Can we enhance cellulose hydrolysis by minimizing enzyme inhibition resulting from pretreatment-derived inhibitors?

by

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Abstract

Any pretreatment process used to enhance the enzymatic deconstruction of lignocellulosic substrates, although opening up and enhancing access to the cellulose, will typically generate inhibitory compounds (i.e. soluble mono/oligomeric sugars, phenolics, furans, extractives, etc.) that will limit or restrict the efficiency of cellulose hydrolysis. To develop more effective inhibition mitigation strategies, it would be beneficial if we had a better understanding of the inhibitory mechanisms of these soluble compounds on the enzyme components of cellulase cocktails. Most of the previous studies which have tried to assess the effects of inhibitors on cellulase enzymes, have used "synthetic mixtures of inhibitors" and "traditional" cellulase preparations such as Celluclast.

The work presented in this thesis assessed the major inhibitory compounds derived from a range of "real-life" lignocellulosic biomass substrates that were steam pretreated at various severities. The major inhibitory mechanisms, such as reversible/irreversible inhibition of the major enzyme activities (e.g. exo/endo-glucanase, β-glucosidase, xylanase activities, etc.), were investigated and potential inhibitor mitigation strategies were evaluated. Initial work showed that, although the more recent cellulase mixture CTec3 was more inhibitor tolerant than the older Celluclast enzyme preparation, they were still strongly inhibited by pretreatment derived inhibitors. Of the various inhibitors, sugars and phenolics were shown to be the major groups that significantly contributed to the observed decrease in cellulose hydrolysis. This was mostly because of the strong inhibition and deactivation of β-glucosidase and cellobiohydrolase activities present within the cellulase mixture. Surprisingly, although hemicellulose derived sugars did not appear to inhibit individual enzyme activities, they did inhibit overall cellulose hydrolysis. Subsequent work suggested that hemicellulose-derived sugars inhibited the

processive movement of cellobiohydrolase Cel7A and thus restricted cellulose hydrolysis. In addition to sugars, pretreatment derived phenolics were shown to be more influential, with the molecular size and carbonyl content of the phenols playing major roles in influencing the extent of phenolic inhibition. However, the phenolics could be modified to minimize enzyme inhibition and allow the use of lower enzyme loadings.

Lay Summary

To convert biomass such as trees or grass to biofuels, a pretreatment process is needed to open up the structure of the plant cell wall so that enzymes can better access and break down the cellulose to sugars. However, pretreatment results in the production of various inhibitory compounds that greatly restrict the action of the deconstruction enzymes. The thesis work better elucidated how soluble compounds hinder enzyme function and suggested ways of reduce this inhibitory effect. The work showed that, although there were likely numerous inhibitory compounds present in the water soluble fraction of pretreated biomass, sugars and phenolics limited enzymatic hydrolysis the most. By modifying the pretreatment process the production of inhibitory compounds could be minimized while concomitantly enhancing the enzyme cocktails ability to deconstruct the biomass to fermentable sugars. The thesis work showed how a likely industrial bioconversion process could be enhanced.

Preface

- **Zhai, R.**, Hu J., Saddler, J. 2016, What are the major components in steam pretreated lignocellulosic biomass that inhibit the efficacy of cellulase enzyme mixtures? (Chapter 3.1, ACS Sustainable Chemistry & Engineering, 4, 3429–3436)
- **Zhai, R.**, Hu J., Saddler, J. The inhibitory effect of water soluble compounds from steam pretreated biomass on cellulases and xylanase activities. (Chapter 3.2, *Manuscript*)
- **Zhai, R.**, Hu J., Saddler, J. Inhibition of high concentration of sugars on cellulose hydrolysis: role of enzyme kinetics and substrate characteristics. (Chapter 3.3, *Manuscript*)
- **Zhai, R.**, Hu J., Saddler, J. Effect of the major structure properties of phenolics that contribute to their inhibitory effects. (Chapter 3.4, *In preparation*)
- **Zhai, R.**, Hu J., Saddler, J. Minimizing the inhibitory effect of water soluble compounds by modifying steam pretreatment. (Chapter 3.5, *In preparation*)

For paper 1 to 5, Rui Zhai, Jack Saddler, and Jinguang Hu contributed to the planning of the experimental work, interpretation of the results, and drafting of the manuscripts. Rui Zhai carried out the laboratory work.

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List of Units and Abbreviations

2N	2-naphthol
HBA	4-hydroxybenzoic acid
AA9	auxiliary activity family 9
AIL	acid-insoluble lignin
Ara	arabinose
ASL	acid-soluble lignin
BG	β-glucosidase
BSA	bovine serum albumin
CBH	cellobiohydrolase
CBM	carbohydrate binding module
CD	catalytic domain
CMC	carboxymethyl cellulose
CNC	cellulose nanocrystal
CrI	Crystallinity index
DAPLP	dilute acid pretreated lodgepole pine
DAPLP-2N	dilute acid pretreated lodgepole pine with addition of 2-naphthol
DAPLP-2N-WS	water soluble fraction derived from dilute acid pretreatment of lodgepole pine with 2-naphthol added
DAPLP-HBA	dilute acid pretreated lodgepole pine with addition of 4-hydroxybenzoic acid
DAPLP-HBA-WS	water soluble fraction derived from dilute acid pretreatment of

	lodgepole pine with 4-hydroxybenzoic acid added
DAPLP-SA	dilute acid pretreated lodgepole pine with addition of syringic acid
DAPLP-SA-WS	water soluble fraction derived from dilute acid pretreatment of lodgepole pine with syringic acid added
DAPLP-VA	dilute acid pretreated lodgepole pine with addition of vanillic acid
DAPLP-VA-WS	water soluble fraction derived from dilute acid pretreatment of lodgepole pine with vanillic acid added
DAPLP-WS	water soluble fraction derived from dilute acid pretreatment of lodgepole pine
DNS	3,5-dinitrosalicylic acid
DP	dissolving pulp
EG	endoglucanase
FPA	filter paper activity
GA	gallic acid
Gal	galactose
Glu	glucose
Man	mannose
PNPC	4-nitrophenyl β-D-cellobioside
PNPG	4-nitrophenyl β-D-glucopyranoside
SA	syringic acid.
SPLP/SPP-WS-5%AC	activated carbon treated water soluble fraction derived from steam pretreated lodgepole pine/poplar

SPLP	steam pretreated lodgepole pine
$SPLP_{low}$-WS	water soluble fraction derived from low-severity steam pretreatment of lodgepole pine
SPLP-SA	steam pretreated lodgepole pine derived from a pretreatment process with syringic acid added
SPLP-SA-WS	water soluble fraction derived from a pretreatment process with syringic acid added
SPLP-WS	water soluble fraction derived from steam pretreated lodgepole pine
SPP	steam pretreatment poplar
SPP_{low}-WS	water soluble fraction derived from low-severity steam pretreatment of poplar
SPLP/SPP-WS-Phe-HP	hydroxypropylation of steam pretreated lodgepole pine/poplar derived phenolics
Xyl	xylose
VA	vanillic acid
WS	water soluble fraction
WS-AH	acid hydrolysed water soluble fraction.
WS-PheA	phenolics (>10 kDa) isolated from water soluble fraction
WS-PheB	phenolics (3-10 kDa) isolated from water soluble fraction
WS-PheC	phenolics (1-3 kDa) isolated from water soluble fraction
WS-PheD	phenolics (<1 kDa) isolated from water soluble fraction
w/w	weight/weight

w/v	weight/volume
FTIR	fourier-transform infrared spectroscopy

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1. Introduction

1.1 Background and rationale for the thesis work

The so-called biorefinery approach to replacing oil derived produces continues to attract considerable interest as it can help to solve many global issues such as energy security concerns and fossil fuel derived carbon emissions that result in climate changes (Chandel et al., 2012; Maity, 2015). In general, the term “biorefinery”, means providing an alternative to traditional oil-based refineries. In this way a wide range of bio-based fuels, chemicals, materials can be produced from renewable lignocellulosic biomass in an integrated process (Chandel et al., 2012).

Although the various biorefinery approaches can produce different product profiles, the so-called “sugar platform”, resulting from biomass deconstruction, has been proven to be one of the most useful platforms as it can be used to make a range of renewable fuels and chemicals (Fernando et al., 2006). However, the biomass deconstruction-to-sugar stage of this overall process currently requires too-high amounts of costly enzymes, limiting the development of economically viable biomass biological degradation processes. To reduce the operating costs and make the forest biomass based biorefinery more commercially competitive a further reduction of enzyme loading is needed. Pretreatment has been shown to significantly reduce the amount of enzyme required to hydrolyse cellulosic substrates by enhancing the access of enzymes to the cellulose in the biomass (Mosier et al., 2005). It also generates various water-soluble compounds that have been shown to inhibit cellulose hydrolysis, significantly increasing the minimum enzyme loading needed to achieve effective hydrolysis. Thus, one of the ways to try to reduce the amount of enzyme required to hydrolyze cellulosic substrates would be to mitigate the influence of enzyme inhibition by the water soluble compounds during enzymatic hydrolysis of pretreated substrates.

Pretreatment; It has been shown that steam explosion can solubilize the majority of hemicellulose as well as redistributing some lignin within the lignocellulosic biomass, consequently increasing cellulose accessibility to the cellulase enzymes (Figure 1) (Arantes et al., 2011; Ohgren et al., 2007). It has proven to be one of the most common pretreatment processes that has been used by companies such as Abengoa, DSM-POET and Iogen, because of the low cost, low energy consumption and limited environmental pollution resulting from steam pretreatment (Buchholz et al., 2012). However, the various soluble components generated during steam explosion, including various degradation products derived from the hemicellulose and lignin fractions, are known to be inhibitory. These include mono/oligosaccharides, organic acids, furans and phenolics, all of which have been shown to inhibit the enzymatic hydrolysis of cellulosic biomass (Hodge et al., 2008; Yang et al., 2010). As a result, extensive washing has been commonly used to try to remove these water soluble components as one way of improving the hydrolysability of steam pretreated substrates (Frederick et al., 2013; Rajan and Carrier, 2014a). However, since this washing step requires the use of significant amounts of water and energy, and can also result in the loss of most of the hemicellulose derived sugars, it has proven difficult to use this type of "detoxification" process at a commercial scale (Bellido et al., 2011; Tengborg et al., 2001a; Um and Van Walsum, 2012; Zakaria et al., 2016). It would also be more economically attractive to include the hemicellulose-rich water-soluble fraction in the total amount of sugars derived from the pretreated biomass (Manzanares et al., 2011). Thus, considering the strong inhibitory effects of the pretreatment-derived, water-soluble inhibitors on cellulase enzymes, we need to develop cost-efficient inhibition mitigation strategies that we can enhance the enzymatic hydrolysis of pretreated biomass slurries using relatively low enzyme loadings. Thus, as will be described in this thesis, a comprehensive understanding of the major

group of inhibitors and their inhibitory mechanisms on the state-of-the-art cellulase enzyme systems would be beneficial.

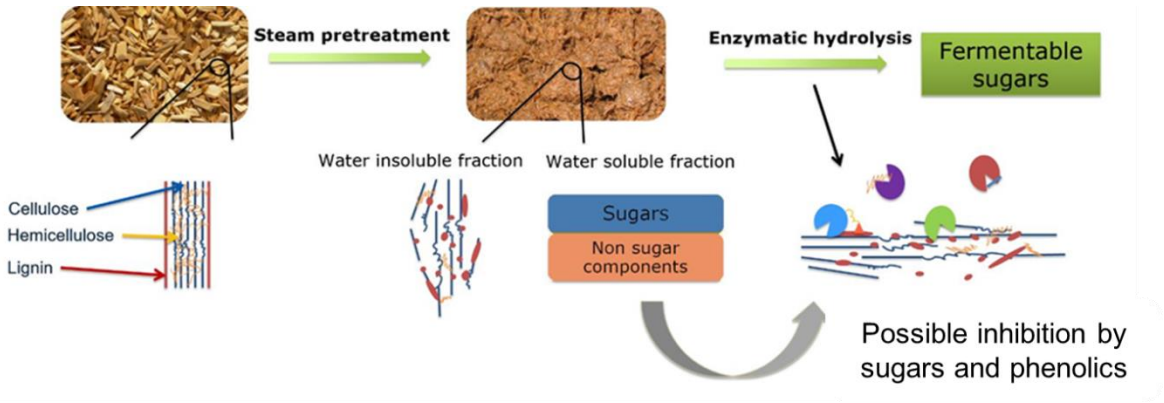

Figure 1 Process diagram of the steam pretreatment/enzyme hydrolysis mediated bioconversion of lignocellulosic biomass to fermentable sugars.

Potential inhibition by water soluble components; As mentioned earlier, the composition of the water-soluble inhibitors will be highly dependent on the nature of the biomass substrate and the pretreatment conditions used. However, several of the previous studies on enzyme inhibition were performed by using the water soluble fractions that were obtained under unrealistic conditions (i.e either too mild, so no inhibitors, or too severe, generating too many inhibitors) (Cantarella et al., 2004a; Palmqvist et al., 1996; Zakaria et al., 2016). Earlier work has shown that compromise pretreatment conditions are required so that a relatively easily hydrolysed cellulose-rich fraction and good hemicellulose sugar recovery can be achieved, thus optimizing the recovery of all of the sugars present in the original biomass (Chandra et al., 2007). Thus, for the majority of the work reported in this thesis, "compromise" steam pretreatment conditions were used, to generate more representative, but potentially inhibitory, soluble compounds. As mentioned earlier, another factor that will likely influence the inhibition studies is the nature of the biomass substrate used (Davison et al., 2013). As different types of biomass can vary in their composition, chemistry and mechanical properties, it is highly likely that the

water-soluble compounds generated from different types of biomass will have different inhibitory effects on the enzymes. One of the objectives in the thesis work was to study how the source of different woody biomass (e.g. hardwood and softwood) pretreated under the compromise conditions might affect the enzyme inhibition mechanisms of the water-soluble fractions.

Deconstruction enzymes; It has been shown that, for the cellulosic component of pretreated biomass to be effectively hydrolysed, a mixture of cellulases and the so called "accessory" enzymes/proteins are usually required. The major cellulases include endoglucanases (EGs), exoglucanases (CBHs), and β-glucosidase (BG), which act synergistically to break down the insoluble cellulose to glucose (Zhang et al., 2006). "Traditional" *Trichoderma reesei* (T. reesei) cellulase mixtures such as Novozymes' Celluclast 1.5L and Genencor International's Spezyme preparations, are primarily composed of various EGs and CBHs, and these preparations have been widely used over the last few decades in much of the published research into cellulose hydrolysis. However, these traditional cellulase mixtures have been shown to have a relatively low hydrolytic potential, often requiring high protein/enzyme loadings to achieve reasonable rates and yields of cellulose hydrolysis (Arantes et al., 2011). It has also been shown that these "traditional" enzymes are inhibited by a range of soluble inhibitors such as glucose, xylooligomeric sugars and phenolics (Kont et al., 2013; Palmqvist et al., 1996; Tengborg et al., 2001a). More recently, the enzyme companies have been able to enhance the hydrolytic potential of commercial cellulase preparations by adding various "accessory" proteins/enzymes which have significantly improved the hydrolytic performance of the traditional cellulase enzyme cocktails such as Celluclast. Accessory proteins/enzymes can include, various hemicellulases, lytic polysaccharide monooxygenases (LPMOs), and non-hydrolytic disrupting enzymes/proteins such as swollenin (Duarte et al., 2012). For example, it has been shown that

hemicellulases, such as xylanases, arabinanases and galactanases, can remove hemicellulose from lignocellulosic biomass, greatly improving the effectiveness of the cellulases by increasing cellulose accessibility (Gusakov, 2013; Hu et al., 2013; Wang et al., 2012). Similarly, copper-dependent LPMOs such as auxiliary activity family 9 (AA9) and auxiliary activity family 10 (AA10) have been shown to cleave the glycosidic bond on the organized crystalline cellulose regions through catalytic oxidation, significantly increasing the reactive sites of cellulose to cellulase enzymes (Hemsworth et al., 2013). The addition of non-enzymatic proteins such as swollenins and expansins can also improve the degradation of cellulose by disrupting the cellulose crystalline structure (Karimi, 2015). Thus, the addition of accessory proteins/enzymes to cellulase mixtures is known to greatly enhance the hydrolytic capability of traditional cellulase mixtures (Eijsink et al., 2008; Hu, 2014; Zhang et al., 2013). The recent cellulase preparations produced by Novozymes (such as Cellic CTec 1, 2 and 3) are rich in β-glucosidases, xylanase and AA9, resulting in the greater hydrolytic potential of these more recent "enzyme cocktails" on a range of cellulosic substrates, compared to the "traditional" enzyme mixtures such as Celluclast and Spezyme CP (Li et al., 2012). It has also been reported that more recent cellulase mixtures, such as Novozymes's CTec3, have better tolerance to glucose, likely because of added, supplemental β-glucosidases (Soderberg and Fuglsang, 2013). However, the tolerance of these enzyme cocktails towards other, soluble, potentially inhibitory compounds derived from the pretreatment process has not yet been determined. Thus, another objective of this study was to investigate whether pretreatment-derived inhibitors are inhibitory towards the more recent enzyme cocktails. We also wanted to determine if there are any specific inhibition mechanisms (such as reversible/irreversible) that affected either individual or the collective action of the cellulases and the "accessory" enzymes.

Potential enzyme inhibitors; As will be confirmed in the main body of the thesis, the biomass derived sugars and phenolics are the two major groups of inhibitors present in the water-soluble fraction that accounted for most of the observed decrease in cellulose hydrolysis. Interestingly, we also found that the type of the sugar that was present influenced sugar-mediated inhibition. As has been reported previously, glucose can directly inhibit β-glucosidase activity, resulting in the accumulation of cellobiose that consequently inhibits cellulase activity (Andrić et al., 2010a; Teugjas and Väljamäe, 2013a). Earlier work had also shown that, while hemicellulose derived sugars (such as xylose and mannose) were not inhibitory to enzyme activities on soluble model substrates, they were strongly inhibitory to the hydrolytic performance of the enzymes on the insoluble cellulose rich substrate (Xiao et al., 2004). As cellulose hydrolysis is an interfacial hydrolysis reaction, where the enzyme-substrate interaction such as enzyme processivity determines the hydrolysis efficiency (Costa et al., 2014; Nakamura et al., 2014; Payne et al., 2013), it is likely that xylose and mannose mediated inhibition was a result of sugar interference with the enzyme-substrate interaction. Previous work has also reported that the structural properties of cellulose, such as accessibility, degree of polymerization, crystallinity and surface morphology, may influence the binding of the deconstruction enzymes to the cellulose (Hidayat et al., 2012; Zhang and Lynd, 2004). This, in turn, likely influences the extent of enzyme inhibition. Thus, one of the other goals of the thesis work was to determine if the released sugars inhibited enzyme-substrate interaction, consequently reducing the extent of cellulose hydrolysis.

In contrast to monomeric sugars, phenolics derived from steam pretreatment are heterogeneous monomer/polymeric aromatic compounds, which have diverse functional groups and varied molecular size (Carrasco et al., 2012; Tejirian and Xu, 2011). Pretreatment-derived phenolics have been shown to be inhibitory to various cellulases (Michelin et al., 2016; Sineiro et al., 1997; Ximenes et al., 2010) and this inhibition has been shown to be more influenced by

the structure of the phenolics rather than their concentrations (Qin et al., 2016; Ximenes et al., 2010). Previous research on protein-phenolic interaction has suggested that the molecular size, and hydroxyl and carbonyl groups present in some phenolics play a key role in the binding of phenolics to protein (Carrasco et al., 2012; Tejirian and Xu, 2011). Thus, another objective of the thesis work was to investigate the influence of the structural properties of phenolics, such as their molecular weight, the nature of their hydroxyl and carbonyl groups, etc., on enzyme inhibition.

As the inhibitory effects of phenolics are likely to be influenced by their structure (Oliva-Taravilla et al., 2016) one of the goals of the thesis work was to assess if their inhibitory effect might be mitigated by modifying the phenolic structure. Ideally, if the phenolics could be modified during pretreatment while still increasing accessibility to the cellulose, this would be doubly beneficial. Previous work has reported that the addition of carbocation scavengers during acid-catalyzed pretreatment could minimize lignin condensation and reduce their inhibitory effect on cellulases (Pielhop et al., 2015). As the derived phenolics are a result of lignin degradation, it was hoped that such a strategy might modify the structure of the phenolics and alleviate their inhibition against cellulases. Thus, the objective of this part of the research was to see if carbocation scavengers could mitigate the inhibitory effect of phenolics. In this way we hoped to improved whole cellulose slurry hydrolysis using relatively low enzyme loading.

1.2 Sugar based biorefinery

Traditional fossil fuels are finite and will eventually become too expensive or will “run out”. Their continued and increasing use is also one of the primary reasons why carbon is accumulating in the atmosphere, resulting in climate change (Khatib, 2012). This is also one of the main motivations driving the development of “renewable fuels/chemicals” in many countries around the world (Smeets et al., 2007). It is worth noting that biomass is more evenly distributed

around the world than oil. Compared to other renewable energy sources such as wind and solar, biomass has the potential to generate energy-condensed liquid fuel and various chemical products that have traditionally been produced by fossil fuels (Smeets et al., 2007). Although the production of biofuels from starch and sugar feedstocks such as corn and sugarcane has been commercialized for some time, there are ongoing food-versus-fuels concerns (Solomon and Johnson, 2009). Thus if the fuels and chemicals could be made from biomass rather than starch, this would be very beneficial. Lignocellulosic biomass is the most abundant organic carbon source on Earth, with an annual production of about 5.64×10^{10} Mg-C (Field et al., 1998).

A typical bioconversion process includes a physicochemical pretreatment step, followed by an enzymatic hydrolysis step to breakdown the polysaccharides (cellulose and hemicellulose) to monomeric sugars. Although the hydrothermal (steam) pretreatment step opens up the cell wall structure and increases the accessibility of cellulose to cellulases, relatively high enzyme loading is still required for to achieve efficient enzymatic hydrolysis of the cellulosic component of the pretreated biomass (Humbird et al., 2011; Kazi et al., 2010). One of the major reasons for the high enzyme dose demand is that various pretreatment-derived inhibitors (mainly resulting from hemicellulose and lignin degradation) inhibit cellulase activities, and thus decrease the hydrolytic potential of the cellulase cocktails. Although previous work has shown that water washing can reduce the toxicity towards the cellulase enzymes (Rajan and Carrier, 2014a; Söderström et al., 2004; Tengborg et al., 2001a), this approach will result in the loss of hemicellulose-derived oligo/monomeric sugars and the need for significantly more water and processing. An alternative to washing might be to assess the potential of using the whole pretreated "slurry" as the substrate for the downstream enzymatic hydrolysis process. To do so, we need to better understand the nature of inhibitory compounds generated during pretreatment

as well as their inhibitory influence on the deconstruction enzyme cocktail. This is the main focus of the thesis work

1.3 Recalcitrance of woody biomass

One of the objectives of the thesis was to better characterise the major inhibitory compounds derived from biomass pretreatment and to assess their possible inhibitory effects on the enzyme-mediated cellulose hydrolysis. To provide some background, the lignocellulosic biomass substrate and its physical properties are briefly discussed in this section.

Biomass sources can include agricultural and woody biomass, municipal solid waste and industrial processing wastes. Woody biomass has attracted a lot of attention as it is found in most parts of the world. Only about 5% of the world's trees are used for various traditional products such as furniture, pulp, paper, clothing and construction materials (Thoen and Busch, 2006). The residues obtained from the processing of these products and the forest residue remaining after tree harvesting will probably be the first sources of biomass used for bioenergy and biofuels production.

Woody biomass can be grouped into softwoods and hardwoods. Some hardwoods, like poplar, have been studied as possible energy crops because of their fast growth and high yield (Gasol et al., 2009). Softwoods tend to be more abundant and evenly distributed in North America and have thus been considered as alternative for biorefinery in these countries and areas (Zhang, 2014). For example, lodgepole pine is one of the most abundant wood species in British Columbia (BC), Canada, and accounts for a quarter of total amount of forest in BC. Over the last 10-15 years, the mountain pine beetle (MPB) has infested a large amount of the lodgepole pine forest in BC. As these trees die and the wood dries out, logs from these infested trees cannot be readily used to produce traditional lumber products (Taylor et al., 2006). However, the

increasing amount of residue obtained when these logs are sawn has produced much of the sawdust feedstock that is used for BC's wood pellet sector. This sawdust residue could also be readily used as a feedstock source for a biorefinery.

Wood has a complex and compact cell wall organization, consisting mainly of cellulose, hemicellulose lignin and extractives. This makes the cell wall extremely recalcitrant to microbial/enzyme destruction (Figure 2). In the following part of the thesis, the major characteristics of these components in lignocellulosic biomass and their effect on cellulose hydrolysis are discussed.

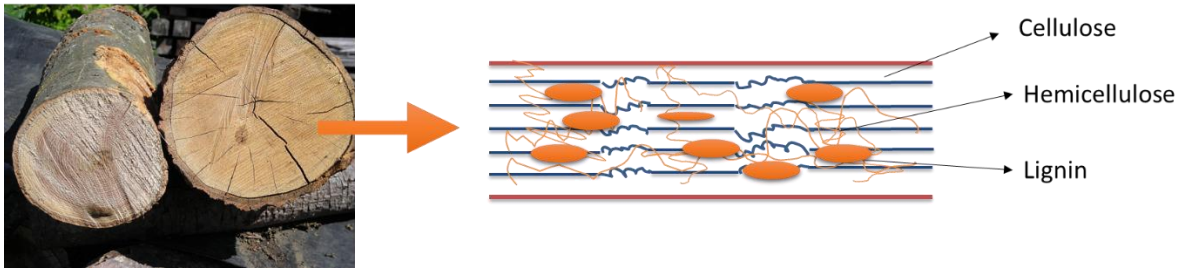

Figure 2 Simple diagram of lignocellulose structure.

Cellulose, like starch, is a homopolysaccharide of D-glucose units, accounting for 40-55% of total dry weight of woody biomass (Kafle et al., 2015). Unlike starch (α (1→4) linked D-glucose), the linear cellulose molecules (β (1→4) linked D-glucose) are pulled together by hydrogen bond and van der Waals force, which greatly limit the accessibility of the glycosidic bonds in cellulose to cellulase enzymes (Figure 3). Some of the properties of the packed cellulose structure include crystallinity, degree of polymerization, surface area and overall accessibility. The most commonly found cellulose crystalline structure is cellulose I, including cellulose Iα and cellulose Iβ. These can be further altered into other allomorphs such as cellulose II and cellulose III during thermochemical treatment (Zhang and Lynd, 2004). The change in the organization of the cellulose molecules affects the binding affinity of cellulases to cellulose. Thus, different cellulose allomorphs exhibit different enzymatic hydrolysis profiles (Lynd et al.,

2002). The degree of polymerization can also affect overall cellulose hydrolysis (Puri, 1984; Zhang and Lynd, 2004). The lower degree of polymerization likely indicates there are more reducing/non-reducing ends available for cellulase enzymes to act on, suggesting the substrate might be more hydrolysable. Besides crystallinity and degree of polymerization, the cellulose surface acid group content may also affect cellulose hydrolysis. Previous work has suggested that the acid groups on the cellulose surface may influence the binding of cellulase enzymes to cellulose (Jiang et al., 2013). However, so far, there is no general agreement on which substrate factor is the main driver that affects the enzymatic hydrolysis of cellulose. It more likely that they all, to varying degrees, influence the interactions of cellulases with cellulosic substrates.

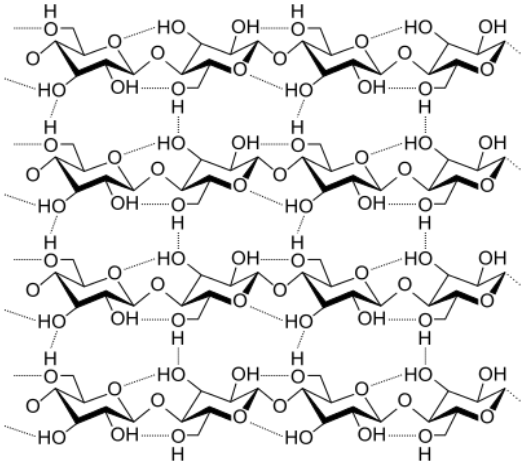

Figure 3 Supramolecular structure of cellulose I with inter- and intramolecular hydrogen bonds (adapted from Credou and Berthelot, 2014).

Hemicellulose constitutes 20-35% of the total woody biomass, which functions as supporting material, strengthening the plant cell wall through interaction with cellulose and with lignin (Figure 4). The hemicelluloses in woody biomass include xylan and mannan. They are frequently acetylated and branched with side chain groups such as 4-O-methyl ester and uronic acid (Gírio et al., 2010; Scheller and Ulvskov, 2010). For hardwoods, glucuronoxylan is the

principal hemicellulose component, constituting 15-30% of the biomass. For softwoods, *O*-acetyl-galactoglucomannan is the major hemicellulose, constituting around 20% of the biomass (Sjostrom, 2013). The xylan and mannan have varied side chains and lower degree of polymerization (50-300). As a result of its flexible and branched structure, hemicellulose often serves as bridge between cellulose and lignin (Salmen et al. 1998). Some sugars in the hemicellulose help form the lignin-carbohydrate complexes (LCC) through covalent linkage, restricting biomass from easy chemical- and/or enzyme-catalyzed deconstruction (Chundawat et al., 2011a). Hemicellulose is thought to interact closely with the cellulose, strongly limiting the accessibility of cellulose to cellulases. Thus, it has been suggested that the removal/recovery of the hemicellulose during the pretreatment process would aid enzyme mediated cellulose hydrolysis.

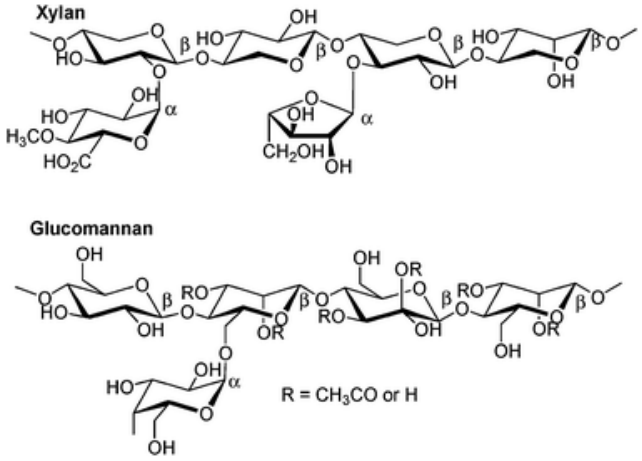

Figure 4 Predominant forms of hemicelluloses in hardwoods and softwoods: (a) xylan, (b) glucomannan (Dutta et al., 2012).

Lignin serves as a physical and chemical barrier in plants, interacting with hemicellulose and cellulose and making the cell wall structure more rigid and impermeable. Lignin is a polyphenolic polymer which is mainly composed of guaiacyl unit (coniferyl, G), syringyl unit (sinapyl, S), and 4-hydroxyphenyl unit (para-coumaryl, H) (Figure 5, 6). The structure and

composition of lignin vary in different types of woody biomass. For example, the lignin from Scots Pine contains 2% H and 98% G, while the lignin from poplar has a 41% G and 59% S content (Baucher et al., 1998). In addition, softwood lignin can contain a higher percentage of β-5 and 5-5 than hardwood lignin, making it more recalcitrant toward acid hydrolysis during the pretreatment process (Table 1). Due to structural differences, softwood lignin tends to experience more condensation during steam pretreatment as a result of a higher ratio of G units and a more cross-linked structure (Li et al., 2009).

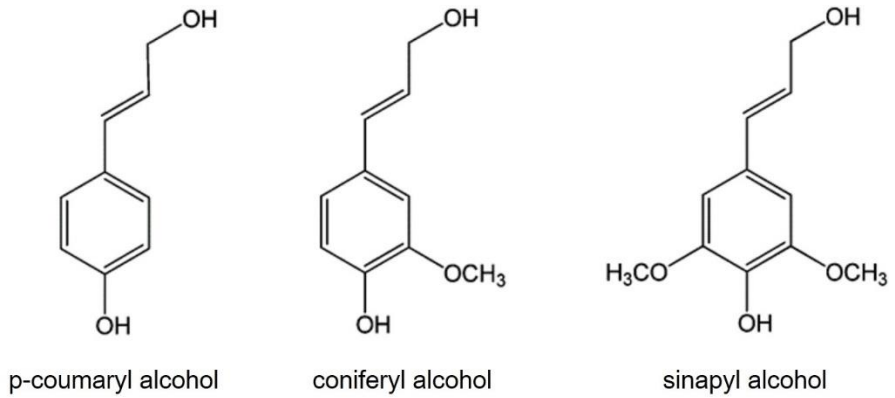

Figure 5 The phenylpropane units that make up the basic structure of lignin (Adler, 1977)

Table 1 Types of linkages and dimeric structures (Adler 1977)

Linkage	Dimer structure	Percent (%) of the total linkages	
		Softwood	Hardwood
β-O-4	Arylglycerol-β-aryl ether	50	60
α-O-4	Noncyclic benzyl aryl ether	2-8	7
β-5	Phenylcoumaran	9-12	6
5-5	Biphenyl	10-11	5
4-O-5	Diaryl ether	4	7
β-1	1,2-Diaryl propane	7	7
β-β	Linked through side chain	2	3

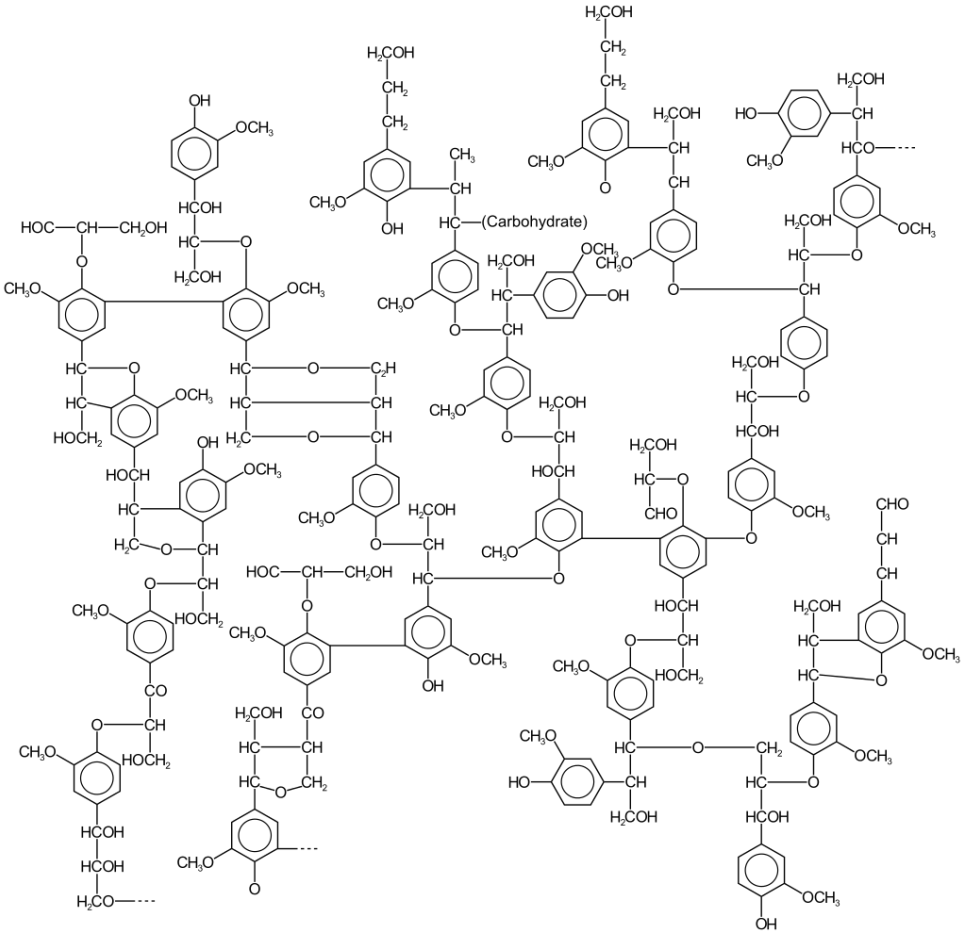

Figure 6 Lignin structure in woody biomass (https://en.wikipedia.org/wiki/Lignin)

Extractives, which account for 2-5 % of the woody material, exist mainly in the heartwood and bark. They include heterogeneous groups of substances such as terpenoids, steroids, fats, waxes, and phenolic constituents (Figure 7) (Sjostrom, 2013). Certain wood extractives that have a similar structure to non-ionic surfactants, can exhibit a positive influence on overall cellulose hydrolysis, while relatively hydrophobic extractives such as triolein and docosane strongly inhibited cellulase activities (Leskinen et al., 2015). These contradictory findings may be related to structural difference in these extractives.

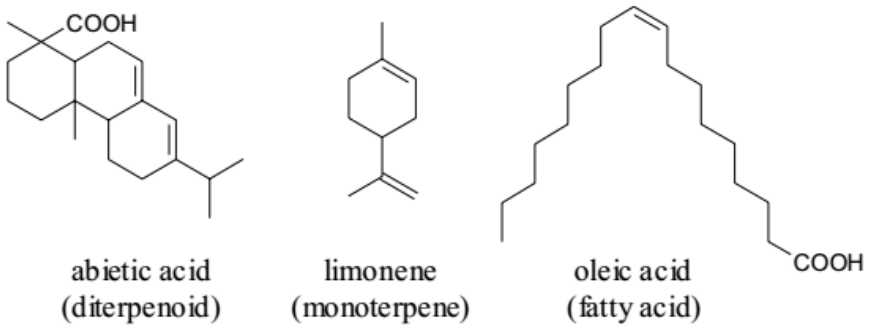

Figure 7 Examples of extractives molecules from different categories: diterpenoids, monoterpenes, and fatty acids (Sjostrom, 2013).

1.4 Steam pretreatment at "compromise" conditions

As mentioned earlier, to overcome the recalcitrance of biomass, a pretreatment step is typically needed to open up the cell wall structure and to increase the accessibility of cellulose to the deconstruction enzyme cocktail (Pribowo et al., 2012). A range of pretreatment methods such as physical, biological, mechanical and chemical methods have been studied to try to improve the digestibility of woody biomass (Chandra et al., 2007). Some widely studied pretreatment methods include steam explosion, dilute acid, hot water, alkaline extraction and ammonium fiber explosion (Hamelinck et al., 2005; Taherzadeh and Karimi, 2007). However, several of these methods are applicable to only certain types of biomass, some have strict operating requirements such as fine particle-size feedstock (sawdust instead of wood chips), and others require high chemical consumption (e.g. around 10:1 solve-biomass ratio are needed for ionic liquid or phosphoric acid pretreatment) (Sathitsuksanoh et al., 2012). In contrast, steam pretreatment works on most lignocellulosic biomass and requires less than 5% chemical loading, which is substantially lower than other pretreatment methods (Galbe and Zacchi, 2012). Steam pretreatment also generates a whole slurry (a mixture of water soluble fraction and water

insoluble fraction) that has a high solid content, which is beneficial if we are to achieve high product concentration in the subsequent processing steps.

During steam pretreatment the biomass is heated, often with addition of an acid catalyst such as sulfur dioxide (1-4%), at high temperatures (180 to 240 °C) and pressures (1 to 3.5 MPa) for a short period of time (5-10 min) (Pandey et al., 2014). Subsequently, these pretreated/cooked biomass material are rapidly released (i.e. exploded) into a collecting tank. During this process, the woody biomass experiences physical and chemical disruption. The destruction of some of the components in the biomass (i.e. sugars to furans) is greatly dependent on the pretreatment severity. Severity can be expressed by a single factor "R_o" (R_o = t exp(T-100)/14.75), that combines the effect of time (t) and temperature (T). At lower severities (Log Ro = 3-3.5), the acid hydrolysis of the glycosidic bonds takes place in the presence of H^+ (Tahiliani, 2007). Initially, the hydrogen atom is attached to the oxygen of the 1-4 glycosidic bond, breaking the glycosidic bond. A proton is then added to one of the oxygen's on a sugar unit and a hydroxyl group added to the pyranose ring of another sugar unit (Tahiliani, 2007).

During pretreatment, part of the amorphous region of the cellulose can be hydrolysed to soluble sugars. The degree of polymerization is also significantly decreased as a result of acid hydrolysis (Sannigrahi et al., 2010). In addition, it is likely that the cellulose surface is also modified with acid groups (Araki et al., 1999). Similarly, the hemicellulose component is hydrolysed to low molecular weight monomers or oligomers, consequently increasing the accessibility of cellulose to the enzymes. The low-molecular-weight hemicellulose-derived sugars are readily dissolved in water and constitute the major soluble components in the water soluble fraction (Shevchenko et al., 2000). When the pretreatment temperature is increased above the glass transition temperature of lignin (100-160 °C), the lignin is softened and

redistributed in the lignocellulosic matrix. This should result in more accessible cellulosic substrates (Selig et al., 2007).

At relatively high pretreatment severities, some of the monomeric sugars such as glucose and xylose are degraded into 5-hydroxymethylfurfural (HMF) and furfural respectively (Bellido et al., 2011; Larsson et al., 1999; Suryawati et al., 2009; Um and Van Walsum, 2012). These furans can be further dehydrated to formic acid and levulinic acid (Arora et al., 2013; Cantarella et al., 2004a; Um and Van Walsum, 2012). In addition, many of the acetyl groups within the hemicellulose are cleaved, releasing acetic acid into the water-soluble fraction. As a result of high pretreatment severity, partial lignin is depolymerized through the cleavage of acid labile linkage such as β-O-4 ether, generating carbocation intermediates (Rajan and Carrier, 2014b). As the carbocation intermediates are unstable, they can either repolymerize with the nucleophiles within the lignin structure or depolymerized into various water soluble phenolics such as "Hibbert's ketones" (phenylpropanoid ketones) (Canilha et al., 2012; Chandel et al., 2013).

As a result of these various mechanisms, the optimum pretreatment conditions where maximum sugar recovery and the most digestible cellulosic component can be obtained are highly substrate and pretreatment dependent. Although lower steam pretreatment severities (Log Ro = 3-3.5) removed most of the hemicellulose and generated less inhibitory compounds (Boussaid et al., 1999; Ewanick et al., 2007), higher pretreatment severities (Log Ro>4.0) result in more hydrolysable pretreated substrates at the expense of the significant degradation of the hemicellulose sugars (Boussaid et al., 2000). Thus, a medium severity has been recommended by many previous studies as a way of compromising sugar degradation while enhancing the digestibility of the cellulosic substrates (Boussaid et al., 2000; Ewanick et al., 2007; Wu et al., 1999).

1.5 Enzyme-mediated cellulose hydrolysis

An effective deconstructive enzyme mixture needs to contain multiple hydrolytic cellulase and accessory enzymes if efficient cellulose hydrolysis of a pretreated substrate is to be achieved. A "typical cellulase mixture" includes endoglucanases (EGs), cellobiohydrolases (CBHs) and β-glucosidases (BG), which work together on the insoluble cellulose to break it down to glucose. The EGs non-processively cleave internal glycosidic bonds in the cellulose chains at less organized position in order to create more glucan chain ends. The CBHs act processively to release mainly cellobiose from the reducing and non-reducing ends of the cellulose. The BG's then further hydrolyse the cellobiose to individual glucose monomers. These three types of "cellulase enzymes" have been shown to synergistically break down the cellulose to glucose (Mba Medie et al., 2012).

"Traditional" enzyme cocktails such Celluclast and Spezyme CP are composed of mainly EGs and CBHs. However, high enzyme loadings of these enzyme mixtures, with supplementation of BG, have often been required to achieve efficient cellulose hydrolysis (Arantes et al., 2011; Tengborg et al., 2001b). In recent years, several accessory proteins/enzymes, which do not directly hydrolyse cellulose but can greatly improve cellulose accessibility to cellulases, have been added to these traditional cellulase enzyme cocktails (Gao et al., 2011; Hu et al., 2013, 2011). This has greatly improved the hydrolytic performance of these enzyme mixtures, especially on the pretreated lignocellulosic biomass. For example, hemicellulases, such as xylanases, arabinanases and galactanases, can remove hemicellulose from lignocellulosic biomass and greatly improve the effectiveness of cellulases by increasing cellulose accessibility (Gusakov, 2013; Hu et al., 2013; Wang et al., 2012). In a complementary fashion, swollenins and expansins seem to be able to enhance cellulose hydrolysis by increasing

the accessibility of cellulose and opening up the structure of the substrate (Arantes and Saddler, 2010; Gourlay et al., 2013; Saloheimo et al., 2002). It has also been shown that auxiliary activity family 9 (AA9) and auxiliary activity family 10 (AA10) enzymes can significantly improve cellulose hydrolysis through opening up the crystalline cellulose region, through an oxidative mechanism, in the presence of a metal cofactor and electron donor (Hemsworth et al., 2013; Horn et al., 2012b). By adding these accessory enzymes to cellulase mixtures, the hydrolytic potential of the traditional cellulase mixtures on various biomass substrates has been improved significantly (Eijsink et al., 2008; Hu, 2014; Zhang et al., 2013).

It is apparent that commercially available cellulase preparations have been improved significantly over the past decade through a better understanding of the roles and functions of cellulases and accessory enzymes. More recently developed enzyme cocktails (such as CTec1, 2 and 3 from Novozymes) all include high β-glucosidase and xylanase content, making these enzyme cocktail much more efficient at hydrolyzing a range of cellulosic substrates when compared to "traditional" enzyme mixtures such as Celluclast and Spezyme CP (Quinlan et al., 2011; Teter, 2012). Besides these accessory activities, the enzyme producers have also claimed that these new enzyme cocktails are more inhibitor-tolerant towards the degradation products derived from pretreatment processes (Soderberg and Fuglsang, 2013). However, as described in the main body of the thesis, to facilitate whole slurry hydrolysis, further studies are still needed to better understand the inhibitor tolerance of these more recent enzyme cocktails.

To better understand the possible inhibitor-tolerance of these more recent enzyme cocktails, it is important to better understand the major function and properties of the major enzyme components within the cocktails. Thus, the mode of action of the deconstructive enzymes are discussed in more detail in the following sections.

1.5.1 Cellobiohydrolases

Cellobiohydrolases (CBHs) attack the 1,4- β-D-glucosidic linkages of cellulose from both the reducing or non-reducing end, releasing mainly cellobiose (Kurašin and Väljamäe, 2011; Medve et al., 1998). It has been shown that CBHI works from the reducing end of cellulose. It typically constitutes about 40-60% of total cellulase protein (Levasseur et al., 2013; Zhang et al., 2006). In contrast, CBHII works from the non-reducing end of the cellulose and typically accounts for 20-30% of total cellulase protein (Barr et al., 1996; Momeni, 2014). According to the database of Carbohydrate-Active Enzymes (CAZy), CBHI and CBHII can also be classified as Cel7A and Cel6A respectively, based on the amino acid sequence similarities (Claeyssens et al., 1989; Vršanská and Biely, 1992). Both enzymes are considered to be processive enzymes that can cleave cellulose chains consecutively for a period of time, without dissociating from the cellulose chain (Davies and Henrissat, 1995; Divne et al., 1994).

Compared to other cellulases, Cel7A is the most abundant enzyme secreted by *T. reesei.* It is thought to be the most essential enzyme which determines the overall efficiency of cellulose hydrolysis (Ilmen et al., 1997). Cel7A (52-kDa) contains a catalytic domain (CD) and a carbohydrate binding module (CBM), which are connected by a highly glycosylated linker. The Cel7A CD contains a tubular active centre that can cleave the glycosidic bonds from the reducing ends of cellulose, with cellobiose as the major product (Divne et al., 1994). The Cel7A CBM has a planar binding surface that can strongly bind to the cellulose surface.

The catalytic action of the Cel7A CD requires hydrogen bonding and stacking interactions with four essential tryptophan residues. They rotate the cellulose chain to locate the β-1,4-glycosidic linkage in the appropriate position for hydrolytic action (Momeni, 2014). Subsequently, the two glutamic acid residues in the active site catalyze the breakage of the β-1,4-

glycosidic linkage, releasing cellobiose through a double displacement retention mechanism (Zechel and Withers, 2000). However, it has been shown that the accumulation of the cellobiose seems able to compete with the cellulose chain for the substrate binding site in the catalytic domain (Bu et al., 2011). It also has been reported that the lignin in the pretreated biomass can also interfere with the action of the Cel7A catalytic domain through strong lignin-enzyme interactions (Strobel et al., 2015). Thus, it is likely that lignin derived phenolics might affect the action of the Cel7A catalytic domain.

It has been proposed that an important role of Cel7A carbohydrate binding module (CBM) is to increase the proximity of the CD to its targeted substrates, thus enhancing the catalytic efficiency of the CD (Lehtiö et al., 2003). It has been shown that the aromatic amino-acid residuals on the planar surface of the CBM are essential for its binding to cellulose through hydrophobic and hydrogen bond interactions. They are also thought to be responsible for the non-productive binding of Cel7A to lignin (Lehtiö et al., 2003). Thus, the strong hydrophobicity of CBM in Cel7A may drive the binding of Cel7A to some hydrophobic compounds, thus affecting overall enzyme activity.

As mentioned early, the two domains (CD and CBM) are linked by glycosylated linker peptides (30–40 amino acid) with the amino acid composition typically rich in proline, threonine, serine and hydroxyl amino acids (Srisodsuk et al., 1993). The linker is usually glycosylated, which improves the binding of enzyme to cellulose (Srisodsuk et al., 1993). The linker peptide also represents flexible hinges between the CD and CBM, facilitating their independent function (Srisodsuk et al., 1993). These workers have shown that the deletion of the linker region significantly decreases the hydrolysis rate of the enzyme on crystalline cellulose even though the Cel7A binds to the substrate (Srisodsuk et al., 1993). This suggests that it is desirable to keep sufficient spatial separation of the two domains (CD and CBM) for the efficient functioning of

the enzyme. However, it seems that the glycosylated linker may participate in non-productive adsorption to lignin, which likely contributes to enzyme inhibition (Strobel et al., 2015). Thus, it is likely that the lignin-derived phenolics in the water-soluble fraction may also interact with the glycosylated linker within Cel7A.

As a result of its special protein structure, Cel7A can move processively on the cellulose surface and release cellobiose as its major product. Thus, the processivity of Cel7A is considered to be an important characteristic as it enhances the Cel7A's ability to degrade crystalline cellulose (Kipper et al., 2005; Momeni, 2014). The processive Cel7A releases mainly glucose from this initial cleavage while producing cellobiose in the following processive movement. Thus, many studies have used the ratio between glucose and cellobiose to indicate the Cel7A processivity. This has proven to be a fairly accurate method when using high resolution microscopy (Fox et al., 2012; Horn et al., 2012a; Nakamura et al., 2014). The processivity of Cel7A is closely related to the binding affinity of the enzyme to the substrate. As indicated earlier, a stronger enzyme-cellulose interaction will usually give a higher processivity of Cel7A (Nakamura et al., 2014). Since the substrate characteristics will affect the enzyme-substrate interaction (Gao et al., 2013), it is reasonable to assume that the substrate characteristics will influence the processivity of Cel7A.

As Cel7A has activity on model cellulosic substrates and soluble model compounds (such as p-nitrophenyl cellobioside and p-nitrophenyl-lactoside) its activity is often measured by using filter paper or Avicel (Sharrock, 1988) or soluble model compounds (Dashtban et al., 2010; Sharrock, 1988). Although using small soluble substrates for enzyme activity may not fully reflect the action of Cel7A on insoluble cellulose substrates, it helps to better understand the catalytic capabilities of Cel7A.

1.5.2 Endoglucanases

Endoglucanases (EGs) include at least five endoglucanases (EGI, EGII, EGIII, EGIV, and EGV) within the cellulolytic system of *T. reesei*. Among these EGs, EGI and EGII account for more than 80% of the endoglucanase activities (Suominen et al., 1993). Specifically, EGI (also known as Cel7B) accounts for 6-10% of total protein produced by *T. reesei* (Viikari et al., 2007), while EGII (also known as Cel5A) constitutes another 1-10% (Markov et al., 2005). EGI has been shown to decrease the degree of polymerization of cellulose (Nakazawa et al., 2008). However, EGII reduces the degree of polymerization of cellulose more efficiently when compared to EGI (Markov et al., 2005).

As EGs randomly hydrolyses glycosidic bonds in the more disorganized regions of cellulose, their activities can usually be determined by using soluble cellulose derivatives such as carboxymethyl cellulose (CMC) or cellulose derivatives substituted with dyes (chromogenic CMC) (Dashtban et al., 2010). It should be noted that glucose and cellobiose are reported to be inhibitory to EGs (Gruno et al., 2004; Murphy et al., 2013). This might be one of the factors that that results in poor cellulose hydrolysis when high sugar concentrations are present during enzymatic hydrolysis.

1.5.3 β-glucosidases

β-glucosidases (BG) are key enzyme cocktail components if efficient cellulose hydrolysis is to be achieved, as these enzymes break down cellobiose to glucose, greatly alleviating the inhibition of cellobiose on CBHs and EGs (Korotkova et al., 2009). However, the original *T.reesei* only produced relatively low amounts of BG, making the cellulase mixtures highly sensitive to end-product inhibition (Korotkova et al., 2009). Therefore, BG's from alternative

sources such as *Aspergillus niger* were commonly added to *T.reesei* cellulase mixtures, to improve their ability to hydrolyze cellulosic substrates (Korotkova et al., 2009). For example, the "traditional" commercial cellulase preparation Celluclast, is typically supplemented with a commercial BG preparation such as Novozyme 188. Considering the importance of BG, many studies have tried to develop more glucose-tolerant BG to further improve cellulose hydrolysis. In addition to glucose, soluble phenolics were found to be highly inhibitory to BG, although the extent of inhibition is highly dependent on the source of commercial enzyme preparations (Ximenes et al., 2010).

1.5.4 Xylanases

Hemicellulases include a wide range of enzymes which can hydrolyse the hemicellulose present in various types of biomass (Gusakov, 2013). As xylan is one of the major hemicellulose components in hardwoods, xylanases have been widely studied (Kulkarni et al., 1999) and have also been added to many recent commercial enzyme preparations such as CTec3 (Ohgren et al., 2007).

Xylanases can be grouped into glycosyl hydrolase families 5, 7, 8, 10, 11 and 43. Among these enzymes, GH10 and 11 xylanases are the most abundant although they have different structures and substrate specificities (Biely et al., 1997). Xylanases from GH11 contain a single catalytic domain with a β-jelly roll structure (Paës et al., 2012), while xylanases from GH10 have a two domain structure with the catalytic domain containing a TIM-barrel structure (Pollet et al., 2010). Because of structural differences, xylanases from GH10 need only two unsubstituted xylose residues for effective activity and they can attack a glycosidic bond near a substituted xylose residue. GH11 xylanases require more than three consecutive, unsubstituted xylose residues for enzymatic hydrolysis (Paës et al., 2012). It has been reported that both GH10 and

GH11 xylanases are strongly inhibited by phenolic compounds, resulting from structural changes to these xylanases (Boukari et al., 2011).

1.6 Inhibition of enzyme mediated cellulose hydrolysis by the water-soluble fractions derived from pretreated wood

As mentioned earlier, the major enzyme components in "cellulase" cocktails can be inhibited by various soluble compounds such as sugars and phenolics. It has also been shown that overall enzymatic hydrolysis is greatly decreased by the water soluble compounds derived from pretreatment which contain large amount of mono/oligomeric sugars and lignin degradation products (Jönsson and Martín, 2015; Jönsson et al., 2013).

Monomeric sugars (such as xylose, mannose, galactose, and glucose) and solubilized oligomeric sugars (such as glucooligomeric, xylooligomeric, and mannooligomeric sugars) are the major components presented in the liquid phase after steam treatment (Figure 8) (Jönsson and Martín, 2016). In addition, phenolic compounds, generated from both lignin and extractives, are another major group of water soluble components that generated during steam pretreatment (Jönsson et al., 2013). However, their content and composition are highly dependent on the nature of the pretreatment conditions, the biomass and the p-hydroxyphenyl/guaiacyl/syringyl (H/G/S) ratio of the original biomass lignin (Klinke et al., 2004). Besides sugars and phenolics, organic acids such as formic and levulinic acids and furans such as furfural and hydroxymethylfurfural (HMF) are also generated due to hexose and pentose degradation during pretreatment (Jönsson et al., 2013; Palmqvist and Hahn-Hägerdal, 2000; Panagiotou and Olsson, 2007). HMF and furfural are formed due to hexose and pentose sugar degradation, while formic

acid and levulinic acid are formed due to furfural and HMF degradation (Duarte et al., 2012; Palmqvist and Hahn-Hägerdal, 2000).

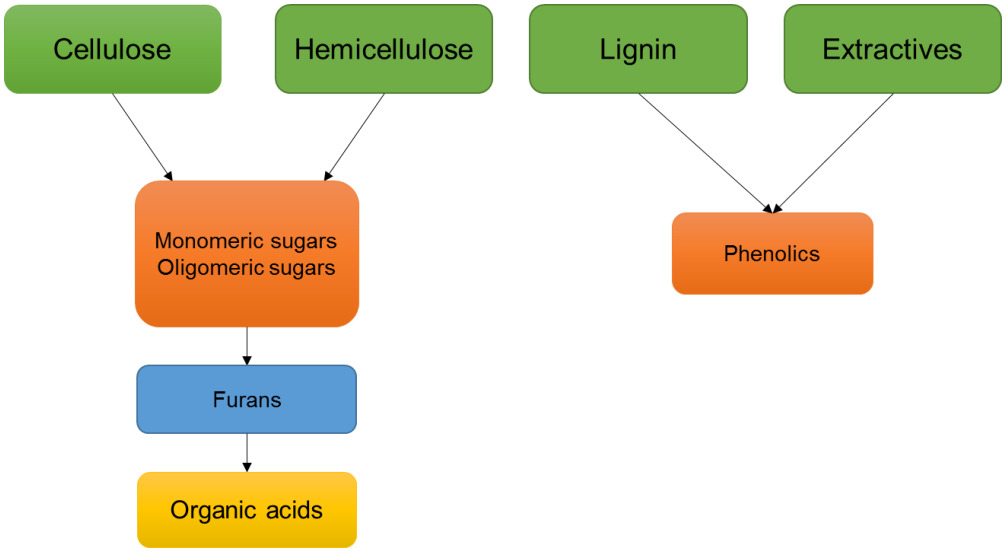

Figure 8 Formation of inhibitors during acid-catalyzed pretreatment of lignocellulosic biomass (Jönsson et al., 2013).

A large portion of these water soluble degradation products will be strongly associated with the pretreated lignocellulosic substrate even after filtration. This, in turn, makes the pretreated biomass less hydrolysable owing to the "toxicity" of this fraction to the enzyme mixture (Cantarella et al., 2014; Humpula et al., 2014; Jing et al., 2009; Kim et al., 2011). Therefore, in much of the previous work in this area, after steam pretreatment, the whole biomass slurry is typically separated into the water insoluble fraction (mainly cellulose and lignin) and the water soluble fraction (mainly hemicellulose derived mono/oligomeric sugars as well as phenolics and sugar degradation products). The water insoluble fraction (WIF) is usually washed extensively with fresh water to remove the residual water-soluble inhibitors, before enzymatic hydrolysis of this cellulose rich fraction (Palmqvist et al., 1996; Söderström et al., 2004; Tengborg et al., 2001a). However, it would likely be more economically attractive to perform the enzymatic hydrolysis using the whole biomass slurry (which includes both the hemicellulose-rich water

soluble and cellulose-rich, water insoluble fractions), not only increasing the concentration of the final monomeric sugars but also eliminating the need for the filtration and washing steps. However, in order to achieve an efficient whole slurry hydrolysis at relatively low enzyme loading, it is important to have a better understanding of enzyme inhibition mechanism by the pretreatment-derived soluble compounds.

Although the inhibitory effect of these soluble components on the downstream fermentation process has been extensively studied (Jönsson et al., 2013; Palmqvist and Hahn-Hägerdal, 2000), the information of their inhibitory effects on enzymatic hydrolysis is still quite limited. Earlier work has shown that the water soluble fraction derived from steam pretreated wood strongly inhibited the enzymatic hydrolysis of cellulose (Mes-Hartree and Saddler, 1983). However, the nature of the inhibitory components was not clearly identified. Later work indicated that monomeric sugars were the major group of inhibitors that accounted for most of the decrease in cellulose hydrolysis (Hodge et al., 2008) while other studies suggested that phenolics were the most inhibitory components (Ximenes et al., 2010). In addition, furans and organic acids have been also been shown to be highly inhibitory to the catalytic activities of cellulase enzymes on cellulosic substrates (Arora et al., 2013). However, since various conditions such as enzyme loading, the nature of the biomass substrate, pretreatment conditions, hydrolysis conditions, etc., have been used in this previous work, it is difficult to determine which groups of soluble compounds are the major groups of inhibitors for enzymatic hydrolysis. It seems that all of the major groups of soluble compounds such as sugars, phenolics, furans or organic acid may all have some inhibitory effect on deconstructive enzyme cocktail (Table 2).

Table 2 Summary of papers describing cellulase enzyme inhibition studies by soluble compounds

Enzyme mixtures	Solid loading (%, w/w) and substrates	Focus/Key Results/Conclusion	Ref.
Cellulase complex of *Trichoderma harzianum* E58	5% Solka Floc	Identification of inhibition effect of specific chemical compounds Key Result: Furans are not inhibitory to the enzymes	(Mes-Hartree and Saddler, 1983)
Celluclast 2L Novozyme 188	5% steam pretreated softwood	Influence of the low concentration of pretreatment liquors from softwood on cellulose conversion Key Result: The cellulose conversion was reduced by up to 36%, when pretreatment liquor is included	(Tengborg et al., 2001a)
Spezyme CP Novozyme 188	4% Pretreated corn stover	Model phenolic compounds inhibition Key Result: Tannic acid is the most inhibitory phenolic compound.	(Ximenes et al., 2010)
Spezyme CP Novozyme 188	1% Pretreated Solka Floc	Inhibitory effect of specific chemical compounds Key Result: The order of inhibitory strength is: phenolic lignin derivatives > sugars > organic acids > furans.	(Kothari and Lee, 2011)
Spezyme CP Novozyme 188	1% Solka Floc	Inhibitory effect of specific chemical compounds Key Result: Phenolic compounds from Hot water pretreated Maple are the most inhibitory chemical compounds	(Kim et al., 2011)

1.7 Factors which might affect the extent of enzyme inhibition by pretreatment derived inhibitors

1.7.1 Pretreatment conditions

As mentioned several times previously, the pretreatment conditions used will greatly affect the composition and concentration of components present in the water-soluble fractions and thus their inhibitory effects on the enzymatic hydrolysis of cellulose. For example, it has been shown that the water-soluble fraction obtained after the use of relatively high pretreatment severities showed much stronger inhibitory effects on enzymatic hydrolysis than those obtained at lower pretreatment severities (Um and Van Walsum, 2012). However, many inhibition studies have been performed when using water-soluble fractions derived from unrealistic pretreatment conditions such as too low/high pretreatment severity and low solid loadings. Thus, to try to assess enzyme inhibition at representative conditions, it is preferable to use the water-soluble fraction derived from pretreatment under commercially relevant conditions. Earlier work has shown that the use of "compromise" steam pretreatment conditions allows good sugar recovery while also generating a relatively digestible cellulose-rich insoluble component (Chandra et al., 2008, 2015). Thus, the inhibition studies described in the thesis were based on the soluble compounds generated when compromise (as described in the material and methods section) steam pretreatment conditions were used.

1.7.2 Properties of biomass

In addition to the pretreatment conditions that are used, the nature of the biomass feedstock will also affect the composition of pretreatment-derived water-soluble compounds, which, in turn, will affect the extent of enzyme inhibition. Different wood species contain

different types of hemicellulose and lignin, which will generate different types of sugar and phenolics in the water-soluble fractions. For example, hardwoods contain mainly glucuronoxylan (accounting for 15-30% of the biomass), while softwoods include *O*-acetyl-galactoglucomannan (constituting around 20% of the biomass) (Sjostrom, 2013). Because of these differences, acid hydrolysis of hardwood will generate xylooligomeric sugars as the major oligomeric sugar components in the water soluble fraction (Ko et al., 2015; Shevchenko et al., 2000). As some studies have shown xylooligomeric sugars to be highly inhibitory in some studies, it might be that hardwood-derived water-soluble fractions may be more inhibitory than softwood-derived water soluble fractions. The lignin structure is also highly heterogeneous in different wood species. For example, softwoods contain a relatively high percentage of G units while hardwoods have relatively high amounts of S units (Baucher et al., 1998). Thus, softwoods tend to form more condensed structures than hardwoods, which results in relatively low concentrations of lignin derived phenolics in the water soluble fractions. The differences in the concentration and composition of phenolics will likely influence their inhibitory effect on cellulose hydrolysis. However, no studies to date have been reported on how the source of biomass might influence the inhibitory effect of the water soluble fraction on the hydrolytic potential of cellulase enzymes. Thus, it would be beneficial if we could further characterize the composition of water soluble fractions obtained from different woody biomass and assess their effects on enzymatic hydrolysis.

Although some studies have used soluble model compounds to assess the influence of the inhibitors on cellulase activities, the extent of inhibition when using the model substrates have not been well correlated with their influence in the presence of realistic, cellulosic substrates. For example, when Teugjas et al. (2013) evaluated the cellobiose inhibition of Cel7A using a ^{14}C labelled substrate and soluble 4-methylumbelliferyl-β-lactoside (Teugjas and Väljamäe, 2013b), they found stronger inhibition (K_I=0.124 mM, 4-methylumbelliferyl-β-lactoside, MUL) when

using the soluble model substrate than when using the solid cellulose substrate (K_I = 2.61 mM, ^{14}C-labeled bacterial cellulose) (Teugjas and Väljamäe, 2013b). It is likely that soluble model substrates reflected the activity of the enzyme on the glycosidic bonds, while the cellulosic substrates reflected, not only the activity of the enzyme on the glycosidic bonds, but also the influence of other substrate-related factors. Thus, it is essential to select a representative substrate for the evaluation of enzyme inhibition by potential inhibitors.

1.7.3 The composition of the enzyme "cocktail"

It is highly likely that the extent of enzyme inhibition will also be influenced by the composition of the enzyme cocktails. For example, "traditional" cellulase mixtures such as Celluclast contain mainly cellulases and are highly sensitive to cellobiose inhibition (Zhang et al., 2006). Thus, the supplementation of Celluclast with beta-glucosidase (BG) significantly increases the tolerance of the enzyme cocktail towards cellobiose inhibition as the BG hydrolyses the cellobiose to glucose, alleviating its inhibitory effects on the CBHs (Zhang et al., 2006; Singhania et al., 2013; Teugjas and Väljamäe, 2013b). Thus, to improve the sugar tolerance, more recently developed enzyme cocktails, such as CTec1, 2 and 3 from Novozymes, include a relatively high content of glucose-tolerant β-glucosidase. As mentioned before, the new enzyme preparations also contain other accessory enzymes such as hemicellulases and lytic polysaccharide monooxygenases. However, the tolerance of these so-called accessory enzymes to pretreatment derived inhibitors has not been studied extensively (Quinlan et al., 2011; Teter, 2012). Thus, the influence of the various water soluble components on the hydrolytic potential of these more recent enzyme preparations was studied as part of the thesis.

1.7.4 Enzyme loading

In addition to enzyme composition, increased enzyme loading has been used as one strategy to try to alleviate enzyme inhibition (Qin et al., 2016). However, it is essential to apply a reasonable enzyme loading, both to reflect economic reality and to better assess inhibition.

For the purposes of the thesis work the "minimum enzyme loading" is defined as the enzyme loading needed to achieve substantial cellulose hydrolysis (~70%) within 72 h, This is thought to be more reflective of the conditions used in an industrial bioconversion process. During the typical enzymatic hydrolysis of cellulose, there is a fast initial rate of hydrolysis followed by an intermediate degree of conversion where, after about 48-72 hrs, about 50-70% of the original substrate has been hydrolyzed. Thereafter, the enzymatic hydrolysis slows down gradually (Arantes et al., 2011). Because long residence times will significantly increase operating costs, as a result of lower reactor capacity and higher overall energy consumption, it is likely more economically attractive to target 70%-80% hydrolysis at the stage before the slowdown takes place (Arantes and Saddler, 2010). Therefore, to achieve effective cellulose hydrolysis (~70%) within 72 h, a "minimum enzyme loading" is required (Arantes and Saddler, 2010; Arantes et al., 2011; Ewanick et al., 2007; Macrelli et al., 2012).

1.8 Possible modes of inhibition of the compounds present in the water-soluble fraction

1.8.1 Overview of inhibition mechanisms

As mentioned earlier, the specific mechanism by which pretreatment-derived inhibitors reduce the hydrolytic potential of cellulase/hemicellulase enzymes has not been fully resolved. In the thesis work we have defined enzyme inhibition as the slowdown of enzyme-catalyzed

reactions. This can be further divided into two types of inhibition, reversible and the irreversible (Smith and Simons, 2004). Reversible enzyme inhibition is typically caused by inhibitors that form non-covalent interaction with various amino acid side chains exposed on the enzyme surface (Maurer and Fung, 2000; Smith and Simons, 2004) and the catalytic performance of the enzyme is usually, readily recovered by system dilution. The inhibition mechanisms that fall into this category typically follow the well-defined Michaelis-Menten kinetics. Inhibition can be further grouped into competitive inhibition, uncompetitive inhibition and non-competitive inhibition/mixed inhibition. Irreversible enzyme inhibition is usually caused by the inhibitors that strongly interact with enzyme by forming covalent bonds which often remain even after complete breakdown of enzymes to amino acids (Smith and Simons, 2004). As irreversible inhibition cannot be easily alleviated, it is often also called enzyme deactivation. Compared to reversible inhibition, irreversible inhibition often forms enzyme-inhibitor complexes, resulting in a loss of enzyme activity in a time-dependent manner (Maurer and Fung, 2000).

1.8.2 Challenges in studying enzyme inhibition mechanisms using classical kinetic analysis

As mentioned earlier, reversible inhibition can be studied by using classical inhibition kinetics and the Michaelis-Menten equation. Inhibition can be further grouped into the four general categories of competitive inhibition, uncompetitive inhibition, non-competitive inhibition, and mixed inhibition. However, Michaelis-Menten assumes the use of a soluble, homogeneous substrate and a single enzyme reaction. The inhibition kinetic behaviors of cellulases on soluble substrates such as oligosaccharides have been shown to fit well with a Michaelis–Menten model (Schou et al., 1993). However, the model has been proven to be over simplified when trying to describe the action of multiple cellulases acting on insoluble cellulosic substrates where the

cellulose hydrolysis happens in a heterogeneous reaction system with the involvement of several groups of enzyme components (Andrić et al., 2010a; Peri et al., 2007).

The difficulty in better elucidating the inhibition mechanism of cellulases is related to the complexity of cellulose degradation process itself. First, various cellulases including CBH, EG, BG and other "accessory" activities are often needed to synergistically degrade insoluble cellulose into the soluble sugars in a heterogeneous reaction system (Hu et al., 2014a). The action of the various cellulases on cellulose follows a complex scenario, where the enzyme has to adsorb onto the cellulose and form a productive enzyme-cellulose binding conformation. For some enzymes, such as cellobiohydrolases, the enzyme needs to processively move along the cellulose surface (Horn et al., 2006). There are several factors that might influence the kinetic profiles of CBHI hydrolysis behavior. For example, only a small fraction of the cellulose chain is actually available for CBHI catalytic performance as it could only react with the available reducing ends of cellulose (Zhang et al., 2006). Similarly, part of the CBHI might bind non-productively within the cellulose chain (Beckham et al., 2011; Gao et al., 2013). The enzyme might also get stuck on or desorbed from the cellulose surface (Igarashi et al., 2011).

As mentioned earlier, the assumptions of Michaelis–Menten kinetics are based on a homogeneous, soluble substrate and a single enzyme reaction (Andrić et al., 2010a). Unfortunately, the action of cellulases on a cellulosic substrate does not satisfy these assumptions. Thus, so far, there is no perfect kinetic model that describes the enzymatic hydrolysis of cellulose. This is probably one of the reasons why there is a large variation among the reported values of the glucose inhibition constant (KI) ranging from 0.1 to 319 g/L (Andrić et al., 2010b).

Besides kinetics, another way to try to better understand cellulase inhibition is to assess the effects of various inhibitors on the various process steps of cellulose degradation such as

enzyme adsorption, productive binding and processive movement. In this way we might better develop a picture of how the action of enzymes on cellulose are influenced by pretreatment derived inhibitors.

1.8.3 Possible inhibition by monomeric sugars

1.8.3.1 Inhibitory effects of monomeric sugars

Monomeric sugars derived from woody biomass contain hexose (mannose, galactose and glucose) and pentose sugars (arabinose and xylose). In previous work, the inhibitory effects of the major monomeric sugars at varied concentrations on the cellulose hydrolysis were assessed (Table 3). It appeared that high concentrations of glucose resulted in the strongest inhibition of cellulose hydrolysis. However, it remains unclear as to what extent the hemicellulose-derived sugars such as xylose and mannose might affect cellulose hydrolysis. Additionally, most previous inhibition studies only evaluated the influence of sugars on the hydrolytic performance of cellulases (Table 3) not any accessory enzymes. It is also still not clear how the sugars might affect the individual enzyme activities, the mechanisms behind such inhibitory effects and how different sugars might have different inhibition effects. It is also not clear how the substrates physicochemical characteristics might influence these inhibitory mechanisms.

Table 3 Previous work which has looked at monomeric sugar inhibition of cellulose hydrolysis

Inhibitory concentration of (g/L)	Decrease in glucose yield (%)	Enzyme sources	Hydrolysis conditions	Ref.
21 g/L xylose	No influence	Spezyme CP Novozyme 188	1% Solka Floc, 168 h	(Kim et al., 2011)
17 g/L xylose, 5 g/L glucose	No influence	Novozym188, Celluclast	10% Steam pretreated poplar wood, 68 h	(Cantarella et al., 2004a)
10 g/L xylose	~5%	Cellulase mixture of Trichoderma harzianum E58	5% Solka Floc, 48 h	(Mes-Hartree and Saddler, 1983)
16g/L xylose, 20 g/L mannose	~5%	Celluclast 2L, Novozyme 188	5% pretreated spruce, 144 h	(Tengborg et al., 2001a)
34 g/L glucose,10 g/L xylose	~15%	Novozyme, Celluclast	10% steam pretreated poplar wood, 68 h	(Cantarella et al., 2004a)
125 g/L monomeric sugars[1]	~20%	Novozyme 188, Celluclast	15% Steam pretreated corn stover, 168 h	(Hodge et al., 2008)
122 g/L monomeric sugars[2]	~30%	Spezyme CP, Novozyme 188	1% Solka Floc, 72 h	(Kothari and Lee, 2011)

1: sugar composition: glucose, 31.2 g/L; xylose, 78.5 g/L; arabinose, 9.5 g/L; galactose, 5.7 g/L.

2: sugar composition: glucose 21.8 g/L; xylose, 74.1 g/L; galactose, 12.4 g/L; arabinose 11.5 g/L; mannose 2.6 g/L.

1.8.3.2 Possible inhibition mechanisms

As mentioned earlier, the inhibition mechanisms of glucose on cellulase hydrolytic performance has been studied in some detail (Andrić et al., 2010a). It is commonly acknowledged that glucose strongly inhibits β-glucosidase activity, which in turn leads to an accumulation of cellobiose and significantly slows down the catalytic performance of the cellulase enzymes (Olsen et al., 2014; Xiao et al., 2004).

Although many previous studies have proven that the build-up of glucose severely inhibits the catalytic capability of cellulases (Andrić et al., 2010a; Du et al., 2010; Gusakov and Sinitsyn, 1992), to date there is no clear understanding of inhibition mechanism of hemicellulose-derived monomeric sugars such as xylose and mannose on cellulose hydrolysis. It has been reported that these hemicellulose sugars, unlike glucose, have almost no influence on BG activity (Xiao et al., 2004). However, interestingly, they have been shown to considerably decrease cellulase hydrolytic performance when hydrolysing cellulosic substrates (Hsieh et al., 2014). Previous work has also suggested that hemicellulose-derived sugars restricted the free water (one of the reactants for cellulose hydrolysis) available to the reaction system, thus decreasing the hydrolytic performance of the cellulases (Hsieh et al., 2015). However, this study did not show how the enzymatic action on cellulose was directly affected by these monomeric sugars. Previous research has indicated that the hemicellulose-derived sugars may interfere with the adsorption kinetics of cellulase monocomponents onto the insoluble cellulose (Pribowo et al, 2014). In addition, a recent work has suggested that some sugars adsorb onto the cellulose surface, generating a thin sugar layer, interfering with enzyme-substrate interactions and thus decreasing cellulose hydrolysis (Hu et al., 2014). Various cellulose properties, such as the degree of polymerization, accessibility, crystallinity and surface charge have also been shown to significantly affect the binding affinity of cellulases to cellulose (Gao et al., 2013; Hall et al.,

2010; Jiang et al., 2013). Thus, it is very likely that some of these substrate characteristics play an important role in sugar-induced inhibition mechanisms.

1.8.4 Inhibition by oligomeric sugars

1.8.4.1 Inhibitory effect on cellulose hydrolysis

Oligomeric sugars have also been shown to be inhibitory to effective enzymatic hydrolysis of cellulose (Qing and Wyman, 2011; Qing et al., 2010; Xin et al., 2015). Among the water-soluble oligomeric sugars, the xylooligomeric sugars (XOSs), derived from pretreated hardwood and agricultural residues have received considerable attention (Table 4). For example, Qing et al. have (2013) claimed that the XOS derived from Birchwood xylan decreased cellulose hydrolysis by up to forty percent (Qing et al., 2013). However, most of these previous studies have only used "synthetic" oligomeric sugars obtained by the acid hydrolysis of extracted xylan. As the xylan was usually produced via an alkaline extraction process, it is highly likely that the extraction had already removed most of the side branches (e.g. arabinose, glucuronic acid, acetic and p-coumaric acids) on the xylan backbone while maintaining a relatively high degree of polymerization of the XOSs (Fengel and Wegener, 2003). However, when using hot-water pretreatment-derived oligomeric sugars from maple wood, other workers observed relatively low levels of enzyme inhibition induced by these XOSs (Kim et al., 2011). It has been suggested that the XOSs derived from acid catalyzed pretreatment are likely to have a lower degree of polymerization while preserving some of the branches on the xylan backbone (Kabel et al., 2007). However, further studies are needed to clarify how the hemicellulose-derived oligomeric sugars from varied sources might affect enzyme mediated cellulose hydrolysis.

Table 4 Previous work which looked at oligomeric sugar inhibition of cellulose hydrolysis

Inhibitor sources	Concentration	Inhibition	Enzyme sources	Hydrolysis conditions	Reference
Hot-water pretreated maple wood	11.2 g/L XOS	~6%.	Spezyme CP, Novozyme 188	1% Solka Floc, 72 h	(Kim et al., 2011)
Birchwood xylan	2 g/L XOS	~16%	Purified enzyme from *Thermoascus aurantiacus*	2% Avicel, 48 h	(Zhang and Viikari, 2012)
Birchwood xylan	10 g/L xylan	~28 %	Cellulase from *Trichoderma harzianum* E58	5% Solka Floc, 24 h	(Mes-Hartree and Saddler, 1983)
Birchwood xylan	12.5 g/L XOS	38%	Spezyme CP, Novozyme 188	2% Avicel, 96 h	(Qing et al., 2010)

1.8.4.2 Inhibition mechanisms

As mentioned earlier, it has been suggested that the XOS's bind to the catalytic domains of the cellulases, resulting in a strong inhibition of cellulase activities. Of the various cellulases, Cel7A has been shown to be competitively inhibited by XOSs, with the "linear" XOSs entering the enzymes catalytic tunnel, resulting in inhibition (Baumann et al., 2011). A second, less-explored potential inhibition mechanism, has suggested that the linear XOSs form a rigid layer on the cellulose surface, possibly through hydrophobic binding interactions, resulting in a

decrease in accessibility to the cellulase enzymes (Linder et al., 2003; Paananen et al., 2004; Selig et al., 2015).

Thus it can be anticipated that the extent of enzyme inhibition by XOS will be highly dependent on the structural properties of the XOSs. For example, XOS's with higher degrees of polymerization might have a stronger inhibitory effect on Ce7A activities owing to their stronger binding affinity to the Cel7A enzymes (Baumann et al., 2011). Alternatively, the highly branched XOSs might exhibit a lower inhibitory effect (Selig et al., 2015), as these branches might sterically prevent the XOS's from entering the catalytic components of the Cel7A. Thus, it might be anticipated that steam pretreatment-derived oligomeric sugars have less inhibitory effects on cellulase enzymes, as acid-catalyzed steam explosion generates shorter and highly substituted oligomers.

1.8.5 Possible inhibition by phenolics

1.8.5.1 Inhibitory effect of phenolics

Various types of phenolic compounds, including monomeric and polymeric phenolics, have been detected in the pretreatment-derived water soluble fractions (Mitchell et al., 2014). Although phenolics are typically found in relatively low concentrations (ranging from ~1 to ~10 g/L) in the water soluble fractions, as compared with mono/oligomeric sugars, they can also inhibit cellulose hydrolysis (Hodge et al., 2008; Kim et al., 2011; Kothari and Lee, 2011; Tengborg et al., 2001a) (Table 5). It has also been shown that phenolic compounds (such as tannic acid and hot-water extracted phenolics) can strongly inhibit and/or deactivate cellulase enzymes during hydrolysis (Kim et al., 2011; Michelin et al., 2016; Ximenes et al., 2010). However, other researchers have claimed that certain types of phenolics, such as hydroxybenzoic acid and vanillic acid, have almost no inhibitory effect on the enzymatic hydrolysis of cellulose

(Cantarella et al., 2004a; García-Aparicio et al., 2006; Hodge et al., 2008). One of the possible reasons for these contradictory observations may be the structural differences of these "phenolics".

Table 5 Previous work which has looked at phenolics inhibition of cellulose hydrolysis

Key results	Sources of inhibitors	Ref.
Tannic acid have a significant inhibitory effect	Selected phenolic compounds (0.9 g/L Tannic acid)	(Tejirian and Xu, 2011)
Tannic acid have the greatest inhibitory effect	Synthetic phenolics 0.2% (w/v) with phenol/enzyme ratios	(Ximenes et al., 2010)
Significant inhibition of hydrolysis	Extracted phenolics from hot water treatment (3.5 g/L)	(Kim et al., 2011)
No inhibition of hydrolysis	Synthetic phenols (9 g/L)	(Hodge et al., 2008)
No inhibition of hydrolysis	Synthetic phenols (2 g/L)	(Cantarella et al., 2004a)

1.8.5.2 Phenolics-protein interactions

Although phenolics inhibition of cellulase activity has been shown to be associated with their enzyme interaction, the nature of the phenolics-cellulase interaction remains unclear. Phenolics have been shown to bind to proteins through either non-covalent (reversible) interaction or covalent (irreversible) interaction (Prigent, 2005). Non-covalent binding mainly involves hydrogen bonding and hydrophobic interactions (De Freitas and Mateus, 2012). Hydrogen bonding is associated with the interaction between the hydroxyl groups of the phenolics and nitrogen or oxygen elements of the amino-acid residuals (Haslam, 1996). In

contrast, hydrophobic interactions likely involve π-π stacking between the phenolics and the amino-acid residuals containing aromatic ring structures (Baxter et al., 1997). Covalent interactions between phenolics and protein is a result of the reaction of oxidized phenolics (radicals/quinones) with peptide chains that contain amino-acid residuals such as cysteine and lysine and histidine (Cilliers and Singleton, 1990). Thus, it is likely that phenolics-cellulase interactions involve hydrogen bonding, hydrophobic interactions and covalent interactions, which, as will be discussed below, will also be influenced by the nature of the phenolic and the enzyme structures (Baxter et al., 1997).

1.8.5.3 Influence of the phenolics structure on phenolics-protein interaction

The interaction between phenolics and proteins will be influenced by properties such as the chemicals degree of polymerization, hydroxylation of phenolics and the overall hydrophobicity of the phenolics. For example, phenolics with an intermediate average degree of polymerization (5-8) have been shown to have a higher affinity to proline-rich proteins than phenolics with a lower degree of polymerization (<4) (Sarni-Manchado et al., 1999). However, other work found that the ability of phenolics to bind protein molecules (such as alpha-amylase or bovine serum albumin) increased until the molecular weight of phenolics reached 3 kDa and decreased afterwards (De Freitas and Mateus, 2002). The authors' rational was that the highly polymerized phenolics might be insufficiently soluble and difficult to fit into the binding site of the protein because of steric hindrance (De Freitas and Mateus, 2002). For the case of pretreatment derived phenolics, some studies have reported that polymeric phenolics are more inhibitory than monomeric phenolics (Tejirian and Xu, 2011; Ximenes et al., 2010). However, other work has suggested that dehydrogenative ferulic acid polymers have less of an inhibitory effect than monomeric ferulic acid (Nakagame et al., 2011). These contradictory findings

indicated that the influence of phenolics on cellulase inhibition might depend on properties other than just molecular weight. For example, function groups such as carbonyl, phenol hydroxyl and methoxy group have been reported to influence the inhibitory effects of phenolics (Berlin et al., 2006; Qin et al., 2016). By using different model phenolic compounds such as 4-hydroxybenzoic acids and p-coumaric acid, recent work has claimed that the presence of carbonyl, phenol hydroxyl and methoxy group significantly increased their inhibitory effects on cellulase enzymes (Qin et al., 2016). A similar result was also observed in work by Berlin et al., (2006) who fractionated two types of lignin from Douglas-fir and found that lignin with the higher amount of carbonyl group and phenolic hydroxyl group was more inhibitory (Berlin et al., 2006). Other researchers have claimed that the addition of certain functional groups can alleviate the inhibitory effect of lignin on cellulose hydrolysis. For example, Nakagame et al (2010) found that the increased carboxylic acid content alleviated the non-productive binding of cellulases to lignin, likely because these acid groups decreased the hydrophobicity of lignin (Nakagame et al., 2011). Overall, it seems that the inhibitory effects of phenolics are closely associated with the major properties of this group of chemicals. Thus, if we could identify the key inhibitory mechanisms, we might be able to modify the structure of the phenolics to make them less inhibitory towards cellulase enzymes.

1.8.5.4 Effect of protein structure on phenolics-protein interaction

It has been shown that protein properties such as amino acid composition can greatly influence their interactions with phenolics (Prigent et al., 2003). Proline has been reported to be a key component that can induce enzyme-phenolics interaction (Baxter et al., 1997; Pascal et al., 2009). This is mainly because proline is not able to fit into the α-helix structure and thus, often results in the formation of a loose and open structure that is highly accessible to various

phenolics (Makkar and Becker, 1998). Additionally, the amino acids that contain aromatic groups such as phenylalanine, tryptophan and tyrosine can readily interact with protein through π-π stacking (Baxter et al., 1997; Charlton et al., 2002). Since different cellulases have different amino acid compositions, it is likely that they will have different binding affinities to phenolics.

In addition to their composition, the surface properties of proteins such as their hydrophobicity will also strongly influence their binding affinity to phenolics (Bandyopadhyay et al., 2012; Qin et al., 2014; Sammond et al., 2014). When dissolved in the water, protein molecules tend to minimize exposure of their hydrophobic groups. However, partial hydrophobic groups are preserved on the surface due to the structure of protein. This increases the hydrophobic interaction between enzymes and other hydrophobic components such as phenolics (Sammond et al., 2014). For example, Roseman et al have found that Cel7B had a stronger binding affinity to lignin than Cel7A because of the higher hydrophobicity of the amino acid side chains of Cel7B (Roseman, 1988). A similar result was reported by Sammond et al, who have found that the extent of exposed hydrophobic clusters on cellulases was a determining factor that governs the binding affinity of cellulases with phenolics and/or lignin (Sammond et al., 2014). Thus, it is expected that the surface hydrophobicity of cellulase enzymes will also influence the extent of phenolic inhibition.

In summary, the extent of phenolics-protein interaction will be determined, not only by the characteristics of the phenolics, but also by the protein structure. Thus, the various differences in the phenolics and enzymes used in the various studies may be the main reason for the diverse observations in these previous enzyme inhibition studies. However, as mentioned earlier, most of these previous studies have used model phenolics compounds and “traditional” enzyme preparations. This might not represent the possible influence of pretreatment-derived phenolics on “newer” enzyme preparations such as CTec 1, 2 and 3. Thus, one of the objectives

of the thesis was to try to better understand how phenolics might cause enzyme inhibition during whole slurry hydrolysis and to identify the major characteristics of phenolics that determine enzyme inhibition.

1.8.6 Inhibition by acetic acid and furans

As mentioned earlier, acetic acid and furans are generated from sugar degradation during acid hydrolysis of lignocellulose. Compared with sugars and phenolics, the inhibitory effects of acetic acid and furans are thought to be much less significant (Kothari and Lee, 2011; Tengborg et al., 2001a). It has been shown that, only at concentrations up to 10 g/L, furfural or hydroxymethylfurfural (HMF) could strongly inhibit enzymatic hydrolysis. While, at the concentrations normally found in the water-soluble fraction (2-5 g/L), furfural or HMF have almost no influence on the enzymatic hydrolysis of cellulose (Jing et al., 2009; Kothari and Lee, 2011; Mes-Hartree and Saddler, 1983; Takagi, 1984; Tengborg et al., 2001a). As the concentrations of furfural or HMF in the work reported in the thesis are only between 1 and 3 g/L (much lower than the reported inhibitory concentrations) in the water soluble fraction, they were not expected to inhibit cellulose hydrolysis.

1.9 Methods for extracting inhibitors

To effectively study the major inhibitors in the water-soluble fraction and investigate their specific inhibition mechanism, it is important to be able to fractionate and identify the major groups of soluble compounds. As mentioned earlier, model or synthetic compounds are not really representative of the real characteristics of these components in the pretreatment liquid. Thus, we had to develop an effective method of fractionating these compounds, derived from

pretreatment, so that their inhibitory effect on cellulose hydrolysis could be assessed more accurately.

Ultracentrifugation has long been used to separate biological macromolecules and components (Schachman, 2013). For example, Humpula et al. (2014) used ultracentrifugation to remove or separate high-molecular-weight oligosaccharides derived from ammonia fibre explosion pretreated corn stover (Humpula et al., 2014). In addition, size-exclusion chromatography could also be used to separate molecules with varied molecular weight (Humpula et al., 2014). Some studies have also tried to separate the oligomeric sugars by ethanol precipitation followed by fractionation via anion exchange columns (Appeldoorn et al., 2010). However, it seems that using these strategies to extract the oligomeric sugars might also result in considerable loss of oligomeric sugars as well as providing relatively impure oligomers. Thus, it is difficult to use these extracted oligomeric sugars to represent the real oligomeric sugars presented in the water-soluble fraction after pretreatment. Alternatively, instead of oligosaccharides extraction, another way of assessing their potential influence would be to hydrolyse the oligomeric sugars to monomeric sugars (by acid hydrolysis) to see if this reduces their inhibitory effect (Shevchenko et al., 2000). By comparing the effects of the original and the acid hydrolyzed water-soluble fraction on enzyme-mediated cellulose hydrolysis, the inhibitory effect of oligomeric sugars might be determined.

The pretreatment-derived phenolics range from monomeric (e.g., hydroxybenzoic acid, vanillic acid) to highly polymerized (e.g., tannic acid) phenolics. Although monomeric phenolics can be extracted through solvent extraction using ethyl acetate, polymeric phenolics cannot be extracted by this type of process. However, solid phase extraction (SPE) has been used to fractionate both the monomeric and polymeric phenolics, based on their polarity and acidity. For example, other workers have used carbon/charcoal, XAD-type resins and C18 phase extraction to

isolate more than 80% of soluble phenolics from the pretreatment liquors derived from a hot-water pretreatment process (Kim et al., 2013, 2011). Thus, it seems the SPE method is more suitable for extracting steam pretreatment-derived phenolics than the solvent extraction method as steam pretreatment has been shown to generate both monomeric and polymeric phenolics (Carrasco et al., 2012).

1.10 Strategies to minimize enzyme inhibition

1.10.1 Developing pretreatment methods which generate less inhibitory components

To minimize enzyme inhibition and improve cellulose hydrolysis, various strategies to modify pretreatment process have already been proposed. For example, some studies have tried to lower the pretreatment severity in order to decrease the inhibitory effects of the pretreatment-derived water soluble compounds on cellulose hydrolysis (Um and Van Walsum, 2012). However, it is likely that this type of strategy will result in the decreased digestibility of the pretreated substrates by the cellulase enzymes. Thus, an additional post-treatment such as mechanical refining is often needed to ensure reasonable substrate hydrolyzability.

Alternatively, an addition of carbocation scavenger during acid catalyzed pretreatment is likely to both suppress the generation of inhibitory compounds and maintain the digestibility of cellulosic substrates. During acidic pretreatment, carbocation intermediates are formed from lignin deionization. As these carbocation intermediates have a very high affinity to the nucleophiles on the lignin structure, a condensed lignin structure could be formed through repolymerization mediated by the carbocations and nucleophiles on the lignin structure (Figure 9). However, the unstable carbocation intermediates, if not reacted with nucleophiles, could

degrade into smaller water-soluble phenolic compounds. To avoid the repolymerization of carbocations with the nucleophiles on lignin structure, carbocation scavenger such as 2-naphthol, which have electron-rich properties, have been used to react with the carbocation intermediates (Li and Gellerstedt, 2008; Li et al., 2007). In this manner, it is likely that carbocation scavengers could prevent the carbocation intermediates from further degradation into smaller water-soluble phenolic compounds (Figure 9, upper pathway). Thus, it may be possibly to alleviate the inhibitory effect of pretreatment-derived phenolics on cellulose hydrolysis.

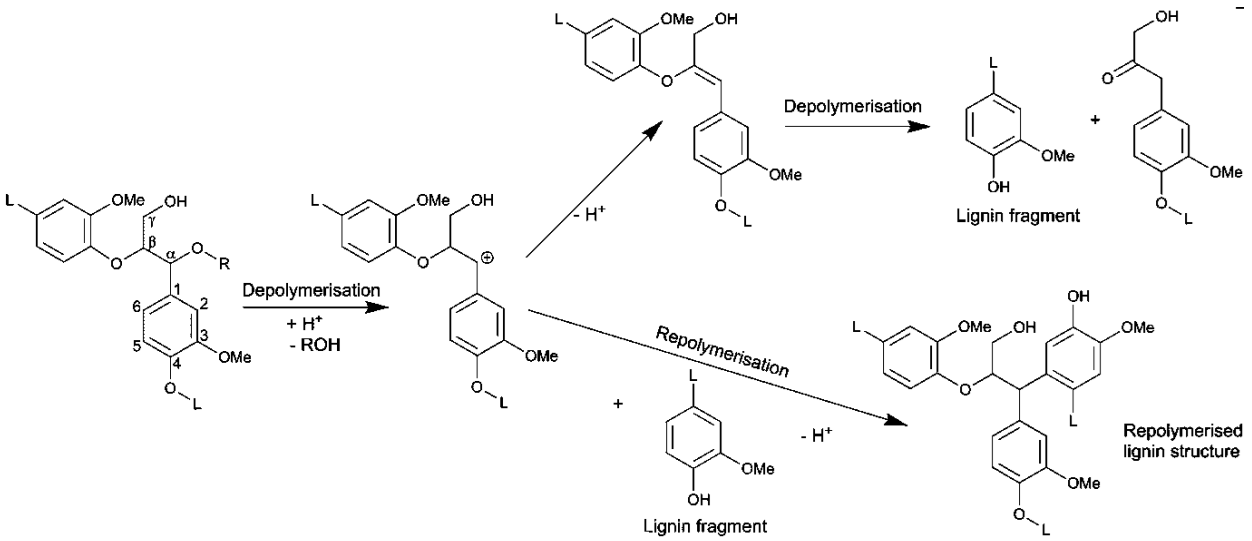

Figure 9 Schematic of the depolymerisation and repolymerisation of lignin under acid conditions (Pielhop et al., 2015).

1.10.2 Developing inhibitor tolerant enzyme components/preparations

In much of the previous inhibitor work, traditional enzyme preparations such as Celluclast and Spezyme CP have been commonly used. However, high protein/enzyme loadings had to be used to achieve reasonable hydrolysis (~70% in 48 h) because of the low hydrolysis efficiency of these traditional enzyme cocktails. Over the past decade, to decrease the enzyme loading needed for efficient hydrolysis, the hydrolytic potential of commercial enzyme cocktails, has been greatly improved (Soderberg and Fuglsang, 2013). In addition, recent enzyme cocktails

such as CTec3 seem to have great tolerance towards glucose, probably because a glucose-tolerant BG was added to the enzyme cocktail (Soderberg and Fuglsang, 2013). Therefore, it is possible that we can further improve the tolerance of the cellulase cocktails by adding more inhibitor-resistant enzyme components to the pretreated whole slurry.

1.10.3 Developing inhibition mitigation strategies

Considerable work has been carried out to alleviate the inhibition of fermentation processes. This includes detoxification methods such as physical, chemical and biological detoxification, in-situ detoxification and in-situ microbial detoxification in term of the fermentation process (Jönsson et al., 2013). However, inhibition mitigation methods that could be used to alleviate enzyme inhibition by the water-soluble fraction have not been explored extensively.

Some of the chemical treatments that have been applied to improve enzymatic hydrolysis efficiency include the addition of specific reducing reagents such as the sulfur oxyanions dithionite and sulfite. These compounds have been shown to improve cellulose hydrolysis in the presence of water soluble compounds (Soudham et al., 2011). In addition, surfactants have been shown to prevent the detrimental adsorption of tannins onto cellulose and the formation of tannin-enzyme complexes, thus alleviating the inhibition (Tejirian and Xu, 2011). Cantarella et al. (2004b) found that, when comparing overliming, water rinsing, water–ethyl acetate two-phase extraction and in situ detoxification with yeast, overliming with $Ca(OH)_2$ was the most effective detoxification method for enzymatic hydrolysis. The likely mechanism was that it reduced the concentration of some inhibitors by precipitation at higher pH's (Cantarella et al., 2004b). However, the main drawbacks of detoxification with $Ca(OH)_2$ are that it increases processing costs and also results in sugar losses in the reaction system (Jönsson et al., 2013; Martinez et al.,

2000). Enzymatic approaches have also been used to try to remove inhibitors. For example, Niu et al. (2009) found that removing phenolics through laccase treatment could greatly alleviate the inhibition of CBH, EG and BG by pretreatment liquors (Niu et al., 2009). However, similar to overliming with $Ca(OH)_2$, this process requires relatively high loadings of laccases to result in the removal of most of the phenolics in the water soluble fractions. This would likely increase the process costs of detoxification.

However, because of the insufficient understanding of enzyme inhibition by the pretreatment-derived inhibitors, it appears that there is still no obvious mitigation strategy that can be used to alleviate the inhibition caused by the soluble compounds present in pretreated biomass. Thus, as tackled within the main body of this thesis, it is necessary to achieve a better understanding of enzyme inhibition in order to develop cost-effective strategies that could mitigate enzyme inhibition caused by pretreatment-derived inhibitors.

1.11 Thesis objectives

The work described in this thesis hoped to provide a better understanding of how pretreatment-derived inhibitors affect cellulose hydrolysis. By better understanding the potential factors that might influence the inhibition we hoped to develop more efficient inhibition mitigation strategies and improve the hydrolysis of the cellulosic fraction present in a pretreated whole slurry. The detailed objectives of each section are described as below.

Chapter 3.1. How much do the water-soluble fractions (WS's) derived from steam pretreated woody biomass inhibit traditional and newly developed cellulase enzyme preparations? Can we identify the major groups of soluble compounds within the WSs that account for the most of enzyme inhibition? In order to do so, the WSs derived from steam pretreated hardwood (poplar) and softwood (lodgepole pine) were isolated and their inhibitory effects were assessed.

Different inhibitor mitigation strategies such as mild acid hydrolysis (to remove oligomeric sugars) and activated carbon treatment (to remove phenolics) were assessed, to try to identify the major groups of inhibitory components and their possible inhibitory effects on both well-established enzyme mixtures (Celluclast) and relatively novel enzyme mixtures, CTec3. It appeared that CTec3 was still inhibited by the inhibitory compounds presented in the water soluble fraction of steam pretreated poplar and lodgepole pine. Monomeric sugars were the major group of inhibitory compounds while phenolics accounted for another part of the inhibition phenomenon.

Chapter 3.2. To develop a better understanding of how potentially inhibitory compounds decrease cellulose hydrolysis. The inhibitory effects of the major pretreatment-derived inhibitors, i.e. sugars and phenolics, on the major groups of enzyme activities within the cellulase enzyme preparation (e.g. endoglucanases, xylanase, cellobiohydrolases and β-glucosidase) were assessed. The specific inhibition mechanisms such as reversible/irreversible inhibition and deactivation/precipitation of the major enzyme components were evaluated. It was apparent that the main enzyme components (CBH and BG) present in CTec3 were strongly inhibited by pretreatment-derived inhibitors. The BG was mainly inhibited by glucose while CBH was mainly affected by phenolics. Interestingly, hemicellulose-derived sugars such as mannose and xylose did not inhibit the major enzyme activities. This suggested different inhibition mechanism might be occurring, other than end product inhibition.

Chapter 3.3. The potential inhibitory influence of different types of major monosaccharides (e.g. xylose, mannose and glucose) within the WS fractions on the enzyme-substrate interactions. This included, enzyme adsorption, productive binding and processive movement while determining the inhibitory effects of different sugar components on cellulose hydrolysis. Various model substrates such as Avicel, cellulose II, nanocellulose, dissolving pulp

with varied cellulose properties were used to try to better understand how substrate characteristics such as crystallinity, degree of polymerization, accessibility and acid group content might affect the monosaccharides inhibition of cellulose hydrolysis.

Chapter 3.4. Identify the main physicochemical characteristics of phenolics that might affect cellulose hydrolysis by cellulase enzymes. The phenolics within the WS fractions of steam pretreated biomass were firstly fractionated and separated into several fractions according to their molecular size. The essential functional groups such as carbonyl group and phenol hydroxyl groups were further modified to try to evaluate how these groups might affect the cellulase hydrolysis kinetics.

Chapter 3.5. Assess the potential application of carbocation scavengers in the acid catalyzed pretreatment of woody biomass as inhibition mitigation strategy to alleviate the inhibitory effect of the water-soluble fraction. Carbocation scavengers were able to reduce the inhibitory effect of phenolics in the water-soluble fractions while improving digestibility of the pretreated lodgepole pine to certain extent.

Summary:

By identifying the components and mechanisms involved the inhibition of enzyme mediated hydrolysis of the cellulose present in pretreated wood slurries, we hoped to facilitate the development of more efficient pretreatment, tolerant enzymes and better inhibition mitigation strategies, resulting in efficient whole slurry hydrolysis using relatively low enzyme loading.

2. Materials and Methods

2.1 Steam pretreatment of softwood and hardwood

Wood chips of *poplar* (hardwood) and *lodgepole pine* (softwood) were used as feedstocks for steam pretreatment. Steam pretreatment of the biomass was performed in a 2 L StakeTech III steam gun (Stake Technologies, Norvall, ON, Canada). The chips were pretreated by SO_2-catalyzed steam pretreatment as reported previously (Arantes et al., 2011; Ohgren et al., 2007). Hybrid poplar (POP) and mountain pine beetle killed lodgepole pine (Pinus contorta) (LPP) were provided by the British Columbia Forest service and FP Innovations respectively.

Prior to pretreatment, the woody biomass was debarked and chipped using a custom designed chipper. The wood chips were then collected and used as the feedstock for steam pretreatment (approximately 2 cm × 2 cm × 0.5 cm). Wood chips from lodgepole pine and poplar (dry weight of 200 g) were impregnated overnight with 4% (w/w) and 3% (w/w) SO_2, respectively, in sealable plastic bags. Subsequently, the samples were kept in the fume hood at room temperature overnight. After incubation, the unabsorbed SO_2 was discharged from the plastic bags in a fume hood. The bags containing wood chips and the adsorbed SO_2 were then weighed to determine the amount of the adsorbed SO_2. The pretreatment was carried out under pre-optimized conditions (200 ^{o}C, 5 min) to maximize overall sugar recovery and to, hopefully, achieve efficient cellulose hydrolysis at relatively low enzyme loading according to previous studies (Bura et al., 2003; Nakagame et al., 2010).

2.2 Analysis of chemical composition of water-insoluble fraction (WIS) and water soluble fraction (WS) after steam pretreatment

After pretreatment, the biomass slurry was separated by filtration into solid and the liquid fractions (water soluble fraction, WS). The solid fraction was then washed extensively with water to remove any remaining soluble compounds, pressed again to remove excess moisture and collected for further usage as the water-insoluble fraction (WIS). Both the WIS and WS fractions were stored at 4 °C.

The composition of the WIS fractions of steam pretreated lodgepole pine (SPLP) was 52.8% glucan, 2.0% mannan, 42.3% Klason lignin, whereas the composition of WIS fractions of poplar (SPP) was 58.7% glucan, 2.2% xylan, 1.4% mannan, 35.1% Klason lignin, and 1.7% acid-soluble lignin (all values are expressed as % total dry weight (w/w) and determined using the modified Tappi T-222 on- 88 method, as described previously (Boussaid et al., 1999; Bura et al., 2003)).

The monomeric sugars in the WS fraction were analyzed by high-performance liquid chromatography (HPLC, ICS-2500), fitted with an AS50 autosampler, ED50 electrochemical detector, GP50 gradient pump, and anion exchange column (Dionex, CarboPac PA1). Degassed water was used as an eluent at 1 mL/min while degassed 0.2 M NaOH was added in the post-column to maintain the baseline stability and detector sensitivity. 1 M NaOH solution was applied to recondition the column. Standards were prepared using arabinose, galactose, glucose, xylose and mannose (Sigma) with fructose as internal standard. All the samples were pre-filtered through a 0.45 µm syringe filter before injection.

The oligomeric sugars were detected by the traditional acid hydrolysis protocol (Shevchenko et al., 2000). Briefly, a mild acid hydrolysis (4% v/v, H_2SO_4, 120 °C and 60 min) was performed to break down all the oligomers. The oligomeric sugar concentration was determined as the difference in monomers sugar concentration before and after acid hydrolysis.

The total phenolics were determined using the Folin-Ciocalteu method and expressed as the equivalent vanillin amount, adapted from the method proposed by Singleton and Rossi (1965). Briefly, the concentration of total phenolics in water soluble fraction was analyzed using Folin-Ciocalteu reagent. Vanillin was used as the standard. 100 µL of the diluted sample was mixed with 250 µL of the Folin-Ciocalteu reagent. After 5 minutes, 750 µL of 20% (w/v) Na_2CO_3 was added into the mixture. Finally, the total volume was brought up to 5 mL using nanopure water. The tubes were then incubated for 2 hours at 22 °C. The absorbance of each reaction was read spectrophotometrically at 760 nm. The reactions were carried out in triplicate for each sample, and the average values reported.

Degradation products such as furfural, 5-hydroxymethyl furfural (HMF) and acetic acid were measured using HPLC (ICS-500) with Aminex HPX-87H column (Bio-Rad, Hercules, CA), an AS3500 auto sampler, and a UV detector at 280 nm. Five mM sulfuric acid (H_2SO_4) was used as an eluent at a flow rate of 0.6 mL/min. The concentration of HMF standards ranged from 0.1 g/L to 4.0 g/L, while that of furfural standards ranged from 0.1 g/L to 2.0 g/L. All of the standards and samples were filtered through a 0.45 µm syringe filter.

2.3 Model cellulosic substrates and their characterization

2.3.1 Model cellulose substrates

Avicel PH 101 was purchased from Sigma. Cellulose nanocrystal (CNC) was a kind gift from the Forest Products Laboratory-USDA Forest Service. Dissolving pulp (DP) produced from hardwood was obtained from Tembec. Cellulose II was prepared from Avicel as described previously (Chundawat et al., 2011b). NaOH (25% w/w, 50 mL) was added to the Avicel (5 g) at 4 ^{o}C and the mixture was kept at 4 ^{o}C overnight. The slurry was then centrifuged and washed with water to result in a neutral pH.

2.3.2 Acid group

The amount of total acid groups on the cellulose was determined using the conductometric titration method (Katz and Beatson, 1984). Briefly, 50 mg (dry weight base) substrate was treated with 0.1 M HCl solution then thoroughly washed with de-ionized water. Subsequently, the substrates were added to 50 mL NaCl (0.001 M) and 20 µL HCl (0.5 M) and then mixed well. The total acid groups were determined by the titration curve (volume of NaOH against conductivity).

2.3.3 Accessibility

The accessibility of the cellulases to the cellulose was estimated by the Simons' stain technique according to the modified procedure of Chandra et al. (2008). The dye used was direct orange (DO-Pontamine Fast Orange 6RN), provided by Pylam Products Co. Inc. (Garden City, NY, U.S.). Prior to use, the dye was filtrated, to remove any aggregates, using an Amicon filtration system. The never-dried samples (10 mg, dry weight) were weighed into six 2-mL

centrifuge tubes. Different amount of direct orange (10 mg/mL) were added to the tubes (0.025, 0.050, 0.075, 0.1, 0.15, and 0.2 mL), followed by the addition of 0.1 mL of phosphate-buffered saline (PBS, pH 6). The total volume was made up to 1 mL with the addition of water. After incubation at 70°C for 6 h, the supernatant was obtained through centrifugation at 10,000 rpm for 5 min. The absorbance of the supernatant was read at 450 nm on a Cary 50 UV-Vis spectrophotometer and used to calculate the amount of adsorbed dye.

2.3.4 Crystallinity

Cellulose crystallinity (CrI) was determined by X-ray diffraction (XRD), as described (Nishiyama et al., 2002). The cellulose samples were filtered and washed to prepare cellulose sheets with a flat surface and placed onto a zero-background plate. Data was obtained by using a Bruker D8-Advance powder X-ray diffractometer. The analysis of the crystallinity was carried out using Bruker TOPAS version 4.2, which used the model from Nishiyama's cellulose Iβ to estimate the crystallinity of the cellulose (Nishiyama et al., 2002). The extent of cellulose crystallinity was calculated according to the equation: 100 x (crystalline area/total area), where the total area = crystalline area + amorphous area.

2.3.5 Degree of polymerization

The intrinsic viscosity [η] of a cellulose sample was measured in a copper ethylenediamine (CED) solution according to the standard TAPPI (Technical Association of Paper and Pulp Industries) method. Briefly, the running time through a capillary-tube viscometer was recorded from the CED and testing solutions. The intrinsic viscosity [η] of each testing sample was calculated based on the running time. The average degree of polymerization of the

cellulose samples was calculated from [η] using the following equation between intrinsic viscosity and degree of polymerization.

$$[\eta] = 1.67 \times N^{0.71} (N \cong [\eta] \times 190)$$

where 1.67 and 0.71 are intrinsic for cellulose/copper ethylene diamine solution and N represents the degree of polymerization.

2.4 Mild acid hydrolysis of oligomeric sugars

The water soluble fractions were treated with mild acid hydrolysis to convert the oligomeric sugars into monomeric sugars as described in previous studies (Shevchenko et al., 2000). Mild acid hydrolysis of the water soluble fractions was carried out in autoclave at 120 °C with 0.5% H_2SO_4 for 1 h. The pH of hydrolysed water soluble (WS) fractions were adjusted with NaOH (4 M) to a final pH of 4.8. The composition of the acid hydrolysed WS was determined.

2.5 Selective removal of phenolic compounds in WS

Activated carbon has been reported to be an efficient adsorbent to remove phenolics from aqueous phases (Dabrowski et al., 2005; Kim et al., 2011). The WS fractions were mixed with 5% or 10% (w/w) powdered activated carbon. The mixture was incubated at room temperature under stirring for 3 h, and then centrifuged to collect the supernatant. Then the supernatant was neutralized with NaOH (4 M) to a final pH of 4.8. The composition of the activated carbon treated WSs was then determined.

2.6 Enzyme preparations and enzyme assay procedures

Commercial cellulase and β-glucosidase preparations (Cellic CTec3, Celluclast and Novozyme 188, respectively) were obtained from Novozyme, North America Inc. (Franklinton,

NC). Cellulase activity was determined using the filter paper assay recommended by the International Union of Pure and Applied Chemists and is expressed in filter paper units (FPUs) (Adney and Baker, 1996). The β-Glucosidase activity was measured using p-nitrophenyl-β-D-glucoside as substrate and is expressed in cellobiose units (CBUs) (Wood and Bhat, 1988). The total protein content of Cellic CTec3 (245 mg protein/mL), Celluclast 1.5 L (58 FPU, 125 mg protein/mL) and Novozyme 188 (390 CBU, 297 mg protein/mL) were determined according to the modified ninhydrin method with bovine serum albumin as the protein standard (Starcher, 2001). The specific activities of endoglucanase, xylanase, β-glucosidase, cellobiohydrolases in CTec3 cocktail are 6, 14, 15, 2 U/mg protein respectively.

β-Glucosidase activity was measured using p-nitrophenyl-β-D-glucoside (Wood and Bhat, 1988). Briefly, 50 µL of PNPG substrate solution (10 mM) was mixed with 50 µL of diluted enzyme preparation and the reaction mixture was incubated at 50 °C for 30 min. The reaction was stopped by the addition of 100 µL of NaOH glycine buffer solution (100 mM). The concentration of p-nitrophenol produced was measured by the absorbance at 410 nm. Cellobiohydrolases (CBH) activity was measured under similar conditions using p-nitrophenyl-β-d-cellobioside (PNPC) as the substrate in the presence of D-glucono-1,5-δ-lactone to inhibit β-glucosidase activity (Deshpande et al., 1984). Although endoglucanase and Cel6A can also hydrolyse the substrate, previous studies have shown that they have considerably lower activity than that of Cel7A. Thus, the CBH activity was mainly due to the Cel7A component. Enzyme activity was expressed as International Units (U), where one U releases one µmol p-nitrophenol per min. Experiments were performed in triplicate and the mean values were calculated.

Endoglucanase (EG) activity was determined as described previously (Miller, 1959). The total reaction mixture (100 µL) contained a suitably diluted enzyme and 0.7% (w/v) of carboxymethyl cellulose (CMC, Sigma-Aldrich) solution in 50 mM acetate buffer (pH=4.8).

Xylanase activity was determined using a 1% xylan solution (birchwood, Sigma–Aldrich) as substrate under similar conditions (Berlin et al., 2006). The enzyme reaction was stopped by adding 200 µL of 3,5-dinitrosalicylic acid (DNS) reagent after 10, 20, and 30 mins of incubation. The microplates were then placed in an oven at 105 °C for 30 min and the reducing sugar content of the samples was analyzed by measuring the absorbance at 540 nm (Miller, 1959). Xylose and glucose were used as standards for calibration. The EG and xylanase activities were expressed in international units (U) defined as 1 µmol of glycoside bonds of the substrate hydrolysed per minute. Experiments were performed in triplicate and the mean values were calculated.

It is difficult (and sometimes impossible) to quantify a small amount of product formed using the DNS method against a high background of reducing sugars added as possible inhibitors (Zhang et al., 2006). Therefore, EG activity and xylanase activity in the presence of hemicellulose sugar rich water soluble fractions (WSs) were estimated using relatively indirect assays based on release of dyes labelled in the matrix of CMC or birchwood xylan. Both substrates (CMC and xylan) were labelled with Remazol brilliant blue (RBB).The RBB-CMC (Remazol brilliant blue labelled carboxymethyl cellulose) and birchwood xylan were prepared according to a modified method of Biely et al. (1985).

2.7 Enzymatic hydrolysis

Prior to studying the effect of the water soluble (WS) fraction on the enzymatic hydrolysis of cellulosic substrates, the pH of the WS fraction was adjusted to pH 4.8 using NaOH (4 M). The WIS (water insoluble) fraction was diluted to 1%-10% solid consistency using the pH adjusted WS fraction. The enzymatic hydrolysis of the pretreated and pure cellulosic substrates was performed using CTec3 or Celluclast 1.5 in 0.05 M sodium acetate buffer (pH 4.8) at 50 °C on a rotary shaker at 150 rpm for 72 h. The Celluclast was supplemented with β-

glucosidase (1 FPU: 1.5 CBU) to avoid product inhibition caused by cellobiose accumulation. Samples were taken periodically and the concentration of glucose in the supernatants was determined using HPLC (Dionex DX-3000, Sunnyvale, CA). Substrate and enzyme blanks were run at the same time by incubating the substrates in buffer/WS without enzymes and by incubating the enzymes alone. The hydrolysis yields (%) of cellulosic substrates were calculated based on the amount of glucose released in the hydrolysate divided by the theoretical cellulose content of the substrates.

2.8 Enzyme-cellulose interaction

2.8.1 Productive binding and adsorption measurement

Model cellulose substrates (10 g/L) were incubated with Cel7A at 30 ^{o}C for 1 h. After the separation of the cellulose bound Cel7A, the concentration of cellulose-free Cel7A in solution was determined by its p-nitrophenyl-β-d-lactoside (PNPL) hydrolysing activity at 30 ^{o}C according to (Jalak and Väljamäe, 2014). The total amount of bound enzyme was determined as the difference between the total Cel7A and cellulose-free Cel7A in solution.

The "productive binding" of Cel7A (also called active site mediated binding of Cel7A) was measured as described by Jalak et al (2014). Briefly, the model cellulose substrate (10 g/L) was incubated with Cel7A and β-glucosidase in 300 µL total volume for 30 min at 30 ^{o}C. Then, 100 µL of PNPL (2 mM) was added, and the reaction mixture was incubated for 30 min and stopped by the addition of a sodium hydroxide solution. Finally, the cellulosic substrate was separated by centrifugation for 5 min and the released PNP in the supernatant measured by the absorbance at 405 nm. Active-site-free Cel7A was determined by the rate of PNPL hydrolysis.

The “productive binding” of Cel7A was defined as the difference between the total concentration of Cel7A and active-site-free Cel7A.

2.8.2 Processivity measurement

As mentioned previously, processivity is an essential characteristic of CBH enzymes. The Cel7A processive cleavage hydrolysis cellulose chains into mainly cellobiose and glucose (Hamre et al., 2014). Previous studies have suggested that glucose could be mainly released from the first cleavage the Cel7A makes after complexing with a cellulose chain, with subsequent cuts releasing only cellobiose (Biely et al., 1981; Nidetzky et al., 1994). Therefore, the degree of processivity of Cel7A under the current experimental conditions could be approximated from the ratio of the glucose and cellobiose (Horn et al., 2012b; Nakamura et al., 2014). In order to determine processivity, a high-performance liquid chromatographic (HPLC) was used to analyze the soluble products (glucose and cellobiose).

2.9 Reversible enzyme inhibition

The water soluble (WS) fractions derived from steam pretreated lodgepole pine and poplar were referred to as SPP-WS and SPLP-WS, respectively. These water soluble (WS) fractions were first neutralized with NaOH to pH 4.8. A series of volumes of water soluble fractions (WSs) were then mixed with a substrate solution and a diluted enzyme solution. Activities were then determined in the presence of the WS fractions, as described previously. The activity assayed in the absence of pretreatment liquors was defined as the control activity of 100%. All reactions were carried out in triplicate and the residual enzyme activity was expressed as the mean.

The kinetics of enzyme inhibition by model inhibitors was tested at varied substrate concentration (PNPG and PNPC) over the range of 0.0625 to 1.25 mM, while the mode of enzyme inhibition was determined from a Lineweaver-Burk plot. According to previous work (Cornish-Bowden, 2014), competitive enzyme inhibition has the same y-intercept but different slopes and x-intercepts between the two data sets. Non-competitive inhibition has the same x-intercept but different slopes and y-intercepts. Uncompetitive inhibition results in different intercepts on both the y- and x-axes.

2.10 Irreversible enzyme inhibition

Pretreatment-derived soluble compounds have been reported to inhibit not only the initial hydrolysis rate but also the extent of hydrolysis. The enzyme activity assays only evaluated the inhibitory effect of pretreatment-derived water soluble fractions on the catalytic action of enzyme over the initial stages of hydrolysis. To investigate the possible deactivation effects of pretreatment-derived soluble compounds on the major groups of enzyme activities during the time course of hydrolysis, the extents of enzyme activity loss were examined using the same enzyme activity assay procedure as described earlier (Ximenes et al., 2010). The deactivation effects of pretreatment liquors on specific activities were determined by pre-incubating the enzyme with the WS fractions (pH 4.8 and 50 ◦C) for 0-72 h. The enzyme mixtures were combined with each WS fraction to achieve a final concentration of 2.8 mg/mL. This was within the range of enzyme loading normally used for high-solid cellulose hydrolysis. After an appropriate period of time, samples of the incubation mixture were withdrawn, diluted 100-1000 fold and then added to the standard assay mixture (PNPG, PNPC, CMC and xylan). The residual activity of BG, CBH, EG and xylanase activity were determined.

2.11 Fractionation of soluble phenolics

Activated carbon was used to selectivity remove the potential inhibitory phenolics within the water soluble fraction from steam pretreatment derived whole slurry (5-10% w/w loading). After incubation of the water soluble fraction with activated carbon overnight at 30 ^{o}C, the adsorbent was filtered through a Whatman No.1 filter paper. The adsorbed phenolics were desorbed at 40 ^{o}C using a solution containing 50%-100% acetone in water. The acetone/water mixture, containing desorbed phenolics, was air dried and re-dissolved in buffer to make a phenolics "stock solution".

2.12 Separation of soluble phenolics according to their molecular size

To study the effect of molecular size on the inhibitory effect of phenolics on enzymatic hydrolysis of cellulose, the fractionation of the extracted phenolics was performed by filtering a phenolics solution through 1, 3 and 10 kDa ultrafiltration membranes (Millipore, Bedford, MA) using an Amicon ultrafiltration system (Amicon, Beverly, MA) under 30 psi nitrogen gas pressure (Humpula et al., 2014). Before use, the membranes were washed twice with nanopure water and incubated in 50% ethanol solution. The phenolics solution was then transferred into the Amicon container and filtered until 10% of the original volume remained. After filtration, the filtrates and retentates were stored at 4 ^{o}C for further use.

2.13 Characterization of phenolics

The extracted phenolics from the water soluble fraction were analyzed by Fourier transform infrared (FTIR) spectroscopy. The analysis was carried out by directly coating a thin

layer of phenolics onto a KBr plate and evaporating the solvent (Kim et al., 1999; Liu et al., 2008). The solid residual on the KBr plate was subjected to FTIR analysis. The FTIR spectra were obtained using a Varian 3100 (Varian Inc. Palo Alto, CA) with an MIRacle Accessory (Pike technologies, Madison, WI). The absorbance was recorded between 400-4000 cm^{-1} at a resolution of 4 cm^{-1} with 32 scan.

2.14 Modification of phenolics

2.14.1 Hydroxypropylation of phenolics

To assess the effect of the contribution of phenolic hydroxyl group to enzyme inhibition, the phenolics were modified by hydroxypropylation, to block free phenolic hydroxyl groups, according to the method described previously (García et al., 2013). The phenolics stock (3 ml, ~20 g/L) was mixed with 180 mg NaOH and vortexed for 1 min. Then 2 mL propylene (PO) was added into the mixture and stirred at room temperature (~22 °C) for 24 h. Subsequently, the reaction mixture was heated to 60 °C to remove potentially unreacted PO. The HCl (6 M) was then added to adjust the pH into 4.8.

2.14.2 Chemical reduction of phenolics

The chemical reduction of the extracted phenolics first involved adjusting the pH of the phenolics solution to 7.0 with a 5 M solution of sodium hydroxide. A certain amount of sodium borohydride (Sigma–Aldrich) was then added directly to the phenolics solution. The reaction was carried out at room temperature for 24 h. After the reaction, the pH of the phenolics solution was adjusted to 4.8 with 6 M HCl to decompose the unreacted $NaBH_4$. The $NaBH_4$ treated phenolics solution was stored at 4 ^oC for further analysis.

3. Results and Discussion

3.1 What are the major components in steam pretreated lignocellulosic biomass that inhibit the efficacy of cellulase enzyme mixtures?

3.1.1 Background

As mentioned previously in the Section 1.7, mild-severity steam pretreatment is the compromised conditions that are used to open up the cell wall structure and increase the accessibility of cellulose to cellulase enzymes. However, even under such pretreatment conditions, various carbohydrates and lignin degradation products such as mono/oligomeric sugars, phenolics, furans and organic acid, will still be generated which are strongly inhibitory to the downstream enzymatic hydrolysis and fermentation processes.

Although the inhibitory effect of these soluble components derived from pretreatment process has been recognized for some time, most of this previous work used "synthetic mixtures of inhibitors" and assessed their possible detrimental effects on cellulose hydrolysis at a low inhibitor concentrations (Qing and Wyman, 2011; Qing et al., 2010; Tejirian and Xu, 2011). The vast majority of this previous work typically used either purified enzyme components or the "older" generation of cellulase preparations such as Celluclast (Novozymes) and Genencor Spezyme (Kont et al., 2013; Rajan and Carrier, 2014b; Tengborg et al., 2001a; Zakaria et al., 2016). Cellulose and hemicellulose derived sugars have been shown to predominantly inhibit cellulase enzymes through end-product inhibition mechanism (Xiao et al., 2004), while non-sugar components such as some polymeric phenolics are believed to deactivate cellulase

enzymes predominantly through the formation of precipitable phenolic-enzyme complexes (Tejirian and Xu, 2011). However, it is still not clear which inhibitory mechanisms predominate and which components derived from the various types of pretreated biomass substrates affect the individual and collective enzyme mixture.

The various enzyme companies have made considerable progress in developing more hydrolytic enzyme mixtures, which allow lower enzyme loadings to be used while achieving effective biomass deconstruction. Enzyme mixtures such as Novozymes Cellic CTec3 (CTec3) are known to contain various accessory enzymes such as xylanase and lytic polysaccharide monooxygenases (LPMO) as well as more glucose-tolerant β-glucosidase (Hu et al., 2014, 2011; Soderberg and Fuglsang, 2013). However, it has not yet been determined if these "next generation enzyme mixtures" have a stronger inhibitor tolerance towards pretreatment derived compounds, particularly as the nature of the pretreatment and the biomass substrate have been shown to significantly influence the composition/concentration of the soluble compounds released (Palmqvist and Hahn-Hägerdal, 2000; Rajan and Carrier, 2014a). For example, previous work has shown that the different ratios of p-hydroxyphenyl/guaiacyl/syringyl (H/G/S) lignin components derived from either softwoods or hardwoods lignin greatly influenced the characteristics and amounts of the phenolic compounds derived after pretreatment (Klinke et al., 2004). Therefore, a more comprehensive study to assess the main inhibitory compounds derived over a range of "real-life" steam pretreated lignocellulosic biomass conditions, especially at high substrate concentrations, would be useful.

In this section, the possible inhibitory effects on enzymatic hydrolysis of the major soluble compounds derived from steam pretreated hardwood (poplar) and softwood (lodgepole pine) cellulases was determined. Different inhibitor mitigation strategies such as mild acid hydrolysis (to remove oligomeric sugars) and activated carbon treatment (to remove phenolics)

were employed to try to identify the major group of inhibitory components and assess their possible inhibitory effects on both well-established enzyme mixtures (Celluclast) and the relatively novel enzyme mixture, CTec3.

3.1.2 Results and discussion

3.1.2.1 The composition of the water soluble fractions and water insoluble fractions derived from steam pretreated lodgepole pine and poplar

As mentioned earlier, the nature and the amount of water-soluble material that can be generated during steam pretreatment are affected by both the nature of the biomass substrate and the conditions used such as temperature, catalyst dosage, time and solids loading. Although high-severity pretreatment typically produces a more accessible/digestible cellulosic substrate (Kang et al., 2013), it also results in more sugar degradation and the production of potentially inhibitory compounds such as phenolics and extractives (Um and Van Walsum, 2012; van der Pol et al., 2014). In the work reported here, a high solids loading steam pretreatment was carried out using pretreatment conditions (200 °C for 5 min using 4% and 3% SO_2 for lodgepole pine and poplar respectively) that had previously been shown to maximize overall sugar recovery from both the cellulose and hemicellulose components of the original biomass (Hu et al., 2014).

After pretreatment, the pretreated slurry was separated by filtration into a solid (water insoluble fraction, WIS) and water soluble fraction (WS). The insoluble fraction was then washed extensively with water to remove any remaining soluble compounds, pressed again to remove excess of moisture and collected for further use (Figure 10). The glucan content of each of the WIS fractions was 51.8% and 58.7% respectively for steam pretreated lodgepole pine (SPLP) and poplar (SPP) (Table 6). The water soluble (WS) fraction contained high concentrations of hemicellulose derived sugars (Table 7). As anticipated, the major

hemicellulose sugars detected in the water soluble fractions of steam pretreated lodgepole pine (SPLP-WS) and steam pretreated poplar (SPP-WS) were respectively mannose/mano-oligomer (~27/8 g/L) and xylose/xylo-oligomer (~52/11 g/L). As milder pretreatment conditions were used, more than 20% of the sugars that were present in each of the water soluble fractions were in an oligomeric form. Phenolic compounds accounted for another ~5% and ~10% of the total mass of the SPLP-WS/SPP-WS fractions respectively (Table 7). The higher concentration of phenolics in the SPP-WS fraction was likely due to the higher percentage of β-O-4 linkage in hardwood lignin, which made it more susceptible to degradation during the thermochemical steam pretreatment process (Higuchi, 1990; Li et al., 2007).

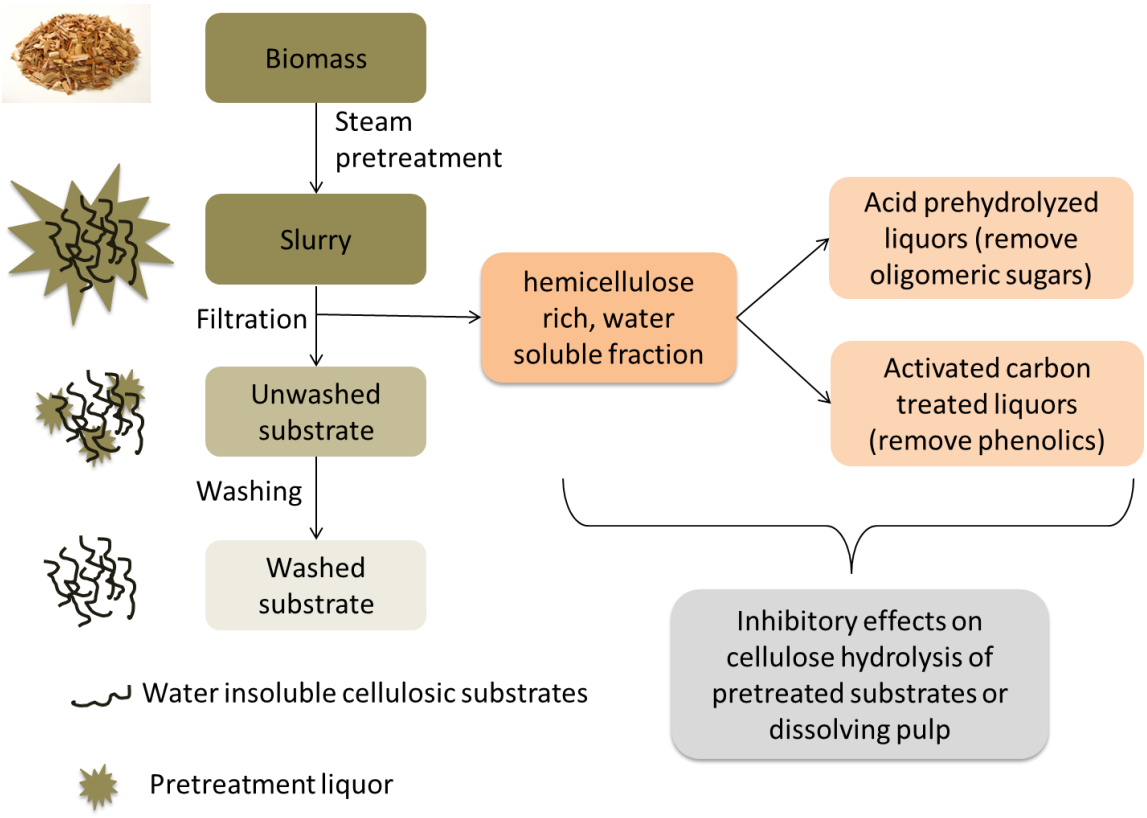

Figure 10 Flow chart of steps performed to obtain the water soluble (WS) fractions from the slurry of steam pretreated lodgepole pine and poplar.

Table 6 Chemical composition of steam pretreated lodgepole pine (SPLP) and poplar (SPP).

Samples	Major sugar composition (%)						Lignin analysis (%)		
	Ara	Gal	Glu	Xyl	Man	Total	AIL	ASL	Total
SPLP	0.2	0.3	51.8	0.5	2.0	54.8	43.3	0.8	44.1
SPP	0.0	0.0	58.7	2.2	1.4	62.3	33.5	1.9	35.4

SPLP/SPP: steam pretreated lodgepole pine/poplar; AIL: acid insoluble lignin; ASL: acid soluble lignin; Ara: arabinose; Gal: galactose. Glu: glucose; Xyl: xylose; Man: mannose. All analyses were carried out in duplicate. The mean values are reported.

Table 7. Chemical composition of the water soluble (WS) fractions from steam pretreated lodgepole pine (SPLP) and poplar (SPP).

WS	Monomeric sugars (g/L)					Oligomeric sugars (g/L)					Others (g/L)			
	Ara	Gal	Glu	Xyl	Man	Ara	Gal	Glu	Xyl	Man	Phe	FF	HMF	AA
SPLP-WS	4.7	8.5	19.8	15.4	27.2	0.4	2	6.5	2.4	7.9	4.5	2.1	0.8	4.4
SPP-WS	1.3	2.7	22.9	52.1	3.9	0.1	0.7	5.8	11.3	1.8	9.8	1.1	3.0	12.4

SPLP/SPP-WS: steam pretreated lodgepole pine/poplar derived water soluble fractions; Ara: arabinose; Gal: galactose. Glu: glucose. Xyl: xylose; Man: mannose; Phe: phenolics; FF: furfural; HMF: hydroxymethylfurfural; AA: acetic acid; nd: not detected. All analyses were carried out in duplicate. The mean values are reported.

3.1.2.2 The possible inhibitory effect of water soluble fractions on the hydrolytic potential of CTec3 and Celluclast

The possible inhibitory effects of the SPLP-WS and SPP-WS on Novozymes Celluclast and CTec3 preparations were initially assessed during the hydrolysis of a relatively pure, cellulosic substrate, dissolving pulp (DP) at increasing enzyme loadings (Figure 11). As anticipated, the CTec3 showed significantly higher hydrolytic activity than did the Celluclast

enzyme mixture, with a respective protein loading of approximately 10 mg and 95 mg per g of cellulose required to achieve 70% hydrolysis of the dissolving pulp after 48 h incubation (Figure 11). Although the hydrolytic potential of both enzyme preparations was significantly inhibited by the addition of SPLP-WS and SPP-WS fractions, the CTec3 was more tolerant to these inhibitory compounds than was the Celluclast, especially at low enzyme loadings (Figure 11).

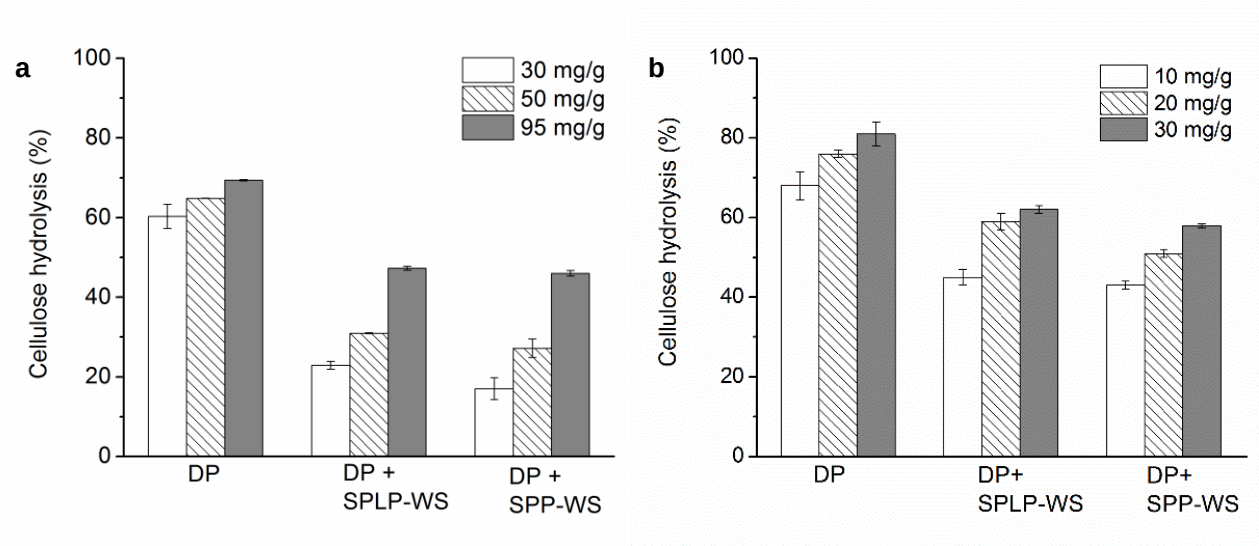

Figure 11 Effect of the water soluble fractions from steam pretreated lodgepole pine (SPLP-WS) and poplar (SPP-WS) on the hydrolysis of dissolving pulp (DP) using (a) Celluclast and (b) CTec 3 at different enzyme doses (mg protein / g cellulose) after 48 h hydrolysis. The statistical significance in cellulose hydrolysis with and without WSs was assessed by one way analysis of variance (ANOVA) followed by Tukey's test. The addition of SPLP-WS and SPP-WS resulted in statistically significant decrease of cellulose hydrolysis when using Celluclast and CTec3 at different enzyme doses ($p < 0.05$). However, the difference between cellulose hydrolysis with SPLP-WS and SPP-WS were statistically insignificant ($p > 0.05$).

As previous studies had reported the strong inhibitory effects of different pretreatment derived soluble compounds on more traditional cellulase mixtures, the significant inhibition of the Celluclast enzyme mixture by the water soluble fractions was expected (Kim et al., 2011;

Panagiotou and Olsson, 2007). However, as the hydrolytic potential of even the CTec3 enzyme preparation was reduced by the pretreatment derived soluble components, we next wanted to determine the nature of the inhibitors and their inhibitory mechanisms.

3.1.2.3 The possible inhibitory effect of the water soluble fractions on the hydrolysis rate and extent of cellulose hydrolysis

Dissolving pulp (DP) was hydrolysed by CTec3 in the presence of either acetate buffer alone or in the pretreatment derived water soluble fractions. Cellulose hydrolysis in the presence of buffer was ~90% after 72 h. However, when the pretreatment derived water soluble fractions were added at the beginning of the hydrolysis, after 12 h incubation, this reduced the rate of cellulose hydrolysis by ~20%. After 48 h, cellulose hydrolysis was reduced by ~20% and ~40% respectively when the SPLP-WS and SPP-WS were added to the reaction (Figure 12). This suggested that the water soluble fractions had a strong inhibitory effect, not only on the rate, but also on the extent of cellulose hydrolysis.

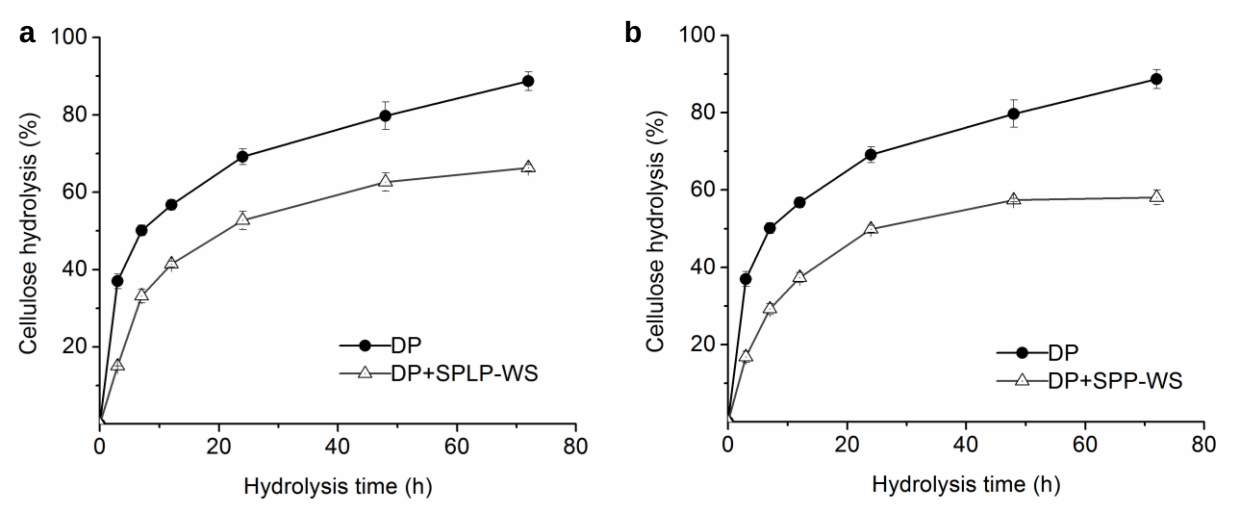

Figure 12 Effect of the water soluble fractions from (a) steam pretreated lodgepole pine (SPLP-WS) and (b) poplar (SPP-WS) on the hydrolysis of dissolving pulp (DP). The enzymatic hydrolysis was performed at 50 ^{o}C using an enzyme loading of 30 mg CTec3/g glucan for 72 h.

3.1.2.4 Possible influence of the monomeric sugars within the water soluble fractions on the hydrolytic potential of the CTec3 enzyme mixture

Previous work had shown that high concentrations of sugars diminished cellulose hydrolysis, primarily because of end product inhibition (Xiao et al., 2004). As indicated earlier (Table 7) the water soluble fractions contained relatively high concentrations of monomeric sugars. Thus, the possible inhibitory effects of monomeric sugars were assessed by adding a "synthetic sugar" solution at the same concentration and composition as detected in the water soluble fractions during the CTec3 mediated hydrolysis of dissolving pulp (Figure 13).

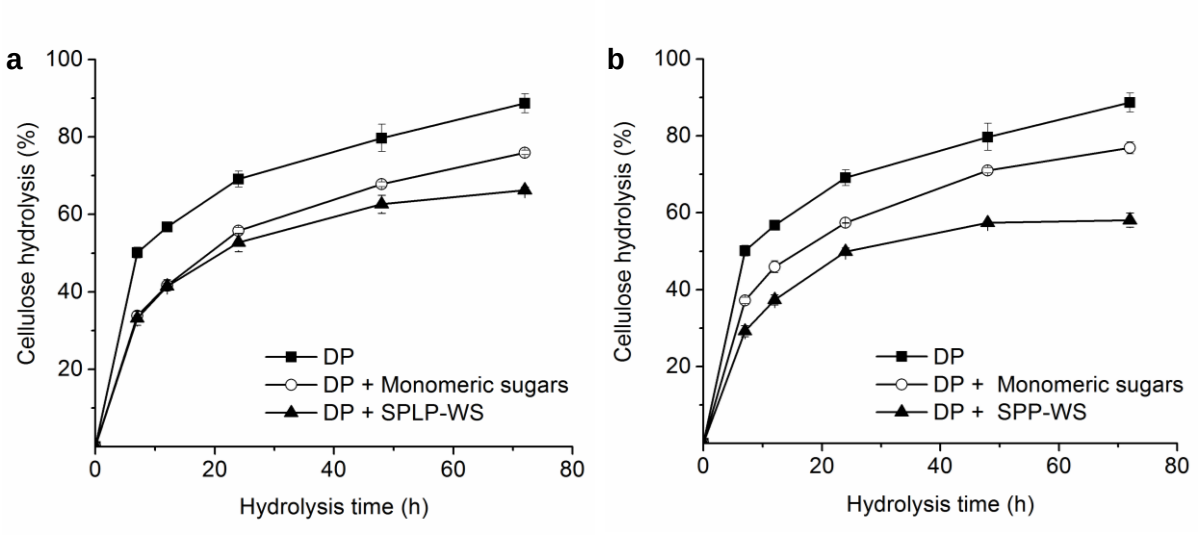

Figure 13 Effect of water soluble fractions from (a) steam pretreated lodgepole pine (SPLP-WS) and (b) poplar (SPP-WS) and the monomeric sugars present in the SPLP/SPP-WS fractions on the hydrolysis of dissolving pulp (DP).

It was apparent that the addition of the synthetic sugar solution inhibited both the rate and extent of cellulose hydrolysis, but to a lesser extent than did the addition of the SPLP/SPP water soluble fractions. The addition of each of the "synthetic sugar" solutions resulted in a similar decrease in cellulose hydrolysis, decreasing hydrolysis by about 15% after 72 h (Figure 13). This implied that inhibition was influenced more by the concentration rather than the exact composition of the monomeric sugars as the composition of the monomeric sugars in SPLP-WS and SPP-WS fractions were quite different while the concentrations were similar (Table 7).

Although the monomeric sugars appeared to be the major inhibitory components within the SPLP/SPP-WS fractions at the initial stages of hydrolysis (Figure 13a, b), they seemed to only account for 60% of the inhibitory effects of SPLP/SPP-WS (Figure 13a, b) after 72 h incubation. This indicated that other potentially inhibitory components, such as oligomeric sugars or phenolics, were also limiting the hydrolytic performance of the CTec3 cellulase mixture, particularly during the latter stages of hydrolysis.

Earlier work has suggested that the inhibitory influence of monomeric sugars on cellulose hydrolysis might be due to a combination of direct end product inhibition and indirect effects such as the reduction of free water and inhibition of enzyme adsorption (Hsieh et al., 2014; Olsen et al., 2014). For example, glucose has been shown to inhibit β-glucosidase activity, consequently resulting in an accumulation of cellobiose. This in turn strongly inhibits the cellulase enzymes, especially exo-glucanases (Xiao et al., 2004). High concentrations of sugars are also thought to inhibit enzyme adsorption by reducing cellulose accessibility to the cellulase enzymes (Kristensen et al., 2009). Recent studies have also suggested that the inhibitory effects of mannose, galactose and xylose are governed by weak interactions with water, as indicated by the correlation between the amount of "free water" and hydrolysis yield (Hsieh et al., 2014).

3.1.2.5 Possible influence of furfural, HMF, acetic acid on cellulose hydrolysis

Since small amounts of sugar degradation products, such as 5-hydroxymethylfurfural (HMF), furfural and some acetic acid were also detected in the water-soluble fractions, we next assessed their possible inhibitory effects on cellulose hydrolysis. The concentrations of these sugar degradation products in the SPLP/SPP-WS fractions were 0.8 and 3.0 g of HMF, 2.1 and 1.1 g of furfural, and 4.4 and 12.4 g of acetic acid, per liter WS, respectively. Consistent with what has been reported previously (Cantarella et al., 2014; Tengborg et al., 2001a), these small amounts of sugar degradation products and their combination showed little or no effect on the enzymatic hydrolysis by cellulase enzymes (Figure 14).

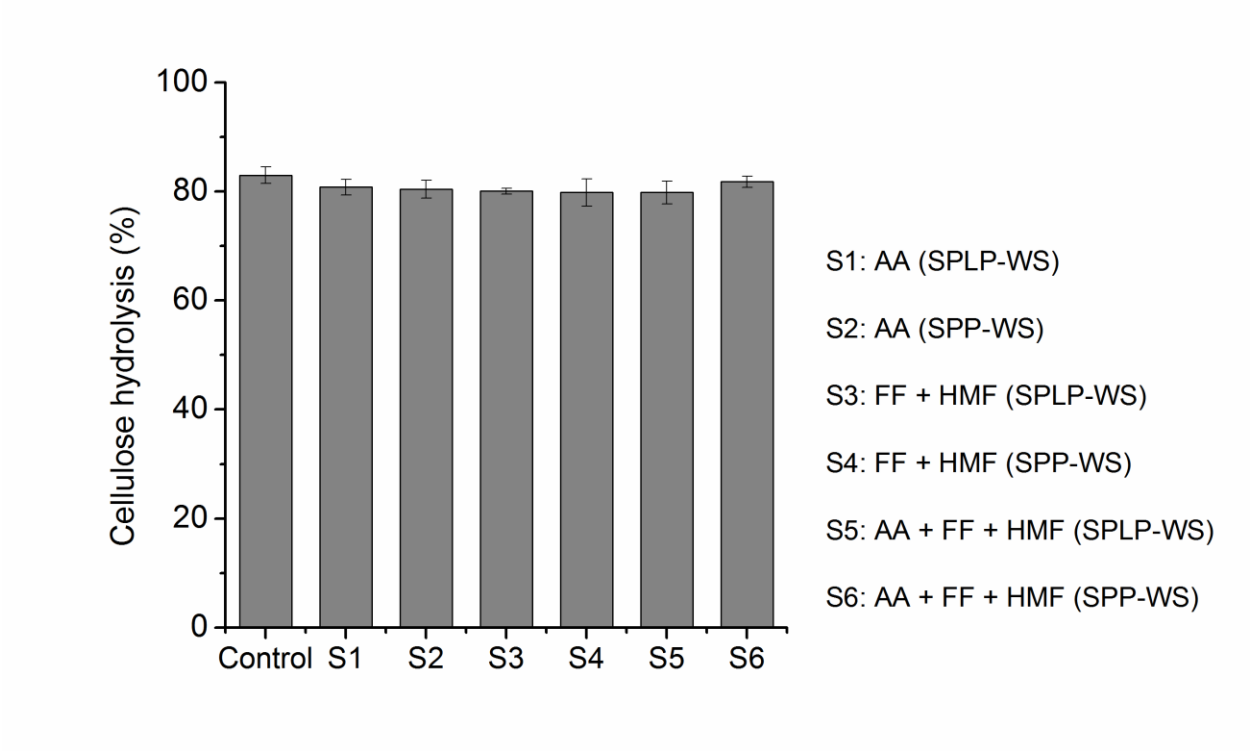

Figure 14 Effect of degradation products within the SPLP/SPP-WS fractions on cellulose hydrolysis. Degradation products were added to reach the same concentration as they were found in SPLP/SPP-WS fractions. AA: acetic acid; FF: furfural; HMF: hydroxymethylfurfural.

3.1.2.6 Possible influence of the oligomeric sugars within the water-soluble fraction on the hydrolytic potential of the CTec3 enzyme mixture

Oligomeric sugars were also generated during steam pretreatment (Table 7) and, as it has been suggested that these sugars limit enzymatic hydrolysis by cellulases (Kumar and Wyman, 2014; Qing et al., 2010), we next wanted to assess the possible inhibitory influence of the oligomeric sugars present in the water-soluble fraction. However, it proved challenging to separate out the oligomeric sugars from other soluble compounds. Therefore, in an alternative approach, the oligomeric sugars were converted to monomeric sugars via mild acid hydrolysis and the possible inhibitory effect of the water-soluble fraction with/without the oligomeric sugars was assessed (Table 8). It was apparent that the acid hydrolysis of oligomers to monomers did

not appear to influence the inhibitory effect of SPLP-WS fraction and even slightly increased the inhibitory effect of the SPP-WS fraction (Figure 15).

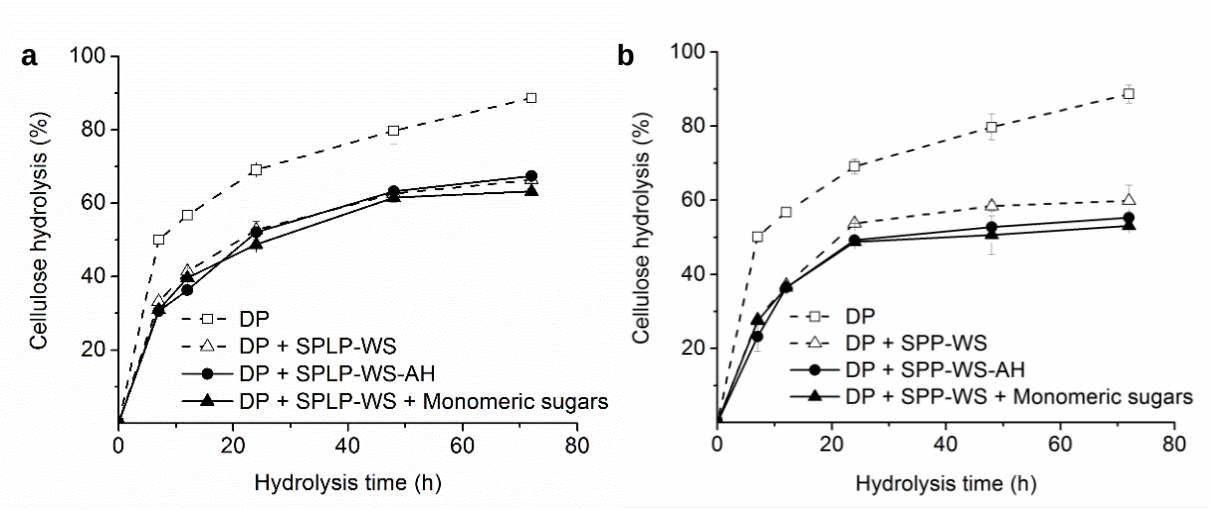

Figure 15 Effect of oligomeric sugar removal by acid hydrolysis in the water soluble fractions from steam pretreated lodgepole pine (SPLP-WS) (a) and poplar (SPP-WS) (b) on the hydrolysis of dissolving pulp (DP). SPLP/ SPP-WS-AH: acid hydrolysed water soluble fraction from steam pretreated lodgepole pine and poplar.

Table 8 Chemical composition of the original and acid hydrolysed (AH) water soluble fraction (WS) from steam pretreated lodgepole pine (SPLP) and poplar (SPP).

WS	Monomeric sugars (g/L)						Oligomeric sugars (g/L)					
	Ara	Gal	Glu	Xyl	Man	Total	Ara	Gal	Glu	Xyl	Man	Total
SPLP-WS	4.7	8.5	19.8	15.4	27.2	75.6	0.4	2	6.5	2.4	7.9	19.2
SPLP-WS-AH	5.1	10.2	25.4	17.5	32.6	90.8	nd	0.2	0.6	nd	0.9	1.7
SPP-WS	1.3	2.7	22.9	52.1	3.9	82.9	0.1	0.7	5.8	11.3	1.8	19.7
SPP-WS-AH	1.4	3.2	27.3	62.2	5.1	99.2	nd	0.1	0.9	nd	0.3	1.3

SPLP/SPP-WS: steam pretreated lodgepole pine/poplar derived water-soluble fraction; SPLP/SPP-WS-AH: steam pretreated lodgepole pine/poplar derived water-soluble fraction that has been acid hydrolysed; WS: Water-soluble fraction; Ara: arabinose; Gal: galactose. Glu: glucose. Xyl: xylose; Man: mannose; Phe: phenolics; nd: not detected.

A possible explanation for the low inhibitory effect of the oligomeric sugars might be the relatively low oligomer to monomer sugar ratio in both of the water-soluble fractions (~1:5, Table 8). Thus, lower severity steam pretreatments were further applied to both the lodgepole pine and poplar chips to produce water-soluble fractions that contained a higher oligomeric to monomeric sugars ratio (Table 9). Although the lower-severity pretreatment generated a higher ratio of oligomeric to monomeric sugars (~1:3), these oligomeric sugars appeared to exhibit only little additional inhibitory effect on cellulose hydrolysis (Figure 16).

Table 9 Chemical composition of the water-soluble fractions from low-severity steam pretreated lodgepole pine ($SPLP_{low}$-WS) and poplar (SPP_{low}-WS).

WS	Monomeric sugars (g/L)					Oligomeric sugars (g/L)					Others (g/L)
	Ara	Gal	Glu	Xyl	Man	Ara	Gal	Glu	Xyl	Man	Phe
$SPLP_{low}$-WS	3	4.3	4.5	9.2	10.2	0.1	1.1	2.1	0.5	6.5	2.2
SPP_{low}-WS	1	1.8	4.6	40.9	2	0.1	0.5	2.1	12.1	2	8.6

WS: water-soluble fraction; Ara: arabinose; Gal: galactose. Glu: glucose. Xyl: xylose; Man: mannose; Phe: phenolics; nd: not detected.

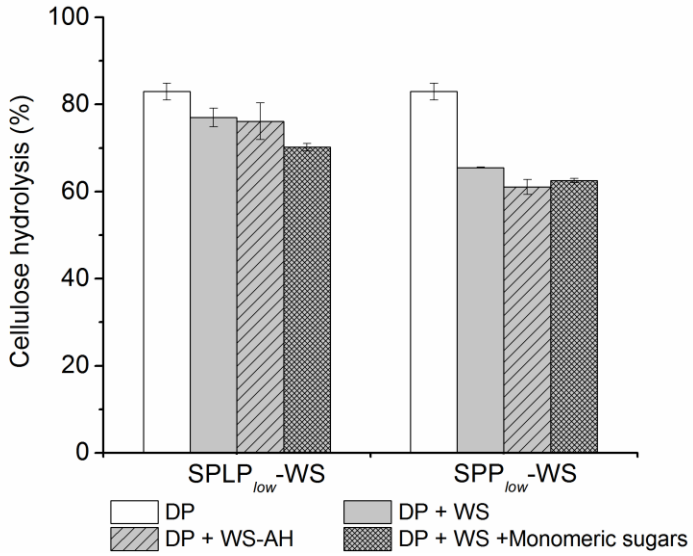

Figure 16 Effect of oligomeric sugar "removal" by acid hydrolysis of the water soluble fractions from low-severity steam pretreated lodgepole pine (SPLP$_{low}$-WS) and poplar (SPP$_{low}$-WS) on the hydrolysis of dissolving pulp (DP). WS: water soluble fraction; WS-AH: acid hydrolysed water soluble fraction.

Although in earlier work, xylooligomers were suggested as being a predominant inhibitory group on cellulase enzymes (Zhang and Viikari, 2012; Zhang et al., 2012), in the work reported here, little or no inhibitory effect was observed from the steam pretreatment derived xylooligomers. However, it is possible that these different inhibition effects might be due to differences in the oligomeric sugar composition, structure, degree of polymerization, substitution, etc. For example, previous work reported that the removal of α-L-arabinofuranosyl side-chains from soluble wheat straw arabinoxylan significantly increased their inhibitory effect on cellulase hydrolysis (Selig et al., 2015). This seemed to indicated that less branched oligomeric sugars were more inhibitory to cellulase enzymes than highly substituted shorter oligomers (Selig et al., 2015). As most of the xylooligomers used in these previous studies were obtained by the alkaline extraction of birchwood, it was highly likely that alkaline extraction already removed most of the branches connected to the xylan backbone while also maintaining a

relatively high degree of polymerization (Fengel and Wegener, 2003; Sjostrom, 2013). In contrast, the water-soluble oligomers derived by the acid catalyzed steam pretreatment of poplar and lodgepole pine used in the current study likely preserved some of the hemicellulose branches and also resulted in oligomers with a relatively low degree of polymerization (Kabel et al., 2007). Related work has also suggested that oligomeric sugars derived from acid based pretreatment processes such as hot-water and dilute acid pretreatment were only slightly inhibitory to cellulase enzymes (Hodge et al., 2008; Kim et al., 2011).

3.1.2.7 Possible influence of the phenolics present in the water-soluble fraction on the hydrolytic potential of the CTec3 enzyme mixture

Past work has shown that, during thermochemical pretreatment, some of the lignin present in the biomass is degraded to various types of phenolics, depending on the biomass source, the pretreatment method and the conditions used (Jönsson et al., 2013). Although phenolics have not tended to be the major inhibitory component generated during pretreatment, they have been shown to inhibit downstream biological processes such as enzymatic hydrolysis and fermentation (Jönsson et al., 2013; Tejirian and Xu, 2011). However, as has been the case in trying to assess the possible inhibitory effects of oligomeric sugars, most of the previous phenolic inhibition studies have used "synthetic compounds". Thus the results have often been inconclusive and sometimes contradictory (Hodge et al., 2008; Kim et al., 2011). To try to assess the possible inhibitory effect of any phenolics present in the water-soluble fraction of the steam pretreatment substrates we added activated carbon to try to selectively remove most of the phenolics.

When activated carbon was added (5% w/w) as an adsorbent, 75% and 47% of the soluble phenolics were respectively removed from the SPLP-WS and SPP-WS fractions with

slight sugar loss detected (Table 10). Although increasing the activated carbon loading to 10% w/w further enhanced phenolics removal, it also resulted in partial removal of the oligomeric sugars, as indicated in Table 10. Although other minor soluble components such as acetic acid, furfural, and hydroxymethylfurfural (HMF) were completely removed after activated carbon treatment, similar to what was observed with the oligomeric sugars, they had almost no effect on the effectiveness of enzymatic hydrolysis (Figure 14).

Table 10 Chemical composition of the original and activated carbon treated (AC) water soluble fraction (WS) from steam pretreated lodgepole pine (SPLP) and poplar (SPP).

WS	Monomeric sugars (g/L)					Oligomeric sugars (g/L)					Others (g/L)
	Ara	Gal	Glu	Xyl	Man	Ara	Gal	Glu	Xyl	Man	Phe
SPLP-WS	4.7	8.5	19.8	15.4	27.2	0.4	2	6.5	2.4	7.9	4.5
SPLP-WS-5%AC	4.3	7.6	19.3	14.8	23.9	0.5	2.3	4.9	1.7	9.8	1.2
SPLP-WS-10%AC	4.2	7.2	18.3	14	22.5	0.3	1.5	2.8	0.5	6.5	0.3
SPP-WS	1.3	2.7	22.9	52.1	3.9	0.1	0.7	5.8	11.3	1.8	9.8
SPP-WS-5%AC	1.2	2.4	21.9	48.8	3.1	0.1	0.8	5.6	7.1	2.9	5.2
SPP-WS-10%AC	1.2	2.4	20.8	45.9	3.3	0.1	0.7	4.6	4.8	2.6	3.1

SPLP/SPP-WS: steam pretreated lodgepole pine/poplar derived water-soluble fraction; SPLP/SPP-WS-5%AC: steam pretreated lodgepole pine/poplar derived water-soluble fraction that has been treated using 5% w/w activated carbon; SPLP/SPP-WS-10%AC: steam pretreated lodgepole pine/poplar derived water-soluble fraction that has been treated using 10% w/w activated carbon. Ara: arabinose; Gal: galactose. Glu: glucose. Xyl: xylose; Man: mannose; Phe: phenolics; nd: not detected.

It was apparent that partial phenolics removal by adding 5% activated carbon significantly increased cellulose hydrolysis, indicating that the phenolics also inhibited the CTec3 enzyme preparation (Figure 17). Interestingly, the activated carbon treated water-soluble fraction resulted in a hydrolysis profile that was almost identical to the one obtained in the

presence of only monomeric sugars (Figure 17). This indicated that a combination of the inhibitory effects of the phenolics and the monosaccharides could account for almost all of the observed inhibition. It appeared that the phenolics present in the SPP-WS fraction were more inhibitory than those present in the SPLP-WS fraction, reducing cellulose hydrolysis by about 18% and 9%, respectively after 72 h (Figure 17). The stronger inhibitory effect of SPP-WS derived phenolics was probably due to the high concentration of phenolics. Unlike monomeric sugar inhibition, the inhibitory effects of phenolics were negligible at the initial stages of cellulose hydrolysis (first 12 h). Inhibition of xylanases and β-glucosidases by simple phenolic compounds, such as vanillic and syringic acids, has been reported previously (Ximenes et al., 2010) with the inhibitory effect gradually increasing with prolonged contact time between the phenolics and the enzymes.

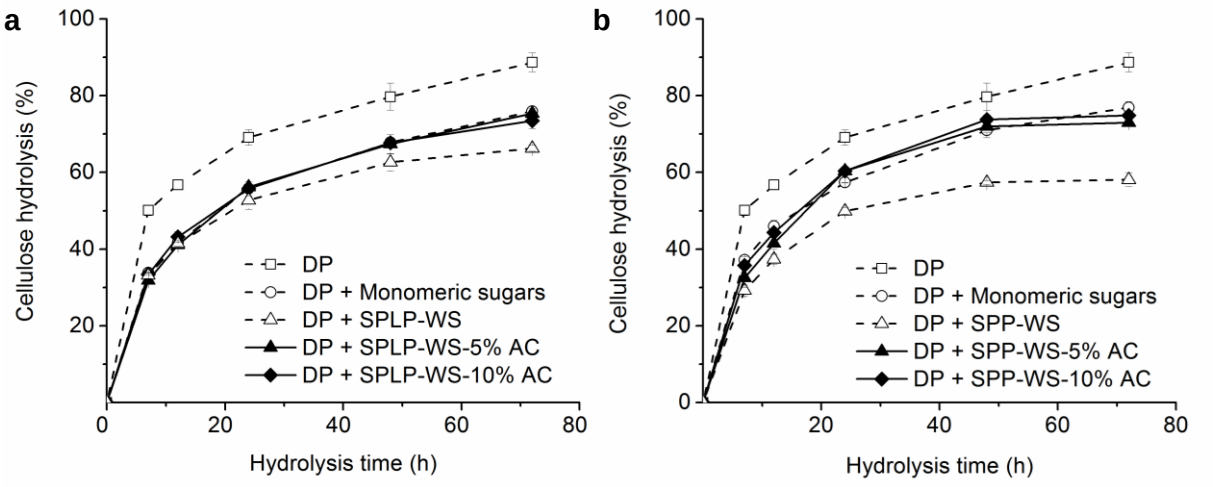

Figure 17 Effect of partial phenolics removal by activated carbon treatment of the water soluble fractions from (a) steam pretreated lodgepole pine (SPLP-WS) and (b) poplar (SPP-WS) on cellulose hydrolysis of dissolving pulp (DP). Phenolics were removed from SPLP/SPP-WS by using 5% and 10% (w/w) activated carbon treatment in order to obtain water soluble fractions with different concentrations of phenolics. These are referred to as SPLP/SPP-WS-5%/10%AC.

3.1.3 Conclusions

It was apparent that the more recent CTec3 enzyme mixture, as compared to the "older" Celluclast cellulase preparations, demonstrated a much better hydrolytic performance on various cellulosic substrates and was also more resistant to the inhibitory components derived from pretreatment. However, the CTec3 was still inhibited, to some extent, by compounds presented in the water-soluble fraction of steam pretreated poplar and lodgepole pine. It appeared that, although monomeric sugars were the major group of inhibitory compounds, phenolics also contributed to inhibition. It also appeared that the inhibitory effect of the monomeric sugars was most influenced by sugar concentration, rather than composition, while the phenolic inhibition was highly dependent on the nature of the biomass source (i.e. softwood and hardwood).

3.2 Which enzyme components are most influenced by the water-soluble compounds derived from steam-pretreated biomass?

3.2.1 Background

The work described in Section 3.1 showed that pretreatment derived water-soluble components such as sugars and phenolics could strongly inhibit the hydrolytic potential of both traditional cellulase preparation (Celluclast) and the recently developed enzyme cocktail (CTec3). As mentioned earlier, most of the previous cellulase inhibition studies used synthetic inhibitors and only assessed their inhibitory effects on either the purified cellulases (Murphy et al., 2013) or the traditional cellulase preparations such as Celluclast and Spezyme CP (Ximenes et al., 2010). However, as indicated in section 3.1, newly developed cellulase enzyme cocktails such as

CTec3 have improved activity, thermostability and inhibitor tolerance. As well as the major cellulases in CTec3 also includes various so-called “accessory enzymes” such as xylanases that likely contribute to its improved hydrolytic performance on various cellulosic substrates (Hu et al., 2013; Shi et al., 2011). Therefore, to try to develop a better inhibition mitigation strategy for the new cellulase cocktail, it would be desirable to further evaluate the influence of these pretreatment derived soluble sugars and phenolics on the major enzyme activities present in the CTec3 enzyme preparation.

Although both the sugars and phenolics present in the water-soluble fraction could strongly inhibit the cellulose hydrolysis, the mechanisms behind this inhibition have yet to be fully resolved (Xiao et al., 2004; Ximenes et al., 2010). For example, sugar inhibition remained at a similar level over the time course of cellulose hydrolysis (Figure 13), while phenolics inhibition developed more sharply at the latter stages of cellulose hydrolysis (Figure 17). In addition, previous research indicated that glucose and cellobiose could strongly inhibit the endo/exo-type of cellulase activities (Murphy et al., 2013), while the phenolic components such tannic acid and gallic acid seemed to selectively inhibit β-glucosidase (BG) activity (Ximenes et al., 2010).

In general, the potential mechanisms of enzyme inhibition can be grouped into either reversible or irreversible inhibition. Reversible enzyme inhibition is thought to be mainly a result of interaction/binding between enzymes and inhibitors, in a concentration-dependent manner (Maurer and Fung, 2000; Smith and Simons, 2004), while irreversible enzyme inhibition, also called enzyme deactivation, is thought to be the result of irreversible binding between inhibitors and enzymes, in a time-dependent manner (Smith and Simons, 2004). For example, glucose can inhibit BG activity reversibly while some phenolics might selectively deactivate the BG activity by binding to the enzyme active sites irreversibly during hydrolysis (Kim et al., 2011; Tejirian

and Xu, 2011). Thus, if we can develop a better understanding of how the major group of inhibitors (sugars and phenolics) restricted the major enzyme activities in CTec3 and the mechanisms behind the inhibition phenomenon, this would likely help us develop more inhibitor-tolerant enzymes cocktails as well as more efficient inhibition mitigation strategies.

The work presented in this chapter further investigated the inhibitory effects of the major pretreatment derived soluble compounds, namely sugars and phenolics (as indicated in Chapter 1), on the major groups of enzyme activities including, exoglucanase (CBH), endoglucanase (EG), β-glucosidase (BG), and xylanase activities within the state-of-the-art cellulase cocktail CTec3. The specific inhibition mechanisms, such as reversible and irreversible inhibition, towards these major enzyme activities, were also evaluated.

3.2.2 Results and discussion

3.2.2.1 Reversible inhibition of specific enzyme activities by pretreatment-derived water-soluble fractions (WSs)

As mentioned earlier, cellobiohydrolases (CBH), endoglucanases (EG), beta-glucosidases (BG) and xylanases are the major types of enzyme activities that govern the overall hydrolytic potential of cellulase preparations on various pretreated lignocellulosic substrates (Van Dyk and Pletschke, 2012). Although the recently identified lytic polysaccharide monooxygenases (LPMOs), which oxidatively cleaves cellulose chain, have also been reported to greatly contribute to the hydrolytic potential of CTec3 preparations, the effect of WSs on LPMO was not covered in the current study due to the lack of accurate methods to measure this activity in an enzyme mixture (Isaksen et al., 2014). Since these major enzyme components (CBH, EG, BG and xylanases) contain quite different amino acid sequences and protein tertiary structures (Levasseur et al., 2013), it was anticipated that they might be influenced by different

mechanisms such as reversible and/or irreversible inhibition by the inhibitors present within the WS fractions. As discussed earlier, reversible enzyme inhibition typically happens immediately after adding the inhibitors and the extent of inhibition follows a concentration-dependent manner, which means the enzyme activity can be recovered once these inhibitors were removed. In contrast, irreversible inhibition takes place after incubation of the enzymes with inhibitors and the enzyme activity cannot be recovered since the inhibitors have bound to the enzyme active sites irreversibly.

As described earlier, reversible enzyme inhibition can be assessed by measuring the specific enzyme activities in the presence of inhibitory compounds at varied concentrations. This is because reversible inhibition decreases enzyme activities quickly and the level of inhibition is positively correlated with inhibitor concentration. To assess potential reversible inhibitory effect of WS fractions, the major enzyme activities (EG, CBH, BG, xylanase activities) of CTec3 were measured with and without the supplementation of varied concentrations (0-100%) of the WS fractions. The model substrates, carboxymethyl cellulose (CMC), p-nithrophenyl-β-d-cellobioside (PNPC), p-nitrophenyl-β-d-glucopyranoside (PNPG) and birchwood xylan were respectively used as the model substrates for the enzyme activities listed above (Figure 18). It was apparent that the WS fractions derived from both the pretreated softwood lodgepole pine (SPLP-WS) and the hardwood poplar (SPP-WS) exhibited similar inhibitory effect towards each of the enzyme activities. Although higher concentrations of the WS fractions decreased the BG and CBH activities to a greater extent (up to more than 50%), the EG and xylanase activities were less affected, even at high WS fraction concentrations (Figure 18). As the CBH and BG are the major enzyme components that account for around 50-70% of the protein content of the cellulase enzyme mixtures (Markov et al., 2005), it was anticipated that the decrease in the CBH

and BG activities would greatly inhibit the overall hydrolytic potential of CTec3 (Gunjikar et al., 2001).

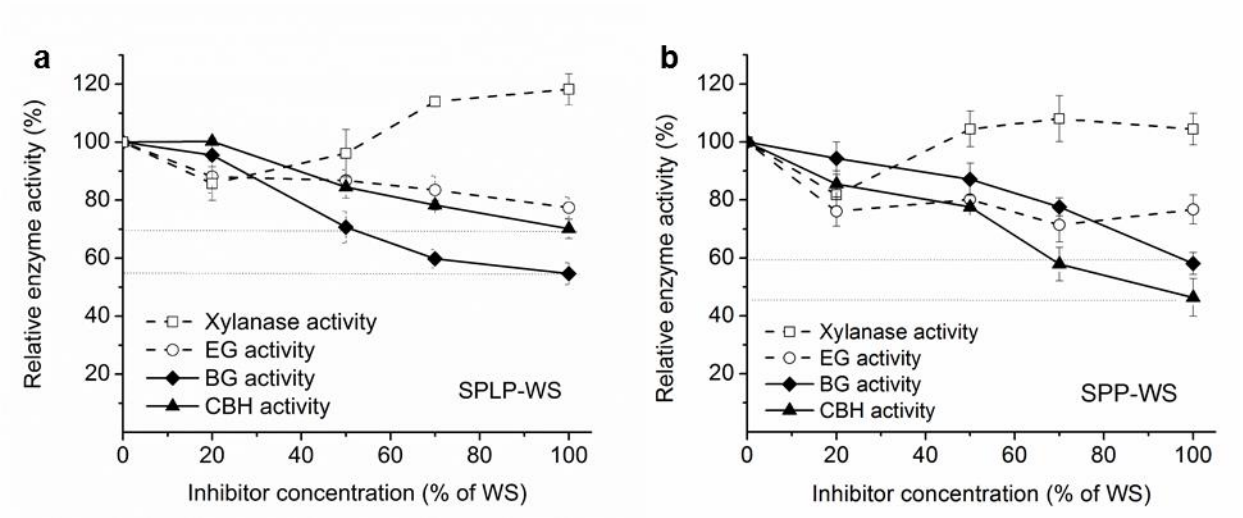

Figure 18 The influence of the water soluble compounds on the xylanase, endoglucanase (EG), β-glucosidase (BG), cellobiohydrolase (CBH) activities within CTec3 at various concentrations of (a) SPLP-WS and (b) SPP-WS. The activities of EG, CBH, BG and xylanase were measured using the model substrates CMC, PNPC, PNPG and xylan, respectively. The water soluble (WS) fractions were added at the start of the assay. 100% EG, CBH, BG, xylanase activities corresponded to 6, 2, 15 and 14 U/mg protein, respectively. These values, measured in the absence of inhibitors, were used as a reference to calculate the loss of enzyme activities due to the presence of inhibitors.

3.2.2.2 Identifying the major inhibitors (sugars and phenolics) that account for the reversible inhibitory effect of the water-soluble fractions (WSs) on the major enzyme activities

As sugars (especially the monomeric sugars) and phenolics were the two major inhibitory compounds within the WS fractions which strongly inhibited the hydrolytic performance of CTec3 enzyme preparation on various cellulosic substrates (Section 3.1), we anticipated they were also the major compounds that significantly inhibited the CBH and BG activities. In order to test this hypothesis, we next measured the specific activities of CBH and

BG in the presence of “synthetic monomeric sugars” (the mixture of pure sugars present in the same sugar concentration/proportion as detected in the WS fraction). As expected, the BG activity (30-40% reduction) was more strongly inhibited than CBH activity (10-20% reduction) by monomeric sugar mixture due to the well-known end product inhibition mechanism (Figure 19). However, when the inhibitory effects of different monomeric sugars such as glucose, mannose and xylose were compared, it was apparent that the hemicellulose-derived monomeric sugars mannose and xylose were not inhibitory, even at concentration up to 100 g/L (Figure 20). This was surprising as these sugars significantly inhibited the cellulase mediated cellulose hydrolysis (Figure 13). This suggested that the hemicellulose-derived sugars might have a different inhibition mechanism to that of glucose.

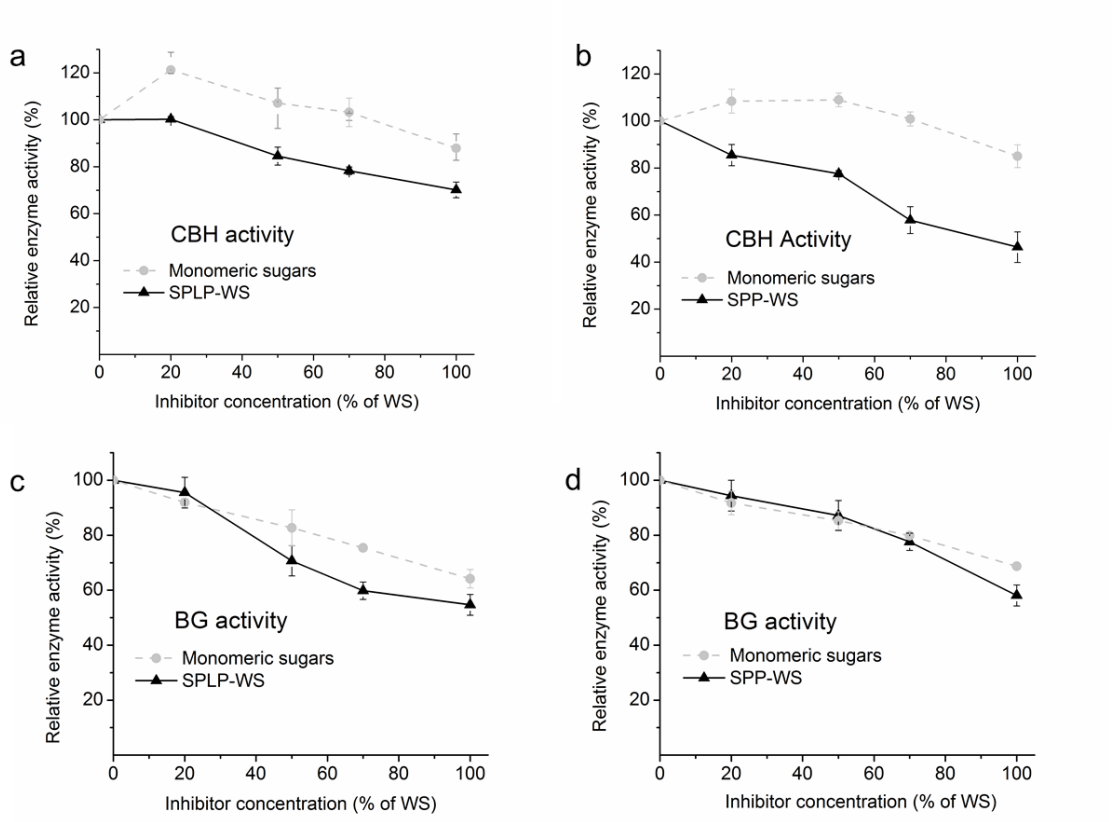

Figure 19 The influence of monomeric sugars from the water soluble fractions (WS) of steam pretreated lodgepole pine and poplar (SPLP/SPP-WS) on the activities of CBH (a-b) and BG (c-d). Inhibitor concentration is expressed as the percentage of original WSs. Inhibitors were added at the start of the assay. 100% CBH and BG activities corresponded to 2 and 15 U/mg protein, respectively. These values, measured in the absence of inhibitors were used as a reference to calculate the loss of enzyme activities due to the presence of inhibitors.

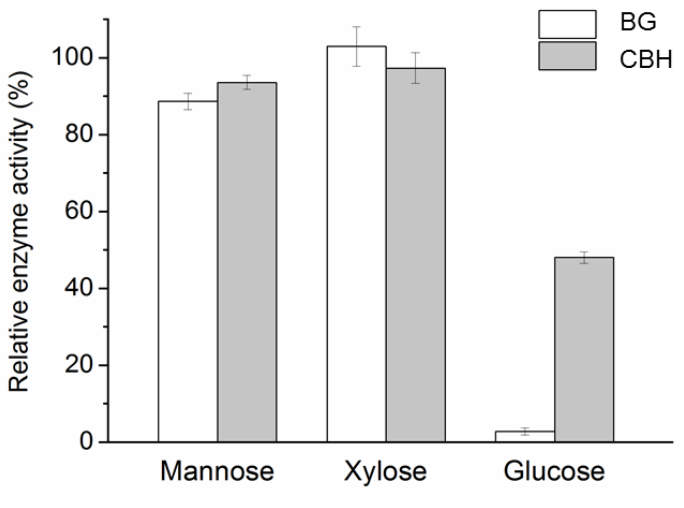

Figure 20 The influence of individual sugars on CBH and BG activities at 100 g/L of mannose, xylose and glucose. Sugars were added at the start of the assay. 100% CBH and BG activities corresponded to 2 and 15 U/mg protein, respectively. These values, measured in the absence of sugars, were used as a reference to calculate the loss of enzyme activities due to the presence of added sugars.

The observed inhibition of cellulase activity by glucose was expected, since previous studies had shown that glucose strongly inhibits BG in a competitive manner, and CBH activity, to a lesser extent, in a non-competitive or uncompetitive manner (Chauve et al., 2010; Teugjas and Väljamäe, 2013b). As a result of the strong inhibition of BG activity by glucose, cellobiose accumulates, which in turn strongly inhibits cellulase activities, leading to a slowdown of enzymatic hydrolysis of cellulose (Oliva-Taravilla et al., 2016; Xiao et al., 2004). However, the results reported here seem to indicate that, although the hemicellulose-derived sugars are not inhibitory to the major cellulase enzymes present within the CTec3 preparation (Figure 21), they do inhibit enzyme mediated cellulose hydrolysis. This suggested that the inhibition mechanism of hemicellulose-derived sugars may be related to other factors rather than the inhibition of specific enzyme activities. As will be described later in the thesis (Chapter 3), further studies were performed to clarify the mechanism behind hemicellulose-derived sugars inhibition.

Since the phenolic components were hard to purify from the pretreatment derived WS fractions, we used activated carbon to try to selectively remove some of the phenolics to try to alleviate their possible inhibition on cellulose hydrolysis (Figure 17). To try to better understand the possible influence of the phenolics fraction on the individual BG and CBH activities, we also used activated carbon (AC) to investigate how partial phenolics removal might alleviate enzyme inhibition. It appeared that partial removal of the phenolics from the SPLP-WS and SPP-WS fractions significantly alleviated inhibition on CBH activity but had little influence on BG activity (Figure 21 a, b). The SPP-WS derived phenolics also seemed to show a stronger inhibitory effect on CBH activity than did the SPLP-WS derived phenolics (Figure 21 a, b). This followed the same trend as observed with the inhibition of cellulose hydrolysis (Figure 12).

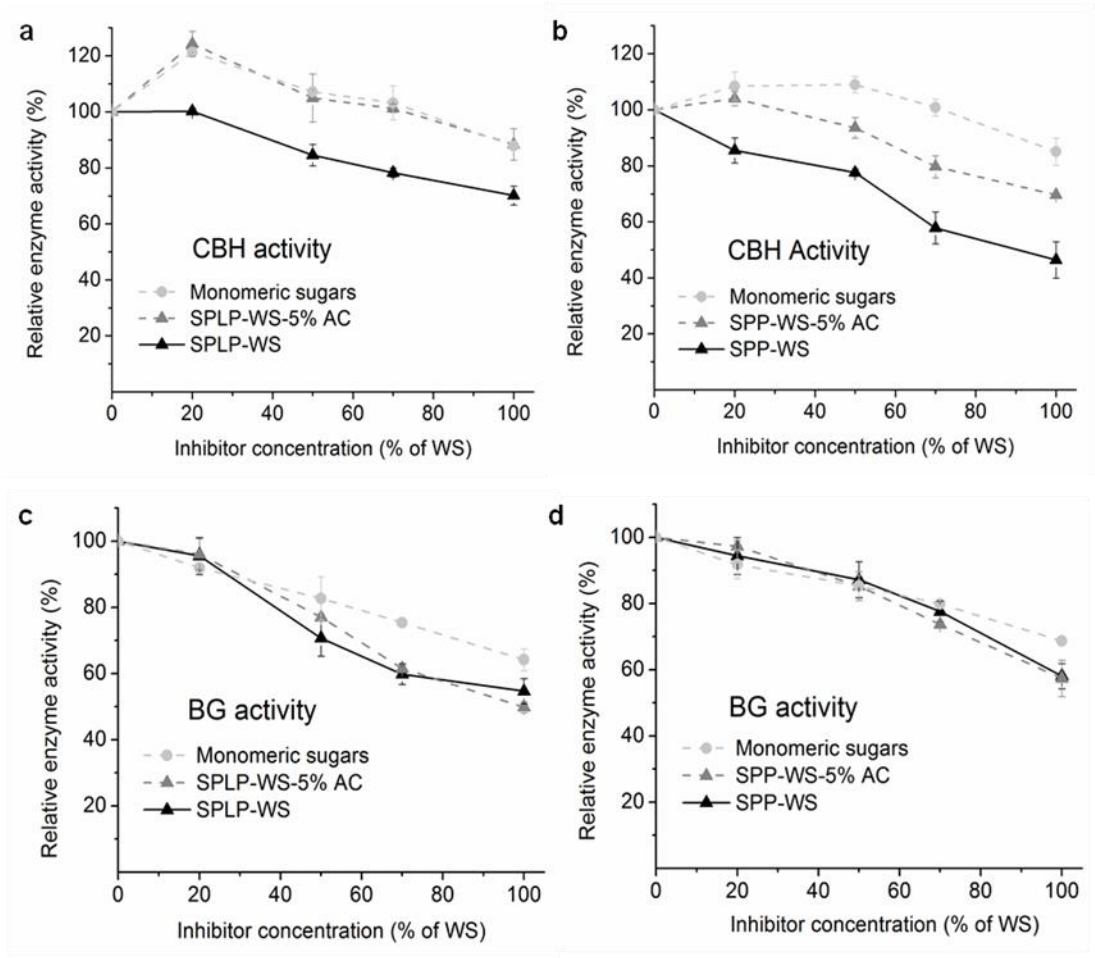

Figure 21 The influence of partial phenolics removal, using activated carbon treatment, from the water soluble fractions (WS) of steam pretreated lodgepole pine and poplar (SPLP/SPP-WS) on the activities of CBH (a-b) and BG (c-d). Phenolics were removed from SPLP/SPP-WS by using 5% (w/w) activated carbon treatment in order to obtain WS fractions with lower concentration of phenolics. These are referred to as SPLP/SPP-WS-5%AC. Inhibitor concentration is expressed as the percentage of original WSs. Inhibitors were added at the start of the assay. 100% CBH and BG activities corresponded to 2 and 15 U/mg protein, respectively. These values, measured in the absence of inhibitors were used as a reference to calculate the loss of enzyme activities due to the presence of inhibitors.

It is possible that the significant phenolic inhibition of CBH activity by might be caused by the strong interaction between CBH and phenolics as the catalytic domain of CBHs contains several tryptophan residues that serve as hydrophobic sugar-binding platforms (Nakamura et al., 2013). The carbohydrate binding module of CBHs also has a planar binding surface which contains several amino-acid residuals with aromatic side chains that interact with the cellulose surface (Momeni, 2014). Thus, it is possible that the aromatic amino-acid residuals in the CBHs interact with aromatic phenolics through π-π stacking interactions. In addition, the linkers in CBHs are rich in proline and also highly glycosylated (Srisodsuk et al., 1993). Since prolines in protein structure could result in the formation of a loose and open structure highly accessible to phenolics, proline has been shown to be a key amino-acid residual that facilitates enzyme-phenolics interactions (Baxter et al., 1997; Pascal et al., 2009). As this glycosylated linker is essential for both the binding of CBH to cellulose and the processivity of CBH during the hydrolysis (Payne et al., 2013), it is also likely that the phenolics decrease CBH activity by binding to their linker regions, consequently interrupting the interaction of the CBH with the cellulose. As BG does not have a similar structure for promoting enzyme binding to phenolics (Dale et al., 1985), this might be one of the reasons for it higher tolerance to phenolics than CBH.

It appeared that phenolics-mediated inhibition is not only dependent on the type of enzyme activity, but also the source of the biomass. For example, the SPP-WS derived phenolics were more inhibitory than the phenolics from SPLP-WS fraction. However, this might be related to different substrate characteristics such as degree of polymerization (De Freitas and Mateus, 2002; Nakagame et al., 2011; Sarni-Manchado et al., 1999), functional groups (Berlin et al., 2006; Qin et al., 2016) and the overall hydrophobicity (Nakagame et al., 2011) of the material. However, further studies will be needed to further clarify the detailed mechanisms of phenolics-mediated inhibition of cellulase enzyme activities.

3.2.2.3 Irreversible inhibition (deactivation) of specific enzyme activities by pretreatment-derived water-soluble fractions (WSs)

As mentioned earlier, compared to reversible enzyme inhibitors, irreversible inhibitors usually interact with different functional groups on the enzyme/protein surface through covalent bonds or through exceptionally strong noncovalent bonds which sometimes remain even after complete protein breakdown (Silverman and Holladay, 2014). Irreversible inhibition is also sometimes referred to as enzyme deactivation and is indicated by a time-dependent loss of enzyme activity (Maurer & Fung, 2000). As the water soluble fractions inhibited the extent of cellulose hydrolysis (cellulose conversion at 72 h) more than the rate of hydrolysis (cellulose conversion at initial 24 h) (Section 3.1, Figure 12), it was anticipated that enzyme deactivation also occurred during cellulose hydrolysis.

Therefore, to try to determine if enzyme deactivation was occurring, the CTec3 preparation was first incubated with either a WS fraction or buffer, for various times to simulate a 'real' hydrolysis scenario. As expected, the enzyme activities of CBH, EG, BG, and xylanase decreased gradually in a time-dependent manner after incubating with the WSs fractions. This indicated that the WS fractions contained soluble compounds that deactivated the major enzyme activities during hydrolysis (Figure 22). For example, the SPP-WS fraction decreased the CBH activity by more than 60% after 72-h incubation (Figure 22b). Although xylanase was thermally unstable, both the SPP-WS and the SPLP-WS fractions significantly accelerated the deactivation of xylanase activity over incubation time (Figure 22 c, d). The WS fractions also deactivated EG and BG activities by 20%-30% after 72 h incubation (Figure 22 e, f). It was apparent that the deactivating effect of the WSs fractions was highly biomass substrate dependent. For example, unlike the effect of the SPP-WS fraction, the SPLP-WS fraction only slightly decreased the CBH activity after 72 h (Figure 22 a). These results indicated that the major enzyme components (such

as CBH, xylanase, EG and BG) were deactivated, to a certain extent, when incubated with the WSs fractions derived after pretreatment.

It is worth noting that the CBH activity (the major enzyme activities in most cellulase preparations) was particularly inhibited by the pretreatment derived WS fractions and that the deactivation correlated to the extent of WS inhibition of overall cellulose hydrolysis (Figure 12). Thus, the deactivation of CBH activity was likely to be one of the major reasons for the slowdown of enzymatic hydrolysis during the later stages of hydrolysis. Although the xylanase activity was also inhibited by the WS fractions, this was not expected to be a major reason for the reduced degree of cellulose hydrolysis as the model cellulosic substrate, dissolving pulp, had a very low hemicellulose content (Figure 12). However, it is likely that xylanase deactivation will cause a significant decrease in the cellulose hydrolysis of pretreated substrates which will contain higher amounts of xylan. Thus, to facilitate the fast, complete hydrolysis of pretreated lignocellulosic substrates, it will be important improve the tolerance of CBH and xylanases to the soluble, inhibitory compounds present in the water soluble (WS) fractions of pretreated biomass substrates.

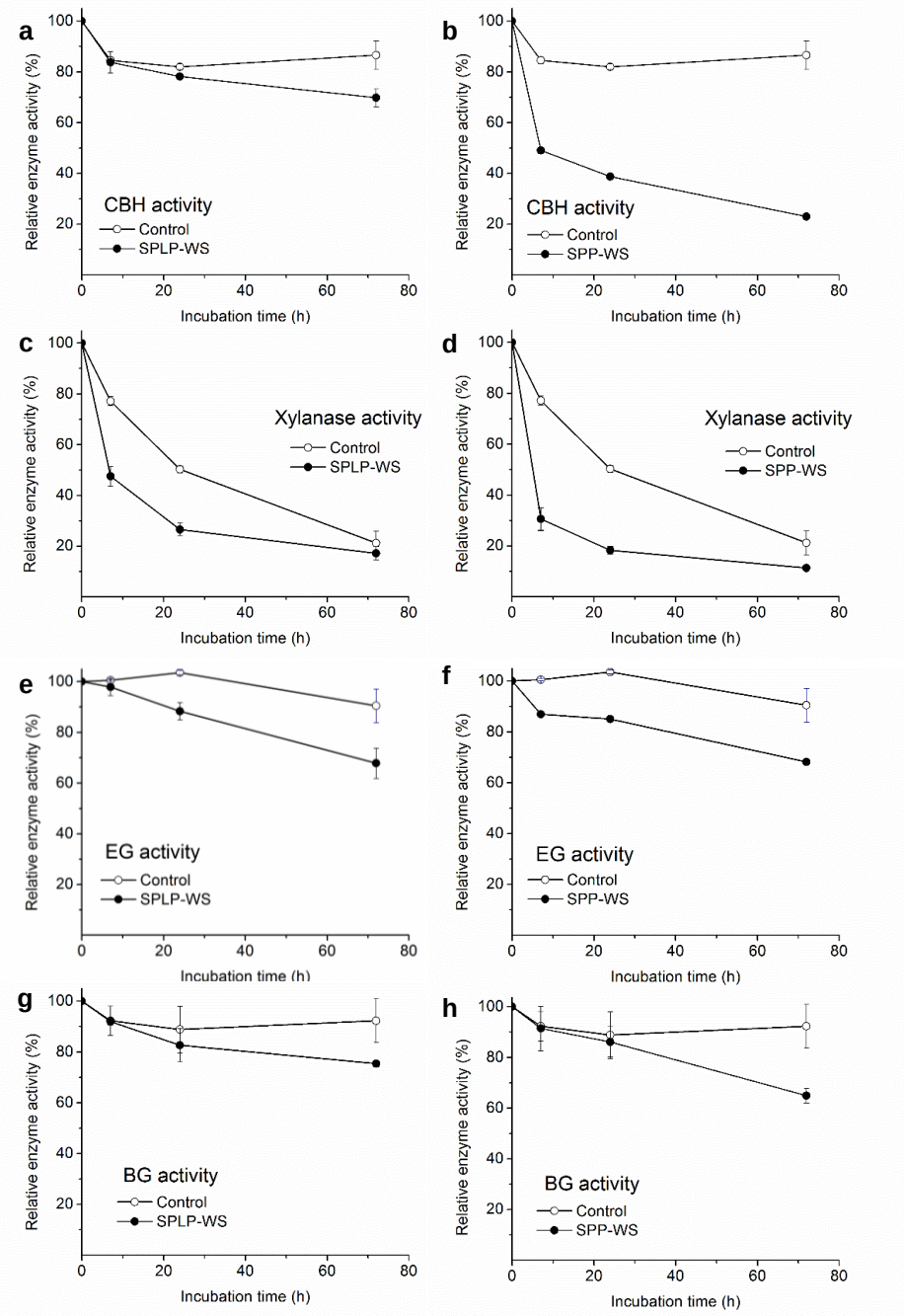

Figure 22 Effect of SPLP/SPP-WS fractions on the residual enzyme activities of (a, b) CBH, (c, d) xylanase, (e, f) EG and (g, h) BG after 72 h incubation at 50 ^{o}C. Controls were performed by incubating enzyme with buffer under the same conditions. The enzyme was incubated with the inhibitors for a certain time at 50 ◦C and 200 rpm. 100% EG, CBH, BG, xylanase activities corresponded to 6, 2, 15 and 14 U/mg protein respectively. These values, measured in the absence of inhibitors were used as a reference to calculate the loss of enzyme activities due to the presence of the possible inhibitors.

3.2.2.4 Identifying the major inhibitors (sugars and phenolics) that account for the irreversible inhibitory effect of the water-soluble fractions (WSs) on the major enzyme activities

Since the WS fractions derived from the pretreated biomass greatly deactivated the major enzyme activities (Figure 23), we next want to assess which of the major soluble compounds within WS fractions was contributing the most to enzyme deactivation. As has been discussed earlier, sugars are more likely to act as reversible inhibitors (Andrić et al., 2010a). As has also been reported earlier, some phenolics, such as tannic acid, can deactivate cellulase enzymes activities and cause enzyme precipitation (Ximenes et al., 2010; Zakaria et al., 2016). As we anticipated that the pretreatment-derived phenolics within the WS fractions deactivated the major enzyme activities during the time course of cellulose hydrolysis we next assessed the deactivating effects of phenolic components on the major enzyme activities present in the CTec3 enzyme cocktail.

As expected, the phenolics present in the water soluble fractions deactivated cellulase enzyme activities in a time-dependent manner (Figure 23), since the activated carbon treated WS fractions (SPLP/SPP-WS-5% AC) showed no deactivating effect as compared to the SPP-WS and SPLP-WS fractions without activated carbon treatment. This was further confirmed when the addition of synthetic monomeric sugars did not result in enzyme deactivation as compared to the control samples (Figure 23 a, b). As was observed before (Figure 22), the cellulase inhibition effect was highly dependent on the nature of the phenolics, as the phenolics within the SPP-WS fraction showed much stronger inhibition effects than those of the phenolics within the SPLP-WS fraction (Figure 23).

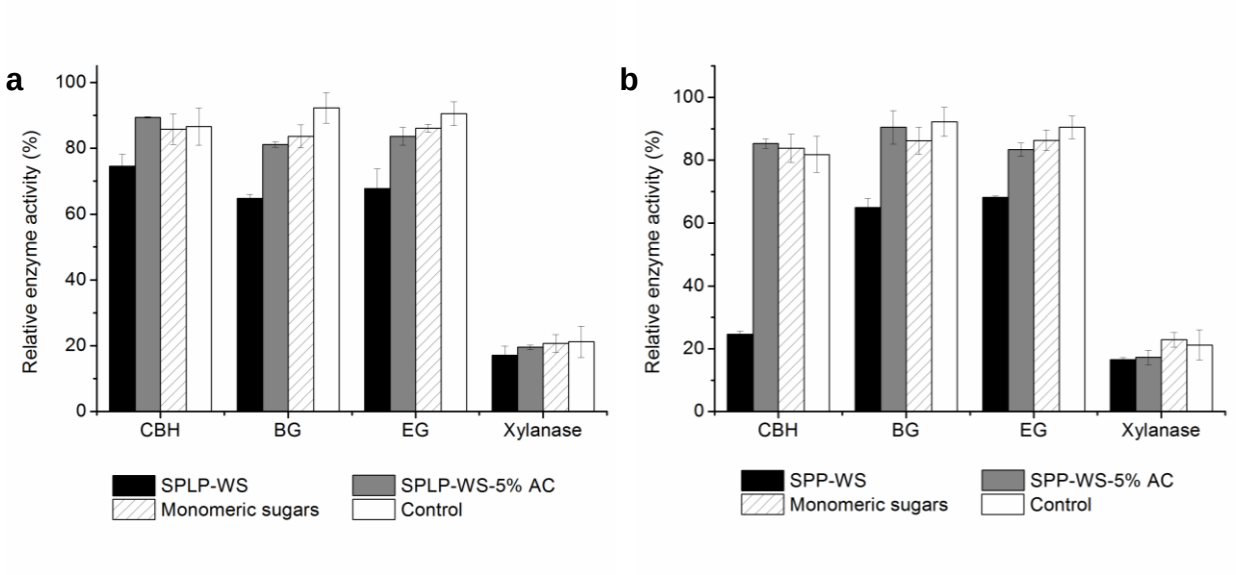

Figure 23 Influence of monomeric sugars and activated carbon (AC) treated water soluble fractions from (a) steam pretreated lodgepole pine (SPLP-WS) and (b) poplar (SPP-WS) on enzyme deactivation within 72 h incubation. 5% activated carbon (AC) was used to treat the water soluble fractions to selectively remove phenolics from the WS fractions (SPLP/SPP-WS-5%AC). The compositions of monomeric sugars were same as the correspondent WS fractions. 100% EG, CBH, BG, xylanase activities corresponded to 6, 2, 15 and 14 U/mg protein respectively. These values, measured in the absence of possible inhibitors were used as a reference to calculate the loss of enzyme activities due to the presence of possible inhibitors.

As it has been shown that activated carbon treatment selectively removes more hydrophobic phenolics (Dąbrowski et al., 2005), it is likely that the more hydrophobic phenolics are the major inhibitory compounds. Although the specific inhibition mechanism of the phenolics was not determined, the work reported here suggested that there might be a possibility of mitigating the inhibitory effect of the phenolics by modifying the upstream pretreatment process to produce less inhibitory (less hydrophobic) phenolics.

3.2.2.5 Proposed mechanism for enzyme inhibition/deactivation by the water-soluble fractions (WSs)

As mentioned in Section 1.5, a group of cellulase enzymes (e.g. CBHs, EGs, and BG) and several key "accessory" enzymes such as xylanases have to work in a synergistic manner to efficiently break down the cellulose within various lignocellulosic biomass into the monomeric sugar platform (Gusakov, 2013; Hu et al., 2013; Wang et al., 2012). In the presence of pretreatment-derived water-soluble fraction, it appeared that the CBH and BG activities were reversibly inhibited by sugars components while the CBH and xylanase activities were mainly irreversibly deactivated by phenolics (as indicated in Figure 24). These combined inhibitory effects contributed to a loss of efficacy of the cellulase preparation towards the cellulosic substrates.

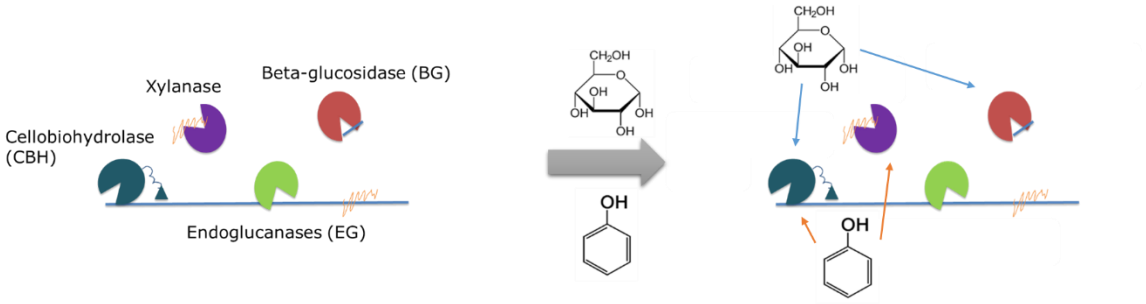

Figure 24 Schematic of the possible inhibitory effect of sugars and phenolics on major individual enzyme activity in CTec3.

3.2.3 Conclusions

Pretreatment-derived inhibitors restrict the efficient enzymatic hydrolysis of various pretreated biomass, especially during whole slurry hydrolysis i.e. water-soluble and water-insoluble fractions combined. In the work reported here, the main enzyme components (CBH and BG) within commercial cellulase preparations were strongly inhibited by pretreatment-derived compounds. It appeared that BG activity was mainly affected by glucose while the CBH

activity was mainly influenced by phenolics. Surprisingly, unlike glucose, the hemicellulose-derived sugars, mannose and xylose, did not appear to inhibit the major enzyme activities. However, they did inhibit cellulose hydrolysis, indicating that a different inhibition mechanism might be occurring in parallel with the end product inhibition caused by glucose. Phenolics are the major enzyme deactivators in the pretreatment-derived soluble fractions. These components resulted in a significant irreversible loss of specific enzyme activities, especially CBH and xylanase activities, in a time-dependent manner. The deactivating influence of phenolics was also highly dependent on the origin of the biomass and the concentration of the phenolics.

3.3 Assessing possible inhibition of cellulose hydrolysis by mannose and xylose and how certain substrate characteristics might also influence hydrolysis

3.3.1 Background

As indicated earlier, the monomeric sugars such as glucose, mannose and xylose that are found in the pretreatment-derived water-soluble fractions, inhibited the hydrolytic potential of both traditional and the newer cellulase enzyme preparations. Unlike glucose, mannose and xylose did not appear to inhibit the individual, major cellulase activities but did decrease the hydrolytic potential of cellulases when they acted on insoluble cellulosic substrates (Figure 13). Although the inhibition mechanism of glucose on cellulase enzyme activities has been well documented, the mechanism of inhibition of mannose and xylose has yet to be clarified. For example, although some researchers have proposed that the hemicellulose derived sugars mainly affected the accessibility of free water (one of the reactants) to the cellulase enzymes (Hsieh et

al., 2015), other workers have suggested that these sugars interrupt the binding of cellulase enzymes to the cellulose surface (Pribowo, 2014). Thus, since xylose and mannose account for the major part of sugars in the pretreatment-derived water-soluble fraction, we next wanted to better determine their specific inhibition mechanisms on cellulose hydrolysis.

As the hemicellulose-derived sugars did not appear to inhibit specific enzyme activities (Figure 20), we anticipated that they might influence the synergistic behavior of the collective cellulase enzymes during cellulose hydrolysis. It is well recognised that to achieve effective cellulose hydrolysis several cellulases need to work together. Typically, of all of the cellulases from *Trichoderma reesei* (*Hypocrea jecorina*), Cel7A accounts for more than half of the total protein present in a cellulase enzyme mixture. Thus, it has been suggested to be the major component responsible for much of the enzyme cocktails effectiveness during cellulose hydrolysis (Zhang et al., 2006). The catalytic behaviour of Cel7A takes place in the solid-liquid interface which involves several key steps such as enzyme adsorption, productive binding, hydrolysis initiation, processive cleavage and enzyme desorption from cellulose. The adsorption/binding of cellulases to cellulose surface is a prerequisite for effective Cel7A catalyzed cellulose hydrolysis (Zhang and Lynd, 2004). This is facilitated by the tunnel-shaped catalytic domain (CD), smaller carbohydrate binding module (CBM) and a glycosylated linker peptide (Stahlberg et al., 1991). It has been shown that the unique tunnel-shaped catalytic site enables processive hydrolysis and movement on crystalline cellulose (Kipper et al., 2005). This step has been considered to key in achieving efficient cellulose degradation (Horn et al., 2012b). Thus, it is likely that the inhibitory effect of mannose and xylose was a result of their influence on any one of these steps during hydrolysis.

As mentioned several times earlier, for effective biomass deconstruction, a pretreatment step is required to open up the cell wall structure to enhance subsequent enzymatic hydrolysis.

Depending on the pretreatment conditions used and the composition of the starting woody biomass, the cellulose within the pretreated substrate will exhibit different physicochemical characteristics (such as accessibility, crystallinity and surface charge) (Zhang and Lynd, 2004). Previous work, that used a wide range of cellulosic substrates with varied cellulose properties, showed that the extent of inhibition was influenced by these substrate characteristics (Andrić et al., 2010a).

In this chapter, to try to better understand the inhibition mechanism of mannose and xylose on cellulose hydrolysis, we evaluated the effects of these sugar components on the kinetics of enzymatic cellulose hydrolysis (e.g. enzyme adsorption/desorption, productive binding, processivity) by using both commercial enzyme preparation and the purified major cellulase Cel7A. A "library" of model cellulosic substrates, which included Avicel, cellulose II, nanocellulose and dissolving pulp, with distinct characteristics, were used to try to evaluate how cellulose characteristics (e.g. crystallinity, degree of polymerization, accessibility and acid group content) might influence hemicellulose sugar-derived enzyme inhibition.

3.3.2 Results and discussion

3.3.2.1 Mannose and xylose strongly inhibit cellulose hydrolysis but do not affect the major enzyme activities present in commercial enzyme mixtures

To try to better clarify the possible inhibition mechanism of hemicellulose-derived monomeric sugars such as mannose and xylose, we first evaluated their inhibitory effects on cellulose hydrolysis and the major enzyme activities. Since different pretreatment strategies and the nature of the biomass feedstock will influence the final sugar concentration present in the whole (combined water soluble and water-insoluble fractions) pretreated liquids, a range of mannose and xylose concentrations (0-100 g/L) were selected to mimic the real, whole slurry

hydrolysis conditions. As expected, both mannose and xylose greatly decreased the cellulose hydrolysis by up to ~30%, depending on the sugar concentration (Figure 25a). However, they did not inhibit the major enzyme activities within the enzyme mixture (e.g. CBH, BG, EG, xylanase activities) (Figure 25b). The control, glucose addition displayed a different inhibition pattern, where it not only inhibited cellulose hydrolysis (Figure 25a), it also greatly reduced the BG and CBH activities (Figure 25b). Thus, it appeared that, unlike glucose, the inhibition of cellulose hydrolysis by mannose and xylose was not directly caused by the inhibition of the major cellulase enzyme activities. Therefore, we wanted to further investigate the specific inhibition mechanism of mannose and xylose on the cellulose hydrolysis.

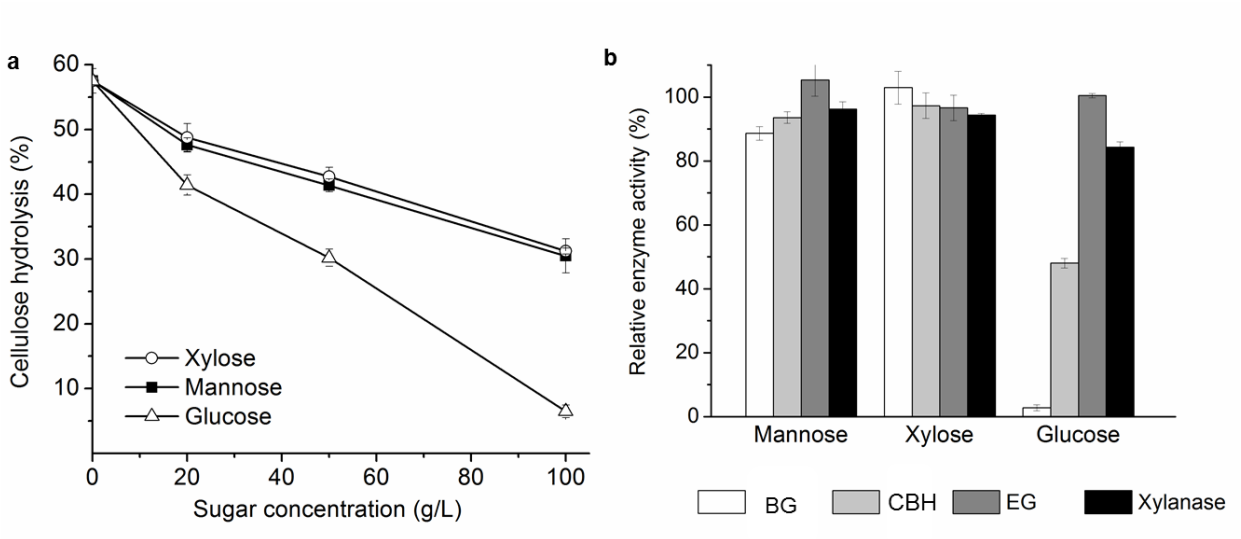

Figure 25 (a) Influence of increasing sugar (glucose, mannose, and xylose) concentration on cellulose hydrolysis. (b) Influence of 100 g/L sugars on the major enzyme activities such as CBH, BG, EG, xylanase. The enzymatic hydrolysis was performed at 50 ^{o}C using an enzyme loading of 30 mg CTec3/g glucan. 100% EG, CBH, BG, xylanase activities corresponded to 6, 2, 15 and 14 U/mg protein respectively. These values, measured in the absence of sugars, were used as a reference to calculate the relative cellulose hydrolysis and enzyme activity due to the presence of possible inhibitors.

3.3.2.2 Mechanism of inhibition of mannose and xylose on purified cellulase

As a commercial "cellulase mixture" includes various enzyme components, the complex enzyme composition introduces various factors that might influence sugar inhibition of cellulose hydrolysis. To try to better understand the mechanism of inhibition, we wanted to assess the influence of sugars on each purified enzyme components. The major cellulase Cel7A, accounts for ~50-60% of total protein secreted by *T. reesei*, and it hydrolyses cellulose from the reducing end in a processive manner (Beckham et al., 2014). It has been also recognized as one of the most important enzyme components that are required in an effective deconstruction "cocktail" that is required for good cellulose hydrolysis. As a result, a considerable amount of work has looked at its mechanistic action in order to improve its performance (Zhang and Lynd, 2004). As the catalytic action of Cel7A on cellulose includes several steps such as adsorption, productive binding, processivity, we anticipated that mannose or xylose might affect these steps, which in turn, influence the overall cellulose hydrolysis. To test this hypothesis, we next studied the effects of mannose/xylose on the kinetic of cellulose hydrolysis by Cel7A.

3.3.2.2.1 Effect of mannose and xylose on Cel7A cellulose hydrolysis and specific activity

Due to the difficulty of measuring minor glucose release in the presence of a high glucose background, this aspect was not studied in detail. However, it is highly likely that glucose does inhibit Cel7A activity as suggested by the data summarised in Figure 25. A recent study also showed that glucose directly inhibited the activity of Cel7A on cellulose by using C_{14} cellulose (Murphy et al., 2013). In the work reported here, the effects of mannose/xylose on the cellulose hydrolysis and the enzyme activity were assessed by using purified Cel7A enzymes. As expected, similar to previous observations (Figure 25), mannose/xylose reduced Cel7A mediated cellulose hydrolysis in a concentration dependent manner (Figure 26). However, when using the soluble

"model" cellulosic substrate such as p-nithrophenyl-β-d-cellobioside (PNPC), Cel7A activity was only marginally influenced with the presence of these sugars even at very high sugar concentrations (Figure 27). Thus, it is likely that cellulose hydrolysis inhibition is not caused by the inhibition of Cel7A activity, but by other factors such as inhibition of adsorption, processivity during cellulose hydrolysis.

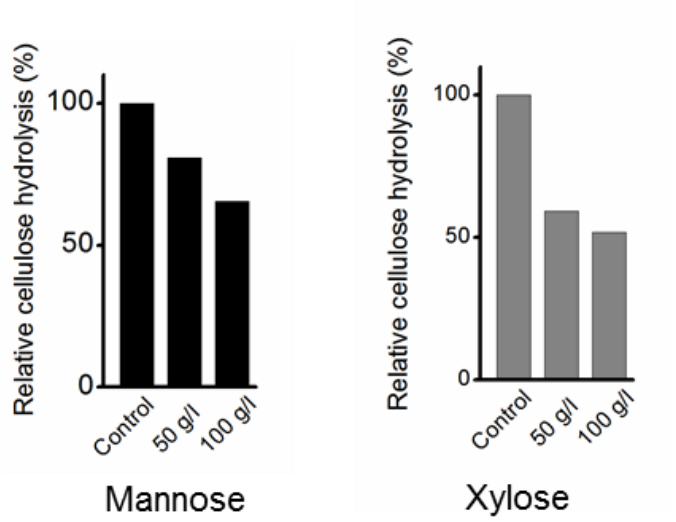

Figure 26 Influence of xylose and mannose on cellulose hydrolysis catalyzed by Cel7A at increasing sugar concentrations. Cellulose hydrolysis was expressed as the relative percentage of hydrolysis obtained in either the presence or absence of added sugar**.** The enzymatic hydrolysis was performed at 50 ºC using an enzyme loading of 30 mg Cel7A/g glucan for 24 h. 100% cellulose hydrolysis corresponded to 16.5% glucan conversion.

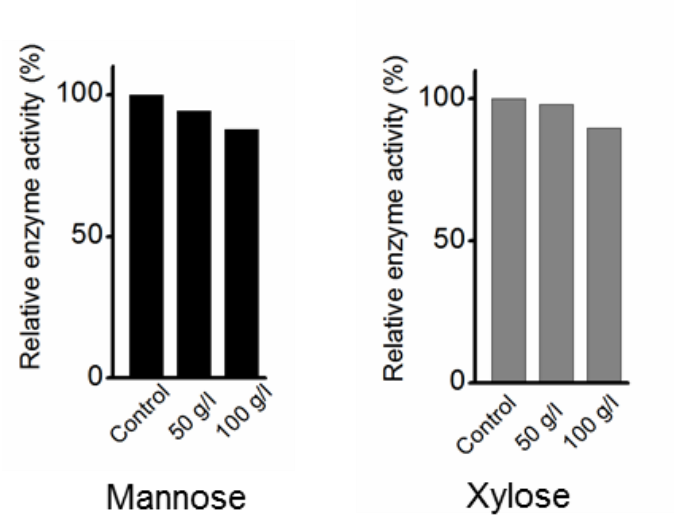

Figure 27 Influence of xylose and mannose on the determined enzyme activity of Cel7A at increasing sugar concentrations. Relative enzyme activity was expressed as the percentage of the parameter tested in the presence of sugar to that without sugars**.** 100% enzyme activity of Cel7A in the stock solution corresponded to 0.03 U/mg protein.

3.3.2.2.2 Effect of mannose and xylose on Cel7A adsorption and productive binding

Previous work has indicated that high concentrations of sugars can interfere with the adsorption/desorption behaviour of cellulase enzymes on cellulose (Pribowo, 2014). However, to what extent the sugars especially mannose and xylose could influence the enzyme adsorption remains unclear. When the effects of mannose and xylose on the absorbability of Cel7A on the "pure" cellulose (dissolving pulp) was assessed, it was apparent that they only had a marginal influence on enzyme adsorption (Figure 28). Thus, the influence of enzyme adsorption was unlikely to be the major reason for their inhibitory effects on cellulose hydrolysis. Recent work has also shown that the adsorption of the cellulase on cellulose was not affected by cellobiose due to the strong binding affinity of carbohydrate binding module (CBM) of cellulases to the cellulose (Jung et al., 2013).

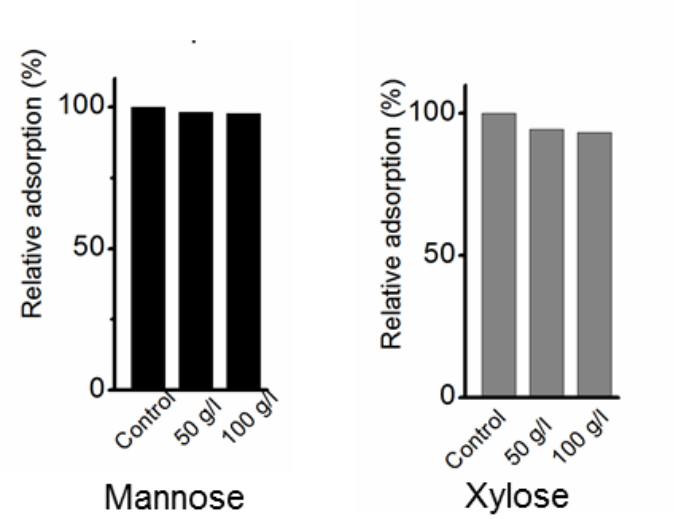

Figure 28 Influence of xylose and mannose on the adsorption of Cel7A at increasing sugar concentrations. Cel7A (30 mg Cel7A/g glucan) was incubated with dissolving pulp (DP) at 30 ^{o}C for 30 min. Relative enzyme adsorption was expressed as the percentage of the parameter tested in the presence of sugar to that without sugars. 100% enzyme adsorption corresponded to 14.0 mg Cel7A/g glucan.

Although adsorption of Cel7A is a prerequisite for its hydrolytic action on cellulose, some studies have suggested that the extent of productive adsorption/binding is actually the determining factor in defining good, enzyme hydrolytic performance (Beckham et al., 2014; Bu et al., 2012; Jung et al., 2013). Productively bound Cel7A enzymes are enzymes that are not only absorbed onto cellulose surface but are also able to access and position the available glucan chains from the reducing ends into their catalytic tunnel (Beckham et al., 2014; Igarashi et al., 2011; Jalak and Väljamäe, 2014). Therefore, it is likely that only these productively bound Cel7A enzymes have the ability to hydrolyse the cellulose substrates. Considering the importance of enzyme productive binding, we next assessed how xylose and mannose might affect the productive binding of Cel7A enzymes on cellulose. We used p-nitrophenyl-β-d-lactoside (PNPL) (a soluble "model" substrate for assessing Cel7A activity) as a probe to determine the unproductive bound Cel7A (these can still react with PNPL due to the free

catalytic site) and the productively bound Cel7A (these can't react with PNPL due to the catalytic site already bind to the glucan chains) to the cellulose surface (Jalak and Väljamäe, 2014). It appeared that 100 g/l of mannose and xylose reduced the amount of Cel7A productive adsorption by ~20% (Figure 29), which indicated that the presence of mannose and xylose could actually decrease the amount of productively bound Cel7A even without influence Cel7A adsorption (Figure 28). Thus, the reduced Cel7A productive adsorption could be one of the reasons that lead to a decrease in cellulose hydrolysis by hemicellulose derived sugars. In addition, xylose seemed to inhibit the productive binding of Cel7A enzymes to a greater extent, indicating that the structure of sugars might also influence the inhibition.

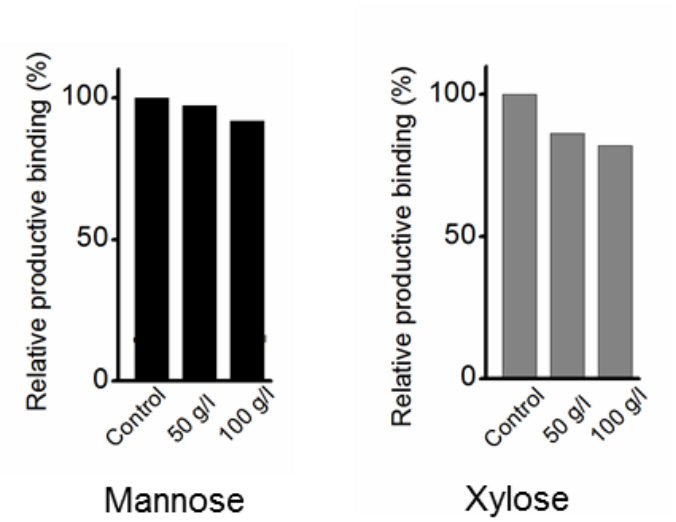

Figure 29 Influence of xylose and mannose on the productive binding of Cel7A at increasing sugar concentrations. Relative productive binding was expressed as the percentage of the parameter tested in the presence of sugar to that without sugars. Cel7A (30 mg Cel7A/g glucan) was incubated with dissolving pulp (DP) at 30 ^{o}C for 30 min. 100% Cel7A productive binding corresponded to 9.9 mg Cel7A/g glucan.

Mannose and xylose reduced the Cel7A productive binding but not adsorption. Thus, it was anticipated that these sugars might only influence the binding of Cel7A catalytic domain (CD) rather than its carbohydrate-binding module (CBM), as the enzyme CDs have much weaker

binding affinity towards cellulose as compared with CBMs (Boraston et al., 2004; Palonen et al., 1999). To test this hypothesis, we next separated and purified the Cel7A CDs and assessed how their cellulose binding affinity might be influenced by mannose and xylose. As expected, mannose and xylose significantly reduced the adsorption of isolated Cel7A CDs on to the cellulose (Figure 30). This further supported the possibility that at least part of the inhibition by mannose and xylose was a result of their interference with the binding of cellulase CDs to cellulose. This, in turn, caused the reduction in productive cellulase binding. A related study also found that cellobiose greatly inhibited the binding of the isolated cellulase CD to cellulose. This was determined by using the single-molecule fluorescence imaging technique (Jung et al., 2013).

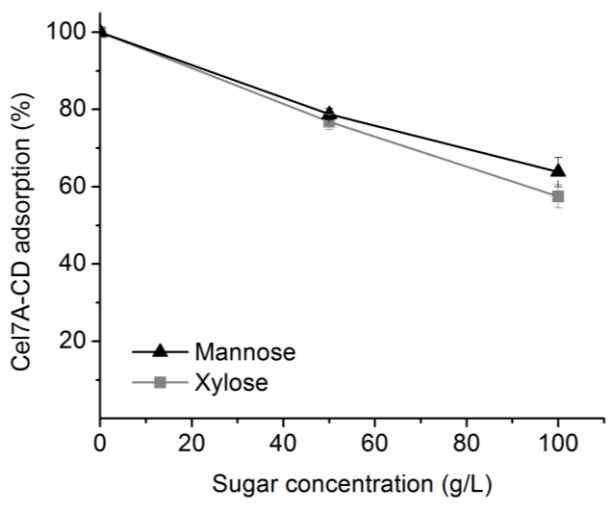

Figure 30 Effect of mannose and xylose on the adsorption of isolated catalytic domain from Cel7A (Cel7A-CD). Cel7A (30 mg Cel7A/g cellulose) was incubated with dissolving pulp (DP) at 30 ^{o}C for 30 min. Relative enzyme adsorption was expressed as the percentage of the parameter tested in the presence of sugar to that without sugars. 100% Cel7A-CD adsorption corresponded to 5.1 mg Cel7A/g glucan.

3.3.2.2.3 Effect of mannose and xylose on Cel7A processivity

Once the Cel7A productively binds to cellulose, it should processively move along the cellulose chain and continuously release cellobiose before dissociating from the chain. This

processive movement of Cel7A is considered to be a determining factor for the efficacy of Cel7A enzymes (Horn et al., 2012b; Nakamura et al., 2014). Therefore, the influence of mannose and xylose on the processivity of Cel7A was further studied to try to better understand their possible inhibition mechanism. Processivity has been typically determined by using the ratio between glucose (from the Cel7A initial cutting) and cellobiose (from the Cel7A processive movement), to reflect the processive movement of Cel7A on the cellulose surface (Fox et al., 2012; Horn et al., 2012b; Nakamura et al., 2014).

It appeared that the presence of mannose and xylose considerably reduced the processivity of Cel7A, up to 50%, during cellulose hydrolysis, depending on the sugar concentration and composition (Figure 31). One of the possible reasons for the strong inhibition of Cel7A processivity was due to the reduced binding affinity of Cel7A CD to cellulose in the presence of these sugars. As mannose or xylose considerably reduced the binding affinity of Cel7A CD (Figure 30), it was reasonable to expect a lower Cel7A processivity in the presence of these sugars. In addition, since xylose showed a stronger influence on the binding of Cel7A CD to cellulose (Figure 30), it would be also expected that xylose had a stronger inhibitory effect on Cel7A processivity than mannose (Figure 31). Other workers have shown that the decreased binding affinity of Cel7A CD to cellulose resulted in a corresponding decrease of Cel7A processivity (Payne et al., 2013). Although we were unable to study the influence of glucose on the processivity of Cel7A, it is likely that glucose would also interfere with Cel7A processivity because of its structural similarity to mannose or xylose. However, since glucose directly inhibits Cel7A and BG activities, its influence on processivity might be a less influential factor.

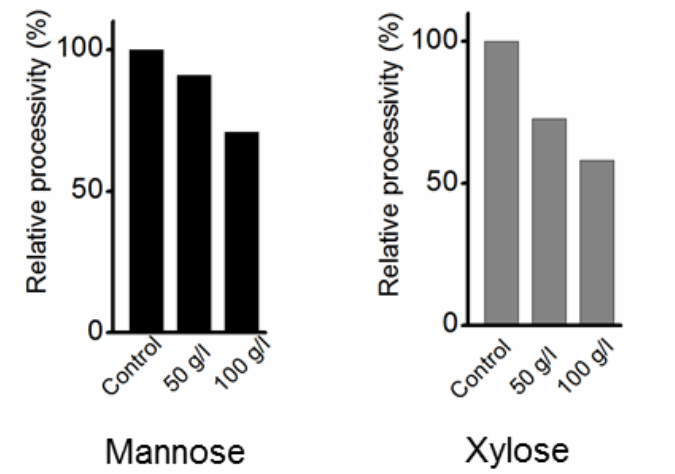

Figure 31 Influence of xylose and mannose on the processivity of Cel7A at increasing sugar concentrations. The enzymatic hydrolysis is performed at 50 ºC using an enzyme loading of 30 mg Cel7A/g glucan for 24 h. Processivity was calculated based on the ratio of cellobiose to glucose. Relative enzyme processivity was expressed as the percentage of the parameter tested in the presence of sugar to that without sugars. 100% Cel7A processivity corresponded to 10.0.

After evaluating the potential influences of mannose and xylose on both the commercial cellulase preparation and the purified major cellulase monocomponent, it was apparent that, even though these hemicellulose derived sugars did not directly inhibit cellulase catalytic ability, they greatly decreased the productive binding of Cel7A on cellulose and its processive movement during hydrolysis (Figure 29 and Figure 31). This was likely the major reason for the observed reduction in the effectiveness of cellulose hydrolysis in the presence of mannose and xylose (Figure 26).

3.3.2.3 To assess the possible influence of some major cellulose characteristics on the extent of cellulase inhibition induced by mannose and xylose

It appears that mannose and xylose inhibit cellulose hydrolysis through the disruption of Cel7A-cellulose interactions such as the binding of the Cel7A catalytic domain to the cellulose surface as well as limiting the processive movement of Cel7A during cellulose hydrolysis.

However, depending on the pretreatment strategy applied and the nature of the biomass the characteristics of the cellulosic component can vary significantly (Zhang and Lynd, 2004). Thus, we next assessed if major cellulose physicochemical characteristics (e.g. crystallinity, degree of polymerization, accessibility and acid group content) might play a role in influencing the inhibition mechanism of mannose and xylose towards cellulase enzymes.

3.3.2.3.1 Properties of model cellulosic substrates characteristics

A "library" of model cellulosic substrates such as cellulose nanocrystalline (CNC), Avicel, dissolving pulp and cellulose II, were selected and their enzymatic hydrolysis was assessed with/without mannose and xylose supplementation. These cellulosic substrates were selected because they have varied properties such as cellulose crystallinity (CrI), degree of polymerization, accessibility and acid group content (Table 11). For example, CNC has a much higher cellulose CrI (72%) and acid group content (151.2 mmol/kg) compared to the other substrates, while Cellulose II has greater cellulose accessibility (61.3 mg dye/g cellulose) and the lowest cellulose CrI (45%) and acid group content (24 mmol/kg). Although Avicel and dissolving pulp (DP) have similar cellulose CrI (~60%), they show significant differences in the degree of polymerization of their cellulose (182 vs. 1061) and accessibility (29.8 vs. 45.3 mg dye/g cellulose) (Table 11). Therefore, these substrates provided representative cellulose "library" that could be used to evaluate the influence of cellulose properties on the inhibition mechanism of mannose and xylose towards cellulase enzymes.

Table 11 Some major characteristics of model cellulose substrates (cellulose nanocrystalline (CNC), Avicel, dissolving pulp (DP) and cellulose II).

Substrate	Degree of polymerization	CrI	Acid group	Accessibility
CNC	104	72%	151.2	22.7
Avicel	182	66%	50.8	29.8
DP	1061	61%	48.4	45.3
Cellulose II	156	45%	24.0	61.3

Degree of polymerization, crystallinity index (CrI), acid group (mmol/kg cellulose), Accessibility (as determined by the amount of adsorbed dye, mg/g cellulose)

3.3.2.3.2 The inhibitory effects of mannose/xylose on the cellulose hydrolysis of varied model cellulosic substrates

To study how the substrate properties might influence the inhibitory effects of mannose and xylose on the cellulose hydrolysis, we firstly assessed the cellulose hydrolysis of these substrates with cellulase enzyme Cel7A in the presence of different amount of mannose/xylose. It appeared that the "model" cellulosic substrates had quite different hydrolysability towards the cellulase enzymes. For example, the same amount of Cel7A could result in more than 40% hydrolysis of Cellulose II but only achieved around 6% hydrolysis on CNC after 24 hours (Table 12). As expected, the addition of mannose and xylose resulted in different influences on the Cel7A hydrolytic performance when added to the various cellulosic substrates. For example, hydrolysis of Cellulose II was strongly inhibited by both sugars, with the addition 100 g/L of xylose significantly reducing hydrolysis by about 60% after 24 h hydrolysis (from 44.6% to 16.8%). The addition of same amount of mannose/xylose had much less of an effect on hydrolysis especially when added to CNC. It is worth noting that, although the inhibitory effects

of xylose were slightly stronger than that of mannose, the overall trend was consistent with previous observations regarding the influence of mannose and xylose on the Cel7A productive binding and processivity (Figure 29 and 31). This further supported the possibility that the inhibition of Cel7A productive binding and processivity by mannose and xylose was the major reason for the observed decrease in cellulose hydrolysis, regardless of the cellulosic substrate properties.

Table 12 Effect of hemicellulose-derived sugars on cellulose hydrolysis (%) by Cel7A after 24 h hydrolysis.

	Control	50 g/L mannose	100 g/L mannose	50 g/L xylose	100 g/L xylose
CNC	6.3%	6.1%	6.2%	5.8%	4.9%
Avicel	13.5%	11.1%	9.0%	9.4%	8.5%
DP	16.5%	13.4%	11.0%	9.9%	9.9%
Cellulose II	44.6%	29.2%	24.5%	20.6%	16.8%

The cellulose hydrolysis was performed at 50 ^{o}C using an enzyme loading of 30 mg Cel7A/g glucan for 24 h.

3.3.2.3.3 The relationship between the substrate characteristics and the inhibitory effects of mannose/xylose on cellulose hydrolysis

As it appeared that the inhibitory effects of mannose and xylose were highly substrate dependent (Table 12), we next wanted to identify the essential substrate characteristics, if any, that governed the extent of enzyme inhibition. A linear regression analysis was employed to correlate the major substrate characteristics (Table 13) with the degree of enzyme inhibition. It seemed that there was a good reverse fit (the R Square and P value were 0.99 and 0.0007 respectively) between the substrate acid group content and the degree of enzyme inhibition

caused by mannose and xylose (Table 13). It is possible that, the higher the acid group content of the cellulosic substrate, the less inhibitory are the mannose and xylose on cellulose hydrolysis.

To try to confirm if the acid group content influenced inhibition of cellulose hydrolysis, we tried to selectively remove the acid groups (without affecting other cellulose properties) from the CNC substrate using dilute alkaline treatment (lower than 0.5% w/w) (Hasani et al., 2008; Lokanathan et al., 2014). As expected, the extent of mannose and xylose inhibition was much higher on the alkaline treated CNC than that on the original CNC (Figure 32). Thus, it did appear that the acid group content was influential in determining the degree of inhibition of mannose and xylose on Cel7A hydrolysis of cellulose.

One of the possible reasons for the lower degree of inhibition of the cellulosic substrates which had a higher acid group content is that the acid group might strengthen the binding of cellulase catalytic domain (CD) to cellulose. This might make cellulose hydrolysis more resistant to the influence of sugars as it has been reported that the acid groups on cellulose might facilitate the formation of electrostatic interaction between the amino-acid resiudals on the CD of the cellulases and the glucan chains on the cellulose surface (Jiang et al., 2013).

Table 13 Linear regression analysis of major substrate characteristics and their possible influence on the inhibition of cellulose hydrolysis.

Substrate	Degree of polymerization	CrI	Acid group	Accessibility
	Correlation with degree of inhibition			
R Square	0.023	0.668	0.9953	0.896
P-value	0.321	0.126	0.0007	0.181

Degree of polymerization, crystallinity (CrI), acid group (mmol/kg cellulose), Accessibility (as determined by the amount of adsorbed dye, mg/g cellulose)

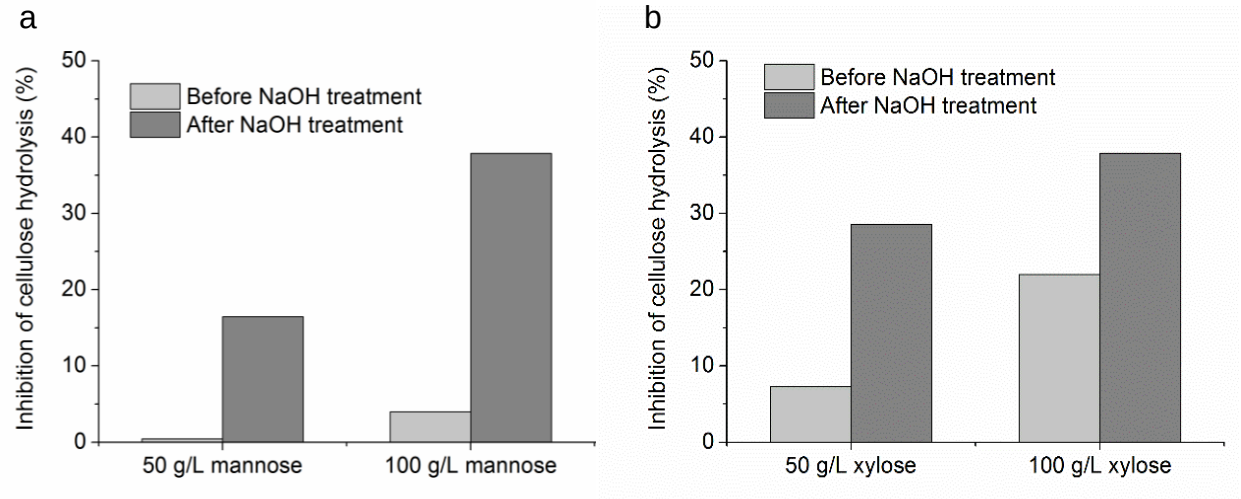

Figure 32 Inhibition of cellulose hydrolysis by mannose and xylose before and after NaOH treatment. Cellulose hydrolysis was performed at 50 ^{o}C using an enzyme loading of 30 mg Cel7A/g glucan for 24 h. The inhibition of cellulose hydrolysis (degree of inhibition) was calculated by dividing the reduced hydrolysis yield by the original hydrolysis yield.

3.3.3. Conclusions

Unlike glucose, mannose and xylose showed negligible influence on the major group of enzymes activities within the CTec3 enzyme preparation. However, they still strongly inhibited the cellulose hydrolysis on various cellulosic substrates. It appeared that the decreased cellulose hydrolysis was closely associated with the reduced productive binding and processivity of Cel7A enzymes. This phenomenon was likely caused by the strong interference of mannose and xylose on the binding affinity of Cel7A catalytic domain (CD) to cellulose surface. In addition, among the various major substrate properties that might influence the interaction between cellulase enzymes and cellulose, it seemed that removing the relatively high acid group content of cellulose could increase the inhibition of mannose/xylose on cellulose hydrolysis.

3.4 What are the major structural properties of phenolics that contribute to their inhibitory effects?

3.4.1 Background

As has been discussed earlier, besides monomeric sugars, water-soluble phenolics derived from pretreatment are another major group of inhibitors which, not only inhibit, but also deactivate commercial cellulase preparations (Section 3.2). It appeared that partial phenolics removal (by activated carbon) could alleviate this phenolics-mediated inhibition of hydrolysis (Figure 17), which indicated that only certain phenolics were strongly inhibitory. However, it was not clear which aspect of the phenolics resulted in this inhibition and which of the major characteristics of the various phenolics predominated in causing this inhibition of cellulose hydrolysis.

During the acid catalyzed steam pretreatment process, the lignin component of the biomass can be either depolymerized or repolymerized, generating soluble phenolics or more condensed lignin respectively. Although the structure of lignin and their effect on cellulose hydrolysis has been extensively studied (Berlin et al., 2006; Gao et al., 2014; Pan, 2008; Rahikainen et al., 2013; Sewalt et al., 1997), the major characteristics of the soluble phenolics that might inhibit cellulase enzyme hydrolytic remains unclear. One of the potential structural properties of phenolics that might relate to its inhibitory effect on cellulases is the molecular size of the compound. For example, some studies have found that polymeric phenolics are more inhibitory than monomeric phenolics (Tejirian and Xu, 2011; Ximenes et al., 2010). However, other work has shown that the dehydrogenative ferulic acid polymers are actually less inhibitory than monomeric ferulic acid (Nakagame et al., 2011). These contradictory results indicated that

the effects of phenolics on cellulase enzymes might be more complicated rather than just a correlation with the molecular weight of the phenolics. For example, some studies have reported that “Hibbert's ketones” type phenolics (e.g. phenylpropanoid ketones) can strongly inhibit microorganisms because of their strong binding affinity to cell membrane proteins (Canilha et al., 2012; Chandel et al., 2013). Thus, these ketone-type phenolics could also inhibit cellulose hydrolysis by strongly binding to cellulase enzymes. Hydroxyl groups on model phenolic substrates have also been shown to be critical components in contributing to the inhibitory effects of lignin on cellulase enzymes (Pan, 2008). However, whether these hydroxyl groups on the pretreatment-derived water-soluble phenolics are also inhibitory towards cellulase enzymes has yet to be determined.

To try to better understand the roles and function of pretreatment derived soluble phenolics on cellulase enzymes, we extracted the phenolics from the water-soluble fractions of steam pretreated lodgepole pine and poplar and assessed their influence on the cellulase hydrolytic performance. The extracted phenolics were further separated into several fractions according to their molecular size to assess the size effects of phenolic on the cellulose hydrolysis. In addition, the effects of essential phenolic functional groups such as carbonyl groups and phenol hydroxyl groups on the cellulase enzymes were also systematically evaluated, to try to better understand the mechanism behind phenolic induced enzyme inhibition.

3.4.2 Results and discussion

3.4.2.1 Effect of phenolics derived from pretreatment on cellulose hydrolysis

The pretreatment derived phenolics were initially fractionated from the water-soluble fraction by using activated carbon as the adsorbent, followed by consecutive washing of with acetone to desorb/recover the adsorbed phenolics (Dąbrowski et al., 2005). After washing, the

phenolics were successively recovered in the solvent system, and then concentrated by solvent evaporation. The concentrated phenolic solutions were freeze-dried and re-dissolved in water to prepare "phenolics stocks" for the following enzyme inhibition studies.

A phenolics concentration of 3 g/L was used in the following enzyme inhibition studies since this was the typical phenolic concentration found in the water-soluble fractions derived after pretreatment (Hodge et al., 2008; Kim et al., 2011; Kothari and Lee, 2011; Tengborg et al., 2001a). When the possible inhibitory effects of these isolated phenolics were assessed on dissolving pulp (DP) with various CTec3 enzyme loadings (10-30 mg/g), it appeared that both the steam pretreated lodgepole pine (SPLP) derived water-soluble phenolics (SPLP-WS-Phe) and the steam pretreated poplar (SPP) derived water-soluble phenolics (SPP-WS-Phe) greatly inhibited cellulose hydrolysis (Figure 33). In addition, the SPLP-WS-Phe fraction seemed to be more inhibitory than the SPP-WS-Phe fraction, especially at high enzyme loading (high cellulose conversion level) (Figure 33). The different inhibitory effect of the phenolics derived from the different biomass substrates was likely due to their component differences. Since softwood lignin contained a higher G and H content and ratio than hardwood lignin, the SPLP-WS-Phe might have more condensed structures than the SPP-WS-Phe fraction. Although the SPLP-WS was less inhibitory to cellulose hydrolysis than the SPP-WS (Section 3.1, Figure 17), this was more likely due to the higher phenolic concentration in the SPP-WS fraction than in SPLP-WS fraction (Figure 17) since the isolated SPLP-WS-Phe was actually more inhibitory than the SPP-WS-Phe fraction (Figure 34).

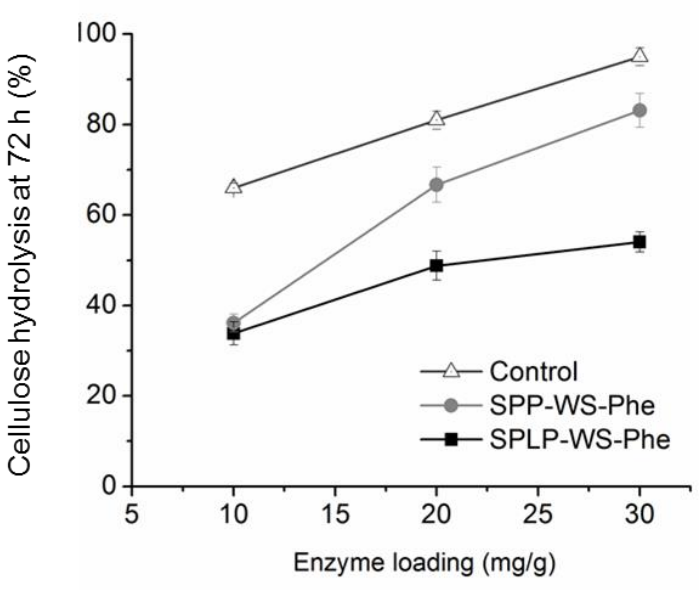

Figure 33 Influence of phenolics present in the water soluble fractions of steam pretreated lodgepole pine and poplar (SPLP/SPP-WS-Phe) on the hydrolysis of dissolving pulp. Enzymatic hydrolysis was performed at 50 ^{o}C using different enzyme loading of CTec3 mg/g glucan after 72 h. The concentration of phenolic compounds was 3 g/L.

It is worth noting that enzyme loading also appeared to play a significant role in determining the extent of enzyme inhibition by the isolated WS-Phe. For example, at the higher enzyme loading, the extent of phenolics inhibition became less severe, especially for the SPP-WS-Phe fraction (Figure 33), likely indicating that the higher ratio of protein to phenolics helped alleviate the inhibitory effects of these phenolics. It is also likely that higher enzyme loadings would ensure enough active enzymes to achieve effective cellulose hydrolysis. However, because softwood lignin tends to be more condensed than hardwood lignin, the SPLP-WS-Phe was still quite inhibitory even when high cellulase loadings were used.

Since low enzyme loadings are preferred in an industrial application, we further studied the effect of phenolics on the kinetic of cellulose hydrolysis when relatively low enzyme loadings (10 mg/g) are used. It appeared that SPLP-WS-Phe and SPP-WS-Phe fractions significantly decreased both the rate of cellulose hydrolysis (cellulose conversion after 12 h) and

the extent of hydrolysis (cellulose conversion after 72), with the extent of the hydrolysis more strongly inhibited (Figure 34). To try to determine whether this phenomenon was phenolic concentration dependent, the inhibitory effects of various concentrations of SPLP/SPP-WS-Phe (from 1 to 6 g/L) were next assessed (Figure 35). As expected, the higher concentration of phenolics leads to greater inhibition of hydrolysis with the extent of hydrolysis more inhibited than the rate of hydrolysis (Figure 35). When the concentration of phenolics, SPLP/SPP-WS-Phe was increased from 1 to 6 g/L, the hydrolysis rate showed almost no change while the extent of hydrolysis decreased by more than 30% (Figure 35). This suggested that phenolics would be more problematic in longer term cellulose hydrolysis. It also appeared that the decrease in cellulose hydrolysis was not linearly proportional to the increased phenolics concentration as, although the inhibitory effects of the phenolics increased with an increase in phenolic concentration from 1 to 3 g/L, it levelled off when the phenolic concentration was increased from 3 to 6 g/L (Figure 35). However, this might be due to the limited availability of enzyme binding sites for the phenolics. If any binding sites on the enzymes were not saturated at low phenolic concentrations, increasing the phenolics concentration would increase enzymes-phenolic interaction, resulting in a rapid decrease in cellulose hydrolysis. However, when most of the potential enzyme binding sites are saturated with phenolics, increasing the concentration of phenolics would not further decrease cellulose hydrolysis.

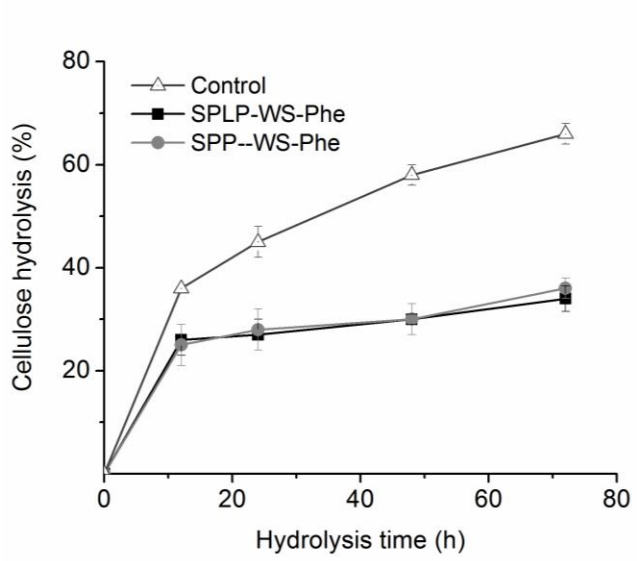

Figure 34 The influence of isolated phenolics from the water soluble fractions of steam pretreated lodgepole pine and poplar (SPLP/SPP-WS-Phe) on the hydrolysis of dissolving pulp. The enzymatic hydrolysis was performed at 50 ^{o}C using an enzyme loading of 10 mg CTec3/g glucan. The concentration of phenolic compounds was 3 g/L.

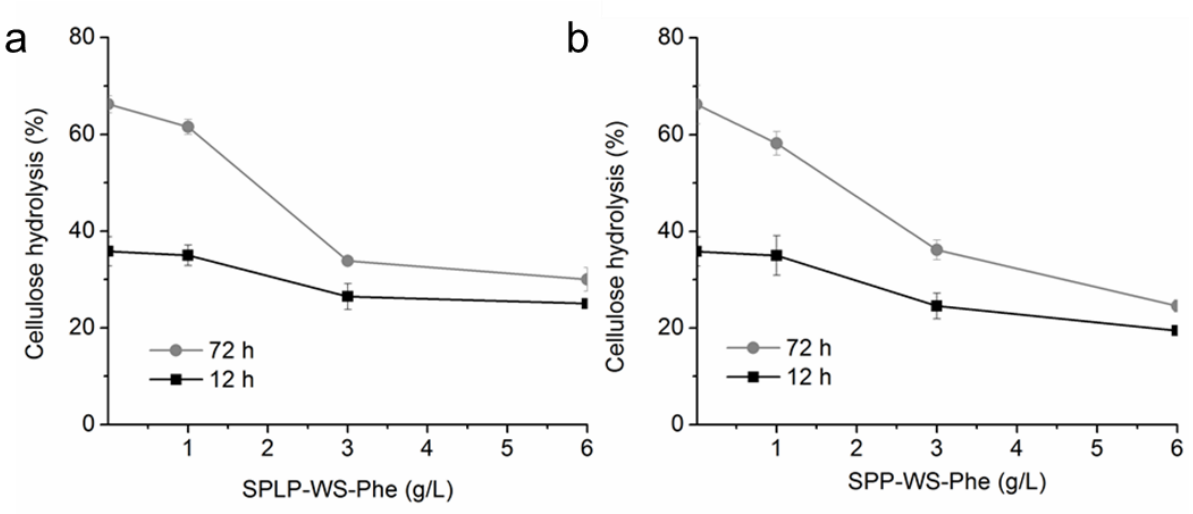

Figure 35 Influence of isolated phenolics from (a) steam pretreated lodgepole pine (SPLP-WS-Phe) and (b) steam pretreated poplar (SPP-WS-Phe) at increasing concentrations (g/L) on the hydrolysis of dissolving pulp. Enzymatic hydrolysis was performed at 50 ^{o}C using an enzyme loading of 10 mg CTec3/g glucan after 12 h and 72 h.

3.4.2.2 Possible influence of the size of the pretreatment derive phenolics on cellulose hydrolysis

In order to study the effect of molecular size of phenolics on the cellulose hydrolysis, the isolated phenolics were further separated into four fractions according to their molecular size, namely WS-PheA (>10 kDa), WS-PheB (3-10 kDa), WS-PheC (1-3 kDa) and WS-PheD (<1 kDa), and their inhibitory effects on cellulose hydrolysis were further assessed respectively.

Of all of the fractions, it appears that the smallest molecular size phenolics (WS-PheD, <1 kDa) had the strongest inhibitory effect for both SPLP-WS-Phe and SPP-WS-Phe fractions (Figure 36). In addition, the phenolics with smallest molecular size (less than 1 kDa) also decreased cellulose hydrolysis more rapidly than the bigger molecular size phenolics did. This was demonstrated by the significant decrease in the initial hydrolysis rate (within 12 h) in the presence of the WS-PheD fraction. It is possible that the small phenolics could enter the active site of cellulase enzymes easily, while the polymerized phenolics compounds have limited access to the catalytic site of cellulases owing to their higher molecular weight. For example, it has been suggested that highly polymerized procyanidins (oligomeric phenolic compounds) bind to alpha-amylase less effectively compared with the low-molecular-weight procyanidins because of the steric hindrance effect (De Freitas and Mateus, 2002).

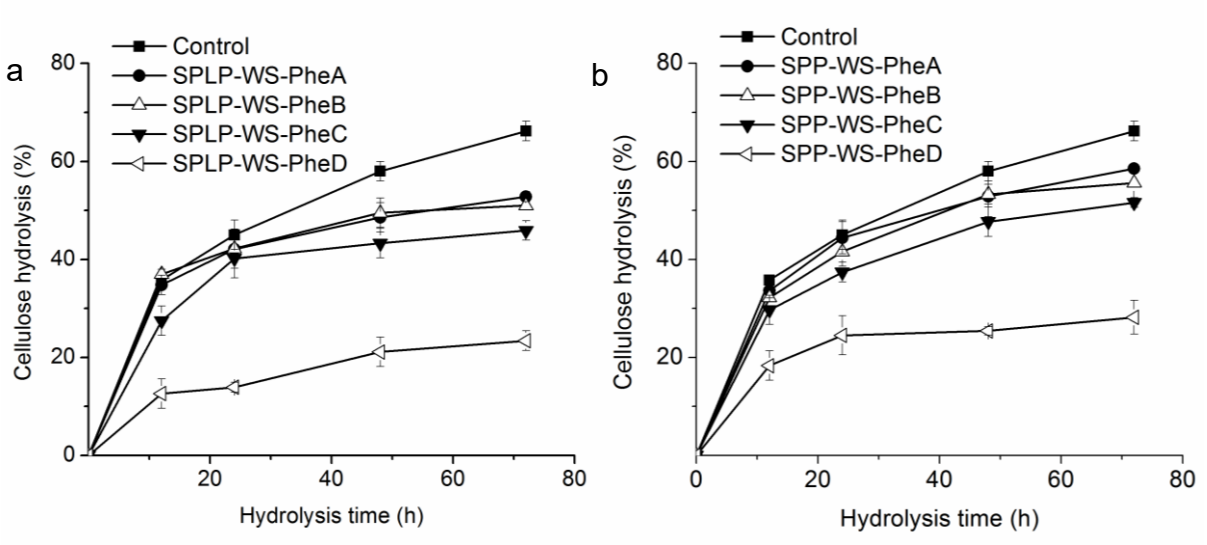

Figure 36 Possible influence of the molecular size of isolated phenolics from (a) steam pretreated lodgepole pine (SPLP) and (b) steam pretreated poplar (SPP) on the hydrolysis of dissolving pulp. Enzymatic hydrolysis was performed at 50 ^{o}C using an enzyme loading of 10 mg CTec3/g glucan. The isolated phenolics were further separated into four fractions, namely WS-PheA (>10 kDa), WS-PheB (3-10 kDa), WS-PheC (1-3 kDa) and WS-PheD (<1 kDa), according to their molecular size. The concentration of the original phenolic compounds was 3 g/L.

3.4.2.3 Possible influence of the phenolic carbonyl groups on cellulose hydrolysis

As mentioned earlier (section 1.8), the β-O-4 linkage is one of the major linkages in the lignin network (Chakar and Ragauskas, 2004). During acid hydrolysis, the cleavage of the β-O-4 linkage gives rise to a mixture of phenylpropanoid ketones (Hibbert's ketones) (Canilha et al., 2012; Chandel et al., 2013). These Hibbert's ketone type phenolics have been shown to be inhibitory to the fermentation process as they tend to interact with nucleophilic compounds such as amines, proteins and DNA (Cilliers and Singleton, 1990). Thus, it was anticipated that the carbonyl group of the pretreatment-derived phenolics might contribute to phenolics-mediated enzyme inhibition.

Carbonyl groups can be modified by either oxidation or reduction. Although oxidative treatment using chemicals such as sodium hypochlorite may also oxidize other function groups

(Sjostrom, 2013), reducing agents such $NaBH_4$ might be milder and more selectively reduce the carbonyl group (Lin and Dence, 2012). Thus, $NaBH_4$ was used to selectively reduce the carbonyl group of phenolics to study their effects on the cellulase enzymes. FTIR analysis was first employed to study the structure change of phenolics before and after $NaBH_4$ treatment (Figure 37). For the FTIR spectrometry, the peak at 3318 cm^{-1} was assigned to hydroxyl groups (phenolics), the peaks at 2973 cm^{-1} to aliphatic CH stretching vibration, and the peak at 1713 cm^{-1} assigned to the carbonyl stretching in unconjugated ketones (Table 14) (Yang et al., 2007). The strong bands at ~1669 cm^{-1}, together with the band at ~1560 cm^{-1} could be attributed to C=O (carbonyl or carboxylic) conjugated with aromatic ring. In addition, the peak at 1632, 1441 and 890 cm^{-1} arising from aromatic C=C stretching and CH bending indicated the presence of phenolics compounds (Table 14). After $NaBH_4$ treatment, the major peaks at 1713, 1669 and 1560 cm^{-1} disappeared while the peak at 1060 cm^{-1} (C–O stretching in alcohols) became more broad and intense. This suggested that the carbonyl groups in the phenolics were indeed reduced to alcohol groups (Figure 37).

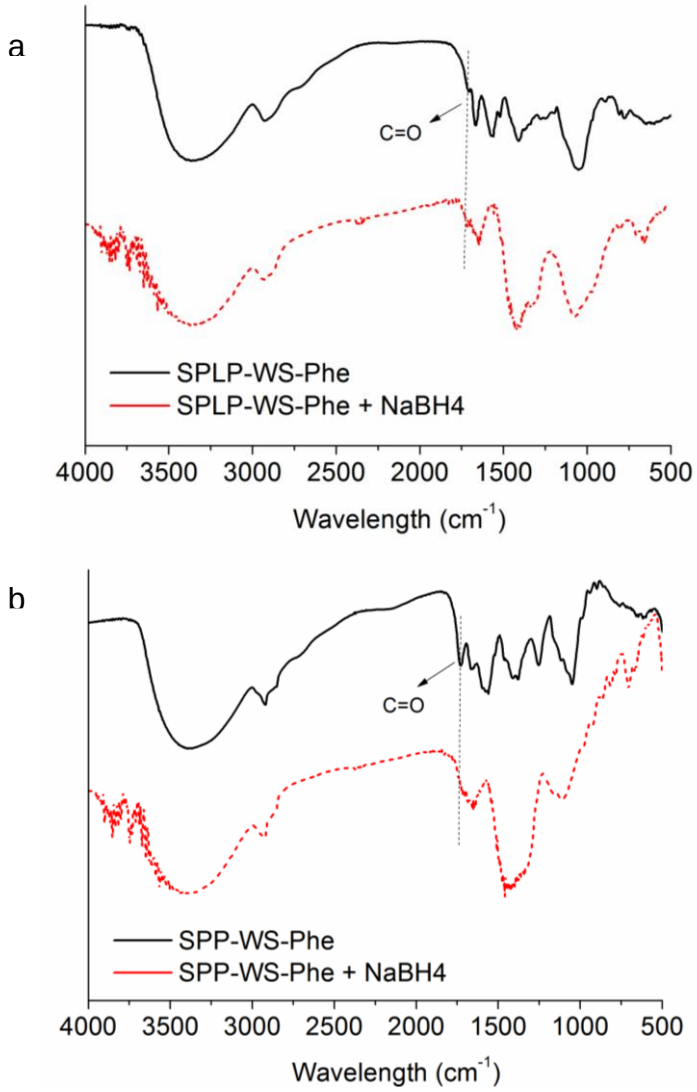

Figure 37 The FTIR spectrums of the phenolics from (a) steam pretreated lodgepole pine (SPLP-WS-Phe) and (b) poplar (SPP-WS-Phe) before and after $NaBH_4$ treatment.

Table 14 Peak assignments for FTIR spectrum of isolated phenolics and reduced phenolics (Yang et al., 2007).

Wavelength (cm^{-1})	Functional groups
3361	O–H stretching in alcohols, phenols or carboxylic acids
2930	C–H vibration of aliphatic carbon
1708	C=O stretch in unconjugated ketones, carbonyl and in ester group
1664, 1562	C=O stretch in conjugation to aromatic ring
1632	Aromatic C=C stretching
1411	Aromatic CH bending
1060	C–O stretching and C–O deformation in alcohol group
890	Aromatic CH stretching

The possible influence of the phenolic derived carbonyl groups were then assessed by comparing the untreated and $NaBH_4$-treated phenolics on cellulose hydrolysis. Surprisingly the $NaBH_4$-treated phenolics (carbonyl group reduced) had almost no inhibitory effect on cellulose hydrolysis, even though both the untreated SPLP-WS-Phe and SPP-WS-Phe fractions strongly inhibited cellulose hydrolysis (Figure 38). Thus, it seemed that the carbonyl group of phenolics might be the major reason for its inhibitory effects on the cellulase enzymes. Previous work has shown that carbonyl phenolic compounds are electrophilic and tend to interact strongly with proteins, nucleic acids, or related biological molecules. This has been shown to result in the inhibition of protein functions, DNA duplication, or even loss of cell activity (Cilliers and Singleton, 1990).

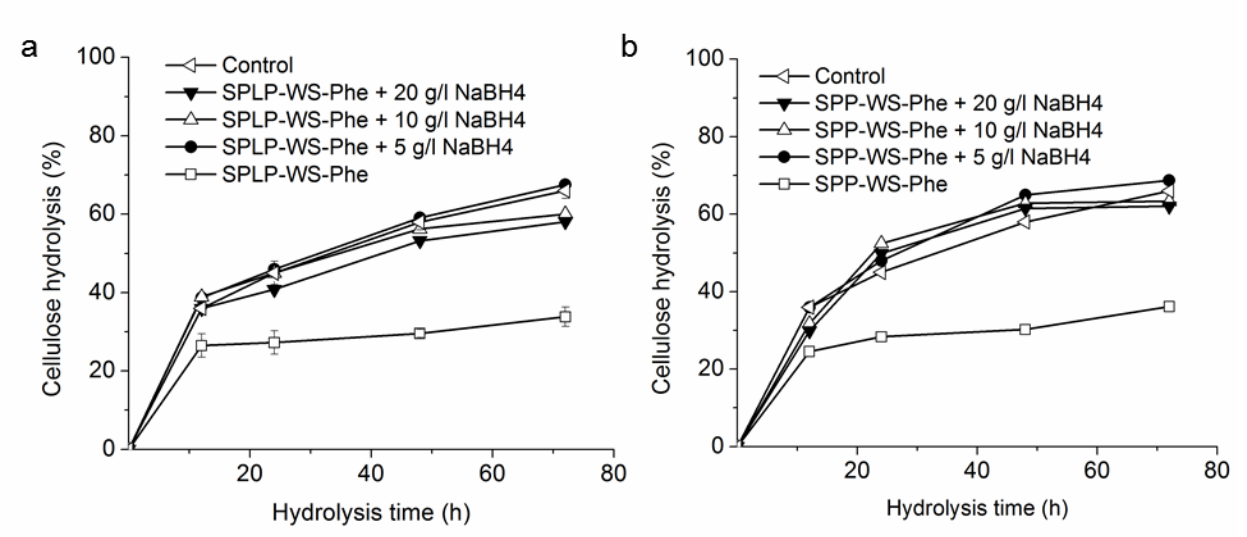

Figure 38 Effect of reductive modification of the (a) steam pretreated lodgepole pine derived phenolics (SPLP-WS-Phe) and, (b) the steam pretreated poplar derived phenolics (SPP-WS-Phe), using varied amount of reducing agent, on the hydrolysis of dissolving pulp. Enzymatic hydrolysis was performed at 50 ^{o}C using an enzyme loading of 10 mg CTec3/g glucan.

To try to confirm the inhibitory effect of the carbonyl groups on cellulose hydrolysis, representative the model phenolic compounds vanillin and syringaldehyde were treated with $NaBH_4$ to produce their corresponding alcohol compounds (confirmed by Gas chromatography/Mass spectrometry) (Figure 39). These phenolic alcohols showed almost no inhibitory effect on cellulose hydrolysis (Figure 40). The carbonyl groups, acting as strong dipoles, may be able to interact strongly with certain essential amino acids within the cellulases or increase the hydrophobicity of the phenolics. This could strengthen the interaction of the phenolics and cellulase enzymes. In addition, the carbonyl compounds could also potentially form covalent bonds with nucleophilic functional groups in protein, which might also be the reason for the decrease in enzyme activity (Cilliers and Singleton, 1990). However, it could be concluded that the carbonyl group of phenolics strongly inhibit the hydrolytic performance of cellulase enzymes.

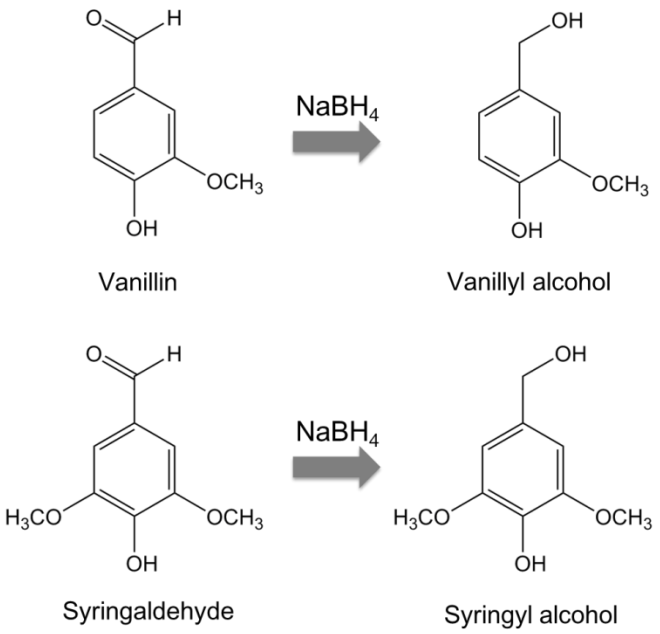

Figure 39 Mode of reduction of the carbonyl groups present in vanillin and syringaldehyde to alcohol groups by $NaBH_4$.

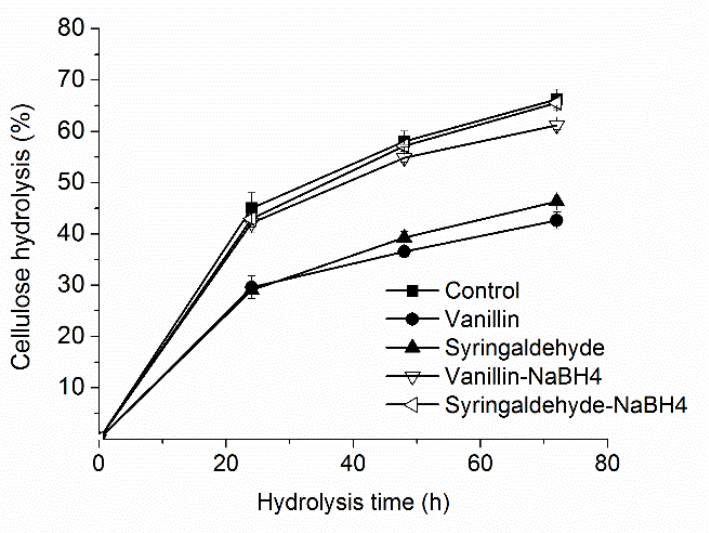

Figure 40 Influence of the reductive modification of model phenolic compounds (vanillin and syringaldehyde) products (17 mM) on the hydrolysis of dissolving pulp. Enzymatic hydrolysis was performed at 50 ^{o}C using an enzyme loading of 10 mg CTec3/g glucan for 72 h. The concentration of model phenolic compounds in the reaction mixture was 17 mM (~ 3 g/L). For the reductive modification of model phenolic compounds, 5 g/L of $NaBH_4$ was used to ensure maximum reduction of the carbonyl groups present in the phenolics.

As we had found that the CBH and BG were more inhibited by phenolics than other enzymes **(**Figure 21), the inhibition kinetics of CBH and BG by phenol aldehyde were next assessed. It appeared that both vanillin and syringaldehyde reversibly inhibited CBH and BG through a mixed-type enzyme inhibition mechanism (Figure 41). This suggested that the carbonyl group increased the binding of phenolics compounds to enzyme sites, influencing its catalytic action.

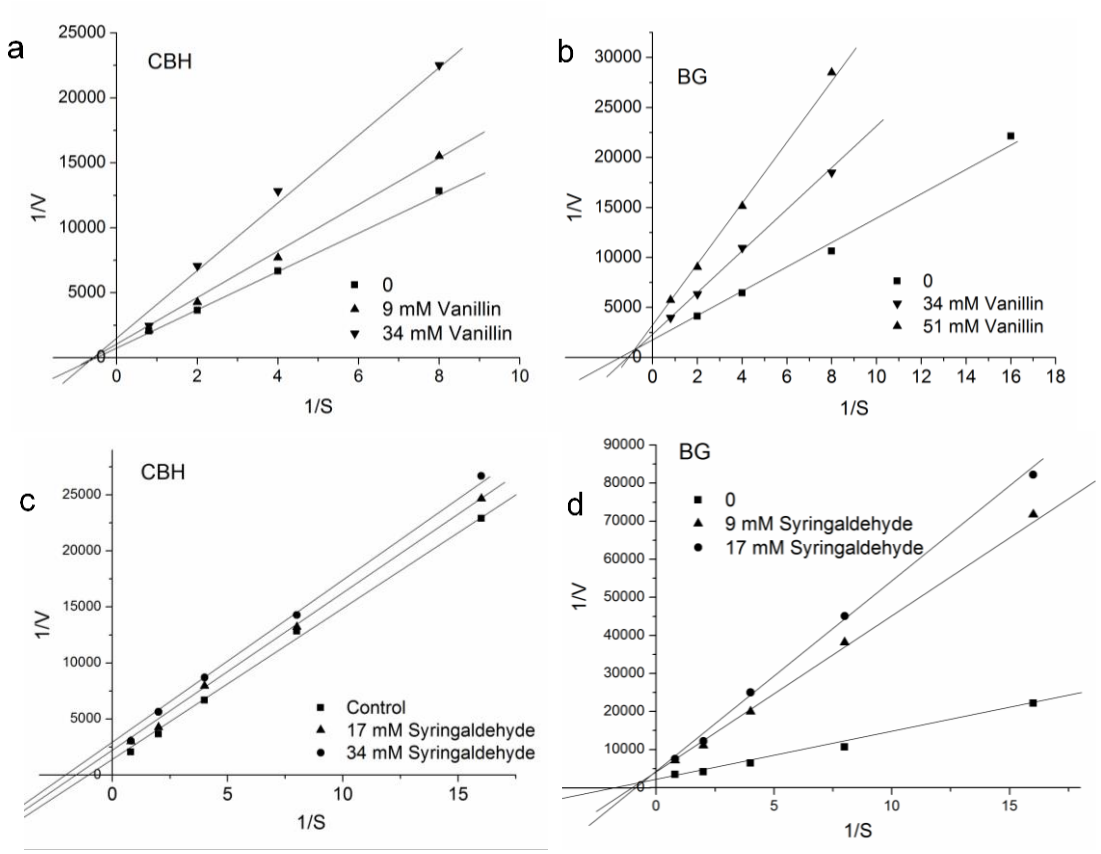

Figure 41 Kinetic analysis of possible inhibition by vanillin and syringaldehyde on two of the major enzyme activities, (a, c) CBH and (b, d) BG, present in CTec3.

Previous work has shown that lignin with more acid groups has a lower binding affinity to cellulase enzyme, thus alleviating enzyme inhibition (Nakagame et al., 2011). It is likely that the conversion of the phenolic carbonyl group to acid groups would alleviate their inhibitory

effect on cellulose hydrolysis. Thus, to better understand the mechanism of phenolic induced enzyme inhibition, the inhibitory effects of phenolics with carbonyl, alcohol and carboxylic acid groups were further studied. Phenolics containing aldehyde group were shown to be the most inhibitory, decreasing cellulose hydrolysis by about 30% after 72 hours (Figure 42). Conversion of the aldehyde groups to alcohol groups greatly improved cellulose hydrolysis, while substitution of the aldehyde groups with carboxylic acid groups alleviated the inhibition to a lesser extent (Figure 42). Thus, it could be promising to use enzymes/chemicals (reducing agent) treatment to reduce these phenolic components if we want to mitigate phenolics inhibition of cellulases (Bollag et al., 1988; Cavka and Jönsson, 2013; Jönsson et al., 2013; Soudham et al., 2011).

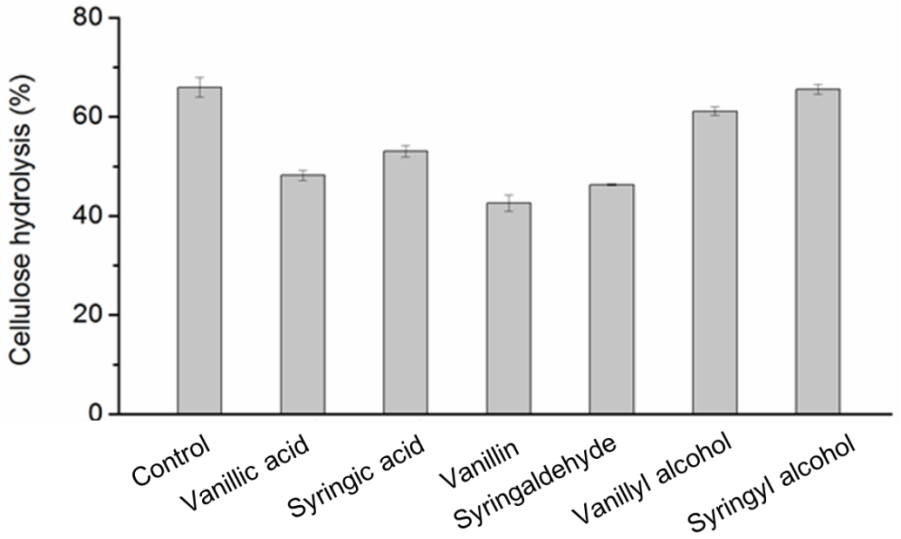

Figure 42 Possible inhibitory effects of the acid, carbonyl and alcohol groups present in model phenolic compounds on the hydrolysis of dissolving pulp. Enzymatic hydrolysis was performed at 50 ^{o}C using 10 mg CTec3/g glucan after 72 h. The concentration of the model phenolic compounds was 17 mM.

3.4.2.4 Effect of hydroxyl groups of phenolics derived from pretreatment on cellulose hydrolysis

Previous work has shown that the phenol hydroxyl groups contribute to the inhibitory effects of lignin on cellulose hydrolysis, possibly through the formation of hydrogen bonds between amino acid within cellulase (Pan, 2008). In a similar fashion, we anticipated that the phenol hydroxyl groups within these water-soluble phenolics might also play a big role in inhibition.

In order to study this possible influence, hydroxypropylation was employed to selectively block the free phenol hydroxyl groups present in the SPLP/SPP-WS-Phes fraction, according to the method described previously (García et al., 2013). Surprisingly, almost no effect was observed after hydroxypropylation treatment. This suggested that the phenol hydroxyl group was not the major function group within the SPLP/SPP-WS-Phe fraction that lead to the strong inhibition of cellulose hydrolysis (Figure 43 a, b). This result was contradictory to the previously observed effect of the lignin hydroxyl groups on cellulases (Pan, 2008). This difference in possible phenolic hydroxyl group inhibition may be related to the 3D network structure of lignin, as the different matrixes might trap the enzyme through different multi-point hydrogen bonding and changes on the enzyme surface (Pan, 2008). The soluble phenolics within the WSs fraction may not have such complex network structure and this might be the reason why their phenolic hydroxyl group seemed to not affect cellulase activity.

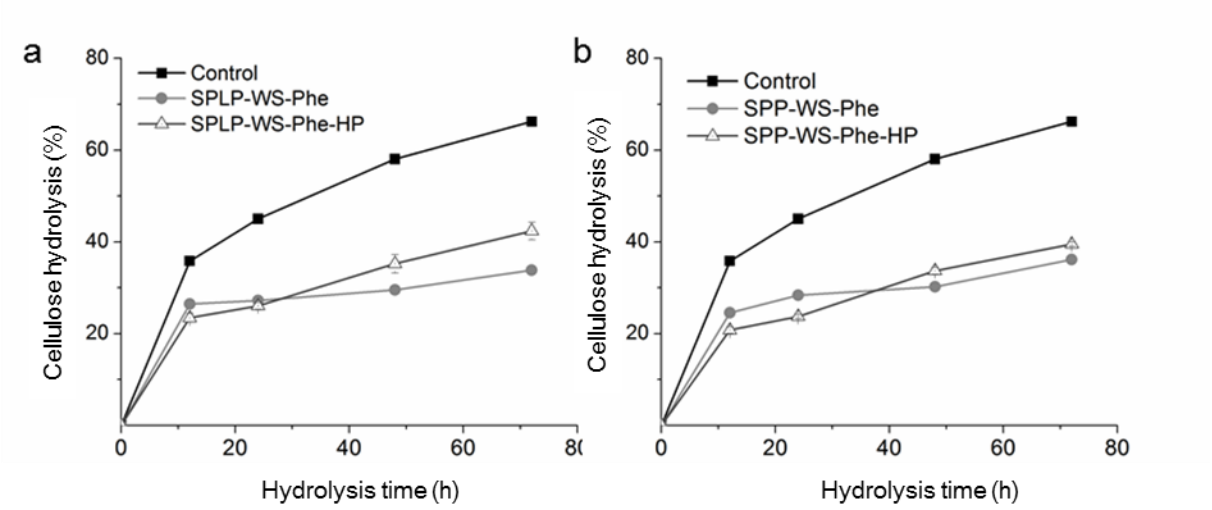

Figure 43 Effect of hydroxypropylation of (a) steam pretreated lodgepole pine derived phenolics (SPLP-WS-Phe-HP) and b) steam pretreated poplar derived phenolics (SPP-WS-Phe-HP) on the hydrolysis of dissolving pulp. The enzymatic hydrolysis was performed at 50 ^{o}C using 10 mg CTec3/g glucan. The concentration of phenolics is 3 g/L.

Model phenolics compounds containing varied amount of phenolic hydroxyl group were next employed to confirm the contribution of phenolic hydroxyl groups to the inhibitory effect of phenolics. It was found that increasing phenolic hydroxyl group content in monomeric phenolics could even slightly improve the overall cellulose hydrolysis (Figure 44). Specifically, hydroxybenzoic acid (HBA) with one hydroxyl group decreased the cellulose hydrolysis by ~20%, while dihydroxybenzoic acid (2HBA) and gallic acid (GA) which has more hydroxyl groups showed less inhibitory effect on cellulose hydrolysis (Figure 45). This further confirmed that the amount of phenol hydroxyl groups was not the major factor that determined the inhibitory effect of the phenolics present in the water soluble fraction derived from pretreatment.

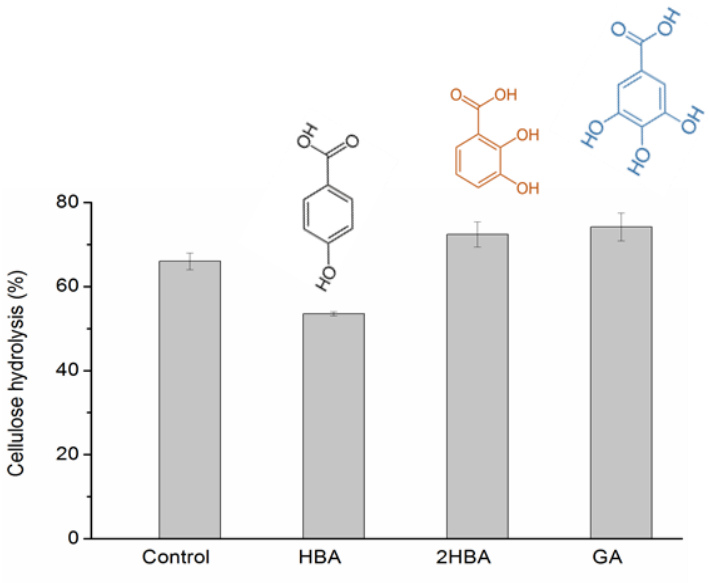

Figure 44 Influence of model phenolic compounds with varied amount of hydroxyl groups on the hydrolysis of dissolving pulp. Enzymatic hydrolysis was performed at 50 ^{o}C using 10 mg CTec3/g glucan for 72 h. The concentration of each model phenolic compound (HBA: hydroxybenzoic acid; 2HBA: dihydroxybenzoic acid; GA: gallic acid) was 17 mM (around 3 g/L).

3.4.3 Conclusions

Steam pretreated lodgepole pine derived phenolics exhibited stronger inhibition on cellulose hydrolysis than did the steam pretreated poplar derived phenolics. The inhibitory effect of phenolics was mostly influenced by the molecular size and the carbonyl content of the phenolics. Specifically, phenolics with a smaller molecular size (less than 1 kDa) containing carbonyl groups showed the strongest inhibitory effect on cellulose hydrolysis.

3.5 Can we minimize the inhibitory effect of water soluble compounds by incorporating carbocation scavengers into steam pretreatment?

3.5.1 Background

In the earlier work in the thesis, we showed that pretreatment-derived water-soluble compounds (WS) especially sugars and phenolics strongly inhibited the hydrolytic potential of both the traditional and the newly developed cellulase enzyme preparations. Although sugar inhibition could be alleviated by various sugar removal processes such as membrane filtration and simultaneous saccharification and fermentation (SSF) (Kovács et al., 2009; Philippidis et al., 1993; Wingren et al., 2003), mitigating the phenolic inhibition was more challenging (Cavka and Jönsson, 2013; Tejirian and Xu, 2011). Some studies have reported that post-detoxification strategies such as reducing agent treatment or laccase polymerization/oxidation treatments could mitigate the inhibitory effect of phenolics (Lee et al., 2012; Soudham et al., 2011). However, applying these detoxification strategies after pretreatment will add extra handling steps that might be relatively expensive for already high-cost bioconversion processes. Since phenolics with certain phenolics structural properties were shown to have relatively low inhibitory effects on cellulase enzymes (Chapter 3.4), modifying the structure of phenolics during pretreatment might provide a better strategy to mitigate phenolic mediated enzyme inhibition.

Water-soluble phenolics were generated from lignin depolymerisation during the acid-catalysed steam pretreatment process. During acid-catalyzed pretreatment, lignin underwent depolymerisation and repolymerisation reactions (also known as condensation reactions). Initially, lignin is depolymerized through the cleavage of acid labile linkage such as β-O-4 ether,

producing carbocations. Carbocations have a high affinity to the nucleophiles within the lignin structure. The repolymerisation between the carbocations and nucleophiles leads to increased molecular weight and stronger inhibitory effect of lignin on cellulose hydrolysis (Li and Gellerstedt, 2008; Li et al., 2007). In contrast, depolymerisation of carbocations will give rise to small size phenolics such as “Hibbert's ketones” (phenylpropanones) that may be inhibitory to cellulose hydrolysis (Canilha et al., 2012; Chandel et al., 2013). Both the depolymerisation and repolymerisation reactions are thought to be mediated through the carbocations that are formed from the benzyl alcohol structures in the lignin. In order to produce less inhibitory phenolics, it would be beneficial to minimize the depolymerisation of carbocations to ketone-type compounds. Some studies have tried to add a carbocation scavenger (carbonium ion scavenger) such as 2-naphthol during pretreatment to minimize lignin repolymerisation (Li et al., 2007; Pielhop et al., 2015), and also to improve the access of enzyme to cellulose. Although these carbocation scavengers were used to minimize lignin condensation, we hoped that the carbocation scavengers might help suppress the depolymerisation of carbocations to various ketone-type compounds because of their high reactivity towards carbocations. As a result, they might alleviate some of the inhibitory effects of the pretreatment derived phenolics.

Although 2-naphthol has been commonly used in previous studies to suppress lignin condensation (Li and Gellerstedt, 2008; Li et al., 2007), the high cost of 2-naphthol (2N) may limit its potential application. Alternatively, phenolic acids such as 4-hydroxybenzoic acid (HBA), vanillic acid (VA), syringic acid (SA) are also electron-rich and reactive toward electrophiles. They can be obtained through the degradation of lignin during various pretreatment processes. Thus, it was possible that these phenolic acids could serve as sustainable sources of carbocation scavengers.

We next assessed the potential of applying carbocation scavengers during pretreatment to minimize the inhibitory effect of pretreatment derived phenolics on the cellulase enzymes. We also evaluated the inhibition mitigating effect of various carbocation scavengers such as 2-naphthol and phenolic acids (4-hydroxybenzoic acid (HBA), vanillic acid (VA), syringic acid (SA). In this way we hoped to develop an alternative direction/approach to minimize the inhibitory effect of the compounds present in the water soluble fraction while facilitating efficient whole slurry hydrolysis at relatively low enzyme loading.

3.5.2 Results and discussion

3.5.2.1 The composition of water-soluble fractions

Since the phenolics derived from softwood lodgepole pine were more inhibitory than those derived from hardwood poplar (section 3.4), lodgepole pine was selected to test the potential inhibition mitigating effect of various carbocation scavengers. Small-scale diluted acid pretreatment was initially employed to mimic the large-scale acid catalyzed steam pretreatment. Dilute acid pretreatment was performed at 180 ^{o}C for 40 min, using 1% H_2SO_4. This had a similar pretreatment severity to the compromised steam pretreatment condition used in previous work (Zhu and Pan, 2010). Four potential carbocation scavengers namely 2-naphthol (2N), 4-hydroxybenzoic acid (HBA), vanillic acid (VA) and syringic acid (SA) (4% w/w biomass) were added to the lodgepole pine biomass prior to pretreatment. After pretreatment, the slurry was separated into solid (water insoluble fraction, WIS) and liquid (water-soluble, WS) fractions through the filtration.

The glucan content of the WIS obtained after dilute acid pretreatment of lodgepole pine (DAPLP) was 29.7%, similar to the glucan content of the DAPLP derived from pretreatment

after prior addition of 2-naphthol (2N), 4-hydroxybenzoic acid (HBA), vanillic acid (VA) and syringic acid (SA). However, the presence of carbocation scavengers in the pretreatment slightly decreased the glucose content and increased the phenolics concentrations within the WS fractions of the pretreated substrates (Table 15). It is possible that the scavengers reacted with the carbocations to prevent the lignin condensation, which leads to an increased soluble phenolics content.

Table 15 The composition of water soluble (WS) fractions derived dilute acid pretreated lodgepole pine (DAPLP) with various scavengers added during pretreatment process.

WS	Glucose (g/L)	Glucose oligomer (g/L)	Acetic acid (g/L)	Phenolics (g/L)
DAPLP-WS	4.8	0.1	4.8	1.2
DAPLP-2N-WS	3.2	0.3	3.1	1.4
DAPLP-HBA-WS	3.4	0.4	3.4	2.2
DAPLP-VA-WS	3.1	0.3	3.1	2.7
DAPLP-SA-WS	3.5	0.4	3.5	2.1

DAPLP-WS: water soluble fraction derived from dilute acid pretreated lodgepole pine;
DAPLP-2N-WS: water soluble fraction derived from dilute acid pretreated lodgepole pine with 2-naphthol added during pretreatment;
DAPLP-HBA-WS: water soluble fraction derived from dilute acid pretreated lodgepole pine with 4-hydroxybenzoic acid added during pretreatment;
DAPLP-VA-WS: water soluble fraction derived from dilute acid pretreated lodgepole pine with vanillic acid added during pretreatment;
DAPLP-SA-WS: water soluble fraction derived from dilute acid pretreated lodgepole pine with syringic acid added during pretreatment.

3.5.2.2 Effect of water-soluble fractions from pretreatment with scavengers on cellulose hydrolysis of pretreated substrates

As expected, the addition of scavengers during pretreatment significantly alleviated the inhibitory effects of the WS fractions on cellulose hydrolysis (Figure 45). It should be noted that the WS of the control experiment, where no carbocation scavengers were added during pretreatment, severely inhibited cellulose hydrolysis (two third reduction as compared with washed substrate), while the inhibitory effects of the WS fractions were significantly decreased when the scavengers were applied (Figure 45). The glucose present in the WS fraction had almost no effect on cellulose hydrolysis due to its low concentrations (data not shown). Thus, the improved cellulose hydrolysis was mainly due to the mitigating effect of the scavengers on the pretreatment derived phenolics. Among all of the carbocation scavengers assessed, phenolic acids such as HBA, VA and SA were more efficient in mitigating the inhibitory effect of the WS fractions.

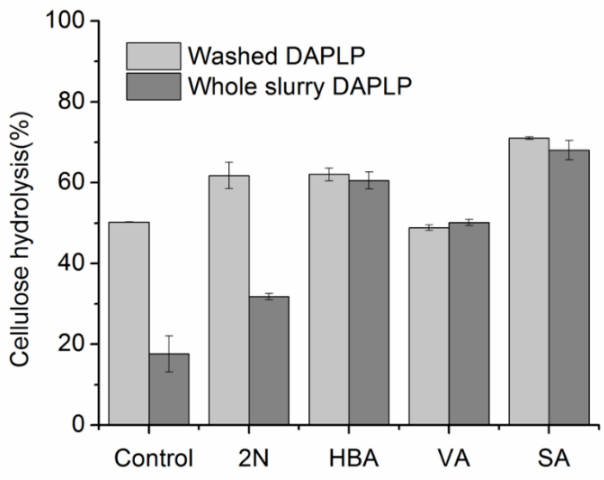

Figure 45 Enzymatic hydrolysis of washed and whole-slurry pretreated lodgepole pine derived after pretreatment which incorporated various carbocation scavengers. Hydrolysis was performed using CTec3 at 40 mg/g glucan for 48 h. DAPLP: dilute acid pretreated lodgepole pine; 2N: 2-naphthol; HBA: 4-hydroxybenzoic acid; VA: vanillic acid; SA: syringic acid.

3.5.2.3 Effect of water-soluble fractions from pretreatment with scavengers on cellulose hydrolysis of model cellulosic substrate

A relatively "pure" cellulosic substrate, dissolving pulp (DP), was next employed to assess the effect of carbocation scavenger on the toxicity of the WSs fractions towards cellulose hydrolysis. As expected, the scavengers could greatly alleviate the inhibitory effect of WS fractions on the DP hydrolysis. The mitigating effect was highly dependent on the enzyme loading applied (Figure 46*)*. It was apparent that, after 24 hours, pretreatment-derived inhibitors without scavengers decreased cellulose hydrolysis by about 50%, while the addition of scavengers helped mitigate inhibition, to only a loss of about 10-20% (Figure 46). Overall, syringic acid (SA) seemed to be the best candidate of all the scavengers tested. (Figure 46). The high efficacy of SA was likely due to the higher electron density of its aromatic ring as the presence of the extra methoxy groups could act as the electron donor on the benzyl ring (Shimada et al., 1997).

These promising results seemed to indicate that lignin derived compounds could serve as efficient carbocation scavengers to mitigate the inhibitory effect of the phenolics that are present in pretreatment derived WS fraction. The possible mechanism for the reduced inhibitory effect due to the addition of scavengers could be due to the production of phenolics with relatively high molecular weight and less carbonyl groups (Li and Gellerstedt, 2008; Li et al., 2007). More specifically, during typical acidic conditions pretreatment, the β-O-4 linkages of lignin are cleaved and new carbonium intermediates are created. These could be either depolymerised into ketone type phenolics or condensed via the electrophiles within the lignin (Li and Gellerstedt, 2008; Li et al., 2007). However, the addition of carbocation scavengers during pretreatment could stabilize these carbonium intermediates (Li et al., 2007; Pielhop et al., 2015), which was likely able to minimize the depolymerisation of carbocation intermediates to the inhibitory small

size ketone-type phenolics (Figure 47). Besides, the acid group in the scavengers might keep enzyme from binding to the pretreatment derived phenolics. Therefore, the addition of carbocation scavengers can alleviate inhibition, thus facilitating the whole slurry hydrolysis of the pretreated substrates.

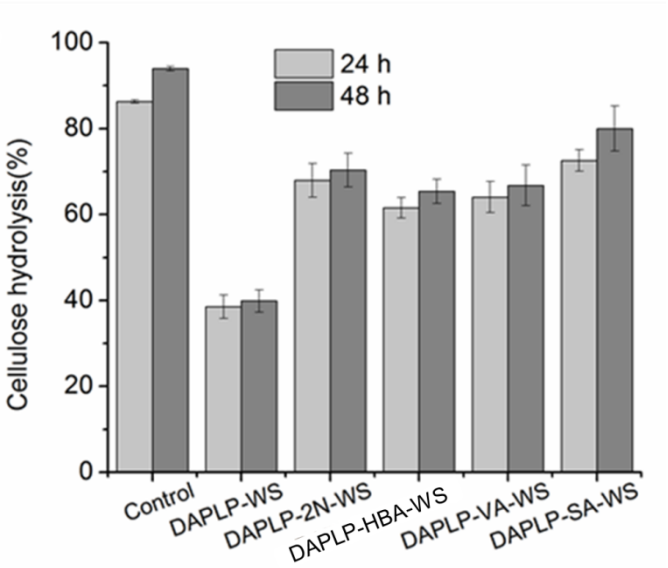

Figure 46 Enzymatic hydrolysis of dissolving pulp (DP) using CTec3 at 40 mg/g glucan in the presence of various water soluble fractions derived from pretreatment process with various scavengers added.

DAPLP-WS: water soluble fraction derived from dilute acid pretreatment of lodgepole pine;
DAPLP-2N-WS: water soluble fraction derived from dilute acid pretreatment of lodgepole pine with 2-naphthol added;
DAPLP-HBA-WS: water soluble fraction derived from dilute acid pretreatment of lodgepole pine with 4-hydroxybenzoic acid added;
DAPLP-VA-WS: water soluble fraction derived from dilute acid pretreatment of lodgepole pine with vanillic acid added;
DAPLP-SA-WS: water soluble fraction derived from dilute acid pretreatment of lodgepole pine with syringic acid added.

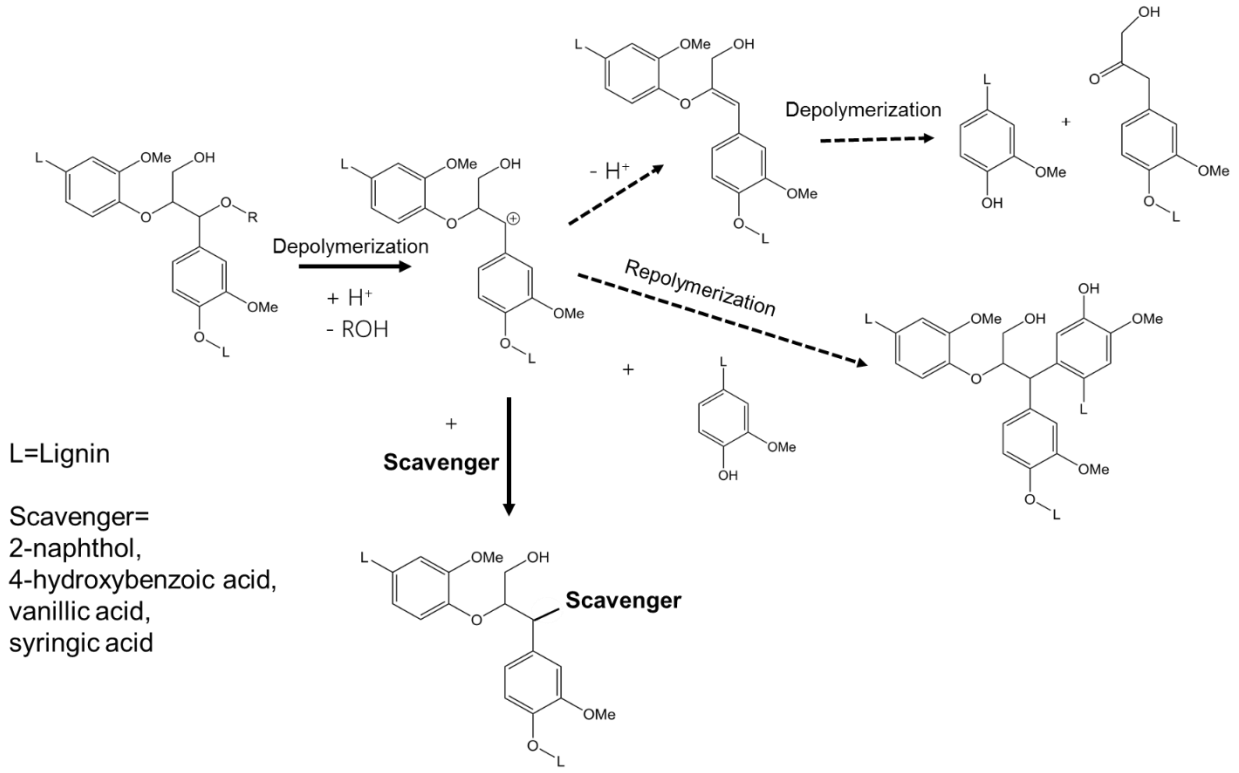

Figure 47 Proposed lignin reaction mechanism during pretreatment with the addition of scavengers. Adapted from from Pielhop et al. (2015).

3.5.2.4 Applying syringic acid (SA) prior to steam pretreatment to achieve relatively high cellulose hydrolysis of steam pretreated whole slurry

As carbocation scavengers improved the cellulose hydrolysis of diluted acid pretreated whole slurry and syringic acid (SA) appeared to be the best scavenger (Figure 46), it was anticipated that the SA would also be able to improve hydrolysis of whole-slurry steam pretreated substrates. Similar to the previously assessed diluted acid pretreated lodgepole pine (Table 15), the chemical composition of steam pretreated lodgepole pine (SPLP) with and without addition of carbocation scavenger were identical. The WS fraction from SPLP-SA (SPLP-SA-WS) contained similar sugars (monomeric/oligomeric sugars) and relatively high phenolics (data not shown). As expected, hydrolysis of the whole slurry SPLP was statistically significant lower than that of washed SPLP (one way ANOVA, $p = 0.009$). However, hydrolysis

efficiency was improved significantly by about 10% when SA was added prior to pretreatment (Figure 48). The effect of SA on whole slurry hydrolysis was shown to be statistically significant (one way ANOVA, p = 0.002). This confirmed that SA could serve as a good scavenger and its addition could improve the efficiency of whole slurry hydrolysis. Similar with dilute acid pretreatment, part of the improvement in the cellulose hydrolysis of whole slurry was a result of the lower inhibitory effect of the WS fraction on the cellulase activities (data not shown). However, compared with dilute acid pretreatment, the mitigating effect of SA was less apparent. This likely indicated that pretreatment conditions (such as time, temperature and the amount of scavengers) still need to be further optimized to result in relatively high cellulose hydrolysis, good sugar recovery and satisfactory mitigating effect by the scavengers for industrial applications.

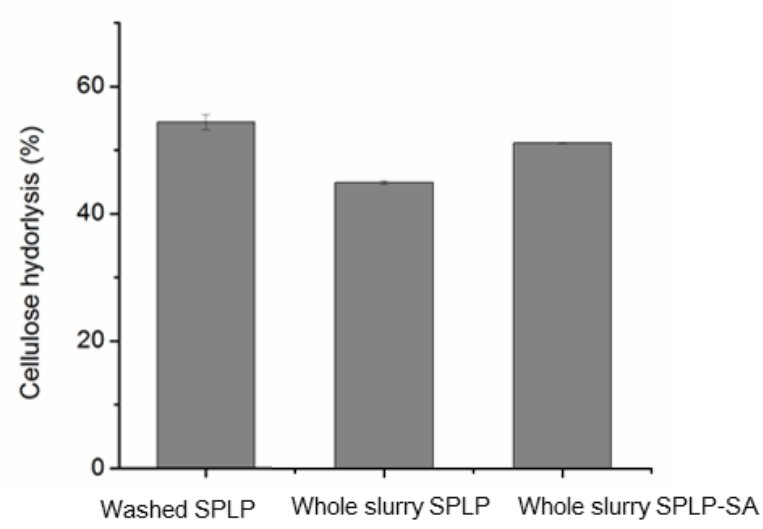

Figure 48 Enzymatic hydrolysis of washed and whole slurry pretreated lodgepole derived from pretreatment process with and without syringic acid added (SPLP and SPLP-SA). The substrates were hydrolyzed with CTec3 (40 mg/g glucan) at 10% (w/v) consistency after 72 h. SPLP: steam pretreated lodgepole pine; SPLP-SA: steam pretreated lodgepole pine derived from a pretreatment process with syringic acid added.

3.5.3 Conclusions

Lignin derived compounds such as phenolics acids could serve as efficient scavengers to reduce the inhibitory effect of phenolics on cellulose hydrolysis. Of the various scavengers studied, syringic acid (SA) showed the best inhibition mitigating effects. By adding syringic acid prior to steam pretreatment of forest biomass, we could achieve relatively good cellulose hydrolysis when using relatively low enzyme loadings.

4. Conclusions and Future Work

4.1 Conclusions

Although previous studies have shown the pretreatment-derived water-soluble (WS) fractions could inhibit both the cellulase enzymes and fermentative microorganism, there have been limited studies that have tried to determine the nature of the major groups of inhibitors and how they might influence cellulose hydrolysis. We systematically assessed the possible inhibitory effects of the major soluble compounds derived from steam pretreated hardwood (poplar) and softwood (lodgepole pine) on hydrolytic potential of the "state-of-the-art" commercial cellulase preparations. It was apparent that even the more recent CTec3 enzyme mixture was inhibited by pretreatment-derived inhibitors despite this relatively novel enzyme mixtures significantly higher resistance when compared to more "traditional cellulase mixtures" such as Novozymes Celluclast mixture. Monomeric sugars were shown to be a major group of inhibitory compounds as were lignin derived phenolics. Contrary to some of the previously published literature, pretreatment-derived oligomeric sugars were found to be non-inhibitory to cellulose hydrolysis.

When the possible inhibition mechanisms (such as reversible/irreversible inhibition) on the major enzyme components (CBH and BG) present in CTec3 were assessed, it appears that BG is mainly inhibited by glucose while CBH is mainly inhibited/deactivated by phenolic compounds. It was also apparent that, although hemicellulose-derived sugars did not affect the major enzyme activity they did inhibit cellulose hydrolysis. As Cel7A accounts for the major part of enzyme mixtures, we next focussed on how hemicellulose-derived sugars might inhibit Cel7A action on the pretreated substrates. It was apparent that hemicellulose derived sugars reduced the productive binding and processivity of Cel7A on cellulose.

When the possible influence of substrate characteristics (such as crystallinity, degree of polymerisation, etc.) on enzyme inhibition were assessed the acid group content of the substrate was found to be the most influential. Thus, by increasing the amount of acid groups on the cellulosic substrate this would likely greatly alleviate the inhibition of cellulose hydrolysis.

The inhibitory/deactivating effects of phenolics on the major enzyme activities were found to be greatly dependent on the source and composition of phenolics. Key structural properties of the phenolics greatly affected their inhibitory effects, particularly their molecular size and their carbonyl group content.

Since sugar inhibition might be alleviated by simultaneous saccharification and fermentation, more efficient strategies to mitigate phenolic-mediated inhibition of cellulose hydrolysis were assessed. Modifying the phenolic structure by carbocation scavengers in the pretreatment step was evaluated to try to minimize their inhibition. It appeared that carbocation scavengers such as syringic acid could potentially enhance whole slurry hydrolysis of steam pretreated lignocellulose when relatively low enzyme loadings are used.

4.2 Future work

4.2.1 Maximizing ethanol concentration through simultaneous saccharification and fermentation (SSF) of whole slurry derived from scavengers supplemented pretreatment

The majority of the work described in the thesis was focused on minimizing enzyme inhibition in order to obtain high concentration of sugars from steam pretreated whole slurry. It was apparent that end-product inhibition due to accumulated sugars was a limiting factor. However, much of the work reported in the thesis could also be potentially extended to try to

produce high concentration of ethanol by adding an extra fermentation step that should alleviate end-product inhibition due to sugars. (Gurram et al., 2011; Klinke et al., 2004; Pienkos and Zhang, 2009). Typically, post-treatment strategies have been assessed to try to detoxify the water soluble fraction so that high concentrations of ethanol from pretreatment derived whole slurry can be achieved. For example, reducing agents like dithionite and sulfite has been used in-situ to detoxify the acid pretreated spruce wood and sugarcane bagasse so as to achieve high ethanol concentrations through separate enzymatic hydrolysis and fermentation (SHF) (Alriksson et al., 2011). It is likely that these reducing agents modify the structure of phenolics and alleviate their inhibitory effect on both cellulases and yeast (Cavka et al., 2011; Jönsson et al., 2013).

In the work reported in the thesis we showed that scavengers could be applied during pretreatment to alleviate the inhibitory effect of phenolics on cellulases (section 3.5). As described in the thesis, it has been suggested that these scavengers reduce the formation of small size ketone-like phenolics during pretreatment, potentially reducing their inhibitory effect on both enzyme and yeast (Chandel et al., 2013; Klinke et al., 2004).Thus, the use of scavengers could be potentially applied to the whole slurry to enhance both hydrolysis and fermentation. In addition, compared with tradition SHF process for ethanol production, simultaneous saccharification and fermentation (SSF) is less time-consuming and more effective as it combines hydrolysis and fermentation in one reactor and can alleviate sugar inhibition through simultaneous removing sugars (Van Dyk and Pletschke, 2012; Wingren et al., 2003). Thus, by utilizing the whole slurry derived from scavenger supplemented pretreatment and a subsequent SSF approach, it may be possible to produce high concentration of ethanol at relatively low enzyme loading over short processing time (i.e. 24 hrs).

4.2.2 Adding reductive enzymes to the steam pretreated whole slurry to mitigate the phenolics inhibition

The thesis work showed that the degree of enzyme inhibition caused by phenolics seemed to be closely related to their structure (i.e. molecular size and carbonyl group content, section 3.4). Although reducing agents such as $NaBH_4$ can decrease the carbonyl group content of phenolic compounds and alleviate their inhibitory effect on cellulose hydrolysis, these chemicals might also result in more detrimental reactions such as reducing the released sugars. Compared to chemical reducing agents, carbonyl reductases might more selectively reduce ketone like phenolics under milder conditions (Ward and Young, 1990; Wohlgemuth, 2014). These enzymes have been widely used for the reduction of a wide variety of structurally diverse carbonyl compounds such as aliphatic and aromatic ketones (Wohlgemuth, 2014). Thus, it would be interesting to assess the use of carbonyl reductases to possibly modify the phenolics in the whole slurry, consequently reducing their inhibitory nature.

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