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BIODEGRADATION OF LIGNIN BY FUNGI, BACTERIA AND LACCASES

by

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Bachelor of Science, Beijing University of Chemical Technology, 2013  
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and Forestry, 2013

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Submitted to the Graduate Faculty

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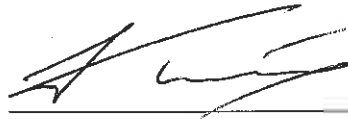
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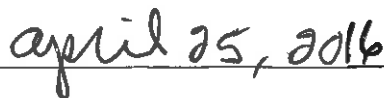


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This thesis is being submitted by the appointed advisory committee as having met all of the requirements of the Graduate School at the University of North Dakota and is hereby approved.



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## ABSTRACT

Lignin, a heterogeneous three-dimensional biopolymer, is one of the building blocks of lignocellulosic biomass. Due to its limited chemical reactivity, lignin is currently processed as a low value by-product in pulp and paper mills. Therefore, valorization of lignin holds great potential as this would provide a readily available source of aromatic compounds for various industrial applications. In this study, indulin AT biodegradation was assessed by comparing the effects of basidiomycetous fungi (*Coriolus versicolor* and *Trametes gallica*) and actinobacteria (*Mycobacterium sp.* and *Streptomyces sp.*) to those of two commercial laccases, those from *T. versicolor* ( $\geq 10$  U/mg) and *C. versicolor* ( $\geq 0.3$  U/mg) while using a suite of chemical analysis methods.

The research results showed that after 54 days of cultivation, microbial (especially fungal) lignin biodegradation is significantly greater than that caused by commercial laccases, reaching a maximum of 20 wt. % degradation for *C. versicolor* by gravimetric analysis. The extent of microbial degradation was further confirmed by thermal carbon analysis (TCA), as all treatments led to changes of the thermal carbon elution profile in the supernatants. However, laccase treatments resulted in only minor changes with increases occurring in the 850 °C and char fractions, thus evidencing the formation of cross-linked polymers. The fungally treated lignin showed a similar change in the thermal carbon elution profile, along with a gradual decrease of the total carbon in the supernatant,

indicating significant lignin mineralization. Bacterial treatment, on the other hand, mainly led to carbon solubilization instead of mineralization.

Chemical characterization of lignin degradation products performed by Thermal Desorption-Pyrolysis-Gas Chromatography-Mass Spectrometry (TD-Pyrolysis-GC-MS) corroborated the carbon fractionation obtained by TCA. The laccase treatments yielded more phenol-based compounds and aromatic hydrocarbons eluting only at higher pyrolytic temperatures, i.e., 700 °C at the expense of monomers eluting at lower temperatures, thus confirming extensive lignin polymerization. The fungal treatments led to similar changes, with a significant consumption of low molecular weight phenolics whereas the bacterial treatments, conversely, facilitated the production of phenolic monomers eluting at low temperatures. Thus, fungi appear to mostly cause significant lignin mineralization combined with polymerization whereas bacteria instead tend to produce phenolic monomers without their further catabolism.

# CHAPTER I

## INTRODUCTION

Lignin, an aromatic natural polymer, represents an essential component of lignocellulose biomass and often underutilized as a by-product in paper industry and as a feedstock for biofuel development. Ideally, one of the efficient ways of lignin valorization is to use chemical or biological treatment to break it down to low molecular weight phenolics of greater values to various industries. However, due to the chemically recalcitrant nature of lignin and our limited knowledge of the structural behavior of lignin during treatment, this has been a challenging task for many researchers over the years. The focus of this thesis is to gain insights into the chemical characterization and changes in carbon mass balance during and after microbial treatment of lignin over an extended period of time.

## 1.1 Motivation

In comparison to chemical treatment processes, biological treatment of lignin features a low energy input thus leading to a significant decrease in operation costs and is considered an environmentally friendly approach. There has been an increasing interest in using natural lignin degraders, including both fungi and bacteria, to break down lignin [1][2][3]. Over the course of microorganism evolution, the natural lignin degraders have been highly adapted to use lignin as a main carbon source for growth. The biodegradation of lignin polymer is carried out through a regulated secretion of various ligninolytic enzymes that target the specific bonds present in lignin.

Published literature has proved a successful microbial degradation of biomass from various sources (see Chapter II). However, industrial lignin, mainly Kraft lignin originated from paper mill dominates the current production of lignin around the world. Kraft lignin undergoes great extent of structural modifications compared to natural lignin present in the lignocellulosic biomass. The focus of my research is to understand the microbial degradation of Kraft lignin at an industrially relevant concentration and the metabolic interactions between the used microorganisms and lignin.

In addition, many papers about microbial degradation of industrial lignin only achieved partial characterization of lignin degraded products using GC-MS, as only phenolic monomers and dimers are volatile enough to elute from GC columns [4]. In order to both qualitatively and quantitatively characterize the microbial lignin degradation products, a comprehensive analysis of microbial lignin degradation products was conducted combining enzyme activity measurement, gravimetric analysis, fractional



TD-Pyr-GC-MS and total carbon analysis (TCA). Because Pyr-GC-MS is an excellent qualitative tool and TCA yields a quantitative carbon evolution profile, a complete mass balance closure of lignin degradation products can be achieved in this study.

## 1.2 Thesis Outline

In this thesis, one review paper (Chapter II) and one research paper (Chapter III) are included. Details on the papers are as follows:

(1) Asina F., Brzonova I., Kozliak E., Kubátová A. & Ji Y. (2016) Microbial treatment of industrial lignin: Successes, Problems and Challenges, *Journal of Renewable & Sustainable Energy Reviews*, under review

(2) Asina F., Brzonova I., Voeller K., Kozliak E., Kubátová A. & Ji Y. (2016) Biodegradation of lignin by fungi, bacteria and laccases, *Manuscript in preparation*

Chapter II introduces the subject of lignin chemical structure and sources, lignin production and processing in biorefineries, industrial lignin sources, ligninolytic enzymes frequently identified among microorganisms and factors responsible for their regulation and induction. A comprehensive literature review on both fungal and bacterial lignin degradation, with a focus on industrial lignin or industrial effluent from paper mill is included. An overview of the characterization of lignin degradation products is also presented.

Chapter III focuses on the study for a direct comparison of fungi (*Coriolus versicolor* (CV) and *Trametes gallica* (TG)), bacteria (*Streptomyces sp.* (S) and

*Microbacterium sp.* (B)), and purified enzymes (laccases). In this chapter, experiment setup, determination of the residual lignin content, measurement of ligninolytic enzyme activities, TCA for carbon mass balance calculation and TD-Pyr-GC-MS analysis for a qualitative characterization of lignin degradation products are included.

Chapter IV highlights the key findings and implications from the study and discussion of guidelines for future work.

## CHAPTER II

### LITERATURE REVIEW

Microbial treatment of industrial lignin: successes, problems and challenges

#### **2.1 Abstract**

Lignin, one of the major components of plant/lignocellulosic biomass, is an irregular 3-D polymer comprised of potentially valuable phenolic monomers. Currently lignin and its colloidal solution in water, black liquor, obtained as by-products in a number of biomass treatment processes, e.g., pulping in paper industry, remain to be considered recalcitrant substrates of a limited commercial value. This study reviews the recent research on both fungal and bacterial lignin degradation, with a focus on the characterization of degradation products. Current research efforts in characterizing lignin degradation products are reviewed with the emphasis on both destructive and non-destructive gas chromatographic methods as they are essential for future detailed kinetic and mechanistic studies. The specific features and biological treatment of industrial lignin and black liquor are detailed along with the degradation conditions employed, complementing other review articles focusing on natural lignin degradation. An overview of ligninolytic enzymes frequently identified among microorganisms is presented, with the emphasis on factors responsible for their regulation and induction including the mediators involved and multi-enzyme systems employed by natural lignin degraders.

Efficient regulation of ligninolytic enzymes can be achieved through the optimization of a cultivation medium composition with supplementation of strain specific stimulatory components such as salts, low molecular weight phenolic compounds and nutrition sources.

Keywords: Lignin, Black liquor, Microbial degradation, Ligninolytic enzyme, Induction, Lignin characterization, Fungi, Bacteria

## **2.2 Background**

In nature, both fungi and bacteria exhibit significant lignin degradation. Therefore, a wide array of microorganisms has been evaluated for their lignin degradative abilities, with a subsequent characterization of degradation products. In this article, we set a goal of connecting the wealth of knowledge accumulated for natural lignin degradation with a survey of less common studies on biological treatment of modified lignin substrates, such as industrial lignin and black liquor. We attempted to provide an integrated interdisciplinary view of lignin biodegradation. The main biochemical topic of this study is an overview of ligninolytic enzymes frequently identified among microorganisms, with the emphasis on factors responsible for their regulation and induction including the mediators involved. From the standpoint of bioengineering, we thus look for practical ways of enabling and enhancing lignin degradation. As accurate and detailed chemical analysis often becomes the limiting factor in applications, we also look into the current research efforts in characterizing lignin degradation products with the emphasis on both destructive and non-destructive gas chromatographic methods.

## **2.3 Lignin chemical structure and sources**

Lignin is an essential plant component comprising up to 30% of plant biomass [1]. As the second most abundant natural polymer on Earth after cellulose and the only aromatic renewable feedstock, lignin plays an important role in providing structural integrity by plant cell wall reinforcement, controlling the fluid flow and enabling resistance to pathogens [2]. Structure-wise, lignin is a complex and structurally diverse aromatic heteropolymer in which its predominant structural components, phenylpropanoid aryl-C<sub>3</sub> units, are linked by a variety of C-O (ether) and C-C bonds [3]. Representative linkages present in lignin and basic structural units are shown in Figure 1. As the predominant linkage in lignin,  $\beta$ -aryl ether ( $\beta$ -O-4) bonds can account for ~50% in softwood and ~80% in hardwood lignin, respectively [4]. Among other linkages, 5-5,  $\beta$ -5, 4-O-5,  $\beta$ -1, and  $\beta$ - $\beta$  are resistant to most degradation processes [5]. In nature, lignin is synthesized by block co-polymerization, i.e., a modular enzymatic dehydrogenative polymerization of three phenylpropanoid units, coniferyl alcohol (with the guaiacyl unit, G), synapyl alcohol (with the syringyl unit, S) and p-coumaryl alcohol (with the p-hydroxyphenyl unit, H) resulting in the formation of lignin backbone of the plant cell wall [6]. These three units share the common basic phenylpropanoid structure but differ in the number of methoxy groups in their phenolic rings [7]. The G unit contains monomethoxy phenoxide, the S unit contains dimethoxy phenoxide, and the H unit contains the non-substituted phenoxide moiety. The G unit is predominantly present in softwood while the S unit is most commonly found in hardwood along with the G unit. The H unit is a common component of grasses [8]. The widely known structure of natural lignin is to be a cross-linked aromatic polymer. In recent years, the linear structure of milled wood lignin isolated from wood was reported to have minimum

branching [9]. One lignin-carbohydrate complex structure (a xylan-rich xylan-lignin-glucomannan complex), isolated from ball milled wood after endoglucanase treatment, was found to have a high degree of linearly coupled  $\beta$ -O-4 structures [10]. The linear structure of xylan-lignin complex obtained from spruce wood comprised 40% of the lignin yield [11].

Unlike *natural* lignin present in lignocellulosic biomass, *industrial* lignins are those isolated as by-products. Industrial lignins have diverse macromolecular structures because they undergo various chemical modifications and contain impurities that are dependent upon the specific processing method involved [12]. Structural properties of lignin can vary greatly depending on the isolation/fractionation method used. Most of cross-linking, which significantly increases the lignin recalcitrance, may result from the lignin isolation/treatment procedure applied. One study reported that the absolute amount of  $\beta$ -O-4 linkages decreased along with the occurrence of condensation reactions when wood is treated during the Kraft pulping process [10].

Azadi et al. summarized the fractionation techniques related to lignin into two groups. First, lignin can be solubilized in the spent liquor and then separated from other biomass components recovered as solid residues. Second, selective hydrolysis of polysaccharide isolates lignin as a solid residue separating it from the liquid phase sugar and degraded carbohydrate products [13]. The former approach is predominant as it is used in pulp and paper industry, which is the main source of industrial lignin. Laurichesse et al. categorized industrial lignin as sulfur-containing lignin and sulfur-free lignin. The former includes lignosulfonate and Kraft lignin (~5 and 1-2wt%, respectively) [7] while the latter refers to soda lignin and organosolv lignin [14]. Alkali lignin refers to lignin

recovered from alkaline pulping which includes Kraft and soda pulping with dominant usage of sodium hydroxide [15]. Indulin AT, a type of lignin most often considered in biodegradation studies, is a purified form of Indulin C, which is a sodium salt of Kraft pine lignin and hemicellulose-free [16][17][18][19]. In the review by Vishtal et al., additional industrial lignin types were also classified including acid hydrolysis lignin, enzymatic hydrolysis lignin and ionic liquid lignin [12]. In the following sections, we discuss the properties of industrial lignin based on the existing lignin classification.

## **2.4 Lignin production and processing in biorefineries**

A biorefinery is aimed to produce multiple intermediate industrial products, fuels and chemicals from a variety of biomass feedstocks [20]. Currently, two platforms for biorefineries have been developed. One is the sugar platform where biomass is broken down into various sugar components for further conversion to fuels and chemicals and the other one is thermal chemical platform whose ultimate products are synthesis gas and pyrolysis oil to be immediately used onsite as fuels [21]. The alternate thermal chemical platform allows for adapting existing infrastructures of petro-chemical refining processes to biorefineries [22]. Meanwhile, for sugar platform biorefineries, traditional pulp and paper mills can also be converted into biorefineries with their well-established pulping processes and infrastructures [23]. Efficient operation of sugar platform biorefineries depends on four main sections including harvest and storage of lignocellulosic biomass, pretreatment and fractionation of biomass components, enzymatic hydrolysis and sugar fermentation [24]. The key step for liquid fuel production via biochemical processes is converting the carbohydrate portion of biomass to monosaccharides, which are further

fermented to yield ethanol [25]. Lignin was found to restrict the cellulose accessibility to enzymes responsible for its treatment by causing unproductive binding [26].

However, future biorefineries will have to adjust to the new requirements of producing not only renewable fuels but also renewable chemicals. These two tasks are inherently intertwined as can be shown by a simple calculation. As the US Department of Energy targets to the replacement of 30% of transportation fuel with biofuels by year 2030, it is estimated that 0.225 billion tons of lignin will have to be produced and then processed [27]. Yet, as little as 40% of the lignin produced is sufficient for the power production by on-site burning for all the needs of a modern lignocellulosic biorefinery including the biomass pretreatment, carbohydrate conversion and product (ethanol) distillation [28]. Thus further utilization of lignin separated from biomass is critically important for the biorefinery economic viability, as it allows for significant improvement in economics of biofuel production.

Thus, either a third platform should be created or the thermal platform has to be adjusted to allow for a comprehensive lignin utilization. Given this, microbial degradation of native lignin as a lignocellulosic biomass component through a wide array of ligninolytic fungi (mainly white rot fungi) and bacteria has been gaining attention for its low energy requirements characteristic for thermal methods. Since any lignin is subject to microbial attacks, these methods are applicable to biomass feedstocks ranging from agricultural crops such as wheat straw or corn stover to various types of soft and hard woods. Efficiencies of various microbial sources in lignin removal are shown in Table 1.



Lignin biodegradation is often accompanied by a simultaneous utilization of cellulose and hemicellulose present in the biomass. When treating synthetic lignin, addition of cellulose slurry facilitated lignin oxidation by peroxidases produced from microorganism [29]. This is the main reason why most of the current lignocellulose biomass treatments start with the carbohydrate removal resulting in lignin isolation as a residual by-product. Yet, several selective lignin-degrading strains were identified that could depolymerize lignin in plant cell walls while keeping cellulose nearly intact to obtain its maximal conversion to ethanol in the downstream process [30]. This review will not cover biorefineries, lignocellulosic biomass pretreatment as well as biodegradation of native lignin, as these topics have been extensively covered by other reviews [28][31]. Instead, we will proceed with reviewing the biodegradation of industrial lignin as a more technically challenging yet currently most practically important topic.

## **2.5 Industrial lignin**

### **2.5.1 Sulfur containing lignin**

#### ***2.5.1.1 Kraft lignin***

The production and chemical properties of the industrial lignins are closely related to the separation technique involved. A summary of common pulping processes is provided in Figure 2. The predominant process employed in pulp and paper mills is called Kraft pulping, in which plant biomass is treated with a concentrated sodium hydroxide and sodium sulfide solution for 1-2 h at 170°C [44]. In order to produce pulp suitable for manufacturing paper products, efficient separation of lignin from cellulosic

fiber is required, during which a considerable volume of effluent, a colloidal aqueous solution of lignin, also known as black liquor, is generated [45]. It has been estimated that near seven tons of black liquor are produced per one ton of pulp production [46]. Although representing merely 15% of waste water amount, black liquor becomes a major pollutant of aquatic ecosystems if the effluent is discharged without any treatment [47]. During production of chemical pulp, 90-95% of lignin is dissolved in black liquor through conventional Kraft cooking along with a significant amount of other organic substances such as hemicellulose and sugar acids [48] [49]. Thus, black liquor should be considered separately from carbohydrate-free industrial lignin despite their similar origin. In addition, around 70kg of organic compounds (mainly lignin) per ton of pulp can be dissolved during the pulp bleaching [50]. Following 90% lignin removal during Kraft pulping, most of the remaining lignin present in the pulp can be dissolved during chlorine treatment and alkali extraction steps in the bleaching process [51]. Chlorolignin produced during the first of these two steps features a lower molecular weight with a higher content of phenolic hydroxyl, carboxyl and conjugated carbonyl groups [52] [53].

Kraft lignin constitutes about 85% of the total world lignin production [54]. Kraft lignin is isolated from black liquor with high alkalinity by bringing pH down to 2-5 with an acid, such as sulfuric acid or carbon dioxide [55][56]. From the standpoint of chemical structure, Kraft lignin is different from the original, naturally occurring lignin. First, lignin condensation/cross-linking reactions take place both during the treatment in a strongly alkaline environment and afterwards, during neutralization, as a result of significant local heat generation. In addition, an extensive cleavage of  $\beta$ -aryl ether bonds occurring at high pH results in lignin modification with phenolic hydroxyl groups while

the concurrent  $\alpha$ -aryl ether cleavage leads to the formation of intermediates with quinonic structure [45][12]. The generation of a free phenolic hydroxyl group makes the remaining  $\alpha$ -aryl ether bonds becoming more susceptible to cleavage compared to  $\beta$ -aryl ether bonds, via a mesomeric effect [7] thus further altering the original native lignin structure.

### ***2.5.1.2 Lignosulfonate / Sulfite Lignin***

In alternate sulfite pulping processes, a partially neutralized sulfurous acid ( $\text{H}_2\text{SO}_3$ ) thus containing bisulfite ion ( $\text{HSO}_3^-$ ), is applied to dissolve lignin. In this process, wood chips are treated at 120-150°C from 500-700 kPa [46]. Sulfite pulping is further classified into four different pulping techniques based on the components of the sulfite cooking liquor (Figure 2). Acid sulfite and bisulfite pulping use high and low  $\text{H}_2\text{SO}_3/\text{HSO}_3^-$  ratios, respectively. The alternate treatment, alkaline sulfite pulping, employs equal amounts of NaOH and  $\text{Na}_2\text{SO}_3$  in the cooking liquor [46]. Neutral sulfite pulping employs a neutral solution of  $\text{Na}_2\text{SO}_3$  and  $\text{Na}_2\text{CO}_3$  to soften the lignin and is often coupled with mechanical pulping for complete lignin removal [57].

Different sulfite treatments lead to different lignin chemical modifications. Preferred cleavage of the  $\alpha$ -ether linkages and  $\beta$ -ether linkages of lignin occurs during acid based sulfite pulping whereas alkaline sulfite pulping presents similar delignification reactions to Kraft pulping [58]. Namely, side-chain cleavages are more pronounced and lignin condensation reactions occur due to the formation of resonance-stabilized benzyl carbocation caused by the cleavage of the aryl ether linkages [7]. Compared to Kraft pulping, sulfite pulping can treat only certain wood species and is completely unable to process wood bark. In addition, the sulfite pulp treatment efficiency can only be as much as 70% of that of Kraft pulping, even for those species to which this method is applicable

[59]. Thus, only around 10% of pulp is currently produced by sulfite pulping worldwide [60]. However, lignosulfonate is currently a raw material considered for industrial application in production of surfactants and dispersing agents, etc., due to its higher water solubility and broader polydispersity compared to Kraft lignin [14]. With a higher molar mass, lignosulfonate can also produce a stronger cement when used as a cement additive [61]. Common lignin reactions occurring during Kraft and sulfite pulping treatments are shown in Figure 3.

Sulfonation of lignin in the solid phase followed by hydrolysis of lignosulfonic acid formed leads to water soluble lignosulfonate [58]. The presence of sulfide has been reported to facilitate breakdown of lignin to soluble fragments while reducing the extent of condensation during Kraft pulping [62]. Sulfide is a strong nucleophilic agent that helps cleave lignin. However, the inevitable result of this treatment is the introduction of sulfur atoms in an irregular pattern, which reduces the subsequent application. For instance, combustion of sulfur-containing lignin produces sulfur oxides thus requiring costly exhaust air purification. The biodegradation of sulfonated lignin could also be restricted because the sulfonated intermediates are unable to enter central metabolic pathways.

## **2.5.2 Sulfur-free Lignin**

### ***2.5.2.1 Soda and Organosolv Lignin***

Soda lignin and organosolv lignin represent a minor portion of industrial lignin production. Soda pulping is mainly used for processing non-wood feedstocks such as bagasse, kenaf and wheat straw[46]. Pulping is conducted at 140–170 °C in the presence

of 13–16 wt% sodium hydroxide [7]. During Soda pulping, anthraquinone can be used as an additive to prevent carbohydrate degradation [46]. The resulting soda lignin from non-wood crops features high percentages of aliphatic hydroxyl group and carboxyl group, originating from oxidation of aliphatic hydroxyl [7]. Compared to Kraft lignin, soda lignin features a less extensive cleavage of  $\beta$ -ether linkages and less reactive phenolic unit along with the abundance of aliphatic hydroxyl groups [62][63].

Lignin can also be extracted from biomass with organic solvents such as ethanol, methanol and organic acids through solubilization. After such an organosolv pulping, the organic solvent can be readily recovered through distillation [46]. Organosolv lignin is relatively less modified compared to Kraft lignin or lignosulfonate [12]. Even though the soda and organosolv treatments are less efficient than those involving sulfur, their greater suitability for operation in small scale paper mills makes these processes viable [46]. A significant advantage of the lignin produced from these processes is their sulfur-free structure, which allows for further processing [7].

#### ***2.5.2.2 Enzymatic Hydrolysis Lignin and Acid Hydrolysis Lignin***

The other sulfur-free lignin, so-called enzymatic hydrolysis lignin, is obtained by selective hydrolysis of biomass using cellulolytic enzymes, which leaves lignin as a solid residue [64]. Compared to Kraft lignin or lignosulfonate, enzymatic hydrolysis leads to high lignin yield and the isolated lignin retains the linkages between lignin and carbohydrates thus keeping the original chemical reactivity of the native lignin [65][66]. However, despite the high content of impurities including carbohydrates and protein residues [66], enzymatic hydrolysis lignin possesses a higher value for its potential applications such as sorbent development [12]. In addition, enzymatic hydrolysis lignin

can be used in the development of new polymers such as epoxy-lignin polymer composites by combining lignin with a synthetic polymer. Both mechanical properties and thermal stability of epoxy composites were found to be strongly influenced by the addition of enzymatic hydrolysis corn straw lignin [67].

Dilute (2-5%) or concentrated acid (10-30%) pretreatment using mostly sulfuric or hydrochloric acids can also break down cellulose and hemicellulose while separating the so called acid hydrolysis lignin as a residue [68][69]. More structural modifications occur during acid hydrolysis than during the enzyme treatments due to harsh conditions, high temperature and acidic environment. Cleavage of  $\beta$ -aryl,  $\alpha$ -aryl and  $\alpha$ -alkyl ether bonds were observed in lignin during acid hydrolysis [70].

### ***2.5.2.3 Ionic liquid lignin***

One additional lignin type is obtained by the application of ionic liquids. Ionic liquids are salts occurring in the liquid state at or below 100° C. The most common cations comprising these salts are trialkyl pyridinium or imidazolium, or tetraalkyl phosphonium or ammonium derivatives while halides and fluorinated organic compounds are often used as counter-anions [71]. Ionic liquids are considered being green solvents due to their low vapor pressure, high thermal stability, relatively low toxicity and non-flammability. In addition, recovery of ionic liquids is relatively easy and so they can often be reutilized [72]. As their ionic nature represents the extreme case of polarity, ionic liquids are suitable solvents for large polar biomolecules, such as cellulose or lignin [73]. Once the whole biomass is dissolved typically at 80-130 °C [74][75], cellulose and lignin can be further separated using so called “antisolvents,” i.e., selective lignin precipitation agents, such as water, acetone-water mixture etc. [73]. Lignin extracted

from ionic liquids shows a certain degree of decomposition and lower thermal stability [76]. Wen et al. reported chemical transformations of alkali lignin during its treatment with a specific ionic liquid, 1-ethyltrimethylimidazolium acetate ([C<sub>2</sub>mim]-[OAc]), namely an increase in the number of phenolic hydroxyl groups due to the thermal cleavage of  $\beta$ -O-4 linkages along with oxidative scission of  $\beta$ - $\beta'$ ,  $\beta$ -5' linkages. At high temperature, the G unit in lignin was preferentially demethoxylated during the pretreatment [77].

Up until now, the production of sulfur-free lignins has been low compared to lignosulfonate and Kraft lignin. However, rapid development of biorefineries may lead to a significant increase of the fraction of those lignins that are produced along with the biomass carbohydrate processing.

## **2.6 Microbial degradation of industrial lignin**

Lignin biodegradation is a complex process, not only due to its multistep nature involving various reactions, but also because, unlike most of the other enzyme catalyzed processes, it produces large amounts of side products. Strong oxidants, such as hydrogen peroxide, are often required and the enzymes involved have a broad specificity to attack any accessible lignin sites, often with the help of even less specific mediators and oxidation reactions. It may result not only in oxygenation (which would eventually lead to lowering the molecular weight) but also dehydrogenation, which may lead to oxidative coupling of two adjacent phenolic moieties creating a new C-C bond [78].

The resulting crosslinked polymer is more recalcitrant; a similar issue is characteristic for thermal, abiotic lignin degradation [79]. However, unlike thermal

degradation, lignin biodegradation occurs at lower, near ambient temperatures. Thus, even the gradually accumulating crosslinked polymers are not as recalcitrant as coke inevitably produced as a by-product of thermal lignin degradation. So lignin can eventually be biodegraded showing a near 100% decomposition [80]. This attractive feature of lignin biodegradation is the main reason why this process is considered for industrial applications despite the unfavorable characteristic features of all bioprocesses, such as slow rate and requirements for conducting the process in batch reactors with aqueous media, i.e., heterogeneous systems.

The ability of microorganisms to grow on lignin cannot be an absolute indicator of efficient lignin degradation. A bacterial strain, *Bacillus* sp. LD003, showed a great ligninolytic potential by decoloration of ligninolytic indicator dyes but exhibited only the minimum growth on lignin among three bacterial strains investigated [81]. When lignin is degraded by microorganisms, its solubilization, degradation and mineralization should be affected in different ways [82]. Therefore an accurate assessment of degradative ability of selected microorganisms is needed during the screening process taking into account all of these factors [81].

### **2.6.1 Biodegradation of model lignin compounds**

Over the years, considerable amount of effort has been placed into biodegradation of synthetic model lignin compounds such as phenolic dimers modeling only certain structural motifs of real-world lignin [83][84][85][86]. The second group of model compounds are phenolic monomers, important intermediates of lignin biodegradation, which are several steps away from entering the common mainstream biochemical



pathways [87]. Studies with model compounds are mainly focused on elucidating the mechanisms employed by certain ligninolytic enzymes, intermediate products and degradation pathways associated with specific microorganisms.

As a result, some catabolic pathways of lignin degradation in various microorganisms have been elucidated with structurally simple lignin model compounds. Bugg et al. described microbial breakdown of model lignin compounds in details including  $\beta$ -aryl ethers, biphenyl, diaryl propanes, phenylcoumarane and ferulic acid. Either vanillin or vanillic acid are claimed to be important degradation intermediates of many catabolic pathways, being further converted to protocatechuic acid via demethylation [88]. A bacterial strain, *Sphingomonas paucimobilis* SYK-6, metabolized  $\beta$ -aryl ether lignin dimer compounds with the aid of specific enzymes such as NAD-dependent dehydrogenase, LigD, and glutathione-dependent  $\beta$ -etherase to ultimately yield vanillic acid [89]. By contrast, a white rot fungus, *Phanerochaete chrysosporium*, breaks down the same model compounds by lignin peroxidase via a  $C_\alpha$ - $C_\beta$  oxidative cleavage to yield vanillin, which is further oxidized to vanillic acid [90].

Given the limited chemical reactivity of phenolics, attacks on such compounds by microorganisms are often carried out with the aid of hydroxylating oxygenases and dioxygenases. Phenolics go through two pathways in order to be used as growth substrates for microorganisms as illustrated in Figure 4. First, microorganisms convert phenolics into a few key intermediates such as catechol (1,2-dihydroxybenzene), protocatechuate (3,4-dihydroxybenzoate) and benzoate in a so-called peripheral or upper pathway [91]. The peripheral/upper pathway shows a broad substrate specificity and serves as a “biological funnel” for decreasing the heterogeneity of carbon [92]. Second,

the intermediates undergo dearomatization through the so-called central pathway and then different intermediary metabolites, such as acetyl-CoA, succinyl-CoA and pyruvate, are produced subsequently entering the tricarboxylic acid cycle [91].

The other way of degrading aromatic compounds employs oxygenases to form non-aromatic epoxides. It is called a hybrid pathway for its partial similarity to anaerobic oxidation [93]. Under anoxic conditions, reduction of aromatic rings is carried out involving de-aromatizing reductases that act on the previously mentioned intermediates [90].

Although efficient biodegradation of model lignin compounds has been demonstrated among various bacterial and fungal strains, the results obtained cannot be directly applied to the degradation of industrial lignin, e.g., that present in paper mill effluents, as industrial lignin are significantly modified compared to the native lignin. For example, sulfonated vanillic acid has a very different biochemical substrate than its non-substituted chemical analog, so it may or may not be further metabolized. Furthermore, every specific set of operational conditions generates a unique industrial lignin, due to its specific modification. In addition, inherent characteristics of industrial lignin such as sulfur content, high alkalinity and lack of easy accessible nutrition source could greatly influence the degradation efficiency and pathway involved. Therefore, it is necessary to conduct lab-scale research on the given whole industrial lignin biodegradation rather than its model compounds.

## 2.6.2 Microbial degradation of sulfur-containing lignin

### 2.6.2.1 Microbial degradation of lignosulfonates

Among the publications on microbial industrial lignin degradation, the use of sulfur-free lignin such as organosolv lignin was limited [94] [95]. Among the sulfur-containing lignin, especially Kraft lignin featuring a smaller sulfur content, is the main substrate under investigation. By contrast, lignosulfonate characterized by a higher sulfur content was studied less frequently. The high sulfur content of lignosulfonate can negatively affect the microbial metabolism, thus hindering efficient lignin degradation. However, sulfur removal prior to microbial degradation could increase the extent of lignosulfonate degradation [96]. Despite the prohibitive effect of sulfur in lignosulfonate, fungal strains such as *Phanerochaete chrysosporium*, *Chrysonilia sitophila*, *Botryosphaeria sp.* were reported to grow on and degrade sodium lignosulfonate [96][97][98][99]. The application of basidiomycetous fungi, *Pycnoporus sanguineus*, *Coriolus pubescens* and *Trametes sp.*I-62, resulted in calcium lignosulfonate decoloration [100]. Meanwhile, a bacterial strain, *Sphingobacterium sp.* HY-H, yielded a 35% removal of COD (Chemical Oxygen Demand) after 5 days of incubation with sodium lignosulfonate as the sole carbon source and *Streptomyces viridosporus* led to a 21% of lignin degradation after 21 days of treatment [101][102]. A mixed culture also led to rapid degradation (50%) of calcium lignosulfonate within a day with two *Trichosporon* yeasts and bacteria of the *Arthrobacter*, *Pseudomonas*, and *Chromobacterium* genera [103].

## ***2.6.2.2 Microbial degradation of Kraft lignin***

### ***2.6.2.2.1. Fungal degradation***

Fungal degradation is equally efficient for natural and industrial lignin, as shown in Table 2. In most studies, lignin was degraded simultaneously with the depletion of carbon and nitrogen source supplemented. In early studies on lignin degradation by a white-rot fungus, *Phanerochaete chrysosporium*, supplemental carbohydrate was found to be key to initiating the process [104][98]. Therefore, lignin degradation by certain white rot fungi can occur simultaneously with that of polysaccharides. Recent studies supported this assertion, with several authors reporting on lignin being degraded by white rot fungi with additional supplements of either carbon or nitrogen source in the medium. Meanwhile, prolonging the duration of incubation may not significantly improve the lignin degradation efficiency as shown in Table 2.

The coupling of fungal lignin biodegradation and growth is a significant factor explaining the popularity of this option. Also, if the biodegradation products are not vitally important for microbial functioning, as for fungi, the minor or even relatively major chemical differences among various lignin feedstocks may be insignificant.

One of the fundamental paradigms explaining the trends of microbial lignin degradation is that it occurs during the *secondary* metabolism [108][111], which involves pathways that are not directly related to the catabolism of phenolics. Secondary metabolism is triggered by depletion (rather than addition) of nutrients, biosynthesis of inducers or supplements and/or by a slow growth rate [112]. Secretion of ligninolytic enzymes can be triggered in response to carbon, nitrogen, manganese or sulfur growth

limitation [113]. Although it is not a rule, nitrogen repression can trigger lignin biodegradation among many fungal species including *Trametes versicolor* and *Phanerochaete chrysosporium* [114]. Therefore, the concept that lignin degradation is characteristic only for secondary metabolism and takes place only in the stationary growth phase is oversimplified, i.e., the primary and secondary metabolism could be pertinent to lignin degradation at the same time[115].

#### **2.6.2.2.2 Bacterial degradation**

In the case of bacterial degradation, lignin itself can also serve as the sole carbon source for bacterial growth, i.e., by *primary* metabolism, since a sizable growth occurs on carbohydrate-free Kraft lignin. Thus, extensive studies of various bacterial strains with respect to the possibility of using Kraft lignin for simultaneous growth and degradation have been conducted as listed in Table 3 with the extent of lignin removal reaching up to 96%. Shi et al. reported that a novel  $\beta$ -proteobacterial strain *C. basilensis* B-8 yielded a 44.4% lignin degradation in 7 days, thus consuming 722.8 mg/L of lignin during the primary growth phase [116]. In another study by the same research group, a bacterial strain, *Pandoraea sp.*B-6, yielded a 46.5% Kraft lignin degradation at the end of a 7 day incubation with lignin serving as a sole carbon source and lignin removal rate reached 795.7mg/L during the first two days of treatment [117]. These studies indicated that bacteria are able to metabolize lignin to provide sufficient carbon and energy for growth and lead to more efficient lignin degradation within a short period of treatment time compared to fungal treatment.

Consistent with this assumption, bacterial strains often exhibit a more enhanced lignin degradation than fungal species in the presence of additional carbon and/or

nitrogen sources at the initial stages of cultivation. Raj et al. achieved a 65% degradation of Kraft lignin with *Bacillus sp. ITRC-S8* after 6 days in a culture medium supplemented with glucose and peptone as carbon and nitrogen sources, respectively. They also found that oxygen inhibition could be dependent on a particular metabolism as low dissolved oxygen content of media did not negatively affect the efficiency of lignin degradation [48]. Another recent study by Lv et al. showed that the maximum lignin degradation efficiency of 96% was achieved with a sucrose supplement of 2g/L in 7 days using a microbial consortium including two indigenous bacteria [*Bacillus sp. (B)* and *Pseudomonas putida (Pp)*] and two fusant strains (PE-9 and Xz6-1) produced by a protoplast fusion between *Pseudomonas putida* and *Psathyrella candolleana*. A further increase of the sugar concentration led to a decline in lignin degradation efficiency thus indicating that an optimum amount of carbon supplement is required [80]. However, the contradictory results obtained by Giroux et al. indicated that lignin degradation started only after a complete depletion of glucose during the bacterial degradation of Kraft Indulin lignin by *Streptomyces viridosporus* and *Streptomyces badius* [129]. Meanwhile, supplementation of the medium with an organic nitrogen source was found to result in an accumulation of depolymerized lignin instead of being metabolized by bacteria [129].

## **2.7 Microbial degradation of lignin in industrial effluent**

### **2.7.1 Microbial decoloration and degradation of paper mill effluent**

Paper mill effluent compliance with discharge regulations is often assessed using its color intensity, so effluent purification is equalized to decoloration [130] [131]. The extent of decoloration and delignification of paper mill effluent are reported separately as the color of paper mill effluent originates from a mixture of organic compounds such as

tannins, resin acids with lignin as the main contributors [132][133]. When treating paper mill effluents with microorganisms, two decoloration mechanisms may be involved, i.e., the enzyme/microorganism action and biosorption [134]. In biosorption, dead microbial cells (mainly fungal biomass) are frequently used instead of live cells which are sensitive to high toxicity. Therefore a continuous supply of nutrients is not needed during the treatment [135][136]. Although efficient decoloration can be achieved, lignin degradation may not occur [137][138].

Concentration of effluent used for microbial degradation studies is often adjusted to lower pollution loads to ensure efficient microbial growth and lignin degradation [139]. In addition, primary growth supporting substrates were shown to be just as important for paper mill effluents' biodegradation as for that of Kraft lignin as shown in Table 4. Santos reported 44.5% maximum lignin removal from paper mill effluent using white rot fungi, *Pleurotus ostreatoroseus*, when the effluent was diluted and enriched with glucose [140]. Optimization of parameters associated with nutrient conditions was investigated extensively to determine the nutrient supplements triggering lignin degradation. When treating effluents with fungal species, *Cryptococcus sp.*, both the carbon and nitrogen sources were optimized through screening experiments. The medium supplementation with a combination of dextrose (1.0% w/v) and tryptone (0.1% w/v) was proved to result in the most significant lignin removal at the optimum temperature (30-35°C), shaking speed (125rpm), pH (5.0) and treatment duration, 24h [132].

### **2.7.2 Microbial degradation of lignin in effluent of various sources**

The majority of biodegradation studies have focused on lignin degradation of black liquor discharged from Kraft pulp and paper mills. Only a few reports showed efficient degradation of spent liquor from different pulping processes. A recent report claimed that depolymerization of spent liquor from sulfite pulping occurred in 2 weeks when treated with a basidiomycete *Irpex consors*[141]. Successful degradation was also shown when a sulfur-free effluent from pulping process was used. For example, a white rot fungus, *Bjerkandera adusta* , led to lignin fragmentation of 10% organosolv black liquor [142]. Decolorization of a soda pulping effluent by white rot fungi, *Trametes versicolor*, was as high as 70% after 4 days of treatment [143]. A bacterial strain, *Streptomyces strain* UAH 51 , led to 80% removal of lignin from a soda pulp mill effluent when the degradation products were detected using GC-MS [144]. Despite the toxicity of chlorolignin , the application of *Ceriporiopsis subvermispora* led to 62% removal in 2 days of treatment[145]. Meanwhile, a pulp bleaching effluent containing chlorolignin was also efficiently degraded by fungal strains including *Trametes villosa* , *Panus crinitus* and *Sporotrichum pulverulentum* [146][51].

### **2.7.3 Microbial treatment of Kraft effluent**

*Coriolus versicolor* and *P.chrysosporium*, the most efficient lignin degraders, were tested for treating black liquor or Kraft pulp under extreme conditions characteristic for real-world paper mill effluents [147] [148] [149] [150]. However, fungi were found to show a weaker performance compared to bacteria when exposed to extreme environmental conditions [125]. The other related practical problem specific for paper mills and biorefineries under operation is that their liquid effluents cannot be stored on site for an extended period of time. Therefore, long cultivation time, usually more than a



week up to few months, has been a major drawback of fungal lignin degradation. Unlike bacteria, which often grow on lignin rapidly from a few hours to a few days, fungi require longer cultivation periods for their primary growth phase. In addition, due to the filamentous nature of fungal growth, solid state fermentation has been favored by fungi as noted in numerous literature reports [32] [34][151] [152] [153]. This inherent feature of fungi hinders their industrial application, which usually involves the use of conventional batch or continuous bioreactors. Despite these setbacks associated with fungal treatment of paper mill effluents, newly isolated fungal strains exhibited equally efficient degradation of paper mill effluent compared to bacterial treatment as shown in Table 4. One of the main reasons specific for using fungal treatment is that more complex compounds can be metabolized during the fungal treatment due to non-specific oxidation reactions of extracellular enzymes [154]. By contrast, bacteria produce target-specific enzymes with a narrower substrate specificity. On the other hand, the eukaryotic nature of fungi renders them more versatile in coping with the presence of inhibitory compounds. Furthermore, hyphal growth and structure of cell walls of fungi is more resistant to inhibitory compounds compared to bacteria [154]. Bacterial treatment of paper mill effluent is often coupled with growth, thus degradation can be easily carried out and controlled in the continuous or batch reactors [167] Optimum nutrition supplement is also critical for most bacteria to exhibit high lignin removal capabilities. In addition, considerable efforts have been made in isolating new strains which could potentially possess a significant ligninolytic capability for industrial applications [168] [169][170][171]. Chandra et al. reported the use of a bacterial consortium comprised of *Serratia marcescens* (GU193982), *Citrobacter* sp. (HQ873619) and *Klebsiella*

pneumoniae (GU193983) to degrade black liquor at high pollutant loads. GC-MS analysis showed a significant consumption of low molecular weight lignin fractions after the treatment confirming that the consortium has a strong ability to utilize lignin constituents as a sole source of carbon, nitrogen and energy [131].

#### **2.7.4 Addressing long treatment times and low process efficiencies**

Regardless of whether fungi or bacteria are used, prolonged time essential for biotreatment along with the stress imposed upon microorganisms when exposed to black liquor hinder the industrial application of microbial lignin degradation. In order to cope with the issue, batch and semi-continuous reactors were used in bacterial treatment of black liquor to achieve the maximum reduction of lignin [172]. Further, novel bioreactor systems were studied in an attempt to implement an industrially viable large-scale waste treatment. Aerobic treatment of waste water from pulp and paper industry often employs aerated, activated sludge treatment and sequencing batch reactors (SBR) [173]. Anaerobic-aerobic systems with high rate bioreactors such as an upflow anaerobic sludge blanket (UASB), filter bioreactor, fluidized bed reactor, membrane bioreactor, etc., achieved high COD removal up to 70% within a short time ranging from a few hours to a few days [174]. Integrated anaerobic–aerobic bioreactors are also under development for increasing degradation efficiency [174]. El-Ashtoukhy et al. used a batch-stirred tank electrochemical reactor for efficient color removal of pulp and paper mill effluent with rice straw as a raw material [175]. Chemical reduction reaction using  $\text{NaBH}_4$  followed by biological oxidation was conducted using hydrogenation-biological batch reactors for chromophores' removal in pulp and paper mill effluents [176]. Lignin or its derivatives could react with the high chlorine content of bleached plant effluent and generate many

recalcitrant compounds. Anaerobic microorganisms demonstrated a higher capacity in terms of chlorinated substances removal allowing for a greater degradation performance by subsequent aerobic fungi and bacterial treatments [163]. Several authors have claimed to achieve an enhanced removal of lignin through a *sequential* treatment of industrial effluents by either fungi or bacteria. Thakur et al. used two step sequential treatments where color removal was carried out by fungi in the first step, with a subsequent effluent treatment by bacteria. A fungal strain *Paecilomyces* sp. alone removed 68% color and 34% color reduction was achieved solely by bacteria, *P. aeruginosa*, in 1 day. By comparison, 82% color reduction was achieved within 3 days when the effluent was treated with a fungal strain followed by *P. aeruginosa* [158]. Singh et al. showed that a combination of an anaerobic process, widely utilized as a secondary treatment of industrial wastewater, followed by an aerobic sequential treatment enhances the biodegradation of Kraft process effluents by either a fungus (*Aspergillus fumigatus*) or a bacterial strain (*Pseudomonas ovalis*)[163].

## **2.8 Ligninolytic enzymes**

### **2.8.1 Laccase: The use of mediators**

The most extensively studied ligninolytic enzymes are laccase, manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP), especially among white rot fungi. Laccase is the most widely distributed and most extensively studied multi-copper oxidase [177]. This enzyme is considered an “ideal green” catalyst because it employs O<sub>2</sub> as a co-substrate and generates water as a non-toxic byproduct. Sufficient oxygen supply was found to be important for achieving an efficient laccase mediated oxidation of industrial lignin [178]. Laccase activity has been discovered in

many fungi and bacteria used for lignin degradation. The most commonly studied laccase is that from white rot fungi of *Trametes sp.* [179] [180][181][182] . Laccase has been reported for the C $\alpha$  oxidation as well as cleavage of aryl-alkyl C-C and C $\alpha$ -C $\beta$  bonds [183]. Oxidation of phenolic substrates involves the removal of one electron from the substrate phenolic hydroxyl groups to form phenyl hydroxyl radicals [183].

With non-phenolic substrates, the use of mediators, i.e., reaction particle transfer “helpers,” is essential for efficient use of laccase [184]. Yet, even a general lignin biodegradation often occurs with the aid of mediators, which are oxidized by ligninolytic enzymes and then diffuse into lignin matrix to serve as effective lignin oxidants rather than the enzymes themselves [1]. Due to a much smaller size of such mediators, potential diffusion problems are overcome. Numerous laccase mediators have been discovered oxidizing the non-phenolic structural moieties of lignin [185]. The most efficient synthetic (non-phenolic) laccase mediators are 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI), violuric acid (VLA), N-hydroxyacetanilide (NHA), N-hydroxyacetanilide (HAA) and 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) (Calcaterra et al., 2008) [179][184][185].

The reaction mechanisms for laccase mediators can be classified as hydrogen atom transfer, electron transfer and ionic mechanisms [186]. For N-OH-type mediators such as HBT, HPI, VLA and NHA, oxidation of lignin substrate employs the electron transfer mechanism where free radicals are generated through monoelectronic oxidation. With the aid of the oxidized form of mediators, N-OH-type mediators can also act on lignin via the hydrogen transfer mechanism, during which the mediator deprotonates to aminoxy anion capable of the abstraction of a hydrogen atom from the substrates with

relatively weak C-H bonds. TEMPO is a laccase mediator functioning through the ionic transfer mechanism. Laccase oxidizes TEMPO to the corresponding oxammonium ion, which subsequently acts on the non-phenolic structural part of lignin to generate an adduct. Then, the removal of a  $\alpha$ -proton occurs to yield the oxidation product, while the reduced TEMPO is oxidized back to TEMPO [187][188]. It was reported that a synergetic effect between two different laccase mediators seems to occur whenever their active species oxidize the same target substrate by different mechanisms [189].

### **2.8.2 MnP and its mediators**

LiP, MnP and VP are classified as heme peroxidases, which act on substrates via multistep electron transfers along with a simultaneous generation of intermediate radicals [184]. Versatile peroxidase (VP) exhibits multi-functionality combining the enzymatic characteristics of MnP, LiP and low redox potential peroxidases thus being capable of oxidizing high redox potential substrates including phenolic and non-phenolic moieties of lignin[190]. MnP is more selective than other peroxidases catalyzing mostly  $C_{\alpha}$ - $C_{\beta}$  cleavages,  $C_{\alpha}$ -H -oxidation and alkyl-aryl C-C bond cleavages of phenolics in lignin structure [191].  $Mn^{2+}$  serves as a reducing substrate for MnP to be oxidized to  $Mn^{3+}$ , which in turn oxidizes phenolic compounds. The  $Mn^{3+}$  formed is released from the enzyme and becomes stabilized through a chelating reaction with  $\alpha$ -hydroxy acids[184].

Without mediators, MnP mainly contributes to the oxidative cleavage of phenolic lignin structures containing phenolic hydroxyl groups, which only accounts for around 10% of the lignin aromatic structure [184][192] [193]. However, the role of manganese peroxidase can be expanded to non-phenolic substrates by using phenolic mediators such

as veratryl alcohol [194]. Veratryl alcohol was also proposed to serve a dual function of being a diffusible mediator for increasing the oxidation capacity of LiP and an electron donor thus preventing LiP from inactivation by  $H_2O_2$ [193]. The second known group of mediators for MnP are unsaturated fatty acids, from which peroxy radicals are generated allowing for MnP to oxidize non-phenolic lignin structures [195] [196][86].

### **2.8.3 LiP: A mediator-free peroxidase**

For LiP, phenoxy radicals can be easily formed in the reduction of LiP-I and LiP-II. LiP degrades non phenolic lignin substrates through the formation of a cation radical via a one-electron oxidation [184]. Classical peroxidases are limited only to the oxidation of strongly activated aromatic compounds, whereas LiP is capable of acting on aromatic compounds just moderately activated by electron donating substituents [197]. In addition, LiP can carry out a long distance electron transfer from an aromatic substrate thus not requiring a direct contact between the oxidized heme and substrate [198].

LiP reacts with  $H_2O_2$  to form an intermediate, LiP- I, which is further converted to LiP-II in the presence of a reducing substrate, such as veratryl alcohol (3,4-dimethoxybenzyl alcohol), via  $1e^-$  reduction. A second one electron reduction by veratryl alcohol returns LiP-II to its active state. However, with excess  $H_2O_2$ , LiP-II can become LiP-III without a reducing substrate. This inactive form of the enzyme can return to its active form through either autooxidation or oxidation by forming veratryl alcohol cation radical [184]. This enzyme plays an important role in the degradation of both phenolic and non-phenolic lignin components through a scission between the  $C\alpha$  and  $C\beta$  atoms of propyl side chains, hydroxylation of benzylic methylene groups and formation of aldehydes or ketones through oxidation of benzyl alcohols [184] [199] [200]. In

general, similar bond cleavages and oxidation reactions can occur with laccase, MnP and LiP treatment of lignin. However, the degradation mechanisms involved differ greatly depending on phenolic or non-phenolic moieties of the lignin structure [199].

#### 2.8.4 Bacterial enzymes

Among the known bacterial enzymes responsible for aromatic compounds' degradation, heme peroxidase DypB was identified as a manganese dependent lignin peroxidase, which decomposes a  $\beta$ -aryl ether lignin model compound [201]. DyP was found to lack a histidine residue axially coordinated to heme, as in common heme peroxidases [202]. It also exhibits a wide substrate specificity with sustained activity under moderately acidic conditions, e.g., at pH near 3.0 [203]. In a study by Ahmad et al., Dyp A and DypB were reported in the bacterial strain *Rhodococcus jostii* RHA1. DypB showed an enhanced activity in the presence of  $\text{MnCl}_2$ , the  $\text{Mn}^{2+}$  is suggested to serve as an oxidative mediator for DypB. DypB was able to selectively act on  $\beta$ -aryl ether lignin model compounds exhibiting a similar behavior to versatile peroxidase [204]. Brown et al. identified a multifunctional dye peroxidase classified as DypC from a lignin degrading bacterium, *Amycolatopsis* sp. 75iv2 [205].

$\beta$ -etherase is another ligninolytic enzyme reported among both fungi and bacteria, which cleaves the aryl glycerol  $\beta$ -aryl ether bonds predominantly present in the lignin structure [205] [206]. One bacterial strain, *S. paucimobilis* SYK-6, is well studied in terms of its lignin metabolic pathway. Three enzymes including LigD, a Ca-dehydrogenase, LigF, a  $\beta$ -etherase and LigG, a glutathione lyase, were reported to be involved in lignin degradation [207]. LigD acts on a  $\beta$ -aryl ether compound containing

C<sub>α</sub>-hydroxyl bond and oxidizes it to a C<sub>α</sub>-carbonyl structure. LigE and LigF account for the reductive cleavage of arylglycerol-β-aryl ether linkages [207].

The unique bond cleavage types and catalytic mechanisms of ligninolytic enzymes are often elucidated *in vitro* by using synthetic model lignin compounds, as the structures of model lignin compounds are much simpler than that of natural lignin. However, to meet industrial demands, ligninolytic enzymes produced from microorganisms need to be equally efficient in treating industrial lignins [208]. *In vitro* treatment of lignosulfonate using laccase from *Cerrena unicolor* led to an efficient degradation with the addition of acetovanillone or acetosyringone as low molecular weight mediators [209]. A peroxidase of *Pleurotus ostreatus* also facilitated the *in vitro* depolymerization of lignosulfonate [210]. The extent of biodegradation reported in these studies was often demonstrated via monitoring changes in molecular weight distribution. However, future studies should also look into the characterization of degraded lignin compounds from *in vitro* enzymatic treatment of industrial lignin.

On the other hand, laccase was also reported to serve as a polymerizing cross-linking agent. For sulfur-containing lignins, concentration of the lignin and extent of sulfonation could be associated with polymerization of lignin structures [211]. Extensive polymerization of lignosulfonate was observed when treated with laccase [212][213]. Kraft lignin also showed an increase in condensed structure when treated with laccase from *Trametes trogii* [214]. It was believed that the sulfonation of terminal group at the α-carbon facilitates cross linking within the lignin phenolic units [211].



Although *in vitro* treatment of industrial lignin by ligninolytic enzymes can be readily controlled and optimized, the degradation efficiency of lignin ultimately depends on synergetic enzymatic systems in the microorganism used [215]. However, the degradation efficiency of ligninolytic enzymes can be negatively affected *in vivo* due to the chemical alteration that have occurred in the native lignin structure during industrial processing [216]. Although an efficient degradation of industrial lignin *in vivo* was previously demonstrated, more research efforts are needed to look at the synergetic effect of ligninolytic enzymes on treating all types of industrial lignin.

## **2.9 Regulation of ligninolytic enzymes**

Enhancement of ligninolytic enzymes' production and induction of enzyme activities are important factors to optimize the lignin degradation process for industrial applications. Successful induction of ligninolytic enzymes requires the achievement of a right combination of selected microorganisms, culture medium and inducers. A wide variety of synthetic or lignocellulosic substrates have been used in culture media for ligninolytic enzyme induction[217] [218]. However, there is still a limitation of obtaining the same induction results when microorganisms are grown on industrial lignin. Low dosage of lignosulfonate and lignosulfonic acid supplements to the culture medium of *Trametes versicolor* and *Stereum ostrea* can act as inducers for both laccase and MnP [219][220]. Vasdev and Kuhad also reported the complementary effect of adding industrial lignin to a malt extract medium for maximum laccase production [221]. With a higher concentration of sulfur-containing lignin, several studies claimed the effect of lignosulfonate based medium on increased production of LiP by a bacterial strain, *Streptomyces viridosporus* [222] [223]. The induction caused by lignosulfonate could be

due to the similarity of its structure to lignin breakdown compounds or antimicrobial agent secreted by other microorganisms, which can often trigger a removal of those toxic chemicals by secreting ligninolytic enzymes [224]. However, reports are lacking on studying the effect of other inducers on the induction of ligninolytic enzymes when a sulfur-containing lignin based culture medium are used. In addition, the specific micro-environment of lignin based culture media, which is equally important during lignin degradation, is not well understood. More research efforts are in need to study whether the extent of sulfonation among various industrial lignin types could affect the induction of ligninolytic enzymes.

### **2.9.1 Metal ion induction**

A commonly used inducer for laccase is  $\text{Cu}^{2+}$  ion, so many studies have been focused on utilizing copper supplements to enhance laccase activity of white rot fungi as shown in Table 5. A copper supplement should be applied during the exponential growth phase rather than stationary phase to achieve the maximal stimulatory effect. However, the addition of copper also leads to an inhibition of the fungal growth [225]. Accurate prediction of optimum nutrient and copper concentrations appears to be critical towards enhancing the fungal laccase production.

The effect of copper ion on the balance between the induction and repression of *D. squalens* laccase activity was found to be influenced by the carbon to nitrogen source ratio. Methylated substrates (3-O-methylglucose and methylcellulose) in combination with casein (phosphoprotein) and copper were reported to enhance both the fungal tolerance and laccase activity [226]. Furthermore, it is usually the concentration of *free*

copper ion in the medium that correlates with the inductive potential of copper supplements. Organic acids such as oxalic acid secreted by white rot fungi might often lead to the decrease of pH in media and complexation of metals thus reducing the induction effect of copper ion on laccase production [227]. Several common medium components such as tartaric acid, thiamine and yeast extract were also found to bind metals. The inductive potential of copper species are reported as free > inorganically complexed > organically complexed > total  $\text{Cu}^{2+}$  [228].

Compared to fungal laccase induction, publications on bacterial induction through the addition of copper supplements are relatively scarce. One of the spore coat proteins (CoA) surrounding endospores of *Bacillus* strains have shown to exhibit laccase activity [229]. Bioelectricity-generating bacteria of *Proteus hauseri* ZMd44 exhibit laccase activity under copper sulfate induction. The identified laccase, which is a typical T1 multi-copper oxidase, was reported to be thermophilic and acidophilic [230]. Samanovic et al. reported that bacteria encode a Cu resistance system to minimize its toxicity when copper is abundant in the medium. Membrane proteins, P-type ATPase, are responsible for the transport of copper ion across membranes [231].

Induction of the MnP activity in microorganisms is also reported to be correlated with the presence of  $\text{Mn}^{2+}$  [232][233]. Previous studies have shown the increased transcription of MnP genes from *Trametes versicolor* PRL 572 with an addition of  $\text{Mn}^{2+}$  ions to the media [234]. Knežević et al. reported the maximum lignin degradation with peaked laccase and MnP activity when the  $\text{Mn}^{2+}$  was present in the media [235]. However, a co-presence of  $\text{Cu}^{2+}$  and  $\text{Mn}^{2+}$  ions leads to a decreased peroxidase activity

for the lack of H<sub>2</sub>O<sub>2</sub> production caused by scavenging of oxygen radicals by the copper ion [236].

However, other studies reported that Mn<sup>2+</sup> is not directly responsible for the expression of MnP, but is rather involved in the post-transcriptional regulation [237]. An accumulation of *intracellular* MnP was reported for the basidiomycete *Ceriporiopsis subvermispora* in a Mn<sup>2+</sup> free culture, suggesting that the system is set up to trigger the initiation of ligninolysis only when the Mn<sup>2+</sup> becomes available [238]. In addition to the typical peroxidase activity of MnP, it also catalyzes the Mn<sup>2+</sup> dependent disproportionation of H<sub>2</sub>O<sub>2</sub>. The catalase activity of MnP was found to be inhibited by a chelating compound, oxalate, probably due to sequestering of Mn<sup>2+</sup>, and is stimulated by the abundance of Mn<sup>2+</sup> [239]. The presence of excess Mn<sup>2+</sup> led to increased catalase activity in MnP thus decomposing H<sub>2</sub>O<sub>2</sub> at the expense of converting Mn<sup>2+</sup> to Mn<sup>3+</sup>. The catalase activity of MnP is believed to function as the means of protection of both MnP and LiP against inactivation when exposed to excess H<sub>2</sub>O<sub>2</sub> [239].

On the other hand, a Mn<sup>2+</sup>- deficient medium is favorable for the induction of lignin peroxidase expression for *Phanerochaete chrysosporium* [240]. Meanwhile, starving a culture with excess oxygen was also reported to trigger the LiP expression and so the LiP gene expression can be induced by the oxidative stress mechanism as well [240]. The LiP production of *Corioloopsis polyzona* was also reported to be repressed by the addition of Mn<sup>2+</sup> [241]. Matityahu et al. suggested the involvement of an increased expression of glyoxal oxidase for generating H<sub>2</sub>O<sub>2</sub>, which is essential for the expression of LiP in Mn<sup>2+</sup> deficient cultures of *Phanerochaete chrysosporium* [242]. However, the

addition of a manganese supplement to nitrogen limited cultures led to an increase in secretion of LiP for a white rot fungus, *Phanerochaete flavido-alba* FPL 106507 [243].

### **2.9.2 Ethanol induction**

Research on induction of ligninolytic enzymes with ethanol is predominantly focused on laccase. As high costs have been a major drawback for industrial application of laccase, the utilization of ethanol, an inexpensive inducer, may significantly improve the process economic sustainability. An ethanol addition may concomitantly increase the membrane permeability by partially disrupting the lipid bilayer [244]. It is also known to preclude the formation of melanin thus increasing the concentration of aromatic monomers of melanin pigments present in the cells, which is believed to be a significant factor for enhanced laccase activity [245].

In a study of ethanol induced response in *Phanerochaete chrysosporium*, a reductive peptide, glutathione, was identified in the ethanol supplemented culture medium in contrast to the blank without ethanol. In turn, glutathione was believed to help degrade a model triaryl methane dye efficiently. The report also suggested the timing of ethanol addition to be an important factor for laccase induction. An ethanol supplement at an initial stage of cultivation hindered the mycelium growth [246]. Meza et al showed that ethanol serves as an inducer rather than a carbon source for a white rot fungus, *P. cinnabarinus*. This induction was proved to occur at the genetic level according to the observation that the expression of the laccase-encoding gene declined and then resumed when the ethanol supplement was removed and then added again, respectively [247].

Despite the qualitative generality of ethanol induction, the quantitative effect of this inducer is strain specific. A rapid increase of laccase gene expression was observed when 25 g/L of ethanol were added to the culture of the basidiomycete *Pycnoporus cinnabarinus* [248]. Ethanol exhibited only a minor influence on the induction of laccase activity and stability of *Trametes versicolor* within a wide concentration range, 0 to 50 g/L [249]. By contrast, the addition of a 35 g/L of ethanol led to a 155-fold increase in laccase production by *Pycnoporus cinnabarinus* yielding a laccase activity of 266600U/L [250]. Yet it should be noted that high ethanol concentrations exceeding or equal to 5% (v/v) ~50 g/L were found to make a negative impact on cell growth of *Ganoderma lucidum* and inhibit the laccase induction [251].

### **2.9.3 Induction via co-cultivation**

Lignin degradation was shown to be enhanced by co-culturing of different microorganisms; this effect was ascribed to the competition for limited space and nutrients, which may result in an improved degradation performance through the elevated ligninolytic enzymes' production [252] [253][254][255][256][257]. Corroborating this hypothesis, corn stover degradation using a co-culture of *T. reesei* and *C. comatus* led to a 2.6-fold induction of laccase activity over monoculture of *C. comatus* [258]. Similarly, a higher activity of ligninolytic enzymes from *S. commune* was observed compared to a co-culture of *S. commune* and *G. incidum* during corn stover degradation [259]. The complementary production of enzymes induced in a mixed culture could lead to enhanced lignin degradation [258][260]. A high degree of decoloration and lignin removal could be obtained for indulin AT lignin with a synergistic effect of three species (*Merulius aureus syn. Phlebia sp.* and an unidentified genus *Fusarium* and *sambucinum Fuckel MTCC*

3788) consortium. However, *Phellinus sp.* – *B. pumilus* and *P. sulphureus* – *B. pumilus* co-cultures were found to be ineffective in increasing the rate of lignin degradation compared to the corresponding monocultures [261].

An advantage of such a co-cultivation induction is the minimal involvement of potentially toxic chemical inducers, which could compromise the robust growth of selected microorganisms. The overproduction of laccase in a fungus, *Panus rudis*, was facilitated in the presence of a non-laccase-producing fungus, *Gongronella sp.*, with a 25 times higher activity compared to a chemical induction using copper/*o*-toluidine [262]. Increased laccase activity was observed at the deadlock interaction zone on an agar medium due to the interspecies interactions of *Phanerochaete chrysosporium* and *Trametes versicolor* [256]. Hiscox et al. reported an increased laccase and MnP activity but no extracellular LiP activity at interaction zones in the mixed culture of *Trametes versicolor* and five other wood decay fungi. However, the gene expression associated with LiP was detected suggesting that LiP accumulated intracellularly after co-cultivation [263]. Specific mechanisms may be involved in various kinds of interspecies' interactions. *Serpula lacrymans* was reported to secrete laccase during the invasion of *T. harzianum* SIWT 25. It is the presence of tyrosine in the medium that could be responsible for the enhanced production of laccase [264]. Presence of particular metabolites from microorganisms could also play a role in enhancing ligninolytic activities. For example, some metabolites from *Gongronella sp.* were likely to serve as a signal of laccase induction in *Panus rudis* [262].

#### 2.9.4 Nutrient Induction

The carbon and nitrogen source selection may induce ligninolytic enzymes, although the role of nutritional regulation needs further research. For example, the presence of peptone in a *P. ostreatus* cultivation medium led to a significant change in the MnP isozyme pattern. However, it is unclear which particular component of peptone stimulated such an alteration of the isozyme production [265]. Ruiz-Duenas et al. showed that the highest molecular weight fraction of peptone with a relatively low content of aromatic amino acids could serve as an inducer for MnP/VP in *Pleurotus eryngii* [266].

Kannaiyan reported that fungal growth capacity did not directly correlate with the induction and suppression of peroxidase activity, but was instead influenced by nutritional conditions. A C/N ratio of 1:10 (arabinose and sodium nitrite) and C/N ratio of 1:1 (glucose and ammonium chloride) in the media increased the Mn<sup>2+</sup> tolerance of *C. subvermispora* and *D. squalens* while showing up to 173% and 90% increase of the MnP activity for these two strains, respectively. Since oxalate as a chelating compound is closely related to the regulation of MnP activity when Mn<sup>2+</sup> is present, sugar regulation associated with the oxalate metabolism might play a role in MnP induction [267].

Increased nitrogen concentrations were shown to suppress the MnP activity of *Phanerochaete chrysosporium* [268]. Similarly, high nitrogen concentrations were claimed to stimulate the laccase production for *Trametes gallica*, whereas the opposite effect was observed for *Trametes versicolor* [179]. The peroxidase production was reported to be inhibited in carbon rich media among all tested mixed cultures including six species of white rot fungi, *Bjerkandera adusta*, *Phlebia radiata*, *Pleurotus ostreatus*, *Dichomitus squalens*, *Hypoxylon fragiforme* and *Pleurotus eryngii* applied in pairs [269].



Anderson et al. reported that the carbon/nitrogen ratio in the medium influenced the laccase isozyme production and secretion by two strains of *Fusarium proliferatum* for both natural and synthetic lignin degradation [270]. The carbon/nitrogen ratio yielding the maximal MnP production in a co-culture of *P. radiata* and *P. ostreatus* was shown to be changing over the cultivation period, decreasing drastically toward the end of cultivation [269]. A simultaneous addition of small regulatory molecules, 5 mM cyclic adenosine 3',5'-monophosphate (cAMP) and 500  $\mu$ M 3'-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, led to an elevated MnP activity by *P. chrysosporium*. This regulation may play a part in a common cAMP signalling cascade, where small molecules trigger both the signal transduction and secondary metabolic pathways [271].

### **2.9.5 Phenolic induction**

A significant reduction of laccase production by *Botryosphaeria* isolates was observed when low-MW phenolic compounds were present in the media. Out of 10 phenolic compounds including ferulic acid, vanillic acid, guaiacol, etc., only syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid) was reported to exhibit no toxic effect on mycelium of *Botryosphaeria* isolates [272]. A physiological role of laccase and common lignin-degrading peroxidases could thus be the removal of potentially toxic phenolic compounds formed as a result of lignin degradation. Namely, this function of laccase was proposed to be associated with the protection of fungal hypha from these compounds by polymerizing the lignin monomers [273].

Corroborating this hypothesis, ferulic acid was shown to be an efficient laccase inducer for many fungal strains such as *Pleurotus sajor-caju* and *Trametes versicolor* [274][180]. By a similar token, a slight but detectable induction of MnP was observed when 10 phenolic compounds including ferulic acid, vanillin and veratric acid were supplemented in the growth medium of three white rot fungi including *Cerrena unicolor*, *Ganoderma lucidum* and *Trametes versicolor* [275]. An endophytic fungus, *Phomopsis liquidambari*, was reported to metabolize ferulic acid as a carbon source. In this process, transcription of laccase gene lacB3 was induced and laccase was believed to be a major contributor of ferulic acid degradation [276]. Syringol-based phenolics were demonstrated to enhance the degradation of the most recalcitrant lignin model compounds by manganese peroxidase from a white rot fungus, *Phlebia sp. Nf b19* [29]. Veratryl alcohol was reported to be involved in the induction of manganese peroxidase on the level of gene transcription for white rot fungi *Clitocybula dusenii*, *Nematoloma frowardii* and a straw degrading strain designated as i63-2 [277].

However, in an earlier study on the ligninolytic system of *Phanerochaete chrysosporium*, the addition of veratryl alcohol led to no stimulatory effect on MnP induction and no induction of the mRNA encoding factor for lignin peroxidase was observed [278]. In addition, phenolic unit structures such as dimeric lignin fragments or phenolic monomers were claimed to inhibit the lignin peroxidase activity of *Phanerochaete chrysosporium* by converting LiP to its inactive form [279]. By contrast, veratryl alcohol was found to reactivate LiP III by converting it to the ferric form [292]. An early report revealed that the reactivation of this enzyme by veratryl alcohol was inhibited by the addition of phenol. Veratryl alcohol was oxidized by LiP to veratryl

alcohol cation radicals, which were involved in the oxidation of phenol. LiPIII accumulated during this time due to the stoichiometric veratryl alcohol cation radicals' consumption [293].

## **2.10 Chemical characterization of lignin degradation products**

### **2.10.1 Methods for assessing the extent of lignin degradation**

#### ***2.10.1.1 Spectrophotometric analysis***

Relatively low concentrations of lignin were used in all reported studies, usually a few grams per liter or even less. Therefore quantification of lignin degradation in most studies was conducted by a method suitable for this concentration range, by measuring the decrease in UV absorption of lignin at 280 nm due to the characteristic absorption band of lignin at this particular wavelength. According to the report by Hatfield et al., the spectrophotometric method has the tendency to overestimate the potential lignin fraction because carbohydrate degradation products, such as furfural and hydroxymethylfurfural, can strongly absorb light within a similar wave length range thus creating interferences, especially in media with a low lignin content [294]. In addition, proteins can also contribute to the absorbance at 280nm [107]. Alternatively, in some studies, Pearl-Benson method was employed to determine the extent of microbial lignin degradation. Acidified sodium nitrite reacts with lignin to form nitrosophenol which can be further converted to quinone mono-oxime when adding alkali [295]. This method involved fluorescence spectroscopy, by which very dilute solutions of lignin can be measured at 430nm [295].

### ***2.10.1.2 Gravimetric analysis***

The simplest gravimetric method is commonly used for quantitative determination of lignin degradation. This method seems to be unassailable and straightforward since the original lignin is insoluble in the culture medium. According to the Klason method developed for lignocellulosic biomass, carbohydrates are dissolved after acid hydrolysis treatment whereas lignin, is filtered, washed, dried and weighed [39] [296].

However, this method may provide superficial or even inaccurate information on the extent of lignin degradation if the original feedstock 1) contains readily biodegradable impurities and 2) degrades partially into a mixture of lower-MW polymers, which is more water-soluble than the original lignin. Even lignin itself becomes soluble in alkaline media, the occurrence of ‘black liquor’ also shows its tendency to form colloids. Both of these trends are expected to become more pronounced for lower-MW lignin fragments, which otherwise remain as recalcitrant as the original lignin. Therefore, gravimetric analyses should be combined with such methods that would assess the formation of lower-MW fragments, such as size exclusion chromatography (SEC).

### ***2.10.1.3 Methods for molecular weight and structural characterization of lignin***

SEC can be used for determination of mass distribution of both natural and synthetic polymers. A wide range of molecular masses can be determined by SEC in a single run through a suitable column [297]. However, secondary separation effects might affect the accuracy of measurement due to adsorption, ionic exclusion, ionic attraction and entrapment [298]. Due to its polar nature, lignin tends to interact non-specifically with any polar column material, thus skewing the results of the separation based on just size exclusion. This tendency may be significantly ameliorated by excessive lignin

acetylation [297], yet the uncertainty remains that some polar functional groups may still remain.

The major weakness of this method is potential deviations from a calibration that correlates the retention time and molecular weights of standards, usually polystyrene standards [299] [300]. Such a calibration is based on the assumption that both the standards and analytes form spherical coils when dissolved [301]. However, while this is true about the standards, rigid aromatic lignin polymers are far from spherical often being branched. Thus even when the analyte-column interactions are insignificant, lignin has a tendency to elute much later than expected because branched polymers may possess a hydrodynamic volume similar to that of a linear polymer with a lower molecular weight [302]. Increased deviation of SEC determined lignin molar masses can be expected when lignin samples with a higher molar mass or polydispersity are analyzed [303]. Accurate molecular weight determination for lignin can be impeded with reactions occurring between lignin and various solvents [304].

Fourier transform infrared spectroscopy (FTIR) is frequently used in microbial lignin degradation studies [305][106], as it provides the fundamental qualitative information regarding the chemical/structural changes through absorption peaks corresponding to the vibrational frequencies of specific chemical bonds [306]. In several studies, lignin degradation was assessed and confirmed by a decrease in the intensity of specific bands corresponding to the aromatic skeleton vibrations [47][106][307]. However, precise identification of specific lignin subunits cannot be obtained via an FTIR analysis as it does not provide the resolution essential to distinguish among individual chemical species [308].

Detailed structural information on changes occurring in lignin could also be obtained through nuclear magnetic resonance spectroscopy (NMR) although this method has similar limitations to FTIR [296]. Namely, it does not provide a resolution on the level of species as only the general functional groups are semi-quantified. Yet, given this limitation, NMR has been a powerful tool in characterizing lignin. FTIR can be combined with NMR to provide a better interpretation of the structural changes of lignin after treatment [309].

Tran et al. studied the modification of  $\beta$ - $\beta$  links in Kraft lignin treated with a specific oxidant 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) using 2D Heteronuclear Single Quantum Correlation/Coherenc (HSQC) NMR (HSQC) 2D-NMR [310]. One disadvantage of 2D (HSQC) 2D-NMR technique is that it is limited to the analysis of soluble lignin fraction for solution state NMR, which has a lower resolution than the solid state NMR [308]. In addition, NMR does not always differentiate between the 4-O-5' and 5-5' subunits of lignin [311]. Development of  $^{31}\text{P}$  NMR spectroscopy allows for a discrimination between the 4-O-5' and the 5-5' condensed forms by using 2-chloro-4, 4', 5, 5'-tetramethyl-1, 3, 2-dioxaphospholane to convert hydroxyl groups to their corresponding phosphites [312]. In addition, characterization of lignin can be conducted without isolating it from biomass by using solid state analysis,  $^{13}\text{C}$  cross-polarization/magic-angle spinning (CP/MAS) solid-state NMR [313][314]. Alekhina et al. characterized softwood Kraft lignin isolated from black liquor by  $^{13}\text{C}$  NMR spectroscopy, which revealed that the carbohydrate content is highest when lignin is precipitated at pH 2.5 with the increasing bands attributed to carbohydrate between 90 and 102ppm. The optimum pH of lignin separation occurred at pH 5.0 [315].  $^{31}\text{P}$  and

$^{13}\text{C}$ - $^1\text{H}$  2D NMR spectroscopy were used in the study by Mbotchak et al. where organosolv wheat straw lignin was chemically modified through replacing phenolic OH groups with more reactive aliphatic OH groups by etherification with 2-chloroethanol under alkaline conditions. Esterification with ethanol and ethane 1, 2-diol with the aid of p-toluenesulfonic acid as a catalyst yielded esterified lignin with a low carboxylic acid content. Meanwhile, a 7-fold decrease in phenol content through hydroxyethylation by 2-chloroethanol proved to be an efficient modification method [316].

## **2.10.2 Methods for identifying degraded products of lignin**

### ***2.10.2.1 Gas Chromatography Mass Spectrometry (GC-MS)***

GC-MS is the most widely employed analytical tool in characterization of lignin degradation products. GC-MS analysis is essential in mechanistic studies, as it provides the composition of the ultimate lignin degradation products, phenolic monomers. Common lignin degraded products reported by previous studies is shown in Figure 5. Wang et al. also proposed potential mechanisms of the lignosulfonate biodegradation by *Sphingobacterium sp.* HY-H through GC-based studies of bacterially degraded samples [307]. Oxidation of hydroxy groups, demethylation of methoxy groups, and decarboxylation of the carboxylic acids might be the predominant mechanisms of lignosulfonate microbial degradation [307]. Acetic acid, readily detectable by GC using derivatization agents is an important lignin degradation intermediate for microorganisms [3]. Its detection could signify the detachment of side chains of phenylpropanoid moieties comprising lignin. Cinnamic acid, if detected by GC, indicates the linkage between lignin and hemicellulose in lignocellulosic biomass whereas the presence of ferulic and p-coumaric acids is evidence of external ester and ether linkages in the original lignin,

respectively [117][317]. The majority of ester linkages are broken during the alkaline extraction within the pulping process while the remaining cinnamic acid bound to lignin by ether linkages may be further broken down by the action of microorganisms [144].

A study of Kraft lignin breakdown by a bacterial strain, *Aneurinibacillus aneurinilyticus*, identified guaiacol, acetovanillone, gallic and ferulic acids as GC-elutable products while *trans*-cinnamic acid, 3,4,5-trimethoxybenzaldehyde and ferulic acid were produced by *Bacillus sp.* These results corroborated the perceived oxidation of coniferylic (G units) and sinapylic (S units) alcohol moieties of the lignin polymer [318].

The GC-detected phenolic monomers formed as a result of lignin degradation could be used as value-added by-products if biochemical methods are developed to assure their accumulation as ultimate products rather than eventual biodegradation. However, there is a biochemical alternative to this route: Valuable *secondary* metabolites can also be obtained through lignin biodegradation by a further processing of phenolic monomers via central metabolic pathways i.e. they are smaller molecules of non-phenolic nature . The treatment of Kraft lignin with a bacterial strain, *Novosphingobium sp. B-7*, produced several low molecular weight alcohols such as ethanediol and 3-methyl-2-butanol, suggesting some potential of developing a more efficient biomass-to-alcohol process involving not only carbohydrates but also lignin [319]. Some of the observed products of microbial degradation of industrial lignin/black liquor are listed in Figure 5.

Chandra and Bharagava analyzed the products of bacterial degradation of Kraft lignin by *Citrobacter freundii* (FJ581026), *Citrobacter sp.* (FJ581023) by GC-MS. 2,4,6-trichloro-phenol, 2,3,4,5-tetrachloro-phenol and pentachloro-phenol were detected



as recalcitrant metabolites of Kraft lignin degradation, which originate from the use of chlorine during wood treatment while *n*-octadecanoic acid and *n*-hexadecanoic fatty acids were observed as apparent secondary metabolites [320]. It should be noted, though, that many studies identified *n*-octadecanoic and *n*-hexadecanoic acids in the blank Kraft lignin/non-inoculated samples thus questioning their relation to the lignin metabolism [321] [125][322]. These ubiquitous fatty acids may instead become entrained/covalently bound to lignin during its processing or produced as a result of microbial contamination.

The GC detection of guaiacol, phenol and acetic acid in non-inoculated lignin samples could be attributed to either a chemical oxidation of lignin due to aeration and agitation [321] [322] or partial degradation caused during alkaline pulping and lignin isolation step. Raj and coworkers also identified propanoic acid, oxalic acid as well as butyric acid in non-inoculated samples, indicating that the anaerobic and aerobic treatments conducted in the industrial process could lead to a partial degradation of Kraft lignin [321][323]. This potential chemical artifact revealed by GC analyses becomes even more pronounced if the medium containing suspended lignin is sterilized prior to inoculation thus leading to a potential biochemical artifact in growth substrate identification as shown in the next paragraph.

Processing of blank samples varied among the available studies on microbial lignin degradation. Some reports used an unsterilized mineral medium with the addition of industrial lignin [125] . Other studies used sterilized whole samples (including lignin) with an adequate comparison with the corresponding blanks [321][318] [108][116]. For black liquor degradation, some researchers used the original black liquor as blank

samples [131][324][161] whereas others considered the *sterilized* black liquor as blank samples [148][325].

In several studies, the presence of lignin related compounds in the non-inoculated samples was attributed to lignin oxidation due to the aeration/agitation and minor degradation during its industrial processing [117][116][307]. However, if Kraft lignin is thermally sterilized in an autoclave before inoculation, the presence of low molecular phenolics in the non-inoculated sample could partially be attributed to the release of thermal degradation products produced under elevated temperature and pressure. Therefore, microorganisms may sustain their growth upon utilizing readily available lignin related compounds in the medium instead of using lignin as a sole carbon and energy source. GC-MS analyses of liginosulfonate partially degraded by a bacterial strain, *Sphingobacterium sp.* HY-H, revealed that low molecular weight aromatic compounds in the non-inoculated sample such as vanillin, 2-methylbenzoic and benzoic acids, disappeared after 2 days of degradation indicating their microbial consumption [307].

Although GC-MS provides the chemical characterization of individual species of lignin degraded compounds, this method is limited to the analysis of volatile compounds, in which case only monomers and dimers among lignin degraded compounds can be detected, thus leaving out higher-MW oligomers [319][320]. In addition, samples for GC-MS analysis are typically extracted by organic solvents. The reason for water to be a “non preferred” solvent includes a large vapor expansion volume easily causing backflash, poor wettability with high surface energy, poor solubility in many stationary phases, potential damage to the stationary phase and the likelihood of extinguishing the flame of a flame ionization detector [326].

Therefore, the product partitioning into the organic phase becomes an additional biasing factor: Only the hydrophobic fraction of phenolics is actually analyzed in their original amounts while partially leaving out hydrophilic compounds which may remain in the aqueous solution. Thus, GC analysis has to be combined with methods characterizing the whole sample and the fraction of GC-analyzed products should be reported with respect to the whole sample.

A potential limitation of GC analysis is that some polar analytes may significantly interact with stationary phase, resulting in such a pronounced tailing of the corresponding GC peaks that they may become indistinguishable from the baseline [327]. Even though this issue is less pronounced for phenols than for alcohols and carboxylic acids [327], the problem is that most of similar analytes may be readily detectable while several others may become “invisible” due to this tailing effect. This problem, though, is readily addressed by the polar analyte derivatization [327]. One other problem of GC-MS is that the MS detectors have different, often unpredictable responses to each individual analyte. Thus, calibration by standards of each detected compound is essential [328][329]. A remedy to this problem is the combination of MS and flame ionization (FID) detection by splitting the carrier gas flow after the column: While the former is used for identification, the latter is used for quantification. The “trick” is that the FID signal is proportional to the number of hydrogen and carbon atoms, so the calibration can be conducted for only selected analytes with an extrapolation to their homologs and analogs [329].

#### ***2.10.2.2 Pyrolysis/gas chromatography/mass spectrometry Pyr-GC-MS***

Pyr-GC-MS is another technique that is widely employed in characterization of lignin degradation products thus helping with elucidation of process mechanisms,

especially among biomass degradation studies [308] [330] [331][332] [333]. As stated in the previous section, the high molecular weight fractions of products are left out in GC analyses. Combining prior pyrolysis with the subsequent GC-MS analyte detection thus expands the analysis capability of GC-MS. In an inert atmosphere, the samples are heated for a few seconds at certain temperatures in a range of 500-1000 °C [334] Therefore no time-consuming sample preparation is required since all compounds end up in the gas phase.

However, most studies using Pyr-GC-MS used only one temperature step, which in many cases cannot distinguish the products of polymer/oligomer pyrolysis from monomers. The usage of multiple temperatures may provide additional mechanistic information. Multiple temperature steps could be employed to accurately characterize the lignin degradation products and to gain insights into the mechanism of specific microbial treatments used. Beranek et al. reported that the sequential use of relatively low (<400 °C ) and then much higher temperatures (e.g., 700 °C) may separate the physically adsorbed low-MW impurities and pyrolytically generated products of lignin degradation [335]. Because of this additional thermal desorption (TD) step, this method should be called TD-Pyr-GC-MS. Brzonova et al., applied the temperatures of 350, 450 and 700 °C and observed that the majority of phenolic products of kenaf degradation eluted in the 450 °C fraction. Subsequent GC analysis of supernatants confirmed that phenolics eluting at 450 °C were indeed microbial lignin degradation products rather than those of thermal decomposition of non-degraded lignin [336].

With sulfur-containing lignin, Li et al. reported a three temperature step sequence suitable for the pyrolysis of lignosulfonate (30–190°C, 190–360°C 360 -650 °C).

Aromatics were also released from 430-450°C [337]. Pyrolysis of the enzymatic hydrolysis lignin from corn stover (sulfur-free lignin) at 350°C led to the release of acids and alcohols due to a side chain cleavage while aromatic compounds were obtained at temperatures  $\geq 450^\circ\text{C}$  [338]. Sulfate present in Kraft lignin was reported to act as an oxidizing agent, as evidenced by the elevated release of carbon dioxide at higher temperature steps of pyrolysis (498-690°C) as opposed to lower temperature step where the release of CO<sub>2</sub> is related to the presence of carboxylic groups [339]. Sulfur dioxide and furan compounds are pyrolysis products of lignosulfonate while monochlorinated 2-methoxyphenols is the main pyrolysis product of chlorolignin from bleaching effluents [338][340][341]. It was also found that the use of either lignosulfonic acid or its calcium/sodium salts can affect the pyrolysis process by altering the product elution profile [341].

Pyr-GC-MS may not only provide sufficient information on identification of degraded compounds but may also help elucidate the degradation mechanisms related to specific microorganisms. Biological treatment of milled wood lignin with *Phanerochaete chrysosporium* and *Ganoderma austral* reported a preferential degradation of syringyl units and it is believed to be a result of syringyl unit connection to the backbone via ether linkages at C-4 rather than C-C linkages [342]. In a study of laccase-mediated biodelignification of *Eucalyptus globulus* (hardwood) Kraft pulp, the Pyr-GC-MS analysis of degradation products at 600 °C indicated that syringyl units are attacked preferentially during biobleaching and pulping with a decrease in the syringyl/guaiacyl (S/G) ratio [333]. In addition, the degradation of softwood (*Pinus pinaster*) and hardwood (*Eucalyptus globulus*) by laccase from *Trametes versicolor* with

violuric acid as a mediator resulted in the formation of ketones and reduction of the amounts of coniferaldehyde and synapaldehyde as pyrolysis products. Since coniferaldehyde and sinapaldehyde are formed through thermal depolymerization and dehydration originating from  $\beta$ -O-4 linked guaiacyl or syringylpropanol units, the presence of both compounds indicates unaltered lignin. Therefore, fungal lignin degradation is confirmed by the alteration of their ratio in the sample through Pyr-GC-MS [343].

## 2.11 Conclusion

During lignocellulosic biomass degradation, lignin is decomposed along with cellulose and hemicellulose. With industrial lignin being the sole substrate, limitations may be imposed on its efficient microbial degradation process, however the low specificity of externally excreted enzymes ameliorates this problem. The results obtained so far on induction of ligninolytic enzymes are highly dependent on specific strains and cultivation conditions, although significant induction has been successfully demonstrated in numerous studies. However, more research is needed to obtain a sufficient induction of ligninolytic enzymes when sulfur-containing lignins are used as base medium. Microbial treatment of industrial lignins to produce valuable chemicals is challenging due to the harsh conditions within the effluents, which is often detrimental to normal growth of microorganisms. For both fungi and bacteria, long treatment periods is a major issue in applying microbial degradation at an industrial scale, although an efficient degradation of lignin was reported within just one day of treatment. The issue concerns the accurate elucidation of the extent of microbial lignin degradation and characterization of degradation products. A reliable and standardized measurement of the extent of lignin

degradation is essential for making studies on microbial degradation comparable among each other.

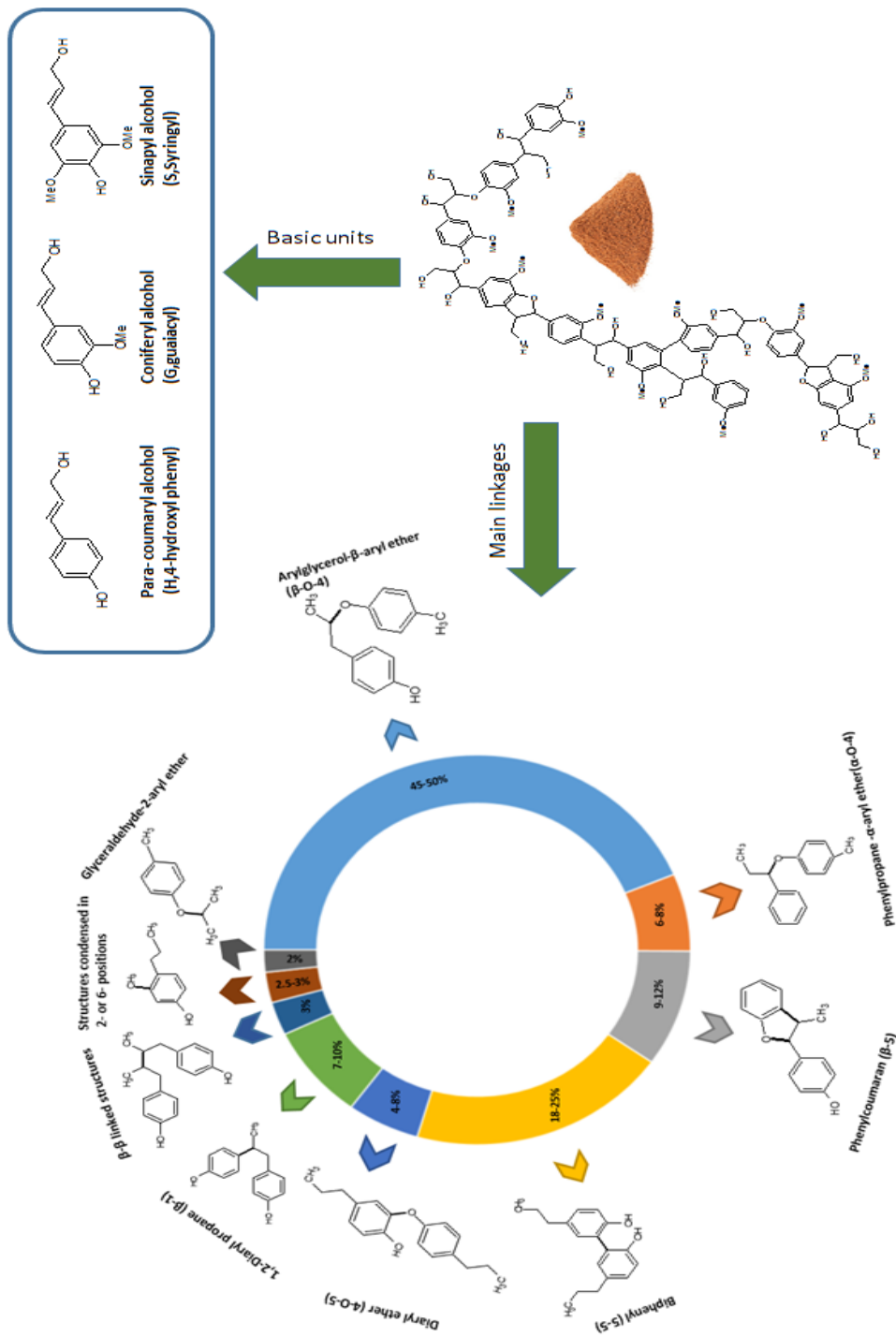


Figure 1. Representative linkages present in lignin and basic structural units found in lignin. Adapted from [295]



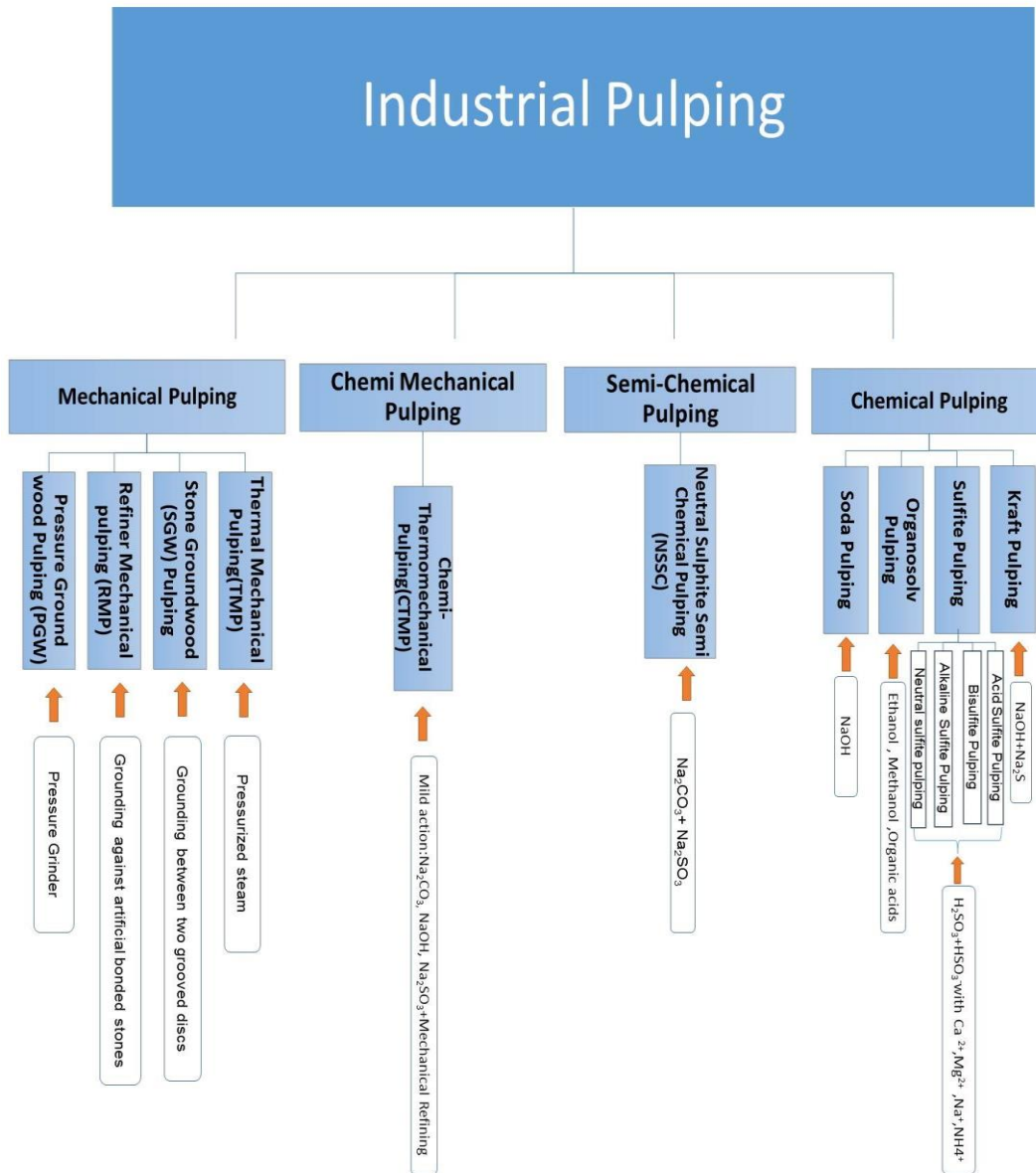


Figure 2. Summary of common pulping processes. Adapted from [46]

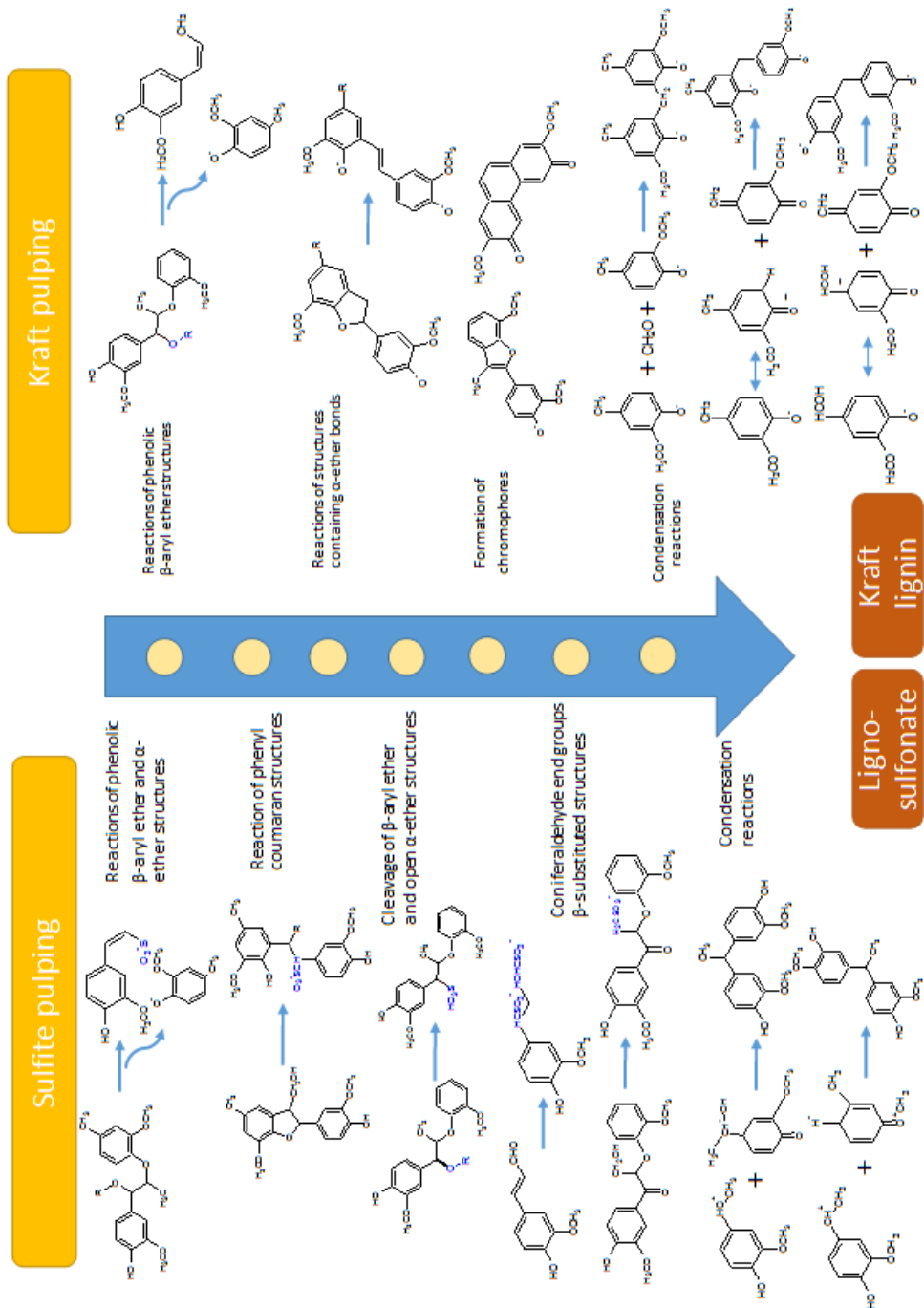


Figure 3. Summary of common lignin reactions during Kraft and sulfite pulping. Adapted from [295]

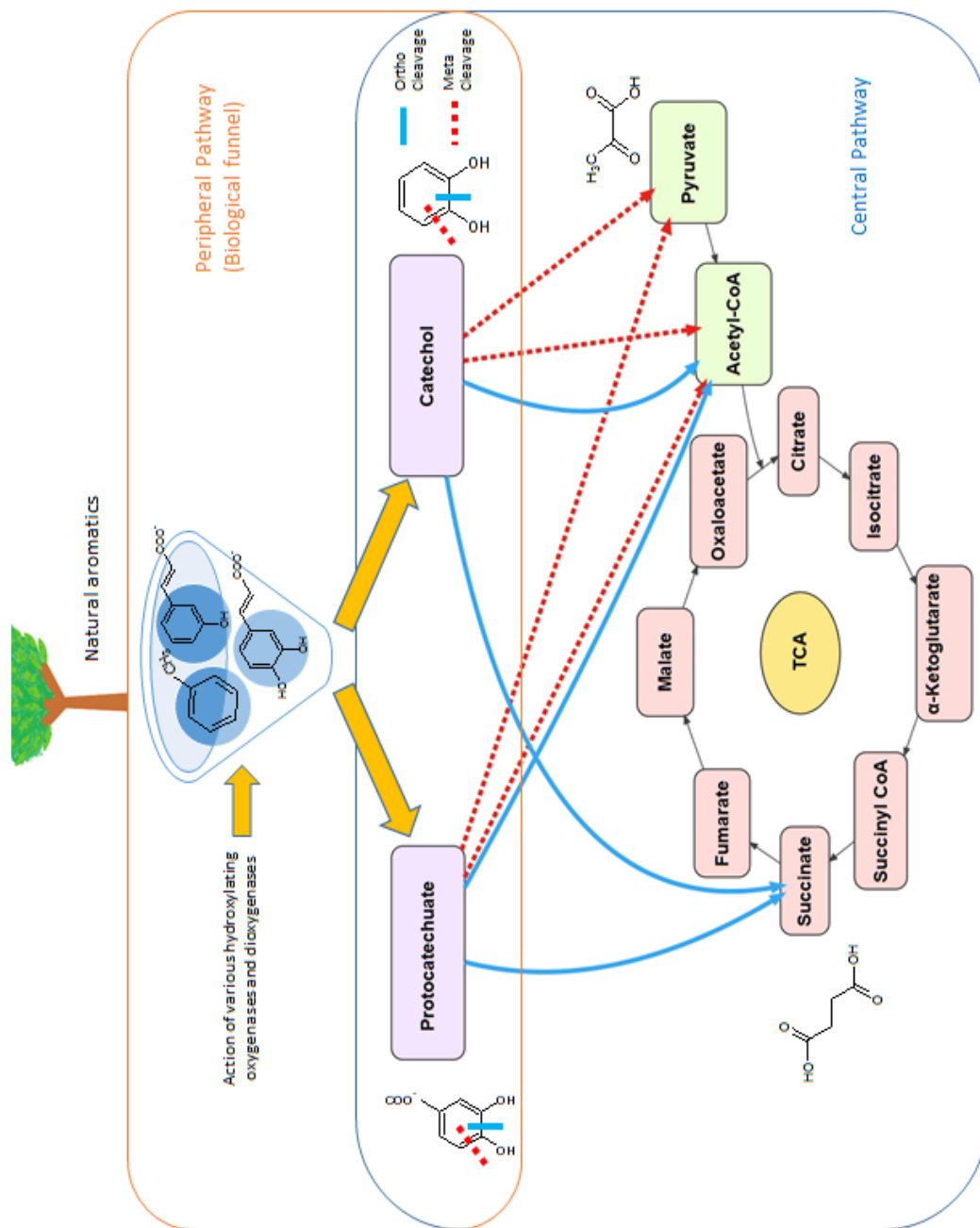


Figure 4. Aerobic pathway involved in microbial degradation of natural aromatic compounds. Adapted from [91][92]

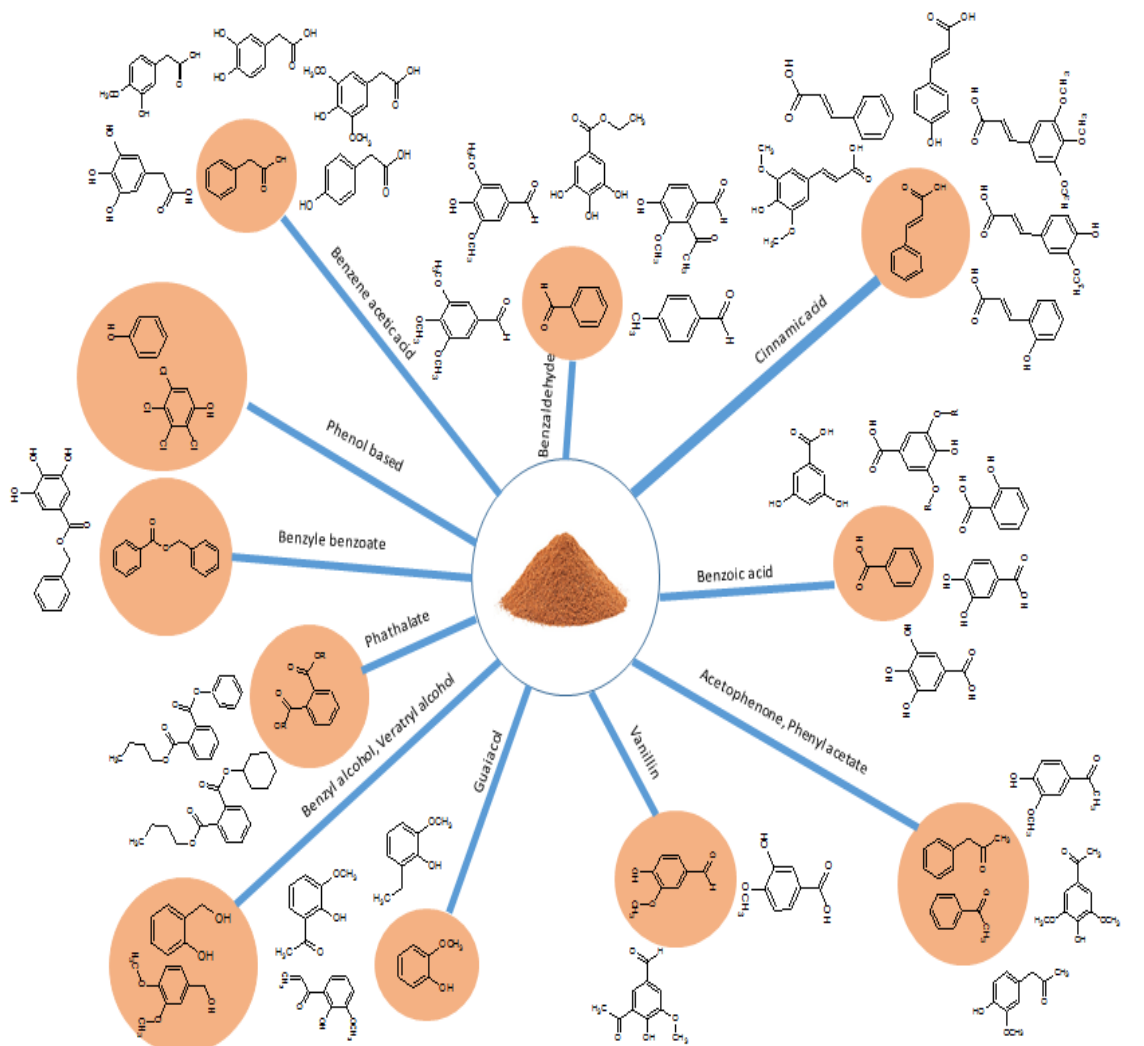


Figure 5. Common lignin degraded products detected through GC-MS in previous studies.

Adapted from [47][117][321][101][318][319][116][108][320][322]

Table 1. Summary of previous studies on microbial degradation of natural lignin in various biomass sources.

Biomass	Fungi	Degradation day	Lignin degradation	Reference
Wheat straw	<i>D. squalens</i>	14	34%	[32]
	<i>P. ostreatus</i>	14	7.1%	
	<i>P. eryngii</i>	14	14.5%	
	<i>L.betulinus</i>	14	28.3%	
	<i>F.pinicola</i>	14	32%	
Japanese beech and cedar wood	<i>T.versicolor</i>	14	20.9%	[33]
	<i>C. subvermispora</i>	84	20 %	
Eucalyptus grandis wood	<i>Ceriporiopsis subvermispora</i>	90	27%	[34]
	<i>Punctularia sp. 1 TUFC20056</i>	84	53.3	[35]
<i>unidentifiedTUFC20057</i>	84	53%		
Bamboo culms	<i>Exidiopsis sublivida TUFC20068</i>	84	30.8%	
	<i>E. sublivida TUFC20069</i>	84	28.2%	
	<i>Dictyopanus sp. TUFC20090</i>	84	27.3%	
Rye straw	<i>Resinicium friabile TUFC20062</i>	84	28.6%	[36]
	<i>Galactomyces geotrichum</i>	60	48%	
	<i>Myrothecium verrucaria</i>	60	42%	
Rice straw	<i>P. chrysosporium</i>	10	28.3%	[37]
	<i>F. moniliforme 821</i>	10	34.7%	
	<i>Fusarium sp.89</i>	10	33.5%	
Bamboo culms	<i>Fusarium sp. 82</i>	10	29.3%	[38]
	<i>T. versicolor G20</i>	120	12%	
	<i>E. taxodii 2538</i>	120	29.14%	
Biomass	Bacteria	Degradation day	Lignin degradation	Reference
Spruce	<i>S. viridosporus T7A</i>	84	30.9%	[39]
Maple	<i>S. viridosporus T7A</i>	84	32%	
Grass	<i>S. viridosporus T7A</i>	84	44.2%	
Spruce	<i>S. setonii 75Vi2</i>	84	34.1%	
Maple	<i>S. setonii 75Vi2</i>	84	29.5%	
Grass	<i>S. setonii 75Vi2</i>	84	39%	
Corn Stover	<i>I. lacteus</i>	28	25.48%	[40]
Rice straw	<i>Bacillus sp. CS-1</i>	10	20%	[41]
Rice straw	Lactic acid bacteria	10	14.3%	
Rice straw	Two step bacillus-lactic acid	10	61.9%	[42]
Corn Stover	<i>S. viridosporuis</i>	42	68.5%	
Rice straw	Microbial consortium XDC-2	12	30.2%	[43]
Wheat straw	Microbial consortium XDC-2	12	40.8%	
Corn stalk	Microbial consortium XDC-2	12	9.6%	

Table 2. Summary of previous studies on fungal degradation of purified lignin

Substrate	lignin content	C source	N source	Fungal strains	Day	Degradation	Analysis
Lignosulfonate	5g/L	-	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> 5g/L	<i>P. sulphureus</i> & <i>Phellinus</i> sp. <i>Phellinus</i> sp. & <i>B. pumilus</i> <i>P. sulphureus</i> & <i>B. pumilus</i>	10	36% 36.52% 36.65%	*
Alkaline non phenolic lignin 520mg/30ml +2g oftwood pulp Alkaline phenolic lignin 380mg/30ml +2g 380mg+2gsoftwood pulp	520mg/30ml +2g 380mg/30ml +2g	Glucose3g/L	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 0.317g/L	<i>Pycnoporus cinnabarinus</i>	90	43.5%	Gravimetric
Alkaline lignin	2g/L	Glucose7g/L	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 2g/L	<i>Cladosporium</i> Bio-1	10	35%	*
Alkali lignin	2g/L	Glucose 10g/L	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 0.5g/L	<i>Aspergillus</i> sp. F-3	8	65%	*
Alkaline lignin	6.7g/L	Glucose 400 ppm	-	<i>Hymenoscyphus ericae</i>	60	42%	Cumulative <sup>14</sup> CO <sub>2</sub> %
Kraft pine lignin	1mg/ml	Glucose 5.5mM	NH <sub>4</sub> NO <sub>3</sub> 25.6mM	<i>P. chrysogenum</i>	30	16.5% 8.7%	* Klason
Kraft lignin	5g/L	Glucose 55.5mM	NH <sub>4</sub> NO <sub>3</sub> 25.6mM	<i>Fusarium proliferatum</i>	30	19.6 % 15.9 14.4 %	* * Klason lignin
Polymeric organosolv lignin	5g/L	Glucose 55.5mM	NH <sub>4</sub> NO <sub>3</sub> 25.6mM	<i>Fusarium proliferatum</i>		14.3 % 4.3% 10%	* * Klason lignin
<sup>14</sup> C-labled lignin	50,000dpm	1% glucose	1mM Ammonium tartrate	<i>Ceriporiopsis subvermispora</i>	29	~30%	Cumulative <sup>14</sup> CO <sub>2</sub> %
Kraft lignin	1g/L	1g/L Glucose	Ammonium tartrate 0.22g/L	<i>Phanerochaete chrysosporium</i>	7	90%	*

\*-Spectroscopic measurement of lignin characteristic band.

Table3. Summary of previous studies on bacterial degradation of purified lignin.

Substrate type	Substrate amount	Bacterial strain	Carbon source	Nitrogen source	Degradation day	Degradation	Analysis	Reference
Kraft Indulin lignin	0.5wt/vol	<i>S. viridosporus</i> <i>S. badius</i>	-	Yeast extract 0.6%	35	3-4%	*	[118]
Softwood Kraft lignin	0.4g/L	<i>Azotobacter</i> sp. HM121.	-	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 0.2g/L	3	27.4%	*	[119]
Hardwood Kraft lignin						41.7%	*	
Kraft lignin	1.2g/L	<i>Acetoanaerobium</i> sp. WJDL-Y2	-	NH <sub>4</sub> Cl 1 g/L	20	24.9% on a COD basis	COD	[120]
Indulin AT	0.1%	<i>Autochthonous Streptomyces</i>	-	Yeast Extract 0.1g/L	10	99%	*	[121]
Kraft Indulin lignin	0.05%	<i>Serratia marcescens</i>	Mannitol0.5% (w/v)	-	1	12%	*	[122]
Kraft lignin	3g/L	<i>Comamonas</i> sp. B-9	-	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 2g/L	7	45%	*	[47]
Kraft lignin	500mg/L	<i>Bacillus</i> sp. ITRC S6,S7 and S8	Glucose 1% (w/v)	Peptone 0.5% w/v	6	40%	*	[48]
Kraft lignin	1 g/L	<i>Aeromonas</i> sp.	-	Yeast Extract 0.01%	5	98%	*	[123]
Alkali lignin	0.05g-2g/L	<i>Bacillus</i> sp.	Glucose 1.0% (w/v)	Yeast extract 0.018% (w/v)	2	61%	*	[41]
Lignin (MW28000)	200mg/L	<i>Bacillus</i> sp. <i>Pseudomonas putida</i> (Pp) Fusants (PE-9) & (Xz6-1)	- Sucrose2g/L	NH <sub>4</sub> Cl 0.5g/L	7	91.2% 96%	* *	[124]
Kraft lignin	600ppm(w/v)	<i>Citrobacter freundii</i> <i>Citrobacter</i> sp. Mixed culture	Glucose 10g/L	Peptone 3g/L	6	49% 54% 62%	*	[125]
Kraft lignin	5g/L	<i>Pandoraea</i> sp	-	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 0.5g/L	5	39.9%	*	[126]
Alkali lignin	2g/L	<i>Streptomyces</i> spp. strains F-6	Glucose 10g/L	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 0.5g/L	12	37.6%	*	[127]
Alkali lignin		<i>Streptomyces</i> spp. strains F-7				38.1%	*	
Alkali lignin		<i>Pleurotus ostreatus</i> strain G5				6%	*	
Kraft lignin	3g/L	<i>Pandoraea</i> sp. B-6	-	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 2g/L	7	46.5%	Color Removal	[117]
Alkali lignin	0.1%	<i>Bacillus</i> sp. EU978470	-	-	6	81.4%	*	[128]

\*-Spectroscopic measurement of lignin characteristic band

Table 4. Summary of previous studies on microbial degradation of paper mill effluent

Substrate	Lignin content	Microorganisms	C source	N source	Day	Deg. %	Analysis	Reference
Effluent from pulp and paper mill	218mg/l	<i>Serratia liquefaciens</i>	glucose (1.0%, w/v)	peptone (0.5%, w/v)	6	58%	Pearl and Benson	[155]
Effluent from a bleached Kraft pulp mill	5.071mg/L	<i>Pleurotus sajor caju</i> <i>P. chrysosporium</i> <i>T. versicolor</i> or <i>Rhizopus oryzae</i>	Glucose 2g/L Glucose 1g/L	0.15g NH <sub>4</sub> Cl	10	25%-46%	*	[156]
Effluent from the first alkaline extractions stage	392-432mg/L	<i>Ceriporiopsis subvermispora</i>	Glucose 1g/L	NH <sub>4</sub> Cl 0.15g/L	2	62%	Modified Klason lignin	[145]
Black liquor	0.92g/l	<i>Phanerochaete chrysosporium</i> <i>Pleurotus ostreatus</i> <i>Lentinus edodes</i> <i>Trametes versicolor</i> White rot fungus S22	Glucose 1g/L	Ammonium tartrate 0.2g/L	16	60% 54% 65% 49% 60%	*	[150]
Effluent generated at the pulping stag	163,741ppm	<i>Emericella nidulans</i> var. <i>nidulans</i>	Dextrose 0.25%	Tryptone 0.1%	1	37%	Pearl Benson	[157]
Kraft pulping liquor	163,741mg/L	<i>Cryptococcus</i> sp.	Dextrose (1.0% w/v)	Tryptone (0.1% w/v)	1	35-40%	Pearl Benson	[132]
Kraft pulp bleached effluents	6716 ppm	<i>Paecilomyces varioti</i> <i>Paecilomyces</i> sp <i>Phoma</i> sp.	-	Fe(CH <sub>3</sub> COO) <sub>3</sub> NH <sub>4</sub> , 0.01g/L	7	56 % 64 % 56 %	Pearl Benson	[158]
Effluent from pulp and paper mill	436+-18mg/L	<i>Paenibacillus</i> sp.	Glucose 0.5% w/v	Peptone 0.25% w/v	6	54%	Pearl Benson	[159]
Effluent from Kraft pulp bleaching	126mg/L	Enterobacter sp.	-	5mM(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .2H <sub>2</sub> O	-	73%	Modified Pearl Benson	[160]
Black liquor	15.5g/l	<i>Comamonas</i> sp. B-9	-	-	10	43.5%	*	[161]
Black liquor	139.41–154.97g/L diluted to 10% BL v/v	<i>Pandoraea</i> B-6, <i>Comamonas</i> B-9 <i>Aspergillus</i> F-1	-	-	7	53.2%	*	[162]
Effluent from pulp and paper mill	4789.00 ± 89.20ppm	<i>Aspergillus fumigatus</i> <i>Pseudomonas ovalis</i>	0.2% sucrose	Urea 0.1%	7	78% 68%	Pearl and Benson	[163]
Pulp and paper mill effluent	6380.56 mg/l	<i>Paecilomyces</i> sp.  <i>Pseudomonas syringae</i> pv <i>myricae</i>	Dextrose 0.1%	Urea 0.02%	7	76.5%	Pearl and Benson	[164]
Pulp and paper mill effluent	-	<i>Microbrevis luteum</i> .	Sucrose0.2%	Urea0.1%	7	69%	Pearl and Benson	[165]
Kraft paper mill effluent	800+-18.4mg/L	<i>Paecilomyces</i> sp. <i>Bacillus subtilis</i> (GU193980) and <i>Klebsiella pneumoniae</i> (GU193981)	Glucose(1%,w/V)	Peptone(0.25%,w/V)	6	86% 58%	*	[166]

\*-Spectroscopic measurement of lignin characteristic band



Table 5. Summary of previous studies on ligninolytic enzyme induction.

Strain	Inducer	Laccase Induction			Cultivation period/day	Reference
		Induction	C&N source	Enzyme activity		
<i>Ganoderma lucidum</i>	tamarind shell plus ethanol 3% (v/v) CuSO <sub>4</sub> 0.4mM Gallic acid 1mM	416 fold	40g/L glucose NH <sub>4</sub> CL 1g/L -	74.84U/mL	15	[251]
<i>Trametes versicolor</i>	40g/L ethanol	24 fold	Glucose 40g/L NH <sub>4</sub> Cl 1g/L	2.6 U/ml	7	[210]
<i>Pycnoporus cinnabarinus ss3</i>	20g/L methanol 20g/L ethanol	5-8.5fold	Sugar cane bagasse	53U/g <sub>ds</sub> 71U/g <sub>ds</sub>	10	[247]
<i>Phanerochaete chrysosporium</i>	10g/L ethanol	4.7 fold	Malt extract 20g/L 20g/L Glucose 1 g/L peptone	92.1U/mg	4	[246]
<i>T. trogii Berk S0301</i>	Cu 1.5mM Tween (80) 7g/L	34.3 fold 5.6 fold	Malachite green 15 mg/L	30.9U/mL 68 U/mL	15	[280]
<i>Paraconiothyrium variabile</i>	Peptone, 2.2 g/L, CuSO <sub>4</sub> , 0.03 g/L, and xyloidine 1.29 mM	17 fold	Glucose 10g/L	16,678 U/L,	12	[281]
<i>G. applanatum</i>	0.5mM Cu <sup>2+</sup>	49.2 fold	12.7 g/L malt extract and 5g/L corn steep liquor	18,830U/g	14	[282]
<i>Peniophora sp.</i>		19.7 fold		11,462U/g		
<i>P. sanguineus</i>		27.7 fold		27,132U/g		
<i>C. versicolor f. antarcticus</i>		7.6 fold		13,304U/g		
<i>Lentinus polychrous</i>	1mM CuSO <sub>4</sub>	300 fold	12.5g/L Glucose 1.25g/L yeast extract	350U/L	14	[283]
	0.3mM CuSO <sub>4</sub>	~2000 fold	20g/L Glucose 5g/L yeast extract 5g/L peptone	2000U/L	14	
<i>Coriolus versicolor</i>	1 mM CuSO <sub>4</sub>	1.5 fold	Glucose 20g/L asparagine 2.5 g/l DL-phenylalanine 0.15 g/l	460U/mL	10	[284]
<i>Trametes versicolor ATCC 32745</i>	1mM CuSO <sub>4</sub>	310 fold	10g/L Glucose Yeast extract 2.5g/L	38.5U/mL	10	[228]
<i>Phlebia radiata</i>	1.5 mM CuSO <sub>4</sub>	20 fold	0.5 % (wt/vol) peptone , 0.2 % (wt/vol) yeast extract, and 0.1 % (wt/vol) (5.6 mM) glucose	22 µkat /l	14	[285]
<i>Trametes versicolor</i>	20g/L corn steep liquor	~2 folds	Glucose 2g/L	633.3U/L	7	[180]

Table 5. cont.

<i>Pestalotiopsis</i> sp. <i>J63</i>	0.09 mmol/L phenol	2.7 fold	8 g/L ammonium sulfate, 3 g-/Lmaltose and 20 g/L rice straw.	5089U/L	13	[286]
<i>P. variabile</i>	Guaiacol 250uM	2.8fold	sabouraud-2%-dextrose broth	835U/L	14	[287]
<i>Stereum ostrea</i>	Veratryl alcohol (0.02%)	1.9 fold	5g wheat bran +70% w/v Koroljova medium	32675U/g dry substrate	12	[220]
	Guaiacol(0.02%)	1.72 fold		29583U/g dry substrate		
<i>Coprinopsis cinerea</i>	2.5%(v/v) <i>Gongronella</i> sp.	900 fold	15g sucrose, 1.5g DL-Asn	1800 ± 119 U/L	10	[257]
<i>C. comatus</i>	<i>T. reesei</i>	2.6 fold	corn cob 5g/100ml , corn stover 2g/100ml, wheat bran 2g/100ml, (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 0.2g/100ml, Urea 0.05 g/100ml	2180U/mL	7	[258]
<i>Phanerochaete chrysosporium</i>	<i>Trametes versicolor</i>	2 fold	Half potato dextrose agar	0.81U/g	8	[256]
<i>T.versicolor</i>	Ultrasound	1.8 fold	Glucose 2g/L NH <sub>4</sub> CL 0.27g/L	588.9U/L	7	[288]
MnP Induction						
<i>Stereum ostrea</i>	Guaiacol 0.02%	1.4 fold	5g wheat bran +70% w/v Koroljova medium	5984U/g dry substrate	12	[220]
	Lignosulfonic acid 0.1% conc.	1.4 fold		5861U/g dry substrate		
<i>Lentinula edodes</i>	Sawdust extract 1g/30mL	~60 fold	2.5 g/L malt extract; 1.0 g/L yeast extract; 1.0 g/L peptone; 5 g/L glucose	60U/mL	50	[289]
White rot fungus strain L-25	Potato-processing wastewater	2.5 fold	20 g/l glucose and 4 g/l potato infusion	7.7U/mL	15	[290]
<i>Phanerochaete chrysosporium</i>	0.01 mmol/L S-adenosyl- methionine	4 fold	2% mannose	8.77 ± 0.23 U/mL	8	[291]
Lip Induction						
<i>Phlebia radiata</i>	3 g (dry weight) milled alder	~28 fold	2mM Asparagine	550 nkat/L	32	[285]
<i>Stereum ostrea</i>	Lignin 0.1%	1.49 fold	5g wheat bran +70% w/v Koroljova medium	318.7U/g dry substrate	12	[220]

## CHAPTER III

### BIODEGRADATION OF LIGNIN BY FUNGI, BACTERIA AND LACCASES

#### 3.1 Introduction

Lignin, a naturally occurring aromatic polymer, is one of the building blocks of lignocellulosic biomass providing structural integrity to plant cell walls [1]. One of the most abundantly produced forms of this biopolymer is Kraft lignin (KL) obtained through an acid precipitation of black liquor, which is generated as a by-product of the alkaline sulfide treatment of lignocellulose characteristic for the paper mill industry [2]. During this process, lignin undergoes structural modifications including the formation of new alkali-stable linkages and various degradation and condensation reactions [3]. Currently, commercial development of lignin based products, such as adhesives and dispersants, accounts only for 2% of the original lignin, with the rest of it mainly contributing to power generation via combustion[4]. Thus, there is a need to increase the commercial potential of industrial lignin.

White and brown rot fungi are well known for their essential role in naturally occurring degradation of lignocellulosic biomass, which is enabled by secretion of extracellular ligninolytic enzymes [5]. Although the lignin degradation potential of white

rot fungi continues being assessed, bacterial KL valorization has gained attention as well, due to a greater adaptability of bacteria to extreme conditions and faster growth rate. Yet compared to fungi, only a limited knowledge has been collected on the enzymology of bacterial lignin breakdown although some laccase and peroxidase activities have been reported [6].

Based on the available literature, efficient KL degradation was reported mainly using bacterial strains [7], as the performance of fungi is often unstable when exposed to harsh industrial treatment conditions [8]. Previous studies focused on the achievement of a significant KL biodegradation within a short period of cultivation and thus did not provide enough detail on the metabolic interactions between the applied microorganisms and lignin, in contrast to microbial degradation of lignocellulosic biomass where such interactions are well documented [9]. In our study, we have selected a longer cultivation time in an effort to gain insights into the degradation pattern of industrial lignin when exposed to various microorganisms. Most of the previous studies used low KL loads thus causing an uncertainty with the scalability of the data obtained. Therefore in our study, a more industrially relevant lignin concentration of 13.3g/L has been used.

GC-MS has been the main method characterizing lignin degradation products. However, elucidation of the degradation processes cannot be fully achieved using just the GC-MS results, as only phenolic monomers and dimers are volatile enough to elute from GC columns. Pyr-GC-MS expands the application of GC-MS to phenolic oligomers by converting them to the gas phase through heating in an inert atmosphere. However, most studies investigating compositional and structural changes of lignin employed a single temperature pyrolytic step [10]. When characterizing lignin degradation products, this

method cannot distinguish between the volatile phenolic monomers/dimers formed through microbial treatment from the pyrolysis products obtained as a result of thermal degradation of phenolic oligomers. A recent study by Brzonova et al. successfully characterized lignin degradation products by using fractional TD-Pyr-GC-MS with three temperature steps to overcome this limitation [11].

The novelty of this study, besides using higher, industrially relevant lignin concentrations, is in the use of comprehensive analysis of lignin degradation products. First, while still using several traditional methods, e.g., measuring enzyme activities and monitoring lignin gravimetrically, we have expanded the application of fractional TD-Pyr-GC-MS to characterization of industrial lignin biodegradation products. As the second innovation, we used a non-chromatographic technique, thermal carbon analysis (TCA). TCA conducted with a thermal optical analyzer is currently applied for analysis of atmospheric aerosol samples [12] to fractionate carbonaceous aerosol particles into ambient air organic (i.e., volatilizable at varied temperatures) and elemental carbon (i.e., non-volatilizable and eluted only upon combustion). TCA and TD-Pyr-GC-MS complement each other, as the former yields a complete quantitative carbon evolution profile (including the elemental carbon not accounted for by TD-Pyr-GC-MS), thus allowing for mass balance closure on carbon among all fractions, whereas the latter provides a qualitative resolution on the level of chemical species.

The use of comprehensive methods of product characterization has allowed us to conduct a direct comparison of fungi, bacteria and purified enzymes (laccases) with respect to lignin degradation. The two fungal strains used, *Coriolus versicolor* (CV) and *Trametes gallica* (TG), are white rot fungi previously applied successfully to black liquor

degradation [13]. Commercial laccases from two similar sources, *Coriolus versicolor* and *Trametes versicolor*, were used separately as a control group for a comparative study of microbial lignin degradation. Among the two bacterial strains used, *Streptomyces sp.* (S) was previously successfully applied to KL degradation [14] [15] while *Microbacterium sp.* (B) was reported to degrade significantly both lignin and its intermediate biodegradation products, phenolics, during Kenaf biomass treatment [11] [16].

## **3.2 Materials and Methods**

### **3.2.1 Microorganisms and lignin**

Two basidiomycetous fungi, *Coriolus versicolor* (CV) and *Trametes gallica* (TG), and two bacterial strains, *Microbacterium sp.* (B) and *Streptomyces sp.* (S) were received from the Department of Biology of Lakehead University, Thunder Bay, Ontario, Canada. The cultures were maintained at 4 °C on agar plates mixed with kenaf provided by the Department of Plant Sciences, North Dakota State University (Fargo, North Dakota, USA). Indulin AT was purchased from MeadWestvaco. Elemental analysis of indulin AT was performed by Atlantic Microlab Inc. ( C% : 64.14, H% : 5.79, N% : 0.46, S% : 1.39). Commercial laccases from *Coriolus versicolor* (0.3U>mg) and *Trametes versicolor* (10U>mg) were purchased from Sigma-Aldrich.

### **3.2.2 Inoculum preparation and cultivation**

For inoculum preparation, both fungal and bacterial cultures were cultivated on agar plates supplemented with Kenaf at room temperature, a week prior to experiments. For laccase treatment, 0.3g of laccase powder was dissolved in 5mL of a buffer solution before experiments. The culture medium (pH 6.0) for microbial treatment contained

KH<sub>2</sub>PO<sub>4</sub> (1.5 g/L), MgSO<sub>4</sub> (0.5 g/L), NaCl (0.5g/L), CuSO<sub>4</sub> (0.2g/L) , ZnSO<sub>4</sub> (0.2g/L), sucrose (1.5 g/L) and FeSO<sub>4</sub> (0.1 g/L). The buffer solution (pH 6.0) for laccase treatments contained KH<sub>2</sub>PO<sub>4</sub> (1.5g/L), CuSO<sub>4</sub> (0.2g/L), ZnSO<sub>4</sub> (0.1g/L) and FeSO<sub>4</sub> (0.05g/L). 1.0 g of indulin AT was autoclaved at 121 °C for 30 min along with 75mL of the medium in Erlenmeyer flasks. After cooling down to room temperature, actively growing fungal colonies were selected and four 20mm × 20mm fragments from the growing edge of fungal cultures were used for inoculation under sterile conditions at room temperature. The bacterial seed cultures were incubated overnight and then 1mL of such a bacterial suspension with an OD 600 of 1.0 was used for inoculation.

For laccase treatments, a suspension containing 1.0 g of lignin in 75mL of the buffer solution was sterilized as described above followed by the addition of 0.5mL of two commercial laccase solutions. To ensure the continuous treatment by active laccase through 54 days, 0.5mL of laccase solutions were repeatedly added under sterile conditions on the days of sample harvesting. As the laccase treatment served merely as a control for comparison with live cultures, no mediators were added to the solution to increase the enzyme productivity. The other control samples included a microbial laccase blanks with just the corresponding media, without any inoculation or enzyme addition. All experiments were performed in triplicate. The incubation with lignin continued for 54 days at 31 °C under shaking at 100rpm. Samples were harvested on days 8, 13, 22, 30, 38, 46, 54 and then the supernatants were separated by centrifugation (1300rpm, 10min) and microfiltration for further analysis.

### **3.2.3 Determination of the residual lignin content**

After 54 days of incubation, the solid lignin portions were separated from the solutions by vacuum filtration. The residual lignin was dried and weighed gravimetrically, as w/w% with respect to the initial lignin.

### **3.2.4 Measurement of ligninolytic enzyme activities**

Selected ligninolytic enzyme activities were measured spectrophotometrically using a UV–Vis Evolution™ 600 Spectrophotometer (Thermo Fisher Scientific, Madison, WI). The laccase activity was recorded by monitoring absorption changes at 436nm reflecting the rate of oxidation of 1.0 mM 2,20-azino-bis-[3-ethyl- thiazoline-6-sulfonate] (ABTS) in 0.1M phosphate buffer (pH 6.0). The reaction mixture with a total volume of 1.0 mL contained the buffer, ABTS and sample. The MnP activity was measured by monitoring the oxidation of DMP (2,6-dimethoxyphenol) at 469nm. The LiP activity was determined by monitoring the oxidation of Azure B at 651nm. Reference blanks contained all components except the enzyme. One unit of enzyme activity was defined as the amount of enzyme that transformed 1.0  $\mu\text{mol}$  of substrate per minute.

### **3.2.5 Thermal carbon analysis**

Samples were analyzed using a Sunset Laboratory Thermal-Optical Carbon Aerosol Analyzer. 10 $\mu\text{L}$  of the supernatant sample were loaded directly onto a quartz-fiber filter with an area of 1.5 $\text{cm}^2$ . The quartz-fiber filters loaded with samples were dried at 40°C for 7 min for moisture removal. Solid samples were dried in an oven at 70 °C overnight and 5mg of the dried sample were transferred to a separate tube adding 1mL of deionized water. The resulting solution was vortexed for 5 min and then 10 $\mu\text{L}$  of a homogeneous sample were transferred to a quartz filter. Samples were subjected to a



stepwise temperature ramp including 200, 300, 400, 500 °C and to a maximum of 850 °C for 6min at each step under a flow of ultrapure (GC-grade) helium (15 cm<sup>3</sup>/min). The carbon evolving from the filter was converted to carbon dioxide in an oxidizing oven by a MnO<sub>2</sub> catalyst and then reduced to methane to be detected with a flame ionization detector (FID). After the ramp, the temperature was dropped down to 550 °C and oxygen (15 cm<sup>3</sup>/min) was flown into the sample with a final temperature being increased to 870 °C, so the leftover char was oxidized to CO<sub>2</sub>, which then evolved and was detected as methane as described above.

### **3.2.6 TD-Pyr-GC-MS**

Supernatants from the samples withdrawn from both the experimental runs and controls at the end of incubation, on day 54, were analyzed using a thermal desorption–pyrolysis unit. The CDS Analytical, Inc. (Oxford, PA, USA) 5000 series pyroprobe was run in a trap mode with Tenax-TATM trap sorbent connecting to an Agilent 7890 gas chromatograph (GC) equipped with an Agilent 5890C mass spectrometer. The 51-m long HP-5MS column used had a film thickness of 0.25 µm and an inner diameter of 0.25 mm. Prior to analysis by the pyroprobe, quartz tubes filled with quartz wool were cleaned at 1200 °C for 5 s and cooled down before the samples were introduced. After the quartz tube loaded with samples (5µL) was inserted into the pyroprobe, it was dried at 50 °C for 60s with a subsequent heating at 10 °C/s to the selected temperature where it was held isothermally for 30 s. The transfer line and valve oven temperatures were set to 300 and 320 °C, respectively. The trap temperature was 45 °C before heating to 300 °C for 2.5 min, while the pyroprobe was held at 300 °C for 2.5 min. Each sample was analyzed with sequential fractional heating at 150, 220, 320, 400 and 700 °C. Based on previous

experiments, 320 °C step served as a transition between thermal desorption and pyrolysis, with consistent and minimum appearance of peaks compared to all other temperature steps. The temperature ramp was set in a way that could have a complete separation of thermal desorption and pyrolysis.

### 3.3 Results and Discussion

#### 3.3.1 Ligninolytic enzyme activities

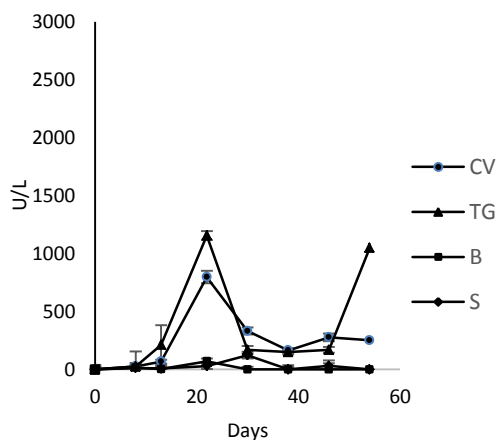


Figure 6-a

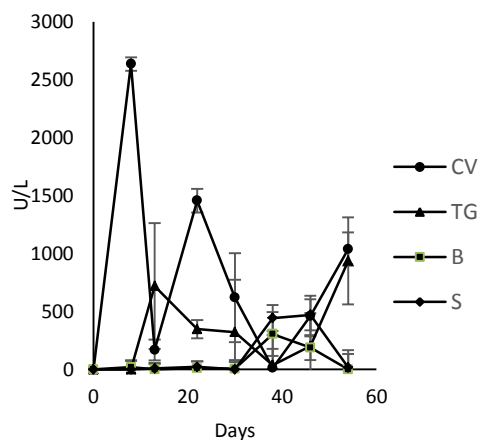


Figure6-b

Figure 6. Ligninolytic enzyme activity profiles over 54 days: Laccase (Figure1-a) , Manganese peroxidase(Figure1-b) The error bars shows variance of mean with one standard deviation obtained from triplicate samples

The profiles of ligninolytic enzyme activity are shown in Fig. 6 for all four strains used. The laccase activities of both fungal strains (CV and TG) turned out to be significantly higher than those of bacteria, reaching the maxima of  $(797\pm 17\text{U/L})$  and  $(1154\pm 172\text{U/L})$ , respectively. The TG and CV laccase activities followed slightly different time patterns, with the former reaching its peak value being higher than previously reported [13]. Predominant fungal laccase activity could be closely associated with lignin polymerization [17], which was confirmed through results obtained from TCA and TD-Pry-GC-MS. The *Streptomyces* bacterial strain did not exhibit a noticeable laccase activity in our study in contrast to the earlier study conducted by Yang et al. where other *Streptomyces* strains showed a significant laccase activity ( $935.4\text{U/g}$  dry cell weight) during the degradation of alkali lignin [14]. Apparently, this enzyme activity expression could be highly dependent on both the substrate and medium selection, which were varied between these two studies. The lack of significant laccase activity may explain the difference in the outcomes of fungal and bacterial treatments described in this study, in terms of the occurrence of cross-linking polymerization.

All four strains investigated showed a pronounced MnP activity throughout all 54 days of cultivation. CV had the highest MnP activity reaching  $1455\pm 101\text{U/L}$  on day 22 while TG had the smallest activity,  $937\pm 375\text{U/L}$ . Compared to fungi, bacteria showed a significant increase of MnP activity toward the second half of the degradation period reaching  $304\pm 189\text{U/L}$  and  $470\pm 134\text{U/L}$  on day 38 and day 46 for B and S, respectively. No LiP was detected among all four strains investigated. A similar pattern, with abundant laccase and MnP activities yet low or no LiP activity was reported among many white rot fungi when treating paper mill sludge and lignocellulosic biomass [18].

On the other hand, a significant LiP activity was reported for many bacterial *Streptomyces* strains [19]. Camarero et al. even claimed that there was no direct correlation between the observed enzyme activity and extent of lignin decomposition [20]. The differences observed between our study and previous reports indicate that lignin biodegradation might involve enzymes other than the three enzymes we investigated.

### 3.3.2 Gravimetric analysis

Table 6. Lignin weight loss in the supernatant after 54 days of treatment

	Lignin weight loss %
<i>C. versicolor</i>	45±8%
<i>T. gallica</i>	29±4%
<i>Mycobacterium sp.</i>	27±9%
<i>Streptomyces sp.</i>	22±12%
Control	25±17%
Laccase I	23±19%
Laccase II	27±20%
Laccase control	21±12%

Results of gravimetric analyses conducted at the end of treatments (day 54) are provided in Table 6. All microbial treatments showed significant differences with the blanks. The fungal treatment, especially with *C. versicolor*, yielded a greater mass decline than bacterial treatments. The greatest lignin weight loss was by the *C. versicolor* treatment reaching 45±8% after 54 days. By contrast, bacterial and laccase treatments only led to a slight decrease in the weight of the treated lignin, if any.

Higher lignin degradation efficiencies by *C. versicolor* (49%) and *Streptomyces* sp. (38%) were previously observed for paper mill effluents and alkali lignin within two weeks of incubation [21][14]. Among the earlier studies on KL microbial treatment, the

extent of lignin degradation was most often measured spectrophotometrically. The Kraft lignin concentrations used in previous reports were significantly lower than those used in this study, thus the spectrophotometric method was more suitable to assess the lignin loss than the gravimetric method. However, measuring the characteristic lignin band at 280nm can be skewed due to the interference imposed by other compounds absorbing at a similar wavelength, e.g., degraded carbohydrates [22]. Phenolics produced during lignin degradation also feature a strong absorption at 280nm [23]. Thus, uncertainty remains on whether the earlier published data reflected the true lignin degradation. For these reasons, the gravimetric method was selected for the use in this study, as higher lignin concentrations tend to increase its accuracy. In addition, the data obtained by gravimetric analyses were verified by using TCA. Gravimetric analysis has been used successfully to assess an industrial lignin biodegradation by a fungus, *Pycnoporus cinnabarinus*. After 90 days, 74% degradation was reported using a combination of alkaline lignin (520mg/30mL) and wood pulp (2g) as a substrate[24]. Thus, gravimetric measurements were conducted in this study for fraction analysis, being supplemented by carbon fractionation.

Yet, it should be noted that even correctly conducted gravimetric measurements do not distinguish either between lignin biodegradation and dissolution or between lignin depolymerization to lower molecular fragments and their ultimate catabolism to CO<sub>2</sub> and water. In this study, this task was accomplished using other methods, primarily TCA as shown in the subsequent sections.

### 3.3.3 Thermal carbon analysis: Carbon mass balance closure

#### 3.3.3.1 The solid product portions

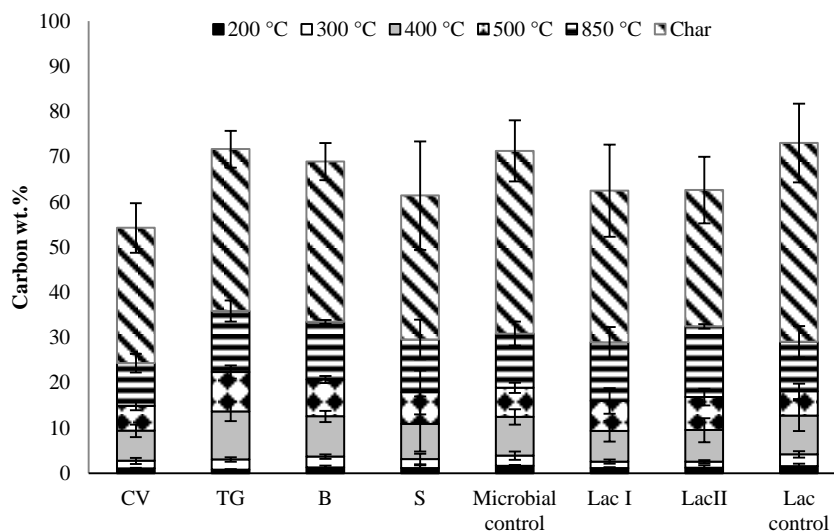


Figure 7-Thermal carbon elution profile of the solid (water-insoluble) lignin portion after 54 days of treatment. The error bars shows variance of mean with one standard deviation obtained from triplicate samples

Figure 7 shows the thermal carbon elution profile of the solid (water-insoluble) lignin portion after 54 days of treatment. The carbon elution profiles were similar for all solid fractions indicating that all (or most) of the changes resulting from treatments occur in the liquid phase: Once lignin is modified by the treatment, it (or the products of its degradation) dissolves.

Yet, a comparison of the *total* carbon in experimental samples and the corresponding controls showed relatively small but statistically significant changes, particularly, for the CV treatment. Furthermore, a decline in the non-volatilizable char precursors (the largest fraction shown on top of the bars in Fig. 7) was mainly accounted for by the total carbon decrease as a result of both microbial and laccase treatment. The

carbon wt.% representing the solid portion of lignin matches that obtained from the gravimetric analysis, Table 7. The match of the gravimetric measurements and total carbon in the TCA validated both of these methods.

These observations indicated significant modification of lignin samples compared to the blanks, including the partial dissolution and apparent biodegradation of the most recalcitrant lignin fraction (char precursors). Further insights into the mechanisms of bacterial and fungal action were obtained by analyzing the water soluble, supernatant portion of the reaction products.

### 3.3.3.2 The supernatant (water-soluble) portions

Table 7. Carbon weight % changes in the supernatant after 54 days of treatment.

	Carbon wt.% in solid (GA) <sup>a</sup>	Carbon wt.% in solid <sup>b</sup>	Carbon wt.% in the supernatant <sup>c</sup>	Total carbon wt.% <sup>d</sup>
<i>C. versicolor</i>	55±8%	54±11%	18±2%	72±8%
<i>T. gallica</i>	71±4%	72±11%	16±1%	88±14%
<i>Mycobacterium sp.</i>	73±9%	69±7%	33±1%	102±9%
<i>Streptomyces sp.</i>	78±12%	61±12%	36±2%	97±11%
Control	75±17%	71±13%	29±5%	100±15%
Laccase I	77±19%	72±17%	34±1%	106±17%
Laccase II	72±20%	63±12%	35±1%	98±12%
Laccase control	79±12%	73±4%	23±1%	96±17%

<sup>a</sup> Carbon wt.% in the solid based on gravimetric analysis

<sup>b</sup> Carbon wt.% in the solid sample on day 54 measured by TCA

<sup>c</sup> Carbon wt.% in the supernatant sample on day 54 measured by TCA

<sup>d</sup> Sum of the values for the solid portion and supernatant, reflecting mass balance closure

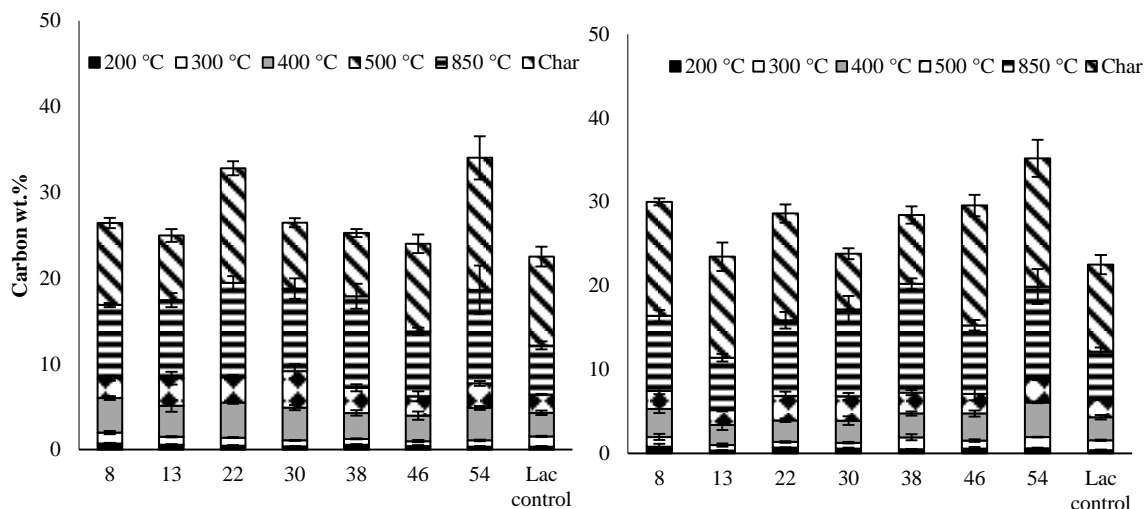


Figure8-a

Figure8-b

Figure 8. The thermal carbon elution profiles of the lignin samples in the supernatant treated with two commercial laccases: a) LacI, b) LacII. The error bars show variance of mean with one standard deviation obtained from triplicate samples.

The total amount of carbon present in the supernatants of control samples did not show any significant changes over 54 days. The elution profiles of laccase treated samples were similar to the blanks in both cases, with increases occurring only in the 850 °C and char fractions. Apparently, laccase produces some water-soluble higher-MW and mostly cross-linked products thus promoting lignin polymerization rather than depolymerization. The total lignin weight loss occurring at the end of experiments may be due to two different factors: 1) Conversion to dissolved or degraded lignin compounds in the supernatant and 2) Conversion to carbon dioxide through microbial metabolism of lignin. In the case of laccase treatments, an increase in the total carbon present in the supernatant was observed along with a corresponding decrease in the solid portion measured by TCA (Table 7, columns b and c), which, in turn, matched the decrease in carbon wt.% loss measured gravimetrically as shown in Table 7, column a. Lignin mineralization is expected to be insignificant for laccase treatments, just as observed.



The laccase treatment featured a suitable carbon mass balance closure, as expected, see Table 7, column d.

For fungally treated samples, carbon wt.% loss on day 54 in the solid portion measured by TCA matched the value obtained from gravimetric analysis. However, this observed decrease in carbon wt.% was not accompanied by a corresponding increase of carbon wt.% in the supernatant on day 54. Instead, there was a significant drop in carbon wt.% in the supernatants of fungally treated samples (Table 7, column c). In contrast to laccase, the thermal carbon elution profiles of the fungally treated lignin changed significantly over 54 days showing a transient decrease of the total carbon present in the supernatants of treated samples (Fig. 9). The total carbon mass balance of fungally treated samples closed only at  $72\pm 8\%$  and  $88\pm 14\%$  of the original carbon input (Table 7, column d). Thus, a significant portion of lignin must be metabolized into volatile products, i.e.,  $\text{CO}_2$ , at least by CV, as the corresponding gap in the carbon mass balance is statistically significant. A similar level of lignin mineralization to  $\text{CO}_2$  was reported when residual lignin in Kraft pulp was treated by *T. versicolor* [25].

The dynamics of fungal lignin treatment shown in Fig. 9 is noteworthy. A significant amount of carbon released at 200 °C and 300 °C in the microbial control is due to the sucrose added to the medium and release of low molecular phenolics during the lignin sterilization. Compared to this control, a significant decrease of the char fraction was observed after the first 8 days of the fungal treatment evidencing lignin breakdown. Surprisingly, the consumption of low molecular weight occurred rather slowly, until day 22-30, followed by an across-the-board consumption of any nutrients in the supernatant. Finally, toward the end of the 54-day incubation period, an increase

occurred in all fractions apparently signifying the dissolution of solid lignin. Delignification by white rot fungi showed similar results in an earlier study, with high molecular weight lignin degrading to yield intermediate molecular weight species while consuming the low molecular weight fraction [26].

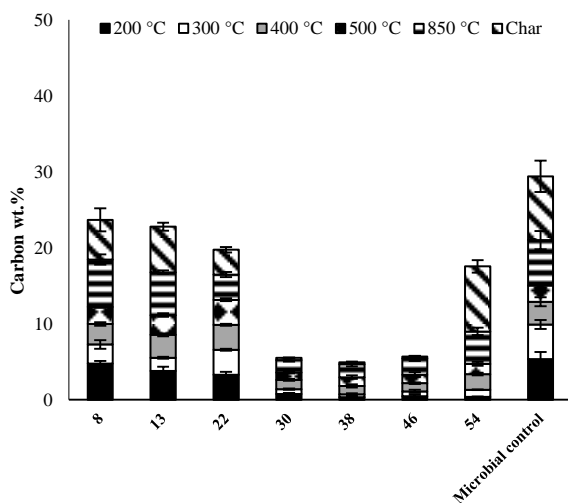


Figure 9-a

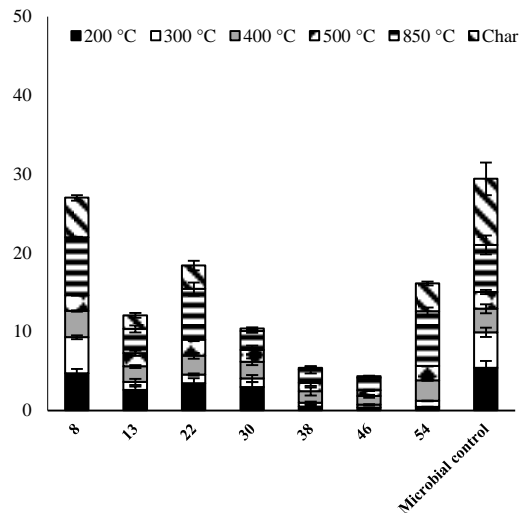


Figure 9-b

Figure 9. The thermal carbon elution profiles of the lignin samples in the supernatant treated with two fungal strains: a) *Coriolus versicolor*, b) *Trametes gallica*. The error bars show variance of mean with one standard deviation obtained from triplicate samples.

Compared to the fungal treatments, the application of ligninolytic bacteria led to a slight yet statistically significant increase of carbon wt.% in the supernatant on day 54 (Table 7, column c). An increase of the total carbon content at the end of experiment indicates that carbon solubilization may not necessarily lead to its mineralization as has been noted previously, especially for actinomycetes [27]. Bacterial solubilization of KL has been previously reported, especially with *Streptomyces* strains [15].

The carbon mass balance closed at  $102 \pm 9\%$  and  $97 \pm 11\%$  for B and S, whereas the carbon wt.% in the solid portion matched that obtained by gravimetric analysis. Unlike

the fungal treatments, only a negligible carbon loss occurred, if any, but 33-36% of the lignin carbon moved into the supernatant. Our results indicate that bacteria may employ a different way of metabolizing lignin for growth to produce more water-soluble products of its biodegradation without significant carbon mineralization. So far, corroborating the results obtained in this work, white rot fungi were mostly reported to mineralize lignin whereas bacteria merely caused structural alterations [28].

The dynamics of changes occurring in the thermal carbon elution profiles as a result of bacterial treatment is shown in Fig. 10. Unlike the fungal treatment, the total carbon amount remained relatively stable and similar to the blank. However, the proportions among the thermally evolving fractions showed significant changes occurring during this process. The observed transient drop in the middle of the incubation period apparently reflects the dissolution of solids followed by the digestion of the resulting dissolved nutrients, low molecular weight fragments of lignin.

Just as for the fungal treatment, the low-MW phenolics evolving at 200 °C and 300 °C exhibited a regular oscillation pattern: They were consumed in the beginning along with the reduction of the total dissolved carbon and then produced again. For the S strain, this process occurred repeatedly until the end of incubation (Fig 10-b). In the case of bacteria the changes in 200 °C and 300 °C fractions are less pronounced and accompanied by different changes in the thermal carbon elution profile. First, the low-MW phenolics do not appear at the end of the fungal incubation as they do for bacteria. Also, the char fraction remains to be significant throughout the entire period of bacterial incubation, unlike the fungal treatment.

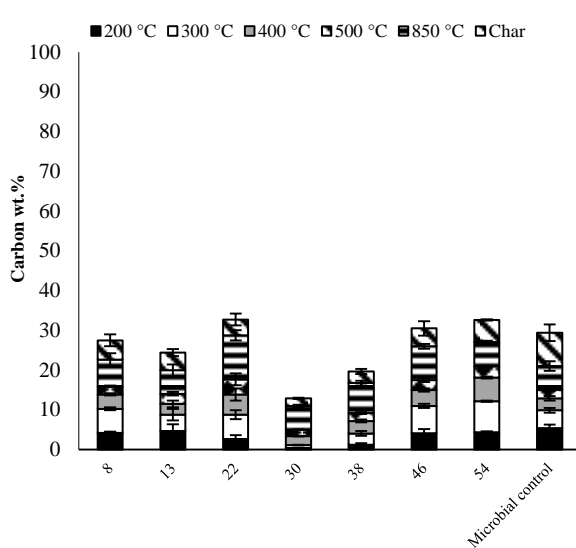


Figure 10-a

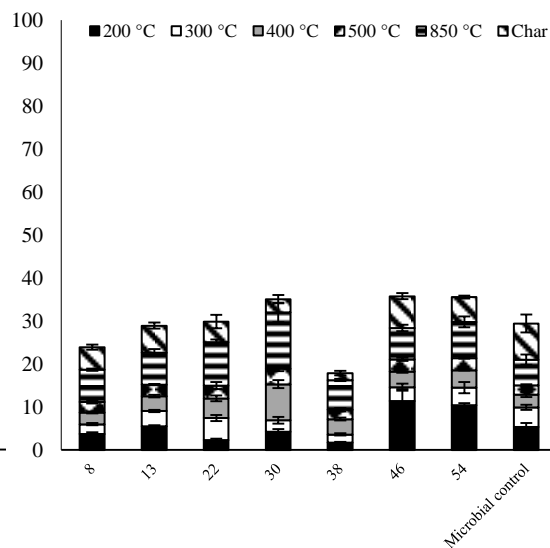


Figure 10-b

Figure 10. The thermal carbon elution profiles of the lignin samples in the supernatant treated with two bacterial strains: a) *Mycobacterium sp.*, b) *Streptomyces sp.* The error bars shows variance of mean with one standard deviation obtained from triplicate samples

### 3.3.4 TD-Pyrolysis-gas chromatography/mass spectrometry Analysis

#### 3.3.4.1 Phenolics elution profile

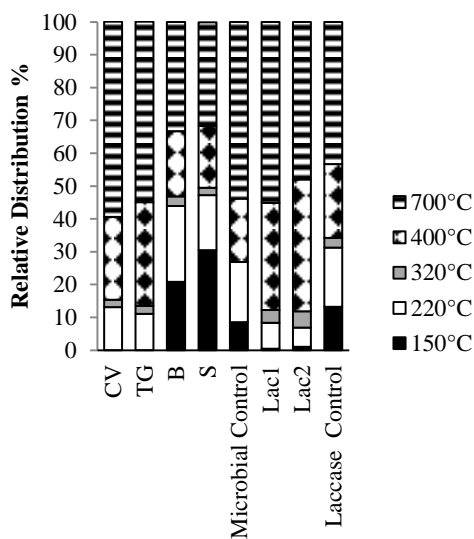


Figure 11-a

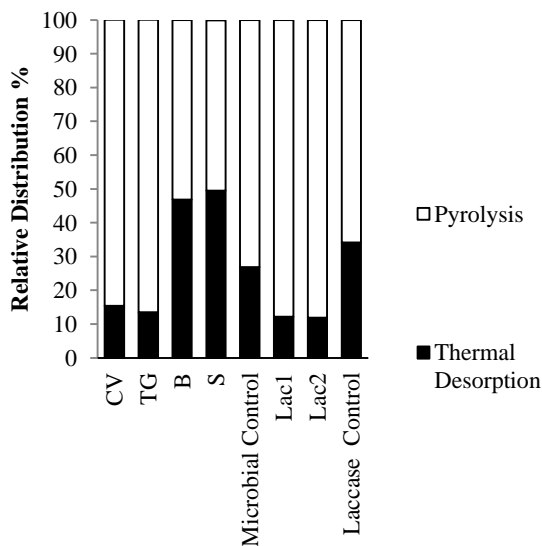


Figure 11-b

Figure 11. Distribution of phenolics elution using TD-Pyr-GC-MS

a) Distribution of phenolics elution across all temperatures steps in the supernatants. b) Distribution of phenolics elution during thermal desorption and pyrolysis after in the supernatants different lignin treatments.

To obtain a more comprehensive characterization of the lignin treatment products, the percentage of phenolics eluting was estimated in all five temperature fractions based on the normalized integrated area after microbial and laccase treatments, as shown in Figure 11.

The profiles of phenolics' elution during thermal desorption and pyrolysis after fungal, bacterial and laccase treatments are shown in Figure 11-b. Given that the 320 °C temperature fraction elutes only traces of chemicals, it marks the boundary between the thermally desorbing and pyrolytic species. To capitalize on this observation, Figure 11-b depicts the sums of thermally desorbing (150-320 °C) and pyrolytic (400-700 °C) products.

Compared to the control, the phenolics' elution shifted from thermal desorption to pyrolysis indicating the polymerization imposed by laccase. With decreases in 150 and 220 °C fraction, phenolics eluting at pyrolysis temperatures, especially the 400 °C fraction, had a significant increase, as can be seen from Figure 11-a. Corroborating this conclusion, a substantial increase in the mean molecular weight was observed when lignin was treated with a purified laccase [29].

The phenolic peak distribution profile after the fungal lignin treatments (Fig. 11-b) appeared similar to that after the laccase treatments, featuring the disappearance of phenolics eluting mainly at 150 °C along with an increase of pyrolysis fraction. This shift

in phenolics' elution suggests that after 54 days of fungal growth, lignin fragments of high molecular weight were further polymerized whereas the low molecular weight fraction was used for fungal growth. The high enzyme laccase activity detected during fungal treatments in this study corroborated this observation. Lara et al. also reported that high molecular weight lignin was further polymerized with a concomitant release of low molecular weight compounds during the treatment of black liquor by white rot fungi, *Trametes elegans* [30]. Several other white rot fungi were shown to further polymerize various kinds of industrial lignin [31].

Although char does not evolve any Pyr-GC-MS detectable organic chemicals, the TCA data considered in the previous section showed that fungi can digest even the presumably cross-linked char fraction resulting in an across-the-board decrease of the supernatant carbon content (among all TCA fractions). Thus, we may conclude that a balance of two opposite trends may occur throughout fungal treatments, between the loosening of cross-linked structure of lignin and re-polymerization of smaller fragments. This balance may be skewed toward one of these trends within certain treatment phases, depending on the specific environmental conditions, e.g., nutrient source and abundance.

Compared to fungal treatments, those conducted with bacteria featured a rather different elution profile, with significantly more phenolics eluting in the thermal desorption fractions (Fig. 11-b). Meanwhile, the fraction of phenolics eluting at 700 °C decreased concomitantly (Figure 11-a), thus indicating once again that bacteria used a different mechanism of metabolizing lignin as a carbon source. Combining this observation with the TCA results shown in Fig. 10 shows that bacteria not only induced the dissolution and degradation of lignin into smaller-MW fragments but also reduced the

char fraction. Thus, some of the observed low-MW phenolics could be produced as a result of loosening the cross-linked lignin matrix.

### 3.3.4.2 Compositional changes of treated lignin

Table 8 Products of lignin degradation after 54 days of microbial and laccase treatment evolving at 150 °C and 220 °C. The marked chemicals were not present in the control samples and appeared after treatment.

Ret Time	150 °C					220 °C					m/z <sup>a</sup>	Identified compounds
	control	CV	TG	S	B	control	CV	TG	S	B		
6.656	+	-	-	-	+	+	+	+	+	+	109, 124, 81	Guaiacol
7.277	-	-	-	-	-	-	-	-	+	-	138,123,95	Methyl guaiacol
7.979	-	-	-	+	+	+	-	+	+	+	150,135,107	Vinyl guaiacol
8.212	-	-	-	-	-	-	-	-	-	+	164, 149, 103	Eugenol
8.282	-	-	-	+	-	-	-	-	-	-	121,122,93,65	Benzaldehyde, 4-hydroxy-
8.406	-	-	-	-	-	-	-	-	-	+	140, 125, 97	2-Methoxyhydroquinone
8.478	+	-	-	+	-	+	+	+	+	-	151,152,81,109	Vanillin
8.707	-	-	-	-	-	+	-	-	+	+	164, 149, 77, 91	Isoeugenol
8.768	-	-	-	-	-	+	-	-	+	+	137, 166, 122	Propyl guaiacol
8.922	+	-	-	+	+	+	-	+	+	+	151,166,123	Apocynin
9.132	-	-	-	+	+	-	-	-	+	+	137, 180, 122	Propan-2-one, 1-(4-isopropoxy-methoxyphenyl)-
9.266	-	-	-	+	+	-	-	-	+	+	137, 138, 123, 182	Vanillyl ethyl ether
9.691	-	-	-	+	+	+	-	-	+	+	137, 182, 138, 122	Homovanillic acid
9.905	-	-	-	+	-	-	-	-	+	-	137, 210, 164	Phenylacetylformic acid, 4-hydroxy-3-methoxy-
10.083	-	-	-	-	-	-	-	-	+	-	137, 196, 122	Homovanillic methyl ester
10.111	-	-	-	-	-	-	-	-	-	+	178, 135, 77, 107	4-Hydroxy-2-methoxycinnamaldehyde

Ret Time	control	LacI	LacII	control	LacI	LacII	m/z <sup>a</sup>	Identified compounds
6.643	-	-	-	+	+	+	109, 124, 81	Guaiacol
7.264	-	-	-	+	+	-	123, 138, 95	Methyl guaiacol
7.761	-	-	-	-	+	-	137, 152, 122	Ethyl guaiacol
7.968	-	-	-	+	+	-	150, 135, 77, 107	Vinyl guaiacol
8.202	+	-	-	-	+	-	164, 149, 77, 91	Eugenol
8.386	-	-	-	-	+	-	140, 125, 97	1,4-Benzenediol, 2-methoxy-
8.466	+	-	-	+	+	+	151, 152, 81, 109	Vanillin
8.696	-	-	-	+	+	-	164, 149, 77, 103	Isoeugenol
8.762	-	-	-	-	+	-	137, 166, 122	Propyl guaiacol
8.91	-	-	-	-	+	+	151, 166, 123	Apocynin

+Present      - Absent      <sup>a</sup>Mass to charge ratio

Previous studies suggested that lignin pyrolysis occurs around 400 °C [32]. In Table 8, all phenolics recovered are listed, which were thermally desorbed from the pyroprobe after different lignin treatments at 150 °C and 220 °C. To further elucidate the compositional changes occurring in lignin as a result of microbial and enzymatic treatments, the phenolics' elution across *all* temperature steps (including the pyrolytic fractions) were classified into 6 groups of compounds as shown in Fig. 12. The origin of

the lignin feedstock used (pine as softwood) explains the ubiquitous abundance of guaiacol (G) among all temperature fractions. Among its derivatives, guaiacol carbonyls (GC) and guaiacol acids (GA) were the major products evolving at lower temperature steps, as they are thermally unstable. Conversely, phenol-based compounds (PH), benzenediol-based compounds (BZ) and aromatic hydrocarbons (AH) were pyrolytic products evolving only at high temperatures [32] [33].

Laccase treatments led to a decrease in G elution at 150 °C along with an increase of the G fraction eluting at 400 °C, compared to the control (Fig. 12). The phenolic peaks shifted toward higher temperatures (Fig. 12) and were more pronounced after the LacI treatment having a lower enzyme activity compared to LacII (Table 8). Corroborating these observations, a high enzyme activity *in vitro* laccase treatment of Kraft lignin was shown to facilitate mostly the polymerization reactions rather than depolymerization [29].

Furthermore, GC showed up in the high temperature fractions after the laccase treatments. Given that these chemicals are thermally unstable, they must be abundant to “survive” pyrolysis. This observation indicates the formation of polymers as a result of laccase treatment, which are more oxidized than the original lignin, so these polymers are comprised of carbonyl guaiacol derivatives rather than guaiacol itself. Corroborating this observation, oxidation, demethylation and hydroxylation of Kraft lignin model compounds were shown to occur when treated by laccase from *T. versicolor* [34].

The amounts of pyrolytic products, BZ (for laccase I) and PH & AH (for laccase II exhibiting a greater enzyme activity) also increased in high temperature fractions being consistent with the formation of more cross-linked polymers than the original lignin.



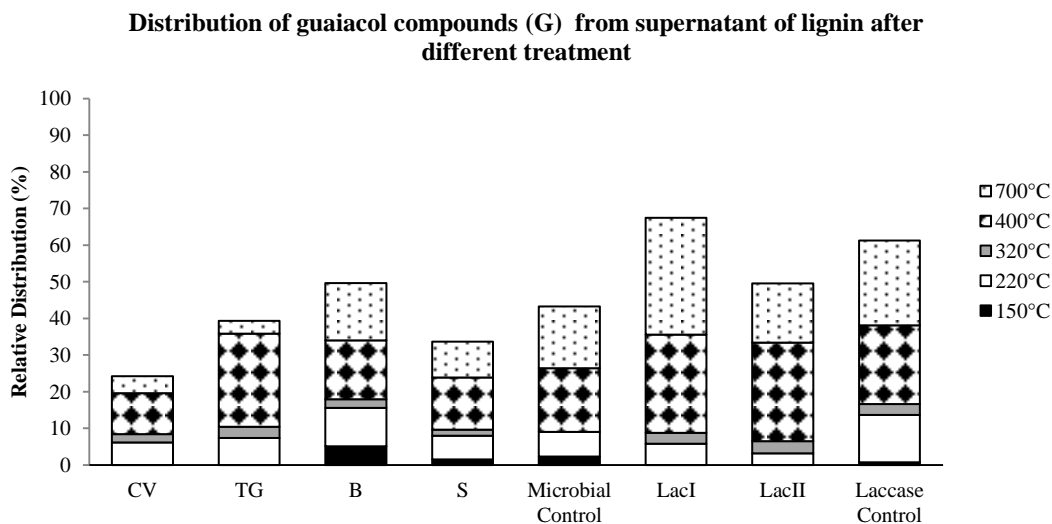
These features corroborate the conclusion made throughout this paper that the prevalent mode of laccase action is oxidative polymerization by cross-linking.

The most significant compositional changes in lignin after *fungal* treatments was an increase in PH and AH along with a corresponding decrease in G eluting at 700 °C (Fig. 12). As we have discussed earlier, pyrolytic phenolics eluting at 400 °C and 700 °C showed a significant increase after the fungal treatments at the expense of monomers eluting at lower temperatures. Therefore, fungi can consume low molecular weight phenolics for growth while converting the remaining lignin fragments present in the supernatant into more condensed, recalcitrant structures. This conclusion is also evidenced by the fact that thermally desorbed phenolics originally present in the control (presumably, due to the sample heating during sterilization) disappeared after the treatment and only a few new compounds were identified (Table 8). The fungal growth thus depends on these intermediates' consumption and mineralization, corroborating the low carbon content of the supernatants on day 54 discussed in the TCA section. A similar observation, i.e., that the majority of aromatic peaks originally present in the medium disappeared after a fungal treatment of black liquor with *Trametes* sp., was reported earlier [35].

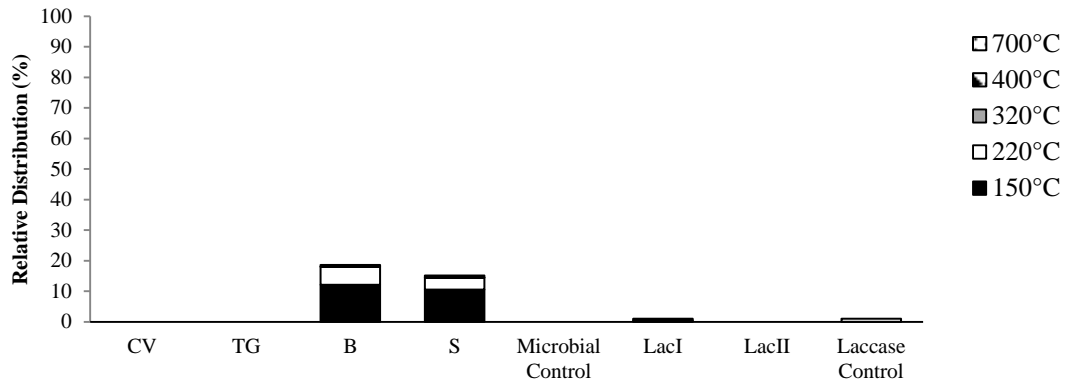
Unlike lignin after a fungal treatment, a decrease in G elution after the *bacterial* treatments did not lead to an increase in the amount of PH and AH evolving at 700 °C. Instead, an increase in BZ eluting at 700 °C was observed. However, the increase of the BZ fraction was less pronounced for bacteria compared to the increase of PH after the fungal treatment. These observations echo a similar difference between a more active laccase II compared to laccase I and may indicate a greater production of oxidative

enzymes by fungi than bacteria. A relatively small change in G compared to the fungal treatments should be considered in combination with the GA elution at low temperatures characteristic only for bacterial samples and observed in stark contrast with the blank (Fig. 12). Apparently, bacteria specifically promote guaiacol oxidation.

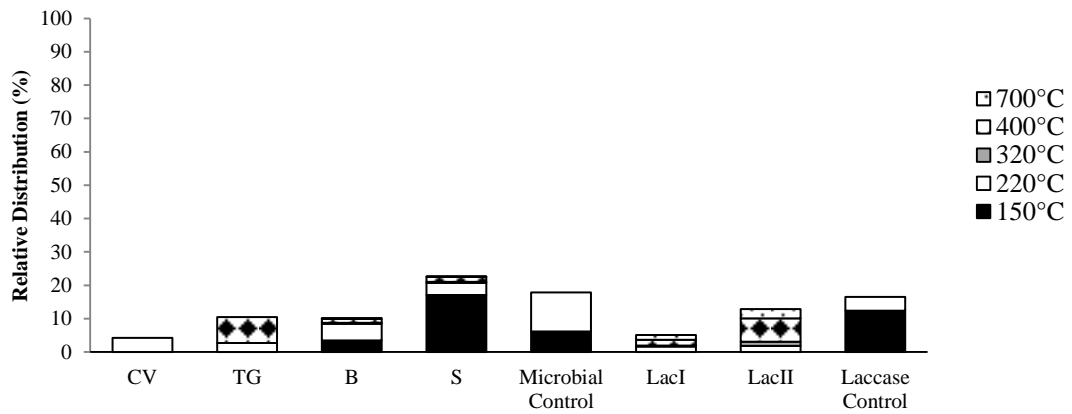
The other specific feature of bacterial treatments was a significant shift in elution of PH and AH compounds at 700 °C towards lower temperature fractions, which suggests a different structural modification imposed on lignin by bacteria that involves loosening of condensed structure and depolymerization. Finally, bacterial supernatants continued to elute phenolics at lower temperatures, unlike both fungal and enzyme-treated samples, as shown in Table 8. Thus, bacteria appear to employ a different approach in using lignin as an energy source, with a lesser extent of mineralization compared to white rot fungi [27] but also with a less pronounced polymerization as shown throughout this study.



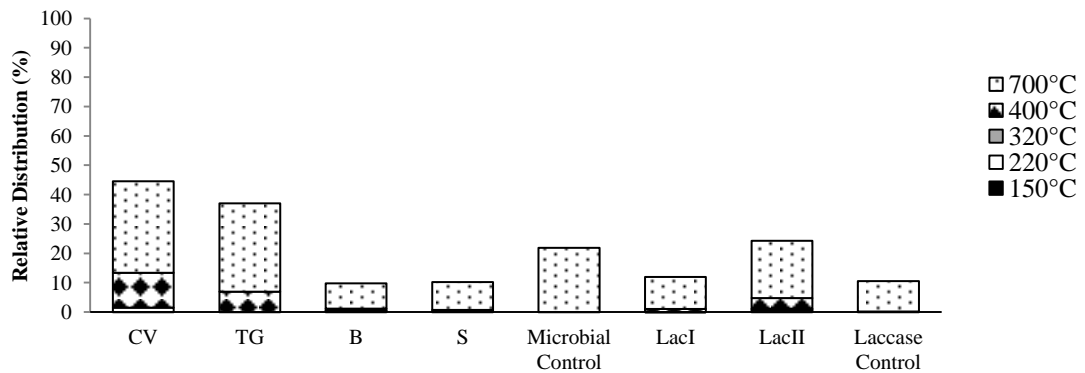
**Distribution of guaiacol acid compounds (GA) from supernatant after different lignin treatment**



**Distribution of guaiacol carbonyl compounds (GC) from supernatant of lignin after different treatment**



**Distribution of phenol based compounds (PH) from supernatant of lignin after different treatment**



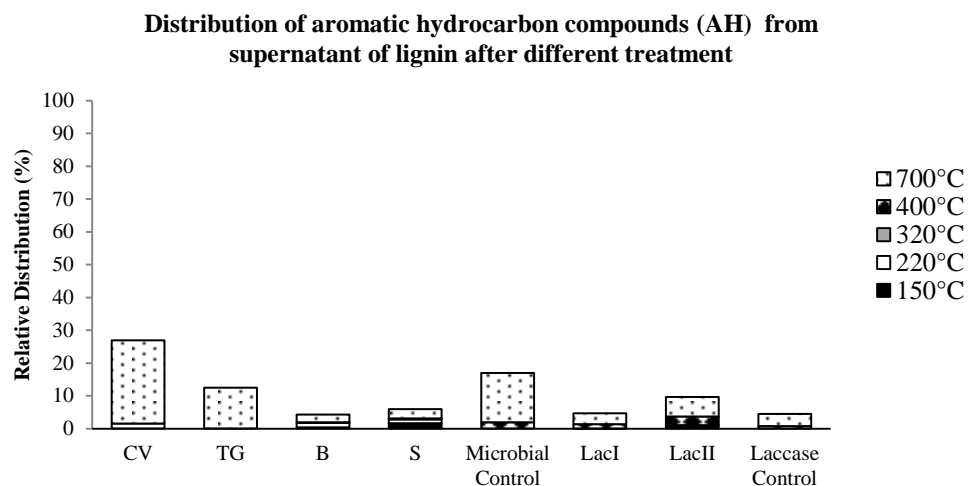
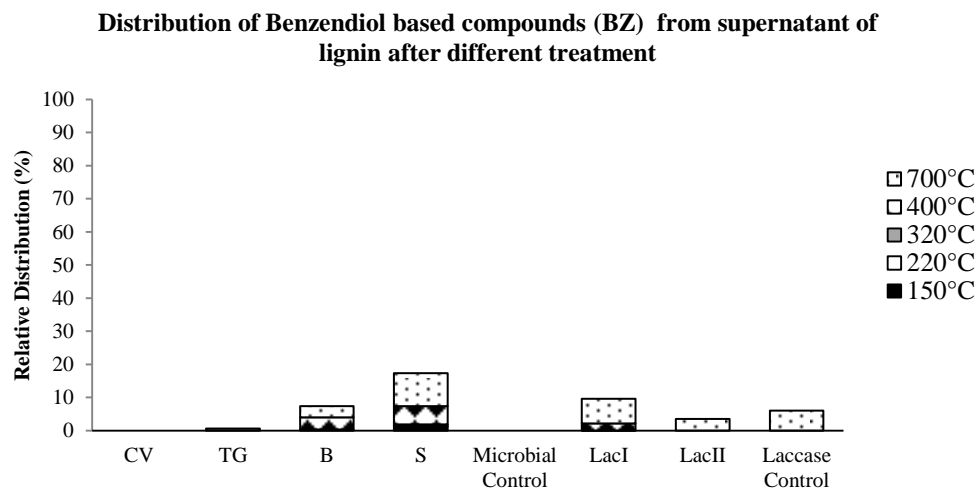


Figure 12. Relative distribution of 6 groups of compounds across five temperature steps after microbial and laccase treatment: Guaiacol and its homologues (G), Guaiacol acids (GA), Guaiacol carbonyls (GC), Phenol-based compounds (PH), Benzenediol-based compounds (BZ) and Aromatic hydrocarbons (AH)

## CHAPTER IV

### CONCLUSION AND FUTURE WORK

#### **4.1 Conclusion**

Gravimetric measurements may be combined with TCA for an accurate assessment of lignin biodegradation distinguishing it from solubilization and providing both the carbon mass balance and fraction analysis by volatilization. A combination of TCA and TD-Pyr-GC-MS yields insights into the resulting product chemical structure.

Different products are produced in the liquid medium depending on the type of treatment (fungal, bacterial or enzymatic) and treatment time. In this study, only fungi mineralized Kraft lignin to a significant extent whereas bacteria degraded and modified this substrate only partially. Both laccase and fungi polymerize a part of lignin with the extent of cross-linking in the resulting polymers being greater for fungi. Yet, fungi appear to be able to break down even the highly cross-linked lignin, with a delicate balance between the cross-linking polymerization and de-polymerization/degradation.

## 4.2 Future Work

In this study, we put our focus on determining the extent of lignin degradation and characterizing lignin degradation products at the very end of treatment on Day 54. Future characterization studies should be conducted for samples harvested on all previous days, which allows for gaining valuable information on metabolic pathways and reaction kinetics associated with industrial lignin degradation.

Our results on carbon mass balance indicated a loss of overall carbon in lignin samples during fungal treatment, which was presumably metabolized to CO<sub>2</sub>. Future study should involve an experimental step to recover the CO<sub>2</sub> during the fungal treatment for a complete mass balance closure. Meanwhile, sugar evolution profile should also be monitored during the microbial treatment to complement the TCA profile of carbon eluting at thermal desorption temperatures.

Given the heterogeneity of industrial lignin, it is very challenging to produce a single compound. However, central intermediates of lignin metabolism such as catechol or protocatechuate can be produced in higher yield by removing the enzymes accountable for subsequent metabolism [1]. With sufficient knowledge of the lignin metabolic pathways, degradation rate can be improved in the future.

Sustainable production of lignin degradation products could be hampered by the inherent toxicity to microorganisms[2]. Efficient separation of aromatic compounds from the medium can increase the degradative ability of microorganisms. In a biorefinery context, such inhibitors are removed using separation techniques such as evaporation, solvent extraction, activated charcoal and ion exchange resins, etc.[2]. In addition,

membranes, chromatography, reactive distillation and crystallization techniques can also be tailored to the recovery of extracellular water soluble phenolics produced during microbial treatment depending on the product of interest [2][3]. Application of an in situ product recovery technique, solvent-impregnated resins containing an ionic liquid, was reported to efficiently extract phenol from a bacterial strain, *Pseudomonas putida* *S12TPL*, fermentation medium and result in 4 fold increased production of phenol [4]. This particular technique could keep the solvent separated from the water phase preventing solvent-toxicity and emulsion formation [4].

Another solution for achieving an efficient degradation of lignin within a short period of time would be to decrease the heterogeneity of industrial lignin stream/black liquor by using genetically modified biomass as a starting feedstock with lignin structures that are easier to be metabolized to produce a few key compounds [3]. For example, genetically modified poplar lignin showed an increased amount of ferulate monomers incorporated into the lignin polymer. This feature introduced an increased amount of ester links which were easily broken down during the pulping process and led to a black liquor stream with high concentration of ferulate [5]. Meanwhile, an upgrade on the existing wild type strains to engineered strains is also an important factor to increase the lignin conversion yield. The engineered strains should exhibit higher growth rates, higher tolerance to oxidative stress with minimum lag time and an enhanced heterologous protein expression [3]. So far, a bacterial strain, *P. putida* *KT2440*, was reported to be engineered to produce a higher yield of a targeted product from aromatic compounds[3][6]. However, more research efforts are needed in applying microbial metabolic engineering to treat complex aromatic substrates such as lignin.

## CHAPTER V

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## Chapter IV

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