INVESTIGATION OF BIOLOGICAL PROCESS FOR THE
CONVERSION OF BARK BIOMASS TO BIO-BASED
POLYPHENOLS

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

Faculty of Forestry

University of Toronto

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Doctor of Philosophy
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Abstract

Due to increasing waste production and disposal problems arising from synthetic polymer production, there is a critical need to substitute these materials with biodegradable and renewable resources. The concept of green polymers has become more appealing due to the presence of large volumes of processing residuals from the timber and pulp industries. This, in turn, supports the idea of developing new polymers based on bark extractives. In this thesis, three comparative treatments i.e., enzymatic, alkaline, and UV/H₂O₂ have been conducted for the extraction of beetle infested lodgepole pine (BILP) and mixed aspen barks polyphenolic extractives. Use of laccases as biocatalysts to affect and enhance the catalytic properties of enzymes has been shown to be a promising solution for bark depolymerization. Furthermore, laccases are suitable for biotechnological applications that transform bark biomass into high valued bark biochemicals. The industrial and biotechnological application of ligninases is constantly increasing due to their multiple uses and applications in a diversity of processes. Bark depolymerization was conducted in submerged fermentation (SF) and we identified polyphenols/polyaromatic compounds after four weeks when the production media (PM) was induced with 50mg/100ml of each type of bark during the lag-phase. During SF where honey was used as a natural mediator substitute (NMS) in the PM, laccase activities were about 1.5 times higher than those found in comparable cultures without honey in the PM. These samples were analyzed by GC-MS. The laccase enzyme was purified using UNO® sphere Q-1 anion exchange chromatography and the molecular weight was determined to be ~50kDa on 10% SDS-PAGE and laccase kinetic parameters including maximal velocity (V_max), Michaelis constant (K_m), and turnover number (K_cat) were calculated from a Lineweaver Burk plot. All calculated kinetic parameters of the laccase activity are substrate (ABTS) specific. Py-GC-MS analysis of bark showed differing effects of fungal activity on bark composition. Polyphenolics were separated in reverse-phase mode using HPLC with two selected wavelengths of 290 and 340 nm to improve separation. The replacement of conventional natural mediators (NM) by monofloral honey in production media, and investigation of the effect of fungi-derived laccases on bark polyphenols are studied for the first time by this thesis work.
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List of Acronyms

ABTS  2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ALIPH  Aliphatic
BF  Balsam fir
BILP  Beetle infested lodgepole pine
BL  Black liquor
bnt  Billion tons
BSA  Bovine serum albumen
CARB  Carbohydrate
α-C  Alpha-cellulose
cat  Catechol
CFIA  Canadian Food Inspection Agency
DIPOA  Departamento Nacional de Inspeção de Productos de Origen Animal
DEAE  Diethylaminoethyl
DHB  2,5-dihydroxy benzoic acid
DTG  Derivative thermogravimetry
FPLC  Fast Protein liquid chromatography
FS  Fermented samples
GC/MS  Gas chromatography-mass spectrometry
gluc  Glucose
H  Honey
HC  Holocellulose
HBT  1-hydroxybenzotriazole
HPLC  High performance liquid chromatography
IU  International unit
K_{cat}  Turnover number
K_m  Michaelis constant
KL  Klason lignin
Lcc  Laccase
LGB  Lignocellulosic green biotechnology
LIG  Lignin
LiP  Lignin peroxidase
LMS  Laccase mediator system
LMW  Low molecular weight
m/z  Mass to charge ratio
MAH  Monocyclic aromatic hydrocarbon
MALDI-TOF/MS  Matrix assisted laser desorption ionization-time of flight-mass spectrometry
MnP  Manganese peroxidase
MS  Methyl syringate
NCOMP  Nitrogen containing compound
NMS  Natural mediator substitute
<table>
<thead>
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<tr>
<td>PAHs</td>
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</tr>
<tr>
<td>PCR</td>
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</tr>
<tr>
<td>PHEN</td>
<td>Phenol</td>
</tr>
<tr>
<td>PIC</td>
<td>Partial ion chromatogram</td>
</tr>
<tr>
<td>PM</td>
<td>Production medium</td>
</tr>
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<td>Py-GC/MS</td>
<td>Pyrolysis gas chromatography-mass spectrometry</td>
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<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SESQUI</td>
<td>Sesquiterpenoid</td>
</tr>
<tr>
<td>SF</td>
<td>Submerged fermentation</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
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<td>$V_{\text{max}}$</td>
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1 Chapter: Introduction

1.1 Motivation and Significance

This chapter describes background, perspective and composition of this thesis. Biotechnology is a promising technology for conversion of biomass materials to value-added products. In this thesis, integrated biological methods are developed in order to obtain bark-derived chemicals for industrial applications.

Worldwide yearly phenol production has increased steadily (almost 2.5% per year), climbing from 8.34 M tons in 2010 to in excess of 8.9 M tons in 2012. The five main phenol-producing states in 2012 were China, USA, Japan, Taiwan and South Korea with a consolidated production of over 5.5 M tons. Ineos Phenol, Mitsui Chemicals, SINOPEC, Cepsa Quimica, Shell Chemical, LG Chem, Honeywell Resins & Chemicals, Versalis S.p.a., Formosa Chemicals and Fiber Corp, Kumho P&B Chemicals, and Sabic Innovative are the main producing companies. The major global firms involved in the phenol market manufacture plastics. Overall yearly production of phenol was expected at over 11.5 M tons in 2012 (Merchant Research and Consulting Ltd Report, 2014).

Chemicals derived from renewable feedstock, e.g., agricultural wastes, lignocellulosic biomass, pulp and paper mill effluents or biomass microorganisms make up the green chemicals market. Market contributors are progressively encountering the need to make a change from petrochemical feedstock to renewable feedstock, on the grounds that the chemicals business sector is subject to the instability in unrefined petroleum costs (Market Publishers Report Database, 2013).

Lignocellulosic biodegradation is a part of the carbon cycle. Development of humus (humic acids) results from decayed lignin deposits through microbial degradation of biomass. Breakdown of lignin in wood allows access to carbohydrates via attack of white-rot fungi on the cell wall. Bond cleavage in lignin by enzymes supported by other ecological impacts (light, temperature, and moisture) occurs in rotting woods or bacterial composting media (Hammel
Production of free radicals results in cleavage of linkages among lignin molecules by naturally occurring lignolytic enzymes (laccases, peroxidases) in white-rot fungi.

Tree bark contains naturally occurring polyphenolic compounds such as tannins. As a result of having numerous biological functions, the polyphenolics are auxiliary metabolites and have a wide range of properties, including anti-carcinogenic, antioxidant, antiviral, and anti-inflammatory properties (Noferi et al., 1997). Tannins have low susceptibility to oxidation because of their polyhydroxylated nature and are generally moderately insoluble in lipophilic media, which conceivably restricts their applications. Diverse alteration techniques have been investigated including esterification to enhance both their lipophilic solvency and relative stability. Both chemical and enzymatic acylation methodologies, used to combine different polyphenolic esters including longer chain unsaturated fatty acids, have been described (Suresh Babu et al., 2005).

The significant source for lignin degrading enzymes is microorganisms. Ligninolytic enzymes manufactured from distinctive microbial sources have been thoroughly recognized. For lignin degrading enzymes, fungi are considered the most effective source. The best known producers of these enzymes are the white rot fungi in contrast to brown rot and the soft rot fungi (Niladevi 2009).

By going with product divisions, the enzyme industry in United States is a multi-million dollar business. Products include: proteases, polymerases, carbohydrases and ligninases. The particular end clients are in distinctive fields like: pharmaceuticals & diagnostics, research/biotechnology, and other applications.

Developments in genetic and protein engineering work are towards improving the stability, specificity, and general application of laccase as a commercial enzyme. It is not surprising that the total of viable applications of this enzyme, not only in textile industry, is expanding consistently, when all the benefits of utilizing laccase are contemplated. The principle clients of the laccase-based advances are developed enterprises, for example, Lion Corporation (Japan), L’Oréal (France), Novo Nordisk (Denmark), and Henkel (Germany), and in addition, the textile industry has been rapid to adopt new catalysts (Polak et al., 2012).

Different aromatic and phenolic compounds can be utilized as dyes, particularly within the textile industry, through catalytic oxidation of laccases yielded from diverse sources. Laccases are considered as green catalysts/enzymes, in biotechnological processes, because of their wide range of substrates, versatile biochemical properties and high protein stability, and are useful for more applications in textile, pulp and paper industries (Polak et al., 2012).

In honey, the main antioxidant compounds are polyphenolics, flavonoids, enzymes (catalase, glucose oxidase), organic acids, ascorbic acid, carotenoid-like substances, amino acids and proteins (Gheldof et al., 2002). Furthermore, it has been reported that manuka honey contains a high amount of several phenolic compounds, such as syringic acid, 4-methoxyphenyllactic acid, methyl syringate, kojic acid, 5-methyl-3-furancarboxylic acid, leptosin, and unedone. These compounds were useful for characterizing manuka honey from the other kinds of investigated monofloral honeys (Oelschlaegel et al., 2012).

The Canadian forest sector is the world’s second largest supplier of woody biomass, behind the United States and as such, an annual supply of more than 200 million m$^3$ of biomass through commercial operations is reported (FAO, 2003; NRCAN, 2003). The pulp and paper industry is the major end user of the forest biomass and has been a major contributor to the North American economy for many years. Recently, the pulp and paper sector has been experiencing a downturn due to global influence of concurrent impacts such as competition from paper industries producing fibers from fast growing species, the high cost of the energy, and the reduced demand for newsprint and pulp (Helmerius et al., 2010).

The pulp and paper industry needs to identify additional economic strategies in the future to revitalize the forest sector and to strengthen its competitiveness in the current global market. The
concept of an integrated forest biorefinery has been suggested as an opportunity for the forest products industry to increase revenues and improve environmental sustainability (Mabee et al. (2005), Towers et al. (2007), Thorpe (2005), Carvalheiro (2008), and Mao et al. (2008). According to this concept, all the components of biomass can be fractionated and be utilized in a most profitable manner to make higher valued chemicals, fuels, materials, heat, and power in addition to the traditional core products (van Heiningen, 2006, Huang et al., 2008).

Currently, chemicals are extracted mostly from petrochemical resources. Due to fossil fuel depletion and climate change, there is a strong demand to develop an alternative woody feedstock and establish green biological methods for extracting biomass-derived chemicals.

Conversion of bark biomass by biological modifications into bark biopolymers/chemicals could be highly advantageous for the forest industry. However, no previous research has been reported that studied identification and characterization of bark-derived chemicals / biopolymers during submerged fermentation and particularly on replacement of natural mediators.

1.2 Scope of Research work

In this thesis study, bark was used as a raw material; bark polyphenolic extractives, fungal ligninases and laccases were examined during the study and bark polyphenolics/polyaromatics were characterized using different analytical and biotechnological approaches. Catalytic oxidation of fungal laccases was enhanced in the presence of honey used as a novel natural mediator substitute (NMS) in production media. Interaction of laccases and bark polyphenolics / polyaromatics eventually modified bark structure to produce bark derived chemicals.

1.3 Hypothesis

The following hypotheses were addressed in this thesis:

1. Ligninolytic fungal laccases could be highly efficient in depolymerize bark-biomass into lower molecular weight polyphenols.

2. A Brazilian isolated fungal strain could produce ligninases and thus is suitable for delignification of lignocellulosic biomass.
3. Honey can be a potentially used as a novel natural mediator substitute for bark depolymerization.

1.4 Research Objectives

The overall objectives of this thesis are to explore fungal derived laccases for depolymerization of bark into bark-derived polyphenols. Based on the research hypothesis, the following specific set of objectives was identified:

1. To elucidate the comparative treatments of bark using fungal derived laccases vs. other chemical conversion methods and to characterize bark polyphenols as a novel biochemical feedstock.

2. To examine the efficacy of a Brazilian isolated fungal strain for ligninases production and to identify the optimum temperature and pH for manganese peroxidase (MnP).

3. To investigate the role of fungal laccases in *Pleurotus pulmonarius* during submerged fermentation which converts bark into bark-derived polyphenols.

4. To conduct comparative studies between conventional phenolic mediator vs. honey as a new natural mediator substitute (NMS).

1.5 Thesis overview

This thesis is divided into eight chapters. Chapter 1 introduces thesis scope, hypothesis and research objectives. Chapter 2 reviews previous literature findings on fungal ligninolytic

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1 In 2012, I was in Brazil as part of an international exchange program, and conducted all experimental work in Embrapa Meio Ambiente, Jaguariuna, Sao Paulo. I found that a Brazilian isolated fungal strain was the best candidate among other fungal strains for producing ligninases, particularly manganese peroxidases. Due to complicated official procedure in transporting fungal strains between two countries authorities, Canadian Food Inspection Agency (CFIA), Canada, and Departamento Nacional de Inspecção de Productos de Origen Animal (DIPOA), Brazil. I was not able to bring the isolated fungal strain here in Canada to continue further work with bark using this particular type of fungal strain.
enzymes, interaction of fungal laccases vs. bark polyphenolics / polyaromatics, use of honey as natural mediator substitute (NMS), and compares synthetic and natural mediators. Knowledge gaps linked to bark biopolymers biological treatment are also summarized. Chapter 3 gives a detailed description of the materials, experimental methods, and microbial/analytical tools used during the study, including: bark polyphenolic extractives, microbial techniques for selection of potential fungal strains, time course study during submerged fermentation, ligninolytic enzyme assays, enzyme purification, characterization techniques e.g., total polyphenolics estimation, HPLC, thermal analysis, GC-MS/MS, MALDI-TOF/MS, and Py-GC/MS.

Chapter 4 focuses on comparative treatment of bark polyphenolic extractives. Chapter 5 and Chapter 6 explore ligninases production, purification, characterization, optimization of process parameters i.e., temperature and pH for extracellular manganese peroxidases and identification of fungal species by 18S rDNA. Chapter 7 investigates monofloral honey as a NMS during submerged fermentation for bark biopolymers characterization. Chapter 8 summarizes the main conclusions of this thesis and provides recommendations for future studies. The main thesis topics and their interrelationship are summarized in Figure 1.1.
Figure 1.1: Thesis overview and organization of thesis chapters.

- Introduction and literature review (chapter 1 & chapter 2)
  - Introduction & background
  - Literature review
  - Significance, hypothesis & objectives

- Materials and Methods (chapter 3)
  - Combined all experimental procedures and strategic plans to achieve specific research targets

- Comparative treatments for bark polyphenolics extractives (chapter 4)
  - Chemical, biological & physical treatments
  - Chemical composition of barks
  - Extractive yields of each bark after treatments

- Ligninases production, purification, optimization and identification of Brazilian isolated fungal strain (chapter 5 & chapter 6)
  - Screening of potential fungal isolates
  - Species identification by 18S rDNA
  - Ligninases production & purification of MnP
  - Optimization of temperature and pH for MnP
  - Morphological characterization and delignification pattern by MALDI-TOF/MS

- Monofloral honey used as NMS during SF for bark biopolymers characterization (chapter 7)
  - Extracellular laccases, purification & kinetic study
  - Honey in production media as NMS
  - Polyphenolics characterization
  - Categorization of bark biopolymers by Py-GC/MS

- Summary, conclusions and future perspectives (chapter 8)
  - Summary and conclusions
  - Recommendations for future work
2 Chapter: Literature review

2.1 Bark

Bark is considered as the outermost layers of stems and roots of woody plants existing outside of the vascular cambium. It mainly consists of the inner and the outer bark. The plants with bark include trees, shrubs, and woody vines. It makes up around 9-15% of the mill log by volume (Harkin et al., 1971). Bark assumes a critical part in a living tree with a complex structure and molecular arrangement. Bark has three primary roles: (1) giving supplement transport from the leaves to the rest of the tree, (2) preventing the inward cambium from drying up, and (3) providing the essential resistance of the tree against out of control conflagration, mechanical wounds brought on by overwhelming wind, and assaults by phytopathogens, phytophagous bugs, bigger beasts, and so on (Hon et al., 2000). In the wood industry, bark is a residue in forest operations and it is mostly burned as a part of the hog fuel.

2.2 Chemical configuration

Bark has relatively similar chemical composition to wood, yet it contains more extractives, higher lignin content, and less holocellulose (Table 2.1). The bark extractives can be organized into polar and non-polar extractives. The quantity of polar extractives, containing flavonoids, phenolics, glycosides, tannins, sugars, and so on, is typically three to five times more abundant than the non-polar compounds, including waxes, resins, lipids, unsaturated fats, sterols, terpenes, and so forth. Chemical composition of bark can be divided into divisions with distinctive polarity through successive extractions utilizing a combination of organic solvents and hot water. To analyze lignin and polysaccharides, bark is usually first extracted using aqueous alkali solutions; followed by hydrolysis of the extractive-free bark by sulfuric acid to give Klason lignin and acid insoluble residue contents (Hon et al., 2000; Harkin et al., 1971).

Table 2.1: General chemical configuration in wood and bark for hardwoods and softwoods (Harkin et al., 1971).
### 2.3 Bark polyphenolics

#### 2.3.1 Lignin

In bark, the polyphenolic part mainly consists of lignin, which is frequently depicted as a complex 3-D polymer having different types of linked phenylpropanoid units. In nature, it has an essential role to encase cellulose chains inside the ultra-structure of plant and wood fiber of the cell walls. Softwood and hardwood lignins may be distinguished by the presence of an extra methoxy group in the ortho-position of phenyl ring in hardwood lignins (Van Langenberg et al., 2010).

Mostly guaiacyl units are found in softwood lignin starting from the precursor, *trans*-coniferyl alcohol (Figure 2.1 (a), whereas hardwood lignin has combined guaiacyl and syringyl units originating from *trans*-coniferyl and *trans*-sinapyl alcohols (Figure 2.1 (b). Overall, hardwood bark lignins are mainly composed of syringyl, guaiacyl and small amounts of *p*-hydroxyphenyl units while softwood bark lignins have quite similar composition of syringyl-guaiacyl ratio but differ in higher proportion of *p*-hydroxyphenyl units, which come from *trans*-p-coumaryl alcohol (Figure 2.1 (c) (Hon et al., 2000).
10

**Figure 2.1:** Phenylpropenoid units in lignin precursors

2.3.2 Lignin valorization

Recently, there are significant efforts worldwide in finding higher valued application for lignin, especially lignin from the kraft pulping operation. Some research even focuses on finding favorable genetic variation in local populations of bioenergy crops and direct manipulation of biosynthesis pathways to create lignin feedstocks with favorable properties for recovery and downstream conversion. Refinement of biomass pretreatment advances has further encouraged lignin recovery. Coupled with biotechnology, there is a growing interests in uncovering new uses for this biopolymer, including carbon fibers, plastics and thermoplastic elastomers, polyurethane foams, biofuels, and biopolymers/chemicals as shown in (Figure 2.2) explained by Ragauskas et al., (2014).
Figure 2.2: Conceptual theme of lignin valorization and application of this renewable resource for value-added products by converting into carbon fibers, biopolymers, biochemicals, and biofuels (Source: Oak Ridge National Laboratory, U.S. Department of Energy, Referred by: Ragauskas, Science Reviews, 2014 with permission.)

2.3.3 Tannins

Tannins, with the molecular weights ranging from 500 to over 3000, are another important type of natural polyphenolic compounds present in a relatively large quantity in coniferous tree barks. The barks of some hardwood species, for example, Quercus, Eucalyptus, Acacia, and Salix also contain a lot of tannin extractives. Tannins can be structured into hydrolysable and condensed tannins based on their configuration and properties (Pizzi 1993; Van Langenberg et al., 2010).

2.3.3.1 Hydrolysable tannins

Polyesters originated from glucose can be considered as hydrolysable tannins, which could be sorted into: (1) gallotannins, which release gallic acid and its related products after acid hydrolysis. (2) ellagitannins, which liberate ellagic and valonic acids upon hydrolysis. Caustic hydrolysis of resorcinolic tannin has been reported to cleave the inter-flavonoid bond and open the etherocyclic ring joining the A and B rings of the flavonoid unit (Figure 2.3). Acid
hydrolysis has been shown to easily open the heterocyclic ring of polyflavonoids with the formation of a carbocation, which is capable of reacting with another nucleophile present (Pizzi 1993; Van Langenberg et al., 2010).

![Flavonoid unit](image)

**Figure 2.3:** Flavonoid unit

### 2.3.3.2 Condensed tannins

Catechins (flavan-3-ols) and leucoantocyanidins (flavan-3,4-diols) recognized as condensed tannins are comprised of flavonoid units. With the average degree of condensation ranging from 4 to 12 flavonoid units, it is commonly present as polymer and does not undergo hydrolysis. The condensed tannins constitute more than 90% of the total world production of commercial tannins.

The main structure of tannin extractives from quebracho, mimosa (black wattle), hemlock and Douglas-fir bark mainly composed of four to six linked flavonoid units where the A-ring is of resorcinol type and B-ring of pyrogallol type units (Figure 2.4), with a few flavonoid units entailing resorcinol A- and catechol B-ring.

In pine (taeda, aleppensis, patula, pinaster, radiata, eliotae, sylvestris, and so forth) species, the flavonoid units are of phloroglucinol A-ring and catechol B-ring (catechin group) mainly connected by four to eight bonds, with flavonoid units of phloroglucinol A-ring and phenol B-ring to a much lesser extent. The structures of the fundamental polymeric constituents of wattle and pine tannins are indicated in Figure 2.4 (Pizzi 1993; Van Langenberg et al., 2010).
Besides the flavonoid units, non-tannins including carbohydrates, hydrocolloid gums, amino and imino acid fractions also exist in the bark tannin extractives. The hydrocolloid gums with hydrophilicity varying in concentration from 3 to 6% contribute significantly to the viscosity of the extractives despite their low concentrations (Pizzi 1993; Van Langenberg et al., 2010).

2.4 Challenges in extraction of bark polyphenolics

Polyphenolic substances include many classes of compounds ranging from phenolic acids, colored anthocyanins, simple and complex flavonoids. Similarly, pine bark has low levels of monomers (Shi et al., 2005). During extraction, a solvent is blended with the plant material (pine bark). Extraction might be accomplished by the evaporation of a solvent and the solvent could be easily removed either by drying, or ultrafiltration (Shi et al., 2005). After any of these methods, the concentrate must be dehydrated to get a powder form and quite a considerable amount of organic solvents is required. Separation of polyphenols by the membrane method is considered more efficient than the organic solvent extraction method. It is important to make effective and productive extraction methods to ensure clean polyphenol product (Shi et al., 2005).

2.4.1 Chemical and physical properties of polyphenolics

Two or more monomers are synthetically reinforced to make oligomeric proanthocyanidins. The two proanthocyanidin monomers are catechin and epicatechin. A couple of procyanidins are shown in (Figure 2.5) with the structures of catechins (monomers). These are made when
dimers, trimers, and tetramers each of these two monomers joined at \( \alpha \) or \( \beta \) position on their molecular structures. Catechin and epicatechin can combine to produce esters, for instance, catechin/epicatechin gallate, comparably, like the bonds between sugars and proteins to make glycosidic and polyphenolic proteins. Around 162 dimers, including, gallic acid and glucose esters that could be made are reported by Bagchi (1999).

![Diagram of polyphenols](image)

**Figure 2.5:** Structures of major identified polyphenols in bark (Source: Shi, J. et al., Food Rev Int, 2005 with permission.)
2.4.2 Polyphenol–protein interactions

Polyphenols interact with protein molecules through hydrophobic or hydrophilic interactions. These interactions lead to formation of soluble or insoluble aggregates that depend on different factors such as pH, temperature and ionic strength. The formations of these aggregates are involved in hydrophobic stacking of aromatic groups of protein and polyphenols, or the interaction between hydroxyl groups of polyphenols with protein chains. Proteins are recognized to play a key role in many physiological activities owing to their stable 3-D structure. Afterwards, unfolding of protein chains, upon binding with polyphenols, is assumed to affect the physiological activity of protein molecules (Bennick, 2002; Naczk et al. 2006; Liang et al. 2008).

Proteins (dry weight ≥ 33%), linked by hydrogen bonding, can be involved in a variety of supramolecular interactions (Loomis, 1969). Other than hydrogen bonding there are different stable bonds that indicate spatial arrangement of polypeptide’s backbone and its subunits. Distinctive substances react non-enzymatically with compounds, such as o-quinones. The materials having amino, thiol, and activated methylene groups could be polymerized, diminished, or maintained by nucleophilic attack (Pierpoint, 1970). Proteins and polyphenolic compounds in this mode have similar bonding characteristics as the quinine and hydrogen bonding reactions.

Polyphenols are known to form complexes with proteins leading to modifications in the structural, functional and nutritional properties of both compounds. The different parameters such as, temperature, pH, protein type, protein concentration, and the structure of phenolic compounds can affect the protein–phenolic interactions (Ozdal et al., 2013). To measure proteins, as, for instance, the haemoglobin, gelatin, and BSA assays, this property of polyphenolic compounds might be utilized.

Several analytical techniques have been developed to characterize the polyphenol–protein complex formation such as fluorescence, circular dichroism (CD) spectroscopy, dynamic light scattering (DLS), Fourier transform infra-red (FTIR) spectroscopy, isothermal titration calorimetry (ITC), and nuclear magnetic resonance (NMR) and mass spectroscopy (ESI-MS) reported by Bandyopadhyay et al. (2012).
Figure 2.6: A model for protein–polyphenol interactions that elucidates having two sides that can join to protein. Proteins are defined as having a fixed number of polyphenol binding sites (Source: Siebert, K.J et al., J. Agric. Food Chem, 1996 with permission.)

A hypothetical model as shown in Figure 2.6 shows that proteins have several sites where polyphenol can bind. In this situation, each polyphenolic molecule should have binding sites where two proteins may attach. However, it is unlikely that there will be enough additional polyphenolic molecules to bridge many of these “sandwiches” or “protein dimers” together. Structure of proteins and polyphenols play a significant role to determine accumulated amount between them.

With an extensive abundance of protein in respect to polyphenol, every polyphenol particle ought to have the capacity to extend between two protein molecules; however it would be unlikely that these proteins would be further connected to others. This would come about for the most part in protein dimers. With excess polyphenol relative to protein, all of the protein binding sites would be occupied, but probably that bridging would occur between polyhydroxyproline contents. Polypeptides with higher percentages of proline tend to form more haze. The amount of haze
formed depends both on the concentrations of protein and polyphenol present in most beverage samples (Siebert K.J et al., 1996).

Currently, the chemical and biological aspects of proteins and polyphenols are challenging because of their applications in food, agriculture and their potential health benefits.

2.4.3 Interaction of low molecular weight (LMW) phenolics

There have generally been few studies conducted using protein and LMW-polyphenol interactions. Certain protein concentrates from sunflower seeds can create an unwanted brown color because of protein binding with the oxidation products of low molecular weight phenolic compounds, such as, chlorogenic acid (Sastry and Rao 1990). Currently, BSA was examined for its interaction with low molecular weight phenols (Bartolomé et al., 2000).

Protocatechuic and caffeic acid showed the most elevated binding for the protein, while p-hydroxybenzoic acid displayed the least binding limit; though, p-coumaric acid and (+)-catechin showed an irrelevant substance for protein-held phenols. The pH and the temperature play important roles in protein-polyphenolics interactions. Phenolic acids with single aromatic rings demonstrated much more significant interaction than multi-aromatic ring isoflavone. BSA-phenolic acid indicated substantial contrast in electrophoretic movement, and displayed total protein when contrasted with BSA (Bartolomé et al., 2000).

2.5 Biopulping

Biopulping is an industrial biotechnology process which utilizes different microorganisms, especially lignin-degrading fungi and enzymes (ligninases and xylanases) for converting wood chips into paper pulp. Biopulping provides an alternative solution to chemical and mechanical pulping. Ligninolytic enzymes attack lignin and decompose it, while xylanases degrade hemicelluloses and make the pulp more penetrable for the removal of remaining lignin (Ali and Sreekrishnan, 2001). Named 'biopulping', this methodology displaces lignin as well as a portion of the wood extractives, while reducing the pitch content and effluent toxicity (Ali and Sreekrishnan 2001).

Biotechnological tools are gradually used to replace chemical processes in a wide range of industries. No pilot-scale biopulping plants are in operation right now as this procedure is still in
its earliest stages (Ali et al., 2001). Mixed hydrolytic and oxidative enzymes have been well documented regarding biopulping and bio-modification of lignin, and thus provide an example which is related to effectiveness, despite the susceptibility of xylanases to deactivate by laccase-generated oxidants (Woolridge, E.M. 2014).

2.5.1 Role of ligninases in pulp delignification

LiP (lignin peroxidase, EC 1.11.1.7) and MnP (manganese peroxidase, EC 1.11.1.7) are Fe^{2+}-containing glycoproteins which necessitate H_2O_2 as an oxidant. The fungal growth releases a few isoenzymes into their development medium, although the enzymes may also be cell wall bound (Lackner et al., 1991). Non-phenolic lignin substructures oxidize LiP to a radical cation followed by a proton loss (Higuchi, 1989).

LiP catalyzes a large variety of reactions e.g., cleavage of β-O-4 ether bonds and Cα-Cβ bonds in dimeric lignin model compounds (Higuchi, 1989). LiP is released during secondary metabolism as response to nitrogen limitation. It is considered as strong oxidizer capable of catalyzing the oxidation of phenols, aromatic amines, aromatic ethers and polycyclic aromatic hydrocarbons (PAHs).

However, the degree of lignin biodegradation mainly depends on the environmental conditions and the fungal species involved (Archibald 1992). Previous studies mainly emphasized the mechanism of fungal degradation of lignin.

2.5.2 Oxidation of PAHs by synthetic LMS

The oxidation of polycyclic aromatic compounds (PAHs) was studied in laccase producing fungi *Trametes versicolor* and synthetic mediators were examined by Johannes et al. (2000). Enzymatic oxidation of acenaphthene, acenaphthylene, anthracene, and fluorene was mediated by different laccase substrates such as phenols and substituted amines or compounds produced and secreted by white rot fungi. There is an option of using wood-decay fungi able to produce hydrogen peroxide in presence of multi-enzyme systems or as mixed fungal cultures to improve decolorization.

The best natural mediators, such as phenol, aniline, 4-hydroxybenzoic acid, and 4-hydroxybenzyl alcohol were as effective as synthetic compounds e.g., ABTS [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] and 1-hydroxybenzotriazole. Natural compounds,
like methionine, cysteine, glutathione that contain sulfhydryl groups, were also observed as mediator compounds.

### 2.6 Fungal extracellular ligninases

Extracellular ligninases may be categorized as either phenol oxidases (laccase) or heme containing peroxidases (lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP)). Commonly, laccases use molecular oxygen as electron acceptors whereas; peroxidases use H$_2$O$_2$ as a co-substrate. White-rot fungi secrete one or more of the lignin-modifying enzymes (LMEs) as well as other compounds required for efficient lignin degradation (Mai et al., 2004).

Non-phenolic aromatic substrates have high redox potential ability to catalytically oxidize and cleave C–C bonds and ether (C–O–C) linkages. Manganese peroxidases (MnP) oxidize Mn(II) to Mn(III), to cleave non-phenolic compounds. Combination of LiP and MnP with a bifunctional part was protected by versatile peroxidases (VP) reported by Wong (2009).

The oxidative enzymes prepared for modifying such complicated and mixed structures are required. It was also felt that these enzymes did not require any cofactors, for example, NADPH since extracellular unit of such cofactors seemed, by all reasons, to be inactive. In 1984, Kuwahara et al. demonstrated that *Phanerochaete chrysosporium* excreted NADP$^+$ into growth media and hypothesized a reactant part for these cofactors play a main role in delignification.

Hall (1980) suggested that delignification can include non-enzymatic attack on the polymer by "activated oxygen" species; H$_2$O$_2$, superoxide, oxygen, or -OH radicals have been found in lignin degradation. Further studies including ligninases purification by use of different spectroscopy methods, and recombinant DNA molecular techniques, have improved our understanding at the molecular level of microbial delignification.

Relative molecular weight ranges between (41,000 to 42,000) had Fe$^{+2}$-containing ligninases defined by Kirk and Tien (1983). The glycoprotein non stereospecific (42,000 Mw) was stated by Tien and Kirk as detached from Cα-Cβ side chain in lignin, which, further oxidized benzyl alcohol to aldehydes or ketones. Therefore, it separated aromatic ring, hydroxylated benzylic methylene groups, and further catalyzed to oxidative coupling of phenols.

Ligninases can similarly catalyze H$_2$O$_2$- related oxidation of heterocyclic sulphur containing compound, thianthrene, to thianthrene monosulfoxide. Kirk and Tien (1983), shown degradation
of β-1 and β-O-4 containing model compounds from lignin. Ligninases oxidize an extensive variety of lignin model compounds. Kuwahara et al. (1984) additionally proved that molecular oxygen factors in the cleavage of the Co-Cβ link of 1-(3',4'-diethoxyphenyl)-1,3-dihydroxy-(4"-methoxyphenyl) propane by ligninolytic enzymes.
**Figure 2.7**: Degradation of oligomeric lignin model compounds by *P. chrysosporium* ligninases (A) β-1 and (B) β-O-4 (Proposed by Tien and Kirk 1984, *Proc Natl Acad. Sci. USA.*, with author permission.)

*Phlebia radiata* produces lignin-modifying enzymes (LMEs), that showed to be an efficient oxidant producing carbon-carbon bond cleavage of a dimeric, non-phenolic lignin model compound (Lundell et al. 1993a). The antigens prepared from these three ligninases (I, II, III) with molecular weights of 42,000, 45,000, and 44,000 cross-reacted with each other. *Pleurotus sajor-caju* produces two flavins containing veratryl alcohol oxidases (Bourbonnais and Paice 1988). The role of the oxidases in biodegradation might be to produce H$_2$O$_2$ and possibly superoxide anion radical (O·⁻₂), during oxidation of lignin fragments (Leonowicz et al., 1999).

Enzymatic systems employed by microorganisms for oxidative transformation of various organic molecules include laccases, ligninases, tyrosinases, monooxygenases, and dioxygenases. The targeted enzymes are important to the carbon-cycle through either transformation or complete mineralization of organic molecules (Sariaslani, 1989).

Microbial enzymatic systems involved in the oxidation of organic molecules were reviewed. Enzymatic systems such as mono- and dioxygenases, with their inherent stereo- and regio specificities, provide powerful tools for generating chemicals that are difficult to synthesize through conventional chemical routes. Successful commercial applications of these catalysts will be possible through new systems, e.g., immobilization of either integral microorganisms or isolated enzyme preparations on various supports, utilization of organic solvents in the reaction mixtures, and genetic engineering technology (Sariaslani, 1989).

### 2.6.1 Reaction mechanism of ligninases

The coupling reactions of a monomer at its β-position to chain polymer are present in β-linked structures (Wong 2009). The attained polymers have 5-5 and 5-O-4 linked structures completing coupling between two lignin oligomers. Changing lignin structure by dimerization to yield β-β (resinol) is rarely found in nature. End groups arising from coupling reactions are not at the side chain β-position of the monomer. The various linkages depend on the relative contribution of monomers to the polymerization process during lignin biosynthesis. For instance, the monolignol coupling in coniferyl alcohol to a guaiacyl (G) unit lignin oligomer/polymer yields G-β-O-4-G and G-β-5-G linkages as shown in (Figure 2.8).
Peroxidases and their structural properties related to the capacity to oxidize substrates of high redox potentials have not been completely recognized. Consideration of specific interactions with
mediators during biotransformation mainly supported that these catalysts are important for delignification. Finally, it was concluded that polymerization is directed by proteinaceous sites on a template, which, specify amongst linkage types and bond arrangements (Wong, 2009).

2.6.2 Laccase–Mediator Systems (LMS)

LMS perform as electron shuttles, offering oxidation of complex substrates (for example lignin polymers) where enzymes can’t access the active sites due to steric interferences. Once it is oxidized and stabilized, the mediators are dispersed far from enzymatic compartment because of their high redox potential (Bourbonnais and Paice, 1990; Kawai et al., 1989). An ideal redox mediator would be a small-size compound which is able to produce stable radicals so it also works in recycling without disintegration (Canas et al., 2010).

Previous studies proved that the *Trametes hirsute* laccase with 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS and Remazol blue used as mediators could oxidize nonphenolic lignin parts with high redox potential to produce veratryl alcohol and 1-(3,4-dimethoxy phenyl)-2-(2-methoxy phenoxy) propane-1,3-diol. Furthermore, laccase with ABTS can disintegrate a model lignin dimer, 1-(3,4-dimethoxyphenyl)-2-phenoxyethane-1,2-diol, to yield veratraldehyde and benzaldehyde. The oxidation of ABTS in presence of laccase is shown in Figure 2.11.

The presence of redox mediators is required for a number of biotechnological applications, to assist the oxidation of complex substrates not oxidized by the enzyme alone. However, it should not hinder the enzymatic response (Johannes et al., 2000). However, the oxidizing mediator could depend on the oxidation mechanism not on the enzyme, so it is possible to oxidize non-phenolic substrates (Fabbrini et al., 2002), subsequently shutting down the sequential catalytic cycle (Figure 2.9).

![Figure 2.9: The sequential catalytic cycle of LMS](Source: Fabbrini, 2002, *J Mol Catal B-Enzym*, with permission.)
The sequential catalytic cycle incorporates a set of several compounds, to meet the condition of several reaction cycles in the redox process.

Distinctive structures of transition segments i.e. (K₃[Mo(CN)₈].H₂O and K₃[W(CN)₈].H₂O) Fe²⁺ complex with o-phenanthroline and 4,4'-dimethylbipyridine, and some natural laccase substrates, e.g., ABTS and 2,2,6,6-tetramethyl-1-piperidinylloxyl (TMPO). For stable nitroxy radical, TMPO is different because it is oxidized by laccase and produced oxoammonium ion (oxidation to >N=O⁺ that correspond to high redox fungal laccase), which follows a non-radical-ionic-mechanism (Fabbrini et al., 2002). Conventional redox potentials have well defined transition metal complexes and can oxidize non-phenolic lignin structures for the most part at low concentrations in the reaction mixture.

ABTS is an organic compound suitable for the expression of "redox mediator". Oxidation of non-phenolic lignin structures offers a way to look at new laccase mediators. The use of eco-friendly mediators, can contribute to the industrial implementation of laccases and in the development of lignocellulosic biorefineries. The following N-OH laccase mediators and their structural formulas (A-E) are shown in Figure 2.10.
Figure 2.10: N–OH type laccase mediators and their chemical structures (A-E); A=1-Hydroxybenzotriazole (HBT); B= N-Hydroxyphthalimide (HPI); C= N-Hydroxyacetanilide (HAA); D= Violuric acid (VA); E= 2,2,6,6-Tetramethylpiperidin-1-oxyl (TMPO).

Figure 2.11: Oxidation of ABTS in presence of laccase (Source: Morozova, 2007, Appl Biochem Microbiol Reviews, with permission.)
2.6.3 Natural mediators / phenolic enhancer

Natural mediators, in presence of laccases, expedite the oxidation of non-phenolics but it depends on the phenolic compound structure, as well as the reactivity vs. stability of phenoxy radicals produced (MS\(^{+} > \) AS\(^{+} > \) SA\(^{+}\)) (Tania et al. 2012). Due to the complex nature of biomass, degrading enzymes, and their interactions, researchers are trying to profile radical-coupling routes involved in the development of several phenolic species that are classified as mediators. Moreover, there is a need to improve relationship between biomass structure, effectiveness of enzymatic hydrolysis and the biomass recalcitrance (Foston et al., 2012).

In presence of redox mediators, laccases enhance their substrate specificity. The enzymatic oxidation of syringyl-type phenolics identified as natural mediators, such as methyl syringate (MS), acetosyringone (AS) and syringaldehyde (SA), participate in phenolic oxidation depending on the negatively charged residues similar to a substrate binding site of the enzyme.

2.6.4 Biotransformation

Biotransformation is a series of biological modifications / conversions made by a living organism on a chemical compound. In the early 1990’s, the oxidation of phenolics by ligninases was investigated for dimethoxylated compounds (veratryl alcohol and 3,4-dimethoxyphenyl acetic acid). In all examples, polyphenolic compounds were found to be exclusively oxidized in contrast with the dimethoxylated compounds. Ligninase catalyzes continuing delignification rather than further polymerization; a model to define the framework is displayed by Harvey et al. (1990).

Furthermore, the ligninases, lignin and phenolic lignin are broken down into small fragments and are connected to radical cations as mediators. If the free phenolic compounds are accumulated then eventually it causes inhibition of ligninases, which prevents lignin cleavage and produces more polyphenolics. Hence temporary inhibition of ligninases by phenols may play some role in metabolic regulation of lignin (Harvey et al., 1990).

2.6.4.1 Coupling routes for polyphenolics oxidation

In the laccase mediator system (LMS), the catalytic cycle demonstrated an interchange of paths including phenoxy radical as an intermediate. When they are oxidized in an enzymatic system, a proton and an electron lead to discharge radicals and release phenolic substrates. In order to oxidize non-phenolic compounds by laccases, the phenoxy radicals are involved in three different possible routes. Moreover, it can either be combined with radical recombination, cross-coupling
or self-coupling to continue with or without release of substituent groups which further change into other coupling structures (Figure 2.12).

**Figure 2.12:** The catalytic cycle of LMS showing different paths implicated in development of several phenolic species (Source: Rosado et al., *Bioresource Technol.*, 2012 with permission.)

The newly identified radical coupling routes which involved several C–O coupling reactions, as well as, different dimeric and trimeric structures were proposed (Tania et al. 2012). The catalytic cycle supports the demand of added phenolic compounds in laccase mediator system which explains the role of phenolics as “mediators” rather than “enhancers” (Díaz-González et al., 2011).

### 2.6.4.2 Coupling routes for non-phenolics oxidation

For oxidation of recalcitrant aromatic compounds, the >N–OH mediators, for instance, 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI), violuric acid (VA) or N-hydroxyacetanilide (NHA) (Xu et al. 2000) are the possible laccase mediators; their mechanism is
shown in (Figure 2.13). Laccase produces an extremely reactive nitroxy radical (> N–O•), owing to the enzymatic removal of an electron by release of a proton and an oxidation of this type of mediators. The target substrate oxidized nitroxy radicals by hydrogen atom transfer (HAT) route (Xu et al., 2000).

A mediator-to-substrate molar ratio can affect the efficiency of oxidation procedure. The low redox potential substrates could be responsible for the oxidation of non-phenolic substrates under non-catalytic conditions (Cantarella et al., 2003).

![Diagram](image)

**Figure 2.13**: Non-phenolic lignin model compound oxidation by laccase mediator system (LMS) ensuing by two different oxidation methods: ET (electron transfer) and HAT (hydrogen atom transfer) (Source: Fabbrini et al., *J. Mol. Catal. B: Enzym*, 2002 with permission.)

### 2.7 Function of laccases with combination of natural mediators (NMs) for lignin biodegradation

The phenolic compounds related to phenolic structures in lignin are able to help oxidation of non-phenolic compounds by radical mechanism. This finding may support the role of laccases in lignin biodegradation with no other mediators. Therefore, oxidation of phenolic lignin units with laccases or ligninolytic peroxidases would release phenolic natural mediators as shown in (Figure
2.14) that could help in start oxidation of more recalcitrant non-phenolic lignin moiety (Canas et al., 2010).

Lignin removal from biomass by using laccases might be achieved by various methods, depending on different types of lignocellulosic material and its further use. Alternatively, the phenolic residues in lignocellulosic material i.e., (p-hydroxycinnamic acids) highly abundant in herbaceous plants, could act as in situ laccase mediators which assist to remove recalcitrant lignin moiety as has already been indicated with model compounds (Cho et al., 2008; Nousiainen et al., 2009).

Figure 2.14: Conceivable function of laccases with combination of phenolic natural mediators (NMs) in decontamination of industrial effluents and soil bioremediation (Source: Canas et al., Biotechnol Adv, 2010 with permission.)
2.7.1 Monofloral honey as mediator

The composition of honey, a natural bee product, mainly depends on different botanical sources, topographical origin and atmospheric conditions (Chen et al., 2000; Vela et al., 2007; Alvarez et al., 2010). In honey, the main antioxidant compounds are polyphenolics, flavonoids, enzymes (catalase, glucose oxidase), organic acids, ascorbic acid, carotenoid-like substances, amino acids and proteins. The antioxidant value varies significantly depending on the floral source (Gheldof et al., 2002). Several phenolic compounds are found in honey, including syringic acid (SA) and methyl syringate (MS). Lately, a compound was obtained having substantial antibacterial activity against *Staphylococcus aureus* (Alvarez-Suarez et al., 2010).

2.8 Usage of laccases and NM for future lignocellulosic biorefineries

The utilization of laccases and their natural mediators represents a promising alternative for environmentally friendly delignification of paper pulp. These enzymatic frameworks could be incorporated at different phases of biopulping and paper production (Camarero et al., 2007). As by-products from pulp production, these natural phenolic mediators can be obtained. Eucalypt kraft pulping black liquor possesses a large amount of some of these phenolic compounds (e.g., acetaldehyde syringone and syringaldehyde) which may be sufficiently separated by little efforts from pulp and paper industrial effluents (Camarero et al., 2005).

Functionalization of wood and non-wood by laccases has not been completely explored (Chandra and Ragauskas, 2002). Figure 2.15 indicates biotechnological application of laccases (and natural mediators), which can contribute most significantly to develop Integrated Lignocellulosic Forest Products Biorefineries (ILFPB). Wood products (boards and panels) used in furniture manufacturing are bonded with high cost adhesives derived from petroleum. Most of these adhesives are formaldehyde-based resins, due to its harmful emissions to limit the reuse of fiberboards (Felby et al., 1997). Therefore, laccases can contribute to reduce (or eliminate) the use of these synthetic adhesives by catalyzing the cross-linking of polyphenolic residues in lignin based materials to produce medium density fiberboards (Canas et al., 2010).

Biografting of phenols onto different types of pulps can provide new physico-mechanical properties to paper such as improved strength due to promotion of new hydrogen bonding and crosslinking between phenoxy groups on pulp surface (Chandra et al., 2004). Laccase-assisted grafting of phenols can also modify optical properties like color. Protection against
antimicrobial activity can be obtained using an enzymatic method to make cellulosic products for food packaging or cleansing materials (Elegir et al., 2008).

**Figure 2.15:** Strategic plan of Integrated Lignocellulosic Forest Products Biorefinery (ILFPB): where, (1) different lignocellulosic reserves; (2) extraction of hemicelluloses to pulping; and (3) biomass gasification. The main features where enzyme technology may help twice as notably to this idea are also shown: (a) assembling of wood (or lignocellulose) products; (b) create of more proficient and environmentally friendly industrial procedures for pulp and paper production; (c) extraction of phenolic mediators and reusing of black liquors; (d) production of cellulosic products with modified characteristics; or (e) enhanced biofuel production (Source: Cañas et al., *Biotechnol Adv*, 2010 with permission.)

In nature, these phenolic compounds (released during lignin depolymerization or present as free acids in plants) perform as natural mediators. Moreover, by the application of these enzymes, sustainable industrial processes are able to move forward by the presence of these environmentally-friendly mediators, easily available from lignocellulosic biomass (Canas et al., 2010). Therefore, there are important economical, ecological and environmental reasons for achieving better understanding of fungal activities and their implementation in lignocellulosic green biotechnology (LGB) (Harms et al., 2011).
3 Chapter: Experimental Methodology

Research approach for objective-1

Synopsis

Comparative studies were conducted among treatments to characterize bark polyphenolic extractives that can be effectively used for green adhesive production, leading to environmental sustainability.

![Diagram of strategic plan](image)

**Figure 3.1:** Design of strategic plan for objective-1. Reproduced by the permission of American Chemical Society (ACS) [http://pubs.acs.org/doi/abs/10.1021/sc400184f](http://pubs.acs.org/doi/abs/10.1021/sc400184f)

3.1 Chemicals and standards

Sodium hydroxide (NaOH), sulphuric acid (H₂SO₄) and acetic acid (CH₃COOH) were purchased from Caledon Laboratory Chemicals. Catechol, tannin, Folin-Denis reagent and sodium chlorite (NaClO₂) were purchased from Sigma-Aldrich. Cellulose extraction thimbles
and grade 42 filters were purchased from Whatman. Mountain pine beetle-infested lodgepole pine and mixed aspen barks were provided by FP Innovations and ground to a particle size below 0.212 mm (US70 mesh size). All of the chemicals used in this study were reagent grade and were used without further purification.

3.2 Chemical Composition of Bark before and after Extraction

3.2.1 Ash Contents in Bark

Ash contents in the bark were determined by oxidizing dry bark at 580°C with a TGA instrument. About 10 mg of oven-dried sample was weighed in a platinum pan and heated from room temperature to 580°C, at a heating rate of 10°C/min using a thermal gravimetric analyzer (TGA-Q500, TA Instruments, USA). The final ignition temperature was 575°C according to the ASTM D1102-84 method-suggested temperature for the determination of ash contents in wood. Bark ash contents were reported as the percentage of residues remaining after treatment. Five samples of each bark were tested to determine the standard deviation.

3.2.2 Preparation of Extractive-Free Bark

Extractive-free bark was prepared according to ASTM D1105-96. Ethanol–toluene extractives and hot water extractives contents in bark were then reported. The ethanol–toluene extractive values listed in this paper refer to the bark extraction with successive ethanol–toluene mixtures and 95% ethanol.

3.2.3 Lignin Content of Bark

The lignin contents of beetle-infested lodgepole pine (BILP) and aspen bark were determined according to a modified procedure for the evaluation of acid-insoluble lignin content in wood and pulp according to standard TAAAPI method T 222 om-02 (TAPPI, 2002).

Briefly, 0.2 g of oven-dried extractive-free bark was combined with 2 mL of 72% sulfuric acid and incubated at 30 °C for 1 h. Fifty mL of distilled water was then added, and the mixture was autoclaved at 120 °C. The solution was then filtered into glass crucibles, grade 40–60, and lignin content was determined using eq 1.
where $m_{be}$ is the oven-dry weight of extractive-free bark, $m_b$ is the oven-dry weight of bark; $m_{lc}$ is the oven-dry weight of crucible and of lignin, and $m_c$ the oven-dry weight of crucible.

3.2.4 Holocellulose and $\alpha$-cellulose content of bark

Holocellulose and $\alpha$-cellulose contents in extracted bark were determined following a procedure developed by Browning, (1967).

0.5 g of oven-dried extractive-free bark was weighed and 16 mL of buffer solution (60 ml of acetic acid and 1.3 g of sodium hydroxide in 1 litre of water) was added. The mixture was placed in a water bath at 70°C and 1 mL of 27% (w/v) NaClO₂ was added every 30 minutes for the following 4 hours. The filtered holocellulose was separated in two sets of experiments. The first set was oven-dried at 65°C and weighed while the second set of filtered holocellulose was kept in the desiccator for 24 hours, and then transferred into beakers for the $\alpha$-cellulose determination. Three mL of 17.5% NaOH solution was added to the prepared holocellulose and incubated at 20°C, followed by another 6 mL after 5 min. The total treatment lasted 45 minutes. Then distilled water was added, and the mixture was allowed to stand for 1 hour. Once the caustic treatment was completed, the solution was filtered under vacuum into 40-60 grade crucibles and washed with 30 mL of distilled water. Crucibles and samples were then oven-dried overnight at 65°C. Holocellulose and $\alpha$-cellulose contents were determined according to equations 2 and 3.

Lignin (%) = \( \left( \frac{m_{be}}{m_b} \right) \times \left( \frac{m_{ic} - m_c}{m_{be}} \right) \)  

Equation 1

\[
\text{Holocellulose} \% = \left( \frac{m_{be}}{m_b} \right) \times \left( \frac{m_{bc} - m_c}{m_{be}} \right) 
\]  

Equation 2

\[
\text{$\alpha$-cellulose} \% = \left( \frac{m_{be}}{m_b} \right) \times \left( \frac{m_{ac} - m_c}{m_{be}} \right) 
\]  

Equation 3
where $m_{hc}$ is the oven-dry weight of holocellulose and crucibles, $m_{ac}$ is the oven-dry weight of $\alpha$-cellulose and of crucible and $m_{c}$ is the oven-dry weight of the crucible.

### 3.2.5 Enzymatic treatment

Enzymatic hydrolysis was performed using a cocktail of three enzymes. They included cellulase (E.C. 3.2.1.4) from *T. reesei* (6.3 U/mg) and $\beta$-glucosidase (E.C. 3.2.1.21) from almonds (7.55U/mg) (both enzymes were purchased from Biochemika-Fluka; the purity of both enzymes is not given in the certificate of analysis describing the product specifications from Sigma-Aldrich (Appendix). Novozym® 51003 laccase (E.C.1.10.3.2) from genetically modified *Aspergillus sp.* (1000 LAMU/g = 3.57 IU/ml/min) was obtained from Novozymes (Franklinton, NC, USA). Enzyme reactions were performed at a solid:liquor ratio of 1:10 (w/v) by inserting ground bark (BILP and aspen bark) in 0.2 M sodium acetate / acetic acid buffer (pH 5.6), containing 10 U of each enzyme, per gram of bark at 45°C for 48 h. The enzymatic treated flasks were agitated in a rotary shaker at 150 rpm. After hydrolysis, 600µl of extractives were removed from the treated barks, passed through a 0.22µm filter and stored for subsequent analysis of phenolic compounds.

### 3.2.6 Alkaline Treatment

Bark powders were extracted using 1% NaOH solution with a solid:liquor ratio of 1:10 (w/v), at 100°C for 120 min. Bark soluble fractions were filtered and the liquid fractions were collected and stored at -10°C. Bark residues were washed with excessive hot water and oven-dried at 65°C to constant weight. The extraction yield was calculated based on equation 4.

\[
\text{Yield} (\%) = \frac{m_b - (m_{bef} - m_f)}{m_b}
\]

Equation 4

Where $m_b$ is the oven-dry bark weight, $m_{bef}$ is the weight of the oven-dried bark after extraction and $m_f$ is the oven-dry filter weight.
3.2.7 UV/H\textsubscript{2}O\textsubscript{2} treatment

Beetle-infested lodgepole pine (BILP) and aspen bark samples were treated with UV/H\textsubscript{2}O\textsubscript{2} in 300 ml Erlenmeyer flasks under room temperature conditions. The ground barks were combined with 1% NaOH at a solid: liquor ratio of 1:10, and then exposed with conventional UV lamps (Philips, 40Watt) for 3 hours with 100mM of H\textsubscript{2}O\textsubscript{2}. Hydrogen peroxide concentration of 100mM was adjusted due to acting as a free-radical eliminator at higher concentrations, as reported by Kusic et al. (2006), which decreases the amount of hydroxyl radicals in the solution.

3.2.8 Sample preparation for HPLC

After three consecutive treatments, the total phenolic contents from bark extract were assessed by dissolving the extractives in hot-water, according to the method reported by Yu et al. (2001). Bark extractive samples (0.1 g) were mixed with 2 mL of hot water in test tubes and heated for 1h in a boiling water bath. The samples were cooled to room temperature and centrifuged at 10000g for 10 min (model Avanti® J-E Centrifuge, Beckman Coulter, USA). Samples were filtered through a 0.45-µm filter and analyzed by HPLC. The treatments selected in this study allowed extracting both polar and non-polar fractions from bark, and, thus, a tiny fraction of the extractives was not completely dissolved as the HPLC sample preparation stage was completed. Consequently, the following study on bark polyphenolic extractives only considered the hot-water soluble fractions.

3.2.9 HPLC analysis

The HPLC system included a Dionex BioLC 20 Series HPLC instrument equipped with a GP50 gradient pump, AS40 auto sampler, AD25 absorbance detector and Chromeleon for data collection and analysis (CMS), all of them from Dionex Technologies (USA). 10µl of sample was injected by the auto-sampler. A Hypersil ODS C18 column (100 mmX4.6 mm, particle size 3µ) from Alltech (USA) was used for chromatographic separation.

For elution, the two mobile phase gradients were used with a flow rate of 1.3mL/min: 0.1% formic acid in aqueous solution as (eluent A) and acetonitrile (ACN) as organic mobile phase (eluent B). The initial isocratic range started from 0-4 min at 5% ACN, and pursued a linear increase until reaching 23% after 10 min. To obtain better separation of polyphenols, an isocratic range from 10-15 min at 23% ACN was applied. ACN concentration was further
increased, linearly, to 50% within 4 minutes to elute the most preserved analytes directly, followed by reverting back to the initial conditions from 50-95% for 1 min. Chromatograms were recorded at three different wavelengths, 280, 310 and 370 nm. The bark polyphenolic extractives were separated and characterized based on their reported retention time ($t_r$) values (Aznar et al., 2011).

### 3.2.10 Folin-Denis method

The Folin-Denis method is a common spectrophotometric method to assess the total phenolic content in the samples. An intense blue colour was produced after 30 min in the reaction mixture due to the reduction of phenol (Iolanda da Cruz et al., 1998). The Folin-Denis reagent is a combination of phosphomolybdic - phosphotungstic acid in alkaline solution. In this study, catechol was used as a reference standard in place of tannic acid or gallic acid. Thus, the total phenolic contents were calculated as catechol equivalents from the calibration curve (mg catechol equiv/g of treated dry extract).

### 3.2.11 SEM for BILP and Aspen bark

Samples of beetle-infested lodgepole pine (BILP) and aspen bark produced after enzyme, alkaline and UV/H$_2$O$_2$ treatments were oven-dried at 50°C for 1 h and a dense layer of samples was kept in the sample-holder fixed on a carbon ribbon. The sample assembly was maintained in plasma for 60 seconds in a SC7620 Mini Sputter Coater (Polaron) purged with Argon to eliminate air from the samples. SEM with a Jeol model JSM-6610LV microscope supplemented by an Oxford/SDD EDS detector was used for observing the bark samples before and after treatments (Ferhan et al., 2012).
Research approach for objective-2

Figure 3.2: Design of strategic plan for objective-2

3.3 Fungal strain isolation

The unknown fungal strain was isolated from the Northeast part of Brazil called Caatinga. It is a Tupi word meaning white-forest and belongs to one of six major ecoregions of Brazil. Caatinga has a semi-arid climate and covers an area of nearly 735,000 km², although < 1% of this semi-arid zone is preserved (da Silva 2003).
The leaf samples of *Combretum leprosum* were collected in the tropical dry forest (Caatinga), in the Brazilian semi-arid region (S05°55′33.7″, W39°31′44.4″) (Santos et al. 2012). The leaf samples were brought to the laboratory in sterile polythene bags and processed within 24 h of collection.

The leaf samples were cut into 2 cm pieces and left on a Petri dish containing PDA (potato dextrose agar) and 150 mg L\(^{-1}\) chloramphenicol with approximately 4–5 pieces on each dish. The material was incubated at 25°C for 10 days and hyphal tips of the fungus protruding from the inner leaf segments on the plates were further purified and transferred to slants. The fungal strain was banked in (CCMAEI) Culture Collection of Microorganisms of Agricultural and Environmental Importance, Embrapa Meio Ambiente, Jaguariuna, SP with accession number: CFE044.

### 3.3.1 Substrate collection and preparation

Sugarcane bagasse and *Eucalyptus* lignin were used for delignification. Sugarcane bagasse and *Eucalyptus* were collected from LWART Quimica, Brazil. All substrates were dried in an oven at 80°C to constant weight and were powdered using an electric grinder and stored in airtight glass containers to keep out moisture.

### 3.3.2 Fermentative organism and culturing conditions

*Pleurotus ostreatus* and *Pycnoporus sanguineus* were obtained from the Mycology Collection Lab, Department of Plant Protection, UNESP, Botucatu, SP, whereas the local fungal strain was isolated from the Northeast part of the country in the Caatinga forest. All fungal strains were plated on malt extract agar medium (malt extract 25 g; agar 20 g; distilled water 1L) containing 0.05% of the dye Remazol Brilliant Blue R (RBBR). Plates were incubated in the dark for 14 days at 25°C. Ligninolytic activity was assessed by scoring for the presence of a halo of decolorized dye surrounding the fungal colonies.

### 3.3.3 Quantification of dye Remazol Brilliant Blue R (RBBR) oxidation in liquid culture medium

Out of ten strains, seven exhibiting ligninolytic activity were then grown in broth culture media containing (2.5% malt extract and 0.05% RBBR). All the cultures were kept in the dark and incubated at 30°C, aerated by constant shaking. The presence of ligninase activity, as evidenced by decolorization following oxidation of polymeric dye, was determined at 12 days by
monitoring the decrease in the absorption peak at $\lambda_{595}$ nm using a Shimadzu UV-1601PC spectrophotometer. Cell biomass was measured gravimetrically by oven-drying at 70°C. All shake flask experiments were tested for ligninolytic activity in replicates of three.

3.3.4 Growth Media preparation

Growth media for *P. ostreatus* contained: Glucose 10g/L, L-Asparagine monohydrate 3g/L, MgSO$_4$.7H$_2$O 0.05g/L, KH$_2$PO$_4$ 0.5g/L, K$_2$HPO$_4$ 0.6g/L, CuSO$_4$.5H$_2$O 0.4mg/L, MnCl$_2$.4H$_2$O 0.09mg/L, H$_3$BO$_3$ 0.07mg/L, NaMoO$_4$.2H$_2$O 0.02mg/L, FeCl$_3$ 1mg/L, ZnCl$_2$ 3.5mg/L, Thiamine-HCl 0.1mg/L, Biotin 5μg/L (Trupkin et al., 2003). The medium was dispersed into 250 mL Erlenmeyer flasks at a rate of 50 mL of medium per flask adjusted pH to 6.5 with 1N NaOH and autoclaved at (121°C) for fifteen minutes. A loop with fungal strains was transferred to the sterilized growth medium under sterile conditions and the flasks were incubated at 30°C in a shaker (130 rpm) with continuous shaking.

3.3.5 Optimization of temperature and pH for MnP activities

During submerged fermentation the process parameters, like temperature and pH, involved in the production of both fungal growth and ligninolytic activities were optimized in shake-flask experiments. Mainly two variable process parameters were examined i.e., incubation temperature (25-35°C), and pH of production media (4.5-7). The two production process parameters of Brazilian isolated fungal strain were optimized mainly for manganese peroxidase (MnP) activities.

3.4 Ligninolytic enzyme assays

The extracellular enzyme manganese peroxidase (MnP) activity was quantified spectrophotometrically at $\lambda_{610}$ nm ($\varepsilon = 4460$ M$^{-1}$cm$^{-1}$) using the methodology described by de Souza Silva, C.M.M. et al., (2005) The reaction mixture / ml contained: 500 μl of culture medium supernatant; 100 μl of phenol red (substrate); 100 μl of 250-mM sodium lactate solution; 200 μl of 0.5% bovine serum albumen (BSA); 50 μl of 2-mM MnSO$_4$; and 50 μl of H$_2$O$_2$ from 2- mM sodium succinate buffer prepared from 20-mM, pH-4.0 stock solution. The reaction mixture was kept in a water bath for 5 min and incubated at 30°C; reactions were terminated by the addition of 40 μl of 2N NaOH solution.

The oxidation of 2,2’-azino-bis(3-ethylbenzthiazoline)-6-sulfonate (ABTS) at $\lambda_{420}$nm ($\varepsilon=36,000$M$^{-1}$cm$^{-1}$), which is indicative of laccase (Lcc) activity, was measured
spectrophotometrically according to the method used by Machado et al., (2006). The reaction mixture included 100µL of 0.3 mM-ABTS (substrate) in 100 mM of NaOAc-buffer (pH-3.5) and 100µL of crude enzyme from culture supernatant solution, was incubated at 40°C for 1 min. The oxidation of ABTS was observed by an increase in absorbance at λ_{420} nm as well as changes in blue colour intensity due to presence of the Cu^{+2} ion in laccases. For all peroxidases, the unit activity is defined as the amount of enzyme required to oxidize 1 μmol of substrate / minute.

Lignin peroxidase (LiP) activity was assayed by measuring the oxidation of veratryl alcohol to veratryl aldehyde by a UV spectrophotometer at λ_{310} nm (ε = 9300 M^{-1}cm^{-1}) as reported by Tien and Kirk (1984). The reaction mixture contained 375µl of 0.33-M sodium tartrate buffer pH-3.0; 125 µl of 4 mM-veratryl alcohol (substrate); 50 µl of 10 mM-H_{2}O_{2}; 450µl distilled water and 250 µl of culture medium supernatant for a final volume of 1250 µL.

### 3.5 18S rDNA amplification and sequencing

The family identification of the unknown fungal strain was carried out on the basis of 18S-rRNA homology carried out by National Centre for Biotechnology Information (NCBI) BLAST service and Gene Bank. The genomic DNA was extracted according to the procedures of Power kit, DNA isolation kit (MOBIO, Carlsbad, USA), and ITS4 (5′ TCCTCGCCGCTTATTGATATGC 3′) - EF4 (5′ GGAAGGGRTGTATTATTTAG 3′) primers set was chosen to amplify a major part of the 18S rDNA, resulted in intense 183 PCR product of approximately 550–820 bp in size for each sample.

Polymerase chain reactions were carried out on a Dyad DNA Engine thermal cycler (MJ Research, Waltham, USA) using 50 ml reaction volumes containing approximately 50 ng of template DNA, 20 pmol of each primer, 2 mM MgCl_{2}, 250 mM of each of dATP, dCTP, dGTP and dTTP, 10 buffer [20 mM Tris–HCl (pH 7.5), 100 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5 % Tween 20 (v/v), 0.5 % Nonidet P40 (v/v), 50% glycerol (v/v)], 1ml of a bovine serum albumin (BSA) solution (20 mg ml^{-1}) and 2.5 U of Expand High Fidelity DNA polymerase. Cycling parameters were: (1) 94 °C for 2 min; (2) 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and (3) 72 °C for 10 min (Santos et al., 2012; White et al. 1990).
The purified PCR products were used in sequencing reactions with the same set of primers using a BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems). Sequencing was performed on an ABI 3100 sequencer (Applied Biosystems). The obtained sequence was subjected to BLAST queries using the “blastn” algorithm implemented at NCBI to determine the putative identity of the strain (www.ncbi.nlm.nih.gov/BLAST). Based on sequence homology the first hit on BLAST was *Dothioraceae* sp. LM572 with accession number EF060858.1 (Mahdi et al., 2008; Ferhan et al., 2013).

### 3.6 Ligninases cocktail partial purification and characterization

The total proteins in 2L of broth culture filtrate were precipitated by the addition of ammonium sulfate (80% saturation); all the steps were carried out at 4°C. The ammonium sulfate precipitate was collected by centrifugation (5000 g x 30 min) and dissolved in 50 mM potassium phosphate buffer, pH 6.5 (buffer A). The dissolved precipitate was dialyzed overnight in Buffer A and then loaded onto a DEAE Sepharose Fast Flow column (10 x 300 mm) equilibrated with buffer A.

The column was washed with buffer A and then eluted at a flow rate of 0.5 ml/min with a 0 to 1.0 M linear gradient of sodium chloride in buffer A. MnP fractions were collected, assayed, and dialyzed overnight against buffer A and then loaded onto a Superdex 75 column (10 x 300 mm), which was also equilibrated with buffer A.

30 ml MnP fractions were collected and concentrated to 5 ml by ultrafiltration with a Centriprep-3 (3 kDa cut-off, Amicon). The concentrated MnP fraction was adjusted to 100 mM sodium chloride and loaded onto a Sephadex G-100 column equilibrated with buffer A, in a fast protein liquid chromatography (FPLC) system (Pharmacia, AKTA purifier). Fractions were observed by a UV detector using Unicorn 5.11 software of Pharmacia. At this phase, the enzyme activity corresponded to a peak of absorbance observed at 280nm and eluted as a single peak. The purified and concentrated enzyme was preserved at -20°C and did not exhibit any noteworthy loss of enzymatic activity over several months (Kenji at al., 2000).
3.6.1 Scanning electron microscopy (SEM)

Sugarcane bagasse and the eucalyptus black liquor samples were kept in an oven and dried at 50°C for 1h; thick layers of the samples were spread on a carbon ribbon in the sample holder. Until analysis, the sample assembly was maintained in a vacuum desiccator. The SEM analysis of lignin samples before and after fungal treatment was observed using a Jeol model JSM-6360LV microscope (Goncalves et al., 1998).

3.6.2 Determination of total proteins

Total proteins were quantified by absorbance at 595 nm according to the Bradford assay method (1976) using BSA as a standard.

3.6.3 Gel electrophoresis and staining

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed as described by (Hofer et al., 1999) to determine the molecular weight of proteins. Gels were stained with Coomassie brilliant blue R-250 and commercially available low molecular weight size markers (Bio-Rad, Munich, Germany) were used as standards.

3.6.4 MS Analysis to determine delignification pattern

An Autoflex III Series MALDI-TOF, (Bruker Daltonic Leipzig, Germany) was used to determine the delignification pattern with pulsed ion extraction of 130 ns. The specification of the mass spectrometer was set up with N₂ laser (337 nm, 3 ns pulse width with pulse energy of 200 mJ). The acceleration voltage of ion source-1=19.48 kV; ion source-2=18.2 kV, Lens: 6.5 kV Reflector: 21 kV Reflector-1= 9.7 kV was 19 kV and reflectron voltage was 15.1 kV. α-cyano-4-hydroxy cinnamic acid (Sigma-Aldrich) was used as the matrix compound.

The matrix compound (10mg/ml) was dissolved 1:1 (v/v) in acetonitrile/Milli-Q-water with a concentration of 2.5% trifluoroacetic acid reagent. The samples were diluted 9:1 (v/v) in acetone/Milli-Q-water with a concentration of 0.1–0.5% (w/v) and mixed 1:1 (v/v) with the matrix compound solution used for analysis. The MS measurements were performed in the reflection mode (Ferhan et al., 2012; Bayerbach et al., 2006).
Research approach for objectives-3 & 4

Synopsis

This is a new green biochemical approach to indicate how we can use monofloral honey as a novel natural mediator substitute which accelerates oxidation process with combination of fungal laccases to convert bark into bark-derived polyphenols.

Figure 3.3: Design of strategic plan for objectives 3 & 4. Reproduced by the permission of Royal Society of Chemistry (RSC)

http://pubs.rsc.org/en/Content/ArticleLanding/2015/RA/c4ra13841d#!divAbstract

3.7 Inoculum preparation

Pleurotus pulmonarius (UAMH-7992) and Guignardia mangiferae (UAMH 11209) cultures were purchased from Microfungus Collection and Herbarium, Faculty of Agricultural, Life, & Environmental Sciences, University of Alberta. In total eight different fungal strains were used in our study, including: Pleurotus pulmonarius, Pleurotus cornucopiae, Pleurotus ostreatus, Phanerochaete chrysosporium, Bjerkandera adusta, Trichoderma harzianum, Trametes versicolor and Guignardia mangiferae.
Out of eight, six cultures were obtained from the Applied Mycology Lab, Faculty of Forestry, University of Toronto. First the cultures were plated on potato dextrose agar media from HiMedia® contains (Potatoes, infusion from 200g; dextrose 20g; agar 15g; water 1L; final pH-5.6) and stored at 4°C. A loop with fungal strains was transmitted into disinfected potato dextrose broth media pH-5.1 under antiseptic conditions and the flasks were incubated at 30°C in an orbital shaking incubator (150 rpm) aerated with continuous shaking.

3.8 Monofloral honey used as a NM-substitute in PM

Production media (PM) for *P. pulmonarius* contained: Monofloral Buckwheat Honey (Burke’s Honey Ltd) mainly used as a substitute for natural mediators along with carbon, sugars, minerals, vitamin source in the production medium (PM) at three different concentrations 5%, 7% and 10% (w/v), L-Asparagine monohydrate 3g/L, MgSO₄·7H₂O 0.05g/L, KH₂PO₄ 0.5g/L, K₂HPO₄ 0.6g/L, CuSO₄·5H₂O 0.4mg/L, MnCl₂·4H₂O 0.09mg/L, NaMoO₄·2H₂O 0.02mg/L, FeCl₃ 1mg/L, Thiamine-HCl 0.1mg/L (Ferhan et al., 2012).

50mL of PM were distributed into each 250 mL Erlenmeyer flasks and pH set to 5.5 with 1N NaOH, autoclaved at (121°C) for 15 minutes. 10% (v/v) inoculum was transferred into PM under sterile conditions and the flasks were incubated at 30°C in a shaker (150 rpm) aerated with constant shaking. Bark containing lignophenols were introduced into the PM after three days during the lag-phase of submerged fermentation.

3.8.1 Time course study during SF

During submerged fermentation, time-courses were run for 24-days and each day samples were stored and kept at 4°C for laccase assays. For optimization, three samples were collected at each time from all various experimental shake flasks contain different concentrations of honey in production medium (5%, 7%, and 10%). During submerged fermentation, the collected samples were centrifuged in a Heraeus Biofuge® Pico for about five minutes at 13,000 rpm and the supernatant was used for laccase assays.

3.9 Laccase purification by UNO® sphere Q-1 column

UNO® sphere Q-1 anion exchange column constructed for single-step polymerization was used. The Q-1 anion exchange column specifications are: column bed volume (1.3mL), maximum protein loading (20 mg), flow rate (0.5ml/min), column dimension (7 x 35mm), and
maximum operating pressure (4.5 MPa). The separation method was initiated by equilibrating the UNO Q-1 column with Buffer A (25mM Tris-HCl; pH-8.1). Crude laccase was injected onto a 50µl pre-column loop, after which, the sample in the loop was loaded onto the UNO Q-1 column using Buffer A.

The fluid flow in the system was incrementally changed to Buffer B (25mM Tris-HCl pH-8.1 + 0.5M NaCl) to elute the proteins captured in the column. At this phase, the laccase activity resembled a peak of absorbance monitored at 280 nm and eluted as a single peak. Fractions were observed at 280 nm using ChromLab™ software. The FPLC system (Bio-Rad, USA) comprised a Biologic Duoflow pump system, BioFrac fraction collector, and UV detector.

The purified and concentrated enzyme was preserved at -20°C and did not show any significant loss of enzymatic activity over several months. The molecular weight of laccase enzyme was determined by running 10% SDS-PAGE as earlier described by Höfer et al., (1999). The protein gel was stained with Coomassie brilliant blue R-250 and commercially available prestained protein ladder (Fermentas, USA) was used as a standard.

3.9.1 Laccase kinetics

Laccase kinetic constants ($V_{max}$, $K_m$, $K_{cat}$ and $K_{cat}/K_m$) were controlled by incubating the set quantity of purified laccase enzyme with different concentrations of substrate (ABTS) ranging from 200-1200 µM at 40°C, pH-3.5 as depicted previously (Li et al., 1999; Mishra et al., 2009).

3.10 Characterization techniques

3.10.1 Estimation of total polyphenolics

The total polyphenolic (TP) content in the samples was estimated by spectrophotometric method using Folin-Denis reagent. 20 µl of sample of standard solution (catechol) was diluted with 1.58 ml of distilled water and thoroughly mixed with 100 µl of Folin-Denis reagent (Fluka Analytical, Sigma-Aldrich, St Louis, MO). The reaction mixture was left for 5 minutes at room temperature and 300 µl of 25% (w/v) Na₂CO₃ stock solution was added into the reaction mixture.
The reaction mixture was incubated at 40°C in a waterbath for 30 minutes and the absorbance measured at $\lambda_{765}$ nm with a UV-160 UV/Vis spectrometer (Shimadzu, USA). A standard curve was plotted using 100, 200, 400, 600, 800 and 1000 ppm (mg/L) concentrations of catechol vs. absorbance at 765 nm. Results were expressed as (mg catechol-equiv/100ml of sample) (Yen-Peng 2011; Ferhan et al., 2013).

### 3.10.2 Sample preparation for HPLC

Fermented bark samples were centrifuged for 10 min at 10,000 rpm. The supernatant was filtered through a 0.45 μm filter and analyzed by HPLC.

### 3.11 HPLC Analysis

The HPLC system comprised a Dionex BioLC 20 Series equipped with a GP50 gradient pump, AS40 auto sampler, AD25 absorbance detector, and Chromleon for information accumulation and examination (CMS); all of them were from Dionex Technologies (USA). Ten μL of sample were injected into a column by an autosampler. A Pinnacle DB Biphenyl C18 column (100 × 4.6 mm, particle size 3 μm) from Restek (USA) was used for chromatographic separation. Two mobile phase gradients were used for elution with a flow rate of 1 mL/min: 5% (v/v) HCOOH in aqueous solution as (eluent A), and CH$_3$OH as organic mobile phase (eluent B). Chromatograms were chronicled at two different wavelengths, 290 and 340 nm (Pyrzynska et al., 2009; Lachman et al., 2010).

The fermented samples were introduced with 50 mg of each bark sample during lag-phase, and polyphenolics were determined spectrophotometrically and characterized based on their reported retention time ($t_r$) values.

Chromatographic separation was performed with gradient elution and the following steps were required (Lachman et al., 2010): 70% eluent A + 30% eluent B by an isocratic elution for 0–15 min, 60% eluent A + 40% eluent B by a linear increase for 16–20 min, 55% eluent A + 45% eluent B by a linear increase for 21–30 min, 40% eluent A + 60% eluent B by a linear increase for 31–50 min, 20% eluent A + 80% eluent B by a linear increase for 51–52 min, 10% eluent A + 90% eluent B by a linear increase for 52–60 min, 10% eluent A + 90% eluent B by an isocratic elution for 61–63 min, 70% eluent A + 30% eluent B by a linear increase for 64–73 min and lastly 70% eluent A + 30% eluent B by an isocratic elution for 74–75 min, respectively.
3.12 Thermal Analysis

Thermogravimetric analysis (TGA) of fungal treated and untreated/control bark samples were performed on a computer controlled (TGA-Q500, TA Instruments, USA). Bark powder samples were loaded into alumina pans and samples were heated from room temperature to 800 °C at a rate of 10 °C min⁻¹ under N₂ atmosphere.

3.13 GC-MS/MS

Fermented bark samples were centrifuged (10,000 rpm for 15 min) to detach cell biomass. Supernatants were acidified with concentrated HCl to pH 2.5–3 and then completely separated with three volumes (1:3 ml) of CH₃COOC₂H₅. An organic layer was collected; moisture was removed over anhydrous Na₂SO₄ and the sample filtered, and then samples were dried over night to keep in the oven at 50 °C. According to the Lundquist method (1971) the ethyl acetate extracts residues were examined as trimethyl silyl (TMS) derivatives. Dioxane and pyridine (10:1 v/v) were added in the samples which were then silylated with 5-ml of silylation derivatizing reagent trimethylchlorosilane (TMCS). To dissolve residual particles, the mixture was heated to 60 °C for 15 min with regular shaking.

An aliquot of 1 ml of silylated compound was injected into a Saturn 2100T GC/MS/MS (Varian, Inc. USA) equipped with a Varian 3900 (GC oven) and Saturn® 200MS workstation software. PE–5MS capillary column (20m x 0.18mm i.d; 0.18 mm film thickness) and helium was utilized as a carrier gas with flow rate of 1ml min⁻¹. The column temperature was maintained (50 °C for 5 min); 50–300 °C (10 °C min⁻¹, hold time for 5 min). 3 min was chosen for solvent delay.

The exchange line and particle source temperatures were sustained at 200 and 250 °C. In full-examine mode, electron ionization (EI) mass spectra within the scope of 30–550 (m/z) were recorded at electron energy of 70 eV (Raj et al., 2007). Characterization of lignin-derived low molecular weight (LMW) compounds, which are isolated from fungal treatment, was interpreted by comparing their retention time (tᵣ) with an existing database of the original compounds.
3.14 MALDI-TOF-MS

Mass spectra of bark samples were analyzed using 4800 MALDI TOF/TOF Analyzer with 4000 Series Explorer Workflow software, by a fixed laser intensity and with an evenly spontaneous spot pattern (Applied Biosystems / MDS Sciex). Before analysis, the spot position was fixed carefully. The positive ion reflector mode for all MS measurements acquired for identical repetition whereas α-cyano-4-hydroxy cinnamic acid (Sigma-Aldrich) was used as the matrix compound (Ferhan et al., 2012).

3.15 Sample preparation for Py-GC-MS

During the time course study, the bark fermented samples were harvested in the late exponential-phase (after 18-20 days of inoculation) and afterwards dried overnight in an oven (Thelco GCA Precision Scientific 18 Mechanical Convection Oven) at 60°C. The oven-dried fermented samples were milled into a fine powder and further analyzed by Py-GC-MS.

3.15.1 Py-GC-MS

Pyrolysis-GC-MS analysis was conducted using a Pt-filament loop test Pyroprobe 5250 pyrolyzer (CDS Analytical, Oxford, USA). For that purpose, 1 mg of specimen was installed in quartz tubes applying glass wool, and pyrolyzed at 650 °C for 10 seconds (heating rate 10 °C/ms), with the pyrolysis valve oven and transfer line set at 325°C.

Typically for thermostable materials such as charcoal or charred condensed tannins 750°C is suggested (Kaal et al., 2009), but 650°C is recommended for uncharred biomass (Eckmeier et al., 2007), which does not need so much energy to pyrolyze adequately. The pyrolysis products were swept online into a 6890N GC instrument, with the inlet set at 325 °C, in splitless mode. The GC instrument was fixed with non-polar 5% phenyl, 95% dimethylpolysiloxane (HP-5MS) column (30 m x 0.25 mm internal diameter; film thickness 0.25 µm) and helium was used as a carrier gas (flow rate 1 ml/min). The GC oven remained heated from 50 to 325 °C (held for 5 min) at 20 °C/min.

The transfer line of GC–MS was maintained at 325 °C. The ion source (electron impact mode, 70 eV) of the 5975 MSD (Agilent Technologies, Palo Alto, USA) was controlled at 230 °C and scanning of quadrupole detector at 150 °C, a range between m/z 50 and 550. Relative
proportions of the pyrolysis products were estimated from their peak areas, built on one or two characteristic or major fragment ions. The aggregate of total quantified peak area (TQPA) was fixed to 100%.
4 Chapter: Comparative treatments of bark to characterize bark polyphenols

4.1 Abstract

This chapter describes the comparison of enzymatic, alkaline and UV/H$_2$O$_2$ treatments for the extraction of polyphenolic extractives from beetle infested lodgepole pine (BILP) and mixed aspen barks. The concept of green polymers has become more appealing due to the presence of large volumes of processing residuals from timber and pulp industries. This, in turn, supports the idea of developing new polymers based on bark extractives. Here we used a chromatographic method to determine the chemical composition of some of the polyphenolic compounds in bark extractives and observed the effect of different extraction methods on extraction yield. Polyphenolic compounds separation was performed using HPLC in reverse-phase mode with an Octadecylsilane (ODS), C18 column (3µm particle size) and an UV detector. Detection wavelengths of 280, 310 and 370 nm were selected to allow better separation of each compound. The comparative studies and effects of enzymatic, alkaline and UV/H$_2$O$_2$ treatments on extractives yield and component contents were investigated. UV/H$_2$O$_2$ treatment exhibited the highest yield with 54% of dry bark weight extracted and was found to degrade larger amounts of lignins/tannins than enzymatic and alkaline treatments. Conversely, enzymatic treatment was good for holocellulose recovery.

4.2 Introduction

Today, we are facing an environmental crisis, and its associated socio-ecological burden, mainly due to the extraction and processing of fossil fuels. Under these circumstances, it is urgent to reduce fossil resource consumption. As such, replacing petrochemical products with green biomaterials based on cost-effective renewable resources appears as a viable solution. Petrochemical compounds such as phenol and its derivatives are produced in quantities over 10 million tons on an annual basis (essentialchemicalindustry.org/chemicals/phenol.html).

Therefore, to alleviate environmental concerns, it is important to find new natural raw materials to produce polyphenolic compounds. Such raw materials are woody biomass and bark, which are available in abundance as by-products from wood conversion industries.
Bark, similar to wood, is composed of cellulose, hemicellulose, lignin, extractives and ash. Compared to wood, bark contains higher amounts of phenolic extractives, such as lignan, and hydrolysable and condensed tannins, which have been of great interest to scientists and green technologists during the last two decades. The main idea of this study to produce value-added chemicals that are rich in phenolic structures, and thus suitable for wood adhesives (Wen-Jau et al., 2003), PF resins (Yong et al., 2012), polyols (D’ Souza et al., 2013), polyurethane foams (Yan et al., 2012), and Novolak resins (Wen-Jau et al., 2008). In addition, the lower toxicity of bark based phenolics as compared to pure phenol would favour resin production (Athanassiadou et al., 2010).

In British Columbia, lodgepole pine (Pinus contorta var. latifolia) accounts for 24% of the total forest growing stock (Reports: COFI 2005). Large numbers of mature lodgepole pines have been damaged by the attack of the mountain pine beetle (Dendroctonus ponderosae) and its fungal associates. The scale of the mountain pine beetle attack in British Columbia is unprecedented. In 2010, the beetle attack had affected over 18 million hectares of forest and had killed 710 million m³ of lodgepole pine (Report: 2000-2010).

Aspen (Populus tremuloides) is considered as a source of abundant forage and important for maintaining biodiversity in western and boreal regions in North America (Hogg et al., 2005). It is one of the main timber sources in Canada, and in some Scandinavian and Baltic nations (Johansson et al., 2002). Aspen accounts for 39.5% of the growing stock volume of the forested land-base in these countries, which is almost equal to the 42.1% of the stock that is attributable to all coniferous species (Forestry Canada 1993). In order to better understand the bark extraction process and to provide better direction for the application of bark extractives, it is important to explore and compare the effect of fundamentally different treatments such as alkaline, enzymatic and UV/H₂O₂ on bark.

Due to the versatile chemical composition of bark, researchers have expended tremendous efforts in developing extraction procedures that allow the isolation of specific phenolic compounds. Among these procedures, extraction using organic solvents such as methanol and ethanol has emerged as one of the most promising techniques for extracting phenolic compounds, especially condensed tannins, with high purity and intact structural properties (Jerez et al., 2009; Maimoona et al., 2011).
However, bark extraction with organic solvents has severe limitations, with notably low extractives yield, generally ranging from 10-20% in bark weight (Sakai, 2001). In this sense, the use of alkaline solvents, such as 1% NaOH, significantly increases the extractives yield and releases large amounts of phenolic compounds (Vazquez et al., 1986; Vazquez et al., 2001).

Nevertheless, extraction with alkaline solvents is a complex process, which involves, as with polar-solvents, the swelling and dissolution of polymers in cell walls, and also hydrolysis reactions. Hence, the extractives obtained using alkaline solvents not only contain phenolic compounds but also fractions of aliphatic acids and hemicelluloses (Yazaki et al., 1988). The low selectivity of alkaline extraction, combined with the large diversity in composition of tree barks, requires a systematic and thorough investigation of extraction process effects on each species of tree bark.

In addition to these extractives, tree bark mainly contains cellulose, hemicellulose and lignin; owing to the very selective nature of enzymes, to proceed to the extraction it is necessary to use a cocktail of enzymes that include exoglucanase, endoglucanase, β-glucosidase and ligninases. These enzymes are already widely used for process integration to reduce the number of process steps and increase yield (Galbe et al. 2002). Using a combination of cellulases, hemicellulases and ligninases initiates hydrolytic degradation of the plant cell wall, which retains phenolics in the polysaccharide-lignin network by hydrogen or hydrophobic bonding. Direct enzyme catalysis is another approach to cleave ether and ester linkages between phenol and plant cell wall polymers, as explained by Pinelo et al. (2008).

The mechanism of photocatalysis by UV/TiO$_2$ has been described by several authors (Selli et al. 1999; Tanaka et al. 1999; Lanzalunga et al. 2000; Castellan et al. 1990; Chang et al. 2004) who have studied the degradation of various wastes, in particular lignin. Because of the multifunctionality of lignin compared to phenol, wavelengths ranges of lower energy are sufficient to initiate the degradation (Castellan et al., 1990). The main reactive species produced in photocatalytic process by UV/H$_2$O$_2$ are hydroxyl and superoxide radicals (Machado et al., 2000), which are proposed to allow the degradation of bark components.

The aim of the work was to determine and compare the effectiveness of an alkaline solution, a cocktail of enzymes and UV/H$_2$O$_2$ treatment of BILP and mixed aspen barks to improve the extraction efficiency and polyphenolic extractive yields. The raw materials considered for this
study were beetle infested lodgepole pine (BILP) bark and mixed aspen bark; both bark species have a world-wide distribution but are most abundant in North America. In addition, we sought to select the most appropriate wavelengths for each phenolic compound in bark polyphenolic extractives. Biotechnological processes enable the discerning separation of main fractions (cellulose, hemicellulose and lignin) from bark constituents.

4.3 Results and discussion

4.3.1 Bark composition

The major components of bark from infested lodgepole pine and aspen are summarized in Table 4.1.

Table 4.1: Chemical composition of beetle infested lodgepole pine and aspen bark.

<table>
<thead>
<tr>
<th>Composition (%)</th>
<th>BILP Bark</th>
<th>Aspen Bark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol-Toluene extractives</td>
<td>19.3 ± 0.5</td>
<td>18.4 ± 0.7</td>
</tr>
<tr>
<td>Water extractives(^a)</td>
<td>9.4 ± 0.4</td>
<td>17.1 ± 1.2</td>
</tr>
<tr>
<td>Total extractives(^b)</td>
<td>28.6</td>
<td>35.5</td>
</tr>
<tr>
<td>Klason Lignin(^c)</td>
<td>33.1 ± 0.6</td>
<td>34.3 ± 0.6</td>
</tr>
<tr>
<td>Holocellulose(^c)</td>
<td>39.2 ± 1.7</td>
<td>31.5 ± 0.7</td>
</tr>
<tr>
<td>(\alpha)-cellulose(^c)</td>
<td>24.8 ± 1.0</td>
<td>19.2 ± 1.4</td>
</tr>
<tr>
<td>Ash contents</td>
<td>4.1 ± 0.7</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>Total extracts(^d)</td>
<td>100.9</td>
<td>101.3</td>
</tr>
</tbody>
</table>

Mean ± S.D (n = 3)

\(^a\) Yield obtained after extraction using ethanol-toluene. \(^b\) Addition of ethanol-toluene and water extractives. \(^c\) Calculated as the percentage of initial dry bark weight. \(^d\) Addition of total extracts, Klason lignin, Holocellulose and Ash contents.
Bark major components analysis was reported in terms of extractives, which correspond to the soluble fractions in ethanol/toluene mixture and 95% ethanol, water and the bark main components, which refer to lignin, holocellulose and α-cellulose. The addition of bark fractions from infested lodgepole pine and aspen resulted in 100.9 and 101.3 % of total bark weight, respectively. The slight deviation observed may be due to some experimental errors. Infested lodgepole pine bark and aspen bark contained 4.1% and 5.7% of ash, respectively, which falls between the values obtained for other softwood and hardwood species such as Tsuga heterophylla and Quercus mongolica (Herrick, 1980; Kofujita et al., 1999).

Infested lodgepole pine and aspen bark total extractives contents were 28.6% and 35.5% of dry bark weight, which is generally high compared to the 16.6% obtained for Pinus pinaster after dichloromethane, ethanol and water extraction and the 12.1% reported for the bark belly of cork oak (Quercus suber L.), after successive extraction with ethanol and water, yet within the range of the 43.40% reported for Populus hybrid bark (Fradinho et al., 2002; Jove et al., 2011; Blankenhorn et al., 1988).

The extractives content in barks was assessed by subtracting the weight before and after extraction and not by direct evaporation of the concentrated extractives. As a result, the values could be slightly overestimated due to possible weight losses during manipulations. In addition, the high extractives values may be due to other effects, such as bark particles size, which drastically affect the fractionation and extraction yield, resulting in general difficulties in precisely comparing the extractives content obtained with values in the literature (Miranda et al., 2013). It is interesting to note that our results revealed higher extractives content in the mixed aspen bark compare to lodgepole pine, which is unusual although such high values are reported for Populus hybrid (Blankenhorn et al., 1988).

Aspen bark exhibited considerably higher amounts of water extractives compared to infested lodgepole pine bark, indicating a larger presence of polar extractives such as lignans, neolignans, and hydrolysable tannins (Sakai, 2001). Infested lodgepole pine and aspen bark’s main components exhibited similar content of klason lignin but aspen holocellulose content was lower (Rowell et al., 2005).
4.3.2 Treated bark composition

Infested lodgepole pine and aspen barks were extracted using alkaline, enzymatic, and UV/H$_2$O$_2$ treatment and the influence of these treatments on bark chemical composition is listed in Table 4.2 as well as shown in Figure 4.1. Infested lodgepole pine bark extractives yield ranged from 49-55% depending on the treatment used, whereas aspen bark extractives yield was slightly lower, ranging from 48.3-53%.

**Table 4.2:** Extraction yield from soluble part and bark residues (insoluble part) of beetle infested lodgepole pine (BILP) and aspen bark after extraction with different methods.

<table>
<thead>
<tr>
<th>Bark species</th>
<th>Extraction method</th>
<th>Soluble fraction after extraction</th>
<th>Insoluble fraction after extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extraction Yield (% w/w)</td>
<td>Klassen Lignin (KL) (% w/w)</td>
</tr>
<tr>
<td>BILP</td>
<td>Alkaline</td>
<td>52.3 ± 1.4</td>
<td>19.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Enzymatic</td>
<td>49.5 ± 2.2</td>
<td>25.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>UV/H$_2$O$_2$</td>
<td>54.1 ± 2.1</td>
<td>14.8 ± 1.7</td>
</tr>
<tr>
<td>Aspen</td>
<td>Alkaline</td>
<td>48.3 ± 0.5</td>
<td>22.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Enzymatic</td>
<td>48.8 ± 2.7</td>
<td>21.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>UV/H$_2$O$_2$</td>
<td>53.6 ± 2.7</td>
<td>15.9 ± 0.8</td>
</tr>
</tbody>
</table>

Mean ± S.D (n = 3)
The α-cellulose value is a fraction of the holocellulose and the percentage (% w/w) of bark residues was calculated based on the relative proportion of total bark weight.
UV/H$_2$O$_2$ was an efficient treatment due to improvement of 1.13-fold extraction yield, as compared to alkaline and enzymatic treatments, but the difference in extraction yields between the different treatments did not exceed 6% in weight. Although only slight differences in extraction yields were observed depending on the treatments used, we found different effects on the main component constituents. The one-way ANOVA showed that the extraction yield of BILP among three treatments represents no significant difference ($p > 0.05$); however, bark residues of BILP (KL, HC and $\alpha$-C) were significantly different ($p < 0.05$). The extraction yield of aspen among three treatments significantly different ($p < 0.05$) and bark residues of aspen (KL, HC and $\alpha$-C) were also significantly different ($p < 0.05$). All the samples were precisely evaluated at the beginning and then at the end of each treatment step to follow the mass balance. All the experiments were performed in triplicate.

Mean ± S.D (n = 3)

**Figure 4.1:** Bark residue composition of Beetle Infested Lodgepole Pine (BILP) and Aspen after three different treatments.
The lower holocellulose and α-cellulose content observed in enzymatic treated bark residues is possibly due to the presence of cellulase and β-glucosidase in the enzyme cocktail, which resulted in the removal of large amounts of polysaccharides from the bark. Matsushita et al. (2010) previously observed the swelling of the phloem parenchyma’s primary cell wall and the presence of many nano-clefts in the phloem fiber’s secondary wall after hydrothermal pre-treatment, which may allow greater access of enzymes to the secondary cell wall cellulose, supporting enzymatic hydrolysis.

In addition, the lower klason lignin content observed after UV/H₂O₂ treatment supports its strong oxidative nature, which would preferentially degrade phenolic structures in bark such as lignin and condensed tannins (Ugurlu et al., 2007). In general, alkaline treatments resulted in the extraction of klason lignin and holocellulose with yields very similar to that of enzymatic treatment, although the α-cellulose content did not vary, which suggests that the decrease in holocellulose is mainly due to the degradation of hemicellulose.

SEM images at different magnifications were obtained for control, alkaline, enzymatically and UV/H₂O₂ treated BILP and aspen barks reported in this work. Figure 4.2 illustrates the surface morphology; the treated bark revealed the surface roughness while the untreated surface appears smooth.
Figure 4.2: Scanning electron microscopy of beetle-infested lodgepole pine (BILP) and aspen barks at different magnifications. (A) Control BILP bark (magnification, x 3,500), (B) treated with alkaline (magnification, x 1,500), (C) treated with cocktail of enzymes (magnification, x 1,000), and (D) treated with UV/H₂O₂ (magnification, x 1,000). (E) Control aspen bark (magnification, x 2,000), (F) treated with alkaline (magnification, x 1,000), (G) treated with cocktail of enzymes (magnification, x 1,000), and (H) treated with UV/H₂O₂ (magnification, x 2,200).

Morphological modifications on the cell wall surface of BILP and aspen bark samples caused by the pre-treatments were analyzed using scanning electron microscope. The observed effects on the fiber structure were very similar for both bark species. These pre-treatment differences can be associated with lignin relocation and amalgamation on the cell wall surface (Lima et al., 2013).

Fromm et al. (2003) showed that more than 50% of the lignin is concentrated in the middle of lamellae. A correlation between the disaggregation of cell bundles and the lignin content of different biomass has been reported (Rezende et al., 2011). The formation of lignin agglomerates on the surface has also been explained in other lignocellulosic biomasses exposed
to steam-explosion, diluted acid or organosolv pretreatments (Koo et al., 2012; Heiss-Blanquet et al., 2011).

Following the different treatments, the total phenolic compounds in aspen and BILP bark extractives were assessed. The given values in the above mentioned tables (Table 4.1 and Table 4.2) are the means of three replicates of each. Enzymatic treatment resulted in $6.95\pm0.40$ mg/g and $8.82\pm0.51$ mg/g, alkali treatment provided $4.3\pm0.66$ mg/g and $4.64\pm0.44$ mg/g, and UV/H$_2$O$_2$ treatment yielded $5.62\pm0.30$ mg/g and $8.0\pm0.52$ mg/g, respectively. Generally, extractives from BILP exhibited higher amounts of phenolic compounds compared to aspen.

The extractive levels in barks are higher than in wood and, similarly, the total phenols level in bark has been found to be higher (Chow et al., 2008), although some studies have shown higher polyphenols levels in wood than in bark (Chang et al., 2001; Wang et al., 2004). The total polyphenolic level attained by sequential extraction of wood of *Maclura tinctoria* was higher than compared to bark, although these results were similar, and the differences when acetone: water was used as extraction solvents were not significant ($P > 0.05$) reported by Lamounier et al., (2012). Bark of *Eucalyptus globulus* has less favourable chemical composition with more extractives, pentosans, and ash contents, although the pulp obtained with tops was similar to the wood pulp in regards to yield and strength properties (Miranda et al., 2012).

The values found in the literature for the total phenols content of methanol: water (80:20, v/v) extracts of Eucalyptus bark (E. camaldulensis, E. globulus, and E. rudis) were in the range of 0.25 to 9.16% (Conde et al., 1997). Vázquez et al. (2008) reported a value of 1.48 mg/g for *Eucalyptus globulus* bark extracted with this solvent. Subsequently, the estimation of total phenolic compounds should be reflected as a polyphenol index, depending upon the applied method.

The values obtained after the different treatments of BILP and mixed aspen bark were generally low compared to the values found in the literature for the total phenols. However, the extraction with organic solvents generally resulted in low extractives yield (20.8%) in bark (Lamounier et al., 2012), obtained with cyclohexane: ethanol (1:2, v/v) solvent mixture compared to the treatments reported in this chapter.

Most co-elutions occurred between the peaks of ferulic acid and quercetin, so the gradient was modified in this part of the chromatogram to allow a progressive elution of components and
minimize overlapping. The chromatograms corresponding to the better separation of polyphenolic extractives treated with a cocktail of enzymes are shown in Figure 4.3 from (A-C) for BILP and (D-F) for aspen bark, respectively. This demonstrates that all analytes can be successfully separated at three different wavelengths. Polyphenolic constituents of the extractives obtained after enzymatic cocktail treatment of BILP and aspen bark exhibited better separation at three different wavelengths as compared to the extractives from other treatments.

In all cases, the yields from enzymatic cocktails were comparable to the other two treatments, but the enzymatic treatment resulted in better separations of polyphenolics, while the chromatograms of the other treatments showed overlapping separations. The separation of polyphenolic complex molecules in bark under acidic mobile phase with the combination of acetonitrile gradient is a common method in reverse-phase liquid chromatography.
Figure 4.3: Chromatograms of BILP bark (A, B, C) and aspen bark (D, E, F) polyphenolic extractives.¹

¹ The BILP bark polyphenolic extractives at $\lambda_{280}$ nm are gallic acid (1.13), p-hydroxybenzoic acid (5.68), syringic acid (9.38), rutin (14.90), taxifolin (15.08), myrecetin (17.5) and kaempferol (22.0); at $\lambda_{310}$ nm are (+)-catechin (6.42), caffeic acid (8.72), syringic acid (9.75), $p$-coumaric acid (12.71), rutin (14.9), taxifolin (15.69), fisetin (18.73), trans resveratrol (19.35, 19.72), apigenin (21.02, 21.48), and kaempferol (22.5); and at $\lambda_{370}$ nm are gallic acid (1.24), p-hydroxybenzoic acid (5.65), ethyl gallate (11.71), myrecetin (17.92), quercetin (20.63), and apigenin (21.8). Aspen bark polyphenolic extractives at $\lambda_{280}$ nm are gallic acid (1.07), $p$-hydroxybenzoic acid (5.53), syringic acid (9.53), and kaempferol (22.1); at $\lambda_{310}$ nm are ethyl gallate (11.64, 11.94), $p$-coumaric acid (12.39), ferulic acid (13.08), myricetin (17.93), fisetin (18.44, 18.86), trans-resveratrol (19.53, 19.93), quercetin (20.34), and apigenin (21.74); and at $\lambda_{370}$ nm are protocatechuic acid (2.31), vanillic acid (7.93), ethyl gallate (11.1), trans-resveratrol (19.48), quercetin (20.64), and kaempferol (22.1). All peak assignments were characterized based on their ($t_r$) values of polyphenolic standards as reported by Aznar et al. 2011.
It was reported that under alkaline conditions, the caffeic and syringic acids may overlap, as can \textit{p}-coumaric acid and vanillic acid. Under alkaline conditions, phenolic acid tends to complex with sodium and prevents an efficient separation, due to alteration of the hydrophilicity of analytes, thereby reducing its interaction with the stationary phase (Hemstrom et al., 2006). Furthermore, under alkaline conditions, syringic acid and ferulic acid were not completely separated; moreover, \textit{p}-hydroxybenzoic acid shifted as an unsymmetrical peak. As a result, syringic acid and ferulic acid, as well as caffeic acid and hydroxycinnamic acid, nearly overlapped (Pomponio et al., 2002).

Although the difficulties in separating analytes may be due to the samples basicity, samples dilution during preparation, as well as the use of 0.1\% formic acid as eluent may prevent these phenomena. The very low selectivity of alkaline and UV/H$_2$O$_2$ compared to enzymatic treatment lead to obtaining a very diverse range of molecules with close structural features, which could also complicate analytes separation.

Degradation of various products treated by AOPs using UV light and H$_2$O$_2$, linked to specific pH dependent mechanisms, produces very reactive hydroxyl radicals (\textbullet OH) in the reaction mixture, thus making it more susceptible to oxidation (Machado et al., 1994). Nevertheless, the chromatogram and the chromatographic peak patterns were comparable to those reported in a previously published article (Aznar et al., 2011).

As a result, these two treatments have low selectivity embedded in very diverse structures that might be difficult to separate during chromatography. Moreover, UV/H$_2$O$_2$ and alkaline treatment lead to highly basic extractives, which could prevent an effective separation due to possible interactions between analytes and column. However, our results indicate that enzymatic treatment, due to its highly specificity, low pH, and cleavage of selective bonds among the molecules can probably affect the polyphenolic compounds in bark.

### 4.4 Conclusions

The effect of three different treatments on the surface morphology and chemical composition of bark residues and polyphenolic extractives from BILP and aspen barks were studied. The total chemical composition of BILP bark was ash (ca. 4\%), extractives (ca. 28\%), Klason lignin (ca. 33\%), holocellulose (ca. 39\%), while mixed aspen bark residues consisted of ash (ca. 5\%), extractives (ca. 35\%), Klason lignin (ca. 34\%), and holocellulose (ca. 31\%), respectively. The
HPLC analysis shows fast, selective, sensitive and reliable determination of the most common polyphenolics in bark extractives. UV/H$_2$O$_2$ treatment was able to preferentially remove phenolic compounds, with limited effect on the sugar concentration of the bark hydrolysates. The removal of phenolic compounds (catechol equivalent) was found to be 0.8% in BILP and 0.6% in aspen bark under UV/H$_2$O$_2$ treatment. Furthermore, these results elucidate the effects of different treatments on bark-derived polyphenols, which can be considered as promising methods for applications in bioconversion and polymer industrial processes.
5 Chapter: Brazilian isolated fungal strain for ligninases production

5.1 Abstract

The potential of ligninases in green processes for effective valorization of lignin can be shown through investigation of enzymatic cocktails containing different lignin degrading enzymes. The present study deals with the screening of potential fungal strains useful for the liquefaction of bark containing lignin. Three different local isolates (Pleurotus ostreatus POS97/14, Pycnoporus sanguineus and the Brazilian isolated fungal strain) were selected out of ten different strains for ligninases production. Maximum production of enzymes was observed in the local isolated fungal strain after ten days in submerged fermentation. The isolated fungal strain produces ligninases, mainly manganese peroxidase (MnP). The enzyme oxidized a variety of the usual MnP substrates, including lignin related phenols. Furthermore, the partial purification for MnP was determined by fast protein liquid chromatography (FPLC) and the molecular weight was evaluated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

5.2 Introduction

Among microorganisms that colonize wood, white-rot fungi are regarded as considerable lignin degraders. They produce extracellular enzymes, such as MnP, LiP and laccase, which play important roles in lignin biodegradation (Mester et al., 2000). Pleurotus ostreatus is a white rot fungus belonging to the basidiomycetes. Pleurotus ostreatus is an edible mushroom, as well as a food supplement for hypercholesterolemia patients due to presence of higher concentrations of lovastatins (Alarcon et al., 2003). It was also noticed that due to lack of lignin peroxidases in some genera of basidiomycetes, such as Pleurotus spp, laccases are involved in the degradation of lignin (Mansur et al., 2003).

The combined action of laccase and aryl alcohol oxidase decreases the molecular weights of soluble lignosulphonates by P. ostreatus (Mansur et al., 2003). Ligninolytic system configuration is complicated and species-restricted (Baldrian 2008). White rot fungi produce ligninases, including MnPs, LiPs and Lcc, which are possible contributors to fungal
ligninolysis. White-rot fungi were mainly found to produce consistent products following the ligninolysis of model lignin compounds (Cullen et al., 2008).

MnP belongs to the family of peroxidases, and the methodical name of this enzyme is Mn(II):H$_2$O$_2$ oxidoreductase. According to protein databank, the MnP containing cofactor is protoporphyrin IX having Fe (C$_{34}$H$_{32}$FeN$_{4}$O$_{4}$) incorporated with Mn$^{2+}$ and Ca$^{+2}$ ions required for enzyme activity.

Eventually, peroxidases oxidize phenolic compounds and reduce molecular oxygen to water (Mester et al., 2000). Ligninases oxidize several environmental pollutants such as polycyclic aromatic hydrocarbons, dyes and chlorophenols. Heme containing enzymes such as LiP and MnP also have catalytic cycles characteristic of other peroxidases. LiP has an ability to oxidize many aromatic compounds, whereas MnP oxidizes Mn (II) to Mn (III) (Sadhasivam et al., 2008; Wang et al., 2009).

Laccases [benzenediol: oxygen oxidoreductase, EC (1. 10. 3. 2)] are multi-copper blue oxidases commonly found in higher plants, several bacteria and some insects. Nevertheless, the well-characterized laccases are fungal in origin (Sadhasivam et al., 2008). Laccases have significant importance in many industrial areas due to their remarkable catalytic properties. Potential applications include immunoassay bio-labeling, biosensors, biocatalysts, and advancement of oxygen cathodes in biofuel cells. In addition, they have good prospects in the environmental sector, including use in textile dye bleaching, pulp delignification and xenobiotic compound degradation due to their wide-ranged substrate specificity (Sadhasivam et al., 2008; Mazumder et al., 2009). The present applications of this enzyme motivated us to do new basic research.

The current research activity of ligninases includes utilizing the local lignin sources (Eucalyptus and sugarcane bagasse) and checking their delignification pattern. The characterization of ligninases (MnP$s$, LiP$s$ and Lcc,) from Brazilian isolated fungal strain, with respect to production, partial purification and time course studies, is reported in this study.

5.3 Results and Discussion

Due to broad substrate specificity, the ligninases potential of white rot fungi has been reported as liable for the alteration and mineralization of organic pollutants that are structurally similar
to lignin. Ligninases are the combination of three peroxidases, MnP, LiP and laccases, which are characterized as lignin degrading enzymes. The distribution of MnP and laccases are very frequent in white rot fungi, while LiP is not (Palaez et al., 1995).

Degradation and decolorization of many organic pollutants by white-rot fungi have been reported by many researchers (Clemente et al., 2001), but MnP and LiP are noted mainly for the degradation of polymeric dyes (Moreira et al., 2001). The white rot fungi have the ability to decolorize polymeric dyes because of existing ligninolytic enzymes. An anthracene derivative like Remazol Brilliant Blue R (RBBR), which is considered to be an organic pollutant and is similar to the lignin polymers, has been used as a model compound by many researchers to measure ligninases activity (Moreira et al., 2001).

Therefore, we examined a petri plate assay to identify ligninases-producing fungal strains, based on their ability to decolorize RBBR. Using this system, we determined that the fungal strains Pleurotus ostreatus, Pycnoporus sanguineus and the new Brazilian isolate all exhibited ligninolytic activity (Figure 5.1).

**Figure 5.1:** Potential screening of three fungal strains out of ten based on the ligninases production and degradation of RBBR dye, where, A - Pleurotus ostreatus POS 97/14, B- Pycnoporus sanguineus and C was Brazilian isolated fungal strain.

### 5.3.1 Ligninases production during time course studies

To better quantify ligninases production, the ligninolytic activity of the aforementioned strains was measured in liquid cultures. During the time course study, triplicate fermentation flasks
were harvested after every 48h and the culture supernatants were analyzed for ligninases activity and cell biomass was also noted. The maximum production of ligninolytic activity by the newly isolated Brazilian fungal strain, MnP (64 IU l\(^{-1}\)), LiP (26.35 IU l\(^{-1}\)) and laccase (5.44 IU l\(^{-1}\)), respectively, was attained in 10 days. In contrast, the ligninolytic activity of *Pleurotus ostreatus*, and *Pycnoporus sanguineus* (Table 5.1) had minimal Remazol Brilliant Blue R (RBBR) decolorization efficiency and surprisingly did not show any peroxidase activity. The low peroxidase activity and decolorization efficiency, especially as compared to that which has been reported for other strains (*Ganoderma* spp, *Stereum ostrea*, and *Trametes versicolor*) (Pointing 2001) raises the possibility that the growing conditions or the growth medium were not optimal for these fungal strains.

**Table 5.1:** Measurement of ligninases activities during the time course study in three different fungal strains.

<table>
<thead>
<tr>
<th>Brazilian isolated fungal strain</th>
<th>Days</th>
<th>Laccase (IU l(^{-1}))</th>
<th>LiP (IU l(^{-1}))</th>
<th>MnP (IU l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>1.42±0.01(^{f})</td>
<td>17.6±0.05(^{e})</td>
<td>16.7±0.21(^{f})</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.17±0.01(^{e})</td>
<td>19.7±0.09(^{d})</td>
<td>36.8±0.09(^{d})</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4.45±0.00(^{d})</td>
<td>21.8±0.03(^{e})</td>
<td>56.8±0.09(^{e})</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5.31±0.01(^{b})</td>
<td>23.8±0.09(^{b})</td>
<td>59.3±0.09(^{b})</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.44±0.01(^{a})</td>
<td>26.4±0.07(^{a})</td>
<td>64.1±0.12(^{a})</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5.22±0.01(^{c})</td>
<td>14.9±0.10(^{f})</td>
<td>30.9±0.14(^{c})</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><em>Pleurotus ostreatus</em> POS97/14</th>
<th>Days</th>
<th>Laccase (IU l(^{-1}))</th>
<th>LiP (IU l(^{-1}))</th>
<th>MnP (IU l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>0.14±0.01(^{d})</td>
<td>0.82±0.07(^{f})</td>
<td>5.17±0.11(^{e})</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.18±0.02(^{d})</td>
<td>2.93±0.05(^{e})</td>
<td>7.62±0.09(^{d})</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.31±0.01(^{c})</td>
<td>4.03±0.07(^{d})</td>
<td>10.09±0.16(^{c})</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.56±0.01(^{b})</td>
<td>5.84±0.04(^{c})</td>
<td>13.32±0.14(^{b})</td>
</tr>
</tbody>
</table>
It was also observed that cell biomass increased with increasing fermentation time. In addition, ligninases activity also increased with increasing fermentation time for 10 days, but a further increase (12 days) in fermentation time (Table 5.1) decreased ligninase activities (Table 5.1). The local isolated fungal strain was the best producer of MnP (64 IU l\(^{-1}\)) as the main enzyme activity, followed by LiP (26.35 IU l\(^{-1}\)) and laccase (5.44 IU l\(^{-1}\)), respectively, as evidenced by the high percentage (94%) of RBBR decolorization in the broth medium. The given values of ligninases activities in Table 5.1 are the mean of three replicates of each. The least significant difference among the experimental values was calculated by ANOVA using Assistat-Statistical Assistance version 7.6-beta software.

During exponential phase, the increase of cell biomass resulted in an increase in secretory proteins in the growth medium. Increase of cell biomass yield was associated with increased extracellular secretory protein in the fermentation medium up to the 12\(^{th}\) day, when biomass started to decrease. However, the specific activities of MnP and LiP constantly increased (Pointing 2001).
5.3.2 Purification of MnP

MnP purification mainly consists of two steps, ammonium sulfate precipitation and size exclusion chromatography. Following chromatography, protein fractions eluted in two peaks as shown in Figure 5.2A.

**Figure 5.2:** Size exclusion chromatography of MnP from isolated fungal strain (A): DEAE fractionations from isolated fungal strain (B): MnP activity was eluted as a single peak.

The eluted peak was monitored at $\lambda_{610}$ nm (MnP) and at $A_{280}$ nm (protein) following size exclusion chromatography (Figure 5.2B). Under reducing and non-reducing conditions, total proteins were run on native-PAGE shown in (Figure 5.3A). Finally, to determine the molecular weight of the purified enzyme, MnP was separated on SDS-PAGE and stained with Coomassie Blue R-250 (Figure 5.3B). The purification procedure is summarized in Table 5.2. The molecular mass of purified MnP was 37kDa as shown in (Figure 5.3B) and the specific activity of the purified enzyme was 3.22-fold.
Figure 5.3: Polyacrylamide gel electrophoresis (5.3A): Ligninases with reduction and non-reduction conditions (5.3B): Purified MnP from isolated fungal strain stained with Coomassie Blue R-250.
Table 5.2: Partial purification summary of MnP produced during submerged fermentation by isolated fungal strain under optimized conditions.

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Activity/L</th>
<th>Protein content (U/mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>4467.8</td>
<td>228.9</td>
<td>98.6</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium Sulphate</td>
<td>1634.9</td>
<td>157.8</td>
<td>182.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Dialysis</td>
<td>1442.6</td>
<td>101.2</td>
<td>190.1</td>
<td>1.9</td>
</tr>
<tr>
<td>1st DEAE-Sepharose Fast Flow column (10 x 300mm)</td>
<td>1381.5</td>
<td>89.0</td>
<td>219.8</td>
<td>2.2</td>
</tr>
<tr>
<td>2nd DEAE Superdex 75 (10 x 300mm)</td>
<td>1254.1</td>
<td>19.9</td>
<td>293.6</td>
<td>2.9</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>1085.9</td>
<td>5.6</td>
<td>317.9</td>
<td>3.2</td>
</tr>
</tbody>
</table>

5.3.3 Electron microscopy

Scanning electron microscopy (SEM) was performed to determine the surface morphology of lignin samples of sugarcane bagasse and eucalyptus black liquor that were treated with the isolated fungal strain (Figure 5.4). Abundant fungal growths were seen on bagasse and eucalyptus black liquor, which indicated the decay of lignin samples. After fungal modification, the fiber structure of bagasse specifically shows the presence of bore holes, indicated by arrows in Figure 5.4 (C and D).
**Figure 5.4:** Scanning electron microscopy of sugarcane bagasse (SCB) and Black liquor (BL) of Eucalyptus. (A & B) Control SCB (magnification, x 400); (C & D) bagasse treated with Brazilian isolated fungal strain grown after 2 weeks (magnification, x 2k); (E & F) Control BL of Eucalyptus fibers (magnification, x 10k); (G & H) BL treated with local fungal isolate grown after 2 weeks (magnification, x 5k); indicates the fungal growth where k denoted by (x1000 magnification).

Sugarcane bagasse contains cellulose (40–50%), hemicellulose (20–30%), lignin (20–25%) and ash (1.5–3.0%). Hemicelluloses are complex polysaccharides that appear in correlation with cellulose in the cell wall. It is a mixture of polysaccharides, comprised of almost all sugars, such as glucose, mannose, xylose, arabinose, methylglucoronic and galaturonic acids, with an average molecular weight < 30,000 Da (Saxena et al., 2009). Mushroom growth is encouraged on sugarcane bagasse in some countries, including Brazil. This is in response to the increase in global demands for ethanol (from 66-125 million m$^3$), that is required in the production of bagasse (Pandey et al., 2000; Balat et al., 2009). By utilizing agricultural wastes and converting them into high valued products, this is an alternative approach to decreasing the waste disposal costs (Sidana et al., 2014).

Moreover, the main monosaccharides (glucose and xylose) were found in Eucalyptus grandis bark (~ 40% glucose, and ~ 9% xylose, respectively) reported by Yu et al. (2010). The edible mushroom production with eucalyptus is considered to be a raw material for the pulp and paper mill. Eucalypt farming competes with agricultural crops, is more suitable to farmers to consider as a substitute for agricultural crops, e.g. cassava. The farmers are also supportive of the idea of eucalypt farming (Reungchai, 1996).

### 5.3.4 MALDI-TOF MS

The MALDI-TOF method provides information regarding the interpretation of lignin composition. This method also helps to determine the lignin structure. After biological modification, it illustrates the effectiveness of fungal attack on the lignin bio-molecule and the impact on its compositional arrangement. The fungal attack generally affected phenolic units of the lignin molecule, as observed by the reduction in the relative frequency of β-O-4-linked H units, which are mainly terminal units with free phenolic groups (Lapierre et al., 1988).

After delignification, the complete structures of molar mass distribution of lignophenols by MALDI-TOF-MS spectra are shown in (Figure 5.5). In delignification spectra, the lignin
monomers specified the dominant signals at m/z 171, 188 (coniferyl aldehyde), m/z 227, 229, 233 (syringyl propene), m/z 334, 378, 397 (phenyl coumaran), m/z 453 (resinol), m/z 655, 715 (dimethoxyphenol) reported by Bayerbach et al. (2006). Most monomers were by-products from guaiacol and syringol. Most dimers were assumed to be a phenylcoumaran structure. Biphenyl and resinol were infrequent.
Figure 5.5: MW distribution to determine the lignin monomers by using MALDI-TOF-MS where α-cyano-4-hydroxy cinnamic acid was used as matrix compound.

5.4 Conclusions

With the increasing global anxiety over fossil fuel use and its environmental footprint, there is a strong interest in using biorenewable materials as substitute feedstocks for making more environmental-friendly biomaterials. The present study screens potential fungal strains for ligninases production, and includes partial purification of MnP and the degradation pattern of local lignin resources found in Brazil. The industrial and biotechnological application of ligninases is constantly increasing due to their multiple uses and applications in a diversity of
processes. Their ability to remove xenobiotic pollutants and produce polymeric products makes them a beneficial tool for bioremediation purposes. The unknown isolated fungal strain has the potential for delignification. Nevertheless, further molecular biology studies are needed to identify the species by 18S-rDNA.
6 Chapter: Optimization of temperature and pH for Manganese Peroxidase (MnP)

6.1 Abstract

In this study, a fungal species selected for ligninases production was investigated by 18S ribosomal DNA sequence analysis. Two primers sets were chosen to amplify a major part of the 18S rDNA, which resulted in intense PCR product of approximately 550–820 bp in size per sample. The results suggest that the 18S rDNA-based approach is a useful tool for identification of unknown potential fungal species for ligninases production. The isolated fungal species produces mainly manganese peroxidase (MnP). It oxidizes a wide range of typical MnP substrates as well as lignin-related polyphenols. The process parameters such as temperature and pH of the medium in shake flask experiments were optimized for the maximum production of manganese peroxidases. Time course studies showed that maximum production of ligninolytic enzymes MnP (64 IUL⁻¹), LiP (26.4 IUL⁻¹), and laccase (5.44 IUL⁻¹), respectively, were achieved after 10 days of cultivation under optimum conditions (temperature and pH). Furthermore, the biological decolorization of Remazol Brilliant Blue R (RBBR) dye following 10 days of cultivation was 94%. NCBI BLAST was used to search for closest matched sequences in the GenBank database and based on sequence homology the first BLAST hit was Dothioraceae sp. LM572 with accession number EF060858.1.

6.2 Introduction

Filamentous fungi play a prominent role in decomposition of lignocellulose. There are three types of fungi living on dead wood that better degrade one or more wood components: soft-rot fungi, brown-rot fungi, and white-rot fungi. Soft-rot fungi (Ascomycetes and Fungi Imperfecti) can decay cellulose as well as also partially degrade lignin (Grazyna et al., 1989).

Extracellular oxidoreductases, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase produced by wood-decomposing microorganism, are directly involved in the degradation of lignin in natural lignocellulosic substrates and various xenobiotic compounds including dyes (Abrahao et al. 2008). Some wood-degrading fungi contain all three classes of ligninases, while the others include only one or two of these ligninolytic enzymes (Dhouib et al. 2005). The ligninases produce a colourless halo around microbial growth in media
containing coloured indicator compounds (Dhouib et al. 2005). So, the lignin degradation capability of microbes is initially screened indirectly on solid media containing different indicator compounds.

Although, wood decay fungi are primarily basidiomycetes, other microorganisms are also involved in the lignocellulosic decaying processes (Nilsson et al. 1989). Wood biodegradation by certain ascomycetes was first described in detail and designated as “soft rot” by Savory (1954). Nilsson et al. (1989) showed that, some higher ascomycetes, mainly *Daldinia concentrica*, degraded aspen wood with the same strength as *Trametes versicolor*, a basidiomycete characteristically classified as white-rot fungus.

The ascomycete, *Chrysonilia sitophila* could degrade rice hull and *Pinus radiata* bark products and also produce ligninolytic and cellulolytic enzymes (Ferraz et al. 1991). However, some of ligninolytic microorganisms have ability to degrade a wide range of pollutant substances which are similar to lignin or its derivative. This is an interesting method due to low cost, specificity and likelihood of a total mineralization of these compounds (Van der Zee et al. 2005).

In this study, an ascomycete producing ligninolytic enzyme, was isolated from leaf samples of *Combretum leprosum* in the tropical dry forest (Caatinga), in Brazil. The fungal species was identified by 18S rDNA and its ability for lignin degradation was characterized by production of ligninases and decolorization of polyaromatic ligninolytic indicator dye, RBBR respectively (as explained above in Chapter 3; Section 3.3).

### 6.3 Results and Discussion

#### 6.3.1 Ligninases production during the time course studies

The Brazilian isolated fungal strain was identified as *Dothioraceae sp.* using 18 S rDNA sequence analysis (as explained above in Chapter 3; Section 3.5) was used for the production of ligninases in submerged fermentation. *Dothioraceae* belongs to Phylum of Ascomycota as recognised by NCBI Taxonomy in the Encyclopedia of Life. The triplicate fermentation flasks were harvested every 48 h, and the culture supernatants were analyzed for ligninases activity and the dry weight of biomass (residue) was also recorded. The results of *Dothioraceae* fungal species during time course study proved that the highest production of MnP (64 IU·l⁻¹), LiP
(26.35 IU\textsuperscript{-1}) and laccase (5.44 IU\textsuperscript{-1}) were achieved in 10 days as shown in (Table 5.1, Figure 6.1).

![Figure 6.1: Time course for ligninases production and cell biomass produced by Dothioraceae sp.](image)

The enzymatic pattern for Dothioraceae fungal species was distinguished among the known strains \textit{P. ostreatus}, and \textit{P. sanguineus}. It showed ligninolytic activities that were exceptional as compared to other strains (\textit{Ganoderma spp, Stereum ostreum}, and \textit{Trametes versicolor}) which had the lowest remazol decolorization efficacy and did not show any peroxidase activity. \textit{Dothioraceae} had the highest percentage of RBBR decolorization (94\%) in liquid medium.

As a control, the fungi were cultivated in broth culture medium without supplementation. During the development period, a record was carried out for biomass and protein yield in the broth culture medium. The concentration of proteins was comparable to the biomass yield, until the 12th day, when biomass started to decrease (Figure 6.2). The specific activities of MnP and LiP conversely continually increased.
Figure 6.2: Plot between incubation time (days) vs. biomass (g/100 ml flask) production among three fungal strains.

The white-rot fungi also required 35°C and 30°C for their optimal growth and ligninases production. The microbial growth and media composition are mainly involved for ligninases production (Fernandez et al., 2014). *Pleurotus ostreatus* also produce peroxidases, but at two different temperatures (10°C and 37°C), started to decrease manganese peroxidase (MnP) activities reported by Fernandez et al., (2014). Mester and Field (1997) optimized the MnP production by *Bjerkandera* sp. at 30°C and pH = 5.2. However, the ligninolytic activities produced by white-rot fungi is supposed to be species and strain dependent (Cupul et al., 2014). The maximum manganese peroxidase activity, as well as cell biomass, was supported at 30°C by *Dothioraceae* sp as shown in (Figure 6.3).
Figure 6.3: Effect of Temperature on MnP production; the test shake-flasks were inoculated with Brazilian isolated fungal strain (*Dothioraceae* sp) and incubated at different temperatures for 12 days in shake flasks. The maximum MnP activities (IU/L) and cell biomass (g/100ml) were observed at 10\(^{th}\) day of inoculation and plotted vs. different temperatures between (25-35 °C). Error bars represent the mean values of ± standard deviation from three replicates of shake-flask experiments. All values were differed significantly at \(p<0.05\).

The exposure of fungal culture to abiotic stress such as elevated temperature (Fink et al., 1999), heavy metals (Elisashvili et al., 2011) and oxidative stress (Jaszek et al., 2006) can be associated with the secretion of fungal oxidative enzymes. The laccase activity can be enhanced by *T. versicolor* in acidic pH-4.0 reported by Wang et al. (2014), while Fernandez et al. (2014) reported that at two different pH values (3.0 and 8.0) can reduce the enzyme activity. The effect of different pH range (4.5-7.0) of production media at 30°C was examined for MnP activities and the pH-6.5 was found to be the optimal pH as shown in (Figure 6.4).
Figure 6.4: Effect of pH on MnP production; the test flasks were inoculated with Brazilian isolated fungal strain (*Dothioraceae* sp) and incubated at 30°C for 12 days in shake flasks. The maximum MnP activities (IU/L) and biomass (g/100ml) were observed at 10th day of inoculation and plotted against vs. different pH range (4.5-7.0). Error bars represent the mean values of ± standard deviation from three replicates of shake-flask experiments. All values were differed significantly at $p \leq 0.05$.

White-rot fungi have been investigated by many scientists for the degradation and decolorization of many organic pollutants (Clemente et al. 2001). Peroxidases like MnP and LiP were generally recognized for the degradation of polymeric dyes (Moreira et al. 2001). The white rot fungi have the ability to decolorize polymeric dyes due to active ligninolytic activity. Selection of the best ligninases producing microorganism was investigated using petri plates with 0.05% Remazol Brilliant Blue R (RBBR) dye, which is similar to the lignin bio-polymer. RBBR is considered to be an anthracene derivative as well as an organic pollutant. In the past, many researchers used it as a model compound and found decolorization effectiveness based on existing ligninases activity (Moreira et al. 2001).
Our results indicated that, the newly isolated ascomycete *Dothioraceae* sp. is the first on record of producing extracellular oxidoreductases including laccase (Lcc), lignin peroxidase (LiP) and manganese peroxidase (MnP). The basidiomycetous white-rot fungi and related litter decomposing fungi are the most efficient lignin-degrading organisms in nature (Frackler et al. 2006). Nevertheless, the degradation of lignocellulose by ascomycetes is the major path for carbon cycling in soil and plant wastes (Nilsson et al. 1989; Liers et al. 2006). Species in this family have broad distribution, and are biotrophic or necrotrophic, usually linked with woody plants (Cannon et al. 2007). The ability to use several lignin model compounds and the decoloration of aromatic dyes by *Dothioraceae* sp. LM572 provided additional evidence of its potential to degrade lignin (Huiju et al. 2011).

### 6.4 Conclusions

The new isolated *Dothioraceae* sp. LM572 had a certain lignin degradation capacity and provided a new microorganism resource for lignin degradation. The ability of this fungal strain could be of interest for lignocellulosic biomass degradation as well as treatments of pulp and paper mill effluent. In addition, it would be more useful to compare the ligninolytic potential of this fungus with other lignin-degrading ascomycetes and basidiomycetes and more research regarding the role of ascomycetes on lignin biodegradation must carried out using *Dothioraceae* sp. LM572 and other related microorganisms.
7 Chapter: Depolymerization of bark with fungal laccases into bark-derived polyphenols and comparative studies between phenolic mediator vs. honey as a natural mediator substitute

7.1 Abstract

Due to increasing waste production and disposal problems arising from synthetic polymer production, there is a critical need to substitute materials with biodegradable and renewable resources. Use of laccases as biocatalysts to affect and enhance the catalytic properties of enzymes has been shown to be a promising solution for bark depolymerization. Initially eight different fungal strains were tested during submerged fermentation (SF) for laccase enzyme production, with the Pleurotus species found to be good producers compared to other fungal strains investigated. P. pulmonarius mainly produces laccase enzyme in production medium (PM) at initial conditions of pH-5.5 and 30°C. Bark depolymerization was conducted in SF and we identified polyphenols/polyaromatic compounds after four weeks when the PM was induced with 50mg/100ml of each bark during the lag-phase. During SF where honey was used as a natural mediator substitute (NMS) in the PM, laccase activities were about 1.5 times higher than those found in comparable cultures without honey in the PM. These samples were analyzed by GC-MS/MS. The laccase enzyme was purified using UNO® sphere Q-1 anion exchange chromatography and the molecular weight was determined to be ~50kDa on 10% SDS-PAGE. The laccase kinetic parameters were determined by using 2,2’-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as a substrate. The maximal velocity (V_{max}), Michaelis constant (K_{m}), and turnover number (K_{cat}) were found to be 76.9μM min^{-1}, 909μM and 739min^{-1}, respectively, from a Lineweaver Burk plot. Furthermore, laccases are suitable for biotechnological applications that transform bark biomass into high value bark biopolymers / biochemicals. The differences observed among identified aromatic compound GC-MS profiles were due to the utilization of two different bark species. Py-GC-MS analysis of bark showed differing effects of fungal activity on bark composition. Polyphenolics were separated in reverse-phase mode using HPLC with a pinnacle DB Biphenyl, C18 column, and UV detector. Two recognition wavelengths of 290 and 340 nm were selected to improve the separation of each single compound in monofloral honey and bark-fermented samples. This study is novel because it replaces natural mediators (NM) with
monofloral honey in PM and bark materials impregnated with honey, and studies the effects of fungi-derived laccases on bark-derived polyphenols.

7.2 Highlights

- Biochemical and catalytic properties of purified fungal laccases.
- Characterization of LMW polyphenols via GC-MS/MS & MALDI-TOF/MS.
- Use of monofloral honey as natural mediator substitute and compared with MS.
- Polyphenols separated by HPLC using two different detection wavelengths.
- Biotransformation of bark biomass into high value biopolymers/biochemicals.
- Integration between laccases vs. bark biopolymers and characterized by Py-GC/MS.

7.3 Introduction

The biobased economy relies on sustainable resources, and is likely to have an enormous impact comparable to the fossil fuel-based economy. The biobased economy is not about simply utilizing renewable resources and applying them to cutting-edge innovations; its principles can be used to modify procedures with far-reaching effects on society (Langeveld et al., 2010).

Nations have set managed goals to replace certain fuel and chemical commodities with biomass to help the biobased economy. Woody biomass provides lignocellulosic feedstock for the energy sector and chemical industry. Because of its inexhaustible accessibility, useful chemical composition, and reasonably low costs, woody biomass can improve products economically and sustainably. Global annual lignocellulosic biomass production was reported by Zhang (2008) to be about 200 billion tons (bnt). Haveren et al. (2008) found that 0.3 billion tons (bnt) is differentiated to synthetic chemicals every year.

In Europe and North America, *P. pulmonarius* is the most cultivated fungal species (Fungal Guide, 2012), and is commonly found throughout the world, particularly in temperate and subtropical forest. This species is frequently found on hardwoods in eastern North American forests; however, it is also found on conifers in the western United States (Stamets, 2000). The cultivation method,
which is comparable to that of other *Pleurotus* species, is by spreading spores in grain and then dispersing the inoculated seeds in cellulosic biomass as substrate, like straw, coffee grounds, wood chips, sawdust and cardboard.

*Pleurotus* is considered an effective lignin degrader that can grow fairly fast on different types of lignocellulosic biomass. Laccase (EC 1.10.3.2) belongs to the multicopper oxidase family. Fungal growth conditions, media composition and cultivation method play an important role for the laccase production (Lee et al., 1999).

The composition of honey, a natural bee product, depends mainly on the botanical source, topographical origin, and dispensing and atmospheric conditions (Chen et al., 2000; Vela et al., 2007; Alvarez et al., 2010). In honey, the main antioxidant compounds are polyphenolics, flavonoids, enzymes (catalase, glucose oxidase), organic acids, ascorbic acid, carotenoid-like substances, amino acids and proteins. The antioxidant value varies significantly depending on the floral source (Ghelfof et al., 2002). Several phenolic compounds are found in honey, including syringic acid (SA) and methyl syringate (MS) found in honey. Lately, a compound was found having substantial antibacterial activity against *Staphylococcus aureus* (Alvarez-Suarez et al., 2010).

The tree species balsam fir (Abies balsamea) is one of the more important conifers of the boreal forest. It is mainly used in pulp, light frame construction, and as well is a food source and shelter for wildlife (http://www.borealforest.org/).

Mixed aspen (Populus tremuloides and Populus grandidentata) are the most widespread forest species for managing biodiversity in the western and boreal zones in North America (Hogg et al., 2005). Polyaromatics are hydrocarbons, containing mainly C and H with multiple aromatic rings in which the electrons are delocalized. They are primarily found in fossil fuels (oil, coal and tar deposits) and are produced by partial burning of organic matter.

Laccase alone does not polymerize, nor depolymerize nor delignify pulp until it consolidates with a mediator like hydroxybenzotriazole (HBT). Laccases, owing to high redox-potential in basidiomycetes from genus *Trametes*, assist in lignin removal when combined with HBT from complete (Gutierrrez et al., 2012) and preserved (Chen et al., 2012) lignocellulosic biomass, producing cellulose available to hydrolysis. Generally examined mediators are synthetic
compounds based on nitrogen heterocycles. Because of high toxicity and high price, it is hard to make laccase-mediator systems (LMS) at an industrial scale.

Natural mediators, in the presence of laccase, facilitate the oxidation of non-phenolics depending on the phenolic compound structure, as well as, the reactivity vs. stability of phenoxy radicals produced (MS‘ > AS‘ > SA‘) reported by Tania et al. (2012). Due to complex nature of woody biomass, degrading enzymes, and their interactions, researchers are trying to profile radical-coupling routes which are involved in the formation of different phenolic species and identified as mediators (Foston et al., 2012).

Molecular properties of bark/biomass combustion (BC) can be assessed by pyrolysis–gas chromatography–mass spectrometry (Py–GC–MS) (De la Rosa et al., 2008; Kaal et al., 2009; Kaal et al., 2009; Fabri et al., 2012). Heat-affected reaction and resulting changes produce structures that may be like the pyrolysis artifacts of BC (Saiz-jimenez 1995; Wampler 1999). Py-GC-MS is considered a quick and quite inexpensive method for the bark characterization. The hydrolyzable bonds are cleaved and, subsequently, CO₂H and OH groups are changed in-situ by pyrolysis to related methyl esters and methyl ethers, respectively, which are more amenable to GC than their underivatized complements (Hatcher et al., 2001; Shadkami et al., 2010). It also provides supplementary data on structure over position of derivatized multifunctional rings (Shadkami et al., 2010).

There is need to focus on bark depolymerization and realizing the effect of honey in production media where it can mainly be used as replacement of natural phenolic mediators.

7.4 Results and discussion

7.4.1 Extracellular laccases and screening of potential strain

Extracellular laccase was produced during submerged growth conditions and screening of the best fungal strain was done based on the laccase production and growth conditions. These experiments were conducted both in solid as well as in liquid media. Among other competitor fungal strains, P. pulmonarius was screened for further examination as it possesses a strong laccase activity. This was confirmed using malt extract agar media plates (malt extract 25g; agar 20g; distilled water 1L, and 0.05% of the dye Remazol Brilliant Blue R), wrapped with aluminum foil to avoid light and incubated at 30°C for two weeks. Ligninolytic activity was assessed on agar plates by observing decolorizing of polyaromatic anthraquinone dye and
scoring attainable halos surrounding the fungus growing colonies as shown in Figure 7.2 (K-M).

After the agar plate prescreening, all fungal strains were transferred into the production media and fungal growth observed during submerged fermentation. 50mg of each bark was introduced in 100ml of production medium (PM) during lag-phase, when the fungus starts to produce laccase activity in the PM; this occurs about five days after the inoculum is placed in the PM. Maximum laccase activity was recorded after 25 days in submerged fermentation as 52 IU/ml/min in *P. pulmonarius*, 46 IU/ml/min in *P. cornucopiae*, 35 IU/ml/min *P. ostreatus*, 28 IU/ml/min in *P. chrysosporium*, 27 IU/ml/min in *T. versicolor*, and 23 IU/ml/min in *G. mangiferae*, respectively as shown in (Figure 7.1).

![Graph](image)

**Figure 7.1:** Representative time course of extracellular laccase activity (IU/ml/min) in the production medium on glucose (10g/L) in shake flask cultures (pH 5.5, 30°C, 150 rpm) following the growth of six different fungal strains induced with aspen bark during lag-phase including: (◊) *P. pulmonaris*, (□) *P. cornucopiae*, (+) *P. ostreatus*, (○) *P. chrysosporium*, (▲) *T. versicolor*, and (▼) *G. mangiferae*. Each point is a mean of three independent experiments. Bars indicate standard deviation among three replicates.
In nature, the lignocellulosic biomass is mainly composed of carbohydrate polymers (cellulose, hemicellulose) and an aromatic polymer (lignin) (Henriksson et al., 2000; Bernards et al., 2001). By utilizing fungal degrading enzymes, the polyphenolic–carbohydrate complex compounds disrupt it into smaller fragments. As lignin retains high antioxidant activity, (Cruz et al., 2001; Oki et al., 2002) hypothetically a decrease in size of lignin-like polyphenolics degraded fungus is able to increase soluble bark phenolic contents (Cohen et al., 2002; Jeandet et al., 2002; McCue et al., 2003).

It was observed that fungal growth increased through mid (10–25 days) to late (20–30 days) phases, with improved soluble phenolic contents. *P. pulmonarius* had the maximum virtual laccase activity among investigated fungal strains. In general, the phenolic compounds oxidized easily due to phenoxy radical formation as compared to non-phenolic compounds.

Increased phenolic concentration and oxygen availability assist polymerization. Lignin degrading rate via feedback control of laccases with phenolics directs an enzyme into a latent catalytic state explained by Harvey et al., 1993. Therefore, it starts a decline in enzyme activity after a certain time period owing to feedback inhibition due to accumulation of secondary metabolites and toxic compounds to a certain level. Morphological characterization of different fungal growth patterns on each bark was observed using AmScope-WF25X/9 (magnification 0.5X) attached with cold-light source haloid lamp 150W (Figure 7.2).
Figure 7.2: Different fungal strains growth on balsam fir (A-E) and mixed aspen bark (F-J) while, *P. cornucopiae, G. mangiferae, P. pulmonarius* (K-M) were exhibiting laccase activity on agar plates containing (2.5% malt extract +0.05% RBBR). From (N-P) shows fungal growth on the bark surface and (Q-R) presenting bark and *P. pulmonarius* surface morphology was observed under AmScope-WF25X/9 (magnification 0.5X).

7.4.2 Laccase purification and kinetics

The extracellular laccases from *P. pulmonarius* cultivated under submerged fermentation were purified. The purification method has already been explained in the methodology section. The sequential purification steps were summed up in (Figure 7.3) and its purification on 10% SDS-PAGE stained with Comassie Blue R-250 (Figure 7.4A). With the help of Lineweaver-Burk plot, the Michaelis constant (*K_m*) and maximal velocity (*V_max*) values of laccase from *Pleurotus pulmonarius* were obtained 909μM and 76.9μMmin⁻¹, respectively, and these constants are specific to a substrate (ABTS) (Figure 7.4B).
Figure 7.3: Laccase purification using FPLC by UNO® sphere Q-1 anion exchange column where (A-D) represents sequential purification steps.

Figure 7.4: Purified laccase molecular weight was determined on 10% SDS-PAGE from *P. pulmonarius* stained with Coomassie Blue R-250 showed Mw~50kDa. Lanes 1–2 are serially purified laccase enzyme, lane-3 shows purified laccase enzyme, *Pp* = total protein from *Pleurotus pulmonarius*, *Pc* = total protein from *Pleurotus cornucopiae*, and TS26616 for protein standard
marker shown in (7.4A); Lineweaver-Burk plot for the calculation of laccase kinetic parameters where ABTS used as a substrate (7.4B).

The turnover number \( (K_{cat}) \) and specificity constant \( (K_{cat}/K_m) \) was 739 min\(^{-1}\) and 0.81 µM\(^{-1}\)min\(^{-1}\) respectively. The similar specific constant was investigated for laccase enzyme from \( P. ostreatus \) where ABTS used as substrate (Palmieri et al., 1997). As a result of high catalytic efficiency and binding affinity with substrate (ABTS) it is possible to compare with other laccase producing microorganisms (Palmieri et al., 1997).

### 7.4.3 Bark decomposition by TG/DTG

Thermal degradation of bark samples was estimated using TGA. All experiments were done under nitrogen atmosphere. Both control and fungal (\( P. pulmonarius \)) treated bark samples were examined (Figure 7.5). Cellulose decomposition is denoted by the main DTG peak, while the shoulder peak mainly represents hemicellulose decomposition assigned at lower temperature (around 160 °C) (Varhegyi et al., 1989) while, lignin starts to decomposes from lower to wide temperature range (200–400 °C) (Jakab et al., 1997).

Aspen and fungal treated aspen bark samples started to decompose at about 190 °C and 140°C respectively, while, balsam fir and fungal treated fir bark samples were activated to decay at about 200°C and 170°C respectively. The solid-lines in both TG/DTG (thermograms- shown in Figure 7.5) indicate control or untreated barks whereas the dotted-lines specify fungal-treated bark samples. The weight loss of fungal degraded bark samples was faster between 140-400°C as also observed by Meszaros et al. (2004).

Relatively large amounts of water, carbon monoxide and carbon dioxide are formed during thermal decomposition of bark materials due to a large number of hydroxyl groups and oxygen atoms present in the natural polymers that form cell wall (cellulose, hemicellulose and lignin). Besides decomposition, smaller amounts of gases are released from the natural polymer. Carbon monoxide is formed around 500 °C, while carbon dioxide is released around 500 °C and also between 600-700°C from the bark. The occurrence of these smaller peaks is characteristic to oxalate decomposition due to presence of higher amount of oxalate ions in the bark compared to wood (Szekely et al., 1987).
Figure 7.5: TG/DTG curves for control/untreated (solid-lines) and *Pleurotus pulmonarius* treated (dotted lines) bark samples (A): aspen, (B): balsam fir.

7.4.4 Mass spectrometry (GC-MS/MS, MALDI-TOF/MS)

Mainly lower molecular weight compounds including polyaromatics and polyphenolics from fungal treatment of each bark species were identified and analyzed by GC-MS/MS and
MALDI-TOF-MS methods. In this study, GC–MS/MS was considered to analyze LMW compounds liberated from fungal decayed bark. The total ion chromatograph (TIC) of GC-MS/MS relating to ethyl acetate extracted compounds from acidified supernatants according to the method reported by Ksibi et al. (2003) were obtained from both control and fungal treated bark samples with *P. pulmonarius* and are shown in (Figure 7.6). Major identified peak retention time \( (t_r) \) values were marked in (green) while, all others in (black) are summarized in Table 7.1.
C  Fungal treated fir  
(Sample collected after three weeks of lag-phase)

D  Control-aspen

E  Fungal treated aspen  
(Sample collected after two weeks of lag-phase)
Figure 7.6: TIC from GC-MS/MS of identified compounds w.r.t. their $t_r$ values characterized from both control and each treated bark species from *Pleurotus pulmonarius* are listed in Table 7.1: where (A) balsam fir, (B and C) fungal treated fir, while, (D) aspen and (E and F) fungal treated aspen. The fungal treated samples of each bark exhibited a variable number of new peaks due to fungal degradation and changes in chemical composition.

Table 7.1: Bark polyphenols/polyaromatics identification.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Polyaromatics/polyphenols</th>
<th>m/z</th>
<th>Polyaromatics/polyphenols</th>
</tr>
</thead>
<tbody>
<tr>
<td>39, 39.6</td>
<td>CH₂=CH anion, Propyne</td>
<td>124.9</td>
<td>Guaiacol, 4-methoxy-1-oxide isocyanato-</td>
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<td>41.2</td>
<td>Methyl Isocyanide</td>
<td>127.83</td>
<td>2-Propenoic acid, Oxiranylnmethyl ester, 4- Pentenoic acid,</td>
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<td>42.1</td>
<td>Propene</td>
<td>128.8</td>
<td>Isoquinoline, 2-propanoic acid</td>
</tr>
<tr>
<td>43.01, 44</td>
<td>Iso-cyanic acid</td>
<td>139.97</td>
<td>1-propanol, 3-phenoxy-</td>
</tr>
<tr>
<td>56.9</td>
<td>CH₂COCH₃</td>
<td>143.84</td>
<td>2-Butenedioic acid, dimethyl ester</td>
</tr>
<tr>
<td></td>
<td>Chemical Name</td>
<td>Molecular Formula</td>
<td>Molecular Mass (Da)</td>
</tr>
<tr>
<td>---</td>
<td>---------------</td>
<td>-------------------</td>
<td>--------------------</td>
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<td>59</td>
<td>CH$_3$COO$^-$, Glyoxal</td>
<td>59.04</td>
<td>150.9, 151</td>
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<td>68</td>
<td>1,3 butadiene, 2-methyl-</td>
<td>74.12</td>
<td>128.8</td>
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<td>69</td>
<td>vinyl Isocyanate</td>
<td>C$_2$H$_3$N=C=O</td>
<td>62.03</td>
</tr>
<tr>
<td></td>
<td>CH$_2$=CHCH=CHO anion</td>
<td></td>
<td>128.8</td>
</tr>
<tr>
<td>74</td>
<td>Methyl propyl ether</td>
<td>C$<em>8$H$</em>{14}$O</td>
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<tr>
<td>77</td>
<td>Phenyl radical, Isopropyl methyl-d3-ether</td>
<td>C$<em>8$H$</em>{15}$O</td>
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<td>2-vinyl-1,3 butadiene</td>
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<td>81</td>
<td>2-furanyl-CH$_2$ anion, C$_6$H$_9$</td>
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<td>2-butene-1,4 diol</td>
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<td>196.2</td>
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<td>furaldehyde</td>
<td>C$_6$H$_5$CHO</td>
<td>96.11</td>
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<td>97</td>
<td>Isoxazole, 3,5 dimethyl-</td>
<td>C$_9$H$_9$N$_2$O</td>
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<td>1,3 butadiene-1-carboxylic acid</td>
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<td>128.18</td>
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<tr>
<td>104</td>
<td>Propanedioic acid</td>
<td>C$_4$H$_6$O$_3$</td>
<td>104.08</td>
</tr>
</tbody>
</table>
The fungal treated chromatographic profiles as shown in (Figure 7.6) are different than those of the controls, implying a strong biochemical ability of fungus on bark to alter bark composition. Apart from aldehyde and ketone types many acid-type complexes were also investigated due to microbial degradation of lignin (Hernandez et al., 2001).

MALDI-TOF analyses were done in order to determine the molecular weight distribution (MWD) of each bark species treated by *P. pulmonarius* as shown in (Figure 7.7); the main peak t_r values were specified in (blue) in Table 7.1. It was observed that mostly bark comprised LMW compounds and their molecular weights were lower than 327g mol^{-1}. The MALDI-TOF technique has been selected because it mainly produces singly charged ions, as well as, does not require any polymer standards for mass calibration. Analysis of low-mass fragments ions in the positive reflector mode offers a “fingerprint” of each bark sample that could be a good strategy to qualitatively identify lower molecular weight polyphenolics and polyaromatics owing to fungal treated depolymerized bark (Richel et al., 2012).
The molecular weight distribution (MWD) patterns were very similar within each bark species. During depolymerization, β-O-4 ether bond is heterolytically cleaved via formation of sodium phenolate and carbenium ion in transition state, which is instantly neutralized by hydroxide ion. In addition, the heterolytic cleavage of β-O-4 ether bond, dealkylation of side chains and hydrolysis of methoxy groups took place and lead to the formation of phenol, cresols and catechol reported by Erdocia et al. (2014).

A

Balsam fir
(Sample collected after two weeks of lag-phase)

B

Balsam fir
(Sample collected after three weeks of lag-phase)
C  Aspen
(Sample collected after two weeks of lag-phase)

D  Aspen
(Sample collected after three weeks of lag-phase)
Figure 7.7: MWD of each bark treated by *P. pulmonarius* from MALDI-TOF/MS, and the values are listed in Table 7.1. Chemical changes observed in fungal treated (A and B) balsam fir, and (C and D) aspen bark samples, where α-cyano-4-hydroxy cinnamic acid was used as the matrix compound.

7.4.5 Honey in PM as natural mediator substitute (NMS)

The effects of different honey concentrations on each bark species, extracellular laccase activity, and total polyphenolic contents were determined during a time course study in *P. pulmonarius*. Extracellular laccase activity by *P. pulmonarius* in response to different bark species are shown in (Figure 7.8). A significant increase in the laccase activity was observed in aspen bark compared to fir with methyl syringate used as a control natural mediator. The obtained results from fermented aspen bark species are comparable with methyl syringate (MS).
**Figure 7.8**: Time course study for laccase activity (IU/ml/min) in *P. pulmonarius* during SF containing 5, 7 and 10% (v/v) of monofloral honey in production media mainly used as a natural mediator substitute induced with 50mg of each bark in 100ml of PM during lag-phase (A)-Balsam fir, (B)-Mixed aspen bark, and (C)-1% Methyl syringate as natural phenolic mediator. Each point is a mean of three independent experiments. Bars indicate standard deviation among three replicates.

In the presence of different applied honey concentrations, after 18-days of incubation, the laccase activity began to slightly decrease. In our study, we used three different (% w/v) concentrations of honey but we observed the highest laccase activity 68 IU/ml/min at 7% (w/v) honey concentration after 18-days of growth (Figure 7.8). During fermentation, a higher substrate concentration in the production medium leads to catabolite repression which ultimately affects enzyme productivity yield. In contrast, it has been suggested that during laccase production in the presence of phenolic compounds, oxidation occurs to form quinone which is considered as toxic to fungal growth (Haars et al., 1980; Arora et al., 1985).

Furthermore, induction with bark during the lag-phase may decrease the extracellular proteolytic activity as well as ligninolytic enzymes including LiP, and MnP; therefore, it may increase the laccase activity. Thus, we can propose that improved laccase activity owing to ready availability of phenolics and aromatic compounds in fungal degraded aspen bark might help to improve enzyme stability (Yang et al., 2013).

In presence of ligninases, the oxidation mechanism of synthetic mediators like hydroxybenzotriazole (HBT) is similar to phenolic type mediators such as methyl syringate. During oxidation, highly reactive phenoxy radicals are produced which assist to remove one proton and one electron from the target substrate (Cantarella et al., 2003). Bark related free radicals and reactive oxygen species (ROS) are associated with laccase and laccase mediator system where honey is used as a natural mediator substitute.

### 7.4.6 Polyphenolic chromatograms

Reversed phase-high performance liquid chromatography (RP-HPLC) has been used for qualitative and quantitative analysis such as identification of separated analytes, which are precisely analyzed by UV/Vis detector and the spectral peaks are identified by their reported retention time (t\(_r\)) values (Chen et al., 2000; Romani et al., 2000; Inoue et al., 2005; Zielinska et al., 2008). Romani et al. (2000) compared electrochemical detection methods with HPLC for
polyphenolics in natural extract. The HPLC procedure was found to be more precise as compared to differential pulse voltammetry method which was suitable for fast screening. The polyphenolics separation was conducted by HPLC and the chromatograms are shown in (Figure 7.9).
(d) FS-Mixed aspen

(e) \(\lambda=340\text{nm};\) Dil. Honey

(f) FS-Mixed fir

(g) FS-Mixed aspen
Figure 7.9: HPLC chromatograms phenolic profiles of bark fermented samples at 7% honey in production media (a-d at $\lambda_{290}$) and (e-h at $\lambda_{340}$). Peak identification: methyl syringate (12.017), pinobanksin (13.100), 8-methoxykeampferol (24.567), pinocembrin (36.467), chrysin (39.62), pinocembrin 7-Me (55.217), tetrochrysin (57.142). All major peaks were characterized based on their $t_r$ values of honey polyphenolics previously reported by Pyrzynska et al. (2009).

Remarkably, it was noticed that the standard methyl syringate (MS) peak appeared at 290nm but vanished at 340nm. Similarly, the diluted honey sample had MS peak at 290nm, but, when the same sample was run at 340nm the peak was missing and different peaks were seen. Our HPLC results confirmed that the wavelength at $\lambda=290$ nm was more suitable for the separation of MS natural mediator compounds. All major peaks in the chromatograms were compared and characterized based on their $t_r$ values of honey polyphenolic compounds as earlier reported by Pyrzynska et al. (2009).
Table 7.2: Total polyphenolics in fermented sample when induced with 50mg of each bark into 100ml of PM during lag-phase.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mean total polyphenolics (mg cat equiv/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM-Gluc</td>
<td>18.4 ± 0.54</td>
</tr>
<tr>
<td>Aspen PM-H</td>
<td>23.7 ± 0.61</td>
</tr>
<tr>
<td>PM-MS</td>
<td>27.8 ± 0.48</td>
</tr>
<tr>
<td>Balsam PM-Gluc</td>
<td>20.1 ± 0.81</td>
</tr>
<tr>
<td>Fir PM-H</td>
<td>28.8 ± 0.36</td>
</tr>
<tr>
<td>PM-MS</td>
<td>32.1 ± 0.74</td>
</tr>
</tbody>
</table>

Mean ± S.D (n = 5); Catechol was used as a standard for the calibration of total polyphenols in fermented samples during late-log phase (after 20-days of fermentation). Calibration curve was prepared by using catechol solutions at concentrations (10 to 50 mg / 100 ml) in distilled water.

Total phenolic content of 0.114 ± 0.09 mg cat equiv/g was found in a buckwheat monofloral honey. Wood degrading fungi play an important role by attacking protein-polyphenolic complexes which possibly changes substrate properties (Bending et al., 1997). Polyphenolics, mainly condensed tannins with protein complexes assist to develop in fungal growth (Mutabaruka et al., 2007). During fermentation in PM with honey, it was also noticed that total polyphenols in each bark species were significantly degraded as compared to glucose and natural mediator (MS) samples because of fungal biomass accumulation.

7.4.7 Py-GC-MS

Py–GC–MS total ion chromatograms (TIC) are represented in Figure 7.10. The major peaks in TIC-fingerprints are the pyrolysis products reported in (Table 7.3), with contrasting retention times (RT), characteristic ion fragments (m/z) utilized for the quantification and their relative proportions. Flags are used to organize the compounds and are mainly divided into seven groups i.e., ALIPH = aliphatic compound, CARB = carbohydrate product, LIG = lignin, MAH
= monocyclic aromatic hydrocarbon, NCOMP = nitrogen-containing compound, PHEN = phenol, SESQUI = sesquiterpenoid. Total quantified peak area (TQPA) values of each group are given in Table 7.4, and the values of each group were calculated based on Table 7.3.

**Figure 7.10:** Total ion chromatograms (TIC) of untreated (control) bark samples of aspen and fir, MS-treated and bark fermented samples in honey production medium. Peak labels refer to peak numbers in Table 7.3.
Table 7.3: List of main pyrolysis products identified from the untreated/control, MS treated and honey fermented bark samples of Aspen and Fir. Percentages refer to the proportion of total quantified peak area (TQPA) accounted for by each compound (sum 100%). Peak numbers refer to labels in (Fig.7.10). Retention time = (tR), Dominant ion fragments = m/z. Flags are used to organize the compounds in main groups: ALIPH = aliphatic compound, CARB = carbohydrate product, LIG = lignin product, MAH = monocyclic aromatic hydrocarbon, NCOMP = Nitrogen containing compound, PHEN = phenol, SESQUI = sesquiterpenoid.

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Pyrolysis product</th>
<th>Retention time (tR) min</th>
<th>m/z</th>
<th>Flag</th>
<th>Mixed Aspen Bark</th>
<th>Mixed Balsam Fir Bark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>MS treated samples</td>
</tr>
<tr>
<td>1</td>
<td>toluene</td>
<td>2.535</td>
<td>91, 92</td>
<td>MAH</td>
<td>23.2</td>
<td>19.7</td>
</tr>
<tr>
<td>2</td>
<td>3/2-furaldehyde</td>
<td>2.918</td>
<td>95, 96</td>
<td>CARB</td>
<td>15.0</td>
<td>11.0</td>
</tr>
<tr>
<td>3</td>
<td>C1-pyridine</td>
<td>3.107</td>
<td>93, 66</td>
<td>NCOMP</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>C2-benzene</td>
<td>3.107</td>
<td>91, 106</td>
<td>MAH</td>
<td>7.3</td>
<td>5.6</td>
</tr>
<tr>
<td>5</td>
<td>2-furanmethanol</td>
<td>3.147</td>
<td>98, 97</td>
<td>CARB</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>6</td>
<td>Styrene</td>
<td>3.404</td>
<td>104, 78</td>
<td>MAH</td>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>7</td>
<td>2,3 dihydro-5-</td>
<td>3.896</td>
<td>98, 55</td>
<td>CARB</td>
<td>6.9</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>methylfuran-2-one</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3-methyl-2-</td>
<td>3.939</td>
<td>110, 109</td>
<td>CARB</td>
<td>1.8</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>furfuraldehyde</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>2-methyl-2-</td>
<td>4.091</td>
<td>96, 67</td>
<td>CARB</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>cyclopenten-1-one</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>phenol</td>
<td>4.234</td>
<td>94, 66</td>
<td>PHEN</td>
<td>15.0</td>
<td>15.9</td>
</tr>
<tr>
<td>11</td>
<td>3-hydroxy-2-methyl-</td>
<td>4.486</td>
<td>112, 55</td>
<td>CARB</td>
<td>2.4</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>2-cyclopenten-1-one</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1,2-</td>
<td>4.583</td>
<td>121, 122</td>
<td>CARB</td>
<td>2.3</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>hydroxybenzaldehyde</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>4-methylphenol</td>
<td>4.749</td>
<td>107, 108</td>
<td>PHEN</td>
<td>4.2</td>
<td>4.6</td>
</tr>
<tr>
<td>14</td>
<td>unidentified</td>
<td>4.766</td>
<td>57, 70</td>
<td>ALIPH</td>
<td>6.5</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>aliphatic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>guaiacol</td>
<td>5.046</td>
<td>109, 124</td>
<td>LIG</td>
<td>5.0</td>
<td>8.7</td>
</tr>
<tr>
<td>16</td>
<td>4-methylguaiacol</td>
<td>5.784</td>
<td>123, 138</td>
<td>LIG</td>
<td>1.7</td>
<td>2.6</td>
</tr>
<tr>
<td>17</td>
<td>4-ethylguaiacol</td>
<td>6.380</td>
<td>137, 152</td>
<td>LIG</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>18</td>
<td>4-vinylguaiacol</td>
<td>6.643</td>
<td>150, 135</td>
<td>LIG</td>
<td>2.6</td>
<td>6.5</td>
</tr>
<tr>
<td>19</td>
<td>C3-guaiacol</td>
<td>6.912</td>
<td>164, 149</td>
<td>LIG</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>20</td>
<td>alkene</td>
<td>7.015</td>
<td>55, 69</td>
<td>ALIPH</td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td>21</td>
<td>C3-guaiacol</td>
<td>7.244</td>
<td>164, 149</td>
<td>LIG</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>22</td>
<td>C3-guaiacol</td>
<td>7.530</td>
<td>164, 149</td>
<td>LIG</td>
<td>0.8</td>
<td>1.5</td>
</tr>
<tr>
<td>23</td>
<td>cf-α-murolene</td>
<td>7.833</td>
<td>105, 204</td>
<td>SESQUI</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>(sesquiterpenoid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>unidentified</td>
<td>10.010</td>
<td>81, 95</td>
<td>ALIPH</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Table 7.4: Main pyrolytic compound groups and their cumulative relative proportions (% TQPA). The calculated (% TQPA) values of each group were based on Table 7.3.

<table>
<thead>
<tr>
<th>Flag</th>
<th>Aspen (% TQPA)</th>
<th>Fir (% TQPA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>MS</td>
</tr>
<tr>
<td>ALIPH</td>
<td>8.1</td>
<td>6.2</td>
</tr>
<tr>
<td>CARB</td>
<td>30.2</td>
<td>23.2</td>
</tr>
<tr>
<td>LIG</td>
<td>13.2</td>
<td>22</td>
</tr>
<tr>
<td>MAH</td>
<td>24.5</td>
<td>27</td>
</tr>
<tr>
<td>NCOMP</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>PHEN</td>
<td>24</td>
<td>21.6</td>
</tr>
<tr>
<td>SESQUI</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

7.4.7.1 Aspen bark

The pyrolysis fingerprints of untreated/control aspen bark is dominated by phenols (compounds 10, 13) and guaiacols (15-19, 21, 22), in addition to an unidentified aliphatic compound (14). The guaiacols originate predominantly from guaiacyl-based lignin, while syringyl-type markers were virtually absent. Such dominance of guaiacyl groups is typical of lignin in bark materials (Marquez et al., 2013).

The MS-treated bark sample from aspen produced a set of pyrolysis products that is rather similar to that of the control sample, even though there are minor differences such as a smaller relative proportion of phenols and higher proportion of guaiacols, especially guaiacol (15) and 4-vinylguaiacol (18). This might suggest that the material that is primarily affected by fermentation in MS is the phenol precursors while the lignin component is relatively unaffected.
The pyrolysis fingerprint of the aspen bark sample fermented in honey medium was very different from the control and MS-treated analogues. It is dominated by a set of furans, furaldehydes and cyclopentenones (2, 5, 7-9, 11), which are typical products of polysaccharides (Pouwels et al., 1989). Guaiacol represents a lignin fraction that is relatively unaffected, but the intensities of the guaiacols are much lower than in the control and MS-treated samples.

Moreover, several N-containing pyrolysis products such as methyl pyridine (compound 3), in combination with the chitin marker acetamide (intensity too low for representation in Figure 7.10, but identified unambiguously) are indicative of a major increase in the amount of microbial biomass in this sample. This makes it very likely that the carbohydrate products do not represent a recalcitrant polysaccharide component but rather that they originate from the honey source. Despite the small proportion of the lignin products, they can be traced from partial ion chromatograms (PIC) as shown in Figure 7.11.
Figure 7.11: Partial ion chromatograms (PIC) of selected compounds of polyphenolic origin from each bark fermented samples in PM with monofloral honey used as NMS analyzed by Py-GC-MS. Selected polyphenolic compounds are: guaiacol (m/z 109+124), 4-methylguaiacol (m/z 123 + 138), 4-vinylguaiacol (m/z 135 + 150) and syringic acid methyl ester (m/z 181 + 212). Chromatograms were plotted between relative peak intensity vs. retention time (t_r).

7.4.7.2 Balsam fir bark

The pyrolysate of the untreated bark material from fir was strongly dominated by guaiacol products (15-19, 21-23), suggesting that the sample consists almost completely of guaiacyl-type lignin material.
The MS-treated sample gave a similar pyrolysis fingerprint, even though minor increases of monocyclic aromatic hydrocarbons (MAHs, 1, 4, 6) can be witnessed. This suggests that fermentation in MS produced some degradation of the lignin backbone. The relative loss of 4-vinylguaiacol in comparison to other guaiacols might indicate that the sample contained a non-lignin polyphenolic material that was preferentially decomposed to a slight extent, as 4-vinylguaiacol is not only a product of guaiacyl-type lignin but also of cinnamic acids (Ralph et al., 1991).

The fir bark material submerged in honey medium produced a very different set of pyrolysis products, dominated by MAHs (1, 4) and furaldehydes (2, 8). Some remains of lignin can still be recognized from the total ion chromatograms (TIC), e.g. 4-methylguaiacol (compound 16) and 4-ethylguaiacol (17), and additional compounds can be traced from partial ion chromatograms (Figure 7.11), but it is clear that this fermentation treatment almost completely degraded the original polyphenolic structures and the sample became dominated by microbial biomass.

The lignin products are very abundant in control/untreated and MS-treated samples, and they can be identified directly from the TIC (with the peak labels). There would be no added scientific value to presenting the partial ion chromatograms (PIC) of these compounds. As compared to bark fermented samples in PM with honey as NMS, the lignin products are not very abundant; therefore, partial ion chromatograms (PIC) were supported for the better separation of polyphenolic compounds. However, most of the syringol (S-type lignin) products were below the detection limit and the syringol was quantified at (m/z 154 and 139 at 6.8 min) as shown in Figure 7.11.

Possibly, the polyphenols in honey production media are below the detection limit of Py-GC-MS or these polyphenols could have been degraded by pyrolysis, producing aromatics such as toluene and benzene. Traces of catechol can be detected by the Folin-Denis method but it should be below approximately ~1mg/100mg of catechol and below the detection limit of the pyrolysis method.

Both control samples produced typical pyrolysis fingerprints of bark materials consisting primarily of lignin and polysaccharides. The MS-treatment seemed to preferentially degrade a non-lignin component in the aspen bark, causing the enrichment of guaiacol markers from
lignin, while the opposite trend was observed for the fir bark fermented in MS medium. This difference may be explained by the differences in original sample composition, with the fir bark being composed almost purely of lignin while the aspen bark sample also contains polyphenolic precursors producing phenols rather than guaiacols and a more abundant aliphatic component.

These additional components in fir bark materials are probably more heavily affected by fermentation in MS than the lignin component. Anyway, the effects of fermentation on the pyrolysis fingerprints of both bark samples were rather small. By contrast, fermentation in honey medium eliminated most of the recognisable polyphenols (lignin) and both samples were almost completely converted into microbial cells which mainly composed of carbohydrates as shown in Figure 7.12 (A).

![Figure 7.12: Representation of the Lignin/Carbohydrate index (A) and Syringol/Guaiacol ratio (B) for the different analyzed samples.](image-url)
The lignin is strongly dominated by guaiacol (G-type lignin) which is why it can be concluded that proportionately very small amount of syringol (S-type lignin) was found (less than 0.20 \%/%) in all samples and the relative proportion of syringol/guaiacol (S/G) ratio of different treated samples are shown in Figure 7.12 (B). MS-treated sample seems to slightly increase (S/G, \%/%) ratio while honey medium has opposite effects.

7.5 Conclusions

In the present study, we demonstrated that monofloral honey can be used as a natural mediator substitute in PM during submerged fermentation for the production of fungal laccases. We also found a methyl syringate in the diluted honey sample through HPLC analysis that separated at a wavelength of 290 nm. The time course study showed diverse laccase production among different fungal cultures. Moreover, GC-MS/MS and MALDI-TOF/MS analyses were found to be suitable methods to analyze LMW polyaromatics and polyphenolics due to fungal degradation of bark. Methyl syringate is considered to be the most effective natural mediator for the oxidation of non-phenolic lignin units, but our results during submerged fermentation with 7\% honey in production medium was comparable to the natural mediator. Py-GC-MS of bark fermented samples in a honey medium indicated evidence of a profound effect on biotic degradation to an extent that a large proportion of the pyrolyzate can be traced back to microbial biomass. This microbial biomass may be related to the formation of thermolabile substances (proteins, polysaccharides, and microbial cell wall components such as chitin) detected by thermogravimetry. The abundance of phenols and unidentified products combined with the polysaccharide fingerprint of degraded material indicate significant fungal degradation and depolymerisation of bark biopolymers. Hence, biotechnological procedures were effectively used for the conversion of bark biomass into high value bark-derived polyphenols.
8 Chapter: Conclusions and Future work

8.1 Major contributions

This thesis describes a comprehensive and systematic study on bark depolymerization during submerged fermentation using monofloral honey, a natural mediator substitute, and investigates the interaction between laccases and bark biopolymers. It incorporates comparative studies of enzymatic, chemical and physical treatments for the extraction of two bark species and characterizes bark polyphenolic extractives. Potential fungal strains for ligninases production were identified, and purification, characterization and effective valorization of lignin and bark were investigated.

The major findings of this thesis are as follows:

1. The HPLC analysis shows fast, selective, sensitive, and reliable determination of the most common polyphenolics in bark extractives. UV/H₂O₂ treatment was able to preferentially remove phenolic compounds, with limited effect on the sugar concentration of the bark hydrolysates. Under these conditions, high removal of phenolic compounds, expressed in terms of catechol equivalent (0.8% in BILP and 0.6% in aspen) bark extractives were observed. Furthermore, these results elucidate the effects of different treatments on bark derived polyphenols, which can be considered as promising methods for applications in bioconversion and polymer industrial processes.

2. Potential fungal strain for ligninolytic activities were screened, MnP partially purified and the degrading pattern of local lignin resources found in Brazil was observed. In addition to this, potential products from various lignin-degrading enzymes and substrates were identified. Application of ligninases in the biotech industry will be possible if these enzymes and mediators are commercially developed. The unknown isolated fungal strain was Dothioraceae sp. LM572 by 18S-rDNA.
3. The process parameters such as temperature and pH of the medium in shake flask experiments were optimized for the maximum production of manganese peroxidases. The maximum manganese peroxidase activity, as well as cell biomass, was supported at 30°C and pH 6.5 by *Dothioraceae* sp.

4. The new isolated *Dothioraceae* sp. LM572 has high lignin degradation capacity and is a new microorganism resource found for lignin degradation. The ability of this fungus strain could be of interest for lignocellulosic biomass degradation as well as for treatment of pulp and paper mill effluent. Furthermore, it would be more informative to compare the ligninolytic capabilities of this fungus with other lignin-degrading ascomycetes and basidiomycetes and further research concerning the role of ascomycetes on delignification should be carried out using *Dothioraceae* sp. LM572 and other microorganisms of this class.

5. GC-MS/MS and MALDI-TOF/MS analyses were found to be suitable methods to analyze LMW polyaromatics and polyphenolics from fungal degradation of bark. Methyl syringate is considered as the most effective natural mediator used for the oxidation of non-phenolic lignin units but our obtained result during submerged fermentation with combination of 7% honey in production media increased laccase activity 1.5-times, which was comparable to the natural phenolic mediator.

6. Pyrolysis-GC/MS of bark fermented samples in honey medium indicated evidence of a profound effect of biotic degradation, to an extent that a large proportion of pyrolyzate (~60-90%) was converted into microbial cells. This microbial biomass may be related to the formation of the thermolabile component detected by thermogravimetry. The abundance of phenols and unidentified products, in combination with the polysaccharide fingerprint of degraded material, are indicative of significant fungal degradation and depolymerisation of bark biopolymers.

In this thesis, significant data about bark depolymerization and for application development are presented. This research has explored the feasibility of bark conversion into bio-based green chemicals; additional research is required to obtain the optimum bioprocess conditions either using chemoenzymatic or biocatalysis approaches applying natural mediator sources.
To further develop this process there has to be more focused research to find low-cost natural mediator sources. Further investigation into effect of concentrations on bark biological modifications is needed to better explore biotransformation routes for green biochemicals production.

### 8.2 Recommendations for Future Work

Further work is needed in the research area of creating new bio-based chemicals and functional biopolymers. There is a need to improve technology through pilot scales to facilitate future applications and commercialization processes. Key areas proposed for further work are outlined as follows:

1. The fungal strains can be improved for industrial scale ligninases production through random mutagenesis (uv-irradiation, gamma-ray irradiation, chemical mutagens), protoplast fusion or by genetic manipulation which might be involved in the re-routing of metabolic pathways. Comparative studies can be conducted between parent and the mutated strains.

2. Another potential research area associated with microbial metabolism is to isolate and identify lignin- and furfural-tolerant bacteria from the gut of wood feeding termites and to screen various ligninolytic enzymes for different industrial bioprocesses. Consequently, in fungus or anaerobic microorganisms, the metabolism of polymeric / furanic compounds which eventually lead to discover alternative metabolic pathways.

3. Scale-up of current process from lab scale to industrial scale, and the optimization of fermentation parameters for the process development of bark depolymerization, with emphasis on honey to produce more polyphenolic compounds and should motivate future research.

4. Moreover, difficulties exist in the purification and recovery of polyphenolic compounds after bark depolymerization during submerged fermentation, which is a big challenge. Nanofiltration membrane might be used for the separation of natural polyphenolic compounds which can be used as food additives, active cosmetics or as drugs.
5. There needs to be more focus on eco-friendly methods of extracting bark biopolymers from bark biomass as an alternative way to synthetically derive molecules. Based on the functionality of materials from bark, they can be developed in applications for adhesives, coatings and plastic additives.
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Appendix

Figure 1. Representative time course of extracellular lignin peroxidases (LiP) production in shake-flask experiments among three fungal strains.

Figure 2. Representative time course of extracellular laccase (Lcc) production in shake-flask experiments among three fungal strains.
Figure 3. Representative time course of extracellular manganese peroxidases (MnP) production and cell biomass in shake-flask experiments among three fungal strains.

Figure 4. Determinations of decolorization ability of RBBR dye in the broth medium.
Figure 5. MW distribution to determine the lignin monomers by using MALDI-TOF-MS, where 2,5-dihydroxy benzoic acid (DHB) was used as matrix compound. From (A-D) Black liquor of Eucalyptus (BL) and from (E-I) Sugarcane bagasse (SCB) samples treated with or without Brazilian isolated fungal strain (O).
# Certificate of Analysis

**Product Name:** CELULLASE FROM TRICHODERMA REESEI, \(-4 U/\text{MG}\)

**Product Number:** 22173  
**Batch Number:** 1086305  
**Product Brand:** Fluka  
**Molecular Formula:**  
**Molecular Mass:**  
**CAS Number:** 9012-54-8  
**Quality Release Date:** 01/\text{APR}/04  
**Date retested:** 24/\text{APR}/06  
**Recommended Retest Date:** AUG/08

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**RESULTS**  

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1 U corresponds to the amount of enzyme which sets free 1 umol glucose from carboxymethylcellulose per minute at pH 5.0 and 57 deg C

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Claudia Geithere  
Manager Quality Control  
Buchs, Switzerland
Certificate of Analysis

Product Name: β-GLUCOSIDASE ALMONDS
                lyophilized, powder, >= 6 units/mg
Product Number: 49290
Batch Number: BCBP9743V
Brand: Sigma
CAS Number: 9001-22-3

Formula:
Storage Temperature: 2-8 °C
Quality Release Date: 28 APR 2015
Recommended Retest Date: AUG 2017

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Dr. Claudia Geltner
Manager Quality Control
Buchs, Switzerland

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Bark depolymerization during submerged fermentation using monoflora\nmoney, a natural mediator substitute, and integration between lac\ncases vs. bark biopolymers, characterized by Py-GC-MS

M. Fehan, a, b N. Yan a, b and M. Sain a, b

Due to increasing waste production and disposal problems arising from \nsynthetic polymer production, there is a critical need to substitute \nmaterials with biodegradable and renewable resources. Attempts to \nuse laccases as a catalyst to enhance the catalytic properties of \enzymes have shown them to be a promising solution for bark \ndepolymerization. In this study, eight different fungal strains were \ntested for laccase enzyme production during submerged fermentation \n(SF). and the Pleurotus species were shown to be the best producers \namong the competing fungal strains. P. pulmonarius mainly produces \nlaccase enzyme in production medium (PM) at initial conditions of \n$pH$ 5.5 and 30 °C. Bark depolymerization was conducted in SF and \nwe identified polynucleic/polynuclear aromatic compounds after four \nweeks when the PM was induced with 50 mg per 100 mL of each \nbark during the lag-phase. During SF where honey was used as a \nnatural mediator substitute (NMS) in the PM, laccase activities were \nabout 1.5 times higher than those found in comparable cultures \nwithout honey in the PM. These samples were analyzed by GC-MS/ \nMS. The laccase enzyme was purified using UNO® sphere Q-1 anion \nexchange chromatography and the molecular weight was determined \nto be $\sim$50 kDa on 10% SDS-PAGE. The laccase kinetic parameters \n$V_{max}$, $K_m$, and turnover number ($K_{cat}$) were found to be 76.9 \n$\mu$m min$^{-1}$, 909 $\mu$m and 739 min$^{-1}$, respectively, from a \nLineweaver–Burk plot. Furthermore, laccases are suitable for \nbiochemical applications that transform bark biomass into high \nvalue bark biopolymers/biochemicals. The differences observed \namong the identified aromatic compound MS/MS profiles were due \nto the utilization of two different bark species. Py-GC-MS analysis of \nbark showed differing effects of fungal activity on bark composition. \nPolyphenolics were separated in reverse-phase mode using \nHPLC with a pinacle DB \nBiphienyl, C18 column, and UV detector. Two recognition \nwave-lengths of 290 and 340 nm were selected to improve the \nseparation of each single compound in monoflora honey and \nimmune-fermented samples. This study was novel because it replaces \nnatural mediators (NM) with monoflora honey in PM and bark \nmaterials impregnated with honey, and studies the effects of fungi-derived \nlaccases on bark biopolymers.

1. Introduction

The biobased economy relies on sustainable resources, and is \nlikely to have an enormous impact comparable to the fossil-\nbased economy. The biobased economy is not about simply \nutilizing renewable resources and applying them to cutting-\nedge innovations; its principles can be used to modify procedures \nwith far-reaching effects on society. J Nations have set \nm\n\n
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Comparison of Enzymatic, Alkaline, and UV/H₂O₂ Treatments for Extraction of Beetle-Infested Lodgepole Pine (BLIP) and Aspen Bark Polyphenolic Extractives

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Center for Biocomposites and Biomaterials Processing (CBBP), Faculty of Forestry, University of Toronto, Toronto, ON, Canada MSS 3B3

ABSTRACT: This paper describes the comparison of enzymatic, alkaline, and UV/H₂O₂ treatments for the extraction of beetle-infested lodgepole pine (BLIP) and mixed aspen barks polyphenolic extractives. The concept of green polymers has become more appealing due to the presence of large volumes of processing residuals from the timber and pulp industries. This, in turn, supports the idea of developing new polymers based on bark extractives. Here, we used a chromatographic method to determine the chemical composition of some of the polyphenolic compounds in bark extractives and observed the effect of different extraction methods on extraction yield. Polyphenolic compounds separation was performed using HPLC in reverse-phase mode with an octadecylsilane (ODS), C₁₈ column (3 μm particle size), and an UV detector. Detection wavelengths of 280, 310, and 370 nm were selected to allow better separation of each compound. The comparative studies and effects of enzymatic, alkaline, and UV/H₂O₂ treatments on extractives yield and component contents were investigated. UV/H₂O₂ treatment exhibited the highest yield with 54% of dry bark weight extracted and was found to degrade larger amounts of lignins/tannins than enzymatic and alkaline treatments. Conversely, enzymatic treatment was good for holocellulose.

KEYWORDS: BLIP, Mixed aspen bark, Polyphenolic extractives, HPLC, UV/H₂O₂, Enzymatic and alkaline treatments

INTRODUCTION

Today, we are facing an environmental crisis, and its associated socio-ecological burden, mainly due to the extraction and processing of fossil fuels. Under these circumstances, it is urgent to reduce fossil resources consumption. As such, replacing petrochemical products with green biomaterials based on cost-effective renewable resources appears a viable solution. Petrochemical compounds such as phenol and its derivatives are produced in quantities over 10 million tons on an annual basis (essentialchemicalsindustry.org/chemicals/phenol.html). Therefore, to alleviate environmental concerns, it is important to find new natural raw materials to produce polyphenolic compounds. One such raw material is woody biomass and bark, which is available in abundance as the byproduct from wood conversion industries.

Bark, similar to wood, is composed of cellulose, hemicellulose, lignin, extractives, and ash. Compared to wood, bark contains higher amounts of phenolic extractives, such as lignan, hydroxylxy, and condensed tannins, which have been of great interest to scientists and green technologists during the last two decades. The main idea of this study is to produce value-added chemicals that are rich in phenolic structures and thus suitable for wood adhesives, PF resins, polyols, polyurethane foams, and Novolak resins. In addition, the lower toxicity of bark-based phenolics as compared to pure phenol would expedite resin production.

In British Columbia, lodgepole pine (Pinus contorta var. latifolia) accounts for 24% of the total forest growing stock. Large numbers of mature lodgepole pines have been damaged by the attack of the mountain pine beetle (Dendroctonus ponderosae) and its fungal associates. The scale of the mountain pine beetle attack in British Columbia is unprecedented. In 2010, the beetle attack had affected over 18 million hectares of forest and had killed 710 million m³ of lodgepole pine.

Aspen (Populus tremuloides) is considered as a source species and is important for maintaining biodiversity in the western and boreal regions in North America. It is one of the main timber sources in North America and in some Scandinavian and Baltic nations. Aspen accounts for 39.5% of the growing stock volume of the forested land base in these countries, which is almost equal to the 42.1% of the stock that is attributable to all coniferous species. In order to better understand the bark extraction process and to provide better direction for the application of bark extractives, it is important to explore and compare the effect of

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Identification of a potential fungal species by 18S rDNA for ligninases production

M. Ferhan, S. N. Santos, I. S. Melo, N. Yan, M. Sain

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Abstract  Fungal species for ligninases production was investigated by 18S ribosomal DNA sequence analysis. Two primer sets were chosen to amplify a major part of the 18S rDNA, which resulted in intense PCR product of approximately 550–820 bp in size per sample. The results suggest that the 18S rDNA-based approach is a useful tool for identification of unknown potential fungal species for ligninases production. The isolated fungal species produces mainly manganese peroxidase (MnP). The enzyme oxidized a variety of the usual MnP substrates, including lignin related polyphenols. Time course studies showed that maximum production of ligninolytic enzymes MnP (64 IU L⁻¹), lignin peroxidase (26.35 IU L⁻¹), and laccase (5.44 IU L⁻¹), respectively, were achieved after 10 days of cultivation under optimum conditions. Furthermore, the biological decolorization of Remazol Brilliant Blue R dye following 10 days of cultivation was 94 %. NCBI BLAST was used to search for closest matched sequences in the GenBank database and based on sequence homology the first BLAST hit was Dothioraeae sp. LM572 with accession number EF060858.1.

Keywords  Dothideomycetes · 18S rDNA · PCR · Ligninases · Ascomycetes

Introduction

Filamentous fungi play a prominent role in the decomposition of lignocellulose. There are three types of fungi living on dead wood that effectively degrade one or more wood components: soft-rot fungi, brown-rot fungi, and white-rot fungi. Soft-rot fungi (Anamorphs of Ascomycota and Basidiomycota) can decay cellulose, but are thought to degrade lignin gradually and partly (Grazyna et al. 1989). Dothioraeae, a family of fungi in the order Dothideales, includes widely distributed species that are biotrophic or necrotrophic, and usually associated with woody plant decay (Cannon and Kirk 2007). Extracellular oxidoreductases, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase produced by wood-decomposing microorganisms, are directly involved in the degradation of lignin in their natural lignocellulosic substrates and various xenobiotic compounds including dyes (Abrahão et al. 2008). Some wood-degrading fungi contain all three classes of lignin-modifying enzymes, while the others contain only one or two of these enzymes (Dhouib et al. 2005). The production of ligninolytic enzymes is observed as a colourless halo around microbial growth (Dhouib et al. 2005). So, the lignin degradation capability of microbes is initially screened indirectly on solid media containing different indicator compounds.

Although, wood decay fungi are primarily basidiomycetes, other microorganisms are also involved in the lignocellulosic decaying processes (Nilsson et al. 1989). Wood biodegradation by certain ascomycetes was first described in detail and designated as “soft rot” by Savory (1954). Nilsson et al. (1989) showed that, some higher ascomycetes, mainly Daldinia concentrica, degraded Aspen wood with the same strength as Trametes versicolor, a basidiomycete characteristically classified as white-rot fungus. The
Ligninases Production and Partial Purification of Mnp from Brazilian Fungal Isolate in Submerged Fermentation

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3Department of Microbiology, Embrapa Environment, Jaguariuna, SP, Brazil

Abstract

The potential of ligninases as a green tool for effective valorization of lignin can be shown through enzymatic cocktails containing different lignin degrading enzymes. The present study deals with the screening of potential fungal strains useful for the liquefaction of bark containing lignin. Three different local isolates (Pleurotus ostreatus POS87/14, Pycnoporus sanguineus and the local isolated fungal strain) were selected out of ten different strains for ligninases production. Maximum production of enzymes was observed in the local isolated fungal strain after ten days in submerged fermentation. The isolated fungal strain produces ligninases mainly for manganese peroxidase (MnP). The enzyme oxidized a variety of the usual MnP substrates, including lignin related phenols. Furthermore, the partial purification for MnP was determined by FPLC and the molecular weight was evaluated by SDS-PAGE.

Keywords: Ligninases; Fungal Strain; Pleurotus ostreatus; P. sanguineus; Submerged Fermentation; FPLC; MnP; LiP; Lcc; Time course studies

Introduction

Among microorganisms that colonize living wood, white-rot fungi are regarded as considerable lignin degraders. They produce extracellular enzymes, such as MnP, LiP and laccase, which play important roles in lignin biodegradation [1]. Pleurotus ostreatus is a white rot fungus belonging to the basidiomycetes and it is also considered to be a cholesterol reducing mushroom [2]. It was also noticed that the deficiency of ligninases started in some genera of Basidiomycetes, such as Pleurotus spp. [3], especially laccinolytic laccases involved in the degradation of lignin. The combined action of laccase and aryl alcohol oxidase decreases the molecular weights of soluble lignosulfonates secreted by P. ostreatus [3]. Laccinolytic system configuration is complicated and species restricted [4]. White rot fungi produce ligninases, including MnPs, LiPs and Lcc, which are possible contributors to fungal lignolysis. White-rot fungi were mainly found to produce consistent products following the ligninolysis of model lignin compounds [5].

MnP belongs to the family of peroxidases, and the methodical name of this enzyme is Mn(II):H₂O₂, oxidoreductase. According to protein databank, the MnP containing cofactor is protoporphyrin IX having Fe(C₃H₄FeNO) immobilized with Mn²⁺ and Ca²⁺ ions required for enzyme activity.

Eventually, peroxidases oxidize phenolic compounds and reduce molecular oxygen to water [1]. Ligninases oxidize several environmental pollutants such as polycyclic aromatic hydrocarbons, dyes and chlorophenols. Heme containing enzymes such as LiP and MnP also have catalytic cycles characteristic of other peroxidases. LiP has an ability to oxidize many aromatic compounds, whereas MnP oxidizes Mn (II) to Mn (III) [6, 7]. Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper blue oxidases commonly found in higher plants, several bacteria and some insects. Nevertheless, the well-characterized laccases are fungal in origin [6]. Laccases have significant importance in many industrial areas due to their remarkable catalytic properties. Potential applications include immunoassay bio-labeling, biosensors, biocatalysts, and advancement of oxygen cathodes in biofuel cells. In addition, they have good prospects in the environmental sector, including use in textile dye bleaching, pulp delignification and xenobiotic compound degradation due to their wide-ranged substrate specificity [6, 8]. The present applications of this enzyme motivated us to do new basic research.

The current research activity of ligninases includes utilizing the local lignin sources (Eucalyptus and sugarcane bagasse) and checking their delignification pattern. The characterization of ligninases (MnP, LiP and Lcc) from Brazilian fungal isolates, with respect to production, partial purification and time course studies, is reported in this study.

Materials and Methods

Strain isolation

The unknown fungal strain was isolated from the Northeast part of Brazil called Caatinga. Caatinga has a semi-arid climate and covers an area of nearly 735,000 km², although 1% of this semi-arid zone is preserved [9].

Substrate collection and preparation

Sugarcane bagasse and Eucalyptus lignin were used for delignification. Sugarcane bagasse and Eucalyptus were collected from LWART Quimica, Brazil. All substrates were dried in an oven at 80°C to constant weight and were powdered using an electric grinder and stored in airtight glass containers to keep out moisture.

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A New Method for Demethylation of Lignin from Woody Biomass using Biophysical Methods

M. Ferhan¹, N. Yan and M. Sain
Center for BioComposites and Biomaterials Processing (CBBP), Faculty of Forestry, University of Toronto, Toronto, ON, Canada

Abstract

Demethylation of softwood kraft lignin from woody biomass to improve the hydroxyl number in modified demethylated lignin and to produce lignin-based polyols was investigated using several biophysical techniques. Lignin is a tremendously under-developed natural polymer co-generated through papermaking and biomass fractionation. Molecular weights of lignins were analyzed by high-performance size-exclusion chromatography (HPSEC). Molecular weights of softwood kraft lignin (SKL) and demethylated lignin (DL) were determined as 1071 and 891 g mol⁻¹, respectively. For demethylation, iodocyclohexane in dimethyl formamide (DMF) was used under reflux conditions, with a resulting yield of 67%. Fourier transform infrared spectroscopy (FTIR) was used to determine characteristic absorption peaks of softwood kraft lignin and demethylated lignin. Significant spectral differences were noticed between the two types of lignin due to changes in chemical structure. Total hydroxyl numbers were determined by titration. Phosphorus nuclear magnetic resonance spectrometry (³¹P-NMR) was employed to analyze the structure of lignin and different types of phenolic hydroxyl units. Nevertheless, further chemical and biological modifications within the lignin molecule are needed for various industrial applications to synthesize polyurethane foam by using chemically modified lignin-based polyols.

Keywords: Demethylation; Softwood kraft lignin (SKL); Demethylated lignin (DL); Hydroxyl numbers; FTIR spectroscopy; ³¹P-NMR

Introduction

Lignin is described as an unsystematic, unstructured, 3-D polymeric complex system that does not possess a regular, precise structure with fixed recurring units. In lignin, the main functional groups are hydroxyl, methoxy, carbonyl, and carboxyl moieties in several amounts, whose components depend on the botanical source and the applied extraction processes [1]. In the plant kingdom, lignin is the most abundant polymeric organic material after cellulose [2]. The lignin biosynthetic pathway utilizes mainly three different types of phenylpropane units to make the lignin molecule. These include a guaiacyl precursor made from coniferol alcohol and guaiacyl-syringyl precursors derived from coniferyl and sinapyl alcohol. Lignin from softwood (conifers) is mainly composed of guaiacyl units, while hardwood (angiosperms) lignin contains guaiacyl-syringyl units [3].

Both lignins contain mainly glycerol-aryl ether (β-O-4) linkages between short and linear chains of phenylpropane units [4]. The monomeric units of lignin are connected by a number of different C-C and ether linkages, which accounts for the complicated 3-D structure of lignin [2,5].

The different functional groups in a complex lignin macromolecule are presented in Figure 1 [3,6]. Lignins are the by-product of pulp and paper industries and are abundant in nature. The uses of lignin in different applications are as a dispersant agent for pesticides, emulsifiers, ion-exchange resins, water treatment agents, pesticide surfactants, heavy metal sequestrant, binders, animal foods, grinding aids, electrolytic refining, and tanning agents, or as a component of composites and copolymers [7-9].

To create value-added applications of lignin, medium- and long-term technologies mainly for the preparation of LMW compounds that can substitute for products produced by the petrochemical industry must be established [10]. Nevertheless, the structure of lignin is not completely recognized yet. Over the last two decades, many studies have been undertaken to understand the industrial applications of lignins [6]. The subjects of study by several research groups are lignin and their advanced applications, including, polyurethanes [10], acrylics [11], epoxies [12], and phenolic resins [13].

For polyurethane synthesis, the utilization of lignin as a macromonomer frequently follows two general methods: direct use of lignin without any major chemical modification, alone or in blends with other polyols [14], or (2) chemical modification, such as depolymerization, esterification and etherification reactions [10,15]. A broad range of lignin-based polyurethane materials (rigid foams, elastomers, sealants) has been produced and the resultant mechanical and thermal properties have been assessed [16].

Due to shared structural similarities, the industrially important organic compound vanillin can be produced from lignin, as has been reported by many scientists [17]. By catalytic degradation of lignin, many other industrially valuable small molecule chemicals can be produced [17]. However, to control for serious practical problems related to the enormous energy costs and basic purification processes, additional research is required to produce small molecule chemicals from this complex natural biopolymer. The second approach for the utilization of lignin is as a primary material in a diversity of green polymer products [18]. Nevertheless, the synthesis of commercial based...