Lignin Degradation by Some Wood-rot Fungi with Emphasis on *Cylindrocladium camelliae*

A thesis presented to

The Faculty of Graduate Studies

of

Lakehead University

by

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In partial fulfillment of requirements
for the degree of
Master of Science in Biology

Date
April, 2012

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Abstract

Thirty wood-rot fungi were screened for their ability to grow on media containing Kraft lignin with a limited amount of glucose. Of these fungi, twenty-three were tested for ligninolytic activity judged by decolorization of lignin. Four species: *Absidia cylindrospora, Piptoporus betulinus*, *Cylindrocladium camelliae* and *Gleoephyllum trabeum* were selected for further studies because they decolorized lignin after 3 weeks of culture by 9, 11, 26, 9%, respectively. Gel permeation chromatography (GPC) analysis showed that significant changes in lignin structure were made by the culture ultrafiltrates of these four species. Further HPLC analysis of *Cylindrocladium camelliae* culture GPC fractions detected changes in low molecular weight UV absorbing products of lignin degradation that were different from those detected in fresh lignin media. Among the four species, *Cylindrocladium camelliae* was found to produce low levels of laccase (0.02 ± 0.02 U/ml) in liquid cultures.

Acknowledgements

First and foremost, I would like to thank the almighty Allah, my Lord and God, for His spiritual strength and guidance. Then, I would like to thank my advisor, Professor L. Malek for allowing me the opportunity to work in his lab and for guiding me through this project. My sincere thanks are due to Drs. H. Schraft and R. F. H Dekker for agreeing to serve on my committee.

I very much appreciate the time taken by Dr. A. Barbosa to help me with the laccase assay. She deserves my warmest thanks. Dr. L. Hutchison is thanked for access to species his fungal collection.

I am also very grateful to Susanne Walford for her help with HPLC analysis. Thanks to all the people at Lakehead University and the Biorefining Research Institute who gave me a helping hand during my work on this project, and who have given me their friendship.

Dedication

I dedicate my thesis to:

- My mum, her support, encouragement, and constant love have sustained me throughout my life.
- My husband, Mohammed, without his care, and support I would never have been able to do this.
- My children, Ayoob, Sara and Yasmin with my love.

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List of abbreviations

AAD	aryl alcohol dehydrogenase
ABTS	2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
CA	caffeic acid
DHBA	3,4-dihydroxy-benzoic acid
DMBA	3,4-dimethoxy-benzoic acid
DMCA	3,4-dimethoxycinnamic acid
FA	ferulic acid
G	guaiacyl
GA	gallic acid
GLOX	glyoxal oxidase
GPC	gel permeation chromatography
HBT	triazole 1-hydroxybenzotrizole
4H3MBA	4-hydroxy-3-methoxy benzoic acid
HPLC	high-performance liquid chromatography
LA	laccase
LiP	lignin peroxidase
LMS	laccase-mediator system
LU	Lakehead University
MnP	manganese peroxidase
PDA	potato dextrose agar
QR	quinin reductase
S	syringyl

TEAA	triethylammonium acetate
UAMH	University of Alberta microfungus herbarium
Van	vanillin
VMS	Vogel's minimal salts
VP	versatile peroxidase
w/v	weight per volume

1.0. Introduction

My research was aimed at investigating lignin-modifying enzymes that are produced by the wood-rot fungi, and at examining their ability to demethylate lignin by removing its methoxy groups. This should result in the release of methanol and the generation of additional free phenolic hydroxyl groups in the residual lignin. My thesis was part of a larger project the objective of which is to enzymatically increase the reactivity of Kraft lignin as a phenol substitute in formaldehyde-polymer applications. Specifically, I was focusing on the cultivation and screening of fungal species from both white and brown-rot groups for potential lignin modification. I was using analytical methods that include ultrafiltration, ultra violet spectroscopy (UV spectroscopy), gel permeation chromatography (GPC), and high-performance liquid chromatography (HPLC). In particular, I intended to characterize the low molecular weight products of lignin degradation. Finally, I examined the production, isolation, and properties of one of the enzymes, laccase, that are known to be involved in lignin modification.

Lignin is one of the most abundant renewable natural products in biosphere, and is the main by-product of the pulp and paper industry. Annually, lignin is produced in tremendous quantities as a waste material and burned. The abundance of lignin has resulted in studies investigating it as a potential source of fuels, or feedstock for a variety of valuable chemicals based on phenols (Harvey *et al.*, 1985; Tien, 1987). A biological approach to woody biomass degradation led to the study of specific enzymes, which may be used to modify lignin. This is seen as an environmentally-friendly approach to modifying lignin, employing specific enzymes produced by fungi or bacteria, which are known to be natural wood decayers. Interest in using these enzymes from white and brown-rot fungi has increased and is reviewed in detail in subsequent sections. The enzymes are generally

suggested for use in a range of various unspecified biotechnological applications, and specifically to form aldehyde polymers from waste lignin.

2.0. Literature review

2.1. Lignin

Lignin, with cellulose, is a major carbon-based material on earth that is found in all vascular tissues of terrestrial plants. Lignin is an aromatic, water insoluble, heterogeneous, recalcitrant biopolymer (Fengel and Wegener, 1989), which provides plants with strength and resistance to microbial degradation (Eriksson *et al.*, 1990). In contrast to cellulose that forms a highly repeating, highly ordered linear structure, lignin forms a relatively amorphous, highly branched, three-dimensional structure (Fig. 1, Brunow, 2001). Lignin is a complex biopolymer composed of repeat phenylpropanoid units that are called monolignols (Xiao *et al.,* 2003). Monolignols are derived from phenylalanine via the shikimic acid pathway, and are characterized by side group additions to the phenolic ring. Based on monolignol units and the number of methoxy substitutions on the aromatic rings, lignin is classified into three major groups that contain three main precursor aromatic alcohols including coniferyl, sinapyl, and *p*-coumaryl alcohol (Fig. 2, Buswell and Odier, 1987). These aromatic precursors form the guaiacyl (*G*), syringyl (*S*) and *p*-hydroxyphenyl (*H*) subunits in the lignin molecule, respectively (Martinez *et al.*, 2005). Their ratios vary greatly in different groups of plants. For example, coniferyl alcohol is the predominant monolignol in gymnosperms, whereas the monomers, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, are all major precursors in grasses. In angiosperms, coniferyl alcohol and sinapyl alcohol are both synthesized in substantial proportions (Brunow, 2001; Higuchi, 2006).

Lignification is attained by the monomers' cross-linking reactions and growth of polymer through the generation of radicals by oxidase enzymes (Wong, 2009; Albersheim *et al.*, 2010).

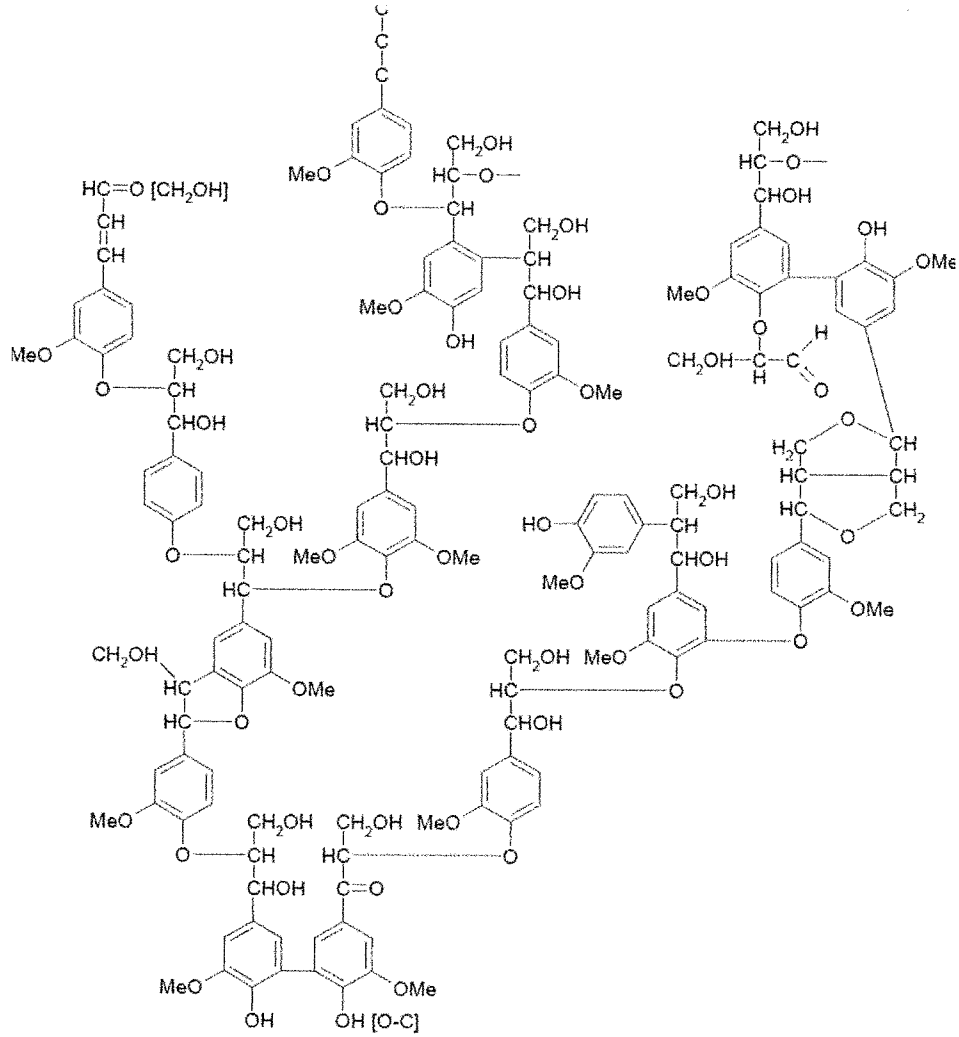


Figure.1. A structural model of softwood lignin (Alder, 1977).

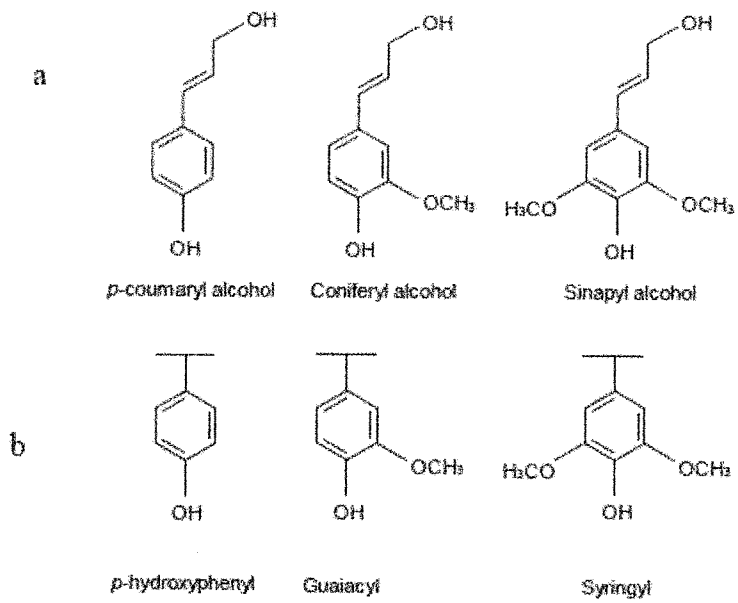


Figure. 2. a) Primary structural units of lignin monomers, b) Lignin monomers, based on Buswell and Odier (1987).

Several oxidative enzymes, including peroxidases and laccases, are known to oxidize monolignols to form free radicals (Albersheim *et al.,* 2010), which then couple oxidatively to form a complex, three-dimensional lignin structure (Wong, 2009). *G*-, *S*-, and *H*- lignin units are mainly interconnected by ether linkages and carbon-carbon linkages. These couplings include β-0-4, β-5, β-β, 5-5, 4-0-5 and α-0-4, where β-0-4 predominates. Lignin biosynthesis initiation takes place at certain sites in plant cell walls, and slowly develops through and between secondary walls (Albersheim *et al.,* 2010).

2.2. Lignin-degrading fungi

The lignin biodegradation process plays a central role in the earth carbon cycle, and is performed by fungi and bacteria. Wood-rot fungi are reported to be the most important group that makes a huge contribution to this process (Eriksson *et al.*, 1990). On the basis of

the appearance of the rotting wood, fungi are generally divided into three groups; white, brown and soft rot fungi.

2.2.1. White-rot fungi

Fungi producing white, papery-appearing rotten wood are comprised mainly of Basidiomycotina, with a few Ascomycotina also causing this type of decay (Eriksson *et al.*, 1990). They are able to degrade lignin as well as all wood polysaccharides (Buswell and Odier, 1987; Blanchette, 1995). Somewhat dogmatically, Kirk and Farrel (1987) stated that both hardwoods and softwoods are degraded by Basidiomycotina, whereas only hardwood is degraded by Ascomycotina. This is not entirely true, as many Ascomycotina are also found on softwoods. White-rot fungi are considered to be the main agents of lignin degradation in nature, and are characterized by their unique ability to degrade lignin using a set of extracellular ligninolytic enzymes (Xiao *et al.*, 2003). Hatakka (1994) reported that white-rot fungi might be divided into three main groups, based on of their typical extracellular ligninolytic enzymes. These are the lignin peroxidase-manganese peroxidase group, the manganese peroxidase-laccase group, and the lignin peroxidase-laccase group. White-rot fungi also are able to degrade lignin either selectively without any marked loss of cellulose, or simultaneously with all major cell wall components (Eriksson *et al.*, 1990). *Phanerochaete chrysosporium* and *Phlebia radiata* are the most studied white-rot fungi. They degrade lignin selectively, whereas the white-rot fungus *Trametes versicolor*, simultaneously degrades lignin and polysaccharides (Hatakka, 1994).

2.2.2. Brown-rot fungi

Brown-rot fungi preferentially degrade hemicelluloses and cellulose of wood, with a limited change in lignin (Kirk and Farrel, 1987; Eriksson *et al.*, 1990). They have the ability to remove methoxy groups from lignin and release methanol, leaving a residue of modified lignin that mainly consists of an aromatic hydroxyl-rich product (Ander *et al.*, 1988; Eriksson *et al.*, 1990). Brown-rotted wood residue is dark brown in color, shrunken, cracked, and broken into cubical fragments. Generally, brown-rot fungi are associated with decay of conifer wood, although they may also decay hardwoods (Blanchette, 1995). The most studied brown-rot fungi are *Gloeophyllum trabeum* (Haider and Trojanowski, 1980; Jensen *et al.*, 2001), *Piptoporus betulinus* (Valaskova and Baldrian, 2006) and *Poria cocos* (Sun *et al.*, 2009).

2.2.3. Soft-rot fungi

Fungi that cause soft rot decay of both hardwood and softwood trees tend to belong to Ascomycotina and Deuteromycotina (Kuhad *et al.*, 1997), which can decompose both wood components: polysaccharides and lignin (Argyropoulos and Menachem, 1997). Soft-rotted wood residue is characterized by a brown, soft appearance that is cracked when dry (Blanchette, 1995). Soft-rot fungi degrade wood under extreme environmental conditions; for example, potentially high or low water content that is too severe for other fungi (Martinez *et al.*, 2005), though they tend to work slower than white or brown-rot fungi (Eriksson *et al.*, 1990). They comprise several genera, including *Chaetomium, Cephalosporium, Allecheria, Graphium, Monodictys, Paecilomyces, Papulospora,* and *Thielavia*. All degrade lignin, except *Allecheria* and *Paecilomyces* degraded the wood polysaccharides preferentially (Eslyn *et al.*, 1975). Sanchez (2009) reported that compared to white and brown-rot fungi, little is known about the lignin degrading enzymatic system of soft-rot fungi.

2.3. Ligninolytic enzymes

Since many wood rotting fungi produce ligninolytic enzymes, they can degrade lignin relatively efficiently (Sanchez, 2009). Extracellular enzymes involved in lignin degradation include three types of peroxidases: lignin peroxidase, manganese peroxidase, and versatile peroxidase, and also phenol oxidase called laccase (Wong, 2009). Some accessory enzymes, glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO) are involved in hydrogen peroxide production for use by the peroxidases.

2.3.1. Peroxidases

Ligninolytic peroxidases are heme-containing glycoproteins, which require hydrogen peroxide (H_2O_2) as an oxidant. Peroxidases are characterized by their high redox potential (Hatakka, 1994).

2.3.1.1. Lignin peroxidases *(LiPs) (1,2-bis (3,4 dimethoxyphenyl) propane -1,3-diol: hydrogen peroxide oxidoreductases, EC 1.11.1.14)*

Lignin peroxidases (LiPs) were the first ligninolytic enzymes discovered in the early 1980's (Glenn *et al.*, 1983; Tien and Kirk, 1983). LiPs are potent oxidants and give reaction catalyzed oxidize phenolic, as well as a variety of non-phenolic lignin structures (Kersten *et al.*, 1990) (Fig. 3, Kirk and Cullen, 1998), The LiPs catalyse oxidation of nonphenolic units of lignin structure by abstraction of one electron and production of cation radicals (Kirk *et al.*, 1986; Kirk and Farrell, 1987) in the presence of H_2O_2 as an oxidant (Hatakka, 1994).

2.3.1.2. Manganese peroxidases *(MnPs) (Mn(II): hydrogen-peroxide oxidoreductases, EC 1.11.1.13)*

Manganese peroxidases, the second type of ligninolytic peroxidases that are involved in lignin degradation, were discovered in *Phanerochaetae chrysosporium* in 1985, and some MnPs have been described recently in other basidiomycetes, such as *Lenzites betulinus* (Hoshino *et al.*, 2002), *Agaricus bisporus* (Lankinen *et al.*, 2001), *Phanerochaete flavido-alba* (de la Rubia *et al.*, 2002), and *Nematoloma frowardii* b19 (Hilden *et al.*, 2008). MnPs are heme-containing glycoproteins (Glenn and Gold, 1985), and catalyze Mn dependent reactions. The main role of manganese peroxidase is to oxidize Mn(II) to Mn(III), using H_2O_2 as an oxidant (Kuwahara *et al.*, 1984), (Fig. 3), which is removed from the active site of the enzyme and forms complexes that have unusually high redox potential. Chelators such as oxalate, and malate optimize the reaction by providing ionic manganese. (Gold *et al.*, 1989; Hatakka, 1994; Wong, 2009).

2.3.1.3. Versatile peroxidases *(VPs) (EC 1.11.1.16)*

Versatile peroxidases were discovered in *Bjerkandera* and *Pleurotus* spp (Mester and Field, 1998; Camarero *et al.*, 1999) as a third type of ligninolytic peroxidase (Martinez *et al.*, 2005). VPs are bifunctional peroxidases that combine the catalytic properties of lignin and manganese peroxidases (Heinfling *et al.*, 1998). Additionally, VPs are able to oxidize Mn(II), phenolic and nonphenolic compounds that are substrates for LiP (Wong, 2009).

2.3.2. Laccases *(LA) (p-diphenol: oxygen oxidoreductases, EC1.10.3.2)*

2.3.2.1. General properties of laccases

Laccases are blue multi-copper-containing oxidases (Wong, 2009), discovered more than one century ago in the Japanese tree *Rhus vernicifera* (Yoshida, 1883). They have

been found to be widely distributed in higher plants, fungi, insects and bacteria. Laccases perform different biological functions in addition to those related to lignin synthesis and degradation, such as humus turnover, detoxification processes (Baldrian, 2006), development of spore resistance and pigmentation (Aramayo and Timberlake, 1993; Williamson *et al.*, 1998).

Laccases are extracellular enzymes, present as several isoenzymes having their own unique substrate specificity (Gavnholt *et al.,* 2002). Laccases are involved in lignin synthesis (O'Malley *et al.*, 1993) as well as in its degradation process; where laccases catalyze the oxidation of phenolic units of lignin to phenoxy radicals by one-electron transfer mechanism (Xu *et al.,* 1996; Piontek *et al.*, 2002). Laccases use molecular oxygen as an oxidant with the concomitant reduction of oxygen to water in a four-electron transfer mechanism (Thurston, 1994), (Fig. 3). Laccases are dimeric or tetrameric glycoprotein containing four copper atoms bound to three redox sites (T1, T2 and T3) (Fig. 4); blue copper center, normal copper and coupled binuclear copper centers, respectively, (Messerschmidt and Huber, 1990; Solomon *et al.,* 1992). Morozova *et al* (2007); Yaropolov *et al* (1994) reported that blue copper (T1 site) is involved in the oxidation of the reducing substrate, then internal electrons transfer from T1 to the trinuclear copper cluster (T2/T3 site), in which O_2 is reduced to water. Bourbonnais and Paice (1990) reported that laccases are only able to oxidize non-phenolic units of lignin in the presence of a redox mediators, such as 2,2'-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS). This also allows oxidation of a broad range of organic substrates, including phenols, polyphenols, anilines (Sakurai, 1992; Xu, 1996). Typically, laccases are produced by most white rot fungi (Kaarik, 1965; Call and Mücke, 1997), and some brown rot fungi (D' Souza *et al.*, 1996; Lee *et al.*, 2004). Baldrian (2006) reported that more than 60 fungal species belonging to Basidiomycetes, Ascomycetes, and Deuteromycetes demonstrated laccase activities.

2.3.2.2. Laccase-mediator system

Mediators are low redox molecules, which can act between enzyme and biopolymer as a sort of electron shuttle, and leads to depolymerization (Rochefort *et al.*, 2004). More than 100 mediator compounds have been tested for being able to oxidize lignin (Sergio, 2006), though the most commonly used ones are 2,2'-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) and the triazole 1-hydroxybenzotriazole (HBT) (Bourbonnais *et al.*, 1997; Camarero *et al.*, 2005). Different laccases oxidize ABTS by free radicals, to the cation radical $ABTS^{+}$ and the concentration of the intensely coloured, green-blue cation radical reports the enzyme activity (Fig. 5). This also leads to new oxidative reactions of laccase towards substrates, which the enzyme by itself cannot attack. However, many substrates should be tested to evaluate laccase activity, because laccases of various species differ in their substrate specificities. (Kunamneni *et al.*, 2008b).

2.3.2.3. Application of laccases

Laccases and laccase-mediator systems (LMS) have received much attention because of their ability to oxidize both phenolic and non-phenolic lignin-related compounds, as well as highly recalcitrant environmental pollutants. Laccases are finding potential applications in several biotechnological processes such as: delignification and biobleaching in the pulp and paper industry (Bourbonnais *et al.*, 1997; Smith *et al.*, 1997; Camarero *et al.*, 2004), modification of fibers and dye-bleaching in the textile and dye industy (Abadulla *et al.*, 2000; Kunamneni *et al.,* 2008c), detoxification of pollutants and bioremediation (Gianfreda and Rao, 2004; Alcalde *et al.*, 2006), treatment of wastewater from industrial plants (Berrio *et al.*, 2007), and in organic synthesis (Fritz-Langhals and Kunath, 1998; Kunamneni *et al.*, 2008a).

2.3.3. Accessory enzymes

Accessory enzymes are other fungal extracellular oxidases also involved in lignin degradation, and their role is to generate H_2O_2, which is required by the peroxidases. These include the enzymes glyoxal oxidase (GLOX) found in *Phanerochaete chrysosporium* and other white rot fungi (Kersten, 1990), aryl alcohol oxidase (AAO), which has been studied in *Pleurotus eryngii* (Guillen *et al.*, 1992), in addition to aryl alcohol dehydrogenase (AAD) and quinone reductase (QR) (Guillen *et al.*, 1997; Gutiérrez *et al.*, 1994).

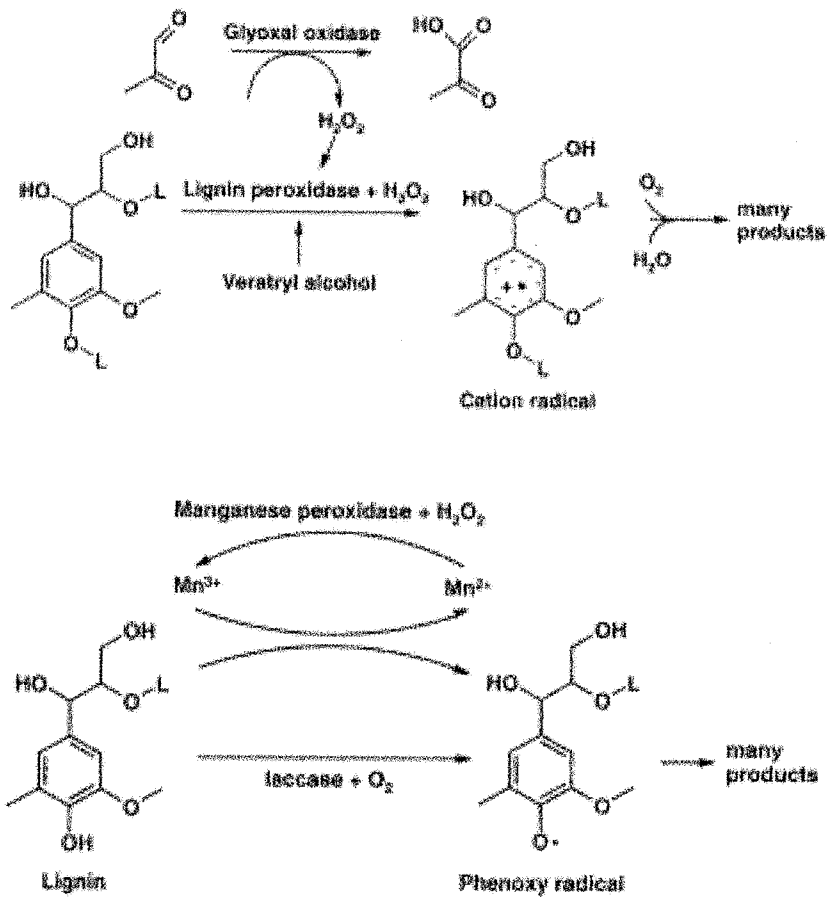


Figure. 3. A scheme for lignin biodegradation via enzymatic reactions (based on Kirk and Cullen, 1998).

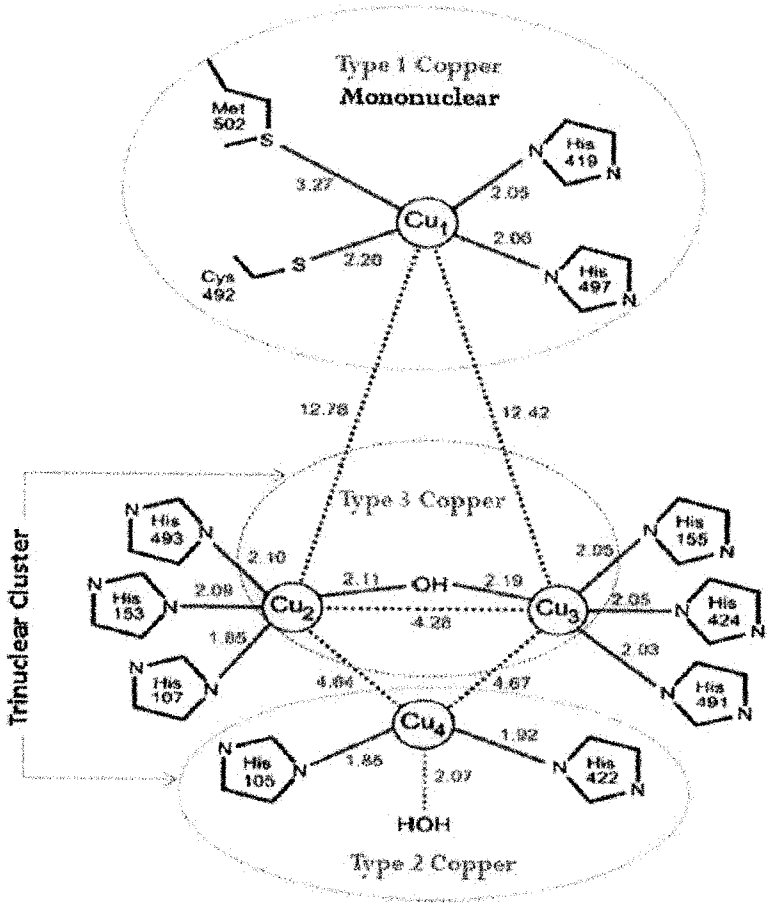


Figure. 4. Active site of laccase including the orientation of the copper atoms (Dwivedi *et al.*, 2011).

Figure. 5. Schematic of laccase enzyme catalysis in the presence of mediator (Kunamneni *et al.*, 2008b).

2.4. Lignin demethylation by brown-rot fungi

Jin *et al.* (1990) reported that brown-rot fungi cause dual lignin modification; demethylation associated with phenolic hydroxyl group formation. In regard to brown rotted lignin, earlier findings showed that the methoxy content was lower than in native lignin (Apenitis *et al.*, 1951; Brown *et al.*, 1968). Later, Kirk *et al.* (1970) stated that demethylation of methoxy groups of lignin polymer will lead to the generation of new phenolic hydroxyl groups. The limited activity of the enzymes that are produced by brown rot fungi appears to degrade lignin partially via removal of its methyl or methoxy groups, resulting in an increase of its phenolic hydroxyl content. This is the type of enzymatic activity our project aims to detect and charecterize further. Sun *et al.* (2009) have analyzed the functional groups of modified lignin from *Pinus massoniana* treated with the brown rot fungus *P. cocos* and reported that the main modifications were partial degradation, oxidation and demethylation. Among nine brown rot fungi that were studied by Haider and Trojanowski (1980), *G. trabeum* was the best in demethylation of radioactively labelled lignin.

2.5. High-performance liquid chromatography (HPLC) technique for analysis degraded lignin products.

A variety of chromatographic techniques such as paper and thin-layer chromatography (Harborne, 1973), gas chromatography (GC) (Kuwatsuka and Shindo, 1973), and high-performance liquid chromatography (HPLC) (Hartely and Buchan, 1976) have been developed for analysis the phenolic compounds in soils and plant materials. Wulf and Nagel (1976) reported that HPLC, compared to the equivalent analysis by older chromatographic techniques, enables small amount of phenolic acids and aldehydes to be estimated more rapidly and with greater accuracy. This modern technique has offered better selectivity, resolution, speed and sensitivity of analysis.

The objectives of my project are as follows:

a) Identify wood-rot fungi that specifically use only lignin as a sole carbon source.

b) Characterize the degree to which particular species modified lignin (ultrafiltration and UV_{280} absorbance).

c) Characterize lignin fragments by gel permeation chromatography (GPC), and high performance liquid chromatography (HPLC).

d) Focus on candidate species and characterize its lignin-modifying enzyme (laccase).

3.0. Materials and methods

3.1. Materials

3.1.1. Microorganisms

Cultures of wood-rot fungi used in my study are listed in Table 1. Twenty-nine wood-rot fungal species were selected from the Boreal forest fungal collection, maintained at the Mycological Herbarium of Lakehead University. Commonly used model brown-rot fungus *Gloeophyllum trabeum* (UAMH 7375) was obtained from Lynne Sigler, University of Alberta Microfungus Herbarium, Edmonton, Alberta.

3.1.2. Media

Stock cultures were grown at room temperature (22 - 25°C), and maintained on Potato Dextrose Agar (PDA, Oxoid).

Experimental cultures were grown on semi solid lignin-containing media as follows:

- 0.25% alkali lignin (Sigma-Aldrich, St. Louis USA, 471003), contains no reducing sugars, low sulfonate content, 10000 M.Wt., soluble in water)

- 2.0% Vogel's minimum salts (Vogel and Bonnerd, 1956)
- Glucose concentrations (0.0%, 0.1% and 1.0% (w/v)) are given for each experiment
- 1.5% agar

Liquid experimental nutrient media were prepared as above, but without agar, and were prepared with 0.25% lignin (w/v), or without, lignin.

Cultivation on PDA and semi solid lignin-containing media was on 20 ml media on 90 mm diameter plastic Petri plates. Forty and 25 ml of liquid media were used in 250 ml and 125 ml Erlenmeyer flasks, respectively. Cultures were maintained at room temperature, liquid cultures were shaken as indicated for each set of experiments.

3.2. Methods

3.2.1. Growth on lignin agar medium

Thirty fungal species were tested for growth on lignin-containing media. They were sub-cultured on PDA and then transferred at 3 weeks to semi-solid lignin agar media with 0.1% (w/v) glucose. Inoculum of one plug (7 mm in diameter) of mycelium attached to PDA agar was cut with a sterile cork borer from the colony's outer edge and placed into the center of the petri dish, the mycelia side of the plug down. The cultures were grown at room temperature and the experiment was performed twice in triplicate. The growth was assessed by measuring radial diameters of the colony after four days, and then every two days until the Petri dish was full, for 30 days maximum or until the culture died. The mean of mycelial growth was then calculated from the six replicates (Weitz *et al.*, 2001).

For the four species, *A. cylindrospora, P. betulinus*, *C. camelliae* and *G. trabeum,* the experiment was repeated on the same media, but with the glucose concentrations of 0.0% and 1.0% (w/v).

3.2.2. Determination of lignin decolorization and fungal biomass

Excluding seven species, which grew poorly on semi-solid media, twenty-three fungal species were tested further for their ability to grow in lignin. The experiment was performed using lignin-containing liquid medium, glucose at 0.1% (w/v). The Erlenmeyer flasks (250 ml) with 40 ml of media were inoculated with three plugs (7 mm in diameter) and shaken at 180 rpm for 21 days. Cell-free supernatants were obtained by centrifugation (average 12000.g/ 20 min) in pre-weighed 50ml tubes and then ultrafiltered on YM5 membrane (Amicon, U.S.), the volumes and the total A_{280} absorbance of supernatant and ultrafiltrate determined from diluted aliquots using a Cary 50 UV-visible spectrophotometer (Varian). To determine fungal biomass, fungal pellet from each liquid culture was dried in an oven at 90°C for 24h, and weighed, subtracting the weight of 3 uninoculated agar plugs.

3.2.3. Gel permeation chromatography (GPC) analysis

The gel filtration matrix, Bio-gel P-2 Gel, fine (BioRad, Mississuaga, Ont, Canada) with a fractionation range of 100-1800 daltons was selected to investigate the degree of lignin modification by fungal growth. Bio-gel P-2 Gel was swollen in distilled water, degassed and packed into a glass chromatography column (25 cm x 1.5 cm). Fifty mM phosphate buffer pH 6.8, 0.02% NaN_3 was pumped at 0.75 $ml.min^{-1}$ by using Econo pump at 10% max speed.

Two milliliters of fresh liquid medium ultrafiltrate and the culture ultrafiltrates of four fungal species; *A. cylindrospora, P. betulinus, C. camellia, G. trabeum* were applied on the gel, and eluted with phosphate buffer. Eluted fractions (2 ml) were collected using Biologic biofrac fraction collector. The absorbances of the eluted fractions were taken at 280 nm, using a Cary 50 UV-visible spectrophotometer (Varian).

Table 1. Fungal species used.

NO	Lakehead Uni NO	Fungal strain
1	LU07	*Absidia cylindrospora*
2	LU10	*Schizophyllum commune*
3	LU12	*Baeospora myosura*
4	LU15	*Piptoporus betulinus*
5	LU16	*Fomes fomenarius*
6	LU22	*Flammulina velutipes*
7	LU25	*Gliocladium roseum*
8	LU31	*Geomyces pannorum*
9	LU33	*Epicoccum purpurascens*
10	LU40	*Laetiporus sulphureus*
11	LU41	*Sphaerobolus stilettos*
12	LU43	*Coprinus comatus*
13	LU48	*Sphaerobolus stellatus*
14	LU50	*Urnula craterium*
15	LU51	*Hypoderma puberum*
16	LU54	*Hyphoderma typhicola*
17	LU60	*Armillaria ostoyae*
18	LU66	*Fomitopsis pinicola*
19	LU72	*Irpex lacteus*
20	LU82	*Sarcosome Mexicana*
21	LU86	*Galerina autumnalis*
22	LU92	*Pholiota populnea*
23	LU93	*Climacodon septentrionale*
24	LU94	*Hypsizygus tessulatus*
25	LU97	*Cerrena unicolor*
26	LU107	*Cytospora pinastri*
27	LU112	*Mycenaoverholtsii*
28	LU113	*Oligoporus leucospongia*
29	LU120	*Cylindrocladium camelliae*
30	UAMH 7375	*Gleoephyllum trabeum*

3.2.4. HPLC analysis

The culture ultrafiltrate of *C. camelliae* fractionated by GPC was analysed by reversed-phase high-performance liquid chromatography. "High" and "low" molecular weight

fractions resulted from the combination of eluted fractions numbers 20-49 and 50-70, respectively. A reversed phase C-18 column was used (Gemini 5 μm, 250 x 4.6 mm, OOG-4435-EO) on Varian system equipped with solvent delivery modules (ProStar 210, USA), autosampler (ProStar 410, USA), UV-Vis detector (ProStar 325, Australia). Mobile phase A was 1.0% Triethyl ammonium acetate (TEAA, pH 5, Sigma-Aldrich, acetic acid-triethylamine solution 1:1) in 99% water, mobile phase B was methanol and acetonitrile; (25:75, v/v) with flow rate of 0.3 ml. min^{-1}. The gradient established was 0% B for 2 min, 40% B for 20min, 100% B for 10 min, 100% C for 10 min, 0% B for 2 min (Meyermans *et al.*, 2000). All solvents were of HPLC grade. Absorption was detected at 280 nm. Data collection and integration were performed with Varian star chromatography software. Unknown samples and eight standards were applied, caffeic acid (CA), 3,4-dihydroxybenzoic acid (DHBA), 3,4-dimethoxybenzoic acid (DMBA), 3,4-dimethoxycinnamic acid (DMCA), 4-hydroxy-3-methoxybenzoic acid (4H3MBA), ferulic acid (FA), gallic acid (GA), vanillin (Van) (Sigma-Aldrich, St. Louis, USA). Injection volumes of samples and standards were 100 μL and 20 μL, respectively.

3.2.5. Laccase assay

Laccase activity in supernatants of fungal cultures (prepared as in section 3.2.2.) was determined spectrophotometrically as described by Barbosa *et al.*, (1996) using 50 mM ABTS, 50 mM citrate-phosphate buffer (McIlvaine's buffer) at pH 3.0, and 40°C. The laccase reaction mixture in a total volume of 1 ml contained 0.05 ml of cell free supernatant and 0.05 ml of ABTS in 0.15 ml of 50 mM citrate-phosphate buffer (pH 3.0). The reaction was monitored by measuring the change at 420 nm for 5 min using Cary 50 UV-visible spectrophotometer. The extinction coefficient of 36,000 M^{-1} cm^{-1} was used for oxidized ABTS.

3.2.5.1. Laccase activity of *C. camelliae*

This experiment was performed adding an inoculum of 4 plugs into lignin-containing liquid media (25 ml in 125 ml Erlenmeyer flasks with 0.1% and 1.0% glucose, and with 0.25% and without lignin, 2.0% VMS). The cultures were incubated at room temperature on a platform shaker (New Exella E_5, Brunswick Scientific, Edison, N.J, USA) 180rpm for 7 days. After incubation, laccase activity was determined as described above, at pH 3.0 and at 37°C. The experiment was done in triplicate.

3.2.5.2. Effect of pH on laccase activity of *C. camelliae*

Different reaction pHs 3.0 to 6.5 at temperature 37°C, 50 mM citrate- phosphate buffer for 5 min were used to determine the optimum pH for laccase activity.

3.2.5.3. Effect of temperature on laccase activity of *C. camelliae*

The assay was performed at different reaction temperatures using incubators set at 37, 40, 45, 50 and 55°C, 50 mM citrate- phosphate buffer for 5 min at pH 3.0.

3.2.6. Spectral analysis of *C. camelliae* degraded lignin

UV spectra were determined for cell filtrates of *C. camelliae* cultures that grew in two culture conditions in the presence of 0.25% lignin and with two glucose concentrations (0.1% and 1.0% (w/v) for 7 days on a shaker at room temperature. The filtrates were diluted (50X). Fresh liquid media at the same dilution were used as a reference. The scanning was carried out using a Cary 50 UV-Vis Spectrophotometer.

4.0. Results

4.1. Growth study

Nearly all of the thirty wood-rot fungal species were able to grow on lignin-containing semi-solid medium supplemented with 0.1% glucose (Fig. 6), but colony sizes of different isolates varied at a particular point in time, i.e. the growth rate varied among species. The best growth was obtained with *A. cylindrospora*, and *C. camelliae*. These reached to the edges of Petri dishes in less than two weeks (Fig. 6A and F). Other species such as *F. fomentarius*, *S. stellatus*, *I. lacteus*, and *C. unicolor* grew slower and needed at least two weeks to reach to the edges of the plates. Colonies of the even slower growing organisms such as *P. betulinus*, *G. pannorum*, *L. sulphureus*, *H. tessulatus* and *G. trabeum* have taken 30 days to reach to the edges of the plates. Some organisms, such as *C. comatus*, *A. ostoyae*, *P. populnea* and *C. septentrionale* grew very slowly and then stopped entirely prior to the termination of the experiment (Fig. 6).

The four fungal species selected for further investigation were the fast growing *A. cylindrospora*, *C. camelliae,* and the slower growing; *P. betulinus*, *and G. trabeum* (Fig. 7). The fast growing *A. cylindrospora* and *C. camelliae* needed two weeks to fill the Petri dish when not supplied with glucose (Fig. 7A), but reached the edges of the dish within 10 days in the presence of 0.1% or 1.0% glucose (Fig. 2B, C). Similarly, the slower growing *P. betulinus* and *G. trabeum* did not fill the plates during 30 days of the experiment without glucose (Fig. 7A), but completed filling the plate by about 30 days in 0.1 % glucose (Fig. 7B), and 20 days in 1.0 % glucose (Fig. 7C).

I observed significant differences in the hyphal density of four selected species in the cultures without glucose and with 1.0% glucose of each species after one week of

growth (Figs. 8 and 9). For *A. cylindrospora* and *C. camelliae,* hyphal density increased and dense hyphae accumulated in the cultures with 1.0 % glucose (Fig. 9), while within the medium not supplied with glucose the hyphae were less branched and more sparse (Fig. 8). Colonies of *P. betulinus* and *G. trabeum* grew as a minor ring of mycelia in the region immediately around the inoculum plug in the culture without glucose (Fig. 8) and also with 1.0% glucose (Fig. 9). The ability of these four species to degrade lignin was investigated in more detail by GPC (Section 4.3).

4.2. Lignin degradation

Among the twenty-three fungal species investigated in liquid cultures, I found that seven fungi *A. cylindrospora*, *S. commune, P. betulinus*, *L. sulphureus, I. lacteus, C. camelliae and G. trabeum* showed noticeable lignin-decolorizing ability which exceeded 7% decrease at A_{280} in the cell free supernatants (Table 2). Five other fungi; *F. fomentarius, F. velutipes, S. stellatus, I. resinosum, P. arcularius* had very low ability to decolorize lignin (Table 2). On the other hand, I observed that growth of some species resulted in increased absorbance of their cell free filtrates compared to the absorbance of fresh medium. These were *U. craterium, H. puberum, S. mexicana, G. autumnalis, H. tessulatus, C. unicolor, C. pinastri, M. overholtsii,* and *O. leucospongia* (Table 2). A_{280} of the less than 5000 M.Wt. ultrafiltrate, representing lignin fragments (Table 2) also declined in the presence of a majority of the fungi, except for *G. autumnalis, C. pinastri,* and *M. overholtsii* where intensity of A_{280} also increased following culture (Table 2).

Growth in liquid culture (Fig. 10) reflected that seen on Petri dishes (Fig. 5). Some species grew well such as *A. cylindrospora, S. stellatus* and *C. camelliae.* Other species such as *S. commune, F. velutipes, S. mexicana, M. overholtsii and O. leucospongia* grew moderately well (Fig. 10), and no growth was observed in *U. craterium, G. autumnalis* liquid

cultures (Fig. 10).

4.3. GPC analysis

The elution of culture 5 kDa ultrafiltrate from the P-2 GPC (gel permeation chromatography, separation range 100 to 1800 Daltons) column was based on the molecular weight, with the largest lignin fragments eluting first and the smallest later. Compared to fresh medium ultrafiltrate (solid lines in Figs 11 to 14), there was a decrease in overall absorbance (as already indicated in Table 2). The decrease was particularly evident in the small lignin fragment region (Fractions 38-70) of the ultrafiltrates that were extracted from the cultures of *A. cylindrospora*, *P. betulinus* and *C. camelliae* (Figs. 11, 12 and 13). In contrast, *G. trabeum* ultrafiltrate (Fig. 14) while showing an overall decrease in absorbance, the peak between fractions 42-45 remained nearly as high as that in the original media.

4.4. HPLC Analysis of *C. camelliae* spent media

The HPLC analysis of commercially available phenolic standards: caffeic acid (CA), 3,4-dihydroxybenzoic acid (DHBA), 3,4-dimethoxybenzoic acid (DMBA), 3,4-dimethoxycinnamic acid (DMCA), 4-hydroxy-3-methoxybezoic acid (4H3MBA), ferulic acid (FA), gallic acid (GA), vanillin (Van) was performed individually and in a mixture to determine retention times of these chemical entities that are potential products of lignin degradation. The reverse-phase chromatogram of a mixture (Fig. 15) shows reasonably good separation of these standard phenolic substances, with the exception of CA and 4H3MBA (peaks 3 and 4, Fig. 15).

Unknown UV absorbing compounds present in large and small fragments prepared by GPC of fresh liquid medium ultrafiltrate, were used as a control (Figs. 16 and 18). HPLC analysis of large fragments shows the main unknown peaks in unmodified fresh medium

and their retention times as: at L1 - 8.57, L2 - 9.09, L3 - 9.40, L4 - 11.75, L5 - 25.81, and L6 - 26.34 min (Fig. 16). The retention times of unknown peaks of small fragments of fresh medium were also determined (Fig. 18), where the first peak was detected at 14 min. These were numbered so as to remain in the same elution order as peaks in fungus modified medium (Fig. 19) as S4 - 14.17, S5 - 25.42, S6 – 28.00, S7 - 29.25, S8 - 33.45 min (Fig. 18). Additional early eluting peaks were present in the fungus modified medium S1 - 8.63, S2 – 9.13, S3 – 11.74 (Fig. 19). Peak S5 (possibly caffeic acid) decreased, while peak S7 (possibly dimethylcinnamic acid) increased following *C. camelliae* culture (Fig. 19).

Compared to the control (Fig. 16), unknown peaks produced from loading large fragments of *C. camelliae's* culture ultrafiltrate (Fig. 17), showed some changes in the lignin products such as reduction in UV absorbance (disappearance) of peaks L3 and L5.

4.5. Laccase activity

4.5.1 Laccase activity of LU07, LU15, LU120 and UAMH 7375

A. cylindrospora and *C. camelliae* incubated on a shaker at 28°C showed good growth under these conditions, while slow growth was obtained with *P. betulinus* and *G. trabeum* (Table 3). An increase from initial pH 5.92 for *A. cylindrospora* and *C. camelliae* to 6.77 and 7.00, respectively, and a slight decrease of initial pH to 5.00 was observed with *P. betulinus* (Table 3). Laccase was detectable only in the culture of *C. camelliae,* which also produced the highest biomass (Table 3). I attempted to characterize this activity further.

4.5.2. Laccase activity of *C.camelliae*

4.5.2.1. Effect of pH and temperature on Laccase activity of *C.camelliae*

Optimum laccase activity of *C. camelliae* was at pH 3.0 (Fig. 20). There was a decrease in activity with an increase in pH and complete inactivation above pH 6.0. Laccase

had highest activity at the assay temperature of 37°C. Incubators at lower temperature were not available. The increase in temperature resulted in decrease in enzyme activity (Fig. 21).

4.5.2.2. Laccase activity of *C.camelliae* in two culture conditions

In this experiment, I examined *C. camelliae* for its ability to grow and produce laccase in potentially improved cultural conditions, increased glucose, and with lignin removed. I observed very good growth in the media with 1.0% glucose, and also some growth in the media with 0.1% of glucose, with 0.25% lignin (Table 4) on a shaker at room temperature for seven days. However, laccase activity did not appear to be enhanced with increased glucose and removal of lignin. Some measurable laccase was detected in cultures containing lignin (Table 4).

4.6. Spectral analysis

There were no changes in the absorption spectrum of liquid media that were inoculated by *C. camelliae* under two culture conditions for one week of incubation (Fig. 22A and B). The spectra only reflect the decreased concentration of UV absorbing material after 7 days of culture. The culture with 0.25% lignin and 1.0% glucose, showed significant decrease in the absorbance, 0.6403 units compared to 1.0830, the absorption of fresh liquid medium as already demonstrated in Fig. 22B, indicating that this difference was greater than that detected in slower growing culture at 0.1% glucose (Fig. 22A)

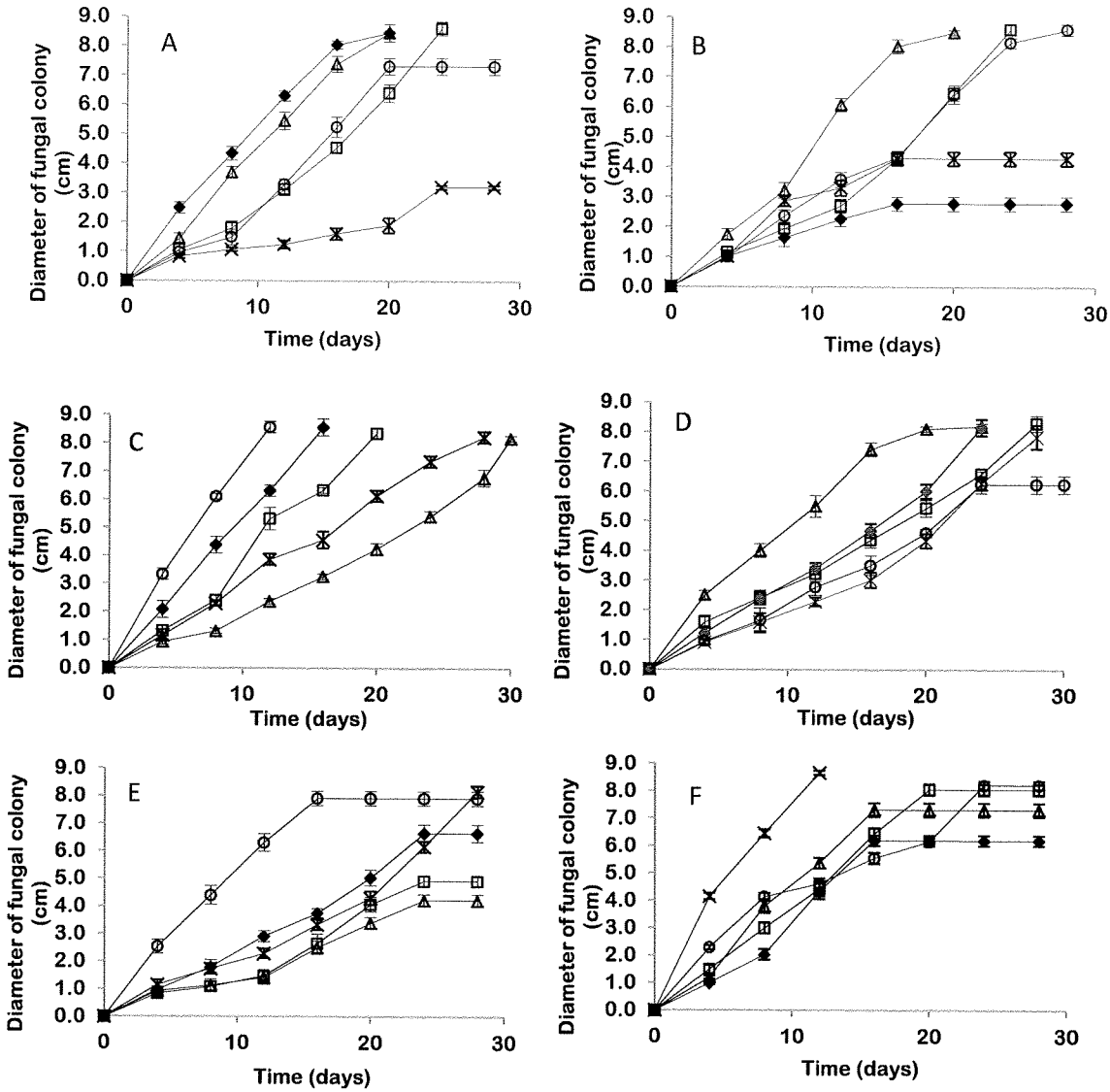


Figure 6. Growth of fungal species on semi-solid media, lignin (0.25%), glucose (0.1%), VMS (2.0%) and agar (1.5 %) for 30 days. Bars represent standard deviation of the mean of 6 cultures each. In A. LU07 *A.cylindrospora* (o), LU10 *S. commune* (□), LU12 *B. myosura* (Δ), LU15 *P. betulinus* (x), LU16 *F. fomenarius* (◆). In B. LU22 *F. velutipes* (□), LU25 *G. roseum* (Δ), LU31 *G. pannorum* (x), LU33 *E. purpurascens* (◆), LU40 *L. sulphureus* (o). In C. LU41 *S. stilettos* (Δ), LU43 *C. comatus* (x), LU48 *S. stellatus* (◆), LU50 *U.* craterium (□), LU51 *H. puberum* (o). In D. LU54 *H. typhicola* (o), LU60 *A. ostoyae* (◆), LU66 *F. pinicola* (□), LU72 *I. lacteus* (Δ), LU82 *S. mexicana* (x). In E. LU86 *G. autumnalis* (◆), LU92 *P. populnea* (□), LU93 *C. septentrionale* (Δ), LU94 *H. tessulatus* (x), LU97 *C. unicolor* (o). In F. LU107 *C. pinastri* (◆), LU112 *M. overholtsii* (□), LU113 *O. leucospongia* (Δ), LU120 *C. camelliae* (x), UAMH7375 *G. trabeum* (o)

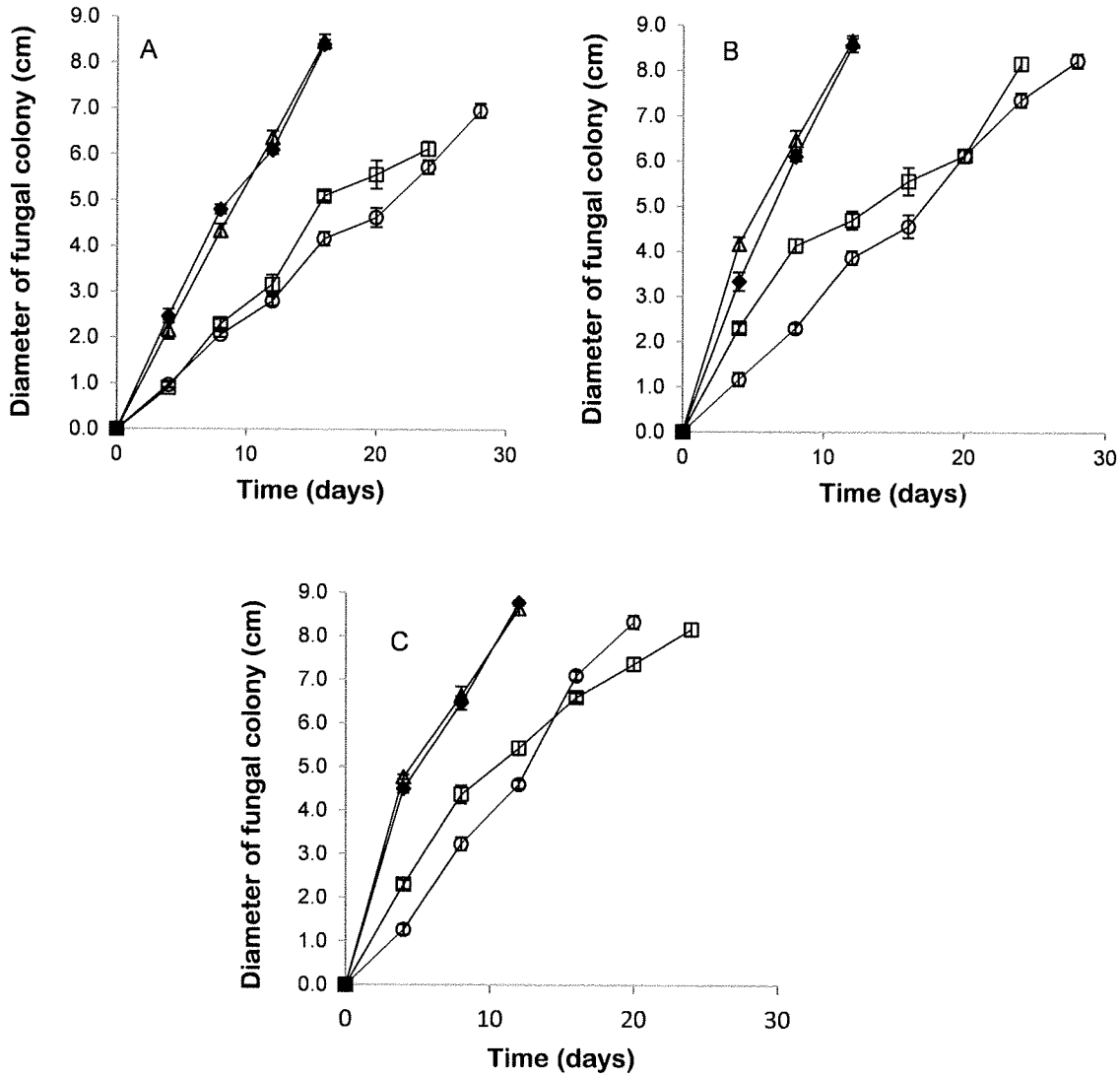


Figure 7. Growth of LU07 *A. cylindrospora* (◆), LU15 *P. betulinus* (o), LU120 *C. camelliae* (Δ), and UAMH 7375 *G. trabeum* (□) on semi-solid media, lignin (0.25%), VMS (2.0%), agar (1.5%) and various glucose concentrations for 30 days. (A) 0.0%, (B) 0.1%, and (C) 1.0% glucose. Three cultures each.

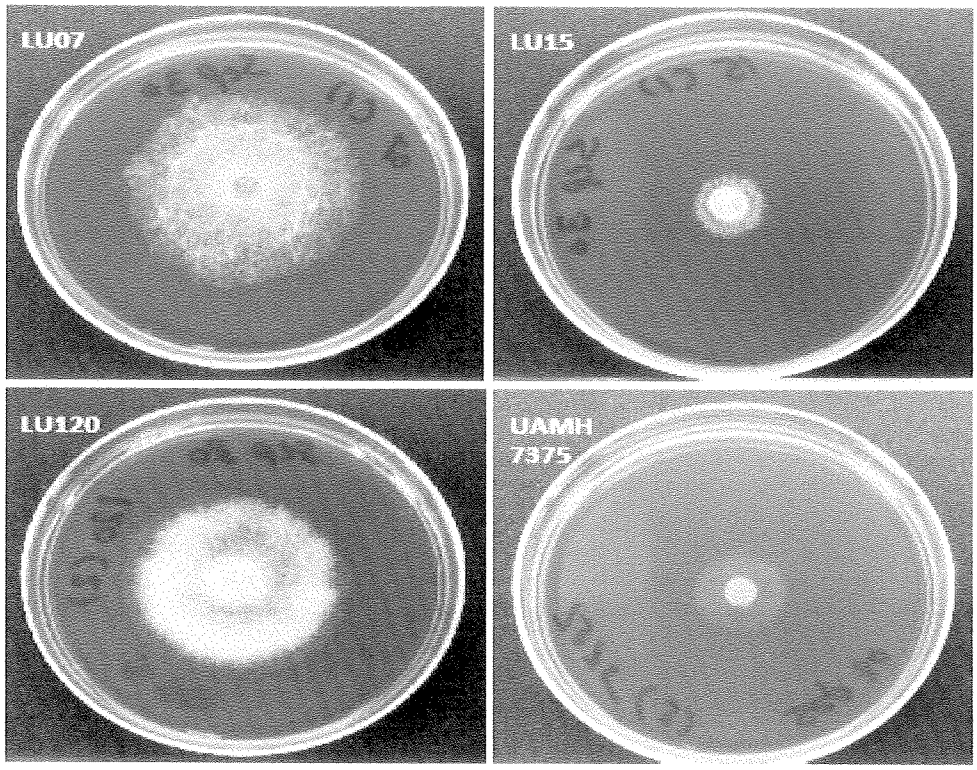


Figure 8. Growth of *A. cylindrospora* (LU07), *P. betulinus* (LU15), *C. camelliae* (LU120), and *G. trabeum* (UAMH 7375) on semi-solid media (0.25% lignin, 0.0% glucose, 2.0% VMS and 1.5% agar) at room temperature for one week.

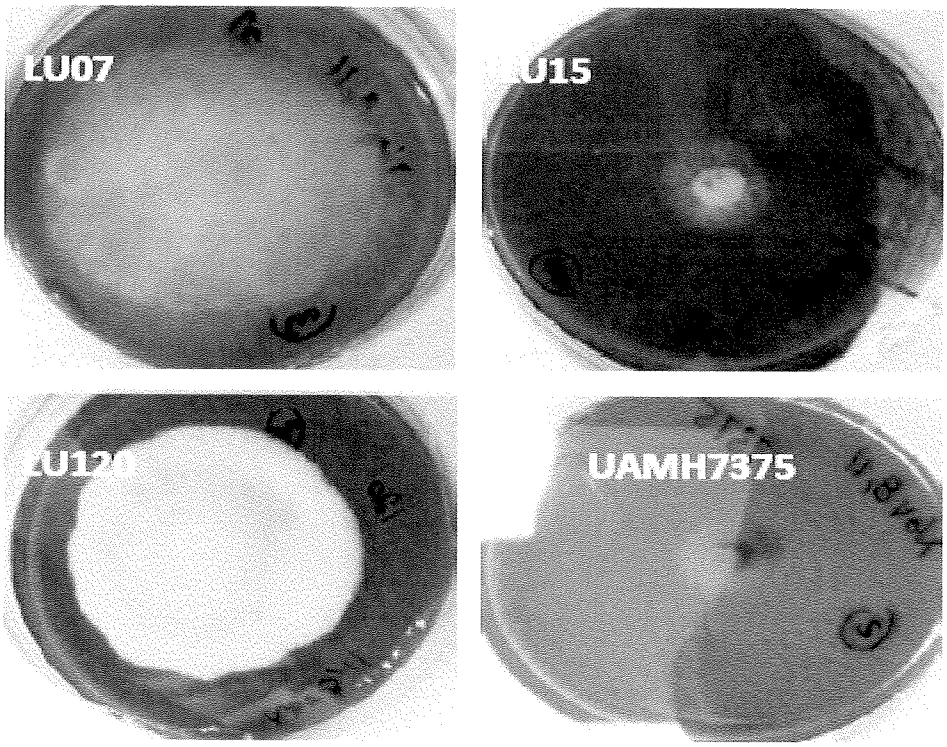


Figure 9. Growth of *A. cylindrospora* (LU07), *P. betulinus* (LU15), *C. camelliae* (LU120), and *G. trabeum* (UAMH 7375) on semi-solid media (0.25% lignin, 1.0% glucose, 2.0% VMS and 1.5% agar) at room temperature for one week.

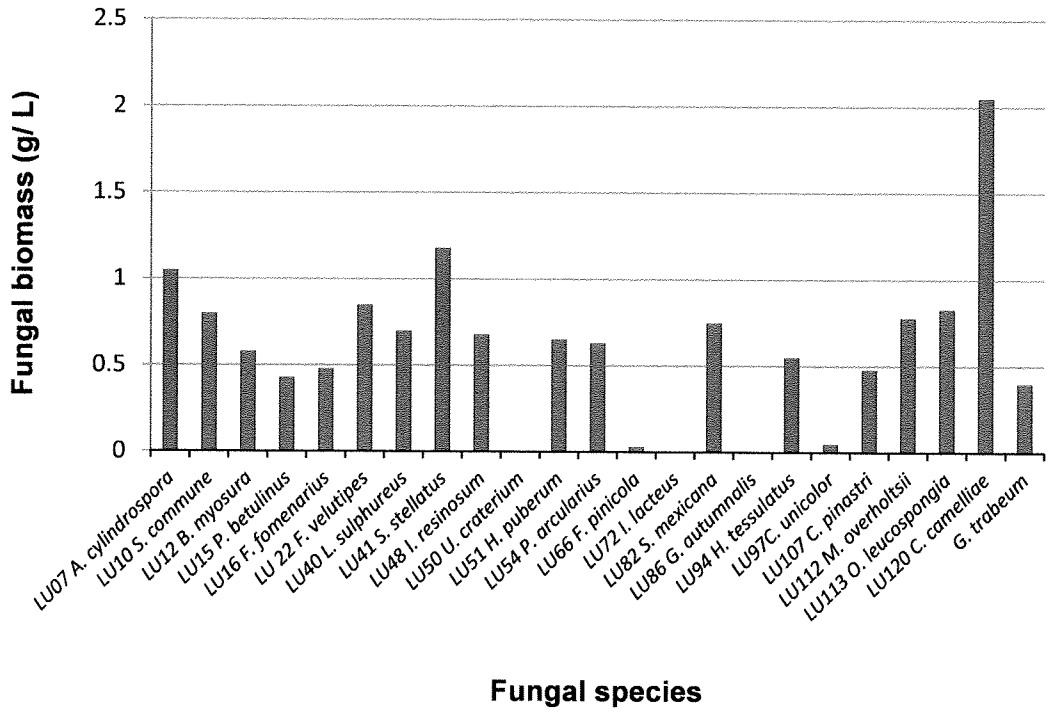


Figure 10. Fungal biomass (g/L) of various species grown in liquid media (lignin 0.25%, glucose 0.1%, 2.0% VMS) on shaker for 21 days at room temperature. Standard deviations are not shown, as they are less than 5% of each average.

Table 2. Change in UV absorbance at 280 nm following incubation on shaker for 21 days in liquid media (lignin 0.25%, glucose 0.1%, 2.0% VMS) at room temperature, based on 6 replicates. Minus signs indicate cultures, which decolorized lignin, while plus signs indicate cultures, which increased absorbance at 280 nm.

Microorganism	**SUPERNATANT**		**ULTRAFILTRATE**	
	Total A_{280}		**Total A_{280}**	
	AVE ± STDEV	**% Change**	**AVE ± STDEV**	**% change**
Lignin-containig medium	1705 ± 110		296 ± 123	
LU07*A. cylindrospora*	1555 ± 125	-9	183 ± 80	-38
LU10 *S. commune*	1577 ± 57	-8	156 ± 27	-47
LU12 *B. myosura*	1601 ± 37	-6	160 ± 44	-46
LU15 *P. betulinus*	1509 ± 56	-11	200 ± 69	-32
LU16 *F. fomenarius*	1680 ± 81	-1	136 ± 20	-54
LU22 *F. velutipes*	1659 ± 5	-3	162 ± 61	-45
LU40 *L. sulphureus*	1579 ± 144	-7	208 ± 51	-30
LU41 *S. stellatus*	1672 ± 86	-2	177 ± 13	-40
LU48 *I. resinosum*	1666 ± 52	-2	149 ± 12	-50
LU50 *U. craterium*	1925 ± 134	+13	144 ± 20	-51
LU51 *H. puberum*	1871 ± 53	+10	199 ± 18	-33
LU54 *P. arcularius*	1649 ± 41	-3	106 ± 79	-64
LU66 *F. pinicola*	1618 ± 67	-5	102 ± 41	-66
LU72 *I. lacteus*	1562 ± 33	-8	53 ± 29	-82
LU82 S. Mexicana	1861 ± 133	+9	195 ± 44	-34
LU86 *G. autumnalis*	1857 ± 176	+8	405 ± 134	+37
LU94 *H. tessulatus*	1798 ± 106	+5	104 ± 8	-65
LU97 *C. unicolor*	1850 ± 104	+9	103 ± 11	-65
LU107 *C. pinastri*	1715 ± 95	+1	369 ± 3	+25
LU112 *M. overholtsii*	1830 ± 12	+7	379 ± 8	+28
LU113 *O. leucospongia*	1771 ± 68	+4	125 ± 20	-58
LU120 *C. camelliae*	1259 ± 29	-26	167 ± 33	-44
UAMH 7375 *G. trabeum*	1560 ± 93	-9	234 ± 96	-21

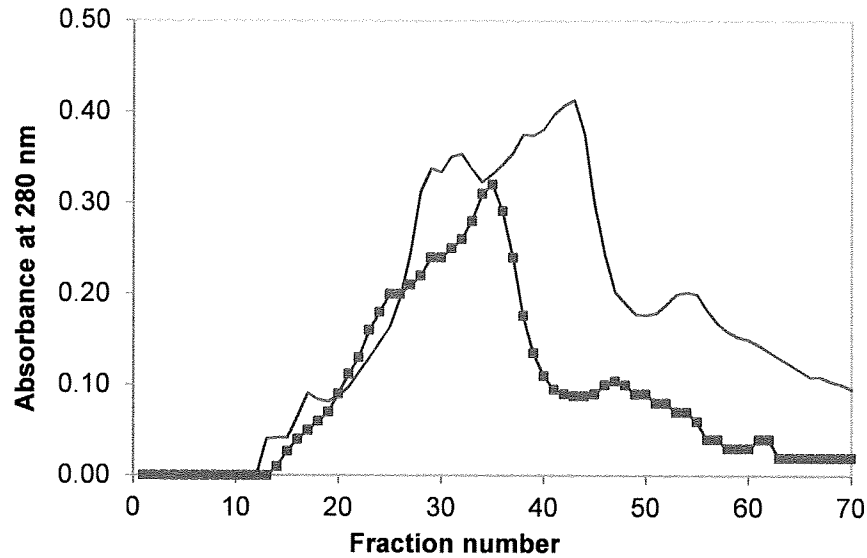


Figure 11. Representative gel filtration chromatography of lignin ultrafiltrates on bio-gel P2, using fresh medium (−) and medium modified after 3 weeks old culture of LU07 *A. cylindrospora* (■) based on three replicates.

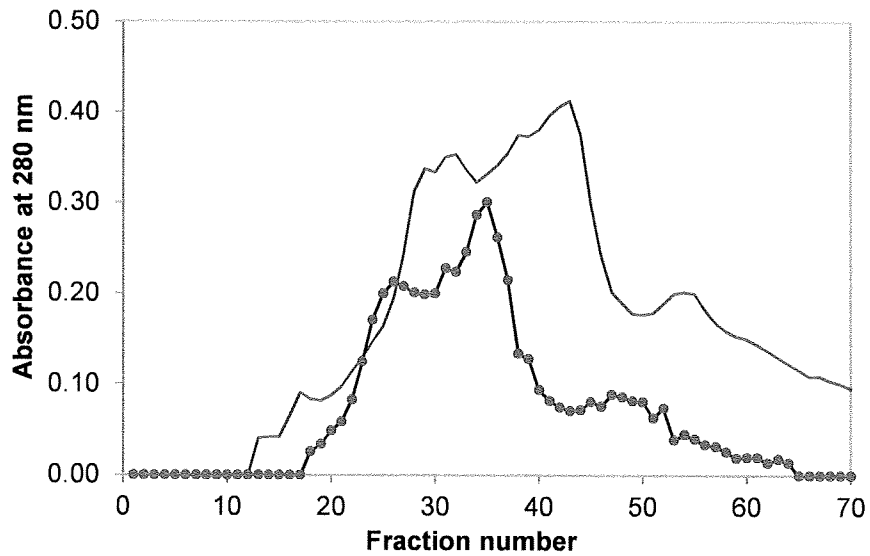


Figure 12. Representative gel filtration chromatography of lignin ultrafiltrates on bio-gel P2, using fresh unused medium (−) and medium modified after 3 weeks old culture of LU15 *P. betulinus* (•) based on three replicates.

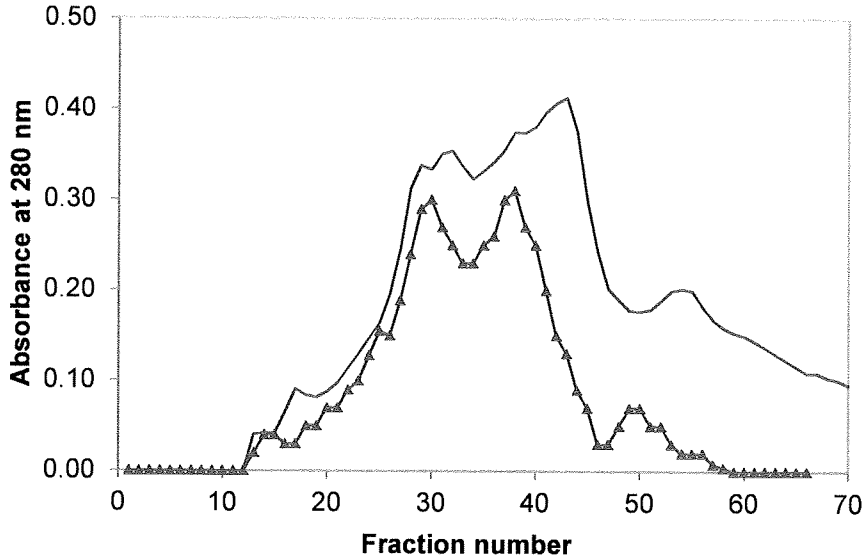


Figure 13. Representative gel filtration chromatography of lignin ultrafiltrate on bio-gel P2, using fresh unused medium (−) and medium modified after 3 weeks old culture of LU120 *C. camelliae* (▲) based on three replicates.

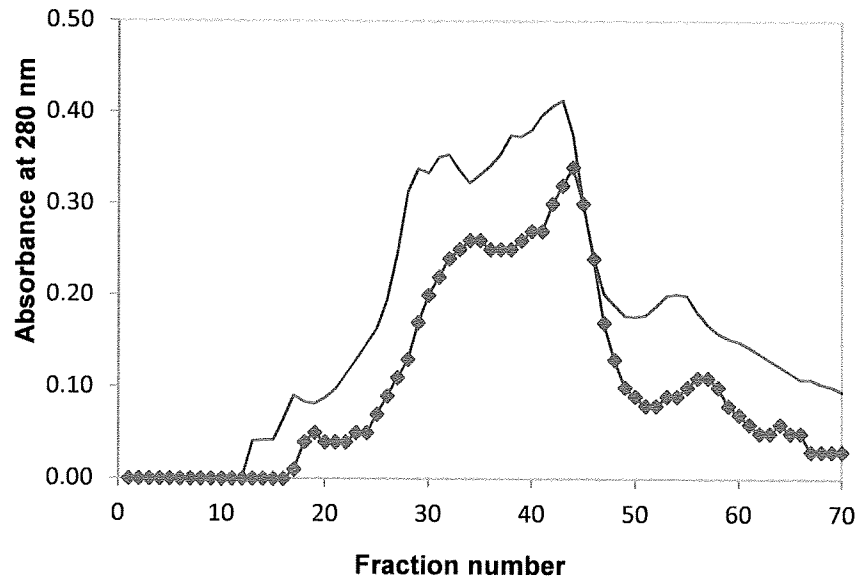


Figure 14. Representative gel filtration chromatography of lignin ultrafiltrate on bio-gel P2, using fresh unused medium (−) and medium modified after 3 weeks old culture of UAMH 7375 *G. trabeum* (◆) based on three replicates..

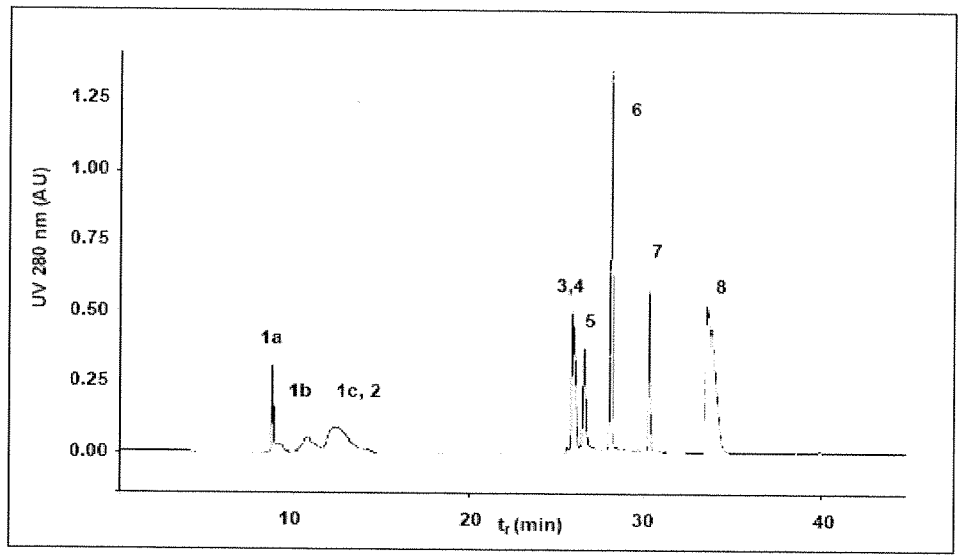
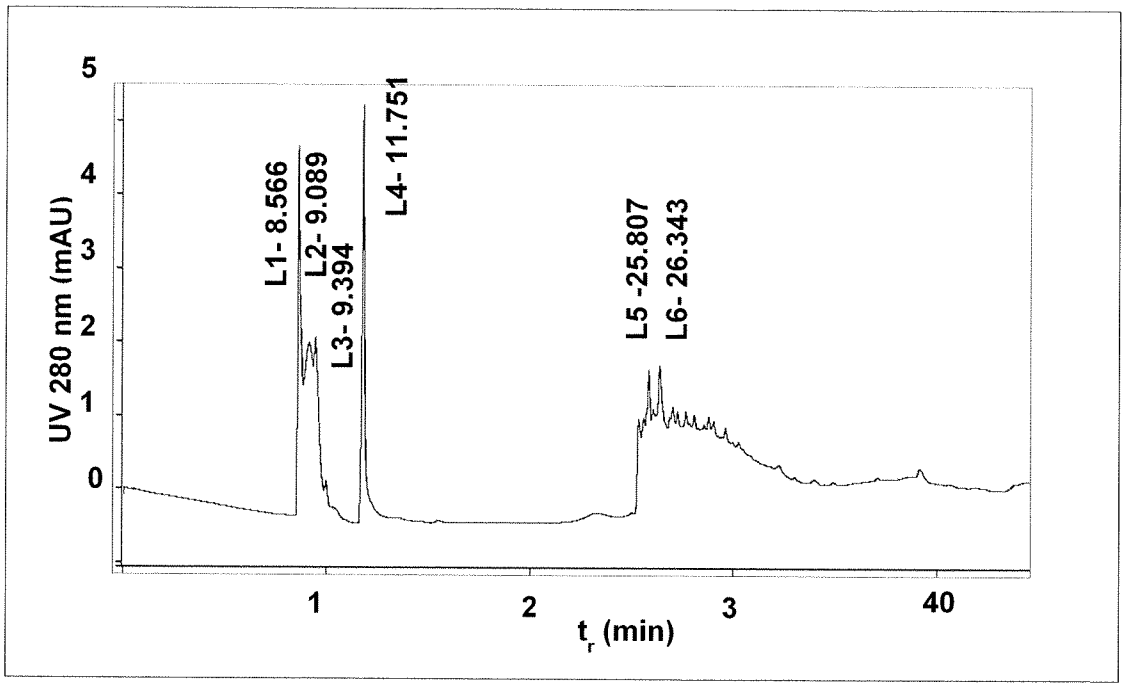
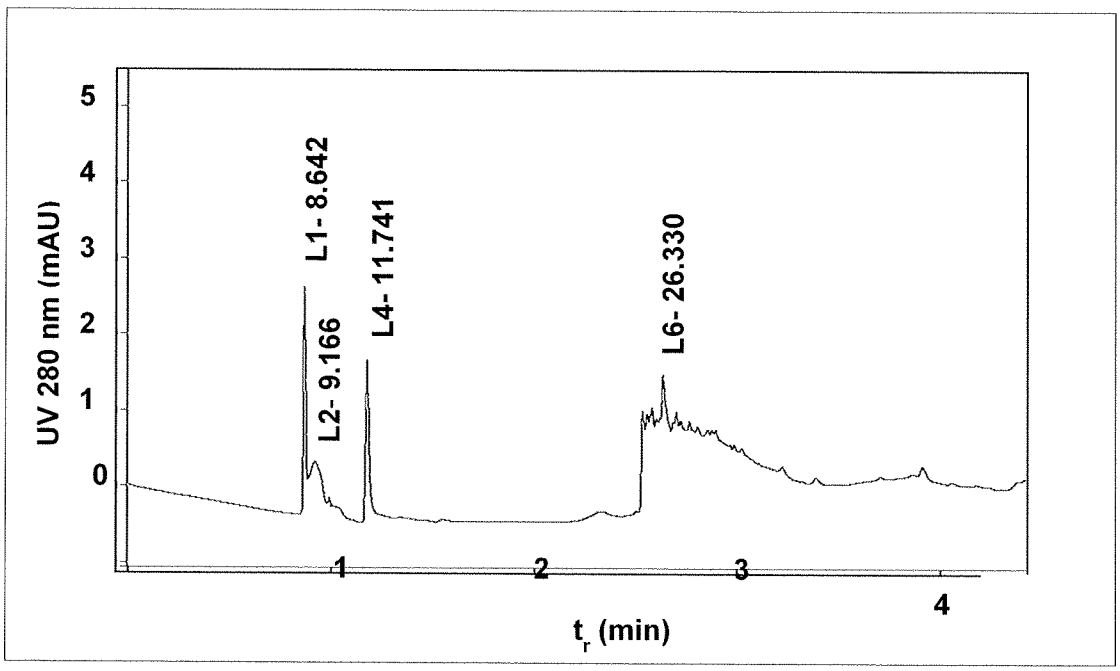
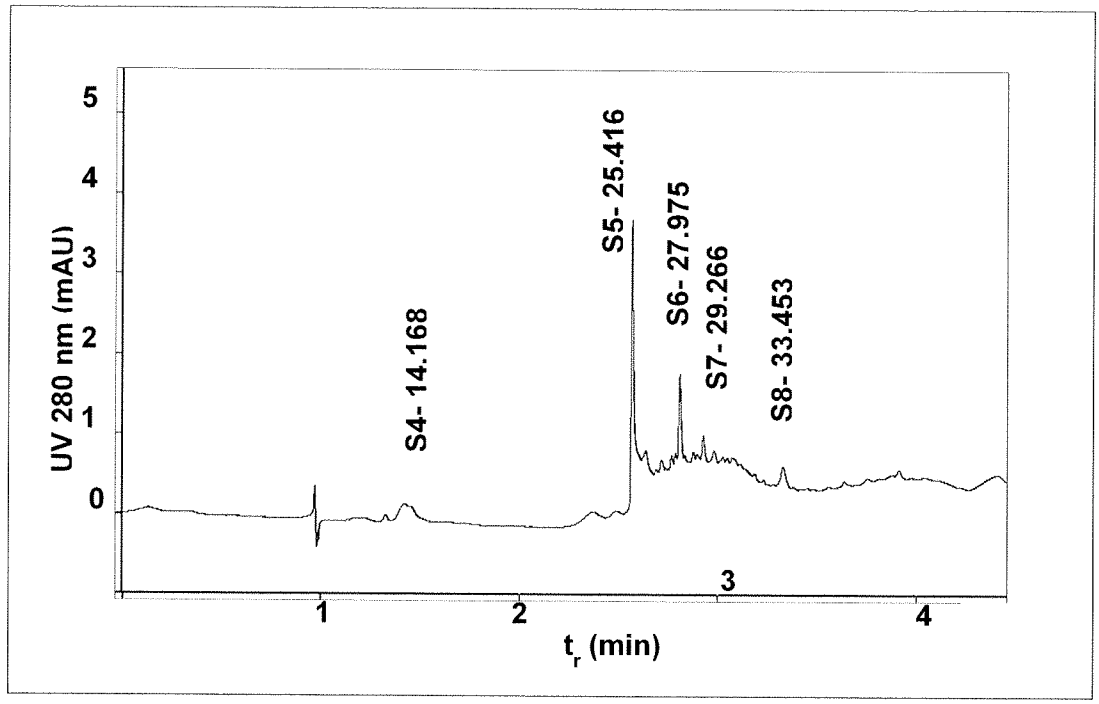


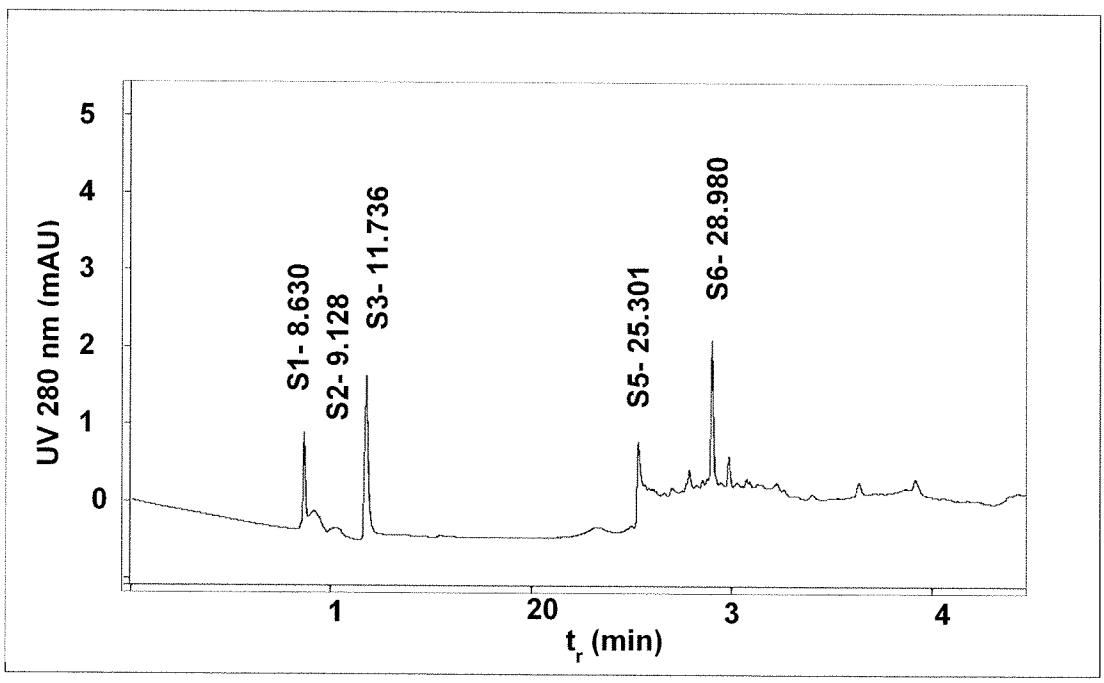
Figure15. HPLC chromatogram of a standard mixture containing 8 commercially available phenolic compounds and their retention times (min). (1) gallic acid; (1a) at 8.72min; (1b) at 10.74 min; (1c) at 12.15 min, (2) 3,4-dihydroxybenzoic acid at 12.40 min; (3) caffeic acid at 25.77 min; (4) 4-hydroxy-3-methoxybenzoic acid at 25.91 min; (5) ferulic acid at 26.46 min; (6) 3,4-dimethoxybenzoic acid at 27.98 min; (7) 3,4-dimethoxycinnamic acid at 30.22 min; (8) vanillin at 33.48 min.





Figures 16, and 17. HPLC chromatograms of high molecular weight fractions (defined in materials and methods, Page 17) (A) fresh lignin-containing medium ultrafiltrate. (B) LU120 *C. camelliae* culture ultrafiltrate. Note the thousand-fold expanded Y-axis, compared to standards.





Figures 18, and 19. HPLC chromatograms of low molecular weight fractions (defined in materials and methods, Page 18) (A) fresh lignin-containing medium ultrafiltrate. (B) LU120 *C. camelliae* culture ultrafiltrate. Note the thousand-fold expanded Y-axis, compared to standards.

Table 3. Fungal culture pH, biomass (g/L) and laccase activity (U/ml) of LU07 *A. cylindrospora,* LU15 *P. betulinus,* LU120 *C. camelliae,* and UAMH 7375 *G. trabeum.* Those were grown in liquid media (0.25% lignin, 2% VMS, 0.1% glucose) Incubated for 21 days on shaker, 180 rpm at 28°C. The experiment was performed in triplicate.

Fungal species	**pH**	**Fungal biomass (g/L)**	**Laccase activity (U/ml)**
Lignin- containing medium	5.92 ± 0.03		
LU07 *A. cylindrospora*	6.77 ± 0.04	1.01 ± 0.01	0.00 ± 0.00
LU15 *P. betulinus*	5.00 ± 0.08	0.60 ± 0.00	0.00 ± 0.00
LU120 *C. camelliae*	7.00 ± 0.02	1.76 ± 0.01	0.02 ± 0.02
UAMH 7375 *G. trabeum*	5.92 ± 0.05	0.40 ± 0.00	0.00 ± 0.00

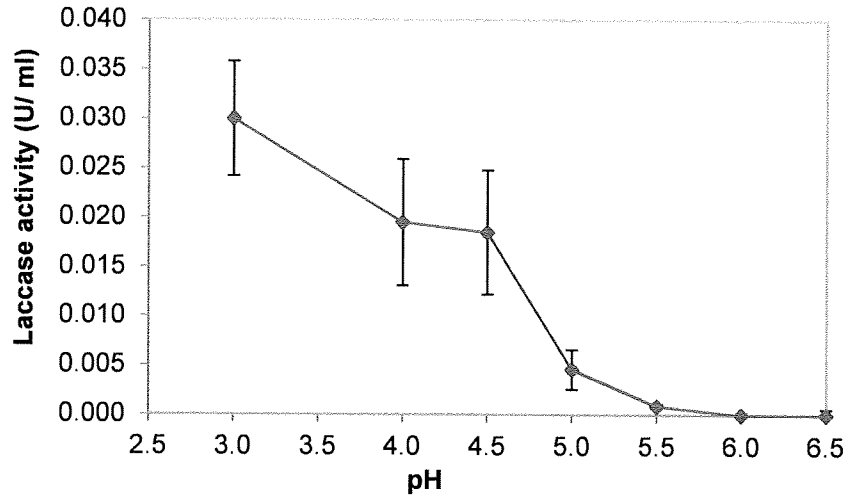


Figure 20. Effect of different pH on LU120 *C. camelliae* laccase activity.

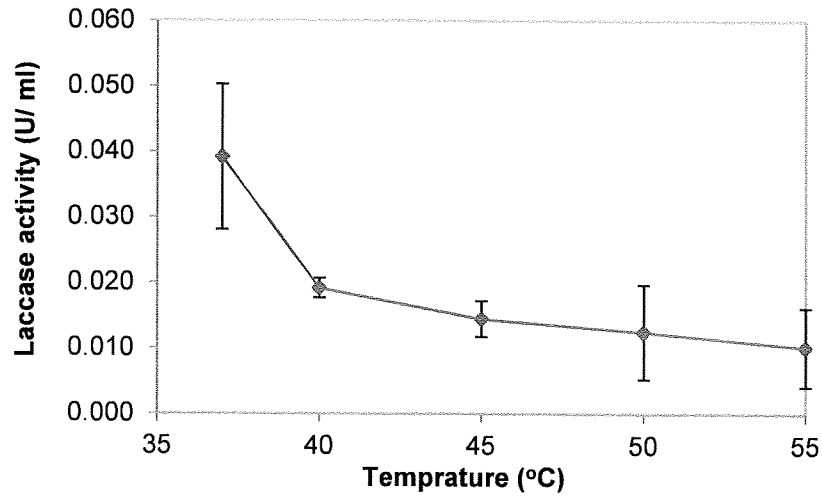
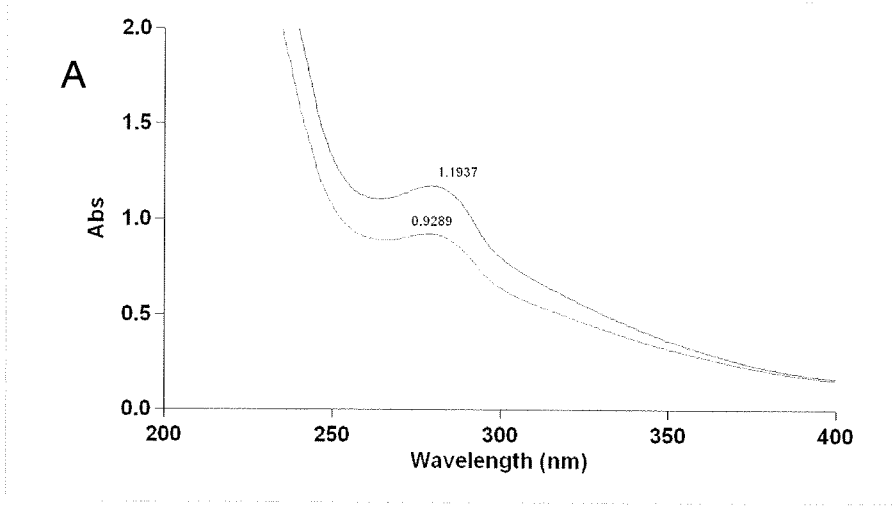


Figure 21. Effect of different temperatures on LU120 *C. camelliae* laccase activity.

Table 4. Culture pH, biomass (g/L) and laccase activity (U/ml) of LU120 *C. camelliae.* Cultures were grown in liquid medium in the absence and presence of 0.25% lignin, and with two different concentrations of glucose (0.1% and 1.0%) and incubated on shaker at 180 rpm for 7 days and performed in triplicate.

	0.1% glucose		**1.0% glucose**	
	0% lignin	**0.25% lignin**	**0% lignin**	**0.25% lignin**
Initial pH	6.67	7.14	6.00	6.12
Final pH	7.02 ± 0.05	7.16 ± 0.01	6.83 ± 0.06	6.86 ± 0.12
Biomass (g/L)	1.40 ± 0.01	2.17 ± 0.01	6.05 ± 0.01	6.48 ± 0.01
Laccase activity (U/ml)	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00



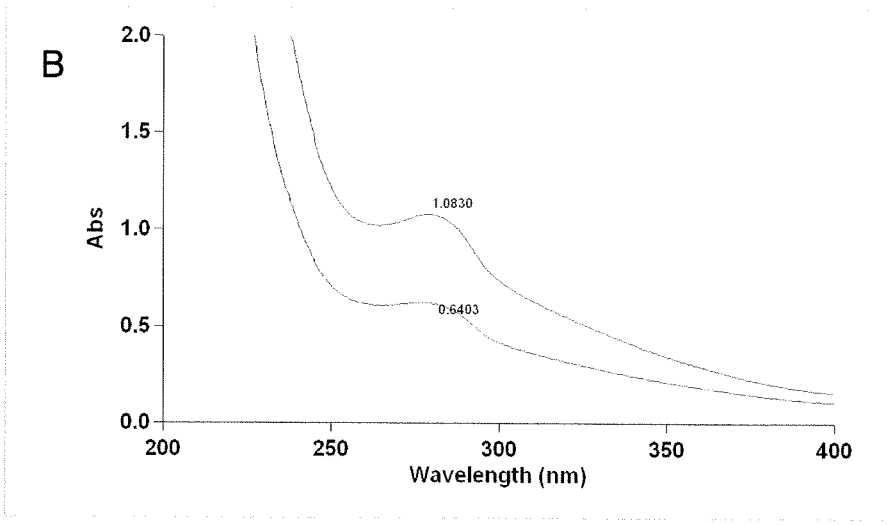


Figure 22. UV spectra of cultural media of LU120 *C. camelliae* that were incubated on a shaker 180 rpm for 7 days at room temperature. Glucose concentration in (A) was 0.1%, and in (B) was 1.0%.

5.0. Discussion

Different growth patterns were obtained by cultivating wood-rot fungi on agar medium containing lignin (Fig. 6). That could be due to differences in the ability of each species to use lignin in the presence of limited amounts of glucose. Among the thirty species tested, *C. camelliae* and *A. cylindrospora* grew best on lignin (as judged by increase in the colony size), and appeared to be able to use lignin as a carbon source in addition to glucose (Fig. 6). The growth of some fungi such as *F. velutipes, H. tessulatus, E. purpurascens* and the brown-rot fungi *P. betulinus* and *G. trabeum* was slow over one month (Fig. 6), and these fungi seemed to have limited ability to use lignin as a carbon source in the presence of low glucose. These findings either emphasize what was previously reported, that certain fungi require a readily useable growth substrate to breakdown lignin (Kirk *et al.*, 1976), and agree with what often been observed in brown-rot fungi that they are very much dependent on the presence of carbohydrates to grow on and degrade lignin (Kuhad *et al.,* 1997; Goodell, 2003). The fungi *A. ostoyae C. comatus, and C. septentrionale* also grew very slowly on lignin and stopped entirely before the end of experiment. That might be due to existence of some inhibitory compounds in the lignin preparation. Moreover, lignin as an aromatic compound, could play an inhibitor role that prevents growth or enzyme production in some species. Highley and Micales (1990) reported that 12 monomeric aromatic compounds inhibited the growth and production of 6 carbohydrate-degrading enzymes produced by the brown-rot fungi *Gleoephyllum trabeum* and *Postia (Oligoporous) placenta*, and the white-rot fungus *Trametes versicolor.*

Based on results in Fig. 6, I selected four microorganisms to be tested for growth with lignin at three levels of glucose (Fig. 7A, B and C). Two of the four, *A. cylindrospora* and *C. camelliae* were selected due to their rapid growth on the lignin medium with limited

amount of glucose. Whereas *P. betulinus* (Reh *et al.*, 1989) and *G. trabeum* were chosen as both of them are well studied brown-rot fungi (Kirk and Adler, 1970; Kerem *et al.*, 1999; Jensen *et al.*, 2001; Cohen *et al.*, 2004). *A. cylindrospora* is a species of the *Mucorales* (Yamada *et al.*, 1982), and *C. camelliae* is known to be a plant pathogen. It causes root rot of tea (Venkataramani and Venkata Ram, 1961), root rot in nutmeg (Rahman *et al.*, 1981), and a leaf spot of *Wisteria sinensis* Sweet (Reddy, 1975). A. *cylindrospora* and *C. camelliae* grew well (Fig. 7B, C) even on the medium with no glucose added (Fig. 7A). The growth of *P. betulinus* and *G. trabeum* was slow but possible without glucose and was slightly enhanced on the medium with 1.0% glucose (Fig. 7C). This is contrary to claims that additional readily metabolized carbon source such as cellulose, hemicellulose (De Jong *et al.,* 1994) or glucose (Lee, 1997) is required to degrade lignin. These results agree with those were reported by Gottlieb and Pelczar, Jr (1951); and Hiror and Eriksson (1976), who found that white-rot fungi can grow with Kraft lignin as a sole carbon source, but presence of carbohydrates accelerate growth and lignin the decomposition. To summarize, all the four species grew in lignin-containing media with and without glucose. The hyphal morphology and the biomass content were altered by increasing the glucose concentration (Figs. 8 and 9). These results seem to be in agreement with previous work indicating that the presence of glucose has a beneficial effect in the growth media, stimulating the growth of fungal biomass and enzyme production (Xiaoyan *et al.*, 2007).

According to Aro *et al* (2005), the possible explanation of my findings concerning the capability of the selected species to grow in different growth conditions that the production of plant cell wall degrading enzymes by fungi is regulated mainly at the transcriptional level. Where the expression of the genes encoding the enzymes is regulated by various environmental and cellular factors. Thus, production on enzymes may only take place under conditions in which the fungus needs to use plant polymers as an energy and carbon

source. When present, glucose may repress lignin utilization genes.

Decolorization of lignin suggests that its degradation is taking place in liquid culture. Most fungal cultures caused some changes in the UV_{280} absorbance of media supernatants (as well as ultrafiltrates) relative to control fresh medium (Table 2). Somewhat surprisingly, some cultures resulted in an increase in absorbance. This could be due to the free phenolic units in lignin (Hong et al., 2006) which can be oxidized enzymatically via laccase or chemically to produce phenoxy radicals and combine into more efficiently UV-absorbing chemical entities (Boerjan *et al.*, 2003; Ralph *et al.*, 2004; Davin *et al.*, 2000). In the majority of cultures, I observed a reduction in A_{280} (Table 2). These changes in the lignin molecules might be due to enzymatic attack on lignin, ligninolytic reactions taking place, leading to release of low molecular weight products as a result of lignin breakdown. In the fungal cultures where growth occurred, there was a decrease in A_{280} of the filtrate, and there was a general positive relationship between the growth and the decrease in A_{280} (Fig. 10 vs. Table 2). *C. camelliae* grew the best (Fig. 10) and its culture resulted in a large decrease in the UV absorbance of 26% in supernatant and 44% in ultrafiltrate (Table 2). Some fungi, such as *G. trabeum* and *P. betulinus* did not grow as well, but did change in the A_{280} by about 10% in the total medium and by 20-30% in the ultrafiltrate (Table 2). These results suggest these organisms did use lignin as a substrate.

I also observed interesting changes in the pattern of molecular weight distribution of the lignin polymer fragments (Fig. 11-14). Decrease in the A_{280} in the small fragments range (fractions 40-70) corresponds to molecular weight range 100-500 Da. This occurred in all four cultures (Fig. 11-14), suggesting that these small fragments were particularly metabolized by the four species studied. This decrease was somewhat less in *G. trabeum* suggesting a slightly different metabolic pathway (Fig. 14). Specific kinetics of lignin

fragments utilization would need to be investigated using labeled mono- and di-lignols (Xiao *et al.,* 2003; Wei *et al.*, 2009; Wong, 2009).

RP-HPLC analysis for a mixture of commercially available standard "phenolic compounds" (Fig. 10) was performed because these phenols are relevant to lignin structure and may be potential degradation products (Jugal *et al.*, 1986; Rybka *et al.*, 1993; Kim *et al.*, 2006). It was possible to distinguish each phenolic compound based on column retention times. I analysed *C. camelliae* spent media by HPLC using small and large fragments from GPC, in trying to investigate the low molecular weight phenolics derived from digested lignin. I found that large fragments of cultured lignin resulted in disappearance of some peaks, compared to the control (Figs. 16, 17) as well as production of new compounds in the small fragments GPC sample. These were retained on the column for 8.630, 9.128, and 11.736 minutes (Fig. 18, 19). Therefore, HPLC analysis confirmed to some extent the biodegradation of lignin by *C. camelliae.* These findings might largely be attributed to the action of putative fungal ligninolytic enzymes that led to the degradation and transformation of lignin substrate.

To initiate the investigation of potential lignin hydrolytic enzymes, laccase assay was performed with *A. cylindrospora, P. betulinus, C. camelliae* and *G. trabeum,* as these represent different taxonomic groups. Only *C. camelliae* produced high biomass and detectable laccase under the culture conditions used, but its activity was low (Table 3). Pelaez *et al.* (1995) concluded that the levels of laccase activities obtained did not necessarily reflect the optimum production for each species. My data agree with the findings of Xavier *et al.* (2001) who found that media which sustain producing high biomass, do not automatically result in high laccase yield. In other words, the production of high levels of the laccase enzyme was not dependent on high biomass yields. However, my observations about low biomass and no laccase production by *G. trabeum* do not agree with the findings

D'Souza *et al.* (1996), who found the brown-rot fungus *G. trabeum* to have laccase genes and produce laccase *in vitro*. Perhaps my culture conditions did not support the growth of *G. trabeum* sufficiently to produce the enzyme.

It is important to note that the conditions for cultivation of the fungus were not optimized for the four different species as these highly influence production of laccase by fungi (Heinzkill *et al.*, 1998). Vasconcelos *et al.* (2000) found that fungi known as laccase producers, produced laccase in low concentration, and that producing higher concentrations of laccase can be attained by adding various specific nutritional components to the medium (Lee *et al.*, 1999). As well, factors such as pH, and temperature can affect production of the enzyme (Kunamneni et al., 2007). Therefore, *C. camelliae* was examined for its ability to produce laccase under different, possibly improved, cultural conditions. However, laccase production remained very low in the presence or absence of lignin with 0.1% glucose and there was also a decrease in biomass production. In the presence and absence of lignin with 1.0% glucose, the fungus grew very well, but laccase production still remained low (Table 4). This suggests that presence of lignin did not induce laccase production to a high level, also glucose in two different concentrations did not change the laccase level. Peroxidases or other enzyme(s) may be involved. My findings conflict with the observation that the addition of xylidene, lignin, or veratryl alcohol as xenobiotic compounds increase and induce laccase activity (Xavier *et al.,* 2001), and agree with findings that the increase of glucose concentration in the cultivation media resulted in reduction (Bollag and Leonowicz, 1984) or a delay (Monteiro and De Carvalho, 1998), of the production of laccase.

Laccase activity was studied at pH 3.0 to 6.5 (Fig. 20). The optimum pH for *C. camelliae* laccase appeared to be 3.0. A further rise in pH was found to deactivate the enzyme. *C. camelliae* laccase had lower optimum pH as compared to laccase produced by *S. commune* that was optimally active at pH 6.0 (Irshad *et al.,* 2011). Laccases from

Trametes versicolor (Stoilova *et al.*, 2010) and *Lentinula edodes* (Boer *et al.,* 2004) had optimal activity at pH 4.5. On the other hand, it has also been reported that laccase produced by *Mauginiella* sp. has optimum acidic pH at 2.4 (Palonen et al., 2003). Therefore, further lowering the pH in my experiment may further increase laccase production.

The effect of temperature on *C. camelliae* laccase was studied at temperatures between 37 and 55°C (Fig. 21). The maximum laccase activity for *C. camelliae* was obtained at the assay temperature of 37°C, which is higher as compared to laccase of *Ganoderma lucidum* (Ko *et al.*, 2001) and lower compared to most fungal laccases, that is usually between 50 and 70°C (Baldrian, 2006). Specifically, Stoilova *et al* (2010) reported that laccase from *Trametes versicolor* had 45°C as an optimum temperature. My findings indicate that with an increase in assay temperature beyond 40°C, laccase activity decreased (Fig. 21). The activity of the enzyme could possibly be higher if the assay was done at temperature lower than 37°C, normal growth temperatures for this species.

6.0. Conclusions and future work

The main conclusions obtained throughout the series of experiments I performed were:

a) Identification of four wood-rot fungi, which were able to grow on lignin as a sole carbon source. This was not previously reported.

b) UV absorbance measurements at 280 nm indicated that different species modified lignin by different pathways, although *in vitro* degradation studies must not be taken as an absolute indication of the behavior of wood-rot fungi in natural environment, and the use of different cultural conditions may change the organism performance (Job, 2002).

c) The best future approach to ligninolytic enzymes production in cultures may be to increase biomass production followed by enzyme induction with lignin or related phenylpropanoids.

d) *C. camelliae* was able to produce laccase at low level under culture conditions used. Other enzymes could be responsible for lignin degradation and these should be investigated further.

e) The goal of identifying novel lignin degrading enzymes was not reached.

f) Since expression of the genes encoding ligninolytic enzymes is likely regulated by various environmental and cellular factors, more research is needed to determine the effect of certain environmental factors such as temperature on the growth of selected fungi. Accidently, I found that *C. camelliae* could grow at room temperature (22-25°C) faster than in the incubator (28°C).

g) The four selected lignin-degrading fungi broke down lignin into smaller fragments, which were likely used for growth as shown by GPC analysis.

h) Likewise, HPLC analysis confirmed a shift in the composition of lignin small fragments due to metabolism by *C. camelliae*.

i) Further work should focus on fungal species showing significant change in lignin and in doing so characterise lignin fragments using analytical methods such as LS-MS.

j) The use of various labeled lignin may provide further details regarding the pathway and kinetics of lignin metabolism.

7.0. References

Abadulla E, Tzanov T, Costa S, Robra K-H, Cavaco-Paulo A, Gübitz GM (2000) Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. Appl. Environ. Microbiol. **66**: 3357-3362

Albersheim P, Darvill A, Roberts K, Sederoff R, Staehelin A (2010) Plant cell walls: from chemistry to biology. Garland Science, New York

Alcalde M, Ferrer M, Plou FJ, Ballesteros A (2006) Environmental biocatalysis: from remediation with enzymes to novel green processes. Trends. Biotechnol **24:** 281-287

Alder E (1977) Lignin chemistry – past, present, and future. Wood Science and Technology **11:** 169-218.

Ander P, Stoytschev I, Eriksson KE (1988) Cleavage and metabolism of methoxyl groups from vanillic acid and ferulic acid by brown-rot and soft-rot fungi. Cellulose Chem. Technol. **22:** 255-266

Apenitis A, Erdtman H, Leopold B (1951) Studies on lignin. V. The decay of spruce wood by brown-rotting fungi. Sven. Kem. Tidskr. **63:** 195-207

Aramayo R, Timberlake, WE (1993) The *Aspergillus nidulans* yA gene is regulated by abaA gene. EMBO J. **12:** 2039-2048

Argyropoulos DS, Menachem SB (1997) Lignin. In: Eriksson, K.E., (Ed.), Advances in Biochemical Engineering/ Biotechnology. Springer Verlag, pp 127-158

Aro N, Pakula T, Penttilä M (2005) Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS. Microbiol. Rev. **29:** 719–739

Baldrian P (2006) Fungal laccases – occurrence and properties. FEMS Microbiol. Rev. **30:** 215-242

Barbosa AM, Dekker RFH, Hardy GE (1996) Veratryl alcohol as an inducer of laccase by an ascomycete, *Botryosphaeria* sp., when screened on the polymeric dye Ploy R-478. Lett Appl. Microbiol. **23:** 93-6

Berrio J, Plou FJ, Ballesteros A, Martinez AT, Martinez MJ (2007) Immobilization of *Pycnoporus coccineus* laccase on Eupergit C: Stabilization and treatment of olive oil mill wastewaters. Biocatal. Biotransform. **25:** 130-134

Blanchette RA (1995) Degradation of lignocelluloses complex in wood. Can. J. Bot. **73:** 999-1010

Boer CG, Obici L, Souza CG, Peralta RM (2004) Decolourization of synthetic dyes by solid state cultures of *Lentinula (Lentinus) edodes* producing manganese peroxidase as the main lignolytic enzyme. Biores. Technol. **94:** 107-112

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu. Rev. Plant Biol. **54:** 519-46

Bollag JM, Leonowicz A (1984) Comparative studies of extracellular fungal laccases. Appl. Environ. Microbiol. **48:** 849-854

Bourbonnais R, Paice MG (1990) Oxidation of nonphenolic substrates - an expanded role for laccase in lignin biodegradation. FEBS Lett. **267:** 99-102

Bourbonnais R, Paice MG, Freiermuth B, Bodie E, Borneman S (1997) Reactivities of various mediators and laccases with Kraft pulp and lignin model compounds. Appl. Environ. Microbiol. **63**: 4627-4632

Brown W, Cowling EB, Falkehag SI (1968) Molecular size distribution of lignins liberated enzymatically from wood. Sv. Papperstidn. **71:** 811-821

Brunow G (2001) Methods to reveal the structure of lignin. In: Hofrichter, M., Steinbuchel, A (Eds.), Lignin, humic substances and coal. John Wiley, Weinheim. **1:** 89-116

Buswell JA, Odier E (1987) Lignin biodegradation. CRC. Crit. Rev. Biotechnol. **6:** 1-60

Call HP, Mücke I (1997) History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozyme®-process). J. Biotechnol. **53:** 163-202

Camarero S, Sarkar S, Ruiz- Duenas FJ, Martinez MJ, Martinez AT (1999) Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. J. Biol. Chem. **274:** 10324- 10330

Camarero S, García O, Vidal T, Colom J, del Río JC, Gutiérrez A, Gras JM, Monje, R, Martínez MJ, Martínez AT (2004) Efficient bleaching of nonwood high-quality paper pulp using laccase-mediator system. Enzyme. Microb. Technol **35:** 113-120

Camarero S, Ibarra D, Martínez MJ, Martínez AT (2005) Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Appl. Environ. Microb. **71:** 1775- 1784

Cohen R, Suzuki MR, Hammel KE (2004) Differential stress-induced regulation of two quinone reductases in the brown rot basidiomycete *Gloeophyllum trabeum.* Appl. Environ. Microbiol. **70:** 324-331

Davin LB, Lewis NG (2000) Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignin biosynthesis. Plant

Physiology. **123:** 453-461

De Jonge E, Field JA, De Bont JAM (1994) Aryl alcohols in the physiology of ligninolytic fungi. FEMS Microbiol. Rev. **13:** 153-188

de la Rubia T, Ruiz E, Perez J, Martinez AJ (2002) Properties of a laccase produced by *Phanerochaete flavido-alba* induced by vanillin. Arch. Microbiol. **179:** 70–73

D'Souza TM, Boominathan K, Reddy CA (1996) Isolation of laccase gene-specific sequences from white rot and brown rot fungi by PCR. Appl. Environ. Microbiol. **62:** 3739–3744

Dwivedi UN, Singh P, Pandey VP, Kumar A (2011) Structure-function relationship among bacterial, fungal and plant laccases. J. Mol. Catal. B: Enzym. **68:** 117-128

Eriksson KE, Blanchette RA, Ander P (1990) Microbial and enzymatic degradation of wood and wood components. Springer Verlag, Berlin

Eslyn WE, Kirk TK, Effland MJ (1975) Changes in the chemical composition of wood caused by six soft rot fungi. Phythopathol. **65:** 473- 476

Fengel D, Wegener G (1989) Wood – Chemistry, Ultrastructure, Reactions. Walter de Gruyter, Berlin

Fritz-Langhals E, Kunath B (1998) Synthesis of aromatic aldehydes by laccase mediator assisted oxidation. Tetrahedron Lett. **39:** 5955-5956

Gavnholt B, Larsen K, Rasmussen SK (2002) Isolation and characterisation of laccase cDNAs from meristematic and stem tissues of ryegrass (*Lolium perenne*). Plant Science. **162:** 873-885

Glenn JK, Morgan MA, Mayfield MB, Kuwahara M, Gold MH (1983) An extracellular H_2O_2 requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete, *Phanerochaete chrysosporium.* Biochem. Biophys. Res. Commun. **114:** 1077-1083

Glenn JK, Gold MH (1985) Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium.* Arch. Biochem Biophys. **242:** 329-341

Gianfreda L, Rao MA (2004) Potential of extracellular enzymes in remediation of polluted soils: a review. Enzyme. Microb. Technol. **35:** 339-354

Gold MH, Wariishi H, Valli K (1989) Extracellular peroxidases involved in lignin degradation by the white rot basidiomycete, *Phanerochaete chrysosporium.* ACS Symp. Ser. **389:** 127-140

Goodell B (2003) Brown-rot fungal degradation of wood: Our evolving view. In: Goodell B, Nicholas DD and Schultz TP (eds.) Wood deterioration and preservation. ACS Symposium series 845, Washington DC, USA, pp. 97-118

Gottlieb S, Pelczar, Jr MJ (1951) Microbial aspects of lignin degradation. Bacteriol. Rev. **15:** 55- 76

Guillen F, Martínez AT, Martínez MJ (1992) Substrate specificity and properties of the arylalcohol oxidase from the ligninolytic fungus *Pleurotus eryngii.* Eur. J. Biochem. **209:** 603-611

Guillen F, Martínez MJ, Munoz C, Martínez AT (1997) Quinone redox cycling in the ligninolytic fungus *Pleurotus eryngii* leading to extracellular production of superoxide anion radical. Arch. Biochem. Biophys. **339:** 190-199

Gutiérrez A, Caramelo L, Prieto A, Martínez MJ, Martínez AT (1994) Anisaldehyde production and arylalcohol oxidase and dehydrogenase activities in ligninolytic fungi from the genus *Pleurotus.* Appl. Environ. Microbiol. **60:** 1783-1788

Haider K, Trojanowski J (1980) A comparison of the degradation of ^{14}C-labeled DHP and corn stalk lignins by micro- and macrofungi and bacteria. In: Lignin biodegradation: microbiology, chemistry, and potential application, TK Kirk, T Higuchi and H-M Chang, (Eds.). CRC Press. pp. 111–134

Harborne JB (1973) Phytochemical methods. Chapmann and Hall, London

Hartley RD, Buchan H (1976) High-performance liquid chromatography of phenolic acids and aldehydes derived from plants or from the decomposition of organic matter in soil. J. Chromatogr. **180:** 139-143

Harvey PJ, Schoemaker HE, Palmer JM (1985) Veratryl alcohol as a mediator and the role of radical cations in ligninlignin biodegradation by *Phanerochaete chrysosporium.* FEBS Lett. **195:** 242–246

Hatakka A (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. **13:** 125– 135

Heinfling A, Ruiz-Duenas FJ, Martínez MJ, Bergbauer M, Szewzyk Martinez AT (1998) A study on reducing substrates of manganese oxidizing peroxidases from *Pleurotus eryngii* and *Bjerkandera adusta.* FEBS Lett. **428:** 141-146

Heinzkill M, Bech L, Halkier T, Schneider P, Anke T (1998) Characterization of laccases and peroxidases from wood-rotting fungi (family *Coprinaceae).* Appl. Environ. Microbiol. **64:** 1601-1606

Highley TL, Micales JA (1990) Effect of aromatic monomers on production of carbohydrate-degrading enzymes by white-rot and brown-rot fungi. FEMS. Microbiol Lett. **66:** 15-21

Higuchi T (2006) Look back over the studies of lignin biochemistry. J. Wood Sci. **52:** 2-8

Hilden KS, Bortfeldt R, Hofrichter M, Hatakka A, Lundell TK (2008) Molecular characterization of the basidiomycete isolate *Nematoloma frowardii* b19 and its manganese peroxidase places the fungus in the *corticioid* genus *Phlebia*. Microbiol. **154:** 2371-2379

Hiror T, Eriksson K (1976) Microbiological degradation of lignin. I. influence of cellulose on the degradation of lignin by the white rot fungus *Pleurotus ostreatus*, Sven. Pappersitidn. **79:** 157

Hong F, Jönsson L, Lundquist K, Wei Y (2006) Oxidation capacity of laccases and peroxidases as reflected in experiments with methoxy-substituted benzyl alcohols. Appl. Biochem. Biotechnol. **129:** 303-319

Hoshino F, Kajino T, Sugiyama H, Asami O, Takahashi H (2002) Thermally stable and hydrogen peroxide tolerant manganese peroxidase (MnP) from *Lenzites betulinus*. FEBS Lett. **530:** 249-252

Irshad M, Asgher M, Sheikh MA, Nawaz H (2011) Purification and characterization of laccase produced by *Schyzophylum commune* IBL-06 in solid state culture of Banana stalks. Bioresour. Technol. **6:** 2861-2873

Jensen KA, Houtman CJ, Ryan ZC, Hammel KE (2001) Pathways for extracellular fenton chemistry in the brown rot basidiomycete *Gloeophyllum trabeum*. Appl. Environ. Microbiol. **67:** 2705-2711

Jin L, Schultz TP, Nicholas DD (1990) Structural characterization of brown rotted lignin. Holzforschung. **44:** 133-138

Job D (2002) Assessment of selected decay basidiomycetes for selective biodefibrillation of Picea abies wood. Mycological Progress. **1:** 123- 130

Jugal K, Gupta JK, Jebsen K, Kneife H (1986) Sinapic Acid Degradation by the Yeast *Rhodotorula glutinis*. J. Microbiolo. **132:** 2793-2799

Kaarik A (1965) The identification of the mycelia of wood-decay fungi by their oxidation reactions with phenolic compounds. Stud. Forest. Suec. **31:** 1- 81

Kerem Z, Jensen KA, Hammel KE (1999) Biodegradative mechanism of the brown rot basidiomycete *Gloeophyllum trabeum:* Evidence for an extracellular hydroquinone-driven fenton reaction. FEBS Lett **446**: 49-54

Kersten PJ (1990) Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase. Proc. Natl. Acad. Sci. USA. **87:** 2936-2940

Kersten PJ, Kalyanaraman B, Hammel KE, Reinhammer B (1990) Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzene. J. Biochem. **268:** 475–480

Kim HJ, Kim EJ, Seo SH, Shin CG, Jin C, Lee YS (2006) Vanillic acid glycoside and quinic acid derivatives from *Gardeniae fructus*. J. Nat. Prod. **69:** 600–603

Kirk TK, Adler E (1970) Methoxyl-deficient structural elements in lignin of sweetgum decayed by a brown-rot fungus. Acta. Chem. Scand **24:** 3379-3390

Kirk TK, Larsson S, Miksche GE (1970) Aromatic hydroxylation resulting from attack of lignin by a brown rot fungus. Acta. Chem. Scand. **24:** 1470-1472

Kirk TK, Connors WJ, Zeikus JG (1976) Requirement of a growth substrate during lignin decomposition by two wood rotting fungi. Appl. Environ. Microbiol. **32:** 192-194

Kirk TK, Croan S, Tien M, Murtaugh K, Farrell R (1986) Production of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of mutant strain. Enzyme Microb. Technol. **8:** 27-32

Kirk TK, Farrell RL (1987) Enzymatic combustion: the microbial degradation of lignin. Ann. Rev. Microbiol. **41:** 465-505

Ko EM, Leem YE, Choi HT (2001) Purification and characterization of laccase isozymes from the white-rot basidiomycete *Ganoderma lucidum*. Appl. Environ. Microbiol. **57:** 98-102

Kögel-Knabner I (2002) The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. Soil. Biol. Biochem. **34:** 139-162.

Kuhad RC, Singh A, Eriksson KEL (1997) Microorganisms and Enzymes involved in the degradation of plant fiber cell walls. In: Eriksson KL (Ed.), Advances in Biochemical Engineering/ Biotechnology. Springer Verlag. pp. 46-125

Kunamneni A, Ballesteros A, Plou FJ, Alcalde M (2007) Fungal laccase-a versatile enzyme for biotechnological applications, in Communicating Current Research and Educational Topics and Trends in Applied Microbiology, A. Mendez-Vilas, Ed., vol. 1, pp. 233–245, Formex, Badajoz, Spain

Kunamneni A, Ghazi I, Camarero S, Ballesteros A, Plou FJ, Alcalde M (2008a) Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers. Process. Biochem. **43:** 169-178

Kunamneni A, Camarero S, García-Burgos C, Plou FJ, Ballesteros A, Alcalde M (2008b) Engineering and Applications of fungal laccases for organic synthesis. Microbial. Cell. Factories. **7:** 32

Kunamneni A, Plou FJ, Ballesteros A, Alcalde M (2008c) Laccases and their applications: A patent review, Recent Pat. Biotechnol. **2:**10-24

Kuwahara M, Glenn JK, Morgan MA, Gold MA (1984) Separation and characterization of two extracellular H_2O_2 dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett. 169, 247-250

Kuwatsuka S, Shindo H (1973) Behavior of phenolic substances in the decaying process of plants. I. Identification and quantitative determination of phenolic acids in rice straw and its decayed product by gas chromatography. Soil Sci. Plant Nutr. **19:** 219-227

Lankinen VP, Bonnen AM, Anton LH, Wood DA, Kalkkinen N, Hatakka A, Thurston CF (2001) Characteristics and N-terminal amino acid sequence of manganese peroxidase from solid substrate cultures of *Agaricus bisporus*. Appl. Microbiol. Biotechnol. **55:** 170-176

Lee J (1997) Biological conversion of lignocellulosic biomass to ethanol. J. Biotech. **56:** 1-24

Lee IY, Jung KH, Lee CH, Park TH (1999) Enhanced production of laccase in *T.versicolor* by addition of ethanol. Biotechnol Lett. **21:** 965-968

Lee KH, Wi SG, Singh AP, Kim YS (2004) Micromorphological characteristics of decayed wood and laccase produced by the brown-rot fungus *Coniophora puteana*. J. Wood. Sci. **50:** 281-284

Liao Y-M (1990) Nutritional and environmental conditions for growth of *Coriolus versicolor*, a wood decaying and medical fungus. J. Agric. Res. **39:** 190-203

Martinez AT, Speranza M, Ruiz-Duenas FJ, Ferreira P, Camarero S, Guillen F, Martinez MJ, Gutierrez A, del Rio JC (2005) Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int. Microbiol. **8:** 195-204

McGinnis MR, Rinaldi MG (1991) Antifungal drugs: mechanisms of action, drug resistance, susceptibility testing, and assays of activity in biological fluids, p. 198–251. In V. Lorian (ed.), Antibiotics in laboratory medicine, 3rd ed. Williams & Wilkins, Baltimore, Md

Messerschmidt A, Huber R (1990) The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin. Modelling and structural relationships. Eur. J. Biochem. **187:** 341-352

Mester T, Field JA (1998) Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. J. Biol. Chem. **273:** 15412-15417

Meyermans H, Morreel K, Lapierre C, Pollet B, De Bruyn A, Busson R, Herdewijn P, Devreese B, Van Beeumen J, Marita JM, Ralph J, Chen C, Burggraeve B, Van Montagu M, Messens E, Boerjan W (2000) Modifications in lignin and

accumulation of phenolic glucosides in poplar xylem upon down-regulation of caffeoyl-coenzyme A *O-methyltransferase,* an enzyme involved in lignin biosynthesis. J. Biol. Chem. **275:** 36899-36909

Monteiro MC, De Carvalho MEA (1998) Pulp bleaching using laccase from *Trametes versicolor* under high temperature and alkaline conditions. Appl. Biochem. Biotechnol. **70:** 983-993

Morozova V, Shumakovich GP, Gorbacheva MA, Shleev SV, Yaropolov AI (2007) Blue laccases. Biochem-Moscow **72:**1136–50

O' Malley DM, Whetten R, Bao W, Chen CL, Seedorf RR (1993) The role of laccase in lignification. Plant J. **4:** 751-757

Palonen H, Saloheimo M, Viikari L, Kruus K (2003) Purification, characterization and sequence analysis of a laccase from the ascomycete *Mauginiella sp.* Enz. Microb. Technol. **33:** 854-862

Peláez F, Martínez MJ, Martínez AT (1995) Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation. Mycol. Res. **99:** 37-42

Piontek K, Antorini M, Choinowski T (2002) Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. J. Biol. Chem. **277:** 37663-37669

Rahman M U, Sankaran KV, Leelavathy KM, Zachariah S (1981) Cylilldrocladium root rot of nutmeg in South India. Plaut Disease. 65: 514-515

Ralph J, Lundquist K, Brunow G, Lu F, Kim H, Schatz PF, Marita JM, Hatfield RD, Ralph SA, Christensen JH, Boerjan W (2004) Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. Phytochem Reviews. **3:** 29-60

Reddy SM (1975) Some new leaf spot diseases caused by hyphomycetes. Proc. Nat. Acad. Sci. India sect. B. **45:** 97-100

Reh U, Kraepelin G, Lamprecht I (1986) Use of Differential Scanning Calorimetry for Structural Analysis of Fungally Degraded Wood. Appl. Environ. Microbiol. **52:** 1101-1106

Rochefort D, Leech D, Bourbonnais R (2004) Electron-transfer mediator systems for bleaching of paper pulp. Green Chem. **6:** 14–24

Rybka K, Sitarski J, Raczy K, Bojanowska S (1993) Ferulic Acid in Rye and Wheat Grain and Grain Dietary Fiber. Cereal Chem. **70:** 55-59

Sakurai T (1992) Kinetics of electron transfer between cytochrome-c and laccase. Biochem. **31:** 9844-9847

Sanchez C (2009) Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol. Adv. **27:** 185-194

Sergio R (2006) Laccases: blue enzymes for green chemistry. Trends Biotechnol. **24:** 219-226

Smith M, Thurston CF, Wood DA (1997) Fungal laccases: Role in delignification and possible industrial applications. In Multi-copper oxidases Edited by: Messerschmidt A. Singapore, World Scientific. 201-224

Solomon EI, Baldwin MJ, Lowery MD (1992) Electronic structures of active sites in copper proteins: contributions to reactivity. Chem. Rev. **92:** 521–542

Stoilova I, Krastanov A, Stanchev V (2010) Properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation. Adv. Biosc. Biotechnol. **1:** 208-215

Sun QN, Qin TF, Li GY (2009) Chemical groups and structural characterization of brown-rotted *Pinus massoniana* lignin. Int. J. Polym. Anal. Charact. **14:** 19-33

Tien M, Kirk TK (1983) Lignin-degrading enzyme from the *Hymenomycete Phanerochaete chrysosporium* Burds. Science. **221:** 661-663

Tien M (1987) Properties of ligninase from *Phanerochaete chrysosporium* and their possible applications. CRC. Crit. Rev. Microbiol. **15:** 141-168

Thurston CF (1994) The structure and function of fungal laccases. Microbiol. UK. **140:** 19-26

Valaskova V, Baldrian P (2006) Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus* - production of extracellular enzymes and characterization of the major cellulases. Microbiolo. **152:** 3613-3622

Vasconcelos AFD, Barbosa AM, Dekker RFH, Scarminio IS, Rezende MI (2000) Optimization of laccase production by *Botryosphaeria* sp. in the presence of veratryl alcohol by the response-surface method. Process. Biochem. **35:** 1131-1138

Venkataramani KS, Venkata Ram CS (1961) A new species of *Cylindrocladium* parasitic on tea roots. Curr. Sci. **30:** 186

Vogel HJ, Bonnerd H (1956) A convenient growth medium for *E. coli* and some other micro-organisms (Medium E). Microbial Genetics Bulletin **13:** 43-44

Wei H, Xu Q, Taylor LE, Baker JO, Tucker MP, Ding SY (2009) Natural paradigms of plant cell wall degradation. Curr. Opin. Biotechnol. **20:** 330-338

Weitz JH, Ballard AL, Campbell CD, Killham K (2001) The effect of culture conditions on the mycelial growth and luminescence of naturally bioluminescent fungi. FEMS

Microbiol. Lett. **202:** 165-170

Williamson PR, Wakamatsu K, Ito S (1998) Melanin biosynthesis in *Cryptococcus neoformans*. J. Bacteriol. **180:** 1570-1572

Wong DW (2009) Structure and action mechanism of ligninolytic enzymes. Appl. Biochem. Biotechnol. **157:** 174-209

Wulf LW, Nagel CW (1976) Analysis of phenolic acids and flavonoids by high-pressure liquid chromatography. J. Chromatogr. **116:** 271-279

Xavier AMRB, Evtuguin DV, Ferreira RMP, Amado FL (2001) Laccase production for lignin oxidase activity. Proceed- ings 8th Int. Conf. Biotechnol. Helsinki, Finland. Environ. Sci. Engin. China. **1:** 89-94

Xiao YZ, Tu XM, Wang J, Zhang M, Cheng Q, Zeng WY, Shi YY (2003) Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete *Trametes* sp. strain AH28-2. Appl. Microbiol. Biotechnol. **60:** 700-707

Xiaoyan Z, Xianghua W, Yan F (2007) Influence of glucose feeding on the ligninolytic enzyme production of the white rot fungus *Phanerochaete chrysosporium*. Front.

Xu F (1996) Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochem-US. **35:** 7608-7614

Xu F, Shin W, Brown S, Wahleithner JA, Sundaram UM, Solomon EI (1996) A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. Biochem. Biophys. Acta. **1292:** 303-311

Yamada H, Oshima Y, Miyazaki T (1982) Characterization of fucomannopeptide and mannoprotein from *Absidia cylin- drospora. Carbohydr.Res.* **110:** 113-126

Yaropolov AI, Skorobogat'ko OV, Vartanov SS, Varfolomeyev SD (1994) Laccase. properties, catalytic mechanism, and applicability. Appl. Biochem. Biotechnol. **49:** 257-280

Yoshida H (1883) Chemistry of lacquer (Urushi), part 1. J. Chem. Soc. 472-486