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Sugarcane bagasse hydrolysis enhancement using BSA

Antonio Carlos Freitas dos Santos
Purdue University

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Michael R. Ladisch
Chair

Nathan S. Mosier

Eduardo A. Ximenes

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SUGARCANE BAGASSE HYDROLYSIS ENHANCEMENT USING BSA

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Antonio Carlos Freitas dos Santos

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For my parents

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance.

BSA: Bovine serum albumin.

CMC: Carboxymethylcellulose.

DM: Dry matter.

FPU: Filter paper units.

HPLC: High performance liquid chromatography.

LHW: Liquid hot water pretreatment.

RPM: Revolutions per minute.

SCB: Sugarcane bagasse.

ABSTRACT

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Lignocellulose is composed of polysaccharides linked to lignin and other aromatic compounds, making the sugars not readily available to fermentation. This entails that biomass must go through the unit operations of pretreatment and enzyme hydrolysis. Pretreatment opens the structure to allow the enzymes to act on and hydrolyze cellulose and hemicellulose to glucose and/or xylose which in turn are fermented to ethanol. Concomitantly, the enzymes interact with soluble phenols and insoluble solids derived from lignin that inhibit hydrolysis. This leads to high enzyme loadings and higher production costs. Soluble phenols can be eliminated through washing. Insoluble lignin, however, demands another approach. Using bovine serum albumin (BSA) and pretreated sugarcane bagasse, the effect of blocking solid lignin from adsorbing enzymes during hydrolysis after 72 hours was evaluated. Hydrolysis was carried using 6.25 FPU (Cellulase 13P) and 12.5 IU (Novozyme 188) /g solids (10 mg protein/g solids) at pH 4.8 and 50°C. The conversion was generally higher when BSA was present, 51% (±1%) vs 42% (±1%) with 1.5% solids loading and 46% (±1%) vs 40% (±1%) with 8% solids loading. The use of BSA produced an increase in the final conversion (p-value < 0.001), but conversion decreased as loadings increased. This has been observed in multiple other

studies and cannot be explained by a single factor. The basis of this phenomenon is being investigated. Initial experiments that adjusted the enzyme preparation to 2.5 FPU/g glucan (10.5 mg protein/g glucan) of Cellic CTEC3 and improved the mixing of the slurry elevated the conversion to 72% (±5%) without BSA and 76% (±10%) with it, at 1% (w/v) solids loading and 73% (±6%) without BSA and 80% (±10%) with it, at 10% (w/v) solids loading.

CHAPTER 1. INTRODUCTION

1.1 Ethanol Production from Biomass

The need for a sustainable, economically viable and local alternative for petroleum based fuels and chemicals has been recognized since the 1970s when two Oil Crisis (1973 and 1979) drove the oil prices to record values.

Biomass was identified as the main source of such *(Ladisch et al., 1979)*. Ethanol is produced by fermenting the sugars that constitute biomass. Due to technological limitations and large supply, sugarcane (Brazil) and corn (U.S.) were used as the source of fermentable sugars. However, cellulosic parts of these crops (sugarcane bagasse and corn stover) were ignored, as the yields to convert them into fermentable sugars are low, making them not cost-effective. The same occurred with other materials, such as grasses, wood, agricultural residuals *(Ximenes et al., 2013, Wyman, 1999).*

Boosted by governmental programs that include tax breaks, investments and mandated ethanol-gasoline blends, ethanol demand rose constantly. In Brazil, the Brazil National Alcohol Program instituted in 1975 lead to the creation and expansion of the biofuel industry. In 1984, 94% of the passenger cars were fueled by ethanol. By 1992, 50% of the country's fleet ran exclusively on ethanol. However, falling oil prices, supply shortages and other political factors lead to the program gradual dismissal *(Hollanda and Poole,2001, Soccol et al., 2010).*

In 2003, the demand rose again. Internally, flex fuel cars that can run in any blend of gasoline and ethanol were introduced. Internationally, an export market was created.

These increases shone a new light in the production limits for sugarcane, and for the first time in more than 20 years Brazil imported ethanol from the United States.

According to the Renewable Fuels Association, the United States is the biggest producer of ethanol in the world, producing 14.7 billion gallons in 2015), Brazil is second with 7.1 billion gallons *(RFA, 2016)*.

Despite the fact that these are record-breaking numbers, oil-based fuels still are dominant. To effectively substitute oil, production and productivity have to grow. The use of cellulosic biomass is a viable option as it allows for higher production in the same planted area (using corn or sugarcane) or high productivity in dedicated crops, including in areas with low agricultural development.

1.2 Challenges to Economic Viability

Ethanol production is a sequence of integrated steps (Figure 1-1) *(US DOE, 2007)*. They are: biomass growth, harvest and transportation to the biorefinery; pretreatment; enzymatic hydrolysis; fermentation; and distillation/purification and distribution.

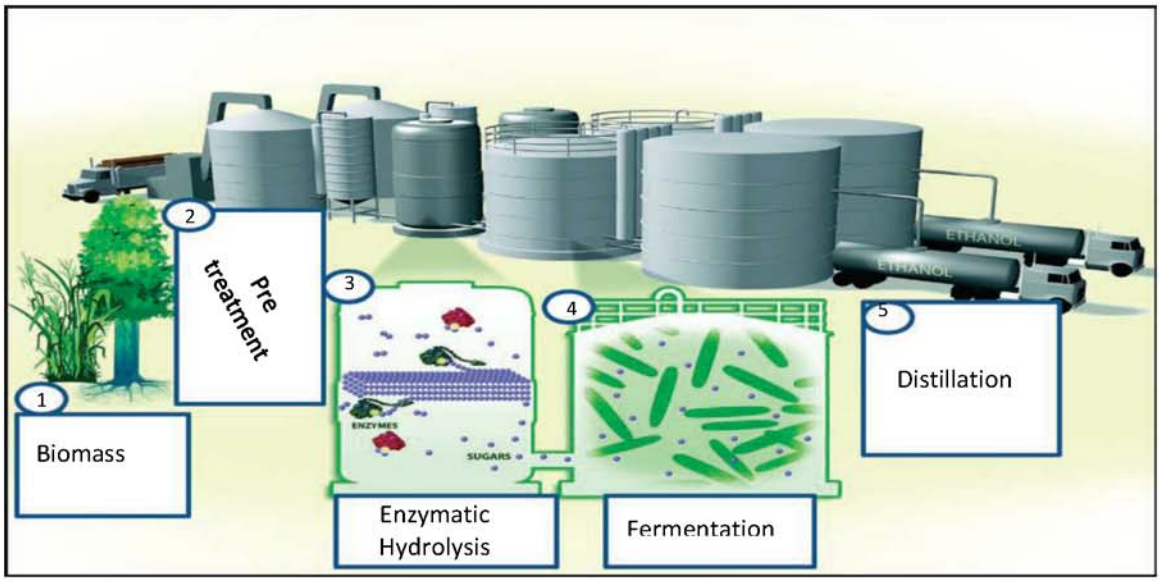

Figure 1-1 Biomass conversion to ethanol general process. From Ximenes et al., 2013, adapted from US DOE, 2007.

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 *(Mosier et al., 2005, Wyman, 1999, Lynd et al., 1999).*

1.3 Lignin and Protein Interactions

After pretreatment, the individual components of biomass are exposed and/or eliminated. This allows the enzymes to act on cellulose and hemicellulose, hydrolyzing and generating fermentable monomers. Concomitantly, lignin is also affected. Its relation

changes in two ways. Part of it is degraded, becoming soluble phenolic compounds and the rest is exposed. The enzymes interact with both phases, being inhibited by both *(Ximenes et al., 2010, Ximenes et al., 2011, Sammond et al., 2014).*

This leads to a need for a higher amount of enzymes on a given process, which raises considerably the production costs *(Kim et al., 2005, Ladisch et al., 1983).*

The mechanisms of enzyme adsorption onto solid lignin have not been elucidated. 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 different rates. The only factor that appears to be universally accepted is hydrophobicity. Other factors such as pore size, surface area area have not been proven to be associated to adsorption *(Sammond et al., 2014, Gao et al., 2014, Guo et al., 2014, Parrek et al., 2013, Ko et al., 2015a).*

These factors indicate that lignin composition, enzyme structure, pretreatment type and severity all contribute to some extent to enzyme inhibition. Limiting the gains obtained in isolated changes, e.g. modifying pretreatment severity.

1.4 Addition of Competitive Protein

A more 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) *(Kim et al., 2015, Ko et al., 2015a, Ko et al., 2015b, Eriksson et al., 2002, Kumar et al., 2012, Kristensen et al., 2007, Yang and Wyman, 2006).* These studies

were carried using spruce, corn stover, hardwood, softwood and wheat straw. Generally, they agree that BSA blocks lignin interaction and leads to higher productivity from the enzymes.

BSA, however, is expensive. Becoming impractical, especially using high purity BSA and high concentration as those used in these studies. To choose another, more feasible blocker, the characteristics of BSA-lignin interaction must be studied, so that, a cheaper alternative can be identified.

1.5 Basis of Experiment

Sugarcane bagasse (SCB) was acquired from a processing plant in Brazil through Universidade Estadual Paulista (São José do Rio Preto). The biomass was then characterized and pretreated using liquid hot water (LHW). The initial parameters (pH, temperature, time, RPM, loading rates) for the adsorption and hydrolysis assays were based on previous studies.

Individual protein adsorption kinetics were identified early and used to guide sample timings, loadings, pH and loading sequencing. In a second stage, BSA and the enzymes were combined to evaluate their influence on each other and on the final glucose concentrations as solids were increased.

CHAPTER 2. LITERATURE REVIEW

2.1 Approach

The production of ethanol from lignocellulose and cellulolytic enzyme action has a significant literature associate with it. Lignin interaction with cellulolytic and related enzymes was identified as the focus of the review and research. Within this area the capabilities of BSA to adsorb on lignin and block adsorption of enzyme proteins were further investigated.

2.2 Lignocellulose

Lignocellulose, also referred as biomass, is a complex structure composed of three major organic components: cellulose, hemicellulose and lignin. Biomass also contains small amounts of minerals and various extractives *(Wyman, 1999)*. 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)*.

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 non-cellulosic polysaccharides of wood, including the related substances, such as uronic acids and their substituents. 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 is a branched polymer 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 & Gibeuat, 1993)*.

Hydrogen bonds are an important characteristic of lignocellulosic materials. 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 gaps of this structure, giving the mature cell wall rigidity and water-impermeability *(Karkonen & 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. The most

successful process so far is the use of a pretreatment (Section 2.5) and a subsequent enzymatic saccharification (Section 2.6).

2.3 Sugarcane Bagasse

Sugarcane is any of 6 to 37 species (depending on taxonomic interpretation) of tall perennial grasses of the genus Saccharum (family Poaceae, tribe Andropogoneae). Sugarcane is native of warm temperate climate, common in the tropical regions as Brazil, India, Africa, and Asia pacific (*Canilha et al., 2012).* The plant is divided into stem (central) and straw (leaves and tops). In a classical setting, the sugarcane stem is the material of interest. It is separated from the straw and milled to obtain a juice which is subsequently used for sugar (sucrose) or alcohol (ethanol) production. Sugarcane bagasse (SCB) is the material left after the extraction. It also has a high yield (approximately 80 t/ha) *(Pandey et al., 2000).* The Brazilian annual production of sugarcane bagasse is currently estimated at 186 million tons of bagasse on a wet basis *(Soccol et al., 2010).* Sugarcane presents a great morphological heterogeneity and consists of fiber bundles and other structural elements such as vessels, parenchyma, and epithelial cells *(Sanjuan et al., 2001).* SCB and sugarcane straw have roughly the same composition in terms of major components. The most important differences among the straw and bagasse components are the much higher potassium content in the straw, mainly in the tops. Potassium could cause deposits on the hot surfaces, corrosion and slagging of the ashes *(Leal et al., 2013).* SCB and sugarcane straw have limited applications, due to low productivity, lack of technological advancements, unprofitable alternatives and outdated legislation *(Janke et al., 2015; Canilha et al., 2012).* SCB is mainly used are as fuel for boilers in the sugar-

industry while open-field burning is still the destination of the straw. New legislations are being imposed to ban this practice due to the health and environmental issues *(Pandey et al., 2000; Paraiso & Gouveia, 2015; Leal et al., 2013).*

SCB is preferred for now as the use of sugarcane straw would require innovations in collection, storage and transportation, a similar process to what happened in the US for corn stover, while SCB is already utilized in the plants *(Leal et al., 2013).* SCB and sugarcane straw are lignocellulose biomass. As such they are composed mainly by cellulose, hemicellulose, and lignin (Table 2.1). In Table 2.2 more general characteristics of sugarcane straw and SCB are listed. Table 2.3 lists the projected crop yields between 2014/15 and 2024/25 *(IBGE, 2015)* and actual crop yield for 2014/15 and 2015/16 *(UNICA, 2015, 2016),* all weights listed are on a wet basis. Sugarcane bagasse and straw productions are based on the following proportions: every ton of sugarcane yields approximately, 0.28 tons of sugarcane bagasse *(Hofsetz & Silva, 2010)* and 0.14 tons of sugarcane straw *(Dias et al., 2012).* The crop yields are projected to increase 30.8% in cultivated area and 3.1% per year in production of sugar between 2014/2015 and 2024/25. These projections also consider that the proportion if sugarcane used for sugar (70.4%) and ethanol production (29.6%) will be maintained. Productivity is also expected to increase from 68.6 tons per hectare (2014/15) to 74.3 tons per hectare (2024/25) *(IBGE, 2015).*

Table 2-1 Sugarcane bagasse or straw composition in % of dry weight.

Component (%)	Reference									
	Da Silva et al., 2010		Canilha et al., 2011	Rocha et al., 2011	Brienzo et al., 2009	Rabelo et al., 2011	Templeton et al., 2010	Kim & Day, 2011	Ladisch et al., 2013	Pratto et al., 2016
Biomass	SCB	Straw	SCB	SCB	SCB	SCB	SCB	SCB	SCB	Straw
Cellulose	38.8	33.6	45.0	45.5	42.4	38.4	39.0	41.6	39	35
Hemicellulose	26.0	28.9	25.8	27.0	25.2	23.2	21.8	25.1	27.7	24
Lignin	32.4	31.8	19.1	21.1	19.6	25.0	24.8	20.3	24.8	20
Ash	2.8	5.7	1.0	2.2	1.6	1.5	3.9	4.8	3.9	8.7
Extractives	-	-	9.1	4.6	-	-	5.7	-	5.7	-
Total	100	100	100	100.4	88.8	88.1	95.2	91.8	101.1	87.7

Table 2-2 Comparison between Sugarcane bagasse and sugarcane straw in % of dry weight (Hassuani et al., 2005)

	Dry leaves	Green leaves	Tops	Bagasse
Moisture content	13.5	67.7	82.3	50.2
Ash	3.9	3.7	4.3	2.2
Fixed carbon	11.6	15.	16.4	18.0
Volatile matter	84.5	80.6	79.3	79.9

Table 2-3 Sugarcane crop yields as is per year

Crop	Total Sugarcane (10^3 tons)	Sugar (10^3 tons)	Sugarcane bagasse (10^3 tons)[a]	Sugarcane straw (10^3 tons)[b]
2014/15[1]	617,520	34,406	172,906	86,453
2014/15[2]	633,927	35,571	177,500	88,750
2015/16[1]	663,845	36,486	185,877	92,938
2015/16[3]	666,824	33,837	186,711	93,355
2016/17[1]	684,185	37,523	191,572	95,786
2017/18[1]	699,177	38,756	195,770	97,885
2018/19[1]	723,585	39,953	202,604	101,302
2019/20[1]	739,866	41,156	207,162	103,581
2020/21[1]	761,405	42,358	213,193	106,597
2021/22[1]	779,931	43,560	218,381	109,190
2022/23[1]	800,009	44,762	224,003	112,001
2023/24[1]	819,357	45,964	229,420	114,710
2024/25[1]	839,014	47,166	234,924	117,462

[a] 0.28 tons per ton of sugarcane. [b] 0.14 tons per ton of sugarcane. [1] IBGE, 2015. [2] UNICA2015. [3] UNICA, 2016.

2.4 Lignin Composition

2.4.1 Overall chemistry

Lignin is a main component of wood cell wall *(Rydholm, 1965)*. It is a phenolic polymer mainly composed by three hydroxycinnamyl alcohols ρ-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol (Figure 2-1). Lignin provides rigidity and structural support to cell wall polysaccharides and makes the cell walls water-impermeable. In addition to developmental lignification, lignin synthesis can be induced after wounding or pathogen attack as a defense response *(Kärkönen & Koutaniemi, 2010)*.

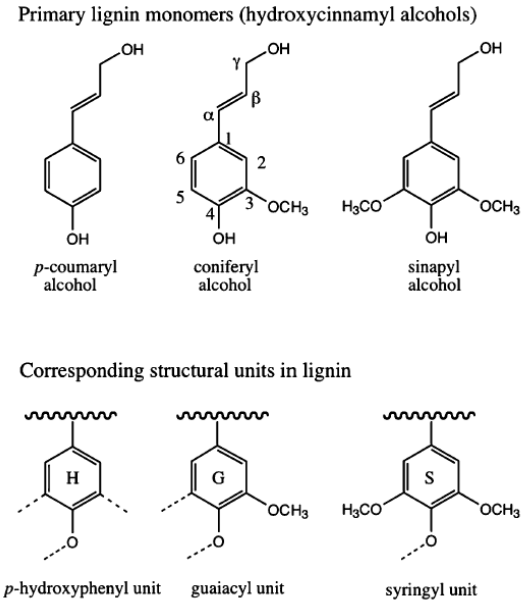

Figure 2-1 Primary lignin monomers and corresponding lignin units. From Wong (2009).

Lignin monomers are synthetized through the phenylpropainoid pathway, starting from phenylalanine ammonia-lyase (PAL) and subsequent series of hydroxylations, methylations and reductions (Figure 2-2). They differ on the number of methoxy substituents present on its aromatic portion.

The three monomers share part of the pathway, the conversion of L-phenylalanine to 4-coumaroyl-CoA, at this point it splits in two directions. The first involves cinnamoyl-CoA reducatase (CCR) and cinnamyl alcohol dehydrogenase (CAD) and ends in ρ-coumaryl alcohol, which becomes H-lignin. The second involves a sequence of enzymes to introduce a second methoxy substituent in the aromatic ring generating Feruloyl-CoA.

After that CCR reduces Feruloyl-CoA to coniferyl aldehyde. At this point the pathway splits again. Coniferyl alcohol (G) is synthetized by CAD reducing coniferyl aldehyde directly. Sinapyl alcohol (S), on the other hand undergoes two extra reactions with ferulate/coniferaldehyde 5-hydroxylase (F5H) and caffeate/5-hydroxyconiferaldehyde O-methyltransferase (COMT) to introduce the third methoxy substituent. CAD subsequently reduces it to its final form *(Kärkönen & Koutaniemi, 2010; Boerjan et al., 2003).*

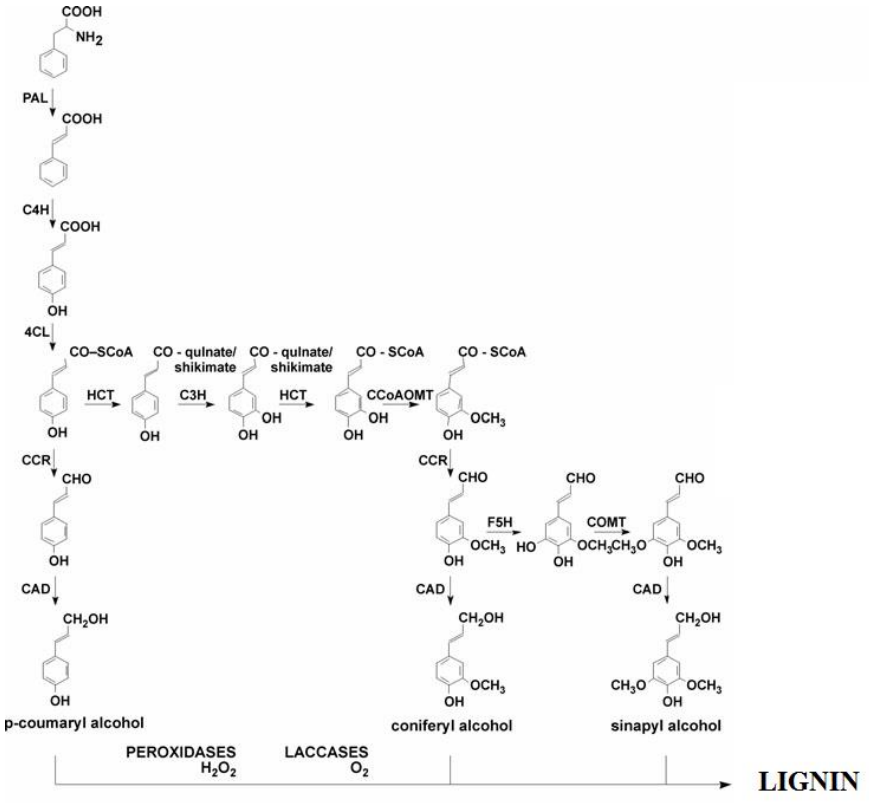

Figure 2-2 Schematic lignin synthesis pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate:CoA ligase; HCT, hydroxycinnamoyl:CoA transferase; C3H, 5-O-(4-coumaroyl)shikimate 3'-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyl transferase; CCR, cinnamoyl-CoA reducatase; CAD, cinnamyl alcohol dehydrogenase, F5H, ferulate/coniferaldehyde 5- hydroxylase; COMT, caffeate/5-hydroxyconiferaldehyde O-methyltransferase. Adapted from Kärkönen and Koutaniemi, 2010.

After secretion into the apoplast, the monolignols are activated to radicals, and are condensed through a series of radical reactions catalyzed by peroxidases and laccases, leading to three different types of lignin (ρ-hydroxyphenyl (H), guaiacyl (G), syringil (S), respectively) *(Kärkönen & Koutaniemi, 2010; Freuderberg, 1959)*. Due to resonance of the radicals, multiple types of interactions are possible between the precursors (Figure 2-3) *(Wong, 2009)*. The most common bond is β-O-4 involving from 50 to 80% of the phenylpropanoid units *(Alder, 1977)*.

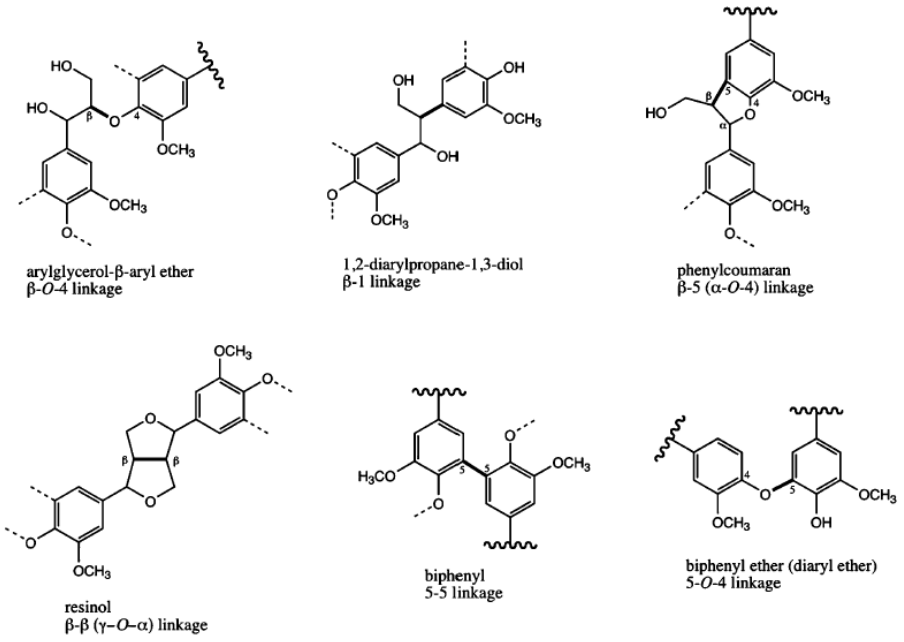

Figure 2-3 Major structural units derived from cross-coupling of monomers. From Wong (2009).

2.5 Pretreatment

Pretreatment is an essential step of the conversion of lignocellulose to ethanol. Specific processes, general considerations and economic evaluations have been

extensively reviewed elsewhere *(Mosier et al., 2005; National Research Council (US), 2000).*

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 2-4) *(Mosier et al., 2005).*

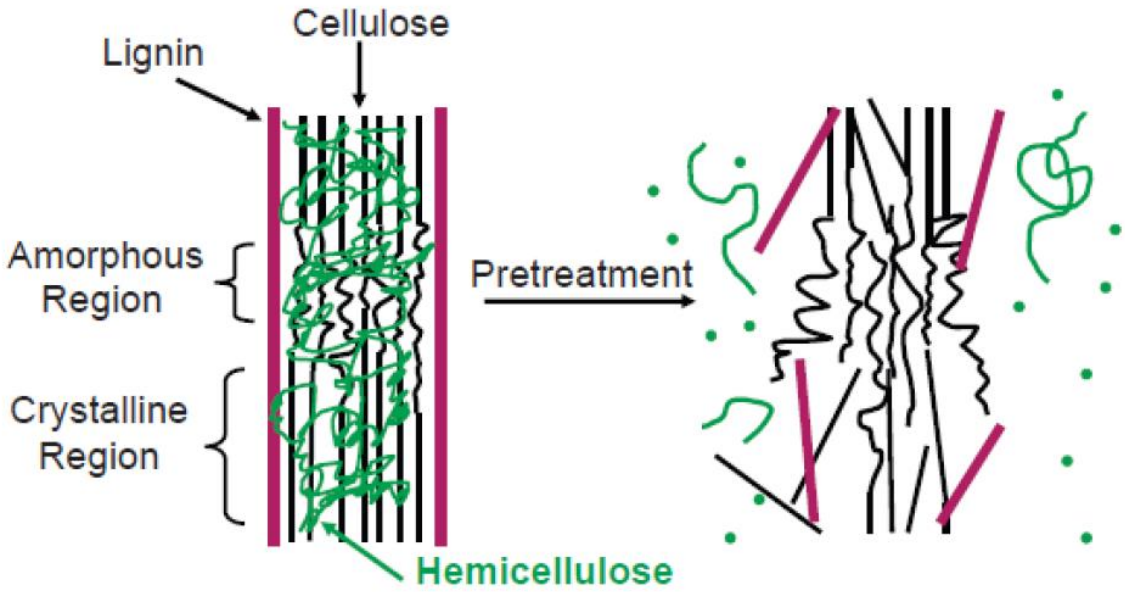

Figure 2-4 Schematic of goals of pretreatment on lignocellulosic material. From Mosier et al., 2005.

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 2-5). Pretreatment has a wide range of effects on the components of lignocellulose depending on the process utilized and various parameters *(Mosier et al., 2005)*

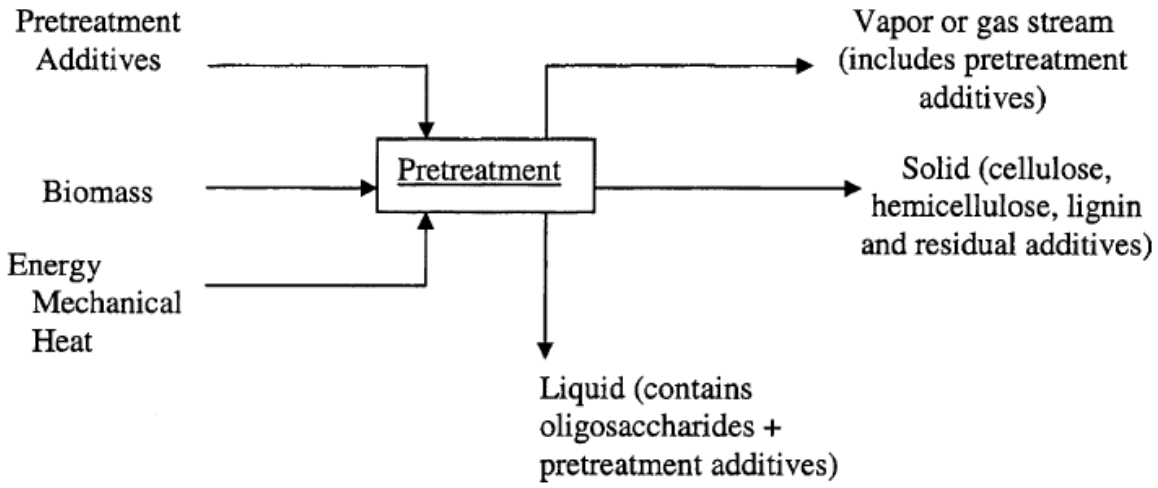

Figure 2-5 Pretreatment process schematic representation. From Mosier et al., 2005.

2.5.1 Liquid hot water pretreatment

Liquid hot water (LHW) pretreatment utilizes water, high pressure and temperatures to hydrolyze ether and hemiacetal bonds. The process consists of maintaining the biomass slurry at a temperature > 140ºC for a determined period, generally 10 to 30 minutes *(Kim et al., 2015; Mosier et al., 2005).*

It has dramatic effect on hemicellulose converting most of it to monomers. However, it has limited effect on removing lignin, although soluble phenolic compounds and solid lignin are generated *(Kim et al., 2015; Mosier et al., 2005).* These compounds are inhibitory for the enzymatic process that follows and fermentation *(Ko et al., 2015b; Ximenes et al., 2011, 2010).*

Solutions for the soluble inhibitors (also including inhibitory sugars) have been researched. Kim et al. (2009) showed that washing pretreated poplar with water at 80–90 °C increases glucose yield upon enzyme hydrolysis by 20% compared to solids

washed with water at 25°C. However, the solid lignin portion is not removed and its inhibitory effect is still present as will be discussed on Section 2.7.

An important feature of LHW is the pretreatment severity. Based on the work of Overend and Chornet (1987), the pretreatment condition is expressed as a severity factor, log R_0 (R_0 = t X exp ((T–100)/ω))) where ω represents an activation energy for pretreatment. Overend and Chornet (1987) identified ω = 14.75. While, this value does not fit empirical data for SCB or hardwood a general correlation may be obtained if the curve is fitted to the data, and appropriate constants determined. Kim et al. (2013) identified an empirical parameter value of ω = 4.6 that accurately correlates the severity factor with pretreatment responses (i.e. cellulose hydrolysis) for hardwood.

2.6 Enzymatic Saccharification

Numerous processes for the saccharification of cellulose have been studied. The enzymatic process has been identified as the most cost-effective *(Wyman, 1999; Mosier et al., 2005).* 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 *(Ladish et al., 1983).*

They 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 2-6. 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 *(Ladish et al., 1983).*

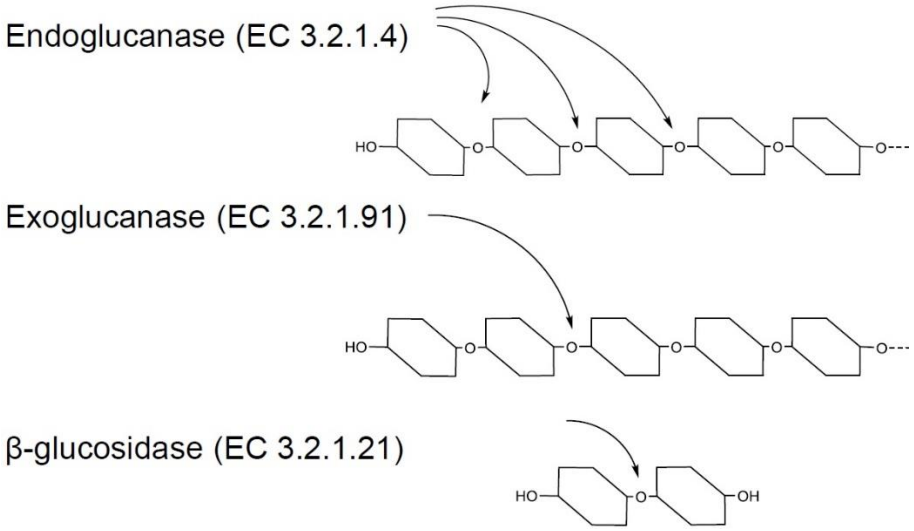

Figure 2-6 Schematic representation of cellulases mechanism.

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,b).*

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 *(Kumar & 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).*

2.7 Inhibition of cellulolytic enzymes

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

Phenols appear to be the strongest inhibitors of enzyme production by different microorganisms as well as the enzyme activities themselves *(Vohra et al., 1980; Martin & Akin, 1988; Paul et al., 2003; Sineiro et al., 1997).* Phenolic compounds can both inhibit and deactivate the enzymes. Although all cellulases and hemicellulases are affected, β-glucosidases are the most sensitive *(Ximenes et al., 2011; Ximenes et al., 2010).* The microorganism that produces it also changes the inhibition, inhibition of enzyme from *A. niger* requires 4× higher concentrations than β-glucosidase from *T.*

reesei. Most of that difference is due to a less susceptible β-glucosidase from *A. niger (Ximenes et al., 2011; Ximenes et al., 2010)*. Polymeric phenols such as tannic acid are more inhibitory than monomeric forms, reducing conversion of cellulose to sugars in up to 60%. Tannic, ferulic and ρ-coumaric acids inactivated both *T. reesei* and *A. niger* β-glucosidases. While inhibition occurs almost immediately, continuing loss of enzyme activity is caused by time-dependent deactivation of endo-, exo- and β-glucosidase enzymes by phenols generated by pretreatment, as described in the discussion section of this thesis *(Ximenes et al., 2011)*.

Xylo-oligomers and to a lesser extent xylose monomers also cause major inhibition. This is explains the presence the hemicellulases in the fungi biochemistry and commercial enzyme preparations *(Ximenes et al., 2007)*. Soluble xylose sugars, both in oligomeric and monomeric forms, inhibit cellulases instantaneously, an effect that predominates over other inhibitors in decreasing the initial hydrolysis rate of cellulose to glucose *(Kim et al., 2011)*.

2.8 Solid lignin/Enzyme interaction

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 *(Sammond et al., 2014; Gao et al., 2014; Guo et al., 2015)*. 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 *(Sammond et al., 2014; Gao et al., 2014; Guo et al., 2015; Pareek et al., 2013).*

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 SO_2-steam pretreated Douglas-fir in different temperatures (190, 200 and 210 °C). At higher severity, higher adsorption was measured, mainly due to changes on the surface of lignin. Guo et al. (2014) analyzed the adsorption of proteins onto lignin of different plants also identified were differences in the composition of lignin that are related to cellulase adsorption, the higher the G/S ratio, the higher the affinity. 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. Utilizing BSA ability as a lignin surface blocker, Ko et al. (2015b) effectively reversed the lignin adsorption inhibition as shown on Figure 2-7.

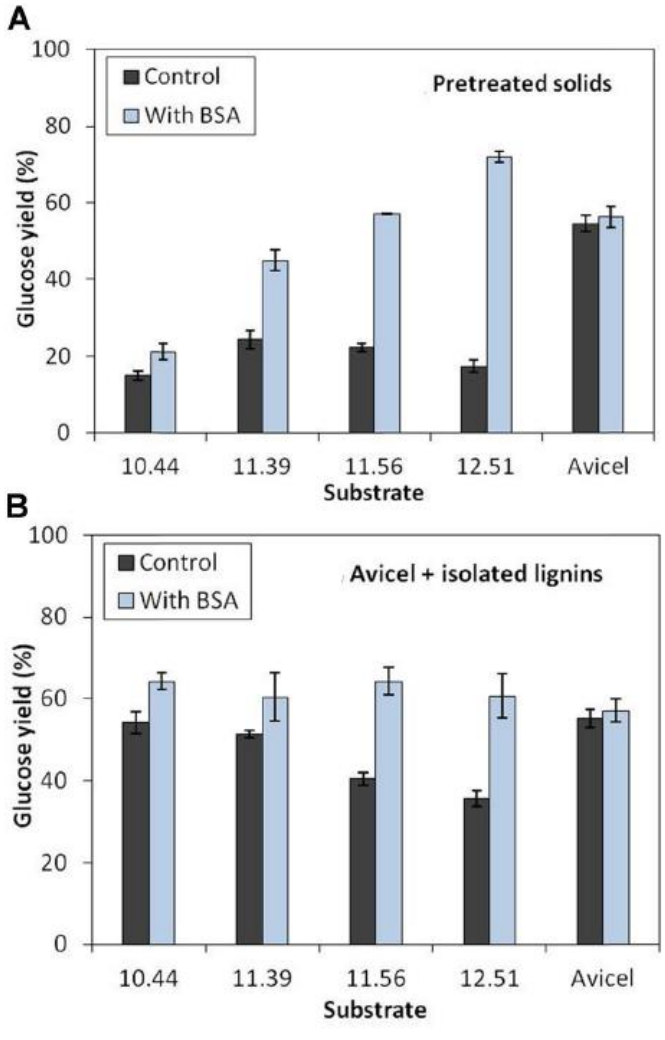

Figure 2-7 Enzymatic hydrolysis of liquid hot water pretreated solids with and without BSA. Hydrolysis conditions: Pretreated solids of severity factor of log R0 = 10.44, 11.39, 11.56 and 12.51 vs. Avicel (A) and Avicel in the presence of isolated lignins (B) were pre-incubated with 50 mg BSA/g dry solid 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.

2.9 Lignin composition and hydrolysis

The lignin composition has been negatively correlated with the enzymatic hydrolysis of biomass cellulose portions. While the amount of lignin impacts the final conversion, the distribution of H, G and S lignin are also important. Although the G/S ratio correlates positively with the final conversion, when H-lignin is high, the G/S ratio is not enough to explain the glucose conversion *(Chen & Dixon, 2007)*. This factor indicates the reason of why different pretreatments generate different adsorption coefficients. Hydrothermal pretreatment alters lignin's structure into more condensed and syringyl deficient forms *(Ko et al., 2015a,b; Nakagame et al., 2011)*.

When G and S-lignins are greatly reduced, and lignin is mainly composed by H-lignin the hydrolysis of biomass is increased both in pretreated and non-pretreated biomass as shown by Bonawitz et al. (2014) and by Van Acker et al. (2013) with genetically modified Arabidopsis.

An analogous effect has been identified in pulping where the lignin from transgenic tobacco plants with higher G/S ratio were more easily removed. In their study, O'Connell et al. (2002) theorized that the higher percentage and presence of free phenolics bound by β-O-4 was explained this observation.

The reasons for this phenomenon are not well known. Davison et al. (2005) identified a higher release of xylose in a Populus with higher G/S ratio when pretreated with diluted acid hydrolysis. Since xylo-oligomers are inhibitory to the cellulases (Kumar et al, 2009), this effect could partially explain this difference.

Vanholme et al. (2012) listed the preferred lignin monomer characteristics for hydrolysis as: monomers that directly produce a readily cleavable functionality in the

lignin polymer; hydrophylic monomers; difunctional monomers and monomer conjugates linked via a readily cleavable functionality; monomers that minimize lignin–polysaccharide cross-linking; monomers that give rise to shorter lignin polymers. In general these monomers should create either more β-O-4 links and/or easier access to internal parts of lignin, making the lignin easier to cleave and eliminate during pretreatment. The main disadvantages of these are the higher generation of soluble phenolic compounds and growth-related problems for the plants.

These studies seem to agree that different lignin composition affects the saccharification, but differ in how and which type of lignin increases and/or decreases the hydrolysis. A probable cause for this disconnection may be the relatively low presence of H-lignin across different species, which makes its contribution hard to quantify and study properly.

2.10 Biomass high solids loading and glucose conversion

For an economically viable lignocellulosic ethanol production, a high biomass loading is necessary *(Wingren et al. 2003; Zacchi & Axelsson, 1989)*. Increasing the initial loading significantly lowers the costs of machinery, energy consumption and labor costs *(Wingren et al. 2003; Zacchi & 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).*

In order to limit and/or counter these issues, many different approaches have been investigated. The most common involves a simultaneous saccharification and fermentation process *(Ramachandriya et al., 2013).* The introduction of the fermenting yeast in a fed-batch mode, reduces the glucose concentration and its inhibitory effect on hydrolysis *(Rudolf et al., 2004; Varga et al., 2004; Geng et al., 2015; Chandra et al., 2011).* However, the enzyme loadings necessary on these processes are still high, 43.5 FPU/g glucan *(Varga et al., 2004)* and 36.5 FPU/g glucan *(Rudolf et al., 2004).*

Geng et al. (2015) achieved 76.4% conversion with 5 FPU/ g-solids, with 15% solids of pretreated corn-stover. However, the main difference from the other studies is the addition of steel balls to improve mixing, which had the biggest impact on the final conversion. Chandra et al. (2011) observed that at 10% solids loading of pretreated corn-stover, a fed-batch strategy is less efficient than batch hydrolysis, especially at low enzyme loadings (5 FPU/ g glucan), with the conversion being only 66%. This difference can be explained by the use of different enzyme preparations and the inclusion of auxiliary enzymes.

Bommarius et al. (2008) suggested that "jamming" occurs when there is a high ratio of cellulase molecules to cellulose molecules. 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.

2.10.1 Mixing

Expanding on this concept, Jørgensen et al. (2007) explored a more efficient mixing pattern, using a five-chambered horizontal reactor (Figure 2-8). The authors used

steam pretreated wheat straw, with concentration up to 40% dry mass. This work showed that the viscosity and biomass accessibility has a 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 2-9).

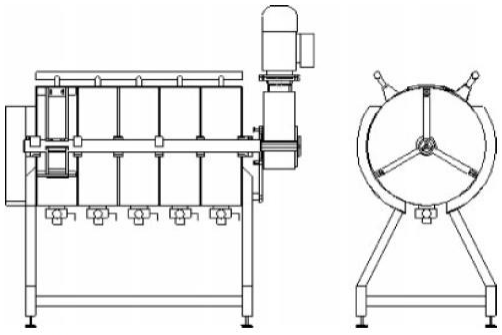

Figure 1. Diagram of the five chamber liquefaction reactor.

Figure 2-8 Diagram of five chamber liquefaction reactor used for high solids liquefaction. From Jørgensen et al. (2007).

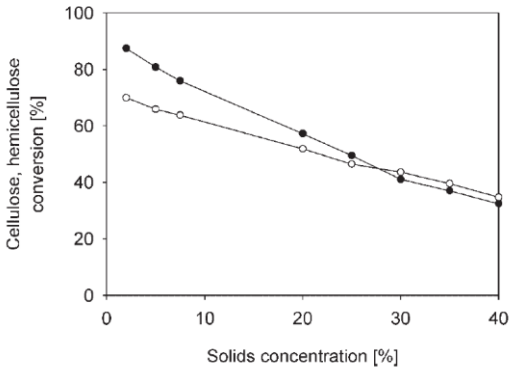

Figure 2-9 Cellulose (•) and hemicellulose (○) conversion after 96 h of liquefaction and sacharification of wheat straw. Experiments at 2%-7.5%(w/w) dry matter were performed in shake flasks. The experiments above 20% dry matter were performed in the liquefaction reactor. From Jørgensen et al. (2007).

Other reactor designs have been explored, Roche et al. (2009) identified roller bottle reactors to be the best for laboratory scale. Peg mixer *(Zhang et al., 2009)*, addition of steel balls *(Geng et al., 2015; Ramachandriya et al., 2013)* helical impellers and Rushton impellers *(Zhang et al., 2010; Palmqvist et al., 2011, 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.

2.10.2 Other factors

The other factors have been less studied. Kristensen et al. (2009) investigated the multiple factors that may lead to lower conversion using filter paper and a fixed enzyme loading of 10 FPU/g dry mass. Product inhibition was evaluated using 50 g/L of glucose.

The inhibition was significant at 5% dry mass loading, but did not account for all the conversion loss at 20% dry mass. When a varying enzyme loading was used, no difference was noted on the relation between biomass loading and conversion. The study concludes that the adsorption of cellulases on cellulose is likely the most important factor on the final conversion.

Based on more recent studies, multiple inhibitors that affect the cellulase-cellulose interaction such soluble phenolic compounds and solid lignin may be the main cause of the lower conversion at high solids *(Ximenes et al., 2011, 2010).* Although the product inhibition from glucose and cellobiose cannot be dismissed, the elimination of these other inhibitors may increase the conversion at higher biomass loadings. Kim et al. (2013) that showed that the soluble phenolic compounds generated after liquid hot water pretreatment can be removed by washing the pretreated biomass with hot water. Ko et al (2015b) then demonstrated that BSA has capacity to block solid lignin and increase glucose conversion. The overall effect generated by high solid loading may be drastically reduced.

2.11 Enzyme loadings and production costs

Wooley et al. (1999) evaluated the industrial production of ethanol from lignocellulosic biomass and identified that the cost could drop by 40 cents per gallon by improving yields and performance of the conversion process. Solomon et al. (2006) projected a cost of 20 cents per gallon due to enzymes alone. That value is lower than the $ 0.50/gal used by Wyman et al. (1999) on which it is based. Davis et al. (2013, 2015) projected an economically feasible process assuming $0.21/gallon gasoline equivalents

which equate to 10 mg protein/g cellulose with a conversion of 90% by 2017 when applied to a 20% (w/v) solids loading. These represent a reduction of 50% of the enzyme loading from the 2012 best conversions: 78-82% with 19mg protein/g glucose. Although the biomasses considered in these studies were not SCB, they provide insight into ranges that these values may be found.

CHAPTER 3. MATERIALS AND METHODS

3.1 Material

3.1.1 Sugarcane bagasse

Sugarcane bagasse was supplied by Usina Alta Mogiana (São Joaquim da Barra, Brazil) through Universidade Estudual Paulista - São Paulo State University – (Rio Preto, Brazil). It was washed to remove all reducing sugars, and then it was dried to lower than 10% humidity at 42 ± 2 ºC. The material was milled and sieved to pass through a sieve size of 1.0 mm.

3.1.2 Other chemicals

Microcrystalline cellulose, Avicel PH101, was purchased from Sigma–Aldrich (St. Louis, MO). Cellulase 13P and DEPOL 692P enzymes were purchased from Biocatalysts Ltd. (Cardiff, Wales). All other chemicals were purchased from Sigma-Aldrich unless noted.

3.2 Methods

3.2.1 Biomass compositional analysis

Raw and pretreated sugarcane bagasse were analyzed following the NREL LAP standard procedures (Sluiter et al., 2008a and Sluiter et al., 2008b).

3.2.2 Liquid Hot Water (LHW) Pretretament

For LHW pretreatment, stainless steel tubes 316 tubes were filled with 6.05g of SCB (moisture content = 4.81% (w/w)) and 32.5 mL of distilled water achieving 15% (w/w) dry solids slurry (Kim et al., 2009b). To minimize heat-up time uncertainty, the tubes were heated to 140ºC for up to one hour then held at 190ºC for 20 min in a Tecam1SBL-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 R_o ($R_o = t \times \exp((T - 100)/\omega)$) (Overend & Chornet, 1987), where the value of ω represents an activation energy associated with the pretreatment (Xu et al., 2011).

3.2.3 Enzyme activities

Enzyme activities were measured using IUPAC method (Ghose, 1987). Filter paper assay determined the total activity of the enzyme preparations. Enzyme optimal pH and temperature were determined in a range of different pHs (3.0 – 6.0) and temperatures (40 – 70 ºC). For Standard Operating Procedures procedure see Appendix.

The endo-glucanase (EG) activity was assayed using 1% carboxymethyl cellulose (CMC) as substrate. 50 µL of the enzyme was added to 200 µL of the substrate and incubated at 50 ºC in a water bath for 15 minutes, the reaction was stopped by the

addition of 1.0 mL of DNS and subsequent boiling for 5 minutes. The final concentration of reducing sugars was determined by spectrophotometry at 540 nm.

The β-glucosidase activity was measured, 10mM of p-nitrophenyl-b-D-glucoside (pNPG) was used as substrate. 100 µL of the enzyme was added to 400 µL of the substrate and incubated at 50 ºC in a water bath for 15 minutes, the reaction was stopped by the addition of 2.0 mL of 1 M Na_2CO_3. The final concentration of released nitrophenols was determined by spectrophotometry at 410 nm.

3.2.4 Soluble protein measurement

Protein concentration in all samples and enzymes was determined using Pierce™ BCA Kit (Thermo-Fisher, Rockford, IL).

3.2.5 Adsorption and enzymatic hydrolysis assays

Both adsorption and enzymatic hydrolysis assays were conducted in an orbital shaker at 200 rpm. Citrate buffer (50 mM, pH 5.0) was used. Final volume was 50 mL. Initial test were done with 1% glucan (w/v). All reactions were run in triplicate. Controls lacking biomass, cellulose, BSA or enzymes were also run in parallel. All samples were immediately frozen and stored at -18ºC.

3.2.5.1 Adsorption assays

BSA adsorption was measured onto raw and pretreated SCB and Avicel under multiple conditions for up to 24 hours. Temperature was set at either 25 or 50ºC. To

evaluate the effect of pH, two citrate buffers were used (pH 5.0 and 6.0). After incubation, the supernatant was separated from the solid fraction by centrifugation at 10,000 rpm for 5 min. Adsorbed protein was determined by subtracting the initial protein added from the remaining soluble protein. Experimental adsorbed protein data was fitted into the following Langmuir equation (Equation 3-1):

Equation 3-1: Langmuir Equation.

$$E_{ads} = \frac{E_{max} \cdot K \cdot E_{free}}{1 + K \cdot E_{free}}$$

Where E_{ads} is the amount of adsorbed enzyme (mg/g lignin), E_{free} the amount of free (non-adsorbed) enzyme in supernatant (mg/mL), E_{max} the maximum amount of adsorbed enzyme (mg/g lignin), and K the Langmuir constant (mL/mg enzyme). The constants were determined by non-linear regression of experimental data using Excel Solver program (Kemmer and Keller, 2010).

3.2.5.2 Enzymatic hydrolysis

Enzymatic hydrolysis was conducted at 50 ºC for up to 72 hours. 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 in a 1.5 mL Eppendorf tube 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.

Samples were also collected to determine protein adsorption and activity loss.

CHAPTER 4. RESULTS

4.1 Cellulase 13P characterization

Cellulase 13P (Biocatalysts Inc.) was characterized in this study as a part of the study of cellulases and their action on sugarcane bagasse. The enzymatic profile at optimum pH (4.8) and temperature (50°C) is described on Table 4.1, compared to the profile of other commercial enzymes as shown by Dien et al. (2008). When the low concentration of the studied solution (2.5% w/w) is accounted for, the profile for Cellulase 13P is similar to the profile of other *Trichoderma sp*. cellulase, besides the much higher xylanase activity that also explains the relatively low FPU/mg protein observed.

However, cellulases that are expressed by wild-type fungal organisms contain hundreds of enzymes and protein components, of which less than 5% are cellulases (Florencio et al., 2016a, 2016b). These other components play a role in lignocellulose hydrolysis, and hence cellulase alone, is only a partial indicator of cellulose hydrolysis activity.

The activity profiles in Table 4.1 are similar to those of enzymes used in studies that addressed high biomass loading and are known to be effective in these conditions.

Table 4-1 Cellulase 13P enzyme profile compared to similar commercial enzymes

Activity	Cellulase 13P	GC 220	Spezyme CP	Novozyme 188	Cellic CTEC3
	Activity units/mL				
Cellulase (FPU[a])	5.3*	92.8	58.2	8.5	58.6*
β-glucosidase	7.5*	99.7	128	665	47976*
Xylanase (OSX[b])	2160*	2782	2622	123	9613*
Endoclucanase (CMC)	52*	Nm	Nm	Nm	3594*
Protein concentration (mg/ml)	20.3*	NA	NA	NA	246.3*

[a] filter paper units; [b] oat spelt xylan; nm: not measured; NA: not available; *this study. All other activities from Dien et al., 2008.

4.2 Changes in Lignin 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 4-2). Raising the temperature and severity resulted in higher proportions of glucan recovered in the solid portion. Conversely, the amount of xylan decreased, with its complete removal at 200 and 210°C. Lignin decreased, while the amount of extractives dramatically increased.

During pretreatment all parts of sugarcane bagasse, cellulose, hemicellulose and lignin, underwent hydrolysis, solubilizing small fractions of them. The hydrolysate constituents were removed during the washing steps or later as extractives depending on their hydrophylicity. After each pretreatment and following washing, 37% of the original mass was removed, the loss could be attributed to the process itself and 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 but in lower amounts. This can be explained by high amount of extractives generated in this condition. The harsher conditions lead to more ethanol soluble small compounds, i.e. more likely to be extracted.

The extractives consists of a large number of compounds that derived from SCB, including xylose and its degradation products, phenolics not removed during washing and ash residues. The presence of the remaining but large fraction of lignin still generates inhibitory effects. As LHW pretreatment preferentially cleaves β-O-4 bonds, it removes a higher proportion of S lignin, increasing the G/S ratio and making the lignin more hydrophobic and with higher affinity to cellulases. At higher severities, more lignin is removed, and the more inhibitory the remaining lignin becomes.

Compared to hardwood *(Ko et al., 2015a)*, 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 lignin composition. SCB has a lower G/S ratio than hardwood and leads to a more readily hydrolyzed biomass. However, after pretreatment this difference decreases to the point where inhibition occurs at comparable levels when the same severity is applied to both.

Table 4-2 Sugarcane bagasse composition

	Untreated	1	2	3
Temperature/time	-	190 °C / 20 min	200 °C / 20 min	210 °C / 20 min
Severity factor[a]	-	9.80	10.74	11.69
Severity factor[b]	-	3.95	4.25	4.54
Glucan (%)	48	61	67	60
Xylan (%)	29	10	0	0
Lignin (%)	23	18	15	14
Ash (%)		1.0	1.1	1.0
Extractives (%)	4	19	23	29
Total (%)	104	109	105	104

[a]Severity factor with ω = 4.6 (Kim et al., 2013). [b]Conventional severity factor with ω = 14.75 (Overend and Chornet, 1987).

4.2.1 BSA adsorption on pretreated and washed sugarcane bagasse

To determine the maximum protein adsorption at pH 5, pretreated sugarcane bagasse was incubated with BSA for 24 hours in varying protein concentration (100, 200 and 300

mg BSA/g glucan). The total free protein was measured at equilibrium and adsorbed protein calculated by difference. The final total adsorbed protein was not significantly different between the different conditions (Figure 4-1).

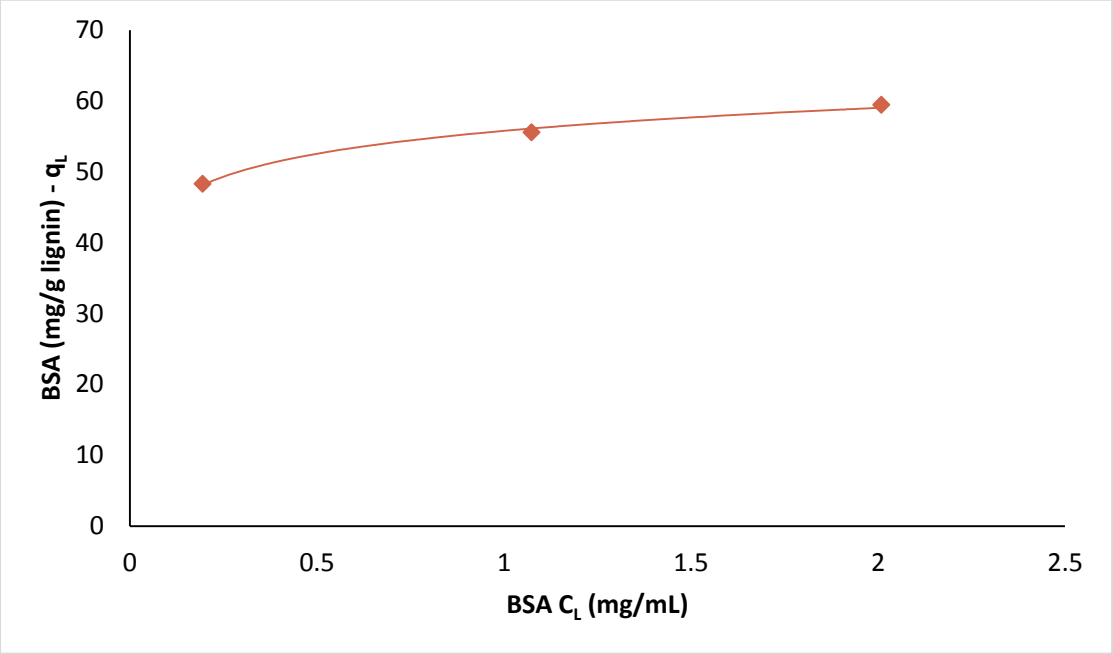

Figure 4-1 Langmuir isotherm adsorption model for BSA adsorption onto pretreated and washed sugarcane bagasse. Calculated constants Q^0 = 61.0 mg BSA/g lignin, K_{eq} = 14.9 mL/mg BSA.

Considering that BSA has a pI of approximatedly 4.6, precipitation at pH 5.0 is a possibility. To evaluate whether that was responsible for the protein loss on solution shown on Figure 4.1, pH effect on adsorption kinetics of BSA on pretreated SCB was evaluated at room temperature. There was no significant difference between pH 5.0 and pH 6.0. Equilibrium was reached after 4 hours at both conditions (Figure 4-2).

Pure cellulose (Avicel) was used as a control. This type of cellulose is derived from acid hydrolysis of wood pulp, and has an average particle size of 50 µm, less than 2%

hemicellulose, and cellulose with a DP of 250 *(Kafle et al., 2015)*. There was no adsorption of BSA when Avicel was used at room temperature. However, when adsorption onto Avicel was measured at 50°C, up to 40% of the BSA was lost with pH 5.0 and none with pH 6.0 probably due to precipitation.

Adsorption data was fitted to the Langmuir equation and adsorption parameters were estimated. Maximum adsorption capacity (Q^0) was estimated at 61.0 mg BSA/g lignin and affinity (K_{eq}) at 14.9 mL/mg BSA. Both parameters are higher than the ones observed by Ko et al. (2015a) for isolated lignin from pretreated hardwood. In that study, Q^0 varied from 36.6 to 44.8 mg protein/g lignin and K_{eq} from 2.8 to 4.9 mL/mg. This difference can be attributed to the fact that this evaluation includes complete biomass and not isolated lignin. Ooshima et al. (1990) compared the adsorption of proteins on complete pretreated hardwood and its isolated lignin and found that Q^0 when lignin from hardwood pretreated at 200°C was 66.6 mg protein/g lignin and K_{eq} was 0.655 mL/mg. When the complete biomass is used Q^0 becomes 30.5 mg protein/g lignin and K_{eq} becomes 12.5 mL/mg. These last parameters are closer to the observed in this thesis and the remaining difference is possibly due to the difference in lignocellulose used.

Therefore to achieve maximum BSA adsorption on lignin, the combination of pH 5.0 and 50°C must be avoided, while the BSA adsorption at 25°C minimizes precipitation. For this study, adsorption was done at pH 5.0 and 25°C, to avoid pH correction before hydrolysis.

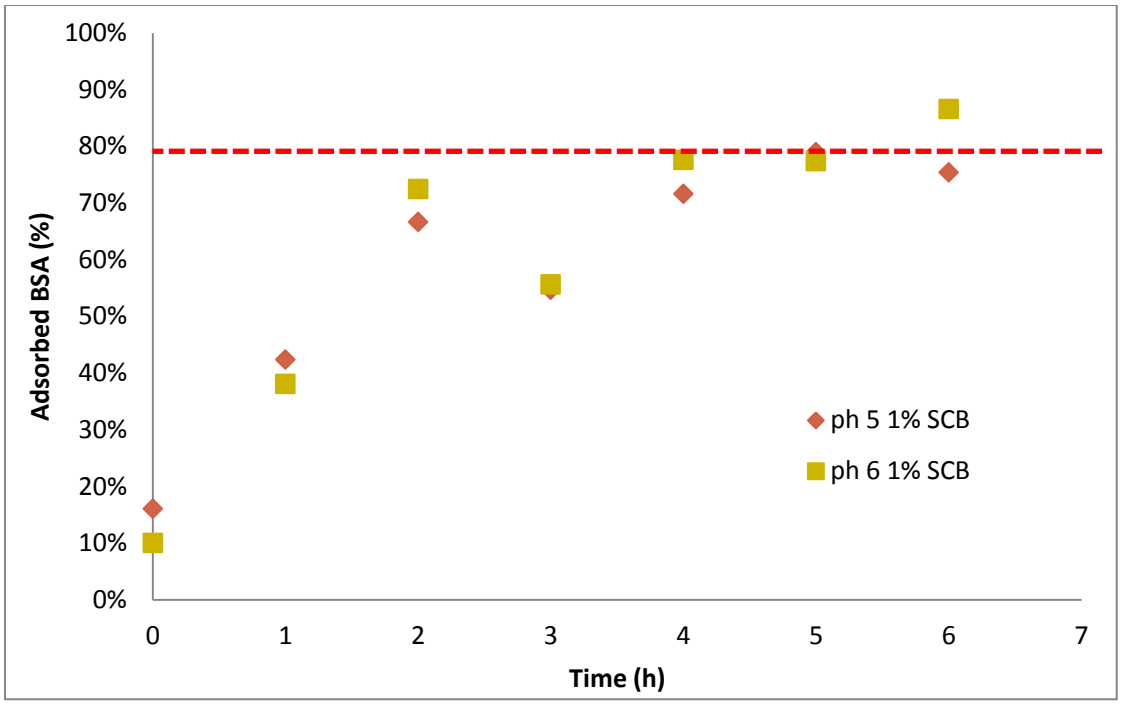

Figure 4-2 BSA adsorption on pretreated sugarcane bagasse at 25ºC. Sugarcane bagasse was pretreated using liquid hot water pretreatment at 190ºC for 20 min. 1% glucan and 100 mg BSA/g glucan were incubated for 24 hours. Citric acid buffer was used on both conditions. Adsorbed BSA determined by subtracting free protein from initial protein concentration.

4.3 Enzymatic hydrolysis

To evaluate impact of pre-adsorption of BSA on enzymatic hydrolysis, SCB was incubated with BSA at 25°C. After the incubation with BSA, the enzymatic solution (10 FPU/g glucan) was added and the reaction tubes were transferred to a shaking incubator at 50°C and 200 RPM to initiate hydrolysis. Glucose concentration was evaluated by liquid chromatography after 72 hours.

At the lowest pretreatment severity (190°C), no gain was observed on final glucose conversion when BSA was used. For SCB obtained from higher severities pretreatments (200°C and 210°C for 20 minutes), the conversion was significantly higher, mostly

because of a lower conversion observed on the absence of BSA (Figure 4-3). However, the conversions observed when BSA was used in these conditions were significantly higher than the ones observed at the lowest severity ($p < 0.01$). It is also notable that there is no difference between 1 hour and 24 hour incubation times for BSA. This relation between severity and higher BSA influence was also observed in hardwood (Ko et al, 2015).

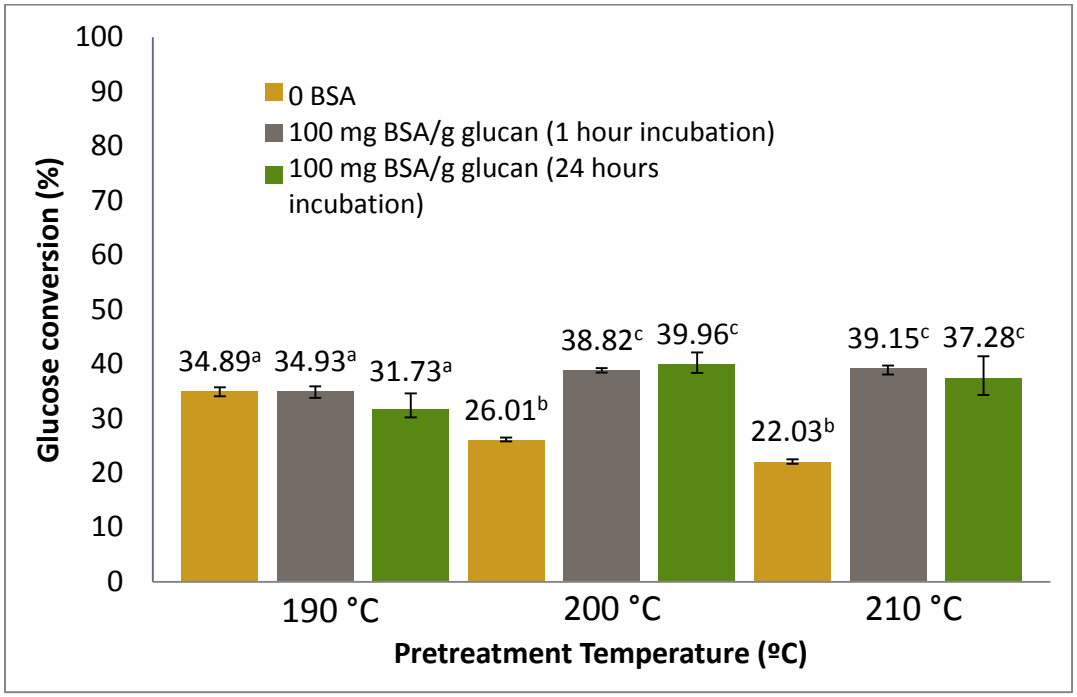

Figure 4-3 Sugarcane bagasse enzymatic hydrolysis. Three different pretreatment conditions were evaluated, 190°C, 200°C and 210°C, all for 20 minutes. Cellulase 13P (5 FPU/g glucan) was used. ANOVA was used to evaluate the final glucose conversion values, three groups were identified a, b, and c ($p < 0.05$).

These values, however, are lower than the observed by Ko et al. (2015b) and others (Section 2). The main difference was that the enzyme used (Cellulase 13P) has a low β-glucosidase activity. When β-glucosidase is supplemented with Novozyme 188

(Novozyme) at the proportion of 1 FPU : 2 IU. The conversion increased when 100 mg BSA/ g glucan was added (40% to 51%) and without BSA addition (26% to 42%). The supplementation has two effects on the final conversion. First, it lowers the inhibition caused by cellobiose by hydrolyzing it. Second, it counters solid lignin inhibition since it is the most affected of the cellulase enzymes by it (Ko et al., 2015a). Therefore, the supplementation is more effective in the situation where lignin is not blocked by BSA. However, this shows that β-glucosidase inhibition by lignin is not the only factor that BSA counters.

For the remaining experiments, the pretreatment parameters were set at 200°C for 20 minutes for two reasons: First, it had the highest conversed cellulose portion and lower severity / temperatures and no gain in conversion was observed at higher severities even with BSA.

4.3.1 – Solids loadings increase

The solids loading was increased to evaluate the BSA impact in these conditions. All other components (enzymes and BSA concentration) were increased at the same rate. Hydrolysis was conducted using 10 FPU + 20 IU (β-glucosidase) / g glucan at pH 5.0, in shaking incubator at 50°C and 200 RPM for 72 hours. Incubation with 100 mg BSA/ g glucan was done at 25°C and 200 RPM. Figure 4.4 shows the conversion curves found with higher solids loading.

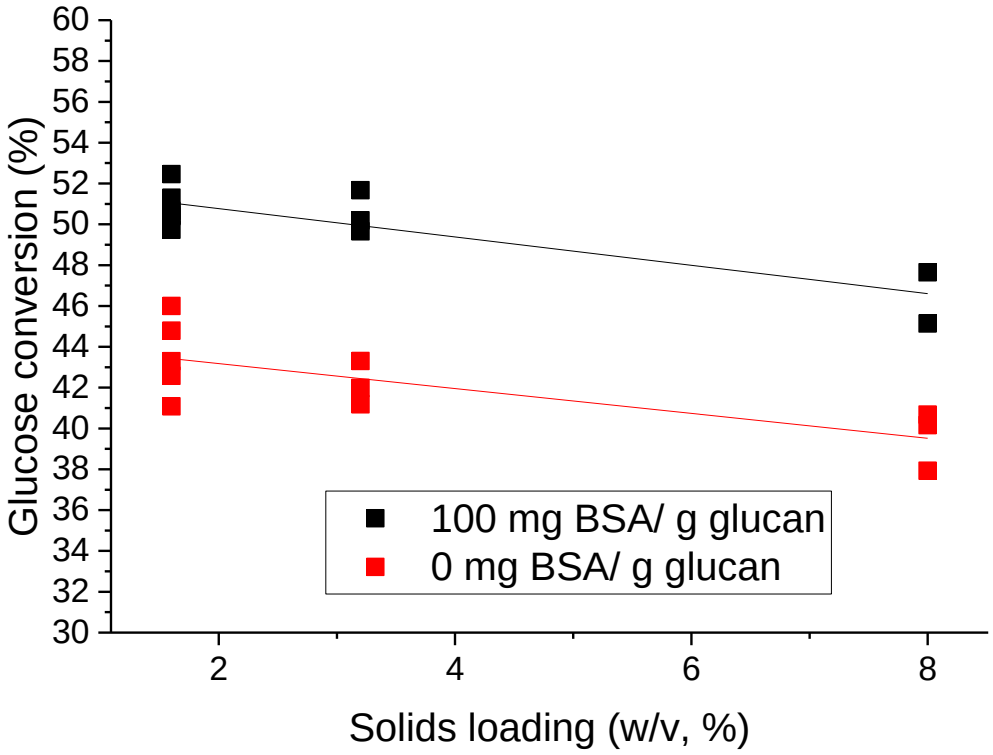

Figure 4-4 Glucose conversion from pretreated and washed sugarcane bagasse (LHW 200ºC, 20 min) as solids loading increases using 10 FPU (Cellulase 13P) + 20 IU (Novozyme 188)/ g glucan.

In this figure we can see that the samples with BSA maintain a higher conversion rate after 72 hours. However, to achieve this, a longer incubation time is needed at 8% solids (24 hours) than at 1.6 and 3.2% solids (1 hour). When only 1 hour is used for 8% solids, the conversion is 41%, closer to the observed when no BSA is added (40%).

When the lines are compared using SAS 9.4, the lines are found to be statiscally different ($p<0.0001$). The lines however are parallel, as they have the same slope with different intercepts ($p<0.0001$). This indicates that some common factor is responsible for the yield loss observed. The general lines given is for BSA = 100 mg/g glucan:

Conversion = 51.9509 – [SOLIDS] * 0.64644; and for BSA = 0 mg/g glucan: Conversion = 44.53380 – [SOLIDS] * 0.64644.

These numbers indicate that initial solids concentration has a negative effect on final glucose conversion. Multiple factors can contribute to this loss, as explained in Section 2.10. It also appears that lignin inhibition is not the main or one of the main factors, since conversion decreased at the same rate with or without BSA.

Mixing is the other factor that needs to be considered, since BSA adsorption got less effective with higher mass, meaning it took more time to reach all lignin sites that it could. However, other factors such as accumulation of product and other inhibitors cannot be discarded.

4.3.2 – Hydrolysis using commercial enzyme preparation (Cellic CTEC3)

In order to evaluate the impact of the enzyme preparation, the glucose yields using Cellulase 13P supplemented with Novozyme 188 were compared to the ones obtained using Cellic CTEC3 (Novozymes, Inc) with 58.6 FPU/ml and 246.3 mg protein/ mL. The initial enzyme loadings were reduced to 2.5 FPU/ g glucan due to the higher efficiency of this preparation. Hydrolysis with Cellulase 13P and Novozyme 188 were carried at the same conditions. These assays were carried using centrifuge tubes with total volume of 1 mL, shaken at 800 RPM. The experiments were repeated 6 times. The results are summarized in Table 4-3.

Table 4-3 Glucose conversion of pretreated and washed sugarcane bagasse with 2.5 FPU/ g glucan in different conditions

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%)*

* Possible outlier excluded.

Glucose yield obtained with Cellic CTEC3 enzyme was much higher than the yield using the other enzymes. No statistical difference was found between the different conditions with this enzyme. However, results may change due to the existence of a possible outlier when 10% solids and 100 mg BSA/g glucan were used. If that observation is removed, that group becomes statically different from the observed yields with 0 mg BSA/g glucan ($p<0.05$). No difference is observed between the conditions when BSA is included - $p=0.42$, with all observations and $p=0.20$, when the possible outlier is removed.

The small sample size for this assays make it almost impossible to determine whether the observation is an outlier as most tests are better suited for bigger samples. Simpler methods such as evaluating interquartile ranges (IQR) suggest this observation to be removed as it falls outside the IQR. IQR = 5.96, lower bound (Q1-1.5xIQR) = 70.29 and higher bound (Q3+1.5xIQR) = 94.13. The observation is 67.52% lower than the lower bound. However, values even lower than that were observed when 1% solids (w/v) and

100 mg BSA/ g glucan was used, suggesting that the inclusion of BSA increases the variability of hydrolysis and this observation is not a outlier.

For Cellulase 13P + Novozyme 188 preparation, the results differed from the higher volume/ higher enzyme loading previously observed. As expected, with lower enzyme loadings all conversions fell. However, the pattern changed. While there was significant loss of conversion between 1.6% solids and 10% solids without BSA ($p = 0.02$), there was no difference when 100 mg of BSA/g glucan was used ($p=0.53$). More interestingly, there was no difference with or without BSA when only 1.6% solids was used ($p=0.83$). At 10% solids, BSA lead to a significant difference.

In this case, there is also a possible outlier with 1.6% solids and 100 mg BSA/g glucan. However, removing it does not change any of the results. Except, the ANOVA analysis of all four groups at same time that goes from a borderline no-difference ($p=0.06$) to a clear presence of a different group ($p<0.01$).

4.3.3 – Hydrolysis using higher β-glucosidase supplementation of Cellulase 13P

The initial supplementation of β-glucosidase activity in Cellulase 13P using Novozyme 188 was done in a 1 FPU: 2 IU proportion based on previous literature. However, that proportion is much lower than the one measured with Cellic CTEC3 which is closer to 1 FPU : 700-800 IU. To test whether this proportion was responsible for the differences observed between the two preparations a new supplementation was done at 1 FPU: 400 IU. This preparation was only tested at 1% solids and a volume of 50 mL, each condition was repeated six times.

Using 10 FPU/g glucan, the average conversion without BSA was 40% and 43% with 100 mg BSA/g glucan. There was no statistical difference between the groups. These numbers are roughly the same as the ones observed when BSA was used in the original preparation. The higher supplementation countered the lignin adsorption at the same rate as BSA. However, it did not increase the yield, indicating that other enzymes are involved in the higher conversion observed with Cellic CTEC3.

At 5 FPU/ g glucan, the conversions observed were 29% and 32% and not statically different, without and with BSA respectively, which are both lower than the observed with the original preparation at 1 mL. Suggesting that the over supplementation increases the generation of glucose and product inhibition at low enzyme loadings that is not dissipated in the inefficient mixing with 50 mL.

4.3.4 – Hydrolysis with Cellic CTEC3 compared to other sugarcane bagasse sample

To understand the effect of SCB on hydrolysis yield, the same conditions were used in a different sample. The composition of that sample is listed on Table 4-4.

Table 4-4 Composition of second sugarcane bagasse sample before and after pretreatment in dry weight %.

Component	No pretreatment (%)	Pretreated (LHW, 200ºC, 20 min) (%)
Cellulose	50.3	52.2
Hemicellulose	24.6	0
Lignin	25.3	13.7
Klason lignin	20.6	12.3
Soluble lignin	4.7	1.8
Ash	1.1	19.2
Extractives	-	34.3
Total	101.3	119.4

The samples have roughly the same composition before pretreatment. However, they reacted to pretreatment in the same conditions (200°C for 20 minutes) in a slightly different way. The main difference was that the second sample did not increase its glucan fraction as much, going from 50 to 52%, compared with a 13 percent point increase of the first. While lignin was removed in a similar rate, going from 25 to 14%, while the original one went from 23 to 15%, the ratio between Klasson lignin and soluble lignin was different; the second sample had a ratio of 7.0, and the original a ratio of 12.7. Hemicellulose was completely removed from both. These differences might be explained by the higher extractive fraction observed after pretreatment in the second sample (34%

vs 23%). This indicates that this pretreatment lead to the solubilization of a greater fraction of the biomass, especially cellulose.

Recovery numbers were also slightly different. Even though, the same 63% overall mass was recovered, only 77% of the cellulose fraction was recovered in the solid of the second sample, while 87% was recovered in the original sample. This value is close to the recovered in when the original sample was pretreated at 210°C for 20 minutes. Meanwhile, lignin was recovered at similar rate in both samples, 40% for both.

When composition was analyzed without the extraction step, the samples were nearly identical Table 4.5. This suggests once again that a significant portion of cellulose in the second sample is already disassociated from the solids and more accessible to enzymes.

Table 4-5 Composition of sugarcane bagasse samples after pretreatment without ethanol extraction in dry weight %.

Component	Original Pretreated Sample (LHW, 200ºC, 20 min) (%)	Second Pretreated Sample (LHW, 200ºC, 20 min) (%)
Cellulose	64.4	63.4
Hemicellulose	2.9	0
Lignin	32.1	31.1
Klason lignin	29.4	28.6
Soluble lignin	2.7	2.5
Ash	18.7	19.1
Total	118.14	113.6

The conditions for hydrolysis tested were the same as above (Section 4.3.3) using Cellic CTEC3 due to its higher overall conversion when compared to Cellulase 13P + Novozyme 188 preparation. Hydrolysis yields are summarized on Table 4-6.

Table 4-6 Glucose conversion of two different pretreated and washed sugarcane bagasse with 2.5 FPU/ g glucan (Cellic CTEC3) in different conditions:

Solids loading (w/v)	Second sample		Original sample	
	0 mg BSA/g glucan	100 mg BSA/g glucan	0 mg BSA/g glucan	100 mg BSA/g glucan
1.6%	91.4%	113.5%	71.8%	76.1%
10%	70.6%	90.5%	72.6%	80.5% (83.1%)*

The differences in composition lead to a number of effects on hydrolysis conversion. All conversion values were significantly different from the original sample, except when initial solids loading was 10% and no BSA was used that was equal to the original sample in the same conditions, being consistently higher. As solid increased, the conversion with and without BSA, that was only seen on the original sample when the Cellulase 13P + Novozyme 188 (10 FPU + 20 IU/ g glucan) was used. Contrary to the observed with the original sample, BSA was not able maintain the same conversion at 1.6% and 10% solids. When BSA was added to 10% solids it only recovered the conversion to the same level that was observed at 1% solids and no BSA. This suggests the existence of another factor that lowers conversion. Another important observation was that conversion superior to

100% was observed with 1% solids and BSA added. This is probably caused by already hydrolyzed glucose and partially hydrolyzed glyco-oligomers that are extracted during composition analysis. If conversion yields are based on the non-extracted composition, no conversion goes above 100%.

All these factors combined show that SCB intra-species variation is important when deciding the optimum process, including pretreatment and enzymatic hydrolysis.

CHAPTER 5. DISCUSSION

5.1 BSA adsorption onto sugarcane bagasse lignin

A clear adsorption of BSA was observed. The calculated constants for the Langmuir adsorption model, Q^0 (maximum BSA adsorption capacity) = 61.0 mg BSA/ g lignin and K_{eq} (affinity) = 14.9 mL/mg BSA were both higher than the observed for cellulose directly on isolated lignin observed by Ko et al. (2015a).

In that study Q^0 varied between 37.0 and 44.8 mg protein/ g lignin and K_{eq} between 2.8 and 4.9 mL/mg protein depending on pretreatment severity. This difference indicates that BSA has higher affinity for lignin and would adsorb faster, which agrees with the projected trends from Sammond et al. (2014).

However, it was also observed that a BSA blocking step was needed before hydrolysis to ensure that it had any impact. Once again the projection from Sammond et al. (2014) help explain it. In that study, β-glucosidase and not BSA had the highest Q^0 while also being the cellulase with the highest affinity. This is corroborated by Ko et al (2015a) that observed that β-glucosidase is the one that is most affected by solid lignin, losing up to 90% of its activity in 25 hours. This could explain why, there is still some conversion losses when BSA is used, and why at higher solids the impact is much higher.

The constants must be used with caution as it might include some BSA adsorption on cellulose or other parts of lignocellulose, even though none was observed when Avicel was used in the place of SCB.BSA impact on final conversion

The BSA impact observed with 1% glucan solid is significant but smaller than Ko et al (2015b) observed. The improvement in the enzymatic hydrolysis yield by the addition of BSA increased significantly as the pretreatment severity increased.

However, comparing both studies directly like this is incorrect. The first difference to observe is that hardwood is used and not SCB. Sugarcane bagasse and hardwood have different compositions. SCB in this study had only 23% lignin before pretreatment, while hardwood has 32%.

This higher lignin content demanded a more severe pretreatment for the best hydrolysis conditions and the differences continued after pretreatment. In Ko et al. (2015a,b), the lignin content of pretreated hardwoods ranged from 36.2 to 40.3% compared to 15% in pretreated SCB. At the highest severity of log R0=12.51, the enzymatic hydrolysis yield increased from 17.4 (±1.6) to 71.9% (±1.5%) in the presence of BSA.

In this work, the best pretreatment conditions have a severity of log R_0 = 10.74. Using SCB pretreated at this condition, conversion increased from 42.3 (±1.5) to 50.9% (± 1.6%) in the presence of BSA. A comparable severity was investigated by Ko et al (2015b) and gives a better comparison. The conversion increases from approximately 15% to 20% with BSA when severity was log R_0 = 10.44. It is important to note that the lignin content was 36.2%. When evaluating higher temperatures and severities, such as 220ºC (the best

condition used in Ko et al, 2015b), biomass melts into a greasy compost and more than half of its mass was lost and most of the carbohydrate content eliminated.

The lignin composition is also different, SCB has 9-35-56 H-G-S lignin distribution *(Pal et al., 1995),* while hardwood has 2-36-62 *(Capanema et al., 2005).* While some variation from the ones used in the hydrolysis and the composition analysis studies is expected, the difference in the H-lignin proportion is also significant. On section 2.9, the impacts of lignin composition were discussed and apply here. The lower lignin content and higher G/S ratio of SCB (0.625 to 0.58) make SCB more readily digestible than hardwood, increasing the baseline conversion to glucose. Additionally, the presence of higher H-lignin may increase the differences even more. Bonawitz et al. (2014) showed that biomass made almost exclusively of H-lignin was more readily hydrolysable and required a less severe pretreatment. This can be verified already higher conversion of SCB when Cellulase 13P was not supplemented with β-glucosidase or incubated with BSA compared to the conversions of hardwood observed by Ko et al. (2015b).

Another important factor is the enzyme preparations used. Ko et al. (2015b) used Cellic CTEC2, while this study used Cellulase 13P supplemented with Novozyme 188. Cellic CTEC2 was carefully designed with multiple accessory enzymes to increase its activity. While Cellulase 13P has a higher than normal xylanase activity – one of the enzymes included on Cellic CTEC2 – it still lacks the other ones, most notably β-glucosidase. The accessory enzymes importance to the conversion have been acknowledged and studied and are difficult to reproduce in laboratory conditions *(Florencio et al., 2016a,b).*

When a more developed enzyme blend was used – Cellic CTEC3, a subsequent product of Cellic CTEC 2 – the hydrolysis conversions were increased to the point of almost full conversion with relatively low loadings (2.5 FPU/g glucan or 10.5 mg protein of enzyme/ g glucan). Interestingly, all gains from BSA disappear when Cellic CTEC3 is used.

5.2 Conversion at increasing solids loadings

As the solids loading increased, the conversion decreased in a linear fashion. This phenomenon has been described by others (see Section 2.7). It is probably a multi-factorial problem as no single approach has canceled this effect. Mixing *(Jorgensen et al., 2007)*, product inhibition *(Kristensen et al, 2009)*, and now the cellulase adsorption cannot solely explain the loss.

This study, showed that even when the adsorption factor is addressed the conversion decreases at the same rate, with and without BSA (Figure 4.4). When volumes were reduced (to 1 mL), this effect mostly disappeared and a flat line is obtained as solids loadings increase, the lone exception being when Cellulase 13P + Novozyme 188 was used without BSA. One important difference is the higher mixing speed used (800 RPM vs 200 RPM), a factor that has been shown to lead higher conversion *(Palmqvist et al., 2011)*.

However, it could be argued that no mixing is actually occurring in such small volume regardless of the speed. This leads to two possible interpretations: either better mixing leads to better interaction between BSA and lignin, and later between enzymes and cellulose; or alternately, mixing leads to less interaction between the enzymes and its

inhibitors. Either way this indicates that agitation is contributing to lower conversion yields.

Another important finding was the longer incubation time required for BSA as solids increased, which can be correlated with the mixing problems already mentioned. The more inefficient mixing leads to a slower lignin-BSA interaction, analogous to the slower rate of cellulase-biomass interaction. Other factors, not yet accounted for are the release and accumulation of other inhibitors as the reaction progresses, including newly exposed solid lignin.

5.3 High variability when BSA is used

In all hydrolysis assays one factor was always present, whenever BSA was added the variance of the glucose yields increased multiple times. This limits the applicability of ANOVA tests to compare different conditions and can lead to erroneous interpretation of the empirical results. One example, already discussed in Section 4.3.2, shows how the increased variability can change the conclusions of the work.

This is especially important in this study as the objective is to create a condition that leads to constant conversion independent of solids loadings. The results here indicate that the use of BSA and Cellic CTEC3 is enough for all sugarcane bagasse samples. However, if the possible outliers are included, it can be argued that BSA is not needed for the main sample studied. By removing outliers, BSA is indicated as necessary.

Other factors that may contribute to the observed variability is the small volume used and the consequent use of small amounts of SCB, enzyme, etc. Internal variability of the samples can also partially explain it. However, those do not explain why, the variance

increases between 5 to 100 times when the presence of BSA is the only difference between the groups evaluated in all volumes, samples and enzyme blends.

5.4 Glucose conversion upper limit

The main SCB sample used appeared to have an upper limit for glucose conversion around 80% of the theoretical yield, regardless of enzyme preparation, use of BSA and time. To go over this yield, much higher enzyme loadings were necessary (>20 FPU/g glucan). This suggests that a fraction of cellulose is still crystalline and inaccessible to the enzymes even after pretreatment. Such behavior has been observed before with Avicel where the degree of polymerization of cellulose did not change after hydrolysis *(Kafle et al., 2015).*

The second SCB sample did not have such limitations and complete conversion (~100%) was achieved with 2.5 FPU/g glucan or 10.5 mg protein/g glucan. This sample's behavior after pretreatment, solubilizing a higher fraction of cellulose than the main sample supports the existence of a more crystalline portion of cellulose in the main sample.

The existence of this upper limit might also explain why BSA had almost effect in this sample by simple ensuring that no significant gain was possible.

5.5 Lignin composition impact on biomass hydrolysis

Literature consensus on lignin variable impact on hydrolysis depends on total lignin content. The relation of lignin composition and hydrolysis, however, is less well defined. The proportions of H-, G-, and S- lignin should indicate how lignin will behave in a given

circumstance. For this work, the relevant characteristics are lignin-hemicellulose interaction and lignin-enzyme interaction. These occur in different times and influence different facets of the process.

S-lignin mainly makes β-O-4 bonds and a small proportion of β-β and β-1. This leads to a very linear structure. G- and H- lignin have a more diverse range of bonds, that leads to more condensed and branched structure (Kishimoto et al., 2010).

The number of branches influences the degradability of biomass in two ways. First, more branches lead to more free phenolics bound through β-O-4s that are easily hydrolyzed during pretreatment (O'Connell et al., 2002). Second, the more linear structure interacts more strongly with the sugar polymers making their hydrolysis harder either through larger contact area or through larger number of free hydrogens that bound to glucan or xylan polymers.

This explains results that indicate higher conversion with higher G/S ratios, since more hemicellulose and lignin – both inhibitory components – are removed from biomass during pretreatment. It also offers an insight on why a predominantly H-lignin structure is easily hydrolyzed even before pretreatment.

After pretreatment, more S-lignin is removed than the others and the G/S ratios increase. With higher severity, more S-lignin is removed and solid lignin becomes more inhibitory (Ko et al., 2015a,b). Even though these two factors may not be related – as surface modifications may explain the correlation between severity and lignin inhibition – after pretreatment higher G/S ratios have lower conversion.

Therefore, an optimum distribution between G and S is not a pure structure of either. The desired balance relies on the efficiency of pretreatment and enzymatic preparations.

Additionally, H-lignin will modify this balance, presumably with a behavior analogous to G-lignin but with a more pronounced effect. As long as other phenotypical characteristics are maintained, such as growth rate and sugar content, lignin modifications must be directed by empirical processing results.

5.6 Remarkable results

While the effect of BSA was minimal, multiple advances in understanding the phenomena were made. First, the best liquid hot water conditions for sugarcane bagasse were identified (200°C, 20 min). Almost complete elimination of hemicellulose, removal of 40% of lignin, while recovering 90% of all cellulose in the solid fraction. Hydrolysis conducted with this pretreated and washed SCB lead to 80% conversion of the theoretical maximum using 2.5 FPU/ g glucose (10.5 mg protein/ g glucose) of Cellic CTEC3. This conversion is close to the target conversion of 90% with 10 mg protein/ g glucose for an economically feasible process.

Second, it clarifies what factors are involved in the loss of conversion when solids loadings increase. Adsorption of cellulases onto solid lignin cannot be discarded since when a better mixing pattern is used, the addition of BSA eliminates the downward slope. However, it is not the most important factor as when it is mostly eliminated (full BSA adsorption) and mixing not corrected the conversion still falls in the same linear fashion. Mixing appears to be most important one as increasing rotational speed reduced the negative slope, even though it did not eliminate it. Additionally, the inefficient mixing limits BSA adsorption on lignin prior to hydrolysis, increasing the overall duration in 24 hours rather than 1 required in low loadings.

Third, it indicates that lignin adsorption might also be countered with accessory enzymes. When these effects were included, most of gains with BSA disappeared. Investing in better enzyme blends is probably better as it excludes the need for an extra step for lignin blocking. Simply increasing the supplementation of β-glucosidase, however, may not be the answer. Even though it eliminated the lignin inhibition it did not increase conversion, indicating that other enzymes/compounds are responsible.

Fourth, the high variability of sugarcane bagasse makes the results hard to generalize the conditions necessary for the process.

CHAPTER 6. CONCLUSION

6.1 Summary

Production of ethanol from sugarcane bagasse is a potential alternative to petroleum fuels. However, it presents technical challenges due to its composition. In this work, we have been able overcome its natural recalcitrance by optimizing the pretreatment conditions, implementing liquid hot water pretreatment (LHW) at 200°C for 20 minutes followed by a hot water washing step. Subsequent hydrolysis reached complete hydrolysis using 2.5 FPU/g glucan (10.5 mg protein/g glucan) when BSA incubation for up to 24 hours was also utilized with solids loading up to 10% (w/v).

6.2 Optimum Pretreatment

Among the multiple tested parameters for LHW, 200°C for 20 minutes was the best condition. It led to highest recovery of the cellulose fraction (87%), complete removal of the hemicellulose fraction and removal of 60% of lignin, with total solids recovery of 63%. This condition also showed highest conversion after enzymatic hydrolysis compared to the others.

6.3 Use of BSA as a blocking agent

Incubation of pretreated SCB with BSA prior to enzymatic hydrolysis led to a limited increase (8%) in conversion compared to the previously observed with hardwood (up to

55%) *(Ko et al., 2015b).* This result suggests that BSA's impact is limited by lignin content, lignin composition and pretreatment severity and how much it changes lignin surface.

6.4 SCB Hydrolysis at high solids

Due to physical limitations, the highest solid loading possible in a single-batch test was 10% w/v. Conversion decreased as solids increased in most cases. When biomass was incubated with BSA and faster rotary shaking was used the downward, conversion became the same across solids loadings. However, if either of these conditions is removed, conversions decrease. This suggests that the loss of yield is a multi-factorial problem.

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APPENDIX

APPENDIX – STANDARD OPERATING PROCEDURES

A.1 SOP 1 - Filter Paper Assay For Saccharifying Cellulase

This procedure was adapted from Mandels, M., Andreotti, R. and Roche, C. 1976.

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Required reagents:

- Dinitrosalicylic acid
- Sodium hydroxide pellets
- Potassium sodium tartarate
- Deionized (DI) water
- Sodium Citrate
- Citric acid
- D-Glucose

Materials:

- Whatman No. 1 filter
- 200 - 1000 µL adjustable pipette
- Brown glass bottle
- Water bath
- 25 mL test tubes
- Vortex mixer
- Spectrometer

Procedure:

1- Prepare DNS solution:
   a. Dissolve 10g of dinitrosalicylic acid, 16g sodium hydroxide pellets, and 300g potassium sodium tartarate in 1.0 L DI water. Let stir until completely dissolved. Store in a brown glass bottle.
2- Cut the Whatman No. 1 filter into 1.0 x 6.0 cm paper strips, equivalent to 50mg.

3- Prepare a 50 mM Sodium Citrate Buffer (SCB) at the desired pH.
4- Prepare a Glucose standard (10g/L) by dissolving 0.5 g of D-glucose in 50 mL DI water.
5- Allow all samples, standards, buffers to adjust to room temperature.
6- Set water bath to 50°C.
7- Prepare triplicates for each sample, using a test tube for each replicate:
    a. Add 1.0 mL SCB, pH 4.8, to a test tube of volume at least 25 mL.
    b. Add 0.5 mL enzyme appropriately diluted, if it is the case.
    c. Add one filter paper strip.
    d. Vortex the test tube, so that the paper strip is on the bottom of the tube.
8- Incubate at 50°C for 60 min.
9- Prepare triplicates for each standard concentration, using a test tube for each replicate:
    a. Add 1.0 mL Sodium Acetate Buffer, pH 4.8, to a test tube of volume at least 25 mL.
    b. Add 0.5 mL Glucose standard appropriately diluted, if it is the case.
10- Add 3.0 mL DNS, mix.
11- Boil for 5.0 min.
12- Add 20 mL either deionized or distilled water. Mix using the vortex mixer.
13- Let the pulp settle for 20 min.
14- Transfer 1000µL from the test tube to a cuvette and measure the absorbance at 540 nm.

<u>Unit Calculation:</u>

1- Construct a linear glucose standard curve using the absolute amounts of glucose (mg/0.5mL).
2- Using this standard curve, translate the absorbance values of the sample tubes into glucose representing the amount of glucose produced during the reaction.
3- Translate the dilutions used into enzyme concentrations.
4- Estimate the concentration of enzyme which would have released exactly 2.0 mg of glucose by plotting glucose liberated against enzyme concentration on semilogarithmic paper.
5- Calculate FPU by dividing 0.37 by the enzyme concentration to release 2.0 mg glucose.

A.2 SOP 2 - Acid Xylanase Assay

Required reagents:

- Dinitrosalicylic acid
- Sodium hydroxide pellets
- Potassium sodium tartarate
- Deionized (DI) water
- Sodium Citrate
- Citric acid
- D-Xylose
- Oat spelt xylan (OSX)
- Sodium hydroxide
- 2N Hydrogen Chloride
- Sodium azide

Materials:

- 200 - 1000 µL adjustable pipette
- Brown glass bottle
- Water bath
- 15 mL test tubes
- Vortex mixer
- Spectrometer

Procedure:

1- Prepare a 50 mM Sodium Citrate Buffer (SCB) at the pH 4.8.
2- Prepare the OSX substrate:
   a. Weigh 4.0 g oat spelt xylan (OSX) and add it to about 40 mL of 0.2 M NaOH. Incubate overnight with agitation. Add DI water to 100mL and adjust pH for 4.8 with 2N Hydrogen Chloride. Add 20mL 1.0 M sodium citrate buffer pH 4.8 (SCB) and bring the volume to 200 mL. Store the clouded suspension in bottle at 4°C, and label it as 2% oat spelt xylan in 100 mM SAB, pH 4.8. Add sodium azide (0.02%) for avoiding microbial contamination.
   b. For assay, the substrate is diluted to 1% with equal water volume. Because of incomplete solubility, the suspension should be shaken to mix before use.

3- Prepare DNS solution:
   a. Dissolve 10g of dinitrosalicylic acid, 16g sodium hydroxide pellets, and 300g potassium sodium tartarate in 1.0 L DI water. Let stir until completely dissolved. Store in a brown glass bottle.
4- Set water bath to 50°C.
5- Set up the standard as described on the following table:

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

6- Prepare triplicates for each sample, using a test tube for each replicate by adding 50 µL of appropriately diluted enzyme to 200 µL 1% OSX.
7- Incubate for 15 min at 50°C.
8- Stop the reaction by adding 1.0 mL DNS reagent.

9- Boil tubes for 5 min.
10- Transfer 1000µL from the test tube to a cuvette and measure the absorbance at 540 nm.

Unit Calculation:

1- Construct a linear xylose standard curve using the absolute amounts of xylose (mg).
2- Using this standard curve, translate the absorbance values of the sample tubes into xylose representing the amount of xylose produced during the reaction.
3- Xylanase activity = [amount of xylose equivalent produced / (150/15/0.05)] x dilution
4- Where: 150 is a conversion factor to µmoles (Mr of xylose is 150) from µg, 15 is a conversion to per min, and 0.05 is related to 50 µL of enzyme used.

A.3 SOP 3 - Acid Cellulase Assay

Required reagents:

- Dinitrosalicylic acid
- Sodium hydroxide pellets
- Potassium sodium tartarate
- Deionized (DI) water
- Sodium Citrate
- Citric acid
- D-Glucose
- Carboximethilcellulose (CMC)
- Sodium hydroxide
- 2N Hydrogen Chloride
- Sodium azide

Materials:

- 200 - 1000 µL adjustable pipette
- Brown glass bottle
- Water bath
- 15 mL test tubes
- Vortex mixer
- Spectrometer

Procedure:

1- Prepare a 50 mM Sodium Citrate Buffer (SCB) at the pH 4.8.
2- Prepare the CMC substrate:
   a. Weigh 10g of CMC and add it to 400mL dH20. Bring the volume to 500 mL with DI water and label it as 2% CMC. The solution must be kept at 4°C and be free of microbial contamination when used.
   b. For assay, the substrate is diluted to 1% CMC working solution in 50 mM SCB, pH 4.8. For example, if 50 mL of working solution is prepared, use 25 mL of 2% CMC, 2.5 mL 1.0M SCB, and 22.5 mL of DI water. Because of incomplete solubility, the suspension should be shaken to mix before use.
3- Prepare DNS solution:
   a. Dissolve 10g of dinitrosalicylic acid, 16g sodium hydroxide pellets, and 300g potassium sodium tartarate in 1.0 L DI water. Let stir until completely dissolved. Store in a brown glass bottle.
4- Set water bath to 50°C.

5- Set up the standard as described on the following table:

	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 SAB pH 4.8 (µL)	50	48	47	45	40	35
Total volume (µL)	250	250	250	250	250	250
Amount of glucose (µg)	0	20	30	50	100	150

6- Prepare triplicates for each sample, using a test tube for each replicate by adding 50 µL of appropriately diluted enzyme to 200 µL 1% CMC.
7- Incubate for 15 min at 50°C.
8- Stop the reaction by adding 1.0 mL DNS reagent.
9- Boil tubes for 5 min.
10- Transfer 1000µL from the test tube to a cuvette and measure the absorbance at 540 nm.

Unit Calculation:

1- Construct a linear glucose standard curve using the absolute amounts of glucose (mg).
2- Using this standard curve, translate the absorbance values of the sample tubes into glucose representing the amount of glucose produced during the reaction.
3- Cellulase activity := [amount of glucose equivalent produced / (180/15/0.05)] x dilution.
4- Where: 180 is a conversion factor to μmoles (Mr of glucose is 180) from μg, 15 is a conversion to per min, and 0.05 is related to 50 μL of enzyme used.

A.4 SOP 4 – β-Glucosidase enzyme assay using ρ-nitrophenyl-glucosidase

Required reagents:

- P-Nitrophenol-glucosidase
- P-Nitrophenol
- Deionized (DI) water
- Sodium Citrate
- Citric acid
- Sodium Carbonate
- Sodium azide

Materials:

- 200 - 1000 μL adjustable pipette
- Brown glass bottle
- Water bath
- 15 mL test tubes
- Vortex mixer
- Spectrometer

Procedure:

1- Prepare a 50 mM Sodium Citrate Buffer (SCB) at the pH 4.8.
2- Prepare a 10mM ρ-nitrophenol-glucosidase in SCB. Stir to dissolve the substrate.
3- Prepare a ρ-Nitrophenol standard: Weigh 139 mg ρ-nitrophenol and dissolve it in 450 mL DI water. Stir to dissolve. Add volume exactly to 500mL. The ρ-NP concentration is 2 mM.
4- Prepare a Sodium Carbonate 1M solution: Dissolve 106 g sodium carbonate (Na2CO3) in 1L dH2O. Cap tightly between uses. pH must be ≥ 10.0.
5- Set water bath to 50°C.

6- Set up the standard as described on the following table:

	1	2	3	4	5
10mM ρ-nitrophenol-glucosidase	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

7- Prepare triplicates for each sample, using a test tube for each replicate by adding 100 µL of appropriately diluted enzyme to 400 µL 10mM ρ-nitrophenol-glucosidase.
8- Incubate for 15 min at 50°C.
9- Stop the reaction by adding 1.0 mL Sodium Carbonate 1M.
10- Transfer 1000µL from the test tube to a cuvette and measure the absorbance at 410 nm.

Unit Calculation:

1- Construct a linear ρ-nitrophenol standard curve using the absolute amount of µmoles of ρ-nitrophenol.
2- Using this standard curve, translate the absorbance values of the sample tubes into ρ-nitrophenol representing the amount of glucose produced during the reaction.
3- β-glucosidase activity = ρ-NP generated/(0.1/15) x dilution factor.
4- Where: 15 is a conversion to per min, and 0.1 is related to 100 µL of enzyme used.

A.5 SOP 5 – Adsorption and Hydrolysis assays

Required reagents:

- Deionized (DI) water
- Sodium Citrate
- Citric acid
- Sodium Carbonate
- Sodium azide
- Bovine Serum Albumin (BSA)
- Cellulase Enzymatic blend

Materials:

- 200 - 1000 µL adjustable pipette
- 20 – 200 µL adjustable pipette
- Brown glass bottle
- Water bath
- 15 mL test tubes
- Vortex mixer
- Spectrometer
- 125 mL Erlenmeyers
- Orbital Shaker
- 50 mL graduated cylinder
- 1.5 mL centrifuge tubes with caps
- 50 mL centrifuge tubes with caps

Preparation and Sampling:

1- Set orbital shaker to the desired temperature (25°C or 50°C) and 200 RPM.
2- Prepare a 50 mM Sodium Citrate Buffer (SCB) at the pH 4.8-5.0.
3- Prepare a 100 mg/mL BSA solution in DI water. Store at 4°C.
4- Allow the biomass, BSA solution and enzyme to adjust to room temperature.
5- Weigh the biomass to the required amount for the assay on a weighting paper.
6- Transfer biomass to 125 mL Erlenmeyer.
7- Add the required BSA solution to the 50 mL graduated cylinder.
8- Add SCB to the 50 mL graduated cylinder to the required total volume (50 mL or 20 mL).
9- Transfer the SCB + BSA solution to the 125 mL Erlenmeyer.
10- Add the cellulase enzyme directly to the 125 mL Erlenmeyer using a 20-200 µL automatic pipette.
11- Close the 125 mL Erlenmeyer with a rubber cap and fix it into place with tape.

12- Put the replicates at the same time into the orbital shaker.
13- Intermediate sampling times:
    a. Remove the replicates from the shaker at the same time.
    b. Let the biomass settle for 30 seconds.
    c. Using a 100-1000 µL automatic pipette transfer 1000µL to a 1.5mL centrifuge tube.
    d. Freeze and store at -4°C.
14- Final sample:
    a. Remove the replicates from the shaker at the same time.
    b. Transfer the whole content to a 50mL centrifuge tube
    c. Freeze and store at -4°C.

Sample treatment:

1- Allow the samples to adjust to room temperature.
2- Centrifuge the samples:
    a. For 1.5mL centrifuge tubes, centrifuge at 10.000 RPM for 5 min.
    b. For 50mL centrifuge tubes, centrifuge at 5.000 RPM for 5 min.
3- Freeze and store at -4°C.

A.6 SOP 6 – HPLC procedure

Materials:

- Auto-pipette with disposable tips
- 1 ml Syringe with 25-mm Nylon 0.2 µm attachment filters

Procedure:

1- Use the samples prepared as described at SOP 5.
2- Using autopipettes, transfer the supernatant to a separate 1.5 mL centrifuge tube.
3- Using autopipettes, dilute with DI water into a separate 1.5 ml centrifuge tube.
4- Using a 1 ml syringe, from the previous (diluted) 1.5 ml centrifuge tubes from the last centrifuging, attach a 25-mm Nylon 0.2 µm filter and filter into an HPLC vial.
5- Switch out filters if filtering becomes too difficult and continue filtering until the HPLC vial is greater than half full. Do not over exert with pressure.
6- Place vials into the HPLC and analyze.