Characterizing the Microbial Degradation of Kraft Lignin and Lignin-Derived Compounds

A Dissertation
Presented to
The Faculty of Graduate Studies
of

Lakehead University
Thunder Bay, ON

by

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Submitted in partial fulfillment of requirements
for the degree of
Doctor of Philosophy, Biotechnology
May 12, 2014

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ABSTRACT

Analytical methods for characterizing the microbial degradation of Kraft lignin and lignin-derived compounds were utilized with the goal of biologically generating demethylated lignin for subsequent industrial applications. Selected ion flow tube mass spectrometry (SIFT-MS) technology was used for the first time with both bacterial and fungal cultures growing on lignin as a sole carbon source. Methanol and other volatile compounds were evaluated using this method and lignin-derived compounds were identified. Methanol oxidation products were found in the headspace of seven microbial cultures, as well as several unknown products not present in the SIFT-MS compound library. An assay was then developed to both confirm the results obtained by the SIFT-MS and help to understand the nature of the microbial demethylation reactions. The Ti(III)-NTA assay was found to be an economical method for rapidly determining the relative degree of lignin demethylation by cultures of microorganisms and their enzymes. Using the Ti(III)-NTA assay, some fungal cultures were found to degrade lignin monomers completely and others to metabolize methanol. Four cultures were then selected for growth optimization; to both maximize vicinal diol generation and methanol formation. By altering variables such as induction day, incubation length, culture agitation, hydrogen peroxide concentration and micronutrient concentrations (known to promote enzyme production), the effect on four fungal species was investigated. Induction with vanillin after 1 week of growth on glucose resulted in the highest demethylation activity. In the final study, culture media from the fungus *Absidia cylindrospora* and the bacterium *Sphingobium* sp. SYK-6 were used to partially purify demethylating activity. The fungal enzyme had higher specific activity than the bacterial

enzyme, but was much less abundant. Further research is needed to purify these enzymes responsible for demethylation.

ACKNOWLEDGEMENTS

I would like to thank everyone who has helped me with the progress of my PhD. The Natural Sciences and Engineering Research Council (NSERC) provided funding for our project and our industrial partner, FPInnovations, helped to support us along the way.

Thanks to Dr. Lada Malek for supervising my PhD and taking me on as his student. His help and the help of members of our BRI and Lakehead University have provided me with much needed support along the way. I would like to thank Dr. Brian Ross for spending many hours to make me not only proficient, but also knowledgeable about SIFT-MS technology. Interpreting mass spectra is not an easy task. I would like to thank Dr. Robert Dekker who helped me during his time at the BRI and thank Dr. Sudip Rakshit for agreeing to be part of my committee and for help with revisions. I also thank Dr. Leonard Hutchison for providing me with access to the mycological collection at Lakehead University.

Several students, in doing collaborative projects over the years, have helped me with both motivation and experimental design. Thank you Steve Uchtenhagen, Vanessa Ivec and Jessica Grochowski. I would also like to thank both, Iryna Kandybovich and Balaji Venkatesagowda, who worked at the BRI and met with me almost weekly to help keep the project on track.

Support from my family and friends made this achievement possible. I am grateful for support from my father Jeff Gibson and also Brenda Winter. I would like to thank my grandparents Blair and Jean Gibson who provided kind words of encouragement, Ron Nelson for his support, and Courtney Reich who helped with her knowledge of the subject, laboratory skills, as well as encouraging me to finish writing. Most of all I would like to thank my mother Colleen Kennedy who helped with support, motivation and enthusiasm.

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LIST OF ABBREVIATIONS

4-AAP - 4-aminoantipyrine

3-HAA - 3-hydroxyanthranilic acid

A_{280} nm - absorbance at 280 nanometers

ABTS - 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

AU - absorbance unit

BRI - Biorefining Research Institute, Lakehead University

C_1, C_3, C_9 - one-carbon, 3-carbon, 9-carbon

^{13}C - isotope of carbon

3D - three dimensional

Da - dalton

ddH_2O - double distilled water

DEAE - sephadex diethylaminoethyl-dextran

DMSO - dimethyl sulfoxide

EDTA - ethylenediamineteraacetic acid

eV - electron volts

FPI - Forest Products Innovations

FS - full scan

FTIR - Fourier transformed infrared spectroscopy

GC - gas chromatography

GC-MS - gas chromatography with mass spectrometer detector

GC-FID - gas chromatography flame ionization detector

HPLC - high pressure liquid chromatography

k - rate constant

kDa - kilodalton

LC-MS - liquid chromatography mass spectrometry

LiP - lignin peroxidase

LU - Lakehead University

3MGA - 3-*O*-methylgallate

MIM - multiple ion monitor

MnP - manganese peroxidase

Mw - molecular weight

m/z - mass-to-charge

N - normality

NHA - *N*-acetyl-*N*-phenylhydroxylamine

NMR - nuclear magnetic resonance

NTA - nitrilotrioacetic acid

0-demethylation - *ortho*-demethylation

OD - optical density

PCA - protcatechuate

PDA - potato dextrose agar

PFTE - polytetrafluoroethylene

ppb - parts per billion

ppm - parts per million

rpm - rotations per minute

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIFT-MS - selected ion flow tube mass spectrometry

Ti(III) - titanium^{3+}

Torr - $^{1}/_{760}$ of a standard atmosphere

µkat - SI unit for enzymatic catalytic activity

UAMH - University of Alberta Microfungus Collection and Herbarium

UV - ultraviolet

VMS - Vogel's minimal salts

VOCs - volatile organic compounds

w/v - mass/volume

1 Introduction

Northwestern Ontario is a region rich in renewable resources and has an economy closely connected to the pulp and paper industry. Issues such as the depletion of these resources, environmental awareness and industry viability have led to an ongoing search for value-added products from this renewable material. Some novel value-added products may originate from the biological conversion of lignin using microorganisms such as fungi and bacteria.

1.1 Background and Literature Review

In the Background and Literature Review, the structure of wood and lignin are described, followed by a description of the Kraft pulping process (p.9), and potential products from lignin (p.15, and particularly Table 1.2). The role of living organisms in metabolizing lignin is dealt with on pp. 22-37, culminating in the introduction of assays needed to investigate lignin modification (p.38) and beyond.

1.1.1 Lignin as a component of wood

1.1.1.1 Lignin

Lignin is a complex, composite biomacromolecule found in the cell walls of all vascular plants (Filley et al., 2000). It is the second most abundant natural polymer in the world after cellulose, rich in renewable aromatic polymer, and resistant to degradation (Zhu et al., 2011). It is estimated that 30% of all organic carbon in plant biomass is contained in lignin and its decomposition is indispensable to the global carbon cycle (Wong, 2009). Researchers have long been studying lignin's potential use in novel

applications, but despite some successes, lignin is still currently a highly under-utilized raw material (Lora & Glasser, 2002).

The term lignin comes from the Latin word, “lignum” which means wood. Anselme Payen (1838), a French chemist and chemical manufacturer, was the first to recognize the heterogeneous nature of wood by conducting experiments that revealed that wood contained “cellulose” and an embedding material that Schulze (1857) later defined as lignin (McCarthy & Islam, 1999).

In higher plants, lignin is one of the three major polymeric components found between cells, within cells and primarily in the cell walls (Sarkanen, 1963). Along with the other two major components, cellulose and hemicellulose, lignin forms a highly efficient composite system representing 20 -30% of the total weight (Zhu et al., 2011). Spatially, lignin is interspersed with hemicellulose forming a matrix that surrounds cellulose microfibrils (Fig. 1.1).

The role of lignin in plants is to act as a gluing material in cell walls, giving the plant structural rigidity for vertical growth and protecting the plant from degradation (Sjöström, 1993). Structural support is achieved by reinforcing the xylem, which strengthens wood in trees. In the xylem cells, lignin also acts as a sealant to prevent the permeation of water, thus aiding in water transport (Crawford & Crawford, 1976). Ether and carbon-carbon linkages found in lignin are not susceptible to hydrolytic attack, making them highly resistant to breakdown. Thus, the encasement of cellulose filaments

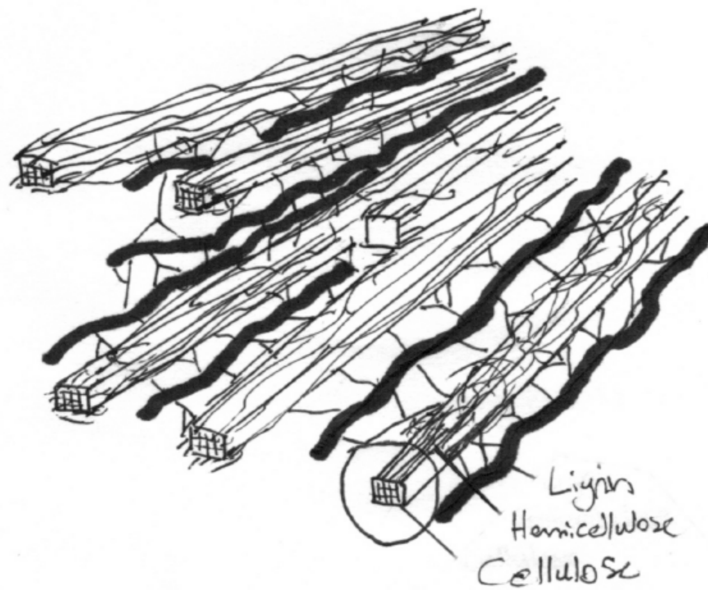


Figure 1.1 Cellulose strands surrounded by hemicellulose and lignin (based on Doherty et al., 2011)

in lignin provides a physical barrier to the breakdown of cellulose and hemicellulose by physical and biological processes (Cullen & Kersten, 2004). These attributes help inhibit microbial attack. Investigations into the degradation of wood showed lignin to be the most resilient component and the last to be degraded (Blanchette, 1991).

1.1.1.2 Lignin Structure

Lignin is not just one compound but many. The name is applied to lignin found in its natural state (also known as protolignin or native lignin) as well as to the products of modified lignin (for example, Klasson lignin, milled wood lignin or Kraft lignin). There is variation to be found in the composition of lignin in plants with different genetic origins and in the different tissues of the same plants (Table 1.1). Any method that isolates lignin also alters it, so the name lignin does not designate a substance with a defined structure, it rather refers to a group of chemically related polymeric combinations (Fengel & Wegener, 1984).

Table 1.1 Lignin content in some representative softwoods and hardwoods (Sjöström, 1993)

Scientific name	Common name	Lignin content (%)
Abies balsamea	Balsam fir	29.1
Tsuga canadensis	Eastern hemlock	29.3
Picea glauca	White spruce	27.5
Acer rubrum	Red maple	25.4
Acer saccharum	Sugar maple	25.2
Betula papyrifera	White birch	21.4

Lignin is a branching polymer network resulting from the dehydrogenative radical polymerization of three monolignols (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) (Fig. 1.2) that are connected via carbon-carbon and ether linkages (Fig. 1.3) (Boeriu et al., 2004). The monomer structures of lignin consist of the same phenylpropanoid skeleton, but differ in the substitution of oxygen on the phenyl ring. The monomers vary in abundance in (1) various plants species (Table 1.1), (2) within the plant itself and (3) even within a cell wall (Campbell & Sederoff, 1996). The 3 corresponding phenylpropanoid units in the lignin polymer are denoted as *p*-hydrophenyl (H), guaiacyl (G) and syringyl (S) units, based on methoxy substitution (Fig. 1.2). The structure of lignin is so complex, it has not been completely described (Masai et al., 2007; Sarkanen, 1963).

Lignin from different plant families are made up of different monolignol ratios, so lignin is classified according to the type of plant from which it originated. The two major classifications are gymnosperm lignin and angiosperm lignin. The lignin in softwood (gymnosperm) tree species consist mainly of G units (95%), hardwood (angiosperm) lignins consist of almost equal amounts of G and S, and grasses contain all three phenylpropanoid units (G,S and H) typically in a 70:25:5 ratio (Wong, 2009). Although some linkages are much more common than others, more than 20 different linkage types have been identified (Sederoff et al, 1999) (Figure 1.3). Ether linkages have been estimated to make up one half to two thirds of the total number of lignin linkages (Chakar & Ragauskas, 2004).

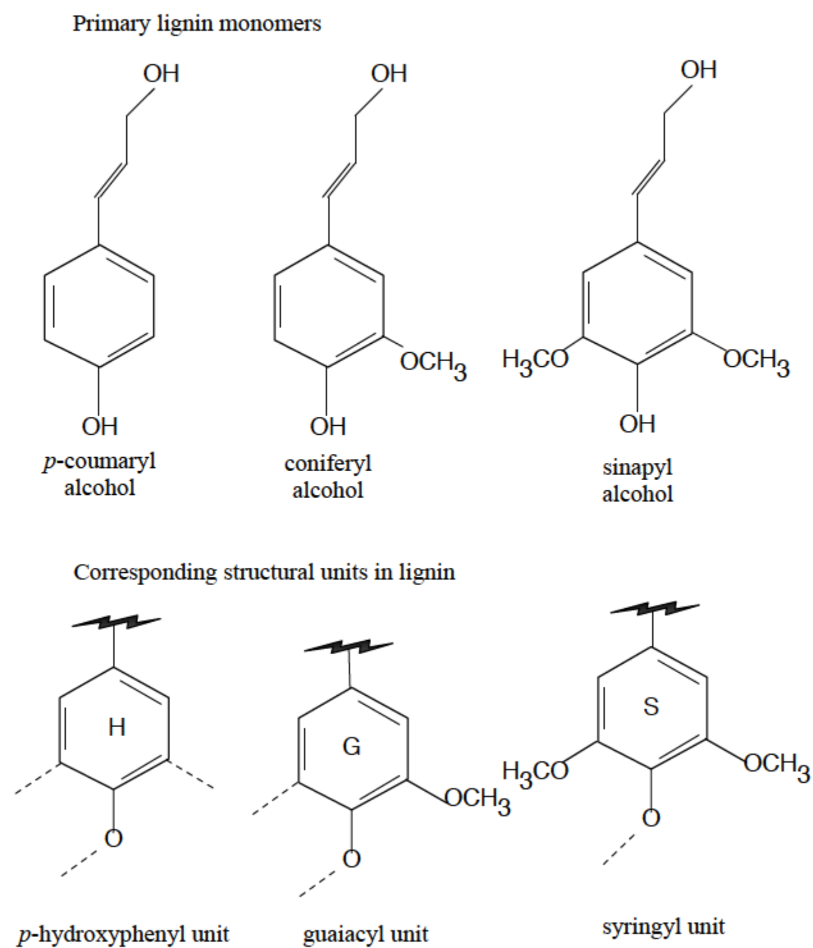


Figure 1.2 Primary lignin monolignols: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, and the corresponding phenylpropanoid units: *p*-hydrophenyl (H), guaiacyl (G) and syringyl (S) units

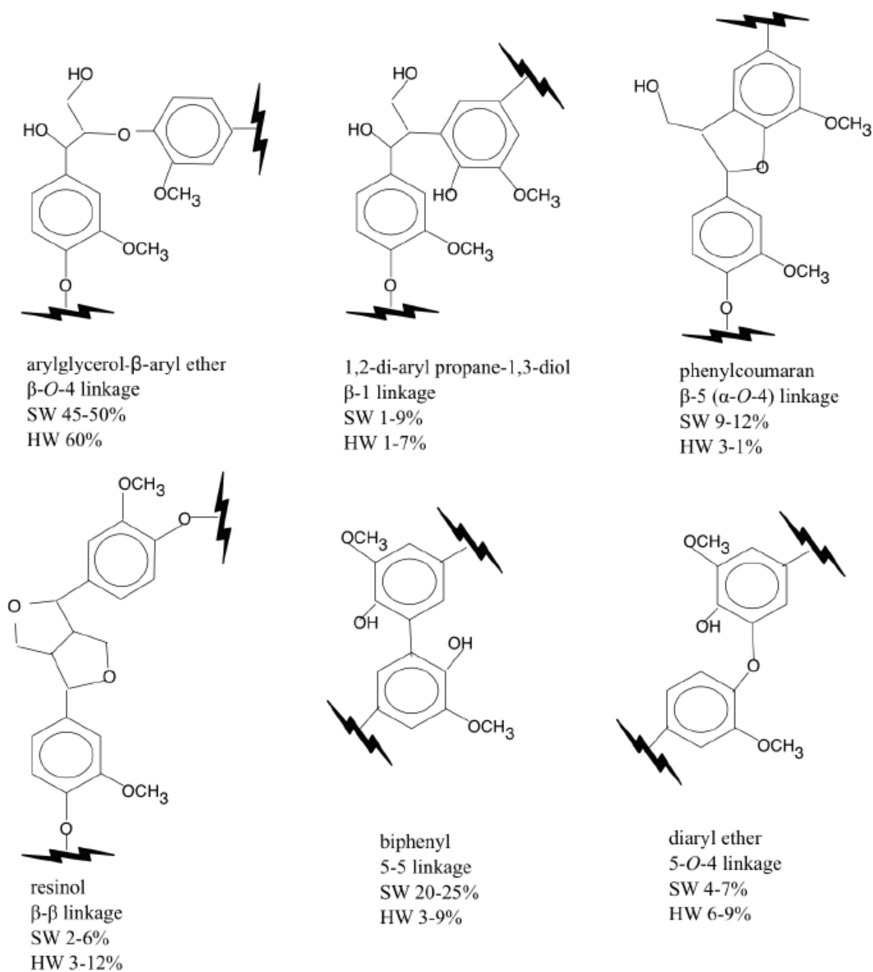


Figure 1.3 Major structural units of lignin with some of the common lignin linkages (β-aryl ether, diaryl propane, phenylcoumaran, resinol, biphenyl and diaryl ether) with percentages of the total lignin linkages for softwoods (SW) and hardwoods (HW) (adapted from Bugg et al., 2011a and Wong, 2009)

Lignins contain several functional chemical groups, such as hydroxyl (phenolic or alcoholic), methoxyl, carbonyl and carboxyl in various amounts depending on the source (Gosselink et al., 2004). The polymeric network structure, as well as the presence of the various chemical side-chains, give lignin its unique functional properties (Boeriu et al., 2004).

1.1.1.3 Lignin Biosynthesis

Monolignols are synthesized in plants by a two-step process. First D-glucose is converted by the shikimate pathway to L-phenylalanine or L-tyrosine then enzymatically converted to p-coumaryl, coniferyl or synapyl alcohol by the cinnamate pathway (Sjöström, 1993). Lignin is formed through oxidation and free radical coupling of phenyl alcohol precursors (Cullen & Kersten 2004). The formation of the linkages in lignin can be explained by radical dimerization or polymerisation reactions of these precursors. Plant peroxidase and laccase enzymes generate phenoxyl radicals, which mediate the single electron oxidation of the alcohol precursors. These exhibit a radical character on the phenolic oxygen, adjacent aromatic carbon and at the β-carbon on the 3–position of the side-chain. All these points on the monomer have the potential to generate linkages. Since monolignols can be linked from three points, branch points are made within the polymer giving lignin a network-like structure. The final lignin polymer composition and crosslinking are determined by the degree of hydroxylation and methylation of the individual units (Zubieta et al., 2002). A continuing debate exists regarding the extent to which lignin polymerization is regulated by the plant. Some suggest that the arrangement of monomers in lignin is highly ordered (Davin et al., 1997; Boudet, 1998), while others suggest the arrangement is in some respects random (Denton, 1998; Sederoff et al, 1999).

1.1.2 Kraft Pulping

1.1.2.1 Isolating Lignin

Isolating lignin can be problematic due to its resilient nature. There are numerous methods for isolating lignin from biomass, from large-scale industrial processes to

laboratory level procedures. Since the main object in an industrial processes such as wood pulping is to separate the lignin from the other plant materials in order to obtain the polysaccharide component or wood fibre for use in making paper products, the processes are optimized to produce fibre with uniform characteristics, while lignin is generally considered to be a by-product waste (Smook, 1982). As a result, the processes can significantly alter the chemical structure of lignin.

Chemical pulping involves cooking wood chips or sawdust in an aqueous solution of pulping chemicals (alkaline, acidic or neutral) to dissolve the lignin component and extract the cellulose component from the wood. Using mild mechanical force the majority of the lignin can be removed from the individual fibres after chemical treatment. The most common process used in the pulp and paper industry is the Kraft process (discussed later), which produces strong pulps for use in corrugated board, liner board and paper bags (Doherty et al., 2011).

The sulfite process, another process that has been used in paper pulping historically, is much less prevalent than Kraft pulping currently (Doherty et al., 2011). In this process wood reacts with sulfurous acid and bisulfite, resulting in the removal of lignin as it is cleaved into smaller and more soluble fragments (Smook, 1982). Specific reactions can be modified by altering the pH, SO_2 concentration, temperature and base cation (Smook, 1982). Lignin produced by the sulfite pulping process is known as lignosulfonates. Over the past 50 years, a few sulphite mills have taken steps towards converting themselves into industrial biorefineries by producing specialty cellulose

products, lignosulphonates, ethanol, vanillin and other high value products (Paleologou, 2011). Other processes used by this industry include solvent pulping, which has the advantage of being more ecologically sound, but the disadvantage of higher cost (Pan & Saddler, 2013).

Laboratory lignin extraction aims to extract the lignin with as little modification as possible to the lignin structure, there is unfortunately no process that will separate lignin from plant polysaccharides without some alteration to the lignin chemistry. Ball milling is a commonly used method that mechanically breaks down plant fibres however, it too has been shown to affect lignin structure. Other methods exist on a laboratory scale but are for specific applications and are not as useful for native lignin chemistry studies (Kirk & Obst, 1988).

1.1.2.2 Kraft Process

The Kraft process is the dominant chemical pulping process, used in 80% of paper production globally (Smook, 1982). The method is also referred to as the sulfate process and has been in use since 1879, when sodium sulphate replaced sodium carbonate (soda process) (Someshwar & Pinkerton, 2000). Sodium hydroxide/sodium sulfite solution (white liquor) is used to create strong alkaline conditions to cleave the ether bonds of lignin. White liquor is combined with either wood chips or milled wood at a 4:1 ratio in a digester (Fig. 1.4).

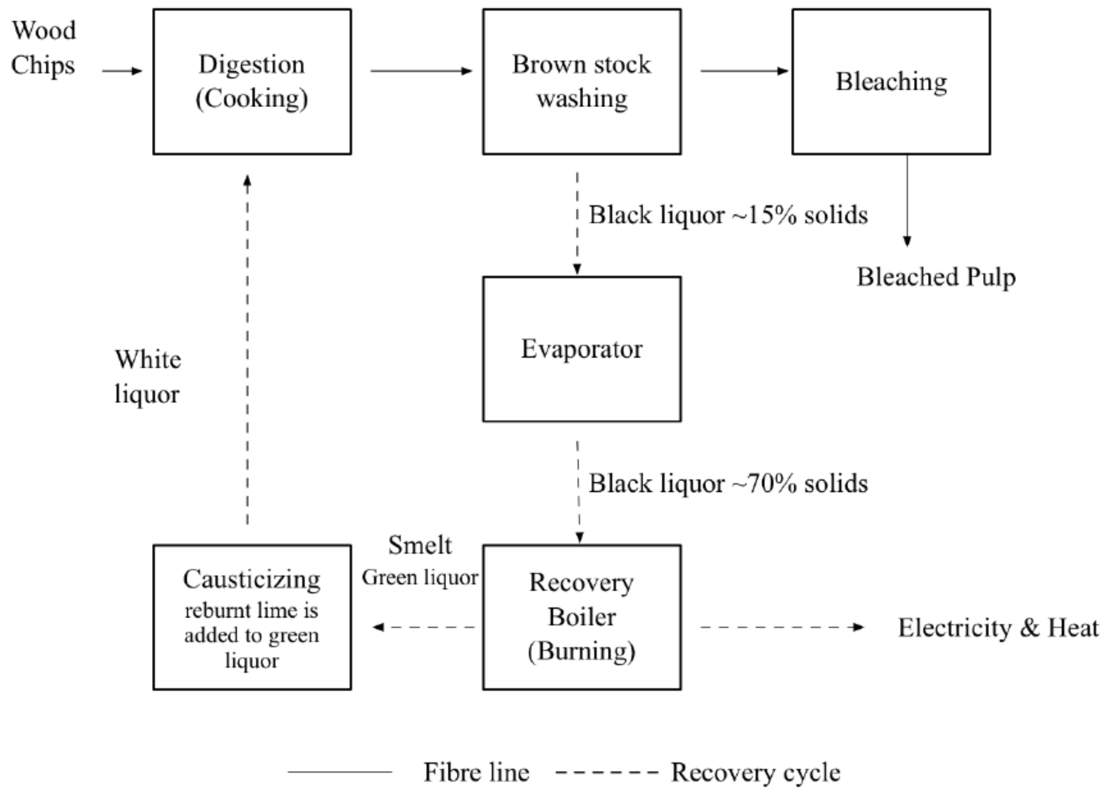


Figure 1.4 Schematic of the Kraft Process (based on Smook, 1982)

Lignin is removed in three distinct stages (Fig. 1.5). The first or initial phase uses diffusion at around 150 ℃ to remove any soluble lignin (Sjöström, 1993). The second or bulk stage is at 150-170 ℃ and the third or residual stage operates at an even higher temperature.

The cooking process takes about 8 hours (70 minutes of that is at the maximum Kraft cooking temperature) and temperatures vary depending on the type of wood used (155 ℃ for hardwoods and 170 ℃ for softwoods) (Smook, 1982). The cooking vessel is heated in an oil bath and rotated end over end to ensure mixing. Most of the lignin (roughly 90%) is removed at the second stage. As a result of the process, pulp and a by-product called black liquor is formed. Black liquor is the combination of removed lignin, water and chemicals used in the extraction process. The lignin in the black liquor can be extracted as well by lowering the pH between 5-7.5 usually using sulfuric acid or carbon dioxide (Koljonen et al., 2004). The goal of this process is to remove enough lignin to separate the cellulosic fibers from each other, producing a pulp suitable for paper and related products.

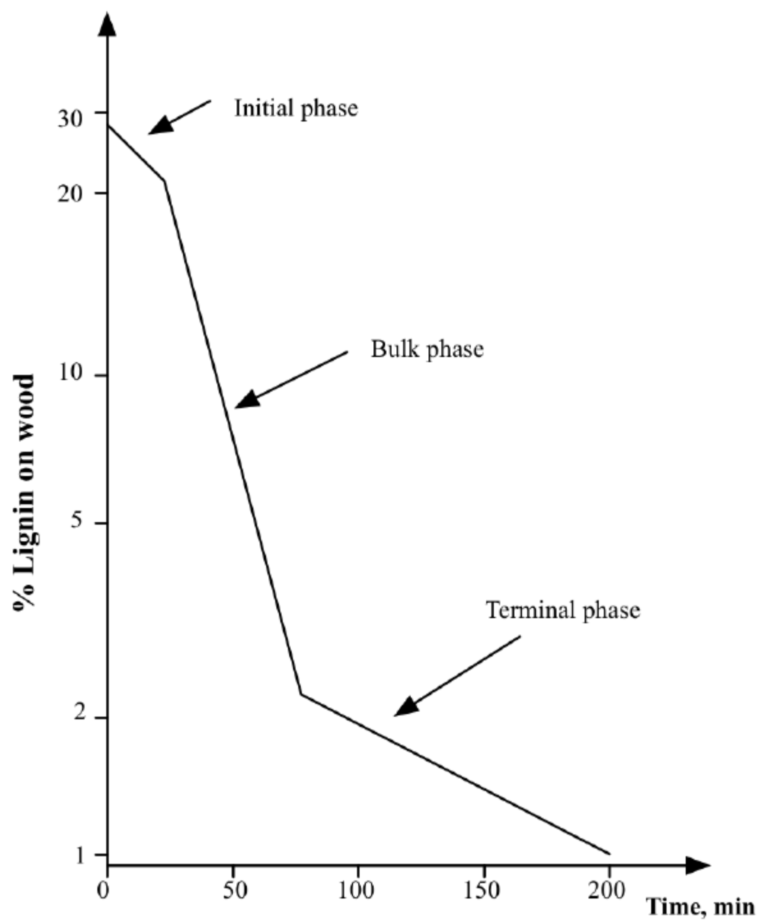


Figure 1.5 Three phases of delignification in cooking process shown as lignin content (% lignin on wood) versus reaction time in softwood Kraft pulping (adapted from Smook, 1982)

1.1.2.3 Kraft Lignin Chemistry and Structure

The reaction chemistry of Kraft pulping is complex and not completely understood (Chakar & Ragauskas, 2004). During the cooking process, lignin is chemically split into fragments by OH^- and SH^- ions present in the pulping liquor. Approximately 80% of the lignin, 50% of hemicellulose and 10% cellulose are dissolved. In Kraft lignin production, linkages are classified as alkali-stable or alkali-sensitive. The alkali-sensitive linkages are readily cleaved under harsh alkaline conditions (i.e. white liquor). The alkali-stable bonds may be enriched and present difficulties when trying to disrupt them at the final stages of processing. During processing it is believed that both degradation and fragmentation are balanced by condensation reactions. Condensation reactions are not desirable because the condensed lignin is more difficult to remove from the fibers. The two main considerations in Kraft lignin reactions are alkali concentration and temperature (Smook, 1982).

1.1.2.4 Potential Industrial Uses for Lignin

Currently, industrial lignin is used in a wide range of low value applications where quantity is more important than quality. The vast majority of lignin recovered by industrial processes is burnt to provide energy for pulp and paper mill operations (Wallberg et al., 2005). The high caloric value of lignin is due to the abundance of aromatic units (Doherty et al., 2011). Only 1-2% of lignin is isolated from spent pulping liquors and used in other applications, such as resins, concrete and adhesives, etc. (Lora & Glasser, 2002).

Other alternatives to the destructive use of lignin in black liquor are of interest because it is so readily available in large quantities (Sena-Martins et al., 2008). The presence of reactive functional groups allows for its use in copolymers (Doherty et al., 2011). It also has good absorbent, ion exchange and adhesive properties (Borges da Silva et al., 2009). Lignin can be a direct source of various phenolytic and aromatic compounds and is harmful to the environment if disposed as waste (Stewart, 2008).

For any of the current industrial uses of lignin, solubility in water is an important requirement and, when chemically modified to become water soluble, the lignin product is known as lignosulphonate. Of the estimated 50 million tons of lignin available from pulping processes world-wide, approximately 1.1 million tonnes is isolated for lignosulphonate production. Of that, 1 million tonnes is derived from the acid sulphite pulping process and only the remaining 100,000 tonnes is derived from the Kraft pulping process (Higson & Smith, 2011). Further modifications to industrial lignin have been explored in an effort to make lignin more reactive and therefore enhance its industrial utility (for summary see Figure 1.6).

The end-use markets for lignin derived products include construction, mining, animal feeds and agriculture. Large market applications include dust control, animal feed and concrete admixtures. Medium market applications include dye dispersants, gypsum wallboard dispersants and vanillin. Smaller market applications include resins, binders, emulsifiers, water treatment, cleaning chemicals, battery expanders, pesticide dispersants and rubber additives (Higson & Smith, 2011).

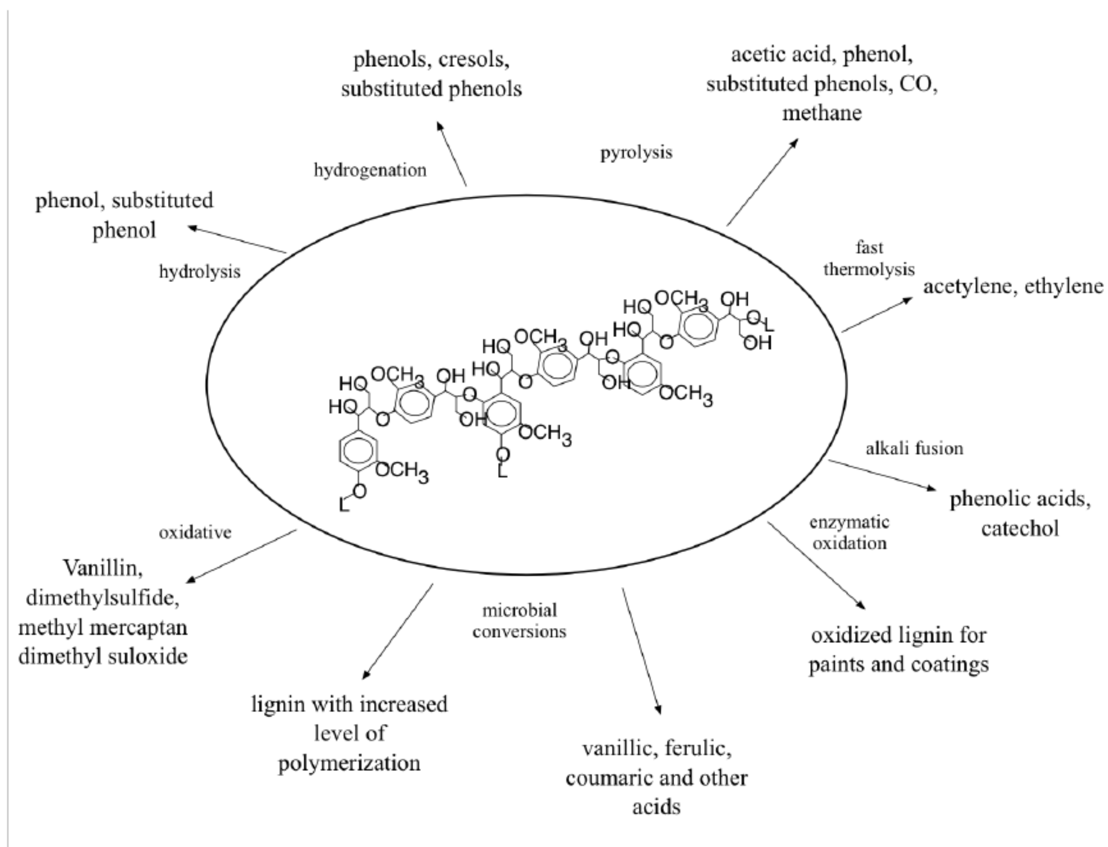


Figure 1.6 Brief summary of catalytic lignin modifications (based on Holladay et al., 2007)

About 67% of world consumption of lignosulphonate in 2008 was for dispersant applications, followed by binder and adhesive applications at 33% (Higson & Smith, 2011). Table 1.2 outlines current applications for modified industrial lignin. Enzymatic approaches for industrial lignin modification are desirable because of the high selectivity and efficiency of enzymatic catalysts, mild operating conditions required, and broad range of substrates and their ability to react under adverse conditions. Challenges exist with the properties of enzymatic proteins, such as their non-reusability unless conveniently immobilized, high sensitivity to several denaturing agents (such as pH, H_2O_2, temperature and mechanical stress) and the potential for adverse sensory and toxicological effects (Durán et al., 2002).

Potential market developments include the use of lignin in chemical production. So far, this production has been limited due to contamination from salts, sugars, particulates, volatiles and the heterogeneity of the lignin molecule (Holladay et al., 2007). The production of high value-added chemicals from lignin is viewed as a longer-term opportunity (Holladay et al., 2007). Any use for chemicals or value-added products would need to be competitive against the simple burning of lignin as fuel and is relative to the price of fossil fuel derived feedstocks. Potential high value chemicals from lignin are shown in Table 1.3.

Table 1.2 Current applications for use of modified lignin

Application	Process	Pro	Con	Ref
Lignin co-polymers	- grafting of industrial lignins to synthetic polymers for preparing a new class of engineering plastic, for ex. thermoplastic blends	- enhanced biodegradability of the new products - higher graft efficiency - better molecular weight control	- mechanism needs to be better understood – compatibility with other polymers represents a major obstacle	Sena-Martins et al., 2008; Lora & Glasser, 2002
Phenolic resins	- lignin used as a phenol substitute in phenol formaldehyde resins	- problems can be addressed by chemically modifying lignin to make it more reactive; adding filler agents such as starch and using lignins derived from processes such as organosolv (organic wood digestion) which are sulphur free and low M_w	- chemical heterogeneity a limiting factor - lignin needs to be sulphur free - adds M_w to the resin - industrial organosolv process is only used to a limited extent	Stewart, 2008; Peng & Reidl, 1994
Epoxies	Epoxy resin is obtained by an epoxidization reaction using Kraft lignin that is modified by methylolation - lignin is used as a crosslinking agent	- thermal stability of lignin increased, which allows for wider temperature range of application - epoxy resin market is large, so there is potential for lignin use to flourish	-lignin must be impurity free (free of salts, water and sugars)	Stewart, 2008; El Mansouri & Salvado, 2006
Adhesives	- modified lignin (enzymatic modification of lignin by laccase, i.e. to increase crosslinking of phenoxy radicals) used to replace urea-formaldehyde	- increasing legislation expected to restrict chemical usage in fibreboard formation (alt. such as the laccase system should benefit from this)	- fibreboard market has large turnover and low profit margins, so difficult to introduce new processes	Stewart, 2008; Felby, 2002
Polyolefins	-lignin used as a feedstock for polymer blends and UV stabilization	- blends incorporating epoxy-modified lignin can be made at lower cost with enhanced properties (ex. thermal, mechanical & surface properties	- some concerns about biodegradability of the blend	Cazacu, 2004

Table 1.2 (continued)

Application	Process	Pro	Con	Ref
Concrete	- lignin and modified lignin are mixed into the concrete	- low levels added yield high performance concrete strength, grinding aid, reduced damage caused by moisture - modified lignins can improve the grindability, particle size distribution and compressive strength of cement pastes	- over the next years, the consumption of lignin for this use is expected to decline (changes in construction techniques and less demand for contrete	Li et al., 2014
Binders for wood composites	- solid wood is fragmented, then supplemented with binder and pressed to form a wood-like structure again (water insoluble lignins are used with the addition of small amounts petrochemical resins as binders)	- formaldehyde free (commercial binders usually release a harmful amount of formaldehyde during manufacture & use)	- chemical heterogeneity of lignin is the limiting factor	Sena-Martins et al., 2008
Chelating complexes	- after enzymatic modification, lignin has improved chelating capacity - metal sequestration can be enhanced by by modifying the lignin with amino and quaternary ammonium groups	- can be used to remove heavy metals from pulps or to treat effluents containing heavy metals - economic interest in this is long term as waste and effluent regulations continue to increase	- better characterization of the macromolecular products obtained needed to find useful applications	Gonçalves & Benar, 2001 Dizhbite et al., 1999 Stewart, 2008
Coatings and Paint	-prepared by using a phenol oxidizing enzyme (ex. laccase) for polymerization or as curing catalyst for lignin	- used for surface protection against photo oxidation and other types of wood degradation		Bolle Aehle 2000 and 2001

Table 1.3 Potential high value applications attained from lignin conversions. Expected difficulty from lignin marked Easily Done (✓), Medium difficulty (n) or Highly difficult to do (✗) (Holladay et al., 2007)

Syngas	Hydrocarbons	Phenols	Oxidized Products	Macromolecules
Methanol (✗)	Cyclohexane (■)	Phenol (■)	Vanillin (✗)	Carbon fibre (■- ✓)
Ethanol (✓)	Styrenes (■-✓)	Substituted phenols	Vanillic acid (■)	Polyelectrolites (■)
Mixed liquid fuels (■)	Biphenyls (✓)	Catechols (✓)	Aromatic acids (✓)	Polymer alloys (■)
		Cresols (✓)	Aliphatic acids (✓)	Fillers, Polymer extender (✓)
		Eugenol (✓)	Syringaldehyde (✓)	Thermosets (✓)
		Syringols (✓)	Aldehydes (✓)	Composites (M- ✓)
		Coniferols (✓)	Quinones (✓)	Formaldehyde-free Adhesives and Binders (✗ -■)
		Guaiacols (✓)	Cyclohexanol/al (✓)	Wood preservatives(✓)
				Pharmaceuticals(■- ✓)

✓ = Easily done - Highly developed
■ = Medium difficulty - Partially developed
✗ = Difficult to do - requires intense effort to develop

1.1.3 Microbial Lignin Degradation

The biodegradation of lignin is achieved in nature primarily by species of wood decay fungi (Reid, 1995). The first report of fungi causing wood decay was in 1863 by Hermann Schacht (Blanchette, 1991). Today, three general types of decay are recognized: white-rot, brown-rot and soft-rot (Blanchette, 1991; Liese, 1970, Worrall et al., 1997).

1.1.3.1 White-rot Fungi

White-rot fungi are a diverse group of fungi that belong to the phylum Basidiomycota. They are the only fungi capable of degrading all cell wall ingredients, including lignin, efficiently (Kirk & Cullen, 1998; Hatakka, 2001). Two forms of white-rot exist: selective delignification where hemicelluloses and lignin are preferentially attacked, especially in the early stages, and simultaneous white-rot where carbohydrates and lignin are attacked more or less uniformly (Worrall, et al., 1997)

Lignin biodegradation by fungi has only slowly been defined chemically and biochemically. One of the main reasons for this was a poor knowledge of the chemical structure of lignin from its discovery in the late nineteenth century until it became better known in the 1960s (Kirk, 1983; Adler, 1977; Eriksson et al., 1990).

Prior to the 1920s, little research was conducted on lignin biodegradation (Kirk, 1983). The first review of lignin degradation research came in 1936 and was written by Arthur Geoffrey Norman, an English biochemist and botanist who was working at the Biochemistry Section and Fermentation Department at the Rothamsted Experimental

Station (Kirk, 1983; Norman, 1934). From that time until mid-century, there was great interest in investigating lignin structure through degradation reactions, biosynthetic work and spectroscopy studies (McCarthy & Islam, 2000). Some findings, summarized in the 1930's are still valid today: firstly that lignin is degraded, but is resistant to degradation, secondly that white-rot fungi degrade lignin in wood, and thirdly that the completely selective removal of lignin (that is, without also removing hemicellulose and/or cellulose) had not been observed (Kirk, 1983).

In the 1950s, in addition to white-rot Basidiomycota fungi, other types of fungi were discovered to degrade or modify lignin including brown-rot Basidiomycota fungi and soft-rot (Hatakka, 2001). The first lignin model compound studies were published in the 1960's (Henderson, 1961) and biodegradation assays based on ^{14}C-lignins were developed in the 1970's (Haider & Trojanowski, 1975; Kirk, 1975; Kirk et al., 1978). Using radioactively labeled isotopes, it was revealed how lignin was optimally degraded under laboratory conditions by white-rot fungi. *Phanerochaete chrysosporium* was used as the main experimental organism in the USA, while in some other laboratories, the anamorph of the same fungus, *Sporotrichum pulverulentum*, had been chosen for lignin biodegradation studies (Ander & Eriksson, 1976; Ander et al., 1980); and before that, *Trametes versicolor* was a popular experimental fungus (Higuchi, 1985).

In the late 1970s and early 1980s many important findings in the physiology of lignin degradation by *P. chrysosporium* were made. The effect of nutrient nitrogen was explored and it was established that low nitrogen was required for lignin degradation,

indicating that the mineralization of lignin occurred during secondary metabolism. The effect of atmosphere was explored and it was established that 100% oxygen gave the highest mineralization, thus demonstrating that lignin degradation is oxidative. The effect of agitation was explored and it was found to be detrimental to lignin mineralization. Finally, it was established that during lignin degradation veratryl alcohol was produced (Kirk, 1975; Kirk et al., 1978).

In 1983-84, a breakthrough in the enzymology of lignin biodegradation occurred with the discovery of the first extracellular enzymes from *P. chrysosporium* involved in the degradation of lignin. Lignin peroxidase (LiP) was found in 1983 (Tien and Kirk, 1983), and manganese peroxidase (MnP) was found in 1984 (Kuwahara et al., 1984).

Since then, there have been a large number of studies on the biochemistry and molecular biology of these enzymes, but they were again strongly concentrated on the enzymes of one fungus, *P. chrysosporium.* The catalytic mechanism of lignin peroxidase was proposed and verified (based on initial one-electron oxidation of the lignin model compounds followed by subsequent breakdown reactions via radical cation intermediates) (Kirk et al., 1986). Numerous publications describing the effect of ligninolytic enzymes on lignin model compounds have also appeared (reviews by Higuchi, 1985; Kirk and Farrell, 1987).

In the 1990s and early 2000s detailed studies on catalytic and enzymatic properties of the lignin-modifying peroxidases as well as their molecular biology were

undertaken. Major lines of research dealt with the potential applications of selected white-rot fungi and their enzymes in bio-pulping (biomechanical pulping) and pulp bleaching (Eriksson et al., 1990; Akhtar et al., 1998; Felby et al., 2002).

The 2000s have seen the completion of massive genomic studies (Gregoriev et al., 2011). In 2004-2006, the genome was published for *P. chrysoporium* (Martinez et al., 2004). The model predicts complex mixtures of extracellular enzymes including 4 lignin peroxidases, 3 lipases, 2 carboxylesterases and 8 glycosyl hydrolases (Vanden Wymelenberg et al., 2006). Since then, the number of fungal species with complete genome sequences has grown exponentially. In the next five years, researchers are aiming to sequence 1000+ fungal genomes from the Fungal Tree of Life (Grigoriev et al., 2011). In addition to providing reference information for research on harnessing fungi for industrial, energy and climate management uses, the genomes are needed for genomic, metabolomics and transcriptomic work to link enzymatic activity and secondary metabolites to carbon polymer degradation.

The extracellular enzymes involved in lignin degradation by white-rot fungi are peroxidases and laccases, with their accessory enzymes (Hatakka, 1994; Kirk & Cullen, 1998; Hammel & Cullen, 2008). Peroxidases include: lignin peroxidases (also known as LiPs, ligninases, EC 1.11.1.14) and manganese peroxidases (aka MnPs, Mn-dependent peroxidases, EC 1.11.1.13) which were discovered in the early 1980s and versatile peroxidases (VPs, EC 1.11.1.16) which were discovered in the 1990s (Martinez et al., 1996) (Table 1.4).

Table 1.4 White-rot fungal enzymes involved in the degradation and modification of lignin (from Hatakka, 2001; Dashtban et al., 2010 Martinez et al., 2005)

Type of enzyme	Main Reaction	Cofactor	Metals or ions	Mediators	Subunits & molecular mass (kDa)	Localization	Ref.
Phenol oxidase							
Laccase (EC 1.10.3.2)	oxidation of phenols to phenoxyl radicals	n/a	Ca^{2+}, Cd^{2+}, Cu^{2+}, H_2O_2, Imidazole, K^+, K_2SO_4, Mn^{2+}, Na_2SO4, $(NH_4)_2SO_4$	Phenols, aniline, 3-HAA, NHA, syringaldehyde, hydroxybenzotri azole , ABTS, and others	Most are monomeric (60-70), dimeric, trimeric and oligomeric (ex. tetramers with ~383 kDa)	mostly extracellular (some intracellular have been found)	Baldrian, 2006; Cullen & Kersten, 2004
Peroxidases							
Lignin Peroxidase (LiP, EC 1.11.1.14)	oxidation of aromatic ring; degrades non-phenolic lignins (up to 90% of the polymer)	heme	Iron	veratryl alcohol	monomeric (38-46)	extracellular	Cullen & Kersten, 2004
Manganese Peroxidase (MnP, EC 1.11.1.13)	generates Mn^{3+} which oxidizes phenolic or non-phenolic lignin units via lipid peroxidation reactions	heme	Ca^{2+}, Cd^{2+}, Mn^{2+}, Sm^{3+}	Organic acid as chelators, thiols, unsaturated lipds	monomeric (32-62.5)	extracellular	Sundara-moorthy et al., 2005; Matsubara et al., 1996

Table 1.4 (continued)

Type of enzyme	Main Reaction	Cofactor	Metals or ions	Mediators	Subunits & molecular mass (kDa)	Localization	Ref.
Versatile Peroxidase (VP, EC 1.11.1.16)	Combines the catalytic properties of LiP and MnP oxidizing phenolic compounds	heme	Mn^{2+}, Ca^{2+}, Cd^{2+}, Iron	Veratryl alcohol, compounds similar to LiP and MnP mediators	monomeric	extracellular	Camarero et al., 1999
Other							
Incl. oxidases such as glyoxal oxidase (GLOX), aryl-alcohol oxidase (AAO), aryl-alcohol dehydrogenase (AAD) and quinone reductases (QR)	- oxidases generate H_2O_2; dehydrogenases reduce lignin-derived compounds	GLOX: glyoxal, methyl glyoxal AAO: anisyl, veratryl alcohol Others: organic compounds			GLOX (68)		Hatakka, 2001; Cullen & Kersten, 2004

1.1.3.2 Brown-rot fungi

Brown-rot fungi, which grow mostly on gymnosperms, represent only 7% of the wood rotting Basidiomycota (Martinez et al., 2005). Yet, they are responsible for the most destructive type of wood decay, which is characterized by preferential degradation of the cellulose and hemicellulose of wood (Fahr et al., 1999). Wood degraded by brown-rot becomes brown in colour and rich in lignin as the cellulose and hemicellulose are removed and the lignin is left more or less intact, only chemically modified (Kirk, 1975; Blanchette, 1995; Hatakka, 2001; Schmidt & Czeschlik, 2006) (Fig.1.7). In contrast to white-rot, where the lignin is extensively mineralized, brown-rot yields a complex, aromatic ring-containing polymer derived from the original lignin, which remains on the forest floor, as it is slowly degraded as a component of humus (Kirk & Adler, 1970; Kirk, 1975; Schilling, 2013).

Lignin in brown-rotted wood was first analyzed by Apenitis et al., 1951, who noted that methoxyl loss was a characteristic feature (Kirk, 1983). In 1975, Kirk proposed that the brown-rot modification of lignin was primarily oxidative, because the ratio of O:C was increased in the remaining lignin (Kirk, 1975). In a study, where the brown-rot fungus *Gloeophyllum trabeum* was used, the methoxyl content of spruce wood lignin was reduced by 35% per C_9-unit compared with sound wood (Kirk, 1975). The presence of carboxyl and carbonyl groups was also apparent, as deduced from increased oxygen content (Kirk, 1975; Jin et al, 1990b).

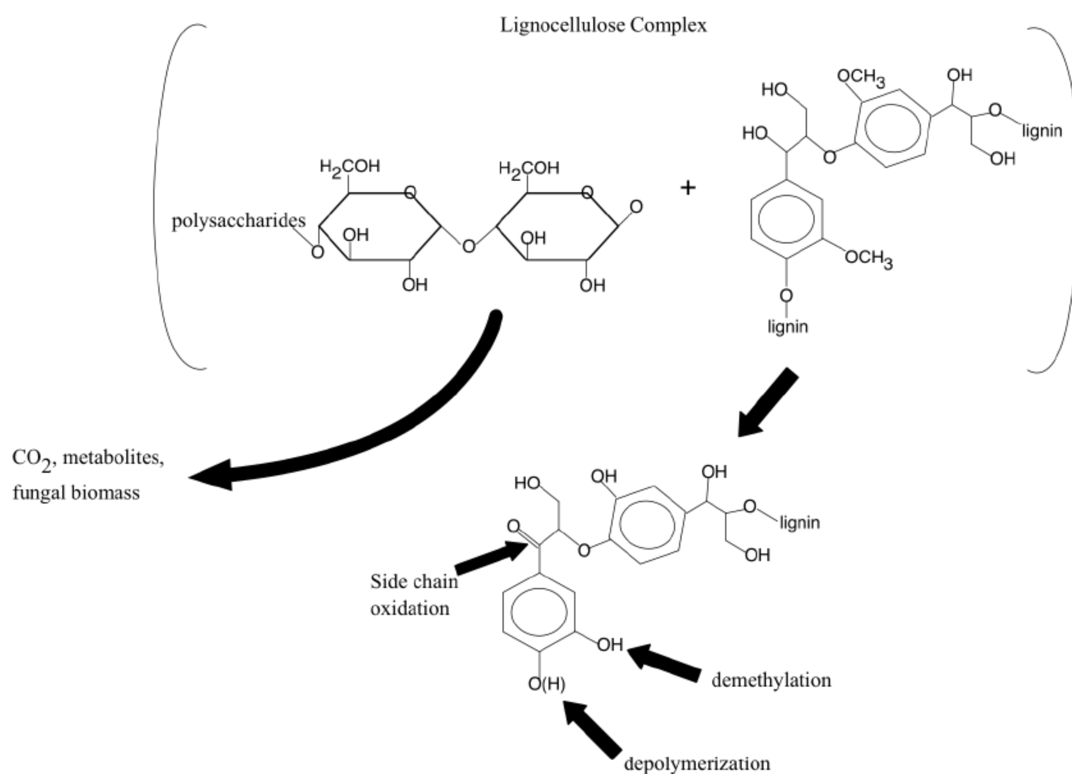


Figure 1.7 Brown-rot fungal degradation. A simplified representation of the principal modification of wood (guaiacyl-based lignocellulose). Metabolism of the cellulose and hemicellulose (polysaccharides) happens early and quickly. Lignin alteration is slower and is thought to be restricted to side chain oxidation, demethylation and to a lesser extent depolymerization (adapted from Filley et al., 2002)

In 1987, analyses of lignin from brown-rotted wood showed that it became oxidized, partially through demethylation of the aromatic rings. This demethylation increased the phenolic hydroxyl content, partially through the introduction of new carbonyl and carboxyl groups (Kirk et al., 1991). Next, several studies showed that brown-rot fungi were able to remove methoxyl groups from lignin and produce methanol, thereby leaving a residue consisting mainly of modified lignin (Ericksson et al, 1990; Jin et al, 1990a; Schmidt & Czeschlik, 2006). In 1992, Niemenmaa et al. found that the demethylation activity of brown-rot fungi was stimulated in the presence of wood (Hatakka, 2001). In 1997, Enoki et al. isolated iron ion containing glycoproteins from *G. trabeum* and *Tyromyces palustris* that showed phenol oxidase activity.

As an understanding of the enzymes involved in white-rot decay emerged in the late 1980s and early 1990s, researchers found that brown-rot fungi do not usually produce the same lignin degrading enzymes as white-rot fungi, although one brown-rot fungus, *Polyporus ostreiformus,* was thought to produce lignin peroxidase and manganese peroxidase (Dey et al., 1994). More recent research has shown that the peroxidases secreted by brown-rot fungi lack essential residues, such as tryptophan in lignin peroxidase or acidic amino acids in manganese peroxidase that are required for catalytic function (Martinez, 2002; Hatakka, 2010). In fact, the genome for the brown-rot fungus, *Postia placenta,* has revealed that the species has no known type of ligninolytic peroxidase encoded (Martinez et al., 2009). According to Niemenmaa et al., 2008, observation of *G. trabeum* showed efficient demethoxylation of dimeric and monomeric compounds, but *P. placenta* could efficiently remove methoxyl groups only from vanillic

acid indicating that these brown-rot fungi have differences in their demethoxylation mechanisms. More research is needed to elucidate the details and better characterize this metabolic system.

Researchers have agreed on two conclusions: firstly that brown-rot fungi have limited capacity to degrade lignin and secondly that the low-molecular weight species they use to initiate decay are oxidants (Erikkson et al., 1990; Hammel et al., 2002). All models suggest that brown-rot decay is based on the generation of hydroxyl radicals and participation of low molecular weight non-protein compounds (ex. phenolate and other types of iron-chelating compounds, i.e. siderophores and oxalate) (Evans et al., 1994; Goodell et al., 1997; Shimada et al, 1997; Schmidt & Czeschlik, 2006). The lignin component of brown-rotted wood was found to be altered by hydroxylation, demethylation and only to a minor extent, depolymerization (Evans et al, 1994) (Fig. 1.7).

The demethylation reactions associated with brown-rot are of interest because the cleaving of lignin methoxyl groups and aromatic hydroxylation leads to increased amounts of phenolic hydroxyl groups, which makes lignin more reactive and thereby potentially useful industrially.

1.1.3.3 Soft-rot Fungi

Soft-rot fungi were discovered in 1950 by Findlay and Savory, who observed this type of decay in wood removed from water-cooling towers (Findlay and Savory, 1954). They isolated the fungus, *Chaetomium globosum* and were able to re-produce the decay in pure culture in the laboratory. They called the decay, "soft-rot" to describe the softening produced in the surface layers of the wood. It was initially characterized by longitudinal chains of cavities with conical tips (Savory, 1954). The decayed wood is brown and soft and the residue is cracked when dry (Hatakka, 2010) Although most recently described, this type of wood decay has proven difficult to define and differentiate from the other decays (Worrall et al., 1997). It is caused by fungal species of the Ascomycota (earlier known as ascomycetes, mitosporic fungi and *Fungi imperfecti)* (Nilsson et al., 1989; Blanchette, 1995).

Soft-rot fungi predominate only in environments that are too severe for the wood-rotting Basidiomycota, such as very wet or very dry environments, or in substrate rich in extractives or preservatives (Blanchette et al., 1991; Blanchette, 1995). These fungi tolerate wider ranges of temperature, pH, and oxygen limitation that white or brown-rot fungi (Blanchette, 1991).

Soft-rot fungi degrade all wood components, but lignin removal is slow and partial with demethylation (Haider & Trojanowski, 1975). All cell wall constituents may be degraded during soft-rot attack but there is usually a preference for carbohydrates, esp.

in hardwoods (Worrall et al. 1997). Knowledge of soft-rot fungi is limited and very little is known about how they degrade lignin (Hatakka, 2010).

1.1.3.4. Bacteria

Since 1940, some actinomycetes and other bacteria have been identified as lignocellulose-degrading microorganisms (Zobell, 1940). These strains come from both aerobic and anaerobic environments including compost soil, land and aquatic ecosystems (Crawford et al., 1973; Gonzalez, 1990).

Wood degrading bacteria have primarily cellulolytic activity (Blanchette, 1995). Some early researchers were skeptical about the ability of bacteria to degrade or modify the structure of lignin (Janshekar and Fiechter, 1982; Blanchette, 1995; Daniel and Nilsson, 1998). All agreed that little was known about which species were capable and that nothing was known about the metabolic system.

The earliest studies with lignin model compounds began in the 1950s with a substance composed of two phenylpropane units (α-conidendrin), metabolized by *Flavobacterium* (Konetzka et al., 1952), *Achromobacter* and *Pseudomonas* (Tabak et al., 1959). Soil bacteria such as *Nocardia* and *Rhodococcus* were identified as being able to breakdown lignin, using an assay with ^{14}C-labelled lignin (Trojanowski et al., 1977; Ahmad et al., 2010). In 1980, Deschamps et al. isolated lignin degrading bacteria using Kraft lignin as the sole carbon source. The different genera were identified as *Corynebacterium, Agrobacterium, Pseudomonas, Aeromonas, Klebsiella* and

Enterobacter demonstrated an ability to assimilate different lignin-related monomers (such as benzoic acid, veratric acid, ferulic acid and vanillin). The ability of bacteria to grow on low-molecular weight lignin oligomers as their sole carbon source indicated that they were producing enzymes catalyzing the cleavage of various inter-monomeric linkages (Gonzalez, 1990).

The lignin degradation mechanism of bacteria is more specific than that of fungi; for example, a single bacterial species may be able to cleave only one type of bond in the lignin polymer (Gonzalez, 1990). Thus, bacteria degrade lignocellulose in mixed bacterial cultures, or more commonly, in bacterial and fungal cultures together (Gonzalez, 1990; Daniel & Nilsson, 1998). In 1988, *Streptomyces viridosporus* T7A was found to be able to depolymerize lignin, using an extracellular lignin peroxidase (Ramachandra et al., 1988). Brunel and Davison used cloning and sequencing of a DNA fragment to learn more about the demethylase involved in the degradation of vanillin. They found that vanillate is a key intermediate in the biodegradation of vanillin (Brunel and Davison, 1988). They knew that the genes *vanA* and *vanB* code for a vanillate demethylase that broke down vanillate to protocatechuate, but the purification of the relevant enzyme components was not achieved, and their properties and mode of action remained to be characterized (Brunel and Davison, 1988). This use of genetics to understand the catabolism of lignin-derived aromatics was key to the success researchers had with *Sphingobium* sp. SYK-6.

In 1987, the bacterial strain *Pseudomonas paucimobilis* SYK-6 (an aerobic Gram-negative soil bacillus) was isolated from a pond for the treatment of waste liquor from a Kraft pulp mill (Masai et al., 2007). Researchers were interested in the species because it was capable of utilizing various types of lignin derived compounds including bi-phenyl, beta-aryl ether, phenylcoumarane, and diarylpropane, as well as various lignin-related mono-aryls including ferulate, vanillin, vanillate, syringaldehyde and syringate. Since that time SYK-6 has been studied extensively for its ability to breakdown lignin-derived fragments (Table 1.5).

The understanding of bacterial enzymology in the degradation of lignin remains far behind that of the white-rot fungi. Researchers have been looking for similar extracellular enzymes to those found in fungi. *S. viridosporus* T7A produces extracelluar peroxidases, which cleave β-aryl ether lignin-derived compounds (Ramachandra et al., 1988).

Bugg and Winfield, 1998 describe two strategies used by micro-organisms for aromatic ring breakdown: the first, by aerobic bacteria, in which there is oxidation of the ring to a dihydroxy-aromatic compound (a catechol or a hydroquinone), followed by oxidative cleavage of the ring, and the second, by anaerobic bacteria in which there is reductive hydrogenation of the ring, followed by fragmentation of the cyclohexane ring skeleton (Bugg & Winfield, 1998).

SYK-6 has a complicated mechanism including catabolic pathways for lignin-derived aromatic compounds including the ferulate catabolic pathway, the PCA 4,5-cleavage pathway, the beta-aryl ether cleavage pathway, the biphenyl catabolic pathway and multiple 3MGA catabolic pathways. These pathways start with either the guaiacyl moiety of guaiacyl lignin or the syringyl moiety of syringyl lignin, which are degraded to syringate and vanillin respectively. From there syringate and vanillin are converted to 3-*O*-methylgallate (3MGA) and protcatechuate (PCA), and these compounds are further degraded through ring cleavage pathways for PCA, 3MGA and gallate (Masai et al., 2007). Although a significant portion of the SYK-6 genes involved in the catabolism of lignin-derived aromatics have been isolated and characterized thus far, there remains much work to get a clear understanding of how the metabolism works (Masai et al., 2012).

Table 1.5 SYK-6 Research by the Masai group, leading to the elucidation of the SYK-genome

Year	Achievement	Ref.
1987	*P. paucimobilis* SYK-6 isolated in kraft mill pond (in Japan). This bacterial species was of interest because it was able to grow on DDVA, syringic acid, vanillate and other lignin dimeric model compounds as a sole carbon source	Katayama et al., 1987
1988	Bacterial metabolism laid out with a degradation pathway from wood lignin to the TCA cycle	Katayama et al., 1988
1989	Eiji Masai joins research team. First detection of β-aryl ether cleaving enzyme.	Masai et al., 1989
1990	First report of cloning and sequencing of the protocatechuate 4,5-dioxygenase gene	Noda et al., 1990
1991	First report of cloning and sequencing of the protocatechuate 4,5-dioxygenase gene	Masai et al., 1991
2000	LigX was found to encode an essential enzyme for DDVA *O*-demethylation in SYK-6 and that this bacteria has two *O*-demethylation systems: one that is an oxygenative demethylation system, and the other is a tetrahydrofolate-dependent methyltransferase system.	Sonoki et al., 2000
2004	The gene DesA was found to encode an enzyme that catalyses the transfer of the methyl moiety of syringate to H_4folate, forming 5-methyl- H_4folate and that desA is essential to syringate degradation for SYK-6.	Masai et al., 2004
2005	More than 20 genes involved in the catabolism of lignin-related aromatics had been characterized by this year. Vanillate *O*-demethylase gene isolated and characterized (its functions and roles in the metabolism of both vanillate and syringate)	Masai et al., 2007; Abe et al., 2005
2007	Award review (written in response to winning The Japan Bioscience, Biotechnology, and Agrochemistry Society Award for the Encouragement of Young Scientists in 2005) – outlined the catabolic pathways in chart form, elucidating with genetic analysis the major pathways of degradation of monoaryls and biaryls via the PCA 4,5-cleavage pathway or multiple 3-*O*-methylgallate pathways.	Masai et al., 2007
2009	A product was produced from their work: 2-Pyrone-4,6-dicarboxylic acid (PDC, a resin) with industrial uses; bacterial name changes to Sphingobium sp. strain SYK-6	Hishida, 2009
2012	SYK-6 genome announcement released	Masai et al., 2012

1.1.4 Lignin Demethylation Assays

1.1.4.1 Ti(III)-NTA

Elemental titanium has received interest for many applications, including colorimetric assays, as a result of the colored complexes which titanium can form, variable oxidation states and compatibility with complex ions (Tseng et al., 2014; Eboka et al., 2003). Titanium is commonly found either as Ti^{+4} [Ti(IV]) or Ti^{+3} [Ti(III)], which absorbs in the green/yellow spectrum and transmits in the violet/blue spectrum (Berg et al., 2007).

Zehnder and Wuhrman (1976) first used Ti(III)-citrate in a buffering system as a media reductant. Their oxidation-reduction based buffering system eliminated all oxygen from the culture medium and served as an indicator for low oxidation-reduction potentials. The medium was used to inhibit the growth of facultative anaerobes, which frequently contaminate anaerobic bacterial cultures.

In 1983, Moench & Zeikus were unable to reproduce the reagent as described in the Zehnder and Wuhrman paper. The inorganic reductant Ti(III)-citrate kept precipitating, either in the culture or during the preparation. Hudson and Morel (1989) used the Ti(III)-citrate, together with EDTA, as a reagent for rapidly dissolving extracellular iron at pH 8, thereby reducing precipitation without causing cell damage or culture toxicity. Ti(III)-citrate alone had a tendency of polymerizing at pH 8, which made it difficult to use. Adding EDTA helped to stabilize the preparation and allowed for minimal precautions against oxygen contamination. This study found that Ti(III)-citrate needed further investigation for effect on different organisms and that the citrate and

EDTA needed to be in excess to prevent precipitation of Ti(III), which can also clog fine filters. Moench and Zeikus (1983) were able to improve the reagent by using nitrilotriacetic acid (NTA) instead of citrate, which proved to be very stable in the presence of oxygen. Later, Premović and Jovanović (1991) conducted a structural study of Ti(III)-NTA and found pH 4-10 to be optimal, with a ratio of Ti(III) to NTA of 1:2.

The Ti(III)-NTA reagent was also found to be useful in detecting demethylation spectrophotometrically by Kreft and Schink (1993). The aerobic degradation of ether compounds was a well-known process (Axelrod, 1956). However, the degradation of phenylmethylethers by acetogenic bacteria was only first reported in 1981 (Bache & Pfennig, 1981). An assay method was needed to investigate demethylation in anaerobic species and Ti(III)-NTA was adapted for this purpose. The colorimetric assay was developed to directly detect *O*-demethylation activity by measuring a yellow complex produced between Ti(III)-NTA and the vicinal diol produced from the *O*-demethylation of syringate.

This anaerobic assay was improved by Liu (1997), who adjusted reagent concentrations to improve linearity at higher product concentrations. The most effective retaining accuracy at both low and high concentrations was achieved at 25 mM Ti(III) and 100 mM NTA. The extinction-concentration curves at various wavelengths were examined and 380 nm was found to be optimal. HEPES pH 8 buffer was used in the reaction mixture at a 1mM final concentration which increased the detection limit.

Most recently I adapted the Ti(III)-NTA for use in aerobic conditions with a detection range of 0.5 to 500 μM of vicinal diols (Gibson et al. (2014). The method was found to be suitable for use with lignin concentrations of up to 0.3% w/v without interference with the accuracy of the assay. At higher lignin concentrations interference was found to limit the detection of vicinal diols. The Ti(III)-NTA assay has been shown to be a simple, rapid, cost effective method to detect vicinal diols in aerobic conditions with samples containing lignin and lignin-derived compounds.

1.1.4.2 HNO_2

A previously published method was investigated by Bashtan-Kandybovich et al. (2012) to quantify demethylation by measuring changes in the concentration of pyrocatechol in lignin samples (Maslennikov and Porivaeva, 1965; Bashtan-Kandybovich et al., 2012). The investigation employed two solutions and measured pyrocatecholic content by titrimetry. Solution A was a red solution containing 0.2mg/mL catechol, 0.5mL $Al_2(SO_4)_3$ (10% w/v), 0.1mL $NaNO_2$ (10% w/v) and 0.5mL NaOH (50%). Solution B contained 1mL of sample (dissolved in DMSO, 2-methoxyethanol or 1,4-dioxane) and 0.5mL $Al_2(SO_4)_3$ (10% w/v). The reaction was prepared by using 1 mL of Solution B, 0.1mL $NaNO_2$ (10% w/v), 0.5mL NaOH (50%) and 3 mL of deionized water. After adding a drop of $K_3(Fe(CN)_6)$ (0.3% w/v), the solution changed from red to yellow. Using Solution A, the yellow solution was titrated back to red. The titration volume was used to calculate the pyrocatecholic content. This method did not work well with lignin present.

1.1.4.3 4-AAP

The 4-aminoantipyrine assay (4-AAP) is commonly used in the detection of phenolics in waste water (Ettinger et al., 1951). Bashtan-Kandybovich et al. (2012) examined this method to detect pyrocatechol indicating demethylation. The reaction mixture contained 0.25 mL of sample (containing lignin) and 1 mL of 4-AAP reagent (1mg/mL). The resulting positive red color can be quantified at 516 nm using a spectrophotometer and comparing the result to a set of standards. This method was examined using a protocol previously established without lignin by LaRue & Blakley, (1964). In practice, there were problems with interference, from lignin and possibly from the fungal pigments produced (data not shown).

1.1.4.4 Other methods

Many methods have been used to determine demethylation of lignin and lignin-derived compounds by both direct and indirect means. Other studies have used instrumentation such as GC-MS, GC-FID, HPLC, LC-MS, ^{31}P NMR and FTIR to study these reactions (Table 1.6). Many of these methods require lengthy sample preparation or conditions that will alter the products analyzed. The instrumental methods are not suitable for rapid, routine enzyme analysis.

Table 1.6 Analytical protocols for characterization of demethylation enzymes

Assay	Substrate	Method Author	Used In
GC-MS	biaryls	Girardin & Metch, 1983	Sonoki et al., 2000
GC-FID	monoaryls	van der Hage, 1993	Nishikawa, 1998
LC-MS	monoaryls	de Jager et al., 2007	Sonoki et al., 2009 Sainsbury et al., 2013
^{31}P NMR	lignin	Argyropoulos, 1994	Granata & Agyropoulos, 1995; Ferhan, 2013
^{1}H NMR	biaryls, lignin	Santos Abreu & Inacio Friere, 1995	Crestini and Argyropoulos, 1998
FTIR	lignin	Ferhan et al., 2013	Ferhan et al., 2013

1.1.5 Selected Ion Flow Tube Mass Spectrometry

The SIFT-MS technique merges gas-phase physical chemistry (ion molecule kinetics), computational chemistry (gas-phase mechanisms) and analytical research (determining analyte concentrations). The technique was originally developed for use in explaining cosmic events, but has been adapted for use in the detection of trace compounds in air, breath, the headspace above biological fluids and the volatile compounds emitted by living cells and bacterial cultures (Smith & Španěl, 2011).

In 1969, a flowing afterglow mass spectrometer (FA) was developed following the discovery that ions in a long glass tube with flowing gas could affect the observed neutral chemistry as far as 1 m downstream of an electrical discharge (Schmeltekopf & Broida, 1963). The device was found to be useful in studying the ion-neutral chemistry of the earth's upper atmosphere (Adams et al., 1975). However, there were problems with unwanted reactant ions in the mass spectra and the presence of electrons in the tube (Adams & Smith, 1976a). Adams and Smith separated the ion creation region from the ion reaction region by adding a second quadrupole mass spectrometer (Adams & Smith, 1976b). The new selected ion flow tube (SIFT) allowed each ion of interest to be selectively injected into the flow tube and reactions of ions having a single *m/z* ratio were used to identify product and calculate ion rate coefficients. This technique was found useful in modeling the more complex reactions, which occur in the interstellar medium (Adams et al., 1989).

In 1996, Španěl and Smith, published research using a SIFT instrument to measure concentrations of a range of analytes in whole air samples (Smith & Španěl, 1996). The technique, called SIFT-MS, reversed the process, which had been in common use for the previous 30 years of determining rate coefficients. They proposed, that if a rate coefficient and product ions were known for an ion-molecule reaction, then the concentration of the neutral analyte could be determined. Since 1996, SIFT-MS has been used for a variety of applications (Table 1.7).

The SIFT-MS technique is based on the chemical ionization, using selected precursor ions, of trace gases in an air sample that is introduced into a fast flowing inert gas (Smith & Španěl, 1996). The selected precursor ions, H_3O^+, NO^+ and O_2^+ are created in a microwave gas discharge. They and are introduced via a quadrupole mass filter and Venturi inlet into the carrier gas (usually helium at approximately 0.5 Torr) flowing along a flow tube at a known velocity. They do not react with the major components of air (N_2, O_2, H_2O, Ar and CO_2) but will react with many volatile compounds (Abbott et al., 2003). The gas mixture to be analyzed is introduced downstream (at a flow rate of approximately 0.3 Torr L sec^{-1}) into this carrier gas/ion swarm and the trace gases in the sample react with the precursor ions producing characteristic product ions (Španěl & Smith, 1999). The ionized products along with remaining precursor ions are then quantified using a downstream quadrupole mass analyzer and the partial pressures of the trace gases in the air sample are determined (Španěl & Smith, 1999).

Table 1.7 SIFT-MS Applications (adapted from Smith & Španěl, 2011)

	Liquid Headspace	Breath analysis	Ambient air, fumes, emissions	Ref
Biomedical				
Volatile biomarkers for cancer and infection in urine	✓			Španěl et al., 1999a; Smith et al., 1999
Ketones in urine	✓			Pysanenko et al., 2009; Wang et al., 2008
Volatile markers of ovulation in urine	✓			Smith et al., 2006; Diskin et al., 2003
Tissue cell cultures	✓			Sulé-Suso et al., 2009; Smith et al., 2003
Bacterial cultures	✓			Pysanenko et al., 2008
Clinical diagnosis:				
renal failure		✓		Španěl et al., 1999b; Španěl et al., 1998
diabetes; celiac disease		✓		Turner et al., 2009; Hryniuk & Ross, 2010
cancer		✓		Španěl & Smith, 2008
Therapeutic monitoring:				
dialysis		✓		Davies et al., 2001
chemo- and radiotherapy		✓		Smith et al., 2010
halitosis		✓		Pysanenko et al., 2008; Ross et al., 2009a
Release of volatile compounds by skin			✓	Turner et al., 2008
Compounds in tobacco and cannabis smoke			✓	Smith & Španěl, 1996
Food Science				
Quantification of aroma Compounds in fermentation	✓			Olivares et al., 2010
Food flavor analyses	✓			Oliveres et al., 2010; Xu and Barringer, 2010
Volatile organic compounds related to sensory qualities			✓	
Environment; health and safety				
Biological monitoring		✓		Španěl et al., 1997
Exhaust gases			✓	Smith et al., 2002; Smith et al., 2004
Emissions from waste incineration, sewage, landfills			✓	Smith et al., 2000; Sovová et al., 2008
Security				
volatile markers of explosives			✓	
Products of fumes of explosions			✓	Sovová et al., 2010

The Profile 3 (Instrument Science, UK) SIFT-MS, located at Lakehead University, uses helium as the carrier gas, and runs model-specific software (see Fig. 1.8) The instrument has two modes of operation: multiple ion monitor (MIM) and full scan (FS) mode. The MIM mode operates in real time and is useful for comparing several samples (Smith & Španěl, 2005). The displayed product mass-to-charge ratio (*m/z*) can be changed to preset values (such as methanol), or individual products can be selected manually. FS mode displays product counts in relation to *m/z* values. The FS mode can be set to sample the headspace for any period of time depending on the protocol (Smith & Španěl, 2005). The range of the scan selected is based on the requirements of the experiment (ie 1-200 *m/z*). The main difference between the two modes is that MIM is a plot of product counts in relation to time while FS relates product counts to *m/z*.

The SIFT-MS has several variables that must be taken into consideration, including temperature, water vapor, pressure, gas flow rate, mass discrimination and diffusion. Pressure is mediated by the turbo pump, which must keep the reaction at a constant pressure in order for the software to properly calculate product counts from the reaction coefficients. Flow rate to the inlet is constant but changes when the length of the tube is changed or the flow is altered in some way. This is corrected for, during the calibration process, and is crucial to obtaining accurate counts because the software calculations are based on the flow rate into the inlet. Mass discrimination settings are used to account for larger ions that are not counted as accurately as smaller ions. Diffusion settings are used to account for ions that strike the tube wall and are not read by the quadrupole mass filter.

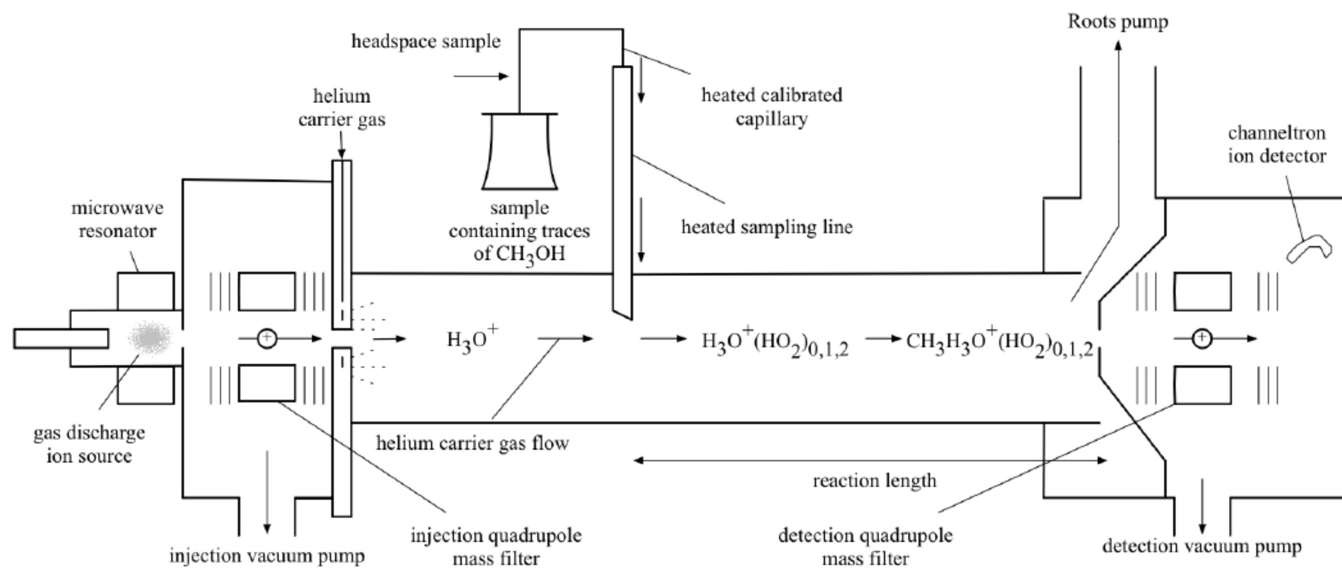
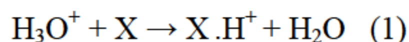


Figure 1.8 A diagram of the Profile 3 SIFT-MS instrument sampling the headspace of a microbial culture flask. The illustration shows the ion chemistry in the analysis of methanol using the H_3O^+ precursor (based on Turner et al., 2006)

The precursor ion is selected to best suit the product that is being analyzed. Depending on the interaction, an incorrect precursor will yield a reaction coefficient that is too slow or may create an ambiguous m/z pattern. Isomeric (same chemical formula and mass) and isobaric compounds (different chemical formula but same mass) can be difficult to distinguish with mass spectrometers. However, SIFT-MS is able to overcome this by switching precursors. A sample that is reacted with a second or third precursor can provide information that verifies or distinguishes it from other probable compounds. The ability to use different precursors for chemical ionization increases the selectivity and allows for the detection of otherwise undistinguishable compounds.

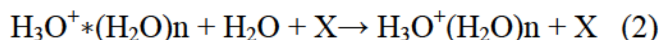
The rate coefficient and characteristic product ions for many compounds have been experimentally determined in previous studies and catalogued in the SIFT-MS product ion library. Previous research has found that the H_3O^+ precursor is best suited for the routine analysis of methanol and is also the most commonly used precursor with SIFT-MS technology (Davis et al, 2005). The reaction of H_3O^+ with a molecule (X) proceeds by proton transfer (Equation 1) (Španěl & Smith, 1997).



$$H_3O^+ + X \rightarrow X.H^+ + H_2O \quad (1)$$

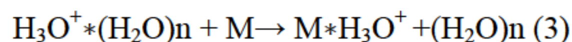
Although several reactions are possible using H_3O^+ precursor, proton transfer is the most common (Equation 1). Depending on the amount of vapor present in the reaction and the compound analyzed, water molecules may form clusters. When the precursor has a water molecule clustered (Equation 2), the molecule will react normally. However, the product ion detected by the quadrupole mass filter will have an additional 18 m/z added

(Španěl & Smith, 2011). The NO^+ precursor may also form water clusters but is much less pronounced in the SIFT-MS spectra as a result of slower rate coefficients (Ikezoe et al., 1986).



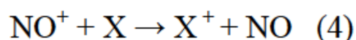
$$H_3O^+ * (H_2O)n + H_2O + X \rightarrow H_3O^+(H_2O)n + X \quad (2)$$

Hydration reactions must be considered when analyzing compounds using SIFT-MS. In previous studies, a 15% error in product concentrations occurred if the hydration products were not taken into consideration (Španěl & Smith, 2000). When all the hydration products were accounted for in the calculation, the error was reduced to less than 5%. Another possible reaction with H_3O^+ is ligand switching shown below in Equation 3. The reaction results in a charged ligand transferred to a neutral analyte.



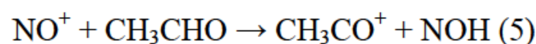
$$H_3O^+ * (H_2O)n + M \rightarrow M * H_3O^+ + (H_2O)n \quad (3)$$

The reactions of NO^+ can be quite varied, commonly undergoing either electron transfer, hydride abstraction or association reactions depending on the ionization energy. If the ionization energy is less than 9.26 eV, then an electron transfer reaction will occur (Smith & Španěl, 2011). In the case of Equation 4, an electron is transferred from a neutral species to form a new cation.



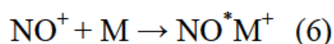
$$NO^+ + X \rightarrow X^+ + NO \quad (4)$$

Hydride abstraction is commonly observed with the NO^+ precursor, leading to a $[M\text{-}H]^+$ cation. Equation 5, shown below, uses acetaldehyde as an example (Španěl & Smith, 1997).



$$NO^+ + CH_3CHO \rightarrow CH_3CO^+ + NOH \quad (5)$$

Association (as shown in Equation 6) is another common reaction of the NO^+ precursor and can occur by a radiative or termolecular pathway (Smith & Španěl, 1997). Radiative association is thought not to occur using SIFT-MS, however termolecular association occurs with the assistance of a carrier atom (i.e. He) to collide with and stabilize the complex at a lower energy than required to dissociate (Bates & Herbst 1988).



$$NO^+ + M \rightarrow NO^*M^+ \quad (6)$$

One of the advantages of SIFT-MS, is that appropriate reagent ion can be chosen from one the three available precursors (H_3O^+, NO^+ and O_2^+), which have been demonstrated to work with ambient air, breath and biological headspaces (Smith & Španěl, 2011). The knowledge gained from initial SIFT-MS studies (see Table 2.1), involving the surveys of ion/molecule reaction kinetics, has laid the ground work for a comprehensive library that identifies in real-time, many volatile organic compounds in headspace gas.

1.2 Objectives

My thesis investigates the biological modification of Kraft lignin using microorganisms such as fungi and bacteria. With access to over 400 fungal species at the Mycological Herbarium at Lakehead University, fungal species were chosen for a novel screening study detailed in Section 2. The results were obtained using SIFT-MS and provided a unique opportunity to demonstrate the technique with the headspace of fungal

cultures using Kraft lignin as a sole carbon source. To verify that the change in methanol was a result of vicinal diol generating demethylation reactions, I developed the Ti(III)-NTA assay to directly measure the change in vicinal diols (Section 3). Taking into consideration several demethylation indicators, Section 4 outlines the optimization of culture conditions for demethylation activity using lignin-derived compounds. The work presented in section 5 is the partial purification and characterization of demethylation enzymes from the fungal species, *Absidia cylindrospora* and bacterial species, *Sphingobium* sp. SYK-6. This section was used to examine and compare the two enzymes: one fungal, the other, bacterial.

Specific objectives targeted by my work are the following:

1. To screen microbial cultures for methanol production, indicative of lignin demethylation
2. To verify fungal demethylation based on model compounds using the Ti(III)-NTA assay
3. To increase demethylation by changing growth conditions using vanillin as a lignin-model substrate
4. To examine and characterize enzyme activity associated with demethylation

2. Use of SIFT-MS to examine the headspace of lignin-degrading organism cultures for the presence of methanol and other volatile compounds

Unpublished:

Gibson, A. [a,b*], Malek, L.[a,b] , Dekker, R.F.H. [ad] and Ross, B.M. [b,c] . 2014. Detecting volatile compounds from Kraft lignin degradation in the headspace of microbial cultures by selected ion flow tube mass spectrometry (SIFT-MS). Manuscript submitted for publication

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Author Contribution

Gibson, A.: completed all writing and experimental

Malek, L., Dekker, R.F.H. and Ross, B.M.: critical evaluation of design and interpretation of data; editing; final approval before publishing

2.1 Abstract

Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) was used to quantify methanol and other volatile compounds in the headspace of one bacterial and 12 fungal lignin degrading microbial cultures. Cultures were grown in 250 mL Erlenmeyer flasks capped with tin foil containing 40 mL of media using Kraft lignin (0.3%,w/v) as the sole carbon source. The analysis was done using SIFT-MS with H_3O^+ and NO^+ precursors. Product ions were identified with multiple ion mode (MIM), while full scan (FS) mode was used to identify other compounds of interest. *Absidia cylindrospora, Ischnoderma resinosum* and *Pholiota aurivella* increased headspace methanol concentration by 210 ppb, 1269 ppb and 352 ppb respectively, while *Flammulina velutipes* and *Laetiporus sulphureus* decreased the concentration below ambient levels by 54 ppb and 63 ppb. *F. velutipes* and *L. sulphureus* were found to produce products of methanol oxidation (formaldehyde and formic acid) and are likely metabolizing methanol. Some additional unidentified compounds generated by the fungal cultures are intriguing and will require further study. The SIFT MS can be used to quantify methanol and other volatile compounds in the headspace of microbial cultures and has the potential to be a rapid, sensitive, non-invasive tool useful in elucidating the mechanisms of lignin degradative pathways.

2.2 Introduction

Lignin is a complex heterogeneous aromatic polymer found in wood, consisting of phenylpropanoid units linked together by ether and carbon-carbon bonds (Sjöström, 1993). It is the most abundant aromatic compound in nature and second only to cellulose

as the most abundant renewable carbon source on earth (Zhu et al., 2011). It is, however, highly resistant to degradation (Blanchette, 1991).

The abundance of lignin and the potential of commercial applications have fueled an interest in lignin degradation research and lignin-derived products for the past several decades (Hu et al., 2011). Currently the vast majority of lignin separated from wood industrially is burnt as fuel. Only 1-2 %, isolated in the Kraft pulping process, is employed in a range of products from materials for car brakes, to wood panel products, biodispersants, polyurethane foams, and epoxy resins (Lora & Glasser, 2002). The enzymatic modification of lignin has immense potential to create more reactive lignin and produce polymers for specialty applications.

In nature, there are a limited number of fungal and bacterial microorganisms capable of playing a role in breaking down this complex compound (Higuchi, 1985). Some types of fungi degrade lignin completely, while others are of special interest because they modify the polymer, leaving the ring structures intact (Kirk, 1975). Much research has focused on the most efficient lignin degraders, the white-rot Basidiomycota (Tien & Kirk, 1983; Kersten & Cullen, 2007). Extracellular lignin-modifying enzymes are thought to be involved, the best characterized of which are laccase, lignin peroxidase and manganese perioxidase (Hatakka, 1994). Less is known about the mechanisms of the brown-rot Basidiomycota, soft-rot Ascomycota, and bacterial degraders, although catabolic pathways for the degradation of lignin fragments have been published for the bacterium *Sphingobium* sp. SYK-6 (SYK-6) (Masai et al., 2007). One potential

mechanism involves demethylation. Fungi that modify lignin (brown-rot and soft-rot for example), rapidly depolymerize cellulose and then slowly remove the methyl and methoxyl groups through hydrolytic cleavage reactions (Cullen & Kersten, 2004). Hardwood and softwood demethylation reactions take place on the coniferyl and synapyl monomers that make up the guaiacyl and syringyl lignin units found in these tree species. Demethylation can occur on both intact lignin and on smaller lignin fragments. Analyses of lignin modified by fungi indicate a high proportion of demethylated lignin resulting in a high phenolic content in the lignin residue (Filley et al., 2000).

Specific enzymes are anticipated to be associated with demethylation. Evidence of these enzymes, based on the methanol released by wood-decay fungi, has been reported in previous studies (Ander & Eriksson, 1985). After lignin or monolignols have been demethylated, two adjacent hydroxyl groups are formed (see Fig. 2.1), making the lignin more reactive (Jin et al., 1990a; Hu et al., 2011). Phenolic lignin has the potential of being the source of industrially relevant derivative compounds (Stewart, 2008). The detection, purification and characterization of any lignin-demethylating enzyme are of interest to elucidate the biological/metabolic mechanisms involved. So far, there is a lack of rapid, cost-effective methods for the detection of this enzymatic activity. To this end, I used selected ion flow tube mass spectroscopy (SIFT-MS) as a screening assay for identifying methanol production by fungal species isolated from decaying wood and held in the Mycological Herbarium at Lakehead University.

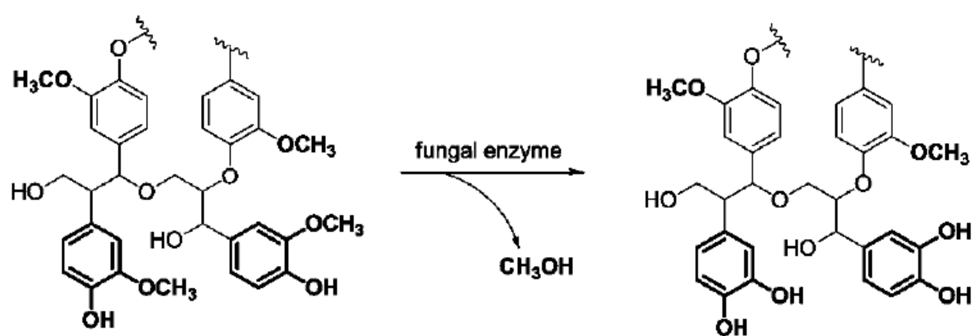

Figure 2.1 Fungal enzyme hypothesized to demethylate lignin while releasing methanol as a product

Selected ion flow tube mass spectroscopy (SIFT-MS) is a sensitive analytical method that detects and quantifies, in real time, trace gases found in air and breath (Španěl & Smith, 2000). The method was first conceived and developed in the late 1970s and, during the past 40 years, has been applied in laboratories world-wide to environmental science (polluted air and exhaust gases), biological science (emissions from feces and urine, rumen gases and soil), food science (emissions from food, food products, and yeast fermentation), and human health science (diagnosing diseases in their early stages from exhaled breath and urine headspace analysis) (Smith & Španěl, 2005, 2011).

The SIFT-MS technique relies on the chemical ionization, using selected precursor ions, of trace gases in an air sample that is introduced into a fast flowing inert gas (Smith & Španěl, 1996). The precursor ions generated by the microwave source are H_3O^+, NO^+ and O_2^+. They do not react with the major components of air (N_2, O_2, H_2O, Ar and CO_2) but will react with many volatile compounds including methanol (Abbott et al., 2003; Davis et al., 2005).

Given the potential use of SIFT-MS as a rapid, non-invasive analytical instrument coupled with an interest in the microbial demethylation of lignin, I aimed to demonstrate in this study, the use of SIFT-MS to quantify methanol and other volatile compounds in the headspace of lignin degrading microbial cultures.

2.3 Materials and Methods:

2.3.1 Organisms: *Absidia cylindrospora* (LU-7, soft-rot), *Fomes fomentarius* (LU-16, white-rot), *Flammulina velutipes* (LU-22, white-rot), *Laetiporus sulphureus* (LU-40, brown-rot), *Ischnoderma resinosum* (LU-48, white-rot), *Irpex lacteus* (LU-72, white-rot) *Pholiota aurivella* (LU-90, white-rot), *Oligoporus leucospongia* (LU-113, brown-rot), *Lentinellus montanus* sp. 1 (LU-115,white-rot), *Lentinellus montanus* sp. 2 (LU-116, white-rot) and *Cylindrocladium camelliae* (LU-120, black-rot) were from the Mycological Herbarium at Lakehead University. *Gleophyllum trabeum* (UAMH 7375, brown-rot) was obtained from University of Alberta Microfungus Collection and Herbarium (UAMH). Bacterium *Sphingobium* sp. SYK-6 was obtained from NITE Biological Resource Center, Kazusakamatari, Kisarazu, Chiba, Japan.

2.3.2 Growth conditions: The fungal stocks were grown on potato dextrose agar (PDA) plates that were incubated at room temperature. Culture flasks were inoculated using 3 mycelium plugs taken from the PDA plate. Fungal cultures were grown in 250 mL wide neck Erlenmeyer flasks containing 40 mL of media and tightly capped with aluminum foil. The media contained 2% Vogel's minimal salts (VMS)(Vogel 1956) and 0.3% w/v lignin. Each culture was grown with 6 replicates at 20 ± 2°C for 3 weeks.

Bacterium SYK-6 was grown on nutrient agar plates (Sigma Aldrich) that were incubated at 20 ± 2°C. A sterile loop from the plate was used to inoculate 200 mL of nutrient broth, and the culture was grown to OD_{550} 1. SYK-6 was grown on 40 mL of minimal W-medium (Peng et al., 1998) in 250 mL wide neck Erlenmeyer flasks using

0.3% w/v lignin as a carbon source. Cultures were grown for 5 days at room temperature before analysis.

2.3.3 SIFT-MS analysis: A Profile 3 SIFT-MS instrument (Instrument Science, Crewe, UK) using H_3O^+ and NO^+ precursors was used in this study. Samples were analyzed at room temperature and pressure with a flow tube pressure at approximately 1 Torr and an inlet flow of 28 SCCM (standard cubic centimeters per minute) (Ross et al., 2009b).

Samples were taken by piercing the tinfoil cap with a needle on the end of a PFTE transfer line heated to 100°C connected to the SIFT-MS. Reaction rate constants were determined in previous studies, as listed in Table 2.1. Multi-ion-monitor (MIM) samples were taken for 30 seconds and full-scan (FS) mass spectra were taken over 2 minutes at an *m/z* range from 10 to 200.

To identify major product ions, the MIM scan from each culture headspace was used for methanol quantification, while the FS mode was used to detect other compounds of interest. To ensure accuracy with methanol analysis, 3 methanol hydrates (*m/z* 33, 51 and 69) were also taken into account using the H_3O^+ precursor. Compounds were tentatively identified using the SIFT-MS library containing known reaction products and rate constants obtained in studies (Španěl & Smith, 1997). A compound was considered "present" if the majority of the flasks contained the product ions for that compound. The SIFT-MS data was normalized from experiment to experiment, by a procedure developed by Hryniuk and Ross (2010).

Further details of the operation of the SIFT-MS are in section 1.2.1.5.

2.3.4 Chemicals: All chemicals were of analytical grade and were obtained from Sigma-Aldrich. Kraft lignin was obtained Forest Product Innovations (FPI Pointe-Claire, QC, Canada).

2.4 Results

Headspace samples were taken from 12 fungal and one bacterial species grown on lignin liquid cultures and measured in triplicate using FS analysis (Table 2.1). Cultures 7, 22, 72, 120 and 7375 grew well using Kraft lignin as sole carbon source. The remaining fungal species (LU 16, 40, 48, 90, 113, 115 and 116) and the bacterium were not able to grow well on lignin alone, although some altered the headspace gas composition (LU 48, 90, 115 and 116). Putative compounds were identified through the SIFT-MS product ion library and by examining the mass spectrum for all characteristic product ions and cross-referencing with both precursors (H_3O^+ and NO^+) if applicable.

The compounds in Table 2.1 were identified from full scan analysis (Fig. 2.2 and 2.3). *A. cylindrospora* produced the most unique set of product ions in comparison to the other species. Significant amounts of formaldehyde (*m/z* 31), formic acid (*m/z* 47) and methanol (*m/z* 33, 51 and 69) were identified in the H_3O^+ spectra (Fig 2.2). The assignments of products were considered as preliminary and must be verified in future work. Each culture was also analyzed using NO^+ precursor to confirm product ions

identified with the H_3O^+ precursor (Fig 2.3), examples include: acetaldehyde (*m/z* 43), ethanol (*m/z* 45), propanal (*m/z* 59). hexanal (*m/z* 99) and 2-hexanone (*m/z* 130).

Formaldehyde and formic acid were tentatively identified using scans of the same sample flasks (Table 2.2). Control flasks (media not inoculated) on average had about 75 ppb of methanol. Formic acid and formaldehyde product ions were not detected in control flasks.

Table 2.1 Summary of putative compounds identified by SIFT-MS in the headspace of 9 fungal species grown on Kraft lignin. Spectra were normalized to precursor ion count rates (see Methods) to aid comparison of different cultures. The values shown below are the product ion *m/z*, which can be discerned on the H_3O^+ spectra along with the putative compound identification. The last column indicates whether the expected NO^+ reaction products are present for the putatively identified compounds

Putative Compounds	Species	H_3O^+ Product Ions (*m/z*)	NO^+ Product Ions (*m/z*)	Ref.
Ammonia	90, 115, 116, 120, 7375	18, 36[1], 54[2]	NR	Španěl & Smith, 1998a
Formaldehyde	7, 22, 72	31	NR	Španěl et al., 1997a
Methanol	7, 72, 90	33, 51[1], 69[2]	NR	Španěl et al., 1997b
Propanol	7	43 ,61, 79[1], 97[2]	59	Španěl et al., 1997b
Acetaldehyde	7, 22, 72	45	43	Španěl et al., 1997a
Unidentified	7, 22, 45, 72, 90, 7375	46	-	-
Ethanol	7, 22, 72, 7375	47, 65[1], 83[2]	45	Španěl & Smith, 1996
Formic Acid	7, 22, 72, 90, 116, 120, 7375	47	NR	Španěl & Smith, 1998b
Unidentified	22, 72, 7375	58	-	-
Acetone	7, 22, 72, 7375	59, 77[1]	88	Španěl et al., 1997a
Unidentified	7, 22, 72	60	-	-
Acetic acid	7, 22, 72	61, 79[1], 97[2]	90	Španěl & Smith, 1998b
Pentanal	7	69, 87[1]	85	Španěl et al., 1997a
Benzene	7, 22, 72, 7375	79	NR	Španěl & Smith, 1998c
Hexanal	7, 72, 7375	83, 101, 119[1]	99	Španěl & Smith, 1999
2-Pentanone	22, 120	87	116	Španěl et al., 1997a
Phenylmethanol	7, 22	91	NC	Wang et al., 2004
2- Hexanone	7, 72, 7375	101	130	Španěl et al., 1997a
Butanal	7, 22, 90, 120, 7375	NR	71	Španěl et al., 1997a
Unidentified	120, 7375	-	135	-
Unidentified	120, 7375	-	144	-

NR: no reaction; for some compounds NO^+ cannot be used to confirm their presence since this precursor ion does not react appreciably with the compound of interest
NC: not confirmed; putative compound has not been confirmed with second precursor
[1,2] Number of hydrates

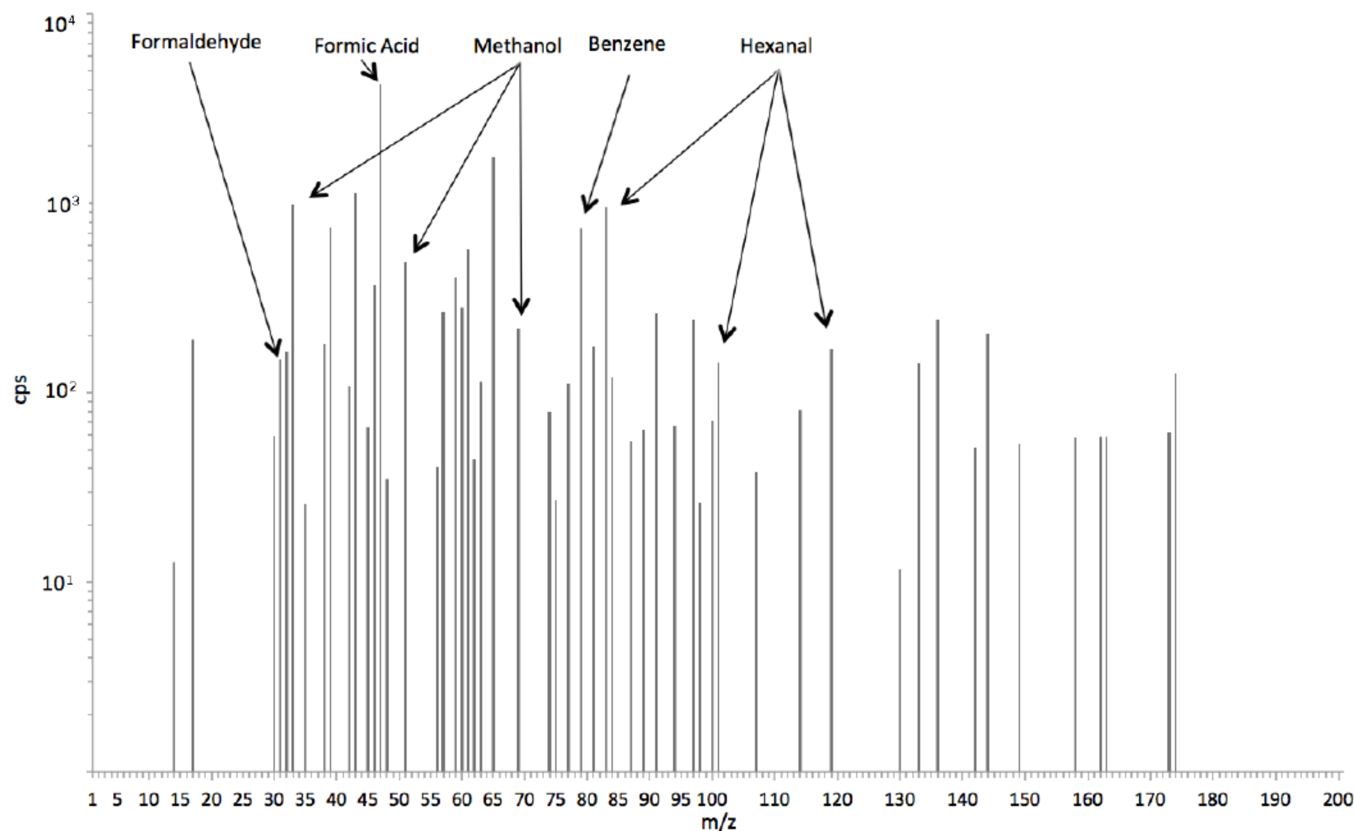

Figure 2.2 Analysis of headspace gas of *A. cylindrospora* grown on lignin supplemented cultures by SIFT-MS. Cultures were grown in 40 mL of media containing 0.3% w/v Kraft lignin and 2% VMS. Fungi were inoculated with 3 fungal plugs from stock plates and grown at room temperature for 3 weeks. The SIFT-MS spectra were generated using H_3O^+ precursor ions showing the mean of 3 measurements from a single culture. Ions produced in a lignin blank flask were subtracted from this data. Product ion *m/z* values consistent with methanol, formaldehyde, formic acid, benzene and hexanal have been indicated

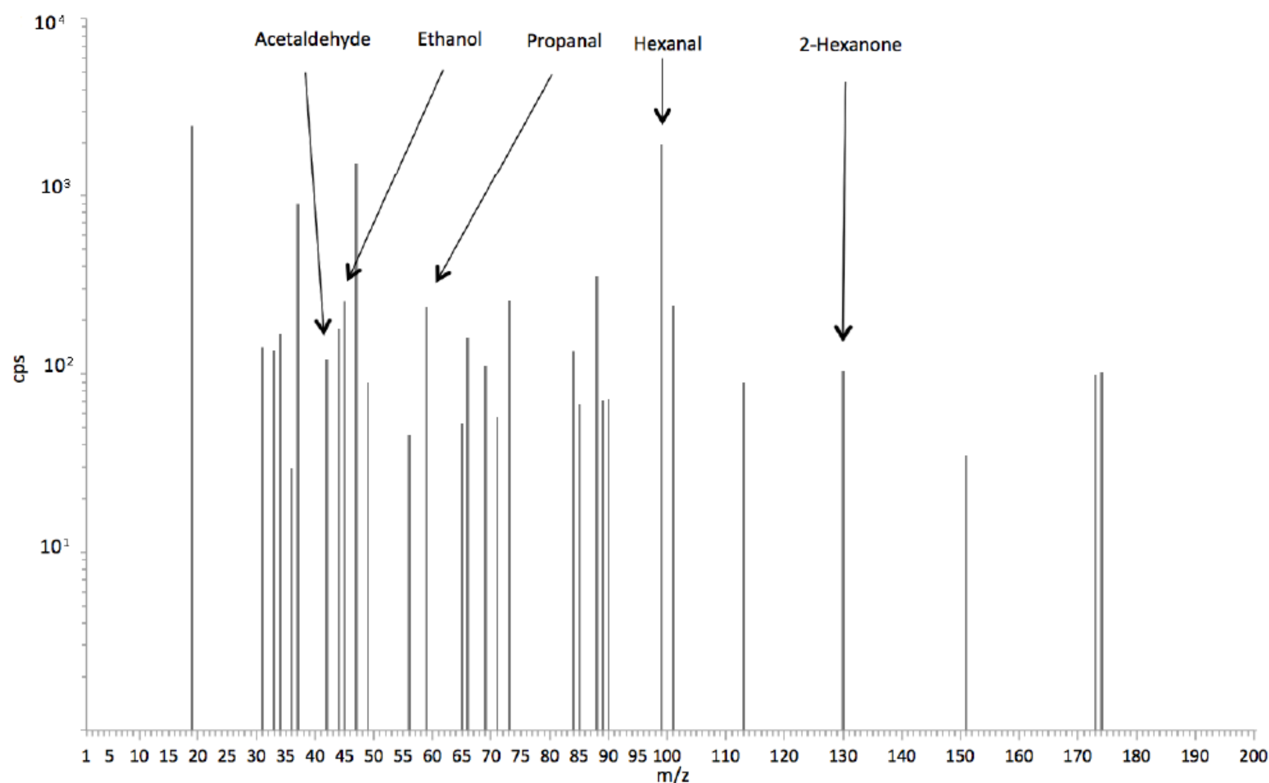

Figure 2.3 Using NO^+ precursor ions, SIFT-MS spectra were generated from headspace gas of *A. cylindrospora* grown on lignin. Cultures were grown in 40 mL of media containing 0.3% w/v Kraft lignin and 2% VMS. Fungi were inoculated with 3 fungal plugs from stock plates and grown at room temperature for 3 weeks. The mean of 3 measurements from a single culture is shown. A lignin blank flask was subtracted from this data. Product ions consistent with acetaldehyde, ethanol, propanol, hexanal and 2-hexanone have been indicated

Table 2.2 Methanol concentration and, formaldehyde and formic acid presence in the headspace of 8 fungal and 1 bacterial species grown on Kraft lignin. Each sample was taken for 30 seconds using the MIM mode of the SIFT-MS, analyzed using the H_3O^+ precursor. Methanol refers to the mean value (in ppb) of the number of samples taken. Subsequent full scans were analyzed for the presence of formaldehyde and formic acid

Species Name	Methanol (ppb)	Standard deviation	Samples	Formaldehyde	Formic Acid
A. cylindrospora	210	26	12	Present	Present
F. fomenarius	116	43	9	Absent	Present
F. velutipes	20	8	9	Present	Present
L. sulphureus	11	1	3	Absent	Present
I. resinosum	1267	47	3	Present	Absent
P. aurivella	352	69	9	Present	Present
O. leucospongia	78	5	3	Present	Absent
C. camelliae	76	8	12	Absent	Present
SYK-6	139	25	12	Present	Absent
Blank media control	74	2	12	Absent	Absent
Ambient*	21	9	4	Absent	Absent

* Ambient level samples were taken from the lab air at the time of each experiment, normalized without the control flask subtracted

2.5 Discussion

2.5.1 Culture headspace spectra using FS mode

Previous studies suggest that the microbial degradation of lignin results in methanol formation (Ander & Eriksson, 1985). Therefore, the headspace of the fungal and bacterial cultures growing on lignin, were examined as a source of methanol and potentially other volatile compounds. Using the FS mode of the SIFT-MS, many common compounds including ammonia, acetaldehyde, acetone and acetic acid, were detected in the headspace of the 9 fungal species growing on lignin liquid cultures (Table 2.1). The slower growing species had fewer product ion counts exceeding the control flask indicating that Kraft lignin was used by these species less effectively as a nutrient.

Several compounds were more abundant in the headspace of actively growing species (*A. cylindrospora*, *F. velutipes*, *I. lacteus*, *C. camelliae* and *G. trabeum*) (Table 2.1). Ammonia was found in species *P. aurivella*, *L. montanus* sp. 1, *L. montanus* sp. 2, *C. camelliae* and *G. trabeum* in levels higher than the control flask and all three characteristic product ions of ammonia (*m/z* 18, 36 and 54) were found in the headspace. The cultures appeared to have released nitrogen. This was surprising because a major limitation for the fungal degradation of lignin in nature is the availability of nitrogen and fungal species have adopted an intricate method of recycling nitrogen where nitrogen from older, disused parts of the mycelium is not released, rather it is translocated to growing hyphae (Dix & Webster, 1995). Therefore, one would not expect to find volatile nitrogenous compounds as a product in the headspace. The source of the ammonia was

likely a result of the fungal metabolism of excess VMS present in the fungal media (which contains NH_4NO_3 and $Fe(NH_4)_2(SO_4)_2$).

Methanol was detected in cultures of only 3 of the species investigated (*A. cylindrospora, I. lacteus* and *P. aurivella*) , using the FS mode. Some cultures were found to have product ion counts less than that of the medium control, while others did not have all of the necessary product ions for methanol identification. Methanol would be an expected metabolite of lignin degradation as lignin consists of roughly 10% methoxyl groups (El Mansouri & Salvado, 2006). A likely explanation for the lack of methanol detected is that it was rapidly metabolized. In that case, one would expect to find metabolism products, such as formaldehyde, formic acid and CO_2 (Ander & Eriksson, 1985).

Both formaldehyde and formic acid were detected using the SIFT-MS (Table 2.2). CO_2 is difficult to quantify using SIFT-MS due to its natural abundance. Formaldehyde was found in a few select cultures (Table 2.2). Formaldehyde, due to its toxicity, would also likely be metabolized quickly by any culture producing it and found at low concentrations (Mitsui et al., 2003). Formic acid product ions (*m/z* 47) were found in 7 species using H_3O^+ spectra. However, *m/z* 47 is shared with ethanol, which was confirmed using both H_3O^+ (*m/z* 47, 65 and 83) and NO^+ (*m/z* 45) spectra. Unfortunately, the NO^+ precursor does not react with formic acid, making it difficult to conclude if the product ions present are a result of both formic acid and ethanol or only one of these (Španěl & Smith, 1998b). Only 4 of the 7 species apparently producing formic acid had all of the

product ions for ethanol. Therefore it is reasonable that fungal species 90, 116 and 120 did in fact have formic acid present in the headspace.

Acetaldehyde was detected in the cultures of species *A. cylindrospora*, *F. velutipes* and *P. aurivella* and confirmed with both H_3O^+ and NO^+ spectra (Table 2.1). This compound is quite common and can result from the partial oxidation of ethanol (Sauer & Ollis, 1996). Acetic acid was also detected in the same cultures and is often formed from the oxidation of acetaldehyde (Venugopal et al., 1967). Acetic acid identity was confirmed with both H_3O^+ and NO^+ spectra (Table 2.1).

Several other major products were identified in the headspace of *A. cylindrospora* cultures that proved to be quite interesting. Propanol was identified using the H_3O^+ spectrum with all 4 hydrates present (*m/z* 43, 61, 79 and 97), and confirmed using the NO^+ spectrum with *m/z* 59 ion present. Propanol and its formation from the degradation of lignin has not been investigated previously, although the 3-carbon propanoid unit is highly abundant in the monolignols that constitute lignin coniferyl alcohol (guaiacyl propanol) and synapyl alcohol (syringyl propanol). Pentanal was also identified in the headspace using both H_3O^+ (*m/z* 69 and 87) and NO^+ (*m/z* 85) spectra. Previously, pentanal has been found to be released from wood piles (Otwell et al., 2000), but has not been associated with the fungal degradation of lignin.

Kraft lignin is a complex mixture of lignin and lignin fragments and is degraded by fungal and bacterial species using an assortment of pathways and a variety of enzyme

systems (Kirk & Farrell, 1987). Using the SIFT-MS, many product ions were identified related to the variety of lignin fragments generated during its degradation. The complex mixture of products present in the headspace including benzene, hexanal, phenyl methanol, 2-hexanone and 2-butanal can be visualized with *A. cylindrospora,* as an example, in Figures 2.2 and 2.3. Benzene, for example, is rarely produced during microbial metabolism and maybe an intermediate in the degradation of lignin. Several products ions, not included in the existing SIFT-MS library, require further investigation. As the SIFT-MS library continues to grow, more of these volatile lignin degradation products will be identified.

The mass spectrum of compounds under *m/z* 200 for the bacterium SYK-6 proved to have fewer product ions than that for the fungal species. SYK-6 had no observable growth using lignin as a sole carbon source, which agrees with previous observations (Masai et al., 2007). When the control flask headspace values were subtracted from the mass spectrum of SYK-6, most of the product ions were eliminated as background (data not shown).

2.5.2 Measurement of methanol and formaldehyde in culture headspace using MIM mode

In order to investigate the formation of methanol in lignin metabolism, the MIM mode of the SIFT-MS was used. Some fungal species increased the concentration of methanol in the headspace (*A. cylindrospora, I. resinosum* and *P. aurivella*) while others decreased the concentration below ambient levels (*F. velutipes* and *L. sulphureus*) (Table

2.2). Our study confirms that methanol was released by some species during lignin degradation. The resulting methoxyl groups may be an important source of C1 compounds for these fungi, as found in bacterial metabolism (Mitsui et al., 2003; Sonoki et al., 2009). It has been established that formaldehyde-fixing systems usually found in methylotrophic organisms play critical physiological roles in the degradation of lignin monomers in non-methylotrophic organisms (Mitsui et al., 2003). Using ^{14}C lignin or phenyl propanoid compound as substrate would help measure the kinetics of metabolic conversions using the SIFT-MS.

Previous studies have found that methanol is consumed by some fungi during lignin degradation, generating formaldehyde followed by further oxidation to formic acid and CO_2 (Ander & Eriksson, 1985). A species that demethylates lignin may release formaldehyde through a reaction catalyzed by a demethylase enzyme. The SIFT-MS was used to measure these reaction intermediates to see if they were present in the headspace. Traces of formaldehyde and formic acid were putatively detected in all fungal cultures with the exception of *O. leucospongia* and *C. camelliae*. Formaldehyde and formic acid were most prevalent in the cultures of species with less than ambient methanol concentrations. This would suggest that the accumulation of methanol does not occur in these species as it was further oxidized. Formaldehyde is present in most of the cultures but at low concentrations, with the exception of *P. aurivella* (Table 2.2). This is not surprising due to the toxicity of this compound at higher concentrations. Formic acid, found in many of the cultures tested, is likely the product of the oxidation of formaldehyde and is probably subsequently oxidized to CO_2.

2.6 Conclusions

Our results show that SIFT-MS can be used to detect and quantify methanol and other volatile compounds in headspace of microbial cultures growing on lignin as a substrate. With an increasing knowledge of lignin and the role microbial organisms play in the breakdown of this complex polymer, more information needs to be gathered to elucidate the mechanism of lignin degradative pathways. Some of the identified compounds in the FS mode, such as benzene, hexanal, phenyl methanol, 2-hexanone and 2-butanal, would not have been expected to be present in the culture headspace. These compounds may originate from the metabolism of lignin ring structures. As well, there were compounds that remain unidentified at this point. Further research is needed to confirm the presence and identity of these compounds, and to elucidate the biochemistry of their formation.

3. Adaptation of Ti(III)-NTA colorimetric assay for use in detecting microbial demethylation of lignin and lignin derived compounds in aerobic conditions

Published as:

Gibson, A. [1,2 *], Dekker, R.F.H. [1] and Malek, L.[1,2] 2014. Adaptation of Ti(III)-NTA colorimetric assay for use in detecting microbial demethylation activity of lignin and lignin derived compounds in aerobic conditions. Journal of Microbiological Methods. **101**: 28-32.

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Author Contribution

Gibson, A.: completed all writing and experimental

Dekker, R.F.H. and Malek, L.: critical evaluation of design and interpretation of data; editing; final approval before publishing

3.1 Abstract

An anaerobic colorimetric assay previously used to quantify demethylation activity during the microbial utilization of phenyl methyl ethers was adapted for aerobic use in the study of lignin and lignin derived compounds. Standard curves were prepared with 0-500 μM pyrocatechol in the absence or presence of 0.3% lignin and the assay was found to work in either case. Lignin, at concentrations over 0.3%, interfered with the assay. Lignin and chemically demethylated lignin were compared using the assay and found to result in a difference of about 1 AU. This method appears suitable for detecting initial demethylation products up to 500 μM without using additional dilutions and provides a rapid, reproducible, low cost approach for studying demethylation activity.

3.2 Introduction

Lignin is an abundant, renewable carbon source derived from wood (Zhu et al., 2011; Schmidt & Czeschlik, 2006; de Jong et al., 1994; Boerjan et al., 2003). It is a complex heterogeneous aromatic polymer consisting of phenylpropanoid units linked together by ether and carbon-carbon bonds (Sjöström, 1993). A number of models exist, but the structure of lignin is so complex it has not yet been completely described (Fengel & Wegener, 1984). The monomers that primarily make up the guaiacyl (G) and syringyl (S) lignin units found in angiosperms and gymnosperms are methylated (Fig. 3.1). The G unit is singly methylated on the 3-hydroxyl group, whereas the S subunit is methylated on both the 3- and 5-hydroxyl moieties.

The abundance of lignin produced in the paper-making process and the alluring potential of its commercial applications has continued to fuel an interest in lignin

degradation research and lignin-derived products for the past several decades (Lora & Glasser, 2002). Two potential products: a modified lignin with a higher phenolic content and biomethanol are of particular interest in respect to Kraft lignin. More than half of the potentially reactive aromatic hydroxyl groups in Kraft lignin are blocked by methyl groups. If these methyl groups could be removed, then the resulting material would have more free phenolic groups and be more reactive (Okamoto et al., 1996). Industrial applications may include use as a phenol substitute in phenol formaldehyde resins (Stewart, 2008).

I am interested in using a microorganism-driven modification of lignin (Higuchi, 1985; Kasai et al., 2004; Jin et al., 1990b). But so far, a rapid, cost-effective method for the detection of this enzymatic activity is lacking. Previous studies of demethylation reactions have relied on high-performance liquid chromatography (HPLC), gas chromatography with flame ionization detector (GC-FID), proton nuclear magnetic resonance (^{1}H NMR) and Fourier transform infared spectroscopy (FTIR) to quantify and detect the transformation of monolignols (Kasai et al., 2004; Girardin & Metche, 1983; Santos Abreu & Inacio Friere, 1995; Ferhan et al., 2013). This is time intensive and costly. In this study, we explored an anaerobic colorimetric assay based on titanium (III) nitrilotriacetic acid [Ti(III)-NTA] and adapted it for aerobic use.

Ti(III)-NTA has been used to quantify microbial anaerobic demethylation of phenyl methyl ethers (Kreft and Schink, 1993). Originally this method was developed as an improvement to an anaerobic media reductant, Ti(III)-citrate (Zehnder & Wuhrmann,

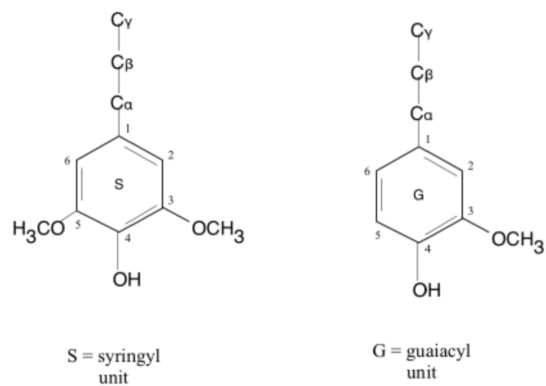

Figure 3.1 Syringyl (S) lignin unit with methyl groups on the 3- and 5-hydroxyl moieties and guaiacyl (G) lignin units with a single methyl group on the 3-hydroxyl group (based on Nurchi et al., 2007)

1976). After several attempts to use this method, Moench and Zeikus in 1983 replaced citrate with NTA (Moench & Zeikus, 1983). Ti(III)-NTA media reductant was then adapted to be used as an assay for vicinal diol detection and quantification in anaerobic cultures and samples (Liu, 1997). Here we report a further adaptation of the assay to detect fungal and bacterial demethylation aerobically, providing a useful, simple and cost-effective screening tool in the search for fungal and bacterial lignin modifying enzymes.

The Ti(III)-NTA assay is able to directly detect demethylation activity by measuring the yellow colour produced when Ti(III)-NTA complexes with vicinal diols (Fig. 3.2, based on ref. (Liu, 1997). In previous studies, the complex formed was stable between 25-100 hrs depending on the substrate (Liu, 1997). Compared to other phenyl methyl ethers, the Ti(III)-NTA assay has the highest sensitivity towards the vicinal diols vanillin and 3,4-dihydroxybenzoic acid (protocatechuate or PCA), with PCA detected in as low concentrations as 10 μM (Liu, 1997). Lignin is very complex and variable and there may be a mixture of methylated and demethylated groups in any given sample. Lignin also provides other challenges, having a high UV light absorbance, varying composition, and difficulty in separating it from protein. I used vanillin as a substrate, simplifying the system and making it easier to detect the demethylation. Also, I investigated the application of this assay aerobically and with Kraft lignin present in the sample. Previous studies using this assay have focused on anaerobic bacterial cultures alone (Kreft & Schink, 1993; Liu, 1997). I developed the assay for use with aerobic fungal cultures, potentially providing a comparison of fungal and bacterial screening.

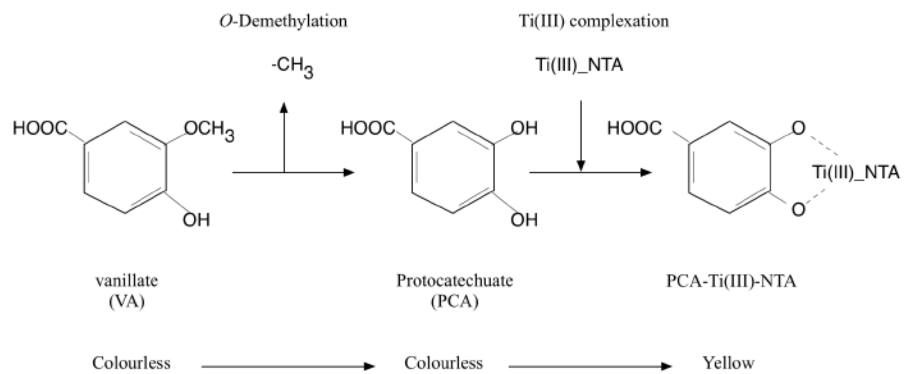

Figure 3.2 Reaction scheme for demethylation and complexation of Ti(III) with vicinal diols (Liu, 1997)

3.3 Materials and Methods

3.3.1 Ti(III)-NTA assay

Ti(III)-NTA solution was prepared based on the method of Moench and Zeikus (1983). A 167 mM NTA solution was prepared using ddH_2O (rather than anaerobic water) and adjusted to pH 9.0 using concentrated NaOH. I then added 0.2688 mL of 12% Ti(III)-Cl to 6 mL of NTA solution. While adding the Ti(III)-Cl, the mixture was kept above pH 2.0 using saturated Na_2CO_3 to avoid precipitation, and the pH of the solution was adjusted to 7.0. The final solution was made up to 10 mL with ddH_2O resulting in a concentration of 25 mM of Ti(III) and 100 mM NTA. The final solution was deep purple and used within thirty minutes.

Vicinal diols were quantified by diluting 100 µL of sample with 800 µL of 50 mM HEPES pH 8.0. One vicinal diol was considered to be equal to one molecule of vanillin or PCA (see Fig. 2). If the sample was too concentrated, a smaller sample volume was used, made up to 100 µL with HEPES buffer to keep the concentration of vicinal diols between 100 µM and 500 µM. A blank was used, using 900 µL of buffer. Each sample was used to zero the spectrophotometer before adding the Ti(III)-NTA reagent. To the 900 µL of sample, 100 µL of Ti(III)-NTA solution was added, giving a final volume of 1 mL. Thirty minutes after addition, the sample absorbance was measured at 380 nm.

3.3.2 Chemically demethylated Kraft lignin

Fresh lignin was chemically demethylated using a method developed by Adler and Hernestam (1955). Kraft lignin at a concentration of 0.3% w/v was suspended in 50 mL water, treated with 50 mL of 0.14 M sodium periodate and acidified with 500 µL of 12N HCl for 24 hr. The acidification was performed with HCl due to SIFT-MS interference from the acetic acid used in the original method (Adler & Hernestam, 1955).

3.3.3 Fungal and bacterial strains

Absidia cylindrospora and *Fomes fomentarius* were used in this study as examples of fungal lignin degraders. The cultures were inoculated by adding 3 fungal plugs from a culture grown on potato dextrose agar plate. The cultures were then grown for 2 weeks at room temperature. Initially, the cultures were grown on VMS supplemented with 1% glucose and at the beginning of the second week, the cultures were induced with vanillin. At the end of the second week, medium with modified substrate was collected by removing the mycelium and filtering through a 20 µm pore nylon sheet.

Fungal lignin medium was made by dissolving 6 g of Kraft lignin into 8 mL of 50% NaOH and 40 mL of ddH_2O for 1 hour. This 12.5% dissolved lignin stock solution was added to fungal cultures to a final volume of 0.3% w/v.

Sphingobium sp. SYK-6 was grown on minimal W-medium using 1 mM vanillin as a carbon source (Peng et al., 1998). A liquid stock of SYK-6 was grown to OD 1 on

nutrient broth media and cultures were inoculated with a sterile loop. Cultures were grown for 5 days at room temperature before vicinal diol analysis.

3.3.4 Reagents and chemicals

All chemicals were of analytical grade and were obtained from Sigma-Aldrich with the exception of 3,4 dihydroxybenzonic acid (PCA), Indofine Chemical Company, Hillsborough, NJ. Kraft lignin extracted by acid precipitation was obtained from FPInnovations, Pointe Claire (Quebec).

3.4 Results

The reaction between saturating amounts of Ti(III)-NTA and increasing concentrations of pyrocatechol (up to 500 μM) led to a linear increase in absorbance at 380 nm (Fig. 3.3, diamonds). Lignin at 0.3% did not affect the linearity of the relationship as vicinal diols could still be quantified accurately (Fig. 3.3, squares).

Absorbance maxima of the aerobic reaction product were at 380 nm for pyrocatechol concentrations of 300 μM and higher (Fig. 3.4a), but were not as defined at lower concentrations. Lignin at 0.3% with absorbance maximum at 280 nm interfered only slightly at 380 nm, increasing the absorbance reading by about 2% at 300 μM pyrocatechol (Fig. 3.4b). Without the Ti(III)-NTA reagent, the background absorbance of lignin is less than 0.1 AU at 380 nm (data not shown). The background absorbance of lignin at 380 nm was subtracted by blanking the spectrophotometer with the sample, as mentioned in the Methods section.

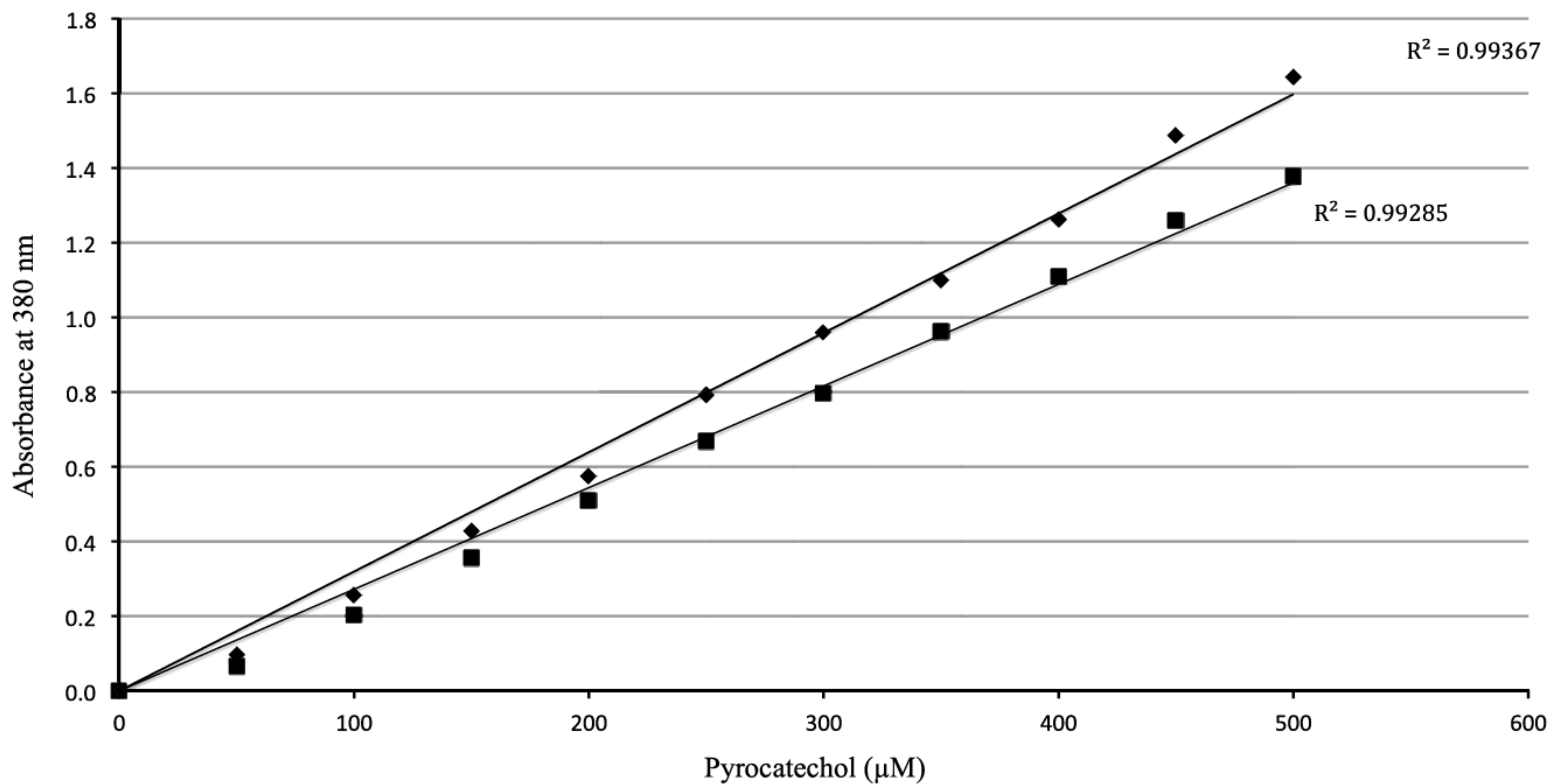

Figure 3.3 Pyrocatechol standard curve at concentrations ranging from 0-500 μM using the Ti(III)-NTA assay. Shown is pyrocatechol reaction product in buffer (squares) and in the presence of 0.3% lignin (diamonds)

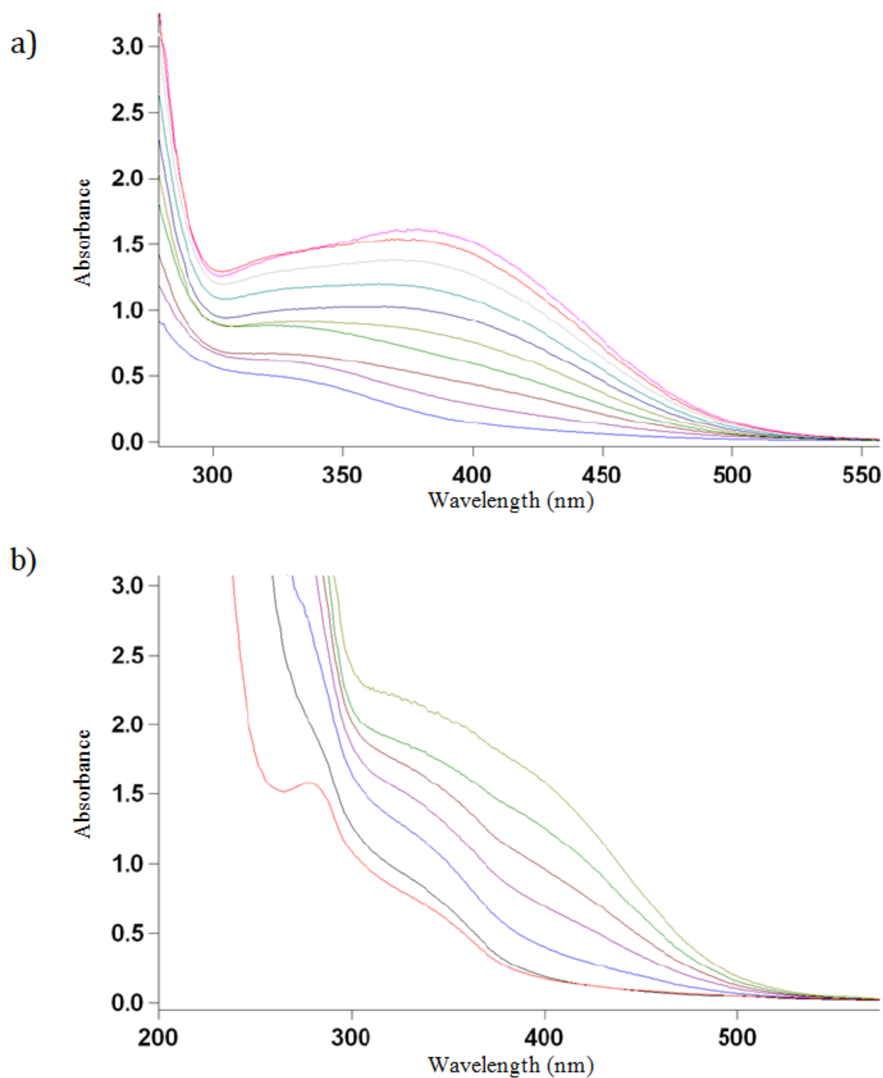

Figure 3.4 (a) Absorbance scans (300-550 nm) of reaction products at concentrations ranging from 0-450 µM of pyrocatechol (in 50 µM increments from bottom to top) in buffer detected with Ti(III)-NTA assay. (b) Absorbance scan (200-500 nm) of reaction products at concentrations of 0, 50, 100, 200, 300, 400 and 500 µM in buffer with 0.3% Kraft lignin

Potential substrate interference was investigated by developing three standard curves of the reaction between PCA and Ti(III)-NTA (Fig. 3.5). Excess vanillin, at twice the highest concentration of PCA used, had no significant effect on the intensity of absorbance by the reaction product (Fig. 3.5, squares). Similarly, adding reciprocal quantities of the substrate to the product (Fig. 3.5, triangles) had no effect. Slight differences in the maximum values obtained with 500 μM PCA were noted between experiments: 1.2 control-diamonds in Fig. 3.5, 1.0 vanillin treatments - triangles and squares in Fig. 3.5; and 1.4 or 1.6 in Fig. 3.3.

The difference in vicinal diol content between Kraft lignin and chemically demethylated lignin was about 1 AU (Fig. 3.6a). The largest difference was observed using 0.1 mL of 0.3% w/v Kraft lignin. The potential difference using the same assay and lignin prepared for use in fungal medium (see method) was also approximately 1 AU (Fig. 3.6b). The solvent used in preparing the 0.3% w/v lignin media (demethylated or not) did not make a statistically significant difference in the obtained absorbance readings (Fig. 3.6a vs. 3.6b).

A. cylindrospora, *F. fomentarius* and SYK-6 were grown on vanillin and tested for vicinal diol generation using the Ti(III)-NTA assay (Fig. 3.7). Total Ti(III)-NTA activity was determined by multiplying the dilution factor by the absorbance of the sample at 380 nm. *A. cylindrospora* demonstrated the most demethylation over three weeks while SYK-6 showed the least, over 5 days of growth. *F. fomentarius* showed less

of an increase in vicinal diols over four weeks than *A. cylindrospora* over three weeks and also grew slower.

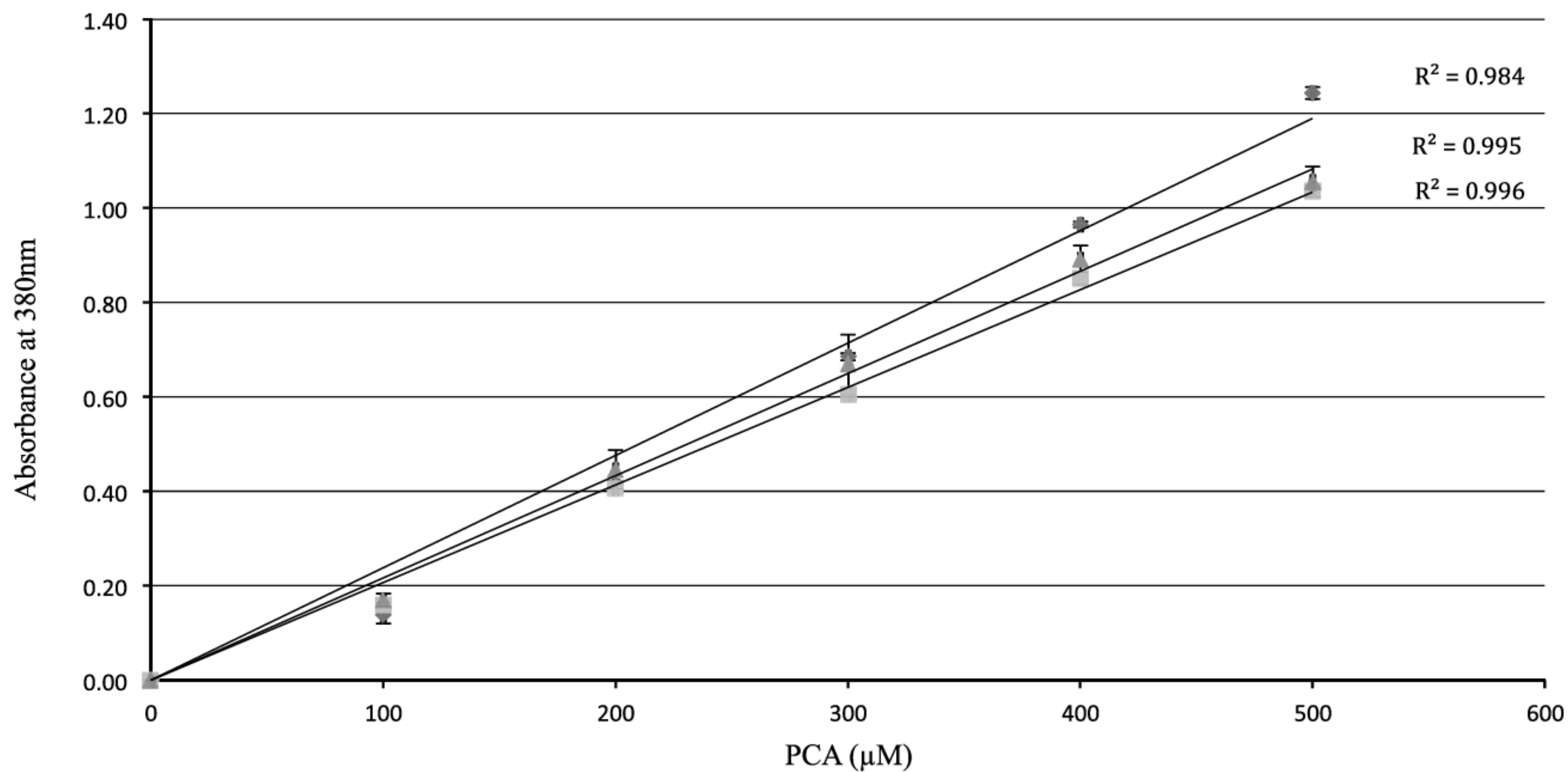

Figure 3.5 Standard curves obtained by analyzing 0-500 µM concentrations of PCA with the Ti(III)-NTA assay. Standard curve without vanillin (diamonds), with constant amount of 1 mM vanillin (squares) and reciprocal amounts of vanillin (i.e. 100 µM PCA and 400 µM vanillin, etc.) (triangles)

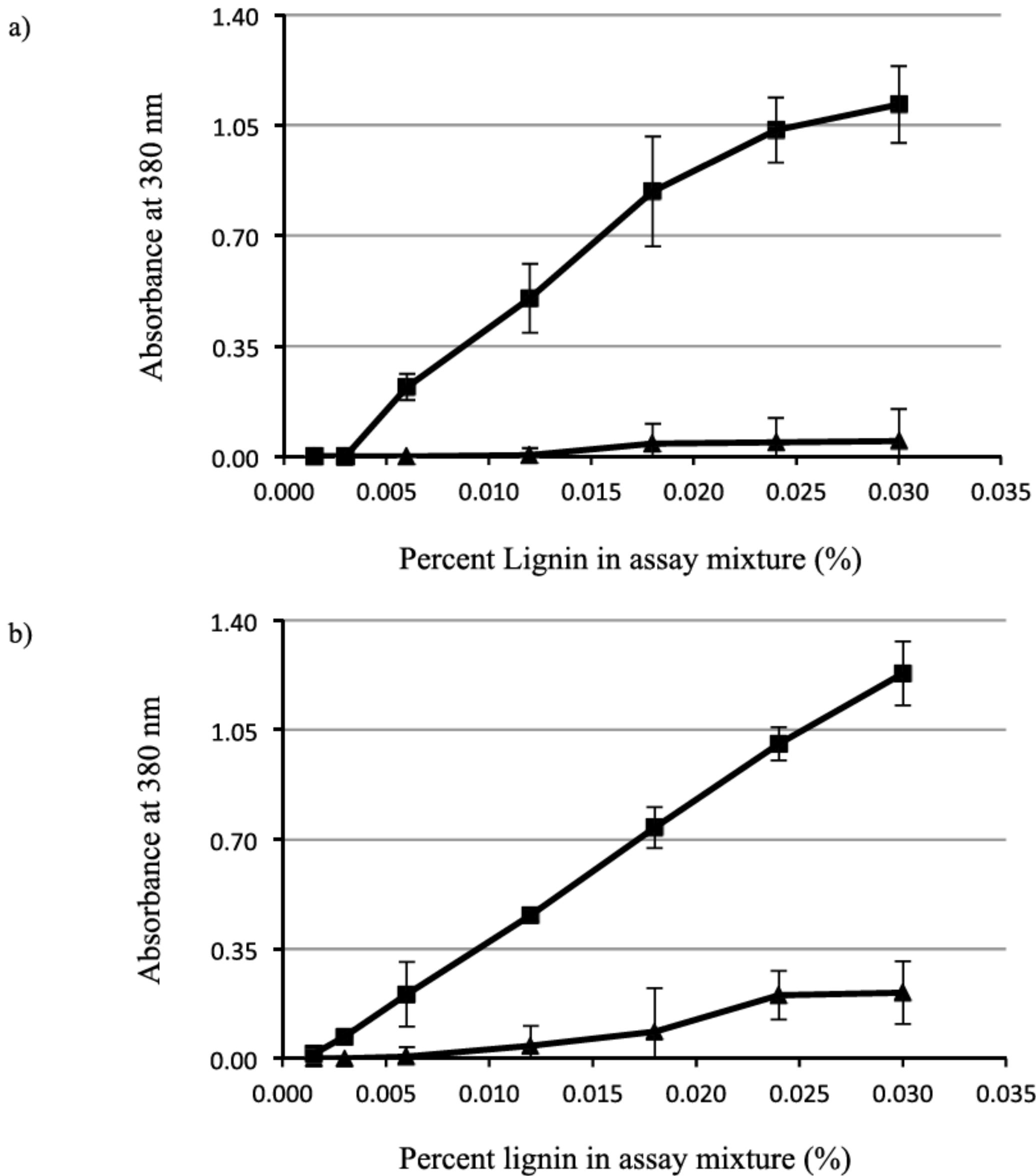

Figure 3.6 (a) The absorbance difference at 380 nm between chemically demethylated lignin dissolved in buffer (squares) and Kraft lignin dissolved in buffer (triangles) following the Ti(III)-NTA assay. (b) The difference in absorption between chemically demethylated lignin dissolved in buffer (squares) and Kraft lignin dissolved in buffer (triangles) made in fresh fungal media. Error bars represent standard deviation from two experiments run in triplicate

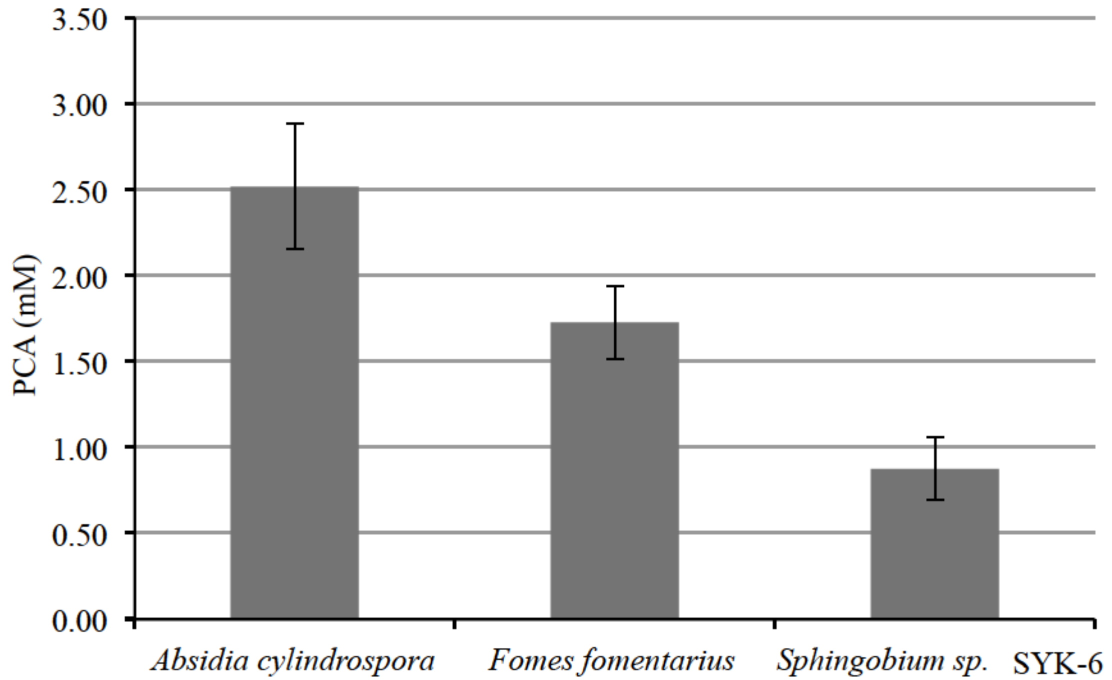

Figure 3.7 The amount of PCA generated by fungal cultures *A. cylindrospora* (3 week culture) and *F. fomentarius* (4 week culture) from 12 mM vanillin supplemented with 1% glucose. Fungal cultures were grown the first week only on glucose. SYK6 was grown for 5 days on W-medium supplemented with 1 mM vanillin

3.5 Discussion

I found that the optimal range for measuring pyrocatechol and PCA in aerobic conditions was between 100 μM and 500 μM (Fig. 3.3). This is in contrast to previous studies, using anaerobic conditions, where a detection limit as low as 10 μM of vicinal hydroxyl groups was observed (Liu, 1997). The 50-100 μM values in figures 3 and 5 fall below the calculated regression line extrapolated to 0, suggesting lower reliability of the assay in this concentration range. When using Ti(III)-NTA reagent in the presence of oxygen, oxidation will occur. The oxidation results in the pH of the solution decreasing, as well as the color of the reagent changing from a deep to a faint purple and eventually to clear. With time, each reagent solution will be differentially oxidized, therefore a standard curve of known concentrations was made before each experiment and the reagent solution was prepared fresh. This accounts for the slight differences between experiments presented in Fig. 3.3 and 3.5.

Previous studies have used monolignols in Ti(III)-NTA reactions (Kreft & Schink, 1993; Liu, 1997). Monolignols such as pyrocatechol, PCA and vanillin are not as chemically complex as lignin and do not usually have an initial colour. I investigated the use of Ti(III)-NTA in the presence of polymeric lignin. Lignin absorbs strongly near 280 nm and may cause interference at other close wavelengths. For this reason, lignin can be problematic in assays relying on colorimetric reactions (Arora & Gill, 2001). Known concentrations of a compound containing a vicinal diol (pyrocatechol) were analyzed with and without lignin (Fig. 3.3). The assay was capable of detecting vicinal diols in both cases with equal sensitivity at low concentration of lignin. At concentrations of

lignin higher than 0.3% w/v, the interference reduced the effectiveness of the assay (data not shown). The main peak in a scan of lignin (280 nm) was shown to interfere with the reading wavelength (380 nm) for the Ti(III)-NTA assay (Fig. 3.4b). The maximum useable concentration of lignin as a substrate for bacterial and fungal species for the Ti(III)-NTA assay was found to be 0.3% w/v. This resulted in about a 10% increase in readings at 380 nm at higher concentrations of pyrocatechol (Fig. 3.4a vs. b, and Fig. 3.3).

Substrate interference was also investigated by adding vanillin to the reaction. A concentration range of 0-500 μM PCA was analyzed using the Ti(III)-NTA assay without vanillin, with 1mM vanillin and with reciprocal amounts of PCA and vanillin (Fig. 3.5). The standard curves with and without vanillin were not significantly different, so interference from the substrate is not likely. However, vanillin is prone to oxidation in air, particularly if heated and in light (Kumar et al., 2012). Therefore, vanillin may cause positive readings if it has been oxidized. Time course experiments using vanillin as substrate and fungal enzyme treatments could potentially be affected by non-biological vanillin oxidation.

Lignin samples with added demethylated lignin monomers may not accurately mimic lignin being degraded by fungal or bacterial enzymes. A method developed in previous research used a sodium periodate solution to chemically strip industrial Kraft lignin of its methyl groups (Adler & Hernestam, 1955). By comparing Kraft lignin to fully demethylated Kraft lignin, using the Ti(III)-NTA assay, the relative difference in

vicinal groups can be estimated. Since Kraft lignin from different sources may be highly variable, vicinal groups are likely to be present in the sample prior to fungal or bacterial modification. Therefore, appropriate lignin blanks were used to account for vicinal diols already present in the fresh media.

It is possible that the method used to chemically demethylate the Kraft lignin might also cleave some of the linkages in lignin (Adler & Hernestam, 1955). However, studies have shown that sodium periodate treatment of lignin leaves the majority of the linkages unchanged and is still structurally representative of the original lignin sample (Cousins, 1976; Meshitsuka & Isogai, 1996).

Fresh lignin medium was also tested. This contained lignin dissolved in NaOH and VMS salts. The results of Ti(III)-NTA assay were not significantly different from those obtained for lignin in buffer (Figs. 3.6a and b, triangles) Demethylated lignin medium was also prepared for the purpose of testing the assay and the relative number of vicinal groups for demethylated lignin in buffer versus fungal media was also found to be the same (Fig. 3.6a and b, squares). Using 0.3% lignin, the difference between Kraft lignin and fully demethylated Kraft lignin is about 1 AU (Fig. 3.6). The exact value for this difference will depend on the specific lignin sample (origin, processing, etc.) and can be very useful in determining what relative percentage of the lignin sample was demethylated by fungal or bacterial enzymes. Absolute demethylation values for lignin may be difficult to determine due to the three dimensional structure of the polymer. The

Ti(III)-NTA reagent may have limited access to some vicinal -OH groups hidden within the polymeric network.

Finally, the Ti(III)-NTA assay was used to assess the demethylation of lignin by fungal and bacterial cultures (Fig. 3.7). The fungal species tested were able to demethylate both intact Kraft lignin (not shown) and monolignols. *A. cylindrospora* was able to demethylate vanillin substantially over the two week incubation. The slower growing *F. fomentarius* was also tested for the demethylation of vanillin, but was less effective than *Absidia cylindrospora*. *F. fomentarius* produced about 10% of the biomass compared to that produced by *A. cylindrospora* in the same growth period (data not shown). SYK-6 appeared to demethylate vanillin more than the fungi, even though the incubation time was shorter (5 days).

In summary, I demonstrated that the relatively straightforward Ti(III)-NTA assay will be a useful and economical method for rapidly determining the relative degree of lignin demethylation by cultures of microorganisms and their enzymes.

4 The effects of changing growth conditions on the demethylation of lignin-related compound by *Absidia cylindrospora*, *Fomes fomentarius*, *Ischnoderma resinosum* and *Cylindorcladium camelliae*

Published as: Gibson, A.[1,2 *], Grochowski, J.[2], Dekker, R.F.H.[1] and Malek, L.[1,2] 2013. The effects of changing growth conditions on the demethylation of a lignin-related compound by *Absidia cylindrospora*, *Fomes fomentarius*, *Ischnoderma resinosum* and *Cylindrocladium camellia.* Research Journal of Biotechnology. **8**(12): 42-48.

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Gibson, A.: completed all writing and experimental

Grochowski, J.: assisted with media preparation and data analysis

Dekker, R.F.H. and Malek, L.: critical evaluation of design and interpretation of data; editing; final approval before publishing

4.1 Abstract

Culture parameters influencing the demethylation of the lignin-related compound para-vanillin in defined media were examined in culture flasks of wood degrading *Absidia cylindrospora, Fomes fomentarius, Ischnoderma resinosum and Cylindrocladium camelliae* with respect to methanol release, vicinal diol generation and protein content. Cultures were grown in 40 mL media containing 2% VMS, 1% glucose and demethylation activity induced with 1 mL of 400 mM para-vanillin. Study of the effect of incubation and induction time indicating longer incubation length did not significantly affect demethylation. Vanillin inhibited fungal growth. Culture agitation at 81 rpm increased methanol released in *A. cylindrospora* and *F. fomentarius* by 27% and 87% respectively. The concentration of vicinal diols, however, decreased in *A. cylindrospora* by 53% with 0-3 mM of hydrogen peroxide added, indicating hydrogen peroxide may have acted as a mediator in vanillin degradation. Changes in medium composition with respect to metals had six-fold decrease in vicinal diol concentration for *F. fomentarius* with 0-2 μM final concentration of copper and 166% increase in methanol concentration for *C. camelliae* with 0-0.5 μM final concentration of iron. Control of culture conditions is essential for optimal ligninolytic activity and will facilitate finding more reactive lignin useful in future industrial applications.

4.2 Introduction

Lignin is a complex aromatic biomacromolecule found in vascular terrestrial plants and is second only to cellulose as the most abundant biological material on earth (Zhu et al., 2011). It is estimated that 30% of all organic carbon in biomass is contained

in lignin (Boerjan et al., 2003). The degradation of lignin is therefore indispensable to the global carbon cycle (Wong, 2009). Lignin is a major component of wood and is found in the lignocellulosic material where it binds plant cellulose and hemi-cellulose like a glue in the cell wall (Schmidt & Czeschlik, 2006). The role of lignin is to give structural rigidity, help in the transport of water and nutrients, as well as protect the plant from microbial attack (de Jong et al., 1994). The structure of lignin is so complex, it has not yet been completely described (Fengel & Wegener, 1984).

Lignin is made of 3 monomers: coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol (Fig. 4.1) (Wong, 2009). These monomer structures consist of the same phenylpropanoid skeleton, but differ in the substitution of oxygen on the phenyl ring (Doherty et al., 2011). Within the lignin molecule, the monomers vary in abundance depending on plant species and even location within the plant (Wong, 2009). The phenylpropanoid units that primarily comprise lignin in soft and hardwoods are syringyl (corresponding to sinapyl alcohol) and guaiacyl (corresponding to coniferyl alcohol) units, based on the methoxy substitution on the aromatic rings.

Lignin has been extensively researched as a source of industrial feedstocks and phenolic products due to its high availability and the tremendous energy content from its aromatic subunits (Zhu et al., 2011). Despite all the potential applications of lignin, relatively few applications have been implemented on an industrial scale. Currently only 1-2% of lignin is used to make other products (Lora & Glasser, 2002). Industrial processes have not become widespread due to its natural variability, inhomogeneity and

the difficulty with lignin separation methods used in the production of pure chemicals (Leisola et al., 2012). Additional industrial uses may come from the modification of lignin, where two potential products, a modified lignin with a higher phenolic content and biomethanol are of particular interest. Lignin has a low reactivity due to its structure, because the ether linkages contained within that structure occupy most of the reactive sites on the lignin molecule. In addition, more than half of the potentially reactive aromatic hydroxyl groups are methylated, inhibiting any reactions with those groups. If these groups were demethylated, then the resulting material would have more free phenolic groups and, therefore, be more reactive (Okamoto et al., 1996). Lignin with a higher phenolic content would be useful in industrial applications as a phenol substitute in phenol formaldehyde resins (Stewart, 2008).

A possible means of modifying lignin for polymer applications is to consider the use of enzymes. Even though lignin is highly resistant to degradation, there are a number of microorganisms, including fungi, capable of breaking down the complex compound (Higuchi, 1985). In nature the breakdown of lignin is thought to be initiated by phenol oxidases secreted by Basidiomycota white-rot species, using enzymes such as lignin peroxidase, manganese peroxidase and laccase (Kasai et al., 2004). Brown rot fungi, soft rot fungi and bacteria in soil and water contribute by modifying or breaking down the lignin and lignin-derived products (Fengel & Wegener, 1984).

Demethylation reactions, which are part of the principal chemical modification of lignin, take place on the syringyl and guaiacyl building blocks that primarily make up

hardwood and softwood lignin (Fig. 4.1) (Filley et al., 2000). Lignin monomers are known to induce ligninolytic enzyme production in fungi and it has been shown that in the presence of p-vanillin (vanillin), certain white-rot fungi actively begin decaying wood (Faison & Kirk, 1985; Lobarzewski & Trojanowski, 1979; Tsujiyama, 2003). In this study, the addition of vanillin to induce ligninolytic activity was examined.

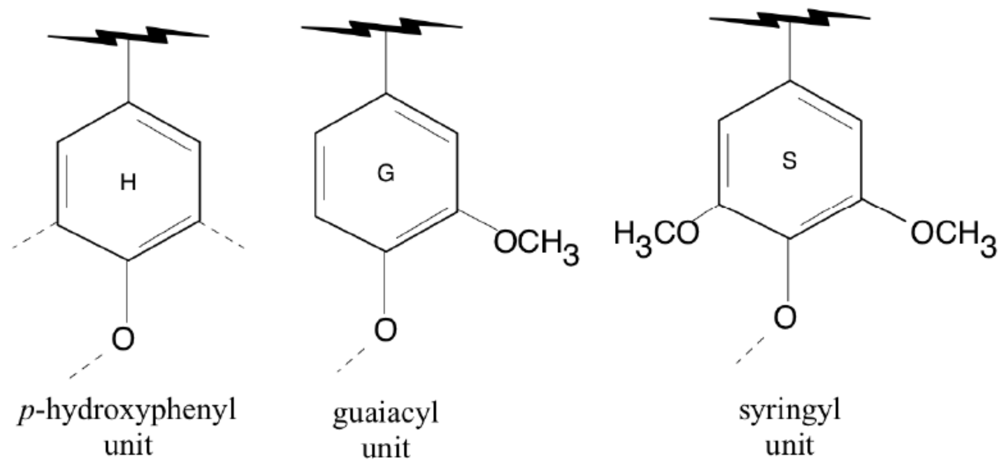

Figure 4.1 Syringyl lignin unit (S) has methyl groups on the 3- and 5-hydroxyl moieties and guaiacyl lignin (G) unit has a single methyl group on the 3-hydroxyl group. The *p*-hydroxyphenyl unit (H) is not methylated (based on Wong, 2009)

Analysis of wood rotted by Basidiomycota fungi indicates extensive demethylation of the lignin monomers released from brown-rot degradation and selective enrichment in an aromatic di-hydroxy-rich lignin residue (Filley et al., 2000). The buildup of vicinal diols in the lignin residue is of interest to this study because it indicates formation of a more reactive lignin. Wood-decay fungi release methanol as they degrade lignin and studies have shown that fungal cultures are able to reduce the methoxyl content of lignin by up to 35% (Jin et al., 1990a; Ander & Eriksson, 1985). Although the biochemistry and physiology of brown-rot and soft-rot fungal demethylation of lignin has not been as extensively studied, previous studies of white-rot fungi have found that, by changing growth conditions, it is possible to accumulate methanol in the fungal medium during lignin degradation, suggesting an increase in demethylation (Kirk et al., 1978). Kirk *et al.* examined the effects of oxygen, agitation, pH and changes in medium composition of *Phanerochaete chrysosporium*, such as glucose and nitrogen concentrations, in order to determine optimal lignin use conditions.

In this paper, I report on the effects of changing incubation and induction times, culture agitation, hydrogen peroxide concentration and metal concentrations with respect to methanol release, vicinal diol generation and protein content in four fungal species: *A. cylindrospora, F. fomentarius, I. resinosum* and *C. camelliae.*

4.3 Materials and Methods:

4.3.1 Organisms: *Absidia cylindrospora* (LU-7, soft-rot), *Fomes fomentarius* (LU-16, white-rot), *Ischnoderma resinosum* (LU-48, white-rot) and *Cylindrocladium camelliae* (LU-120, black rot) from the Mycological Herbarium at Lakehead University.

4.3.2 Growth conditions: The fungal stocks were grown on potato dextrose agar (PDA) plates that were kept at room temperature. Culture flasks were inoculated using 3 mycelium plugs taken from the PDA plate. Fungal media flasks were 40 mL and contained 2% Vogel's minimal salts (VMS) and 1% glucose (Vogel, 1956). Each culture was grown with 6 replicates. One week after the cultures were inoculated, p-vanillin was added to the culture with a final concentration of 10 mM to induce demethylating activity. Three of the six flasks were incubated at room temperature for two additional weeks and the other three flasks were incubated for three additional weeks. The mycelium from each flask was removed from the media by vaccum filtration (through 20 μm pore nylon sheet). The filtrate was used for further analysis.

After the fungi were inoculated, half of the flasks were placed on a shaker at about 80 rpm while the other half were left stationary. Different concentrations of hydrogen peroxide, added to cultures (0 mM, 1 mM, 2 mM and 3 mM final concentration), were also investigated. Induction time with vanillin was changed by adding it to the culture from the inoculation day, one week later, and two weeks later. Copper and iron concentrations in the VMS solution were also changed. The copper concentrations used were 0 μM, 0.5 μM, 1 μM and 2 μM. The iron concentrations

used were 0 μM, 0.125 μM, 0.25 μM and 0.5 μM. Each variable that was changed was measured in triplicate and the average activities were plotted. Stocks of hydrogen peroxide, $CuSO_4$ and $Fe(NH_4)_2(SO_4)_2$ were added to give final concentrations shown in relevant tables.

4.3.3 Analysis

4.3.3.1 Vicinal Diols: Change in vicinal diol generation was measured using the titanium(III) nitrilotriacetic acid assay, adapted from the procedure described by Liu (1997). A 167 mM NTA solution was prepared using ddH_2O and adjusted to pH 9.0 using concentrated NaOH. Subsequently, 0.2688 mL of 12% Ti(III)-Cl was added to 6 mL of NTA solution. The final stock solution was made up to 10 mL with ddH_2O after pH adjustments (see below). While adding the Ti(III)-Cl, the solution was kept above pH 2.0 using saturated Na_2CO_3 to avoid precipitation. The final pH of the solution was adjusted to 7.0 using saturated Na_2CO_3. Final concentration was 25 mM of Ti(III) and 100 mM NTA. The final solution was deep purple and used within 30 minutes. To determine vicinal diol concentration 0.1 mL of Ti(III)-NTA was added to 1 mL of sample. A standard curve with known concentrations of protocatechuic acid from 0-500 μM was used to determine vicinal concentration.

4.3.3.2 Methanol: The methanol released was measured using a procedure adapted from Klavons and Bennett (1986). Two solutions were required for the assay. Solution A contained diluted alcohol oxidase (EC 1.13.13, 1 unit) in 50 mM phosphate buffer pH 7.4. Solution B was 0.02 M 2,4 pentanedione in 2 M ammonium acetate and 0.05 M

acetic acid. First, 250 µL of solution A was added to 250 µL of sample and mixed. After 15 minutes at 25 °C, 250 µL of Solution B was added and incubated at 60°C for 15 minutes. Absorption was measured on a spectrophotometer in 96 well plates at 412 nm. A standard curve was made using 0-20 µg of methanol.

4.3.3.3 Protein: Content was determined using the Bradford assay, using bovine serum albumin (BSA) as a standard (Bradford, 1976). The assay was performed using an appropriate dilution to be in a range of 0-20 µg of protein. The reaction mixture contained 0.2 mL Bradford reagent (BioRad, Missisauga, Canada) and 0.8 mL sample. The absorbance was measured after five minutes at 595 nm.

4.3.4 Chemical Reagents: All chemicals were of analytical grade and were obtained from Sigma-Aldrich.

4.4 Results and Discussion

4.4.1 Effect of culture incubation time and induction time on demethylation: The culture extract grown in liquid media containing 1% glucose, 2% VMS and 10mM p-vanillin (vanillin) final concentration, was analyzed for vicinal diol concentration, methanol concentration and protein content with an incubation of two and three weeks after induction by vanillin. In the three-week samples of *C. camelliae,* the methanol concentration increased by 24% and the protein content increased by 16%, whereas it remained the same in the other three cultures (Fig. 4.2). The addition of vanillin was

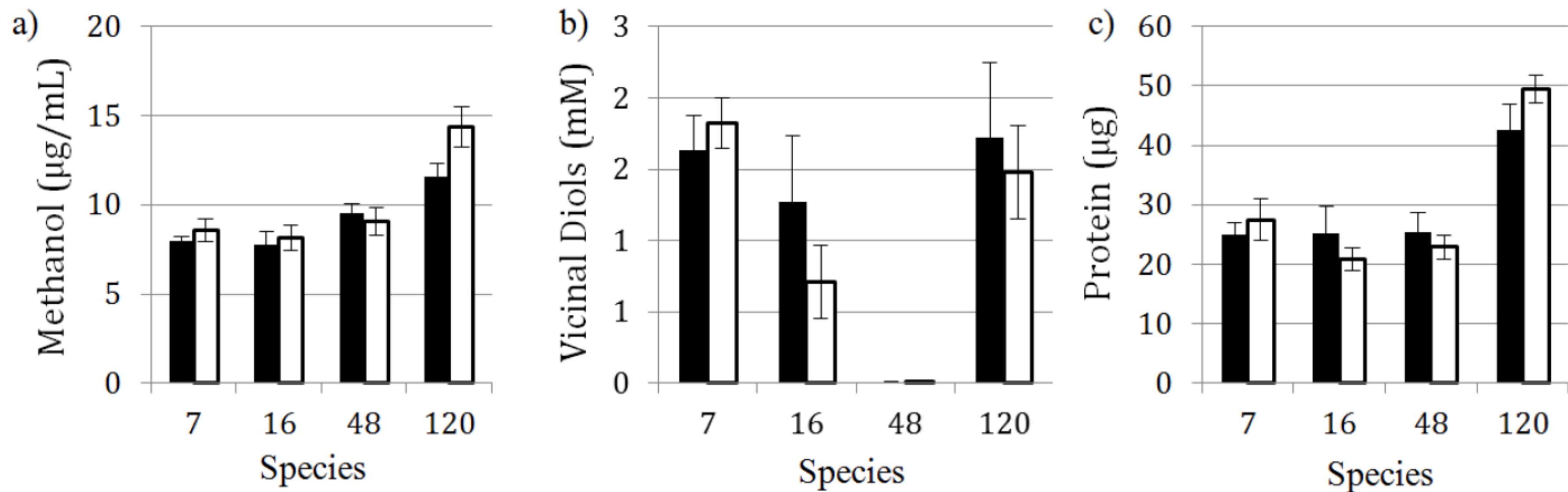

Figure 4.2 Changing incubation duration. Samples harvested two weeks after induction with p-vanillin are shown with black bars while samples harvested three weeks after induction with p-vanillin are shown with white bars. Shown in: a) change in methanol concentration (in µg/mL), b) change in vicinal diol concentration (mM), and c) change inprotein content (in µg/mL). Error bars represent the mean of 6 sample flasks. Species tested were *A. cylindrospora* (7), *F. fomentarius* (16), *I. resinosum* (48) and *C. camelliae* (120)

inhibitory to the growth of the cultures where the methanol concentration and protein content remained the same (Fig. 4.3). Once these cultures were induced, relatively little growth occurred. This is contrary to some previous studies where activation and enhanced enzyme production after vanillin addition was observed in white-rot fungi and in agreement with others where vanillin inhibited growth in black rot fungi (Tsujiyama, 2000; Tsujiyama, 2003; Lopez-Malo et al., 1995).

The timing of the addition of vanillin was altered to see its effect on the methanol released, vicinal diol concentration, and protein content. Vanillin was added on day 0 (same day as fungal inoculation), day 7, and day 14 (Fig. 4.3). When vanillin was added to the samples on day 0, the methanol released was very low in comparison to those samples that were induced later. In the case of *A. cylindrospora* an 11-fold increase of methanol was observed between day 0 and day 7 induction. Protein content increased as the day in which the sample was induced increased, except for *F. fomentarius*, which had the same protein content from day 0 to day 14 induction. Since vanillin inhibited the growth of the fungal cultures, adding vanillin at day 0 resulted in little methanol and vicinal diol formation because the fungal culture had not grown sufficiently.

In the incubation and induction time experiments, some cultures had a reduction in vicinal diols while generating a higher methanol concentration. For example, *A. cylindospora,* had a 2-fold reduction in vicinal diols along with the above mentioned 11-fold increase in methanol. The degradation of the vanillin ring structure after demethylation, reported in previous bacterial studies, would account for the decrease in vicinal diols in this soft-rot fungus (Masai et al., 2007). The enzymes responsible for

demethylation would continue to degrade the substrate past the monitored product of demethylation: 3,4-dihydroxybenzoic acid. In the case of *I. resinosum,* a 20% increase in vicinal diols and a 27% decrease in methanol concentration was observed. In a fungal culture containing active alcohol oxidases, vicinal diol concentration may be high but methanol concentration will be low, ambient or even sub-ambient because the methanol is oxidized to formaldehyde, formic acid and CO_2 (Ander & Eriksson, 1985).

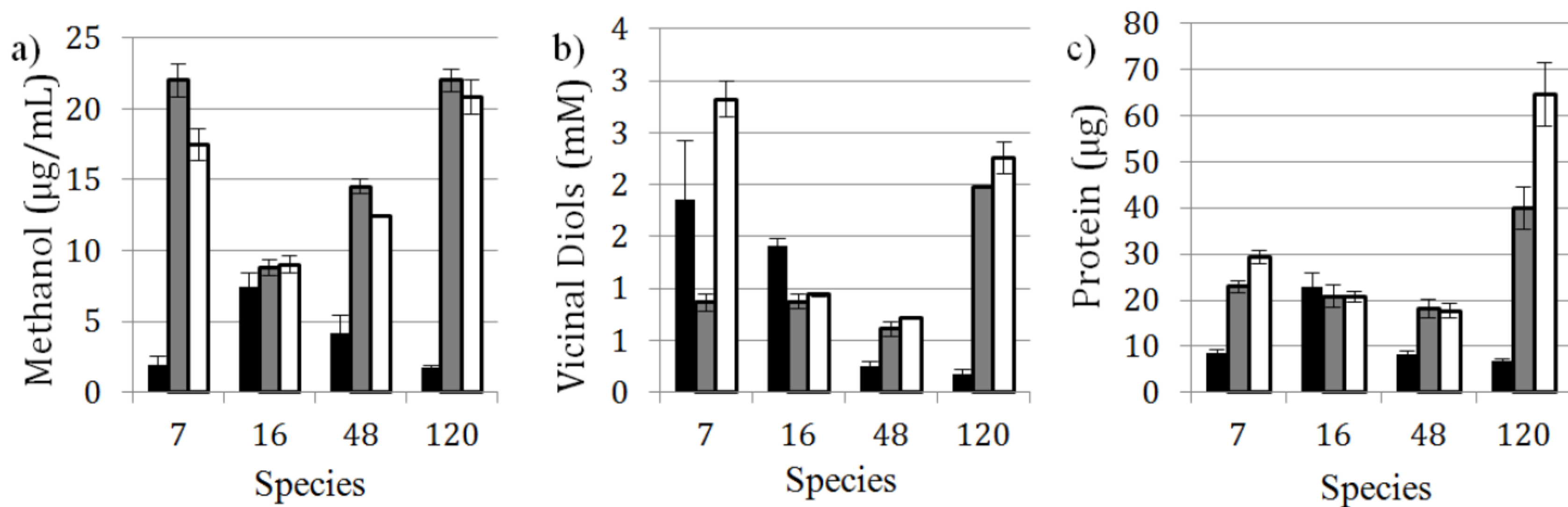

Figure 4.3 Varying induction day with p-vanillin. The black bars represent the samples that were induced with vanillin the same day and the fungi were inoculated. The cultures were induced at 7 days after inoculation (grey bars) at 14 days after inoculation (white bars). Shown in: a) change in methanol concentration (in µg/mL), b) change in vicinal diol concentration (mM), and c) change in protein content (in µg/mL). Error bars represent the mean of 6 sample flasks. Species tested were *A. cylindrospora* (7), *F. fomentarius* (16), *I. resinosum* (48) and *C. camelliae* (120)

4.4.2 Effect of agitation on methanol released, vicinal diol concentration, and protein content: Agitation using an orbital shaker increases the amount of dissolved oxygen in the cultivation medium (Purwanto et al., 2009). Previous studies have found that excessive agitation produces greater mechanical stress that could damage fungal mycelia and pellets, leading to cell destruction and resulting in a of lowering enzyme activity (Porcel et al., 2005; Purwanto et al., 2009). In this experiment the agitation speed was kept at 80 rpm, which is slower than other studies to minimize damage to the fungal mycelia and pellets (Kirk et al., 1978; Purwanto et al., 2009). The effect of agitation on methanol released and vicinal diol concentration varied for each of the four fungal species, while protein content did not change appreciably (Fig. 4.4). Methanol concentration measured in *A. cylindrospora* and *F. fomentarius* increased 27% and 87% respectively, when the samples were agitated, suggesting increased demethylation. *C. camelliae* showed 3 times higher levels of vicinal diols when kept stationary perhaps due to increased enzyme activity. *F. fomentarius* methanol concentrations increased while vicinal diol concentrations decreased, suggesting the vanillin degradation product, 3,4-dihydroxybenzoic acid, had been degraded further. Conversely, *C. camelliae* had a decreasing methanol concentration while an increasing vicinal diol concentration, suggesting the removal of methanol either through agitation or alcohol oxidases.

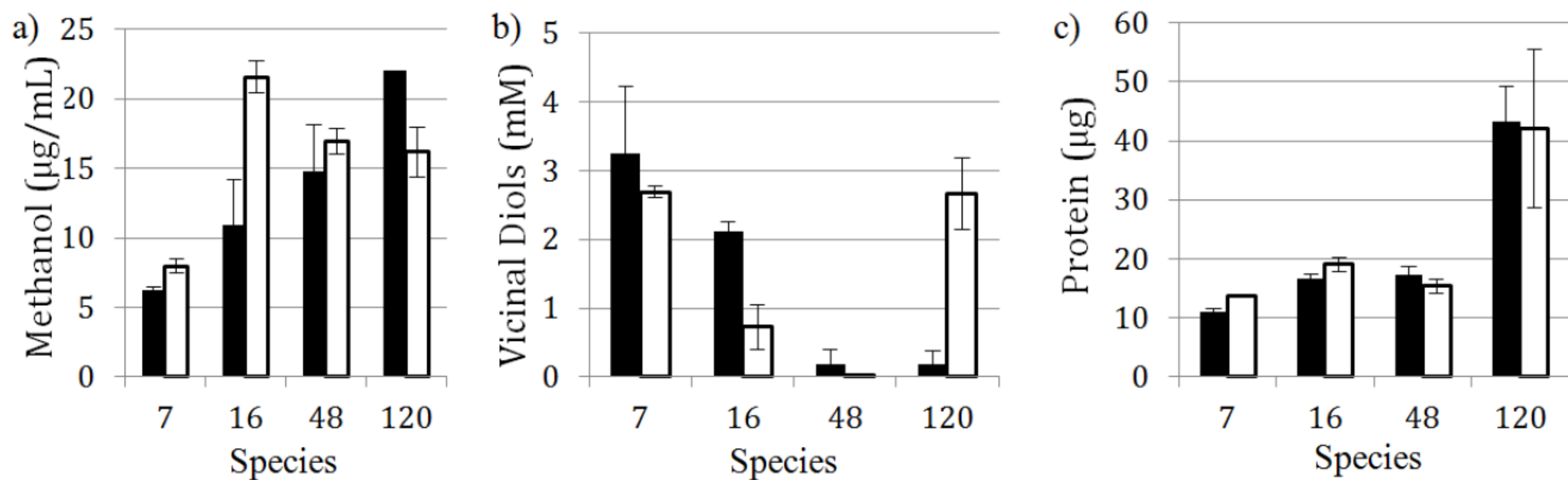

Figure 4.4 Agitation of samples. Samples agitated at about 80rpm are shown with black bars and samples kept stationary are shown with white bars. Shown in: a) change in methanol concentration (in µg/mL), b) change in vicinal diol concentration (mM), and c) change inprotein content (in µg/mL). Error bars represent the mean of 6 sample flasks. Species tested were *A. cylindrospora* (7), *F. fomentarius* (16), *I. resinosum* (48) and *C. camelliae* (120)

4.4.3 Effect of hydrogen peroxide concentrations on methanol released, vicinal diol concentration, and protein content: Because hydrogen peroxide has been shown to be involved in the degradation of wood by white-rot fungi, changes to concentration were examined as a variable (Bes et al., 1983; Forney et al., 1982). The concentration of vicinal diols decreased in *A. cylindrospora* and *C. camelliae* by 53% and 45% respectively with 0 to 3 mM of hydrogen peroxide added (Table 4.1). For *A. cylindrospora*, with the increased methanol released mentioned above, hydrogen peroxide may have acted as mediator in the degradation of vanillin. For *C. camelliae* the hydrogen peroxide simply inhibited growth, as the protein content and methanol released decreased by 123% and 62% respectively. For the other 2 cultures, hydrogen peroxide had relatively no effect on growth, methanol released or vicinal diol concentration.

4.4.4 Effect of metal concentrations on methanol released, vicinal diol concentration, and protein content. The metals necessary for fungal growth include copper, iron, manganese, molybdenum, zinc, and nickel. Copper requirements for fungal growth are usually satisfied by very low concentrations, in the order of 1 to 10μM (Cervantes & Gutierrez-Corona, 2004). Copper present in higher concentrations of its free, cupric form is toxic to microbial cells (Labbé & Thiele, 1999).

Table 4.1 The effect of hydrogen peroxide on protein content and demethylation indicators (methanol release and vicinal diol formation). Methanol concentration, vicinal diol concentration and protein content were measured after 3 weeks growth. Fungal cultures were grown for 1 week in 40 mL cultures containing 2% VMS and 1% glucose, then they were induced vanillin (0.3% final concentration) and incubated for 2 more weeks

	Fungal Species											
	Absidia cylindrospora			***Fomes fomentarius***			***Ischnoderma resinosum***			***Cylindrocladium camelliae***		
Hydrogen Peroxide Concentration	Methanol (μg/mL)	Vicinal OH (mM)	Protein (μg/mL)	Methanol (μg/mL)	Vicinal OH (mM)	Protein (μg/mL)	Methanol (μg/mL)	Vicinal OH (mM)	Protein (μg/mL)	Methanol (μg/mL)	Vicinal OH (mM)	Protein (μg/mL)
0mM	15.3	3.4	23.1	11.2	1.6	20.0	19.8	0.0	17.6	22.8	1.6	34.2
1mM	14.7	2.6	23.6	12.7	1.7	26.1	21.0	0.1	17.3	20.2	1.5	17.4
2mM	15.0	2.6	24.1	12.7	1.7	19.6	21.3	0.1	17.5	15.7	1.6	16.1
3mM	16.1	1.6	21.3	11.3	1.5	17.2	21.4	0.0	16.6	14.0	0.9	15.3

4.4.4.1 Copper concentration: Protein content in *F. fomentarius* and *I. resinosum* was not affected by the change in copper concentration however, with *F. fomentarius* the methanol and and vicinal diol concentration resulted in a six-fold decrease (Table 4.2) without copper. *C. camelliae* had almost a five-fold increase in protein content at a 0 to 2 μM final concentration of copper. Demethylation decreased with the increased growth of *C. camelliae,* as both methanol and vicinal diol had roughly a three-fold decrease in concentration.

4.4.4.2. Iron concentration: A 47% and 166% increase in methanol concentration was observed in *F. fomentarius* and *C. camelliae,* respectively, at a 0 to 0.5 μM final concentration of iron (Table 4.3). The protein content remained relatively the same through the increase in iron while vicinal diol concentration decreased with the exception of *C. camelliae* which had a two-fold increase in protein as iron increased 0 to 5 μM. Iron is used both in vital processes such as amino acid biosynthesis, energy production, and lipid metabolism, as well as in certain classes of methyltransferases (Kosman, 2003; Masai et al., 2007; Rutherford & Bird, 2004). The correlation between increasing methanol released and vicinal diol concentration decreasing and can be seen in both *F. fomentarius* and *C. camelliae*. An increase in demethylation activity generating more methanol and also degrading vanillin to smaller molecular weight fragments would explain the trend observed.

Table 4.2 The effect of copper on protein content and demethylation indicators (methanol release and vicinal diol formation). VMS was modified to contain no copper other than the added copper, otherwise medium and calculation as in Table 4.1

	Fungal Species								
	Fomes fomentarius			***Ischnoderma resinosum***			***Cylindrocladium camelliae***		
Copper Concentration	Methanol (µg/mL)	Vicinal OH (mM)	Protein (µg/mL)	Methanol (µg/mL)	Vicinal OH (mM)	Protein (µg/mL)	Methanol (µg/mL)	Vicinal OH (mM)	Protein (µg/mL)
0µM	17.9	2.4	21.1	12.3	0.1	14.0	34.0	3.0	18.3
0.5µM	13.6	1.9	21.3	15.8	0.0	14.3	34.0	2.1	46.6
1µM	8.8	0.9	20.8	14.5	0.6	18.2	22.0	2.0	40.0
2µM	3.0	0.4	20.2	10.5	0.1	14.1	14.0	0.9	83.0

Table 4.3 The effect of iron on protein content and demethylation indicators (methanol release and vicinal diol formation). VMS was modified to contain no iron other than the added iron, otherwise medium and calculation as in Table 4.1

	Fungal Species								
	Fomes fomentarius			***Ischnoderma resinosum***			***Cylindrocladium camelliae***		
Iron Concentration	Methanol (µg/mL)	Vicinal OH (mM)	Protein (µg/mL)	Methanol (µg/mL)	Vicinal OH (mM)	Protein (µg/mL)	Methanol (µg/mL)	Vicinal OH (mM)	Protein (µg/mL)
0µM	12.7	1.6	18.6	16.4	0.0	19.6	9.17	3.2	21.9
0.125µM	14.0	0.8	18.6	16.5	0.0	19.6	21.3	1.8	48.7
0.25µM	15.8	0.9	20.8	14.5	0.0	18.1	22.0	1.9	40.0
0.5µM	18.4	0.8	18.4	17.5	0.0	18.8	24.5	1.7	44.9

4.5 Conclusion

Generally, cultures required one week of growth prior to induction and demethylation indicators did not benefit from incubation longer than two weeks after induction. Culture conditions with respect to media composition were found to be species-specific and affected each of the four fungal species studied quite differently. In order to increase demethylation activity one should increase hydrogen peroxide in the media for *A. cylindrospora* and increase iron in the media for *F. fomentarius* and *C. camelliae*. As well, agitation with *F. fomentarius* and *C. camellia* was found to be beneficial.

Control of culture conditions is essential for optimal ligninolytic activity and will facilitate finding a more reactive lignin useful in future industrial applications. We are continuing our studies of demethylation of both lignin and lignin-related compounds and are searching for evidence of novel demethylase enzyme activity in the fungi of the northern Ontario boreal forest.

5. Characterization and partial purification of vanillin demethylases from *Absidia cylindrospora* and *Sphingobium* sp. SYK-6 (unpublished)

Unpublished:

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Author Contribution

Gibson, A.: completed all writing and experimental

Dekker, R.F.H. and Malek, L.: critical evaluation of design and interpretation of data

5.1 Abstract

We investigated the enzymes involved in demethylation of lignin-derived compounds from the fungal species, *Absidia cylindrospora* and the bacterial species *Sphingobium* sp. SYK-6. *A. cylindrospora* was grown in 800 mL of media containing 2% VMS and 1% glucose in 1.8 mL Fernbach flasks capped with aluminium foil. SYK-6 was grown in 40 mL of minimal W-medium in 250 mL Erlenmeyer flasks. Demethylation activity was induced by the addition of vanillin to a final concentration of 1 mM. Culture fluid was purified using a 30 kDa ultrafiltration membrane and a DEAE sephadex cartridge. Enzyme activity was characterized by SDS-PAGE and Ti(III)-NTA assay. *A. cylindrospora* produced much less enzyme than SYK-6 however the specific activity of its demethylation was much higher, 336.51 μkat mg^{-1} to 39.15 μkat mg^{-1} respectively. SDS-PAGE of the partially purified SYK-6 enzyme and the *A. cylindrospora* enzyme confirmed that the two demethylation systems are different. For the first time, a 3-D model of this enzyme, vanillin *O*-demethylase, was constructed. Further work is needed to fully characterize and purify both of these enzymes associated with the microbial demethylation of lignin.

5.2 Introduction

Lignin, the most abundant aromatic ring polymer present in the biosphere by far, is found in the secondary cell walls of plants (particularly trees); providing mechanical strength and making the plant resistant to microbial attack because the lignin is relatively resistant to degradation (Zhu et al, 2011). Some organisms, though, are capable of degrading lignin, and research has focused on white-rot fungi and such ligninolytic

enzymes as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccases (Cullen & Kersten, 2004). Brown-rot and soft-rot fungi as well as bacteria have received less attention because they do not degrade lignin completely or as efficiently (Eriksson et al, 1990; Worrall et al., 1997). However, there is continuing interest in the ability of these species to modify lignin by producing ligninolytic enzymes capable of demethylating methoxyl groups, while retaining the polymeric lignin structure in a more reactive phenolic form (Hu et al., 2011).

Demethylation reactions take place on the guaiacyl and syringyl phenylpropanoid units that primarily make up wood lignins (Sjöström, 1993). Analyses of wood rotted by Basidiomycota brown-rot species indicate a high proportion of demethylated lignin resulting in an aromatic-rich lignin residue. White-rot fungi, in contrast, leave a residue characterized by significant side-chain oxidation and aromatic ring cleavage (Worrall et al., 1997). Figure 5.1 shows the types of chemical changes imparted to lignin during degradation (Filley et al., 2002).

The mechanisms of lignin demethylation by fungi and bacteria are complicated, so the chemical pathways have been investigated using lignin model compounds (for example SYK-6, see figure 5.2, Godden et al, 1992), Fenton reaction experiments (for example *G. trabeum* by Shimada, 1997; Hyde and Wood, 1997) and cultivation

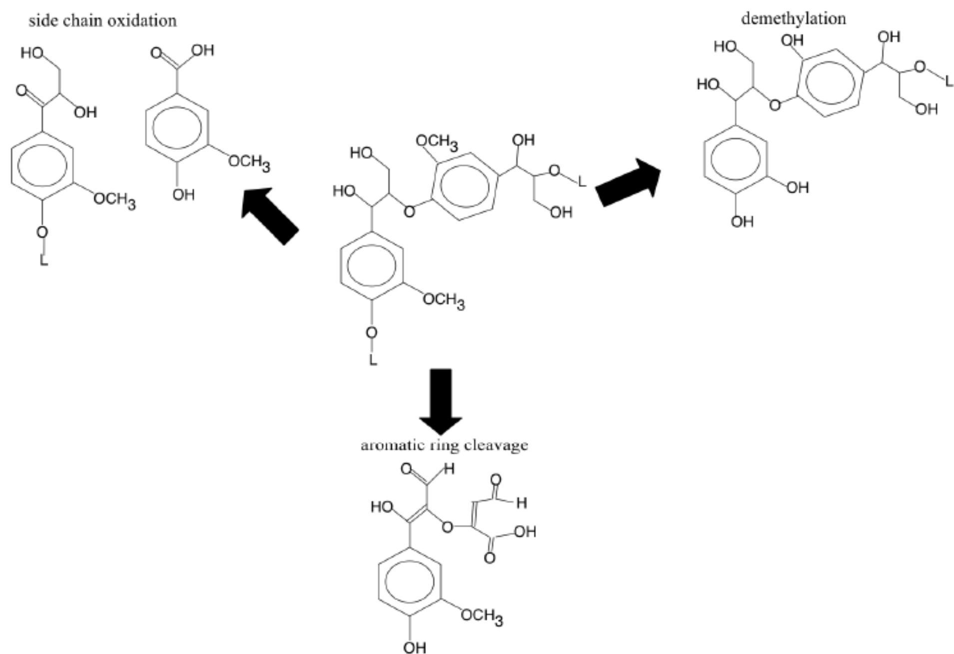

Figure 5.1 Examples of the types of chemical changes occurring during lignin degradation by fungi and bacteria showing side-chain oxidation, aromatic ring cleavage (associated with white-rot fungi) and demethylation (associated with brown and soft-rot fungi and bacteria). *L* is the lignin macromolecule

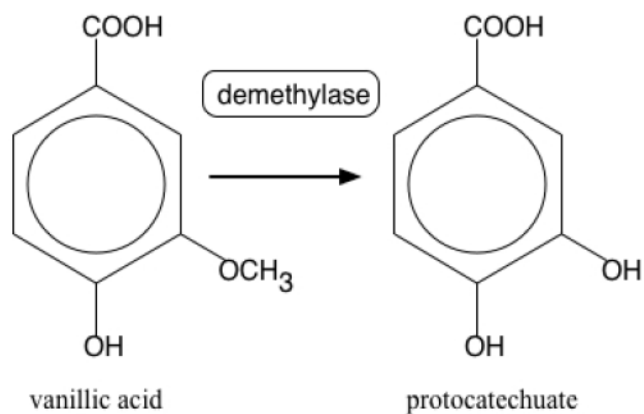

Figure 5.2 Reaction scheme for the demethylation of vanillic acid to protocatechuate. The enzyme responsible for demethylation of this lignin-derived compound was proposed for SYK-6

optimization experiments (Jin et al 1990b). Overviews of previous research on the biodegradation of lignin by fungi and bacteria have been written by Hatakka and Bugg, but to date an enzyme solely involved in the demethylation of lignin has not been described or isolated (Hatakka, 2001; Bugg et al., 2011b).

Some lignin-degrading enzymes (for example laccase and MnP), involved in the demethylation of lignin, use a free-radical process that releases methanol by a mechanism that has not been characterized (Paice et al., 1993). A previous study specifically reported methanol production by *P. chrysosporium* K-3 from the demethoxylation of lignin-related substances, and demonstrated that methanol formation occurred together with delignification (Ander & Eriksson, 1985). They suggested that some kind of unknown peroxidase or other enzyme may be involved. These findings show evidence for the existence of specific enzymes involved in the demethylation of lignin. This is further supported by evidence that laccase-catalyzed oxidation of vanillyl glycol (a lignin model compound) produces methanol, arising from demethylation (Lundquist et al., 1985).

In this study, we attempted to isolate and compare the enzymes responsible for demethylation of the lignin-related compound vanillin, in the soft rot fungus, *Absidia cylindrospora* and the bacterium SYK-6.

5.3 Materials and Methods:

5.3.1 Organisms: *Absidia cylindrospora* (LU-7, soft-rot) was obtained from Mycological Herbarium at Lakehead University and *Sphingobium* sp. SYK-6 (SYK-6) was obtained from NITE Biological Resource Center, Kazusakamatari, Kisarazu, Chiba, Japan.

5.3.2 Growth conditions: *A. cylindrospora* was maintained on potato dextrose agar (PDA) plates and incubated at room temperature. Culture flasks were inoculated using 9 mycelium plugs taken from the PDA plate. Fungal cultures were grown in 1.8 L Fernbach flasks containing 800 mL of media and capped with aluminum foil. The media contained 2% Vogel's minimal salts (VMS) (Vogel 1956) and 1% glucose. The culture was grown at 20 ± 2 °C for 1 week prior to adding 2 mL of 400 mM vanillin to a final concentration of 1 mM, and grown for an additional 2 weeks.

SYK-6 was grown on nutrient agar plates that were incubated at 20 ± 2 °C. A sterile loop from the plate was used to inoculate 200 mL of nutrient broth, and the culture was grown to OD_{550} 1. SYK-6 was grown on 40 mL of minimal W-medium (Peng et al., 1998) in 250 mL wide neck Erlenmeyer flasks using 1mM vanillin as a carbon source. Cultures were grown for 5 days at 20 ± 2 °C before analysis.

5.3.3 Enzyme preparation: Once the fungal culture was grown for 2 weeks after vanillin addition, the 800 mL culture flask was filtered through a coarse nylon mesh (20 µm pore size). The fungal filtrate (800 mL) and the bacterium culture fluid (40 mL) were ultrafiltered using a 30 KDa filter and filter residue resuspended in 5 mL of 50 mM Tris

pH 8 buffer. A 5 mL DEAE (Bio-Scale Mini Macro-Prep DEAE Cartridge, Bio-rad) column was used and 3 mL of sample was loaded onto the column. A flow rate of approximately 1 mL/min was used and the column was washed for 20 minutes. A stepwise elution was used with 50mM Tris pH 8 buffer containing increasing salt concentrations from 0.05M, 0.1 M, 0.15 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M, each for 15 minutes. The fraction collector was programed for 5 minute fractions. After the elution steps the column was washed with 1M NaCl and the first two fractions were retained for analysis. Each fraction was analyzed for protein using Bradford protein assay and vicinal diol concentration using the Ti(III)-NTA assay.

5.3.4 Enzyme characterization: Precast polyacrylamide gels (8-16% Precise Tris-HEPES 10 x 8.5 cm, Thermo Fisher Scientific) were used for SDS-PAGE with a 20-118 kDA protein molecular weight ladder (Fermentas #SM0441). Both the ultrafiltrate crude enzyme sample and fraction 33 from the DEAE separation were run in duplicate (30 µL per well). SDS-PAGE was performed as described by Hames (1986). Images were taken by a Bio-rad XR gel documentation apparatus.

Using the genomic sequence obtained from previous studies, a 3D model was generated for vanillin *O*-demethylase from SYK-6 using Geno3d Pôle BioInformatique Lyonnais (Combet et al., 2002; Masai et al., 2012). Using full model analysis, 10 possible models were generated. The model shown in Fig. 5.6 was selected based on similarity of protein sequence and stereochemical quality (Ramchandran plot, disallowed portions etc.).

5.3.5 Degradation of lignin model compounds: Demethylation activity was measured using the titanium(III) nitrilotriacetic acid assay as described previously (Gibson et al., 2014), by examining the change in vicinal diols. A 167 mM NTA solution was prepared using ddH_2O and adjusted to pH 9.0 using concentrated NaOH. Ti(III)-Cl (0.2688 mL of 12%) was added to 6 mL of NTA solution and the final stock solution was made up to 10 mL with ddH_2O after pH adjustments (see below). While adding the Ti(III)-Cl, the solution was kept above pH 2.0 using saturated Na_2CO_3 to avoid precipitation. The final pH of the solution was adjusted to 7.0 using saturated Na_2CO_3. Final concentration was 25 mM of Ti(III) and 100 mM NTA. The final preparation was deep purple and used within 30 minutes. Ti(III)-NTA (0.1 mL) was added to 1 mL of sample to determine vicinal diol concentration. A standard curve with known concentrations of protocatechuic acid (PCA) (0-500 µM) was used to determine the concentration of vicinal diol groups (1µM PCA is equivalent to 1µM vicinal diol).

5.3.6 Chemicals: All chemicals were of analytical grade and were obtained from Sigma-Aldrich.

5.4 Results

Enzyme mixture from concentrated medium of SYK-6 produced a maximum PCA concentration of 207 µM at 30 min., while enzyme from concentrated medium of *A. cylindorspora* produced a maximum PCA concentration of 30 µM at 120 minutes (Fig. 5.3). However, this activity stopped between 2 and 3 hours of incubation. SYK-6 showed a decline in phenolic content (A_{280} nm) at 30 min. followed by a rapid steady decline with

no substrate remaining after 2hrs (Fig 5.3) while *A. cylindrospora* maintained a relatively constant phenolic content, which decreased gradually, to about 90% of original after 180 minutes. Following DEAE cartridge fractionation, fraction 33 had the highest demethylation activity, as well as the unbound wash, fractions 1-10, with a concentration of 47 μM PCA (Fig. 5.4 dotted line). This fraction was eluted at 30% NaCl and contained 6.6 μg protein. The partially purified culture fluid exhibited a major protein bands at approximately 35, 50 and 110 kDa as determined by SDS-PAGE (Fig. 5.5). The 3D model of vanillin *O*-demethylase from SYK-6 genomic data revealed a globular protein with about equal amounts of α-helices and β-sheets, and thiol groups on the outer structure (Fig. 5.6). Table 5.1 summarizes the results of purification of 80 mL of culture fluid from SYK-6 and 800 mL of culture fluid from *A. cylindrospora*. Purification for the SYK-6 demethylase was approximately three-fold and 1.5-fold for *A. cylindrospora*.

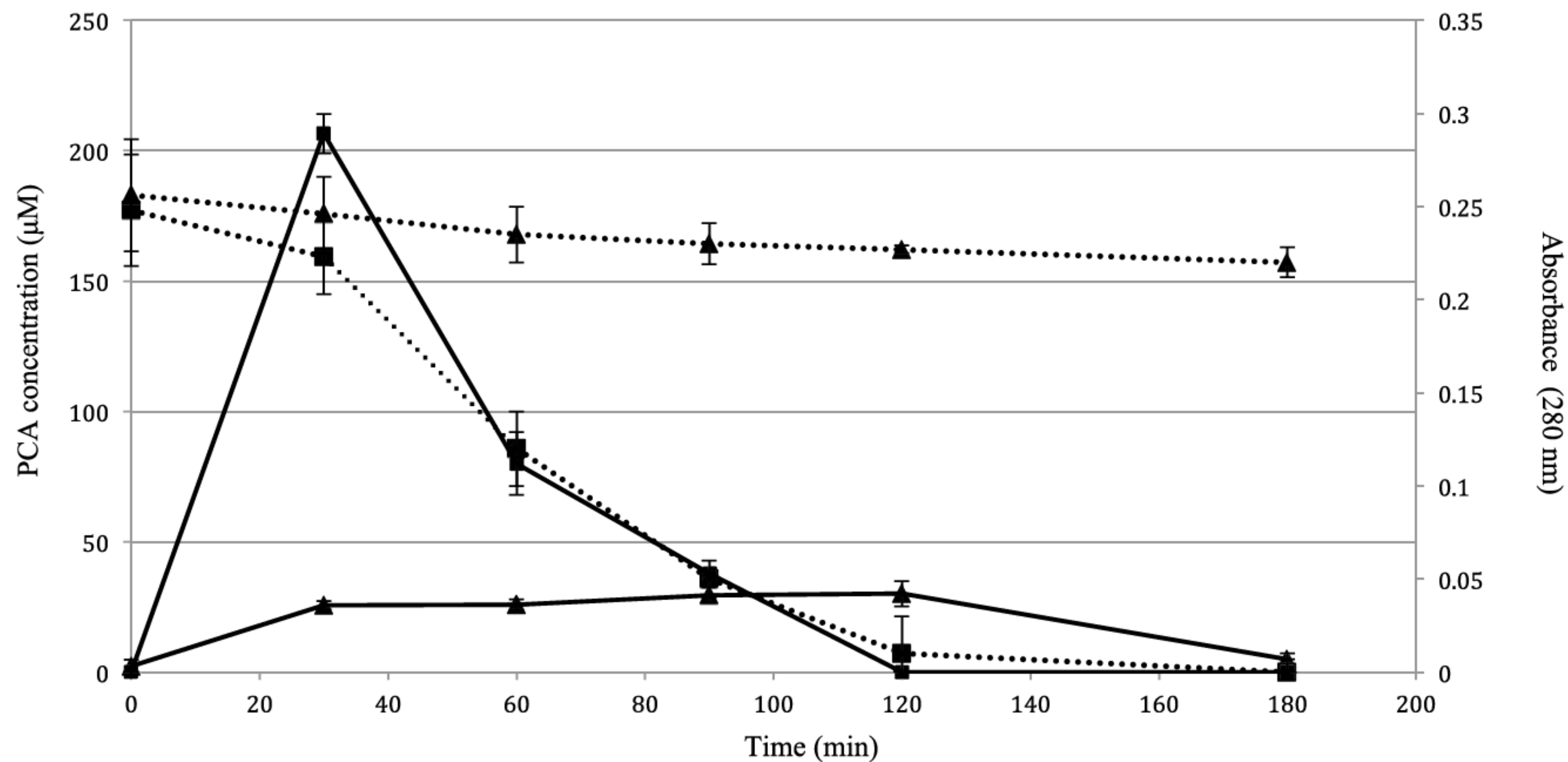

Figure 5.3 Concentrated culture fluid from *A. cylindrospora* (triangles) and SYK-6 (squares) analyzed for demethylation activity by Ti(III)-NTA assay (solid lines) and phenolic content by spectrophotometry (A_{280} nm) (dotted lines). Demethylation activity expressed by the formation of PCA from vanillin. Standard error bars reflect variation around the mean of three samples analyzed

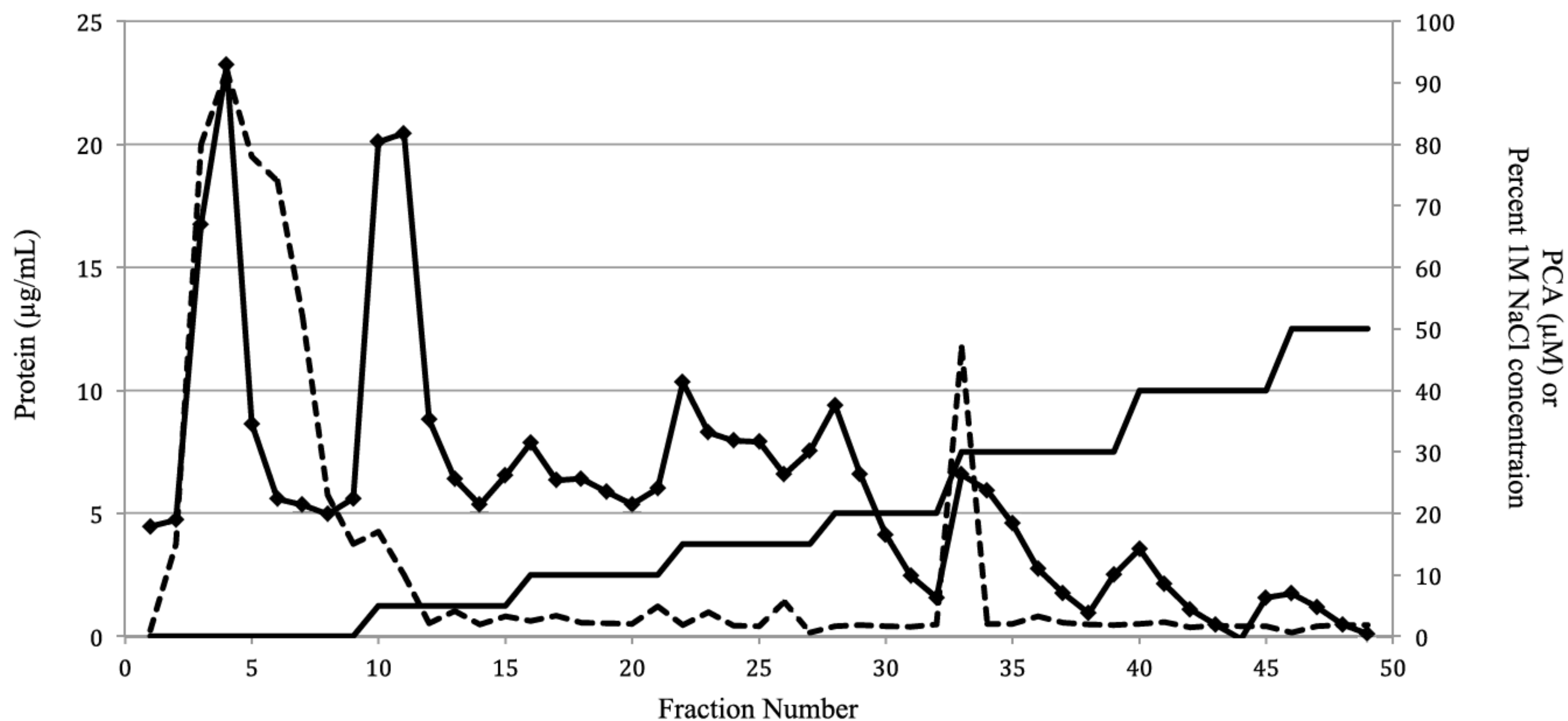

Figure 5.4 DEAE chromatography of the concentrated culture fluid from SYK-6 using 50 mM Tris pH 8 buffer. Protein was eluted stepwise with buffer containing increasing % of 1 M NaCl (solid line). Fractions were analyzed for both demethylation activity by Ti(III)-NTA assay (dashed line) and protein content by Bradford assay (diamonds). Demethylation activity expressed by the formation of PCA from vanillin using a standard curve to determine concentration

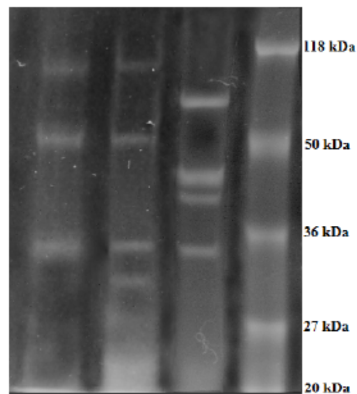

Figure 5.5 SDS-PAGE of the partially purified demethylase and concentrated culture fluid from SYK-6 and the concentrated culture fluid from *A. cylindrospora* (kDa); Lane 1, 30 µL partially-purified demethylase (after DEAE) from SYK-6; Lane 2, 30 µL concentrated culture fluid from SYK-6; Lane 3, 30 µL concentrated culture fluid from *A. cylindorspora*; Lane 4, 10 µL marker protein

Figure 5.6 3D model of vanillin *O*-demethylase from SYK-6. α-Helices and are shown as coiled ribbons, while β-sheets are shown as flat strands. Several thiol groups appear to be exposed at the surface of the protein (not highlighted). Model generated from SYK-6 genomic data using Geno3D (http://geno3d-pbil.ibcp.fr)

Table 5.1 Purification steps of demethylase from SYK-6 and *A. cylindrospora.*

	Volume (mL)	Total Protein (mg)	Total Activity (µkat)	Specific Activity (µkat mg^{-1})	Fold	Yield (%)
SYK-6						
Culture medium	80	50.15	658	13.12	1.00	100.0
UF concentrate	5	1.943	34.42	17.71	1.35	5.2
DEAE sephadex	5	0.04	1.566	39.15	2.98	0.2
A. cylindrospora						
Culture medium	800	30	6666	222.20	1.00	100
UF concentrate	5	0.63	212	336.51	1.51	3

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5.5 Discussion

5.5.1 Production and purification of the demethylase from *Absidia cylindrospora* and *Sphingobium sp*. SYK-6

Concentrated culture fluids (containing a mixture of enzymes) from both *A. cylindrospora* and SYK-6 were analyzed over 180 minutes for their demethylation capacity and residual phenolic content. Vanillin was chosen as a lignin model substrate to reduce sample variability and to specifically isolate the enzyme associated with demethylation. The demethylation product, PCA, was detected by the Ti(III)-NTA assay indicating demethylation. Shown in Figure 5.3, *A. cylindrospora* degraded the substrate at a slower rate than SYK-6 (Fig 5.3). Metabolism of *A. cylindrospora* is likely much slower than SYK-6 which explains the difference in activity. While SYK-6 had a peak concentration of PCA at 30 minutes followed by a sharp decline, indicating that PCA was being metabolized by the bacteria. As shown in previous research, the degradation of vanillin continues past PCA and involves phenyl ring cleavage (Masai et al., 2007). After this ring cleavage the Ti(III)-NTA assay would not be expected to detect the subsequent degradation products and one would expect a decrease in absorbance as reported in the results. Examining the absorbance at 280 nm provides an estimate of phenolic structures, which showed a sharp decline, presumably a decrease in PCA concentration in SYK-6.

Following the next step in purification on DEAE Sephadex (Fig 5.4), a small portion of the demethylase was retained in fraction 33 yielding only 0.2% of the original protein content. The majority of the demethylase, perhaps in a different form, did not bind to the cartridge. Using different column material or changing ionic conditions of the

buffer system may help to purify this protein. *A. cylindrospora* only had activity in the concentrated culture fluid (data not shown). The lack of activity in *A. cylindrospora* could suggest that the enzyme is easily denatured or may involve a multi-enzyme unit, which dissociates during purification.

5.5.2 Physical properties

With SDS-PAGE the concentrated culture extract from both *A. cylindrospora* and SYK-6 were compared with the partially purified SYK-6 fraction (Fig. 5.5). On both the samples from SYK-6 a band around 50kDa was observed which is consistent with the molecular weight calculated from the gene products identified by Masai et al. The DEAE fraction from SYK-6 had other fragments clustered up to 20 kDa band but none as defined as the 50 kDa band suggesting the defined band was the dominant protein in the sample. The enzyme was only considered to be partially purified due to the presence of the other proteins. Additional purification steps would be needed to purify this enzyme. This 50 kDa band was not prominent in the concentrated culture fluid from *A. cylindrospora*. Little is known about the demethylation mechanisms of soft rot fungi. Given these results another enzyme system from the one used by SYK-6 is likely used in *A. cylindrospora* to demethylate vanillin.

Subsequent purification steps were attempted after DEAE with the partially purified SYK-6 enzyme, which proved to be unsuccessful. After examining the proposed 3D model of the putative protein a large concentration of thiol groups were found on the surface (Fig 5.6.). Thiol groups have been found to reduce the stability of the enzyme and

may explain the difficulty purifying this enzyme (Sigalov et al., 2001). The 3D model of the vanillin *O*-demethylase from SYK-6 has not been previously modeled and was generated based on genomic data (Masai et al., 2012). Further studies may focus on the stabilization of these functional groups to retain enzyme activity through purification.

5.5.3 Catalytic properties

A purification table was developed based upon activity generated at each purification step and is shown in Table 5.1. Total protein was much higher in SYK-6, however specific activity was higher in *A. cylindrospora* indicating a more efficient demethylation system in the soft-rot fungus compared to the bacterial species. More research is needed to confirm this finding.

6. Conclusions and Future Work

SIFT-MS was demonstrated to be a useful analytical instrument for investigating methanol production and other volatile compounds in the headspace of lignin degrading microbial cultures. As our knowledge of lignin and the role microorganisms play in the breakdown of lignin continues to increase, more information needs to be gathered to elucidate the mechanism of lignin degradative pathways. In the SIFT-MS study (Section 2), a number of the compounds identified, including benzene, hexanal, phenyl methanol, 2-hexanone and 2-butanal, were not expected to be present in the culture headspace. They are not components associated with the current understanding of microbial lignin metabolism and highlight the complexity of the mechanisms involved. As well, there were compounds that remain unidentified due to limitations of the SIFT-MS library and will require further investigation. Once identified, they will be of interest to research in the area of bacterial and fungal metabolic pathways.

The Ti(III)-NTA assay was developed to rapidly determine the relative degree of lignin demethylation by cultures of microorganisms and their enzymes. This method detects methylation products up to 500 μM. As well, it can be used to measure vicinal diol product formation over time, to quantify enzyme activity (Section 5). In practice, the assay not only confirmed the findings of the SIFT-MS study (Section 3), but helped to interpret the findings with respect to the relationship between methanol formation and vicinal diol generation (Section 5).

Culture conditions affected by incubation, induction time, agitation, hydrogen peroxide and metal concentrations were found to be species specific and affected each of the four fungal species studied in Section 4 quite differently. The effects on methanol, protein and vicinal diol concentration confirm that control of culture conditions is essential for optimal ligninolytic activity.

Enzyme activity for the fungal species *A. cylindrospora* and the bacterial species SYK-6 was examined in Section 5. Many studies have reported lignin demethylation reactions by various fungal and bacterial species. A specific enzyme has not yet been characterized, although many uses have been suggested for demethylated (modified) lignin as a precursor to chemical feedstocks and biofuels. *A. cylindrospora* produced much less enzyme than SYK-6, however the specific activity of its demethylation was much higher. The SDS-PAGE of the fungal and bacterial enzymes confirmed that the two demethylation systems are different. Further work is needed to fully characterize and purify both of these enzymes associated with the microbial demethylation of lignin.

My PhD project has focused on a number of tools for investigating lignin degradation that will be of use in future studies. It has also identified a number of fungi from the Mycological Herbarium at Lakehead University capable of growing on lignin as a sole carbon source. The enzyme mechanisms of these fungi need further study and their enzymes need to be purified. We also identified a number of novel compounds generated by microbial lignin degradation in the headspace of fungal cultures, demonstrating the usefulness of the SIFT-MS. It is hoped that the tools developed and investigated here will

be of use in the development of methods to modify lignin enzymatically for conversion into non fossil fuel based bio-polymers and other products with industrial significance.

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