Investigating the role of laccase and laccase mediator systems to improve the saccharification of biomass for bioethanol production

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy (PhD) in the Faculty of Engineering and Physical Sciences

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Lucy Heap

School of Chemistry

Manchester Institute of Biotechnology

Table of Contents

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Abstract

Investigating the role of laccase and laccase mediator systems to improve the saccharification of biomass for bioethanol production

Lucy Heap

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As global energy demands increase, there is a requirement to decrease our dependency on fossil fuels due to their finite supply and negative environmental impacts. Alternative sources of energy are required that offer sustainability, reduced cost and environmental benefits. Second generation biofuels remove the 'food vs fuel' drawback of the first generation. They utilise lignocellulosic biomass, providing cheap and abundant starting materials for energy production. The major biotechnological challenge associated with lignocellulosic processing is the natural recalcitrance of the substrate to sugar conversion (saccharification). This recalcitrance is largely associated with lignin, an aromatic heteropolymer that encases cellulose. To improve bioethanol yields, there is a need for cost effective and environmentally friendly pretreatment methods that can remove lignin.

An enzymatic pretreatment strategy was investigated using laccase from the fungus *Trametes versicolor* (TvL). Expansion of the laccase substrate range towards non-phenolic substrates was explored by screening of a panel of synthetic and naturally derived phenolic compounds as potential redox mediators with laccase. Both groups enabled decolourisation of the recalcitrant dye (RB-5) to varying degrees, which laccase alone was unable to achieve. In the case veratryl alcohol, a lignin model substrate, synthetic compounds 1-HBT, ABTS and violuric acid proved effective laccase mediators. On this basis, TvL with 1-HBT was selected as the most successful laccase mediator system (LMS) and was further explored as a biomass pretreatment method.

The effects of LMS treatments towards the saccharification of acid hydrolysed wheat straw were extensively investigated. Optimisation revealed that when both TvL and a TvL LMS of synthetic origin (1-HBT, violuric acid) were applied, saccharification was improved. The observed increase in glucose release was only detected when a second lignin removing technique was applied in succession. Both alkaline-peroxide and organosolv extractions were successfully used to demonstrate the role of laccase/LMS in saccharification improvement, with improvements reaching up to 44.6%. The effect was further demonstrated with additional substrates (corn and sorghum stover) and additional laccases (*Pleurotus ostreatus, Agaricus bisporus and Rhus vernicifera*). Further studies using β-O-4 structures, py-GC/MS and FTIR analyses provide further information on the structural actions of an LMS towards lignin, including strong evidence for Cα-Cβ cleavage and Cα hydroxyl oxidation mechanisms.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright statement

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Abbreviations

1-HBT	1-Hydroxybenzotriazole
3-HAA	3-Hydroxyanthranilic acid
Abs	Absorbance
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AEC	Anion exchange chromatography
AOX1	Alcohol oxidase 1
AS	Acetosyringone
BMM	Buffered complex media containing methanol
BMGY	Buffered complex media containing glycerol
BSA	Bovine serum albumin
CBH	Cellobiohydrolase
d.w	Dry weight
E°	Redox potential
E. coli	*Escherichia coli*
ET	Electron transfer
FTIR	Fourier Transform InfraRed
g/dw	grams per dry weight
GOX	Glucose oxidase
HIC	Hydrophobic interaction chromatography
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
kDa	kilodalton
Lcc	Laccase
Lccβ	Laccaseβ (from *T. versicolor*)
Lccδ	Laccaseδ (from *T. versicolor*)
LMS	Laccase mediator system
MW	Molecular weight
MWCO	Molecular weight cut off
P. pastoris	*Pichia pastoris*
PAHs	Polyaromatic hydrocarbons
PC	*p*-coumaric acid

PCR	Polymerase chain reaction
PoL	*Pleurotus ostreatus* laccase (commercial prep)
PR	Phenol red
Py-GC/MS	Pyrolysis-Gas chromatography/Mass Spectrometry
RB-5	Reactive black-5
RBB	Remazol brilliant blue
RPM	Revolutions per minute
SA	Syringaldehyde
S. cerevisiae	*Sacchromyces cerevisiae*
SEC	Size exclusion chromatography
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel
T. versicolor	*Trametes versicolor*
T1, T2 or T3-Cu	Type 1, Type 2 or Type 3 copper
TEMPO	2,2',6,6'-tetramethylpiperidine
TMAH	Tetramethylammoniumhydroxide
TvL	*Trametes versicolor* laccase (commercial prep)
U/mg or U/g	Enzyme units per milligram or per gram
U/mL or U/L	Enzyme units per millilitre or per litre
UF	Ultrafiltration
V	Volts
v/v	volume per volume
VAlc	Veratryl alcohol
VAld	Veratryl aldehyde
w/w	weight per weight
YPD	Yeast extract peptone dextrose
YPDS	Yeast extract peptone dextrose with sorbitol
ε	Extinction coefficient

Chapter one

1.0 General Introduction

1.1 Energy production

1.1.1 Non-renewable energy production

As the world's population and economy continue to grow, there is an increased demand to provide the resources required to sustain this growth. According to the International Energy Agency's (IEA) World Energy Outlook (WEO) factsheet for 2013 [1], the world's primary energy demands are estimated to increase by 33% from 2011 to 2035. The report predicts that emerging economies will account for >90% of this estimated net increase. In 2009, China overtook the United States as the largest consumer of energy. In the current decade, China is predicted to continue to drive this increase in demand, with India expected to prevail as the principle source of growth during the 2020s [1].

In the present day it is predominately the use of non-renewable resources (coal, oil and natural gas) that fulfil the growing increase in energy demand. Although non-renewable fossil fuels may sustain our current energy requirements well into the future [2], a general rise in the cost of crude oil and the eventual decrease in the availability of fossil fuels has forced the world to consider more sustainable and economically feasible alternatives to producing energy. The share of fossil fuels in today's 'energy mix' (the energy that is available to us) is 82%, which is the same proportion as it was 25 years ago. With the rise of renewable energy sources, this share is expected to decrease to 75% by 2035, with nuclear and renewable sources aiming to meet ~40% growth in energy demand [3].

Further to cost and availability, the use of fossil fuels has negative impacts towards our environment. The burning of fossil fuels in the energy sector has been directly linked to the release of greenhouse gases (GHGs) which include carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). GHGs are the primary causative agents of global warming and levels of GHGs have already passed the threshold of 450ppm (parts per million, CO_2), which is classified as dangerously high [4]. By 2035, energy related CO_2 emissions are expected to increase by 20%, which is linked to an average world temperature increase of 3.6°C, 1.6°C higher than the internationally agreed climate target [1]. Consequences of this increase include climate change, rising sea levels and a loss of biodiversity.

In the 2010 IEA WEO [5] it was revealed that crude oil production would never increase and that in order to keep the production of crude oil constant, huge investments would need to be made. However, even if the production of crude oil was maintained at a constant level, it would still not be sufficient to meet the growing global demands.

Historical trends reveal that globally, crude oil is our major energy source. This is largely due to the increased growth of our transportation sector, which almost completely relies on petroleum. The transportation sector currently dominates over half of the global demand for crude oil [6,7]. Data from the Energy Information Administration (EIA) [8] reported that in 2010, the transportation sector was responsible for around 70% of the total crude oil consumed within the U.S.A, highlighting the urgent requirement for crude oil alternatives to support this sector (Figure 1).

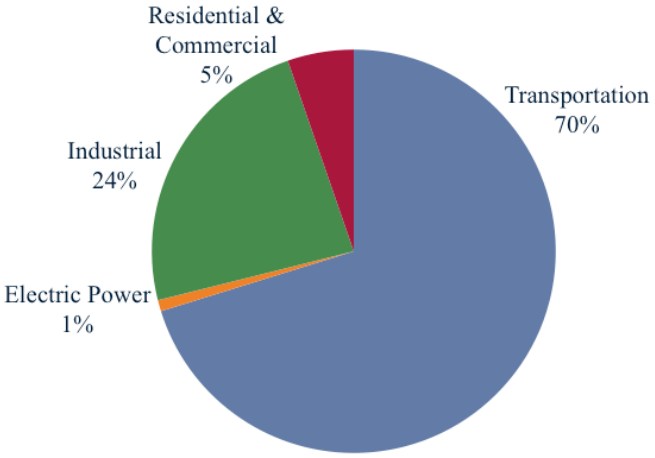


Figure 1: U.S petroleum consumption by sector in 2010 taken from the EIA monthly energy review [8].

In the 2011 IEA WEO, [9] data was published to represent the estimated trends in the world's liquid energy (oil) supply as presented in Figure 2.

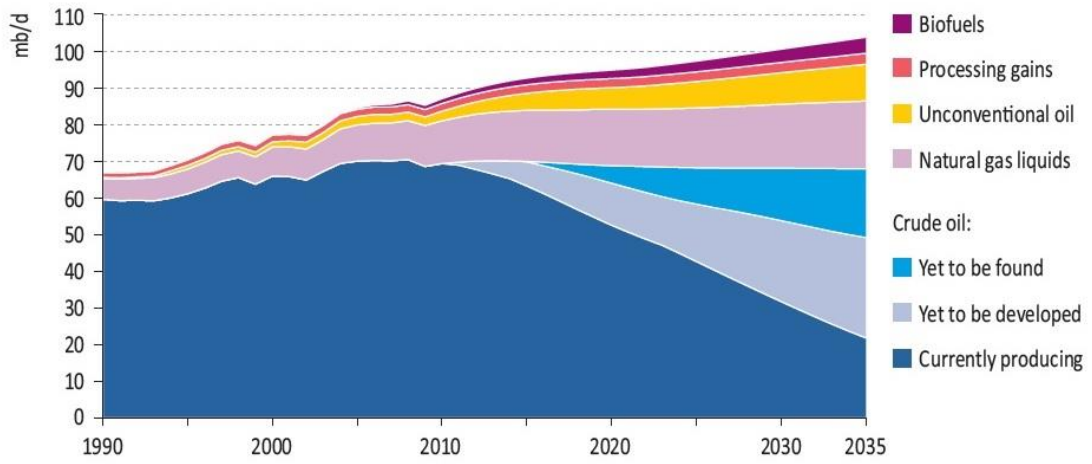


Figure 2: The world's liquid fuel supply by type in the New Policies Scenario as published by the IEA in their 2011 WEO [9].

According to these data, crude oil production will be maintained up to 2035 through the discovery and development of reserves that have or have yet to be found. This estimation appears optimistic, due to reserves depleting at a rate of around 5.5% per year [10], suggesting that constant drilling and identification of new wells would be required. A growth in the production of natural gas liquids (NGLs) is anticipated, with 1.7 million of the 5.8 million b/d (barrels/day) growth in oil production (since 2005) represented by NGLs (in this context 'oil' includes unconventional oils, shale oil and NGLs). Whilst there is value in NGLs, they are not the main feedstock for the production of transportation fuel (gasoline and diesel), despite the availability of autogas/liquid petroleum gas from propane and butane. Furthermore one gallon of NGL only contains around 64% of the energy available from one gallon of gasoline [11].

Aside from NGLs, the scarcity of crude oil reserves has led to an increase in the development of alternative sources of oil such as 'unconventional oils'. The term unconventional oil relates to oils that are difficult to extract, and require unconventional production technologies such as horizontal drilling, thermal processing and hydraulic fracturing (as opposed to standard oil well extraction). Unconventional oils include extra heavy oil, natural bitumen (oil sands) and kerogen oil (not to be confused with unconventional liquids that include biofuels, gas to liquids and coal to liquids) [9,12]. Production costs of unconventional oils are much

greater in comparison to conventional (crude) oil production due to the greater energy requirements associated with the extraction technologies.

One of the key recent developments in oil resourcing is the production of light tight oil (also known as shale oil or simply tight oil) and oil production from ultra-deepwater fields. The success of the USA in the production of light tight oil will eventually enable the country to overtake Saudi Arabia as the world's largest oil producer. The burning of cleaner fuels such as tight light oil offers the benefit of reducing CO_2 emissions, however despite this, concerns currently lie within the methods used to obtain such products (hydraulic fracturing and drilling). These methods are tainted by the uncertainty surrounding possible environmental pollution by toxic chemicals (e.g. methane release), land disruption/degradation and a risk of aquatic oil spills (in the case of ultra-deep water fields) [13,14]. It is also predicted that, once the production in oil has peaked, a decline in output will eventually follow [6], suggesting that these alternatives will not provide longer term solutions.

To summarise, it is essential that in order to provide cleaner fuels, preserve our declining oil supply and to dramatically reduce potential increases in GHG emissions [15], sustainable fuel alternatives must continue to be developed.

1.1.2 Renewable sources of energy & biomass

A rapid expansion in the development of renewable power generation (hydropower, wind and solar) has taken place over recent years due to the reasons discussed in Section 1.1.1 and the requirements of the European Union's Renewable Energy Directive and national targets. This expansion is expected to continue well into the future with renewable sources predicted to rival coal as a primary source of global electricity [6,16]. Aside from the 'free' availability of resources such as wind and solar, their sustainability is questionable and they are generally considered to be unsuitable for meeting the increased demands for power due to their requirements for a continuous supply (of wind or sunlight).

In addition to these sources of renewable power, globally (land and sea), there is an abundance of different types of biomass available. The term biomass refers to organic matter that is available on a renewable or recurring basis, and includes dedicated energy crops and trees, wood and wood residues, agricultural food and feed crop residues, animal wastes, aquatic plants (microalgae) and grasses [17]. Utilising

biomass as a renewable carbon source in combination with current efficient technological advances can enable the sustainable production of bioenergy, biofuel and bio-based products in a biorefinery-type system as presented in Figure 3. The economic benefits of a biorefinery can be enhanced by the selection of feedstocks that are not required for other economical processes (e.g. food such as corn and wheat) [17].

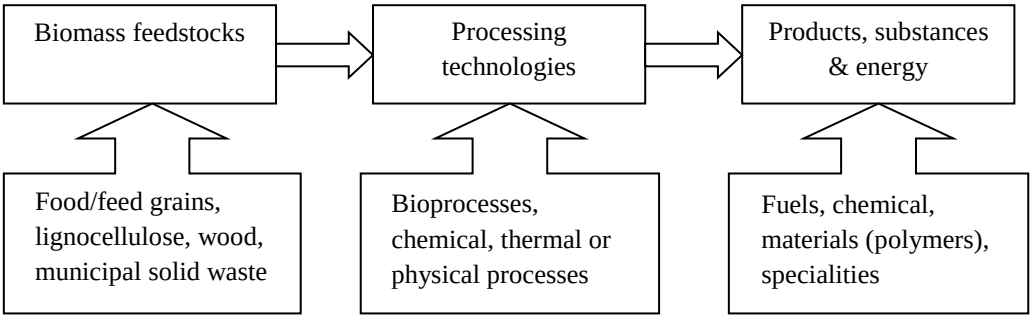


Figure 3: The basic principles of a biorefinery adapted from Kamm & Kamm [17].

1.2 Biofuels

1.2.1 General

The demand for fossil fuels has risen in line with an increase in industrialisation and motorisation. Their gradual depletion and the associated effects of increased GHG emissions (climate change, raised sea level and loss of biodiversity) mean that sustainable and renewable alternatives are required. Promising alternatives that currently exist include biofuels, hydrogen, syn gas and natural gas. Although there are many advantages within each alternative, biofuels are particularly attractive due to the environmentally friendly benefits they offer, especially regarding renewability and biodegradability [18].

The term ‘biofuel’ relates to a range of fuels that can be derived from biomass including bioalcohols (such as ethanol, methanol and butanol), biodiesel (FAME: Fatty acid methyl esters, FAEE: Fatty acid ethyl esters), biogas (methane), Fischer-tropsch biodiesel and biohydrogen. The use of biofuels produced from renewable resources offers the potential to reduce the burning of fossil fuels and promote a more carbon neutral environment. Worldwide, the production of biofuels is set to rapidly

increase, with the IEA predicting that their usage will triple, from 1.3 million barrels of oil equivalent per day (mboe/d) in 2011 to 4.1 mboe/d in 2035 [1].

1.2.2 Classification of biofuels

Biofuels are commonly classified as either primary or secondary dependent on how they are used (primary: non-processed, secondary: processed). Additionally, liquid fuels have been further classified according to the type of feedstock used for production, namely 'first generation' or 'second generation' (second generation are often referred to as advanced biofuels). Research is also active around the production of 'third generation' biofuels. Figure 4 presents a summary of the typical feedstocks used within each biofuel classification. The advantages and disadvantages of each generation are presented in Table 1.

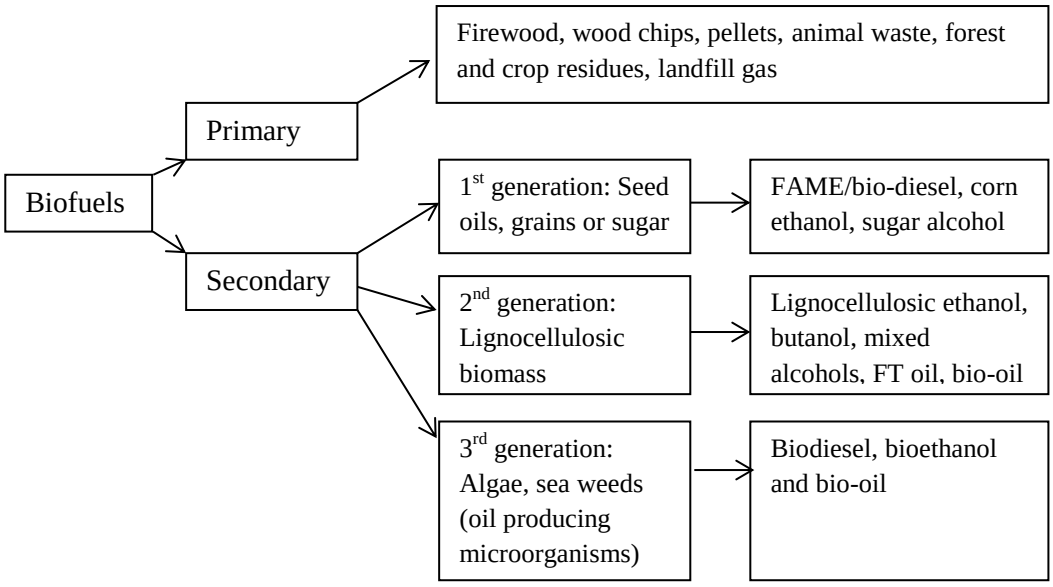


Figure 4: The classification of biofuels initially by processing (primary or secondary), and secondly by feedstock (1st, 2nd or 3rd generation).

Table 1: Advantages and disadvantages of 1st, 2nd and 3rd generation biofuels.

Classification	Advantages	Disadvantages
1st generation	+Offers net benefits to reducing GHG emissions and promoting a carbon neutral energy balance	-Contribute to higher food prices due to the decrease in land available for food production -Accelerate deforestation -Negatively affect biodiversity
2nd generation	+Avoids conflict with food production +Little land requirement as many biomass sources are from forestry wastes +Offers higher land use efficiency than 1st generation +Offers net benefits in reducing GHG emissions and promoting a carbon neutral energy balance	-Sophisticated technologies required in processing which can lead to the requirement of expensive equipment
3rd generation	+Small land area requirements +Algae will grow in salt and waste waters which are in abundance +Sequestration of CO_2 +Avoids conflict with food production +Algae can accumulate both lipids and carbohydrate for biodiesel and ethanol production respectively	-High energy input. Energy conversion is lower than for rape seed oil, palm & Jatropha -Lipid extraction is a high energy process -Technology still in infancy
References used: [18-21].		

1.3 Lignocellulosic biomass

Lignocellulose is by volume, the most significant biologically produced substance worldwide. Lignocellulosic raw materials include straw, reed, grass, wood and paper waste. Lignocellulosic materials are highly abundant and not used for the production of food; therefore they can be considered as waste products making them a highly attractive feedstock. The raw material costs are low, for example in 2006 in the U.S, the cost of corn stover (the leaves and stalks left after harvest) and straw was 30 US$/ton in comparison to the 110$/ton cost of corn [22]. Lignocellulose is an increasingly popular feedstock for use in biorefineries for a multitude of industries because a wide variety of platform chemicals and products can be produced as demonstrated in Figure 5.

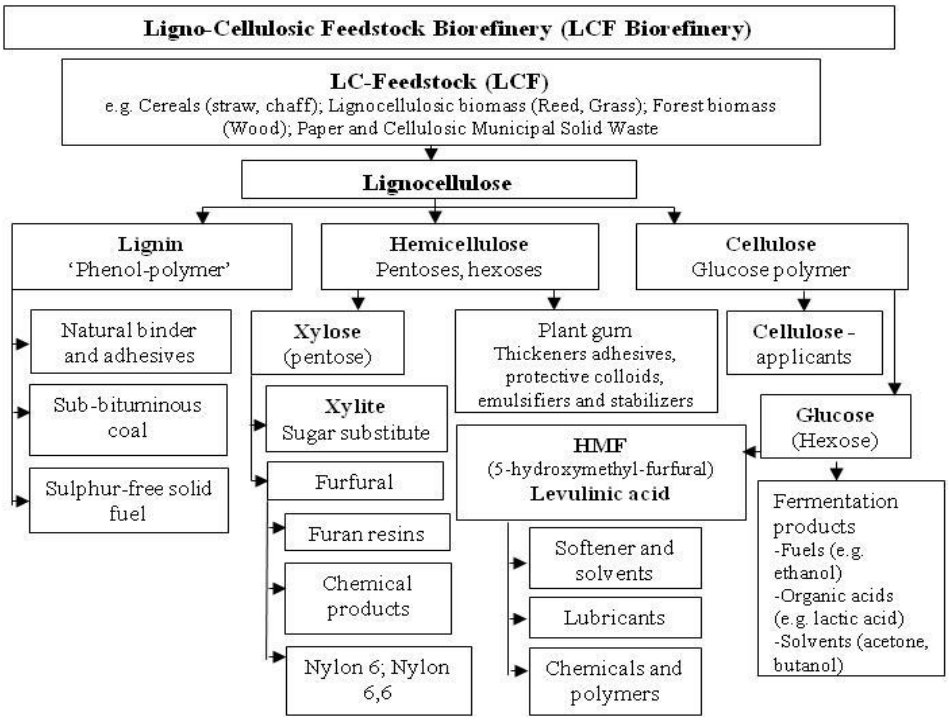


Figure 5: Potential products from a lignocellulosic feedstock biorefinery, taken from Kamm *et al.* [23].

Lignocellulose is made up of three major components, cellulose, hemicellulose and lignin. The ratio of each component varies between the different types of lignocellulosic substrate (hardwood, softwood, grasses or agricultural residues), but typical proportions are around 35-50% (% of the total dry mass) for cellulose, 35% for hemicellulose and 25-40% lignin [2,24]. Compounds such as pectin, protein, non-structural sugars, nitrogenous compounds and chlorophyll are also present in lignocellulosic biomass but in smaller quantities [25]. Each major component will be discussed in further detail.

1.3.1 Cellulose

Cellulose is found in the primary and secondary cell walls of plants. The function of cellulose is to give plant cells strength and rigidity and to prevent cells from over swelling. Cellulose is a linear homopolymer of anhydrous D-glucose monomers. The glucose monomers are linked by β-1,4 glycosidic bonds (Figure 6) [26] to form a glucan chain. Glucan chains align parallel and are held together by strong hydrogen bonding and Van der Waals forces to form crystalline microfibrils. Inter- and intra-molecular hydrogen bonds stabilise the microfibrils which explains why plant cells have high tensile strength and are resistant to cellulose depolymerisation [27-29]. Additionally, cellulose microfibrils play a pivotal role in the direction of elongation of

a growing cell as they are found located transversely to the elongation axis and thus control the shape of the plant cell [30].

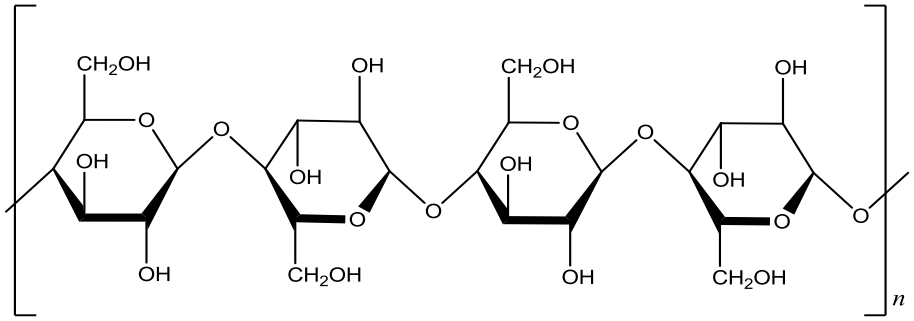


Figure 6: Structure of a β-1,4-linked cellulose polymer (*n*: number of repeating units).

Isolation of cellulose from lignocellulosic biomass is desirable as a wealth of products can be obtained. It is well established that cellulose hydrolysis produces glucose, the starting material for yeast fermentation into bioethanol, a current source of biofuel. Glucose is also a starting material for fermentations to yield high value products such as succinic acid, lactic acid, acetone and butanol [23]. Furthermore, glucose can undergo dehydration to produce 5-hydroxymethyl-furfural (HMF), a useful intermediate in the production of fuels and chemicals. Subsequent rehydration of HMF produces levulinic acid, another platform chemical with high potential [23,31,32].

1.3.2 Hemicellulose

Hemicellulose, unlike cellulose, is a heteropolymer. A hemicellulose polymer can be composed of pentose (C5) sugars such as xylose, galactose, mannose, arabinose and rhamnose and the hexose (C6) sugar glucose. The composition of the sugar units and the structure of the hemicellulose chains vary widely within plant species. The biological function of hemicellulose is in strengthening of the plant cell wall. This is achieved by the multiple hydrogen bonding between hemicellulose, cellulose microfibrils and other cell matrix polymers such as lignin [33]. Compared to cellulose, the polymer chains are much shorter, branched and easier to hydrolyse [34]. Hemicellulose is randomly acetylated, and different forms of hemicelluloses have different degrees of acetylation. The presence of the acetyl groups reduces the

degradation of hemicellulose by enzymatic attack due to steric hindrance. Glucuronoxylan (the polysaccharide of xylan backbone with glucoronic acid) is the predominant hemicellulose found in hardwood, whereas for softwood, this is glucomannan (glucose and mannose polysaccharide) [34]. The sugars derived from hemicellulose can also be fermented to produce ethanol, which adds to the efficiency and economic benefits of a lignocellulosic biorefinery by improving yields of bioethanol [35]. The xylose monomers from hemicellulose can undergo dehydration under high temperatures and acidic conditions to produce furfural, a precursor in the production of Nylon 6, Nylon 6,6 and other valuable chemical products [23,36].

1.3.3 Lignin

Lignin is the second most abundant polymer in nature (after cellulose). It is a complex and heterogeneous polymer, composed of aromatic hydroxyphenylpropanoid units. Once the hydroxyphenylpropanoids are deposited in the cell wall, the cell wall becomes rigid and impenetrable [37,38]. It is the presence of lignin in plant cell walls that confers recalcitrance and it is the recalcitrance of lignin that is a major limitation for the conversion of biomass into bioethanol. For this reason, research into the degradation or utilisation of lignin is a large and active area. Figure 7 shows the typical structure of lignin from softwood.

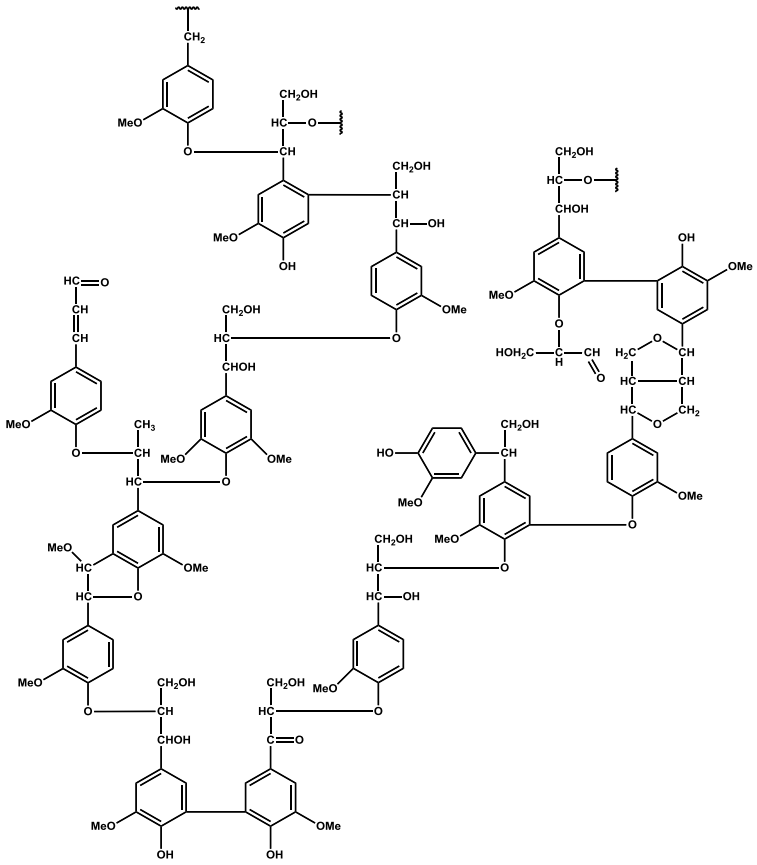


Figure 7: A proposed structure of softwood lignin adapted from Adler [39].

The main building blocks of lignin are the three hydroxycinnamyl alcohols, often referred to as monolignols, coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol (Figure 8). In the lignin structure, they are referred to as guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) units [37]. The frequency of each monolignol in lignin is highly dependent on the source of biomass. The biosynthesis of lignin is a result of the oxidative coupling reactions of the 4-hydroxyphenylpropanoids (monolignols) [38,40].

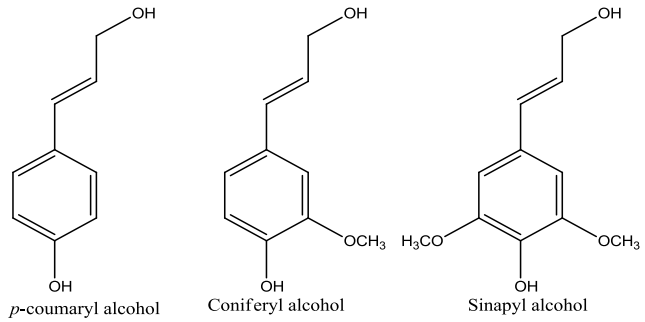


Figure 8: The three structural units (monolignols) within lignin, *p*-coumaryl alcohol (hydroxyphenyl, H), coniferyl alcohol (guaiacyl, G) and sinapyl alcohol (syringyl, S).

In the final stages of biosynthesis, the monolignols are oxidised by plant oxidative enzymes to form phenoxyl radicals. Coupling reactions between two radicals produce dilignols and further oxidation results in the formation of a polymer of the monolignol radical subunits. Due to the stability and high frequency of the coupled radicals in the polymer, ether linkages form between the phenolic carbons (C4) and the side chains or aromatic ring carbons from each monolignol precursor. This accounts for the high degree of non-phenolic linkages in a lignin polymer, and hence the resistance to degradation [37,41]. Due to this free radical polymerisation mechanism of synthesis, lignin polymers have a fairly diverse series of intermolecular linkages, and the linkages differ in proportion depending upon the origin of the lignin. Table 2 shows the composition of intermolecular linkages for softwood and hardwood and the structures of these linkages are presented in Figure 9. The β-O-4 is the predominant linkage accounting for ~50% total linkages. It is therefore the cleavage of this linkage that is important for the degradation of lignin [38,42-44].

Table 2: The composition of linkages within hardwood and softwood lignin by Adler [39].

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

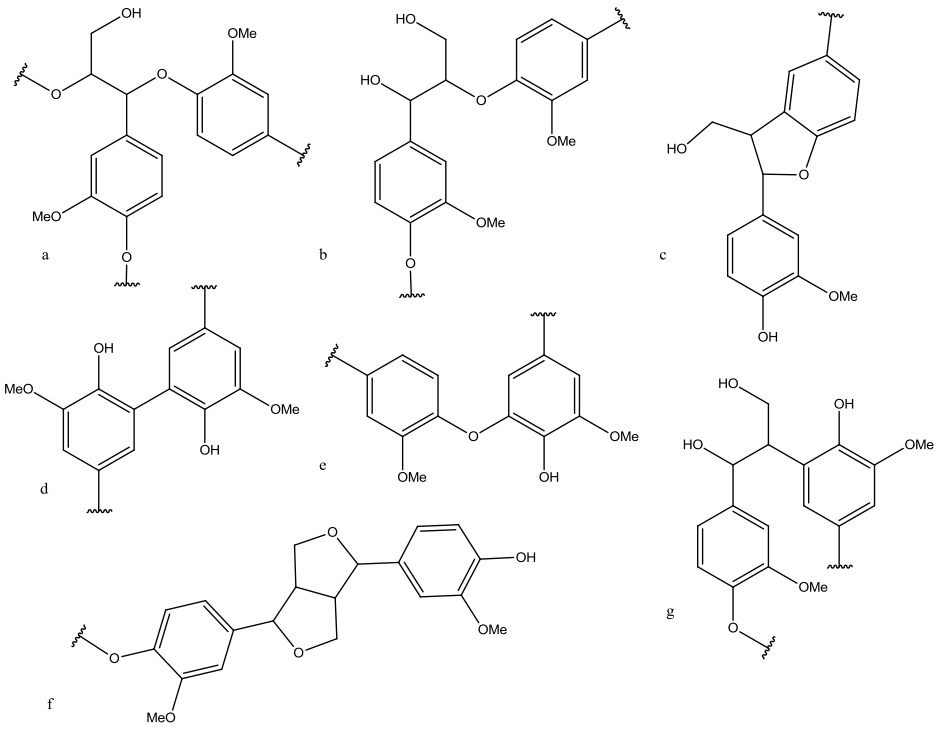


Figure 9: Common structural linkages within a lignin polymer; a) α-O-4'; b) β-O-4'; c) β-5'; d) 5-5'; e) 4-O-5'; f) β-β'; g) β-1'.

In wheat straw, lignin is cross-linked to the arabinose side chains of xylan in hemicellulose via ether bonds, mainly the α-benzyl ether linkage; however, a small percentage of wheat straw lignin links to the glucuronic acid residues or 4-O-methyl glucoronic acid of hemicellulose by ester linkages [45,46]. The bonding between lignin and hemicellulose produces a matrix in which the cellulose fibrils are embedded into, protecting the cellulose from degradation [47]. Removal of lignin is required in order to access the carbon from cellulose for the production of bioethanol and other industrially valuable products.

Lignin can be depolymerised in a controlled process to produce aromatic compounds [2] which are a good source of starting materials for many chemical processes. If lignin was isolated in an economically efficient way to yield its mono-aromatic hydrocarbons, it could be utilised as an adhesive or binder, for example, in particle woodboard and in phenol-formaldehyde resins thus adding significant market value to the processing of lignocellulose [23]. In biorefinery systems where the lignin recovered is not suitable for the production of high value chemicals, it is burnt to provide power to the biorefinery.

1.4 Bioethanol production from lignocellulosic biomass

The production of bioethanol offers a sustainable and attractive alternative energy source for our transportation sector. Its usage would contribute to lowering pollution levels and providing a cleaner environment. The use of highly abundant and lower costing starting materials such as lignocellulosic feedstocks for ethanol production would provide a more environmentally friendlier competitor to current non-renewable fuel sources [48].

Generally speaking, to obtain bioethanol from lignocellulosic feedstocks, the initial focus is to gain access to the glucose-rich cellulose portion by breaking apart the lignin-hemicellulose shield by the application of a pretreatment strategy. Once the cellulose is made more accessible, it is converted into monomeric glucose by hydrolysis. Solubilised hemicellulose (in addition to cellulose) can also be used as a sugar source for ethanol production. Microorganisms are then applied to utilise the sugars *via* fermentation to yield ethanol which is separated by distillation [25]. Figure 10 presents a generalised flow chart outlining the key steps involved in the conversion of lignocellulosic biomass to bioethanol. The hydrolysis and fermentation steps can successfully be performed simultaneously under optimised conditions.

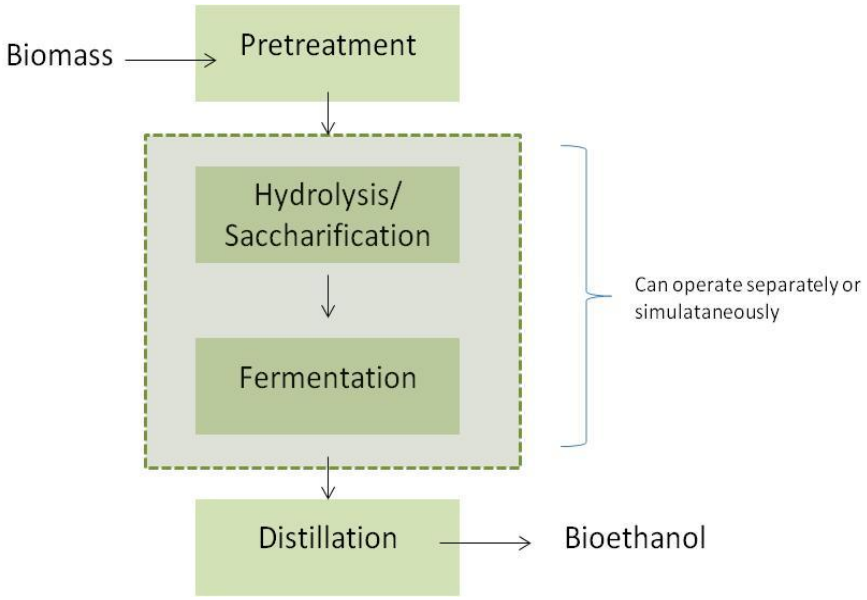


Figure 10: A flow chart to highlight the stages involved in the conversion of lignocellulosic biomass to bioethanol.

The process of converting cellulose into glucose, known as saccharification, is a critical step in bioethanol production. Saccharification can be achieved both chemically by the use of acids [49,50] and biologically using enzymes, although the use of enzymes are generally preferred due to their greater specificity [51]. Unfortunately saccharification is hindered by the naturally recalcitrant nature of lignocellulose to enzymatic hydrolysis unless a pretreatment strategy is applied prior to hydrolysis. When lignocellulosic biomass is left untreated, higher concentrations of hydrolytic enzymes are required to carry out saccharification [52]. Each stage involved in the conversion of lignocellulose to bioethanol will be discussed further.

1.4.1 Lignocellulosic pretreatment

A variety of structural and compositional factors contribute to the recalcitrant nature of lignocellulose to enzymatic hydrolysis. As mentioned previously, cellulose is a highly crystalline structure which consequently results in a low surface area, reducing the accessibility of hydrolytic enzymes (cellulases). Furthermore, cellulose access is hindered by the presence of hemicellulose and lignin. Pretreatment is required to remove hemicellulose and lignin, to reduce the crystallinity of cellulose and increase the porosity of the biomass making the hydrolysis process more efficient. Pretreatment is usually the most expensive step in the overall process of converting biomass into fermentable sugars [28,52].

A schematic representation adapted from Hsu *et al*. [53] has been widely used in the literature to describe the aim or outcome of pretreatment on the structure of lignocellulose [25,28,54] and is presented in Figure 11. The figure however, misrepresents the majority of pretreatment methods as it assumes that the application of a pretreatment method will successfully achieve separation of all 3 components within lignocellulose. Furthermore, it suggests that lignin is fragmented and that cellulose chains are disrupted. In reality most pretreatment methods successfully enable the separation of just one component, for example the isolation of lignin or solubilisation of hemicellulose. Lignin often still remains linked to carbohydrates after pretreatment, and cellulose chains can be left in-tact.

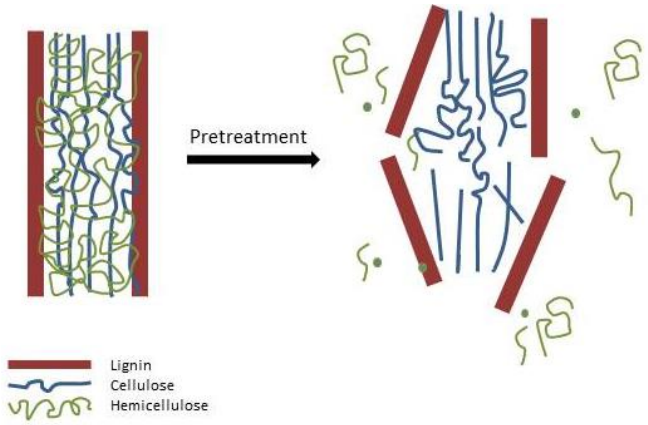


Figure 11: A common schematic representation outlining the effects of pretreatment on the structure of lignocellulose adapted from Hsu *et al.* [53].

1.4.1.1 Pretreatment methods

According to Sun & Cheng [55], for a pretreatment method to be viable it must meet the following requirements:

1) It should improve the formation of sugars from biomass, or improve the effectiveness of hydrolysis to form sugars.

2) It should not lead to the subsequent loss of carbohydrate or degradation.

3) It should avoid the formation of by-products that can inhibit downstream processes (e.g. hydrolysis and fermentation).

4) It should be cost effective.

There are many well developed pretreatment methods available, based on physical, physico-chemical, chemical and biological processes. Some of the most established methods are detailed in Table 3, however there is an extensive range of literature reviews describing the majority of all established methods in more detail [25,28,55,56]. Table 3 summarises some of the most commonly applied pretreatment technologies and highlights the components that are solubilised/removed, and some of the advantages and disadvantages of their use.

Table 3: Summary of the most commonly applied pretreatment technologies and their advantages and disadvantages

Category	Method	Action	Component(s) affected	Advantages	Disadvantages
Physical	Mechanical	Techniques employed include chipping, milling and grinding. Aim is to reduce the size of biomass, increase surface area and therefore improve the digestibility of hemicellulose and cellulose.	N/A	• Effective to increase surface area and improve digestibility	• High energy input
Thermal	Pyrolysis	Heating of biomass to high temperatures (400-650°C) in the absence of oxygen to enable thermal degradation of lignocellulose and the production of solid char and volatile gases. Quenching of gaseous vapours allows formation of pyrolysis/bio-oils.	N/A	• Process reduces oxygen content of biomass, providing higher energy density products • Low production costs	• Product steam often a complex mixture of products • Bio-oil cannot currently be used in existing fossil fuel infrastructure due to composition and is difficult to store, transport and upgrade
Physico-chemical	Steam explosion	Biomass is treated with high pressure steam (160-260^{o}C, 0.69-4.83 MPa) before a pressure reduction to atmospheric induces an explosive decompression of the biomass. Release of acetic and other organic acids from the biomass promote hemicellulose hydrolysis. Lignin transformation and redistribution occurs as an effect of the high temperatures.	Hemicellulose hydrolysis and lignin transformation /redistribution	• Lower energy requirement than mechanical methods • No recycling or environmental associated costs	• Less effective for softwoods than hardwoods • Generation of inhibitory compounds • Destructs a portion of the xylan fraction
Physico-chemical	Ammonia Fibre Expansion (AFEX)/ Ammonia Recycle Percolation (ARP)	AFEX: Biomass exposed to liquid ammonia (1-2kg/dw biomass) at high pressure, 30min, 90°C. Pressure is reduced. Hemicellulose is deacetylated and degraded to oligomeric sugars. ARP: Biomass exposed to 10-15% (weight) aqueous ammonia, 150-170°C, 14min. Leads to lignin depolymerisation and lignin-carbohydrate linkage cleavage.	Changes structure of hemicellulose Lignin and some hemicellulose removal	• Method does not lead to the production of inhibitory compounds • Lower temps usually required compared with steam explosion	• AFEX is not very effective towards substrates with higher lignin contents (~25% e.g. woods and nut shells)

Continued on next page.

Category	Method	Action	Components affected	Advantages	Disadvantages
Physico-chemical	CO_2	Use of supercritical CO_2 under pressure and at a temperature above its critical point (it is in a liquid state). Carbonic acid production from the dissolved CO_2 in water increases hydrolysis. The explosion of CO_2 due to pressure release disrupts the cellulose structure and increases surface area for hydrolysis.	Cellulose disruption	• Lower operating temperature which prevents decomposition of monosaccharides • Lower cost than steam explosion and potentially lower than AFEX	• Evidence of reduced saccharification yields compared to steam explosion and AFEX
Chemical	Ozonolysis	Treatment of biomass with ozone. Ozone is reactive towards highly electron rich compounds and those containing conjugated double bonds therefore lignin can be oxidised and solubilised.	Lignin (hemicellulose to lesser extent)	• Can operate at room temperature and ambient pressures • Ozone is easily decomposed so process can be designed to minimise environmental pollution	• Large amounts of ozone required • Can be costly due to amount of ozone
Chemical	Acid hydrolysis	Concentrated or dilute acid is reacted with biomass typically at a high temperature (up to 200°C). H_2SO_4 is the most commonly employed acid. The treatment increases substrate porosity as hemicellulose is solubilised. Xylose is predominantly recovered.	Hemicellulose	• Can achieve high reaction rates (good removal of hemicellulose) • Proven to improve cellulose hydrolysis	• Corrosive nature of acids leads to requirement for costly resistant reactors • Formation of inhibitory compounds • To make the process feasible, acid must be recovered • Neutralisation or water washing required as additional steps before hydrolysis
	Alkaline hydrolysis	Biomass is reacted with basic solutions (such as sodium, calcium, potassium and ammonium hydroxides) at temperatures typically less than 150°C. NaOH treatment has been demonstrated to induce biomass swelling, increasing surface area and a decreasing the degree of polymerisation and crystallinity.	Lignin removal or disruption Some hemicellulose removal	• Operates at lower temperatures than most pretreatments • Less degradation of sugar monomers than acidic treatments • Caustic salts recovered	• Usually requires longer pretreatment times (hours) compared to other methods which require minutes

Continued on next page

Category	Method	Action	Components affected	Advantages	Disadvantages
Chemical	Organosolv	Biomass is heated in an organic/aqueous organic solvent mixture. Common organic solvents include methanol, ethanol and acetone. Lignin can be precipitated from the organic phase whilst the addition of inorganic acid catalysts (HCl, H_2SO_4) allows for cleavage of lignin-hemicellulose linkages.	Lignin (extracted in organic phase) Hemicellulose (with acid catalyst)	• Can enable separation of all 3 components (insoluble cellulose. ethanol organosolv lignin and water soluble hemicellulose sugar)	• Formation of inhibitory compounds when acid is added • Recycling or removal of solvent required

References [25,28,55].

In addition to the more established and explored methods listed in Table 3, the employment of ionic liquids and biological agents as alternative pretreatment techniques are increasingly receiving attention due to their 'greener' properties. Ionic liquids have successfully been demonstrated to solubilise cellulose [57], hemicellulose [58] and lignin [59]. Cellulose can be decrystallised whilst lignin and hemicellulose are simultaneously removed. Ionic liquids offer a reduced toxicity compared to traditional solvents and can be used at lower pressures and temperatures due to the discovery of some ionic liquids at room temperature [54].

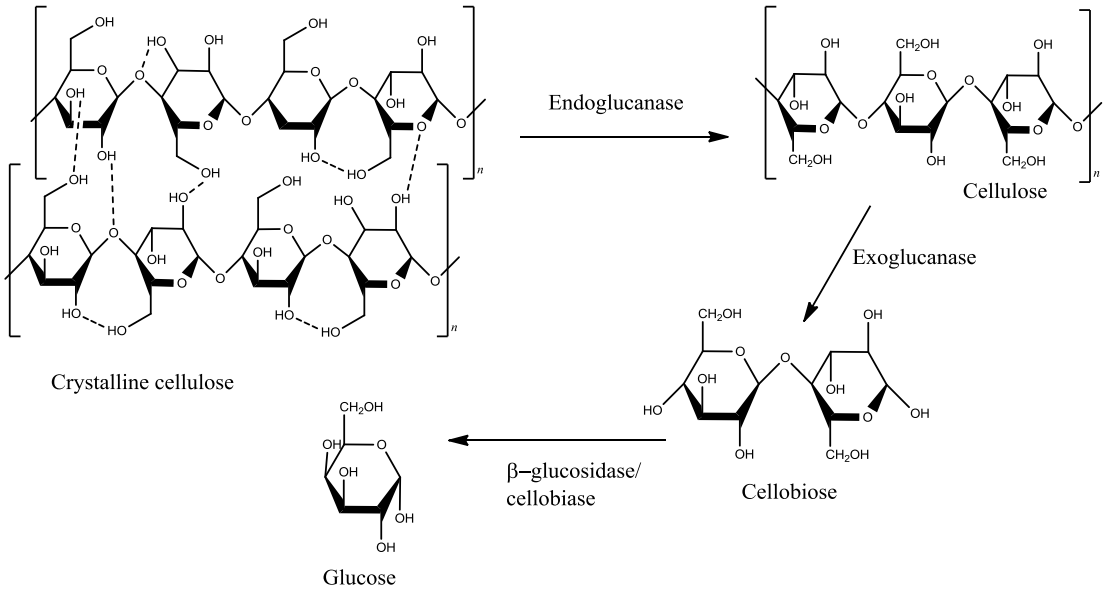
Biological pretreatment offers an attractive 'chemical-free' alternative to existing pretreatment methods due to the lower associated energy requirements and operating costs. This method will be discussed in greater detail in Section 1.5.2.

1.4.2 Cellulose hydrolysis

Following the pretreatment of lignocellulosic biomass, the cellulose (solid fraction) is subjected to enzymatic hydrolysis by cellulases.

Enzymatic hydrolysis of the solid fraction is performed using a cocktail of three cellulases: 1) Endo-β-1,4-glucanases (EG), 2) Exoglucanases/cellobiohydrolases (CBH) and 3) β-glucosidases/cellobiases, in order to produce monomeric glucose.

The role of EG is to hydrolyse the 1,4-glycosidyl linkages of the polysaccharide to produce reducing and non-reducing chain ends. CBH's hydrolyse the 1,4-glycosidyl linkages at either the reducing or non-reducing ends to form water soluble glucose dimers known as cellobiose. EG's and CBH's act synergistically to produce cellobiose (Figure 12) [60,61]. Finally, cellobiases act on the dimers to split the cellobiose into two glucose molecules. Without cellobiase, cellobiose dimers would accumulate and eventually inhibit the actions of EG and CBH [62]. A schematic representation of the hydrolysis of cellulase using these different enzymes is presented in Scheme 1.

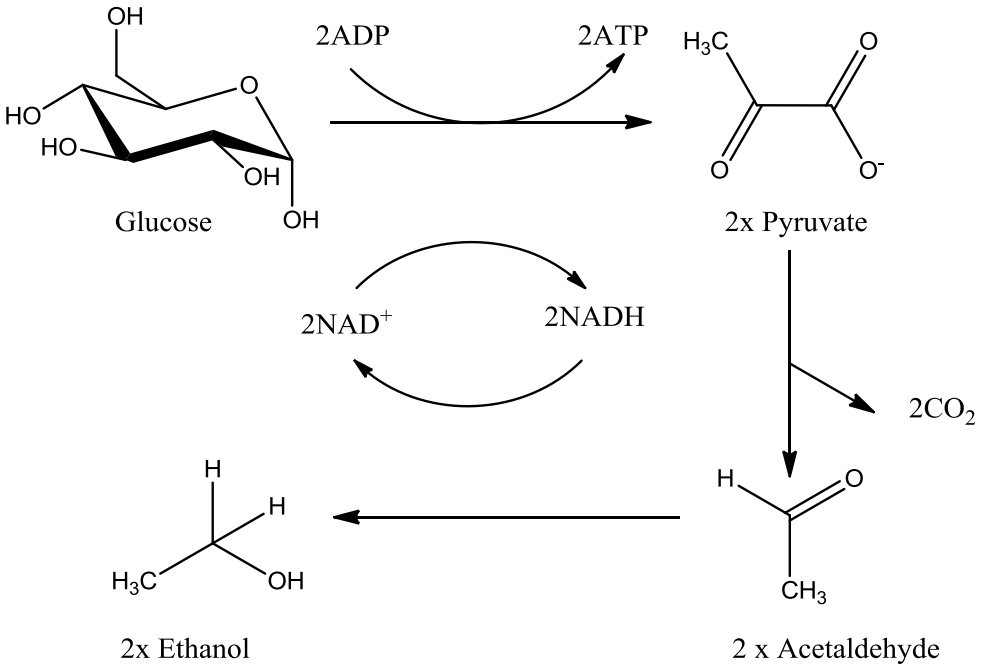


Scheme 1: The production of monomeric glucose from crystalline cellulose by a cocktail of cellulases. The internal bonds of crystalline cellulose are cleaved by endoglucanases (EG) to produce a cellulose chain that can be converted to cellobiose (2 x glucose monomers) by exoglucanase (CBH). β-glucosidases (also referred to as cellobiases) then cleave the glycosidic bond of cellobiose to produce single glucose monomers.

1.4.3 Fermentation of hexose sugar

Following the generation of glucose from cellulose, glucose can be converted to ethanol by microbial fermentation. Wild type *Sacchromyces cerevisiae* is the most commonly employed yeast to perform the anaerobic fermentation of glucose due to its high ethanol tolerance and ability to produce high ethanol yields [63]. The bacterium *Zymomonas mobilis* has also demonstrated suitability; however studies have only been performed on laboratory scale [64].

Within *S. cerevisiae* is a family of proteins known as the Hexose Transporter (HXT) family. It is this group of proteins that enable the fermentation to ethanol to take place [65]. Glucose is taken up by the cells by facilitated diffusion therefore all that is initially required is a glucose concentration gradient and a HXT carrier protein across the cell membrane. Glucose is metabolised in the cell following the Embden Meyerhof glycolysis pathway. In brief, glucose is phosphorylated into glucose-6-phosphate and then, following several metabolic enzymatic steps, is converted into pyruvate, ATP and NADH. Pyruvate is decarboxylated to produce acetaldehyde which is reduced to yield ethanol [66] (Scheme 2).



Scheme 2: A simplified scheme of the Embden-Meyerhoff pathway (glycolysis) employed by yeast such as *Sacchromyces cerevisiae* during alcohol fermentation.

A limitation of the use of wild-type *S. cerevisiae* for sugar fermentation is the inability to ferment pentose sugars such as hemicellulose-derived xylose. In an attempt to overcome this, recombinant strains of *S. cerevisiae* have been constructed to carry the genes required for xylose assimilation. These include the heterologous genes encoding xylose reductase (XR) and xylose dehydrogenase (XDH) from *Pichia stipitis*, and xylulose kinase (XK) from *Thermus thermophilus* that are absent in the wild type [67-69]. However despite this, reported ethanol yields produced by these engineered strains are lower than those by the wild type with glucose, and of xylose fermentation by *Pichia stipitis* [70,71].

In addition to pentose sugars, hydrolysates produced from the bioprocessing of biomass often reach the fermentation stage containing additional hexose sugars such as fructose and mannose. The metabolism of fructose by wild type *S. cerevisiae* employs different HXT proteins than those used for glucose transport. Mannose and glucose however, compete for the same HXT transporters so it is ultimately the concentration of each sugar within the hydrolysate that determines which substrate is utilized [65].

The hexose sugar galactose can also be fermented by *S. cerevisiae*, but growth is much lower when compared to growth on glucose. Furthermore, *S. cerevisiae* cannot

successfully co-ferment glucose and galactose due to repression of the GAL genes required for galactose metabolism (via the Leloir pathway) by glucose [72,73]. Research to promote the rate of galactose metabolism and achieve glucose and galactose metabolism in the same medium is relatively promising. Bro *et al*. [74] increased the consumption rate of galactose by 70% by overexpression of the *PGM*2 gene in *S. cerevisiae* whilst Ostergaard *et al*. [75] demonstrated that deletion of three *GAL* genes (*GAL*16, *GAL*80 and *MIG*1) improved the ability of the yeast to consume both glucose and galactose.

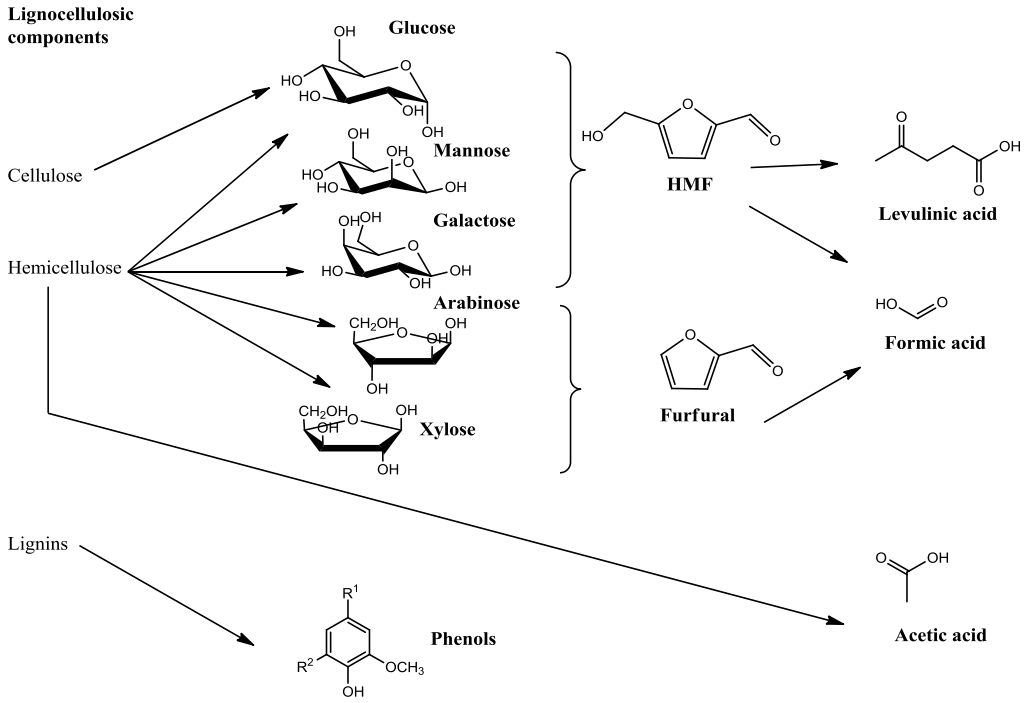
The fermentation process can be carried out either as a separate process to the hydrolysis (separate hydrolysis and fermentation, SHF) or as a simultaneous process alongside hydrolysis (simultaneous saccharification and fermentation, SSF). In SHF, both reactions are performed in separate vessels under their own optimal conditions. This strategy is unfortunately associated with drawbacks such as end product enzymatic inhibition and microbial contamination. In SSF, both processes are performed simultaneously within the same vessel, allowing the sugars produced from hydrolysis to be quickly consumed and converted into ethanol, avoiding sugar accumulation and product inhibition. SSF allows the overall processing of biomass to be carried out quicker (as both processes are carried out at the same time) and cheaper (through use of one reaction vessel), however the optimal operational conditions of both processes are not usually the same. Hydrolytic enzymes (e.g. cellulases) usually have optimal activities around 50°C whereby fermenting yeasts operate at their optimum around 30°C [63,76]. Higher product yields are usually obtained when SHF is carried out. In SSF, more than one microorganism is usually required and compromises in temperature and pH are often made to make the process viable [77].

As well as sugars, other products generated from commonly applied pretreatment methods can be carried over in the hydrolysate for fermentation. These can include weak acids, furan derivatives and phenolics and can exhibit inhibit detrimental effects towards fermentation.

1.4.4 Lignocellulose derived inhibitors

1.4.4.1 Inhibitors generated from pretreatment

As highlighted in Table 3, many pretreatment procedures successfully degrade or solubilise hemicellulose and lignin. A drawback to some of these methods include the generation of toxic compounds that can elicit inhibitory effects during the downstream processing of lignocellulosic biomass for bioethanol production. These inhibitory compounds have been found to contaminate both the liquid phase (referred to as the pre-hydrolysate) following pretreatment and the solid phase (by remaining within the cellulose rich biomass) [62,78,79]. The production of pretreatment derived inhibitory compounds has been extensively studied [80-84] and it has been reported that both the concentration and properties of the inhibitory products produced depend on the pretreatment method used (temperatures employed and length of treatment), the starting material (hardwood, softwood, plant) and whether an acidic catalyst is added [78]. Three groups of known inhibitors are produced during lignocellulosic pretreatment, their origins of generation and the effects they elicit during the later stages of bioprocessing are presented in Scheme 3 and described in Table 4.



Scheme 3: The routes of generation for known inhibitors produced during lignocellulosic pretreatment. Inhibitory furans (hydroxymethylfurfural (HMF) and furfural) and aliphatic acids (levulinic acid, formic acid and acetic acid) are produced *via* the degradation of carbohydrates. Inhibitory phenols are derived via the degradation of lignin (R^1: Aldehyde, ketone or carboxylic acid group, R^2: OCH_3).

Table 4: A summary of the groups of inhibitors, their generation and how they inhibit fermentation.

Group	Examples	Generation	Effect
Furans	Furfural 5-hydroxymethyl furfural (5-HMF)	Pentose dehydration (e.g. Xylose) at low pH. Hexose sugar dehydration (e.g. Glucose, fructose) [85].	Prolong the lag phase during the growth of fermentative yeasts and thus reduce volumetric ethanol production [86,87].
Weak acids	Acetic acid Levulinic acid, formic acid	Conversion of acetyl groups of hemicellulose during steam explosion pretreatment. Acid catalysed degradation of polysaccharides and from the degradation of 5HMF and furfural [85,88].	Acids decrease the cytosolic pH of the fermentative yeast cells. If acid concentrations are too high, the proton exporting activity of the ATPase membrane protein cannot maintain neutral intracellular pH, resulting in and cell death [89,90].
Phenolics	Phenolic aldehydes, ketones, alcohols and acids (e.g. Vanillin, 4-hydroxybenzaldehyde, *p*-coumaric acid)	Degradation of lignin during pretreatment.	Aromatic groups derived from lignin are toxic to yeast, preventing successful growth and sugar assimilation [88,90,91].

The order of relative toxicity for these inhibitors with respect to ethanol fermentation can be summarised as phenolics>furans>HMF>weak acids [92]. The degree of toxicity of the compounds is a direct result of their concentration, with inhibition greatest when all groups of inhibitors are present. Another factor that is important when determining the inhibitory nature of such compounds is the resistance of the fermenting microorganism to the inhibitors. Research is developing in the engineering of microorganisms to tolerate and resist levels of lignocellulose derived fermentation inhibitors [93-97].

The washing and filtration of biomass following pretreatment offers a solution for the removal of contaminating inhibitory compounds before hydrolysis and fermentation. Separation of the solid and liquid phase post-pretreatment can be performed, however these additional steps generally increase operational costs and the total running time of the overall process.

1.4.4.2. Lignin as an inhibitor

Many of the pretreatment methods discussed in Section 1.4.1 focus on the removal of lignin, however these methods tend to involve higher operational costs or longer treatment times. The most widely used pretreatment method is steam explosion [85,98], which does not successfully degrade/solubilise lignin.

When lignin is not effectively removed from lignocellulosic substrates prior to hydrolysis and fermentation, its presence can negatively affect both processes by the following actions:

Hydrolysis:

1. Lignin acts as a physical barrier to cellulose reducing the accessibility of the substrate towards cellulases during saccharification. If lignin remains bound to cellulose (and hemicellulose) after pretreatment, the reduction in surface area of the cellulose sites available for hydrolysis may cause lower yields of glucose. This reduction therefore leads to a requirement for higher cellulase loadings and increased hydrolysis running times [99].

2. Lignin (bound or unbound to cellulose) can non-specifically adhere to cellulases, thus decreasing their catalytic activity towards cellulose by potentially blocking active sites and preventing the binding of cellulases [100].

Fermentation:

1. Soluble aromatic phenols produced by the depolymerisation of lignin during pretreatment inhibit fermentation of sugars in the production of bioethanol. Small concentrations of these inhibitors have been found to destroy the integrity of the yeast membrane systems preventing growth and sugar assimilation [80,88,90,91].

As mentioned previously, the washing and filtration of biomass can successfully remove contaminating phenols prior to fermentation. However, cheaper and more environmentally friendly methods for removing and degrading native lignin within lignocellulose are required in order to improve the economics involved in the saccharification of biomass.

1.5 Lignin removal

As a component of lignocellulosic biomass, lignin is an important consideration for any lignocellulosic biorefinery. As the commercialisation of lignocellulose-to-bioethanol processing increases, the utilisation of lignin as a waste product will become a critical consideration for the overall sustainability of a biorefinery. Some industries make use of separated lignin waste for use as a low grade fuel source to power the biorefinery, however there are alternative potential uses for waste lignin. Lignin can be pyrolysed or gasified to produce char, pyrolysis oils and syngas (hydrogen and carbon monoxide). The

recombining of the gas from gasified lignin can further produce alcohols such as methanol [101]. Lignin is most likely to be the only renewable material for the production of monomeric aromatic compounds. Lignin pyrolysis can therefore generate value added compounds such as aromatic acids, aldehydes which can be even further processed to produce benzene, phenol and toluene [102]. Figure 12 summarises how different pyrolysis methods, as well as oxidation and combustion, can generate useful products from lignin.

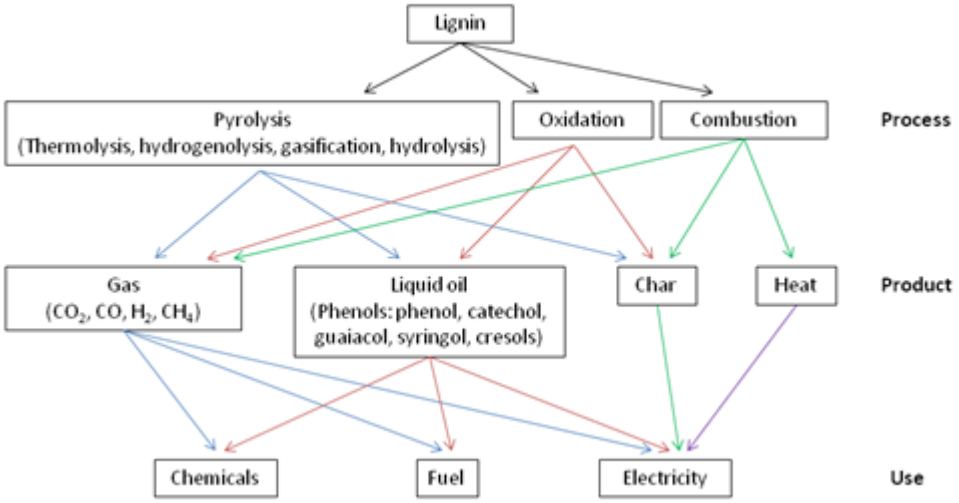


Figure 12: Thermochemical conversions of lignin and the associated products and usages, adapted from Pandey & Kim [102].

In the context of this work, the separation of lignin is a requirement for the improvement of the saccharification and fermentation processes in the production of bioethanol.

1.5.1 Existing lignin removal techniques

Historically lignin removal was developed as a technology for the production of cellulose enriched pulps during the manufacturing of paper and fibre board. Some of the leading and well established methods to remove lignin are summarised in Table 5:

Table 5: Common techniques for the isolation of lignin.

Process	Conditions	Action	Product
Kraft	Wood treated with high pH, NaOH and Na_2S, 150-180°C, ~2h [102].	Fragmentation of lignin via cleavage of ether linkages. Ionization of phenolic hydroxyl groups solubilises lignin [103].	Kraft lignin in black liquor (with hemicellulose). Lignin precipitated by reducing pH, followed by filtration and washing.
Sulphite	Wood treated with salts of sulphurous acid (counter ion can be Na^+, Ca^{2+}, K^+, Mg^{2+} or NH_4^+), 130-160°C [102].	Sulfonic acid groups attach to lignin making it water soluble [102].	Lignosulfonate (lignin is sulfonated).
Organosolv	Lignocellulose is heated in organic or aqueous organic solvent (+/- acid catalyst), 100-250°C [102].	Hydrolyses internal lignin bonds and the lignin-hemicellulose bonds (ethers and 4-O-methylglucuronic acid esters that link to Cα of lignin) [104].	Low molecular weight and high purity lignin [104].

Kraft and sulfite technologies are optimised processes for obtaining high cellulose yield. Other common methods include the Bjorkman process, in which milled wood lignin is purified from an aqueous dioxane extraction of finely milled wood; and the Klason process, whereby lignin is recovered as the insoluble condensed residue following polysaccharide hydrolysis with sulfuric acid [105].

The methods summarised above have advantages relating to their ability to isolate lignin for the production of power (kraft and sulphite) and for the production of high purity lignin (organosolv). However, all of these methods suffer from drawbacks including expensive running costs (organosolv), water and air pollution effects (kraft and sulphite) and the alteration of lignin to an extent whereby the 'isolated' lignin structure is poorly representative of native lignin (Klason) [105]. All of these methods make use of elevated temperatures and chemicals including flammable solvents, acids and bases. To increase the environmentally friendly and sustainable requirements of a biorefinery, (for bioethanol production in this case), there is a requirement for cleaner, and cheaper methods for the removal of lignin from lignocellulosic biomass.

1.5.2 Biological pretreatment

As outlined previously (Table 3) there are an abundance of established methods available that can improve the hydrolysis of biomass by the removal of one or several components of lignocellulose. However, common drawbacks of these methods include high operational costs as a result of high energy inputs and costly equipment, especially for

physical and physico-chemical technologies. As a move towards more safer and environmentally friendlier technologies for the processing of biomass, biological methods of pretreatment are being increasingly explored. Biological pretreatment strategies take advantage of microorganisms and their enzymatic productivities, in particular from white and brown rot fungi from the basidiomycete phyla. Enzymes are attractive for use in biotechnological applications due to the reduced or absent need for chemicals and the milder operational conditions (including temperature and lower energy inputs) that they generally require.

White rot fungi represent over 90% of all wood rotting basidiomycetes and can effectively decompose all 3 components of lignocellulose [47,106]. Brown rot fungi (which represent 7% of wood rotting basidiomycetes) attack the polysaccharide components of wood but degrade and modify lignin to a much lesser extent [106,107]. Soft rot fungi, (which include microorganisms from the ascomycete and deuteromycete phyla), also secrete polysaccharide degrading enzymes, however they do not degrade lignin as effectively as white rot basidiomycetes [107].

Many studies have demonstrated fungal mediated lignin degradation by employing solid state fermentations (SSF) [25,108-111]. In SSF, white rot fungi are grown directly on the solid lignocellulosic material. The aim of SSF is to utilise the secreted lignin and polysaccharide degrading enzymes to modify/degrade lignocellulose as outlined in Figure 13. Microorganisms can be selected based on their enzymatic profiles to target the required components of lignocellulose (e.g. lignin and hemicellulose removal, or predominantly cellulose solubilisation).

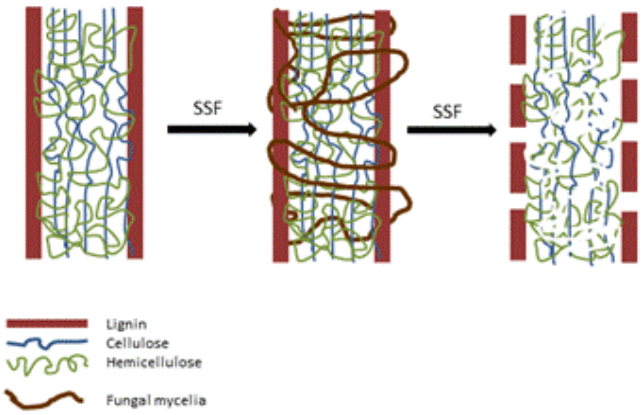


Figure 13: A schematic representation of the structural changes that may occur following biological pretreatment during SSF with white rot fungi. Adapted from Isori *et al*. [112].

Major drawbacks associated with biological pretreatment methods include lengthy processing times, increased space requirements to accommodate large scale microbial growth, and the continual monitoring of both cell growth and enzyme production [25]. With respect to lignin depolymerisation, enzymes are currently criticised for achieving low rates of hydrolysis, however research is currently active in the discovery of new and efficient ligninolytic microorganisms [113-115]. Advances in the discovery of lignin degrading species, and the potential for the ligninolytic enzymes to work in combination with other pretreatment methods, proposes a strong likelihood for the inclusion of enzymes in future developments of biotechnologies for biofuel production [55,85].

1.5.2.1 Enzymes involved in lignin degradation

Due to their ability to degrade cellulose, hemicellulose and lignin, white rot fungi are the most well studied microorganisms for the biological treatment of lignocellulose. In addition to the production of cellulases, xylanases and various hemicellulases, white rot fungi secrete a panel of extracellular oxidative ligninolytic enzymes that enable lignin degradation and modification.

Ligninolytic enzymes include the peroxidases lignin peroxidase (LiP, EC 1.11.1.14) and manganese peroxidase (MnP, EC 1.11.1.13) which were discovered in the mid 1980's in the extracellular medium of *Phanerochaete chrysosporium* [116]. More recently, a third ligninolytic peroxidase known as versatile peroxidase (VP EC 1.11.1.16) was been discovered in *Pleurotus eryngii* [117,118]. MnP is reportedly the most common ligninolytic enzyme produced by white rot fungi. Almost all fungi are found to secrete MnP whilst only some produce LiP [107]. The production and secretion of these enzymes is regulated by metabolic responses to carbon and nitrogen limitation [25,106,119]. Brief overviews of the catalytic properties of the peroxidases are presented in Table 6.

Table 6: A summary of the activities of the three peroxidases involved in the biodegradation of lignin.

Enzyme	Co-factor/substrate	Action
Lignin Peroxidase (LiP)	H_2O_2 Veratryl alcohol (proposed redox mediator)	Oxidation of co-substrate veratryl alcohol enables production of a diffusible oxidant. A mobile *trp*171 residue makes long range electron transfer possible. Oxidation of non-phenolic substrates by a one electron abstraction to produce aryl radical cations. Leads to Cα-Cβ cleavage and ring opening reactions.
Manganese Peroxidase (MnP)	H_2O_2 Mn Organic acid (chelator)	Oxidation of Mn^{2+} to Mn^{3+} by MnP and H_2O_2. Stabilised Mn^{3+} acts as a diffusible oxidant, allowing oxidation of phenolic structures to generate radical species. Results in Cα-Cβ and alkyl-phenyl cleavage reactions.
Versatile Peroxidase (VP)	H_2O_2 Mn	Combined activities of LiP and MnP. Oxidation of Mn^{2+} to Mn^{3+}. Has a tryptophan residue (*trp*164) analogous to *trp*171 found in LiPA isozyme. Enables oxidation of phenolic and non-phenolic substrates.
References: [25,106,107,112].		

Accessory enzymes have also been identified that are involved in the generation of the H_2O_2 required for the peroxidases. They include glyoxal oxidase (GLOX, EC 1.2.3.5) and aryl alcohol oxidase (AAO, EC 1.1.3.7) [107]. GLOX and AAO generate H_2O_2 by coupling the reduction of O_2 with the concomitant oxidation of a co-substrate [112]. The co-substrates are typically extracellular fungal metabolites including aldehydes (for GLOX) [120] and chlorinated anisyl alcohols (for AAO) [121].

In addition to fungi, bacteria are also capable of degrading lignin. The ligninolytic activities of bacterial enzymes are reported to be lower than those of white rot fungi, however recent studies have demonstrated a comparable level of lignin degradation in *Psuedomonas putida* and *Rhodococcus jostii* RHA1 with known lignin degrading fungi [122]. Studies into the enzymology of bacterial lignin degradation are not as advanced as fungal systems, however there is evidence to suggest that bacteria make use of extracellular peroxidases such as lignin peroxidase [123] and a dyp-type peroxidase [122].

Another group of enzymes suggested to play a role in lignin degradation/modification include the H_2O_2 independent enzymes laccases (benzenediol: oxygen oxidoreductase EC 1.10.3.2). Laccases have received recent attention as useful enzymes for industrial applications due to their catalytic requirement for molecular oxygen as opposed to H_2O_2. This property gives laccases a potential for increased stability over peroxidases which can suffer from H_2O_2 deactivation [124].

1.6. Laccases

1.6.1 General

Laccases are multi-copper containing enzymes that belong to the blue oxidase group. They catalyse the mono-electronic oxidation of a range of organic and inorganic substrates including mono-, di- and polyphenols, amino phenols, methoxyphenols aromatic amines, diamines, and ascorbate. Four molecules of substrate are oxidised (in a four electron transfer mechanism) at the expense of molecular dioxygen which is in turn reduced to produce two molecules of water [125-128].

Laccase was first described in 1883 by Yoshida who extracted the enzyme from the exudates of the Japanese lacquer tree *Rhus vernicifera*. Laccase was later discovered to exist in fungi by both Bertrand and Laborde [129,130], and since then has mostly been isolated from fungi although laccase is also produced by bacteria [131].

Laccases are typically present as homodimer proteins with molecular weights in the region of 60-100kDa. Approximately 10-45% of this weight is accounted for by glycosylation. Glycosylation is required for secretion of the protein as well as to promote thermal stability, copper retention, activity and protection from proteolytic degradation [129,132]. Most fungal laccases are extracellular and have their isoelectric points (pI) between 3-7 and pH optimums in the range of 3.6-5.2. These properties differ from the intracellular-produced plant laccases which operate at pIs up to 9 and have their pH optimum in the physiological range [129]. These differences are likely to reflect the different roles that the laccases perform. Plant laccases are primarily involved in the biosynthesis of lignin by catalysing the dimerisation of monolignols. In contrast, fungal laccases have a predominant role in the removal of toxic phenols and (more controversially), in the degradation of lignin.

Despite laccases exhibiting lower redox potentials than ligninolytic peroxidases (typically 450-800mV and >1V respectively), they are easily extractable enzymes that offer great versatility due to their capabilities to oxidise a large range of substrates. Examples of the application of laccases from different fungi in a variety of industrial processes are outlined in Table 7.

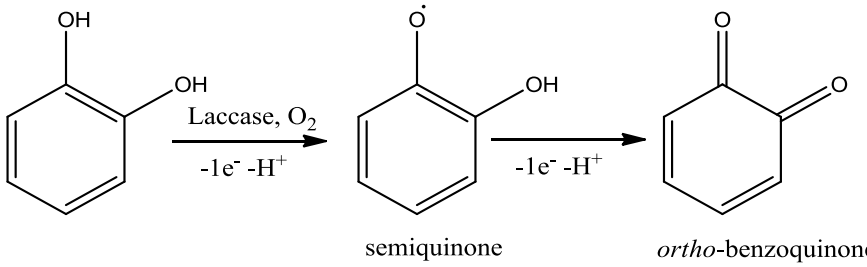
Table 7: Applications of laccases from different microorganisms.

Application of laccase	Organism (origin of laccase)
Dye decolourisation	*Trametes hirsuta, Trametes versicolor, Trametes villosa, Trametes trogii, Stereum ostrea, Laetiporous sulphurous*
Xenobiotic degradation	*Strophana rugosoannulata, Strophana coronilla, Coriolopsis polyzona, Rigidoporus lignosus*
Biodegradation/bioremediation	*Cerrena unicolor, Trametes versicolor, Streptomyces ipomoea, Ganoderma lucidum*
Effluent treatment	*Trametes versicolor, Trametes trogii, Botrytis cinerea, Lentinula edodes, Lentinus tigrinus*
Biosensors	*Cerrena unicolour, Trametes hirsuta, Trametes versicolor*
Biopulping	*Trametes versicolor, Trametes villosa, Lentinula edodes, Botrytis cinerea*
Examples taken from Desai & Nityanand [133].	

1.6.2 Laccase catalysed reactions

Laccase catalysed reactions have been divided into the following 3 categories as described by Polak and Jarosz-Wilkolazka [134]: 1) Direct oxidation of phenolics; 2) Coupling of radical species generated by laccase oxidation; and 3) Laccase-mediated reactions employing mediator compounds.

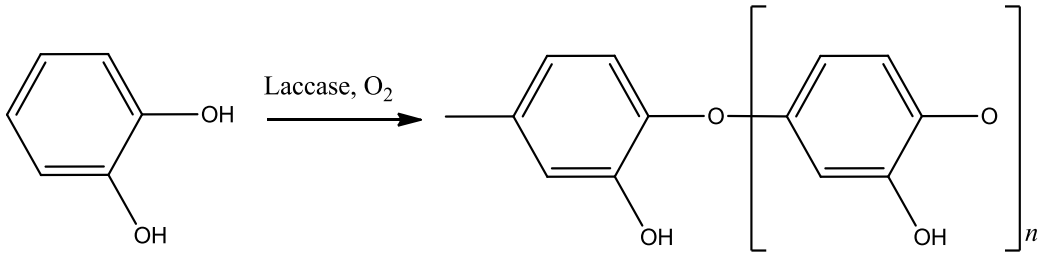
For the direct oxidation of phenolic substrates, laccases deprotonate the phenolic hydroxyl group to produce an unstable phenoxyl radical. Further enzymatic oxidation by laccase can produce quinoid derivatives as outlined in Scheme 4. Quinone formation has been reported to induce alkyl-aryl cleavage in lignin [129].



Scheme 4: The oxidation of catechol to *ortho*-benzoquinone via a semiquinone intermediate by laccase as an example of the direct oxidation of phenolic substrates.

Alternatively, an enzyme independent polymerisation reaction can take place, whereby the unstable phenoxyl radicals produced by direct oxidation dimerise with each other to eventually produce polymeric structures (Scheme 5) as occurs *in vivo* during the biosynthesis of lignin. Additionally, oxidative coupling can be applied *in vitro* between

phenolic monomers and polymer surfaces for polymer modification and to introduce new characteristics onto non-laccase substrates [135].



Scheme 5: The laccase catalysed polymerisation of catechol (*n*: number of repeating units)

Although the predominant route following the oxidation of phenolic substrates is polymerisation, phenoxyl radical formation has also been reported to result in Cα oxidation, Cα-Cβ cleavage, aryl-alkyl and aromatic ring cleavage [136,137].

In the third laccase catalysed reaction described by Polak and Jarosz-Wilkolazka [134], laccases oxidise low molecular weight compounds that are then able to behave as mediators for the transfer of electrons between laccases and alternative substrates. This mechanism allows laccases to indirectly oxidise substrates that would otherwise not be possible, due to reasons such as redox potential differences or steric hindrance. The concept of laccase-mediated oxidations will be discussed in greater detail in Section 1.6.4.

1.6.3 The laccase catalytic mechanism

Laccases, like other multi-copper oxidases (which include cerulosplasmin, ascrobate oxidase, cyctochrome *c* oxidase and nitrile reductase) are characterised by their copper containing active centres. A typical laccase active centre consists of four copper atoms which are classified in accordance with their spectroscopic features. Type 1 copper (T1 Cu) is oxidised in its resting state and is responsible for the blue-green colour of laccases at 610nm. The T1 Cu is also detectable by Electronic Paramagnetic Spectroscopy (EPR). Type 2 copper (T2 Cu) is not coloured but can be detected by EPR, and type 3 coppers (T3 Cu) exist as a pair of atoms that produce a weak absorbance in the near UV range but are not detected by EPR [133].

Close arrangement of the (1x) T2 Cu and the (2x) T3 Cu atoms within the laccase active site allow for the formation of a 'trinuclear cluster' (Figure 14). The trinuclear cluster provides the site for the reduction of O_2 and is therefore critical for the overall catalytic mechanism [125,128]. The T2 Cu atom coordinates with 2x histidine ligands and each T3 Cu atom coordinates 3x histidine ligands. The T1 Cu, which lies approximately 12-13Å away from the trinuclear cluster, is trigonal coordinated with 2x histidine ligands and 1x cysteine. In the bacterial laccase CotA from *Bacillus subtilis*, the axial ligand is a methionine. Structural studies have revealed that fungi lack this methionine, and the axial ligand is either a leucine or a phenylalanine and is unable to coordinate to copper [138,139].

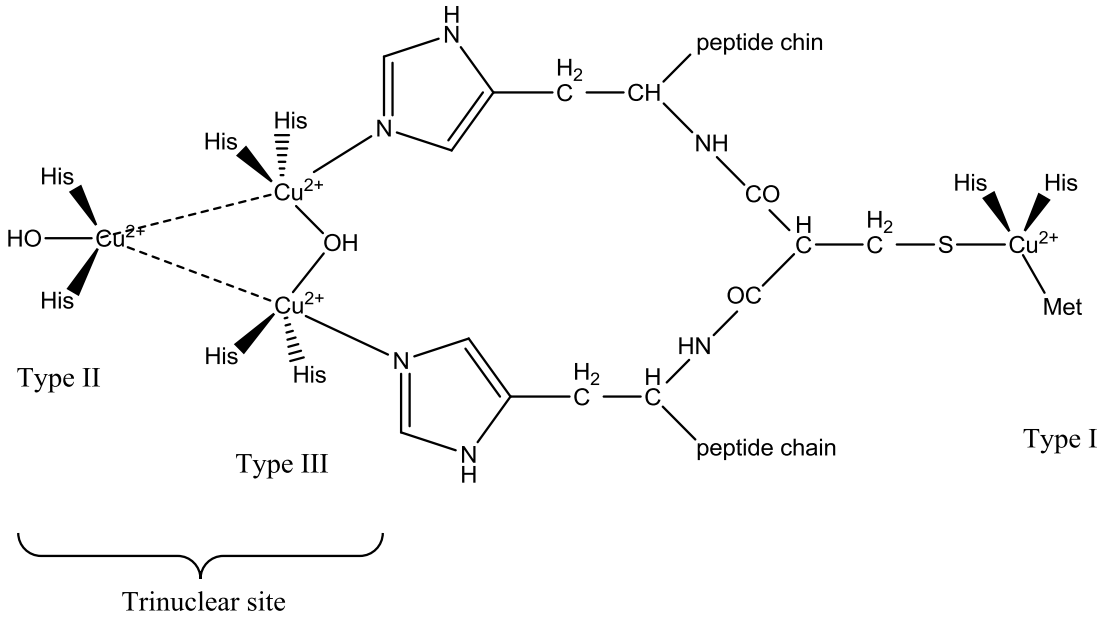


Figure 14: Structural representation of the active site of laccase, focusing on the connection between the T1, T2 and T3 coppers, adapted from Octavio *et al*. [128]. This structure represents the active centre of CotA laccase from *Bacillus substilis*, for fungal laccases the methionine coordinated to Type I Cu would be replaced with either an uncoordinated leucine or phenylalanine.

The catalytic mechanism of laccase is proposed to follow three important stages:

1) The binding and oxidation of the substrate to the T1 Cu site
2) The transfer of electrons from the T1 Cu to the T2/T3 trinuclear cluster
3) The activation of molecular oxygen and its reduction to water

Each event will be discussed in further detail.

1.6.3.1 Substrate oxidation

The T1 Cu is the site responsible for substrate oxidation. An electron is abstracted from the substrate to produce a free radical and a reduced T1 Cu site. The limiting step of the total reaction rate is the rate of electron removal, and this is determined by the redox potential of the substrate relative to the T1 Cu [128,140]. Since laccases perform a one electron oxidation but require four electrons to reduce O_2, laccases essentially store electrons from each reaction until four substrates have been oxidised and O_2 can be fully reduced [133].

1.6.3.2 Electron transfer from T1 to T2/T3 cluster

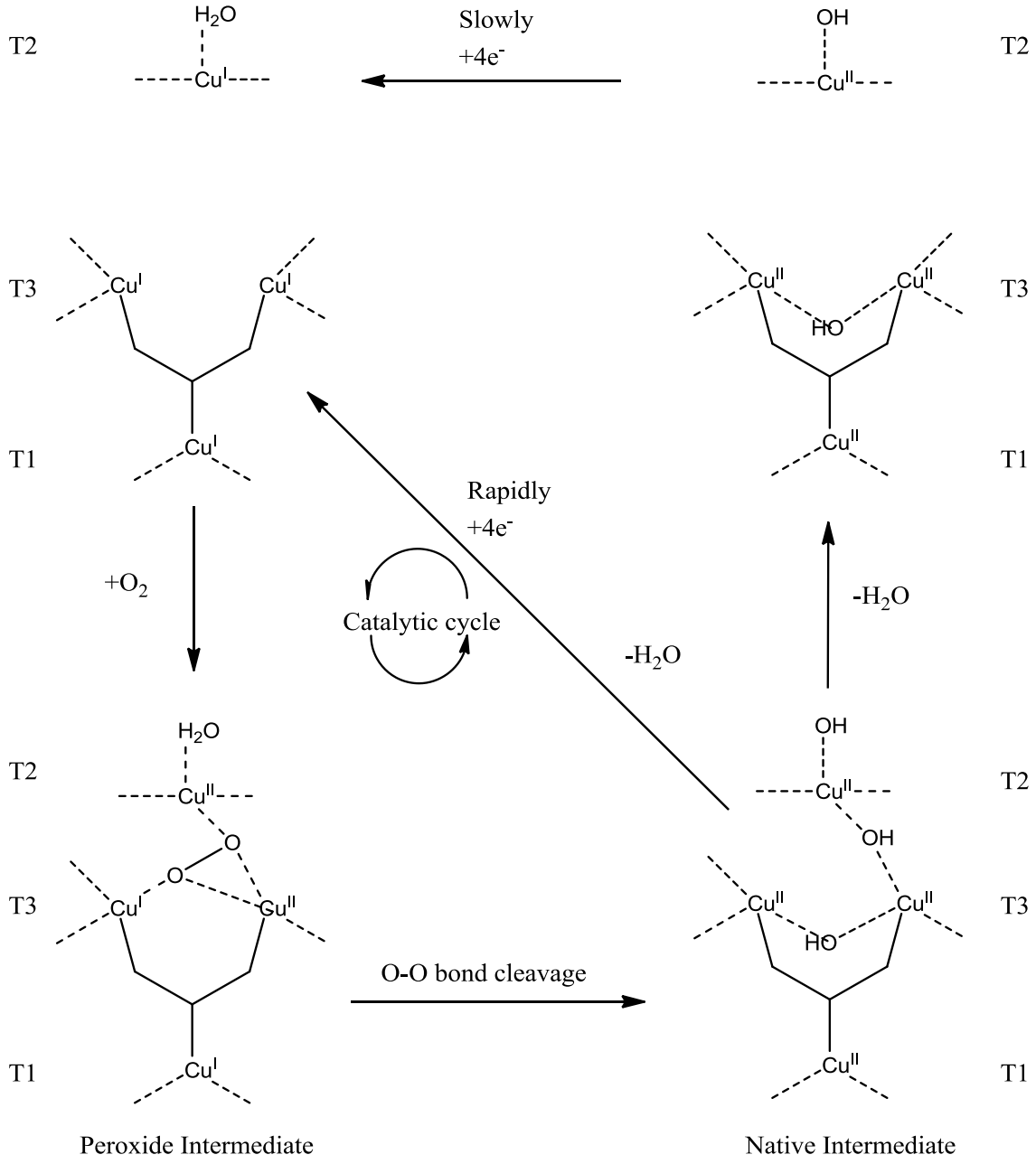
Solomon *et al*. [139] proposed two possible mechanisms for the electron transfer from the T1 Cu to the T2/T3 Cu cluster. In the first, the T1 Cu transfers its abstracted electron to the T2 and is re-reduced by abstracting another electron from the substrate. The T1 and T2 transfer an electron each to both T3 Cu and T1 is reduced by abstracting a 3rd electron from the substrate. The 3rd electron is transferred to re-reduce T2 before T1 is re-reduced and the enzyme is in a fully reduced form storing 4 electrons. This mechanism is supported by the observation that when the laccase is in its 'resting' oxidised form, the T3 site accepts two electrons only. In the second proposed mechanism, the sequential reduction of the trinuclear cluster by three one electron transfers from the T1 site would allow the T3 Cu to no longer act as two electron acceptors. Slow decay of a native intermediate would result in an oxidised resting centre. There is little known about the native intermediate and its reduction of the coppers therefore further research in this area is required.

It may appear that different electron transfer mechanisms exist between T1 Cu and the trinuclear cluster in laccases from different origins. Bento *et al*. [125] studied the dioxygen reduction in *Bacillus subtilis* laccase (CotA) and reported the transfer of electrons across a T1 Cu co-ordinating cysteine to two histidines that co-ordinate the T3 copper sites in the trinuclear cluster. Farver *et al*. [141] reported that for *Trametes hirsuta* laccase, only 3 electrons were required to fully reduce the T1 Cu and that the electron transfer was directly from the T1 Cu coordinating cysteine to the histidines coordinating the 2x T3 Cu. Farver further implied that the redox potential of the T2 Cu (~400mV) [142] was too low to drive electron transfer from T3 Cu to T2 Cu therefore the site did not occupy an electron. In the same study, it was established that 4 electrons were

required to fully reduce a laccase from *Rhus vernicifera* and that calculation of the redox potentials of all Cu sites enabled it plausible that the T2 Cu could occupy an electron.

1.6.3.3 The reduction of oxygen to water by the trinuclear cluster

To date, little is known about the mechanism of proton transfer during the reduction of oxygen to two molecules of water however it is being studied extensively. It is proposed that a dioxygen molecule interacts with the T2 and T3 coppers in their reduced state to produce a peroxide intermediate [143]. In a two electron process, one oxygen atom is bound to the T2 and T3 copper ions, whilst the other oxygen atom is bound within another T3 copper ion. A second two electron process takes place and the peroxide bond is split to produce a fully oxidised native intermediate [144]. In this state all three coppers are bridged by the H_2O products following O_2 reduction. Szilagyi and Solomon *et al*. [145] confirmed this intermediate of laccase by magnetic circular dichroism and x-ray spectroscopy. A schematic representation of the mechanism of O_2 reduction in the trinuclear cluster is presented in Scheme 6.



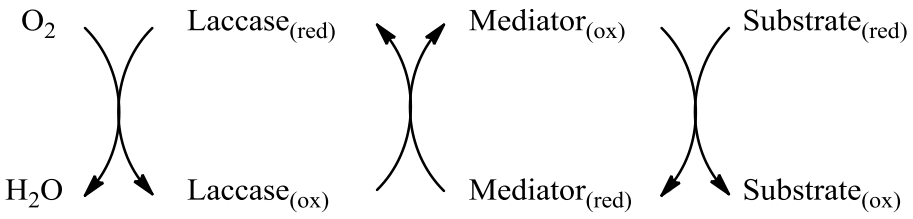
Scheme 6: The proposed mechanism for the four electron reduction of oxygen to water in the laccase trinuclear cluster during the catalytic cycle. Reaction adapted from Witayakran & Ragauskas [146].

Research in support of this mechanism includes the consistent findings of the role of T2 copper in the stabilisation of the intermediary in the reduction of oxygen to water [147]. Laccases show catalytic inhibition at increased pH levels and this is suggested to be due to formation of a T2-OH^- copper complex, therefore the T3 copper cannot be reduced until dissociation of the OH^- or its conversion to water has occurred. Winkler *et al*. [148] reported that peroxide only binds to one T3, and if T2 is not present, hydroperoxide is only weakly bound suggesting that T2 stabilises the T3-hydroperoxide complex prior to

reduction to water. What is still largely unknown is how the hydroxyl or water molecules migrate past the T2 copper before they leave the enzyme via the exit channel.

1.6.4 Mediated catalysis

A third type of reaction that laccases are capable of achieving (as mentioned in Section 1.6.2), and one that makes them interesting to explore, is known as mediated catalysis (or mediator-assisted oxidation). Redox mediators, or simply 'mediators' as they are commonly known, are low molecular weight compounds that act as electron shuttles between laccases and substrates that are not oxidised by laccase alone. A mediator, once oxidised by laccase and released from the active site, can perform oxidative reactions on substrates using mechanisms that are unachievable by laccases. Mediators can therefore, expand the substrate scope of laccases by promoting the oxidation of non-phenolic substrates [149]. The general concept of a laccase-mediator catalytic system is outlined in Scheme 7.



Scheme 7: The role of a mediator in the catalytic cycle of laccases. Oxidised laccase is reduced by the four electron oxidation of four mediator molecules. The oxidised mediators are capable of oxidising substrates that cannot be oxidised by laccase.

Mediator compounds have been identified of both natural and synthetic origin. Bourbonnais and Paice [150] first described the ability of a synthetic compound, 2,2'-azino-bis-3-ethylthiazoline-6-sulfonate (ABTS), to act as an effective mediator with laccase for the oxidation of non-phenolic lignin model compounds with high E°. Since then, other artificial mediators have been identified including 1-hydroxybenzotriazole (1-HBT), N-hydroxypthalimide (HPI), violuric acid (VA), 2,2,6,6-tetra methyl-1-piperidinyloxy free radical (TEMPO) and 1-nitroso-2-naphth-3,6-disulfonic acid (NNDS) [151]. ABTS and 1-HBT are used industrially in pulp delignification and for the decolourisation and detoxification of textile dyes [128]. Structures of these synthetic mediators are shown in Figure 15.

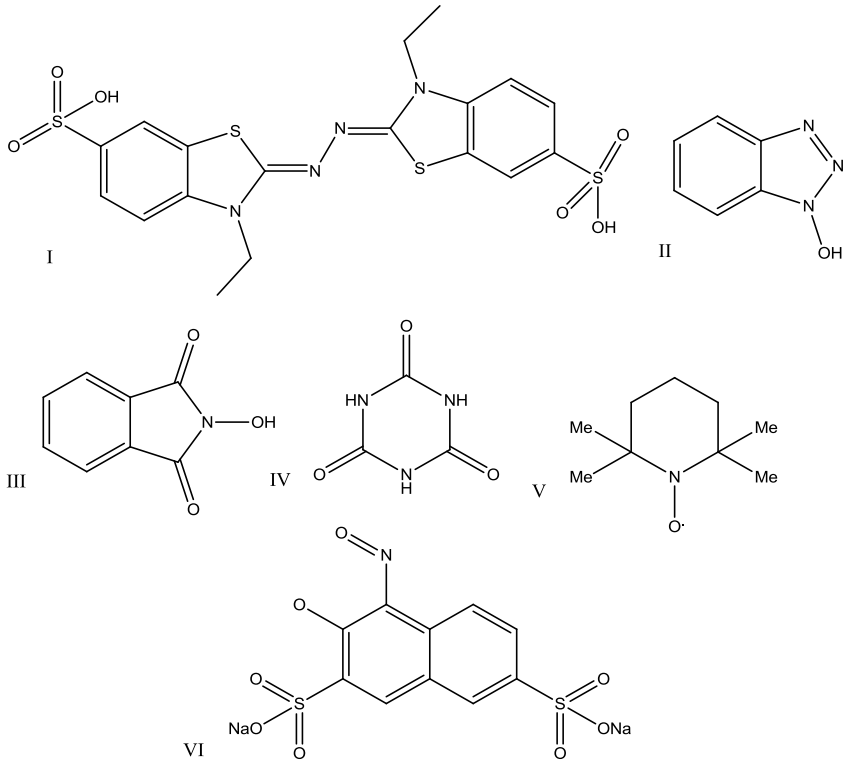


Figure 15: Structures of some established synthetic laccase redox mediators I: 2,2'-azino-bis-3-ethylthiazoline-6-sulfonate (ABTS); II: 1-Hydroxybenzotriazole (1-HBT); III: N-Hydroxyphthalimide (HPI); IV: Violuric acid (VA); V: 2,2,6,6-tetra methyl-1-piperidinyloxy free radical (TEMPO) and; VI: 1-Nitroso-2-naphth-3,6-disulfonic acid (NNDS).

The natural environment also produces a suite of compounds that are suggested to behave as redox mediators during natural processes such as the delignification of wood by white rot fungi. These 'natural mediators' are often referred to as 'enhancers' due to their poor stability as true mediators [152], and are, in most cases phenolic structures. Some of most commonly referenced structures are presented in Figure 16.

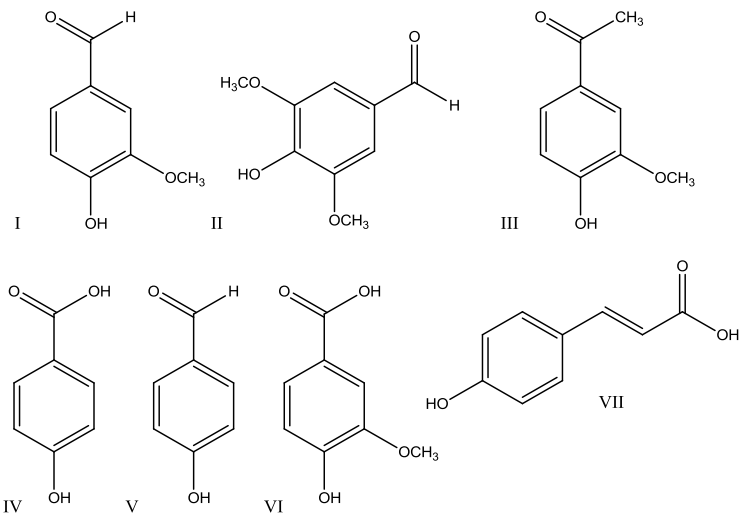
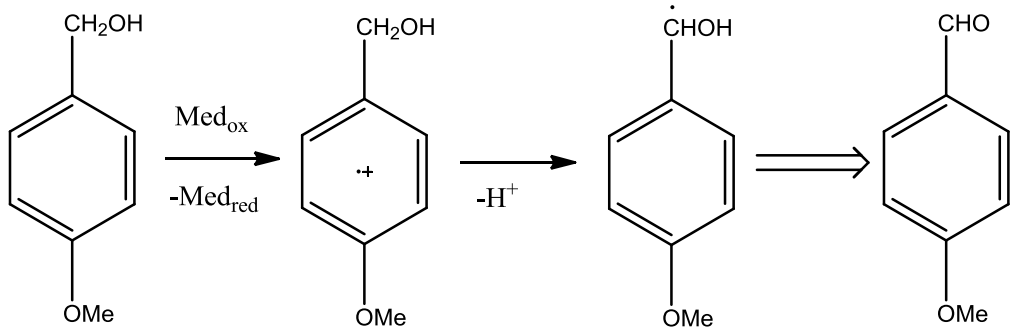


Figure 16: Structures of phenolic 'natural' laccase redox mediators I) Vanillin II) Syringaldehyde III) Acetovanillone IV) 4-Hydroxybenzoic acid V) 4-Hydroxybenzaldehyde IV) Vanillic acid VII) *p*-Coumaric acid.

The oxidation of substrates by oxidised laccase mediators predominantly take place *via* two mechanisms; an electron transfer (ET) route, as in the case of ABTS, or by a hydrogen atom transfer (HAT) route (also known as the radical route) as is demonstrated by 1-HBT. Both mechanisms will be discussed in further detail, as well as a third mechanism, the ionic route, which is unique to the laccase mediator TEMPO.

1.6.4.1 ABTS and the electron transport mechanism

As previously discussed, ABTS was the first synthetic mediator to demonstrate the oxidation of non-phenolic compounds when reacted with laccase [150]. ABTS is oxidised by laccase to produce the stable green coloured $ABTS^{\bullet+}$ cation which is then slowly oxidised to the dication, $ABTS^{2+}$. Oxidised ABTS follows an electron transfer (ET) mechanism to enable the oxidation of non-phenolic substrates. In this mechanism, an electron is abstracted from the substrate mono-electronically, which is followed by the loss of H^+ [153,154] (Scheme 8).



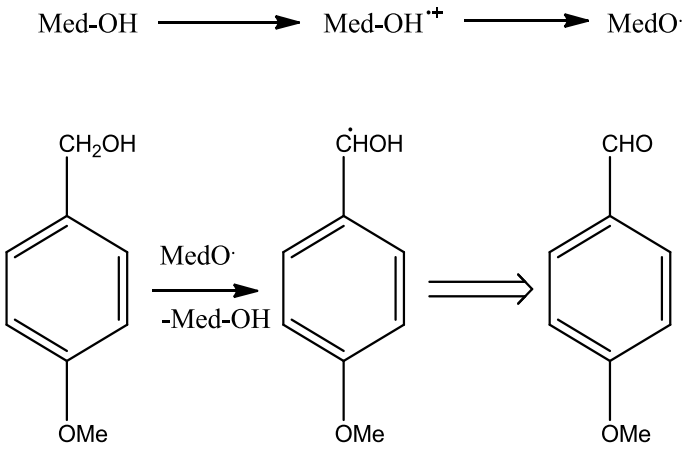
Scheme 8: The oxidation of 4-methoxybenzyl alcohol to 4-methoxybenzaldehyde *via* the laccase-ABTS electron transfer route [154].

The ABTS cation has previously been demonstrated to insufficiently react with non-phenolic substrates [150,155]. It is therefore widely believed that the dication is the responsible species for performing the electron transfer step required for oxidation. This is supported by the redox potentials of the cation and dication which have been established as 0.69V and 1.1V respectively [154,155].

The exact mechanism of how $ABTS^{2+}$ oxidises non-phenolic substrates is still unknown. Branchi *et al*. [155] suggest that by products from the degradation of $ABTS^{\bullet+}$ and $ABTS^{2+}$ are formed by hydrolysis during the reaction between laccase and ABTS and take part in the oxidation of non-phenolic structures. The mechanism of the laccase-ABTS mediator system towards non-phenolic substrates is discussed in further detail in Chapter 3 Section 3.2.2.1.

1.6.4.2 The radical hydrogen atom transfer mechanism

The most efficient laccase mediator system is the radical mechanism of oxidation. This applies to the generation of phenoxyl ($PhO^{\bullet}$) and nitroxyl (>N-$O^{\bullet}$) radicals. Nitroxyl radicals are generated from the oxidation by laccase of synthetic mediators such as 1-HBT, HPI, violuric acid (VLA) and *N*-hydroxyacetanilide (NHA). Phenoxyl radicals are generated from the laccase oxidation of phenolic compounds, for example 3-hydroxyanthranilic acid (3-HAA). Both radicals oxidise non-phenolic substrates by a hydrogen atom transfer mechanism (HAT). In this pathway, laccase oxidises the mediator to produce a radical species [156]. The radical species then abstracts a hydrogen atom from the substrate to produce a radical species substrate (in the case of benzylic substrates, a benzylic radical will be produced). The substrate reacts with oxygen to produce the final oxidation products (Scheme 9) [157,158].



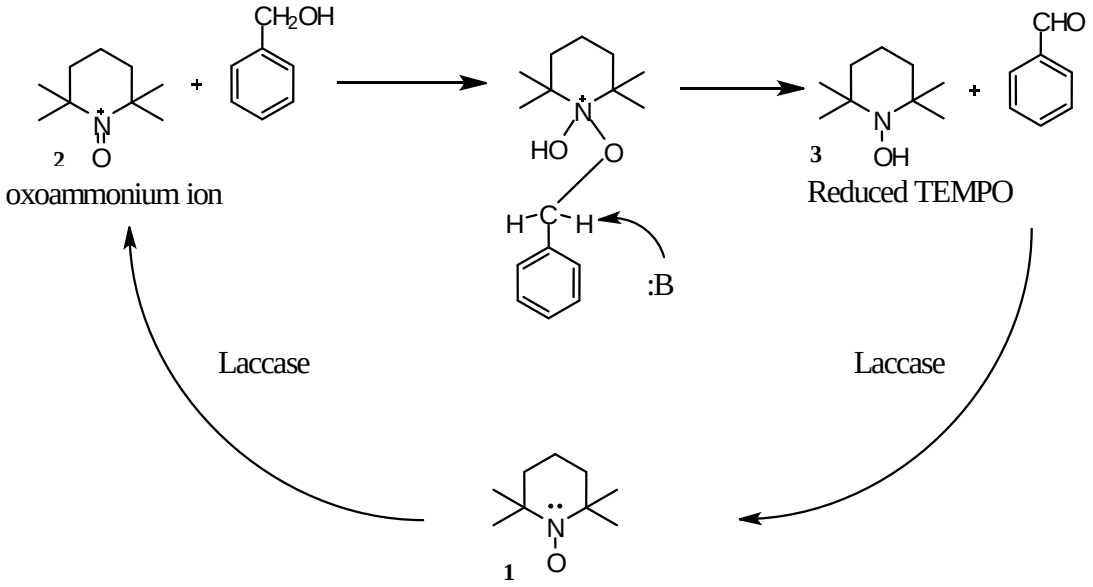
Scheme 9: The oxidation of 4-methoxybenzyl alcohol to 4-methoxybenzaldehyde *via* the laccase-1-HBT hydrogen atom transfer mechanism (the radical route) [154].

Despite their high efficiency, >N-O$^{\bullet}$ radicals are reported to inactivate laccase overtime due to their decay into inactive species. On the other hand PhO$^{\bullet}$ radicals can benefit biotechnological reactions by protecting the laccase enzyme from inactivity by harsh conditions. The reactive phenoxyl radicals can undergo oxidative coupling reactions to produce phenolic dimers, which can in turn behave as mediators or, in the case of the phenolic compounds such as 3-hydroxyanthranilic acid (3-HAA), deactivators. Cinnabarinic acid, a compound with no phenolic hydroxyl group, is produced via the dimerisation of oxidised 3-HAA. Loss of the phenolic hydroxyl functionality inhibits the dimerised mediators to perform oxidation [159]. The HAT mechanism, with specific focus on 1-HBT, will be discussed in more detail in Chapter 3 Section 3.2.2.2.

1.6.4.3 TEMPO and the ionic route

2,2’6,6’-tetramethylpiperidine-*N*-oxyl (TEMPO) is a stable aminoxyl radical which acts as a laccase mediator but follows a different mechanism to allow oxidation of non-phenolic substrates. An ionic oxidation route is followed and this reaction is employed for the oxidation of primary alcohols.

Laccase (and other chemical oxidants) oxidise TEMPO to a stable oxoammonium ion. The radical then attacks the substrate as a nucleophile and removes an α-proton. This produces the oxidised substrate and a reduced TEMPO (N-OH), which can be oxidised back to TEMPO (N-O$^{\bullet}$) and then follows a cycle to form the oxoammonium ion again (Scheme 10) [160].



Scheme 10: The oxidation of benzyl alcohol to benzaldehyde by the laccase-TEMPO ionic route of oxidation. The 'stable' *N*-oxyl radical of TEMPO (**1**) is oxidised by laccase to the oxoammonium ion (**2**) which is believed to be responsible for the oxidation of the alcohol to the aldehyde. TEMPO is then regenerated from its hydroxylamine form (**3**) by laccase, adapted by Fabbrini *et al*. [154] (B: Base form of buffer for deprotonation of the adduct at the α-C-H benzylic bond).

TEMPO follows a different mechanism to the radical HAT route followed by other >N-OH mediators due to thermochemical preferences. A radical route is not supported for TEMPO because the O-H bond that would be produced would be weak. The oxidation to the oxoammonium ion is enthalpically favourable and occurs readily. The O-H bond formed following HAT by other aminoxyl radicals is strong, and the oxidation of these mediators to the oxoammonium ion would require redox potentials >1.3V which is too great for laccase to achieve [161].

1.7 Natural mediator systems and lignin degradation

With respect to lignin degradation, laccases are capable of oxidising only the phenolic units of lignin due to their lower redox potentials (compared to other ligninolytic enzymes). A redox potential (E°) refers to the tendency of a chemical species to acquire electrons and subsequently be reduced. A higher redox potential increases the ability of a chemical species to gain electrons, whereas a lower redox potential results in the likely loss of electrons from a species [162]. Non-phenolic substrates (80-90% of a lignin polymer) have redox potentials >1.3V which explain why laccases cannot oxidise them directly. Since the phenolic composition of lignin is approximately 10-20%, the majority of the lignin polymer is theoretically unavailable for laccase oxidation. Another issue that hinders laccases from effectively oxidising lignin directly is steric hindrance. The

polymeric structure of lignin is too large to allow simple access to the active site of laccase [163]. Furthermore, laccases are too large to diffuse into the cell walls of plants to cause delignification.

It is widely believed that naturally-derived mediators are employed in nature to overcome these issues. Oxidised phenolic compounds usually have higher redox potentials than laccases and therefore have the potential to enable oxidation of non-phenolic components. In addition, the small molecular weight structures of naturally produced mediators can both fit into the active site of a laccase, and diffuse into the cell walls of plants to carry out further oxidations.

The natural mediator system concept is still unclear, however, fungi are believed to 'self-generate' their mediators for enzymatic oxidation and this is supported by their secretion of phenol, aniline, 3-hydroxyanthranilic acid (3-HAA), 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol [151,164]. In addition to this, lignin degradation itself produces compounds that have the potential to act as oxidative mediators, including acetosyringone, vanillin, syringaldehyde and ferulic acid [151,165]. The stable phenoxyl radicals produced by both natural processes are suggested to take part in further oxidative reactions towards the non-phenolic components within lignin as summarised below in Figure 17.

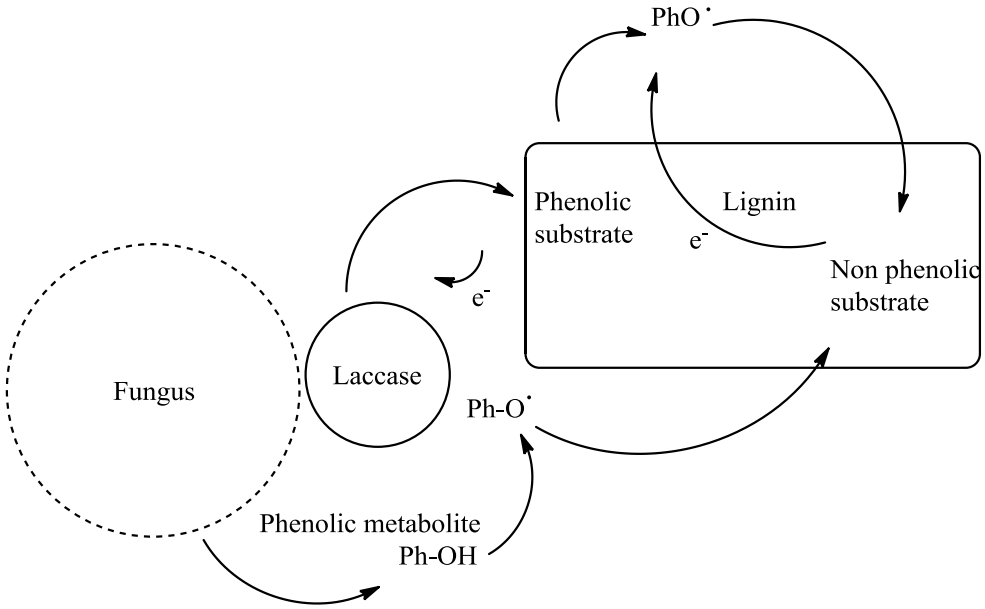


Figure 17: A representation of the laccase-mediated reactions that are suggested to take place in nature, whereby the oxidation of both lignin phenolic subunits and secreted fungal phenolic metabolites by laccase generates phenoxyl radicals that have the potential to attack the non-phenolic portion of lignin.

Mediators of natural origin are becoming increasingly popular options for industrial processes such as pulp bleaching and poly-aromatic hydrocarbon removal due to their high abundance and low cost, therefore offering to increase the economic benefits of a biotechnological process as well as making it more environmentally friendly [127]. On the other hand, synthetic mediators such as 1-HBT and ABTS, despite their effectiveness in oxidations, are limited by their high cost and related toxicity.

In this work the natural lignin degradation mechanism is considered for application in the production of bioethanol from lignocellulosic biomass. If the phenolic residues within the biomass can be oxidised by laccases to form the radicals capable of performing non-phenolic oxidation of the biomass, then the costly requirement for synthetic mediators would not be needed.

1.7.1 Laccase activity in delignification

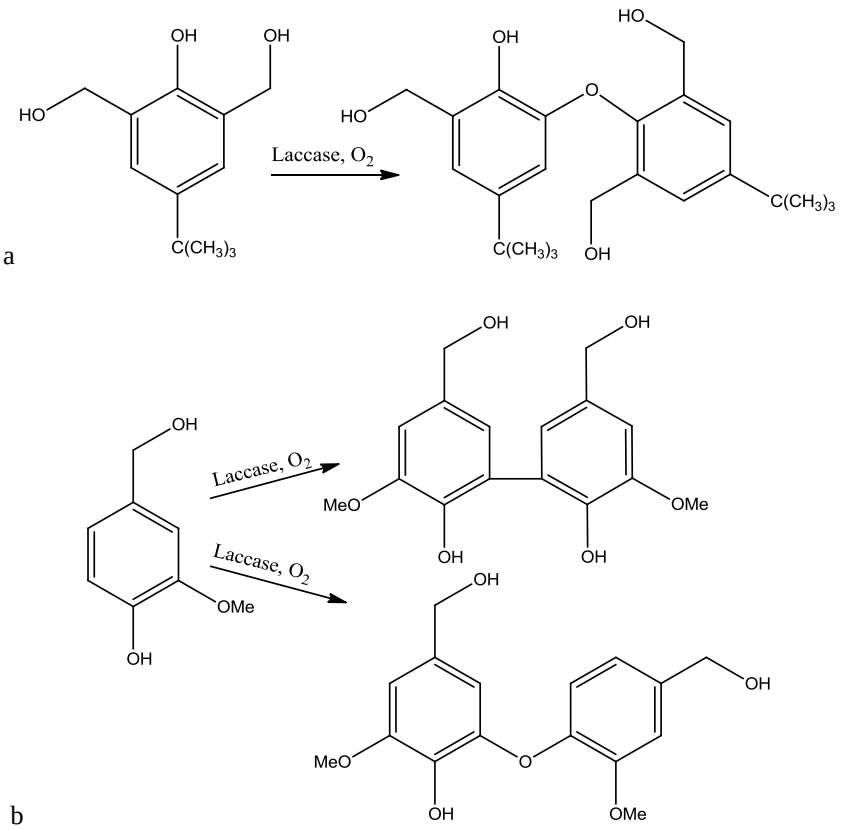
Despite an increase in the number of studies investigating the enzymatic activities of laccases, the role of laccases with respect to lignin degradation currently remains unclear. Due to the prominent role of laccases in the biosynthesis of lignin, laccases are often considered to not partake in lignin degradation. This belief is supported by studies with some strains of *Phanerochaete chrysosporium* which demonstrate lignin degradation in the absence of laccase [130,166]. On the contrary, laccase has been identified as the only ligninolytic enzyme produced by a lignin degrading strain of *Pyncoporous cinnabarinus* [164,167]. Further evidence towards laccase involvement in lignin degradation has been provided by Ander & Erikkson [168] and Srebotnik *et al*. [169] who demonstrated lignin degradation by a peroxidase deficient *Sporotrichum pulverulentum* mutant by the addition of laccase from *Trametes versicolor* [168] and by a LiP deficient strain of *Ceriporiopsis subvermispora* [169].

1.7.2 Application of laccase in biomass pretreatment

Laccases have recently attracted attention as candidate enzymes for the biological pretreatment of biomass. Due to their likely role as lignin degrading enzymes and their ability to function using molecular oxygen, they offer the potential for a highly specific and environmentally friendly method of removing lignin.

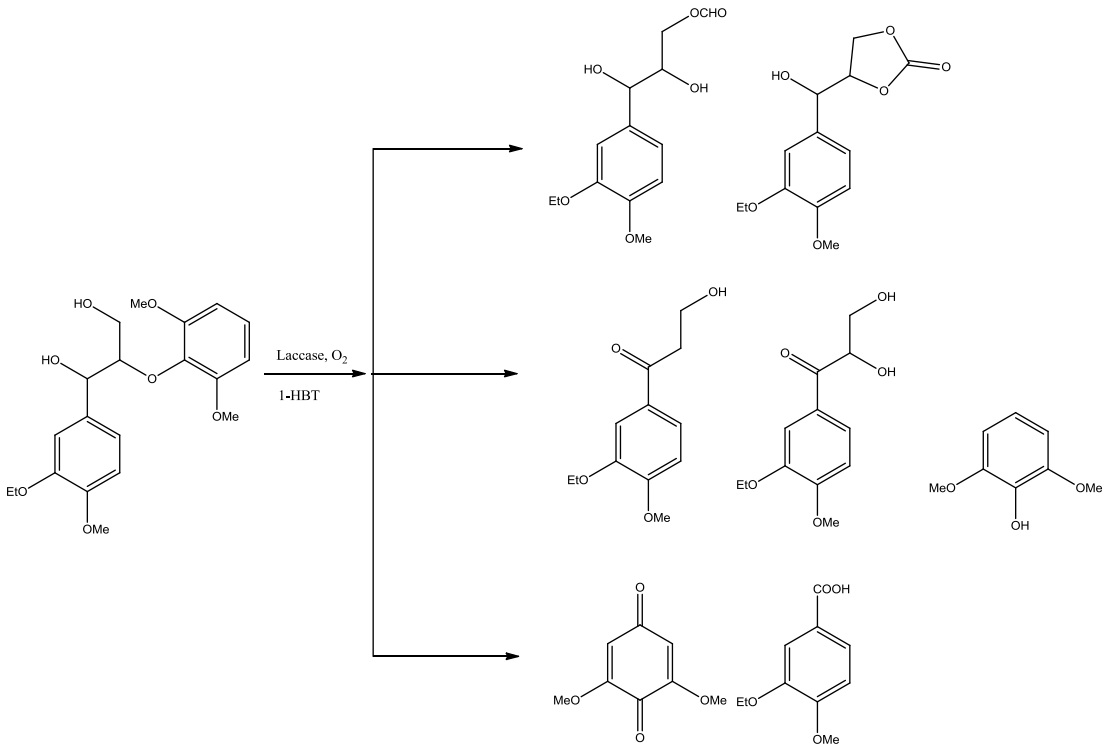
Earlier studies have investigated the reactivity of laccases by the use of lignin model compounds. These studies generally conclude that in the absence of mediators, laccases predominantly couple phenolic compounds by 5,5'coupling [170-172], although other

coupling routes have been reported [173-175] (Scheme 11), in addition to ring-opening reactions [136,174].



Scheme 11: Examples of phenolic coupling reactions by laccase with phenolic lignin model compounds; a) Coupling of a phenolic model substrate as demonstrated by d'Acunzo *et al*. [174]; b) Dimerisation of vanillyl alcohol by laccase *via* 5,5' (top) and 5-O-4' (bottom) coupling as reported by Areskogh *et al*. [175].

Laccases alone have thus demonstrated very poor or non-existent oxidations of non-phenolic compounds including monomeric benzyl alcohols and dimeric β1, β-O-4 compounds [150]. However following the inclusion of mediator compounds, laccases have demonstrated the ability to oxidise non-phenolic model compounds by Cα oxidation, Cα-Cβ cleavage and ring opening mechanisms [176] (Scheme 12).



Scheme 12: Product profile from the oxidation of a β-O-4 linked dimer by *T. versicolor* laccase and the mediator 1-HBT as reported by Kawai *et al*. [176]. Products are derived from different oxidation mechanisms including Cα oxidation, Cα-Cβ cleavage and ring cleavage.

In more recent years, especially with the increasing focus on sustainable biotechnology, studies have moved towards assessing the effect of laccases with lignocellulosic substrates. To date, studies with lignocellulosic biomass have demonstrated contradictory effects regarding the improvement of biomass saccharification following pretreatment with laccases. Some studies have reported an impairment of saccharification [79,99,177], whereby others have reported an improvement [99,178]. Palonen & Viikari [179], expanded the scope of these studies by investigating the effect of a synthetic mediator *N*-acetyl-*N*-phenylhydroxylamine (NHA) with laccase on the enzymatic hydrolysis of softwood, and reported enhancements in polysaccharide conversion of 21%. Similar improvements have also been reported with 1-HBT [180,181], and very recently, and for the first time, with the phenolic mediator methyl syringate [182]. The majority of these successful studies have combined the laccase/LMS treatments in combination with non-biological pretreatment methods (e.g. steam explosion) however these studies are still in their infancy.

Currently there is a lack of research that attempts to link laccase studies with model compounds to reactions towards natural lignin. It would be a mistake to make assumptions regarding the reactivity of laccases towards real lignin based on mediator studies alone. Furthermore, research is fairly limited in applying laccase mediator systems to biomass prior to saccharification, especially with regards to employing mediators other than 1-HBT. To date investigations with natural mediators in lignocellulosic saccharification reactions are very limited [182,183] and will be further explored in this work.

1.8 Project aims

Due to the current uncertainty of the role of laccase in the biodegradation of lignin by fungal systems, the preliminary aims of this project are focused on assessing the role of laccase activity with respect to lignin degradation. An investigation into the reactivity of laccase will be carried out both in the presence and absence of a mediator, and using both lignin model compounds and lignin within lignocellulosic biomass. Laccase-mediator systems will be investigated of both synthetic and natural origin, with the hope to find a successful and low cost naturally derived mediator for lignin studies.

The main focus of this work is to assess whether laccases (both with and without a successful mediator system) can be applied to biomass as an enzymatic pretreatment method to improve saccharification. This will be investigated using the lignocellulosic substrates wheat straw (*Triticum aestivum*), and the stover of corn (*Zea mays*) and sorghum (*Sorghum bicolor*) and methods to determine the effects towards saccharification will be explored.

Furthermore, this study aims to establish the mechanisms by which laccase and/or the laccase-mediator system degrade or modify the lignin structure to affect saccharification to contribute towards existing knowledge in this area.

1.9 Objectives

1. To successfully express a high redox potential laccase in a suitable host for high volumetric laccase productivity for use for all laccase-related studies.

2. To explore a wide range of redox mediators for use with *T. versicolor* laccase

- Investigate the oxidative ability of existing identified synthetic laccase mediators.
- Examine a panel of naturally derived phenolic mediators to identify compounds that can be used with laccase for the oxidation of non-phenolic substrates and lignin.

3. To examine the effect of the best laccase mediator systems on the saccharification of biomass

- Establish methods to quantify glucose release from cellulose and lignocellulose.
- Assess effect of laccase treatment alone, or with a mediator on the saccharification of pure cellulose substrate.
- Determine whether the effects are reproducible in additional lignocellulosic substrates.

4. To establish whether the reaction of laccase towards lignin model compounds is representative to that of natural lignocellulose.

5. To explore a variety of analytical techniques to investigate any structural changes by laccase and/or a laccase mediator system towards lignin.

Chapter two

2.0 Expression of *Trametes versicolor* laccases in *Pichia pastoris* and the development of laccase activity assays.

2.1 Introduction

From a biotechnological viewpoint, the most useful laccases are of fungal origin. The high redox potentials of fungal laccases, in comparison to laccases of bacterial origin, make them especially attractive [124]. Large quantities of enzyme are usually required for a successful bioprocess, however due to the typically low yields of laccase production when wild-type organisms are used, homologous expression systems are considered economically undesirable. Heterologous expression systems offer a platform to express different laccases in one host. The successful expression of active fungal laccase in a range of filamentous fungi and yeasts has been reported, for example in *Aspergillus oryzae* [184], *Aspergillus niger* [185,186], *Sacchromyces cerevisiae* [187,188], *Pichia pastoris* [186,189-191] and *Pichia methanolica* [192]. As glycosylation is required for laccase function (for secretion, activity, thermal stability and copper retention), expression by prokaryotic systems such as *Escherichia coli* is not favoured. In addition, differences in the use of chaperones and codons in *E. coli* further limit this expression system for laccase production [124,193].

Generally speaking, protein expression in yeast is favoured over filamentous fungi due to the lower yields of protease that are generally secreted. Furthermore, fungal systems often require more time consuming genetic manipulation procedures [194]. The methylotrophic yeast *Pichia pastoris* is an attractive candidate host for the expression of foreign proteins such as laccases due to the potentially high expression levels that are achievable and the ability to perform post-translational modifications such as glycosylation [192,195]. *P. pastoris* can grow to high cell densities in defined minimal medium and offers efficient secretion systems that allow for rapid protein isolation from the cells, avoiding the requirement for time consuming cell lysis procedures [195]. *P. pastoris* has the advantage over *S. cerevisiae* in that it can use the strongly controlled and highly inducible alcohol oxidase (AOX1) promoter to control protein expression [196]. Induction by the AOX1 promoter is driven by the addition of methanol as a sole carbon source, which allows for a simple, cost effective and scalable expression procedure. However, yeast cell death can occur as a direct result of methanol accumulation in the medium, therefore methanol addition is usually a carefully controlled process to avoid high concentrations of methanol [195,196].

Trametes versicolor is a well-studied white rot basidiomycete [196] due to its production of laccases with high redox potentials ($E°$) (T1 Cu $E°$ ~800V) . In 2002 Bertrand *et al*. [197] published the crystal structure of a laccase isozyme from *T. versicolor* which provided the first crystal structure of a laccase in its active form (Figure 18).

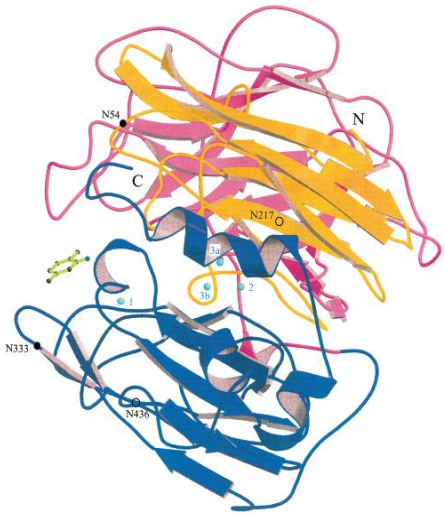


Figure 18: A ribbon representation of the structural folding of a *T. versicolor* laccase determined by Bertrand *et al*. [197]. Each domain is represented by a different colour (domain 1: orange, domain 2: magenta and domain 3: blue) and the copper ions of the active site are represented by blue spheres (and labelled 1 for T1 Cu, 2 for T2 Cu and 3a and 3b for 2x T3 Cu). The substrate 2,5-xylidine can be observed within the active site.

Multiple isozymes of *T. versicolor* laccases have been identified and enzyme assignments have been based on chromatographic elution profiles such as isoelectric point (IP). The secreted isozymes have been assessed for their efficiency in the degradation of polyaromatic hydrocarbons (PAHS) [198,199], biobleaching [200,201] and for the dechlorination of chlorophenols [202]. However inconsistencies within the literature regarding the nomenclature of the different laccase isozymes has caused confusion, with a range of nomenclature being used for each laccase, such as LacI, LccI, Lcc2, LaccaseIII, LacIIIb and LccIV [187,191,197,203,204] (Table 8). Necochea *et al*. [204] attempted to clarify this confusion by establishing a common nomenclature based on the phylogenetic analysis of ten reported laccase sequences The analysis, which was consistent with southern genomic experimental analysis by Mikuni *et al*. [205] allowed assignation of four distinct isozyme groups as α, β, δ and γ which share sequence identities of ~97% within group and ~70% between groups. (Table 8, Figure 19).

Table 8: Phylogenetic distribution of *T. versicolor* laccase isozymes and their characteristic properties, adapted from Necochea *et al.* [204].

Nomenclature by Necochea *et al*. [204]	Accession number	Previous given name	Amino acid length	Isoelectric point	Reference
α	23200086	TvL	499	5.9	[206]
	1172163	Lcc1	519	6.5	[207]
	15617226	Lac1	519	6.7	[191]
β	19848919	LaccaseIII	520	4.6	Unpublished
	4218523	Lcc2	520	4.7	[187]
	15778441	LacIIIb	520	4.7	[197]
	2388516	CVL3	520	4.5	[205]
δ	886718	Lcc1	520	4.2	[203]
γ	1172164	LccIV	527	4.2	[207]
	2598856	CVLG1	526	4.1	[205]

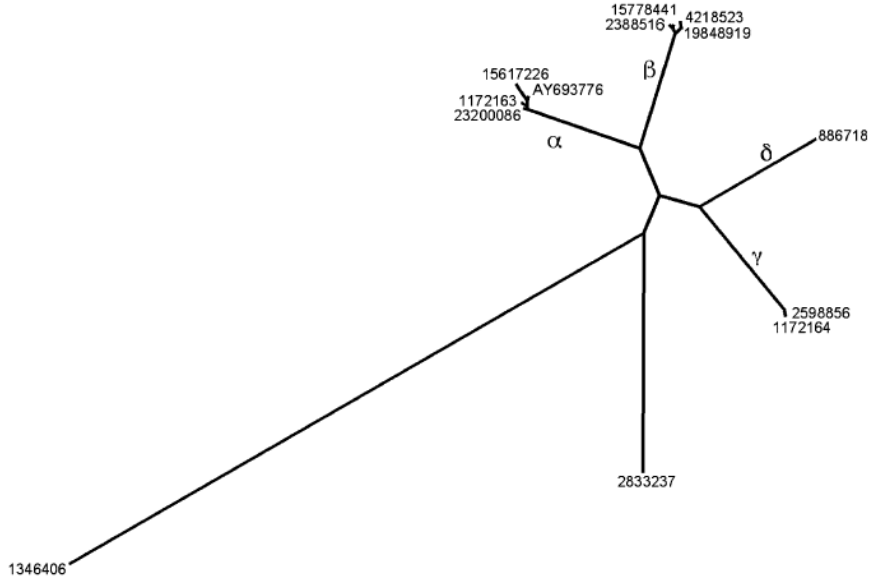


Figure 19: The phylogenetic classification of *T. versicolor* laccase sequences into four groups (α, β, δ and γ). Accession number AY693776 represents a *T. versicolor* laccase sequence explored by Necochea *et al*. [204], 1346406 represents a *Neurospora crassa* laccase and 2833237 represents a laccase from *Pleurotus ostreatus.* The remaining accession numbers relate to the *T. versicolor* isozymes presented in Table 8. Taken from Necochea *et al.* [204].

The aim of this study was to produce an active laccase from *T. versicolor* using *P. pastoris* with the view to use the enzyme preparation for all further studies towards investigating the reactivity of laccase towards lignin. In order to monitor and optimise successful protein expression, methods are required for the detection and quantification of laccase activity during expression.

2.2 Results & discussion

2.2.1 Expression of pPICZA-Lccβ and pPICZαA-Lccδ by *P. pastoris*

Four plasmids (pPICZαA-Lccα, pPICZA-Lccβ, pPICZA-γ and pPICZαA-Lccδ) were received from the research group of Prof. V. Urlacher (University of Dusseldorf, Germany) which each carried a laccase gene from each of the four isozyme groups described by Necochea *et al*. [204] (α, β, δ and γ) [198]. The two plasmids pPICZA-Lccβ and pPICZαA-Lccδ (Appendix Figures 75-76) were selected for expression studies due to reported differences between the activities of the expressed laccases. Koschorreck *et al.* [198] reported that of the four laccases expressed in *P. pastoris*, Lccβ was found to display the highest oxidative activity towards PAHs. Lccδ was reported to have a stronger function towards the phenolic C-C coupling reactions of sinapic acid. These functional differences between the proteins made them of interest to study.

The expression of each protein was previously optimised by Koschorreck *et al.* [198] using both the native laccase signalling peptides and the α-mating factor signal peptide from *S. cerevisiae*. Greatest expression levels of Lccβ were reported with the native signal peptide, whilst for Lccδ, the α-mating factor signal peptide produced greater expression levels. Transformation of each plasmid into *E. coli* DH5α allowed for purification of the DNA required for sequencing and transformation into *P. pastoris.*

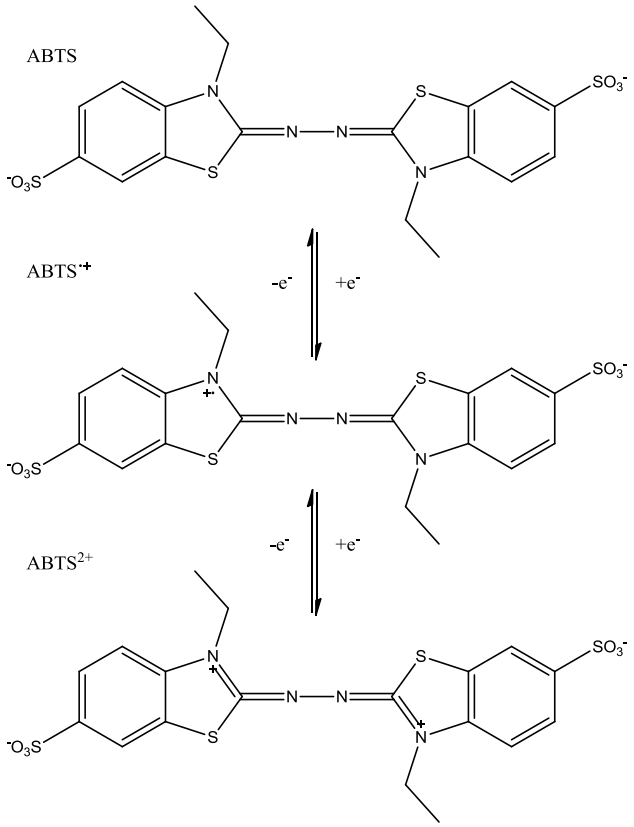
Primers were designed as previously described by the original authors (Chapter 7 Section 7.2 for primer sequences). Primers were designed based on the published genomic sequences of each laccase as found in GenBank, NCBI (GenBank Accession Numbers Y18012 for Lccβ, previously named as Lcc2 [187], and X84683 for Lccδ, previously named as Lcc1 [204]). Use of NCBI BLAST and Gentle software for the alignment of nucleotide and amino acid sequencing data obtained for Lccβ revealed a total of eleven point mutations when compared to the published GenBank sequence Y18012, nine of which were silent. Regarding the two mutations that resulted in an amino acid change, contact with the original authors revealed that one of the mutations (a C-terminal arginine to leucine) was introduced by the primer used for gene amplification, thus leaving an aspartic acid to asparagine mutation unaccounted for.

Alignment of the α-mating factor signal to the sequenced Lccδ gene revealed that the laccase gene directly followed on from the signal peptide as expected. Nucleotide and amino acid alignments revealed many point mutations (45), however only three mutations resulted in an amino acid change, with all three identified as isoleucine to valine substitutions. Communication with the original authors revealed that the published sequence from Jonsson *et al.* [196] (Lcc1) was used for alignment, which differed slightly to the sequence referenced in their work (Accession no X84683). Upon alignment of the sequencing data with the published laccase sequence it was revealed that two of the isoleucine to valine substitutions were present in the published sequence, leaving just one mutation unaccounted for. Transformation of both plasmids into the expression host *P. pastoris* was carried out and the sequence differences were noted for future use.

Linearised pPICZA-Lccβ and pPICZαA-Lccδ were transformed into the genome of *P. pastoris* X33 by electroporation. Successful integration of Lccβ was confirmed by PCR using purified genomic DNA as template and primers described earlier. PCR reactions using different combinations of primers for Lccδ failed to produce an amplification product. This suggested that either the transformation was unsuccessful or that optimal PCR conditions were not met. Small scale expression (50mL) was carried out to investigate and confirm successful integration of both genes using the optimised expression protocols reported by Koschorreck *et al*. [198]. pPICZA and pPICZαA vectors not carrying laccase genes were transformed alongside the laccase containing vectors as negative controls. Expression was monitored by the assessment of laccase activity using the methods described below.

2.2.1.2 Solid phase screening to monitor laccase expression

A laccase activity assay was developed to monitor laccase secretion during the expression time course and to confirm activity. The electron rich, chromogenic substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was chosen for the assay because it can be rapidly oxidised by peroxidases and laccases to the green coloured cation $ABTS^{+\bullet}$ (before further oxidation to the dication, Scheme 13). The high stability of the cation makes ABTS a popular substrate for the assessment of laccase expression systems [153].



Scheme 13: The oxidation states of ABTS upon oxidation by laccases. ABTS is first oxidised to the stable cation $ABTS^{+\bullet}$, and is then slowly oxidised to the dication $ABTS^{2+}$.

A solid plate assay (buffered medium incorporating ABTS solidified by agarose) was developed to provide a qualitative assessment of laccase expression within the expression medium after cell removal by centrifugation. Addition of the (cell-free) expression medium into wells within the plate allowed for contact of the medium with the ABTS. A positive result was indicated by a colour change around the well from colourless to green. The size and intensity of the green zones, as well as the speed of colour change of the agar gave an indication of the amount of oxidising enzyme present, although this assay was not quantitative.

The assay was used to monitor the expression of Lccβ. Aliquots of expression medium were removed throughout the expression time course and cell-free supernatants were added to the ABTS plate. Laccase activity was detected in the supernatants 24 hours after methanol induction. Productivity appeared to increase at 72 hours and was maintained up to 192 hours as indicated by the increased size and density of the green zones in the wells representing 72-192 hours (post methanol induction) suggesting higher laccase concentration (Figure 20).

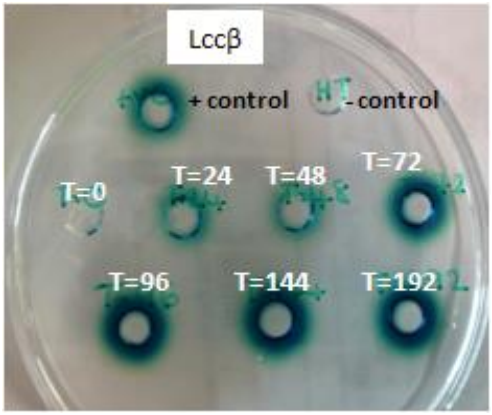


Figure 20: Solid-phase ABTS assay plate used to identify and monitor expression of Lccβ from *P. pastoris* culture supernatants over time. Commercial *T. versicolor* laccase (TvL) was used as a positive control and a heat treated TvL preparation was used as a negative control.

For Lccδ, the production and secretion of laccase appeared to follow a different trend to that of Lccβ expression. Laccase activity was detected in the sample collected 48 hours after methanol induction and appeared to peak at 72 hours. Activity appeared to decrease at 96 hours and there was no further increase in activity after 144 hours (Figure 21).

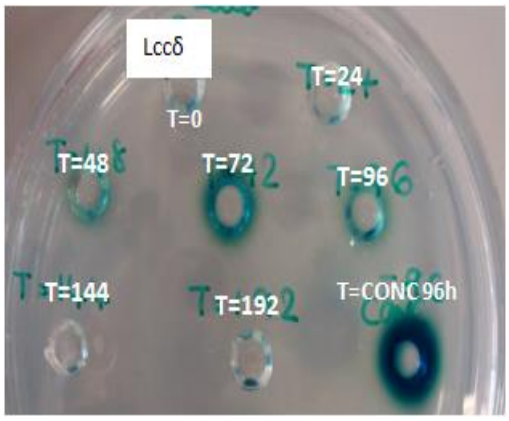


Figure 21: Solid-phase ABTS assay plate used to identify and monitor expression of Lccδ from *P. pastoris* culture supernatants over time. Commercial *T. versicolor* laccase (TvL) was used as a positive control and a heat treated enzyme preparation was used as a negative control.

The results observed by the solid plate ABTS activity assay suggest that Lccδ was successfully transformed into *P. pastoris* and secreted into the expression medium up to 96 hours post induction. However after 96 hours, the expression of laccase appears to cease which could be due to possible inhibition of protein expression or

degradation/instability of the secreted protein. The supernatant collected 96 hours after induction was concentrated ~20x using a 10kDa MWCO and assayed on the ABTS activity plate to demonstrate the dilute nature of the supernatants (Figure 21).

The pPICZA and pPICZαA vectors not carrying laccase genes were expressed alongside both pPICZA-Lccβ and pPICZαA-Lccδ to demonstrate that the colour changes observed were due to the laccase gene product and no other vector products. No colour change was observed during the expression time course of both empty vectors. Solid plate assays of the supernatants taken 96 hours post methanol induction of the empty vectors are presented in Figure 22.

Figure 22: Solid phase ABTS assay plate of wells containing cell supernatants from the expression of pPICZαA and pPICZA 96h post induction to demonstrate negative laccase activity.

A disadvantage of this solid phase ABTS assay is the requirement for the continued sampling and separation of the cell suspensions. A second solid-phase assay was therefore developed for faster and simpler determination of laccase expression. In the second solid assay, the minimal medium used for expression (BMM) was solidified with agar and was supplemented with 0.5% methanol (once cooled) to induce expression, and 0.2mM ABTS to visualise activity. Transformed cells (with pPICZA-Lccβ, pPICZαA-Lccδ, pPICZA and pPICZαA) were plated and incubated at 30°C for a minimum of 3 days.

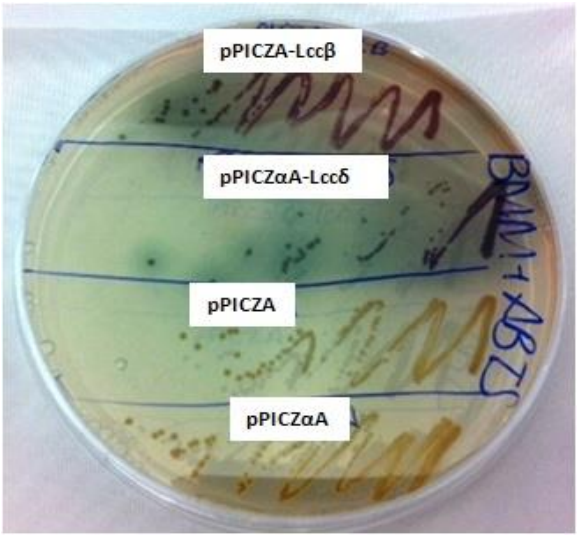


Figure 23: A BMM plate with X33 *P. pastoris* cells expressing Lccβ and δ, and empty vector controls grown at 30°C. The green zone around the colonies corresponds to oxidation of ABTS due to laccase expression during growth. Negative controls do not produce the green zone.

The empty vector negative controls (pPICZA and pPICZαA) grew colonies that were beige in colour and did not change the colour of the surrounding growth medium. For *P. pastoris* expressing Lccβ and δ, the solid medium changed from colourless to green indicating ABTS oxidation by laccase secretion (Figure 23). In addition the cells appeared purple, which is an observation that has not been previously reported, however it has been suggested in the literature that the dication of ABTS is purple in colour [153] which could explain this observation. The BMM plate, together with the solid phase ABTS plate were effective tools for confirming and monitoring laccase expression and activity, however they cannot be used for quantification, in which case a liquid phase assay was developed as described in Section 2.2.2.

2.2.1.3 Optimisation of Lccβ and Lccδ expression

For successful expression of heterologous proteins in yeast systems, certain parameters are crucial. These include the use of multi-copy transformants, the avoidance or reduction of proteolytic degradation and maintenance of a constant environment for the cells with respect to temperature and pH [208,209].

The production of multi-copy transformants was screened for using the ABTS solid plate assay by comparing the laccase activity zones of supernatants from different transformants at the same time point during expression. Transformants were selected that displayed greater laccase activity in their supernatants as demonstrated by the

larger and darker green oxidation zones at a given time point. The higher laccase activity observed by one transformant compared to another is often due to a higher copy number of the expression plasmid within the *Pichia* genome. No successful episomal vectors for *P. pastoris* have been developed to enable the production of stable expression strains, therefore integration occurs into the genome of *P. pastoris*. Integration within the AOX1 promoter can promote repeated recombination events which can lead to multiple plasmid integration and a higher copy number of the gene within the *Pichia* genome [209,210].

The loss of oxidative activity during the expression time course of Lccδ can be explained and restored by pH optimisation. It has been reported that a rapid decrease in pH is detrimental to the production of laccase during fermentation and that the expression of laccase from *T. versicolor* in *P. pastoris* can be improved by maintaining the pH at 6.0 [187,196]. Throughout the expression study of Lccβ and δ, the pH of the expression medium was found to decrease from pH 6.0 to pH 3.0 as the experiment progressed. It is thought that the production of acidic end products from the metabolism of methanol cause this pH reduction [191]. Koschorreck *et al.* [198] found that Lccδ from *T. versicolor* was completely inactivated at pH 3.0 after 3 hours suggesting that the enzyme was not stable at lower pH. The same was not reported for Lccβ, which retained 85-95% activity after 20 hours incubation at pH 3.0. This explains the continual detection of laccase activity throughout the total duration of Lccβ expression despite the drop in pH. Work by O'Callaghan *et al.* [191] revealed that when D/L-alanine was supplemented into the expression medium at 0.8%, not only was the pH maintained at 6.0, but the laccase activity was increased 2-3 times in comparison with cultures not supplemented with D/L-alanine. It was later found that only the L-isomer was effective in maintaining the pH. It is suggested that the metabolism of alanine releases ammonia which can neutralise the acidity produced following methanol metabolism [191]. Furthermore, a pH decrease has been linked to an increase in proteolytic activity throughout *P. pastoris* fermentations. Most proteases work under acidic optimum therefore a higher pH is often favoured for *P. pastoris* fermentation [195]. These findings help to understand why pH maintenance is critical and also explain the loss of activity observed during Lccδ expression.

The stabilisation and improvement of laccase activity by the addition of L-alanine was investigated. Alanine was added at 0.8% into the expression medium of *P. pastoris* expressing both pPICZA-Lccβ and pPICZα-Lccδ. An alanine omitted control was also set up. Surprisingly, although the pH decrease from 6.0 to 3.0 did not drastically affect the stability of Lccβ, a greater oxidative activity against ABTS was observed on the plates when the media was supplemented with alanine. At later time points of expression (216 and 288 hours), when a pH decrease would usually affect stability (Figure 24), laccase activity was improved. This suggests a role for alanine to improve the stability of proteins that are not highly vulnerable to pH change.

Figure 24: *Left:* Laccase activity zones on the ABTS activity plate during Lccβ expression at 48, 144, 216 & 288h with (+) and without (-) 0.8% alanine. *Right:* Laccase activity is observed throughout the 165h expression of Lccδ with the addition of 0.8% alanine.

Following the addition of alanine into the expression medium of *P. pastoris* cells expressing Lccδ, laccase activity was detected throughout the full duration of the expression time course (165 hours) (Figure 24). This was not observed in the absence of alanine as previously found, suggesting that maintenance of the expression medium pH is an essential consideration for the production of a stable and active Lccδ protein.

2.2.2 ABTS assay for quantifying laccase activity

A disadvantage of the solid plate ABTS assay is that it only allowed for the collection of qualitative data. A liquid based ABTS assay for laccase activity was developed in order to calculate enzyme activities. A method was developed based on the work of Niku-Paavola *et al.* [211] which enabled the absorbance change at 420nm to be monitored during the oxidation of ABTS following the colour development from colourless to green (Figure 25).

Figure 25: The colour change of ABTS with commercial TvL at (L-R) 0, 0.03 0.06, 0.13 and 0.25mM ABTS concentration.

2.2.2.1 Calculation of the extinction coefficient for oxidised ABTS

The published molar extinction coefficient (ε) for the oxidised ABTS cation is 3.6 x 10^4 M^{-1} cm^{-1} [212,213] however, for this study, to calculate a system-specific ε was calculated for use in all future studies using this assay. ε was determined using the Beer Lambert Law (Equation 1).

$$A = \varepsilon \, x \, c \, x \, L$$

Equation 1: The Beer Lambert equation whereby whereby A = absorbance (A/U), ε = extinction coefficient (M^{-1} cm^{-1}) concentration (M) L = path length (cm).

ε is a measurement of how strongly a given chemical species absorbs light at a specified wavelength and should be fixed for a given pH and temperature. The concentration of ABTS was varied to produce a standard curve which was used to record the maximum absorbance of ABTS oxidised by commercial *T. versicolor* laccase (TvL) at 420nm, pH 3.0 and 20°C (Figure 26). The linear range at 420nm produces a gradient that is equal to the extinction coefficient x path length.

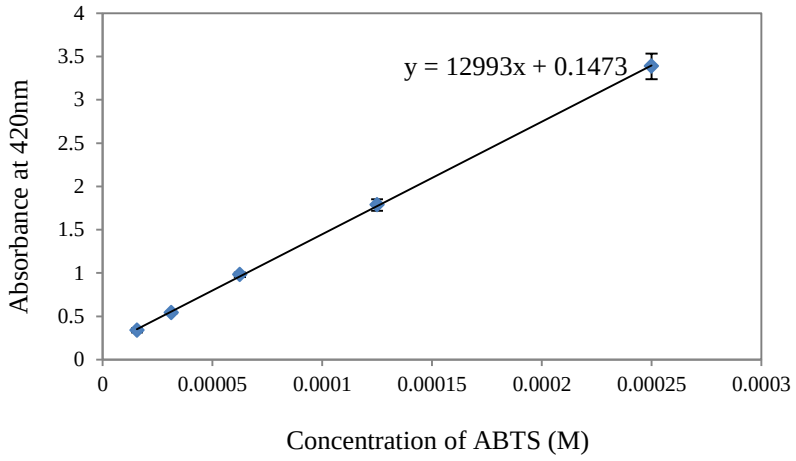


Figure 26: Standard curve of oxidised ABTS concentration against absorbance for the calculation of ε. Each data point represents the mean of 3 replicates with error bars representing the standard error.

The path length for a given set of reaction volumes was previously calculated within the research group [214]. A 200µl reaction in a microtitre plate well gave L = 0.61 therefore the average ε calculated from three replicates was 2.1 x 10^4 M^{-1} cm^{-1}. This value is lower than the published ε (3.6 x 10^4) which is reported for assay conditions at pH 5.0 instead of pH 3.0. The ABTS activity assay developed was adapted from that of Niku-Paavola *et al*, [211] with the exception that in this current system a wavelength of 420nm was used instead of 436nm. The published ε at 436nm is 2.9 x 10^4 M^{-1} cm^{-1}.

2.2.2.2 Calculation of the specific activity of commercial TvL

The specific activity of the commercial TvL preparation (Sigma) was calculated by the manufacturers with respect to catechol. The specific activity was recalculated in this study using ABTS due to the high sensitivity of the developed activity assay which is ideal when measuring enzyme activities in dilute culture supernatants. In addition, ABTS appears to be the oxidative substrate of choice for calculating laccase activity according to the majority of published research, therefore the use of ABTS will keep the data in this work in line and comparable to the work of other researchers.

1mg/mL solutions of TvL were prepared in triplicate and assayed using the ABTS method described above. Samples were diluted until the absorbance change per minute could be measured. Protein concentration was determined using the BCA assay and the specific activities calculated using the previously established ε. The calculated specific activity for the commercial preparation was found to be 13U/mg (Table 9).

Table 9: Calculation of the specific activity of commercial TvL against ABTS

	Protein concentration (mg/mL)	Abs change per min at 420nm	Specific activity (U/mg)
Replicate 1	0.0136	1.1829	13.120
Replicate 2	0.0141	1.2433	13.294
Replicate 3	0.0146	1.2211	12.643
Mean	0.0141	1.2158	13.019
Standard error of mean	0.0003	0.0176	0.1946

2.2.2.3 Calculation of the specific activities of expressed Lccβ and Lccδ

Volumetric activity, protein concentration and specific activity was measured throughout the expression time course of both Lccβ and δ. Typical values from the small scale shake flask expression experiment are presented in Table 10. The volumetric and specific oxidative activities against ABTS were consistently higher in the supernatants from the cells secreting Lccβ compared to Lccδ. The same results were observed by Koschorreck *et al*. [198]. In addition Bohlin *et al.* [186] reported 3-4x higher activities from the culture supernatants expressing Lcc2 compared with Lcc1. As mentioned previously, the sequences of Lccβ and Lccδ used in this study correspond to the same proteins termed Lcc2 and Lcc1 respectively from previous studies [187,204] suggesting that Lcc2 and Lcc1 in the work of Bohlin may correspond to Lccβ and Lccδ in this study.

Table 10: Volumetric activity, measured protein concentration and specific activity of Lccβ and δ during a 240h expression time course in shake-flasks.

	Lccβ				Lccδ			
Time h	Average ▲ Abs/min	Vol activity U/L	Protein conc[b] mg/ml	Specific activity U/mg	Average ▲ Abs/min[a]	Vol activity U/L	Protein conc[b] mg/ml	Specific activity U/mg
18	0.003	0.5 (0.02)	0.01	0.05	0.006	0.9 (0.03)	0.006	0.14
43	0.093	14.5 (0.27)	0.02	0.73	0.007	10.4 (0.03)	0.02	0.52
70	0.205	32.1 (1.96)	0.02	1.61	0.135	21.0 (0.07)	0.03	0.70
96	0.466	72.8 (0.92)	0.04	1.82	0.320	49.9 (0.23)	0.04	1.25
120	0.665	103.8 (1.49)	0.04	2.60	0.482	75.2 (0.06)	0.04	1.88
144	0.886	138.3 (0.37)	0.05	2.76	0.565	88.9 (0.17)	0.05	1.78
168	1.083	165.2 (4.58)	0.05	3.30	0.583	91.6 (3.29)	0.06	1.53
192	1.417	221.3 (0.59)	0.06	3.69	0.862	134.5 (1.07)	0.07	1.90
216	1.555	242.7 (0.38)	0.07	3.47	0.732	114.3 (0.21)	0.08	1.43
240	1.764	275.3 (0.46)	0.10	2.75	0.902	140.9 (1.01)	0.11	1.28

Parentheses represent the standard error of the standard deviation of three biological replicates
[a] absorbance measured at 420nm
[b] determined by the BCA assay

The highest measured volumetric activity of Lccβ from the shake flask expression experiment was 275.3U/L, which is greater than that reported by Koschorreck *et al*, [198] (105U/L). Bohlin *et al*, [186] recorded a lower activity of the expressed Lcc2 protein (1.3U/L) in *P. pastoris* however the expression system in this case was under

the control of a different promoter (Glyceraldehyde-3-phosphate dehydrogenase (GAP)).

For Lccδ, the highest volumetric activity recorded was 140.9U/L which is >5x higher than the reported activity by Koschorreck *et al.* [198] in shake flask experiments. A lower expressed activity of 23.9U/L was also reported following the expression of a cloned laccase from *T. versicolor* in *P. pastoris* LP8 [191]. Other researchers have reported activities up to 12600U/L for the expression of a *T. versicolor* laccase in *P. methanolica* [192] and up to 5470U/L in *P. pastoris* GS115 [215] under highly optimised experimental conditions. It is difficult to compare results with those of other researchers due to the inconsistencies associated with the nomenclature of the named laccases and differences in the ε values used in activity calculations.

2.2.3 Purification of expressed Lccβ

A purification strategy was developed for the concentrated supernatant collected following a 7 day expression of Lccβ (specific activity: 1.75U/mg). Lccβ was chosen over Lccδ due to its higher volumetric activity. The method was adapted from existing published protocols [186,189,190,198,216]. Methods for the purification of secreted laccases by *P. pastoris* have been previously reported and generally consist of four purification steps which include ultrafiltration (UF), anion exchange chromatography (AEC), hydrophobic interaction chromatography (HIC) and size exclusion chromatography/gel filtration (SEC). The sequence and number of purification steps used has been found to differ between some of the existing research published (Table 11). The sequence used in this study is briefly summarised in Figure 27.

Table 11: Purification strategies and sequences employed by existing studies for the purification of laccases expressed by *P. pastoris*.

Purification steps and sequence	Reference
AEC, HIC, SEC	[186]
AEC, SEC, HIC	[189]
UF, AEC, HIC, SEC	[190]
UF, HIC, SEC	[198]
AEC, HIC, SEC	[216]

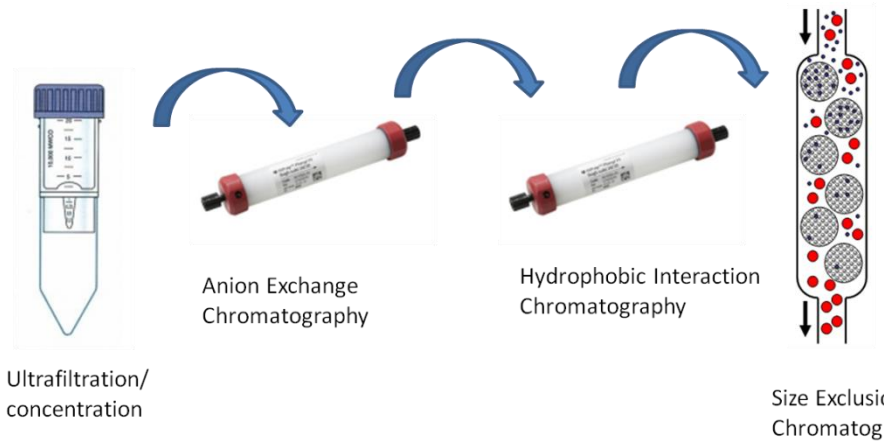


Figure 27: The four stages used for the purification of Lccβ from the dilute expression medium following expression and secretion by *P. pastoris.*

The specific activity of the concentrated protein solution used for the purification experiment was relatively low (1.94U/mg, when compared to the greatest specific activity recorded previously for Lccβ, 2.75U/mg), however for the demonstration of laccase purification this was not highly important. Many problems were encountered whilst developing the purification method, the biggest problem being the loss of active enzyme during the loading of the protein to the columns at both the AEC and HIC stage. To minimise the loss of laccase, the flow through following each loading stage were re-loaded onto the column in 3 cycles to improve binding. Laccase elution was monitored by assaying eluted fractions by the solid ABTS activity plate. During the anion exchange step, laccase activity was detected following elution with 0.1-0.2M NaCl and the fractions were pooled and concentrated before loading onto the HIC column. Laccase activity was detected as soon as 1.7M NH_4SO_4 was applied to the column and the fractions containing activity were pooled and concentration prior to size exclusion.

The purification strategy was successful in improving the specific activity of the protein preparations following each purification stage (Table 12) by removing protein fractions that did not contain laccase. However, due to the low starting concentration of the protein preparation and the loss of active protein during each purification stage, the procedure was not ideal for the production of suitable quantities of purified active laccase for use in further studies. As shown in Table 3, 11.1U of purified protein was recovered in 0.5ml (22.2U/mL). Typical laccase reactions on lignin-related substrates usually require high concentrations of enzyme. This procedure for the crude

production and purification of active laccase was therefore deemed too time consuming and inefficient with respect to the low quantities of enzyme produced at the final stage.

Table 12: Specific and total activities of Lccβ following purification.

Purification step	Volume (ml)	Protein conc[a] (mg/ml)	Specific activity (U/mg)	Total activity (U)
Ultrafiltration	16.0	1.94	1.75	54.3
Anion exchange	3.0	1.70	3.39	17.1
HIC	1.0	1.48	7.50	11.1
SEC	0.5	0.24	92.3	11.1

[a] determined by the BCA assay

2.2.4 Characterisation of expressed laccase

Biochemical characterisation of both Lccβ and δ has been previously carried out by Koschorreck *et al*. [198]. The enzymes are reported to have different pH and temperature optimums with Lccβ demonstrating optimal activity against ABTS at pH 2.3 and 80°C, whilst the optimal activity of Lccδ is reported to be at pH 3.1 and 45°C. Lccβ is reported to have a higher thermal stability than Lccδ and as previously discussed, retains greater activity in the pH range of 3.0-5.0 which represent the typical pH environment of the large majority of laccase reactions. It was further discovered by Koschorreck *et al*. [198] (as briefly mentioned earlier) that of the four laccases of *T. versicolor* (α, β, γ and δ), Lccδ displayed the highest activity in the oxidative coupling of sinapic acid (98% conversion to the dimer in 20 minutes) and that Lccβ showed the greatest activity towards the oxidation of three polyaromatic hydrocarbons (PAHs) (18-50% conversion in 72 hours). In the same study, Lccδ showed no oxidative ability towards the PAHs. It is interesting that the enzymes have different catalytic preferences. Since the aims of this study aims are focused on the depolymerisation of lignin and lignin-like substrates and not polymerisation reactions, it was believed that Lccβ would be more suitable for future investigations.

For confirmation of laccase expression and for the characterisation of laccase molecular weight, SDS-PAGE gel analyses were performed. Unfortunately, the identification of both laccases on a standard Coomassie stained SDS gel was not straight-forward. The theoretical molecular weights (Mw) of Lccβ and δ are 55.4 and 55.6kDa respectively however due to glycosylation they are reported to lie around

72kDa. To improve the weak protein staining by Coomassie blue due to the dilute nature of the supernatants and low protein concentrations, all protein preps were concentrated before analysis.

The cell-free supernatants of four clones (2x Lccβ and 2x Lccδ) following expression were investigated, along with the empty vector negative controls and commercial TvL as a positive control. Staining revealed a dense band around 65-70kDa, however this band was also present in the empty vector controls that did not express oxidative activity towards ABTS therefore the band was unlikely to represent laccase (Figure 28 a). The commercial TvL protein stained around 65-70kDa indicating that the expressed laccases were expected to be present within the region of the dense band staining and were likely to be co-migrating with another protein. Native PAGE analysis of the expressed laccases stained with ABTS revealed dark green bands indicating the presence of a protein oxidising ABTS (Figure 28b). The activity staining was greater for Lccβ expressing clones, supporting the previously calculated specific activity data. The empty vector negative controls did not stain green. The oxidative bands stained with ABTS were large and 'smeary' and covered the MW region of 65-75kDa. Corresponding native coomassie blue stained gels that were run alongside the activity stained gels failed to reveal protein bands in the same region as the activity stain, suggesting that the proteins were poorly stained by coomassie most likely due to their low concentrations.

Currently, the use of a semi-denaturative protocol has provided this research with the best method for locating the laccase protein on a gel. The method does not fully denature proteins therefore some of the tertiary structure is not destroyed and a degree of enzymatic activity remains. The method allows for the co-staining of the SDS-PAGE gel with coomassie and ABTS. The ABTS stain must be applied before the coomassie blue, the method was not found to work the other way round. Following visualisation of a green ABTS oxidation band, the stain is removed and the gel is incubated with coomassie blue (Figure 28 c, d). As the blue protein stain develops the green oxidised ABTS stain washes off therefore in order to capture the location of the laccase with respect to protein migration there is a small window of time in which both stains can be observed together.

In the current work only Lccβ produced a green coloured oxidised ABTS stain on the semi-denaturative gel and this is due to the greater activity of this enzyme towards ABTS compared to Lccδ (Figure 28, c, d). Under semi-denaturative conditions, some of the protein is denatured and the activity is lowered. Lccδ has a much lower oxidative activity and hence staining compared to Lccβ even under native conditions, so it is not a surprise that the protein does not stain on the gel.

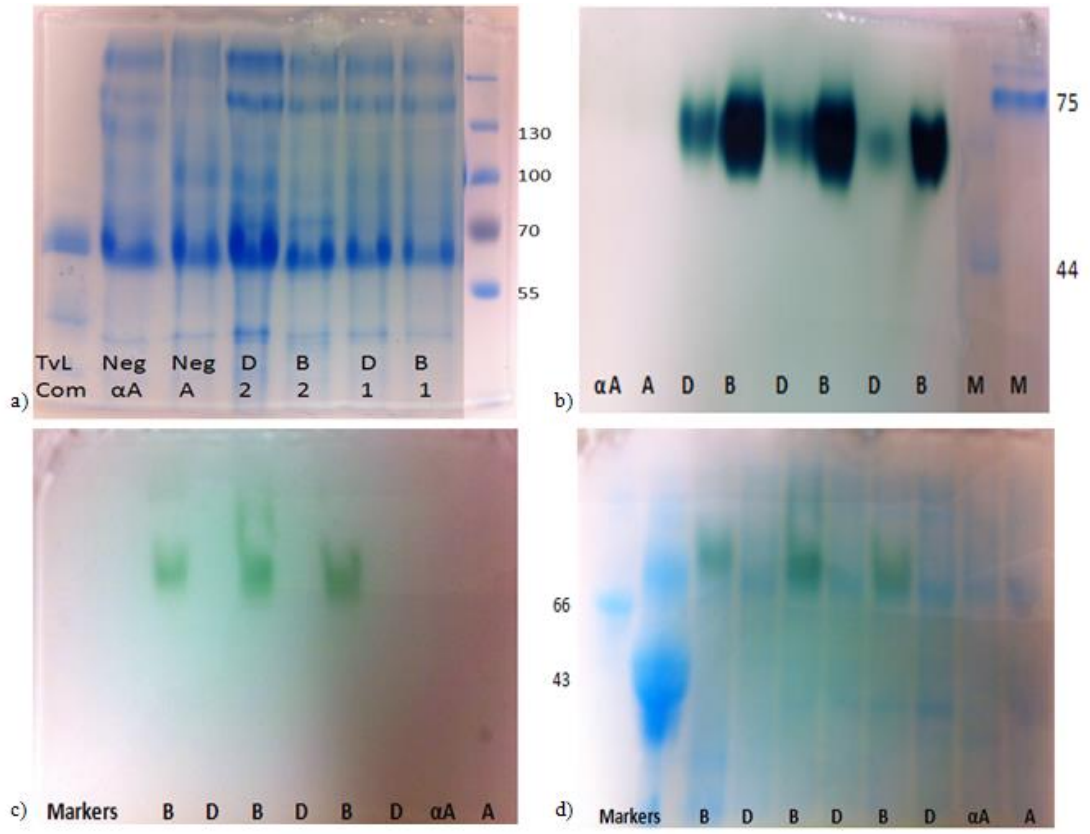


Figure 28: a) SDS-PAGE analysis of two clones expressing Lccβ and δ (labelled on all images as B (β) and D (δ)) and empty vector negative controls pPICZαA and pPICZA (labelled as Neg αA and Neg A respectively). TvL com represents the commercial TvL positive control. All samples contain the same protein band(s) that stain heavily around 70kDa b) Native PAGE stained with ABTS to locate laccase activity in three Lccβ (B) and δ (D) expressing clones plus the empty vector negative controls (αA and A) c) Semi-denaturative PAGE of three Lccβ (B) and δ (D) expressing clones initially stained with ABTS d) Semi-denaturative PAGE upon the incubation with the second stain, coomassie blue to visualise proteins, at this stage the ABTS activity bands and protein bands can both be observed. Markers represent protein Mw in kDa.

SDS and native PAGE analysis following purification showed removal of the contaminating protein in the 70kDa region to a small extent. A tryptic digest was performed by the Protein Identification Facility at The University of Manchester in

order to elucidate the identities of proteins in the stained 70kDa region from Lccβ analysis. Protein identification was assigned using Mascot software (Matrix science) and the UniProt database. Three proteins were identified whereby the number of matched peptides >4 (which relates to an almost certain protein match). The highest match represented *Trametes versicolor* lcc2, with the other two contaminating proteins being identified as cell wall and putative uncharacterised proteins from *P. pastoris.* Protein identification and the activity assays confirmed successful and active laccase expression. Lccδ was not sequenced for identification due to the poor staining and visualisation of this protein band.

2.3 Conclusions

The expression of two selected laccases from *T. versicolor*, Lccβ and Lccδ was successfully achieved using a methanol induced *P. pastoris* expression system. Laccase activities were detected in the supernatants from as early as 24 hours and volumetric activities reached up to 275.3U/L and 140.9U/L (β and δ respectively). Purification of expressed Lccβ resulted in some removal of the contaminating proteins and this was confirmed by the increase in specific activity. The purification strategy was however a multi-step and time consuming procedure which produced a protein concentration that was higher than the crude extracts, but in too small a collected volume to provide enough activity for future multiple laccase-based experiments. Purification of fungal laccases are noted to be almost always purified following methods similar to the one described in this work, although tagged purification methods are published for bacterial laccases [217-219]. Tagged purification is a simpler option however this is not a method of choice for fungal laccases. The reason why is not discussed in the literature, but it is assumed that the presence of histidines in a His-tag will affect the properties of the expressed enzyme by interacting with the divalent copper ions of the T1 Cu in the laccase active site. This explanation could apply to the lack of success of other tagging methods, for example it was found that the incorporation of a StrepII coding purification tag during the expression of an archael laccase in bacteria was found to significantly decrease the activity of the laccase produced [220].

It was discovered by gel analyses that the *P. pastoris* expression system secreted other native proteins in addition to laccase which made the isolation and detection of the expressed proteins problematic. Correspondence with K. Koschorreck (first author of paper in which expression and purification methods were extracted from), revealed that ~10U laccase is required for loading onto a typical SDS-PAGE gel well for successful visualisation. To gain an understanding of this challenge, 11U of Lccβ activity was recovered in 0.5ml after a 7 day expression and lengthy multi-step purification. The concentration of proteins on the gel was therefore never high enough for detection and even if this was possible, little enzyme would be available for other studies. To improve yield, enzyme production on a very large volumetric scale would be required.

The best method for obtaining higher protein yields and therefore increasing laccase concentrations is to switch the expression protocol to large scale fermentation strategies in bioreactor systems. This has been done by many researchers in order to obtain high protein productivity for characterisation [186,189,190,195,196,198]. Volumetric laccase activities have been reported to increase by up to 12-32 times in comparison to shake flask systems [190,195,198]. The production of high amounts of heterologous protein is difficult in shake flasks due to limitations in elements such as volume, oxygen transfer and the addition of substrate (e.g. methanol) which is generally performed manually and therefore poorly controlled [221].

The equipment for a 2L fermentation of *P. pastoris* expressing Lccβ was available at Shell Technology Centre, Thornton (UK). Following previously reported fermentation methods [196,198] and the protocol described by Invitrogen Life Technologies [222], a laccase expression system was established and laccase activity was detected within 2 hours of methanol induction. At 18 hours the volumetric activity reached 10.9U/L, which was x21 higher than previously observed for Lccβ during the shake flask expression. Unfortunately, the growth of the cells could not be maintained, and the volumetric activity of the laccase in the supernatant decreased significantly. At 120 hours, the fermentation was terminated. Due to the lack of *P. pastoris* fermentation expertise available and the time consuming nature of the work, it was decided not to pursue the fermentation strategy further.

The intention of this work was to produce enough active protein to investigate the reactivity of two laccases towards lignin model substrates and eventually lignocellulose. It was unfortunate that the shake flask expression system did not produce enough protein to enable this however the lower activities from the crude supernatants following concentration could still be investigated for this purpose. If time had not been a limiting factor, strategies could have been investigated in order to obtain higher enzyme yields such as optimisation of the purification method, exploring different tagging methods and by scale up of the expression system.

Investigation into the expression systems did however provide an opportunity for successful laccase activity assays to be developed. These assays were useful for assessing an increase in integration events following transformation, for monitoring laccase expression during cell growth, and for calculating specific activities. For

future studies requiring high concentrations of laccase, the commercial preparation of laccase (TvL) will be used.

Chapter three

3.0 The assessment and screening of laccase and laccase mediator systems for lignin degradation studies

3.1 Introduction

A large number of compounds are available that do not only react with laccase as substrates, but upon oxidation can produce oxidised species that have the potential to mediate further oxidative reactions towards substrates that cannot react with laccase alone. The assessment of the reactivity of laccase alone and with potential laccase mediators towards lignin is challenged by the heterologous and complex structural features of a typical lignin polymer. Lignin studies can be incredibly time consuming due to the preparation of lignocellulosic substrates especially when multiple analyses are required. Furthermore, some lignin analysis methods can include run times of over an hour per sample (e.g. py-GC/MS). To screen all of the potential laccase mediator systems (LMS) against lignocellulosic substrates would be an inefficient and time consuming strategy and this is why many researchers make use of lignin model compounds.

Lignin model compounds can be phenolic, non-phenolic, monomeric, dimeric or polymeric depending on the lignin reaction and key linkages that are being investigated (e.g. polymerisation, depolymerisation). Examples of some model compounds are provided in Figure 29.

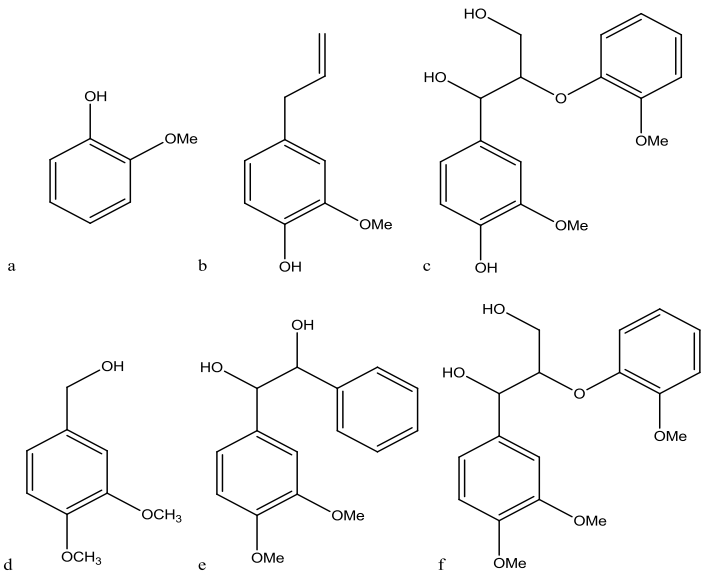


Figure 29: Examples of phenolic (a-c) and non-phenolic (d-f) lignin model compounds. Structures a (2-methoxyphenol) and b (eugenol) represent monomeric phenolic model compounds whilst structure d (veratryl alcohol) represents a monomeric non-phenolic model. Structures c (1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol) and f (1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol) represent β-O-4 linked phenolic and non-phenolic dimer models respectively whilst structure e (1-(3,4-dimethoxyphenyl)-2-phenylethane-1,2-diol) represents a non-phenolic β1 linked dimer.

A typical lignin polymer is approximately 90% non-phenolic in composition [223,224] therefore for lignin degradation studies, non-phenolic model compounds are very useful. Non-phenolic model compounds aim to represent the recurring structural motifs/moieties that make up lignin and are therefore usually benzylic. Making use of lignin model compounds for laccase and LMS studies provides a method of simplifying the detection of oxidative catalytic activity and the identification of reaction products to provide an insight into laccase-mediator reaction mechanisms. Lignin model compound and laccase substrate studies can be used for the assessment of laccase and LMS reactivity and they can be used to identify mediators for potential use in industrial processes. Some processes that have been proven to gain benefits from an LMS include the decolourisation/degradation of dyes [225,226], pulp bleaching [201,227,228] and the oxidation of recalcitrant substrates such as polyaromatic hydrocarbons [151,198,199,229] and kraft lignin [230,231].

Synthetic mediators such as 1-hydroxybenzotriazole (1-HBT) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) are already known for their ability to act as effective mediators to expand the reactivity of laccases for industrial applications [201,232,233]. They therefore represent good starting materials for the search for successful mediators. However, despite advantages regarding their high oxidative capabilities, issues including the high price and associated toxicity of synthetic mediators presents an opportunity for the identification and development of cheaper and less toxic alternatives [151].

The concept of exploring ‘natural mediators’ as substitutes for synthetic compounds has received recent interest due to their availability from renewable and natural sources [151,165,230,234,235]. An LMS of natural origin has been suggested following reports that some white rot basidiomycetes are found to degrade lignin or lignin model compounds in the absence or deficiency of lignin peroxidase (LiP) and/or manganese peroxidase (MnP) [164,167,169,236]. This would imply a predominant role for laccase with respect to lignin depolymerisation in nature. It is speculated that in nature fungi use laccases to degrade the non-phenolic portion of lignin either by:

1) The generation of reactive phenoxyl radicals following laccase oxidation of the phenolic subunits within lignin or from existing lignin degradation products generated by other ligninolytic enzymes [167,237,238].

or by;

2) The secretion of phenolic metabolites such as 3-hydroxyanthranilic acid (3-HAA) which are believed to act as natural phenolic mediators for the laccase catalysed generation of oxidised reactive radicals [164].

Both of the speculated mechanisms indicate a possible role for phenolic compounds to act as laccase mediators whether derived from the existing lignin substrate or from the fungal metabolome. Phenolic compounds make good substrates for laccase due to a match between the redox potentials of most laccases (0.5-0.8V) [139,239] and phenolic substrates (0.5-0.9V) [240]. The successful demonstration of these lignin-related phenolic compounds to act as redox mediators towards lignin and lignin model substrates could provide alternative mediator options that have added environmental and economic advantages over synthetic mediators.

Ideally, a high throughput method is required in order to screen a large panel of potential natural mediators to assess their reactivity with laccase and towards the oxidation of substrates that are not oxidised by laccase alone. Previous work by Camarero *et al*. [165] made use of the recalcitrant dyes Azure B and Reactive Black-5 (RB-5) for the screening of phenols with *P. cinnabarinus* and *T. villosa* laccase (Figure 30).

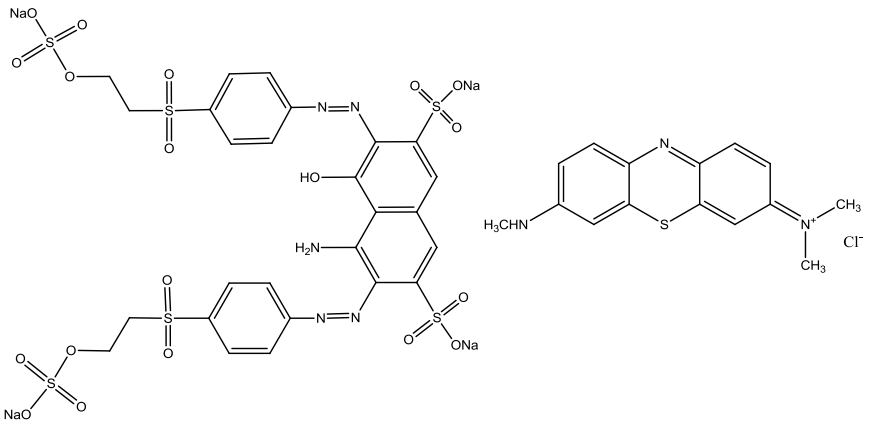
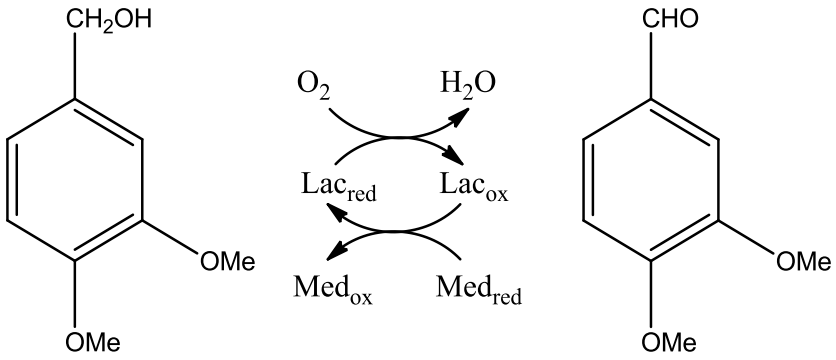


Figure 30: Structures of the diazo type dye Reactive Black-5 (RB-5) (left) and the heterocyclic dye Azure B (right).

Structurally the two dyes do not represent lignin, but the reported lack of oxidation of either dye by laccase alone [165] provides a good basis for an assay that can detect the oxidative activities of the oxidised mediators only. The decolourisation of both dyes can be followed by monitoring the absorbance decrease at 647nm (Azure B) and 592nm (RB-5).

A lignin model compound that is more structurally related to lignin is required for the further screening of any natural mediators that might demonstrate a high oxidative activity against the recalcitrant dye(s). The electron rich non-phenolic substrate veratryl alcohol (3,4-dimethoxybenzyl alcohol) was selected due to its high solubility in aqueous systems and its frequent reported usage in the assessment of LMS efficiencies and reactivities [150,160,235,237]. The redox potential of laccase alone is too low to successfully oxidise veratryl alcohol [241], therefore the observed oxidation of veratryl alcohol to veratryl aldehyde (Scheme 14) should provide an indication towards the oxidative ability of the mediator system employed.



Scheme 14: The oxidation of veratryl alcohol to veratryl aldehyde by a laccase mediator system in the presence of O_2.

Due to the structural similarity of veratryl alcohol to the reoccurring monomeric moieties within lignin, it is assumed that an LMS that is successful for the oxidation of a lignin model compound such as veratryl alcohol will have good reactivity towards lignin in a real lignocellulosic environment. This assumption and relationship will also be investigated in this work. In addition, the effect of some natural and synthetic oxidised mediators on the stability of commercial *T. versicolor* laccase (TvL) will be explored. An ideal mediator should not severely inactivate the laccase and should be recycled successfully to have a stable, continued catalytic action [152].

3.2 Results & discussion

3.2.1 Azure B and RB- 5 screening of potential mediators with TvL

Initial studies using 25µM Azure B, 200µM 1-HBT and a variety of concentrations of TvL (0-600mU) revealed a fast visual decolourisation and a measureable reduction in absorbance at 647nm (Figure 31).

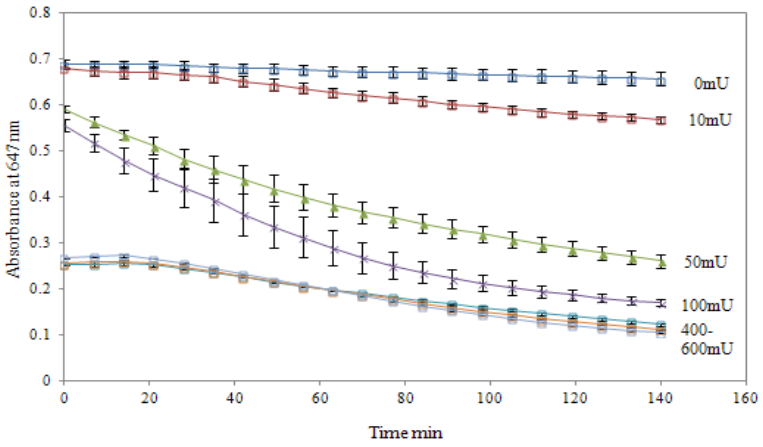


Figure 31: Measured absorbance profiles of 25µM Azure B following incubation with 0-600mU TvL with 200µM 1-HBT over time. Each point represents the mean value of 3 replicates and the error bars represent the standard error.

However, both visual decolourisation and an absorbance decrease were observed when TvL was reacted with Azure B without 1-HBT (Figure 32 well 5, top). This was unexpected due to a previous report claiming Azure B to be poorly oxidised by laccase alone [165]. A second commercially available fungal laccase from *Pleurotus ostreatus* (PoL) was tested without a mediator and the same reactivity was observed (Figure 32 well 5, bottom).

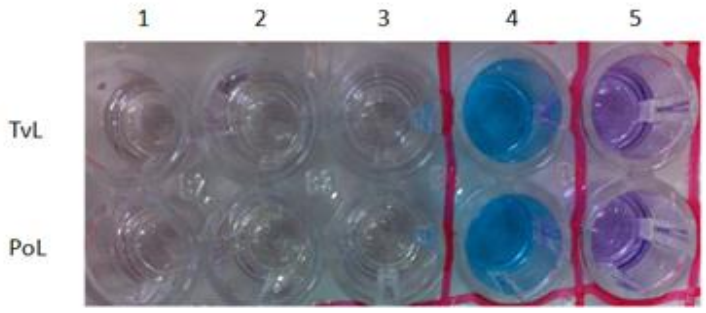


Figure 32: Microtitre plate assay to monitor the decolourisation of Azure B in the presence of 100mU laccase (TvL and PoL) and 200µM 1-HBT (triplicate analysis) (1-3), with no laccase and no mediator (4) and with 100mU TvL or PoL only (5).

When 1-HBT is present the decrease in absorbance continues beyond the maximum absorbance decrease for TvL without 1-HBT, indicating that the decolourisation reaction is further enhanced by the mediator (Figure 33). Analysis of the initial decrease in absorbance from both reactions during the first 60 minutes of incubation shows that the decolourisation rate is faster when 1-HBT is not present. This can be explained by substrate competition which will occur between the mediator and the dye for the active site of TvL, which will slow down the rate of interactions between TvL and Azure B.

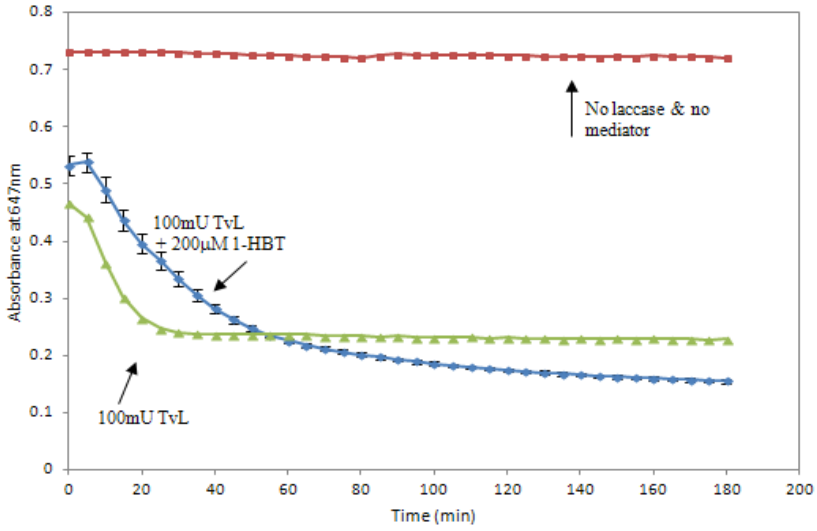


Figure 33: Absorbance profiles at 647nm following the oxidation of Azure B with TvL with and without 1-HBT, a laccase and mediator free reaction was included as a negative control.

Due to the background oxidation of Azure B by both TvL and PoL, it was decided that the dye was not suitable for high throughput mediator screening experiments therefore RB-5 was explored.

Initial experiments with RB-5 revealed that no observable colour change or significant absorbance decrease (at 592nm) was detected when RB-5 was incubated with TvL only, making RB-5 a good candidate dye substrate for investigating decolourisation as a direct result of the oxidised mediator. The difference between the reactivity of RB-5 and Azure B with laccase is likely to be due to the different redox potentials of each substrate. The decolourisation (oxidation) of Azure B by laccase suggests that the redox potential of Azure B is lower than that of RB-5, making Azure B more easy to oxidise by the T1 Cu of TvL (~800mV) [206]. When RB-5 was incubated with TvL in the presence of 200μM 1-HBT, a rapid decolourisation was observed that appeared to be complete within 18 hours (Figure 34).

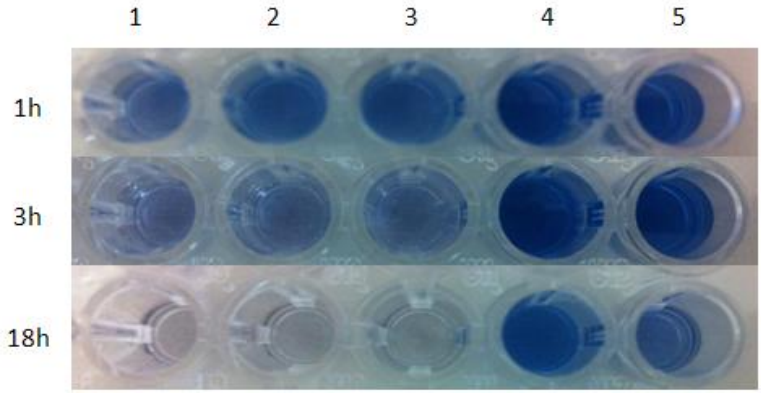


Figure 34: Microtitre plate assay to monitor the decolourisation of RB-5 over 18 hours in the presence of 200mU TvL and 200μM 1-HBT (in triplicate analysis) (1-3), with no laccase and no mediator (4) and with and 200mU TvL only (5).

Spectrophotometric analysis of the RB-5 reaction with various TvL concentrations (0-600mU) and 200μM 1-HBT revealed that the rate of decolourisation was fairly slow at concentrations of 50-100mU TvL (maximum decolourisation achieved within 110 minutes). The rate of (percentage) decolourisation increased as the TvL concentration increased and 200mU was chosen as the best concentration of TvL to use for the mediator screen due to the fast reaction rate (maximum decolourisation

reached within 30 minutes), that also allowed for measureable sampling at 5 minute intervals (Figure 35).

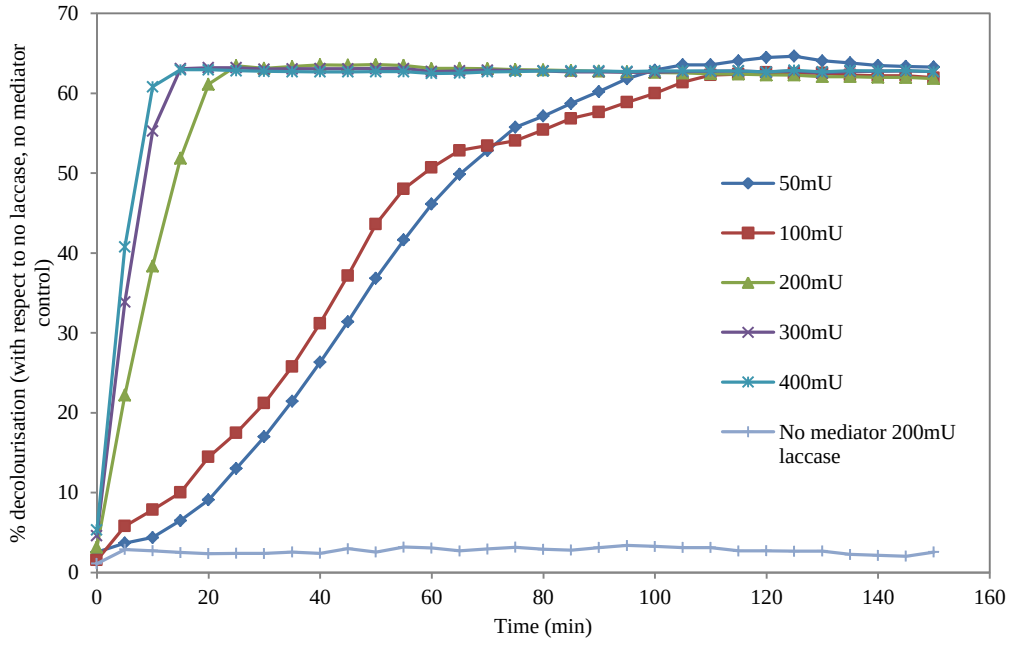


Figure 35: The decolourisation of RB-5 with a range of TvL concentrations (0-400mU) with 1-HBT expressed as a percentage with respect to the no laccase and no mediator negative control.

30 phenolic compounds deriving from either syringyl (S), guaiacyl (G) or p-hydroxyphenyl (H) lignin units (differentiated by the presence of 0, 1 or 2 methoxy groups in *ortho* position to the phenolic hydroxyl group respectively) were selected for screening along with two synthetic mediators previously identified as good laccase substrates (1-HBT and violuric acid) [134,153,154,157,160,234]. Four additional compounds (5-aminopyrimidine-2,4-diol, 2-hydroxynicotinic acid, *N*-methylpiperin-4-ol and 2-chloro-2-pyridinol) were included due to their availability in the laboratory and the presence of a phenolic hydroxyl group within their structure (Figure 36).

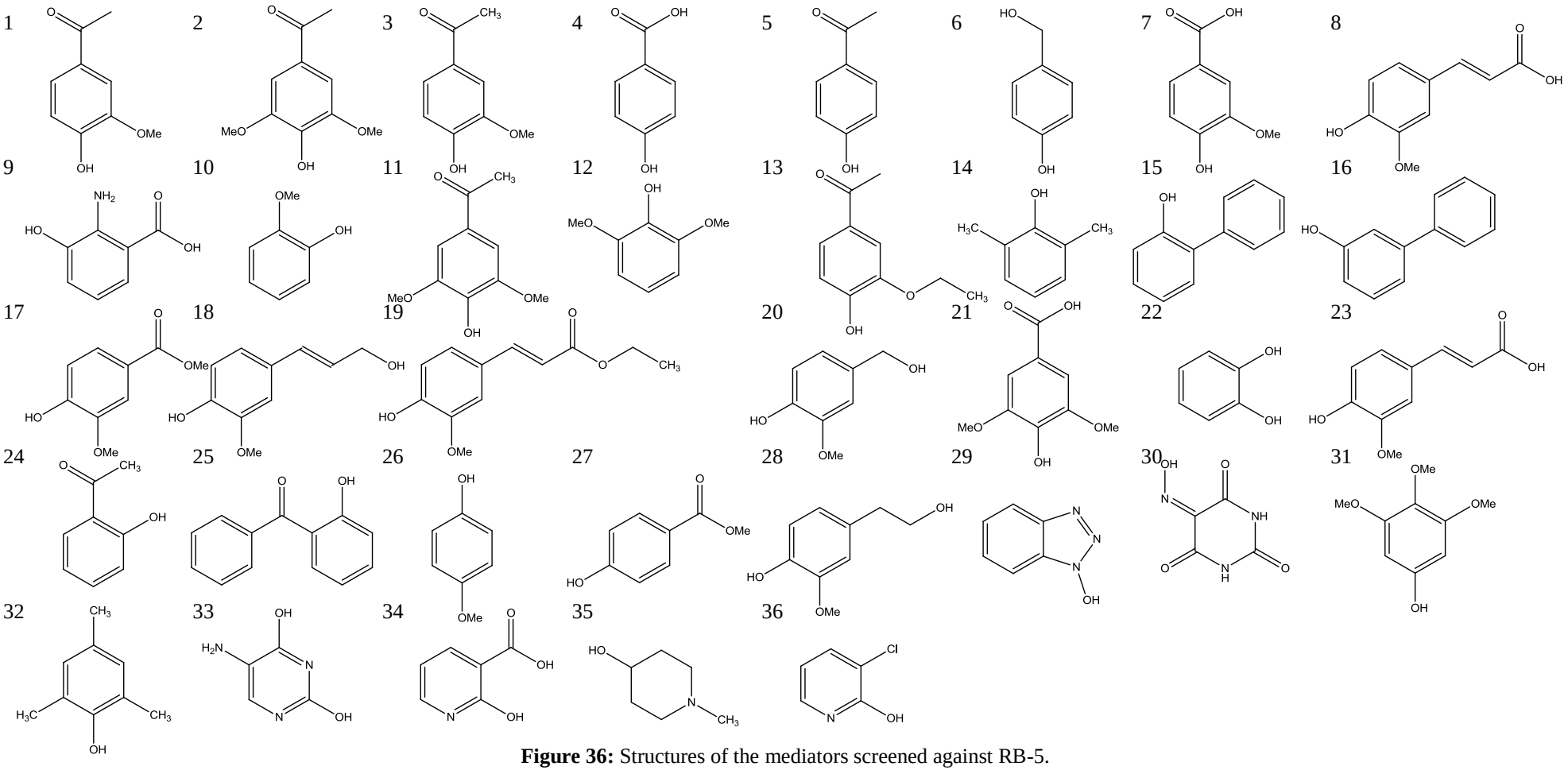
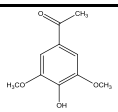
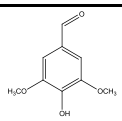
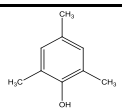
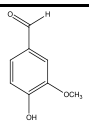
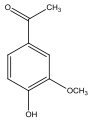
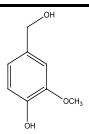
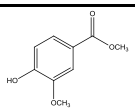
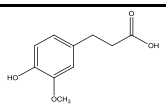
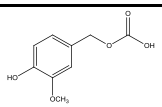
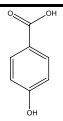
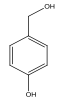
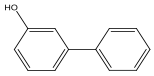
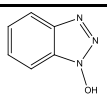
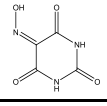


Figure 36: Structures of the mediators screened against RB-5.

1. Vanillin 2. Syringaldehyde 3. Acetovanillone 4. 4-Hydroxybenzoic acid 5. 4-Hydroxybenzaldehyde 6. 4-Hydroxybenzyl alcohol 7. Vanillic acid 8.*p*-Coumaric acid 9.3-HAA 10. Guaiacol 11. Acetosyringone 12. 2,6-Dimethoxyphenol 13. Ethyl vanillin 14. 2,6-Dimethylphenol 15. 2-Hydroxybiphenyl 16. 3-Hydroxybiphenyl 17. Methyl vanillate 18. Coniferyl alcohol 19. Ethyl ferulate 20. Vanillyl alcohol 21. Syringic acid 22. Catechol 23. *Trans*-ferulic acid 24. 2-Hydroxyacetophenone 25. 2-Hydroxybenzophenone 26. 4-Methoxyphenol 27. Methyl-4-hydroxybenzoate 28. Homovanillyl alcohol 29. 1-HBT 30. Violuric acid 31.3,4,5-Trimethyoxyphenol 32. 2,4,6-Trimethylphenol 33. 5-aminopyrimidine 34. 2-Hydroxynicotinic acid 35. N-Methylpiperin-4-ol 36.2-chloro-2-pyridinol

Screening was set up against RB-5 at three different dye:mediator ratios (1:1, 1:5 and 1:10). Potential mediators that exist as coloured compounds in either their oxidised or reduced states were omitted due to possible assay interferences (e.g. ABTS). Overall, the majority of the phenolic compounds screened were found to decolourise RB-5 to varying degrees within 18 hours (at a 1:1 ratio, 32 of the 36 compounds screened were found to decolourise RB-5 whilst at 1:5 and 1:10 this increased to 34), with many compounds showing decolourisation after as little as 15 minutes (at 1:1, 1:5 and 1:10 ratios this was observed in 22, 28 and 25 of the 36 compounds screened respectively). The 12 best phenolic mediators for this reaction and their decolourisation abilities (compared to the TvL negative control) are presented in Table 13 along with 1-HBT and violuric acid at all three dye:mediator concentrations.

Table 13: Percentage decolourisation of the best phenolic mediators against RB-5 at different dye:mediator ratios compared to TvL only control. Parentheses represent standard error of 3 replicates.

Mediator*	Structure	Ratio dye:med	Percentage Decolourisation	
			3h	18h
1. Acetosyringone		1:1	**83.6 (0.1)**	**83.9 (0.2)**
		1:5	74.0 (0.1)	71.8 (0.1)
		1:10	69.0 (0.6)	67.7 (0.7)
2. Syringaldehyde		1:1	**81.0 (0.7)**	**83.2 (0.3)**
		1:5	76.1 (0.9)	75.2 (0.8)
		1:10	71.0 (0.8)	71.4 (0.6)
3. 2,4,6-Trimethylphenol		1:1	46.2 (1.4)	61.3 (1.3)
		1:5	**78.1 (0.8)**	**81.7 (0.1)**
		1:10	76.9 (0.8)	78.4 (0.2)
4. Vanillin		1:1	29.6 (1.2)	36.9 (1.0)
		1:5	**41.6 (1.0)**	**52.5 (0.8)**
		1:10	**39.1 (1.0)**	**56.0 (1.0)**
5. Acetovanillone		1:1	23.4 (0.6)	31.4 (1.3)
		1:5	**31.9 (0.4)**	**39.2 (0.7)**
		1:10	**26.4 (1.66)**	**40.2 (1.23)**
6. Vanillyl alcohol		1:1	17.5 (1.0)	23.8 (1.0)
		1:5	**19.9 (0.7)**	**34.3 (0.4)**
		1:10	12.1 (0.5)	26.9 (0.3)
7. Methyl vanillate		1:1	25.5 (1.4)	30.7 (1.1)
		1:5	31.4 (0.4)	36.7 (0.4)
		1:10	**34.9 (0.6)**	**42.4 (0.61)**
8. *p*-coumaric acid		1:1	9.25 (0.5)	18.4 (1.3)
		1:5	**24.7 (0.2)**	**40.2 (0.4)**
		1:10	19.7 (0.5)	40.8 (1.4)
9. Ethyl vanillin		1:1	32.0 (0.8)	38.7 (0.5)
		1:5	39.5 (1.5)	50.0 (1.4)
		1:10	**39.3 (0.33)**	**51.6 (0.3)**
10. 4-Hydroxybenzoic acid		1:1	1.2 (0.4)	17.2 (0.5)
		1:5	**12.9 (1.4)**	**46.8 (2.1)**
		1:10	5.2 (1.45)	44.2 (1.06)
11. 4-Hydroxybenzyl alcohol		1:1	14.3 (1.3)	22.8 (1.5)
		1:5	**20.2 (0.9)**	**24.2 (0.2)**
		1:10	**14.7 (1.1)**	**25.9 (1.3)**
12. 3-Hydroxybiphenyl		1:1	16.7 (0.6)	41.6 (0.5)
		1:5	20.2 (1.3)	52.6 (1.0)
		1:10	**21.6 (0.96)**	**57.6 (0.9)**
13. 1-Hydroxybenzotriazole		1:1	**66.1 (1.0)**	**69.5 (0.6)**
		1:5	65.1 (0.7)	68.0 (0.4)
		1:10	62.8 (0.3)	68.8 (0.9)
14. Violuric acid		1:1	42.0 (2.0)	55.9 (1.2)
		1:5	**80.5 (0.2)**	**81.9 (0.2)**
		1:10	79.3 (0.7)	80.4 (0.7)

* Mediators listed according to structural similarities (e.g. syringyl, guaiacyl or *p*-hydroxyphenyl).

Interestingly, the best three phenolic compounds for the decolourisation of RB-5 with TvL (acetosyringone (AS), syringaldehyde (SA) and 2,4,6-trimethylphenol (2,4,6-TMP)) share the same structural similarity of having two substituent groups in both *ortho* positions to the phenolic hydroxyl group. Decolourisation was in the range of 81-84% with these three compounds, which was greater than that observed with 1-HBT (maximum decolourisation 69.5%) and of a similar level to that observed with violuric acid (81.9%). In a similar screening experiment by Camarero *et al*. [165] using *Pycnoporous cinnabarinus* laccase, AS and SA were also found to be the greatest mediators for decolourising RB-5. Additionally, Andreu & Vidal [242] found increased delignification effects following the use of AS and SA with *P. cinnabarinus* laccase on kenaf pulp compared to three other natural mediators.

Structurally, the presence of two substituent groups on either side of the hydroxyl group is suggested to induce a stabilisation effect on the phenoxyl radical produced following laccase oxidation of the hydroxyl group. Diaz-Gonzalez *et al*. [235], used cyclic voltammetry to investigate the electrochemistry of a panel of phenolic compounds as mediators and found that oxidation was easier when chemical groups that are electron-donating such as OCH_3 were present due to reducing the redox potential of the substrate and increasing radical stabilisation effects.

However, the size of the substituent groups *ortho* to the phenol is also important for the efficient oxidation by laccase. The inability of laccase to oxidise 2,4,6-tri-tert-butylphenol due to steric hindrance caused by the bulky groups in *ortho* positions to the phenol has been reported, providing evidence that not all phenols are easily oxidised [241]. Phenoxyl radicals are highly reactive and generally unstable due to the spontaneous radical coupling reactions that proceed following oxidation of their phenolic precursor. The coupling reactions can lead to the production of dimers (and often, eventually, polymers) which cannot be reduced back to their original state by non-phenolic substrates [134,238]. The rapid decolourisation rate of RB-5 (<15 minutes) in this system suggests that dimerisation of the reactive radicals is likely to occur after the majority of radicals have reacted with RB-5. The differences observed in the reactivity of each oxidised phenolic mediator against RB-5 are likely to be due to a combination of both the accessibility of the structure towards the laccase active site and the stability of the radical species generated.

Additionally, it is also suggested that the pK_a value of the phenolic substrate affects its oxidation rate by laccase [165]. SA and AS have relatively low pK_a values (7.34 and 7.88 respectively), which is interesting when comparing to the pK_a values of some of the phenols screened that did not perform as well, for example homovanillyl alcohol (pK_a 10.09), coniferyl alcohol (pK_a 9.54), ferulic acid (pK_a 9.39), syringic acid (pK_a 9.49) and 2,6-dimethoxyphenol (pK_a 9.98) [243]. Production of a phenoxyl radical is promoted by the existence of the phenolate form therefore a lower pK_a will induce faster oxidation [165]. The structural and chemical properties of the successful compounds identified from this screen can therefore be used to provide a useful starting point for the identification of future novel laccase mediators.

3.2.2 Veratryl alcohol oxidation by TvL & LMS

The UV absorbance of veratryl alcohol and the oxidation product veratryl aldehyde at 210, 230 and 280nm was investigated using HPLC. Standards (0-3mM) were run in order to construct a standard curve to determine the best wavelength for analysis. The alcohol and aldehyde did not absorb at a 1:1 ratio at any of the wavelengths tested, with the greatest difference in absorbance observed at 280nm (Figure 37). The smallest difference between the standard curves was observed at 210nm, but this wavelength consistently produced unclean chromatograms with unstable baselines and broad peaks after 15 minutes. The factor of difference (RF value) between the slopes of the standard curves measured at 230nm was x1.3. A method was created to standardise the data collected from the oxidation reactions by multiplication of the peak area at 230nm (for veratryl alcohol) by 1.3 before calculating conversion.

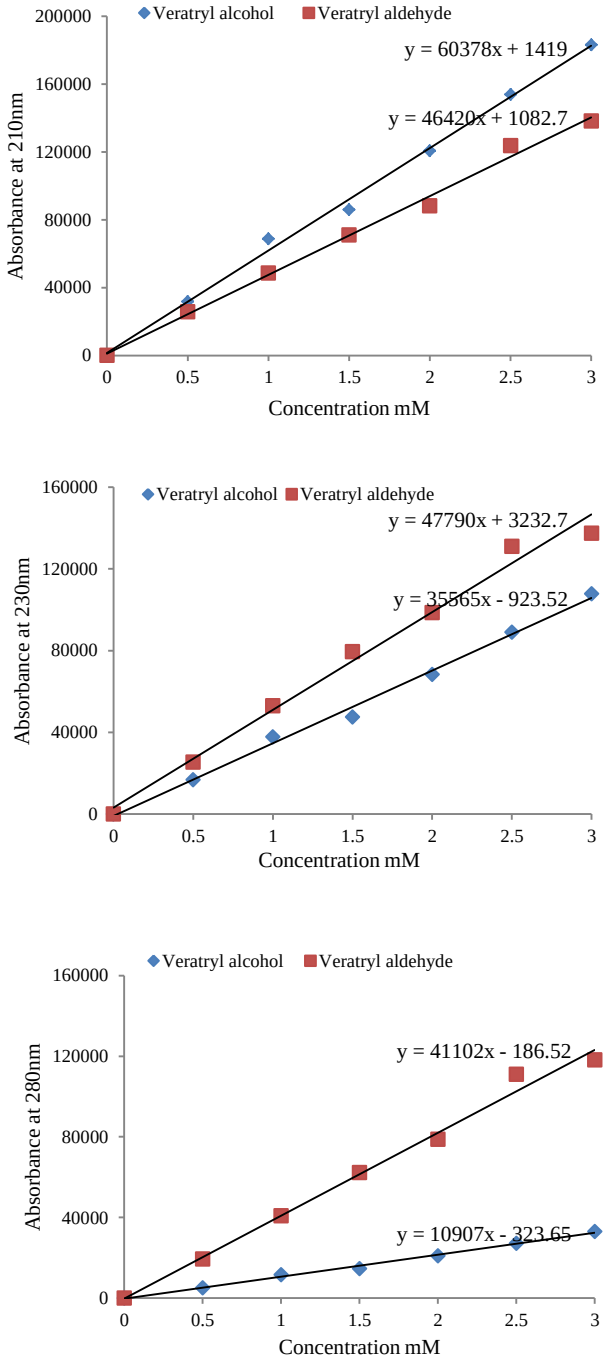


Figure 37: Standard curves of veratryl alcohol and veratryl aldehyde at (top) 210nm, (middle) 230nm and (bottom) 280nm.

Initial studies using TvL and veratryl alcohol demonstrated that laccase alone cannot oxidise veratryl alcohol to the aldehyde, in agreement with previous studies [153,241]. The addition of 1-HBT and ABTS at a substrate-mediator ratio 3:1 with 0.05U/mL TvL revealed the production of the aldehyde from 4 hours (conversion of

8% and 9.5% for 1-HBT and ABTS respectively) with maximum conversions of 98.5% with 1-HBT after 312 hours and 96.8% with ABTS after 672 hours.

A screening assay was set up to investigate veratryl alcohol oxidation with the 12 phenolic mediators that produced the greatest decolourisation of RB-5 in the initial screen (Table 13). 3-hydroxyanthranilic acid (3-HAA) was also included in the screen despite its poor performance in the decolourisation assay due to a previous report which provided evidence for the production of this metabolite by *P. cinnabarinus* to enable the oxidation of non-phenolic substrates [164]. This claim has however been challenged by the work of Li *et al*. [244] who reported only the oxidative coupling of 3-HAA to produce the non-reactive product cinnabarinic acid, with no further reactions observed. Johannes and Majcherczyk [151], provided further evidence against this metabolite acting as a natural mediator in lignin degradation by reporting the failure of 3-HAA to mediate the degradation of polyaromatic hydrocarbons (PAHs).

All mediators were investigated at a higher mediator concentration with respect to the substrate (substrate-mediator ratio of 1:5) due to the higher decolourisations observed at 1:5 and/or 1:10 during the RB-5 reaction (with the exception of syringaldehyde and acetosyringone which were investigated at their optimal ratio of 1:1). It is thought that in nature the phenolic compounds are released in high concentrations in order to enable efficient oxidations [151]. It is worth noting that it is problematic in the laboratory to work with high concentrations (>10mM) of these compounds due to their poor solubility in aqueous systems. This can be solved if required, by using mixed solvent systems using solvents such as dioxane [197,241]. Synthetic mediators 1-HBT and violuric acid were included in the study as positive controls.

The absence of veratryl aldehyde in the HPLC traces following the reactions between veratryl alcohol, TvL and the 12 phenolic compounds suggested that all of the phenolic compounds were unsuccessful in mediating the oxidation of veratryl alcohol. A small peak was detected at a retention time around 16 minutes in almost all of the mediators screened, however this same small product peak was also observed in the negative controls (substrate only, substrate + laccase and substrate + 1-HBT) suggesting a small degree (<2%) of background oxidation of the substrate. Similar findings were published by Larson *et al*. 2012 [245] who reported trace amounts of

veratryl aldehyde following the reaction of a range of laccases with three of the phenolic mediators screened here. Diaz-Gonzalez *et al*. [237] screened a similar panel of phenols with *T. villosa* laccase and found that alcohol conversions were generally <1% with the exceptions of methyl syringate and acetosyringone (9% and 5% respectively). The study supports the previously referenced studies reporting 3-HAA as a poor laccase mediator for lignin oxidation.

The observation (in this case) of the phenols acting as unsuccessful redox mediators for the oxidation of veratryl alcohol might be explained both by the rate of the reaction and the ability of laccase to produce highly reactive and unstable species from phenols that can then undergo undesired non-catalytic routes. Presumably veratryl alcohol is less effective at reducing the intermediate phenoxyl radicals produced by laccase compared with RB-5. This will explain the slower reaction rate observed with veratryl alcohol which would promote undesired non-catalytic mechanisms leading to radical coupling and polymerisation, thus preventing the conversion to veratryl aldehyde.

A study was set up to further assess the three synthetic mediators 1-HBT, ABTS and violuric acid. Reactions were set up to investigate two substrate-mediator ratios (1:1 and 3:1) and two TvL concentrations (1.6U/mL and 0.4U/mL) (Table 14). In addition, a panel of dyes were screened following reports of various dye compounds acting as laccase substrates [165,238,241]. The panel included phenol red, azure blue, toluidine blue, methyl blue, ponceau X, indigo carmine and remazol brilliant blue.

Table 14: The oxidation of veratryl alcohol to veratryl aldehyde using TvL and a panel of synthetic mediators at two enzyme concentrations and two substrate-mediator ratios

Mediator	Percentage Conversion to veratryl aldehyde[a]			
	A	B	C	D
1-HBT	**99**	94	98	92
ABTS	74	61	87	**88**
Violuric acid	31	71	24	**74**
Phenol red	**50**	19	17	33
Remazol Brilliant Blue	21	9	22	**52**

Oxidation of 3mM veratryl alcohol under four different enzyme-mediator conditions. A: 1.6U/mL TvL, 3:1 substrate-med ratio, B: 1.6U/mL TvL, 1:1, C: 0.4U/mL TvL, 3:1 D: 0.4U/mL 1:1 [a] % conversion was determined by LC-MS after 24 hours, optimal conversions for each mediator are shown in bold.

The results revealed that 1-HBT was the best laccase mediator for the oxidation of veratryl alcohol, with 99% conversion observed in 24 hours at the higher laccase concentration (1.6U/mL) and the higher substrate-mediator ratio (3:1), although conversions were high in all experimental conditions investigated (92-98%). The second most successful mediator was ABTS, with the greatest conversions observed at the lower laccase concentration (0.4U/mL) (87-88%). The data from the investigation with violuric acid revealed that oxidation was favoured at the lower substrate-mediator concentration, with oxidations at a 1:1 ratio producing conversions that were more than twice as high than when the substrate is in excess of the mediator (71-74% at 1:1 compared with 24-31% at 3:1).

Of the dyes investigated, only phenol red (PR) and remazol brilliant blue (RBB) acted as potential mediator substrates for TvL, resulting in 50 & 52% conversion respectively. Bourbonnais and Paice [150] reported the oxidative ability of RBB towards veratryl alcohol with laccase, and like the findings in Table 14, observed a lower oxidation rate with RBB compared to ABTS. PR is structurally a phenolic compound but unlike the majority of the phenolic compounds tested in this study, it is of synthetic origin. Due to its structural features, PR is reported to produce a resonance stabilised phenoxyl radical which enables the longevity that is required to allow non-phenolic substrate oxidation instead of self-coupling reactions and polymerisation [149]. Calcaterra *et al*. [246] studied the mechanism of the oxidative reaction by PR and found evidence to suggest that the phenoxyl radical ($PhO^{\bullet}$) follows a H-abstraction mechanism analogous to that reported by the nitroxyl ($>N\text{-}O^{\bullet}$) radicals. PR has also been reported to act as a mediator with *Polyporus pinsitus* laccase for the oxidation of 4-methoxybenzylalcohol and 2,4,6-tri-tert-butylphenol [238,241].

The study with veratryl alcohol revealed that 1-HBT was the best mediator for overcoming the redox differences between the laccase T1 Cu site and the non-phenolic substrate. 1-HBT has been previously demonstrated to produce higher rates of oxidation compared to ABTS in similar studies [153,154,232,247]. The oxidation of both mediators by laccase leads to the production of reactive intermediates that enable the oxidation of non-phenolic substrates by different oxidative mechanisms.

3.2.2.1 ABTS and the ET route

ABTS has been demonstrated to oxidise non-phenolic substrates such as veratryl alcohol by following an electron transfer (ET) mechanism [155,248]. Evidence to support the proposed ET route of oxidation has been investigated by studying substrate reactivity patterns. If an ET route is to take place, substrates with lower redox potentials should be more easily oxidised and oxidation should take place at a faster rate. This was proven by Fabbrini *et al*. [154] who reported the redox potentials ($E°$) on studying the oxidation of benzyl alcohol ($E°$=2.68V *vs.* Normal Hydrogen Electrode (NHE) in acetonitrile, and ~2.4V in H_2O), 4-methoxybenzyl alcohol ($E°$=1.98V *vs.* NHE in acetonitrile, and ~1.7V in H_2O) and veratryl alcohol ($E°$=1.36V *vs.* NHE in H_2O; in acetonitrile it should be ~1.6V). From a redox standpoint, veratryl alcohol should be the easiest to oxidise, followed by 4-methoxybenzyl alcohol and benzyl alcohol. In the study, a laccase/ABTS system was found to produce a negligible amount of benzaldehyde, whilst the yield of veratryl aldehyde was found to be higher than that of 4-methoxybenzaldehyde, supporting the ET mechanism.

Although the reaction between laccase, ABTS and non-phenolic substrates is well studied, there is still some uncertainty in the exact nature of the reaction. What is apparent is that the reaction of ABTS with laccase produces two oxidative species, the radical cation $ABTS^{\bullet+}$, and the dication $ABTS^{2+}$. There is strong evidence to suggest that it is the dication $ABTS^{2+}$ that is responsible for the reaction with non-phenolic substrates, with the most convincing evidence deriving from the $E°$ determined for each species. Cyclic voltammograms have revealed $E°$=0.69V for $ABTS^{\bullet+}$/ABTS and $E°$=1.1V $ABTS^{2+}$/$ABTS^{\bullet+}$ *vs*. NHE [154,155]. Given that the $E°$ for veratryl alcohol is 1.35V NHE [249], oxidation by the cation would be unfavourable from a redox viewpoint. Further evidence for this comes from the work of Fabbrini *et al*. [154] and Bourbonnais & Paice [232] who have reported an increased yield of 4-methoxybenzaldehyde from 4-methoxybenzyl alcohol when the pH of the reaction was decreased from pH 5.0 to 3.5. $ABTS^{2+}$ has been reported to have a greater stability at more acidic pH [153], which suggests that this species is more likely to be involved in the oxidation of non-phenolic substrates than the cation.

Despite the strong evidence in favour of the dication species having responsibility for the oxidation of non-phenolics, work by Majcherczyk *et al*. [199] demonstrated an

enzyme-free oxidative ability of the cation ($ABTS^{\bullet+}$) against aromatic alcohols and claimed that the cation alone could produce high yields of veratryl aldehyde from the alcohol under high substrate and low mediator concentrations. There are further reports that suggest that the conditions that can generate the $ABTS^{2+}$ species and successfully maintain its stability in order to perform oxidations are unsuitable for laccase operation, for example a solvent system of 2M H_2SO_4 [155].

This has lead to investigations into the possibility of other species that may be responsible for the oxidations other than $ABTS^{2+}$, possibly deriving from the degradation of the oxidised mediator. Marjasvaara *et al*. [250] used electrospray ionisation (ESI) to investigate the products generated following ABTS incubation with TvL and found that the cation predominates over the dication, suggesting that the amount of dication generated could be too low to be responsible for oxidation. Furthermore, they identified a molecular ion of m/z 258 (in negative mode) that increased in concentration as the reaction time progressed and proposed that it could be a potential oxidative agent produced from the interaction of laccase with ABTS. Cantarella *et al*. [157] proposed a similar theory after a laccase/ABTS system failed to oxidise a panel of alkylbenzenes with high $E°$ (1.5–2.5 V *vs.* NHE range) [251]. The results suggest that the redox potentials of these substrates are too high for oxidation by $ABTS^{2+}$ making the reaction thermodynamically unfavourable [155]. Redox potentials of the diazonium-like products derived from ABTS breakdown are reported to have $E°$>1.2V (*vs.* NHE) [252] which is greater than that of $ABTS^{2+}$ ($E°$=1.1V), supporting a possibility for their role in oxidation.

Finally, ABTS mediated oxidation of veratryl alcohol has been shown to be greatest at lower concentrations of mediator with respect to the substrate [150,157,248]. This has been explained by the theory that at higher mediator concentrations, ABTS can act as a competitor for the laccase active site along with veratryl alcohol, slowing down the rate of oxidation, whereby at lower concentrations, its role is to activate the enzyme [248]. The results in Table 14 do not support this, with greatest percentage conversion to the aldehyde at the higher mediator concentration with respect to the substrate (1:1) when the lower concentration of TvL was used. Conversely, at the higher TvL concentration, a lower concentration of mediator with respect to the substrate (3:1) appeared to enhance oxidation. A study by Diaz-Gonzalez *et al*. [237] revealed the same percentage of veratryl alcohol oxidation with ABTS and *Trametes villosa*

laccase at both 10:1 and 1:1 substrate-mediator concentrations making it difficult to confirm the optimum ABTS concentration with respect to the substrate.

It can be concluded that inconsistencies within the literature with respect to the exact oxidative mechanism by laccase and ABTS, together with the irregularities with the reported optimum mediator concentrations, make interpreting the data difficult. One point that could be explained by the data in Table 14 is the greater percentage oxidation when 0.4U/mL TvL is used over 1.6U/mL. It is believed that laccase eventually degrades ABTS, therefore removing it from the catalytic cycle [253]. There is little information available on this elimination event; however it could explain why the experiments at lower TvL concentrations perform better oxidations, as higher TvL concentrations could result in greater degradation of the mediator. However there is currently not enough evidence to claim this.

3.2.2.2 1-HBT and the HAT route

For *N*-hydroxyl (>N-OH) mediators such as 1-HBT, the opposite is true of the laccase mediator system compared to ABTS. In this case, it is the oxidised mediators that eventually inactivate the laccase. This was demonstrated previously by Ibarra *et al*. [233] who reported a 50% inactivation of laccase activity following the incubation with 1-HBT. The results in Table 14 show that at both 1.6U/mL and 0.4U/mL TvL, the greatest conversion percentage to the aldehyde was observed when the mediator concentration was in deficiency with respect to the substrate, as demonstrated by Bourbonnais and Paice [153]. Unlike the oxidation of ABTS, which produces the stable $ABTS^{\bullet+}$ cation, laccase oxidation of 1-HBT generates the nitroxyl radical (>N-O$^{\bullet}$) which has lower stability [234]. However, the high conversions of veratryl alcohol by TvL with 1-HBT seen in Table 14 indicate that the radical must survive long enough to allow oxidation to take place, therefore in this case the issue of low stability is not a problem.

The reaction mechanism of non-phenolic substrate oxidation by oxidised 1-HBT follows a hydrogen atom transfer (HAT) route which is initiated by the production of a >N-O$^{\bullet}$ species following laccase oxidation. The radical abstracts a hydrogen atom from the benzylic substrate in the reaction to produce a benzylic radical. This radical then further reacts with oxygen to transform into oxidation products [158,253,254]. Evidence for the HAT mechanism has been determined by electrochemical studies

[153,154] and by investigations into the substrates that are oxidised by this system. The same study that demonstrated the ET mechanism for the laccase/ABTS system by investigating the oxidation of the three benzylic alcohols with increasing redox potentials [154] revealed the same pattern of reactivity for the laccase/1-HBT system. However, differences were observed in the yield of the aldehydes produced from all alcohols, which was in all cases greater than with TvL/ABTS, especially for the alcohol with the highest $E°$ (benzyl alcohol, in which the yield of the aldehyde produced was 2% with oxidised ABTS and 30% for oxidised 1-HBT). This suggests that substrate oxidation by 1-HBT takes place via a different mechanism than by oxidised ABTS. The reaction of laccase/1-HBT with a range of alkylarenes and polyaromatic hydrocarbons that were poorly oxidised by laccase/ABTS revealed moderate oxidation yields, providing further evidence towards the laccase/1-HBT oxidation system utilising a different oxidative mechanism than laccase/ABTS, and one that can achieve the oxidation of substrates with much higher $E°$ [157].

The oxidation of 1-HBT by laccase is a very slow reaction, approximately 85 times slower than the oxidation of ABTS to its cation which takes place within an hour [248]. This may appear surprising due to the increased rate of oxidation of veratryl alcohol by laccase/1-HBT, however it is likely that the following reaction from the cation to the dication is comparably as slow as the oxidation of 1-HBT [232]. It appears that the HAT mechanism of oxidation towards veratryl alcohol must be more effective than the ET route, however if this was the case, the oxidation of veratryl alcohol by laccase/violuric acid would surely be higher than with ABTS. Fabbrini *et al*. [154] reported a comparable yield of veratryl aldehyde with that produced following laccase/1-HBT oxidation, supporting the theory that the HAT route is more efficient. There is currently some confusion regarding the reactive mechanism carried out by oxidised violuric acid in order to oxidise non-phenolic substrates. Its *N*-hydroxyl structure like 1-HBT would suggest a HAT mechanism of oxidation, and this has been proposed by Astolfi *et al*. [255]. However electrochemical experiments by Arzola *et al*. [231] suggest that an ET route is followed, indicated by the production of a stable radical cation following oxidation by an electrode. In a study by Li *et al*. [256] violuric acid was found to inactivate laccase to a greater extent than 1-HBT, which might also, in addition to a difference in mechanism, provide an explanation for the lower oxidation of veratryl alcohol observed in Table 14.

3.2.3 Oxidation of veratryl alcohol by expressed Lccβ and Lccδ

The veratryl alcohol oxidation reaction was used to investigate the reactivity of the expressed laccases Lccβ and Lccδ from *T. versicolor* in *Pichia pastoris* (Chapter 2 Sections 2.2.2-2.2.4). Both crude enzyme preps (following ultracentrifugation and concentration using a 10kDa MWCO membrane) and the purified preparation of Lccβ were reacted with veratryl alcohol with the chosen synthetic mediators 1-HBT or ABTS.

Table 15: The oxidation of veratryl alcohol to veratryl aldehyde using expressed and commercial *T. versicolor* laccase with synthetic mediators 1-HBT and ABTS.

Enzyme	Mediator[a]	% Conversion to veratryl aldehyde[b]	
		90h	308h
0.13U Lccβ	1-HBT	34.3 (1.0)	80.3 (3.1)
	ABTS	64.6 (1.2)	92.5 (0.9)
0.3U Lccδ	1-HBT	2.6 (0.4)	3.6 (0.3)
	ABTS	6.7 (0.3)	8.7 (0.4)
0.1U TvL	1-HBT	99.0 (0.3)	98.5 (0.1)
	ABTS	70.3 (1.0)	97.9 (0.1)
0.02U purified Lccβ	1-HBT	35.8 (1.6)	81.4 (1.1)

[a] Substrate-mediator ratio of 3:1 [b] % conversion determined by HPLC. Background oxidation by laccase only and heat treated controls was calculated as 0.9%. Parentheses represent standard error of 2 replicates.

The results show that both expressed laccase preparations were successful in the oxidation of the alcohol to the aldehyde by comparison to the laccase only and heat treated negative control (0.9%) (Table 15). Lccβ was previously found to have a greater specific activity towards ABTS than Lccδ (Chapter 2, section 2.2.2.3), and to reflect this, a higher concentration of Lccδ was applied to the reaction (0.13U for Lccβ compared to 0.3U of Lccδ). The conversions achieved using Lccβ were found to be much greater than those achieved using Lccδ however they were lower than those achieved using the commercial preparation (0.1U TvL). Generally, with regards to Lccβ, conversions were high (80.3-92.5% after 308 hours). The same was not true for the reactions with Lccδ with either ABTS or 1-HBT whereby conversions were very low (3.6-8.7% after 308 hours).

The results are fitting with the observations by Koschorreck *et al*. [198] who found that Lccδ was more suited to radical coupling reactions than the oxidation of polyaromatic hydrocarbons which were oxidised the greatest by Lccβ. The poor

stability of Lccδ (Chapter 2 Section 2.2.4) is likely to play a part in its low oxidation towards veratryl alcohol. The high conversions achieved using the crude preparation of Lccβ demonstrate the potential use of the *P. pastoris* crude extracellular medium post expression without the requirement for any further purification. However, the high conversions achieved using the purified Lccβ preparation at the very low concentration of 0.02U demonstrates that removal of contaminating proteins significantly increases the activity of the enzyme preparation. Therefore a lower concentration of enzyme can be used to yield the same high conversions (Table 15).

The results obtained with the expressed laccases do not demonstrate the previously observed finding in that the use of 1-HBT with laccase leads to an increased oxidation of veratryl alcohol over ABTS. The findings in Table 15 show the reverse (greater conversions using ABTS compared to 1-HBT), but the reason for this remains unclear.

3.2.4 Stability studies of TvL with synthetic and natural mediators

The results from the model compound study have demonstrated that synthetic mediators are promising candidates for lignin degradation studies, in particular the *N*-hydroxyl mediator 1-HBT. The stability of 1U/mL TvL in the presence of two *N*-hydroxyl mediators 1-HBT and violuric acid was investigated over 72 hours along with the two natural mediators that previously demonstrated the greatest decolourisation of RB-5, acetosyringone (AS) and syringaldehyde (SA). ABTS was omitted due to its use as the colorimetric substrate for the activity test.

Table 16: Inactivation of TvL activity in the presence of two synthetic and two natural mediators.

Enzyme/mediator concentration (mM)	Percentage activity loss[a] 20h	48h	72h
TvL with no mediator	42 (0.45)	64 (0.45)	75 (0.45)
TvL + 1-HBT			
0.05	59 (0.52)	74 (0.52)	83 (1.38)
0.1	71 (0.53)	85 (0.29)	90 (0.05)
0.5	88 (0.13)	97 (0.21)	98 (0.05)
1.0	92 (0.05)	98 (0.13)	98 (0.19)
2.0	95 (0.18)	98 (0.11)	99 (0.08)
3.0	95 (0.44)	98 (0.05)	99 (0.21)
TvL + VA			
0.05	32 (3.18)	62 (0.20)	77 (1.05)
0.1	57 (0.24)	72 (2.41)	82 (0.10)
0.5	94 (0.14)	97 (0.07)	97 (1.22)
1.0	97 (0.15)	99 (0.46)	98 (0.53)
2.0	99 (0.12)	99 (0.20)	99 (0.06)
3.0	99 (0.03)	99 (0.15)	99 (0.08)
TvL + AS			
0.05	32 (0.71)	73 (0.19)	80 (1.09)
0.1	35 (0.43)	72 (0.87)	77 (0.87)
0.5	60 (0.41)	74 (0.41)	77 (2.19)
1.0	60 (1.85)	76 (0.88)	82 (0.42)
2.0	83 (0.00)	88 (0.31)	91 (0.16)
3.0	84 (0.05)	88 (0.04)	94 (0.06)
TvL + SA			
0.05	53 (0.42)	71 (0.85)	80 (0.73)
0.1	49 (2.55)	70 (1.11)	75 (1.45)
0.5	49 (0.79)	67 (0.46)	75 (0.20)
1.0	56 (0.44)	68 (1.92)	79 (0.44)
2.0	68 (0.99)	80 (1.12)	88 (0.08)
3.0	70 (0.35)	84 (0.92)	88 (0.69)

[a] Activity measured by ABTS oxidation assay with absorbance measured at 420nm. % loss calculated from time zero controls
Parenthesis represents standard error of 3 replicates

The data from the stability study showed that the activity of TvL in the absence of a mediator decreased slowly (42% decrease in 20 hours) and reached a 75% reduction after 72 hours (Table 16). In the presence of 1-HBT, the decrease in laccase activity was greater than when TvL was incubated without a mediator, even at the lowest concentration (0.05mM). The same was observed during TvL incubation with violuric acid, with the exception of the lowest concentration of violuric acid (0.05mM), whereby the decrease in activity was similar to the enzyme without mediator. The greatest decrease in laccase activity was observed in just 20 hours with 2.0mM violuric acid (99%).

In the case of 1-HBT, the greatest decrease in activity (99%) occurred following 72 hours incubation of 2.0mM 1-HBT with TvL, supporting the claim that violuric acid deactivates laccase faster than 1-HBT [256]. Despite this observation, the degree of laccase inactivation by both mediators was generally similar and followed the same trend whereby inactivation was increased in accordance with increased mediator concentration. When the phenolic mediators AS and SA were incubated with TvL, the inactivity levels were initially lower/comparable to TvL without a mediator when lower concentrations were used (0.05-0.5mM). Higher mediator concentrations (1-3mM) were found to increase TvL deactivation, suggesting an inhibitory effect on laccase activity due to the phenols. The maximum percentage decrease in laccase activity in the presence of natural mediators was lower than with the synthetic mediators, with a maximum decrease of 94% (3mM AS), and 88% (2mM and 3mM SA) at 72 hours. This result was expected due to the likely event of laccase induced radical coupling of the oxidised phenols, which could eventually remove the reactive species and prevent their interaction with the enzyme, preventing further inactivation.

This effect should not take place with nitroxyl radicals, which are reported to continue to degrade laccase until they eventually form inactive species such as benzotriazole [257]. The mechanism of inactivation of laccase by nitroxyl radicals is not thoroughly understood, but is thought to involve a reaction between the radical species and the aromatic amino acid residues in the laccase active site. A disappearance of laccase tyrosine and tryptophan residues has been reported following laccase incubation with 1-HBT [258]. In the same study, an increase in molecular weight of the enzyme was reported, suggesting possible bonding between the free radicals and the amino acid residues within the enzyme. For LMS optimisation, site-directed mutagenesis could potentially be explored to improve laccase stability. Furthermore, laccase stability could be improved by the addition of other laccase substrates, as demonstrated by the addition of PR to laccase inactivated by violuric acid [256] and the addition of unbleached pulp to laccase/1-HBT and laccase/*p*-coumaric acid systems [259].

3.3 Conclusions

By investigating the reactivity of a range of phenolic compounds with TvL against a dye substrate and a lignin model compound, there is evidence to suggest that phenolic compounds are unlikely to act as good mediators in lignin degradation studies. This is most likely due to their instabilities in the catalytic cycle when used with substrates that are not oxidised rapidly such as veratryl alcohol, which is a simpler structure than a lignin polymer. The fast decolourisation reaction by many of the phenolic mediators screened demonstrates a potential usage for these laccase mediator systems in the dye decolourisation/detoxification industry.

The synthetic mediators 1-HBT and violuric acid were successful in both the decolourisation of RB-5 and for the oxidation of veratryl alcohol (in which ABTS also proved to be suitable). 1-HBT was overall (with TvL), the most successful synthetic mediator against veratryl alcohol and is a promising mediator to be explored in studies with real lignocellulosic substrates. The ability of oxidised phenolic compounds to act as effective radical mediators should not be excluded on the basis of the model compound studies, however, due to differences in the reaction environments between a lignin model compound reaction and a reaction with a natural substrate. Further work with natural substrates should be performed before full conclusions can be drawn.

Chapter four

4.0 Investigating the effect of laccase and laccase mediator systems on the enzymatic saccharification of cellulose and lignocellulosic substrates

4.1 Introduction

The conversion of biomass into bioethanol requires the successful breakdown or solubilisation of the sugar containing polymers hemicellulose and cellulose within lignocellulose (saccharification). The breakdown or solubilisation of these components yields the valuable C-5 or C-6 monosaccharide substrates that can be converted into ethanol during fermentation. It is the saccharification stage of biomass processing that is the most challenging due to the recalcitrant structure of lignocellulose as a result of the presence and interaction of the non-sugar polymer lignin with cellulose and hemicellulose. Lignin significantly contributes to the recalcitrance associated with lignocellulosic hydrolysis by mechanisms discussed in Chapter 1. The conversion of native, recalcitrant lignocellulose into a form that can be effectively hydrolysed is achieved by the application of a pretreatment method.

Pretreatment strategies are designed to increase the liberation of monosaccharides from cellulose and hemicellulose by altering the structure of the substrate or by the separation or removal of one or all three lignocellulosic components. Different pretreatment methodologies have been developed which affect the biomass substrate in a variety of ways to improve saccharification. Mechanical and physical pretreatments, which include ball milling and chipping focus on decreasing particle size to increase surface area and to reduce substrate crystallinity. Chemical and physicochemical methods have been introduced such as steam explosion and (dilute) acid hydrolysis, which aim to achieve hemicellulose solubilisation. Alternatively, chemical and physicochemical methods may utilise alkalis, peroxides or organic solvents to successfully remove or alter lignin structures (the different types of pretreatment were discussed in Chapter 1 Section 1.4.1.1).

Unfortunately, despite the success of the pretreatment strategies mentioned, there are disadvantages with their use including harsh operating conditions (as a result of the high pressures and temperatures required), the corrosive and often toxic nature of the catalysts employed (e.g. sulfuric acid or ammonia), a lack of selectivity and high associated costs (operational/equipment/expensive solvents). It is unlikely that one ‘ideal’ pretreatment option exists for all lignocellulosic substrates. Due to the pretreatment stage being one of the most expensive during the processing of biomass

to bioethanol, there is a pressing need to reduce the costs associated with improving lignocellulosic saccharification.

Employing biological catalysts (enzymes) in place of traditional chemical methods for many industrial processes can offer economic and environmental advantages. These advantages result from the milder operating conditions that can often be achieved with enzymes and the replacement of toxic chemicals and solvents with aqueous buffered systems. Use of biological pretreatment methods as an alternative strategy to existing chemical methods for the pretreatment of biomass is therefore biotechnologically favourable. Biological pretreatment methods most commonly utilise white rot fungi and the suite of ligninolytic enzymes that are secreted during their growth, which can degrade lignin. Disadvantages of biological pretreatments with fungi include the long incubation periods required to support the successful fungal growth and secretion of active enzymes (typically 10-14 days), the complex growth conditions and space required for large scale growth, and the lack of selectivity offered due to the ability of some white rot fungal species to attack both hemicellulose and cellulose, which may or may not be desired [260]. Despite these disadvantages, the economic and environmental benefits offered by biological routes are incredibly attractive in order to establish a highly desired, more eco-friendly biorefinery concept. Biological pretreatments currently offer a plethora of enzymatic pretreatment options that are worth investigating alone, or in combination with pre-existing technologies.

Of the panel of enzymes secreted by fungi that are widely believed to be involved in lignin degradation within natural systems (lignin peroxidase, manganese peroxidase, laccase, versatile peroxidase), laccases have attracted biotechnological interest due to their catalytic dependence on molecular oxygen as opposed to hydrogen peroxide. This dependence results in the higher stability of most laccases compared to peroxidases which suffer from a potential deactivation by hydrogen peroxide [124]. Researchers have recently started exploring the potential of applying ligninolytic enzymes to biomass as a biological pretreatment method, with the majority of studies focusing on the solid state cultivation of fungi with the selected biomass substrate [261-266]. Although promising results have been reported, in addition to the previously discussed lengthy incubation periods involved it is also difficult to establish which enzymes and mechanisms are responsible for the observed improved saccharification, making optimisation a challenge. Delignification and biobleaching

studies using purified and concentrated preparations of the ligninolytic enzymes manganese peroxide [224,267-272] and lignin peroxidase [122,201,273-276] have been reported. However in recent years, research has increasingly become more focused on establishing the effect of applying laccase preparations with lignocellulosic substrates (in combination with existing pretreatment strategies). Some of these studies are presented in Table 17.

Table 17: Recent studies using laccase treatments with lignocellulosic substrates and the overall effect on saccharification

Substrate	Laccase/ mediator	Other pretreatments	Outcome	Reference
Spruce wood	*Trametes hirsute* +/- mediators NHA* and NHA-Ac**	Steam explosion	Sequential and simultaneous laccase treatments +/- mediator enhanced enzymatic hydrolysis and polysaccharide conversion	[179]
Wheat straw	*Pycnoporus cinnabarinus*	Acid pretreatment, steam explosion	Simultaneous laccase and 1-HBT treatment impaired enzymatic hydrolysis	[177]
Wheat straw	*Trametes villosa Coriolopsis rigida*	Steam explosion	Sequential laccase treatments impaired sugar recovery following enzymatic hydrolysis	[79]
Spruce wood and giant reed	*Coriolopsis unicolor*	Steam explosion	Sequential laccase treatment found to improve enzymatic hydrolysis of spruce wood but impair hydrolysis of giant reed	[99]
Eucalyptus wood and elephant grass	*Trametes villosa* +/- 1-HBT	Alkaline-peroxide extraction	Sequential laccase treatments with and without 1-HBT and alkaline peroxide extractions improved enzymatic hydrolysis for both substrates	[180]
Corn stover	*Trametes versicolor* + 1-HBT	Ensilage	Sequential laccase treatments with 1-HBT following ensilage increased cellulose conversion	[277]
Wheat straw	*Sclerotium* sp	Steam explosion	Sequential and simultaneous laccase treatments improved enzymatic hydrolysis	[178]
Wheat straw	*Pycnoporous cinnabarinus* and *Trametes villosa*	Steam explosion	Laccase treatment before hydrolysis impaired enzymatic hydrolysis but improved ethanol yield via fermentation after hydrolysis	[278]
Eucalypt wood	*Mycliophthora thermophila* + methyl syringate	Alkaline-peroxide extraction	Up to 40% improvement in saccharification following enzymatic treatments	[182]

* NHA: *N*-hydroxy-*N*-phenylacetamide; ** NHA-Ac: *N*-acetoxy-*N*-phenylacetamide

The existing research as outlined in Table 17 covers a range of lignocellulosic substrates and fungal laccases, however research exploring the additional effect of laccase mediators is limited. Furthermore, the results from the literature are contradictory, with some studies reporting an enhancement of hydrolysis by laccase [178-180,182,277], whilst others report the impairment of hydrolysis [79,177,278] or mixed effects depending on the substrate investigated [99].

Due to the predominance of non-phenolic structures within lignin, a successful laccase-mediator system (LMS) has the potential to initiate further oxidative reactivity than just a laccase alone. The current shortage of research into the effects of an LMS towards lignocellulosic substrates offers an opportunity to investigate the incorporation of potential synthetic and natural mediators into a laccase mediated bioprocess.

In this study the effect of *Trametes versicolor* laccase (TvL) alone and as part of an LMS was investigated with respect to the saccharification of pure cellulose and the agricultural residue wheat straw (*Triticum aestivum*). Potential mediators of synthetic and natural origin were selected following previous investigations with TvL as described in Chapter 3. The improvement in saccharification was, for the main part, examined using TvL with and without redox mediators as a biological pretreatment in combination with several existing pretreatment methods. This approach was taken as the majority of lignocellulosic substrates were supplied in their dilute acid pretreated form (as slurries). Due to pre-removal of hemicellulose, the focus of this work was the improvement of saccharification with respect to cellulose. The study was expanded by determining the applicability of the laccase/LMS treatments towards two other agricultural residue substrates, the stovers of corn (*Zea mays*, also known as maize) and sorghum (*Sorghum bicolor*) (stover referring to the leaves and stalks that are left over when the grain is harvested). Furthermore, 3 additional laccases were assessed, and methods were developed for the identification and quantification of cellulose and hemicellulose derived sugars.

4.2 Results & discussion

4.2.1 GC220 cellulase

The cellulase preparation GC220 (Genencor) was used in all hydrolysis reactions. GC220 represents a cocktail of endogluconases, exoglucanases and β-glucosidases from *Trichoderma* sp. The protein concentration of the GC220 cellulase mix from Genencor was determined by the BCA method [279]. Enzyme dilution 1:10 in distilled water and buffer exchange using a PD10 column removed any stabilisers and other non-protein components present in the preparation to allow accurate quantification. The preparation had a measured concentration of 196mg/mL. For initial hydrolysis reactions with pure cellulosic substrate Avicel, GC220 was investigated at 5, 10 and 15mg/g/cellulose. The following calculation was used to determine the amount of GC220 to add to each reaction (Equation 2)**.**

$$Enzyme\ volume\ (mL) = \frac{Substrate(g)}{Enzyme(mg/mL)}\ x\ Enzyme\ amount\ (mg/g\ substrate)$$

Equation 2: Calculation of the volume of enzyme for use in the cellulose hydrolysis reaction.

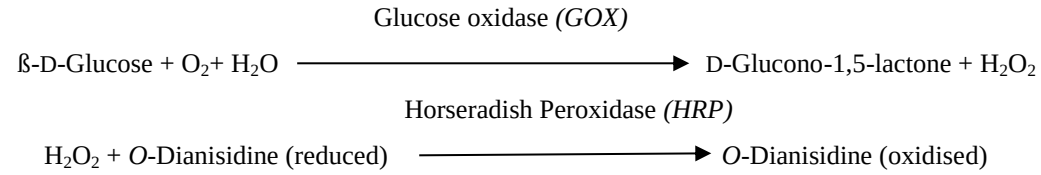
The accurate determination of cellulose concentration within natural lignocellulosic substrates can be both complex and time consuming, making the expression of cellulose concentration in mg/g cellulose difficult. To overcome this, the concentration of GC220 was additionally measured in terms of the filter paper unit (FPU) according to the method by Ghose [280]. The concentration of GC220 was determined to be 69FPU/mL (Appendix Figure 77, 78 and Equation 3) and a concentration of GC220 in FPU was used in all experiments with lignocellulosic substrates as stated. The consistency of adding the same concentration of GC220 across all lignocellulosic samples within an experiment is critical for result comparison.

4.2.2 Development of glucose analysis methods

4.2.2.1 GOX colorimetric assay

A spectrophotometric assay was developed using glucose oxidase (GOX) from *Aspergillus niger* for the quantification of glucose released from cellulosic substrates. GOX catalyses the oxidation of glucose to D-glucono-1,5-lactone and hydrogen peroxide as presented in Scheme 15. This reaction is linked to the decomposition of

hydrogen peroxide with HRP and the subsequent oxidation of the chromogenic substrate *o*-dianisidine. Oxidised *o*-dianisidine is amber in colour and its production can be monitored by measuring the change in absorbance at 500nm.



Scheme 15: Reaction scheme for the glucose oxidase reaction

Due to the high sensitivity of the assay, glucose standards of low concentration (0-1mM) were used to establish a standard curve. The absorbance values at 500nm following 120 seconds incubation from the reaction were plotted against concentration (Figure 38).

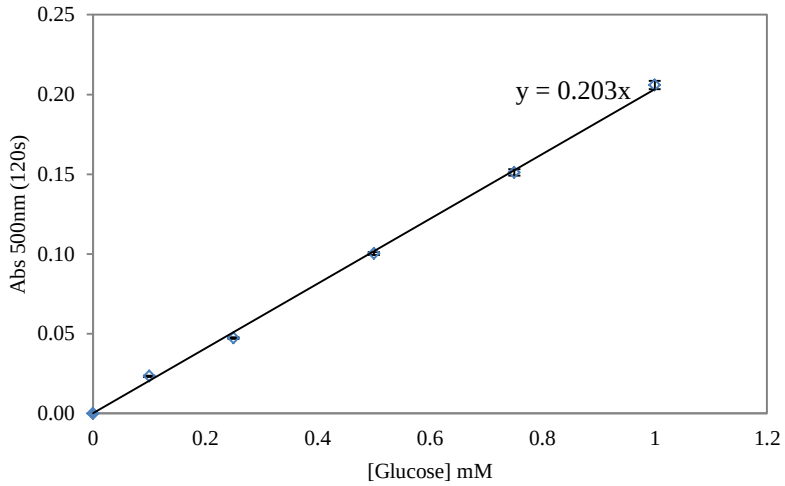


Figure 38: Standard curve generated from the mean absorbance values recorded during the oxidation of glucose standards (0-1mM) by glucose oxidase (GOX). Error bars represent the standard error of the mean of 3 replicates.

To test the applicability of the assay, hydrolysis experiments with 0.2g Avicel and 5, 15 and 30mg/g/cellulose GC220 were set up. The GOX assay allowed for the quantification of the released glucose as presented in Table 18.

Table 18: Released glucose concentrations from Avicel hydrolysis using different concentrations of GC220 as determined by the GOX method.

	Enzyme concentration (mg/g/cellulose)		
	5mg/g GC220	15mg/g GC220	30mg/g GC220
[Glucose][a] mM	49 (2.60)	54 (2.67)	76 (0.01)
[Glucose][a] g/L[b]	8.82	9.72	13.68

[a] Hydrolysis conditions: 50°C, pH 5.0, 200rpm, 144h. Parentheses represent standard error of 3 biological replicates [b] Conversion of average glucose concentration in mM to g/L.

Problems associated with the system included the pre-requirement of high sample dilution (up to x20) prior to analysis due to the high concentrations of glucose liberated during hydrolysis. The multi-step transfer of both the enzyme and sample mix during the assay already increases sampling errors and encourages sample variability. Furthermore, the carcinogenic nature of *o*-dianisidine made the assay less favourable for frequent high throughput analysis of high sample numbers.

During process development, it was likely that laccase may be present within saccharification reactions or that traces of laccase activity could be carried over. To investigate the effect of laccase carry-over within the GOX assay, TvL was added to GOX reactions with glucose standards (0-2mM) at three concentrations (0.01, 0.1 and 1U/mL). Comparison of the standard curves produced under each set of conditions revealed that all three concentrations of laccase interfered with the GOX assay due to the ability of laccase (in addition to HRP) to oxidise *o*-dianisidine (Figure 39). In the presence of 1U/mL TvL, the oxidation of *o*-dianisidine occurred so rapidly that the absorbance increase could not be measured under the reaction conditions and after 120 seconds the absorbance values significantly decreased and lower values were recorded. Due to the much slower oxidation of *o*-dianisidine with 0.1U/mL and 0.01U/mL TvL, the increase in absorbance could successfully be measured however the absorbance values were slightly higher than those measured when no TvL was present due to the additional oxidant.

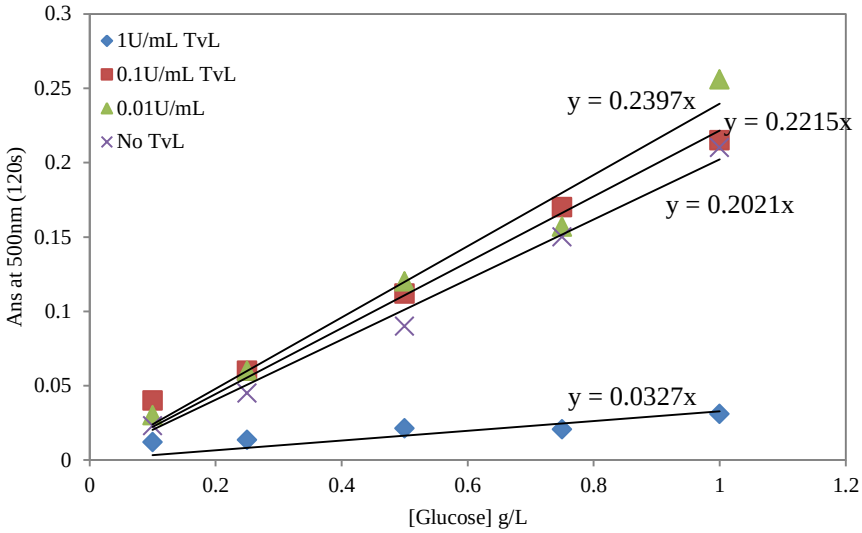


Figure 39: Standard curves generated by the oxidation of glucose standards (0-1mM) by glucose oxidase (GOX) in the presence of 3 laccase concentrations (0.01, 0.1 and 1U/mL) and a laccase-free negative control.

The data suggests that the GOX assay is unsuitable when high laccase concentrations are present. However even low concentrations present a risk of producing falsely elevated glucose concentration readings. This issue could be resolved by either the complete denaturation of laccases by heating, or by the thorough washing of substrates prior to hydrolysis. Due to the additional experimental errors and toxicity effects associated with this method, a second method of analysis, HPLC with refractive index detection, was investigated.

4.2.2.2 HPLC for glucose analysis

A method was developed using HPLC with Refractive Index (RI) detection for the analysis of sugars derived from biomass based on the published method by NREL [281]. Glucose standards (1-12g/L) were pre-diluted 1:10 with sulfuric acid (10mM) to reflect the same conditions of treatment required for the analytical samples (Figure 40).

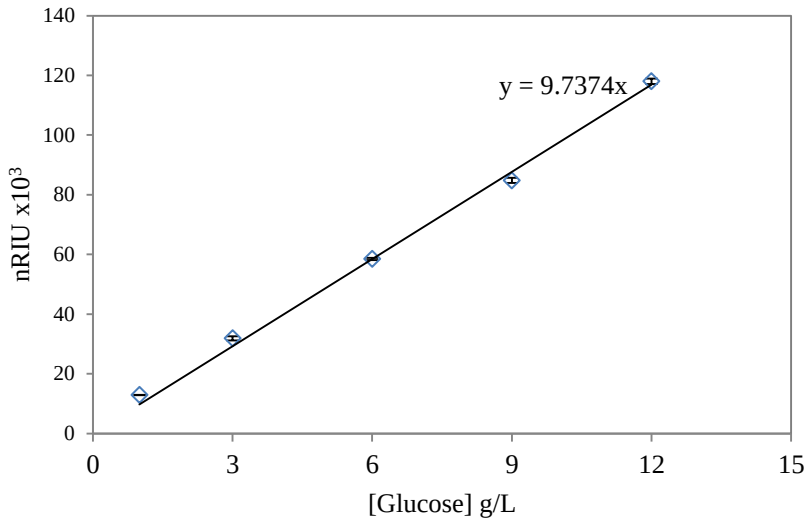


Figure 40: Standard curve generated by quantification of peak areas from glucose standards (1-12g/L) using HPLC with RI detection. Each data point represents the mean of 3 biological replicates and error bars represent the standard error of the replicates.

This method was selected for the analysis of glucose in hydrolysed cellulose and lignocellulosic substrates. The method was preferred over the spectrophotometric assay due to its reduced toxicity, reduced risk of sampling errors due to reduced multi- pipetting steps and the absence of a multi component enzyme reagent, which minimises the risk of interference by reaction components such as laccase or coloured oxidised products.

4.2.3 Laccase & LMS on cellulose saccharification

2% Avicel was incubated with 1.3U/mL TvL in the presence and absence of 1mM 1-HBT during enzymatic hydrolysis with GC220 (laccase and mediator concentration selected based upon the concentrations used in the veratryl alcohol assay which was on a similar scale). Control conditions included cellulose without both laccase and a mediator and cellulose incubated with denatured (by heat) TvL. At given time points during the reaction, samples were taken and quenched 1:10 with sulfuric acid (10mM) and analysed by HPLC with RI detection as described by NREL [281].

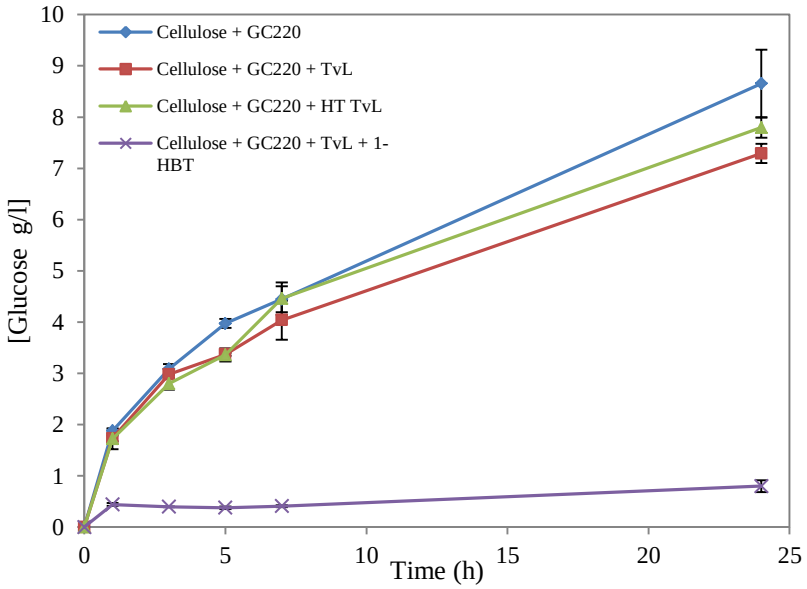


Figure 41: Glucose concentrations following the hydrolysis of 0.2g Avicel with GC220 only (blue), GC220 with 1.3U/mL TvL (red), GC220 with denatured TvL (green) and GC220 with 1.3U/mL TvL and 3mM 1-HBT (purple). 30mg/g/cellulose GC220 used in all conditions. Glucose concentration measured by HPLC-RID. Error bars represent standard error of 3 biological replicates.

The quantification of released glucose following hydrolysis over 24 hours revealed that the presence of the LMS is inhibitory to the saccharification of cellulose (Figure 41). The amount of glucose released following 24 hours hydrolysis in the presence of the LMS reached 0.8g/L which was >10x lower than the released glucose concentration in the LMS-free negative control (8.86g/L after 24 hours hydrolysis). The effect of TvL without the mediator in the hydrolysis reaction (7.29g/L glucose) appeared only slightly inhibitory compared to the LMS free control (8.86g/L glucose) and the denatured TvL control (7.79g/L glucose). If the action of laccase alone is inhibitory, then it would be expected that the glucose concentration released from the LMS free control and the denatured TvL control would be very similar. It is difficult to determine whether the difference observed between the two conditions (1.07g/L glucose after 24 hours hydrolysis) is insignificant due to the high variability between the replicates in the LMS free control following 24 hours hydrolysis. It is possible however that even the presence of denatured TvL could be inhibitory to the reaction to a small degree. This could be due to the inactive protein reducing the accessibility of cellulose towards GC220 by blocking potential binding sites on cellulose, or by participating in non-specific interactions with the cellulases, thus decreasing their efficiency. The differences in the glucose concentrations from each set of reaction

conditions appears to increase as the reaction time course progresses, suggesting that the inhibitory nature of the reaction environment due to TvL with and without 1-HBT increases as the reaction continues.

The hydrolysis experiment with cellulose was repeated to evaluate whether a second synthetic mediator, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), was also inhibitory to the reaction with TvL. The study was extended to investigate a panel of phenolic compounds previously investigated as natural mediators with veratryl alcohol (Chapter 3 Section 3.2.2). Glucose quantification following 44 hours hydrolysis revealed that the TvL + ABTS LMS was similarly as inhibitory as the TvL + 1-HBT LMS, releasing 0.53g/L and 0.47g/L glucose respectively compared to 9.91g/L in the LMS free control (Table 19). Again, the presence of TvL in the absence of a mediator caused only a small reduction in glucose yield compared to the control, confirming that its presence has a lower inhibitory action compared to the LMS with synthetic mediators. When phenolic compounds were included in the hydrolysis reaction with TvL, saccharification was inhibited to varying degrees (Table 19). The reaction between TvL and syringaldehyde (SA) appeared to induce the greatest inhibitory effect on the reaction (2.00g/L glucose released after 44 hours hydrolysis) in comparison with other phenolic compounds (4.28-9.66g/L glucose).

Table 19: Concentrations of glucose released following Avicel hydrolysis by 30mg/g/cellulose GC220 in the presence/absence of TvL with synthetic and natural mediators

Hydrolysis condition[a]	Glucose conc g/L[b]
Cellulose + GC220	9.91 (0.18)
Cellulose + GC220 + TvL	9.60 (0.36)
Cellulose + GC220 + TvL + 1-HBT	0.47 (0.08)
Cellulose + GC220 + TvL + ABTS	0.53 (0.03)
Cellulose + GC220 + TvL + VAN	5.48 (0.30)
Cellulose + GC220 + TvL + SA	2.00 (1.72)
Cellulose + GC220 + TvL + AV	6.90 (0.31)
Cellulose + GC220 + TvL + 4-HBAc	4.28 (0.70)
Cellulose + GC220 + TvL + PC	8.49 (0.79)
Cellulose + GC220 + TvL + 4-HBAld	9.66 (0.51)

[a] Reaction conditions 0.2g Avicel, 1.3U/mL TvL, 3mM mediator, pH 5.0, 30mg/g/cellulose GC220, 44h [b] determined by HPLC with RI detection. VAN: Vanillin, SA: Syringaldehyde, AV: Acetovanillone, 4-HBAc: 4-Hydroxybenzoic acid, PC: *p*-coumaric acid, 4-HBAld: 4-Hydroxybenzaldehyde. Parentheses represent standard error of 3 biological replicates.

Interestingly, SA was found to be one of the best phenolic mediators when reacted with TvL during the decolourisation of Reactive Black-5 (RB-5, Chapter 3, Section 3.2.1), most likely due to its lower redox potential and the increased stability of its

phenoxyl radical. The results with 1-HBT and ABTS suggest that the inhibition of saccharification by GC220 is due to the generation of oxidised mediator radical species. Regarding the results with the phenolic compounds, it is logical to assume that the more stable the reactive species produced following laccase oxidation, the greater the inhibitory effect towards the cellulases. This can be demonstrated by the order of hydrolysis inhibition reported in Table 19 (SA > 4HBAc > VAN > AV > PC > 4-HBAld) which is similar to the ability of the compounds to act as effective mediators for RB-5 decolourisation (SA > VAN > 4-HBAc > AV = PC > 4-HBAld). The inhibition of cellulose hydrolysis by any laccase generated phenoxyl radicals appeared lower than the inhibition observed with oxidised ABTS and 1-HBT. This is likely to be due to the occurrence of coupling reactions between the phenoxyl radicals generated, leading to the production of dimers and polymers that cannot be further oxidised by laccase as described in Chapter 3.

4.2.4 Cellulase inhibition studies by LMS

Inhibition studies were set up to investigate the activity of the cellulolytic enzymes that are included within the GC220 enzyme mix to determine which enzyme(s) are affected by the oxidised mediator species and to what degree. *p*-nitrophenyl-β-D-cellobioside (*p*-NPC) (Figure 42) is a synthetic substrate that is used in assay procedures to estimate exoglucanase activity. It has been used ever since it was found to be hydrolysed by a β-glucosidase-free preparation of *Irpex* cellulose [282]. It can be used in a semi-quantitative manner to look at exo- and endoglucanases as they work in synergy. Exoglucanases such as cellobiohydrolase (CBH), act by removing cellobiose from the non-reducing end of cellulose. Towards *p*-NPC, exoglucanases will hydrolyse the agluconic bond (the linkage between the *p*-nitrophenol and the disaccharide moiety, Figure 42) to release *p*-nitrophenol (*p*-NP). The addition of anhydrous sodium carbonate induces a colour change from colourless/pale yellow to bright yellow. Measurement of agluconic hydrolytic activity can be established by measuring the absorbance of this bright yellow compound at 410nm. Endoglucanases and β-glucosidases also act on the agluconic bond of *p*-NPC, as well displaying activity for the holosidic bond (the linkage between the two glucose molecules of cellobiose, Figure 42), however the release of *p*-NP is a measure of agluconic bond cleavage only.

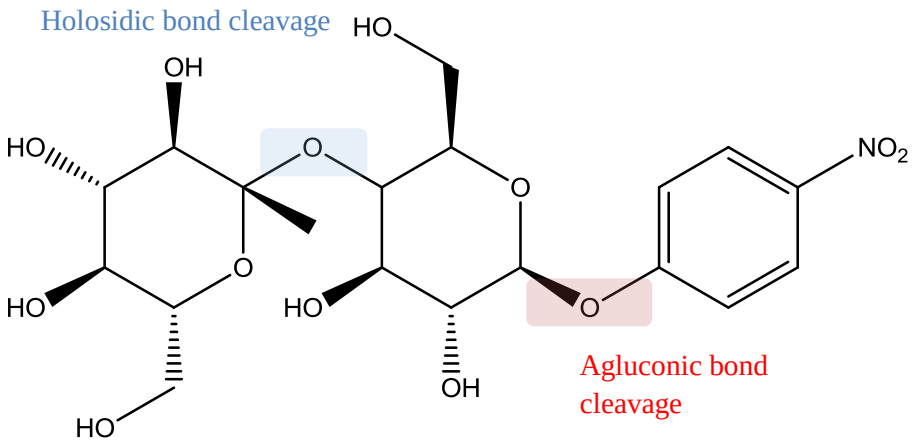


Figure 42: The structure of *p*-nitro-β-D-cellobioside (*p*-NPC). The area shaded red indicates where agluconic bond cleavage occurs, whilst the blue area highlights where holosidic bond cleavage occurs.

D-glucono-1,5-δ-lactone (gluconolactone) is a known inhibitor of β-glucosidases and the addition of this compound to the *p*-NPC assay at a concentration of 0.1-0.5mg/mL is reported to be satisfactory for the inhibition of β-glucosidase activity without strongly inhibiting exo- and endoglucanases [283-285]. Deshpande *et al*. [283] reported up to 20% inhibition of agluconic bond cleavage by 3 purified exoglucanases with gluconolactone at concentrations >0.4mg/mL. Gluconolactone at a low concentration is therefore considered a useful additive for this assay especially in the assessment of hydrolytic activity in crude and mixed cellulase preparations that might have high β-glucosidase content. As mentioned previously, endoglucanases also hydrolyse agluconic bonds therefore the data collected herein reflects the combined activities of both the exo- and endoglucanases present in the GC220 preparation. Experimentation with purified exo- and/or endoglucanases would overcome this issue however in this work, purified enzymes were not readily available. Experimentation omitting D-glucono-1,5-δ-lactone from the reaction allowed for the assessment of 'total agluconic activity'.

Cellulose hydrolysis assays were set up with TvL and the inhibitory mediators ABTS and 1-HBT as described earlier. Aliquots of the hydrolysis reactions were taken at 0 and 24 hours and centrifuged to remove any insoluble cellulose. The supernatants were assayed against *p*-NPC for determination of enzyme activity.

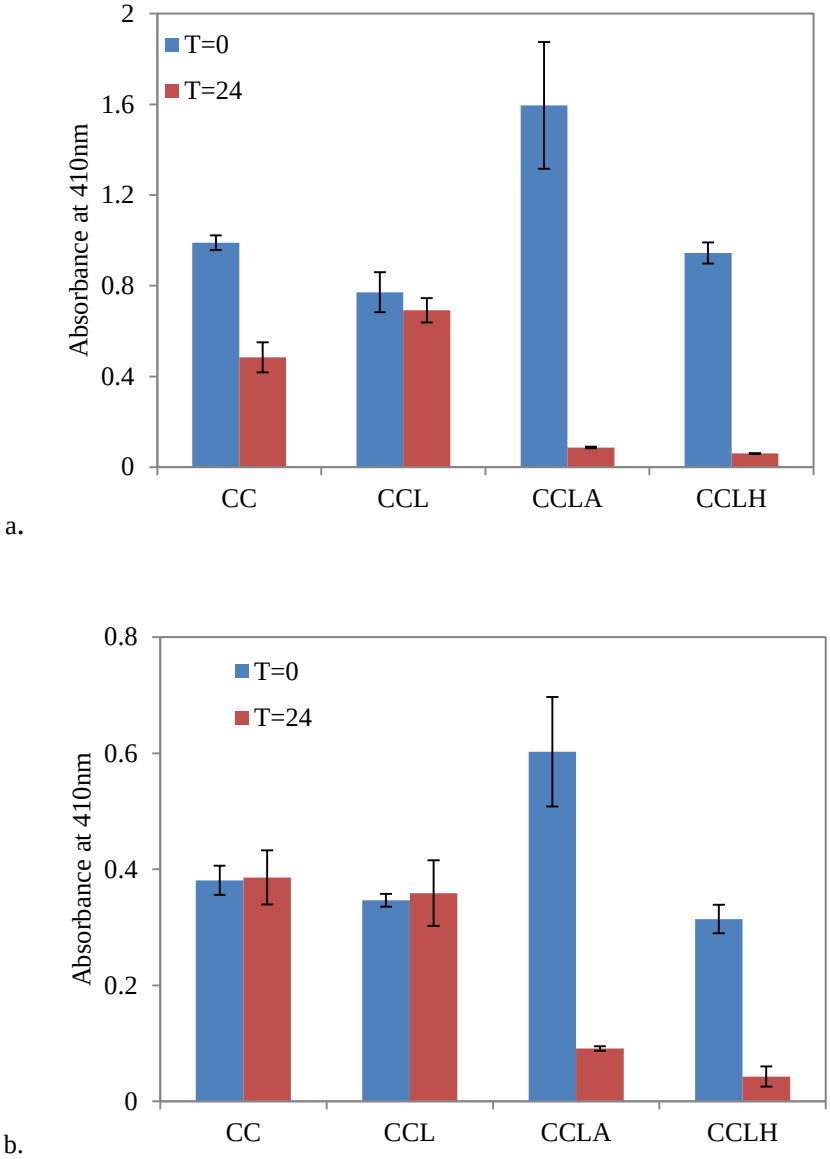


Figure 43: Absorbance values represent the release of *p*-NP from *p*-NPC hydrolysis by cellulolytic enzymes within GC220 preparations taken from Avicel hydrolysis reactions after 0h and 24h under the following conditions CC: Cellulose + GC220, CCL: Cellulose + GC220 + TvL, CCLA: Cellulose + GC220 + TvL + ABTS, CCLH: Cellulose + GC220 + TvL + 1-HBT. 30mg/g/cellulose GC220 and 1.3U/mL and 3mM mediator present in all Avicel hydrolysis reactions. a: No gluconolactone added therefore data represents 'total agluconic bond cleavage' b: Results with 0.4mg/mL gluconolactone therefore representing agluconic bond cleavage by endo- and exoglucanases only. Error bars represent standard error of 3 biological replicates.

When gluconolactone was omitted from the reaction (Figure 43a), the absorbance increase (released *p*-NP) represented agluconic bond cleavage by the combined action of all three types of cellulases. In all four hydrolysis conditions tested (CC: Cellulose plus GC220, CCL: Cellulose plus GC220 and TvL, CCLA: Cellulose plus GC220, TvL and ABTS, CCLH: Cellulose plus GC220, TvL and 1-HBT), the absorbance

values are seen to decrease between 0 and 24 hours hydrolysis indicating a reduction in released *p*-NP and hence the total hydrolytic activity of GC220. The decrease in total hydrolytic activity was much greater in the conditions with both LMS (CCLA and CCLH), providing evidence towards the inhibitory action of cellulase activity in general as a result of the oxidised mediators as previously suspected. The activity decrease observed in the CC and CCL reactions is most likely due to a natural loss of cellulase activity over time.

The same experiment with the addition of 0.4mg/mL gluconolactone in theory reveals the agluconic bond cleavage activities of just the endo- and exoglucanases within GC220 (Figure 43b). In this experiment the absorbance values are much lower than those recorded in the absence of gluconolactone demonstrating that a degree of hydrolytic activity towards *p*-NPC has been inhibited which is believed to be that of β-glucosidase. The absorbance change and hence activity change observed between 0 and 24 hours was negligible in the CC and CCL hydrolysis reactions. In the case of CCLA and CCLH there was a noticeable decrease in the absorbance recorded after 24 hours, suggesting an inactivation of exoglucanase and/or endoglucanase activity. The results of this study further support an inhibitory role of oxidised synthetic mediators towards exo- and endoglucanases.

A second colorimetric substrate, *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) (Figure 44), was used to determine the effect of TvL and both LMS on β-glucosidase activity within GC220. The action of β-glucosidase is to cleave the β-1,4-glycosidic bond of cellobiose to produce two glucose monomers in the final stage of cellulose hydrolysis. A defect in this enzymatic reaction can lead to the accumulation of cellobiose which can in turn inhibit both exoglucanase and endoglucanase activity. β-glucosidase activity is therefore critical for the reaction rate of cellulose hydrolysis, and to ensure the efficient regulation of the exo- and endoglucanase activities [286,287]. The enzyme is active on a range of additional synthetic chromogenic substrates such as *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl -β-D-1,4-glucopyranoside, β-naphthyl-β-D-glucopyranoside, 6-bromo-2-naphthyl-β-D-glucopyranoside, and 4-methylumbelliferyl-β-D-glucopyranoside [288], however *p*-NPG was selected due to reports claiming maximal activity of β-glucosidase towards this substrate [286].

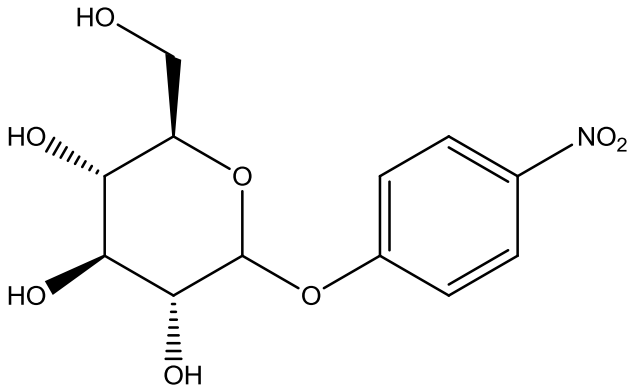


Figure 44: Structure of *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG)

As with *p*-NPC, hydrolysis of *p*-NPG releases *p*-NP which is detected by production of the bright yellow compound on the addition of sodium carbonate. This colour change can be monitored by measuring absorbance at 430nm.

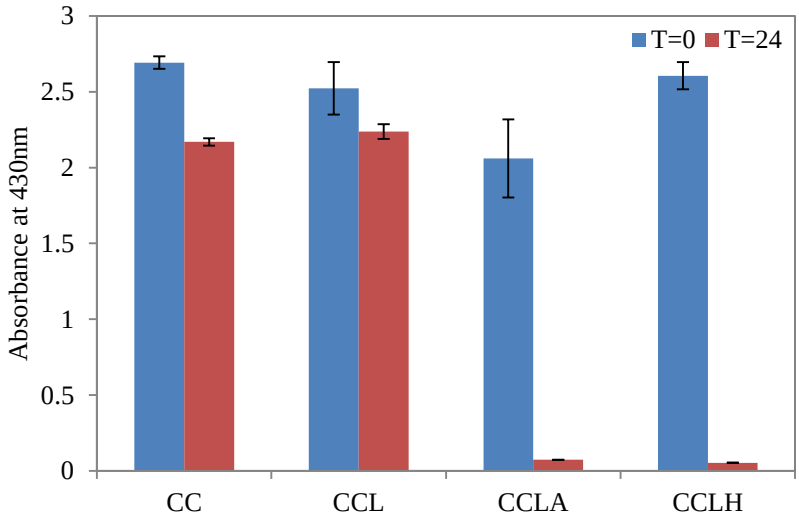


Figure 45: Absorbance values represent the release of *p*-NP from *p*-NPG hydrolysis by β-glucosidase within GC220 preparations taken from the hydrolysis reactions of Avicel cellulose at 0h and 24h under the following conditions; CC: Cellulose + GC220, CCL: Cellulose + GC220 + TvL, CCLA: Cellulose + GC220 + TvL + ABTS, CCLH: Cellulose + GC220 + TvL + 1-HBT. 30mg/g/cellulose GC220 and 1.3U/mL and 3mM mediator present in all Avicel hydrolysis reactions. Error bars represent standard error of 3 biological replicates.

The results from the *p*-NPG hydrolysis assay show a small decrease in β-glucosidase activity after 24 hours in the CC and CCL control reactions, as indicated by the relatively small absorbance decreases at 430nm (Figure 45). The absorbance decreases from 2.06 to 0.07 in the reaction with CCLA and from 2.6 to 0.05 with

CCLH indicate a high loss of β-glucosidase activity compared to the CC and CCL controls. The results provide evidence for the inhibitory action of the oxidised mediators towards the β-glucosidase enzymes within GC220 and explain why very low levels of glucose were measured in the previous hydrolysis reactions with both LMS. Additional studies using ABTS and 1-HBT in the absence of laccase oxidation revealed that the presence of unoxidised mediators did not affect hydrolytic activity (data not shown).

The panel of natural mediators used in the cellulose hydrolysis experiment with Avicel were also screened against *p*-NPG and *p*-NPC with TvL (Appendix Figures 79-80). The *p*-NPC hydrolysis assay with gluconolactone revealed that SA, AV, 4-HBAc and PC inhibited exo- and endoglucanase activity to a similar degree as 1-HBT and ABTS. The inhibitory effect of 4-HBAld was lower which correlated with a lower degree of hydrolysis inhibition observed with this mediator towards Avicel hydrolysis (Table 19). On the contrary, the *p*-NPG assay for β-glucosidase activity revealed that none of the oxidised phenolic compounds with the exception of SA and PC caused a reduction in the hydrolytic activity of β-glucosidase. This reduced β-glucosidase inhibition explains why cellulose hydrolysis in the presence of these compounds was not affected to the same degree as when 1-HBT and ABTS were included. Oxidised SA was found to inhibit β-glucosidase activity to the same extent as oxidised ABTS and 1-HBT. To summarise, the three mediators with the greatest inhibition of β-glucosidase activity (SA, ABTS, and 1-HBT) were those found to inhibit cellulose hydrolysis the most, confirming the importance of β-glucosidase in the effective release of glucose from cellulosic substrates.

4.2.5 The effect of laccase & LMS on the lignocellulosic substrate wheat straw

4.2.5.1 Total phenol assay

The ability of laccases to act as phenol oxidases has been observed amongst many phenolic substrates such as guaiacol, catechol and 2,6-dimethoxyphenol [127]. The generation of phenoxyl radicals by laccase and the subsequent polymerisation into high molecular mass products creates a potential role for laccase in the removal of phenolic compounds generated from pretreatment methods (such as steam explosion and acid pretreatment), which can be inhibitory to downstream fermentations. The ability of TvL to remove phenols within lignocellulosic substrates was investigated using acid pretreated wheat straw (received from NREL; for acid hydrolysis protocol

see Chapter 7 Section 7.21) that was either washed (WWS: washed wheat straw) or unwashed (WSS: wheat straw slurry).

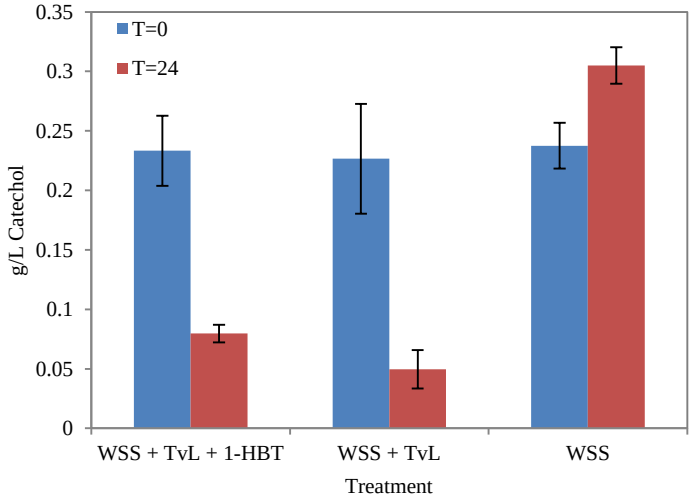


Figure 46: Total phenol concentration (expressed as g/L catechol) measured in the soluble fractions of reactions with 0.5g (d.w) wheat straw slurry (WSS) with or without 10U/g TvL and 5mM 1-HBT. Phenol assay performed following 0h and 24h incubation using the Folin ciocalteau assay [289]. Error bars represent standard error of 3 replicates.

The folin-ciocalteau assay [289] revealed that phenol concentration (expressed as g/L catechol), was similar in all three experimental conditions at 0 hour within the unwashed substrate (WSS) (Figure 46). Following the 24 hour incubation of wheat straw with both laccase and the LMS (WSS + TvL and WSS + TvL + 1-HBT), a decrease in the overall phenol concentration was observed supporting the theory of laccase catalysed oxidation and polymerisation of free phenols in the biomass slurry. In the control reaction without any enzymatic pretreatment (WSS), the free phenol content increased after 24 hours. This was not expected and suggests that a small amount of phenol was released into the soluble fraction under non-enzymatic conditions at 30°C. The decrease in phenol concentration observed from the laccase treated samples was greater when the mediator 1-HBT was absent (78% decrease in WSS + TvL and 65% decrease in WWS + TvL + 1-HBT) and this is likely to be due to 1-HBT acting as a competitor towards the active site of laccase, decreasing the interactions between laccase and free phenols. Further experiments investigating the effect of laccase dosage using 0.8-100U/g TvL (units of TvL per gram dry weight of washed wheat straw) revealed that detoxification occurred at a greater rate when higher laccase concentrations were used (phenol removal could be detected as early as

2 hours). However, the overall degree of phenol removal was essentially the same at all concentrations after 22 hours. This suggests that lower concentrations of TvL are suitable for this reaction (Appendix, Figure 81).

When the same experiment was performed using washed wheat straw (WWS), lower starting concentrations of phenol were measured compared to the slurry (WSS) due to the removal of most of the inhibitory free phenols during the prior wash step. The very low phenol concentrations made the data more difficult to interpret however a similar trend was still observed when data from the slurries (WSS) was compared. An increase in free phenol was detected in the control reaction under no enzymatic conditions (WWS), consistent with the results observed with the unwashed substrate. An increase in free phenol was also observed following the incubation with laccase and 1-HBT (WWS + TvL + 1-HBT), but to a much smaller degree, suggesting laccase removal of free phenols that may be released from the material (Figure 47). When laccase was incubated with the washed wheat straw without 1-HBT (WSS + TvL), a decrease in the concentration of free phenol after 24 hours was observed.

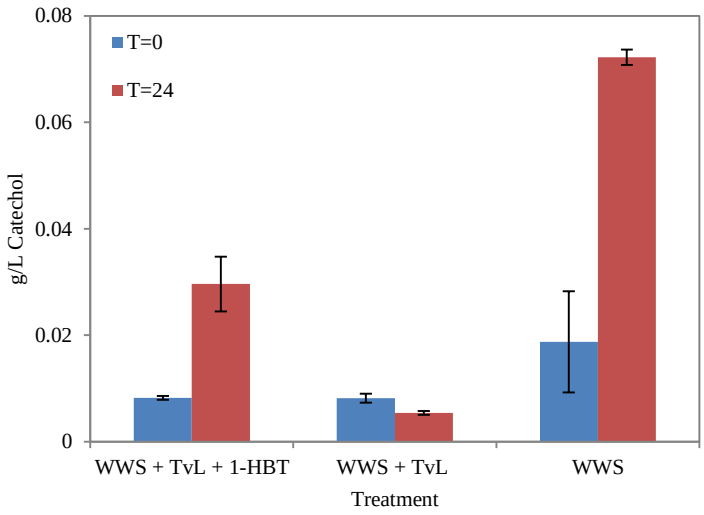


Figure 47: Total phenol concentration (expressed as g/L catechol) in the soluble fractions of reactions with 0.5g (d.w) washed wheat straw (WWS) with or without 10U/g TvL and 5mM 1-HBT. Phenol assay performed following 0h and 24h incubation using the Folin ciocalteau assay. Error bars represent standard error of 3 replicates.

The combined results from both WSS and WWS substrates provide evidence towards the laccase-based removal of free phenols from acid pretreated wheat straw. This free phenolic polymerisation activity of laccase could be applied towards the removal of

inhibitory phenols that can be liberated from lignin following acid pretreatment of biomass. These inhibitory phenols largely include the acid, aldehyde and ketone form of the phenolic compound (e.g. syringic acid, syringaldehyde and acetosyringone). Other commonly detected phenols in acid hydrolysates include vanillin, vanillic acid, acetovanillone, 4-hydroxybenzoic acid, guaiacol and ferulic acid [290]. Following acid pretreatment, phenolic compounds have been detected in the hydrolysates of sugarcane bagasse (2.75g/L) [291], corn stover (0.08g/L) [292], spruce (0.44g/L) [293], *Eucalyptus globules* (2.23g/L) [294], and *Saccharum spontaneum* (2.01g/L) [295]. The presence of these phenolic compounds in the downstream fermentation process is inhibitory due to the toxicity of the compounds towards fermenting microorganisms. Small concentrations of these inhibitors have been found to destroy the integrity of the yeast membrane systems preventing growth and sugar assimilation [88,90]. Laccase treatment could be considered as an alternative method to costly water washing steps for the removal of these inhibitory phenols by enabling the oxidative coupling and polymerisation reactions that lead to their removal.

Washed wheat straw was used in all further investigations due to the success of this additional step in the removal of free inhibitory phenols and the neutralisation of the previously acidified slurry. The aim of the remaining studies was to investigate the action of laccase and LMS on the non-phenolic portion of lignin for saccharification purposes therefore the application of laccase towards inhibitory compound removal was not further explored.

4.2.5.2 Hydrolysis of TvL and LMS treated wheat straw

Following the investigation into the oxidative reactivity of TvL towards lignocellulosic derived phenols, the actions of an LMS towards the non-phenolic portion of lignin (~90% of lignin polymer) was explored. The analysis of the effect an LMS exhibits towards the subsequent saccharification of lignocellulosic material can provide an indirect method for the determination of the potential actions of laccase and the LMS. The hydrolysis of cellulose could in theory be improved by the oxidation, modification or cleavage of lignin that can potentially be achieved by laccase or an LMS. These reactions would result in the removal or disruption of some of the lignin that encases cellulose, thus increasing the accessibility of cellulose towards cellulases. Increased accessibility of the substrate would in turn result in an

improved saccharification reaction indicated by an increase in released glucose when compared to lignocellulosic substrates not treated with laccase or an LMS.

To investigate the effect of the TvL and 1-HBT LMS towards saccharification, 0.5g (d.w) WWS (washed wheat straw) was incubated under three conditions: 1) In the absence of both laccase and mediator; 2) With TvL (50U/g d.w biomass) only and; 3) With TvL (50U/g) and 1-HBT (2.5% w/w of d.w WWS) for 24 hours at 28°C. Initial studies were set up to allow consecutive laccase and hydrolysis treatment, whereby GC220 was added to the wheat straw slurries following 24 hours laccase/LMS incubation without substrate washing or enzyme inactivation. Aliquots of the hydrolysis reaction were taken every 24 hours for 3 days. A low concentration of GC220 was selected to promote a slow hydrolysis reaction, in order to allow observation of any differences in glucose concentration over the course of the hydrolysis reaction.

Table 20: Concentration of glucose released from wheat straw hydrolysis in the presence of laccase and LMS (consecutive laccase and cellulase treatments).

Pretreatment[a]	[Glucose] g/L[c]		
	24h	48h	72h
NLNM (no laccase no mediator)	14.6 (0.37)	18.9 (0.35)	20.8 (0.72)
50U/g TvL	14.2 (0.11)	18.1 (0.70)	20.3 (0.32)
50U/g TvL + 1-HBT[b]	13.3 (0.23)	17.1 (0.19)	18.6 (0.32)

[a] 0.5g d.w WS, 28°C, 24h, 200rpm, followed by hydrolysis conditions using 2.9FPU GC220, pH 5.0, 50°C [b] 2.5 % 1-HBT [c] determined by HPLC with RI detection. NLNM: No laccase no mediator. Parentheses represent standard error of 3 biological replicates.

The data revealed that the incubation of TvL and TvL plus 1-HBT with wheat straw before hydrolysis negatively affected saccharification when compared to the untreated control (Table 20), supporting the findings of previous studies [79,177,278]. Interestingly, the LMS (TvL plus 1-HBT) was not found to inhibit wheat straw hydrolysis to the same extent as it was found to inhibit Avicel hydrolysis. This is most likely to be due to the presence of lignin in the wheat straw substrate, which potentially offers alternative sites for the oxidised 1-HBT radicals to react, reducing the radical attack of the cellulases. A similar finding was reported by Palonen & Viikari [179] with steam pretreated softwood, laccase and the mediator *N*-hydroxy-*N*-phenylacetamide (NHA).

The same procedure was repeated with minor adjustments. The pH of the laccase treatment step was reduced to pH 4.0 in line with the optimal pH range of 3-4.5 for TvL [198,216,296]. Furthermore, two wash steps were incorporated after the enzymatic pretreatment to remove the laccase and oxidised mediator for the elimination of potential inhibitory effects. GC220 was added in a separate reaction and the pH was increased to pH 5.0, (optimal pH for GC220). Quantification of glucose revealed again that at all three time points investigated during hydrolysis (24, 48 and 72 hours), the incubation of WWS with TvL and TvL plus 1-HBT failed to increase glucose release during saccharification. This is shown in Figure 48, which presents the measured glucose concentrations released from the wheat straw after 48 hours hydrolysis.

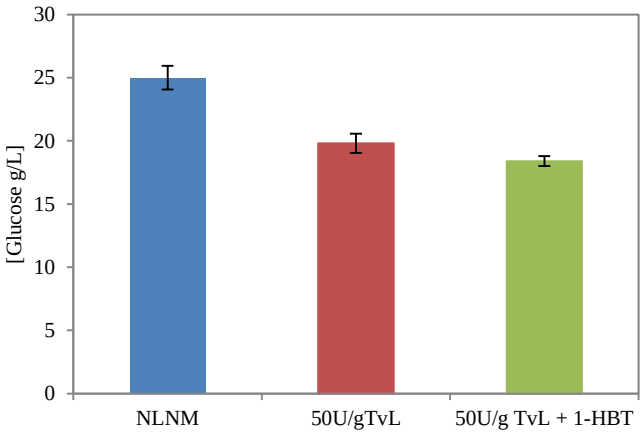


Figure 48: Concentrations of glucose released from 0.6g (d.w) wheat straw hydrolysed by 2.9FPU GC220 after 48h following pretreatment with/without TvL and TvL with 2.5% 1-HBT (w/w biomass). Error bars represent the standard error from 3 biological replicates.

It appears that despite the removal of the enzyme and mediator, hydrolysis is still inhibited by the addition of TvL and the LMS, as demonstrated by the lower concentrations of glucose released under these conditions. It can therefore be concluded that the action of TvL alone, or with its previously demonstrated successful mediator 1-HBT, does not induce the structural modifications or changes to the lignin polymer of wheat straw that are required in order to improve saccharification.

4.2.5.3 Alkaline-peroxide extractions following TvL and LMS treatment

Recent studies by Gutierrez *et al.* [180,182] have reported an increase in polysaccharide hydrolysis for eucalypt wood and elephant grass following treatment with two different LMS (1-HBT and methyl syringate), after the incorporation of an

alkaline-peroxide extraction (APE) step. This step, involving incubation of the biomass with hydrogen peroxide (3%) and sodium hydroxide (1%) (both w/w d.w WWS) at 80°C for one hour, was applied in three sequential steps to all wheat straw slurries following pretreatments with TvL (50U/g) and TvL (50U/g) plus 1-HBT as described previously. Wash steps were applied following the extractions to remove any reactive oxidised hydrogen peroxide and to neutralise the biomass before hydrolysis. Aliquots removed at 24 hour intervals during hydrolysis were analysed for glucose concentration and interestingly, the results collected at three time points revealed the opposite trend to those observed previously without the additional APE step. TvL and TvL plus 1-HBT treated slurries were now found to release higher concentrations of glucose following hydrolysis compared to the untreated negative control (NLNM: No laccase no mediator) (Figure 49). This result demonstrates for the first time that TvL and TvL plus 1-HBT treatments can be used alongside other pretreatment strategies to improve the saccharification of wheat straw.

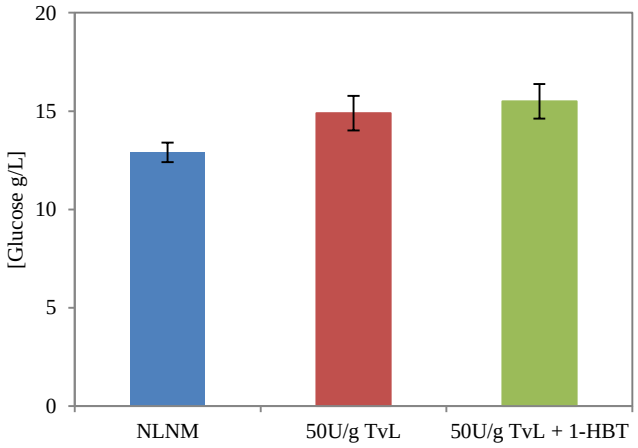
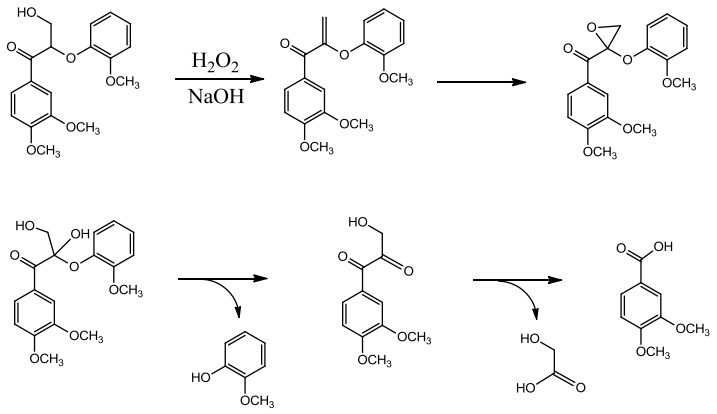


Figure 49: Concentration of glucose released from 0.6g (d.w) wheat straw cellulose hydrolysed by 2.9FPU GC220 after 48h following pretreatment with/without TvL and TvL with 2.5% 1-HBT (w/w biomass) and subjected to APE. Error bars represent the standard error from 3 biological replicates.

APE is a lignin removal technique that includes an alkali treatment stage (sodium hydroxide) which solubilises lignin by the ionisation of carboxylic acid and phenolic groups. The addition of hydrogen peroxide to this process provides further delignification through its decomposition and generation of hydroperoxide anions (HOO^-). The hydroperoxide anions are reported to attack carbonyl structures within lignin and result in C-C bond cleavage [297]. Furthermore, the reaction of undissociated hydrogen peroxide with the hydroperoxide anion produce the highly

reactive hydroxyl radical ($OH^{\bullet}$) and superoxide ($O_2^{\bullet}$) which are also claimed to oxidise structures within lignin, producing carboxyl groups and inducing bond cleavage and lignin dissolution [298,299]. Gierer *et al*. [300] studied the reaction of APE towards phenyacylaryl ethers and proposed a degradation pathway involving Cα-Cβ cleavage, as presented in Scheme 15.



Scheme 15: The proposed pathway for the degradation of a phenacylaryl ether (involving cleavage of the Cα-Cβ bond) by alkaline-peroxide treatment as investigated by Gierer *et al*. [300].

The increased saccharification of laccase and LMS treated wheat straw following APE suggests a possible role for both treatments in the facilitation of the solubilisation of lignin by APE.

It is evident from the data in Figures 48 and 49 that the application of APE leads to a general decrease in overall glucose concentration both in the presence and absence of laccase when compared to the glucose concentration ranges reported without APE. A plausible reason for this could include the loss of biomass achieved due to the increased sample handling and washing stages associated with repeated APE. Furthermore, the alkali oxidative environment of APE is reported to introduce carbonyls within the cellulose chain [299,301]. This effect can induce the unwanted side reaction of cellulose cleavage, further explaining the observed reduction in glucose concentration [301]. In this work, the experimental aim is focused around the effect of laccase and an LMS towards saccharification, therefore important observations include the differences observed between experimental conditions and the consistency of trends reported across different experiments. For this purpose the

overall reduction in biomass is not largely important. However, if this process was introduced industrially this factor would cause problems because the aim of bioethanol production from biomass is to produce high sugar and consequently ethanol yields as possible. Potential loss of substrate throughout the bioprocess would be detrimental to the overall yield of bioethanol. Reports have demonstrated the use of magnesium sulphate to reduce this loss by preventing cellulose cleavage. The mechanism is not fully understood but it is widely accepted that the magnesium ions form metallic magnesium-carbohydrate complexes with the partially degraded cellulose chain thus preventing further damage to the fibres [301]. Gavriliu *et al.* [301] reported that a magnesium sulphate concentration of 0.5% (d.w/biomass) was sufficient to prevent this unwanted side effect of APE. This claim was briefly investigated by subjecting two samples of acid pretreated wheat straw to the APE process with and without magnesium sulphate (0.5%) in addition to a negative control that was not subjected to APE. All samples were hydrolysed and glucose was quantified (Figure 50).

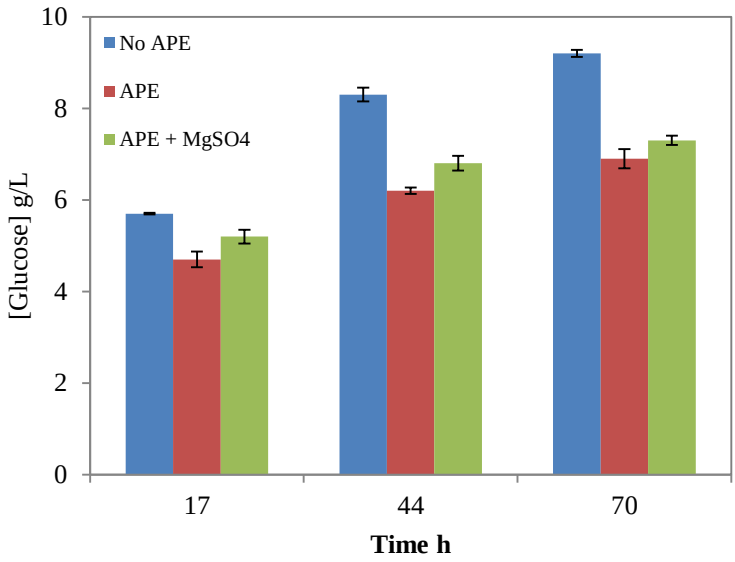


Figure 50: Concentrations of glucose released from the hydrolysis of 0.4g d.w wheat straw over 70h with 1.4 FPU GC220 following APE with and without $MgSO_4$ (0.5%).

The results confirmed that the APE procedure reduces the concentration of released glucose (Figure 50). The addition of magnesium sulphate was found to reduce this loss by 4.4-10.8% during the hydrolysis reaction. However, a reduction in glucose concentration of up to 20.6% was still observed compared to wheat straw without

APE treatment. This difference could be due to non-optimal concentrations of magnesium sulphate, or simply by the loss of biomass through the repeated extractions and wash steps involved. As previously mentioned, for the purpose of this study this loss is not critical, however the amount of magnesium sulphate used and the reduction of biomass loss are two areas that could be further optimised should this process be developed further.

To confirm the importance of both the dilute acid and the APE pretreatment steps, dry wheat straw with no prior acid pretreatment was incubated with and without TvL and 1-HBT. In one experiment, the wheat straw was directly subjected to hydrolysis, whilst in the other experiment the APE step was applied before hydrolysis. Under both conditions the measured glucose concentrations following 88 hours hydrolysis were low (<2.5g/L) considering the starting mass of substrate, confirming the requirement for prior hemicelluloses hydrolysis (Table 21). When the APE was applied, glucose concentrations were higher in all three experimental conditions, highlighting the role of this lignin removal technique in the improvement of hydrolysis yield. Under both conditions the LMS treatment lead to an increase in saccharification, however the difference in glucose concentration was not significant in the study without APE due to the high error associated with the negative control. A significantly higher glucose concentration 0.44g/L (23.4%) was recorded compared to the untreated control when APE was applied. Both conditions failed to demonstrate an improvement in saccharification when TvL without the mediator was used, which could potentially be explained by the reduced binding of laccase to lignin in the presence of hemicellulose.

Table 21: Glucose release following wheat straw hydrolysis with and without acid pretreatment, APE and laccase treatments

Pretreatment[a]	[Glucose] g/L[c] from 88h hydrolysis. No acid pretreatment, - APE	[Glucose] g/L[c] from 88h hydrolysis. No acid pretreatment, + APE
NLNM	0.85 (0.19)	1.88 (0.02)
150U/g TvL	0.84 (0.06)	1.83 (0.05)
150U/g TvL + 1-HBT[b]	0.95 (0.13)	2.32 (0.14)[++]

[a] 0.5g d.w WWS, 40h, 28°C, 200rpm [b] 2.5% 1-HBT (w/w WS) [c] determined by HPLC with RID following hydrolysis with 3FPU GC220, 50°C, pH 5.0. Parenthesis represent standard error of 2 biological replicates [++] result is significantly different when compared to NLNM and 150U/g TvL as determined by paired t-test $t(4) = -4.3026$ $p=0.05$

Following the success of the developed process for demonstrating the positive effect of TvL and TvL when 1-HBT on enzymatic saccharification, further studies were carried out to explore the effect of laccase concentration, mediator concentration and type of mediator. The process followed in each experiment was as presented in Figure 51.

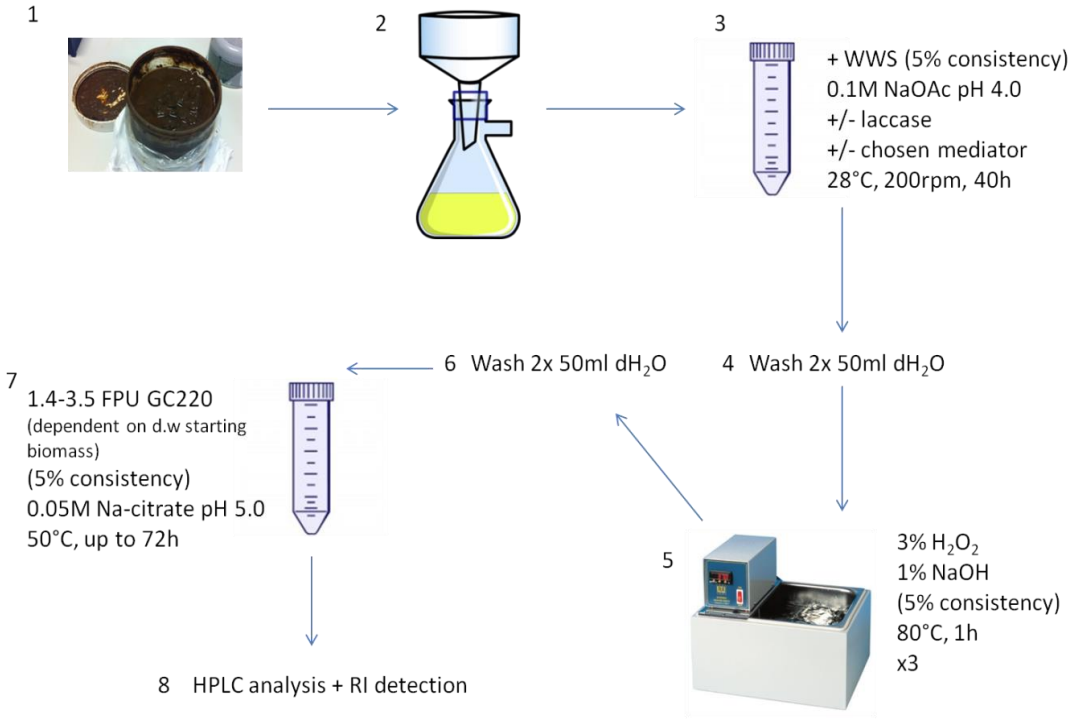


Figure 51: The bioprocess developed for investigating the effect of laccase and LMS on biomass saccharification. 1. Starting substrate (in most cases this was acid pretreated material). 2. Water washing of substrate with dH_2O using Buchner funnel filtration of the soluble material. After this step a portion of the material is removed and dried for determination of dry weight. 3. Set up and incubation of laccase and LMS treatments 4. After 40h incubation, samples are centrifuged to separate the liquid fraction and the biomass is washed with 2x 50mL dH_2O (the washing steps). 5. Alkaline-peroxide extraction 6. After the final extraction, samples are centrifuged and the liquid fraction discarded, biomass was washed with 2x 50mL dH_2O (washing steps). 7. Cellulose hydrolysis reaction. 8. Aliquots removed, centrifuged and the soluble fraction diluted 1:10 H_2SO_4 (10mM) before analysis by HPLC with RI detection.

4.2.5.4 The effect of increasing laccase concentration

The effect of laccase concentration was investigated due to the variation of concentrations used in previous research (20-80,000U/g d.w biomass) [179,180,277,302]. Two concentration ranges of TvL were investigated for the saccharification of WWS with 1-HBT (2.5% w/w biomass). At the lower enzyme

concentration range, 50, 100 and 150U/g TvL (d.w WWS)) were tested. In a separate experiment, high TvL concentrations were investigated (500-4000U/g) following the work of Chen *et al.*[277] who reported optimal laccase loadings of 4400U/g towards ensiled (stored and fermented) corn stover.

The results from both experiments revealed that 500U/g TvL was optimal for increased saccharification, with the greatest increase in glucose reported at this concentration after 44 hours hydrolysis (2.42g/L, 44.6%) compared to the NLNM negative control (Figure 52). In the experiment using lower TvL concentrations, the effect of laccase on saccharification appeared to be dependent on concentration, whereby at TvL concentrations above 500U/g this was not observed, suggesting that the concentration of laccase is important up until a maximum.

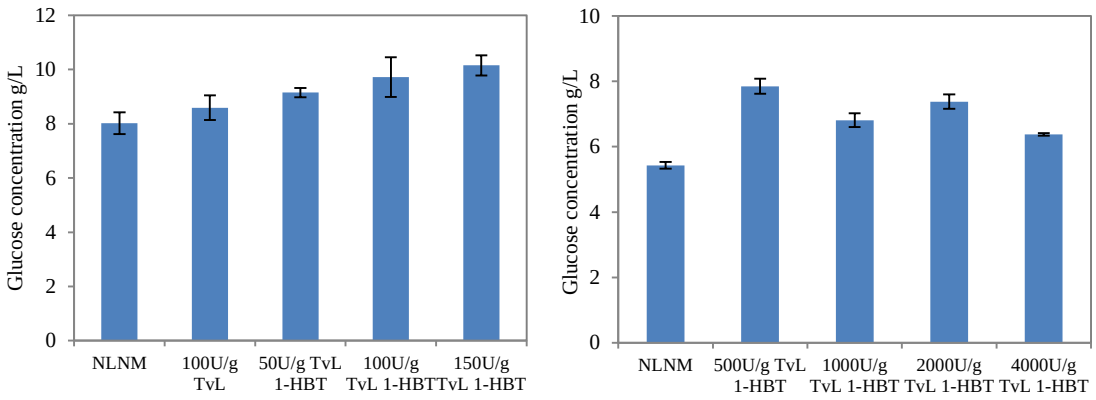


Figure 52: Concentrations of glucose released from 0.5g (d.w) (*left*) and 0.4g (d.w) (*right*) acid-pretreated wheat straw subjected to pretreatment with different concentrations of TvL and 2.5 % (w/w biomass) 1-HBT prior to APE and 48h hydrolysis with 3FPU GC220. Laccase concentration was investigated at both lower ranges (50-150U/g, *left*) and higher ranges (500-4000U/g, *right*).

The increase in glucose release following 44 hours hydrolysis with 150U/g TvL plus 1-HBT was also high compared to the NLNM control (2.13g/L, 26.6% increase). Due to high commercial enzyme costs, a compromise was established and all future experiments were carried out using 150U/g of commercial enzyme.

4.2.5.5 The effect of the mediator concentration

The optimum concentration of the redox mediator 1-HBT was determined by examining the concentration of glucose release following hydrolysis after WWS incubation with 2.5, 5 and 7.5% (w/w biomass) 1-HBT. WWS treated with 5%

1-HBT and 150U/g TvL lead to the greatest improvement in saccharification (1.4g/L,13% increase in glucose) after 64 hours hydrolysis compared to the TvL only (mediator free) negative control (Table 22). 5% (w/w) 1-HBT was therefore selected for the majority of the future investigations, although experiments with 2.5% 1-HBT (Figures 48, 49, 51 and Table 21) previously demonstrated the potential of using a lower concentration.

Table 22: Investigating the optimum concentration of 1-hydroxybenzotriazole (1-HBT) with 150U/g TvL to improve wheat straw saccharification

Biomass treatment[a]	Glucose concentration g/L[b]
150U/g TvL	10.8 (0.13)
150U/g TvL + 2.5% 1-HBT	11.0 (0.23)
150U/g TvL + 5.0% 1-HBT	12.2 (0.37)
150U/g TvL + 7.5% 1-HBT	11.2 (0.80)

[a] 0.8g d.w acid pretreated wheat straw [b] glucose measured by HPLC-RID following 64h hydrolysis with 3FPU GC220, 50°C, pH 5.0. Parentheses represent standard error of two biological replicates.

4.2.5.6 Investigating the use of different mediators

Based on the mediator screening data collected in Chapter 3, additional synthetic mediators and a selection of phenolic compounds were applied to the optimised bioprocess outlined in Figure 51 to assess suitability and reactivity towards wheat straw lignin. Rico *et al*. [182] recently reported the increased saccharification of *Eucalyptus globulus* by pretreatment with *Myceliophthora thermophilia* laccase and the phenolic 'natural' mediator methyl syringate with APE.

Synthetic mediators ABTS and violuric acid were used at molar concentrations equal to the molar concentration of 5% 1-HBT (20mM with respect to 0.6g d.w WWS). Natural mediators syringaldehyde (SA) and acetosyringone (AS) were used at the lower concentration of 7mM due to their poor solubility at higher concentrations. The results in Table 23 show that 1-HBT was the most effective mediator in this experiment, increasing the concentration of released glucose following hydrolysis by 2.3g/L (35%) compared to the NLNM negative control. Violuric acid also demonstrated an effective mediator in this experiment increasing glucose concentration by 1.7g/L (26%).

Table 23: Investigating glucose release from wheat straw hydrolysis following incubation with TvL and a range of synthetic and natural mediators

Mediator	[Glucose] g/L[c]
NLNM	6.6 (0.03)
150U/g TvL	7.8 (0.19)[+]
150U/g TvL 1-HBT[a]	8.9 (0.20)[++]
150U/g TvL ABTS[a]	7.7 (0.22)[+]
150U/g TvL violuric acid[a]	8.3 (0.10)[+]
150U/g TvL syringaldehyde[b]	7.7 (0.08)[+]
150U/g TvL acetosyringone[b]	7.5 (0.25)[+]

[a] [1-HBT] 5% (w/w) d.w biomass (0.6g d.w wheat straw), [ABTS] and [violuric acid] matched on a molar ratio to 1-HBT, [b] [natural mediators] 7mM (due to low solubility) [c] as determined by HPLC-RID following 42h hydrolysis with 1.4FPU GC220. Parentheses represent standard error of 3 biological replicates. [+] Result is statistically different when compared to NLNM control, [++] compared to both NLNM and TvL no mediator control as determined by paired t-test $t(4) = -2.7764$ $p<0.05$.

Use of the potential natural mediators AS and SA did not improve saccharification when compared to the mediator free control. This result was expected following the previous unsuccessful oxidation of the model compound veratryl alcohol by both LMS (Chapter 2 Section 3.2.2). Surprisingly, the reaction of TvL with the synthetic mediator ABTS did not improve saccharification despite the excellent conversion of veratryl alcohol to veratryl aldehyde by this LMS as described in Chapter 3 (Section 3.2.2). A similar result was also observed by Chen [181] who reported little differences between the measured water soluble carbohydrate concentration released from hydrolysed ensiled corn stover after TvL and ABTS treatment. This observation is further supported by the application of 1-HBT as an effective mediator for the delignification and biobleaching of lignocellulose in the paper and pulp industry [152,201] and the lower reported efficiency of ABTS as a mediator in the flax pulp bleaching process compared to 1-HBT [201].

These results, along with the previously collected data further demonstrate the ability of laccase treatment alone to improve wheat straw saccharification. This effect of laccase has been previously reported towards a range of wood and non-wood substrates [99,179,180]. Laccase is thought to positively affect saccharification by two mechanisms; 1) By binding to lignin sites within the biomass thus blocking available sites for the non-specific binding of cellulases, and 2) By modification of the lignin structure making the lignin less able to bind proteins such as cellulases [99]. To test this, Moilanen [99] incubated steam pretreated spruce (SPS) and giant reed (SPGR)

with denatured laccase, active laccase and BSA. All three pretreatments enhanced the release of sugars from the SPS during hydrolysis, supporting mechanism 1. However, incubation with active laccase was found to improve hydrolysis to the greatest extent, suggesting that the activity of the laccase with SPS induced structural changes to the lignin that further improved hydrolysis, supporting mechanism 2. The opposite effect was observed with SPGR, with active laccase treatment negatively affecting hydrolysis. This highlights a possibility that lignin from different sources can exhibit different binding properties towards cellulases, most likely due to differences within their composition (syringyl, guaiacyl or *p*-hydroxyphenyl type lignins). In further work by the same researchers, SPS was found to adsorb lower amounts of cellulases after laccase treatment compared to the untreated control. In contrast, the results with SPGR revealed a higher adsorption of endoglucanases and an increased amount of lignin bound enzymes compared to SPS, supporting the hydrolysis data.

To investigate the structural effects of laccase towards SPS, Palonen and Viikari [179] used electron spectroscopy for chemical analysis (ESCA) and reported an increase in the presence of acidic groups within lignin after laccase treatment. The increase in negative charge to the lignin surface and hence reduction in the hydrophobicity as a result of laccase treatment is thought to induce a 'repulsion effect' towards cellulases. The study did not however confirm the reduced binding of cellulases as did Moilanen's study [99]. In addition to the reported negative effect of laccase on SPGR hydrolysis, Jurado *et al*. [79] and Tabka *et al*. [177] both reported a reduction in glucose release from steam exploded wheat straw incubated with *Pycnoporus cinnabarinus* [177] and *Coriolopsis ridiga* [79] laccases without mediators. Both authors put this effect down to the release of phenolic compounds from the lignin following laccase treatment, which possibly inhibited cellulose activity. This is a plausible explanation for the work of Tabka *et al*. [177] since the laccase and hydrolysis reactions were run simultaneously in the same vessel. In the work of Jurado *et al*. [79] the laccase reaction with wheat straw was centrifuged and the supernatant separated from the biomass before hydrolysis. This separation step should theoretically remove the majority of any inhibitory free phenols prior to hydrolysis, however even small concentrations carried over could be responsible for inhibition. Tabka *et al*. [177] suggested that removal of the supernatant followed by washing steps could remove the inhibitory phenols, however as observed by Figures 48 and 49,

laccase treatment (with and without mediator) was still found to inhibit hydrolysis despite the incorporation of washing steps unless the APE step was applied afterwards. It cannot be ruled out however, that in this work that the washing steps did not effectively remove all the phenols, thus causing inhibition. However, it is more likely that the pretreatment with laccase can produce structural modifications to the lignin which in turn increases cellulase adsorption.

The study was expanded to investigate additional phenolic compounds as mediators that were successful in the previous decolourisation of RB-5 (*p*-coumaric acid (PC), methyl vanillate (MV), and 4-hydroxybenzoic acid (4-HBAc), (Chapter 3 Section 3.2.1)). Like SA and AS, their reaction with TvL did not increase saccharification (Table 24). Glucose concentrations after 72 hours hydrolysis suggest an inhibitory effect on saccharification following their use. This could even be explained by a possible carry-over of the polymerised phenols into the saccharification reaction, or the trapping of these polymers within the biomass. Inhibitory effects of the inactive polymers could include the non-specific interaction with cellulases or the blocking of accessible cellulose sites for hydrolysis. The presence of oxidised (and therefore highly likely to be polymerised) phenols was previously demonstrated to inhibit the hydrolysis of cellulose to variable degrees (Table 19).

Table 24: Glucose release from wheat straw following incubation with laccase and a panel of mediators plus APE

Mediator	[Glucose] g/L T48h[c]	Glucose (g/L) T72h[c]
NLNM	5.04 (0.27)	5.31 (0.36)
150U/g TvL	5.43 (0.20)	6.50 (0.26)
150U/g TvL HBT L [a]	6.19^{++} (0.02)	6.85 (0.46)
150U/g TvL HBT H [b]	6.94^{++} (0.29)	7.50^{++} (0.21)
150U/g TvL PC	5.03 (0.09)	5.69 (0.08)
150U/g TvL MV	5.46 (0.35)	5.87 (0.12)
150U/g TvL 4-HBAc	5.61 (0.10)	6.12 (0.45)

[a] [HBT] L = 2.5%, [b] [HBT] H = 5% dw biomass (0.4g dw WS) [c] as determined by HPLC-RID following hydrolysis with 1.4FPU GC220. Parentheses represent standard error of 3 biological replicates $^{++}$result is statistically different when compared to the NLNM negative control and the TvL no mediator control as determined by paired t-test statistical analysis. Conditions t(4)=- 2.7764 p= 0.05.

In separate experiments, three additional compounds were selected for investigation as potential mediators with TvL to improve the saccharification of WWS; 2,2,6,6-tetramethylpiperidinyloxy (TEMPO), Phenol Red (PR) and Remazol Brilliant

Blue (RBB). PR and RBB were selected following previous observations of their oxidative capabilities towards veratryl alcohol (Chapter 2 Section 3.2.2). TEMPO is a well referenced synthetic laccase mediator and was included following a report of the compound acting as a highly efficient synthetic mediator in the oxidation of a lignin model compound [154].

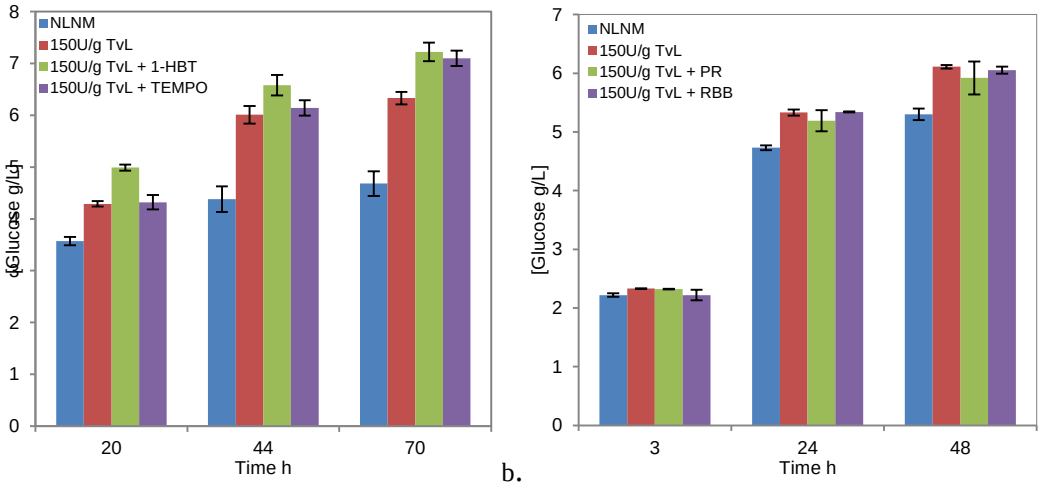


Figure 53: Released glucose concentrations over time following the hydrolysis of 0.4g (d.w) acid pretreated wheat straw incubated with 1.4FPU GCC20 following treatment with and without TvL and mediators; a: 1-HBT and TEMPO; b: PR and RBB. Error bars represent the standard error of 3 biological replicates.

The increased concentration of glucose released during the later stages of hydrolysis following TvL and TEMPO incubation (Figure 53a) suggest that there is a potential for TEMPO to be considered a useful synthetic mediator along with violuric acid for the improvement of wheat straw saccharification. Despite this, TEMPO would not be an ideal synthetic mediator for this application due to its reported ability to oxidise cellulose in the presence of laccase. The oxidation of cellulose by TEMPO results in the conversion of hydroxyl groups to aldehydes thus increasing the carboxyl content of the polymer [303,304].

The results presented in Figure 53b reveal that the saccharification of WS slurries is not improved by pretreatment with TvL and PR or RBB when compared to slurries treated with TvL only. This observation echoes previous findings with TvL plus ABTS (Table 23), whereby successful oxidative activity towards veratryl alcohol did not correspond to increased saccharification of lignocellulose. It can therefore be concluded that the ability of an LMS to successfully oxidise a lignin model compound

such as veratryl alcohol does not always correlate to the reactivity of the LMS towards natural lignocellulose. This effect could be due to differences between the experimental conditions during lignin model compound oxidation reactions and those with natural lignocellulose. Lignin model compounds do not represent the true chemical nature of lignin. A reaction with a lignin model compound such as veratryl alcohol contains few components, namely the substrate, enzyme, mediator and buffer components. As an example, phenoxyl radical species that are generated in lignin model compound reactions have little to react with therefore the rapid dimerisation with other phenoxyl radicals is highly likely to occur, resulting in dimerisation and eventual polymer formation. In lignocellulosic natural systems, the polymerisation of phenoxyl radicals may occur at a much slower rate due to the presence of additional substrates such as cellulose and hemicellulose. The oxidised species may become trapped within the substrate thus reducing the interactions with other radical species and prolonging the lifespan and exposure of the radicals to the substrate, allowing oxidative activity to occur. The presence of lignocellulosic substrates could also have the opposite effect, and oxidised radical species such as ABTS may struggle to modify lignin due to side reactions with additional lignocellulosic components or other stability related issues.

4.2.5.7 TvL and LMS effect on lignin removal by organosolv

Following the success of several LMS with acid pretreated wheat straw and APE to improve saccharification, the effect of the TvL and 1-HBT LMS was investigated in combination with a second lignin removal technique, organosolv. Organosolv is a biomass pretreatment procedure that is primarily used for the extraction of lignin, although hemicellulose can also be hydrolysed through this method. The process involves treatment of the biomass with an organic solvent (usually alcohols or organic acids), with water as a co-solvent, at temperatures as high as 200°C.

An initial study was set up as outlined in Figure 51 whereby the APE step was replaced with ethanol organosolv treatment. The three previously investigated conditions for WWS treatment were set up (an untreated negative control: NLNM, a laccase only control: 150U/g TvL, and the optimised LMS treatment: 150U/g TvL plus 1-HBT). Following the enzymatic incubation step and subsequent washing, equipment was set up to allow the biomass to reflux in an organosolv solvent (60:40

(%) ethanol:water). The biomass was mixed in the solvent (1:10 solid:liquid) and heated to 150°C for 90 minutes. The set up was as displayed in Figure 54.

Figure 54: Set up of the organosolv procedure to allow three extractions to run simultaneously

When the reaction was terminated, the solid biomass was collected, air dried and hydrolysed with 3.5FPU GC220. Lignin within the organosolv solvent fraction was precipitated out as described in Chapter 7 Section 7.26, separated by centrifugation and freeze-dried for further analysis.

Table 25: Released glucose concentrations and weights of precipitated lignin following wheat straw saccharification after laccase and LMS treatments with organosolv

Treatment[a]	[Glucose] g/L[b]			Weight of precipitated lignin (mg)
	24h	46h	93h	
NLNM	12.8	15.4	15.6	11.2
150U/g TvL	13.2	17.1	20.7	15.5
150U/g TvL + 1-HBT	16.0	20.7	20.4	20.0

[a] 40h incubation of 2g d.w WS at 28°C, 200rpm, pH 4.0 [b] 5% 1-HBT [c] measured by HPLC-RID following hydrolysis with 4FPU GC220.

The incubation of acid-pretreated WWS with TvL and TvL with 1-HBT prior to organosolv was found to increase saccharification as outlined in Table 25. The difference between the untreated control and the LMS treated substrate after 46 hours hydrolysis was 5.3g/L (34.4% increase). Treatment with TvL alone also increased glucose release compared to the negative control, as previously observed following

APE. Dried precipitated lignin was weighed following the organosolv reaction revealing a greater mass of precipitated lignin following both laccase treatments compared to the untreated control. The greatest mass of lignin was recorded following LMS treatment, suggesting increased lignin removal by this pretreatment. This initial study therefore revealed that the pre-incubation of wheat straw with TvL and TvL with 1-HBT affects or changes the wheat straw in such a way that subsequently positively influences lignin removal by organosolv.

To confirm these results, the experiment was reproduced with the inclusion of three biological replicates and revealed the same trend in saccharification as the previous study (Figure 55). The increase in glucose concentration released from the LMS treated WWS compared to the untreated negative control was lower compared to the previous study (increase of 2.74g/L (21.8%) after 48 hours and 3.37g/L (44.8%) after 72 hours) highlighting the problem of variability between different washed batches of biomass. The increase however was still high and confirmed the success of the pretreatment methods for improving saccharification.

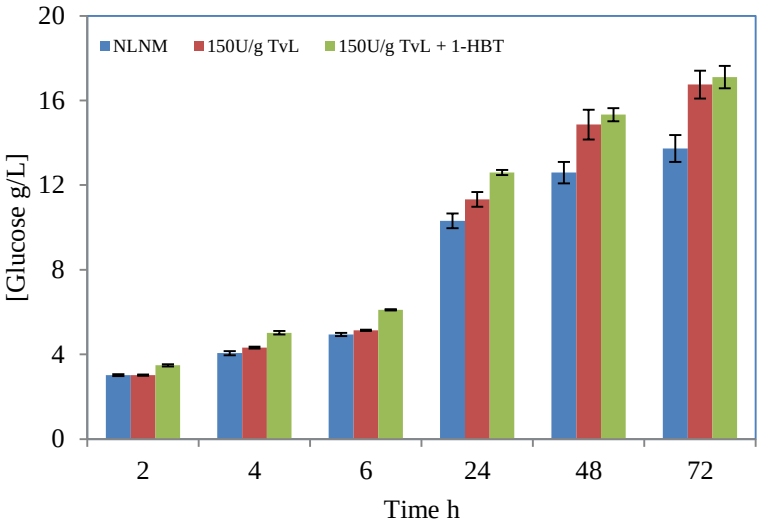


Figure 55: Released glucose concentrations following the hydrolysis of 0.8g (d.w) acid pretreated wheat straw over time with 4FPU GC220 following incubation with and without TvL and 5% 1-HBT and an ethanol organosolv procedure at 150°C for 1h. Error bars represent the standard error of 3 biological replicates.

Despite these promising results, several factors make this process time consuming, including the set up and operation of the reflux equipment, and the subsequent separation and drying of the biomass. This procedure is therefore not ideal for the high throughput analysis of different substrates, enzymes and mediators with replicate analyses. To overcome this, a second method was set up using glass pressure tubes (as seen in Figure 56) and a priorclave. In this case, the conditions for the organosolv reaction were restricted by the limits of the priorclave settings therefore the maximum temperature that could be used was 126°C. This temperature was investigated with a reaction time of one hour. The small volume within the pressure tubes limited the amount of biomass that could be used in each experiment to <1g d.w.

Figure 56: Glass pressure tube used for organosolv treatment of biomass (Note: Not to scale, dimensions: 25mm x 105mm (outer diameter and length)).

Under the adjusted organosolv conditions, hydrolysis of the acid-pretreated WWS was again improved by applying the laccase and LMS pretreatments (Table 26). The glucose concentrations released and the average increases observed between the untreated and the LMS treated substrate (2.06g/L (20.8%) glucose after 22 hours, 1.89g/L (15.7%) glucose after 44 hours) were however lower, than those reported in the previous study, suggesting that the adjusted conditions were less successful for improving saccharification. This is likely due to the reduced temperature and lack of stirring function in the priorclave. Despite this, the average difference in released glucose concentrations are still relatively high, and are significantly increased when the LMS is applied as determined by paired t-test analysis (Table 26).

Table 26: Released glucose concentrations from wheat straw saccharification following laccase and LMS pretreatment and organosolv in glass pressure tubes

Treatment[a]	[Glucose] (g/L)[c] 1h	[Glucose] (g/L)[c] 4h	[Glucose] (g/L)[c] 22h	[Glucose] (g/L)[c] 44h
NLNM	2.20 (0.01)	4.53 (0.01)	9.91 (0.01)	12.06 (0.26)
150U/g TvL	2.12 (0.02)	4.46 (0.04)	10.24^{+} (0.06)	12.62 (0.33)
150U/g TvL + 1-HBT [b]	2.41 (0.03)	5.10^{++} (0.08)	11.97^{++} (0.23)	13.95^{++} (0.13)

[a] 1.0g d.w WWS, 28°C, 200rpm, 40h, pH 4.0 [b] 5% 1-HBT, [c] determined by HPLC-RID after hydrolysis with 4FPU GC220, 50°C, pH 5.0. Parentheses represent standard error of 3 biological replicates. [+] Result is statistically different when compared to NLNM control or [++] compared to both NLNM and TvL with no mediator as determined by paired t-test $t(4)= -2.7764$ $p=0.05$.

Despite the reduced effect on saccharification under milder organosolv conditions, from an industrial viewpoint, the use of lower temperatures and shorter reaction times is highly beneficial for process cost reduction. The ability of both laccase treatments to demonstrate improved saccharification under milder conditions is biotechnologically advantageous. The high costs associated with the use of a synthetic mediator (such as 1-HBT), would hinder its industrial application. To investigate the effects of reduced mediator concentrations in addition to milder operating conditions, the organosolv reaction was repeated with 2.5% 1-HBT (Table 27).

Table 27: Released glucose concentrations from wheat straw saccharification following laccase and LMS treatment with organosolv using glass pressure tubes and a lower temperature

Treatment[a]	[Glucose] (g/L)[c] 20h	[Glucose] (g/L)[c] 96h
NLNM	4.80 (0.13)	7.22 (0.14)
150U/g TvL	5.19 (0.17)	$8.21\ (0.17)^{+}$
150U/g TvL + 1-HBT[b]	5.36 (0.09)	$8.69\ (0.13)^{+}$

[a] 0.5g d.w WWS, 28°C, 200rpm, 40h, pH 4.0 [b] 2.5% 1-HBT, [c] determined by HPLC-RID following hydrolysis with 4FPU GC220, 50°C, pH 5.0. Parenthesis represent standard error of 3 biological replicates [+] result is statistically different when compared to the NLNM control as determined by paired t-test $t(4)=-2.7764$ $p=0.05$.

The hydrolysis results after 96 hours show statistically significant increases in glucose concentration from both TvL and TvL with 1-HBT pretreated wheat straw compared to the untreated control. These results are promising considering that less than optimal conditions were used and demonstrates how the addition of laccase and a LMS can

enhance the saccharification of biomass through the improvement of a lower cost organosolv and pretreatment procedure.

Another cost reducing strategy was explored by investigating the necessity of the acid hydrolysis step prior to laccase treatment in an organosolv process. Reducing the number of individual stages in a bioprocess can potentially reduce overall costs and sample losses. Acid can be incorporated into an organosolv procedure to allow simultaneous hemicellulose solubilisation and lignin removal at lower temperatures. When higher temperatures are used (e.g. 185-210°C), organic acids that are generated from the substrate act as catalysts for the disruption of lignin-carbohydrate complexes [305]. Two experimental conditions were explored in this study. In the first (condition 1), wheat straw was treated with TvL with and without 1-HBT (including an untreated negative control NLNM), and all organosolv reactions were supplemented with hydrochloric acid (0.02M). This acid type and concentration were selected following a report by Huijgen *et al*. [306], which reported the optimal catalytic conditions for xylan solubilisation and lignin removal in wheat straw by organosolv. In the second (condition 2), dry wheat straw was first acid pretreated with sulfuric acid (following an optimised procedure described in Chapter 7 Section 7.21) before laccase treatment and organosolv. Acid was omitted during the subsequent organosolv procedure in this condition.

Table 28: Released glucose concentrations from wheat straw saccharification after acid catalysed organosolv or acid pretreatment prior to organosolv

Pretreatment[a]	[Glucose] g/L[c] 72h Condition 1[d]	[Glucose] g/L[c] 72h Condition 2[d]
NLNM	3.16 (0.07)	10.80 (0.18)
150U/g TvL	3.04 (0.04)	11.65 (0.26)
150U/g TvL + 1-HBT[b]	3.69 (0.09)[+]	12.04 (0.08)[+]

[a] 1.5g d.w WWS following acid pretreatment, 2g d.w when acid pretreatment stage was omitted, 28°C, 200rpm, pH 4.0, 40h [b] 2.5% 1-HBT [c] determined by HPLC-RID following hydrolysis with 4FPU GC220 50°C, pH 5.0 [d] 100°C, 1h, 60:40 ethanol:H_2O. Parenthesis represent standard error of 3 biological replicates [+] result is statistically different when compared to the NLNM control determined by paired t-test $t(4)= -2.7764$ $p=0.05$

Glucose quantification after 72 hours hydrolysis revealed that acid pretreatment prior to laccase incubation (condition 2) lead to significantly higher concentrations of glucose release compared to acid catalysis during hydrolysis (condition 1) (Table 28). This suggests that the acid hydrolysis step prior to laccase treatment is more

successful at increasing the saccharification of wheat straw by the removal of hemicellulose derived sugars (concentrations of glucose, xylose and arabinose in the hydrolysate were 12.7, 90.2 and 13.2g/L respectively). In addition, hemicellulose removal prior to laccase pretreatment will increase the accessibility of the laccase and oxidised mediator towards lignin. Under condition 1, LMS treatment improved hydrolysis by 0.53g/L glucose (16.8%) compared to the negative control. A small decrease in hydrolysis (0.13g/L, 4.1%) was observed following treatments with TvL in the absence of mediator, which was unusual considering the trends from previous data. Saccharification of the wheat straw following condition 2 released glucose concentrations in all 3 treatments that followed the previously observed trend in this work, supporting the ability of TvL and TvL with 1-HBT to improve saccharification. In condition 2, the increase in glucose release following LMS treatment compared to the negative control (1.24g/L, 16.8%), was lower than what has been typically observed. The surface area of the substrate used in this experiment is likely to be responsible for this reduced increase. The batch of wheat straw used in this study was received dry (not as an acid pretreated slurry) and the size of the chips was noticeably larger than observed in previous batches. A reduced surface area could possibly negatively affect all three stages of the process by reducing the area of substrate sites exposed to pretreatment. The study confirms the combined success of the treatment of wheat straw with laccase/LMS organosolv and prior acid-hydrolysis. The addition of hydrochloric acid (0.02M) did not benefit the process and was not considered further.

4.2.6 The effect of other laccases and LMS on wheat straw saccharification

In order to establish whether the observed behaviour previously described was specific to TvL, the use of other fungal laccases were investigated. Due to the difficulties experienced in expressing active TvL at concentrations high enough for use in saccharification experiments (Chapter 2), three commercially available laccases were selected; laccases from the fungi *Pleurotus ostreaus* (PoL) and *Agaricus bisporus* (AgL) and a laccase from the Japanese lacquer tree *Rhus vernicifera* (RvL).

The calculated specific activity of the commercial preparation of PoL was determined using the previously developed ABTS assay and was found to be 67U/mg. This allowed for higher concentrations to be applied to the biomass without using a significantly greater mass of enzyme than when TvL was used. 250U/g (d.w biomass) PoL and 1-HBT (2.5%) were used in the procedure outlined in Figure 51 which

incorporates the APE method. Glucose quantification revealed that the incubation of WWS with PoL with and without 1-HBT increased saccharification by 56.3% (2.7g/L glucose increase) when compared to the untreated negative control (NLNM) (Table 29). A high increase in saccharification (1.7g/L, 35.4%) was also observed after 65 hours hydrolysis when PoL was incubated in the absence of 1-HBT. The study confirmed that in addition to TvL, the treatment of PoL both with and without 1-HBT can improve the saccharification of WS when combined with acid pretreatment and APE.

Table 29: Released glucose concentration during wheat straw saccharification following incubation with/without PoL and 1-HBT

Treatment[a]	[Glucose] g/L[c] 40h	[Glucose] g/L[c] 65h
NLNM	4.1 (0.21)	4.8 (0.16)
250U/g PoL	5.5 (0.20)$^{+}$	6.5 (0.29)$^{+}$
250U/g PoL 1-HBT[b]	6.3 (0.08)$^{++}$	7.5 (0.13)$^{++}$

[a] 0.5g d.w WWS, 28°C, 200rpm, pH 4.0, 40h [b] 2.5% (w/w d.w WWS) 1-HBT, [c] determined by HPLC-RID following hydrolysis with 1.4FPU GC220 50°C, pH 5.0. Parenthesis represent standard error of 3 biological replicates $^{+}$ result is statistically different compared to the NLNM control $^{++}$ compared to the TvL no mediator control as determined by paired t-test $t(4)= -2.7764$ $p=0.05$

The calculated specific activities of RvL and AgL were found to be much lower than those of TvL and PoL at 2.3U/mg and 5.4U/mg respectively. Due to the small quantities provided and the high commercial cost of these enzymes, (especially in the case of RvL), lower concentrations were used in the biomass saccharification experiments (12.5U/g RvL and 125U/g AgL). The results shown in Table 30, following 72 hours hydrolysis revealed that both enzymes were successful in improving the saccharification of WWS (increased glucose concentration of 0.4g with RvL and 1.6g/L AgL to negative control). However it appeared that the addition of 1-HBT (2.5%) did not further improve saccharification as previously observed with both TvL and PoL. It is unlikely that the lower 1-HBT concentration is responsible for this effect, since previous studies with TvL and PoL have shown increased saccharification with only 2.5% 1-HBT when compared to laccase only treatment. It is possible that the low concentrations of the laccases used were not sufficient to successfully regenerate and recycle the oxidised mediator, although other issues such as low enzyme stability in the presence of radical species should not be ruled out. Due

to the high costs of both enzymes the study was not further investigated although it was successful in demonstrating the potential of both laccases to positively impact wheat straw saccharification.

Table 30: Released glucose concentrations during wheat straw saccharification following incubation with RvL, AgL and 1-HBT

Treatment[a]	[Glucose] g/L[c] 72h
NLNM	4.1 (0.16)
12.5U/g RvL	4.5 (0.16)
12.5U/g RvL + 1-HBT[b]	4.4 (0.20)
125U/g AgL	5.7 (0.15)[+1]
125U/g AgL + 1-HBT[b]	5.5 (0.06)[+2]

[a] 0.4g d.w WWS, 28°C, 200rpm, 40h, [b] 2.5% (w/w d.w WWS) 1-HBT, [c] determined by HPLC-RID following hydrolysis with 1.4FPU GC220, 50°C, pH 5.0. Parenthesis represent standard error of 3 biological replicates [+] result is statistically different when compared with the NLNM control as determined by paired t-test [1] $t(3)= -3.1824$ $p=0.05$ 2 $t(2)= -4.3026$ $p=0.05$.

4.2.7 The effect of laccase and LMS on the saccharification of other substrates

To test the applicability of the developed bioprocesses towards other lignocellulosic substrates, experiments were set up using TvL and PoL +/- 1-HBT with the agricultural residues corn and sorghum stover. Both APE and organosolv procedures were investigated. Corn and sorghum stover substrates were received, and used, as dry milled chips with larger surface areas than that of the acid pretreated wheat straw previously used. Acid pretreatment was performed on both substrates using sulfuric acid at 126°C. Sulfuric acid was chosen because it is the most widely used acid for hemicellulose hydrolysis [28,307,308] and it has successfully been demonstrated to yield the xylose required for furfural production [309,310]. 126°C was selected as this was the maximum operational temperature for the priorclave. Two acid concentrations were investigated, 1% and 2% sulfuric acid, alongside a water negative control. Both substrates were pretreated (1:9 w/w biomass:acid) for one hour and the sugars released were quantified in the filtrates following Buchner funnel filtration. Xylose was the most abundant sugar identified in the filtrates, which was expected due to its reported predominance in the composition of corn and sorghum stover hemicellulose and hydrolysates compared with other C-5 (arabinose) and C-6 (glucose, galactose, mannose) hemicellulose derived monosaccharides [311-313]. Sugar analysis using HPLC-RID detected xylose, arabinose and glucose at low

concentrations following treatment without an acid catalyst (water only) for both substrates. The highest sugar concentrations were detected following the addition of 2% sulfuric acid (Appendix, Table 47) suggesting that hemicellulose solubilisation was more effective at higher acid concentrations (although differences between the sugar concentrations released using 2% and 1% acid were small). It is worth noting that increasing the severity of acid pretreatment conditions (e.g. increasing concentration from 1 to 2% in this case) increases the chance of producing hemicellulose degradation products such as furfural and hydroxymethyl furfural which are inhibitory to downstream fermentations. In this work, hemicellulose derived sugar fermentation was not under investigation, and water wash steps were performed on all acid pretreated materials which will remove inhibitors, therefore this was not an issue. The effect of acid concentration on furfural and hydroxymethyl furfural production should be investigated if the hemicellulose hydrolysate is to be considered for fermentation studies.

Both substrates were treated with 150U/g TvL with and without 5% 1-HBT and the APE step as described previously. The data demonstrated a consistent trend to that observed with wheat straw following TvL incubation (with and without 1-HBT) prior to APE and hydrolysis. The addition of 1-HBT was again found to further improve hydrolysis as shown in Figure 57.

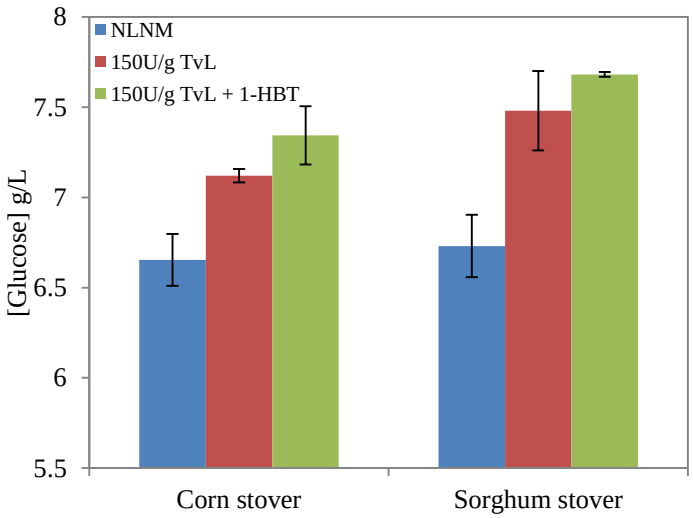


Figure 57: Released glucose concentrations from 0.4g (d.w) acid pretreated corn stover and sorghum stover following 70h hydrolysis with 2FPU GC220 following treatment with TvL and 1-HBT.

Saccharification increases were lower than those typically observed following wheat straw treatment. With corn stover, TvL pretreatment with and without 1-HBT increased glucose concentrations by 0.47g/L (7.1%) and 0.69g/L (10.4%) respectively. Similar increases were observed with sorghum stover, with TvL both with and without 1-HBT increasing glucose concentrations by 0.75g/L (11.1%) and 0.95g/L (14.1%) respectively compared to the untreated control. This reduction in saccharification improvement is likely to be due to the lower surface area of both substrates used compared to the more finely milled wheat straw used from previous batches of material. A higher surface area will increase the number of sites available for laccase and/or LMS reactions to occur, resulting in biomass that is more amenable to lignin removal techniques (APE or organosolv extraction) and cellulose hydrolysis.

The experiment was repeated with sorghum stover and PoL at the higher concentration of 250U/g as previously used with wheat straw (Table 31). A greater saccharification increase between the LMS and negative control was observed with PoL at 250U/g compared with TvL at 150U/g (1.6g/L (28.2%) and 1.1g/L (19.4%) respectively after 72 hours hydrolysis).

Table 31: Released glucose concentrations during the saccharification of sorghum stover following treatment with PoL and 1-HBT with APE.

Treatment[a]	[Glucose] g/L[c]		
	15h	40h	72h
NLNM	4.53 (0.31)	5.38 (0.37)	5.68 (0.32)
250U/g PoL	5.25 (0.06)	6.03 (0.40)	6.77 (0.05)$^{+}$
250U/g PoL + 1-HBT[b]	5.50 (0.07)$^{++}$	6.66 (0.06)$^{+}$	7.28 (0.10)$^{++}$

[a] 0.35g (d.w) sorghum stover, incubated at 28°C, 200rpm, pH 4.0, 40h, followed by alkaline-peroxide extraction [b] 5% (w/w) 1-HBT [c] determined by HPLC-RID following hydrolysis with 1.4FPU GC220 50°C, pH 5.0. Parenthesis represent standard error of 3 biological replicates $^{+}$ result is statistically different when compared to the NLNM control $^{++}$ compared to both the NLNM and TvL with no mediator control as determined by paired t-test $t(4)= -2.7764$ $p=0.05$.

The same experiment was repeated with corn stover (employing a higher concentration of GC220) (Table 32). The greatest differences in released glucose concentration between the 3 treatments were observed more rapidly (after 15 hours of hydrolysis), presumably due to the increased reaction rate associated with higher cellulase loading. The saccharification increases were 1.7g/L (28.1%) and 1.2g/L (19.8%) for PoL with and without 1-HBT respectively compared to the untreated control. The differences in glucose release observed using both substrates were again

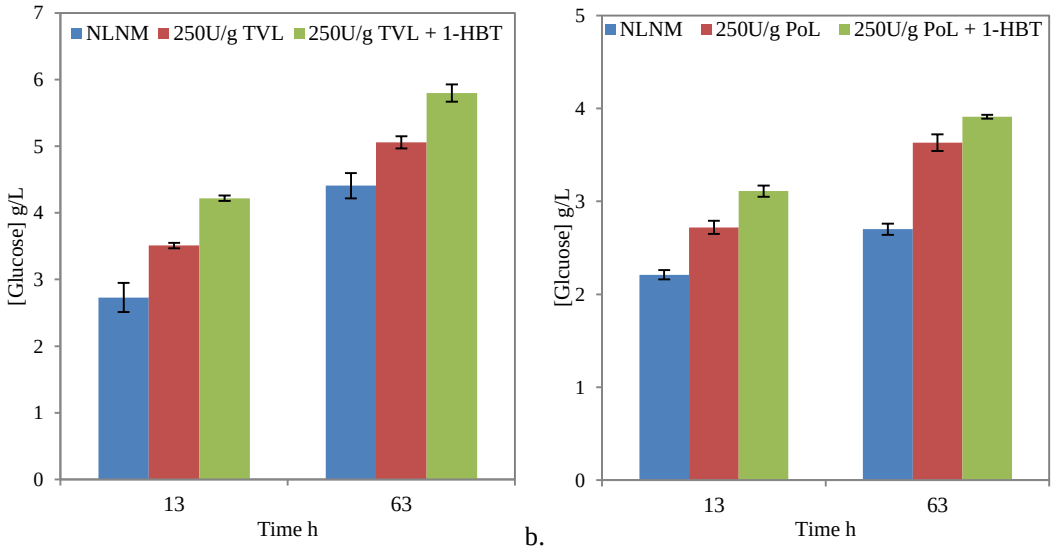
lower than those recorded with wheat straw under the same conditions (Table 29). However the results were promising regarding the improvement of saccharification for alternative lignocellulosic substrates with lower surface areas.

Table 32: Released glucose concentrations during the saccharification of corn stover following treatment with PoL and 1-HBT with APE

Treatment[a]	[Glucose] g/L[c]		
	15h	40h	72h
NLNM	6.04 (0.31)	6.46 (0.37)	6.85 (0.32)
250U/g PoL	7.23 (0.06)[+]	7.23 (0.40)	7.37 (0.05)
250U/g PoL + 1-HBT[b]	7.75 (0.07)[++]	7.76 (0.06)[++]	8.01 (0.10)[++]

[a] 0.4g (d.w) corn stover, incubated at 28°C, 200rpm, pH 4.0, 40h followed by alkaline-peroxide extraction [b] 5% (g/g) 1-HBT [c] determined by HPLC-RID following hydrolysis with 2FPU GC220 50°C, pH 5.0. Parenthesis represent standard error of 3 biological replicates [+] result is statistically different when compared with the NLNM control [++] compared with the NLNM and TvL with no mediator control as determined by paired t-test $t(4)= -2.7764$ $p=0.05$.

The improved saccharification of corn stover following laccase and LMS treatment was further studied by replacing the APE with organosolv. In this study, both TvL and PoL were used at 250U/g. The organosolv procedure was performed using the glass pressure tubes in the priorclave due to the high sample numbers and biological replicates.



a. b.

Figure 58: Released glucose concentrations from 0.3g (d.w) acid pretreated corn stover hydrolysis with 2FPU GC220; a: TvL and 1-HBT; b: PoL and 1-HBT with organosolv at a lower temperature.

The results confirmed that both laccases with corn stover under the lower temperature organosolv process conditions increased saccharification. Increases with TvL with and without 1-HBT were found to peak at 1.5g/L (54.9%) and 0.71g/L (26.0%) respectively after 13 hours hydrolysis time compared to the negative control (Figure 58a). When the experiment was conducted with PoL, the greatest glucose increases were observed after 63 hours hydrolysis time and were 1.21g/L (44.8%) and 0.93g/L (34.4%) with and without mediator respectively (Figure 58b).

4.2.8 Estimation of saccharification as % conversion of cellulose

The data obtained from the saccharification experiments in this work have been presented as glucose concentrations expressed in g/L. Many studies have presented saccharification data as percentage conversion or hydrolysis [99,178-180,182]. This approach was not chosen for this work due to the time consuming nature of the procedure for cellulose compositional determination for high throughput experimentation. To establish an estimation of the increases in cellulose conversion as a result of laccase and LMS treatment, an experiment was set up using acid pretreated corn stover and the compositional information provided by NREL (Appendix, Table 48). The conversion values were calculated based on an estimation of the percentage of cellulose within the substrate prior to hydrolysis before and after APE.

Table 33: Conversion of cellulose during the saccharification of corn stover after laccase and LMS pretreatment and APE

Treatment	Average starting mass (g)[a]	Average mass post treatment and APE (g)[b]	Average loss in mass (%)[c]	[Glucose] g/L post hydrolysis[d]	% Conversion **1**	% Conversion **2**
NLNM	0.64g	0.58g	9%	16.1g/L (0.24)	50.1%	42.8%
150U/g TvL	0.64g	0.58g	9%	18.1g/L (0.31)	56.1%	47.9%
150U/g TvL + 1-HBT	0.64g	0.58g	9%	19.6g/L (0.17)	60.9%	52.0%

[a] average from 6 measurements (SE 0.003) [b] average from 6 measurements (SE 0.004) [c] average from 6 experiments (SE 0.97) [d] following 72h hydrolysis with 3FPU GC220. Parentheses represent standard error of 3 biological replicates. 1: Estimated % conversion based on 50% cellulose prior to hydrolysis 2: Estimated % conversion based on 59% cellulose presuming 9% lignin removal by APE.

The data in Table 33 presents estimated cellulose conversions by two calculations. The first calculation assumed that the 9% loss of biomass during APE did not change the composition of cellulose prior to hydrolysis (therefore the substrate is 50%

cellulose). The second calculation assumed that the 9% loss did not include cellulose (e.g. it was lignin removal) and that the cellulose composition was 59% prior to hydrolysis (previous evidence of cellulose removal by APE, Figure 50, would make this unlikely). It was estimated that 100% conversion would yield 32.2g/L glucose *via* conversion 1. This is based on a 10ml hydrolysis reaction where 50% of 0.58g would yield 0.29g, therefore 2.9% cellulose. Complete conversion of this amount of cellulose (2.9%) can be calculated as follows: 2.9% x 1.11 (which takes into account the weight gained during cellulose hydrolysis i.e. glycosidic bond cleavage) which equals 3.22% and 32.2g/L. For conversion 2, a value of 37.7g/L is estimated. Although the results herein do not accurately represent exact conversions, they successfully demonstrate increased cellulose conversions following laccase and LMS treatments in line with existing studies.

4.2.9 Ethanol fermentation

Monosaccharides produced from biomass processing methods (such as enzymatic hydrolysis) are converted to ethanol by yeast fermentations. It is expected that the higher concentrations of glucose produced from biomass pretreated with the successful laccases and LMS presented in this work will yield higher concentrations of ethanol during fermentation. This assumption was briefly investigated by the demonstration of small-scale fermentation reactions using an industrial strain of *Sacchromyces cerevisiae* (Thermosacc) and hydrolysates from the saccharification reactions of TvL and TvL plus 1-HBT pretreated corn stover. Hydrolysates (containing the released glucose) were diluted 1:1 in a defined medium described by Roca & Olsson [314] to provide the nutrients required for growth of the yeast. Initial concentrations of glucose and the quantified concentrations of ethanol are presented in Table 34.

Table 34: Ethanol production from corn stover hydrolysates after laccase and LMS pretreatments with APE

Pretreatment[a]	[Glucose] g/L [b]	[Ethanol] g/L[c]
NLNM	12.68 (0.05)	8.86 (0.39)
500U/g TvL	13.16 (0.09)	9.59 (0.81)
500U/g TvL + 5% 1-HBT	14.29 (0.18)	10.24 (0.66)

[a] 2g d.w (5.5g w.w) acid pretreated corn stover, 5% consistency, pH 4.0, 200rpm, 28°C [b] concentration determined by HPLC-RID once diluted 1:1 with growth media following 48h hydrolysis with 12FPU GC220, [c] concentration determined by HPLC-RID according to ethanol standard curve.

As expected, the hydrolysates containing higher glucose concentrations as a result of the laccase treatments yielded higher ethanol concentrations (as determined by use of an ethanol standard curve, Appendix, Figure 82), demonstrating the beneficial application of laccase treated biomass. As this was a demonstration, the fermentations were performed in falcon tubes and not in optimised fermentation vessels. Various experimental conditions were trialled to promote an anaerobic environment to encourage anaerobic fermentation over aerobic (although it is known that *S. cerevisiae* can produce ethanol even under aerobic conditions [315,316]). However, due to the lack of an appropriate carbon dioxide removal system, the pH of the growth media was found to decrease over the fermentation time course due to the production of carbonic acid from dissolved carbon dioxide. This study was used to demonstrate increased ethanol production as a result of improved saccharification under non-optimised fermentation conditions. For higher ethanol yields, further work would be required with optimised fermentation systems.

4.3 Conclusions

Hydrolysis studies with a cellulosic substrate (Avicel) revealed an inhibitory effect of oxidised mediator species towards all three of the enzymes present in a typical cellulase preparation (endoglucanase, exoglucanase and β-glucosidase). The oxidised mediators displayed the greatest degree of inhibition towards β-glucosidase activity (within GC220) and this was demonstrated by impairment in cellulose hydrolysis as characterised by very low concentrations of glucose. The degree of inhibition was found to differ depending on the nature of the mediator species investigated (nitroxyl or phenolic). The same level of inhibition was not observed with the natural lignocellulosic substrate wheat straw. This is believed to be due to the alternative reaction sites offered by the substrate that are available for the oxidised radical species.

The saccharification of acid pretreated wheat straw was not improved following pretreatment with TvL with and without 1-HBT only. However, upon the incorporation of an APE step, saccharification was subsequently improved by the pretreatment with TvL and TvL with 1-HBT. This effect suggests that both enzyme treatments act on the biomass in such a way that the alteration or removal of lignin by APE is improved positively affect cellulose hydrolysis. Lignin derived phenolic compounds reported to act as mediators in nature did not improve wheat straw hydrolysis. Of the synthetic mediators tested, 1-HBT and violuric acid produced the greatest effects. TvL pretreatment with and without 1-HBT further demonstrated a potential for the improvement of saccharification by increasing hydrolysis when APE is replaced with a second pretreatment method responsible for lignin removal (organosolv).

In addition to TvL, three other laccases demonstrated an ability to improve biomass saccharification (PoL, AgL and RvL), with encouraging results observed with PoL. The increased saccharification of corn and sorghum stover with both TvL and PoL with and without 1-HBT opens up a potential to expand the applicability of the enzyme systems towards other lignocellulosic substrates.

Overall, a panel of laccases have been shown to improve the saccharification of cellulose and to theoretically increase ethanol yield when used in combination with mediators and existing biomass pretreatment methods.

Chapter five

5.0 Understanding the reactivity of laccase and a laccase-mediator system towards lignin

5.1 Introduction

5.1.1 General

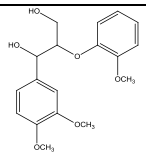
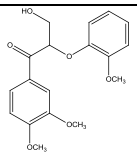
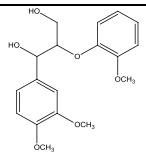
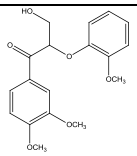
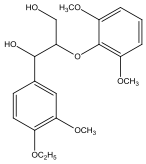
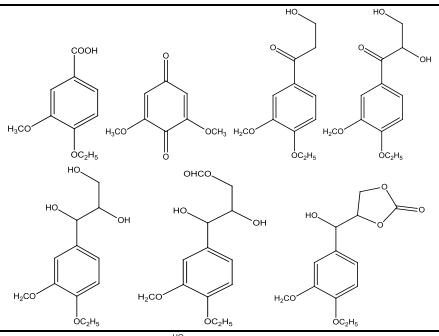
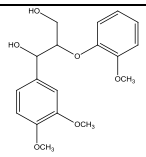
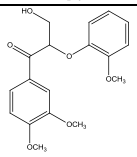
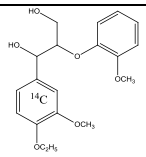
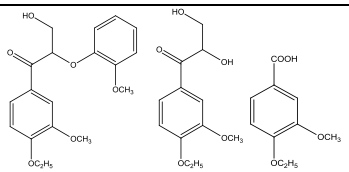
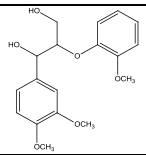
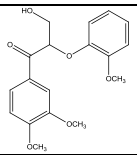
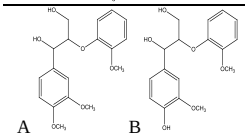
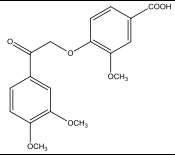
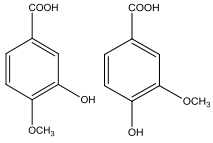
The oxidative function of *Trametes versicolor* laccase (TvL) with selected redox mediators was previously successfully demonstrated with the monomeric non-phenolic substrate veratryl alcohol (Chapter 3 Section 3.2.2). Expanding the substrate range towards lignocellulosic materials (wheat straw, corn and sorghum stover) revealed significant improvements in saccharification following quantification of the hydrolysed glucose from cellulose when pretreatment with TvL and three other laccases were applied (+/- mediator, Chapter 4 Sections 4.2.5-4.2.7). Despite the contribution of other chemical pretreatment methods into the bioprocess (e.g. acid hydrolysis, organosolv and alkaline-peroxide extraction), the only variation within the processing of each sample was in every case the addition of laccase or the laccase mediator system (LMS). The results strongly indicate that the consistently observed difference in glucose concentrations released between samples during saccharification was solely due to the effects of the enzymatic pretreatments. In order to ascertain and/or understand the mode of action of laccase towards lignin with and without a mediator, further analytical studies are required.

5.1.2 Lignin model compound studies

Veratryl alcohol provided a useful lignin model structure for screening the oxidative reactivity of a panel of synthetic and natural mediators with TvL (Chapter 3). However, the ability of the investigated LMS towards the oxidation of the alcohol did not always correlate to an improvement in the subsequent saccharification of the natural lignocellulosic substrate wheat straw (Chapter 4). A probable explanation for this could be the poor structural representation of veratryl alcohol to natural lignin due to its simple monomeric structure. Lignin is a heterogeneous and complex polymer biosynthesised by the radical polymerisation of coumaryl, coniferyl and sinapyl alcohols. Coupling of the monolignol radicals is favoured at the β position and yields a network of β-β, β-1', β-0-4 and β-5' linkages (in addition to 5-5', 4-O-5 and α-O-4 linkages) [37]. Substrates such as veratryl alcohol provide limited information into the delignification of lignin *via* inter-unit bond cleavage as they lack the presence of lignin linkages that are cleaved during degradation. The most abundant linkage within lignin is the β-O-4 which typically represents ~55% of the total linkages [317]. A good representative structure for the assessment of lignin degradation would include

this arylglycerol-β-arylether (β-O-4 linked) structural motif. In light of this, several researchers have already used β-O-4 linked dimers to evaluate the reactivity of laccases with different mediators as summarised in Table 35. In most cases, the major product detected is the Cα ketone derivative [150,256,317-319], however, LMS reactions have also revealed additional products that indicate oxidative cleavage of the dimer [176,319]. In this work, use of such structures offers the potential to provide an insight into the reaction(s) that might occur within the lignocellulosic substrates used in Chapter 4.

Table 35: Products identified in existing studies with β-O-4 dimers, laccase and mediators

Substrate(s)	Enzyme/ mediator	Product(s)	Reference
	*T. versicolor* laccase + ABTS		[150]
	Laccase (unnamed) + various mediators		[318]
	*T. versicolor* + 1-HBT		[176]
	*T. villosa*, *M. thermophila*, *P. cinnabarinus*, *B. cinerea* laccase +1-HBT and violuric acid		[256]
	*T. villlosa* laccase + 1-HBT		[319]
	*T. villosa* laccase + various mediators		[317]
 A B	*T. villosa*	A: No oxidation with laccase only B: Coupling reactions	[320]
	*C. unicolor* laccase + acetovanillone & acetosyringone		[321]

5.1.3 Lignin analytical techniques

Lignin model compounds such as β-O-4 dimers can be useful for providing information on the mechanistic actions of enzymes such as laccases towards lignin-composed substrates. Furthermore, analytical techniques also exist that can be used to extend our knowledge by enabling structural information to be obtained on substrates such as lignin before and after enzymatic treatments. These methods exist as either non-destructive (mostly spectroscopic) or destructive (those utilising chemical or thermal degradation and subsequent fragment analysis) techniques. A summary of the most commonly employed techniques and their advantages and disadvantages is presented in Table 36. In this work, pyrolysis-gas chromatography-mass spectrometry (py-GC/MS) both with and without the addition of tetramethylammonium hydroxide (TMAH) and Fourier Transform-Infrared (FTIR) spectroscopy with and without pyrolysis, were selected as analytical tools to support the β-O-4 dimer study for the investigation of lignin changes by TvL and the LMS of TvL with 1-hydroxybenzotriazole (1-HBT).

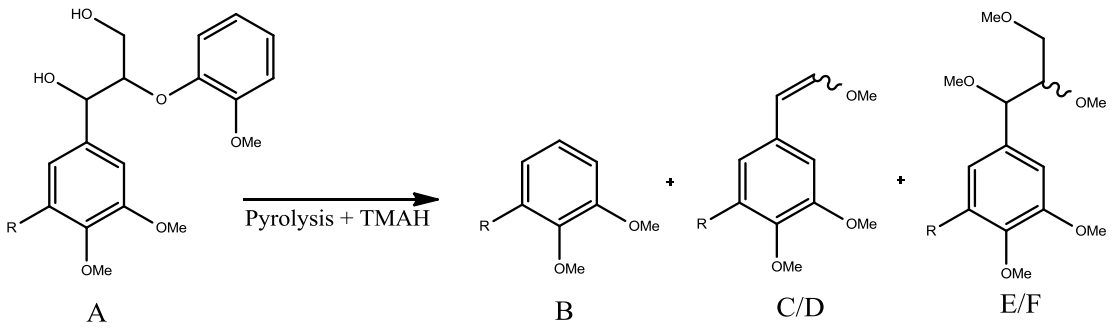
Table 36: Techniques commonly employed for lignin structural analysis and their advantages and disadvantages. Contents adapted from Brunow *et al*. [322,323], Adapa *et al*. [324] Gierlinger *et al*. [325] and Galletti *et al*. [326].

Method	Description	Advantages	Disadvantages
Acidolysis	Acid catalysed hydrolysis of arylglycerol-β-aryl ethers. Lignin refluxed in HCl or HBr in dioxane: water.	+ Small samples required + Simple degradation procedure	- Syringylglycerol-β-aryl ethers stable in acidiolytic cleavage reducing yield of syringyl type monomers
Thioacidolysis	Cleavage of arylglycerol-β-aryl ethers with BF_3 and ethanethiol. Monomers substituted with thioethyl groups (GC analysis). Dimers desulfurised by Raney-nickel reduction.	+ Higher product yields than acidolysis + Allow for detection of 5-5' linked dimers	- Incomplete cleavage - Foul smelling reagent
Permanganate oxidation	Methylation of phenolic groups. Side chain conversion to carboxyl groups attached to aromatic ring.	+ Provides structural information that is usually difficult to obtain	- Only analyses phenolic content - Error prone due to low monomeric acid yield - Laborious
Nitrobenzene oxidation	Lignin reaction with alkaline nitrobenzene. Produces aromatic aldehydes and to a lesser extent, acids.	+ High yield of degradation products + semi-quantification of G/S/H units	- Laborious - Error prone
Ozonolysis	Alkaline hydrolysis. Degradation of aromatic groups producing a mixture of intact side chain fragments for analysis by GC.	+ Allows for distribution of diasteromers to be determined	- Expensive
Derivatization followed by reductive cleavage (DFRC)	Selective β-aryl ether cleavage. Bromination of benzylic positions and acetylation of hydroxyl groups. Reductive cleavage of brominated intermediate.	+ Cleaner reaction conditions and simpler products than acidolysis	- Incomplete degradation
UV	UV-absorbance measured at usually 280nm for estimated lignin concentration.	+ No isolation or disruption of lignin required	- Exact structure and molar absorptivity of chromophores in lignin unknown
FTIR	Fourier Transform InfraRed absorption spectra collected on lignin.	+ Allows detection of differences between samples + Non-destructive and minimal sample prep	- Samples must be in dry form only -Quantification of monomers difficult
Py-FTIR	As above but with pyrolysis of the lignin before FTIR analysis.	+ As above but more structural information can be obtained	- Longer analysis times - Quantification of monomers difficult -Questionable reproducibility of derivatisation
Pyrolysis-GC/MS	Thermal fission of sample in absence of O_2. Liberated fragments detected by GC-MS.	+ Small samples required + Published identification of liberated lignin moieties for rapid identification	- Lengthy analysis times - Often requires pre-hydrolysis with chemical methods - Sample is destroyed
+ TMAH	As py-GC/MS but with TMAH derivatisation.	- As above but often more detailed structural information can be obtained	-As above but derivatisation agents such as TMAH are very toxic
NMR	Analysis of lignins and acetylated lignins by ^{13}C and HSQC NMR, phosphorylated lignin analysis by ^{13}P NMR.	+ ^{13}P NMR can be combined with DFRC for quantification of arylglycerol-α-ether linkage + Used collectively can provide structure of whole macromolecule	- Large sample size and high acquisition time of ^{13}C NMR - High cost of instrumentation and expert analysis required
Raman spectroscopy/ imaging	Monitoring molecular vibrations to produce spectra that can provide information of lignin structure. Sequential measurement of spectra by scanning adjacent points of sample can produce an image	+ Minimal sample prep + Analysis of very small samples	- Substances must be pure and concentrated due to lower sensitivity than IR

5.1.4 Pyrolysis-GC/MS

Detailed structural information can be obtained on complex biomacromolecules such as lignin by combining pyrolysis with gas chromatography (GC) and mass spectrometry (MS). During pyrolysis, the macromolecular structure of lignin is broken down by heat in an inert atmosphere (typically under nitrogen or helium gas) and the smaller volatile fragments or moieties liberated are transferred to a GC column where they are separated and identified by MS. A disadvantage of py-GC/MS is the reduced detection of highly reactive and polar moieties such as carboxyl (COOH) and hydroxyl (OH) groups [327]. To overcome this, thermally assisted hydrolysis and methylation (THM) methods have been developed (initially by Challinor [328]) that allow the methyl derivatives of compounds with these polar moieties to be detected [329]. The most widely used THM reagent is tetramethylammonium hydroxide (TMAH).

TMAH-THM followed by GC/MS has rapidly developed as a tool for characterising the relative proportions of lignin monomers in plant material [330]. Pyrolysis of lignin with TMAH is reported to induce cleavage of propyl-aryl-ether bonds (β-O-4) and the methylation of both aromatic and alkyl side chain hydroxyl groups [331]. Filley *et al*. [332] explored the mechanism of TMAH thermochemolysis towards lignin by treating a β-O-4 linked dimer with ^{13}C-labelled TMAH. The authors proposed that β-O-4 cleavage occurs *via* an intermolecular epoxide formation with phenoxide displacement with α or γ hydroxyl groups acting as nucleophiles in a nucleophilic reaction. This reaction was found to only occur when the β-O-4 bond contained an adjacent hydroxyl group to the alkyl side chain. Kuroda *et al*. [333] demonstrated THM pyrolysis with TMAH using the *erythro* β-O-4 dimer (**A**) in Scheme 16 and identified the cleavage products **B-F** in greatest abundance. Although the *erythro* dimer was of guaiacyl origin, the authors proposed the same mechanism for syringyl components.



B: R = H *(guaiacyl)* R = OMe *(syringyl)*
C/D: R = H *Z/E (guaiacyl)* R = OMe *Z/E (syringyl)*
E/F: R = H *erythro/threo (guaiacyl)* R = OMe *erythro/threo (syringyl)*

Scheme 16: Main products (**B-F**) derived from cleavage of a β-aryl ether linked dimer (**A**) by TMAH pyrolysis reported by Kuroda *et al*. [333].

Py-GC/MS with TMAH has been widely applied in research studying the degradation of lignin in the natural environment by pure cultures of fungi [334-336]. In this work, the technique was explored for the determination of structural changes within wheat straw lignin as a result of TvL and TvL + 1-HBT pretreatment prior to an organosolv lignin extraction. Figure 59 shows the set-up of the pyrolysis unit used with the GC/MS system.

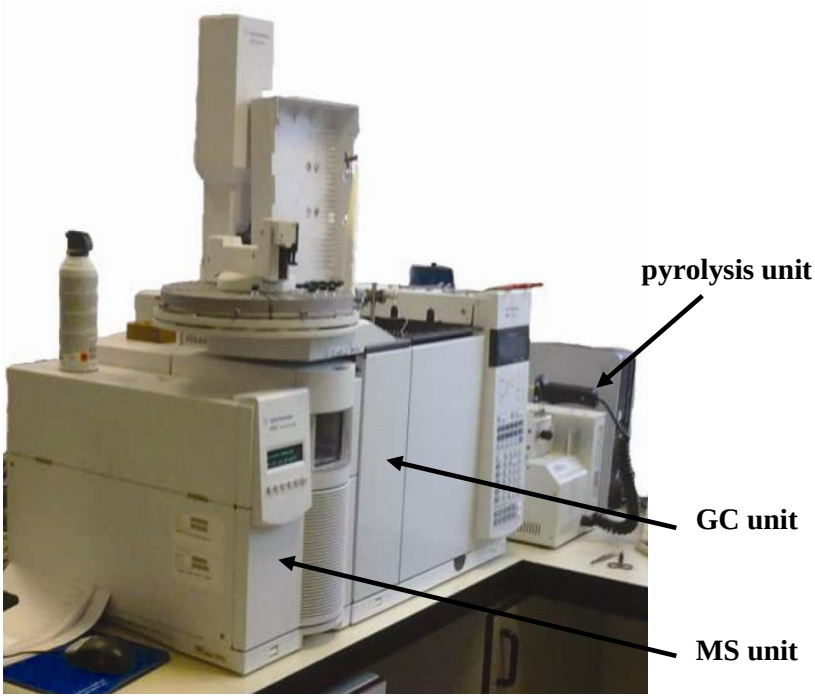


Figure 59: A labelled photograph of the pyrolysis-GC/MS set-up.

The identification of functional groups within lignin can be useful due to reports of an increase in aromatic acids within lignin during the decay of wood by fungi [337,338]. Differences in the proportions of specific lignin moieties released from laccase, laccase-mediator treated and untreated wheat straw lignin can provide information on the degradative state of the wheat straw as a result of these treatments. Py-GC/MS was additionally carried out in the absence of TMAH to obtain information that might provide further insight into the reactivity of the enzymatic pretreatments.

5.1.5 ATR-FTIR and pyrolysis FTIR

Fourier Transform-Infrared (FTIR) is a non-destructive spectroscopic technique employing mid infrared regions of radiation to enable molecular substituents of an irradiated sample to be determined by functional and fundamental vibrations [339]. FTIR has provided a successful platform for the analysis of biomass for example in the determination of the chemical composition of wood [340-342], the discrimination of wood from different tree species [343] and the distribution and structural modification of lignin within biomass [344-347]. Attenuated Total Reflectance (ATR) has been developed to improve the FTIR analysis of solid matter such as biomass derived materials by removing the requirement of sample preparation within a transparent KBr pellet [348]. Instead, the ATR crystal is pressed onto the sample, allowing the measuring beam to directly interact with the sample to enable direct reflection of the attenuated radiation to the spectrometer [339].

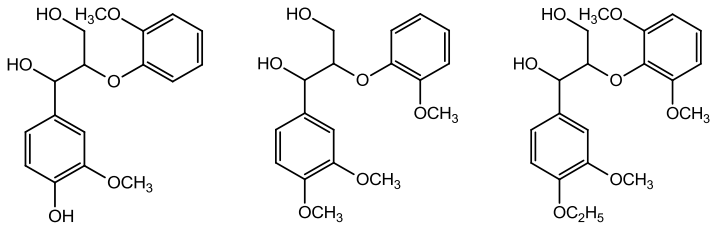
In this study, ATR was coupled with FTIR for the initial analysis of extracted lignin from wheat straw. Additionally, the pyrolysis unit used for the py-GC/MS analyses was also coupled to the FTIR to enable a ‘destructive’ method of analysis of the pyrolysed products from lignin, in a similar way to thermo-gravimetric analysis (TGA-FTIR). TGA-FTIR is a technique that has been applied to successfully analyse volatile compounds released within the vapours of pyrolysed substrates and has been demonstrated with lignin from pine [349], birch and fir [350]. The technique differs from the pyrolysis method investigated in this work since TGA-FTIR typically utilises a slower heating mode to enable the identification of released products over time. The method was investigated to establish whether more detailed structural information could be obtained from the pyrolytic breakdown of lignin.

Common to all lignin IR spectra is the broad band ~3500-3100cm^{-1} assigned to the O-H stretch of alcohol and phenol groups. Hegert [351] assigned the bands at 2920 and 2850cm^{-1} to the C-H stretch of methoxy groups, although Terajo *et al.* [352] split this region into two band assignments, 2960-2925cm^{-1} (C-H of CH_3 and CH_2) and 2850-2840cm^{-1} (C-H from C also attached to methoxy groups). Other consistently assigned bands for lignin include the unconjugated and conjugated carbonyl (C=O stretch) around 1720 and 1660cm^{-1} respectively, and the aromatic C-C stretch region 1600-1500cm^{-1} [351]. Assignments of the remaining bands within typical lignin spectra, in particular within the fingerprint region, are less straightforward due to many differences in band assignments as will be demonstrated and discussed along with the result interpretation.

5.2 Results and discussion

5.2.1 β-O-4 dimer degradation studies

β-O-4 linked dimers (**1-3**) (Figure 60) were used in an effort to gain an understanding of potential structural changes occurring within lignin as a result of laccase/LMS treatment. 1-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (dimer **1**) and 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol (dimer **2**) were commercially obtained whilst 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (dimer **3** *threo*: *erythro* 5:2) was synthesised by a modified method of Kawai *et al*. [353,354] as described in Chapter 7 Section 7.28.



1 **2** **3**

Figure 60: Structures of β-O-4 dimers **1-3** used in the study.

All 3 dimers were incubated with TvL both with and without 1-HBT. Immediate sampling of the reaction of the phenolic dimer **1** with TvL in the absence of 1-HBT and subsequent analysis by LC-MS revealed a product peak *m*/*z* 661 (Figure 61, middle chromatogram) consistent with oxidative dimerisation of **1**. After 24 hours, complete consumption of substrate **1** and the initially formed dimerisation product were observed (Figure 61, bottom chromatogram), presumably as a result of further polymerisation as anticipated with oxidised phenolic substrates. The same effect was observed when 1-HBT was included in the reaction (data not shown).

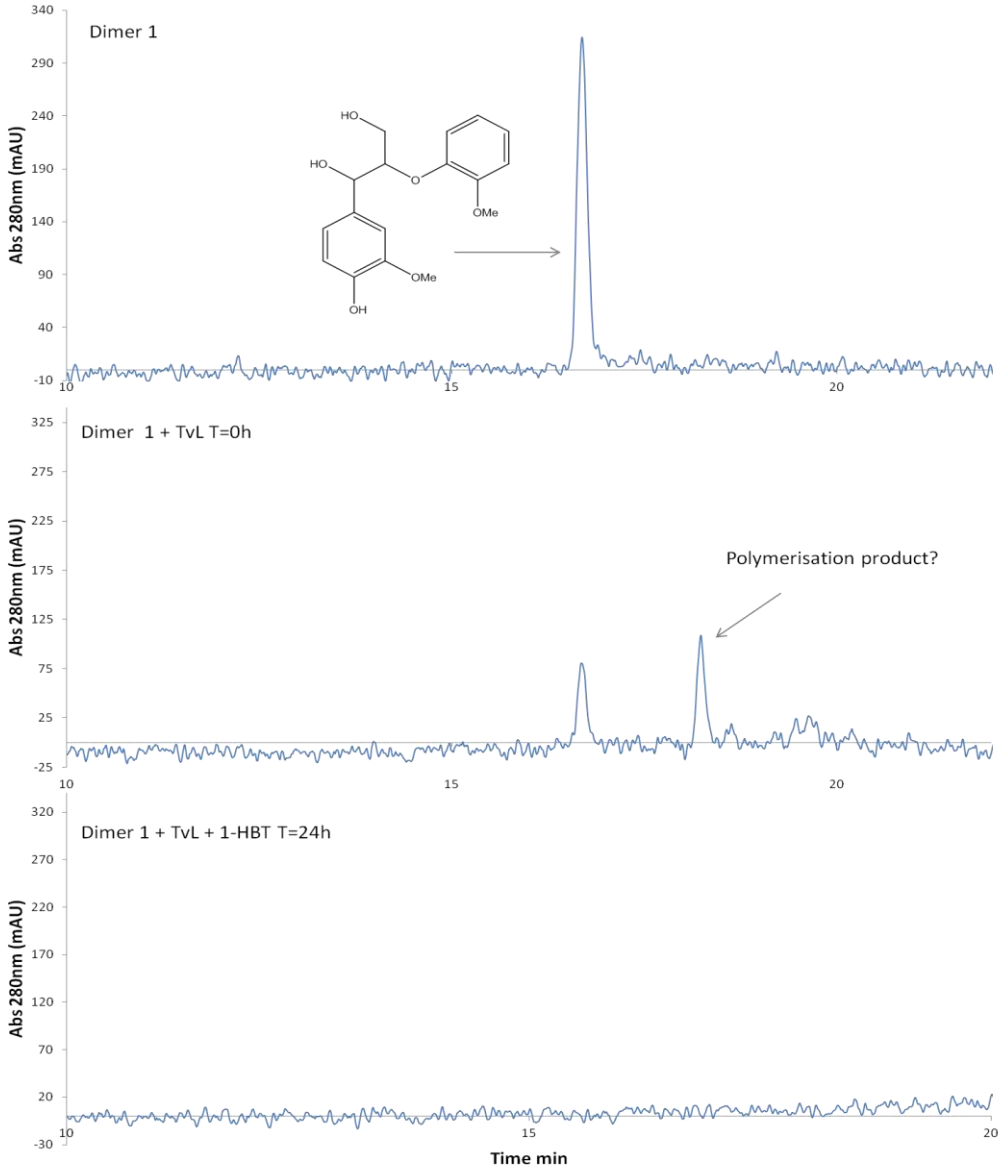


Figure 61: UV traces following the oxidation of dimer **1**. Top: The retention time of the standard compound of **1**. Middle: UV trace of dimer **1** upon addition of TvL whereby the disappearance of **1** is observed and a dimerisation product appears. Bottom: The complete disappearance of **1** after 24h incubation with TvL and no single products are detected.

As expected, the reactions of non-phenolic dimers **2** and **3** with TvL in the absence of 1-HBT failed to result in the formation of oxidation products, with only starting material observed by LC-MS. Consistent with previous studies [150,256], oxidation of dimer **2** with TvL in the presence of 1-HBT led to the formation of the uncleaved ketone product **4** as the sole oxidation product, providing evidence for a Cα hydroxyl oxidation mechanism (Figure 62). Ketone product **4** was confirmed following preparative scale HPLC and subsequent NMR analysis (Chapter 7 Section 7.30 and Appendix, Figure 86).

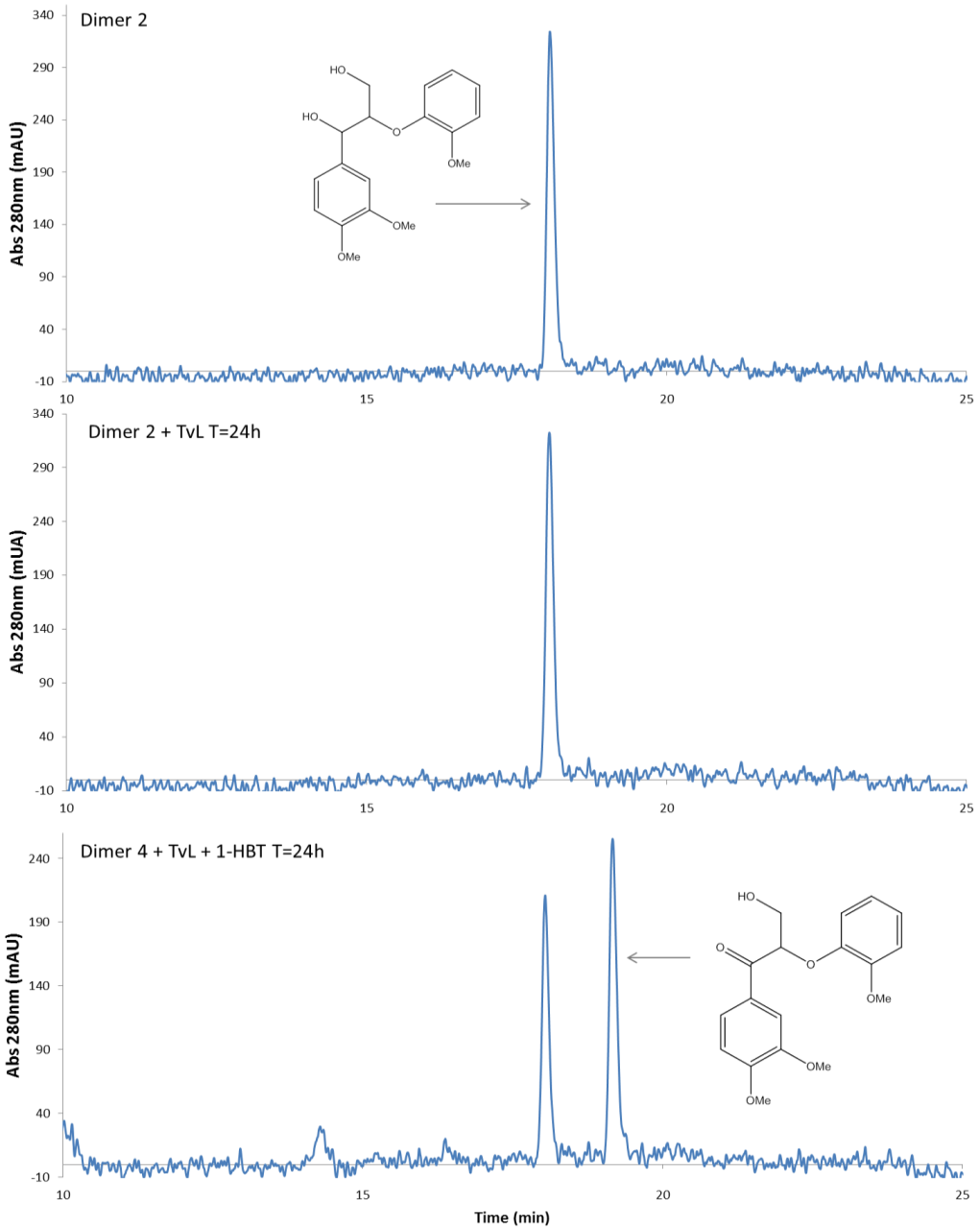


Figure 62: UV traces following the oxidation of dimer **2**. Top: The retention time of the standard compound of **2**. Middle: UV trace of **2** upon addition of TvL after 24h whereby no products are detected. Bottom: The reaction of **2** with TvL and 1-HBT whereby the Cα oxidation product **4** was identified after 24h (by preparative HPLC and subsequent NMR analysis).

The analogous Cα oxidation product ketone **5** was observed when dimer **3** was reacted with TvL + 1-HBT (as confirmed by comparison of both retention time and MS profile with that of an authentic standard), however, several other peaks were also observed on the LC trace at earlier retention times in addition to substrate **3** and ketone **5** (Figure 63).

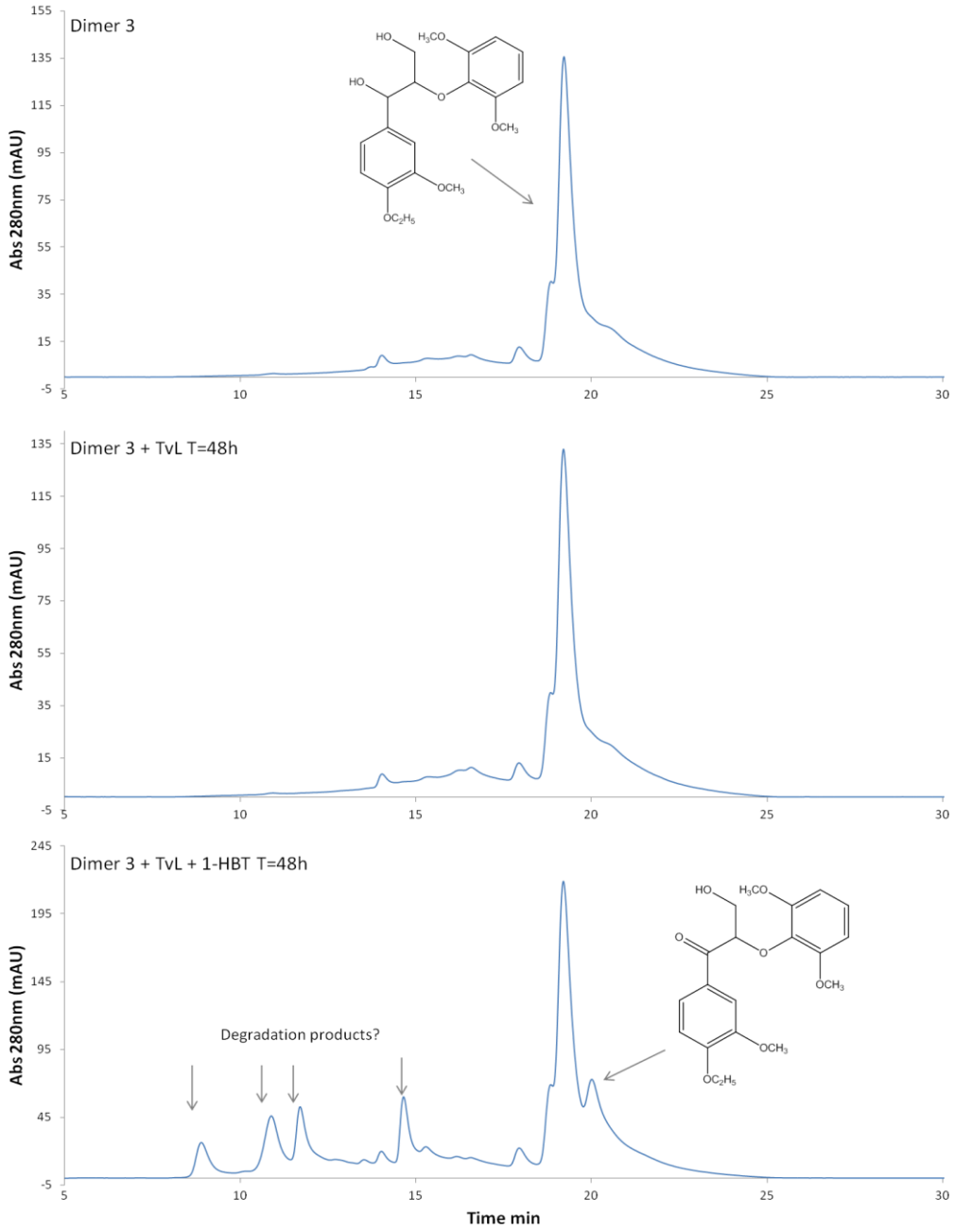


Figure 63: UV traces following the oxidation of dimer **3**. Top: The retention time of the standard compound of **3**. Middle: UV trace of **3** upon addition of TvL whereby no oxidation products are detected after 48h. Bottom: The reaction of **3** with TvL and 1-HBT whereby the production of a Cα oxidation product (ketone **5**) was observed (as confirmed by comparison of RT and MS profile with that of the authentic standard) in addition to products of greater polarity.

The additional peaks detected were not observed in the TvL only and untreated negative control and potentially represent a complex mixture of more polar degradation products. Presumably these degradation products are derived from oxidation of the more electron rich aromatic ring in dimer **3** which bears an additional methoxy substituent compared to dimer **2**. MS analysis allowed for the *m*/*z* values

detected in the peak areas to be compared against the suite of oxidation products characterised previously by Kawai *et al*. [176] following experimentation with the same dimer (dimer **3)**. This enabled potential identification of products **6**-**10** (*m*/*z* 265, 293, 263, 247 and 291) (Figure 64).

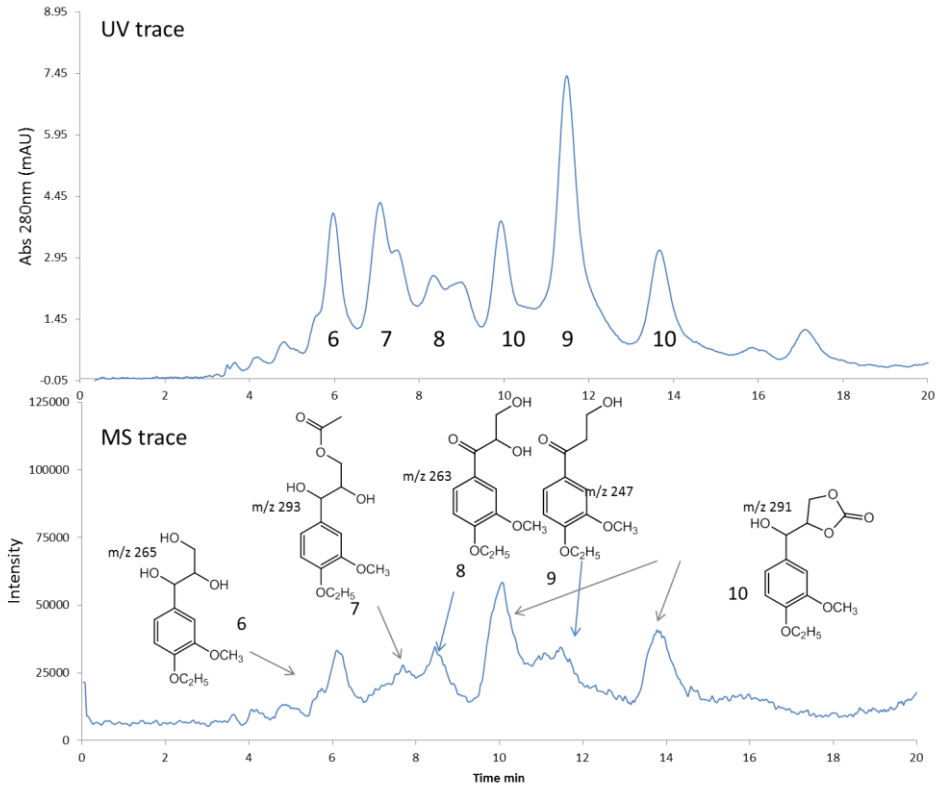


Figure 64: LC-MS traces from the oxidation of dimer **3** with TvL and 1-HBT. The top UV chromatogram represents the UV region whereby additional unconfirmed degradation products were eluted. The bottom chromatogram represents the corresponding MS trace to the UV chromatogram. Regions whereby particular masses corresponding to previously referenced degradation products were picked up are annotated by the possible structures and arrows.

The production of Cα oxidation products **4** and **5** from the non-phenolic dimers **2** and **3** may explain how the alkaline-peroxide extraction (APE) procedure (Chapter 4, Section 4.2.5.3, Scheme 15) resulted in the improvement of saccharification following LMS treatment. During APE, the hydroperoxide anions produced are reported to react with the carbonyl structures within lignin resulting in C-C bond cleavage [297,355]. An increased presence of carbonyl structures in lignin following treatment with TvL and 1-HBT may correlate with increased lignin degradation by C-C bond cleavage following APE. The β-O-4 studies described here, with non-phenolic dimers **2** and **3**,

may therefore assist to provide mechanistic insights into the role of a LMS towards lignin degradation.

5.2.2 Py-GC/MS with TMAH derivatisation

Pyrolysis-GC/MS with TMAH derivatisation was used to investigate and compare the composition of organosolv extracted wheat straw lignin following pretreatment with TvL (150U/g) and TvL + 1-HBT (5% w/w d.w WS). Wheat straw incubated with buffer and water was used as a negative control (no laccase and no mediator: NLNM). A collection of thermochemolysis products (of guaiacyl (G), syringyl, (S) and *p*-hydroxyphenyl (P) origin) were liberated from lignin samples and were identified by library searches (NSIT software) and by the comparison of peak positions and characteristic mass ions with published data [334], as outlined in Table 37.

Table 37: List of identifiable TMAH thermochemolysis products adapted from Vane *et al*. [334].

Label	Molecular weight	Assignment	Characteristic ions
G4	166	3,4-Dimethoxybenzaldehyde	151, 165, 166
G5	180	3,4-Dimethoxyacetophenone	137, 165, 180
G6	196	Methyl, 3,4-dimethoxybenzoate	165, 181, 196
S4	196	3,4,5-Trimethoxybenzaldehyde	125, 181, 196
G7	194	*cis*-2-(3,4-Dimethoxyphenyl)-1-methoxyethylene	151, 179, 194
G8	194	*trans*-2-(3,4-Dimethoxyphenyl)-1-methoxyethylene	151, 179, 194
G10	208	*cis*-1-(3,4-Dimethoxyphenyl)-1-methoxy-1-propene	165, 193, 208
P18	161	*trans*-3-(4-Methoxyphenyl)-3-propenoate	161, 192, 133
S5	195	3,4,5-Trimethoxybenzoate	195, 210, 139
S6	226	Methyl, 3,4,5-trimethoxybenzoate	226, 211, 195
G13	208	*trans*-1-(3,4-Dimethoxyphenyl)-3-methoxy-1-propene	91, 177, 208
S7	209	*cis*-1-(3,4,5-Trimethoxyphenyl)-2-Methoxyethylene	209, 224, 181
S8	209	*trans*-1-(3,4,5-Trimethoxyphenyl)-2-methoxyethylene	209, 224, 181
G14	181	*threo/erythro*-1-(3,4-Dimethoxy phenyl)-1,2,3-trimethoxypropane	166, 181, 270
G15	181	*threo/erythro*-1-(3,4-Dimethoxy phenyl)-1,2,3-trimethoxypropane	166, 181, 270
S10	223	*cis*-1-(3,4,5-Trimethoxyphenyl)-methoxyprop-1-ene	223, 238, 195
G18	222	*trans*-3-(3,4-Dimethoxyphenyl)-3-Propenoate	222, 207, 191
S14	211	*threo/erythro*-1-(3,4,5-Trimethoxy-phenyl)-1,2,3-trimethoxypropane	211, 181, 300
S15	211	*threo/erythro*-1-(3,4,5-Trimethoxy-propenoate	211, 181, 300

All of the lignin derived TMAH thermochemolysis products referenced by Vane *et al*. [334] were identified in the chromatograms with the exception of G10 (*cis*-1-(3,4-Dimethoxyphenyl)-1-methoxy-1-propene)) and S16 (*cis*-1-(3,4,5-Trimethoxyphenyl)-1,3-dimethoxyprop-1-ene)). Labelled chromatograms from wheat straw lignin pyrolysis following treatment in all three conditions are shown in Figures 65, 66 and 67.

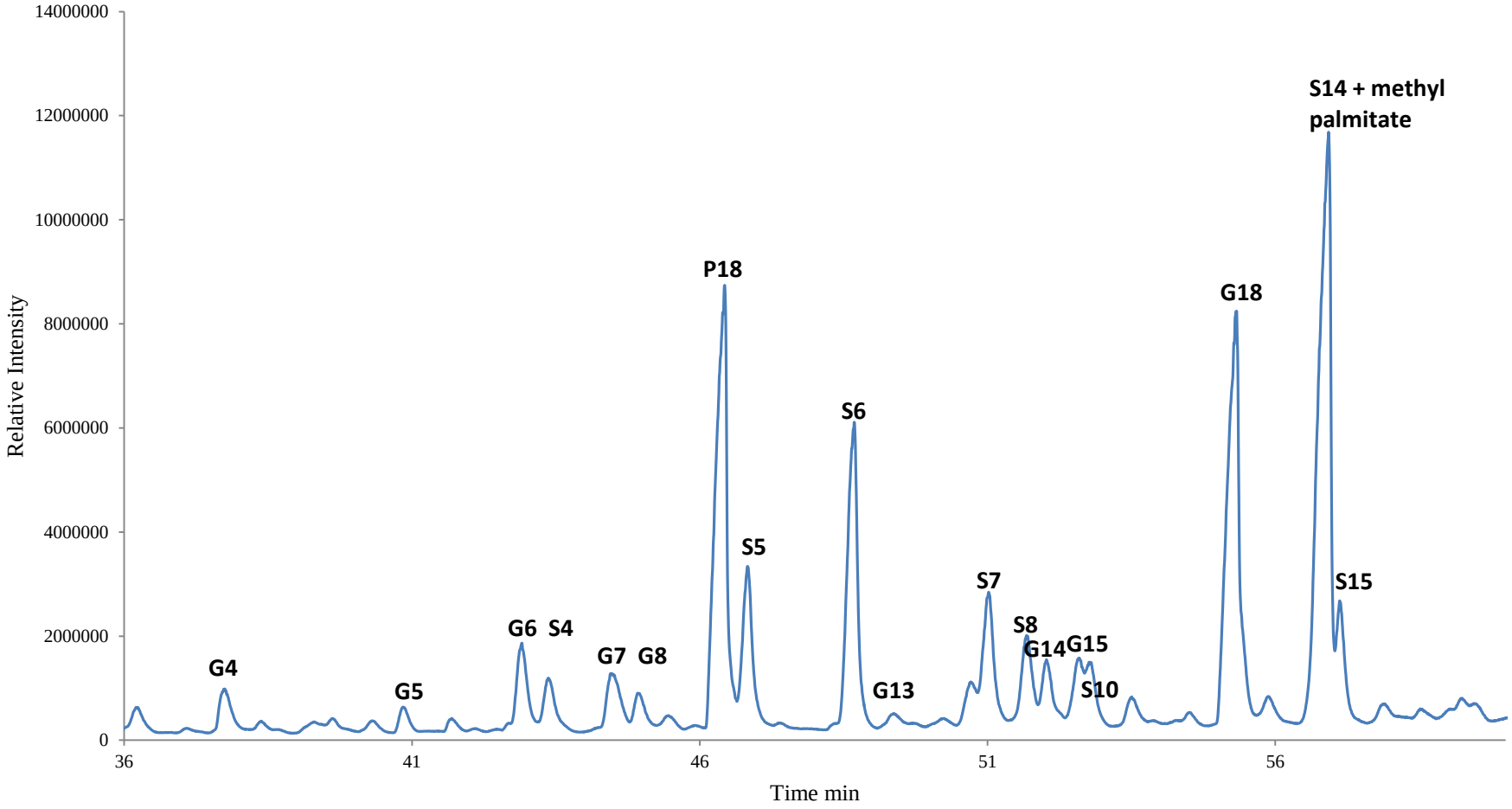


Figure 65: Partial chromatogram of the total ion current (TIC) for the TMAH thermochemolysis products from organosolv extracted wheat straw lignin with no laccase or mediator treatment (NLNM). Products were identified from the information in Table 37 and by library searches (G:Guaiacyl, S:Syringyl and P: *p*-hydroxyphenyl).

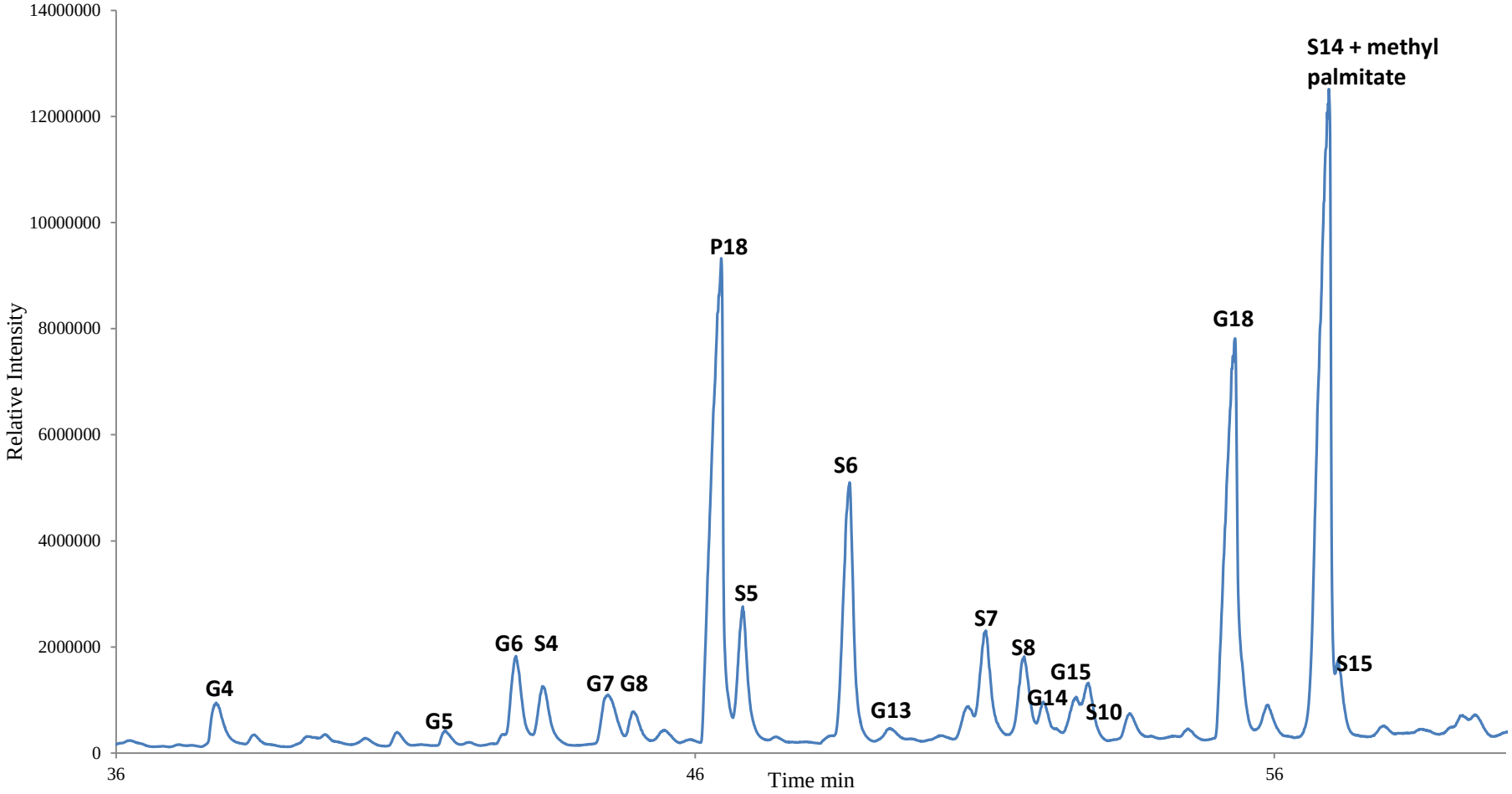


Figure 66: Partial chromatogram of the total ion current (TIC) for the TMAH thermochemolysis products from organosolv extracted wheat straw lignin with laccase treatment in the absence of mediator (TvLNM). Products were identified from the information in Table 37 and by library searches (G: Guaiacyl, S: Syringyl and P: *p*-hydroxyphenyl).

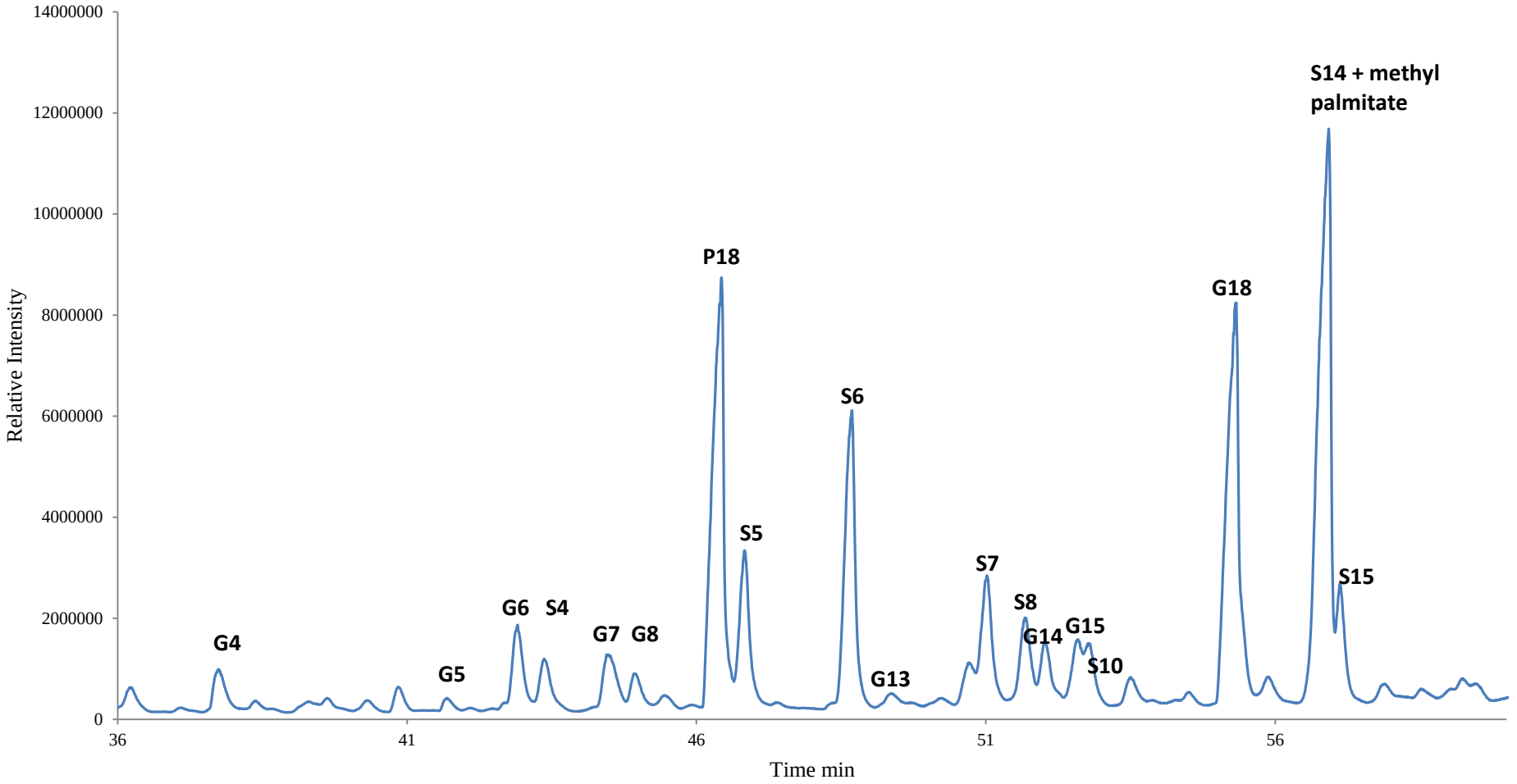


Figure 67: Partial chromatogram of the total ion current (TIC) for the TMAH thermochemolysis products from organosolv extracted wheat straw lignin with laccase treatment and 1-HBT (TvL+1HBT). Products were identified from the information in Table 37 and by library searches (G:Guaiacyl, S:Syringyl and P: *p*-hydroxyphenyl).

The identified structures are as shown in Figure 68.

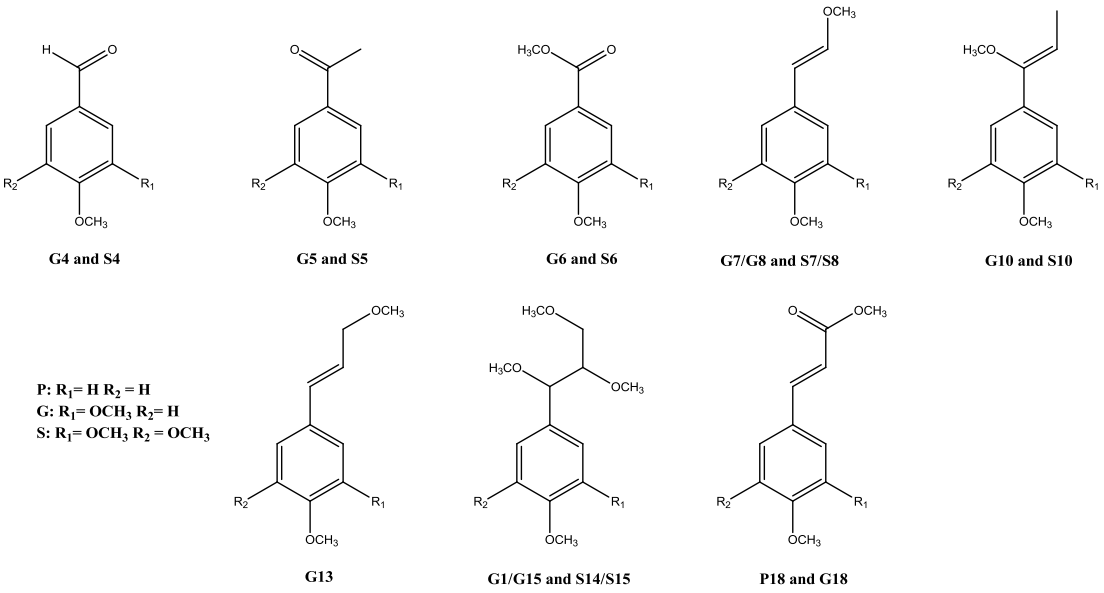


Figure 68: Structures of identified products from the TMAH thermochemolysis of wheat straw lignin. Structures are labelled as identified by mass spectrometric fragmentation patterns (Table 37) and as identified within the peaks in Figures 65, 66 and 67.

The analytical procedure (wheat straw pretreatment with TvL with and without 1-HBT followed by organosolv and py-GC/MS with TMAH) was repeated on two further occasions, both several months apart from the previous experiment and both using different batches of washed wheat straw. This was in order to obtain a triplicate data set and to generate consistent and reproducible conclusions (Appendix, Figures 88-93).

Overlaying the chromatograms from each treatment in each experiment allowed for the abundances of thermochemolysis products to be compared. The most significant observations from the total ion currents (TIC) that were consistent amongst all 3 separate experiments included the increased peak intensities of methyl 3,4,5-trimethoxybenzoate (**S6**) and methyl 3,4-dimethoxybenzoate (**G6**) from both of the laccase treated samples compared to the untreated negative control (NLNM). In all three cases, the **S6** peak intensity was further increased following the addition of 1-HBT and in 2/3 experiments, the same was observed for **G6** as shown in Figure 69.

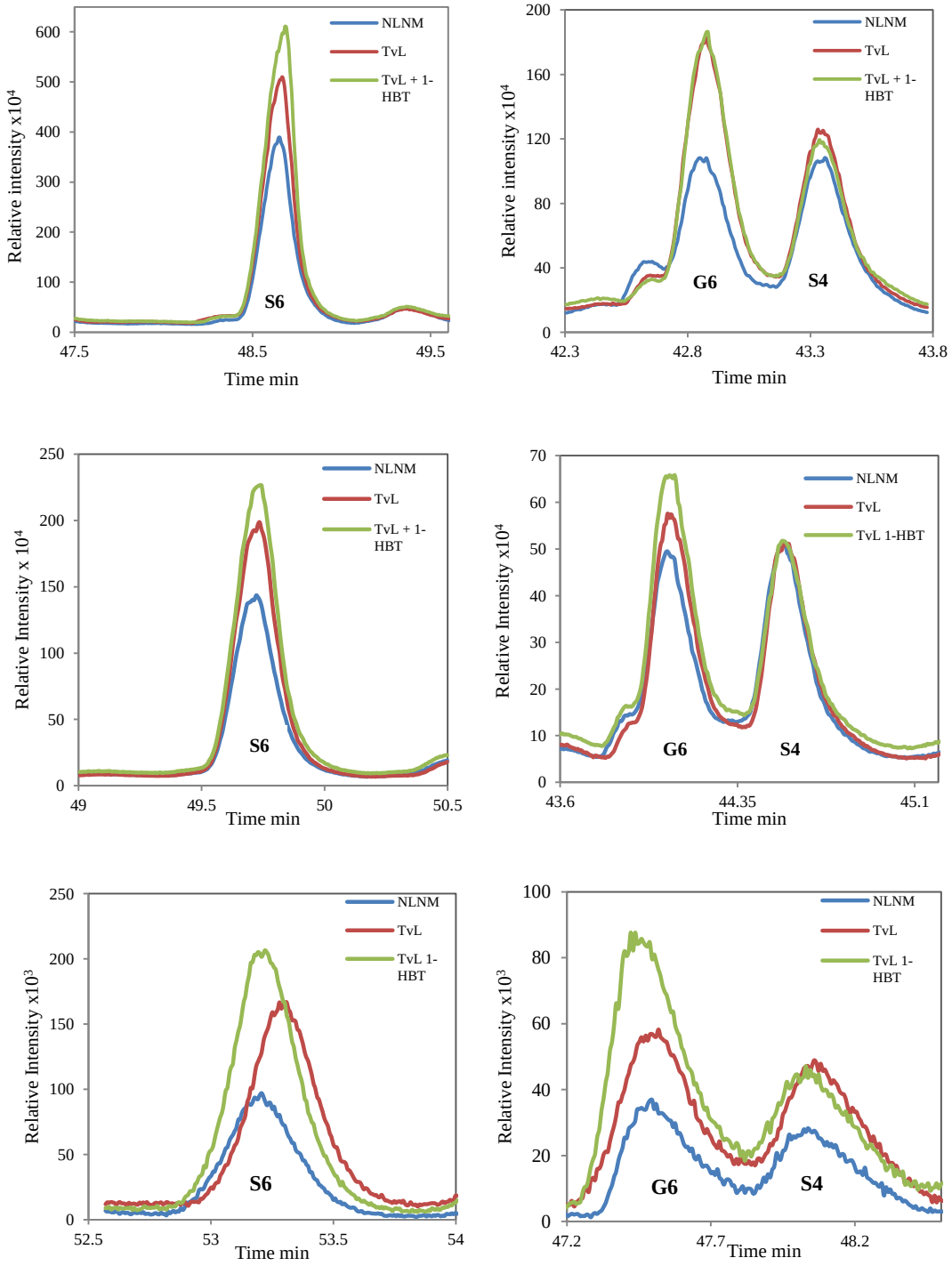


Figure 69: Partial chromatograms of the total ion current (TIC) for the TMAH thermochemolysis products methyl, 3, 4, 5-trimethoxybenzoate (**S6**), methyl, 3,4-dimethoxybenzoate (**G6**) and 3,4,5-trimethoxybenzaldehyde (**S4**) for both laccase and laccase mediator treated wheat straw lignins and the untreated control. The top, middle and bottom graphs represent results from each individual experiment (experiments were performed on 3 separate occasions).

The increased abundance of **G6** and **S6** from TvL and TvL + 1-HBT treated wheat straw lignin provides information regarding the actions of the enzymatic/chemoenzymatic treatments. Oxidative lignin degradation mediated by white rot fungi such as *T. versicolor* is reported to occur via Cα-Cβ side chain cleavage at the Cα position. This oxidative cleavage can lead to the production of aromatic aldehydes such as 3,4,-dimethoxybenzaldehyde (**G4**) and 3,4,5-trimethoxybenzaldehyde (**S4**) from alcohol groups which are reported to undergo further oxidation to their carboxylic acids (such as **G6** and **S6**) [223,334,356]. Researchers examining the ratio between the guaiacyl (G) and syringyl (S) unit acids (**G6** and **S6**) and the aldehydes (**G4** and **S4**) by calculating [Ac/Ald]G and [Ac/Ald]S have found increased ratios in fungal-degraded lignin compared to native lignin [334-336]. Increased ratios have also been found when fungal-degraded lignin was analysed *via* alternative methods such as solid state ^{13}C-NMR, alkaline CuO and nitrobenzene oxidation [332,356,357]. Calculation of these ratios in the analyses presented in Figures 65, 66 and 67 revealed an increase in the [Ac/Ald]G ratio from 0.79 (NLNM) to 1.22 (TvL) and a further increase to 1.55 (TvL+1-HBT). A similar trend was observed in the case of [Ac/Ald]S whereby the ratio increased from 3.44 (NLNM) to 4.91 (TvL) and 5.69 (TvL+1-HBT). This increasing trend of [Ac/Ald] ratios for both G- and S-type lignin structures following laccase and LMS treatment was consistently observed in all 3 experiments (Table 38). This observed trend could potentially provide supporting evidence towards the Cα-Cβ oxidative lignin cleavage mechanisms reported by fungal enzymes [223,334]. However, it is more likely that the increased [Ac/Ald] ratios are a result of the formation of Cα oxidation products previously demonstrated to be produced by laccase-mediator treatments (Section 5.2.1). Cα oxidation may occur in the absence of a synthetic mediator in natural lignin by the *in situ* generation of reactive natural mediators.

Table 38: [Ac/Al] ratios of both G and S units from organosolv extracted wheat straw treated with and without TvL and TvL with 1-HBT

	Treatment[a]	[Ac/Al]G	[Ac/Al]S
Exp 1	NLNM	0.79	3.44
	150U/g TvL	1.22	4.91
	150U/g TvL+1-HBT[b]	1.55	5.69
Exp 2	NLNM	0.76	3.96
	150U/g TvL	1.67	5.21
	150U/g TvL+1-HBT	1.82	6.97
Exp 3	NLNM	1.13	4.80
	150U/g TvL	1.67	5.36
	150U/g TvL+1-HBT	1.82	7.62
		Average increase in [Ac/Al]G	Average increase in [Ac/Al]S
	NLNM to TvL	0.63 (0.15)	1.09 (0.27)
	NLNM to TvL + 1-HBT	0.84 (0.11)	2.69 (0.23)
	TvL to TvL + 1-HBT	0.21 (0.06)	1.6 (0.43)

[a] Incubation of wheat straw for 40h, at 28°C, pH 4.0 and 200rpm [b] 5% (w/w) d.w biomass. Parentheses represent standard error of three biological replicates.

Aside from the observed increase in intensity of both the **G6** and **S6** peaks following laccase and LMS pretreatment, further differences in the peak intensities of other products were not consistently observed in all three replicate experiments. Overlaying the chromatograms of thermochemolysis products offers a quick and useful method of identifying changes in product abundance, however, despite subjecting the same mass of substrate (in this case 1mg lignin) to the pyrolysis tube, inaccuracies associated with the weighing and transfer of such small quantities of substrate make this method of comparison unreliable and misrepresentative of product abundances. To overcome this, researchers have made use of other ratios in addition to the [Ac/Ald] ratio for G and S to establish differences between native and degraded lignin [334,336]. The S/G ratio has been used to provide information on the oxidative reactivity of S-type lignin moieties relative to G-type during lignin degradation/biomass fungal decay. In this study, S/G ratios calculated from the three separate experiments with wheat straw lignin failed to reveal a reproducible trend (Table 39), with both decreases and increases observed following laccase and LMS treatment. The inconsistency of the calculated S/G ratios limits a conclusion to be drawn for the preferential oxidative activity of TvL towards S- or G-type lignin moieties. It is assumed that the S/G ratio of lignin will decrease during oxidative lignin degradation due to the assumption that S-units are more susceptible to oxidative degradation than G-units (due to a lower redox potential and degree of condensation) [358]. Decreased S/G ratios have been

previously reported in fungal degraded biomass in support of this assumption [334,336,359,360], however, these referenced studies focus on lignin degradation by fungal systems and not by individual enzymatic systems. Skyba *et al*. [361] recently reported the preferential degradation of guaiacyl units in poplar wood by a brown rot fungus previously identified as secreting laccase [362]. Further to this, studies with laccase employing both synthetic and natural mediators have revealed an increase in S- derived monomers supporting G- type structure removal [180,182]. The increase in the S/G ratio following TvL + 1-HBT pretreatment in 2 of the experiments here is therefore not entirely unexpected.

Table 39: Calculated S/G ratio from the thermochemolysis products from wheat straw lignin after TvL treatment with and without 1-HBT

	S/G Ratio*		
	Experiment 1	Experiment 2	Experiment 3
NLNM	1.18	1.97	2.77
TvL	1.13	2.32	2.72
TvL + 1-HBT	1.01	2.24	2.82
Trend (relative to NLNM)	Decrease	Increase	Decrease/increase
* Calculated by (sum of **S4**-**S15**)/(**G4**-**G15**)			

Researchers have additionally made use of the C/G ratio (cinnamyl/guaiacyl) and r value (ratio of **G6** to the sum of **G14** and **G15**) to establish structural changes in degraded lignin. C/G ratios were previously reported to decrease in degraded wheat straw compared to native wheat straw [334]. *p*-Coumaric acid (**P18**) and ferulic acid (**G18**) cross link hemicelluloses to lignin by ether or ester bonds. A decreased C/G ratio could indicate cleavage of the hemicellulose linkages to lignin [334]. In this study, a decrease was calculated in both TvL and TvL + 1-HBT pretreated samples in 2 of the 3 experiments. The calculated r values were established for both S and G-type lignin moieties (**G6**/**G14**+**G15**, **S6**/**S14**+**S15**) and were found to be increased in both TvL and TvL + 1-HBT pretreated samples compared to untreated wheat straw lignin in 2 of the 3 experiments analysed. A previous study reported a much larger increase of r for guaiacyl moieties (r: 1.0 and 10.9 for native and degraded biomass respectively), however the study focused on degradation by growth of the fungal organism in the presence of the substrate, and not with isolated individual enzymes. **G14**, **G15** (*threo/erythro*-1-(1-(3,4-dimethoxyphenyl)-1,2,3- trimethoxypropane) and **S14**, **S15** (*threo/erythro*-1-(3,4,5-trimethoxy-phenyl)-1,2,3-trimethoxypropane) are

derived from lignin moieties with an intact alkyl side chain. It has been rationalised that a decrease in these products relative to products such as **G6** increases r and represents of a shortening of side chains by Cα-Cβ oxidative cleavage. White rot fungal decay is reported to be characterised by a reduction of lignin moieties with phenylpropane alkyl side chains such as **G14**, **G15**, **S14** and **S15** [334].

Table 40: Calculated C/G ratio and r from the thermochemolysis products observed in wheat straw lignin after TvL treatment with and without 1-HBT

	C/G Ratio*		
	Experiment 1	Experiment 2	Experiment 3
NLNM	3.26	4.28	3.16
TvL	3.19	3.90	1.41
TvL + 1-HBT	3.37	3.27	1.69
Trend	Decrease/increase	Decrease	Decrease
		Γ**	
NLNM	(G) 0.88 (S) 2.46	(G) 0.57 (S) 0.93	(G) 0.22 (S) 2.50
TvL	(G) 0.81 (S) 2.02	(G) 2.51 (S) 1.89	(G) 0.42 (S) 3.41
TvL + 1-HBT	(G) 0.88 (S) 2.46	(G) 1.24 (S) 1.30	(G) 0.52 (S) 6.56
Trend	Decrease/no change	Increase	Increase

* Calculated by (**P18+G18**/sum of **G4-G16**) **Calculated by(**G6**/**G14**+**G15**, **S6**/**S14**+**S15)**

Additional wheat straw samples were subjected to py-GC/MS with TMAH following pretreatment with either buffer (NLNLM), TvL, or TvL + 1-HBT without any other further pretreatment or extraction (APE or organosolv). The pyrograms contained a higher abundance of peaks at earlier retention times due to the presence of cellulose within the samples. Due to contamination of product **G4** by the increased concentrations of products eluting around ~38 minutes, the molecular ion *m/z* 166 was used and selected for its quantification. Calculation of the [Ac/Ald]G and S ratios initially revealed an increase following TvL and TvL + 1-HBT treatment (ratio increase from 1.66 to 2.38 and 2.62 respectively for G, and ratio increase from 2.66 to 3.34 and 3.44 respectively for S, which is lower than the increases observed following extraction). However a second investigation did not confirm this trend. Furthermore, reproducible trends were not observed for the S/G ratio in both studies making the information difficult to interpret. The identification of cellulose derived thermochemolysis products from previous work [327,329,363] allowed for cellulose/lignin ratios to be calculated (Appendix, Table 49). The cellulose/lignin ratio

was not found to increase during both experiments after TvL or TvL treatment with 1-HBT which would be expected if the enzymatic treatments were removing lignin. The information collected from these analyses can be used in part to provide support for the results found in Chapter 4 (Section 4.2.5.2) whereby saccharification was not improved by both TvL treatments in the absence of another pretreatment method such as APE or organosolv.

5.2.3 Py-GC/MS (no TMAH)

Py-GC/MS was repeated with organosolv extracted wheat straw lignin following pretreatment with buffer (NLNM negative control), TvL or TvL+ 1-HBT without TMAH. Pyrolysis fragments were identified by comparison of the mass spectral data from each peak with those of published lignocellulose derived pyrolysis products [326,364]. Figure 70 (A-C) shows the partial chromatograms (TIC) of the extracted lignins following treatment by all 3 pretreatment conditions (NLNM, TvL and TvL with 1-HBT). Table 41 details the products represented by each peak, together with the structure, *m/z* ions and the overall percentage of each product with respect to the total abundance of all products.

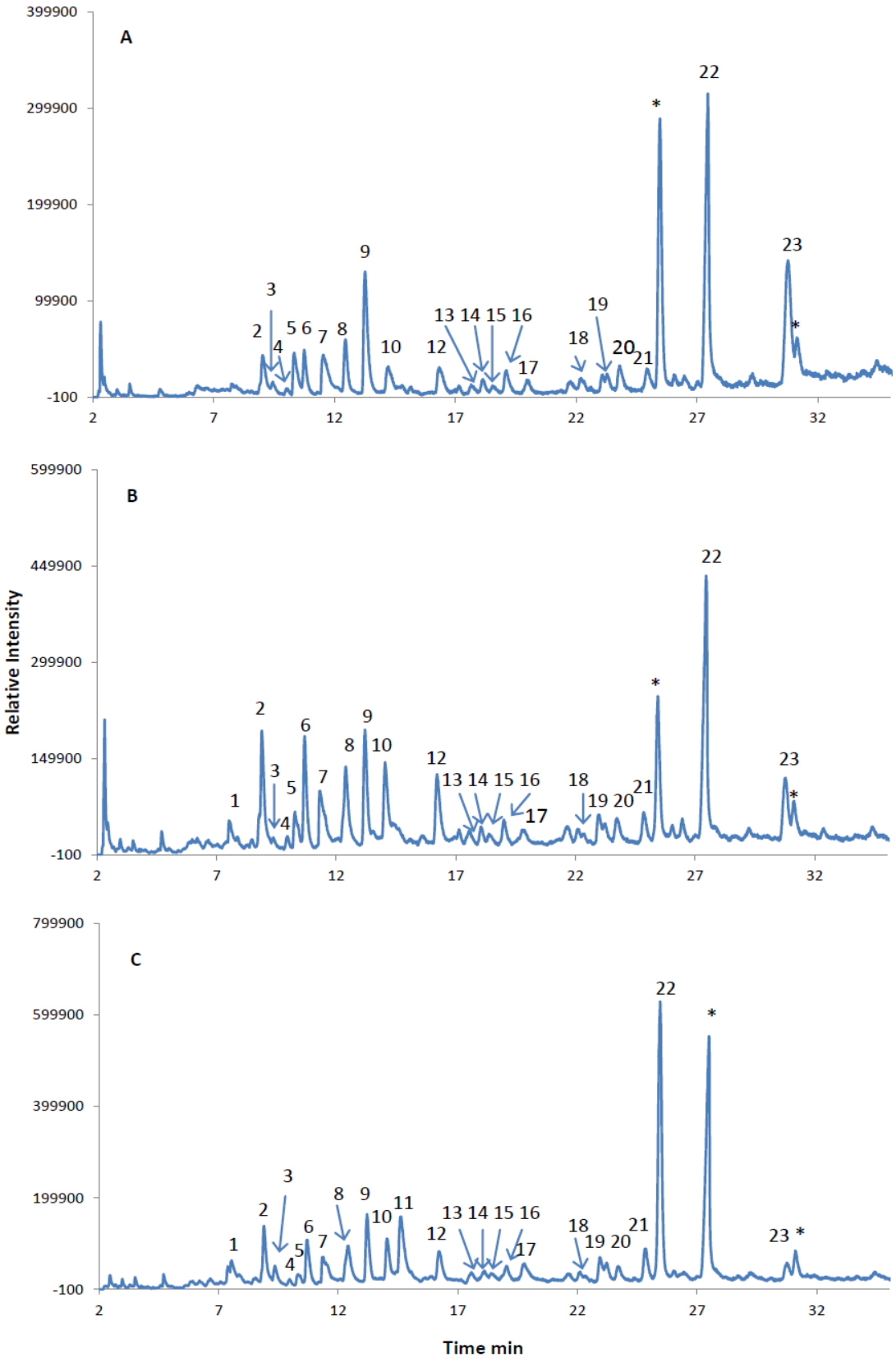


Figure 70: Partial chromatograms of the TIC showing the pyrolysis products from organosolv extracted wheat straw lignin following treatment with; A: no laccase or mediator (NLNM); B: TvL with no mediator and C: TvL with 5% 1-HBT. Peak identification is as according to Table 41.

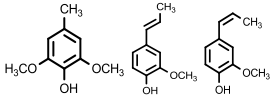
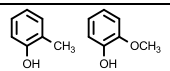
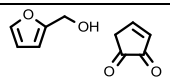
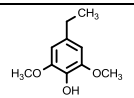
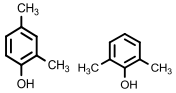
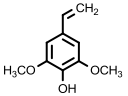
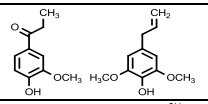
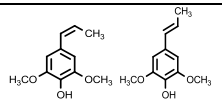
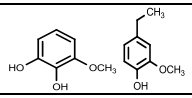
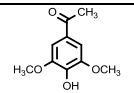
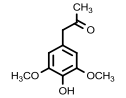
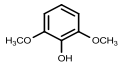
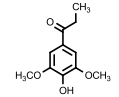
Peak No #	Identified product(s) /mass ions	Structure	% abundance[a]	Peak No #	Identified product(s) /mass ions	Structure	% abundance[a]
1	Phenol / 66, 94		In TvL and TvL + 1-HBT treated lignin only	12	2,6-dimethoxy-4-methylphenol / 125, 153, **168** Cis/trans isoeugenol77, 103, 131, 149, **164**		NLNM: 6.6 TvL: 9.6 TvL + 1-HBT: 7.5
2	2-methylphenol (*o*-cresol) / 79, 90, 107, 108 2-methoxyphenol (*o*-guaiacol) / 81, 109, **124**		NLNM: 6.7 TvL: 13.2 TvL + 1-HBT: 10.4	13	Acetovanillone / 123, **151,** 166		NLNM: 1.7 TvL: 2.0 TvL + 1-HBT: 2.6
3	2-hydroxymethylfuran / 81, 98 Cyclopent-1-ene-3,4-dione / 68, 69		NLNM: 1.2 TvL: 0.7 TvL + 1-HBT: 3.0	14	4-ethyl-2,6-dimethoxyphenol / 107, 167, **182**		NLNM: 2.1 TvL: 1.7 TvL + 1-HBT: 1.7
4	2,4/2,6-dimethylphenol / 77, **107**, 122		NLNM: 1.0 TvL: 1.1 TvL + 1-HBT: 1.1	15	Guaiacylacetone / 122, **137**, 180		NLNM: 1.4 TvL: 1.3 TvL + 1-HBT: 2.7
5	4-ethylphenol / 77, 91, 107, 122		NLNM: 6.9 TvL: 3.9 TvL + 1-HBT: 2.2	16	2,6-dimethoxy-4-vinylphenol / 122, 137, 165, **180**		NLNM: 4.3 TvL: 3.3 TvL + 1-HBT: 4.0
6	4-methylguaiacol / 95, 123, **138**		NLNM: 5.5 TvL: 10.4 TvL + 1-HBT: 9.3	17	Propiovanillone / 151, 180 4-ally-2,6-dimethoxyphenol / **194**		NLNM: 3.3 TvL: 3.3 TvL + 1-HBT: 1.2
7	4-vinylphenol / 65, 91, 120		NLNM: 10.0 TvL: 9.0 TvL + 1-HBT: 7.8	18	Cis/trans 2,6-dimethoxy-4-propenylphenol / 119, 151, 179, **194**		NLNM: 2.5 TvL: 1.1 TvL + 1-HBT: 1.1
8	3-methoxy catechol / 97, 125, 140 4-ethylguaiacol / 122, 137, **152**		NLNM: 8.2 TvL: 11.2 TvL + 1-HBT: 10.3	19	Acetosyringone / 153, 181, **196**		NLNM: 1.3 TvL: 2.1 TvL + 1-HBT: 3.2
9	4-vinylguaiacol / 77, 107, 135, **150**		NLNM: 22.3 TvL: 11.2 TvL + 1-HBT: 12.7	20	Syringylacetone / 123, **167**, **210**		NLNM: 4.9 TvL: 3.1 TvL + 1-HBT: 3.6
10	2,6-dimethoxyphenol / 96, 139, **154**		NLNM: 5.4 TvL: 7.8 TvL + 1-HBT: 7.9	21	Propiosyrigone / 123, 151, 181, **210**		NLNM: 4.7 TvL: 4.2 TvL + 1-HBT: 7.7
11	Unknown?		In TvL + 1-HBT treated lignin only	[a] % abundance expressed as % peak area of product(s) relative to total peak area of all other products 22: hexadecanoic acid 23: octadecanoic acid * Peaks did not represent lignocellulosic products that could be identified by literature comparison			

Table 41: Identified lignin derived products from wheat straw lignin +/- TvL and TvL + 1-HBT with their structures and fragment mass ions. % abundance of each product was calculated as a % of the total peak area of all identified products.

Almost all of the peaks were successfully identified as pyrolysis products derived from lignin (peaks labelled **1**-**21)**. In some cases two products were found to co-elute within the same peak area (peaks **2**, **12** and **14**). The identification of peak **11** could not be established by the NIST library search and comparison with published data despite the *m*/*z* information collected (mass ions 133, 105, 90, 71). This was unfortunate as peak **11** was only observed in the chromatogram of lignin following treatment with TvL + 1-HBT. Additionally, the product(s) within peak **17** could not accurately be determined due to the predominant mass ion observed (*m*/*z* 151) being present in several lignin derived pyrolysis products reported by Ralph *et al*. [364]. The two structures with the closest mass ion fragmentation patterns to those observed within the peak area were included as possible products for peak **17**.

The results were initially interpreted by the expression of each product peak as a percentage of abundance relative to the total peak area of all products as presented in Table 41. Peaks **1** and **11** were not included due to their absence in some of the chromatograms. Observation of the percentage increase/decrease of peak areas in this way revealed many changes following both TvL and TvL + 1-HBT treatments with the greatest including the increase in percent abundance of peak **2** (2-methylphenol & 2-methoxyphenol from 6.7% (NLNM) to 13.2% and 10.4% (TvL and TvL + 1-HBT respectively)), the decrease in the percent abundance of peak **5** (4-ethylphenol from 6.9% (NLNM) to 3.9% and 2.2% (TvL and TvL+ 1-HBT)) and the percentage decrease of peak **9** (4-vinylguaiacol from 22.3% (in NLNM) to 11.2% and 12.7% (in TvL and TvL + 1-HBT)). However, interpretation of the data in this way can be misleading due to co-elution of more than one product within one peak area and the risk of missing a decrease of a product by the increase of the other product within the same peak area. To account for this, and to allow for comparison of the data with that of a recently published study [365], products were also quantified by extraction of the single mass ion (SIM, as highlighted in bold in Table 41 for the specific products investigated). The SIM data was used to calculate the results presented in Table 42.

The ratios of S lignin pyrolysis products to G lignin pyrolysis products were calculated and a small increase in ratio was revealed from untreated (S/G: 0.45) to TvL (S/G: 0.62) and TvL + 1-HBT (S/G: 0.56) treated lignin. The significance of this increase is questionable without a repeated analysis however it is consistent with the small increases observed in two of the three studies previously carried out with

TMAH derivatisation (Table 42). The calculated ratios are much lower than those reported in a recent study by Du *et al*. [365]. This is most likely due to the referenced study using eucalyptus pulp with a higher S content than wheat straw. In the same study, an increased S/G ratio was reported following py-GC/MS without TMAH when *Myceliophthora thermophilia* (MtL) laccase was used with the phenolic mediator methyl syringate. This increase was not observed with *Pycnoporus cinnabarinus* (PcL) laccase and 1-HBT, suggesting that different laccase mediator systems may have different actions towards lignin. The results from this study provide further support, along with two other recent studies using py-GC/MS [182,366], against the assumptions that S units are preferentially attacked by laccase. Rico *et al*. [182] also reported an increased S/G ratio with (MtL) laccase in the absence of mediator as found in this study.

Calculation of the percent abundance of Cα carbonyl structures revealed little difference between the untreated lignin and TvL treated lignin in the absence of 1-HBT (13.2% and 13.3% respectively). However when TvL was applied with 1-HBT, the percentage of Cα carbonyl structures increased to 24.3%, a trend that was also observed in the study by Du *et al*. [365] with PcL and 1-HBT. The same study using MtL and methyl syringate did not reveal the same increase using py-GC/MS however a more recent study with this LMS reported an increase in Cα carbonyl structures when investigated by 2D NMR analysis [182]. This observation is in further support of the observations revealed from the data using β-O-4 linked dimers **4** and **5** (Section 5.1.2) whereby the reaction with TvL + 1-HBT produced a Cα carbonyl derivative of the dimers that was not detected with TvL in the absence of 1-HBT.

To establish whether Cα-Cβ cleavage had occurred, further calculations were performed to determine the proportion of phenolic structures with longer side chain carbons such as Ph-C2 (2 carbons on side chain) and Ph-C3 (3 carbons on side chain) (Table 42). A reduction of total Ph-C2 structures (S & G-type) was calculated following TvL treatment with and without 1-HBT suggesting Cα-Cβ cleavage, however this trend was not observed with the Ph-C3 structures, with an increase in the overall percentage of these structures calculated after TvL treatment with 1-HBT. The analysis of both Ph-C2 and C3 structures for S and G fragments separately revealed mixed results, with decreases in both S and G Ph-C2 structures observed following both TvL treatments (with and without 1-HBT) compared with the untreated control.

TvL treatment plus 1-HBT revealed an increase in G type Ph-C3 structures and a decrease in S type Ph-C3 structures (TvL treatment without 1-HBT also decreased S Ph-C3), as shown in Table 42.

Table 42: Calculations performed from the abundances of lignin pyrolysis products (SIM) identified from untreated lignin (NLNM), laccase treated lignin (TvL) and the LMS treated lignin (TvL + 1-HBT)

	NLNM[a]	TvL[a]	TvL + 1-HBT[ab]
S/G ratio	0.45	0.62	0.56
Cα carbonyl structure (%)	13.2	13.3	24.3
Total Ph-C2 (%)	53.9	35.2	35.2
Total Ph-C3 (%)	17.0	13.4	22.6
G Ph-C2 (%)	61.6	41.3	38.7
G Ph-C3(%)	14.2	14.4	27.6
S Ph-C2 (%)	36.7	25.4	29.0
S Ph-C3(%)	23.1	12.0	13.9

[a] Treatment with either no laccase or mediator (NLNM), or with 150U/g TvL +/- 1-HBT, 40h, 200rpm, 28°C, pH 4.0 [b] 5% 1-HBT (w/w d.w WS).

It is difficult to draw accurate conclusions regarding Cα-Cβ cleavage by TvL due to the inconsistent trends observed in the abundance of Ph-C2 and Ph-C3 structures. Overall, the data revealed that the abundance of both G and S lignin Ph-C2 structures decreased following wheat straw incubation with TvL with and without 1-HBT, suggesting that Cα-Cβ cleavage is possible in the absence of a supplemented mediator such as 1-HBT, or that cleavage may be possible due to the generation of *in situ* mediators that might be produced within the wheat straw lignin following laccase treatment (echoing the effect of natural mediators produced in the environment). Du *et al*. [365] reported a decrease in both Ph-C2 and Ph-C3 structures as a result of pretreatment with MtL and methyl syringate, however this decrease was not reported following PcL and 1-HBT pretreatments, again suggesting different mechanisms by each LMS. The data presented here for TvL with 1-HBT supports the proposed mechanism by Du *et al*. of Cα oxidation by PcL and 1-HBT and is in partial support of the Cα-Cβ cleavage mechanism by MtL and methyl syringate.

Another interesting observation from the py-GC/MS study included the % decrease in the *p*-hydroxyphenyl (H) Ph-C2 structures 4-ethylphenol (peak **5**) and 4-vinylphenol (peak **7**) after TvL treatment (6.9% to 3.9% peak **5** and 10.0% to 9.0% peak **7** after TvL). This reduction was even greater upon 1-HBT addition (peak **5** decreased to 2.2% and 7.8% for peak **5** and **7** respectively). Only two other possible H-derived structures could be detected within the pyrograms which were phenol (peak **1**) and

2-/4-methylphenol (observed as the small shoulder to the left of peak **2**). Interestingly phenol was not detected in the untreated lignin pyrogram (Figure 70A) and furthermore, 2-/4-methylphenol could not be quantified due to its presence in such small abundance (NB. to obtain quantitative information on 2-/4-methylphenol, a selected ion extraction at *m*/*z* 108 was used due to the high contamination of 2-methoxyphenol). In contrast, phenol was detected within the area of peak **1** in the TvL and TvL + 1-HBT treated lignins and the quantification of 2-/4-methylphenol (by selection of *m*/*z* 108) was possible and enabled percentages of 0.17 and 0.21% respectively to be calculated (relative to total peak area of all peaks and not the extracted ion data). The decrease in H type Ph-C2 structures and the subsequent increase in phenol and 2-/4-methylphenol (with reduced carbon side chain number) in both laccase treated wheat straw compared to the untreated lignin suggested potential Cα-Cβ cleavage by TvL +/- 1-HBT. These H-type structures were not investigated by Du *et al*. [365] however an increase in H units has been previously reported in a different study following fungal lignin degradation [367].

The decrease of 4-vinylguaiacol (peak **9**) reported in this study is consistent with observations reported by two other studies [201,368]. Saiz-Jimenez *et al*. [368] reported a decrease in *trans iso*eugneol in biodegraded spruce which was also observed in this study when quantification was performed *via* the selected *m/z* 164 (due to contamination of the product peak with 2,6-dimethoxy-4-methylphenol which increased following laccase treatment). Other observations reported in these studies include the decrease in 4-methylguaiacol (peak **6**) which in contrast appeared to increase in this work according to calculated % abundance and the decrease in 2,6-dimethoxy-4-vinylphenol (peak **16**) following flax pulp treatment with *P. cinnabarinus* laccase and 1-HBT as reported by Camarero *et al*. [201] which was also calculated to decrease in this study but to a much lesser extent.

5.2.4 FTIR of lignin

Lignin structural change as a result of TvL treatment with and without 1-HBT was further assessed by FTIR. The technique was performed in the first instance without pyrolysis (intact lignin) using ATR and secondly, coupled to pyrolysis (no ATR). Lignin from wheat straw previously incubated with buffer (NLNM), laccase (TvL) and the LMS (TvL plus HBT) was extracted by organosolv and dried prior to analysis. Figure 71 shows the ATR-FTIR spectra of all three extracted lignins without

pyrolysis, including an overlay of the 3 spectra (Figure 71b) and an analysis of the fingerprint region (Figure 71c). The fingerprint region ~1500-600cm^{-1} is more difficult to analyse due to most bands arising from vibration modes from more than one contributing vibration. Band assignment is shown in Table 43 as previously reported by Terajo *et al.* [352].

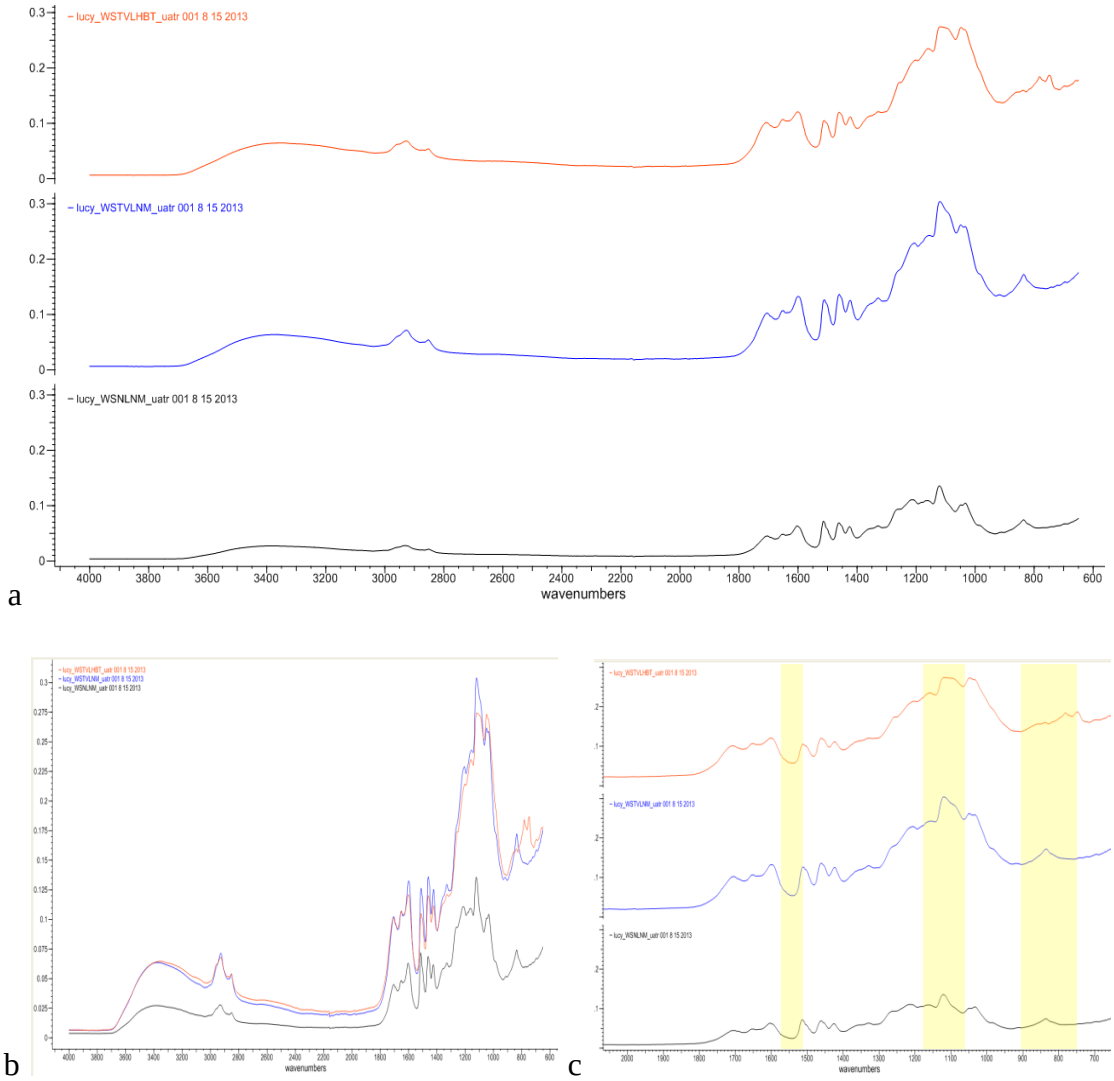


Figure 71: ATR-FTIR spectra of three organosolv extracted lignins from wheat straw following treatment with buffer (NLNM, negative control, black), TvL only (blue), and with the LMS TvL + 1-HBT (red); a) Full spectra of all three treated lignins; b) All 3 spectra overlaid; and c) The fingerprint regions of the spectra from all 3 treatments, key differences between the 3 spectra are highlighted in yellow (Y axis for a, b and c = Absorbance).

Table 43: FT-IR band assignment by Tejado *et al*. [352].

Band (cm^{-1})	Vibration	Assignment
3420-3405	st O-H	Phenolic OH + aliphatic OH
2960-2925	st C-H	CH_3 + CH_2
2850-2840	st C-H	OCH_3
1705-1715	st C=O	Unconjugated C=O
1650	st C=O	Conjugated C=O
1600	st C-C	Aromatic skeleton
1515	st C-C	Aromatic skeleton
1460	$\delta_{asymmetric}$ C-H	CH_3 + CH_2
1425	st C-C	Aromatic skeleton
1365	O-H	Phenolic OH
1326	st C-O	S
1270	st C-O	G
1220	st C-O(H) + C-O(Ar)	Phenolic OH + ether
1125	δ Ar C-H	G
1115	δ_{ip} Ar C-H	S
1030	st C-O(H) + C-O (C)	1^{st} order aliphatic OH + ether
855	δ_{op} Ar C-H	G
825	δ_{op} Ar C-H	S
810	δ_{op} Ar C-H	G

st: Stretching vibration δ_{ip}: In-plane deformation vibration δ_{op}: Out-of-plane deformation vibration

Analysis of the FTIR spectra was performed non-quantitatively by visual interpretation of the spectra. As observed in the overlaid spectra (Figure 71b), the majority of bands from lignin treated with TvL +/- 1-HBT appeared to be at a greater intensity/abundance than the untreated negative control. Despite using the same mass of lignin (5mg) for each analysis, it cannot be guaranteed that increases in band intensity are not a result of potential variation within sample mass, therefore differences in band height, shape and ratio relative to surrounding bands were compared in each sample.

The most noticeable differences between the three spectra include a decrease in the band at 1515cm^{-1} in the LMS treated sample, a change in ratio/distribution of bands in the 1140-1000cm^{-1} region between all three spectra and the diminished broad band around 835cm^{-1} in the LMS treated sample, which was followed by an introduction of two bands at 781 and 748cm^{-1}, which were not present in the other two spectra. The band at 1515cm^{-1} corresponds to the C-C aromatic skeletal vibrations and has been previously observed as diminished during the biodegradation of wheat straw by *Phanerochaete chyrosporium* [367]. The band at 1121cm^{-1} in the buffer treated

negative control significantly changed shape following TvL treatment with the peak developing a shoulder to the right (as observed in the middle yellow highlighted region within Figure 71c). This effect was more pronounced when 1-HBT was used with TvL, with the band appearing flatter due to the appeared reduction of the band at 1121cm^{-1} and the peak shifting to 1115cm^{-1}. According to Tejado *et al*. [352] this region represents the aromatic C-H in-plane deformation of guaiacyl (~1125cm^{-1}) and syringyl (~1115cm^{-1}) units. Changes in the ratio or shape of bands within this region could provide information into the proportions of both structures within lignin. In light of this the spectra implied that the guaiacyl content is decreased following TvL treatment, with this effect appearing further augmented following the LMS treatment. This observation provides further evidence towards the preferential attack of guaiacyl structures over syringyl by laccase, with previous results revealing inconsistencies to this trend (Tables 39 and 42). Durie *et al.*[369] however assigned the band at 1115cm^{-1} to the C-O stretch of aliphatic alcohols and ethers.

It is difficult to interpret the exact changes that occur regarding the disappearance of the band around ~835cm^{-1} in the LMS treated lignin that is present in the buffer and TvL treated lignin. This is because both guaiacyl (855cm^{-1}) and syringyl (825cm^{-1}) out of plane aromatic C-H vibration bands should be present within the region of 795-890cm^{-1}. According to the publication by Smith [370], this area is likely to represent *para* substituted aromatics (860-790cm^{-1}), with bands in the regions of 810-750 and 770-735cm^{-1} reported to represent *meta* and *ortho* substitution patterns respectively. The bands present in the TvL + 1-HBT treated lignin spectra at 781 and 748cm^{-1} may therefore represent C-H vibrations from *meta* and *ortho* substituted aromatics. It is not clear which precise structures are represented by the bands at 748, 781 and within 795-890cm^{-1}, or whether they are mono, di or tri-substituted. However the spectra suggests that lignin treated with TvL and 1-HBT has a lower proportion of *para*-substituted structures and an increased presence of *meta* and *ortho*-substituted structures when compared to the untreated and TvL only spectra.

Figure 72 shows the FTIR spectra from the same three treated lignins which in this instance were subjected to pyrolysis before FTIR analysis. Figure 73 shows the fingerprint region of the 3 spectra overlaid.

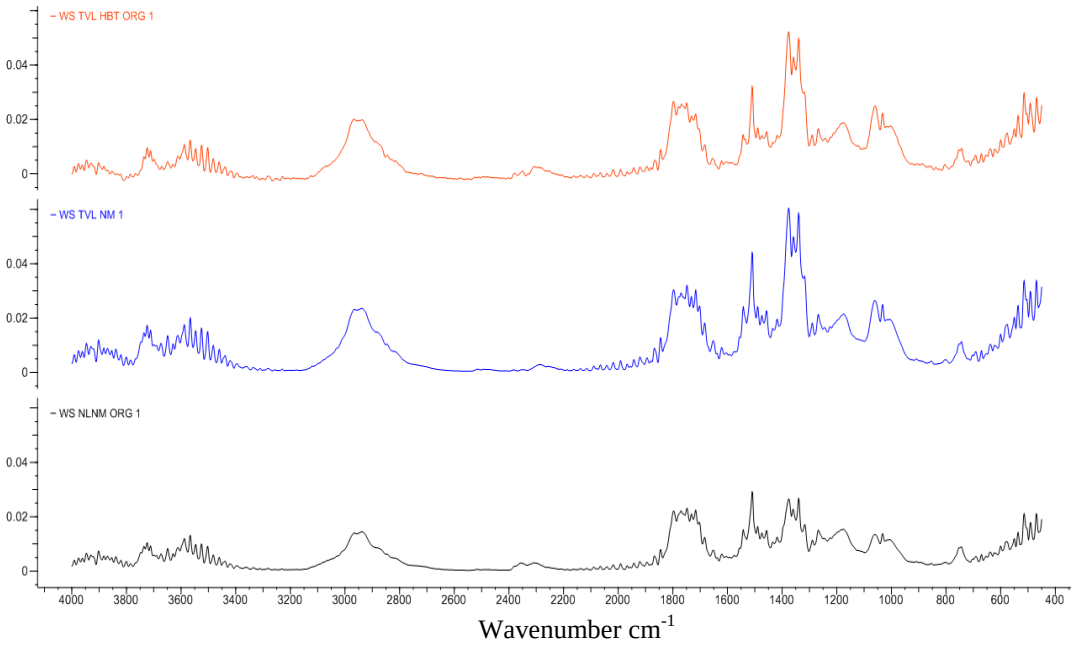


Figure 72: Pyrolysis-FTIR spectra of organosolv extracted lignin following wheat straw pretreatment with buffer (NLNM, negative control, black) TvL only (blue) and TvL with 5% 1-HBT (red) (Y axis = Absorbance).

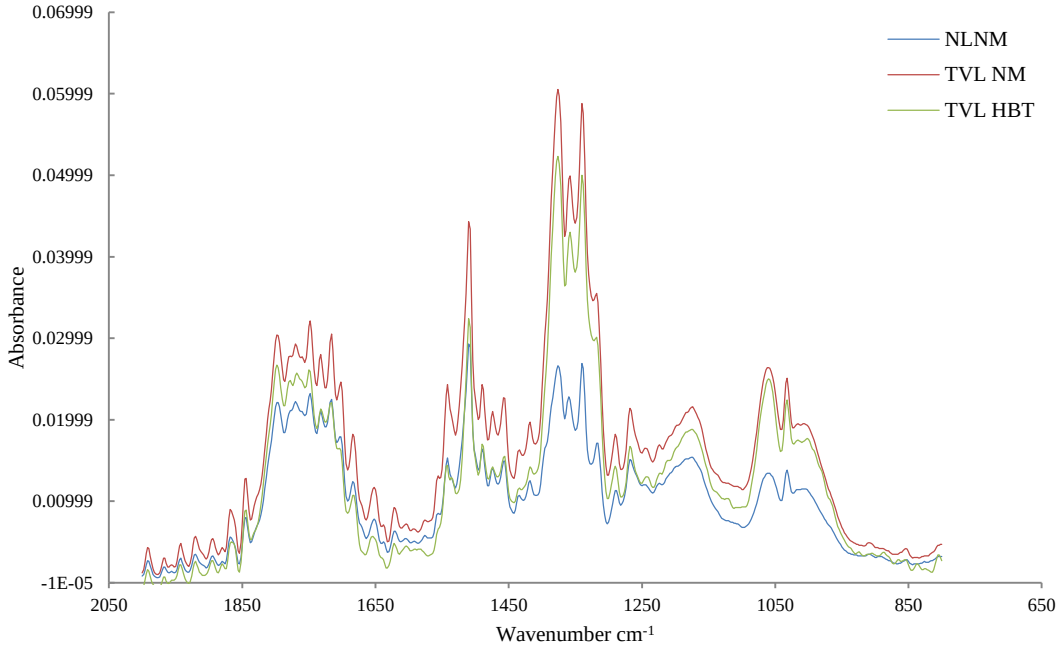


Figure 73: Pyrolysis FTIR spectral overlay of the fingerprint region of organosolv extracted wheat straw lignin following pretreatment with TvL (red), TvL + 1-HBT (green) or buffer (negative control, blue).

As with the spectra obtained without pyrolysis, a general increase in intensity for the majority of bands following TvL and TvL + 1-HBT treatment was observed. The

overlaid spectrum in Figure 73 demonstrates this, with the biggest increases in band intensity occurring in the 1400-1300cm^{-1} region. Visual interpretation of the 3 spectra revealed several differences regarding band shape and intensity in comparison with neighbouring bands; these are highlighted yellow as presented in Figure 74.

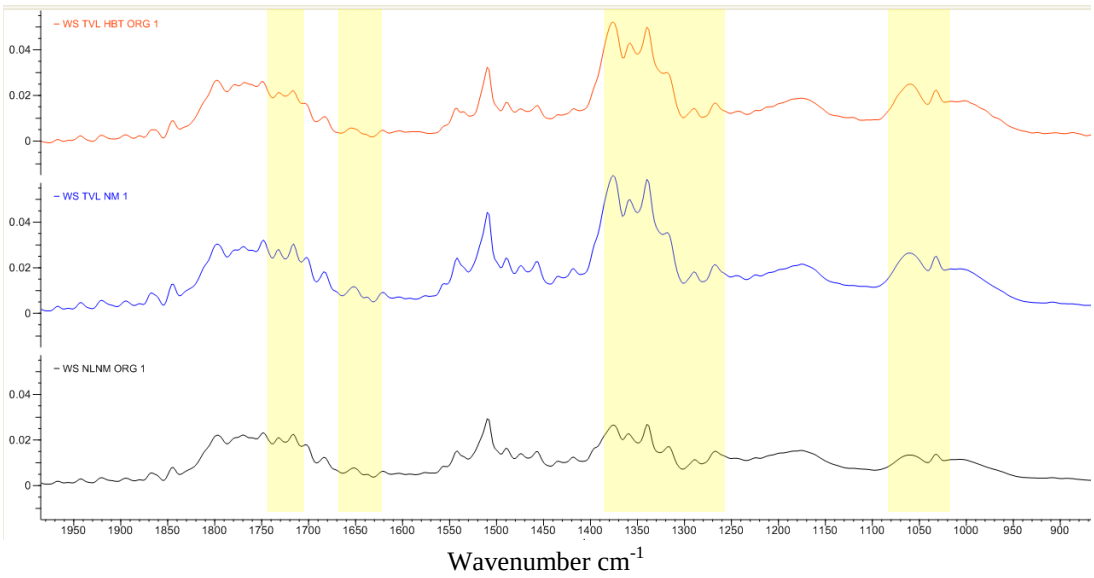


Figure 74: Pyrolysis-FTIR spectra of wheat straw lignin treated with buffer (black), TvL (blue) and TvL with 5% 1-HBT (red). Areas highlighted in yellow represent identified areas of spectral differences between samples (Y axis = Absorbance).

The FTIR spectrum generated from the pyrolysed lignin pretreated with the LMS revealed a decrease in intensity of bands within the region of ~1740-1700cm^{-1}. This region corresponds to the unconjugated C=O carbonyl stretch and was also found to be decreased in a previous study investigating lignin structure following TvL treatment with ABTS [345]. In the same study, an increase in the conjugated C=O stretch at 1646cm^{-1} was reported, however this effect was not observed in this study (conjugated C=O ~1652cm^{-1}). In fact, the TvL treated lignin (without 1-HBT) appeared to produce the greatest band intensity in this region. It was surprising to not observe a greater increase of this vibration in the LMS treated lignin due to the previously observed formation of the Cα carbonyl structure from non-phenolic β-O-4 structures treated with the same LMS (Section 5.1.2). However, a separate study with wheat straw reported this same band to be diminished following fungal biodegradation [367].

In the 1400-1300cm^{-1} region of the spectra (assigned generally as the O-H band from alcohols and phenols [350]), a degree of variation was observed between the 3 treated lignins with respect to the ratio of the bands at 1376, 1358 and 1340cm^{-1}. Specific assignments of the individual bands were not successfully achieved by the KnowItAll AnalyzeIt IR software although it was used to confirm the presence of hydroxyl groups around ~1365cm^{-1}. Sarknen *et al*. [371] previously assigned bands 1380 and 1340cm^{-1} to phenolic hydroxyls therefore the bands in this region may represent differences in phenol abundance due to enzymatic treatment. 1370-1376cm^{-1} has previously been assigned to the C-H bending in cellulose [372-374] which might suggest contamination of cellulose within the extracted lignin preparations.

Assignment of bands corresponding to the syringyl C-O stretch was difficult due to an absence of bands within the region of 1326cm^{-1}. Despite this, two peaks at either side of this wavenumber (1340 and 1316cm^{-1}) were prominent. The software was unsuccessful at assigning both peaks and contradiction was found within the literature, with some sources assigning wavenumbers 1340-1330cm^{-1} and 1340cm^{-1} to syringyl C-O stretches and syringyl ring vibrations [375,376] whilst others assigned the region of 1315-1317cm^{-1} to the syringyl C-O stretch [377-379], CH vibrations [380,381] or the CH_2 wagging of cellulose [372]. Due to the uncertainty of the band assignment within this region it was not possible to accurately conclude the exact changes that may have occurred to the lignin structure as a result of the enzymatic pretreatments. However, it can be suggested that the treatments generally increased the overall alcohol/phenolic content of the lignin due to the increase of the three bands (1376, 1358 and 1340cm^{-1}) within this region following treatment with TvL and TvL with 1-HBT.

The band at 1317cm^{-1} appeared of greater intensity from the spectra from lignin treated with TvL with and without 1-HBT, and additionally appeared as more of a shoulder peak to the right of the band at 1340cm^{-1}. The increase of this band with respect to surrounding bands when compared to the untreated lignin suggests an increase in abundance. If this band was to represent the syringyl C-O functionality then the spectra may support the observed decrease in guaiacyl structures as observed in Figure 71.

Additional observations from visual interpretation of the spectra include a change in ratio between bands at 1290 and 1268cm^{-1}, and 1063 and 1033cm^{-1}. The band at 1290cm^{-1} is referenced as the C-O stretch of phenols or esters [379], with 1268cm^{-1} representing the C-O stretch of guaiacyl structures [352]. It is difficult to establish whether the C-O guaiacyl stretch is decreasing in the laccase treated lignin spectra or if the band at 1290cm^{-1} is increasing. Changes within this region following laccase and LMS treatment may suggest attack of the guaiacyl structures; however the data in Figure 74 does not demonstrate this clearly. Both bands at 1063 and 1033cm^{-1} appear to have increased in height following laccase treatment with and without 1-HBT (Figures 73-74). The band at 1063 is reported to represent the C-OH deformation in cellulose [382] and the 1033cm^{-1} band has been assigned to the aliphatic stretch of C-O in alcohols and ethers [369]. In other work, the band area of 1030cm^{-1} has been assigned to represent the aromatic in plane C−H vibrations of guaiacyl units, with additional complex vibrations associated with the C-O, C-C and C-OH bends of polysaccharides [383]. The potential effect of cellulose contamination limits the interpretation of this band area with regards to lignin structural change.

The spectral region 960-720cm^{-1} was analysed to establish whether differences existed within the aromatic C-H out of plane deformations of syringyl and guaiacyl structures. Syringyl (S) and guaiacyl (G) bands were identified according to Table 43 and previous reports [352,384-386] as labelled in Figure 75.

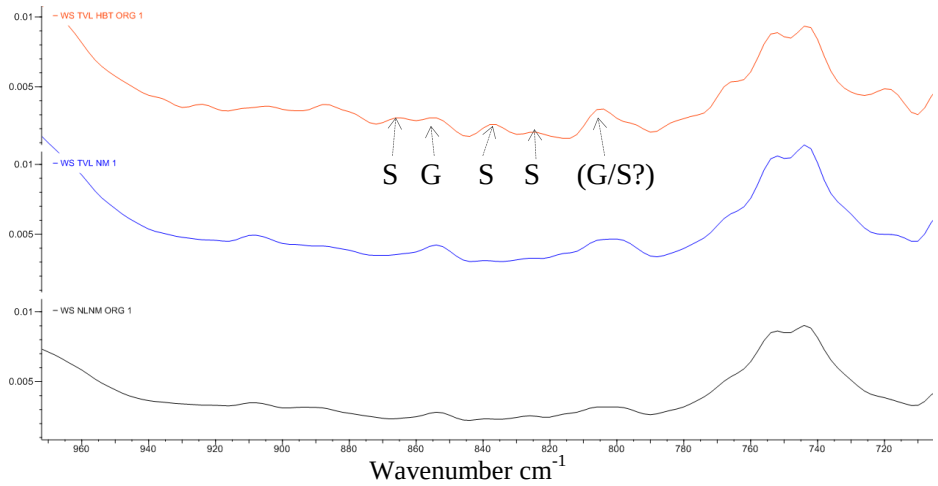
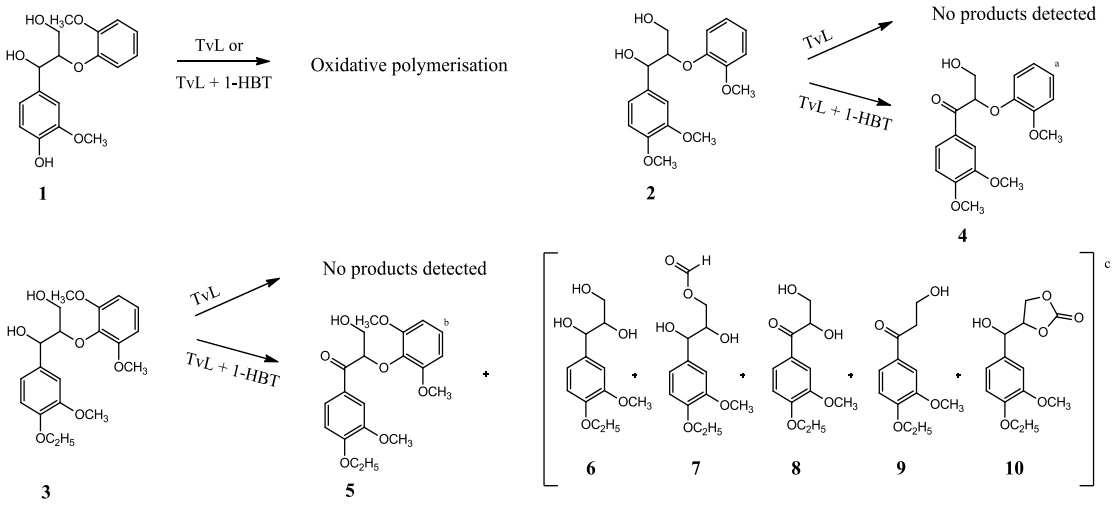


Figure 75: Py-FTIR spectra (wavenumbers 960-720cm^{-1}) from organosolv extracted wheat straw lignins treated with buffer only (NLNM, black), TvL (blue) and TvL + 5% 1-HBT (red). The bands are labelled underneath the spectra of TvL + 1-HBT treated lignin in an attempt to assign band regions as guaiacyl (G, at 855-853cm^{-1} and possibly 810cm^{-1}) or syringyl (S, at 863cm^{-1}, 834-833cm^{-1}, 825cm^{-1} and possibly 810cm^{-1}) structures (y axis= intensity).

Comparison of spectra within this region for all three lignin samples revealed a presence of more bands in the LMS treated lignin (Figure 75, red) than observed in the TvL and buffer treated lignins. Assignments of these additional bands suggest an increase in syringyl type out of plane aromatic C-H vibrations. These data therefore provides evidence of an LMS treatment affecting the proportion of syringyl and guaiacyl structures within lignin. Although an increase of syringyl structures was evident, the guaiacyl band at ~855cm^{-1} did not subsequently appear decreased. The band at 810cm^{-1} was not assigned to represent the guaiacyl structure despite the information referenced in Table 43. This is because there was contradicting information within the literature regarding the assignment of 810cm^{-1} including its reference as a syringyl structure [387].

5.3 Conclusions

Three analytical strategies (β-O-4 linked dimers, py-GC/MS and FTIR) were explored with a view to examine and identify potential structural changes within lignin as a result of treatment with *Trametes versicolor* laccase with and without the synthetic mediator 1-HBT. The reaction of a phenolic β-O-4 linked dimer with TvL demonstrated the role of laccase in the oxidation of phenolic groups and the production of dimers as a result of spontaneous coupling reactions between phenoxyl radicals. The inability of laccase to oxidise non-phenolic lignin structures was confirmed using 2 non-phenolic β-O-4 linked dimers. The reaction of 1-HBT with TvL with the same substrates produced a Cα carbonyl derivative of both dimers, providing evidence for Cα hydroxyl oxidation. Further potential oxidation products were detected with masses in strong correlation with degradation structures reported in a previous study by Shingo *et al*. [176]. Further work is required to confirm the presence of these structures, and to confirm degradation of the non-phenolic dimer by mechanisms such as Cα oxidation, Cα-Cβ, β1 and/or aromatic ring cleavage. However, this study was successful for highlighting expansion of TvL reactivity by the addition of 1-HBT. The complete findings are summarised in Scheme 17.



Scheme 17: Reactions of lignin model β-O-4 dimers 1, 2,and 3 [a] Product 4 characterised by MS and NMR following purification [b] Product 5 characterised by MS and by comparison with an authentic standard [c] Degradation products were identified by LC-MS and comparison with degradation products as reported in the literature [176].

The analysis of extracted wheat straw lignin products by py-GC/MS confirmed the production of Cα carbonyl structures within the natural substrate (as opposed to the synthetic β-O-4 dimers) following treatment with TvL and 1-HBT. In addition to Cα oxidation, the data further suggests an occurrence of Cα-Cβ cleavage reactions due to a reduction of Ph-C2 structures for both syringyl and guaiacyl structures. TMAH derivatisation during pyrolysis allowed for calculation of ratios that are used by researchers to investigate fungal degradation mechanisms of lignin. In all three experimental repeats, both [Ac/Ald] ratios for guaiacyl and syringyl structures increased, in support of the proposed mechanism of oxidative lignin degradation in nature.

The results in this chapter have produced trends that are consistent with research into lignin degradation by whole white-rot fungal systems. In addition to laccase, most white-rot fungi secrete lignin peroxidase (LiP) and manganese peroxidase (MnP). Both enzymes are reported to degrade lignin by Cα hydroxyl oxidation, Cα-Cβ cleavage and alkyl bond cleavage mechanisms following investigations with β-aryl ether compounds [106,388,389]. The results revealed herein provide evidence for a role in lignin degradation by laccases by the same mechanisms used by alternative ligninolytic enzymes.

The greater increase in [Ac/Ald] ratio for syringyl units than guaiacyl in this work suggests a preferential oxidative attack by laccase towards syringyl structures. Despite this, calculated S/G ratios did not support this, with 2/3 experiments reporting an increased ratio following treatment with TvL and 1-HBT indicative of preferential guaiacyl attack. FTIR (with ATR and pyrolysis), allowed further information to be collected regarding changes in proportion of guaiacyl (G) and syringyl (S) structures within lignin, with differences in band patterns and intensities for both structures observed. In general, the FTIR spectra collectively suggested an increase in syringyl structures and a decrease in guaiacyl structures following TvL treatment with 1-HBT. However, difficulties in data interpretation were experienced due to the appearance of contributing bands (potentially originating from contaminating cellulose or hemicellulose). Furthermore, due to the complex and heterogeneous structure of lignin, in many instances multiple vibrations could be assigned to a wavenumber/range, making assignment challenging.

Surprisingly, despite the association of TvL and 1-HBT for the production of Cα carbonyl functionalities (as determined by β-O-4 dimer studies, and lignin py-GC/MS analyses), the vibration of C=O carbonyl groups (acids, ketones and aldehydes) was not noticeably increased during FTIR analysis of lignin following the LMS treatment.

Studies with natural lignin (py-GC/MS +/TMAH and (py) FT-IR), revealed data of similar trends when TvL was applied with and without 1-HBT (compared to untreated controls). Examples include the consistent increases in both G and S [Ac/Al] ratios and the decrease in the C/G ratio by (TMAH enhanced) py-GC/MS, the increase in S/G and the decrease in Ph-C2 and C3 demonstrated by py-GC/MS, and the similarities of band ratios and patterns from FT-IR analysis. These similarities between the two pretreatment conditions would not be expected when the results of the β-O-4 study are considered (no oxidation of the non-phenolic lignin model dimers). This work further highlights a difference between the use of natural substrate systems that 'mimic' nature within a laboratory setting (e.g. studies with biomass) and synthetic lignin model substrate studies. Lignin model substrate reactions are highly controlled, and despite having use in representing lignin for the establishment of degradation mechanisms, they poorly represent lignin degradation as it occurs in nature. In nature, β-O-4 and Cα-Cβ linkages could be cleaved by laccase secretion and the subsequent generation of lignin derived oxidative species that act as natural mediators. In the laboratory, reactions with biomass and TvL without 1-HBT may also generate the same natural mediators that are suggested to be produced in nature, leading to similar effects towards lignin that are observed when mediators such as 1-HBT are applied. Supporting evidence for this comes from the study by Du *et al*. [365], whereby a decrease in Ph-C2 and Ph-C3 structures was observed with the addition of a natural mediator (methyl syringate) with laccase. In this work, TvL without a mediator reduced these structures to a similar extent, suggesting a similar mechanism of Cα-Cβ cleavage as the natural mediator system investigated by Du *et al*. [365].

Chapter six

6.0 Overall conclusions

6.1 Laccase production & activity screening

This study initially investigated the heterologous expression of two laccase isozymes from *Trametes versicolor* (Lccβ and Lccδ), which previously demonstrated different activities towards the oxidation of various PAHs [198]. A shake flask expression strategy using the methylotrophic yeast *Pichia pastoris* was successful for the extracellular expression of both laccases, as determined by both solid and liquid phase developed ABTS activity assays.

The solid phase ABTS assay enabled the secretion of both laccases to be monitored throughout the expression time course, whilst the liquid phase assay was successful for the determination of volumetric and specific activity. Ultimately, purified protein yields produced by this expression strategy were too low to allow a stock of active laccase to be produced for multiple activity studies. Despite this, both crude Lccβ and Lccδ, and purified Lccβ were found to catalyse the oxidation of veratryl alcohol to veratryl aldehyde in the presence of a laccase mediator to varying degrees. In agreement with Koschorreck *et al*. [198] Lccβ demonstrated greater oxidative activity than Lccδ and therefore provided a good candidate enzyme for use with a mediator for the oxidation of non-phenolic substrates such as lignin.

Greater protein yields and hence higher volumetric laccase activities could be achieved by up-scaling the expression using increased volume bio-reactors (controlled fermentations). However for the general characterisation of laccases (e.g. for temperature stability, pH optimum and small scale oxidations), shake flask expressions can provide effective routes to production.

The multi-step procedure for the purification of fungal laccases was demonstrated to be reasonably successful in this work. However the loss of enzyme experienced in between purification steps and the time consuming nature of the overall procedure was a limitation for this process to produce laccase of reasonable purity. The development of simpler purification strategies for expressed fungal laccases in yeasts would be beneficial. Many researchers have made use of commercially available laccase preparations [180,182,237,277,365] most likely due to these reasons. This approach was the considered course of action for this work, to allow experiments to be generated using high concentrations of laccase.

6.2 Identification of suitable *T. versicolor* laccase mediator systems

Preliminary screening of phenolic compounds (with commercial *T. versicolor* laccase, TvL) representing naturally occurring laccase mediators revealed (in the majority of cases) that successful oxidation and decolourisation of a recalcitrant dye (Reactive Black-5 (RB-5)) was possible. Synthetic mediators 1-hydroxybenzotriazole (1-HBT) and violuric acid (VA) proved effective laccase mediators for the reaction, however in some cases they were not as effective as the phenolic compounds tested (e.g. acetosyringone and syringaldehyde).

Further work with veratryl alcohol, a non-phenolic compound more structurally related to lignin than RB-5, demonstrated phenolic compounds as insufficient mediators for non-phenolic model compound oxidation. On the contrary, and in agreement with existing studies, synthetic mediators 1-HBT, VA and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) provided excellent mediating capabilities for the oxidation of veratryl alcohol to veratryl aldehyde. Furthermore two other laccase substrates, the dyes Phenol Red (PR) and Remazol Brilliant Blue (RBB) were able to mediate the TvL catalysed oxidation to a reasonable degree.

The data collected confirmed that naturally occurring phenols react with laccase to produce radicals capable of rapidly oxidising RB-5, but their reactivity towards other non-phenolic substrates is hindered. Differences in redox potentials ($E°$) and radical stabilities are most likely to explain this. It is known that the oxidative ability of phenols is increased when a less negative difference in $E°$ exists between the phenoxyl radical and substrate. Furthermore, the more difficult the substrate is to oxidise, the more likely the radicals are to undergo oxidative coupling and produce inactive dimers and polymers. Phenolic compounds in this instance demonstrate a potential to be used as co-substrates with laccase for dye decolourisation or to reduce effluent toxicity as previously demonstrated [165,226,390,391].

This study provided a basis for the selection of synthetic mediator compounds over natural phenolic compounds for the oxidation of non-phenolic lignin structures. This strategy is unfortunately less advantageous for a biorefinery concept, whereby more costly and toxic compounds like 1-HBT, VA and ABTS would be disfavoured. Further work in this area could focus on increasing the longevity of phenoxyl radicals

(radical stabilisation) to increase their oxidative activity towards substrates with a higher $E°$, or sourcing alternative phenolic compounds with structural similarities to acetosyringone and syringaldehyde. Furthermore, applying a combination of natural and synthetic mediators to a reaction with laccase and a non-phenolic substrate has not been exploited and could be investigated in an attempt to decrease the amounts of synthetic mediator applied.

As determined in this work, an increased concentration of oxidised synthetic mediator leads to a faster inactivation of laccase, another limitation of their use industrially. An opportunity to decrease laccase inactivation by engineering laccases to increase their tolerance to radical attack could be explored.

6.3 Application of laccase/LMS as a biomass pretreatment strategy

Expansion of the study from the lignin model compound veratryl alcohol to the lignocellulosic substrate wheat straw revealed that single-step pretreatments with TvL and the optimised LMS (with 1-HBT) impaired saccharification. Use of a combined pretreatment strategy, whereby the laccase and LMS reaction preceded a lignin-solubilising alkaline-peroxide extraction (APE) step, was found to reverse this impairment, with both treatments improving saccharification.

This saccharification improvement was consistently observed and was reproduced with alternative substrates corn and sorghum stover. An alternative synthetic mediator (VA) used with TvL also demonstrated the same improvement, and alternative laccases, *Pleurotus ostreatus* (PoL) (with and without 1-HBT), *Agaricus bisporus* and *Rhus vernificera* (without 1-HBT) reproduced the same trends observed with TvL. The versatility of this laccase-induced saccharification improvement was revealed by replacement of APE with a second lignin solubilising method, organosolv, which produced the same increased trends.

Despite the multi-step treatments applied to the biomass, the only difference between the treatments in all investigations was in every case the incubation of laccase or a LMS, thus directly linking the increase in saccharification to the addition of laccase or LMS. Another consistent observation included the enhanced improvement by the LMS in comparison to laccase without 1-HBT. Three conclusions can be drawn from this study:

- Laccase and LMS treatment without APE does not improve saccharification; suggesting that the oxidative activities of TvL and TvL with 1-HBT towards lignin within wheat straw does not induce lignin structural changes that positively impact cellulose hydrolysis.

- Biomass treatment with laccase and LMS prior to APE or organosolv does improve saccharification; suggesting that the oxidative activities of laccase and LMS towards lignin within the biomass positively impact the APE or organosolv step, indirectly improving cellulose hydrolysis.

- The addition of 1-HBT (and VA) with TvL (and PoL) further improves saccharification compared to TvL or PoL used alone; suggesting that the oxidised mediators induce additional/further oxidative effects towards lignin that positively impact the overall effect of APE or organosolv.

The findings from this work build upon those of Gutierrez *et al*. [180] who in 2012, demonstrated for the first time the use of *Trametes villosa* laccase and a LMS with APE to improve the sacharrification of a wood (Eucalyptus) and non-wood (elephant grass) substrate. This work confirmed these improvements with the same synthetic mediator (1-HBT) but with different lignocellulosic substrates, different origins of laccase, an additional synthetic mediator (violuric acid) and by an alternative lignin-solubilisation technique (organosolv). Furthermore this study achieved comparable improvements in saccharification following a single application of enzymatic treatment (at a higher concentration) compared to the four successive applications reported by Gutierrez *et al*. [180], thus reducing the overall lenght of the process.

Very recently the same researchers [182] published similar improvements in saccharification with, for the first time, a phenolic mediator (methyl syringate). The research described in this current work did not confirm saccharification improvements when acetosyrigone, syringaldehyde and several other phenolic compounds were investigated as 1-HBT alternatives with wheat straw. However, full optimisation of reaction conditions was not explored. Further work regarding the optimisation of phenolic mediator concentration, laccase concentration, length of enzymatic reaction

and mediator dosing could be explored. Additionally, the inclusion of alternative phenolic compounds not investigated in this work screened with both TvL and PoL could, in the future, identify additional phenolic mediators that may improve saccharification like methyl syringate.

The process developed in this work successfully demonstrated the benefit of incorporating laccase and LMS treatments prior to biomass saccharification, which would subsequently increase ethanol yields following fermentation. However, the process developed in this study included multiple steps, which industrially presents limitations such as high costs and long operational durations. Removal of the prior acid hydrolysis step in this work revealed low glucose yields, even when the APE step was included. This implied that the succession of all three steps (acid hydrolysis, laccase/LMS and APE) was required for increased saccharification by laccase/LMS. Future improvements could focus on reducing the number of steps involved or the replacement of the existing steps with more environmentally friendly processes. This could be achieved by investigating the use of hemicellulases (e.g. xylanases) to solubilise hemicellulose as opposed to the use of acid hydrolysis. Combination of a dual biological system has already been demonstrated for saccharification improvements by Chen [181], who pretreated corn stover with enzymatic ensilage enzymes (hemicellulases and cellulases) prior to laccase and LMS treatments.

Although the combined use of the laccase and LMS treatments with existing chemical/thermochemical pretreatments is lengthy, this added biological step may benefit a biorefinery not only by increasing sugar and ethanol yield, but by reducing the severity and operational durations of the chemical/thermochemical processes. Further important areas in the development of this multi-step biochemical process should focus on reducing the substrate loss between each step and the prevention of cellulose oxidation during APE (by $MgSO_4$ as explored) to ensure that maximum yields of sugar are achieved.

The project has contributed further knowledge towards the existing uncertainty and contradictory research surrounding the involvement of laccases in the degradation of lignin. Two β-O-4 linked non-phenolic dimers revealed Cα hydroxyl group oxidation by the LMS, in support of previous research claims and proposed LMS catalysed lignin degradation pathways. Py-GC/MS provided further evidence of this reaction by

the TvL and 1-HBT LMS in wheat straw lignin. Both methods generated data to suggest that further cleavage reactions may occur, by the detection of additional products arising from the reaction of one β-O-4 dimer with the LMS, and the calculated decrease in Ph-C2 structures by Py-GC/MS. Further confirmation of this proposed Cα-Cβ cleavage mechanism by the LMS is required, due to the low yields of potential cleavage products (from the β-O-4 reaction) generated and the inconsistent reduction of Ph-C3 structures observed (Py-GC/MS). Multiple analyses in future investigations would supply more evidence. Py-GC/MS with TMAH provided data to support Cα-Cβ cleavage by revealing increased acid:aldehyde ratios of both guaiacyl and syringyl products after laccase and LMS treatments. The preferential oxidative attack by laccase towards syringyl or guaiacyl structures could not be confirmed due to inconsistencies within the results from different investigations (Py-GC/MS and FTIR).

The range of information collected from this study highlights the requirement for more than one analytical technique for determining the action of laccase and LMS towards lignin, and the combined use of model compounds and natural lignin to establish mechanisms. Model compound studies do not represent the natural substrate and the poor correlation of results collected between both substrates (veratryl alcohol and wheat straw) in this work confirmed this.

As a final conclusion, this work has expanded the scope of information available regarding the inclusion of laccases and their respective mediators into bioprocesses for future biorefinery technologies. More specifically, two laccases in particular, TvL and PoL, with and without a synthetic mediator (1-HBT and the less investigated VA) have demonstrated a potential to improve lignocellulosic saccharification for the application of bioethanol production. Further expansion of this work using soft and hardwood substrates, laccases of different redox potentials (possibly of bacterial origin), phenolic mediators, additional analytical assessments and alternative biological processing treatments (e.g. xylanases) would further provide a broad and in depth evaluation of their industrial applications towards lignocellulose.

Chapter seven

7.0 Materials and methods

7.1 Chemicals and reagents

Unless stated otherwise, all chemicals were of analytical grade and used as received from the supplier (Sigma-Aldrich, Dorset, UK). All solvents used were from commercial suppliers (Sigma-Aldrich, Fisher Scientific or Romil) and were of analytical grade.

All colorimetric assays were monitored on a Tecan Infinite M200 plate reader.

7.2 Transformation of pPICZA-Lccβ and pPICZα-Lccδ into *E. coli* DH5α and *Pichia pastoris* X33

Plasmids pPICZA-Lccβ and pPICZαA-Lccδ were received as gifts from the research group of Vlada Urlacher, University of Dusseldorf, Germany (Table 44). Empty pPICZA and pPICZαA vectors were used as negative controls and were received from Professor Simon Turner at the University of Manchester.

Table 44: Plasmids used for this study

Plasmids	Relevant properties	Source/Reference
pPICZ A	Zeocin resistance gene *Sh ble*, 5' *AOX1* promoter	Invitrogen
pPICZαA	Zeocin resistance gene *Sh ble*, 5' *AOX1* promoter, α-factor secretion signal	Invitrogen
pPICZA-Lccβ	Zeocin resistance gene *Sh ble*, 5' *AOX1* promoter	Koschorreck *et al*. [198]
pPICZαA-Lccδ	Zeocin resistance gene *Sh ble*, 5' *AOX1* promoter, α-factor secretion signal	Koschorreck *et al*. [198]

Chemically competent *E. coli* DH5α cells (Table 45) were transformed with 10 µl plasmid. Cells were incubated on ice for 30 min and heat shocked at 42°C for 90 s. 500 µL low salt lysogeny broth (LB: 1% tryptone, 0.5% yeast extract and 0.5% sodium chloride) with zeocin (25 µg/mL) was added and cells were incubated at 37°C for 60 min whilst shaking at 250 rpm. Aliquots were plated onto low salt LB with zeocin (25 µg/mL). Colonies were selected and the plasmids purified using the Qiagen Midiprep Kit and protocol. Plasmid DNA was quantified at 260 nm on the Nanodrop 2000 (Thermo scientific).

Table 45: Strains used for this study

Strain	Genotype	Source/Reference
E. coli DH5α	*supE*44 DEL*lac*U169 (phi80 *lacZ*DM15) *hsdR recA*1 *endA*1 gyrA96 *thi*-1 *rel*A1	Hanahan [392]
P. pastoris X33	Wild type Mut^+	Invitrogen

To confirm the identity of the genes, Lccβ and Lccδ were excised from the plasmid DNA (pPICZA and pPICZαA) by a *Bst*BI-*Eco*RI digestion and analysed by agarose gel electrophoresis. All restriction enzymes were obtained from New England Biolabs. The procedure was carried out as instructed by the manufacturer's protocols. The plasmids were sequenced by GATC using sequencing primers as shown in Table 46.

For integration of the plasmid DNA into the *P. pastoris* genome, plasmid DNA was linearised with *Pme*I prior to transformation. Using the protocol described by Invitrogen [222], *P. pastoris* X33 cells were transformed *via* electroporation using the BioRad GenePulser Xcell using parameters set by the manufacturer for *P. pastoris.* The electroporated cells were plated onto YPDS plates with Zeocin (100 µg/mL). Colonies were selected and the DNA purified by a standard genomic DNA purification method taken from Invitrogen Life Technologies [222]. The purified DNA was treated with RNase and analysed by agarose gel electrophoresis.

PCR amplification was performed to confirm the integration of laccase into the *P. pastoris* genome using primers outlined in Table 46. A 50 µL reaction was set up to contain 1 µg DNA, 50 pmol of each primer and 2x DFS GenTaq PCR Mastermix (2.5 U DNA polymerase) according to the protocol by Bioron GmbH. Initial denaturation at 95^{o}C for 5 min was followed by 30 cycles of 95^{o}C for 30 s, the annealing temperature as shown in Table 46 for 30 s, 72^{o}C for 1.5 min and a final extension at 72^{o}C for 7 min in an Eppendorf Mastercycler Gradient PCR machine (Stevenage, UK). The conditions were the same for laccase δ amplification with the exception of 1.25U DreamTaq DNA polymerase. PCR products were analysed on an agarose gel.

Table 46: Oligonucleotide primers used for PCR and sequencing

Primers[a]	Sequence (*5'-3'*)	Annealing Temperature (^{o}C)	Source/Reference
pPICZAForward	CAAGCTTTTGATTTTAACGAC	52.0	This study
pPICZAReverse	GAGTTTTTGTTCGGGCCCAAGC	62.1	This study
BF2	ACGACCACCGCGGACCTC	62.8	This study
BF3	CGGTCTACAACTACGACAAC	57.3	This study
BR2	GATGGCCAGGTCAACACCAC	61.4	This study
BR3	CGGACCCCTCAGACCATCAC	63.5	This study
DF2	CGACGGCTTCACTCGTGCC	63.1	This study
DF3	CCGTTTCGCCGGTGGCGACAG	67.6	This study
DF4	CAGATCCTTAGCGGCACCAC	61.4	This study
DR2	GGTGATGGGGAACGAGATCTC	61.8	This study
DR3	AGACATAGTTGGGGTCGCAAG	59.8	This study
DR4	TGGAAGTTGTCGCCCTGTGG	61.4	This study

[a] F: Forward primer, R: Reverse primer, B: Lccβ, D: Lccδ.

7.3 Laccase expression in *P. pastoris*

Media preparation and the expression protocol were carried out as described by Koschorreck *et al*. [198]. During pH investigation, 0.8% alanine was added to the expression medium at the start of expression as described by O'Callaghan *et al*. [191]. Methanol was added at 0.5% (v/v) daily. For initial expression studies 1 mL samples were taken daily, centrifuged (5 min at 17,900 x *g*/13,000 rpm) and supernatants stored at -20^{o}C for further analysis. Expression was carried out up to 9 d. Supernatants were concentrated ~20x using a 10KDa MWCO filtration system (Vivaspin, Sartorius stedim).

7.4 Solid-phase liquid ABTS activity assay

Laccase activity was measured using a solid phase plate assay by the incorporation of 0.5% agarose into the ABTS substrate mix as described by Scrinvasan *et al*. [393]. Plates were incubated in the dark for one hour at room temperature (25°C). Commercially sourced laccase (Sigma Aldrich) from *Trametes versicolor* was used as a positive control, with heat treated laccase (1 U/mL) and water used as negative controls. A green halo around the well was considered a positive test for laccase activity.

A second solid phase laccase activity assay was developed using a solidified preparation (0.5% agarose) of the minimal media used previously for expression (containing 0.3 mM $CuSO_4$, 0.5% methanol and 0.04 mg/mL biotin). The medium was supplemented with 1 mM ABTS as described by Brown *et al*. [189] and He *et al*. [394]. Transformed *P. pastoris* colonies were transferred from the YPDS plates (+Zeocin) onto the minimal media plates and incubated for up to 72 h at 30°C. The appearance of green zones around growing colonies was indicative of laccase secretion.

7.5 Liquid-phase laccase ABTS activity assay

Laccase activity was measured with a spectrophotometer based on the method described by Niku-Paavola *et al*. [228]. The reaction mixture (total volume 200 µl in microtitre plate well) contained glycine-HCl buffer pH 3.0 with 1.4 mM ABTS (100 µl) and commercial laccase or culture supernatant (100 µl). A system specific ε for the oxidised ABTS product was calculated using the Beer Lambert equation and ABTS standards of different concentrations. One unit of enzyme activity was defined as the amount of enzyme that oxidised 1 µM ABTS in one minute.

7.6 Laccase purification

Concentrated culture supernatant was dialysed against 10 mM sodium acetate buffer pH 5.0 (buffer A) overnight at 4°C. Protein sample was loaded onto a DEAE FF (GE Healthcare) anion exchange column pre-equilibrated with buffer A. After sample loading the column was washed with 5x column volumes of buffer A before a stepped gradient elution using buffer A with increasing concentration of ammonium sulphate (10, 20, 40, 60, 80 & 100 mM). The ABTS solid plate assay was used to identify laccase containing fractions and all fractions showing activity were pooled and concentrated 2x. The laccase fractions were dialysed overnight in 10 mM sodium acetate buffer pH 6.0 containing 2.0 M NH_4SO_4 (buffer B) and then loaded onto a Phenyl Sepharose FF (GE Healthcare) hydrophobic interaction column pre-equilibrated with buffer B. Protein was eluted with a 2-0 M step gradient (2, 1.7, 1.4, 1, 0.75, 0.5 & 0 M) NH_4SO_4 and fractions containing laccase were pooled and concentrated. Fractions were desalted using buffer exchange into 10 mM sodium acetate pH 5.0. Protein estimation was carried out on the fractions via the BCA

method and laccase specific activity was calculated against ABTS (methods as described in 7.5).

7.7 SDS-PAGE laccase analysis

Aliquots (20 µl) of the supernatants during/following expression were dissolved with 2x Laemmli SDS loading buffer, loaded onto a gradient 4-20% SDS Polyacrylamide gel (NuSep) and ran according to Laemmli [395]**.** Gels were stained with Instant Blue (a Coomassie Brilliant Blue based dye, Expedeon Protein Solutions). The gels were calibrated with the PageRuler^{TM} prestained protein ladder (Fermentas).

7.8 Native and semi-denaturative PAGE

For native protein analysis, samples were analysed by native PAGE using an 8% (wt/vol) acrylamide resolving gel and a 4% (wt/vol) acrylamide stacking gel at room temperature according to the method previously described by Laemmli [395]. Laccase activity staining was carried out after fixing the proteins in 10%:40% acetic acid:methanol with 50 mM glycine-HCl pH 3.0 containing ABTS (2.7 mg/mL) as described by Niku-Paavola *et al*. [228]. The gels were calibrated with 1 mg/mL BSA.

Semi-denaturative conditions were used to co-stain proteins with Coomassie and ABTS as adapted from the method by Gonclaves, [396]. Protein samples were incubated at 37°C for 15 min in Laemmli buffer. After electrophoresis the gel was washed twice with 50 mM sodium acetate pH 4.0 for 50 min, then stained in the same buffer containing 50 µM ABTS.

7.9 Protein concentration determination

Protein concentration was determined using the Bicinchioninic Acid (BCA) protein assay reagent using bovine serum albumin (BSA) as standards (Pierce). The method is as described by Smith *et al*. [279].

7.10 Fermentation of *P. pastoris*

Cell biomass was generated by preparation of a starter culture of *P. pastoris* X33-Lccβ. Cells were inoculated into YPDS and incubated at 30°C and 200 rpm. Cells were harvested in log phase and inoculated into a sterile 2L bioreactor (Applikon Biotechnology) containing 1.5 L basal salts medium (composition (per L): H_2PO_4 (85%) 26.7 mL, $CaSO_4$ 0.93 g, K_2SO_4 18.2 g, $MgSO_47H_20$ 14.9 g, KOH 4.13

g, Glycerol 40.0 g) to an OD between 0.5-1.0 to initiate the batch phase growth. The medium was supplemented with 4.35 mL/L PTM_1 trace salts (as described by Invitrogen), 0.5 mL antifoam and pH was automatically maintained at 5.0 by continual supplementation with 28% NH_4OH. Following the exhaustion of glycerol, induction was initiated by decreasing the temperature to 25°C and feeding methanol (+1.2% PTM_1) to achieve a maintained concentration of 0.5%. Air and oxygen flow rates were set to 1.0 and 0.25 mL/L/min respectively. Agitation was set in the range of 750-1000 rpm to maintain a dissolved oxygen concentration of 25%. Samples were removed periodically to assess the production of laccase *via* the ABTS assay. pH, temperature, stirring speed, air flow, and dissolved oxygen levels were monitored by the ez-Control module (Applikon Biotechnology).

7.11 Mediator screening using TvL and Azure B

Reactions were set up in 96-well plates. 25 μM Azure B was incubated with 200 μM 1-HBT and a range of TvL concentrations (0, 10, 50, 100, 400, 500, 600 mU). Reactions were followed in a plate reader by recording absorbance readings at 647 nm every minute. For visual analysis of colour change, reactions were set up with 25 μM Azure B, 200 μM 1-HBT and100 mU TvL or *Pleurotus ostreatus* laccase (PoL). Both laccase without mediator and laccase and mediator free negative controls were set up by replacement of each component with water.

7.12 Phenolic and synthetic mediator screening using TvL and RB-5

Reactions were set up in 96-well plates. 50 μM RB-5 was incubated with 100 mU *Trametes versicolor* laccase (TvL) in the presence of a panel of mediators at dye:mediator concentrations of 1:1, 1:5 and 1:10. Reactions were covered with aeroseals and were incubated at 25°C and 150 rpm. At given time points the reactions were analysed by measuring absorbance at 598 nm. No laccase-no mediator (NLNM), and laccase without mediator (TvL NM) controls were included and percent decolourisation was calculated based on both controls to produce two separate sets decolourisation data.

7.13 Oxidation of veratryl alcohol by TvL and LMS

Oxidation reactions were performed using 3 mM veratryl alcohol in 0.1 M sodium acetate buffer pH 5.0. Commercial TvL was used at either high (1.6 U/mL) or low (0.4 U/mL) concentrations. Synthetic mediators and dyes/indicators were investigated

at both 3:1 and 1:1 (substrate:mediator) ratios. Phenolic mediators were investigated using their optimal concentration established from the RB-5 screen (1:1, 1:5 or 1:10 dye:mediator). Reactions were incubated at 25°C at 200rpm for 24 hours. Reactions were acidified to pH 2.0 with 1M HCl and extracted twice using 2x volumes of ethyl acetate. Ethyl acetate was evaporated using the Genevac EZ and residues were resuspended in 75% methanol. Veratryl aldehyde production was monitored at 230nm by reversed phase HPLC on an Agilent 1100 series HPLC + LC/MSD SL ESI mass spectrometer. Separation was achieved using an isocratic mobile phase of 60% methanol in super pure water both with 0.1% formic acid at a flow rate of 0.2 mL/min for 20 minutes using a C:18(2) 250 x 2.00 mm 5 μ column. MS was performed in positive mode using a mass range 100-1500 MW (MS spray chamber conditions: Drying gas flow 12.0 L/min^{-1}, nebuliser pressure 50 psi, drying gas temperature 350°C and capillary voltage 5000 V). Authentic standards of veratryl alcohol & veratryl aldehyde were used for the identification and quantification of substrate and product. Conversion was calculated based on the difference between the peak areas of the substrate and the product.

Expressed laccases Lccβ and Lccδ were reacted with veratryl alcohol at 0.1 U and 0.3 U respectively. In the same experiment, commercial TvL and a purified preparation of Lccβ were used at 0.1 U and 0.02 U respectively.

7.14 Laccase stability studies

Reactions were set up in deep well microtitre plates to contain 1U/mL TvL in 0.05M NaOAc buffer pH 4.0. Four mediators were investigated, two synthetic (1-HBT and violuric acid) and two phenolic (acetosyringone and syringaldehyde). Mediators were added to the reactions at increasing concentrations (0.05, 0.1, 0.5, 1.0, 2.0 and 3.0 mM) and reactions were incubated at 28°C at 200 rpm. Aliquots of each reaction were removed daily (0, 20, 48, and 72 h) and assayed for laccase activity using the ABTS activity assay previously described. Activity was calculated from the maximum change in absorbance (at 420 nm) per minute and expressed as a percentage decrease based on the activity recorded at 0 h (initial activity).

7.15 Calculation of GC220 concentration

The concentration of cellulase GC220 (Genencor) expressed in filter paper units was determined following the filter paper method as described by Ghose, [280]. Glucose standard were used in the range of 0-5 mg/0.5 mL, GC220 dilutions were prepared in the range 0.0025-0.0087 mg/0.5 mL. HPLC with RID detection was used to quantify glucose release as described in 7.18.

7.16 Avicel hydrolysis with GC220 and laccase/LMS treatment

2% Avicel cellulose was hydrolysed with 0, 5, 15 and 30mg/g cellulose GC220 in 50mM sodium citrate/acetate buffer with 0.5% sodium benzoate at pH 5.0. Reactions were incubated at 50^{o}C and 250 rpm (Infors Multitron standing incubator). Aliquots (200µL) were taken at the time points 0, 1, 2, 4, 6, 25, 48, 72 and 144 h and centrifuged (5 min at 17,900 x *g*/13,000 rpm) to remove any insoluble cellulose. The reaction was either quenched with the addition of H_2SO_4 (900 µL), or heat treated at 95°C for 10 min to denature the enzyme.

To investigate the effect of laccase and an active laccase mediator system on the hydrolysis of cellulose, commercial *T. versicolor* laccase was added at 1.3 U/mL with 1mM synthetic mediator ABTS or 1-HBT. The following natural mediators were screened in the reaction at a concentration of 3 mM: vanillin, vanillic acid, *p*-coumaric acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, syringaldehyde and acetovanillone.

7.17 GOX method for glucose determination

Glucose oxidase (GOX) was used to quantify glucose release from cellulose according to the method of Bergmeyer. [397] with slight modifications. The final concentration of the reaction mix contained 48 mM sodium acetate buffer pH 5.1, 0.16 mM *o*-dianisidine, 4.2 U/mL HRP, and 2 U/mL GOX. 100 µl of hydrolysed cellulose sample was mixed with 100 µL reaction mix and the absorbance was measured at 500 nm for 20 min at 35^{o}C. D-glucose standards were used to construct a standard curve and the absorbance after 2 min of reaction was used to determine glucose concentration.

To determine the effect of laccase on the GOX assay, TvL was added to the reaction at concentrations of 0, 0.01, 0.1 and 1 U/mL. 21.8 U laccase was denatured by heating

at 98^{o}C for 1, 5 and 15 min and added to the reaction to analyse enzyme deactivation and to determine optimum denaturation time.

7.18 Glucose quantification with HPLC-RID

Samples from saccharification experiments (quenched in H_2SO_4) were filtered through a 0.2 μM membrane (Millipore) before analysis on an Agilent 1200 series HPLC with RID detection using an Aminex HPX 87H column at 55°C. Each run was 15 min with 5 mM H_2SO_4 mobile phase and a 0.6 mL/min flow rate. D-glucose standards were prepared in the range 0-12 g/L and a calibration curve was produced to quantify glucose from hydrolysis samples.

7.19 Detection of exo- & endoglucanase activity

Method as described by Deshpande *et al*. [283], but downscaled for microtitre plate analysis. 180 μL of a 1 mg/mL solution of *p*-nitrophenyl-β-D-cellobioside (*p*-NPC) in 0.05 M sodium acetate buffer pH5.0 was incubated with 20 μL of enzyme supernatant taken from the cellulose hydrolysis reaction at 50°C for 30 min. 100 μL of the reaction mix was removed and mixed with 100 μL 2% Na_2CO_3 before spectrophotometric analysis of released *p*-nitrophenol at 410 nm. Addition of 0.5 mg/mL gluconolactone to the *p*-NPC solution was used as reported by Deshpande *et al*, [283] to inhibit the activity of β-glucosidase.

7.20 Detection of β-glucosidase activity

Method as described by Ghose [280], but adapted for microtitre plate analysis. 1.8 mL of 0.1 M sodium acetate buffer pH 5.0 was equilibrated with 1.0 mL 5 mM *p*-nitrophenyl-β-D-1,4-glucopyranoside (*p*-NPG) at 50°C. 0.2 mL enzyme supernatant from the cellulose hydrolysis reaction was added and the reaction left for 30 minutes at 50°C. 100 μL of reaction mix was removed and mixed with 100 μL 0.4 M glycine buffer pH 10.8 for spectrophotometric analysis of released *p*-nitrophenol at 430 nm.

7.21 Acid-pretreated wheat straw and corn stover (NREL)

Acid-pretreated wheat straw and corn stover used in all biomass studies were received from NREL after subjection to the following acid hydrolysis protocol (information received from Shell):

Corn stover: 158°C, 5 min residence time, 17 mg acid/g dry biomass at approximately 30% total solid concentration. A fraction of this material was subjected to a hydrolysis

step to convert xylo-oligomers to monomeric sugars. The reaction conditions for the oligomer conversion step were 130°C, 20 min residence time with an additional acid loading of 7.5 mg acid/g dry biomass.

Wheat straw: 152°C, 5 min residence time, 17 mg acid/g dry biomass at approximately 30% total solid concentration, and then the slurry was subjected to oligomeric conversion at the same conditions mentioned above.

For the acid-pretreatment of dry substrates (corn and sorghum stover), a method was developed whereby biomass was mixed 1:10 (solid:liquid) with 2% H_2SO_4 and heated at 126°C for 1 h in a priorclave.

7.22 Acid-pretreated wheat straw washing

Acid-pretreated wheat straw (WS) slurries were washed three times with distilled H_2O at a biomass to water ratio 1:100 to remove products from acid pretreatment. Solids were filtered through a Buchner funnel under a vacuum pump and collected on filter paper. Dry weight estimation was determined by drying biomass in an oven at 80°C for 24 h.

7.23 Folin-ciocalteau assay for total phenol estimation

Total phenol content was determined by modification of the Folin-Ciocalteau method [289]. 20 μL sample was mixed with 80 μL water and 50 μL Folin's reagent. The reaction was incubated for 3 min at room temperature. 250 μL 20% sodium carbonate was added and the reactions were incubated for 30 min in the dark. Absorbance was measured at 725 nm. Total phenol was expressed as g/L based on catechol standards.

7.24 Studies with laccase and acid-pretreated wheat straw

For successive laccase and cellulase treatments, acid-pretreated WS (0.5 g d.w WS) was washed and treated with 50 U/g (d.w WS) TvL with and without 2.5% (w/w d.w) 1-HBT in 0.05 M sodium citrate buffer pH 5.0 at 5% consistency. Reactions were incubated at 28°C and 200 rpm. An enzyme and mediator free reaction was set up as a negative control. 2.9 FPU GC220 was added after 24 h and the temperature increased to 50°C. Aliquots were taken at 24 h intervals over 72 h.

Laccase and mediator optimisation studies were carried out on washed WS using the above treatment conditions except 0.1 M sodium acetate pH 4.0 was used instead of citrate buffer at pH 5.0 because pH 4.0 is optimal for laccase activity and pH 5.0 is

optimal for hydrolysis. 3X wash steps after the laccase/LMS treatment with dH_2O was included to remove the enzyme and associated soluble products to create a step-wise laccase and cellulase treatment. Different concentrations of TvL (50-150 U/g and 500-4000 U/g d.w WS) and 1-HBT (2.5, 5 and 7.5% w/w WS) were investigated for reaction optimisation.

Optimised laccase and laccase-mediator system (LMS) biomass pretreatment experiments were set up to typically contain 0.6-1g (d.w WS) at 5% consistency (d.w was consistent for each individual experiment), 0.1 M sodium acetate buffer pH 4.0, 28°C, 200 rpm for 40 h. An optimal TvL concentration of 150 U/g TvL (g/d.w WS) and 5% mediator (w/w WS) was used in all experiments unless otherwise stated. All experimental conditions were performed in triplicate and data expressed as an average.

All reactions with acid-pretreated corn stover were as described with WS. All experiments with dry substrate were as described with acid-pretreated substrates but with no prior acid-hydrolysis step.

7.25 Alkaline-peroxide extractions

An alkaline-peroxide extraction (APE) procedure was developed based on previous work [180]. After enzymatic treatments, samples were subjected to an alkaline extraction step reinforced with peroxide using 1% NaOH and 3% H_2O_2 (both w/w d.w WS) at 80°C for 1 h. Samples were centrifuged (15 min at 20,000 x g) to remove the liquid fractions and the extraction step was repeated twice. After the last extraction, three water washing steps were applied to remove lignin soluble products, H_2O_2 and to neutralise the biomass before saccharification. For optimisation studies, $MgSO_4$ was included in the extractions at a concentration of 0.5% (w/w biomass).

7.26 Organosolv pretreatment

Two strategies were used for organosolv fractionation. In the first case, reactions were performed in 200 mL round bottom flasks and heated to 150°C using hot plates and condensers to allow solvent refluxing. Biomass samples were mixed 1:10 (biomass:solvent) with organosolv solvent (60:40 EtOH:H_2O) and refluxed at 150°C for 1.5 h. In the second set up, for higher throughput, smaller scale reactions were set up in glass pressure tubes and carried out for one hour at 100-126°C in a priorclave. Upon completion, solid and liquid fractions were separated either by Buchner funnel

filtration or centrifugation. Lignin was precipitated from the liquid fraction by dilution with acidified refrigerated water (3:1 water:liquid fraction) as described by Huigen *et al*. [398].

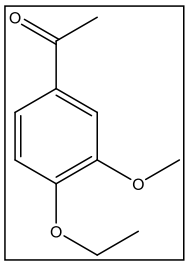
7.27 Saccharification

Biomass samples were hydrolysed with a cellulase preparation GC-220 (69 FPU/mL) (Genencor) containing a mix of the three hydrolytic enzymes for cellulose hydrolysis (endo-β-1,4-glucanase, exo-glucanase and cellobiohydrolase). Reactions were run at 5% consistency with 0.5 M sodium citrate buffer pH 5.0 at 50°C and 200 rpm. GC-220 was added in the activity range of 1.4-3.5 FPU depending on the dry mass of starting material in each experiment or as specified in each reaction. Reactions were monitored daily by sampling of the liquid fraction and stopping the reaction by quenching 1:10 10 mM H_2SO_4 for either immediate analysis or freezing for later analysis. All experimental conditions were ran in triplicate and data expressed as an average.

7.28 Synthesis of 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (dimer 3)

NMR spectra for **S1**, **S2**, **S3** and ketone **5** were recorded on a Bruker Advance 400. NMR analysis for dimer **3** was recorded on a Bruker Advance 500.

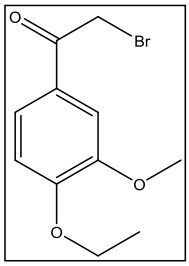
1-(4'-ethoxy-3'-methoxyphenyl)ethan-1-one (**S1**)



To a solution of acetovanillone (3.0g, 18.1 mmol) in DMF (60 cm^{-3}) was added K_2CO_3 (3.0g, 21.7 mmol). After 20 min at room temperature, ethyl iodide (1.75 cm^{-3}, 21.7 mmol) was added and the resulting mixture was heated at 50°C for 20 h. After cooling to room temperature, diethyl ether (250 cm^{-3}) and aqueous NaOH (1 M, 250 cm^{-3}) were added. After separation of the organic phase, the aqueous phase was extracted with diethyl ether (2 x 200 cm^{-3}). The combined organics were washed with water (200 cm^{-3}), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give the title compound **S1** (2.86 g, 81%) which required no further purification.

δ_H (400MHz, $CDCl_3$) 7.53 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 6.85 (d, *J* = 8.4 Hz, 1H), 4.14 (q, *J* = 7.0 Hz, 2H), 3.90 (s, 3H), 2.54 (s, 3H), 1.48 (t, *J* = 7.0 Hz, 3H). δ_C (100MHz, $CDCl_3$) 196.9, 152.8, 149.1, 130.3, 123.3, 110.9, 110.3, 64.5, 56.1, 26.3, 14.7.

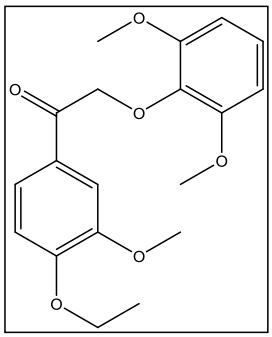
2-bromo-1-(4'-ethoxy-3'-methoxyphenyl)ethan-1-one (**S2**)



A mixture of ketone **S1** (2.86 g, 14.7 mmol) and $CuBr_2$ (5.25 g, 23.5 mmol) in ethyl acetate (25 cm^{-3}) was heated at reflux for three hours before cooling to room temperature. Filtration with copious ethyl acetate washings and concentration under reduced pressure afforded the crude product **S2** along with starting material **S1** in a 15 : 2 ratio (3.53 g) which was used in the next step without further purification.

δ_H (400MHz, $CDCl_3$) 7.55 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 6.86 (d, *J* = 8.4 Hz, 1H), 4.38 (s, 2H), 4.15 (q, *J* = 7.0 Hz, 2H), 3.89 (s, 3H), 1.47 (t, *J* = 7.0 Hz, 3H).

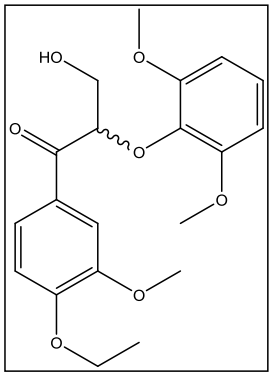
2-(2",6"-dimethoxyphenoxy)-1-(4'-ethoxy-3'-methoxyphenyl)ethan-1-one (**S3**)



A mixture of syringol (2.6 g, 16.9 mmol) and K_2CO_3 (2.3 g, 16.9 mmol) in acetone (35 cm^{-3}) was stirred at room temperature for 30 min before the addition of crude bromide **S2** (3.53 g) in acetone (10 cm^{-3}). After 3 h at room temperature, ethyl acetate (200 cm^{-3}) and aqueous NaOH (1 M, 200 cm^{-3}) were added. After separation of the organic phase, the aqueous phase was extracted with ethyl acetate (2 x 100 cm^{-3}). The combined organics were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Flash chromatography (5 : 1 then 7 : 2 cyclohexane : ethyl acetate) afforded the title compound **S3** (2.16 g, 42% over 2 steps).

δ_H (400MHz, $CDCl_3$) 7.69 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.64 (d, *J* = 2.0 Hz, 1H), 7.01 (t, *J* = 8.4 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 1H), 6.58 (d, *J* = 8.4 Hz, 2H), 5.15 (s, 2H), 4.17 (q, *J* = 7.0 Hz, 2H), 3.94 (s, 3H), 3.81 (s, 6H), 1.50 (t, *J* = 7.0 Hz, 3H). δ_C (100MHz, $CDCl_3$) 193.7, 153.3, 152.8, 149.1, 136.7, 128.2, 124.1, 123.0, 111.0, 110.8, 105.3, 75.3, 64.4, 56.1, 56.1, 14.6.

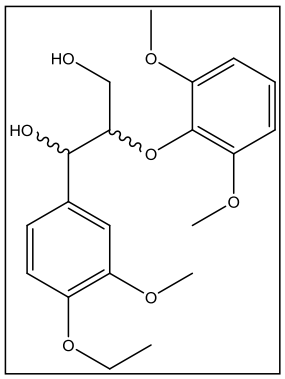
2-(2",6"-dimethoxyphenoxy)-1-(4'-ethoxy-3'-methoxyphenyl)-3-hydroxypropan-1-one (ketone **5**)



To a mixture of ketone **S3** (500 mg, 1.44 mmol) and paraformaldehyde (63 mg, 2.16 mmol) in DMSO (9.3cm^{-3}) was added K_2CO_3 and the resulting solution stirred at room temperature for 20 h before the addition of diethyl ether (200 cm^{-3}) and water (200 cm^{-3}). After separation of the organic phase, the aqueous phase was extracted with ethyl acetate (2 x 100 cm^{-3}). The combined organics were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the title compound ketone **5** (400 mg, 74%).

δ_H (400MHz, $CDCl_3$) 7.69 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.65 (d, *J* = 2.0 Hz, 1H), 7.02 (t, *J* = 8.4 Hz, 1H), 6.86 (d, *J* = 8.4 Hz, 1H), 6.57 (d, *J* = 8.4 Hz, 2H), 5.10 (dd, *J* = 7.9, 3.0 Hz, 1H), 4.17 (q, *J* = 7.2 Hz, 2H), 4.00 (dd, *J* = 12.2, 7.9 Hz, 1H), 3.93 (s, 3H), 3.82 (dd, *J* = 12.2, 3.0 Hz, 1H), 3.72 (s, 6H), 1.50 (t, *J* = 7.2 Hz, 3H).

2-(2",6"-dimethoxyphenoxy)-1-(4'-ethoxy-3'-methoxyphenyl)propane-1,3-diol (dimer **3**)



To a solution of ketone **5** (400 mg, 1.06 mmol) in methanol (8 cm^{-3}) at 0°C was added $NaBH_4$ (84mg, 2.2 mmol). After 2 h at 0°C, the reaction mixture was partitioned between diethyl ether (100 cm^{-3}) and water (100 cm^{-3}). After separation of the organic phase, the aqueous phase was extracted with diethyl ether (2 x 100 cm^{-3}). The combined organics were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the title compound dimer **3** (230 mg, 57%) as a 5:2 ratio of *threo* : *erythro* isomers. The major and minor isomers were assigned based on comparison of the ^{13}C chemical shifts with those of a closely related structure (2-(2",6"-dimethoxyphenoxy)-1-(4'-methoxy-3'-methoxyphenyl)propane-1,3-diol) [399].

δ_H (500MHz, DMSO, 50°C) 7.06 (d, *J* = 1.8 Hz, 0.71H), 7.00 (br. s, 0.29H), 6.97 (t, *J* = 8.4 Hz, 1H), 6.94 (dd, *J* = 8.3, 1.8 Hz, 0.71H), 6.88-6.85 (m, 1.29H), 6.66 (d, *J* = 8.4 Hz, 0.58H), 6.66 (d, *J* = 8.4 Hz, 1.42H), 4.93 (d, *J* = 4.8 Hz, 0.71H), 4.86 (d, *J* =

4.8 Hz, 0.29H), 4.17 (q, *J* = 4.7 Hz, 0.29H), 4.08 (q, *J* = 4.8 Hz, 0.71H), 3.99 (q, *J* = 7.0 Hz, 2H), 3.77-3.72 (m, 9.29H), 3.65 (dd, *J* = 11.5, 5.0 Hz, 0.71H), 3.45 (dd, *J* = 11.8, 4.1 Hz, 0.29H), 3.27 (dd, *J* = 11.5, 4.6 Hz, 0.71H), 1.31 (t, *J* = 7.0 Hz, 3H). δ_C (125MHz, DMSO, 50°C) 152.9 (minor), 152.8 (major), 148.6 (minor), 148.4 (major), 146.9 (minor), 146.9 (major), 136.3 (major), 135.8 (minor), 135.0 (minor), 134.8 (major), 123.3 (major), 123.2 (minor), 119.1 (minor), 118.9 (major), 112.8 (minor), 112.8 (major), 111.1 (minor), 111.1 (major), 105.9 (minor), 105.8 (major), 86.8 (major), 85.9 (minor), 72.0 (minor), 71.4 (major), 63.8 (minor), 63.8 (major), 60.2 (major), 59.7 (minor), 55.9 (minor), 55.9 (major), 55.4 (major + minor), 14.7 (major + minor).

7.29 β-O-4 dimer oxidation studies

1-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (dimer **1**) (TCI) and 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol (dimer **2)** (Fluorochem, UK) were prepared as 10 mM stock solutions in dimethylforamide (DMF). Oxidation reactions were performed as described by Li *et al*. [256] with some modifications. Each reaction contained 1 mM dimer **1** or **2**, 1 mM 1-HBT, and 2U/mL commercial *T. versicolor* laccase in 50 mM sodium acetate buffer pH 4.5. Reactions were carried out at 25°C and 150 rpm agitation. 500 μL aliquots were taken after 24 h and acidified with 1 M HCl. The extraction and analysis procedure was the same as described for the veratryl alcohol oxidation reaction. Oxidation studies of 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (dimer **3**) with TvL and 1-HBT were carried out according to Kawai *et al*. [176]. Reactions were analysed and monitored by reversed phase HPLC on an Agilent 1100 series HPLC + LC/MSD SL ESI mass spectrometer. Separation was achieved using an isocratic mobile phase of 60% methanol in super pure water both with 0.1% formic acid at a flow rate of 0.2 mL/min for 30 min using a C:18(2) 250 x 2.00 mm 5 μ column. MS was performed in positive mode using a mass range 100-1500 MW (MS spray chamber conditions: Drying gas flow 12.0 mL/min^{-1}, nebuliser pressure 50 psi, drying gas temperature 350°C and capillary voltage 5000 V).

7.30 Characterisation of ketone 4

Due to the lack of an authentic standard for the ketone product **4**, scale-up followed by preparative HPLC and NMR analysis was required. The biotransformation as previously described for dimer **2** was scaled up using 1 mM dimer **2**, 10 U TvL and 1 mM 1-HBT. The reaction ran for 72 h and product formation was monitored by LC-MS as described procedure 7.29. The reaction was acidified to pH 2.0 and extracted with 2x volumes ethyl acetate. The combined organics were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Following resuspension in 65% methanol, preparative HPLC was used to isolate the ketone product **4** for NMR analysis. Preparative reverse phase HPLC was performed on an Agilent 1200 series HPLC with UV/Vis detection at 280 nm using a C:18 250 x 21.20 mm Hyperclone 5 μ ODS column with separation achieved with an isocratic mobile phase of 60% methanol in super pure water at a flow rate 3.0 mL/min for 30 min. Fractions were collected coinciding with the elution of ketone **4** (21.5-23.2 min) and pooled before evaporating under reduced pressure.

δ_H (400MHz, $CDCl_3$) 7.75 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.62 (d, *J* = 2.0 Hz, 1H), 7.00 (ddd, *J* = 8.0, 7.2, 1.6 Hz, 1H), 6.92 (dd, *J* = 8.0, 1.6 Hz, 1H), 6.89 (d, *J* = 8.4 Hz, 1H), 6.89 (dd, *J* = 8.0, 1.6 Hz, 1H), 6.83 (ddd, *J* = 8.0, 7.2, 1.6 Hz, 1H), 5.41 (t, *J* = 5.3 Hz, 1H), 4.07 (d, *J* = 5.3 Hz, 2H), 3.95 (s, 3H), 3.92 (s, 3H), 3.86 (s, 3H). δ_C (100MHz, $CDCl_3$) 195.0, 154.0, 150.5, 149.2, 147.0, 128.1, 123.7, 121.2, 118.5, 112.3, 111.0, 110.1, 84.6, 63.8, 56.1, 56.0, 55.8.

MS of **4** was generated by liquid chromatography followed by electrospray ionization (ESI). The *m/z* of **6** is 332.13. ESI generated the molecular ion $[MH^+]$ = 333.0 (332.12 + 1), [M+Na] = 355.0 (332.12 + 22.9) and [2M+Na] = 687.3 (332.12 (x2) +22.9).

7.31 Characterisation of ketone 5

Ketone **5** was produced via step 4 in the synthesis of dimer **3** therefore was used as an authentic standard for the LC-MS analysis of the biotransformation of dimer **3**. MS of **7** was generated by liquid chromatography followed by electrospray ionization (ESI). The *m/z* of **5** is 376.15. ESI generated the molecular ion $[MH^+]$ = 377.0 (376.15 + 1), [M+Na] = 399.0 (376.15 + 22.9) and [2M+Na] = 775.3 (376.15 (x2) +22.9).

7.32 Lignin extraction by organosolv for py-GC/MS

Lignin was extracted from the solid WS substrate fraction following enzymatic treatments by organosolv delignification. The substrate was mixed with 60:40% w/w ethanol:H_2O at a solid/liquid-ratio of 1:10 (g d.w WS) for 2 h at 126°C. After 2 h the solid and liquid fraction were separated by centrifugation and lignin was precipitated from the liquid fraction by dilution with acidified refrigerated water (3:1 water:liquid fraction) as described by Huijegen *et al*. [398].

7.33 Py-GC/MS +/- TMAH

1mg of extracted wheat straw lignin from laccase treated samples was placed into a quartz pyrolysis sampling tube with quartz wool capped at the end. 7 μL tetramethylammoniumhydroxide (TMAH) was added to the lignin before loading onto the Chemical Data System (CDS) 5200 series pyroprobe pyrolysis unit. The probe was heated to 600°C for 10 s. Pyrolysed organic lignin fragments were analysed using an Agilent 7890A GC fitted with a HP-5 fused capillary column (J+W Scientific; 5% diphenyldimethypolysiloxane; 30 m length, 0.32 m internal diameter, 0.25 μm film thickness) coupled to an Agilent 5975 MSD single quadrupole mass spectrometer operating in electron ionisation (EI) mode (scanning a range of m/z 50 to 600 at 2.7 scans s^{-1}, ionisation energy 70eV). The pyrolysis transfer line and injector temperatures were set at 350°C, the heated interface at 300°C, the EI source at 230˚C and the MS quadrupole at 150°C. Helium was used as the carrier gas and the compounds were introduced in split mode (split ratio 50:1) with a split flow rate of 50 mL/min. The oven temperature started at 40°C and was held for 2 min and increased to 220˚C at 2.5˚C min^{-1}, then held at this temperature for one min before being heated to 300˚C at 20˚C min^{-1} whereby it was held at this temperature for 11 min. The total run time was 90 min. Products were identified and named by comparison to previously published data [330,334].

For studies without TMAH derivatisation, TMAH was omitted and 3 mg lignin sample was pyrolysed as described above but with pyroprobe heating to 600°C for 20 s instead of 10. GC/MS parameters were as described above but with changes to the split ratio (40:1), flow rate (40 mL/min) and the oven heating programme (40°C for 3 min, increase to 100 at 30°C min^{-1}, then increase to 300°C at 5°C min^{-1} for 5 min) and a reduced run time of 60 min.

7.34 FTIR analysis

FTIR analyses were performed on a Perkin Elmer Frontier unit with the Attenuated Total Reflectance (ATR) accessory. Spectra were collected in the 4000-450 cm^{-1} range with 4 cm^{-1} resolution and 2 mg extracted lignin was applied to the ATR crystal. The spectra reported an average of 16 scans that were subjected to background subtraction. Spectra were analysed using the Bio-rad KnowItAll Informatics System 8.2 Multi-Technique database and with literature as referenced in this work.

For FTIR with pyrolysis, the same pyroprobe conditions were employed as described for py-GC/MS in 7.33.

Appendix I: Supporting information

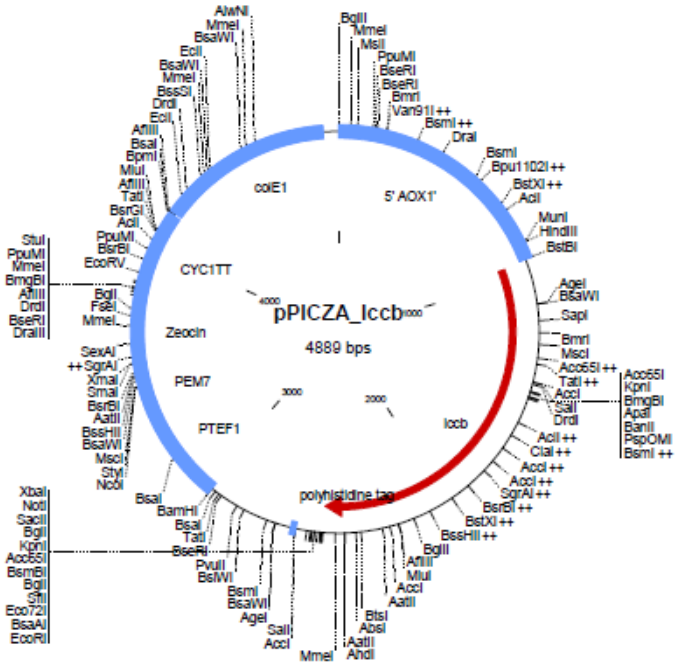


Figure 75: Plasmid map of the pPICZA-Lccβ plasmid (used for transformation into *P. pastoris* and for expression of Lccβ protein). Obtained through correspondence with K. Koschorreck (University of Dusseldorf).

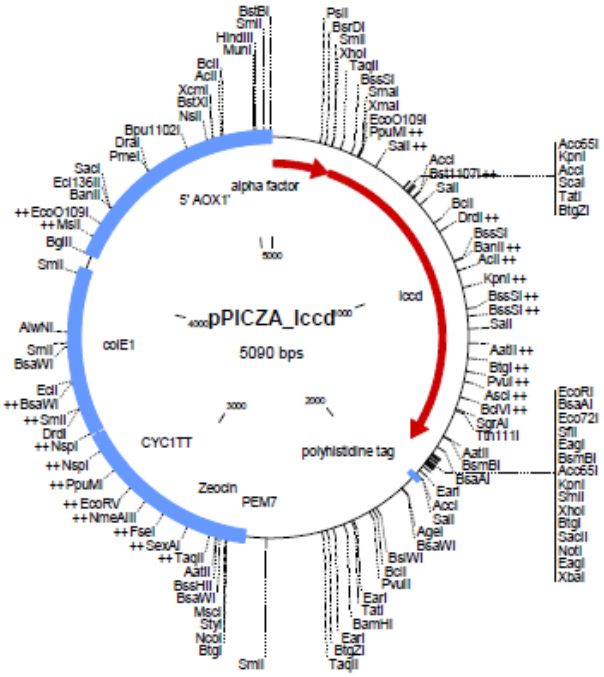


Figure 76: Plasmid map of the pPICZαA-Lccδ plasmid (used for transformation into *P. pastoris* and for expression of Lccβ protein). Obtained through correspondence with K. Koschorreck (University of Dusseldorf).

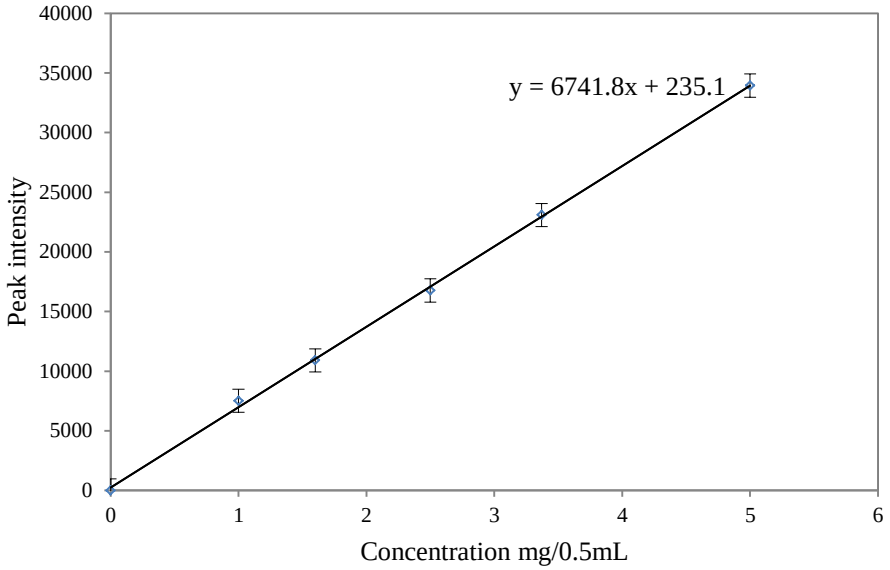


Figure 77: Glucose standard curve used for the determination of cellulase GC220 concentration in filter paper units/mL (FPU/mL), glucose standards used: 0, 1.00, 1.65, 2.50, 3.37 and 5.00mg/0.5mL.

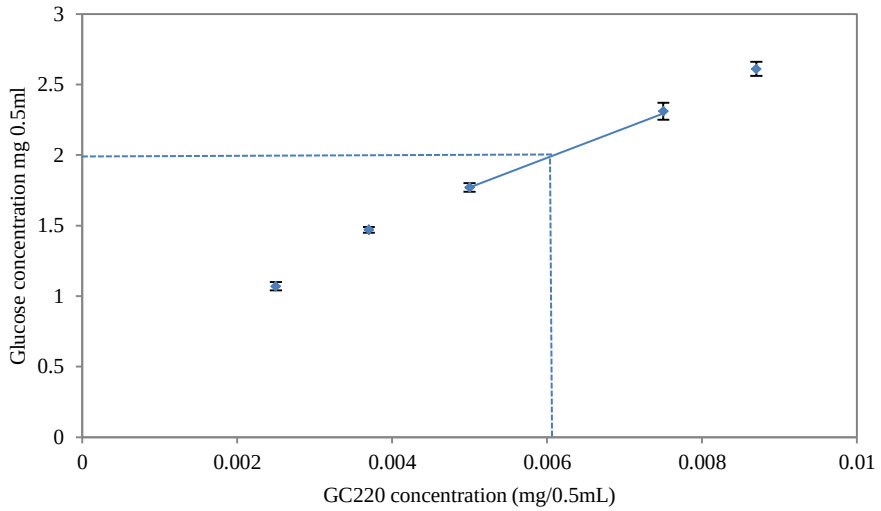


Figure 78: Glucose concentrations (mg/0.5mL) released during the hydrolysis of filter paper with GC220 in the range of 0.0025-0.0087mg/0.5mL. To determine the concentration of the GC220 preparation in FPU/mL, the estimated concentration of GC220 which would release 2mg/0.5mL glucose was determined by applying a trend line to both standards releasing just over and just under 2mg glucose (0.5mL) as described by Ghose [280]. Once this value was determined a calculation was applied (equation 3).

$$FPU/mL = \frac{0.37}{Enzyme\ concentration\ to\ release\ 2.0mg\ glucose}$$

$$\frac{0.37}{0.0061} = 61FPU/mL$$

Equation 3: Calculation of the FPU concentration of GC220. Calculation as described by Ghose [280]. 0.37 represents the amount of enzyme required (in units) to release 2.0mg glucose in an FPU reaction (calculation for the production of this value as described by Ghose [280]).

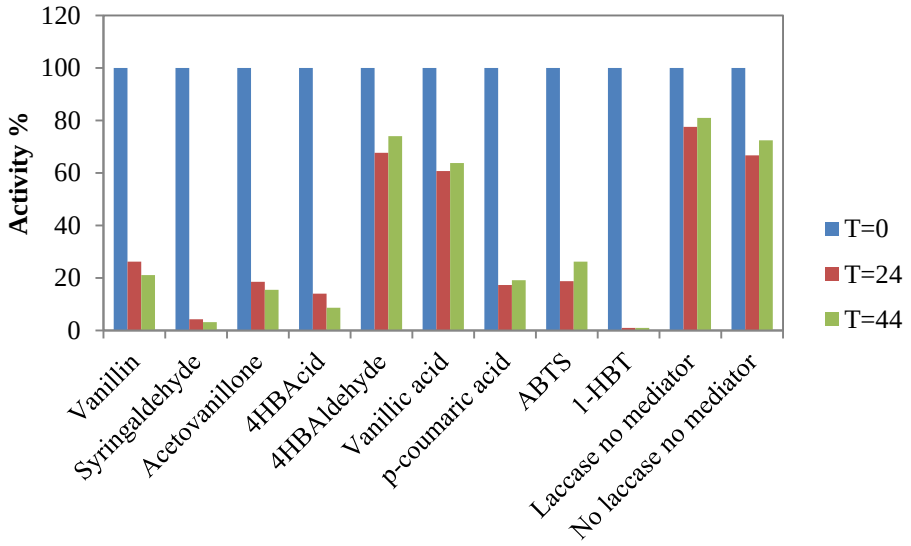


Figure 79: *p*-NPC assay to determine the combined endo and exoglucanase activity of GC220 during cellulose hydrolysis assays in the presence of an LMS (+ gluconolactone) with phenolic and synthetic mediators.

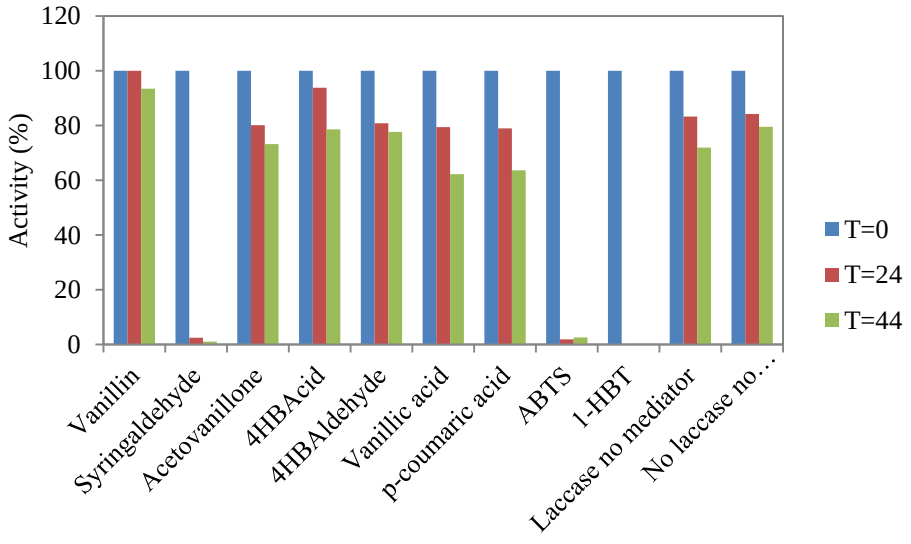


Figure 80: *p*-NPG assay to determine the β-glucosidase activity of GC220 during cellulose hydrolysis assays in the presence of an LMS (+ gluconolactone) with phenolic and synthetic mediators.

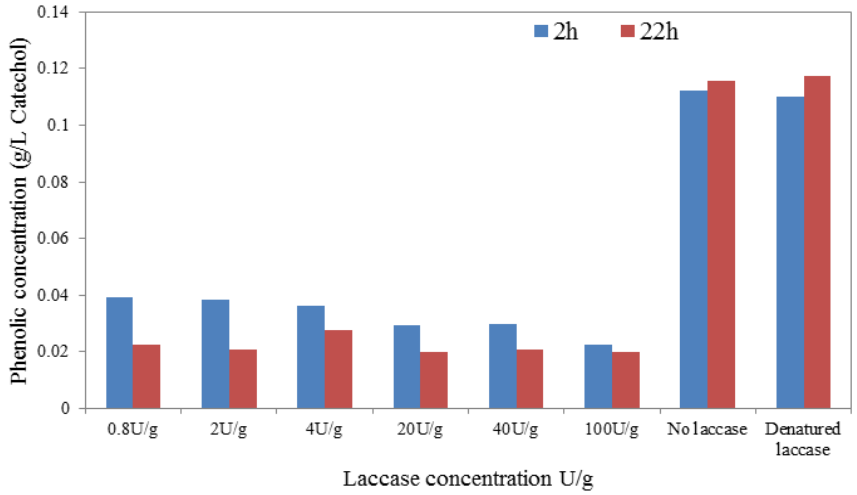


Figure 81: Phenol content (expressed as g/L catechol) from acid-treated wheat straw slurries treated with different concentrations of TvL. No laccase and denatured laccase samples were used as negative controls.

Table 47: Concentrations of sugars released following acid pretreatment of sorghum and corn stover

Substrate	Pretreatment[a]	[Glucose] g/L[b]	[Xylose] g/L[b]	[Arabinose] g/L[b]
Sorghum stover	H_2O	2.46 (0.14)	6.11 (0.19)	0.44 (0.15)
	1% H_2SO_4	4.75 (0.16)	22.57 (1.26)	3.12 (0.30)
	2% H_2SO_4	5.22 (0.03)	23.52 (2.06)	3.55 (0.19)
Corn stover	H_2O	0.46 (0.05)	0.14 (0.10)	0.08 (0.01)
	1% H_2SO_4	2.61 (0.19)	22.55 (1.52)	3.39 (0.28)
	2% H_2SO_4	3.12 (0.20)	25.70 (1.04)	3.74 (0.51)

[a] 1:10 (biomass:pretreatment solution), 1h, 126°C [b] Determined by HPLC-RID

Table 48: Biomass composition (raw and acid pretreated) as supplied by NREL.

Component	Raw Corn Stover	Raw Wheat Straw	Pretreated Corn Stover	Pretreated Corn Stover with Oligomer Hold Step	Pretreated Wheat Straw with Oligomer Hold Step
Cellulose	35.5	30.6	54.6	53.1	50.3
Xylan	22.4	16.8	2.4	2.2	3.0
Arabinan	4.0	2.3	0.8	0.7	0.7
Galactan	1.9	1.0	0.0	0.3	0.7
Lignin	10.8	12.5	31.5	33.1	19.5
Acetyl	5.5	1.0	0.3	0.2	1.4
Ash	5.0	13.8	5.3	6.3	14.2
Protein	1.8	1.9	2.4	2.3	1.5
Sucrose	6.1	0.1	-	-	-
Other Water Extractives	8.1	16.2	-	-	-
Ethanol Extractives	2.7	1.6	-	-	-

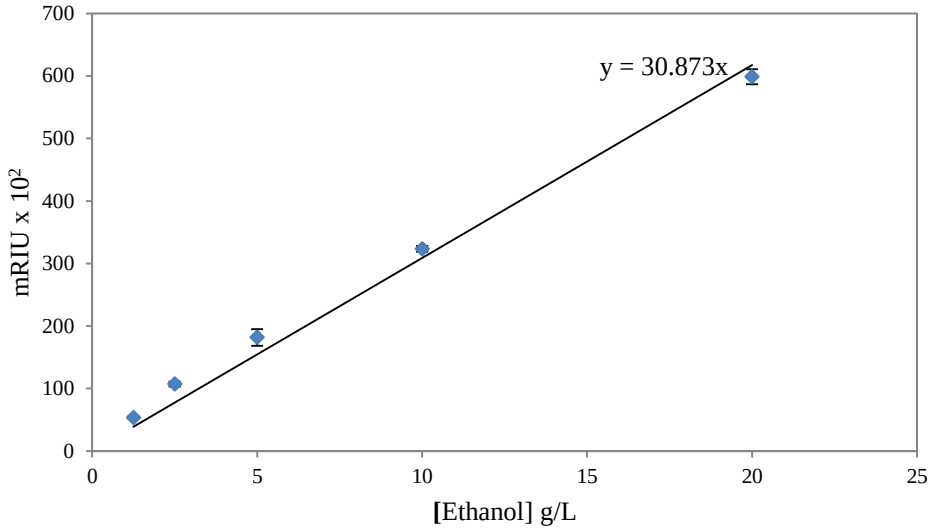


Figure 82: A standard curve for ethanol concentration (generated using standards of 1.25-20g/L ethanol).

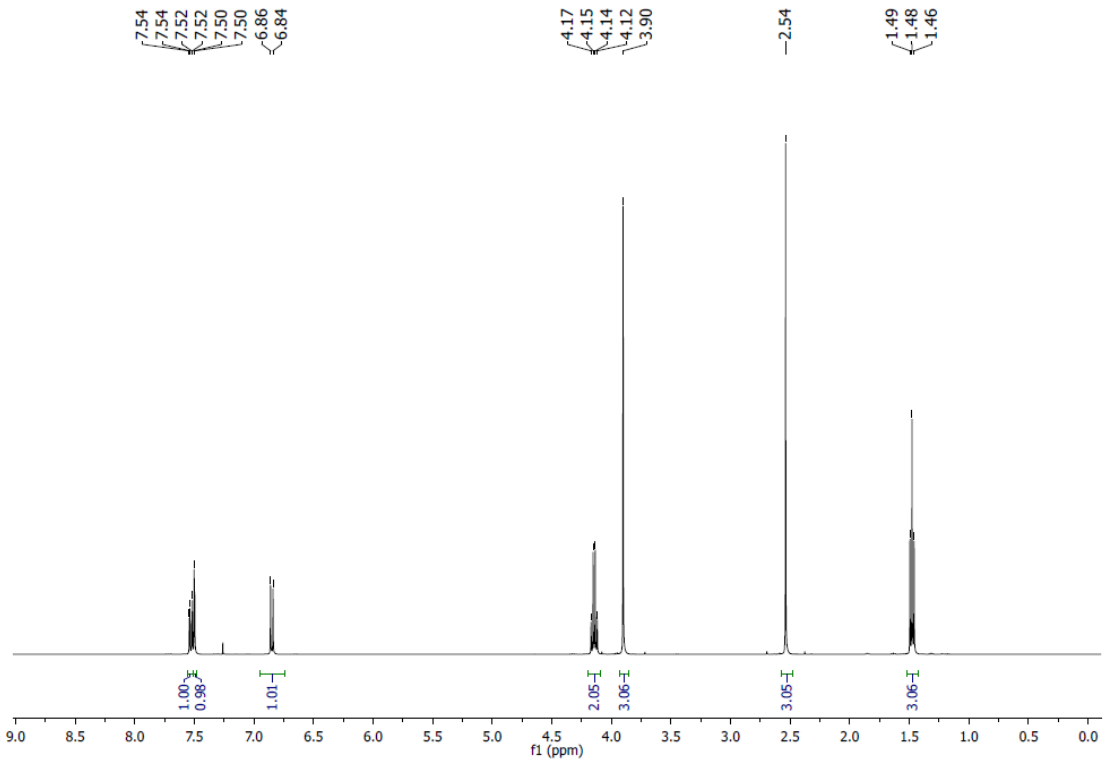


Figure 83: NMR of product from S1 of the synthesis of dimer 3.

δ_H (400MHz, $CDCl_3$) 7.53 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 6.85 (d, *J* = 8.4 Hz, 1H), 4.14 (q, *J* = 7.0 Hz, 2H), 3.90 (s, 3H), 2.54 (s, 3H), 1.48 (t, *J* = 7.0 Hz, 3H).

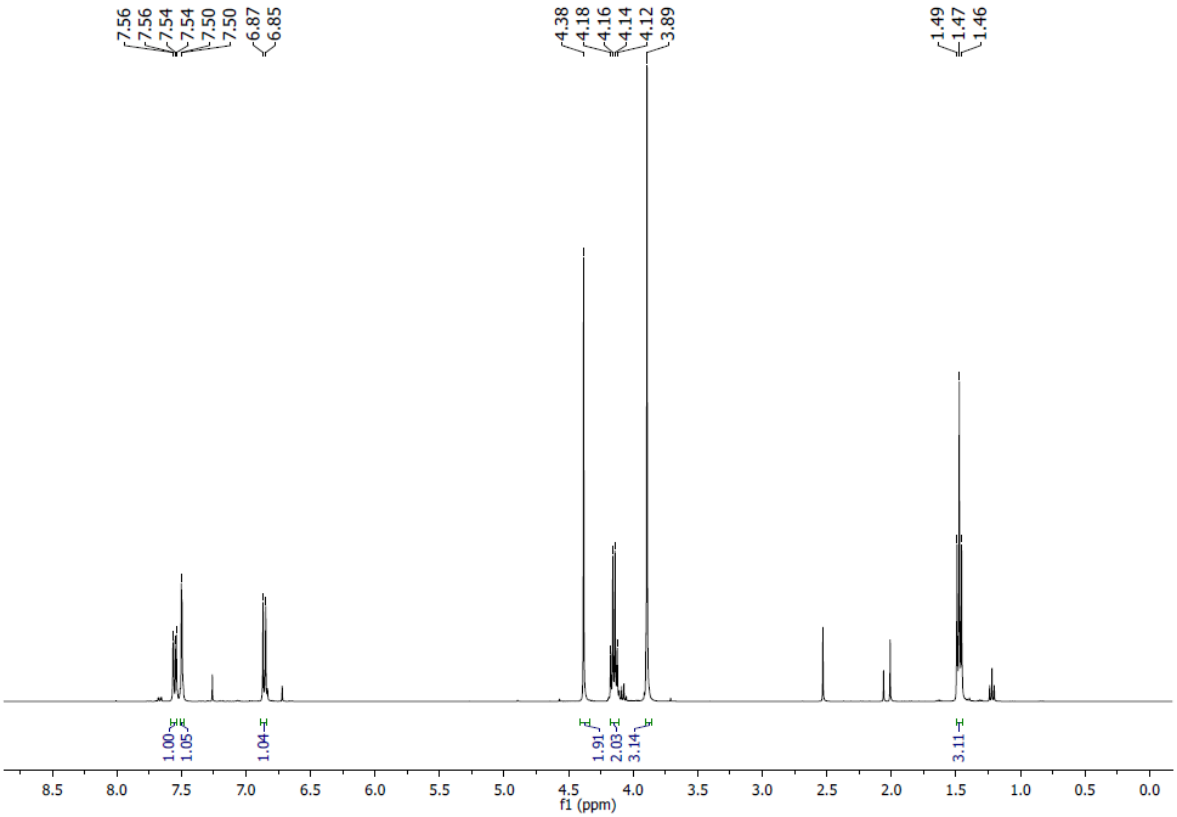


Figure 84: NMR of product from S2 of the synthesis of dimer 3.

δ_H (400MHz, $CDCl_3$) 7.55 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 6.86 (d, *J* = 8.4 Hz, 1H), 4.38 (s, 2H), 4.15 (q, *J* = 7.0 Hz, 2H), 3.89 (s, 3H), 1.47 (t, *J* = 7.0 Hz, 3H).

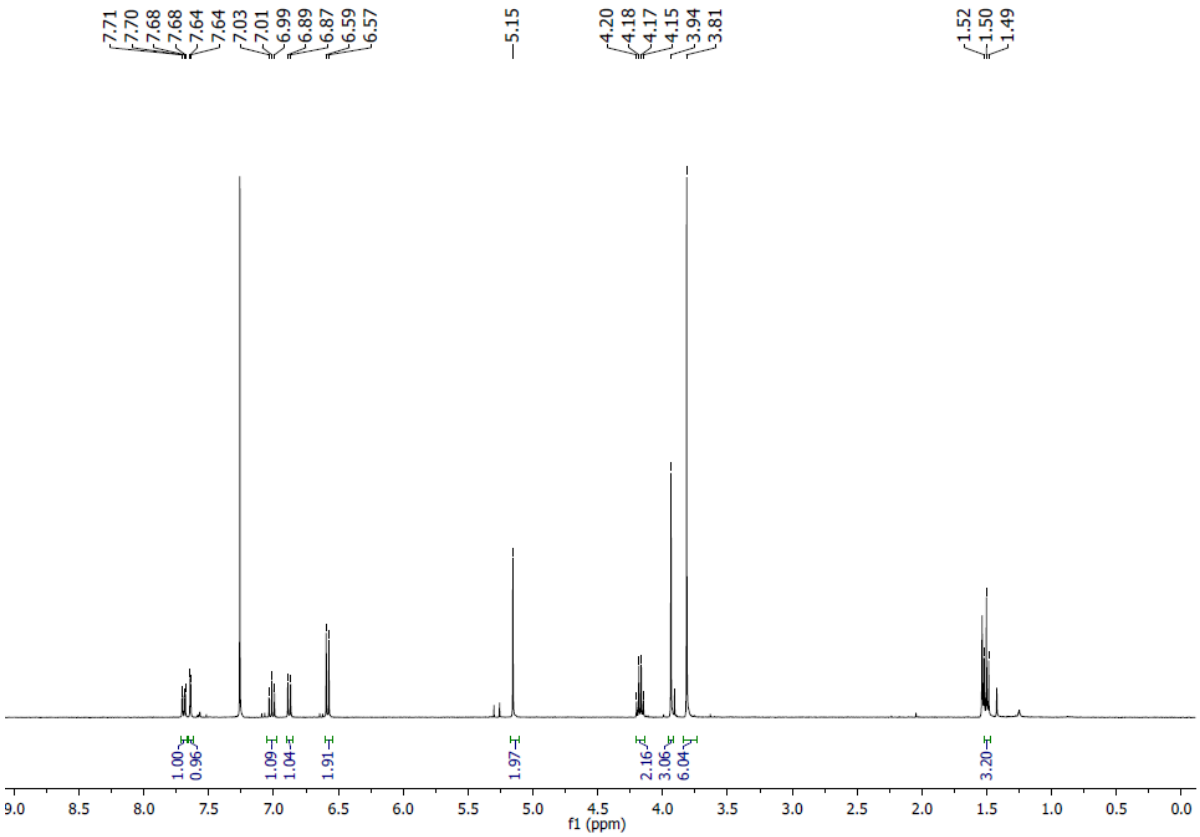


Figure 85: NMR of product from S3 of the synthesis of dimer 3.

δ_H (400MHz, $CDCl_3$) 7.69 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.64 (d, *J* = 2.0 Hz, 1H), 7.01 (t, *J* = 8.4 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 1H), 6.58 (d, *J* = 8.4 Hz, 2H), 5.15 (s, 2H), 4.17 (q, *J* = 7.0 Hz, 2H), 3.94 (s, 3H), 3.81 (s, 6H), 1.50 (t, *J* = 7.0 Hz, 3H).

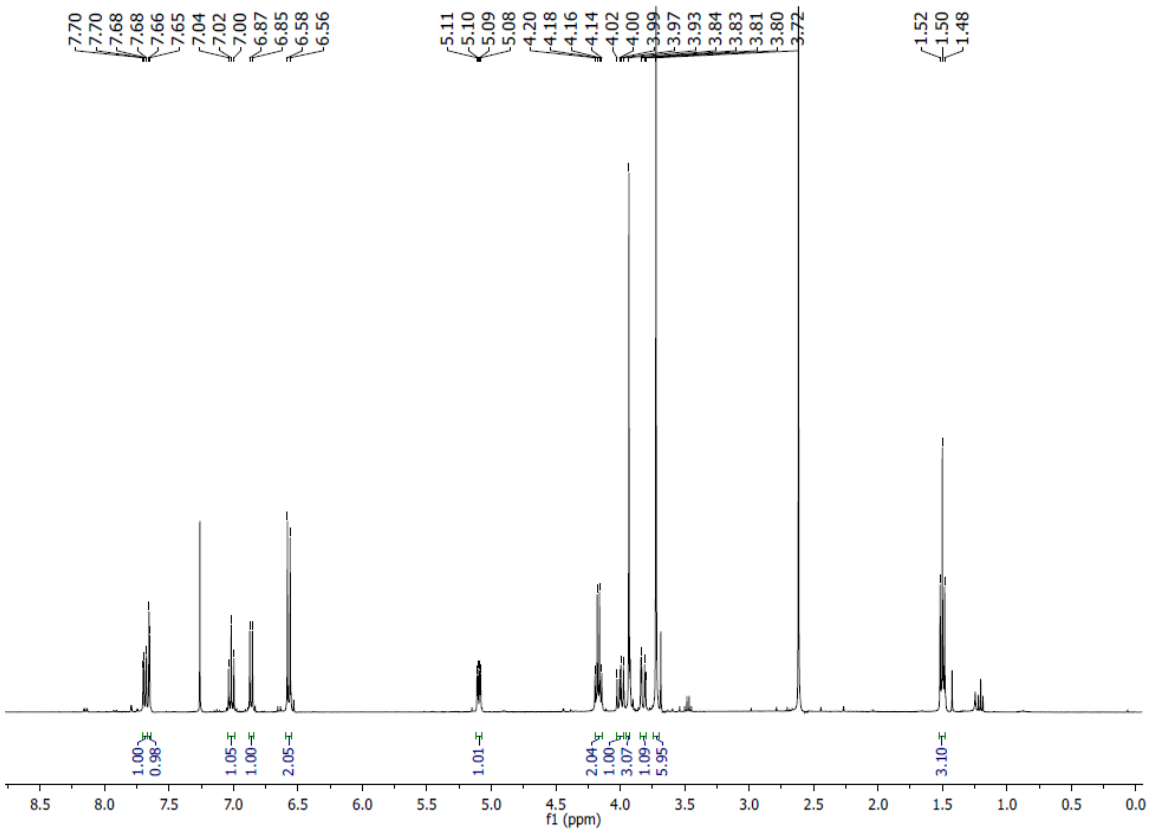


Figure 86: NMR of product from S4 of the synthesis of dimer 3 (ketone 5)

δ_H (400MHz, $CDCl_3$) 7.69 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.65 (d, *J* = 2.0 Hz, 1H), 7.02 (t, *J* = 8.4 Hz, 1H), 6.86 (d, *J* = 8.4 Hz, 1H), 6.57 (d, *J* = 8.4 Hz, 2H), 5.10 (dd, *J* = 7.9, 3.0 Hz, 1H), 4.17 (q, *J* = 7.2 Hz, 2H), 4.00 (dd, *J* = 12.2, 7.9 Hz, 1H), 3.93 (s, 3H), 3.82 (dd, *J* = 12.2, 3.0 Hz, 1H), 3.72 (s, 6H), 1.50 (t, *J* = 7.2 Hz, 3H).

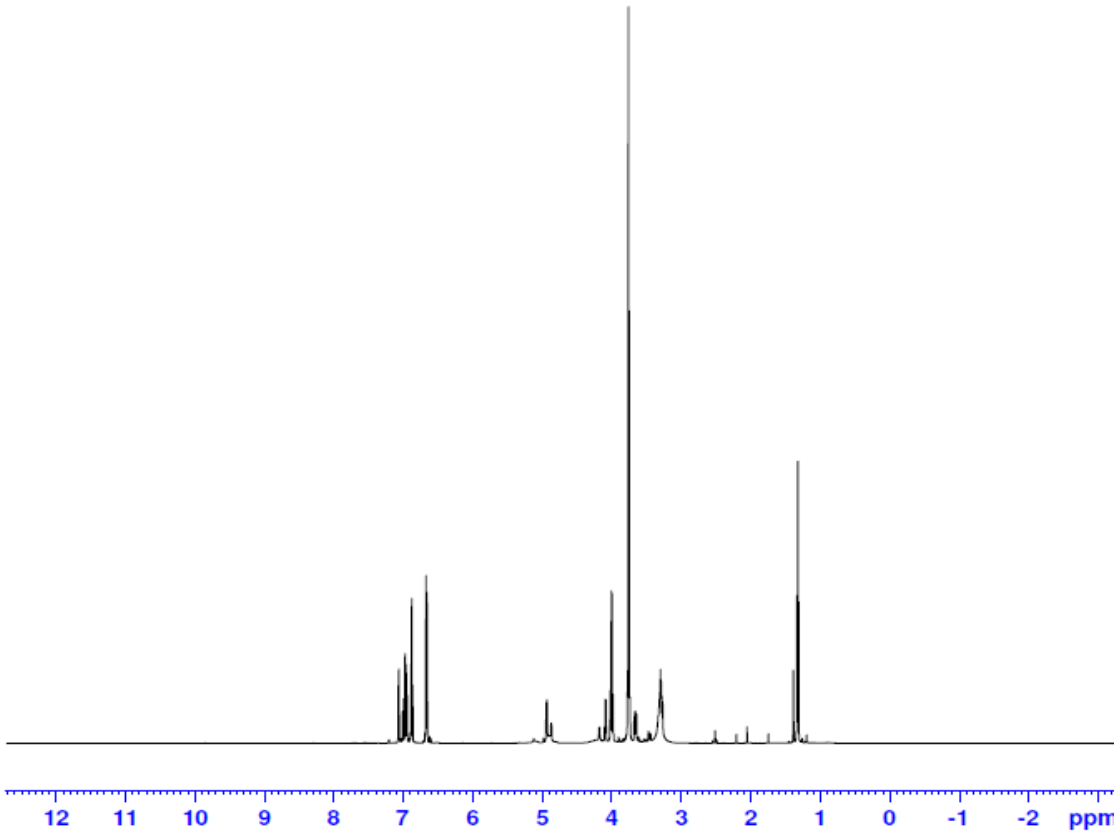


Figure 87: NMR of product from S5 of the synthesis of dimer 3.

δ_H (500MHz, DMSO, 50°C) 7.06 (d, *J* = 1.8 Hz, 0.71H), 7.00 (br. s, 0.29H), 6.97 (t, *J* = 8.4 Hz, 1H), 6.94 (dd, *J* = 8.3, 1.8 Hz, 0.71H), 6.88-6.85 (m, 1.29H), 6.66 (d, *J* = 8.4 Hz, 0.58H), 6.66 (d, *J* = 8.4 Hz, 1.42H), 4.93 (d, *J* = 4.8 Hz, 0.71H), 4.86 (d, *J* = 4.8 Hz, 0.29H), 4.17 (q, *J* = 4.7 Hz, 0.29H), 4.08 (q, *J* = 4.8 Hz, 0.71H), 3.99 (q, *J* = 7.0 Hz, 2H), 3.77-3.72 (m, 9.29H), 3.65 (dd, *J* = 11.5, 5.0 Hz, 0.71H), 3.45 (dd, *J* = 11.8, 4.1 Hz, 0.29H), 3.27 (dd, *J* = 11.5, 4.6 Hz, 0.71H), 1.31 (t, *J* = 7.0 Hz, 3H).

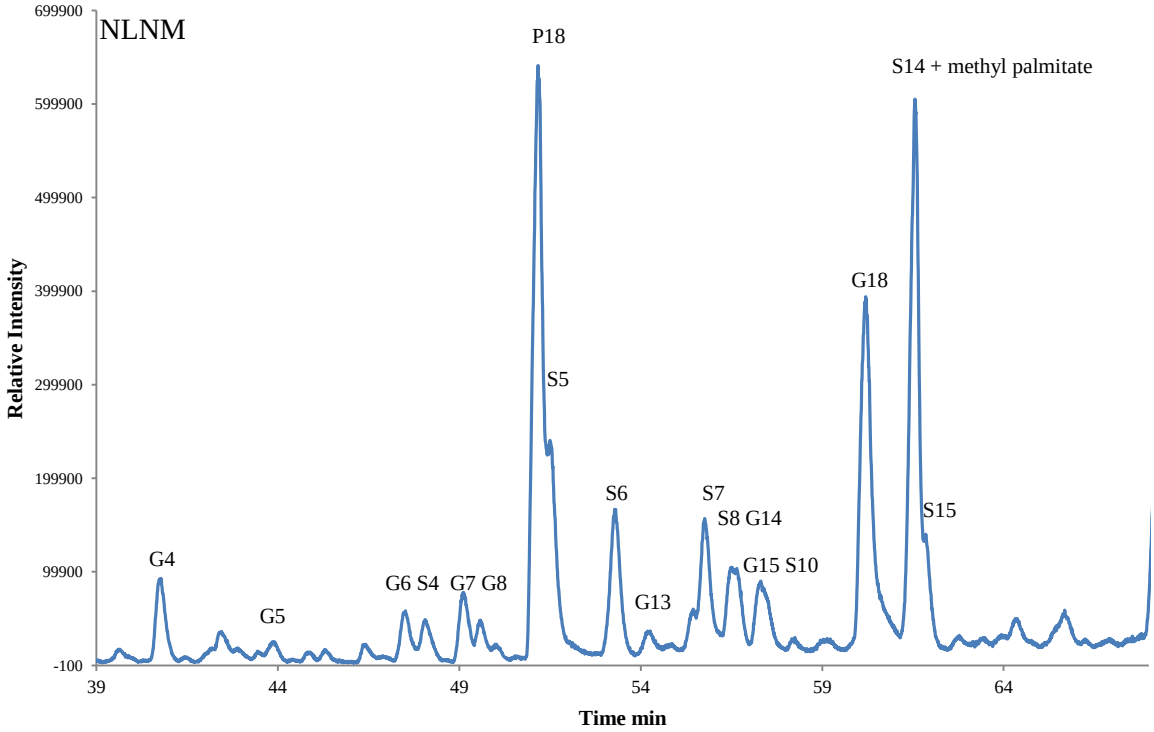


Figure 88: Partial chromatogram of the TIC for the TMAH thermochemolysis products from organosolv extracted wheat straw lignin without laccase or mediator treatment (NLNM). Products were identified from the information in Table 37 and by library searches (G: Guaiacyl, S: Syringyl and P: *p*-hydroxyphenyl).

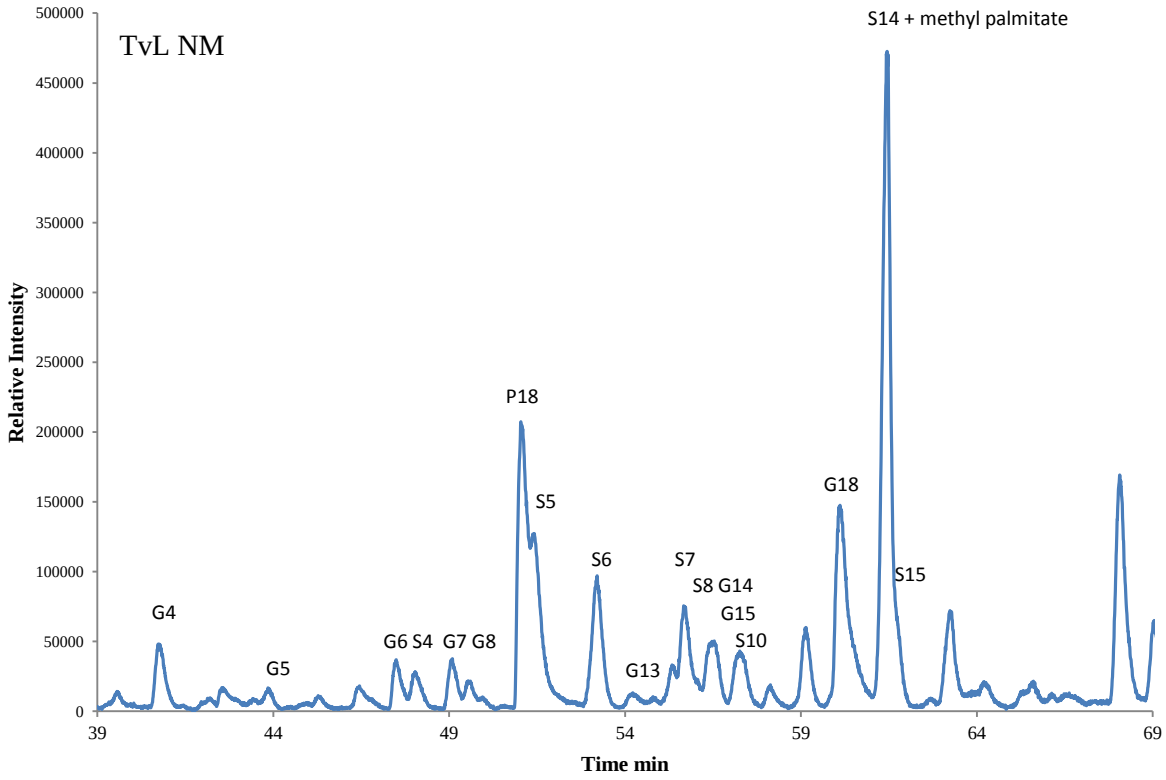


Figure 89: Partial chromatogram of the total ion current (TIC) for the TMAH thermochemolysis products from organosolv extracted wheat straw lignin with laccase treatment in the absence of mediator (TvLNM). Products were identified from the information in Table 37 and by library searches (G: Guaiacyl, S: Syringyl and P: *p*-hydroxyphenyl).

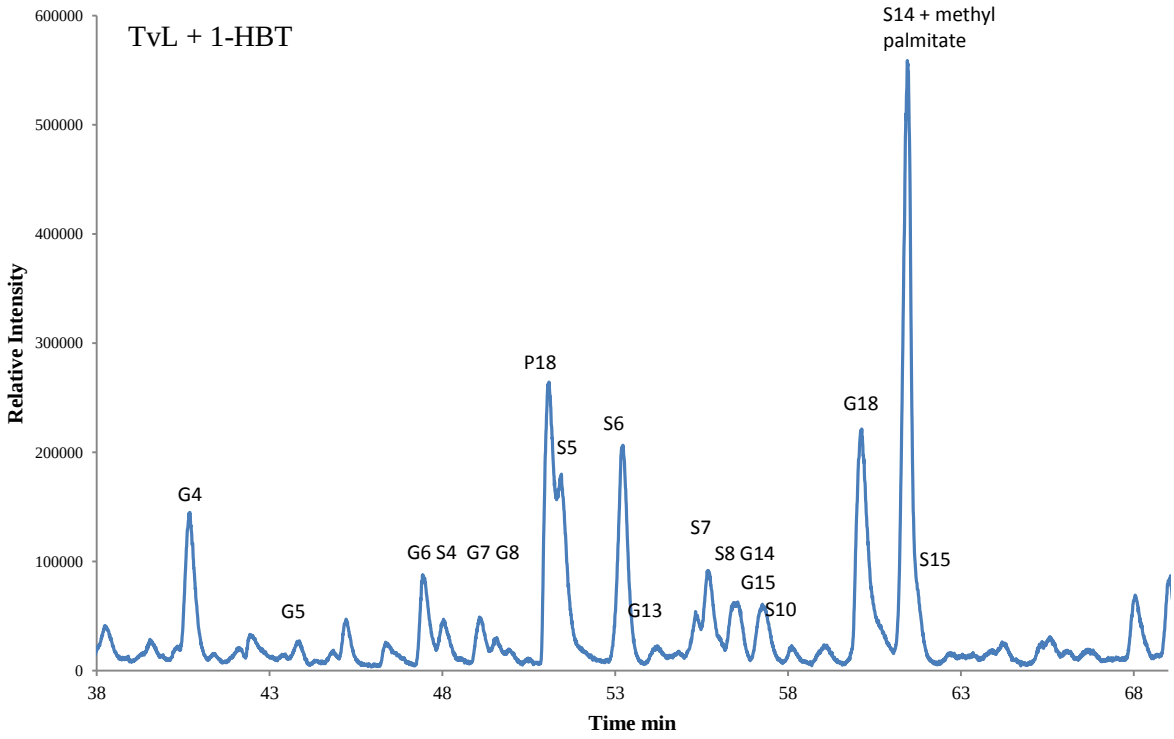


Figure 90: Partial chromatogram of the total ion current (TIC) for the TMAH thermochemolysis products from organosolv extracted wheat straw lignin with laccase treatment in the presence of mediator (TvL + 1-HBT). Products were identified from the information in Table 37 and by library searches (G: Guaiacyl, S: Syringyl and P: *p*-hydroxyphenyl).

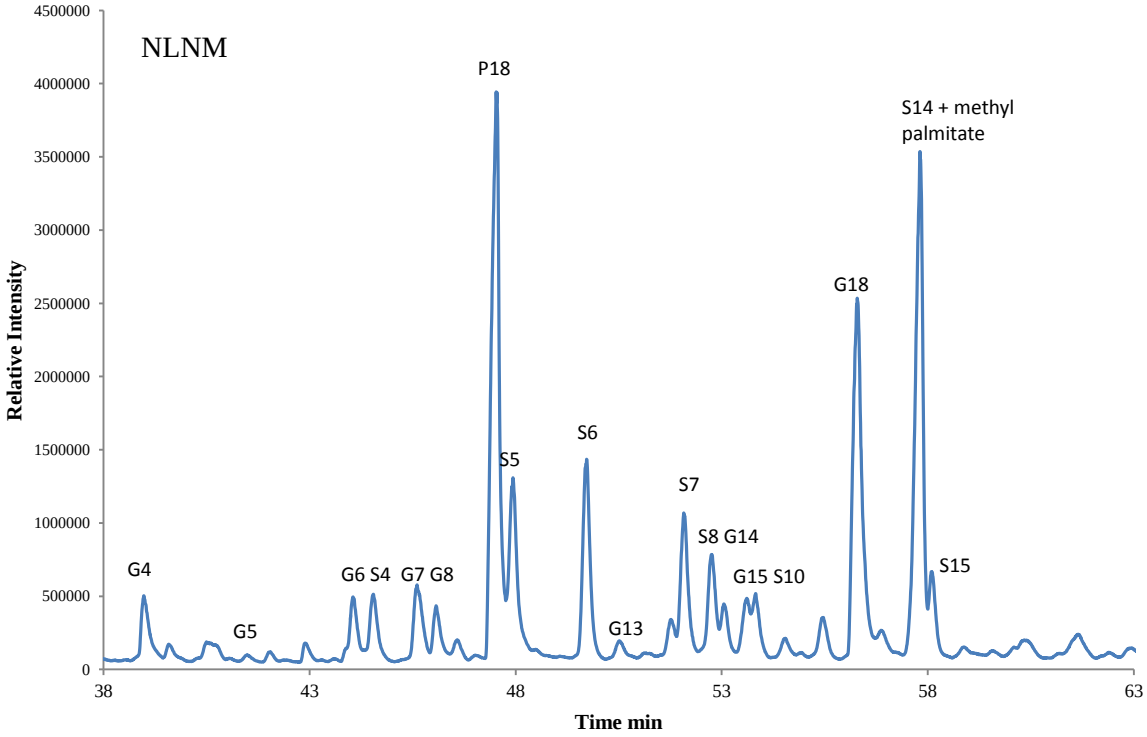


Figure 91: Partial chromatogram of the TIC for the TMAH thermochemolysis products from organosolv extracted wheat straw lignin without laccase or mediator treatment (NLNM). Products were identified from the information in Table 37 and by library searches (G: Guaiacyl, S: Syringyl and P: *p*-hydroxyphenyl).

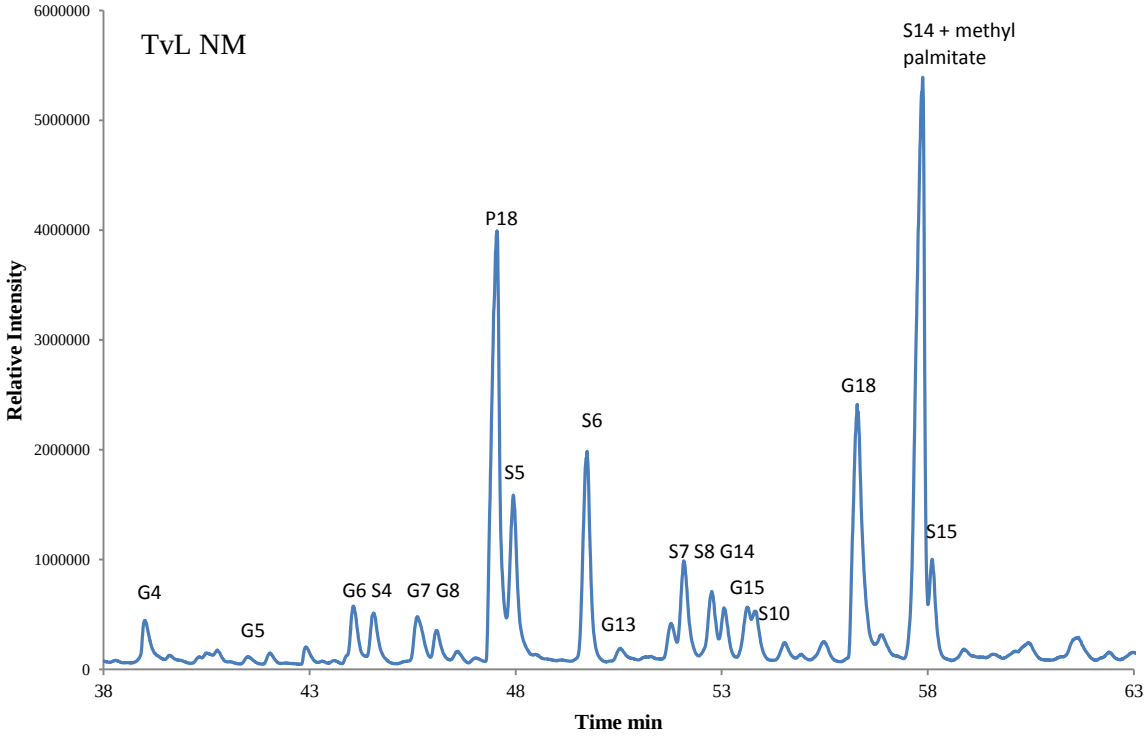


Figure 92: Partial chromatogram of the TIC for the TMAH thermochemolysis products from organosolv extracted wheat straw lignin with laccase treatment in the absence of mediator (TvLNM). Products were identified from the information in Table 37 and by library searches (G: Guaiacyl, S: Syringyl and P: *p*-hydroxyphenyl).

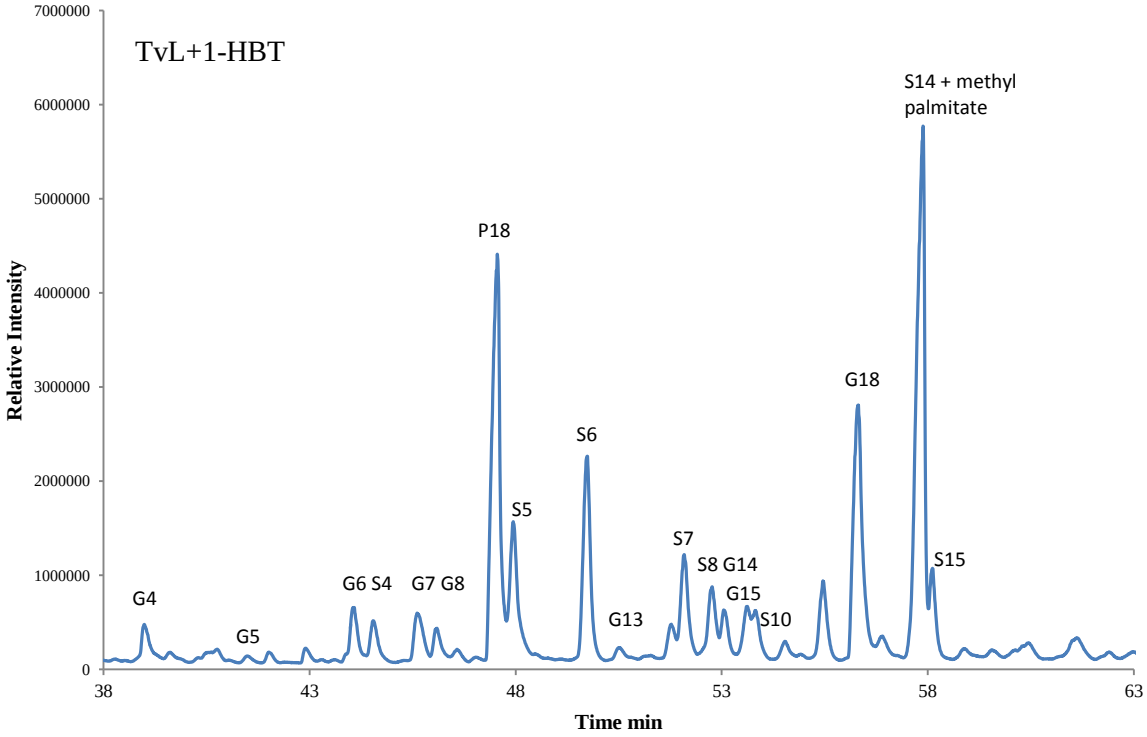


Figure 93: Partial chromatogram of the TIC for the TMAH thermochemolysis products from organosolv extracted wheat straw lignin with laccase treatment in the presence of mediator (TvL + 1-HBT). Products were identified from the information in Table 37 and by library searches (G: Guaiacyl, S: Syringyl and P: *p*-hydroxyphenyl).

Table 49: Cellulose and lignin thermochemolysis product peak areas and calculation of cellulose:lignin ratio

Cellulose marker	Peak area NLNM		Peak area TvL NM		Peak area TvL + 1-HBT	
	Exp 1	Exp 2	Exp1	Exp 2	Exp 1	Exp 2
Furfural	6314098	1525862	4140795	418382	4104657	3713111
2-methoxytoluene	2236812	884842	1818222	1561188	1457168	1044031
Guaiacol	14910740	685985	11269581	15408725	9464548	13044579
1,4-dimethoxybenzene	6099505	4635260	6884516	4696883	5271403	3555721
4-methoxybenzaldehyde	1158923	745827	1024148	1191044	839042	960693
3,5-dimethoxyphenol	28088519	23942741	23292336	25335410	28205946	20829669
1,2,3-trimethoxybenzene	3683451	8064991	4306153	9916247	3085090	7449477
1,2,3-trimethoxy-5-methylbenzene	3475687	497212	2533210	1593672	1901912	989915
Lignin marker						
G4	6570253	3134168	5616780	6979221	4861702	5163632
G5	25489045	4670277	10170563	20824057	8393909	15249622
G6	12098261	8261404	14420346	17318163	14165098	13939011
S4	20147606	9712373	14288573	20039223	13837496	15873345
S5	46365501	27869330	38537930	53093022	37474556	43768580
S6	49961392	37930408	51893272	54138681	52479011	50387533
Cell:Lignin ratio*	0.29	0.31	0.29	0.26	0.29	0.26

(*total peak area cellulose markers/ total peak area of lignin markers)

References

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Role of Laccase as an Enzymatic Pretreatment Method to Improve Lignocellulosic Saccharification

Lucy Heap[a], Anthony Green[a], David Brown[b], Bart van Dongen[c] and Nicholas Turner*[a]
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The recalcitrant nature of lignocellulose, in particular due to the presence of lignin, is found to decrease the efficiency of cellulases during the saccharification of biomass. The efficient and cost effective removal of lignin is currently a critical biotechnological challenge in order to improve the enzymatic digestibility of cellulose for bioethanol production. In this study the role and reactivity of laccase from *Trametes versicolor* (TvL) was assessed with and without mediators for the improved saccharification of acid-pretreated wheat straw. Lignin model compound studies using veratryl alcohol and β-O-4 dimers revealed that 1-hydroxybenzotrizole (1-HBT) was the most effective mediator. Combination of TvL and TvL+1-HBT treatments with an alkaline-peroxide extraction step increased the released glucose concentration following hydrolysis by up to 2.3g/L compared to an untreated control. Pyrolysis-gas chromatography-mass spectrometry (py-GC-MS) with tetramethylammonium hydroxide (TMAH) thermochemolysis analysis of the extracted lignin revealed structural changes that are consistent with lignin degradation mechanisms typical of fungi.

Introduction

Lignocellulosic substrates are attractive feedstocks for second generation biofuel production due to their high abundance and low cost. They are widely considered as waste or by-products from forestry and agricultural industries and unlike first generation biofuel feedstocks such as corn, they do not compete with food [1]. The removal of lignin from lignocellulosic substrates remains a critical issue in biomass processing for the production of bioethanol. The presence of residual lignin following conventional pretreatment methodologies is reported to negatively affect ethanol production via several mechanisms:

1) Lignin can bind non-specifically to the cellulases employed for saccharification, resulting in the reduced catalytic activity of these enzymes due to the potential blocking of active sites and the prevention of cellulose binding [2-4].
2) Lignin that remains bound to cellulose following pretreatment causes a reduction in the surface area of the cellulose that is available for enzymatic hydrolysis [4].
3) Lignin derived products such as aromatic phenols, acids and aldehydes are often toxic to the yeasts that perform the ethanol fermentation. Small concentrations of these inhibitors have been found to destroy the integrity of the yeast membrane systems preventing growth and sugar assimilation [5-8].

The first two issues directly affect the efficiency of cellulose hydrolysis during biomass processing and contribute to the recalcitrance-related obstacles that currently make second generation bioethanol production a costly and energy intensive process. There is a need for effective pretreatment technologies that can remove or reduce this recalcitrance issue whilst still maintaining cost competitive fuel production.

Biocatalytic approaches to lignin removal are currently being explored as greener alternatives to pre-existing chemical, thermal and physical pretreatment strategies. White-rot fungi from the basidiomycete phyla degrade all components in wood including lignin. Lignin degradation is carried out via the oxidative activities of a panel of ligninolytic enzymes from the fungal secretome. The majority of these enzymes are peroxidases such as lignin peroxidase (LiP EC 1.11.1.14) and manganese peroxidase (MnP EC 1.11.1.13), however laccases (EC 1.10.3.2; benzenediol: oxygen oxidoreductase) are also suggested to play a role in lignin degradation and are attractive enzymes industrially due to their catalytic dependence on molecular oxygen as opposed to hydrogen peroxide. This dependence results in the higher stability of most laccases compared to peroxidases which suffer from deactivation by hydrogen peroxide [9]. Laccases are phenol-oxidases that catalyse the one electron oxidation of phenolics, aromatic amines, di-amines and other electron rich substrates via the four-electron reduction of oxygen to water [9-11]. Interestingly the substrate range of laccase can be expanded through the oxidation of lower molecular weight molecules known as mediators or enhancers. Oxidation of these mediators by laccase generates charged intermediates which are able to act as chemical oxidants, overcoming the steric and redox limitations that prohibit laccases from oxidising bulky and/or nonphenolic substrates [12-14].

Interest into the role of laccases on lignin degradation has developed due to reports of some white rot basidiomycetes that are found to degrade lignin or lignin model compounds in the absence or deficiency of LiP and/or MnP [15-18]. This observation suggests that degradation mechanisms by other enzymes such as laccase or aryl-alcohol oxidase (AAO) can predominate in lignin depolymerisation. However, a typical lignin polymer is 10-15% phenolic in composition, rendering up to 90% of the polymer unreactive to laccase. Fungi are assumed to use laccase for non-phenolic lignin degradation through self-generation of reactive phenoxy radicals which act as natural mediators. This is said to occur from the laccase catalysed oxidation of phenolic lignin components and or/existing lignin degradation products [18-20] or through the oxidation of their secreted phenolic metabolites [16].

In this study we initially investigated the development of an effective laccase-mediator system (LMS) using laccase from *Trametes versicolor* (TvL) in the presence of both synthetic and natural mediators. Selected systems were thereafter evaluated for their

ability to improve the saccharification reaction of acid-pretreated wheat straw by analysing the concentration of glucose released. The mechanism of laccase and laccase-mediated lignin modification and degradation was explored using a series of model lignin β-O-4 linked dimers and py-GC-MS with TMAH.

Results and Discussion

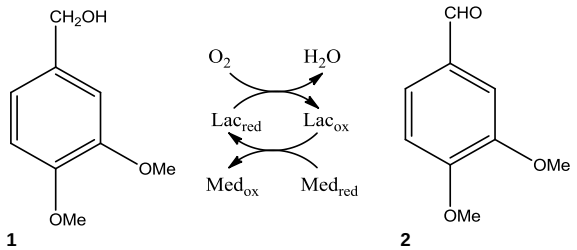
Redox mediator screening for lignin studies

A panel of 30 phenolic compounds were selected for assessment of their suitability as natural redox mediators with Trametes versicolor laccase (TvL redox potential ~800mV [21]). A preliminary high throughput screening assay was developed based on the previous work of Camarero *et al.*, [22] using the recalcitrant dye Reactive Black-5 (RB-5). The data revealed that under optimised mediator and dye concentrations most of the 30 phenolic compounds screened were successful at decolourising RB-5 to varying degrees within 3h. Syringaldehyde, acetosyringone and 2,4,6-trimethylphenol were found to be the best mediators for decolourising RB-5 (see SI Table 1). No decolourisation was observed in the absence of mediators.

The best 13 phenolic compounds from the decolourisation of RB-5 were further screened against the lignin model compound veratryl alcohol 1 along with synthetic compounds 1- 25 hydroxybenzotriazole (1-HBT), 2,2'-azinobis(3- ethylbenzthiazoline-6-sulphonic acid) ABTS, and violuric acid which are reported to be effective mediators for laccase catalysed oxidations [14, 23, 24]. In addition, several dyes/indicators were screened following reports of these compounds acting as laccase substrates and mediators [20, 22, 25]. 1 is a widely utilised monomeric lignin model compound for laccase mediator studies [12, 13, 24, 26-28]. Oxidation leads to the formation of a single product veratryl aldehyde 2, which is simple to detect by HPLC**.** Laccase is unable to oxidise **1** in the absence of a mediator making this a suitable candidate for assessing the non-phenolic oxidation ability of laccase mediators. Analysis of the oxidation of **1** over time showed that 1-HBT, violuric acid and ABTS were efficient redox mediators for this reaction, with conversions to **2** reaching up to 99% after 24h under optimal conditions (Table 1). Of the dyes investigated, only phenol red (PR) and remazol brilliant blue (RBB) acted as potential mediator substrates for TvL, resulting in 50 & 52% conversion respectively. No oxidation of **1** was observed in the absence of mediators.

In agreement with previous studies [19, 29], the natural phenolic mediators were not found to oxidise **1** to **2** in significant quantities which is in contrast to the results observed with RB-5. Presumably substrate **1** is less effective at reducing the intermediate phenoxy radicals produced by laccase compared with RB-5. This will explain the slower reaction rate observed with **1** which would promote undesired non-catalytic mechanisms leading to radical coupling and polymerisation, thus preventing the conversion of **1** to **2** [20, 30]. Under the assumption that lignin model compounds such as **1** can provide a reasonable indication of laccase reactivity towards natural lignin, then naturally occurring phenolic structures such as those investigated here would be expected to act poorly as mediators due to their instability in the catalytic cycle. The rapid decolourisation of RB- 5 suggests a role for these compounds in technologies linked with dye detoxification/decolourisation in the textile industry.

Table 1: Oxidation of veratryl alcohol (**1**) by TvL in the presence of a redox mediator



1 **2**

Mediator	% Conversion to **2**[a]			
	A	B	C	D
1-HBT	**99**	94	98	92
ABTS	74	61	87	**88**
Violuric acid	31	71	24	**74**
Phenol red	**50**	19	17	33
Remazol Brilliant Blue	21	9	22	**52**

Oxidation of 3mM **1** under four different enzyme-mediator conditions. A: 1.6U/ml TvL, 3:1 lac-med ratio, B: 1.6U/ml TvL, 1:1, C: 0.4U/ml TvL, 3:1 D: 0.4U/ml 1:1[a]% conversion was determined by LC-MS after 24h, optimal conversions are shown in bold.

The effect of laccase and LMS on lignocellulosic saccharification

In order to determine the effectiveness of the successful laccase mediator systems (LMS) investigated with **1** towards a lignocellulosic bioprocess, laccase and LMS treatments were carried out on wheat straw that had previously been treated with dilute sulfuric acid. Dilute acid hydrolysis is a commonly employed pretreatment method that effectively solubilises the hemicellulose component within lignocellulose. Removal of hemicellulose improves the digestibility of cellulose by increasing the porosity of the substrate [31]. Wheat straw slurries treated with different concentrations of TvL were initially analysed for free phenol content using the Folin-ciocalteu total phenol assay [32]. In accordance with a previous study [33], a decrease in soluble phenol content was observed in wheat straw slurries treated with TvL (see SI, Figure 2) supporting the role of laccase in the oxidation of phenolic structures within biomass and the removal of the reactive intermediate phenoxy radicals by polymerisation. A cellulase preparation (Genencor GC-220) was added to the slurries following treatment with the different TvL concentrations to allow hydrolysis of the cellulose fraction. Following quantification of the released glucose it was revealed that no increase in glucose concentration was observed in the TvL treated slurries compared to the laccase free and denatured laccase negative controls. The reaction was further investigated with TvL and the addition of synthetic mediator 1-HBT. An increase in glucose release was not observed following hydrolysis despite the incorporation of wash steps to remove potential inhibitory compounds and both the enzyme and mediator. In fact, it appeared that laccase treated slurries were inhibiting the saccharification process (Figure 1 top graph).

A recent study by Gutierrez *et al.*, [34] reported an increase in polysaccharide hydrolysis for eucalypt wood

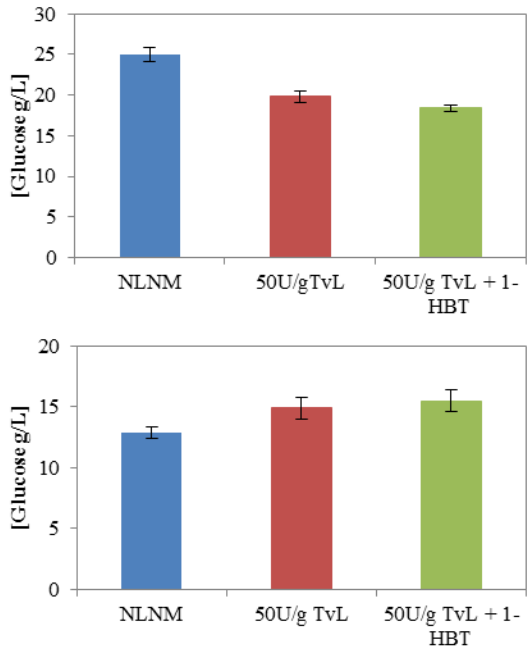


Figure 1. Concentration of glucose released from 0.6g (d.w) wheat straw hydrolysed by 2.9FPU GC220 for 48h following treatment with/without TvL and TvL+1-HBT. Top: No APE step after laccase treatment. Bottom: APE treatment performed after laccase treatment. Error bars represent standard error of three biological replicates.

Table 2: Investigating the optimum concentration of 1-hydroxybenzotriazole (1-HBT) with TvL to improve wheat straw saccharification

Biomass treatment[a]	Glucose concentration g/L[d]
150U/g[b] TvL	10.8 (0.13)
150U/g[b] TvL + 2.5%[b] 1-HBT[c]	11.0 (0.23)
150U/g[b] TvL + 5%[b] 1-HBT[c]	**12.2** (0.37)
150U/g[b] TvL + 7.5%[b] 1-HBT[c]	11.2 (0.80)

[a] 0.5g d.w wheat straw incubation for 40h, 28°C, 200rpm [b] enzyme units per g dry weight wheat straw biomass [c] % (g/g) mediator per dry weight wheat straw biomass [d] glucose concentration determined by HPLC-RID following 64h hydrolysis with 2.9 FPU GC220. Parentheses represent standard error of three biological replicates. Bold indicates most successful treatment.

Table 3: Screening of synthetic and natural mediators with TvL for the improvement of wheat straw lignocellulosic saccharification

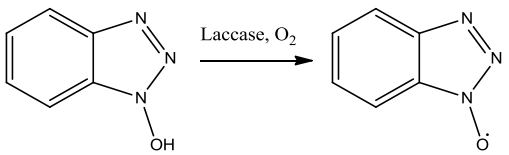
Biomass treatment[a]	Glucose concentration g/L[d]
NLNM	6.6 (0.03)
150U/g TvL	7.8 (0.19)
150U/g TvL 1-HBT[b]	**8.9** (0.20)
150U/g TvL ABTS[b]	7.7 (0.22)
150U/g TvL violuric acid[b]	8.3 (0.10)
150U/g TvL syringaldehyde[c]	7.7 (0.08)
150U/g TvL acetosyringone[c]	7.5 (0.25)

[a] 0.5g d.w wheat straw incubation for 40h, 28°C, 200rpm [b] mediator concentration of 1-HBT was 5% (g/g) d.w biomass, molar concentration of ABTS and violuric matched to molar concentration of 1-HBT [c] mediator concentration 7mM [d] glucose concentration determined by HPLC-RID following 42h hydrolysis with 2.9 FPU GC220. Parentheses represent standard error of three replicates. Bold indicates most successful treatment.

and elephant grass treated with *T. villosa* laccase and 1-HBT after the incorporation of an alkaline-peroxide extraction (APE) step. This step was therefore applied to all wheat straw slurries following laccase treatments. Quantification of released glucose during hydrolysis following this additional step revealed that slurries pretreated with TvL and TvL+1-HBT now released higher concentrations of glucose following hydrolysis compared to the no laccase no mediator negative control (NLNM) (Figure 1 bottom graph). This LMS can induce a positive effect on the saccharification process of wheat straw. The results in Figure 1 also demonstrate that use of an APE step leads to a decrease in released glucose concentration both in the presence and absence of laccase (Figure 1). This can be explained by the additional biomass handling steps during three consecutive extractions and subsequent wash steps which results in biomass loss. In addition, the alkali oxidative environment can induce undesired cellulose cleavage reactions. The addition of magnesium sulphate has been reported to minimise peroxide induced cellulose degradation suggesting the opportunity to minimise these undesired reactions during process optimisation and development [35].

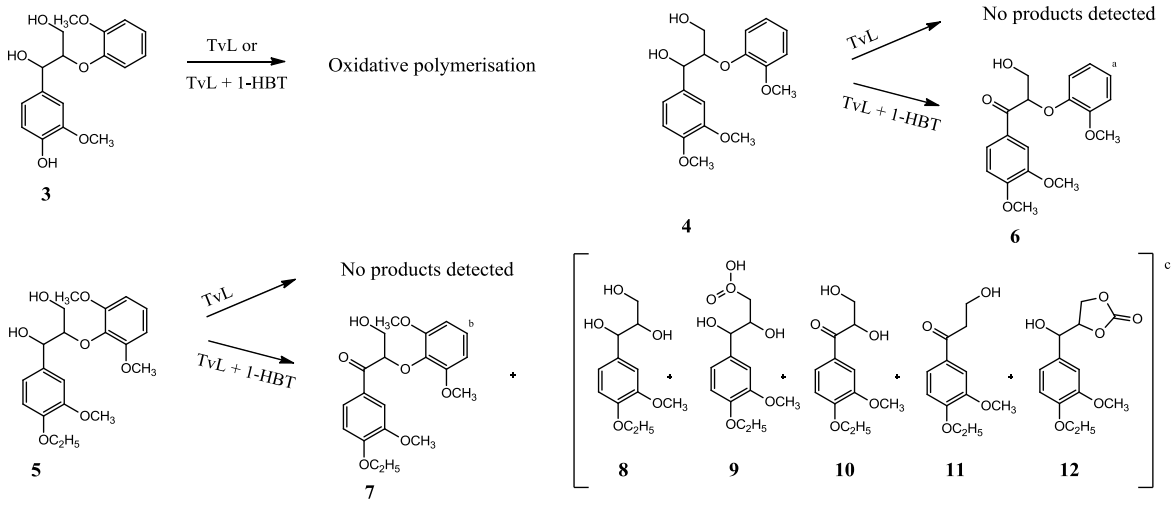
Optimisation of laccase loading and mediator concentration revealed that 500U/g d.w WS (units TvL per gram dry weight of wheat straw) was optimal for increased saccharification (see SI, Figure 4) however due to commercial enzyme costs, subsequent experiments were carried out using 150U/g (d.w WS). Use of the redox mediator 1-HBT at 5% (w/w) with 150U/g TvL proved optimal and lead to an increase of 1.4g/L (13%) glucose after 64h hydrolysis compared to the mediator free control (Table 2). Based on the mediator screening data, some synthetic and natural mediators were applied to the optimised bioprocess to assess their suitability and reactivity on wheat straw lignin. 1-HBT was found to be the most effective mediator, increasing the concentration of released glucose following hydrolysis by 2.3g/L (35%) compared to the NLNM negative control (Table 3). This is in support of previous studies whereby 1-HBT was reported to be the most efficient mediator in the delignification and biobleaching of lignocellulose [14, 36-38].

Violuric acid also proved to be an effective mediator, increasing glucose concentration by 1.7g/L (26%). 1-HBT and violuric acid belong to the hydroxylamine laccase mediator class and share the N-OH structural feature. Previous studies have revealed that these mediators undergo laccase catalysed oxidation to produce aminoxyl radicals following deprotonation as outlined with 1- HBT in Scheme 1. Oxidation reactions promototed by these aminoxyl radicals are reported to follow a hydrogen-atom transfer (HAT) mechanism by abstraction of the benzylic hydrogen of the reduced substrate [28, 39].



Scheme 1. The oxidation of 1-hydroxybenzotriazole (1-HBT) by laccase to produce the corresponding nitroxy radical

The use of potential TvL mediators acetosyringone and syringaldehyde did not improve saccharification when compared to the mediator free control. This result was expected following the previous unsuccessful oxidation of model compound 1 by both LMS. Surprisingly, the reaction of TvL with the synthetic mediator ABTS did not improve saccharification despite the excellent conversion



Scheme 2: Reactions of lignin model β-O-4 dimers **3**, **4**,and **5** [a] Product **6** characterised by MS and NMR following purification [b] Product **7** characterised by MS and by comparison with an authentic standard [c] Degradation products were identified by LC-MS and comparison with degradation products reported in the literature [40].

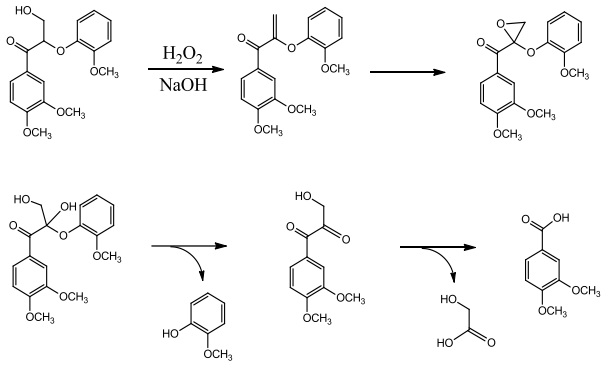
of **1** to **2** using this LMS (Table 1). A similar result was also observed by Chen [37], who reported little differences between the measured water soluble carbohydrate concentration released from hydrolysed ensiled corn stover after TvL and ABTS treatment. Similarly, the application of PR and RBB as mediators failed to result in an increased glucose concentration compared with the mediator free control (see SI, Figure 6). These observations suggest that the ability of a LMS to successfully oxidise the monomeric guaiacyl (G-type) lignin model compound **1**, does not necessarily correlate with an ability of the LMS to react with lignin to improve cellulose hydrolysis. Monomeric substrates such as **1** are likely to represent poor lignin model compounds due to the absence of β-linked structures that are predominant in natural lignin. Furthermore, the structural complexity of lignin regarding the heterogeneously linked G-type, S-type (syringly) and H-type (*p*-hydroxylphenyl) sub-structures highlights the challenges associated with drawing meaningful conclusions from model studies.

Interestingly, the incubation of wheat straw with TvL in the absence of mediator consistently improved saccharification. This increase in glucose release following laccase treatment alone has been previously reported [4, 34, 36]. It has been speculated that the binding of laccase to lignin sites within biomass competes with, and reduces the non-specific binding of cellulases therefore improving saccharification. Furthermore, electron spectroscopy for chemical analysis (ECSA) has been used to probe surface modifications of spruce lignin following treatment with *Trametes hirsuta* laccase [36]. The study revealed an increase in carboxylic acid residues following laccase treatment and suggests that this modification to lignin may decrease the non-specific adsorption of negatively charged cellulases due to electrostatic repulsion. Conversely, the treatment of lignin with laccase has been reported to increase undesired cellulase binding thus inhibiting saccharification [4]. Although the mechanism of this has not been described, this effect may explain the decrease in saccharification following TvL and TvL + 1-HBT treatment without APE reported in this study. Jurado *et al*., and Tabka *et al*., [33, 41] reported the same inhibitory effect with steam exploded wheat straw and two different fungal laccases, however they did not investigate the use of APE.

The results obtained in this study consistently revealed an improvement of saccharification following the incubation of wheat straw with TvL and a successful LMS followed by APE, demonstrating the reproducibility of this effect even when different batches of washed substrate were used. To further investigate reproducibility, the optimised bioprocess with TvL, 1- HBT and APE was repeated with two other agricultural residues, corn and sorghum stover. The same trends regarding the increase in glucose release was observed with both substrates (see SI Figure 7) demonstrating the success of the laccase and 1-HBT treatments with alternative substrates.

Investigating the reaction of TvL and TvL + 1HBT with lignin model β-O-4 linked dimers

β-O-4 linked dimers (**3**-**5**) were used in an attempt to gain an understanding of potential structural changes occurring within lignin as a result of laccase/LMS treatment. Dimers **3** and **4** were commercially available whilst dimer **5** was synthesised by a modified method of Kawai *et al*., [40, 42] (see SI section 1.9). Such structures have been used extensively to study lignin degradation mechanisms due to the predominance of this β-O-4 linkage within lignin [42-46]. All 3 dimers were incubated with TvL both with and without 1-HBT. Immediate sampling of the reaction of the phenolic dimer **3** with TvL in the absence of 1-HBT and subsequent analysis by LC-MS revealed a product peak *m/z* 661, consistent with oxidative dimerisation of **3**. After 24h, complete consumption of starting material **3** and initially formed dimerisation product was observed (see SI, Figure 8), presumably as a result of further polymerisation as anticipated with oxidised phenolic substrates. As expected, the reactions of non-phenolic dimers **4** and **5** with TvL in the absence of 1-HBT failed to result in the formation of oxidation products, with only starting material observed by LC-MS. Consistent with previous studies [13, 47], oxidation of dimer **4** with TvL in the presence of 1-HBT led to the formation of the uncleaved



Scheme 3. The proposed pathway for the degradation of a phenacylaryl ether (involving cleavage of the Cα-Cβ) by alkaline-peroxide treatment as investigated by Gierer *et al.*, [50]

ketone product **6** as the sole oxidation product, providing evidence for a Cα oxidation mechanism (Scheme 1). The analogous Cα oxidation product was also observed when dimer **5** (*threo*: *erythro* 5:2) was reacted with TvL + 1-HBT, however, in this instance a complex mixture of degradation products were also observed by LC-MS that were not present in the TvL and substrate only negative controls. Presumably these degradation products are derived from oxidation of the more electron rich aromatic ring in dimer **5** which bears an additional methoxy substituent. Comparison of the *m*/*z* values with the suite of oxidation products characterised by Kawai *et al*., [44] from the same dimer allowed identification of degradation products 8- 12 (*m*/*z* 265, 293, 263, 247 and 291) (Scheme 2, refer to SI figures 8-11 for all LC-MS traces and product characterisation). The production of Cα oxidation products 6 and 7 from the non-phenolic dimers **4** and **5** may provide a plausible explanation for the role of APE in the improvement of saccharification following LMS treatment. During APE, the hydroperoxide anions produced are reported to react with the carbonyl structures within lignin resulting in C-C bond cleavage [48, 49]. The mechanism of degradation of phenyacylaryl ethers (e.g. structure 6) hydrogen peroxide in alkaline media has been studied previously and is outlined in Scheme 3 [50]. An increased presence of carbonyl structures in lignin following treatment with TvL + 1-HBT may correlate with increased lignin removal by C-C bond cleavage following APE. These studies conducted with non-phenolic dimers **4** and **5** may provide important mechanistic insights into the role of LMS towards lignin degradation.

Pyrolysis GC-MS with TMAH

Thermally-assisted hydrolysis and methylation using tetramethylammonium hydroxide (TMAH) thermochemolysis followed by gas chromatography-mass spectrometry (GC-MS) has rapidly developed as a tool for characterising the relative proportions of lignin monomers in plant material [51]. Pyrolysis of lignin with TMAH is reported to induce cleavage of propyl-aryl-ether bonds (β-O-4) and the methylation of both aromatic and alkyl side chain hydroxyl groups [52]. This technique has been used by researchers studying the degradation of lignin in the natural environment or by pure cultures of fungi [53-55] however in this study it is explored for the determination of the action of laccase and LMS on the lignin structure. The technique was used to investigate and compare the composition of organosolv extracted wheat straw lignin

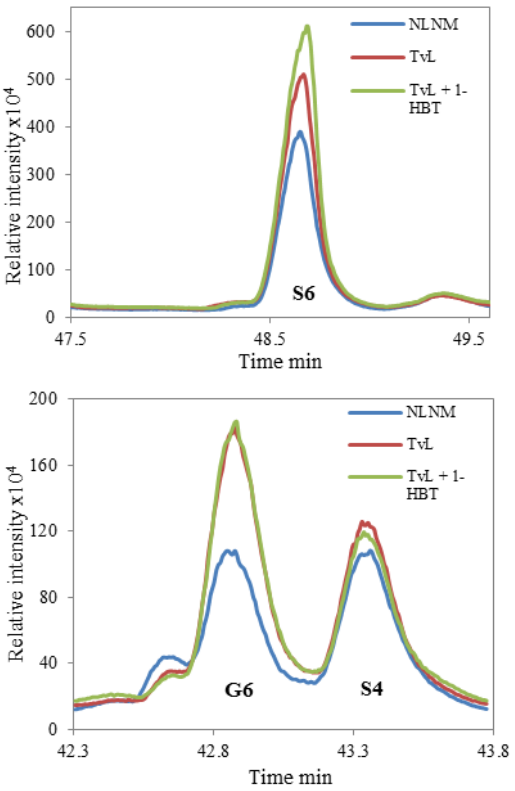


Figure 2: Partial chromatograms of the total ion current (TIC) for the TMAH thermochemolysis products methyl, 3, 4, 5-trimethoxybenzoate (S6) (top), and methyl, 3,4-dimethoxybenzoate (G6) and 3,4,5-trimethoxybenzaldehyde (S4) (bottom) for both laccase and laccase mediator treated wheat straw lignins and the untreated control.

following pretreatment with TvL, TvL + 1-HBT and no enzyme and no mediator (NLNM).

A collection of guaiacyl (G) and syringyl (S) thermochemolysis products were liberated from the lignin samples. Peaks were identified by library searches and by the comparison of peak positions and characteristic mass ions from published data [53] (See SI Table 2). A peak of high abundance derived from the *p*-hydroxylphenyl (H) monolignol was also observed. Wheat straw lignin is reported to contain all 3 mono-lignols (G, S and H) with a predominance of G and S-type lignins (H-type <10%) [56, 57] and S/G ratios around 1.2-1.4 [53, 58]. A partial chromatogram displaying the identified TMAH thermchemolysis products of organosolv extracted wheat straw lignin without enzymatic pretreatment is provided in the SI (Figure 12).

The most significant observation from the total ion currents (TIC) was the differences observed in the peak intensities of methyl 3,4,5-trimethoxybenzoate (S6) and methyl 3,4- dimethoxybenzoate (G6) between the laccase treated samples and the untreated control (Figure 2). Oxidative lignin degradation mediated by white rot fungi such as *T. versicolor* is reported to occur via Cα-Cβ side chain cleavage at the Cα position. This oxidative cleavage leads to the production of aromatic aldehydes such as 3,4,-dimethoxybenzaldehyde (G4) and 3,4,5-trimethoxybenzaldehyde (S4) from alcohol groups which are reported to undergo further oxidation to their carboxylic acids (such as G6 and S6) [53, 59, 60]. Researchers examining the ratio between the G and S unit acids (G6 and

Table 4. [Ac/Al] ratios of both G and S units from organosolv extracted wheat straw treated with/without TvL/TvL+1-HBT

	Treatment[a]	[Ac/Al]G	[Ac/Al]S
Exp 1	NLNM	0.79	3.44
	150U/g TvL	1.22	4.91
	150U/g TvL+1-HBT[b]	**1.55**	**5.69**
Exp 2	NLNM	0.76	3.96
	150U/g TvL	1.67	5.21
	150U/g TvL+1-HBT	**1.82**	**6.97**
Exp 3	NLNM	1.13	4.80
	150U/g TvL	1.67	5.36
	150U/g TvL+1-HBT	**1.82**	**7.62**
		Average increase in [Ac/Al]G	Average increase in [Ac/Al]S
	NLNM to TvL	0.63 (0.15)	1.09 (0.27)
	NLNM to TvL + 1-HBT	0.84 (0.11)	2.69 (0.23)
	TvL to TvL + 1-HBT	0.21 (0.06)	1.6 (0.43)

[a] Incubation of wheat straw for 40h, at 28°C and 200rpm [b] 5% (g/g) d.w biomass. Parenthesis represent standard error of three biological replicates. Bold represents highest ratio.

S6) and the aldehydes (G4 and S4) by calculating [Ac/Ald]G and [Ac/Ald]S have found increased ratios in fungal-degraded lignin compared to native lignin [53, 55]. The same ratio increase was found when fungal-degraded lignin was analysed via alternative methods such as solid state 13C-NMR, alkaline CuO and nitrobenzene oxidation [60-62]. Calculation of these ratios revealed an increase in the [Ac/Ald]G ratio from 0.79 (NLNM) to 1.22 (TvL) and a further increase to 1.55 (TvL+1-HBT). A similar trend was observed for [Ac/Ald]S whereby the ratio increased from 3.44 (NLNM) to 4.91 (TvL) and 5.69 (TvL+1-HBT) (Table 4). The trend was reproducible across three separate experiments set up using wheat straw from different washed batches. The increase in both ratios following preincubation with TvL and TvL + 1-HBT is consistent with the reported Cα-Cβ oxidative cleavage mechanism of lignin by fungal enzymes [53, 59].

Conclusions

The saccharification of acid-pretreated wheat straw was successfully improved by the development of a bioprocess incorporating an alkaline-peroxide extraction step following the incubation with laccase and a laccase-mediator system. Mediator screening studies using the lignin model compound veratryl alcohol revealed that a correlation between the activity towards the substrate and real lignin could not be established for all mediators. 1-HBT was proven to be the most successful mediator for the bioprocess. Studies using non-phenolic β-O-4 dimers with the LMS provided evidence for a Cα oxidation mechanism and possible β-O-4 cleavage. We suggest that the increased formation of the Cα oxidised groups within lignin following LMS treatment positively assists with lignin removal by alkaline peroxide extractions. Extracted lignin from laccase and LMS treated wheat straw was found to contain a higher proportional of syringyl and guaiacyl acid monomers by py-GC-MS with TMAH thermochemolysis when compared to the aldehyde counterpart. This observation is consistent with reported fungal-mediated lignin degradation mechanisms providing further evidence for the role of laccase and a laccase mediator system in lignin degradation.

Notes and References

[a] *School of Chemistry , University of Manchester, Manchester Institute of* [45] *Biotechnology (MIB), 131 Princess street, M17DN, UK. Fax: +44(0)161 275 1311; Tel: +44(0)161 306 5173; E-mail: Nicholas.Turner@manchester.ac.uk* [b] *Shell International Exploration and Production, Westhollow Technology Centre, 333 Highway 6 South, Houston, TX, USA Tel: +128 1928 3254*
[c] *School of Earth, Atmospheric & Environmental Sciences and Williamson Research Centre for Molecular Environmental Science, The University of Manchester, Williamson Building, Oxford Rd, Manchester, M139PL, UK*
† Electronic Supplementary Information (ESI) available: See ESI for supporting experimental results and all full experimental procedures. See DOI: 10.1039/b000000x/

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