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Enzymatic Inhibition By Lignin During Second Generation Ethanol Production

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ENZYMATIC INHIBITION BY LIGNIN DURING SECOND GENERATION ETHANOL PRODUCTION

by

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"Unbowed, unbent, unbroken"
Words of the House Martell
Game of Thrones – George R. R. Martin

This dissertation is dedicated to my parents that taught me to never give up and Emma Brace who showed me that hope existed.

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ABSTRACT

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An important hindrance to commercialization of lignocellulosic ethanol is the high cost of enzymes. Reducing enzyme loadings is necessary to reduce costs. Knowledge of the inhibitors of these enzymes is necessary to better inform enzyme development and process design. Five factors have been identified: soluble inhibitors, solid lignin adsorption, product inhibition, mixing efficiency and oxygen deactivation of the enzymes that hydrolyze cellulose to glucose. These factors are intertwined and properly assessing them individually require the elimination of the others. Isolating each factor, however, has not been done throughout literature leading to lumped constants. Soluble inhibitors reduce conversion sharply leading to high enzyme loadings and impeding the evaluation of any of the other factors. Through washing, the soluble inhibitors may be eliminated, and only washed biomass (either sugar cane bagasse or corn stover) was used to study the other factors. This work further investigates adsorption on lignin, mixing, and the effect of air on washed pretreated sugarcane bagasse and corn stover.

Studies of enzyme adsorption on lignin, showed lignin/enzyme interaction was temperature dependent and proportional to pretreatment severity. Lowering reaction temperatures to 30°C, eliminated enzyme adsorption and was opposite to what was expected, indicating a possible entropic process. On a practical basis, the additional free enzyme partial makes up for the lower activity of the enzyme mixture that occurs due to reduced reaction rate at the lower temperature of 30°C. Lower hydrolysis rates also require longer reaction times to achieve the same extent of conversion to glucose.

An alternative to counter adsorption of enzyme on lignin occurs by regulating the amount of lignin exposed by adjusting pretreatment conditions. At higher temperatures a large portion of lignin is solubilized and redeposited, increasing lignin exposure and adsorption is higher. At a lower severity, lignin is less exposed, and adsorption is lower. However, higher severity is needed to increase the accessibility of cellulose, thereby facilitating accessibility and conversion.

For sugarcane bagasse a 10.74 severity pretreatment (200°C for 20 minute) using liquid hot water resulted in minimal protein adsorption and therefore was interpreted to coincide with a small extent of lignin exposure, as qualitatively confirmed using SEM. Efficient conversion (71-76%) was achieved when hydrolysis with 6.5 mg of Cellic CTEC3 / g total solids. A more recalcitrant biomass would require a more intense pretreatment to be hydrolyzed at satisfactory levels.

Mixing and product inhibition were more intricately linked than the others. When efficient mixing was achieved, product inhibition was decreased relative to cases where mixing was not readily achieved. In these runs, concentrations of pretreated and washed corn stover were at initial concentrations of 10 to 200 g/L. At 200 g/L, the higher efficiency led to faster liquefaction of biomass in the early stages. Faster liquefaction resulted in significantly high glucose conversions (up to 47% final yields) after 72 hours of hydrolysis compared to minimally liquefied material where conversion was 34%.

Efficient mixing allowed deactivation due to air to be evaluated properly. This factor is the least understood in the literature and has a potentially major effect on the amount enzyme required for a given level of hydrolysis. Deactivation was isolated and observed by measuring conversion in a mixed 1 L reactor either in the presence of absence of air, except in these experiments with a different enzyme formulation, Cellic CTEC2 was used at 3.6 mg protein (Cellic CTEC2) / g solids. Cellic CTEC2 has lower activity, and the lower amount ensured that differences between the two conditions would be more obvious. Air was shown to decrease conversion by 10 to 15% with lower loss of activity corresponding to high solids loading.

The impact of unfavorable conditions (presence of lignin, inefficient mixing and inadequate air exposure) can be minimized by adjusting the biomass pretreatment and hydrolysis processes. The extent of adsorption of cellulolytic enzymes on lignin can be reduced by lowering hydrolysis temperature or reducing pretreatment severity. Efficient mixing facilitates liquefaction and increases final glucose conversion from cellulose compared to inefficient mixing methods. Limiting the presence of air increases enzyme activity and the associated final conversions. Adoption of the combined adjustments reduced enzyme loading by 50% (from 6 FPU to 3 FPU / g solids) for the enzyme Cellic CTEC2.

CHAPTER 1. INTRODUCTION

1.1 Motivation

"Presently, ethanol production from biomass is a sequence of integrated steps (Figure 1-1). They are: biomass growth, harvest and transportation to the biorefinery; pretreatment; enzymatic hydrolysis; fermentation; and distillation/purification and distribution. In each of these stages, there is room for improvement and cost savings (DOE 2007). However, the most impactful reductions are in the pretreatment and enzymatic hydrolysis units (Ladisch et al. 1978, Ladisch et al. 1983, Wyman 1999). These processes are important due to the characteristics of lignocellulose" (dos Santos 2016).

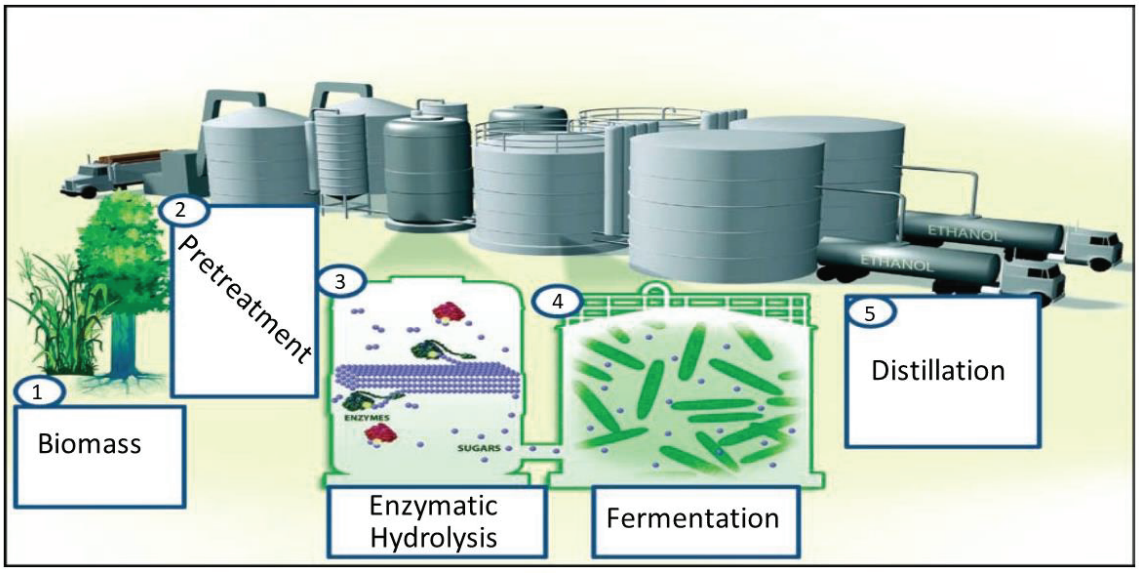

Figure 1-1. Biomass conversion to ethanol general process. From Ximenes et al., 2013, adapted from US DOE, 2007.

Biomass is recalcitrant and include natural inhibitors that cause the loss of enzymatic efficiency. This loss is more pronounced in high solids loadings that are necessary for an economically feasible production (Mosier et al. 2005; M. Ladisch et al. 2013).

Five factors have been identified as causes of low cellulolytic enzyme efficiency:

(1) Product inhibition

Cellulases are sensitive to product inhibition caused by glucose. It predominantly affects β-glucosidase. Product inhibition is an important factor on limiting enzyme efficiency, especially at high solids (Hong et al. 1981; Kristensen, Felby, and Jørgensen 2009; M. R. Ladisch, Gong, and Tsao 1980). Simultaneous saccharification and fermentation is the main method to counteract this effect (Öhgren et al. 2007; Philippidis, Smith, and Wyman 1993).

(2) Inhibition by other soluble components

Soluble inhibitors are generated from the solubilization of lignocellulose during pretreatment. These inhibitors include hemicellulose and cellulose degradation products, but the main cause of inhibition are phenolic compounds derived from lignin (Kim et al. 2011; Ximenes et al. 2010b). They both inhibit and deactivate cellulases. Deactivation is the most significant mechanism of interaction. This factor has been thoroughly investigated and washing was identified as an acceptable solution (Kim et al. 2009; McMillan et al. 2011).

(3) Inhibition due to insoluble components

Insoluble inhibitors are lignin redeposited on pretreated material and that adsorb enzymes deactivating them (Ximenes et al. 2010a). The addition of lignin blockers such as BSA, surfactants and soybean meal have been successfully used to reduce this effect (Florencio, Badino, and Farinas 2016; Jin et al. 2016; Kim et al. 2015; Ko, Ximenes, et al. 2015; Ko, Kim, et al. 2015; Kristensen et al. 2007; Kumar et al. 2012; Li et al. 2016; Siqueira et al. 2017; Sun et al. 2017; Yang and Wyman 2006).

(4) Mixing

At high solids loadings, mixing becomes inefficient as biomass rheological proprieties become harder to handle (Viamajala et al. 2009). Numerous methods have been investigated to counteract these proprieties. While these different mixers increased conversions at high solids, they failed in eliminating the negative relationship between glucose conversion and solids loadings (Jørgensen et al. 2007; Roche, Dibble, and Stickel 2009; J. Zhang et al. 2009; X. Zhang

et al. 2009; Geng et al. 2015; Ramachandriya et al. 2013; Palmqvist, Wiman, and Lidén 2011; Palmqvist and Lidén 2012).

(5) Enzyme deactivation

Deactivation caused by contact with air is known to denature proteins but has not been thoroughly studied for cellulases. This effect, presumably due oxygen, has been described (Scott et al. 2016). Lignocellulose hydrolysis carried out after N_2 purge increased cellulose conversions.

While these factors have not been studied individually, it is difficult to separate each of these factors as independent parameters in an experimental design, since changes in one factor affects – or is affected by – the others. The interactions between these are unknown and, in many instances, their relative importance has not been determined. In order to achieve glucose conversion at high solids loadings, a multifactorial approach is required so that dominating forms of inhibition may be identified, and then related to the overall characteristics of lignocellulosic biomass in a cellulose hydrolysis process all factors must be considered and the most crucial eliminated.

1.2 Summary of Work

This dissertation describes the development of methods to eliminate individual factors that reduce enzyme efficiency. Understanding and counteracting each factor leads to a process of lignocellulose hydrolysis that maintains high effectiveness regardless of solids loading. By counteracting the deleterious effects of lignin, rheology and other inhibitors present individually we can study each by itself. Additionally, by addressing each of the individual factors, a framework was developed that provides guidance on combinations of biomass, pretreatments, enzymes, and processing conditions that enable meaningful solids loadings while achieving high conversion and ultimately ethanol titers production.

The literature has described conditions that minimize inhibitor generation, or that remove inhibitors once formed. Soluble inhibitors were successfully removed through washing. Regarding insoluble lignin, bovine serum albumin was used as a blocker. However, it was

identified that BSA had minimal effect on hydrolysis efficiency. This effect is due the low pretreatment severity used for this biomass and pretreatment method.

These observations allowed for rheology to be addressed individually. As more solids are added to the process, the mixing properties become more important. When using shaker flasks, conversion decreased as mixing becomes less efficient and the interaction between enzymes and biomass less likely.

Hydrolysis was then carried out in 1L vessels, with mixing being carried out by dual marine impellers. This configuration can be used in industrial applications. Its adoption in larger scales will depend largely on power requirements necessary to achieve the same mixing efficiency. This combination of impellers was able to efficiently mix biomass slurry with solids concentrations. Efficient mixing appeared to eliminate product inhibition effects. With efficient mixing the conversions observed are not congruent with product inhibition. These two factors are intrinsically linked. These are likely due to the local concentrations of inhibitors and enzymes being significantly decreased with efficient mixing.

With all other factors eliminated dissolved oxygen deactivation was observed. When hydrolyzing biomass at low solids concentration, oxygen saturates the liquid, which is contrasted with the almost anaerobic conditions at high solids loadings. Hydrolysis was carried out again at strictly anaerobic conditions and efficiency was recovered and increased at all conditions.

Based on these observations, cellulose hydrolysis independent of solids loadings was achieved with dual impeller mixing at anaerobic conditions. These conditions are scalable and meet the requirements of efficiency for an economically feasible process.

CHAPTER 2. LITERATURE REVIEW

This chapter updates and expands on previously published literature review of Antonio dos Santos (2016) of which parts are incorporated in this chapter.

2.1 Introduction

Presently, ethanol production from biomass is "a sequence of integrated steps (Figure 2-1) (DOE 2007, Ladisch et al. 2010). They are: biomass growth, harvest and transportation to the biorefinery; pretreatment; enzymatic hydrolysis; fermentation; and distillation/purification and distribution" (dos Santos 2016).

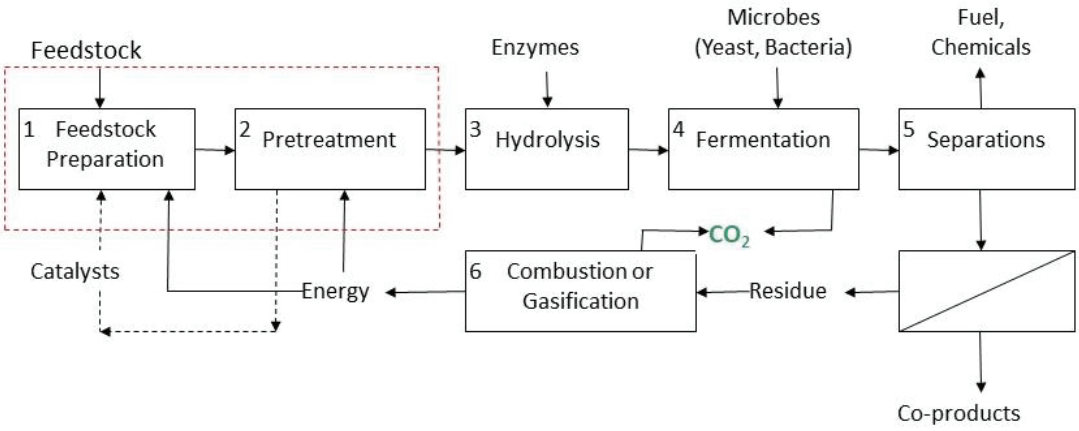

Figure 2-1. Classical cellulose to ethanol conversion schematic. Combined bioprocessing combines steps 3 and 4. Adapted from Ladisch et al. 2010.

"In each of these stages, there is room for improvement and cost savings. However, the most impactful reductions are in the pretreatment and enzymatic hydrolysis units. These processes are important due to the characteristics of lignocellulose. First, lignocellulose is composed of polysaccharides linked to lignin and other aromatic compounds, making the sugars not readily available to fermentation. Making said sugars fermentable in an effective way can decrease the prices to the point that they can compete with oil-based fuels (Lynd, Wyman, and Gerngross 1999; Mosier et al. 2005; Wyman 1999)" (dos Santos 2016).

2.2 Lignocellulose

"Lignocellulose, also referred as biomass, is a complex structure composed of three major organic components: cellulose, hemicellulose and lignin. They are mainly encountered in the secondary cell wall in plants and are formed through photosynthesis from carbon dioxide and water. As the cells grow and mature, cellulose, hemicellulose and lignin are deposited (Rydholm 1965). Biomass also contains small amounts of minerals, various extractives. Ash is also present due to harvesting methods (Wyman 1999; L. Zhang et al. 2017).

Rydholm (1965) defines cellulose as a linear polysaccharide, of sufficient chain length to be insoluble in water or dilute alkali and acids at room temperature, containing only anhydrous glucose units linked together with 1-4-β-glucosidic bonds. Likewise, hemicellulose is defined as the consisting of heteropolymers of 5 and 6 carbon sugars and related substances, such as uronic acids and their substituents, as well as pectins. Lignin is the aromatic polymer of wood, consisting of four or more substituted phenylpropane monomers per molecule. Extractives are the low-molecular compounds of various types, extractable from the biomass with water or organic solvents, excluding components that, by definition, belong to hemicellulose and lignin (Rydholm 1965).

These definitions have not changed significantly since, although hemicellulose definitions now include the carbohydrate component in lignocellulose which depends on the species. Hemicellulose include xylan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan. They are branched polymers of glucose or xylose, substituted with arabinose, xylose, galactose, fucose, mannose, glucose, or glucuronic acid. Some of the side chains may also contain acetyl groups of ferulate (Carpita and Gibeaut 1993).

Lignin is a phenolic polymer mainly composed by three hydroxycinnamyl alcohols: ρ-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol that are polymerized through a series of radical reactions catalyzed by peroxidases and laccases, leading to three different types of lignin (ρ-hydroxyphenyl (H), guaiacyl (G), syringyl (S), respectively) (Freudenberg 1959; Kärkönen and Koutaniemi 2010; Wong 2009) (Figure 2-2)" (dos Santos 2016).

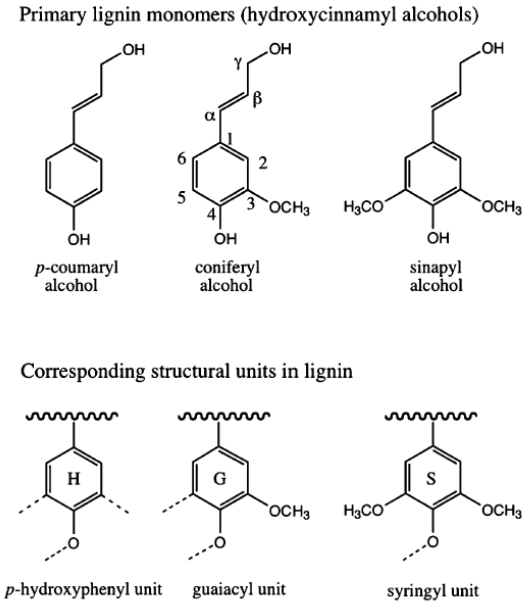

Figure 2-2. Primary lignin monomers and corresponding lignin units. From Wong (2009).

Hydrogen bonds are an important characteristic of lignocellulosic materials. They hold the long chains of cellulose chains tightly together. "Cellulose to cellulose bonds create crystallinity in cellulose microfibrils limiting the access to glucose, making its hydrolysis very difficult (Wyman 1999). Hemicelluloses also make these bonds to cellulose, building the structural backbone of the cell wall (Mosier et al. 2005). Lignin is deposited in the secondary thickened cell walls, giving the mature cell wall structural support, rigidity and water-impermeability (Kärkönen and Koutaniemi 2010).

The fermentable sugars from cellulose and hemicellulose are the components of interest in the lignocellulose. The crystallinity of cellulose, accessible surface area, protection of cellulose by lignin, the heterogeneous character of biomass particles, and cellulose sheathing by hemicellulose all contribute to the recalcitrance of lignocellulosic biomass to hydrolysis. The variability in these characteristics accounts for the varying digestibility between different sources of lignocellulosic biomass (Mosier et al. 2005).

Much of the processing research and development for lignocellulose used for bioethanol production has been focused in overcoming this recalcitrance" (dos Santos 2016).

2.3 Pretreatment

Pretreatment is, in principle, a process to disrupt these barriers so that hydrolytic enzymes can access the carbohydrates and generate the monomers used downstream (Himmel et al. 2007; M. Ladisch et al. 2013; Mosier et al. 2005). This makes pretreatment a critical unit operation as it impacts all other subsequent processing steps to pretreatment. The effect of pretreatment and the subsequent cellulose hydrolysis also depends on the types of changes that occur to lignin structure, which in turn interferes with and hinders enzyme hydrolysis of cellulose. Hence, study of lignin synthesis, structure, function, and its association with the plant cell walls that make up the plant tissues in lignocellulosic biomass (Anderson et al. 2015; Chapple, Ladisch, and Meilan 2007; X. Li et al. 2010) are of specific interest.

"Pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic chemical composition and structure so that hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields (Figure 2-3) (Mosier et al. 2005)" (dos Santos 2016).

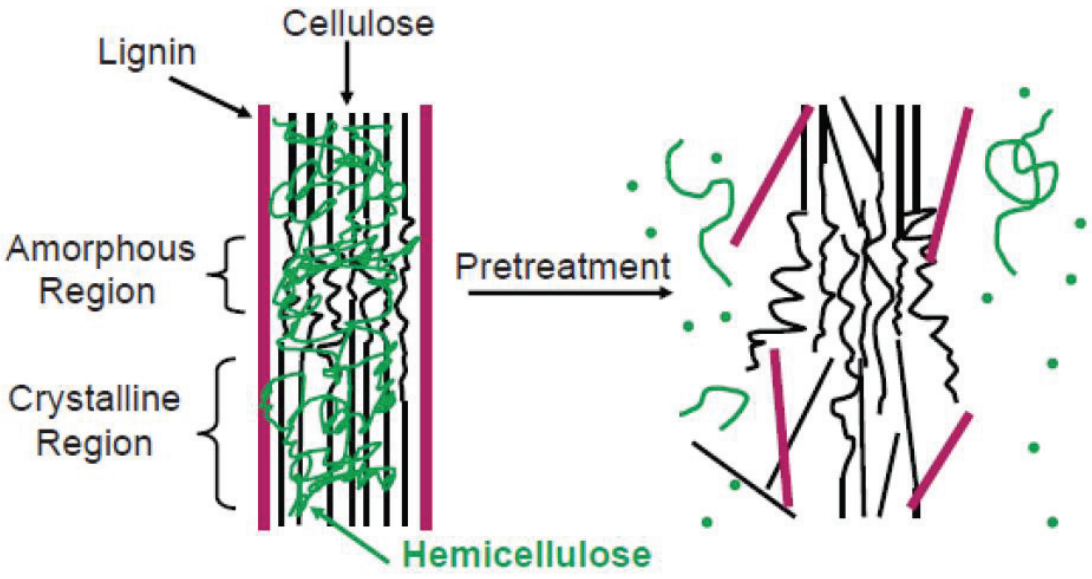

Figure 2-3. Schematic of goals of pretreatment on lignocellulosic material. From Mosier et al., 2005.

"Generally, the process itself utilizes pretreatment additives and/or energy to form solids that are more reactive than native material and/or generate soluble oligo- and monosaccharides (Figure 2-4). Pretreatment has a wide range of effects on the components of lignocellulose depending on the process utilized and various parameters (Mosier et al. 2005)" (dos Santos 2016).

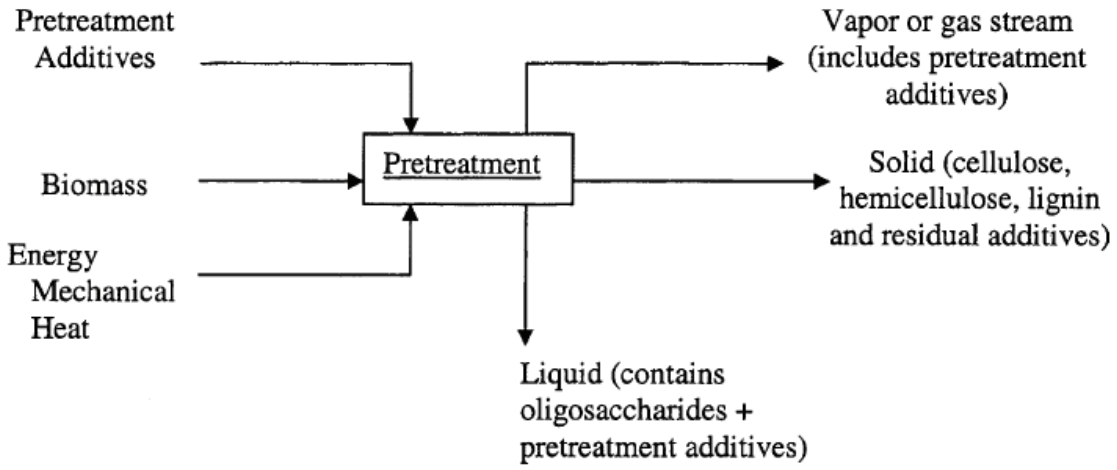

Figure 2-4. Pretreatment process schematic representation. From Mosier et al., 2005.

2.4 Enzymatic Saccharification

"Numerous processes for the saccharification of cellulose have been studied. The enzymatic process has been identified as the most cost-effective (Mosier et al. 2005; Wyman 1999). The cellulase enzyme system consists in three major components: 1,4-D-glucan glucanohydrolase (EC 3.2.1.4), 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). They are commonly called, respectively, Endoglucanase, Exoglucanase and cellobiase. The established optimum conditions for the cellulose saccharification are pH 4.8-5.0 and temperature of 50°C (Ladisch et al. 1983).

"Cellulases are generally produced by fungi, mainly *Trichoderma sp*. and *Aspergillus sp*., as part of their biochemical system to utilize lignocellulose as a carbon source. Their individual mechanism and synergistic action has been elucidated as shown in Figure 2-5. Endoglucanase hydrolyzes cellulose into glucose, cellobiose or cellulose chains of any length. Its action is not strictly random and form non-reducing ends into the cellulose chain. Exoglucanase has a more

specific action, generating only cellobiose, from the non-reducing end of cellulose. β-glucosidase is the most specific of the three, having one substrate (cellobiose) and one product (glucose). β-glucosidase enhances the output of Exoglucanase by eliminating its product which is also an inhibitor. Endoglucanase acts creating more sites for Exoglucanase and breaking down larger chains (Ladisch et al. 1983).

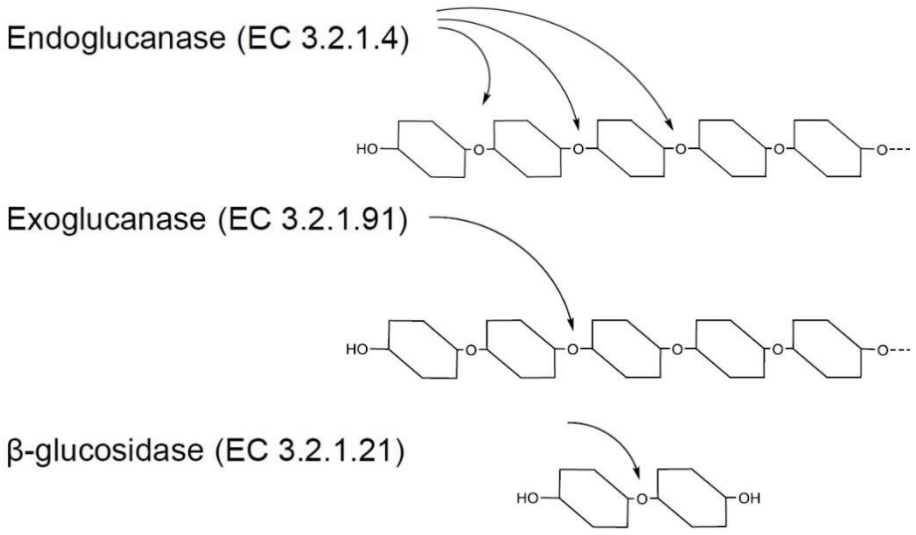

Figure 2-5. Schematic representation of cellulases mechanism.

"The enzymatic hydrolysis can also be done by fermentation using fungi. Studies have shown that these processes increase enzyme titers with high specific activities. This approach may be preferred as it includes more accessory activities including feruloyl esterases, xylanases and other auxiliary hemicellulolytic enzymes (Florencio et al. 2016a, 2016b).

"Recently, other enzymes have been introduced into the enzyme complex to increase productivity. Xylosidases were added to hydrolyze the extensive xylo-oligomers that inhibit cellulase activity (R. Kumar and Wyman 2009). Lytic polysaccharide monooxygenase (LPMO) have also been shown to improve hydrolysis by introducing carboxyl groups in surface-exposed crystalline areas of cellulose allowing cellulases to hydrolyze otherwise highly recalcitrant areas (Eibinger et al. 2014)" (dos Santos 2016).

2.5 Soluble inhibitors and washing

"Liquid hot water, steam explosion, and dilute acid pretreatments generate soluble inhibitors which hamper enzymatic hydrolysis as well as fermentation of sugars to ethanol (Hodge et al. 2008; Jing, Zhang, and Bao 2009; Y. Kim et al. 2011; E. Palmqvist et al. 1996; E. Palmqvist and Hahn-Hägerdal 2000; Tengborg, Galbe, and Zacchi 2001; Ximenes et al. 2010a, 2010b). The inhibitory and toxic compounds and their concentration depend on the specific pretreatments used and its parameters. Soluble sugars, furan derivatives, organic acids, and phenolic compounds have been identified as inhibitory and/or toxic (Hodge et al. 2008; Ximenes et al. 2010a, 2010b).

"Phenols appear to be the strongest inhibitors of enzyme production by different microorganisms as well as being inhibitors and deactivators of enzyme activities themselves (Martin and Akin 1988; Paul et al. 2003; Sineiro et al. 1997; Vohra et al. 1980). Although all cellulases and hemicellulases are affected, β-glucosidases are the most sensitive (Ximenes et al. 2010a, 2010b). The microorganism that produces it also changes the inhibition pattern, i.e., inhibition of enzyme from *Aspergillus niger* requires 4× higher concentrations than β-glucosidase from *Trichoderma reesei*" (dos Santos 2016). The deactivation rates for *T. ressei* and *A. niger* β-glucosidase, respectively are: with tannic acid, 0.068 h^{-1} and 0.045 h^{-1}; with gallic acid, 0.046 and 0.006 h^{-1}; ρ-coumaric, 0.060 and 0.004 h^{-1} (Ximenes et al. 2010b).

The most efficient method to eliminate these soluble inhibitors is through washing. This method has been shown on liquid hot water pretreated poplar (Y. Kim et al. 2009), diluted acid pretreated corn stover (McMillan et al. 2011), diluted acid pretreated poplar (Frederick et al. 2014) and lime pretreated switchgrass (Xu et al. 2010). These methods however require large water usage and the added costs associated with its recycling and treatment.

2.6 Solid lignin/Enzyme interaction

Pretreatment temperatures melt lignin in the secondary cell wall, which extrudes and is redeposited as lignin spheres (Figure 2-6). The high temperatures promote structural changes of lignins to form more condensed, hydrophobic, syringyl deficient forms, accompanied by melting and redeposition into spheres that adsorb proteins (Figure 2-6). An example is given by dilute

acid pretreatment of corn stover and liquid hot water pretreatment of hardwood at high temperature (Selig et al. 2007).

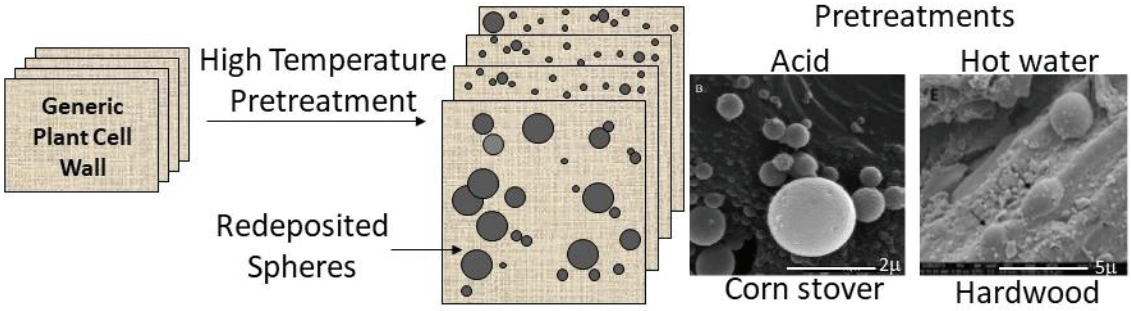

Figure 2-6. Schematic redeposition of lignin spheres after high temperature pretreatment. Acid pretreated corn stover SEM image from Selig et al. (2007). Liquid hot water pretreated hardwood SEM image from Ko et al. (2014b).

Even after washing there is still a large portion of lignin left on the pretreated biomass. The "adsorption of cellulases onto lignin has been appointed as a mechanism of inhibition of the enzymatic function. The mechanism as to why this happens has not been defined (Gao et al. 2014; Guo et al. 2014; Sammond et al. 2014). The possible mechanisms investigated are electrostatic interactions, hydrophobic interactions and interactions of specific regions (carbohydrate-binding modules) with lignin. The interaction appears to be multi-factorial, as hydrophobicity, charge and the presence of CBM are involved in some capacity. The only factor that appears to be universally accepted is hydrophobicity. Other factors such as pore size, surface area have not been proven to be associated to adsorption (Gao et al. 2014; Guo et al. 2014; Pareek, Gillgren, and Jönsson 2013; Sammond et al. 2014).

"Most of the studies are done by analyzing the final adsorption of protein on lignin after equilibrium is reached. Nakagame et al. (2011) analyzed the adsorption of cellulases onto isolated lignin from SO2-steam pretreated Douglas-fir in different temperatures (190, 200 and 210°C) (Nakagame et al. 2011). At higher severity, higher adsorption was measured, likely due to changes on the surface of lignin. Guo et al. (2014) analyzed the adsorption of proteins onto lignin of different plants and identified differences in the composition of lignin that were correlated to cellulase adsorption, the higher the G/S ratio, the higher the affinity. However, other factors could explain the differences in adsorption between samples. The comparison between different species include changes on lignin quantity. Gao et al. (2014) focused on the

free enzyme concentrations during hydrolysis after three pretreatments (Ammonia Fiber Expansion, dilute acid, ionic liquid). Acid pretreated biomass had the highest levels of non-recoverable cellulases, while ionic liquid pretreated biomass had the highest overall cellulase recovery. Sammond et al. (2014) identified the relationship of solvent-exposed hydrophobic clusters to the adsorption of enzymes onto lignin. Lignin was extracted from switchgrass (*Panicum virgatum*) using a solvent-solvent extraction and adsorption of proteins onto it determined using a Quartz crystal microbalance.

"Pareek et al. (2013) measured the adsorption of enzymes (Celluclast 1.5L and Novozyme 188) onto lignin of Norway spruce (*Picea abies*) and black cottonwood (*Populus trichocarpa*) after multiple pretreatments. The experiment underwent up to 72 hours at two different temperatures (4ºC and 45º). Novozyme 188 (mainly β-glucosidase) reaches equilibrium by 2 hours, and Endoglucanase in much less than one hour. The study focused on surface chemistry and found that hydrophobicity is an important characteristic.

"Ko et al. (2015a, 2015b) evaluated the enzyme adsorption onto lignin from hardwood after LHW pretreatment. A close relationship between increasing pretreatment severity and enzyme adsorption was found. The non-productive adsorption led to loss of activity and resulted in dramatically reduced hydrolysis efficiency. Additionally, β-glucosidase, especially from Trichoderma sp. had the highest affinity for lignin. Supplementation with additional β-glucosidase activity from *Aspergillus niger* increased hydrolysis by a factor of 2" (dos Santos 2016).

The adsorption characteristics were generally analyzed by incubating proteins with lignocellulosic material for 2-48 hours, followed by the measurement of the enzyme activity left in supernatant liquid (Guo et al. 2014; Ko, Ximenes, et al. 2015; Ko, Kim, et al. 2015; Rahikainen et al. 2013; Várnai et al. 2011; Zheng et al. 2013). This approach, however is time-consuming, labor intensive and requires large amounts of biomass, especially lignin extraction is involved. Additionally, the preparation of the samples can modify lignin characteristics (Ko, Ximenes, et al. 2015; Rahikainen et al. 2013). While it is a reasonable approach when measuring enzyme adsorption on lignin, it is not an effective method to evaluate a large number of substances. A rapid and reliable method is necessary.

Rahikainen et al. (2013) and Sammond et al. (2014) both utilized quartz crystal microbalance to determine the adsorption of enzyme on lignin film, which is less time consuming and allows for real time measurements when dissipation monitoring is used. However, this method required ammonia solubilization of lignin and the development of the film, which altered the lignin structure, increased complexity and imported imprecision before adsorption experiments.

Lignin blocking by protein can counteract enzyme adsorption caused by lignin exposed after pretreatment, increasing hydrolysis efficiency (Figures 2-7 and 2-8). "This concept has been proven in multiple studies using bovine serum albumin (BSA). These studies were carried out using spruce, corn stover, hardwood, softwood, wheat straw and sugarcane bagasse (Y. Kim et al. 2015; Ko, Ximenes, et al. 2015; Ko, Kim, et al. 2015; Kristensen et al. 2007; L. Kumar et al. 2012; Siqueira et al. 2017; Yang and Wyman 2006)" (dos Santos 2016). Soybean protein extract has also been used as a blocker on the hydrolysis of steam pretreated sugarcane bagasse (Florencio, Badino, and Farinas 2016). The relationship between pretreatment and protein blocking effect was explored on sugarcane bagasse (Siqueira et al. 2017). In this work, chemical pretreatments that modify lignin surface with redeposition were inhibitory.

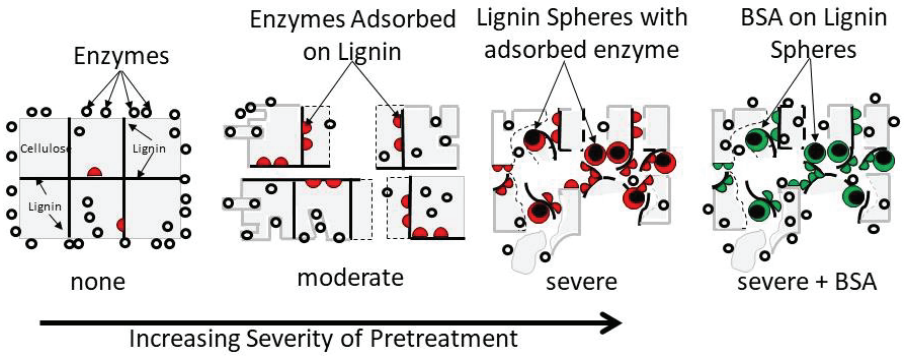

Figure 2-7. Schematic representation of the relationship between pretreatment severity, lignin exposure and protein adsorption.

For recalcitrant biomasses, where high-severity pretreatments are necessary, the protein blocking effect is critical to reducing enzyme loading for a given extent of hydrolysis. Bhagia et al. (2017) evaluated BSA impact on poplar pretreated with liquid hot water and diluted acid in batch and flow-through methods with similar results. BSA was more impactful on diluted acid pretreated material. The BSA effect is restricted to cellulose conversion with no impact on

hemicellulose conversion, which is consistent with β-glucosidase being the most adsorbed enzyme (Bhagia, Kumar, and Wyman 2017; Ko, Ximenes, et al. 2015).

Surfactants are an alternative to proteins as lignin blockers. Their application has been demonstrated with lignin added to pure cellulose (Y. Li et al. 2016), steam or liquid hot water pretreated common reed (Jin et al. 2016), steam pretreated Miscanthus (Sun et al. 2017). Surfactants also block lignin avoiding enzyme adsorption (Y. Li et al. 2016).

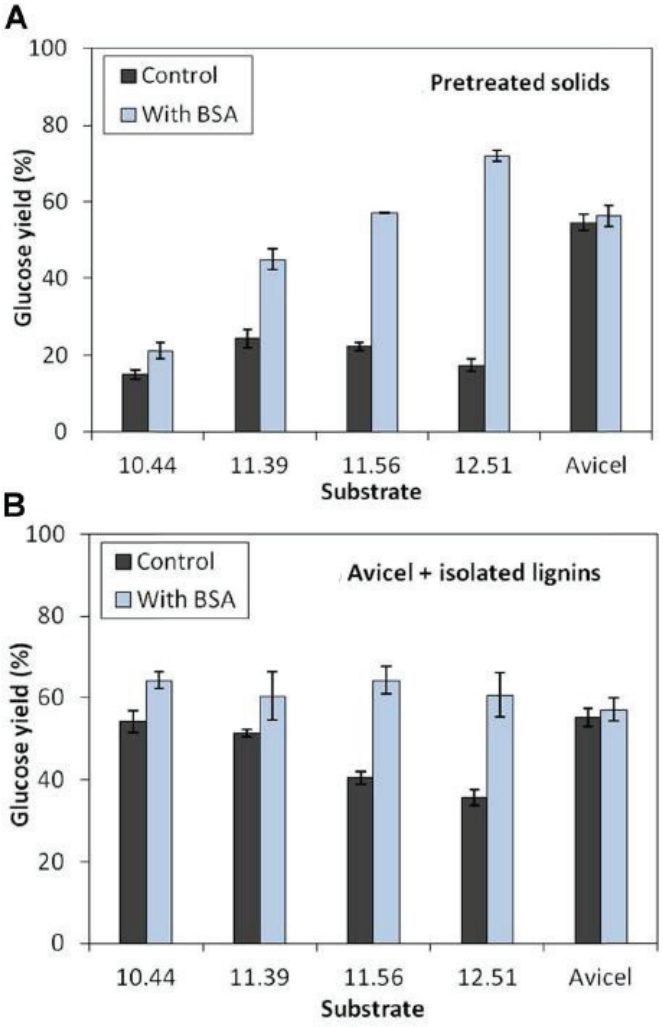

Figure 2-8. Enzymatic hydrolysis of liquid hot water pretreated solids with and without BSA. Hydrolysis conditions: Pretreated solids of severity factor of log R0 = 10.44, 11.39, 11.56 and 12.51 vs. Avicel (A) and Avicel in the presence of isolated lignins (B) were pre-incubated with 50 mg BSA/g dry solid for 1 h at 25°C and 200 RPM. After pre-incubation Cellic Ctec2 of 5 FPU/g glucan was added to reaction mixture and further incubated for 72 h at 50°C and 200 RPM. From Ko et al. 2015b.

2.7 Enzyme/air interaction

Cellulase loss of activity due to interaction with air was first described in 1982 (M. H. Kim et al. 1982). In this work, enzyme deactivation was observed when cellulases when exposed to shear with or without air interface (Figure 2-9). The deactivation was limited with the use of surfactants (Figure 2-10).

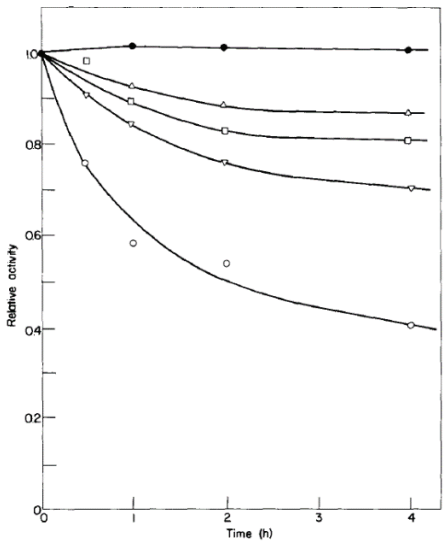

Figure 2-9. Combined effect of air-liquid interface and shearing. Shear rate ($\dot{\gamma}$) = 850 s^{-1}: ●, E_0 = 0.2 mg ml^{-1} without interface; ○, E_0 = 0.2mg ml^{-1} with interface; ▽, E_0 = 0.39mg ml^{-1} with interface; □, E_0 = 0.78 mg ml^{-1} with interface; △, E_0 = 0.98 mg ml^{-1} with interface. From Kim et al. (1982).

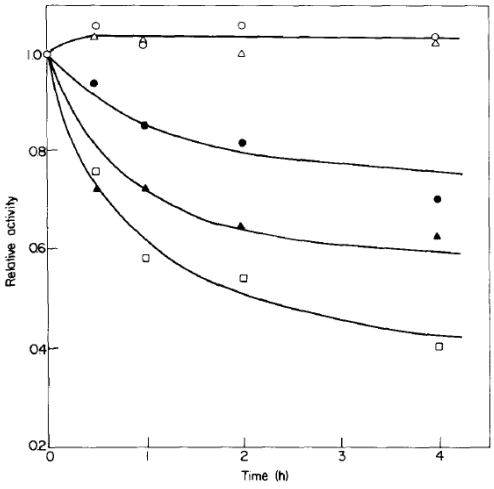

Figure 2-10. Stabilizing effect of Zonyl FSN in the presence of air-liquid interface. Enzyme concentration (E_0) and shear rate ($\dot{\gamma}$) are 0.2 mg ml^{-1} and 850 s^{-1}, respectively: □, control; ▲, 0.02 mg ml^{-1}; ●, 0.1 mg ml^{-1}; △, 1 mg ml^{-1}; ○, 10 mg ml^{-1} of Zonyl FSN solution. From Kim et al. (1982).

The same results were replicated by Bhagia et al. (2018), this time with a newer enzyme preparation and while hydrolyzing pure cellulose (Avicel) and pretreated poplar. In both cases, the lower the enzyme loading the more impactful is the deactivation. In this work, BSA and soy flour had similar effect (Bhagia et al. 2018).

Deactivation of cellulases by O_2 was described by Scott et al. (2016). In this case, the effect was attributed to the presence of a lytic polysaccharide monooxygenase (LPMO). This group showed that maintaining an anaerobic ambient using N_2 reduced the deactivation constant from 17.7 x 10^{-3} ± 2.7 h^{-1} to 1.2 ± 2.1 x 10^{-3} h^{-1} and increased the halftime from 39 to 582 hours. The hypothesis was seemingly confirmed by the addition of a catalase to deactivate LMPOs leading to complete elimination of deactivation when used in an anaerobic ambient (Figure 2-11) (Scott et al. 2016).

These works suggest that air, or oxygen specifically, deactivate cellulases over time and are an important factor in reducing enzyme efficiency.

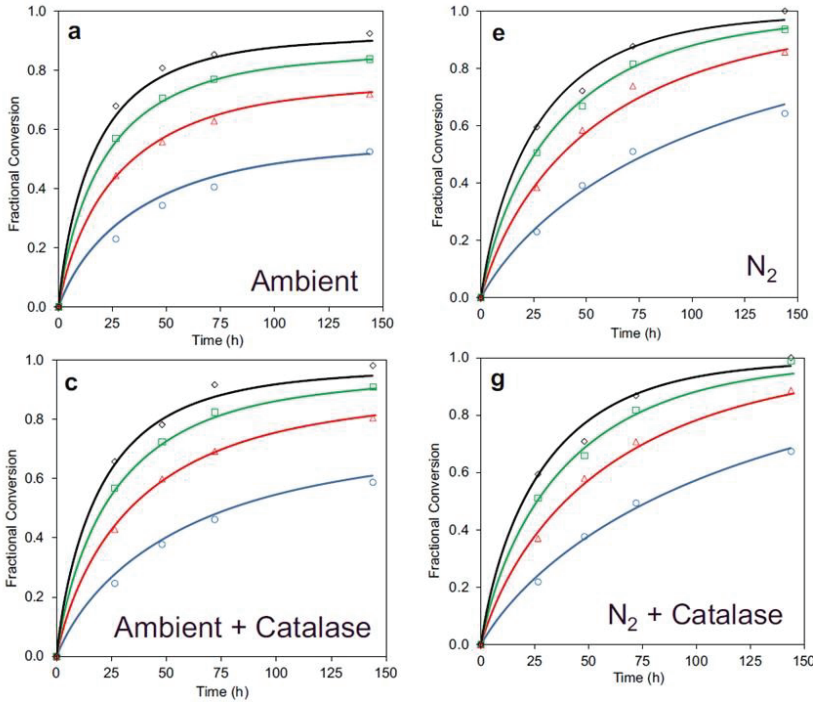

Figure 2-11. Effects of O_2 limitation and catalase addition on CTec3 cellulose hydrolysis progress curves. Pretreated wheat straw was incubated with 2.8 (blue circles), 5.6 (red triangles), 8.4 (green squares) and 11.2 mg protein/g cellulose (black diamonds) of CTec3 for 144 h at 50°C, pH 5. Reactions were carried out in ambient air (panels a and c) and O_2-deprived (panels e and g) conditions. Similarly, the effects of adding catalase under each of these conditions are shown in panels c and g, respectively. Adapted from Scott et al. (2016).

2.8 Liquefaction of biomass

The process of converting biomass to ethanol requires the handling of slurries with high solids concentrations. Slurries of solids concentrations of about 30% (w/v) are needed for efficient ethanol yield (Y. Kim et al. 2015). Slurries high solids concentrations usually exhibit thick, paste-like properties at this stage that make them difficult to handle and transport from one unit operation to the next. This leads to issues with mixing the slurries to achieve adequate mass and heat transfer along with enzyme contact with substrate during the enzyme hydrolysis step (Viamajala et al. 2009). At approximately 20% insoluble solids, the yield stress is on the order of 1,000 Pa. However, pretreated corn stover is considered "pourable" at a concentration of 10% insoluble solids, which corresponds to a yield stress of approximately 10 Pa (Roche, Dibble, and Stickel 2009).

A further complicating aspect of biomass slurries is that biomass can absorb water and therefore discerning distinct volume fractions for the solid and fluid phases is not straightforward. This absorption of water within the biomass particles may cause the bulk to become unsaturated and our visual observations suggest that this unsaturation (i.e., absence of ''free" bulk water continuous phase) occurs at insoluble solid concentrations of 30–40% (w/w). When the slurry becomes unsaturated at high solids' concentrations, portions of the ''void" volume contain air instead of liquid and the biomass now behaves as a wet granular material. This material is highly compressible and the wet particles easily ''stick" to each other and agglomerate. Once there is no free water in the system, the material becomes difficult to shear and uniformly mix (Viamajala et al. 2009). While the exact mechanism leading to pseudoplasticity in biomass slurries remains unknown, the change of the conformation of the lignocellulosic macromolecular chains influenced by the shear rates could be the reason. Highly entangled structures of lignocellulose are formed because it is insoluble in water and difficult to be hydrolyzed (X. Du, Gellerstedt, and Li 2013).

The main liquefaction techniques that have been investigated are thermochemical and enzymatic (Cunha et al. 2014; Jørgensen et al. 2007; Ladisch, Mosier, and Kim 2016). The objective in both processes is decreasing particle size and chain length without generating end-products. Thermochemical liquefaction is defined as process at 250–400°C and high-pressure (5–20 MPa) during which the biomass is converted into bio-oil fraction, a gas fraction and a

solid residue fraction, in water or another suitable solvent (Huang and Yuan 2015). Ladisch et al. (2016) designed a process for liquefaction using relatively low concentrations of maleic acid. An advantage of this process is the selective removal of xylo-oligomers that are highly inhibitory to enzyme activity (Ximenes et al. 2010b).

When enzymatic liquefaction is considered, most experiments have been carried after pretreatment (Cunha et al. 2014; J. Du et al. 2014; Jørgensen et al. 2007), which does not address the transport and handling of biomass before it. These experiments also required large enzyme loadings and were focused solely on viscosity/mixing properties (J. Du et al. 2014; Jørgensen et al. 2007) and the production of enzymes (Cunha et al. 2014). These results however indicate that a fed-batch approach is more efficient, and the only activity needed is Endoglucanase (Cunha et al. 2014).

2.9 Rheology of non-Newtonian fluids

Operating at high-solids concentrations does present several challenges. In addition to product inhibition of the enzyme, there are difficulties with mixing and material handling (Jørgensen et al. 2007). Therefore, an understanding of how these high-solids systems deform and flow, that is, their rheology, during the conversion of the biomass to simple sugars will provide insight into the processing challenges (Roche, Dibble, and Stickel 2009). Viamajala et al. (2009) examined the rheology of acid hydrolyzed corn stover using a Brookfield viscometer and determined that the slurry behaved like a yield stress fluid and that the yield stresses decreased with decreased particle size and increased with solid concentration then became independent. While the relationships are the same before pretreatment, the yield stresses found are generally higher, presumably due to higher particle size (Ehrhardt et al. 2010).

Determining the viscosity of these slurries is a challenge by itself. Yield stress have been evaluated using parallel disks, torque rheometry, helical impeller and cone-and-plate. The values found in these different methods and geometries vary wildly (Ehrhardt et al. 2010; Viamajala et al. 2009; Pimenova and Hanley 2003; Roche, Dibble, and Stickel 2009)

The rheology and yield stress are important as it closely relates to torque and power consumption (Collias and Prud'homme 1985). The relationship has been determined with

Newtonian fluids with the extrapolation to non-Newtonian fluids rely on average viscosity, which depend on empiric measurements (Collias and Prud'homme 1985). The relationships are explained by the Equations 2.1-5, where T is dimensionless torque, τ is torque, P_0 is the power number, Re is the Reynolds number, ρ is the fluid density, D is impeller diameter, N is impeller rotational rate and μ is fluid viscosity. Figures 2-12 and 2-13, show these relationships using model fluids. The model fluids are listed in Table 2-1 (Collias and Prud'homme 1985).

$$P_0 = \frac{P}{\rho N \alpha} \text{ (2.1)}; \quad _e = \frac{\rho N^{\ 2}}{\mu} \text{ (2.2)}; \; T = \frac{\tau \rho}{\mu^2} \text{ (2.3)}; \; 2\pi T = P_{0\alpha} \, \frac{2}{e} \text{ (2.4)}; \; \gamma_{aver\alpha} = 11 * N \text{ (2.5)}$$

Table 2-1. Model fluid composition (Collias & Prud'homme, 1985).

Model fluid	Polyacrylamide polymer concentration (ppm)	Viscosity (Pa.s)
A	0	5.97
A1	150	4.70
A2	300	5.56
B	0	1.72
B1	150	1.94
B2	300	2.07

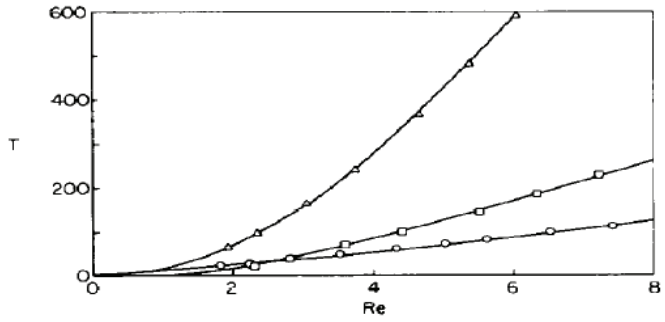

Figure 2-12. Torque number as a function of Re, in the large baffled vessel, for fluids A, IA, 2A. Symbols: ○, fluid A; □, fluid 1A and Δ, fluid 2A (Collias & Prud'homme, 1985).

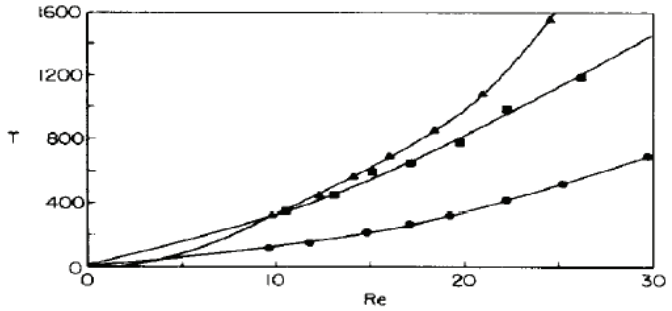

Figure 2-13. Torque number as a function of Re, in the large baffled vessel, for fluids A, IA, 2A. Symbols: ○, fluid B; □, fluid 1B and Δ, fluid 2B (Collias & Prud'homme, 1985).

The yield stress (normalized) relationship with shear rate is shown in Figure 2-14 and the relationship of power and rotational speed is shown in Figure 2-15. As shown in Equation 2.5, there is a direct correlation between those.

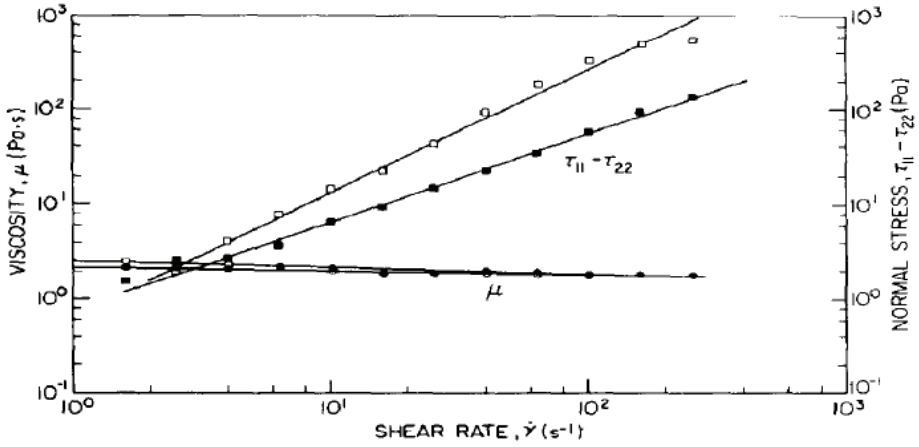

Figure 2-14. Viscosity and primary normal stresses for fluids 1B and 2B. Symbols: ○, μ fluid 2B; □, τ_{11}-τ_{22} fluid 2B; ●, μ fluid 1B and ▪, τ_{11}-τ_{22} fluid 1B (Collias & Prud'homme, 1985).

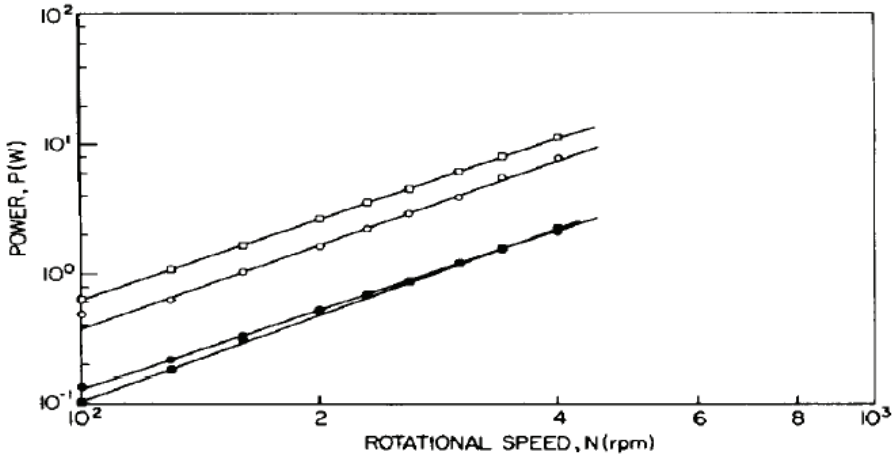

Figure 2-15. Power as a function of the rotational speed of the shaft for Newtonian fluids agitated with a Rushton turbine. Symbols: □, fluid A in large vessel; ▪, fluid A in small vessel; ○, 4.0 Pas Newtonian fluid in large vessel and ●, 4.0 Pas Newtonian fluid in small vessel.

The relationship between yield stress, when pretreated corn stover was used, against biomass conversion during hydrolysis and particle volume fraction, yield stresses are shown in Figures 2-16 and 2-17, respectively. Based on these, torque and power consumption can be estimated, and it becomes clear that higher solids loadings, the power required is high, thus confirming the need to lower the particle size and decreasing the insoluble fraction of biomass.

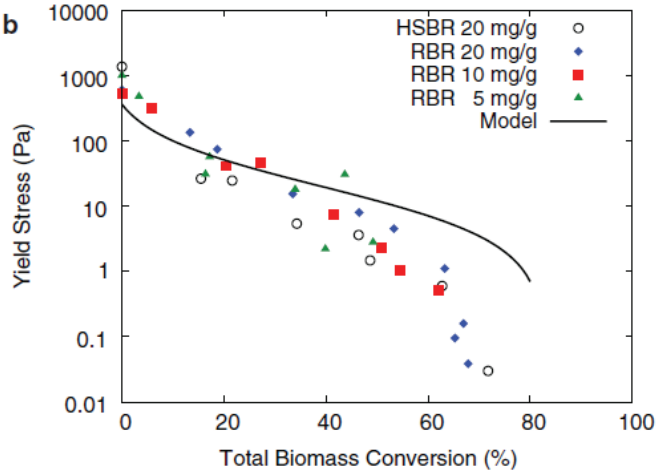

Figure 2-16. Yield stress evolution through the course of enzymatic hydrolysis of pretreated corn stover, starting at 20% insoluble solids content, as a function of conversion and enzyme loading. RBR, roller bottle reactor; HSBR, high solid bioreactor (Roche et al., 2009).

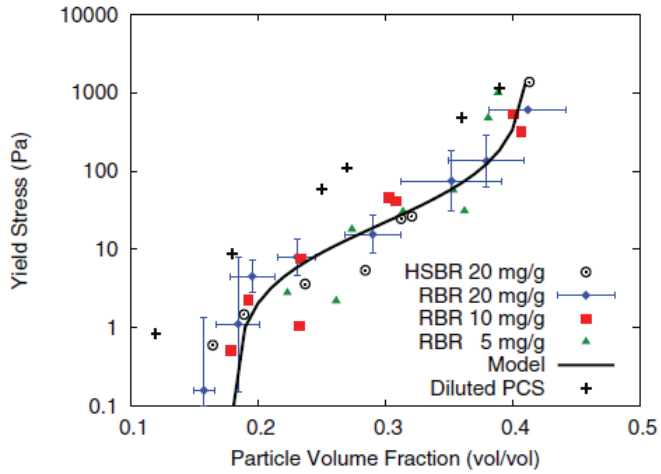

Figure 2-17. Yield stress decreases with decreasing particle volume fraction. The solid line is a model fit. The error bars for the RBR 20 mg/g represent one standard deviation of the mean for each sample point. Standard deviations were determined on a log scale for the yield stress values. RBR, roller bottle reactor; HSBR, high solid bioreactor (Roche et al., 2009).

2.10 Biomass high solids loading and glucose conversion

"For an economically viable lignocellulosic ethanol production, a high biomass loading is necessary (Wingren, Galbe, and Zacchi 2008; Zacchi and Axelsson 1989). Increasing the initial loading significantly lowers the costs of machinery, energy consumption and labor costs (Wingren, Galbe, and Zacchi 2008; Zacchi and Axelsson 1989).

"However, the insolubility of lignocellulose in water, recalcitrance to hydrolysis, accumulation of inhibitors, and inhibitory products, lower the conversion to glucose leading to higher enzyme loadings when compared to laboratory experiments (Alvira et al. 2013; Jørgensen et al. 2007).

"Bommarius et al. (2008) suggested that "jamming" - interference of cellulase action by crowding on proximate cellulose fibers - occurs when there is a high ratio of cellulase molecules to cellulose molecules (Bommarius et al. 2008). Considering that ratio increases as cellulose is hydrolyzed, "jamming" is expected to increase as hydrolysis progresses. Geng et al. (2015) suggested that the lack of free water to diffuse enzymes to other regions of cellulose leads to local accumulation of enzymes and loss of activity (Geng et al. 2015)" (dos Santos 2016). A

related phenomenon is observed when lignocellulose is dried and rewetted, called hornification. This effect leads to smaller pores and stiffening of the polymer structures lowering the efficiency of enzymatic conversion (Fernandes Diniz, Gil, and Castro 2004; Luo and Zhu 2011).

"Expanding on this concept, Jørgensen et al. (2007) explored a more efficient mixing pattern, using a five-chambered horizontal reactor (Figure 2-18). The authors used steam pretreated wheat straw, with concentration up to 40% dry mass. This work showed that the viscosity and biomass accessibility have significant effect in the conversion, and the enzyme loading was lowered to 7.5 FPU/g dry mass. It also demonstrated the close relationship between higher solids loadings and lower conversion, despite the improved mixing (Figure 2-19).

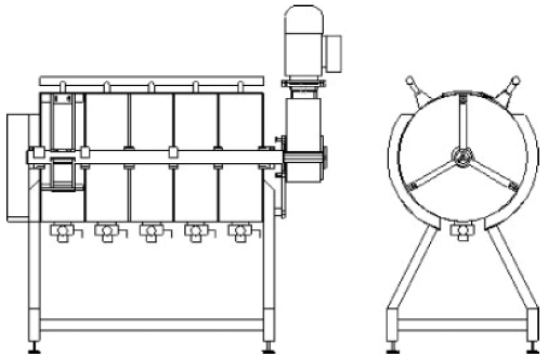

Figure 2-18. Diagram of five chamber liquefaction reactor used for high solids liquefaction. From Jørgensen et al. (2007).

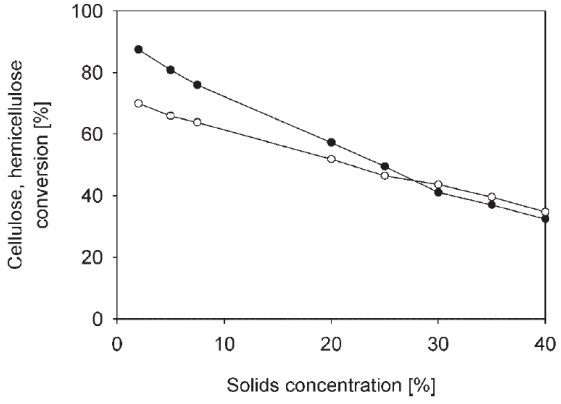

Figure 2-19. Cellulose (•) and hemicellulose (○) conversion after 96 h of liquefaction and saccharification of wheat straw. Experiments at 2%-7.5%(w/w) dry matter were performed in shake flasks. The experiments above 20% dry matter were performed in the liquefaction.

"Other reactor designs have been explored; Roche et al. (2009) identified roller bottle reactors to be the best for laboratory scale. Peg mixer (X. Zhang et al. 2009), addition of steel balls (Geng et al. 2015; Ramachandriya et al. 2013) helical impellers and Rushton impellers (J. Zhang et al. 2009; B. Palmqvist, Wiman, and Lidén 2011; B. Palmqvist and Lidén 2012) were also explored. Despite increasing the final yield across biomass loadings, none of these approaches modified the negative correlation between biomass loading and glucose conversion.

"In two studies, Palmqvist et al. (2011, 2012) evaluated the relationship between impeller settings (speed and torque, respectively) and hydrolysis of steam-pretreated spruce. They observed that conversion and impeller speed are positively correlated. When power input was fixed, and solids loadings increased, initial torque, initial and final speed and glucose conversion fell. When speed was fixed, glucose conversion increased unexpectedly, suggesting that higher power input and higher torque to overcome the viscosity observed lead to higher conversion.

"Viscosity appears to be closely related with the final conversion. Viscosity is related to both particle size and solids loading. As solids increase, viscosity increases linearly until it hit a plateau, at which point there is no free water and the mixture of biomass and buffer becomes a wet granular material rather than slurry (Viamajala et al. 2009). This creates two challenges: first, there is a limit to solids loadings; and higher solids loadings require higher energy input to maintain optimal mixing" (dos Santos 2016).

CHAPTER 3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Sugarcane Bagasse

Sugarcane bagasse from Usina Alta Mogiana S.A. (Brazil) was washed, oven dried at 45°C for 48h, ground in an agricultural crusher and sieved to a particle size fraction of 0.25mm to 2mm and stored at room temperature away from light until use.

3.1.2 Corn stover

Loose corn stover was provided by Forrest Concepts, LLC. The material was ground to pass a 4 mm screen. Pelletized corn stover was provided by Idaho National Laboratory and was kept at room temperature in a sealed container until use.

3.1.3 Enzymes

Enzyme preparations used were Cellulase 13P, Cellic CTEC2, Cellic CTEC3 and Novozymes 188. All preparations were stored at 4°C until use. Portioned samples were transferred to a centrifuge tube using a mechanical pipet. These samples were brought up to room temperature and mixed with the buffer before temperature was adjusted to experiment specifications.

Two batches of Cellic Ctec2 were used. Batch 1 (90 FPU/mL, 247 mg protein/mL) was provided by Novozyme, North America Inc. (Franklinton, NC); Batch 2 (150 FPU/mL, 180 mg protein/mL) purchased from Sigma-Aldrich (St. Louis, MO).

Two batches of Novozyme 188 were used. Batch 1 (381 ρNPGase/mL, 203 mg protein/mL) and Batch 2 (665 ρNPGase/mL, 203 mg protein/mL) was purchased from Sigma-Aldrich (St. Louis, MO).

A single batch of Cellic CTEC3 (59 FPU/mL, 246 mg protein/mL) was provided by Novozyme, North America Inc. (Franklin, NC).

A single batch of Cellulase 13P (0.26 – 0.36 FPU/ mg protein) was purchased from Biocatalysts Ltd. (Cardiff, Wales).

3.1.4 Other Chemicals

All other chemicals were purchased from Sigma-Aldrich unless noted and stored according to the manufacture's recommendation.

3.1.5 Citrate buffer preparation

Citrate buffer (50 mM) was prepared at pH 4.8. Both sodium citrate and citric acid were added to DI water and mixed on plate stirrer at room temperature until all solids are dissolved. pH was confirmed using a Beckman pH meter calibrated with slope accuracy of >90%. Buffer was prepared immediately before any other experiments to avoid contamination. All unused buffer was discarded.

3.2 Methods

3.2.1 Liquid hot water pretreatment

Biomass was pretreated in a Tecam® SBL-1 fluidized sand bath using 1-inch stainless steel reaction tubes. The tubes are filled with biomass and DI water to achieve the desired dry solids while keeping approximately 20% free space above the liquid/slurry level in the tube in order to accommodate thermal expansion of the slurry. Two methods were done to account for heat-up time. The tubes were either kept at 140°C for up to one hour in the fluidized bath before transferring to another bath set to the pretreatment temperature; or directly inserted into the bath with an additional 5 minutes. After pretreatment, the pretreated tubes were immediately placed in water for 5 min for mild quenching. The liquid fraction was separated by vacuum filtration using Whatman #1 filter paper. The filtered solid was washed with 2.5 times the volume of the pretreatment reactor of hot DI water (temperature ≥ 90°C). The washing step was repeated to completely remove the phenolic inhibitors. Biomass was dried overnight and stored at room temperature when used for compositional analysis, lignin extraction, adsorption assays and sugarcane bagasse hydrolysis. Corn stover was immediately used on hydrolysis, surplus pretreatment corn stover was stored at 4°C. The combined effects of pretreatment temperature (T) and time (t) were investigated based on the severity factor equation; log Ro (Ro = t x exp ((T

– 100)/ω)) (Overend, Chornet, and Gascoigne 1987), where the value of ω represents an activation energy associated with the pretreatment (Y. Kim et al. 2014).

3.2.2 Preparation of Lignin Fractions

Milling of liquid hot water pretreated sugarcane bagasse in a Wiley mill® at room temperature with a 20 mesh (0.84 mm) sieving screen was followed by enzymatic hydrolysis in 50 mM citrate buffer at pH 4.8 and 50°C rpm for 7 days in an incubator shaker at 200 rpm or acid hydrolysis.

Enzyme hydrolysis of liquid hot water pretreated sugarcane bagasse was carried out with 0.52 mL enzyme (corresponding to 81.6 mg protein/g pretreated sugarcane bagasse, dry weight basis), of an enzyme mixture prepared from 2.27 mL (331 mg protein) Spezyme CP and 0.5 mL (102 mg protein) Novozyme 188. A large excess of cellulase enzyme was used to ensure maximal hydrolysis of the cellulose to obtain a fraction enriched in lignin for further study.

Following enzyme hydrolysis, the protease Protex TM 7L (54 mg protein/mL) from *Bacillus amyloliquefaciens* (Genencor Division of Danisco, Palo Alto, CA) was used to hydrolyze and remove adsorbed protein from the pretreated sugarcane bagasse. Protease was added at an enzyme concentration of 0.29 mg/mL (equivalent to 5.4 mg protein per g bagasse) (Ko, Kim, et al. 2015). The lignin fraction was then dried at 40°C for 24 hrs and stored at 8°C. Protein adsorbed on the solids washed with water and buffer was measured using the micro Kjeldahl method and corresponded to a nitrogen content (N) of 1.2% (elemental analysis based on mass). After protease treatment, the nitrogen content decreased to 0.4%, which was comparable to the nitrogen content associated with the pretreated bagasse before it was contacted with cellulase.

Acid hydrolysis of liquid hot water pretreated bagasse was carried out using a two-step sulfuric acid treatment following NREL standard laboratory procedures with temperatures and hold times of 30°C (for 60 min) and 120°C (for 60 min) (Sluiter et al. 2008). After the second hydrolysis step, samples were cooled, and solids were washed with water and recovered by vacuum filtration through a porcelain-filtering crucible. The solids were then dried at 40°C for 24 hrs.

3.2.3 Brunauer, Emmett, and Teller (BET) Surface Area Analysis

BET surface areas of isolated lignins and LHW pretreated sugarcane bagasse were determined by nitrogen adsorption, using a Micromeritics TriStar II 3020 at Micromeritics Analytical Services (Norcross, GA) according to the multi-point BET procedure. Samples were dried under vacuum at 40°C overnight before analysis. Data was based on single measurements.

3.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra of lignins were obtained by using a Thermo-Nicolet FTIR (Nexus 470) with OMNIC software. Spectra of each sample ranging from 4000 to 800 cm-1 were averaged from 128 scans at a spectral resolution of 4 cm-1. Peak height and baseline spectra were automatically corrected when determining absorbance intensity.

3.2.5 Scanning Electron Microscopy (SEM)

The liquid hot water pretreated sugarcane bagasse and isolated lignin fractions were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h at room temperature. The samples were then washed with distilled water and post fixed in 0.5% (v/v) osmium tetroxide for 30 min at room temperature, dehydrated in an ethanol series, critical point-dried with CO2, and sputter coated with gold using a Bal-Tec SCD 050. The samples were examined using a FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) with an accelerating voltage of 12.5.

3.2.6 Enzyme Activity Assays

3.2.6.1 Filter Paper Assay for Saccharifying Cellulase

FPU was determined using an adapted procedure from Mandels et al. (1976). A DNS solution was prepared immediately before the assays. In 1 L of DI water, 10g of dinitrosalicylic acid, 16g sodium hydroxide pellets, and 300g potassium sodium tartrate were added in small increments. The solution was stirred until completely dissolved. Surplus DNS was stored in a brown glass bottle. Whatman No. 1 filter 1.0 x 6.0 cm paper strips were used, equivalent to 50mg. Each sample and buffer was allowed to adjust to room temperature.

Into a 30 mL test tube for each replicate, the following were added: 1.0mL of citrate buffer, pH 4.8; 0.5 mL of appropriately diluted enzyme, one filter paper strip. The test tubes were vortexed to push the paper strips to the bottom. All tubes were incubated at 50°C for 60 min. A glucose standard curve was also made with a 0.5 mL of glucose concentration instead of enzyme sample.

After incubation, 3.0 mL of DNS was added and mixed using the vortex. The new solutions were boiled on water bath on hot plate for 5.0 min. To the boiled tubes, 20 mL of DI water was added and mixed using the vortex. Pulp settled over 20 min. Using a mechanical pipet, 1000µL from the test tube was transferred to a cuvette and measure the absorbance at 540 nm.

3.2.6.2 Acid Cellulase Assay

A DNS solution was prepared immediately before the assays. In 1 L of DI water, 10g of dinitrosalicylic acid, 16g sodium hydroxide pellets, and 300g potassium sodium tartrate were added in small increments. The solution was stirred until completely dissolved. Surplus DNS was stored in a brown glass bottle. A 2% solution of carboxymethyl-cellulose (CMC) dissolved in DI water. The solution was kept at 4°C. For the assay, the CMC solution was diluted to 1% using equal parts of the solution and 50 mM citrate buffer (pH 4.8). Using a test tube for each replicate, 50 µL of appropriately diluted enzyme was added to 200 µL 1% CMC. The test tubes were incubated for 15 min at 50°C. Immediately after incubation, 1.0 mL of DNS was added, and tubes were boiled on water bath on hot plate for 5 min. Using a mechanical pipet, 1000µL from the test tube was transferred to a cuvette and measure the absorbance at 540 nm. All samples and standards were run in triplicate. Table 3-1 shows an example of standard curve composition.

Table 3-1. Standard preparation for acid cellulase assay.

	1	2	3	4	5	6
1.0% CMC working suspension, pH 4.8 (µL)	200	200	200	200	200	200
10 mg/mL glucose solution (µL)	0	2	3	5	10	15
50 mM citrate buffer pH 4.8 (µL)	50	48	47	45	40	35
Total volume (µL)	250	250	250	250	250	250
Total glucose (µg)	0	20	30	50	100	150

3.2.6.3 β-Glucosidase enzyme assay using ρ-nitrophenyl-glucosidase

A substrate solution of 10 mM ρ-nitrophenol-glucopyranoside in citrate buffer was prepared immediately before the assay. ρ-Nitrophenol standard solution was prepared in DI water at a concentration of 2 mM. A 1M sodium carbonate solution was prepared in DI water. pH was measured to assure a pH > 10. Using a test tube for each replicate, 100 µL of appropriately diluted enzyme was added to 400 µL of 10 mM ρ-nitrophenol-glucopyranoside. The test tubes were incubated for 15 min at 50°C. Immediately after incubation, 1.0 mL of 1M sodium carbonate was added to stop the reaction. Using a mechanical pipet, 1000µL from the test tube was transferred to a cuvette and measure the absorbance at 410 nm. All samples and standards were run in triplicate. Table 3-2 shows an example of standard curve composition. One unit is defined as the amount of enzyme releasing 1 µmol of ρ-nitrophenol per min under specified conditions (Dien et al. 2008).

Table 3-2. Standard preparation for β-glucosidase activity.

	1	2	3	4	5
10mM ρ-nitrophenol-glucopyranoside (µL)	400	400	400	400	400
2mM ρ-NP	0	10	20	50	100
Buffer (µL)	100	90	80	50	0
Na_2CO_3 (mL)	1	1	1	1	1
ρ-NP (µmole)	0	0.02	0.04	0.10	0.2

3.2.7 Protein Adsorption Experiments

Adsorption measurements of cellulolytic enzymes on the isolated lignin fractions prepared from enzyme hydrolyzed sugarcane bagasse were carried out at a protein concentration of 0.5 mg/mL (equivalent to initial concentrations of 50 mg protein/g dry lignin) using the procedures of Ko et al. (2015a). For assays comparing adsorption of non-purified (Novozyme 188) and purified β-glucosidase (both from *A. niger*), protein concentrations were selected based on maintaining an initial constant ratio of β-glucosidase activity to lignin solids equivalent to 3040 ρNPGase/g lignin. This activity corresponds to concentrations of 203 mg/mL and 0.3 mg/mL for non- purified and purified β-glucosidases, respectively, which is equivalent to 1622 and 10 mg protein/g lignin solids, respectively. All adsorption reactions were run in triplicate, with values averaged for reporting purposes.

Adsorption assays were carried out using 1.5 mL centrifuge tubes containing 10 mg/mL of lignin, enzymes and 0.05 M citrate buffer (pH 4.8) for a total volume of 500μL. Lignin was dispersed by sonication at room temperature for 1 min using a Branson 2510 sonicator. The slurry was gently mixed in a hybridization incubator (FinePCR, GyeongGi, Korea) rotating at 20 inversions per min at 30°C and 45°C for 1.5 h, 24 h and 48 h, respectively. After incubation, the supernatant was separated from the solid fraction by centrifugation at 4700 x g for 10 min and ambient temperature. Controls consisted of enzyme in buffer and confirmed that thermal inactivation and binding of enzymes on tube walls did not occur during the 48 hour time period over which adsorption measurements were carried out.

Proteins in the supernatant were measured using a bicinchoninic acid (BCA) protein assay reagent kit (Thermo-Scientific, Rockford, IL). The amount of protein adsorbed was measured by difference between the protein initially in solution and that remained after contact with added lignin. Changes in enzyme activity in solution were fitted by the model of Sandana and Henley (1987), and differences determined with software Origin Pro 2015 (OriginLab, Northampton, MA).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor changes in enzyme compositions remaining in solution after contact with lignin. The samples were prepared from supernatant, 10 μL was loaded to precast 12% TGX mini-gel (Bio-

Rad) and run using Mini-PROTEIN II cell electrophoresis system at room temperature. The gel was visualized with a silver staining kit (Bio-Rad).

3.2.8 Enzymatic hydrolysis

Enzymatic hydrolysis was conducted in pH 4.8 citrate buffer (50 Mm) at 50ºC for 72 hours. Selected enzyme was mixed with the buffer at the selected concentrations before hydrolysis was started. The substrate (biomass, pure cellulose, and/or lignin fraction) were added directly to the reaction vessels. Then, the enzyme-buffer solution was added to the vessel and moved into the shaker/bioreactor. For 1mL reaction, centrifuge tubes were used, reactions were carried on a Vortemp 56 orbital shaker (Labnet, Edison, NJ) at 800 RPM. For 50 mL shaker flasks were used and reactions carried on a New Brunswick™ Innova® 44 shaker (Eppendorf North America, Hauppauge, NY). For the 600 mL reactions, three 1L Bioflo® glass vessels were used, temperature and mixing were controlled by a BioFlo® 115 control tower using a heating jacket and an overhead mixer. For anaerobic reactions, the headspace was purged using N_2 for 2 hours flowing at 1.5L/hr.

Two mL of thoroughly mixed sample was then taken at the end of the hydrolysis and was boiled for 5 min to deactivate the enzymes. To remove solids from the liquids, the sample was centrifuged at 13000 rpm for 10 min. The centrifuged supernatant was further filtered through a nylon syringe filter (0.2 mm, Acrodisc1) for analysis by HPLC. An Aminex-h87 column was used with a 0.5M H_2SO_4 solution as mobile phase. Run-times were 30 minutes.

3.2.9 Model simulations

Simulations were carried using Microsoft Excel 2013, using Microsoft Visual Basic for Application to input and set the ordinary equations. This software was utilized due to its familiarity and capacity to carry first-order equations quickly. All simulations run on Windows 10.

3.2.10 Mathematical and Statistical analysis

All calculations, data analysis, and graphs were done using Origin Pro 2015 (OriginLab Corp., Northampton, MA). Statistical significance was set at $\alpha = 0.05$. Data fitting was done with a Levenberg Marquardt algorithm.

CHAPTER 4. LIGNIN ADSORPTION OF ENZYMES ON PRETREATED SUGARCANE BAGASSE

This chapter includes material from recently published journal paper by Zanchetta, dos Santos et al. (2018) of which parts are incorporated verbatim in this chapter.

4.1 Introduction

Lignocellulose is an intricate and recalcitrant complex of polysaccharides linked to lignin and other aromatic compounds, making the sugars not readily available to fermentation. "Lignin confers rigidity to the cell wall and protects the cellulose and hemicellulose against hydrolytic attack by plant pathogens (Bermek and Eriksson 2009; Papinutti and Forchiassin 2007). The stable aromatic structure of lignin is a major obstacle to the hydrolysis of lignocellulose and necessitates pretreatment of lignocellulose to make the cellulose accessible and susceptible to enzyme hydrolysis (Bonawitz et al. 2014; Ko et al. 2009; Ladisch, Ladisch, and Tsao 1978)" (Zanchetta et al. 2018).

During lignocellulose pretreatment, "lignin and hemicellulose are solubilized; cellulose crystallinity, degree of polymerization, and particle size are reduced and porosity and accessibility of cellulose to enzymes are increased. For hardwood pretreated in liquid hot water (Weil et al. 1997; Weil et al. 1998), there is a particularly strong correlation of increased enzyme hydrolysis with decreased particle size and cellulose degree of polymerization (Y. Kim et al. 2015; Ximenes et al. 2017). These changes allow greater access of enzymes to cellulose and hemicellulose but may also alter how cell wall components and particularly lignin, interact with enzymes (Ximenes et al. 2017).

"Pretreatment carried out at temperatures above 160°C cause lignin to melt. Upon cooling lignin is redeposited as micron-sized beads or globular granules on plant cell wall structures and cellulose fibers (Donohoe et al. 2008). This increases both exposed hydrophobic surface area of lignin and the adsorption of protein resulting in significant loss of enzyme activities (Kaparaju and Felby 2010; Ko, Ximenes, et al. 2015). Prior work showed that liquid hot water (LHW) pretreatment changed hardwood derived lignin resulting in a more condensed and syringyl

deficient form with higher guaiacyl content that adsorbed more enzymes, mainly β-glucosidases at 45°C (Ko, Ximenes, et al. 2015; Ko, Kim, et al. 2015).” (Zanchetta et al. 2018).

The current work addresses the effect of temperature on enzyme adsorption on lignin-rich fractions derived from liquid hot water pretreated sugarcane bagasse and impact of the addition of bovine serum albumin (BSA) to prevent lignin adsorption. Enzyme adsorption was minimal at 30°C and moderate at 45°C when β-glucosidase, exoglucanases, and endoglucanases from *Asperigillus niger* and *Trichoderma reesei* were contacted with the lignin. Acid hydrolyzed liquid hot water pretreated sugarcane bagasse lignin completely adsorbed cellulases at 45°C. FTIR showed minimal difference between lignin from liquid hot water pretreatment compared to lignin in bagasse prior to pretreatment, while acid hydrolysis generated hydrophobic surfaces in residues essentially devoid of hemicellulose and cellulose (Zanchetta et al. 2018).

Lower temperatures reduced or eliminated enzyme adsorption but also lower the reaction rates, requiring longer reaction times to reach completion. Higher rates require the reaction to be carried at the enzyme optimum temperature in which adsorption is higher. A direct approach to solving this problem is the use another protein that can interact with lignin with higher affinity, effectively blocking the lignin and keeping more enzymes on the solution. This concept has been proven in multiple studies using bovine serum albumin (BSA). These studies were carried using spruce, corn stover, hardwood, softwood and wheat straw (Y. Kim et al. 2015; Ko, Ximenes, et al. 2015; Ko, Kim, et al. 2015; Kristensen et al. 2007; L. Kumar et al. 2012; Siqueira et al. 2017; Yang and Wyman 2006). In this work we evaluate the impact of BSA on pretreated sugarcane bagasse during hydrolysis and identified the factors that modulate lignin adsorption.

4.2 Materials and Methods

4.2.1 Sugarcane Bagasse

Sugarcane bagasse from Usina Alta Mogiana S.A. (Brazil) was washed, oven dried at 45°C for 48h, ground in an agricultural crusher and sieved to a particle size fraction of 0.25mm to 2mm and pretreated using liquid hot water or dilute acid (Zanchetta et al. 2018). Raw and pretreated sugarcane bagasse were analyzed following the NREL LAP standard procedure (Sluiter et al. 2008). Compositions of the sugarcane bagasse before and after pretreatments are given in Table 4-1.

4.2.2 Liquid Hot Water (LHW)

"Sugarcane bagasse was pretreated in a Tecam® SBL-1 fluidized sand bath using 1-inch stainless steel reaction tubes filled with 6.1g of sugarcane bagasse (7.8% moisture) and 31.4 mL of deionized water to achieve 15% (w/w) dry solids with approximately 20% free space above the liquid level in the tube in order to accommodate thermal expansion of the liquid hot water (Y. Kim et al. 2009, 2014)" (Zanchetta et al. 2018). To minimize heat-up time uncertainty, the tubes were heated to 140ºC for up to one hour then held at 190, 200 or 210 ºC for 20 min in a Tecam1 SBL-1 fluidized sand bath (dos Santos, 2016). The pretreated tubes were immediately placed in water for 5 min for mild quenching. The liquid fraction was separated by vacuum filtration using Whatman #1 filter paper. The filtered solid was washed with 100mL of hot DI water (temperature ≥ 90°C). The washing step was repeated to completely remove the phenolic inhibitors dried overnight and stored at room temperature until use. The combined effects of pretreatment temperature (T) and time (t) were investigated based on the severity factor equation; log Ro (Ro = t x exp ((T – 100)/ω)) (Overend, Chornet, and Gascoigne 1987), where the value of ω represents an activation energy associated with the pretreatment (Y. Kim et al. 2014).

4.2.3 Preparation of Lignin Fractions

"Milling of liquid hot water pretreated sugarcane bagasse in a Wiley mill® at room temperature with a 20 mesh (0.84 mm) sieving screen was followed by enzymatic hydrolysis in 50 mM citrate buffer at pH 4.8 and 50°C rpm for 7 days in an incubator shaker at 200 rpm or acid hydrolysis. Enzyme hydrolysis of liquid hot water pretreated sugarcane bagasse was carried out with 0.52 mL enzyme (corresponding to 81.6 mg protein/g pretreated sugarcane bagasse, dry weight basis), of an enzyme mixture prepared from 2.27 mL (331 mg protein) Spezyme CP and 0.5 mL (102 mg protein) Novozyme 188. A large excess of cellulase enzyme was used to ensure maximal hydrolysis of the cellulose to obtain a fraction enriched in lignin for further study.

"Following enzyme hydrolysis, the protease Protex TM 7L (54 mg protein/mL) from Bacillus amyloliquefaciens (Genencor Division of Danisco, Palo Alto, CA) was used to hydrolyze and remove adsorbed protein from the pretreated sugarcane bagasse. Protease was added at an enzyme concentration of 0.29 mg/mL (equivalent to 5.4 mg protein per g bagasse) (Ko, Kim, et al. 2015). The lignin fraction was then dried at 40°C for 24 hrs and stored at 8°C.

Protein adsorbed on the solids washed with water and buffer was measured using the micro Kjeldahl method and corresponded to a nitrogen content (N) of 1.2% (elemental analysis based on mass). After protease treatment the nitrogen content decreased to 0.4%, which was comparable to the nitrogen content associated with the pretreated bagasse before it was contacted with cellulase.

"Acid hydrolysis of liquid hot water pretreated bagasse was carried out using a two-step sulfuric acid treatment following NREL standard laboratory procedures with temperatures and hold times of 30°C (for 60 min) and 120°C (for 60 min) (Sluiter et al. 2008). After the second hydrolysis step, samples were cooled, and solids were washed with water and recovered by vacuum filtration through a porcelain-filtering crucible. The solids were then dried at 40°C for 24 hrs.

"Liquid hot water pretreatment of the sugarcane bagasse removed some hemicellulose and extractives, resulting in increases in the glucan (cellulose) and lignin fractions in the remaining solids (Table 4-1). The ratio of acid insoluble lignin (AIL) to acid soluble lignin remained constant during pretreatment but increased by 2 or 30 fold when the pretreated material was hydrolyzed using enzyme or acid, respectively. The lignin content of acid treated bagasse was about 2.3 x higher than that of enzyme treated lignocellulose (Table 4-1)." (Zanchetta et al. 2018).

4.2.4 Brunauer, Emmett, and Teller (BET) Surface Area Analysis

"BET surface areas of isolated lignins and LHW pretreated sugarcane bagasse were determined by nitrogen adsorption, using a Micromeritics TriStar II 3020 at Micromeritics Analytical Services (Norcross, GA) according to the multi-point BET procedure. Samples were dried under vacuum at 40°C overnight before analysis. Data was based on single measurements" (Zanchetta et al. 2018).

4.2.5 Fourier Transform Infrared Spectroscopy (FTIR)

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra of lignins were obtained by using a Thermo-Nicolet FTIR (Nexus 470) with OMNIC software. Spectra of each sample ranging from 4000 to 800 cm-1 were averaged from 128 scans at a spectral resolution of

4 cm-1. Peak height and baseline spectra were automatically corrected when determining absorbance intensity (Ko et al. 2014).

4.2.6 Scanning Electron Microscopy (SEM)

The liquid hot water pretreated sugarcane bagasse and isolated lignin fractions were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h at room temperature. The samples were then washed with distilled water and post fixed in 0.5% (v/v) osmium tetroxide for 30 min at room temperature, dehydrated in an ethanol series, critical point-dried with CO2, and sputter coated with gold using a Bal-Tec SCD 050. The samples were examined using a FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) with an accelerating voltage of 12.5.

4.2.7 Enzymes for Adsorption Studies

Cellic Ctec2 (90 FPU/mL, 247 mg protein/mL) was provided by Novozyme, North America Inc. (Franklinton, NC). Novozyme 188 (381 ρNPGase/mL, 203 mg protein/mL) was purchased from Sigma-Aldrich, St. Louis, MO. Cellic CTEC3 (59 FPU/mL, 246 mg protein/mL) was provided by Novozyme, North America Inc. (Franklin, NC). Novozyme 188 (665 ρNPGase/mL, 203 mg protein/mL) was purchased from Sigma-Aldrich (St. Louis, MO). Cellulase 13P (0.26 – 0.36 FPU/ mg protein) was purchased from Biocatalysts Ltd. (Cardiff, Wales). The activity of purified β-glucosidase from Megazyme (Wicklow, Ireland) was 80 U/mg at 40°C and pH 4.0 with p-nitrophenyl β-glucoside as substrates. SDS-gel electrophoresis gave a single band of MW = 121,000, with pI = 4.0 as determined by isoelectric focusing (dos Santos, 2016; Zanchetta et al. 2018).

4.2.8 Enzyme Activity Assays

"Endoglucanase activity was measured using 1% (w/v) carboxymethyl cellulose (CMC, Sigma-Aldrich, St. Louis, MO) as substrate (Dien et al., 2008). Exoglucanase and β-glucosidase activities were measured using 2.5 and 10 mM of ρ-nitrophenyl-β-D-cellobioside (pNPC, Sigma-Aldrich, St. Louis, MO) and ρ-nitrophenyl-β-D-glucopyranoside (pNPG, Sigma-Aldrich, St. Louis, MO), respectively, as substrates. One unit is defined as the amount of enzyme that releases 1 μmol of ρ-nitrophenol per min under specified conditions (Dien et al., 2008). Proteins in the supernatant were measured using a bicinchoninic acid (BCA) protein assay reagent kit

(Thermo-Scientific, Rockford, IL). The amount of protein adsorbed was measured by difference between the protein initially in solution and that remained after contact with added lignin. Changes in enzyme activity in solution were fitted by the model of Sandana and Henley (1987), and differences determined with software Origin Pro 2015 (OriginLab, Northampton, MA)" (Zanchetta et al. 2018).

4.2.9 Protein Adsorption Experiments

"Adsorption measurements of cellulolytic enzymes on the isolated lignin fractions prepared from enzyme hydrolyzed sugarcane bagasse were carried out at a protein concentration of 0.5 mg/mL (equivalent to initial concentrations of 50 mg protein/g dry lignin) using the procedures of Ko et al. (2015a). For assays comparing adsorption of non-purified (Novozyme 188) and purified β-glucosidase (both from *A. niger*), protein concentrations were selected based on maintaining an initial constant ratio of β-glucosidase activity to lignin solids equivalent to 3040 ρNPGase/g lignin. This activity corresponds to concentrations of 203 mg/mL and 0.3 mg/mL for non- purified and purified β-glucosidases, respectively, which is equivalent to 1622 and 10 mg protein/g lignin solids, respectively. All adsorption reactions were run in triplicate, with values averaged for reporting purposes.

"Adsorption assays were carried out using 1.5 mL Eppendorf tubes containing 10 mg/mL of lignin, enzymes and 0.05 M citrate buffer (pH 4.8) for a total volume of 500µL. Lignin was dispersed by sonication at room temperature for 1 min using a Branson 2510 sonicator. The slurry was gently mixed in a hybridization incubator (FinePCR, GyeongGi, Korea) rotating at 20 inversions per min at 30°C and 45°C for 1.5 h, 24 h and 48 h, respectively. After incubation, the supernatant was separated from the solid fraction by centrifugation at 4700 x g for 10 min and ambient temperature. Controls consisted of enzyme in buffer and confirmed that thermal inactivation and binding of enzymes on tube walls did not occur during the 48 hour time period over which adsorption measurements were carried out.

"Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor changes in enzyme compositions remaining in solution after contact with lignin. The samples were prepared from supernatant, loaded to 12% TGX mini-gel (Bio-Rad) and run using Mini-PROTEIN II cell electrophoresis system. The gel was visualized by staining with silver (Bio-Rad). The supernatant fractions of enzyme of lignin controls were run in parallel.

4.2.10 Enzymatic hydrolysis

"Enzymatic hydrolysis using biomass was conducted at 50 ºC for 72 hours in an orbital shaker (200 RPM) using a total volume of 50 mL. Enzymatic hydrolysis using lignin fractions were conducted at 30 or 45°C for 72 or 168 hours in an orbital shaker (800 RPM) using a total volume of 1 mL. Initial solids concentration was 1% glucan (w/v) unless otherwise noted. Citrate buffer (pH 4.8) was used. One milliliter of thoroughly mixed sample was then taken at the end of the hydrolysis and was boiled for 5 min to deactivate the enzymes. To remove solids from the liquids, the sample was centrifuged at 13000 rpm for 2 min. The centrifuged supernatant was further filtered through a nylon syringe filter (0.2 mm, Acrodisc1) for analysis by HPLC" (dos Santos 2016).

4.2.11 Mathematical and Statistical analysis

Data was analyzed and fitted to a polynomial model using Levenberg Marquardt algorithm. All calculations and graphs were done using Origin Pro 2015 (OriginLab Corp., Northampton, MA).

4.3 Results and Discussion

4.3.1 Changes in Composition of Liquid Hot Water Pretreated Sugarcane Bagasse

"In order to understand the impact of LHW pretreatment on sugarcane bagasse, biomass composition was analyzed before and after pretreatment (Table 4-1). Raising the temperature and severity resulted in higher proportions of cellulose recovered in the solid portion. Conversely, the amount of hemicellulose decreased, with its complete removal at 200 and 210°C. Lignin content decreased, while the amount of extractives dramatically increased. After each pretreatment and washing, 37% of the original mass was removed, due to removal of water soluble parts. When using 200°C for 20 minutes, 87% of the original cellulose was recovered in solid portion, while only 40% of lignin was recovered. At more severe conditions (210°C for 20 minutes), all components were recovered in lower amounts. Compared to hardwood (Ko, Kim, et al. 2015), SCB was less recalcitrant to the pretreatment. Solid recovery was smaller, between 71.7% to 93.6% of the hardwood mass was recovered depending on severity; xylan (hemicellulose) recovery was also smaller, at the highest severity, hardwood still recovered

17.5% of it; and on the opposite trend, less lignin was recovered, 76.1% to 91.2% of all lignin was still present in pretreated hardwood. This difference was already expected and is explained by the differences in cell wall composition" (dos Santos 2016).

Table 4-1. Compositional Analysis of untreated and LHW pretreated sugarcane bagasse and extracted lignin

	Untreated	LHW pretreated				Lignin from enzyme hydrolysis of LHW bagasse	Lignin from acid hydrolysis of acid pretreated bagasse
Pretreatment condition	-	190°C, 15 min	190°C, 20 min	200°C, 20 min	210°C, 20 min		
Severity factor[a]	-	9.60	9.80	10.74	11.69		
Severity factor[b]	-	3.90	3.95	4.25	4.54		
Cellulose	35.2 ± 2.2	44.1 ± 0.2	58.8 ± 4.4	57.6 ± 2.5	57.6 ± 2.9	42.7 ± 0.2	0.4 ± 0.0
Hemicellulose							
Xylose	18.6 ± 1.6	14.3 ± 0.4	6.4 ± 1.0	0	0	12.7 ± 0.1	1.8 ± 0.1
Arabinose	1.7 ± 0.2	1.5 ± 0.7	-	-	-	0.8 ± 0.1	1.1 ± 0.0
Acetyl groups	3.4 ± 1.0	3.1 ± 1.4	-	-	-	1.4 ± 0.0	0.0 ± 0.0
Total Hemicellulose	23.7	18.9	6.4	0	0	15.0	2.9
Lignin	24.7 ± 0.4	28.0 ± 0.7	32.5 ± 1.8	30.6 ± 3.3	35.1 ± 1.5	40.3	93.9
Acid Insoluble (AIL)	20.7	23.8	28.4	27.7	31.33	36.8	93.35
Acid Soluble (ASL)	4.0	4.1	4.1	2.9	3.75	3.5	0.5
ASL/AIL	5.2	5.8	6.9	9.5	8.3	10.5	186.7
Ash	3.4 ± 0.1	3.6 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	2.5 ± 0.2	3.5 ± 0.1
Total	87.0[1]	94.6	98.7	89.2	93.7	100.5	100.7

[1]Extractives consisting of waxes, nucleic acids, and plant cell wall cytoplasm contents make-up the difference. All analysis ran in triplicate.

4.3.2 Adsorption of *T. Reesei* and *A. niger* Enzymes at 30°C

"Adsorption of enzymes at 30°C onto liquid hot water pretreated and enzyme hydrolyzed bagasse was examined at 30°C, since this temperature is within the range where simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP) with yeast is carried out. The lower temperature must be used in these fermentations since many microorganisms have limited thermal stability. We found that endoglucanase and β-glucosidase from *T. reesei* and *A. niger* were not significantly adsorbed on enzyme derived lignin as indicated by minimal changes in enzyme activity and total protein concentration at 30°C. The only activity that changed was exoglucanase (cellobiohydrolase), which initially decreased by 20% within 1.5 hours of incubation, but then remained stable. Hence, temperature provided an approach to reducing loss in enzyme activity by reducing enzyme adsorption on lignin. More importantly, for SSF or CBP the temperature of about 30°C has unintentionally offered a viable option for mitigating lignin-derived inhibition effects when liquid hot water pretreatment is used to prepare the lignocellulose feedstock for hydrolysis" (Zanchetta et al. 2018).

4.3.3 Adsorption of Enzymes on Lignin from Liquid Hot Water Pretreated and Enzyme Hydrolyzed Sugarcane Bagasse at 45°C (Enzyme Derived Lignin)

"Endoglucanase activity in the supernatant decreased to 34% of its original activity after 1.5 hours incubation with liquid hot water pretreated and enzyme hydrolyzed bagasse at pH 4.8 in 50 mM citrate buffer. After 1.5 hours, adsorption leveled out (Fig. 4-1(A)) indicating inhibition (Mosier and Ladisch 2009; Sadana and Henley 1987). Exoglucanase activity was 73% after 24 hours and decreased to 60% at 48 hours with the continuing decrease in activity indicating deactivation (Fig. 4-1(B)). *A. niger* β-glucosidase activity decreased by only 2% (dark circles, Fig. 4-1(D)) confirming its previously reported stability (Ko, Ximenes, et al. 2015). Differentiation between inhibition and deactivation was based on fitting the data with the first-order unimolecular irreversible reaction (Eq. 4.2) and a pseudo first-order reaction (Eq. 4.1) (Sadana and Henley 1987). Fits were evaluated using an F-test with a 0.05 significance level. The pseudo first-order model (Eq. 4.1) gave the best fit as shown by the lines in Fig. 4-1(A) to (D):

$$A = (1 - \alpha_1) * \exp(-k_1 t) + \alpha_1 \qquad (4.1)$$

$$A = A_0 * \exp(^{-t}/_{\tau})\alpha \qquad (4.2)$$

where t = time; τ = characteristic deactivation time; k_1 = kinetic stability constant; α_1 = ratio two different forms of the same enzyme; A0 = initial activity of enzyme" (Zanchetta et al. 2018).

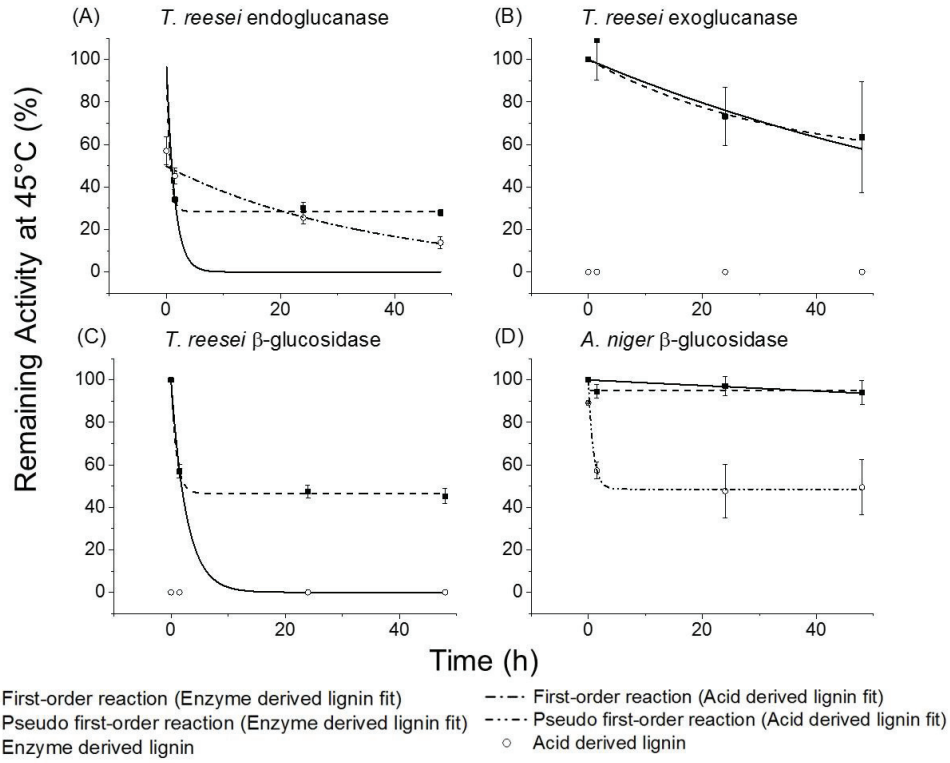

Figure 4-1. Comparison of different inhibition models and experimental data for activity after exposure to enzyme hydrolysis residual lignin (dark symbols) and acid hydrolysis (open symbols) residual lignin at 45°C in 50 mM citrate buffer at pH 4.8, over time (reproduced from Zanchetta et al. 2018). A-C, CTEC2; D, Novozyme 188. First-order reaction model and Pseudo first-order reaction fitted to the experimental data are represented by lines as indicated in the figure. Values of fitted constants for indicated best fit lines are:

(A) Endoglucanase on enzyme-derived lignin, Eq. (4.1), Prob (F) < 0.05: $\alpha_1 = 0.28424$; $k_1 = 1.70156$;

and on acid derived lignin, Eq. (4.2), Prob (F) < 0.05: $\tau = 36.5575$; $A_0 = 49.71113\%$

(B) Exoglucanase on enzyme-derived lignin, Eq. (4.2), Prob (F) = 0.671: $\tau = 87.74496$, $A_0 = 100\%$;

(C) *T. reesei* β-glucosidase on enzyme derived lignin, Eq. (4.1), Prob (F) < 0.05: $\alpha_1 = 0.46477$; $k_1 = 1.08436$;

(D) *A. niger* β-glucosidase on enzyme derived lignin, Eq. (4.2), Prob (F) = 0.054: $\tau = 743.90164$; $A_0 = 100\%$;

and on acid derived lignin, Eq. (4.1), Prob (F) < 0.05: $\alpha_1 = 0.48565$; $k_1 = 1.17438$.

4.3.4 Adsorption of Enzymes on Lignin from Liquid Hot Water Pretreated and Acid Hydrolyzed Sugarcane Bagasse at 45°C (Acid Derived Lignin)

The same experiment carried out with the lignin prepared through acid hydrolysis resulted in a decrease from 0.5 ± 0.01 g/L to 0.3 ± 0.02 g/L, almost immediately after lignin and *A. niger* and β-glucosidase were combined. The activity then remained constant for the next 48 hours. "Endoglucanase activity from *T. reesei* decreased to 45, 26, and 14% of its initial activity, respectively, after 1.5, 24 and 48 hours of incubation indicating deactivation (Fig. 4-1(A)). Complete disappearance of *T. reesei* exoglucanase and β-glucosidase activities from the supernatant occurred almost immediately indicating deactivation after contacting the acid treated lignin (open circles in Fig. 4-1(B), 4-1(C)). Acid treated lignin caused significant inhibition (open circles, Fig. 4-1(D)) for *A. niger* β-glucosidase which decreased to 57% within 1.5 hrs and leveled off at 43% after 24 hours, again indicating an inhibition effect (open circles in Fig. 4-1(D))" (Zanchetta et al. 2018).

4.3.5 Comparison of Enzyme Activities Adsorbed by Enzymatic and Acid Hydrolyzed Lignin at 45°C

"The gel in Fig. 4-2 is divided into 10 lanes as indicated in the horizontal direction from left to right, and 12 bands identified between lanes 1 and 2, and lanes 4 and 5. Cellulase proteins shown by the bands in Lane 2 are endoglucanase (band 9), exoglucanase (band 7) and β-glucosidase (bands 5 for *T. reesei* in lane 2; 12 and 13 for *A. niger* in lane 5) (Himmel et al. 1993; Uniprot Consortium 2017). Molecular weights of the proteins ranged from 6.5 to 200 kD based on comparison to protein standards in lanes 1 and 10. Pretreated acid hydrolyzed bagasse (i.e., acid derived lignin, Table 4-1) adsorbs *T. reesei* proteins to a greater extent than lignin derived from enzyme hydrolysis (compare lane 2 against lane 3 and 4, respectively). This is consistent with Rahikainen et al. (2011) who found that lignin from acid hydrolyzed softwood adsorbed > 95% of *T. reesei* proteins while the residual lignin from enzyme hydrolysis adsorbed 70% protein (Rahikainen et al. 2011).

"Bands 12 and 13 in lanes 5, 6 and 7 representing protein from *A. niger β*-glucosidase (Novo 188) are about the same indicating minimal adsorption. Differences in activity levels of β-glucosidases (Fig. 4-1), however, show losses of activities and confirm that the interaction of enzymes with lignin depends on the microbial source from which the enzyme is derived,

consistent with previous reports (Berlin et al. 2006; Ximenes et al. 2010a). β-glucosidase from *A. niger* is also stable with respect to soluble, lignin derived inhibitors unlike *T. reesei* β-glucosidase which is inhibited (Haven and Jørgensen 2013; Ko, Ximenes, et al. 2015; Ximenes et al. 2010b, 2010a).

"Both ionic and hydrophobic interactions of β-glucosidase with lignin determine adsorption. β-glucosidase from *T. reesei* has pI values of 5.7-6.4, compared to 4.6 for *A. niger* (Ko, Ximenes, et al. 2015). At pH 4.8, *T. reesei* β-glucosidase is positively charged, which promotes binding with negatively charged lignin (Nakagame et al. 2011; Rahikainen et al. 2013) unlike *A. niger* β-glucosidase which is negatively charged at the same pH (compare Fig. 4-1(C) and 4-1(D)). Ko et al. (2015b) showed that when the pH was increased from 4.0 to 6.0, the extent of adsorption of β-glucosidase from *T. reesei* decreased, and when NaCl was added, some β-glucosidase activity was desorbed from the lignin, thus confirming electrostatic interactions. Since 60% of β-glucosidases remained bound to lignin at 200 mM NaCl, hydrophobic interactions between protein and lignin were also indicated to play a role" (Zanchetta et al. 2018).

4.3.6 Specific Surface Area and Surface Characteristics of Lignocellulose Particles

"Liquid hot water pretreated bagasse had a specific surface area of 1.7 m^2/g compared to enzyme hydrolysis derived lignin at 2.5 m^2/g. The surface area attributed to small pores is more pronounced for sulfuric acid hydrolysis derived lignin with a BET specific surface area of 86 m^2/g. Similar results were reported for spruce with 80 m^2/g vs. 2.5 m^2/g for acid and enzyme hydrolyzed softwood, respectively (Rahikainen et al. 2011). Gama et al. (1994) showed that cellulolytic enzymes do not enter into the micropores for five studied celluloses and concluded that hydrolysis occurs initially at the external surface of the fibers (Gama, Teixeira, and Mota 1994). Lin et al. (1985) measured wet pore sizes and pore size distributions in corn stover and also showed that external or macropore surface properties determine the extent of interaction between enzymes and substrate, where the enzymes in this case were from cellulolytic bacteria and the biomass was corn stover (Lin et al. 1985). Since the small pores cannot be penetrated by enzymes from *T. reesei* with molecular weights between 20 and 70 kD (Gong, Ladisch, and Tsao 1979), we would expect the intraparticle access of enzyme preparations in this work (20 to 150

kD, Fig. 4-2) to be limited and the external area to be a key determinant for contact of enzyme with substrate" (Zanchetta et al. 2018).

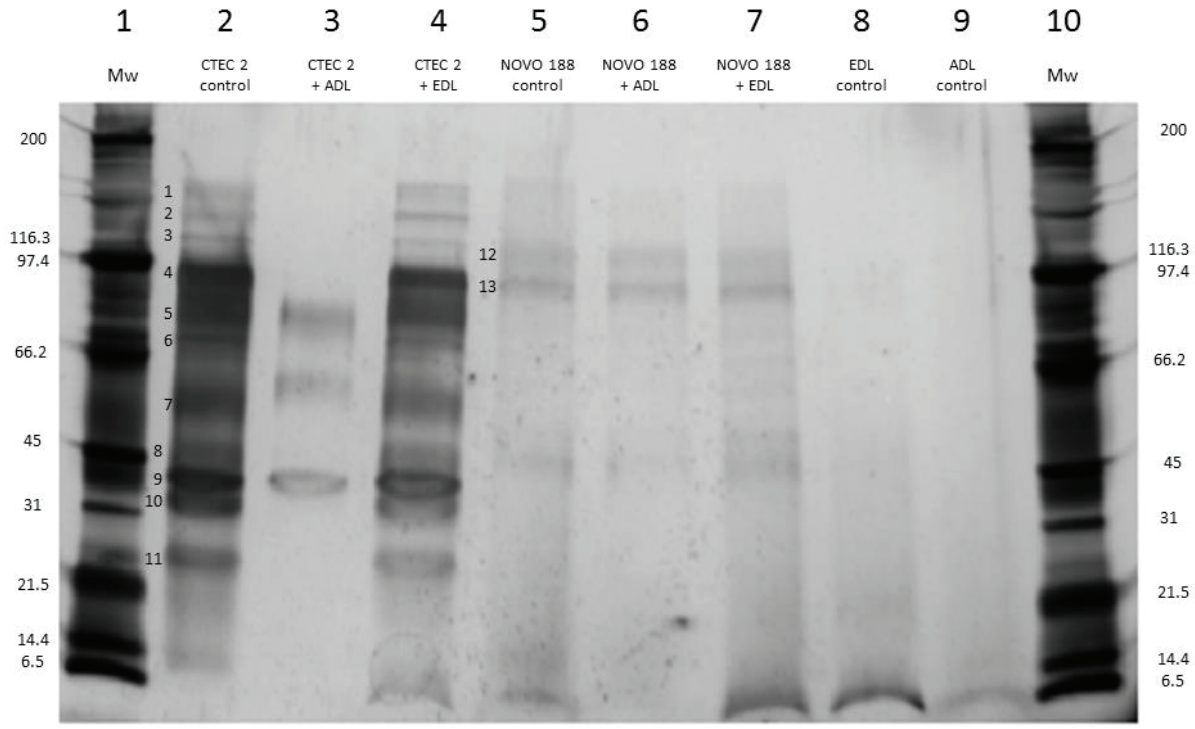

Figure 4-2. SDS-PAGE analysis of free proteins (Cellic Ctec2 and Novozyme 188) in the supernatant after the 1.5 h-adsorption experiment with acid or enzyme hydrolysis residual lignin at 45°C. The band numbers indicate the proteins of the specific molecular weights of between 6.5 and 200 kD. ADL denotes acid digested lignin and EDL enzyme digested lignin. Lanes are 1 and 10 - standard protein mixture; 2 - Cellic CTEC 2; 3, 4 - Cellic CTEC 2 after incubation with acid derived lignin and EDL, respectively, 5- Novozyme 188; 6, 7 - Novozyme 188 after incubation with EDL and ADL, respectively; 8, 9 - washed ADL and EDL controls showing absence of proteins (reproduced from Zanchetta et al. 2018)

"The scanning electron micrographs of liquid hot water pretreated sugarcane bagasse show delamination and separation of fibers on the particle's surface (arrows in Fig. 4-3(A) and 4-3(B)), and a smooth appearance when hydrolyzed by cellulolytic enzymes for 48 hours (Fig. 4-3(C)). Delamination becomes more evident after enzyme digestion (compare Fig. 4-3 (D) to Fig. 4-3 (B)), although dramatic changes in structure are not evident, and indicate adsorption occurs due to exposure of additional surface area as particle size decreases rather than major changes in the internal morphology of the lignocellulose. This mechanism has been noted previously for microcrystalline cellulose (Ladisch et al. 1992). Hence, the effectiveness of acid treatment in

accelerating kinetics of enzyme hydrolysis reflects the smaller particle sizes with uneven surface structures (Fig. 4-3(E) and 4-3(F)) that coincide with a BET specific area (86.2 m^2/g) that is 50 times larger than the specific surface area of liquid hot water pretreated bagasse (1.7 m^2/g) and 34 times larger than enzyme derived lignin obtained from pretreated sugarcane bagasse (2.5 m^2/g). A decrease in particle size with a corresponding increase in the number of particles per weight of biomass (i.e., an increase in specific surface area) increased total surface area available for adsorbing enzymes" (Zanchetta et al. 2018).

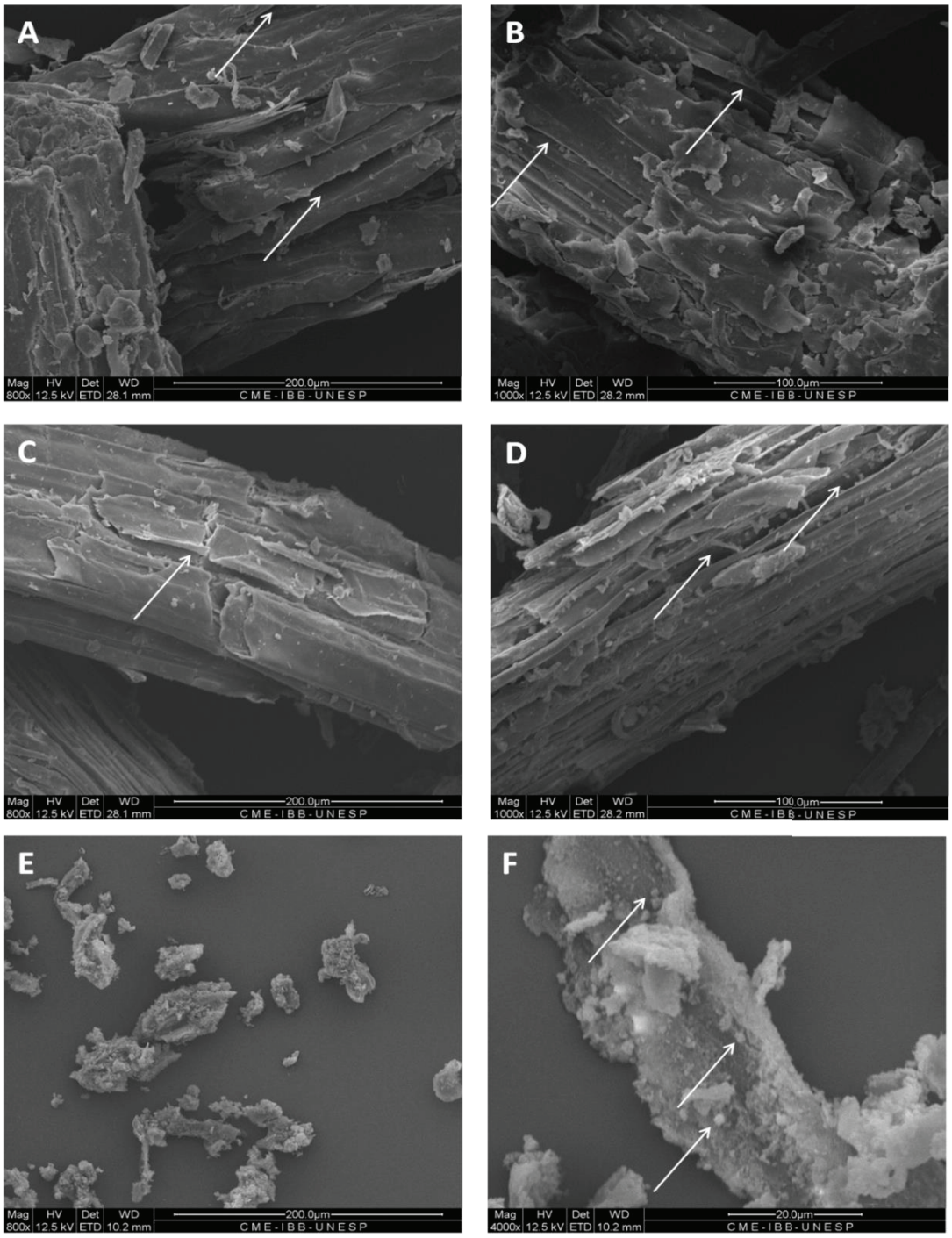

Figure 4-3. SEM micrographs of LHW pretreated sugarcane bagasse at (A) severity of log R0 = 9.64 (magnification 800X – bar = 200 µm) and (B), magnification 1000X – bar = 100 µm) - arrows indicate separation of the fibers; (C) Lignin derived from liquid hot water pretreatment followed by enzyme hydrolysis (at magnification 800X – bar = 200 µm and (D), magnification 1000X – bar = 100 µm - arrows indicate separation of the fibers; (E) Acid treated lignin at magnification 800X – bar = 200 µm and (F) magnification 4000X – bar = 20 µm, reproduced from Zanchetta et al. 2018.

4.3.7 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR absorption spectra for acid treatment derived lignin and liquid hot water pretreated bagasse before and after enzyme hydrolysis (i.e., enzyme derived lignin) further confirmed that differences in these lignin containing solids account for enzyme adsorption behavior observed in this work. Lignin isolated by acid hydrolysis (Fig. 4-4, curve marked ADL) had a low carbohydrate content (Table 4-1) and also lacked the bands 1321 cm^{-1} and 1029 cm^{-1} associated with syringyl and guaiacyl units (Ko, Kim, et al. 2015; Pandey 1999). The condensed form of G and S units coincided with larger extents of enzyme adsorption compared to lignin from liquid hot water pretreated and enzyme digested biomass. Studies with lignin derived from *Miscanthus* isolated using different concentrations of sulfuric acid showed similar results (J.-Y. Kim et al. 2015). In the case of enzyme digested lignin, (denoted as EDL in Fig. 4-4) bands related to carbohydrates (1700-1722, 1160 and 898 cm^{-1}) were the same as the bands for liquid hot water pretreated bagasse before enzyme hydrolysis.

"Previous reports showed that increasing temperature of dilute acid pretreatment resulted in 8 x lower adsorption of *T. reesei* cellulase by lignin (Ooshima, Burns, and Converse 1990) and 6 x higher adsorption on cellulose at 50°C (Lynd et al. 2002). Conversely Zheng et al. (2013) found adsorption of enzymes by lignin to be 10 x higher at 50°C than at 4°C (Zheng et al. 2013). We found acid treatment causes a significant increase in the fractional amount of lignin remaining in the bagasse, an increase in accessible specific surface area, and a decrease in structural carbohydrates that partially shield the lignin from the contact with enzymes. Hence, enzyme adsorption is rapid and extensive" (Zanchetta et al. 2018).

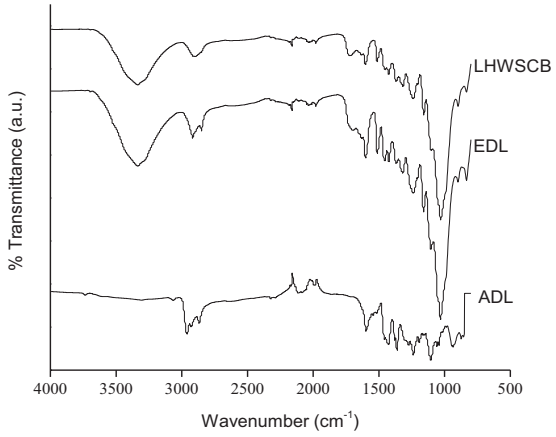

Figure 4-4. FTIR spectra of sugarcane bagasse pretreated in liquid hot water (LHW SCB); sugarcane bagasse pretreated in liquid hot water and hydrolyzed with *T. reesei*/*A. niger* enzymes (EDL); sugarcane bagasse pretreated in liquid hot water and digested with Sulfuric acid per NREL procedures (ADL), reproduced from Zanchetta et al. 2018.

4.3.8 Hydrolysis of Cellulose and Pretreated Sugarcane Bagasse at 30°C and 45°C

"The lack of enzyme adsorption at 30°C indicates that this temperature reaction would be favored compared to the losses of up to 80% of activities observed at 45°C. However, based on a simple Arrhenius approximation that each 10°C reduction in temperature reduces reaction rate 2 x, cellulases would be approximately 3 x less active at 30°C than at 45°C, leading to lower rates of hydrolysis. We tested the hypothesis that lower temperature and longer reaction times mitigate inhibitory effects of lignin for large proteins since these proteins have small diffusion coefficients of 10^{-6} cm^2 and kinetically hindered adsorption (Ladisch 2001). Pure cellulose (solka floc) combined with lignin residues and pretreated sugarcane bagasse were hydrolyzed using 5 FPU or 13 mg protein/g glucan (Cellic CTEC2) for 72 and 168 hours at 30 and 45°C (Fig. 4-5). Enzyme loadings are based on total glucan in the reaction mixture. The amount of added solka floc was adjusted against the residual cellulose in the enzyme or acid digested lignin to give equivalent total glucan. When only solka floc was used, hydrolysis in the absence of inhibitors gave cellulose conversion to glucose of 24% at 30°C vs. 72% at 45°C after 72 hours. After a total of 168 hours, hydrolysis at 30°C caught up to hydrolysis at 45°C (Fig.4-5(i) and 4-5(j)).

"Addition of enzyme hydrolyzed lignin had a minimal effect (Fig. 4-5(c) and 4-5(d)) after 168 hours although the slower rate at 30°C was evident at 72 hours. Addition of acid derived lignin dramatically decreased maximum conversion, with lower yields evident after 168 hours (Fig. 4-5(a) and 4-5(b)). While the presence of inhibitors derived from biomass or generated during pretreatment lowers the overall extents of cellulose hydrolysis (Ximenes et al. 2010a, 2010b), these soluble molecules may be removed by washing (Y. Kim et al. 2013, 2015). Hydrolysis of liquid hot water pretreated sugarcane bagasse (at log R_0 = 9.67 or log R_0 = 10.74) (Fig. 4-5(e), 4-5(f) or 4-5(g), 4-5(h)), give smaller differences in hydrolysis since restricted access of the cellulase to the lignin as well as recalcitrance due to structural features (Fig. 4-5) is a factor, unlike the runs with solka floc as the cellulose substrate. The smaller rates observed with pretreated sugarcane bagasse are still significant and require extending the hydrolysis" (Zanchetta et al. 2018).

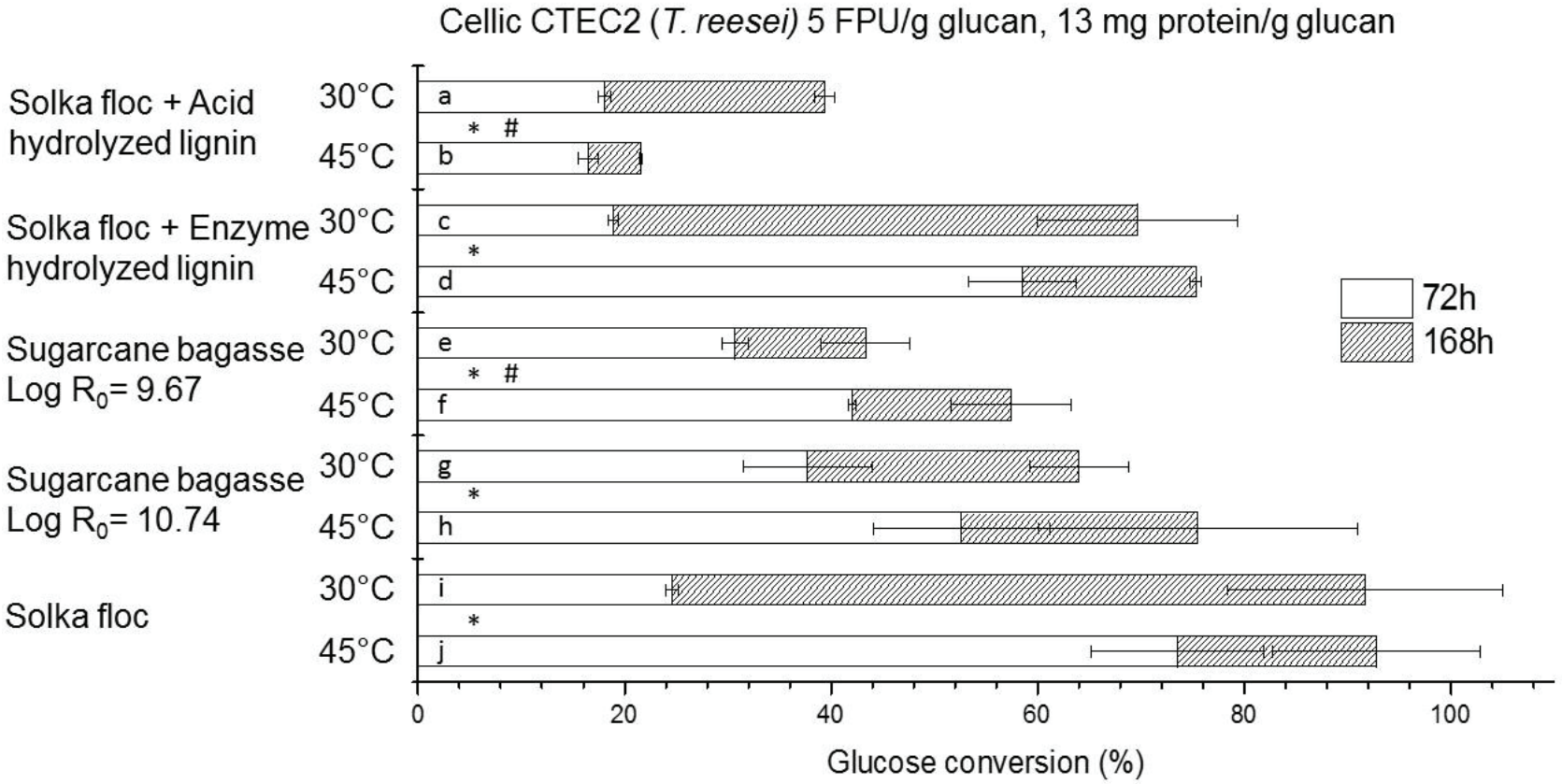

Figure 4-5. Comparison of cellulose and sugarcane bagasse hydrolysis at 30 and 45°C glucose conversion. * indicates significant difference in conversion after 72 hours (p-value < 0.05). # indicates significant difference in conversion after 168 hours (p-value < 0.05). Enzyme loading was 5 FPU/g glucan or 13 mg protein/g glucan, adapted from Zanchetta et al. 2018.

4.3.9 Enzymatic hydrolysis at 50°C and BSA impact

The lower rates of hydrolysis observed at lower temperatures increase the reaction time. Maintaining the rates require higher temperatures. Preventing adsorption will lead to more free active enzymes and complete the hydrolysis at shorter, more manageable time. Lignin blocking with BSA can used. The conditions related to BSA efficiency were then evaluated to design the optimum conditions for hydrolysis of sugarcane bagasse.

Variable impact of solid lignin based on pretreatment severity was evaluated by introducing bovine serum albumin (BSA) as a blocking agent (Ko, Kim, et al. 2015). Pretreatment conditions impact on hydrolysis was screened using Cellulase 13P (10 FPU/g glucan, Table 4-2). Lower protein loadings are more susceptible to the effects of lignin adsorption and allows us to study its effects with more clarity. Pretreated sugarcane bagasse was incubated with BSA at 25°C for 1 or 24 hours. After the incubation with BSA, the enzymatic solution was added, and the reaction tubes were transferred to a shaking incubator at 50°C and 200 RPM to initiate hydrolysis.

Table 4-2. Enzyme loadings conversion chart

Pretreatment	**Enzyme loading (FPU/g glucan)**	**Enzyme loading (FPU/g solids)**	**Enzyme loading (mg protein/g glucan)**	**Enzyme loading (mg protein /g solids)**
190°C, 20 min	**10**	**6.1**	**27.8**	**16.9**
200°C, 20 min	**10**	**6.7**	**27.8**	**18.6**
210°C, 20 min	**10**	**6.0**	**27.8**	**16.7**

Hydrolysis of unwashed pretreated material led to conversions of 13% with and without BSA addition over all pretreated samples. For washed biomass, glucose conversion, when BSA was not used, fell with increased pretreatment severity (31% to 20%, from 190°C to 210°C pretreated biomass). Conversely, when BSA is included, no gain was observed with the lowest pretreatment severity (190°C), while it was significantly higher with higher severities pretreatments, reaching 35% with BSA (200°C and 210°C for 20 minutes) (Figure 4-6). It is also notable that there is no difference between 1 hour and 24-hour incubation periods for BSA.

This relation between severity and higher BSA influence was also observed in hardwood (Ko, Kim, et al. 2015).

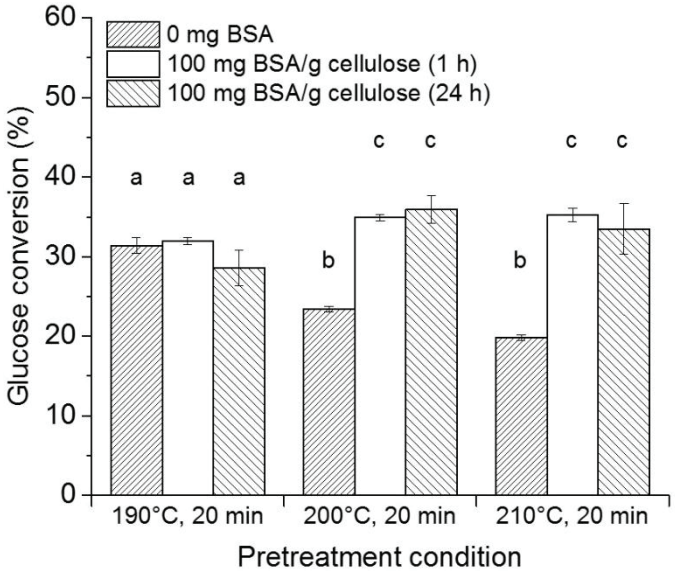

Figure 4-6. Sugarcane bagasse enzymatic hydrolysis. Three different pretreatment conditions were evaluated, 190°C, 200°C and 210°C, all for 20 minutes. Cellulase 13P (10 FPU/g glucan) was used. ANOVA was used to evaluate the final glucose conversion values, three groups were identified a, b, and c ($p < 0.05$). Reproduced from dos Santos (2016).

To further explain the BSA impact on hydrolysis, different enzyme preparations were used to hydrolyze SCB pretreated at 200°C (Figure 4-7). Supplementation with β-glucosidase (Novozyme 188) at the proportion of 1 FPU : 2 IU led to increased conversions with BSA treatment (40 to 51%) and without additional protein (26 to 42%). The supplementation is more effective when lignin was not blocked (p-value < 0.001), conversions increased an average of 11.5 p.p. with no supplementation and only 8.6 p.p. with supplementation. This result confirms previous reports that β-glucosidase is the primarily inhibited enzyme (Ko, Ximenes, et al. 2015; Ko, Kim, et al. 2015; Ximenes et al. 2010a). However, this shows that β-glucosidase inhibition by lignin is not the only factor that BSA can counter. A more developed enzyme - Cellic CTEC3 - was also evaluated. When high protein concentrations were used (20 mg protein/ g solids), complete conversion was observed in all cases. At lower protein loadings (6.5 mg protein/g solids) conversion reached 71 and 76% without and with BSA, respectively. This preparation has high β-glucosidase activity and multiple other accessory enzymes which leads to the higher conversions (Florencio et al. 2016a, 2016b). The conversions observed are close to previously reported for sugarcane bagasse (Table 4-3) (Siqueira et al. 2017). When Celluclast 1.5L

supplemented with β-glucosidase, the highest conversion with BSA was 44% and without BSA only 15% of the steam exploded sugarcane bagasse was hydrolyzed. This combination is similar to Cellulase 13P supplemented with β-glucosidase, which had at the same loading conversions of 40 and 50%, without and with BSA, respectively. Both studies used Cellic CTEC3 and at similar loadings, the conversion in this study (71 and 76%) versus Siquiera et al. (2017) (69 and 70%), without and with BSA, respectively.

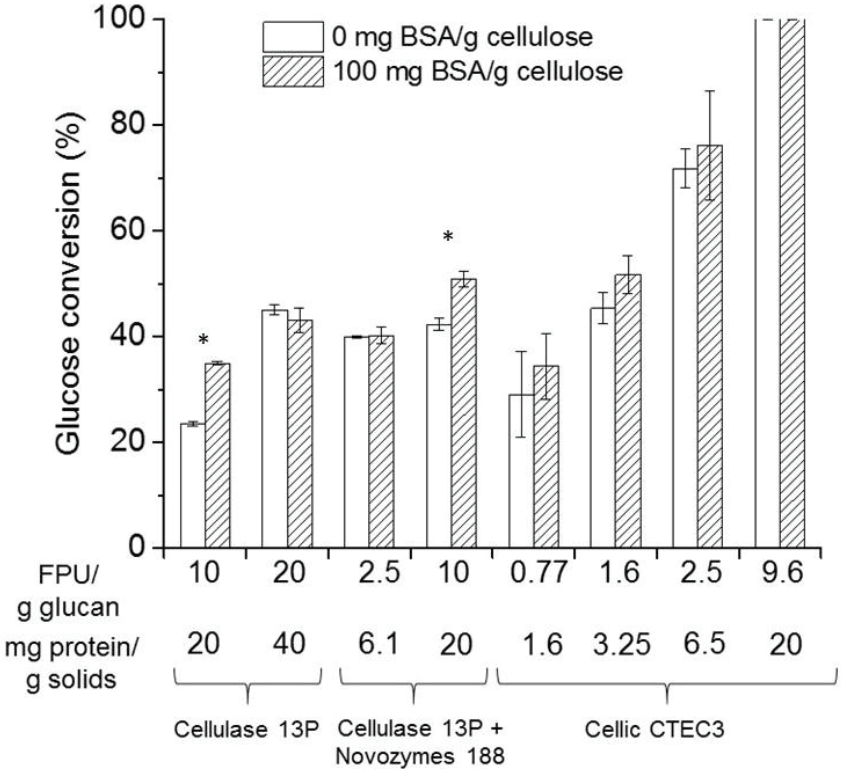

Figure 4-7. Enzymatic hydrolysis of pretreated sugarcane bagasse with and without BSA using different enzymes. Hydrothermal pretreatment was conducted at 200°C for 20 minutes. Biomass was incubated with 0 or 100 mg BSA/g cellulose at 25°C for 1 hour before enzyme was added. Hydrolysis was conducted for 72 hours. * indicates significant increase in conversion with BSA ($p < 0.05$).

"Ko et al. (2015a, 2015b) and Kim et al. (2014, 2015) clearly explained the role of BSA in adsorbing on lignin and blocking non-productive binding of cellulolytic enzymes for liquid hot water pretreated hardwood. Subsequently, Siqueira et al. (2017) confirmed similar phenomena for sugarcane bagasse pretreated by other methods. Studies with cellulose binding domain (CBM), bovine serum albumin (BSA), soy proteins, and surfactants (Tween or polyethyleneglycol) show these act to block protein adsorption (Florencio, Badino, and Farinas 2016; Ooshima, Burns, and Converse 1990). These macromolecules when added to the enzyme

competed with the cellulases for lignin adsorption, and significantly decreased non-productive binding between lignin and cellulolytic enzymes (Liu et al. 2016; Zhang et al. 2017). Hydrophobic interactions of these agents hinderered adsorption of enzymes on lignin and increased the amount of active cellulases and β-glucosidases left in the supernatant (Florencio, Badino, and Farinas 2016; Haven and Jørgensen 2013; R. Kumar et al. 2013)" (Zanchetta et al. 2018).

The impact of BSA was compared to previous reports (Table 4-3). Cellulose conversion increases generated by the addition of BSA is dependent on both lignin and hemicellulose content (Figure 4-8). In general, the higher the proportion of either of these fractions the higher the impact of BSA. However, the conditions used are widely different. The experiments in this work helped bridge some of those differences by exploring different enzyme preparations and loadings.

Newer enzyme preparations were developed considering the loss of enzyme activity observed in these works. This translates to newer preparations being less affected by lignin adsorption and reducing the impact of BSA. When BSA is added to Celluclast supplemented with β-glucosidase the conversions increase up to 136%, but only 48% when Cellic CTEC3 is used on those conditions (Siqueira et al. 2017). When liquid hot water pretreated sugarcane bagasse is used, the BSA-related increases in increase with Cellulase 13P are 49% (20 mg protein/g solids); when supplemented with β-glucosidase, the improvement is of 20% (10 mg protein/g solids) and not statistically significant when Cellic CTEC3 is used.

Pretreatment type impacts the results as their mechanisms dictate how much lignin is removed in relation to the other portions (Mosier et al., 2005). Pretreatments that remove other portions preferably to lignin – dilute acid, liquid hot water, steam explosion – lead to higher BSA gains. Hardwood pretreated with liquid hot water had up to 3 times conversions when BSA was added (Ko et al. 2014). A more direct comparison with corn stover shows that the hydrolysis of diluted acid pretreated was 12% more efficient with BSA while AFEX pretreated corn stover hydrolysis only improved 7% (Yang and Wyman 2006).

Pretreatment severity is also impactful, higher temperatures and longer pretreatments exacerbate this effect. The correlation of hemicellulose to BSA impact is likely caused by variations on severity that remove more hemicellulose. Higher hemicellulose fractions indicate less efficient pretreatments which expose less lignin and reduce BSA impact. Hardwood data

from Ko et al. (2014) shows that BSA impact goes from none to 3 times the conversion with increasing severity of liquid hot water pretreatment. Figure 4-6 also demonstrate this relationship with no impact observed at lowest severity and almost double the conversions at the highest severity.

Pretreatments that preferably remove lignin (AFEX and hydroxide pretreatments) also have low lignin, high hemicellulose and low BSA impact. These effects together show that the observed impact of BSA on the hydrolysis of lightly liquid hot water pretreated sugarcane bagasse should be expected.

Table 4-3. Summary of previously reported lignin blocking impact on lignocellulose hydrolysis

Biomass	Pretreatment	Biomass Composition (Cellulose-Hemicellulose - Lignin)	Enzyme (loading, FPU/g glucan)	Added BSA	Cellulose conversion (%)	Source
Corn Stover	Diluted Acid	56-11-28	Celluclast + Novozyme 188 (15)	0	82	Yang & Wyman (2006)
Corn Stover	Diluted Acid	56-11-28	Celluclast + Novozyme 188 (15)	1% (w/w)	92	Yang & Wyman (2006)
Corn Stover	AFEX	40-33-17	Celluclast + Novozyme 188 (15)	0	77	Yang & Wyman (2006)
Corn Stover	AFEX	40-33-17	Celluclast + Novozyme 188 (15)	1% (w/w)	82.5	Yang & Wyman (2006)
Douglas Fir	Diluted Acid	56-8-46	Celluclast + Novozyme 188 (20)	0	54	Yang & Wyman (2006)
Douglas Fir	Diluted Acid	56-8-46	Celluclast + Novozyme 188 (20)	1% (w/w)	73.5	Yang & Wyman (2006)
Sugarcane Bagasse	NaOH (5%)	45-26-24	Celluclast + Novozyme 188 (2.5)	0	12	Siqueira et al. (2017)
Sugarcane Bagasse	NaOH (5%)	45-26-24	Celluclast + Novozyme 188 (2.5)	250 mg/g glucan	25	Siqueira et al. (2017)
Sugarcane Bagasse	Na_2SO_3/NaOH	51-26-20	Celluclast + Novozyme 188 (2.5)	0	30	Siqueira et al. (2017)
Sugarcane Bagasse	Na_2SO_3/NaOH	51-26-20	Celluclast + Novozyme 188 (2.5)	250 mg/g glucan	37.5	Siqueira et al. (2017)
Sugarcane Bagasse	Diluted Acid	53-14-29	Celluclast + Novozyme 188 (2.5)	0	17	Siqueira et al. (2017)
Sugarcane Bagasse	Diluted Acid	53-14-29	Celluclast + Novozyme 188 (2.5)	250 mg/g glucan	17	Siqueira et al. (2017)
Sugarcane Bagasse	$NaHSO_3/H_2SO_4$	54-10-28	Celluclast + Novozyme 188 (2.5)	0	22	Siqueira et al. (2017)

Table 4-3 continued

Biomass	Pretreatment	Biomass Composition (Cellulose-Hemicellulose - Lignin)	Enzyme (loading, FPU/g glucan)	Added BSA	Cellulose conversion (%)	Source
Sugarcane Bagasse	$NaHSO_3/H_2SO_4$	54-10-28	Celluclast + Novozyme 188 (2.5)	250 mg/g glucan	22	Siqueira et al. (2017)
Sugarcane Bagasse	Steam Explosion	56-2-36	Celluclast + Novozyme 188 (2.5)	0	15	Siqueira et al. (2017)
Sugarcane Bagasse	Steam Explosion	56-2-36	Celluclast + Novozyme 188 (2.5)	250 mg/g glucan	44	Siqueira et al. (2017)
Sugarcane Bagasse	NaOH (5%)	45-26-24	Cellic CTEC 3 (2.5)	0	19	Siqueira et al. (2017)
Sugarcane Bagasse	NaOH (5%)	45-26-24	Cellic CTEC 3 (2.5)	250 mg/g glucan	39	Siqueira et al. (2017)
Sugarcane Bagasse	Na_2SO_3/NaOH	51-26-20	Cellic CTEC 3 (2.5)	0	69	Siqueira et al. (2017)
Sugarcane Bagasse	Na_2SO_3/NaOH	51-26-20	Cellic CTEC 3 (2.5)	250 mg/g glucan	70	Siqueira et al. (2017)
Sugarcane Bagasse	Diluted Acid	53-14-29	Cellic CTEC 3 (2.5)	0	26	Siqueira et al. (2017)
Sugarcane Bagasse	Diluted Acid	53-14-29	Cellic CTEC 3 (2.5)	250 mg/g glucan	28	Siqueira et al. (2017)
Sugarcane Bagasse	$NaHSO_3/H_2SO_4$	54-10-28	Cellic CTEC 3 (2.5)	0	23	Siqueira et al. (2017)
Sugarcane Bagasse	$NaHSO_3/H_2SO_4$	54-10-28	Cellic CTEC 3 (2.5)	250 mg/g glucan	32	Siqueira et al. (2017)
Sugarcane Bagasse	Steam Explosion	56-2-36	Cellic CTEC 3 (2.5)	0	22	Siqueira et al. (2017)
Sugarcane Bagasse	Steam Explosion	56-2-36	Cellic CTEC 3 (2.5)	250 mg/g glucan	40	Siqueira et al. (2017)

Table 4-3 continued

Biomass	Pretreatment	Biomass Composition (Cellulose-Hemicellulose - Lignin)	Enzyme (loading, FPU/g glucan)	Added BSA	Cellulose conversion (%)	Source
Hardwood	Liquid Hot Water	54-8-36	Cellic CTEC 2 (5)	0	18	Ko et al. (2014)
Hardwood	Liquid Hot Water	54-8-36	Cellic CTEC 2 (5)	100 mg/g glucan	20	Ko et al. (2014)
Hardwood	Liquid Hot Water	56-6-37	Cellic CTEC 2 (5)	0	20	Ko et al. (2014)
Hardwood	Liquid Hot Water	56-6-37	Cellic CTEC 2 (5)	100 mg/g glucan	42	Ko et al. (2014)
Hardwood	Liquid Hot Water	58-4-40	Cellic CTEC 2 (5)	0	20	Ko et al. (2014)
Hardwood	Liquid Hot Water	58-4-40	Cellic CTEC 2 (5)	100 mg/g glucan	58	Ko et al. (2014)
Hardwood	Liquid Hot Water	?-0-40	Cellic CTEC 2 (5)	0	18	Ko et al. (2014)
Hardwood	Liquid Hot Water	?-0-40	Cellic CTEC 2 (5)	100 mg/g glucan	78	Ko et al. (2014)

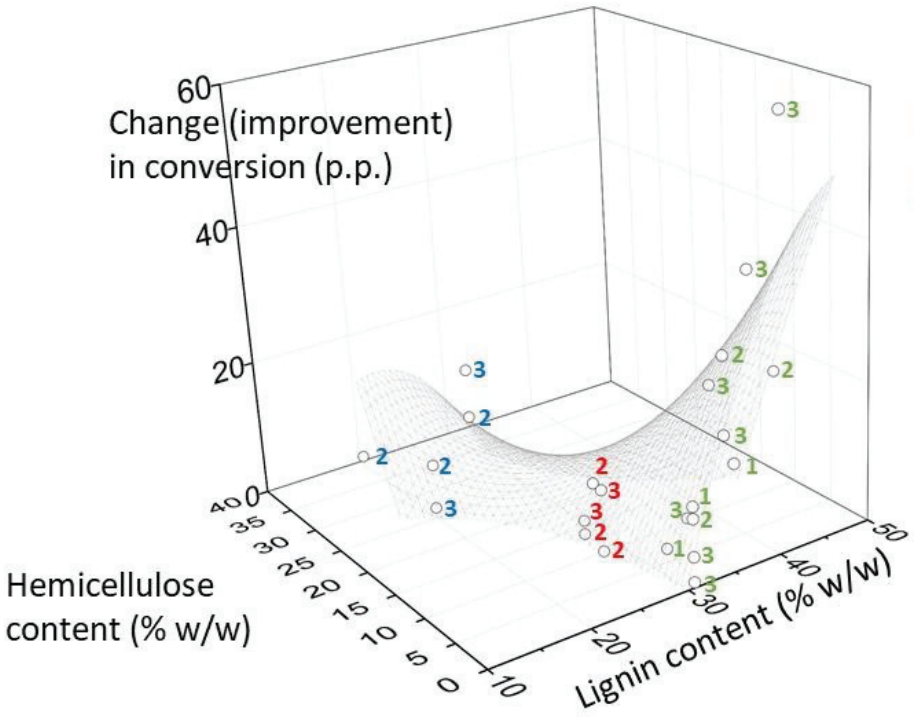

Figure 4-8. Cellulose conversion increase due to BSA addition in varying hemicellulose and lignin composition. Open circles are data points extracted from Table 4-3 and Figures 4-6 and 4-7. Numbers indicate the enzyme preparation used: 1 – Cellulase 13P; 2 – Cellulase 13P or Celluclast 1.5L supplemented with Novozyme 188; 3 – Cellic CTEC2 or Cellic CTEC3. Colors indicate the pretreatments used: blue are alkali based pretreatment; red are acid based pretreatment; green are liquid hot water pretreatment. The surface equation is: Conversion Increase = 8.23202 * [Hemicellulose] + 13.93146 * [Lignin] – 2.04518 * [Hemicellulose]^2 – 14.34222 * [Hemicellulose]^2 – 25.12173 * [Hemicellulose] * [Lignin] – 2.87731.

4.4 Conclusion

"Extents of adsorption of cellulase enzymes on lignin in sugarcane bagasse were an inverse function of incubation temperature and varied with type of pretreatment. At 45°C, lignin derived from acid hydrolyzed liquid hot water pretreated bagasse completely adsorbed cellulolytic enzymes from *Trichoderma reesei* within 90 min. Enzyme extracted lignin from liquid hot water pretreated bagasse adsorbed only 60% of *T. reesei* endoglucanase, exoglucanase and β-glucosidase activities. β-glucosidase from *Aspergillus niger* was not adsorbed. At 30°C, adsorption of all enzymes was minimal" (Zanchetta et al. 2018). However, hydrolysis rates at 30°C are significantly lower than at 45°C. When directly compared, the lower temperature

required a longer reaction time (168 hours) to have comparable conversions to the ones observed at 45°C.

Lower rates and longer reaction times are undesirable features. Hence the necessity to evaluate alternative methods to reduce adsorption and maintain high reaction rates.

Enzyme adsorption on lignin and the related BSA blocking improvements to hydrolysis are proportional to exposed lignin on pretreated biomass. Lignin content, pretreatment mechanism and parameters influence how much lignin is solubilized, redeposited and exposed.

Liquid hot water removes less than 10% of lignin with redeposition being proportional to severity. The hydrolysis conversions were inversely proportional to the severity without BSA. Three pretreatment conditions were evaluated (190, 200 and 210°C) for 20 minutes. Glucose conversions with Cellulase 13P (20 mg protein/g solids) decreased when no BSA was added (35, 26 and 22%) as severity increased. Addition of BSA increased the conversion of sugarcane bagasse pretreated at higher temperatures (39% for both 200 and 210°C), but no change was observed when the lowest temperature was used.

The impact of lignin is also dependent on enzyme composition. Enzyme preparations that are supplemented with β-glucosidase are less affected. When sugarcane bagasse pretreated at 200°C for 20 minutes was hydrolyzed with Celullase 13P supplemented with β-glucosidase (10 mg protein/g solids), conversions were of 42 without BSA and 50% with BSA. Other protein and accessory enzymes also reduce lignin impact. When a better developed enzyme (Cellic CTEC3, 6.5 mg protein/g solids) was used conversions reached 71% without BSA and 76% when BSA was added.

The addition of bovine serum albumin (BSA) increased hydrolysis of pretreated sugarcane bagasse in small amounts. These results demonstrate that lignin adsorption can be modulated by careful choice of pretreatment, pretreatment conditions and enzyme preparation. A relative mild pretreatment can expose enough cellulose to hydrolysis while limiting lignin redeposition and exposure. For maximum conversion of sugarcane bagasse, the use of a protein blocker at optimum temperature may not be necessary as long as enzyme composition is correct.

CHAPTER 5. EFFECT OF MIXING, PRODUCT INHIBITION AND DEACTIVATION BY AIR ON CELLULOLYTIC ENZYMES

5.1 Introduction

For an economically viable lignocellulosic ethanol production, a high biomass loading is necessary. However, the low densities of biomass often make the material difficult to store, transport, and interface with biorefinery infeed systems. The densification process is critical for producing a feedstock material suitable as a commodity product (Tumuluru et al. 2011).

Densified material allows pretreatment and subsequent processes to be carried at higher concentrations. "Increasing the initial loading significantly lowers the costs of machinery, energy consumption and labor costs (Wingren, Galbe, and Zacchi 2008; Zacchi and Axelsson 1989). However, the insolubility of lignocellulose in water, recalcitrance to hydrolysis, accumulation of inhibitors, and inhibitory products, have been correlated with lower conversions to glucose leading to higher enzyme loadings when compared to laboratory experiments (Alvira et al. 2013; Jørgensen et al. 2007)" (dos Santos 2016) because the actual reason for the loss of hydrolysis efficiency as solids loadings increase is still unknown, the combined effect is referred as "solids loadings effect" (Kristensen et al. 2009). Another possible factor responsible for this effect is enzyme deactivation due to air interaction (Scott et al. 2016; Bhagia et al. 2018; M. H. Kim et al. 1982).

In this work we evaluate the impact of improved mixing and identify that product inhibition is the most likely cause for the loss of conversion at high solids loadings. The use of double marine impellers at high speed eliminated this effect and allow for the study of deactivation caused by air. Which was addressed by conducting hydrolysis under anaerobic conditions.

5.2 Materials and Methods

5.2.1 Pelletized corn stover

Pelletized corn stover was provided by Idaho National Laboratory. Biomass was kept at room temperature in a sealed container until use. Loose, pelletized and pretreated corn stover

were analyzed following the NREL LAP standard procedure (Sluiter et al. 2008). Composition of the pelletized corn stover before and after liquid hot water pretreatment is given in Table 5-1.

5.2.2 Liquid Hot Water (LHW) Pretreatment

For LHW pretreatment, stainless steel tubes 316 tubes were filled with 15.25g of pelletized corn stover (moisture content = 8.35% (w/w)) and 23.25 mL of distilled water achieving 36% (w/w) dry solids slurry (Y. Kim et al. 2009). Pretreatment was carried at 190°C for 20 min (log R_0=9.80) in a Tecam1 SBL-1 fluidized sand bath. The pretreated tubes were immediately placed in water for 5 min for mild quenching. The liquid fraction was separated by vacuum filtration using Whatman #1 filter paper. The filtered solid was washed with 100mL of hot DI water (temperature ≥ 90°C). The washing step was repeated to completely remove the phenolic inhibitors. The combined effects of pretreatment temperature (T) and time (t) were investigated based on the severity factor equation; log Ro (Ro = t x exp ((T – 100)/ω)) (Overend, Chornet, and Gascoigne 1987), where ω = 4.6 as calculated by Kim et al. (2014) (dos Santos 2016, Y. Kim et al. 2014).

5.2.3 Enzymatic hydrolysis

Enzymatic hydrolysis was conducted in pH 4.8 citrate buffer (50 Mm) at 50 ºC for 72 hours. Cellic CTEC2 (150 FPU/mL, 180 mg protein/mL) purchased from Sigma-Aldrich (St. Louis, MO) was used at 3.6 mg protein/g solids. Initial solids concentrations were 10, 100 and 200 g/L. A 1 L bioreactor equipped with two marine impellers as shown in Figure 5-1, mixing at 290 RPM or without mixing. For anaerobic reactions the headspace was purged using nitrogen. Reactions were carried at orbital shakers (0 or 200 RPM) with total volume of 50 mL as control. Two mL of "thoroughly mixed sample was then taken at the end of the hydrolysis and was boiled for 5 min to deactivate the enzymes. To remove solids from the liquids, the sample was centrifuged at 13000 rpm for 2 min. The centrifuged supernatant was further filtered through a nylon syringe filter (0.2 mm, Acrodisc1) for analysis by HPLC" (dos Santos 2016).

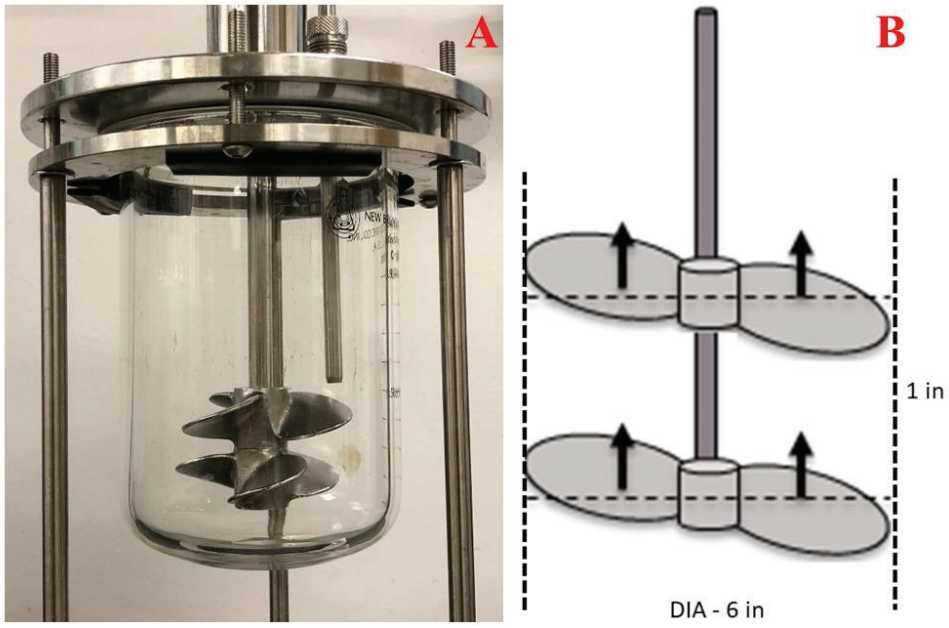

Figure 5-1. (A) Bioreactor setup for enzymatic hydrolysis. (B) Impellers configuration and sizes.

5.3 Results and Discussion

5.3.1 Changes in Composition of Pretreated Pelletized Corn Stover

In order to understand the impact of LHW pretreatment on pelletized corn stover, biomass composition was analyzed before and after pretreatment (Table 5-1). No difference in pretreatment efficiency was observed by either increasing the solids concentration from 150 g/L to 360 g/L or caused by pelletization. Increasing pretreatment solids loadings also reduce the size of vessels and the amount of water required for the process. The pretreatment has relative low severity, even lower than the ones evaluated on Chapter 4. Lignin redeposition and exposure is minimal.

Table 5-1. Composition of pelletized corn stover before and after hydrothermal pretreatment.

	Untreated Corn Stover	**Pretreated Corn Stover (150 g/L)**	**Untreated Pelletized Corn Stover**	**Pretreated Pelletized Corn Stover (360 g/L)**
Cellulose	30 – 34	45 – 49	34 – 37	49 – 51
Hemicellulose	19 – 22	12 – 13	20.2 – 20.3	7.9 – 12
Insoluble lignin	22 – 26	26 – 29	16 – 17	33 – 34
Soluble lignin	1.9 – 2.1	1.7 – 1.8	2.1 – 2.3	1.4 – 2.0
Total lignin	24 – 28	27 – 31	18 – 19	34.7 – 35.4
Extractives	10	-	14 – 17	-
Ash	6 – 6.5	5.0 – 5.1	2 – 4	5.0 – 5.2
Total	89 - 100	87- 98	90 – 92	97 – 102

5.3.2 Impact of improved mixing on conversion at high solids loadings

Glucose conversions behave differently when shaker flasks and bioreactors are used. Enzyme concentration (3.6 mg protein/g solids) was chosen to highlight the differences between conditions. Shaker flasks conversions are summarized in Figure 5-2. Bioreactor conversions are summarized in Figure 5-3. The first observation is that when mixing was not present (0 RPM on Figures 5-2 and 5-3), the conversions follow a different trend from when mixing is present. For shaker flasks, mixing increases conversion only when 10 g solids/L are hydrolyzed ($p < 0.001$). At higher solids loadings, shaker flask mixing has no effect (Figure 5-2). When bioreactors volumes and shaker flasks are compared, the absence of mixing causes a loss of efficiency (Fig. 5-2 vs. 5-3). Presumably this is caused by local accumulation of inhibitors, products and enzymes. A similar result was observed for the hydrolysis of pure cellulose (Solka floc) at 360 g/L (Lavenson et al. 2012). In that study no mixing had approximately 16% conversion after 128h compared to 34% with mixing. These conversions are relatively low despite the use of 18 FPU/g cellulose. However, magnetic resonance imaging (MRI) studies showed that the mixed reactions had uniform liquefaction while unmixed ones did not, indicating ineffective diffusion of enzymes.

Conversion of 51% after 72 hours at 10 g solids/L using 3.6 mg protein/g solids equivalent to 3 FPU/g solids or 6 FPU/g glucan in a shaker flask is the closest condition to the previously described on literature. Liquid hot water pretreated corn stover hydrolyzed with Spezyme CP supplemented with β-glucosidase at roughly 15 FPU/g glucan (5.4 FPU/g solids) hydrolyzed 64.2 to 69.6% of cellulose (Zeng et al. 2006). Cellic CTEC2 (7.0 FPU/g glucan or 3.5 FPU/g solids) supplemented with hemicellulases achieved 60% conversion after 72h (Cao et al 2015). On a more recent study with Cellic CTEC2 at 5 FPU/g glucan but much higher protein loading (16.6 mg protein/g solids) reached 72% conversion (Kim et al. 2016). However cellulose composition of pretreated corn stover were much lower than the ones observed here, only 35% versus 50% here. That is likely an underestimation to the high extractives observed. Considering that corn stover pretreated with liquid hot water pretreatment has roughly 50% cellulose when extractives are not removed, the adjusted conversions from that work would 50.4%, roughly the same as observed here.

When only mixed reactions are considered, the conversions when using shaker flasks fell from 51% with 10 g/L initial solids loadings to 37% with 200 g/L (200 RPM on Figure 5-2) ($p = 0.002$). On the other hand, hydrolysis carried in the bioreactor increased from 42% at 10 g/L to 47% at 100 and 200 g/L ($p = 0.04$) (290 RPM on Figure 5-3).

This result contradicts previous reports of improved mixing methods for high solids loadings (Table 5-2). These results show that even at very high enzyme loadings, the conversions fall. The addition of steel balls to shaker flasks was the closest to equal conversion at all solids loadings, ranging from 78.4% at 10g/L to 73.4% at 150g/L (Geng et al. 2015). The conditions were slightly different from the ones tested here. Hydrolysis was carried for 96 hours and the enzyme preparation included Cellic CTEC2 supplemented with xylanase and had twice as much enzyme added (Geng et al. 2015). "Despite increasing the final yield across biomass loadings compared to shaker flasks, none of these approaches modified the negative correlation between biomass loading and glucose conversion" (dos Santos 2016). This indicates all previous reported methods did not achieve efficient mixing.

Table 5-2. Summary of hydrolysis of pretreated biomass at increasing solids loadings

Biomass	Pretreatment	Enzyme (loading)	Mixing process	Solids loading	Conversion at 72 hours unless noted	Reference
Wheat straw	-	Celluclast 15L + Novozyme 188 (7 FPU/g solids)	Horizontal reactor	20 g/L	87.5%	Jørgensen et al. 2007
				200 g/L	60%	
				400 g/L	40%	
Corn stover	Diluted acid	GC220 (20 mg protein/g cellulose)	Roller bottles	150 g/L	80%	Roche et al. 2009
				200 g/L	70%	
				300 g/L	50%	
Hardwood pulp	-	Celluclast 15L + Novozyme 188 (20 FPU/g cellulose)	Peg Mixer	20 g/L	100%	X. Zhang et al. 2009
				200 g/L	80%	
Corn stover	Diluted acid	Cellic CTEC2 + Cellic HTEC2 (5 FPU/g solids, 7.21 mg protein/g solids)	Added steel balls	50 g/L	78.4% (96 h)	Geng et al. 2015
				100 g/L	76.3% (96 h)	
				150 g/L	73.2% (96 h)	
Eastern redcedar	Diluted acid	Accelerase® 1500 (46 FPU/g glucan)	Added steel balls	20 g/L	82.4% (96 h)	Ramachandriya et al. 2013
				160 g/L	82.5% (96h)	
				200 g/L	75.2% (96h)	

Table 5-2 continued.

Biomass	Pretreatment	Enzyme (loading)	Mixing process	Solids loading	Conversion at 72 hours unless noted	Reference
Arundo donax	Steam explosion	Cellic CTEC2 (0.1g solution/g solids)	Anchor impeller (controlled power input)	100 g/L	40.8%	Palmqvist and Lidén 2012
				150 g/L	35.1%	
				200 g/L	30.3%	
Norway spruce				100 g/L	44.0%	
				150 g/L	34.4%	
				200 g/L	27.6%	

An increase of conversion with increasing solids loadings was observed. This effect is not caused by a loss of enzyme activity due to impeller shear. A similar phenomenon was observed when higher enzyme concentrations are used (6 or 12 mg protein/g solids) in shaker flasks. The conversions reached complete conversions at 100 and 200 g/L, while the conversions at 10 g/L are limited to 65% (6 mg protein/g solids) and 75% (12 mg/ g solids). The lack of efficient mixing in these conditions also demonstrate that high enough enzyme loadings can counteract the high solids loading effect. Lower enzyme loadings are necessary to properly analyze the solids effect. The lower enzyme activity added limits the maximum conversions during hydrolysis of corn stover but expose differences more clearly.

Efficient mixing eliminates the solids loadings effect when high solids loadings (> 100g/L) are used. In the absence of soluble inhibitors derived from pretreatment, the inhibitors present are product of the hydrolysis. Glucose and xylose and their oligomers can all inhibit cellulases (Ximenes et al. 2010). Considering the relative low amount of residual hemicellulose, xylose and xylose oligomer impact are likely low. However, xylanase supplementation will increase conversion of pretreated corn stover and could lower the differences seen between solids loadings (Cao et al. 2015). The impeller configuration and high speed used likely reduces the local concentration of inhibitors and enzymes, leading to an apparent lower product inhibition. Kinect studies have used non-efficient mixing so far. This leads to overestimation of the inhibition constants, making them specific to the reactor used. This specificity is problematic for scaling up and comparing data between reports.

However, product inhibition does not explain the lower conversion at 10 g/L compared to 100 to 200 g/L. If only inhibitors were present, conversion would have been equal at all solids loadings. Taken together these results suggest that another factor is influencing glucose conversion.

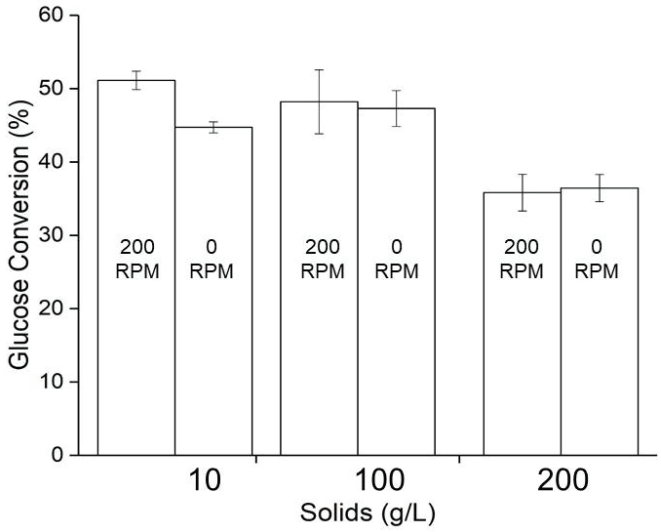

Figure 5-2. Enzymatic hydrolysis of pretreated pelletized corn stover at different solids loadings. Enzyme (Cellic CTEC2) loading of 3.6 mg protein/g solids. Hydrolysis conducted for 72 hours, pH 4.8 (50 mM citrate buffer) in shaker flasks (50 mL).

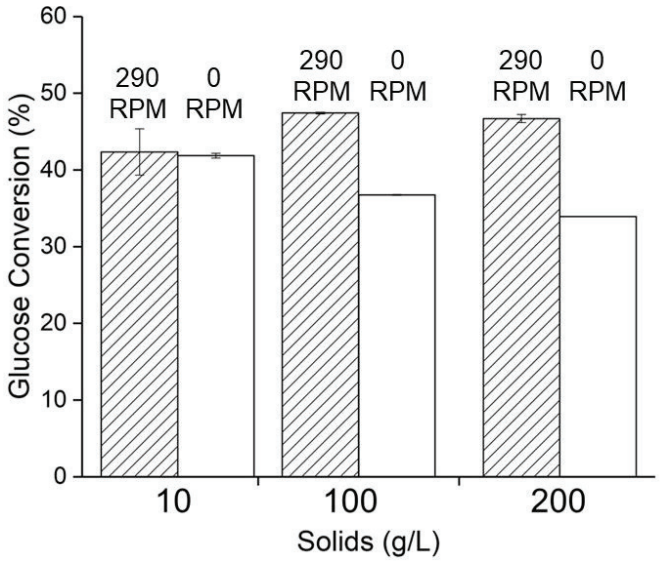

Figure 5-3. Enzymatic hydrolysis of pretreated pelletized corn stover at different solids loadings. Enzyme (Cellic CTEC2) loading of 3.6 mg protein/g solids. Hydrolysis conducted for 72 hours, pH 4.8 (50 mM citrate buffer) in bioreactor (600 mL).

5.3.3 Rheology impact of solids loadings

Improvements of mixing efficiency could be caused by changes on viscosity and related increase on protein and inhibitor diffusion (Kristensen et al. 2009). Figures 5-4 and 5-5 show viscosity and shear stress measured against shear rate. These parameters were measured using 50 mm parallel plates. Biomass concentration of 200g/L was used in all tests. As the distance

between plates increase, apparent viscosity and shear stress also increase. Apparent viscosity decreases with shear rate as the biomass is pushed away and out from under the plates. Both effects from parallel plates has been previously described and increase variability of final results (Knutsen and Liberatore, 2009). Material expulsion from under the plates, limited to shear rates > 100 1/s, was also described by Stickel et al. (2009).

In order to reduce breaking and expulsion, rough parallel plates (25 mm) were used (Figures 5-6). However, wall slips were still evident and expulsion still present. Up to a shear rate of 1 (1/s) the apparent shear stress is stable between 3500 and 4000 Pa. Previous attempts to measure viscosity and shear rate on biomass had similar difficulties on these geometries (Knutsen and Liberatore, 2009, Stickel et al. 2009). Both works determined that the shear stress at 15% and 17% solids is between 100 and 1000 Pa. Torque rheometers are the most accurate method described (Stickel et al. 2009, Ehrhardt et al., 2010). Using this method, biomass behaves as a Bingham plastic. The apparent shear stress of pretreated biomass increases nonlinearly, with a sharper increase until an apparent shear rate of 50 1/s is reached then stabilizes. Apparent viscosity decreases linearly with increasing apparent shear rate.

Yield stress is the rheological characteristic that has been more accurately described and is a function of solids loading and particle size. They are generally measured with narrow-gap vanes. When 300g/L pretreated corn stover was measured under these conditions, a yield stress of approximately 3700 Pa was measured (Figure 5-7). Previous reports range from 1000 Pa to 40 kPa depending on solids concentration and particle size. Higher solids loadings increase yield stress measured by vanes logarithmically (Knutsen and Liberatore, 2009, Stickel et al.. 2009, Ehrhardt et al. 2010). Smaller particle sizes reduce yield stress (Viamajala et al. 2009, Erhardt et al. 2010). The particle sizes used in previous studies are all much smaller than the ones used here. This size difference explains the higher propensity of this material to fracture as the material is less cohesive.

Values described here are in the same range as the ones previously described, when roughened parallel plates and vane geometries are used. However, this ignores the supposed impact of particle size as the values should have been much larger proportionally to particle size. Accurate measurements of rheological proprieties are still needed.

The impact of viscosity, shear stress and yield stress as it relates to this study are still unknown. The improved mixing pattern was capable of inducing a laminar flow through the initial hours at high solids and liquefaction of pretreated corn stover was reached within 12 hours. Visual confirmation of particle movement is due to the yield stress being reached by the dual marine impellers at 290 RPM. The final conversions of glucose from cellulose are dependent on the early liquefaction of biomass. The impact of early liquefaction on overall conversion has been described using peg-mixers (Zhang et al. 2009). In that work, liquefaction occurred within 1 hour compared to 40 hours using shaker flasks. The effect, however, did not recover efficiency completely at high solids loadings resulting in loss of conversion going from 100% (20 g/L) to 80% (200 g/L). This hypothesis was tested by hydrolyzing pretreated corn stover with no mixing on the first 24 hours followed by 48 hours of mixing. Conversions were the same as not having any mixing through the whole experiment (45% at 10 g/L and 100 g/L and 35% at 200g/L).

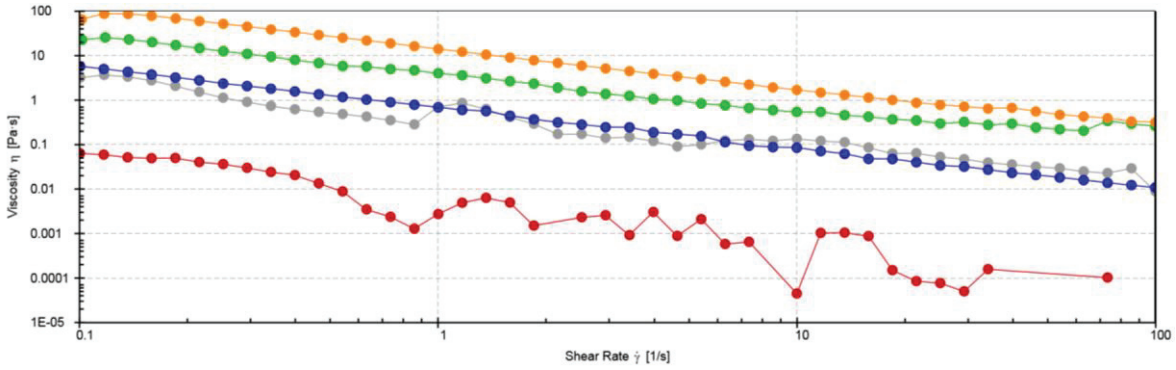

Figure 5-4. Apparent viscosity of 2 mg of 200 g/L slurry of pretreated corn stover as shear rate increases. Color indicates parallel plate distance. Red: 1.5 mm; Grey: 1.25 mm; Blue: 1.20 mm; Green: 1.1 mm; Orange: 1.0 mm.

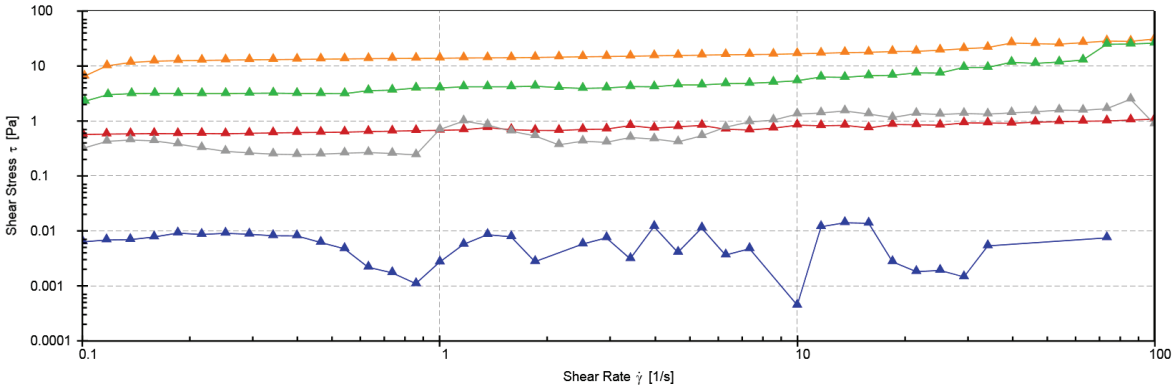

Figure 5-5. Apparent shear stress as shear rate increases of 2 mg of 200 g/L slurry of pretreated corn stover. Color indicates parallel plate distance. Red: 1.5 mm; Grey: 1.25 mm; Blue: 1.20 mm; Green: 1.1 mm; Orange: 1.0 mm.

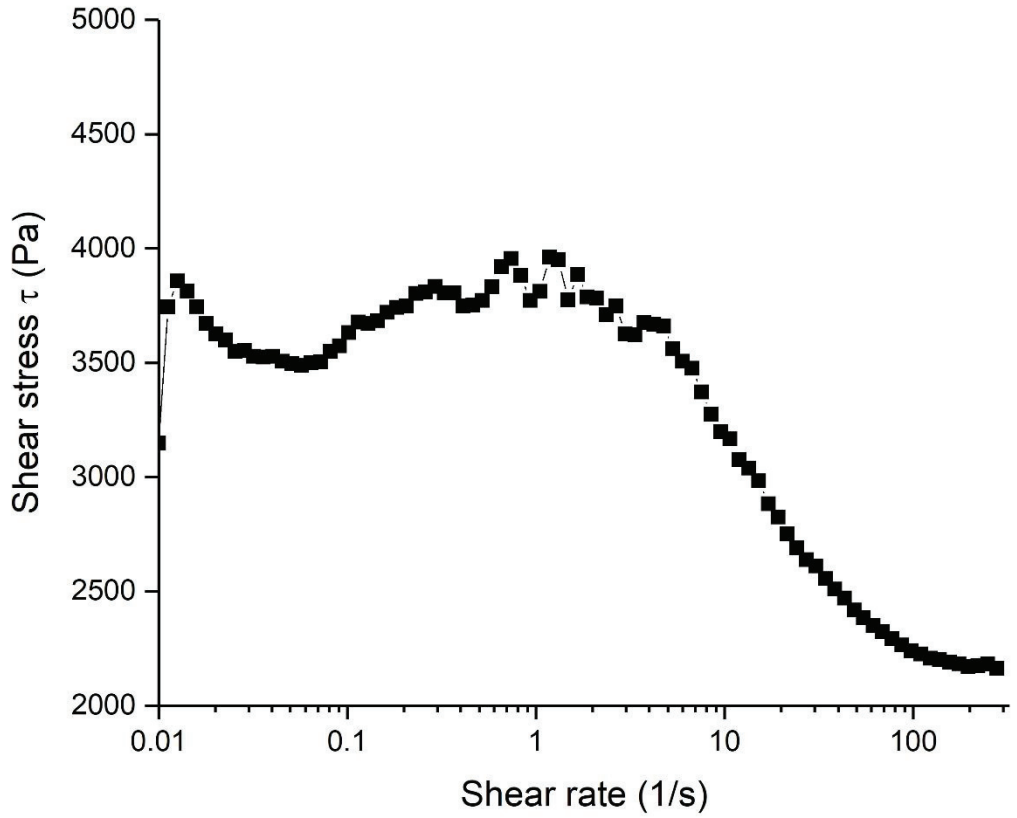

Figure 5-6. Apparent shear stress as shear rate increases of 2 mg of 300g/L of pretreated corn stover. Measurement made with 25mm roughened parallel plates. Plate gap set to 1 mm.

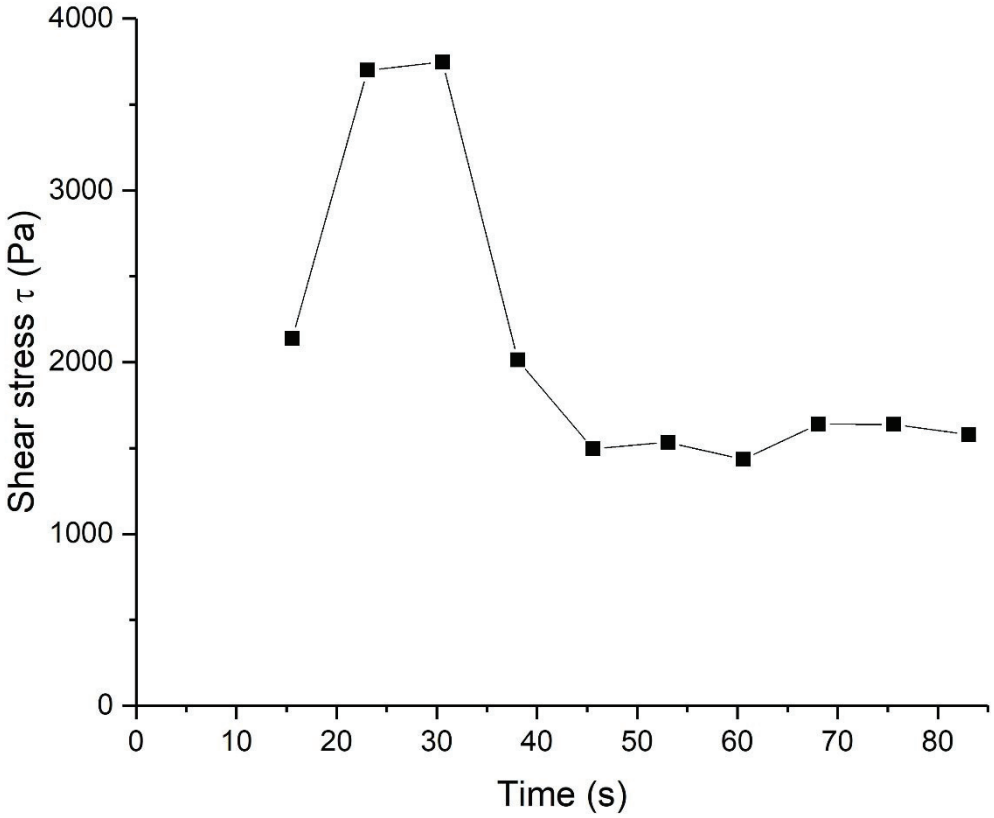

Figure 5-7. Apparent shear stress of 300 g/L pretreated corn stover changes with vane displacement. Narrow-gap vanes at 1.67 rpm. Measurements are average of 7.5 seconds each.

5.3.4 Lignin blocking at high solids

The low severity of the pretreatment suggests that lignin adsorption would be small as previously seen in Chapter 4. The effect of solids loadings over lignin adsorption, however, was not known. The addition of BSA across solids loadings was evaluated in shaker flasks. Biomass was incubated with 100 mg BSA/g solids for an hour. Enzymatic hydrolysis was carried out in the same conditions as above. The results are summarized on Figure 5-8. The conversions with or without BSA were not statistically different at 10 g/L or 100 g/L solids ($p > 0.05$). However, at 200 g solids/L, the difference the conversion with BSA (41%) vs without BSA (37%) is significant ($p=0.01$). Lignin adsorption is, therefore, responsible for a small portion of the solids loading effect.

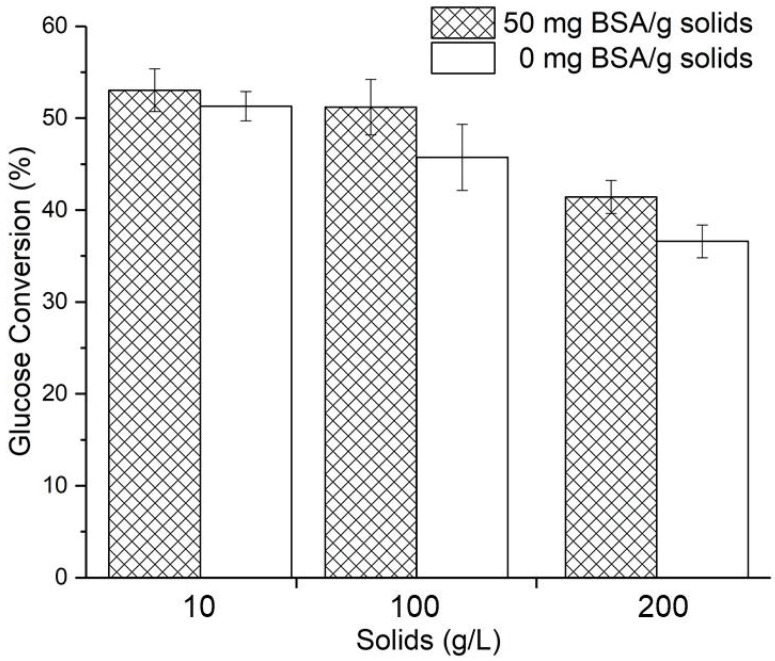

Figure 5-8. Enzymatic hydrolysis of pretreated pelletized corn stover at different solids loadings with or without BSA addition. Enzyme (Cellic CTEC2) loading of 3.6 mg protein/g solids. Hydrolysis conducted for 72 hours, pH 4.8 (50 mM citrate buffer) in shaker flasks (50 mL, 200 RPM).

This small difference is similar to the ones observed with sugarcane bagasse (Chapter 4). When the same experiment was done with sugarcane bagasse pretreated with liquid hot water, the efficiency increases are the same at 10 g/L and 80 g/L with a different enzyme preparation, Cellulase 13P supplemented with β-glucosidase (Novozyme 188). These results are summarized in Figure 5-9. The resulting lines were found to be parallel, having the same slope ($p < 0.0001$). Indicating that the same factor or factors are causing the loss on efficiency and that the inclusion of BSA does not affect it. When Cellic CTEC3 (6 mg protein/g solids) was used the conversions were higher and the conversion increased with BSA at similar levels at 10 and 100 g/L (Table 5-3).

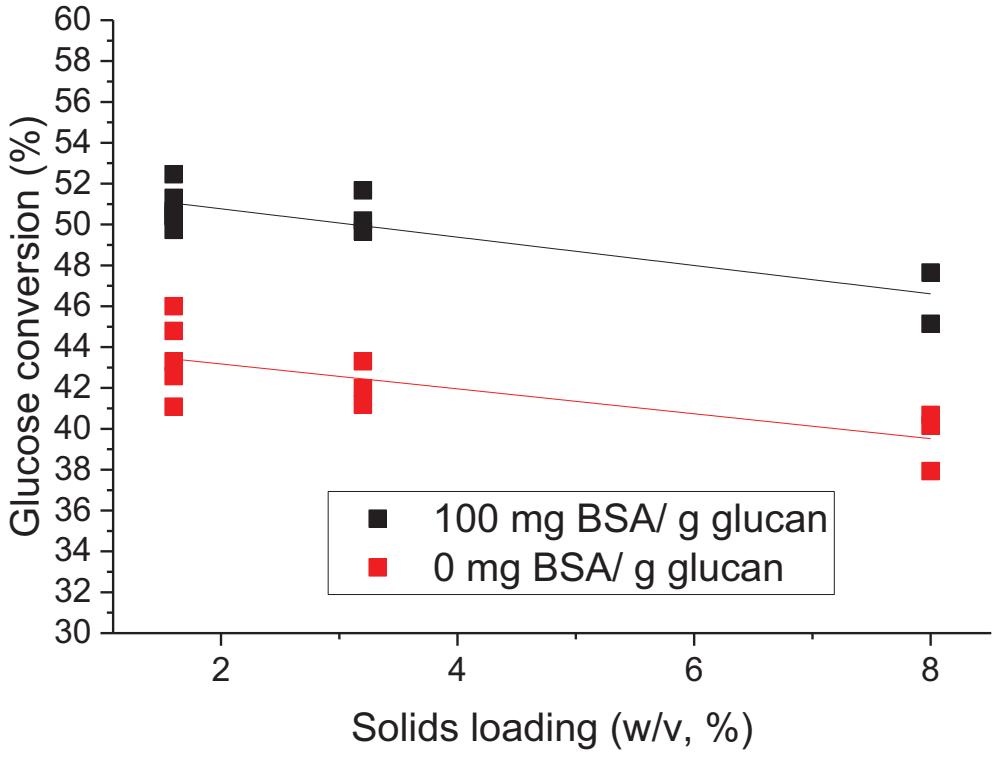

Figure 5-9. Glucose conversion from pretreated and washed sugarcane bagasse (LHW 200ºC, 20 min) as solids loading increases using 10 FPU (Cellulase 13P) + 20 IU (Novozyme 188)/ g glucan. Reproduced from (dos Santos 2016).

Table 5-3. Glucose conversion of pretreated and washed sugarcane bagasse with 2.5 FPU/ g glucan in different conditions. Reproduced from (dos Santos 2016).

Solids loading (w/v)	Cellulase 13P + Novozyme 188 (1 FPU : 2 IU)		CTEC3	
	0 mg BSA/g glucan	100 mg BSA/g glucan	0 mg BSA/g glucan	100 mg BSA/g glucan
1.7%	37.7%	36.8%	71.8%	76.1%
10%	29.7%	39.5%	72.6%	80.5% (83.1%)*

These results reaffirm that enzyme composition plays an important role on lignin adsorption. Both Cellic CTEC2 and CTEC3 are supplemented with β-glucosidase which reduces the impact of lignin blocking, but do not explain the whole effect, as explained on section 4.4.9. They also confirm that there is no difference between 10 and 100 g/L regarding lignin adsorption.

5.3.5 Enzyme hydrolysis at anaerobic conditions

Once it was established that no other inhibitor caused the lower conversion at 10 g solids/L when efficient mixing, the only remaining factor was deactivation caused by air interaction.

Deactivation by air interaction was evaluated using the same bioreactor configuration used in Section 5.3.2. Air was removed by purging the headspace with N_2. The glucose conversions under air and nitrogen are compared in Figure 5-10. Under anaerobic conditions, enzyme efficiency increased at all solids loadings ($p < 0.05$). When pelleted corn stover is pretreated with liquid hot water (190°C, 20 minutes), washed, mixed with two marine impellers and hydrolysis conducted under anaerobic conditions, glucose conversion is independent of solids loadings.

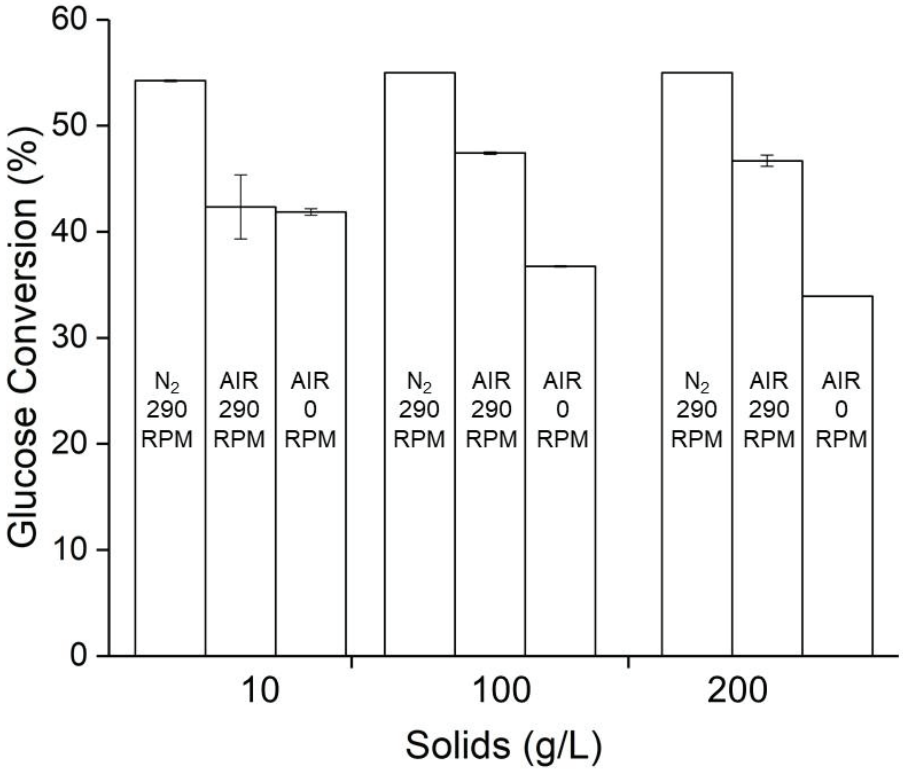

Figure 5-10. Enzymatic hydrolysis of pretreated pelletized corn stover at different solids loadings. Enzyme (Cellic CTEC2) loading of 3.6 mg protein/g solids. Hydrolysis conducted for 72 hours, pH 4.8 (50 mM citrate buffer) in bioreactor (600 mL).

At the conditions tested, Cellic CTEC2 (3.6 mg protein/g solids) hydrolyzed 55% of cellulose. Cellic CTEC2 and CTEC3 contains lytic polyssacharide monooxygenases (LPMO) activity. This enzyme creates new ends on cellulose in a similar manner to endoglucanase and requires oxygen to operate (Villares et al. 2017). Restricting oxygen should eliminate this activity. However, when an enzyme not containing LPMOs undergoes hydrolysis under anaerobic conditions, it does not hydrolyze cellulose at the same rate as the LPMO containing ones (Scott et al. 2016). LPMOs, therefore, are necessary for the action of the combined activities. Another by-product of LPMOs is the generation of reactive oxygen species (ROS) that can deactivate the cellulases (Kittle et al. 2012; Kjaergaard et al. 2014; Scott et al. 2016). These ROS also impact the measurement of dissolved oxygen using a chemical probe (Figure 5-11). Such probes are sensitive to those and generate seemingly impossible values indicating the presence of more oxygen than the saturation maximum. These results indicate a very fast generation of ROS on the early stages followed by a reduction towards equilibrium after the biomass is liquefied. They also demonstrate an inverse correlation between available oxygen and solids loadings. The difference is due to mass transfer of oxygen into the solution. This relationship can explain the differences on impact of ROS in different solids loadings.

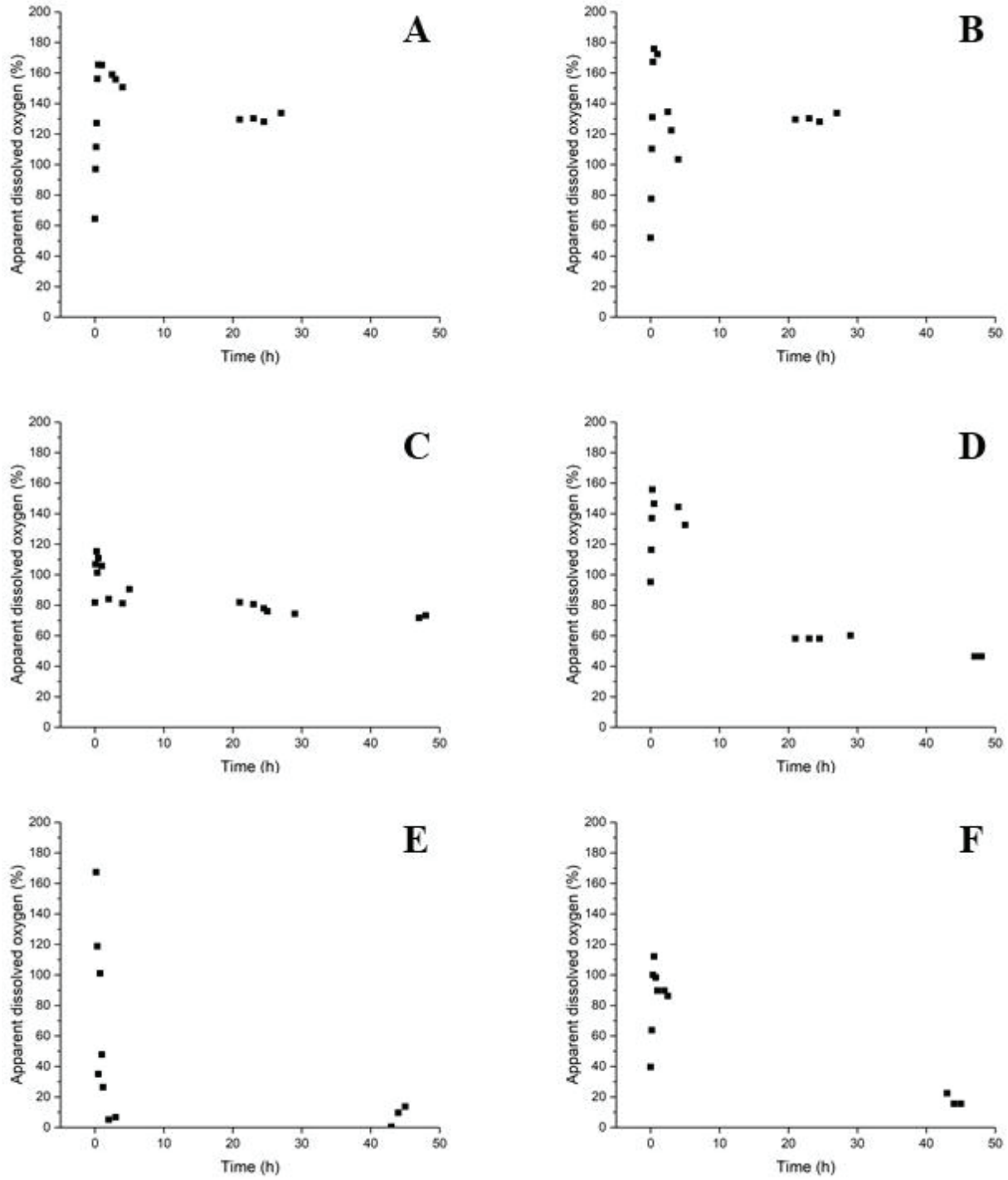

Figure 5-11. Apparent dissolved oxygen measured during enzymatic hydrolysis at different solids loadings (50°C, pH 4.8). All measurements relative to oxygen saturated DI water, 50°C. A, C and E: pretreated corn stover; B, D and F: solka floc. A and B: 10 g/L; C and D: 100 g/L; E and F: 200 g/L.

The restriction of oxygen during hydrolysis would reduce the deleterious effects of monooxygenases on the other enzymes. The extra cellulases and lower activity monooxygenase hydrolyzes cellulose to glucose at higher rates than fully active LPMOs with lower cellulase activity or full cellulase activity with no LPMOs (Scott et al. 2016).

A previous report of high solids loadings hydrolysis under anaerobic conditions had the opposite results. Anaerobic conditions reduced conversions from approximately 65% to 41% at 100 g/L and 63% to 39% at 200 g/L (Muller et al. 2015). In that work, Cellic CTEC2 was also used, although at 5 mg protein/g solids. The two main difference are the use of "free falling mixing" and the addition of L-cysteine hydrochloride monohydrate to remove residual oxygen. Free falling mixing is the same technique used on horizontal reactors that could not counter the solids loading effect (Jørgensen et al. 2007). A lesser mixing method highlights the impact of LPMOs on early liquefaction compared to preparations without it. The use of a more efficient mixing distributed the deleterious by-products of LPMOs, combined with a enhancement of liquefaction across solids loadings led to a overall negative impact on cellulases. The impact of L-cysteine hydrochloride directly on cellulases is not known. However, L-cysteine is an oxygen stripper and complete removal of residual oxygen completely eliminated the activity of LPMOs, thus reducing the overall conversion.

These findings demonstrate that the role of O_2 and air is not as simple as previously thought and are dependent like all the other factors on mixing. The ideal O_2 concentration is still unknown, but it is not as high as saturation of the reaction or as low as complete absence. Additional research in which the dissolved oxygen levels are controlled will clarify the optimum conditions.

The use of an efficient mixing method altered the relationships of enzymes and their inhibitors. For product inhibitors the impact was positive as they became less locally accumulated reducing the contact with cellulases. For ROS, the impact was negative. With inefficient mixing, high LPMO action facilitates liquefaction early counteracting cellulase deactivation. Meanwhile, the efficient mixing facilitates liquefaction (through all activities) and oxygen mass transfer into the reaction. This increases LPMO activity, generating more ROS and their deactivation.

The results here, indicate that with efficient mixing the considerations for lignocellulose hydrolysis are changed. All previous studies had inefficient mixing and their results and conclusions are heavily influenced by it. The role of mixing is, therefore, much bigger than previously suggested. Interactions between enzymes, inhibitors and products are so dependent on mixing that results cannot be compared between different mixing methods. This creates a huge difficulty for scaling up and transference of research to industrial applications. The development of future technologies will have to consider mixing and its impact.

Early liquefaction is highly correlated to final glucose conversions. Efficient mixing facilitates liquefaction by distributing enzymes, especially endoglucanases that linked to reducing viscosity (Cunha et al. 2014). LPMOs can also have that capability as they have an analogous mechanism to endoglucanase. When mixing is inefficient and endoglucanase less available, LPMO activity must be maximized to facilitate liquefaction. When mixing is efficient, oxygen availability must be controlled to maximize the cellulase activities. The positive impact of early LPMO activity on slurry liquefaction points toward another possible development. LPMOs could be applied by themselves on a separate liquefaction process prior to hydrolysis. In that way its activity is better utilized with reduced deactivation impact on the hydrolysis.

5.4 Conclusion

Biomass densification through pelletization allowed hydrothermal pretreatment to be efficiently done at high solids concentration. Pelletized corn stover was pretreated at 190 °C for 20 min in liquid hot water at initial solids loadings of 360 g/L compared to 150 g/L for non-pelletized corn stover. A higher concentration at this stage reduces the size of the pretreatment reactors and eliminates the need to dry biomass to achieve high solids on subsequent steps.

Utilizing washed, liquid hot water (190°C, 20 minutes) pretreated corn stover, the impact of mixing on cellulose hydrolysis after 72 hours was evaluated. The application of marine impellers to mix enzymatic hydrolysis led to efficient mixing. Efficient mixing eliminated product inhibition, and higher glucose conversion (47%) at high solids (100 – 200 g/L) with a relatively low enzyme loading (3.6 mg protein/g solids). In contrast, hydrolysis at the same conditions in 250 mL shake flasks gave lower conversions at the higher solids loadings, 40% at 200 g/L, then lower solids loadings, 51% at 100 g/L. Efficient mixing led to uniform distribution

of enzymes and avoided local accumulation of inhibitors, increasing activity. This increased activity impact especially early liquefaction. An efficient liquefaction greatly impacts the overall conversion positively.

Efficient mixing allowed to isolate air related deactivation as a factor. Glucose conversion was higher under anaerobic conditions (55%) across all solids loadings compared to 42 – 47% when air was present. The combination of efficient mixing and anaerobic conditions led to hydrolysis efficiency independent of solids loadings. The experiment design allowed for a clear separation of the multiple factors that reduce cellulose hydrolysis efficiency. It demonstrated the impact of inefficient mixing on the interpretation of the confounding inhibition factors. Multiple previous observations are the product of the interaction between mixing and the factor studied. Future kinect studies will have to incorporate ideal mixing in order to measure accurate inhibition constants. In this case, the efficient mixing increased the negative impact of LPMO by-products that deactivate other enzymes, limiting the maximum conversion possible.

The methods described here can better inform the design of bioprocesses and enzymes that will be used on biorefineries. In order to maximize cellulose conversion, a very small amount of oxygen is necessary when mixing is efficient. Otherwise, oxygen may be needed to activate LPMOs early and achieve fast liquefaction.

CHAPTER 6. SIMULATIONS FOR SIMULTANEOUS SACCARIFICATION AND FERMENTATION INCORPORATING ALL MODES OF INHIBITION

6.1 Introduction

One of the obstacles to scaling-up ethanol from lignocellulose production is the lack of reliable and accurate models that can predict the multiple unitary processes as one. This deficiency forces the scaling-up to be strictly experimental, which increases costs and the fail rate of pilot plants. Models have been developed to predict hydrolysis (Philippidis, Smith, and Wyman 1993), fermentation of glucose (Maiorella, Blanch, and Wilke 1983), simultaneous saccharification and fermentation (Morales-Rodriguez et al. 2011). All models require sweeping assumptions and does not allow for observation of individual factors due to the extensive use of lumped constants.

6.2 Materials and Methods

6.2.1 Model simulations

Simulations were carried using Microsoft Excel 2013, using Microsoft Visual Basic for Application to input and set the ordinary equations. This software was utilized due to its familiarity and capacity to carry first-order equations quickly.

6.2.2 Enzyme Activity Assays

"Endoglucanase activity was measured using 1% (w/v) carboxymethyl cellulose (CMC, Sigma-Aldrich, St. Louis, MO) as substrate (Dien et al. 2008). Exoglucanase and β-glucosidase activities were measured using 2.5 and 10 mM of ρ-nitrophenyl-β-D-cellobioside (pNPC, Sigma-Aldrich, St. Louis, MO) and ρ-nitrophenyl-β-D-glucopyranoside (pNPG, Sigma-Aldrich, St. Louis, MO), respectively, as substrates. One unit is defined as the amount of enzyme releasing 1 μmol of ρ-nitrophenol per min under specified conditions (Dien et al. 2008). Proteins in the supernatant were measured using a bicinchoninic acid (BCA) protein assay reagent kit (Thermo-Scientific, Rockford, IL)" (Zanchetta et al. 2018).

6.2.3 Mathematical models for SSF

The SSF model utilizes 4th order Runge-Kuntta method to solve higher-order equations using first-order ones (Mosier and Ladisch 2009). The model was based on two preexisting models to describe hydrolysis of cellulose (Philippidis, Smith, and Wyman 1993) and fermentation of glucose (Maiorella, Blanch, and Wilke 1983). The hydrolysis of hemicellulose and the fermentation of xylose were constructed based on these models with the relevant adaptations.

6.2.4 Estimation of parameters

6.2.4.1 Pretreated material composition

Liquid hot water pretreatment was used to base the model. Sugarcane bagasse and corn stover were analyzed following the NREL LAP standard procedure (Sluiter et al. 2008). Hardwood composition was determined using the same procedure by Ko et al. (2015). Biomass composition for hardwood, corn stover and sugarcane bagasse under optimum conditions found in Table 6-1.

Table 6-1. Pretreated biomass composition

Biomass fraction (%)	**Hardwood**		**Corn Stover**		**Sugarcane Bagasse**	
Optimum condition	210 °C, 15 minutes		190°C, 20 minutes		200°C, 20 minutes	
	Untreated	Pretreated	Untreated	Pretreated	Untreated	Pretreated
Cellulose	39.8	57.5	33.6	47.7	48.2	66.7
Hemicellulose	16.6	4	20.6	13.1	28.5	0
Lignin	31.8	39.9	26.0	29.5	23.1	30.3
Solid recovery	100	72.7	100	60.1	100	63.3

6.2.4.2 Enzymatic hydrolysis

The constants used through the model were estimated based on literature and previous works. These values were compared to improved conditions listed on Chapter 5.

6.3 Results and Discussion

6.3.1 Estimation of product inhibition constants and comparison of effect

Estimating product inhibition during enzymatic hydrolysis, especially at high solids is challenging. Experimentally, a time course is necessary. Due to the reaction progress, more samples for the early parts of the process are needed. This is made difficult due to long processing time of each sample. Sampling, especially at early stages, is not necessarily representative of the whole reaction. Modelling the reaction is, therefore, a better method to estimate product inhibition. In this work, data from Kristensen et al. (2009) with pure cellulose is used to estimate the constants (Kristensen, Felby, and Jørgensen 2009).

The model was developed based on the mathematical derivations (Ladisch, Gong, and Tsao 1980; Hong et al. 1981). These were created for β-glucosidase only. It is applicable for the whole cellulase action considering β-glucosidase is the most sensitive to glucose inhibition. The overall reaction is summarized in Reaction (6.1), where E is enzyme; n(G_{anh}) is cellulose, represented as a n anhydrous glucose; G is glucose; k_3 is the reaction constant. Reaction 6.2 represents the product inhibition as EG represents the glucose-enzyme complex and $K_{i,1}$ is the inhibition constant. Reaction 6.3 represents a second type of inhibition where glucose interacts with the intermediate enzyme-cellulose and $K_{i,2}$ is the constant of inhibition for this interaction. Equation 6.4 represents the integration of all constants. In order to accurately represent the concentration of cellulose, the timely cellulose concentration is given by the known initial concentration minus glucose. Equation 6.6 is the integration of Equation 6.4 applying Equation 6.5, where the velocity is described on Equation 6.7.

$$E + n(G_{anh})_\alpha \rightleftharpoons EG_{anh} \underset{k\alpha}{\rightarrow} E + G \tag{6.1}$$

$$E + G \underset{K_{i,1}}{\rightleftharpoons} EG \tag{6.2}$$

$$E + n(G_{anh}) \rightleftharpoons EG_{anh} + G \underset{K_{i,2}}{\rightleftharpoons} EG_{anh}G \tag{6.3}$$

$$V\int_{0\alpha}^{t} dt = \int_{0\alpha}^{G\alpha}\left[\left(1 + \frac{G}{K_{i,1\alpha}}\right) + \left(1 + \frac{G}{K_{i,2\alpha}}\right)\frac{K}{nG_{anh\alpha}}\right] dG \tag{6.4}$$

$$nG_{anh} = (nG_{anh})_0 - G \tag{6.5}$$

$$Vt = \frac{G^2}{2K_{i,1\alpha}} + \left(1 - \frac{K}{K_{i,2\alpha}}\right)G - K\left(1 + \frac{(nG_{anh})_0}{K_{i,2\alpha}}\right)\ln\left(1 - \frac{G\alpha}{(nG_{anh})_{0\alpha}}\right) \tag{6.6}$$

$$V = k_3 E_{tot} \tag{6.7}$$

Figure 6-1 shows the application of this model on the data from Kristensen et al. (2009) with the constants on Table 6-2.

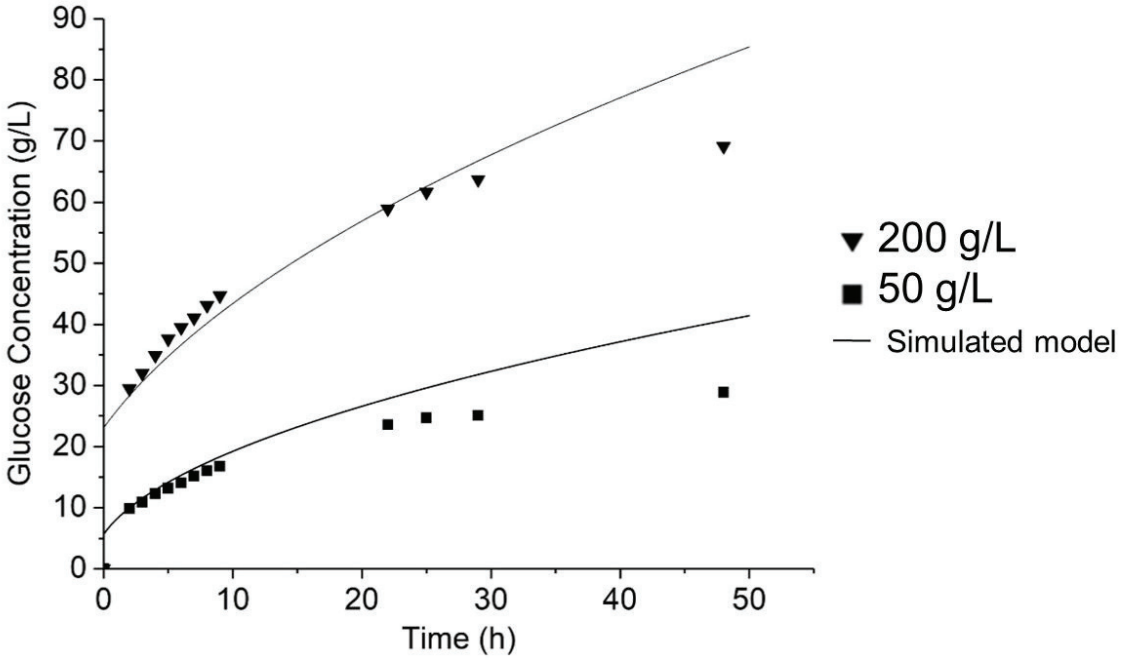

Figure 6-1. Hydrolysis simulation for product inhibition compared to data from Kristensen et al. (2007).

Table 6-2. Constants used on hydrolysis simulation for product inhibition compared to data from Kristensen et al. (2007).

Constant	**Value (calculated)**
K	4 mM
$K_{i,1}$	0.045 mM
$K_{i,2}$	0.5 mM
K_3	290 mM

The fitness of each model was evaluated by comparing and reducing the sum of squared errors (SSE). The lower SSE, the better the model fits the data. This first model only fits the early stages of either group of data points. The sum of squared errors (SSE) are 272.9552 (200 g/L) and 233.7568 (50 g/L), with most of the error coming from later points on the time course. At 200 g/L, 79% of the SSE is caused by the error at 48 hours. Similarly, 96% of the SSE at 50 g/L is due to the data after 22 hours. This suggests that the model is not appropriate. Based on the observations from Chapter 5, a time dependent deactivation of enzymes was added. This

approach was previously used by Scott et al. (2016) to describe this phenomenon. Reaction 6.8 and Equation 6.9 represent the deactivation as a first-order reaction. The fit is described on Figure 6-2 and Table 6-3 (Scott et al. 2016).

$$E \xrightarrow[K_d]{} E_d \qquad (6.9)$$

$$V = k_3 E_{tot} e^{(-K_d * t)} \qquad (6.10)$$

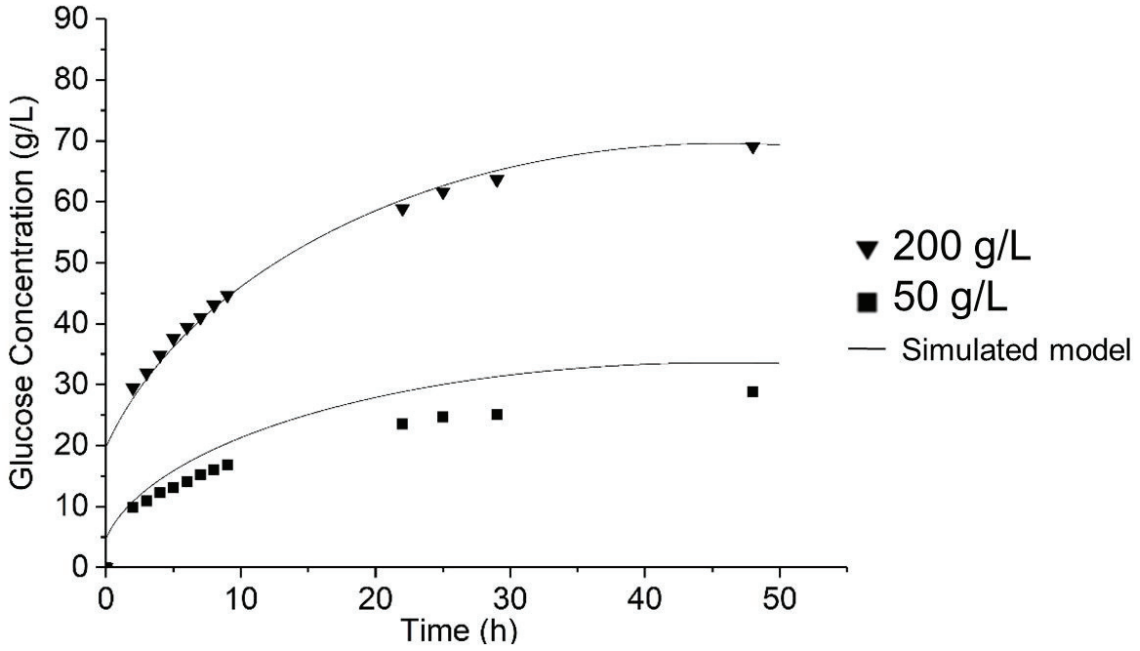

Figure 6-2. Hydrolysis simulation for product inhibition and time-dependent deactivation compared to data from Kristensen et al. (2007).

Table 6-3. Constants used on hydrolysis simulation for product inhibition and time-dependent deactivation compared to data from Kristensen et al. (2007).

Constant	**Value (calculated)**
K	4 mM
$K_{i,1}$	0.075 mM
$K_{i,2}$	0.5 mM
K_3	290 mM
K_t	5.88 *10^{-3} min^{-1}

The mathematical estimation of reaction constants fitted to the experimental data indicate lower inhibition product inhibition and lower enzyme activity that are modified by the time-dependent deactivation. The fit is good at high solids (SSE = 14.3), but not good at low solids

(SSE = 177.5). Taken together with the observations of the higher dissolved oxygen at low solids than at high solids and a possible relationship between dissolved oxygen and deactivation, deactivation is likely not related to time but dissolved oxygen. Modifying Reactions and Equations 6.8 and 6.9 to include a variable for oxygen concentration as shown in Equation 6.10. This data was extracted from the experiments described on Chapter 5. The dissolved oxygen values relative to 200 g/L is listed on Table 6-3. Relative values are used to account to shifts due to bubbles, buffer and impeller interference. The new fit is better (Figure 6-3). At 200 g/L, SSE was reduced to 6.32 and at 50 g/L, SSE fell to 14.41. This model, however, lacks to robustness to make definite claims for the mechanism of deactivation and the constants observed. An exception is the $K_{i,2}$ constant that does not affected the results in any way when changed by orders of magnitude.

$$V = k_3 E_{tot} e^{(-K_d * t * \frac{[O]}{[O_0]})} \qquad (6.10)$$

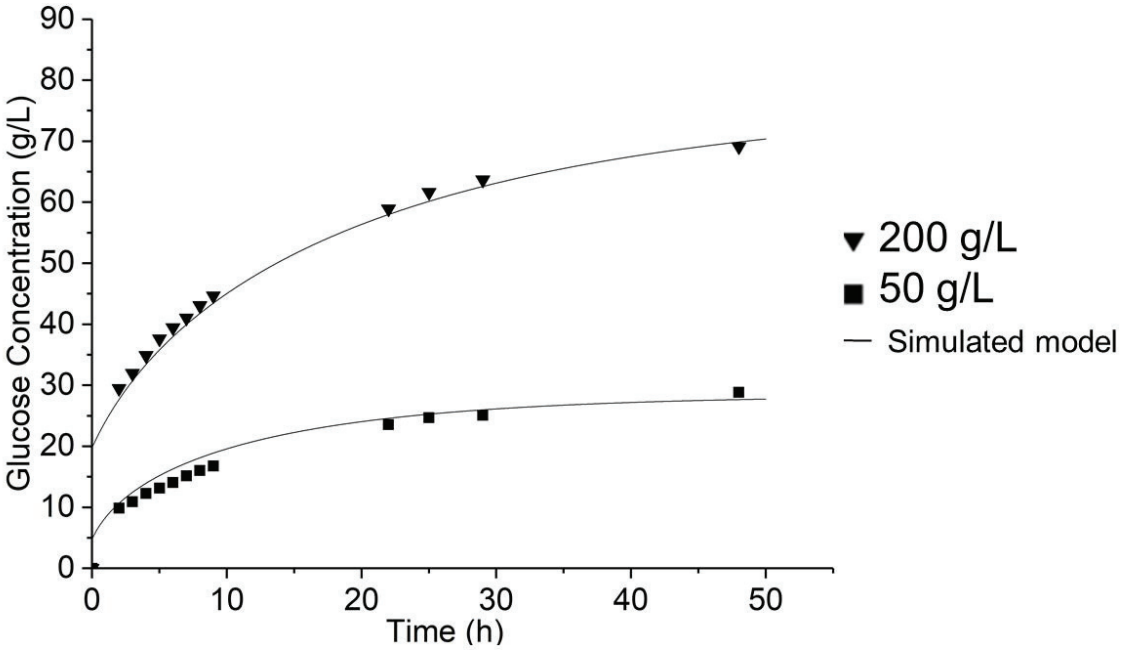

Figure 6-3. Hydrolysis simulation for product inhibition and time-dependent deactivation relative to dissolved oxygen compared to data from Kristensen et al. (2007).

Table 6-4. Relative dissolved oxygen during hydrolysis at equilibrium relative to 200 g/L with pure cellulose

Solids Loading (g/L)	Relative dissolved oxygen (Pure Cellulose)	Relative dissolved oxygen (Pelleted Corn Stover)
10	1.62	1.75
100	1.56	0.94
200	1	0.27

This simulation was compared to the values obtained on Chapter 5. Figure 6-4 compares the expected hydrolysis conversions versus the values obtained experimentally. The model does a good job of simulated anaerobic conditions, final conversions around 56% versus 55% observed experimentally. For aerated hydrolysis, the model overestimates 200g/L. These disparities are likely due to differences on enzyme composition on these conditions (Figure 5-11). The lack of LPMOs on the enzyme used by Kristensen et al. (2009) makes the impact of oxygen inaccurate. This also shows that the deactivation is dependent on LPMOs and that under aerobic conditions LPMO activity increases conversions.

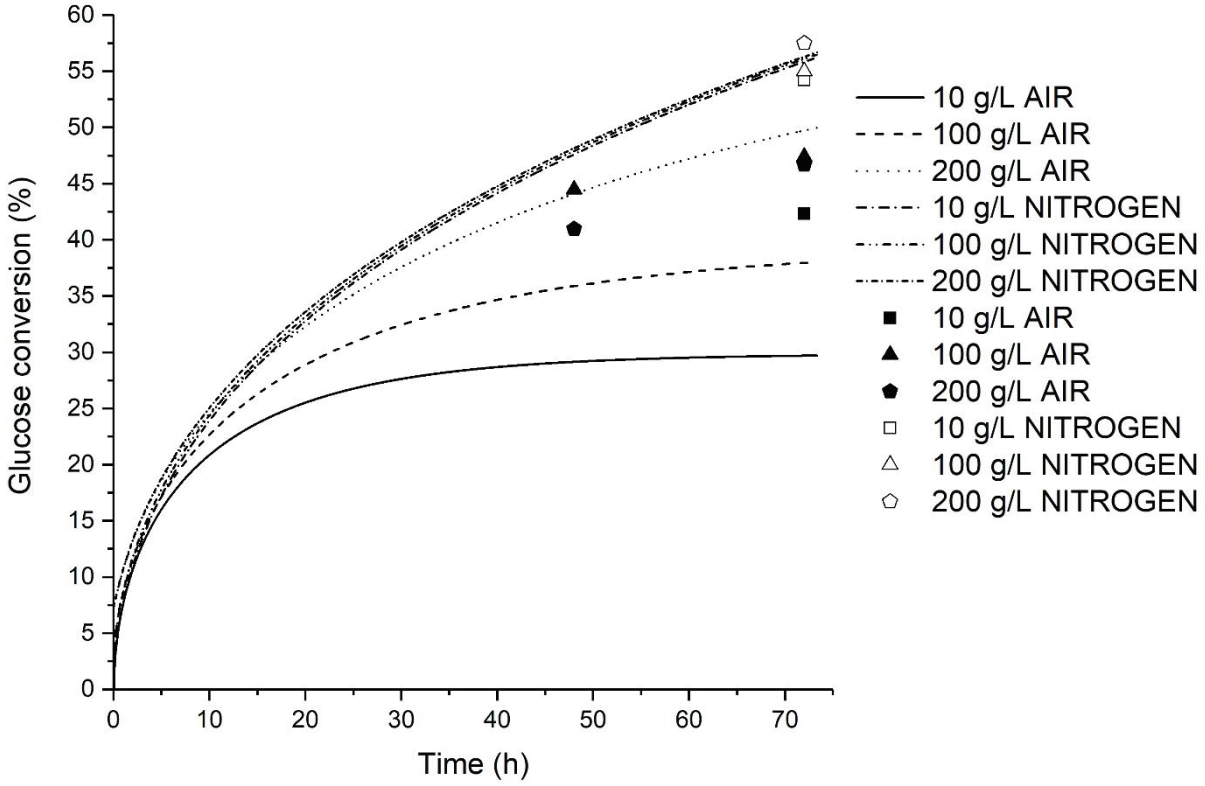

Figure 6-4. Hydrolysis simulation for product inhibition and time-dependent deactivation relative to dissolved oxygen compared to experiments described on Chapter 5.

6.3.2 Final model description

6.3.2.1 Enzymatic hydrolysis model

The hydrolysis model is based on the complete model from Phillippidis et al. (1993) for hydrolysis of cellulose to glucose, including cellobiose: $\frac{dC\alpha}{dt} = -r_1$ (5.11), $\frac{dB}{dt} = 1.056r_1 - r_{2\alpha}$ (5.12), $\frac{dB}{dt} = 1.053r_2$ (5.13). C is cellulose concentration, B, cellobiose concentration and G, glucose concentration. r_1 is the rate of conversion of cellulose to cellobiose and r_2 is the rate of cellobiose to glucose. 1.053 and 1.056 account for the incorporation of water into the molecules.

$$r_1 = \alpha \frac{k_1 \alpha_1 \Phi e_{c\alpha}}{(K_e + e_c)\left(1 + \frac{B}{K_{1B}} + \frac{G}{K_{1G}}\right)} \frac{K_{1E}}{K_{1E} + E} * (1 - K_{1L}L) \text{ (5.14) and } r_2 = \alpha \frac{k_2 B e_{g\alpha}}{K_m\left(1 + \frac{G}{K_{2G}}\right) + B\left(1 + \frac{B}{K_{2B}}\right)} \frac{K_{2E\alpha}}{K_{2E} + E} * \alpha$$

$(1 - K_{2L}L)$ (6.15).

"k_1 and k_2 are the specific rates of cellulose and cellobiose hydrolysis, respectively; with the rates of the hydrolytic action of cellulase (e_c) and β-glucosidase (e_g), K_e, is the equilibrium

constant for cellulase adsorption to cellulose; K_m is the Michaelis constant for β-glucosidase; K_{1L} and K_{2L} are constants for cellulase and β-glucosidase adsorption to lignin, respectively; and K_B, K_G, and K_E are the inhibition constants for cellulase (subscript 1) and β-glucosidase (subscript 2) by cellobiose (B), glucose (G), and ethanol (E), respectively" (Philippidis et al. 1993). As noted in the previous section a single inhibition constant for glucose is enough to describe the effect. The negligible adsorption of enzymes onto lignin at low temperature employed in the SSF (30-32 °C) (Zanchetta et al. 2018), lets us ignore the factors related to it. Considering the enzyme preparation to be Cellic CTEC 3, k2 >>> k1 and cellobiose (B) concentration and inhibition K_{2B} negligible, eq. (6.12 and 6.13) become: $\frac{dG}{dt} = r_1/0.9$ (6.16) and eq 6.14 becomes: $r_1 = \alpha \frac{k_1 \alpha_1 \Phi e_{c\alpha}}{(K_e + e_c)\left(1+\alpha\frac{G\alpha}{K_{1G\alpha}}\right)} \frac{K_{1E\alpha}}{K_{1E}+E}$ (6.17). Because the quality and initial concentration of cellulose, cellulase, and β-glucosidase do not vary from experiment to experiment, then α_1 (surface area of cellulose available), Φ (reactivity coefficient of cellulose), e_c (endoglucanase deactivation) and K_e (equilibrium constant for cellulase adsorption to cellulose) in Eq. (6.14) can be lumped together as k_1' and eq. 6.17 becomes $r_1 = \alpha \frac{k_1'^{\alpha}}{\left(1+\alpha\frac{G}{K_{1G\alpha}}\right)} \frac{K_{1E}}{K_{1E}+E}$ (6.18). The inhibition of xylose is incorporated into eq. 6.17 resulting in eq. 6.19 $r_1 = \alpha \frac{k_1'}{\left(1+\alpha\frac{G}{K_{1G\alpha}}+\alpha\frac{X\alpha}{K_{1IX\alpha}}\right)} \frac{K_{1E}}{K_{1E}+E}$.

The inhibition of cellulases by xylo-oligomers and soluble phenols liberated during pretreatment was ignored as the biomass was considered to be washed before reaction (Kim et al. 2009). Anaerobic conditions were assumed as those conditions are the ideal for *Saccharomyces cerevisiae* and incidentally eliminates the air related deactivation.

6.3.2.2 Co-fermentation model

The ethanol production model is based on Maiorella et al. (1983), with co-fermentation of xylose added to it. The additional constants used are based on Athmanathan el al. (2010). The equations used were $\nu = \nu_{max}\left[\alpha\frac{S\alpha}{S+C_{M\alpha}}\right]\left[1-\alpha\frac{P\alpha}{P_{max\alpha}}\right]^n$ (6.19) and $\mu = E\nu$ (6.20).

Table 6-5. Combine model constants

Constant	Explanation	Glucose	Xylose
νmax	Maximum specific ethanol production rate*	1.5 g ethanol/g cell h[1]	0.621 g xylose/g cell h[2]
Ks or Cm	Monod Constant	0.315 g/L[1]	16.7 g/L[2]
n	Toxic power constant	0.36[1]	1[2]
Pmax	Maximum product concentration	87.5 g/L[1]	121 g/L[2]
Yps	g of ethanol/g substrate	0.434[1]	0.514[2]
Yxs	g of cells/g substrate	0.07[1]	0.017[2]
E	Efficiency of substrate utilization for cell	0.249[1]	-
Ygly	Metabolic glycerol yield g glycerol / g substrate	0.054[3]	0.012[3]
Yxyl	Metabolic glycerol yield gxylitol / g substrate	-	0.137[3]
KmXaa	Km for acetic acid (cell growth)	2.395[4]	8.377[4]
K_{GI}	Glucose inhibition constant to cellulose hydrolysis	53.16 g/L[5]	-
K_{EI}	Ethanol inhibition constant to cellulose hydrolysis	50.35 g/L[5]	-
K_{IX}	Xylose inhibition constant to cellulose hydrolysis	0.1[7]	-
K_{GI}	Glucose inhibition factor of xylose consumption (g/L)	10[3]	-
Y_{aaS}	Metabolic acetate + acid acetic yield g/ g substrate	0.00139[6]	

[1](Maiorella, Blanch, and Wilke 1983)
[2](Athmanathan et al. 2011).
[3](Casey et al. 2013).
[4](Casey et al. 2010), calculated, pH 5.
[5](Philippidis, Smith, and Wyman 1993).
[6](Eliasson et al. 2000).
[7](Morales-Rodriguez et al. 2011).

6.3.2.3 Model simulations results

All simulations are carried on a per liter basis and set to run until 196 hours. The model biomasses compositions are summarized on Table 6-1. Enzyme was set at 2.5 FPU/g solids. The yeast inoculum was set at 1 g/L.

6.3.2.4 Batch simulations

For batch simulations, initial solids concentration was 200 g/L. Figure 6-4 shows Hardwood and it reaches 59.19 g/L of ethanol with 98% of cellulose was converted and all

hemicellulose is converted. Figures 6-5 and 6-6 shows Corn Stover and Sugarcane Bagasse reaching similar values and patterns. The main difference was the precipitous drop in total solids when higher hemicellulose is present while holding cellulose conversion due to its inhibition.

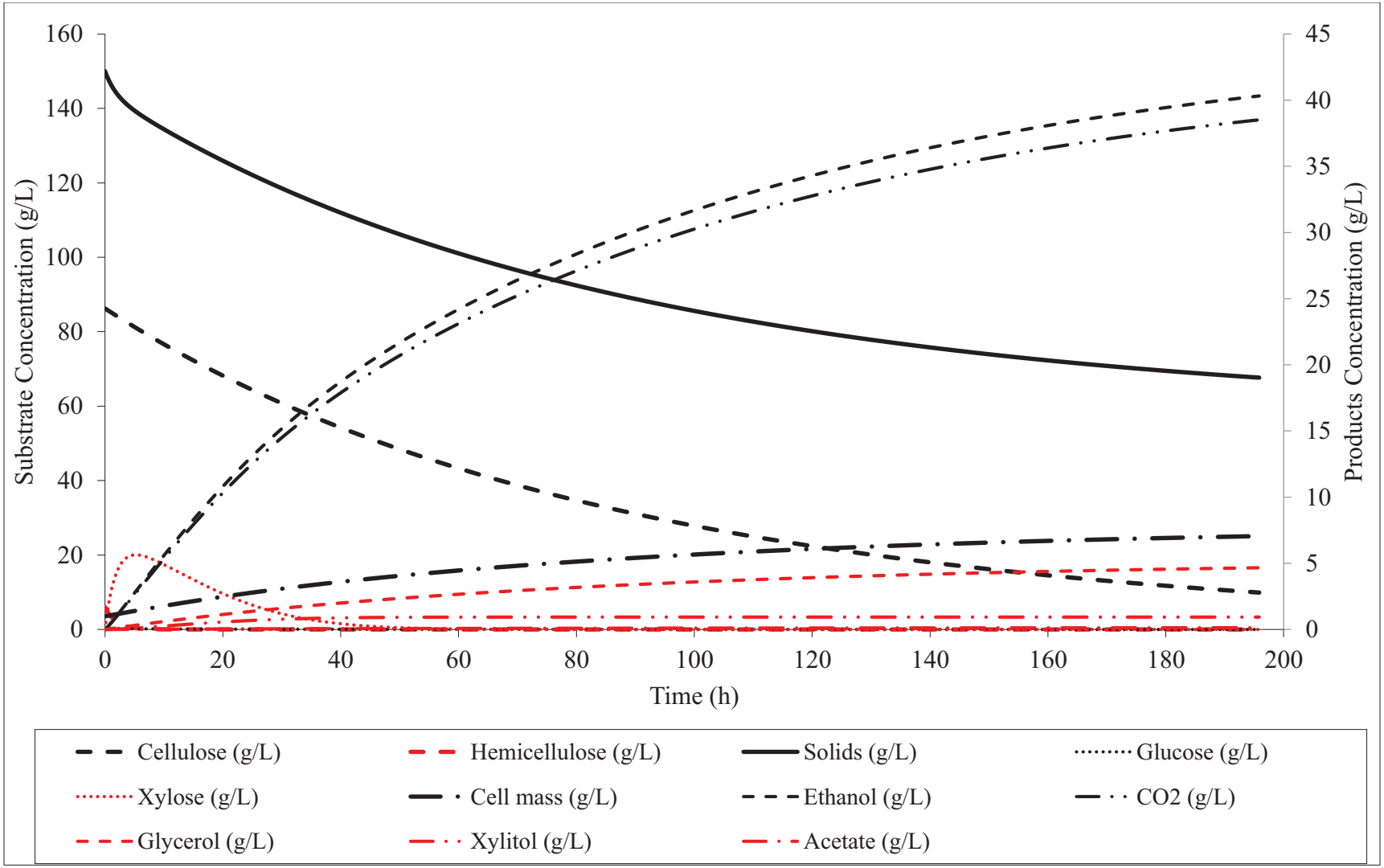

Figure 6-5. Model simulation for hardwood, batch.

Left axis: Solids, Cellulose and Hemicellulose; Right axis: all others.

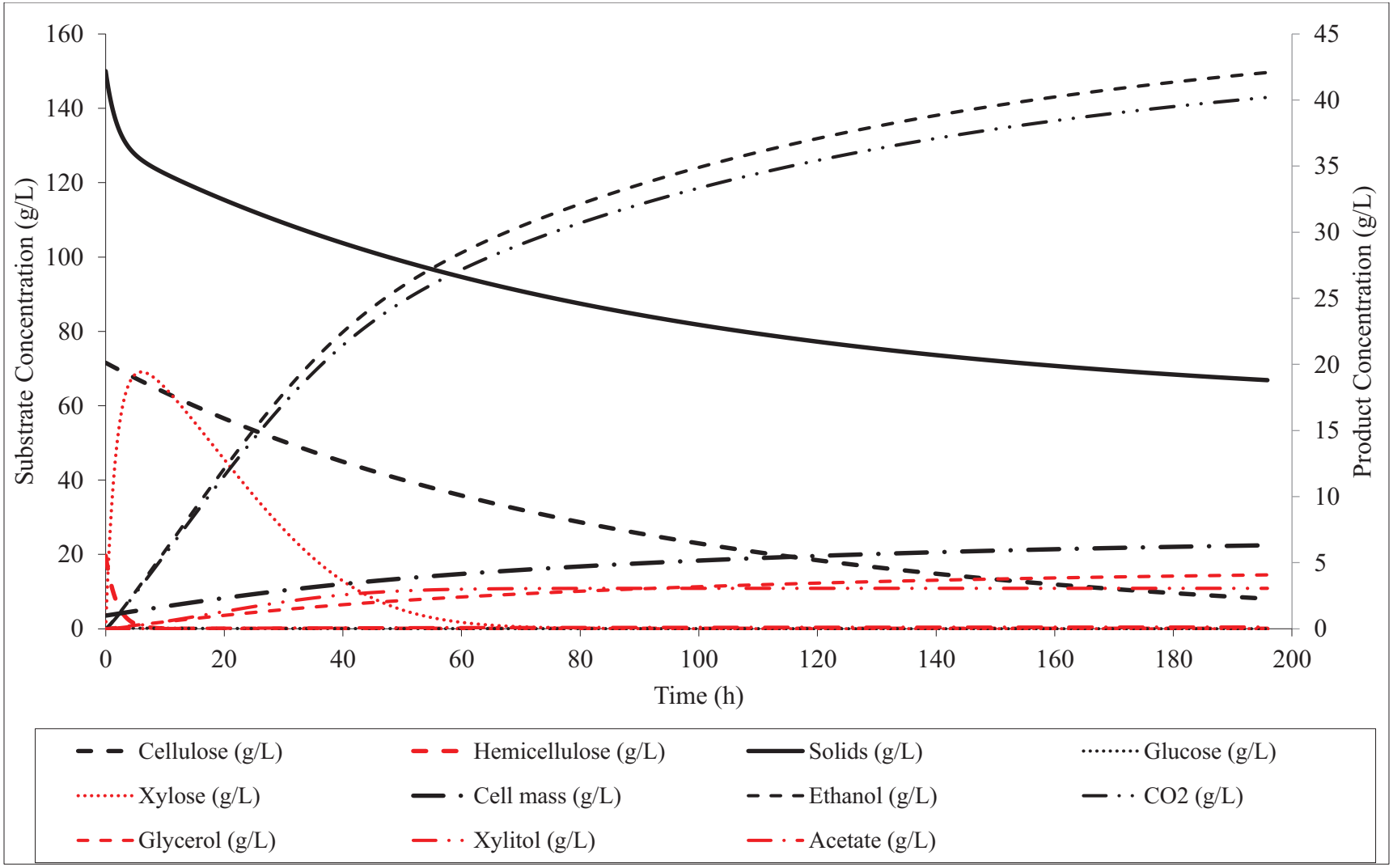

Figure 6-6. Model simulation for corn stover, batch.

Left axis: Solids, Cellulose and Hemicellulose; Right axis: all others.

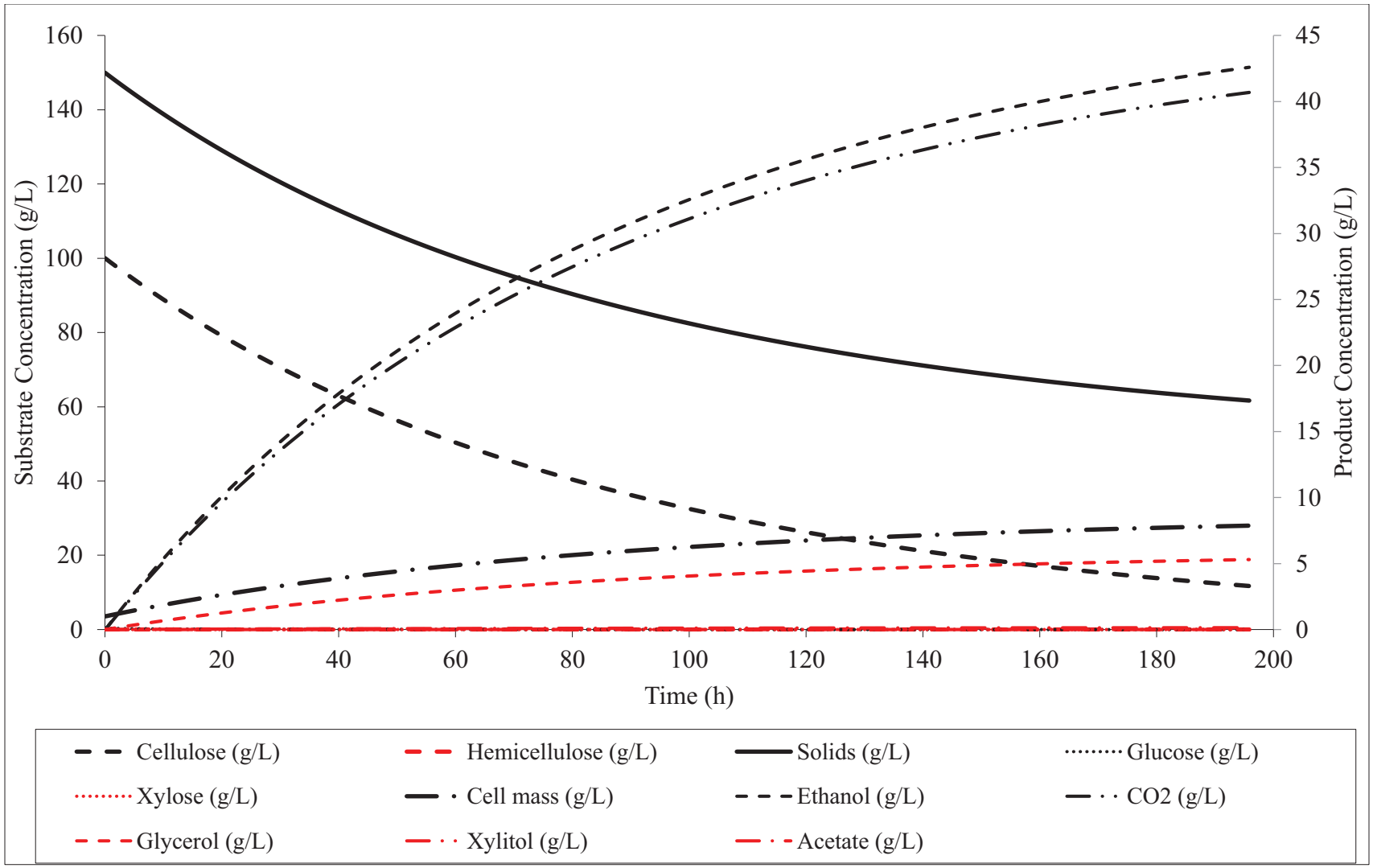

Figure 6-7. Model simulation for sugarcane bagasse, batch.

Left axis: Solids, Cellulose and Hemicellulose; Right axis: all others.

6.3.2.5 Fed-Batch simulations

To achieve higher ethanol titers, fed-batch must be used. For fed-batch simulations, initial solids concentration was 150 g/L, with two additions of 75 g/L added in 48 and 96 hours. Figure 6-7 shows Hardwood and it reaches 85.23 g/L of ethanol with 94.0% of cellulose was converted and all hemicellulose is converted. Figure 6-8 shows corn stover reaching similar values and patterns. Figure 6-9 shows sugarcane bagasse which reaches the maximum ethanol concentration (87.50 g/L) at 172 hours and the remaining glucose accumulates reaching 7.02 g/L at 196 hours.

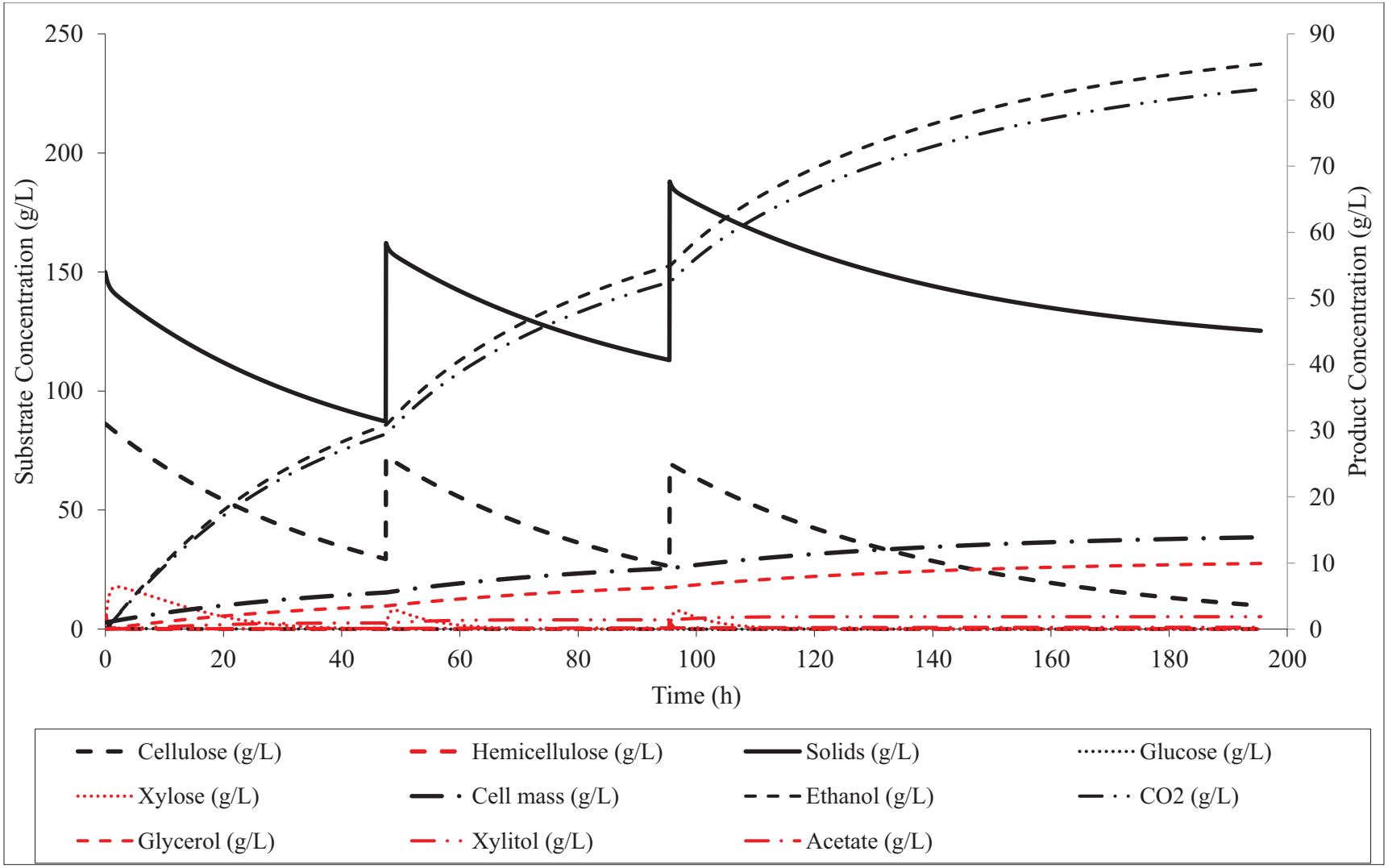

Figure 6-8 Model simulation for hardwood, fed-batch.

Left axis: Solids, Cellulose and Hemicellulose; Right axis: all others.

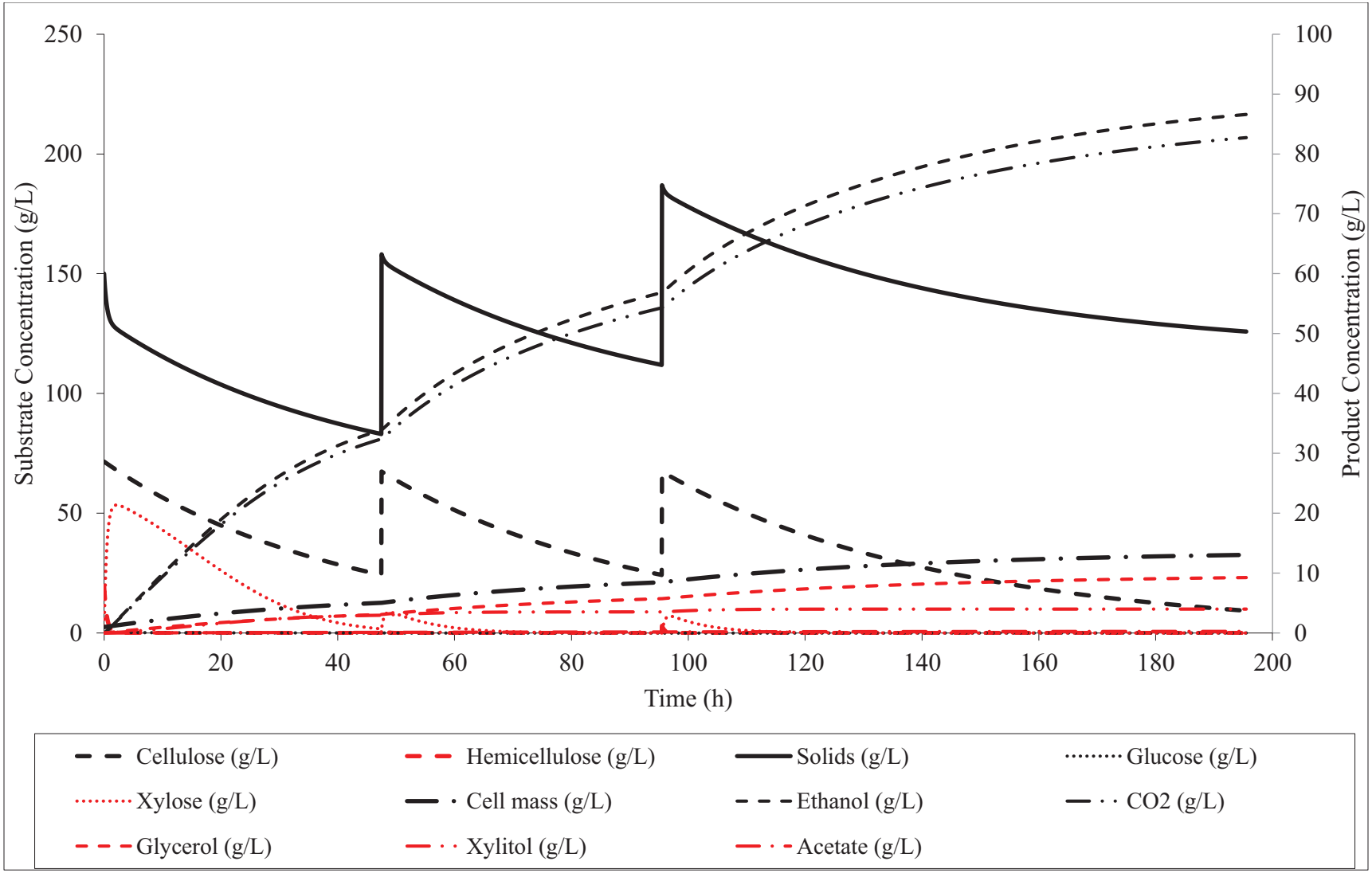

Figure 6-9 Model simulation for corn stover, fed-batch.

Left axis: Solids, Cellulose and Hemicellulose; Right axis: all others.

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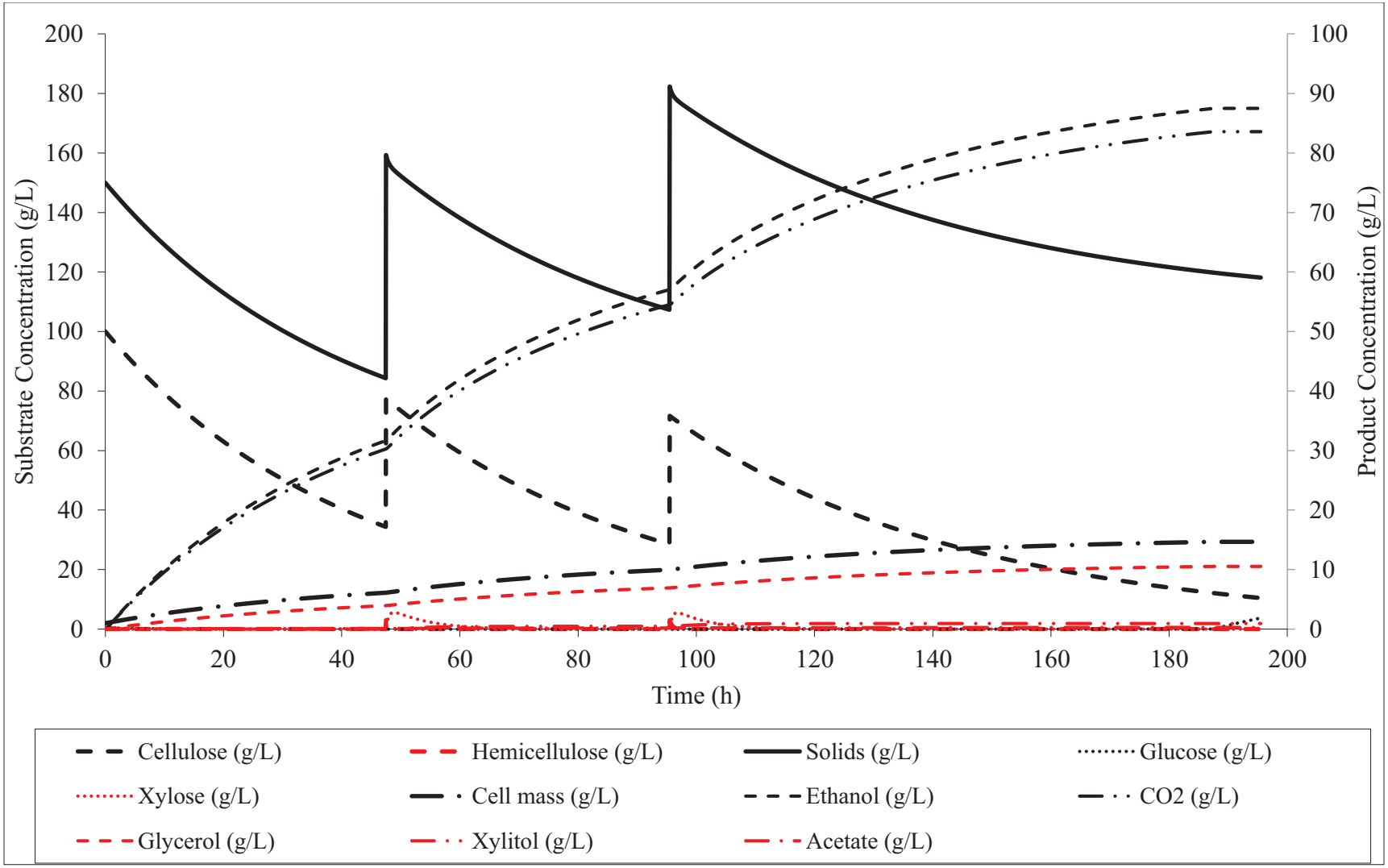

Figure 6-10 Model simulation for corn stover, fed-batch.

Left axis: Solids, Cellulose and Hemicellulose; Right axis: all others.

6.3.3 Model discussion

This model introduces the hydrolysis of hemicellulose into the production of bioethanol modelling. However, it underestimates the impact of xylose and xylo-oligomers based on the high amounts of xylanase used. The model, also, overestimates ethanol production rate from xylose, as the consumption is slightly faster than the xylose generation. Also, the model considers that all hemicellulose is converted to xylose. The total ethanol produced from xylose should be accurate since all xylose is consumed. It underestimates the impact of acetic acid on ethanol production as it is only factored on cell growth. However, the positive impact of low concentration of acetic acid is also missing.

The model uses lumped constants for hydrolysis rather than individual constants for every protein involved. While counterintuitive, this allows the model to account for the variable composition of enzyme preparations, reproducing more accurately their rates. Accessory enzymes have significant influence on the conversion of biomass, that cannot be directly correlated to the standardized tests (Florencio et al. 2016a, 2016b).

Accurate constants at anaerobic conditions are still necessary to define an accurate time course. The aerobic constants available have air time dependent deactivation lumped into them, leading to a cascade of inaccuracy through the time course. However, the model does project final time points accurately.

6.4 Conclusion

A mathematical model for co-saccharification and co-fermentation was developed. The model combined and adapted existing and validated models. The parameters adequacy for hydrolysis was evaluated, and the model includes only the most relevant factors. The model describes the reaction progress at ideal conditions (anaerobic and at 30°C with efficient mixing). Modifications can be done to account to deviations from these conditions. However, these would be more important with separate hydrolysis and fermentation. The higher temperature for hydrolysis (leading to lignin adsorption) and oxygen dependent deactivation lead to lower productivity. Accurate parameters still have to be estimated to account for oxygen deactivation

of cellulases and simultaneous activation of monooxygenases. Besides ethanol, cell growth and other co-products are estimated. The model can be used to evaluate ethanol production in real-time and provide reliable comparison of specific conditions.

CHAPTER 7. CONCLUSIONS

7.1 Conclusions

In this work, a method to hydrolyze lignocellulose to sugar monomers independent of solids loadings was developed. The reaction was carried using dual impellers and under anaerobic conditions. After 72 hours, the conversions were the same at all loadings.

Towards this goal, five factors were identified, and each eliminated. Incremental improvements achieved by addressing each factor add up to achieve higher conversions. The factors are: soluble inhibitors, insoluble inhibitors, product inhibition, mixing and oxygen deactivation. Initial evaluations observed that soluble inhibitors are the most impactful. Unwashed biomass require very large enzyme loadings. The other factors could not be evaluated in their presence. These inhibitors, however, are easily removed with washing. This extra step is so impactful that it must be included as an assumed part of the pretreatment.

In Chapter 4, insoluble inhibitors were evaluated using sugarcane bagasse. It was demonstrated that lignin adsorption can be modulated by changing hydrolysis temperature, pretreatment severity and additional of non-catalytic protein. Sugarcane bagasse is less recalcitrant than wood, requiring lower pretreatment severities. The use of biomass pretreated with lower severity for hydrolysis, limited the impact of lignin blocking with BSA treatment. This effect is caused by lignin not redepositing on the fibers under lower conditions. Redeposition exposes lignin and this larger exposed area adsorbs more enzymes. Avoiding redeposition leads to less deactivation and lower impact of lignin blocking. Lower severities, however, may not be usable on all biomasses. More recalcitrant lignocelluloses would not be hydrolysable with a bland pretreatment. The adequacy of protein blockers depends on the biomass and pretreatment used. Unless an extremely cheap blocker is identified, it is not reasonable to include one on sugarcane bagasse hydrolysis. The same limited impact of lignin blocking is observed with corn stover (Chapter 5), which requires an even less severe pretreatment. Additionally, hydrolysis temperature changed the adsorption capacity and affinity of lignin. When incubate at 30°C, the adsorption was negligible. This effect is another

advantage of a simultaneous saccharification and fermentation of lignocellulose. However, at lower temperatures reaction rates are lower and require longer hydrolysis reactions.

Chapter 5 evaluated solutions to the more elusive mixing factor. The reasons for the loss of enzyme efficiency at high solids with insufficient mixing were not known. When dual impellers are used at high speed (290 RPM) and torque, glucose conversions increase with solids loadings. This observation is at odds with previous reports and is incongruent to product inhibition. An improved mixing method is likely avoiding local accumulation of products and enzymes and inhibitors not removed with washing.

Despite improving enzyme efficiency at high solids loadings, efficient mixing is not capable of making cellulose conversion independent of it. It reversed the pattern, with the low solids conditions having lower conversion. This observation indicated that another factor is present. Deactivation due to air interaction was identified as the possible explanation after deactivation caused by impeller shear was discarded by reproducing the results in shaker flask with higher enzyme loadings. When oxygen is eliminated through nitrogen purge, the conversions increase in all conditions and became independent of solids loadings.

While the impeller configuration is scalable, important questions on the power required to achieve efficient mixing at larger scales still need to be clarified. The power and torque used here are high and focused only achieving efficient mixing. At a larger scale the amount of power may be too large, leading to a process that is too costly or less efficient due to water vaporization and cavitation of the slurry. Further evaluation of the scale-up will determine whether the parameters described here are feasible for industrial application. The conversions observed in this work are relatively low (55% when independent of solids). This is caused by the enzyme preparation used. Cellic CTEC2 does not have the highest specific activity of commercial enzymes and that lead to lower conversion with low enzyme loadings. A more developed enzyme preparation (Cellic CTEC3) is 50% more efficient than the one used here, and it should lead to conversions close to 90%. All the bioprocessing improvements should affect this other enzyme in the same way, based on similar results reported with this preparation.

In Chapter 6, the hypothesis that product inhibition is linked to the mixing effect was evaluated through modelling. The model identified that product inhibition is present under

inefficient mixing but did not explain all efficiency losses at high solids. When a time dependent deactivation was included the losses were better explained. The deactivation was also linked to the dissolved oxygen available during the reaction. The dissolved oxygen is related to solids loading and type of substrate used. It appears that more oxygen is available during the hydrolysis of low solids. This difference is less pronounced when pure cellulose is used. When the constants were adjusted to accommodate oxygen deactivation, product inhibition was smaller than expected. The estimated inhibition constant is a lumped estimation of all factors and splitting a factor should lower it. The reaction constant was also smaller, presumably being originally overestimated due to the model being inaccurate. The simulations can guide future research by demonstrating the gaps in knowledge and the opportunities for improvement of the process.

A final model combining all factor was developed to incorporate all solutions and estimate a simultaneous saccharification and fermentation process. The model predicts that if all negative factors are removed, the ethanol product is close to the theoretical maximum.

The challenge of evaluating the five factors is that they are related and, in many cases, cannot be isolated. Soluble inhibitors are the most important and prevent the others of being studied. Next is oxygen deactivation that has been present in virtually all studies and is likely a source of errors on all other observations. Product inhibition and mixing appear to be more closely related than previously thought. This is likely due to the presence of unwashed soluble inhibitors on previous tests made with lignocellulose and the lack of efficient mixing (if any) on ideal test of enzyme activities. In this work, solving the mixing problems meant eliminating product inhibition. By doing so, we exposed oxygen related deactivation that impacted the conversions at all loadings.

Insoluble inhibition, on the other hand, was demonstrated to be a factor of lignocellulose composition and origin and pretreatment used. This allowed for it to be ignored when evaluating the other factors. That does not mean that this lignin deactivation is irrelevant. Its impact can be modulated before hydrolysis by fine tuning pretreatment processes.

Achieving glucose conversion independent of solids loadings require that all factors being addressed. The solutions include bioreactor design - dual impeller mixing (mixing and product

inhibition), lower air-liquid interface (oxygen deactivation); pretreatment adjustments - washing (soluble inhibitors), lower severity (lignin deactivation); bioprocessing parameter adjustment - anaerobic conditions (oxygen deactivation), simultaneous saccharification and fermentation (product inhibition), lower temperatures (lignin deactivation); and chemical additives - lignin blockers (lignin deactivation), catalase (oxygen deactivation). Selecting the solutions will depend on biomass used and enzyme preparation available.

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