Understanding the Relative Importance of the Effects of Soluble and Insoluble Inhibitors on the Enzyme Digestibility of Steam Pretreated Radiata Pine

A thesis

submitted in fulfilment

of the requirements for the degree

of

Doctor of Philosophy

in Chemistry

at

The University of Waikato

by

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THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2018

Abstract

Lignocellulosic biomass use is one of the keys to growth and development of future biofuels and biochemicals. Amongst the lignocellulosic feedstocks, softwoods are particularly attractive as they have the potential to produce high sugar yields and are prevalent in many forests globally, currently they make up 95% of New Zealand's forest estate.

One of the most promising approaches to the production of lignocellulosic biofuels is the sugar pathway. This pathway involves using enzymes to hydrolyse the carbohydrate polymers in the biomass to monomeric sugars, and then converting these sugars to ethanol. High sugar yields during enzymatic hydrolysis requires an effective pretreatment to disrupt and/or remove some of the lignin and hemicelluloses, so that the cellulose is more accessible to enzymes. Softwoods, such as *Pinus radiata,* are amongst the most recalcitrant substrates towards enzymatic hydrolysis, typically, they require more severe pretreatment conditions and higher enzyme doses than hardwood or agricultural residues.

Steam pretreatment is one simple and cost-effective pretreatment. Steam pretreatment involves heating the biomass in steam or water (150ºC to 250ºC), often in the presence of added acid catalysts. One disadvantage of steam pretreatment is that it can produce compounds that inhibit the subsequent enzymatic hydrolysis. During enzymatic hydrolysis, these inhibitors, particularly lignin, lower the digestibility of the cellulose by non-productively binding the hydrolysis enzymes. This means that high enzyme doses are required increasing the conversion costs.

How these inhibitors are formed, how they work and what types of inhibitors are most deleterious during enzymatic hydrolysis is not well understood. The main objective of this thesis was to understand the relative roles of soluble and insoluble fibre components as inhibitors of enzyme hydrolysis of steam pretreated *P. radiata* as a function of pretreatment severity.

Samples: *P. radiata* sawdust was pretreated using steam explosion under six different pretreatment conditions and separated to produce both water-soluble (filtrate) and insoluble (substrate) samples. These samples were used for the rest of the research.

Insoluble inhibition: The digestibility of the insoluble substrates was examined using a commercial enzyme cocktail (Novozymes Cellic® CTec2). Results showed the digestibility of these substrates increased with increasing pretreatment severity due to increases in accessibility. However, when the substrates were tested after ball-milling to a common cellulose accessibility, as determined by Simons' stain measurements, the digestibility decreased with increasing pretreatment severity. This showed that while increasing pretreatment severity led to greater enzyme inhibition, the inhibition was more than compensated for by increases in the accessibility.

As part of this work, modifications were made to the Simons' stain method to ensure a robust and reliable method was available for measuring cellulose accessibility.

Soluble inhibition: The inhibitory effects of filtrates were investigated by comparing changes in the digestibility of both bleached kraft pulp and the insoluble steam pretreated substrates in the presence and absence of filtrates. The results showed that the inhibitory effects of the filtrates were minimal or non-existent under most conditions. It was therefore concluded that the insoluble components of steam pretreated *P. radiata* were more inhibitory than soluble components.

With the commercial enzyme cocktail CTec2, adding the filtrates back during hydrolysis led to small enhancements in digestibility. The results of subsequent experiments were consistent with the enhancements being due to components in the filtrates acting as reductants for the oxidative cellulase enzymes present in this cocktail.

Inhibition by lignin: Lignins were isolated from three insoluble substrates of differing pretreatment severity and their inhibitory effects on the digestibility of bleached kraft pulp were determined. Results showed: (i) that the inhibitory effects of added lignins increased with the amount of lignin added; (ii) that more severe pretreatments led to more inhibitory lignins; (iii) that adding the surfactant polyethylene glycol could overcome the inhibitory effects of these lignins. Additionally, the more severe pretreatments produced lignins with greater condensation and more phenolic groups, these in turn negatively correlated to digestibility, indicating the structural composition of lignin plays a role in the extent to which lignin is inhibitory.

Publications, conferences and poster presentations

Publications

MacAskill, J.J., Suckling, I.D., Lloyd, J.A., Manley-Harris, M. Unravelling the effect of pretreatment severity on the balance of cellulose accessibility and substrate composition on enzymatic digestibility of steam pretreated softwood. *Journal of Biomass and Bioenergy* **2018**, 109C, 284-290.

Conferences and poster presentations

Jessica MacAskill, Merilyn Manley-Harris, Ian Suckling and John Lloyd. *Measuring the cellulose accessibility of steam-exploded Pinus radiata wood using the Simons' stain procedure*. Presented at the Advanced Biofuels Research Network (ABRN) 2014, Rotorua.

Jessica MacAskill. *Reducing the amount of enzymes used in biofuel production from Radiata Pine*. Presentation at the 2015 APPITA NZ section new speakers competition. (Oral presentation, Runner-up)

Jessica MacAskill, Merilyn Manley-Harris, Ian Suckling and John Lloyd. *Unravelling the relative importance of enzyme accessibility and enzyme inhibition on the digestibility of mild steam pretreated softwoods.* Extended abstract and poster presented at the *18th* International Symposium on Wood, Fiber and Pulping Chemistry (18th ISWFPC), at BOKU University Vienna, September 2015.

Jessica MacAskill, Merilyn Manley-Harris, Ian Suckling and John Lloyd. *Unravelling the relative importance of enzyme accessibility and enzyme inhibition on the digestibility of mild steam pretreated softwoods.* Verbal presentation of a poster at the November 2015 ABRN conference, Hamilton. (Winner of Top Poster Presentation sponsored by BECA AMEC)

Jessica MacAskill, Merilyn Manley-Harris, Ian Suckling and John Lloyd. *Effect of water soluble inhibitors from steam pretreated Pinus radiata on enzymatic digestibility*. Poster presented at the 2016 ABRN conference, Rotorua.

Acknowledgements

First and foremost, I wish to extend my most sincere gratitude and thanks to my two supervisors at Scion; Ian Suckling and John Lloyd. Your time, patience, guidance and knowledge have all been invaluable and instrumental in getting this research to the high quality that it is. You have both taught me many valuable lessons over the last four years, that pertain to both science and life.

To Merilyn Manley-Harris, thank you for agreeing to be my supervisor. Your support and guidance throughout this research has been greatly appreciated. I also wish to thank you for all the opportunities you have provided me over the years. Thanks also to Joseph Lane, for his help throughout this thesis.

To Ian, John and Merilyn, I would like to take this opportunity to not only thank you for your support of my academic and science endeavours, but for permitting me to be flexible with my time thus allowing me to pursue my other passion, Table Tennis. The transferable skills from playing and coaching sport at NZ and international level such as, perseverance, time-management, decision making and teamwork, were fundamental in my work-ethic and being able to complete this thesis to a high standard.

I would like to extend my sincere gratitude to Scion, for providing me with a PhD scholarship and allowing me to conduct my research on site at the Rotorua campus. The facilities were wonderful and it was beneficial being in an environment surrounded by like-minded people who are striving to achieve excellence in their various fields. I also acknowledge Scions' support that has allowed me to attend various conferences over the last four years.

I wish to thank all those at Scion who have helped me over the duration of this research: Kendra, Sunita, Katrina, Kaye, Pat, Kyra, Keryn, Gavin, Alankar, Kirk, Greg, Jamie, Gill (and others); whether it was conducting lab inductions, helping me locate chemicals, or providing assistance with equipment and/or analyses, it was all appreciated. Special thanks must go to: Peter for his assistance with the steam explosion of the radiata pine; Sylke, for showing me how to perform enzyme hydrolysis; Lloyd for his expertise and help with the microscopy; and both Stefan and Daniel for their assistance with the NMR analyses.

I am also extremely grateful to the Sir Edmund Hillary Scholarship Programme, not only for awarding me a Sir Edmund Hillary Higher Degree Scholarship but for their continued support in all 'three pillars' whilst I have been a student.
Thanks, must also go to the New Zealand Federation of Graduate Women Charitable Trust Waikato Branch for awarding me a Merit Award for Doctoral Study.

To Cheryl Ward the science librarian, thank you for all your help. Whether it was helping me track down a journal for inter-loaning, or answering questions/helping with formatting and endnote, you were always happy to help.

To Claire and her boys, thank you for allowing me to share your home and for providing a quiet, study-friendly environment outside of the office. Living with kind and friendly people made my time in Rotorua more enjoyable.

To my friends: Armi, Steph, Jem, Jade, Rach, Megan, Maria, Mark, James, Allanah, Andrew, Ngahina, Charlotte, Katie and Michael, thank you for everything you have all done. Whether it was fitting in a catch-up around my hectic schedule, cooking me dinner, being my gym buddy, or listening to me talk about science while you had no idea what I was talking about, it was all appreciated. Additional thanks must go to Bianca, who helped proof read this thesis when I could not persuade or bribe any of my other friends to take on the task. Most importantly thank you all for not asking the question 'is the thesis done yet' but for reminding me 'you will finish your PhD and it will be worth it in the end'.

To John and Tanya Page, thank you for all your support. Thank you for the house key which meant I always had somewhere to stay in Hamilton, while I lived in Rotorua. I know the key was intended to be used rarely; my apologies, that my definition of rarely was every week-end, we should have clarified that in advance. Without your support, I would not have been able to balance my research and Table Tennis, or achieve a fraction of what I have. You have been like a second set of parents to me, so I thank you from the bottom of my heart.

Lastly, but in no way the least, I wish to thank my family for all their support over the years. Thank you for everything, whether it was being just a text or phone call away, sending me care packages or making my infrequent once-yearly visits home enjoyable. To Mum and Dad, thank you for your support throughout this stage of my life. It has been a hectic and intense journey, one for which I am grateful to have had your unwavering love, encouragement, understanding and patience throughout. Your encouragement, support and love are, and always will be, treasured.

This thesis is dedicated to my Mum and Dad who taught me that: perseverance and hard-work can help you achieve anything in life; and that you should always engage yourself in things you are passionate about.

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α	alpha (anomeric configuration)
β	beta (anomeric configuration)
ε	extinction coefficient
γ	gamma (anomeric configuration)
δ	chemical shift (ppm)
λ	wavelength (nm)
°C	degrees Celsius
ara	arabinose
AIL	acid insoluble lignin
ASL	acid soluble lignin
ATR	attenuated total reflectance
BKp	bleached kraft pulp
BM	ball-milled
C	carbon
COV	coefficient of variance
CSF	combined severity factor
EMAL	enzymatic mild acidolysis lignin
FPU	filter paper units
FPU/g	filter paper units per gram of substrate
FTIR	Fourier transformed infrared spectroscopy
g	gram
gal	galactose
GC	gas chromatography
GC-FID	gas chromatography flame ionisation detection
GGM	galactoglucomannan
gluc	glucose
h	hour(s)
H_2SO_4	sulfuric acid
HCl	hydrochloric acid
HMF	hydroxymethyl furfural
HPLC	high performance liquid chromatography
ICP-MS	inductively coupled mass spectrometry
IU	International unit

kDa	kilodalton
kN	kilonewtons
kPa	kilopascal
L	Litre
LHW	liquid hot water
LPMO	lytic polysaccharide monooxygenase
man	mannose
min(s)	minute(s)
µL	microlitre
µm	micrometre
µM	micromolar
ML	middle lamella
mm	millimetre
M	molar
MPa	megapascal
Mw	molecular weight
MWL	milled wood lignin
nm	nanometre
NMR	nuclear magnetic resonance
NZ	New Zealand
O.D.	oven dried
O/N	overnight
PEG	polyethylene glycol
ppm	parts per million
PSI	pounds per square inch
PTL	protease treated lignin
R49TBM300 minutes	steam exploded control pulp tumble ball-milled 300
RPM	revolutions per minute
S1, S2, S3	secondary cell wall layers one, two and three
SD	sawdust
SF	severity factor
SO_2	sulfur dioxide
t	time
T	temperature (degrees Celsius)

UV	ultra violet
v/v	volume per volume
VFA	volatile fatty acid
w/w	weight per weight
w/v	weight per volume
xyl	xylose

1 Introduction

1.1 Background

Lignocellulosic biomass use is one of the keys to growth and development of future biofuels. Potential lignocellulosic biomass feedstocks include wood, wood residues, agricultural residues such as corn stover or sugarcane bagasse and dedicated energy crops such as miscanthus [1]. Softwoods are prevalent forest species in many areas of the world and the possibility for high sugar yields from hydrolysis makes them an attractive biomass for bioconversion. In New Zealand, currently 95% of forest plantations are softwoods, mainly radiata pine. Because plantation forests can be grown on lower-value land, they provide a good feedstock for scalable biofuel production in New Zealand [2,3].

One of the most promising approaches to the production of lignocellulosic biofuels is the sugar pathway, which involves using enzymes to hydrolyse the carbohydrate polymers in a substrate to monomeric sugars, and then fermenting these sugars to ethanol [4]. High sugar yields during enzymatic hydrolysis rely on an effective pretreatment to disrupt and/or remove the lignin and hemicelluloses, thus making the cellulose more accessible to enzymes [5-9]. One of the simplest and most cost-effective pretreatments is heating the biomass in water or with steam. Throughout this thesis all such pretreatment options will be referred to as ‘steam pretreatments’. There are many variants on the approach with most steam pretreatments heating the biomass to between 150ºC and 250ºC, often in the presence of acid catalysts [5,6,8,10,11]. Steam pretreatments do however have a number of disadvantages. Under acidic conditions, the hemicelluloses may be hydrolysed and degraded and some of the degradation products such as the furans and acetic acid can inhibit subsequent fermentation [12]. Other degradation products such as pseudo lignin can deposit on cellulose and retard enzymatic hydrolysis [13]. Furthermore, during the pretreatment, the lignin can be modified to produce compounds that inhibit the subsequent enzymatic hydrolysis and fermentation [12,14,15]. The presence of chemicals that inhibit enzymatic hydrolysis means that high enzyme doses are often required adding to the conversion costs.

Softwoods, such as *Pinus radiata,* are amongst the most recalcitrant lignocellulosic substrates towards enzymatic hydrolysis. Typically they require not only more severe pretreatment conditions but also higher enzyme doses than hardwood or agricultural residues [9,16,17]. Although a number of pretreatments have been investigated for bioconversion of softwoods, those based on steam pretreatment remain industrially appealing providing that the processes can be scaled up in a cost-effective manner.

1.2 Need for this research

Pretreatment and enzyme costs are currently major contributors to the overall high processing costs of converting lignocellulosic biomass to sugars [18,19]. Hence, many current studies focus on improving pretreatment efficiency and reducing enzyme use. One important area in the processing of softwoods that has received less attention and where knowledge is lacking, is the understanding of inhibitor formation and what types of inhibitors are most deleterious during enzymatic hydrolysis. Inhibitors formed during steam pretreatment of wood may be present in either or both the water-insoluble (insoluble inhibitors) or water-soluble phases (soluble inhibitors), but little is known about their relative importance, their modes of action and remediation options. A better understanding of inhibitors and consequent enzyme use could lead to reductions in the cost of softwood biosugar production processes, specifically to produce biofuels and or biochemicals.

1.3 Objectives of this thesis

The main objective of this thesis was to understand the relative roles of soluble and insoluble fibre components as inhibitors of enzyme hydrolysis in the conversion of steam pretreated *P. radiata* wood to sugars. This included understanding the relative roles and degree of inhibition of the components as a function of pretreatment severity. Components responsible for the inhibitory effects were to be identified, isolated and further investigated. These findings will help explain why the costs of producing sugars from softwood are so high, particularly in terms of enzyme use.

1.4 Thesis structure

Chapter 2 is a detailed literature review which summarises the key research in this field. It provides background information on biochemical and biofuel production from lignocellulosic biomass (including *Pinus radiata*), reactivity of lignocellulosic polymers under acidic conditions and the formation of potential inhibitors of enzymatic hydrolysis. Chapter 2 also provides a review of the current research into the inhibitory nature of lignin, particularly the non-productive binding of enzymes to lignin.

Chapter 3 describes the preparation and chemical characterisation of the various samples and control materials that were used throughout this study. The set of samples covers a wide range of pretreatment conditions (and severity factors) and allowed for the effects of pretreatment conditions temperature, time and pH to be compared. Chapter 3 also introduces some of the key methods used throughout the research such as enzyme hydrolysis.

Chapter 4 describes and discusses modifications made to the Simons' stain procedure to ensure that a robust analytical method was available for the reliable determination of the cellulose accessibility of substrates used in this study. This method was fundamental in being able to hold constant one of the key variables that influences the enzyme digestibility of substrates. The approach of holding cellulose accessibility constant is one that has not been reported previously in literature.

Chapters 5 and 6 evaluate whether soluble (filtrate), insoluble (substrates) or both types of components have inhibitory effects on the enzyme digestibility of lignocellulosic biomass (*P. radiata*). Chapter 5 also investigates some of the key parameters known to inhibit enzyme digestion of substrates. The findings revealed that lignin was influencing digestibility and, as such, understanding lignin inhibition was the focus of latter research and chapters. Chapter 6 shows that the inhibition by filtrate components was negligible in comparison to substrate inhibition.

Chapter 7 investigates the effects of lignin location, content and composition on inhibition by the solid substrate. The central focus was the isolation of 3 lignins from different pretreatment conditions across the pretreatment severity range studied. These lignins were vital for lignin inhibition studies, inclusive of the lignin content study and the chemical compositional studies.

Finally, Chapter 8 presents general conclusions and gives suggestions for future research.

2 Literature Review

2.1 Introduction

Today, nearly the entire transportation sector is reliant on fossil-based fuels, accounting for ~60% of world oil consumption. However, given limited petroleum reserves, increasing demand for petroleum-based fuels by emerging economies and growing political and environmental concerns, there is substantial interest in developing economical and energy efficient processes for sustainable fuel and chemical production [20-22].

Currently, biofuels comprise only 4% of total liquid fuel use worldwide [23], with the main biofuels being ethanol (~82%) and biodiesel (~18%) [24]. The former is primarily produced from sugarcane or corn and the latter from vegetable oils or animal fats [25]. Future investment and research is looking to utilise more sustainable and environmentally friendly biomass alternatives.

Lignocellulosic biomass sources, comprised of cellulose, hemicellulose and lignin (see **section 2.3**), are key feedstocks for future biofuels. Lignocellulosic feedstocks are available in plentiful quantities and have the potential to satisfy society's large fuel demand [26]. The advantages of lignocellulosic biomass over other feedstocks include: being more sustainable; contributing to reducing greenhouse gas emissions; and, permitting the use of land that is unsuitable for growing food crops [20,27]. Examples of common lignocellulosic biomass are shown in **Table 2.1**.

Table 2.1 Examples of common lignocellulosic biomass.

• **Purpose grown crops** wood, grasses, energy crops
• **By-products** cereal, straw, sugarcane bagasse, forest residues from forest clearings and mills
• **Wastes** organic components of municipal solid wastes

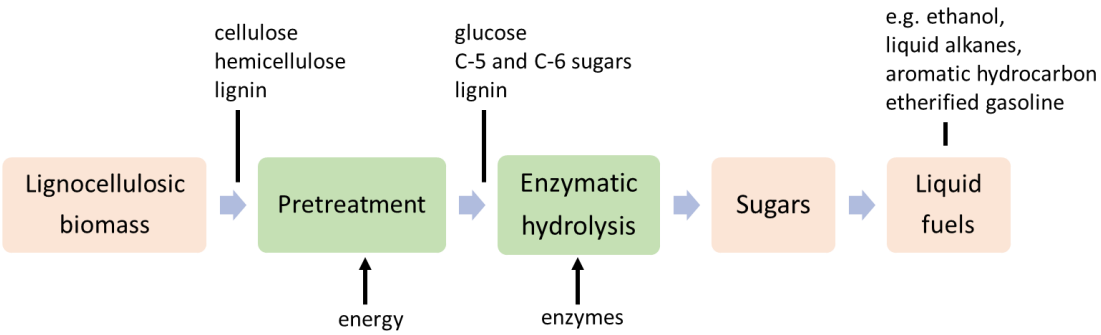


Figure 2.1 Overview of the production processes involved in converting lignocellulosic biomass to liquid fuels or chemicals *via* the sugar pathway.

An overview of the production processes required to produce liquid fuels or chemicals *via* the sugar pathway can be seen in **Figure 2.1**. Pretreatment opens cell walls enabling access to cellulose and hemicelluloses, while hydrolysis is achieved using enzymes. The upgrading of sugars to liquid fuels or chemicals can then occur [1,20,25]. The two most expensive processing steps are pretreatment and enzymatic hydrolysis. This is because, unlike starchy or non-lignocellulosic feedstocks, a major component in cell walls of the lignocellulosic biomass is lignin. The presence of lignin means that more severe and costly pretreatments are required to make the sugars accessible for successful enzymatic hydrolysis [1,20,25,28,29]. Another disadvantage of pretreatment is that degradation of biomass components (cellulose, hemicellulose, lignin) occurs, forming compounds that inhibit the subsequent enzymatic hydrolysis and fermentation stages. Consequently, higher enzyme doses are often required to ensure effective enzymatic hydrolysis. The inhibitors that form vary depending upon the initial biomass, pretreatment technology and pretreatment conditions [10]. The cost of enzymes used during enzymatic hydrolysis of woody biomass is a significant factor in the overall costs of obtaining biochemical or biofuels *via* the sugar pathway. However, woody biomass has such potential for high sugar yields that some of this cost is mitigated [28].

2.2 Trees/*Pinus radiata* as a biomass source

In New Zealand there is the potential to grow biomass in abundance for biofuel production, particularly on lower-value land [2]. A particular focus in New Zealand is on woody feedstocks. This is because most of the land is unsuitable for growing food crops such as corn or sugarcane; these crops are also suitable for biofuel production. The softwood *P. radiata* affords potential benefits such as high growth rates and effective growth turnover times, as well as being grown in forests/plantations (~95% of species in NZ's forest estate) [3]. All of these benefits contribute to New Zealand having potential international economic and environmental advantages in the production of *P. radiata* as a crop for fuel [30].

2.3 Chemical composition of lignocellulosic biomass

Lignocellulosic biomass consists of three main types of polymers: cellulose, hemicelluloses and lignin. These polymers are interwoven and connected by both non-covalent and covalent linkages [25,31]. The proportions of these polymers vary between and within groups of feedstocks, for example the composition of softwoods and hardwoods is quite different (**Table 2.2**). In addition, all woods contain smaller amounts of extractives, or components which can be extracted using various solvents such as methanol, dichloromethane and acetone [31]. Subsequent discussion focuses mainly on softwoods, as these are the focus of this thesis.

Table 2.2 Composition of lignocellulosic biomass feedstocks [20,32,33].

Lignocellulosic biomass	Cellulose (%)	Hemicelluloses (%)	Lignin (%)
Hardwoods	40-50	25-35	18-25
Softwoods	40-45	20-30	25-35
Nut shells	25-30	25-30	30-40
Corn stover	30-40	20-35	15-23
Corn cobs	45	35	15
Sugarcane bagasse	35-45	25-34	18-24
Grasses	25-40	35-50	10-30
Wheat straw	30	50	15
Newspaper	40-55	25-40	18-30
Waste papers from chemical pulps	60-70	10-20	5-10

2.3.1 **Cellulose**

Cellulose is the main component of wood making up approximately 40 to 50% of its dry weight (**Table 2.2**) [34]. Cellulose has the general formula $(C_6H_{10}O_5)_n$ and is comprised of between 100 to 10,000 β-D-glucopyranose units linked (1-4) by glycosidic bonds (**Figure 2.2**). Cellulose chains align themselves regularly with hydrogen bonding between hydroxyl groups; this forms bundles of cellulose molecules which in turn form microfibrils [31].

Microfibrils consist of highly organised crystalline regions alternating with less ordered amorphous regions; the aggregation of microfibrils in turn forms the basis of fibrils. Cellulose forms the framework of cell walls and it is interwoven with, and form bonds with, hemicellulose and lignin. The presence of numerous hydroxyl groups is responsible for the affinity of cellulose to water and consequently the hygroscopic properties of wood [34,35].

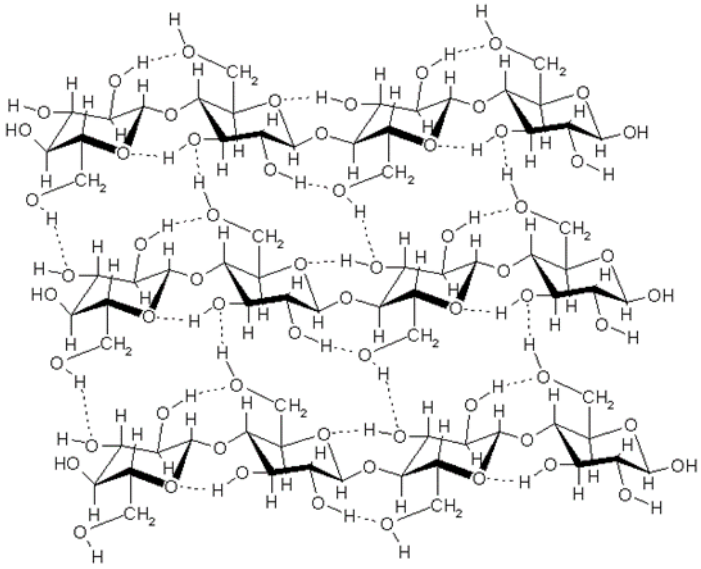


Figure 2.2 Structure of cellulose showing the β-(1-4) glycosidic linkages between D-glucopyranose units, as well as both inter- and intra- chain hydrogen bonding.

2.3.2 **Hemicelluloses**

Hemicelluloses are the second major carbohydrate component of woods; primarily acting as a support matrix for the cellulose microfibrils of cell walls. In softwoods, hemicelluloses make up approximately 20 to 30% of the dry wood weight and are found as short chain (100 to 200 units) branched polysaccharides [34-36]. Unlike cellulose, hemicelluloses are polymers formed from a variety of five- and six-carbon sugars (**Figure 2.3**). Some hemicelluloses contain uronic acid units like glucuronic acid, and acetyl substituents [31,34,36,37]. The chemical properties of hemicellulose differ from those of cellulose; for example, hemicelluloses are more soluble and more easily hydrolysed to their monosaccharide constituents than cellulose [31].

The principal sugar moieties in hemicelluloses are D-glucose, D-galactose, D-mannose, L-arabinose, D-xylose, and 4-*O*-methyl-D-glucuronic acid (**Figure 2.3**) [34,36,37]. The main hemicellulose found in softwoods is galactoglucomannan, whereas hardwood hemicelluloses consist mainly of xylan (**Table 2.3**) [34].

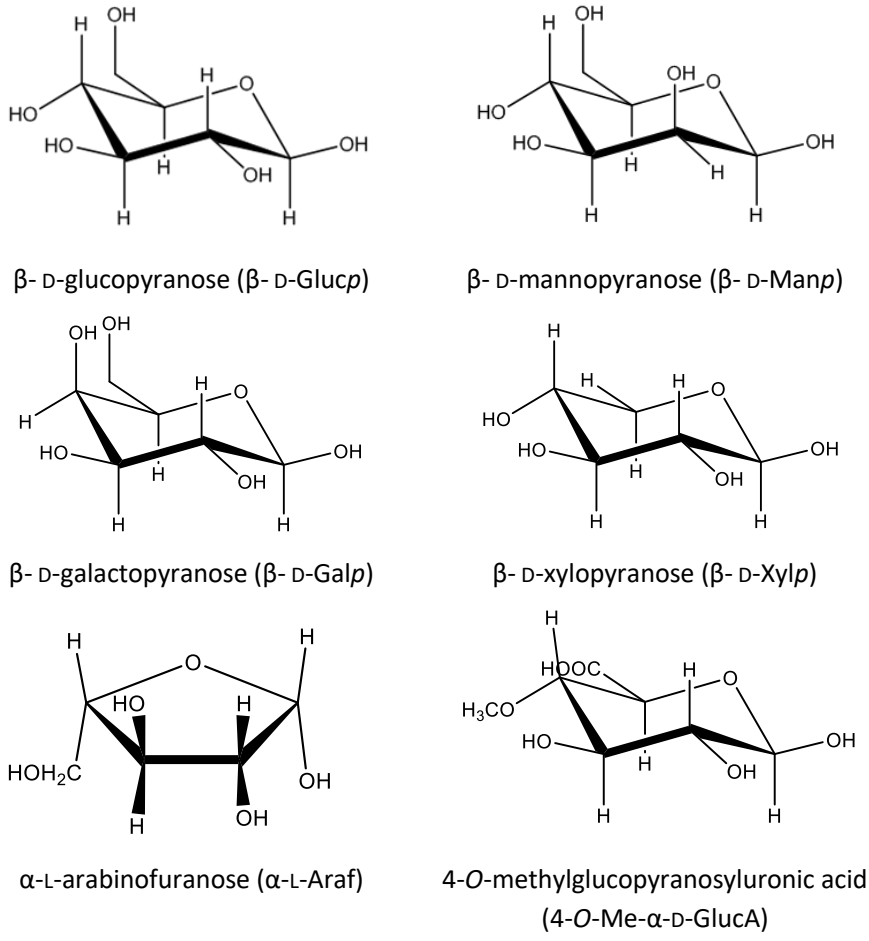


Figure 2.3 The main sugar moieties of hemicelluloses [31,34,36,37].

Table 2.3 Major hemicelluloses found in softwoods and hardwoods [34,36].

Hemicellulose type (degree of polymerisation)	Occurrence	% in wood	Units	Molar ratio	Linkage
Galactoglucomannans (~100)	Softwood	5-8	β- D-Man*p*	3	(1-4)
			β- D-Gluc*p*	1	(1-4)
			β- D-Gal*p*	1	(1-6)
			Acetyl	1	
Galactoglucomannans (~100)	Softwood	10-15	β- D-Man*p*	4	(1-4)
			β- D-Gluc*p*	1	(1-4)
			β- D-Gal*p*	0.1	(1-6)
			Acetyl	1	
Arabinoglucuronoxylans (~100)	Softwood	7-10	β- D-Xyl*p*	10	(1-4)
			4-*O*-Me-α-D-GlucA	2	(1-2)
			α-L-Araf	1.3	(1-2) & (1-3)
Glucuronoxylans (~200)	Hardwood	15-30	β- D-Xyl*p*	10	(1-4)
			4-*O*-Me-α-D-GlucA	1	(1-2)
			Acetyl	7	
Glucomannans (~200)	Hardwood	2-5	β- D-Man*p*	1-2	(1-4)
			β- D-Gluc*p*	1	(1-4)

Note: Arabinogalactan is another softwood hemicellulose; however, it is only present in small quantities other than larch-wood (5 to 35%), so has not been considered.

2.3.3 **Galactoglucomannan**

Galactoglucomannan (GGM), is the major hemicellulose in softwoods (approximately 20%), it is comprised of a β-(1-4) linked backbone of D-glucopyranose and D-mannopyranose units, with D-galactopyranose side branches linked α-(1-6) (**Figure 2.4**). There are two fractions of this polymer: a low galactopyranose fraction and a high galactopyranose fraction. These have Gal*p*:Gluc*p*:Man*p* ratios of 0.1:1:4 and 1:1:3, respectively [34]. The C-2 and C-3 positions of the backbone sugars (Man*p* and Gluc*p*) units are partially substituted by acetyl groups, on average one acetyl per three to four hexose units [34-36]. In *P. radiata*, the average Gal*p*:Gluc*p*:Man*p* ratio is 0.13:1:3.7 [38].

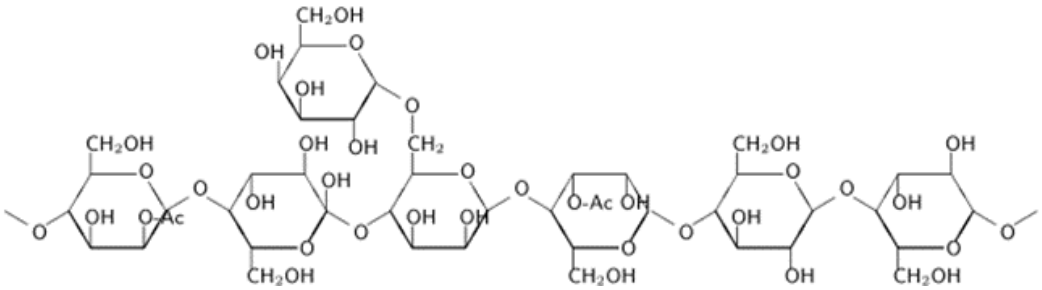


Figure 2.4 Structure of galactoglucomannan.

2.3.4 **Xylan**

The term ‘xylan’ is often used to describe the xylose-based hemicelluloses in both softwoods and hardwoods, however the composition of the xylan in the two wood types is different. The major xylan in softwoods is arabinoglucuronoxylan (7 to 10%), it is comprised of a β-(1-4) linked backbone of D-xylopyranose units with 4-*O*-methyl-D-glucuronic acid side branches. It also contains α-linked L-arabinofuranose substituents (~1.3 residues per 10 xylans) at the C-2 or C-3 positions on xylopyranose units (**Figure 2.5**) [34,36]. In hardwoods the major xylan is glucuronoxylan (15-30%) [34,36].

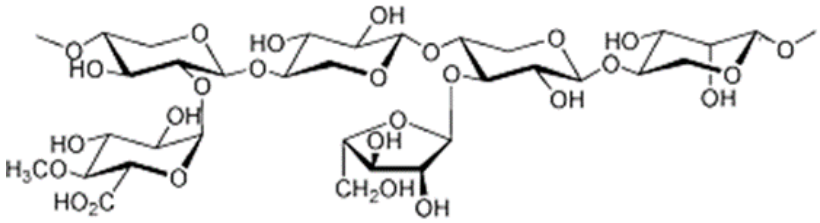


Figure 2.5 Structure of arabinoglucuronoxylan.

2.3.5 Lignin

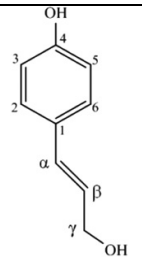
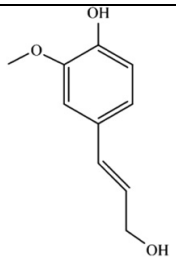
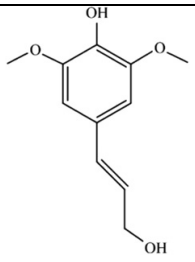
Lignin plays a vital role in increasing the mechanical strength of woods and contributes between 20 to 35% of wood biomass (**Table 2.2**). Softwoods generally contain more lignin than hardwoods, usually 25 to 35% [34,36]. Lignin provides structural support to the wood and decreases water permeation of cell walls. It can also act as a defence mechanism preventing destructive enzymes from penetrating into the cell wall [26,27].

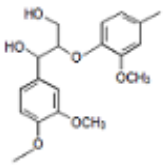
2.3.5.1 Lignin structure

Lignin is a complex amorphous cross-linked three-dimensional copolymer. It is comprised of three different phenylpropane precursors (monolignols): coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These monolignols link together with either C-C and C-O-C bonds [26,27,34,36,39-41]. The three monolignols differ in their degree of methoxylation with either zero, one or two methoxyl groups attached to the aromatic ring (**Table 2.4**) [27,41]. The major monolignol in softwood lignin is coniferyl alcohol, whereas both coniferyl and sinapyl alcohol precursors dominate in hardwoods [34,41].

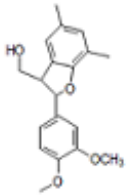
The major monolignol linkages and approximate ratios for softwood can be seen in **Figure 2.6**. The predominant C-O-C and C-C linkages are β-O-4 and β-5, respectively [26,27,36,41].

Table 2.4 Major monolignols present in lignin and their presence in important lignocellulosic biomass sources [27,42].

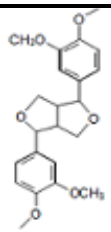
	Lignin (wt %)	Phenylpropane unit		
Structure				
		p-coumaryl alcohol (%)	coniferyl alcohol (%)	Sinapyl alcohol (%)
Lignin type		*p*-hydroxyphenyl (H)	guaiacyl (G)	Syringyl (S)
Softwood	25-35	< 5	90-95	None or trace
Hardwood	20-25	0- 8	25-50	46-75
Grasses	15-25	5-33	35-80	20-55



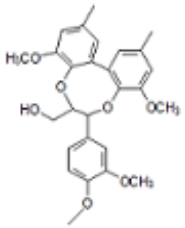
β-aryl ether
(β-O-4)
40-60%



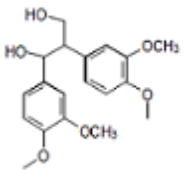
phenylcoumaran
(β-5)
9-12%



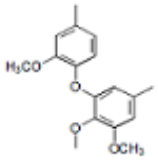
β-β linked structures (pinoresinol)
(β-β)
< 5%



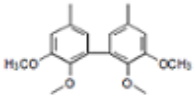
dibenzodioxocin
(5-5)
~10%



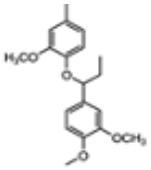
1,2-diaryl
propane
(β-1)
1-10%



diaryl ether
(4-O-5)
3-8%



biphenyl
(5-5)
~10%



α-aryl ether
(α-O-4)
6-8%

Figure 2.6 Proportions of major interunit linkages in softwood lignins [27,34,39].

2.3.6 Extractives

Extractives typically account for 1 to 8% of the weight of wood. They are so named because they can be extracted from wood, using either water or organic solvents, such as acetone, ethanol or dichloromethane [34]. Extractives are chemically diverse and include: tannins, resin acids, essential oils, fats, terpenes, flavonoids, quinones, carbohydrates, lignans, glycosides and alkaloids [31,34-36]. Some extractives are responsible for the colour, durability or smell of the wood. Although extractives are only present as a small proportion of the wood, they are nevertheless important due to the problems they cause during wood processing. For example, if not removed, extractives can decrease pulp yields, cause problems with re-slushing or contribute to effluent toxicities [36]. The latter is relevant to this study as it is intended that filtrates be reused or added back to wood to determine inhibitory effects upon hydrolysis enzymes.

2.4 Wood cell wall ultrastructure

The cell wall is comprised of different layers that can be seen under an electron microscope (**Figure 2.7**). The visible differences in the cell wall layers are due to the differing chemical compositions and orientation of structural elements. The middle lamella (ML) is the thin layer between cells that binds all the cells together. This layer is almost completely cellulose free and is mainly comprised of lignin [34]. The primary cell wall consists of cellulose and hemicellulose (and lignin). It is thin and flexible and allows the cell to grow. The secondary wall is comprised of three layers (S1, S2 and S3) that differ in composition and cellulose fibre orientation. All three secondary layers contain lignin [31,34,43]. The dominant secondary layer is the S2 layer. Its thickness varies between wood types (early and late wood) and the overall characteristics of the S2 layer strongly influence the wood's properties such as stiffness.

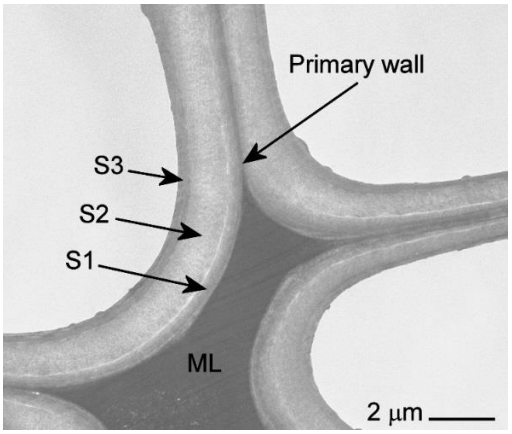


Figure 2.7 Wood cell wall structure: middle lamella (ML), primary wall, secondary walls (S1, S2, S3).

2.5 Biofuel and biochemical production from lignocellulosic biomass

Currently, there are no well-established commercial technologies for converting lignocellulosic biomass into biofuels. However, biofuel production is an evolving field of technology undergoing highly active research and development [22]. In general, lignocellulosic biofuel production requires that biomass is deoxygenated to produce high-energy liquid fuel.

There are three primary pathways whereby lignocellulosic biomass can be converted into liquid fuels: the Syn-Gas pathway, the liquid bio-oil pathway and the sugar pathway (**Figure 2.8**). Within each pathway there are numerous routes and approaches currently under investigation. These involve both thermochemical and biochemical processes [22,44]. This research focuses on converting the softwood radiata pine to sugars *via* the sugar pathway. The sugars can then be further processed into fuels or chemicals. Producing sugars economically is a significant focus of researchers globally [33,45].

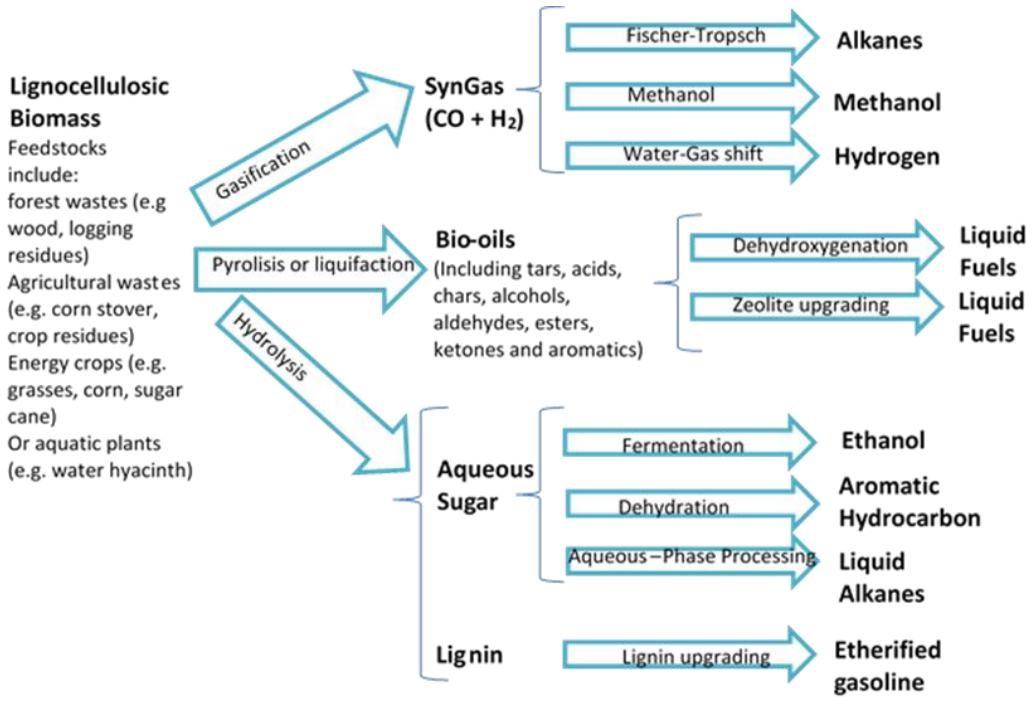


Figure 2.8 Overview of the pathways and processes for biofuel and chemical production from lignocellulosic biomass, based on Huber *et al.* [21].

2.5.1 The sugar pathway

In the sugar pathway, the carbohydrate polymers (cellulose and hemicellulose) are hydrolysed into their constituent sugars, using either acid or enzymes. Other hydrolysis processes have also been proposed, but will not be discussed due to their lack of commercial significance [20]. These include hydrolysis in supercritical water, using gamma rays, electron beams or microwave radiation.

Hydrolysis of cellulose produces glucose, while hemicelluloses are hydrolysed to a mixture of C-5 and C-6 sugars, the composition of which is dependent upon the initial biomass source. The sugars can then be fermented to produce ethanol or other alcohols, or upgraded using catalytic or biological means to produce hydrocarbons, the latter are then converted to fuels (**Figure 2.9**). The by-product, lignin, is either burnt as a source of process energy or occasionally it is recovered and sold as a by-product for upgrading (**Figure 2.8**).

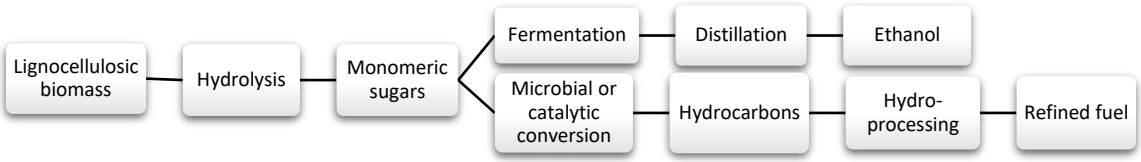


Figure 2.9 Pathway for sugars after hydrolysis; either fermentation or catalytic upgrading [46].

2.5.2 **Hydrolysis**

Lignocellulosic biomass is hydrolysed using acids or treatment with enzymes (**Figure 2.10**) [33]. Acid hydrolysis is well established and can be performed using either dilute acid (high temperature, short time) or concentrated acid (low temperature). Dilute acid hydrolysis gives low sugar yields, while concentrated acid hydrolysis requires the acid to be recovered. Both acid hydrolysis options have a major limitation; they are known to yield a variety of compounds that inhibit the subsequent steps, such as fermentation, and this is highly undesirable [47].

As a result, enzymatic hydrolysis is often preferred because the milder conditions (pH ~4.8 and T = 45-50°C) result in less carbohydrate degradation and fewer inhibitors being formed [28,33]. Enzymes are also advantageous as at optimum conditions, they can have high conversion efficiencies [48].

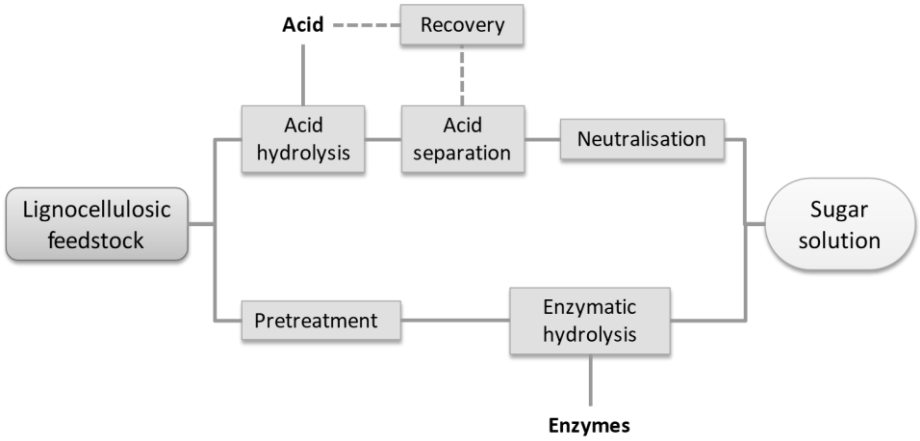


Figure 2.10 The primary routes of hydrolysing lignocellulosic biomass; either using acids or enzymes [46].

2.5.2.1 Enzymatic hydrolysis of lignocellulosic biomass

Enzymatic hydrolysis of starch- and sucrose-based feedstocks is already carried out on an industrial scale. However, employing cellulases to hydrolyse cellulose to glucose in lignocellulosic biomass is technically more difficult. This is because structural and compositional factors make lignocellulosic biomass recalcitrant and difficult to hydrolyse using enzymes (**section 2.5.3**). Thus, in order for effective enzymatic hydrolysis of lignocellulosic biomass, a pretreatment step is essential. This will be discussed in **section 2.5.4**. Nonetheless, enzyme hydrolysis is considered a promising way to convert biomass to sugars due to continual decreases in enzyme costs, lack of equipment corrosion (as with acid alternatives) and the potential for enzymes to be recycled [49].

Currently, softwoods are one of the most difficult lignocellulosic feedstocks to hydrolyse using enzymes [9,48]. This is primarily due to the composition of softwoods, in particular their high lignin content (**section 2.2**, **Table 2.2**) [9]. As a result, softwoods require higher enzyme doses and more severe pretreatments than other lignocellulosics in order to expose the cellulose and obtain efficient hydrolysis [29]. These two requirements add substantially to the overall processing cost but the potential for higher sugar yields and reduced costs with continued research makes enzymatic hydrolysis a viable route of investigation [28].

2.5.2.2 Enzymes used for enzymatic hydrolysis

Multiple enzymes are required for effective enzymatic hydrolysis [50]. These include cellulases, both endo- and exo-glucanases, β-glucosidase and hemicellulases, such as endo-xylanases and endo-mannanases [51]. Each enzyme has its own function in terms of hydrolysing different linkages.

Enzymes are produced by a variety of bacteria and fungi, including aerobic, anaerobic, mesophilic and thermophilic microorganisms [33,51]. Cellulases are most commonly produced by fungi, such as *Trichoderma spp*, *Penicillium spp* and *Aspergillus spp* [33,51]. In commercial applications, proprietary enzyme mixes (usually from fungi) are used, generally incorporating a mix of cellulases and hemicellulases.

2.5.2.3 **Cellulases**

The hydrolysis of cellulose to glucose requires a number of enzymes, each with their own function. The primary function of cellulases is to break down the β-1,4-D-glucan linkages of the cellulose polymer to yield glucose as the main product [25]. The endo-glucanases act on the main cellulose polymer chain and expose both reducing and non-reducing ends. Following this, the exo-glucanases attack the exposed reducing and non-reducing ends to produce smaller units of cello-oligosaccharides, cellobiose and glucose. Finally, β-glucosidases complete the hydrolysis by hydrolysing cellobiose to glucose [25].

2.5.2.4 **Hemicellulases**

Since hemicelluloses are more complex than cellulose, hydrolysis by enzymes involves the action of a larger variety of enzymes. In general, hemicellulases can be classified into three groups: 1) endo-acting enzymes, which internally cleave polysaccharide chains 2) exo-acting enzymes, which cleave either the non-reducing or reducing ends and 3) side-chain-cleaving enzymes ("accessory enzymes"), such as acetyl-esterases [25,37]. The specific hemicellulases selected for individual circumstances depend upon the initial biomass source. For example, if hydrolysing softwood where galactoglucomannans are a significant component, then enzymes capable of degrading these sugars, such as mannanases are beneficial. In contrast, for a hardwood source, xylanases are essential due to the high xylan content.

2.5.2.5 **Standard industrial enzyme cocktails**

In industrial settings, proprietary enzyme mixes 'cocktails' are used. Within each cocktail, the composition, source of enzymes and concentrations of enzymes varies. Hence, depending on the initial biomass source the most suitable cocktail in terms of cost versus efficiency must be evaluated. In some cases, combinations of cocktails are used to further maximise benefits. The disadvantage of many of the current commercial enzyme cocktails is that the cocktails have been developed for treating non-wood lignocellulosics and hardwoods. Therefore, they are not optimised for hydrolysis of softwoods.

In this study, the main enzyme cocktail used was Cellic® CTec2. This cocktail contains cellulase, β-glucosidase, xylanases, cellobiose, endo-glucanase and lytic polysaccharide monooxygenases (LPMOs) [52].

2.5.2.6 **Lytic polysaccharide monooxygenase (LPMO) enzymes**

Current commercial enzyme cocktails contain lytic polysaccharide monooxygenases (LPMOs) [53]. LPMOs are an important class of enzymes that have been recently found to be effective at boosting the hydrolysis of cellulose in lignocellulosic biomass. Several LPMOs have been characterised and shown to be active on crystalline substrates such as cellulose [54-58]. In contrast to typical cellulases which are hydrolytic enzymes, LPMOs are metalloenzymes [53] that operate *via* an oxidative mechanism. For their activity, they require an oxygen containing species that can act as an electron donor for reduction of their divalent copper cofactor [59,60]. The generic mechanism when LPMO (Cu(I)) is present is $-RH + H_2O_2 \rightarrow -R-OH + H_2O$, where R is the crystalline glycoside [57,59]. LPMOs cleave β-(1-4) glycosidic linkages in cellulose leaving the C-1 or C-4 carbons oxidised, thus improving the access that other hydrolytic enzymes such as cellulases have to cellulose [59,61-64]. More recent studies have also found that some LPMO's can act upon non-crystalline polysaccharides such as the hemicelluloses [59,65].

LPMO activity has mainly been investigated using reducing agents that have the ability to act as electron donors, such as ascorbic acid or gallic acid [58,60,66]. However, recent studies [67-69] suggest that addition of external electron donors may not be required for the LPMO activity on lignocellulosic biomass. It is proposed that the lignin itself may act as an electron donor, but substantial evidence of how lignin acts as an electron donor is yet to be seen [58]. There is also the possibility that lignin and carbohydrate degradation products are acting as effective electron donors. However, due to the current limitations in understanding of the occurrence, type and mechanisms of LPMO activity, it is difficult to predict the extent of the advantageous outcomes that LPMO presence can have on the enzymatic digestibility of lignocellulosic biomass.

2.5.3 Factors affecting enzymatic hydrolysis

The complex chemical and physical nature of lignocellulosic biomass means there are multiple parameters to consider when trying to obtain effective enzyme hydrolysis of lignocellulosic biomass. These parameters can be categorised into two main groups: substrate related parameters and enzyme related parameters. In general terms, the former is associated with cellulases accessibility to cellulose, while the latter relates to cellulose reactivity.

2.5.3.1 Substrate related parameters

Substrate related factors that affect hydrolysis include: cellulose crystallinity; degree of polymerisation; particle/substrate size; available/accessible surface area; structural organisation (both macrostructure-fibre and microstructure-microfibril); native (untreated) lignin content and structure; and the extent of lignin and/or hemicellulose removal by pretreatment [25,70,71].

2.5.3.2 Enzyme related parameters

Factors that have been related to the reactivity of cellulase enzymes during the hydrolysis process include: enzyme concentration, enzyme adsorption, synergism, mechanical deactivation (fluid shear stress), thermal inactivation, end-product inhibition and irreversible (non-productive) adsorption of enzymes to lignin [25,70].

2.5.4 Pretreatment of lignocellulosic biomass

One well-known characteristic of lignocellulosic biomass is its recalcitrant nature towards enzymatic hydrolysis. As a result, pretreatment of lignocellulosic biomass prior to hydrolysis is essential for obtaining high yields of sugars [21]. For example, with no pretreatment, hydrolysis yield of sugars from polysaccharides is approximately 20%, whereas with pretreatment, hydrolysis yields can be around 90% [6,21,44]. The downside of pretreatment is that it is one of the most expensive processing steps of producing sugar from biomass.

2.5.4.1 Goals and actions of pretreatment technologies

The overall goal of pretreatment is to improve the digestibility of the lignocellulosic biomass [71]. This is achieved by modifying the structure of the lignocellulosic biomass so that enzyme hydrolysis efficiency can be improved.

Pretreatment can increase the access enzymes have to cellulose in various ways (**Figure 2.11**) [26,28]: 1) fragmentation and cracking of wall components (hemicellulose and lignin) occurs, increasing the accessible surface area; 2) hemicelluloses are hydrolysed (and solubilise) this also increases the accessible surface area of cellulose; and 3) lignin undergoes structural changes and wood is delignified to a certain degree, lignin relocation can also occur [28]. The extent to which these changes occur depends on the pretreatment technology used.

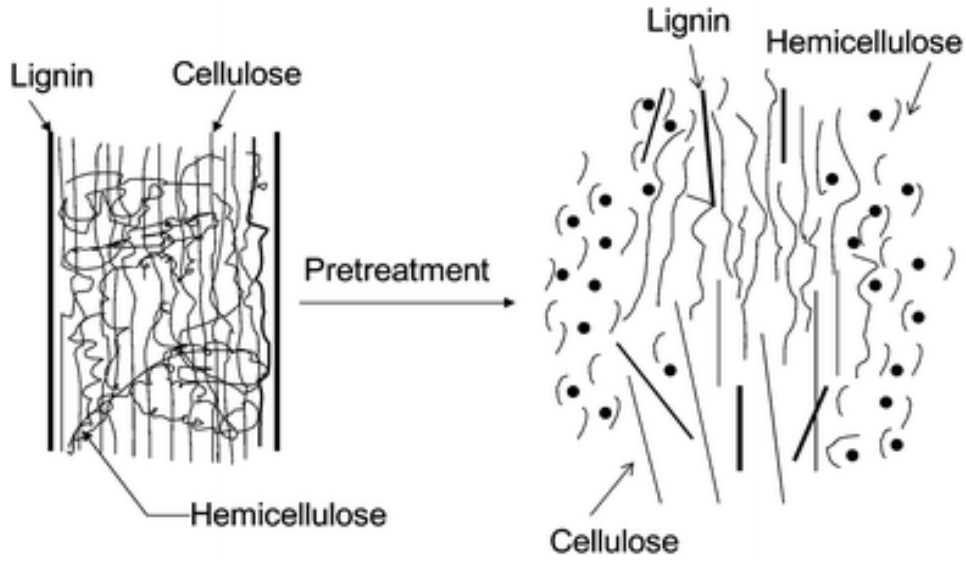


Figure 2.11 Depiction of the increased cellulose accessibility as a result of pretreatment, as based on Kumar *et al.* [26].

2.5.4.2 Current lignocellulosic pretreatment technologies

Currently, there are a variety of pretreatment technologies for lignocellulosic biomass including physical, physio-chemical, chemical and biological options [45,50]. Reviews of the various available pretreatments and their inherent advantages and disadvantages are given by Alvira *et al.* [48], Chiaramonti *et al.* [45], Hendriks and Zeeman [71], Kumar *et al.* [26], Mosier *et al.* [11] and Sun and Cheng [33].

No single pretreatment is suitable for all types of biomass, this is because of the complexity and differing composition of biomass sources. Most successful pretreatments rely on either removing or modifying the hemicelluloses and/or lignin in order to increase the enzymes accessibility to the cellulose. The accessibility of enzymes to cellulose is crucial for efficient enzyme hydrolysis to occur. Since every pore of a substrate has a critical width, estimated to be 40 to 60 nm, enzyme diffusion can be limited. This is because cellulases are ellipsoid with diameters of 4 to 6.5 nm and lengths of 18 to 21.5 nm [72-74]. Researchers believe even substrates with bigger pores of up to 200 nm may also result in limited enzyme diffusion, this is because bound enzymes may contribute to confinement effects [75].

Although severe pretreatments are often required to enhance enzyme digestibility, higher severity pretreatments conditions cause greater degradation [6]. In practice, there is always a trade-off between increasing pretreatment severity to enhance digestibility and decreasing pretreatment severity to minimise hemicellulose and lignin degradation. Hence, research into pretreatments is still ongoing to obtain the best balance between these competing effects, while also being economical and feasible at large scale.

Pretreatments that have been used on woods and their inherent advantages and disadvantages can be seen in **Table 2.5**. For softwoods, extra consideration must be taken when deciding upon an effective pretreatment, owing mainly to their higher lignin contents and concomitant higher recalcitrance. Nonetheless, pretreatments that have been found to be effective on softwoods include milling and mild acid pretreatments such as dilute acid, liquid hot water and steam explosion [76].

Table 2.5 Advantages and disadvantages of pretreatment methods that are effective for woods [1,48,76,77].

Pretreatment technology	Advantages	Disadvantages
Milling	• Reduces cellulose crystallinity and increases carbohydrate accessibility	• High energy consumption
Steam explosion	• Causes lignin transformation and hemicellulose solubilisation • Higher yield of cellulose and hemicellulose in the two-step method	• Generation of toxic compounds • Partial hemicellulose degradation
Dilute acid	• Hemicelluloses are hydrolysed into pentose sugars and the downstream hydrolysis of cellulose is improved • Fewer problems than concentrated acid • Less formation of inhibitors	• Generation of degradation products especially if high temperatures are used • Low sugar concentration in exit stream
Liquid hot water (LHW)	• Requires no catalyst and low-cost reactor	• High water demand • High energy requirements • Low solids processing during pretreatment
Organosolv*	• Causes lignin and hemicellulose hydrolysis	• High cost • Solvents need to be recycled
Ozonolysis*	• Reduces lignin content • Does not imply generation of toxic compounds	• High cost of large amount of ozone needed

*Pretreatment technology that can be used, but is rarely utilised due to cost and limited effectiveness.

2.5.4.3 **Steam pretreatments**

Steam pretreatments are the most extensively studied pretreatment technologies for use on softwoods, they include liquid hot water, dilute acid and steam explosion. They are recognised as being low cost processes for making lignocellulosic biomass more accessible to enzymes for efficient hydrolysis.

Steam pretreatments typically involve heating substrates in water or steam in the presence of acid catalysts under pressure at temperatures of 150°C to 250°C [78]. Pretreatments using only water can be effective due to autohydrolysis reactions driven by the acetic acid that is produced by cleavage of the polysaccharide acetyl groups.

Steam explosion is a mild steam pretreatment that uses rapid decompression after a substrate has been heated in steam, it is of most interest as it is the pretreatment used in this study. Steam explosion can be performed without added catalysts, as discussed already, with enzymatic hydrolysis improvements primarily attributed to hemicellulose removal. Alternatively, it can be performed in the presence of added acids, such as sulfuric acid (H_2SO_4) or sulfur dioxide (SO_2). Acid catalysed pretreatments give further hydrolysis improvements, but at the cost of greater degradation [28]. Numerous studies investigating hydrolysis and fermentation have been performed using various steam pretreated softwoods [29,79-87]; of particular relevance is Clark and Mackie's studies on *P. radiata* [82,83].

2.5.4.4 Severity factor as a consideration during selection of pretreatment conditions

The severity of a pretreatment can be evaluated using a 'severity factor', which compares pretreatments and their conditions by evaluating the harshness of the reaction conditions (Equation **2.1**).

$$\text{Severity factor (SF)} = log\left[t \times \exp\left(\frac{T - T_{ref}}{14.75}\right)\right] \quad \textbf{(2.1)}$$

Where t is the pretreatment or hydrolysis exposure time, T is the reaction temperature in degrees Celcius and T_{ref} is the reference temperature, set to 100°C [6,28].

The greater the severity factor, the greater the disturbance/degradation of the individual components of the biomass. This in turn yields a greater number and concentration of components that can act as inhibitors of the subsequent processing stages such as, enzymatic hydrolysis and fermentation [6,25,28]. The severity factor allows different pretreatment conditions such as time and temperature to be compared. Any given severity factor can be achieved in different ways. For example, a high temperature and short time can be set to have the same severity factor as another treatment of low temperature but longer time.

For acid catalysed pretreatments, the combined severity factor is used (Equation **2.2**). The combined severity factor (CSF) takes pH into consideration, with typical values for acid catalysed steam pretreatments of softwood being between 2 and 4 [28,88].

$$\text{Combined severity factor (CSF)} = \log\left[t \times \exp\left(\frac{T - T_{ref}}{14.75}\right)\right] - pH \quad \textbf{(2.2)}$$

Where pH is that of the acid catalyst used.

2.6 Chemical reactivity of lignocellulosic components under acid conditions

2.6.1 Summary of bonds present in lignocellulosic biomass

As already stated, lignocellulose is a complex substrate, consisting of three polymers: cellulose, hemicellulose and lignin. The individual polymers are held together by intra- and inter-polymer bonds. The four main types of inter-/intra-polymeric bonds are ether, ester, carbon-to-carbon and hydrogen bonds and are summarised in **Table 2.6**.

Table 2.6 Intra- and inter-polymer linkages of lignocellulosic biomass.

Type of bond	Intra-polymer linkage	Inter-polymer linkage
Ether (C-O-C) bond	Lignin, (hemi)cellulose	Cellulose-lignin Hemicellulose-lignin
Ester bond	Hemicellulose	Hemicellulose-lignin
Carbon to carbon (C-C) bond	Lignin	
Hydrogen bond	Cellulose	Cellulose-hemicellulose Cellulose-lignin Hemicellulose-lignin

2.6.2 Acid catalysed reactions of wood

Reactions under mild acid conditions such as those used in steam explosion are important as they cause cleavage of cellulose, hemicellulose and lignin bonds [89]. The primary degradation reactions that occur are the acid catalysed hydrolysis of glycosidic linkages in polysaccharides and the cleavage of α- and β-ether linkages in lignin [90]. These reactions are discussed in the following sections.

2.6.3 Cellulose reactions

Acid hydrolysis of glycosidic linkages of cellulose involves the protonation of the glycosidic oxygen, followed by decomposition of the conjugate acid (**Figure 2.12**) [89]. However, cellulose is unlike other polysaccharides, due to its crystalline nature, it is more resistant to hydrolysis than hemicelluloses. Thus, hydrolysis of cellulose usually requires harsher conditions than needed for hemicelluloses.

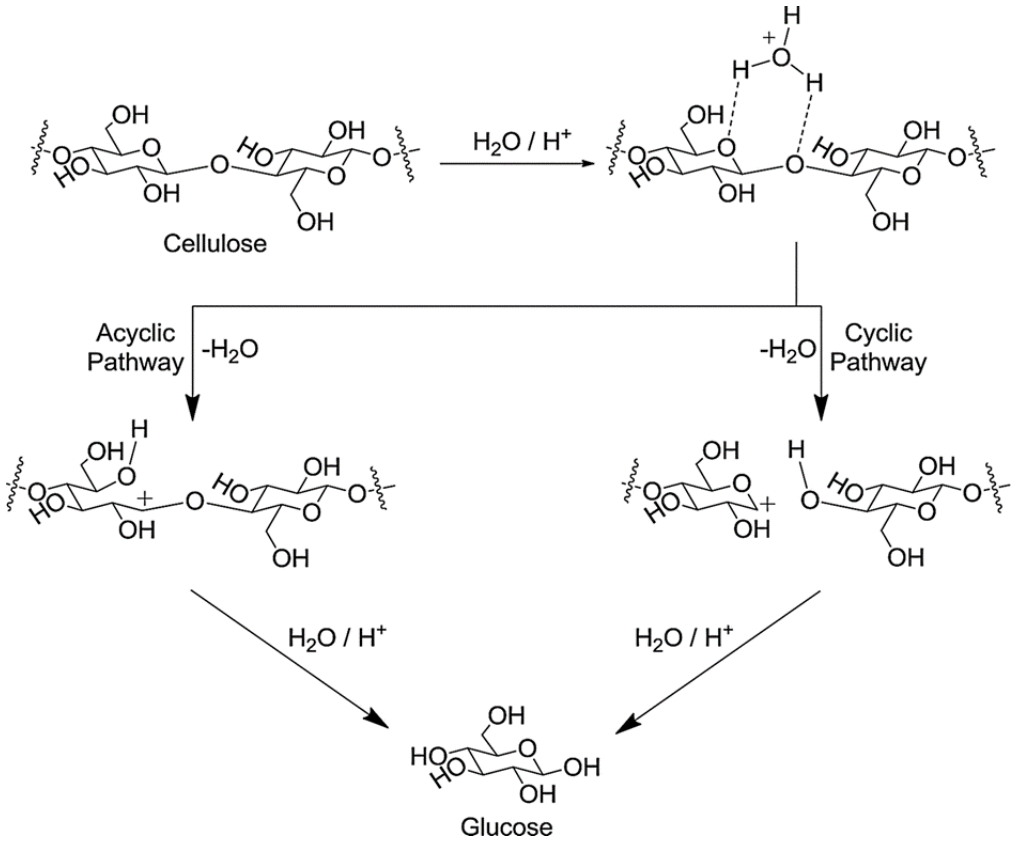


Figure 2.12 Acid hydrolysis of the glycosidic linkage of cellulose to form glucose.

2.6.4 **Hemicellulose reactions**

The glycosidic linkages of hemicelluloses are hydrolysed in an analogous manner to those of cellulose **(Figure 2.12**). Hemicelluloses are more reactive than cellulose under acidic conditions, owing mainly to their chemical composition and amorphous structure. The rate of hydrolysis of glycosidic linkages varies depending upon: the composition of the solution and whether the interactions are of the same phase; and the exact composition of the biomass [90]. Nonetheless, most hemicelluloses can be removed from cellulosic substrates by selectively choosing appropriate pretreatments, such as steam or dilute acid.

The acetyl groups present in galactoglucomannans of softwoods and xylans of hardwoods can be hydrolysed by water or acids at elevated temperatures. The advantage is that the acetic acid released contributes to the acidity, and thus catalyses other hydrolytic reactions (essentially enabling autohydrolysis) [90].

2.6.5 Hexose and pentose sugar reactions

Hydrolysis of cellulose and hemicellulose results in the formation of both hexose and pentose sugars. Sequential dehydration of hexoses and pentoses generates two main furan derivatives, of 5-hydroxymethylfurfural (HMF) and 2-furaldehyde (furfural), respectively (**Figure 2.13**) [34,36]. Successive degradation of these furans can produce levulinic and formic acids, as well as condensation products (**Figure 2.13**) [36].

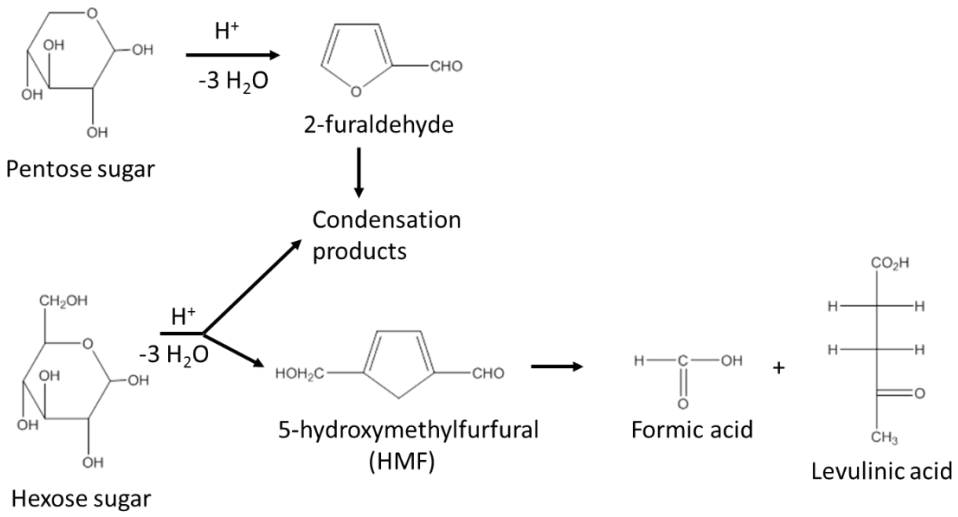


Figure 2.13 Sequential degradation of pentose and hexose sugars.

2.6.6 Lignin reactions

The main reaction of lignin under mildly acidic conditions is cleavage of the β- and α-ether linkages. These cleavage reactions are believed to be largely responsible for the formation of low molecular weight components. Additionally, cleavage of lignin-carbohydrate bonds (ether and glycosidic bonds) can occur.

2.6.6.1 Cleavage of β-O-4 (ether) linkages

Cleavage of β-O-4 linkages of lignin is the most dominant reaction under acidic conditions [91-99]. The cleavage mechanism of the β-O-4 linkage can be seen in **Figure 2.14**. During the cleavage reaction, the β- position of the β-O-4 link (**1**), converts to a benzylium cation intermediate (**2**). Following this, the reaction can proceed *via* either of the competing routes (**A** or **B**). In route **A**, an enol ether (**3**) forms and the β-O-4 linkage of this structure is subsequently hydrolysed to form a

new phenolic lignin unit (**4**) and a Hibbert's ketone type structure (**5**). In the alternative route (**B**), formaldehyde is cleaved from the γ-position of (**2**) to form the enol ether (**6**). The β-O-4 linkage of (**6**) is hydrolysed in an analogous manner as (**3**) is, to yield both a phenolic structure (**4**) and an aldehyde (**7**). The predominant pathway for β-O-4 cleavage has been shown to depend on the acid used, for example, route **A** dominates when hydrochloric acid is used whereas route **B** dominates when sulfuric acid is the catalyst [91,100-102].

In route **B**, formaldehyde is release *via* cleavage of the C-C bond between the β- and γ-carbons. Cleavage of other lignin C-C linkages can also occur, for example between β-1 and β-5 linkages. Since formaldehyde release can encourage acid catalysed condensation reactions of lignin, it must be noted that the order of formaldehyde release as a result of C-C cleavage occurs as β-1 > β-5 > β-β [90].

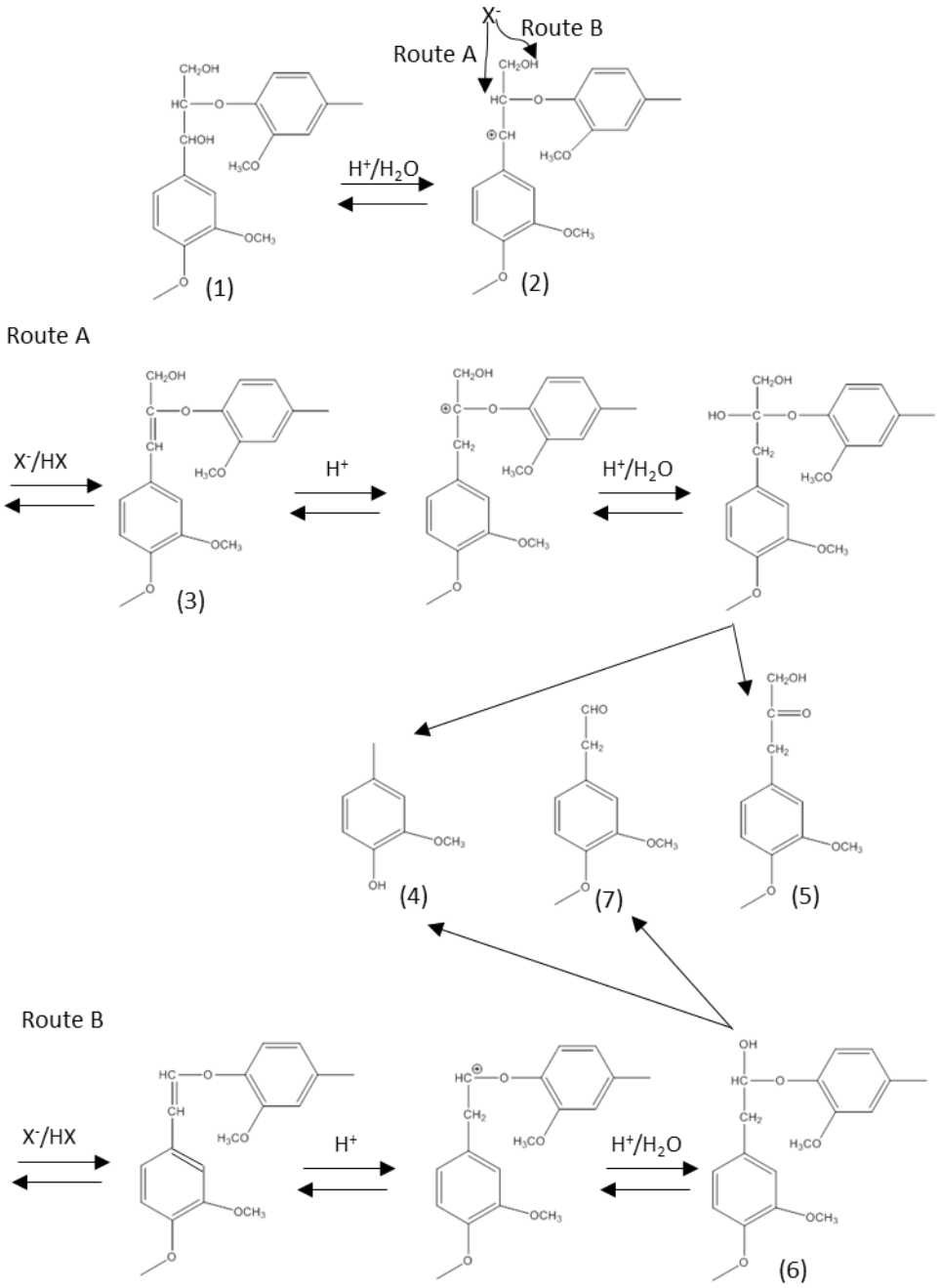


Figure 2.14 Mechanism of the β-O-4 bond cleavage based on Lundquist and Lundgren's research [95].

2.6.6.2 **Cleavage of *α*-O-4 (ether) linkages**

The cleavage of α-ether bonds occurs *via* protonation and formation of benzylic carbonium ion intermediates (**14** and **15**, **Figure 2.15**) in a similar manner to the cleavage and hydrolysis of β-ether linkages. These benzyl cations then undergo subsequent reactions, such as condensation. Under acidic conditions, the rate of reaction of α-linkages has been reported to be higher than that of the equivalent β-ether linkage [90].

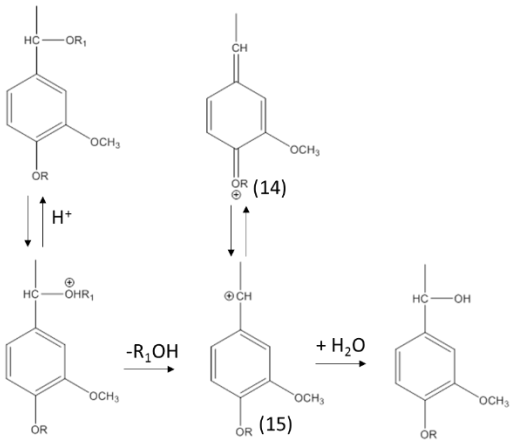


Figure 2.15 Cleavage of α-O-4 ether linkages, based on Lai [90].

2.6.6.3 **Condensation reactions**

Condensation reactions of lignin readily occur even under mildly acidic conditions due to the high reactivity of the benzyl hydroxyl groups. Typically, condensation reactions between the benzylic carbonium ion and aromatic nuclei particularly at position 6 (**Figure 2.16**) [103]. Another type of condensation reaction that is important is the one that produces either methylene cross linked aromatics or 1,3-dioxane derivatives due to the presence of formaldehyde (**Figure 2.16**) [90]. This is important as formaldehyde was previously shown to result from cleavage of C-C bonds, mainly from the loss of Cγ.

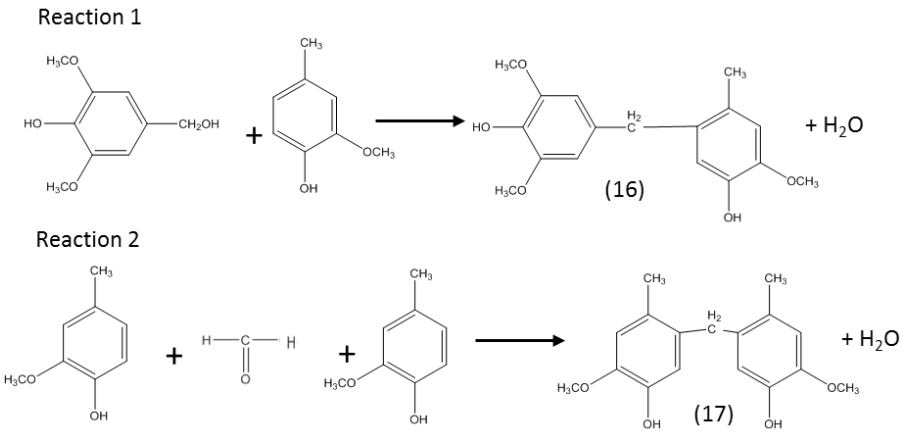


Figure 2.16 Examples of condensation reactions to produce methylene cross linked aromatics (16 and **17), based on Lai [90].**

2.7 Inhibitors

2.7.1 Enzyme inhibition

For enzymatic hydrolysis to occur, cellulases must first adsorb onto the cellulose. However, in competition with this, cellulases can also bind non-productively to other components in the substrate resulting in a decrease in the enzyme activity. Thus, it is vital to identify and reduce/eliminate 'inhibitors' that non-productively bind enzymes during the conversion of biomass to sugars as their presence can result in lower than desired sugar yields, or higher enzyme doses being required.

2.7.2 Formation and consequences of inhibitors

Although pretreatments improve sugar yields, they also result in degradation of the biomass and the formation of by-products. Many of the by-products are inhibitory to the enzymes or microorganisms used in the subsequent enzymatic hydrolysis and fermentation processes [25].

Inhibitors may be components of the fibre that remain in the water-insoluble fraction (insoluble inhibitors), particularly lignin, or water-soluble components (soluble inhibitors) from the pretreated substrate, such as phenolics, organic acids or sugars. There are possible chemical, biological and physical detoxification procedures to remove inhibitors, however these can increase costs, make the process more complex and produce extra waste products [14,104-106]. Moreover, the best types of remediation processes are often unable to be implemented due to lack of identification of specific inhibitory compounds.

Although the pretreated material is often treated to remove some of the inhibitors; another approach is to separate the pretreated slurry into a water-insoluble solid fraction (lignin + cellulose) and a water-soluble liquid fraction (filtrate which contains hemicellulose derived sugars, other sugars, lignin degradation products, organic acids and other compounds). The solid fraction is then washed before enzymatic hydrolysis; however, some inhibitors remain in the solid fraction even after washing. Although the fractions can be separated, economically it is often better to include the filtrate in the enzymatic hydrolysis process, as it simplifies the process and increases the concentration of sugars and thus potentially biofuel and/or biochemical yields [107,108].

The concentration and composition of inhibitors varies with the biomass used and the conditions of the pretreatment including time, temperature and pressure [109]. A further consideration is that not all inhibitors are of equal potency: A compound present in a lower concentration may actually exert greater inhibition of certain enzymes, compared with another compound present in higher concentration [110].

2.7.3 **Soluble inhibitors:**

The main soluble inhibitory compounds formed during pretreatments can be categorised into two groups: 1) sugar degradation products, such as monomeric and oligomeric hemicellulose components, and; 2) non-sugar components including lignin and extractive degradation products, such as organic acids, furans and phenolics [48,111]. An overview of formation of some of the soluble inhibitors is presented in **Figure 2.17**. The structure, molecular weight and names of a variety of chemicals known to form as by-products during lignocellulosic biomass processing can be found in **Table 2.7**.

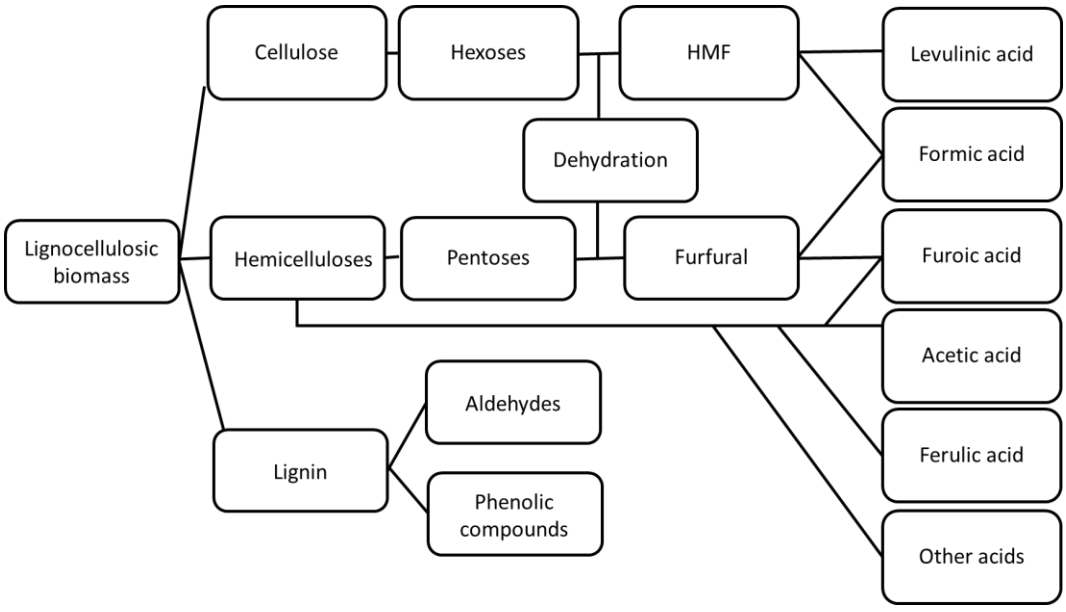


Figure 2.17 Figure of the formation of soluble inhibitors from lignocellulosic biomass, based on Vertes *et al.* [112].

2.7.3.1 **Sugar degradation products**

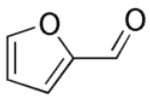
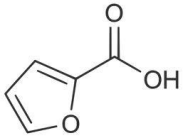
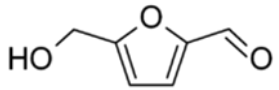
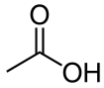
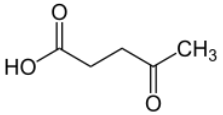
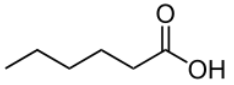
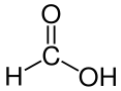
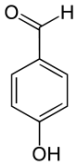
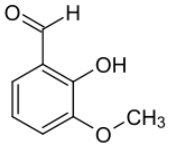
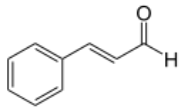
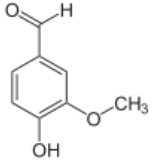
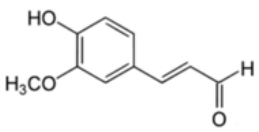
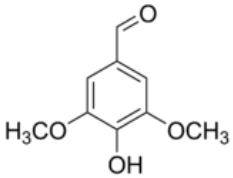
As described in **section 2.6.5**, the hydrolysis of cellulose and hemicellulose and sequential dehydration and degradation can result in the formation of 5-hydroxymethylfurfural (HMF), 2-furaldehyde (furfural), and various aliphatic acids including levulinic, formic, furoic and acetic acids. Pretreatments using acidic conditions can result in elevated concentrations of these compounds [25].

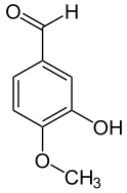
At high concentrations, HMF and furfural are known to exert inhibitory effects on fermentation microorganisms such as yeast, resulting in reduced ethanol yields from sugars [12,113]. Neither HMF nor furfural are believed to inhibit hydrolysis enzymes [114].

Formic acid has been shown to reduce the yield of glucose during the enzymatic hydrolysis of cellulose; however, the exact mode of enzyme inhibition remains unclear [115]. In contrast, acetic acid is not considered to be a hindrance to hydrolysis enzymes at the levels typically found following biomass pretreatments. Levulinic acid, another decomposition product of HMF, is usually found in much lower concentrations than acetic acid or formic acid (**Figure 2.17**), and it has not been reported as being inhibitory to enzyme hydrolysis [25,112].

Although not all acids have been shown to inhibit hydrolytic enzymes by non-productively binding the enzymes; high levels of organic acids can reduce sugar yields by reducing the pH during enzyme hydrolysis, thus taking the enzymes away from their optimum operating pH.

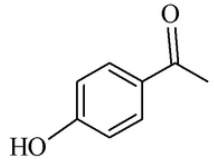
Table 2.7 Chemical compounds that form during pretreatment and/or hydrolysis of lignocellulosic biomass [109,112].

Chemical structure	Chemical information: Chemical name (common name) Molecular formula Molecular weight (Mr)	Chemical structure	Chemical information: Chemical name (common name) Molecular formula Molecular weight (Mr)	Chemical structure	Chemical information: Chemical name (common name) Molecular formula Molecular weight (Mr)
Furan derivatives					
	Furan-2-carbaldehyde (furfural) $C_5H_4O_2$ Mr = 96.09		Furan-2-carboxylic acid (2-furoic acid) $C_5H_4O_3$ Mr = 112.08		5-(hydroxymethyl) furan-2-carbaldehyde (5-(hydroxymethyl) furfural; HMF) C6H6O3 Mr = 126.11
Aliphatic acids					
	Acetic acid $C_2H_4O_2$ Mr = 60.05		4-Oxopentanoic acid (levulinic acid) $C_5H_8O_3$ Mr = 116.12		Hexanoic acid (caproic acid) $C_6H_{12}O_2$ Mr = 116.16
	Formic acid CH_2O_2 Mr = 46.03				
Phenolic compounds					
Aldehydes					
	4-hydroxybenzaldehyde (HBA) $C_7H_6O_2$ Mr = 122.12		2-hydroxy-3-methoxybenzaldehyde (*ortho* vanillin) $C_8H_8O_3$ Mr = 152.15		(2*E*)-3-phenylprop-2-enal (cinnamaldehyde) C_9H_8O Mr = 132.16
	4-hydroxy-3-methoxy-benzaldehyde (vanillin) $C_8H_8O_3$ Mr = 152.15		(2*Z*)-3-(4-hydroxy-3-methoxyphenyl-2-prop-2-enal (coniferyl aldehyde) $C_{10}H_{10}O_3$ Mr = 178.18		4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde) $C_9H_{10}O_4$ Mr = 182.17

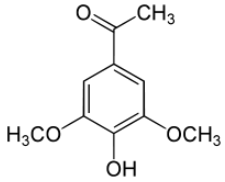


3-hydroxy-4-methoxybenzaldehyde (isovanillin)
$C_8H_8O_3$
Mr = 152.15

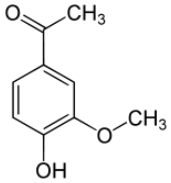
Ketones



1-(4-hydroxyphenyl) ethanone (4-hydroxyacetophenone)
$C_8H_8O_2$
Mr = 136.15

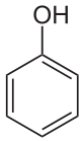


1-(4-hydroxy-3,5-dimethoxyphenyl) ethanone (acetosyringone)
$C_{10}H_{12}O_4$
Mr = 196.20

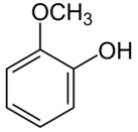


1-(4-hydroxy-3-methoxyphenyl) ethanone (acetovanillone)
$C_9H_{10}O_3$
Mr = 166.17

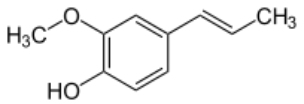
Phenols



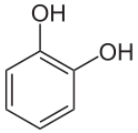
Phenol
C_6H_6O
Mr = 94.11



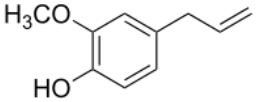
2-methoxyphenol (guaiacol)
$C_7H_8O_2$
Mr = 124.14



2-methoxy-4-[(1*E*)-prop-1-en-1-yl] phenol (isoeugenol)
$C_{10}H_{12}O_2$
Mr = 164.20

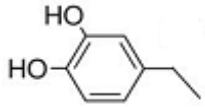


Benzene-1,2-diol (catechol)
$C_6H_6O_2$
Mr = 110.11

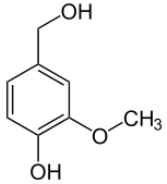


2-methoxy-4-(prop-2-en-1-yl) phenol (eugenol)
$C_{10}H_{12}O_2$
Mr = 164.20

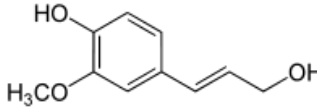
Benzene-1,4-diol (hydroquinone)
$C_6H_6O_2$
Mr = 110.11



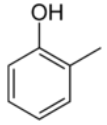
4-ethylbenzene-1,2-diol (ethylcatechol)
$C_8H_{12}O_2$
Mr = 138.16



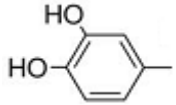
4-(hydroxymethyl)-2-methoxyphenol (vanillyl alcohol)
$C_8H_{10}O_3$
Mr = 154.16



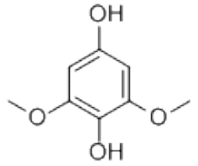
4-[(1*E*)-3-hydroxyprop-1-en-1-yl]-2-methoxyphenol (coniferyl alcohol)
$C_{10}H_{12}O_3$
Mr = 180.20



2-methylphenol (*o*-cresol)
C_7H_8O
Mr = 108.14

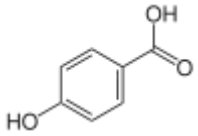


3-methylbenzene-1,2-diol (methylcatechol)
$C_7H_8O_2$
Mr = 124.14

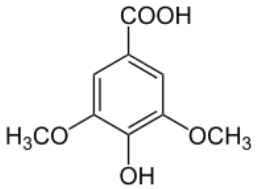


2,6-dimethoxybenzene-1,4-diol (2,6-dimethoxy-hydroquinone)
$C_8H_{10}O_4$
Mr = 170.16

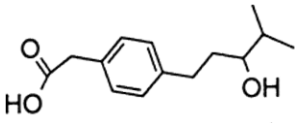
Organic acids



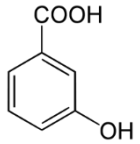
4-hydroxybenzoic acid
$C_7H_6O_3$
Mr = 138.12



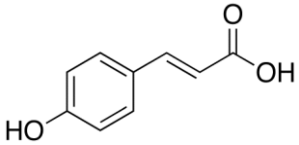
4-hydroxy-3,5-dimethoxybenzoic acid
(syringic acid)
$C_9H_{10}O_5$
Mr = 198.17



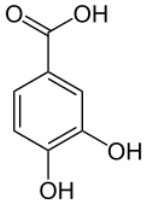
Hydroxy(4-hydroxy-3-methoxyphenyl)-acetic acid
(guaiaclyglycolic acid)
$C_9H_{10}O_5$
Mr = 198.17



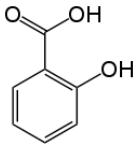
3-hydroxybenzoic acid
$C_7H_6O_3$
Mr = 138.12



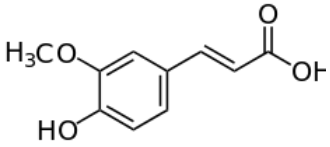
(2*E*)-3-(4-hydroxyphenyl)-prop-2-enoic acid
(4-hydroxycinnamic acid)
$C_9H_8O_3$
Mr = 164.16



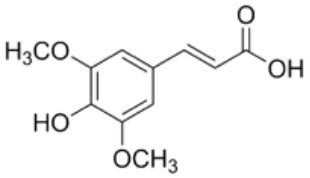
3,4-dihydroxybenzoic acid
(protocatechuic acid)
$C_7H_6O_4$
Mr = 154.12



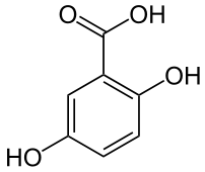
2-hydroxybenzoic acid
(salicylic acid)
$C_7H_6O_3$
Mr = 138.12



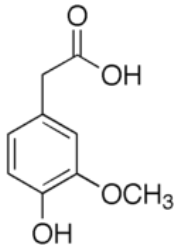
(2*E*)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-enoic acid
(ferulic acid)
$C_{10}H_{10}O_4$
Mr = 194.18



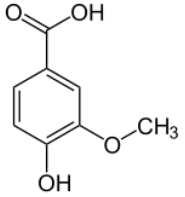
(2*E*)-3-(4-hydroxy-3,5-dimethoxyphenyl)-prop-2-enoic acid
(sinapinic acid)
$C_{11}H_{12}O_5$
Mr = 224.21



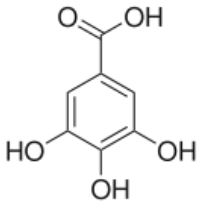
2,5-dihydroxybenzoic acid
(gentisic acid)
$C_7H_6O_4$
Mr = 154.12



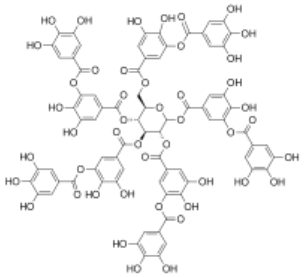
(4-hydroxy-3-methoxyphenyl)-acetic acid
(homovanillic acid)
$C_9H_{10}O_4$
Mr = 182.17



4-hydroxy-3methobenzoic acid
(vanillic acid)
$C_8H_8O_4$
Mr = 168.15



3,4,5-trihydroxybenzoic acid
(gallic acid)
$C_7H_6O_5$
Mr = 170.12



2,3-dihydroxy-5-({((2*R*,3*R*,4*S*,5*R*,6*R*)-3,4,5,6-tetrakis({3,4-dihydroxy-5-((3,4,5-trihydroxyphenyl) carbonyloxy]phenyl}carbonyloxy)oxan-2-yl)methoxy}carbonyl)phenyl 3,4,5-trihydroxybenzoate
(tannic acid)
$C_{76}H_{52}O_{46}$
Mr = 1701.02

2.7.3.2 **Phenolic compounds**

Pretreatment of lignocellulosic biomass can result in the formation of a wide range of lignin-derived phenolic compounds including aldehydes, ketones, phenols and organic acids (**Table 2.7**) [116]. The concentration and type of phenolic compounds are highly dependent upon the initial biomass source and the hydrolytic conditions. For example, phenolic aldehydes are favoured under oxidative conditions, while the hydrolysis of esterified hemicellulose and lignin yields a mix of phenols, such as coumaric, ferulic and cinnamic acids [25]. Some of the more abundant phenolic compounds that can be formed include 4-hydroxybenzoic acid, vanillic acid, 4-hydroxybenzaldehyde, vanillin, syringaldehyde, syringic acid and catechol (**Table 2.7**).

Various simple polymeric and oligomeric phenolics, have been shown to inhibit cellulase enzymes. Simple phenolics shown to inhibit cellulase enzymes include vanillin, caffeic acid and catechol [117]. Two simple phenolics; vanillin and ferulic acid, have both been shown to reduce the growth and production of the *Trichoderma reesei* enzymes; β-glucosidase, Avicelase and carboxymethylcellulase [118]. Low molecular weight phenolics vanillin, trans-cinnamic acid, syringaldehyde, and hydroxybenzoic acid have been shown to inhibit cellulases, especially β-glucosidase [15,119-124]. Terijan and Xu [122] found oligomeric phenols exert greater inhibitory effects than simple phenolics, this was because they can inactivate enzymes by reversibly forming complexes with them.

Duarte *et. al.* [116] investigated the deactivation of cellulase enzymes from *Trichoderma reesei* and *Aspergillus niger* by monomeric and polymeric phenols; they showed that within the group of phenols studied tannic acid was most inhibitory and that the β-glucosidase from *A. niger* was the most resistant enzyme to inhibition. Additionally they found, that even low concentrations of phenolics can deactivate β-glucosidase and negatively impact the overall cellulose hydrolysis yields.

While phenolics are inhibitory to cellulase enzymes, it is difficult to compare the inhibitory responses, this is because studies often test different phenolic compounds, concentrations of phenolics, and use different enzymes. Moreover, industrial

cellulase enzyme cocktails contain multiple enzymes. This means that while reduced sugar yields may be seen, it is often unknown which enzyme or enzymes in the cocktail are being inhibited. Whereas, studies often use individual enzymes to try and understand to what extent certain enzymes are inhibited, however these effects may not be identical to the patterns of enzymes within cocktails [125].

Nonetheless, Jing *et al.*[110] found that the inhibition of the cellulase, Spezyme CP for model compounds to be in the order of lignin derivatives (vanillin, syringaldehyde, 4-hydroxybenzaldehyde) > furan derivatives (furfural and HMF) > organic acids (acetic, formic and levulinic acids) > ethanol.

2.7.3.3 **End product inhibition of hydrolysis enzymes**

Some of the sugars or intermediate products formed during sugar hydrolysis are also capable of acting as enzyme inhibitors [33,49,126,127]. For example, cellobiose is a strong inhibitor of cellulases [85]. Therefore, the inclusion of enzymes such as β-glucosidases within cellulase cocktails is essential for efficient hydrolysis of cellulose [25]. In addition, β-glucosidase can be inhibited by high concentrations of glucose (which is the desired product) [128]. The latter can be overcome by combining hydrolysis and fermentation of the glucose to ethanol into a single stage.

2.7.4 **Insoluble inhibitors**

Insoluble components in pretreated biomass can also act as enzyme inhibitors. As with the soluble inhibitors, identification and removal of insoluble inhibitors is a high priority if the costs of sugar production from lignocellulosics, in terms of enzyme usage, are to be lowered.

2.7.4.1 **Lignin as an inhibitor**

Lignin is the most abundant non-carbohydrate substituent of wood and is widely accepted as a major inhibitor during enzymatic hydrolysis of cellulose [129]. In addition lignin can physically restrict the accessibility of cellulases to cellulose [130].

The mechanism by which lignin inhibits hydrolysis *via* non-productive adsorption has been the focus of a number of studies [131-133]. These studies have suggested that cellulases can bind to lignin *via* hydrophobic interactions [134], electrostatic interactions [135,136], and *via* hydrogen bonding [137]. Hydrophobic interactions have been found to occur between lignin and enzymes because both are relatively hydrophobic components in the aqueous hydrolysis medium [134,138,139]. The hydrophobicity of lignin is greater than cellulose, therefore the more hydrophobic the enzymes the more readily they will adsorb to lignin than cellulose.

Borjesson *et. al*.[140] showed that when two different enzymes were used to digest a common substrate the extent to which digestion occurred was related to the hydrophobicity of the lignin in the substrate and presence of the catalytic binding module of enzymes. Additionally, Berlin *et al.* [135], showed that soluble lignin decreased the degree of hydrolysis by a greater amount than an insoluble residual enzyme lignin. They attributed this to the lower levels of carboxylic acid and aliphatic hydroxyl functional groups of the soluble lignin lowering the hydrophobicity of the lignin.

Electrostatic interactions arise from the fact that both the enzyme and substrate surfaces carry charges [133]. The charges arise from groups such as the carboxylic acid group and amino acids and depend on the aqueous hydrolysis environment (pH, pretreatment type etc). The groups may be positively or negatively charged and if the charge on the enzyme groups and substrate groups are opposite; one is positive and the other is negative the enzyme can adsorb onto the substrate. Typically, lignocellulosic biomass fibres contain a negative charge in water at the pH used during enzymatic hydrolysis (~4.8) mainly due to the carboxylic acid groups on the hemicelluloses and phenolic hydroxyl groups in the lignin being ionised [135,141,142].

Hydrogen bonding occurs when a hydrogen atom is attached to an electron attracting atom such as O, or N, the hydrogen atom is at positive end of the dipole and it is attracted to an atom at the negative end of dipole of another molecule [143]. The hydroxyl groups on cellulose and lignin have been reported to be able to hydrogen-bond to the cellulases during enzyme hydrolysis [137,144]. Although, carboxylic acid groups may be involved in hydrogen bonding, very few studies

mention them, this is because the acid groups are more likely to be ionised at the pH of enzyme hydrolysis (pH ~4.8). Nonetheless, Berlin *et al.* [135] mention the possibility of carboxylic acids being involved in hydrogen bonding.

Many studies highlight lignin as a significant barrier to improving cellulose hydrolysis at low enzyme loadings [109,130-132,145-148]. This is because although pretreatments are used to overcome the recalcitrance of lignocellulosic biomass, the pretreatment only partially removes lignin; the cost of complete lignin removal is high. Thus, non-productive bonding of cellulases is inevitable [136,149].

Studying the inhibitory effects of insoluble lignins during enzymatic hydrolysis of lignocellulosic biomass is particularly challenging [109]. Many studies either use model compounds or isolated lignins to evaluate the extent to which lignin can bind enzymes during enzymatic hydrolysis of lignocellulosic biomass. Ko *et al.* [146,147], found that as lignin content increased and more structural changes occurred to the lignin due to more severe pretreatments, the digestibility of cellulose decreased. Nakagame *et al.* [148,150], found that more severe pretreatment conditions resulted in more condensed lignins and the more condensed lignins reduced the hydrolysis yields to a greater extent; they also showed that the cellulases interacted with lignin *via* electrostatic and hydrophobic interactions.

The amount of free and bound enzymes during hydrolysis of cellulosic substrates in the presence of isolated lignins has been studied, with results indicating that enzyme-lignin interactions are dependent upon the type of biomass, pretreatment type and pretreatment severity that the lignin is isolated from [109]. It has also been shown that isolated softwood lignins adsorb cellulases more strongly than hardwood or agricultural lignins and are therefore more inhibitory towards cellulose hydrolysis [148,150].

Ko *et al.*[146] and Nakagame *et al.* [130], have suggested that the strong inhibition of softwood lignin on the enzyme hydrolysis of cellulose may be due to the condensed and hydrophobic structure, which result in greater hydrophobic interactions between enzymes and the lignin. Nakagame *et al.*[130,148], have also reported that the strength of enzyme-lignin interactions increases as pretreatment severity increases.

To date, there are three major strategies that have been investigated to try and minimise the inhibitory effects of lignin: development of weak lignin-binding enzymes; post-delignification or modification processes; and the use of surfactants to block/prevent non-productive binding [151]. One approach of interest, due to operational feasibility, is that of using surfactants to block potential sites on lignin where enzymes may bind [151]. Surfactants that have been investigated include non-ionic surfactants/polymers such as PEG, Tween and Triton X100, as well as anionic and cationic surfactants, and proteins [152-165]. The disadvantage of surfactants is the additional costs involved, however, if the additional sugar yields are greater than the input cost of additives, the overall feasibility is promising [162].

3 Preparation of steam pretreated *Pinus radiata* samples and the enzyme digestibility of the pretreated substrates

3.1 Introduction

Lignocellulosic biomass is well-known to be recalcitrant towards enzymatic hydrolysis, thus pretreatment is essential. Although the pretreatment stage can be one of the most expensive processing steps, it is essential for high sugar yields [21,44,71]. The overall goal of pretreatment is to improve digestibility by modifying the lignocellulosic structure so that the efficiency of enzymatic hydrolysis is improved [70].

There are a variety of pretreatments used with lignocellulosic biomass including physical, chemical, physico-chemical and biological options [6,11,26,45,48,71]. For softwoods, such as *P. radiata*, more severe disruption of the lignocellulosic biomass is required, due to higher lignin contents and their resultant higher recalcitrance.

Pretreatments suitable for softwoods include milling, dilute acid, liquid hot water and steam explosion [11]. Mild acid pretreatments typically involve heating the biomass in water or steam at temperatures of 150°C to 250°C under pressure (1 to 3.5 MPa) for various times. Steam explosion is essentially a form of a mild acid pretreatment where rapid decompression of the steaming vessel is used to rupture and destructure the steamed substrate [166]. Steam explosion can be performed either in batch or continuous modes with or without the addition of acid catalysts, such as sulfuric acid (H_2SO_4) or sulfur dioxide (SO_2) [166].

The severity of the steam explosion reaction conditions affects the extent to which the biomass is modified. During steaming, acetic acid is released from the wood into the surrounding water. This increases the acidity within the substrate and assists in hydrolysing cell wall components such as the hemicelluloses [167]. An acid catalyst may also be added to the biomass prior to steaming to accelerate hydrolysis of the hemicelluloses [81,166,168,169]. The harshness of reaction conditions used

during steam explosion can be expressed as a combined severity factor (CSF), which takes the temperature, time and treatment pH into consideration [88].
Steam explosion has advantages and disadvantages as a pretreatment option for wood [1,25,26,48,76,170]. The advantages are: it is more cost effective for softwoods than some other pretreatments; higher yields of cellulose and hemicellulose are possible; chemical use and thus costs of chemicals can be avoided and acid recycling and equipment corrosion can be minimised, and; good disruption of wood to individual fibres can occur. The downside is that steam explosion can result in degradation and/or modification of lignin and hemicelluloses to produce components that are inhibitory to further processing stages such as enzymatic hydrolysis.

The inhibitory components formed during pretreatment may be compounds that solubilise in the water, or components that remain or become imbedded in the insoluble fibre [107].

The purpose of the research described in this chapter was to produce a set of samples and control materials that could be used throughout the research. The set of samples included both water-insoluble (substrate) and water-soluble (filtrate) samples from different pretreatment conditions across a range of pretreatment severities.

3.2 Materials and methods

3.2.1 *Pinus radiata* sawdust

Fresh radiata pine (*Pinus radiata*) sawdust was collected from McAlpine's sawmill, Rotorua, New Zealand. This mill processes logs from plantation-grown radiata pine forests having a typical rotation time of 20-25 years. The sawdust was fractionated using a vibratory Williams' chip screen (round holes) and the fraction that passed a 3.00 mm screen, but was retained on a 1.29 mm screen, was kept. The sawdust was manually debarked and stored in closed plastic bags at 4°C until required.

3.2.2 Control materials

3.2.2.1 Bleached kraft pulp (BKp)

Bleached kraft pulp (BKp) used for this study was obtained from a sheet of pressed and dried commercial *P. radiata* bleached kraft pulp. BKp (12 g) was added to water (1 L) containing sodium azide (0.01% w/*v*) and the pulp was reslushed by vigorous mixing in a pulp disintegrator for 10 minutes. The reslushed pulp was filtered with a Büchner funnel to remove excess water; two recycles of the water occurred to ensure all fines were captured. The BKp was manually fluffed followed by additional fluffing in a Ralta Kitchen Wizz containing circular prongs instead of blades. The Bkp was stored in a sealed bag at 4°C until required. The BKp had a glucose content of 83.7%.

3.2.2.2 Ball-milled control substrate (R49TBM300)

Substrate R49TBM300 from another related study was used as a control. R49TBM300 was prepared by steaming radiata pine wood chips at 172°C for 72 minutes [171]. The steamed substrate was then passed through a compression screw to remove most of the hemicellulose-rich aqueous fraction while the residual solids fraction was defibred by feeding it through a disc refiner. The fibre fraction was then diluted with water to 5% solids and treated in a tumble ball mill for 300 minutes to reduce the mean particle size. The product (R49TBM300) had a glucose content of 51.4%.

3.2.3 Chemicals

Citric acid, sulfuric acid and sodium azide were obtained from Sigma Aldrich. The commercial cellulase enzyme cocktail Cellic® CTec2 (88.5 FPU/g solution) was sourced from Novozymes (Franklinton, NC, USA). MilliQ water (18.2 MΩ) was obtained from a Sartorius Arium Pro® system.

3.2.4 Steam explosion

3.2.4.1 Sample preparation

Never-dried sawdust (300 g O.D) was prepared for steam explosion by overnight (O/N) soaking in 2.5 L of either MilliQ water or dilute acid (9 mg/mL citric acid or 7.75 mg/mL sulfuric acid). The concentrations of acid solutions were selected to ensure an acid to solid uptake of 2% *w/w*. After the overnight soak, excess water/dilute acid was removed by filtration. **Figure 3.1** is a schematic of the process.

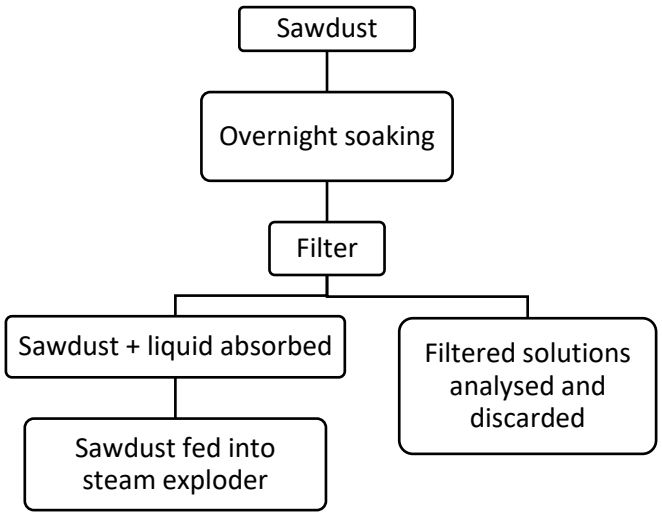


Figure 3.1 Pre-steam explosion sawdust preparation.

3.2.4.1.1 Determination of sulfuric acid concentration

The concentration of sulfuric acid in the initial and post-soaking solutions (20 mL) was determined in triplicate by titration of the with sodium hydroxide (0.1M) to pH 7, using an 888 Titrando Metrohm autotitrator. The acid uptake was calculated from the difference between the two concentrations and expressed as a percent by weight of the sawdust soaked.

3.2.4.1.2 Determination of citric acid concentration

The concentration of citric acid in pre-soaking and post-soaking solutions was determined by high performance liquid chromatography (HPLC) using a Phenomonex ROA organic acid column [172]. The column was eluted isocratically with aqueous sulfuric acid (5 mM) at 80°C and at a flow rate of 0.6 mL/min; a 20 µL injection was used. Ultra-violet detection was at a wavelength of 210 nm. The acid uptake was calculated from the difference between the two concentrations and was expressed as a percent of oven-dry sawdust.

3.2.4.2 Steam exploder mode of operation

A batch reactor with a 2 L reaction vessel was used for the steam explosion, **Figure 3.2 [82]**. The soaked sawdust was placed in the reactor vessel with the bottom exit valve shut. The upper valve was closed and the side valve from the boiler was opened. This allowed steam to enter the reaction vessel for the desired reaction time at the pre-set pressure and temperature. After the required reaction time, the bottom valve was opened allowing the steam saturated sample (slurry) to be rapidly discharged to atmospheric pressure into the cyclone. The entire steam exploded product was then collected. The container was in place throughout the process, so any liquid that leaked was also collected. Leaking tended to occur with longer treatment times.

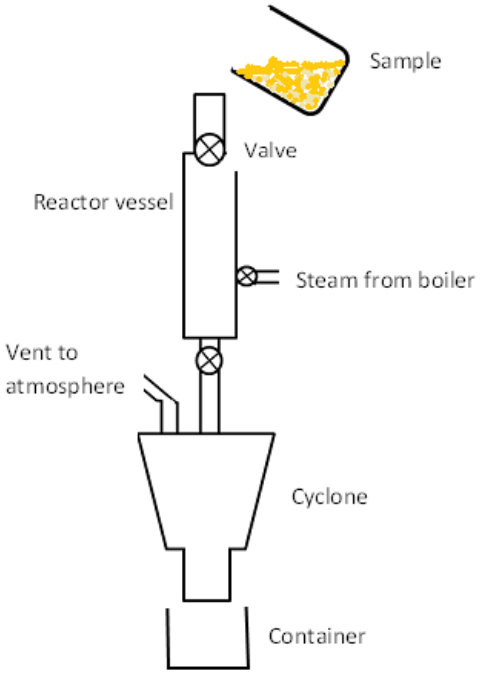


Figure 3.2 Schematic of the steam exploder set-up.

3.2.4.3 Steam pretreatment conditions

Steam explosions were performed at temperatures regarded as "high" (215°C) and "low" (180°C). Two residence times for each temperature were chosen so that both temperatures met at two different severity factors, 3.69 and 4.39. Citric acid and sulfuric acid treatments were performed at 215°C for 2 minutes, 3.69 severity factor. Duplicate steam explosions were performed for each set of conditions and each steam exploded slurry was processed separately. The steam explosion conditions are summarised in **Table 3.1**.

Table 3.1 Steam explosion conditions.

Severity factor (SF)*	Soaking liquid	Temperature (°C)	Time (min)
4.39	Water	180	108
	Water	215	10
3.69	Water	180	21.5
	Water	215	2
	Citric acid	215	2
	Sulfuric acid	215	2

*Severity factor (*SF*): [$SF = \log (t * e^{[T-100/14.75]})$]; where t = time and T = temperature

3.2.4.4 Sample recovery and processing

An overview of the post-steam explosion sample processing is shown in **Figure 3.3**.

The slurry collected from each steam explosion treatment was separated into a liquid (filtrate) and a solid fraction (substrate) by filtration using 100-micron nylon cloth on a Büchner funnel. The filtered substrate was then pressed three times (1 x 50 kN, 2 x 60 kN) to remove the excess filtrate, which was added to the initial Büchner filtrate. The resulting filtrate was stabilised against microbial deterioration by adding sodium azide (0.01% *w/w*) and stored at 4°C.

The pressed substrates were exhaustively washed to remove the water-soluble organic material. Washing was performed by suspending the substrate in water (4 L, 60°C) for 20 minutes, followed by the filtering and discarding of the water. This washing process was carried out a further three times. A sub-sample of the fourth wash-water was collected and the total organic carbon content (TOC) analysed (as described in **section 3.2.4.4.1**) to ensure adequate washing had occurred. Washing

was assumed complete when the TOC level in the wash water was less than 3% of the original TOC of the steam pretreated filtrates. The substrate remaining after washing was pressed twice (60 kN) to remove excess water, it was then thoroughly mixed and stored at 4°C.

3.2.4.4.1 Total organic carbon (TOC)

Total organic carbon (TOC) measurements were determined using a Sievers InnovOx TOC Analyser. The TOC analyser gives a TOC value as the difference when total inorganic carbon (TIC) is subtracted from the total carbon (TC) measurement (TOC = TC-TIC). The internal calibration range for TOC was between 0-1000 ppm. All samples were diluted by a factor of 100 to ensure measurements were within these limits.

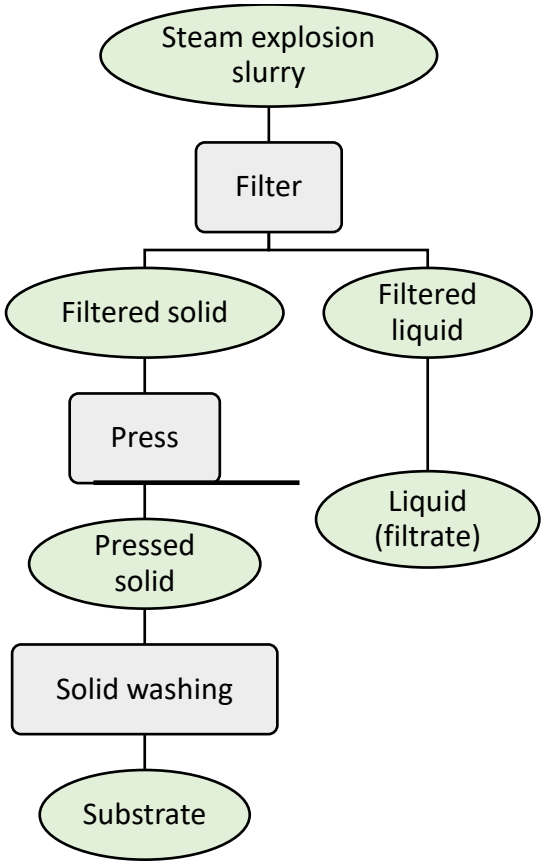


Figure 3.3 Post steam explosion sample processing overview.

3.2.5 Consistency determination

3.2.5.1 Solids and substrates

Sample was weighed into a pre-weighed and dried crucible. The crucible containing the sample was dried in an oven (104°C) overnight and the dry weight recorded. The consistency of the sample was determined as shown in Equation **3.1**.

$$\text{Consistency (\%)} = \frac{\text{Dry weight (g)}}{\text{Wet weight (g)}} \times 100 \qquad \textbf{(3.1)}$$

3.2.5.2 Filtrate

The filtrate was weighed into a pre-weighed jar and frozen at -20°C, then -80°C. The frozen filtrate was freeze-dried overnight in a LabConco Freezone® Plus 6 Liter freeze-dryer. The consistency of the filtrate was determined using Equation **3.1**.

3.2.5.3 Yield determination

The insoluble and soluble solids yields were determined according to Equation **3.2**, with the consistency of filtrate and solids being determined by Equation **3.1**. The total solids yield was calculated as the sum of the insoluble solids yield and the soluble solids yield.

$$\text{Yield (\%)} = \frac{\text{Consistency x weight sample (filtrate or substrate in g)}}{\text{Weight of sawdust steam pretreated(g)}} \times 100 \qquad \textbf{(3.2)}$$

3.2.6 Ash determination

Crucibles were cleaned and pre-conditioned by heating in a muffle furnace at 525°C for 1 hour; they were cooled in a desiccator to room temperature and weighed. Sample with a known moisture content, was placed in a pre-conditioned crucible and the wet weight of the sample was recorded. The crucible with sample was placed in the furnace and charred at 525°C for at least 2 hours to ensure that full combustion had occurred (TAPPI method T 211 om-93). The crucible was removed, cooled to room temperature in a desiccator and weighed. The ash content of the sample was represented as a percentage according to Equation **3.3**.

$$\text{Ash (\%)} = \frac{\text{Weight of ash(g)}}{\text{Oven dry (O. D.) weight of sample (g)}} \times 100 \qquad \textbf{(3.3)}$$

3.2.7 Compositional analyses

3.2.7.1 Extractives

Substrates were air-dried overnight and ground in a Wiley mill to pass a 40-mesh screen. Sub-samples (2 g air-dried) were extracted (1 h boil, 55°C, 40 min rinse), in a Soxtec apparatus (Soxtec System HT extraction unit) using dichloromethane (DCM) as the solvent. The moisture contents of the air-dried substrate were determined and used to calculate the oven-dry weight of the substrate extracted. The percent of the extractives was calculated according to Equation **3.4**. All substrates were analysed in duplicate and a quality control material of known extractives content was analysed with each batch of samples to ensure reproducible results.

$$\text{Extractives (\%)} = \frac{\text{Weight of extractives (g)}}{\text{Oven dry (O. D.) weight of sample (g)}} \times 100 \qquad \textbf{(3.4)}$$

3.2.7.2 Lignin analysis

The lignin content of solid substrates was determined following TAPPI standard methods T 222 om-88 (acid insoluble) and UM 250 (acid soluble).

Primary hydrolysis was performed by weighing the sample (~0.25 O.D. g) into a tapered Schott bottle (100 mL) and adding 72% sulfuric acid (3 mL). The Schott bottle was then placed in a water bath (30°C, 1 h) and stirred every 15 minutes. At the end of 1 hour the Schott bottle was removed and distilled water (84 mL) was added to suspend the hydrolysis reaction. Primary hydrolysis is both temperature and time dependent so when analysing a batch of samples, each sample was offset in time when placed in the water bath (e.g., 2 mins apart). A blank control sample containing no solid was run with each batch.

Secondary hydrolysis was performed by placing the Schott bottles in an autoclave at 15 psi (103 kPa, 121°C) for 1 hour. Autoclaving ensured that oligomeric sugars were fully cleaved into monomers for sugar analysis.

3.2.7.2.1 Acid insoluble lignin

Glass fibre filter papers (GC 50) were dried in an oven (105°C, 3 h), cooled in a desiccator and weighed. After secondary hydrolysis, the solution was filtered through a weighed filter paper and the filtrate was retained for acid soluble lignin and carbohydrate content analyses. The filter paper containing all unhydrolysed solids was oven-dried (105°C) overnight, cooled and weighed. The acid insoluble lignin content was calculated as shown in Equation **3.5**.

$$\text{Acid insoluble lignin (\%)} = \frac{\left(\text{Weight of lignin + filter paper (g)}\right) - \text{Weight filter paper (g)}}{\text{Oven dry (O. D.)weight of sample (g)}} \times 100 \quad \textbf{(3.5)}$$

3.2.7.2.2 Acid soluble lignin content

A 2 mL sub-sample of the filtrate retained from the secondary hydrolysis was diluted using sulfuric acid (8 mL of 3 % w/w H_2SO_4). The absorbance of the sample was measured in duplicate at 205 nm on a Shimadzu UV spectrophotometer (UV-1800) equipped with a sipper unit. All samples were corrected with a blank control. If any absorbance was greater than 0.700 a further dilution was performed. The acid soluble lignin content was calculated according to Equation **3.6**, assuming a coefficient of extinction for mature pine lignin of 110 L/g.cm [173].

$$\text{Acid soluble lignin (\%)} = \frac{\text{Dilution factor} \times \text{Volume (L)} \times 100}{\text{Coefficient of extinction} \times \text{Dry weight of sample(g)}} \quad \textbf{(3.6)}$$

3.2.7.3 Carbohydrate analysis using ion chromatography (IC)

Analysis of the five sugars in the secondary hydrolysate: L-arabinose, D-glucose, D-galactose, D-mannose and D-xylose, was performed by ion chromatography on a Dionex ICS 3000 system [174]. Samples (25 µL) were separated with a PA-10 column (300 mm) at 30°C, eluted isocratically at 1.1 mL/min with potassium hydroxide (2 mM).

A series of standards were made as follows; a sugar stock solution (100 mL, standard A) was made by weighing the necessary weight of solids in **Table 3.2**. The stock solution was subsequently diluted according to **Table 3.3**, to form a series of concentrations that were used to produce calibration curves. The standards were subjected to the hydrolysis process as in **section 3.2.7.2**.

Table 3.2 Sugar weights for stock solution of IC sugar standard.

Sugar	Weight (g)
Arabinose	0.18
Galactose	0.22
Glucose	5.17
Xylose	0.62
Mannose	1.64

Table 3.3 Sugar stock solution dilution scheme for IC sugar analysis.

	Volume of Standard A (mL)	Final volume (mL)
Standard B	20	25
Standard C	15	25
Standard D	10	25
Standard E	5	25

The carbohydrates in the filtrates and standards were analysed before (monomeric sugars) and after (total sugars) hydrolysis. Prior to IC analysis, all samples and standards were diluted 100 times and L-fucose added as an internal standard to a final fucose concentration of 5 mg/L. Samples were filtered through a 0.45 µm nylon filter before IC analysis.

The carbohydrate results were the average of duplicates that agreed within ± 5% of each other. The results were reported on an anhydro sugar-basis by using conversion factors of 0.90 and 0.88 for hexoses and pentoses, respectively. The method measured hydrolysed sugars, but conversion to anhydrous sugars was needed as some of the sugars in the filtrates/starting substrate were oligomeric.

The cellulose and hemicellulose contents were calculated according to Equations **3.7** and **3.8**, in which glucose, mannose, arabinose, xylose, galactose were measured as a percentage on an anhydro sugar-basis in the respective sample and the percent yield was the solids yield after pretreatment [175].

$$\text{Cellulose on original O.D. wood (\%)} = (\text{Glucose} - 0.27 \times \text{Mannose}) \times \%\ \text{yield} \quad (\mathbf{3.7})$$

$$\text{Hemicellulose on original O.D. wood (\%)} = (\text{Arabinose} + \text{Glucose} + \text{Galactose} + (1.1 \times \text{Xylose}) + (1.27 \times \text{Mannose})) \times \%\ \text{yield} \quad (\mathbf{3.8})$$

3.2.7.4 Carbon and Nitrogen (C and N) composition

The carbon and nitrogen contents of solids were determined by elemental analysis using a Leco TruMac CN analyser at a furnace temperature of 1250°C.

3.2.7.5 Trace element analysis

Inductively coupled plasma mass spectrometry (ICP-MS) analysis was performed using a method based on EPA method 200.8 modified for the available equipment. Samples (250 mg or 250 µL) were digested in nitric acid (4 mL of 69% HNO_3 + 1 mL peroxide 30%) using a CEM MARS 6 microwave reaction system. The resulting solution was analysed using a PerkinElmer NexION 300X ICP-MS with collision cell technology. Calibration curves were obtained by analysing standards of known concentration and using the instrument's software. There were two types of check standards; standard reference material (Scion processed pulp) and a commercial check standard solution. Elements reported were: Na, Mg, K, Ca, V, Cr, Fe, Co, Mn, Ni, Cu, As, Cd (Appendix A1).

3.2.7.6 Volatile fatty acids

Volatile fatty acids (VFA) were determined by an in-house Scion method involving pH correction with formic acid, followed by capillary gas chromatography with flame ionisation detection (GC-FID). The column used was a nitroterephthalic acid modified polyethylene glycol capillary column, DB-FFAP (250ºC, 30 m x 530 µm x 0.5 µm, ramped from 40ºC to 180ºC). Butan-1-ol solution was used as the internal standard and turbid samples were filtered through a nylon syringe filter (0.45 µm) before analysis. The method was also used to determine the levels of methanol and ethanol. The VFA results of filtrates can be found in Appendix A2.

3.2.7.7 Furfural, hydroxymethylfurfural (HMF), citric acid and acetic acid

The concentrations of furfural, hydroxymethylfurfural (HMF), acetic acid and citric acid were determined using an HPLC equipped with an Aminex HPX-87H Ion exclusion column (300 mm, 7.8 mm diameter) with 10 µL injections [176]. The column was eluted isocratically with MilliQ water containing sulfuric acid (5 mM) at a flow rate of 0.6 mL/min and at 55°C. Ultra-violet detection was at wavelengths of 210 nm for the acids and 280 nm for the aldehydes. Calibration curves were

obtained by measuring the peak areas of the four components in five different standards of known concentrations. A control with known concentrations was analysed in parallel with the samples.

3.2.8 **Enzyme hydrolysis**

Enzyme hydrolysis of never-dried substrates was carried out at 1.5% *w/v* solids loading in a citrate buffer (50 mM) at pH 4.5 in an incubator (50°C) with constant mixing (rotary shaker, 180RPM) [162]. Enzyme digestions were performed at both a low and high enzyme loading; 2.5 FPU/g substrate and 15 FPU/g substrate. All enzyme digestions were performed using Novozymes Cellic® CTec2 enzyme cocktail for 72 h, unless otherwise stated. Enzyme digestion was stopped by plunging tubes into a boiling water bath and the mixture was then centrifuged (4000 RPM, 10 min, 25ºC). The glucose concentration in the supernatant was determined using a YSI 2700 glucose analyser and the degree of conversion of glucan to glucose was calculated (digestibility (%)) [162]. The values were corrected for the presence of glucose in the enzyme cocktail as well as for glucose released from the undigested substrate. Each batch of enzyme hydrolysed samples included a control substrate (R49TBM300) with a known glucose yield. The control result had to be within ± 5 % of expected value(s) for results from the batch to be accepted.

Statistical analysis in the form of a Student T-test with 95% confidence interval was performed on all samples that were enzymatically hydrolysed in order to ensure they were significantly different to the control of sample with which they were compared. Figure captions state what the error bars represent, which is usually ± two standard deviations of a triplicate analysis, unless otherwise stated.

3.3 Results and discussion

P. radiata wood (< 3 mm, 300 g O.D.) was treated and steam exploded in duplicate using six different conditions, **Table 3.4**. Steam pretreatments without added acid catalyst were compared at combined severity factors of -2.61 and -3.31 [88]. At each CSF, the treatments were carried out both at 215°C for short times and at 180°C for longer times. This allowed the effects of pretreatment severity, temperature and time to be isolated and analysed. Pretreatments with added acid catalyst, both organic and inorganic, are considerably more severe than uncatalysed pretreatments at the same temperature/time due to the lower pH during pretreatment. To expand the range of CSF's for the study and to understand the impact of acid catalysts, additional steam pretreatments were conducted at 215°C for 2 minutes in the presence of 2% *w/w* citric acid or sulfuric acid. These correspond to CSF levels of 1.57 and 2.67, respectively.

Table 3.4 Steam pretreatment conditions and yield recoveries after pretreatment.

Sample #	Catalyst	Temperature (°C)	Time (min)	Combined severity factor (CSF)[a]	Yield (%)[b]		
					Insoluble	Soluble	Total
1 (Sawdust)					100		100
2	Water	215	2	-3.31	83.3, 81.7	9.5, 9.6	92.8, 91.9
3	Water	180	21.5	-3.31	77.3, 79.9	8.5, 5.9	85.8, 85.8
4	Water	215	10	-2.61	71.1, 75.8	8.1, 9.1	79.3, 84.9
5	Water	180	108	-2.61	69.9, 70.4	3.2, 4.2	73.1, 74.7
6	Citric acid	215	2	1.57	72.7, 74.9	18.0, 17.2	90.7, 92.1
7	Sulfuric acid	215	2	2.67	64.4, 63.3	21.5, 20.9	86.0, 84.2

[a] Using pH values: Water 7, Citric acid 2.12, Sulfuric acid 1.02.

[b] Yields are on a *w/w* basis of original wood and duplicates are represented as individual values.

3.3.1 Effect of pretreatment conditions on yield, carbohydrate and lignin content

Steam pretreatment of sawdust resulted in partial dissolution of the wood mass (**Table 3.4**). In addition to the dissolution of carbohydrate oligomers and monomers, lignin and extractives, a series of secondary reactions can occur involving depolymerisation, condensation, decomposition and rearrangements of wood components. These reactions are all driven by the high temperatures and the increasing acidity from the hydrolysis of the acetyl groups [90,94,95,177]. In

addition, these reactions proceed at different rates so that the proportions of soluble and insoluble fractions, and their chemical compositions vary depending on the severity of the steaming stage.

Insoluble solid yields from the six pretreatments ranged from 63% to 83% of the starting mass (**Table 3.4**), with more severe pretreatments tending to give lower solids yields. For pretreatments without added acid catalysts, the insoluble solid yields decreased from 81% to 72% as the CSF increased from -3.31 to -2.61. At a given CSF, yields were lower for the low temperature/long time pretreatments than for the high temperature/short time pretreatments. This difference could possibly be due to the different activation energies required for each degradation reaction and/or a heating time lag in the short duration runs [82]. As expected, due to the higher CSF, addition of an acid catalyst resulted in a substantial increase in dissolution and a corresponding decrease in the insoluble solids yield [82,83]. The insoluble solids yield for the citric acid and sulfuric acid catalysed pretreatments were 74% and 64%, respectively.

Plots of the cellulose, lignin and hemicelluloses remaining in the insoluble solids after pretreatment show that the decrease in solid yields as CSF increases can be mainly attributed to dissolution of hemicelluloses from the wood (**Figure 3.4**). As the pretreatment severity increased, the total carbohydrate content of the pretreated substrates decreased (**Table 3.5**). For example, the total carbohydrate content decreased from 67.5 g/100 g in untreated pine sawdust to between 56.5 and 36.4 g/100 g for the pretreated substrates. More severe pretreatments resulted in greater carbohydrate removal; the percentage hemicellulose removed from substrates increased from 47% to 99% as pretreatment severity increased. This trend is consistent with Clarke and Mackie's studies, where it was found the more severe pretreatment conditions removed more carbohydrates from steam pretreated *P. radiata* [82,83]. There were also small amounts of cellulose and lignin removed from the substrates as the pretreatment severity increased, with the percent losses being 1% to 16% for cellulose and 7% to 19% for lignin.

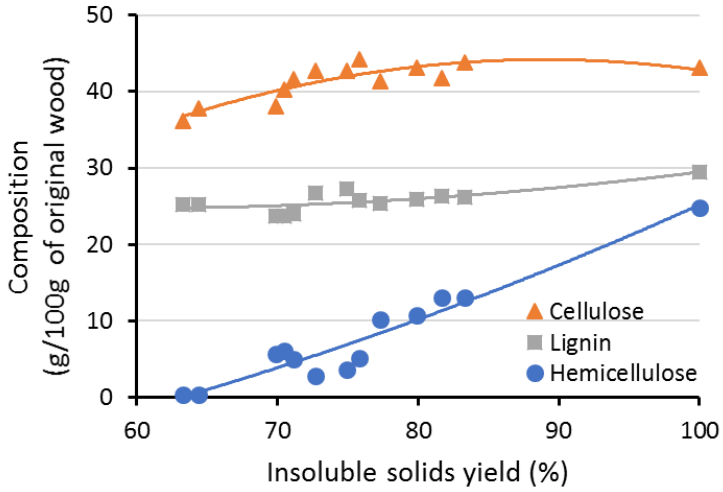


Figure 3.4 Comparison of cellulose, lignin and hemicellulose levels versus insoluble solids yield of pretreated substrates. Steam pretreated experimental duplicates represented as individual points.

The two major hemicelluloses in softwoods, galactoglucomannan and arabinoglucuronoxylan were substantially removed (solubilised and degraded) during pretreatment as supported by the significant decreases in arabinose, galactose, glucose, mannose and xylose contents of the substrates after pretreatment (**Table 3.5**). These decreases were mirrored by increases in these sugars in the corresponding filtrates (**Table 3.6**). The changes are measured as total sugar concentrations, inclusive of both free and oligomeric sugars.

The levels of hemicellulose and cellulose removed from the insoluble solid fractions were greater than the levels of corresponding sugars in the filtrates, indicating that some sugars were lost and/or degraded during the steam explosion pretreatment. Unaccounted mass was in a 7% to 27% range for uncatalysed pretreatments; the unaccounted mass increased as pretreatment severity increased and was highest for long duration pretreatments (**Table 3.4**). Addition of 2% citric acid did not result in higher unaccounted mass, although the severity was substantially greater. However, 2% sulfuric acid addition did increase mass loss relative to the equivalent uncatalysed pretreatment mass. Consistent with this, total recovered solid yields after steam explosion varied between 73% and 93%, depending on the conditions. The mass losses reported here were consistent with those reported previously [82,178].

Also consistent with carbohydrate degradation, low levels of two carbohydrate degradation products, furfural and hydroxymethylfurfural (HMF) were found in the filtrates (**Table 3.6**). Although these two furans are well-known products of carbohydrate degradation under acidic conditions [179], they are both volatile and reactive under these conditions [179,180]. It is anticipated some of the volatile components may have also been lost to the atmosphere when sawdust was exploded into the cyclone.

Table 3.5 Adjusted compositions (g/100 g of original wood) of untreated sawdust and the insoluble solids of steam pretreated *Pinus radiata*.[a]

Pretreatment Conditions	(g/100 g starting material)					Carbohydrate (g/100 g of original wood)[c]						
	Acid soluble lignin	Acid insoluble lignin	Total Lignin[b]	Extractives	Ash	Arabinose	Galactose	Glucose	Xylose	Mannose	Cellulose	Hemicellulose
Untreated sawdust	0.44	29.0	29.4	0.64	0.39	1.50	3.16	46.0	5.98	10.7	43.1	24.9
215°C/2 min Water	0.35, 0.36	25.8, 26.0	26.2, 26.3	0.37, 0.35	0.17, 0.18	0.25, 0.25	1.42, 1.41	45.4, 43.4	3.76, 3.71	5.71, 5.71	43.8, 41.8	13.1, 13.0
180°C/21.5 min Water	0.30, 0.34	25.0, 25.5	25.4, 25.9	0.45, 0.43	0.19, 0.18	0.29, 0.35	0.80, 0.88	42.6, 44.4	3.29, 3.40	4.30, 4.53	41.4, 43.2	10.2, 10.7
215°C/10 min Water	0.24, 0.26	23.8, 25.5	24.0, 25.7	0.71, 0.88	0.13, 0.13	0.04, 0.04	0.38, 0.37	42.2, 44.8	1.84, 1.86	1.98, 2.10	41.6, 44.3	4.95, 5.13
180°C/108 min Water	0.25, 0.25	23.5, 23.5	23.7, 23.8	0.43, 0.44	0.14, 0.14	0.10, 0.11	0.40, 0.47	38.8, 40.9	1.87, 2.01	2.44, 2.59	38.1, 40.2	5.64, 6.06
215°C/2 min Citric acid	0.29, 0.32	26.6 27.0	26.7, 27.3	0.70, 0.71	0.12, 0.12	0.04, 0.09	0.23, 0.29	42.9, 43.0	1.49, 1.69	0.69, 1.09	42.7, 42.7	2.79, 3.63
215°C/2 min Sulfuric acid	0.23, 0.25	25.0, 25.0	25.3, 25.3	1.36, 1.52	0.10, 0.11	0.03, 0.03	0.01, 0.01	37.8, 36.1	0.26, 0.25	0.00, 0.00	37.8, 36.1	0.32, 0.32

[a] Duplicate steam pretreatments represented as individual values.

[b]Total lignin = acid soluble lignin + acid insoluble lignin.

[c]Anhydro sugar units.

Table 3.6 Adjusted compositions of filtrates as g/100 g of original wood.[a]

Pretreatment conditions	Carbohydrate (g/100 g of original wood)[b]								Furans (mg/100 g original wood)	
	Arabinose	Galactose	Glucose	Xylose	Mannose	Total sugars	Cellulose	Hemicellulose	Furfural[c]	HMF[c]
215°C/2 min Water	0.88, 0.87	1.16, 1.14	1.31, 1.27	1.99, 1.93	4.17, 4.02	9.66, 9.37	0.19, 0.18	9.52, 9.23	12.1, 12.5	8.40, 6.70
180°C/21.5 min Water	0.55, 0.49	1.05, 1.09	0.97, 1.01	1.45, 1.53	3.02, 3.17	7.15, 7.40	0.15, 0.15	7.03, 7.29	23.7, 23.9	15.1, 16.9
215°C/10 min Water	0.47, 0.47	1.01, 1.06	1.26, 1.44	1.58, 1.54	3.30, 3.55	7.74, 8.19	0.37, 0.48	7.41, 7.75	84.3, 93.1	105, 98.2
180°C/108 min Water	0.44, 0.47	0.52, 0.60	0.43, 0.56	0.70, 0.84	1.11, 1.46	3.27, 4.01	0.13, 0.16	3.14, 3.85	15.7, 19.7	23.0, 29.5
215°C/2 min Citric acid	1.08, 1.11	1.99, 1.99	2.60, 2.46	3.66, 3.50	7.49, 7.37	17.1, 16.7	0.58, 0.46	16.6, 16.3	30.8, 24.5	48.4, 32.3
215°C/2 min Sulfuric acid	0.93, 0.85	1.89, 1.74	5.46, 5.34	3.56, 3.23	7.11, 6.38	19.2, 17.8	3.54, 3.62	15.8, 14.2	214, 230	385, 420

[a] Duplicate steam pretreatments are represented as individual values.
[b] Sugars are reported as total anhydro units from free and oligomeric sugars.

3.3.2 Additional compositional analysis

In addition to the compositional data above, trace elements, volatile fatty acids, citric and acetic acids were determined (Appendices A1 and A2).

3.3.3 Effect of pretreatment conditions on cellulose digestibility

The digestibility as measured by percentage glucan conversion of the pretreated substrates was assessed at both a low and a high enzyme dose: 2.5 FPU/g substrate and 15 FPU/g substrate. Two enzyme loadings were used to increase the possibility of observing the effects of enzyme inhibition. Although the higher enzyme dose (15 FPU/g) gave sugar yields of greater practical interest (5% to 25% compared to < 8%), treatments at low enzyme doses may be more affected by non-productive binding of the enzymes to the lignin or other inhibitors, due to the lower enzyme to inhibitor ratio.

The digestibility of the pretreated substrates increased as pretreatment severity increased for both enzyme doses evaluated (**Figure 3.5**). This was consistent with previous research on softwoods [81,181]. As expected, the glucose yields from the untreated radiata pine were low, 3.4% and 5.3% for the 2.5 FPU/g and 15 FPU/g enzyme doses, respectively. Pretreatment increased glucose yields up to 5.4% for the low enzyme dose and 19.9% for the high enzyme doses (**Figure 3.5**).

In the absence of acid catalysts, the digestibility of solids pretreated at the same severity but under different conditions showed similar percentage levels of digestibility (**Figure 3.5**). This is consistent with previous research [88]. The digestibilities of substrates from both of the low severity conditions at 215°C/2 min and 180°C/21.5 min (-3.31 CSF) were ~3.6% and 10% for 2.5 and 15 FPU/g substrate, respectively (**Figure 3.5**).

Additionally, the substrates from the higher severity factor, -2.61 CSF, but from the two different pretreatment conditions 215°C/10 min and 180°C/108 min also gave similar percentage levels of digestibility at the low enzyme loading of 2.5 FPU/g ~4.3%. However, the digestibilities at the higher enzyme loading of 15 FPU/g differed slightly and were ~15% and ~17% for 215°C/10 min and 180°C/108 min, respectively (**Figure 3.5**).

Addition of 2% citric acid or sulfuric acid prior to steam pretreatment at 215°C/2 min increased the digestibility of the pretreated substrates in comparison with the substrate pretreated without added acid catalyst. The higher the CSF for the pretreatment, the higher the digestibility observed. Thus, the order of digestibility for both low and high enzyme doses was; sulfuric acid > citric acid > water (**Figure 3.5**). The higher digestibilities were consistent with more severe pretreatments removing more hemicelluloses and thus improving the enzymes access to the cellulose. Consistent with the higher digestibility of substrates being associated with preferential removal of hemicelluloses, the digestibility of solids increased as the insoluble solid yield decreased (**Figure 3.6A**), and as the proportion of lignin remaining in the insoluble solid increased (**Figure 3.6B**).

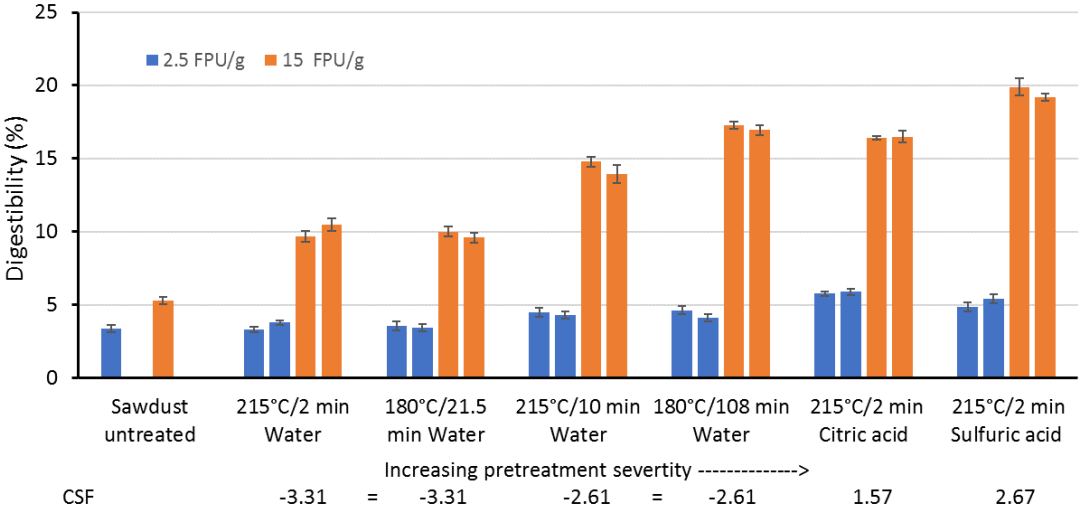


Figure 3.5 Digestibility as measured by percentage glucan conversion of steam pretreated substrates using Cellic® CTec2 at both 2.5 FPU/g and 15 FPU/g (Filter paper units/gram substrate). Triplicate determinations of steam pretreated experimental duplicates, the latter represented as individual bars, error bars represent ± two standard deviations.

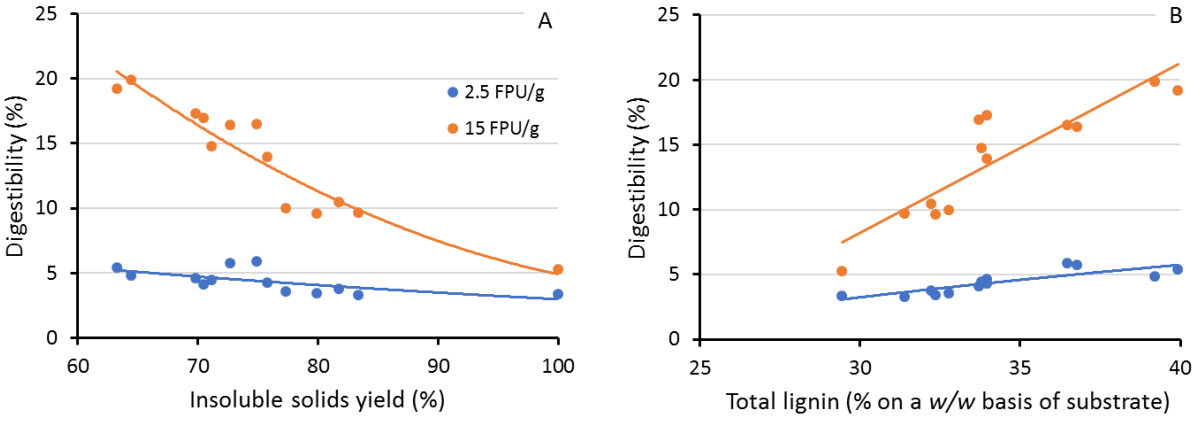


Figure 3.6 Digestibility of steam pretreated substrates versus: insoluble solids yield (A); total lignin (B). For exact yield and lignin values, refer to Tables 3.4 and 3.5. Steam pretreated experimental duplicates represented as individual points.

3.4 Summary

Twenty-five samples were prepared for use throughout this research. The samples included thirteen solid substrates: untreated *Pinus radiata* sawdust and the duplicate steam exploded *P. radiata* samples from the six different pretreatment conditions. The twelve filtrates containing the water-soluble components produced during steam pretreatment were also isolated. The pretreatment conditions used to produce samples covered a range of pretreatment severities, allowing for the effects of temperature, time and acid catalysts to be determined (for conditions see **Table 3.1**).

The chemical compositions of both the substrates and filtrates were determined. The effects of pretreatment conditions on solid yields, carbohydrate and lignin contents were assessed. The general observation was that more severe pretreatment conditions resulted in lower insoluble and total yields. This observation was attributed to the fact that more severe pretreatment conditions lead to greater hemicellulose solubilisation/degradation, as reflected in the carbohydrate compositions of both the substrates and filtrates.

The enzyme digestibility of the steam pretreated substrates was determined by treating them with two different enzyme loadings of the CTec2 enzyme cocktail.
It was found that pretreatment conditions of lower temperatures for longer time periods were more beneficial than high temperatures for short times, as they gave higher digestibilities.

4 Method development for determining cellulose accessibility

4.1 Introduction

4.1.1 Factors affecting enzyme hydrolysis

Several substrate properties are known to influence the rate and/or extent of enzymatic hydrolysis of lignocellulosic biomass [5,149]. These include: cellulose accessibility, particle size, pore volume, cellulose crystallinity, hemicellulose and lignin content of substrates.

This study aimed to evaluate the relative importance of factors influencing the digestibility of steam pretreated radiata pine, in particular, the effect of pretreatment severity on the balance between increasing cellulose accessibility and inhibitor formation. It was therefore vital that a robust and reliable method was available to evaluate the cellulose accessibility. This chapter presents modifications made to the Simons' stain procedure to ensure a suitable method was available for evaluating the cellulose accessibility of the various samples used in this study.

4.1.2 Analytical techniques used to assess cellulose accessibility

Various analytical techniques are available to assess the relative accessibility that cellulase enzymes have to the cellulose in substrates. They are based on determining pore size/volume or measuring the accessibility that molecular probes of similar size to cellulase enzymes, have to the cellulose in a substrate. Each of the techniques has its own inherent advantages and disadvantages, as summarised in **Table 4.1**.

Table 4.1 Analytical techniques for determining cellulose accessibility of substrates and their inherent advantages and disadvantages (Table based on data provided in [182,183]).

Technique	Principle	Advantages	Disadvantages	Ref
Simons' stain	Two dyes of different colour (blue and orange), molecular size and cellulose binding are adsorbed onto substrates. The adsorbed amount is determined by Langmuir adsorption equations. The maximum amounts and ratios of the two dyes gives an estimation of the relative porosity and overall cellulose accessibility.	• Fast, simple and sensitive • Measures both exterior and interior surface areas • Wet samples can be used	• Affected by pore shape and tortuosity • Semi-quantitative	[184,185]
Nitrogen adsorption	The quantity of nitrogen adsorbed onto substrate surfaces and within the pores of substrate is determined and used to calculate surface area by the Brunauer-Emmett-Teller (BET) model.	• Highly precise and fast	• Partial irreversible collapse of pores • May overestimate cellulose accessibility • Substrate needs to be dried • Actual inner pore size is not determined	[186,187]
Mercury porosimetry	Similar to nitrogen adsorption with applied pressure and pore volumes being calculated by the Washburn equation.	• Wet substrates can be used • Gives lots of information: total pore volume, pore size distribution, surface area permeability, fractal dimensions, etc	• Requires the use of mercury	[188,189]
Solute exclusion	Accessibility of probe molecules of different sizes to substrate pores determined and volume distribution calculated using the concentration of a set of solute solutions with various molecular sizes.	• Relatively simple procedure • Can use a wide range of solute sizes	• Laborious • Not specific to cellulose • Not fully quantitative • Does not account for external surface area • May be affected by pore shape • Not acceptable for absolute pore size and volume distribution	[190-192]
Protein adsorption	Cellulase accessibility to cellulose quantified by Langmuir adsorption of a fusion protein containing a green fluorescent protein and cellulose binding module (CBM).	• Use of CBM makes it ideal for enzymatic hydrolysis	• Total exposed surface area to probe molecules includes non-cellulosic surfaces, e.g. lignin.	[192]

Technique	Principle	Advantages	Disadvantages	Ref
NMR Cryoprometry	Cryoporometry takes advantage of the fact that small crystals formed from liquid within pores melt at a lower temperature than bulk liquid known as melting point depression caused by enthalpic interaction with the pore surface. Hydrated samples are cooled to negative temperature to completely freeze all the adsorbed water, and the intensity of the NMR signal which represents the amount of unfrozen water at a specific temperature is measured by a Carr–Purcell–Meiboom–Gill sequence (CPMG) as temperature increases to generate the melting curves. The melting point depression of the liquid can be related to the pore size through the Gibbs–Thompson equation	• Non-destructive and quantitative determination of pore size distribution • Measurement can be done in wet state	• Pore size determination range is limited by the temperature control • Expensive • Requires complicated set-up and long experiment time	[183]
NMR Relaxometry	NMR relaxometry provides information pertaining to the molecular mobility within a porous system. The spin–spin (T2) relaxation curve obtained via a CPMG sequence is used to investigate the changes in biomass–water interactions and subsequent accessibility. The degrees of freedom of water in the pores increase as the T2 relaxation time increases, causing a decrease in the proportion of amount of water located at pore surface versus the pore interior. Therefore, in systems of increasing average pore size, the pore surface area to volume ratio will decrease and is therefore detected by an increase in the T2 relaxation time. It is also well known that liquid molecules near a solid surface will have different spin–lattice (T1) relaxation profiles from that of the bulk liquid because of the interactions at the solid–liquid interface. As a result, the observed average T1 time of the adsorbed water could also reflect the surface area to volume ratio of the pores	• Non-destructive measurement • Not affected by pore inlet size or shape	• Expensive • Requires complicated equipment set-up	[183]

4.1.3 Simons' stain test and principles of action

After assessing the advantages, disadvantages and potential application to samples to be used in this study, the analytical technique chosen for this study to determine cellulose was the Simons' stain test. The most compelling reason for this decision was that the Simons' stain test can be used on wet, never-dried substrates, which is especially important for the lignocellulosic samples because they can undergo significant structural changes upon drying [193,194].

The Simons' stain test is a relatively simple semi-quantitative procedure based on the competitive adsorption of two different dyes, orange and blue (O and B) to substrates.

When a substrate is stained with a mixture of the two dyes, the larger orange dye molecules only penetrate larger pores in the substrate, whereas the smaller blue dye molecules can access all pores. If the orange dye is able to access pores, it can displace/replace the smaller blue dye molecules due to its higher affinity for the hydroxyl groups on the cellulose [194,195]. The dyes bond to cellulose *via* hydrogen bonding between the polar groups on the dye molecules and the hydroxyl groups on the cellulose [185,196,197]. The Simons' stain test has been shown to be a good indicator of the distribution of small and large pores in various substrates independent of the substrate type [198].

4.1.3.1 Simons' stain test and interpretation of results

The Simons' stain test has been modified over time to improve the throughput of samples, as well as the reproducibility and accuracy of results.

The blue dye used had a well-defined chemical formula ($C_{34}H_{24}N_6Na_4O_{16}S_4$, Mr = 992.82), whereas the orange dye was a condensation product of 5-nitro-*O*-toluenesulfonic acid in aqueous alkali, and it did not have a well-defined chemical formula or structure. The original method used orange dye as received; however, the low molecular weight fraction of the orange dye has a similar affinity to cellulose as the blue dye, meaning that fractionation and isolation of the high molecular weight fraction of orange dye is essential [195,198].

The original method used a 1:1 ratio of orange and blue dyes [198-200]. A single 25 mg sample was treated and a lengthy procedure that involved adsorption of the dye followed by a pyridine stripping step prior to the determination of the maximum adsorbed orange and blue dye was used. A 1:5 ratio of orange and blue dyes was trialled [194,195], but no significant advantage over the 1:1 ratio was reported, other than ensuring that the blue dye always reached maximum adsorption in the case of samples that had very few large pores.

The biggest advances in efficiency of the Simons' stain test occurred when Chandra *et al*. [193] developed the modified Simons' stain test. The modified test stains a substantially larger amount of sample (100 mg), with a series of 6 samples being needed per result, that is a total sample size of 600 mg compared to 25 mg for the original method [193]. Increasing volumes (0.25, 0.50, 0.75, 1.0 1.5 and 2.0 mL) of both the orange and blue dyes (10 mg/mL) are added to each sample in the series, thus creating a set of samples with a 1:1 mixture of orange and blue dyes at increasing concentrations. The incubation time decreased from 48 hours to 6 hours, and the hazardous step of pyridine stripping was removed [193]. The amount of dye adsorbed onto each substrate was determined by calculating the difference in the concentration of the initial dye added and the concentration of the dye in the supernatant assuming Beer-Lambert law [194]. The required extinction coefficients were calculated by preparing standard calibration curves of each dye, and measuring the slopes of their absorbance at wavelengths of both 425 nm and 620 nm. Langmuir adsorption isotherm equations were used to determine the maximum amounts of blue and orange dye adsorbed, and consequently the total dye adsorbed and the ratio of orange to blue (O:B) dye adsorbed. This modified procedure gave results for the same parameters as the original method, i.e., maximum adsorbed blue and orange dyes and consequently the O:B ratio.

4.1.3.2 **Best Simons' stain parameter for predicting digestibility**

The ability of the Simons' stain test to indicate the ease with which lignocellulosic materials can be enzymatically hydrolysed has been investigated. The cellulose accessibility of a substrate as measured by dye adsorption is indicative of the potential levels of hydrolysis, with cellulase accessibility to smaller pores being responsible for higher levels of hydrolysis [185,190,193,194,201-203].

Attempts to correlate Simons' stain results with cellulose digestibility have to date used a variety of parameters: maximum orange dye adsorbed; total maximum dye adsorbed (blue plus orange); and the ratio of orange to blue (O:B) dyes adsorbed. It has been shown that the total dye adsorbed is a good indicator of hydrolysis; however, this was in a study where a diverse set of substrates from different pretreatment types was used [193]. The total orange dye adsorbed or the O:B ratio are the more frequently used options to predict accessibility for hydrolysis [185,193,194,201].

Interpretation and comparison of Simons' stain results reported in the literature needs to be undertaken with caution because of the wide range of substrate types and pretreatments, as well as the differing methodologies for staining the substrates that may have been implemented. Additionally, the way in which the staining results are used to predict digestibility may also differ. For example, maximum orange dye, maximum total dye or O:B dye ratio are each used to predict digestibility. The crucial filtration of the orange dye also leads to significant experimental differences and makes comparisons of studies difficult [185]. Thus, a recommendation is to select one methodology and only make comparisons within a study, not between studies.

4.2 Materials and methods

4.2.1 Chemicals

Direct blue dye (Chicago sky blue 6B) was obtained from Sigma Aldrich and direct orange dye (pontamine fast orange 6RN) was obtained from Pylam Products Co. Inc (Garden City, NY). MilliQ water (18.2 MΩ) was obtained from a Sartorius Arium Pro® system.

4.2.2 Substrates

Bleached kraft pulp (BKp) was prepared as per **section 3.2.2.1**. Steam exploded samples were prepared as per **section 3.2.4**.

4.2.3 Ultra-visible (UV) spectrophotometry

4.2.3.1 Simons' stain test measurements

A Shimadzu UV spectrophotometer UV-1800 equipped with a sipper unit was used to determine absorbance measurements at 425 nm and 620 nm. These wavelengths correspond to the maximum absorbance wavelength of the orange and blue dyes, respectively.

4.2.4 Ball-milling (BM)

Ball-milling was performed for the desired time(s) in a Schwingmühle VIBRATOM vibratory ball-mill by treating samples (6 O.D g, 5% *w/v*, 0.01% *w/v* sodium azide) in a ceramic vessel (0.6 L) containing two hundred 15 mm alumina balls. All composition and digestion values of ball-milled samples were corrected for ash content.

4.2.5 Ash determination

The ash content of samples was determined as described in **section 3.2.6**.

4.3 Results and discussion

4.3.1 Final version of Simons' stain test

The final Simons' staining procedure used in this study was based on modifications of the method developed by Chandra et al. [193]; all modifications made are justified in the sections that follow.

The fractionation of the orange dye was based on the procedure described by Esteghalian *et al*. [194]. A 1% *w/v* solution of orange dye was filtered under nitrogen pressure (28 PSI) through a 100 kDa ultrafiltration membrane using an Amicon ultrafiltration apparatus. The orange dye solution (1% *w/v*) was ultrafiltered until about 20% of the original volume remained. To determine the concentration of the remaining dye fraction, a known volume (1.0 mL) was freeze-dried overnight and the weight of the solid residue was measured. This value was then used to dilute the highly concentrated dye fraction to the concentration required for the staining procedure (10 mg/mL). All samples subjected to the

Simons' stain test were analysed in duplicate using the same batch of filtered orange dye to reduce experimental error [185,193].

Substrate (200 mg, O.D.) was weighed into vials (six per series) and 1 mL of phosphate buffered saline solution (pH 6, 0.3 M PO_4^{3-}, 1.4 mM NaCl) was added to each. To each series of six vials, both orange (stock concentration = 10 mg/mL) and blue (stock concentration = 10 mg/mL) dye solutions were added in a series of increasing volumes (0.25, 0.5, 0.75, 1, 1.5, 2 mL). The volume was made up to 10 mL with distilled water and vials were incubated (65°C, 16 h) with continual shaking (180 RPM). After incubation, the vials were centrifuged (4000 RPM, 10 min) and the absorbance of the supernatant was measured after appropriate dilution, at both 425 nm and 620 nm. The amount of dye adsorbed onto samples was determined as the difference in concentration of the original dye added and the dye concentration in the supernatant using the Beer-Lambert law [194]. The extinction coefficients calculated and used in this study were εO425 = 52.07, εO620 = 0.12, εB425 = 7.59 and εB620 = 81.18 L/g cm. Langmuir adsorption isotherms were used to determine adsorption parameters.
The Langmuir expression is represented by the equation:

$$QD=(C_mK[D])/(1+K[D])$$

Where QD is equal to the quantity of the dye adsorbed in mg/g sample; C_m is the maximum quantity of dye adsorbed in mg/g sample; K is the constant of the Langmuir adsorption, and; [D] is the dye concentration equilibrium in mg/mL [193]. Adsorption isotherms are obtained by plotting the concentration of dye adsorbed (mg/g sample) against the free dye (mg/mL) for the six points in the series of samples. The isotherm is then linearised by plotting [D]/QD against [D], which allows for the parameters K and Cm to be determined, with K = slope/intercept and the maximum adsorbed dye (C_m) in mg/g [204]. The maximum orange and blue dye adsorbed then allows for an orange to blue (O:B) dye ratio to be obtained.

4.3.2 Absorbance maxima of orange and blue dyes

Solutions of orange dye, blue dye and mixtures of both orange and blue dyes at varying concentrations were analysed by UV spectrophotometry. The measured absorbance maxima of the dyes used in this study were 425 nm and 620 nm, for the orange and blue dyes, respectively. These vales differ from absorbance maxima reported in the literature; of 455 nm for the orange dye, and 624 nm for the blue dye [193,194]. The difference in absorbance maximum for the orange dye was most likely due to the age of the orange dye used in the study. The solutions of different concentrations were used to make calibration curves and extinction coefficients were calculated from the slope of the calibration curves for both dyes at 425 nm and 620 nm.

4.3.3 Temperature of the staining procedure

The effect of temperature on the Simons' stain test had previously been reported [193,205-207]. At lower temperatures (30 to 40ºC), greater amounts of dye can adsorb onto substrates because some of the dye is present as aggregates and not as separate molecules, but at higher temperatures this aggregation is not present [193,205]. To avoid dye aggregation during the test, the temperature is usually held at 70ºC. However, in the current study, the highest stable temperature was 65ºC, so all staining treatments were performed at 65ºC.

4.3.4 Time of staining procedure

The original Simons' staining method required an incubation time of 48 hours for sufficient substrate staining to occur [194,198]. However, the method reported by Chandra *et al*. [193] increased sample throughput by reducing incubation time to 6 hours, as based on conditions tested by Inglesby and Zeronian [206,207]. In this study, for ease of sample preparation and prompt UV analysis of samples, the incubation time was set to 18 hours.

4.3.5 Stability of orange and blue dyes

4.3.5.1 Blue dye

The stability of blue dye solutions and preparation reproducibility were checked by preparing a series of standards (5 concentrations) from a stock solution of blue dye (10 mg/mL). The UV absorbance of the individual standards in the series were measured after 0, 1, 2 and 5 days. The calibration slopes from plotting the UV absorbance against dye concentration for the standard sets did not vary significantly from day to day, with the coefficient of variance being 0.38% and 0.42% for 425 nm and 620 nm wavelengths, respectively (**Table 4.2**). This indicated the dye was stable over time. This result meant there was no urgency to analyse samples immediately.

The single batch variation when preparing multiple sets of calibration standards from the same set of dye (**Table 4.4**), and batch to batch variation of calibration curves from new stock solutions of the blue dye (**Table 4.3**), were also assessed. The batch to batch coefficients of variation were 0.78% and 0.82% for 425 nm and 620 nm, respectively. These values were larger than those observed for single batch variation; the coefficients of variation were 0.43% and 0.54% for 425 nm and 620 nm, respectively. The reason for higher errors was most likely due to additional weighing and pipetting errors. Batch to batch variation was likely traced to the weighing of the solid dye. Therefore, to minimise experimental error, one batch of blue dye was used for all samples analysed.

Table 4.2 Calibration slope values for a set of standards over a period of 5 days.

	Calibration slope at wavelength (nm)	
	425	**620**
Day 0	7.40	80.7
Day 1	7.44	80.7
Day 2	7.38	80.6
Day 5	7.38	80.0
σ	0.03	0.34

Table 4.3 Calibration slope values for different batches of blue dye used, determination of batch to batch variation.

	Calibration slope at wavelength (nm)	
	425	**620**
Batch 1	7.39	81.3
Batch 2	7.48	81.0
Batch 3	7.45	80.0
Batch 4	7.53	79.9
σ	0.06	0.66

Table 4.4 Calibration slope values for determining variation within a batch of dye.

	Calibration slope at wavelength (nm)	
	425	**620**
1	7.38	80.0
2	7.38	80.2
3	7.45	80.9
4	7.39	80.7
σ	0.03	0.43

4.3.5.2 **Orange dye**

The orange dye was comprised of molecules across a range of molecular weights. It is now standard practice to fractionate the dye and only use the high molecular weight fraction that has been proven to have increased affinity for cellulose [198]. The fractionation procedure for the orange dye was simple [185], yet because the filtration was stipulated to continue until approximately 20% of original volume of the 1% *w/v* solution remained, there was considerable chance for variation in the final percentage of dye retained and therefore the molecular weight range. Therefore, it was not surprising that the batch to batch variation for the orange dye used for staining (**Table 4.5**) was considerably larger than that previously observed for the blue dye; coefficients of variation for the orange dye were 8.4% and 13.1% for 425 nm and 620 nm, respectively, compared to blue dye values of 0.78% and 0.82%. The coefficients of variation (COV's) for the calibration slopes for four different sets of samples diluted from the same batch of filtered orange dye were found to be 5.8% and 5.2% (**Table 4.6**). These values were considerably lower than the COV's for batch to batch variation (**Table 4.5**).

The variation for orange dye from day to day, was not useful to measure owing to the instability and aggregation of the orange dye which visibly occurred at room temperature, even over a short time period of 2 days.

Table 4.5 Calibration slope values for different batches of orange dye used, determination of batch to batch variation.

	Calibration slope at wavelength (nm)	
	425	**620**
Batch 1	50.2	0.12
Batch 2	59.7	0.10
Batch 3	49.8	0.13
Batch 4	53.4	0.09
Batch 5	49.2	0.10
σ	4.39	0.01

Table 4.6 Calibration slope values for determining variation within a batch of orange dye.

	Calibration slope at wavelength (nm)	
	425	**620**
1	52.5	0.10
2	55.4	0.11
3	49.3	0.10
4	48.9	0.10
σ	3.00	0.005

Only one batch of orange dye was filtered and used for all samples in the final experiment. The decision to use one batch was due to the orange dyes ability to aggregate at low temperatures, its instability over time, and taking into consideration, the significant error from experimental preparation [193,205]. This was consistent with the findings of Chandra *et al*. [185,193], who found that when using the orange dye, batch to batch variation was the greatest cause of data comparison problems and experimental error.

4.3.6 **Repeatability of the dye calibration curves**

Calibration curves of both the orange and blue dyes, at both 425 nm and 620 nm were used as the first method repeatability check for the Simons' staining procedure.

A stock solution (10 mg/mL) of the blue dye was diluted to prepare six series of five different standards (0.002, 0.004, 0.006, 0.01, 0.12 mg/mL) and the UV absorbance was measured at both 425 nm and 620 nm. For the blue dye at 425 nm, the acceptable calibration slope range was 7.332 to 7.498 and at 620 nm, the acceptable slope range was 79.836 to 81.916 (mean ± 2 σ).

For the orange dye, five different stock solutions (10 mg/mL) were prepared from the same batch of high molecular weight fraction of dye. A series of standards (0.002, 0.005, 0.01, 0.012, 0.016, 0.018, 0.2, 0.4, 0.6, 0.8 mg/mL) were then prepared from each of these stock solutions. The six lowest concentration standards were used to produce the calibration curve at 425 nm, and the highest four concentrations were used to produce a calibration curve at 620 nm. The acceptable calibration curve ranges were: 47.746 to 60.475 and 0.082 to 0.115 for 425 nm and 620 nm (mean ± 2 σ), respectively. Examples of calibration curves for the blue and orange dyes can be seen in **Figure 4.1**.

If upon dilution the final solutions of blue and orange dyes to be used for Simons' stain test did not give calibration slopes that fitted in the acceptable range, the dye was deemed to have significant preparation error and was discarded.

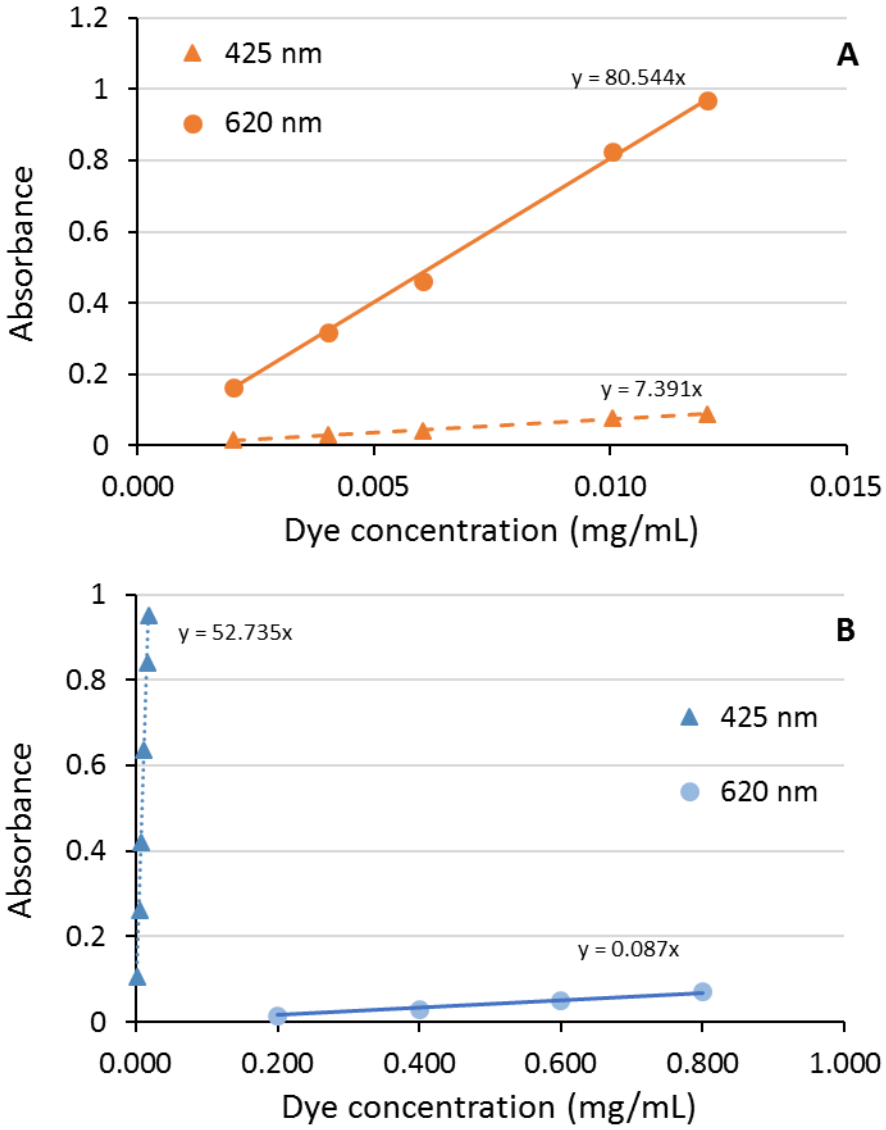


Figure 4.1 Calibration curves of the orange (A) and blue (B) dyes used in the Simons' stain test.

4.3.7 Repeatability of method

The repeatability of the Simons' stain test was assessed by analysing both bleached kraft pulp (BKp) and sawdust (SD) five times. The results are presented in **Table 4.7**. The COV's for both samples were considerably lower than those reported in the literature for similar substrates. Literature values were 10.8% and 11.1% for orange and blue dye variances, respectively [185,193]. The COV's for SD were higher than those for BKp. This is believed to be a direct result of the greater inhomogeneity of the SD.

Table 4.7 Reproducibility of Simons' staining procedure.[a]

	Bleached kraft pulp (BKp)				Sawdust (SD)			
Replicate	**O:B ratio**	**Maximum adsorbed blue dye (mg/g)**	**Maximum adsorbed orange dye (mg/g)**	**Total dye adsorbed (mg/g)**	**O:B Ratio**	**Maximum adsorbed blue dye (mg/g)**	**Maximum adsorbed orange dye (mg/g)**	**Total dye adsorbed (mg/g)**
1	2.13	18.57	39.55	58.12	0.66	35.16	23.21	58.37
2	1.82	18.87	34.34	53.21	0.55	34.25	18.84	53.09
3	1.92	20.03	38.46	58.49	0.59	29.98	17.69	47.67
4	2.07	21.56	44.63	66.19	0.61	31.54	19.24	50.78
5	1.83	21.20	38.80	60.00	0.53	35.40	18.76	54.16
Average	1.95	20.05	39.16	59.20	0.59	28.60	19.55	52.81
Σ	0.14	1.34	3.67	4.66	0.05	2.39	2.12	3.98
COV (%)	7.19	6.69	9.37	7.88	8.71	8.36	10.87	7.53

[a] Sample size O.D 100 mg.

4.3.7.1 **Improved repeatability by increasing sample mass**

The nature and inhomogeneity of many of the samples used in this study made it difficult at times to get a good representative sample at O.D. mass of 100 mg. Thus, five replicates of BKp and SD were subjected to Simons' stain test using a sample size of 200 mg (**Table 4.8**). The coefficients of variance for the maximum adsorbed orange and blue dyes, as well as the total adsorbed dye and dye ratios, improved for both SD and BKp. This indicated that a larger initial sample size delivers more repeatable results for the type of samples used in this study. Therefore, it was decided to increase the sample size to 200 mg.

In this study, results were deemed acceptable if:

a) Repeatability of a control material had a COV < 10% for all measurements (maximum blue and orange dye absorbed, total dye adsorbed and orange:blue (O:B) ratio) and the control material fell within ± 5% of the expected values.

b) Triplicate measurements for the sample agree within a COV of < 10%.

Any results greater than the desired ranges were rejected.

Table 4.8 Repeatability of Simons' staining procedure with a sample size of 200 mg (O.D).

	Bleached kraft pulp (BKp)				Sawdust (SD)			
Replicate	**O:B ratio**	**Maximum adsorbed blue dye (mg/g)**	**Maximum adsorbed orange dye (mg/g)**	**Total dye adsorbed (mg/g)**	**O:B ratio**	**Maximum adsorbed blue dye (mg/g)**	**Maximum adsorbed orange dye (mg/g)**	**Total dye adsorbed (mg/g)**
1	1.97	22.30	43.93	66.23	0.59	31.97	18.86	50.83
2	1.82	23.50	42.77	66.27	0.65	33.00	21.45	54.45
3	2.03	20.80	42.22	63.02	0.54	35.60	19.22	54.82
4	1.86	21.12	39.28	60.40	0.63	28.70	18.08	46.78
5	2.02	19.97	40.34	60.31	0.55	31.00	17.05	48.05
Average	1.94	21.54	41.71	63.25	0.59	32.05	18.93	50.99
Σ	0.10	1.38	1.88	2.95	0.05	2.54	1.64	3.64
COV (%)	4.90	6.40	4.50	4.66	8.14	7.93	8.64	7.14

4.3.8 **Sensitivity of different study substrates to Simons' stain test**

The Simons' stain test has been used to determine cellulose accessibility of a wide range of substrates, both before and after various pretreatments [183,185,193,194,201]. Substrates include diverse materials such as softwoods and hardwoods and their pulping products, non-wood species and cellulose preparations such as Avicel. Pretreatments investigated include steam, thermomechanical and solvent pretreatments. The assessment of substrates under different drying conditions: air-dried, freeze-dried, oven-dried and never-dried states has also been investigated [193,194].

A preliminary experiment was performed to determine whether the Simons' stain test could give measurable differences in the dye adsorption across a range of samples of the type used in this study. The substrates tested were two steam exploded samples prepared at the extremities of anticipated severity conditions for this study (215ºC/2 min, 180ºC/108 min) and a substrate that had been extensively ball-milled (R49TBM300). The results (**Table 4.9**) showed that there were significant differences in Simons' stain results across the substrates and most importantly between the two steam pretreated samples. This result was consistent with literature [185,201], and indicated that the Simons' stain test could distinguish differences between the samples used in this study.

Table 4.9 Evaluation of the suitability of Simons' stain test on different substrates.

	Replicate	O:B ratio	Maximum adsorbed blue dye (mg/g)	Maximum adsorbed orange dye (mg/g)	Total dye adsorbed (mg/g)
	1	0.74	34.25	25.35	59.6
	2	0.82	29.21	23.95	53.16
215°C	3	0.85	31.5	26.78	58.28
2 min	Average	0.8	31.65	25.36	57.01
	Σ	0.06	2.52	1.41	3.4
	COV (%)	7.08	7.97	5.57	5.96
	1	1.05	24.68	25.91	50.59
	2	1.13	23.97	27.09	51.06
180°C	3	1.1	21.41	23.55	44.96
108 min	Average	1.09	23.35	25.52	48.87
	σ	0.04	1.72	1.8	3.39
	COV (%)	3.7	7.37	7.06	6.94
	1	2.13	18.57	39.55	58.12
	2	1.82	18.87	34.34	53.21
R49TBM300	3	1.92	20.03	38.46	58.49
	Average	1.96	19.16	37.45	56.61
	σ	0.16	0.77	2.75	2.95
	COV (%)	8.09	4.03	7.34	5.2

4.3.9 Use of the Simons' stain test on ball-milled substrates

Ball-milling is well-known to decrease particle size and, in this study, it was selected as a tool that would not only decrease particle size but also alter the cellulose accessibility. It was unknown how substrate cellulose accessibility would be affected by ball-milling, because in previous research, ball-milling has not been used as a tool to alter cellulose accessibility whilst using the Simons' stain test to monitor any changes.

Since ball-milling was to be used to alter cellulose accessibility of the substrates in this study without changing the inhibitors (Chapter 5), it was necessary to demonstrate that ball-milling would increase the accessibility and that Simons' stain could assess these changes. To check this, three steam pretreated substrates were wet ball-milled in a vibratory ball-mill and analysed using the Simons' stain method.

Figure 4.2, shows that the substrates respond differently to ball-milling and that the Simons' stain test can evaluate the changes in cellulose accessibility. This was shown by the differing slopes that were independent of the parameter (maximum orange dye adsorbed, total dye adsorbed and O:B ratio).

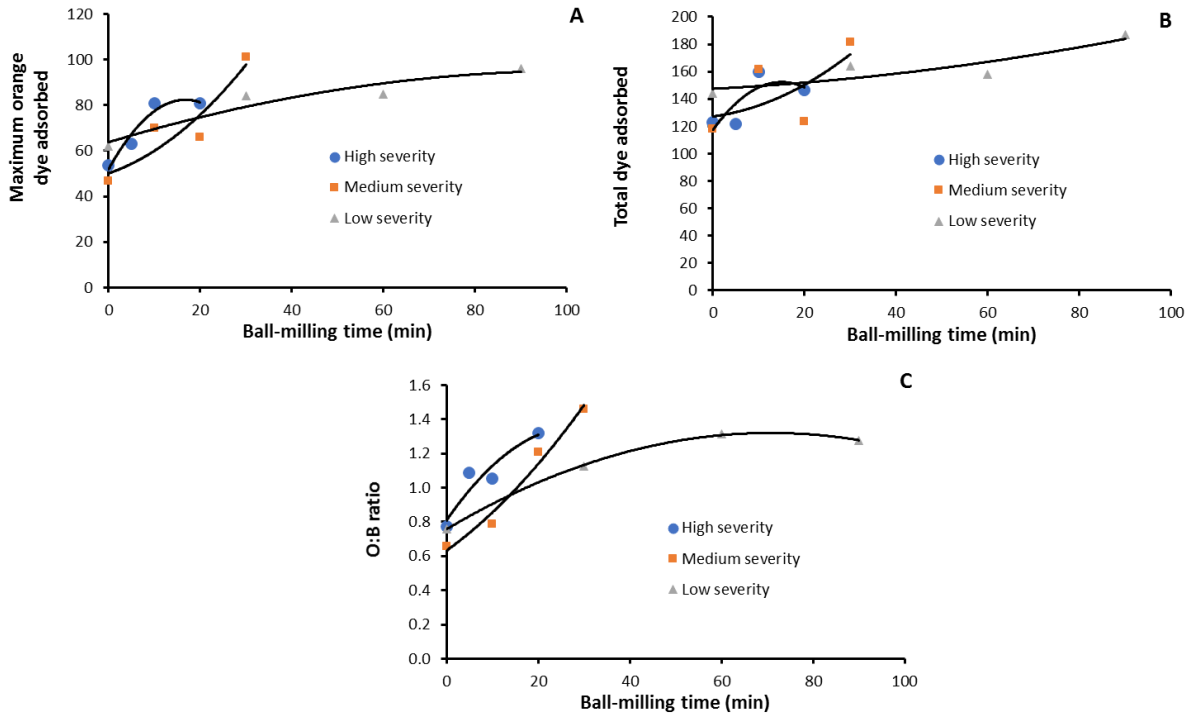


Figure 4.2 The effect ball-milling different pretreatment severity substrates had on their accessibility as measured by maximum orange dye adsorbed (A), total dye adsorbed (B), and orange to blue (O:B) dye adsorbed ratio.

The digestibility of substrates, as expected, increased with longer ball-milling times (**Figure 4.3**) and this coincided with changes in Simons' stain values of total dye adsorbed, orange dye adsorbed and the O:B dye ratio.

Simons' stain measurements are often used as a predictor of cellulose digestibility [185,193,194]. In this study, the O:B dye ratio was chosen as the parameter to estimate potential digestibility. The justification for this was that the O:B dye ratio is often used in literature and, in a trial using ball-milled substrates that compared the three parameters, the O:B ratio gave the best correlation (R^2) (**Figure 4.4).**

The most important finding of the preliminary ball-milling tests was that by selectively choosing ball-milling times, it would be possible to obtain substrates of the same accessibility (**Figure 4.2**).

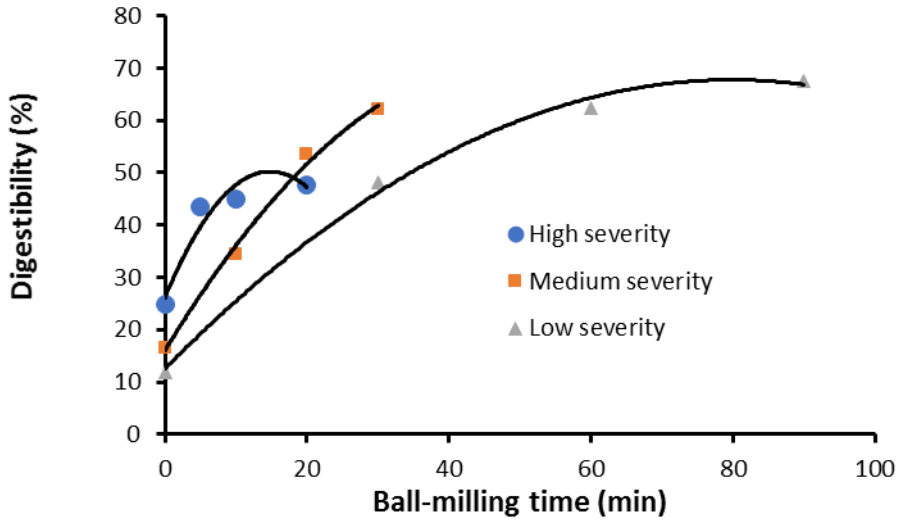


Figure 4.3 Effect of ball-milling on the digestibility of steam pretreated substrates using and enzyme loading of 15 FPU/g substrate.

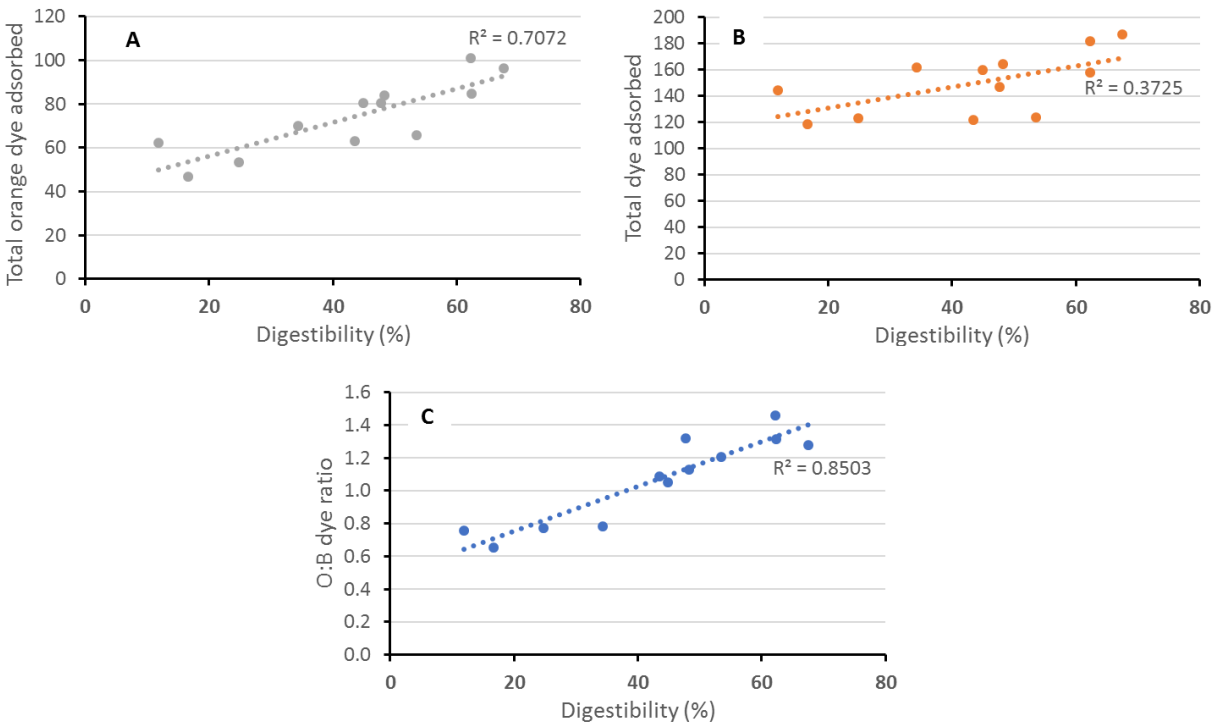


Figure 4.4 Evaluation of the Simons' stain measurements against the digestibility of ball-milled steam pretreated substrates. The plots show the best predictor of digestibility was the O:B dye ratio, as indicated by the R^2 correlation values.

In summary, the trial confirmed that:

a) the Simons' staining procedure would be a suitable method for evaluating cellulose accessibility of various substrates, including differences that would arise by using ball-milling.

b) ball-milling would be a suitable tool to be used in this study to alter cellulose accessibility.

c) the parameter to be used to predict the digestibility of samples in this study would be the Simons' stain O:B dye ratio.

4.3.10 **Effect of the presence of ash resulting from ball-milling**

Ball-milling results in increased ash content of the substrates. The amount of ash transferred to the substrate during ball-milling depends on the substrate type and ball-milling time. Since the amount of dye absorbed during the Simons' stain test is calculated based on weight of the substrate, it was important to adjust the measured result for the ash content of the sample.

It was also necessary to determine if ash itself could adsorb dye and affect the overall results. Ash from ball-milling two control materials, BKp and R49TBM300 was subjected to the Simons' stain test using different weights of ash (1, 5, 10, 20, 30 mg). These values coincided with and exceeded the amount of ash that was present in samples used in this study (2 to 20 mg). The concentration of dye remaining in solutions after the Simons' staining procedure was comparable to the blank control with no ash. This was true for all ash weights and both ash types. This indicated that ash did not adsorb any of the blue or orange dyes and thus would not affect results.

4.4 Summary

The Simons' stain test, as described by Chandra *et al*. [193], was evaluated and modified to obtain results that would be accurate, repeatable and reproducible for the types of samples in this study. The final modified method was effective in showing differences in cellulose accessibility of substrates of different pretreatment severities, as well as the changes that occur to a substrate upon ball-milling. Hence, with the modifications made, the method was ideal for use in this research.

Key modifications made were:
1) only one batch of filtered orange dye was used for samples to decrease error.
2) staining temperature was 65ºC.
3) staining time was 18 hours.
4) an increased sample size of 200 mg was used.

Repeatability of results was ensured by:
1) using the acceptable calibration ranges for both the orange and blue dyes at both 425 nm and 620nm.
2) ensuring sample replicates had a COV of < 10%
3) samples were only accepted if triplicates of the control sample values for maximum orange dye adsorbed, blue dye adsorbed and total dye adsorbed and the O:B ratio all had a COV of < 10% and the values fell within ± 5 % of the expected values.

5 Assessment of enzyme inhibition from steam pretreated solids

The contents of this chapter as well as the relevant sample preparation data from Chapter 3, form the contents of the following published research paper "Unravelling the effect of pretreatment severity on the balance of cellulose accessibility and substrate composition on enzymatic digestibility of steam pretreated softwood", published in the *Journal of Biomass and Bioenergy* (Appendix D).

5.1 Introduction

Pretreatment to modify the structure and/or the chemical composition of a substrate is essential for effective digestibility of lignocellulosic biomass [26]. Short high-temperature (180-250ºC) steam treatments are generally effective for pretreating agricultural and hardwood biomass, whereas softwoods require harsher conditions such as the presence of an acid catalyst during steam pretreatment [9,10,48].

Several substrate properties are known to influence the rate and/or extent of enzymatic digestion [5,149]. Removal of hemicelluloses and lignin to increase accessible cellulose surface area or pore volume is almost always beneficial [149], while low cellulose crystallinity [208] or small particle size [209] often result in a high digestion yield and/or rate. However, the response of a given biomass to enzyme treatment cannot be predicted with certainty from the value of any single property.

Pretreatments that increase the accessible surface area of the substrate, leaving more cellulose accessible for the enzymes to act upon, can deliver higher sugar yields [149]. Accessibility can be increased by either removing hemicelluloses or lignin from the substrate, or by mechanically opening up the fibre to increase the exposure of cellulose within.

Substrates may be made more digestible by increasing the severity of the steaming pretreatment [10]. However, this needs to be balanced against a lower overall sugar

yield at high severity due to the dissolution or degradation of the biomass. The presence of degradation products introduces possible complications in downstream processing, because some of these degraded phenolics can be inhibitory towards cellulase enzymes. The type and concentration of these inhibitory chemicals is believed to increase as pretreatment severity increases [14]. The inhibitors may be components of the fibre that remain in the water-insoluble fraction, particularly lignin or pseudo lignin [77,110,135,144,210], or water-soluble components from the pretreated substrate such as phenolics [116].

Lignin is known to inhibit enzyme digestibility in two main ways: firstly, it can physically hinder the accessibility of enzymes to cellulose [211]; and secondly, it can bind to cellulases [133,212-214]. Both of these actions limit the efficiency of enzymes crucial for the degradation of insoluble polysaccharides. The non-productive binding of enzymes to lignin is a widely studied and accepted phenomenon, but is still poorly understood [77,135,144,150,210,215,216].

The influence of particle size on enzyme digestibility of substrates has previously been investigated [217-219]. Particle size is often a good indicator of enzyme digestibility, since smaller particles have an increased surface area which gives enzymes easier access to the polysaccharides. Pretreatments that produce smaller particles can therefore result in higher sugar yields, but this is not always the case [218,219].

Cellulose crystallinity has also been suggested to influence the digestibility of lignocellulosic substrates, but the effects are inconsistent [70]. Some studies show cellulose crystallinity have a large effect on digestibility [208,220,221], while other studies show little or no relationship between cellulose crystallinity and digestibility [84,222-224].

The steam pretreatment conditions of temperature, time and pH used are a balancing act. On one hand, the pretreatment conditions should maximise the accessibility of the enzymes to the cellulose and/or reduce cellulose crystallinity. On the other hand, yield losses and inhibitor formation that occurs from the degradation and

modification of sugars and/or lignin should be minimised. A better understanding of this balance could lead to lower enzyme usage, and therefore lower biofuel production costs.

This chapter describes an evaluation of the factors that influence the enzyme digestibility of radiata pine that has been steam pretreated across a range of severity factors; using different acid catalysts, pretreatment temperatures and times. Understanding what factors influence the digestibility of steam pretreated radiata pine will enable both: 1) a better understanding of enzyme inhibition, and 2) the use of the best pretreatment conditions in future studies.

5.2 Materials and methods

5.2.1 Steam pretreated solids

Seven samples in total were used: untreated sawdust and the samples that were steam pretreated at the six different pretreatment conditions (in duplicate), as described in Chapter 3, **section 3.2.4**.

5.2.2 Enzyme hydrolysis

Enzyme hydrolysis was performed as described in **section 3.2.9**.

5.2.3 Compositional analysis

Carbohydrates, lignin, ash C and N contents were analysed as described in **section 3.2.8.**

5.2.4 Ball-milling

Ball-milling was performed for the desired time(s) in a Schwingmühle VIBRATOM vibratory ball mill by treating samples (6 O.D g, 5% *w/v*, 0.01% *w/v* sodium azide water) in a ceramic vessel (0.6 L) containing two hundred 15 mm alumina balls. All compositions and digestion values of ball-milled samples were corrected for ash content because the milling action erodes the alumina balls and the ceramic chamber.

5.2.5 Cellulose accessibility

The cellulose accessibility of substrates was determined using a modified version of the Simons' stain test developed by Chandra *et al*. [193]. For the final method used, refer to **section 4.3.1**.

5.2.6 Particle size analysis

Particle size analyses were performed in duplicate using a Malvern Mastersizer 2000 particle analyser.

5.3 Results and discussion

5.3.1 Digestibility of steam pretreated substrates

The digestibility of steam pretreated substrates has previously been evaluated in Chapter 3. The results showed that as the pretreatment severity increased, the digestibility of the substrates also increased (**Figure 5.3A**). It was also found that pretreatment conditions of lower temperatures for longer time periods were more beneficial, as they gave higher digestibilities. This chapter describes the relationships between substrate factors and their influence on digestibility.

5.3.2 Relationship between cellulose digestibility and enzyme accessibility

The positive correlations observed between the digestibility of the pretreated substrates and the accessibility of the cellulose, as measured by the Simons' stain O:B dye ratio [201], can be seen in **Figure 5.1** (both $R^2 > 0.8$). The positive correlations suggest, in agreement with earlier studies [187,224,225], that the accessibility of the cellulose is an important determinant of substrate digestibility. In this study, increased accessibility was mainly due to greater hemicellulose dissolution as a result of increased pretreatment severity (**Table 3.5 and 3.6**).

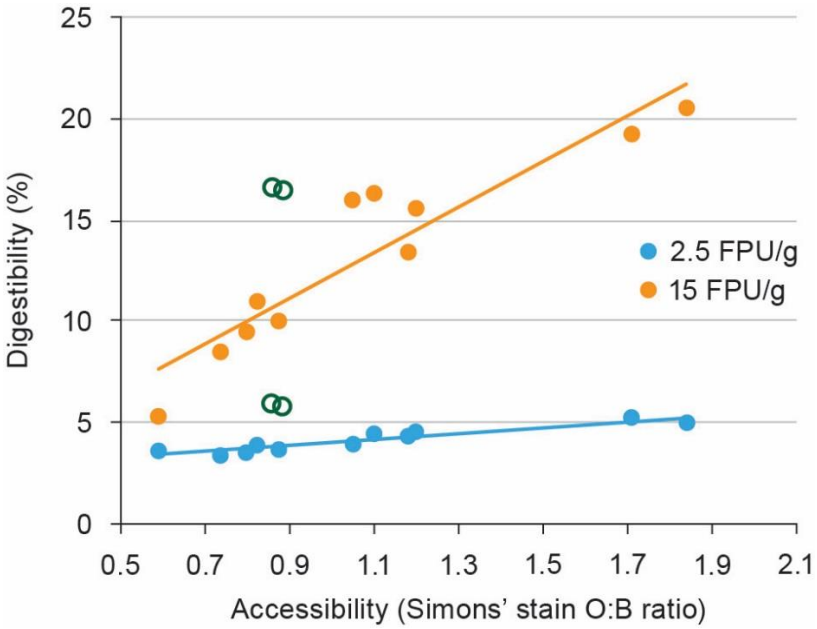


Figure 5.1 Digestibility of steam pretreated substrates versus accessibility, as determined by Simons' stain orange to blue (O:B) dye ratio. Citric acid samples represented as open green symbols. R^2 slope correlations are 0.87 and 0.81 for 15 FPU/g and 2.5 FPU/g enzyme loadings, respectively. Steam pretreated experimental duplicates represented as individual points.

Although the positive correlations indicated that cellulose accessibility was a significant factor contributing to cellulose digestibility, the poor scatter of points indicated there might be a competing and/or a combination of factors contributing to the overall extent of cellulose digestibility.

The citric acid catalysed samples (open green symbols in **Figure 5.1**) were the poorest fitting points, lying farthest from the potential line of best-fit. It is proposed that the reason the digestibility of these substrates was greater than predicted by their accessibility was because, although citric acid is a relatively weak acid (pKa1=3.13 pKa2=4.76 pKa3=6.40) it is very effective at hydrolysing and causing dissolution of the hemicelluloses [149]. As a result, these samples may have had lower levels of acid-catalysed lignin modification at a given accessibility, meaning the lignin in these samples were less inhibitory to enzymes.

The positive correlation between accessibility and digestibility confirmed that the different pretreatment conditions should not be directly compared, because the accessibility of the samples might be different. Thus, if the level of inhibition as a function of pretreatment severity was to be compared, the changes in accessibility would need to be considered.

5.3.3 Obtaining pretreated solids of a common cellulose accessibility

To help understand the relative importance of enzyme inhibition and accessibility as a function of pretreatment severity, the digestibilities of pretreated substrates at a common cellulose accessibility were compared. Wet ball-milling was used to adjust the accessibility of the substrates up to a common level. Wet ball-milling mechanically disrupts fibre walls, thus improving the accessibility of enzymes to cellulose and therefore the overall digestibility [217]. It was assumed that the mechanical forces during ball-milling increase the accessibility of the substrate without inducing chemical changes. Ball-milling is widely used to obtain milled wood lignin (MWL), which is considered the best representative of native lignin; the milling is performed under harsh conditions which include dry-milling for extensive time periods of up to three days. In this study, milling was performed on a wet-basis and for comparatively short time periods, so any change in composition would be expected to be minimal. A recent study [171], confirmed that only very minor lignin degradation occurs when wet ball-milling was undertaken under conditions similar to those used in this study.

Steam pretreated substrates were wet ball-milled for 10, 30 and 60 minutes to determine how cellulose accessibility for each of the substrates changes as a function of ball-milling time (**Figure 5.2**). Each pretreated substrate responded to ball-milling to a different rate and extent, with duplicates of experimental steam pretreatments giving similar values (for individual results, refer to Appendix B2). These different behaviours are probably due to the fact that the more severe steam pretreatment conditions remove more hemicelluloses (as previously shown in Chapter 3), and thus elicit a faster response to ball-milling. This observation has been shown previously with a study on ball-milled substrates [226]. Although the substrates responded to ball-milling differently, by selecting the appropriate ball-milling time (dashed line, **Figure 5.2**), it proved possible to adjust all samples to a common cellulose accessibility of 1.8 ± 0.06, as estimated by the Simons' stain O:B ratio. The required ball-milling time decreased as the pretreatment severity increased, from 110 minutes for the original wood to zero for the wood pretreated in the presence of sulfuric acid (**Table 5.1**).

Table 5.1 Sample pretreatment conditions and required ball-milling times to obtain common cellulose accessibility value of 1.8 as measured by the Simons' stain O:B dye ratio.

Sample #	Steam pretreatment conditions			Common accessibility ball-milling time (min)
	Catalyst	Temperature (°C)	Time (min)	
1 (Sawdust)				110
2	Water	215	2	60
3	Water	180	21.5	28
4	Water	215	10	12
5	Water	180	108	17
6	Citric acid	215	2	13
7	Sulfuric acid	215	2	0

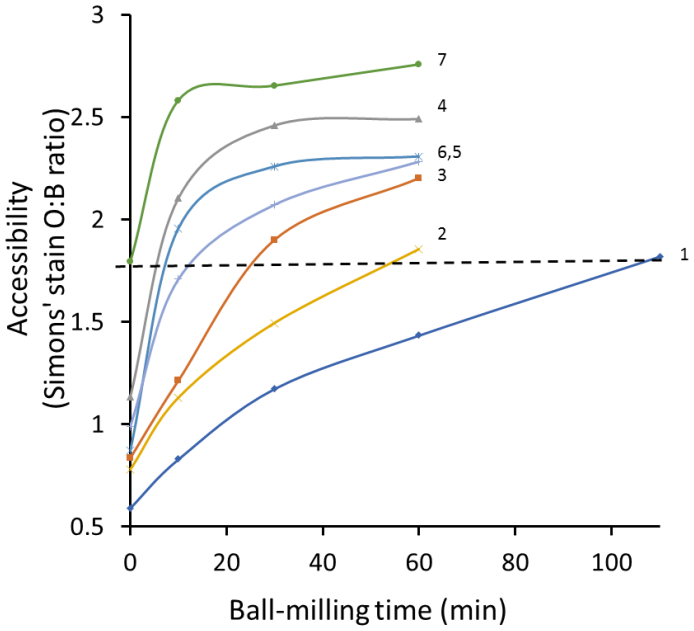


Figure 5.2 Steam pretreated substrates accessibility response as a function of ball-milling time (mean of duplicate samples); #1-7 correspond to pretreatment conditions as seen in Table 5.1. The dashed line indicates the cellulose accessibility O:B dye ratio of 1.8 ± 0.06.

5.3.4 Digestibility of pretreated samples at a common cellulose accessibility

As expected, the digestibilities of substrates at the common cellulose accessibility were higher than for the un-milled substrates (**Figure 5.3B**), as ball-milling is known to open up the fibers and increase the accessibility of the cellulose. For example, sawdust digested at 15 FPU/g enzyme loading gives a digestibility of 5%,

compared to 53% once ball-milled to the common cellulose accessibility (O:B dye ratio of 1.8).

At a common cellulose accessibility, the digestibility of substrates decreased as the pretreatment severity increased (**Figure 5.3B**). This is the opposite trend to that observed prior to ball-milling (**Figure 5.3A**). This suggested that inhibition occurred and increased with increasing pretreatment severity. The inhibition was likely due to non-productive binding of the enzymes to lignin. The incomplete digestion of the original untreated wood suggested that the native lignin in *P. radiata* was also capable of binding the enzymes. This was consistent with an earlier study which had found the digestibility of Avicel, a pure cellulose, decreased when isolated spruce wood lignin was added during digestion [215].

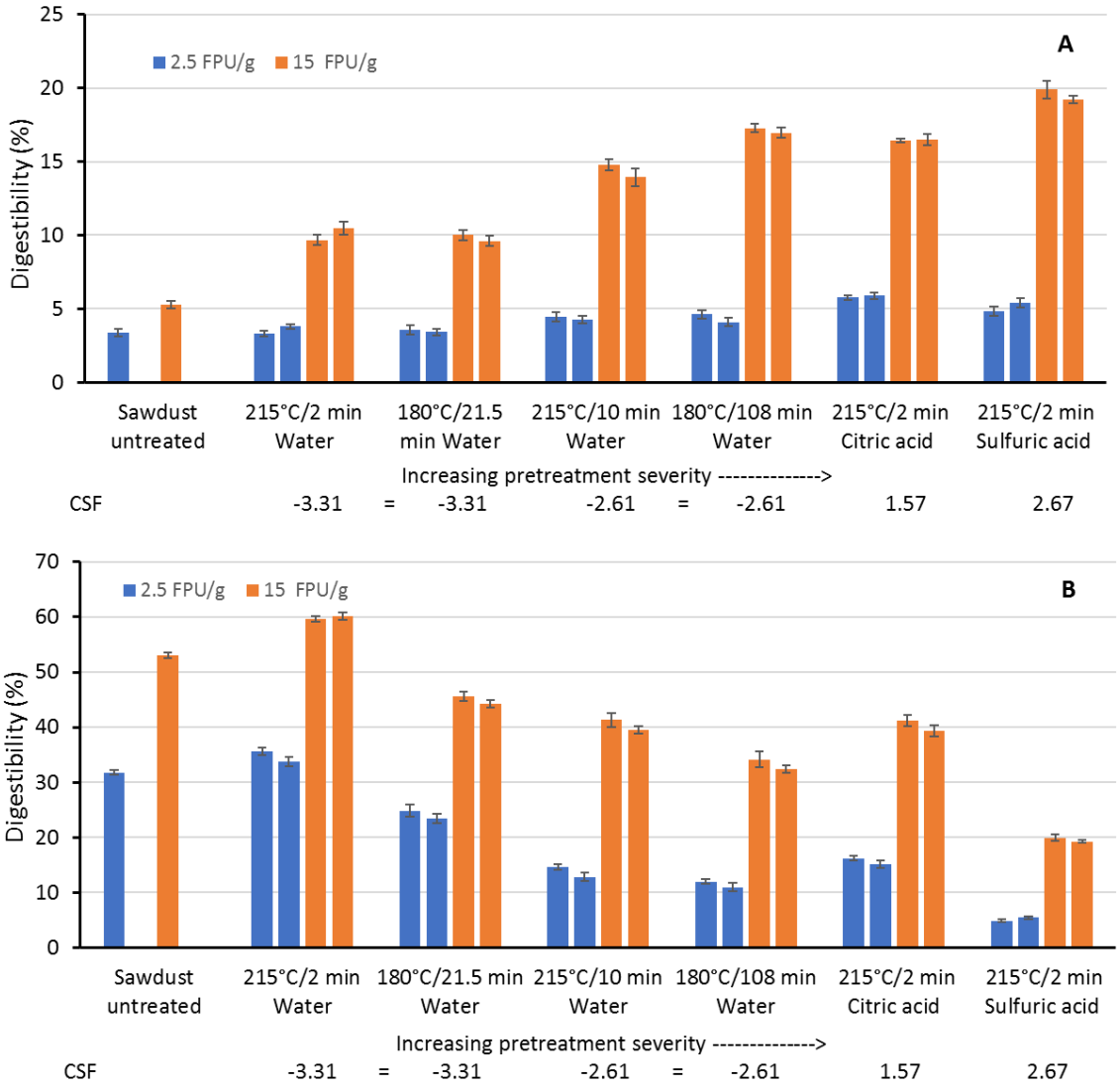


Figure 5.3 Digestibility as measured by percentage glucan conversion of steam pretreated substrates using Cellic® CTec2 at enzyme loadings of 2.5 FPU/g and 15 FPU/g: after steam pretreatment (A), after ball-milling to a common cellulose accessibility O:B dye ratio of 1.8 (B). Triplicate determinations of steam pretreated experimental duplicates, the latter represented as individual bars, error bars represent ± two standard deviations.

Overall, the results indicated that accessibility was a major determinant of substrate digestibility, and that non-productive binding of enzymes to lignin increased in parallel with (or probably more slowly than) accessibility as the pretreatment severity increased. Any pretreatment chosen would thus be a compromise between these two effects. This was in agreement with other studies [187,225,227], in which the increased accessibility seen at higher pretreatment severities more than compensated for increases in enzyme inhibition.

The digestibilities of the substrates at a common cellulose accessibility decreased as the lignin content of the substrates increased (**Figure 5.4**). This may have been a result of greater non-productive binding due simply to the presence of more lignin in the substrate. Alternatively, increasing pretreatment severity may have led to lignin modification resulting in stronger non-productive binding of cellulase enzymes to lignins [77,150,210]. It has been reported that lignins isolated from pretreated substrates reduce the digestibility of pure cellulose more than lignins isolated from original wood [210,215], so lignin modification may explain the results shown in **Figure 5.4**. The scatter seen in **Figure 5.4** would suggest that lignin content was not the only factor.

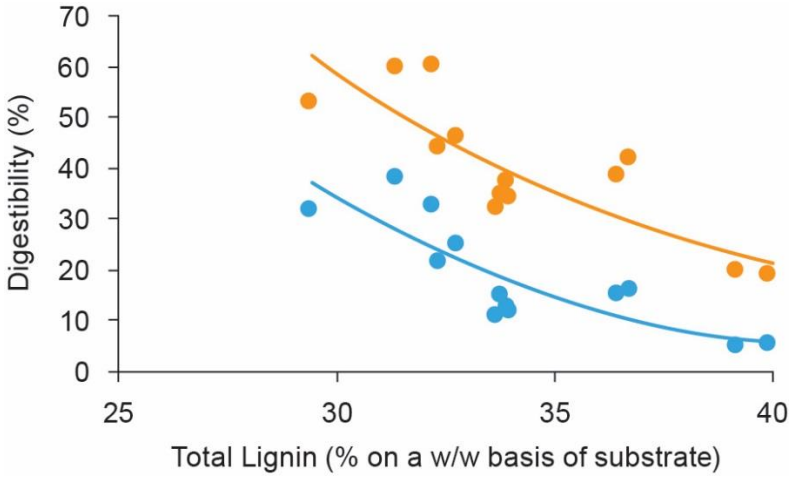


Figure 5.4 Comparison of the digestibility of steam pretreated substrates at a common cellulose accessibility (O:B dye ratio of 1.8) and the total lignin content of the substrates. Steam pretreated experimental duplicates represented as individual points.

5.3.5 Assessment of other factors that may influence enzyme digestibility

Particle size and an indicative cellulose crystallinity value were assessed for substrates obtained from steam pretreatments, as well as for the substrates at the common cellulose accessibility, **Table 5.2**.

5.3.5.1 **Particle size**

Particle size distribution plots were obtained for all steam pretreated samples prior to ball-milling and the corresponding sample at the common cellulose accessibility of Simons' stain O:B ratio of 1.8. An example can be seen in **Figure 5.5** and all other plots can be found in Appendix B1.

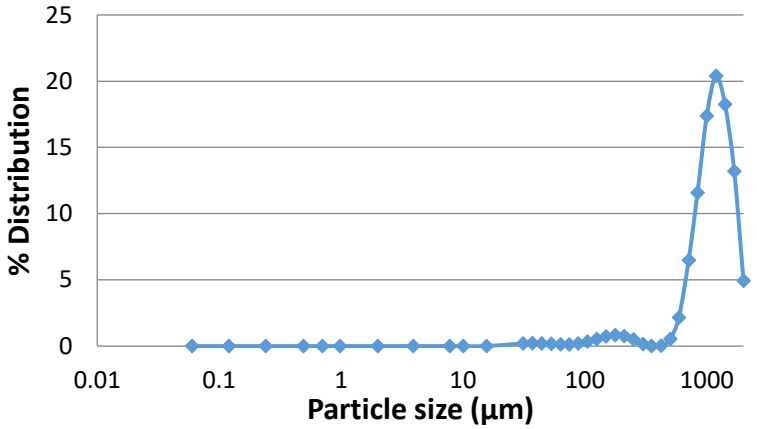


Figure 5.5 Particle size distribution of sawdust.

The mean particle size of steam pretreated solids decreased with increasing pretreatment severity. As particle size decreased, the cellulose accessibility increased (**Table 5.2, Figure 5.6**). This would be expected, given that smaller particle size increases the surface area of a substrate, which in turn can make more cellulose accessible. As expected smaller particle sizes were corresponded to higher levels of digestibility.

Table 5.2 Particle size, crystallinity and accessibility values of pretreated substrates and pretreated substrates ball-milled to a common cellulose accessibility level.

	After pretreatment			At a common cellulose accessibility[a]		
Pretreatment conditions	**Particle size[b,*] (µm)**	**Crystallinity[#] Index**	**Dye ratio [c,*]**	**Ball-mill time (min)**	**Particle size[b,*] (µm)**	**Crystallinity[#] Index**
Sawdust untreated	1200	4.5, 5.4, 5.0	0.59	110	31	4.1
215°C/2 min Water	1100	6.6, 5.9	0.78	12	40	5.6, 5.6
180°C/21.5 min Water	1100	5.9, 5.9	0.84	17	37	5.2, 5.8
215°C/10 min Water	1050	6.9, 6.0	1.13	60	37	5.3, 5.8
180°C/108 min Water	1050	6.5, 6.0	0.99	13	40	5.6, 5.6
215°C/2 min Citric acid	1050	6.8, 6.1	0.95	13	40	5.6, 5.6
215°C/2 min Sulfuric acid	840	6.5, 6.0	1.79	0	840	-----

[a] Value of 1.8 ± 0.06 O:B ratio as determined by Simons' stain

[b] Particle size is represented by the median size

[c] Dye ratio is the O:B ratio as measured by Simons' stain procedure

[*] Measurements are the average of 4 measurements; duplicates of experimental duplicates

[#] For the crystallinity index method used in this study refer to Appendix B3. Error is ±0.4, with values representing the different duplicate steam explosion samples. Sawdust was analysed in triplicate for error.

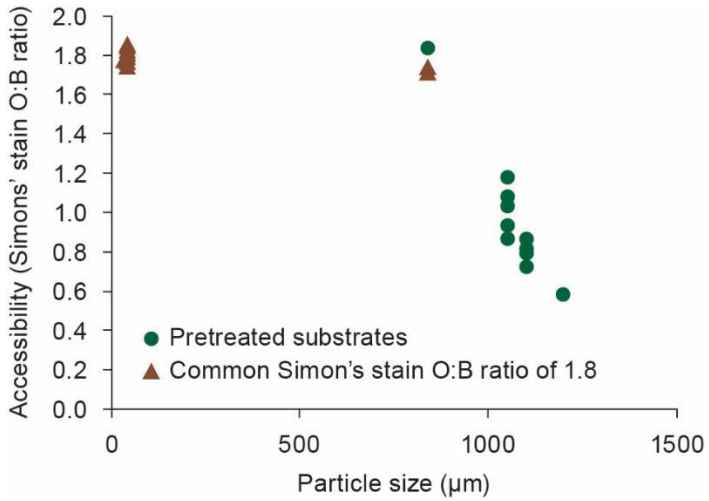


Figure 5.6 Relationship between particle size and accessibility as measured by Simons' stain O:B dye ratio. Experimental duplicates are represented as individual points.

Ball-milling the substrates to a common cellulose accessibility reduced mean particle size substantially, with the mean particle size of pretreated substrates decreasing from ~1100 µm prior to ball-milling to ~30-40 µm once ball-milled, irrespective of ball-milling time. The exception was the substrate from the sulfuric acid catalysed treatment which did not require ball-milling; its particle size remained the same. Mean particle size of the substrate from the sulfuric acid pretreatment was lower than that of the other samples at 840 µm. The exact reason for this significant particle size difference is unknown. However, the smaller

particle size is likely to be attributed to a more brittle material due to a combination of the harshness of the acid catalyst, as well as considerably larger carbohydrate removal.

The particle size of substrates at the common cellulose accessibility were compared to digestibility values, and the correlation was poor for both enzyme loadings (**Figure 5.7**, data for 15 FPU/g not shown**)**. Although at a common cellulose accessibility most samples have a particle size of ~30-40 µm, the digestibilities varied considerably between 11% and 35% (**Figure 5.7**). This indicated that particle size was not the major determinant of digestibility; other factors such as accessibility and enzyme inhibition are more significant. This was consistent with a previous study on poplar wood that showed below a mean particle size of 420 µm, particle size had little effect on biomass digestibility [129].

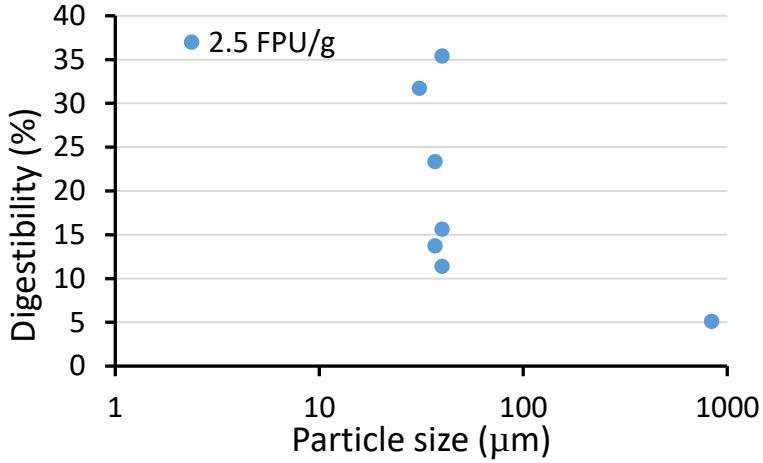


Figure 5.7 Relationship between particle size and digestibility of steam pretreated substrates at a common cellulose accessibility (O:B ratio of 1.8), using CTec2 enzyme and an enzyme loading of 2.5 FPU/g.

The use of ball-milling to alter particle size and accessibility, meant that observing changes in crystallinity was important; as an alteration to one condition always results in changes to other properties. Indicative crystallinity values of substrates did not correlate well to digestibility at either enzyme loading, before or after ball-milling (data not shown, see Appendix B3). Therefore, it would seem that cellulose crystallinity was not a major factor influencing the diverse digestibilities shown in **Figure 5.3**.

5.4 Summary

Cellulose accessibility and enzyme inhibition were both found to be major factors contributing to the digestibility of steam-pretreated *P. radiata* wood. Under the conditions studied, as pretreatment severity increased, increasing accessibility was the dominant factor. The increases in accessibility more than compensated for increases in enzyme inhibition.

It was also found that there is a need to carefully choose pretreatment conditions to optimise the opposing effects of increasing accessibility and increasing enzyme inhibition in order to maximise digestibility.

6 Assessment of enzyme inhibition by filtrates

6.1 Introduction

Softwoods are appealing feedstocks in the sugar pathway for bioethanol production owing to their potential to give high sugar yields compared to hardwoods. However, softwoods are recalcitrant and require a pretreatment step for high sugar yields to be obtained [48]. Steam pretreatment is attractive due to its limited use of chemicals [84], and steam explosion was the pretreatment investigated in this study.

As previously discussed, steam pretreatment disrupts the lignin and removes hemicelluloses, both of which increase enzyme access to cellulose and thus improve the overall sugar yields [228]. One undesirable aspect of steam pretreatment is that some of the carbohydrates and lignin are degraded under the hot, acidic conditions. This produces chemicals capable of inhibiting subsequent enzyme hydrolysis [229]. Solubilisation of extractives during pretreatment may also produce potential inhibitory compounds [108].

Inhibitors have previously been reported in steam pretreated filtrates and biomass [108,229-231]. The type and concentration of inhibitors vary significantly depending upon the pretreatment conditions of temperature, time and pH used, as well as the biomass feedstock source (hardwood, softwood, plants) [1].

The inhibitors formed during steam pretreatment may be found either in the water-soluble filtrate or the water-insoluble substrate, depending on their water solubility and molecular weight [107]. These inhibitory compounds have the potential to affect different stages of the bioconversion process. Some may act as inhibitors to the hydrolytic enzymes used to break down the polysaccharides to monomeric sugars, while others retard the glucose conversion during fermentation [85]. Consequently, the most common processing option is to separate the pretreated slurry into two fractions: an insoluble fraction that contains the lignin and cellulose, and the liquid fraction (filtrate) that contains partially degraded hemicellulose and hemicellulose-derived sugars, lignin degradation products, acetic acid and other compounds including potential inhibitors [85]. The insoluble fraction is always fed forward for enzymatic hydrolysis and fermentation, while the filtrate is either fed forward or discarded. From both economic and environmental

perspectives, the use of the filtrate is preferable, given it can a) increase the yield of sugars and thus potentially give higher ethanol yields, and b) reduce the processing costs associated with handling, separating and washing the insoluble fraction.

The water-soluble organics can be grouped into two main groups: sugar components, which includes hemicellulose derived monomeric and oligomeric sugars, and; non-sugar components, which include lignin and extractive degradation products such as furans, organic acids and phenolics [45,232]. The inhibitory effect of soluble components derived from pretreatment processes has been recognised for some time [114]. Much of the previous work on this has been done using 'synthetic/model' mixtures of inhibitors [108,126] and using either purified or 'older' generation enzymes, such as Novozymes Celluclast or Genencor Spezyme [233].

Cellulose- and hemicellulose-derived sugars have been shown to exert inhibitory effects on cellulase enzymes *via* an end-product mechanism [127]. The non-sugar components, such as polymeric phenolics, are postulated to form precipitable phenolic-enzyme complexes that essentially deactivate cellulase enzymes [122,123,234,235]. However, it still remains unclear which components from steam pretreated woods exert the greatest inhibitory effects on enzymes mixtures and which inhibitory mechanisms dominate [233].

Enzyme companies continue to develop hydrolytic enzymes that can contribute to lower enzyme loadings whilst still being able to achieve high levels of biomass deconstruction [233]. Newer enzyme cocktails such as Novozymes Cellic® CTec2 and CTec3 are known to contain additional accessory enzymes such as xylanase and lytic polysaccharide monooxygenase (LPMO), and more glucose tolerant β-glucosidase [236,237]. However, it is yet to be determined if the newer cocktails have stronger inhibitor tolerance. This is especially true since the nature of pretreatment and the biomass source are known to influence the composition and concentration of pretreatment-derived compounds [107,233,238].

Although there have been previous studies on the effect of inhibitory components upon enzymatic hydrolysis and fermentation steps of softwoods and hardwoods

[12,85,107,180,229,235], there are limited studies on enzymatic hydrolysis inhibitors produced during pretreatment of *P. radiata*. The aim of the work described in this chapter was to investigate the inhibitory effects of filtrates produced from various steam pretreatment conditions of radiata pine. The results were to enable a relative comparison of soluble and insoluble inhibition to be made.

6.2 Materials and methods

6.2.1 Control material

Bleached kraft pulp (BKp) was prepared as described in **section 3.2.2.1**.

6.2.2 Filtrates and steam pretreated substrates

Filtrates and steam pretreated substrates were prepared as described in **section 3.2.4**.

6.2.3 Chemicals

The commercial cellulase enzyme Celluclast 1.5L and cellulase enzyme cocktail Cellic® CTec2 (88.5 FPU/g enzyme) were sourced from Novozymes (Franklinton, NC, USA), while β-glucosidase was obtained from Sigma Aldrich. MilliQ (18.2 MΩ) water was obtained from a Sartorius Arium Pro® system.

6.2.4 Enzyme hydrolysis

Enzyme hydrolysis was performed as described in **section 3.2.8** using enzyme loadings of 2.5 FPU/g or 15 FPU/g and the specified enzyme, either CTec2, or Novozymes Celluclast 1.5L supplemented with β-glucosidase (1 FPU/g:1.25 IU/g ratio).

6.2.5 Filtrate dose back experiments

The filtrates were added to the desired solid (BKp or steam pretreated substrate) at the same level and concentration in which they were in the substrate immediately after steam pretreatment. This was done by setting the liquid to solid ratio equal to that after each respective steam pretreatment (**Table 6.1**). For example, if filtrate from 215ºC/2 min was being dosed back to either the substrate from steam

pretreatment at 215ºC/2 min or BKp, and assuming enzyme hydrolysis was performed on 75 mg of solid, then 285 µL and 300 µL would be the filtrate volumes used for the experimental filtrate duplicates. Enzyme hydrolysis was then performed as in **section 6.2.4**.

Table 6.1 Filtrate to solid dose back ratios.

Steam pretreatment conditions	Filtrate to solid ratio*
215 °C/2min Water	3.8, 4.0
180 °C/21.5min Water	4.6, 4.5
215 °C/10min Water	4.4, 4.6
180 °C/108min Water	5.0, 5.3
215 °C/2min Citric acid	3.9, 4.1
215 °C/2min Sulfuric acid	4.8, 4.7

*Values for experimental duplicates at a given pretreatment condition as A,B.

6.2.6 Gallic acid optimised digestibility

Enzymatic hydrolysis using CTec2 was performed as described in **section 3.2.8** with gallic acid added to give a concentration of 0.01M. In a trial, this was the concentration that gave maximum enhanced digestibility (see Appendix C1).

6.3 Results and discussion

The effects of filtrate presence on the digestibility of steam pretreated substrates and of a BKp control were assessed at both a high (15 FPU/g) and low (2.5 FPU/g) enzyme loading. BKp was selected as the control material over Avicel as it is more representative of steam pretreated substrates but is lignin-free, which precludes potential inhibitory effects that may arise from the presence of lignin. Two enzyme doses were used because it was theorised that high enzyme loadings might overcome small inhibitory effects, whilst treatments at the low enzyme loading may be more affected due to the lower enzyme to inhibitor ratio.

6.3.1 Effect of filtrates on the digestibility of BKp

The digestibility of the control BKp in the presence of water with no added filtrate was 47% and 84% for CTec2 enzyme loadings of 2.5 FPU/g and 15 FPU/g, respectively (**Figure 6.1**).

The digestibility of the BKp remained unchanged within experimental error (95% confidence) when digested with 2.5 FPU/g CTec2 in the presence of the filtrates for all but one of the different steam pretreatment conditions (**Figure 6.1A**). The exception was the filtrate from the most severe pretreatment in which the sawdust was treated with sulfuric acid prior to steam pretreatment, this filtrate reduced digestibility from 47% to 41%. This suggests that in the most severely pretreated filtrate, inhibitory components were either present in higher concentrations or the filtrate contained additional inhibitors.

The BKp digestibility pattern was somewhat different for the 15 FPU/g digestions. Mirroring the pattern observed for 2.5 FPU/g, the filtrate from the most severe sulfuric acid pretreatment produced some inhibition as digestibility was reduced from 84% for the control down to 80% in the presence of the filtrate (**Figure 6.1B**). Surprisingly, however, small increases in digestibility were observed in the presence of all the other filtrates. Digestibility of the BKp increased with increasing severity of pretreatment reaching a maximum of 89% for the citric acid pretreated filtrate. Although, the increased digestibility on adding filtrates was initially surprising, it was hypothesised that this was due to components present in the filtrates acting as reducing cofactors for the lytic polysaccharide monooxygenase (LPMO) enzymes present in the CTec2 enzyme cocktail and leading to greater cellulose hydrolysis [236].

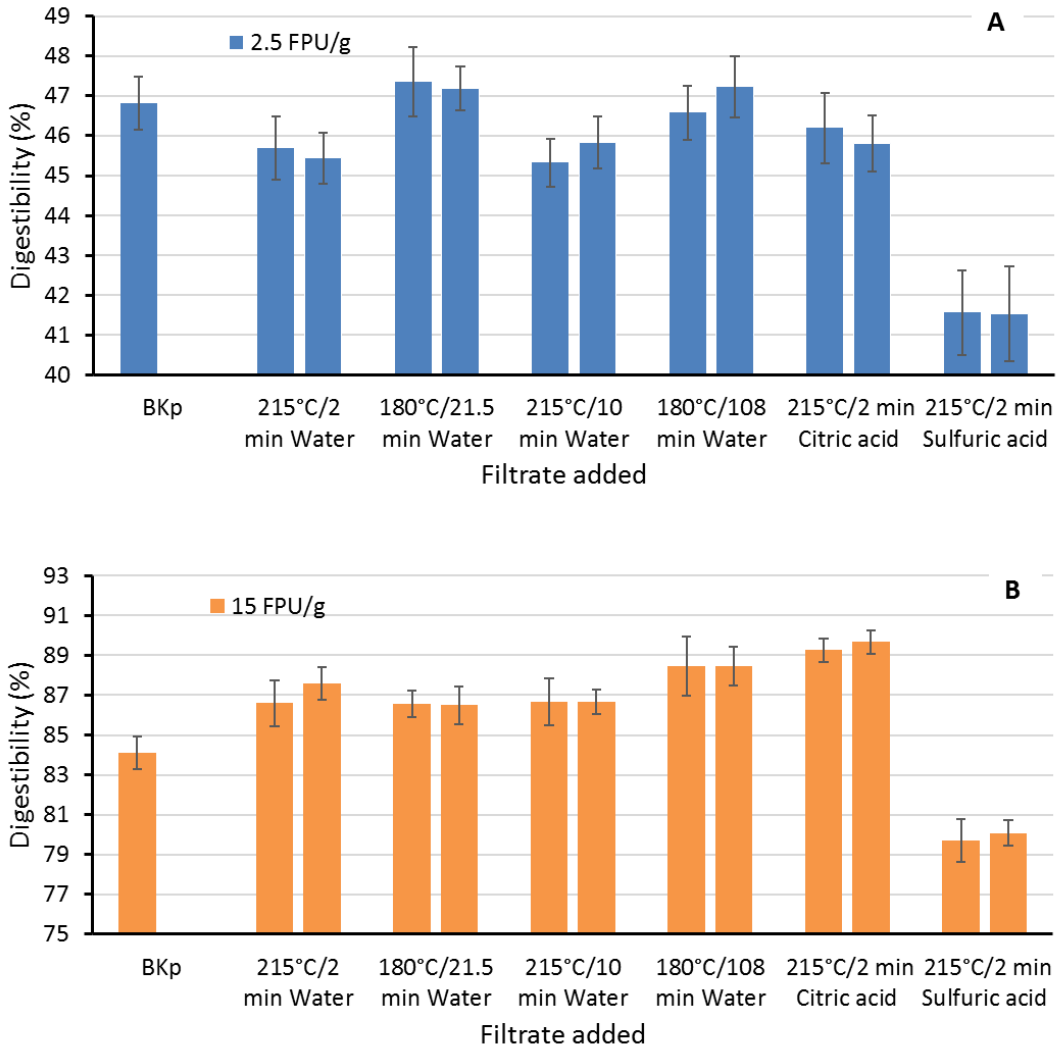


Figure 6.1 Digestibility of bleached kraft pulp (BKp) alone and with filtrates dosed back for CTec2 enzyme cocktail treatments at enzyme loadings of: 2.5 FPU/g (A) and 15 FPU/g (B). Triplicate determinations of steam pretreated experimental duplicates, the latter represented as individual bars, error bars represent ± two standard deviations.

To confirm that the filtrate components were acting as reducing cofactors for LPMO enzymes and were therefore responsible for the enhanced digestibility of BKp in the presence of filtrate, the experiment was repeated using an older commercial enzyme product that does not have any LPMO activity; Celluclast 1.5L supplemented with β-glucosidase [239]. The digestibility trends for the Celluclast enzyme cocktail differed from those observed for CTec2 cocktail (**Figures 6.1** and **6.2**), and showed that neither enzyme dose of Celluclast had enhanced digestibility (**Figure 6.2**). This coincides with the findings of Canella *et al.* [239], who also found improved efficiency of CTec2 relative to Celluclast. They showed this to be due to the production of *C*1-oxidised products, such as aldonic acids.

Figure 6.2A shows that all the filtrates, except for the filtrate from 180ºC/21.5 min reduced the digestibility of the bleached kraft pulp when treated with 2.5 FPU/g Celluclast supplemented with β-glucosidase, but the reductions were small. At the higher 15 FPU/g Celluclast enzyme loading, digestibilities in the presence of the filtrates were not significantly different from the control digestion at the 95% confidence interval (**Figure 6.2B**). This indicated that higher enzyme doses could overcome the small levels of enzyme inhibition found in the filtrates from the steam pretreatment conditions used in this study. This was expected given the inhibitor to enzyme ratio would be smaller than at a lower enzyme dose and justified the use of two enzyme loadings throughout the study.

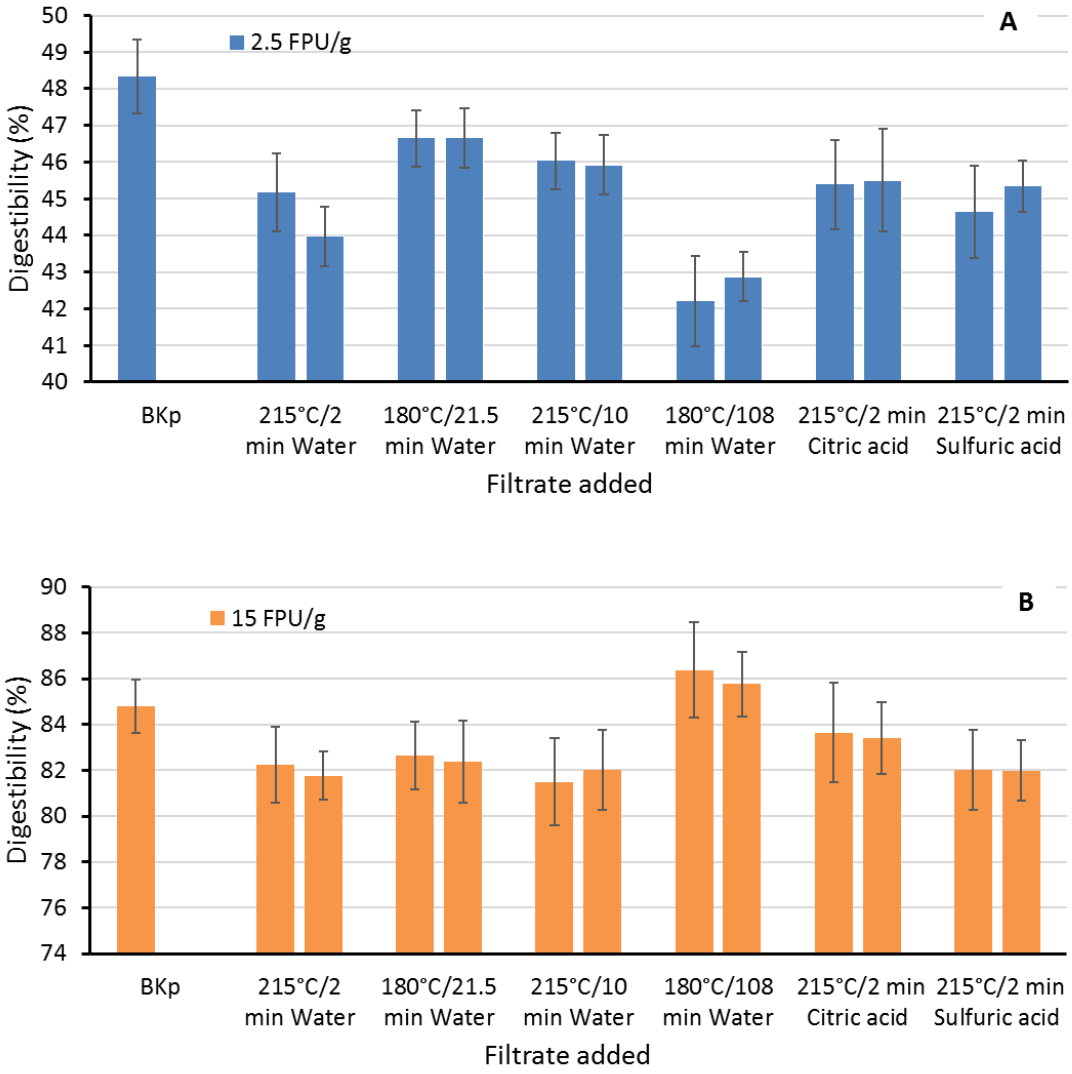


Figure 6.2 Digestibility of bleached kraft pulp (BKp) alone and with filtrates dosed back using Celluclast cocktail supplemented with β-glucosidase (1:1.125 ratio) at enzyme loadings of: 2.5 FPU/g (A); 15 FPU/g (B). Triplicate determinations of steam pretreated experimental duplicates, the latter represented as individual bars, error bars represent ± two standard deviations.

Overall, the inhibitory effects of the filtrates on the digestibility of BKp were minor for most enzyme, enzyme loading and pretreatment filtrate combinations. This indicated that economically there would be no significant or detrimental effect on the enzymatic hydrolysis stage of forward feeding the filtrates during processing.

6.3.2 Filtrate inhibition accounting for LPMO activity

The enhanced digestibility seen when using CTec2 enzyme raised the question of whether it was possible to isolate and differentiate between enzyme inhibition and enzyme enhancements. It was hypothesised that the true inhibitory effects of filtrate components could be isolated by ensuring a reducing agent was present in excess during enzymatic hydrolysis. If filtrate components were in fact acting as reducing agents for the LPMO enzymes, no digestion enhancements would be expected in the presence of additionally added reducing agents. Additionally, any inhibitory effects of the filtrates would not be masked by the activity of the LPMO enzymes. Various organic acids have been found in literature to be beneficial co-factors, including gallic acid and ascorbic acid [58,65,240], and the reducing agent of choice in this study was gallic acid.

The digestibility of BKp was assessed using CTec2 enzyme cocktail in the presence of gallic acid. The digestibility of BKp in the presence of the filtrates and 0.01M gallic acid when treated with an enzyme dose of 15 FPU/g showed that the presence of filtrates had no inhibitory or enhancing effects upon the digestibility of the BKp control that contained no filtrate (**Figure 6.3B**). At the lower enzyme loading of 2.5 FPU/g, the digestibility of BKp in the presence of gallic acid and filtrates again showed no observable enhancements and only two samples (215ºC/2 min and sulfuric) gave slightly lower digestion levels than the control. These findings along with the digestibility data using Celluclast (**Figures 6.2**) confirmed that the enhanced digestibility observed for CTec2 (**Figure 6.1B**) was consistent with filtrate components acting as reducing co-factors for the LPMO enzymes.

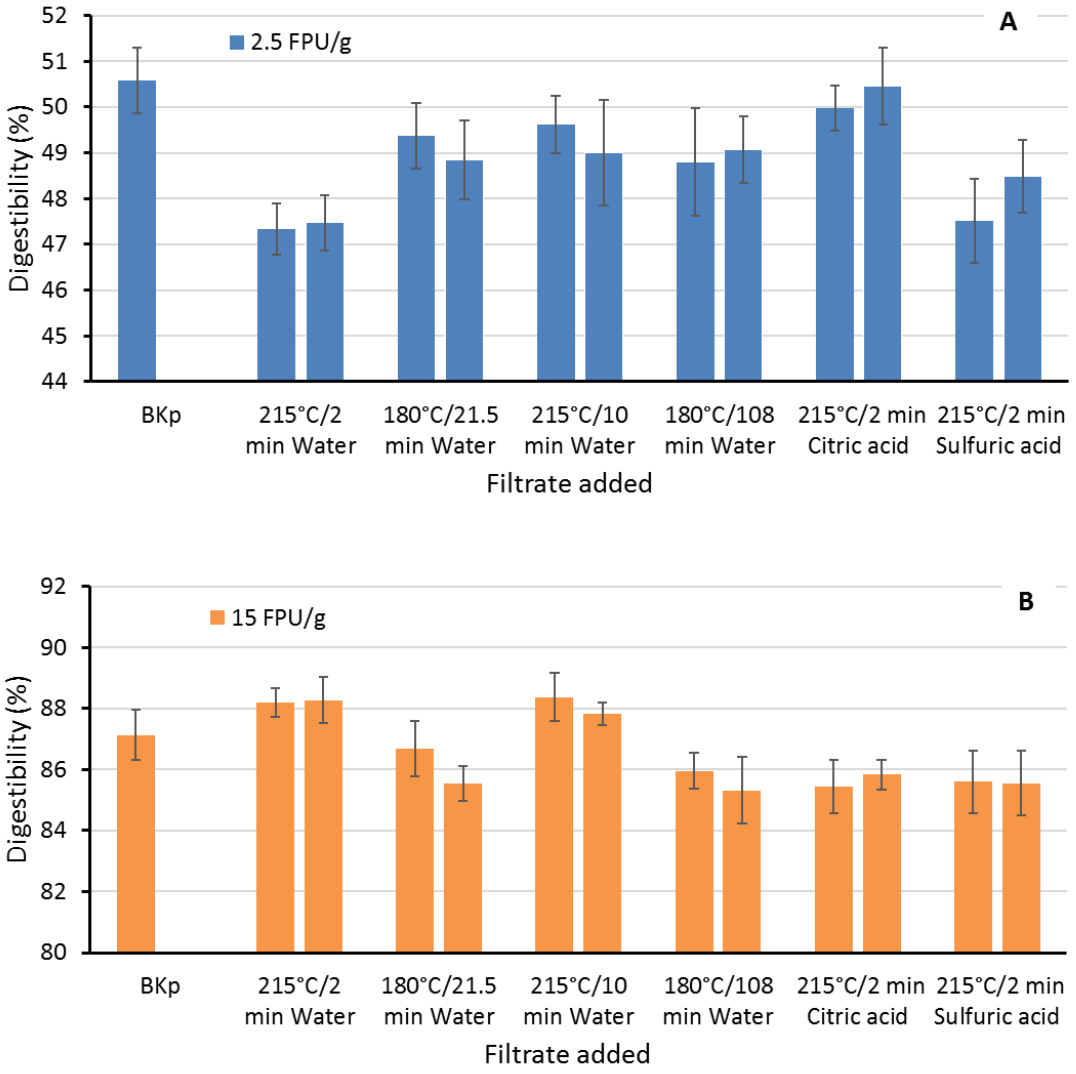


Figure 6.3 Digestibility of bleached kraft pulp (BKp) with the corresponding filtrate dosed back in the presence of 0.01M gallic acid using CTec2 enzyme at enzyme loadings; 2.5 FPU/g (A) and 15 FPU/g (B). Triplicate determinations of steam pretreated experimental duplicates, the latter represented as individual bars, error bars represent ± two standard deviations.

6.3.3 Effect of filtrates on the digestibility of steam pretreated substrates

Although the filtrates in this study had only small inhibitory effects, it was not known whether the inhibitory effects of these filtrates are the same for different substrates. Non-uniform inhibition could result from different physical and chemical environments of substrates, e.g., one substrate may contain lignin, another may contain no lignin, and the lignin itself may chemically or physically influence potential enzyme activity once combined with filtrate components. To determine

this, the effects of the filtrates on the digestibility of the corresponding steam pretreated substrates were also assessed.

Filtrates were dosed back to their corresponding pretreated substrate that had been ball-milled to a common cellulose accessibility (1.8 O:B dye ratio). The effects from the filtrate were then determined by comparing the differences in digestibility of the solid with filtrate to the digestibility of the substrate with no added filtrate. Since previous experimental duplicates of substrates and filtrates gave the same digestibilities and inhibition levels within experimental errors (95% confidence interval), only one of the experimental duplicate substrates and its corresponding filtrate was represented (both experimental duplicates were analysed and agreed within experimental error, data not shown).

The reason for using the ball-milled substrates of a common cellulose accessibility, instead of the substrates directly from the steam pretreatment, was to ensure both inhibition and enhancements of digestibility could be observed. This observation was predicted to be easier for substrates of common cellulose accessibility, as the level and range of digestibilities were of more practical interest. For example, the digestibility range for pretreated substrates at common accessibility using CTec2 enzyme loading of 15 FPU/g were 20% to 60% compared to 5% to 20% for steam pretreated substrates not ball-milled. If pretreated substrates had been chosen, any changes to digestibility would have needed to be large to be noted as true observations.

The digestibilities of the substrates at common cellulose accessibility when digested with 2.5 FPU/g CTec2 in the presence of the filtrates varied (**Figure 6.4A**). The digestibility was enhanced for the 180ºC/21.5 min steam pretreated substrate from 24% to 27% in the presence of its filtrate. The most severely pretreated filtrate (sulfuric acid) was the only filtrate to show inhibition, as seen by the slightly lower digestibility ~3.5% compared to the digestibility of the control substrate with no filtrate added of ~5% (**Figure 6.4A**).

At the higher CTec2 enzyme loading of 15 FPU/g only very minimal differences (1% to 5%) in the digestibility of substrates with and without filtrate were noted: two filtrates (180ºC/21.5 min and 215ºC/10 min) gave enhanced digestion, whilst

the sulfuric acid pretreated filtrate was the only filtrate to give inhibition as shown by its lowered digestibility (**Figure 6.4B**).

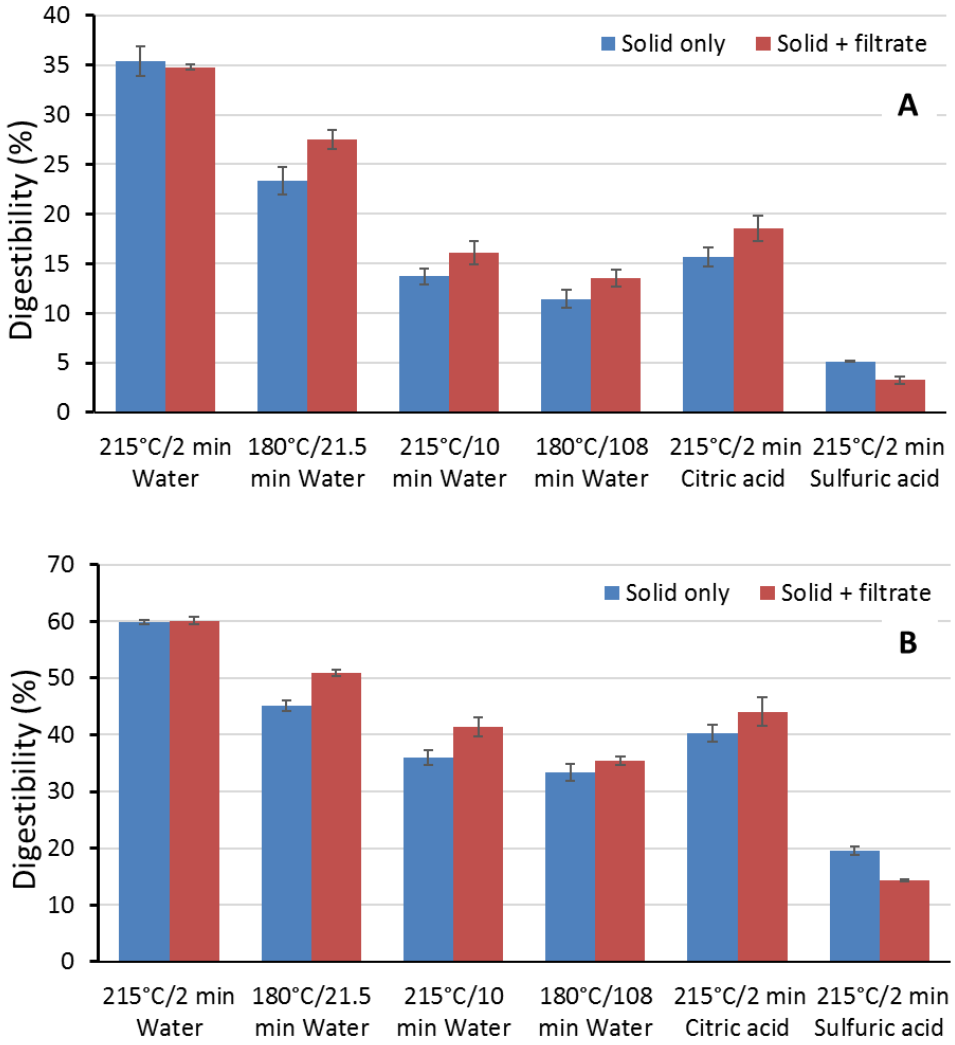


Figure 6.4 Digestibility of steam pretreated substrates at a common cellulose accessibility of O:B ratio of 1.8 with and without the corresponding filtrate dosed back at concentration obtained from pretreatment using CTec2 enzyme at enzyme loadings: 2.5 FPU/g (A) and 15 FPU/g (B). Error bars represent ± two standard deviations of triplicate analyses.

The small enhancements in digestibility were again believed to be due to LPMO activity. To prove this, filtrate dose back experiments were also performed using Cellulclast 1.5L supplemented with β-glucosidase (**Figure 6.5**). The digestibilities of the pretreated substrates with no added filtrates and substrates with filtrates remained unchanged within experimental error for all filtrates at both enzyme loadings (**Figures 6.5A** and **6.5B**).

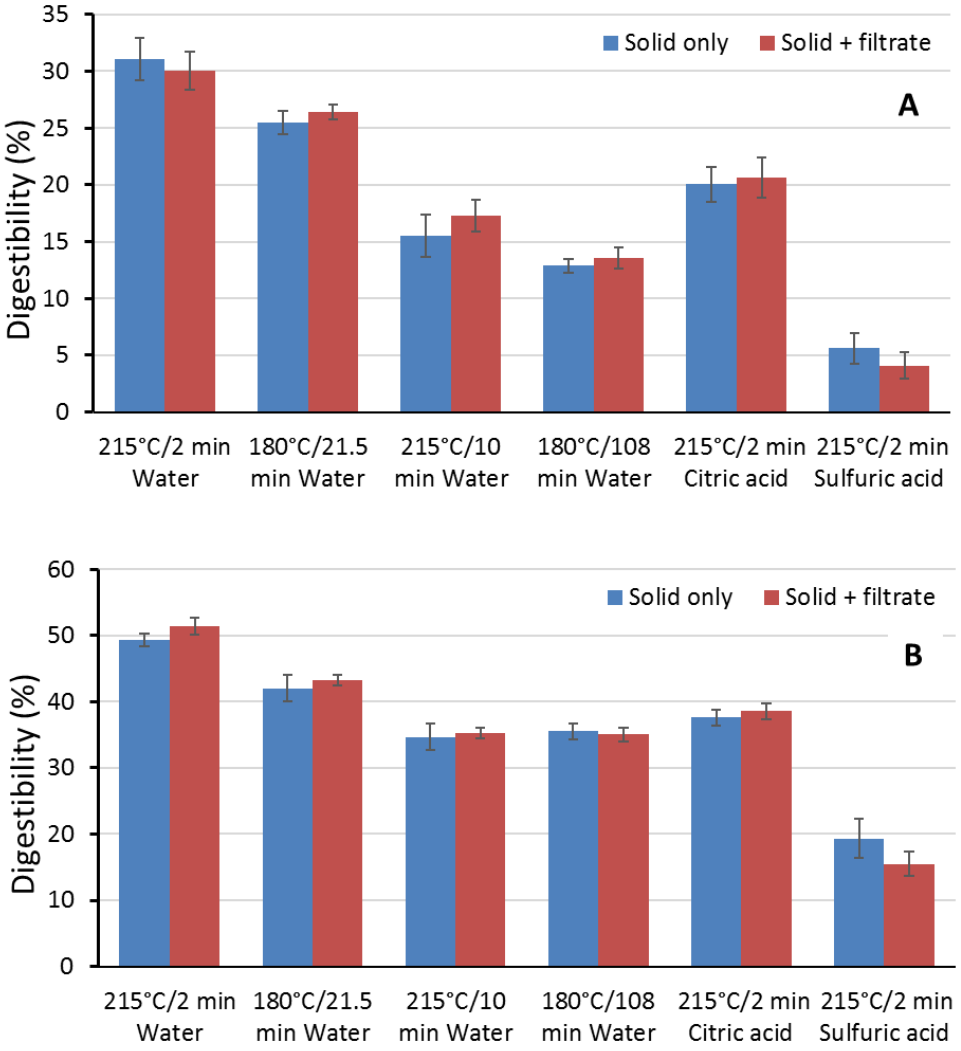


Figure 6.5 Digestibility of steam pretreated substrates at a common cellulose accessibility of O:B ratio of 1.8 with and without the corresponding filtrate dosed back at concentration obtained from pretreatment using Celluclast supplemented with β-glucosidase (1:1.25 ratio) at enzyme loadings: 2.5 FPU/g (A) and 15 FPU/g (B). Error bars represent ± two standard deviations of triplicate analyses.

The above findings indicated that any enzyme inhibition which arose from the filtrates produced in this study was minimal. This being the case then there would be no need to separate the filtrate from the solid fraction before enzyme hydrolysis. However, the possibility remains that some components in the filtrates may have detrimental effects on later fermentation. This falls outside the scope of this investigation and would need to be determined in a separate study.

6.3.4 Relative importance of substrate and filtrate inhibition

Figures 6.4 and **6.5** show the relative effects that water-soluble filtrate components and insoluble substrates had on the enzymatic digestion of steam pretreated radiata pine. Overall, the insoluble substrates exerted greater inhibitory effects than the soluble filtrate components.

An example that highlights the relative effects, was that when using CTec2 as the enzyme (15 FPU/g), the digestibility of the low severity pretreated (215°C/2 min) substrate at a common cellulose accessibility, had digestibilities of around 60%, this decreased to 20% for the most severely pretreated (sulfuric acid) substrate. This indicated that increases in pretreatment severity results in substrates that have considerable levels of enzyme inhibition. As across the pretreatment severity used in this study the digestibility fell by 40%.

If the filtrates had significant relative inhibition, it would be expected that the digestibility of substrates in the presence of the filtrates, would be significantly reduced. However, this is not the case, instead when filtrates were dosed back the digestibility of the substrates was similar to the digestibility levels without filtrate presence. This observation was consistent for both the CTec2 and Celluclast enzymes, at both the high and low enzyme loadings (**Figures 6.4** and **6.5**).

6.3.5 Filtrate inhibition and composition

The composition of sugars, furfural and HMF, in the filtrates have been reported in Chapter 3 (**Table 3.6**). The intention at the start of this study was to identify which components in filtrates were responsible for inhibition of enzymatic hydrolysis. However, since the inhibition attributable to filtrates was small, a decision was made not to explore links between decreases in digestibility and filtrate composition. As such, the components in the filtrates that are contributing to enhancing LPMO activity are unknown and will remain unknown without comprehensive compositional analysis and testing of individual components.

6.4 Summary

Overall, the inhibitory effects of the filtrates were minor for most enzyme, enzyme loading and pretreatment filtrate combinations. The digestions with CTec2 did not show any significant inhibition, except with the most severely pretreated (sulfuric acid) filtrate.

Although the enhancements of digestibility at a high CTec2 enzyme loading were initially surprising, the observation was shown to be due to filtrate components acting as reductants for the LPMO enzymes in CTec2. This was confirmed by using an enzyme that contained no LPMO enzymes and by maximising the potential LPMO effects whilst using CTec2 in the presence of reductants.

The most important finding was that, under the pretreatment conditions used in this study, the inhibitory effects of the filtrates were much less significant than those of the substrates (Chapter 5).

7 Lignin inhibition, isolation, and characterisation

7.1 Introduction

Lignin is widely accepted as one of the primary components that interferes with enzymatic hydrolysis of lignocellulosic biomass. Many studies show negative correlations between hydrolysis yields of cellulose and lignin content [77,110,116,135,144,210,215,241,242]. Additionally, delignification has been shown to increase the digestibility of substrates [243,244]. However, the inhibitory role that lignin has on enzyme hydrolysis is incompletely understood.

Lignin can hinder enzyme hydrolysis of cellulose in two ways: steric hindrance wherein lignin restricts enzyme access to cellulose; non-productive inhibition where the lignin binds with the enzymes, particularly through hydrophobic interactions [129].

The inhibitory effect of residual lignins is believed to be associated with the physiochemical properties and functional groups present in the lignin [144,245]. Various studies have tried to correlate hydroxyl, carboxyl and other functional groups of lignin to the inhibitory nature of lignin; however, the results are often contradictory and/or inconclusive due to the variety of pretreatment types, lignocellulosic biomass sources, and pretreatment conditions used [150,227,246]. Phenolic hydroxyl groups have been shown to be crucial for the negative impact that model lignin compounds had on the enzyme hydrolysis of Avicel [144] and increased aliphatic hydroxyl groups have been correlated to increases in the adsorption capacity of lignin and enzymes [247]. In contrast, Yu *et al.* [245], found the total hydroxyl groups in lignin to have positive effects on enzyme hydrolysis. The presence of carboxylic and sulfonic acid groups in lignin have been proposed to reduce non-productive binding through electrostatic repulsions and therefore increase hydrolysis yields [227].

The type of lignin can also influence the extent to which cellulose hydrolysis is inhibited, with softwood lignin being found to be more inhibitory than hardwood lignin [150].

The pretreatment has been shown to influence the inhibitory response of lignin. During steam pretreatment, the lignin structure is modified and functional groups that can non-productively bind to enzymes can form. While washing removes the soluble lignin that may be inhibitory, most of the lignin remains in the insoluble substrate [146]. Higher severity pretreatments have been reported to produce more inhibitory lignins, irrespective of whether the starting material is softwood or hardwood [246]. Non-productive binding of enzymes by lignin has been investigated indirectly through the use of surfactants and exogeneous proteins, which act as blocking agents, blocking sites on lignin that might otherwise bind enzymes [156,157,162,165]. Nonetheless, how steam pretreatment changes lignin structure in particular that of softwoods remains unclear and certainly the functional groups responsible for enzyme inhibition are still not well-understood.

In this chapter, an investigation of the lignin-enzyme interactions of steam pretreated *P. radiata* is described. The objective was to demonstrate that the lignin present in substrates after steam pretreatment of *P. radiata* wood is inhibitory towards cellulase enzymes and then to determine whether lignin location, content or composition has the greatest inhibitory influence.

7.2 Materials and methods

7.2.1 Chemicals

Dioxane, hydrochloric acid, sodium bicarbonate, proteinase bacterial lyophilised powder (type XXIV, 10 units/mg solid) and polyethylene glycol (PEG, Mw 4000) were obtained from Sigma Aldrich.

7.2.2 Microscopy-lignin location

Untreated and steam pretreated samples (as prepared in **section 3.2.4**) were sectioned in the transverse plane with a razor blade. Samples that had been ball-milled to a common cellulose accessibility were analysed without sectioning. Sections were mounted in 50% glycerol in phosphate buffer at pH 9, and examined with a Leica SP5 II confocal microscope using a 63x glycerol immersion lens [248]. Lignin autofluorescence was examined using 488 nm excitation and 500 nm to 650 nm emission. In some cases, widefield polarised light images were also

gathered to determine cellulose distribution on the same field of view. Fluorescence spectra were acquired from each sample using 355 nm or 488 nm excitation and 400 nm to 700 nm or 500 to 700 nm emission respectively in 5 nm steps with a slit width of 10 nm.

7.2.3 Polyethylene glycol (PEG) loading

Polyethylene glycol 4000 (PEG) was dosed to samples (pretreated substrates and pretreated substrates with common cellulose accessibility of 1.8 O:B Simons' stain ratio) by weighing enzyme(s) into a solution of PEG (1% *w/v*). Enzyme digestion was performed as described in **section 3.2.8**, with the required volume of enzyme being added to give the correct enzyme loading (2.5 FPU/g or 15 FPU/g) and a final PEG concentration of 0.1% *w/v*. The final PEG to substrate concentration was 0.067 g/g.

7.2.4 Lignin isolation

Lignin was isolated from three steam pretreated samples (sawdust, 215°C/10 min and 215°C/2 min sulfuric acid); an overview of the process can be seen in **Figure 7.1**.

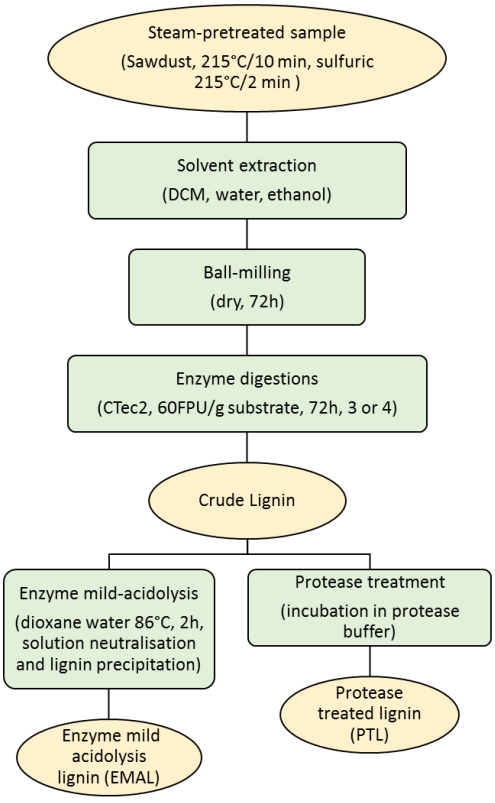


Figure 7.1 Lignin isolation overview.

The extractives were removed from the three steam pretreated samples (100 g O.D.) by sequential 24-hour extractions using dichloromethane (DCM, 45°C), water (110°C), and ethanol (85°C). The extractive-free samples were air dried overnight (O/N) prior to being ground (20 mesh, then 40 mesh) and air dried again (O/N). Ball-milling was then performed under nitrogen, on the extractive-free ground material (40 g O.D. solid per vessel) for 72 hours using a Schwingmühle VIBRATOM vibratory ball-mill with ceramic vessels (0.6 L) that contained two hundred 15 mm alumina balls.

Enzymatic hydrolysis of the ball-milled substrates were then carried out at 5% *w/v* solids loading in a citrate buffer (50 mM) at pH of 4.5 in an incubator with constant mixing [249,250]. The hydrolysis was performed at 60 FPU/g substrate using Novozymes Cellic® CTec2 enzyme cocktail. After 48 hours, the supernatant was decanted and the digestion step was repeated. After each hydrolysis, an indicative glucan to glucose conversion was determined by measuring glucose in the decanted supernatant using a YSI-2700 glucose analyser. The idea was to ensure the glucose levels were becoming as low as possible, indicating successful carbohydrate removal. Three digestion steps were required for the sawdust and 215°C/10 min samples, while four digestions were required for the 215°C/2 min sulfuric acid pretreated sample. After the supernatant from the final digestion was decanted, the solid residue remaining was washed twice using pH 2 (1 M HCl) water. This residue was the crude lignin.

7.2.4.1 **Enzymatic mild-acidolysis lignin (EMAL)**

EMAL lignins were isolated from the crude lignin according to the procedure of Wu and Argyropoulos [250]. Crude lignin (12-20 g) was placed in dioxane water (85:15 *v/v*, 0.01 M HCl) on a 20% *w/v* basis and heated at 86°C for 2 hours. The solution was filtered and the supernatant neutralised using sodium bicarbonate (0.1 M). The neutralised solution was added dropwise to pH 2 water (1.5 L, 1 M HCl) and left overnight to ensure full precipitation. This solution was then centrifuged and filtered to obtain the enzymatic mild-acidolysis lignin (EMAL). The EMAL was washed twice with water, freeze-dried, sieved through a mesh (<160 µm) and stored for future use.

7.2.4.2 **Protease treated lignin (PTL)**

Protease treated lignin was obtained using a method based on previous reports [135,251]. Crude lignin (~2.3 g) was incubated (37°C) overnight in a 50 mM phosphate buffer (pH 7) containing proteinase (10 units/mg lignin). The solution was filtered and protease treated lignin (PTL) was washed extensively three times with water (pH 2.5, 1 M HCl) followed by two washes using distilled water. The PTL was freeze-dried, sieved through a mesh (<160 µm) and stored for future use.

7.2.5 **Compositional analysis**

The compositions of the residual carbohydrates in the EMAL and PTL lignins were determined as described in **section 3.2.7.3**. The consistency, carbon, hydrogen and nitrogen (C, H and N) contents were determined on a micro-scale at the Campbell Microanalytical Laboratory, University of Otago.

7.2.6 **Lignin inhibition**

Enzymatic hydrolysis of BKp was performed as described in **section 3.2.8** in the presence or absence of lignin. All experiments specified which lignin was used, EMAL or PTL, as well as enzyme type (CTec2 or Celluclast supplemented with β-glucosidase) and enzyme loading. The amount of lignin used was 37.5 mg, unless otherwise stated. Glucose readings were corrected for: the glucose in the enzyme solution; the residual soluble glucose in the unhydrolysed substrate, and; the glucose released from hydrolysing the residual carbohydrates of the isolated lignin. Each sample was analysed in triplicate and values had to agree within ± 5 % of each other to be accepted. Additionally, a control substrate (R49TBM300), with a known glucose yield had to be within ± 5 % of expected value(s) for the results from the batch to be accepted.

7.2.6.1 **Percent lignin inhibition**

Lignins (PTL) were dosed in increasing amounts (7.5, 15, 22.5, 30, 37.5 mg) to BKp and enzyme hydrolysis was performed as described in **section 7.2.6**, using Celluclast supplemented with β-glucosidase (1:1.25 FPU/IU ratio) at an enzyme dose of 2.5 FPU/g substrate.

7.2.7 Lignin characterisation

7.2.7.1 Quantitative ^{31}P NMR spectroscopy

A quantitative ^{31}P NMR spectroscopy method was adapted from Granata and Argyropoulos [252]. An accurately weighed lignin sample (30 mg); an internal standard, *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide (135 mM in DMF, 100 µL) in a solution of pyridine and $CDCl_3$ (1.6:1 *v/v*, 400 µL stored over molecular sieves), [253]; and a relaxation agent, chromium acetylacetonate (16.35 mM in pyridine/$CDCl_3$ (1.6:1 *v/v*), 100 µL) were added to a reaction vial and stirred. Once the lignin had completely dissolved the phosphitylating agent, 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP, 100 µL) was added. The solution was mixed briefly for several seconds at ambient temperature and then transferred to a 5 mm NMR tube and analysed immediately by ^{31}P NMR on a Bruker Avance AV III 400 MHz spectrometer using a CryoProbe (Prodigy BBO). Spectra were collected using 256 scans, a 10 second delay, and processed with exponential line broadening of 2 Hz. The chemical shift scale was calibrated on the signal from phosphitylated water (132.2 ppm). Integration of the peaks was based on the following chemical shift regions: internal standard (152.14-151.48 ppm); aliphatic OH (149.80-144.60 ppm); condensed guaiacyl phenolic (144.60-140.50 ppm); uncondensed guaiacyl phenolic (140.50-138.45 ppm); H-type (138.45-137.40), and; carboxylic OH (136.00-133.50 ppm).

7.2.7.2 2-D Heteronuclear single quantum coherence NMR (HSQC)

The method for 2-D heteronuclear single quantum coherence (HSQC) NMR experiments was based on that used by Rahikainen *et. al* [215]. EMAL lignin (~40 mg) was dissolved in 1.0 mL of DMSO-d_6. The solution was filtered through glass wool to remove any fine undissolved particles. Although the amount of undissolved particles was not determined this was estimated to be less than 3% of the sample. The sample was analysed on a Bruker Avance III 400 MHz spectrometer. The spectral widths for ^{1}H and ^{13}C were 9328 Hz (13.3 ppm) and 29930 Hz (170 ppm), respectively. The number of scans was 40, and 256-time increments were recorded. The number of collected data points was 1024 for the ^{1}H dimension with a recycle delay of 1.5 s. The $^{1}J_{CH}$ used was 145 Hz. All data processing was performed using the Bruker Topspin NMR software.

7.2.7.3 **Fourier transform infrared spectroscopy (FTIR)**

The chemical groups of the isolated EMAL lignins were analysed by FTIR spectroscopy using a Bruker Tensor 27 instrument equipped with a single bound diamond attenuated total reflectance (ATR) cell (Bruker). The lignin samples were directly added into the sample cell and pressed uniformly against the diamond surface using the spring-loaded anvil. The IR spectra were obtained by averaging 128 scans from 400 to 4000 cm^{-1} [148]. Spectra were baseline corrected, and normalised using OPUS 7.2 software (Bruker). The intensity of the peak at 1500 cm^{-1}, corresponding to the C-C vibrations of the aromatic ring, was set to 2 (software default) and relative intensities of specific chemical shifts were calculated [148].

7.3 Results and discussion

7.3.1 **Indicative lignin inhibition**

At a common cellulose accessibility, it was found that steam pretreated radiata pine substrates from more severe pretreatment conditions had lower digestibilities (Chapter 5). The objective of this section was to demonstrate that the lignin present in substrates after steam pretreatment of *P. radiata* wood was inhibitory towards cellulase enzymes by using a surfactant and assessing changes in digestibility. If the digestibility increased as a result of surfactant presence, then the lignin is inhibitory to enzymes. This assumption was based on literature reports that show surfactants block sites on lignin that could otherwise bind enzymes [162,254]. If no changes to digestibility were observed the assumption would be that the lignin must not be responsible for the observed inhibitory responses.

Steam pretreated substrates, after steam explosion and after removal of soluble material by washing (as is), and substrates after ball-milling to a common cellulose accessibility (CA, Simons' stain O:B ratio 1.8), were digested using both CTec2 and Celluclast (supplemented with β-glucosidase) at low and high enzyme loadings of 2.5 FPU/g and 15 FPU/g. Digestions were performed in the absence and presence of polyethylene glycol (PEG, 0.067 g PEG/g substrate). Results are presented in **Figures 7.2-7.5**.

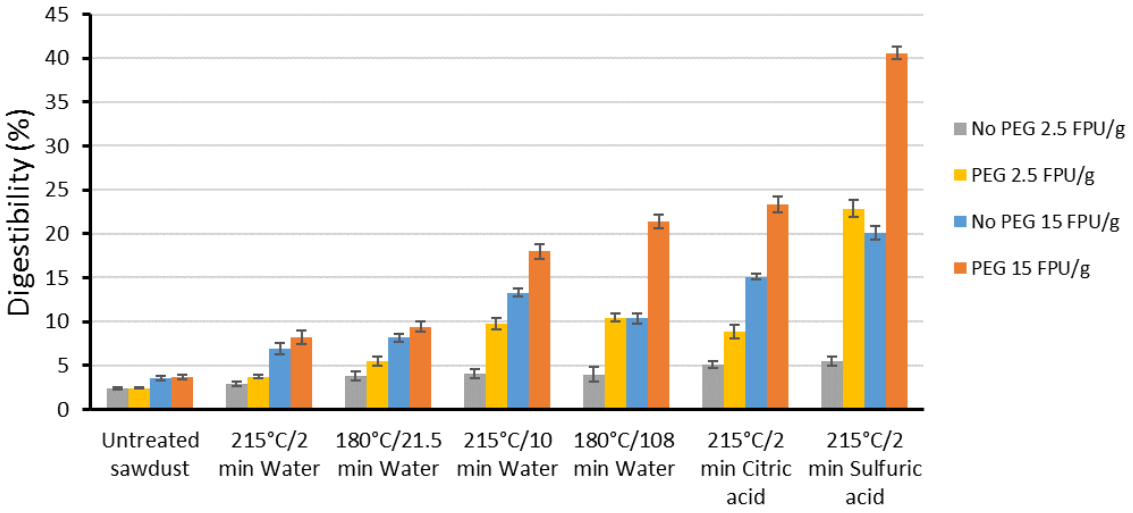


Figure 7.2 Digestibility of steam pretreated substrates using Celluclast (at two different enzyme loadings) supplemented with β-glucosidase in the presence and absence of polyethylene glycol (0.1% *w/v*). Error bars represent ± two standard deviations of triplicate analyses.

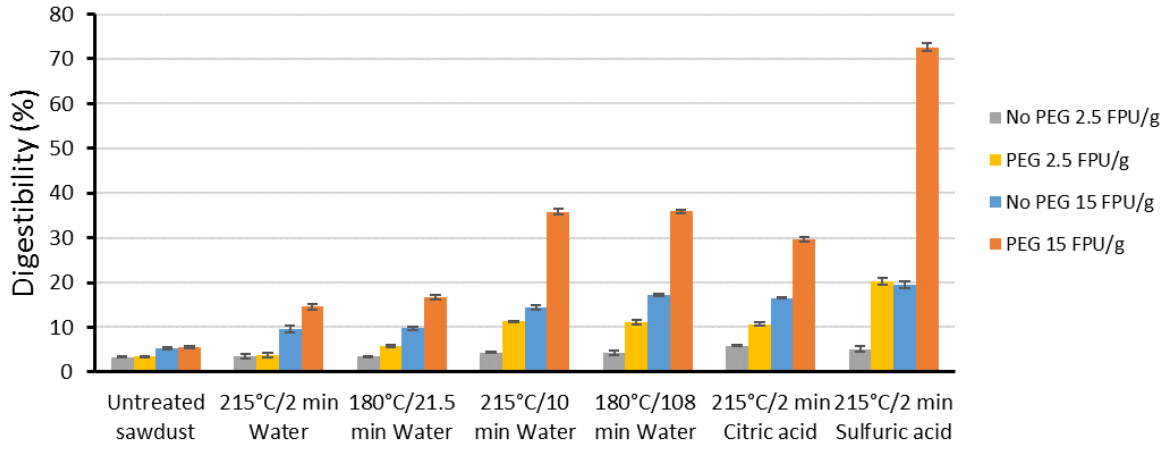


Figure 7.3 Digestibility of steam pretreated substrates using CTec2 (at two different enzyme loadings) in the presence and absence of polyethylene glycol (0.1% *w/v*). Error bars represent ± two standard deviations of triplicate analyses.

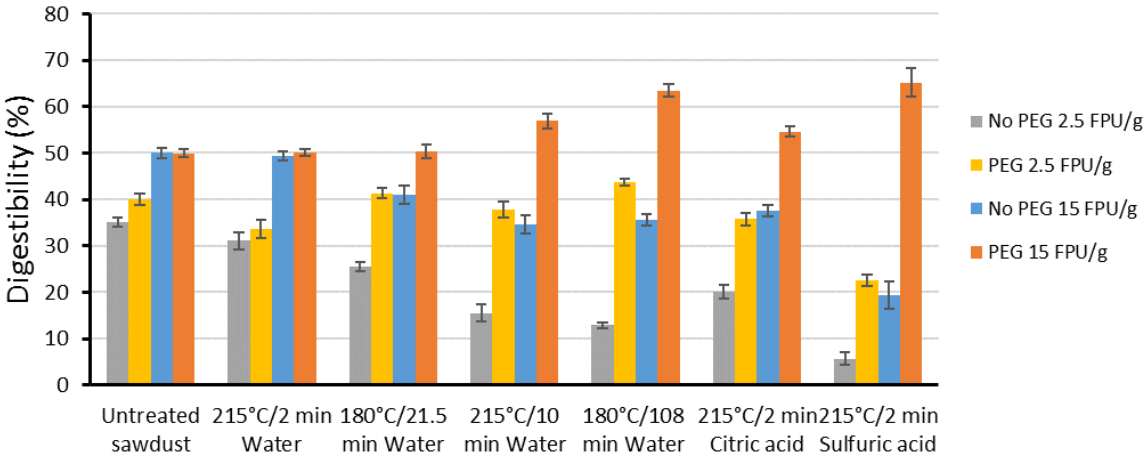


Figure 7.4 Digestibility of steam pretreated substrates at a common cellulose accessibility (O:B ratio of 1.8 as per Simons' stain procedure) using Celluclast (at two different enzyme loadings) supplemented with β-glucosidase in the presence and absence of polyethylene glycol (0.1% *w/v*). Error bars represent ± two standard deviations of triplicate analyses.

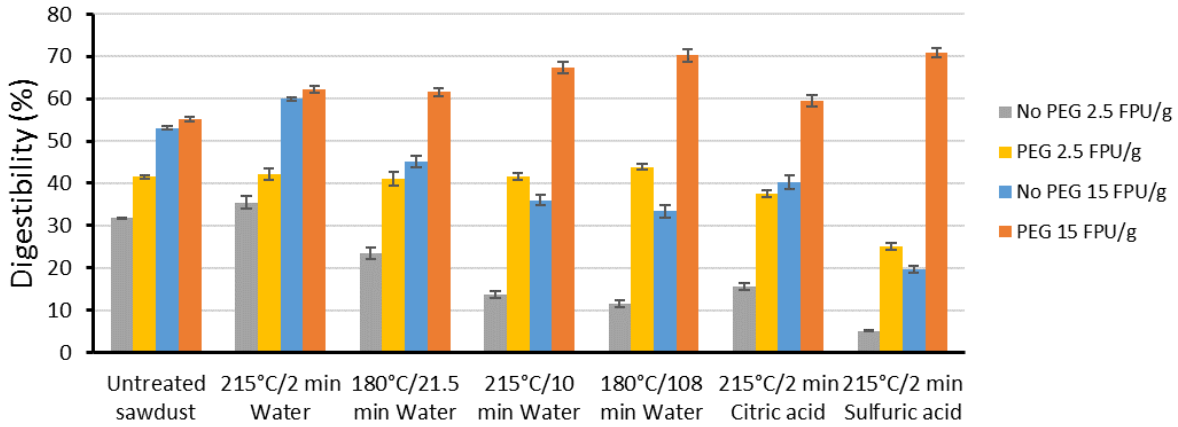


Figure 7.5 Digestibility of steam pretreated substrates at a common cellulose accessibility (O:B ratio of 1.8 as per Simons' stain procedure) using CTec2 (at two different enzyme loadings) in the presence and absence of polyethylene glycol (0.1% *w/v*). Error bars represent ± two standard deviations of triplicate analyses.

The presence of PEG enhanced the digestibility of most steam pretreated substrates and substrates of a common cellulose accessibility for both enzymes and at both the high and low enzyme doses (**Figures 7.2-7.5**). The increased digestibility due to PEG presence tended to be greater for the substrates that had been pretreated under more severe pretreatment conditions, the exception being the samples that were treated in the presence of citric acid. The increases in digestibility on adding PEG (% PEG responses) correlated well with the yield of insoluble solids after pretreatment (data not shown).

The greater PEG responses for the more severely pretreated substrates is believed to be due to removal of more hemicelluloses. This is because the removal of hemicelluloses not only increases the accessibility of enzymes to cellulose but also makes the lignin more accessible, thus increasing the probability of non-productive binding of the enzymes.

This concurs with the findings here. Not only did PEG improve digestibility, but it was more effective at doing so when in the presence of more severely pretreated substrates (**Figures 7.6** and **7.7**), this is in agreement with other studies [162,165].

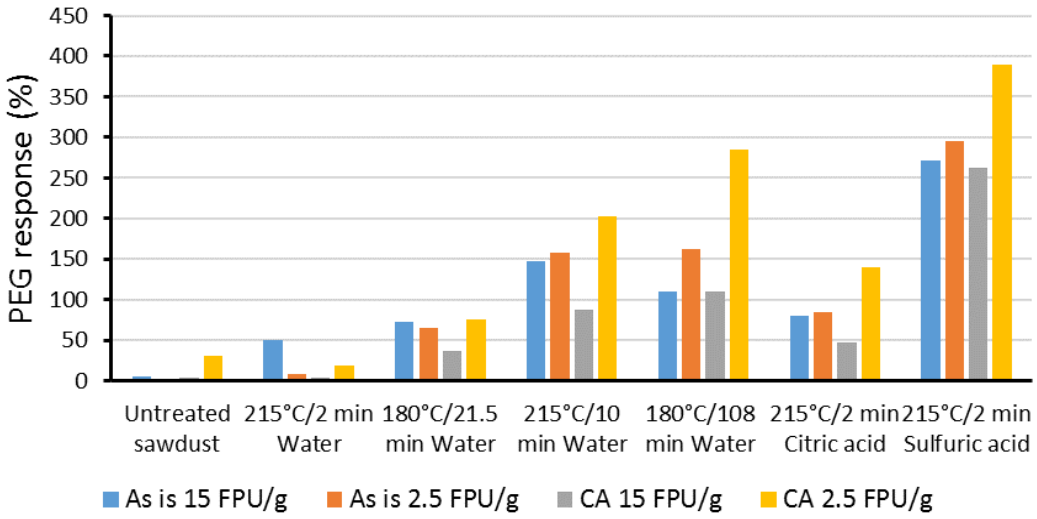


Figure 7.6 Effect of PEG as percentage increase in digestibility during enzyme digestion using CTec2 enzyme with different enzyme loadings. Substrates are steam pretreated (As is) and ball-milled to a common cellulose accessibility (CA) of Simons' stain O:B ratio of 1.8.

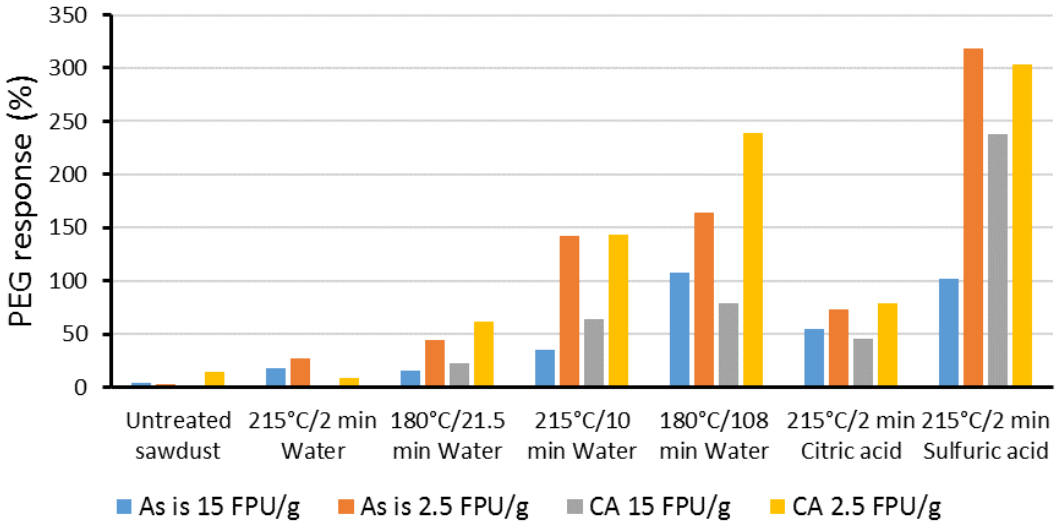


Figure 7.7 Effect of PEG as percentage increase in digestibility during enzyme digestion using Celluclast supplemented with β-glucosidase (1:1.25) with different enzyme loadings. Substrates are steam pretreated (As is) and ball-milled to a common cellulose accessibility (CA) of Simons' stain O:B ratio of 1.8.

In general, the benefit of adding PEG tended to be greater at the lower enzyme loading of 2.5 FPU/g than at the higher enzyme loading of 15 FPU/g, most likely because at the lower enzyme loading, the inhibitor to enzyme ratio was higher.

Although enhanced digestion was observed for both enzyme cocktails in the presence of PEG, the benefits from adding PEG during enzyme digestions using CTec2 were generally greater than the benefits with the Celluclast cocktail. The reason for this is unknown, but was probably due to the different enzymes in the two commercial cocktails and therefore their different responses to PEG.

The enhancements in digestibility when PEG was used were quite significant in terms of digestibility. However, future economic evaluations are required to determine which cost is most significant in order to get the same levels of increased digestibility; the cost of adding PEG or the cost of additional enzyme loadings. The cost of additives and resultant trade-offs have previously been highlighted by others as important in the economy of obtaining high sugar yields [162].

7.3.2 **Lignin location**

Lignin in the steam pretreated radiata pine substrates has been shown to be inhibitory; however, it is yet to be determined what effects lignin location plays on the overall inhibitory role of lignin. This section therefore provides an understanding of if, and how, the location of lignin impacts the digestibility of substrates.

The location of lignin in steam pretreated samples and in the untreated sample was observed *via* autofluorescence microscopy. The main location of the lignin was in the secondary walls and middle lamella as shown by high fluorescence areas in **Figure 7.8**. Varying the pretreatment conditions did not appear to alter this pattern.

It appeared that the two acid catalysed (citric acid and sulfuric acid) pretreatments resulted in swelling/expansion of the cell walls. This observation has previously been reported when using strong acid catalysts [220,221], and may also explain why the sulfuric acid pretreated sample had a higher cellulose accessibility than the other pretreated samples. Wood contains a mix of thin-walled earlywood cells laid

down during spring and thicker-walled latewood cells laid down during summer/autumn, so it is conceivable that thicker-walled latewood samples were examined in these two cases. However similar effects were seen in other images of the same sample making this explanation unlikely.

The lignin location in samples ball-milled to a common cellulose accessibility was also evaluated by autofluorescence microscopy. Ball-milling resulted in defibrillation of fibres and smaller particles being formed. This means obtaining a cross section of samples at a common cellulose accessibility was not possible, and the samples had to be analysed as received. Nonetheless, lignin was still identifiable by autofluorescence and appeared not to have shifted; lignin was present in both the secondary wall and middle lamella (**Figure 7.8**).

The location of lignin in steam pretreated substrates at a common cellulose accessibility did not aid in providing an explanation for why more severely pretreated substrates had lower digestibilities. Nor did lignin location explain why the PEG response was greater for the more severely pretreated substrates. Overall from this limited study, there was no evidence that lignin location was a significant factor influencing the diverse digestibilities previously seen.

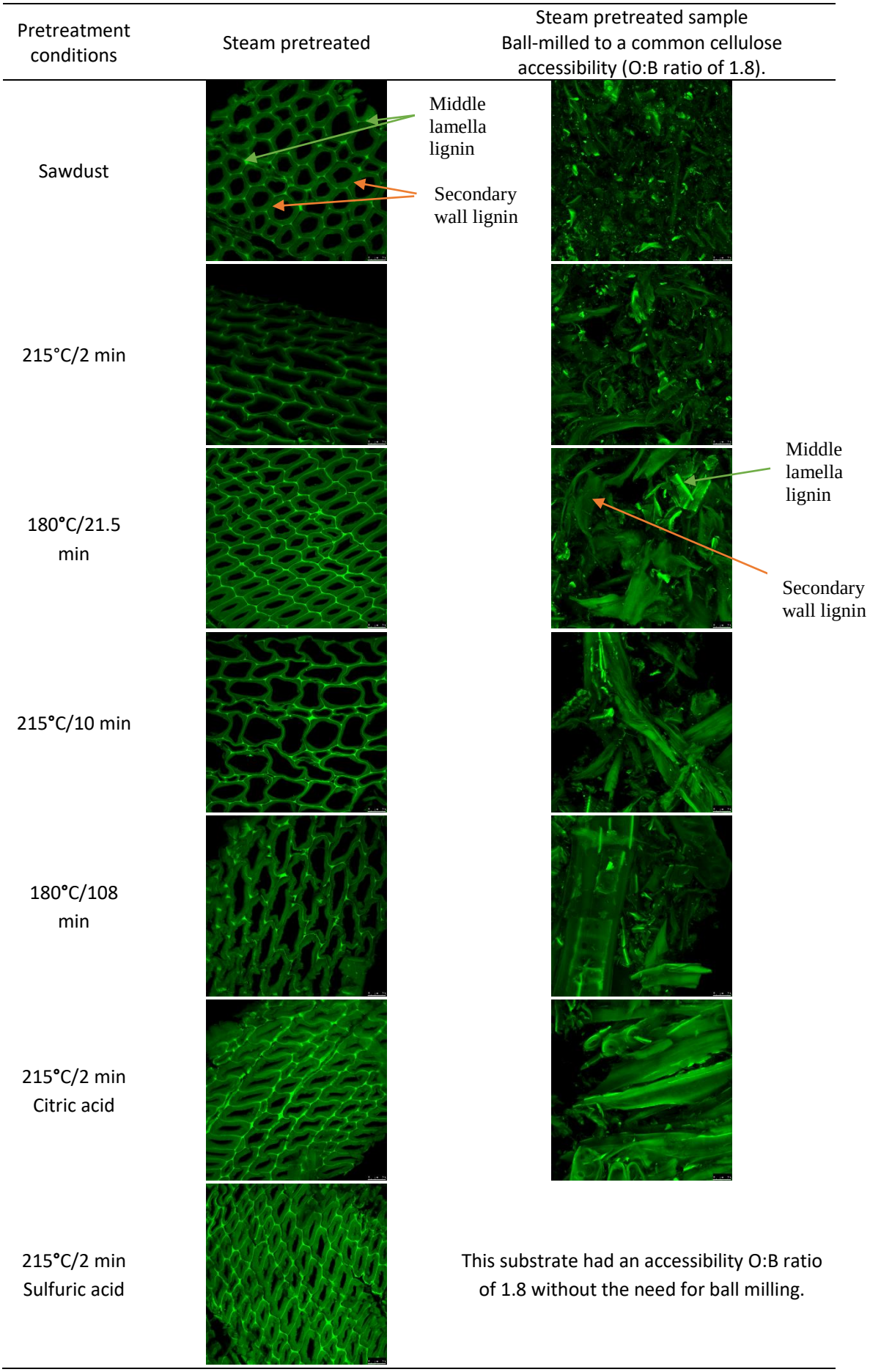


Figure 7.8 Autofluorescence images showing lignin location in steam pretreated substrates and substrates at a common cellulose accessibility (Simons' stain O:B ratio of 1.8).

Polarisation images were obtained of the pretreated substrates and ball-milled pretreated substrates to illustrate that there is a close correlation between the location of the lignin and cellulose [248,255]. An example can be seen in **Figure 7.9**, which shows the position of cellulose and lignin often coincides. This supports the notion that lignin may be limiting cellulose hydrolysis, either sterically, chemically or by a combination of both.

Consistent with our hypothesis that differences in the chemical composition play a role in determining the inhibitory effects of the lignins, distinctly different fluorescent emission spectra were obtained for the excitation of lignin at 355 nm and 488 nm (Data not shown). The different fluorescent emission profiles arise from differing chemical environments.

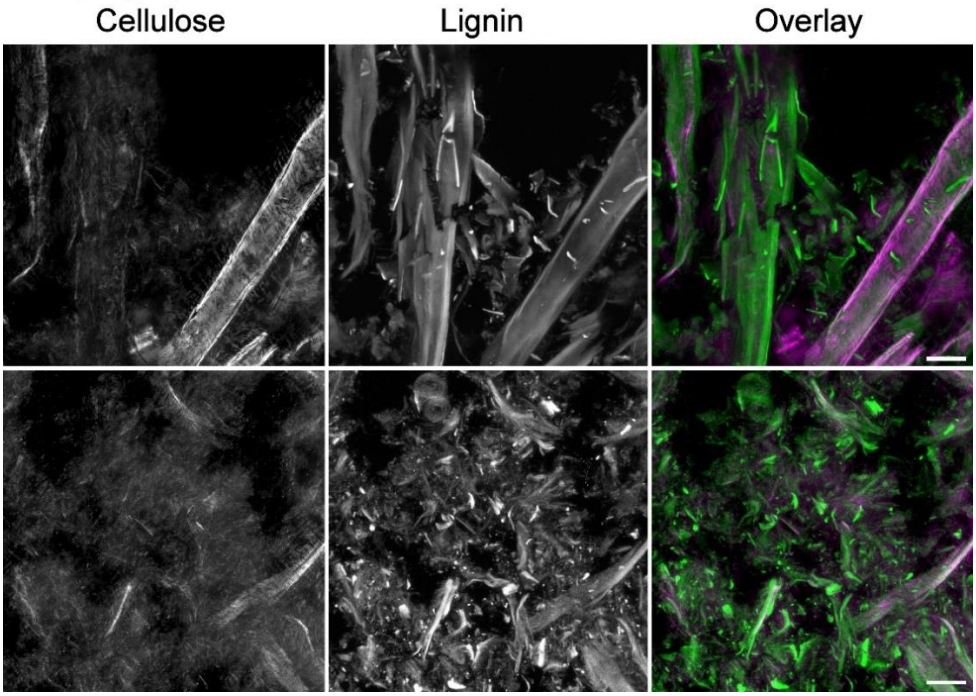


Figure 7.9 A comparison of cellulose and lignin in steam pretreated substrates ball-milled to a common cellulose accessibility of O:B ratio of 1.8 as measured by Simons' stain (top row 215ºC/10 min, bottom row sawdust). Cellulose is localised by polarised light (extended depth of focus image) while lignin is localised by autofluorescence (excitation 488 nm, emission 500-650 nm, maximum intensity projection). In the overlay image lignin is green and cellulose magenta. Scale bar = 30 µm.

7.3.3 Lignin isolation

Lignins were next isolated from three representative steam pretreated substrates to evaluate the effects of lignin amount on digestibility and to determine their chemical composition.

The isolation of lignin from biomass for characterisation needs to be accomplished with minimal structural changes. To date various approaches are used, and each approach has advantages and disadvantages [249]. Milled wood lignin is the most widely used type of lignin, however, it results in low lignin yields of ~10% of lignin in original wood [256]. A more modern approach of enzymatic mild acidolysis lignin (EMAL) uses enzymes during isolation and mild acid treatment. The EMAL isolation procedure usually gives 2 to 5 times greater lignin yields than the MWL procedure [256]. High yields of lignin are a benefit as it means the lignin is more representative of the original lignin in a substrate. Although EMAL may contain some protein impurities, it has a low carbohydrate content and provides a good lignin for structural analysis [256]. The acid hydrolysis step also aids in cleaving lignin-carbohydrate bonds, something that often impedes lignin being able to be isolated in high yields [256]. Protease can be used to assist in removing some of the protein impurities after lignin is milled and treated with enzymes. Such protease treated lignin (PTL) gives the highest yields (~90%), but are often contaminated with carbohydrates, as lignin-carbohydrate bonds are not extensively degraded. Consequently, PTL lignins are less useful for analysis by NMR [148,227].

In this study both enzymatic hydrolysis lignin (EMAL) and protease treated lignin (PTL) were isolated from three of the steam pretreated samples: untreated sawdust, 215°C/10 min, and 215°C/2 min sulfuric acid. Both PTL and EMAL were isolated, as it is known that although PTL is representative of the original lignin in a sample and can be obtained in high yields, it is not very soluble in organic solvents and thus chemical characterisation using NMR is not possible [148,227].

7.3.3.1 **Composition of lignin**

The composition of the PTL and EMAL lignins including carbohydrates, elemental composition, acid soluble and acid insoluble lignin content are shown in **Table 7.1**. The EMAL and PTL lignins were isolated in reasonably high purity, with lignin content being 80-96%. The carbohydrate content was less than 10% for all lignins except for the untreated sawdust PTL which had a carbohydrate content of 13.7%. As expected, the EMAL lignins had lower carbohydrate contents than the corresponding PTL lignins, this was due to the additional carbohydrate removal and purification stages of the EMAL isolation.

During the isolation of the EMAL and PTL lignins the residual protein content decreased (based on nitrogen content); the protein of the crude lignin prior to protease or mild acidolysis treatment was 1.18%, 2.18% and 2.37% for the sawdust, 215°C/10 min and 215°C/2 min sulfuric acid samples, respectively. The PTL procedure decreased protein content by approximately one-third to half, which was a similar reduction to that reported by Rahikainen *et al.* [210]. The protein content was lower for the EMAL lignins due to the additional purification steps in the EMAL isolation.

Although the EMAL lignins appear to be purer than the PTL lignins, it must be noted that the overall EMAL lignin yields were considerably lower than the yields of PTL lignin. The yields of PTL from sawdust, 215°C/10 min and 215°C/2 min sulfuric acid lignins were 80%, 83% and 85%, respectively. While the EMAL lignin yields were only 14%, 10%, 4% for sawdust, 215°C/10 min and 215°C/2 min sulfuric acid, respectively.

Table 7.1 Chemical composition of isolated enzymatic mild acidolysis lignins (EMAL) and protease treated lignins (PTL).

		Carbohydrates (g/100g)*						Lignin (g/100g)			Elemental (%)		
Pretreatment Conditions		Ara	gal	gluc	xyl	man	Total sugars	ASL	AIL	Total	C	H	N
Untreated sawdust	EMAL	0.06	0.99	0.78	0.99	1.52	4.33	0.20	90.2	90.4	59.6	6.10	0.50
	PTL	0.77	4.52	3.12	2.38	2.94	13.7	0.53	79.6	80.1	57.2	6.00	0.64
215 °C/10min	EMAL	0.00	0.48	0.63	0.18	0.90	2.19	1.14	90.7	91.9	61.7	5.83	0.73
	PTL	0.00	1.07	3.47	0.27	1.85	6.68	1.64	87.8	89.4	58.9	5.89	1.69
Sulfuric 215 °C/2min	EMAL	0.00	0.04	0.42	0.05	0.21	0.72	1.37	96.4	97.8	62.3	5.73	1.02
	PTL	0.00	0.18	8.15	0.10	0.73	9.17	1.68	85.5	87.1	58.7	5.57	1.69

*ara=arabinose, gal=galactose, gluc=glucose, xyl=xylose, man=mannose.

ASL = acid soluble lignin, AIL = acid insoluble lignin.

7.3.4 Effect of lignin on the digestibility of bleached kraft pulp (BKp)

The lignin in steam pretreated substrates was previously shown to be inhibitory to enzymes. This section showed the isolated steam pretreated EMAL and PTL lignins were inhibitory to enzymes during hydrolysis, and therefore proved lignin is the likely component of steam pretreated substrates responsible for inhibition.

The inhibitory effects of the lignins isolated from the various steam pretreated substrates were assessed by determining the digestibility of BKp using Celluclast or CTec2 enzyme cocktails in the presence and absence of the EMAL and PTL lignins (**Figure 7.10**). PTL lignins were assessed using both Celluclast and CTec2, whereas EMAL lignins were assessed only using CTec2, due to the limited quantity of EMALs isolated. A low enzyme loading of 2.5 FPU/g was used to ensure that potential inhibition could be seen, since a high enzyme to inhibitor ratio could obscure inhibition. The lignins were added at a set mass of 37.5 mg, corresponding to the typical level of lignin present in the pretreated substrates (~29-39%).

Adding PTL lignins during enzymatic hydrolysis decreased the digestibility of BKp for all lignins. PTL lignins from the more severely pretreated substrates reduced the digestibility of BKp by a greater amount, regardless of whether CTec2 or Celluclast was used as the enzyme. For example, when using CTec2, the digestibility of BKp in the presence of no added lignin was ~48%; the presence of sawdust, 215°C/10 min and 215°C/2 min sulfuric acid PTL lignins reduced the digestibility to 45%, 43% and 38%, respectively. Interestingly, the PTL lignins were more inhibitory towards the Celluclast cocktail than the CTec2 cocktail, the exact reason for this is not clear.

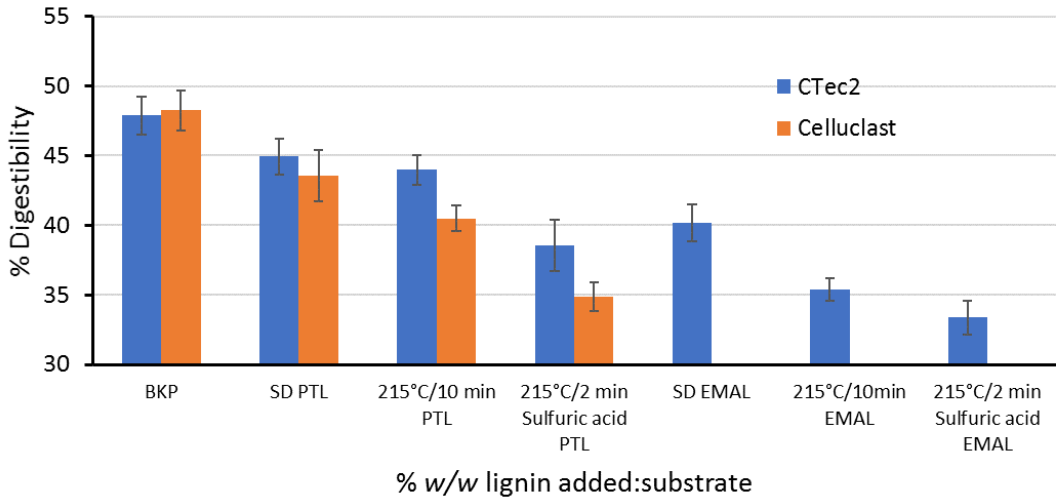


Figure 7.10 Digestibility of BKp in the presence and absence of EMAL and PTL lignins using CTec2 or Celluclast supplemented with β-glucosidase at enzyme loadings of 2.5 FPU/g BKp. Error bars represent ± two standard deviations of triplicate analyses.

The inhibition of EMAL lignins followed the trend of PTL lignins, that is the more severely pretreated lignin exerted greater inhibitory effects upon the digestibility of BKp (**Figure 7.10**). The overall effect of more severely pretreated lignins exerting greater inhibitory effects was consistent with previous studies [148,215].

When BKp was digested using the CTec2 enzyme cocktail, EMAL lignins were more inhibitory than the corresponding PTL lignins. This might have been because the EMAL lignins are cleaner than the corresponding PTL lignins having lower residual protein and carbohydrate contents (**Table 7.1**) thus, allowing greater non-productive binding of the enzymes.

The reduced digestibility of BKp in the presence of the isolated PTL and EMAL lignins provided good evidence that the lignin in steam pretreated substrates was indeed inhibitory to CTec2 and Celluclast enzymes during enzymatic hydrolysis of these substrates.

7.3.5 Lignin amount

It was evident from literature and from the findings of this study so far that lignin acts as an inhibitor during the enzymatic digestibility of substrates. It was also previously seen in **section 5.3.4** that lignin amount may be influencing the digestibility of steam pretreated substrates.

To determine the impact that lignin amount had on the digestibility, the PTL lignins were added back to BKp at a range of lignin to substrate ratios. Since Celluclast had previously been shown to be more susceptible to inhibition, this cocktail was used for this experiment. The highest lignin to substrate ratio was set so that it exceeded the level at which lignin was found in pretreated substrates (29-39%).

Figure 7.11 shows that, regardless of which lignin was added, the higher the amount of lignin present, the greater the inhibition of the digestibility of BKp. For example, the digestibility of BKp in the absence of added lignins was ~48%, this decreased to 43%, 40% and 35% when 50 mg of, respectively, sawdust, 215°C/10 min and 215°C/2 min sulfuric acid PTL lignins were present during the digestion.

Secondly, **Figure 7.11** shows that when the equal amounts of the three lignins were added the inhibitory effects on the digestibility of BKp were not identical. Consequently, the composition of the lignin must also be playing a role in determining the level of inhibition.

Other studies support the notion that although lignin amount plays a role in the inhibition of cellulase enzymes during hydrolysis, the chemical composition of lignin also contributes to the overall extent to which lignin is inhibitory. Nakagame *et al*. [150], showed that when using the same amount of lignin during hydrolysis, the biomass source and pretreatment type strongly influenced the extent to which lignin was inhibitory: Lodgepole pine lignin was more inhibitory than corn stover or poplar lignins; and organosolv pretreatment of lodgepole pine produced more inhibitory lignin than steam pretreatment. Yang and Pan [257] also suggested that the composition of lignin playing a more significant role in enzyme inhibition than amount.

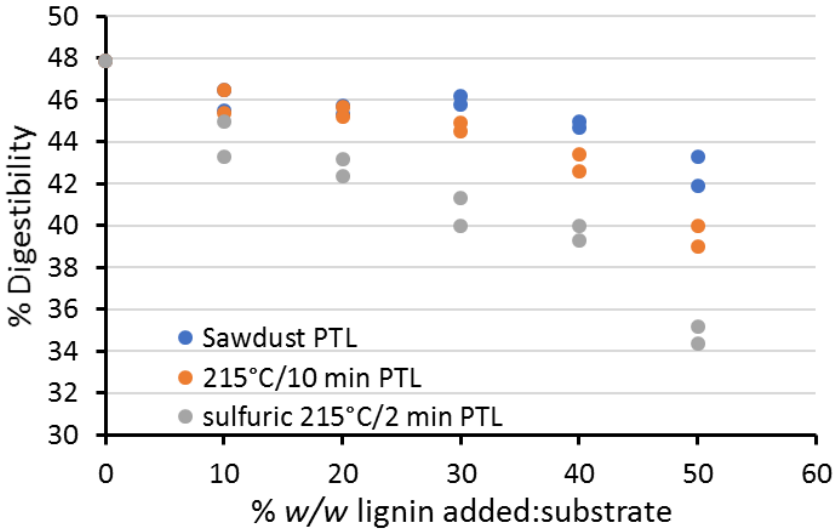


Figure 7.11 Effect of increasing amounts of PTL lignins on the digestibility of BKp using Cellculast supplemented with β-glucosidase as the enzyme, and an enzyme loading of 2.5 FPU/g substrate. Experimental duplicates represented as individual points; the amount of lignin required meant triplicate analyses were not possible here.

7.3.6 Effect of PEG as a surfactant to reduce lignin inhibition

The inhibition of lignins from steam pretreated substrates was believed in part to be due to the ability of lignins to non-productively bind enzymes. This section showed with the use of a surfactant, PEG, that lignins do in fact non-productively bind enzymes.

The effect of adding the surfactant, polyethylene glycol (PEG), to improve the digestibility of BKp in the presence of the pretreated lignins was investigated (**Figure 7.12**). The lowered digestibility of BKp on addition of PTL lignins was overcome by the addition of PEG (**Figure 7.12**).

The enhancement in digestibility as a result of the addition of PEG was greatest for the more severely pretreated lignins: ~ 3%, 4% and 10%, for sawdust, 215ºC/10 min and 215ºC/2 min sulfuric acid PTL's, respectively. The greater enhancements in digestibility indicated that the more severely pretreated lignins non-productively bound enzymes to a greater extent, than the less severely pretreated lignins. Thus, indicating that chemical composition plays a role in the inhibitory mechanism of lignins towards enzymes.

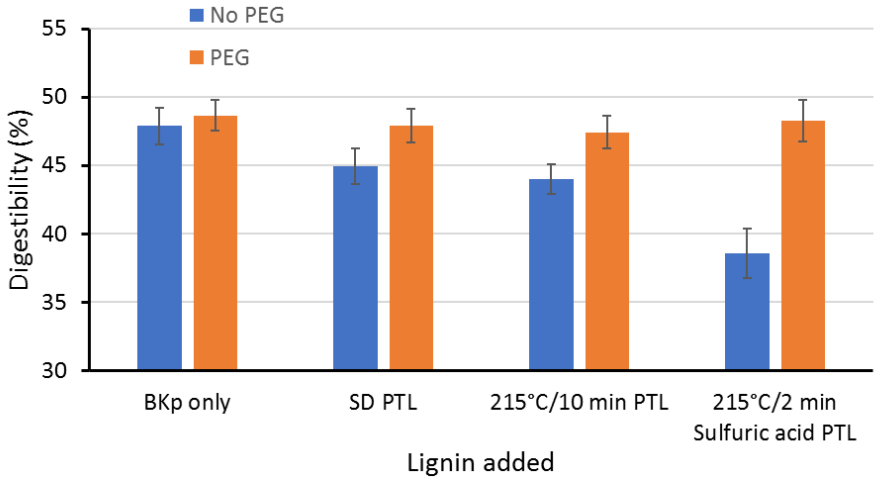


Figure 7.12 Effect of using PEG as a surfactant on the digestibility of BKp in the presence and absence of PTLs using CTec2 enzyme and an enzyme loading of 2.5 FPU/g substrate. The amount of lignin added to BKp was 37.5 mg (50 % *w/w*). Error bars represent ± two standard deviations of triplicate analyses.

7.3.7 Chemical composition of EMAL lignins

To gain a better understanding of how lignin was inhibitory to enzymes and why the more severely pretreated lignins exerted greater inhibitory effects, the chemical composition of the lignins were determined. Only EMAL lignins were characterised due to the limited solubility of PTL lignins in organic solvents.

7.3.7.1 Inter-unit linkages

The HSQC NMR spectra and structures of identified lignin sub-units can be seen in **Figure 7.13**. Signal assignments were based on previous research [246,258-260]. The β-aryl-ether (β-O-4, **A**), resinol substructures (β-β, **B**) and phenylcoumaran (β-5, **C**) were identified by their cross peaks at δ_C/δ_H 71.8/4.78 (**A**$_\alpha$), 84.3/4.30 (**A**$_\beta$), 60.5/3.59 (**A**$_\gamma$), 85.5/4.62 (**B**$_\alpha$), 54.1/3.04 (**B**$_\beta$), 71.5/4.15 & 3.75 (**B**$_\gamma$), 87.5/5.45 (**C**$_\alpha$), 53.5/3.46 (**C**$_\beta$), 63.2/3.70 (**C**$_\gamma$), respectively.

Table 7.2 Approximate amount of inter-unit linkages in the EMAL lignins as evaluated by 2-D HSQC NMR signal integration based on the method used by Sette *et al.* [260].

	Inter-unit linkages (%)*		
	β-O-4	β-5	β-β
Untreated sawdust	62	19	5
215°C/10 min	42	22	5
215°C/2 min sulfuric acid	27	17	6

*Expressed as number per 100 C_9 units.

The HSQC NMR spectra (**Figure 7.13**, **Table 7.2**) show the levels of linkages in the untreated sawdust lignin to be comparable to the linkages in native spruce EMAL [215]. However, it should be noted that the amount of β-5 **(C**) linkages is higher than the typical reported range of 9-12% for softwoods [34]. The amount of β-O-4 (**A**) linkages in lignin decreased from 62% in untreated sawdust to 42% and 27% for 215°C/10 min and 215°C/2 min sulfuric acid steam pretreated lignins, respectively. This indicated that significant cleavage of β-O-4 linkages occurred during steam pretreatment, with the amount of cleavage increasing as pretreatment severity increased. There were only minor changes in the amount of β-β (**B**) and β-5 **(C**) between the lignin samples (**Table 7.2**).

NMR evidence suggests that condensation occurs as a result of steam pretreatment. While the C5 to C2 ratio was constant at 0.67 for all 3 lignins, the C6 to C2 ratios were 0.55, 0.45 and 0.49 for the sawdust, 215°C/10 min and 215°C/2 min sulfuric acid steam pretreated lignins, respectively. This is consistent with previous studies which have shown that lignin condensation under acidic conditions occurs predominantly at C6 [90,94,95,103].

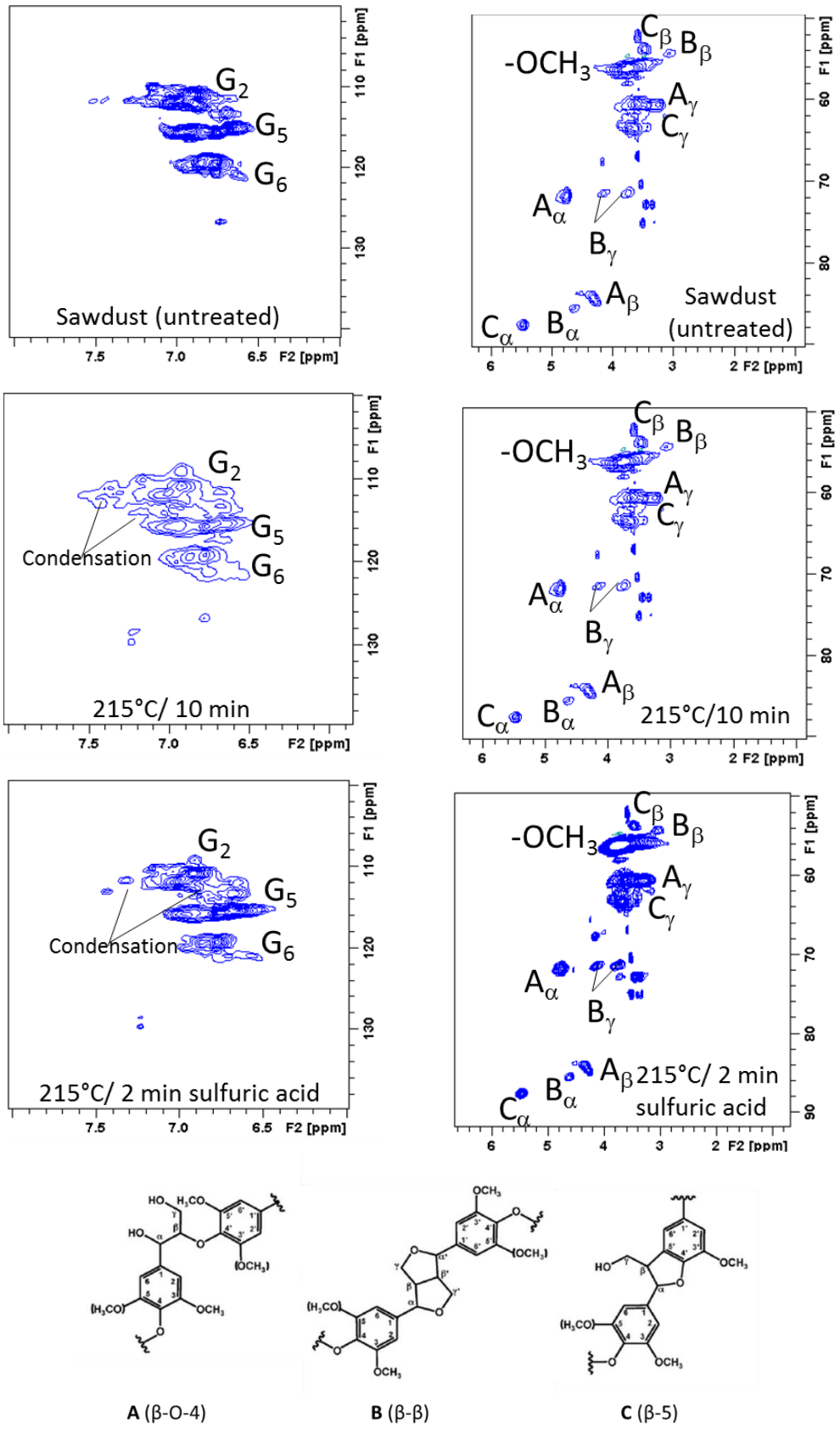


Figure 7.13 2-D HSQC NMR spectra of steam pretreated EMAL lignins: aromatic region δC/H 100-140/6-8 ppm; side chain region δC/H 50-90/1-6.5 ppm.

7.3.7.2 Lignin molecular weight

Gel permeation chromatography (GPC) analysis showed that the molecular number (M_n), molecular weight (M_w), and polydispersity (M_w/M_n), of the isolated lignins decreased as the pretreatment severity increased (**Table 7.3**). The M_w of the isolated lignins decreased from 18,680 g/mol in untreated sawdust to 15,667 g/mol and 10,603 g/mol for 215°C/10 min and 215°C/2 min sulfuric acid lignins, respectively. The decrease suggested that steam pretreatment cleaves inter-unit linkages, with the extent of degradation being dependent upon the pretreatment conditions/severity. Similar results have been previously reported for hydrothermal pretreatment of aspen [246] and switchgrass [261]. However, some studies, such as that of Rahikainen *et al.* [215] or that of Li *et al.* [262], showed that steam pretreatment increased the M_w of residual lignins (spruce and aspen, respectively). The contrasting findings highlight the fact that during pretreatment a combination of two competing types of reactions occur; degradation and condensation. The balance of which obviously depends upon biomass type, pretreatment type and pretreatment conditions/severity.

Table 7.3 EMAL lignin molecular weight and number averages.

	Molecular average (g/mol)		
Pretreatment Conditions	**Number (M_n)**	**Weight (M_w)**	**Polydispersity (M_w/M_n)**
Sawdust (untreated)	2,927 (116)	18,680 (1190)	6.4 (0.3)
215°C/10 min	2,430 (71)	15,667 (939)	6.4 (0.2)
215°C/2 min sulfuric acid	1,989 (31)	10,603 (321)	5.3 (0.1)

Numbers in brackets represent standard deviation of analyses.

7.3.7.3 Lignin hydroxyl content

Phosphorus (^{31}P) NMR spectroscopy is a well-known method that allows for quantitative analysis of various hydroxyl groups in lignin [263]. This includes acidic and aliphatic hydroxyl groups, condensed and uncondensed guaiacyl hydroxyl groups, *p*-hydroxyphenyl phenolic hydroxyl groups and consequently the total phenolic hydroxyl [148,246,263]. It involves phosphitylation of the hydroxyl groups of lignin with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP), followed by measurement and quantification of phosphorylated hydroxyl groups against an internal standard.

In this study, the effects of pretreatment severity on the major hydroxyl groups of steam pretreated lignins was assessed (**Figure 7.14**, **Table 7.4**).

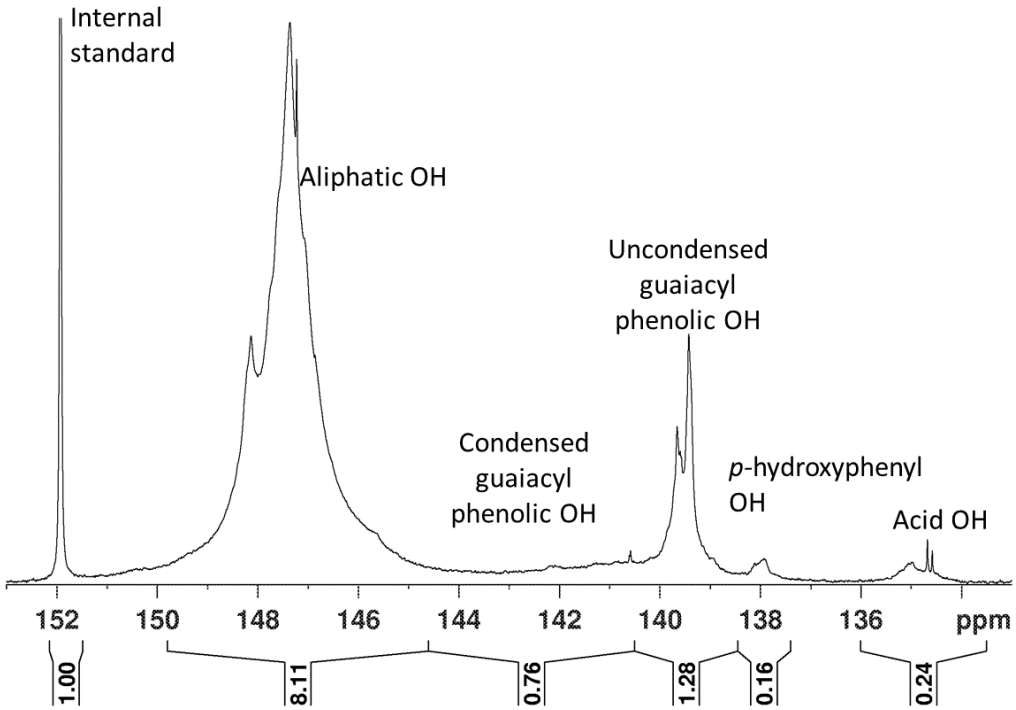


Figure 7.14 ^{31}P NMR spectra of untreated sawdust EMAL lignin showing peak integration ranges.

Table 7.4 Quantification of the different hydroxyl group species (mmol/g) in the EMAL lignins isolated from steam pretreated radiata pine using a quantitative ^{31}P NMR method.

	Acid OH	Aliphatic OH	Total phenolic OH	Condensed guaiacyl OH	Uncondensed guaiacyl OH	*p*-hydroxyphenyl OH phenolic
Sawdust (untreated)	0.13	4.37	1.18	0.41	0.69	0.09
215°C/10 min	0.22	3.57	1.96	0.75	1.08	0.13
215°C/2 min sulfuric acid	0.23	2.88	2.13	0.79	1.18	0.15

Table 7.5 Number of phenolic hydroxyl units per 100 C9 lignin units.

	OH units per 100 C_9 lignin units*	
Pretreatment Conditions	**Etherified**	**Phenolic**
Sawdust (untreated)	79	21
215°C/10 min	65	35
215°C/2 min sulfuric acid	62	38

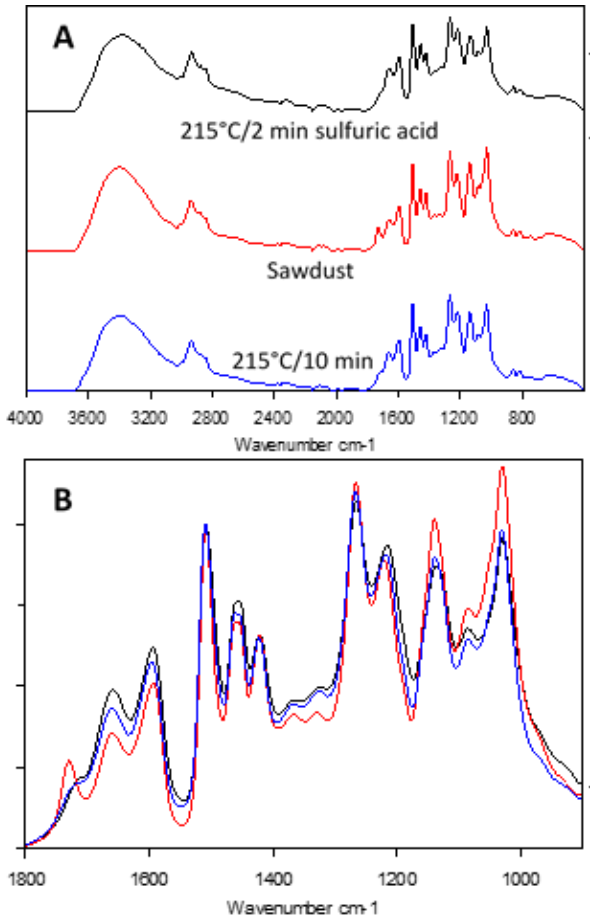
*Units calculated from ^{31}P NMR data in Table 7.4, assuming 5.55 mmol of C9 units/g lignin [264].

The levels of uncondensed, condensed and total phenolic hydroxyl contents all increased with pretreatment severity (**Table 7.4**). The increased phenolic hydroxyl content was consistent with cleavage of β-O-4 linkages as a result of steam pretreatment, with a greater amount of cleavage occurring at the more severe pretreatment conditions (**Table 7.4** and **7.5**). Similar observations have been reported for various pretreated woods, including poplar [265], spruce [215] and aspen [246]. The significant decrease in etherified phenolics also supported the suggestion that significant cleavage of β-O-4 and α-O-4 linkages occurs as pretreatment severity increases [264].

In contrast, the aliphatic hydroxyl content decreased from 4.37 mmol/g in sawdust to between 3.57 mmol/g and 2.88 mmol/g for the steam pretreated lignins. This suggested greater fragmentation of lignin side-chains occurred as the pretreatment severity increased [246,266]. However, caution must be exercised as the reduced aliphatic hydroxyl content may also have been due to differences in carbohydrate content of the lignins (**Table 7.1**).

7.3.7.4 **Fourier transform Infrared spectroscopy**

Attenuated total reflectance Fourier transform Infrared (ATR FTIR) spectra were obtained for the three isolated EMAL lignins (**Figure 7.15**). The peak that corresponds to the hydroxyl groups at 3300-3400 cm^{-1}, decreased as the pretreatment severity increased. This correlated well with the ^{31}P NMR data, and could have been the result of structural changes but was more likely due to the decrease in the carbohydrate content of the lignins (**Table 7.1**) [148]. The peaks at both 1610 cm^{-1} and 1650 cm^{-1} increased with increasing pretreatment severity (**Figure 7.15B**). These signals correspond to, C=O stretches (unconjugated) and aromatic skeletal stretching + C=O stretching. The increase at 1650 cm^{-1} would be consistent with more Hibbert ketone-type substructures (e.g (**5**)) being formed in the residual lignin as a result of degradation of the β-O-4 linkages. The increase of the peaks ~1700 cm^{-1}, was consistent with an increased presence of aldehyde or carboxylic acid groups, the formation of which could have occurred during lignin degradation [148,264]. The spectrum also provided evidence that condensation reactions occurred, as the peak that corresponded to guaiacyl C-H and ether-O- (1140 cm^{-1}) decreased for the more severely pretreated lignins (**Figure 7.15B**).



Band assignment (cm^{-1})	Vibration	Assignment
3420-3405	O-H stretch	Phenolic OH + aliphatic OH
2960-2925	C-H stretch	$CH_3 + CH_2$
2850-2840	C-H stretch	$CH\text{-}OCH_3$
1705-1715	C=O stretch	Unconjugated C=O
1650	C=O stretch	Conjugated C=O
1610	C-C stretch	Aromatic skeleton
1500	C-C stretch	Aromatic skeleton
1460	$\delta_{asymmetric}$ C-H	$CH_3 + CH_2$
1425	C-C stretch	Aromatic skeleton
1365	$\delta_{in\ plane\ deformation}$ O-H	Phenolic OH
1326	C-O stretch	Guaiacyl (G)
1270	C-O stretch	Guaiacyl (G)
1220	C-O (*p*-H) + C-O (Ar)	phenolic OH +ether
1140	$\delta_{in\ plane\ deformation}$ Ar-C-H (G)	Guaiacyl (G)
1090	$\delta_{in\ plane\ deformation}$ Ar-C-H (G)	Guaiacyl (G)
1030	C-O (*p*-H) + C-O (C)	Aliphatic OH + ether
855	$\delta_{out\ of\ plane\ deformation}$ Ar-C-H (G)	Guaiacyl (G)
810	$\delta_{out\ of\ plane\ deformation}$ Ar-C-H (G)	Guaiacyl (G)

Figure 7.15 ATR FTIR spectrum of isolated EMAL lignins (A); zoomed in region of 900-1800 cm^{-1} region (B). Intensity at 1500 cm^{-1} was set to 2, so the relative intensities of specific chemical groups could be compared. Peak assignments based on those of Pandey [267], and Tejado *et al.*[264].

7.3.7.5 Correlation of hydroxyl groups and inhibition

A key finding earlier in this chapter was that adding the more severely steam pretreated lignins decreased the digestibility of BKp to a greater extent, than the less severely pretreated lignins (**section 7.3.4**, **Figure 7.10**). This indicated that greater non-productive binding between the enzymes and more severely pretreated lignins occurred. Previous research has suggested the inhibitory effects are associated with the amount of hydroxyl groups on the lignin [144]. The amounts of different hydroxyl groups were therefore correlated to the digestibility of the BKp in the presence of the lignins (**Figure 7.16**).

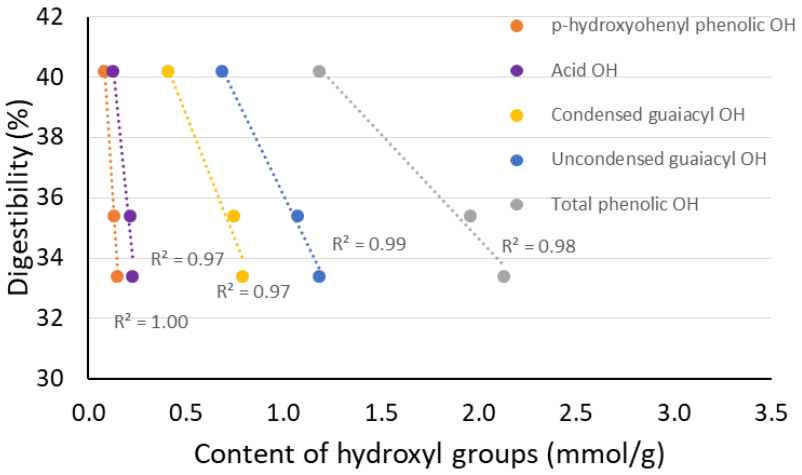


Figure 7.16 Correlation between the hydroxyl group content in steam pretreated EMAL lignins and the digestibility of BKp in the presence of those lignins using CTec2 enzyme and an enzyme loading of 2.5 FPU/g substrate.

Figure 7.16 shows that there are strong negative correlations between the digestibility and the levels of phenolic hydroxyl and carboxyl groups of the lignins. This observation is consistent with previous suggestions that phenolic hydroxyl groups may play a role in the non-productive binding of enzymes to lignin; increases in the phenolic hydroxyl groups, increases enzyme inhibition, as it increases the amount on non-productive binding [215]. The observations of this study are also consistent with findings reported in other studies with both condensed phenolics [246] and total phenolic contents [245] having been shown to strongly correlate to digestibility levels.

It is important to note that it is not possible to conclude from such correlations that phenolic hydroxyl groups in the lignin are responsible for the inhibition as steam pretreatment induces multiple and possibly highly correlated changes to the lignin. In particular, hydrophobic interactions have been reported to be one of the main factors contributing to the non-productive binding of enzymes to lignin [129,133-135] and steam pretreatment has been shown to increase the hydrophobicity of the lignins due to condensation occurring [150]. Perhaps too, lignin can bind enzymes *via* a combination of effects as suggested by Sun *et al*. [246], who propose the more condensed aromatic rings enhance hydrophobic interactions, whilst the phenolic hydroxyls bring rings closer together *via* hydrogen bonding. Furthermore, surface area and particle size of the lignin might also play a role in the hydrophobic interactions between cellulases and lignin [246].

The total OH content of lignins showed a weak positive correlation to the digestibility levels in this study (R^2 =0.40, data not shown). This could have been due to the impact of the decreasing aliphatic OH content (**Table 7.4**), which in turn, was significantly influenced by the carbohydrate content of the lignins (**Table 7.1**), as suggested by Sun *et al.* [246].

The carboxylic acid content of the steam pretreated lignins increased with increasing pretreatment severity (**Table 7.4**). A negative correlation was observed between the carboxylic acid hydroxyl content and the digestibility of BKp in the presence of EMAL lignins (**Figure 7.16**, R^2 = 0.97). This was in contrast to other reports [227,246], where it was found that increased polar carboxylic acid content of lignin increased digestibility by reducing the hydrophobic interactions between enzymes and lignins. The contrasting results of this study may be because other changes to the lignin on steam pretreatment have a larger impact than the levels of carboxylic acid groups.

7.4 Summary

Lignin was confirmed as being a likely component responsible for the inhibitory effects of steam pretreated radiata pine. This was demostrated using PEG as a surfactant, whereby potential sites of lignin that could otherwise bind to enzymes were blocked by PEG: The digestibility of steam pretreated substrates improved in the present of PEG, with the digestibility of the more severely pretreated substrates improving to the greatest extents. Furthermore, in the presence of PEG the inhibitory effects of isolated fibre lignins on BKp digestibility disappeared.

No evidence was found to show that lignin location was a significant factor contributing to enzyme inhibition: The location of lignin in the steam pretreated substrates did not change significantly on steam pretreatment, whereas the digestibility of the substrates did change with changes in pretreatment severity.

To further investigate inhibitory effects, the isolation of three lignins, which were representative of the range of pretreatment conditions and severity used in this study were performed (both EMAL and PTL). The digestibility of BKp decreased more in the presence of the more severely pretreated lignins. An increase in the added amount of all three lignins was shown to increase the observed inhibition, however the inhibition was not the same for all three lignins, providing evidence that lignin composition plays a significant role in the inhibition of enzymes during enzyme digestion.

The chemical composition and functional groups of the isolated EMAL lignins were investigated using various methods; ATR FTIR, ^{31}P NMR, GPC and 2-D HSQC NMR. This showed that as the pretreatment severity increased both depolymerisation and condensation reactions increased. The most significant change seen in the residual lignins was β-O-4 cleavage, as supported by the NMR and GPC data. The amount of condensation and phenolic hydroxyl functional groups of the EMAL lignins were both shown to negatively correlate to the digestibility of BKp, suggesting that levels of phenolic hydroxyl groups may contribute to the non-productive binding by that lignin. However, the exact mechanism requires further understanding.

8 General discussion, conclusions and future research

8.1 Discussion and conclusions

The main objective of this thesis was to understand the relative roles of soluble and insoluble fibre components as inhibitors of enzyme hydrolysis in the conversion of steam pretreated *P. radiata* wood to sugars. This included understanding the relative roles and degree of inhibition of the components as a function of pretreatment severity.

Insoluble inhibition: The digestibility of the insoluble steam pretreated substrates was examined using a commercial enzyme cocktail (Novozymes Cellic® CTec2). Results showed the digestibility of these substrates increased with pretreatment severity due to increases in accessibility. However, when the substrates were tested after ball-milling to a common cellulose accessibility, the digestibility decreased with increasing pretreatment severity. This showed that while increasing pretreatment severity led to greater enzyme inhibition, the inhibition was more than compensated for by increases in the accessibility.
substrates.
The key findings supporting this were:

1) The digestibility of steam-pretreated *P. radiata* substrates increased with pretreatment severity.
    a) At a common severity factor, pretreatments at lower temperatures for longer times gave higher digestibilities.
2) At a common cellulose accessibility, the inhibitory response was greater for the substrates obtained at higher pretreatment severities: The digestibility decreased with increasing pretreatment severity.
3) Modifications to the Simons' stain method ensured that a robust and reliable method with improved repeatability was available for measuring the cellulose accessibility of substrates of the nature used in this research.

Soluble inhibition: The inhibitory effects of filtrates on the digestibility of bleached kraft pulp (BKp) and steam pretreated substrates were investigated. The results showed that the inhibitory effects of the soluble filtrate components were minimal or non-existent under most conditions. It was therefore concluded that the insoluble components of steam pretreated *P. radiata* were more inhibitory than the soluble components.

The key findings supporting this were:

1) The changes in the digestibility levels of substrates or BKp in the presence of filtrates were minimal for most enzymes, enzyme loadings and filtrate combinations.
2) In some cases when the commercial enzyme cocktail CTec2 was used, adding the filtrates back during hydrolysis led to small enhancements in digestibility. Subsequent experiments showed the enhancements were likely due to components in the filtrates acting as reductants for the oxidative cellulase enzymes present in the CTec2 enzyme cocktail.

Inhibition by lignin: To further investigate the inhibition by insoluble components, lignins were isolated from three insoluble substrates of differing pretreatment severity and their inhibitory effects on the digestibility of bleached kraft pulp were determined. Results showed that both the amount and composition of the lignins influenced the extent to which lignin was inhibitory to hydrolysis enzymes.

The key findings supporting this were:

1) There was no evidence to indicate that lignin location played a role in inhibition.
2) Both *in situ* (lignin in substrate) and isolated lignins were shown to be inhibitory towards hydrolysis enzymes.
3) The inhibitory effects of added lignins increased with the amount of lignin added.
4) The inhibitory effects of added lignins were greater for the more severely pretreated lignins.
5) Chemical characterisation of the isolated lignins showed that steam pretreatment resulted in both depolymerisation and condensation of the lignin.
6) Lignin composition plays a role in the extent to which lignin is inhibitory to hydrolysis enzymes, with the main mechanism for lignin inhibition of enzymes in steam pretreated radiata pine being *via* non-productive binding.

The findings in this thesis led to the following key conclusions:

Conclusions:

- Both insoluble (substrate) and soluble (filtrate) components are inhibitory to enzymatic hydrolysis enzymes, at least under the steam pretreatment conditions used in this study.

- Insoluble inhibitors exert far greater levels of inhibition than the soluble inhibitors.

- Substrates from more severe pretreatment conditions result in greater levels of inhibition. While increasing pretreatment severity led to greater enzyme inhibition, the inhibition was more than compensated for by increases in the accessibility.

- Lignin is one of the insoluble components likely responsible for enzyme inhibition: Lignin is inhibitory as it non-productively binds enzymes; with both the amount and composition of the lignin playing a role in its overall inhibitory effect.

8.2 Future research

This section proposes some recommendations for future research based on the key finding and conclusions of this thesis.

Although a range of steam pretreatment conditions were investigated in this study, only two temperatures were investigated. Additionally, the use of acid catalysts was only investigated at a single selected temperature and time. Therefore, to gain a broader understanding of the effects pretreatment conditions have on the digestibility of substrates and to further understand whether adding acid catalysts is beneficial during the steam pretreatment of softwoods, some recommendations for future studies are to use:

- a wider range of pretreatment temperatures and times
- other acid catalysts
- the acid catalysts in conjunction with a variety of temperature and times
- the same set of pretreatment conditions and evaluate the effects on other softwoods

The modifications to the Simons' stain procedure were particularly important in providing a reliable method for determining the cellulose accessibility of substrates; the use of the method on ball-milled substrates was an approach that had not been used previously. Cellulose accessibility was shown to be an important factor in determining the extent to which substrates could be hydrolysed. Thus, using the Simons' stain in other applications may assist in providing a greater understanding of the impacts of both cellulose accessibility and residual lignin on overall enzyme digestibility. Questions of interest are:

- Is the Simons' stain method able to assess the cellulose accessibility of a more diverse range of lignocellulosic substrates that are subjected to ball-milling?
- Can the Simons' stain assess the accessibility of residual cellulose in isolated lignin preparations? This could assist in providing evidence for why certain lignins are more inhibitory than others, especially in *in situ* environments.

Although the soluble filtrate components were found to have negligible inhibition when dosed back to enzyme digestions of various substrates under the conditions of this study, it was noted that some of the filtrate components had positive effects on the digestion levels when the commercial CTec2 enzyme cocktail was used. This was believed to be due to the presence of the LPMO enzyme. To gain a better understanding of the effects that steam pretreated soluble components have on LPMO activity a recommendation is to:

Isolate and characterise the components in filtrates from both extremes of the severity scale and test for LPMO interaction.

Lignin was the principal likely insoluble component responsible for enzyme inhibition during hydrolysis of steam pretreated radiata pine. Lignin non-productively bound enzymes, with both the amount and composition of the lignin playing a role in its overall inhibitory effect. The inhibition of lignin requires further characterisation and attention, and the exact inhibitory mechanism of steam pretreated radiata pine lignin also requires further investigation. Thus, the following areas need attention:

1) What proportion of the enzymes are non-productively bound by the lignins?
    a) The amount of free and bound enzymes during hydrolysis should be undertaken.
    b) The lignin contact angles should be measured; this is a recent approach that helps predict the extent to which lignins bind enzymes and also which lignins are able to bind enzymes.
2) What chemical functional groups of the lignin are responsible for binding the enzymes?
    a) More in-depth chemical analysis of isolated lignins is required.
        i) Degradative techniques to determine additional lignin linkages and the molecular formula.
        ii) Use of NMR to further characterise the lignins inclusive of ^{13}C and ^{1}H NMR.
        iii) The degree of lignin condensation arising from pretreatment should be determined.

b) Isolate and characterise lignins from a greater variety of pretreatment conditions to gain a better understanding of small chemical differences, e.g. phenolic hydroxyl groups, carboxylic groups, etc.

3) Can the specific enzymes of the CTec2 or Celluclast cocktail that are being inhibited be determined?
   a) Comprehensive binding studies need to be performed to assist in understanding:
      i) Which specific enzymes lignin is inhibitory towards.
      ii) The amount of each enzyme non-productively binds to the various lignins.
         (1) The amount of free and bound enzymes.
         (2) What percentage of the lignin functional groups are involved in non-productive binding.

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Appendices

A1: Elemental analysis data

A2: Volatile fatty acid (VFA) data

B1: Particle size distribution supplementary figures

B2: Supplementary ball-milling data

B3: Indicative crystallinity data

C1: Optimisation of gallic acid as a reductant for hydrolysis

D1: Journal Publications: Corrected proof copy of: MacAskill, J.J., Suckling, I.D., Lloyd, J.A., Manley-Harris, M. Unravelling the effect of pretreatment severity on the balance of cellulose accessibility and substrate composition on enzymatic digestibility of steam pretreated softwood. Journal of Biomass and Bioenergy 2018, 109C, 284-290.

A1: Elemental analysis data

Elemental data for filtrates.

Pretreatment conditions	Na ppm	Mg ppm	K ppm	Ca ppm	V ppm	Cr ppm	Fe ppm	Co ppm	Mn ppm	Ni ppm	Cu ppm	As ppm	Cd ppm	Li ppm	Al ppm	Zn ppm	Sr ppm	Ba ppm	Pb ppm	S ppm
180°C/21.5 min Water	59.9	18.1	151	90.1	<0.05	0.16	12.5	<0.05	2.92	0.21	0.72	0.91	0.34	<0.05	3.28	1.90	0.30	0.28	0.13	718
180°C/21.5 min Water	61.8	20.4	159	121	<0.05	0.20	15.5	<0.05	3.63	0.22	1.26	0.70	0.29	<0.05	3.08	2.23	0.33	0.30	0.12	724
215°C/10 min Water	59.4	18.4	165	109	<0.05	0.16	15.7	<0.05	3.37	0.21	0.76	0.48	0.32	0.05	3.07	2.33	0.35	0.32	0.37	713
215°C/10 min Water	56.3	21.3	159	117	<0.05	0.19	16.5	<0.05	3.42	0.21	0.70	0.49	0.30	<0.05	3.00	2.00	0.35	0.31	0.36	719
215°C/2 min Water	60.4	24.9	193	112	<0.05	0.20	52.8	<0.05	4.66	0.31	0.75	0.52	0.32	<0.05	3.43	2.59	0.46	0.38	0.29	713
215°C/2 min Water	60.8	24.8	193	122	<0.05	0.18	16.8	<0.05	4.48	0.21	0.62	0.49	0.31	<0.05	3.31	2.34	0.43	0.38	0.30	736
215°C/2 min Citric acid	57.0	14.3	148	80.8	<0.05	0.44	28.2	<0.05	2.03	0.23	1.11	0.43	0.32	0.05	3.73	2.17	0.39	0.39	0.81	735
215°C/2 min Citric acid	53.1	13.4	148	78.4	<0.05	0.42	28.8	<0.05	2.08	0.24	0.62	0.40	0.32	<0.05	3.55	1.68	0.35	0.35	0.79	724
215°C/2 min Sulfuric acid	68.1	14.1	147	78.9	<0.05	0.49	36.7	0.06	2.16	0.31	1.18	0.33	0.29	<0.05	3.86	2.58	0.20	0.10	0.58	997
180°C/108 min	62.1	15.3	150	82.1	<0.05	0.13	9.23	0.05	2.00	0.22	0.62	0.42	0.32	<0.05	2.49	1.42	0.22	0.23	0.12	710
180°C/108 min	53.3	13.2	141	74.9	<0.05	0.13	7.93	<0.05	1.53	0.15	0.86	0.31	0.32	<0.05	2.19	1.30	0.18	0.18	0.12	717
215°C/2 min Sulfuric acid	57.6	12.4	143	77.0	<0.05	0.45	39.4	0.05	1.94	0.37	1.61	0.35	0.31	<0.05	3.68	2.79	0.19	0.10	0.39	1014

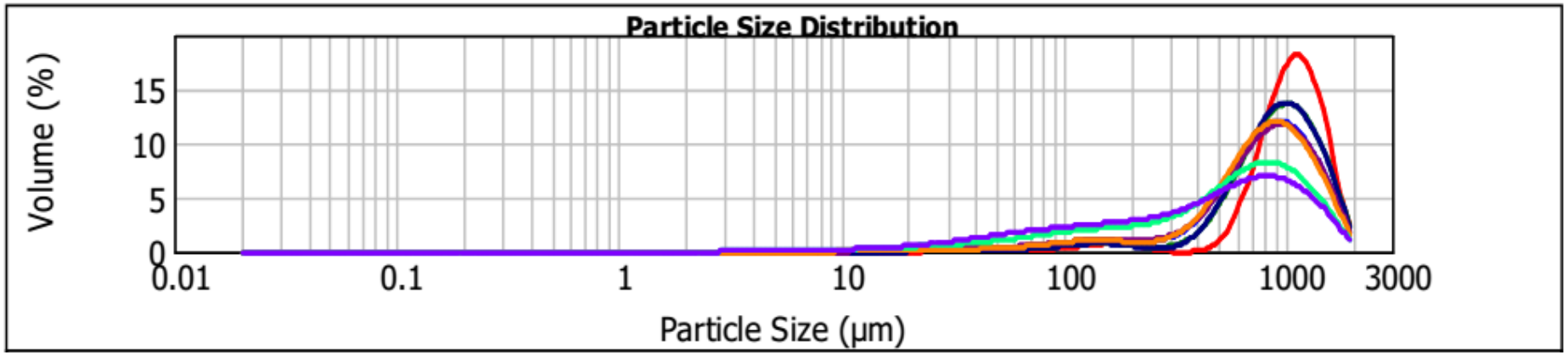
Elemental data for steam pretreated substrates, untreated sawdust and BKp.

Sample Id	Na mg/L	Mg mg/L	K mg/L	Ca mg/L	V mg/L	Cr mg/L	Fe mg/L	Co mg/L	Mn mg/L	Ni mg/L	Cu mg/L	As mg/L	Cd mg/L	Li mg/L	Al mg/L	Zn mg/L	Sr mg/L	Ba mg/L	Pb mg/L	S mg/L
180°C/21.5 min Water	75.4	48.0	127	156	<0.05	0.40	12.6	0.06	2.23	1.58	2.81	0.27	0.31	0.10	7.05	5.34	0.58	1.76	1.09	729
180°C/21.5 min Water	79.1	44.7	124	146	<0.05	0.38	15.1	0.05	2.38	1.47	3.22	0.22	0.31	0.10	8.89	5.88	0.56	1.86	0.88	771
215°C/10 min Water	50.1	31.9	113	113	<0.05	0.55	26.7	<0.05	0.79	0.78	4.51	0.24	0.33	0.06	5.78	6.09	0.35	0.63	1.18	754
215°C/10 min Water	52.5	28.0	116	85.4	<0.05	0.60	13.8	0.08	0.73	0.67	4.24	0.36	0.30	0.07	6.56	5.51	0.33	1.24	0.56	726
215°C/2 min Water	72.1	40.4	118	141	<0.05	0.36	13.8	<0.05	2.79	0.60	3.95	0.14	0.34	0.10	5.40	6.82	0.58	0.80	1.24	724
215°C/2 min Water	78.4	51.7	122	176	<0.05	0.28	13.7	<0.05	3.36	0.61	4.21	0.22	0.32	0.10	6.14	7.02	0.68	0.67	1.54	744
215°C/2 min Citric acid	63.7	32.4	115	110	<0.05	0.39	11.0	0.06	0.24	3.04	3.06	0.27	0.32	0.09	5.05	4.29	0.35	0.43	0.30	745
215°C/2 min Citric acid	70.6	25.4	120	82.8	<0.05	0.42	17.1	0.05	0.23	2.15	2.99	0.23	0.31	0.10	4.96	4.10	0.31	0.33	0.37	748
215°C/2 min Sulfuric acid	50.6	20.4	114	70.2	<0.05	0.58	12.5	0.06	0.11	0.77	4.80	0.27	0.32	0.06	5.41	5.13	0.25	0.36	0.39	735
180°C/108 min	48.0	29.4	117	93.9	<0.05	0.30	14.3	0.05	1.14	1.47	4.18	0.21	0.33	0.06	8.60	4.81	0.37	0.47	0.78	745
180°C/108 min	54.3	38.1	116	116	<0.05	0.36	13.2	0.05	1.58	1.51	4.37	0.20	0.34	0.08	7.43	6.17	0.49	0.60	0.78	744
215°C/2 min Sulfuric acid	50.4	21.1	114	69.3	<0.05	0.57	11.4	0.07	0.17	1.38	3.51	0.21	0.33	0.06	7.00	4.52	0.28	0.38	0.62	742
Sawdust untreated	24.8	58.6	392	240	<0.05	0.22	22.4	<0.05	12.27	0.21	1.02	0.18	0.32	<0.05	12.86	4.20	1.20	1.11	0.07	758
BKP	69.7	54.5	124	115	<0.05	0.22	8.0	<0.05	1.78	0.59	8.26	0.25	0.30	0.09	14.32	10.81	0.46	0.43	0.89	762

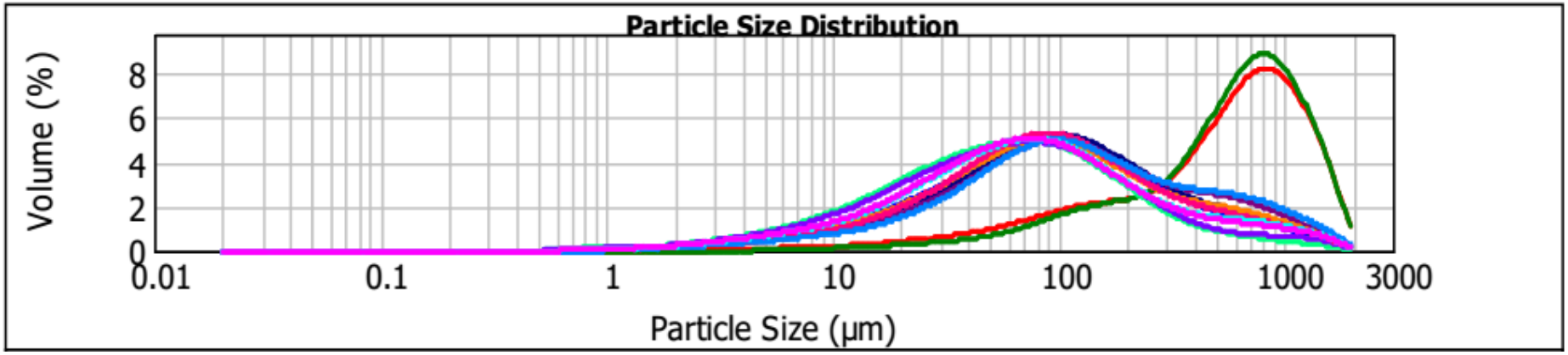
A2: Volatile fatty acids (VFA) data.

Steam pretreated filtrate conditions	Average Concentrations (ppm) Alcohol		Acids						Dilution factor
	Methanol	Ethanol	Acetic	Propionic	Iso-butyric	N-butyric	Pentanoic	Hexanoic	
180°C/21.5 min Water	71	0	279	2	0	0	0	1	20
180°C/21.5 min Water	76	0	349	4	0	0	0	2	20
215°C/10 min Water	152	0	568	5	1	1	0	3	20
215°C/10 min Water	149	0	706	7	1	1	0	4	20
215°C/2 min Water	116	0	252	4	0	0	0	3	20
215°C/2 min Water	111	0	266	6	0	0	0	4	20
215°C/2 min Citric acid	90	0	1086	9	0	1	0	4	20
215°C/2 min Citric acid	73	0	747	8	0	1	0	4	20
215°C/2 min Sulfuric acid	159	0	2759	7	1	0	0	0	50
180°C/108 min	36	0	192	6	0	2	0	3	20
180°C/108 min	22	0	131	4	0	1	0	3	20
215°C/2 min Sulfuric acid	171	0	2830	4	2	2	0	0	50

B1: Particle size distribution supplementary figures.



Particle size distribution of steam pretreated substrates:
Red, untreated sawdust; dark green, 180°C/21.5 min; dark blue, 215°C/2 min; orange, 180°C/108 min; blue, 215°C/10 min; light green, 215°C/2 min citric acid; 215°C/2 min sulfuric acid.



Particle size distribution of steam pretreated substrates ball-milled to common cellulose accessibility O:B ratio of 1.8.
Red and dark green, 215°C/2 min sulfuric acid experimental duplicates. All other pretreatment and duplicates are of similar particle distribution; 180°C/21.5 min, 215°C/2 min, 180°C/108 min, 215°C/10 min, 215°C/2 min citric acid.

B2: Supplementary ball-milling data

		Duplicate 1			Duplicate 2		
Ball-milling time(min)	Sample	Digestibility (%) 2.5 FPU/g	15 FPU/g	O:B ratio	Digestibility (%) 2.5 FPU/g	15 FPU/g	O:B ratio
0	Sawdust untreated	3.9	5.3	0.59			
10		4.6	9.3	0.83			
30		11.6	18.1	1.17			
60		26.9	39.3	1.43			
110				1.80			
0	180°C/21.5 min Water	3.6	10.0	0.87	3.4	9.5	0.80
10		11.0	24.4	1.35	12.4	22.0	1.07
30		25.6	49.7	1.90	27.7	52.1	1.90
60		33.9	60.1	2.29	36.3	60.0	2.12
0	215°C/10 min Water	4.5	15.5	1.09	4.3	13.4	1.18
10		13.7	32.0	1.82	15.1	36.6	2.40
30		23.0	53.3	2.40	23.4	57.2	2.52
60		30.3	60.9	2.41	27.9	64.7	2.57
0	215°C/2 min Water	3.2	8.4	0.74	3.9	10.9	0.82
10		8.9	15.2	1.01	9.9	20.6	1.25
30		23.5	45.0	1.47	30.0	45.9	1.51
60		33.5	52.8	2.05	39.1	60.9	1.66
0	215°C/2 min Citric acid	5.8	16.4	0.88	5.9	16.5	0.86
10		21.0	41.6	2.11	19.8	36.1	1.81
30		31.0	57.7	2.27	35.4	58.4	2.25
60		35.3	67.1	2.32	40.5	68.9	2.29
0	180°C/10 8 min	4.6	17.3	1.03	3.9	16.9	0.95
10		12.4	37.9	1.66	13.4	34.6	1.76
30		18.1	49.9	2.12	18.3	44.7	2.02
60		22.9	60.2	2.21	22.1	51.7	2.35
0	215°C/2 min Sulfuric acid	6.1	23.2	1.88	8.5	40.4	1.71
10		17.8	42.5	2.59	19.6	56.0	2.57
30		19.9	53.5	2.73	21.8	60.6	2.57
60		22.2	59.0	3.01	24.8	62.2	2.51

B3: Indicative crystallinity data

Indicative cellulose crystallinity indexes (CrI) were determined by solid state ^{13}C NMR, as the area of the peak at 89 ppm attributable to C4 for cellulose crystalline interiors as a proportion of the total area under the spectrum (0-200 ppm). This is an unorthodox way of determining 'crystallinity' however, in this study the measurement is intended as a rapid guide opposed to obtaining a more definitive value *via* more intensive and time-consuming techniques such as X-ray diffraction (XRD).

The CrI for untreated sawdust (4.7) increased to 5.1-6.9 for the substrates subject to the various steam pretreatment conditions (**Figure B3-1**). Upon pretreatment, the CrI's were expected to increase, this is because the more severe pretreatments result in greater hemicellulose solubilisation; the CrI measurement can be influenced by this due to measuring the full 0-200 ppm range. Nonetheless, this does not override the fact the CrI values did not correlate well to digestibility at either enzyme loading, before or after ball-milling (**Figure B3-1**). Therefore, it would seem that cellulose crystallinity is not a major factor influencing the diverse digestibilities observed in this thesis, specifically in **Figure 5.3**.

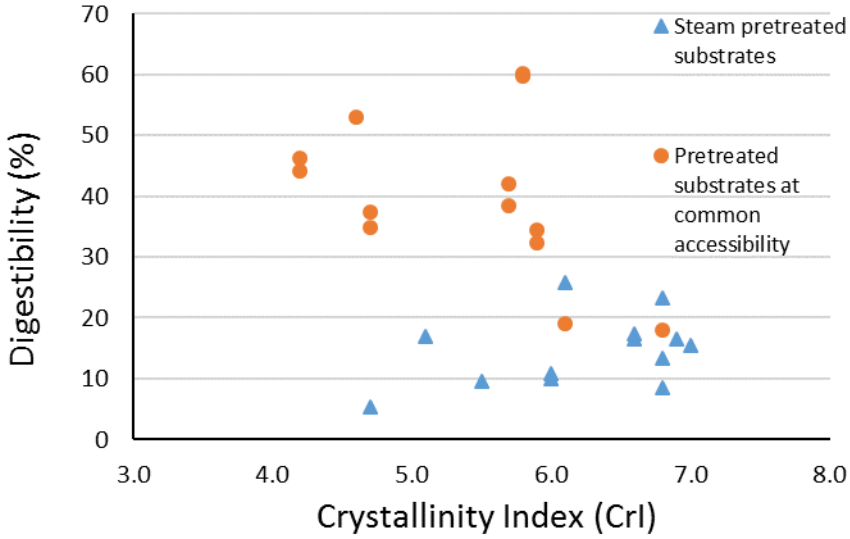


Figure B3-1: Relationship between digestibility and crystallinity indexes at an enzyme loading of 15 FPU/g substrate. Experimental duplicates are represented as individual points.

C1: Optimisation of gallic acid as a reductant for hydrolysis

A digestibility trial was conducted with CTec2 in which gallic acid was dosed back to BKp at differing concentrations (0.005 to 0.06 M) and the digestibility of BKp was determined. The digestibility of the BKp increased at low addition levels of gallic acid before decreasing at higher levels (> 0.02 M). The rapid decrease in digestibility beyond 0.02 M was associated with a significant decrease in pH. The optimal concentration of gallic acid to enhance the LPMO effect and the resulting digestibility was 0.01 M (**Figure C1**).

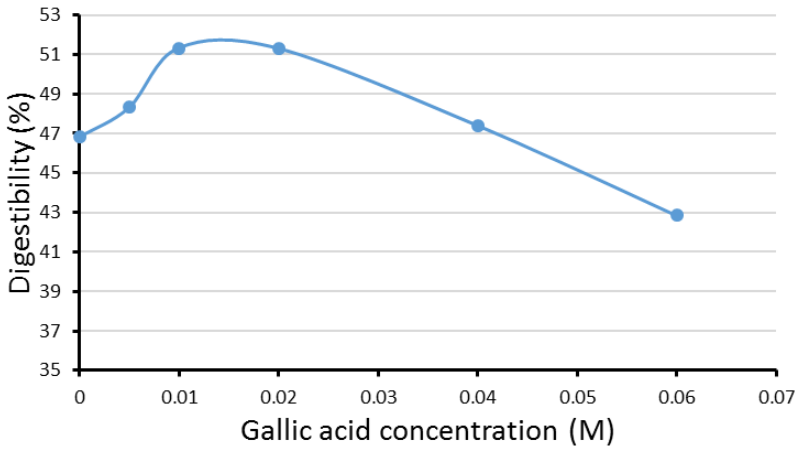


Figure C1: The effect on digestibility of BKp from adding gallic acid as a reducing co-factor. Optimal concentration of gallic acid addition is 0.01M.

D1: Journal publication

The following pages are the corrected proof copy of: MacAskill, J.J., Suckling, I.D., Lloyd, J.A., Manley-Harris, M. Unravelling the effect of pretreatment severity on the balance of cellulose accessibility and substrate composition on enzymatic digestibility of steam pretreated softwood. Journal of Biomass and Bioenergy 2018, 109C, 284-290.

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: www.elsevier.com

Research paper

Unravelling the effect of pretreatment severity on the balance of cellulose accessibility and substrate composition on enzymatic digestibility of steam-pretreated softwood

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ARTICLE INFO

Keywords:
Cellulose accessibility
Enzyme hydrolysis
Digestibility
Enzyme inhibition
Pretreatment
Softwood
Radiata pine

ABSTRACT

Pretreatment is essential for effective enzymatic digestion of lignocellulosic biomass. Steam pretreatments increase the digestibility by increasing the accessibility of the carbohydrates to the enzymes. However, they can also cause yield loss and lowered digestibility *via* increased non-productive binding of enzymes to lignin. The relative importance of these effects is not well defined, especially for softwoods which require more severe pretreatments than other types of biomass. *Pinus radiata* wood was steam pretreated at 180 °C and 215 °C to Combined Severity Factors of −3.31 or −2.61 and the digestibilities of the washed insoluble fractions examined before and after ball-milling to a common accessibility, as determined by Simons' stain measurements. Pretreatments at 215 °C for 2 min with citric and sulfuric acid catalysts were also investigated. Results showed that the digestibility of the pretreated substrates increased with pretreatment severity, rising from ~5% with no pretreatment to ~20% after the most severe pretreatment. However, when the substrates were ball-milled to a common accessibility, the digestibility decreased on increasing pretreatment severity. At a common accessibility and low enzyme dose the digestibility dropped six-fold from ~30% for the original wood to ~5% for the most severely pretreated substrate. This showed that while increasing pretreatment severity does lead to greater enzyme inhibition, this was being overridden by increases in the accessibility.

1. Introduction

Pretreatment to modify the structure and/or the chemical composition of a substrate is essential for effective digestibility of lignocellulosic biomass [1]. Short high-temperature (180–230 °C) steam treatments are generally effective for pretreating agricultural and hardwood biomass, whereas softwoods require harsher conditions such as the presence of an acid catalyst during the steam pretreatment [2–4].

Several substrate properties have been reported to influence the rate and/or extent of enzymatic digestion of pretreated substrates [5,6]. These include: the area of cellulose accessible to the cellulase enzymes; the size of the particles; the cellulose crystallinity; and inhibition of the enzymes by non-productive binding of the enzymes to the lignin or carbohydrate degradation products. In spite of being the subject of many studies, the relative importance of these different factors is still not clearly understood. What has however become clear is that the response of a given pretreated biomass to enzyme treatment is the result of a balance between two or more of these factors.

Pretreatments that increase the surface area of the substrate, leaving the cellulose more accessible to the enzymes, frequently lead to higher sugar yields [6]. Accessibility can be increased by either removing hemicelluloses or lignin from a lignocellulosic substrate, or by mechanically opening up the fibre to increase the exposed carbohydrates [7–9].

Components in the original biomass, or formed during biomass pretreatment can inhibit the enzymes by non-productively binding to the enzymes and therefore reducing their effectiveness. These inhibitors may be components of the fibre that remain in the water-insoluble fraction, particularly lignin [10–13], or water-soluble components from the pretreated substrate such as phenolics, organic acids or sugars [14]. Lignin is known to inhibit enzyme digestibility by binding to cellulases [15,16]. Non-productive binding of enzymes to lignin is a widely studied and accepted phenomenon, but is still poorly understood [10,12,13,16–19].

A number of studies have shown that particle size is a good predictor of digestibility [20,21]. This is due to the fact that smaller particles result in increased surface area, which gives enzymes easier access to

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https://doi.org/10.1016/j.biombioe.2017.12.018
Received 1 June 2017; Received in revised form 19 December 2017; Accepted 21 December 2017
Available online xxx
0961-9534/

the polysaccharides. It has been shown that increasing external surface area by reducing particle size is particularly important when a substrate is not well pretreated and has a low accessibility.

Cellulose crystallinity has also been suggested to influence the digestibility of lignocellulosic substrates, but the effects are inconsistent [22]. Many studies have shown that accessibility and lignin inhibition are the dominant factors and crystallinity is only a major contributor to digestibility for pure cellulosic substrates [6].

All of the above factors are affected by the pretreatment conditions. The pretreatment conditions of temperature, time and pH used to maximise overall sugar yields are a balancing act. On one hand, the pretreatment conditions should aim to maximise the accessibility of the enzymes to the cellulose and/or reduce cellulose crystallinity. At the same time yield losses due to dissolution or degradation of the biomass and inhibitor formation that occurs from the degradation and modification of sugars and/or lignin should be minimised. The relative importance of accessibility and inhibition at any given pretreatment severity ultimately should determine the sugar yield. A better understanding of this balance could lead to lower enzyme usage, as well as less expensive polysaccharide conversion and lower biofuel production costs.

This study evaluates the relative importance of factors controlling the digestibility of steam-pretreated radiata pine and in particular, the effect of pretreatment severity on the balance between the insoluble inhibitors present in pretreated wood and increasing cellulose accessibility. To do this, the digestibility of the insoluble fractions of steam-pretreated *P. radiata* wood derived from various pretreatment conditions was examined both before and after wet ball-milling to a common cellulose accessibility, as determined by Simons' stain measurements [23]. Comparisons of the digestibility of substrates at a common cellulose accessibility allowed for the impact of enzyme inhibitors to be evaluated independent of changes in the cellulose accessibility. The effect of particle size on digestibility was also evaluated.

2. Experimental

2.1. Materials

Fresh radiata pine (*P. radiata*) sawdust was collected from McAlpines Sawmill, Rotorua, New Zealand. This mill processes logs from plantation-grown radiata pine forests having a typical rotation time of 25–30 years. This material had a composition typical of that expected for radiata pine sapwood [24]. The sawdust was screened on a Williams vibratory screen (round holes) to collect the fraction of 1.29–3 mm in size and any bark particles removed. Citric acid, sulfuric acid, sodium azide and direct blue (Chicago Sky Blue 6B) were obtained from Sigma Aldrich. Direct orange dye (Pontamine Fast Orange 6RN) was obtained from Pylam Products Co. Inc (Garden City, NY). The commercial cellulose enzyme cocktail Cellic CTec2® (88.5 FPU g^{-1} enzyme) was sourced from Novozymes (Franklinton, NC, USA).

2.2. Sample preparation-steam pretreatment

Fresh sawdust (300 g oven dried, (O.D.) equivalent of 1.29–3 mm fraction) was soaked overnight in either water, or a solution of citric acid or sulfuric acid of required concentration, to deliver a loading of 20 kg m^{-3} acid on sawdust (oven-dry basis) after filtration and removal of excess liquid. Duplicate samples were steam exploded after heating for the temperature and times shown in Table 1 following the procedure of Clarkand Mackie[25]. Table 1. The Severity Factor (SF) and Combined Severity Factor (CSF) of each treatment was calculated using Equations (1) and (2), where t is time in minutes, T is temperature in degrees Celsius, and pH is of the soaking liquid [26,27].

$$\text{Severity Factor } (SF) = \text{Log } (R_0) = \text{Log}\left[t\right.$$

$$\text{Combined Severity Factor } (CSF) = \text{Log } ($$

The slurry produced from steam explosion was filtered using 100 μm nylon cloth on a Büchner funnel to give a solid and a liquid fraction (filtrate). The solid fraction was pressed to raise solids content and improve recovery of the filtrate, it was then thoroughly washed with hot water (60 °C) to remove any remaining soluble components. The wash-water was discarded. The filtrates were preserved using 0.1 g of sodium azide per litre of filtrate and both the filtrates and solids were stored at 4 °C.

2.3. Chemical composition

Solid fractions (air-dried) were ground in a Wiley mill to pass a 40 mesh screen and extracted with dichloromethane in a Soxtec apparatus. The extracted substrate was analysed in duplicate for acid insoluble lignin by TAPPI Standard Method T 222 om-88 (scaled down to 250 mg), and for acid-soluble lignin by TAPPI UM 250. Ash contents were measured using TAPPI Standard Method T 211 om-93.

Fucose was added to the hydrolysate from the lignin analysis as an internal standard and the carbohydrates (L-arabinose, D-glucose, D-galactose, D-mannose and D-xylose) were analysed by anion chromatography [28]. Carbohydrates (total sugars) in the filtrates were analysed after hydrolysis with 4% sulfuric acid at 121 °C for 60 min. All sugar results are the average of duplicates that agree within ±5% and are reported on anhydro sugar-basis by using conversion factors of 0.90 and 0.88 for hexoses and pentoses, respectively.

Table 1
Steam-pretreatment conditions and yield recoveries after pretreatment.

Sample #[a]	Catalyst	Temperature (°C)	Time (min)	CSF[b]	Yield (%)[c]		
					Insoluble	Soluble	Total
2	–	215	2	−3.31	83.3, 81.7	9.5, 9.6	92.8, 91.9
3	–	180	21.5	−3.31	77.3, 79.9	8.5, 5.9	85.8, 85.8
4	–	215	10	−2.61	71.1, 75.8	8.1, 9.1	79.3, 84.9
5	–	180	108	−2.61	69.9, 70.4	3.2, 4.2	73.1, 74.7
6	Citric acid	215	2	1.57	72.7, 74.9	18.0, 17.2	90.7, 92.1
7	Sulfuric acid	215	2	2.67	64.4, 63.3	21.5, 20.9	86.0, 84.2

[a] Sample 1 was untreated sawdust.
[b] Using initial pH values of soaking solution: Water 7.00, Citric acid 2.12, Sulfuric acid 1.02.
[c] Yields are a mass fraction of the original wood and duplicates are represented as individual values.

The cellulose and hemicellulose mass fractions of the original wood were calculated by means of equations (3) and (4) [29].

$$\text{Cellulose}(\%) = (\text{Glucose content} - 0.27$$

$$\text{Hemicellulose}(\%) = (\text{Arabinose} + \text{Galact}$$

2.4. Enzyme digestion

Enzymatic digestions on never-dried substrates were performed using 2.5 and 15 filter paper units of CTec2 per gram of substrate (FPU g^{-1}) at 1.5 g of solids loading per 100 cm^3 in a citrate buffer (10 kg m^{-3}) at pH 4.5 and 50 °C on a rotary shaker for 72 h. Digestion was stopped by plunging tubes into a boiling water bath and the mixture was centrifuged (3440 RCF, 10 min, 25 °C). The degree of conversion of glucan to glucose was determined by measuring glucose in the supernatant using a YSI-2700 analyser. The values were corrected for glucose contained in the enzyme solution, as well as glucose released from undigested substrate.

2.5. Ball-milling

Ball-milling was performed on wet, never-dried solid fractions (5% solids, 12 g, 0.1 kg m^{-3} sodium azide) in a 600 cm^3 ceramic vessel containing two hundred 15 mm diameter alumina balls on a Schwingmühle VIBRATOM vibratory ball mill. All ball-milled samples were corrected for ash content.

Particle size of substrates before and after ball-milling were determined in duplicate using a Malvern Mastersizer 2000 particle size analyser.

2.6. Simons' stain procedure

A modified version of the Simons' staining procedure developed by Chandra et al. [30] was used. The fractionation of the orange dye was performed according to Esteghalian et al. [31]. All samples were analysed in duplicate and the same batch of filtered orange dye was used for all experiments to reduce experimental error [30,32]. The substrate (200 mg, O.D. equivalent) was weighed into vials (six per series) and 1 cm^3 of phosphate buffered saline solution (pH 6, 28.5 kg m^{-3} PO_4^{3-}, 0.09 kg m^{-3} NaCl) added to each. To each vial both orange (stock conc = 10 mg cm^{-3}) and blue (stock conc = 10 mg cm^{-3}) dye solutions were added in a series of increasing volumes (0.25, 0.5, 0.75, 1, 1.5, 2 cm^3). These vials containing 1:1 mixtures of orange and blue dyes at increasing concentrations were used to measure the dye adsorption isotherms. The volume was made up to 10 cm^3 with distilled water and vials were incubated (65 °C, 16 h) with continual shaking. After incubation, the vials were centrifuged (3440 RCF, 10 min) and after appropriate dilution, the absorbance of the supernatant at both 425nm and 620nm was measured using a Shimadzu UV-1800 analyser. The two wavelengths 425 nm and 620 nm represent λ_{max} of the orange and blue dyes respectively. The amount of dye adsorbed onto samples was determined as the difference in concentration of the original dye added and the dye concentration in the supernatant using the Beer-Lambert law [31]. Extinction coefficients were calculated from the slope of calibration curves for each dye at 425 and 620 nm. The values calculated and used in this study were $\varepsilon O_{425} = 52.07$, $\varepsilon O_{620} = 0.12$, $\varepsilon B_{425} = 7.59$ and $\varepsilon B_{620} = 81.18$ L g^{-1} cm^{-1}. In this study, Simons' stain O:B ratio was selected over the total orange dye adsorbed to represent the cellulose accessibility. This is because although the two follow the same trends, the O:B ratio gave better R^2 correlations to digestibility (data not shown).

3. Results and discussion

P. radiata sawdust was steam pretreated in duplicate using six different conditions (Table 1). Steam pretreatments without any added acid catalyst were performed at Combined Severity Factors of −2.61 and −3.31. At each CSF the treatments were carried out at both 215 °C for short times and at 180 °C for longer times. This allowed the effects of treatment severity, temperature and time to be isolated and analysed. Then, to understand the impact of adding acid catalysts, additional steam pretreatments were conducted at 215 °C for 2 min in the presence of 20 kg m^{-3} citric or sulfuric acid. Although it has limitations, and other measures have recently been proposed, the Combined Severity Factor is a widely-used empirical way to predict pretreatment severity [27,33,34].

3.1. Effect of pretreatment conditions on yield, carbohydrate and lignin content

Steam pretreatment of the sawdust results in partial dissolution of the wood mass (Table 1). Insoluble solid yields from the six steam pretreatment conditions ranged from 63 to 83% of the starting mass. For treatments without added catalyst the average insoluble yields decreased from 81% to 72% as CSF increased from −3.31 to −2.61. At a given CSF, yields were lower for low temperature/long time pretreatments than for the high temperature/short time pretreatments. This difference could possibly be due to the different activation energies required for each degradation reaction and/or a heating time lag in the short duration runs. As expected, due to the higher CSF, addition of an acid catalyst resulted in a substantial increase in dissolution and a corresponding decrease in the insoluble solid yields.

Plots of the cellulose, lignin and hemicelluloses remaining in the insoluble solids after pretreatment show that the decrease in solid yields as CSF increases can be mainly attributed to dissolution of hemicelluloses from the wood (Fig. 1). As the pretreatment severity increased total carbohydrate contents of pretreated radiata pine solids decreased (Table 2). For example, the total carbohydrate mass fraction decreased from 67.3% in untreated pine to between 55.5 and 37.2% for the pretreated samples. More severe pretreatments resulted in greater carbohydrate removal; the percentage hemicellulose removed from solids increased from 47 to 99% as pretreatment severity increased. There were also small amounts of cellulose and lignin removed from solids as the pretreatment severity increased (student t-test, 95% confidence level).

The two major hemicelluloses of softwoods, galactoglucomannan and arabinoglucuronoxylan are substantially removed during pretreat-

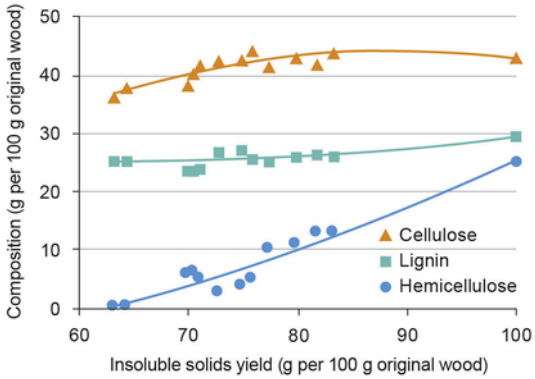

Fig. 1. Comparison of cellulose, lignin and hemicellulose levels versus insoluble solids yields of pretreated substrates. Experimental duplicates represented as individual points.

Table 2
Adjusted component mass fraction of the untreated sawdust and pretreated insoluble solids.[a]

Sample #	Pretreatment conditions	Total lignin[b]	Carbohydrate mass fraction of the original wood (%)[c]						
			Arabinose	Galactose	Glucose	Xylose	Mannose	Cellulose	Hemicellulose
1	Untreated sawdust	29.4	1.50	3.16	46.0	5.98	10.7	43.1	24.9
2	215 °C/2 min Water	26.2, 26.3	0.25, 0.25	1.42, 1.41	45.4, 43.4	3.76, 3.71	5.71, 5.71	43.8, 41.8	13.1, 13.0
3	180 °C/21.5 min Water	25.4, 25.9	0.29, 0.35	0.80, 0.88	42.6, 44.4	3.29, 3.40	4.30, 4.53	41.4, 43.2	10.2, 10.7
4	215 °C/10 min Water	24.0, 25.7	0.04, 0.04	0.38, 0.37	42.2, 44.8	1.84, 1.86	1.98, 2.10	41.6, 44.3	4.95, 5.13
5	180 °C/108 min Water	23.7, 23.8	0.10, 0.11	0.40, 0.47	38.8, 40.9	1.87, 2.01	2.44, 2.59	38.1, 40.2	5.64, 6.06
6	215 °C/2 min Citric acid	26.7, 27.3	0.04, 0.09	0.23, 0.29	42.9, 43.0	1.49, 1.69	0.69, 1.09	42.7, 42.7	2.79, 3.63
7	215 °C/2 min Sulfuric acid	25.3, 25.3	0.03, 0.03	0.01, 0.01	37.8, 36.1	0.26, 0.25	0.00, 0.00	37.8, 36.1	0.32, 0.32

[a] Duplicate steam pretreatments represented as individual values.
[b] Total lignin = Acid soluble lignin + Acid insoluble lignin.
[c] Anhydro sugar units.

ment as shown by the significant decreases in arabinose, galactose, glucose, mannose and xylose contents of the solids after pretreatment (Table 2). These decreases were mirrored by increases of these sugars in the corresponding filtrates (Table 3).

The levels of hemicellulose and cellulose removed from the insoluble solids fractions were greater than the levels of corresponding sugars in the filtrates, indicating that some sugars are lost and/or degraded during steam explosion. Unaccounted mass was in a 7–27% range for uncatalysed pretreatments; the unaccounted mass increased as pretreatment severity increased and was highest for long duration pretreatments (Table 1). Addition of 20 kg m^{-3} citric acid did not result in higher unaccounted mass, although the severity was substantially greater. On the other hand, 20 kg m^{-3} sulfuric acid addition did increase mass loss relative to the equivalent uncatalysed pretreatment mass. Consistent with this, total recovered water insoluble solid yields after steam explosion varied between 73 and 93%, depending on the conditions. The mass losses reported here are similar to those reported in other studies [25,35]. Also consistent with carbohydrate degradation, low levels of furfural and hydroxymethylfurfural (HMF), were found in the filtrates (Table 3). Although these two furans readily form under acidic conditions [36], they are both volatile and reactive under these conditions [36,37].

3.2. Effect of pretreatment conditions on cellulose digestibility

The percentage glucan conversion (digestibility) of the pretreated substrates was assessed at both a high and low enzyme dose; 15 and 2.5 FPU g^{-1} substrate. Two enzyme doses were used to increase the possibility of observing the presence of enzyme inhibition. This was because although the higher enzyme dose gives sugar yields of greater practical interest, treatments at low enzyme doses may be more affected by non-productive binding of the enzymes to the lignin or other inhibitors, due to the lower enzyme to inhibitor ratio in the substrate.

The digestibility of the pretreated substrates increased as pretreatment severity increased for both enzyme doses (Fig. 2A). This is consistent with previous research [6,38,39] and coincides with the findings of Zhou et al. [40], where glucose yields of softwoods were found to positively correlate with hemicellulose removal. As expected, the glucose yields from the untreated radiata pine were low, 3.4 and 5.1% for the 2.5 and 15 FPU g^{-1} enzyme doses, respectively. Pretreatment increased glucose yields up to 5.3 and 19.6% for the low and high enzyme doses, respectively.

In the absence of acid catalysts, the digestibility of solids pretreated at the same severity but under different conditions, showed comparable digestibilities. For example, the digestibilities of both of the low severity conditions; 215 °C/2 min and 180 °C/21.5 min were ~4% and 9% for the respective 2.5 and 15 FPU g^{-1} enzyme doses (Fig. 2A). This observation is consistent with previous research [27].

At 215 °C/2 min, the addition of 20 kg m^{-3} citric or sulfuric acid increased the digestibility of the pretreated solids in comparison with the solid fraction that was not acid catalysed. The more severe the acid catalyst used in the pretreatment the higher the digestibility observed: Digestibility for both low and high enzyme doses is, sulfuric acid > citric acid > water (Fig. 2A). The higher digestibilities are consistent with more severe pretreatments removing more hemicelluloses and thus improving the access by enzymes to cellulose.

Consistent with the higher digestibility of substrates being associated with preferential removal of hemicelluloses, the digestibility of solids increased as the insoluble solid yield decreased (Fig. 3A). The higher digestibilities of substrates also correlated with increasing lignin contents, but this was a result of the proportion of lignin remaining in a substrate increasing as the hemicelluloses were removed (Fig. 3B).

3.3. Relationship between cellulose digestibility and enzyme accessibility

The accessibility of the cellulose in the substrates was assessed by using the Simons' stain procedure following the method of Chandra et al. [30,32]. Simons' stain procedure uses two different dyes. These dyes differ in their molecular size, colour (orange and blue) and affinity to bind to cellulose. The maximum amount and ratio of the two dyes adsorbed can be measured and gives an estimation of the overall cellulose accessibility of a substrate. A good correlation ($R^2 > 0.8$) was observed between the digestibility of the pretreated substrates at both enzyme doses and the accessibility of the cellulose, as measured by the Simons' stain orange to blue (O:B) dye ratio (Fig. 4). This suggests, in agreement with earlier studies [6,41–44], that increases in the accessibility of the cellulose is an important determinant of substrate digestibility. In this study, increased accessibility can be mainly attributed to hemicellulose dissolution that increased with pretreatment severity (Tables 2 and 3).

However, it is apparent from the scatter in the data that cellulose accessibility is not the only factor contributing to digestibility. In particular, the samples pretreated with citric acid are more digestible than predicted by their accessibility (Fig. 4, open markers). It is hypothesized, that although citric acid is very effective at solubilising hemicelluloses [6], it is a relatively weak acid so only causes low levels of acid-catalysed lignin modification.

Table 3
Adjusted component mass fractions of filtrates.[a]

Sample #	Pretreatment conditions	Carbohydrates mass fraction of the original wood (%)[b]							Furans (mg kg^{-1})	
		Arabinose	Galactose	Glucose	Xylose	Mannose	Cellulose	Hemicellulose	Furfural	HMF
2	215 °C/2 min Water	0.88, 0.87	1.16, 1.14	1.31, 1.27	1.99, 1.93	4.17, 4.02	0.19, 0.18	9.52, 9.23	121, 125	840, 670
3	180 °C/21.5 min Water	0.55, 0.49	1.05, 1.09	0.97, 1.01	1.45, 1.53	3.02, 3.17	0.15, 0.15	7.03, 7.29	237, 239	151, 169
4	215 °C/10 min Water	0.47, 0.47	1.01, 1.06	1.26, 1.44	1.58, 1.54	3.30, 3.55	0.37, 0.48	7.41, 7.75	843, 931	1050, 982
5	180 °C/108 min Water	0.44, 0.47	0.52, 0.60	0.43, 0.56	0.70, 0.84	1.11, 1.46	0.13, 0.16	3.14, 3.85	157, 197	230, 295
6	215 °C/2 min Citric acid	1.08, 1.11	1.99, 1.99	2.60, 2.46	3.66, 3.50	7.49, 7.37	0.58, 0.46	16.6, 16.3	308, 245	484, 323
7	215 °C/2 min Sulfuric acid	0.93, 0.85	1.89, 1.74	5.46, 5.34	3.56, 3.23	7.11, 6.38	3.54, 3.62	15.8, 14.2	2140, 2300	3850, 4200

[a] Duplicate steam pretreatments are represented as individual values.
[b] Sugars reported as total anhydro units from free and oligomeric sugars.

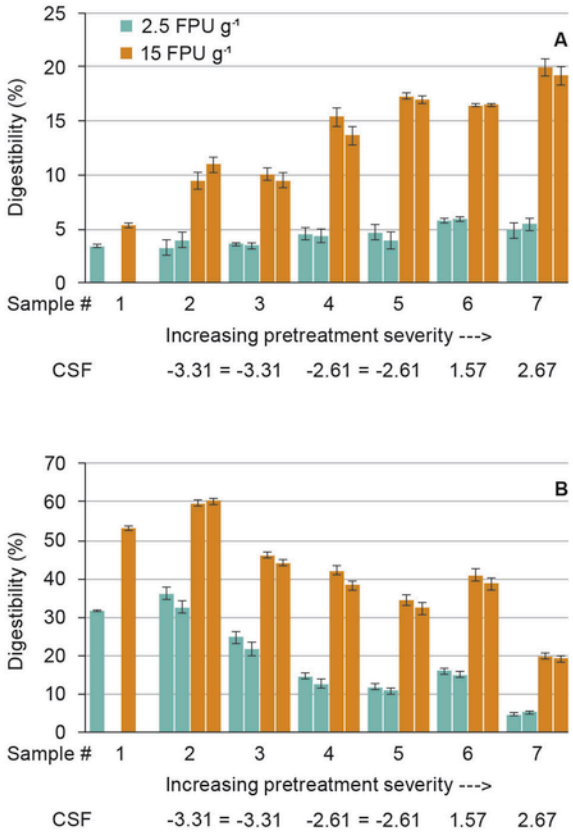

Fig. 2. Digestibility of steam pretreated solids as measured by percentage glucan conversion of steam pretreated substrates using enzyme loadings of 2.5 and 15 FPU g^{-1}: after steam pretreatment (A); after ball-milling to a common Simons' stain accessibility (O:B ratio of 1.8) (B). Experimental duplicates represented as individual bars. Error bars represent ± a standard deviation of triplicate determinations. See Table 1 for details of pretreatment conditions.

3.4. Cellulose digestibility of pretreated samples at a common accessibility

To help understand the relative importance of enzyme inhibition and accessibility as a function of pretreatment severity it was important to compare the digestibility of pretreated samples at a common cellulose accessibility. Wet ball-milling was used to adjust the accessibility of the substrates up to a common level. Wet ball-milling of lignocellulosic materials mechanically disrupts fibre walls and decreases particle size thus improving the accessibility of enzymes to cellulose. As a result, the overall digestibility of a substrate is increased [9]. It was assumed that the mechanical forces during ball-milling increased the accessibility of the substrate without inducing chemical changes. Ball-milling is widely used to obtain milled wood lignin (MWL), which is the best representative of native lignin. The milling is performed under harsh conditions, which includes dry-milling for extensive time periods of up to 3 days. In this study milling was performed on a wet-basis and for comparatively short time periods. A recent study [45], showed only very minor lignin degradation when wet ball-milling was used under conditions similar to those used in this study.

To get the pretreated substrates to a common accessibility, they were wet ball-milled for various times to determine how cellulose accessibility changes as a function of ball-milling time (Fig. 5). Each pretreated substrate responded to ball-milling at a different rate and extent. This is likely due to the fact that steam pretreatment and ball-milling have different mechanisms for increasing cellulose accessibility [9], and the ball-milling was performed on substrates that had been steam-pretreated. This showed by selecting the appropriate ball-milling

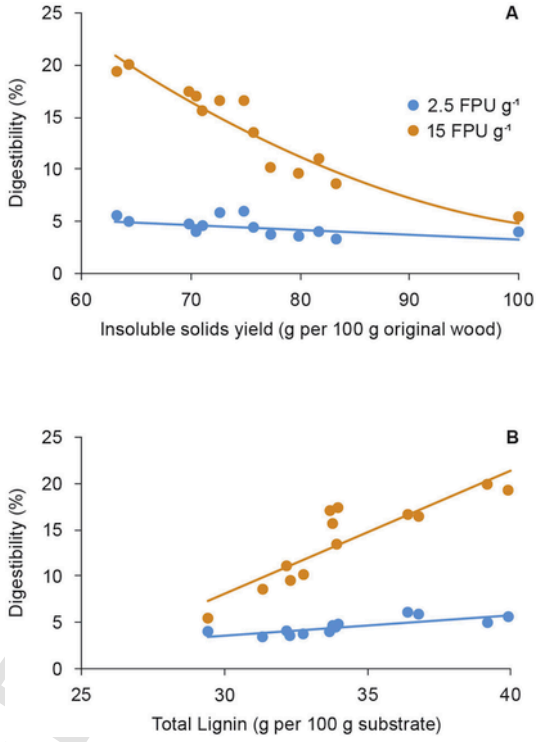

Fig. 3. Digestibility of steam pretreated substrates as measured by percentage glucan conversion of steam pretreated substrates using enzyme loadings of 2.5 and 15 FPU g^{-1} versus: insoluble solids yield (A); total lignin (B).

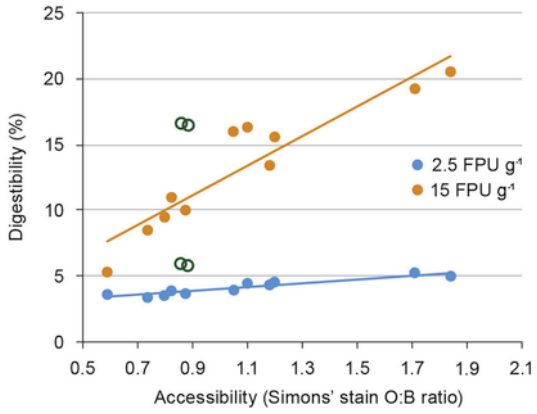

Fig. 4. Digestibility of steam pretreated substrates as measured by percentage glucan conversion of steam pretreated substrates using enzyme loadings of 2.5 and 15 FPU g^{-1} versus accessibility, as determined by Simons' stain orange to blue (O:B) dye ratio; citric acid samples represented as open markers. R^2 correlations were >0.8 for both the high and low enzyme doses, these correlations exclude the citric acid samples.

time (dotted line, Fig. 5), it was possible to take all substrates to a common cellulose accessibility of 1.8 ± 0.06 as estimated by the Simons' stain O:B ratio. The required ball-milling time decreased as the pretreatment severity increased; 110 min being required for the original wood decreasing to zero minutes for the sulfuric acid treated substrate.

At a common accessibility, the digestibility decreased as the pretreatment severity increased (Fig. 2B). This is opposite to the trend observed prior to ball-milling, suggesting that inhibition via non-productive binding of the enzymes to lignin occurs and increases with increasing pretreatment severity. The incomplete digestion of the original untreated *P. radiata* wood may indicate that the native lignin in *P. radiata* is capable of binding the enzymes. This is consistent with earlier work which found the digestibility of Avicel, a pure cellulose, decreased

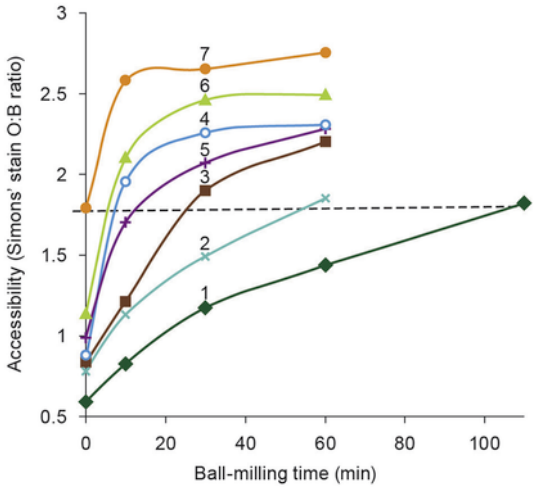

Fig. 5. Accessibility of steam-pretreated substrates as a function of ball-milling time. Substrates 1–7 are identified in Table 1. The dotted line indicates a common accessibility Simons' stain O:B ratio of 1.8 and thus the ball-milling times required to attain this common accessibility.

when isolated spruce milled wood lignin was added during digestion [17].

Overall, the results suggest that accessibility is a major determinant of digestion levels and that non-productive binding of enzymes to lignin increases in parallel with, or possibly more slowly than, accessibility as the pretreatment severity increases. Any pretreatment chosen will thus be a compromise between these two effects. These results are in agreement with other studies [41,42,46], in which the increased accessibility seen at higher pretreatment severities more than compensates for increases in enzyme inhibition.

The digestibilities of the substrates at a common cellulose accessibility decreased as the lignin content of the substrates increased (Fig. 6). This may be a result of greater non-productive binding due simply to the presence of more lignin in the substrate or increasing pretreatment severity leading to lignin modification and stronger non-productive binding of cellulase enzymes to lignins [12,13,46]. It has been reported that lignins isolated from pretreated substrates reduce the digestibility of pure cellulose more than lignins isolated from original wood [13,17], so lignin modification may explain the results shown in Fig. 6. In this study it is not possible to distinguish between the effect of lignin content and composition. However, the scatter seen in Fig. 6, would suggest that lignin content is not the only factor.

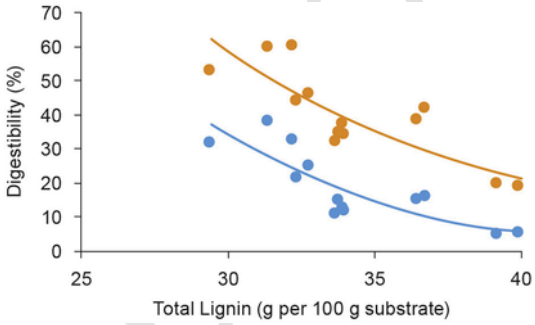

Fig. 6. Comparison of the digestibility as measured by percentage glucan conversion of steam pretreated substrates using enzyme loadings of 2.5 and 15 FPU g^{-1} of steam pretreated substrates at a common accessibility (Simons' stain O:B ratio of 1.8) versus lignin content.

3.5. Particle size

For the samples which were not ball-milled, the mean particle size of steam-pretreated solids decreased with increasing pretreatment severity. As particle size decreased, the cellulose accessibility increased (Fig. 7). This would be expected, given that smaller particle size increases the surface area of a substrate, which in turn can make more cellulose accessible. Consequently, the particle size also correlated well with digestibility (data not shown).

Ball-milling the substrates to a common cellulose accessibility reduced mean particle size substantially, with the mean particle size of pretreated substrates decreasing to ~30–40 μm once ball-milled, irrespective of ball-milling time. However, when the particle size of substrates at the common cellulose accessibility were compared to digestibility values, the correlation was poor for both enzyme loadings (data not shown). This indicates particle size is not the major determinant of digestibility; other factors such as accessibility and enzyme inhibition are more significant. The above finding is not surprising given there is evidence from a previous study on poplar wood showing that below 40 mesh (< 420 μm), particle size has little effect on biomass digestibility [47].

4. Conclusions

This study is consistent with cellulose accessibility and enzyme inhibition being major factors contributing to the digestibility of steam-pretreated *P. radiata* wood. Under the conditions studied, as pretreatment severity increased, increasing accessibility was the dominant factor. The increases in accessibility more than compensated for increases in enzyme inhibition. It was also found that higher temperatures for shorter time periods were more beneficial as they gave higher digestibilities even though fewer hemicelluloses were extracted from the wood. At 215 °C for 2 min and at a common cellulose accessibility, pretreatment without the acid catalyst gave a more digestible substrate. There is a need to carefully choose pretreatment conditions to optimise the opposing effects of increasing accessibility and increasing enzyme inhibition in order to maximise digestibility.

Acknowledgements

JJM acknowledges financial support from Scion and both the Sir Edmund Hillary Scholarship and the Waikato Graduate Women's Trust through The University of Waikato. We also acknowledge partial funding support from MBIE via Scion core funding.

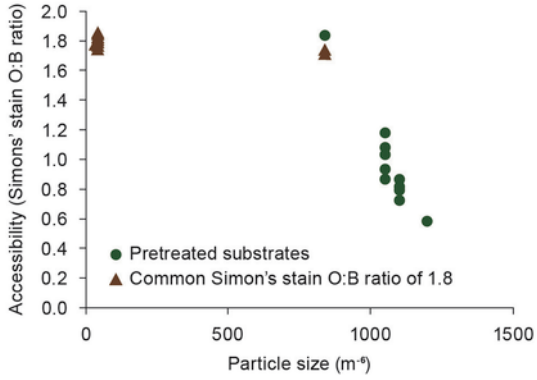

Fig. 7. Relationship between particle size and accessibility as measured by Simons' stain O:B ratio. Experimental duplicates are represented as individual points.

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