelson-Somogyi assay, glucose-oxidase assay, etc).

The CMCase activity is measured using the procedure proposed by Mandels et al (1976). The procedure measures the reducing sugars released in 30 minutes when diluted enzymes are added to CMC at a pH 4.8 and 50°C. The endoglucanase activity has also been measured by staining CMC with various dyes on agar plates because these dyes can only be adsorbed by long chain polymeric sugars (Jang et al., 2003; Kim et al., 2000; Ten et al., 2004). One unit (IU) of EG activity is calculated as the amount of enzyme, which releases one μmol of glucose per minute under specified assay conditions.

1.5.1.2. Exoglucanase activity

As discussed earlier, the CBH enzymes cleave reducing and non-reducing chain ends of cellulose to liberate glucose molecules and cellobiose. Avicel is used to measure the CBH activity because of its lower DP and is quite inaccessible to the actions of the EG enzymes though notwithstanding some amorphous regions that act as substrates for both the EGs and CBH (Dashtban et al., 2010). Wood and Bhat (1988) and Sharrock (1988) reported that there is no such a specific substrate for the CBH activity assay. Thus, different assays have suggested different substrates for measuring the CBH activity, although these assays are faced with some limitations. For instance Van Tilbeurgh et al. (1985) proposed the 4-methylumbelliferyl-β-d-lactoside

substrate and reported that it was effective for assaying the CBH 1 activity of *Trichoderma reesei*, but was not an effective substrate when the CBHII activity was assayed.

Similarly, Deshpande et al. (1984) developed an assay that can selectively determine the CBH activity within a cellulase mixture. The assay is based on CBH enzyme precisely hydrolysing the agluconic bond between p-nitrophenyl and the disaccharide moiety of p-nitrophenyl-β-d-cellobioside liberating cellobiose and p-nitrophenol as products and not holosidic bond (between the two glucose units of cellobiose). The interference of β-glucosidase, which hydrolyses both agluconic and holosidic bonds, was inhibited by adding of D-glucono-1,5-δ-lactone (Deshpande et al., 1984). The interference from the EGs, which also acts on the agluconic and holosidic bonds, was overcome by standardizing the assay procedure with purified EGs from the studied cellulase mixture. This assay also has several limitations. For example, the CBHII enzyme activity could not be measured using *p*-nitrophenyl-β-D-cellobioside substrate, the specific activity of purified EGs is not representative of all the different types of EGs in the enzyme mixture and the hydrolysis products released by the CBH might have been influenced by the action of EGs.

1.5.1.3. β-glucosidases activity

As discussed earlier, β-glucosidases hydrolyses soluble cellobiose to glucose units. The β-glucosidases activity is measured using a wide array of substrates such as β-D-1,4-glucopyranoside (Deshpande et al., 1984), 4-methylumbelliferyl-β-D-glucopyranoside (Tsvetkova et al., 1996), β-naphthyl-β-D-glucopyranoside (Decker et al., 2001), and 6-bromo-2-naphthyl-β-D-glucopyranoside (Polacheck et al., 1987). Similarly, the β-glucosidases activity can be evaluated using cellobiose which cannot be hydrolysed by EGs and CBH (Zhang et al., 2006; Zhang and Lynd, 2004). The β-glucosidases activity method has been standardized using *p*-nitrophenol-β-

glucoside (pNPG) substrate and has been used in several studies (Araujo et al., 1983; Gao et al., 2012; Karnchanatat et al., 2007; Kim et al., 2007; Korotkova et al., 2009; Strahsburger et al., 2017; Yan and Lin, 1997). Briefly, (1-5 mM) of pNPG substrate is added to a buffer at optimal pH containing enzyme dilutions and incubated for around 10 minutes. After 10 minutes, the reaction is terminated by adding sodium tetraborate solution. The absorbance of the colour formation is then read at a wavelength of 405 nm. One unit (IU) of β-glucosidase activity is then determined against p-nitrophenol standard as the amount of enzyme which releases 1 μmol of p-nitrophenol per minute under assay conditions (Chandra et al., 2009).

1.5.1.4. Total cellulase activity

Several methods have been proposed to measure total cellulase activity of cellulase enzyme cocktails (Sharrock, 1988). However, the filter paper assay (FPA) has gained international acceptance as the standard measure for total cellulase activity (Eveleigh et al., 2009; Ghose, 1987; Mandels et al., 1976). The FPA was developed by Mandels et al.(1976) and was standardized by International Union of Pure and Applied Chemistry (IUPAC) in 1984 to be widely used as the measure of cellulase activity. Traditionally, the FPA uses a 50 mg (1 × 6 cm) strip of Whatman no. 1 filter paper as the standard substrate because it's a reproducible substrate, has a uniform thickness, and its degradation requires the action of a complete cellulase mixture (Eveleigh et al., 2009; Mandels et al., 1976). The FPA has been reviewed by Ghose (1987) to quantify the amount of cellulase enzymes which releases 2 mg glucose (3.6% cellulose conversion) from 50 mg Whatman no. 1 filter paper one hour (Ghose, 1987). The filter paper activity is measured in terms of filter paper units (FPU), defined as the μmol of glucose equivalent released per minute by a cellulase mixture under assay conditions of temperature and pH.

The reducing sugar yield after 60 minutes is estimated using Miller's (1959) DNS assay. The DNS assay is based on the redox reaction between glucose and 3,5-Dinitrosalicylic Acid (DNS) reagent and/or other reducing sugars. The method is based on the oxidizing property of carbonyl carbon on glucose to a carboxyl group. The DNS reagent used in the assay contains sodium-potassium tartrate used to increase the ion concentration thereby preventing the DNS reagent from dissolving oxygen. The phenol increases the colour intensity during the colour development reaction. The sodium bisulphite stabilizes the colour obtained in the presence of phenol. Finally, the sodium hydroxide is a prerequisite for the redox reaction between 3,5-Dinitrosalicylic Acid (DNS) and the reducing sugars.

1.6. Deficiencies of the FPA to quantify total cellulase activity

Although the FPA is used widely for measuring total cellulase activity, it is also well-known for its lack of good reproducibility and not being very sensitive (Dashtban et al., 2010). This is because most natural cellulase mixtures have low levels of β-glucosidase activity, yet the DNS assay is not sensitive to cellobiose which "builds-up" due to deficiencies in the β-glucosidase content of the enzyme mixture (Breuil et al., 1986; Sharrock, 1988). As such, low or "erroreneous" absorbance readings are measured by the DNS assay. In fact Coward-Kelly et al. (2003) reported that the FPA could be improved by supplementing cellulase mixtures with β-glucosidase. They realized a 56% boost in the filter paper activity when a cellulase mixture was supplemented with β-glucosidase (Coward-Kelly et al., 2003). Additonally, the preparation of the DNS reagent is quite tedious since it requires optimal mixing of the different chemicals (Dashtban et al., 2010). The DNS reagent is toxic, and requires appropriate temperature control during storage and when running the FPA to allow accurate color formation and stability which so difficult to maintain (Miller, 1959).

To try to circumvent the errors associated with the calorimetric DNS assay, the high-performance liquid chromatography (HPLC) instrument and the Yellow Springs Instrument (YSI) glucose analyser are often used to measure the reducing sugars. The HPLC is by far the most accurate, sensitive and precise method for measuring reducing sugars though consumes a lot of time and is quite expensive to run. The FPA has also been reported to be timeconsuming, labor-intensive, and requires large quantities of reagents and materials (Dashtban et al., 2010). Most of the recent research on the FPA have mainly focussed on trying to modify the FPA to be easy, fast, practical, efficient and consume less reagents (Camassola and Dillon, 2012; Decker et al., 2003; King et al., 2009; Xiao et al., 2004). Such improvement on the FPA have enabled researchers to assay a large number of samples concurrently in a short time and also downsize on the volume of reagents and substrates used (Dashtban et al., 2010). Interestingly, Xiao et al. (2004) reported a no significant difference in the filter paper activity between their modified FPA and the IUPAC FPA.

However, most of the studies referenced above have yet to study the fundamental issues with the FPA related to the substrate and cellulase enzyme cocktail, which has evolved over time. Issues such as the fact that Whatman No.1 filter paper substrate has been dried plus Whatman No.1 filter paper a pure cellulosic substrate while more relevant and realistic substrates will typically not be dried and contain hemicellulose and lignin. In addition, since filter paper is almost pure cellulose the contribution of hemicellulases and ligninases to enzymatic hydrolysis may not be assessed by the FPA. The FPA is typically conducted for 1 hour, yet complete hydrolysis of most realistic cellulosic substrates requires at least 24 hours. Thus, it is apparent that the FPA might not assess the influence of substrate and enzyme factors, which influence enzymatic hydrolysis beyond the 1 hour. Hence, the total enzyme activity derived from the FPA may not reflect the hydrolytic performance of complexed cellulase enzymes on realistic pretreated

lignocellulosic biomass. As described in the main body of the thesis, developing an accurate and reproducible assay for real lignocellulosic substrates is still desired (Wu et al., 2007).

1.7. Enzyme related factors which may not be assessed by the FPA

As noted by several researchers a gradual loss of enzyme activity is exhibited as the hydrolysis time increases. As well as substrates related factors, a decrease in cellulase activity with time has been partly attributed to enzyme factors such as enzyme thermal inactivation, enzyme synergism, end-product inhibition and irreversible adsorption of the cellulases to lignin (Mansfield et al., 1999). As stated earlier, end-production inhibition can partially be overcome by adding sufficient β-glucosidase to the cellulase mixtures. Baker et al. (Baker et al., 1997) also suggested the use a membrane- reactor saccharification assay under simultaneous hydrolysis and fermentation conditions to overcome end product inhibition. Thermal inactivation, irreversible adsorption of the cellulases and synergism are still by far the most predominant enzyme factors that influence enzymatic hydrolysis (Mansfield et al., 1999) and tend to occur as hydrolysis time is increased. It is apparent that the FPA, which is a one-hour assay, may not assess the above-mentioned factors.

1.8. Substrate related factors which may not be assessed by the FPA

It is apparent that the substrate physio-chemical properties of the different cellulosic and lignocellulosic substrate greatly affect the ability of the FPA to predict hydrolytic performance of cellulase enzymes. While the hydrolytic efficiency of cellulase cocktails on a realistic substrate cannot be correlated to a single physio-chemical characteristic, it is likely that our ability to predict the hydrolytic potential of cellulase mixtures primarily depends on the enzymes ability to access the cellulose fibers in any given substrate (Arantes and Saddler, 2011; Saddler, 1986). Many

substrate properties play a vital role in determining cellulose accessibility, and most of these properties are dependent on each other.

Physical characteristics which include DP, crystallinity, pore size and distribution, degree of swelling and available surface area, and chemical factors such as residual lignin and hemicellulose, have been shown to play a significant effect on enzymatic hydrolysis (Mansfield et al., 1999; Mooney, 1998; Zhang and Lynd, 2004). Thus, they also influence the FPA. Several workers have shown that the presence of the above factors in cellulosic and lignocellulosic substrates makes them heterogeneous and also restricts cellulose accessibility (Hall et al., 2010; Nakagame et al., 2011b; Öhgren et al., 2007). The various substrate characteristics and their effects on enzyme activity together with their mechanisms of limiting cellulose accessibility are discussed below.

1.8.1. Influence of hemicellulose and lignin on the FPA

As mentioned earlier, the FPA has mostly been based on using model substrates such as Avicel, PASC, filter paper, solka floc, bacterial microcrystalline cellulose and other cellulose derivatives (Ghose, 1987; Henrissat et al., 1988, 1985; Mandels et al., 1976; Nidetzky et al., 1994b; Sineiro et al., 1995; Wood, 1992, 1968). These types of model substrates have helped us elucidate the mechanisms of action of both individual and total cellulase enzymes in breaking down pure cellulosic substrates. However, hemicellulose removal has been reported by earlier workers to ease the enzymatic hydrolysis of realistic lignocellulose (Berlin et al., 2005a; Gübitz et al., 1997; Hu et al., 2011; Li et al., 2010; Öhgren et al., 2007).

In addition, the lignin content influences cellulose hydrolysis in two ways. 1) forming a resisting block around the cellulose and hemicellulose in the cell wall, thus constraining swelling and accessibility of the cellulose during enzymatic hydrolysis (Chu et al., 2017) and 2) lignin

adsorbs cellulase enzymes irreversibly, thus limiting the amount of cellulases availability to hydrolyze cellulose (Berlin et al., 2005a; Chandra et al., 2016, 2007; Kumar et al., 2012; Nakagame et al., 2011b). The extent to which cellulase enzyme adsorb to the lignin is dependent on the type of lignin (guaiacyl, syringyl, or *p*-hydroxyphenyl) with guaiacyl lignin having a higher adsorption capacity (Guo et al., 2014). The inability of the FPA to reflect the effects of lignin and hemicellulose on cellulase activity calls for development of a new enzyme assay that can predict how cellulase enzymes will perform on a range of pretreated lignocellulosic substrates.

1.8.2. Influence of cellulose physical characteristics on the FPA

Even when the lignin and hemicellulose effects have been eliminated from lignocellulosic substrates, cellulose accessibility on purified celluloses can still be a big challenge. Chandra and Saddler (2012) used the Simons' staining technique to assess cellulose accessibility on pretreated cellulosic substrates. Their study reported different cellulosic substrates (PASC, bleached organosolv, bleached steam pretreated softwood, long, medium, microgranular, and Avicel) adsorbed different amounts of Direct Orange (DO) which also correlated with the rate and extent of hydrolysis exhibited by the substrate (Chandra and Saddler, 2012). This indicated that, although cellulosic substrates can possess the same chemical composition they could still exhibit different levels of accessibility due to their differences in physical characteristics of DP, crystallinity, particle size, etc.

1.8.3. Influence of particle size on enzymatic hydrolysis

Particle size plays a vital role in determining the accessible surface area of cellulose, and hence has an impact on cellulose hydrolyzability (Jeoh et al., 2007). The accessible surface area greatly influences enzymatic hydrolysis since a higher ratio of accessible surface area to substrate weight implies that there are more cellulase adsorption sites per mass of the substrate

(Mansfield et al., 1999). As a result, these types of substrate will hydrolyze faster (Chandra et al., 2007). Previous studies have showed that small/fine particles hydrolyze better than the large particles because of their higher accessible surface area (Laivins and Scallan, 1996; Mansfield et al., 1999; Mooney, 1998). Even with lignocellulosic substrates, pretreatment increases the substrates accessible surface area by reducing the particle size leading to an increase in cellulolytic activity (Meehnian et al., 2016).

1.8.4. Influence of degree of crystallinity on enzymatic hydrolysis

The degree of crystallinity is yet another factor that has a tremendous effect on enzymatic hydrolysis. Most of the model substrates (PASC, avicel, sigmacell, filter paper and solka floc) used in measuring cellulase activity are manufactured from commercial bleached pulps and are a blend of amorphous and crystalline cellulose (Zhang and Lynd, 2004). This designates that these different cellulosic substrates might possess different degrees of crystallinity and hence will hydrolyze differently. Earlier studies have reported that enzymatic hydrolysis of amorphous cellulose is 3 to 30 times faster, compared to crystalline cellulose (Fan et al., 1980; Lynd et al., 2002; Zhang and Lynd, 2004).

1.8.5. Influence of degree of polymerization on enzymatic hydrolysis

The other factor that greatly affects enzymatic hydrolysis of cellulosic substrates is a degree of polymerization (DP), though, the exact effect of DP on the susceptibility of any cellulosic substrate to enzymatic hydrolysis is still not clearly understood (Pan et al., 2007; Zhang et al., 2006). Some studies have suggested that substrates with a lower DP hydrolyse better than those with a higher DP (Martínez et al., 1997; Puri, 1984). However, producing substrates with a lower DP required some severe conditions which might affect other substrates characteristics such as those that contribute to cellulose accessibility (Chandra et al., 2007). Different cellulosic substrates

have different DP and vary from <100 to >15,000 depending on how the substrate was prepared and its origin (Zhang and Lynd, 2004). For instance, the DP of natural cotton is 15,000 (Zhang and Lynd, 2004), a high MW dissolving pulp has a DP of approximately 1000 (Swatloski et al., 2002), the DP of filter paper made from cotton is approximately 1640 while that from microcrystalline substrates is approximately 220 (Kongruang et al., 2004). This implies that different celluloses may hydrolyse differently and show different activities if substituted in the FPA.

1.8.6. Influence of drying and paper-making on enzymatic hydrolysis

The opposite of "opening up" the cellulose structure to increase cellulose accessibility is to actually close the fibre structure, a process which is thought to occur during drying of pulps (Esteghlalian et al., 2001; Jeoh et al., 2007). Drying of cellulosic substrates has a detrimental effect on cellulose hydrolysis as it restricts cellulose accessibility to the cellulases (Fernandes Diniz et al., 2004; Hubbe, 2014). It is thought that drying collapses and shrinks the pores in the cell wall of cellulosic fibers by removing the inter-fibril water resulting in an increased degree of crosslinking between the fibers thus reducing cellulose accessibility (Esteghlalian et al., 2001; Minor, 1994). This process is called hornification and is typically irreversible upon rewetting of the cellulose fibers. It has also been suggested that enzymatic hydrolysis is more effective when the pore size of the substrates is large enough to accommodate cellulase enzymes (Converse et al., 1988; Tanaka et al., 1988). If the pore sizes are too small, then the large molecular cellulase enzymes will be isolated, and thus the synergistic cooperation of a complete cellulase system will be reduced (Esteghlalian et al., 2001).

It is likely that a higher enzyme loading is needed to result in the FPA's 3.6% cellulose hydrolysis expectation in 1 hour and that the needed synergism among cellulase mono-components may be minimal because the larger molecular enzymes are unable to access the

cellulose. Therefore, using dried Whatman No.1 filter paper in the FPA may result in "erroneous" low cellulase activity, which may not be representative of the enzyme action on never-dried substrates. Earlier workers have reported lower hydrolytic efficiencies of enzymes mixtures when working with dry substrates (Fernandes Diniz et al., 2004; Luo and Zhu, 2010; Welf et al., 2005).

Additionally, the process of making paper may contribute to restricting cellulose accessibility to the cellulases. With reference to the TAPPI Test Method T 205 "Forming Handsheets for Physical Tests of Pulp.", during papermaking, the cell wall fibers are pressed between flat plates to remove the remaining water and ensure a uniform thickness. This pressing process aggregates the fibers and, as a result, may lead to the lowering of cellulose accessibility. After pressing the fibers, are dried in a process that further closes the pores of cellulosic fibers. Mechanical refining has been shown to reverse the effect of drying and has a great influence on enzymatic hydrolysis (Nazhad et al., 1995). Mechanical refining increases fiber swelling and yields fine fibers, thus, increasing the specific surface area of cellulose so that it can easily be hydrolyzed by cellulases. Mechanical refining of dried fibers has been suggested as an effective way of partially restoring dried fibers to their never-dried conditions (H. Chen et al., 2012).

1.9. Pretreatments for bioconversion of lignocellulose to reducing sugars

As mentioned earlier, the recalcitrance of lignocellulosic substrates resulting from how hemicellulose and lignin encrust cellulose is a great challenge to cellulose accessibility to the cellulase enzymes. As a result, a pretreatment step prior to enzymatic hydrolysis is required to alter the physio-chemical characteristics of the complex lignocellulosic matrix in order to increase accessibility of the cellulose to cellulases (Mosier et al., 2005). Pretreatment is a vital step in a "biorefinery or biomass-to-ethanol process" as it has the ability to recover and fractionate the hemicellulose and lignin, while increasing cellulose accessibility to cellulase enzymes (Chandra

et al., 2016, 2007). Additionally, pretreatment reduces the cellulose degree of polymerization, particle size, the degree of crystallinity, remove as much lignin without degrading the hemicellulose, and increase cellulose accessible surface area.

Ideally, any pretreatment step should be cost-effective, produce a high cellulose-rich fraction, which can be easily degraded using low enzyme loadings and be effective on a range of lignocellulosic substrates. Concerning the latter, as will be explained in more detail, steam pretreatment has been shown to be effective on a wide range of substrates, providing a cellulose-rich fraction that can be easily hydrolyzed, and a good recovery of the lignin and hemicellulose fractions (Bura et al., 2003a). Over the years, a range of pretreatments methods have been developed covering mechanical, chemical and biological pretreatments (Biermann, 1996; Bura et al., 2003a, 2003b; Fengel and Wegener, 1989; Kim et al., 2012; Saritha et al., 2012; Sasmal et al., 2011; Sindhu et al., 2014). Concerning the work reported in this thesis, the pretreatments used were subdivided into the steam explosion, chemi-mechanical, and pulping based pretreatments.

1.9.1. Steam explosion pretreatment

Among the physio-chemical pretreatments, steam explosion pretreatment has received substantial attention in pretreating lignocellulosic substrates for bio-ethanol production (Taherzadeh and Karimi, 2008). Steam explosion pretreatment is the most commonly used pretreatment process on a commercial scale, for example by Dupont, Abengoa Bioenergy/Iogen, DSM-Poet, and Mascoma Inc. US (IEA Task 39, 2011). The concept of steam explosion pretreatment was introduced in 1926 by William H. Mason (Mason, 1926) and was dubbed as the Masonite process. The Masonite process was introduced to serve as an alternative to mechanical pulping, where wood chips were treated with steam heated at high temperatures (~285^0C) and pressure (7 MPa) (Boehm, 1930). However, this process did not result in significant lignin

removal. Thus, later work modified the Masonite process to incorporate chemical treatments (Kokta et al., 1993).

Currently, steam explosion pretreatment employs acids like sulphuric acid or sulfur-dioxide to enhance delignification at low severity conditions. The steam, pressure, and moisture make lignin reach its glass transition temperature, flow, and re-precipitate in form of droplets on top of fibers upon cooling. This process redistributes lignin on the fiber surface and hence exposes cellulose microfibrils (Shevchenko et al., 2001). The redistribution of lignin increases cellulose accessibility to the cellulase, and as a result increases reducing sugar yield upon hydrolysis (Kabel et al., 2007). Grous and coworkers (1986) realized 92% cellulose hydrolysis after hydrolyzing steam pretreated poplar for 48 hours as compared to the 17% cellulose hydrolysis for the untreated poplar.

Steam explosion pretreatment has several advantages such as low energy and water requirements, it's cost-effective and recovers most of the cellulose-rich and hemicellulose fractions without prior mechanical refining (Bura et al., 2003a). Steam explosion is effective on a range of biomasses especially hardwood and agricultural residues. On the other hand, steam explosion pretreatment has some shortcomings, for instance, forms inhibitory products such as acetic acid, furfural and 5-hydroxymethylfurfural which hinder effective hydrolysis and fermentation (Kabel et al., 2007). Additionally using high severity steam pretreatment conditions leads to complete hemicellulose solubilization and lignin condensation, which in addition to increasing pretreated biomass recalcitrance, limits the development other value-added products of hemicellulose and lignin (Shevchenko et al., 2001). Utilizing low severity conditions often results in high amounts of residual lignin and hemicellulose which restrict cellulose accessibility in pretreated substrates (Pryor and Nahar, 2010). Consequently, steam pretreatment conditions are

regularly a compromise between ensuring maximum lignin and hemicellulose removal, while maximizing total sugar recovery.

1.9.2. Mechanical pretreatment (Deacetylation)

Mechanical pretreatment involves the application of mechanical energy to disintegrate the cell wall fibers thereby increasing fiber porosity, swelling, external and internal fibrillation etc. (Koo et al., 2011). Typically, mechanical pretreatment retains all the cell wall constituents in a water-soluble fraction. Thus, despite the high lignin preservation, mechanical pretreatment provides a good avenue for overall carbohydrate recovery. Previous studies have evaluated mechanical pretreatment and have shown that mechanical refining enhances the accessibility of the carbohydrate to the carbohydrate degrading enzymes (Chen et al., 2013; Jones et al., 2013). However, in these studies, high enzyme loadings were used to achieve complete cellulose hydrolysis, largely due to the presence of retained lignin in the substrates (Chandra et al., 2007). The retained lignin after mechanical refining makes pretreated substrates recalcitrant, restricts fibre swelling and forms a physical barrier which restricts cellulose accessibility (Berlin et al., 2006a; Chandra et al., 2016).

To achieve efficient enzymatic saccharification of the cellulose fraction at lower enzyme loadings, the National Renewable Energy Laboratory (NREL) developed a novel chemi-mechanical approach involving de-acetylation and mechanical refining (DMR) (Chen et al., 2014; X. Chen et al., 2012). The DMR involves de-acetylating the hemicellulose and partial removal of lignin using a dilute alkaline de-acetylation step followed by a low energy disk refining. The DMR pretreatment resulted in 13% and 19% improvement in glucose and xylose yields respectively of the disc refined corn stover at high solids loading (15 and 20 wt.%) and low enzyme loading (17–

22 mg protein/ g cellulose). These results suggested that the DMR pretreatment is a promising development for use in bio-refineries (Chen et al., 2014)

1.9.3. Organosolv Pretreatment

Organosolv pretreatment encompasses treating wood chips at high temperatures and pressure with a mixture of water and organic solvents such as ethanol, acetone, or methanol, and sometimes with/without the addition of a base or acid catalyst with a goal of removing lignin (Zhao et al., 2009). During organosolv pretreatment, the lignin-carbohydrate complex bonds, alpha and beta linkages, as well as glucoside linkages are cleaved resulting in the solubilization of hemicellulose and lignin, while the cellulose remains intact (Muurinen, 2000). Organosolv pretreatment is often conducted in acid catalyzed organosolv conditions in bioconversion research (Del Rio et al., 2012, 2010).

Similar to steam explosion pretreatment, organosolv pretreatment is effective on hardwoods and agricultural residues and often requires no addition of catalyst due to high acetyl groups present in those residues. During organosolv pretreatment of hardwoods and agricultural residues, delignification is enhanced by the formation of acetic acid from acetyl groups on the hemicellulose which provides acid for removal of lignin and solubilization of the hemicellulose (Zhao et al., 2009). Interesting to note is that without the addition of acid to the pretreatment, high temperatures of greater than 200^0C must be applied (Pye and Lora, 1991). In the case of this thesis, organosolv pretreatment was used to pretreat hardwood maple prior to enzymatic hydrolysis. The use of ethanol as a pretreatment solvent dates back to 1983 when Neilson et al. (1983) reported ethanol delignification to be an effective pretreatment method for enzymatic hydrolysis. Ethanol organosolv pretreatment is widely used to increase the susceptibility of lignocellulosic substrates to enzymatic hydrolysis (Pan et al., 2007, 2006).

1.9.4. Kraft pulping

Kraft pulping based pretreatment is mainly focused on complete lignin removal to produce cellulose-rich water-insoluble substrates (Fengel and Wegener, 1989). The Kraft process is a full chemical pulping method, which uses sodium hydroxide (NaOH) and sodium sulfide (Na_2S) at a pH above 12, temperature of 155-180^{0}C and steam pressure of 800 kPa as the main cooking liqour to break down wood chips into pulp (Biermann, 1996; Fengel and Wegener, 1989; Smook, 1989). During Kraft cooking, the wood fibers are swollen and lignin gets split into hydroxyl (OH-) and hydrosulfide (SH-) ions. Most lignin is dissolved in the cooking liquor as phenolate and carboxylate ions.

Additionally, small amounts of hemicellulose are degraded and dissolved in the cooking liquor leaving the cellulose fiber intact. The resultant brown pulp is washed, screened, and then bleached by a chlorite post-treatment. Both softwood and hardwoods can be pulped by the Kraft process (U.S. Congress Office of Technology Assessment, 1989). Kraft pulps from both hardwoods and softwoods were used to study how substrate heterogeneity determines the choice of accessory enzymes to bed used to hydrolyze them and how such cocktails are predicted in the FPA.

1.10. Rationale and objective of the thesis

Due to the challenges faced by the traditional FPA, as described earlier in this introduction, the thesis work is primarily focused on elucidating how the hydrolytic potential of a complex cellulase mixture on a range of cellulosic and lignocellulosic substrates pretreated under different conditions might be best assayed. The specific objectives of the two research sections are;

1) To assess the physical and chemical characteristics of the Whatman No.1 filter paper substrate, with regard to the assay's ability to predict the hydrolytic performance of cellulase mixtures on cellulosic and lignocellulosic substrates, and
2) To assess whether the FPA assay is able to predict the hydrolytic potential of commercial cellulase mixtures. Particularly the contribution that accessory activities (specifically hemicellulases) and lignin (using Bovine Albumin Serum (BSA) to block enzyme binding to lignin) might play in influencing the efficacy of the Filter Paper Assay.

In section 3.1, the heterogeneity of the physical characteristic of cellulosic materials was investigated by evaluating whether different cellulosic substrates with a similar chemical composition to that of the Whatman No.1 filter paper would show similar hydrolysis rates and extents when they were hydrolyzed with a commercial cellulase mixture (CTec 3). We hoped to illustrate whether differences in physical characteristics of DP, crystallinity, pore size, particle size among different cellulosic substrates would influence both enzymatic hydrolysis and the FPA.

We also tried to better understand the effects of using the dried Whatman No.1 filter paper on the ability to predict how well a cellulase enzyme mixture might hydrolyse a substrate that has never been dried. As we could not access the never-dried substrate that was used to make the Whatman No.1 filter paper, we made our own never-dried pre-hydrolysed Kraft pulp. As shown within the thesis, this had a similar chemical composition to that of the Whatman No.1 filter paper. We then used the FPA, enzymatic hydrolysis, water retention value and the Simons' staining techniques to determine how drying might affect the susceptibility of a dry filter paper to enzymatic hydrolysis. By changing the never-dried PHK fibres through drying, rewetting and disintegrating, we hoped to better understand the substrate characteristics that might influence the efficacy of the FPA.

To our understanding, no study has tried to elucidate how lignin and hemicellulose affect the FPA. Consequently, we made a lignocellulose filter paper from organosolv-pretreated hardwood and used it to substitute for the Whatman No.1 filter paper in the FPA. We hypothesized that a "filter paper" made after organosolv pretreatment of a hardwood would reflect the hydrolytic efficacy of cellulase mixtures on a substrate that contains lignin and hemicellulose.

In section 3.2, the interaction between commercial cellulase mixtures (CTec 3) with accessory enzymes [hemicellulases preparations (Multifect xylanase and endo-mannanase) was assessed on pretreated lignocellulosic substrates. We had initially hypothesized that the FPA may not be an effective predictor of the hydrolytic potential of cellulase cocktails when hydrolysing realistic pretreated lignocellulosic substrates, especially when accessory activities replace a portion of the commercial cellulase preparation (CTec 3). We, therefore, identified a heterogeneous set of pretreated lignocellulosic substrates with varying amounts of cellulose, hemicellulose and lignin. These substrates included organosolv-pretreated hardwood, steam pretreated softwood, hardwood and softwood bleached Kraft pulp, de-acetylated corn stover and a chlorite treated thermomechanical pulp (TMP).

The synergistic interaction between cellulase and accessory enzymes was assessed by replacing the CTec 3 cocktail with 10%, 20%, 50% and 100% accessory enzymes on a protein basis. Synergism was evaluated by monitoring changes in the enzyme activity under FPA conditions, and hydrolysis yields on the range of lignocellulosic substrates stated earlier. We also hydrolysed the Whatman No.1 filter paper using the CTec 3 replacement strategies described above to evaluate the influence that a cellulase cocktail replaced with hemicellulases and BSA might have on the enzymatic hydrolysis of filter paper.

By evaluating the interaction between cellulases and various accessory enzymes, we hoped to assess whether the FPA is a reliable predictor of the hydrolytic efficacy of cellulase mixture on a range of substrates. We also hoped to develop a better assay that could predict if a given cellulase preparation was likely to be effective on a targeted substrate. In section 3.2, we discuss potential ways of measuring enzyme performance on a range of cellulosic and lignocellulosic substrates. However, as will be discussed in the main body of the thesis and in the conclusions section specifically, despite its many inadequacies, the filer paper assay will likely remain the main method used to compare the hydrolytic potential of "cellulase cocktails" and the method used to develop the "optimum" enzyme loading used for dosing cellulases to lignocellulosic substrates.

2. Materials and methods

2.1. Substrates used in this study

Most of the cellulosic substrates (Avicel, microgranular cellulose, sigmacell cellulose, fibrous medium cellulose, and fibrous long cellulose) used in this study were purchased from Sigma (MO, USA). The dissolving pulp was a kind gift from Fortress Paper, Canada. The Whatman No.1 filter paper was purchased from Sigma-Aldrich. Fortress Paper, Canada, also provided the maple, aspen and birch chips, which were used to make pre-hydrolyzed Kraft pulp and organosolv pulp. The softwood bleached Kraft pulp were donated from a commercial pulp mill (Weyerhaeuser Canada Ltd), while the hardwood bleached Kraft pulp was donated from Fortress Paper, Canada

2.2. Commercial enzyme preparation

The commercial enzyme cocktail was CTec 3, which was a kind gift from Novozymes.

2.2.1. Total enzyme activity measurement

The total enzyme activity was measured using the filter paper assay (FPA) following the International Union of Pure and Applied Chemistry (IUPAC) guidelines (Ghose, 1987). Briefly, 50 mg of the Whatman No.1 filter paper strip was rolled and placed into 25 mL test tubes. 1 mL of 50 mM of sodium citrate buffer, pH 4.8 was added to the test tube to saturate the filter paper. An aliquot (0.5 mL) of diluted enzymes was weighed and then added to the test tubes and incubated in a water bath at 50^0C for 1 hour. At least two dilutions were made with one dilution capable of releasing slightly more than 2 mg of glucose, while the second one having a capability of releasing slightly less than 2 mg of glucose equivalent. Controls such as, a) reagent blank, b) substrate blank, and c) enzyme blank were also incubated together with the enzyme assays to take

their effects into consideration. Glucose standards (from 1-4 mg) were also prepared in parallel to be used for calibration.

After 1 hour, the reaction was stopped by adding 3 mL of 3, 5-Dinitrosalicylic Acid (DNS) to all the test tubes and mixing gently. The tubes were then boiled for 5 minutes at 105^0C in an oil bath, containing adequate oil to cover the portion of the test tube occupied by the reaction mixture for colour development. All the enzyme assays, blanks, and glucose standards were boiled together. After boiling, the tubes were transferred to an ice-cold water bath, left to cool and to allow the pulp to settle for 30 minutes. Samples of 0.2 mL were drawn from all of the tubes (enzyme assays, blanks and glucose standards), and were diluted in 2.5 mL of water in spectrophotometer cuvettes. Finally, the colour formation was determined by reading the absorbance of the samples at 540 nm. The glucose concentration of the enzymes assays was calibrated against a glucose standard. The two data points from enzyme dilutions that were closest to 2 mg of glucose on the standard curve were considered to interpolate the actual enzyme dilution that released 2 mg glucose. This enzyme dilution was then used to calculate the filter paper activity using equation 2.1.

$$\text{Filter Paper Activity} = \frac{0.37}{\text{Original undiluted Enzyme releasing 2 mg glucose}} \text{ FPU/g enzyme} \qquad (2.1)$$

The filter paper activity was expressed in terms of filter paper units per gram of enzyme preparation (FPU/g enzyme preparation), where one FPU is equal to the amount of original undiluted enzyme which released 2 mg glucose.

2.2.2. Determination of Protein Concentration

The protein concentration of CTec 3 was determined using the modified ninhydrin assay as modified by Mok et al. (2015). Briefly, 100 µl of CTec 3 enzyme samples diluted to concentration ranging between 0 – 800 µg/g protein were first incubated with 50 µl of $NaBH_4$ for

1 hour at a ratio of 1:3 $NaBH_4$/total sugar (w/w) in a screw cap microcentrifuge tube (0.5 ml) using BSA as the protein standard. The centrifuge bottles were then opened and 300 µl of 9M HCl was added to the reaction mixture. The tubes were sealed and incubated in a heating block at 130^0C for 2 hours. After 2 hours, the samples were left to cool down and 100 µl of the hydrolysate was transferred to 1.5 ml Eppendorf tubes. The reaction was then neutralized with 100µl of 5M NaOH solution. After neutralization 200 µl of 2% ninhydrin reagent was added to the Eppendorf tubes and the reaction mixed thoroughly. The tubes were sealed and incubated in a heating block at 100^0C for 10 minutes. The Eppendorf tubes were left to cool down, thereafter 500 µl of 50% (v/v) ethanol was added. Finally, 200µl of the coloured solution was vortexed and transferred to 96-well microplates and their absorbance read at 560 nm. All of the samples were performed and analyzed in triplicate and the mean values and standard deviations reported.

2.3. Preparation of Pretreated substrates

2.3.1. Pre-hydrolysis and Kraft pulping pretreatment

The pre-hydrolyzed, never dried Kraft pulp was a kind gift which was previously prepared by Xiaoli Dou (2017). The pre-hydrolyzed Kraft pulp was prepared from a mixture of never-dried maple, aspen and birch chips (mass ratio of maple: aspen: birch was 7:2:1). Briefly, the never-dried wood chips were first left to sit in water under vacuum overnight to remove air prior to pre-hydrolyzing them. Two hundred (200) grams of the wood chips, water, and 0.4% w/w sulfuric acid were then added to the custom-built four vessels (2L each) rotating digester (Aurora products Ltd. Savona, BC, Canada) at a wood: liquid ratio of 1:4. The digester was pre-warmed to 80°C for 27 minutes, followed by heating to the maximum temperature of 170°C for one (1) hour. It was then kept at 170°C for another one and a half (1.5) hours before cooling in cold water until the pressure dropped to zero. The digester was then opened and the cooking liquor filtered from the pre-hydrolyzed wood chips.

This was followed by Kraft cooking of the pre-hydrolyzed wood chips within the same digester. The pre-hydrolyzed wood chips were again left to sit in water under vacuum overnight to remove air prior the Kraft cooking process. An equivalent of 200 g oven-dried weight (ODW) of pre-hydrolyzed wood chips, water and NaOH was added to the vessel at a wood: liquid ratio of 1:4. The NaOH loading to the chips was 16%, 20% and 24% w/w. The digester was pre-warmed to 110℃ for 37 minutes, followed by heating to a maximum temperature of 170℃ for 25 minutes. It was then kept at 170℃ until the final H factor reached 1000. The digester was then cooled in cold water until the pressure dropped to zero. The digester was then opened, and the cooking liquor filtered from the pre-hydrolyzed pulp.

2.3.2. Organosolv pretreatment

The organosolv pulp was made from maple chips, using a custom, four-vessel (2 L capacity each) rotating digester (Aurora Products, Savona, BC, Canada). Briefly, 200 g (ODW) of never-dried maple chips were pre-incubated overnight in a solution containing water and 65% v/v of ethanol at a liquid: wood chips ratio of 7:1. Sulfuric acid was then added under the two conditions of, low severity (LS) and, high severity (HS) with 0.5 and 1.0 (H_2SO_4 %w/w) respectively. The samples were pretreated at 170°C for one (1) hour at a heating rate of 3°C/min.

After pretreatment, the digester was cooled to room temperature in a water bath. After cooling the digester was opened and pretreated maple chips together with the spent liquor were homogenized in a standard British disintegrator for 15 minutes. They were later separated from one another using vacuum filtration. The resultant pulp was washed three times using 1 L of water in a Buchner funnel. The rejects (un-pulped wood chips) from both severity pretreatments were hand-picked from the top of homogenized settled pulp. The organosolv pretreated pulps were then stored in sealed plastic bags at 4°C for determination of the chemical composition, paper and

enzymatic hydrolysis. The low severity pulp was used to make 0.5% acid organosolv paper, while the high severity pulp was used to make 1% acid organosolv paper.

2.3.3. Deacetylation and screw refining pretreatment

The de-esterification of acetylated xylan present in corn stover was catalyzed using NaOH following the procedure, with some modifications, as described by Chen et al. (2012). Briefly, 25 g (ODW) of never-dried corn stover was suspended in 313 ml of a solution consisting of 4.8% (w/v) 0.1 M NaOH in thermoplastic bags. The mixture was sufficiently mixed to ensure that NaOH impregnates the corn stover fibers. The thermoplastic bags containing the solution was incubated at 80^0C in a water bath for 3 hours. The pH was approximately 12. After deacetylation, the NaOH was extracted from the corn stover by filtration in a Buchner funnel and then washed thoroughly. The deacetylated corn stover was then refined in an Angel Juicer AG- 5500, followed by collection in sealable bags and stored at 4^0C for chemical composition determination and enzymatic hydrolysis.

2.3.4. Chlorite pretreatment

The chlorite pretreatment for delignification of the thermo-mechanical pulp (TMP) and the pre-hydrolyzed Kraft pulp was carried out using the procedure as described by Pulp and Paper Technical Association of Canada's (PAPTAC) Useful methods G10.U. Briefly, 25 g (ODW) of never-dried TMP was suspended in 250 ml of a solution comprising of 5% (w/v) $NaClO_2$ dissolved in 1% (v/v) glacial acetic acid. The reaction mixture was incubated overnight in darkness at room temperature. The chlorite treated TMP was washed thoroughly with water by filtering using a Buchner funnel. The resulting chlorite treated TMP and pre-hydrolyzed pulp was then collected into sealable bags and kept at 4^0C for chemical composition determination and enzymatic hydrolysis.

2.3.5. Steam pretreatment

Steam pretreatment was carried out in a 2-liter StakeTech III steam gun (Stake Technologies, Norvall, ON, Canada) at the FPB/B laboratory as previously described (Hu, 2014). Briefly, 300 g (ODW) of softwood (lodgepole pine) chips were impregnated overnight with SO_2 in sealed plastic bags. The bags were then opened for 1 hour in a fume hood to release the unabsorbed SO_2. A batch of 50 g for each substrate was loaded in the steam gun and pretreated using the conditions of: temperature; 190^0C, time; 5 minutes, and SO_2%w/w; 3%, that were previously optimized by Bura et al. (2003b) to recover maximum sugars (cellulose and hemicellulose).

2.4. Chemical compositional analysis of pretreated substrates

The pretreated substrates were analyzed for their Klason lignin content using the Technical Association of the Pulp and Paper Industry's (TAPPI) standard T-22 om-88 method. The hydrolysate was retained for determination of the reducing sugar composition and acid-soluble lignin. The acid soluble lignin was determined by reading the absorbance at 205 nm on a Cary 50 UV-Vis spectrometer according to the previously described method (Dence, 1992). The reducing sugar composition was determined using a DX-3000 high-performance anion exchange chromatography system (Dionex, Sunnyvale, CA), equipped with an anion exchange column (Dionex CarboPac PA1) and an ED40 electrochemical detector, with fucose as an internal standard. The reducing sugars (arabinose, galactose, glucose, xylose, and mannose) derived from the substrates were calibrated and determined against monomeric standards.

2.5. Papermaking, air-drying and disintegration process

Paper handsheets were made from either never-dried pre-hydrolyzed Kraft pulp (PHK) and organosolv pulp. The hand sheets had a target weight of 1.5 g (ODW), similar to that of the Whatman No.1 filter paper (diameter of 150 mm, Cat No. 1001-150), according to TAPPI

Test Method T 205 “Forming Handsheets for Physical Tests of Pulp.” Briefly, 7.5 g (ODW) of pulp was suspended in distilled water at 0.375% consistency [weight fiber/ (weight fiber + weight water)] for 15minutes. The solution was then disintegrated in a standard British disintegrator at 3000 rpm for 5 minutes at room temperature until all the fibers were dispersed. This was followed by stirring the pulp stock thoroughly and measuring out 400 mL of the pulp stock for each handsheet to be made. For each sheet, the measured pulp stock was poured into the handsheet making machine. Water was added to a depth of 350 mm above the surface of the paper forming wire. A perforated stirrer was used to stir the suspension. The water was subsequently drained from the handsheet making machine at a rapid speed under suction from the water leg. The sheet was then removed from the machine, using couch plates, and pressed in the paper-pressing machine. After wet pressing twice, all of the handsheets were dried at a temperature (30^0C) and relative humidity (50%) controlled room for three days. The dried paper sheets were then stored at room temperature for enzymatic hydrolysis and enzyme activity measurements.

Air-dried PHK fibers were also prepared and used to study the effect of drying on enzyme activity and fiber hornification. Briefly, 10 g (ODW) of never-dried fibers were spread on an aluminum plate and left to dry at a room temperature of 25^0C and a relative humidity of 50% for seven days. After drying, the moisture content was 3.2%. The air-dried fibers were then stored in plastic bags for subsequent enzyme hydrolysis, enzyme activity, Simons’ staining, and water retention value measurements.

Some dried PHK handsheets were rewetted at 1% consistency [weight fiber/ (weight fiber + weight water)] using de-ionized water, then disintegrated for 15 minutes in a Hamilton Beach DrinkMaster Two-Speed Stainless Steel Drink Mixer. After disintegration, the moisture

content of the PHK disintegrated paper was measured. The paper was stored at 4^0C for subsequent enzyme hydrolysis, enzyme activity, Simons' staining and water retention value measurements.

2.6. Enzymatic hydrolysis

The enzymatic hydrolysis of the pure cellulosic and pretreated substrates used in sections 3.1 and 3.2 was carried out in duplicate at 5% (w/v) solids loading in sodium acetate buffer (50 mM, pH 4.8), at 50 °C, with a shaking of 150 rpm in a MaxQ 4000 Incubator. The reaction was conducted in 50 mL capacity septa stoppered bottles (aluminum crimp-top) in 10 mL reaction volumes. Cellulase enzymes (Cellic CTec 3, Novozymes, Bagsvaerd, Denmark) were added based on the protein loading per g cellulose present in the substrate using dosages of 10 mg protein/g cellulose and 20 mg protein/g cellulose for section 3.1 and 15 mg protein/g cellulose and 30 mg protein/g cellulose for section 3.2.

The enzymatic hydrolysis was run for 48 h in section 3.1 and 72 hours in section 3.2 and 100 µl samples were periodically taken at 2, 4, 8, 12, 24 and 48 h for reducing sugar analysis. The samples were heated at 100°C for 10 min in a heating block to inactivate the enzymes followed by centrifugation at 13000 rpm for 10 minutes and stored at 4^0C for reducing sugar yield analysis. For the synergistic experiments described in section 3.2, the protein loading was fixed at 30 mg protein/g cellulose and replaced by either 10%, 20%, 50% or 100% of xylanase, mannanase or BSA used cellulase-accessory enzyme replacement strategies illustrated in Table 2.1. Initial experiment involved using 50% of the above fixed protein loading (15 mg protein/g cellulose) to show the role that accessory enzymes play in enhancing enzymatic hydrolysis of pretreated lignocellulosic substrates. For each replacement experiment, samples were taken at 4, 24 and 72 hours for reducing sugar analysis.

Table 2.1: Enzyme characteristic, experimental design and cellulase replacement strategies with hemicellulases and BSA.

Enzyme characteristics	CTec 3	Xylanase	Mannanase	BSA
Protein concentration (mg/mL)	180.3	37.1	20.3	20.0
Filter paper activity (FPU/g)	139.6	NA	NA	NA
$C_{0\%}$ (mg/g cellulose)	30	0	0	0
$CX_{10\%}$ (mg/g cellulose)	27	3	NR	NR
$CX_{20\%}$ (mg/g cellulose)	24	6	NR	NR
$CX_{50\%}$ (mg/g cellulose)	15	15	NR	NR
$CX_{100\%}$ (mg/g cellulose)	NR	30	NR	NR
$CM_{10\%}$ (mg/g cellulose)	27	NR	3	NR
$CM_{20\%}$ (mg/g cellulose)	24	NR	6	NR
$CM_{50\%}$ (mg/g cellulose)	15	NR	15	NR
$CM_{100\%}$ (mg/g cellulose)	NR	NR	30	NR
$CB_{10\%}$ (mg/g cellulose)	27	NR	NR	3
$CB_{20\%}$ (mg/g cellulose)	24	NR	NR	6
$CB_{50\%}$ (mg/g cellulose)	15	NR	NR	15
$CB_{100\%}$ (mg/g cellulose)	NR	NR	NR	30
$CXM_{10\%}$ (mg/g cellulose)	27	1.5	1.5	NR
$CXM_{20\%}$ (mg/g cellulose)	24	3.0	3.0	NR
$CXM_{50\%}$ (mg/g cellulose)	15	7.5	7.5	NR
$CXM_{100\%}$ (mg/g cellulose)	NR	15	15	NR

FPU- filter paper units; NR- not replaced; BSA- bovine serum albumin; NA- not analyzed; C- cellulase; X; xylanase, and M- mannanase

2.7. Determination of cellulose and hemicellulose hydrolysis

Cellulose hydrolysis was determined as glucose released during enzymatic hydrolysis using the YSI sugar analyzer (Yellow Springs Instruments Co., YSI 2700 SELECT

Biochemistry Analyzer) for section 3.1, while in section 3.2, the high performance liquid chromatography (HPLC) was used to determine the extent of cellulose and hemicellulose hydrolysis. The cellulose and hemicellulose hydrolysis yield (%) was calculated from the measured glucose and mannose and xylose concentration as a percentage of the theoretical carbohydrate available in the substrate using equation 2.2 and 2.3 respectively.

$$\text{Cellulose and mannan hydrolysis (\%)} = \frac{\text{Glucose released } \left(\frac{\text{g}}{\text{L}}\right) \times 0.9}{\text{Solids loading} \times \text{cellulose\%}} \times 100\% \quad (2.2)$$

$$\text{Xylan hydrolysis (\%)} = \frac{\text{Glucose released } \left(\frac{\text{g}}{\text{L}}\right) \times 0.88}{\text{Solids loading} \times \text{xylan\%}} \times 100\% \quad (2.3)$$

Where the glucose released is the maximum concentration after enzymatic hydrolysis; cellulose% is the theoretical amount of cellulose present in the substrate; 0.9 is the conversion factor of cellulose and mannan to glucose, 0.88 is the conversion factor of xylan to glucose; the solids loading used in this study was 5%w/v. The cellulose hydrolysis experiments were conducted in duplicate and their mean values and standard deviations reported. Substrate and enzyme blanks were run in parallel to consider their effects.

2.8. Fiber characterization methods

2.8.1. Simons staining

To determine likely enzyme accessibility to cellulose, Direct Orange (DO) staining was performed according to the modified Simons' Staining Technique (Chandra and Saddler, 2012). DO dye (DO, Pontamine Fast Orange 6RN) was purchased from Pylam Products Co. Inc. (Garden City, NY). The DO dye was then fractionated to isolate the high molecular weight (HMW) molecules that have a high affinity for cellulose from low molecular weight molecules (LMW) following the procedure reported previously (Esteghlalian et al., 2001).

For each substrate, 10 mg (ODW) was weighed into a series of six 1.5 ml centrifuge tubes. To each tube, 0.1 ml of phosphate-buffered saline (PBS) solution (pH 6) was added and left to sit overnight at room temperature. The DO dye (10 mg/ml HMW) was added to each tube in increasing series of (0.025, 0.05, 0.075, 0.1, 0.15, and 0.2 ml). Distilled water was then added to make the final volume of the solution up to 1ml. The tubes were then incubated at 70^0C for 12 hours in a rotary shaker at 200 rpm.

After incubation, the tubes were removed from the rotary shaker and left to cool at room temperature. One millilitre (ml) of sample was drawn from each tube and centrifuged at 13,000 rpm for 5 minutes. The respective absorbance supernatant from each tube was read at 450 nm on a Cary 50 UV-Vis spectrophotometer. The amount of dye adsorbed onto the fibers was calculated using the Langmuir isotherm by subtracting the concentration of the initial added DO dye from the concentration of the DO dye in the supernatant according to the Beer-Lambert law. The extinction coefficients of the DO dye were determined by preparing standard curves for the dye and measuring its slope at an absorbance of 450 nm. The value calculated and used in this thesis was $\varepsilon O_{450} = 34.35$.

2.8.2. Water retention value

The extent of fiber hornification for each substrate was determined using the water retention value (WRV) technique, according to Technical Association of the Pulp and Paper Industry's (TAPPI) useful method UM 256. Briefly, 0.4-1.5 g (ODW) of each substrate was suspended in 5 ml of deionized water and vortexed vigorously to break the pulp apart. The pulp suspension was left to sit overnight at room temperature, to ensure uniform fiber saturation. The pulp suspension was then filtered through a 200-mesh screen in a centrifuge cup. This was followed by recirculation of the filtrates three times, to prevent fiber loss. The resultant pulps were

then centrifuged for 900 G at 21^0C. The wet samples from each substrate were weighed and then oven dried at 105^0C overnight. The oven-dry weight was also noted and WRV was calculated using Equation 2.4.

$$\text{WRV}\ (\%) = \frac{M_w - M_d}{M_d} \times 100\% \qquad (2.4)$$

Where M_w is the mass of the wet substrate and M_d is the mass of the wet substrate.

3. Results and Discussions

3.1. Does substrate heterogeneity affect the standardized enzyme activity assay ability to predict hydrolysis of a range of substrates?

3.1.1. Background

The ability to predict the hydrolytic efficiency to effectively "dose" cellulase enzyme mixtures on pretreated substrates plays a pivotal role in the commercialization of biochemical processes to convert biomass to fuels and chemicals. Currently, the standardized measure of the hydrolytic efficiency of cellulase mixtures is based on the filter paper assay (FPA) which quantifies the amount of cellulase enzymes required to cause a 3.6% cellulose conversion (2 mg glucose) of a 50 mg Whatman No. 1 filter paper in 60 minutes (Ghose, 1987). Several studies have modified the FPA to be carried out using microplates so that we can assess multiple enzyme cocktails at a high throughput (Berlin et al., 2006b; Camassola and Dillon, 2012; Chundawat et al., 2008; Decker et al., 2003; King et al., 2009; Xiao et al., 2004). Baker et al. (1997) proposed a new membrane reactor saccharification assay for measuring enzyme activity under simultaneous saccharification and fermentation (SSF) conditions, while Urbánszki et al. (2000) standardized the traditional FPA to predict the hydrolytic potential of cellulase enzymes under the conditions used for simultaneous saccharification and fermentation. However, these studies have yet to investigate fundamental issues with the FPA that are related to the substrate. The main issue with the FPA is that, although the assay is effective at indicating the hydrolytic potential of a given cellulase enzyme cocktail when applied to filter paper, the assay is not effective when estimating hydrolytic potential of enzyme cocktails when applied to complex, pretreated lignocellulosic substrates.

In this section of the thesis the factors that were investigated included the effects of drying the cellulose that predominates in the Whatman No.1 filter paper substrate during its manufacture, the high cellulose content of the filter paper substrate compared to lignocellulosic

substrates and the differences in the ease of hydrolysis of Whatman No.1 filter paper compared to other cellulosic substrates.

When other wood components such as lignin and hemicellulose are associated with cellulose they form a recalcitrant matrix. Thus, the assessment of the hydrolytic performance of cellulase enzymes on Whatman No.1 filter paper is not indicative of the hydrolytic performance of cellulase enzymes when hydrolyzing lignocellulosic substrates. In addition, the considerable heterogeneity of lignocellulosic substrates caused by different pretreatment methods and condition make realistic substrates a continuous moving target. Hence it is very difficult to standardize a cellulase activity assay.

The process of papermaking which involves the forming, pressing and drying of cellulosic fibres alter their physical characteristics including the substrate pore size and volume which decreases cellulose accessibility (Hubbe, 2014). Pressing of cellulosic fibres removes inter-fibre water and causes fibre aggregation, a process, which restricts cellulose accessibility. Subsequent drying also leads to fibre hornification rendering dried substrates less susceptible to enzymatic hydrolysis (H. Chen et al., 2012). Earlier work has shown that drying-induced fiber hornification lowers enzymatic hydrolysis yields of cellulosic biomass (Fernandes Diniz et al., 2004; Luo and Zhu, 2010; Luo et al., 2011; Welf et al., 2005). Therefore, since the pulp utilized to produce filter paper undergoes both pressing and drying during the manufacturing process, it is likely that the hydrolysis of the collapsed and hornified fibres of the Whatman No.1 filter paper during the FPA may not be representative of the enzyme performance when a never dried substrate is subjected to enzymatic hydrolysis. As well as changes in the fibres undergone during the papermaking process, other characteristics such as cellulose crystallinity, pore volume and degree of polymerization that have been shown to influence the ease of hydrolysis of cellulosic substrates

are expected to differ among various types of purified celluloses such as Avicel, sigma cell, cotton etc., (Fan et al., 1981; Zhang et al., 2006).

Therefore, these differences will also limit the ability of the FPA to predict the hydrolytic potential of a cellulase cocktail when the cocktail is subsequently applied to other types of purified celluloses. These issues with the FPA are likely further compounded when trying to predict the hydrolytic potential of cellulases on the many types of biomass feedstocks and pretreatments that are currently being investigated. Finally, the FPA is conducted within a timeframe of 60 minutes, yet complete hydrolysis of most lignocellulosic substrates typically requires at least 24 hours or longer. Thus, the FPA might not reveal the changes in cellulase activity during a prolonged enzymatic hydrolysis.

Based on these considerations the major goal of the thesis research section was to illustrate the potential deficiencies in the ability of the FPA by assessing the effects of the differences between the filter paper and "real" lignocellulosic substrates that are likely to be "never-dried" and contain cellulose that is accompanied by lignin and hemicellulose. Therefore, the initial experiments involved replacing the Whatman No.1 filter paper with never-dried cellulosic substrates that had a similar chemical composition to the filter paper, to assess the effects of both papermaking and drying on the FPA. Subsequently, the Whatman No.1 filter paper was replaced with paper made from organosolv pretreatment to evaluate how the FPA reflects cellulase activities on a substrate that contains cellulose, lignin, and hemicellulose.

3.1.2. Effects of papermaking on the hydrolytic efficiency of cellulase mixtures

It was hypothesized that the cellulase activity measured on dry Whatman No.1 filter paper during the FPA was not a true representation of the enzyme activity on a substrate that has not undergone the drying and papermaking process. During the papermaking process, the cell walls

in the pulp fibres collapse and form extensive hydrogen bonds during the forming, pressing and drying steps. Drying often causes an irreversible hardening of the cellulose-hemicellulose-lignin matrix referred to as “hornification”. Hornification has been shown to limit cellulose accessibility to the cellulases thereby compromising hydrolysis yields (Esteghlalian et al., 2001; Fernandes Diniz et al., 2004; Ioelovich and Morag, 2011). Therefore, we hypothesized that the pressing and drying steps during the paper making process used to produce filter paper would have a detrimental effect on the ability of the FPA to predict the hydrolytic potential of real cellulosic substrates that typically do not undergo pressing and drying.

To delineate the effects of papermaking and drying, the FPA using the regular Whatman no.1 filter paper was initially tested and compared to the FPA performed with filter paper that was disintegrated prior to being used in the assay. We also postulated that suspending the Whatman No.1 filter paper in the sodium citrate buffer for 24 hours prior to adding the enzymes would help to “open up” or swell the hornified fibers so that the cellulase enzymes could access the cellulose. Therefore, four sets of cellulase activity measurements were set up involving substitituion of Whatman No. 1 filter paper with a pre-wetted (for 24 hours) Whatman filter paper, disintegrated Whatman filter paper and disintegrated Whatman filter paper with pre-wetting (for 24 hours) (Figure 3.1).

As anticipated, disintegrating the Whatman No.1 filter paper increased the filter paper units (FPU) of CTec 3 by 48% FPU/ g enzyme preparation (Figure 3.1). It is apparent that disintegrating the Whatman No.1 filter paper “opened up” the cellulose fibers and increased the surface area available for the cellulases to hydrolyze. Similarly, substrate disintegration reduced the protein loading which released 2.0 mg reducing sugar in 60 minutes by 27% compared with 2.13 mg protein of CTec 3 enzyme mixture for the dry Whatman No.1 filter paper used in the

standardized FPA. However, suspending the Whatman No.1 filter paper in sodium citrate buffer for 24 hours prior to adding the cellulase enzymes (pre-wetted filter paper and pre-wetted disintegrated filter paper) did not influence the filter paper activity measured when compared to the samples that had not undergone the pre-wetting process.

These results indicated that pressing and drying processes during papermaking compromise the FPA of the cellulase mixtures. Previous work on drying and papermaking revealed that bleached Kraft pulps at a moisture content below 18%, exhibited irreversible changes in their fiber due to hornification, and the effect was more pronounced on fibres which contain low amounts of lignin and hemicellulose (Newman, 2004).

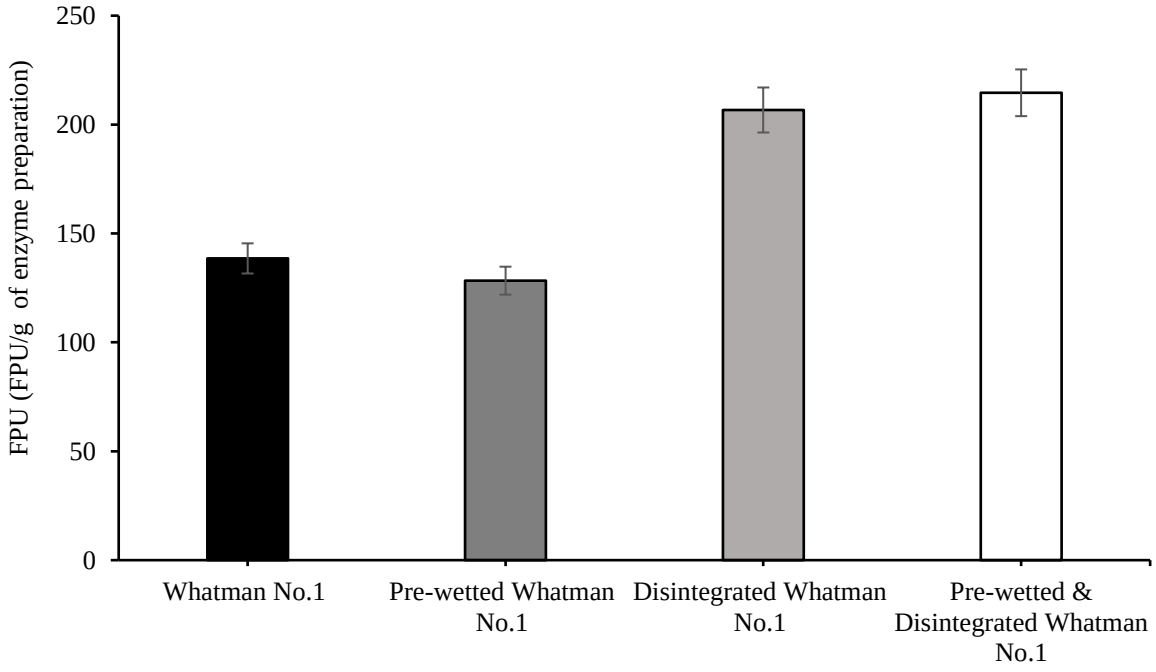

Figure 3.1: Filter paper units (FPU) of CTEC 3 using different substrates (Whatman No.1 filter paper, pre-wetted Whatman No.1 filter paper, disintegrated Whatman No.1 filter paper, prewetted, and disintegrated Whatman No.1 filter paper in 50 mM sodium citrate buffer. Hydrolysis conditions: 50^0C for exactly 60 min. Error bars indicate the standard deviation (n=3).

While disintegrating the Whatman No.1 filter paper increased the FPU of CTec 3 during the 1-hour FPA, it was also of interest to investigate whether the same effect would be

observed over an extended duration of enzymatic hydrolysis. Similar to the results observed with the FPA, when dry and disintegrated Whatman No.1 filter papers were subjected to enzymatic hydrolysis, an increase in hydrolysis yields were observed when using the disintegrated Whatman no.1 filter paper (Figure 3.2). After 48 hours, hydrolysis yields reached up to 62% and 86% cellulose hydrolysis for disintegrated filter paper at 10 and 20 mg protein /g cellulose respectively. It should also be noted that during the first 4 hours of hydrolysis, the reducing sugar yields for both the substrates at an identical protein loading were similar. However, when the duration of the enzymatic hydrolysis was extended, both substrates exhibited different hydrolytic profiles. This indicated that the FPA, which is carried out over 60 minutes, might not be representative of the hydrolytic potential of cellulase mixtures over the 24-48 hours which is typically necessary to obtain conversion of >70% of the cellulose to glucose.

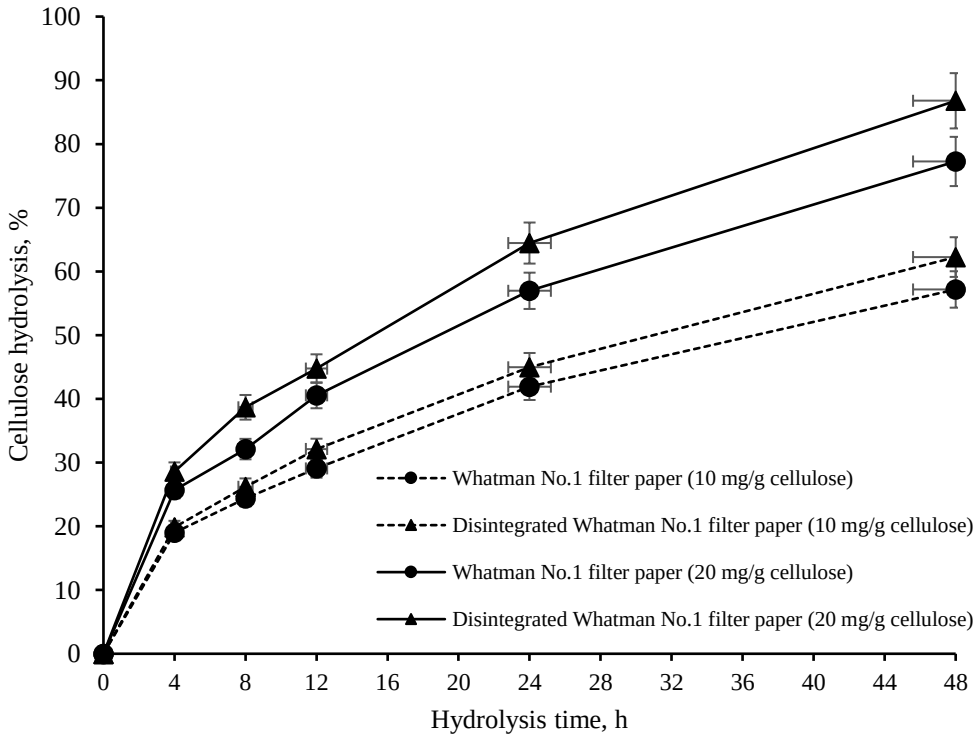

Figure 3.2: Cellulose hydrolysis of Whatman No.1 filter paper and disintegrated Whatman No.1 filter paper at 10 mg/g cellulose and 20 mg/g cellulose for 48 hours. Hydrolysis conditions: 50^0C, 5% solids loading and 150 rpm shaking.

3.1.3. Changes in cellulase activity when hydrolyzing cellulosic substrates from their never-dried state to air-dried and paper state

To evaluate the effects of drying on the filter paper activity, never dried fibres were compared to those that had undergone drying under FPA conditions. Initially the chemical composition of the Whatman filter paper was measured indicating that the substrate consisted of 96% cellulose and 4% hemicellulose (Table 3.1). Bleached pre-hydrolyzed Kraft pulp (PHK) was used as a substrate to mimic the Whatman No.1 filter paper substrate since it had a similar chemical composition to the Whatman No.1 filter paper (Table 3.1). To assess the effects of drying, the PHK pulp was used and substituted in the FPA and compared to PHK fibres that were air dried in a fume hood prior to assessment using the FPA. The PHK pulp was formed into sheets and subsequently disintegrated to again assess the effects of papermaking on the FPA.

Table 3.1: Carbohydrate and lignin composition of cellulosic substrates

Substrate	**Compositional analysis of cellulosic substrate (%)**						
	Ara	**Gal**	**Glu**	**Xyl**	**Man**	**AIL**	**ASL**
Whatman No.1 FP	0.5±0.0	0.1±0.0	96.0±0.8	1.6±0.0	1.7±0.0	bdl	0.1±0.0
PHK pulp	0.4±0.1	0.3±0.2	94.5±0.4	3.6±0.6	0.0±0.0	bdl	0.3±0.1
Dissolving pulp	0.7±0.2	0.1±0.0	95.4±0.3	2.8±0.5	2.5±0.4	bdl	0.2±0.1

Mean ± Standard deviation; Ara - Arabinan; Gal – Galactose; Glu – Glucan; Xyl – Xylan; Man – Mannan; AIL – Acid insoluble lignin; ASL – Acid insoluble lignin; bdl – below detectable levels; FP – filter paper

Surprisingly, it was apparent that the filter paper activity results using the PHK formed paper (148 FPU/ g of enzyme preparation) were almost the same as those obtained with Whatman no 1 filter paper (139 FPU/ g of enzyme preparation). Therefore, the PHK pulp seemed to be a good representation of a substrate that has a similar response during the FPA as the Whatman no. 1 filter paper. On average, air-drying and papermaking reduced the CTec 3 activity

on PHK pulp by 60% FPU/g enzyme preparation (Figure 3.3). There is no big difference in the FPU of PHK air-dried fibers and PHK filter paper, which indicated that the reduction in CTec activity was mostly caused by drying rather than the papermaking process. When the PHK filter paper was disintegrated, the CTec 3 activity increased but could not reach the same level as the FPU results obtained using the never-dried fibres.

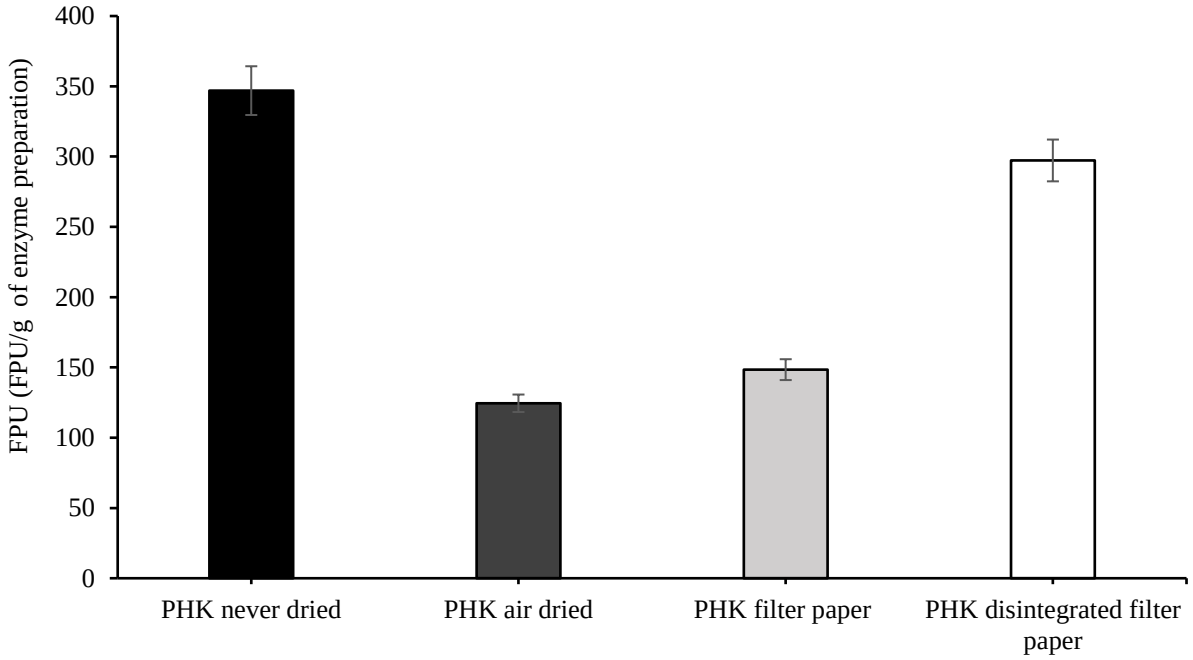

Figure 3.3: Filter paper units of CTEC 3 using different substrates (PHK never-dried fibers, PHK air-dried fibers, PHK filter paper, and PHK disintegrated filter paper) in 50 mM sodium citrate buffer. Hydrolysis conditions: 50^0C for exactly 60 min. Error bars indicate the standard deviation (n=3).

The high CTec 3 activity when hydrolyzing the never dried PHK fibers was indicative of the higher substrate accessibility of the never dried fibres, which was lost when the fibres had undergone drying either via air drying or during the production of paper sheets. The reduction in CTec 3 activity exhibited by air-dried pulp and filter paper is likely due to fibre hornification, which has been shown to decrease cellulose accessibility via the formation of fibre aggregates. It was apparent that disintegration only partially restored the cellulose accessibility of

the PHK paper, thus the effects of hornification were only partially reversible. Since never-dried pretreated biomass will typically be used for enzymatic hydrolysis, the drying undergone by fibres during the production of the Whatman filter paper appears to severely compromise the ability to estimate the true activity of cellulases on never dried substrates.

Although drying-induced fiber hornification significantly reduced CTec 3 activity on PHK within the 60-minute timeframe of the FPA, it was unclear whether the hydrolysis yields would be affected if the hydrolysis was extended over a longer time. When the hydrolysis time for the PHK substrates with and without drying and disintegration was extended to 48 hours using an enzyme loading of 20 mg/g cellulose (Figure 3.4), the trend observed for the hydrolysis yields was similar to the trend observed after 60 minutes during the FPA (Figure 3.3). A similar trend was observed in earlier work which assessed the effects of drying on never dried fully bleached Kraft pulp and its subsequent ease of enzymatic hydrolysis (Esteghlalian et al., 2001).

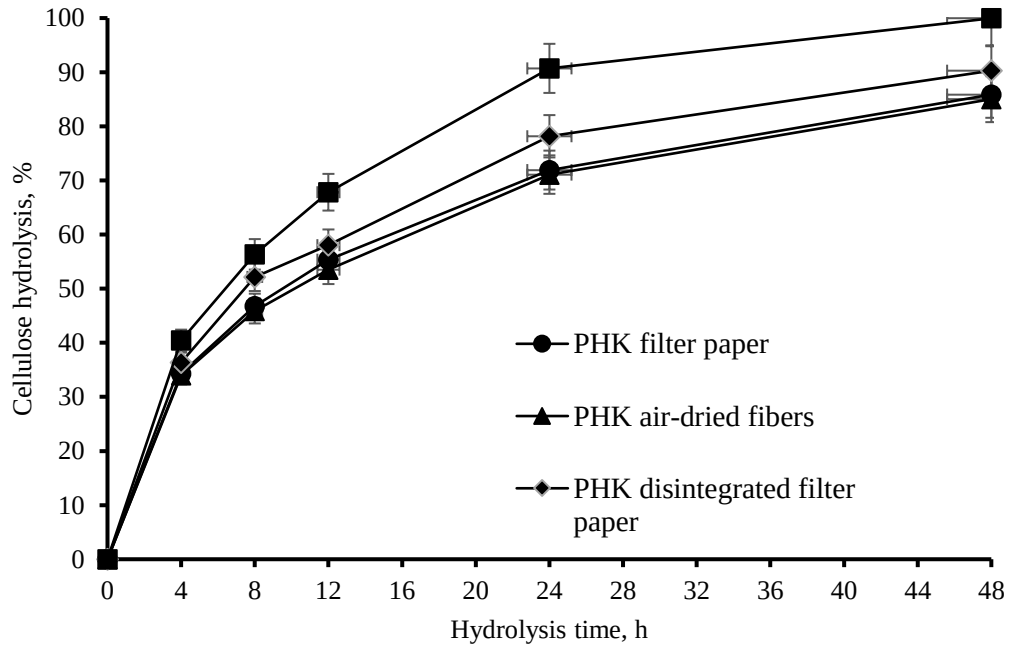

Figure 3.4: Cellulose hydrolysis of PHK filter paper, PHK air-dried fibers, PHK disintegrated filter paper and PHK never-dried fibers at 20 mg/g cellulose for 48 h hydrolysis. Hydrolysis conditions: 50^0C, 5% solids loading and 150 rpm shaking.

It was apparent that, after 48 hours of hydrolysis, the PHK never-dried fibers hydrolyzed better than the PHK disintegrated filter paper, filter paper and air-dried fibers (Figure 3.4). However, the PHK paper and air-dried fibres showed the same hydrolysis profiles, which differed slightly from what was observed in the FPA measurement as the PHK filter paper had a slightly higher FPA than did the PHK air-dried pulp. This difference could likely be attributed to the effects of shaking at 150 rpm during the 48-hour hydrolysis, which likely improved the mixing of the air-dried fibres compared to the static hydrolysis conditions employed during the standard FPA procedure.

3.1.4. Characterizing the effect of hornification on enzymatic hydrolysis

It has been reported that the cell wall lumens of never-dried pulps are swollen, porous and accessible (Laivins and Scallan, 1996). Drying and pressing causes collapse of the swollen lumens and increases the degree of cross-linking of adjacent microfibrils inter-linked by strong hydrogen bonds (Häggkvist et al., 1998). This "hornification" results in a reduction in the accessible surface area of cellulose, which inhibits effective enzymatic hydrolysis (Häggkvist et al., 1998; Laivins and Scallan, 1996). The effects of fiber-induced hornification and drying of cellulosic substrates has often been measured using the water retention value (WRV) technique.

The WRV concept was introduced by Jayme (1944) to evaluate the pore closure changes that pulps and fibers undergo during drying and rewetting processes. This technique attempts to quantify the water trapped in the inner pores of cellulosic substrates after centrifugation. Centrifugation removes excess water from cellulose fibers, leaving behind only water inside the cell wall (Jayme, 1944). Thus, we used the WRV to assess the extent by which cellulose accessibility was affected by drying. The degree of hornification was defined as the

reduction in WRV expressed as grams of water retained in fibers per gram of wetted fibers (g water/g fiber).

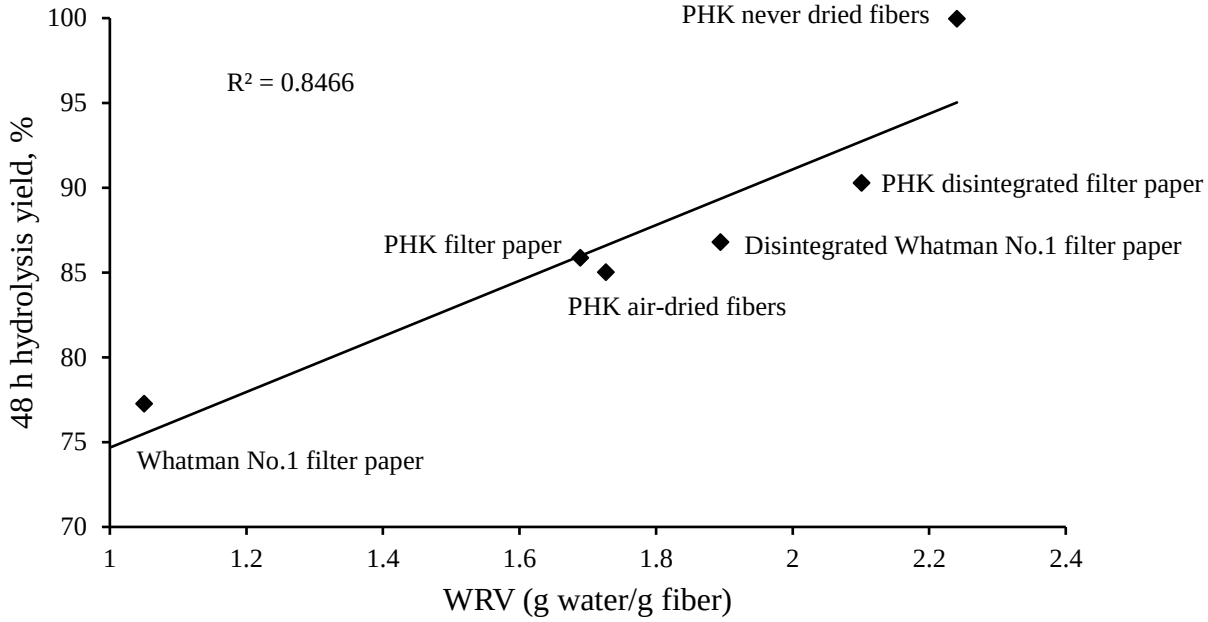

Figure 3.5: The extent of fibre swelling of various cellulosic substrates contrasted to their ease of enzymatic hydrolysis. Cellulose hydrolysis experiments were performed at 5% solids loading using a cellulase loading of 20 mg/g substrate cellulose, 50^0C, and 150 rpm.

The never-dried PHK pulp exhibited the highest WRV of 2.3 g water/g fiber as compared to the PHK air-dried pulp and PHK filter paper (Figure 3.5). Disintegration increased the WRV of the Whatman No.1 filter paper and PHK filter paper from 1.1-1.9 g water/g fiber, and 1.7-2.1 g water/g fiber (Figure 3.5). It is apparent that disintegration partially restored the WRV of both the Whatman and PHK filter paper. Similarly, Chen et al. (2012), mechanically refined oven-dried bleached hardwood and softwood pulps which consequently increased the WRV of the pulp substrates. The hydrolysis yields (after 48h) of the never-dried, air-dried, paper and disintegrated fibers were also correlated with the WRV results (Figure 3.5).

It appeared that the increased cellulose hydrolysis observed at 48 hours correlated well with the WRV. It was also apparent that drying of the substrates compromised the accessibility of the pulp to water as well as enzymes. Therefore, it may be more representative to use never dried pulp substrates in the FPA to obtain a more accurate assessment of the hydrolytic potential of a given cellulase cocktail. However, the use of wet substrates will present other difficulties since their moisture content would need to be standardized and these wet substrates would be sensitive to microbial degradation during storage.

As well as using WRV to assess accessibility, several other studies have suggested that the Simons' staining technique can provide a good estimate of cellulose accessibility. It has been shown that this assay is a good predictor of how well a given substrate will be hydrolyzed by cellulase enzyme mixtures (Arantes and Saddler, 2011; Chandra et al., 2008, 2016; Chandra and Saddler, 2012; Hu et al., 2011). The Simons' staining method is based on the adsorption of a Direct Orange dye onto the cellulose fibers since. The >100kDa portion of the dye has been shown to have a diameter of 5-7 nm which is similar to the rate limiting pore size for enzymatic hydrolysis (Grethlein et al., 1984; Stone et al., 1969). Hence, the adsorption capacity of the orange dye molecule by cellulosic fibers has been suggested to better represent how cellulase enzyme components might access the cellulose.

Similar to previous work, the Simons staining values reflected the ease of hydrolysis of the substrates closely with an r^2 value of 0.93 (Figure 3.6). Again it was apparent that drying severely compromised enzymatic hydrolysis while disintegration partially recovered some cellulose accessibility (Figure 3.6). Thus, it was very likely that the detrimental effects of drying played a key role in limiting the effectiveness of the FPA in predicting the ease of hydrolysis on pretreated substrates that are typically "never-dried".

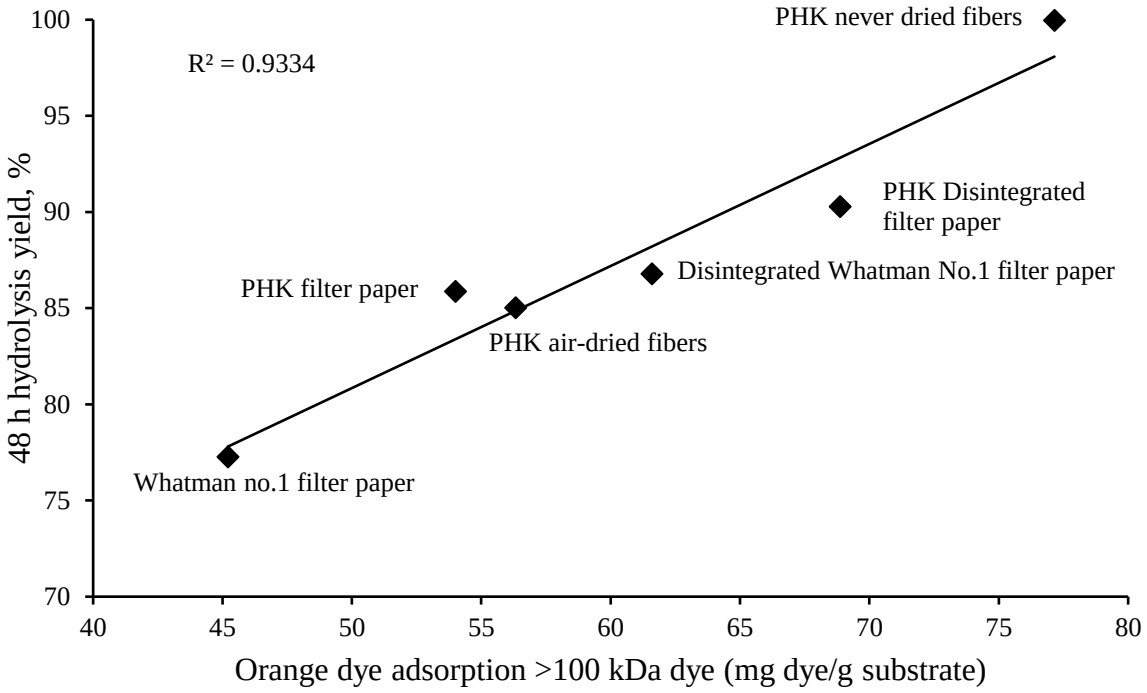

Figure 3.6: The adsorption of the enriched (> 100 kDa) DO dye of various cellulosic substrates contrasted to their ease of enzymatic hydrolysis. Cellulose hydrolysis experiments were performed at 5% solids loading using a cellulase loading of 20 mg/g substrate cellulose, 50^0C, and 150 rpm.

3.1.5. Comparing the use of other pure cellulosic substrates to the Whatman No.1 filter paper in the FPA

In addition to issues with the filter paper substrate itself, the chemical composition of the Whatman No.1 filter paper indicates that the substrate is mainly composed of highly purified cellulose (Table 3.1). However, the various methods that "pulp" and/or "bleach" to obtain purer form of cellulose will results in a range of cellulosic substrates which will vary in their properties. Therefore, although different cellulosic substrates might possess a similar amount of cellulose, the hydrolytic activity of cellulase mixtures acting on these substrates might be variable due to differences in their physio-mechanical characteristics imparted by processing.

Thus, the FPA may be limited in its ability to predict the hydrolytic potential of cellulase enzymes even within sets of highly purified cellulose samples. To test this hypothesis,

an initial enzymatic hydrolysis was conducted on the commercial cellulosic substrates, dissolving pulp, and Whatman No.1 filter paper at 5% solids loading for 48 hours at 20 mg/g cellulose protein loading. The Whatman No. 1 filter paper and dissolving pulp possessed a relatively similar glucan content of over 95%dwt (Table 3.1). In previous work other commercial cellulosic substrates (avicel, microgranular cellulose, sigmacell cellulose, fibrous medium cellulose, and fibrous long cellulose) were shown to contain more than 90% cellulose (Chandra and Saddler (2012).

With the same enzyme and solids loading, the various cellulosic substrates resulted in significantly different hydrolytic profiles (Figure 3.7). When the hydrolysis time was extended to 48 hours, the sigmacell cellulose, dissolving pulp and Whatman No.1 filter paper hydrolyzed far better than the Avicel, microgranular, fibrous medium and fibrous long cellulose. Thus, it was apparent that each of the different types of cellulose hydrolyzed to varying extents despite having similar chemical composition. These results are similar to those of Chandra and Saddler (2012) who used the Simons' staining technique to assess the cellulose accessibility of various cellulosic substrates.

It was apparent that the FPA, which uses the Whatman No.1 standardized filter paper substrates, may not adequately assess the hydrolytic performance of cellulase mixtures on substrates even when they have a similar chemical composition. It was also likely that the presence of lignin and hemicellulose might further compound the issues of the FPA in predicting the ability of cellulase cocktails to hydrolyze real lignocellulosic substrates. Due to the high cost of removing lignin, realistic pretreated substrates will typically contain some amount of residual lignin as well as hemicellulose. Therefore, we next investigated the impact that the presence of lignin and hemicellulose might have on the efficacy of the FPA.

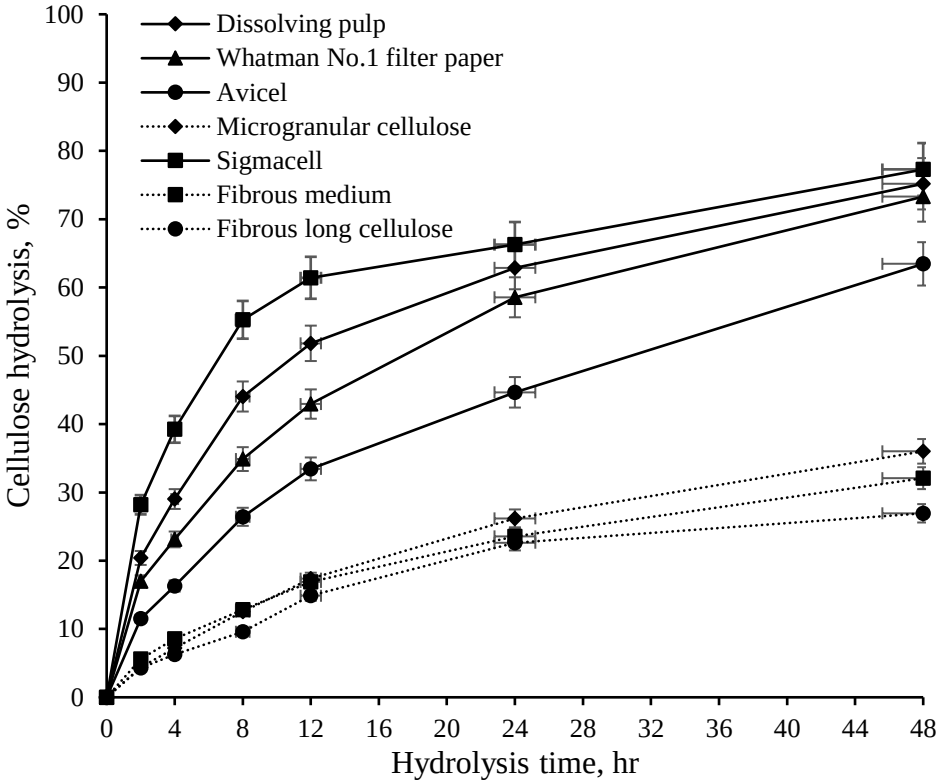

Figure 3.7: Enzymatic hydrolysis of pure cellulosic substrates at 5% solids loading and protein loading of 20 mg protein/ g cellulose in 50 mM sodium acetate buffer. Hydrolysis conditions: 50^0C, 150 rpm. Error bars indicate the standard deviation (n=2).

3.1.6. Influence of lignin and hemicellulose within the substrate on the efficacy of the filter paper assay

The large number of combinations of pretreatments and biomass types will result in a large number of biomass substrates (Chandra et al., 2007; Maurya et al., 2013; Sun et al., 2016). Therefore, it is challenging to recommend a standardized lignocellulosic substrate that might be representative of other substrates when used in a standardized cellulase activity assessment. Therefore, to assess the possible effects of hemicellulose and lignin on the FPA, an organosolv pulp was produced using conditions and steps described earlier in section 2.3.2. The organosolv substrates provided fibres that contained lignin but could still be readily formed into paper sheets at the same target weight as the Whatman No.1 filter paper.

Table 3.2: Carbohydrate and lignin composition of Whatman No.1 filter paper and Organosolv pretreated substrates

Substrate	**Compositional analysis of cellulosic substrate (%)**						
	Ara	**Gal**	**Glu**	**Xyl**	**Man**	**AIL**	**ASL**
Whatman No.1 FP	0.5±0.0	0.1±0.0	96.0±0.8	1.6±0.0	1.7±0.0	bdl	bdl
0.5% acid	0.3±0.0	0.2±0.0	53.4±1.8	11.35±0.5	2.9±0.3	25.5±5.1	0.1±0.0
1% acid	0.4±0.0	bdl	61.09±3.1	5.78±0.5	1.7±0.0	28.9±2.8	0.1±0.0

Mean ± Standard deviation; FP – filter paper; 0.5% acid - 0.5% acid pretreated organosolv maple; 1% acid - 1% acid pretreated organosolv maple; Ara - Arabinan; Gal – Galactose; Glu – Glucan; Xyl – Xylan; Man – Mannan; AIL – Acid insoluble lignin; ASL – Acid insoluble lignin; bdl – below detectable levels.

The 0.5% acid pretreated organosolv maple (0.5% acid) and 1% acid pretreated organosolv maple (1% acid) were formed into filter paper that contained 53.4%dwt and 61.1%dwt cellulose content respectively (Table 3.2). The 1% acid had a higher lignin content (28.9%dwt) as compared to 0.5% acid (25.5%) likely due to the hydrolysis of the hemicellulose component at the higher acidity, which increased the proportion of lignin in the substrate. The total hemicellulose-derived sugars (arabinose, galactose, mannose, and xylose) present in the 0.5% acid and 1% acid treated substrates was 14.8%dwt and 7.9%dwt respectively (Table 3.2). These substrates were quite different when compared to the Whatman No.1 filter paper, which contained 96%dwt cellulose and 4%dwt total hemicelluloses (Table 3.1).

When assessing the standardized FPA using the Whatman No.1 filter paper, a result of 139 FPU/ g enzyme preparation was obtained (Figure 3.8). In the presence of the hemicellulose and lignin that were contained in the organosolv substrates, the filter paper activity on the filter paper made using the 1% acid, and 0.5% acid organosolv treated substrates decreased to 79 and 66 FPU/ g of enzyme preparation respectively. The filter paper derived from the 0.5% acid organosolv substrate appeared more recalcitrant when compared to the paper derived from the organosolv treatment employing a 1% acid charge.

It was apparent that the higher severity organosolv conditions using 1% acid filter paper (section 2.3.2) removed more hemicellulose and likely decreased the cellulose DP, consequently reducing substrate recalcitrance. Previously, Pan et al. (2008) also showed that using a higher acid concentration during organosolv pretreatment contributed to a lower degree of polymerization of cellulose, lower cellulose crystallinity, and a smaller fiber size. This resulted in an increase in cellulose hydrolysis. It was evident that the presence of lignin and hemicellulose in both the 0.5% acid, and 1% acid filter paper reduced the CTec 3 activity by approximately 47% FPU/ g enzyme preparation (Figure 3.8). This indicated the higher recalcitrance of pretreated lignocellulosic substrates that contained lignin and hemicellulose.

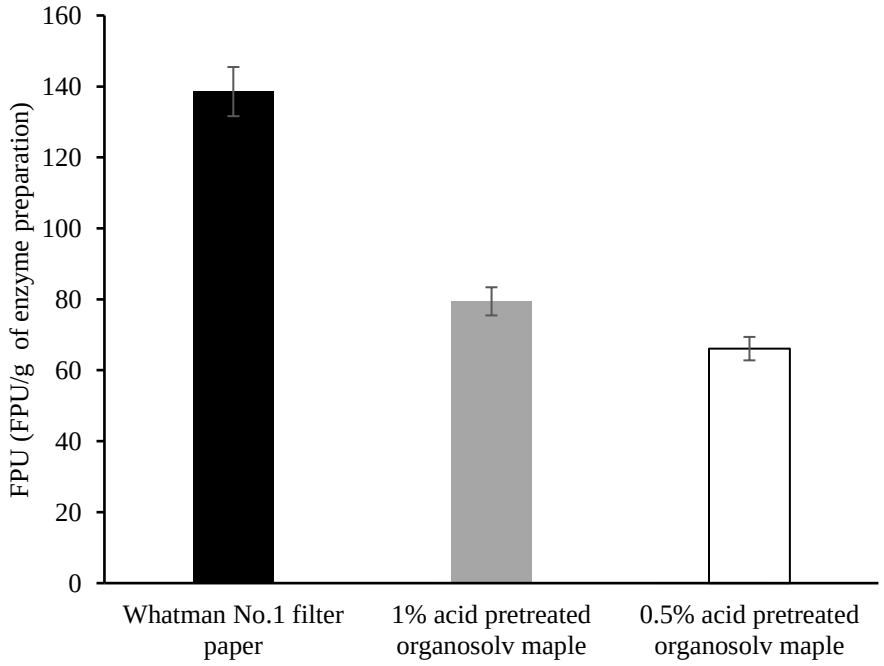

Figure 3.8: Filter paper units of CTEC 3 using different substrates (Whatman No.1 filter paper, 1% acid pretreated organosolv maple filter paper, and 0.5% acid pretreated organosolv maple filter paper) in 50 mM sodium citrate buffer. Hydrolysis conditions: 50^0C for exactly 60 min. Error bars indicate the standard deviation (n=3).

When the Whatman No.1 filter paper, and paper made from 0.5% acid, and 1% acid pulps were disintegrated and subjected to prolonged hydrolysis at a 5% solid loading with 20 mg

protein/g cellulose for 48 hours, the disintegrated filter paper from the organosolv pulp produced using 0.5% acid was the most recalcitrant as only a 63% hydrolysis yield was obtained after 48 hours. However, the disintegrated sheet which used the organosolv pulp using 1% acid actually hydrolyzed to a higher yield than did the disintegrated Whatman No.1 filter paper (Figure 3.9), even though the FPA of the organosolv substrate was just over half that of the filter paper (Figure 3.8). Earlier work by Pan et al. (2008) showed that the use of a higher acid concentration during organosolv pretreatment contributed to a lower degree of polymerization of cellulose, lower cellulose crystallinity, and a smaller fiber size, thus resulting in an increase in cellulose hydrolysis. This indicated that, at a fixed solids (%w/v) and protein loading using an extended hydrolysis residence time, the FPA results did not reflect the hydrolytic potential of CTec 3 cellulase preparation on these substrates. Consequently, the FPA might not be representative of the hydrolytic potential of the same enzyme on realistic lignocellulosic biomass.

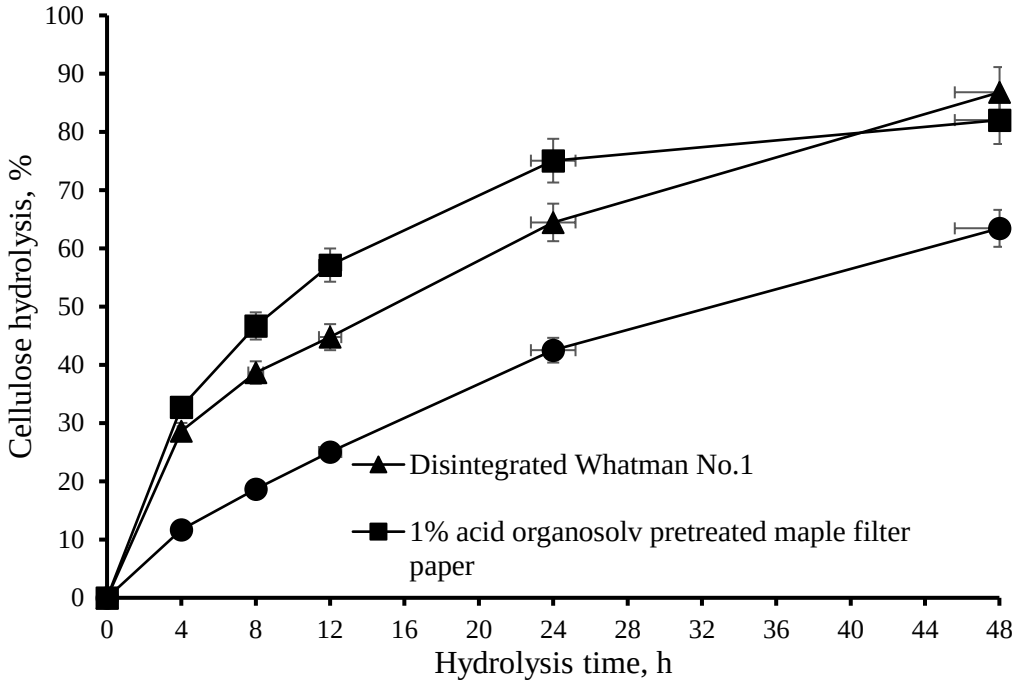

Figure 3.9: Cellulose hydrolysis of Whatman No.1 filter paper, 1% acid pretreated organosolv maple filter paper, and 0.5% acid pretreated organosolv maple filter paper at 20 mg/g cellulose for 48-hour hydrolysis. Hydrolysis conditions: 50^0C, 5% solids loading and 150 rpm shaking.

3.1.7. Conclusions

In this section of the thesis, we tried to elucidate the role that substrate heterogeneity will likely play in influencing our ability to accurately predict the hydrolytic potential of cellulase enzyme mixtures on a range of substrates. Although Whatman No.1 filter paper might have a similar chemical composition to that of other cellulosic materials, the hydrolytic performance of CTec 3 on other, "purer", cellulosic substrates the results were quite different. The fact that the Whatman No.1 filter paper used in the standardized FPA is dry greatly influenced its ability to predict how a cellulase mixture might hydrolyze a never dried substrate. When lignin and hemicellulose are present in a substrate, the substrate becomes more heterogeneous making it even more difficult to standardize the "cellulase" assay. It was also apparent that the current one-hour assay used in the FPA was not representative of the sugar released over longer hydrolysis times.

3.2. Can the filter paper assay assess the enhancement in hydrolysis yields when cellulases are supplemented with accessory enzymes to better hydrolyze pretreated lignocellulosic substrates?

3.2.1. Background

It was apparent that the nature of the cellulose, the processing conditions (forming, pressing and drying during papermaking), and the presence of lignin and hemicellulose all influenced the cellulase activity measured using the FPA. In all cases the filter paper activity was significantly lower when a substrate containing hemicellulose and lignin was substituted in the FPA for Whatman no 1. Filter paper. As discussed earlier, both lignin and hemicellulose have been shown to limit the accessibility of cellulase enzymes to cellulose (Berlin et al., 2005b; Scheller and Ulvskov, 2010; Siqueira et al., 2017).

Therefore, to try to deal with lignin and hemicellulose limiting cellulose hydrolysis, enzyme cocktails have been developed to include hemicellulose and/or lignin degrading/modifying enzymes (accessory enzymes) that act synergistically with the cellulases to breakdown the complexed pretreated lignocellulosic substrates (Berlin et al., 2005a; Gao et al., 2011; Hu et al., 2014, 2013, 2011; Sun et al., 2015; Várnai et al., 2011). The addition of these accessory enzymes has been shown to have a tremendous effect on reducing the overall cellulase loading required to hydrolyze pretreated lignocellulosic substrates (Hu et al., 2011). However, it was likely that the FPA might be insensitive to the beneficial contributions of accessory enzymes and other, potentially "lignin blocking" proteins, such as BSA, in the cellulase cocktail.

It has been suggested that accessory enzymes hydrolyse hemicellulose resulting in increased cellulose accessibility to the cellulases (Arantes and Saddler, 2011; Gourlay et al., 2013; Hu et al., 2011). The synergism among the cellulase enzymes (cellobiohydrolases, endoglucanases, and β-glucosidase) act in concert to break down cellulose (Henrissat et al., 1985;

Kostylev and Wilson, 2012), while the synergism among cellulases and accessory enzymes (LPMOs, swollenin, xylanases, mannanase, BSA, pectinase, esterase) specifically breakdown pretreated lignocellulosic substrates (Gourlay et al., 2013; Hu et al., 2011; Jung et al., 2015; Li et al., 2014; Selig et al., 2008; Várnai et al., 2011; Yang and Wyman, 2006).

Different pretreatments produce lignocellulosic substrates with different compositions, which may require varying enzyme requirements for complete hydrolysis. Recently the newly advanced cellulase cocktails such as the CTec series (Cellic CTec 1, CTec 2, and Cellic CTec 3) from Novozymes have been developed. These enzyme mixtures have made a significant impact by enhancing hydrolysis using lower protein loading, especially when hydrolyzing xylan rich substrates (Sun et al., 2015). Though the Novozymes approach has been beneficial in improving the cellulase cocktails to hydrolyze complex lignocellulose, Rana and Ahring (2015) reported that CTec 2 cellulase preparations were not effective when hydrolyzing softwood (loblolly pine) as compared to corn stover. The same study also revealed that supplementation of CTec 2 with fungal strains from *T. reesei* RUT-C30 enhanced the mannan and cellulose hydrolysis (Rana and Ahring, 2015). Other workers (Hu et al., 2011) replaced cellulase enzymes by up to 86% with xylanases when hydrolyzing steam pretreated cornstover and realized gains of up to 19% in cellulose and xylan hydrolysis. These findings support the notion of adding specific accessory enzymes to cellulase cocktails that target specific substrates components. For instance, a cellulase enzyme cocktail enriched with xylanase may be best suited for the hydrolysis of a deacetylated corn stover substrate that contains up to 20-30% xylan (Chen et al., 2014; X. Chen et al., 2012).

The synergistic interaction of commercial cellulase mixture (CTec 3) with accessory enzymes [hemicellulases preparations (xylanase and endo-mannanase)] and Bovine Albumin Serum (BSA) was next assessed during the hydrolysis of a range of pretreated lignocellulosic

substrates. It was thought that, although a synergistic enhancement in the hydrolysis yield could be realized using a cellulase cocktail that contained accessory enzymes, these enhancements would not be shown as an increase in cellulase activity when the activity of the same cocktail was quantified using the standardized FPA.

3.2.2. Experimental design

The pretreated substrates were chosen based on the type/amount of hemicellulose and lignin they contained. They were then matched with respective accessory enzymes that were anticipated to function synergistically with cellulases to enhance their hydrolysis. Bovine Serum albumin (BSA) was used to replace a portion of cellulase enzymes in the lignin-rich steam pretreated softwood because of its ability adsorb and "block lignin", consequently decreasing non-specific binding of cellulases (Kim et al., 2014; Yang and Wyman, 2006). Mannanases and xylanases were used to replace a portion of the cellulase mixture to hydrolyze the mannan and xylan rich pretreated substrates (Hu et al., 2011; Malgas et al., 2015).

The protein loading was fixed at a minimum cellulase loading required to achieve greater than 70% cellulose hydrolysis for all of the substrates (Table 3.3). The cellulase cocktail was then replaced with 10%, 20%, 50% and 100% of each of the accessory enzymes and BSA on a protein basis. For each replacement, the changes in total cellulase activity under FPA conditions was measured and correlated to hydrolysis yields. Finally, the Whatman No.1 filter paper that was used in the FPA was hydrolysed using the cellulase cocktail that was amended with accessory enzymes. This was done to assess the influence of accessory enzymes on their ability to hydrolyze the cellulose-rich filter paper substrate.

Table 3.3: Monosaccharide and lignin composition of pretreated lignocellulosic substrates and Whatman No.1 filter paper (% dry weight).

Substrate	**Compositional analysis of cellulosic substrate (%)**						
	Ara	**Gal**	**Glu**	**Xyl**	**Man**	**AIL**	**ASL**
Whatman FP	0.5±0.0	0.1±0.0	96.0±0.8	1.6±0.0	1.7±0.0	bdl	bdl
Organosolv maple	0.4±0.0	bdl	64.1±3.1	5.8±0.5	1.7±0.0	28.9±2.8	0.1±0.0
SPS	0.4±0.0	bdl	55.5±0.8	1.1±0.0	bdl	37.6±5.1	0.4±0.8
SBKP	0.3±0.0	0.3±0.0	92.7±9.8	8.2±0.5	5.9±0.6	bdl	bdl
HBKP	0.3±0.0	bdl	87.3±3.2	16.6±0.8	bdl	bdl	bdl
PD-TMP	0.8±0.0	1.8±0.0	47.5±0.2	4.9±0.1	16.1±0.3	15.6±0.3	0.3±0.0
DACS	2.3±0.0	0.7±0.0	49.9±0.1	17.1±0.1	bdl	16.1±0.2	0.2±0.0

Mean ± Standard deviation; FP – filter paper; SPS-Steam pretreated softwood; SPCS-Steam pretreated corn stover; SBKP-Softwood bleached Kraft pulp; HBKP-Hardwood bleached Kraft pulp; PD-TMP- partially delignified TMP; DACS-De-acetylated corn stover; Ara - Arabinan; Gal – Galactose; Glu – Glucan; Xyl – Xylan; Man – Mannan; AIL – Acid insoluble lignin; ASL – Acid insoluble lignin; bdl – below detectable levels; %dwt - % dry weight.

Table 3.4: Enzymatic hydrolysis of pretreated lignocellulosic substrates for 72 hours using CTec 3 loaded at 15 mg/ g cellulose, shaking: 150 rpm, pH 4.8 and 5% solids loading, assessed for percentage of cellulose, xylan and mannan hydrolysis and total reducing sugar yield.

Substrate	**Hydrolysis, %**			**Total reducing sugar, g/l**
	Cellulose	**Xylan**	**Mannan**	
Organosolv maple	83.4	86.6	NA	32.3
SPS	48.7	NA	NA	14.9
SBKP	73.3	69.6	50.8	42.7
HBKP	78.7	82.6	NA	46.5
PD-TMP	39.6	NA	17.5	11.9
DACS	53.1	46.4	NA	19.0

Standard deviation: 0.1-0.8 (n=2); SPS-Steam pretreated softwood; SBKP-Softwood bleached Kraft pulp; HBKP-Hardwood bleached Kraft pulp; PD-TMP- partially delignified TMP; DACS-De-acetylated corn stover; NA – Not Assessed.

3.2.3. Synergistic effect of cellulase, xylanase, mannanase, and BSA on pretreated lignocellulosic substrates

3.2.3.1. Xylan-rich substrates

a) Deacetylated Corn Stover

The de-acetylated corn stover (DACS) was chosen since it contained approximately 17% xylan. Therefore, the cellulase cocktail (CTec3) was partially replaced with a xylanase enzyme preparation (multifect xylanases) which was anticipated to enhance the hydrolysis yields of the de-acetylated corn stover. Multifect xylanase has been shown to exhibit high xylanase and xylosidase activities (Hu et al., 2011). The xylan in xylan rich substrates is thought to be covalently bonded to acetyl groups (branched) by ester bonds (Agger et al., 2010). Successful debranching of xylan (removal of acetyl groups) through de-acetylation under alkaline conditions has a significant impact on enhancing the rate and extent of enzymatic hydrolysis (X. Chen et al., 2012). The enhancement in hydrolysis yields via de-acetylation has been attributed to an increase in cellulose accessibility by removing the steric hindrance presented by acetyl groups (Grohmann et al., 1989).

The synergism between cellulase (CTec 3) and multifect xylanase enzymes when hydrolyzing de-acetylated corn stover was investigated after a hydrolysis time of 4, 24 and 72 hours (Figure 3.10). After 4 hours, replacing 20% of the cellulase cocktail (20% Repl) increased the hydrolysis by 5.7% (Figure 3.10A) while a 50% replacement of CTec 3 presented a similar cellulose hydrolysis yield to the original enzyme cocktail (0% Repl) indicating that replacing >50% of the cellulases perhaps limited the ability of the cocktail to hydrolyze cellulose. However, replacing the cellulase cocktail with 20 and 50% xylanases increased the xylan hydrolysis by 23% (20% Repl and 50% Repl respectively).

After 72 hours, it was apparent that substituting 50% of the cellulases led to a similar hydrolysis yield as the cellulase cocktail without replacement although xylan hydrolysis was

complete (Figure 3.10C). Interestingly, the use of 50% of CTec 3 (15 mg CTec 3/ g cellulose) without xylanase supplementation when hydrolyzing DACS resulted in approximately a 2-fold reduction in xylan and cellulose hydrolysis as well as the total reducing sugar (Table 3.4) as compared to when xylanase was supplemented to CTec 3 at 1:1 enzyme ratio (Figure 3.10C-D). This implied that CTec 3 synergistically cooperated with multifect xylanase to enhance cellulose and xylan hydrolysis when commercial CTec 3 was replaced by up to 50%.

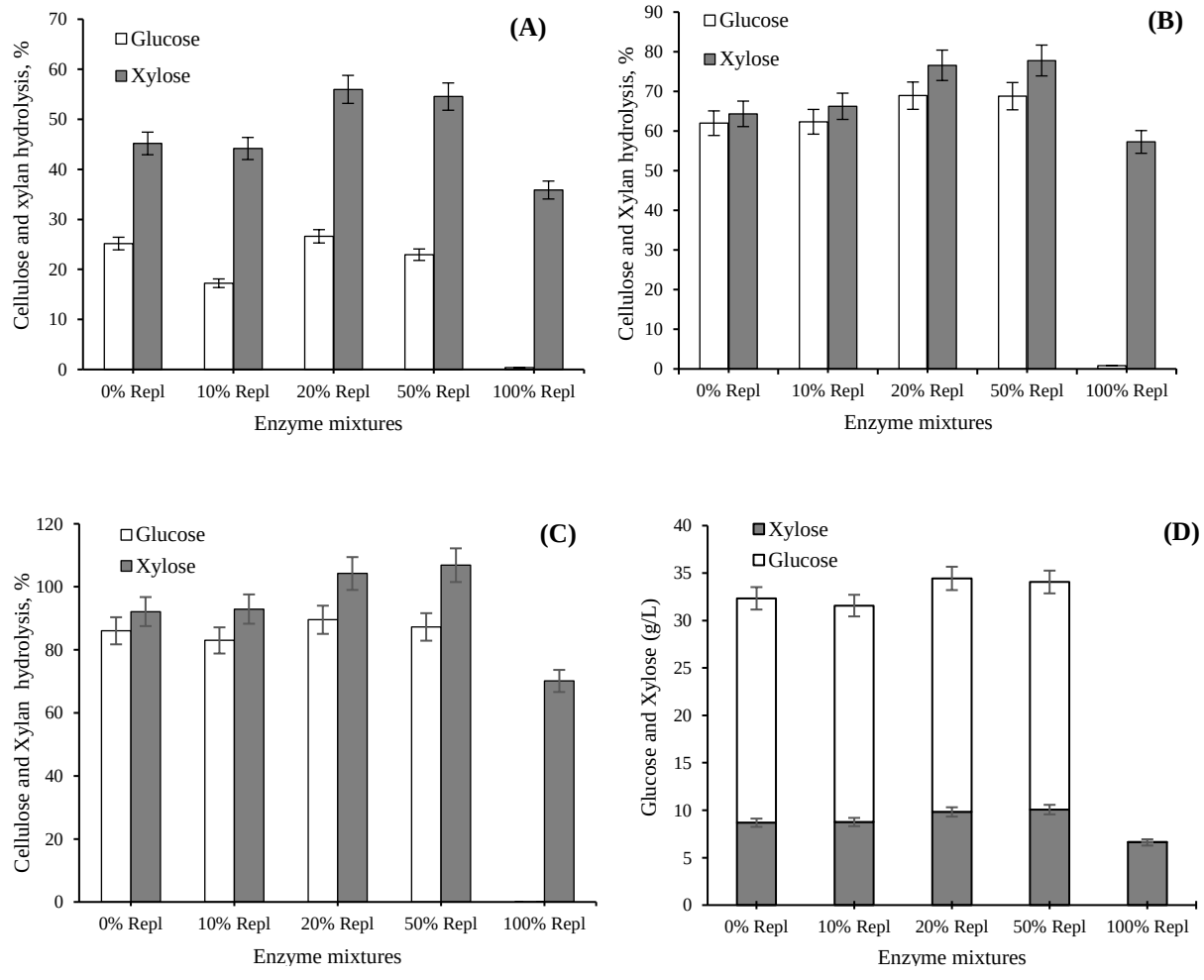

Figure 3.10: Synergistic effect of cellulase and xylanase (A–D) on deacetylated corn stover with the corresponding hydrolysis time for (A) and (B), and (C) being 4 h, 24 h, and 72 h respectively. (D) is the total reducing sugar yield from different cellulase-xylanase replacement strategies. Error bars indicate the standard deviation (n = 2). Repl - % of CTec 3 replaced by xylanases.

b) Organosolv pretreated maple

The synergism between CTec 3 cellulase mixture and multifect xylanase was further investigated using the organosolv pretreated maple substrate that was detailed in section 2.3.2. The organosolv pretreated maple had a lower xylan content (~6% xylan) compared to the de-acetylated corn stover (Table 3.3). The organosolv pretreatment often results in a higher cellulose yield in the solid phase, while the hemicellulose and lignin fractions are solubilized in the cooking liquor (Nitsos et al., 2018; Zhao et al., 2009). This has been shown to improve the susceptbility of organosolv substrates to enzymatic hydrolysis (Karnaouri et al., 2018).

After 4 hours, the cellulose hydrolysis of the organosolv pretreated maple increased by 3.2% when CTec 3 was replaced by up to 10% xylanase (10%Repl) (Figure 3.11A). However, when the hydrolysis time reached 72 hours, replacing 0%, 10%, 20% and 50% (0%Repl, 10%Repl, 20%Repl and 50%Repl) of the cellulase cocktail with xylanases resulted in relatively the same cellulose and xylan hydrolysis yields as the cellulase cocktail without replacement (Figure 3.11C). It was apparent that, likely due to the lower amount of xylan in the organosolv pretreated maple substrate, that the replacement with xylanase was less effective in improving cellulose hydrolysis yields. Previous work by Bura et al. (2009) showed that corn stover and poplar substrates that contained a higher amount of xylan were far more responsive to xylanase treatments than substrates that which contained <5% xylan.

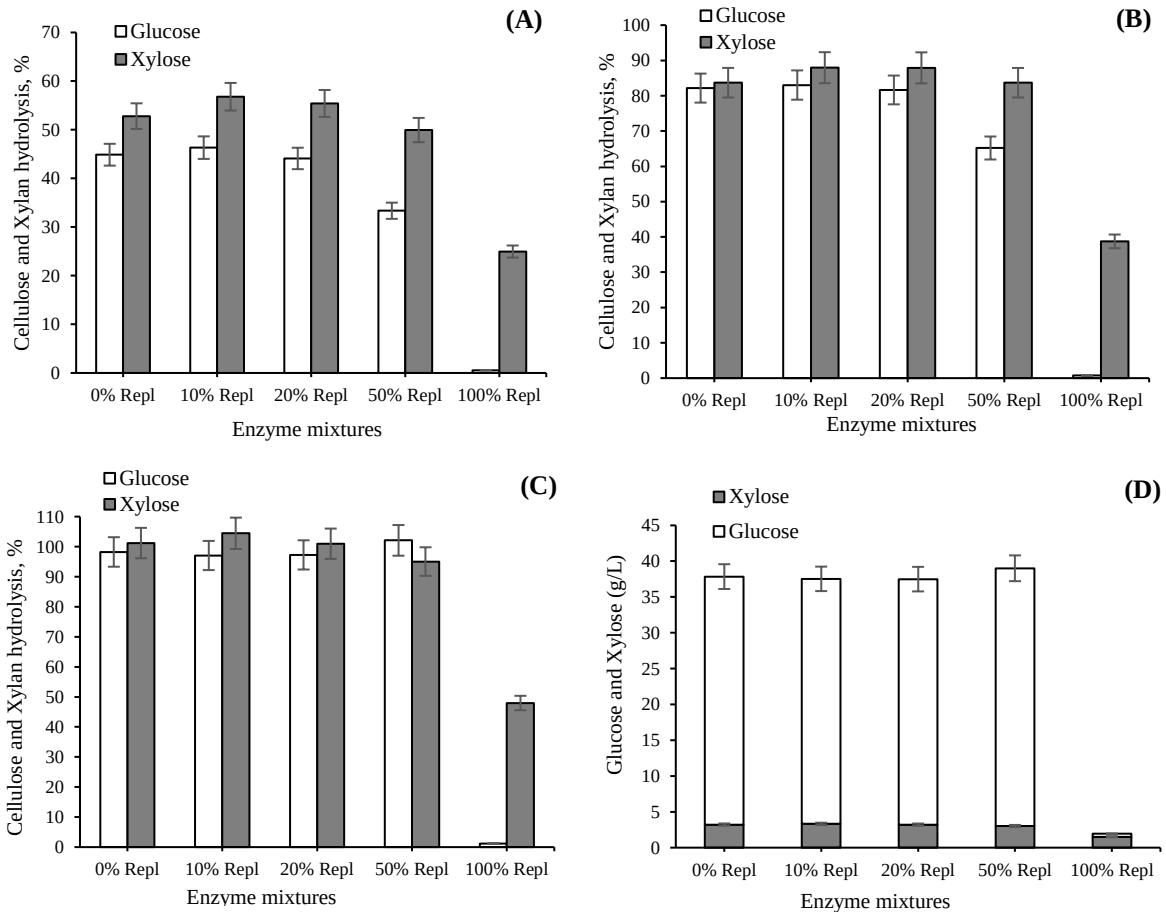

Figure 3.11: Synergistic effect of cellulase and xylanase (A–D) on organosolv pretreated maple with the corresponding hydrolysis time for (A) and (B), and (C) being 4 h, 24 h, and 72 h respectively. (D) is the total reducing sugar yield from different cellulase-xylanase replacement strategies. Error bars indicate the standard deviation (n = 2). Repl - % of CTec 3 replaced by multifect xylanase.

c) Hardwood bleached Kraft pulp

In contrast to the de-acetylated corn stover and organosolv pretreated maple, the hardwood bleached Kraft pulp contained a high proportion of xylan (~17%) and was virtually lignin free (Table 3.3). Thus, it was anticipated that the xylanases could potentially play a greater role in acting synergistically with the cellulases since lignin was not present to compromise the accessibility of enzymes to the cellulose and the hemicellulose. The Kraft pulping process

removes lignin under highly alkaline conditions, which likely also results in the deacetylation of the hemicellulose component in the hardwood. This is a similar effect to the targeted deacetylation of the corn stover in the case of the DASC substrate, discussed earlier.

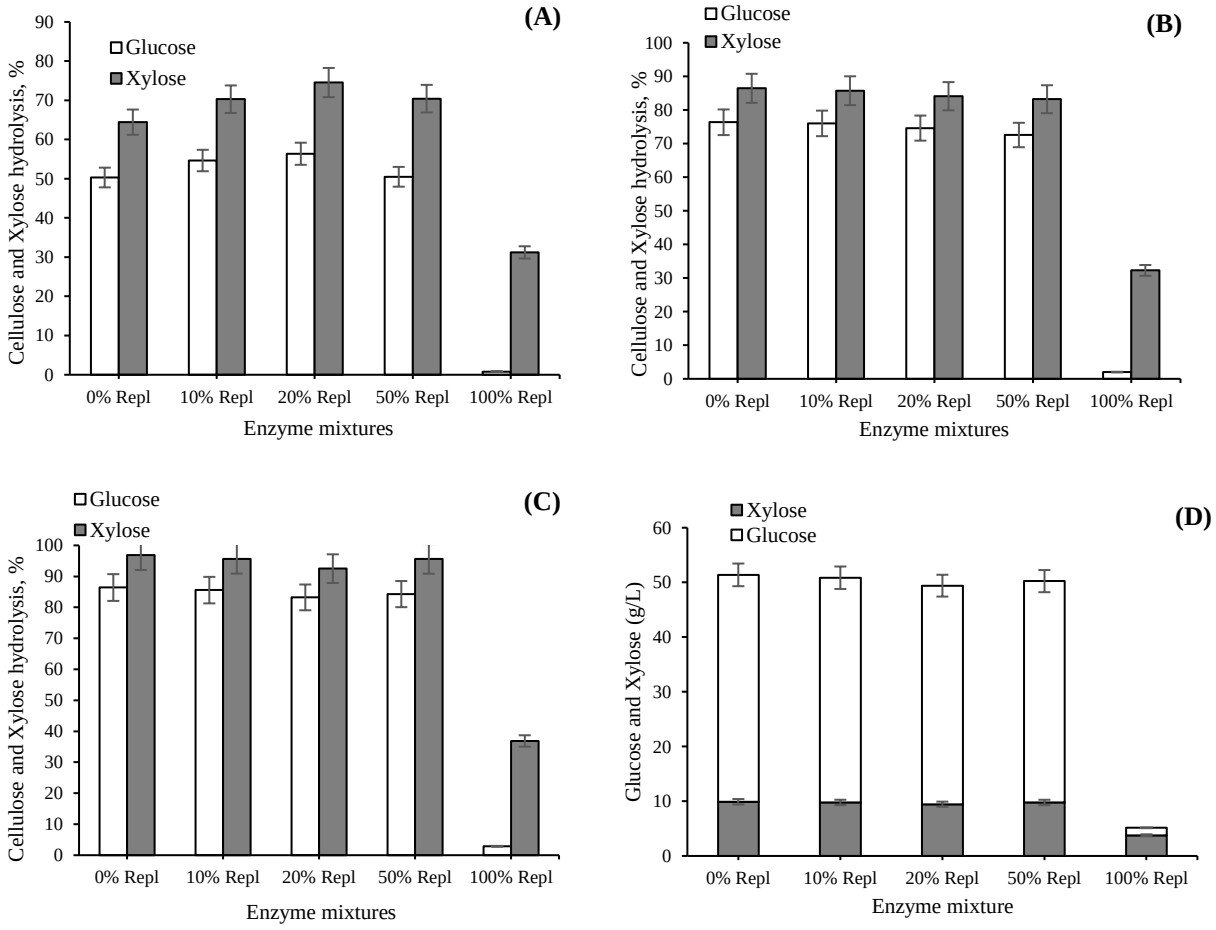

Figure 3.12: Synergistic effect of cellulase and xylanase (A–D) on hardwood bleached Kraft pulp with the corresponding hydrolysis time for (A) and (B), and (C) being 4 h, 24 h, and 72 h respectively. (D) is the total reducing sugar yield from different cellulase-xylanase replacement strategies. Error bars indicate the standard deviation (n = 2). Repl - % of CTec 3 replaced by xylanases.

Enzymatic hydrolysis results with partial replacement of the cellulase cocktail with xylanases showed that the partial replacement of the cellulases with xylanase was beneficial during the initial stages of hydrolysis (Figure 3.12A). However when the hydrolysis reaction time was extended beyond 4 hours, the cellulases synergistically cooperated with the xylanase to maintain

the same cellulose and xylose hydrolysis yields for all of the tested enzyme cocktails (0%Repl, 10%Repl, 20%Repl, and 50%Repl) constant (Figure 3.12B-C).

These results suggested a high degree of synergism between the cellulases and xylanases as substituting 50% of the cellulase cocktail with xylanases still resulted in the hydrolysis of 80 and 100% of the cellulose and xylan respectively. Suprisingly, when 50% of the initial cellulase loading (15 mg CTec 3/ g cellulose) was used without xylanase supplementation to hydrolyze HBKP, there was a ~10% and ~25% reduction in cellulose and xylan hydrolysis (Table 3.4) as well as the total reducing sugar. This seemed to indicate that the xylanase was helpful in facilitating the hydrolysis of both the cellulose and the hemicellulose.

d) Determining the "overall" cellulase activity of the cellulase-xylanase enzyme mixture

Supplementing a commercial cellulase cocktail (CTec 3) with xylanases to hydrolyze a range of substrates that differed in their xylan and lignin contents revealed the important role played by xylanase in facilitating the hydrolysis of the cellulose and hemicellulose especially in the case of xylan rich substrates. However, although the substitution of a portion of the cellulases with xylanases might aid in the hydrolysis of xylan rich substrates, it was likely that this enhancement might not be reflected in an increase in filter paper activity, as measured by the FPA. It was therefore of interest to assess the effects of replacing a portion of cellulase cocktail (CTec 3) with xylanases on the ability of the FPA to predict the hydrolytic potential of a cellulase-xylanase mixture that was shown to effectively hydrolyze xylan rich substrates. Therefore, the cellulase activity of a cellulase cocktail substituted with the various proportions of xylanases was measured using the FPA.

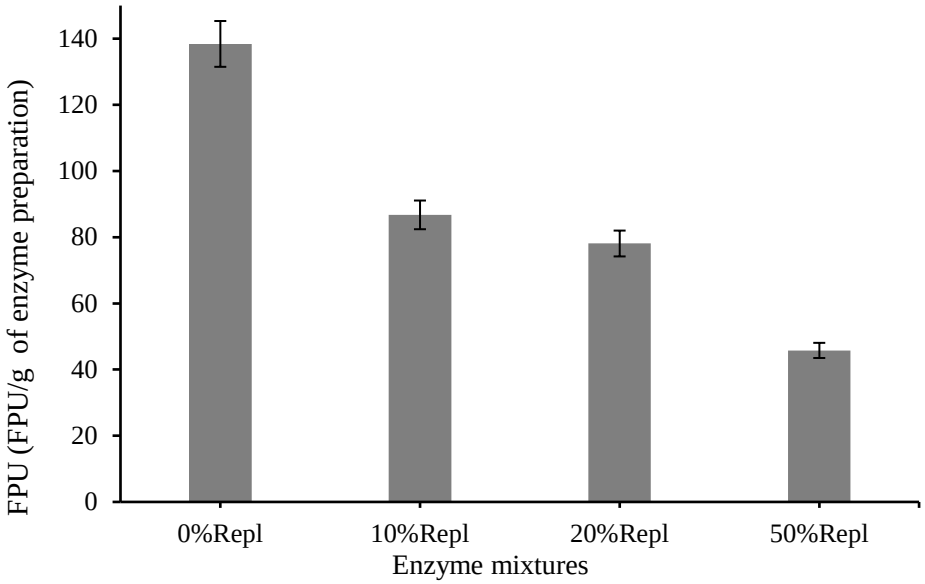

Figure 3.13: Filter paper units (FPU) of CTec 3 with xylanase. Error bars indicate the standard deviation (n = 3). %Repl – percentage (%) of CTec 3 replaced by Xylanase

It was apparent that the replacement of the cellulase cocktail with xylanase resulted in a decrease in total filter paper activity (Figure 3.13). Surprisingly, replacing only 10% of the cellulase cocktail with xylanases (10% Repl) resulted in a 40% decrease in the measured FPU. Further decreases, while replacing 50% of the cellulase cocktail, actually decreased the activity to <50% of the cellulase cocktail without xylanase replacement (Figure 3.13). Thus, unlike the "real" xylan rich substrates studied in the previous sections where there was synergism between cellulases and xylanases, the cellulase enzymes seemed to be the only "essential components" involved in the hydrolysis of the cellulose rich Whatman No.1 filter paper used in the FPA.

3.2.3.2. Lignin-rich substrates

a) Steam pretreated softwood

Steam-pretreated softwood (SPS) substrates were used to asssess the possible synergy between cellulases and the addition of BSA limiting enzyme binding to the lignin

component (Table 3.3). Previous work had reported that, after steam pretreatment of softwoods, the water-insoluble lignin and cellulose content of the substrate increases due to solubilization of the hemicellulose in the liquid stream (Chandra et al., 2007; Nakagame et al., 2011a; Shevchenko et al., 2001). The residual lignin in steam pretreated softwoods has been shown to play a pivotal role in the ease of hydrolysis of the cellulose component as lignin removal from these substrates has been shown to result in enhanced hydrolysis of the cellulose.

Mooney et al. (1998) suggested that the lignin present in pretreated softwoods inhibits cellulose hydrolysis through steric hindrance of the cellulose, while other workers have attributed it to unproductive binding of cellulases onto lignin rather than the cellulose (Berlin et al., 2006a; Kumar et al., 2012; Nakagame et al., 2011b; Siqueira et al., 2017). The effects of lignin on enzymatic hydrolysis are not readily alleviated through the synergistic action of accessory enzymes. Therefore, rather than adding auxiliary enzyme activities to the enzyme cocktail, bovine serum albumin (BSA) was used to replace a portion of the cellulase (CTec 3) to prevent unproductive binding of cellulases to the surface of lignin as was demonstrated in previous studies (Kim et al., 2014; Siqueira et al., 2017; Yang and Wyman, 2006).

Partial replacement of the cellulase cocktail with BSA resulted in similar hydrolysis yields to the cellulase cocktail without replacement. However, when 50% of the cellulase cocktail was replaced by BSA the hydrolysis yield underwent a slight decrease (50%Repl), (Figure 3.14A-C). Although the partial replacement of the cellulases with BSA did not result in increases in cellulose hydrolysis, it should be noted that when 50% of the maximum cellulase (15 mg CTec 3/ g cellulose) loading was used alone (without BSA supplementation) the cellulose hydrolysis yield decreased to only ~49% (Table 3.4) as compared to the 65% when the BSA was added (Figure 3.14). These results suggest that the BSA was helpful in aiding the hydrolysis of the SPS substrate,

but it is likely that the 38% lignin (Table 3.3) contained in the substrate presented a physical barrier inhibiting hydrolysis while the BSA typically only helps partially alleviate non-productive binding.

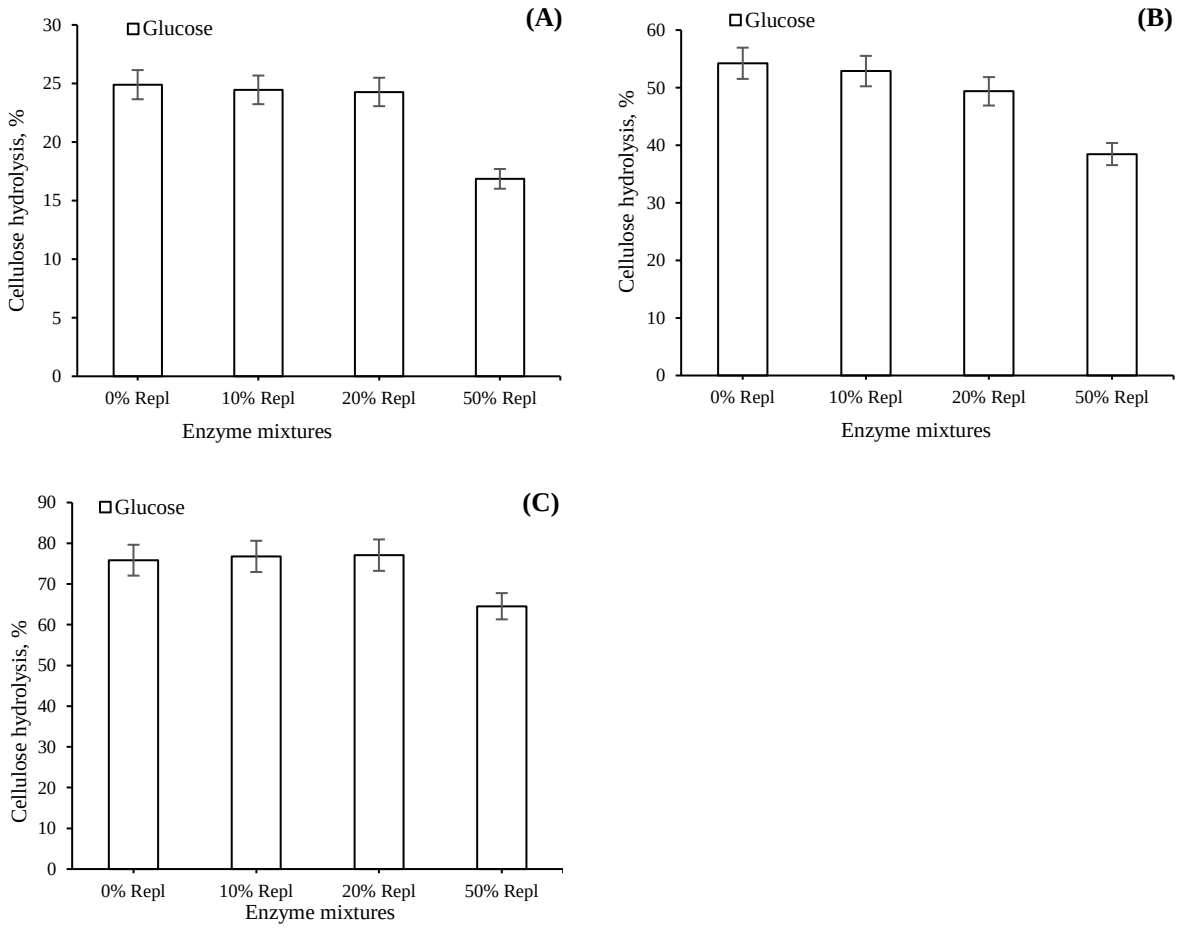

Figure 3.14: Synergistic effect of cellulase and BSA (A–C) on Steam pretreated softwood with the corresponding hydrolysis time for (A) and (B), and (C) being 4 h, 24 h, and 72 h respectively. Error bars indicate the standard deviation (n = 2). Repl - % of CTec 3 replaced by BSA.

b) Assessing total cellulase activity for the cellulase-BSA complexed enzyme mixture

Similar to the experiments with xylan rich substrates in the previous sections where the replacement of cellulases in the enzyme cocktail resulted in decreased filter paper activity when

the modified cocktail was measured using the FPA, the activity of the cellulase cocktails substituted with BSA were also measured using the FPA. Similar to what was observed with xylanase enzyme, replacement of CTec 3 with BSA reduced the total cellulase (CTec 3) activity in the FPA (Figure 3.15). The cellulase activity, decreased by 25%, 34% and 58% when the cellulases were replaced with 10%, 20% and 50% with BSA respectively.

These results were anticipated since the Whatman No.1 filter paper is a highly cellulosic substrate and BSA does not contribute enzyme activity, but rather acted as a lignin-blocking agent. This is consistent with the findings of Yang and Wyman (2006) who found that only a small amount of BSA was adsorbed by Avicel cellulose. Therefore, it was apparent that the addition of protein to the enzyme cocktail, to decrease possible enzyme binding to the lignin within "real" lignocellulosic substrates, such as steam pretreated softwoods, are not reflected in an increase in the filter paper activity.

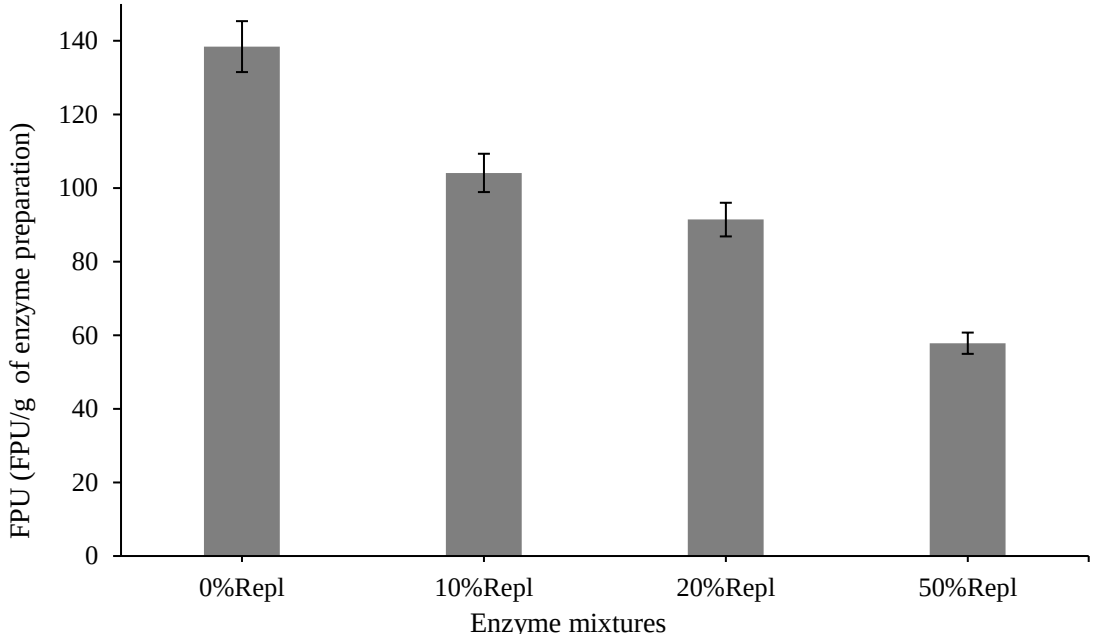

Figure 3.15: Filter paper units (FPU) of CTec 3 with BSA. Error bars indicate the standard deviation (n = 3). %Repl – percentage (%) of CTec 3 replaced by BSA.

3.2.3.3. Mannan containing substrates

a) Partially delignified TMP

Compared to xylan rich substrates, there has been far fewer studies detailing the ability of hydrolytic enzymes to breakdown mannan containing substrates. Due to their lower degree of acetylation compared to xylans, galactoglucomannans are typically more labile and are thus partially solubilized under highly alkaline Kraft pulping conditions. Similarly, the acidic conditions employed during steam, organosolv or dilute acid pretreatments tend to hydrolyze and solubilize the mannan component. Therefore, in order to preserve the hemicellulose in the water insoluble substrate fraction, a thermomechanical pulp was used. Thermomechanical pulps involve a pre-steaming of wood chips with subsequent mechanical defibration. However, these softwood TMP pulps have been shown to be highly recalcitrant to enzymatic hydrolysis due to their high lignin content (Boussaid and Saddler, 1999; Mooney et al., 1998).

Therefore, the TMP was subjected to partial delignification using sodium chlorite, which reduced the lignin content and provided a substrate that contained approximately 16% mannan (Table 3.3). It was predicted that replacement of a portion of cellulase cocktail with 10%, 20%, and 50% mannanase might boost the hydrolysis yields of a partially delignified TMP. Previous work had shown that endo-β-mannanase have a high specificity for galactoglucomannan, breaking it down to mannose at the reducing end, resulting in improved cellulose accessibility (Katsimpouras et al., 2016; Tenkanen et al., 1997; Várnai et al., 2011).

Similar to the results observed earlier with the steam pretreated softwood, a 50% substitution of the cellulase cocktail with mannanases resulted in a decrease in cellulose hydrolysis but 10 and 20% replacements maintained the same hydrolysis yields as the initial 100% cellulase

cocktail (0%Repl) with slight increases to the mannan hydrolysis (Figure 3.16). Employing a 50% loading of the cellulase cocktail without mannanases resulted in a yield of only 39% (Table 3.4) compared to the hydrolysis yield of >70% with the cocktail that contained 50% mannanases. Therefore, similar to the results observed when replacing cellulases with xylanases in the enzyme cocktail used to hydrolyze xylan rich substrates, it was evident that the mannanases and cellulases worked together to increase the total glucose and mannose yields when applied to mannan rich substrates.

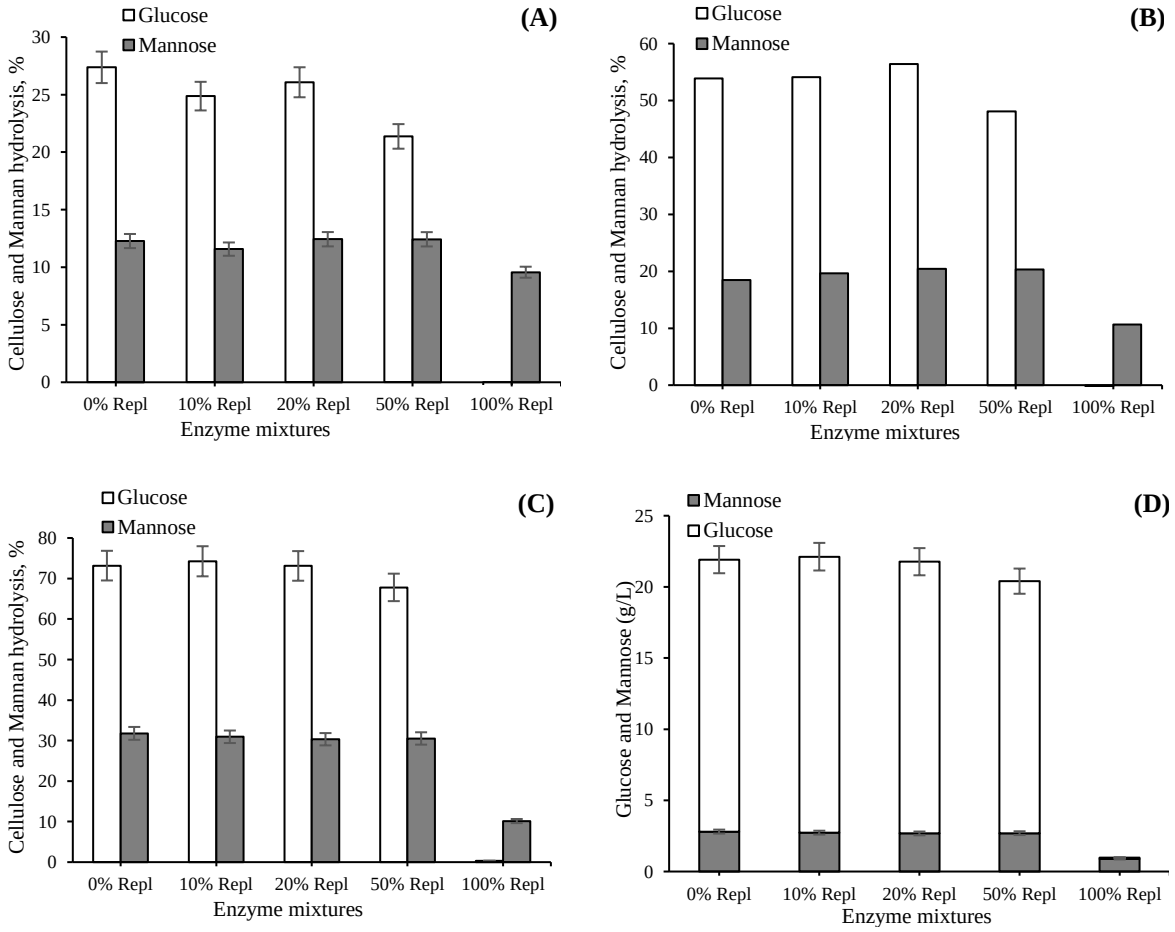

Figure 3.16: Synergistic effect of cellulase and mannanase (A–D) on partially delignified TMP with the corresponding hydrolysis time for (A) and (B), and (C) being 4 h, 24 h, and 72 h respectively. (D) is the total reducing sugar yield from different cellulase-mannanase replacement strategies. Error bars indicate the standard deviation (n = 2). Repl - % of CTec 3 replaced by mannanase.

b) Softwood bleached Kraft pulp

Compared to the partially de-lignified softwood TMP described above that contained 15% lignin, the softwood bleached Kraft pulp (SBKP) was assessed as a mannan containing substrate that was virtually lignin free, similar to the xylan-rich hardwood Kraft pulp discussed earlier (Table 3.3). However, the SBKP also contained, 8% xylan, the cellulase cocktail was substituted with both xylanases and mannanases to determine if these enzymes could act synergistically with the cellulases to improve hydrolysis yields. It was apparent that after 72 hours, substitution of the cellulase cocktail with the 50:50 mixture of xylanases and mannanases resulted in an increase in cellulose, mannan and xylan hydrolysis compared to the 100% cellulase cocktail (0% Repl). (Figure 3.17D).

It was also evident that the replacement of 20% of the cellulase cocktail with the 50:50 xylanase: mannanase mix was the most effective in increasing the total hydrolysis yields. When the cellulase loading was reduced to 50% of the initial loading, the cellulose, mannan and xylan hydrolysis yield all decreased by approximately 9%. Therefore, it was apparent that partially substituting the cellulases with the xylanases and mannanases improved enzymatic hydrolysis yields.

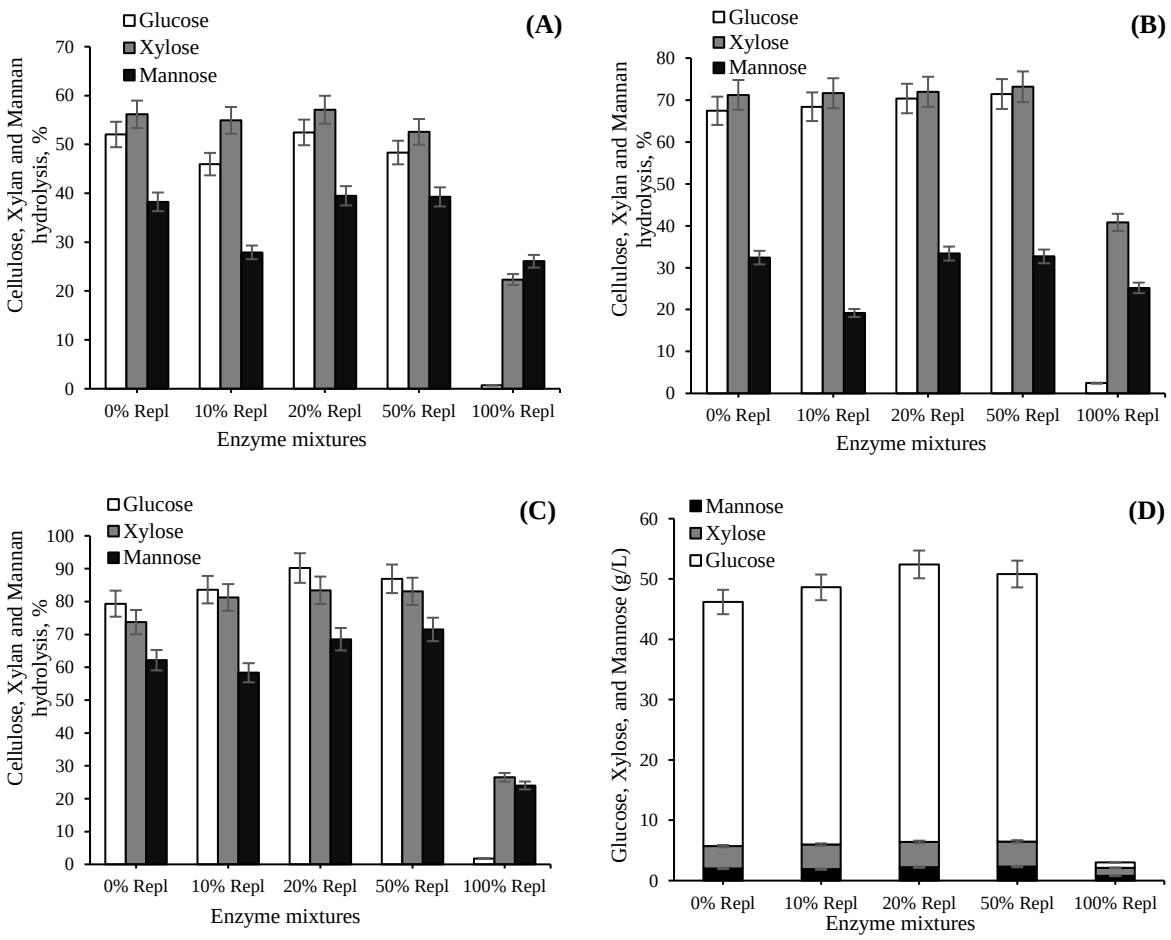

Figure 3.17: Synergistic effect of cellulase, xylanase, and mannanase (A–D) on softwood bleached Kraft pulp with the corresponding hydrolysis time for (A) and (B), and (C) being 4 h, 24 h, and 72 h respectively. (D) is the total reducing sugar yield from different cellulase-Xylanase-mannanase replacement strategies. Error bars indicate the standard deviation (n = 2). Repl - % of CTec 3 replaced by Xylanases and mannanases.

c) Assessing the total cellulase activity for the cellulase-xylanase-Mannase enzyme mixture

Since the previous section showed that the partial replacement of the cellulase cocktail (Ctec 3) with a combination of xylanases and mannanases enhanced the total reducing sugar yield of a softwood bleached Kraft pulp, similar to previous sections the activity of the cellulase-mannanase-xylanase cocktail was quantified in the FPA. Unlike the actual enzymatic

hydrolysis of the softwood bleached Kraft pulp and partially delignified TMP shown in the previous sections (where mannanases and a mannanase-xylanase combination increased hydrolysis yields), replacement of cellulases (CTec 3) with the mannanases and xylanases resulted in a decreased measured FPA activity (Figure 3.18). The decrease in cellulase activity in the FPA using the partially substituted cellulase cocktails was consistent with every substrate that was studied in this section. Although it has been shown here and in other work that the adjustment of cellulase cocktails based on the chemical composition of pretreated lignocellulosic substrates can significantly reduce total cellulase loading and increase cellulase activity on lignocellulose, the FPA seems insensitive to the enhancements provided by accessory enzymes. This is primarily due to the differences between the cellulose-rich Whatman No.1 filter paper utilized in the FPA and "real" pretreated lignocellulosic substrates that contain lignin and hemicellulose (Himmel et al., 2017).

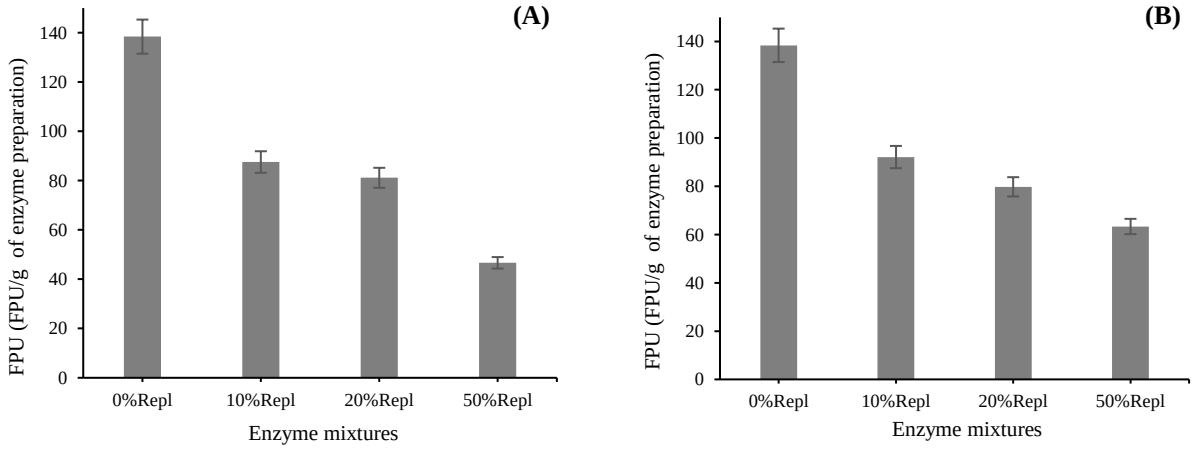

Figure 3.18: Filter paper units (FPU) of CTec 3 with (A) mannanase, and (B) xylanase and mannanase. Error bars indicate the standard deviation (n = 3). Repl - % of CTec 3 replaced by mannanase, and xylanase and mannanase.

3.2.4. Hydrolysis of Whatman No.1 filter paper with different CTec 3 replaced cocktails

Since the FPA is a one-hour assay, yet complete hydrolysis of cellulose takes at least 24 hours, it was of interest to assess whether the standardized FPA is representative of the

hydrolysis yields obtained after hydrolyzing the Whatman No.1 filter paper for 72 hours using the different cellulase cocktails. Similar to what was observed in the FPA results of a cellulase preparation (CTec 3), the cellulose hydrolysis when cellulase enzymes were replaced with mannanase, xylanase and BSA decreased with an increase in cellulase replacement for all the different cocktails (Figure 3.19).

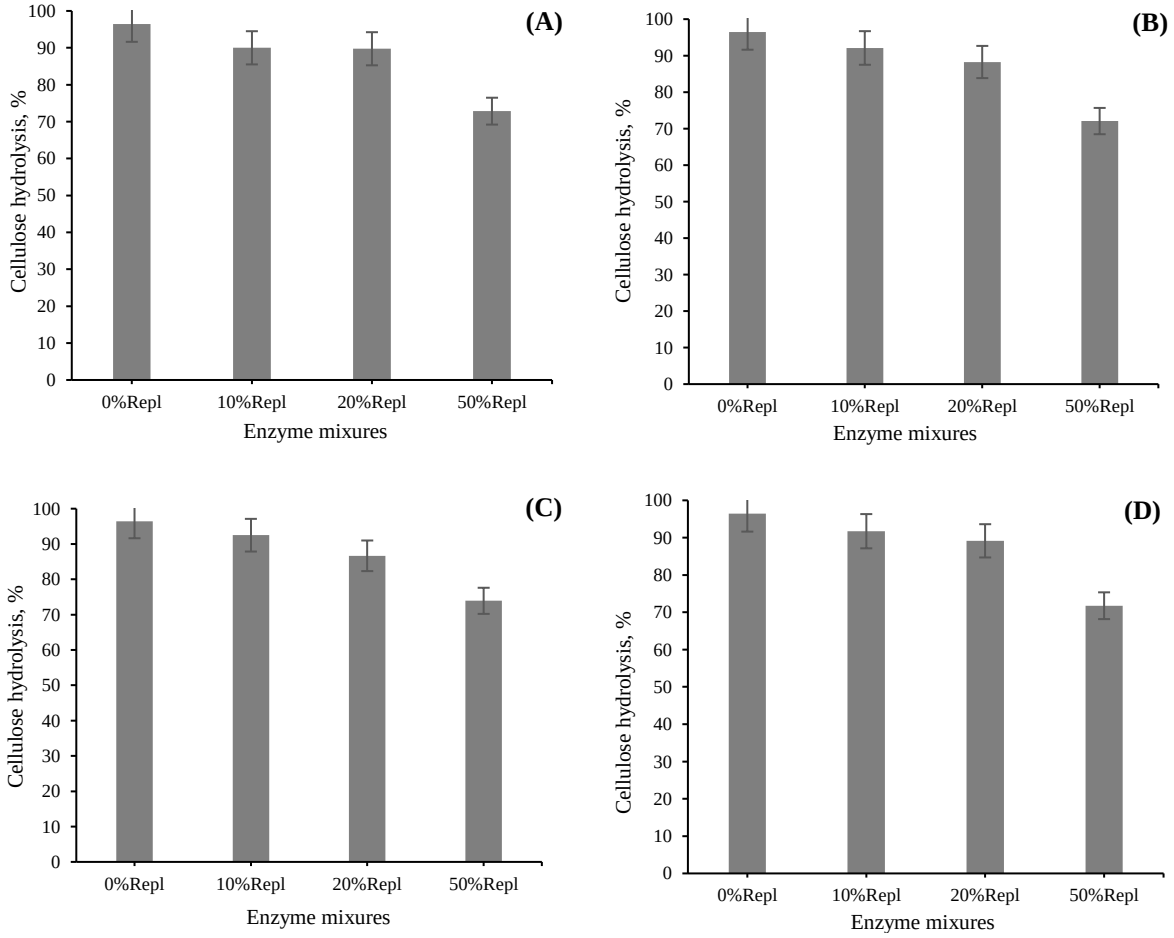

Figure 3.19: Synergistic effect of cellulase, xylanase (A), BSA (B), mannanase (C), and xylanase and mannanase (D) when hydrolyzing Whatman No.1 filter paper corresponding to a hydrolysis time of 72 hours. Error bars indicate the standard deviation (n = 2). Repl - % of CTec 3 replaced by Xylanases, BSA, and mannanase. Hydrolysis conditions: 50^0C, 150 rpm shaking, pH 4.8.

This again supports our earlier assertion that, when hydrolyzing a highly cellulosic substrate like the Whatman No.1 filter paper, cellulases are the key enzymes. As such, the FPA is

not reflective of the hydrolytic performance of certain enzyme mixture especially those which have been developed to work on more realistic lignocellulosic substrates.

3.2.5. Conclusions

Substituting cellulase enzymes with accessory enzyme activities that are known to enhance enzymatic hydrolysis of actual pretreated substrates was assessed. Lignocellulosic substrates were produced to contain different amounts of cellulose, xylan, mannan and lignin. They were subsequently used to assess how the addition of xylanase and mannanase, as well as the ability of BSA to block enzyme adsorption to lignin, consequently increasing enzymatic hydrolysis yields when these proteins were substituted for cellulases in the enzyme cocktail. The substitution of the cellulase cocktails with accessory enzymes was shown to enhance or at least maintain hydrolysis yields even though the enzyme cocktail contained a lower amount of cellulases. However, it was evident that when the activity of the same enzyme cocktails containing accessory enzymes were assessed using the FPA, the increases observed during the actual enzymatic hydrolysis were not reflected in an increase in overall FPU activity. It was apparent that the high cellulose content of the Whatman no.1 filter paper compromises the ability of the FPA to effectively quantify the activities of accessory enzymes such as mannanases and xylanases on more realistic lignocellulosic substrates.

4. General conclusions and future work

4.1. Conclusions

The work in this thesis shed light on the shortcomings of the FPA and its ability to predict the hydrolytic potential of a given cellulase enzyme cocktail. The limitations of the FPA arise mainly from the characteristics of the Whatman No. 1 filter paper substrate that is used in the assay. The process of drying and sheet-forming undergone by the Whatman No.1 filter paper used in the assay decreases this substrates accessibility, resulting in an underestimation of cellulase activity. Consequently, this compromises the prediction of cellulase activity on substrates that are typically not dried or formed into sheets prior to enzymatic hydrolysis. The work also showed that the Whatman No.1 filter paper substrate has a cellulose content >95%. The FPA exhibited behaviour similar to that obtained with the hydrolysis of a pre-hydrolyzed Kraft pulp. This is a high value purified cellulose feedstock typically used for the production of cellulose derivatives, not for lower value applications such as enzymatic breakdown to sugar. This high cellulose content of the filter paper substrate also compromises the ability of the FPA to predict the ease of hydrolysis of typical pretreated lignocellulosic substrates that contain both hemicellulose and lignin. When the Whatman No.1 filter paper used in the FPA was substituted with paper produced using organosolv pretreated hardwood substrates, which contained up to 15% hemicellulose and 29%dwt lignin, it was likely that the lignin and hemicellulose compromised accessibility to the cellulose.

We assessed the potential of changing the cocktail mix as one way of increasing hydrolysis and lowering the cellulase enzyme protein loading required to obtain effective enzymatic hydrolysis of real pretreated lignocellulosic substrates. These accessory enzymes have been shown to work synergistically with cellulases to hydrolyze the non-cellulosic components, such as hemicellulose, to increase the accessibility of cellulases to cellulose. This consequently

increases the cellulose hydrolysis yield and also the overall sugar yield from the biomass (cellulose and hemicellulose). However, due to the highly pure cellulose used to produce the Whatman No 1 filter paper, it was likely that the filter paper assay did not effectively predict the hydrolytic potential of cellulase cocktails when accessory enzymes were added. Therefore, in the second section of the thesis substrates were prepared which varied in their xylan, mannan and lignin content. The CTec 3 cellulase cocktail was then partially substituted with xylanases, mannanases and BSA to potentially enhance the hydrolysis of the respective xylan, mannan and lignin rich substrates. The addition of these accessory enzymes increased cellulose hydrolysis and overall sugar yields and allowed for up to 50% of the cellulase cocktail to be substituted with the accessory enzymes while maintaining virtually the same cellulose hydrolysis yield as the original cellulase cocktail. However, when the cellulase cocktails that had been substituted with accessory enzymes were tested in the FPA, it was apparent that, even though similar or increased hydrolysis yields were obtained with these cocktails when they were applied to real substrates, these modified cocktails showed reduced FPA reactivity. Unfortunately, it was apparent that the filter paper assay was unable to predict the hydrolytic capability of these modified enzyme cocktails on real pretreated lignocellulosic substrates.

The thesis work highlighted the shortcomings of the FPA with regard to predicting the hydrolysis of real lignocellulosic substrates. However, the work also emphasised the high level of variability that can result with regard to the substrates being hydrolysed by cellulase cocktails. The substrate variability that arises from the numerous variables including, biomass heterogeneity, processes such as drying or pressing, the type/conditions of pretreatments/pulping processes employed, etc., all have an effect. As a result, it continues to be a significant challenge to develop a “universal” assay to predict cellulase activity.

4.2. Future work

4.2.1. Optimization of the "cellulase cocktail" for specific lignocellulosic substrate

The replacement of commercial cellulase preparation with accessory enzymes enhanced reducing sugar yield upon hydrolysis. Thus, it was apparent that accessory enzymes improved the cellulase activity of CTec 3 when hydrolysing lignocellulosic substrates, though this improvement was not reflected in the standardized FPA. It was also apparent that adding accessory enzymes based on substrate composition did result in an increase in the hydrolytic potential of that particular enzyme mixture for that particular substrate. For instance, xylanase and mannanase enzymes boosted the reducing sugar yields of xylan-rich and mannan-rich substrates respectively. Though a chemical composition analysis was conducted on pretreated lignocellulosic substrates, the commercial cellulase preparation (CTec 3) was not characterized for its composition in terms of specific enzyme activity. Sun et al. (2015) characterized the CTec 3 enzyme to be rich in xylanase and β-glucosidase enzymes. However the same study never assessed the composition of other accessory enzymes such as the LPMOs in the cocktail, CTec 3 tolerance to inhibitors and lignin effects and its thermos-stability (Sun et al., 2015). This should be done. This will enable us to envisage how different cellulase cocktails can hydrolyse a range of substrates for which they were custom-made.

4.2.2. Development of a prolonged throughput assay for lignocellulosic substrates

The current one-hour FPA assay does not predict the hydrolysis yields obtained when the hydrolysis time is extended to a more realistic 48 to 72hours. Hence, further research should be conducted to assess the influence of modifying/optimising enzyme cocktails and the appropriate biomass substrates over a longer hydrolysis time preferably measuring cellulase activity at 12 hours, using high substrate concentrations.

References

Aden, A., Foust, T., 2009. Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. Cellulose 16, 535–545. doi:10.1007/s10570-009-9327-8

Agger, J., Viksø-Nielsen, A., Meyer, A.S., 2010. Enzymatic xylose release from pretreated corn bran arabinoxylan: Differential effects of deacetylation and deferuloylation on insoluble and soluble substrate fractions. J. Agric. Food Chem. 58, 6141–6148. doi:10.1021/jf100633f

Andersen, N., Johansen, K.S., Michelsen, M., Stenby, E.H., Krogh, K.B.R.M., Olsson, L., 2008. Hydrolysis of cellulose using mono-component enzymes shows synergy during hydrolysis of phosphoric acid swollen cellulose (PASC), but competition on Avicel. Enzyme Microb. Technol. 42, 362–370. doi:10.1016/j.enzmictec.2007.11.018

Arantes, V., Saddler, J.N., 2011. Cellulose accessibility limits the effectiveness of minimum cellulase loading on the efficient hydrolysis of pretreated lignocellulosic substrates. Biotechnol Biofuels 4, 3. doi:10.1186/1754-6834-4-3

Araujo, E.F., Barros, E.G., Caldas, R.A., Silva, D.O., 1983. Beta-glucosidase activity of a thermophilic cellulolytic fungus, Humicola sp. Biotechnol. Lett. 5, 781–784. doi:10.1007/BF01386502

Arola, S., Linder, M.B., 2016. Binding of cellulose binding modules reveal differences between cellulose substrates. Sci. Rep. 6, 35358. doi:10.1038/srep35358

Baker, J.O., Adney, W.S., Thomas, S.R., Nieves, R.A., Chou, Y.-C., Vinzant, T.B., Tucker, M.P., Laymon, R.A., Himmel, M.E., 1996. Synergism Between Purified Bacterial and Fungal Cellulases. pp. 113–141. doi:10.1021/bk-1995-0618.ch009

Baker, J.O., Vinzant, T.B., Ehrman, C.I., Adney, W.S., Himmel, M.E., 1997. Use of a new membrane-reactor saccharification assay to evaluate the performance of celluloses under simulated SSF conditions : effect on enzyme quality of growing Trichoderma reesci in the presence of targeted lignocellulosic substrate. Appl. Biochem. Biotechnol. 63–65, 585–595. doi:10.1007/BF02920456

Barr, B.K., Hsieh, Y.L., Ganem, B., Wilson, D.B., 1996. Identification of two functionally different classes of exocellulases. Biochemistry 35, 586–592. doi:10.1021/bi9520388

Beckham, G.T., Bomble, Y.J., Matthews, J.F., Taylor, C.B., Resch, M.G., Yarbrough, J.M., Decker, S.R., Bu, L., Zhao, X., McCabe, C., Wohlert, J., Bergenstråhle, M., Brady, J.W., Adney, W.S., Himmel, M.E., Crowley, M.F., 2010. The O-Glycosylated linker from the Trichoderma reesei family 7 cellulase is a flexible, disordered protein. Biophys. J. 99, 3773–3781. doi:10.1016/j.bpj.2010.10.032

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., Saddler, J., 2006a. Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. J. Biotechnol. 125, 198–209. doi:10.1016/j.jbiotec.2006.02.021

Berlin, A., Gilkes, N., Kilburn, D., Bura, R., Markov, A., Skomarovsky, A., Okunev, O., Gusakov, A., Maximenko, V., Gregg, D., Sinitsyn, A., Saddler, J., 2005a. Evaluation of novel fungal

cellulase preparations for ability to hydrolyze softwood substrates - Evidence for the role of accessory enzymes. Enzyme Microb. Technol. 37, 175–184. doi:10.1016/j.enzmictec.2005.01.039

Berlin, A., Girkes, N., Kurabi, A., Tu, M., Kilburn, D., Saddler, J., 2005b. Weak lignin-binding enzymes a novel approach to improve activity of cellulases for hydrolysis of lignocellulosics. Appl. Biochem. Biotechnol. 121, 163–170. doi:10.1385/abab:121:1-3:0163

Berlin, A., Maximenko, V., Bura, R., Kang, K.-Y., Gilkes, N., Saddler, J., 2006b. A rapid microassay to evaluate enzymatic hydrolysis of lignocellulosic substrates. Biotechnol. Bioeng. 93, 880–886. doi:10.1002/bit.20783

Bezerra, R.M.F., Dias, A.A., 2005. Enzymatic kinetic of cellulose hydrolysis. Appl. Biochem. Biotechnol. 126, 49–59. doi:10.1007/s12010-005-0005-5

Bezerra, R.M.F., Dias, A.A., 2004. Discrimination among eight modified michaelis-menten kinetics models of cellulose hydrolysis with a large range of substrate/enzyme ratios: inhibition by cellobiose. Appl. Biochem. Biotechnol. 112, 173–84.

Bhat, M.K., Hazlewood, G.P., 2001. Enzymology and other characteristics of Cellulases and Xylanases. Enzym. Farm Anim. Nutr. 32. doi:10.1079/9780851993935.0011

Bhattacharya, A.S., Bhattacharya, A., Pletschke, B.I., 2015. Synergism of fungal and bacterial cellulases and hemicellulases: a novel perspective for enhanced bio-ethanol production. Biotechnol. Lett. doi:10.1007/s10529-015-1779-3

Biermann, C.J., 1996. Handbook of Pulping and Papermaking, 2nd ed. Elsevier Inc. doi:10.1016/B978-012097362-0/50008-X

Boehm, R.M., 1930. The Masonite Process. Ind. Eng. Chem. 22, 493–497. doi:10.1021/ie50245a019

Boisset, C., Fraschini, C., Schülein, M., Henrissat, B., Chanzy, H., 2000. Imaging the enzymatic digestion of bacterial cellulose ribbons reveals the endo character of the cellobiohydrolase Cel6A from Humicola insolens and its mode of synergy with cellobiohydrolase Cel7A. Appl. Environ. Microbiol. 66, 1444–1452. doi:10.1128/AEM.66.4.1444-1452.2000

Boussaid, A., Saddler, J.N., 1999. Adsorption and activity profiles of cellulases during the hydrolysis of two Douglas fir pulps. Enzyme Microb. Technol. 24, 138–143. doi:10.1016/S0141-0229(98)00096-9

Breuil, C., Mayers, P., Saddler, J.N., 1986. Substrate conditions that influence the assays used for determining the β-glucosidase activity of cellulolytic microorganisms. Biotechnol. Bioeng. 28, 1653–1656. doi:10.1002/bit.260281109

Bura, R., Bothast, R.J., Mansfield, S.D., Saddler, J.N., 2003a. SO 2 -Catalyzed Steam Pretreatment of Corn Fiber Optimization of SO 2 -Catalyzed Steam Pretreatment of Corn Fiber for Ethanol Production. Appl. Biochem. Biotechnol. 105108.

Bura, R., Bothast, R.J., Mansfield, S.D., Saddler, J.N., 2003b. Optimization of SO2-catalyzed steam pretreatment of corn fiber for ethanol production. Appl. Biochem. Biotechnol. 105–108, 319–335. doi:10.1385/ABAB:106:1-3:319

Bura, R., Chandra, R., Saddler, J., 2009. Influence of Xylan on the enzymatic hydrolysis of steam-pretreated corn Stover and hybrid poplar. Biotechnol. Prog. 25, 315–322. doi:10.1002/btpr.98

Camassola, M., Dillon, J.P.A., 2012. Cellulase Determination: Modifications to Make the Filter Paper Assay Easy, Fast, Practical and Efficient. J. Anal. Bioanal. Tech. 01, 10–13. doi:10.4172/scientificreports.125

Chandra, M., Kalra, A., Sharma, P.K., Sangwan, R.S., 2009. Cellulase production by six Trichoderma spp. fermented on medicinal plant processings. J. Ind. Microbiol. Biotechnol. 36, 605–609. doi:10.1007/s10295-009-0544-9

Chandra, R., Ewanick, S., Hsieh, C., Saddler, J.N., 2008. The characterization of pretreated lignocellulosic substrates prior to enzymatic hydrolysis, part 1: A modified Simons' staining technique. Biotechnol. Prog. 24, 1178–1185. doi:10.1002/btpr.33

Chandra, R.P., Bura, R., Mabee, W.E., Berlin, A., Pan, X., Saddler, J.N., 2007. Substrate Pretreatment: The Key to Effective Enzymatic Hydrolysis of Lignocellulosics?, in: Biofuels. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 67–93. doi:10.1007/10_2007_064

Chandra, R.P., Chu, Q.L., Hu, J., Zhong, N., Lin, M., Lee, J.S., Saddler, J., 2016. The influence of lignin on steam pretreatment and mechanical pulping of poplar to achieve high sugar recovery and ease of enzymatic hydrolysis. Bioresour. Technol. 199, 135–141. doi:10.1016/j.biortech.2015.09.019

Chandra, R.P., Saddler, J.N., 2012. Use of the Simons' Staining Technique to Assess Cellulose Accessibility in Pretreated Substrates. Ind. Biotechnol. 8, 230–237. doi:10.1089/ind.2012.0016

Chang, L., Ding, M., Bao, L., Chen, Y., Zhou, J., Lu, H., 2011. Characterization of a bifunctional xylanase/endoglucanase from yak rumen microorganisms. Appl. Microbiol. Biotechnol. 90, 1933–1942. doi:10.1007/s00253-011-3182-x

Chen, H., 2014. Biotechnology of lignocellulose: Theory and practice, Biotechnology of Lignocellulose: Theory and Practice. doi:10.1007/978-94-007-6898-7

Chen, H., Venditti, R.A., Jameel, H., Park, S., 2012. Enzymatic hydrolysis of recovered office printing paper with low enzyme dosages to produce fermentable sugars. Appl. Biochem. Biotechnol. 166, 1121–1136. doi:10.1007/s12010-011-9498-2

Chen, X., Kuhn, E., Wang, W., Park, S., Flanegan, K., Trass, O., Tenlep, L., Tao, L., Tucker, M., 2013. Comparison of different mechanical refining technologies on the enzymatic digestibility of low severity acid pretreated corn stover. Bioresour. Technol. 147, 401–408. doi:10.1016/j.biortech.2013.07.109

Chen, X., Shekiro, J., Elander, R., Tucker, M., 2012. Improved Xylan hydrolysis of corn stover by deacetylation with high solids dilute acid pretreatment. Ind. Eng. Chem. Res. 51, 70–76. doi:10.1021/ie201493g

Chen, X., Shekiro, J., Pschorn, T., Sabourin, M., Tao, L., Elander, R., Park, S., Jennings, E., Nelson, R., Trass, O., Flanegan, K., Wang, W., Himmel, M.E., Johnson, D., Tucker, M.P., 2014. A highly efficient dilute alkali deacetylation and mechanical (disc) refining process for the conversion of renewable biomass to lower cost sugars. Biotechnol. Biofuels 7, 98.

doi:10.1186/1754-6834-7-98

Chu, Q., Chandra, R.P., Kim, C.S., Saddler, J.N., 2017. Alkali-Oxygen Impregnation Prior to Steam Pretreating Poplar Wood Chips Enhances Selective Lignin Modification and Removal while Maximizing Carbohydrate Recovery, Cellulose Accessibility, and Enzymatic Hydrolysis. ACS Sustain. Chem. Eng. 5, 4011–4017. doi:10.1021/acssuschemeng.6b03169

Chundawat, S.P.S., Balan, V., Dale, B.E., 2008. High-throughput microplate technique for enzymatic hydrolysis of lignocellulosic biomass. Biotechnol. Bioeng. 99, 1281–1294. doi:10.1002/bit.21805

Converse, A.O., Matsuno, R., Tanaka, M., Taniguchi, M., 1988. A model of enzyme adsorption and hydrolysis of microcrystalline cellulose with slow deactivation of the adsorbed enzyme. Biotechnol. Bioeng. 32, 38–45. doi:10.1002/bit.260320107

Corazza, F.C., Calsavara, L.P. V, Moraes, F.F., Zanin, G.M., Neitzel, I., 2005. Determination of Inhibition in the Enzymatic Hydrolysis of Cellobiose Uing Hybrid Neural Modelling. Brazilian J. Chem. Eng. 22, 19–29.

Coward-Kelly, G., Aiello-Mazzari, C., Kim, S., Granda, C., Holtzapple, M., 2003. Suggested improvements to the standard filter paper assay used to measure cellulase activity. Biotechnol. Bioeng. 82, 745–749. doi:10.1002/bit.10620

Dashtban, M., Maki, M., Leung, K.T., Mao, C., Qin, W., 2010. Cellulase activities in biomass conversion: Measurement methods and comparison. Crit. Rev. Biotechnol. 30, 302–309. doi:10.3109/07388551.2010.490938

Dashtban, M., Schraft, H., Qin, W., 2009. Fungal bioconversion of lignocellulosic residues; Opportunities & perspectives. Int. J. Biol. Sci. 5, 578–595. doi:10.7150/ijbs.5.578

Decker, C.H., Visser, J., Schreier, P., 2001. β-Glucosidase multiplicity from Aspergillus tubingensis CBS 643.92: Purification and characterization of four β-glucosidases and their differentiation with respect to substrate specificity, glucose inhibition and acid tolerance. Appl. Microbiol. Biotechnol. 55, 157–163. doi:10.1007/s002530000462

Decker, S.R., Adney, W.S., Jennings, E., Vinzant, T.B., Himmel, M.E., 2003. Automated Filter Paper Assay for Determination of Cellulase Activity. Appl. Biochem. Biotechnol. 3, 689–703.

Del Rio, L.F., Chandra, R.P., Saddler, J.N., 2012. Fibre size does not appear to influence the ease of enzymatic hydrolysis of organosolv-pretreated softwoods. Bioresour. Technol. 107, 235–242. doi:10.1016/j.biortech.2011.12.057

Del Rio, L.F., Chandra, R.P., Saddler, J.N., 2010. The effect of varying organosolv pretreatment chemicals on the physicochemical properties and cellulolytic hydrolysis of mountain pine beetle-killed lodgepole pine. Appl. Biochem. Biotechnol. 161, 1–21. doi:10.1007/s12010-009-8786-6

Dence, C.W., 1992. The Determination of Lignin. In: Lin S.Y., Dence C.W. (eds) Methods in Lignin Chemistry. Springer Series in Wood Science. Springer, Berlin, Heidelberg, pp. 33–61. doi:10.1007/978-3-642-74065-7_3

Deshpande, M. V., Eriksson, K.-E., Göran Pettersson, L., 1984. An assay for selective determination of exo-1,4,-β-glucanases in a mixture of cellulolytic enzymes. Anal. Biochem. 138, 481–487. doi:10.1016/0003-2697(84)90843-1

Din, N., Gilkes, N.R., Tekant, B., Miller, R.C., Warren, R.A.J., Kilburn, D.G., 1991. Non–Hydrolytic Disruption of Cellulose Fibres by the Binding Domain of a Bacterial Cellulase. Bio/Technology 9, 1096–1099. doi:10.1038/nbt1191-1096

Divne, C., Ståhlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, J.K., Teeri, T.T., Jones, T.A., 1994. The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from Trichoderma reesei. Science 265, 524–8.

Dou, X., 2017. Elucidating the pulp properties that influence the ability of enzymes to facilitate the conversion of hardwood Kraft pulp to dissolving-grade pulps. Thesis submitted to the University of British Columbia for partial fulfillment of requirement of Degree of Philosphy in Forestry. doi:10.14288/1.0340609

Duedu, K.O., French, C.E., 2016. Characterization of a Cellulomonas fimi exoglucanase/xylanase-endoglucanase gene fusion which improves microbial degradation of cellulosic biomass. Enzyme Microb. Technol. 93–94, 113–121. doi:10.1016/J.ENZMICTEC.2016.08.005

Eriksson, T., Börjesson, J., Tjerneld, F., 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microb. Technol. 31, 353–364. doi:10.1016/S0141-0229(02)00134-5

Esteghlalian, A.R., Bilodeau, M., Mansfield, S.D., Saddler, J.N., 2001. Do enzymatic digestibility and Simons' stain reflect the differences in the available surface area of lignocellulosic substrates. Biotechnol Prog 17, 1049–1054. doi:10.1021/bp0101177

Eveleigh, D.E., Mandels, M., Andreotti, R., Roche, C., 2009. Measurement of saccharifying cellulase. Biotechnol. Biofuels 2, 21. doi:10.1186/1754-6834-2-21

Fägerstam, L.G., Pettersson, L.G., 1980. The 1.4-β-glucan cellobiohydrolases of Trichoderma reesei QM 9414: A new type of cellulolytic synergism. FEBS Lett. 119, 97–100. doi:10.1016/0014-5793(80)81006-4

Fan, L.T., Lee, Y.-H., Beardmore, D.H., 1980. Mechanism of the enzymatic hydrolysis of cellulose: Effects of major structural features of cellulose on enzymatic hydrolysis. Biotechnol. Bioeng. 22, 177–199. doi:10.1002/bit.260220113

Fan, L.T., Lee, Y.-H., Beardmore, D.R., 1981. The influence of major structural features of cellulose on rate of enzymatic hydrolysis. Biotechnol. Bioeng. 23, 419–424. doi:10.1002/bit.260230215

Fengel, D., Wegener, G., 1989. Wood: chemistry, ultrastructure, reactions., Wood: chemistry, ultrastructure, reactions. doi:10.1007/BF02608943

Fernandes Diniz, J.M.B., Gil, M.H., Castro, J.A.A.M., 2004. Hornification - Its origin and interpretation in wood pulps. Wood Sci. Technol. 37, 489–494. doi:10.1007/s00226-003-0216-2

Gao, D., Uppugundla, N., Chundawat, S.P., Yu, X., Hermanson, S., Gowda, K., Brumm, P., Mead,

D., Balan, V., Dale, B.E., 2011. Hemicellulases and auxiliary enzymes for improved conversion of lignocellulosic biomass to monosaccharides. Biotechnol. Biofuels 4, 1–11. doi:10.1186/1754-6834-4-5

Gao, Z., Van Hop, D., Yen, L.T.H., Ando, K., Hiyamuta, S., Kondo, R., 2012. The production of β-glucosidases by Fusarium proliferatum NBRC109045 isolated from Vietnamese forest. AMB Express 2, 49. doi:10.1186/2191-0855-2-49

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268. doi:10.1351/pac198759020257

Gilad, R., Rabinovich, L., Yaron, S., Bayer, E.A., Lamed, R., Gilbert, H.J., Shoham, Y., 2003. CelI, a noncellulosomal family 9 enzyme from Clostridium thermocellum, is a processive endoglucanase that degrades crystalline cellulose. J. Bacteriol. 185, 391–8. doi:10.1128/JB.185.2.391-398.2003

Gilkes, N.R., Jervis, E., Henrissat, B., Tekant, B., Miller, R.C., Warren, R.A., Kilburn, D.G., 1992. The adsorption of a bacterial cellulase and its two isolated domains to crystalline cellulose. J. Biol. Chem. 267, 6743–9.

Gourlay, K., Hu, J., Arantes, V., Andberg, M., Saloheimo, M., Penttil??, M., Saddler, J., 2013. Swollenin aids in the amorphogenesis step during the enzymatic hydrolysis of pretreated biomass. Bioresour. Technol. 142, 498–503. doi:10.1016/j.biortech.2013.05.053

Grabber, J.H., Mertens, D.R., Kim, H., Funk, C., Lu, F., Ralph, J., 2009. Cell wall fermentation kinetics are impacted more by lignin content and ferulate cross-linking than by lignin composition. J. Sci. Food Agric. 89, 122–129. doi:10.1002/jsfa.3418

Grethlein, H.E., Allen, D.C., Converse, A.O., 1984. A comparative study of the enzymatic hydrolysis of acid-pretreated white pine and mixed hardwood. Biotechnol. Bioeng. 26, 1498–1505. doi:10.1002/bit.260261215

Grohmann, K., Mitchell, D., Himmel, M., 1989. The role of ester groups in resistance of plant cell wall polysaccharides to enzymatic hydrolysis. Appl. Biochem. Biotechnol. 20, 45–61.

Grous, W.R., Converse, A.O., Grethlein, H.E., 1986. Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar. Enzyme Microb. Technol. 8, 274–280. doi:10.1016/0141-0229(86)90021-9

Gübitz, G.M., Hayn, M., Sommerauer, M., Steiner, W., 1996. Mannan-degrading enzymes from Sclerotium rolfsii: Characterisation and synergism of two endo β-mannanases and a β-mannosidase. Bioresour. Technol. 58, 127–135. doi:10.1016/S0960-8524(96)00093-4

Gübitz, G.M., Lischnig, T., Stebbing, D., Saddler, J.N., 1997. Enzymatic removal of hemicellulose from dissolving pulps. Biotechnol. Lett. 19, 491–495. doi:10.1023/A:1018364731600

Guo, F., Shi, W., Sun, W., Li, X., Wang, F., Zhao, J., Qu, Y., 2014. Differences in the adsorption of enzymes onto lignins from diverse types of lignocellulosic biomass and the underlying mechanism. Biotechnol. Biofuels 7. doi:10.1186/1754-6834-7-38

Häggkvist, M., Li, T.-Q., Ödberg, L., 1998. Effects of drying and pressing on the pore structure in the cellulose fibre wall studied by 1H and 2H NMR relaxation. Cellulose 5, 33–49.

doi:10.1023/A:1009212628778

Hall, M., Bansal, P., Lee, J.H., Realff, M.J., Bommarius, A.S., 2010. Cellulose crystallinity - A key predictor of the enzymatic hydrolysis rate. FEBS J. 277, 1571–1582. doi:10.1111/j.1742-4658.2010.07585.x

Haynes, C.A., Norde, W., 1994. Globular proteins at solid/liquid interfaces. Colloids Surfaces B Biointerfaces. doi:10.1016/0927-7765(94)80066-9

Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100, 10–18. doi:10.1016/J.BIORTECH.2008.05.027

Henrissat, B., 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 280 (Pt 2), 309–16.

Henrissat, B., Bairoch, A., 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 293, 781–788. doi:10.1042/bj2930781

Henrissat, B., Driguez, H., Viet, C., Schülein, M., 1985. Synergism of Cellulases from Trichoderma reesei in the Degradation of Cellulose. Bio/Technology 3, 722–726. doi:10.1038/nbt0885-722

Henrissat, B., Vigny, B., Buleon, A., Perez, S., 1988. Possible adsorption sites of cellulases on crystalline cellulose. FEBS Lett. 231, 177–182. doi:10.1016/0014-5793(88)80726-9

Himmel, M.E., Abbas, C.A., Baker, J.O., Bayer, E.A., Bomble, Y.J., Brunecky, R., Chen, X., Felby, C., Jeoh, T., Kumar, R., McCleary, B. V., Pletschke, B.I., Tucker, M.P., Wyman, C.E., Decker, S.R., 2017. Undefined cellulase formulations hinder scientific reproducibility. Biotechnol. Biofuels 10, 283. doi:10.1186/s13068-017-0974-y

Hoshino, E., Shiroishi, M., Amano, Y., Nomura, M., Kanda, T., 1997. Synergistic actions of exo-type cellulases in the hydrolysis of cellulose with different crystallinities. J. Ferment. Bioeng. 84, 300–306. doi:10.1016/S0922-338X(97)89248-3

Howard, R.L., Abotsi, E., Jansen, van R.E.L., Howard, S., 2003. Lignocellulose biotechnology: issues of bioconversion and enzyme production. African J. Biotechnol. 2, 602–619. doi:10.5897/AJB2003.000-1115

https://www.novozymes.com/en/news/news-archive/2012/02/advanced-biofuels-becoming-reality-with-novozymes-new-enzyme-technology [WWW Document], 2012. URL https://www.novozymes.com/en/news/news-archive/2012/02/advanced-biofuels-becoming-reality-with-novozymes-new-enzyme-technology (accessed 1.12.18).

Hu, J., 2014. The role of accessory enzymes in enhancing the effective hydrolysis of the cellulosic component of pretreated biomass. Thesis submitted to the University of British Columbia for partial fulfillment of a Degree of Philosophy in Forestry.

Hu, J., Arantes, V., Pribowo, A., 2014. Substrate factors that influence the synergistic interaction of AA9 and cellulases during the enzymatic hydrolysis of biomass. Energy Environ. Sci. 7, 2308–2315. doi:10.1039/C4EE00891J

Hu, J., Arantes, V., Pribowo, A., Gourlay, K., Saddler, J., 2012. How Improved Pretreatment And Enzyme Complexes Can Help Us Evolve From A Hydrocarbon Based Economy To A Carbohydrate / Biomass Based Society. Proc. 55th Int. Conv. Soc. Wood Sci. Technol. 1–11.

Hu, J., Arantes, V., Pribowo, A., Saddler, J.N., 2013. The synergistic action of accessory enzymes enhances the hydrolytic potential of a "cellulase mixture" but is highly substrate specific. Biotechnol. Biofuels 6, 112. doi:10.1186/1754-6834-6-112

Hu, J., Arantes, V., Saddler, J.N., 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? Biotechnol. Biofuels 4, 36. doi:10.1186/1754-6834-4-36

Hubbe, M.A., 2014. Prospects for Mantaining Stength of Paper and Paperboard Products While Using Less Forest Resources: A Review. Bioresour. Technol. 9, 1–131.

Humbird, D., Mohagheghi, A., Dowe, N., Schell, D.J., 2010. Economic impact of total solids loading on enzymatic hydrolysis of dilute acid pretreated corn stover. Biotechnol. Prog. 26, 1245–1251. doi:10.1002/btpr.441

IEA Task 39, 2011. Status of second generation biofuel demonstration plants.

Ioelovich, M., Morag, E., 2011. Effect of cellulose structure on enzymatic hydrolysis. BioResources 6, 2818–2835. doi:10.15376/BIORES.6.3.2818_2835

Irwin, D.C., Spezio, M., Walker, L.P., Wilson, D.B., 1993. Activity studies of eight purified cellulases: Specificity, synergism, and binding domain effects. Biotechnol. Bioeng. 42, 1002–1013. doi:10.1002/bit.260420811

Jalak, J., Väljamäe, P., 2014. Multi-mode binding of cellobiohydrolase Cel7A from Trichoderma reesei to Cellulose. PLoS One 9, e108181. doi:10.1371/journal.pone.0108181

Jang, S.J., Ham, M.S., Lee, J.M., Chung, S.K., Lee, H.J., Kim, J.H., Chang, H.C., Lee, J.-H., Chung, D.K., 2003. New integration vector using a cellulase gene as a screening marker for Lactobacillus. FEMS Microbiol. Lett. 224, 191–5.

Jayme, G., 1944. Mikro-Quellungsmessungen an Zellstoffen. Der Pap. für Pap. 42, 187–794.

Jeoh, T., Ishizawa, C.I., Davis, M.F., Himmel, M.E., Adney, W.S., Johnson, D.K., 2007. Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. Biotechnol. Bioeng. 98, 112–122. doi:10.1002/bit.21408

Jeoh, T., Wilson, D.B., Walker, L.P., 2006. Effect of Cellulase Mole Fraction and Cellulose Recalcitrance on Synergism in Cellulose Hydrolysis and Binding. Biotechnol. Prog. 22, 270–277. doi:10.1021/bp050266f

Jeoh, T., Wilson, D.B., Walker, L.P., 2002. Cooperative and Competitive Binding in Synergistic Mixtures of Thermobifida fusca Cellulases Cel5A, Cel6B, and Cel9A. Biotechnol. Prog. 18, 760–769. doi:10.1021/bp0200402

Ji, L., Yang, J., Fan, H., Yang, Y., Li, B., Yu, X., Zhu, N., Yuan, H., 2014. Synergy of crude enzyme cocktail from cold-adapted Cladosporium cladosporioides Ch2-2 with commercial xylanase achieving high sugars yield at low cost. Biotechnol. Biofuels 7, 130.

doi:10.1186/s13068-014-0130-x

Jones, B.W., Venditti, R., Park, S., Jameel, H., Koo, B., 2013. Enhancement in enzymatic hydrolysis by mechanical refining for pretreated hardwood lignocellulosics. Bioresour. Technol. 147, 353–360. doi:10.1016/j.biortech.2013.08.030

Jung, S., Song, Y., Kim, H.M., Bae, H.J., 2015. Enhanced lignocellulosic biomass hydrolysis by oxidative lytic polysaccharide monooxygenases (LPMOs) GH61 from Gloeophyllum trabeum. Enzyme Microb. Technol. 77, 38–45. doi:10.1016/j.enzmictec.2015.05.006

Kabel, M.A., Bos, G., Zeevalking, J., Voragen, A.G.J., Schols, H.A., 2007. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresour. Technol. 98, 2034–2042. doi:10.1016/j.biortech.2006.08.006

Karnaouri, A., Topakas, E., Matsakas, L., Rova, U., Christakopoulos, P., 2018. Fine-tuned enzymatic hydrolysis of organosolv pretreated forest materials for the efficient production of cellobiose. Front. Chem. 6, 128. doi:10.3389/fchem.2018.00128

Karnchanatat, A., Petsom, A., Sangvanich, P., Piaphukiew, J., Whalley, A.J.S., Reynolds, C.D., Sihanonth, P., 2007. Purification and biochemical characterization of an extracellular β-glucosidase from the wood-decaying fungus Daldinia eschscholzii (Ehrenb.:Fr.) Rehm. FEMS Microbiol. Lett. 270, 162–170. doi:10.1111/j.1574-6968.2007.00662.x

Katsimpouras, C., Dimarogona, M., Petropoulos, P., Christakopoulos, P., Topakas, E., 2016. A thermostable GH26 endo-β-mannanase from Myceliophthora thermophila capable of enhancing lignocellulose degradation. Appl. Microbiol. Biotechnol. 100, 8385–8397. doi:10.1007/s00253-016-7609-2

Kim, I.J., Lee, H.J., Choi, I.G., Kim, K.H., 2014. Synergistic proteins for the enhanced enzymatic hydrolysis of cellulose by cellulase. Appl. Microbiol. Biotechnol. 98, 8469–8480. doi:10.1007/s00253-014-6001-3

Kim, S.-J., Lee, C.-M., Kim, M.-Y., Yeo, Y.-S., Yoon, S.-H., Kang, H.-C., Koo, B.-S., 2007. Screening and characterization of an enzyme with beta-glucosidase activity from environmental DNA. J. Microbiol. Biotechnol. 17, 905–12.

Kim, S., Park, J.M., Seo, J.W., Kim, C.H., 2012. Sequential acid-/alkali-pretreatment of empty palm fruit bunch fiber. Bioresour. Technol. 109, 229–233. doi:10.1016/j.biortech.2012.01.036

Kim, Y.S., Jung, H.C., Pan, J.G., 2000. Bacterial cell surface display of an enzyme library for selective screening of improved cellulase variants. Appl. Environ. Microbiol. 66, 788–93.

King, B.C., Donnelly, M.K., Bergstrom, G.C., Walker, L.P., Gibson, D.M., 2009. An optimized microplate assay system for quantitative evaluation of plant cell wall-degrading enzyme activity of fungal culture extracts. Biotechnol. Bioeng. 102, 1033–1044. doi:10.1002/bit.22151

Kleywegt, G.J., Zou, J.-Y., Divne, C., Davies, G.J., Sinning, I., Sta, J., Hlberg, Ê., Reinikainen, T., Srisodsuk, M., Teeri, T.T., Jones, T.A., 1997. The Crystal Structure of the Catalytic Core Domain of Endoglucanase I from Trichoderma reesei at 3.6 A Ê Resolution, and a Comparison with Related Enzymes.

Kokta, B. V., Ahmed, A., Esdale, B., 1993. Steam explosion pulping of kenaf: A preliminary study on pulp characteristics and a comparison with simulated cmp and ctmp of kenaf. J. Wood Chem. Technol. 13, 213–236. doi:10.1080/02773819308020515

Kongruang, S., Han, J., Isela, C., Breton, G., Penner, M.H., 2004. Analysis of Cellulose-Reducing Ends Quantitative Analysis of Cellulose-Reducing Ends. Appl. Biochem. Biotechnol. 113116.

Koo, B.W., Treasure, T.H., Jameel, H., Phillips, R.B., Chang, H.M., Park, S., 2011. Reduction of enzyme dosage by oxygen delignification and mechanical refining for enzymatic hydrolysis of green liquor-pretreated hardwood. Appl. Biochem. Biotechnol. 165, 832–844. doi:10.1007/s12010-011-9301-4

Korotkova, O.G., Semenova, M. V., Morozova, V. V., Zorov, I.N., Sokolova, L.M., Bubnova, T.M., Okunev, O.N., Sinitsyn, A.P., 2009. Isolation and properties of fungal β-glucosidases. Biochemistry. (Mosc). 74, 569–577. doi:10.1134/S0006297909050137

Kostylev, M., Wilson, D., 2012. Synergistic interactions in cellulose hydrolysis. Biofuels 3, 61–70. doi:10.4155/bfs.11.150

Kumar, L., Arantes, V., Chandra, R., Saddler, J., 2012. The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. Bioresour. Technol. 103, 201–208. doi:10.1016/j.biortech.2011.09.091

Kumar, R., Wyman, C.E., 2013. Physical and Chemical Features of Pretreated Biomass that Influence Macro-/Micro-Accessibility and Biological Processing, in: Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals. John Wiley & Sons, Ltd, Chichester, UK, pp. 281–310. doi:10.1002/9780470975831.ch14

Kumar, R., Wyman, C.E., 2009. Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. Biotechnol. Prog. 25, 302–314. doi:10.1002/btpr.102

Laivins, G. V., Scallan, A.M., 1996. The influence of drying and beating on the swelling of fines. J. pulp Pap. Sci. 22, 178–184.

Lehtiö, J., Sugiyama, J., Gustavsson, M., Fransson, L., Linder, M., Teeri, T.T., 2003. The binding specificity and affinity determinants of family 1 and family 3 cellulose binding modules. Proc. Natl. Acad. Sci. U. S. A. 100, 484–9. doi:10.1073/pnas.212651999

Li, H., Saeed, A., Jahan, M.S., Ni, Y., van Heiningen, A., 2010. Hemicellulose Removal from Hardwood Chips in the Pre-Hydrolysis Step of the Kraft-Based Dissolving Pulp Production Process. J. Wood Chem. Technol. 30, 48–60. doi:10.1080/02773810903419227

Li, J., Zhou, P., Liu, H., Xiong, C., Lin, J., Xiao, W., Gong, Y., Liu, Z., 2014. Synergism of cellulase, xylanase, and pectinase on hydrolyzing sugarcane bagasse resulting from different pretreatment technologies. Bioresour. Technol. 155, 258–265. doi:10.1016/J.BIORTECH.2013.12.113

Li, S., Yang, X., Yang, S., Zhu, M., Wang, X., 2012. Technology prospecting on enzymes: application, marketing and engineering. Comput. Struct. Biotechnol. J. 2, e201209017. doi:10.5936/csbj.201209017

Long, W.L., Faizal, Y., Mohamad, S.M.A., 2015. Modeling of Sago Starch Hydrolysis Using Glucoamylase Modeling of Sago Starch Hydrolysis Using Glucoamylase. Sains Malaysiana 44, 973–977.

Luo, X., Zhu, J.Y., 2010. Effects of drying-induced fiber hornification on enzymatic saccharification of lignocelluloses. Enzyme Microb. Technol. 48, 92–99. doi:10.1016/j.enzmictec.2010.09.014

Luo, X.L., Zhu, J.Y., Gleisner, R., Zhan, H.Y., 2011. Effects of wet-pressing-induced fiber hornification on enzymatic saccharification of lignocelluloses. Cellulose 18, 1055–1062. doi:10.1007/s10570-011-9541-z

Lynd, L.R., Weimer, P.J., Van Zyl, W.H., Pretorius, I.S., 2002. Microbial Cellulose Utilization: Fundamentals and Biotechnology. Microbiol. Mol. Biol. Rev. 66, 506–577. doi:10.1128/MMBR.66.3.506–577.2002

Malgas, S., van Dyk, J.S., Pletschke, B.I., 2015. A review of the enzymatic hydrolysis of mannans and synergistic interactions between β-mannanase, β-mannosidase and α-galactosidase. World J. Microbiol. Biotechnol. doi:10.1007/s11274-015-1878-2

Malherbe, S., Cloete, T.E., 2002. Lignocellulose biodegradation: Fundamentals and applications. Rev. Environ. Sci. Biotechnol. 1, 105–114. doi:10.1023/A:1020858910646

Mandels, M., Andreotti, R., Roche, C., 1976. Measurement of saccharifying cellulase. Biotechnol. Bioeng. Symp. 6, 21–33.

Mansfield, S.D., Mooney, C., Saddler, J.N., 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnol. Prog. 15, 804–816. doi:10.1021/bp9900864

Markov, A. V, Gusakov, A. V, Kondratyeva, E.G., Okunev, O.N., Bekkarevich, A.O., Sinitsyn, A.P., 2005. New effective method for analysis of the component composition of enzyme complexes from Trichoderma reesei. Biochemistry. (Mosc). 70, 657–63.

Martínez, J.M., Reguant, J., Montero, M.Á., Montané, D., Salvadó, J., Farriol, X., 1997. Hydrolytic Pretreatment of Softwood and Almond Shells. Degree of Polymerization and Enzymatic Digestibility of the Cellulose Fraction. Ind. Eng. Chem. Res. 36, 688–696. doi:10.1021/ie960048e

Mason, W., 1926. Process and apparatus for the disintegration of wood and the like. United States patent. 1578609.

Maurya, D.P., Singla, A., Negi, S., 2013. An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. 3 Biotech 3, 415–431. doi:10.1007/s13205-015-0279-4

Medve, J., Karlsson, J., Lee, D., Tjerneld, F., 1998. Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from Trichoderma reesei: adsorption, sugar production pattern, and synergism of the enzymes. Biotechnol. Bioeng. 59, 621–34.

Meehnian, H., Jana, A.K., Jana, M.M., 2016. Effect of particle size, moisture content, and supplements on selective pretreatment of cotton stalks by Daedalea flavida and enzymatic saccharification. 3 Biotech 6, 235. doi:10.1007/s13205-016-0548-x

Menon, V., Rao, M., 2012. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. Prog. Energy Combust. Sci. 38, 522–550. doi:10.1016/j.pecs.2012.02.002

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31, 426–428. doi:10.1021/ac60147a030

Minor, J.L., 1994. Hornification -Its origin and meaning. Progrress Pap. Recycl. 3, 93–95. doi:10.1017/CBO9781107415324.004

Mok, Y.K., Arantes, V., Saddler, J.N., 2015. A NaBH4 Coupled Ninhydrin-Based Assay for the Quantification of Protein/Enzymes During the Enzymatic Hydrolysis of Pretreated Lignocellulosic Biomass. Appl. Biochem. Biotechnol. 176, 1564–1580. doi:10.1007/s12010-015-1662-7

Mooney, C.A., 1998. Substrate Factors Limiting the Enzymatic Hydrolysis of Softwood Substrates by.

Mooney, C.A., Mansfield, S.D., Touhy, M.G., Saddler, J.N., 1998. The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods. Bioresour. Technol. 64, 113–119. doi:10.1016/S0960-8524(97)00181-8

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol. doi:10.1016/j.biortech.2004.06.025

Mosier, N.S., Hall, P., Ladisch, C.M., Ladisch, M.R., 1999. Reaction kinetics, molecular action, and mechanisms of cellulolytic proteins. Adv. Biochem. Eng. Biotechnol. 65, 23–40.

Muurinen, E., 2000. Organosolv pulping- a review and distillation study related to peroxyacid pulping 1–314. doi:10.1016/0960-8524(91)90105-S

Nakagame, S., Chandra, R.P., Kadla, J.F., Saddler, J.N., 2011a. The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose. Bioresour. Technol. 102, 4507–4517. doi:10.1016/J.BIORTECH.2010.12.082

Nakagame, S., Chandra, R.P., Saddler, J.N., 2011b. The influence of lignin on the enzymatic hydrolysis of pretreated biomass substrates, in: ACS Symposium Series. pp. 145–167. doi:10.1021/bk-2011-1067.ch006

Nakamura, A., Tasaki, T., Ishiwata, D., Yamamoto, M., Okuni, Y., Visootsat, A., Maximilien, M., Noji, H., Uchiyama, T., Samejima, M., Igarashi, K., Iino, R., 2016. Single-molecule imaging analysis of binding, processive movement, and dissociation of cellobiohydrolase trichoderma reesei Cel6A and its domains on crystalline cellulose. J. Biol. Chem. 291, 22404–22413. doi:10.1074/jbc.M116.752048

Nakazawa, H., Okada, K., Kobayashi, R., Kubota, T., Onodera, T., Ochiai, N., Omata, N., Ogasawara, W., Okada, H., Morikawa, Y., 2008. Characterization of the catalytic domains of Trichoderma reesei endoglucanase I, II, and III, expressed in Escherichia coli. Appl. Microbiol. Biotechnol. 81, 681–689. doi:10.1007/s00253-008-1667-z

Nazhad, M.M., Ramos, L.P., Paszner, L., Saddler, J.N., 1995. Structural constraints affecting the

initial enzymatic hydrolysis of recycled paper. Enzyme Microb. Technol. 17, 68–74. doi:10.1016/0141-0229(94)00057-X

Neilson, M.J., Shafizadeh, F., Aziz, S., Sarkanen, K. V., 1983. Evaluation of organosolv pulp as a suitable substrate for rapid enzymatic hydrolysis. Biotechnol. Bioeng. doi:10.1002/bit.260250225

Newman, R.H., 2004. Carbon-13 NMR evidence for cocrystallization of cellulose as a mechanism for hornification of bleached kraft pulp. Cellulose 11, 45–52. doi:10.1023/B:CELL.0000014768.28924.0c

Nidetzky, B., Steiner, W., Haynt, M., Claeyssenst, M., 1994a. Cellulose hydrolysis by the cellulases from Trichoderma reesei: a new model for synergistic interaction. Biochem. J 298, 705–710.

Nidetzky, B., Zachariae, W., Gercken, G., Hayn, M., Steiner, W., 1994b. Hydrolysis of cellooligosaccharides by Trichoderma reesei cellobiohydrolases: Experimental data and kinetic modeling. Enzyme Microb. Technol. 16, 43–52. doi:10.1016/0141-0229(94)90108-2

Nitsos, C., Rova, U., Christakopoulos, P., 2018. Organosolv fractionation of softwood biomass for biofuel and biorefinery applications. Energies 11, 50. doi:10.3390/en11010050

Obeng, E.M., Adam, S.N.N., Budiman, C., Ongkudon, C.M., Maas, R., Jose, J., 2017. Lignocellulases: a review of emerging and developing enzymes, systems, and practices. Bioresour. Bioprocess. 4, 16. doi:10.1186/s40643-017-0146-8

Öhgren, K., Bura, R., Saddler, J., Zacchi, G., 2007. Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. Bioresour. Technol. 98, 2503–2510. doi:10.1016/j.biortech.2006.09.003

Pan, X., Gilkes, N., Kadla, J., Pye, K., Saka, S., Gregg, D., Ehara, K., Xie, D., Lam, D., Saddler, J., 2006. Bioconversion of hybrid poplar to ethanol and co-products using an organosolv fractionation process: Optimization of process yields. Biotechnol. Bioeng. 94, 851–861. doi:10.1002/bit.20905

Pan, X., Xie, D., Yu, R.W., Lam, D., Saddler, J.N., 2007. Pretreatment of lodgepole pine killed by mountain pine beetle using the ethanol organosolv process: Fractionation and process optimization, in: Industrial and Engineering Chemistry Research. pp. 2609–2617. doi:10.1021/ie061576l

Pan, X., Xie, D., Yu, R.W., Saddler, J.N., 2008. The bioconversion of mountain pine beetle-killed lodgepole pine to fuel ethanol using the organosolv process. Biotechnol. Bioeng. 101, 39–48. doi:10.1002/bit.21883

Poeta, P., Dias, A.A., Igrejas, G., Silva, V., Bezerra, R., 2018. Selection, engineering, and expression of microbial enzymes. Enzym. Hum. Anim. Nutr. 1–29. doi:10.1016/B978-0-12-805419-2.00001-0

Polacheck, I., Melamed, M., Bercovier, H., Salkin, I.F., 1987. beta-Glucosidase in Candida albicans and its application in yeast identification. J. Clin. Microbiol. 25, 907–10.

Pryor, S.W., Nahar, N., 2010. Deficiency of cellulase activity measurements for enzyme

evaluation. Appl. Biochem. Biotechnol. 162, 1737–1750. doi:10.1007/s12010-010-8955-7

Puri, V.P., 1984. Effect of crystallinity and degree of polymerization of cellulose on enzymatic saccharification. Biotechnol. Bioeng. 26, 1219–1222. doi:10.1002/bit.260261010

Pye, E.K., Lora, J.H., 1991. The AlcellTM process: a proven alternative to Kraft pulping. Tappi J. 74, 113–118.

Ramos, L.P., Breuil, C., Saddler, J.N., 1992. Comparison of steam pretreatment of eucalyptus, aspen, and spruce wood chips and their enzymatic hydrolysis. Appl. Biochem. Biotechnol. 34–35, 37–48. doi:10.1007/BF02920532

Rana, V., Ahring, B., 2015. Evaluating the impact of boosting on-site enzymes produced by Trichoderma reesei RUT C30 and Aspergillus saccharolyticus with commercial Enzymes in lowering the use of commercial cellulases. Mol. Enzymol. Drug Targets 1, 1–11. doi:10.21767/2572-5475.10003

Reese, E.T., Siu, R.G., Levinson, H.S., 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol. 59, 485–497. doi:10.1111/j.1750-3841.2010.01868.x

Riedel, K., Ritter, J., Bronnenmeier, K., 2006. Synergistic interaction of the Clostridium stercorarium cellulases Avicelase I (CelZ) and Avicelase II (CelY) in the degradation of microcrystalline cellulose. FEMS Microbiol. Lett. 147, 239–243. doi:10.1111/j.1574-6968.1997.tb10248.x

Saddler, J.N., 1986. Factors limiting the efficiency of cellulase enzymes. Microbiol. Sci. 3, 84–7.

Sánchez, C., 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi. Biotechnol. Adv. doi:10.1016/j.biotechadv.2008.11.001

Saritha, M., Arora, A., Lata, 2012. Biological pretreatment of lignocellulosic substrates for enhanced delignification and enzymatic digestibility. Indian J. Microbiol. 52, 122–30. doi:10.1007/s12088-011-0199-x

Sasmal, S., Goud, V. V., Mohanty, K., 2011. Optimisation of the acid catalysed pretreatment of areca nut husk fibre using the Taguchi design method. Biosyst. Eng. 110, 465–472. doi:10.1016/j.biosystemseng.2011.09.013

Scheller, H.V., Ulvskov, P., 2010. Hemicelluloses. Annu. Rev. Plant Biol. 61, 263–289. doi:10.1146/annurev-arplant-042809-112315

Schülein, M., 1997. Enzymatic properties of cellulases from Humicola insolens. J. Biotechnol. 57, 71–81. doi:10.1016/S0168-1656(97)00090-4

Schwarz, W.H., 2001. The cellulosome and cellulose degradation by anaerobic bacteria. Appl. Microbiol. Biotechnol. 56, 634–649. doi:10.1007/s002530100710

Selig, M.J., Knoshaug, E.P., Adney, W.S., Himmel, M.E., Decker, S.R., 2008. Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities. Bioresour. Technol. 99, 4997–5005. doi:10.1016/j.biortech.2007.09.064

Shallom, D., Shoham, Y., 2003. Microbial hemicellulases. Curr. Opin. Microbiol. 6, 219–228. doi:10.1016/S1369-5274(03)00056-0

Sharrock, K.R., 1988. Cellulase assay methods: a review. J. Biochem. Biophys. Methods 17, 81–105. doi:10.1016/0165-022X(88)90040-1

Shevchenko, S., Chang, K., Dick, D.G., Gregg, D.J., Saddler, J., 2001. Structure and properties of lignin in softwoods after SO2 - Catalyzed steam explosion and enzymatic hydrolysis, Cellulose Chemistry and Technology.

Sindhu, R., Kuttiraja, M., Binod, P., Sukumaran, R.K., Pandey, A., 2014. Physicochemical characterization of alkali pretreated sugarcane tops and optimization of enzymatic saccharification using response surface methodology. Renew. Energy 62, 362–368. doi:10.1016/j.renene.2013.07.041

Sineiro, J., Dominguez, H., Núñez, M.J., Lema, J.M., 1995. Hydrolysis of microcrystalline cellulose by cellulolytic complex of Trichoderma reesei in low-moisture media. Enzyme Microb. Technol. 17, 809–815. doi:10.1016/0141-0229(94)00107-3

Singh, R., Hu, J., Regner, M.R., Round, J.W., Ralph, J., Saddler, J.N., Eltis, L.D., 2017. Enhanced delignification of steam- pretreated poplar by a bacterial laccase. Nat. Publ. Gr. doi:10.1038/srep42121

Siqueira, G., Arantes, V., Saddler, J.N., Ferraz, A., Milagres, A.M.F., 2017. Limitation of cellulose accessibility and unproductive binding of cellulases by pretreated sugarcane bagasse lignin. Biotechnol. Biofuels 10, 1–12. doi:10.1186/s13068-017-0860-7

Smook, G.A., 1989. Handbook for Pulp and Paper Technologists, Tappi Press.

Sousa, R., Carvalho, M.L., Giordano, R.L.C., Giordano, R.C., 2011. Recent Trends in the Modelling of Cellulose Hydrolysis. Brazilian J. Chem. Eng. 28, 545–564.

Ståhlberg, J., Johansson, G., Pettersson, G., 1991. A New Model For Enzymatic Hydrolysis of Cellulose Based on the Two-Domain Structure of Cellobiohydrolase I. Nat. Biotechnol. 9, 286–290. doi:10.1038/nbt0391-286

Stone, J.E., Scallan, A.M., Donefer, E., Ahlgren, E., 1969. Digestibility as a simple function of a molecule of similar size to a cellulase enzyme, in: Advances in Chemistry Series. pp. 219–241. doi:10.1021/ba-1969-0095.ch013

Strahsburger, E., de Lacey, A.M.L., Marotti, I., DiGioia, D., Biavati, B., Dinelli, G., 2017. In vivo assay to identify bacteria with β-glucosidase activity. Electron. J. Biotechnol. 30, 83–87. doi:10.1016/j.ejbt.2017.08.010

Sun, F.F., Hong, J., Hu, J., Saddler, J.N., Fang, X., Zhang, Z., Shen, S., 2015. Accessory enzymes influence cellulase hydrolysis of the model substrate and the realistic lignocellulosic biomass. Enzyme Microb. Technol. 79–80, 42–48. doi:10.1016/j.enzmictec.2015.06.020

Sun, S., Sun, S., Cao, X., Sun, R., 2016. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. Bioresour. Technol. 199, 49–58. doi:10.1016/j.biortech.2015.08.061

Swatloski, R.P., Spear, S.K., Holbrey, J.D., Rogers, R.D., 2002. Dissolution of cellose with ionic liquids. J. Am. Chem. Soc. 124, 4974–4975. doi:10.1021/ja025790m

Taherzadeh, M.J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. Int. J. Mol. Sci. doi:10.3390/ijms9091621

Tanaka, M., Ikesaka, M., Matsuno, R., Converse, A.O., 1988. Effect of pore size in substrate and diffusion of enzyme on hydrolysis of cellulosic materials with cellulases. Biotechnol. Bioeng. 32, 698–706. doi:10.1002/bit.260320515

Teeri, T.T., 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends Biotechnol. 15, 160–167. doi:10.1016/S0167-7799(97)01032-9

Ten, L.N., Im, W.-T., Kim, M.-K., Kang, M.S., Lee, S.-T., 2004. Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates. J. Microbiol. Methods 56, 375–382. doi:10.1016/j.mimet.2003.11.008

Tenkanen, M., Makkonen, M., Perttula, M., Viikari, L., Teleman, A., 1997. Action of Trichoderma reesei mannanase on galactoglucomannan in pine kraft pulp. J. Biotechnol. 57, 191–204. doi:10.1016/S0168-1656(97)00099-0

Thurston, C.F., 1994. The structure and function of fungal laccases. Microbiology. doi:10.1099/13500872-140-1-19

Tsvetkova, I. V, Karpova, E.A., Dudukina, T. V, Voznyi, Y. V, 1996. 4-Pentafluoroethylumbelliferyl-beta-D-glucoside as a new fluorogenic substrate for acid beta-D-glucosidase. Clin. Chim. Acta. 248, 125–33.

Tuka, K., Zverlov, V. V, Velikodvorskaya, G.A., 1992. Synergism between Clostridium thermocellum cellulases cloned in Escherichia coli. Appl. Biochem. Biotechnol. 37, 201–7.

U.S. Congress Office of Technology Assessment, 1989. Technologies for Reducing Dioxin in the Manufacture of Bleached Wood Pulp, OTA-BP-O-54, in: Washington, DC: U.S. Government Printing Office. pp. 17–26.

Urbánszki, K., Szakács, G., Tengerdy, R.P., 2000. Standardization of the filter paper activity assay for solid substrate fermentation. Biotechnol. Lett. 22, 65–69. doi:10.1023/A:1005654514980

Van Dyk, J.S., Pletschke, B.I., 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy. Biotechnol. Adv. 30, 1458–1480. doi:10.1016/J.BIOTECHADV.2012.03.002

van Tilbeurgh, H., Pettersson, G., Bhikabhai, R., De Boeck, H., Claeyssens, M., 1985. Studies of the cellulolytic system of Trichoderma reesei QM 9414. Reaction specificity and thermodynamics of interactions of small substrates and ligands with the 1,4-beta-glucan cellobiohydrolase II. Eur. J. Biochem. 148, 329–34.

Várnai, A., Huikko, L., Pere, J., Siika-aho, M., Viikari, L., 2011. Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. Bioresour. Technol. 102, 9096–9104. doi:10.1016/j.biortech.2011.06.059

Várnai, A., Siika-aho, M., Viikari, L., 2013. Carbohydrate-binding modules (CBMs) revisited: reduced amount of water counterbalances the need for CBMs. Biotechnol. Biofuels 6, 30. doi:10.1186/1754-6834-6-30

Vlasenko, E., Schülein, M., Cherry, J., Xu, F., 2010. Substrate specificity of family 5, 6, 7, 9, 12, and 45 endoglucanases. Bioresour. Technol. 101, 2405–2411. doi:10.1016/j.biortech.2009.11.057

Wald, S., Wilke, C.R., Blanch, H.W., 1984. Kinetics of the Enzymatic Hydrolysis of Cellulose. Biotechnol. Bioeng. 26, 221–230.

Watt, D.K., Ono, H., Hayashi, K., 1998. Agrobacterium tumefaciens β-glucosidase is also an effective β-xylosidase, and has a high transglycosylation activity in the presence of alcohols. Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol. 1385, 78–88. doi:10.1016/S0167-4838(98)00046-6

Welf, E.S., Venditti, R.A., Hubbe, M.A., Pawlak, J.J., 2005. The Effects of Heating Without Water Removal and Drying on the Swelling as Measured by Water Retention Value and Degradation as Measured by Intrinsic Viscosity of Cellulose Papermaking Fibers. Prog. Pap. Recycl. 14.

Wilson, D.B., 2004. Studies of Thermobifida fusca plant cell wall degrading enzymes. Chem. Rec. 4, 72–82. doi:10.1002/tcr.20002

Wood, T.M., 1992. Fungal cellulases. Biochem. Soc. Trans. 20, 46–53. doi:10.1042/BST0200046

Wood, T.M., 1969. The cellulase of Fusarium solani. Resolution of the enzyme complex. Biochem. J. 115, 457–64.

Wood, T.M., 1968. Cellulolytic enzyme system of Trichoderma koningii. Separation of components attacking native cotton. Biochem. J. 109, 217–27. doi:10.1042/BJ1090217

Wood, T.M., Bhat, K.M., 1988. Methods for measuring cellulase activities. Methods Enzymol. 160, 87–112. doi:10.1016/0076-6879(88)60109-1

Wood, T.M., McCrae, S.I., 1986. The cellulase of Penicillium pinophilum. Synergism between enzyme components in solubilizing cellulose with special reference to the involvement of two immunologically distinct cellobiohydrolases. Biochem. J. 234, 93–9.

Wood, T.M., McCrae, S.I., 1978. The cellulase of Trichoderma koningii. Purification and properties of some endoglucanase components with special reference to their action on cellulose when acting alone and in synergism with the cellobiohydrolase. Biochem. J. 171, 61–72.

Wood, T.M., McCrae, S.I., 1972. The purification and properties of the C 1 component of Trichoderma koningii cellulase. Biochem. J. 128, 1183–92.

Woodward, J., Lima, M., Lee, N.E., 1988. The role of cellulase concentration in determining the degree of synergism in the hydrolysis of microcrystalline cellulose. Biochem. J. 255, 895–899. doi:10.1042/bj2550895

Wu, B., Zhao, Y., Gao, P.J., 2007. A New Approach to Measurement of Saccharifying Capacities of Crude Cellulase. BioResources 1, 189–200. doi:10.15376/biores.1.2.189-200

Xiao, Z., Storms, R., Tsang, A., 2004. Microplate-based filter paper assay to measure total cellulase activity. Biotechnol. Bioeng. 88, 832–837. doi:10.1002/bit.20286

Yan, T.R., Lin, C.L., 1997. Purification and characterization of a glucose-tolerant beta-glucosidase from Aspergillus niger CCRC 31494. Biosci. Biotechnol. Biochem. 61, 965–70.

Yang, B., Wyman, C.E., 2006. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnol. Bioeng. 94, 611–617. doi:10.1002/bit.20750

Yang, M., Zhang, K.-D., Zhang, P.-Y., Zhou, X., Ma, X.-Q., Li, F.-L., 2016. Synergistic Cellulose Hydrolysis Dominated by a Multi-Modular Processive Endoglucanase from Clostridium cellulosi. Front. Microbiol. 7, 932. doi:10.3389/fmicb.2016.00932

Zhang, P.Y.H., Himmel, M.E., Mielenz, J.R., 2006. Outlook for cellulase improvement: screening and selection strategies. Biotechnol. Adv. 24, 452–81. doi:10.1016/j.biotechadv.2006.03.003

Zhang, Y.-H.P., Lynd, L.R., 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. Biotechnol. Bioeng. 88, 797–824. doi:10.1002/bit.20282

Zhao, X., Cheng, K., Liu, D., 2009. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Appl. Microbiol. Biotechnol. doi:10.1007/s00253-009-1883-1

Zhiwei, C., Friedland, G.D., Pereira, J.H., Reveco, S.A., Chan, R., Park, J.I., Thelen, M.P., Adams, P.D., Arkin, A.P., Keasling, J.D., Blanch, H.W., Simmons, B.A., Sale, K.L., Chivian, D., Chhabra, S.R., 2012. Tracing determinants of dual substrate specificity in glycoside hydrolase family 5. J. Biol. Chem. 287, 25335–25343. doi:10.1074/jbc.M112.362640

Zhong, L., Matthews, J.F., Crowley, M.F., Rignall, T., Talón, C., Cleary, J.M., Walker, R.C., Chukkapalli, G., McCabe, C., Nimlos, M.R., Brooks, C.L., Himmel, M.E., Brady, J.W., 2008. Interactions of the complete cellobiohydrolase I from Trichodera reesei with microcrystalline cellulose Iβ. Cellulose 15, 261–273. doi:10.1007/s10570-007-9186-0