Nutrient Regulation and Combined Pretreatment Strategies to Improve the Rate of Delignification by White Rot Fungi

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Nutrient Regulation and Combined Pretreatment Strategies to Improve the Rate of Delignification by White Rot Fungi

by

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Abstract

Delignification is necessary for efficient conversion of lignocellulosic biomass to biofuels. Microbial delignification by white rot fungi is achieved by the action of lignin degrading enzymes, laccase and peroxidase. As the microbial process of delignification is very slow, two strategies have been employed to achieve effective delignification in a short duration. In the first strategy, nutrient regulation of lignin degrading enzymes was exploited to identify the specific carbon and nitrogen sources, their ratio and inducer concentrations and to optimize the nutrients for maximum ligninolytic activity. In the second strategy, fungal delignification was combined with hydrothermal process.

A dual effect of copper on *Dichomitus squalens* laccase activity, inducing and suppressing was revealed. Maximum laccase activity is a function of fungi tolerance to copper and media composition. Some nutrients improved tolerance of cells and laccase-producing system to copper load. Methylated substrates in combination with casein and copper demonstrated high potential to support fungi tolerance and enhanced laccase activity.

In addition to induction, the suppression of peroxidase activity by Mn$^{2+}$ (Manganese sulfate) has been observed in *D. squalens* and *Ceriporiopsis subvermispora*. The induction and suppression were independent of fungal growth, but depended on the nutritional conditions. Manipulation of carbon and nitrogen sources shifted the peroxidase suppression by Mn$^{2+}$ to high concentrations and, hence, increased the peroxidase tolerance to Mn$^{2+}$ and, consequently, peak peroxidase activities.
Optimized nutrient parameters from studies with the monocultures of *D. squalens* and *C. subvermispora* were used to design the experiments for co-cultivation of the above microorganisms. Cross regulations of laccase and peroxidase activities was observed for arabinose, casein, copper and manganese. Optimization resulted in the production of 1378 and 1372 U/g protein of laccase and peroxidase respectively. Crude enzyme mixture from optimized media degraded 16.9% of lignin in wheat straw.

To test the second strategy, raw wheat straw was exposed to hydrothermal process followed by fungal hydrolysis. On substrate cooling, by-products of lignin degradation via hydrothermal treatment recondensed on the cellulose fibers. The following fungi treatment enhanced lignin hydrolysis by degradation of the residual by-products. The combined approach is an effective method to prepare substrates for downstream fermentation of cellulose to biofuels.
I dedicate this thesis to the Almighty and my Family
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Abbreviations

C/N Carbon to Nitrogen ratio
LC/LN Low Carbon/Low Nitrogen
LC/HN Low Carbon/High Nitrogen
HC/LN High Carbon/Low Nitrogen
HC/HN High Carbon/High Nitrogen
MSC Minimum Growth Suppressive Concentration of Copper
MEC Minimum Growth Eradication Concentration of Copper
MLIC Minimum Laccase Inductive Concentration of Copper
PC Copper Concentration responsible for peak laccase activity
MLSC Minimum Laccase Suppressive Concentration of Copper
MIC Minimum Inducing Concentration of Manganese sulfate
PC Manganese sulfate Concentration responsible for peak peroxidase activity
MSC Minimum total peroxidase Suppressive Concentration of Manganese sulfate

Symbols

$E_0, E_t$ Enzyme concentration initially and at time, t (mg/L)
$S_0, S_t$ Substrate concentration initially and at time, t (mg/L)
$P$ Product concentration (mg/L)
$t$ Time (min)
$k_1$ Kinetic constant associated with substrate decomposition (min$^{-1}$)
$k_2$ Kinetic constant associated with enzyme deactivation (min$^{-1}$)
Chapter One: Introduction

1.1 Overview

The global energy consumption will increase by 56\% between 2010 and 2040 according to the statistics delivered by Energy Information Administration. With the inevitable decline in the fossil based fuels and the rising energy demand, it is necessary to seek alternate energy sources as part of the solutions for energy crisis. In addition to the energy demand, growing environmental concern and energy security also play a role in motivating the research in renewable energy resources. Biomass based fuels are one such renewable energy resource which can be relied upon for a sustainable energy supply. The benefits of biomass based fuels are: increased energy supply; reduced green house gas emissions; less dependence on imported oil; and increasing the markets for agricultural products worldwide.

Cellulosic biomass such as corn is utilised for the production of first generation biofuels [1]. However, a limitation of cellulosic food crops is that it interferes with food supply. Hence, in order to overcome this conflict between food and fuel supply, cellulosic biomass from crops can be replaced with lignocellulosic waste for the production of second generation biofuels [2]. Residues from agriculture, forest and industry contribute to lignocellulosic biomass. Lignocellulosic biomass can be utilized, in addition to biofuel generation, for production of a vast range of chemicals, including tulipalin, a plant extractive used in polymer applications and α-pinene, extracted from pine trees as a ‘green’ solvent [3]. Lignocellulosic biomass consists of
the polymers, cellulose, hemicellulose and lignin. Lignin encapsulates the carbohydrate matrix and offers structural stability to the polymer, and hence enhances recalcitrance to environmental factors [4]. In other words, lignin inhibits the susceptibility of encrusted cellulose to saccharification [5]. Thus delignification is required to disrupt the fibrous structure and increase the receptiveness of the carbohydrate substrate towards effective cellulose hydrolysis [6]. The choice of suitable delignification approaches is the critical step in biofuel production because it determines the efficiency of the subsequent hydrolysis, fermentation and further downstream processes for product recovery [7].

The physical and chemical delignification techniques such as steam explosion, ammonia fibre explosion, acid/alkali treatment offers disadvantages such as inhibitor formation, high cost of solvents/catalysts, recycling cost, and high energy/pressure requirements [8-11]. These drawbacks can be overcome by implementing an alternative technique which might result in negligible inhibitors and waste emissions; low maintenance cost; and low energy requirement. One such technique is the microbial biodegradation of lignocellulosic biomass [12, 13]. However, the application of microbial delignification is limited due to very low rate of biodegradation [14]. Hence it is essential to develop new strategies to increase the rate of biodegradation by modifying the existing microbial processes.

Microbial treatment, primarily by white rot fungi such as *Dichomitus squalens*, *Ceriporiopsis subvermispora* and *Cyathus stercoreus* involves selective lignin degradation, leaving back a cellulose-rich biomass residue [15, 16]. Fungal delignification is preferable owing to the advantages of mild operating conditions, low energy consumption, no chemical
requirements for depolymerization or neutralization, zero chemical recycling cost, minimum waste generation, negligible inhibitor formation and complete lignin mineralization to carbon dioxide [17]. Lignin depolymerization by white rot fungi occurs by the action of extracellular-oxidative ligninolytic enzymes – lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and laccase. However, even with all these positive attributes, biological delignification has not been developed to a larger scale because the complete biodegradation takes place over a period of weeks to months and makes it a less attractive technique [18]. The lignin degradation by white rot fungi is a function of ligninolytic enzyme activities [19] and the stimulation of the latter to higher levels by certain carbon and nitrogen sources and by the addition of inducers to the fungal growth media can reflect in increased rate of lignin biodegradation. Also, co-cultivation of two white rot fungi can result in enhancement of lignin degrading enzymes due to the synergistic relationship between the white rot fungi [20]. In addition, the fungal treatment can be combined with an appropriate physical approach to increase the effectiveness of delignification in terms of rate and cellulose availability.

A subcritical hydrothermal process is one such physical approach which involves the heating of the lignocellulosic material with hot water under high pressure (150-400°C under 5-25 MPa) to maintain water in the liquid state. Under these conditions water becomes an excellent solvent for hydrophobic organic compounds. Complete hemicelluloses and part of lignin are solubilised under 180-200°C for residence time of 5-15 minutes [21]. Optimum choices of reaction temperature, residence time and particle size can lead to less corrosion, low operational cost, zero waste generation, and low inhibitors generation [22]. Prolonged residence time and higher reaction temperatures are responsible for formation of inhibitors in hydrothermal process.
Even at a low temperature of 180°C, lignin depolymerization products re-condense and accumulate on the substrate surface alongside cellulose and prevent the cellulases from reaching the active sites of cellulose for hydrolysis [23]. Hence a lignin removal step is to be incorporated after hydrothermal treatment, before enzyme hydrolysis and fermentation. This intermediate step can be accomplished with selective lignin-degrading white rot fungi.

In this research, several objectives were undertaken to understand the underlying mechanisms involved in the nutrient regulation of lignin degrading enzymes and to identify the scope of co-cultivation and combined delignification. A wide range of carbon sources and nitrogen sources were used along with appropriate inducer compounds to enhance the secretion of lignin degrading enzymes. Copper being an inducer of laccase, is also toxic to white rot fungi at high concentrations. Studies were undertaken to understand how the carbon and nitrogen sources regulate the tolerance of white rot fungi, \textit{D. squalens} and \textit{C. subvermispora} to copper (Chapter 2 and Appendix A respectively). The effect of manganese as an inducer of manganese peroxidase of the above said fungi was studied in the presence of various carbon and nitrogen sources (Chapter 3). To investigate the effect of co-cultivation on the lignin degrading enzymes, white rot fungi, \textit{D. squalens} and \textit{C. subvermispora} were combined in the presence of specific carbon and nitrogen sources and inducers for simultaneous enhancement of laccase and peroxidase. In addition, the potential of crude enzymes on lignin degradation was explored (Chapter 4). To improve the efficiency of treatment of lignocellulosic biomass, fungal delignification was combined with hydrothermal process (Chapter 5). Finally, in order to gain an understanding of kinetics of enzymes on lignin degradation, laccase was used to degrade lignin in wheat straw. The corresponding mathematical model was developed (Chapter 6).
Consequently, this thesis addresses the shortcomings of the current fungal delignification of lignocellulosic waste in terms of low production of lignin degrading enzymes and low rate of degradation, so as to improve the prospect of fungi-based technology for large scale applications.

1.2 Literature Review

The focus of this section is to present the current scenarios related to delignification of lignocellulosic biomass. Emphasis is given to fungal (enzymatic) lignin degradation, subcritical hydrothermal treatment and combination of these approaches. The limitations identified from previous studies are highlighted. This thesis is presented in a paper-based format of four articles in four chapters. As each chapter includes a detailed review, the literature review in this section will be condensed, so as to avoid repetition.

1.2.1 Necessity for Delignification of Lignocellulosic Biomass

The increase in green house gas emissions due to the use of fossil based fuels and the related environmental concern leads to the research in alternative fuel sources. Biomass is one such resource which could be effectively exploited for sustainable biofuel generation. Lignocellulosic biomass is preferred over cellulosic biomass because of the advantages such as not interfering with food supply, cheap and easily available as agricultural waste, forest residues, and wood-based industry waste. Lignocellulosic biomass is a composite material composed of mainly three main polymers: cellulose, hemicelluloses and lignin linked together by ether and ester bonds [24]. Cellulose, the world’s abundant renewable carbon source, is a linear polymer of D-glucopyranosyl-β-1,4-D-glucopyranose units that represents about 40-50% of the plant cell wall.
Cellulose monomers are linked together by β-1,4 linkages forming a crystalline structure that is highly resistant to hydrolysis. Hemicelluloses composed of pentoses and hexoses, forming a branched short chain polymer and represents to about 20-30% of total weight. Lignin, the second abundant polymer is an aromatic, three-dimensional network of three p-hydroxycinnamyl alcohols; non-methoxylated (p-hydroxyphenyl alcohol), monomethoxylated (coniferyl alcohol) and dimethoxylated (syringyl alcohol) phenylpropanoid units linked together by ether and C-C bonds. The percentage of lignin in wood biomass is around 10 to 25%.

The intact lignin coating coupled with hemicelluloses protects the cellulose fibrils from chemical and enzymatic hydrolysis. Hence, it is essential to break the linkages between lignin and carbohydrate matrix to liberate cellulose. A pretreatment process makes the cellulose polymers accessible for enzymatic hydrolysis by the removal of hemicelluloses and lignin [17]. Wyman et al., [25] has postulated the following list of desirable attributes for an effective pretreatment technique:

- Low cost of chemicals for pretreatment, neutralization, and subsequent conditioning.
- Minimal waste production.
- Limited size reduction because biomass milling is energy-intensive and expensive.
- Fast reactions and/noncorrosive chemicals to minimize pretreatment reactor cost.
- The concentration of hemicellulose sugars from pretreatment should be above 10% to keep fermentation reactor size and reasonable level and facilitate downstream recovery.
- Pretreatment must promote high product yields in subsequent enzymatic hydrolysis or fermentation operations with minimal conditioning cost.
Hydrolysate conditioning in preparation for subsequent biological steps should not form products that have processing or disposal challenges.

Low enzyme loading should be adequate to realize greater than 90% digestibility of pretreated cellulose in less than 5 days and preferably 3 days.

Pretreatment should facilitate recovery of lignin and other constituents for conversion to valuable co-products and to simplify downstream processing.

The conventional physical and chemical pretreatments, such as acid and alkaline pretreatments, steam explosion, ammonia fiber explosion, milling and grinding are used for partial delignification and cellulose exposure for enhanced enzymatic hydrolysis. But, all these processes require extreme operating conditions such as high pressure, high temperature, corrosion-resistant reactors, high use of chemicals and/or energy, and results in release of inhibitors such as weak acids, furan and phenolic compounds which reduce hydrolysis and fermentation rates and overall biofuel yield [10] and is also accompanied by notable waste emissions. Moreover, the existing pretreatment processes result in insufficient exposure of cellulose primarily due to re-condensation of lignin fragments on the surface of the pretreated substrate [21] and hence result in reduced yield of fermentable sugars. For example, considering the high temperature dilute acid hydrolysis, a conventional pretreatment being practiced on large scale, does not fully remove lignin, and it is believed that re-condensed lignin derivatives precipitate on the cellulose surface and inhibit the hydrolysis process through a combination of binding with the cellulase enzymes and blocking the progress of the enzymes along the glucose chains [21, 23]. Hence the re-condensed lignin fragments on substrate surface must be removed.
by solvent extraction as an intermediate step between acid pretreatment and cellulose hydrolysis in order to increase the yield of glucose monomers.

The implementation of lignin-selective fungi degraders for the removal of lignin from lignocellulosic substrate promises to overcome a majority of the disadvantages of conventional pretreatment techniques [26, 27]. Low rate of lignin removal [28] is the major limitation of fungal pretreatment, which can be overcome by inducing the ligninolytic enzyme activity [29].

1.2.2 Biological Delignification of Lignocellulosic Biomass

Biological delignification is based on enzymatic degradation of lignin via activity of microorganisms. Basidiomycetes, a group of higher fungi are natural wood decomposers, which modifies the chemical composition and structure of the lignocellulosic biomass and makes it more amenable to enzyme digestion. There are white rot and brown rot wood degrading basidiomycetes, of which the latter degrades polysaccharides and leaves back non-oxidized lignin [30]. White rot fungi are well known for their efficient lignin degrading ability of lignocellulosic materials. Even though the rate of fungal degradation of lignin is low, it is highly selective and efficient [18]. There are two patterns of decomposition of lignocellulosic biomass by white rot fungi: (a) simultaneous degradation of lignin and polysaccharides, which is known for *Phanerochaete chrysosporium, Trametes versicolor* [31, 32] and (b) selective degradation of lignin and hemicelluloses, leaving behind a cellulose-rich biomass residue, which is known for *C. subvermispora* and *D. squalens* [16, 33]. The selective lignin degraders have wide applications such as biobleaching, biofuel generation, and animal feed production [27, 34, 35].
P. chrysosporium, D. squlaens, C. stercoreus, C. subvermispora, Pycnoporus cinnarbarinus and Pleurotus ostreaus are some of the white rot fungi examined on different lignocellulosic biomass showing high delignification efficiency [16, 27, 36]. Though some white rots result in effective lignin removal, the cellulose availability was also considerably reduced due to simultaneous cellulose-lignin degradation. Bak et al. [16] reported 38% lignin removal efficiency accompanied with 32% glucan removal which would result in very low biofuel yield. Lignin removal efficiency and cellulose digestability of various lignocellulosic substrates are highly specific in terms of the choice of the lignocellulosic substrate, fungi, and mode (submerged or solid state cultivation) and duration of treatment [37-39]. The maximum cellulose digestibility of corn stover by cellulases, pretreated 26 days with C. stercoreus was 37.7% and 35 days with C. subvermispora was 66.9% [12, 40]. Submerged and solid state pretreatment of cotton stalks resulted in a lignin degradation of 19.38% and 35.53%, respectively [41]. Lignin and glucan content of rice straw pretreated with P. chrysosporium was decreased by 6.3% and 16.1% respectively [42] On the other hand, for the same substrate (rice straw), lignin and glucan content was decreased by 7.5% and 11.9% when pretreated with D. squlaens [16]. This variation is due to the different wood degrading pattern of two white rot fungi, P. chrysosporium and D. squlaens. Hence, it is essential to optimize the delignification conditions for the specific lignocellulosic substrate and the specific fungi.

1.2.3 Lignin Degrading Enzymes

The mode of attack of lignin by white rot fungi is mediated by extracellular oxido-reductases that modify the lignin polymer by demethylation, hydroxylation, and ring cleavage to
release low molecular weight fragments which are further degraded to carbon dioxide [43]. The fate of lignin in the presence of white rot fungi includes degradation and/or modification, such as loss of C–C, C–O, and C=O stretching, reduction in the lignin substructures such as dibenzodioxacin and cinnamyl alcohol, and cleavage of aromatic ring [44]. The major ligninolytic enzymes are lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), laccase (EC 1.10.3.2) and versatile peroxidase (EC 1.11.1.16). Lignin peroxidase (LiP) and manganese peroxidase (MnP) are both heme containing peroxidases that oxidize lignin through a series of electron transfer steps and intermediate radical formation [45]. Laccases oxidize their substrates through one electron oxidation of the substrate with concomitant reduction of oxygen to water [19]. LiP degrades both phenolic and non-phenolic lignin substructures, whereas both MnP and laccase degrade the phenolic subunits of lignin [19, 45]. However, both these enzymes have been shown to degrade non-phenolic substrates in the presence of mediators such as Tween 80 and 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) respectively [46, 47]. Versatile peroxidase has properties of both lignin peroxidase and manganese peroxidase and hence degrades both phenolic and non-phenolic subunits of lignin [48]. White rot fungi mostly express any two of the lignin degrading enzymes. For example, *P. chrysosporium*, and *T. versicolor* secrete LiP and MnP [49, 50], whereas, *D. squalens* and *C. subvermispora* secrete laccase and peroxidase (MnP) [51, 52].

1.2.4 Factors affecting ligninolytic activity of white rot fungi

Lignin degradation by fungi occurs during the secondary metabolism, which indicates that sufficient growth is essential to enter the secondary metabolic stage and synthesize the lignin
degrading enzymes [53]. The rate of lignin degradation by lignin-selective basidiomycetes which has low/negligible cellulase activity can be increased by providing a readily available carbon source for growth as the carbohydrates in lignocelluloses is initially locked inside the lignin matrix [53]. Despite the fact that these fungi are capable lignin degraders, the enzymes responsible for lignin depolymerization are constitutively produced in insufficient quantities [38] whence posing a barrier to increasing available cellulose for subsequent processes. A variety of factors such as nutrient composition, type of fermentation and species selection influence the growth and enzyme secretion of white rot fungi. The optimum choice of the afore-mentioned factors might aid in improving the production of ligninolytic enzymes and hence the lignin degradation rate by white rots. During the initial stages of fungal degradation, low surface area of lignocellulosic substrate prevents the diffusion of the lignin degrading enzymes to the site of attack and result in a prolonged treatment for effective lignin removal [54].

**Type of Cultivation**

The type of cultivation of white rot fungi on lignocellulosic substrate also plays a vital role in the secretion of extracellular ligninolytic enzymes. The two modes of growth conditions for the cultivation of white rot fungi on a substrate are, (1) submerged cultivation in which fungi grows on substrate in the presence of excess water, and (2) solid-state fermentation in which fungi grows on substrate containing a thin layer of water on its surface [55]. Solid state fermentation (SSF) offers more advantages such as good aeration and natural environment for the fungal growth, which greatly influences the secretion of high concentrations of ligninolytic enzymes [56]. The patterns of lignin degrading enzymes were quite different in liquid and solid
culture of *Phlebia radiata*. LiP3, the major isoform of LiP in liquid cultures was not expressed in solid cultures and additional MnP isoforms were expressed in solid cultures [57].

**Effect of carbon and nitrogen sources**

Ligninolytic enzyme productivity can be stimulated by many factors including aeration, shear stresses, and media composition [58]. In particular, natural and concentrated sources of carbon and nitrogen have been reported as critical factors regulating ligninolytic enzyme production and activity [59, 60]. Studies have shown that easily metabolizable carbon sources and low concentrations of nitrogen have an inducing effect on ligninolytic enzyme [61, 62]. However, there is discrepancy in the choice of carbon and nitrogen source and their ratios, which varies according to fungal species [55, 63]. Mikiashvili et al. tested two strains of *P. ostreatus* against a variety of carbon sources including wastes such as mandarin peel, in the presence of yeast extract, and a variety of nitrogen sources in the presence of glucose [61]. Depending on the strain, the highest MnP activity was obtained in the presence of xylan, mandarin peel, peptone or casein. On the contrary, mandarin peel and carboxy-methylcellulose provided high laccase activity [61]. Laccase activity of *P. eryngii* and *P. ostreatus* was maximum in the presence of mandarine peels and grapevine sawdust as carbon sources and ammonium sulfate as the nitrogen source [64]. MnP activity of *Lentinus kauffmanni* was explored in media supplemented with different carbon and nitrogen sources [65]. The maximum MnP activity was observed in the presence of fructose or peptone. Songulashvilli et al. reported that glucose enhanced MnP titres of *Phellinus robustus* but repressed that of *Ganoderma adspersum* [66]. Ammonium sulfate and
ammonium tartrate were shown to have no impact on MnP activity but increased the laccase titles of P. robustus [66].

**Effect of carbon to nitrogen ratio**

Laccase activity of P. ostreatus, L. edodes and Agaricus blazei decreased and fungal growth increased at high carbon to nitrogen ratio [67]. High glucose and low ammonium chloride have been shown to be ideal for maximum laccase production in Cerrena unicolor MTCC 5159 [68]. MnP activity of L. edodes was suppressed by high levels of ammonium nitrate and asparagine at defined glucose levels [69]. High MnP activity was obtained in the presence of high concentrations of glucose and low ammonium tartrate concentrations in Trametes ssp. [70]. On the other hand, P. flavido-alba exhibited high MnP activity under carbon limitation and high levels of ammonium tartarate [71]. In this case, glucose was substituted with glycerol to accomplish carbon limitation. But, no clear idea of the effect of glucose limitation on MnP activity was postulated. Thus, nutrient regulation of laccase and MnP activity is a function of carbon and nitrogen sources and fungal strain.

**Effect of metallic cofactors**

Metal ions are involved in the regulation of the lignin degrading enzymes, laccase and MnP, which are most commonly encountered in selectively lignin degrading white rot fungi such as D. squalens and C. subvermispora [54, 72, 73]. The active site of laccase consists of four copper atoms. Copper acts as the transcriptional regulator of laccase and also regulates its activity [51, 62, 74]. However, low concentrations of copper have been shown to be limiting the
fungal growth due to its toxicity [75]. This limitation in fungal growth reflects in low enzyme production. In the presence of various carbon and nitrogen sources, copper has been reported to increase laccase activity. In liquid culture of malt agar, copper increased the laccase activity of *T. versicolor* [76]. Laccase isozymes were reported to be differentially regulated by copper in *P. ostreatus* [77]. It was also shown that free copper ions cause oxidative stress and hence delay the laccase transcription [78]. However, there are no reports on the regulation of copper effect on laccase activity by various carbon and nitrogen sources.

In the presence of H$_2$O$_2$, MnP catalyzes the oxidation of Mn$^{2+}$ to Mn$^{3+}$. Mn$^{3+}$ combines with an organic chelator such as oxalate, tartarate and malonate to form a low molecular weight, diffusible redox-mediator that targets the phenolic subunits of lignin [51, 79]. MnP regulation by Mn$^{2+}$ was also reported to be sensitive to the type and concentration of carbon and nitrogen sources. Regulatory effects of Mn$^{2+}$ on *P. chrysosporium* MnP activity was better under ammonium tartrate limitation, but glucose had no impact on Mn$^{2+}$ [80]. Low manganese concentrations have been shown to be beneficial for lignin degradation by *P. chrysosporium* [81]. Manganese not only regulates the genetic transcription of MnP isozymes, but also controls the secretion of MnP to the extracellular medium [82]. Yet, there is a limited knowledge on the effect of carbon and nitrogen sources on Mn$^{2+}$ regulation of MnP activity.

**Effect of co-cultivation**

Microorganisms belonging to different classes exist in close interaction with one another in the natural environment. The interactions between the microorganisms may be symbiotic or competitive. Decaying of trees is caused by one such interaction, which is due to the lignin and
polysaccharide degradation by basidiomycetes. White rot fungi degrades wood through a symbiotic relationship with other white rots, brown rots and soft rots. This close relationship between the basidiomycetes results in complex enzyme pattern which in turn promotes wood deterioration primarily by lignin degradation. When *P. chrysosporium* was co-cultured with *Aspergillus niger* and *A. oryzae*, the production of β-glucosidase, α-cellobiohydrolase, β-galactosidase, laccase and β-xylosidase were higher than the respective monocultures [83]. Lignin degradation has been reported to be synergistic in the presence of mixed cultures [84]. Compared to the monocultures, laccase activity was enhanced in the co-culture of *D. squalens* and *P. radiata* [20]. Co-cultivation also modifies the isoform composition of the lignin degrading enzymes [85]. The critical parameters regulating the synthesis of ligninolytic enzymes in co-cultivation are nutrient media composition and fungal speciess chosen for mixed culture [86]. Though there are studies on co-cultivation for production of lignin degrading enzymes, there is no defined media recommended for simultaneous production of lignin degrading enzymes using mixed cultures. Therefore, it is necessary to optimize a defined media for enhanced production of ligninolytic enzymes by co-cultivation.

**Statistical approach for optimization**

Response surface methodology (RSM) is a statistical tool used to optimize a range of factors for maximum production of a single response or multiple responses [87]. RSM helps in understanding the individual effects of factors and interaction effects between factors and to determine the factors that have significant influence on the response and hence to optimize the significant factors for maximum response. RSM allows the concurrent analysis of multiple
variables in less number of experiments which in turn results in less use of chemicals and related costs. Laccase activity of *Coriolus versicolor* NCIM 996 was increased by 7.6-times using a nutrient medium optimized by RSM [88]. By the application of parameter optimization by RSM, nutrient media was optimized separately for LiP, MnP and laccase of *P. chrysosporium* [89]. RSM based experimental design was used to optimize nutrient concentrations in growth media for maximum laccase and MnP of *Fomes sclerodermeus* [90]. Levin et al., optimized the media constituents in solid state fermentation for maximum laccase production of *T. trogii* [91]. However, there are no reports on optimized media for maximum production of lignin degrading enzymes using mixed cultures.

### 1.2.5 Hydrothermal treatment

Hydrothermal treatment is normally conducted at subcritical and supercritical temperatures of water and the pressure is maintained in the range of 10 to 25 MPa [92, 93]. As shown in the phase diagram (Figure 1-1), water exists in the liquid state under these conditions and is the main reactant that degrades the biomass. The polar nature of water at standard state involves the potential charge differential within the molecule, and is responsible for the dissolution of polar compounds only. But non-polar compounds are not soluble in water under these conditions [94]. Superheated water at elevated pressure and temperature close to the critical point exhibits properties entirely different from water at standard conditions [7]. The thermo-physical properties of subcritical water such as high density, high diffusivity, low viscosity, high compressibility and a high dissociation constant mimic the acid-catalyzed reactions [95]. Ionic reactions such as dehydration of carbohydrates and alcohols are favored in the subcritical range.
due to the relatively high density and high dissociation constant [96]. Hence, all these characteristics make the application of subcritical water as one of the alternative for the dissolution of hemicelluloses and partial disintegration of hydrophobic lignin fraction of the lignocellulosic substrate [93].

![Phase diagram of water in hydrothermal process](image)

Figure 1-1. Phase diagram of water in hydrothermal process

Table 1-1 shows the properties of the subcritical water in comparison to normal water. Hydrothermal treatment of lignocellulose substrate is operated at the same reaction temperature as that of dilute acid hydrolysis, and the resultant pretreated biomass had compatible characteristics as that of dilute acid pretreatment [97]. Since no chemical is used, there is no cost...
of chemical recycling or hazardous waste generation and also supports moderate operating
temperature and pressure (170-230°C and 5 MPa). The main drawback of hydrothermal
treatment is the rapid corrosion of the reactor due to the acidic and oxidizing conditions.
Optimum choices of reaction temperature and residence time can lead to less corrosion, low
operational cost, less waste generation, and low inhibitors generation [22].

Table 1-1. Thermo-physical properties of water

<table>
<thead>
<tr>
<th>Properties [95]</th>
<th>Normal water</th>
<th>Subcritical water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>25</td>
<td>250</td>
</tr>
<tr>
<td>Pressure (MPa)</td>
<td>0.1</td>
<td>5</td>
</tr>
<tr>
<td>Density (g cm⁻³)</td>
<td>1</td>
<td>0.80</td>
</tr>
<tr>
<td>Dielectric constant (F m⁻¹)</td>
<td>78.5</td>
<td>27.1</td>
</tr>
<tr>
<td>Ionic product pKᵢₚ</td>
<td>14.0</td>
<td>11.2</td>
</tr>
<tr>
<td>Heat capacity (kJ kg⁻¹ K⁻¹)</td>
<td>4.22</td>
<td>4.86</td>
</tr>
<tr>
<td>Dynamic viscosity (mPa s)</td>
<td>0.89</td>
<td>0.11</td>
</tr>
</tbody>
</table>

The reactions involved in the hydrothermal degradation of lignocellulosic biomass are
depolymerization, decomposition of monomers and re-combination of decomposition products
[98]. The operating conditions and reaction time are selective to the degradation of the specific
constituents in the lignocellulose material. Rogalinski et al. [99] have shown that hydrothermal
decomposition of pure cellulose to monosaccharides occurs between 240 and 310°C at 25 MPa,
but the rate was lower than that of starch. At temperatures below 350°C, the rate of cellulose
hydrolysis was much lower than the decomposition rate of glucose and leads to the formation of glucose decomposition products such as 5-hydroxymethyl furan and furfural which are the inhibitory compounds of the subsequent fermentation stage [99]. Hemicelluloses being amorphous due to the presence of side chains and non-uniform structure is easily solubilised at temperatures above 180°C [95]. Hemicellulose degradation conducted on various woody and herbaceous biomass revealed that almost 100% of hemicellulose was degraded by hydrothermal pretreatment at 230°C [100]. Further heating beyond the complete hydrolysis of hemicelluloses results in the formation of decomposition products such as glycolaldehyde, glyceraldehydes, and dihydroxyacetone [101]. Hydrothermal degradation of lignin is caused by the hydrolysis of ether bonds. Studies conducted on pure lignin revealed that the decomposition products were catechol, 2-methoxyphenol, 1,2-benzenediol, 4-methyl-1,2-benzenediol, 3-methyl-1,2-benzenediol, and phenol [102, 103]. In addition, solid yield due to the re-condensation of decomposition products of lignin was very high during the hydrothermal degradation of lignin [103]. Several phenol derivatives were formed during the hydrothermal processing of walnut shells at reaction temperatures of 200 to 300°C [104].

During the hydrothermal treatment of wheat straw, complete hemicelluloses and part of lignin were solubilised at 180-200°C for a residence time of 5-15 minutes [21]. Beech wood degradation conducted under hydrothermal conditions of 130-220°C and 15-180 min showed that hemicelluloses were completely solubilised and lignin was partially solubilised and re-located, resulting in increased surface area and total pore volume [105]. Hydrolyzates from hydrothermal treatment of lignocellulosic biomass mainly contain hemicelluloses derived sugars, furan derivatives, acetic acid and low molecular weight lignin derivatives [106].
Lignin depolymerization products re-condense together and accumulate on the substrate surface alongside cellulose [21, 23]. Kristensen et al. [21] reported that hydrothermal treatment of wheat straw between 190-200°C for 6 minutes resulted in wax removal, partial hemicelluloses solubilisation, lignin re-localisation and undisturbed fibrillar cellulose. Though lignin is re-localized, its mere presence restricts the access of cellulase for cellulose hydrolysis [107, 108]. Hence, after hydrothermal treatment of biomass, additional delignification step is necessary before proceeding to the enzymatic hydrolysis of cellulose. Delignification of hydrothermally treated lignocellulose materials are commonly conducted by alkali treatment or solvent extraction and hence contribute to high chemical cost and recycling cost [21].

The high temperature and high pressure requirements for dissolution of lignocellulosic substrate and the concurrent re-polymerization of lignin due to the formation of inhibitors such as furan and phenolic derivatives during hydrothermal treatment results in significant decrease in the availability of fermentable sugars. However, hydrothermal treatment conducted at relatively low temperature and reaction time could result in dissolution of hemicelluloses and lignin but with formation of less inhibitors. Also, for efficient enzymatic hydrolysis of cellulose in the treated substrate, the re-condensed lignin should be removed by employing an intermediate delignification process.

1.2.6 Combined effect of various pretreatments

Although fungal delignification is highly efficient and selective in lignin degradation, the long treatment time makes its application less desirable for biofuel generation from
lignocellulosic biomass. Due to their large molecular size, the penetration of lignin degrading enzymes into the lignocellulosic substrate is restricted [109]. Other delignification methods such as dilute acid treatment, hydrothermal treatment, steam explosion result in effective lignin degradation but are also accompanied by formation of products which inhibit the downstream enzymatic hydrolysis and fermentation [10, 21, 110, 111]. Hence, it would be beneficial to combine delignification techniques to maximize lignin removal and reduce inhibitors production.

Ball milling and microwave radiation were combined to enhance the enzymatic hydrolysis of microcrystalline cellulose [112]. *P. chrysosporium* treatment in association with mild acid treatment increased the cellulose hydrolysis of *Glycyrrhiza uralensis* residue by decreasing the inhibitor concentrations [113]. Dilute acid treatment was followed by aqueous ammonia treatment and resulted in increased yield of fermentable sugars from rice straw [114]. However, there are no reports on combining fungal treatment with hydrothermal treatment for effective lignin degradation and increased enzymatic hydrolysis to obtain fermentable sugars.

1.3 Objectives and Layout of the Thesis

The overall objective of this thesis is to increase the rate of fungal biodegradation and extent of delignification. Hence, two strategies have been put forth for investigation. The first strategy deals with enzyme induction and the second with combination of fungal treatment with hydrothermal process. Based on these strategies, the specific objectives of this thesis are as follows:
• To implement and understand the mechanism of ligninolytic enzyme induction in monocultures of white rot fungi.
• To simultaneously enhance the laccase and peroxidase activities of co-cultivation of two white rot fungi.
• To combine fungal treatment with hydrothermal process to improve the extent of delignification of a lignocellulosic substrate.

Chapter 1 deals with the introduction to this thesis, literature review and objectives. Fungal delignification of lignocellulosic biomass is efficient and selective. However, the constitutive enzyme synthesis by white rot fungi is low and hence the rate of lignin degradation is very low compared to other physical and chemical methods. Therefore, the ligninolytic enzymes must be induced to get high concentrations and hence rapid and effective lignin degradation.

Laccase activity of white rot fungi is induced by copper at low concentrations. But, at high concentrations, copper toxicity results in decreasing the fungal growth and laccase activity. The hypothesis for the first objective is that specific carbon and nitrogen sources and ratios could increase the copper tolerance of fungal growth and laccase activity. Chapter 2 focuses on the first objective which is to evaluate the copper induction and suppression of laccase activity in the white rot fungus, *Dichomitus squalens*, in the presence of various carbon and nitrogen sources at a range of copper, carbon and nitrogen concentrations and ratios. Similar study was also conducted with another white rot fungus, *Ceriporiopsis subvermispora* and the details are given in Appendix A. This study helps in identifying the appropriate carbon and nitrogen sources and
the copper concentration which would result in increase of the laccase tolerance to copper and hence allow obtaining high laccase activity.

The role of manganese as a cofactor of the enzyme, manganese peroxidase is indispensable. Though manganese does not have detrimental effect on fungal growth, its inducing effect on peroxidase activity in the presence of different carbon and nitrogen sources is unknown. The hypothesis for the second objective is that manganese induction of peroxidase activity could be increased in the presence of a specific carbon and nitrogen source. Chapter 3 will focus on the second objective which is to understand the induction and suppression behavior of manganese on peroxidase activity of two white rot fungi, D. squalens and C. subvermispora in the presence of various carbon and nitrogen sources. This study helps in identifying the manganese concentration and the right carbon and nitrogen sources that would result in high peroxidase activity.

The best carbon and nitrogen sources and the relevant concentrations, copper and manganese concentrations will be chosen from the previous two studies and if appropriate, the two white rot fungi will be co-cultivated in order to increase the simultaneous activity of laccase and peroxidase. Chapter 4 will focus on the co-cultivation of two white rot fungi for enhanced laccase and peroxidase activity. The nutrient parameters chosen from the first two studies will be optimized by the application of response surface methodology for enhanced activity of laccase and peroxidase using the co-culture of D. squalens and C. subvermispora.

White rot fungi degrades lignin effectively, but the process is very slow. On the other hand, hydrothermal treatment degrades lignocelluloses rapidly, but results in lignin re-deposition on
the substrate surface, which in turn blocks the cellulase penetration towards cellulose. Therefore, a co-treatment strategy with hydrothermal process as the first step followed by the fungal delignification will be explored and evaluated for effective lignin degradation and cellulose availability. *Chapter 5* will focus on the combined treatment of wheat straw using hydrothermal process and fungal delignification.

*Chapter 6* will be a synthesis chapter which specifies the connections between the chapters 2, 3, 4, and 5. In addition, kinetic modeling of laccase catalyzed degradation of phenolic content in wheat straw will be included. Finally, conclusions and recommendations for future work will be elaborated in *Chapter 7*. 
1.4 References


Chapter Two: Enhancement of Dichomitus squalens Tolerance to Copper and Copper-associated Laccase Activity by Carbon and Nitrogen Sources

2.1 Presentation of the Article

This article is concerned with the understanding of the effect of various carbon and nitrogen sources combined with the laccase inducer, copper sulfate on laccase activity and fungi growth and viability. The carbon sources chosen ranged from hexoses such as glucose, mannose; pentoses such as arabinose, xylose; disaccharides such as cellobiose; polysaccharide such as methylcellulose and methyl substituted sugar such as 3-O-methylglucose. The rationale for choosing a wide range of carbon sources is to investigate if there is any specific impact of the chosen carbon source on the behavior of laccase activity and the related fungal growth. Organic (casein), inorganic (sodium nitrate) and ammoniated (ammonium chloride) sources were chosen as various nitrogen sources in order to understand the effect of the nature of the nitrogen sources on laccase activity. Previous reports have stated different toxic levels for copper depending on the fungal species. Copper toxicity varied between 0 and 1 mM and hence this range of copper sulfate was chosen for this study. The results obtained from the above study showed that copper toxicity was laccase-specific and fungal growth-specific. The specific combination of glucose and casein at a C/N ratio of 1:10 resulted in alleviating the copper toxicity on fungal growth. 2.5-3 fold higher laccase activity was observed when casein was combined with methylated sugars compared to that of glucose.
Enhancement of Dichomitus squalens Tolerance to Copper and Copper-associated Laccase Activity by Carbon and Nitrogen Sources

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2.2 Abstract

This study revealed a dual effect of copper on *D. squalens* laccase activity, inducing and suppressing. The addition of copper sulfate led to enhancement of the enzyme activity until it reached a maximum value at peak copper sulfate concentration (PC) followed by suppression and even complete termination of the enzyme activity. Maximum laccase yield is a function of fungi tolerance and media composition. Nutrients had a significant impact on copper-independent and copper-induced laccase activity. Some nutrients also improved tolerance of cells and laccase-producing system to copper load. The effect of specific nutrient depended on the ratio of carbon and nitrogen sources. This research can be used to predict the effective copper concentration and nutrient composition to stimulate the laccase production without compromising the fungi growth. The methylated substrates (3-⁴-methylglucose, methylcellulose) in combination with casein and copper demonstrated high potential to support fungi tolerance and enhanced laccase activity.
2.3 Introduction

The production of biofuels, e.g. bioalcohols, via the conversion of biomass is a rapidly growing industry. However, current biofuel generation faces supply-limitation pressures since most bioconversion technologies rely on the fermentation of ‘food crop’-derived sugars to bioalcohols and, hence, interfere with the food supply chain. An attractive approach is to replace food crops used for biofuel generation with lignocellulosic raw materials. Lignocellulosic materials constitute a large portion of the wastes produced by different industries including forestry, pulp and paper, and agriculture. The basic element of this technology is the hydrolysis of biomass cellulose to sugars with subsequent sugar fermentation to bioalcohol. The utilization of waste cellulose increases the amount of biofuel that can be produced sustainably without competing with the food supply. The principal challenge to the ‘cellulosic’ biofuel generation is the complex structure of lignocellulose which locks the fermentable sugars within the lignin-hemicellulose matrix [1]. Lignin-hemicellulose networks normally protect the embedded cellulose from interaction with water, solvents and enzymes, and, hence, prevent cellulose hydrolysis to simple sugars and further fermentation to bioalcohols. Thus, efficient conversion of lignocellulosic waste to biofuel requires the pre-removal of lignin and hemicellulose.

Chemical delignification, widely used in biofuel generation, is expensive, energy consuming, and results in the release of inhibitors such as weak acids, furan and phenolic compounds which reduce hydrolysis and fermentation rates and overall biofuel yield [2]. Alternatively, lignin can be degraded by the action of lignin-degrading enzymes produced by white-rot fungi [3]. This approach is based on naturally occurring delignification and has the
advantage of: low-energy demand; minimal waste and toxic compounds production; and negligible environmental impact. Some white-rot basidiomycetes such as Dichomitus squalens selectively degrade lignin [4] in a wide range of substrates such as hard or soft wood, and grass [5, 6] without removing the cellulose subsequently needed for sugar release and fermentation [7].

The main D. squalens enzyme participating in delignification is laccase (EC.1.10.3.2). The activity of laccase is determined by copper, which is the active center and transcriptional regulator of the enzyme [8-10]. However, copper is toxic for fungi at relatively low concentrations and limits fungi growth, and consequently laccase production [11]. On the other hand, some carbon and nitrogen sources have been reported to enhance laccase activity in copper-containing media [12, 13]. The mechanism for laccase activity enhancement by media modification is not elucidated. We hypothesize that some carbon and nitrogen sources and their combinations could impact fungi tolerance to copper and, hence, facilitate copper-associated laccase activity. To this end, D. squalens growth capacity, tolerance of laccase producing system, and laccase activity in response to copper sulfate load in the presence of different carbon and nitrogen sources at different relative proportions in the media were investigated. The goal of this investigation is to identify the possibility to enhance fungi tolerance to copper and associated laccase activity by modification of media composition and set up optimal cultivation conditions for effective laccase production by D. squalens.
2.4 Materials and Methods

2.4.1 Microorganism and chemicals

*Dichomitus squalens* ATCC 201541 was purchased from American Type Culture Collection (ATCC), USA. All chemicals were purchased from Sigma-Aldrich.

2.4.2 Cultivation conditions

Fungi were incubated in basic mineral medium containing KCl - 0.56 g/l, MgSO$_4$$\cdot$7H$_2$O - 0.78 g/l, FeSO$_4$$\cdot$7H$_2$O - 8 mg/l, KH$_2$PO$_4$ - 2.22 g/l and thiamine - 1 mg/l (pH 5). Standard (control) medium was supplemented with 1% of glucose and 20 mM of sodium nitrate. For experimental purposes, the basic medium were supplemented with a range of carbon sources including glucose, 3-O-methylglucose, methylcellulose, cellobiose, mannose, arabinose and xylose at high level (1%) and low level (0.1%) with sodium nitrate as nitrogen source at high level (20 mM) and low level (2 mM). Alternatively, basic media with different nitrogen sources such as sodium nitrate (20 mM and 2 mM), ammonium chloride (20 mM and 2 mM), and casein hydrolysate (0.5% and 0.05%) were supplemented with glucose as carbon source at 1% and 0.1%. Copper sulfate was added to the media as an inducer in the range of 0 to 1 mM. The 24-well microplates were filled with 2 ml of challenge media and inoculated with agar plug of 3 mm in diameter obtained from the edge of actively growing fungal colonies on potato-dextrose agar (PDA). The plates were incubated at 25°C with agitation at 150 rpm for seven days. Cell-free media with extracellular enzymes from each well was collected on the seventh day and were analyzed for laccase activity. Fungi grown on agar plugs in each well were transferred on to fresh PDA plates to measure growth capacity.
2.4.3 Dynamics of copper-induced laccase production

To determine copper impact on fungi growth and laccase activity, standard medium was supplemented with copper sulfate at concentrations ranging from 0.06 mM to 1 mM. The plates were incubated at 25°C with agitation at 150 rpm for seven days. At that point, fungi growth and laccase production were determined as function of copper concentration.

2.4.4 Impact of carbon-to-nitrogen ratios

To determine the impact of carbon-nitrogen (C/N) ratios on fungi growth and laccase activity, four challenge media were designed as follows: (i) Low Carbon and Low Nitrogen sources (LC/LN), (ii) Low Carbon and High Nitrogen (LC/HN), (iii) High Carbon and Low Nitrogen (HC/LN), and (iv) High Carbon and High Nitrogen (HC/HN). High levels designate concentrations 10 times higher than those for low levels (actual individual nutrient concentrations are presented in section 2.3.2), relative C/N ratios were: 1:1 for LC/LN, 1:10 for LC/HN; 10:1 for HC/LN; and 10:10 for HC/HN. Additionally, challenge media were supplemented with copper sulfate at concentration ranging from 0 to 1 mM to determine copper interaction with carbon and nitrogen sources at different levels. The plates were incubated at 25°C with agitation at 150 rpm for seven days. Fungi growth and laccase production in response to challenge media were determined as described below.
2.4.5 Tolerance and biomass units

To determine fungi viability and growth capacity, fungal mycelium grown in challenge media was transferred to fresh PDA plates. Plates were incubated at 25°C under static conditions. Radial growth rates were quantified by the increase in the fungal colony radius (in mm) per hour, and used to determine viable biomass units for calculation of laccase activity (section 2.3.6), and fungi tolerance to copper represented by minimum suppressive (MSC) and minimum eradication (MEC) concentrations. The MSC were determined as the minimum level of copper sulfate, which reduced fungal growth rate compared to corresponding copper-free media. The MEC was determined as the minimum level of copper sulfate, which completely kills the exposed fungi population (no radial growth observed).

2.4.6 Copper impact on laccase activity

Laccase activity was determined by monitoring the oxidation of 2, 6-dimethoxyphenol (DMP) to an orange brown dimer [14]. The reaction mixture (200 µl) containing 2 mM of DMP in 0.1 M phosphate buffer (pH 6.0) and 50 µl of supernatant was incubated at 30°C for 15 min [15]. The absorbance at 468 nm was monitored in microplates (path length 0.6 cm) with Spectramax Multimode microplate reader [16]. Laccase activity was expressed in activity units (AU), which are international units per liter (U/l) per biomass units (mm/h). One international unit of laccase activity is defined as the amount of enzyme that oxidizes 1 µmol of DMP (molar extinction coefficient 4.96 × 10⁴ M⁻¹cm⁻¹) per min [14]. Biomass units were determined as radial (mm/h) growth of fungal biomass on PDA after exposure to challenged media. The laccase activity dynamics as function of copper load was characterized with minimum laccase inductive
(MLIC), peak (PC) and minimum laccase suppressive (MLSC) concentrations. The MLIC was determined as the minimum level of copper sulfate, which increased laccase activity compared to corresponding copper-free media. The PC was determined as copper sulfate level, which provided maximum laccase activity. The MLSC was determined as minimum copper sulfate level, which reduced laccase activity compared to corresponding copper-free media.

2.4.7 Statistical analysis

All the experiments were carried out in triplicate. One way ANOVA, $t$-test and correlation analysis were used to estimate statistical differences in growth rate and enzyme activity between experimental conditions.

2.5 Results

2.5.1 Impact of carbon and nitrogen sources on fungi tolerance to copper

The copper effect on the $D. squalens$ growth was studied in standard and challenge media in order to understand whether the tolerance to copper is a function of media composition. The types and proportion of tested carbon and nitrogen sources in copper-free media did not impact the growth capacity of $D. squalens$ in copper-free media. The average growth rate was around $0.15$ mm per hour. Addition of copper sulfate at very low concentrations did not impact the $D. squalens$ growth rate, but further increase in the copper concentration resulted in growth suppression compared to copper-free media at a certain copper sulfate level, referred to as the minimum suppressive concentration (MSC). Further increase in copper concentration resulted in
more adverse effect in fungi growth rate towards total destruction of the growth. The minimum level of copper sulfate, which eradicated fungal culture, was defined as the minimum eradication concentration (MEC). Although the general trend observed for fungi response to copper load under all tested conditions were qualitatively similar, the effective copper sulfate concentrations (MSC and MEC) depended on challenge media composition (Table 2-1).

Table 2-1. Growth rates of *Dichomitus squalens* in response to different carbon-to-nitrogen ratios for a range of carbon and nitrogen source

<table>
<thead>
<tr>
<th>Carbon /Nitrogen source</th>
<th>Carbon-to-nitrogen ratios $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:10</td>
</tr>
<tr>
<td>Glucose $^b$</td>
<td>0.13±0.02 $^a$</td>
</tr>
<tr>
<td>3-0-methylglucose</td>
<td>0.20±0.04</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>Ammonium chloride $^c$</td>
<td>0.18±0.04</td>
</tr>
<tr>
<td>Casein</td>
<td>0.21±0.01</td>
</tr>
</tbody>
</table>

$^a$ Radial growth rates were quantified by the increase in fungal colony radius (in mm) per hour (See section 2.3.5).

$^b$ Different carbon sources were tested in combination with sodium nitrate as standard nitrogen source.

$^c$ Ammonium chloride and casein were tested with glucose as standard carbon source.

$^d$ C/N ratios: 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN.
Table 2-2 shows the MSC and MEC values obtained for various C/N ratios for each of carbon and nitrogen combinations. The MSC value for *D. squalens* incubated in standard medium supplemented with 1% of glucose and 20 mM of sodium nitrate (10:10 C/N ratio) was 0.18 mM of copper sulfate. The ten-fold reduction of the concentrations of one or both nutrients (10:1, 1:1 and 1:10 ratios) did not impact *D. squalens* tolerance. This trend was also observed for *D. squalens* response to copper in the presence of mannose and methylcellulose. However, substitution of glucose with equivalent amounts of 3-O-methylglucose or xylose reduced *D. squalens* tolerance to copper compared to standard media; while, cellobiose and arabinose enhanced it. However, a ten-fold decrease of 3-O-methylglucose and xylose concentration regardless of the sodium nitrate level (1:10 and 1:1 ratios) increased *D. squalens* tolerance to the level observed in glucose-containing media. In contrast, reduced concentration of cellobiose (1:10 and 1:1 ratios) decreased *D. squalens* tolerance to copper below the glucose level. In the media supplemented with arabinose and sodium nitrate, reduction of one or both nutrients (10:1, 1:10 and 1:1) resulted in decreased *D. squalens* tolerance, for 1:1 ratio, again, below the glucose level.

The substitution of sodium nitrate (in the presence of glucose at C/N ratio of 10:10) with casein resulted in increase of MSC values up to 1.0 mM (Table 2-2), while ammonium chloride reduced *D. squalens* tolerance up to 0.06 mM. However, ten-fold decrease in casein concentration regardless the level of glucose (10:1 and 1:1 ratios) led to decrease of MSC values up to 0.25 mM, albeit still was higher than in corresponding standard media. For ammonium chloride-containing media, reduction of one of the nutrients (ratios of 1:10 and 10:1) led to
enhancement of MSC values up to 0.25 mM; whereas, low (1:1) level of both compounds resulted in low *D. squalens* tolerance equivalent to standard (10:10) ratio.

The sensitivity of MEC values to the media composition was lower than MSC response. The eradication of *D. squalens* was observed at 0.25 mM of copper sulfate in the media containing glucose, mannose, methylcellulose, and at 0.5 mM in the media containing 3-O-methylglucose, cellobiose, xylose, arabinose and ammonium chloride irrespective of C/N ratios. In the media supplemented with glucose and casein, *D. squalens* tolerance to copper load depended on casein levels. At low casein concentrations, the viability of *D. squalens* was similar to other tested media. However, at high level of casein, the copper sulfate MEC exceeded 1.0 mM, which was the highest tolerance level observed.

Thus, suppression of the *D. squalens* growth was observed at relatively low copper sulfate concentrations for most tested conditions, but MSC values significantly increased in the presence of high level of cellobiose regardless of the concentration of nitrate. Similar trend in MSC value was observed for high level of casein irrespective of glucose concentration. Similarly, MSC values were also high for media with arabinose at high sodium nitrate level, and in the glucose/ammonium chloride media only when one of the nutrients was supplemented at high level (Table 2-2). The MEC values were independent of the nutrient type and C/N ratios. Only high concentrations of casein enhanced the MEC values to above 1mM, which is the maximum tested concentration.
Table 2-2. Minimum suppressive (MSC) and eradication (MEC) concentrations of copper sulfate in response to different C/N carbon-to-nitrogen ratios for a range of carbon and nitrogen source

<table>
<thead>
<tr>
<th>Carbon/nitrogen source</th>
<th>Effective concentrations</th>
<th>Carbon-to-nitrogen ratios $^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10:10</td>
</tr>
<tr>
<td>Glucose $^a$</td>
<td>MSC $^c$</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>MEC $^d$</td>
<td>0.25</td>
</tr>
<tr>
<td>3-O-methylglucose</td>
<td>MSC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.5</td>
</tr>
<tr>
<td>Mannose</td>
<td>MSC</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.25</td>
</tr>
<tr>
<td>Xylose</td>
<td>MSC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.5</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>MSC</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.5</td>
</tr>
<tr>
<td>Arabinose</td>
<td>MSC</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.5</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>MSC</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.25</td>
</tr>
<tr>
<td>Ammonium chloride $^b$</td>
<td>MSC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.5</td>
</tr>
<tr>
<td>Casein</td>
<td>MSC</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>$&gt;1.0$</td>
</tr>
</tbody>
</table>

$^a$ Different carbon sources were tested in combination with sodium nitrate as standard nitrogen source.

$^b$ Ammonium chloride and casein were tested with glucose as standard carbon source.

$^c$ MSC is the minimum level of copper sulfate, which reduced fungal growth rate compared to corresponding copper-free media.

$^d$ MEC is the minimum level of copper sulfate, which completely kills the exposed fungi population.

$^e$ C/N ratios: 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN. bold indicates maximum tolerance level observed.
2.5.2 Impact of carbon and nitrogen sources on laccase activity

Laccase activity was investigated for *D. squalens* incubated in standard and challenge media supplemented with different carbon and nitrogen sources. Although growth rate of *D. squalens* in copper-free media did not depend on media composition (as indicated in section 2.4.1), specific laccase activity was a function of carbon and nitrogen source type and proportion in the media. The laccase activity obtained in the standard copper free medium supplemented with 1 % glucose and 20 mM sodium nitrate (C/N ratio of 10:10) was 0.54 AU (Table 2-3). The substitution of glucose in the standard media with arabinose, cellobiose and xylose resulted in increasing the laccase activity by 1.4 – 4-fold. 3-O-methylglucose and mannose increased laccase by 8 and 13 folds, respectively. In contrast, methylcellulose reduced the laccase activity by 50 %. When sodium nitrate in the standard media was substituted with ammonium chloride and casein, laccase activity increased drastically: by 62 and 54-folds, respectively.
Table 2-3. Laccase activity in response to different carbon and nitrogen sources and their ratios under copper-free conditions

<table>
<thead>
<tr>
<th>Carbon/nitrogen source</th>
<th>Carbon-to-nitrogen ratios&lt;sup&gt;c&lt;/sup&gt;</th>
<th>10:10</th>
<th>10:1</th>
<th>1:10</th>
<th>1:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>0.54±0.01</td>
<td>0.66±0.04</td>
<td>0.27±0.01</td>
<td>0.34±0.08</td>
</tr>
<tr>
<td>3-O-methylglucose</td>
<td></td>
<td>4.26±0.81</td>
<td>1.82±0.07</td>
<td>1.07±0.17</td>
<td>2.25±0.03</td>
</tr>
<tr>
<td>Mannose</td>
<td></td>
<td>6.96±0.34</td>
<td>2.72±0.21</td>
<td>3.56±0.03</td>
<td>0.75±0.05</td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td>1.19±0.33</td>
<td>1.05±0.17</td>
<td>1.13±0.31</td>
<td>0.66±0.04</td>
</tr>
<tr>
<td>Cellobiose</td>
<td></td>
<td>2.09±0.07</td>
<td>2.22±0.03</td>
<td>0.42±0.02</td>
<td>0.67±0.02</td>
</tr>
<tr>
<td>Arabinose</td>
<td></td>
<td>0.94±0.6</td>
<td>0.79±0.2</td>
<td>1.72±0.82</td>
<td>0.59±0.3</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td></td>
<td>0.05±0.00</td>
<td>0.31±0.04</td>
<td>0.05±0.00</td>
<td>0.11±0.00</td>
</tr>
<tr>
<td>Ammonium chloride&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>33.28±0.74</td>
<td>13.70±0.77</td>
<td>23.10±1.26</td>
<td>15.81±1.15</td>
</tr>
<tr>
<td>Casein</td>
<td></td>
<td>29.38±1.92</td>
<td>29.06±1.41</td>
<td>14.95±1.2</td>
<td>9.71±0.15</td>
</tr>
</tbody>
</table>

<sup>a</sup> Different carbon sources were tested in combination with sodium nitrate as standard nitrogen source

<sup>b</sup> Ammonium chloride and casein were tested with glucose as standard carbon source

<sup>c</sup> C/N ratios: 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN

The ten-fold reduction of one or both nutrients, in most cases, led to reduction or improvement of laccase activity (Table 2-3). In particular, laccase activity increased by 22% when sodium nitrate level was decreased by ten-fold in the presence of high glucose level (C/N ratios of 10:1). Reducing the glucose by ten-folds resulted in two-fold decreasing of the laccase activity irrespective of the sodium nitrate concentration (1:10 and 1:1). Laccase activities also decreased under all conditions expect 10:10 C/N ratios for 3-O-methylglucose and mannose.
Laccase activity did not significantly changed when either xylose or sodium nitrate reduced, but the activity decreased to 50% when both the nutrients reduced by ten-folds. The ten-fold reduction of cellobiose concentration led to decrease of laccase activity irrespective of the nitrate concentration, but lowering the sodium nitrate level did not show any significant effect on laccase activity.

In the case of arabinose with sodium nitrate, C/N ratio of 1:10 resulted in two-fold increase in laccase activity compared to the standard 10:10 ratio. Reduced methylcellulose or sodium nitrate concentrations resulted in 2-4 fold laccase activity increase. The highest activity occurred when both methylcellulose and sodium nitrate were at their low level (1:1). For the case of glucose-ammonium chloride, all other C/N ratios except the standard 10:10 ratio tend to reduce the laccase activity. In the case of glucose-casein, decreasing the glucose concentration resulted in reducing the laccase activity irrespective of casein level (1:10, 1:1).

Thus, *D. squalens* laccase activity varied according to the type and proportion of carbon and nitrogen sources in the media. In general, replacement of either the standard carbon source (glucose) or the standard nitrogen source (sodium nitrate) with alternative carbon or nitrogen sources resulted in enhancement of laccase activity. However, reduction of the nutrients’ level led to reduction of laccase activity. The highest yield (33 AU) was observed for the media supplemented with glucose and ammonium chloride at 10:10 ratio.
2.5.3 Copper-associated enhancement of laccase activity

For all tested conditions, addition of copper sulfate to the media significantly increased *D. squalens* laccase activity (Table 2-4). The minimum copper sulfate concentration required to induce the laccase activity (Minimum Laccase Inducing Concentration, MLIC) under all tested conditions was below 0.06 mM, the minimum copper sulfate level analyzed in this study. The increase in copper sulfate concentration resulted in increasing laccase activity until it reached a maximum values at certain copper sulfate levels defined as Peak Concentrations (PC). Further increase in copper sulfate concentration tended to reduce the laccase activity to a level when the laccase activity was lower than that observed under copper-free conditions. This critical copper sulfate concentration was referred to as Minimum Laccase Suppressive Concentration (MLSC). PC and MLSC depend on the type and relative proportion of media carbon/nitrogen sources (Table 2-4).

The PC values in standard and challenge media were relatively low 0.06 – 0.12 mM and varied insignificantly in response to changes in C/N ratios. The only exception was arabinose-nitrate media, where the PC increased up to 0.25 mM at C/N ratio of 10:10. In contrast to PC, maximum laccase activity changed significantly with the media composition (Table 2-5). Irrespective of sodium nitrate concentration, media supplemented with glucose, xylose, and cellobiose provided twice higher peak laccase activity at high carbon concentrations (ratios of 10:10 and 10:1) than at low carbon level (1:10, 1:1).
Table 2-4. Peak (PC) and Minimum laccase suppressive (MLSC) concentrations of copper sulfate in response to different carbon-to-nitrogen ratios for a range of carbon and nitrogen source

<table>
<thead>
<tr>
<th>Carbon/nitrogen source</th>
<th>Copper (mM)</th>
<th>Carbon-to-nitrogen ratios ³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10:10</td>
</tr>
<tr>
<td>Glucose ⁴</td>
<td>PC  ⁵</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MLSC ⁶</td>
<td>0.18</td>
</tr>
<tr>
<td>3-O-methylglucose</td>
<td>PC</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.25</td>
</tr>
<tr>
<td>Mannose</td>
<td>PC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.12</td>
</tr>
<tr>
<td>Xylose</td>
<td>PC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.37</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>PC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.25</td>
</tr>
<tr>
<td>Arabinose</td>
<td>PC</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.37</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>PC</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.18</td>
</tr>
<tr>
<td>Ammonium chloride ⁷</td>
<td>PC</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.25</td>
</tr>
<tr>
<td>Casein</td>
<td>PC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.25</td>
</tr>
</tbody>
</table>

⁴ Different carbon sources were tested in combination with sodium nitrate as standard nitrogen source.
⁵ PC (Peak concentration), minimum level of copper sulfate, which provides maximum laccase activity.
⁶ MLSC (Minimum laccase suppressive concentration), minimum level of copper sulfate, which suppress the laccase activity compared to copper-free conditions.
⁷ C/N ratios: 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN.
Table 2-5. Maximum laccase activity obtained at various C/N ratios for a range of carbon and nitrogen sources

<table>
<thead>
<tr>
<th>Carbon/nitrogen source</th>
<th>Carbon-to-nitrogen ratios c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:10</td>
</tr>
<tr>
<td>Glucose a</td>
<td>3.20±0.24</td>
</tr>
<tr>
<td>3-O-methylglucose</td>
<td>42.59±37.03</td>
</tr>
<tr>
<td>Mannose</td>
<td>15.90±0.86</td>
</tr>
<tr>
<td>Xylose</td>
<td>37.29±2.69</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>39.60±0.19</td>
</tr>
<tr>
<td>Arabinose</td>
<td>22.81±6.2</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>26.15±0.49</td>
</tr>
<tr>
<td>Ammonium chloride b</td>
<td>57.67±1.57</td>
</tr>
<tr>
<td>Casein</td>
<td>29.38±1.91</td>
</tr>
</tbody>
</table>

a Different carbon sources were tested in combination with sodium nitrate as standard nitrogen source.
b Ammonium chloride and casein were tested with glucose as standard carbon source.
c C/N ratios: 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN.

In contrast, peak laccase activity was twice higher in the media with 3-O-methylglucose, mannose and methylcellulose at low carbon concentration (1:10, 1:1) than those observed at high carbon level (10:10, 10:1) independent of nitrogen level. Two-fold higher laccase was expressed with arabinose at C/N ratio of 10:1 compared to other conditions. In the media with glucose and ammonium chloride, low carbon conditions (1:10, 1:1) provided higher peak activity, irrespective of the nitrogen concentration. Glucose with casein resulted in three-fold higher peak
activity at low casein level (1:1 and 10:1) compared to high nitrogen conditions. The highest laccase activity (about 70 AU) was determined in media supplemented with glucose and either ammonium chloride and casein. Among carbon group, 3-O-methylglucose and methylcellulose provided the highest yield, about 60 AU.

### 2.5.4 Copper-associated suppression of laccase activity

As discussed above, increasing the copper sulfate concentration eventually resulted in suppression of the laccase activity compared to copper-free media. This level was characterized by MLSC. As for other tested parameters, MLSC values were also dependent on the nature and concentration of the carbon and nitrogen sources (Table 2-4). In particular, MLSC in the media supplemented with glucose, mannose and methylcellulose was 0.12 – 0.18 mM irrespective to C/N ratios. Suppression of laccase activity in the media supplemented with 3-O-methylglucose and arabinose was observed at MLSC of 0.25 – 0.37 mM, again irrespective to C/N ratios. Similar trend was also observed in the presence of glucose and ammonium chloride with MLSC of 0.25 mM. In contrast, MLSC values in the presence of xylose and cellobiose were as low as 0.12 – 0.18 mM at 1:10 C/N ratio, but increased up to 0.25 – 0.37 mM at other tested C/N ratios. In the media supplemented with glucose and casein, even the minimum tested copper sulfate concentration, 0.06 mM was found to suppress the laccase activity at C/N of 10:10. However, laccase activity was suppressed at 0.37 mM of copper sulfate when either or both nutrient concentrations were ten-fold decreased. The highest MLSC value was observed for arabinose in the presence of sodium nitrate (0.37mM for all tested ratios).
2.5.5 Laccase activity in response to casein in combination with alternative sugars

Media with glucose and casein was observed to be the best in regard to high fungi tolerance to copper sulfate and related laccase activity; whereas, 3-O-methylglucose and methylcellulose with sodium nitrate provided the highest laccase activity among carbon sources tested. In this experiment, glucose in casein media was substituted with either 3-O-methylglucose or methylcellulose in order to enhance laccase activity. Sugars and casein were added to media at different C/N ratios: 10:10, 10:1, 1:10 and 1:1. Fold increases in laccase activity in media supplemented with alternative sugars compared to glucose-containing media at corresponding ratio are presented in Table 2-6.

Table 2-6. Fold increase in laccase activity in response to replacement of glucose in the media supplemented with casein on 3-O-methylglucose and methylcellulose

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Carbon-to-nitrogen ratios a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:10</td>
</tr>
<tr>
<td>3-O-methylglucose</td>
<td>2.34</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>1.04</td>
</tr>
</tbody>
</table>

a C/N ratios: 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN
Both alternative sugars allowed enhancement of laccase activity compared to glucose. However, the level of improvement depended on sugar/casein ratio. The lowest enhancement was observed for 3-O-methylglucose and casein at 1:1 ratio. At high levels of casein, but low levels of 3-O-methylglucose, laccase activity increased 1.6-fold compared to glucose media at corresponding ratios. Approximately, 2.5-fold increase was observed when glucose was substituted by 3-O-methylglucose at 10:10 and 10:1 ratios. Methylcellulose provided 2.5 and 3.0-fold enhancement of laccase activity when substituting glucose at 10:1 and 1:1 ratios, respectively and 1.9-fold at 1:10 ratio. For high levels of both sugar and casein, no difference was observed between glucose and methylcellulose.

2.6 Discussion

Copper is essential for production and activity of laccase, one of the important lignin-modifying enzymes [17]. However, the present study clearly demonstrated dual effects of copper on the specific laccase activity: inducing and suppressing. The induction of D. squalens laccase activity required copper sulfate addition as low as 0.06 mM. Further increase of the copper sulfate concentration led to enhancement of the laccase activity until it reached a maximum values at certain copper sulfate level defined as Peak Concentration (PC) followed, however, by suppression and even complete termination of the enzyme activity. Mechanism of the laccase induction by copper is associated with its role as laccase active center [11], and its participation in the regulation of laccase genes transcription and post-transcription modification of enzyme [13]; whereas, copper toxicity is attributed to the interaction of copper ions with proteins,
enzymes, nucleic acids, and metabolites associated with cell functions and viability, and due to oxidative stress [18, 19].

The switch from copper inducing to suppressing effect was observed to be a function of fungi tolerance to copper, and was regulated by the type and ratio of carbon and nitrogen sources. Two factors associated with fungi tolerance to copper were identified: growth tolerance (the competence of fungi to grow in the presence of copper without undergoing any significant difference in the growth capacity compared to copper-free conditions [20]) and biochemical tolerance (the competence of the laccase-producing system to function at the level observed for copper-free conditions), and their impacts on specific laccase activity were investigated.

Fungi growth inhibition by copper is known [11, 13, 21]. In contrast to other essential metals, copper is known to inhibit fungi growth capacity at very low concentrations [11]. For example, D’Souza-Ticlo et al. [22] and Patel et al. [23] independently reported that copper sulfate concentrations above 0.3 mM led to the reduction in C. unicolor and P. otsreatus growth rates. In the present paper, growth tolerance was characterized by the copper sulfate minimum suppressive concentrations (MSC, the minimum levels of copper sulfate, which reduced fungal growth rate compared to corresponding copper-free media). Along with its impact on fungi growth, copper was observed to be toxic for specific (independent of growth) laccase activity. Fungi response to this type of copper toxicity was termed biochemical (in respect to laccase-production system) tolerance and characterized by the copper sulfate minimum laccase suppressive concentrations (MLSC, the minimum concentrations of copper sulfate, which suppressed laccase activity compared to copper-free conditions).
The MSC values for *D. squalens* was below 0.2 mM for most tested carbohydrates, but MSC values significantly increased in the presence of high concentrations of cellobiose and arabinose. In media with either casein or ammonium chloride, enhanced tolerance was observed even at low levels, and further increased at higher levels. The impact of enhanced MSC on laccase activity was not uniform. The increase of MSC values improved specific laccase activity ($r = 0.91$) in cellobiose-containing media, reduced it ($r = -0.94$) in the presence of casein, and had no impact on laccase activity in the presence of arabinose and ammonium chloride. The carbohydrates that provided lower levels and range (0.06 mM to 0.18 mM depending on C/N ratios) of tolerance also influenced the laccase activity, and their impacts again were not uniform. 3-O-methylglucose and methylcellulose enhanced both tolerance and enzyme activity ($r$ values 0.9 and 0.5, respectively) when nutrients’ concentration decreased. Glucose and xylose provided higher tolerance when added at low levels, but enhanced laccase activity at high levels ($r$ values -0.76 and -0.97, respectively).

The simplest explanation for nutrient-induced tolerance is inactivation of the copper ions via complex formation as reported for cellobiose, ammonium chloride and casein [24, 25]. This scenario is attributed to the ability of the nutrient to bind and, hence, reduce bioavailability of the copper ions and their toxic effect on fungi culture. And, this property is a function of nutrient concentration: higher the level of nutrient, higher the binding capacity. However, reduced bioavailability of copper would lead to higher copper demand to support laccase production, and, hence, results in reduced laccase activity. Casein seems to be the only nutrient tested in this study involved in copper complex formation under tested conditions. Casein was observed to increase fungi tolerance at higher concentrations, but enhanced laccase activity at low concentrations. The
complex formation also results in reduced copper impact on fungi cultures in the presence of casein. For instance, copper induction of laccase production was reduced in the presence of the complex (3-5 fold enhancements vs. 10–50 fold under other conditions), whereas fungi growth was positively affected by the formation of the complex (MSCs of 1 mM for casein media vs. 0.06 – 0.25 mM for other conditions). In addition, the eradication of fungi growth occurred at higher concentration of copper (MECs of >1 mM vs. 0.25 - 0.5 mM for other conditions).

The PC values, indicating copper levels associated with peak enzyme activity were determined to be similar (0.06 – 0.12 mM) for all tested conditions, but, were equal or slightly lower than the corresponding MSC values. This observation points to a high probability that the maximum activity occurs during the idiophase. At the MSC, biomass growth rate slows down and the fungus starts to release laccase, a secondary metabolite, and hence leads to an increased laccase activity. The biochemical tolerance (MLSC) levels were equal or higher than growth tolerance (MSC) levels for most tested conditions with the exception of casein-containing media where MLSC values were lower than MSC values at high concentrations of casein. This observation indicates that the laccase-producing system still functions properly even when the fungi culture growth is slowing-down. In other words, increased growth tolerance levels would allow appropriate performance of laccase-producing system at higher copper load, and, hence, would improve laccase activity as it has been observed for cellobiose, mannose, xylose and casein ($r = 0.44 – 0.92$). However, nutrient-associated regulation of biochemical tolerance was different from growth tolerance regulation mechanisms. The laccase suppressing copper impact (MLSC) was delayed (compared to standard media) by 3-O-methylglucose, arabinose, cellobiose and ammonium chloride at any C/N ratios, as well as high level of xylose and low
level of casein. In the case of MLSC, even casein did not show impact of copper-binding on the laccase-producing system tolerance. Nevertheless, the carbon and nitrogen sources obviously have an impact on fungi tolerance (both growth and biochemical) to copper and copper-associated laccase activity and, this impact is a function of the nutrient’ type and concentration in the media. However, no general trend was observed and mechanisms underlying behind the influence of carbon and nitrogen sources on laccase activity requires further investigation.

The present study demonstrated that a variety of carbon and nitrogen sources (with the exception of methylcellulose) induced laccase activity (compared to standard media containing glucose and sodium nitrate) without participation of copper. In particular, 2 to 10-fold enhancement of laccase activity was observed after substitution of glucose with alternative sugars and 15 – 50 folds when ammonium chloride and casein replacing sodium nitrate. The addition of copper sulfate further enhanced laccase activity, while the level of enhancement again depended on the type and ratio of carbon and nitrogen sources. At the peak level, the laccase activity of *D. squalens* incubated in standard (supplemented with glucose and sodium nitrate) media increased by around 5 folds compared to copper-free conditions. The enhancement of the laccase activity by 10 – 50 folds was achieved when media with alternative sugars was supplemented with copper sulfate and by 2 – 5 folds when copper sulfate was added to media with ammonium chloride or casein. Methylcellulose, which, as mentioned above, had no impact on copper-free laccase activity, enhanced copper-associated laccase yield by around 500 folds. Finally, the highest laccase activity was achieved via the synergistic effect of copper sulfate and carbon/nitrogen sources. 3-0-methylglucose, methylcellulose, ammonium chloride and casein at low levels were the most productive in the enhancement of the laccase activity in combination
with copper sulfate. However, laccase activity enhancement had no clear correlation with enhanced copper load. As mentioned above, the corresponding PC values were similar (0.06 – 0.12 mM) for all tested conditions, but laccase activity varied from 2 AU to 70 AU in response to the type and C/N ratios. Thus, in this system, copper load (determined by PC values) was not the only contributing factor in laccase yield as expected from enzyme structure (each laccase center contains four copper ions, and hence, laccase activity is supposed to be a function of copper load) and copper participation in laccase transcription (increasing the copper concentration in the growth media results in increase in laccase transcripts and, hence, the corresponding laccase activity [26]). The nutrient conditions had a significant impact on copper-free laccase activity and laccase activity associated with copper. Since the copper load (PC values) is more or less constant, we can expect similar numbers of active centers involved in the process and no significant changes in enzyme activity under different nutrient conditions. The enhancement of laccase activity under this scenario could be attributed to (i) reinforcement of copper center by nutrients; (ii) substitution of copper in active center; and/or (iii) substitution of copper in transcription and post-transcriptional regulation of enzyme production. However, these proposed mechanisms need to be further investigated.

Thus, the interaction of carbon and nitrogen sources with laccase production is remains poorly understood and requires additional research to understand the mechanisms of the laccase activity enhancement. Nevertheless, experimental data obtained in the present study clearly demonstrate the potential for enhancement of fungi tolerance and laccase activity by manipulation of the media composition. We observed that methylated substrates (3-O-methylglucose, methylecellulose) are more attractive for laccase production in combination with
casein and copper; where casein plays the role of an effective nitrogen source which also protects the cell from toxicity imposed by excess copper, while methylated carbon sources enhanced both copper-independent and copper-associated laccase activity.

2.7 Conclusions

Laccase production can be improved by enhancing the inducing level of copper sulfate. Induction of laccase by copper can be varied by the manipulation of the culture media with different carbon and nitrogen sources, so as to increase the copper tolerance. The C/N ratio was also found to be a crucial factor in deciding the copper tolerance and related laccase activity. Changing the growth related-copper tolerance shifts the copper induction and suppression of laccase activity. The suppression of laccase activity is independent of the copper toxicity towards fungal growth, but it is dependent on the nature of the carbon and nitrogen sources and their corresponding ratios in the culture medium. This research can be used as a preliminary study to predict the effective copper concentration and media composition which would stimulate the laccase production without compromising the fungi growth.
2.8 References


Chapter Three: Induction and Suppression of Dichomitus squalens and Ceriporiopsis subvermispora Peroxidase Activity by Manganese Sulfate in Response to Carbon and Nitrogen Sources

3.1 Presentation of the Article

This article deals with the investigation of various carbon and nitrogen sources and their effect on the enhancement of peroxidase activity and fungal growth in the presence of peroxidase inducer, manganese sulfate. In order to comprehend the effect of carbon sources on peroxidase activity, carbon sources were chosen from various groups such as simple hexoses, pentoses, disaccharides, polysaccharides and substituted sugars. Similarly, the impact of the nitrogen sources on peroxidase activity was tested in the presence of nitrogen sources belonging to organic, inorganic and ammoniated groups. Based on previous reports, manganese sulfate concentration higher than 1 mg/ml had no impact on peroxidase activity and hence its concentration was varied between 0 and 1 mg/ml in this study. It is found from this study that at low concentrations, manganese has enhancing effect on peroxidase activity, but at high concentrations, it suppressed the enzyme activity. The effect of various carbon and nitrogen sources on peroxidase activity in the presence of manganese sulfate was species specific.
Induction and Suppression of Dichomitus squalens and Ceriporiopsis subvermispora

Peroxidase Activity by Manganese Sulfate in Response to Carbon and Nitrogen Sources

This article has been accepted by the Canadian Journal of Chemical Engineering.

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3.2 Abstract

Delignification is critical in the production of biofuel from a potentially abundant renewable resource, lignocellulosic waste. One of the classes of ligninolytic enzymes are peroxidases. Manganese sulfate (Mn²⁺) has a significant impact on the peroxidase activity of ligninolytic fungi. Along with induction, the suppression of peroxidase activity by Mn²⁺ has been observed. Peroxidase regulation is governed by three critical Mn²⁺ concentrations: the minimum inductive concentration (MIC), the peak concentration (PC), and the minimum suppressive concentration (MSC). The induction and suppression of enzyme activity were not associated with fungal growth capacity, but with a specific enzyme response to the nutritional conditions. Manipulation of the carbon and nitrogen sources shifted the peroxidase suppression by Mn²⁺ to high concentrations and, hence, increased the peroxidase tolerance to Mn²⁺ and, consequently, peak peroxidase activities. The manipulation of carbon and nitrogen sources allowed increasing the peak concentrations of Mn²⁺ and corresponding peroxidase activity up to 0.5 mg/ml and
180.70 AU (173% increase compared to standard) in *C. subvermispora*, and up to 0.05 mg/ml and 151.23 AU in *D. squalens* (91% increase compared to standard). Thus, manipulation of carbon and nitrogen sources is a useful tool in enhancing fungi peroxidase tolerance to Mn$^{2+}$ and improving the delignification of lignocellulosic biomass by fungi to prepare an easily-consumable substrate for economically viable biofuel production.

### 3.3 Introduction

The current demand for a sustainable and eco-friendly energy supply motivates the research in alternative energy resources such as biofuels derived from a renewable source, biomass. The biofuel generation is based on the conversion of biomass to fermentable sugars and further into ethanol or butanol. The utilization of food crops for biofuel production has a limited capacity to satisfy energy demands as it uses only part of the available biomass and competes with the food supply. Lignocellulosic biomass (switchgrass, miscanthus, byproducts of lawn and tree maintenance, agricultural residues, wood residues, municipal waste) is an abundant renewable resource and offers a good alternative to food crops. However, lignocellulosic raw materials require a greater amount of pre-processing to liberate fermentable sugars. Lignocellulose biomass is a composite of cellulose embedded in a matrix of hemicellulose and lignin [1]. Thus, hydrolysis of polysaccharides to glucose and xylose requires delignification of raw materials. The conventional chemical methods currently used for the lignin removal generate byproducts such as weak acids, furan and phenolic compounds which negatively impact hydrolysis and fermentation rates and thus overall biofuel yield [2]. A biological approach to biomass delignification utilizing lignin degrading enzymes is a promising method which
overcomes most of the disadvantages of chemical delignification [3]. Laccase, manganese
peroxidase, lignin peroxidase, and versatile peroxidase are the most common ligninolytic
enzymes secreted by white rots [4], including Ceriporiopsis subvermispora [5] and Dichomitus
squalens [6], which are known for their selective lignin degradation without removing the
cellulose which is subsequently needed for glucose release and fermentation[7, 8]. The activities
of both manganese-dependent and manganese-independent peroxidases are subjected to
nutritional regulation.

While Mn$^{2+}$ is known to be the main regulator of manganese-dependent peroxidase activity
[7-9], there are several publications reporting regulation of non-specific peroxidase production
and activity by means of different carbon and nitrogen sources. Nevertheless, the role of
nutritional regulation of peroxidase activity is complex, poorly understood, and sometimes
ambiguous to interpret. For example, the highest peroxidase activity in Pleurotus ostreatus was
reported in the presence of xylan, mandarin peel, peptone or casein which depended on the
species [10]. The highest Lentinus kauffmanni peroxidase was observed in the presence of
fructose or peptone [11]. Studies have shown that glucose enhanced peroxidase titres of
Phellinus robustus but repressed that of Ganoderma adspersum [12]. Thus, the types of carbon
and nitrogen sources play a significant role in the regulation of peroxidase activity which also
appears to be strain dependent.

Along with nutrient type, nutritional regulation appears to be controlled by nutrient
concentration and the carbon-to-nitrogen (C/N) ratio. For example, Lentinula edodes peroxidase
production was suppressed by high levels of ammonium nitrate and asparagine at defined
glucose levels [13]. High concentrations of glucose at low ammonium tartrate concentrations were also reported to provide higher Trametes ssp. peroxidase activity [14]. In contrast, high peroxidase activity for Phanerochaete flavido-alba was obtained under carbon limitation and high levels of ammonium tartrate [15]. Manganese peroxidase regulation by the specific substrate Mn\(^{2+}\) was also reported to be sensitive to the type and concentration of carbon and nitrogen sources. For example, regulatory effects of Mn\(^{2+}\) on P. chrysosporium peroxidase activity was better under ammonium tartrate limitation, but glucose had no impact on peroxidase response to Mn\(^{2+}\) [16].

Thus, regulation of peroxidase activity by coupled effect of Mn\(^{2+}\) and carbon and nitrogen sources is a promising way to improve delignification of lignocellulosic waste and enhance biofuel production. However, limited and sometimes conflicting information on nutritional regulation of peroxidase activity does not allow further development of this technology. The conditions in different studies do not allow the direct comparison and interpretation of nutrient regulation on peroxidase activity. Hence, in order to gain a deeper understanding of nutrient regulation of peroxidase activity, a variety of nutrients were investigated under similar conditions. The present study investigates the Mn\(^{2+}\) regulation of peroxidase activity in response to different carbon and nitrogen sources and their C/N ratio for two speciess of white rot fungi with selective ligninolytic activity: D. squalens and C. subvermispora. To explain the variability in the impact of different types of nutrients on Mn\(^{2+}\) regulation of peroxidase activity, the tolerance of fungi to Mn\(^{2+}\) was investigated as a function of carbon and nitrogen source types and ratios. The enhancement of the tolerance level of the fungal peroxidases to Mn\(^{2+}\) by means of
nutrient regulation allows increasing the productivity of ligninolytic enzyme, and subsequent biofuel production.

3.4 Materials and Methods

3.4.1 Microorganism and chemicals

Dichomitus squalens ATCC 201541 and Ceriporiopsis subvermispora ATCC 90467 were purchased from American Type Culture Collection (ATCC), USA. All chemicals were purchased from Sigma-Aldrich Canada Co. Oakville, Ontario.

3.4.2 Cultivation conditions

Fungi were incubated in basic mineral medium containing KCl – 0.56 g/l, MgSO₄•7H₂O – 0.78 g/l, FeSO₄•7H₂O – 8 mg/l, KH₂PO₄ – 2.22 g/l and thiamine – 1 mg/l (pH 5). The standard (control) medium was supplemented with 1% of glucose and 1.7 g/l of sodium nitrate. For experimental purposes, the basic medium were supplemented with a range of carbon sources including glucose, 3-O-methylglucose, methylcellulose, cellobiose, mannose, arabinose and xylose at high level (1%) and low level (0.1%) with sodium nitrate as nitrogen source at high level (1.7 g/l) and low level (0.17 g/l). Alternatively, basic media with different nitrogen sources such as sodium nitrate (1.7 g/l and 0.17 g/l), ammonium chloride (1.1 g/l and 0.11 g/l), and casein hydrolyzate (0.5% and 0.05%) were supplemented with glucose as carbon source at 1% and 0.1%. Manganese sulfate was added to the media as an inducer in the range of 0 to 1 mg/ml. The 24-well microplates were filled with 2 ml of standard or challenge media and inoculated...
with 3 mm potato-dextrose agar (PDA) plugs covered with fresh fungal mycelium. Experiments were conducted in triplicate with fungal inoculums taken on different days but of the same age. We have verified that the results were consistent for the different inoculum sources. The plates were incubated at 25°C with agitation at 150 rpm in an orbital shaker for seven days. Cell-free media with extracellular enzymes from each well was collected on the seventh day and analyzed for peroxidase activity. Fungi grown on agar plugs in each well were transferred on to fresh PDA plates to measure fungi growth capacity.

### 3.4.3 Impact of carbon-to-nitrogen ratios

To determine the impact of carbon-nitrogen (C/N) ratios on fungi growth and peroxidase activity, four challenge media were designed with relative C/N ratio as follows: (i) 1:1 (Low Carbon and Low Nitrogen), (ii) 1:10 (Low Carbon and High Nitrogen), (iii) 10:1 (High Carbon and Low Nitrogen), and (iv) 10:10 (High Carbon and High Nitrogen). High levels designate concentrations 10 times higher than those for low levels (actual individual nutrient concentrations are presented in the section: cultivation conditions). Additionally, the challenge media were supplemented with manganese sulfate at concentrations of 0.01, 0.05, 0.1, 0.5 and 1.0 mg/ml to determine the impact of manganese on peroxidase activity in response to the type and concentration of carbon and nitrogen sources. The plates were incubated at 25°C with agitation at 150 rpm for seven days. The supernatant for the enzyme assay was taken from the 2 ml of the media in the microplate wells. Fungi growth and peroxidase activity in response to challenge media were determined as described below.
3.4.4 Peroxidase activity

Peroxidase activity was determined by monitoring the oxidation of 2, 6-dimethoxyphenol (DMP) to an orange brown dimer, coerulignone [17]. To the reaction mixture (250 µl) containing 0.5 mM of DMP and 0.1 mM manganese sulfate in 0.1 M sodium tartrate buffer (pH 5.0) and 5 or 10 µl of supernatant, 0.05 mM hydrogen peroxide was added to initiate the reaction and the absorbance kinetics was monitored at 469 nm for 5 minutes at 30°C. Kinetic measurements were carried out in microplates (path length 0.69 cm) with a Spectramax Multimode microplate reader [18]. Peroxidase activity was expressed in activity units (AU), which are international units per liter per biomass units. One international unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 µmol of DMP (molar extinction coefficient 27500 M⁻¹cm⁻¹) per min [17]. Peroxidase activity was corrected for laccase activity which was determined in the absence of manganese sulfate and hydrogen peroxide. Biomass units were determined as the rate of fungi radial growth (mm/h) on PDA (as below).

3.4.5 Biomass Units

To determine the growth capacity, fungal mycelium grown in challenge media was transferred to fresh PDA plates. The plates were incubated at 25°C under static conditions. Radial growth rates were quantified by the increase in the fungal colony radius (in mm) per hour, and used to determine viable biomass units for calculating peroxidase activity. One unit of viable biomass is equivalent to 1 mm radial growth per hour.
3.4.6 Statistical analysis

All the experiments were carried out in triplicate with fungal inoculums taken on different days but of the same age. One way ANOVA and \(t\)-test were used to estimate statistical differences in growth rates and enzyme activity between experimental conditions.

3.5 Results

3.5.1 Dichomitus squalens

3.5.1.1 Effect of various carbon and nitrogen sources and their ratios on peroxidase activity

The peroxidase activity of *D. squalens* was measured in the standard media consisting of glucose and sodium nitrate at 10:10 ratio and challenge media where either glucose or sodium nitrate were substituted with alternative carbon or nitrogen sources at the same C/N ratio. Additionally, the impact of varying the C/N ratio in the standard and challenge media (e.g., 10:1, 1:10 and 1:1 C/N ratios) on peroxidase activity was investigated.
Table 3-1. Total peroxidase activity (AU) of *D. squalens* under different nutrient composition in the presence and absence of manganese sulfate

<table>
<thead>
<tr>
<th>Carbon/Nitrogen Source</th>
<th>10:10</th>
<th>10:1</th>
<th>1:10</th>
<th>1:1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard with Mn²⁺</td>
<td>Standard with Mn²⁺</td>
<td>Standard with Mn²⁺</td>
<td>Standard with Mn²⁺</td>
</tr>
<tr>
<td><strong>Glucose</strong> a</td>
<td>0.0±0.0d</td>
<td>79±5</td>
<td>10.5±0.4</td>
<td>62±4</td>
</tr>
<tr>
<td><strong>Arabinose</strong></td>
<td>9.7±0.5</td>
<td>18.0±0.4</td>
<td>13±1</td>
<td>22±2</td>
</tr>
<tr>
<td><strong>Xylose</strong></td>
<td>3.0±0.1</td>
<td>20±1</td>
<td>1.33±0.05</td>
<td>45±4</td>
</tr>
<tr>
<td><strong>Cellobiose</strong></td>
<td>1.0±0.0</td>
<td>4.09±0.07</td>
<td>0.0±0.0</td>
<td>3.64±0.09</td>
</tr>
<tr>
<td><strong>3-O-methylglucose</strong></td>
<td>11.2±0.9</td>
<td>92±2</td>
<td>11.5±0.9</td>
<td>22±2</td>
</tr>
<tr>
<td><strong>Methylcellulose</strong></td>
<td>2.7±0.1</td>
<td>22.6±0.9</td>
<td>0.0±0.0</td>
<td>29±4</td>
</tr>
<tr>
<td><strong>Mannose</strong></td>
<td>0.0±0.0</td>
<td>4.1±0.0</td>
<td>0.0±0.0</td>
<td>49±2</td>
</tr>
<tr>
<td><strong>Ammonium chloride</strong> b</td>
<td>0.0±0.0</td>
<td>18.1±0.0</td>
<td>0.0±0.0</td>
<td>29±2</td>
</tr>
<tr>
<td><strong>Casein</strong></td>
<td>4.0±0.0</td>
<td>15.0±0.0</td>
<td>3.0±0.0</td>
<td>20±2</td>
</tr>
</tbody>
</table>

a Carbon sources combined with sodium nitrate as the standard nitrogen source
b Nitrogen sources combined with glucose as the standard carbon source
c 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN
d ± refers to "mean ± standard error"
The level of peroxidase activity in the standard media (10:10 C/N ratio) was undetectable (Table 3-1). However, reducing either glucose or sodium nitrate by 10-fold, resulted in peroxidase activity of 11 AU. Peroxidase activity dropped to 3.4 AU when both the nutrients were reduced by 10-fold. Similarly, peroxidase activity was not detected when glucose was substituted with mannose and when sodium nitrate was substituted with ammonium chloride at a 10:10 C/N ratio. In the presence of mannose, peroxidase activity increased up to 3.9 AU when the sugar level was reduced regardless the sodium nitrate level (C/N ratios of 1:10 and 1:1). Glucose-to-ammonium chloride ratios did not show any impact on *D. squalens* peroxidase activity. Cellobiose in combination with sodium nitrate also provided very low (< 1.5 AU) peroxidase activity regardless of C/N ratios.

Replacement of glucose with methylcellulose, and sodium nitrate with casein, resulted in peroxidase activity of 2.7 – 4.0 AU. However, enzyme activity significantly decreased when nutrient levels were reduced. Xylose provided 5.4 AU at C/N of 1:10, but 1.33 – 3.0 AU at other ratios. Arabinose supplementation resulted in 13 AU at C/N ratios 10:1 and 1:1, and 9.7 and 12 AU at 10:10 and 1:10 C/N ratios. 3-**O**-methylglucose enhanced the peroxidase activity to 11.5 AU at high carbon level, but only 2.0 – 3.57 AU at low carbon level.

3.5.1.2 Induction of peroxidase activity by manganese sulfate in response to carbon and nitrogen sources

To investigate the manganese regulation of peroxidase activity in response to nutrient composition, the standard and challenge media were supplemented with 0.01 mg/ml of manganese sulfate (Mn^{2+}). While the addition of Mn^{2+} increased peroxidase activity under all
tested conditions, the level of activity enhancement depended on carbon and nitrogen sources and their ratios. In particular, manganese sulfate enhanced peroxidase activity by 20- and 80-fold in the presence of glucose and sodium nitrate at 10:10 and 1:1 C/N ratios. However, the reduction in the concentration of one of the nutrients resulted in low (less than 5-fold) peroxidase activity enhancement by Mn$^{2+}$. In the presence of xylose and sodium nitrate, Mn$^{2+}$ induced 10- to 20-fold increases in enzyme activity regardless of the C/N ratios. 3-O-methylglucose supported 10-fold induction of peroxidase activity by Mn$^{2+}$ at all tested ratios, except 10:1. Methylcellulose supported enzyme induction at high sugar levels regardless of the nitrogen concentration, while mannose in combination with sodium nitrate supported enzyme induction when one or both of the nutrients were supplied at low levels. In the media with ammonium chloride and glucose, 10 to 15-fold enzyme induction was observed at high sugar levels; but in the presence of casein and glucose, peroxidase activity was induced by Mn$^{2+}$ when either of the nutrients was at low levels (8-fold) and especially when both nutrients were at low levels (59-fold). In contrast, addition of Mn$^{2+}$ to the media with arabinose or cellobiose enhanced peroxidase activity by less than 5-fold at all C/N ratios.

The resultant enzyme activity in most tested media was higher than in standard media. Peroxidase activity was identical in media supplemented with glucose and either sodium nitrate or ammonium chloride at a C/N ratio of 1:1. 3-O-methylglucose at 10:10 ratio provided the highest enzyme activity observed, 92 AU.
3.5.2 Ceriporiopsis subvermispora

3.5.2.1 Effect of various carbon and nitrogen sources and their ratios on peroxidase activity:

Peroxidase activity of *C. subvermispora* in the media supplemented with glucose and sodium nitrate was approximately 5 AU when one or both nutrients were at high levels, but decreased when both nutrients were at low levels (Table 3-2). Arabinose showed the same enzyme activity trends, but enhanced peroxidase titres up to 8.1 AU at a 1:1 C/N ratio. Other tested carbon sources: xylose, cellobiose, 3-*O*-methylglucose, methylcellulose and mannose enhanced the peroxidase activity by 2 to 4-fold compared to the standard media at a 10:10 ratio. Mannose in combination with sodium nitrate supported the same level of peroxidase activity at all C/N ratios. Cellobiose, xylose and methylcellulose supported high peroxidase activity at high sodium nitrate level, but activity decreased when sodium nitrate concentration was reduced, regardless of the sugar level. 3-*O*-methylglucose enhanced enzyme activity at high sugar levels regardless of the sodium nitrate concentration. Casein and ammonium chloride provided very low peroxidase activity at all tested C/N ratios.

3.5.2.2 Induction of peroxidase activity by manganese sulfate in response to carbon and nitrogen sources

Addition of Mn$^{2+}$ in standard and challenge media resulted in enhancement of *C. subvermispora* peroxidase activity. The level of enhancement was a function of media composition. In contrast to *D. squalens*, the extent of peroxidase induction by Mn$^{2+}$ (0.01 mg/ml) in *C. subvermispora* was very low (by 1.5 to 5-fold) in most of the tested media. Significant (8 to 15-fold) enhancement was observed only for xylose and cellobiose at a 10:1 C/N ratio; for methylcellulose at 10:1 and 1:10 ratios; and for 3-*O*-methylglucose at a 1:1 ratio. In the presence of casein, Mn$^{2+}$ had no impact on peroxidase activity for all tested C/N ratios.
Table 3-2. Total peroxidase activity (AU) of *C. subvermispora* under different nutrient composition in the presence and absence of manganese sulfate

<table>
<thead>
<tr>
<th>Carbon/Nitrogen Source</th>
<th>Carbon to Nitrogen ratios&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:10</td>
</tr>
<tr>
<td>Standard with Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Standard with Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4±0.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arabinose</td>
<td>4.9±0.4</td>
</tr>
<tr>
<td>Xylose</td>
<td>10.8±0.5</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>17±1</td>
</tr>
<tr>
<td>3-&lt;i&gt;O&lt;/i&gt;-methylglucose</td>
<td>14.3±0.4</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>13.9±0.7</td>
</tr>
<tr>
<td>Mannose</td>
<td>14.7±0.0</td>
</tr>
<tr>
<td>Ammonium chloride&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6±0.0</td>
</tr>
<tr>
<td>Casein</td>
<td>1.30±0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> Carbon sources combined with sodium nitrate as the standard nitrogen source

<sup>b</sup> Nitrogen sources combined with glucose as the standard carbon source

<sup>c</sup> 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN

<sup>d</sup> ± refers to "mean ± standard error"
3.5.3 Nutritional regulation of the peak peroxidase activity

Peroxidase activity was tested at six Mn\(^{2+}\) concentrations (0, 0.01, 0.05, 0.1, 0.5 and 1.0 mg/ml) over the range of carbon and nitrogen sources at different C/N ratios. A typical trend was observed in the peroxidase activity dynamics as a function of manganese sulfate concentration. Manganese sulfate at 0.01 mg/ml (the minimum manganese sulfate level analyzed in this study) induced peroxidase activity of both the species studied under all tested conditions. This level was defined as the Minimum Inducing Concentration of manganese sulfate (MIC). An increase in Mn\(^{2+}\) concentration resulted in increased peroxidase activity until it reached a maximum value for which the corresponding Mn\(^{2+}\) levels are defined as the Peak Concentrations (PC). Further increase in Mn\(^{2+}\) concentration tended to reduce the peroxidase activity to a level where the enzyme activity was lower than that observed under Mn\(^{2+}\)-free conditions. This critical Mn\(^{2+}\) concentration corresponds to the Minimum Suppressive Concentration (MSC).

The determination of PC of Mn\(^{2+}\) and the corresponding nutritional conditions is the main factor in the optimization of enzyme yield. The PC values for each combination of carbon and nitrogen sources are presented in Table 3-3. In general, PC values were regulated by the specific carbon and nitrogen sources and their ratios.
Table 3-3. Maximum total peroxidase activity (AU) and the corresponding C/N ratio and manganese sulfate peak concentration (PC, mg/ml)

<table>
<thead>
<tr>
<th>Carbon/Nitrogen Source</th>
<th><strong>D. squalens</strong></th>
<th></th>
<th></th>
<th><strong>C. subvermispora</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peroxidase(_{\text{max}})</td>
<td>PC</td>
<td>C/N ratio(^c)</td>
<td>Peroxidase(_{\text{max}})</td>
<td>PC</td>
<td>C/N ratio(^c)</td>
</tr>
<tr>
<td>Glucose(^a)</td>
<td>91±7(^d)</td>
<td>0.1</td>
<td>1:1</td>
<td>66±6</td>
<td>0.10</td>
<td>10:10</td>
</tr>
<tr>
<td>Arabinose</td>
<td>53±1</td>
<td>0.05</td>
<td>1:1</td>
<td>181±6</td>
<td>0.50</td>
<td>1:10</td>
</tr>
<tr>
<td>Xylose</td>
<td>65±2</td>
<td>0.05</td>
<td>1:10</td>
<td>64±2</td>
<td>0.1</td>
<td>10:10</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>8.3±0.3</td>
<td>0.05</td>
<td>1:1</td>
<td>174±4</td>
<td>0.5</td>
<td>1:10</td>
</tr>
<tr>
<td>3-O-methylglucose</td>
<td>93±3</td>
<td>0.01</td>
<td>10:10</td>
<td>23.4±0.3</td>
<td>0.50</td>
<td>1:10</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>29±3</td>
<td>0.01</td>
<td>10:1</td>
<td>81±5</td>
<td>0.05</td>
<td>10:10</td>
</tr>
<tr>
<td>Mannose</td>
<td>28±2</td>
<td>0.05</td>
<td>10:10</td>
<td>90±7</td>
<td>0.10</td>
<td>10:10</td>
</tr>
<tr>
<td>Ammonium chloride(^b)</td>
<td>151±5</td>
<td>0.05</td>
<td>1:1</td>
<td>54±2</td>
<td>0.50</td>
<td>10:1</td>
</tr>
<tr>
<td>Casein</td>
<td>60±4</td>
<td>0.01</td>
<td>1:1</td>
<td>2.9±0.2</td>
<td>0.01</td>
<td>1:10</td>
</tr>
</tbody>
</table>

\(^a\) Carbon sources combined with sodium nitrate as the standard nitrogen source
\(^b\) Nitrogen sources combined with glucose as the standard carbon source
\(^c\) 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN
\(^d\) ± refers to "mean ± standard error"

*D. squalens* peroxidase activity reached maximum values when carbon and nitrogen sources were limited (C/N of 1:1) in the media supplemented with glucose, arabinose, cellobiose, ammonium chloride and casein. Corresponding PC values were 0.01 mg/ml for casein, 0.1 mg/ml for glucose and 0.05 mg/ml for rest of the nutrients. Xylose provided maximum enzyme activity at 1:10 C/N ratio and 0.05 mg/ml of Mn\(^{2+}\); methylcellulose – at 10:1 C/N ratio and 0.01 mg/ml. Mannose and 3-O-methylglucose supported peroxidase activity when both carbon and
nitrogen sources were supplemented at high levels (10:10) with 0.05 mg/ml and 0.01 mg/ml of Mn$^{2+}$, respectively.

For *C. subvermispora*, the maximum peroxidase activity in media with glucose, xylose, methylcellulose and mannose was observed at 10:10 C/N ratio and Mn$^{2+}$ concentrations ranging from 0.05 mg/ml to 0.1 mg/ml. The maximum enzyme activity for other carbon sources was obtained at 1:10 C/N ratio and 0.5 mg/ml of Mn$^{2+}$. Maximum peroxidase activity in the presence of ammonium chloride was observed at 10:1 C/N ratio and 0.5 mg/ml of Mn$^{2+}$; whereas it was at 1:10 C/N and 0.01 mg/ml of Mn$^{2+}$ for casein.

High peroxidase activity (91 – 151 AU) was observed for *D. squalens* in response to glucose, 3-O-methylglucose and ammonium chloride; whereas, high peroxidase activity (90 – 181 AU) of *C. subvermispora* was in response to arabinose, mannose and cellobiose.

### 3.5.4 Suppressive effect of manganese sulfate on peroxidase activity in response to nutrient composition

Peroxidase activity is said to be suppressed when the observed activity drops below that for the corresponding Mn$^{2+}$-free media. The suppressive effect of Mn$^{2+}$ was characterized by the Minimum Suppressive Concentrations (MSC) presented in Table 3-4. The MSC levels were specific for the type of carbon and nitrogen sources and C/N ratios.
Table 3-4. Minimum total peroxidase suppressive concentration (MSC) of manganese sulfate (mg/ml)

<table>
<thead>
<tr>
<th>Carbon/Nitrogen Source</th>
<th>Carbon to Nitrogen ratios&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D. squalens</td>
</tr>
<tr>
<td></td>
<td>10:10</td>
</tr>
<tr>
<td>Glucose&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.1</td>
</tr>
<tr>
<td>Xylose</td>
<td>1</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>1</td>
</tr>
<tr>
<td>3-O-methylglucose</td>
<td>1</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>0.1</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.5</td>
</tr>
<tr>
<td>Ammonium chloride&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Casein</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Carbon sources combined with sodium nitrate as the standard nitrogen source

<sup>b</sup> Nitrogen sources combined with glucose as the standard carbon source

<sup>c</sup> 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN

For *D. squalens*, methylcellulose, arabinose and mannose were highly sensitive to the suppressive effect of Mn<sup>2+</sup> (MSC values of 0.05 – 0.1 mg/ml) in terms of peroxidase activity. However, MSC values increased up to 0.5 mg/ml in the presence of arabinose at C/N ratio of 1:1 and mannose at 10:10 ratio. Under other nutritional conditions, MSC values were equal or exceeded 0.5 mg/ml. In the presence of glucose in combination with either sodium nitrate or ammonium chloride, there was no suppression in the peroxidase activity even at 1.0 mg/ml of
Mn\textsuperscript{2+}, the maximum tested concentration. In the glucose/casein medium, suppression in the peroxidase activity was observed only for 1:10 C/N ratio (within the concentration threshold tested).

For *C. subvermispora*, the suppression effect of Mn\textsuperscript{2+} on the enzyme activity was not observed for most of the tested carbon and nitrogen sources except for 3-O-methylglucose/sodium nitrate and glucose/casein media. In the first case, MSC value of 1 mg/ml was determined at 10:1. In the glucose/casein media, the suppressive effect was observed only at 1:10 C/N ratio (MSC of 0.05 mg/ml) and at 1:1 ratio (MSC of 0.5 mg/ml).

### 3.6 Discussion

Peroxidases are important lignin degrading enzymes that appear ubiquitously in almost all the white rot fungi [19]. There are two types of lignin-degrading peroxidases: Mn-dependent and Mn-independent. Manganese (Mn\textsuperscript{2+}), the specific substrate of manganese peroxidase (MnP) is not only essential for the manganese-dependent peroxidase activity [20], but also is involved in the transcription of the *mnp* (manganese peroxidase) genes and secretion of the enzyme into the extracellular medium [9]. Therefore, it was reasonable to observe induction of peroxidase activity in the white rot fungi, *D. squalens* and *C. subvermispora*, by manganese sulfate at very low concentration (0.01 mg/ml). Further increase in the Mn\textsuperscript{2+} concentration resulted in a steady increase in the peroxidase activity that reached a maximum value at a critical Mn\textsuperscript{2+} level (peak concentration, PC), and was followed, however, by suppression of enzyme activity. No impact on fungi growth was observed; therefore, high Mn\textsuperscript{2+} levels have imposed toxicity only towards
peroxidase production and/or activity. A similar effect was observed for response of *D. squalens* laccase to copper sulfate [21]. Authors also reported that both inducing and suppressing effects of copper on laccase activity were regulated by the type and proportion of carbon and nitrogen sources in the media.

The present study was conducted to investigate the impact of the nutritional regulation on Mn$^{2+}$ - peroxidase interaction. It has revealed that both the type of carbon and nitrogen sources and carbon-to-nitrogen ratios influence peroxidase response to Mn$^{2+}$. At low (0.01 mg/ml) levels, manganese sulfate significantly induced *D. squalens* peroxidase activity in the presence of glucose and sodium nitrate. The highest activity was observed at a 10:10 C/N ratio (referred to as standard media). Under challenge conditions (low level of one or both nutrients or alternative types of carbon and nitrogen source), levels of Mn$^{2+}$-induced peroxidase activity increased. At the peak values, Mn$^{2+}$ had higher impact on peroxidase activity in the presence of glucose, arabinose, cellobiose, xylose, sodium nitrate, casein and ammonium chloride at low levels. Changes in nutrient demand were associated with increased concentration of Mn$^{2+}$. In particular, glucose provided maximum peroxidase activity at 0.1 mg/ml of manganese sulfate and for the rest of the nutrients at 0.05 mg/ml. Methylcellulose allowed maximum peroxidase activity at high levels when supplemented with low concentration level of both manganese sulfate (0.01 mg/ml) and sodium nitrate (0.17 g/l). Mannose and 3-O-methylglucose supported peroxidase activity when both carbon and nitrogen sources were supplemented at high levels (10:10) with low Mn$^{2+}$ level. These data indicate that peroxidase productivity directly correlates with tolerance of *D. squalens* peroxidase to Mn$^{2+}$, and both parameters are functions of the type of carbon and nitrogen source and their combination. The tolerance of *D. squalens* in the presence
of mannose, methylcellulose and 3-O-methylglucose was very low as indicated by PC of 0.01 mg/ml. Under these conditions, high concentrations of nutrients are required to support Mn\(^{2+}\)-induced peroxidase activity. 3-O-methylglucose and methylcellulose seem to provide compensatory effect because they were observed to enhance peroxidase activity in Mn\(^{2+}\)-free media when supplemented at high levels. The mechanism of mannose impact on the Mn\(^{2+}\)-peroxidase system is unclear. Mannose induced Mn\(^{2+}\) - independent peroxidase activity at low levels, but a high concentration of mannose was necessary to support Mn\(^{2+}\) - induced peroxidase activity. This observation may indicate participation of mannose in Mn\(^{2+}\) mediator activity or Mn\(^{2+}\) - associated peroxidase transcription regulation. In the presence of other nutrients, tolerance of *D. squalens* increased up to 0.05 – 1.0 mg/ml of manganese sulfate. However, high tolerance and maximum activity can be achieved when nutrients are supplied at low concentrations. The nutrients themselves (without Mn\(^{2+}\)) had a different impact on peroxidase activity. The highest level of activity was observed in the presence of high levels of arabinose. Glucose, xylose, casein and sodium nitrate favored peroxidase activity at low levels; while cellobiose and ammonium chloride did not have any effect on the enzyme activity in Mn\(^{2+}\)-free media. The impact of these nutrients on Mn\(^{2+}\) - peroxidase interaction is unknown. However, our data indicate that, under most tested conditions, starvation/nutrient limitation plays a role in peroxidase activity at high (probably toxic) Mn\(^{2+}\) level, and under Mn\(^{2+}\)-free conditions. It is reasonable because lignin degradation is a stationary phase metabolism associated with limitation of the primary substrate. The main purpose of lignin degradation is to provide the microbial culture with new portions of cellulose and hemicellulose.
One of the possible mechanisms involved in nutritional regulation of Mn$^{2+}$ - peroxidase interaction is the stabilization of manganese ions within carbohydrates and casein complexes [22, 23]. Binding Mn$^{2+}$ within the complex reduces its availability and interaction with peroxidase, which in turn results in increased tolerance of peroxidase, but reduced enzyme activity. To support sufficiently high tolerance, but also high activity, some optimum concentrations of nutrients are required. In most cases, low levels of nutrients was high enough to support Mn$^{2+}$ availability for enzyme activity, but low enough to limit toxic effects. However, the impact of mannose, methylcellulose and 3-O-methylglucose contradicts this trend. These carbohydrates enhanced enzyme activity but reduced tolerance at high levels when Mn$^{2+}$ availability should be reduced via complex formation, and, hence, activity should be low, but tolerance high. The effect of these nutrients along with arabinose, which also demonstrated higher *D. squalens* peroxidase activity in Mn$^{2+}$-free media may be attributed to direct interaction of sugar with enzyme.

One more indicator of tolerance is the suppression of enzyme activity below levels in Mn$^{2+}$-free media, which is determined by the Minimum Suppressive Concentration (MSC): the higher MSC value, the higher the tolerance. MSC values also indicate possible Mn$^{2+}$ load without compromising enzyme activity. The mechanism of peroxidase activity suppression by Mn$^{2+}$ is unclear. Reports show that in excess of free Mn$^{3+}$, peroxidase is involved in catalysis of H$_2$O$_2$ disproportionately, which was accompanied by a decline in peroxidase activity [24]. Thus, the suppression may be attributed to a switch in the enzymatic activity. However, the magnitude of the reaction switch depends on the nutritional background. Under all tested conditions, the maximum possible Mn$^{2+}$ load (MSC) exceeded 0.5 mg/ml with the exception of methylcellulose, in the presence of which *D. squalens* peroxidase activity was suppressed by 0.05-0.1 mg/ml of
manganese sulfate. For most cases, nutrient levels associated with higher PC provided higher MSC values as well. However, higher MSC values were observed in the presence of high concentrations of xylose, while higher PC values at low xylose levels. In contrast, 3-O-methylglucose supported higher PC at high levels, but higher MSC at low levels. The effect of glucose/sodium nitrate and glucose/ammonium chloride levels on MSC is impossible to predict under these conditions since MSC values exceeded the maximum tested concentration of manganese sulfate (1 mg/ml). How nutrients impact the reaction switch or enzyme suppression is unknown. Oxalate has been shown to inhibit the catalase activity of peroxidase and stimulate the Mn$^{2+}$-dependent peroxidase functions of the enzyme [24]. Thus, sugars may regulate Mn$^{2+}$-peroxidase interaction via the participation in oxalate metabolism.

Another important observation in the present work is that peroxidases from different fungal species follow the same pattern in enzyme response to Mn$^{2+}$: increasing manganese sulfate concentration induces peroxidase activity to the maximum value and then suppresses it. However, there are species-dependent variations in tolerance, productivity and response to nutritional regulation. In general, productivity and tolerance of *C. subvermispora* peroxidase activity was greater than observed for *D. squalens*. No suppressive effect of Mn$^{2+}$ on peroxidase activity of *C. subvermispora* was observed for most of the tested cases except for glucose/casein and 3-O-methylglucose/sodium nitrate within the concentration threshold tested in this study. However, induction of *C. subvermispora* peroxidase required higher Mn$^{2+}$ load (MICs of 0.01 mg/ml - 0.5 mg/ml). Maximum activity values at low nutrient levels were determined only for arabinose, cellobiose and 3-O-methylglucose. However, PC values under these conditions increased by 10 to 20-fold compared to *D. squalens* requirements. Two-fold increase in PC
values and high nutrient concentrations were required to maximize peroxidase activity in the presence of other nutrients. In Mn\(^{2+}\) - free media, most of the nutrients provided better peroxidase activity compared to glucose-sodium nitrate media. The highest activity values were observed at high nutrient concentrations, again, in contrast to \textit{D. squalens}. These differential regulations may be explained by differences in the peroxidase metabolism. \textit{D. squalens} secretes two isoforms of peroxidase only in the presence of Mn\(^{2+}\) [25]. In contrast, \textit{C. subvermispora} is able to express seven isoforms [5]. Each isoform of peroxidase has been reported to be differentially regulated by Mn\(^{2+}\), which might ensure the stability of peroxidase activity even at high Mn\(^{2+}\) concentrations. Oxalic acid is synthesized in the fungal mitochondria by oxidative degradation of cell wall components [26]. When oxalic acid is surrounded by excess Mn\(^{2+}\), peroxidase enzyme exhibits a catalase type-behavior which results in decomposition of H\(_2\)O\(_2\) [24]. Decomposition of H\(_2\)O\(_2\) requires Mn\(^{3+}\), superoxide and hydroxyl radicals, thereby decreasing the Mn\(^{2+}\) availability. Oxalate decarboxylase (ODC) secreted by \textit{D. squalens} is another Mn\(^{2+}\) dependent enzyme which decomposes oxalic acid to formic acid.[27] ODC activity increases with Mn\(^{2+}\) and hence results in lowering the oxalate concentration [28]. In the presence of excess Mn\(^{3+}\) and low concentration of chelating compound such as oxalate, Mn\(^{3+}\) disproportionates into MnO\(_2\) which precipitates as black or brown deposits on the fungal mycelia. The MnO\(_2\) deposits create a concentration gradient between the mycelia and liquid culture that further promotes the abstraction of Mn\(^{2+}\) from the liquid culture [29]. \textit{C. subvermispora} lacks the ODC activity and this may contribute to the insignificant peroxidase activity suppression.
Thus, both manganese-dependent peroxidase and manganese-independent peroxidases contribute to the total peroxidase activity in the fungi under study, and both are regulated by nutrient composition: types and concentrations of carbon and nitrogen sources. The influence of Mn$^{2+}$ is highly pronounced in both fungi cultures which indicate the significance of Mn$^{2+}$ ions in regulation of peroxidase activity in response to variation in nutrient composition. However, manipulation in carbon and nitrogen sources also had a significant impact on Mn-independent peroxidases activity.

The nutritional regulation of peroxidase activity is a complex process, the mechanism of which requires additional investigation. However, the present paper clearly demonstrated that there is a differential response of peroxidases to Mn$^{2+}$: at low level, Mn$^{2+}$ induces the peroxidase activity; and at high level – suppresses the same. Suppression of the enzyme activity is not associated with fungi viability, but with specific response of the enzyme to Mn$^{2+}$ effect. Magnitude of peroxidase activity and corresponding Mn$^{2+}$ concentrations are functions of types and concentrations of carbon and nitrogen sources, and fungal species. Analysis of nutritional impact of Mn$^{2+}$ - induced peroxidase activity allows better understanding of the mechanisms involved in regulation of ligninolytic activity and optimizing it for a particular species. For example, the present study determined that maximum D. squalens peroxidase activity may be achieved in media supplemented with glucose and ammonium chloride at 1:1 ratio in the presence of 0.05 mg/ml of manganese sulfate; whereas, arabinose/sodium nitrate at ratio of 1:10 with 0.5 mg/ml Mn$^{2+}$ provided the highest peroxidase activity in C. subvermispora.
3.7 Conclusions

The main contribution of this research was the investigation of the nutritional impact and fungal species on Mn\(^{2+}\) regulated peroxidase activity. This study revealed that the Mn\(^{2+}\) regulation of peroxidase activity includes not only induction of the enzyme, but also suppression. The behavioral trend of peroxidase activity in regards to Mn\(^{2+}\) was dependent on the specific ligninolytic fungi and carbon and nitrogen sources. Beyond the peak concentration, Mn\(^{2+}\) supplementation lowered the peroxidase activity of both the fungi species.

The findings of this study underline the fact that induction and suppression of peroxidase activity by Mn\(^{2+}\) is not associated with fungi growth capacity, but with a specific enzyme response to nutritional regulation. This finding will motivate molecular level research to gain an understanding of the mechanisms related to the toxicity of Mn\(^{2+}\) on peroxidase activity. The results from this research led to the identification of the key areas requiring further investigation to enhance the peroxidase activity. The insignificant suppression of peroxidase activity of *C. subvermispora* makes this fungus highly tolerant to Mn\(^{2+}\) supplementation. However, the low Mn\(^{2+}\) PC for maximum peak activity makes *D. squalens* a suitable candidate for high production of peroxidases. Knowledge of the critical concentrations (peak concentration and minimum suppressive concentration) of Mn\(^{2+}\) is indispensable to the establishment the optimal culture conditions for enhanced peroxidase activity and, hence, effective lignin degradation.
3.8 References


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Chapter Four: Nutrient Media Optimization for Simultaneous Enhancement of the Laccase and Peroxidases Production by Co-culture of *Dichomitus Squalens* and *Ceriporiopsis Subvermispora*

4.1 Presentation of the Article

This article aims at optimizing the simultaneous enhancement of laccase and peroxidase activity in a co-culture of *Dichomitus squalens* and *Ceriporiopsis subvermispora*. From the first study which deals with enhancement of laccase activity, *D. squalens* resulted in highest laccase activity in the presence of glucose, casein and copper sulfate. Similarly, from the second study, it was observed that *C. subvermispora* resulted in the highest peroxidase activity in the presence of arabinose, sodium nitrate and manganese sulfate. Therefore, the above two fungal species were co-cultivated in the presence of glucose, arabinose, sodium nitrate, casein, copper sulfate and manganese sulfate. These nutrients were chosen because the highest enzyme activities were observed in their presence. Response surface methodology was used to determine the nutrient factors having significant impact on enzyme activities and to optimize the significant factors for maximum activities. The optimized co-cultivation nutrient media resulted in 2 and 3-fold higher laccase and peroxidase activity compared to that of respective monocultures which in turn led to increased lignin degradation.
Nutrient Media Optimization for Simultaneous Enhancement of the Laccase and Peroxidases Production by Co-culture of Dichomitus Squalens and Ceriporiopsis Subvermispora

This article has been submitted to the Biochemical Engineering Journal.

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4.2 Abstract

A co-culturing strategy was explored for the simultaneous augmentation of laccase and peroxidase production using the two white rot fungi, *Dichomitus squalens* and *Ceriporiopsis subvermispora*. The nutrient parameters chosen from our previous studies with the monocultures of *D. squalens* and *C. subvermispora* were used to design the experiments for the simultaneous enhancement of laccase and peroxidase using the co-cultivation of the above microorganisms. Glucose, arabinose, sodium nitrate, casein, copper sulfate and manganese sulfate were combined in different proportions based on a central composite design and used as the incubation media for the co-cultivation. The tested concentrations of glucose and sodium nitrate did not alter enzyme activity. These nutrients provided 800 U per g protein as the basic level of activity of each of the enzymes, laccase and peroxidase, and regulated impacts of the other nutrients on the ligninolytic activities. Cross regulations of enzymes activity was observed for arabinose, casein, copper
sulfate and manganese sulfate, which controlled laccase and peroxidase activity as a function of concentrations. The optimized nutrient levels correspond to incubation media containing 1% glucose, 0.1% arabinose, 20 mM sodium nitrate, 0.27% casein, 0.31 mM copper sulfate, and 0.07 mM manganese sulfate. The cultivation of co-culture of *D. squalens* and *C. subvermispora* in this media resulted in the production of 1378 U/g laccase and 1372 U/g peroxidase. The treatment of wheat straw with the optimized crude enzyme mixture resulted in a lignin degradation of 16.9%.

4.3 Introduction

The present-day emphasis on renewable energy resources is motivated by the need to meet the growing energy demand while reducing the environmental impact. Biofuels is a renewable energy source obtained from cellulosic or lignocellulosic biomass. Biofuels have the added advantage of energy efficiency, sustainability and environmental-friendly. Processing of cellulosic biomass for biofuels is simple and easy. However, cellulosic biomass belongs to the food-crops and hence its exploration interferes with food supply. This competition between food and fuel can be overcome by opting for the lignocellulosic waste for biofuel generation. Agricultural byproducts such as straw and waste from wood-based industries are, but a few examples of lignocellulosic biomass which is mainly composed of lignin, cellulose and hemicellulose. Lignin, a phenyl-propanoid based polymer, encases the cellulose and hemicellulose matrix, such that these polysaccharides are not available for the fermentation process. Hence, as the preliminary step towards biofuel generation, the lignin polymer must be
fragmented to release the polysaccharides. Polysaccharides are hydrolyzed to monomeric sugar molecules and then fermented to obtain the biofuel yield.

Currently, delignification is performed with acid, alkali, steam, ammonia. However, chemical delignification produces weak acids, furan and phenolic compounds as byproducts which have undesirable effects on the subsequent hydrolysis and fermentation steps. Biological delignification based on the activity of white rot fungi has been shown to overcome these drawbacks. White rot fungi secrete extracellular ligninolytic enzymes such as laccase, lignin peroxidase, manganese peroxidase and versatile peroxidase. These enzymes are non-specific oxido-reductases which degrade the lignin polymer into fragments of phenolic and non-phenolic low molecular compounds. Laccase (EC.1.10.3.2) is the ligninolytic enzyme with copper as the active center and the transcriptional regulator [1]. Manganese peroxidase (E.C.1.11.1.13) is a heme-containing enzyme which consists of Mn-binding sites [2], catalyzes the oxidation of Mn$^{2+}$ to Mn$^{3+}$ in the presence of H$_2$O$_2$. The highly reactive Mn$^{3+}$ combines with organic chelating compounds such as oxalic acid and acts as low-molecular weight, diffusible redox-mediator targeting the oxidation of the phenolic components of lignin. Mn$^{2+}$ has been shown to play the key role in the transcriptional phase as well as in the activation of the peroxidase enzyme. Both microelements have an important role in the regulation of either laccase or peroxidase activity [3, 4] causing either induction or suppression of the enzyme activity, depending on the concentration of nutrients. The effects of copper and manganese are controlled by the type and relative ratios of carbon and nitrogen sources. The prevailing enzyme in a fungal species and its nutritional regulation were strain-dependent. For example, *D. squalens* is a better producer of laccase [3]. In contrast, *C. subvermispora* is a better producer of peroxidases with low laccase production.
Among the variety of carbon and nitrogen source tested, the highest laccase activity in *D. squalens* was observed in the presence of glucose and casein supplemented with copper sulfate [3]. The peroxidase activity in *C. subvermispora* was enhanced to a greater level in the presence of arabinose and sodium nitrate along with manganese sulfate [4]. These observations were done for monocultures of fungi.

In the natural environment, microorganisms of different phenotype and genotype exist in a close interaction. Most of the ecological processes are either symbiotic or competitive and require the close relationship of different biological organisms. One such natural process is the wood decaying of trees primarily caused by the lignin degradation which is in turn brought about by the mutual interaction between different white rot fungi, soft rot fungi and brown rot fungi. This leads to the possibility of alteration in the enzyme synthesis pattern due to the complex metabolic process caused by the interaction between the mixed cultures. For instance, Hu et al [5], reported that *Aspergillus niger* and *A. oryzae* when co-cultured with *Phanerochaete chrysosporium* produce higher quantities of β-glucosidase, α-cellulbiohydrolase, β-galactosidase, laccase and β-xylosidase than monocultures. In addition, Sundman et al., [6] have shown that the lignin degradation was synergistic in the presence of mixed cultures. Chen et al., [7] reported that the combination of *D. squalens* and *Pleurotus radiata* resulted in higher laccase production than the related monocultures; and MnP was stimulated in the co-cultures of *P. radiata* and *P. ostreatus*. Based on Chi et al., [8], co-cultivation modifies the ligninolytic enzymes secreted and also the related isoform composition. In general, the strategy of co-cultivation seems to be beneficial for increased ligninolytic enzyme production and hence improved lignin degradation.
The nutrient environment and the specific microorganisms chosen for mixed culture are the key parameters governing the effectiveness of the co-cultivation [9].

Though co-cultivation has been studied for the synthesis of ligninolytic enzymes, no media has been suggested for the optimum simultaneous production of enzyme mixtures. The goal of this work is to utilize a co-cultivation strategy to establish the optimum nutrient conditions for the production of both laccase and peroxidase using a single media. Response surface methodology (RSM) was used to understand the individual and interaction effects of nutrients and hence to optimize the nutrients conditions for maximum simultaneous laccase and peroxidase production. Central composite design (CCD) was used to design the experiments [10]. The nutrient factors used in the experiment were chosen from our previous studies with the monocultures of *D. squalens* and *C. subvermispora*.

### 4.4 Materials and methods

#### 4.4.1 Microorganisms and Chemicals

*Dichomitus squalens* ATCC 201541 and *Ceriporiopsis subvermispora* ATCC 90467 were purchased from American Type Culture Collection (ATCC), USA. Fungi were maintained on potato dextrose agar (PDA) and stored at 4°C. Prior to experiments, fungi were grown on fresh PDA plates at 25°C for 5 days. All chemicals were purchased from Sigma-Aldrich, USA.
4.4.2 Cultivation conditions

The fungi were cultivated in 24-well plates with 2 ml of the nutrient media consisting of the basic mineral medium (KCl – 0.56 g/l, MgSO₄•7H₂O – 0.78 g/l, FeSO₄•7H₂O – 8 mg/l, KH₂PO₄ – 2.22 g/l and thiamine – 1 mg/l, pH 5) in addition to the glucose, arabinose, sodium nitrate, casein, CuSO₄ and MnSO₄ according to the central composite design. Each well was inoculated with two agar plugs (3 x 3 mm), each obtained from the edge of actively growing D. squalens and C. subvermispora colonies on PDA plates. The plates were incubated at 25 °C on an orbital shaker at 150 rpm for seven days. Each experiment was replicated at least thrice. Cell-free media with extracellular enzymes was separated from the fungal biomass at the end of seventh day. Seven day incubation period was chosen because it has been reported that this time period will provide sufficient fungal growth and maximum enzyme production for several other white-rot fungal species irrespective of the media/substrate composition and type of incubation [11, 12]. The supernatant was analyzed for laccase and peroxidase activities. The fungal biomass was analyzed for protein content.

4.4.3 Laccase and peroxidase assays

Laccase and peroxidase activity were determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) to an orange brown dimer, coerulignone [13]. The reaction mixture (250 µl) for laccase activity contained 0.25 mM of DMP in 0.1 M sodium tartarate buffer (pH 5.0) and 10 µl of culture media. Absorbance was monitored for 5 minutes at 468 nm and 30 °C. To determine the peroxidase activity, the reaction mixture (250 µl) contained 0.5 mM of DMP and 0.1 mM manganese sulfate in 0.1 M sodium tartrate buffer (pH 5.0) and 5 or 10 µl of
supernatant, 0.05 mM hydrogen peroxide was added to initiate the reaction and the absorbance kinetics was monitored at 468 nm for 5 minutes at 30 °C. Kinetic measurements were carried out in microplates (path length 0.69 cm) with Spectramax Multimode microplate reader [14]. One international unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 µmol of DMP (molar extinction coefficient 27500 M\(^{-1}\) cm\(^{-1}\)) per min at the conditions of the experiment [13]. Peroxidase activity was corrected for laccase activity which was determined in the absence of manganese sulfate and hydrogen peroxide. Both laccase and peroxidase activities were normalized with respect to the protein content in the fungal biomass and hence represented in terms of U per g of protein.

4.4.4 Protein assay

Fungal biomass was sonicated (VWR model 150HT) at 40 KHz in lysis buffer for 20 min in ice and then placed in ice for another 20 min before centrifugation to separate the supernatant for protein analysis. The total protein content of the fungal biomass was determined by the Biorad DC protein assay using Bovine Serum Albumin (BSA) as the standard according to manufacturer instructions. Briefly, sample was incubated for 15 min in a solution of alkaline copper tartarate and Folin reagent and the absorbance was read at 750 nm. The calibration curve was plotted using BSA as the standard and the protein content of the samples was calculated from the calibration curve.
4.4.5 Experimental Design

Basic mineral media were supplemented with glucose, arabinose, sodium nitrate, casein, CuSO$_4$ and MnSO$_4$ according to the face centered ($\alpha = 1$), central composite design (CCD) of resolution V. In case of resolution V design, none of the main effect or two-factor interaction is aliased with any other main effect or two-factor interaction; but two-factor interactions are aliased with three-factor interactions. All factors were varied at three levels as shown in Table 4-1. The CCD was constructed on a cubic domain with 12 axial points, 22 factorial points and one center point, replicated 8 times. Table 4-1 shows the 42 experimental trials carried out to study the effect of the six factors on the responses, laccase and peroxidase activity of the co-culture (U/g of protein). The relationship between the responses and the six factors can be modelled by a second order quadratic equation,

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j$$  

(4-1)

where $Y$ represents the predicted response (either laccase or peroxidase activity), $\beta_0$ is the constant coefficient which is equal to $Y$ when all the independent factors are zero, $\beta_i$ is the linear coefficient for each factor, $\beta_{ii}$ is the square coefficient for each factor and $\beta_{ij}$ is the interaction or cross coefficient between factors.

4.4.6 Monoculture versus Co-culture

To compare the effect of monocultivation and co-cultivation on laccase and peroxidase activities, eight random experimental conditions (various nutrient concentrations) were chosen (Table 4-4). As mentioned in section 4.4.2, 2 ml of different nutrient media was taken in each
well of a 24-well microplate and two agar plugs (3 x 3 mm) were added to each well. For monoculture experiments, both the agar plugs were taken from either *D. squalens* or *C. subvermispora* colonies on PDA plates. For co-culture experiments, one agar plug was taken from *D. squalens* and one from *C. subvermispora* colonies. After incubation for seven days at 25°C on an orbital shaker at 150 rpm, cell-free media with extracellular enzymes was separated from the fungal biomass. The cell-free media was analyzed for laccase and peroxidase activities and the fungal biomass was analyzed for protein content. Each experiment was replicated at least thrice.

**4.4.7 Delignification study using optimized nutrient medium**

Design-Expert 8.0.6 software, Stat-Ease Inc. was used to determine the optimum nutrient levels for simultaneous enhancement of laccase and peroxidase activity in the co-culture based on numerical optimization. The nutrient media was prepared based on the optimum factor levels and the co-culture was incubated in this media for seven days at 25°C and 150 rpm in an orbital shaker. For comparison purpose, monocultures of *D. squalens* and *C. subvermispora* were incubated in the above optimized nutrient medium. The resultant crude enzyme extract from both co-cultivation and mono-cultivations after centrifugation was used to delignify wheat straw. The crude enzyme extract was analyzed for laccase and peroxidase activities. The delignification study of wheat straw was also carried out using crude enzymes obtained from standard media containing 1% glucose and 20 mM sodium nitrate. 60 ml of crude enzyme extract was added to 250 ml capacity flask containing 5 g dried wheat straw. The mixture was placed in rotary shaker at 130 rpm for a period of 6 h under room temperature. 10 μM H$_2$O$_2$ and 10 μM MnSO$_4$ were
added for every 2 h as the requirement of manganese and hydrogen peroxide is mandatory for the completion of the catalytic cycle of the peroxidase enzyme [2, 15]. At a concentration of 10 µM, H₂O₂ was found not to inhibit laccase activity. On the contrary, it has been reported that during in vitro experiments, lignin breakdown was promoted in the presence of both laccase and H₂O₂ [16]. At the end of the treatment period, the liquid and solid fractions were separated by vacuum filtration. The solid fraction was dried at 50 °C for 24 h and then subjected to lignin determination by two-stage sulfuric acid hydrolysis method [17]. The method involves the acid hydrolysis of polysaccharides followed by gravimetric analysis of lignin in the wheat straw.

4.5 Results

4.5.1 Central composite design

Central composite design (CCD) provides important information regarding the optimum level of each variable along with their interaction with other variables and their impact on enzyme activity. Based on the regression analysis for monocultures/monoenzyme conditions [3, 4], glucose (X₁), arabinose (X₂), sodium nitrate (X₃), casein (X₄), copper sulfate (X₅) and manganese sulfate (X₆) were selected for CCD optimization of simultaneous production of laccase and peroxidase by the co-culture of D. squalens and C. subvermispora. The concentrations of these variables and the corresponding experimental and predicted data are shown in Table 4-1. The experimental results: either laccase or peroxidase activity, were fitted by a second-order polynomial function (Eqn. 4-1). ANOVA was performed on the response surface reduced quadratic model to incorporate all the individual effects and the significant interaction and higher order effects. The values of the corresponding regression coefficients were
calculated and the equations were fitted (Eqn. 4-2 and Eqn. 4-3) for predicting the enzyme activity as shown below.

\[
Y (\text{Laccase}) = 835.85 + (15.72 X_1) – (1.36 X_2) - (3.86 X_3) + (174.74 X_4) - (309.25 X_5) - (56.17 X_6) + (187.76 X_2 X_6) + (338.54 X_2^2) – (402.51 X_4^2) – (353.59 X_5^2) \quad (4-2)
\]

\[
Y (\text{Peroxidase}) = 837.96 – (52.24 X_1) - (10.46 X_2) – (46.05 X_3) + (36.50 X_4) - (221.49 X_5) – (45.63 X_6) + (279.41 X_4 X_5) + (91.87 X_4 X_6) + (120.80 X_5 X_6) + (374.45 X_2^2) - (358.16 X_4^2) – (517.64 X_5^2) \quad (4-3)
\]

where \( Y \) is the response value (the enzyme activity), \( X_1 \) – glucose (%), \( X_2 \) – arabinose (%), \( X_3 \) – sodium nitrate (mM), \( X_4 \) – casein (%), \( X_5 \) – copper sulfate (mM), \( X_6 \) – manganese sulfate (mM).

The coefficient of determination \( (R^2) \) representing the goodness of fit [18] of the empirical model for laccase activity was 0.7011 and for peroxidase activity 0.8131, which indicates that 70% and 81% of the total variation in the data around the average is explained by the fitted models given in Equation (4-2) and (4-3) for laccase and peroxidase respectively.
Table 4-1. Experimental conditions of the six factor face centered central composite design with the corresponding experimental and predicted responses

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Variable Level</th>
<th>Laccase activity (U/g)</th>
<th>Peroxidase activity (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td>Experimental</td>
<td>Predicted</td>
</tr>
<tr>
<td>1</td>
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<td>1104</td>
<td>914.8</td>
</tr>
<tr>
<td>2</td>
<td>1 0.1 20 0.5 1 0.05</td>
<td>215.1</td>
<td>261.9</td>
</tr>
<tr>
<td>3</td>
<td>3 0.1 50 0.05 0.13 3</td>
<td>522.1</td>
<td>419.5</td>
</tr>
<tr>
<td>4</td>
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<td>1436</td>
<td>1262</td>
</tr>
<tr>
<td>5</td>
<td>3 0.1 20 0.05 0.13 0.05</td>
<td>1162</td>
<td>913.1</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
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</tr>
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</tr>
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<td>32.97</td>
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<td>949.1</td>
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<td>Run No.</td>
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<td>Laccase activity (U/g)</td>
<td>Peroxidase activity (U/g)</td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
<td>------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td></td>
<td>X₁ X₂ X₃ X₄ X₅ X₆</td>
<td>Experimental</td>
<td>Predicted</td>
</tr>
<tr>
<td>27</td>
<td>3 0.01 20 0.5 1 0.05</td>
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<td>3 0.06 35 0.28 0.56 1.52</td>
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<td>2 0.06 35 0.28 1 1.52</td>
<td>31.63</td>
<td>58.91</td>
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</table>

* X₁ – Glucose(%) ; X₂ – Arabinose(%) ; X₃ – Sodium nitrate(mM) ; X₄ – Casein(%) ; X₅ – Copper sulfate(mM) ; X₆ – Manganese sulfate(mM).
The corresponding analysis of variance (ANOVA) is shown in Table 4-2 and Table 4-3. ANOVA explains whether the model adequately fits the variations in the enzyme activity with the designed levels of variables. If the $F$-test for the model is significant at the $5\%$ level ($p<0.05$), then the model is fit and can adequately explain the variation observed. In general, the model provides a good prediction of the experimental results if the calculated $F$ value is greater than the tabulated $F$ value. In our models, the calculated $F$ values of 9.38 and 14.99 were greater than the corresponding tabulated $F$ values of 5.72 and 5.57, respectively, at $p$ level of 0.0001. This indicated that the model was highly significant.

### Table 4-2. Analysis of Variance for Response Surface Reduced Quadratic Model of Laccase activity

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-value</th>
<th>p value Prob &gt; F</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
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<td>4965615</td>
<td>551735</td>
<td>9.38</td>
<td>&lt;0.0001</td>
<td>Significant</td>
</tr>
<tr>
<td>Error</td>
<td>32</td>
<td>2113500</td>
<td>66046.88</td>
<td>3.14</td>
<td>0.0784</td>
<td>Not significant</td>
</tr>
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<td>Total</td>
<td>41</td>
<td>7079115</td>
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</tbody>
</table>

$R$ - squared 0.7011

### Table 4-3. Analysis of Variance for Response Surface Reduced Quadratic Model of Peroxidase activity

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-value</th>
<th>p value Prob &gt; F</th>
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<tbody>
<tr>
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<td>&lt;0.0001</td>
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<td>Total</td>
<td>41</td>
<td>6636600</td>
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</table>

$R$ - squared 0.8131
4.5.2 Effect of nutrient factors on laccase activity

The three-dimensional response surface plots were used to demonstrate the interactions of media compounds and to determine the optimum levels of compounds in the media to enhance enzyme activity. The response surface plots for laccase activity and peroxidase activity are shown in Figure 4-1 and Figure 4-2, respectively.

Glucose and sodium nitrate supported the enzymes’ activities of 835 U/g regardless the nutrients concentrations (at least at the levels tested in this study); whereas, variation in levels of arabinose, casein, copper sulfate and manganese sulfate regulated the enzymes activities. The lowest concentration of arabinose (0.01%) resulted in increasing the laccase activity to 1100 U/g in the presence of glucose. Increase in arabinose to 0.05%, lowered the laccase activity. However, at the highest concentration tested, laccase activity similar to 0.01% was observed (Figure 4-1). In the presence of sodium nitrate, 0.03% arabinose provided laccase activity of 920 U/g, which increased to 1171 U/g at 0.01 U/g and 0.1 U/g. 0.06% arabinose minimized the laccase activity in the presence of casein, copper sulfate and manganese sulfate. Increasing and decreasing the arabinose levels led to increasing the laccase activity. The lowest enzyme activity was observed at 0.06% arabinose and 0.05% casein (267 U/g), 1 mM copper sulfate (179 U/g) and 3 mM manganese sulfate (779 U/g).
Figure 4-1. 3D surface plot of laccase activity as function of the nutrient factors (Continued)
Figure 4-1. 3D surface plot of laccase activity as function of the nutrient factors (Continued)
Figure 4-1. 3D surface plot of laccase activity as function of the nutrient factors

(A) X₁ vs X₂; (B) X₁ vs X₃; (C) X₁ vs X₄; (D) X₁ vs X₅; (E) X₁ vs X₆; (F) X₂ vs X₃; (G) X₂ vs X₄; (H) X₂ vs X₅; (I) X₂ vs X₆; (J) X₃ vs X₄; (K) X₃ vs X₅; (L) X₃ vs X₆; (M) X₄ vs X₅; (N) X₄ vs X₆; (O) X₅ vs X₆

X₁ – Glucose (%); X₂ – Arabinose (%); X₃ – Sodium nitrate (mM); X₄ – Casein (%); X₅ – Copper sulfate (mM); X₆ – Manganese sulfate (mM).
Laccase activity in response to casein in the presence of glucose and sodium nitrate increased until 0.28% casein reaching 837 U/g and 836 U/g, respectively and then decreased to 608 U/g and 614 U/g at 0.5% casein. Casein with copper sulfate provided maximum laccase activity (899 U/g) at 0.28% and 0.32 mM, respectively. Increasing the nutrients’ concentrations beyond these levels reduced laccase activity. The minimum (zero) enzyme activity was observed at 1 mM copper sulfate irrespective of casein level. The maximum (891 U/g) laccase activity due to casein-manganese sulfate interaction was observed at 0.28% casein and 0.05 mM manganese sulfate. The minimum level of enzyme activity (205 U/g) resulted from interaction of 0.28% casein and 3 mM manganese sulfate.

Copper sulfate-induced laccase activity in the presence of glucose and sodium nitrate increased until 0.32 mM copper sulfate reaching 898 U/g, and then gradually reduced up to 175 U/g at 1 mM copper sulfate. In the presence of glucose and sodium nitrate, manganese sulfate provided maximum laccase activity (892 U/g) at 0.05 mM manganese sulfate, which reduced to 779 U/g at 3 mM. Interaction of copper sulfate and manganese sulfate resulted in highest laccase activity at 0.05 mM manganese sulfate and 0.32 mM copper sulfate, and minimum activity at 0.05 mM manganese sulfate and 1 mM copper sulfate.

4.5.3 Effect of nutrient factors on peroxidase activity

The response of peroxidase to nutrient regulation has similar trend as laccase. A peroxidase activity of 837 U/g was observed in glucose-sodium nitrate case regardless the concentrations of the nutrients (Figure 4-2). Peroxidase activity in response to 0.06% arabinose in the presence of glucose or sodium nitrate was 837 U/g, and then increased to 1212 U/g at 0.01% and 0.1% arabinose. Peroxidase activity in response to casein in the presence of glucose
or sodium nitrate increased from 442 U/g at 0.05% casein to 837 U/g at 0.28% casein and reduced to 530 U/g at 0.5%. Copper sulfate-induced peroxidase activity increased from 554 U/g at 0.13 mM to 857 U/g at 0.51 mM and then dropped to 104 U/g at 1 mM due to interaction with glucose or sodium nitrate. Manganese sulfate provided the highest enzyme activity (875 U/g) at 0.05 mM, which decreased at higher manganese concentrations.

The interaction of arabinose and casein resulted in the highest peroxidase activity (1207 U/g) at 0.28% casein and either 0.01% or 0.1% arabinose. The minimum enzyme activity of 444 U/g was observed at 0.06% arabinose and 0.05% casein. Arabinose and copper sulfate provided the highest peroxidase (1220 U/g) at 0.01%/0.1% and 0.51 mM, respectively. The lowest activity (109 U/g) was observed at 0.06% arabinose and 1 mM copper sulfate. Arabinose and manganese sulfate provided the highest peroxidase (1245 U/g) at 0.01%/0.1% and 0.05 mM, respectively. The lowest activity (799 U/g) was observed at 0.06% arabinose and 3 mM manganese sulfate.
Figure 4-2. 3D surface plot of peroxidase activity as function of the nutrient factors (Continued)
Figure 4-2. 3D surface plot of peroxidase activity as function of the nutrient factors (Continued)
Figure 4-2. 3D surface plot of peroxidase activity as function of the nutrient factors

(A) $X_1$ vs $X_2$; (B) $X_1$ vs $X_3$; (C) $X_1$ vs $X_4$; (D) $X_1$ vs $X_5$; (E) $X_1$ vs $X_6$; (F) $X_2$ vs $X_3$; (G) $X_2$ vs $X_4$; (H) $X_2$ vs $X_5$; (I) $X_2$ vs $X_6$; (J) $X_3$ vs $X_4$; (K) $X_3$ vs $X_5$; (L) $X_3$ vs $X_6$; (M) $X_4$ vs $X_5$; (N) $X_4$ vs $X_6$; (O) $X_5$ vs $X_6$

$X_1$ – Glucose(%) ; $X_2$ – Arabinose(%) ; $X_3$ – Sodium nitrate(mM) ; $X_4$ – Casein(%) ; $X_5$ – Copper sulfate(mM) ; $X_6$ – Manganese sulfate(mM).
The maximum (773 U/g) peroxidase activity was achieved at 0.39% casein and 0.51 mM copper sulfate. The minimum (zero) enzyme activity resulted from 0.13 mM copper sulfate and 0.5% casein, 1 mM copper sulfate and either 0.05% or 0.16% casein. 0.28% casein provided the highest peroxidase activity (875 U/g) with 0.05 mM manganese sulfate. The minimum activity (405 U/g) was observed at 0.05% casein and 3 mM manganese sulfate. Manganese and copper sulfates interaction resulted in the maximum peroxidase activity (906 U/g) at 0.05 mM and 0.51 mM, respectively, and minimum enzyme activity (28 U/g) at 0.05 mM manganese sulfate and 1 mM copper sulfate.

4.5.4 Optimization of nutrient factors

The Design expert 8.0.6 software was used to determine the optimum nutrient levels based on numerical optimization. The software generates the possible solutions along with the desirability factor. Desirability is an objective function which ranges from zero to one, the latter being closer to the set goal. To obtain the maximum laccase activity of 1437 U/g, the optimum solution is 2.95% glucose, 0.1% arabinose, 20.05 mM sodium nitrate, 0.3 mM casein, 0.25 mM copper sulfate, and 0.05 mM manganese sulfate with a desirability factor of 1.0. Similarly, for a maximum peroxidase activity of 1417 U/g, the optimum factor levels are 1% glucose, 0.1% arabinose, 20 mM sodium nitrate, 0.23 mM casein, 0.39 mM copper sulfate, and 0.08 mM manganese sulfate associated with a desirability factor of 0.91. However, the main objective of this work was to establish the optimum conditions for simultaneous enhancement of laccase and peroxidase activity. The design expert software allowed setting goals for both the responses for simultaneous optimization. Hence the goal was set to be a maximum for both the responses with equal importance. The optimum factors for a maximum laccase of 1378 U/g and peroxidase 1372
U/g are 1% glucose, 0.1% arabinose, 20 mM sodium nitrate, 0.27% casein, 0.31 mM copper sulfate, and 0.07 mM manganese sulfate with a desirability of 0.918.

4.5.5 Monocultures versus co-culture

In order to elucidate whether the co-cultivation is advantageous over the monocultures for ligninolytic enzymes production, eight random experimental trials were chosen for the comparison of the ligninolytic activity of the co-cultivation of *D. squalens* and *C. subvermispora* against their respective monocultures. Table 4-4 shows the factor levels for the eight random trials and the laccase and peroxidase activities of the co-culture and monocultures. For most of the tested conditions, both the laccase and peroxidase activities were 2 – 4-fold higher in the co-cultivation when compared to the monocultures of *D. squalens* and *C. subvermispora*. 
Table 4-4. Laccase and peroxidase activities of the co-culture and monocultures for eight random trials

<table>
<thead>
<tr>
<th>Run</th>
<th>Experimental conditions</th>
<th>Laccase (U/g)</th>
<th>Peroxidase (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X₁ (Glucose(%))</td>
<td>X₂ (Arabinose(%))</td>
<td>X₃ (Sodium nitrate(mM))</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0.1</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.06</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.03</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>0.01</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>0.05</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>1.5</td>
<td>0.01</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>0.1</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>1.2</td>
<td>0.1</td>
<td>20</td>
</tr>
</tbody>
</table>

a X₁ – Glucose(%); X₂ – Arabinose(%); X₃ – Sodium nitrate(mM); X₄ – Casein(%); X₅ – Copper sulfate(mM); X₆ – Manganese sulfate(mM)
4.5.6 Delignification of wheat straw

Laccase and peroxidase activities obtained from monocultures and co-cultures incubated in optimized media are shown in Table 4-5. The lignin content of the untreated wheat straw was 201 g/kg (Table 4-6). Standard media (1% glucose and 20 mM sodium nitrate) based crude enzyme extract lowered the lignin content by 6.5% compared to untreated wheat straw. Lignin content in wheat straw was decreased by approximately 5 and 9% by crude enzyme extracts of optimized medium obtained from monocultures of *D. squalens* and *C. subvermispora*, respectively (as in section 4.4.2). Crude enzyme extract obtained from co-cultivation of *D. squalens* and *C. subvermispora* decreased lignin content by 16.9% in wheat straw.

Table 4-5. Laccase and peroxidase activities in crude enzyme extract of optimized medium

<table>
<thead>
<tr>
<th>Fungal Culture</th>
<th>Laccase (U/g)</th>
<th>Peroxidase (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. squalens</em></td>
<td>547.5</td>
<td>306.3</td>
</tr>
<tr>
<td><em>C. subvermispora</em></td>
<td>409.0</td>
<td>355.5</td>
</tr>
<tr>
<td>Co-culture</td>
<td>1027</td>
<td>948</td>
</tr>
</tbody>
</table>
Table 4-6. Delignification of wheat straw

<table>
<thead>
<tr>
<th>Wheat straw sample</th>
<th>Lignin (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>201</td>
</tr>
<tr>
<td>Exposed to crude enzymes of D. squalens in optimized media</td>
<td>191</td>
</tr>
<tr>
<td>Exposed to crude enzymes of C. subvermispora in optimized media</td>
<td>183</td>
</tr>
<tr>
<td>Exposed to crude enzymes from co-cultivation in standard media</td>
<td>188</td>
</tr>
<tr>
<td>Exposed to crude enzymes from co-cultivation in optimized media</td>
<td>167</td>
</tr>
</tbody>
</table>

4.6 Discussion

Delignification is a complex process based on lignin oxidation by a variety of enzymes including laccase, Mn-dependent and Mn-independent peroxidases. Individual and multiple enzymes production depend on fungi and nutritional conditions. As previously reported, while fungal strains produce different ligninolytic enzymes, they, nevertheless, specialize on production of particular enzyme. For example, *D. squalens* is a better producer of laccase, while *C. subvermispora* is a better producer of peroxidases [3, 4] in the presence of appropriate carbon and nitrogen sources and inducers. The combination of 1% glucose with 0.05% casein provided the maximum laccase activity in *D. squalens* [3], while 0.1% arabinose and 20 mM sodium nitrate were the best combination for high peroxidase activity in *C. subvermispora* [4]. To regulate the transcription and functionality of laccase, copper (which is the active center of the enzyme) is required [19, 20]. The acceptable (non-toxic) copper level providing maximum productivity depends on the carbon and nitrogen sources. The optimum copper sulfate level in glucose-casein medium was 0.12 mM. Similarly, manganese ions are involved in the regulation
of the transcription of manganese peroxidase and also mediate the extracellular activity of the enzyme [2, 21]. The manganese sulfate impact on peroxidase activity is a function of nutrients composition. The highest peroxidase activity in *C. subvermispora* culture was observed at 0.05 mM manganese sulfate in arabinose-sodium nitrate media. Thus, our preliminary experiments allowed determining the nutritional factors, which may contribute to simultaneous enhancement of laccase and peroxidase production in the co-culture of *D. squalens* and *C. subvermispora*: glucose, arabinose, sodium nitrate, casein, copper sulfate and manganese sulfate. The nutrient levels for the experimental design were chosen based on the regression analysis of the results obtained from monoculture experiments. Positive regression coefficients were observed for all but arabinose and hence the levels were increased or decreased accordingly. The highest levels for copper and manganese were based on toxicity levels of these factors [3, 4].

While the concentrations of glucose and sodium nitrate used in this study did not change the enzymatic activity, both glucose and sodium nitrate impacted the enzymes’ responses to other nutrients. Also, cross regulations of the enzymes’ responses to nutrients were observed for arabinose, casein, copper sulfate and manganese sulfate, which controlled laccase and peroxidase activities according to their concentrations. Compared to the basal laccase and peroxidase activity of 835 U/g, both induction and suppression of the enzymes’ activities due to interaction with other nutrients were observed. Both enzymes activities increased when casein, copper sulfate and manganese sulfate levels increased until the critical concentrations, and then reduced with further increase of the nutrients levels. These critical nutrient levels depended on the interactions with other nutrients. A critical concentration of 0.28% casein was required for maximum laccase under most tested conditions. Under most conditions, copper sulfate provided
the highest level of laccase activity at 0.32 mM in the presence of glucose, sodium nitrate, arabinose, and casein. Irrespective of other nutrients, 1 mM copper sulfate suppressed laccase activity, which may be due its toxic effect on fungal growth [22]. Manganese sulfate provided the highest level of laccase activity at 0.05 mM in the presence of glucose, sodium nitrate, arabinose, and casein.

Casein provided highest peroxidase activity at 0.28% in the presence of glucose, sodium nitrate, and arabinose. This influence of casein on peroxidase activity had also been reported for *Trametes versicolor* [23]. 0.51 mM copper sulfate provided maximum peroxidase activity in the presence of glucose, sodium nitrate, arabinose, and manganese sulfate. A critical concentration of 0.39% casein and 0.51 mM copper was required for maximum peroxidase activity. Two unique cases were observed for the effect of casein and copper on peroxidase activity: (1) lowering copper and increasing casein concentrations resulted in lowering peroxidase activity; (2) similar effect was observed at high copper and low casein levels. This may be due to the complex formation between copper and casein [24], which in the first case may have reduced the required copper for increasing peroxidase and in the second case, high level of free copper may have inhibited the fungal growth and hence lowered the peroxidase activity. The above impact of copper sulfate on the peroxidase activity demonstrates the significant role played by copper on the regulation of the peroxidase activity. The maximum peroxidase activity in response to manganese sulfate was observed at 0.05 mM under most test conditions. However, increasing concentrations of copper sulfate increased critical concentrations of manganese sulfate to obtain high peroxidase activity. This may be due to the alleviation of copper toxicity due to the interaction between manganese and copper ions [25].
The interesting observation resulting from the present study is the regulation (both inductive and suppressive) of peroxidase activity by copper sulfate and laccase activity by manganese sulfate. Though copper has been traditionally known to regulate the laccase activity [26] and manganese is known to regulate the peroxidase activity [4], we found the cross regulation of ligninolytic enzymes by copper and manganese ions. Although copper was previously reported to enhance peroxidase activity in *T. trogii* [27], the cross regulation of laccase and peroxidase activity by copper and manganese is reported for the first time. Hence, the interactions between manganese and copper metal ions play a significant role in shifting the suppressive concentration of copper sulfate to a higher concentration which in turn results in minimizing the copper toxicity and hence enhancing the enzyme activities.

The observed laccase and peroxidase responses to nutrients and their interactions allowed optimizing the conditions for simultaneous enhancement of enzymes productions by co-culture of *D. squalens* and *C. subvermispora*, which was the main goal of this study. The optimization process allowed determining the optimum concentrations of the six nutrients which were found to regulate both the laccase and peroxidase activity. The optimization of the nutrients also resulted in a reduction of their toxic effects on ligninolytic enzymes activities. In our previous work [3], 0.12 - 0.25 mM copper sulfate were observed to suppress the laccase activity of monocultures of *D. squalens* and *C. subvermispora*. This suppression in laccase activity due to copper sulfate is attributed to copper ions toxicity against laccase production and activity. However, the presence of fungi co-culture and the modified nutrient conditions have increased the laccase tolerance to copper load almost by 2-folds, thereby enhancing the laccase production. The suppressive effects of manganese ions on monocultures were observed at manganese sulfate
concentrations as low as 0.1 – 0.5 mM [4]. The suppression of peroxidase activity in co-culture incubated in modified media was observed only at 1.5 – 2.0 mM. Moreover, reduction of enzyme activity was insignificant.

Lignin degradation using crude enzyme extract from the co-cultivation of *D. squalens* and *C. subvermispora* was approximately 3.5 and 2-folds higher than that obtained using crude extracts from *D. squalens* and *C. subvermispora* mono-cultures, respectively. Thus, the crude enzyme mixture obtained from co-cultivation degraded lignin more efficiently than mono-cultivation. Compared to the co-cultivation in standard media, optimized media co-cultivation resulted in 2.6-folds higher lignin degradation. The enzymes production was regulated by nutrients and mediators, and hence optimized media enhanced simultaneous production and activity during co-cultivation. Therefore, high ligninolytic activity of the crude enzyme mixture obtained from the co-cultivation of *D. squalens* and *C. subvermispora* on optimized media can be effectively utilized for the lignin degradation of lignocellulosic biomass.

### 4.7 Future Work - Mechanism of Nutritional Regulation

The enhancement of laccase and peroxidase activity in co-culture conditions may be due to reinforcement of active center by carbon and nitrogen sources, substitution of copper and manganese during transcription and post-transcriptional regulation of enzyme production, and formation of various isoforms of enzymes. It is required to further investigate these proposed mechanisms in order to obtain a deeper understanding of the regulation of ligninolytic activity by nutrients in the presence of co-culture. In the next phase of this study, the influence of carbon and nitrogen sources and their interactions with copper and manganese on laccase and
peroxidase will be assessed by analyzing the molecular structure of the respective enzymes. Future experiments will be focused on SDS-PAGE to separate the proteins, RT-PCR to qualitatively detect gene expressions for transcriptional regulation, northern blot to study gene expression by detection of mRNA.

4.8 Conclusions

The CCD design and statistical analysis of the nutrient conditions for co-culture of the two white rot fungi, *D. squalens* and *C. subvermispora* with the purpose of simultaneous enhancement of the activity of two ligninolytic enzymes, laccase and peroxidases, led to (i) understanding synergistic and antagonistic interactions between the significant nutrient factors obtained from mono-culture based study; (ii) the optimization of the incubation media addressing requirements of both fungal cultures and both enzymes; (iii) the production of 1370 U/g crude enzymes by co-culture incubated in optimized media; (iv) 16.9% enhancement of lignin degradation in wheat straw by produced enzymes; and (iv) enhancement of the fungal tolerance to copper and manganese resulting in higher enzymatic activity.
4.9 References


Chapter Five: Enhanced Delignification of Wheat Straw by the Combined Effect of Hydrothermal and Fungal Treatments

5.1 Presentation of the Article

This article deals with a two-step pretreatment procedure for enhanced delignification of wheat straw. Hydrothermal process causes rapid decomposition of lignocellulosic biomass, but the byproducts from this process inhibit the downstream processes such as cellulose hydrolysis and fermentation. On the other hand, lignin degradation by white rot fungi is effective but requires long duration for the pretreatment. Therefore, in this work, hydrothermal process was combined fungal treatment to overcome the disadvantages of each of the pretreatment methods. An extensive range of temperatures were investigated for hydrothermal process, along with the influence of hydrothermal temperature on fungal delignification. Hydrothermal process was carried out in a 30 ml stainless steel reactor which was sealed on both ends. The treatment residence time for hydrothermal process was chosen as 5 min based on the extent of decomposition of lignocellulosic biomass. Higher the residence time, higher the decomposition of primary products, which would lead to the formation of secondary products which are the main inhibitory compounds for the downstream processes. Hydrothermal temperature was found to be the controlling factor for both hydrothermal process and fungal delignification. The combined approach resulted in 2-fold higher delignification and higher cellulose content than the individual methods under the conditions tested.
Enhanced Delignification of Wheat Straw by the Combined Effect of Hydrothermal and Fungal Treatments

This article has been submitted to the Bioenergy Research Journal.

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5.2 Abstract

Pretreatment/delignification is the most crucial step during the conversion of lignocellulosic biomass to biofuel. In this work, wheat straw delignification was enhanced by a two-step pretreatment process, comprising hydrothermal and fungal treatments. The effect of a range of hydrothermal treatment temperatures on fungal delignification has been extensively investigated. Wheat straw was exposed to hydrothermal treatment at various subcritical temperatures and then subjected to fungal treatment. Lignocellulose hydrolysis rate was significantly higher during the hydrothermal treatment compared to the considerably slower fungal delignification. However, by-products of lignin degradation via hydrothermal treatment were re-deposited on the cellulose fibers as the substrate was cooled to room temperature. It is shown that post-treatment fungi can enhance delignification by degradation of the residual lignin by-products. The effect of fungi on delignification of hydrothermally treated substrate was a function of temperature of the hydrothermal process. Compared to the hydrothermal treatment, the novel combined approach, proposed in this study, resulted in two-fold higher lignin removal
and hence promises to be an effective method for delignified substrate preparation for downstream fermentation of cellulose to biofuel.

5.3 Introduction

The increasing fossil-based fuel energy consumption rapidly leads to depletion of oil reserves and increases rate of greenhouse gas (GHG) emissions. Underpinned by the effort to reduce GHG emissions and provide enough energy, attention is growing on the utilization of alternative energy resources, such as biofuel production from waste biomass. Waste biomass is an abundant by-product of agriculture and wood-based industries, and municipal waste. These resources do not compete with the food supply as the conventional crop-based biofuel, which gives waste biomass a great advantage in energy production. Lignocellulosic waste biomass is mainly composed of three polymers, cellulose, hemicellulose and lignin. Lignin encapsulates the energy rich cellulose [1] and hence has to be broken to release the cellulose. Delignification can be achieved by either a physical, chemical or biological method.

The physical and chemical methods presently used for the lignin degradation encompass severe operating conditions and generate by-products such as weak acids, furan and phenolic compounds. These by-products act as inhibitors to the downstream processes: hydrolysis and fermentation, and hence, suppress the overall biofuel yield [2]. Recently hydrothermal treatment at subcritical levels was introduced as a physical method for delignification [3, 4]. In this approach, substrate (e.g., lignocellulosic material) is exposed to liquid water under very high pressure (20 MPa) and high temperature (150-374°C). Under these conditions water becomes a highly effective solvent for hydrophobic organic compounds such as lignin. Complete
Hemicelluloses and part of lignin were reported to be solubilized under 180-200°C for residence time of 5-15 minutes [5]. However, products of the hydrothermal-induced lignin depolymerization re-condense together and accumulate on the substrate surface alongside cellulose [5, 6]. Varnai et al. showed that, though lignin is re-localized, its mere presence restricts the hydrolysis of cellulose [7]. Hence, it is essential to remove the lignin aggregates from the substrate surface after hydrothermal pretreatment to ensure effective downstream processing. Delignification of hydrothermally treated substrates is commonly conducted by alkali treatment or solvent extraction and hence contributes to high recycling cost and waste emissions [5].

Alternatively, ligninolytic activity of white rot fungi can complete subcritical hydrothermal delignification [8]. The common lignin degrading enzymes are peroxidases and laccase [9]. The reactions involved in lignin degradation by these extracellular oxidoreductases include demethylation, hydroxylation, and cleavage of aromatic rings in the large intact lignin polymer and cause the release of low molecular weight fragments which are further degraded to carbon dioxide [10]. White rots such as *Dichomitus squalens* [11], *Cyathus stercoreus* and *Ceriporiopsis subvermispora* [12] selectively degrade lignin and leave cellulose-rich substrate for hydrolysis and fermentation. However, lignin degradation using white rot fungi call for lengthy incubation periods and hence limit the application of this method at industrial scale. Here, we propose that an improvement in processing rates and product yield quality could be achieved by first implementing a subcritical hydrothermal treatment followed by a biological treatment to prevent relocation of lignin residuals.
The objective of this work was to enhance the delignification of wheat straw by the combination of hydrothermal and fungal pretreatments. The combined treatment is expected to facilitate delignification by means of reducing the treatment time via hydrothermal pretreatment, and enhanced removal of lignin residuals by fungi. This is the first time that a range of hydrothermal temperatures have been investigated to evaluate the effect of fungal delignification on the lignocellulosic substrate. The individual and combined effects of the hydrothermal and biological treatments on the wheat straw delignification was evaluated based on compositional analysis of solid and liquid fractions, solid-state NMR analysis to assess the structural changes and SEM analysis for morphological changes caused by the treatments.

5.4 Materials and Methods

5.4.1 Materials

Wheat straw (Triticum aestivum) samples obtained from southern Saskatchewan, Canada were used in this study. The samples were ground and sieved to obtain average particle size of 900 µm. Further, the samples were air-dried and stored in air tight containers until use. All chemicals were purchased from Sigma-Aldrich, Canada Co. Oakville, Ontario.

5.4.2 Microorganisms

Dichomitus squalens ATCC 201541 was purchased from American Type Culture Collection (ATCC), USA.
5.4.3 Subcritical Hydrothermal Delignification

Hydrothermal treatment of wheat straw was carried out in the sub-critical temperature range (150 – 250°C) in a salt-bath (KNO₃) equipped with a stirrer and a temperature and pressure controller. Based on the phase diagram, the appropriate pressure was fixed for a given temperature and the agitation was maintained at 1000 rpm. 2 g wheat straw was added to 20 ml of distilled water in a 30 ml vertical stainless reactor. Reactor was placed in the salt-bath at a specific temperature for 5 min. At the end of the reaction time, the reactor was immediately cooled down in water and the contents were separated by vacuum filtration into solid and liquid fractions. Solid fraction was dried at 100°C for 12 h and subjected to fungal pretreatment. Both the solid and liquid fractions were stored for analysis.

5.4.4 Fungal Delignification

Fungi grown on fresh potato-dextrose agar (PDA) plates for a period of five days were used for the treatment of hydrothermally pretreated wheat straw. Hydrothermally pretreated wheat straw obtained from different operating temperatures was subjected to solid-state fermentation (SSF) as followed: 2 g hydrothermally treated wheat straw were moistened with 11.33 ml of distilled water (85% moisture); wet wheat straw was supplemented with five agar plugs (4x4 mm each) from the growing edges of 5-day old D.squalens culture; SSF was performed at 25°C with agitation at 150 rpm over 15 days. To observe the effects of individual biological treatment, raw wheat straw was treated with fungal culture as above. At the end of the incubation period, 30 ml of 0.1M acetate buffer (pH 7.0) was added to the SSF contents and
extracted at room temperature for 2 h. Extraction was followed by centrifugation at 3000 rpm for 30 min for the separation of the solid pellet and liquid hydrolysate. Liquid hydrolysate was further analyzed for laccase activity, peroxidase activity, glucose and phenolic contents.

5.4.5 Analysis of laccase and peroxidase activity

Laccase and peroxidase activity were determined by monitoring the oxidation of 2, 6-dimethoxyphenol (DMP) to an orange brown dimer, coerulignone [13]. The reaction mixture (250 µl) for laccase activity contained 0.25 mM of DMP in 0.1 M sodium tartarate buffer (pH 5.0) and 10 µl of culture media. Absorbance was monitored for 5 minutes at 468 nm at 30°C. To determine the peroxidase activity, the reaction mixture (250 µl) contained 0.5 mM of DMP and 0.1 mM manganese sulfate in 0.1 M sodium tartrate buffer (pH 5.0) and 5 or 10 µl of supernatant, 0.05 mM hydrogen peroxide was added to initiate the reaction and the absorbance kinetics was monitored at 468 nm for 5 minutes at 30°C. Kinetic measurements were carried out in microplates (path length 0.69 cm) with Spectramax Multimode microplate reader [14]. One international unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 µmol of DMP (molar extinction coefficient 27500 M-1cm-1) per min at the conditions of the experiment [13]. Peroxidase activity was corrected for laccase activity which was determined in the absence of manganese sulfate and hydrogen peroxide.
5.4.6 Analysis of liquid hydrolyzate for glucose and phenol

Glucose concentration was determined using anthrone assay [15]. Briefly, 150 µl of anthrone reagent was added to 50 µl of the hydrolysate and after mixing was incubated at 80°C for 30 min. The mixture was cooled to room temperature before reading the absorbance at 625 nm. The glucose concentration related to the measured absorbance was obtained from the calibration curve plotted using glucose as the standard.

Total phenolic content in the liquid hydrolysate which is a measure of lignin dissolution was monitored by Folin-Ciocalteau assay with gallic acid as the standard [16]. Briefly, 50 µl of Folin-Ciocalteau reagent was added to 25 µl of liquid hydrolysate followed by 200 µl of sodium carbonate. All the contents were mixed thoroughly and incubated at room temperature for 2 h and the final absorbance was measured at 765 nm. Calibration curve plotted using gallic acid as the standard was used to determine the total phenolic content from the absorbance values.

5.4.7 Analysis of carbohydrate and lignin content in wheat straw

Raw wheat straw (control), hydrothermally treated wheat straw and fungal treated wheat straw were analyzed for the carbohydrate and lignin content by the two-step sulfuric acid hydrolysis method [17]. Briefly, 72% sulfuric acid was used to hydrolyze the wheat straw for 1 h. After 1 h, the acid content was diluted to 4% by adding distillated water and the resultant mixture was autoclaved at 121°C and 15 psi for 60 min. The contents were separated into solid and liquid fractions by vacuum filtration. The liquid fraction was analyzed for glucose and acid soluble lignin. Glucose was monitored as mentioned in section 5.3.6. The percentage acid soluble lignin was calculated from the absorbance of the sample measured at 205 nm. The solid
residue which mainly consists of lignin was oven dried at 105°C for 12 h and weighed for acid insoluble lignin. Total lignin content is represented as the sum of the acid soluble and acid insoluble lignin fractions.

5.4.8 Scanning electron microscopic analysis

Treated and control wheat straw were oven dried at 105°C for 24 h, sputter coated with gold and then were analyzed for surface morphologies with FEI XL30 scanning electron microscope.

5.4.9 NMR analysis

NMR spectrometry was used to characterize the structural components of the biomass. Wheat straw samples were oven dried at 105°C for 24 h and ground to obtain homogeneous samples. Solid state NMR spectroscopic analysis of samples was carried out in Bruker AMX300, equipped with solid probe BL7 and a rotor of 7mm outer diameter. NMR spectra were obtained based on the cross-polarization pulse program with a contact time of 1 ms and a relaxation delay of 5s.

5.4.10 Statistical analysis

All the experiments were carried out in triplicate. Student t-test was used to estimate statistical significance at the 95% confidence interval.
5.5 Results

5.5.1 Analysis of liquid fraction

Wheat straw was subjected to hydrothermal treatment, fungal treatment, and hydrothermal treatment followed by fungal delignification. As a result of the treatments, certain portion of the wheat straw was hydrolyzed and extracted in the liquid phase. Quantification of lignin and cellulose in the liquid phase is essential to determine the effectiveness of the above treatments. Therefore, liquid hydrolysates were analyzed for total phenolic and glucose contents, respectively. Table 5-1 shows the total phenolic and glucose contents obtained after wheat straw hydrolysis with various treatment approaches.

Table 5-1. Total phenolics and glucose in liquid hydrolyzate

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Total phenolics (mg/L)</th>
<th>Glucose (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydrothermal</td>
<td>Combinedb</td>
</tr>
<tr>
<td>150</td>
<td>99.2±5.9 a</td>
<td>115.4±7.3</td>
</tr>
<tr>
<td>170</td>
<td>119±7.5</td>
<td>138.6±8.27</td>
</tr>
<tr>
<td>190</td>
<td>165±13</td>
<td>187.3±14.9</td>
</tr>
<tr>
<td>210</td>
<td>225±10</td>
<td>253.5±11.7</td>
</tr>
<tr>
<td>230</td>
<td>267±8.1</td>
<td>301.4±11</td>
</tr>
<tr>
<td>250</td>
<td>570±9.3</td>
<td>615.9±12.8</td>
</tr>
<tr>
<td>Fungi only</td>
<td>26.7±0.85</td>
<td></td>
</tr>
</tbody>
</table>

a ± refers to “mean ± Standard Error.”

b Results obtained after fungal treatment on hydrothermally treated substrate
Delignification of wheat straw with fungi culture resulted in the release of 27 mg/L phenolic compounds (products of lignin degradation). Delignification during hydrothermal process, in terms of phenolic hydrolysis increased linearly with respect to temperature. For the temperature range, 150°C to 250°C, phenolic content increased in the range of 4 to 21-folds. The total phenolic content in the hydrolysates increased from 99 mg/L to 570 mg/L as temperature increased from 150°C to 250°C. Subsequent treatment of the hydrothermally degraded wheat straw with fungi culture further improved delignification efficacy, which again depended on the temperature applied during hydrothermal process. 16 mg/L of phenolic compounds was additionally released after biological treatment of the wheat straw exposed to 150°C, and increased up to 46 mg/L, when the pretreatment temperature increased to 250°C. Combined treatment allowed releasing 115 mg/L to 615 mg/L of phenolic compounds due to lignin degradation in wheat straw. This increase in the removal of phenolic compounds could lead to increased cellulose availability.

Similar trends were observed for cellulose degradation by biological, hydrothermal and combined treatments, which were determined by glucose contents in the corresponding hydrolysates. Due to the activity of fungal culture, 2 mg/L of glucose was released. Glucose content in these hydrolysates may reflect the net glucose content which is the difference between glucose released from cellulose and glucose consumed by fungi over the incubation. Glucose content in the hydrolysates from hydrothermal treatment increased from 10 mg/L (150°C) to 66 mg/L (250°C). Subsequent biological treatment released additional 5 – 25 mg/L glucose. Thus, due to combined treatment, 91.5 mg/L glucose may be released from wheat straw for the downstream biofuel fermentation.
5.5.2 Analysis of enzyme activity

The liquid hydrolysates obtained from the biological and combined treatment approaches were subjected to enzyme activity analysis on the end of the incubation period. Laccase activity on the raw wheat straw was 9.9 U/L of hydrolysate, (Table 5-2). Laccase activity on hydrothermally pretreated wheat straw was lower than those on substrate, but increased as process temperature increased: from 0.9 U/L after treatment at 150°C to 5.6 U/L at 250°C.

Table 5-2. Laccase and peroxidase activity in liquid hydrolysate

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Enzyme Activity (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laccase</td>
</tr>
<tr>
<td>Fungi only</td>
<td>9.87±0.33 a</td>
</tr>
<tr>
<td>150</td>
<td>0.962±0.07</td>
</tr>
<tr>
<td>170</td>
<td>0.406±0.02</td>
</tr>
<tr>
<td>190</td>
<td>2.34±0.16</td>
</tr>
<tr>
<td>210</td>
<td>4.53±0.43</td>
</tr>
<tr>
<td>230</td>
<td>6.45±0.23</td>
</tr>
<tr>
<td>250</td>
<td>5.58±0.18</td>
</tr>
</tbody>
</table>

a ± refers to “mean ± Standard Error.”
Peroxidase activity on raw wheat straw was 11.1 U/L. Peroxidase activity on hydrothermally pretreated wheat straw again depended on temperature. Peroxidase activity of samples treated at 150°C to 190°C was lower than those obtained with raw wheat straw. For the rest of the samples, peroxidase activity was equivalent to or higher than in fungi only treated samples.

5.5.3 Analysis of solid fraction

The solid fractions in raw wheat straw, and wheat straw treated with biological, hydrothermal and combined approaches were analyzed for lignin and cellulose (presented by glucose units in the assay) contents (Table 5-3). Raw wheat straw contained 20% lignin and 643 mg/L glucose. After treatment with fungal culture, the lignin content in wheat straw was lowered by 18% compared to that of the raw substrate. Lignin content of hydrothermal treated wheat straw was lowered by 2.5% at 150°C with further improvement up to 22% at 210°C. At 230°C, the lignin removal dropped to 7% improvement. In contrast to all the temperatures tested, hydrothermal treatment at 250°C increased the lignin content of the sample, higher than that of raw wheat straw.

The subsequent fungal delignification of hydrothermally treated samples resulted in further lignin degradation. The combined treatment with 150°C cycle increased the lignin removal by 3.5-fold compared to the corresponding hydrothermal treatment. Comparing the percentage lignin degradation between the hydrothermal and combined treatments, it was observed that for the cases of 170°C, 190°C, 210°C and 230°C cycles, the lignin degradation in
combined treatment was approximately 2-folds higher. 14% of lignin was removed from the 250°C hydrothermal treated sample by the combined treatment.

Table 5-3. Lignin and glucose in solid fraction of wheat straw

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Lignin (%)</th>
<th>Glucose (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydrothermal</td>
<td>Combined b</td>
</tr>
<tr>
<td>150</td>
<td>19.6±0.54 a</td>
<td>18.3±0.09</td>
</tr>
<tr>
<td>170</td>
<td>18.2±0.63</td>
<td>16.9±0.42</td>
</tr>
<tr>
<td>190</td>
<td>16.7±0.92</td>
<td>13.9±0.21</td>
</tr>
<tr>
<td>210</td>
<td>15.1±0.36</td>
<td>12.2±0.80</td>
</tr>
<tr>
<td>230</td>
<td>18.5±0.40</td>
<td>16.8±0.40</td>
</tr>
<tr>
<td>250</td>
<td>22.1±0.58</td>
<td>19.1±0.30</td>
</tr>
<tr>
<td>Raw wheat straw</td>
<td>20.1±0.65</td>
<td>643±39</td>
</tr>
<tr>
<td>Fungi</td>
<td>16.4±0.59</td>
<td>741±46</td>
</tr>
</tbody>
</table>

a ± refers to “mean ± Standard Error.”
b Results obtained after fungal treatment on hydrothermally treated substrate

The polysaccharide content of the untreated and treated wheat straw is expressed in terms of glucose. The glucose content of the raw wheat straw was 583 mg/L. The fungal activity on wheat straw resulted in 1.3-fold increase in the glucose content. The hydrothermal treatment of 150°C and 170°C samples improved the glucose content in the residual solid by approximately 1.2-folds. At 190°C, the glucose content increased by 1.4-folds. For the sample pretreated at
210°C, the increase in glucose dropped to 1.1-fold. The glucose content of samples pretreated at higher temperatures, 230°C and 250°C, was lowered by 2-folds approximately. The subsequent fungal delignification of 150°C, 170°C and 190°C samples lowered the glucose content by 1.5-fold approximately. The glucose content of 210°C sample was lowered by 1.2-fold. In contrast to the above samples, the glucose content of the samples treated at 230°C and 250°C was improved by 1.5-fold by the combined treatment.

5.5.4 SEM analysis

Figure 5-1 shows the SEM images obtained for the raw wheat straw and for the samples that were subjected to hydrothermal, fungal and combined treatments. At low temperatures, 150°C and 170°C, minor degradation of wheat straw was observed. Further increase in temperature up to 250°C resulted in swelling of the biomass. In addition to defibration of wheat straw, tiny droplets were observed on the surface of the biomass at temperatures 230°C and 250°C. The fungi-only treatment resulted in swelling and partial destruction of the wheat straw. The subsequent fungal delignification of the wheat straw resulted in further deterioration of the biomass, which included swelling and defibration. The tiny droplets which were observed on 230°C and 250°C hydrothermally treated wheat straw had disappeared after the fungal delignification.
Figure 5-1. SEM images of wheat straw (Continued)

No fungi        Fungi-treated

25°C

A               H

150°C

B               I

170°C

C               J

190°C

D               K
5.5.5 NMR analysis

NMR analysis was performed on raw wheat straw, and wheat straw treated with fungi, hydrothermal (at 150°C, 210°C and 250°C) and combined approaches. The peaks on NMR spectra at 88 ppm represents C-4 carbons of crystalline cellulose; 84 ppm represents C-4 carbons of amorphous cellulose; 56 ppm represents the methoxyl groups in lignin and 21 ppm represents the acetyl groups in hemicelluloses [18-20].
Table 5-4 shows the spectral data obtained for various samples. The crystallinity of the cellulose represented by the C-4 carbon at 88 ppm was very low for the control wheat straw and was increased by 2.5-fold by the fungi only treatment. The hydrothermal treatment at 150°C improved the crystallinity, but the increase was lower than that caused by the fungi only treatment. Both 230°C and 250°C hydrothermally treated samples increased the crystallinity higher than the fungi only treatment, but the 250°C resulted in 5-fold increase compared to the raw wheat straw. The subsequent fungal delignification of the 150°C and 210°C hydrothermally treated samples resulted in further increase in the crystallinity. But for the sample treated at 250°C, the crystallinity was slightly lowered by the fungal delignification. The amorphous cellulose represented by the C-4 carbons at 84 ppm was the lowest for the raw wheat straw. The fungi only treatment increased the amorphous carbon by 2-folds. All the hydrothermally treated samples resulted in higher amorphous carbon than the raw wheat straw. However, with increase in the treatment temperature, the amorphous carbon level in wheat straw decreased. The subsequent fungal delignification of the samples increased the amorphous carbon level compared to the hydrothermal treatment.
### Table 5-4. Chemical shifts and intensities in the NMR spectra of different samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>88 ppm</th>
<th>84 ppm</th>
<th>56 ppm</th>
<th>21 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw wheat straw</td>
<td>1.93</td>
<td>3.79</td>
<td>1.48</td>
<td>0.721</td>
</tr>
<tr>
<td>Fungi only</td>
<td>4.86</td>
<td>6.79</td>
<td>1.64</td>
<td>1.07</td>
</tr>
<tr>
<td>150°C HT &lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.51</td>
<td>7.69</td>
<td>1.95</td>
<td>0.972</td>
</tr>
<tr>
<td>210°C HT</td>
<td>5.46</td>
<td>7.29</td>
<td>2.28</td>
<td>0.933</td>
</tr>
<tr>
<td>250°C HT</td>
<td>9.65</td>
<td>5.39</td>
<td>2.23</td>
<td>0.215</td>
</tr>
<tr>
<td>150°C fungi &lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.75</td>
<td>8.41</td>
<td>1.88</td>
<td>1.39</td>
</tr>
<tr>
<td>210°C Fungi</td>
<td>6.44</td>
<td>7.78</td>
<td>1.56</td>
<td>0.804</td>
</tr>
<tr>
<td>250°C Fungi</td>
<td>9.44</td>
<td>6.83</td>
<td>1.60</td>
<td>0.842</td>
</tr>
</tbody>
</table>

<sup>a</sup> HT – Hydrothermal treatment

<sup>b</sup> Combined treatment - Results obtained after fungal treatment on hydrothermally treated substrate

Methoxy group represented at 56 ppm was slightly higher in the wheat straw treated by fungi compared to that of the raw wheat straw. With increase in the treatment temperature of the hydrothermal process, the methoxy group level increased. But at 250°C, the intensity was slightly lower than that of 210°C. The subsequent fungal delignification decreased the methoxy levels of all the hydrothermally treated samples. Compared to the 210°C and 250°C samples, the decrease in methoxy level by the fungal delignification was lower for the 150°C sample. The acetyl groups represented at 20 ppm was increased by the fungi only treatment by 1.5-fold. Hydrothermal treatment at 150°C and 210°C resulted in higher acetyl level than the raw wheat straw but lower than the fungi only treatment. At 250°C subcritical process, the acetyl level was
decreased by 3.4-fold compared to the raw wheat straw. The subsequent fungal delignification of 150°C and 250°C samples increased the acetyl level by 2-fold and 1.16-fold compared to that of the raw wheat straw. For the 210°C sample, the acetyl groups were decreased by the subsequent fungal delignification.

### 5.6 Discussion

The combination of the subcritical hydrothermal process with fungal treatment has been explored for delignification of wheat straw. The lignin and cellulose degradation in wheat straw due to the hydrothermal, biological and combined processes were monitored by total phenolic and glucose content released in the liquid hydrolysate, respectively, and residual lignin and cellulose contents in wheat straw solid fraction. According to liquid hydrolysates analysis, hydrothermal treatment provided significantly higher delignification rate than fungi treatment. The post-hydrothermal application of fungi facilitated degradation of residual lignin by-products. Efficiency of both hydrothermal and combined delignification increased with the increase in the process temperature. These observations are in consensus with other publications. Lignin dissolution was previously reported to be higher after hydrothermal treatment than fungal treatment owing to the treatment severity of the former [21]. The low phenolic content from the fungi treatment is related to the low rate of biological degradation [22]. Combined treatment was effective in terms of lignin dissolution only when the hydrothermal treatment was carried out at or above 210°C. Due to the availability of recondensed lignin on the surface of the hydrothermally treated wheat straw [5, 23], the lignolytic activity of *D. squalens* was high and hence dissolved the recondensed lignin components during the subsequent downstream process.
In addition, it has been shown that *D. squalens* preferentially degrades lignin and hence the effective removal of lignin from hydrothermally treated wheat straw [24]. However, the trend in lignin dissolution represented by phenolic content in liquid hydrolyzate contradicts with lignin content in residual solid fraction. With the increase in process temperature, total phenolic content gradually increased in liquid hydrolysates obtained from hydrothermal and combined processes. Whereas, residual lignin in wheat straw decreased, when the temperature increased from 150°C to 210°C, and then increased again. Moreover, lignin content in wheat straw treated with fungi was lower than that in hydrothermally treated substrate. According to NMR analysis, the number of methoxyl groups of lignin in hydrothermally treated wheat straw increased with increasing temperature of the process and were higher than in fungi treated substrate and raw wheat straw. Similar trends were observed for cellulose hydrolysis. While release of glucose gradually enhanced with increasing temperature of the treatment, residual cellulose in wheat straw increased when the temperature increased from 150°C to 190°C and then decreased. NMR analysis revealed that amount of crystalline cellulose increased, but amount of amorphous cellulose reduced when process temperature increased. Under most conditions, residual cellulose content was higher than in raw substrate, but lower than in wheat straw treated with fungi.

The lignin and polysaccharides hydrolysis in the present research was achieved by chemical (hydrothermal) and enzymatic (fungi) catalysis. In the hydrothermal process, wheat straw was exposed to hot water under high pressure (20 MPa) which allows maintaining water in the liquid state at the temperature above 150°C. Under these conditions, water becomes a highly effective catalyst for lignin and cellulose degradation [5]. However, the repolymerization of the
lignin fragments on the surface of the wheat straw lead to the increase in the lignin content of wheat straw treated at high temperatures [5, 23]. However, repolymerization and relocation of lignin by-products on substrate cannot explain the corresponding high release of phenolics in liquid hydrolysates. The possible explanation for high residual lignin could be a relative increase in the amount of lignin due to the removal of hemicelluloses [5, 25, 26]. Another possible mechanism is repolymerization of polysaccharides degradation products (such as furfural) and/or polymerization with lignin to form lignin-like material termed pseudo-lignin (which, however, is determined as lignin with lignin monitoring assays) [27, 28].

The effect of the hydrothermal process on cellulose is a function of temperature. Hydrothermal process conducted at low temperatures, (150 – 190°C) increased the cellulose content of wheat straw above that of raw substrate. At these temperature conditions, hemicelluloses and lignin are removed partially and expose the encrusted cellulose, hence increasing the cellulose content of substrate [29, 30]. The exposed cellulose has not been hydrolyzed effectively and hence the low glucose yield. At higher temperatures (210 – 250°C), cellulose content decreased and was lower than that of raw wheat straw. This is due to the increase in cellulose decomposition at higher temperatures [31]. Under these conditions, reduction of cellulose well correlated with increase in glucose release into liquid hydrolyzate.

Fungi treatment led to low release of phenolics at high delignification of substrate with very low rate of cellulose hydrolysis. This phenomenon could be attributed to the mechanisms of enzymes actions. The activity of fungi is more specific and is due to the lignin degrading enzymes such as laccase and peroxidase [11, 12]. These enzymes catalyze oxidation of lignin leading to cleavage of the phenolic rings [32]. As a result, products of the fungi-induced lignin
degradation are non-phenolic, and cannot be determined by the used assay. Low cellulose
degradation is a function of *D. squalens* which is selective lignin degrader [33]. Thus, fungal
treatment improved delignification while the cellulose was mostly unaffected, which is the
requirement for downstream processes.

Morphological changes caused by the various pretreatment strategies were revealed by
SEM analysis complemented to the findings obtained from chemical and NMR analysis. The
deterioration of wheat straw by the fungi-only pretreatment was equivalent to that of 190°C
hydrothermally treatment. Increasing the process severity of hydrothermal pretreatment resulted
in increasing the defibration of wheat straw. At temperatures higher than 170°C, biomass
swelling occurred. Swelling results in increase in the internal surface area and decrease in the
crystallinity of biomass [34]. The formation of lignin droplets on the surface of wheat straw
hydrothermally treated at 230°C and 250°C was due to the repolymerisation of lignin fragments
at high temperature conditions [5, 23]. In general, the fungal delignification increased the
defibration of the biomass. The effect of fungal delignification was evident from the
disappearance of lignin droplets on the surface of 230°C and 250°C hydrothermally treated
samples. Lignin re-condensation obstructed the food supply necessary for fungal growth. This
carbon limitation resulted in increasing the lignolytic activity and hence, removed lignin from the
surface of wheat straw.

Thus, the combination of hydrothermal pretreatment with fungal delignification had
improved the pretreatment efficiency. Lignin dissolution and cellulose preservation in the
biomass has been enhanced by this co-pretreatment strategy. Repolymerization of lignin
components occur during hydrothermal pretreatment at temperatures above 230°C. The problem
caused by lignin re-condensation in hydrothermal pretreatment has been effectively overcome by incorporating a downstream fungal delignification process.

5.7 Conclusions

Two different pretreatment strategies for wheat straw have been compared separately and in combination. Biological pretreatment using white rot fungi is effective in selective lignin degradation, but is very slow. Hydrothermal pretreatment is rapid, but results in the formation of re-condensed by-products on the surface of the treated substrate, which inhibits the downstream processes. Therefore, a new strategy in which hydrothermal pretreatment combined with fungal delignification was explored. Fungal delignification of hydrothermally treated substrate aided in removal of re-condensed by-products and hence improved the pretreatment efficiency. The hydrothermal temperature was found to be the critical factor which determines the extent of degradation in hydrothermal and combined treatments. Both the hydrothermal and fungal delignification complemented one another and hence the combined strategy assisted in overcoming the disadvantages of the individual pretreatments.
5.8 References


[34] H.H. Brownell, J.N. Saddler, Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis, Biotechnology and Bioengineering, 29 (1987) 228-235.
Chapter Six: Synthesis

6.1 Overview

In Chapters 2-5, four papers each focusing on specific objective have been presented. The purpose of this chapter is to summarize and explain the conceptual connection between the four papers. The important findings will be highlighted and the contributions of this thesis will be brought together in the context of the motivation for this research.

6.2 Synthesis of the thesis

The primary objective of this research is to increase the rate and efficacy of delignification by white rot fungi. In this regard, enzymatic hydrolysis of lignin using white rot fungi as a baseline treatment was chosen based on the advantages of the method: low energy requirement; negligible waste generation; low carbon footprint; no chemical and associated recycling cost. However, the main limitation of the microbial process is the low rate of degradation. Hence, the main focus of this research is to devise strategies to increase the rate of biodegradation by white rot fungi. White rot fungi degrades lignin using the action of extracellular ligninolytic enzymes. These enzymes are secreted naturally at low concentrations and hence it is necessary to induce the enzyme production using an appropriate inducer compound. The first hypothesis is that manipulating the nutrients in the growth medium of white rot fungi could facilitate enzyme induction and hence increase the enzyme productivity. To this end, a range of carbon and nitrogen sources were tested at various proportions along with a range of copper sulfate and manganese sulfate concentrations for the enhancement of laccase (Chapter 2) and peroxidase
activities (Chapter 3), respectively. In this line, a co-cultivation strategy was also explored to determine the nutrient parameters that would enhance both laccase and peroxidase activities simultaneously (Chapter 4). As the fungal pretreatment on its own lacks the characteristics for large scale applications, it can be combined with a physical pretreatment approach to increase the overall efficiency of delignification. Hence, the second hypothesis was devised as: combining the fungal hydrolysis with a subcritical hydrothermal process would increase the extent of delignification and thus lead to enhanced glucose recovery during the subsequent enzymatic hydrolysis (Chapter 5).

6.2.1 *Enhancement of lignin degrading enzymes by nutrient manipulation*

The first goal of this thesis relates to the first hypothesis that nutrient manipulation can enhance the activity of lignin degrading enzyme activities. Although there have been several studies on influence of nutrients on laccase activity, there is significant discrepancy in the results pertaining to the chosen carbon and nitrogen sources, carbon to nitrogen ratio, mode of cultivation and fungi [1-3]. Moreover, copper being the active center of laccase, induces the enzymes’ activity, but at certain critical concentration, it becomes toxic to fungal growth [4, 5]. Therefore, it is essential to understand how various nutrients influence the laccase activity of specific white rot fungi in the presence of copper. Seven different carbon sources and three different nitrogen sources were tested in different combinations and proportions along with a range of copper sulfate concentrations. The study in Chapter 2 illustrated that carbon and nitrogen sources and their ratios influence fungi tolerance to copper and, hence, facilitate copper-associated laccase activity. The main finding of this study is that there are two types of copper
toxicity – growth related and laccase activity related. The suppression of laccase activity is independent of the growth related -copper toxicity, but depends on the nature of the carbon and nitrogen sources and their corresponding ratios in the growth medium. The results from this study can be used to predict the suitable copper concentration and nutrient composition which would induce laccase production without compromising the fungi growth.

The next goal elaborates on the first hypothesis of the thesis to enhance the manganese peroxidase activity of white rot fungi. Nutrient impact on peroxidase activity has been studied in several publications [6, 7]. However, the knowledge of the underlying mechanism of nutrient regulation is inadequate and unclear. Manganese being the specific substrate of peroxidase has been used to enhance peroxidase activity [8, 9]. However, the interaction between the carbon and nitrogen sources and manganese has not been elucidated clearly. The information from literature is contradictory and makes the direct comparison and interpretation of nutrient regulation on peroxidase activity very difficult. Hence, it is necessary to investigate different carbon and nitrogen sources in the presence of manganese in order to get a deeper understanding of involvement of manganese on nutrient regulation of peroxidase activity. In this regard, a study was carried out in the presence of seven different carbon sources, and three different nitrogen sources at various proportions along with six different manganese concentrations to observe the peroxidase activity in two white rot fungi, *D. squalens* and *C. subvermispora* (Chapter 3). The influence of various nutrients on manganese regulation of peroxidase activity was assessed by the fungal tolerance to manganese. The main finding of this study is that manganese not only induces the peroxidase activity, but also suppresses the enzyme activity at critical concentrations. Manganese concentration responsible for induction or suppression can be shifted by modifying
the carbon and nitrogen supplementation, so as to obtain maximum possible peroxidase activity. The enzyme activity suppression caused by manganese was independent of fungal growth. On comparison, *C. subvermispora* was found to be highly tolerant to manganese than *D. squalens*. Knowledge of manganese concentrations responsible for maximum peroxidase activity and suppression of the same helps in the formulation of optimal nutrient medium for enhanced peroxidase activity.

From the first study (Chapter 2), maximum laccase activity was observed in *D. squalens* monoculture, in the presence of glucose, casein and copper sulfate. Similarly, from the second study (Chapter 3), monoculture of *C. subvermispora* resulted in maximum peroxidase activity in the presence of arabinose, sodium nitrate and manganese sulfate. *D. squalens* and *C. subvermispora* resulted in maximum laccase activity and peroxidase activity respectively. Co-cultivation of the above two species could result in enhanced production of both laccase and peroxidase. Though, previous studies on co-cultivation of white rot fungi have reported that co-cultivation aids in increasing the enzyme activities, there have been no defined media prescribed for optimum production of both laccase and peroxidase [10]. Therefore, these two species were co-cultivated in the presence of glucose, arabinose, sodium nitrate, casein, copper sulfate and manganese sulfate in order to obtain a defined nutrient media for optimum production of both laccase and peroxidase (Chapter 4). A statistical approach, response surface methodology was applied to identify the significant factors and interactions between factors. The main findings of this study are 1) identification and understanding of synergistic and antagonistic effects between significant factors, and 2) nutrient composition for optimum simultaneous production of laccase and peroxidase. The optimized crude culture extract was used for the delignification of wheat
straw and it resulted in 16.9% lignin degradation in 6 h. The extent of lignin degradation obtained in this study was highly significant considering the low duration of treatment.

6.2.2 Combination of fungal delignification with hydrothermal pretreatment

The second hypothesis of the thesis is to combine a physical pretreatment with white rot fungi delignification in order to increase the rate of lignin degradation and achieve effective delignification (Chapter 5). Hydrothermal pretreatment has been shown to solubilise hemicelluloses completely and cellulose and lignin partially at a temperature range, 180 – 200°C [11]. This method uses no chemicals and the process is rapid compared to fungal treatment. However, the main drawback of this method is that the products from lignin degradation re-polymerize and deposit on the substrate surface and hence lower the glucose yield during the subsequent enzymatic hydrolysis [11, 12]. With respect to the fungal treatment, even though white rot fungi efficiently delignifies, it takes long period of time to completely degrade lignin [13]. Hence, the white rot fungi can be employed to delignify the re-deposited lignin residues on the surface of hydrothermally treated biomass. Previous studies are reported on combination of fungal hydrolysis with dilute acid pretreatment [14]. There are no reports on the effect of fungal delignification on the hydrothermal lignin residuals. Therefore, in this study, hydrothermal pretreatment of wheat straw was followed by fungal delignification. The combined two-step process not only increased lignin degradation but also increased the glucose availability for the subsequent enzymatic hydrolysis.

In summary, two different strategies were explored to increase the rate of lignin degradation by white rot fungi. The first strategy focused on enhancing lignin degrading enzyme
activities by the selection of suitable nutrients and by co-cultivating two white rot fungi, so as to aid in increasing the lignin degradation rate. The second strategy combined the hydrothermal process with the delignification capability of white rot fungi and hence increased the rate and extent of lignin degradation.

6.3 References


Chapter Seven: Conclusions and Recommendations for Future Studies

7.1 Conclusions

The objective of this research was to increase the rate of fungal aided lignin degradation for the purpose of biofuel generation from lignocellulosic biomass. In order to address this objective, two different strategies were explored. The first one was to enhance the lignin degrading enzymes synthesized by white rot fungi by nutrient manipulation. This was carried out in three parts – laccase activity enhancement, peroxidase activity enhancement, and simultaneous enhancement of both laccase and peroxidase using co-culture. The second strategy included the combination of physical pretreatment with the fungal delignification in order to increase the rate and extent of delignification.

The study carried out on laccase activity enhancement of *Dichomitus squalens* and *Ceriporiopsis subvermispora* in the presence of various carbon and nitrogen sources and copper resulted in understanding that: 1) copper related toxicity was of two types, growth related and laccase activity related; 2) the appropriate choice of carbon and nitrogen sources and their ratio helps to shift the level of copper tolerance and induction and hence result in increased laccase activity. This aspect of the copper effect on laccase activity had not been explored in the literature [1, 2]. The findings from this study add to the existing literature by considering the prediction of effective copper concentrations that increase the laccase activity without adverse effect on fungal growth.
The study of peroxidase activity enhancement in *D. squalens* and *C. subvermispora* in the presence of carbon and nitrogen sources and manganese led to the understanding that: 1) manganese suppression of peroxidase activity was independent of fungal growth; 2) manganese suppression depends on carbon and nitrogen sources and their ratios and the specific white rot fungi. Though the inducing effect of manganese on peroxidase activity has been reported, the suppression effect of manganese on peroxidase activity has not been described in the literature [3, 4]. The findings from this study provide a deeper insight on the manganese effect on peroxidase and helps in acquiring the critical manganese concentrations which enhance the peroxidase activity.

Based on the outcomes of the above two studies, carbon and nitrogen sources were chosen appropriately and combined with copper and manganese in order to formulate and optimize a nutrient composition for simultaneous enhancement of both laccase and peroxidase activities in the co-cultivation of *D. squalens* and *C. subvermispora*. Both the enzyme activities from co-cultivation were higher than that could be obtained from monocultures. The treatment of wheat straw using the crude culture extract produced from the co-culture resulted in 16.9% lignin degradation. Previous reports on co-cultivation have been on whether this strategy improves lignin degrading enzyme production [5]. The results from this study contribute to those in literature by highlighting the optimization of nutrient composition for enhanced production of both laccase and peroxidase using coculture.

Wheat straw pretreatment by hydrothermal method followed by fungal delignification resulted in increased lignin degradation and glucose availability. The challenge that was highlighted in previous works was the presence of re-condensed lignin fragments on the surface
of hydrothermally treated substrate which decreased the glucose availability for the subsequent enzymatic hydrolysis [6]. The study undertaken in this thesis demonstrates a new approach combining the fungal delignification with the hydrothermal pretreatment for effective lignin removal and increased glucose availability. The fungal hydrolysis of hydrothermally treated wheat straw aided in the removal of re-condensed lignin from the biomass surface and hence led to the increase in the glucose availability.

The contributions from this thesis towards biofuel generation from lignocellulosic biomass primarily focus on the delignification which is the most critical stage during the conversion of lignocelluloses to biofuels. Fungal based hydrolysis of lignocelluloses depends on the lignin degrading enzymes produced by white rot fungi. As the natural production of these enzymes is very low, the rate of lignin degradation is also very low. Hence, in this thesis, efforts were made to enhance the laccase and peroxidase activity of D. squalens and C. subvermispora. The inducing and toxic effect of the metallic cofactors, copper and manganese on laccase and peroxidase, respectively depended up on the nature and ratio of carbon and nitrogen sources. This finding is very important as it helps to enhance the ligninolytic activity in addition to eliminating the metal toxicity by careful choice of carbon and nitrogen sources and inducer concentrations. In addition, nutrient media for co-cultivation of D. squalens and C. subvermispora was optimized for enhanced production of both laccase and peroxidase enzymes, which in turn could increase the fungal hydrolysis of lignin. Combining the fungal hydrolysis with hydrothermal treatment is a novel approach to increase the biofuel yield by effective lignin removal from the lignocellulosic biomass.
7.2 Recommendations for future work

Lengthy duration of degradation process is the major disadvantage of fungal delignification. In this thesis, various strategies have been explored to enhance the rate of lignin biodegradation. A new dimension was shown on the effect of copper on laccase activity, including the influence of carbon and nitrogen sources and their ratios. Though there are some presumptions on the interaction between copper and carbon/nitrogen nutrients, the actual interactions are still unidentified. Hence further investigation on molecular level research on the interactions between copper and carbon/nitrogen is essential to aid in understanding the mechanism of enzyme induction and suppression, so as to establish the critical copper concentrations for optimum production of laccase. Similarly, the suppressive effect of manganese and carbon/nitrogen sources on peroxidase activity which was independent of fungal growth was poorly understood and needs further molecular level investigation. The future research should address the why and how does manganese above a critical concentration suppress the peroxidase activity with respect to various carbon/nitrogen sources. Understanding the mechanism lying behind the enzyme induction and suppression might well help in enhancing the lignin degrading enzyme activities and hence, the rate of lignin degradation by white rots.

The findings from the co-cultivation study motivate further research on the understanding of the impact of nutrients on the synergistic effect between white rot fungi for the secretion of lignin degrading enzymes. The mode of cultivation – liquid or solid conditions is one of the critical factors that determine the ligninolytic activity in the co-cultivation of white rot fungi. Therefore, future studies are recommended for the comparison of the ligninolytic behavior of co-
culture in liquid and solid cultivation conditions so as to establish the best cultivation conditions for enhanced secretion of lignin degrading enzymes.

The outcomes from the combined delignification study extend further research on the effect of crude lignin degrading enzymes on the delignification of physically pretreated biomass. It is also recommended to study the other combination - fungal delignification followed by hydrothermal process in order to assess the quality of final product for enzymatic hydrolysis. Lignin – derived compounds are mostly value-added products such as carbon fibers, polymer fillers, and phenol. Hence, future studies could also be conducted on the evaluation of various strategies for the isolation of lignin derivatives.

Scale up studies should be conducted in future for exploring the potential of the production of large quantities of lignin degrading enzymes. Process conditions such as aeration, agitation, temperature, moisture content should all be included in the mathematical modeling to understand the mass and heat transfer phenomena and hence to design a bioreactor for large scale processing.
7.3 References


APPENDIX A: ENHANCEMENT OF CERIPORIOPSIS SUBVERMISPORA LACCASE ACTIVITY VIA SIMULTANEOUS EFFECTS OF COPPER, CARBON AND NITROGEN SOURCES

A.1 Abstract

Copper and several carbon and nitrogen sources were investigated with respect to their individual and combinatorial effects on growth, viability and laccase activity of *C. subvermispora*. Copper sulfate did not affect fungi growth up to concentrations of 0.06 – 0.5 mM. Higher concentrations of copper reduced viable fungi populations. Fungi sensitivity to copper depended on the type and concentration of carbon and nitrogen sources in the media. Carbon and nitrogen sources alone did not impact fungal growth in copper free media, but enhanced laccase production by fungi. *C. subvermispora* sensitivity to changes in media composition was low. Among the tested alternative carbon and nitrogen sources, 3-O-methylglucose, xylose, cellobiose, arabinose and casein improved laccase activity of *C. subvermispora* compared to glucose and sodium nitrate. Activity depended on ratios of carbon and nitrogen sources, and the copper concentration. Laccase activity was a function of copper sulfate in which the critical copper concentrations varied according to nature of the carbon and nitrogen sources and their ratios in the media. Copper showed the highest impact on laccase activity for both species and also affected the demands for carbon and nitrogen sources in respect to laccase activity enhancement.
A.2 Introduction

Biofuel is a quickly growing industry, which is based on conversion of biomass into alternative energy sources, e.g. bioalcohols. Due to the utilization of food-crop for biofuel production, the technology has not been developed in a large scale. This is because of interference with food supplies and biodiversity. Alternative technologies aim to substitute food crops used for biofuel generation with lignocellulosic biomass and hence there is no intervention with food supply. Lignocellulosic materials in general are obtained from the wastes produced by different industries sectors such as forestry, pulp and paper, and agriculture. Biofuel generation includes the hydrolysis of cellulose from lignocellulosic biomass to sugars and fermentation of sugars to bioalcohol. However, the challenge in the use of lignocellulosic biomass is that the availability of cellulose is restricted for hydrolysis due to the intervening lignin-hemicellulose matrix [1]. The purpose of the lignin-hemicellulose matrix includes protecting the encrusted cellulose from interacting with external agents such as water, solvents and enzymes, and, hence, obstructs cellulose hydrolysis to sugars. Therefore, it is essential to remove lignin and hemicellulose for efficient conversion of lignocellulosic biomass to biofuel.

Conventional delignification employs physical or chemical methods such as steam explosion and ammonia. However, these methods are expensive, energy consuming, and also release byproducts such as weak acids, furan and phenolic compounds which act as inhibitors and lower the extent of hydrolysis and fermentation [2]. On the other hand, lignin removal can be accomplished by the application of lignin-degrading enzymes produced by white-rot fungi [3]. This method is advantageous than the conventional methods owing to the low energy consumption, low/negligible waste generation, and being eco-friendly. *Dichomitus squalens* and
Ceriporiopsis subvermispora belong to the category of white rot fungi which selectively degrade lignin [4] from lignocellulosic biomass such as hard wood, soft wood, and grass without affecting the cellulose availability for enzymatic hydrolysis and fermentation [5, 6].

In basidiomycete fungi, laccases (EC.1.10.3.2) are constitutively produced in small amounts [7]. The nature and concentration of carbon and nitrogen sources have been reported as critical factors regulating laccase production and activity [8]. However, there is discrepancy in the reported literature as to the choice of carbon and nitrogen sources and nutrient concentration [9, 10]. One more important factor inducing laccase production and activity is copper [11, 12]. The effective copper concentration again varies according experimental conditions and species.

This study is part of a continuous effort to design an effective delignification approach for lignocellulosic wastes. The purpose of this research is to understand the effect of different carbon and nitrogen sources, and copper sulfate on laccase activity of C. subvermispora, and select the optimum combination of media components for future studies addressing the pretreatment of lignocellulosic biomass. A complex interaction between media components poses a challenge to interpreting impacts of an individual component on laccase activity and predicting optimum concentrations of individual components. In this study, the effect of media composition on laccase activity was investigated with a variety of approaches, including changing the levels of individual nutrients and nutrient ratios.
A.3 MATERIALS AND METHODS

A.3.1 Microorganisms and chemicals

*Ceriporiopsis subvermispora* ATCC 90467 were purchased from American Type Culture Collection (ATCC), USA. All chemicals were purchased from Sigma-Aldrich Canada Co. Oakville, Ontario.

A.3.2 Cultivation conditions

Fungi were incubated in basic mineral medium containing KCl 0.56 g/l, MgSO<sub>4</sub>•7H<sub>2</sub>O 0.78 g/l, FeSO<sub>4</sub>•7H<sub>2</sub>O 8 mg/l, KH<sub>2</sub>PO<sub>4</sub> 2.22 g/l and thiamine 1 mg/l (pH 5). For experimental purposes, the basic medium were supplemented with a range of carbon sources including glucose, 3-O-methylglucose, glycerol, methylcellulose, cellobiose, mannose, arabinose and xylose at high level (1%) and low level (0.1%) with sodium nitrate as nitrogen source at high level (20 mM) and low level (2 mM). Alternatively, basic media with different nitrogen sources such as sodium nitrate (20 mM and 2 mM), ammonium chloride (20 mM and 2 mM), and casein hydrolysate (0.5 % and 0.05%) were supplemented with glucose as the standard carbon source at 1 % and 0.1 %. Copper sulfate was added to the media as an inducer in the range of 0 to 1 mM. The 24-well microplates were filled with 2 ml of challenge media and inoculated with agar plug of 3 mm in diameter obtained from the edge of actively growing fungal colonies on PDA. The plates were incubated at 25°C with agitation at 150 rpm for seven days. Cell-free media with extracellular enzymes from each well was collected on the seventh day and were analyzed for laccase activity. Fungi grown on agar plugs in each well were transferred on to fresh PDA plates to measure growth capacity.
A.3.3 Individual effects of nutrients

Challenge media were designed such that copper-free media containing selected carbon and nitrogen sources at low levels were used as standard. To determine the individual nutrient effects, the changes in laccase activity were estimated in response to an increase in the concentration of the nutrient of interest (10 fold for carbon and nitrogen sources and up to 1 mM for copper sulfate) in standard medium at fixed concentration of other components.

A.3.4 Carbon-to-nitrogen ratios

To determined the impact of carbon-nitrogen (C/N) ratios, four challenge media were designed as follows: (i) Low Carbon and Low Nitrogen sources (LC/LN), (ii) Low Carbon and High Nitrogen (LC/HN), (iii) High Carbon and Low Nitrogen (HC/LN), and (iv) High Carbon and High Nitrogen (HC/HN). High levels designate concentrations 10 times higher than those for low levels (actual individual nutrient concentrations are presented in section A.2.2), relative C/N ratios were: 1:1 for LC/LN, 1:10 for LC/HN; 10:1 for HC/LN; and 10:10 for HC/HN. Additionally, challenge media with different C/N ratios were supplemented with copper sulfate at concentration ranging from 0 to 1 mM to determine copper interaction with carbon and nitrogen sources at different levels.

A.3.5 Laccase activity assay

Laccase activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) to an orange brown dimer [13]. The reaction mixture (200 µl) containing 2 mM of DMP in 0.1 M phosphate buffer (pH 6.0) and 50 µl of supernatant was incubated at 30°C for 15 min
The absorbance at 468 nm was monitored in microplates (path length 0.6 cm) with Spectramax Multimode microplate reader [15]. Laccase activity was expressed in activity units (AU), which are international units per liter (U/l) normalized by biomass units (mm/h). One international unit of laccase activity is defined as the amount of enzyme that oxidizes 1 µmol of DMP (molar extinction coefficient $4.96 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$) per min [13]. Biomass units were determined as radial (mm/h) growth of fungal biomass on PDA after exposure to challenged media (as below).

A.3.6 Viability and growth

To determine viability and growth capacity of fungal mycelium, cultures immobilized on agar plugs and incubated in challenge media (as above) for seven days were transferred on fresh PDA plates. Plates were incubated at 25°C under static conditions. Radial growth rates were quantified by the increase in the fungal colony radius (in mm) per hour. The copper sulfate minimum suppressive concentrations (MSC) were determined as the minimum level of copper sulfate, which reduced fungal growth rate compared to corresponding copper-free media. The minimum eradication concentration (MEC) was determined as the minimum level of copper sulfate, which completely kills the exposed fungi population.

A.3.7 Influence of copper on laccase activity

The laccase activity dynamics due to copper were described in terms of critical concentrations, 1) Peak Concentration (PC) – corresponds to copper concentration that is responsible for maximum laccase activity; 2) Minimum Laccase Suppressive Concentration
(MLSC) – corresponds to copper concentration that decreased laccase activity lower than the copper-free conditions.

A.3.8 Statistical analysis

All the experiments were carried out in triplicate. One way ANOVA was used to estimate statistical differences in growth capacity and enzyme activity between experimental conditions.

A.4 RESULTS AND DISCUSSION

A.4.1 Impact of media composition on fungal growth and viability

Different types and concentrations of carbon and nitrogen sources, and especially copper concentrations can impact fungal growth and activity [8, 16]. To determine the impact of different media components and their concentrations on growth and viability of *C. subvermispora*, fungal mycelium immobilized on agar plugs were incubated in challenge media for seven days, since the seven day incubation period has been reported to provide sufficient fungal growth and maximum laccase production for several other white-rot fungal species irrespective of the media/substrate composition and type of incubation [9, 16]. Viable populations for fungi incubated in challenge media were assessed by radial growth rates after reseeding the agar plugs with attached fungal biomass on fresh PDA plates (Table A-1).

Table A-2 shows the MSC and MEC of copper related to fungal growth. Substitution of either glucose or sodium nitrate in media with other carbon or nitrogen sources reduced *C. subvermispora* growth rate. The tolerance of *C. subvermispora* to copper under most tested conditions was very low. Only in the presence of casein, fungal tolerances improved. Mannose
and 3-O-methylglucose provided better tolerance for *C. subvermispora* compared to *D. squalens* [17]. Copper is one of the trace metals essential for fungal growth and activity [12]. In contrast to other essential metals, copper is toxic to most fungi at very low concentrations [18]. Copper toxicity is attributed to the interaction of copper ions with proteins, enzymes, nucleic acids, and metabolites associated with cell functions and viability, and due to oxidative stress [19]. D’Souza-Ticlo et al. [20] and Patel et al. [16] independently reported that increase in copper sulfate concentration above 0.3 mM led to a reduction in *C. unicolor* and *P. otsreatus* growth rates.

The aforementioned observations suggest that fungal tolerance to copper is governed by the type and concentration of the carbon and nitrogen sources in the species-specific manner. However, significant enhancement in the fungal tolerance to copper was achieved by supplementing casein, xylans (xylose, arabinose) and 3-O-methylglucose (listed in the descending order of tolerance threshold levels). Casein-mediated tolerance can be attributed to the potential formation of casein-copper complexes, and hence reduction of the toxic effect of copper at higher concentrations [21]. The mechanisms of tolerance induction by xylans and 3-methylgluocse are unknown.
Table A-1. Growth rates of *Ceriporiopsis subvermispora* in response to different carbon-to-nitrogen ratios for a range of carbon and nitrogen source

<table>
<thead>
<tr>
<th>Carbon /Nitrogen source</th>
<th>Carbon-to-nitrogen ratios $^d$</th>
<th>10:10</th>
<th>10:1</th>
<th>1:10</th>
<th>1:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose $^b$</td>
<td></td>
<td>0.38±0.04 $^a$</td>
<td>0.43±0.02</td>
<td>0.44±0.06</td>
<td>0.42±0.04</td>
</tr>
<tr>
<td>3-O-methylglucose</td>
<td></td>
<td>0.20±0.01</td>
<td>0.19±0.02</td>
<td>0.26±0.01</td>
<td>0.26±0.03</td>
</tr>
<tr>
<td>Mannose</td>
<td></td>
<td>0.25±0.02</td>
<td>0.24±0.00</td>
<td>0.23±0.02</td>
<td>0.25±0.01</td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td>0.27±0.01</td>
<td>0.27±0.02</td>
<td>0.27±0.03</td>
<td>0.26±0.01</td>
</tr>
<tr>
<td>Cellobiose</td>
<td></td>
<td>0.17±0.02</td>
<td>0.22±0.01</td>
<td>0.20±0.01</td>
<td>0.21±0.03</td>
</tr>
<tr>
<td>Arabinose</td>
<td></td>
<td>0.18±0.00</td>
<td>0.17±0.01</td>
<td>0.21±0.02</td>
<td>0.17±0.04</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td></td>
<td>0.34±0.01</td>
<td>0.29±0.04</td>
<td>0.31±0.03</td>
<td>0.31±0.02</td>
</tr>
<tr>
<td>Ammonium chloride $^c$</td>
<td></td>
<td>0.33±0.02</td>
<td>0.32±0.00</td>
<td>0.30±0.03</td>
<td>0.32±0.03</td>
</tr>
<tr>
<td>Casein</td>
<td></td>
<td>0.28±0.02</td>
<td>0.27±0.01</td>
<td>0.24±0.01</td>
<td>0.28±0.02</td>
</tr>
</tbody>
</table>

$^a$ Radial growth rates were quantified by the increase in fungal colony radius (in mm) per hour.  
$^b$ Different carbon sources were tested in combination with sodium nitrate as standard nitrogen source.  
$^c$ Ammonium chloride and casein were tested with glucose as standard carbon source.  
$^d$ C/N ratios: 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN.
Table A-2. Minimum suppressive (MSC) and eradication (MEC) concentrations of copper sulfate in response to different carbon-to-nitrogen ratios for a range of carbon and nitrogen source

<table>
<thead>
<tr>
<th>Carbon/Nitrogen source</th>
<th>Effective concentrations</th>
<th>Carbon-to-nitrogen ratios&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10:10</td>
</tr>
<tr>
<td>Glucose&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MSC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MEC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.25</td>
</tr>
<tr>
<td>3-O-methylglucose</td>
<td>MSC</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>1</td>
</tr>
<tr>
<td>Mannose</td>
<td>MSC</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.5</td>
</tr>
<tr>
<td>Xylose</td>
<td>MSC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.25</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>MSC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.25</td>
</tr>
<tr>
<td>Arabinose</td>
<td>MSC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.12</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>MSC</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.12</td>
</tr>
<tr>
<td>Ammonium chloride&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MSC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>0.5</td>
</tr>
<tr>
<td>Casein</td>
<td>MSC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MEC</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Different carbon sources were tested in combination with sodium nitrate as standard nitrogen source.

<sup>b</sup> Ammonium chloride and casein were tested with glucose as standard carbon source.

<sup>c</sup> MSC is the minimum level of copper sulfate, which reduced fungal growth rate compared to corresponding copper-free media.

<sup>d</sup> MEC is the minimum level of copper sulfate, which completely kills the exposed fungi population.

<sup>e</sup> C/N ratios: 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN.
A.4.2 Laccase activity in response to carbon sources

Modulation of laccase activity by carbon sources has been widely reported. Glucose, a main carbon source in the mineral medium, has been reported to effectively support fungal growth and laccase activity in several white rot fungi [8, 22]. In this study, *C. subvermispora* incubated in the basic mineral media supplemented with 0.1 % glucose and 2 mM sodium nitrate provided laccase activity equivalent to 0.1 AU (Table A-3). As an easily assimilable compound, glucose has been suggested to improve constitutive laccase production via enhanced biomass accumulation, but, at a certain critical glucose concentrations, repression of laccase transcription occurs in several fungi [23, 24]. Herein, an increase of glucose concentration from 0.1 % to 1 % did not affect the growth of either fungus (Table A-1), but resulted in doubling laccase activity (Table A-3), indicating that tested concentrations of glucose are lower than the critical concentration necessary for catabolic repression. In comparison, Patel et al. [16] and D’Souza-Ticlo et al. [20] reported 1% as a critical concentration of glucose for *P. otsreatus* and *C. unicolor*; while Mansur et al. [25] demonstrated that reduced expression of laccase genes, for example *lcc3*, occurs at glucose concentration lower than 1 % glucose. Galhaup et al. [26] observed that glucose depletion during fungal growth resulted in a slow increase of laccase production, which drastically increased when glucose was depleted below the critical concentration. Similarly, in this study, laccase production by *C. subvermispora* incubated in media supplemented with glucose slowly increased until 10 – 12 days of incubation, at which time a significant jump in laccase activity was observed.
Table A-3. Laccase activity (AU) of *Ceriporiopsis subvermispora* in response to different carbon-to-nitrogen ratios for a range of carbon and nitrogen sources under copper-free conditions

<table>
<thead>
<tr>
<th>Carbon /Nitrogen source</th>
<th>Carbon-to-nitrogen ratios $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:10</td>
</tr>
<tr>
<td>Glucose $^a$</td>
<td>0.13±0.00</td>
</tr>
<tr>
<td>3-$O$-methylglucose</td>
<td>0.97±0.13</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.28±0.02</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.24±0.08</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.24±0.06</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>0.05±0.00</td>
</tr>
<tr>
<td>Ammonium chloride $^b$</td>
<td>0.42±0.04</td>
</tr>
<tr>
<td>Casein</td>
<td>4.46±0.44</td>
</tr>
</tbody>
</table>

$^a$ Different carbon sources were tested in combination with sodium nitrate as standard nitrogen source.

$^b$ Ammonium chloride and casein were tested with glucose as standard carbon source.

$^c$ C/N ratios: 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN.

Thus, the laccase activity monitored in the present study after 7 day incubation more likely reflects partial repression. Catabolic repression of laccase transcription in fungal cultures in the presence of glucose limits its application, and requires new approaches to support fungal growth and laccase production.

A possible mechanism to avoid catabolic repression is fed-batch cultivation to maintain glucose levels below the critical repression concentration [27]. Another way to avoid catabolic
repression is to replace glucose with an alternative carbon source [25]. Substitution of glucose by starch, fructose, cellobiose or glycerol was previously reported to enhance laccase production by several fungi [24, 25]. Replacement of glucose with 3-\(O\)-methylglucose, xylose, cellobiose and arabinose increased laccase activity 2 – 5 fold. However, mannose and methylcellulose did not affect laccase activity.

Along with protection from glucose-associated catabolic repression, alternative carbon sources may directly impact laccase expression and/or performance. In particular, cellobiose, xylose and arabinose metabolisms are associated with lignin degradation. Cellobiose induces extracellular cellobiose dehydrogenase (CDH, EC 1.1.99.18) production [28]. Using cellobiose as a reducing substrate, CDH are able to produce hydroxyl radicals, and hence improve laccase performance [29]. Xylose and arabinose are main components of xylan in the lignin-carbohydrate matrix encrusting the cellulose microfibrils [1]. Xylans hydrolysis is parallel to lignin degradation, which may contribute to higher laccase production in the presence of xylose and arabinose [8]. Increased concentration of cellobiose, xylose and arabinose can also indicate high level of hydrolysis of cellulose and hemicellulose, and hence, their reduced availability. Under these conditions, laccase production may be induced to increase access to polysaccharides for hydrolysis. In the present study, increased concentrations of cellobiose, xylose and arabinose did not enhance laccase activity of *C. subvermispora* (Table A-3). This observation can be related to poor hemicellulases and cellulases production by *C. subvermispora*, and subsequently poor response to xylan and glucan compounds.

The response of the presently studied fungi to methylated compounds differed. Methylcellulose did not impact *C. subvermispora* laccase activity when compared to glucose
cultures; whereas, 3-O-methylglucose significantly enhanced it. Increased MC and MG concentrations resulted in better *C. subvermispora* laccase activity. Methylated substrates are preferential for laccase [30]. Moreover, demethylation is one of the functions of this enzyme [31]. Thus, the presence of methyl groups may enhance laccase production as it was observed for *C. subvermispora*.

In general, *C. subvermispora* demonstrated very low sensitivity to changes in media composition in this study. Different carbon sources directly or indirectly regulate laccase expression. As a result, significant improvement in laccase activity can be achieved by selecting a suitable carbon source and concentration. *C. subvermispora* laccase showed the best activity in the media supplemented with high concentrations of 3-O-methylglucose.

### A.4.3 Laccase activity in response to nitrogen sources

Nitrogen is another important element regulating laccase production by white rot fungi. In contrast to peroxidases, production of which is activated by nitrogen depletion and repressed by higher nitrogen levels [32], laccase production is facilitated under non-limiting nitrogen conditions [33]. In the present study, 10 fold increases of sodium nitrate, ammonium chloride or casein concentrations led to 2 – 4 fold enhancement of *C. subvermispora* laccase activity (Table A-3). The preference of amino acids-derived (casein, yeast extract, peptone, pure amino acids) nitrogen was previously reported to enhance laccase production by *P. sajor-caju*, *P. ostreatus*, and *C. subvermispora* [22, 34, 35]. Patel et al. [16] reported high level of laccase production by *P. ostreatus* in the presence of either L-asparagine or ammonium nitrate, which, however, was maximum when a combination of organic and inorganic nitrogen was used. Stajic et al. [8],
demonstrated that preference of nitrogen sources (peptone vs. ammonium salts) for better laccase production was species-dependent.

In general, the impact of nitrogen sources on laccase production may be attributed to the fact that nitrogen is an intrinsic part of lignocellulose materials, and absorption of nitrogen from lignocellulose by white-rot fungi is associated with lignin-carbohydrate complex degradation [36]. Moreover, nitrogen is necessary for build-up of cells and enzymes. In this sense, assimilability of nitrogen sources plays a critical role in fungal growth and enzyme production. Among the tested nitrogen sources, nitrate was identified as less assimilable by fungi [37]. However, no differences in growth rates of the tested fungi incubated with either sodium nitrate, ammonium chloride or casein were observed (Table A-1) indicating that enhancement of *C. subvermispora* laccase production by ammonium chloride and/or casein can be attributed to the direct impact of these nitrogen sources on laccase production, rather than increased biomass build-up.

Thus, the replacement of the conventional nitrogen source nitrate with ammonium chloride and amino acids (casein), which are inherent part of white-rot fungi substrate (wood and grass), allows significant improvement in laccase production by switching metabolic pathways. Application of this approach along or in combination with a variety of carbon sources provides good perspectives for improving delignification processes and subsequent biofuel production.
A.4.4 Laccase activity in response to copper sulfate

Another way to improve delignification is to supplement media with copper. Copper was reported to be a strong inducer of laccase production. A number of white rot fungi, P. ostreatus, T. versicolor, T. hirsute increase the synthesis of laccase in the presence of copper ions [12, 38, 39]. In order to assess the effect of copper on laccase production, the basal media containing low levels of carbon and nitrogen sources with 0.06 mM copper sulfate. This concentration of copper sulfate was below toxic level (See section A.4.1). The copper supplement to media with glucose and sodium nitrate resulted in 20 fold increase in the C. subvermispora laccase activity. It has been observed for the first time that manipulation of carbon and nitrogen sources influenced the level of inducing effects of copper. Copper addition to the media supplemented with glucose and mannose had similar effects and resulted in approximately 20 fold increase in laccase activity compared to copper-free media. Addition of copper to xylose-, cellobiose- and methylcellulose-containing media enhanced C. subvermispora laccase activity 4 – 8 fold, but had no impact on enzyme performance in the presence of 3-O-methylglucose, and even reduced it in the presence of arabinose and casein. The most significant effect was achieved when copper sulfate was supplied into media containing ammonium chloride (65 fold increase). Table A-4 shows the PC and MLSC of copper related to laccase activity. PC and MLSC are functions of carbon to nitrogen ratios and nature of carbon and nitrogen sources.

The mechanism underlying these differences is not elucidated. Nevertheless, the general impact of copper on laccase activity can be attributed to its function as an active center of the enzyme [18], and its participation in the regulation of laccase genes transcription and post-transcription modifications of enzyme [12].
Table A-4. Peak (PC) and Minimum laccase suppressive (MLSC) concentrations of copper sulfate in response to different C/N carbon-to-nitrogen ratios for a range of carbon and nitrogen source

<table>
<thead>
<tr>
<th>Carbon /Nitrogen source</th>
<th>Effective concentrations</th>
<th>Carbon-to-nitrogen ratios $^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10:10</td>
</tr>
<tr>
<td>Glucose $^a$</td>
<td>PC $^c$</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>MLSC $^d$</td>
<td>0.25</td>
</tr>
<tr>
<td>3-O-methylglucose</td>
<td>PC</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.06</td>
</tr>
<tr>
<td>Mannose</td>
<td>PC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.38</td>
</tr>
<tr>
<td>Xylose</td>
<td>PC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.12</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>PC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.18</td>
</tr>
<tr>
<td>Arabinose</td>
<td>PC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.09</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>PC</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.09</td>
</tr>
<tr>
<td>Ammonium chloride $^b$</td>
<td>PC</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>0.38</td>
</tr>
<tr>
<td>Casein</td>
<td>PC</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>MLSC</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ Different carbon sources were tested in combination with sodium nitrate as standard nitrogen source.

$^b$ Ammonium chloride and casein were tested with glucose as standard carbon source.

$^c$ PC (Peak Concentration), minimum level of copper sulfate, which provides maximum laccase activity.

$^d$ MLSC (Minimum laccase suppressive concentration), minimum level of copper sulfate, which suppress the laccase activity compared to copper-free conditions.

$^e$ C/N ratios: 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN.
To summarize, although copper induced laccase activity, the effect of copper on laccase activity is highly dependent on the particular carbon and nitrogen sources in a species-specific manner. Only ammonium chloride reinforced copper impact on *C. subvermispora* laccase activity compared to nitrate; whereas, copper-mediated laccase activity in response to alternative carbon sources was equal or lower than in the presence of glucose. These observations raise a necessity of detail investigation of copper response to nitrogen and carbon sources and their proportion in respect to laccase activity enhancement.

**A.4.5 Laccase activity in response to carbon-to-nitrogen ratios**

Laccase activity in response to changes in C/N ratios is presented in Table A-5. Maximum laccase activity was achieved for *C. subvermispora* incubated in media containing casein and glucose, associated with a C/N ratio of 1:10. In the media supplemented with increased 3-0-methylglucose, arabinose, or methylcellulose levels simultaneously with reduced sodium nitrate concentrations (C/N ratio – 10:1). These carbon and nitrogen sources provided better laccase performance when their concentrations increased at low level of the second nutrient. These conditions are relevant to C/N ratios of 10:1 for carbon source and 1:10 for nitrogen sources. Thus, optimum conditions predicted by changing the individual component levels were consistent with ratio assay for abovementioned nutrients. However, for other carbon-nitrogen combinations, maximum levels of laccase activity were achieved when concentrations of the second component increased. In particular, the media supplemented with mannose and sodium nitrate or glucose and ammonium chloride, maximum *C. subvermispora* laccase activity was observed at C/N of 10:10. Moreover, the highest laccase activity in media with high level of
sodium nitrate required low concentrations of glucose and cellobiose for *C. subvermispora*. Laccase activity was induced in the media supplemented with xylose and sodium nitrate regardless C/N ratio.

Table A-5. Maximum laccase activity obtained at various carbon-to-nitrogen ratios for a range of carbon and nitrogen sources.

<table>
<thead>
<tr>
<th>Carbon /nitrogen source</th>
<th>Carbon-to-nitrogen ratio (^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:10</td>
</tr>
<tr>
<td>Glucose (^a)</td>
<td>0.57±0.03</td>
</tr>
<tr>
<td>3-O-methylglucose</td>
<td>0.97±0.13</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.2±0.08</td>
</tr>
<tr>
<td>Xylose</td>
<td>2.28±1.7</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>2.37±0.14</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.28±0.2</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>0.38±0.01</td>
</tr>
<tr>
<td>Ammonium chloride (^b)</td>
<td>2.8±0.01</td>
</tr>
<tr>
<td>Casein</td>
<td>10.66±1</td>
</tr>
</tbody>
</table>

\(^a\) Different carbon sources were tested in combination with sodium nitrate as standard nitrogen source.  
\(^b\) Ammonium chloride and casein were tested with glucose as standard carbon source.  
\(^c\) C/N ratios: 10:10 for HC/HN; 10:1 for HC/LN; 1:10 for LC/HN; and 1:1 for LC/LN.
Thus, the impact of carbon and nitrogen sources strongly depends on their proportion in the media. Although experiments based on changing the concentration of one component while fixing the level of another component allowed identifying the possible impact of a single nutrient factor on laccase activity, these results did not allow predicting optimum media composition, which is determined by nutrients’ interactions and their ratios.

A.4.6 Copper sulfate mediation of laccase activity

The challenge media with different C/N ratios were supplemented with copper sulfate at concentration ranging from 0 to 1 mM to determine the copper interaction with carbon and nitrogen sources when present at different levels. Copper addition significantly enhanced fungi activity under most experimental conditions. In general, laccase activity increased up to a critical peak concentration (PC) of copper sulfate. Further increase in copper concentration resulted in reduction of laccase activity. The copper concentration which suppresses the laccase activity below that of copper-free conditions is referred to as minimum laccase suppressive concentration (MLSC). These data are consistent to observations of other researches. D’Souza-Ticlo et al. [20] and Patel et al. [16] reported 0.3 mM as peak or optimum copper concentration for *C. unicolor* and *P. otsreatus*, respectively. In the present study, the peak copper sulfate concentrations and relevant laccase activity values varied according to the nutrient and their ratios in the media. Typically, peak laccase activity occurred at copper concentrations equal or slightly below MSCs, the minimum levels of copper sulfate, which reduced fungal growth rate compared to corresponding copper-free media. However, in the presence of glucose with sodium nitrate, all at low levels (1:1), as well as glucose with ammonium chloride regardless of C/N ratios, peak
values for *C. subvermispora* laccase activity were observed at copper sulfate concentrations exceeding MSC values. In the presence of mannose with sodium nitrate at 1:1 ratio, laccase activity reached a peak below MSC value, but did not change until the eradication concentration of copper sulfate.

In the media containing glucose and mannose in combination with sodium nitrate, and glucose in combination with ammonium chloride, the highest *C. subvermispora* peak laccase activity was observed at 1:1 C/N ratios. In the media supplemented with 3-O-methylglucose or arabinose with sodium nitrate, the highest laccase activity was determined at C/N of 10:1 although there was no difference in peak copper levels. Glucose and casein provided the highest *C. subvermispora* peak laccase activity at C/N of 1:10. The peak activity of *C. subvermispora* laccase in the media supplemented with sodium nitrate and xylose, cellobiose or methylcellulose did not depend on nitrogen and carbon ratios.

The mechanism for the observed laccase response to C/N ratios is unclear. A general trend in carbon and nitrogen source interactions, and their impact on copper-mediated laccase activity could not be determined. C/N ratios providing higher laccase activity in copper free media correlated poorly with C/N ratios associated with the highest peak activity in the presence of copper that indicates complex interaction between media components and their combinatorial impact on laccase activity. While the mechanism for this interaction is unclear, optimizing nutrient types and relative proportions can achieve better delignification performance. Maximum laccase activities for *C. subvermispora* observed under tested conditions are presented in Table A-5. The medium supplemented with 0.1% glucose, 0.5% casein and 0.25 mM copper sulfate was identified as optimum for laccase production by *C. subvermispora*. 

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A.5 Conclusions

Most of the alternative carbon sources tested in this study provided better laccase activity due to elimination glucose-mediated laccase gene repression. A direct impact on laccase activity was observed for xylose, cellobiose, 3-O-methylglucose and methylcellulose. Among the tested nitrogen sources, only casein had direct impact on laccase activity. Laccase activity was observed to be a function of concentration of the individual nutrients, their ratio and copper supplementation. Copper had the most pronounced impact on laccase activity. However, due to copper toxicity, laccase activity had a peak at critical copper sulfate concentration followed by suppression of activity. The fungal tolerance to copper and laccase mediation by copper were governed by the type and concentration of the carbon and nitrogen sources and their ratios. These observations motivate more detailed investigations of copper response to nitrogen and carbon sources and their proportion in respect to laccase activity enhancement. This study demonstrated a strong interaction between copper, carbon and nitrogen sources with regards to their impact on fungi laccase activity. Overlapping in nutrients impact on laccase activity poses a challenge in the interpretation of individual effects and predicting optimum concentrations of individual components. The optimization of media composition under these conditions requires the investigation of synergism and antagonism between copper, carbon and nitrogen sources.
A.6 References


Laccases are copper containing oxidoreductases secreted by white rot fungi [1]. It catalyzes oxidation of the phenolic units of lignin [2]. Due to the wide substrate specificity, laccases have found various applications such as biobleaching of dye effluents, delignification of pulp, and detoxification of industrial effluents and bioremediation of contaminated soil [3]. It is essential to understand the kinetics of an enzyme system in order to design bioreactors for industrial applications. Studies on kinetic analysis of laccase detoxification of phenolic compounds so far have been shown to follow Michaelis-Menten model [4, 5]. Michaelis-Menten model can be applied only if the following criteria are satisfied: the ratio of substrate to enzyme concentration is very high; no enzyme inactivation; no enzyme inhibition; formation of enzyme-substrate complex at steady state. Pertaining to these conditions, Michaelis-Menten kinetics can be used to predict the initial rate of the reaction. However, this model cannot be used to predict the kinetics of an in vitro reaction over an extended period of time owing to the practical situations such as interference from other compounds present in the reaction system, reaction not following the exact sequence as assumed and enzyme inactivation [6]. Hence, it is necessary to develop a kinetic model that closely follows the practical behavior of laccase-catalyzed, phenolic degradation in wheat straw.

The objective of the present study is to develop a kinetic model for laccase-catalyzed, phenolic degradation of hydrothermally pretreated wheat straw. This model involves an appropriate formulation of differential equations for the reaction system and integrating the
equations over a period of time to obtain a model equation that would closely follow the experimental behavior.

B. Materials and Methods

B.1 Materials

Laccase from *Rhus verinifera* was purchased from Sigma-Aldrich, Oakville, Ontario. All chemicals were purchased from Sigma-Aldrich, Canada Co. Oakville, Ontario.

B.2 Hydrothermal process

Hydrothermal process of wheat straw was carried at 250°C in a salt-bath equipped with a stirrer and a temperature and pressure controller. 2 g wheat straw was combined with 20 g of distilled water in a 30 ml vertical stainless reactor. Reactor was placed in the salt-bath for a reaction time of 5 min. At the end of the reaction time, the reactor was immediately cooled down in water and the contents were separated by vacuum filtration into solid and liquid fractions. The solid fraction was dried at 100°C for 12 h and subjected to laccase detoxification.

B.3 Laccase detoxification – Kinetic study

Wheat straw (solid fraction) obtained from hydrothermal process (1 g) was combined with 50 ml of sodium tartarate buffer (pH 5.0) and laccase (50 U) and placed in a rotary shaker at 150 rpm and 25°C. Liquid hydrolyzate samples were collected at 5 min interval for phenolic content analysis.
B.4 Phenolic content determination

Phenolic content in the liquid hydrolyzate was analyzed by Folin-Ciocalteau assay using gallic acid as the standard [7]. 50 µl of Folin-Denis reagent was added to 25 µl of liquid hydrolyzate followed by 200 µl of sodium carbonate. All the contents were mixed thoroughly and incubated at room temperature for 2 h and the final absorbance was measured at 765 nm. Calibration curve of absorbance versus gallic acid was used as the standard and used to determine the total phenolic content from the absorbance values.

B.5 Model development

As laccase catalyzes one electron oxidation of the substrate with concomitant reduction of oxygen to water, the following reaction was written.

$$E + O_2 + S \rightarrow E + P + H_2O \quad \text{(B-1)}$$

E represents the enzyme (laccase), S is substrate (phenolics), and P is product. The enzyme concentration and substrate concentration at time t =0 are known.

The rate of substrate degradation is given as follows:

$$- \frac{d[S]}{dt} = k \cdot [E_t] \cdot [O_2] \cdot [S] \quad \text{(B-2)}$$

where k is the kinetic constant.

Assuming that laccase inactivation follows first order kinetics [8],

$$\frac{d[E]}{dt} = -k_2 \cdot [E] \quad \text{(B-3)}$$
\[
\frac{d[E]}{[E]} = -k_2 \, dt
\]  
(B-4)

Integrating the above equation with limits \([E_0]\) to \([E_t]\) and 0 to \(t\),

\[
\ln\left(\frac{[E_t]}{[E_0]}\right) = -k_2 t
\]  
(B-5)

\[
[E_t] = [E_0] \cdot e^{-k_2 t}
\]  
(B-6)

subscript \(t\) represents enzyme concentration at time, \(t\) and subscript \(0\) indicates enzyme concentration at time, \(0\). \(k_2\) is the kinetic parameter associated with enzyme inactivation.

Substituting eqn. (6-6) in eqn. (6-2),

\[
-\frac{d[S]}{dt} = k_1 [E_0] \cdot [O_2] \cdot [S] \cdot e^{-k_2 t}
\]  
(B-7)

Assuming that oxygen availability is in excess, concentration of oxygen in the reaction system can be considered to be constant,

\[
k_1 = k_1 [E_0] \cdot [O_2]
\]  
(B-8)

\(k_1\) is the kinetic parameter associated with decomposition of substrates.

Therefore,

\[
-\frac{d[S]}{dt} = k_1 [S] \cdot e^{-k_2 t}
\]  
(B-9)

Integrating the above equation with limits \([S_0]\) to \([S]\) and 0 to \(t\),

\[
\ln\left(\frac{[S]}{[S_0]}\right) = \frac{k_1}{k_2} \left( e^{-k_2 t} - 1 \right)
\]  
(B-10)

\[
[S] = [S_0] \cdot e^{\left(\frac{k_1}{k_2}\right) \left( e^{-k_2 t} - 1 \right)}
\]  
(B-11)

The kinetic parameters \(k_1\) and \(k_2\) were estimated from experimental data by non-linear regression analysis using Minitab 16 statistical software.
B.2 Results and Discussion

Hydrothermal pretreatment of lignocellulosic biomass results in formation of furan and phenolic derivatives that re-condense and deposit on the substrate surface [9]. Hence, detoxification is necessary to get rid of the phenolic derivatives from the substrate surface in order to obtain maximum yield in enzymatic hydrolysis. Laccase was used to detoxify the hydrothermally treated wheat straw. The degradation of phenolic content in wheat straw by laccase was studied to develop a kinetic model. The experimental data did not fit well with the single exponential model ($[S] = [S_0] \cdot e^{(-kt)}$), which follows the first order kinetics (Figure B-1).

Figure B-1. Single exponential model for degradation of phenolic content. Solid line represents model prediction and dotted line represent experimental data. SER represents the standard error of the regression.
The discrepancy between the model and experimental data was high (Standard Error of Regression SER = 18.78), indicating that some more effect should be taken into account. Figure 6-2 shows the exponential decomposition model of phenolic content degradation with respect to time. From the figure, it can be seen that the concentration of phenolic content becomes almost a constant after 70 min, which may be because of laccase inactivation. Therefore, laccase inactivation was taken into account in addition to phenolic degradation while the model was developed. The kinetic constants were determined by non linear regression analysis using Guass Newton method (Figure B-2).

Table B-1. Model parameters obtained from kinetic data by non-linear regression

<table>
<thead>
<tr>
<th>$k_1$ (min$^{-1}$)</th>
<th>$k_2$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0433</td>
<td>0.0312</td>
</tr>
</tbody>
</table>

The model adequacy was checked by comparing the model output with the experimental data (Table B-1). The correlation between the predicted and experimental values was 0.98 and the SER was 0.03, indicating that the developed kinetic model describes the laccase-catalyzed phenolic degradation very well. The standard error of regression which is a measure of overall model fit was very low for the proposed model compared to the single exponential model. The model developed in this study postulates that phenolic degradation and laccase inactivation follow exponential decomposition.
Though there have been studies on kinetic analysis of pure phenol [10], kinetic studies on phenolic content degradation in wheat straw is scarce. In contrast to Gianfreda et al. [4] who developed a kinetic model based on Michaelis-Menten equation which takes into account only the initial rate of degradation, the model developed in this thesis could be applied over extended period of degradation. During the degradation of wheat straw by laccase, the phenolic sub-units of lignin are oxidized. There might be interferences from other molecules present in the composite lignocellulose, which may lead to inactivation of laccase. In spite of the interferences, the model in this study closely predicts the degradation of phenolic content in wheat straw over extended period of time. Hence, it can be concluded that this exponential decomposition model
can be used to simulate laccase-catalyzed phenolic degradation in complex lignocellulosic biomass.

Hydrothermally treated wheat straw was detoxified using laccase. Laccase treatment can be incorporated as the intermediate step between pretreatment and enzymatic hydrolysis owing to the high rate of degradation. A kinetic model was developed for laccase-catalyzed phenolic degradation which followed exponential decomposition pattern. Close correlation between the experimental and predicted data shows that the developed model is of appropriate accuracy. The model can be used as a tool to simulate and design bioreactors for phenolic degradation in complex lignocellulosic biomass by laccase.

B.6 References


