ENZYME PRODUCTION AND ACTIVITIES OF LIGNOCELLULOLYTIC FUNGI CULTIVATED ON AGRICULTURAL RESIDUES

by

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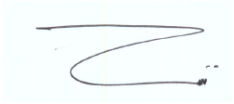
Enzyme Production and Activities of Ligninolytic Fungi cultivated on Agricultural Residues

I declare that the above dissertation/thesis is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

SIGNATURE



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08 November 2016

Abstract

A total of 30 fungal fruiting bodies were collected from decaying plant materials (barks and litter) from the wild based on morphological variations. Nine of these fungi purified to monoaxenic cultures were included in the present study and also a type strain *Ganoderma lucidum* ATCC- 32471. These fungi were screened for lignocellulolytic activities, five of these organisms produced ligninolytic enzymes when exposed to two different concentrations of guaiacol (0.02% and 0.2%) on two different media (MEA and PDA). All ten fungal isolates screened for cellulolytic activity were positive for the production of the cellulase enzyme. The fungal isolates were characterised using morphological and molecular methods. Molecular characterization using ITS1 and ITS4 primers was able to identify these fungal isolates to degrees of accuracy ranging from 98% to 100%. The phylogenetic and lineage analysis showed that the species varied amongst phylum Basidiomycota, Ascomycota and early diverging fungal lineages Mucormycotina. Both monocultures and dual cultures of these 10 fungal species were cultivated for the purpose of spectrophotometrically quantifying and evaluating enzyme production on agricultural waste residues; corn cob, sugar cane bagasse and wheat straw. A pattern of antagonistic invasion interaction was identified to demonstrate increased enzyme production on dual cultures. Four of these fungal species, *Trichoderma* sp. KN10, *Rhizopus microsporus* KN2, *Fomitopsis* sp. KN1 and *Coriolopsis* sp. KN6 demonstrated tendencies of invasion and replacement in co-cultures. The fungi and their dual cultures showed varying levels of enzyme production. Analysis of mean showed dual culture interactions involving KN10 with values for MnP production approximately at 1.46U/ml compared to monoculture of 0.06U/ml. Further, dual laccase values approximately at 0.09U/ml compared to monocultures of 0.05U/ml. Overall the highest enzyme activity was observed using wheat straw. This study demonstrated and proved that agricultural waste residues can be used for lignocellulytic enzyme production and that antagonistic invasion by some fungi (in particular *Trichoderma* sp. KN10) in co-cultures can increase production of one or more of the three enzyme laccase, lignin peroxidase and manganese peroxidase.

Keywords: fungi**,** dual cultures, competition, antagonism, ligninolytic enzymes, agricultural residues

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Dedication

To the memory of my father,

Isaac Chijioke Ijoma

1943 – 2003

I did as you persuasively asked…….

Ditto…

Felicia Ngozi Odinnma Ijoma (neé Amolo)

1953 – 2017

You were here….

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List of Abbreviations

AAC	American association for clinical chemistry
ABTS	2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid
ACMA	Acidified cornmeal agar
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
ARC	Agricultural research council
ASBC	American society of brewing chemists
ATCC	American type culture collection
BLASTn	Basic local alignment search tool nucleotide
CBP	Consolidated bioprocessing
CCA	The corn cob agar
CDH	Cellobiose dehydrogenase
CV	Coefficient of Variance
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytisine triphosphate
dGTP	Deoxyguanosine triphosphate
DHN	1,8 dihydroxynaphthalene
DM	Dry mass
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DP	Degree of polymerization
dTTP	Deoxythymidine triphosphate
EtBr	Ethidium bromide
GDHB	γ-glutaminyl-3,4 – dihydroxybenzene
GHG	Greenhouse gases
GlcNAc	1,4-β-linked *N*-acetyl-D-glucosamine
HSD	Honest significant difference
ITS	Internal transcribed spacer
Lacc.	Laccase
LC50	Median lethal concentration

L-Dopa	L-dihydroxyphenylalanine
LiP	Lignin peroxidase
LSU	Large subunits
MEA	Malt extract agar
ML	Maximum likelihood
MnP	Manganese-dependent peroxidase
MPT	Mitochondrial permeability transition
NCBI	National Centre for Biotechnology Information
OD	Optical density
OH	Hydroxyl radical
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
QR	Quinone reductases
RBB-R	Remazol brilliant blue-R
RFLP	Restriction fragment length polymorphism
RISA	Ribosomal intergenic spacer analysis
ROS	Reactive oxygen species
rRNA	Ribosomal Ribonucleic acid
SBA	Sugarcane bagasse agar
SD	Standard deviation
SEM	Scanning electron microscopy
SSF	Solid state fermentation
SSU	Small subunits
UNEP	United Nations Environmental Programme
UV	Ultra violet
VOCs	Volatile organic compounds
VP	Versatile peroxidase
WSA	Wheat straw agar

List of Conference and Research Article Manuscript Submitted

GN Ijoma and M Tekere. 'Increased Enzyme Activity during Antagonistic Invasion Interaction of Fungi grown on Corn Cob' (Oral Presentation and Article publication). 8th International Conference on Environmental Science and Technology. American Academy of Science, Houston, Texas, USA. June, 2016

GN Ijoma and M Tekere. 'Increased Production of Ligninolytic Enzymes using dual cultures of Fungi on Corn Cob and Sugarcane Bagasse Media' (Oral Presentation). 6th International Symposium on Energy from Biomass and Waste. Venice, Italy. November, 2016

GN Ijoma, Ramganesh Selvarajan and M. Tekere. 'Evaluating the potential of different fungi as biological inducers in dual cultures to promote increased ligninolytic enzymes production on agricultural residues'(Research Article submitted to International Biodeterioration and Bioremediation)

GN Ijoma and M Tekere. 'Potential microbial applications of co-cultures involving ligninolytic fungi in the bioremediation of recalcitrant xenobiotic compounds' (Review Article published in International Journal of Environmental Science and Technology)

Chapter 1

Overview

Lignocellulose are regarded as the most abundant renewable organic matter on earth (Giovannozzi-Sermanni *et al.*, 2001) and are potentially a great feedstock reserve for the production of biofuels and chemicals. However, the presence of lignin as its primary constituents makes it under-utilized at present and in most cases commercially non-viable; this is because of the presence of covalent lignin-carbohydrate linkages connecting sugar hydroxyl of hemicellulose and phenylpropane subunits in lignin making the sugars less biologically available. The primary role of lignocellulose is that it protects the plant against degradation that could happen due to the presence of autochthonous enzymes and microorganisms. The ability to break down the lignin would effectively make the carbohydrate more accessible for efficient bioconversion.

Several pre-treatments currently exist that seek to render lignocellulosic materials more accessible to enzymes. However these pre-treatments primarily increase process cost, and some are considered extremely harsh and produce inhibitory compounds that are not environmentally friendly and in most cases prove harmful to the degrading microorganisms (Palmqvist & Hahn-Hagerdal, 2000; Howard *et al.*, 2003). Current research attempts to address these problems by refining the integration of pre-treatment technologies and downstream enzyme applications, as well as minimizing the production of compounds that inhibit fermentation processes (Kabel *et al.*, 2006; Zhao *et al.*, 2009; Kumar *et al.*, 2011; Zheng *et al.*, 2014); however these processes do not necessarily prove inexpensive.

A shift towards environmentally friendly fuel production is paramount; thus it is necessary to make concerted efforts to develop economically viable alternatives that include the application of microorganisms in modification of lignocellulose. Other alternatives include genetic modification of microorganism to increase their lignocellulolytic abilities; and the use of purified enzymes, such as cellulases and hemicellulases. The latter have already found notable application in textile and pulp and paper making industries. However, some authors have noted the economic effects in terms of increased market prices and capital costs of production associated with greater quantities demand of purified enzymes which will serve as a deterrent to the commercial application of this processes (Yang & Wyman, 2007; Sainz, 2011; Kuhad *et al.*, 2011).

One major microbial resource available for the degradation of lignocellulose is the white-rot basidiomycetes. They are able to degrade lignin efficiently, although, this is greatly dependent on the environmental conditions and the fungal species involved (Hatakka & Hammel, 2010; Isroi *et al.*, 2011). Studies aimed at the elucidation of lignin degradation mechanisms by fungal species revealed the complexity of the enzymatic systems; this is attributed to the diverse pathways to lignin degradation and the enzymatic machinery of the various microorganisms (Hatakka, 1994). The use of these white-rot fungi is a potential strategy that considers both economics and natural environmental remediation.

The co-cultivation of lignocellulose degrading fungi with the associated high activity of lignin modifying enzymes (Laccase [Lac], Manganese-dependent peroxidases [MnP], Lignin peroxidase [LiP] and versatile peroxidase [VP]) (Solarska, 2009; Mahajan, 2011) due to their synergistic and antagonistic actions is necessary to increase performance, efficiency and end-product yield (Mata *et al*., 2005; Flores *et al*., 2009; Bader *et al*., 2010; Dwivedi *et al*., 2011).

The use of fungal co-cultures can significantly improve lignocellulose hydrolysis as it minimizes the need for purified enzymes and its associated cost implications. It would also mean that there is better product optimization as most of the component sugars (cellulose and hemicellulose) are utilized in this way (Dashtban *et al.*, 2009). This is further supported by the fact that in nature, lignocellulose degradation is achieved by a complex mix of co-existing lignocellulolytic microorganisms (Bennet *et al*., 2002; Cheng & Zhu, 2012). This principle has found wide application in several industrial processes including antibiotics, enzyme and fermented food productions (Gutierrez-Correa & Tengerdy, 1998). The use of fungal co-cultures has several advantages when compared with single strain use in lignocellulose degradation, including improved productivity, adaptability and substrate utilization (Dashtban *et al.*, 2009). Although the effects of co-culturing fungi for lignocellulose biodegradation is poorly understood (Chi *et al*., 2007; Qi-He *et al.*, 2011; Hays *et al*., 2015), the understanding of these interactions between associated strains is important. Currently, very little research has been done making it a credible research area (Cheng & Zhu, 2012).

Furthermore, the usefulness of interspecific interactions as a strategy to improve hydrolysis and maximize the use of substrates thereby increasing product yields (Cheng & Zhu, 2012) should not be ignored in lignocellulose bioconversion. Moreover, the economic viability of this strategy and the significant reduction in the problem of inhibition between product and enzyme

is considered an incentive for its application. The use of co-cultures for improving fungal cellulolytic activities has been studied by several authors; Juhász *et al*. (2003) investigated the use of co-cultures of *Aspergillus niger* and *Trichoderma reesei* as a way to increase β-glucosidase production with significant results. Hamelinck *et al.* (2005) and Shrestha *et al*. (2008) demonstrated that after 1 to 3 days pre-treatment of the maize straw using the co-culture system of *Saccharomyces cerevisiae* and white-rot fungi, more ethanol can be gained than using mono-culture, where the ethanol yield can reach 0.03 g/g. Similarly, Hu *et al*. (2011) demonstrated that co-cultivation of *A. niger* and *A. oryzae* as well as co-cultivation with *Magnaporthe grisea* or *Phanerochaete chrysosporium* can improve the production of extracellular ligninolytic enzymes. Mostafa *et al.* (2014) screened several marine-derived fungal isolates and demonstrated the co-cultivation of three isolates (*A. flavus, Cladosporium sphaerospermum* and *Epicoccum purpurascens*) remarkably improved xylanase production when cultivated on a mixture of wheat bran and saw dust.

Similarly, this study screened at least 30 different fungal species obtained from the Kloofendal Nature Reserve, Roodeport, Johannesburg and included a type strain *Ganoderma lucidum* ATCC- 32471 that was obtained from the Agricultural Research Council (ARC), Pretoria, South Africa for their potential to produce ligninolytic enzymes in monocultures. The main aim of the study was to ascertain whether co-cultivation would improve enzyme production and on commonly available lignocellulosic agricultural waste biomass. In particular the research hypothesis tested was that: competition and antagonism will promote or induce the expression of ligninolytic enzymes in interspecific interactions of fungi on different lignocellulosic substrates.

It is hoped that the results of this study will address the challenge of substrate preferences in lignocellulose degrading organisms. The elucidation of physical and chemical properties of commonly found lignocellulose wastes in South Africa is essential for the design of subsequent process operations and application. Furthermore it is necessary to consider more than a single substrate to accommodate the regional differences in substrate availability. Whilst one type of lignocellulosic waste materials may be available in one region, some other region may have waste derived from other agricultural produce due to climatic changes and soil differences that accommodate certain types of crops and not the other. The biodiversity of ligninolytic fungi in all regions presents an opportunity to find a consortium that can be suitably adapted to

biodegrade these different agricultural residues for the purposes of biofuel production and the generation of enzymes for other applications.

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Chapter 2

Literature review

2.1 Lignocellulose: Nature's tightly wrapped gift

Lignocellulose are renewable energy resources that are abundant and found naturally (Perez *et al*., 2002). They are a natural part of all plants and are generated as waste products of forestry, agriculture and agro-industry. Their accumulation tends to create a problem of waste disposal. Therefore it can be utilised as an inexpensive feedstock in the production of chemicals and biofuels without placing undue stress on scarce food resources that could lead to food prices increase particularly as they are considered to be inedible materials (Balan, 2014; Searchinger & Heimlich, 2015). Moreover, the utilization of lignocellulosic agricultural residues has the potential to provide intermediate building blocks chemicals and second generation biofuels without negative competition for land (Walford, 2008; Searchinger & Heimlich, 2015).

Lignocellulosic materials do not contain readily accessible monosaccharides. Considerable efforts in researches are focused on the bioconversion of lignocellulosic materials into platform sugars that can be readily fermented to fuels and chemicals (Mahajan, 2011). One major limitation to its use in the production of bioethanol is its recalcitrant nature to degradation; this means that unlike starch and sugar, lignocellulose require pre-treatment before fermentation. In addition, the structural compositions of lignocellulose are complex and variable and the likelihood of formation of inhibitors to fermenting microorganisms further increases technical challenges (Mosier *et al.*, 2005a; Petersson, *et al.*, 2007; Jönsson & Martín, 2016) and production cost involved in fermentation processes towards the production of bioethanol and biogas alike. Therefore, working with lignocellulosic biomass makes it necessary to factor in the digestibility of the main constituent because it is only with pre-requisite treatment can the cellulose in the plant fibres become exposed for degradation (Kumar *et al.*, 2009).

2.2 Composition of Lignocellulose

Plant cell are composed mainly of lignocellulose which comprise of three major groups of polymers – cellulose, hemicellulose and lignin (Mussatto & Teixeira, 2010). The cellulose framework is surrounded by hemicellulose and lignin.

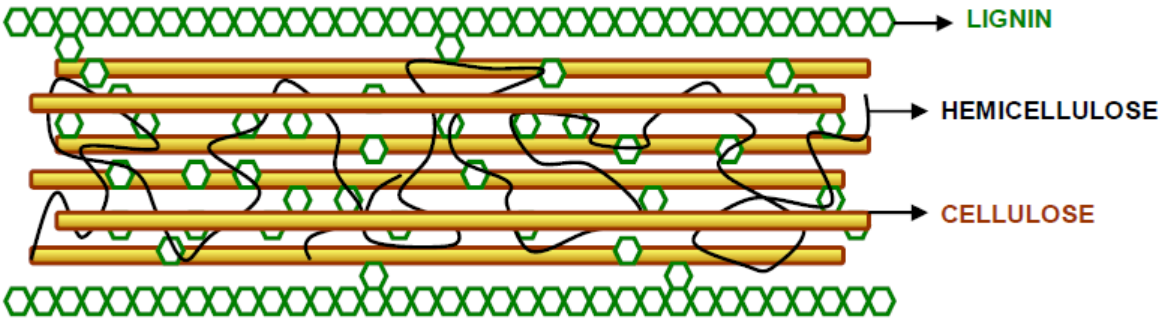


Figure 2.1: Representation of lignocellulose structure showing cellulose, hemicellulose and lignin fractions (Mussatto & Teixeira, 2010)

The composition of these constituents can vary from one plant species to another (Table 1) (Kumar *et al.*, 2009; Sorek *et al.*, 2014). Furthermore, the ratios between constituents within a single plant vary with age, stage of growth and other environmental conditions (Perez *et al.*, 2002). Except for cellulose, the other polymers are synthesized within the cell and extruded to cell membrane where they are organized in a matrix-like arrangement; with the primary cell deposited first. This is characterized by relatively amorphous cellulose structure, followed by the synthesis of the secondary cell wall after cell differentiation characterized by cellulose microfibrils with higher crystallinity and altered hemicellulose content (Ding & Himmel, 2006).

2.2.1 Cellulose

The plant cell wall is primarily composed of cellulose which is synthesized by a cellulose synthase complex that is found within the cytoplasmic membrane of plant cells (Taylor *et al.*, 2000; Li *et al.*, 2014). It is a linear homopolysaccharide with repeated units of cellobiose (two anhydrous glucose rings joined via a β-1, 4 glycosidic linkages) (Beguin & Aubert, 1994; Klemm *et al.*, 1998; Li *et al.*, 2014).

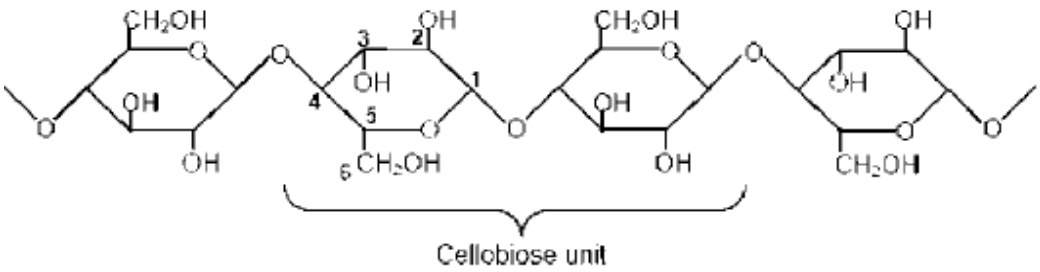


Figure 2.2: Diagram of cellulose chain (Kumar *et al.*, 2009)

These cellobiose units are linked by hydrogen and van der Waals bonds which cause the cellulose to be packed into microfibrils that is made up of highly organized crystalline regions and unorganized amorphous regions (Ha *et al*., 1998; Khazraji & Robert, 2013) intertwined with both hemicellulose and lignin. Crystalline cellulose is less soluble and less degradable and makes up the larger ratio as compared to amorphous cellulose. The arrangement of crystalline and amorphous cellulose gives plant the unique properties of dual rigidity and flexibility; the hydrogen bond promotes insolubility in most solvents and is partly responsible for the resistance of cellulose to microbial degradation (Jørgensen *et al*., 2007).

Crystalline cellulose can be degraded to fermentable D-glucose using acids or enzymes that break the glycosidic bonds; however the amorphous form of cellulose is more susceptible to enzymatic degradation (Beguin & Aubert, 1994; Yang *et al*., 2011). The enzymes for cellulose degradation belong predominantly to hydrolases; cellulose is hydrolyzed by cellulase (endoglucanase), 1,4-β-cellobiosidase and β-glucosidase (Schmidt, 2006).

2.2.2 Hemicellulose

Hemicellulose is a general term used to represent a group of complex heterogenous polysaccharides characteristically made up of different sugars, such as D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose, as well as other components such as acetic, glucoronic and ferulic acids.

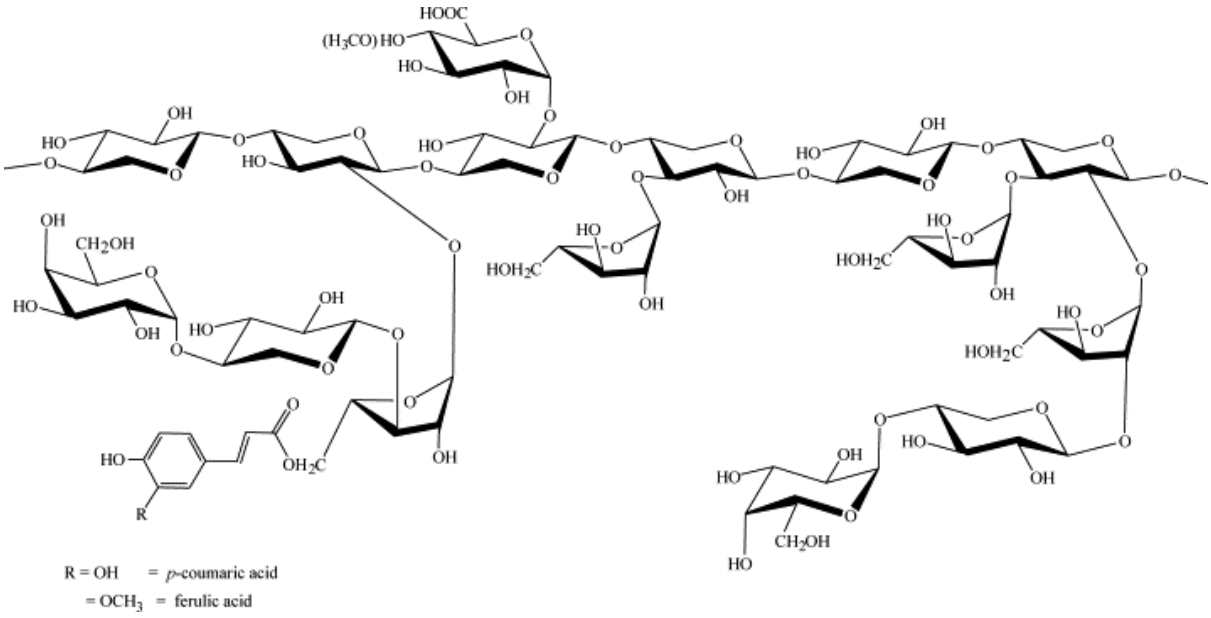


Figure 2.3: Diagram of hemicellulose structure (Chaikumpollert *et al*., 2004)

Hemicellulose is similar to cellulose in that – their basic structural compositon is primarily β-1, 4-linked sugars, although, the particular sugar composition of hemicellulose is dependent on the source of polysaccharide. This also means that the main sugar residue in its basic structural composition determines the classification of hemicellulose, for example, xylans, mannans, glucans, glucuronoxylans, arabinoxylans, glucomannans, galactomannans, galactoglucomannans, β-glucans, and xyloglucans (Fengel & Wegener, 1989). Hayashi and Kaida (2011) describes this aptly using xyloglucan which is the main hemicellulose of primary cell walls and thus consists of a β-1, 4-linked glucose backbone that is surrounded by xylose, galactose and in some instances fructose branching sugars. By contrast the main hemicellulose in secondary cell walls of hardwoods and softwoods is xylan and galactoglucomannan respectively. Xylan is composed of β-1, 4-linked xylose that can be substituted by arabinose and glucuronic acid; xylan can also be acetylated. Xylan can be degraded by endo-1, 4-β-xylanase and 1, 4-β-xylosidase to xylose (Jørgensen *et al.*, 2003). Furthermore, wood-derived mannans are composed of β-1, 4-linked mannose and glucose that can be substituted by galactose (Stenius & Vuorinen, 1999; Liepman *et al.*, 2007).

Fengel & Wegener (1989) summarize the difference between hemicellulose and cellulose in terms of sugar-units composition by stating that – hemicelluloses possess shorter chains consisting of different sugars that branch off the main chain molecules; this makes them amorphous and thus makes the structure easier to hydrolyze than cellulose. These monosaccharide chains include pentoses (xylose, rhamnose and arabinose), hexoses (glucose, mannose and galactose) and uronic acids (4-*o*methylglucuronic, D-glucuronic, and D-galactouronic acids) linked by -1, 4-glycosidic bonds and sometimes by -1, 3-glycosidic bonds (Kuhad *et al.*, 1997).

Hemicelluloses have a lower degree of polymerization (DP 100 – 200) and a lower crystallinity than cellulose (Rowell, 2005). While cellulose is synthesized by cellulose synthases located within the cytoplasmic membrane, hemicelluloses are synthesized in the Golgi complex, and then secreted to the plant cell wall (Keegstra, 2010). The heterogenous nature of hemicellulose compared to cellulose, implies that a complex mixture of enzymes is required for its degradation, such as endoxylanases, β-xylosidases, endomannanases, β-mannosidases, α-L-arabinofuranosidases and α-galactosidases (Jørgensen *et al.*, 2005).

The hydrolysis of hemicellulose portions of the lignocellulosic biomass produces not only hexose sugars but pentose sugars as well as potential microbial inhibitors such as uronic, ferulic and acetic acids which exerts undue stress on the fermenting microorganisms, leading to poor cell growth and low ethanol yields. Although progress has been made to ameliorate the effects of these inhibitors and increase the sugar level and enhance overall fermentability of lignocellulosic hydrolysate, it still remains a challenge in exploring lignocellulosic biomass as fermentation substrates (Blaschek & Ezeji, 2010; Parawira & Tekere, 2011).

2.2.3 Lignin

Lignin is a complex macromolecule that is structurally composed of three phenyl propane units [*p*-coumaryl alcohol (*p*-hydroxyphenyl propanol), coniferyl alcohol (guaiacyl propanol) and sinapyl alcohol (syringyl alcohol)] linked together by carbon-to-carbon (C–C) and ether (C–O–C) linkages (Humphreys & Chapple, 2002; Mussatto & Teixeira, 2010). Its structure is believed to be the result of radical polymerization (Blanchette *et al.*, 1997; Ralph *et al.*, 2004).

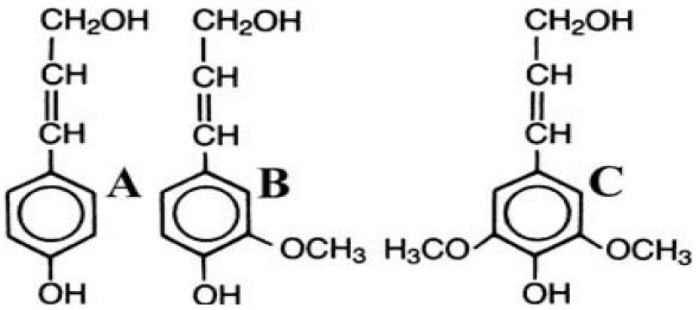


Figure 2.4a: Monomers of lignin; (A) p-coumaryl alcohol, (B) coniferyl alcohol, (C) sinapyl alcohol (Schmidt, 2006)

The structure of lignin varies widely within species. There is a general consensus that plants such as grasses have the lowest contents of lignin, whereas soft woods have the highest lignin contents. In grasses *p*-coumaryl alcohol is found predominantly causing the formation of H-lignin, while in soft wood coniferyl alcohol is the main monolignol forming G-lignin, and in hardwood G/S-lignin contains both sinapyl and coniferylmonolignols (Perez *et al.*, 2002; Humphreys & Chapple, 2002).

Lignin is present in the cell wall and is tightly bound to cellulose and hemicellulose; it confers rigidity and structural cohesion to the cell wall, it also acts as a reinforcing component to connect cells and harden xylem vessels, thus, conferring water impermeability but also improves transportation of water from roots to leaves. It forms an amorphous complex with

hemicellulose enclosing cellulose and thus prevents the microbial degradation of accessible carbohydrates within wood cell wall (Perez *et al.*, 2002; Barcelo *et al.*, 2004; Schmidt, 2006; Ruiz-Dueñas & Martínez, 2009).

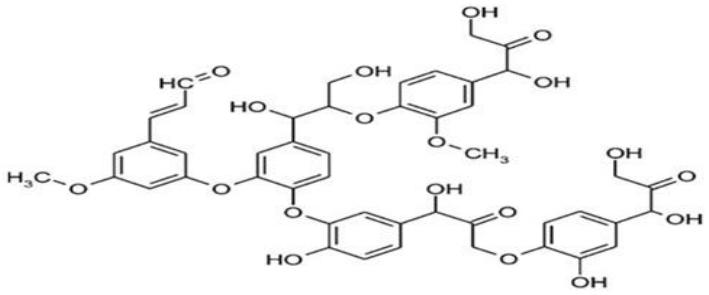


Figure 2.4b: Structure of lignin (Chhabra, 2014)

In contrast to cellulose and hemicellulose; the three-dimensional configuration of lignins makes it highly hydrophobic causing extreme resistance to chemical and enzymatic degradation (Palmqvist & Hahn-Hägerdal, 2000).The degradation of cellulose and hemicellulose portions of lignocellulosic materials produces a significant percentage of fermentable sugars for ethanol production, but the contrary is the case with lignin degradation which is recognized as a potential source of microbial inhibitors (Ezeji *et al.*, 2007); but it can be used as an ash free solid fuel for production of heat and electricity (Galbe & Zacchi, 2002).

Table 2.1: Main constituents of some lignocellulosic wastes of agriculture (Nigam *et al.*, 2009)

Lignocellulosic waste	Cellulose (wt %)	Hemicellulose (wt %)	Lignin (wt %)
Barley straw	33.8	21.9	13.8
Corn cobs	33.7	31.9	6.1
Corn stalks	35.0	16.8	7.0
Cotton stalks	58.5	14.4	21.5
Oat straw	39.4	27.1	17.5
Rice straw	36.2	19.0	9.9
Rye straw	37.6	30.5	19.0
Soya stalks	34.5	24.8	19.8
Sugarcane bagasse	40.0	27.0	10.0
Sunflower stalks	42.1	29.7	13.4
Wheat straw	32.9	24.0	8.9

2.3 Lignocellulosic Agricultural residues used in this study

The understanding that lignocellulosic agricultural waste potentially contain significant concentrations of soluble carbohydrates and inducers that promote the growth of fungi and the efficient production of ligninolytic enzymes has made these waste residues an attractive option as platform ingredients in fermentation technology (Sun *et al.*, 2004; Rosales *et al.*, 2005; Kachlishvili *et al.*, 2006; Winquist *et al.*, 2008; Elisashvili *et al.*, 2009). The implication of exploiting agricultural residues could potentially reduce production cost and serve as an effective method of agro-industrial waste recycle. Furthermore, most ligninolytic fungi have been observed to demonstrate increased enzyme activity when growing on these agricultural residues as compared to the low enzyme activity on defined medium (Bollag & Leonowicz, 1984; Elisashvili *et al.*, 2006; Songulashvili *et al.*, 2007; Magan *et al.*, 2010).

Three of these agricultural residues were the focus of this study because of their preponderance in South Africa. Furthermore it is necessary to consider more than one substrate to accommodate the regional differences in substrate availability. This is necessary because each region cultivates a particular crop based on its climatic conditions and soil differences. The elucidation of physical and chemical properties of commonly found lignocellulose wastes in South Africa is essential for the future design of subsequent processing operations schemes.

2.3.1 Corn Cob

The largest agricultural biomass in South Africa comes from corn production, with the total area planted per year varying between 3.8 and 4.8 million hectares which represents approximately 25% of the country's total arable land (National Department of Agriculture, n.d). The direct implication of corn production is the generation of waste biomass (corn plant residue), which in South Africa alone, accounts for 8, 900 thousand metric tonnes of waste annually (Nation Master, 2005). These waste products are potential source of feedstock for bio-fuels.

Approximately 50% of the weight of the total corn plant is residue with the above ground corn plant, stover, consisting of stalk, leaf, cob and husk that are potentially useful as biomass feedstock (Graham *et al.*, 2007). Approximately 66% of fermentable sugars can be obtained from corn stover, of which approximately 38% and 28% come from cellulose and hemicellulose portions, respectively. Producing ethanol from corn stover hydrolysates will increase the possibility of ethanol production on a larger scale (Blaschek & Ezeji, 2010). Table

2.2 indicates the dry matter (D.M) distribution in corn residue at or immediately following corn harvest.

The removal of corn stover after harvest should be done with consideration as this crop residue contributes to organic matter and nutrient depletion (Follet, 2001; Wilhelm *et al.*, 2004). Corn stover provide top cover on agricultural land and acts as buffers to falling rain and wind force that dislodge soil particles subsequently causing erosion (Wilhelm *et al.*, 2004). As such the removal of crop residue should be balance against the overall environmental impact (soil erosion); maintenance of nutrient and soil organic matter levels and preservation of productivity levels (Wilhelm *et al.*, 2004). One strategy to quell concerns regarding corn stover removal will be to rather implement partial stover collection (Zych, 2008). Such partial stover collection will involve the collection of only corn cobs leaving other corn residues. Moreover, the low nutrient content of corn cobs implies that a much lower cost of macro-nutrient replacement is incurred when it is removed compared to grain and stover. This reduced cost of nutrient replacement for cob collection promotes greater profit potential in feedstock collection (Zych, 2008).

Table 2.2: Dry matter (DM) distribution in corn residue (Myers & Underwood, 1992)

Corn Residue	% Moisture	% of Residue DM Basis
Stalk	70-75	50
Leaf	20-25	20
Cob	50-55	20
Husk	45-50	10

2.3.2 Sugar cane bagasse

Sugar cane is used worldwide as a feedstock for sugar and ethanol production. The sugarcane plant consists of stem and straw. The sugarcane straw has three components – fresh leaves, dry leaves, and tops. The sugarcane stems are extracted for cane juice leaving fibrous residues of cane stalks after crushing and extraction. These residues are referred to as sugarcane bagasse.

Sugarcane bagasse is about 25% of the total weight of the sugar cane plant and contains 60% to 80% of carbohydrates (Betancur & Pereira, 2010). It is an abundant and cheap source of lignocellulosic biomass with high cellulose content. The sugar cane bagasse contains between

34 – 45 % cellulose, 24 – 25 % hemicellulose, and 20 – 25 % lignin (Fox *et al.*, 1987; Kim & Day, 2011).

Countries such as Brazil and the United States produce significant quantities of ethanol fuel from sugar cane juice. This transformation of sugarcane into ethanol generates two main by-products, thermal energy and bagasse (Smeets *et al.*, 2008). Approximately 1 ton of sugarcane stem generates 280 kg of bagasse after juice extraction (Sun *et al.*, 2004). The Brazilian government establishment Conab (2012) estimates that more than 602 million tons of sugarcane will be harvested in the 2012/13 season, from which about 39 million tons of sugar and 24 million litres of ethanol will be produced. However this leaves an estimated 169 million tons of sugarcane bagasse and 84 million tons of straw. South Africa produces an estimated average 2.2 thousand million tons of sugar per season (SASA, 2012) and with this also comes the concomitant residue waste as well. The importance of increasing biofuel yield cannot be over emphasized particularly with the increasing demand of this form of energy. Therefore the utilization of sugarcane bagasse for the production of second-generation biofuels is an attractive option.The fermentation of carbohydrates obtained from the bagasse could significantly improve bioethanol productivity and sustainability (Zhang & Lynd, 2004; Himmel *et al.*, 2007; Pauly & Keegstra, 2008; Betancur *et al.*, 2010).

One major advantage to using bagasse in ethanol production is that unlike corn stover, bagasse collection is incorporated into the sugar production process, therefore a separate harvest process is unnecessary. In addition it is also physically ground as part of the extraction process (Fox *et al.*, 1987); thereby reducing process costs which makes a significant difference in the economic viability of its use. The processing of sugarcane bagasse for ethanol production can be annexed to the sugar production process (Canilha *et al.*, 2012).

Although, traditionally bagasse is often used to supply fuels for the sugar mills for boilers and as such it effectively balances the economics of the sugar production (Pandey *et al.*, 2000). Nevertheless, in countries with high sugar cane yields and with the improvement of better energy-saving and efficient boilers in the industries, there tends to be surplus sugar bagasse. In Brazil, these surplus sugarcane bagasse and straw are routinely burnt in the open agricultural field and less frequently utilized for ethanol production (Ensinas *et al.*, 2009; Canilha *et al.*, 2012). Additionally, quantities required for the production of enzymes and other products (e.g. drugs) that utilize bagasse as solid substrate/support require relatively small fractions of total

bagasse which will not necessarily affect its use in energy generation in the sugar mills (Pandey *et al.*, 2000).

Hydrolytic biodegradation of bagasse is a pre-requisite for their conversion into fermentable sugars, thereby ensuring this renewable feedstock to be used for biofuel production (Mosier *et al.*, 2005a; Himmel *et al.*, 2007). Successful saccharification of bagasse has been achieved using a variety of micro-organisms including bacteria, yeasts and fungi. However filamentous fungi, especially basidiomycetes are considered the preferred choice for enzyme production and protein enrichment and they have been most widely employed (Pandey *et al.*, 2000).

2.3.3 Wheat straw

Wheat is regarded as the second most important grain crop in the world. The world annual wheat production has increased by 85%, within 2004 to 2009 from 585 to 682 million tons, with average postharvest production of straw recorded as 1kg per 1.3kg of grain threshed. South Africa produces at least 3 million tons per hectare annually although it is dependent on whether it is farmed on dry or irrigated land, the latter tends to increase production quantities (DAFF, 2010). The by-product of threshing wheat is a dry stalk left over after the grain and chaff have been collected, referred to as wheat straw (Gubitz *et al.*, 1998; Singh *et al.*, 2009).

The overall estimate of wheat straw produced was 524 million tons in 2009; an amount that justifies its consideration as a complimentary source of raw material for the production of bioethanol (Petersen, 1987; FAOSTAT, 2009). The low cost and wide availability of wheat straw are reasons it is considered to have the greatest potential as a source of platform sugars for fermentation (Kim& Dale, 2004; Sarkar *et al.*, 2012).

Wheat straw is the residual waste generated from the harvesting of wheat and is one of the most abundant agricultural by-products of low commercial value as its major use is for cattle feed and bedding; even at that a significantly large proportion of wheat straw is left on the fields or disposed of as waste (Sarkar *et al.*, 2012). Wheat straw is rich in carbohydrates and contains about 35-40% cellulose, 20-30% hemicellulose, and 8-15% lignin (Saha *et al.*, 2005). Wheat straw is also rich in bioactive compounds and vitamins (Slavin, 2003). However, there are variations in macro and micronutrients composition resulting from various factors including stages in plant growth, the characteristic of soil and fertilizer, climatic conditions and type of cultivar (Safdar *et al.*, 2009; Yasin *et al.*, 2010). Wheat straw is an efficient substrate because

of its better air circulation, its structure allows for sturdy binding ability and effective fungal mycelial penetration, therefore making it a cost effective substrate in fermentation industry (Khan & Mubeen, 2012).

2.4 Pre-treatment of Lignocellulosic Biomass

The structural compositions of lignocellulosic biomass increase the recalcitrant nature of lignocellulosic biomass and deter the hydrolysis of the cellulose component. The overall objective of hydrolysis is to cleave the polymers of cellulose and hemicellulose to fermentable monomeric sugars (Taherzadeh & Karimi, 2007; Himmel *et al*., 2007; Kumar *et al.*, 2009). Pre-treatments seek to expose the cellulose fibres and make them accessible to enzymes (Sun & Cheng, 2002) thereby increasing the yields of sugar for subsequent fermentation to ethanol (Mosier *et al*., 2005a). This is achieved by disrupting the lignocellulosic matrix causing a reduction in the amount of lignin and hemicellulose and modifying the crystalline structure of cellulose making it more susceptible to enzymatic attack (Silverstein *et al.*, 2007). The absence of pre-treatments leads to a low yield of glucose from lignocellulosic material conversion; therefore it is vital for this step to occur before enzymatic hydrolysis. Furthermore, the ability to utilize all the sugars including hexose and pentose sugars present in the lignocellulosic feedstock is necessary for the efficient production of ethanol (Luo *et al*., 2010).

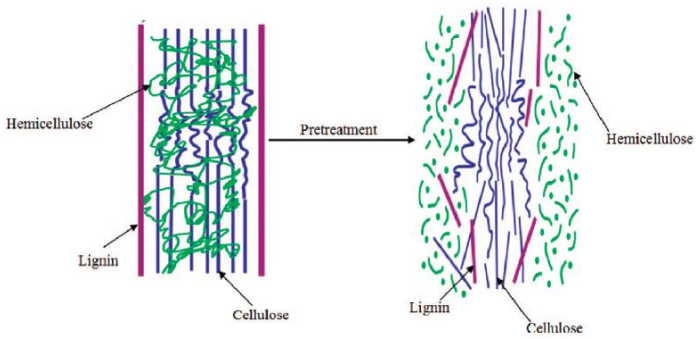


Figure 2.5: A schematic representation of the role of pre-treatment in the conversion of lignocellulosic biomass to platform sugars (Hsu *et al*., 1980)

Selecting an effective pre-treatment is crucial because it determines the performance of microorganism during the subsequent step of fermentation; if high levels of inhibiting by-products are present, it would mean prerequisite steps must be taken to reduce or eliminate such inhibitors and this in turn increases the overall processing cost. Another reason the type of pre-treatment selected may also affect fermentation yield is that – some pre-treatment

methods may lead to the loss of valuable fractions as a result of partial hydrolysis of these fractions in the biomass (Mussatto & Teixeira, 2010).

Alvira *et al.* (2010) summarizes that an effective pre-treatment strategy should meet the following requirements:

i. *overcome lignocellulosic biomass recalcitrance, deconstructing the three-dimensional structure of lignocellulose, and breaking down the semi-crystalline cellulose and hemicellulose;*
ii. *afford high yields to sugars or chemicals and/or give highly digestible pre-treated solid;*
iii. *avoid carbohydrates degradation and in particular preserve the utility of pentosan(hemicellulose) fraction;*
iv. *avoid the formation of inhibitory toxic by-products;*
v. *allow lignin recovery and exploitation to give valuable co–products;*
vi. *be cost–effective, involving reasonable size reactors, low wastes amount and low energetic requirements.*

Ligninocellulosic materials pre-treatment techniques for bioethanol production are broadly classified into four distinct categories: physical, physicochemical, chemical and biological pre-treatments.

2.4.1 Physical Pre-treatment methods

Physical pre-treatment methods primarily function to increase the accessible surface area and decrease the crystallinity and polymerization degree of cellulose. Several types of physical processes including milling, grinding, extrusion, and irradiation have been developed.

2.4.1.1 Mechanical Comminution

Mechanical comminution achieves size-reduction through a combination of chipping, grinding, and/or milling with the overall objective of reducing cellulose crystallinity and in this way increase reaction surface area, thus reducing reaction time. The process makes cellulose readily accessible to cellulases. Furthermore, it is presumed that disrupting the lignin-carbohydrate complexes will aid enzymatic hydrolysis (Mais *et al.*, 2002). The size of the materials is usually 10 – 30mm after chipping and 0.2 – 2mm after milling or grinding (Sun & Cheng, 2002). Although this form of pre-treatment is usually necessary, its primary limiting factor is the high energy consumption required (Miao *et al*., 2011); the smaller the particle size the more

the energy requirements making certain particle size, economically unfeasible (Hendriks & Zeeman, 2009). In addition further reduction of biomass particle size does not necessarily have significant effect on the rates and yields upon hydrolysis (Chang *et al.*, 1997; Draude, Kurniawan & Duff, 2001). For optimized economic consideration it is necessary to balance comminution against microbial and chemical reactions surface area requirements.

2.4.1.2 Pyrolysis and Torrefaction

Pyrolysis and torrefaction are forms of thermal treatment used on lignocellulosic biomass. When cellulose is treated at temperatures greater than 300^{o}C, it decomposes rapidly to gaseous products and char; although decomposition is much slower at lower temperatures and products formed are less volatile (Kilzer & Broido, 1965; Shafizadeh & Bradbury, 1979). The pyrolysis of cellulose produces levoglucosan-containing tar which when condensed the products can be readily hydrolyzed to glucose with sometime yields as high as 85% depending on the substrate. Although the use of acid-treated cellulose samples is preferred for best yields; with wood inorganic acids are best (Shafizadeh, 1983).

Recently it is re-emerging as a promising method to convert biomass into a number of products (such as syn-gas, liquid fuel and charcoal) (Nhuchhen *et al*., 2014). Lignocellulosic wastes such aswood and forest residues, bagasse as well as straw and agricultural residues have been treated with pyrolysis. Some authors have shown through several studies that certain parameters affect the quality of the final product including feedstock composition, type of reactor, final temperature, heating rate, sweeping gas flow rate, vapours residence time and particle sizes (Rengel, 2007; Shen *et al.*, 2011).

Torrefaction involves heating to moderate temperatures within the range of 200 and 300^{o}C, working under inert or nitrogen atmosphere (Prins *et al.*, 2006a; 2006b; Englisch, 2010) which would lead to an initial reduction and subsequent elimination of moisture content with simultaneous depolymerisation of the long polysaccharides. This renders the once hygroscopic raw biomass, hydrophobic and reduces inhibition in successive enzymatic hydrolysis. Pre-treatment by torrefaction is considered more attractive than pyrolysis (Kumar *et al.*, 2009), especially using light and mild temperatures between 220 and 250^{o}C.

2.4.1.3 Microwave irradiation

The use of microwave is considered an alternative pre-treatment to conventional heating. It incorporates direct interaction between the material that needs heating and applied electromagnetic field to generate heat unlike the conventional heating that uses superficial heat transfer.In this way the microwave generates higher heating efficiency and therefore process operations are made easier (Binod *et al.*, 2012). Furthermore, the electromagnetic field used in the microwave is believed to cause non-thermal effects that also accelerate destruction of crystalline structure (De la Hoz *et al*., 2005).

Its advantages include short reaction times consequently it minimizes operation time and energy and less inhibitory compounds are likely to be generated. Ooshima *et al.* (1984) reported a significantly higher lignin and hemicellulose removal from rice straw in shorter pre-treatment time when a combination of microwave and alkali pre-treatment as compared to using alkali-only pre-treatment. Similarly Diaz *et al.* (2015) reported more significant modification when microwave irradiation was applied to enhance enzyme hydrolysis of corn straw and rice immersed in medium such as water, aqueous glycerol or alkaline glycerol.

Physical pre-treatments most often are inadequate when used alone and usually are used in combination with chemical pre-treatments to improve the process efficiency (Keshwani, 2009). Microwave can be combined with chemicals such as dilute acids to further improve sugar yield from lignocellulosic substrates (Balcu *et al.*, 2011). Keshwani and Cheng (2010) investigated microwave-based alkali pre-treatment; this amalgamated technique involved immersing the biomass in dilute alkali reagents and exposing the slurry to microwave radiation at 250 watt for residence times ranging from 5 minutes to 20 minutes. Sodium hydroxide showed the most effect in improving the production of fermentable sugars for the microwave pre-treatment of switch grass and coastal Bermuda grass.

2.4.2 Physico-chemical Pre-treatment methods

2.4.2.1 Steam explosion

Steam explosion is a widely accepted method of pre-treatment for lignocellulosic materials (Jacquet *et al.*, 2015). Initially the biomass is treated with high-pressure saturated steam (0.69 – 4.83 MPa, 160 – 260^{o}C) for a short period of time (several seconds to no longer than 10 minutes) which wets the material; this is followed by a sudden reduction in pressure that causes the material to experience an explosive decompression (Kumar *et al.*, 2009). Even though this

method can be used alone, incorporating acetic acid during de-pressurization promotes the hemicellulose hydrolysis and solubilisation and decreases the production of compounds (Clark & Mackie, 1987; Sun & Cheng, 2002). Steam pre-treatment can also incorporate sulphur dioxide (SO_2) with the aim of improving the recovery of both hemicellulose and cellulose fractions. Eklund *et al.* (1995) obtained a 95% maximum glucose yield when SO_2 and H_2SO_4 were incorporated into steam pre-treatment of willow; the process employed 1% SO_2 at 200^oC. However, the yield of xylose recovery by SO_2 was not as high as pre-treatment with dilute sulphuric acid. More recently Tutt *et al.* (2014) employed a combination of steam explosion at 200^oC in combination with enzymatic hydrolysis to pretreat floodplain meadow hay with ethanol yield of 115g kg^{-1}. Observation of the plant cell wall using scanning electron microscope showed a disintegration that exposed cellulose fibres.

The major disadvantages to the use of steam explosion is that the lignin-carbohydrate matrix is only partially disrupted and in addition the reaction generates compounds such as furan derivatives – furaldehyde and 5-hyroxymethyl-2-furaldehyde and phenolic compounds that may be inhibitory to microorganisms (Sun & Cheng, 2002). However, Martín and coworkers (2008) observed that in comparison to pretreatment by oxidation, steam explosion generated less inhibitory by products. It becomes necessary to wash pre-treated biomass with water which reduces the saccharification as soluble sugars derived from hemicellulose hydrolysis are lost in this way (Ballesteros *et al*., 2011).

2.4.2.2 Liquid Hot Water Pre-treatment

Liquid hot water pre-treatment is similar to steam explosion, although the explosion does not occur but rather it involves immersing the biomass in liquid hot water. The method uses compressed liquid hot water (180 – 230^oC; pressure > saturation point; solids concentration < 20wt %) which results in the formation of acids from the hydrolysis of acetyl and uronic groups, originally present in the hemicellulose that subsequently catalyzes hydrolysis of links between hemicellulose and lignin as well as between the carbohydrates resulting in release of glucose and xylose monomers in a phenomenon referred to as autohydrolysis (Weil *et al*., 1997; Mosier *et al*., 2005b). Residence time is usually 10 – 15 minutes. The process is able to hydrolyze hemicellulose, in minutes with high yields, low by-products formation and no significant lignin solubilisation (Carvalheiro, Duarte & Girio, 2008). Some techniques utilize small quantities of H_2SO_4, CO_2 or SO_2 as catalyst to enhance recovery of hemicellulose sugars and improve the enzymatic hydrolysis on the solid residue as well as decrease the production of inhibitors (Sun

& Cheng, 2002; Mosier *et al*., 2005b). Its major advantage is the absence or limited use of chemicals consequently there is no requirement for corrosion-resistant equipment; it does not require an initial step of comminution, thus reducing energy costs (Taherzadeh & Karimi, 2008). Hendriks *et al.* (2009) reported the dissolution of 50% total biomass, an almost complete removal of hemicellulose, increase cellulose digestibility (5 – 20% removal of cellulose) and the removal of 30 – 60% removal of lignin. The primary reason for the lower concentrations of inhibitory derivatives is due to the higher volumes of water input. It is regarded as a simple and environmentally friendly approach (Raspolli-Galletti & Antonetti, 2009). Its drawback stems from the high volumes of water needed; this increases the energy demand (Agbhor *et al.*, 2011).

2.4.2.3 Ammonia fibre explosion

Ammonia fibre explosion (AFEX) is similar to steam explosion; lignocellulosic biomass are exposed to liquid ammonia at high temperature and pressure, subsequently the pressure is rapidly reduced. Process parameters include a dosage of 1 – 2 kg of ammonia/kg of dry biomass, temperature at 90^{o}C and the residence time is between 10 – 30minutes (Alizadeh *et al*., 2005; Bals *et al*., 2010). It is remarkable that the hemicellulose and lignin portion remain intact during this treatment, it has been suggested that deacetylation and degradation of hemicellulose is the probable reason for the hemicellulose remaining insoluble (Gollapalli, Dale & Rivers, 2002). However, this form of treatment changes the material structure that results in increased water retention capacity and higher digestibility (Galbe & Zacchi, 2007). AFEX pre-treatment simultaneously reduces lignin content and removes some hemicellulose while decrystallizing cellulose. AFEX technology has been used for the pre-treatment of alfalfa, wheat straw, wheat (Mes-Hartree, Dale & Craig, 1988; Bensah & Mensah, 2013) barley straw, corn stover, rice straw (Vlasenko *et al*., 1997; Harun *et al*., 2013), municipal solid waste, softwood newspaper, kenaf newspaper (Holtzapple, Lundeen, & Sturgis, 1992a), coastal Bermuda grass, switchgrass (Reshamwala, Shawky & Dale, 1995), aspen chips (Tengerdy & Nagy, 1988), and sugarcane bagasse (Holtzapple *et al*., 1991). AFEX reduces the use of water in the down stream biological processes because it does not produce inhibitors therefore a water wash is unnecessary (Mes-Hartree *et al.*, 1988).

However, AFEX pretreatment must factor in the cost of ammonia and ammonia recovery, the latter being absolutely necessary due to environmental reasons.This additional costs make the process economically unfeasible for large scale production (Eggeman & Elander, 2005; Mosier

et al., 2005a). Teymouro *et al*. (2004) reported that the quantity of ammonia vapourized during AFEX would usually depend on temperature. When the temperature is elevated, a great quantity of ammonia vapour would flash leading to a significant loss of ammonia and therefore increasing production cost as well as a greater disruption of the biomass fibre structure. Temperature was also reported to affect the system pressure, therefore making it a crucial component of this process.

2.4.2.4 Carbon dioxide explosion

Carbon dioxide explosion was developed to offset the limitation due to high temperatures and the high costs associated with Steam explosion and AFEX respectively. The mechanism is facilitated by high pressure. CO_2 explosion uses CO_2 as a supercritical fluid; such fluids, even though gaseous, possess liquid-like density under compression at temperatures above its critical point.

$$\mathbf{CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO^{3-} \Leftrightarrow 2H^+ + CO_3^{2-}}$$

The presumption with CO_2 explosion was that there will be an increased rate of hydrolysis as a result of the formation of carbonic acid when CO_2 is dissolved in water; because the molecular size of CO_2 is comparable to water and ammonia; they should be able to penetrate small pores accessible to water and ammonia molecules (Zheng *et al.*, 1995). Zheng *et al*. (1998) did a comparative analysis on CO_2 explosion with steam and ammonia explosion for the pre-treatment of recycled paper mix, sugar bagasse and re-pulping waste of recycled paper and established that CO_2 was more cost-effective than ammonia explosion and did not cause formation of inhibitory compounds that could occur in steam explosion. In addition, CO_2 explosion is considered advantageous because it is non-toxic, non-flammable, it is easily recovered after extraction and overall it is environmentally friendly (Huang *et al.*, 2008; Schacht *et al.*, 2008; Luterbacher *et al.*, 2010; Srinivasan & Ju, 2010). Its high capital costs for the high pressure equipment is a limiting factor to the commercialization of this technique (Agbhor *et al.*, 2011).

2.4.3 Chemical Pre-treatment

2.4.3.1 Acid Pre-treatment

Pre-treatments using acids may be performed using dilute or concentrated acids; however the latter is hazardous with high cost implications on the long run from the corrosion of equipment and recovery processes (Sivers & Zacchi, 1995). This form of pre-treatment favours the formation of degradation and inhibitory compounds (Alvira *et al.*, 2010). The use of dilute

acids has been extensively studied for the hydrolysis of hemicellulose (Mussatto & Teixeira, 2010; Hu & Ragauskas, 2012); wherein diluted acids (< 4wt %), under high temperatures ranging from 140 to 215^{o}C is usually adequate for hemicellulose with little decomposition of sugar (McMillian, 1994; Carvalheiro, Duarte & Girio, 2008). Although, sulphuric acid (H_2SO_4)is most commonly preferred, other acids such as hydrochloric acid (HCl), nitric acid(HNO_3) and phosphoric acid (H_3PO_4) as well as organic acids(maleic and oxalic acids) are also used. Acid hydrolysis using H_2SO_4 enhances digestibility of cellulose in the residual solids (Mosier *et al.*, 2005a), with high xylan to xylose conversion yields that is necessary to increase productivity as xylan accounts for one-third of the total carbohydrate in most lignocellulosic materials (Hinman, Schell, Riley, Bergeron & Walter, 1992). Despite the low energy costs associated with dilute acid hydrolysis (Gírio, *et al.*, 2010); it is usually necessary to neutralize hydrolysates before fermentation (Mosier *et al.*, 2005a). However, its most significant limitation is the formation of inhibitory by-products (furans, furfural, carboxylic acids, formic, levulinic and acetic acids and phenolic compounds) that affects the microbial fermentation; this makes a detoxification step necessary (Palmqvist & Hahn-Hägerdal, 2000; Jonsson & Martin, 2016).

2.4.3.2 Organosolv Pre-treatment

The organosolv process employs organic liquid (such as methanol, ethanol, phenols, acetone, ethylene glycol and tetrahydrofurfuryl alcohol) and water, sometimes with the addition of catalysts (oxalic, salicylic and acetylsalicylic acids) (Sarkanen, 1980) to reduce the operating temperature; thus resulting in the dissolution of lignin with cellulose residual solid materials (Chum *et al.*, 1988; Thring *et al.*, 1990; Sun & Cheng, 2002). The removal of lignin increases the surface area and volume of the material for further degradation of enzymes; thus improving process efficiency and levels of fermentable sugars. The addition of catalysts is usually not necessary at temperatures higher than 185^{o}C (Sarkanen, 1980; Aziz & Sarkanen, 1989; Taherzadeh & Karimi, 2008).

Less quantities of chemicals are required to neutralize hydrolysates from the organosolv process with equally less amount of waste generated (Taherzadeh & Karimi, 2008). The drawback in this process is the need to drain from the reactor used solvents as well as the evaporation, condensation and recycling steps that must be done to reduce costs. In addition these steps are necessary as the solvents might be inhibitory to the growth of microorganisms in the downstream operation (Kumar *et al.*, 2009). Sidiras and Salapa (2015) evaluated five

solvents (ethanol, methanol, diethylene glycol, acetone and butanol) using the organosolv pretreatment process for wheat straw, catalysis was achieved using H_2SO_4 at 160^oC for 20 minutes, the highest delignification was 59% w/w using acetone with a maximum cellulose concentration yield of 72% w/w and 95% w/w of xylan hydrolysed to xylose.

2.4.3.3 Alkaline Pre-treatment

Alkaline pre-treatment employs alkaline solutions such as sodium hydroxide (NaOH), calcium hydroxide or slake lime ($Ca(OH)_2$) or ammonia for the treatment of lignocellulosic biomass. NaOH is considered a preferred choice and the most studied for the delignification of agricultural residues (Kim *et al*., 2016) with associated digestion of significant amounts of hemicellulose. Dilute NaOH causes swelling consequently increasing internal surface area and a simulataneous reduction in both the degree of polymerization and crystallinity. In addition it leads to a separation of structural linkages between lignin and carbohydrates as well as disruption of the lignin structure (Fan *et al.*, 1987). The end-product is dissolved in the form of a liquor rich in phenolic compounds that represents the process effluent (Fengel & Wegener, 1989; Mussatto *et al.*, 2007). Advantages of alkaline pre-treatment overother pre-treatment techniques include utilization of lower temperatures and pressures (Mosier *et al.*, 2005a), although residence time are relatively longer; in comparison with acid processes, there is less sugar degradation and most of the caustic salts can be regenerated and/or recovered. In a study done by Iroba *et al.* (2013) where optimization involved temperature variations (70, 80 and 90^oC) against different concentration ratios of NaOH solution, it was observed that the ratio 1:6 concentration at four different temperatures proved to be optimal at all of these temperatures.

The degradation of portions of the hemicellulose is considered a major drawback (Mussatto & Teixeira, 2010) and the high cost implications associated with recycling the high concentrations of expensive salts that are employed in the process, waste water treatment and residual handling tends to make this technique commercially non-viable on the long-run (Hamelinck *et al*., 2005; Zheng *et al.*, 2009). Although, the option of using calcium hydroxide (slake lime) is reported to be an effective pre-treatment agent and is the least expensive per kilogram of hydroxide; its recovery can also be achieved from an aqueous reaction system as insoluble calcium carbonate by neutralizing it with inexpensive carbon dioxide upon which it is regenerated using established lime kiln technology (Kumar *et al.*, 2009).

Ammonia is a very promising pre-treatment reagent, in particular for biomasses with low lignin contents such as agricultural residues and herbaceous feedstock. It is suitable for simultaneous saccharification and co-fermentation because the treated biomass retains the cellulose and hemicellulose portions (Kim & Lee, 2005a). Yoon *et al.* (1995) describe ammonia recycle percolation (ARP) process where ammonia is applied to biomass at temperatures between 160 and 180^{o}C. Kim and Lee (2005a; 2005b) in two separate studies using corn stover, reported approximately 50% of the xylan and >75% of the lignin was removed, which enhanced digestibility of the remaining solids.

2.4.3.4 Oxidizing Pre-treatment – Hydrogen peroxide

The decomposition of lignocellulosic materials can be hastened with H_2O_2 at temperatures higher than 100^{o}C. The reaction is similar to alkaline pre-treatment because the lignin solubilisation by H_2O_2 causes the partial removal of hemicellulose from the biomass (Nigam, Gupta & Anthwal, 2009).Peroxidase enzyme under alkaline conditions is able to catalyse lignin biodegradation in the presence of H_2O_2 (Azzam, 1989; Sun & Cheng, 2002) whereby highly reactive radicals such as hydroxyl radicals (HO) and superoxide anions (O_2^-) are produced and are responsible for the degradation of lignin. Oxidative delignification (as it is often known), not only de-lignifies the biomass but causes the chemical swelling of cellulose thereby improving enzymatic saccharification (Yamashita *et al*., 2010). This technique is used in the paper and pulp industries to bleach and de-lignify the biomass, thus, improve the brightness of pulp as it reacts with coloured carbonyl-containing structures in the lignin (Nigam *et al.*, 2009). It has been applied to a large variety of biomass including corn stover, barley straw, wheat straw, bamboo, rice straw and sugar cane bagasse (Banerjee *et al*., 2011). Azzam (1989) achieved about 50% of the lignin and most of the hemicellulose was solubilized with 95% efficiency of glucose production from cellulose when sugarcane bagasse was pre-treated with 2% H_2O_2 at 30^{o}C within 8 hours. A continuous flow operation that incorporates high biomass loading (≈ 40% solids) and low H_2O_2 loading has been used successfully to achieve oxidative delignification; though it is still a relatively less explored method compared to other thermochemical pre-treatments (Banerjee *et al.*, 2011). This pre-treatment is promising as it takes little or no toll on the environment, however, the operational cost for large scale production can be limiting (Nigam *et al.*, 2009).

2.4.3.5 Wet Oxidation

Wet oxidation was offered as an alternative to steam explosion in the early 1980s (McGinnis, 1983). It is a process of treating lignocellulosic biomass with water and air or oxygen at temperature ranges of 140 – 200^{o}C under pressure for about 30 minutes. Varga *et al*. (2003) used wet oxidation in the pre-treatment of corn stover with the following parameters: 195^{o}C, 2gl^{-1} Na_2CO_3, 12 bar O_2, 15 minutes. Similar parameters have been found optimal for wet oxidation of wheat straw (Klinke *et al*., 2003). Oxygen participates in the degradation reaction, enhancing the generation of organic acids and allowing operations at comparatively low temperatures (Taherzadeh & Karimi, 2007). Martín and Thomsen (2007) identified that the fractionation of the lignocellulosic biomass, formation of sugars and by-products and cellulose enzymatic convertibility is greatly affected by oxygen pressure and the type of raw material used. Bjerre *et al.* (1996) combined wet oxidation and alkaline hydrolysis for the pre-treatment of wheat straw and achieved 85% conversion yield of glucose with no formation of inhibitory derivatives. Petersson *et al.* (2007) produced ethanol using *Saccharomyces cerevisiae* from winter rye, oilseed rape, faba bean straw after wet oxidation pre-treatment with theoretical yields of 66%, 70% and 52% respectively. Wet oxidation is amongst the simplest process in terms of equipment, energy and chemicals required for operations (Chum *et al*., 1985). However it is only suitable for substrates with low lignin content as yield decrease with increased lignin content; also the lignin produced from wet oxidation cannot be used for fuel consequently reducing income that can be generated from by-products in large-scale ethanol production from lignocellulose (Galbe & Zacchi, 2002).

2.4.3.6 Ozonolysis

Ozone, a known powerful oxidant that is soluble in water, can be used to effectively degrade lignin and part of hemicellulose. It is highly reactive toward conjugated double bonds and functional groups associated with high electron density; the large quantities of C=C bonds guarantees an immediate reaction with lignin moieties. Ozone attacks lignin with an associated reduction in solution pH from 6.5 to 2 thereby releasing soluble compounds of low molecular weight, such as organic acids, formic and acetic acids (Garcìa-Cubero, 2009). In the reaction because lignin functions as a scavenger, it consumes most of the ozone but the residual ozone may also attack the cellulose and hemicellulose components attached to the lignin. It is presumed that cellulose degradation may be as a result of a direct reaction of ozone with the glycosidic linkage and partly a free radical mediated oxidation of hydroxyl groups in glucose (Nigam *et al*., 2009; Johansson, Lind & Ljunggren, 2000). Ozonolysis can be carried out at

room temperature and normal pressure, with the added benefit that it does not produce any toxic residues for the enzymatic processes (Vidal & Molinier, 1988). Ozone is easily decomposed by using a catalytic bed or increasing with slight modification in process design consequently minimizing environmental pollution (Kumar *et al.*, 2009). A major limitation to the use of this pre-treatment is the large quantities of ozone required that makes the process expensive (Kumar *et al.*, 2009; Mussatto & Teixeira, 2010).

2.4.4 Biological Pre-treatment

The cost implication of physical and chemical pre-treatments necessitates seeking alternatives such as biological pre-treatments. There are present in nature organisms including bacteria, brown-, soft- and white-rot fungi (Galbe & Zacchi, 2007; Zheng *et al.*, 2009; Hatakka & Hammel, 2010). These organisms are able to degrade the lignin, and hemicellulose, thus making the cellulose readily available for enzymatic hydrolysis (Sarkar *et al.*, 2012). As a result of the low energy requirement and mild environmental conditions associated with biological treatment with its concomitant reduction in sugar loss (Sun & Cheng, 2002; Hamelinck *et al.*, 2005; Tian *et al.*, 2012), its potential application cannot be ignored. Although there are challenges that need to be overcome such as the exceedingly long residence time and large space requirements which make its use in the industry less attractive. Moreover the inevitable consumption of carbohydrate fractions (Agbhor *et al.*, 2011) by microorganisms means that the overall yield of product is affected. Never-the-less, these limitations can be overcome with the use of biological pre-treatment in combination with other treatments (Hamelinck *et al.*, 2005) or on its own if the biomass has low lignin content (Magnusson *et al.*, 2008). Itoh *et al.* (2003) in their analysis of the impact of biological pre-treatment using white-rot fungi in a combination pre-treatment of beech stated that biological pre-treatment saved 15% of the electricity needed for ethanolysis of beech wood undergoing bio-organosolv pre-treatment. Wang *et al.* (2012) also combined fungal pre-treatment with liquid hot water to enhance the enzymatic hydrolysis of *Populus tomentosa.*

2.4.4.1 Bacteria

There are few bacteria species available in nature that can degrade lignocellulose; in fact the degradation of hemicellulose is more common in wood fungi than in bacteria (Schmidt, 2006). Some aerobic and anaerobic bacteria have the competency to digest cellulose and hemicellulose although these organisms are regarded as secondary lignocellulose degraders (Walker & Wilson, 1991). The simultaneous activity of cell-associated and free extracellular

cellulases and hemicellulases is required to initiate aerobic degradation as observed with *Thermobifida fusca* and *Cellulomonas composti* (Walker & Wilson, 1991; Beguin & Aubert, 1994). However, anaerobic degraders usually attach relevant hydrolase activities to the cell through a cellulosome complex. Cellulosomes are multi-enzyme complexes that bind to the bacterial cell wall and promote the uptake of solubilised sugar by the hydrolytic organisms (Bayer *et al.*, 2008). *Clostridium thermocellum* and *C. cellulolyticum* are among the best-studied anaerobic, cellulose degrading bacteria (Bayer *et al.*, 2008). Lynd *et al.* (2002) extensively evaluated these two anaerobic bacteria for their potential to promote simultaneous saccharification and fermentation (SSF) or consolidated bioprocessing (CBP). Streptomycete species have also been shown to degrade low levels of lignin (Watanabe *et al.*, 2003; Zeng *et al.*, 2013; Majumdar *et al.*, 2014).

2.4.4.2 Brown rot fungi

Brown-rot fungi are basidiomycetes that are characterized by their ability to degrade wood, producing a brown, shriveled end-product that demonstrates a pattern of cubical crack that disintegrate easily upon handling (Hatakka & Hammel, 2010). Brown rot primarily attack cellulose while soft- and white-rot fungi attack both cellulose and lignin (Sun & Cheng, 2002). This involves the rapid degradation of hemicellulose, with removal of almost all the cellulose leaving behind a complex aromatic ring-containing polymer derived from the original lignin (Hatakka & Hammel, 2010). The partial oxidation of lignin is presumably achieved through demethylation of the aromatic rings, this increase the phenolic hydroxyl content as well as the partial introduction of new carbonyl and carboxyl groups, although the lignin remains polymeric *in situ* (Kirk & Adler, 1970; Kirk, 1975). Owing to the lignin remaining intact after so-called activity of brown rot fungi it is concluded that they are inefficient degraders of lignin and the prime candidates implicated in its degradation of lignocellulosic materials are low-molecular-weight species which it employs in oxidation, these compounds possess oxygen-centred free radicals such as the hydroxyl radical (OH) (Koenigs, 1974; Eriksson *et al.*, 1990; Hammel *et al.*, 2002; Kamada *et al.*, 2002). Examples of extensively studied brown rot fungi include *Postia placenta* (also known as *Poria monticola*), *Tyromyces palustris, Gloeophyllum trabeum, Serpula lacrymans, Fomitopsis palustris, Laetiporus portentosus* and *Piptoporus betulinus* (Jensen *et al.*, 2001; Kamada *et al.*, 2002; Högberg *et al.*, 2006; Valášková & Baldrian, 2006; Yoon *et al.*, 2007; Lindner & Banik, 2008; Martinez *et al.*, 2009; Watkinson & Eastwood, 2012; Kojima *et al.*, 2016).

2.4.4.3 Soft-rot fungi

The soft-rot decay of wood is characterized by the wood becoming brown and soft where the residue is cracked when dry. This is caused by ascomycetes and mitosporic fungi (Nilsson *et al.*, 1989; Blanchette, 1995). Soft-rot fungi form invaginations within secondary walls or erosion although the middle lamella are unaffected (Hatakka & Hammel, 2010). In comparison to basidiomycetous fungi, the knowledge about lignocellulose degradation by ascomycetes is sparse and very little is known about how they degrade lignin (Nilsson *et al.*, 1989; Martinez *et al.*, 2005). *Trichoderma reesi* is one of the best studied examples of mesophilic soft-rot fungus with its ability to degrade plant biomass and cellulose; it has been employed in the production of cellulases and hemicellulases in various industrial applications. It has also long been a model system for the degradation of plant cell wall polysaccharides; however it is unable to degrade lignin (Martinez *et al.*, 2008; Hatakka & Hammel, 2010).

2.4.4.4 White-rot fungi

White-rot fungi are from the family basidiomycetes that are able to degrade lignin in wood (Hatakka, 2001; Dashtban *et al.*, 2010); there are however, some white-rot fungi found amongst the ascomycetes (Schmidt, 2006). The decayed wood is characteristically bleached pale in colour, brittle, spongy, soft and fibrous in texture (Erikkson *et al.*, 1990). White-rot fungi may be classified according to their degradation pattern as simultaneous or non-selective degradation and selective delignification (Erikkson *et al.*, 1990; Blanchette, 1995). Simultaneous degradation involves the white-rot fungi colonizing cell lumina, causing cell wall erosion; where the eroded zones amalgamate as decay progresses and large cavities filled with mycelium are formed with a further accumulation of calcium oxalate and MnO_2 (Blanchette, 1995). Examples of fungi with such non-selective degradation include *Fomes fomentarius*, *Phellinus robustus*, and *Trametes versicolor* (Blanchette, 1984; Blanchette, 1994). Conversely, some white-rot degrade lignin in wood in relatively larger proportions than cellulose, in this case they are referred to as selective white-rot fungi which are typically observed as white-mottled type of rot as seen with *Phellinus nigrolimitatus* (Blanchette, 1995; Stokland & Kauserud, 2004). This selective degradation is often seen in wood as small elongated craters within wood tissue such that decayed regions are surrounded by tissues that appear healthy (Blanchette, 1984; Stokland & Kauserud, 2004). A typical fibrous texture is observed with the progressive delignification of the middle lamella and primary cell wall (Schmidt, 2006). *Ceriporiopsis subvermispora* and *Phlebia radiata* are perhaps the best studied fungi to elicit selective white-rot decay (Hatakka *et al.*, 2002; Uusi-Rauva & Hatakka, 2006; Fackler *et al.*,

2007; Fernandez-Fueyo *et al.*, 2012). The type of wood being decayed, the stage of wood degradation and the particular strain of fungi employed – are significant factors that determine the form of degradation that ensues (Blanchette, 1992; Messner & Srebonik, 1994).

2.5 Ligninolytic Enzymes

The production of ethanol from cellulosic materials requires the removal of lignin to significantly improve digestibility, in addition lower lignin content would considerably reduce enzyme loadings thus reducing costs (Milagres *et al.*, 2011). A consequence of delignification is the swelling and disruption of the lignin structure thereby increasing the internal surface area and median pore volume (Zhu *et al.*, 2008).

White-rot fungi have unique mechanisms to degrade lignins. This is facilitated by enzymes that are collectively termed as "ligninases." Ligninases are divided into two main groups: phenol oxidases (laccase) and heme peroxidase [lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP)] (Martinez *et al.*, 2005). They secrete one or more of the lignin modifying enzymes in varying proportions and in some cases more than one isoform of the ligninases are expressed by different taxa under different culture conditions (Wesenberg *et al.*, 2003). These enzymes have lower substrate specificity (making them useful in degrading other recalcitrant pollutants) than typical biological catalysts, in addition, other compounds are secreted to aid effective lignin degradation (Solarska, 2009; Dashtban *et al.*, 2010).

Recall that the compact structure of lignin makes it difficult for most substances including proteins to penetrate it; for this reason lignin modifying enzymes are secreted extracellularly as well as low molecular weight mediators that aid the penetration of wood, which otherwise would not be possible due to the large molecular size of the enzymes (Evas *et al.*, 1994). These low molecular weight mediators are secreted during fungal metabolism and include veratryl alcohol (VA), oxalate, malate and fumarate (Cho *et al.*, 2004). In addition to these, H_2O_2-utilising enzymes such as glyoxal oxidase (a copper-radical protein) and aryl alcohol oxidase (a flavoprotein) are also excreted (Solarska, 2009).

Ligninolytic enzymes are highly oxidative in nature and act synergistically to mineralise lignin by catalysing a one-electron oxidation with the formation of radicals that undergo further spontaneous reactions (Lonergan, 1992; Kluczek-Turpeinen, 2007). The production of ligninolytic enzymes are promoted under limited nutrient availability (C: N ratio) or secondary

metabolism as lignin oxidation provides no net energy to the fungus (Kirk & Farrell, 1987; Wesenberg *et al.*, 2003; Kaštovská *et al*., 2012). The depolymerisation of lignin allows the fungi to access cellulose and hemicellulose which they can utilise as carbon and energy sources. Lignin degradation causes the formation of water-soluble compounds and where mineralisation occurs it leads to the formation of CO_2. Apart from the effects nitrogen limitation gas has on ligninolytic enzymes production, it is also the limiting nutrient for fungal growth in most wood and soils (Kirk & Farrell, 1987; Watkinson *et al.*, 2006).

2.5.1 Laccases

Laccases (EC 1.10.3.2, benezenediol:oxygen oxidoreductase) are glycosylated blue copper-containing oxidase that effects the complete catalysis of one molecule of dioxygen to two molecules of water using four-electron reduction with a concomitant electron withdrawal from the reducing phenolic, aromatic amines as well as other electron-rich substrates such as arylamines, anilines, thiols and lignin, although the latter can only proceed with the inclusion of a mediator (Thurston, 1994; Call & Mücke, 1997; d'Souza *et al.*, 1999; Madhavi & Lele, 2009).

Most monomeric laccase (Lacc) molecules contain four copper atoms in their catalytic centre that mediate redox process, the classification based on the copper's co-ordination and spectroscopic properties whichare as follows: type-1 (T1) is responsible for the intense blue colour of the enzyme; type-2 (T2) is colourless and type-3 (T3 and T3') is a pair of copper that are not easily detectible with spectroscopic signals (Palmieri *et al.*, 1998; Van Der Merwe, 2002; Dashtban *et al*., 2010). The T2 and T3 copper sites are in close proximity and form a tri-nuclear centre (Leontievsky *et al.*, 1997) that are involved in the catalytic mechanism of the enzyme (Jones & Solomon, 2015).

Laccase catalyzes the cleavage of the C_α – C_β bonds in phenolic subunits (β-1 and β-O-4 dimers) leading to C_α oxidation (Figure 2.6); this is achieved by splitting the aryl-alkyl bonds (Picart *et al*., 2015). This oxidation results in an oxygen-centred free radical that can undergo numerous spontaneous reactions consequently resulting in various bond cleavages of aromatic rings and other bonds and later polymerisation reaction with the formation of quinone (Thurston, 1994; Leonowicz *et al*., 2001).

Laccase cannot directly oxidize non-phenolic lignin units for primarily two reasons – the large size which restrict penetration into the enzyme active site and the relatively high redox potential (> 1.2V) of these substrates (Tadesse *et al.*, 2008). Therefore, it employs suitable chemical mediators such as 2, 2' –azinobis-(3-ethylbenthiazoline-6-sulfonate) (ABTS) (Figure 3.1) that act as an intermediate substrate for laccase in this way boosting the otherwise low redox protential of laccase; the oxidized radicals formed are then able to react with the high redox potential substrates (Arora & Sharma, 2010).

In fungi, the laccase-mediator system is usually involved in a range of physiological functions that are unrelated to lignolysis (Thurston, 1994; Divya & Sadasivan, 2016). They include morphogenesis and differentiation of sporulating and resting structures in basidiomycetes (Robene-Soustrade & Lung-Escarmant, 1997); pigmentation in mycelia and fruiting bodies, improves cell-to-cell adhesion, assists rhizomorph formation and it is also responsible for the formation of polyphenolic adhesion that binds hyphae together (Thurston, 1994).

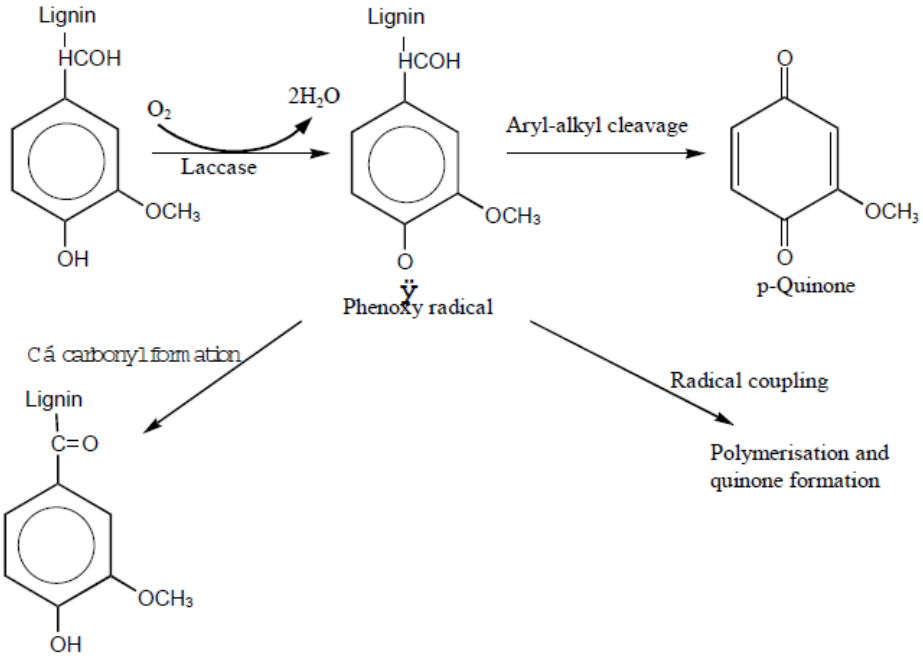


Figure 2.6: Oxidation of phenolic subunits of lignin by laccase (Archibald *et al.*, 1997)

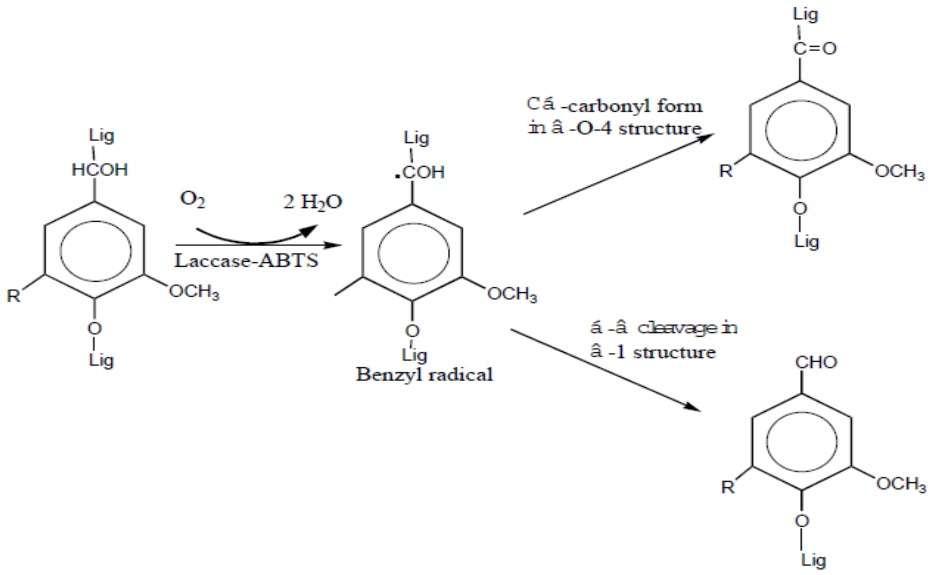


Figure 2.7: Oxidation of non-phenolic subunits of lignin by laccase and ABTS (Archibald *et al.*, 1997)

Laccases have also been implicated in plant pathogens; where extracellular laccases are produced that enable the fungus to overcome the immune response of the host (Thurston, 1994). It is also able to facilitate the detoxification of the plant tissue via the oxidation of antifungal phenols or deactivation of phytoalexins (Assavanig *et al.*, 1992; Robene-Soustrade & Lung-Escarmant, 1997). More recently it has been suggested that it is likely to be involved in defense against antagonistic organisms (Divya & Sadasivan, 2016). The production of low concentrations of laccase is constitutive in white-rot fungi, but the higher concentrations can be promoted using inducers in the cultivation media such as metals and aromatic compounds (Van der Merwe, 2002).

Laccase was first discovered in the sap of the Japanese lacquer tree *Rhusvernicifera* and described in 1883 by Yoshida (as cited by Call & Mücke, 1997). It has since been found to be widespread in nature, for example in plants, insects, bacteria, and to very large extents in fungi (Claus, 2004; Durao *et al.*, 2006; Ferraroni *et al.*, 2007). Although found predominantly in white-rot fungi such as *Lentinus tigrinus* (Ferraroni *et al.*, 2007), *Pleurotus ostreatus* (Pozdniakova *et al.*, 2006), *Cerrena unicolor* strain 137 (Michniewicz, *et al.*, 2006), *Trametes versicolor* (Necochea *et al.*, 2005), *Trametes* sp. strain AH28-2 (Xiao *et al.*, 2003), *Trametes pubescens* (Shleev *et al.*, 2007) and *Cyathus bulleri* (Salony *et al.*, 2006). It has also been reported in brown rot fungi including *Coniophora puteana* (Lee *et al.*, 2004) and also in Ascomycetes such as *Melanocarpus albomyces* (Hakulinen *et al.*, 2006), *Chaetomium thermophile* (Ishigami & Yamada, 1986), *Magnaporthe grisea* (Iyer & Chatto, 2003),

Myrothecium verrucaria 24G-4 (Sulistyaningdyah *et al.*, 2004) and *Neurospora crassa* (Germann *et al.*, 1988).

Laccase has found application in construction of biosensors, textile decolourisation, pulp bleaching, effluent detoxification, removal of phenolic compounds in beverages, food processing and in bioremediation (Couto & Toca-Herrera, 2006; 2007; Osma *et al.*, 2010).

2.5.2 Peroxidases

Lignolytic peroxidases include lignin peroxidase (LiPs), manganese-dependent peroxidases (MnPs) and the most recently discovered versatile peroxidases (VPs) (Martínez, 2002) which appears to be a hybrid of LiPs and MnPs (Hatakka & Hammel, 2010). These fungal peroxidases are all heme-containing glycoproteins that require hydrogen peroxide as an oxidizing agent. Peroxidases possess heme (protoporphyrin IX) as their prosthetic group coordinated by two highly conserved histidine residues (Martínez, 2002). The heme co-factor is located within an internal cavity connected by two access channels; with the main channel used by H_2O_2 and the second site is the reserved oxidation site for MnP and VP to oxidize Mn^{2+} and Mn^{3+} (Ruiz-Dueñas *et al.*, 2009). A common strategy employed by both LiP and MnP to oxidize corresponding substrates is the two consecutive one-electron oxidation steps with intermediate cation radical formation (Sanchez, 2009).

2.5.2.1 Lignin Peroxidase

Lignin peroxidase (1, 2-bis (3,4 di-methoxyphenyl) propane-1,3-diol: hydrogen peroxide oxidoreductases, EC 1.11.1.14) degrades non-phenolic lignin units (such as di-arylpropane), β-O-4 non-phenolic lignin model compounds) in an oxygen-dependent reaction by abstracting one electron and generating unstable aryl cation radicals that undergo spontaneous degradation (Kirk & Farrell, 1987; Sayadi & Odier, 1995; Ruiz-Dueñas & Martínez, 2009). However, with phenolic substrates, LiP oxidises them to products similar to those produced by peroxidases generally (Kluczek-Turpeinen, 2007).

LiPs are able to catalyse these reactions because of their unusually high redox potential which is responsible for their ability to oxidize non-phenolic aromatic substrates without the participation of mediators (Wang *et al.*, 2008; Wong, 2009). Unlike their counterpart MnP which generates Mn^{3+} whose action being as a diffusible oxidant allows it to degrade both phenolic and non-phenolic lignin unit (Hofrichter, 2002; Cullen & Kersten, 2004).

In studies involving the *P. chrysosporium* it was observed that characteristically for LiP activity, the amino acid tryptophan, Trp171, in the isozyme LiPA (LiP H8) is essential. This tryptophan found on the LiP protein surface is conserved in LiP sequences, this is similar to those on versatile peroxidases (VPs) (Martínez, 2002; Pérez-Boada *et al.*, 2005). It is believed that it participates in long-range electron transfer (LRET) from a protein radical at the surface of the enzyme, which would act as the substrate oxidizer to the heme co-factor (Ruiz-Dueñas & Martínez, 2009); making it possible for the enzyme to oxidize large substrates such as polymeric lignin that cannot directly contact the oxidized heme in the active site of LiP or VP (Hatakka & Hammel, 2010).

Several strains of *P. chrysosporium* (Renganathan *et al.*, 1985; Howard *et al.*, 2003; Wymelenberg *et al*., 2006) that have shown LiP activity. Moreover other white-rot fungi such as *T. versicolor* (Hossain & Anantharaman, 2006) and fungal species including *Panus* sp., *Pycnoporus sanguineus* and *Perenniporia medulla-panis* (Pointing *et al.*, 2005) have demonstrated LiP activity. Remarkably, bacteria such as *Streptomyces viridosporus* T7A (Gottschalk, Bon & Nobrega, 2008) and *Acinetobacter calcoaceticus* NCIM 2890 (Ghodake *et al*., 2009) have also shown LiP activity.

LiPs oxidize the substrate in a cyclic multi-step electron transfers involving the oxidation of native Fe (III) enzyme by H_2O_2 to LiP compound I (Figure 2.8). The primary of oxidation of LiPs is initiated by H_2O_2 with the initial production of a two-electron-deficient compound 1involving a one-electron reduction of compounds such as veratryl alcohol (VA). The intermediate compound I removes one electron from the donor substrate VA resulting in the formation of reduced compound II and a cation radical intermediate. This compound II then undergoes oxidation using the second molecule of the donor substrate, also via a one-electron transfer producing a cation radical intermediate and the resting state of the peroxidase. These cation radical intermediates spontaneously break into smaller units. In the presence of excess H_2O_2, compound II can be further oxidized to compound III (Dosoretz *et al*., 2004). The presence of H_2O_2 and VA will cause compound III to return to the native state of LiP. However, excessive H_2O_2 inactivates compound III. VA is vital in protecting LiP from the likelihood of H_2O_2-dependent inactivation by providing intermediate VA^+ that is capable of reducing compound III to its native state (Wan & Li, 2013).

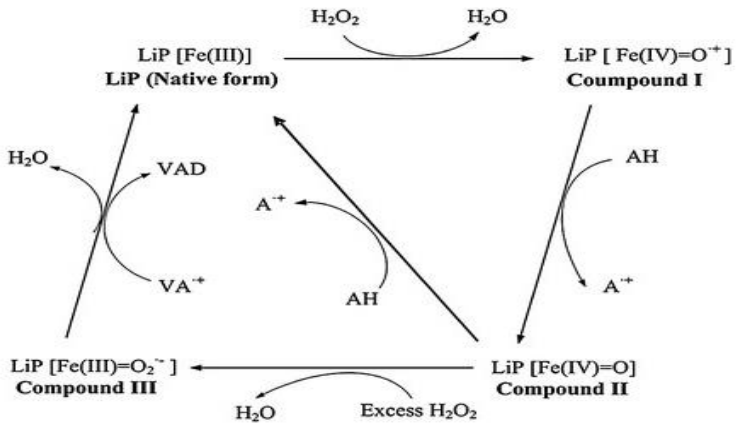


Figure 2.8: Oxidative mechanism of lignin peroxidase (Wong, 2009)

Schmidt (2006) illustrated the mechanism LiP utilises to initiate various non-enzymatic reactions such as the cleavage of C_α – C_β bond and aryl C_α bonds in the side chain, cleavage of β-O-4 bond between side chain and next ring and cleavage of aromatic ring, demethylation and phenolic oxidation (Figure 2.9)

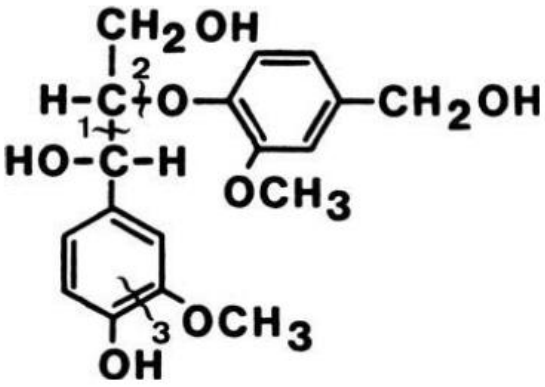


Figure 2.9: Reactions initiated by lignin peroxidase, (1) cleavage of C_α– C_β bond in the side chain, (2) β-O-4 bond between side chain and next ring and (3) cleavage of aromatic ring (Schmidt, 2006).

2.5.2.2 Manganese Peroxidases

Some authors have implied that manganese peroxidases (Mn (II): hydrogen-peroxide oxidoreductases, EC 1.11.1.13) is more commonly expressed than LiPs (Hatakka, 1994; Hofrichter, 2002; Wesenberg *et al.*, 2003) in many basidiomycetous fungi such as *P. chrysosporium* (Urek & Pazarlioglu, 2007), *Bjerkandera* sp. (Palma *et al.*, 2000), *Agaricus bisporus* (Lankinen *et al.*, 2001), *Phanerochaete flavido-alba* (de la Rubia *et al.*, 2002), *Lenzites betulinus* (Hoshino *et al.*, 2002), *Panus tigrinus* (Lisov *et al.*, 2003) and *Nematoloma*

frowardii b19 (Hilden *et al.*, 2008). However, there are seemingly no concrete evidences of manganese peroxidase (MnP) production in bacteria, yeasts and moulds, nor in mycorrhiza-forming basidiomycetes (Hofrichter, 2002). In fact Hatakka and Hammel (2010 p.333) go so far as to assert that any such reports should be regarded with suspicion as there is a possibility that analysis are based on incorrectly identified fungi or there is a lack thereof of insufficient or incorrect enzyme assays.

The expression of MnP in the fungal culture is dependent on Mn which regulates the *mnp* gene transcription (Ma *et al.*, 2004). Similar to LiPs, MnPs are secreted extracellularly in multiple isoforms by most white-rot fungi and litter decomposing species (Wesenberg *et al.*, 2003; Asgher *et al.*, 2008). These isoforms contain one molecule of heme as iron protoporphyrin IX (Asgher *et al.*, 2008). The catalytic cycle of MnP is also similar to LiP and other peroxidases but the presence of Mn^{2+} is necessary. MnP mediates initial steps in the degradation of high-molecular-weight lignin (Schlosser & Höfer, 2002).

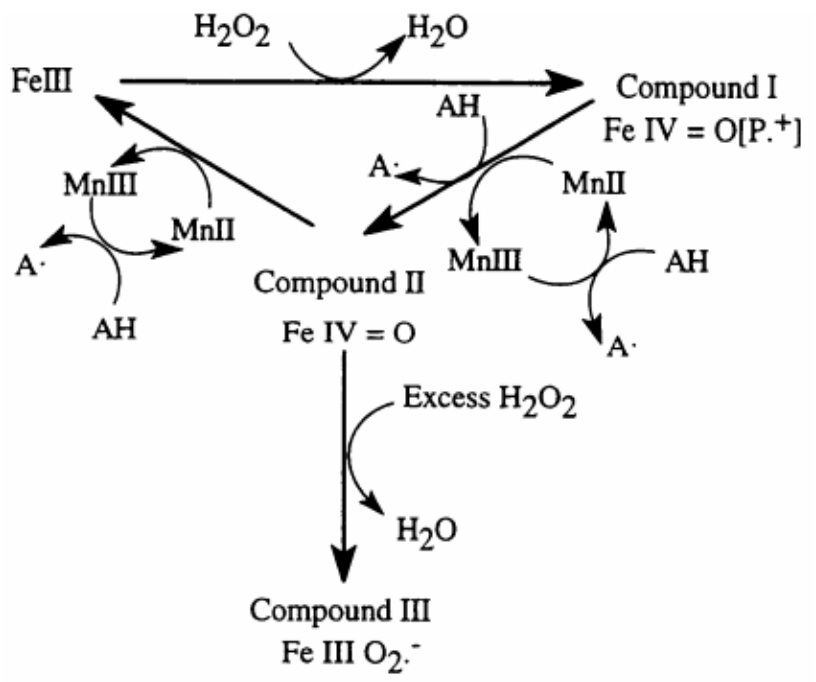


Figure 2.10: Catalytic cycle of MnP (Adapted from Hofrichter *et al.*, 2002)

The cycle is peroxide-dependent and is initiated by the binding of H_2O_2 to the native ferric enzyme and the formation of an iron peroxide complex (Figure 2.10). Consequently, two-electron is transferred from the heme to cleave the oxygen-oxygen bond in the peroxide and form MnP compound I (Fe^{4+}-oxo-porphyrin-radical complex). Subsequent reduction of MnP compound I cause the simultaneous formation of Mn Compound II (Fe^{4+}-oxo-porphyrin

complex) and another Mn^{3+} from Mn^{2+}, this leads to the re-generation of native enzyme with the release of water molecule (Hofrichter *et al.*, 2002). Wariish *et al.* (1988) observed that high concentrations of H_2O_2 cause reversible inactivation of MnP and the formation of compound III. Mn^{3+} is subsequently released from the surface of the enzyme in complex with oxalate or with other chelators such as malate, lactate or malonate which helps to stabilise it as well as promote their release from the enzyme into the surrounding environment (Hofrichter *et al.*, 2002). The chelated Mn^{3+} is able to oxidize various compounds including lignin by single electron oxidation (Schmidt, 2006); it acts as a reactive low molecular weight, diffusible redox-mediator of phenolic substrates not only for the depolymerisation of lignins, but also for compounds such as simple phenols, amines, dyes and dimmers (Wesenberg *et al.*, 2003; Asgher *et al.*, 2008; Wong, 2009); successively oxidising the phenolic rings of lignin to unstable phenoxy radicals with later spontaneous disintegration (Hofrichter, 2002). These highly reactive phenoxy radicals are also involved in the cleavage of $C_\alpha - C_\beta$ bonds, and similarly alkyl-phenyl bonds, with a resultant formation of smaller intermediates including quinones and hydroxyquinones (Solarska, 2009).

The presence of a secondary mediator such as oxalate and malonate is required for the oxidation of non-phenolic lignin units by Mn^{3+}, this leads to the production of carbon-centred reactive radicals such as acetic acid, peroxyl, superoxide and formate radicals (Wariishi *et al.*, 1989; Wesenberg *et al.*, 2003; Asgher *et al.*, 2008; Wong, 2009). These radicals can be used by MnP in fungi that lack H_2O_2 generating oxidases, as a source of peroxides and increase the lignin-degrading efficiency (Hofrichter *et al.*, 1998; Wesenberg *et al.*, 2003; Asgher *et al.*, 2008).

MnP has found potential application in aspects including biomechanical pulping, pulp bleaching, dye decolorization, bioremediation and production of high-value chemicals from residual lignin from biorefineries and pulp and paper side-streams (Maijala, 2005)

2.5.2.3 Versatile Peroxidases

Versatile peroxidases (VPs) (EC 1.11.1.16) were discovered in the 1990s and are considered hybrids of LiPs and MnPs (Martinez, 2002). VPs have the capacity to oxidize characteristic substrates of other basidiomycetes peroxidases such as Mn^{2+} (typical MnP substrate), 2,6-dimethoxyphenol (DMP) and veratyl alcohol (VA) (typical LiP substrates) (Wesenberg *et al.*, 2003; Asgher *et al.*, 2008; Ruiz-Dueñas *et al.*, 2009). The dual oxidative abilities of VPs are

most likely due to the co-existence of different catalytic sites on a single protein (Camarero *et al.*, 2000).

Ruiz-Dueñas and Martinéz (2009) allude to the point that VP has so far only been isolated from *Pleurotus* sp. and *Bjerkandera* sp. Busse *et al.* (2013) demonstrated that VP isolated from *Bjerkandera adusta* could facilitate a H_2O_2-dependent degradation of Alderol, a biopolymer of lignin; in reviewing the peroxidase reaction mechnanism they were able to recommend strategies to prevent premature enzyme losses associated with reactions. Several authors have established the additional ability to oxidise high redox-potential compounds such as dyes and a wide variety of phenols including hydroquinones (Heinfling *et al.*, 1998; Gómez-Toribio *et al.*, 2001; Veitch, 2004). Furthermore, Salvachúa *et al.* (2013) demonstrated that VP could become a vital tool for generating new biomolecules with higher molecular mass that could be exploited for biotechnological applications such as lignans and peptides; this they proved could be achieved by enzyme cross-linking as a result of VP's high redox-potential capable of oxidative activity on a wide variety of substrates. The VP used in their study was extracted from *Pleurotus eryngii*.

2.5.2.4 Accessory Lignin degrading enzymes

There are other fungal extracellular enzymes which act as accessory enzymes in lignin degradation. These enzymes include H_2O_2-generating enzymes such as aryl alcohol oxidase (AAO, EC 1.1.3.7) which provide the hydrogen peroxide required by peroxidases, they are found in variety of fungi including *P. eryngii* (Hernández-Ortega *et al.* 2012); glyoxal oxidase (GLOX), a copper radical protein found in *P. chrysosporium*and pyranose-2 oxidase (EC 1.1.3.10) (Hatakka, 2001; Kersten & Cullen, 2007; Wongnate & Chaiyen, 2013; Yin *et al.*, 2015).

Aryl-alcohol dehydrogenases (AAD) is a flavoprotein and quinone reductase have been shown to be involved in lignin degradation by fungi (Ferreira *et al.* 2009). Similarly, cellobiose dehydrogenase (CDH) is also involved in lignin degradation but requires H_2O_2 and chelated Fe ions as observed in a variety of fungi under cellulolytic conditions. CDH is believed to indirectly generate hydroxyl radicals by reducing Fe^{3+} to Fe^{2+} and O_2 to H_2O_2. Hydroxyl radicals are subsequently generated by a Fenton type reaction; it is possible for these hydroxyl radicals to then react with various wood compounds, including lignin (Henriksson *et al.*, 2000).

2.6 Other enzymes involved in lignocellulose degradation – Cellulolytic enzymes

The complexity of cellulose means that its complete saccharification requires synergistic interactions between groups of enzymes responsible for the cleaving of the different linkages found within their structures. These enzymes are collectively known as cellulolytic enzymes or cellulases (Strakowska *et al*., 2014).

2.6.1 Cellulases

There are at least 3 major classes of enzymes involved in cellulose degradation to glucose, they are predominantly hydrolases and are endo-glucanase, exo-glucanase and β-glucosidase (Wyman, 1996; Schmidt, 2006).

Endo 1,4-β-glucanase(EC 3.2.1.4) hydrolyze glycosidic bonds internally by randomly cleaving the chains thereby creating free end chains (Taherzadeh & Karimi, 2007) preferably acting mainly on the amorphous regions of cellulose (Carere *et al.*, 2008). Conversely exo-1,4-β-D-glucanase (EC 3.2.1.91) or cellobiohydrolase typically act upon crystalline cellulose and can release cellobiose from either the reducing or non-reducing end of cellulose. However these released cellobiose act as a competitive inhibitor to cellobiohydrolases. Both endo-cellulase and cellobiohydrolase are often associated with cellulose-binding modules to promote their activity on polymeric substrates (Kirk & Cullen, 1998). Finally, 1,4-β-D-glucosidase (EC 3.2.1.21) hydrolyze cellobiose to D-glucose as described in Figure 2.11.

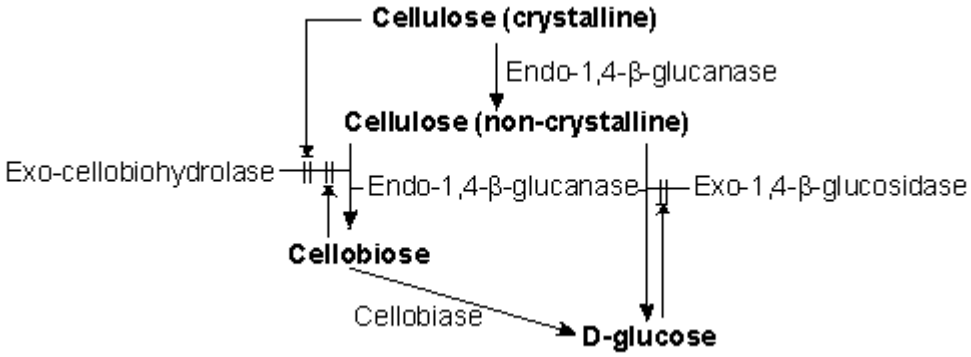


Figure 2.11: Cellulose degradation to glucose

(Source: http://www1.lsbu.ac.uk/water/enztech/cellulose.html Access date: 10-10-2016)

2.6.2 Hemicellulases

As a consequence of the heterogeneous nature of hemicellulose; its degradation requires several enzymes including glycoside hydrolases and carbohydrate esterases (Jørgensen *et al.*, 2005). Similar to cellulases, these enzymes synergistic activities are associated with carbohydrate binding molecules that usually promote enzyme activity on polymeric substrates (de Vries *et al.*, 2000; Hervé *et al.*, 2010). The degradation of hemicellulose is more widespread in wood fungi than in bacteria (Schmidt, 2006).

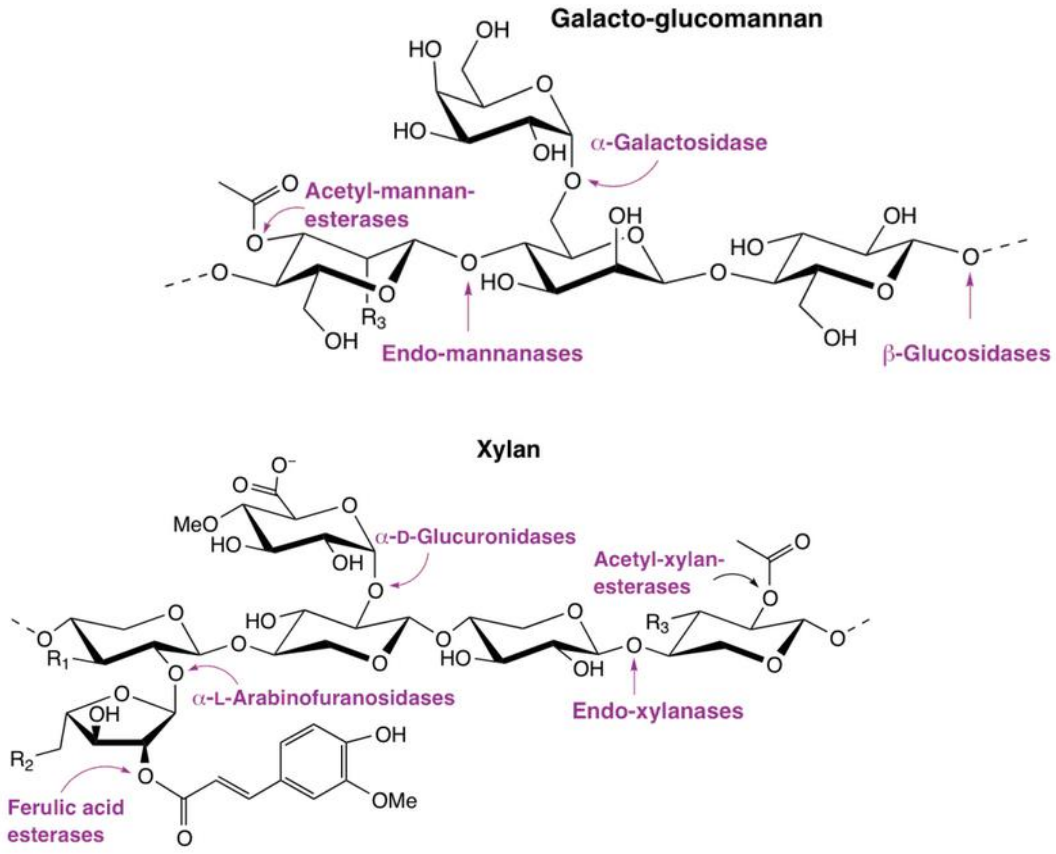


Figure 2.12: Enzymes involved in hemicellulose degradation (Deboy *et al.*, 2008)

Recall that xylan, xyloglucan and galacto-glucomannan are the major constituents of hemicellulose with β-1,4 glycosidic back bone linkages; therefore the backbone is hydrolyzed by endo-1,4-β-xylanase (EC 3.2.1.8) to xylobiose, and 1,4- β-xylosidase (EC 3.2.1.37) hydrolyses it to xylose (Jørgensen *et al.*, 2003; Scheller & Ulvskov, 2010). The side chains linked to the xylan backbone, with the acetyl group and 4-*O*-methylglucoronic acid are cleaved by acetylesterase (acetic ester acetylhydolase, EC 3.1.1.6) and xylan α-D-1,2-(4-*O*-methyl) glucoronohydrolase (EC3.2.1.131) respectively (Figure 2.3 and 2.12). Other enzymes involved in hemicellulose degradation include endomannanases, β-mannosidases, α-L-

arabinofuranosidases, α-galactosidases, deacetylases, and feruloyl esterases (Jørgensen *et al.*, 2005).

2.7 Factors that influence enzyme production by ligninolytic fungi

2.7.1 Substrate concentration

It is noteworthy to highlight some factors that affect enzymatic hydrolysis of cellulose and hemicellulose portions of lignocellulosic materials as specific parameters are necessary to guarantee efficient hydrolysis and increase sugar yields for subsequent fermentation. A significant factor is substrate concentration of the hydrolysates; although primarily increasing substrate concentration tends to increase the rate of reaction. This is because the increased number of substrate molecules will ensure more collision with enzyme molecules, thus guaranteeing the formation of more products. However, several authors have reported that high concentrations of substrate can cause substrate inhibition (Sun & Cheng, 2002; Misra, 2006; Sharma, 2012). This adversely affects the rate of hydrolysis; bearing in mind that substrate inhibition is dependent on the ratio of total substrate to total enzyme (Huang & Penner, 1991; Penner & Liaw, 1994). Sun & Cheng (2002) further highlighted that although an increase in the dosage of cellulases to a certain level would improve the yield and rate of hydrolysis; this carries with it cost implications. Ideally in laboratory conditions the dosage is 10FPU/g as this provides optimal glucose yields in a reasonable time frame (48 to 72 hours) and enzyme cost (Gregg & Saddler, 1996). It is hoped that with better enzyme recovery strategies during lignocellulose bioconversion to ethanol, cost can be reduced and make this approach much more feasible. Currently the addition of surfactants is employed during hydrolysis to modify the cellulose surface property and minimize the irreversible binding of cellulose to cellulose; this in turn lowers the enzyme loading. Some of these surfactants include non-ionic Tween® 20 and 80 and polyoxyethylene glycol, they are believed to be most effective in enhancing cellulose hydrolysis (Park *et al.*, 1992; Wu & Ju 1998). Park *et al.* (1992) attested to increased enzyme desorption from the binding site on the substrate surface (used newspaper) after the completion of saccharification at the site when non-ionic surfactants were employed. The addition of polyoxyethylene glycol to lignocellulose substrates increased the enzymatic conversion from 48% to 72% in 16 hours (Börjesson *et al.*, 2007). Other factors that affect enzymatic hydrolysis include pH of fermentation broth, fermentation temperature, applied pre-treatment techniques and mixing as well as nutrient composition (Taherzadah & Karimi, 2007).

However the optimized production of particularly ligninolytic enzymes is highly regulated by nutrients such as nitrogen, copper and manganese. Furthermore, the nature of the nitrogen source and the presence of inducers, mediators and organic acids such as citric, oxalic and tartaric acids may also contribute to efficiency of enzymatic activity (Ryan *et al.*, 2007; Wen *et al.*, 2009; Iqbal *et al.*, 2011).

2.7.2 Nutrient composition

A significant number of studies on the production of ligninolytic enzymes carried out using defined media has clearly demonstrated that basidiomycetes display varying responses to carbon sources and their concentration in nutrient medium (Galhaup *et al.*, 2002; Mikiashvili *et al.*, 2005; Elisashvili *et al.*, 2006; Wang *et al.*, 2008). In some instances ligninolytic gene expression is activated only by the depletion of nutrient carbon as was observed with *Phanerochaete chrysosporium* (Wang *et al.*, 2008). Similar observation was seen with *Trametes pubescens* when laccase secretion only commenced when glucose concentration in the media reached low, critical concentration (Galhaup *et al.*, 2002). It is also noteworthy that *Fomes fomentarius* showed a capability to secrete high levels of laccase in the presence of polysaccharides (2481Ug^{-1}biomass) in medium containing carboxymethyl cellulose (Elisashvili & Kachlishvili, 2009). There is enough indication that some carbohydrates and the carbon source appear to regulate the laccase expression in white-rot basidiomycetes (Revankar & Lele, 2006; Bettin *et al.*, 2008) and as such it is deemed important that fungus-specific carbon source should be elucidated maximally to enhance enzyme synthesis (Elisashvili & Kachlishvili, 2009).

The carbon/nitrogen ratio is considered an important factor to balance biomass and production of primary metabolites and idiolites (also known as secondary metabolites). The excess or lack of nitrogen content in the substrate may be a limiting factor to the growth of fungus particularly in large scale production of fungal enzymes (Mantovani *et al.*, 2007; Rivera-Hoyos *et al.*, 2013). One of the best studied white-rot fungi *P. chrysosporium* was observed to produce LiP and MnP in synthetic medium only under nitrogen-limited conditions (Reddy *et al.*, 1994). However, several authors have found that high nitrogen content in the presence of lignocellulosic substrate stimulates the production of ligninolytic enzymes (Saparrat *et al.*, 2002; Kapich *et al.*, 2004; Songulashvili *et al.*, 2007). Similarly, a high nitrogen content in synthetic media produced increased laccase activity in *Lentinus edodes*, *Rigidoporus lignosus*, and *Trametes pubescens*. Conversely, the nitrogen-limited conditions enhanced enzyme

production in *Pycnoporus cinnabarinus*, *P. sanguineus*, and *Phlebia radiata* (Mester & Field, 1997; Gianfreda *et al.*, 1999; Tekere *et al.*, 2001; Galhaup *et al.*, 2002).

There are indications that fungi respond differently to diverse nitrogen sources in the culture medium (D'Agostini *et al.*, 2011; Janusz *et al.*, 2013). Furthermore, there are studies that establish a link between secondary metabolism and nitrogen catabolite repression system (Poussereau *et al.*, 2001; Sotero-Martins *et al.*, 2003; DeMoura *et al.*, 2004; Jiang *et al.*, 2009; Parente *et al.*, 2010). Eggert *et al.* (1996) observed that the production of laccase in submerged cultures was enhanced by the presence of inorganic nitrogen from simple mineral source such as ammonium nitrate and ammonium sulphate. However it is noteworthy, that complex organic sources such as amino acids, peptone and yeast extract has the similar effect of increased laccase production in some fungi (Thongkred *et al.*, 2011; Khanam *et al.*, 2012; Sarnthima *et al.*, 2013). Kalisz *et al.* (1986) reported high laccase yield when complex organic nitrogen was added to the medium in contrast with the use of simple mineral nitrogen; Mikiashvili *et al.* (2006) also observed a similar effect with *Pleurotus ostreatus*. Similar findings were observed with *Trametes hirsuta* and *Trametes pubescens* (Strong, 2011; Bakkiyaraj *et al.*, 2013).

2.7.3 Inducers and Mediators

The use of synthetic inducers such as metals and aromatic compounds are known to increase enzyme activities in white-rot fungi as well as the secretion of isoenzymes. Bourbonnais and Paice (1990) first described that it is possible for laccases to assume the function of LiP provided there is the presence of a suitable redox mediator 2,2,9-azino-bis(3-ethylthiazoline-6-sulfonate) (ABTS) as they observe from *T. versicolor* when it oxidized non-phenolic model compounds. Several authors have since reported an increase in laccase activities with the addition of the aromatic compound 2,5-xylidine (Min *et al.*, 2001; Elisashvili *et al.*, 2002; Revankar *et al.*, 2007). Other aromatic compounds that have been used with success to increase laccase expression include ferulic acid, guaiacol, veratryl alcohol and 1-hydroxybenzotriazole (Collins & Dobson, 1997; Revankar & Lele 2006; Kocyigit *et al.*, 2012). Kuhar *et al.* (2014) and Wang *et al.* (2014) utilized ferulic acid as inducer to enhance laccase activity in *G. lucidum* and *T. versicolor* respectively. Elisashvili and Kachlishvili (2009) reported using a variety of inducers including 2,4,6-trinitrotoluene (TNT), vanillic acid pyrogallol in medium containg ethanol production residues for the cultivation of *Cerrena unicolor* with varying results in laccase activity. The same authors used dimethoxyphenol (2,6-DMP) that yielded a 2-fold increase in MnP activity. In addition, vanillic acid, ferulic acid, pyrogallol and veratric acid

increased MnP activity by more than 50% (Elisashvili & Kachlishvili, 2009). An increase in MnP activity was observed in ligninolytic fungi when Tween 80 with the highest activity demonstrated by *P. chrysosporium* (Novotný *et al.*, 2004). Tween 80 is among a group of surfactants that increases the bioavailability of less soluble substrate for fungus, in this way it enhances the production by allowing the MnP permeate from the cell into the medium, thus stimulate the growth of spores (Zheng & Obbard, 2001; 2002). There was an increase in the expression of ligninolytic enzymes in the mushroom *Sterum ostrea* (Usha *et al.*, 2014) when Tween 80 was used. Munoz *et al.* (1997) had previously identified two isoenzymes when they studied laccase activity of *P. eryngii* in glucose medium that contained ammonium tartrate.

Although often dimethyl sulfoxide and ethanol are used as solvents to dissolve water-insoluble compounds for the determination of enzyme activities; it has also been found to actively induce enzyme activity (Shah *et al.*, 2006). An increase in laccase activity was observed in *T. versicolor* when ethanol was added to medium containing glucose as the carbon source (Lee *et al.*, 1999). The same authors observed mediator effect of ethanol on laccase production by *Grifola frondosa* and *Coriolus hirsutus*. Although little is known about the mechanism of induction by ethanol, it has however been identified as a cheap and less toxic inducer for laccase production (Valeriano *et al.*, 2009). There are speculations that ethanol is able to induce enzyme expression by causing oxidative stress, usually by disrupting the cell membrane or by segregating intracellular Ca^{2+} which acts a secondary messenger and induces laccase gene expression (Lomascolo *et al.*, 2003). However there are mixed reactions to the use of ethanol as an inducer as some authors have reported negative effects for the use of ethanol (Barreto *et al.*, 2007; Eugenio *et al.*, 2010). It is presumable that the effectiveness of ethanol as an inducer is related to the unique culture conditions (Hernández *et al.*, 2015).

Some heavy metals especially manganese and copper are necessary for fungi. However an excess of these metals are toxic. Notably, the threshold of toxicity is rather small (Hughes *et al.*, 1991; Baldrian, 2003). Copper plays a key role as a metal activator by inducing both laccase transcription and expression as was observed in *P. ostreatus* (Palmieri *et al.*, 2000; Tinoco *et al.*, 2011). Copper is also a co-factor in the catalytic centre of laccase. It has been reported that the combination of 2,5-xylidine and $CuSO_4$ had a synergic and positive effect on laccase production in the presence of organic nitrogen sources (Strong, 2011; Bakkiyaraj*et al.*, 2013). This effect can be explained with the understanding that there is the presence of metal responsive elements present in several laccase genes (Baldrian, 2003). The addition of $CuSO_4$

to broth culture resulted in a substantial increase in the total laccase activity and the production of isozymes (Giardina *et al.*, 1999). More recently, Khammuang *et al.* (2013) observed increased laccase activity in *Lentinus polychrous* when copper was added to a liquid fermentation medium. Manganese plays a regulatory role in the expression of LiP, MnP and Lac and in the degradation of lignin (Gadd, 1993; Shimada, 1997). Gill and Arora (2003) observed an improved MnP production and stimulation of enzymatic activity by white-rot fungi following the addition of the inducer $MnSO_4$. Jarvinen *et al.* (2012) reported that although the production of MnPs varied in different species of fungi, it is clearly a function of inducers such as Mn^{2+}, veratryl alcohol and malonate. Qin *et al.* (2014) found that oxalic acid, veratryl alcohol and 2,6-Dimethoxyphenol can stimulate the producton of MnP in the white-rot fungus *Irpex lacteus* CD2. The novel enzyme (CD2-MnP) discovered in their study was demonstrated to be capable of decolourising several dyes.

2.7.4 Organic Acids

Organic acids perform the role of chelators and include oxalate, lactate, malonate and citrate. Oxalate and malonate are secreted by the fungus *P. crysosporium* (Wariishi *et al.*, 1992). MnP enzyme competes with chelators for free Mn^{2+}. Optimal chelators, such as malonate facilitate Mn^{3+} dissociation from the enzyme thereby stabilizing Mn^{3+} in aqueous solution. Furthermore malonate have relatively low Mn^{2+} binding constant (Wariishi *et al.*, 1992; Wong, 2009). Moreira *et al.* (1998) reported with *Bjerkandera* sp.that the addition of simple physiological organic acids (e.g., glycolate, glyoxylate, oxalate and others) may extend simply beyond providing pH-buffering but could stimulate the production of MnP and LiP as well as increase physiological concentrations of veratryl alcohol and oxalate which ultimately increased the biobleaching effects and pulp delignification. Several authors have shown that chelators such as oxalate and malonate are vital for the oxidation of non-phenolic compounds involving Mn^{3+}; these organic acids function as secondary mediators in the production of reactive radicals like carbon-centred radicals such as acetic acid radicals ($COOHC\boldsymbol{.}H_2$), peroxyl radicals ($COOH\text{-}CH_2OO\boldsymbol{.}$), superoxide ($O_2\boldsymbol{.}$) and formate radicals ($CO_2\boldsymbol{.}$) (Wesenberg *et al.*, 2003; Asgher *et al.*, 2008; Wong, 2009). It is presumed that in the absence of H_2O_2 particularly with fungi that lack theH_2O_2-generating oxidases, these radicals can be employed by MnP as a source of peroxides thereby increasing the lignin-degrading efficiency (Hofrichter *et al.*, 1998; Wesenberg *et al.*, 2003; Wong, 2009). Hofrichter *et al.* (1999) in their study of the basidiomycetous fungus *Nematoloma frowardii* observed the predominant production of MnP and low levels of laccase and lignin peroxidase in the biodegradation of straw residue; however the production of

enzymes were associated with the detection of high levels of organic acids in the fermented straws such as malate, fumarate and oxalate, which they presumed rendered the MnP effective in the mineralization of the straw. Kurek and Gaudard (2000) as well as Makela *et al.* (2009) reported the involvement of oxalate decarboxylase and the regulation of oxalate concentration in CDH catalyzed reaction involving *Dichomitus squalens.*

2.8 Fungal Interspecific Interactions in Mixed Cultures

A vast amount of the knowledge about lignin biodegradation comes from studies done on pure cultures of white-rot basidiomycete fungi. However in nature, most fungi will exist in mixed communities with other fungi and even bacteria. This inevitably will lead to competition for nutrient and space. Fungi engaged in such competition frequently produce idiolites, extracellular phenol-oxidizing enzymes and differentiated structures in the zone of conflict (Cooke & Rayner, 1984; Dyer *et al.*, 1992; Griffith *et al.*, 1994). Competition in natural environments plays a key role in determining the distribution and abundance of fungal species (Widden, 1997). These responses are not only critical in determining the outcome of a biocontrol treatment but can be exploited in the induction of enzyme production. It is suggested that the competition for space and nutrient leads to increased degradation of lignin and elevated production of ligninolytic enzymes such as laccase and MnP (Sundman & Näse, 1972; Asiegbu *et al.*, 1996; Baldrian, 2004). It is common in nature that biotransformations tend to utilize a combination of metabolic pathways from different microorganisms. Numerous examples of the co-existence of different microorganisms have been observed, such as forest soils, compost piles, insect gut and mammalian intestines (Mai & Morris, 2004; Belenguer *et al.*, 2006).

In recent times, most of the fungal enzymes used in industrial biotechnology are produced by processes involving single microbial strains (Bader *et al.*, 2010). However one major drawback to utilizing ligninolytic fungi in industrial scale fermentation is the low productivity and high cost implication for the production of the constitutive extracellular enzymes produced by these organisms (Flores *et al.*, 2009; Rivera-Hoyos *et al.*, 2013). Chemical induction of laccase and MnP by the addition of compounds related to lignin or lignin derivatives such as phenolic and aromatic compounds, copper and ethanol have been studied extensively (Baldrian, 2003; Gill & Arora, 2003; Lomascolo et *al.*, 2003; Shah *et al.*, 2006; Revankar *et al.*, 2007; Kocyigit *et al.*, 2012; Kuhar *et al.*, 2014; Wang *et al.*, 2014). Moreover, the use of chemical inducers makes the industrial process economically non-feasible and in some cases these chemical inducers are toxic.

Therefore any industrial process that would in future exploit the use of ligninolytic enzymes must produce economical and safe laccase and MnPs. For this reason, this objective has been the focus of most enzyme research work in the past few decades (Flores *et al.*, 2009). Using co-cultures appears to be gaining considerable attention where in the past the focus had been single microorganism cultures. The reason can be linked to the potential to utilize synergism and even surprisingly antagonism between the metabolic pathways of the strains involved in the co-culture (Baldrian, 2004; Bader *et al.*, 2010; Dwivedi *et al.*, 2011; Pandya & Albert, 2014). However an elucidation of the type of interactions and mechanism of interaction demonstrated during fungi-fungi interaction is pertinent towards any future exploitation.

2.8.1 Competition

Extremely intense interactions occur when individual organisms occupy a common habitat with other organisms owing to the desperate need to use available resources. The relative success of each organism in meeting its needs will determine its survival or death. This process tends to lead to natural selection and its outcome affects the population and ultimately the composition of the community. Interactions tend to influence metabolic activities of individual organisms. Wood decay fungi demonstrate a broad spectrum of interaction types with varying impact including neutralistic, mutualistic, and combative/antagonistic (Moore-Landecker, 2002).

However, a vast majority of these interactions are competitive. Keddy (1989) defines competition as 'the negative effects which one organism has upon another by consuming, or controlling access to a resource that is limited in availability.' Two major types of competition are recognized – these are exploitation and interference competition (Lockwood, 1992; Wicklow, 1992). Exploitation competition occurs when one organism depletes the resources without preventing access of other organisms to the same resources; however the former organism reduces availability to the latter organism. Usually the presence of certain characteristics that are favourable for exploitative competition contributes to success, this include rapid germination of spores, a rapid growth rate, and an ability to quickly consume the available nutrients. Due to the large quantities of cellulose and lignin nutrients present in plant residues, it is pertinent that a fungus has good enzymatic machinery to be at a competitive advantage when utilizing plant residue (Moore-Landecker, 2002). On the other hand, interference refers to the situation where one organism inhibits another. For example a fungus may be directly attacked by parasitic or predatory organisms. Such interference could induce antibiosis or cause hyphal interference (Wicklow, 1992; Moore-Landecker, 2002).

2.8.1.1 Outcomes of competitive interspecific interactions

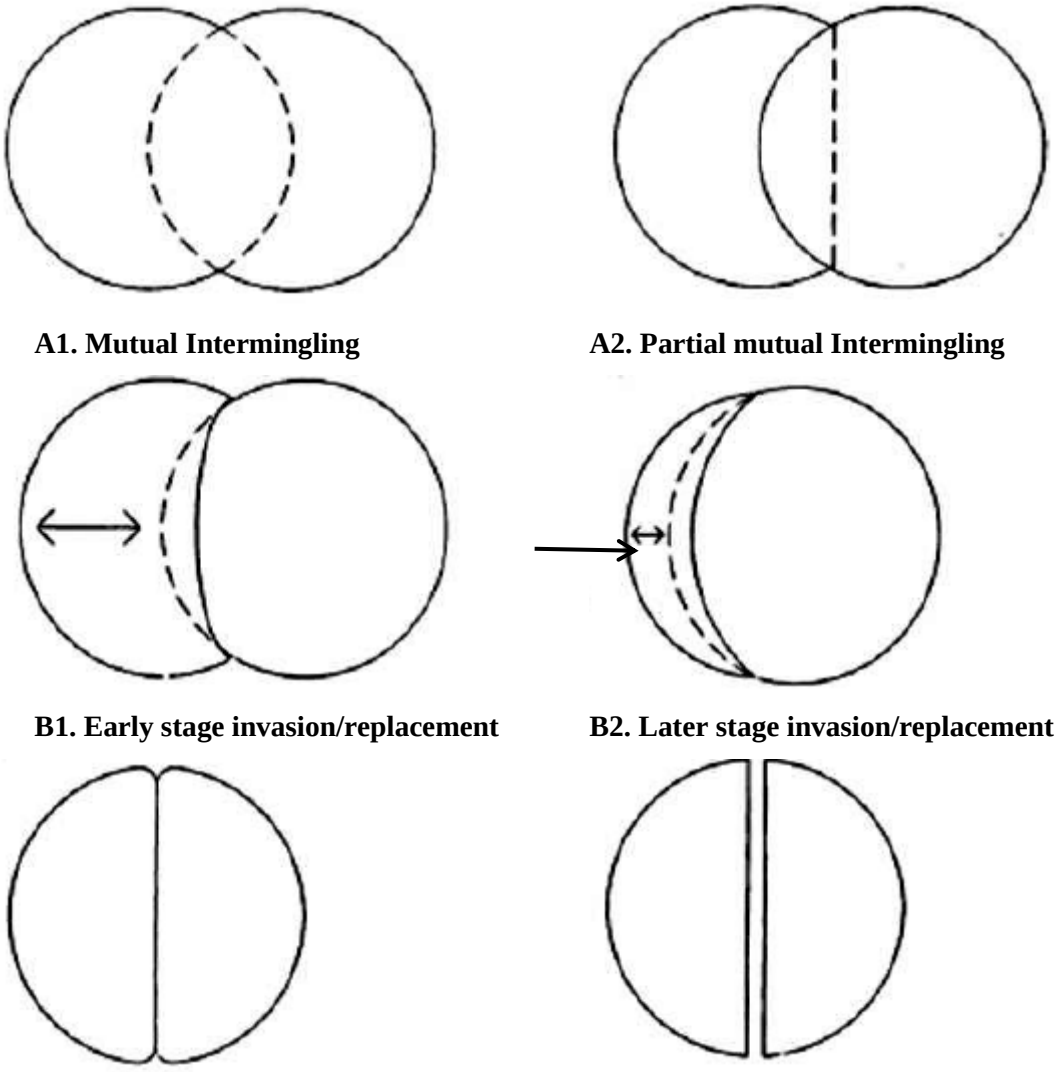
In any given fungal community combative antagonistic interactions will ultimately result in either deadlock or replacement. Although there are slight variations to these two outcomes, the results tend to follow either of these two patterns. Deadlock occurs when neither species gains headway. It is possible for both species to progress on the substratum until they meet and mycelia from one population touches the other, when this occur it is regarded as deadlock at touching point. There may not be any significant physiological difference between the fungi, so that such interaction does not produce any 'winners' or 'losers' (Moore-Landecker, 2002). Deadlock at touch point could also result in the formation of barrages at the point of interaction, to prevent further invasion into their respective territories (Rayner *et al.*, 1995). However another variation to deadlock is when a clear zone of inhibition is established between the two combating organisms, this is referred to as deadlock at a distance (Stahl & Christensen, 1992; Woodward & Boddy, 2008).

There are two conditions under which replacement can occur in the natural environment that is when an existing fungal population may decline in a substratum as a result of nutrient depletion to the extent that the substratum environment is unfavourable for growth, this may lead to the disappearance of the species. Replacement will occur if the arriving fungus is capable of utilizing the remaining organic compound, or because it can tolerate the prevailing conditions (Moore-Landecker, 1996).

Alternatively in an environment where the two fungal populations are present; one fungal population may prove to be a better competitor and replace the other as a consequence. The extent of replacement may vary, when one species captures some but not all of the antagonist's territory, this is referred to as partial replacement, and this tends to lead to a stalemate (Boddy, 2000; Ujor, 2010). Mutual replacement occurs when one species takes some of the territory formerly occupied by the other or vice versa (Boddy, 2000). It is equally possible in later stages of replacement that the entire substratum is occupied by one fungal population leading to the death of the other population (Stahl & Christensen, 1992).

The ability of one organism to successfully replace the other is very much dependent on the combative machinery available to one or both of the interacting species. The populations can engage in exploitative competitions, with factors such as differential growth rates or interference competition, in which the one of the combatants successfully utilize antibiosis,

mycoparasitism, or hyphal interference to capture the food substratum (Moore-Landecker, 1996). It is possible that with replacement one individual will completely overwhelm the other as a preamble to complete replacement. Replacement may induce autophagy, a mechanism that cells employ to degrade and recycle aged proteins and organelles in eukaryotic cells (Yorimitsu & Klionsky, 2005). Pinan-Lucarre *et al.* (2005) validate the presence of key genes involved in autophagy in filamentous fungi. For successful complete replacement, the membrane of the weaker colonies of fungi will lyse (cellular necrosis), thereby releasing the hyphal content that can subsequently be used by the antagonist (Falconer *et al.*, 2008).



A1. Mutual Intermingling **A2. Partial mutual Intermingling**

B1. Early stage invasion/replacement **B2. Later stage invasion/replacement**

C1.Inhibition/Deadlock (at touching point) **C2. Inhibition/Deadlock (at distance)**

Figure 2.13: Alternative types of interaction between two fungal populations competing for the substratum after one week incubation period on MEA (based on observation of Porter (1924) as cited by Skidmore and Dickinson (1976) and Stahl and Christensen (1992).

It was observed that the pairing of *Peniophora lycii* with *Coriolus versicolor* in twenty replicates resulted in overgrowth of *P. lycii* by *C. versicolor* in two cases, while *P. lycii* replaced *C.versicolor* in the other pairings (Rayner *et al*., 1995). However, under laboratory conditions it has been observed that repeated pairings between the same fungi do not always produce the same outcome (Boddy, 2000).

Although not a commonly observed phenomenon, it is noteworthy to mention a form of interaction when two populations of fungi intermingle. It is often confused with partial and mutual replacement and occurs when fungi species are closely related; such interactions may be neutral with no gain by the two fungi co-existing or result in synergism (Stahl & Christensen, 1992; Moore-Landecker, 1996; Boddy, 2000).

2.8.2 Mechanisms employed in antagonistic interspecific interactions

Interspecific interactions lead to complex and morphological, physiological and biochemical changes that are dependent on species present and environmental conditions (Rayner & Boddy, 1988; Griffith *et al*., 1994; Boddy, 2000; Baldrian, 2004; Iakovlev *et al*., 2004; Woodward & Boddy, 2008). A broad classification can be summarised into two main strategies – these are development of attack or defence mechanisms and improvements in nutrient uptake (Hiscox *et al*., 2010). However numerous examples of interaction-specific responses include rapid cell division, branching, hyphal aggregation, aerial growth, autolysis, pigment production, pigment loss, release of volatile signal chemicals, diffusible enzymes, toxins and antifungal metabolites (Griffith *et al*., 1994; Boddy, 2000; Baldrian, 2004; Hynes *et al*., 2007; Evans *et al*., 2008; Woodward & Boddy, 2008). These physiological and biochemical changes determine the outcome of the interaction (Hiscox *et al*., 2015).

Although studies on molecular level changes are sparse, there are varied explanations for the changes demonstrated during antagonistic interspecific interaction. Studies done by Iakovlev *et al.* (2004) using mRNA differential display to probe gene expression patterns to non-self-mycelia showed that the interaction of mycelia induced secondary metabolism and oxidative stress. On the other hand, Adomas *et al.* (2006) used macroarray technology to show that nutrient acquisition mechanism is significantly employed by the antagonistic species. More recently Arfi *et al.* (2013) used PCR analysis and transcriptome sequencing to suggest that broad array of functions is used by the antagonistic fungi, *Pycnoporus coccineus* in replacing competing fungi with the major role being that of secondary metabolites detoxification.

The utilization of antagonistic interspecific interaction holds great promise not only as a screening tool for the efficacy of biocontrol fungi against plant pathogenic and wood rot fungi but they can also be used as bio-control agents (Holdenrieder & Grieg, 1998; Savoie & Mata, 1999; Fravel, 2005; Costa *et al*., 2013). They have also been exploited to enhance wood-pulping (Baldrian, 2004; Chi *et al*, 2007) and for optimizing enzyme production as well as screening for novel industrially useful enzymes (Score *et al.*, 1997; Parke *et al*., 2001; Zhang *et al.*, 2006; Gregorio *et al.*, 2006).

2.8.2.1 Morphological response to antagonistic interspecific interactions

2.8.2.1.1 Gross mycelial contact

Gross mycelial contact is often demonstrated among wood decay fungi (Webber & Heger, 1986; Boddy, 2000), causing a rather obvious morphological and associated physiological changes. The most obvious change being the formation of defensive mycelial barrages on agar plates, at the contact points in resistance to invasion. With some other fungi such invasion could cause the formation of mycelial fans and cords, and rhizomorphic structures (Boddy, 2000). Such a shift in mycelial distribution could cause a release of hydrophobic metabolites and a consequential increase in the secretion of phenol-oxidizing activity (laccases and peroxidases) (De Vries *et al.*, 1986; White *et al.*, 1992; Griffith *et al.*, 1994a; 1994b; Score *et al.*, 1997; Gkarmiri *et al*., 2015). In some other cases changes may occur in inter- and intracellular pigmentation as well as a barricade of the mycelial front at contact-points (Griffith *et al.*, 1994b; Boddy, 2000; Gregorio *et al.*, 2006; Peiris, 2009). These reactions are considered to be the after-effects of resistance to axial deformation by confronting mycelia (Rayner *et al*., 1995). It is presumed that a reciprocal relationship between tyrosinase and laccase enzyme activity is responsible, often an indication is seen with the formation and suppression of aerial mycelium respectively. Notably there are marked differences in the release of hydrophobic metabolites in extracts from interacting cultures at different stages of development (Griffith *et al.*, 1994b; 1994c; Rayner *et al.*, 1994). Tyrosinase production in fungi is associated with melanin production which is part of the defense mechanism employed under stress in abiotic stress (UV radiation, free radicals, gamma rays, dehydration and extreme temperature changes) and it also contributes to to the fungal cell wall resistance against hydrolytic enzymes thereby preventing cellular lysis (Beel & Wheeler, 1986; Duarte *et al*., 2012).

2.8.2.1.2 Hyphal Interference

Contact and fusion of the two combating fungal hyphae is required for hyphal interference. It is commonly observed in closely related species of the Basidiomycota. It follows a programmed sequence of cytoplasmic destruction that begins with a change in membrane permeability, and this is followed by the appearance of lipid globules and vesicles. Organelles such as mitochondria and nuclei become swollen. The swollen mitochondria disintegrate, forming vacuoles, and eventually the entire cell becomes vacuolated as well. The zone of hyphal interference is distinguished by having various types of vesicles radiating outside the plasmalemma. Lysis and death of the hyphae is the final outcome that could affect either one or both of the contacting hyphae (Ikediugwu *et al*., 1970; Ikediugwu, 1976; Rayner & Webber, 1984; Boddy, 2000; Behrendt & Blanchette, 2001; Ujor, 2010). The phenomenon of hyphal interference has been observed in several species; however, the exact mechanism is presently unknown. Furthermore, the exact mechanism with which fungi differentiates self from non-self that stimulates the productionoftoxic substances leading to hyphal death remains inexplicable. However, there are hypothetical explanations offered with two major differing opininons: that it could be an ancient phenomenon conserved in fungi and others propose that it could possibly be the result of convergent evolution (Silar, 2012).

A rather similar occurrence to hyphal interference is 'mycelial interference', however the latter involves cord systems which when they make contact with other antagonising cord systems the area of contact is often observed to have a marked yellow or brown discoloration with associated lytic responses in one or both cord segments involved (Boddy, 2000). It has been suggested that hyphal interference is facilitated by non-enzymic diffusible metabolites, which are secreted when non-self mycelia are detected at close range (Boddy, 2000). Moreover, the process may also involve secretion of both active and passive non-enzymic toxins in the presence and absence of confronting fungal mycelia as reported in *Hypomyces aurantius* and *Phanerochaete magnolia* respectively (Rayner & Boddy, 1988; Ainsworth *et al*., 1991; Jeffries, 1997; Boddy, 2000).

Hyphal interference is prevalent amongst wood-decaying basidiomycetes, the most extensively studied being *Heterobasidion annosum* hyphae in contact with *Phlebiopsis gigantea* because of its application to biocontrol (Rayner & Boddy, 1988; Jeffries, 1997; Boddy, 2000).

Although laboratory media have been used extensively to study fungal interactions, Boddy (2000) warns that one must treat such results and its interpretation with caution. Several authors support this argument by indicating that a wider range of factors influence interactions in natural substrata, therefore, outcomes of such interactions would likely vary from those on agar (Dowson *et al.*, 1988; Pearce, 1990; Griffith & Boddy, 1991; Hiscox *et al.*, 2010). Dowson *et al*. (1988) showed that whereas *Hypholoma fasciculare* replaced *Steccherinum fimbriatum* under ambient conditions on agar, it deadlocked with it in soil and was replaced by the latter in wood. Water activity, gaseous regimes, and to a lesser extent temperature, have been shown to have a sizeable effect on the outcomes of interactions in agar cultures (Boddy *et al*., 1985; Boddy *et al*., 1987; Griffith & Boddy, 1991). Such premise is the basis for studies involving dual culture interactions on specific substrate such as a variety of agricultural residues.

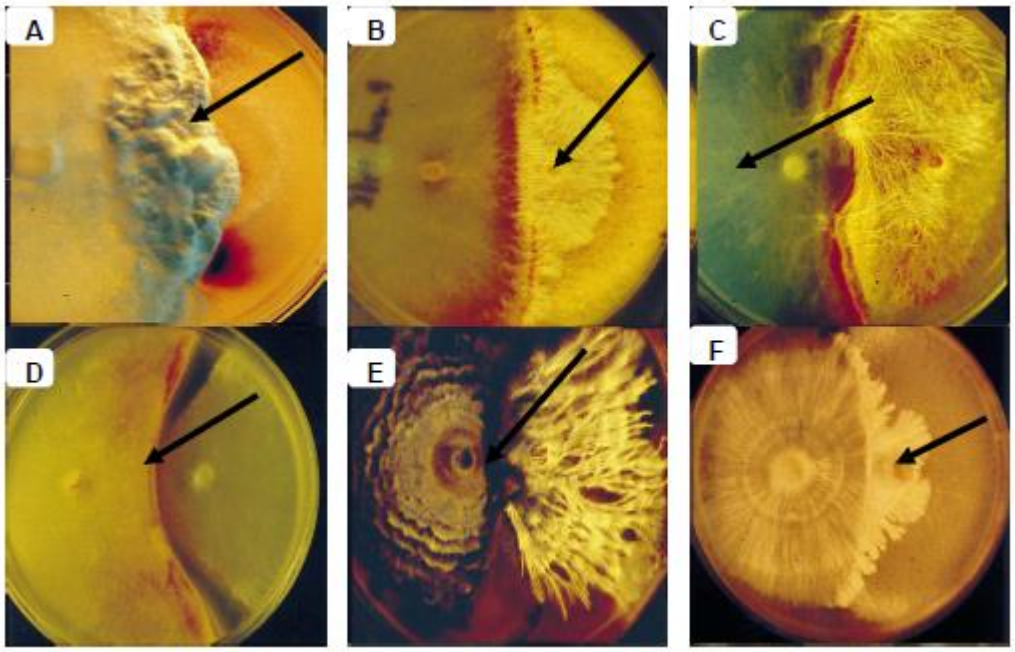


Figure 2.14: Agar plate images showing macroscopic morphological changes associated with interspecific mycelial interactions in fungi (adapted from Boddy, 2000). A: development of invasive mycelial front by *Phanerochaete velutina* replacing *Stereum hirsutum* at 25 °C; B: formation of mycelial fans by *P. velutina* replacing *S. hirsutum* at 10 °C; C: reduction of mycelial density by *Hypholoma fasciculare* replacing *Coriolus versicolor*; D: Complete replacement of *Armillaria gallica* by the ascomycete, *Xylaria hypoxylon*; E: mycelial interference of *P velutina* by *Resinicium bicolor* on soil; F: replacement of *S. hirsutum* by *Psathyrella hydrophillum*.

2.8.2.2 Physiological response to interspecific interactions

2.8.2.2.1 Accelerated secondary metabolism

Several authors have reported that a major consequence of non-self fungus-fungus interaction is a switch to secondary metabolism (Rayner *et al*., 1994; Griffith *et al*., 1994; Rayner 1997; Baldrian, 2004; Maijala, 2005). The abiotic stress conditions associated with nutrient limitation, inability to absorb or metabolise nutrients tends to trigger the initiation of secondary metabolism (Rayner *et al.,* 1995). It is presumed that disruptions in nutrient uptake may result in a decrease in energy levels, with a corresponding rise in the levels of secondary messenger, cAMP (Rayner *et al.,* 1995). Such changes in physiology and metabolism within the cell is demonstrated in increased production of secondary metabolites including phenolics as well as inhibition of ATP synthesis which maybe a consequence of uncoupling oxidative phosphorylation and electron transport with associated rise in the levels of free radicals (Rayner *et al.,* 1995). Earlier reports showed a relationship between oxidative phosphorylation and the activity of phenoloxidases (Lyr, 1958; 1963). Inferences can be made that such reasoning could explain why the exposure of single species to the uncoupling agent 2,4-dinitrophenol resulted in a similar reaction as non-self fungus-fungus confrontation (Griffith *et al.,* 1994; Rayner *et al.,* 1994). Further inferences can be made that non-self mycelial interaction have a significant impact on nutrient metabolism, thereby stimulating a switch of mycelial growth to secondary metabolism with a direct effect on the mitochondrion and its ability to generate energy (Rayner *et al.,* 1995). Iakovlev *et al.* (2004) provided evidence to support this earlier assumptions, they demonstrated the repression of a gene encoding a fimbrin protein and a gene that codes for a mitochondrial import component in the mycelia of *Physisporinus sanguinolentus*, and up-regulation of a gene homologous to the *Coprinus cinereus* (*Coprinopsis cinerues) recA* in *Heterobasidion annosum* during antagonistic interactions between both species. Because fimbrin protein is involved in the polarization of the actin cytoskeleton, it repression in *P. sanguinolentus* was an indication of arrest of cell growth (Iakovlev *et al*., 2004). Furthermore, Iakovlev *et al.* (2004) proposed that the fluctuations in mitochondrial energy production may be attributed to the repression of a gene (*recA*) that codes for a protein (RAD51) involved in mitochondrial import which is often associated with secondary metabolism. It was observed that RAD51 is strongly induced by irradiation with a consequential induction of hydroxyl radicals. Presumably the up-regulation of the gene, *recA*, could be the reason for the possible increase in the production of free radicals during interspecific mycelial interactions. There are studies that however implicate reactive oxygen species (ROS) as a major compound produced during non-self fungus-fungus interactions (Baker & Orlandi, 1995; Li *et al*., 1995; Ruiz-

Dueñas *et al*., 1999; Hammel *et al*., 2002). During abiotic stress, it is suggested that the production of ROS may be a component of the stress exerted on interacting fungal mycelia. However it is unclear if it is produced as an aftermath of interspecific mycelial contact or as a result of the induction of secondary metabolism (Ujor, 2010; Breitenbach *et al*., 2015).

2.8.2.3 Biochemical response to interspecific interactions

2.8.2.3.1 Antibiosis

Fungi can modify their environment (antibiosis) by secreting volatile or diffusible metabolic wastes or by-products. This form of interference competition may include the production of carbon dioxide, alcohols, acids, and other simple compounds that have a nonspecific effect. It is also possible that by-products such as high molecular weight antibiotic compounds with specific toxic effects on susceptible micro-organisms are produced to ward-off attack or invasion of territory. Because these compounds are diffusible or volatile in nature, the effect of such antagonism can be felt at a distance (Sonnenbichler *et al*., 1994). More often than not, the antibiotic producer is not immune to its effects but may have a high tolerance (Moore-Landecker, 1996) which is why it is commonly observed as mutual inhibition on agar plates with clear zones of inhibition that neither organism can grow upon. Reactions and responses vary depending on species combinations suggesting a reciprocal exchange of chemical signals and recognition at a distance (Rayner & Webber, 1984). On agar plates it is clear to distinguish mutual inhibition for 15mm or more, but the distances over which such mechanisms operate in the natural environment are unknown, though they are likely to be shorter in organic substrata than those observed on plates (Boddy, 2000).

2.8.2.3.2 Mycoparasitism

Majority of knowledge on the biology of mycoparasitism are based on laboratory observations rather than actual field studies (Moore-Landecker, 1996). However pioneer work on this phenomenon done by Barnett and Binder (1973) showed that mycoparasites vary significantly in their method of obtaining nutrient and in some other biological attributes. But a clear demarcation in describing will be that there are two kinds of mycoparasite-types: one that are highly specialized and cause no harm to their hosts known as ‘biotrophs’ and the other, are mycoparasites that kill their host either before penetration or shortly after. Either way, there are distinct phases in parasitism by a fungus over another or it involves host-directed mycelial growth, host recognition and attachment, pronounced synthesis and secretion of cell wall lytic enzymes and antibiotics, penetration and lysis of the host fungus (Markovich & Kononova,

2003). Some studies have demonstrated that host recognition is mediated by cell wall lectins (Barak & Chet, 1986; Inbar & Chet, 1994; Jeffries, 1997; Chet *et al*., 1997; Omann & Zeilinger, 2010; Cooper- Driver *et al*., 2013).

Rayner *et al.* (1987) reported that parasitism in order to obtain nutrition was a temporary measure for *Lenzites betulina* (parasite) that facilitated the possession of the large mycelial domain occupied initially by the host *Coriolus* species. Once the parasite had established itself in the host environment it began obtaining its own nutrition by decomposing the wood and even interacted aggressively with other fungi by gross mycelial contact. A similar observation was made with *Pseudotrametes gibbossa* which is parasitic on *Bjerkandera* species (Rayner *et al*., 1987; Boddy 2000).

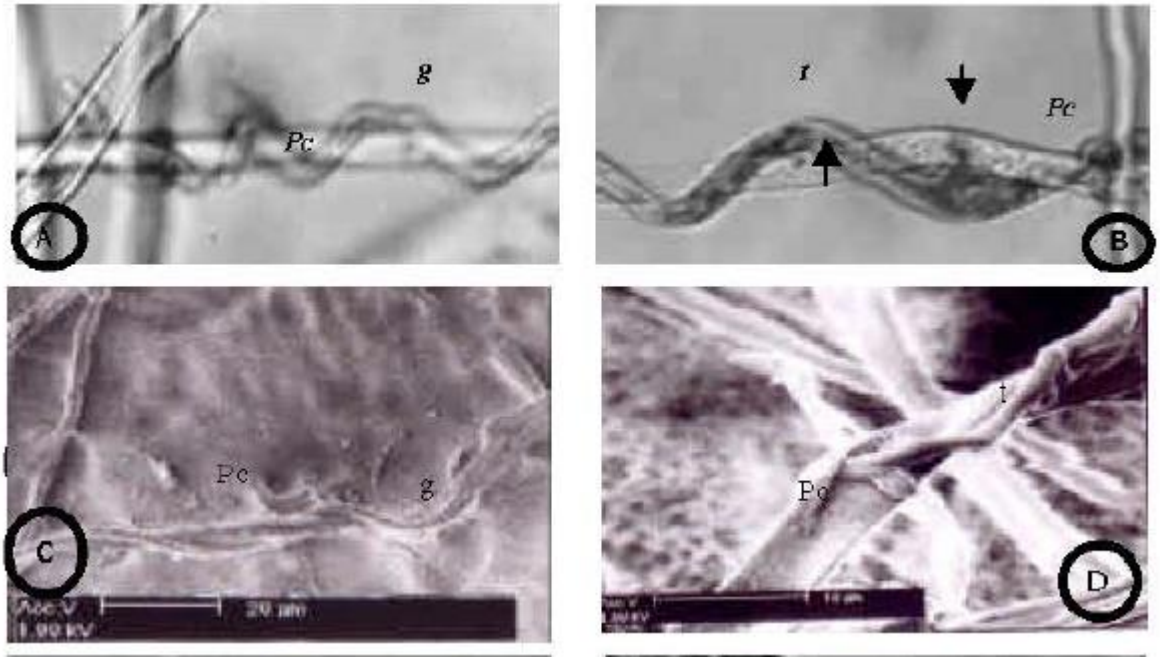


Figure 2.15: Mycoparasitic interaction of fungal isolates of *Phytophthora cinnamon* (Pc). (A) Coiling by *Gliocladium penicillioides* (g); (B) Penetration by *Trichoderma* sp. (t) with appresorium (arrow); (C) coiling observed with SEM; (D) Hyphal penetration by *Trichoderma* sp. observed with SEM (adapted from Aryantha & Guest, 2006)

By far the most extensively studied mycoparasitic fungi are the members of the genus *Trichoderma*. Their ubiquity in nature makes isolation relatively non-tedious, they have been isolated from soil, decaying wood and other forms of plant-related organic materials (Zeilinger & Omann, 2007). Various mechanisms seem to be exploited by *Trichoderma* species in

mycoparasitism and they include: cell wall hydrolytic enzymes - chitinases, proteases, glucanases and laminarases (Kubicek *et al*., 2001; Brunner *et al*., 2003; Howell, 2003; dos Reis Almeida *et al*., 2007; Gruber *et al*., 2011; Reithner *et al*., 2011); the use of advanced growth and nutrient acquisition mechanisms to gain competitive edge and foothold (Chet, 1987; Harman, 2006), and secretion of antifungal metabolites/peptides – peptaibols/mycotoxins (Schirmbröck *et al*., 1994; Lorito *et al*., 1996; Jaworski *et al*., 1999; Mukherjee *et al*. 2012). *Trichoderma* species have also been known to use morphological adaptive mechanisms such as coiling around the host and the development of antagonistic appressoria-like structures (Elad *et al*., 1983; Lu *et al*., 2004). Often these mechanisms are employed in a synergistic manner to damage the host or in the utilization of host components (Zeilinger & Omann, 2007). The initial presumption that only chitinases were involved in mycoparasitism (Patil *et al*., 2000) was refuted by Kubicek *et al*. (2001); they demonstrated that the median lethal concentration (LC50) values of *Trichoderma* chitinases are too high to explain mycoparasitism exclusively on the basis of their action. Their claim was further supported by the identification of antifungal peptides (peptaibols) a mycotoxin produced by *Trichoderma* species during interactions with host fungi (Kubicek *et al*., 2001).

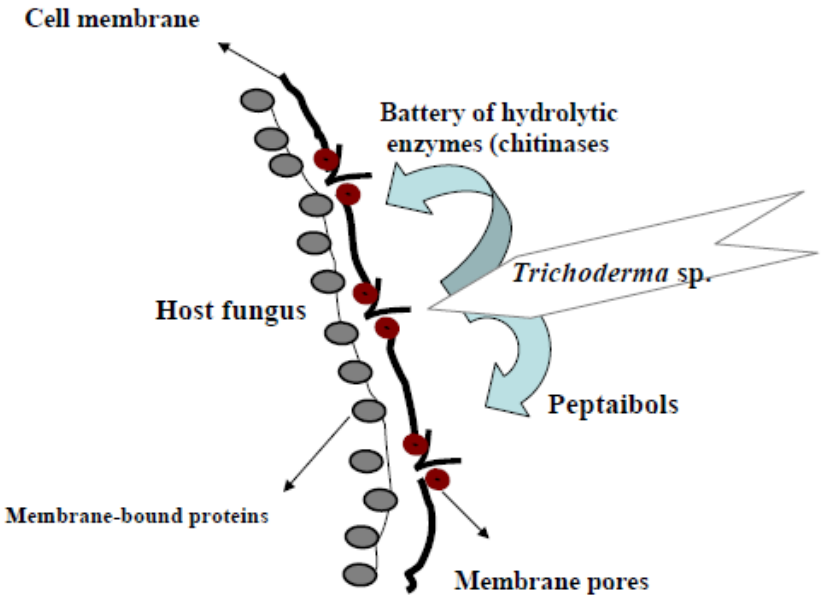


Figure 2.16: Hypothetical model of synergism between *Trichoderma* cell wall hydrolases and membrane disturbing compounds such as peptaibols employed in mycoparasitism (adapted from Kubicek *et al*., 2001 and cited by Ujor, 2010)

These peptaibols are linear oligopeptides which form voltage-gated ion channels in lipid membranes thereby modifying membrane permeability and inhibiting the activities of

membrane-bound synthases of the cell wall (El Hajj *et al.*, 1989; Kubicek *et al*., 2001). Although chitinases reduce cell wall rigidity (Reyes *et al.*, 1990; Patil *et al.*, 2000; Lindahl & Finlay, 2006), without peptaibol antibiotics to impair the ability of the hyphae to repair cell wall damage to the host, mycoparasitic penetration of the host cell will not be achieved. It is presumable that other mechanisms may be included in the synergistic lysis of host cell wall (Ujor, 2010).

Several authors have shown a correlation between the production of chitinases by *Trichoderma* species and abiotic stress conditions such as prolonged carbon and nitrogen starvation, acidic pH, cold temperature (4°C), high temperature (40°C), high osmotic pressure and the presence of ethanol (Carsolio *et al*., 1994; Garcia *et al*., 1994; Margolles-Clark *et al*., 1996; Bhushan, 1998; Mach *et al*., 1999; Hamid *et al.*, 2013). This allows for the inference that host fungi may be recognised by *Trichoderma* species as a source of physiological stress therefore the expression of chitinases is a direct response. Moreover Inbar and Chet (1995) showed that *Trichoderma harzianum* produced different combinations of chitinase isozymes in response to different host/confronting species. This lends credence to the antagonistic adaptability of *Trichoderma* species and could be attributed to variations that often exist in host cell wall composition and varying antagonistic pressures that may be presented by different hosts (Ujor, 2010).

2.8.2.3.3 Pigmentation

A majority of the pigments found in higher fungi are quinones or similar conjugated structures classified based on the perceived biosynthetic pathways, reflecting their structure, therefore to pigments derived from (i) the shikimate (chorismate) pathway, (ii) the acetatemalonate (polyketide) pathway, (iii) the mevalonate (terpenoid) pathway, and (iv) pigments containing nitrogen (Gill & Steglich, 1987; Gill, 1994;1996;1999; 2003;Hanson 2008a; 2008b; Räisänen, 2009; Zhou & Liu 2010;Velíšek & Cejpek, 2011).

However a wide range of compounds including arylpyruvic, cinnamic and benzoic acids which serve as building blocks for many pigments of higher fungi requires phenylalanine and tyrosine as precursors, therefore the shikimate pathway is often used for these pigmentations. The shikimate pathway provides a route to these essential amino acids, phenylalanine, tyrosine and tryptophan via the central intermediates shikimic and chorismic acids (Velíšek & Cejpek, 2011). In the case of melanin, it is generally accepted that ascomycetes fungi produce melanin

from 1,8 dihydroxynaphthalene (DHN), while basidiomycetes fungi usually produce catechol melanin from γ-glutaminyl-3,4 – dihydroxybenzene precursor, also known as GDHB melanin, and more rarely from L-dihydroxyphenylalanine (L-Dopa) (Turner, 1971, Wheeler & Bell, 1985).

The fungal metabolism is affected by the nutrient availability and chemical composition of the wood substrate (Schmidt, 2006). Pigmentation tends to vary with age, and in some cases distinctive colour changes are associated with bruising. Pigmentation may offer protection against UV damage and unfavourable environmental conditions. The production of pigments is often a response to antagonistic interactions during fungus-fungus contact. Pigmented substances are produced during secondary metabolism that functions as physical and chemical barriers in the wood substrate (Tudor *et al*., 2013). Development of pigments in cultures of interspecifically paired fungi has been strongly attributed to increased synthesis and secretion of secondary metabolites (Rayner & Boddy, 1988; White & Boddy, 1992; Griffith *et al*., 1994a; 1994b; 1994c; Rayner *et al*., 1994; Score *et al*., 1997; Peiris *et al*., 2008; Hynes *et al*., 2007; Peiris, 2009). Li (1981) observed black lines of demarcation or pigmented zones which were formed on agar plates by two isolates of *Phellinus weirii* and *P. weirii* in response to the presence of an antagonist. Furthermore the activity of enzymes from the zone lines when compared with that of activity of enzymes from the mycelial tissue adjacent to the zone lines showed that zone lines produced stronger phenoloxidase and peroxidase reactions than those of the adjacent tissues. Pigmentation during combative interactions is thought to be mainly an after effect of the interplay between phenolics and phenol-oxidase activities (Rayner *et al*., 1994; Griffith *et al*., 1994a; 1994b; 1994c; Boddy, 2000; Gregorio *et al*., 2006; Peiris *et al*., 2008; Peiris, 2009). This assumption was supported by studies done by Peiris *et al. (*2008), who reported predominance of metabolic peaks with mass spectra which strongly implied the possession of aromatic structures such as 1-methyl-3,5-dihydroxybenzene and 1,2-dihydroxyanthraquinone, potent inducers of laccase/manganese peroxidase, during combative interactions between *Stereum hirsutum* and its two competitors *Coprinus micaceus* and *Coprinus disseminatus*.

The synchronized detection of volatile organic compounds (VOCs) particularly sesquiterpenes and quinolinium-type compound and induction of pigment accumulation in paired cultures of *H. fasciculare* and *Resinicium bicolor* by Hynes *et al.* (2007) further lends credence to the supposition that pigmentation is anactive component of antagonistic interactions and that

phenolics and phenol-oxidases are involved. Tsujiyama and Minami (2005) concluded that phenol-oxidizing enzymes play a central role in the detoxification of harmful compounds during fungus-fungus interactions, as species with superior phenol-oxidase-secretory abilities in their study were more predominant during mycelial conflicts. As most of these enzymes tend to be secreted during oxidative and abiotic stresses, it has been suggested that they are part of the defensive machinery used in the detoxification of reactive oxygen species (ROS) (De Vries *et al.*, 2007; Hynes *et al.*, 2007; Evans *et al.*, 2008).

2.8.2.3.4 Enzymatic activities

Most of the studies on enzymes involved in interspecific antagonistic interactions have focused on the *Trichoderma* species. Examples include *T. harzianum*, *T. viride* and *T. reesei* which are all known producers of a variety of hydrolytic enzymes including cellulases chitinases, glucanases, lipases and proteases used in mycoparasitism (Haran *et al.*, 1996). During intra- and interspecific interactions in fungi, enzymes are known to play critical roles in detoxification, attack/defence and nutrient acquisition that overall influence the outcome of competition (Score *et al.*, 1997; Cortes *et al.*, 1998; Zeilinger *et al.*, 1999 ; Boddy, 2000; Iakovlev & Stenlid, 2000; Kubicek *et al.*, 2001; Brunner *et al.*, 2003; Tsujiyama & Minami, 2005; Adomas, *et al.*, 2006; Gregorio *et al.*, 2006; Chi *et al.*, 2007; Zhang *et al.*, 2006; Peiris, 2009; Kushwaha & Verma, 2014).

Chitinases are employed by fungi in degrading chitin which is an unbranched homopolymer of 1,4-*β*-linked *N*-acetyl-D-glucosamine (GlcNAc). Chitin is the second most abundant polymer in nature after cellulose. It is the prevalent structural polymer found in most fungi and insects (Havukkala, 1991; Howell, 2003). Therefore, for successful antagonistic activity it is vital that chitinase be secreted to hydrolyze the chitinous cell wall of the fungi under attack causing the lysis of the hyphae (Lorito *et al.*, 1994; Carsolio *et al.*, 1999; Sangle & Bambawale, 2004; Lindahl & Finlay, 2006).Sahai and Manocha (1993) divided chitinolytic enzymes into three principal types: (a) 1,4-*β*-*N*-acetyl-D-glucosamindases (EC3.2.1.30) which split the chitin polymer into GlcNAc monomers in an exo-type fashion; (b) endochitinases (EC3.2.1.14), which randomly cleaves at internal sites over the entire length of the microfibril and (c) exochitinases (EC3.2.1.14), which catalyse the successive release of diacetylchitobiose in a step-wise fashion such that no monosaccharides or oligosaccharides are formed. Harman *et al.* (1993) referred to enzyme exhibiting this activity as ‘chitobiosidase’.

It is well known that a number of *Trichoderma* isolates secrete 1,3-β- and 1,6-β-glucanases (Del Rey *et al.*, 1979; de la Cruz *et al.*, 1995; Howell, 2003; Glese *et al.*, 2011). Glucanases prompt chitinases and proteases to bring about lysis of several phytopathogenic during mycoparasitic process (Elad *et al.*, 1982). The structural composition of fungi cell wall determines the extent of degradation; therefore the cell wall structural matrix of chitin and β-glucan implies that more than one is require for its successful degradation. Moreover, chitin seems to be protected by β-glucan and is not readily accessible to chitinases (Cherif & Benhamou, 1990). Therefore, it most likely that chitinase activity is preceded by, or coincides with the hydrolytic activity of other enzymes in particular 1,3-β- and 1,6-β-glucanases. Lorito *et al.* (1994) provided evidence of the involvement of glucanases in the mycoparasitic process when they introduced purified 1,3-β-glucanases obtained from *T. harzianum* strain P1 on to *Botrytis cinerea*. This purified enzymes inhibited spore germination and germ-tube elongation of *B. cinerea* with at least 50%-effective dose values. De La Cruz *et al.* (1995) used purified 1,6-β-glucanases in combination with other cell-wall-degrading enzymes such as 1,3-β-glucanases, chitinases, all obtained from *T. harzianum* strain CECT 2413, to hydrolyse filamentous fungal cell walls and also inhibit the growth of several fungi tested including *B. cinerea*, *Gibberella fujikuroi*, *Phytophthora syringae* and *Saccharomyces cerevisiae*.

Filamentous fungal cell also contains lipid and protein (Hunsley & Burnett, 1970). As such proteolytic enzymes are required by antagonistic fungi for complete lysis of host cell wall (Howell, 2003). This is because the key components of fungal cell wall and/or fibrils of β-glucans are embedded into a protein matrix (Wessels 1986; Peberdy, 1990); therefore extracellular proteases play a vital role in the degradation of host cell wall (Nygren *et al.*, 2007). Elad *et al.* (1999) suggested that proteases secreted by *Trichoderma* species may be involved in the inactivation of vital extracellular enzymes of their host cells, especially those involved in nutrient hydrolysis and absortption.

Although phenol-oxidases have been studied extensively in monocultures and the early parts of this literature review has highlighted their structure and application; it is noteworthy that it be highlighted in literature involving interspecific antagonistic interactions. Their exceptional ability to detoxify a wide range of xenobiotics *in vitro*, particularly those with phenolic/aromatic structures with subsequent production of pigments/discolouration (Thurston, 1994; Reinhammer & Malstrom, 1981; Guillen, *et al*., 1994; Eggert *et al*., 1996) has strengthened the assumption that their major role during fungal interactions is to oxidise

toxic compounds produced by the same or confronting species during mycelial conflict (Rayner *et al*., 1994; Griffith *et al*., 1994a; 1994b;1994c;White & Boddy, 1992; Tsujiyama & Minami, 2005; Gregorio *et al*., 2006). Several authors have reported an increase in laccase activity (Freitag & Morrell, 1992; Baldrian, 2004; Tsujiyam & Minami, 2005; Gregorio *et al*., 2006; Elisashvili & Kachlishvili, 2009). Tsujiyama and Minami (2005) detected the predominant presence of phenol-oxidase activity in the zones of confrontation and underneath mycelia overgrowing another fungus during inter-specific combat involving *Pleurotus ostreatus*, *Trametes versicolor*, *Pynoporus coccineus*, *Ganoderma applantum* and *Schizophyllum commune* on agar. The study demonstrated that species that were more efficient at phenol-oxidase expression were more successful in replacing the less efficient ones.

In recent times, most of the fungal enzymes used in industrial biotechnology are produced by processes involving single microbial strains (Bader *et al*., 2010). However one major drawback to utilizing ligninolytic fungi in industrial scale fermentation is the low productivity and high cost implication for the production of the constitutive extracellular enzymes produced by these organisms (Flores *et al*., 2009; Rivera-Hoyos *et al*., 2013). Chemical induction of laccase and MnP by the addition of compounds related to lignin or lignin derivatives such as phenolic and aromatic compounds, copper and ethanol have been studied extensively (Baldrian, 2003; Gill & Arora, 2003; Lomascolo *et al*., 2003; Shah *et al*., 2006; Revankar *et al*., 2007; Kocyigit *et al*., 2012; Kuhar *et al*., 2014; Wang *et al*., 2014). However, the use of chemical inducers makes the industrial process economically non-feasible and in some cases these chemical inducers are toxic.

Therefore any industrial process that would in future exploit the use of ligninolytic enzymes must produce economical and safe laccase and MnPs. For this reason this has been the objective of most enzyme researches in the past few decades (Flores *et al*., 2009). Using co-cultures appears to be more successful than using single microorganism cultures, because of the potential to utilize synergisms and even surprisingly antagonism between the metabolic pathways of the strains involved in the co-culture (Baldrian, 2004; Bader *et al*., 2010; Dwivedi *et al*., 2011; Pandya & Albert, 2014).

2.9 Application of antagonistic interspecific interactions

2.9.1 Biological Control

In the past, farmers have often relied on the application of chemical pesticides to reduce or eliminate incidences of plant diseases (Baker, 1987; Agrios, 1988). However, recent changes in public opininons – a consequence of environmental pollution caused by the excessive use of agrochemicals have prompted a shift to more safe agricultural practices. Furthermore, the implementation of stricter regulations on chemical pesticides application has caused an increase in the efforts to develop alternative methods of plant pests and disease control. Biological control exploits antagonistic interactons that exist in nature to reduce the damage caused mainly to plants, by pathogen and pests (Perotto *et al.*, 2013). Several authors have described examples of fungal antagonism used for the biocontrol of fungal plant pathogens, nematodes, weeds and insects (Marco *et al.*, 2003; Barron, 2004; Vega *et al.*, 2009; Güerri-Agulló *et al.*, 2011).

A strain of *Trichoderma viride* has shown significant success in the biological control of *Crinipellis perniciosa* the causal agent of the "witches' broom" disease of cocoa in South America (Bastos, 1991; Marco *et al.*, 2003). Similarly in India, *Trichoderma* sp. has proved effective as biological control against the stem rot disease of groundnut caused by *Sclerotium rolfsii* (Parmar *et al.*, 2015). In the Scandinavia, Korhonen (1993) isolated a strain of *Phlebiopsis gigantea* this is commercially used in Finland (Rostop®, product of Verdera Oy), Poland (PG IBL® from Biofood s.c.) and Great Britain ((PG® suspension made by Forest Research Surrey, UK) for the control of *Heterobasidion* species in spruce and pine stumps (Adomas *et al*., 2006).

2.9.2 Biopulping in Paper Production

The paper and pulp industry carry out two major process steps – pulping and bleaching that essentially generate environmental pollutants (Thompson *et al.*, 2001; Sumathi & Hung, 2006; Bahar *et al.*, 2011). Traditionally, the kraft process uses sodium hydroxide (NaOH) and sodium sulphide (Na_2S) to pulp wood creating black liquor as a by-product. Although efforts are made at the recovery of these chemicals it comes with concomitant pollution problems of air and water. Moreover, the bleaching process needed to remove the remaining fractions of lignin and whiten the pulp also uses chlorinated bleaching agents that also generate unwholesome effluents. Since the 1970s efforts have been made to improve technologies using biopulping and biobleaching as alternative to reduce chemical pollutions. Several authors have described

the benefits of pre-treatments involving white-rot fungi (Eriksson *et al.*, 1990; Kashino *et al.*, 1993; Messner & Srebotnik, 1994; Akhtar *et al.*,1998; Yu *et al.*, 2010). However the incubation time of between 2–4 weeks required todegrade the wood lignin limits the feasible industrial application of this alternative. Co-fungal cultures have been suggested as a cheaper alternative for biopulping process (Hatakka *et al.*, 2001; Chi *et al.*, 2007) based on abundant evidences that has demonstrated that fungal competition can improve lignin degradation (Sundman & Näse, 1972; Asiegbu *et al.*, 1996) and increased secretion of phenol-oxidising enzymes (Baldrian, 2004; Iakovlev & Stenlid, 2000; White & Boddy, 1992; Score *et al.*, 1997). Chi *et al.* (2007) demonstrated that co-culturing involving *Ceriporiopsis submervispora*, *Physisporinus rivulosus*, *Phanerochaete chrysosporium* and *Pleurotus ostreatus* in different combinations resulted in varied patterns of lignin degradation thereby suggesting that there is potential practical application in terms of shorter incubation times in exploring fungal interactions for the biopulping process.

2.9.3 Improved Enzyme Production

Exhaustive literature have shown that interspecific antagonistic interactions in fungi enhances the production of extracellular enzymes including chitinase, glucanases, xylanase, cellulase, laccase and phenol-oxidases (Zeilinger *et al.*, 1999; Boddy, 2000; Kubicek *et al.*, 2001; Baldrian, 2004; Tsujiyama & Minami, 2005; Gregorio *et al.*, 2006; Zhang *et al.*, 2006; Peiris, 2009; Flores *et al.*, 2010; Bader *et al.*, 2010; Dwivedi *et al.*, 2011; Albert *et al.*, 2011; Mucha, 2011; Cupul *et al.*, 2014). The implication of these studies is that co-cultivations of fungi can offer a cheap alternative to increasing enzyme production for several industries that employ enzymatic processes such as food, textile, paper and detergent making industries as well as in effluent treatment strategies (Savoie *et al.*, 1998; Hatvani *et al.*, 2002; Chi *et al.*, 2007). The cost of enzymes has always been a crucial limiting factor in the implementation of industrial bio-processes and has often driven industries towards practices that tend to generate often hazardous effluents in the bid to cut costs. Therefore studies that move us closer to finding ways to increase enzyme production must be continuous. The hope of this present study was that work done will improve our understanding of interspecific interactions amongst ligninolytic fungi and findings would assist with the development of a cost-effective, economically viable alternative for the hydrolysis of a variety of agricultural residues.

2.10 Aims and Objectives of Study

The literature reviewed for the purpose of this study has revealed that there are several aspects of both agricultural and industrial application that requires the increased production of enzymes. Furthermore, several studies have been done on interspecific interactions amongst fungal species, the biodiversity of the fungi community implies that there are several interactions that have not yet been studied. Moreover there is strong evidence that interspecific interactions can cause an acceleration into secondary metabolite production. The primary objective of this study is to investigate interspecific interactions amongst different fungi obtained from nature and to determine if such interactions increased enzyme activity. This study also investigated if such interactions are duplicated on different agricultural waste residues commonly generated in the South African region and whether substrate type will affect enzymatic activities with the potential for application in improving enzyme production.

Specific objectives included the following:

- To isolate, and screen different ligninolytic fungi obtained from the Kloofendal Nature Reserve for their ligninolytic activities
- To characterize and identify the different ligninolytic fungi using biochemical and molecular methods.
- To analyse the composition of the different agricultural residues (corn cob, sugarcane bagasse and wheat straw) and use them as substrates for the production lignin peroxidase, manganese peroxidase and laccase enzymes production
- To investigate patterns of growth in both monocultures and interspecific interactions on dual culture on the different agricultural residue media.
- To quantify and compare the enzymatic activities and production of three of the enzymes produced by the different ligninolytic fungi on different substrates.

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Chapter 3

Isolation, screening and molecular characterization of ligninolytic fungi

3.0 Abstract

A total of 30 fungal fruiting bodies were collected from decaying plant materials (barks and litter) from the Kloofendal Nature Reserve based on morphological variations. Nine of these fungi were included in the present study and a type strain *Ganoderma lucidum* ATCC- 32471. Preliminary screening showed that five of these organisms produced ligninolytic enzymes when exposed to two different concentrations of guaiacol (0.02% and 0.2%) on two different media (MEA and PDA). All ten fungal isolates screened for cellulolytic activity were positive for the production of the cellulase enzyme. Molecular characterization using ITS1 and ITS4 primers was able to identify these fungal isolates to degrees of accuracy ranging from 98% to 100%. The phylogenetic and lineage analysis showed that the species varied amongst phylum Basidiomycota, Ascomycota and early diverging fungal lineages Mucormycotina. This study demonstrated that the production of ligninolytic enzymes is not limited to Basidiomycetes as members of the Mucorales and Ascomycetes also demonstrated ligninolytic activity.

3.1 Introduction

The natural environment is comprised of microbial population that is very diverse, such that it is near impossible to identify all species found in it (Kirk *et al*., 2004; Anastasi *et al.*, 2013). On almost routine-like basis, new microorganisms are discovered. Such discovery of novel microorganism or new strains of previously identified microorganisms enhance our knowledge of a particular region's biodiversity thereby making it possible to obtain novel biological compounds that prove useful in industrial applications. To ease this task there is need to not only develop new isolation and characterization techniques (Stanbury *et al*., 1995; Wietse *et al*., 2005; Hays & Van Leeuwen, 2012) but to also modify existing techniques to enable us to coax otherwise fastidious microorganisms to grow under laboratory conditions. Several techniques have been used to detect incipient decay in wood, which include isolation and culturing fungi (Nicolotti *et al*., 2009), chemical staining (Jasalavich *et al*., 2000), nuclear magnetic resonance (Fackler & Schwanninger, 2012) and electrical resistance as well as serological methods, such as immunoblotting and enzyme-linked immunosorbent assay (ELISA) (Kim *et al*., 1991). The development of the DNA-based PCR and taxonomic specific

primers has made the detection and study of fungi in their natural habitat (substrate) and even on isolate media easier (Jasalavich *et al*., 2000; Nicolotti *et al*., 2009; Horisawa *et al*., 2009; Kirker, 2014).

In the forest ecosystem, wood decay fungi are crucial because of their pertinent role in the carbon nitrogen cycling to convert organic debris into the humus layer of soil. Some of these fungi are pathogenic to living trees; others are saprophytes that attack dead wood and forest floor leaf litter. Wood decaying basidiomycetes colonize and degrade wood using enzymatic and non-enzymatic processes (Osono & Takeda 2002; Berg & McClaugherty, 2003; Lindahl *et al*. 2007). Therefore, the identification of new fungal species or known fungi from different habitats that have capabilities of producing enzymes that could be employed in the degradation of xenobiotic compounds and recalcitrant lignocellulose towards the production of second generation biofuels needs is important particularly with the growing awareness for environmental rehabilitation (Magan *et al*., 2010; Bansal *et al*., 2011; Marco-Urrea & Reddy, 2012; Philbrook *et al*., 2013; Matínez *et al*., 2015)

It is typical that fungal collections from natural environments would initially yield mixed microbial cultures. This is because in their natural environment fungi tend to be in close association with both bacterial and other fungal species (Scherlach *et al*., 2013). It is often necessary to execute several sub-culturing processes until mono-axenic cultures are derived on agar plates (Burdass *et al*., 2006; Lee *et al*., 2015). The isolation procedure can be designed in such a way that the characteristics of the desired organism are the basis of the selection (Kaur *et al*., 2012; Nigam, 2013). Isolation method depending on the use of desired characteristics as selective factor comprise of enrichment cultures, use of selective and differential media (Stanbury *et al*., 1995; Copetti *et al*., 2009; Singer *et al*., 2011).

Definitive taxonomic identification of microorganisms is essential for further applications of fungal species. In recent times, the most commonly used method of identification involves characterizing the microorganism using DNA, with currently available techniques and burgeoning databases. Identification can be achieved at genus- and species-levels (Iotti *et al*., 2005; Sharma & Pandey, 2010; Gontia-Mishra *et al*., 2013; Upentra *et al*., 2013; Majid *et al*., 2015). Therefore the present study isolated and characterized a variety of fungi from the Kloofendal Nature Reserve in the Rooderpoort suburbs of Johannesburg, South Africa.

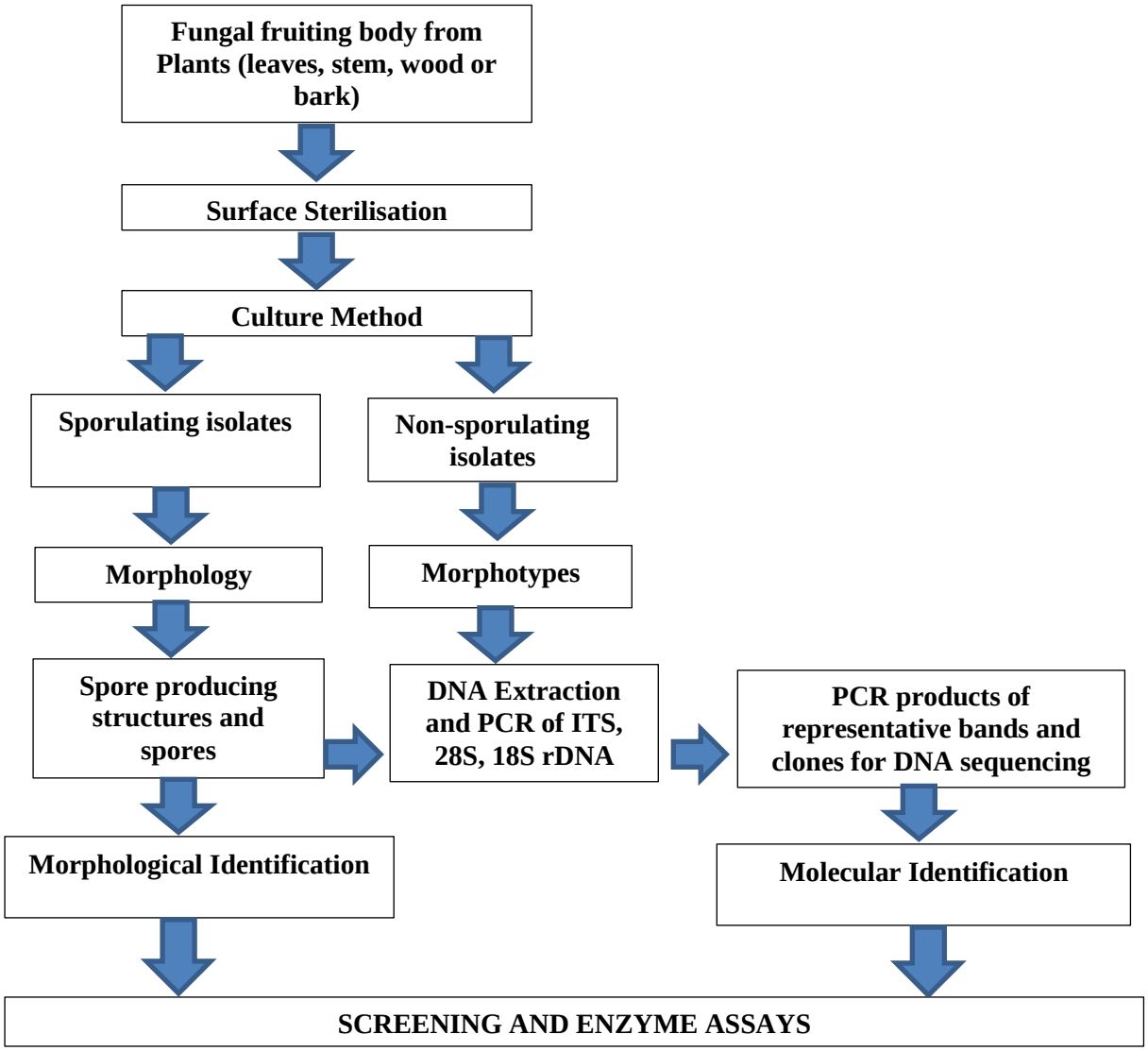

Figure 3.1: Schematic map of the research route used for this present study to isolate ligninolytic fungi (adapted from Sun & Guo, 2012)

3.2 Literature Review

3.2.1 Isolation of Ligninolytic Fungi

Fungi can be cultured on a variety of media types, although culture media type for fungal evaluation must be highly selective to suppress fast growing bacterial contamination and limit the growth rate and spread of fungal mycelia (Sharma & Pandey, 2010; Ravimannan *et al*., 2014). Moreover the type of media could affect colony morphology and determine whether particular structures are formed, in some cases some fungi may not grow because they lack the necessary enzymes to utilize different carbon sources (Tsudome *et al*., 2009; Jun *et al*., 2011). These media can be either solid or liquid depending on the experiment or desired biomass or product required (Ryan, 2008). The pre-requisite conditions are extremely varied and include

a medium containing a high carbohydrate source, nitrogen and vitamin sources, a pH range of 5–6 as well as the ability to remain stable at temperature range of 15 – 37°C (Weitz *et al*., 2001; Patel *et al*., 2009; Hubballi *et al*., 2010; Prasher & Chauhan, 2015). Other conditions that may be necessary for the more fastidious fungal species may include the maintenance of light/dark cycles, humidity and aeration (Hubballi *et al*., 2010).

Since no one medium sustains the growth and detection of every fungal species; it is often necessary to bait endophytic and epiphytic fungi with selective nutrient media (Kiziewicz, 2005; Copetti *et al*., 2009; Sharma & Pandey, 2010, Lahli *et al*., 2013). These baits are specific natural substrates such as broth made up of plant leaves or stems as well as other lignocellulosic agricultural residues (Sidana & Farooq, 2014; Ballhausen *et al*., 2015). An example of such a media is the V-8 juice agar medium which is composed of a mixture of eight different vegetables and calcium carbonate immobilized on agar (Quintana-Obregon *et al*., 2013). The V-8 juice agar belongs to a group of natural media which is characterized by the variation and the difficulty in duplicating the exact compositions of their ingredients. Other examples include corn meal agar, potato dextrose agar, malt extract agar and dung agar (Basu *et al*., 2015). Traditionally, Potato Dextrose Agar (PDA) is preferred for general quantification of fungi, although it does not always present an adequate nutritive source for the selective growth of some fungi with certain characteristics (Griffith *et al*., 2007; Ravimannan *et al*., 2014), nor does it provide the specific elemental requirements for growth and reproduction amongst fastidious fungal species. It should be noted that generally the requirements for growth are less stringent than for sporulation. Although fungi tend to thrive on PDA, it is considered too rich for many fungi, thus excessive mycelial growth is achieved at the expense of sporulation (Islam & Ohga, 2013, Basu *et al*., 2015).

The second classification of media, known as synthetic (defined) media contain ingredients of known composition and duplication is achieved with ease and exactness every time they are made (Basu *et al*., 2015; Koley & Mahapatra, 2015). They contain defined amounts of carbohydrates, nitrogen and vitamin sources. Examples include Czapek-Dox medium, Saborauds medium, glucose-asparagine, *Fusarium* medium and *Neurospora crassa* minimal medium. Salts of elements such as Fe, Zn and Mn are often added to defined media but are usually excluded from common media used for routine culture (Mello *et al*., 2004).

Fungi have natural deficiencies for vitamins; although the addition of vitamins is easily satisfied by the addition of minute quantities of the deficient vitamins in ranges of µM to nM (Griffith, 1996). The most common naturally occurring vitamin deficiency is thiamin and biotin (Beguin, 2010). Deficiency in these vitamins is quite common among the Ascomycota (Walker & White, 2011). It is usually not necessary to add these vitamins to organic nutrients such as glucose as they tend to be contaminated with vitamins sufficient to supply the growth requirements of fungi (Crinnion, 2010). It may also be necessary to use supplements such as dyes and antibiotics to inhibit undesirable microorganisms (Ryan, 2008; Copetti *et al*., 2009; Kaur *et al*., 2015) although some authors argue that fungi will grow in spite of microbial contamination and it is even possible that these antibiotics may inhibit the growth of fungi (Choi *et al*., 1999; Ryan, 2008). However it is common to include antibiotics such as penicillin, streptomycin, crystomycin and chloramphenicol to prevent bacterial contamination (Jafaril *et al*., 2006; Scognamiglio *et al*., 2010; Msogoya *et al*., 2012). When antibiotics are included in growth media, they are usually added after sterilization when media is cooled to 50–55^{o}C (Lahli *et al*., 2013; Jung *et al*., 2013; Hussain *et al*., 2014). An alternative to the use of antibiotic media is to create an unfavorable environment in the isolation such as low pH in which fungus will grow but the bacteria will be inhibited. An example is the use of acidified cornmeal agar (ACMA) which works well for the isolation of fungi from various types of plant material. However it is important to use surface-sterilisation of materials intended for inoculation in conjunction with ACMA (Hauser, 2006).

Various other amendments may be used that favour the isolation of certain groups of fungi, for example, benlates as well as dichloran rose of Bengal inhibits the growth of ascomycetes and deuteromycetes thus enhancing the isolation of basidiomycetes (Tello *et al*., 2009). With more fastidious xerophilic fungal growth that were limited by water activity, the use of dichloran glycerol agar 18% (Aw 0.95) is preferred because the traditional media tend to have Aw 0.99 that will otherwise limit their growth due to the rapid growth and development of other species that are better adapted to the later water activity (Williams & Hallsworth, 2009).

3.2.2 Molecular Characterisation of Fungi

Once an axenic fungal culture is obtained through isolation; it used to be that the traditional method of identification relied on morphological characteristics; this included both the macro- and micro-physiological characteristics (Allen, 1991; Dix & Webster, 1995; Kendrick, 2000; Amicucci *et al*., 2001). However morphological characterisation tends to be a tedious and time

consuming process that might only yield ambiguous results because although there are many diverse fungal species, many of them have similar characteristics (Stevens, 2002). Often at least 8 weeks is required under ideal culture conditions to observe the different life cycle of particular fungi and the appearance of fruiting bodies to enable easy identification. Furthermore the estimated 8 weeks is not guaranteed as growth optimisation may be required before actual observation of life cycle can be achieved. In other cases fungal fruiting bodies are inconspicuous and easily overlooked and some may not even produce fruiting bodies (Keller *et al.*, 2008). Moreover, morphological identification requires significant levels of experience and expertise to even deduce the phylum, let alone genus or species of the organism (Balajee *et al*., 2007).

In recent time, the most common and definitive method for identifying fungi employs molecular characterisation (Glaeser & Lindner, 2010). It relies on the understanding of genetic sequences including ribosomal RNA (rRNA) operon, the second largest ribosomal subunit of RNA polymerase II, and domains D1/D of nuclear-encoded large subunit ribosomal RNA genes (nLSU-rDNA) (Xie *et al*., 2007). The four rRNA genes: the 26 to 28S, 18S, 5.8S and 5S genes are arranged as head-to-tail tandem repeats separated by the space regions which include the internal transcribed spacer regions (ITS). The ITS region is defined as the unit containing ITS1 spacer, 5.8S rRNA gene and ITS2 spacer. In fungi, the ITS region is typically 650–900 base pairs (bp) in size, including the 5.8 gene and is usually amplified by the universal primer pair ITS1 and ITS4 designed by White *et al.* (1990). This understanding allows for the use of ribosomal intergenic spacer analysis (RISA) to identify fungal species (Xie *et al*., 2007) which targets the length and sequence variations of the non-coding ITS regions between the 18S small subunits (SSU) and the 28S large subunits (LSU) rRNA genes thus making it possible to distinguish closely related fungal species (Jensen *et al*., 1993; Fisher & Triplett, 1999; Kendrick, 2000; Schmidt *et al*., 2012). This method of molecular identification of fungi to the species level is primarily based on the variable nature of ITS regions and compares sequences from one fungi species with another (Baura *et al*., 1992; Chen *et al*., 1992; Lee & Taylor 1992; Peterson, 1995; Horton & Bruns 2001; Romanelli *et al.*, 2010; Delgado-Serrano *et al.*, 2016). The high degree of variation even between closely related fungal species is a consequence of low evolutionary pressure acting on non-functional sequences (Gardes *et al*., 1991; Anderson & Stasovski 1992; Baura *et al*., 1992; Chen *et al*., 1992; Lee & Taylor 1992; Liu *et al.*, 2010).

Ribosomal intergenic spacer analysis has great potential for the rapid detection and identification of fungi for medical, scientific and commercial purposes (Iwen *et al*., 2002; Oechsler *et al*., 2009) because the highly variable nature of the ITS1 and ITS2 can be exploited to generate restriction fragment length polymorphism (RFLP) patterns that makes for easy identification of fungi. Furthermore, this RFLP patterns makes it easier to design taxon-specific primers for PCR amplification (Jasalavich *et al*., 2000). It is noteworthy that the D1/D2 regions of the nLSU are known to be variable regions within the nLSU-rDNA also contain useful phylogenic information (Fell *et al*., 2000; Moncalvo *et al*., 2000).

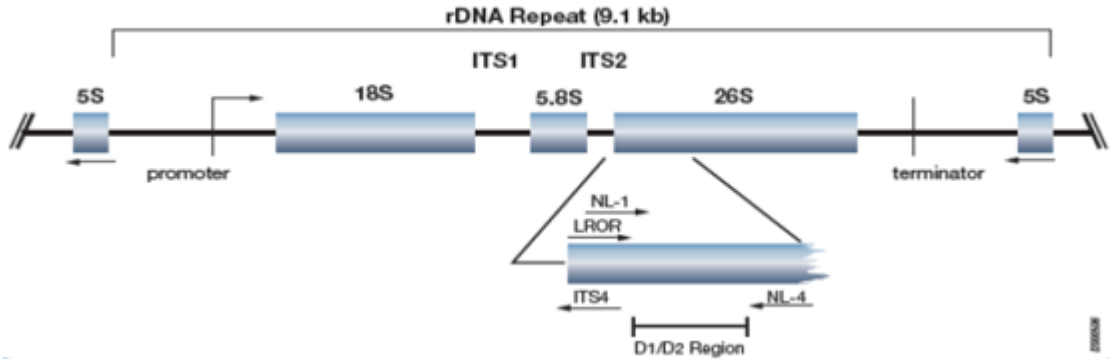

Figure 3.2 rDNA sequence. The fungal genome is unique in that it contains 50 to 100 identical copies of this gene in its genome. (Source: https://courses.cns.utexas.edu/BIOP411/wp-content/uploads/2012/09/BPLM-Chapter-9-Fungi-Isolation-Molecular-Characterization-Preservation-2013.pdf; accessed 12 June 2016)

Despite the biases that are introduced by traditional isolation and cultivation techniques, it must be emphasized that pure-culture experiments are indispensable for detailed analysis and molecular characterisation (Xie *et al*., 2007). Therefore the DNA extraction and amplification of targeted region using Polymerase chain reaction (PCR) as well as universal primers in protocols are vital to molecular characterisation (Horton & Bruns, 2001; Melo *et al*., 2006). DNA is extracted from pure fungal cultures using molecular biology methods; once the DNA is purified it can be amplified by PCR. Either DNA fragment or PCR amplicons can be submitted for sequencing. A variety of bioinformatics techniques can be employed to determine the fungus species based on the sequence result which is compared to that of known species. These DNA sequences are usually disseminated to the National Centre for Biotechnology Information (NCBI) to increase the body of data that enhances faster identification for future researchers. The NCBI has readily available local alignment search tool nucleotide (BLASTn) program for alignment with highly similar sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Although there are quite a number of DNA based identification methods that are available, the

ITS sequencing of fungi has one major advantage; the increasing quantity of information associated with ITS sequencing means that it is easier to correlate sequences with available data (Schmidt *et al*., 2012).

3.2.3 Screening of Ligninolytic Fungi

The screening of microorganisms for the production of desired metabolites is by far the most important aspect of biotechnology (Steele & Stowers, 1991). Although dependent on the type of microorganisms, the standard procedure for screening is typically similar for bacteria and fungi. Screening employs the use of highly selective procedures to allow for the detection and isolation of only those microorganisms which are of interest from among a large microbial population (Stanbury *et al*., 1995); thereby discarding the myriads of valueless microorganisms that are routinely isolated from nature. This is achieved by isolating microorganism from nature, in some instances from special ecological niches (Kubicek & Druzhinina, 2007; Abo-Shadi *et al*., 2010). Screening is executed to select a fungus with desired characteristics intended for various application e.g. fermentation, bioremediation and enzyme production (Nam *et al*., 2011). Screening programs are more often based on classical microbiological techniques of enrichment and mutagenesis (Steele & Stowers, 1991; Schaechter, 2009). However there are no universal screening methods and success of any screening program is dependent on the organisms and the methods for detection of activity (Waites *et al.*, 2001). Moreover screening can never be considered a routine activity as it is vital that methods must continuously be adapted to newest techniques, available instrumentation and knowledge. Ultimately, the objective is to detect and identify new substances or variations in existing products and to isolate them from those that are not useful.

Screening could utilize either the shotgun or objective strategies. With the shotgun approach, samples of free living microorganisms, biofilms or other microbial communities are collected from sources including animal, plant material, soil, sewage, water and effluents particularly from unusual and natural habitat. These isolates are then screened for desirable traits using metagenomics analysis (mass genome sequencing) (Neelakanta & Sultana, 2013). However, the objective approach takes sample from specifically targeted sites where organisms with the desired characteristics are most likely to be resident with the natural microflora (Waites *et al*., 2001). Screening holds limitless potential as it is very likely that only a small fraction of the world's estimated 5.1 million fungi have been identified (O'Brien *et al*., 2005; Taylor *et al*., 2010). Moreover, even with the approximately 100,000 known fungi that have demonstrated

production of products; there are still yet to be discovered natural products from these identified fungi (Kirk *et al*., 2008; Blackwell, 2010).

Screening involves preliminary and secondary processes, where the preliminary screening does not necessarily target product activity or concentration but rather the presence of the desired product. Ideally, the primary screening should be rapid, inexpensive, predictive, specific but effective for a broad range of compounds (Singh *et al.*, 2015). However, primary screening is by nature time consuming and tedious since a large number of isolates have to be screened to identify a few potential microorganisms (Sukesh, 2010; Tilay & Annapure, 2012). Once the product is determined to be present, a more comprehensive screening process follows which will also include screening for important features, such as stability and where necessary non-toxicity (Waites *et al.*, 2001).

Recently, the focus of screening researches has been to find new and alternative fungal strains that produce value-added and efficient ligninolytic enzymes (Erden *et al.*, 2009) because of the vast potential of these enzymes in areas of biofuel production and bioremediation of xenobiotic compounds. Several screening programs have focused mainly on white-rot basidiomycetes (Saparrat *et al*., 2002; Dhouib *et al*., 2005) as well as other groups of fungi capable of producing laccase which include the ascomycetes and deuteromycetes (Shraddha *et al*., 2011). Generally, most of the screening methods are based on colorimetric method whereby the colour changes of synthetic chemicals introduced into the media are associated with specific fungal ligninolytic enzyme activities (Nam *et al.*, 2011). Thus, fungi that possess ligninolytic ability are capable of degrading screening reagents with similar structure to lignin (Glenn & Gold, 1983; Esposito *et al*., 1991; Wunch *et al*., 1997; Gowthaman *et al*., 2001). Previously these screening reagents used to be tannic and gallic acid (Nishida *et al.*, 1988; De Jong *et al*., 1992; Pointing, 1999) but they have been replaced with synthetic phenolic reagents such as guaiacol (Coll *et al.*, 1993; Kiiskinen *et al.*, 2004;Viswanath *et al.*, 2008; Mabrouk *et al*., 2010; Sasidhar & Thirunalasundari, 2014; Devasia & Nair, 2016), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Floch *et al*., 2007; Hao *et al*., 2007; Sivakumar *et al*. 2010; Yadav *et al*., 2014), syringaldazine (Floch *et al.*, 2007; Kumar *et al*., 2011; El-Batal *et al*., 2015), and polymeric dyes such as Remazol Brilliant Blue-R (RBB-R) (D'Souza *et al*., 1999; Machado & Matheus, 2006; Narkhede *et al*., 2013).

Typically in the presence of phenol oxidases, the colourless guaiacol and ABTS are oxidized to form reddish brown (Kiiskinen *et al.*, 2004) and blue-green coloured compounds, respectively (De Jong *et al.*, 1992; Doerge *et al.*, 1997; Solis-Oba *et al.*, 2005; Yadav *et al.*, 2014). The pale yellow colour of syringaldazine is oxidized to purple coloured compound in the presence of laccase (Harkin *et al.*, 1974; El-Batal *et al.*, 2015). The blue RBB-R is decolourised through the combined actions of peroxidases and hydrogen peroxide-producing oxidases (Glen & Gold, 1983; Mtui & Masalu, 2008; Narkhede *et al.*, 2013).

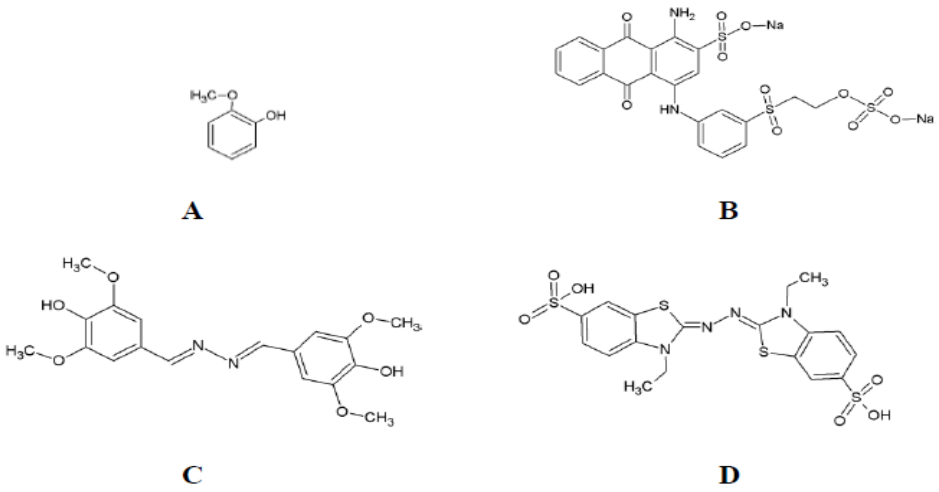

Figure 3.3: Phenolic structures of commonly used reagents in screening for ligninolytic enzymes. A: Guaiacol; B: Remazol Brilliant Blue-R (RBB-R); C: Syringaldazine; D: 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Source: Nam *et al.*, 2011)

In the present study, an integrated experimental approach that combined traditional culture-based techniques and ribosomal intergenic spacer analysis was used to identify different fungi that were isolated from their natural environment. The specific objective was to screen these fungal species once identified for the presence of ligninolytic enzymes to enable subsequent investigation involving quantification of enzymes in dual cultures to be executed.

3.3 Materials and Method

3.3.1 Sample Area

The sampling area for the present study was the Kloofendal Nature Reserve, Roodepoort, Johannesburg (26.1275S 27.8806E). It is situated in the West Rand and covers an area of 150 hectares. It consists of pristine Savanna-type vegetation with rocky quartzite outcrops. The common wildlife in this area is birds and a variety of small mammals including mountain

reedbuck, mongoose and hedgehog. There are many tree species within the reserve and it's the geological site of an old gold mine known as the Conference Reef and it is bordered by a dam.

Figure 3.4: Map showing the Kloofendal Nature Reserve **(Source:** http://www.footprint.co.za/images/Kloofendaldirectionslg.jpg; accessed 15 June 2016)

3.3.2 Isolation of Fungal Species and Cultivation Conditions

A total of 30 fungal fruiting bodies were collected from decaying plant materials (barks and litter) based on morphological variations and a type strain *Ganoderma lucidum* ATCC- 32471 was obtained from the Agricultural Research Council (ARC), Pretoria, South Africa. Fruiting bodies were spray-rinsed with 70% ethanol to remove debris and soil. To propagate the mycelia of the collected species, tissues were sliced from the fruiting bodies and transferred to Potato Dextrose agar (PDA) and Malt Extract agar (MEA) solid media and incubated at 25°C for 6 days under humidified conditions. All the isolated cultures, including the type strain were purified by repeated transfer onto agar plates. All culture plates were stored at 4°C for a maximum of 10 days before use.

3.3.3 Screening

3.3.3.1 Cellulolytic Activity

To test for cellulolytic activity, the fungal isolates were grown on PDA medium amended with 0.5% Na-carboxymethyl cellulose and the plates were incubated for 3 days at 25°C. The plate with young mycelial growth was flood with 0.1% Congo red and destained with 1M NaCl. Plates were left at room temperature for 15 minutes and observed for clear halo around the colony; an indication of cellulose activity.

3.3.3.2 Ligninolytic Activity

To test for ligninolytic activity, the fungal isolates were grown on MEA and PDA amended with 0.02% and 0.2% guaiacol, respectively. Both concentrations were chosen based on several literatures citing them as the ideal concentrations for ligninolytic activity screening. The plates were incubated for 5 – 7 days (Coll *et al*., 1993; Eggert *et al*., 1996). After incubation the fungal colonies were observed for coloured zone around and below surface of the colony.

3.3.3.3 Morphological Characterisation – Microscopy

Fungal mycelial were exercised using a scapel. A drop of 0.1 % Congo-red stain (Slifkin & Cumbie, 1988) was added. Samples were washed with distilled water after 2 minutes, blotted dry before covering with cover slip. Microscopic observations were done at X4 and X10 objective magnifications on an Olympus CX41™ Light Microscope.

3.3.3.4 Molecular Characterisation

3.3.3.4.1 Genomic DNA Extraction

The DNA extraction was carried out using mycelium grown on PDA plates. Approximately 100 mg wet weight of fungal cells samples was re-suspended in phosphate-buffered saline and was added directly to a 0.1-0.5 mm ZR BashingBead™ Lysis Tube. Lysis was achieved by securing the bead beater with a 2 ml tube holder and the assembly was processed at maximum speed for 10 minutes on a standard benchtop vortex. Exactly 750 µl Lysis solution was added to the tube. The mixture in the ZR BashingBead™ Lysis Tube was centrifuged at 10, 000 x *g* for 1 minute. Exactly 400 µl of the supernatant was transferred to a Zymo-Spin™Spin Filter (Orange Top) in a collection tube and centrifuged at 7, 000 x *g* for 1 minute. Exactly 1, 200 µl of manufacturer supplied Fungal/Bacterial DNA Binding Buffer was added to the filtrate now present in the Collection Tube. Exactly 800 µl of this mixture was transferred to a Zymo-Spin™IC Column in a Collection Tube and centrifuged at 10, 000 x *g* for 1 minute. The flow

through from the previous step was discarded and the mixture was again centrifuged 10, 000 x *g* for 1 minute. Exactly 200 µl of manufacturer supplied DNA Pre-Wash Buffer was added to the Zymo-Spin™IC Column in a new Collection Tube and centrifuged 10, 000 x *g* for 1 minute. Exactly 500 µl of manufacturer supplied Fungal/Bacterial DNA Wash Buffer was added to the Zymo-Spin™IC Column in a new Collection Tube and centrifuged 10, 000 x *g* for 1 minute. The Zymo-Spin™IC Column was transferred to a clean 1.5 ml micro-centrifuge. Exactly 10 µl of manufacturer supplied DNA Elution Buffer was added directly to the column matrix. The mixture was left to stand for 3 minutes and then centrifuged at 10, 000 x *g* for 30 seconds to elute the DNA.

3.3.3.4.2 DNA Amplification

Eluted pure DNA was amplified using Ribosomal DNA known to be conserved among fungal taxa. This was achieved by amplifying the internal transcribed spacer (ITS) region. The Primers ITS1 and ITS4 were used (Sigma-Aldrich, USA). The sequences of the primers were as follows: ITS1 (forward primer): 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4 (reverse primer): 5'-TCC TCC GCT TAT TGA TAT GC-3' (White *et al*., 1990). Amplification was performed in 20 µl of reaction mixture containing 1µl of DNA template of fungi, 2 µl 10X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP (dATP, dTTP, dGTP and dCTP), 0.4 µM of both primers, 1 unit Taq polymerase (Sigma-Aldrich, USA) and double-distilled water. The Polymerase Chain Reaction was run using Bio-Rad MJ Mini Thermal Cycler. The PCR Cycler initiated with denaturation for 5 minutes at 95^{o}C. This was followed by 32 cycles of 30 secs-denaturation at 94^{o}C, annealing for 2 minutes at 55^{o}C and extension for 1 minute at 72^{o}C. Further extension was then carried out for 10 minutes at 72^{o}C before it was maintained at 4^{o}C. The PCR product was run on 1% agarose gel, stained with ethidium bromide (EtBr) and visualized under UV light. The images were photographed using gel documentation system (SynGene). The PCR products were sequenced by using forward and reverse directions on the ABI PRISM™ 3500xl Genetic Analyser. Sequences were analysed using Sequence editor software followed by a BLAST search provided by NCBI. The ITS sequences obtained in this study was deposited in the NCBI GenBank and were ascribed accession numbers (see Table 3.2).

3.3.3.4.3 Phylogenetic Analysis

The programs utilised for the phylogenetic analyses of the complete ITS1-5.8S-ITS2 region are included in MEGA6 software package as described by Tamura *et al*. (2013). This allowed

the inference of evolutionary relationship of homologous sequences. Multiple sequence alignment was achieved using ClustalW algorithm, parsimony and maximum likelihood (ML) employing the PhyML programme as described by Tamura *et al.* (2013) in order to construct the phylogenetic trees. The robustness of the tree topologies was evaluated by bootstrap analysis based on 500 re-samplings of the sequence alignment.

3.4 Results

3.4.1 Isolation of Fungal Species and Screening for Cellulose and Ligninolytic Activities

A total of 30 different specimens of fungi were collected from the Kloofendal Nature Reserve, based on visual morphological differences observed. Monoaxenic cultures that showed significant activities for the presence of these enzymes were selected. Ten of the isolated fungi (shown in Figure 3.4.2a and 3.4.2b) were selected for this study.

Table 3.1: Ligninolytic and cellulolytic activity screening in isolates

Initial notations	**Ligninolytic Activity Screening MEA 0.02% Guaiacol**	**Ligninolytic Activity Screening PDA 0.2% Guaiacol**	**Cellulolytic Activity Screening MEA 0.5% Na-CMC**
A2	--	--	++
B2	--	--	++
C2	++	++	++
D2	++	++	++
E2	++	++	++
F2	++	++	++
G2	--	--	++
H2	--	--	++
I2	--	--	++
J2	++	++	++

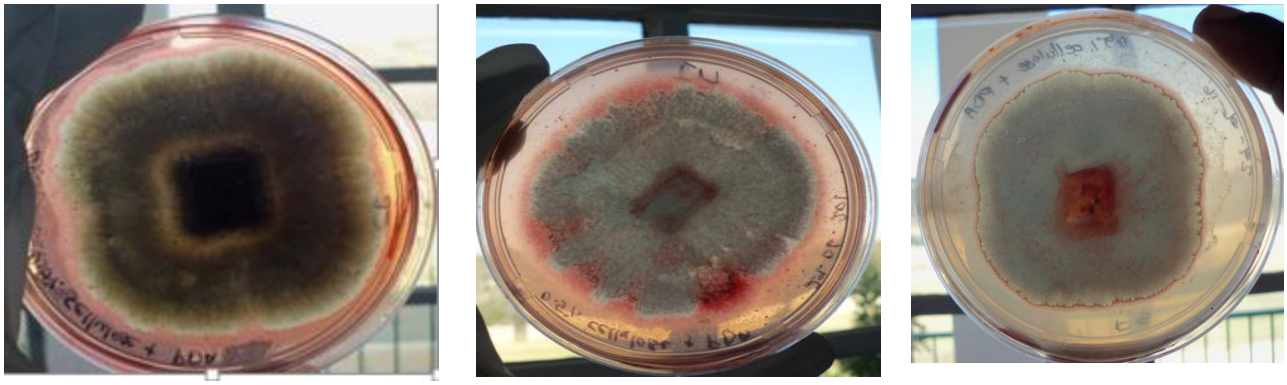

Figure 3.4.1a: Cellulolytic activity screening using 0.1% Congo Red Stain, observable clear halo surrounding the fungi colony

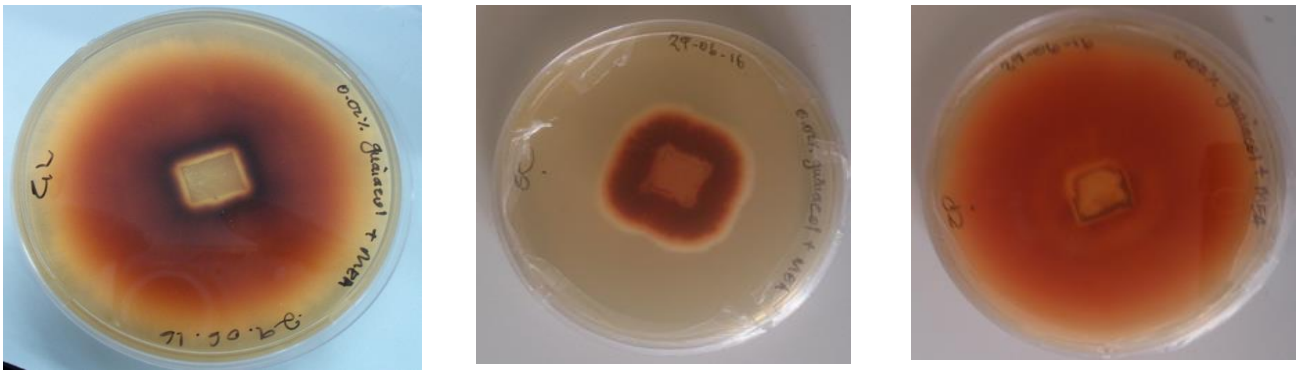

Figure 3.4.1b: Ligninolytic activity screening on MEA plates amended with 0.02% Guaiacol, red coloured colony growth after 3 – 5 days incubation

Of the 10 organisms chosen for the study, five monoaxenic cultures (C2, D2, E2, F2 and J2) demonstrated visible colouration on amended MEA plates containing 0.02% guaiacol (as shown in Figure 3.4.1). However E2 did not demonstrate visible colouration or grow on PDA plates that were amended with 0.2% guaiacol. All plates amended with 0.5% Na-carboxymethyl cellulose (Na-CMC) showed clear halo zones around the edges of colonies when stained with 0.1% Congo red and de-stained with 1M NaCl.

3.4.2 Microscopic Examination of Fungal Isolates

Microscopic examinations of monoaxenic plate cultures of fungal species showed profuse hyphal growths and in some species clearly visible septal junctions, protoplasmic components and healthy intertwining interactions between self-mycelial extensions. Fungal micrograph B2 showed clear sporangial head as well as a profuse number of spores within the interstitial spaces between mycelia.

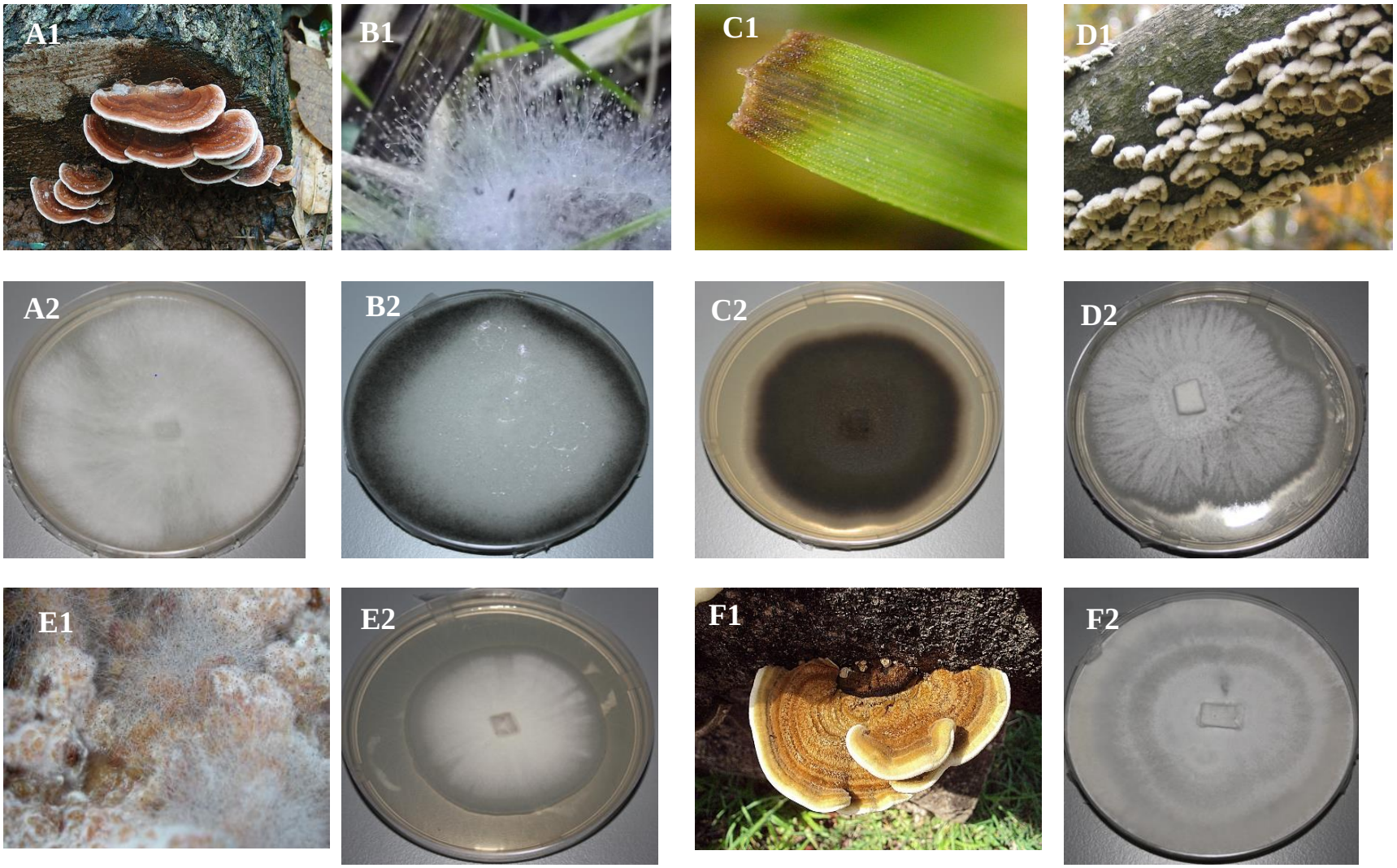

Figure 3.4.2a: Fruiting bodies and monoaxenic cultures of fungi isolates used in the study

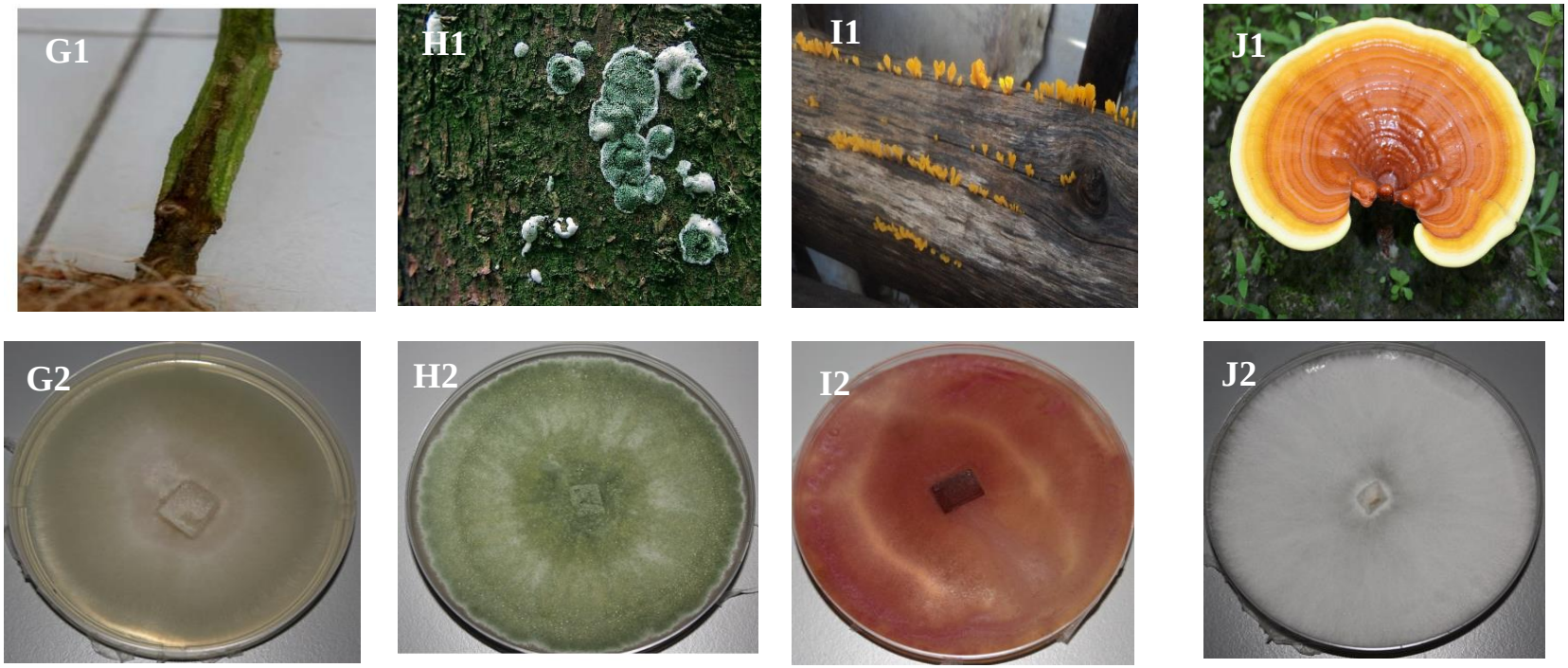

Figure 3.4.2b: Fruiting bodies and monoaxenic cultures of fungi isolates used in the study

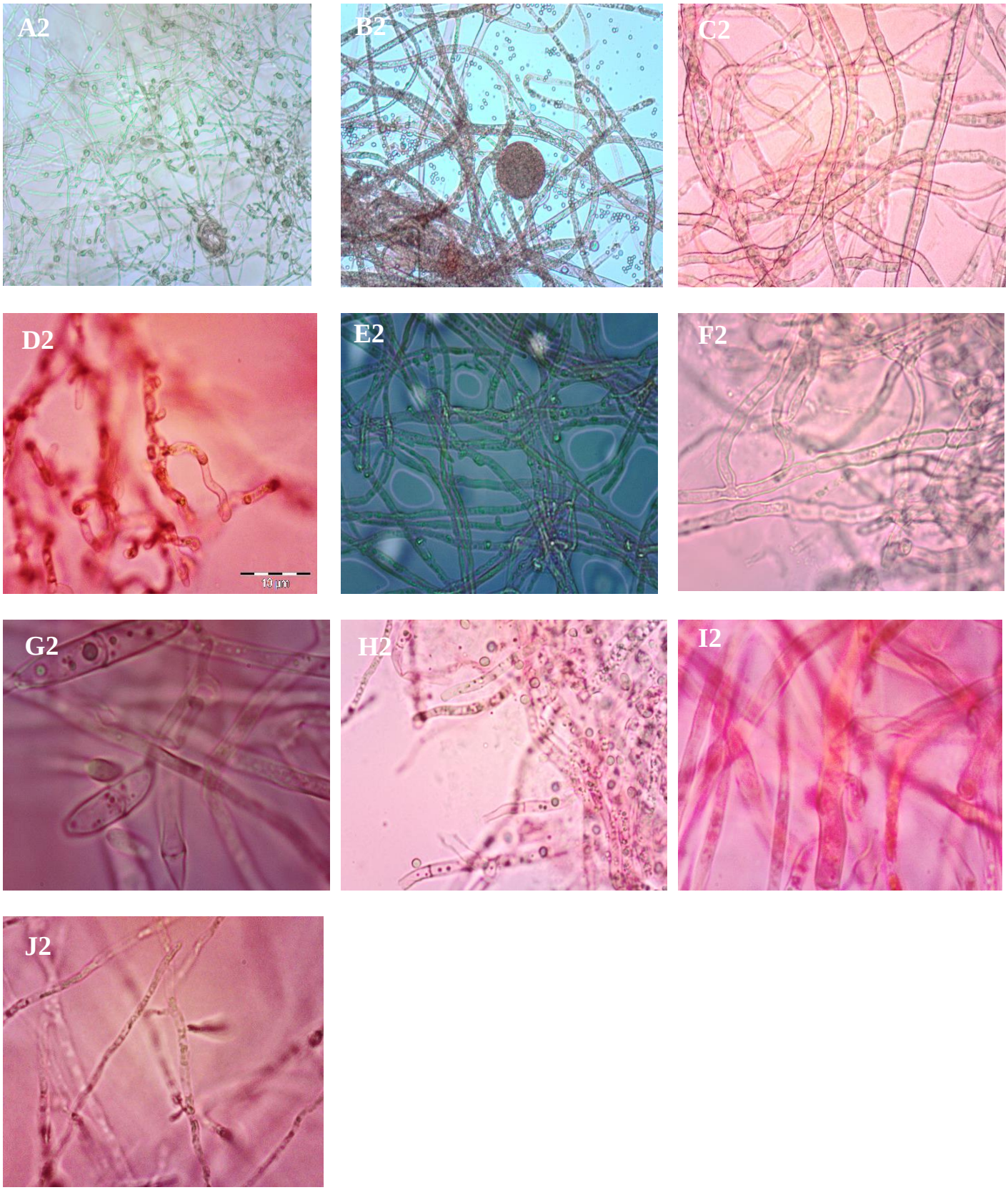

Figure 3.4.3: Fungal micrographs of monoaxenic plate cultures using light microscopic at X10 object lens magnification

3.4.3 Genomic DNA Extraction and PCR amplification

PCR amplification of the fungal isolates with the ITS primers conceded effective amplification for all isolates with sizes of ~650 bp (Figure 3.4.4). All fungi species corresponded to similarities between the sequences queried and the biological sequences within the NCBI database. However, only 10 fungi species subjected to BLAST were used in the study (Table 3.2).

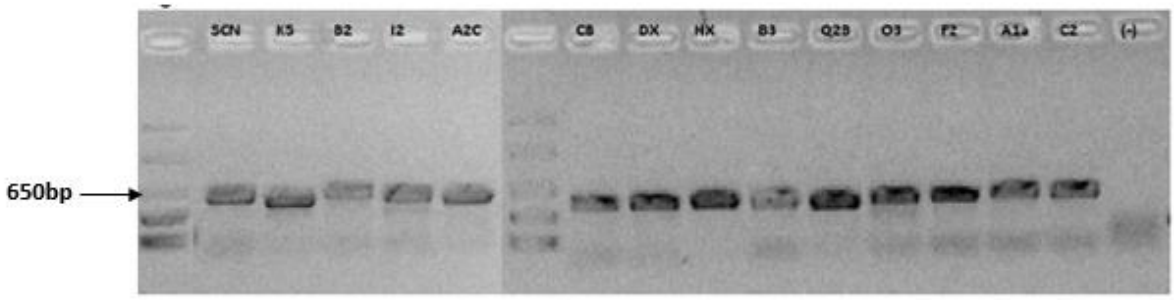

Figure 3.4.4: A photographic image of an agarose gel indicating the amplification of the ITS target

Table 3.2: Taxonomic identification of screened fungal species based on ITS gene sequence analysis

S. No.	Initial notations	Strain code	Total base pairs	Query Description	Close relatives	Similarity	Accession Number
1	A1, A2	KN1	606	18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence?	*Fomitopsis meliae* voucher SRM-209	98.84	KU253767
2	B1, B2	KN2	636	Internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence?	*Rhizopus microsporus* strain SHLSYD	100	KU253769
3	C1, C2	KN3	549	18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence?	*Curvularia borreriae* strain FMR 11523 isolate	100	KU253770
4	D1, D2	KN4	583	Internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence; mitochondrial?	*Schizophyllum commune* isolate GBJ8	99.66	KU253771
5	E1, E2	KN5	408	18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence?	*Umbelopsis isabellina* strain CBS 250.95	99.66	KU253772
6	F1, F2	KN6	573	Internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence?	*Coriolopsis polyzona* strain Cof143	99.83	KU253773
7	G1, G2	KN9	517	18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence?	*Fusarium sp.*VSMU-S001	100	KU253776
8	H1, H2	KN10	557	Internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	*Trichoderma harzianum* strain BHU-BOT-RYRL10	99.83	KU253777
9	I1, I2	KN12	511	Internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.	*Myrmaecium rubricosum* strain VRJ	99.61	KU253779
10	J1, J2	NA	NA	Identified previously	*Ganoderma lucidum* ATCC- 32471	NA	NA

Note: Organism Number 10 was obtained from the Agriculture Research Council therefore it had previously been sequenced and identified

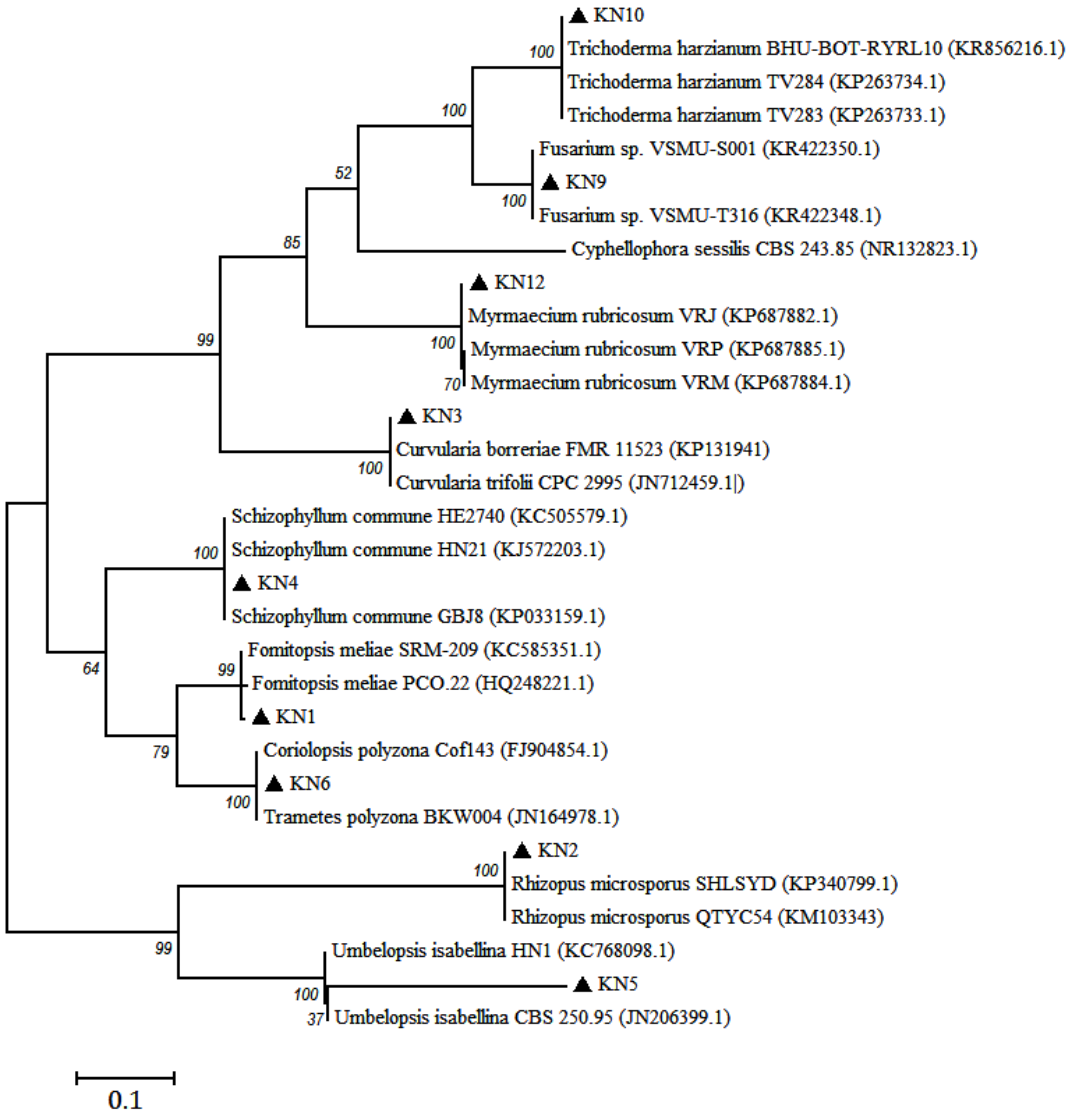

Figure 3.5: Phylogenic tree showing the relationships among the screened fungal species and the most similar sequences based on the ITS rRNA gene (ML analysis, 500 rounds of bootstrap resampling).

The outcome of the submitted sequences affirmed that all isolated speciess belonged to Basidiomycota, Mucorales and Ascomycota species. The phylogenetic relationship of all isolated fungi was inferred using the maximum likelihood [ML] analyses. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted using the MEGA6 software. Based on the analysis of ITS rDNA gene sequence, the isolates KN1, KN2, KN3, KN4, KN5, KN6, KN9, KN10 and KN12 were identified as *Fomitopsis* sp., *Rhizopus microsporus.*, *Curvularia borreriae*, *Schizophyllum* sp., *Umbelopsis* sp., *Coriolopsis* sp., *Fusarium* sp., *Trichoderma* sp., and *Myrmaecium* sp., respectively.

Table 3.3: Lineage analysis of screened fungal isolates

Strain code	Fungal Isolates	Accession Number	Lineage : Domain; Kingdom; Phylum; Sub-phylum; Class; Order; Family
KN1	*Fomitopsis meliae* voucher SRM-209	KU253767	Eukaryota; Fungi; Dikarya; Basidiomycota; Agaricomycotina; Agaricomycetes; Polyporales; Fomitopsis
KN2	*Rhizopus microsporus* strain SHLSYD	KU253769	Eukaryota; Fungi; Fungi incertae sedis; Early diverging fungal lineages; Mucoromycotina; Mucorales; Mucorineae; Rhizopodaceae; Rhizopus
KN3	*Curvularia borreriae* strain FMR 11523 isolate	KU253770	Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Pleosporales; Pleosporineae; Pleosporaceae; Curvularia.
KN4	*Schizophyllum commune* isolate GBJ8	KU253771	Eukaryota; Fungi; Dikarya; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Agaricales; Schizophyllaceae; Schizophyllum
KN5	*Umbelopsis isabellina* strain CBS 250.95	KU253772	Eukaryota; Fungi; Zygomycota; Fungi incertae sedis; Early diverging fungal lineages;Trichomycetes; Mucoromycotina; Mucorales; Umbelopsidaceae; Umbelopsis
KN6	*Coriolopsis polyzona* strain Cof143	KU253773	Eukaryota; Fungi; Dikarya; Basidiomycota; Agaricomycotina; Agaricomycetes; Polyporales; Trametes/Coriolopsis
KN9	*Fusarium* sp.VSMU-S001	KU253776	Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Nectriaceae; Fusarium
KN10	*Trichoderma harzianum* strain BHU-BOT-RYRL10	KU253777	Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreaceae; Trichoderma
KN12	*Myrmaecium rubricosum* strain VRJ	KU253779	Eukaryota; Fungi; Dikarya; Ascomycota;Pezizomycotina;Dothideomycetes; Dothideomycetes incertae sedis; Valsariales; Valsariaceae; Myrmaecium
GL	*Ganoderma lucidum* ATCC- 32471	NA	Eukaryota; Fungi; Dikarya; Basidiomycota; Agaricomycotina; Agaricomycetes; Polyporales; Ganodermataceae; Ganoderma

Discussion

Managed forests and grasslands similar to the Kloofendal nature reserve where nine of the ten fungi used in this study were isolated have a significant portion of litter present within their natural ecosystem (Geethanjali, 2012). This litter contains approximately 20 – 50% lignin that occurs in infinite association with cellulose and hemicellulose, thus providing structural strength and protecting the polysaccharides by its biodegradation-resistant barrier (Atlas & Bartha 1998). This litter that makes up the surface layer of the forest floor consists of freshly fallen leaves, needles, twigs, stems, barks and fruits in various stages of decay (Brady & Well, 2005). The degradation of lignin is commonly associated with fungal species belonging to the Basidiomycetes (Rao, 2008), although lignin degrading ability have also been found in species other than Basidiomycetes. Moreover there is established data placing an approximate estimate of over 600 species of Basidiomycete as having been found to be ligninolytic and being able to mineralize lignin to CO_2 by secreting extracellular lignin peroxidase, manganese-dependent peroxidase and laccase enzymes (Kumar & Gupta, 2006).

Of the 10 organisms chosen for the study, five of them (*Curvularia borreriae, Schizophyllum commune, Umbelopsis isabellina, Coriolopsis polyzona* and *Ganoderma lucidum*) demonstrated visible red colouration due to the oxidation of guaiacol indicating ligninolytic activity (Table 3.1). Three of these fungi belong to the Basidiomycete and with the other two belonging each to the Ascomycetes and Mucorales phyla. All 10 fungi cultures demonstrated cellulolytic activity. Results are similar to studies done by Naik *et al.* (2012). The structural proximity of lignin and cellulose implies that more often, it is expedient that organisms produce both enzymes to be able to degrade the lignocellulosic structure of most plants. However it must be added that in previous studies, there are examples of ligninolytic fungi such as *Trametes versicolor* and *Phellinus* sp. that have demonstrated low cellulolytic activity but very high lignin degrading capabilities thereby suggesting that lignin degradation is of more interest to these fungal species (Liew *et al.*, 2011).

The ligninolytic activity as demonstrated by *S. commune*, *C. polyzona* and *G. lucidum* are not surprising, as these fungal isolates all belong to Basidiomycetes phylum. There are numerous studies that have shown these fungi producing lignin-degrading enzymes (Novotný *et al*., 2004; Irshad & Asgher, 2011; Pozdnyakova, 2012; Asgher *et al*., 2013;Vrsanska *et al*., 2015; Zhu *et al*., 2016). However there are sparingly few studies that show members of the phyla Ascomycetes and Mucorales as producers of ligninolytic enzymes. *Umbelopsis isabellina*

(also known as *Mortierella isabellina*) belonging to the *Micromucor/Umbelopsis* clade, has gone through several taxonomic changes with the last known change being done by Meyer and Gams (2003) after RFLP analysis that exploited ITS1 and ITS2 primers. Furthermore, there is confusion as to its ability to produce lignin-degrading enzyme. In one study it was referred to as non-ligninolytic (Janicki *et al.*, 2016) whereas previous studies espouse that *Umbelopsis isabellina* produced manganese-dependent peroxidase, a ligninolytic enzyme (Yang *et al.*, 2003) in the decolourisation of synthetic dyes and in addition there are studies that have implicated the previously known nomenclature *Mortierella isabellina* in the production of ligninolytic enzymes and bioremediation of the pesticide, diuron (Tixier *et al*., 2000; Tixier *et al*., 2001). The present study also observed ligninolytic activity with *Umbelopsis isabellina* as shown in Table 3.1.

Although little is known about the decay mechanism employed by Ascomycetes in plant litter and soil, it is generally agreed that some of these soft-rot producing fungi are capable of degrading lignin; therefore an enzymatic system must be employed in the process (Nilsson *et al*., 1989; Dix & Webster, 1995; Worrall *et al.*, 1997; Shary *et al.*, 2007). To further compound the problem of the poor elucidation of ligninolytic activity amongst Ascomycetes is that there are few reports on these groups of fungi and fewer still on *Curvularia* sp. However enough evidence in literature exists of their ability to degrade dye effluents (Senthilkumar *et al.*, 2012; de Miranda *et al*., 2013; Neoh *et al*., 2014; Neoh *et al*., 2015). In a series of studies done by Neoh *et al.* (2014; 2015); they observed the production of manganese-dependent peroxidase, lignin peroxidase and laccase enzymes with *C. clavata* in the decolourisation of recalcitrant dyes and palm oil effluents. Similarly de Miranda *et al.* (2013) showed that *C. lunata* produced ligninolytic enzymes as well during the decolourisation of textile effluent. This present study indicates that *C. borreriae* is capable of ligninolytic activity.

Conclusion

A bioprospecting study was done on Kloofendal Nature Reserve which yielded nine fungi isolates, a type strain *G. lucidum* ATCC- 32471 was obtained from the Agricultural Research Council, South Africa. This preliminary research and screening work done on 10 fungal isolates showed that three of the basidiomycetes isolated were capable of producing ligninolytic enzymes. One Ascomycetes and one Mucorales species also produced ligninolytic activity. Further investigation is necessary to ascertain the type of enzyme produced, quantification and optimisation of production of these enzymes.

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Chapter 4

Macroscopic and microscopic changes during antagonistic interaction of fungi

4.0 Abstract

Interspecific interactions amongst fungi usually cause morphological changes that are a consequence of biochemical reactions involving protein expressions during these interactions. This study investigated dual cultures of 10 different fungal species with a focus on previously established antagonistic interactions observed on Potato Dextrose Agar (PDA). Approximately 20 different fungal combinations were cultured and investigated on different substrates including PDA, Corn Cob Agar, Sugarcane Bagasse Agar and Wheat straw Agar. *Trichoderma* sp. KN10, *Rhizopus microsporus* KN2, *Fomitopsis* sp. KN1 and *Coriolopsis* sp. KN6 consistently demonstrated tendencies of invasion and replacement in dual cultures. The plate interactions examined for macroscopic and microscopic morphological changes demonstrated varying morphological changes similar to those previously described in literature. This study demonstrated that interspecific interactions particaularly antagonism does cause morphological changes as well as a change in the biochemical responses from the interacting fungal species.

4.1 Introduction

When fungi are introduced to new territory, there is a tendency for individual fungi to occupy distinctive domains. However over a time period, either due to the exhaustion of nutrients within the immediate vicinity or the need for mycelial extension, these changes could cause an overlap between domains that leads to mycelial interactions (Boddy, 2000). It seems plausible that the interaction between hyphae is possibly significant in determining the pattern of colonization of substrate (Ikediugwu & Webster, 1970a; 1970b; Pečiulytė, 2002). Tlalka *et al.* (2008) explains that with filamentous fungi hyphae, such taxis is necessary to facilitate penetration of complex substrates and often it produces aggregated hyphae to allow it pass through unfavorable habitats to interconnect occupied nutrient sources. Such connections of several resource areas, allows fungi to translocate energy from nutrient-rich substrate to other parts of its mycelium, for example in its utilization of substrate such as wood. Therefore a circumstance that hinders these networks of hyphae is detrimental to the well-being of the fungi.

Interaction between two combatant fungi usually activates intense changes in mycelial morphology that are observable, particularly with antagonistic interactions, as the production of barrages and invasive cords as well as metabolic changes that include up-regulation and secretion of antifungal toxins, metabolites and oxidative enzymes (Boddy, 2000; Baldrian, 2004;Heilmann-Clausen and Boddy, 2005; Hiscox *et al*., 2010; 2016).

One of the most commonly observed morphological changes during fungus-fungus interaction is hyphal interference. It is characterised as observed in the interaction between *Phlebiopsis gigantea* and *Heterobasidion annosum*, by the rapid localised disruption of the cytoplasm and cell membrane of *H. annosum* upon its hyphae making contact with that of *P. gigantean* (Adomas *et al*., 2006).

Interactions involving *Trichoderma* species invariably involves this fungus using a process referred to as mycoparasitism whereby it extends profuse quantities of hyphae towards host mycelia and the development of specialized parasitic structures such as appressoria and aggressive coiling, around host hyphae causing strangulation in nutrient uptake. As a result of the potential application of *Trichoderma* species in the biological of plant pathogen, this mechanism has been studied extensively (Boddy, 2000; Steyaert *et al*., 2003; White & Traquair, 2006). It should however be noted that mycoparasitism should not be regarded as primarily a mechanism by this organisms for nutrient acquisition but rather it is a temporary strategy exploited to gain territory from a host (Boddy, 2000).

The production of barrages as stated previously is associated with another form of interspecific fungi interaction referred to as gross mycelial contact. It is observed on rich laboratory agar media as a development of dense aerial hyphae, which form a thickened tuft at the interaction interface (barrages) (Boddy, 2000; Adomas *et al*., 2006; Peiris *et al*., 2008; Peiris, 2009; Ujor, 2010). This morphological modification by the fungi provides a physical barrier against invasion by the opposing fungal species (Boddy, 2000). Possibility exist that the barriers can be surmounted and such phenomenon has been observed in decaying wood (Boddy, 2000). Other morphological changes associated with gross mycelial contact include the formation of invasive mycelial fronts, mycelial fans, and linear structures – cords and rhizomorphic structures (Boddy, 2000).

Several authors have reported that mycelial contact in interspecific interactions is commonly associated with changes in pigmentation, sometimes yellowish/brownish to dark discolouration of mycelia that begins from the interface between the two combatant fungi and spreads inwards into the domain occupied by individual species (Peiris *et al.*, 2008, Gregorio *et al*., 2006; Peiris, 2009, Tudor *et al*., 2013). Furthermore, microscopic examination had shown other morphological changes observed during these interspecific mycelial struggles including vacuolation, swelling of hyphal filaments and protoplasmic degeneration, pronounced invagination of plasmalemma, loss of recognisable mitochondria, and appearance of dense intracellular streak-like aggregates within the cytoplasm (Ikediugwu, 1976; Iakovlev & Stenlid, 2000; Gregorio *et al*., 2006; Li *et al*., 2010).

In the present study, 10 fungi species previously identified and characterised were paired using statistically derived combinations on different substrates (PDA and agricultural residues). A comprehensive literature review of interspecific interactions has been executed in Chapter 2 of this thesis. The objective of this study was to investigate the macroscopically and microscopically changes that resulted from the competitive interactions.

4.2 Materials and Method

4.2.1 Preparation of Media

Media plates for surface growth analysis of fungi and crude enzyme extraction was prepared using corncob, sugarcane bagasse and wheat straw powder with media composition calculation based on their standardized glycaemic index values that approximated PDA to achieve a similar consistency. The corn cob agar (CCA), sugarcane bagasse agar (SBA) and wheat straw agar (WSA) were prepared by adding 5.2 g of corncob powder, 5.88 g of sugarcane bagasse powder and 6.52 g of wheat straw powder respectively with 18 g of agar and made up to 1000 ml with distilled water. The CCA, SBA and WSA were autoclaved at 121^{o}C for 15 mins and allowed to cool to 55^{o}C. These sterile media was poured into petri-plates and allowed to solidify.

4.2.2 Culture Combination and Dual Culture Interactions

A total of 10 identified fungal species were cultivated on PDA plates at 25^{o}C for 6 days as individual monocultures and in 45 statistically derived possible combinations for the dual cultures were derived by using statistical permutations adapted from the Punnett square as described by Punnett in 1905 (Bateson, 1902). Approximately 10 mm wide plugs from monoaxenic plate cultures were cut and placed at the edge of the plates with a distance of 50mm

between the two plugs of interacting fungal mycelia to ensure that competing mycelia met at the centre. These interactions were repeated on all the different agriculture residue plate media. After 6 days of inoculation, the growth state of both self and interspecies pairing was observed, growth was allowed to extend for 14 days.

4.2.3 Microscopic Examination of Dual Culture Interactions

Fungal mycelial were exercised using a scapel from the interaction zones of whole cultures in Petri dishes as described by Ujor (2010) (refer to Figure 4.1). A drop of 0.1 % Congo-red stain (Slifkin & Cumbie, 1988) was added. Samples were washed with distilled water after 2 minutes, blotted dry before covering with cover slip. Microscopic observations were done at X4 and X10 objective magnifications on an Olympus CX41™ Light Microscope.

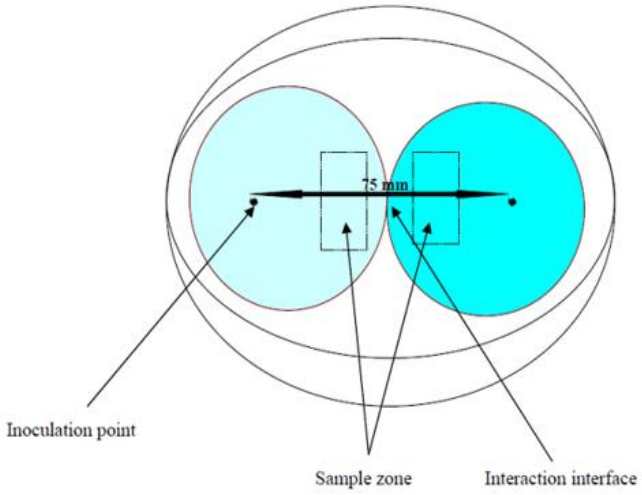

Figure 4.1: Schematic representation of and agar plate interaction assay between self and non-self-fungal colony. Note: sample zones as indicated by Ujor (2012).

4.3 Results

4.3.1 Culture Combination and Dual Culture Interactions

Table 4.1 describes the various dual combinations that were possible for the 10 fungal species in the present study. Observations were reported for day 14 of incubation. Two types of interactions were observed on PDA in all 45 combinations – deadlock and replacement. Although deadlock interactions were not the focus of the study it is an important outcome of competition. About 55% of the total number of dual culture fungi interactions demonstrated varied types of deadlock antagonism (Table 4.2). Figure 4.2 shows the different types of deadlock observed with the interspecific interactions done within the study.

Table 4.1: Factorial combinations of the isolated fungal treatments

Fungi	KN1	KN2	KN3	KN4	KN5	KN6	KN9	KN10	KN12	GL
KN1	KN1-KN1	KN1-KN2	KN1-KN3	KN1-KN4	KN1-KN5	KN1-KN6	KN1-KN9	KN1-KN10	KN1-KN12	KN1-GL
KN2	KN1-KN2	KN2-KN2	KN2-KN3	K2-KN4	KN2-KN5	KN2-KN6	KN2-KN9	KN2-KN10	KN2-KN12	KN2-GL
KN3	KN1-KN3	KN2-KN3	KN3-KN3	KN3-KN4	KN3-KN5	KN3-KN6	KN3-KN9	KN3-KN10	KN3-KN12	KN3-GL
KN4	KN1-KN4	KN2-KN4	KN3-KN4	KN4-KN4	KN4-KN5	KN4-KN6	KN4-KN9	KN4-KN10	KN4-KN12	KN4-GL
KN5	KN1-KN5	KN2-KN5	KN3-KN5	KN4-KN5	KN5-KN5	KN5-KN6	KN5-KN9	KN5-KN10	KN5-KN12	KN5-GL
KN6	KN1-KN6	KN2-KN6	KN3-KN6	KN4-KN6	KN5-KN6	KN6-KN6	KN6-KN9	KN6-KN10	KN6-KN12	KN6-GL
KN9	KN1-KN9	KN2-KN9	KN3-KN9	KN4-KN9	KN5-KN9	KN6-KN9	KN9-KN9	KN9-KN10	KN9-KN12	KN9-GL
KN10	KN1-KN10	KN2-KN10	KN3-KN10	KN4-KN10	KN5-KN10	KN6-KN10	KN9-KN10	KN10-KN10	KN10-KN12	KN10-GL
KN12	KN1-KN12	KN2-KN12	KN3-KN12	KN4-KN12	KN5-KN12	KN6-KN12	KN9-KN12	KN10-KN12	KN12-KN12	KN12-GL
GL	KN1-GL	KN2-GL	KN3-GL	KN4-GL	KN5-GL	KN6-GL	KN9-GL	KN10-GL	KN12-GL	GL-GL

The combinations in grey fonts are repeats and therefore were not plated.

Table 4.2: Fungi dual cultures interactions showing deadlock

	Fungi dual cultures	Observation
KN1-KN12	*Fomitopsis* sp. +*Myrmaecium* sp.	deadlock at touching point
KN1-KN4	*Fomitopsis* sp.+ *Schizophyllum* sp.	deadlock at touching point
KN1-KN6	*Fomitopsis* sp.+ *Coriolopsis* sp.	deadlock at touching point
KN1-KN9	*Fomitopsis* sp.+ *Fusarium* sp.	deadlock at touching point
KN2-KN10	*R. microsporus* + *Trichoderma* sp.	deadlock at touching point
KN3-KN4	*C. borreriae* + *Schizophyllum* sp.	deadlock at touching point
KN3-KN5	*C. borreriae*+ *Umbelopsis* sp.	deadlock at a distance with clear zone of inhibition, darkening of *Umbelopsis* sp. at the edge closest to zone
KN3-KN6	*C. borreriae* + *Coriolopsis* sp.	deadlock at touching point with *Coriolopsis* sp. covering 75% surface area
KN3-KN9	*C. borreriae* + *Fusarium* sp.	deadlock at a distance with clear zone of inhibition
KN3-KN12	*C. borreriae* + *Myrmaecium* sp.	deadlock at a distance with marginal zone of inhibition
KN3-GL	*C. borreriae* + *G. lucidum*	deadlock at a distance with clear zone of inhibition
KN4-KN5	*Schizophyllum* sp. + *Umbelopsis* sp.	deadlock at touching point
KN4-KN6	*Schizophyllum* sp. + *Coriolopsis* sp.	deadlock at touching point with *Coriolopsis* sp. covering 75% surface area
KN4-KN9	*Schizophyllum* sp. + *Fusarium* sp.	deadlock at touching point
KN4-KN12	*Schizophyllum* sp. + *Myrmaecium* sp.	deadlock at touching point
KN4-GL	*Schizophyllum* sp. + *G. lucidum*	deadlock at touching point
KN5-KN9	*Umbelopsis* sp.+ *Fusarium* sp.	deadlock at a distance with clear zone of inhibition; *Fusarium sp* darkened to black; *Umbelopsis sp* kills by darkening
KN5-KN12	*Umbelopsis* sp.+ *Myrmaecium* sp.	deadlock at a distance with clear zone of inhibition
KN5-GL	*Umbelopsis* sp.+ *G. lucidum*	deadlock at touching point
KN6-KN9	*Coriolopsis* sp.+ *Fusarium* sp.	deadlock at touching point
KN6-KN12	*Coriolopsis* sp.+ *Myrmaecium* sp.	deadlock at touching point with *Coriolopsis* sp. covering 75% surface area
KN6-GL	*Coriolopsis* sp.+ *G. lucidum*	deadlock at touching point
KN9-KN12	*Fusarium* sp. + *Myrmaecium* sp.	deadlock at a distance with clear zone of inhibition; *Fusarium* sp. darkened to black
KN9-GL	*Fusarium* sp. + *G. lucidum*	deadlock at touching point
KN12-GL	*Myrmaecium* sp.+ *G. lucidum*	deadlock at touching point

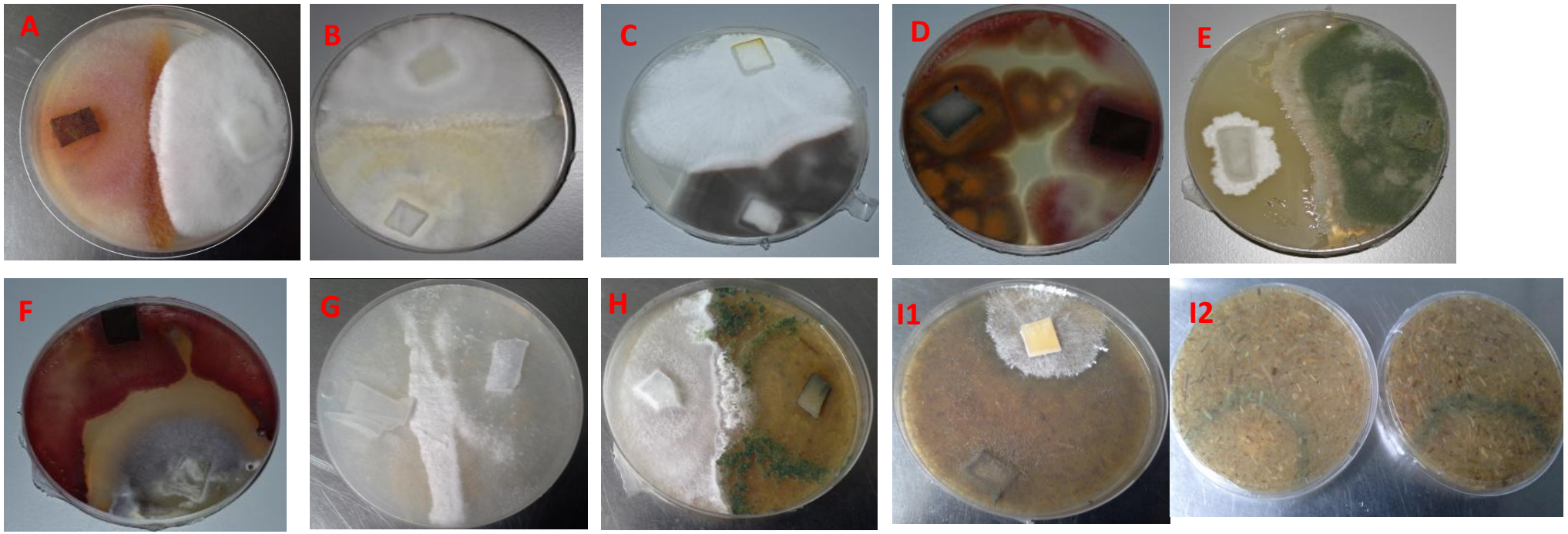

Figure 4.2: Various types of deadlock observed on different substrates. A – *Fomitopsis* sp. KN1 and red pigmented *Myrmaecium* sp. KN12 showing deadlock at touch point; B – *Fomitopsis* sp. KN1 and *Trametes* sp. KN6 showing deadlock at touch point and yellow discolouration in KN6; C – *Ganodermalucidum* encroaching on *Umbelopsis* sp. KN5 with deadlock at touch point and blackening of KN5; D – Clustered growth of *Curvularia sp.* KN3 around *Myrmaecium sp.* KN12 but with deadlock and varying degrees of zones of inhibition; E –*Fomitopsis* sp. KN1 and *Trichoderma* sp. KN10 in struggle observable clear zone of inhibition with obvious production of metabolites; F – *Fusarium* sp. KN9 and *Myrmaecium* sp. KN12 at deadlock with clear zone of inhibition; G – interaction on CCA involving*Fomitopsis* sp. KN1 and *Trametes* sp. KN6 showing deadlock and the formation of barrage; H – interaction on WSA involving *Trametes* sp. KN6 and *Trichoderma* sp. KN10 showing deadlock with the formation of barrage; I1 and I2 – interaction on WSA involving the encroachment of *Rhizopus microsporus* KN2 on *Schizophyllum* sp. KN4, the underside of the agar plate (I2) is showing the inception of green pigmentation around the point of contact. It should be noted that this pigment is not associated with monoaxenic cultures of either fungi.

Table 4.3: Fungi dual cultures showing invasion/replacement

	Fungi dual cultures	**Observation**
KN1-KN2	*Fomitopsis* sp. + *R. microsporus*	*R. microsporus* invaded
KN1-KN3	*Fomitopsis* sp. + *C. borreriae*	*Fomitopsis* sp. invaded
KN1-KN5	*Fomitopsis* sp. + *Umbelopsis* sp.	*Fomitopsis* sp. invaded
KN1-KN10	*Fomitopsis* sp. + *Trichoderma* sp.	*Trichoderma* sp. invaded
KN1-GL	*Fomitopsis* sp. + *G. lucidum*	*Fomitopsis* sp. invaded
KN2-KN3	*R. microsporus* + *C. borreriae*	*R. microsporus* invaded
KN2-KN4	*R. microsporus* + *Schizophyllum* sp.	*R. microsporus* invaded
KN2-KN5	*R. microsporus* + *Umbelopsis* sp.	*R. microsporus* invaded
KN2-KN6	*R. microsporus* +*Coriolopsis* sp.	*R. microsporus* invaded
KN2-KN9	*R. microsporus* + *Fusarium* sp.	*R. microsporus* invaded
KN2-KN12	*R. microsporus* + *Myrmaecium* sp.	*R. microsporus* invaded
KN2-GL	*R. microsporus* + *G. lucidum*	*R. microsporus* invaded
KN3-KN10	*C. borreriae*+ *Trichoderma* sp.	*Trichoderma* sp. *invaded*
KN4-KN10	*Schizophyllum* sp.+ *Trichoderma* sp.	*Trichoderma* sp. *invaded*
KN5-KN6	*Umbelopsis* sp.+ *Coriolopsis* sp.	*Coriolopsis* sp.invaded
KN5-KN10	*Umbelopsis* sp.+ *Trichoderma* sp.	*Trichoderma* sp. *invaded*
KN6-KN10	*Coriolopsis* sp.+ *Trichoderma* sp.	*Trichoderma* sp. *invaded*
KN9-KN10	*Fusarium* sp. + *Trichoderma* sp.	*Trichoderma* sp. *invaded*
KN10-KN12	*Trichoderma* sp. + *Myrmaecium* sp.	*Trichoderma* sp. *invaded*
KN10-GL	*Trichoderma* sp. + *G. lucidum*	*Trichoderma* sp. *invaded*

Approximately 45% of the dual cultures demonstrated replacement interactions (Table 4.3). Four of these fungal species, *Trichoderma* sp. KN10, *Rhizopus microsporus* KN2, *Fomitopsis* sp. KN1 and *Coriolopsis* sp. KN6 demonstrated tendencies of invasion and replacement in dual cultures. However, monoculture of *Myrmaecium* sp. KN12 did not grow on CCA, SBA and WSA; monoculture of *Umbelopsis* sp. KN5 did not grow on SBA therefore it was impossible to test the dual culture combinations involving these organisms on these media. Figure 4.2 show some examples of the antagonistic invasion outcomes that resulted from the interspecific interactions of the fungi used in this study.

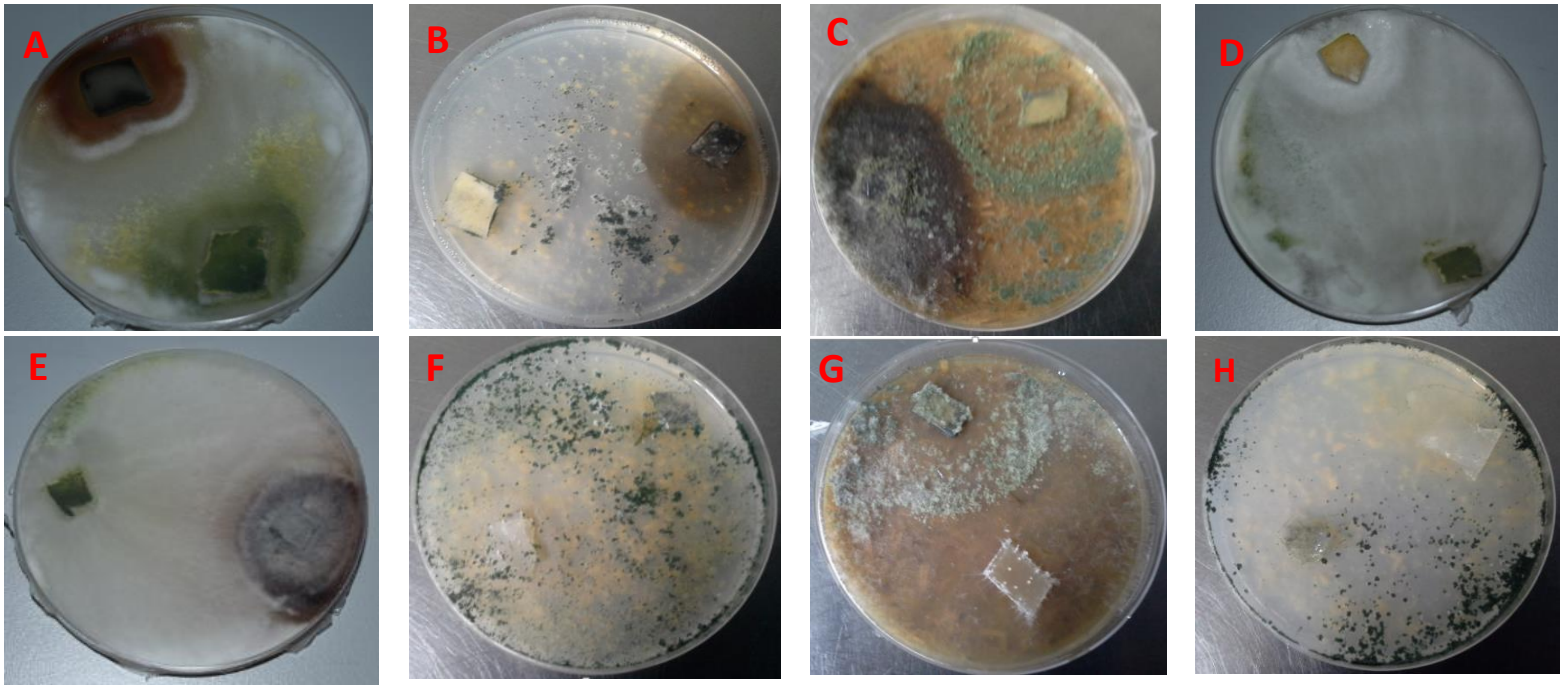

Figure 4.3a: Various antagonistic invasion interactions involving *Trichoderma* sp. KN10. A, B and C – KN10 invading *Curvularia* sp. KN3 on different substrates, PDA, CCA and WSA respectively, with observable changes in morphology as a consequence. On PDA, KN10 show a loss of pigmentation but with a uniform distribution of mycelia around the plate, however on CCA and WSA although it retained its green pigmentation its mycelial distribution on plates are uneven. E, F and G – KN10 invading *Fusarium* sp. KN9 on PDA, CCA and WSA. Complete loss of pigmentation on PDA and a blackening of KN9. On CCA and WSA, mycelia are uneven but green pigmentation is retained. D and H are invasion on *Ganoderma lucidum* on PDA and CCA with the same pattern described previously.

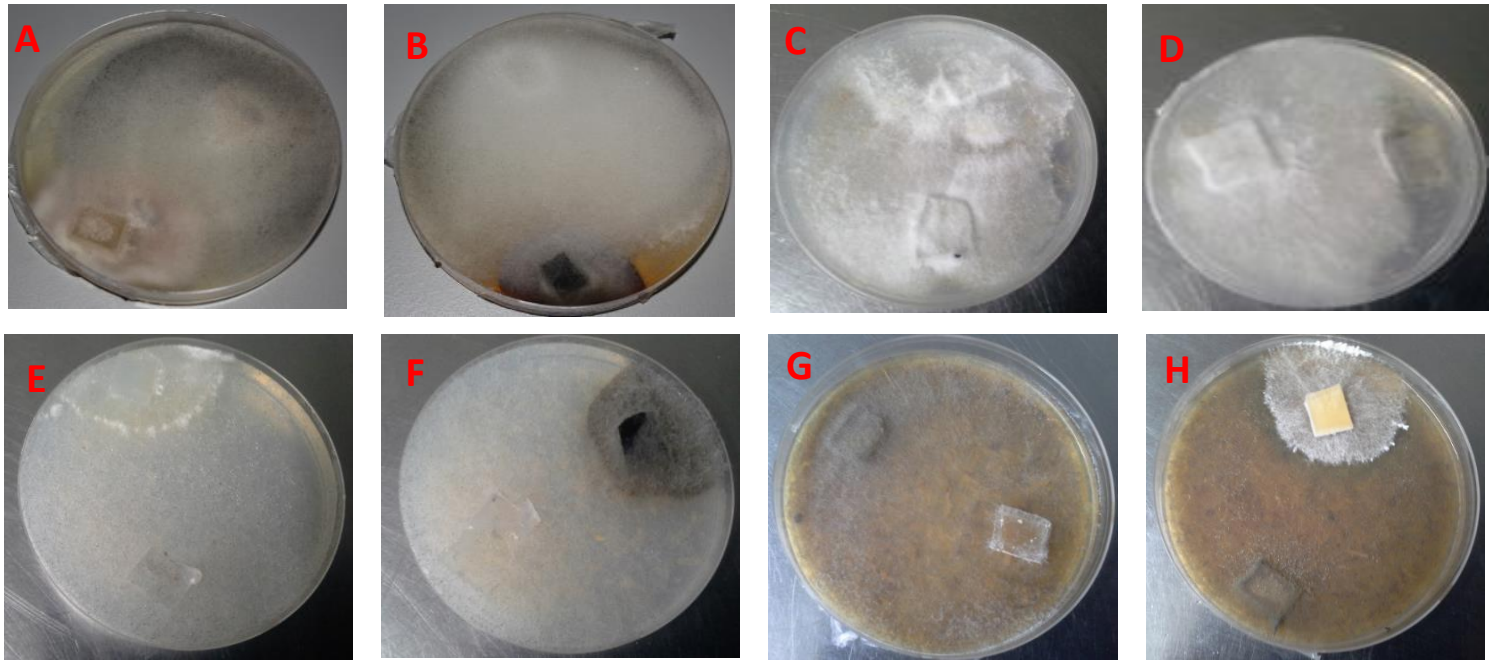

Figure 4.3b: Various antagonistic invasion interactions involving *Rhizopus microsporus* KN2. A and B – KN2 invading *Fusarium* sp. KN9, *Curvularia* sp. KN3 respectively on PDA, observed luxuriant spread of rhizoid tufts of mycelial spread with some black pigments on media; C and D – invasion on SBA, however the invading fungi were *Coriolopsis* sp. KN6 and *Fomitopsis* sp. KN1; E and F – KN2 invaded *Schizophyllum* sp. KN4 and *Curvularia* sp. KN3 respectively on CCA with observed difference in rhizoid mycelial distribution compared to PDA; G and H – KN2 invading *Fusarium* sp. KN9 and *Schizophyllum* sp. KN4 respectively on WSA with observed difference in rhizoid mycelial distribution compared to PDA.

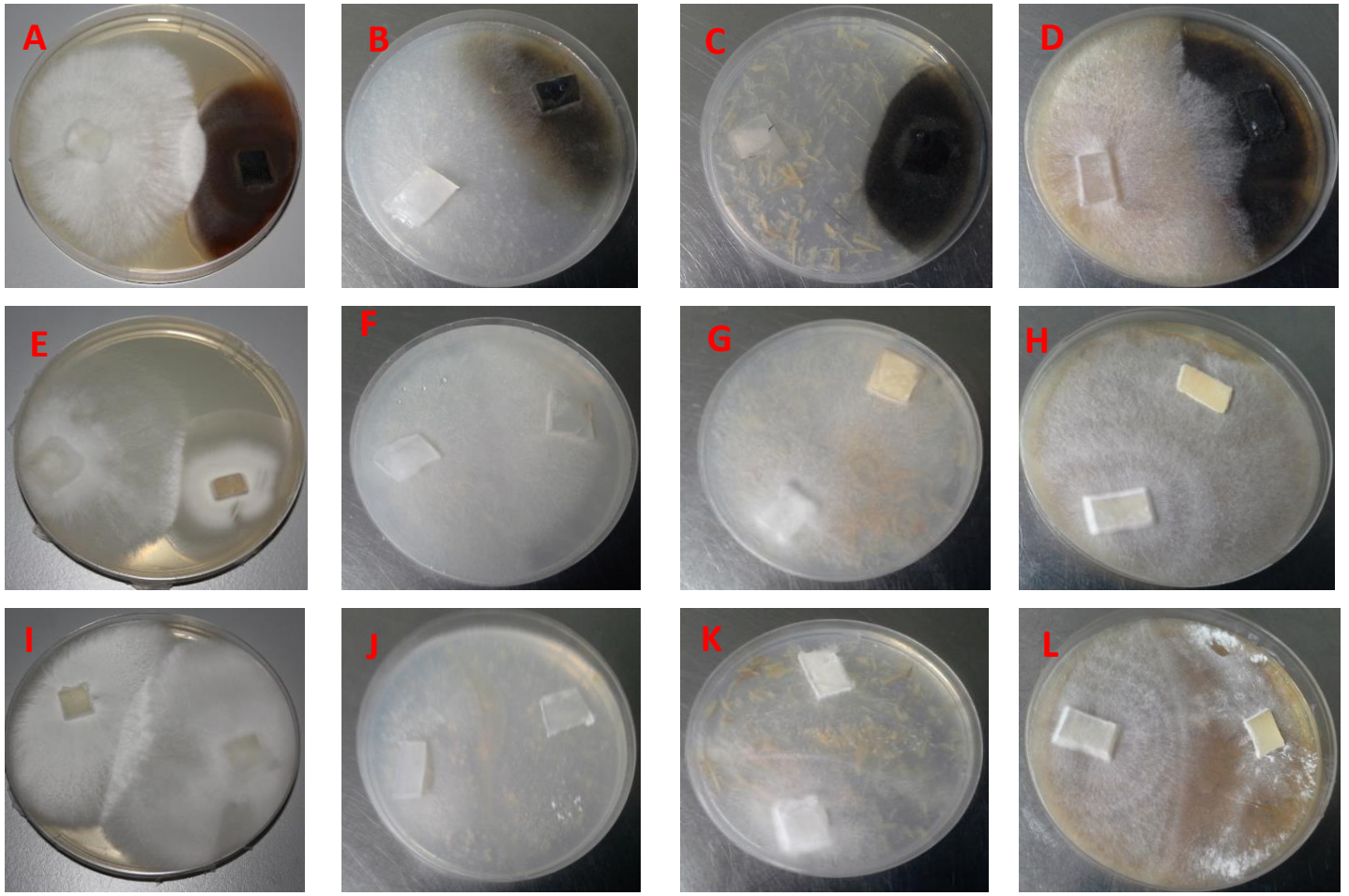

Figure 4.3c: Various antagonistic invasion interactions involving *Fomitopsis* sp. KN1. A, B, C and D – KN1 invading *Curvularia* sp. KN3 on PDA, CCA, SBA and WSA respectively; E, F, G and H – KN1 invading *Umbelopsis* sp. KN5on PDA, CCA, SBA and WSA respectively; I, J, K and L – KN1 invading *Ganodermalucidum*on PDA, CCA, SBA and WSA respectively observable variation in morphology

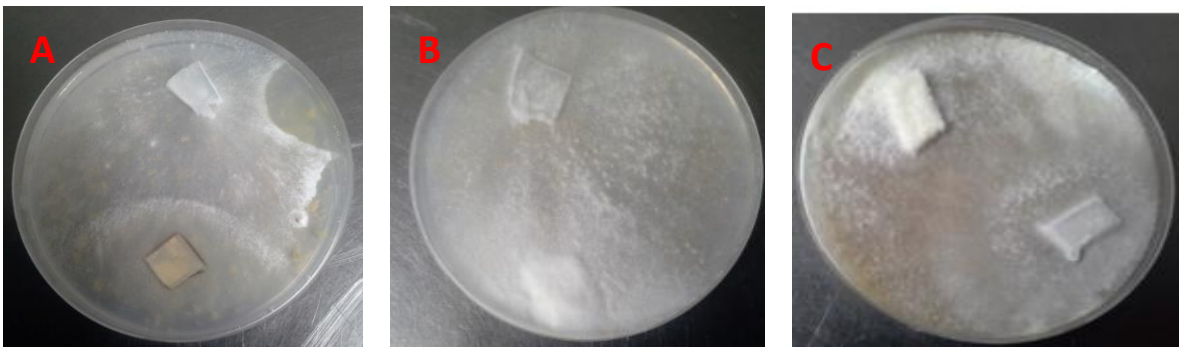

Figure 4.3d: Antagonistic invasion interaction involving *Corioplosis* sp. KN6. A, B and C – KN6 invading *Umbelopsis* sp. KN5 on CCA, SBA and WSA respectively observable variation in morphology

4.3.2 Microscopic Examination of Dual Culture Interactions

Figure 4.4a – 4.4g are micrographs of the interaction zones of whole cultures demonstrating varying examples of antagonistic interspecific interactions. A common observation was situations of hyphal and protoplasmic degeneration. Further protoplasmic leakages was also identified with older cultures showing necrosis within the hypha. Remarkably, but specific to antagonistic invasion interaction involving *Rhizopus microsporus* KN2 invading *Fusarium* sp. KN9 coiling of KN2 around the hypha of KN9 was observed.

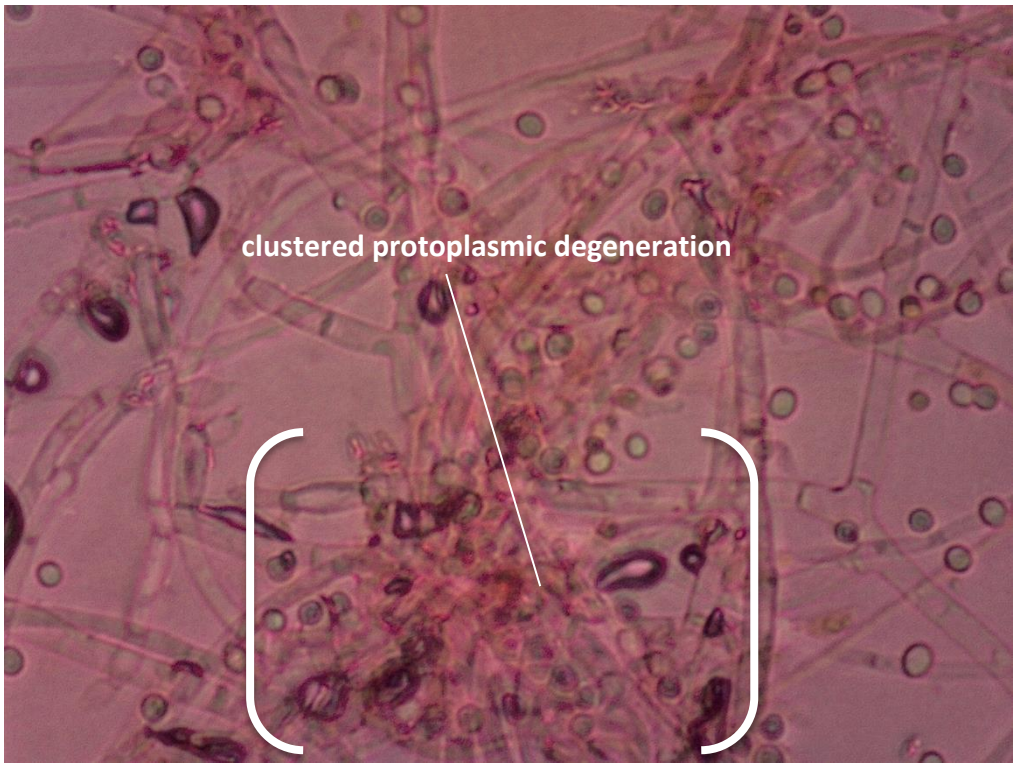

Figure 4.4a: Microscopic examination of antagonistic invasion interaction involving *Trichoderma* sp. KN10 invading *Curvularia* sp. KN3 (Total Magnification = X100)

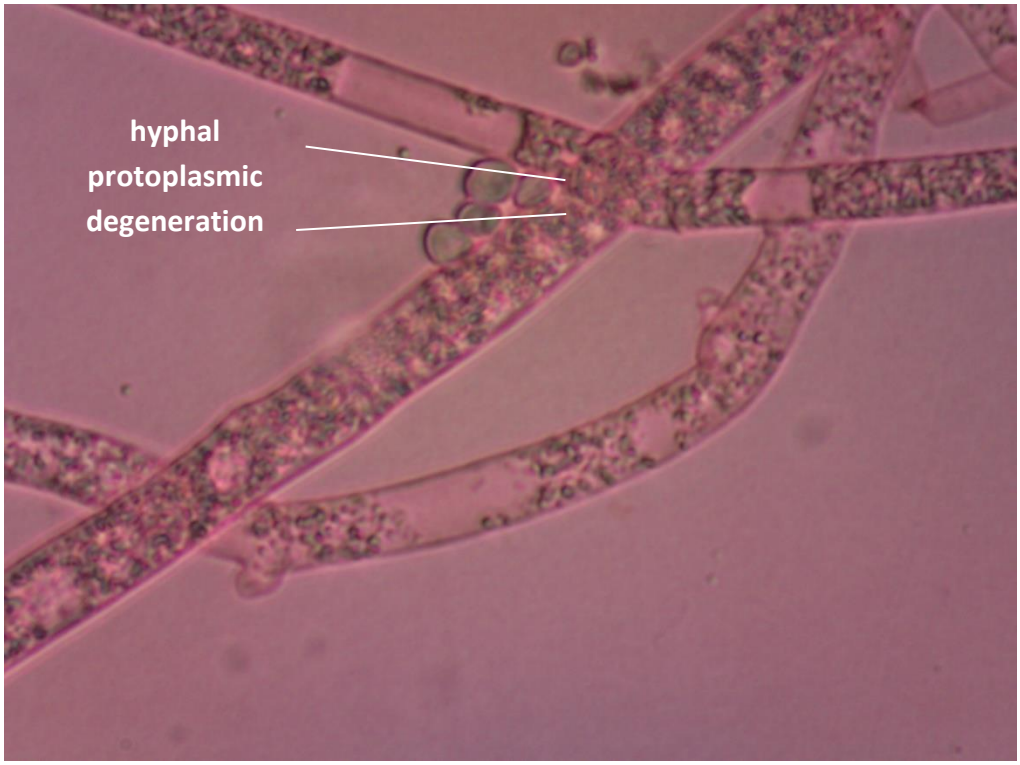

Figure 4.4b: Microscopic examination of antagonistic invasion interaction involving *Fomitopsis* sp. KN1 invading *Ganoderma lucidum;* showing hyphal protoplasmic degeneration and relative enlargement of hypha (Total Magnification = X100)

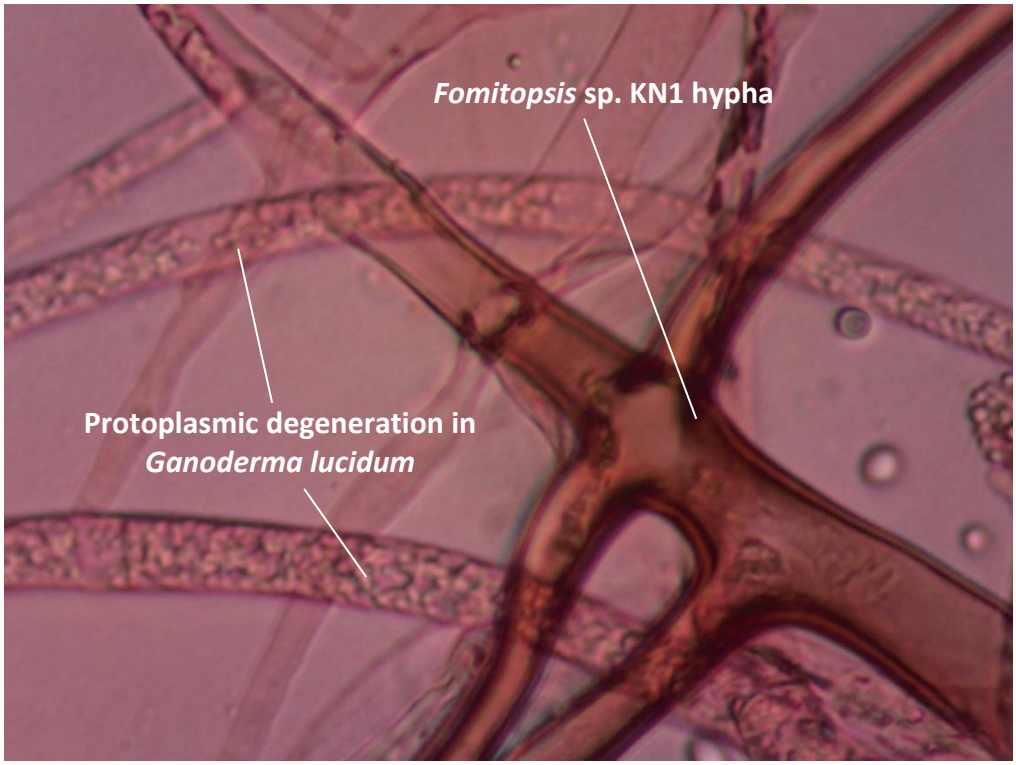

Figure 4.4c: Microscopic examination of antagonistic invasion interaction involving *Fomitopsis* sp. KN1 invading *Ganoderma lucidum;* showing hyphal protoplasmic degeneration at points of hyphal contact with KN1 (Total Magnification = X100)

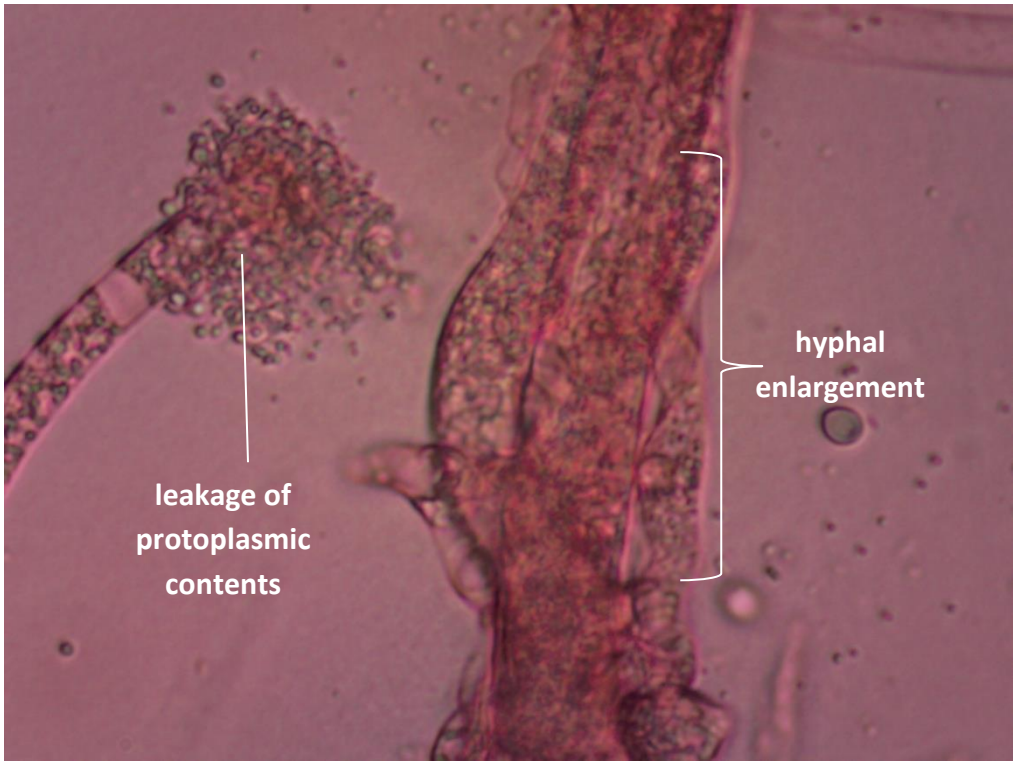

Figure 4.4d: Microscopic examination of antagonistic invasion interaction involving *Fomitopsis* sp. KN1 invading *Ganoderma lucidum;* showing hyphal disintegration and leakage of protoplasmic contents (left) and enlargement of hyphae (right) in *Ganoderma lucidum* (Total Magnification = X100)

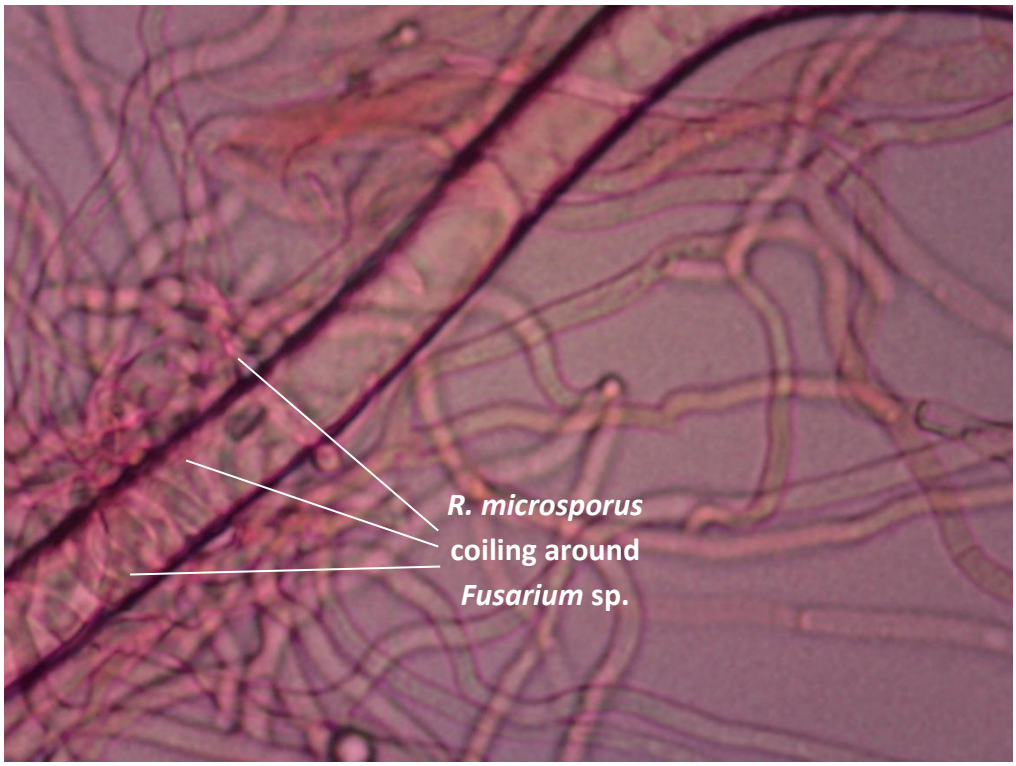

Figure 4.4e: Microscopic examination of antagonistic invasion interaction involving *Rhizopus microsporus* KN2 invading *Fusarium* sp. KN9*;* showing coiling of KN2 around the hypha of KN9 (Total Magnification = X100)

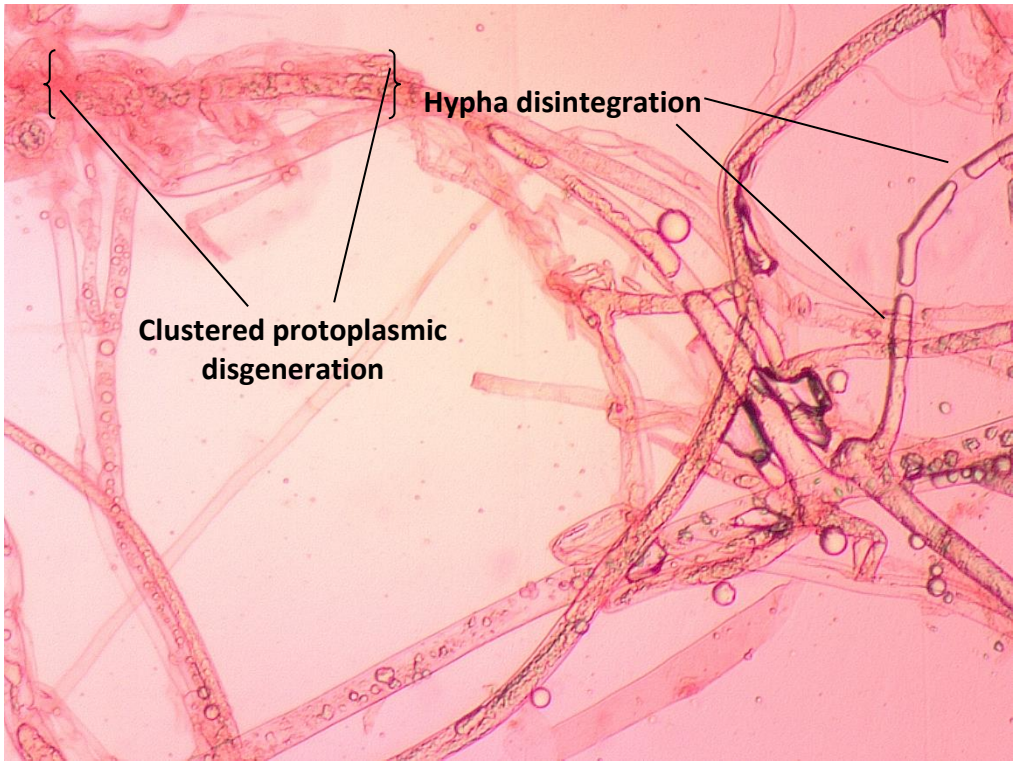

Figure 4.4f: Microscopic examination of antagonistic invasion interaction involving *Rhizopus microsporus* KN2 invading *Ganoderma lucidum;* showing hyphal disintegration and clustered protoplasmic degeneration (Total Magnification = X100)

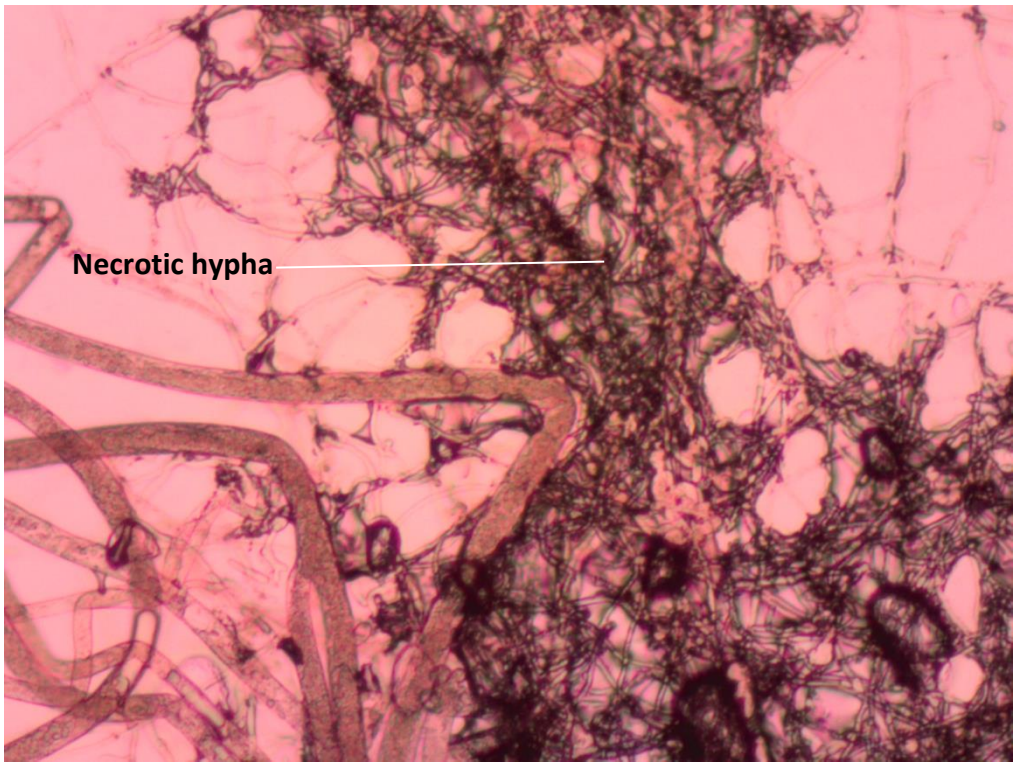

Figure 4.4g: Microscopic examination of antagonistic invasion interaction involving *Rhizopus microsporus* KN2 invading *Schizophyllum sp* KN4*;* showing necrosis of KN4 (Total Magnification = X100)

Discussion

All possible dual combinations for the 10 fungal species used in this study were derived by applying statistical permutations based on the Punnet Square (Bateson, 1902). Although the use of the Punnet Square is considered a simple and effective method to achieve all possible statistical combinations of dual fungi interaction, it lends itself to redundancy as there are several repetitions as well as duplication of self-to-self interactions. However a generalized permutation formula can be used that eliminates repetition and redundancy is ${}_nP_r = n!/(n-r)!$; where n is number of fungal species and r is equal to 1 (McCaffey, 2006). Nevertheless, 90 combinations were generated; only 45 of these possible interactions were used from the permutations of dual interactions of the 10 selected fungal species in this study.

The primary focus of the present study was invasion-replacement interactions (Table 4.3). The conceptual hypothesis is that competition and antagonism will promote or induce the expression of ligninolytic enzymes in interspecific interactions of fungi on different lignocellulosic substrates. This is in agreement with work done by Hiscox *et al*. (2015) which reported that such antagonistic mechanisms in wood territory tend to affect the rate of resource use and therefore increase decay rate to provide the necessary carbon and energy for metabolism. On PDA plates, it was observed that almost all invading fungi covered the entire plate surface area within the 6 days of incubation; in most cases killing off the weaker fungi. However complete invasion was slower on agricultural waste residue than on PDA with most occurrences happening between the 9^{th} and 18^{th} day of incubation. It is most likely that this happened because the ligninolytic gene expression necessary for substrate enzyme production is only triggered by the depletion of nutrient carbon (Wang *et al.*, 2008). Furthermore, on some previously deadlocked interactions between day 18 and day 21 some of the barrages initially observed disappeared, most likely as a consequence of the invading fungi overwhelming the physical defences of the weaker fungi. This peculiar phenomenon observed in this study had been previously alluded to by Boddy (2000) however observations were on wood colonies. In the present studies this ability to overcome an initial deadlocked situation was observed on the different substrates by different fungal combinations. It is also most likely the reason Boddy (2000) asserted that it is difficult to replicate interactions. However this study demonstrated that often when investigations are concluded before the final outcomes of interspecfic interactions it is possible that conflicting results may be reported, thereby creating the assumption that interactions are not replicated.

More often, similar patterns of invasion that were observed on PDA (which was employed as a positive control in this study) were replicated on all three agricultural residues except in cases where monocultures of the fungi were unable to grow on the agricultural residues, most likely due to a lack of substrate affinity. It must be noted that the primary habitat of the fungi used in this study were forest litter and although these are lignocellulosic material they are likely to be of different biomass composition as compared to the agricultural residues used in this study (Kumar *et al*., 2009; Sorek *et al*., 2014). Nevertheless, three of the fungi *Fomitopsis* sp. KN1, *R.microsporus* KN2 and *Trichoderma* sp. KN10 tended to invade the others in co-cultures, but, in one co-culture combination, *Coriolopsis* sp. KN6 was the invader when in combination with *Umbelopsis* sp. KN5.

Remarkably, macroscopic examinations of invasion-replacement interactions involving *Trichoderma* sp. KN10 showed several variations in pigmentation and morphological distribution of mycelia on the different substrates. On PDA, KN10 demonstrated a loss of pigmentation but had a uniform spread of mycelial on plates, however on agricultural substrates it tended to retain its pigmentation but its mycelial distribution became uneven to the extent that in some cases signs of invasions could only be concluded with speckled growth on top of the other fungal species mycelial spread, in the first few days. However usually by the 17th day its invasions were complete on the plate, often with associated death of the other fungi observed by the blackening of some of these other fungi. Several authors have associated the changes in pigmentation during fungus-fungus interactions to the activity and changes in the levels of phenoloxidases (Boddy, 2000; Crowe & Olsson 2001; Iakovlev *et al*. 2004; Rayner *et al*., 1994). Griffith *et al.* (2007) even go so far as to suggest a link between pigment formation and copper quantities within substrates. A correlation can be made between the retaining of pigmentation by *Trichoderma* sp. KN10 on all agricultural residues during the interspecific invasion and the presence of copper in these substrates (details of these can be found in Chapter 5 compositional analysis of these substrates). However it must be noted that there was a loss of pigmentation observed in the same interspecific invasion interaction (Figure 4.3a) on PDA which contains no copper. Most authors are in agreement that interspecific interactions often results in a change in the morphology of the fungi involved (Boddy, 2000; Baldrian, 2004; Heilmann-Clausen & Boddy, 2005; Hiscox *et al*., 2010; 2016). In the present study, one fungi that consistently demonstrated these variations in its morphology, particularly on the different substrates was *Fomitopsis* sp. KN1 (Figure 4.3c), thereby exemplifying this assertion.

Microscopic examination at the points of contact of fungi involved in these interspecific interactions showed similar characteristics as previously described by several authors (Howell, 2003; Lu *et al*., 2004; Marra *et al*., 2006; Ujor, 2010). *Trichoderma* sp. KN10 demonstrated the typical mycoparasitic properties associated with its invasion during interspecific interaction in most of the interaction sample zones examined (Ujor, 2010) including as seen in Figure 4.4a. It is presumed that cell wall-degrading enzymes are responsible in this mycoparasitic strategy (Howell, 2003; Lu *et al*., 2004; Marra *et al*., 2006). A major recurrent morphological change that was observed in almost all interspecific interactions in the present study, was hyphal protoplasmic degeneration (Figures 4.4a and 4.4b) which was characterized by the formation of protoplasmic aggregates within the affected mycelial which often led to eventual necrosis. Ujor (2010) noted that a distinction exists between early onset of intracellular protoplasmic content-aggregation and hyphal interference as the former required other physiological reaction before hyphal disintegration can commence which is not the case with typical hyphal interference. Halliwell and Aruoma (1991) suggest the possibility that some protoplasmic degeneration could be attributed to oxidative damage such as lipid peroxidation of cell membranes, partially a consequence of the likely disruption of mitochondrial functioning that allows electron leakage (Iakovlev *et al*., 2004). This link is not far-fetched if one considers that the mitochondria functions in the electron transport chain; therefore an aggregation of intracellular components would inadvertently change the mitochondrial membrane potential (Ujor, 2010). It was possible to visualize hyphal disintegration and leakage of protoplasmic content in Figure 4.4d where *Fomitopsis* sp. KN1 was invading *Ganoderma lucidum.* It must also be noted that with most samples examined there usually was an enlargement of hyphae before mycelia lysis. It is presumed that the lysis of the cell wall may be associated with pigmentation as well as the up-regulation of phenol compounds. However, it is established that these phenol compounds are produced as a consequence of the expression of phenylalanine ammonia lyase (PAL), an enzyme that is commonly produced during the stationary phase in plants, fungi and actinomycetes. Its importance stems from PAL being the catalyzing enzyme of its specific compounds in the phenylpropanoid pathway which is the major source of phenol compounds (Kalghatgi & Rao, 1976; Kurosaki *et al*., 1986; Kim *et al*., 2001; Gomez-Vasquez *et al*., 2004). Moreover, it can be deduced that since wounding is one of the stress factors that can activate PAL synthesis, it is indeed plausible that, if one factors in substrate induction, that is possibly a consequence of the different agricultural residues that were used and the stress conditions associated with dual culture interactions, in all probability PAL maybe responsible

for the increased production of phenol compounds, which promote the synthesis and secretion of phenoloxidases during interspecific interactions that involve lysis (Crowe & Olsson, 2001).

In Figure 4.4e, antagonistic invasion interaction involving *Rhizopus microsporus* KN2 invading *Fusarium* sp. KN9 showed coiling of KN2 around the hyphae of KN9 in a mechanism similar to *Trichoderma* species when involved in mycoparasitic relationships (Lu *et al.*, 2004; Aryantha & Guest, 2006). Although, *Rhizopus* sp. is a known pathogen and *R. oryzae* have in the past been investigated in interspecific interactions (Jeffries, 1995; Bonfante & Anca, 2009); the present study observed a range of antagonistic invasion interactions with *Rhizopus* sp. KN2 invoking responses and increased enzyme activity as a consequence of this interspecific interaction, therefore this requires further study. With *Trichoderma* sp. such coiling and development of antagonistic structures known as 'appressoria' was determined to be associated with the accumulation of polyols proposed to be necessary to a certain extent in the production of inner hydrostatic turgor pressure that is a pre-requisite in the development of the said structure around host mycelia (Ujor, 2010).

Ultimately, most invasion replacement interactions tend to end with cellular necrosis as shown in Figure 4.4g. Yorimitsu and Klionsky (2005) suggest that replacement could induce autophaghy which is a normal degradation mechanism in eukaryotic cells that a cell employs in recycling aged proteins and other organelles. Pinan-Lucarre *et al.* (2005) identified the presence of key genes involved in autophagy during interspecific interactions. It is presumed that the objective of this attack (replacement) could be that the lysis of the weaker fungi will release hyphal contents that can subsequently be used by the antagonist (Falconer *et al.*, 2008).

There are copious evidence that apoptosis is a stress response in fungi, examples that can be easily associated with interspecific invasion interaction will include the production of antifungal agents (Leiter *et al.*, 2005) and carbon starvation (Emri *et al.*, 2005; Sharon *et al.*, 2009) particularly in confined spaces with limited nutrient supply as typified by plate cultivation in this present study. Thus far, one of the best studied case of fungal apoptosis still remains that *Saccharomyces cerevisiae* (Sharon *et al.*, 2009). There are evidence to support that apoptosis in fungi is associates with an intrinsic-like pathway. This form of apoptotic response is mediated by the mitochondria in a series of complex reactions involving several mitochondrial proteins released when the mitochondrial permeability transition (MPT) pore is opened (Daugas *et al.*, 2000; Li *et al.*, 2001; Jiang and Wang, 2004). However, knowledge of

apoptosis in other interspecific interactions of fungi remains limited and the elucidation of molecular components and cellular mechanisms involved is needed.

Conclusion

The present study is a part of studies undertaken to understand the interspecific interactions identified within the dual combinations of fungal species chosen for this research. Macroscopic and microscopic examinations of the interspecific interactions demonstrated that there are significant changes that are a consequence of these interactions and would likely influence biochemical activities such as enzyme production in response to antagonism. The outcome of the interactions in substrate utilisation and enzyme production is discussed in Chapter 5.

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Chapter 5

Comparative evaluation of enzyme production efficiency of monocultures and paired interactions of fungi on different agricultural substrates

5.0 Abstract

There is a renewed interest in finding sustainable energy sources with particular focus on agricultural waste residues. The justification for this perspective is that this eliminates the need to cultivate already scarce land mass and has the added advantage of utilising agricultural residues that otherwise would have presented problems of waste management. However the use of agricultural waste residue is significantly hindered by the difficulty in degradation of lignocellulose components of its structure. Ligninolytic fungi have the ability to degrade these agricultural waste residues but enzymes employed in the degradation process are produced in limited quantities and more often during the secondary metabolism by these organisms. The objective of this study was to investigate a strategy that could improve the production of these enzymes and likely accelerate the organisms into secondary phase enzyme production mode. Dual culture combinations of 10 fungi that had previously demonstrated the ability to produce ligninolytic enzymes were cultivated on PDA to ascertain their interspecific interaction and also on three agricultural residues, corn cob, sugarcane bagasse and wheat straw. Spectrophotometric analysis of the enzyme activities of laccase, manganese peroxidase and lignin peroxidase demonstrated that observed antagonistic invasions yielded an increased enzyme activity in dual cultures on all the substrates. However the highest ligninolytic enzyme production was observed in invasion/replacement interactions that involved *Trichoderma* sp. KN10 with average mean value of MnP production was approximately 1.46U/ml compared to all monocultures of 0.055U/ml. Similarly Lacc mean value was 0.10U/ml compared to monocultures value of 0.05U/ml. This study demonstrated and proved that antagonistic invasion by some fungi in co-culture, although dependent on substrate affinity, can increase production of one or more of the three enzymes laccase, lignin peroxidase and manganese peroxidase.

5.1 Introduction

One of the most difficult problems in these contemporary times is managing the waste generated as a consequence of the various human activities. In our quest to improve the quality

of life, we have exploited and transformed natural resources to provide more food, better living conditions and promote longevity (Hall *et al*., 2009). However such endeavors have left a cornucopia of environmental problems in its wake. Agriculture is considered one of the greatest generator of waste. Globally, 140 billion metric tons of biomass is generated every year from agriculture (UNEP, 2009). The large volume of agroforestry waste presents a challenge as to finding ways for disposal that does not negatively impact the environment. The usual practice of burning or leaving the waste products of agriculture to rot is deemed unwholesome to humans and the environment alike.When waste management is not handled efficiently, it can lead to disastrous consequences that leave a lasting effect (Levis *et al*., 2010). Therefore it is necessary to find effective waste disposal methods that have the added advantage of being environmentally friendly as well as cost effective. Thus, the current focus of environmental management is the conversion of these agricultural waste residues into useful products as this provides incentive for the continual practice of such a method of waste disposal.

Moreover the utilization of agricultural waste residue is regarded as highly sustainable because it is routinely generated thus guaranteeing energy security and climate change mitigation. The enormous volume of biomass generated in agriculture offers readily available annual resource for raw materials and energy production. The United Nations Environmental Programme (2009) estimates that approximately 50 billion tons of oil can be produced annually from agricultural biomass waste which has the potential to substantially displace fossil fuel. The positive spin-off will be a reduction in greenhouse gases (GHG) emission, the provision of renewable energy to an estimated 1.6 billion people in developing countries as well as offering cheap raw materials to large-scale industries and community-level enterprises.

Our dependence in the past century on fossil derived energy has taken its toll on the environment and has brought with it a realization that there is an urgent need to seek renewable sources to meet our burgeoning energy demands. Exacerbating this problem is the predicted decline of global crude oil production; Campbell and Laherrere (1998) attest to this pending reduction in fossil fuel generation, with an earlier prediction that oil production will decline from 25 billion to approximately 5 billion barrels in 2050. Such predictions are credible when one looks at the underpinning fact that crude oil is a limited non-renewable resource.

The use of these fossil derived fuels such as coal and petroleum releases CO_2 which is regarded as a major contributor to global warming (Yat *et al*., 2008). Most countries around the world

have joined in the global combat against climate change with a pledge to reduce their GHG emissions to help reduce the negative impact of global warming One way to be able to fulfill such pledges is to seek alternative energy sources that reduce the carbon footprint and are environmentally friendly.

The most popular renewable fuel alternative at present is ethanol-derived fuels, particularly with its on-going success in emerging economies such as Brazil (Watanabe 2009; Villarreal 2013). Currently the most convenient source of ethanol-derived fuels is from maize and sugarcane (first-generation) (Figure 5.1). However there is a fierce debate regarding "food versus fuel" exploitations of these raw material base (UNEP, 2009). Credible concerns are raised that these ethanol-derived fuels currently competes negatively with the human use as food source and that it would on the long run impact food security and drive world market prices; creating similar problems associated with demand as is seen with petroleum currently. Furthermore, the dependence on petroleum has led to most countries' economic crisis especially with the concomitant fluctuations in market prices. One solution that immediately presents itself is the use of agriculture-derived waste products (second-generation derived biofuels), which are readily available in all countries and more often the lack of use of these waste residues tend to pose both environmental and health problems due to uncontrolled decomposition process.

Moreover, the use of second-generation derived biofuels not only promotes independence from outside suppliers but it also promotes rural development and reduction in GHGs (Demirbas, 2005). Countries like the United States through its Department of Energy in 2007 invested over US$1 billion for lignocellulosic bioethanol projects, with a goal of making the fuel cost competitive at US$1.33 per gallon by the year 2012 (Slade *et al*., 2009). Presently the United States Department of Energy partners with developing countries such as India with large agricultural base towards developing efficient second generation biofuels (DOE, 2015). Owing to the abundance and sustainability of lignocellulosic materials; they present an attractive, low-cost feed stock for energy production (Ren *et al*., 2009). This is justifiable in the light of an estimated worldwide annual yields of 140 billion metric tons of biomass generated from agriculture (UNEP, 2009).

The shift towards the use of lignocellulosic agricultural residues will only be possible if economically viable pretreatment methods are readily available. At present few

microorganisms have the innate capability to degrade lignocellulose. Some ligninolytic fungi are able to produce a group of enzymes that degrades the recalcitrant constituent lignin thereby exposing the cellulose and hemicellulose fractions that are more readily useful as platform sugars in fermentation. The use of these ligninolytic fungi is a potential strategy that considers both economics and environmental remediation (Mahajan, 2011). The use of these biological pretreatment strategies is limited by several factors which include the poor production of these enzymes particularly since most studies have focused on mono-culture production. Studies on microbial consortia and their mixed enzyme systems could provide an important foundation for the understanding of the complex interactions of lignocellulosic degradation in composting, anaerobic digestion and enzymatic biomass saccharification (Wongwilaiwalin *et al*., 2010).

Furthermore, the use of microbial co-cultures or complex communities has in more recent times been proposed as a highly efficient approach because it avoids the problems of biotechnological applications, feedback regulations as well as metabolite repression posed by isolated single strains (Haruta *et al*., 2002). Moreover most fungi during their natural life cycle exist in mixed communities that expose them to complex interactions such as symbiosis and competition (Hu *et al*., 2011). Although considered a tedious exercise; the understanding of the interactions between associated strains in a co-culture is quite important. Currently, very little research has been done, thus, this offers new avenues for future research work (Cheng & Zhu, 2012).

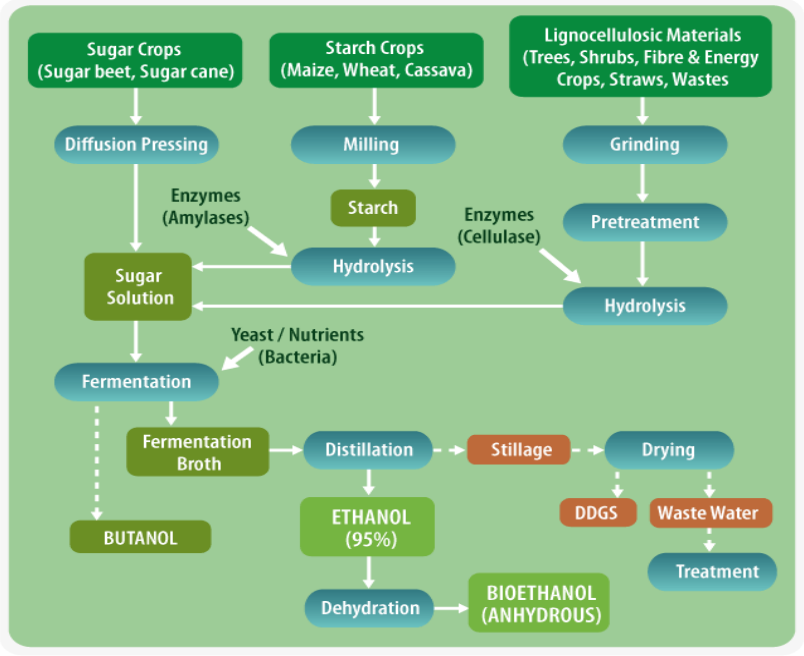

Figure 5.1 Schematic representation of steps involved in the conversion of first and second generation to bioethanol (adapted from Sun & Guo, 2012).

5.2 Literature Review

5.2.1 First generation versus second generation biofuels

Currently the vast majority of biofuels used in vehicles and petrol engines are made using first generation bioethanol produced from resources such as wheat, sugar cane, sugar beet and maize. Second generation biofuels are a response to 'food vs fuel' debate and necessary to avoid land use conflict with the food and feed sectors which also use first generation resources in their production. The distinction between first and second-generation biofuels is somewhat blurred wherein the only clear classification is based on the feedstock used in production (Larson, 2008). Generally apart from the feedstock source of first generation biofuels, which is mainly sugars, grains or seeds; it tends to require relatively simple processing to produce fuel. In comparison, second generation biofuels would generally be produced from non-edible lignocellulosic biomass, including residues of agricultural crops or forestry production including corn cobs, rice husks, sugarcane bagasse, wheat straw, forest thinning, saw dust e.t.c and whole plant biomass such as energy crops including switchgrass, jatropha poplar and other fast growing trees and grasses. Such energy crops do no directly compete with crops for high quality land as such they are regarded as second-generation biofuels (Carriquiry *et al*., 2010).

The issues of sustainability raised regarding the possibility of undue competition for land and water used in the production of first generation biofuels has made it clear that they cannot be considered a substitute for oil-products neither can it be viewed as a strategy for climate change mitigation. Furthermore it is regarded as likely to have detrimental effect on economic growth as gains observed in environmental management is eroded with the likely effect it will have on food prices of these cash crops. Moreover, some authors criticize the so-called environmental management counter-measures as potentially likely to cause the production of monocultures and deforestation to accommodate the demand for these crops (Searchinger *et al*., 2008; Fargione *et al*., 2008; Mitchell, 2008; Sims *et al*., 2008).

With these increasing criticisms of the sustainability of many of the first-generation biofuels there has been a necessary paradigm shift toward second generation biofuels. Depending on the feedstock chosen, and the cultivation technique, second generation biofuels offer considerable benefits, the most important being the added benefit of efficient waste management and the use of non-arable land. Furthermore, because biomass is a renewable resource particularly those biomass that are generated as by-products of agricultural activity;

its use is considered to be carbon-neutral, thus it can credibly displace fossil fuels and reduce GHG emissions and at the same time close the carbon cycle loop (Sims *et al*., 2008; UNEP 2009).

However, in recent times cost estimates for the production of second generation biofuels have shown that they are two or three times more expensive than petroleum fuels on an energy equivalent (Carriquiry *et al*., 2011); although this is attributed to the process technological development still being at the early stages (Balan, 2014). There are several challenges highlighted by Balan (2014) in his review that will be necessary to address in attempting to reduce production cost of lignocellulosic biomass conversion to biofuels. These challenges include the following aspects in processing: (i) feedstock production; (ii) feedstock logistics; (iii) development of energy efficient technologies (pretreatment, enzyme hydrolysis, and microbial fermentation); (iv) co-products development; (v) establishment of biofuel and biochemical standards; (vi) biofuel distribution; (vii) societal acceptance; and (viii) environmental impact minimization (Hoekman, 2009; Menon & Rao, 2012; Luo *et al*., 2010; Balan, 2014).

The present study focuses on only one aspect of these challenges which is the identification of a possible strategy to increase enzyme production necessary for the biological pretreatment of lignocellulosic biomass towards exposing platform sugars that can be utilized in microbial fermentation and the production of liquid biofuels. It is advantageous to use biological pretreatment because it can be operated under mild conditions and it requires low capital costs when compared to expensive reactor systems required for physical / chemical pretreatment processes (Balan *et al*., 2008).

Several kinds of fungi (soft, brown and white-rot fungi) have been used in the biological pretreatment of biomass. These microorganisms are very effective in degrading lignin as a result of the production of enzymes (peroxidases and laccases) (Sánchez & Cardona, 2008). Biological pre-treatments can be achieved with mild conditions and low capital costs when compared to usual expensive reactor systems that are necessary for physical/chemical pre-treatment processes. Although, it must be noted that biological processes are relatively slow processes. Additionally, microbial bio-treatment causes lower sugar conversion compared to chemical pre-treatment. Often, an initial biological pretreatment followed by chemical pre-

treatment is found to be effective and reduces the severity of pre-treatment conditions usually needed for effective chemical hydrolysis of biomass (Balan *et al.*, 2008).

The production of ligninolytic enzymes in industrially feasible quantities is a critical aspect that has limited the exploitation of the biological pre-treatment. In spite of its advantages over physical and chemical pre-treatment process, even when biological pre-treatments are applied it is often in combination with other methods. Sections of the literature review in this thesis have expanded on the factors that affect enzyme production.

In the present study, previously isolated and characterised fungi that had demonstrated the ability to produce ligninolytic enzymes were cultivated in dual cultures. The study focused of antagonistic interactions. The premise was that such interaction would require an increased metabolic activity (enzyme activity) for the invading fungi to be able to achieve territorial superiority and an increased utilization of available nutrients. The specific objective was to quantify the enzyme produced to enable the determination to be made, whether these dual culture combinations caused an increase in enzyme activity and production. In addition the efficiency of enzyme production in dual cultures was determined on three different agricultural residues using culture-based methods. It is important to note that although methodology and overall objective of the study is the same for both mono- and dual cultures evaluation and quantification, however to establish coherence in data analysis and interpretation of the results in this chapter, results will be presented as follows:

- Data analysis and interpretation of fungi monocultures
- Data analysis and interpretation of fungi dual cultures
    - Invasion interactions involving *Trichoderma* sp. KN10
    - Invasion interactions involving *Rhizopus microsporus* KN2
    - Invasion interactions involving *Fomitopsis* sp. KN1
    - Invasion interactions involving *Coriolopsis* sp. KN6

5.3 Methodology

Preparation of Agricultural Waste Residues – Corncob and Sugarcane bagasse

The corncobs, sugarcane bagasse and wheat straw were comminuted to approximately 2 mm mesh size using initially a garden shredder and then milled with a commercial coffee grinder (Sunbeam Coffee Grinder SCG-250). Samples were oven-dried at 55^{o}C for 24 hours before

further use.

5.3.1 Compositional Analysis of Agricultural Waste Residues

Compositional analyses were carried out for all agricultural residue samples at Nutrilab, Pretoria (South Africa).

Preliminary dry matter (DM) content of samples were determined by weighing the corncob, sugarcane bagasse and wheat straw samples and drying overnight at 105^{o}C in an oven. Ash content was derived by weighing samples before and after ashing at 550^{o}C for two hours in a Muffle Furnace.

5.3.1.1 Starch and Protein Analysis

Total starch analysis was carried out using Mega-zyme starch assay kit based on the use of thermostable α-amylase and amyloglucosidase (McCleary *et al*., 1997) according to a method that was adopted (with modifications) from AOAC Method 996.11 and AACC Method 76.13. The Protein content was quantified by measuring the nitrogen content using Dumas method correspondent to the ASBC-AOAC Method 997.09. The protein content in the corn cob, sugarcane bagasse and wheat straw samples were determined indirectly by thermal decomposition through combustion in a tube at high temperatures (between 900 – 1200^{o}C) in the presence of oxygen.

5.3.1.2 Fibre Content Analysis

The composition of cellulose, hemicellulose and lignin were determined gravimetrically using methods described by Van Soest *et al*. (1991).

5.3.1.3 Elemental Analysis

The Elemental Analysis for all substrates was done using the Shimadzu Atomic Absorption and Flame Emission Spectrophotometer using methods adopted from AOAC 968.08

5.3.2 Preparation of Media

Media plates for surface growth analysis of fungi and crude enzyme extraction were prepared using corncob, sugarcane bagasse and wheat straw powder with media composition calculation based on their standardized glycaemic index values that approximated PDA to achieve a similar consistency. The CCA, SBA and WSA was prepared by adding 5.2 g of corncob powder, 5.88 g of sugarcane bagasse powder and 6.52 g of wheat straw powder respectively with 18 g of

agar and made up to 1000 ml with distilled water. The CCA, SBA and WSA was autoclaved at 121ºC for 15 mins and allowed to cool to 55ºC. These sterile media was poured into petri-plates and allowed to solidify.

5.3.3 Culture Combination and Dual Culture Interactions

A total of 10 identified fungal species were cultivated on PDA plates at 25ºC for 6 days as individual monocultures and in 45 statistically derived possible combinations for the dual cultures were derived by using statistical permutations adapted from the Punnett square as described by Punnett in 1905 (Bateson, 1909). Approximately 10 mm wide plugs from monoaxenic plate cultures were cut and placed at the edge of the plates with a distance of 50mm between the two plugs of interacting fungal mycelia to ensure that competing mycelia met at the centre. These interactions were repeated on all the different agriculture residue plate media. After 6 days of inoculation, the growth state of both self and interspecies pairing was observed, growth was allowed to extend for 14 days.

5.3.4 Crude Enzyme Extraction for Enzyme Assays

Only combinations that demonstrated antagonistic interactions were replicated and assayed for enzyme activity. The crude enzymes were harvested from both monoculture and co-culture plates by washing the mycelia growth on plates in double distilled water, the mycelia was scraped and filtered through glass-wool and diluted to MacFarland 0.5 and verified using a spectrophotometer at OD650. Samples were stored at 4°C prior to enzyme analysis.

5.3.5 Crude Enzyme Assays

5.3.5.1 Lignin Peroxidase Activity

Lignin peroxidase activity was determined in a reaction mixture containing 0.5 mL of 8 mM veratryl alcohol, 1 mL of 0.3 M citrate/phosphate buffer at pH 4.5, 100 µl of crude enzyme extract and 3.15 mL of double distilled water. These contents were shaken and the reaction was activated by the addition of 250 µl of 5 mM of hydrogen peroxide at room temperature of 26ºC. Absorbance was immediately observed at room temperature and read after 1 min at 310 nm (Kirk *et al*., 1986).

5.3.5.2 Manganese Peroxidase Activity

Manganese peroxidase activity was determined in a reaction mixture containing 1 mL of 0.1 M manganese sulphate, 1 mL of 50 mM sodium tartrate at pH 5, 0.5 mL of crude enzyme

extract and 1.5 mL of double distilled water. These contents were shaken and the reaction was activated by the addition of 1 mL of 50 mM of hydrogen peroxide at room temperature of 26°C. Absorbance was immediately observed at room temperature and read after 1 min at 350 nm (Leonowicz *et al*., 1999).

5.3.5.3 Laccase Activity

Laccase activity was determined in a reaction mixture containing 3 mL of 100 mM guaiacol dissolved in double distilled water, 1 mL of 100 mM of sodium acetate buffer at pH 5 and 1 mL of crude enzyme extract. These contents were shaken and incubated at 30°C for 2 minutes. Absorbance was immediately observed and read at room temperature at 470 nm (Collins & Dobson, 1997).

Enzyme activity for all samples was calculated as follows: $\boldsymbol{E.A = A * V/[t * e * v]}$

E.A = Enzyme activity

A = Absorbance

T = Total volume of mixture [ml]

t = incubation time

e = extinction coefficient

v = volume of enzyme

5.3.6 Statistical Analysis

Data collected for enzyme assays in both monocultures and dual cultures of fungi were analysed using the Statistix^{TM} 10.0 Analytical Soft Ware. Multiple comparisons amongst the different substrates and mono- and dual cultures enzyme production efficiency were executed using the Tukey's HSD tests with its statistical significance establish at $\alpha = 0.05$ and the Analysis of Variance (ANOVA) were analysed using a two-way test model.

5.4 Results

5.4.1 Compositional Analysis of Agricultural Waste Residues

Preliminary ('as is') analysis of agricultural residues showed the lowest values for moisture, ash and crude protein contents were observed in sugarcane bagasse. The lignin and cellulose contents were highest in wheat straw. Although the hemicellulose content is highest in corn cob, starch content was lowest in wheat straw. Varied element composition for individual

elements, notably the highest quantities of copper and manganese quantities were observed sugarcane bagasse (4.68mg/Kg and 31.9mg/Kg respectively).

Table 5.1: Moisture and ash content of lignocellulosic biomass

***AS IS* Basis**	**Moisture Content g/100g**	**Ash Content g/100g**	**Crude Protein g/100g**
Corn cob	6.81	2.10	5.71
Sugarcane bagasse	3.4	1.80	1.91
Wheat Straw	7.79	5.59	3.25

Table 5.2: Lignocellulosic biomass composition analysis

100% DM basis	**Starch g/100g**	**Hemicellulose g/100g**	**Cellulose g/100g**	**Lignin g/100g**
Corn cob	1.02	33.85	44.44	7.97
Sugarcane bagasse	1.20	12.45	40.43	5.67
Wheat straw	0.82	23.23	70.31	9.76

Table 5.3: Lignocellulosic biomass element composition analysis

100% DM basis	**N g/100g**	**Ca g/100g**	**P g/100g**	**Mg g/100g**	**K g/100g**	**Na g/100g**	**Cu mg/Kg**	**Fe mg/Kg**	**Mn mg/Kg**	**Zn mg/Kg**
Corn cob	0.98	0.025	0.161	0.083	0.34	0.22	4.28	39.6	12.9	39.6
Sugarcane bagasse	0.31	0.042	0.042	0.095	0.25	0.031	4.68	73.8	31.9	23.4
Wheat straw	0.56	0.027	0.052	0.106	1.16	0.17	3.79	74.2	13.3	10.8

5.4.2 Culture Combination and Dual Culture Interactions

The dual culture interaction types (Deadlock and Invasion/Replacement) have been discussed previously in Chapter 4. The previous chapter also provided data of the different combinations (Table 4.3) used in the enzyme assays with the intent of determining the efficiency of enzyme production by comparing mono- and dual cultures. Enzyme quantities produced using the three substrates investigated in this study and PDA are outlined in tables in Appendix D.

5.4.3 Data Analysis and Interpretation of Fungi Monocultures

The ANOVA test used in this study implemented a factorial design that considered two factors notably, the fungal species and the substrate with the enzyme activity being the outcome of the

treatments. The results were reported using specific enzyme activities mean variance as the categories. Results showed a consistency in the P value (0.0000) indicating that overall significant differences existed amongst the actual quantities of enzyme. Furthermore the Pearson's analysis of correlation showed a 20.84% correlation between LiP and MnP and 83.28% correlation between MnP and Lacc (within the Cronbach's $\alpha = 0.3678$) (Appendix E).

Table 5.4: Statistical analysis of mean values for enzyme activities in monocultures using ANOVA

ANOVA	**Lignin Peroxidase (LiP)**	**Manganese Peroxidase (MnP)**	**Laccase (Lacc)**
Grand Mean	2.1203	0.05537	0.0509
Coefficient of Variance (CV)	0.70	1.95	3.86

The Tukey HSD all-pairwise tests showed that there were no significant difference amongst the fungi with respect to the production of LiP however similarities in enzyme activities was more common to KN9, KN1, KN4, and KN3 respectively, and GL and KN2 respectively (Appendix E). The highest response in LiP activity when compared with the optimized commercial substrate PDA was observed with KN3 cultivated on WSA and the lowest enzyme activity was observed in KN9 and KN3 cultivated on WSA and SBA respectively as well as with KN2 cultivated on WSA. The exclusion of KN5 and KN12 must be noted, although they are reflected on the Tukey test, they did not grow on the agricultural residues. With regards to, MnP production there was no significant difference across the fungi, however greater similarities were observed with KN4 and KN10. Nine of the 10 fungal species (except KN12) used in the study cultivated on WSA produced greater quantities of MnP compared to all other substrates including PDA. There were also no significant differences in Lacc production across the 10 fungi species. However KN9, GL and KN10 cultivated on WSA produced the greatest quantities of Lacc compared to PDA and the other two substrates.

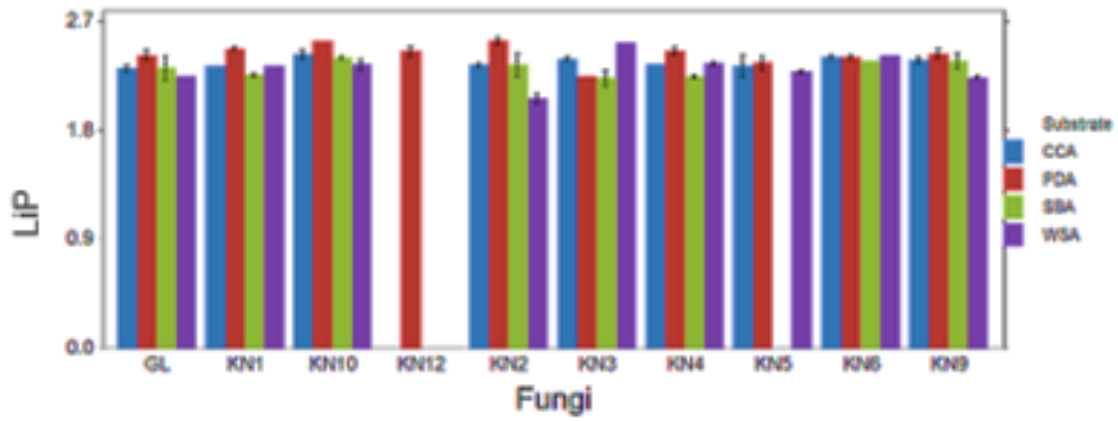

Figure 5.2 Lignin Peroxidase (LiP) Activities (U/ml) in monoculture of fungi species on the different substrates.Values were measured in triplicates (n=3) and are reported as the mean ±SD

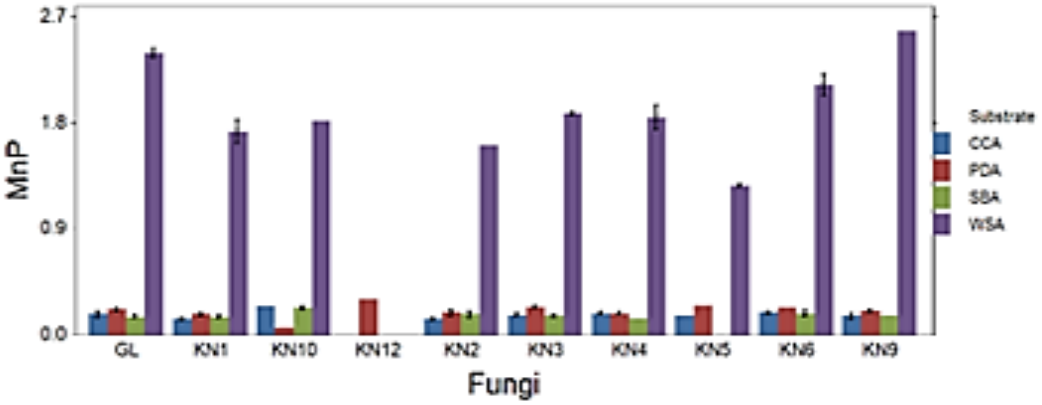

Figure 5.3 Manganese Peroxidase (MnP) Activities (U/ml) in monoculture of fungi species on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

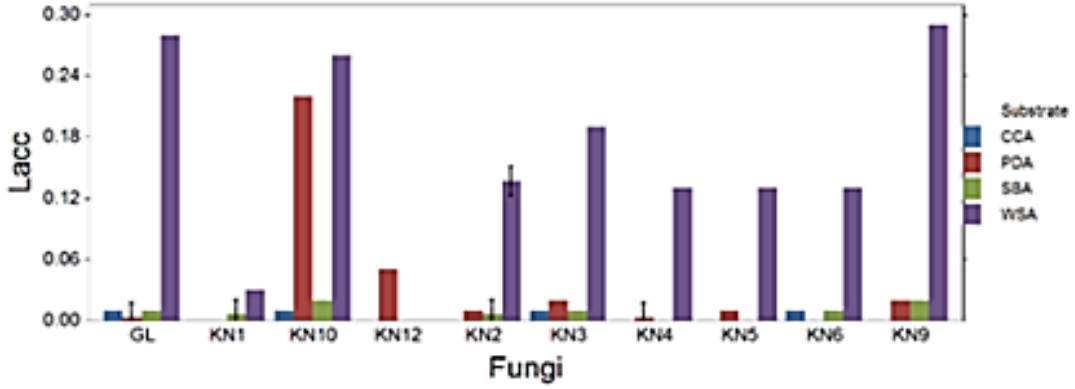

Figure 5.4 Laccase (Lacc) Activities (U/ml) in monoculture of fungi species on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

5.4.4 Data Analysis and Interpretation of Fungi Dual Cultures (Antagonistic invasion/replacement interactions)

Four of the fungal species used in the study, *Trichoderma* sp. KN10, *Rhizopus microsporus* KN2, *Fomitopsis* sp. KN1 and *Coriolopsis* sp. KN6 demonstrated tendencies of invasion and replacement in dual cultures. However, monoculture growths of *Myrmaecium* sp. KN12 did not grow on CCA, SBA and WSA; monoculture of *Umbelopsis* sp. KN5 did not grow on SBA therefore it was impossible to test the dual culture combinations involving these organisms on these media. The exclusion of statistical analysis of PDA results of enzyme activity should be noted. The primary objective of this study was to ascertain the feasibility of the three agricultural residues. Thus comparison was done to determine the most suitable agricultural substrate for increased enzyme production. The ANOVA analysis are reflected in tables below; however summary analysis and discussion that ensues focused on the Tukey tests carried out for the different invasion/replacement interactions as it provides clarity with comparisons involving multiple means and factors presented in this study. The choice of Tukey HSD tests was because it is able to prevent the occurrence of inherent Type I error rates common to ANOVA analysis in multi-factorial analysis (Wilkinson, 1999).

5.4.4.1 Invasion interactions involving *Trichoderma* sp. KN10

The P value (0.0000) indicated that there was not an overall significant difference amongst the actual quantities of enzyme (Appendix E). Analysis using the Tukey HSD tests demonstrated that there was greater production of LiP in dual cultures of KN3-KN10 cultivated on CCA when compared to KN3 monocultures which had demonstrated the highest production of LiP on WSA, most of the high performing enzyme activities with respect to LiP were also observed on CCA in dual cultures and interactions involving GL, KN6, KN4, KN3 and KN9. Remarkably the lowest LiP performance was observed with dual cultivations on WSA involving GL, KN5 and KN4.

Table 5.5: Statistical analysis of mean values for enzyme activities in dual cultures representing antagonistic invasions involving *Trichoderma* sp. KN10 using ANOVA

ANOVA	Lignin Peroxidase (LiP)	Manganese Peroxidase (MnP)	Laccase (Lacc)
Grand Mean	1.9798	1.4580	0.0976
Coefficient of Variance (CV)	0.85	1.30	8.37

With regard to MnP production several dual cultures on WSA produced greater quantities of MnP, this included interactions with KN3, KN5, KN9, KN1, KN6, GL and KN4 respectively as compared to the highest monoculture production observed in GL cultivated on WSA. Moreover the lowest production of MnP in dual cultures significantly surpasses most of the monocultures on the different substrates. The expression patterns of MnP with invasion interactions involving KN10 was at least a 2-fold increase compared to all other invasion interactions across all substrates. Overall laccase production was highest in invasion interactions involving *Trichoderma* sp. KN10 on all agricultural substrates compared to all other invasion interactions. It should be added that in general this increased laccase production in interactions was best on WSA particularly with GL, KN4, KN3 and KN5 respectively.

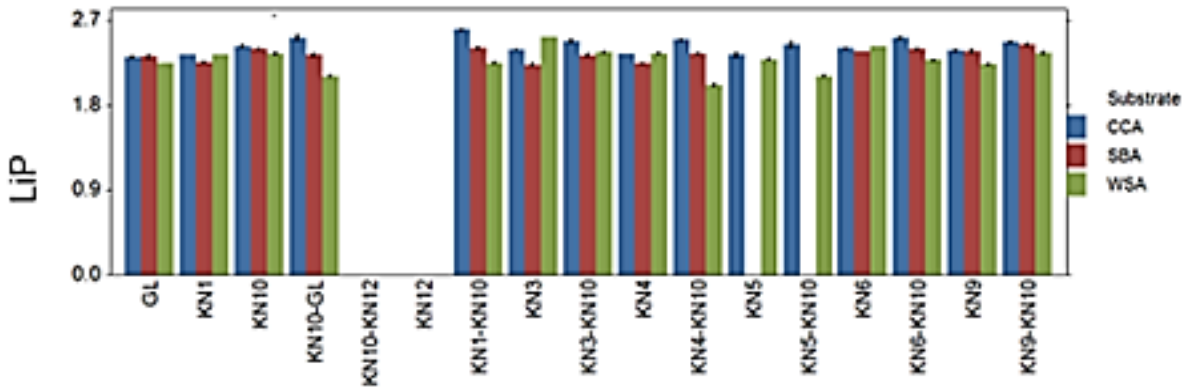

Figure 5.5a Lignin Peroxidase (LiP) Activities (U/ml) involving antagonistic/replacement by *Trichoderma* sp. KN10 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

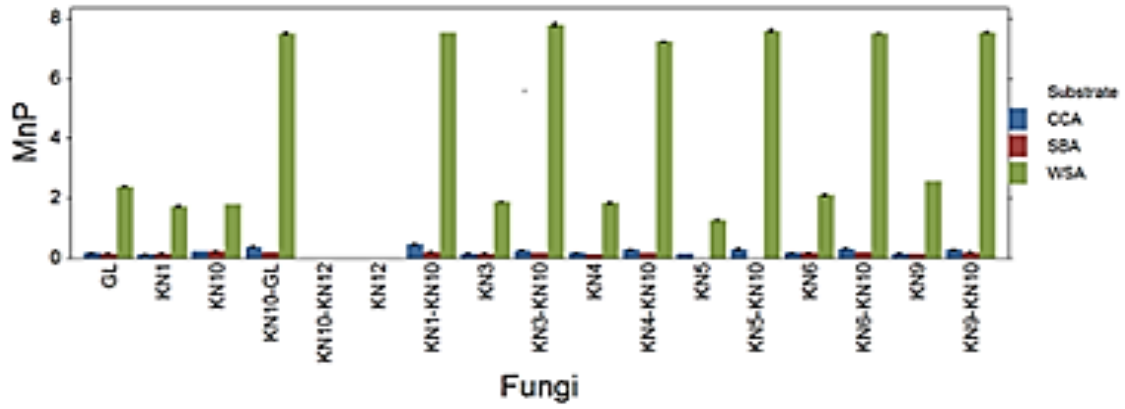

Figure 5.5b Manganese Peroxidase (MnP) Activities (U/ml) involving antagonistic/replacement by *Trichoderma* sp. KN10 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

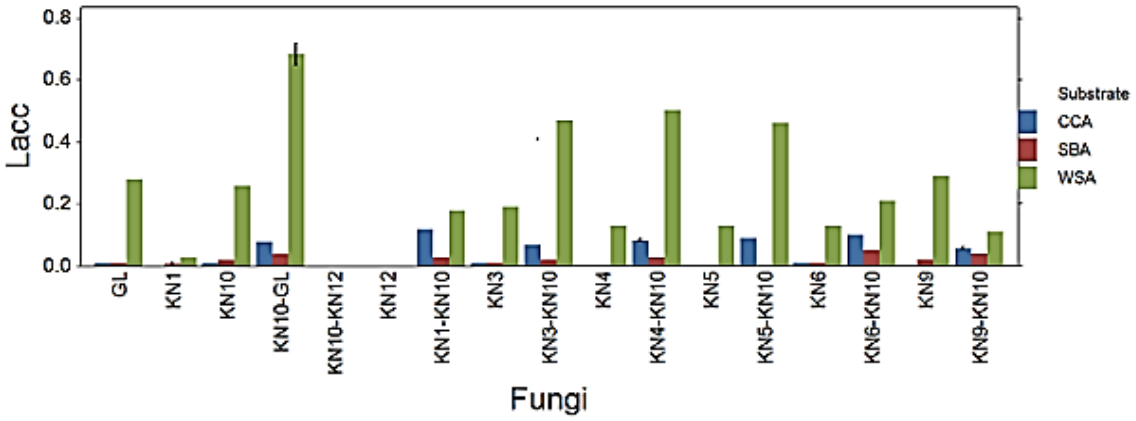

Figure 5.5c Laccase (Lacc) Activities (U/ml) involving antagonistic/replacement by *Trichoderma* sp. KN10 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

5.4.4.2 Invasion interactions involving *Rhizopus microsporus* KN2

The highest value of LiP production was observed in invasion/replacement interaction involving KN2-KN5 cultivated on CCA, although, this value did not surpass monocultures of KN3 and KN6 cultivated WSA nor KN6 cultivated on CCA. Similarly highest MnP production was observed with KN2-KN6 interactions cultivated on WSA but it also did not surpass monoculture of KN9 cultivated on WSA. However, with Lacc production there were several interactions with KN3, GL, KN6 and KN5 respectively, which were cultivated on WSA that produced an increase in enzyme activity greater than the highest monoculture production, KN9, which was observed previously on WSA. It should be noted that KN5 did not grow on SBA.

Table 5.6: Statistical analysis of mean values for enzyme activities in dual cultures representing antagonistic invasions involving *Rhizopus microsporus* KN2 using ANOVA

ANOVA	**Lignin Peroxidase (LiP)**	**Manganese Peroxidase (MnP)**	**Laccase (Lacc)**
Grand Mean	1.9380	0.6598	0.0767
Coefficient of Variance (CV)	0.79	1.90	4.86

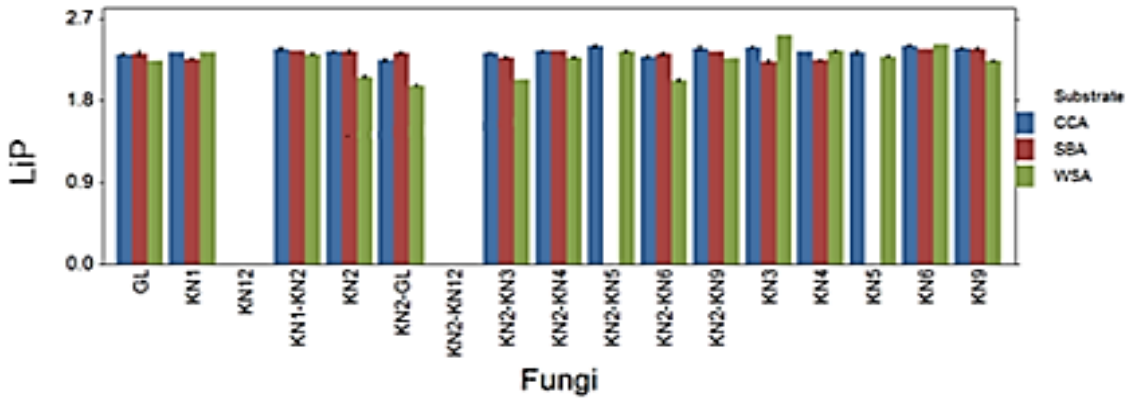

Figure 5.6a Lignin Peroxidase (LiP) Activities (U/ml) involving antagonistic/replacement by *Rhizopus microsporus* KN2 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

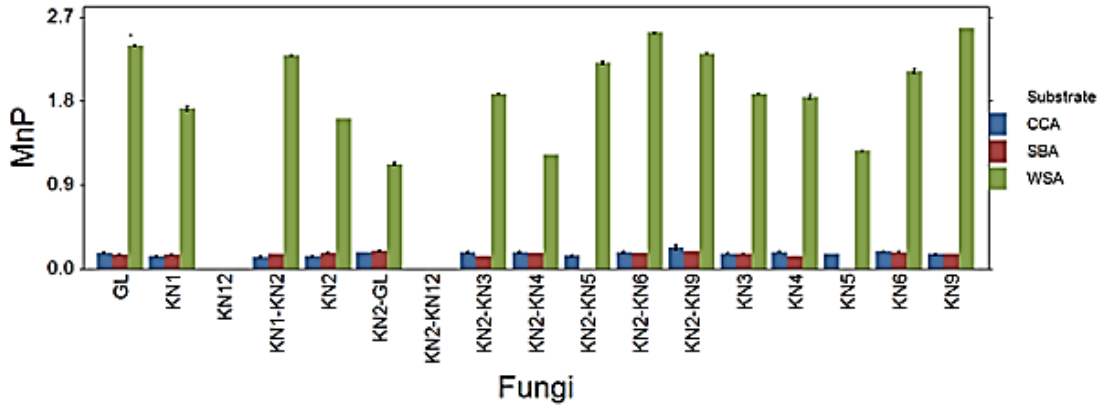

Figure 5.6b Manganese Peroxidase (MnP) Activities (U/ml) involving antagonistic/replacement by *Rhizopus microsporus* KN2 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

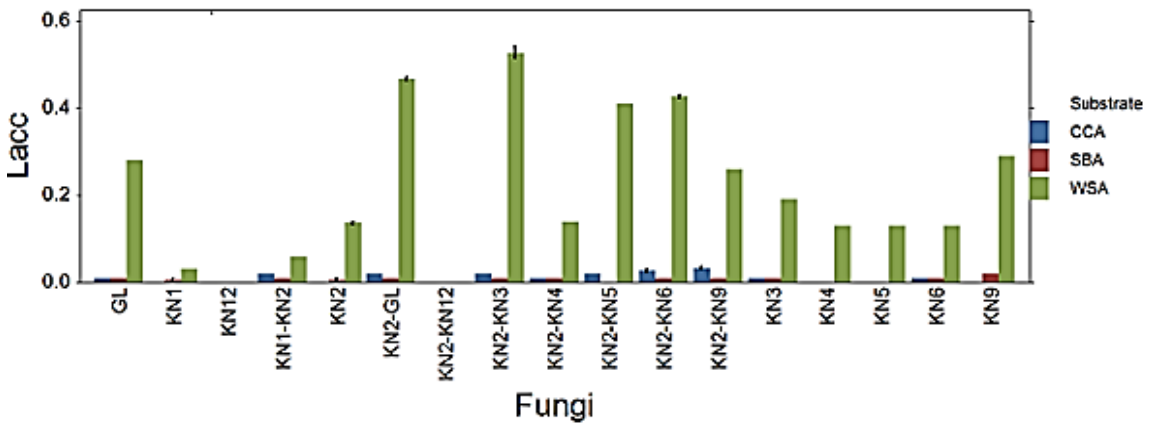

Figure 5.6c Laccase (Lacc) Activities (U/ml) involving antagonistic/replacement by *Rhizopus microsporus* KN2 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

5.4.4.3 Invasion interactions involving *Fomitopsis* sp. KN1

The highest value of LiP production was observed in invasion/replacement interaction involving KN1-GL cultivated on CCA, although, this value did not surpass monocultures of KN3 cultivated on WSA. The Tukey tests did not provide clear comparative values for MnP production, although it is obvious that none of the dual cultures surpassed monocultures of KN1 and GL in their MnP activities on all substrates (Appendix E). Nevertheless, dual cultures of KN1-KN5 and KN1-GL cultivated on WSA had greater Lacc activities when compared to the highest Lacc activity which was observed in monoculture of GL cultivated on WSA. It must be noted that there were fewer invasion interactions observed with KN1 when compared to KN10 and KN2.

Table 5.7: Statistical analysis of mean values for enzyme activities in dual cultures representing antagonistic invasions involving *Fomitopsis* sp. KN1 using ANOVA

ANOVA	Lignin Peroxidase (LiP)	Manganese Peroxidase (MnP)	Laccase (Lacc)
Grand Mean	2.0959	0.7952	0.0919
Coefficient of Variance (CV)	0.65	1.52	2.32

Note: SS are marginal (type III) sums of squares

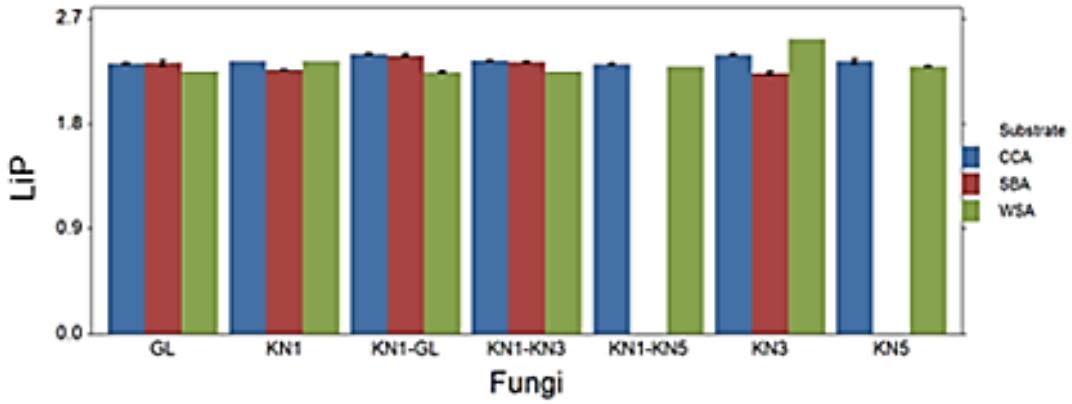

Figure 5.7a Lignin Peroxidase (LiP) Activities (U/ml) involving antagonistic/replacement by *Fomitopsis* sp. KN1 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD. Correct LiP on axis

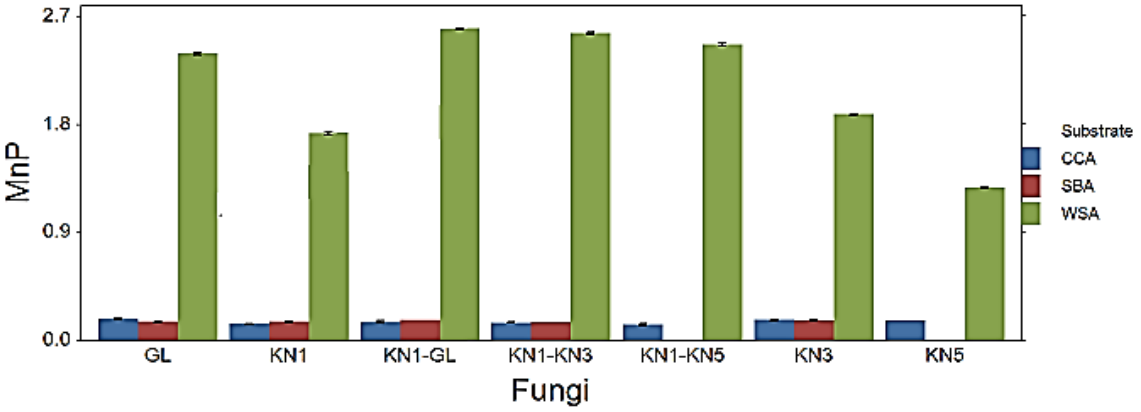

Figure 5.7b Manganese Peroxidase (MnP) Activities (U/ml) involving antagonistic/replacement by *Fomitopsis* sp. KN1 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

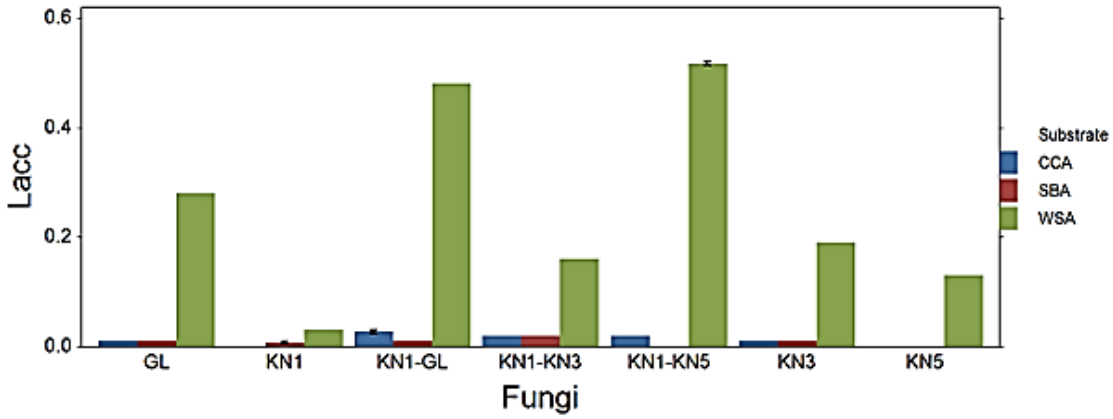

Figure 5.7c Laccase (Lacc) Activities (U/ml) involving antagonistic/replacement by *Fomitopsis* sp. KN1 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

5.4.4.4 Invasion interactions involving *Coriolopsis* sp. KN6

Invasion/replacement interaction involving *Coriolopsis* sp. KN6 was only observed when it was in dual cultures with *Umbelopsis* sp. KN5 and this was consistent on all three substrates. However it did not cause an increase in LiP enzyme activity. Comparisons were difficult to ascertain because KN5 did not grow on SBA, furthermore the monocultures enzyme activities on CCA, were very insignificant. However the invasion/replacement interaction caused an increase in MnP and Lacc quantities on WSA.

Table 5.8: Statistical analysis of mean values for enzyme activities in dual cultures representing antagonistic invasions involving *Coriolopsis* sp. KN6 using ANOVA

ANOVA	Lignin Peroxidase (LiP)	Manganese Peroxidase (MnP)	Laccase (Lacc)
Grand Mean	1.8144	0.7289	0.0489
Coefficient of Variance (CV)	1.43	1.65	***

*** Mean square too insignificant, data possibly fits the model exactly

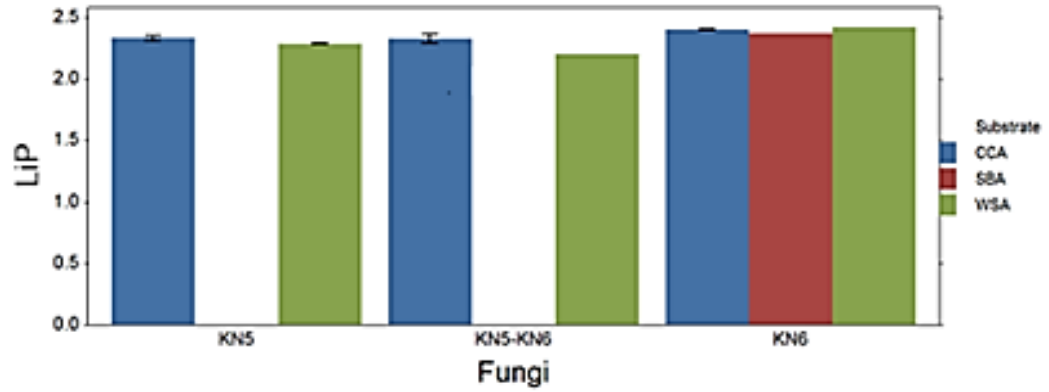

Figure 5.8a Lignin Peroxidase (LiP) Activities (U/ml) involving antagonistic/replacement by *Coriolopsis* sp. KN6 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

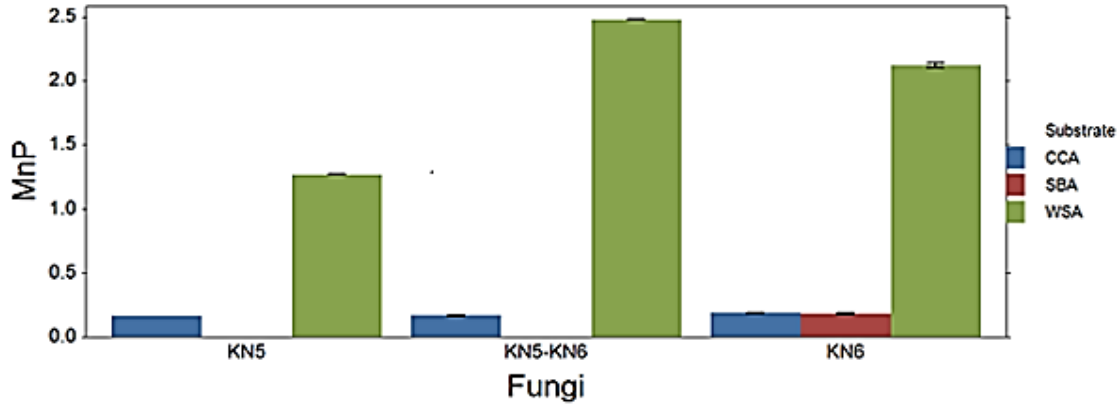

Figure 5.8b Manganese Peroxidase (MnP) Activities (U/ml) involving antagonistic/replacement by *Coriolopsis* sp. KN6 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

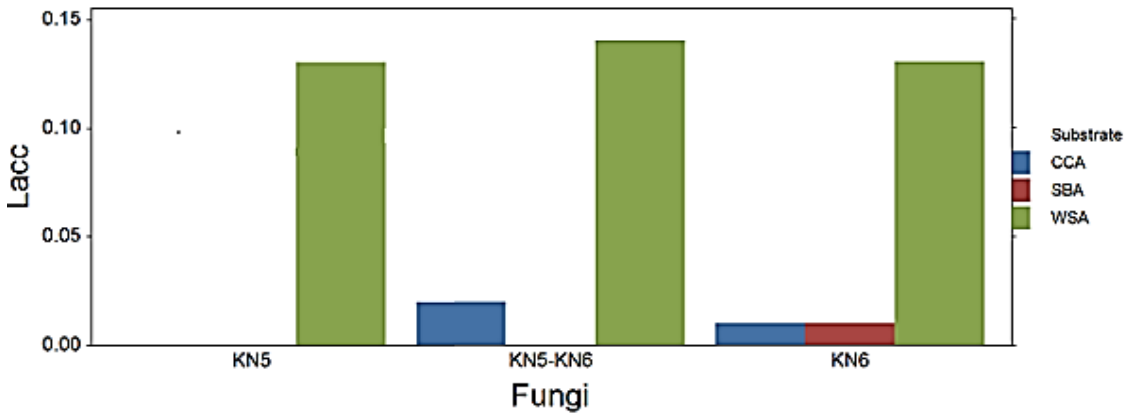

Figure 5.8c Laccase (Lacc) Activities (U/ml) involving antagonistic/replacement by *Coriolopsis* sp. KN6 on the different substrates. Values were measured in triplicates (n=3) and are reported as the mean ±SD

Discussion

The ability to degrade wood requires that fungi possess all three classes of lignin-modifying enzymes (LiP, MnP and Lacc), however it is not always the case that all three are present, usually there are variations where one or two of these enzymes may be present (Hatakka, 1994; Dhouib *et al*., 2005). These variations in enzyme production or the presence or absence of these enzymes critically determines the degradation efficiency of ligninolytic fungi. The fungi selected for this study demonstrated these variations in the expression of the three enzymes, with some producing one or at least two in monocultures. Furthermore, statistical analysis of the surface response to various substrates used in the study demonstrated that a higher correlation (83.28%) seemingly exists between the production of MnP and Lacc production. This is in agreement with study done by Bonugli-Santos *et al*. (2010) on Brazilian marine-derived fungi to determine the quantities of laccase, manganese peroxidase and lignin peroxidase. Their work like the present study demonstrated that changes in substrate concentrations affected the MnP and laccase activities. Moreover it was established by statistical analysis that a linear relationship can be deduced, thus, an increase in MnP expression corresponded to an increase in Lacc expression.

This study was intended to give an indication of the type of potential interactions demonstrated by these fungi in dual cultures. This was achieved by carrying out confrontation studies of paired interaction on agar plates. Keller and Surrette (2006) suggested that interactions within co-cultures may cause one strain to be enhanced or inhibited by the activities of the other

microorganisms; similarly the formation of primary and secondary metabolites is also affected by such interactions. This can be regarded as a unique characteristic of the co-cultivation processes (Qi-He *et al*., 2011). Morover, paired interaction between fungi are more akin to the type of interaction that are often found in the natural habitat of these organism(Moore-Landecker, 2002).

To ascertain whether the fungi could express these ligninolytic enzymes, it was necessary to carry out preliminary investigations using an optimized commercial substrate (PDA) that has been established to support the growth of most fungal species. All ten fungi selected for the study were able to grow luxuriantly on PDA and titre values of the three enzyme expressions were measured spectrophotometrically. This served as basis of comparison for the agricultural residues (corn cob, sugar cane bagasse and wheat straw) that were used by fungi in this study for the production of ligninolytic enzymes. For all substrates used in the study both monocultures and dual cultures enzyme expressions were measured. Although, it was observed that titre values obtained from PDA were not always the optimal values possible for enzyme production, some of the fungi on agricultural residues, particularly WSA, produced quantities significantly greater than was obtained from PDA. This can be attributed to the compositional differences in substrates (Tables 5.2 and 5.3). Some of the elements such as calcium, copper, manganese, iron and zinc, present within the agricultural residues are established inducers (Levin *et al*., 2002; Gomaa & Momtaz, 2015; Vrsanska *et al*., 2015) as such their presence in optimum quantities required by individual fungi can be presumed to have promoted growth and enzyme expression. In contrast, the growth of *Umbelopsis* sp. KN5 was not supported on SBA and *Myrmaecium* sp. KN12 was not able to grow on all three agricultural residues. It is likely that the quantities of the elemental inducers may have reached toxic levels for the two fungi that did not grow (Sharma & Pandey, 2010). It should be noted that individual fungi strains have different nutrient requirements therefore a nutritional requirement that may promote and even enhance enzyme expression in one fungus may not necessarily do the same for another. As a caution, it should be added that variation in concentration is an established limitation in the use of non-defined media (Basu *et al*., 2015; Koley & Mahapatra, 2015) in which all three agricultural residues can be typically classified. Nevertheless, WSA, in the present study produced the best responses in terms of enzyme production in contrast to SBA which was the poorest performing media, it is very likely that the composition of the sugarcane bagasse (Table 5.2 and 5.3) played a role in its performance as observations showed that it had significantly higher quantities of most of the known enzyme inducer elements.

Results of the enzyme quantification obtained from monocultures were used to compare dual cultures enzyme assays. Significant numbers of the dual cultures interactions, particularly with antagonistic invasions involving *Trichoderma* sp. KN10 (Figures 5.4b and 5.4c) demonstrated an increased activity for at least two of the ligninolytic enzymes analysed, suggesting that co-cultivation likely triggered the induction of these enzymes. Hu *et al*. (2011) proposed that this inductive effect is likely dependent on the carbon source. In general invasion interactions with *Trichoderma* sp. KN10 and *Ganoderma lucidum*, *Curvularia* sp. KN3 and *Schizophyllum* sp. KN4 produced profound results for all three enzymes in different substrates.

To ascertain efficiency in enzyme production in dual cultures it was necessary to exclude dual culture interactions that did not produce titre values that are greater than monoculture enzyme expression values. Therefore most values that seemingly were significantly greater than the individual monocultures were excluded because their values did not surpass the highest monoculture values for the same enzyme expression on the same media or even on a different media. Nevertheless there were values that were remarkable with invasion involving *Rhizopus* sp. KN2, *Fomitopsis* sp. KN1 and *Coriolopsis* sp. KN6 (Figures 5.5a – c and 5.6 a – c).

Conclusion

Dual cultivation of fungi and the exploitation of interspecific interaction even antagonism, shows considerable promise as a strategy in enhancing enzyme production. Although *Trichoderma* sp. has been identified in past as a biocontrol agent, much work has not been done on aspects of its use as biological inducer in terms of enzyme production. This study demonstrated that with the appropriate substrate and other favourable conditions, it is possible for some ligninolytic fungi particularly *Trichoderma* sp. to be paired with a variety of other fungi with resulting increase in enzyme production. The extremely biodiverse nature of fungi implies that echo studies can be done using a variety of indigenous agricultural residues thereby guaranteeing that efficient enzyme producing consortia of fungi can be developed specifically for different substrates. However the elucidation of the highly complex enzymatic systems and mechanisms employed, particularly during interspecific interactions is crucial to any progress in developing the degradation potential of ligninolytic fungi.

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Chapter 6

Synthesis and Conclusion

This study addressed a single hypothesis:

Competition and antagonism will promote or induce the expression of ligninolytic enzymes in interspecific interactions of fungi on different lignocellulosic substrates.

Figure 6 describes schematically the synthesis of information flow derived from the three experiments applied to achieving the research objectives (see Segment 2.10 in Chapter 2).

To characterize fungi isolates obtained from the Kloofendal Nature Reserve, a series of culture transfers were necessary to obtain monoaxenic cultures of the 10 fungi used for the study. It was established that optimal growth conditions for these fungi was 25^{o}C, furthermore luxuriant growth was achieved under humidified conditions. Although incubators used for this study did not have humidifying features, they were adapted to be able to provide adequate moisture simulating the initial habitat from which these fungi were obtained.

Preliminary screening in Chapter 3 was used to eliminate fungi that were unable to produce ligninolytic fungi. Molecular characterization involved the use of ITS1 and IT4 primers to identify the fungal isolates used in this study to degrees of accuracy ranging from 98% to 100%. Based on the analysis of ITS rDNA gene sequence, the isolates KN1, KN2, KN3, KN4, KN5, KN6, KN9, KN10 and KN12 were identified as *Fomitopsis* sp., *Rhizopus microsporus.*, *Curvularia borreriae*, *Schizophyllum* sp., *Umbelopsis* sp., *Coriolopsis* sp., *Fusarium* sp., *Trichoderma* sp., and *Myrmaecium* sp., respectively. Furthermore, phylogenetic relationship were derived for these fungal species and a lineage analysis revealed that there were species varying amongst phylum Basidiomycota, Ascomycota and early diverging fungal lineages Mucormycotina. Studies in Chapter 3 profoundly demonstrated that ligninolytic enzymes production is not limited to Basidiomycetes as members of the Mucorales; Ascomycetes also demonstrated ligninolytic activity.

The result of antagonistic interspecific interactions amongst the 10 identified fungal species was investigated in Chapter 4, these morphological changes are a consequence of biochemical reaction involving protein expressions during these interactions. All fungi isolates were

cultivated in monocultures and dual cultures of 45 varying combinations initially on Potato Dextrose Agar and the three different media preparations of the agricultural residues, Corn cob Agar, Sugar cane bagasse Agar and Wheat straw Agar. A trend of antagonistic invasion/replacement was established with 20 combinations as it was repeated across all substrates. *Trichoderma* sp. KN10, *Rhizopus microsporus* KN2, *Fomitopsis* sp. KN1 and *Coriolopsis* sp. KN6 consistently demonstrated tendencies of invasion and replacement in dual cultures and plate interactions. Macroscopic and microscopic observation of surface plate responses of stained samples showed that dual cultures interspecific interactions caused morphological changes and biochemical changes that inevitably led to necrosis in most cells.

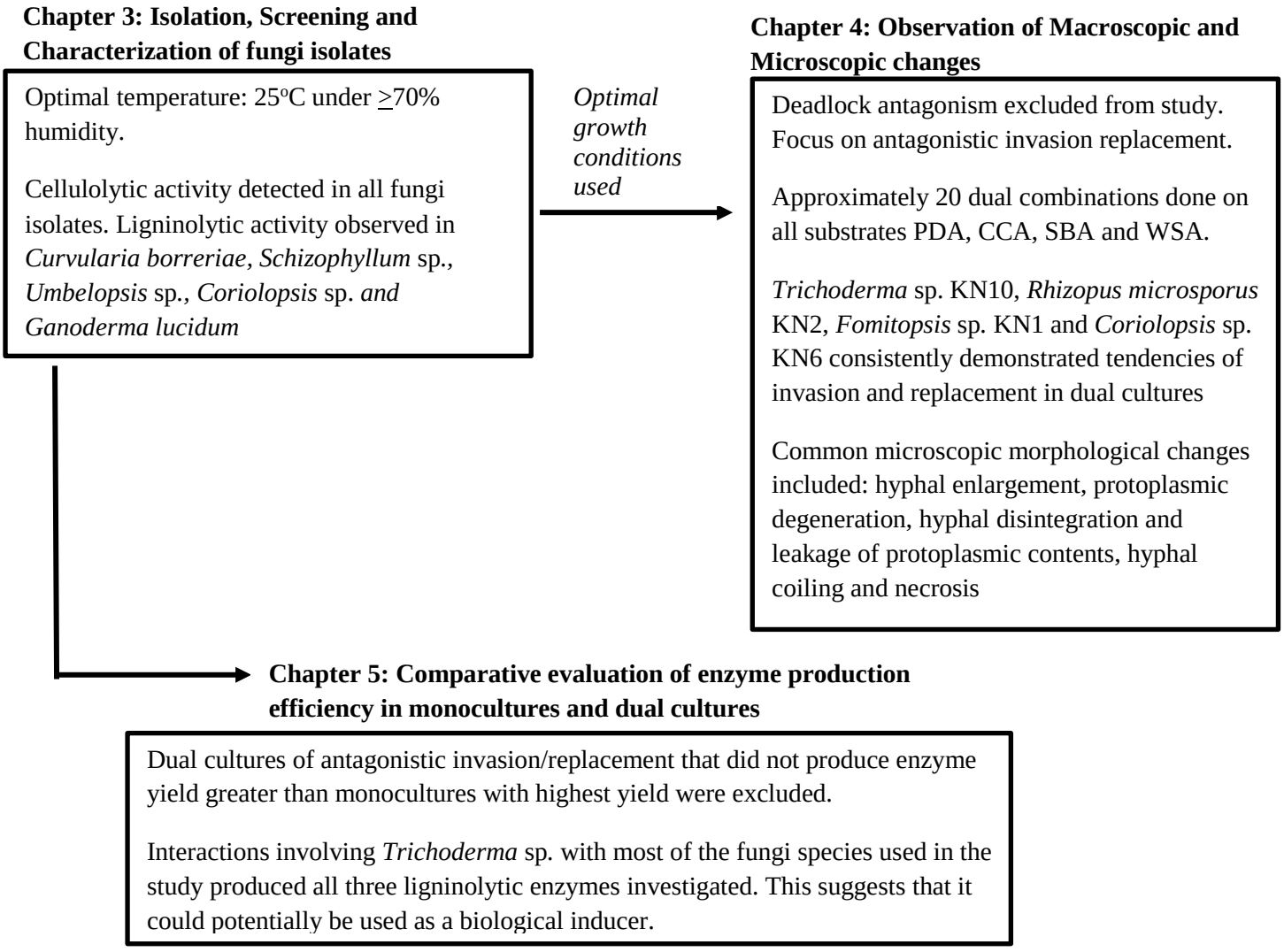

Figure 6 Conclusion and synthesis of information flow description of the main highlights of thesis

In Chapter 5, the 20 dual culture combinations that had demonstrated antagonistic invasion/replacement interactions were evaluated for their enzyme production efficiency on all substrates using spectrophotometric analysis. Although there were several

invasion/replacement interactions observed, to obtain a true reflection of increased enzyme activity in dual cultures, it was necessary to make the determination of increased enzyme production only in dual cultures that demonstrated increased enzyme yield greater than values observed with the highest possible value obtained in monoculture. Therefore on this basis several dual cultures involving invasions by *Trichoderma* sp. showed great potential such as with *Ganoderma lucidum*, *Curvularia* sp. KN3 and *Schizophyllum* sp. KN4 produced on all three enzymes in different substrates. This suggested potential use of *Trichoderma* sp. as a biological inducer for the production of ligninolytic enzymes. This study demonstrated and proved that antagonistic invasion by some fungi in co-culture, although dependent on substrate affinity, can increase production of one or more of the three enzymes laccase, lignin peroxidase and manganese peroxidase.

APPENDICES

APPENDIX A

Media

Preparation of Media

A. Potato Dextrose Agar (PDA) (g/l)

Dextrose	20
Potato	4
Agar	15

To prepare agar plates: 39 g of PDA was suspended in 1000 ml distilled water. It was heated for 5 minutes to dissolve the media completely. Media solution was sterilized in the autoclave at 121^{o}C for 15 minutes. After sterilization, media was left to cool to 55^{o}C. Approximately 20 ml of partially cooled media was dispensed into each plate and left to stand in the laminar airflow chamber to solidify before plugging with fungi mycelia.

B. Malt Extract Agar (MEA) (g/l)

Malt Extract	12.75
Dextrin	2.75
Glycerin	2.35
Gelatin Peptone	0.78
Agar	15

To prepare agar plates: 33.6 g of MEA was suspended in 1000 ml distilled water. It was heated for 1 minute to dissolve the media completely. Media solution was sterilized in the autoclave at 121^{o}C for 15 minutes. After sterilization, media was left to cool to 55^{o}C. Approximately 20 ml of partially cooled media was dispensed into each plate and left to stand in the laminar airflow chamber to solidify before plugging with fungi mycelia.

C. Carboxymethyl Cellulose Agar (CMC) (g/l)

$(NH_4)_2SO_4$	1.0
$MgSO_4.7H_2O$	1.0
$CaCl_2.2H_2O$	1.0
$FeCl_3$	0.2
K_2HPO_4 (Filtered)	1.0
Casitone	2.0
Carboxymethyl cellulose	15
Agar	15

To prepare agar plates: 40 g of CMC was suspended in 1000 ml distilled water. It was heated for approximately 5 minute to dissolve the media completely. Media solution was sterilized in the autoclave at 121^{o}C for 15 minutes. After sterilization, media was left to cool to 55^{o}C. Approximately 20 ml of partially cooled media was dispensed into each plate and left to stand in the laminar airflow chamber to solidify before plugging with fungi mycelia.

APPENDIX B

Reagents

Preparation of Reagents and Stock Solutions

A. 0.1% Congo Red Solution

Exactly 0.1g of Congo red was dissolved in distilled water and further diluted to 100 ml.

B. 1 M Sodium Chloride (NaCl) Solution

Exactly 58.5 g of NaCl was dissolved in 1000 ml distilled water.

C. 0.5 M Sodium Succinate buffer

An equal quantity ratio-wise of 0.5 M succinic acid was added to 0.5 M NaOH. The pH of the solution was tested and depending on the pH either the succinic acid or NaOH was added until a pH of 4.5 was reached.

D. 0.5 M Sodium Hydroxide (NaOH) Solution

Exactly 20 g of NaOH was dissolved in 1000 ml distilled water.

E. 0.5 M Succinic Acid Solution

Exactly 5.91 g of succinic acid powder was dissolved in 100 ml double distilled water.

F. 20 mM Guaiacol Solution

Exactly 2.8 ml of Guaiacol was added in 1000 ml double distilled water. The mixture was warmed at 55^{o}C in a warm bath for 10 minutes. Solution was stored in aluminum foil-wrapped brown bottle at 27^{o}C for a maximum of 3 months. Serial-fold dilutions was used to obtain lower concentrations.

G. 1 mM Manganese Sulphate Monohydrate ($MnSO_4.H_2O$) Solution

Exactly 1.69 g of $MnSO_4.H_2O$ was dissolved in 1000 ml distilled water.

H. 1 mM Hydrogen Peroxide (H_2O_2) Solution

Exactly 11.3 µl of 30% concentrated H_2O_2 was diluted in 100 ml double distilled water. Fresh solutions were prepared daily.

I. 5 mM Hydrogen Peroxide (H_2O_2) Solution

Exactly 56.5 µl of 30% concentrated H_2O_2 was diluted in 100 ml double distilled water. Fresh solutions were prepared daily.

J. 8 mM Veratryl Alcohol Solution

Exactly 115 µl of Veratryl alcohol was added to 100 ml double distilled water. Mixture is vortexed for 30 seconds before pipetting. Fresh solutions were prepared daily.

K. 0.3 M Citrate-Phosphate buffer

An equal quantity ratio-wise of 0.3 M citric acid was added to 0.4 M dibasic sodium phosphate. The pH of the solution was tested and depending on the pH either the citric acid or dibasic sodium phosphate was added until a pH of 4.5 was reached.

L. 0.3 M Citric Acid Solution

Exactly 5.76 g of citric acid powder was dissolved in 1000 ml double-distilled water.

M. 0.4 M Dibasic Sodium Phosphate Solution

Exactly 5.68 g of succinic acid powder was dissolved in 100 ml double distilled water.

N. 0.1 M Sodium Acetate Buffer Solution

An equal quantity ratio-wise of 0.1 M acetic acid was added to 0.1 M sodium acetate. The pH of the solution was tested and depending on the pH either the acetic acid or sodium acetate was added until a pH of 5.0 was reached.

O. 0.1 M Acetic Acid Solution

Exactly 5.71ml of concentrated glacial acetic acid powder was diluted in 1000 ml double-distilled water.

P. 0.1 M Sodium Acetate Solution

Exactly 0.82 g of sodium acetate powder was dissolved in 1000 ml double distilled water.

Q. 0.5 M Potassium-Sodium Tartrate Buffer Solution

Dropwise concentrated hydrochloric acid (HCl) is added to a quantity of 0.5 M of potassium-sodium tartrate. The pH of the solution was tested and depending on the pH either the HCl or potassium-sodium tartrate was added until a pH of 5.0 was reached.

R. 0.5 M Potassium-Sodium Tartrate Solution

Exactly 14.11 g of potassium-sodium tartrate powder was dissolved in 1000 ml double distilled water.

APPENDIX C

Fasta Files

Fungal Basiodocarps collected from the Kloofendal Nature Reserve 26.1275S 27.8806E, on 28 January 2015, Fasta Files submissions to NCBI and issued accession numbers

Strain Code	**Total Base Pairs**	**Fasta File**	**Isolate name**	**Similarity**	**Accession Numbers**
KN1	606	GTTGTTGCTGGCCGTCAGCGGCATGTGCACGCCCTGATCACTATCCATCTCACACACCTG TGCACACACTGTAGGTCGGTTTGTGGCTGGAGTGGGCACTCTGTGTCTGCTTTGGTTGTA GGCCTTCCTATGTTTTATTACAAACTACTTCAGTTTAAAGAATGTCACTCTTGCGTCTAA CGCATTTAAATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCA GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC ACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGGAATTCTCAAC TCTATTTGCTTTTGTGAATAGAGCTTGGACTTGGAGGTTTATTGCCGGTACATCTGTGAT CGGCTCCTCTTGAATGCATTAGCTCGAACCTTTGTGGATCAGCTATCGGTGTGATAATTG TCTACGCCGTTGCTGTGAAGCATGTTAATGGGATCGGCTTCTAATCGTCCTTTTACTCGA GGACAATGACTTTGACCTTTGACCTCAAATCAGGTAGGATTACCCGCTGAACTTAAGCAT ATCATA	*Fomitopsis meliae* voucher SRM-209	98.84	KU253767
KN2	636	TTTACTGGGATTTACTTCTCAGTATTGTTTGCTTCTATACTGTGAACCTCTGGCGATGAA GGTCGTAACTGACCTTCGGGAGAGACTCAGGACATATAGGCTATAATGGGTAGGCCTGTT CTGGGGTTTGATCGATGCCAATCAGGATTACCTTTCTTCCTTTGGGAAGGAAGGCGCCTG GTACCCTTTACCATATACCATGAATTCAGAATTGAAAGTATAATATAATAACAACTTTTA ACAATGGATCTCTTGGTTCTCGCATCGATGAAGAACGTAGCAAAGTGCGATAACTAGTGT GAATTGCATATTCGTGAATCATCGAGTCTTTGAACGCAGCTTGCACTCTATGGATCTTCT ATAGAGTACGCTTGCTTCAGTATCATAACCAACCCACACATAAAATTTATTTTATGTGGT GATGGACAAATTCGGTTAGATTTAATTATTATACCGATTGTCTAAAATACAGCCTCTTTG TAATTTTCATTAAATTACGAACTACCTAGCCATCGTGCTTTTTTGGTCCAACCAAAAAAC ATTTAATCTAGGGGTTCTGCCAGCCAGCAGATATTTTAATGCTCTTTAACTATGATCTGA AGTCAAGTGGGACTACCCGCTGAACTTAAGCATATC	*Rhizopus microsporus* strain SHLSYD	100	KU253769
KN3	549	GCTGTACGCGGCTGGGCTCTCGGGCGCAGTGCTGCTGAGGCTGGATTATTTATTCACCCT TGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCCCGCCGCCAGGACCACACCATA AACCTTTTTTTATGCAGTTGCAATCAGCGTCAGTACAACAAATGTAAATCATTTACAACT TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTA GTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGT ATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGG	*Curvularia borreriae* strain FMR 11523 isolate	100	KU253770

		GCGTCTTGTCTTTTGGCTCTTTGCCCAAAGACTCGCCTTAAAACGATTGGCAGCCGGCCT ACTGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAAGAGGACGGCACTCC ATCAAGACTCCTTCTCACGTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC ATATCAATA			
KN4	583	TTCATCTTGTTCTGATCCTGTGCACCTTATGTAGTCCCAAAGCCTTCACGGGCGGCGGTT GACTACGTCTACCTCACACCTTAAAAGTATGTTAACGAATGTAATCATGGTCTTGACAGA CCCTAAAAAGTTAATACAACTTTCGACAACGGATCTCTTGGCTCTCGCATCGATGAAGAA CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA ACGCACCTTGCGCCCTTTGGTATTCCGAGGGGCATGCCTGTTTGAGTGTCATTAAATACC ATCAACCCTCTTTTGACTTCGGTCTCGAGAGTGGCTTGGAAGTGGAGGTCTGCTGGAGCC TAACGGAGCCAGCTCCTCTTAAATGTATTAGCGGATTTCCCTTGCGGGATCGCGTCTCCG ATGTGATAATTTCTACGTTGTTGACCATCTCGGGGCTGACCTAGTCAGTTTCAATAGGAG TCTGCTTCTAACCGTCTCTTGACTGAGACTAGCGACTTGTGCGCTAACTTTTGACTTGAC CTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCATA	*Schizophyllum commune* isolate GBJ8	99.66	KU253771
KN5	408	ATCAAGTTTGAAATGAGTTTTCTGTATTATCAAGACTGATTTCCAGAAGCAAATGTAGCC AAAGATTTGACAAAAGTCAAAACTATTAGGCCAAACTGCTGTAGTAGGAAACAGCCAAGA GCTACATTTTAGGAGTGCCGACTAGTAAAAATCGGCGCATCCCAATTCCACATCATAACC ACAAAGGTTAGGAGTGAGAGTGCTCATGATACTGAAACAGGCATACTCCTCGGAATACCA AGGAGTGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACG TATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTT GTTTTAAACAGATTATTAAAATCATTGTTTAGTTGTTCAGTAAGTTTT	*Umbelopsis isabellina* strain CBS 250.95	99.66	KU253772
KN6	573	GGTTGTAGCTGGCCTTCCGAGGCATGTGCACACCCTGCTCATCCACTCTACACCTGTGCA CTTACTGTAGGTTGGCGTGGGCTTCGGACCTCCGGGTTCGAGGCATTCTGCCGGCCTATG TACACTACAAACTCCGAAGTAACAGAATGTAAACGCGTCTAACGCATCTTAATACAACTT TCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTA TTCCGAGGAGCATGCCTGTTTGAGTGTCATGGAATTCTCAACCCATAGATCCTTGTGGTC TACGGGCTTGGATTTGGAGGCTTGCCGGCCCTTACACGGGGTCGGCTCCTCTTGAATGCA TTAGCTTGATTCCGTGCGRATCGGCTCTCAGTGTGATAATTGTCTACGCTGTGGCCGTGA AGCGTTTGGCGAGCTTCTAACCGTCCGTTAGGACAACTTCTTGACATCTGACCTCAAATC AGGTAGGACTACCCGCTGAACTTAAGCATATCA	*Coriolopsis polyzona* strain Cof143	99.83	KU253773
KN9	517	TCATCAACCCTGTGAACATACCTATAACGTTGCCTCGGCGGGAACAGACGGCCCCGTAAC ACGGGCCGCCCCCGCCAGAGGACCCCCTAACTCTGTTTCTATAATGTTTCTTCTGAGTAA ACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAA CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA ACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCC TCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGCGGGCACAACGCCGTC CCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACACCTCGCAA CTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACTTCTGAATGTTGACCTCGAATCA GGTAGGAATACCCGCTGAACTTAAGCATATCAATAAG	*Fusarium* sp.VSMU-S001	100	KU253776

KN10	557	TGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGCAGCCCC GGACCAAGGCGCCCGCCGGAGGACCAACCAAAACTCTTTTTGTATACCCCCTCGCGGGTT TTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACT TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGT ATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGTCG GCGTTGGGGATCGGCCCTCCCTTAGCGGGGGCCGTCTCCGAAATACAGTGGCGGTCTCGC CGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGAGCGCGGCGCGTCCACAGC CGTTAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACT TAAGCATATCAATAAGC	*Trichoderma harzianum* strain BHU-BOT-RYRL10	99.83	KU253777
KN12	511	GGGCGCGCGCACCTTCCAACCCCTTGAATCGTCACACCCGACCCGGTCGCTCCCYYCGCT CGCGGGGGGCGCTCCAGTCCAACTCGCGTCTCGAAACGTTGCCGTCTGAGTCAACACGAC AAATCAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC ATTGCGCCCCCTGGCAGTCCGGGGGGCACATCTGTTCGAGCGTCATTACAACCCTCAAGC TCTGCTTGGTCTTGGGCGTCCCGTCCCCGCCTCGCGCGGCGGACGCGCCTCAAACTCCTC GGCGGTCCGCACCGGCTTCGAGCGTAGCAATCGCACCTCGCTCACGGAGTCCGGCTCGGG TCCTGCCGCATGACGACACACCGTTTCATTTCGAAAGGTTGACCTCGGATCAGATGGGGA TACCCGCTGAACTTAAGCATATCAATAAGCG	*Myrmaecium rubricosum* strain VRJ	99.61	KU253779

APPENDIX D

Enzyme Assays

Monocultures Enzyme Assay Results on PDA

LIGNIN PEROXIDASE ASSAY ON PDA (Potato Dextrose Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
KN10	0.27	0.471	2.355	0.002532258	0.025322581	2.532258065	**2.53**
KN10	0.27	0.471	2.355	0.002532258	0.025322581	2.532258065	**2.53**
KN10	0.27	0.47	2.35	0.002526882	0.025268817	2.52688172	**2.53**
KN2	0.274	0.474	2.37	0.002548387	0.025483871	2.548387097	**2.55**
KN2	0.274	0.471	2.355	0.002532258	0.025322581	2.532258065	**2.53**
KN2	0.274	0.471	2.355	0.002532258	0.025322581	2.532258065	**2.53**
KN1	0.265	0.462	2.31	0.002483871	0.02483871	2.483870968	**2.48**
KN1	0.265	0.46	2.3	0.002473118	0.024731183	2.47311828	**2.47**
KN1	0.265	0.46	2.3	0.002473118	0.024731183	2.47311828	**2.47**
KN4	0.255	0.457	2.285	0.002456989	0.024569892	2.456989247	**2.46**
KN4	0.255	0.457	2.285	0.002456989	0.024569892	2.456989247	**2.46**
KN4	0.255	0.454	2.27	0.00244086	0.024408602	2.440860215	**2.44**
KN12	0.276	0.457	2.285	0.002456989	0.024569892	2.456989247	**2.46**
KN12	0.276	0.457	2.285	0.002456989	0.024569892	2.456989247	**2.46**
KN12	0.276	0.452	2.26	0.002430108	0.024301075	2.430107527	**2.43**
KN9	0.28	0.453	2.265	0.002435484	0.024354839	2.435483871	**2.44**
KN9	0.28	0.453	2.265	0.002435484	0.024354839	2.435483871	**2.44**
KN9	0.28	0.449	2.245	0.002413978	0.024139785	2.413978495	**2.41**
GL	0.279	0.453	2.265	0.002435484	0.024354839	2.435483871	**2.44**
GL	0.279	0.448	2.24	0.002408602	0.024086022	2.408602151	**2.41**

GL	0.279	0.448	2.24	0.002408602	0.024086022	2.408602151	**2.41**
KN6	0.274	0.448	2.24	0.002408602	0.024086022	2.408602151	**2.41**
KN6	0.274	0.447	2.235	0.002403226	0.024032258	2.403225806	**2.40**
KN6	0.274	0.447	2.235	0.002403226	0.024032258	2.403225806	**2.40**
KN5	0.269	0.442	2.21	0.002376344	0.023763441	2.376344086	**2.38**
KN5	0.269	0.436	2.18	0.002344086	0.02344086	2.344086022	**2.34**
KN5	0.269	0.436	2.18	0.002344086	0.02344086	2.344086022	**2.34**
KN3	0.254	0.417	2.085	0.002241935	0.022419355	2.241935484	**2.24**
KN3	0.254	0.417	2.085	0.002241935	0.022419355	2.241935484	**2.24**
KN3	0.254	0.417	2.085	0.002241935	0.022419355	2.241935484	**2.24**

MANGANESE PEROXIDASE ASSAY ON PDA (Potato Dextrose Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X3451 X0.5**	**FX10D.F**	**GX100**	**Enzyme Activity**
KN12	0.466	0.104	0.52	0.000301362	0.003013619	0.301361924	**0.30**
KN12	0.466	0.104	0.52	0.000301362	0.003013619	0.301361924	**0.30**
KN12	0.466	0.102	0.51	0.000295567	0.002955665	0.295566502	**0.30**
KN5	0.184	0.085	0.425	0.000246305	0.002463054	0.246305419	**0.25**
KN5	0.184	0.085	0.425	0.000246305	0.002463054	0.246305419	**0.25**
KN5	0.184	0.085	0.425	0.000246305	0.002463054	0.246305419	**0.25**
KN3	0.238	0.084	0.42	0.000243408	0.002434077	0.243407708	**0.24**
KN3	0.238	0.081	0.405	0.000234715	0.002347146	0.234714575	**0.23**
KN3	0.238	0.081	0.405	0.000234715	0.002347146	0.234714575	**0.23**
KN6	0.244	0.081	0.405	0.000234715	0.002347146	0.234714575	**0.23**
KN6	0.244	0.08	0.4	0.000231817	0.002318169	0.231816865	**0.23**
KN6	0.244	0.08	0.4	0.000231817	0.002318169	0.231816865	**0.23**
GL	0.132	0.075	0.375	0.000217328	0.002173283	0.217328311	**0.22**
GL	0.132	0.072	0.36	0.000208635	0.002086352	0.208635178	**0.21**
GL	0.132	0.072	0.36	0.000208635	0.002086352	0.208635178	**0.21**
KN9	0.141	0.071	0.355	0.000205737	0.002057375	0.205737467	**0.21**
KN9	0.141	0.07	0.35	0.00020284	0.002028398	0.202839757	**0.20**
KN9	0.141	0.07	0.35	0.00020284	0.002028398	0.202839757	**0.20**
KN4	0.152	0.064	0.32	0.000185453	0.001854535	0.185453492	**0.19**
KN4	0.152	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
KN4	0.152	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
KN1	0.102	0.061	0.305	0.00017676	0.001767604	0.176760359	**0.18**
KN1	0.102	0.061	0.305	0.00017676	0.001767604	0.176760359	**0.18**
KN1	0.102	0.058	0.29	0.000168067	0.001680672	0.168067227	**0.17**
KN2	0.126	0.068	0.34	0.000197044	0.001970443	0.197044335	**0.20**
KN2	0.126	0.061	0.305	0.00017676	0.001767604	0.176760359	**0.18**

KN2	0.126	0.061	0.305	0.00017676	0.001767604	0.176760359	**0.18**
KN10	0.158	0.326	0.098	5.67951E-05	0.000567951	0.056795132	**0.06**
KN10	0.158	0.326	0.098	5.67951E-05	0.000567951	0.056795132	**0.06**
KN10	0.158	0.326	0.096	5.5636E-05	0.00055636	0.055636048	**0.06**

LACCASE ASSAY ON PDA (Potato Dextrose Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**A X 5**	**E / 2 X 6740X1**	**FX10D.F**	**GX100**	**Enzyme Activity (X10)**
KN10	0.751	0.599	2.995	0.000222181	0.00222181	0.222181009	**0.22**
KN10	0.751	0.591	2.955	0.000219214	0.002192136	0.21921365	**0.22**
KN10	0.751	0.591	2.955	0.000219214	0.002192136	0.21921365	**0.22**
KN12	0.014	0.126	0.63	4.67359E-05	0.000467359	0.046735905	**0.05**
KN12	0.014	0.125	0.625	4.6365E-05	0.00046365	0.046364985	**0.05**
KN12	0.014	0.125	0.625	4.6365E-05	0.00046365	0.046364985	**0.05**
KN9	0.078	0.045	0.225	1.66914E-05	0.000166914	0.016691395	**0.02**
KN9	0.078	0.045	0.225	1.66914E-05	0.000166914	0.016691395	**0.02**
KN9	0.078	0.044	0.22	1.63205E-05	0.000163205	0.016320475	**0.02**
KN3	0.09	0.043	0.215	1.59496E-05	0.000159496	0.015949555	**0.02**
KN3	0.09	0.042	0.21	1.55786E-05	0.000155786	0.015578635	**0.02**
KN3	0.09	0.042	0.21	1.55786E-05	0.000155786	0.015578635	**0.02**
KN2	0.116	0.031	0.155	1.14985E-05	0.000114985	0.011498516	**0.01**
KN2	0.116	0.031	0.155	1.14985E-05	0.000114985	0.011498516	**0.01**
KN2	0.116	0.03	0.15	1.11276E-05	0.000111276	0.011127596	**0.01**
KN5	0.02	0.027	0.135	1.00148E-05	0.000100148	0.010014837	**0.01**
KN5	0.02	0.027	0.135	1.00148E-05	0.000100148	0.010014837	**0.01**
KN5	0.02	0.025	0.125	9.273E-06	9.273E-05	0.009272997	**0.01**
GL	-0.006	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
GL	-0.006	0.013	0.065	4.82196E-06	4.82196E-05	0.004821958	**0.00**
GL	-0.006	0.013	0.065	4.82196E-06	4.82196E-05	0.004821958	**0.00**
KN4	-0.016	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
KN4	-0.016	0.012	0.06	4.45104E-06	4.45104E-05	0.004451039	**0.00**
KN4	-0.016	0.012	0.06	4.45104E-06	4.45104E-05	0.004451039	**0.00**
KN1	-0.013	0.009	0.045	3.33828E-06	3.33828E-05	0.003338279	**0.00**
KN1	-0.013	0.009	0.045	3.33828E-06	3.33828E-05	0.003338279	**0.00**

KN1	-0.013	0.009	0.045	3.33828E-06	3.33828E-05	0.003338279	**0.00**
KN6	-0.023	0.007	0.035	2.59644E-06	2.59644E-05	0.002596439	**0.00**
KN6	-0.023	0.007	0.035	2.59644E-06	2.59644E-05	0.002596439	**0.00**
KN6	-0.023	0.007	0.035	2.59644E-06	2.59644E-05	0.002596439	**0.00**

Dual Cultures Enzyme Assays on PDA with Antagonistic Invasion Interactions Results

DUAL CULTURES LIGNIN PEROXIDASE ASSAY ON PDA (Potato Dextrose Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
When *Rhizopus microsporus* KN2 invaded							
KN2-KN9	0.879	0.459	2.295	0.002467742	0.024677419	2.467741935	**2.47**
KN2-KN9	0.879	0.456	2.28	0.002451613	0.024516129	2.451612903	**2.45**
KN2-KN9	0.879	0.456	2.28	0.002451613	0.024516129	2.451612903	**2.45**
KN2-KN4	0.385	0.407	2.035	0.002188172	0.02188172	2.188172043	**2.19**
KN2-KN4	0.385	0.407	2.035	0.002188172	0.02188172	2.188172043	**2.19**
KN2-KN4	0.385	0.407	2.035	0.002188172	0.02188172	2.188172043	**2.19**
KN2-KN5	0.401	0.406	2.03	0.002182796	0.021827957	2.182795699	**2.18**
KN2-KN5	0.401	0.405	2.025	0.002177419	0.021774194	2.177419355	**2.18**
KN2-KN5	0.401	0.405	2.025	0.002177419	0.021774194	2.177419355	**2.18**
KN2-KN3	0.384	0.403	2.015	0.002166667	0.021666667	2.166666667	**2.17**
KN2-KN3	0.384	0.401	2.005	0.002155914	0.02155914	2.155913978	**2.16**
KN2-KN3	0.384	0.401	2.005	0.002155914	0.02155914	2.155913978	**2.16**
KN2-KN12	0.413	0.39	1.95	0.002096774	0.020967742	2.096774194	**2.10**
KN2-KN12	0.413	0.392	1.96	0.002107527	0.021075269	2.107526882	**2.11**
KN2-KN12	0.413	0.392	1.96	0.002107527	0.021075269	2.107526882	**2.11**
KN2-GL	0.354	0.388	1.94	0.002086022	0.020860215	2.086021505	**2.09**
KN2-GL	0.354	0.388	1.94	0.002086022	0.020860215	2.086021505	**2.09**
KN2-GL	0.354	0.386	1.93	0.002075269	0.020752688	2.075268817	**2.08**
KN1-KN2	0.431	0.394	1.97	0.00211828	0.021182796	2.11827957	**2.12**
KN1-KN2	0.431	0.394	1.97	0.00211828	0.021182796	2.11827957	**2.12**
KN1-KN2	0.431	0.394	1.97	0.00211828	0.021182796	2.11827957	**2.12**
KN2-KN6	0.329	0.426	2.13	0.002290323	0.022903226	2.290322581	**2.29**
KN2-KN6	0.329	0.425	2.125	0.002284946	0.022849462	2.284946237	**2.28**

KN2-KN6	0.329	0.42	2.125	0.002284946	0.022849462	2.284946237	**2.28**
When *Fomitopsis* sp. KN1 invaded							
KN1-KN3	0.292	0.436	2.18	0.002344086	0.02344086	2.344086022	**2.34**
KN1-KN3	0.292	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
KN1-KN3	0.292	0.433	2.165	0.002327957	0.02327957	2.327956989	**2.33**
KN1-KN5	0.306	0.41	2.05	0.002204301	0.022043011	2.204301075	**2.20**
KN1-KN5	0.306	0.41	2.05	0.002204301	0.022043011	2.204301075	**2.20**
KN1-KN5	0.306	0.408	2.04	0.002193548	0.021935484	2.193548387	**2.19**
KN1-GL	0.319	0.404	2.02	0.002172043	0.02172043	2.172043011	**2.17**
KN1-GL	0.319	0.404	2.02	0.002172043	0.02172043	2.172043011	**2.17**
KN1-GL	0.319	0.402	2.01	0.00216129	0.021612903	2.161290323	**2.16**
When *Coriolopsis* sp. KN6 invaded							
KN5-KN6	0.407	0.389	1.945	0.002091398	0.020913978	2.091397849	**2.09**
KN5-KN6	0.407	0.386	1.93	0.002075269	0.020752688	2.075268817	**2.08**
KN5-KN6	0.407	0.386	1.93	0.002075269	0.020752688	2.075268817	**2.08**
When *Trichoderma* sp. KN10 invaded							
KN10-KN12	0.891	0.423	2.115	0.002274194	0.022741935	2.274193548	**2.27**
KN10-KN12	0.891	0.423	2.115	0.002274194	0.022741935	2.274193548	**2.27**
KN10-KN12	0.891	0.425	2.125	0.002284946	0.022849462	2.284946237	**2.28**
KN6-KN10	0.61	0.418	2.09	0.002247312	0.022473118	2.247311828	**2.25**
KN6-KN10	0.61	0.418	2.09	0.002247312	0.022473118	2.247311828	**2.25**
KN6-KN10	0.61	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN4-KN10	0.514	0.417	2.085	0.002241935	0.022419355	2.241935484	**2.24**
KN4-KN10	0.514	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN4-KN10	0.514	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN1-KN10	0.497	0.409	2.045	0.002198925	0.021989247	2.198924731	**2.20**
KN1-KN10	0.497	0.408	2.04	0.002193548	0.021935484	2.193548387	**2.19**
KN1-KN10	0.497	0.408	2.04	0.002193548	0.021935484	2.193548387	**2.19**

KN10-KN9	0.597	0.4	2	0.002150538	0.021505376	2.150537634	**2.15**
KN10-KN9	0.597	0.4	2	0.002150538	0.021505376	2.150537634	**2.15**
KN10-KN9	0.597	0.398	1.99	0.002139785	0.021397849	2.139784946	**2.14**
KN5-KN10	0.586	0.395	1.975	0.002123656	0.021236559	2.123655914	**2.12**
KN5-KN10	0.586	0.395	1.975	0.002123656	0.021236559	2.123655914	**2.12**
KN5-KN10	0.586	0.394	1.97	0.00211828	0.021182796	2.11827957	**2.12**
KN3-KN10	0.52	0.387	1.935	0.002080645	0.020806452	2.080645161	**2.08**
KN3-KN10	0.52	0.387	1.935	0.002080645	0.020806452	2.080645161	**2.08**
KN3-KN10	0.52	0.387	1.935	0.002080645	0.020806452	2.080645161	**2.08**
KN10-GL	0.608	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
KN10-GL	0.608	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
KN10-GL	0.608	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**

DUAL CULTURES MANGANESE PEROXIDASE ASSAY ON PDA (Potato Dextrose Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
When *Rhizopus microsporus*KN2 invaded							
KN2-KN9	0.304	0.092	0.46	0.000266589	0.002665894	0.266589394	**0.27**
KN2-KN9	0.304	0.092	0.46	0.000266589	0.002665894	0.266589394	**0.27**
KN2-KN9	0.304	0.091	0.455	0.000263692	0.002636917	0.263691684	**0.26**
KN2-KN12	0.291	0.087	0.435	0.000252101	0.002521008	0.25210084	**0.25**
KN2-KN12	0.291	0.087	0.435	0.000252101	0.002521008	0.25210084	**0.25**
KN2-KN12	0.291	0.087	0.435	0.000252101	0.002521008	0.25210084	**0.25**
KN2-KN5	0.139	0.074	0.37	0.000214431	0.002144306	0.2144306	**0.21**
KN2-KN5	0.139	0.074	0.37	0.000214431	0.002144306	0.2144306	**0.21**
KN2-KN5	0.139	0.074	0.37	0.000214431	0.002144306	0.2144306	**0.21**
KN2-KN4	0.19	0.073	0.365	0.000211533	0.002115329	0.211532889	**0.21**
KN2-KN4	0.19	0.073	0.365	0.000211533	0.002115329	0.211532889	**0.21**
KN2-KN4	0.19	0.07	0.35	0.00020284	0.002028398	0.202839757	**0.20**
KN2-GL	0.183	0.07	0.35	0.00020284	0.002028398	0.202839757	**0.20**
KN2-GL	0.183	0.07	0.35	0.00020284	0.002028398	0.202839757	**0.20**
KN2-GL	0.183	0.07	0.35	0.00020284	0.002028398	0.202839757	**0.20**
KN2-KN3	0.169	0.076	0.38	0.000220226	0.00220226	0.220226021	**0.22**
KN2-KN3	0.169	0.07	0.35	0.00020284	0.002028398	0.202839757	**0.20**
KN2-KN3	0.169	0.07	0.35	0.00020284	0.002028398	0.202839757	**0.20**
KN1-KN2	0.214	0.076	0.38	0.000220226	0.00220226	0.220226021	**0.22**
KN1-KN2	0.214	0.076	0.38	0.000220226	0.00220226	0.220226021	**0.20**
KN1-KN2	0.214	0.076	0.38	0.000220226	0.00220226	0.220226021	**0.20**
KN2-KN6	0.151	0.065	0.325	0.000188351	0.001883512	0.188351203	**0.19**
KN2-KN6	0.151	0.065	0.325	0.000188351	0.001883512	0.188351203	**0.19**
KN2-KN6	0.151	0.065	0.325	0.000188351	0.001883512	0.188351203	**0.19**
When *Fomitopsis* sp. KN1 invaded							

KN1-KN5	0.132	0.082	0.41	0.000237612	0.002376123	0.237612286	**0.24**
KN1-KN5	0.132	0.082	0.41	0.000237612	0.002376123	0.237612286	**0.24**
KN1-KN5	0.132	0.082	0.41	0.000237612	0.002376123	0.237612286	**0.24**
KN1-KN3	0.063	0.069	0.345	0.000199942	0.00199942	0.199942046	**0.20**
KN1-KN3	0.063	0.069	0.345	0.000199942	0.00199942	0.199942046	**0.20**
KN1-KN3	0.063	0.069	0.345	0.000199942	0.00199942	0.199942046	**0.20**
KN1-GL	0.174	0.066	0.33	0.000191249	0.001912489	0.191248913	**0.19**
KN1-GL	0.174	0.066	0.33	0.000191249	0.001912489	0.191248913	**0.19**
KN1-GL	0.174	0.066	0.33	0.000191249	0.001912489	0.191248913	**0.19**
When *Coriolopsis* sp. KN6 invaded							
KN5-KN6	0.129	0.064	0.32	0.000185453	0.001854535	0.185453492	**0.19**
KN5-KN6	0.129	0.064	0.32	0.000185453	0.001854535	0.185453492	**0.19**
KN5-KN6	0.129	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
When *Trichoderma* sp. KN10 invaded							
KN1-KN10	0.513	0.12	0.6	0.000347725	0.003477253	0.347725297	**0.35**
KN1-KN10	0.513	0.118	0.59	0.00034193	0.003419299	0.341929875	**0.34**
KN1-KN10	0.513	0.118	0.59	0.00034193	0.003419299	0.341929875	**0.34**
KN6-KN10	0.213	0.091	0.455	0.000263692	0.002636917	0.263691684	**0.26**
KN6-KN10	0.213	0.088	0.44	0.000254999	0.002549986	0.254998551	**0.25**
KN6-KN10	0.213	0.088	0.44	0.000254999	0.002549986	0.254998551	**0.25**
KN10-KN9	0.255	0.086	0.43	0.000249203	0.002492031	0.24920313	**0.25**
KN10-KN9	0.255	0.083	0.415	0.00024051	0.0024051	0.240509997	**0.24**
KN10-KN9	0.255	0.083	0.415	0.00024051	0.0024051	0.240509997	**0.24**
KN5-KN10	0.212	0.085	0.425	0.000246305	0.002463054	0.246305419	**0.25**
KN5-KN10	0.212	0.085	0.425	0.000246305	0.002463054	0.246305419	**0.25**
KN5-KN10	0.212	0.085	0.425	0.000246305	0.002463054	0.246305419	**0.25**
KN3-KN10	0.229	0.084	0.42	0.000243408	0.002434077	0.243407708	**0.24**
KN3-KN10	0.229	0.083	0.415	0.00024051	0.0024051	0.240509997	**0.24**

KN3-KN10	0.229	0.083	0.415	0.00024051	0.0024051	0.240509997	**0.24**
KN4-KN10	0.237	0.076	0.38	0.000220226	0.00220226	0.220226021	**0.22**
KN4-KN10	0.237	0.076	0.38	0.000220226	0.00220226	0.220226021	**0.22**
KN4-KN10	0.237	0.073	0.365	0.000211533	0.002115329	0.211532889	**0.21**
KN10-KN12	0.519	0.109	0.545	0.00031585	0.003158505	0.315850478	**0.32**
KN10-KN12	0.519	0.107	0.535	0.000310055	0.003100551	0.310055057	**0.31**
KN10-KN12	0.519	0.109	0.545	0.00031585	0.003158505	0.315850478	**0.32**
KN10-GL	0.423	0.109	0.545	0.00031585	0.003158505	0.315850478	**0.32**
KN10-GL	0.423	0.109	0.545	0.00031585	0.003158505	0.315850478	**0.32**
KN10-GL	0.423	0.106	0.53	0.000307157	0.003071573	0.307157346	**0.31**

DUAL CULTURES LACCASE ASSAY ON PDA (Potato Dextrose Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
When *Rhizopus microsporus* KN2 invaded							
KN2-KN9	0.018	0.014	0.07	1.03858E-05	0.000103858	0.010385757	**0.01**
KN2-KN9	0.018	0.014	0.07	1.03858E-05	0.000103858	0.010385757	**0.01**
KN2-KN9	0.018	0.014	0.07	1.03858E-05	0.000103858	0.010385757	**0.01**
KN2-GL	-0.014	0.016	0.08	1.18694E-05	0.000118694	0.011869436	**0.01**
KN2-GL	-0.014	0.016	0.08	1.18694E-05	0.000118694	0.011869436	**0.01**
KN2-GL	-0.014	0.016	0.08	1.18694E-05	0.000118694	0.011869436	**0.01**
KN2-KN5	-0.009	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN2-KN5	-0.009	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN2-KN5	-0.009	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN2-KN3	-0.006	0.014	0.07	1.03858E-05	0.000103858	0.010385757	**0.01**
KN2-KN3	-0.006	0.014	0.07	1.03858E-05	0.000103858	0.010385757	**0.01**
KN2-KN3	-0.006	0.014	0.07	1.03858E-05	0.000103858	0.010385757	**0.01**
KN2-KN4	-0.012	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN2-KN4	-0.012	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN2-KN4	-0.012	0.01	0.05	7.4184E-06	7.4184E-05	0.007418398	**0.01**
KN2-KN12	-0.001	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN2-KN12	-0.001	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN2-KN12	-0.001	0.01	0.05	7.4184E-06	7.4184E-05	0.007418398	**0.01**
KN1-KN2	-0.018	0.015	0.075	1.11276E-05	0.000111276	0.011127596	**0.01**
KN1-KN2	-0.018	0.015	0.075	1.11276E-05	0.000111276	0.011127596	**0.01**
KN1-KN2	-0.018	0.014	0.07	1.03858E-05	0.000103858	0.010385757	**0.01**
KN2-KN6	-0.004	0.031	0.155	2.2997E-05	0.00022997	0.022997033	**0.02**
KN2-KN6	-0.004	0.031	0.155	2.2997E-05	0.00022997	0.022997033	**0.02**
KN2-KN6	-0.004	0.024	0.12	1.78042E-05	0.000178042	0.017804154	**0.02**
When *Fomitopsis* sp. KN1 invaded							

KN1-GL	0.014	0.008	0.04	5.93472E-06	5.93472E-05	0.005934718	**0.01**
KN1-GL	0.014	0.008	0.04	5.93472E-06	5.93472E-05	0.005934718	**0.01**
KN1-GL	0.014	0.006	0.03	4.45104E-06	4.45104E-05	0.004451039	**0.00**
KN1-KN5	-0.02	0.022	0.11	1.63205E-05	0.000163205	0.016320475	**0.02**
KN1-KN5	-0.02	0.022	0.11	1.63205E-05	0.000163205	0.016320475	**0.02**
KN1-KN5	-0.02	0.02	0.1	1.48368E-05	0.000148368	0.014836795	**0.01**
KN1-KN3	-0.015	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN1-KN3	-0.015	0.01	0.05	7.4184E-06	7.4184E-05	0.007418398	**0.01**
KN1-KN3	-0.015	0.01	0.05	7.4184E-06	7.4184E-05	0.007418398	**0.01**
When *Coriolopsis* sp. KN6 invaded							
KN5-KN6	0.019	0.01	0.05	7.4184E-06	7.4184E-05	0.007418398	**0.01**
KN5-KN6	0.019	0.01	0.05	7.4184E-06	7.4184E-05	0.007418398	**0.01**
KN5-KN6	0.019	0.014	0.07	1.03858E-05	0.000103858	0.010385757	**0.01**
When *Trichoderma* sp.KN10invaded							
KN10-KN12	0.243	0.062	0.31	4.59941E-05	0.000459941	0.045994065	**0.05**
KN10-KN12	0.243	0.062	0.31	4.59941E-05	0.000459941	0.045994065	**0.05**
KN10-KN12	0.243	0.061	0.305	4.52522E-05	0.000452522	0.045252226	**0.05**
KN1-KN10	0.066	0.032	0.16	2.37389E-05	0.000237389	0.023738872	**0.02**
KN1-KN10	0.066	0.032	0.16	2.37389E-05	0.000237389	0.023738872	**0.02**
KN1-KN10	0.066	0.032	0.16	2.37389E-05	0.000237389	0.023738872	**0.02**
KN6-KN10	0.146	0.037	0.185	2.74481E-05	0.000274481	0.027448071	**0.03**
KN6-KN10	0.146	0.037	0.185	2.74481E-05	0.000274481	0.027448071	**0.03**
KN6-KN10	0.146	0.037	0.185	2.74481E-05	0.000274481	0.027448071	**0.03**
KN5-KN10	0.128	0.036	0.18	2.67062E-05	0.000267062	0.026706231	**0.03**
KN5-KN10	0.128	0.036	0.18	2.67062E-05	0.000267062	0.026706231	**0.03**
KN5-KN10	0.128	0.036	0.18	2.67062E-05	0.000267062	0.026706231	**0.03**
KN4-KN10	0.114	0.033	0.165	2.44807E-05	0.000244807	0.024480712	**0.02**
KN4-KN10	0.114	0.033	0.165	2.44807E-05	0.000244807	0.024480712	**0.02**

KN4-KN10	0.114	0.035	0.175	2.59644E-05	0.000259644	0.025964392	**0.03**
KN3-KN10	0.113	0.049	0.245	3.63501E-05	0.000363501	0.036350148	**0.04**
KN3-KN10	0.113	0.049	0.245	3.63501E-05	0.000363501	0.036350148	**0.04**
KN3-KN10	0.113	0.05	0.25	3.7092E-05	0.00037092	0.037091988	**0.04**
KN10-KN9	0.1	0.038	0.19	2.81899E-05	0.000281899	0.028189911	**0.03**
KN10-KN9	0.1	0.038	0.19	2.81899E-05	0.000281899	0.028189911	**0.03**
KN10-KN9	0.1	0.036	0.18	2.67062E-05	0.000267062	0.026706231	**0.03**
KN10-GL	0.221	0.053	0.265	3.93175E-05	0.000393175	0.039317507	**0.04**
KN10-GL	0.221	0.053	0.265	3.93175E-05	0.000393175	0.039317507	**0.04**
KN10-GL	0.221	0.053	0.265	3.93175E-05	0.000393175	0.039317507	**0.04**

Monocultures Enzyme Assay Results on CCA

LIGNIN PEROXIDASE ASSAY ON CCA (Corn Cob Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
KN6	0.338	0.447	2.235	0.002403226	0.024032258	2.403225806	**2.40**
KN6	0.338	0.446	2.23	0.002397849	0.023978495	2.397849462	**2.40**
KN6	0.338	0.448	2.24	0.002408602	0.024086022	2.408602151	**2.41**
KN9	0.331	0.441	2.205	0.002370968	0.023709677	2.370967742	**2.37**
KN9	0.331	0.444	2.22	0.002387097	0.023870968	2.387096774	**2.39**
KN9	0.331	0.44	2.2	0.002365591	0.023655914	2.365591398	**2.37**
KN3	0.355	0.444	2.22	0.002387097	0.023870968	2.387096774	**2.39**
KN3	0.355	0.444	2.22	0.002387097	0.023870968	2.387096774	**2.39**
KN3	0.355	0.443	2.215	0.00238172	0.023817204	2.38172043	**2.38**
KN2	0.333	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN2	0.333	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
KN2	0.333	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN4	0.269	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN4	0.269	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN4	0.269	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN1	0.279	0.433	2.165	0.002327957	0.02327957	2.327956989	**2.33**
KN1	0.279	0.433	2.165	0.002327957	0.02327957	2.327956989	**2.33**
KN1	0.279	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
KN5	0.333	0.426	2.13	0.002290323	0.022903226	2.290322581	**2.29**
KN5	0.333	0.439	2.195	0.002360215	0.023602151	2.360215054	**2.36**
KN5	0.333	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
GL	0.342	0.432	2.16	0.002322581	0.023225806	2.322580645	**2.32**
GL	0.342	0.428	2.14	0.002301075	0.023010753	2.301075269	**2.30**
GL	0.342	0.428	2.14	0.002301075	0.023010753	2.301075269	**2.30**
KN12	0	0	0	0	0	0	**0.00**

KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**

MANGANESE PEROXIDASE ASSAY ON CCA (Corn Cob Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X3451 X0.5**	**FX10D.F**	**GX100**	**Enzyme Activity**
KN10	0.158	0.084	0.42	0.000243408	0.002434077	0.243407708	**0.24**
KN10	0.158	0.084	0.42	0.000243408	0.002434077	0.243407708	**0.24**
KN10	0.158	0.083	0.415	0.00024051	0.0024051	0.240509997	**0.24**
KN6	0.114	0.066	0.33	0.000191249	0.001912489	0.191248913	**0.19**
KN6	0.114	0.061	0.305	0.00017676	0.001767604	0.176760359	**0.18**
KN6	0.114	0.065	0.325	0.000188351	0.001883512	0.188351203	**0.19**
KN4	0.053	0.064	0.32	0.000185453	0.001854535	0.185453492	**0.19**
KN4	0.053	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
KN4	0.053	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
GL	0.118	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
GL	0.118	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
GL	0.118	0.056	0.28	0.000162272	0.001622718	0.162271805	**0.16**
KN3	0.107	0.059	0.295	0.000170965	0.001709649	0.170964938	**0.17**
KN3	0.107	0.058	0.29	0.000168067	0.001680672	0.168067227	**0.17**
KN3	0.107	0.054	0.27	0.000156476	0.001564764	0.156476384	**0.16**
KN9	0.082	0.059	0.295	0.000170965	0.001709649	0.170964938	**0.17**
KN9	0.082	0.055	0.275	0.000159374	0.001593741	0.159374094	**0.16**
KN9	0.082	0.053	0.265	0.000153579	0.001535787	0.153578673	**0.15**
KN5	0.059	0.056	0.28	0.000162272	0.001622718	0.162271805	**0.16**
KN5	0.059	0.056	0.28	0.000162272	0.001622718	0.162271805	**0.16**
KN5	0.059	0.054	0.27	0.000156476	0.001564764	0.156476384	**0.16**
KN1	0.053	0.049	0.245	0.000141988	0.001419878	0.14198783	**0.14**
KN1	0.053	0.045	0.225	0.000130397	0.00130397	0.130396986	**0.13**
KN1	0.053	0.045	0.225	0.000130397	0.00130397	0.130396986	**0.13**
KN2	0.064	0.045	0.225	0.000130397	0.00130397	0.130396986	**0.13**
KN2	0.064	0.044	0.22	0.000127499	0.001274993	0.127499276	**0.13**

KN2	0.064	0.047	0.235	0.000136192	0.001361924	0.136192408	**0.14**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**

LACCASE ASSAY ON CCA (Corn Cob Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**A X 5**	**E / 2 X 6740X1**	**FX10D.F**	**GX100**	**Enzyme Activity (X10)**
KN10	0.034	0.024	0.12	8.90208E-06	8.90208E-05	0.008902077	**0.01**
KN10	0.034	0.022	0.022	1.63205E-06	1.63205E-05	0.001632047	**0.01**
KN10	0.034	0.02	0.02	1.48368E-06	1.48368E-05	0.00148368	**0.01**
KN6	0.011	0.02	0.1	7.4184E-06	7.4184E-05	0.007418398	**0.01**
KN6	0.011	0.025	0.125	9.273E-06	9.273E-05	0.009272997	**0.01**
KN6	0.011	0.02	0.1	7.4184E-06	7.4184E-05	0.007418398	**0.01**
KN4	0.015	0.008	0.04	2.96736E-06	2.96736E-05	0.002967359	**0.00**
KN4	0.015	0.007	0.035	2.59644E-06	2.59644E-05	0.002596439	**0.00**
KN4	0.015	0.007	0.035	2.59644E-06	2.59644E-05	0.002596439	**0.00**
GL	0.009	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
GL	0.009	0.02	0.1	7.4184E-06	7.4184E-05	0.007418398	**0.01**
GL	0.009	0.02	0.1	7.4184E-06	7.4184E-05	0.007418398	**0.01**
KN3	0.009	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
KN3	0.009	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
KN3	0.009	0.015	0.075	5.5638E-06	5.5638E-05	0.005563798	**0.01**
KN9	0.009	0.01	0.05	3.7092E-06	3.7092E-05	0.003709199	**0.00**
KN9	0.009	0.011	0.055	4.08012E-06	4.08012E-05	0.004080119	**0.00**
KN9	0.009	0.01	0.05	3.7092E-06	3.7092E-05	0.003709199	**0.00**
KN5	0.02	0.009	0.045	3.33828E-06	3.33828E-05	0.003338279	**0.00**
KN5	0.02	0.007	0.035	2.59644E-06	2.59644E-05	0.002596439	**0.00**
KN5	0.02	0.01	0.05	3.7092E-06	3.7092E-05	0.003709199	**0.00**
KN1	0.02	0.008	0.04	2.96736E-06	2.96736E-05	0.002967359	**0.00**
KN1	0.02	0.008	0.04	2.96736E-06	2.96736E-05	0.002967359	**0.00**
KN1	0.02	0.006	0.03	2.22552E-06	2.22552E-05	0.002225519	**0.00**
KN2	0.01	0.007	0.035	2.59644E-06	2.59644E-05	0.002596439	**0.00**
KN2	0.01	0.006	0.03	2.22552E-06	2.22552E-05	0.002225519	**0.00**

KN2	0.01	0.01	0.05	3.7092E-06	3.7092E-05	0.003709199	**0.00**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**

Dual Cultures Enzyme Assays on CCA with Antagonistic Invasion Interactions Results

DUAL CULTURES LIGNIN PEROXIDASE ASSAY ON CCA (Corn Cob Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
When *Trichoderma* sp. KN10 invaded							
KN1-KN10	1.385	0.485	2.425	0.002607527	0.026075269	2.607526882	**2.61**
KN1-KN10	1.385	0.484	2.42	0.002602151	0.026021505	2.602150538	**2.60**
KN1-KN10	1.385	0.481	2.405	0.002586022	0.025860215	2.586021505	**2.59**
KN6-KN10	0.763	0.467	2.335	0.002510753	0.025107527	2.510752688	**2.51**
KN6-KN10	0.763	0.47	2.35	0.002526882	0.025268817	2.52688172	**2.53**
KN6-KN10	0.763	0.464	2.32	0.002494624	0.024946237	2.494623656	**2.49**
KN4-KN10	0.699	0.463	2.315	0.002489247	0.024892473	2.489247312	**2.49**
KN4-KN10	0.699	0.465	2.325	0.0025	0.025	2.5	**2.50**
KN4-KN10	0.699	0.462	2.31	0.002483871	0.02483871	2.483870968	**2.48**
KN10-KN9	0.557	0.459	2.295	0.002467742	0.024677419	2.467741935	**2.47**
KN10-KN9	0.557	0.456	2.28	0.002451613	0.024516129	2.451612903	**2.45**
KN10-KN9	0.557	0.462	2.31	0.002483871	0.02483871	2.483870968	**2.48**
KN3-KN10	0.592	0.458	2.29	0.002462366	0.024623656	2.462365591	**2.46**
KN3-KN10	0.592	0.455	2.275	0.002446237	0.024462366	2.446236559	**2.45**
KN3-KN10	0.592	0.466	2.33	0.002505376	0.025053763	2.505376344	**2.51**
KN5-KN10	0.649	0.448	2.24	0.002408602	0.024086022	2.408602151	**2.41**
KN5-KN10	0.649	0.446	2.23	0.002397849	0.023978495	2.397849462	**2.40**
KN5-KN10	0.649	0.465	2.325	0.0025	0.025	2.5	**2.50**
KN10-GL	0.755	0.465	2.325	0.0025	0.025	2.5	**2.50**
KN10-GL	0.755	0.462	2.31	0.002483871	0.02483871	2.483870968	**2.48**
KN10-GL	0.755	0.477	2.385	0.002564516	0.025645161	2.564516129	**2.56**
When *Coriolopsis* sp. KN6 invaded							
KN5-KN6	0.306	0.444	2.22	0.002387097	0.023870968	2.387096774	**2.39**

KN5-KN6	0.306	0.432	2.16	0.002322581	0.023225806	2.322580645	**2.32**
KN5-KN6	0.306	0.42	2.1	0.002258065	0.022580645	2.258064516	**2.26**
When *Fomitopsis* sp. KN1 invaded							
KN1-GL	0.323	0.443	2.215	0.00238172	0.023817204	2.38172043	**2.38**
KN1-GL	0.323	0.445	2.225	0.002392473	0.023924731	2.392473118	**2.39**
KN1-GL	0.323	0.446	2.23	0.002397849	0.023978495	2.397849462	**2.40**
KN1-KN3	0.292	0.436	2.18	0.002344086	0.02344086	2.344086022	**2.34**
KN1-KN3	0.292	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
KN1-KN3	0.292	0.433	2.165	0.002327957	0.02327957	2.327956989	**2.33**
KN1-KN5	0.313	0.43	2.15	0.002311828	0.02311828	2.311827957	**2.31**
KN1-KN5	0.313	0.428	2.14	0.002301075	0.023010753	2.301075269	**2.30**
KN1-KN5	0.313	0.427	2.135	0.002295699	0.022956989	2.295698925	**2.30**
When *Rhizopus microsporus* KN2 invaded							
KN2-KN9	0.346	0.447	2.235	0.002403226	0.024032258	2.403225806	**2.40**
KN2-KN9	0.346	0.446	2.23	0.002397849	0.023978495	2.397849462	**2.40**
KN2-KN9	0.346	0.433	2.165	0.002327957	0.02327957	2.327956989	**2.33**
KN2-KN5	0.378	0.443	2.215	0.00238172	0.023817204	2.38172043	**2.38**
KN2-KN5	0.378	0.444	2.22	0.002387097	0.023870968	2.387096774	**2.39**
KN2-KN5	0.378	0.45	2.25	0.002419355	0.024193548	2.419354839	**2.42**
KN2-KN4	0.349	0.438	2.19	0.002354839	0.023548387	2.35483871	**2.35**
KN2-KN4	0.349	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN2-KN4	0.349	0.437	2.185	0.002349462	0.023494624	2.349462366	**2.35**
KN2-KN3	0.35	0.433	2.165	0.002327957	0.02327957	2.327956989	**2.33**
KN2-KN3	0.35	0.431	2.155	0.002317204	0.023172043	2.317204301	**2.32**
KN2-KN3	0.35	0.431	2.155	0.002317204	0.023172043	2.317204301	**2.32**
KN2-GL	0.341	0.423	2.115	0.002274194	0.022741935	2.274193548	**2.27**
KN2-GL	0.341	0.413	2.065	0.00222043	0.022204301	2.220430108	**2.22**
KN2-GL	0.341	0.417	2.085	0.002241935	0.022419355	2.241935484	**2.24**

KN2-KN6	0.329	0.426	2.13	0.002290323	0.022903226	2.290322581	**2.29**
KN2-KN6	0.329	0.425	2.125	0.002284946	0.022849462	2.284946237	**2.28**
KN2-KN6	0.329	0.42	2.1	0.002258065	0.022580645	2.258064516	**2.26**
KN1-KN2	0.34	0.441	2.205	0.002370968	0.023709677	2.370967742	**2.37**
KN1-KN2	0.34	0.447	2.235	0.002403226	0.024032258	2.403225806	**2.40**
KN1-KN2	0.34	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
COMBINATION NOT DONE DUE TO NON-VIABILITY OF KN12 ON CCA							
KN1-KN12	0	0	0	0	0	0	**0.00**
KN2-KN12	0	0	0	0	0	0	**0.00**
KN10-KN12	0	0	0	0	0	0	**0.00**

DUAL CULTURES MANGANESE PEROXIDASE ASSAY ON CCA (Corn Cob Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
When *Trichoderma* sp. KN10 invaded							
KN1-KN10	0.883	0.163	0.815	0.000472327	0.004723269	0.472326862	**0.47**
KN1-KN10	0.883	0.156	0.78	0.000452043	0.004520429	0.452042886	**0.45**
KN1-KN10	0.883	0.17	0.85	0.000492611	0.004926108	0.492610837	**0.49**
KN6-KN10	0.514	0.109	0.545	0.00031585	0.003158505	0.315850478	**0.32**
KN6-KN10	0.514	0.108	0.54	0.000312953	0.003129528	0.312952767	**0.31**
KN6-KN10	0.514	0.11	0.55	0.000318748	0.003187482	0.318748189	**0.32**
KN5-KN10	0.375	0.105	0.525	0.00030426	0.003042596	0.304259635	**0.30**
KN5-KN10	0.375	0.102	0.51	0.000295567	0.002955665	0.295566502	**0.30**
KN5-KN10	0.375	0.108	0.54	0.000312953	0.003129528	0.312952767	**0.31**
KN4-KN10	0.326	0.101	0.505	0.000292669	0.002926688	0.292668792	**0.29**
KN4-KN10	0.326	0.107	0.535	0.000310055	0.003100551	0.310055057	**0.31**
KN4-KN10	0.326	0.095	0.475	0.000275283	0.002752825	0.275282527	**0.28**
KN10-KN9	0.295	0.1	0.5	0.000289771	0.002897711	0.289771081	**0.29**
KN10-KN9	0.295	0.092	0.46	0.000266589	0.002665894	0.266589394	**0.27**
KN10-KN9	0.295	0.097	0.485	0.000281078	0.002810779	0.281077948	**0.28**
KN3-KN10	0.316	0.095	0.475	0.000275283	0.002752825	0.275282527	**0.28**
KN3-KN10	0.316	0.094	0.47	0.000272385	0.002723848	0.272384816	**0.27**
KN3-KN10	0.316	0.087	0.435	0.000252101	0.002521008	0.25210084	**0.25**
KN10-GL	0.045	0.134	0.67	0.000388293	0.003882932	0.388293248	**0.39**
KN10-GL	0.045	0.136	0.68	0.000394089	0.003940887	0.39408867	**0.39**
KN10-GL	0.045	0.126	0.63	0.000365112	0.003651116	0.365111562	**0.37**
When *Coriolopsis* sp. KN6 invaded							
KN5-KN6	0.076	0.057	0.285	0.00016517	0.001651695	0.165169516	**0.17**
KN5-KN6	0.076	0.054	0.27	0.000156476	0.001564764	0.156476384	**0.16**
KN5-KN6	0.076	0.06	0.3	0.000173863	0.001738626	0.173862649	**0.17**

When *Fomitopsis* sp. KN1 invaded							
KN1-KN5	0.077	0.047	0.235	0.000136192	0.001361924	0.136192408	**0.14**
KN1-KN5	0.077	0.04	0.2	0.000115908	0.001159084	0.115908432	**0.12**
KN1-KN5	0.077	0.045	0.225	0.000130397	0.00130397	0.130396986	**0.13**
KN1-GL	0.072	0.054	0.27	0.000156476	0.001564764	0.156476384	**0.16**
KN1-GL	0.072	0.054	0.27	0.000156476	0.001564764	0.156476384	**0.16**
KN1-GL	0.072	0.046	0.23	0.000133295	0.001332947	0.133294697	**0.13**
KN1-KN3	0.063	0.05	0.25	0.000144886	0.001448855	0.14488554	**0.14**
KN1-KN3	0.063	0.051	0.255	0.000147783	0.001477833	0.147783251	**0.15**
KN1-KN3	0.063	0.049	0.245	0.000141988	0.001419878	0.14198783	**0.14**
When *Rhizopus microsporus* KN2 invaded							
KN2-KN9	0.119	0.096	0.48	0.00027818	0.002781802	0.278180238	**0.28**
KN2-KN9	0.119	0.071	0.355	0.000205737	0.002057375	0.205737467	**0.21**
KN2-KN9	0.119	0.069	0.345	0.000199942	0.00199942	0.199942046	**0.20**
KN2-KN4	0.107	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
KN2-KN4	0.107	0.066	0.33	0.000191249	0.001912489	0.191248913	**0.19**
KN2-KN4	0.107	0.06	0.3	0.000173863	0.001738626	0.173862649	**0.17**
KN2-GL	0.103	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
KN2-GL	0.103	0.062	0.31	0.000179658	0.001796581	0.17965807	**0.18**
KN2-GL	0.103	0.062	0.31	0.000179658	0.001796581	0.17965807	**0.18**
KN2-KN3	0.085	0.062	0.31	0.000179658	0.001796581	0.17965807	**0.18**
KN2-KN3	0.085	0.065	0.325	0.000188351	0.001883512	0.188351203	**0.19**
KN2-KN3	0.085	0.058	0.29	0.000168067	0.001680672	0.168067227	**0.17**
KN2-KN5	0.086	0.051	0.255	0.000147783	0.001477833	0.147783251	**0.15**
KN2-KN5	0.086	0.048	0.24	0.00013909	0.001390901	0.139090119	**0.14**
KN2-KN5	0.086	0.049	0.245	0.000141988	0.001419878	0.14198783	**0.14**
KN2-KN6	0.08	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
KN2-KN6	0.08	0.067	0.335	0.000194147	0.001941466	0.194146624	**0.19**

KN2-KN6	0.08	0.059	0.295	0.000170965	0.001709649	0.170964938	**0.17**
KN1-KN2	0.125	0.04	0.2	0.000115908	0.001159084	0.115908432	**0.12**
KN1-KN2	0.125	0.041	0.205	0.000118806	0.001188061	0.118806143	**0.12**
KN1-KN2	0.125	0.047	0.235	0.000136192	0.001361924	0.136192408	**0.14**
COMBINATION NOT DONE DUE TO NON-VIABILITY OF KN12 ON CCA							
KN1-KN12	0	0	0	0	0	0	**0.00**
KN2-KN12	0	0	0	0	0	0	**0.00**
KN10-KN12	0	0	0	0	0	0	**0.00**

DUAL CULTURES LACCASE ASSAY ON CCA (Corn Cob Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
***When Trichoderma* sp.KN10 invaded**							
KN1-KN10	0.538	0.167	0.835	0.000123887	0.001238872	0.12388724	**0.12**
KN1-KN10	0.538	0.164	0.82	0.000121662	0.001216617	0.121661721	**0.12**
KN1-KN10	0.538	0.16	0.8	0.000118694	0.001186944	0.118694362	**0.12**
KN6-KN10	0.422	0.135	0.675	0.000100148	0.001001484	0.100148368	**0.10**
KN6-KN10	0.422	0.133	0.665	9.86647E-05	0.000986647	0.098664688	**0.10**
KN6-KN10	0.422	0.13	0.65	9.64392E-05	0.000964392	0.096439169	**0.10**
KN5-KN10	0.037	0.123	0.615	9.12463E-05	0.000912463	0.091246291	**0.09**
KN5-KN10	0.037	0.121	0.605	8.97626E-05	0.000897626	0.089762611	**0.09**
KN5-KN10	0.037	0.121	0.605	8.97626E-05	0.000897626	0.089762611	**0.09**
KN4-KN10	0.263	0.115	0.575	8.53116E-05	0.000853116	0.085311573	**0.09**
KN4-KN10	0.263	0.105	0.525	7.78932E-05	0.000778932	0.077893175	**0.08**
KN4-KN10	0.263	0.105	0.525	7.78932E-05	0.000778932	0.077893175	**0.08**
KN3-KN10	0.221	0.095	0.475	7.04748E-05	0.000704748	0.070474777	**0.07**
KN3-KN10	0.221	0.095	0.475	7.04748E-05	0.000704748	0.070474777	**0.07**
KN3-KN10	0.221	0.095	0.475	7.04748E-05	0.000704748	0.070474777	**0.07**
KN10-KN9	0.162	0.078	0.39	5.78635E-05	0.000578635	0.057863501	**0.06**
KN10-KN9	0.162	0.076	0.38	5.63798E-05	0.000563798	0.056379822	**0.06**
KN10-KN9	0.162	0.073	0.365	5.41543E-05	0.000541543	0.054154303	**0.05**
KN10-GL	0.302	0.114	0.57	8.45697E-05	0.000845697	0.084569733	**0.08**
KN10-GL	0.302	0.114	0.57	8.45697E-05	0.000845697	0.084569733	**0.08**
KN10-GL	0.302	0.11	0.55	8.16024E-05	0.000816024	0.081602374	**0.08**
When *Coriolopsis* sp. KN6 invaded							
KN5-KN6	0.013	0.029	0.145	2.15134E-05	0.000215134	0.021513353	**0.02**
KN5-KN6	0.013	0.027	0.135	2.00297E-05	0.000200297	0.020029674	**0.02**

KN5-KN6	0.013	0.028	0.14	2.07715E-05	0.000207715	0.020771513	**0.02**
***When Fomitopsis* sp.KN1 invaded**							
KN1-GL	-0.013	0.036	0.18	2.67062E-05	0.000267062	0.026706231	**0.03**
KN1-GL	-0.013	0.035	0.175	2.59644E-05	0.000259644	0.025964392	**0.03**
KN1-GL	-0.013	0.033	0.165	2.44807E-05	0.000244807	0.024480712	**0.02**
KN1-KN3	-0.019	0.03	0.15	2.22552E-05	0.000222552	0.022255193	**0.02**
KN1-KN3	-0.019	0.024	0.12	1.78042E-05	0.000178042	0.017804154	**0.02**
KN1-KN3	-0.019	0.023	0.115	1.70623E-05	0.000170623	0.017062315	**0.02**
KN1-KN5	-0.01	0.032	0.16	2.37389E-05	0.000237389	0.023738872	**0.02**
KN1-KN5	-0.01	0.031	0.155	2.2997E-05	0.00022997	0.022997033	**0.02**
KN1-KN5	-0.01	0.029	0.145	2.15134E-05	0.000215134	0.021513353	**0.02**
When *Rhizopus microsporus* KN2 invaded							
KN2-KN9	0.014	0.05	0.25	3.7092E-05	0.00037092	0.037091988	**0.04**
KN2-KN9	0.014	0.045	0.225	3.33828E-05	0.000333828	0.033382789	**0.03**
KN2-KN9	0.014	0.04	0.2	2.96736E-05	0.000296736	0.029673591	**0.03**
KN2-GL	0.004	0.027	0.135	2.00297E-05	0.000200297	0.020029674	**0.02**
KN2-GL	0.004	0.026	0.13	1.92878E-05	0.000192878	0.019287834	**0.02**
KN2-GL	0.004	0.026	0.13	1.92878E-05	0.000192878	0.019287834	**0.02**
KN2-KN5	-0.014	0.027	0.135	2.00297E-05	0.000200297	0.020029674	**0.02**
KN2-KN5	-0.014	0.028	0.14	2.07715E-05	0.000207715	0.020771513	**0.02**
KN2-KN5	-0.014	0.025	0.125	1.8546E-05	0.00018546	0.018545994	**0.02**
KN2-KN3	-0.012	0.026	0.13	1.92878E-05	0.000192878	0.019287834	**0.02**
KN2-KN3	-0.012	0.023	0.115	1.70623E-05	0.000170623	0.017062315	**0.02**
KN2-KN3	-0.012	0.023	0.115	1.70623E-05	0.000170623	0.017062315	**0.02**
KN2-KN4	-0.019	0.015	0.075	1.11276E-05	0.000111276	0.011127596	**0.01**
KN2-KN4	-0.019	0.014	0.07	1.03858E-05	0.000103858	0.010385757	**0.01**
KN2-KN4	-0.019	0.013	0.065	9.64392E-06	9.64392E-05	0.009643917	**0.01**
KN2-KN6	-0.011	0.034	0.17	2.52226E-05	0.000252226	0.025222552	**0.03**

KN2-KN6	-0.011	0.037	0.185	2.74481E-05	0.000274481	0.027448071	**0.03**
KN2-KN6	-0.011	0.03	0.15	2.22552E-05	0.000222552	0.022255193	**0.02**
KN1-KN2	-0.017	0.027	0.135	2.00297E-05	0.000200297	0.020029674	**0.02**
KN1-KN2	-0.017	0.025	0.125	1.8546E-05	0.00018546	0.018545994	**0.02**
KN1-KN2	-0.017	0.024	0.12	1.78042E-05	0.000178042	0.017804154	**0.02**
COMBINATION NOT DONE DUE TO NON-VIABILITY OF KN12 ON CCA							
KN1-KN12	0	0	0	0	0	0	**0.00**
KN2-KN12	0	0	0	0	0	0	**0.00**
KN10-KN12	0	0	0	0	0	0	**0.00**

Monocultures Enzyme Assay Results on SBA

LIGNIN PEROXIDASE ASSAY ON SBA (Sugar Cane Bagasse Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
KN10	0.496	0.447	2.235	0.002403226	0.024032258	2.403225806	**2.40**
KN10	0.496	0.446	2.23	0.002397849	0.023978495	2.397849462	**2.40**
KN10	0.496	0.444	2.22	0.002387097	0.023870968	2.387096774	**2.39**
KN9	0.331	0.444	2.22	0.002387097	0.023870968	2.387096774	**2.39**
KN9	0.331	0.442	2.21	0.002376344	0.023763441	2.376344086	**2.38**
KN9	0.331	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN6	0.288	0.441	2.205	0.002370968	0.023709677	2.370967742	**2.37**
KN6	0.288	0.441	2.205	0.002370968	0.023709677	2.370967742	**2.37**
KN6	0.288	0.44	2.2	0.002365591	0.023655914	2.365591398	**2.37**
KN2	0.347	0.431	2.155	0.002317204	0.023172043	2.317204301	**2.32**
KN2	0.347	0.43	2.15	0.002311828	0.02311828	2.311827957	**2.31**
KN2	0.347	0.442	2.21	0.002376344	0.023763441	2.376344086	**2.38**
GL	0.285	0.426	2.13	0.002290323	0.022903226	2.290322581	**2.29**
GL	0.285	0.426	2.13	0.002290323	0.022903226	2.290322581	**2.29**
GL	0.285	0.439	2.195	0.002360215	0.023602151	2.360215054	**2.36**
KN1	0.278	0.42	2.1	0.002258065	0.022580645	2.258064516	**2.26**
KN1	0.278	0.42	2.1	0.002258065	0.022580645	2.258064516	**2.26**
KN1	0.278	0.418	2.09	0.002247312	0.022473118	2.247311828	**2.25**
KN3	0.297	0.418	2.09	0.002247312	0.022473118	2.247311828	**2.25**
KN3	0.297	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN3	0.297	0.409	2.045	0.002198925	0.021989247	2.198924731	**2.20**
KN4	0.278	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN4	0.278	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN4	0.278	0.418	2.09	0.002247312	0.022473118	2.247311828	**2.25**
KN5	0	0	0	0	0	0	**0.00**

KN5	0	0	0	0	0	0	**0.00**
KN5	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**

MANGANESE PEROXIDASE ASSAY ON SBA (Sugar Cane Bagasse Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X3451 X0.5**	**FX10D.F**	**GX100**	**Enzyme Activity**
KN10	0.226	0.078	0.39	0.000226021	0.002260214	0.226021443	**0.23**
KN10	0.226	0.078	0.39	0.000226021	0.002260214	0.226021443	**0.23**
KN10	0.226	0.075	0.375	0.000217328	0.002173283	0.217328311	**0.22**
KN6	0.12	0.065	0.325	0.000188351	0.001883512	0.188351203	**0.19**
KN6	0.12	0.064	0.32	0.000185453	0.001854535	0.185453492	**0.19**
KN6	0.12	0.06	0.3	0.000173863	0.001738626	0.173862649	**0.17**
KN2	0.088	0.061	0.305	0.00017676	0.001767604	0.176760359	**0.18**
KN2	0.088	0.061	0.305	0.00017676	0.001767604	0.176760359	**0.18**
KN2	0.088	0.054	0.27	0.000156476	0.001564764	0.156476384	**0.16**
KN3	0.076	0.057	0.285	0.00016517	0.001651695	0.165169516	**0.17**
KN3	0.076	0.056	0.28	0.000162272	0.001622718	0.162271805	**0.16**
KN3	0.076	0.056	0.28	0.000162272	0.001622718	0.162271805	**0.16**
KN9	0.1	0.056	0.28	0.000162272	0.001622718	0.162271805	**0.16**
KN9	0.1	0.056	0.28	0.000162272	0.001622718	0.162271805	**0.16**
KN9	0.1	0.056	0.28	0.000162272	0.001622718	0.162271805	**0.16**
GL	0.068	0.054	0.27	0.000156476	0.001564764	0.156476384	**0.16**
GL	0.068	0.053	0.265	0.000153579	0.001535787	0.153578673	**0.15**
GL	0.068	0.053	0.265	0.000153579	0.001535787	0.153578673	**0.15**
KN1	0.054	0.054	0.27	0.000156476	0.001564764	0.156476384	**0.16**
KN1	0.054	0.053	0.265	0.000153579	0.001535787	0.153578673	**0.15**
KN1	0.054	0.053	0.265	0.000153579	0.001535787	0.153578673	**0.15**
KN4	0.056	0.047	0.235	0.000136192	0.001361924	0.136192408	**0.14**
KN4	0.056	0.047	0.235	0.000136192	0.001361924	0.136192408	**0.14**
KN4	0.056	0.047	0.235	0.000136192	0.001361924	0.136192408	**0.14**
KN5	0	0	0	0	0	0	**0.00**
KN5	0	0	0	0	0	0	**0.00**
KN5	0	0	0	0	0	0	**0.00**

KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**

LACCASE ASSAY ON SBA (Sugar Cane Bagasse)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**A X 5**	**E / 2 X 6740X1**	**FX10D.F**	**GX100**	**Enzyme Activity (X10)**
KN10	0.16	0.063	0.315	2.3368E-05	0.00023368	0.023367953	**0.02**
KN10	0.16	0.062	0.31	2.2997E-05	0.00022997	0.022997033	**0.02**
KN10	0.16	0.062	0.31	2.2997E-05	0.00022997	0.022997033	**0.02**
KN9	0.051	0.049	0.245	1.81751E-05	0.000181751	0.018175074	**0.02**
KN9	0.051	0.049	0.245	1.81751E-05	0.000181751	0.018175074	**0.02**
KN9	0.051	0.047	0.235	1.74332E-05	0.000174332	0.017433234	**0.02**
KN6	0.011	0.03	0.15	1.11276E-05	0.000111276	0.011127596	**0.01**
KN6	0.009	0.028	0.14	1.03858E-05	0.000103858	0.010385757	**0.01**
KN6	0.011	0.028	0.14	1.03858E-05	0.000103858	0.010385757	**0.01**
GL	-0.003	0.015	0.075	5.5638E-06	5.5638E-05	0.005563798	**0.01**
GL	-0.003	0.015	0.075	5.5638E-06	5.5638E-05	0.005563798	**0.01**
GL	-0.003	0.015	0.075	5.5638E-06	5.5638E-05	0.005563798	**0.01**
KN3	-0.013	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
KN3	-0.013	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
KN3	-0.013	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
KN1	-0.018	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
KN1	-0.018	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
KN1	-0.018	0.013	0.065	4.82196E-06	4.82196E-05	0.004821958	**0.00**
KN2	-0.017	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
KN2	-0.017	0.014	0.07	5.19288E-06	5.19288E-05	0.005192878	**0.01**
KN2	-0.017	0.011	0.055	4.08012E-06	4.08012E-05	0.004080119	**0.00**
KN4	-0.018	0.009	0.045	3.33828E-06	3.33828E-05	0.003338279	**0.00**
KN4	-0.018	0.008	0.04	2.96736E-06	2.96736E-05	0.002967359	**0.00**
KN4	-0.018	0.008	0.04	2.96736E-06	2.96736E-05	0.002967359	**0.00**
KN12	0	0	0	0	0	0	0.00
KN12	0	0	0	0	0	0	0.00

KN12	0	0	0	0	0	0	0.00
KN5	0	0	0	0	0	0	0.00
KN5	0	0	0	0	0	0	0.00
KN5	0	0	0	0	0	0	0.00

Dual Cultures Enzyme Assays on SBA with Antagonistic Invasion Interactions Results

DUAL CULTURES LIGNIN PEROXIDASE ASSAY ON SBA (Sugar Cane Bagasse Agar)							
Ligninolytic Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
When *Trichoderma* sp. KN10 invaded							
KN10-KN9	0.521	0.455	2.275	0.002446237	0.024462366	2.446236559	**2.45**
KN10-KN9	0.521	0.455	2.275	0.002446237	0.024462366	2.446236559	**2.45**
KN10-KN9	0.521	0.45	2.25	0.002419355	0.024193548	2.419354839	**2.42**
KN1-KN10	0.538	0.45	2.25	0.002419355	0.024193548	2.419354839	**2.42**
KN1-KN10	0.538	0.447	2.235	0.002403226	0.024032258	2.403225806	**2.40**
KN1-KN10	0.538	0.447	2.235	0.002403226	0.024032258	2.403225806	**2.40**
KN6-KN10	0.565	0.447	2.235	0.002403226	0.024032258	2.403225806	**2.40**
KN6-KN10	0.565	0.445	2.225	0.002392473	0.023924731	2.392473118	**2.39**
KN6-KN10	0.565	0.445	2.225	0.002392473	0.023924731	2.392473118	**2.39**
KN4-KN10	0.515	0.438	2.19	0.002354839	0.023548387	2.35483871	**2.35**
KN4-KN10	0.515	0.438	2.19	0.002354839	0.023548387	2.35483871	**2.35**
KN4-KN10	0.515	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN3-KN10	0.392	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN3-KN10	0.392	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN3-KN10	0.392	0.427	2.135	0.002295699	0.022956989	2.295698925	**2.30**
KN10-GL	0.549	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN10-GL	0.549	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN10-GL	0.549	0.43	2.15	0.002311828	0.02311828	2.311827957	**2.31**
When *Fomitopsis* sp. KN1 invaded							
KN1-GL	0.327	0.445	2.225	0.002392473	0.023924731	2.392473118	**2.39**
KN1-GL	0.327	0.445	2.225	0.002392473	0.023924731	2.392473118	**2.39**
KN1-GL	0.327	0.439	2.195	0.002360215	0.023602151	2.360215054	**2.36**

KN1-KN3	0.289	0.433	2.165	0.002327957	0.02327957	2.327956989	**2.33**
KN1-KN3	0.289	0.432	2.16	0.002322581	0.023225806	2.322580645	**2.32**
KN1-KN3	0.289	0.432	2.16	0.002322581	0.023225806	2.322580645	**2.32**
When *Rhizopus microsporus* KN2 invaded							
KN2-KN4	0.359	0.438	2.19	0.002354839	0.023548387	2.35483871	**2.35**
KN2-KN4	0.359	0.437	2.185	0.002349462	0.023494624	2.349462366	**2.35**
KN2-KN4	0.359	0.437	2.185	0.002349462	0.023494624	2.349462366	**2.35**
KN2-KN9	0.369	0.436	2.18	0.002344086	0.02344086	2.344086022	**2.34**
KN2-KN9	0.369	0.436	2.18	0.002344086	0.02344086	2.344086022	**2.34**
KN2-KN9	0.369	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN2-GL	0.365	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN2-GL	0.365	0.429	2.145	0.002306452	0.023064516	2.306451613	**2.31**
KN2-GL	0.365	0.429	2.145	0.002306452	0.023064516	2.306451613	**2.31**
KN2-KN3	0.318	0.425	2.125	0.002284946	0.022849462	2.284946237	**2.28**
KN2-KN3	0.318	0.425	2.125	0.002284946	0.022849462	2.284946237	**2.28**
KN2-KN3	0.318	0.421	2.105	0.002263441	0.022634409	2.26344086	**2.26**
KN1-KN2	0.325	0.437	2.185	0.002349462	0.023494624	2.349462366	**2.35**
KN1-KN2	0.325	0.437	2.185	0.002349462	0.023494624	2.349462366	**2.35**
KN1-KN2	0.325	0.437	2.185	0.002349462	0.023494624	2.349462366	**2.35**
KN2-KN6	0.318	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
KN2-KN6	0.318	0.428	2.14	0.002301075	0.023010753	2.301075269	**2.30**
KN2-KN6	0.318	0.428	2.14	0.002301075	0.023010753	2.301075269	**2.30**
When *Coriolopsis* sp. KN6 invaded							
COMBINATION NOT DONE DUE TO NON-VIABILITY OF KN12 AND KN5 ON SBA							
KN5-KN6	0	0	0	0	0	0	**0.00**
COMBINATION NOT DONE DUE TO KN10E NON-VIABILITY OF KN12 AND KN5 ON SBA							
KN5-KN10	0	0	0	0	0	0	**0.00**
KN2-KN5	0	0	0	0	0	0	**0.00**

KN1-KN5	0	0	0	0	0	0	**0.00**
KN1-KN12	0	0	0	0	0	0	**0.00**
KN2-KN12	0	0	0	0	0	0	**0.00**
KN10-KN12	0	0	0	0	0	0	**0.00**

DUAL CULTURES MANGANESE PEROXIDASE ASSAY ON SBA (Sugar Cane Bagasse Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X3451 X0.5**	**FX10D.F**	**GX100**	**Enzyme Activity**
When *Trichoderma* sp. KN10 invaded							
KN1-KN10	0.157	0.076	0.38	0.000220226	0.00220226	0.220226021	**0.22**
KN1-KN10	0.157	0.076	0.38	0.000220226	0.00220226	0.220226021	**0.22**
KN1-KN10	0.157	0.072	0.36	0.000208635	0.002086352	0.208635178	**0.21**
KN6-KN10	0.18	0.076	0.38	0.000220226	0.00220226	0.220226021	**0.22**
KN6-KN10	0.18	0.076	0.38	0.000220226	0.00220226	0.220226021	**0.22**
KN6-KN10	0.18	0.075	0.375	0.000217328	0.002173283	0.217328311	**0.22**
KN10-KN9	0.171	0.068	0.34	0.000197044	0.001970443	0.197044335	**0.20**
KN10-KN9	0.171	0.068	0.34	0.000197044	0.001970443	0.197044335	**0.20**
KN10-KN9	0.171	0.065	0.325	0.000188351	0.001883512	0.188351203	**0.19**
KN4-KN10	0.136	0.067	0.335	0.000194147	0.001941466	0.194146624	**0.19**
KN4-KN10	0.136	0.067	0.335	0.000194147	0.001941466	0.194146624	**0.19**
KN4-KN10	0.136	0.065	0.325	0.000188351	0.001883512	0.188351203	**0.19**
KN3-KN10	0.103	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
KN3-KN10	0.103	0.063	0.315	0.000182556	0.001825558	0.182555781	**0.18**
KN3-KN10	0.103	0.062	0.31	0.000179658	0.001796581	0.17965807	**0.18**
KN10-GL	0.049	0.068	0.34	0.000197044	0.001970443	0.197044335	**0.20**
KN10-GL	0.049	0.068	0.34	0.000197044	0.001970443	0.197044335	**0.20**
KN10-GL	0.049	0.068	0.34	0.000197044	0.001970443	0.197044335	**0.20**
When *Fomitopsis* sp.KN1 invaded							
KN1-GL	0.068	0.06	0.3	0.000173863	0.001738626	0.173862649	**0.17**
KN1-GL	0.068	0.059	0.295	0.000170965	0.001709649	0.170964938	**0.17**
KN1-GL	0.068	0.059	0.295	0.000170965	0.001709649	0.170964938	**0.17**
KN1-KN3	0.049	0.052	0.26	0.000150681	0.00150681	0.150680962	**0.15**
KN1-KN3	0.049	0.051	0.255	0.000147783	0.001477833	0.147783251	**0.15**

KN1-KN3	0.049	0.051	0.255	0.000147783	0.001477833	0.147783251	**0.15**
When *Rhizopus microsporus*KN2 invaded							
KN2-KN9	0.123	0.066	0.33	0.000191249	0.001912489	0.191248913	**0.19**
KN2-KN9	0.123	0.065	0.325	0.000188351	0.001883512	0.188351203	**0.19**
KN2-KN9	0.123	0.065	0.325	0.000188351	0.001883512	0.188351203	**0.19**
KN2-GL	0.167	0.069	0.345	0.000199942	0.00199942	0.199942046	**0.20**
KN2-GL	0.167	0.064	0.32	0.000185453	0.001854535	0.185453492	**0.19**
KN2-GL	0.167	0.064	0.32	0.000185453	0.001854535	0.185453492	**0.19**
KN2-KN4	0.084	0.06	0.3	0.000173863	0.001738626	0.173862649	**0.17**
KN2-KN4	0.084	0.06	0.3	0.000173863	0.001738626	0.173862649	**0.17**
KN2-KN4	0.084	0.06	0.3	0.000173863	0.001738626	0.173862649	**0.17**
KN2-KN3	0.058	0.048	0.24	0.00013909	0.001390901	0.139090119	**0.14**
KN2-KN3	0.058	0.048	0.24	0.00013909	0.001390901	0.139090119	**0.14**
KN2-KN3	0.058	0.048	0.24	0.00013909	0.001390901	0.139090119	**0.14**
KN2-KN6	0.071	0.057	0.285	0.00016517	0.001651695	0.165169516	**0.17**
KN2-KN6	0.071	0.057	0.285	0.00016517	0.001651695	0.165169516	**0.17**
KN2-KN6	0.071	0.057	0.285	0.00016517	0.001651695	0.165169516	**0.17**
KN1-KN2	0.081	0.056	0.28	0.000162272	0.001622718	0.162271805	**0.16**
KN1-KN2	0.081	0.056	0.28	0.000162272	0.001622718	0.162271805	**0.16**
KN1-KN2	0.081	0.054	0.27	0.000156476	0.001564764	0.156476384	**0.16**
When *Coriolopsis* sp.KN6 invaded							
COMBINATION NOT DONE DUE TO NON-VIABILITY OF KN12 AND KN5 ON SBA							
KN5-KN6	0	0	0	0	0	0	**0.00**
COMBINATION NOT DONE DUE TO NON-VIABILITY OF KN12 AND KN5 ON SBA							
KN5-KN10	0	0	0	0	0	0	**0.00**
KN2-KN5	0	0	0	0	0	0	**0.00**
KN1-KN5	0	0	0	0	0	0	**0.00**
KN1-KN12	0	0	0	0	0	0	**0.00**

KN2-KN12	0	0	0	0	0	0	**0.00**
KN10-KN12	0	0	0	0	0	0	**0.00**

DUAL CULTURES LACCASE ASSAY ON SBA (Sugar Cane Bagasse Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**A X 5**	**E / 2 X 6740X1**	**FX10D.F**	**GX100**	**Enzyme Activity**
When *Trichoderma* sp. KN10 invaded							
KN6-KN10	0.184	0.066	0.33	4.89614E-05	0.000489614	0.048961424	**0.05**
KN6-KN10	0.184	0.066	0.33	4.89614E-05	0.000489614	0.048961424	**0.05**
KN6-KN10	0.184	0.064	0.32	4.74777E-05	0.000474777	0.047477745	**0.05**
KN10-KN9	0.151	0.059	0.295	4.37685E-05	0.000437685	0.043768546	**0.04**
KN10-KN9	0.151	0.059	0.295	4.37685E-05	0.000437685	0.043768546	**0.04**
KN10-KN9	0.151	0.059	0.295	4.37685E-05	0.000437685	0.043768546	**0.04**
KN1-KN10	0.104	0.037	0.185	2.74481E-05	0.000274481	0.027448071	**0.03**
KN1-KN10	0.104	0.037	0.185	2.74481E-05	0.000274481	0.027448071	**0.03**
KN1-KN10	0.104	0.035	0.175	2.59644E-05	0.000259644	0.025964392	**0.03**
KN4-KN10	0.096	0.039	0.195	2.89318E-05	0.000289318	0.028931751	**0.03**
KN4-KN10	0.096	0.037	0.185	2.74481E-05	0.000274481	0.027448071	**0.03**
KN4-KN10	0.096	0.037	0.185	2.74481E-05	0.000274481	0.027448071	**0.03**
KN3-KN10	0.049	0.024	0.12	1.78042E-05	0.000178042	0.017804154	**0.02**
KN3-KN10	0.049	0.023	0.115	1.70623E-05	0.000170623	0.017062315	**0.02**
KN3-KN10	0.049	0.023	0.115	1.70623E-05	0.000170623	0.017062315	**0.02**
KN10-GL	0.158	0.057	0.285	4.22849E-05	0.000422849	0.042284866	**0.04**
KN10-GL	0.158	0.055	0.275	4.08012E-05	0.000408012	0.040801187	**0.04**
KN10-GL	0.158	0.055	0.275	4.08012E-05	0.000408012	0.040801187	**0.04**
When *Fomitopsis* sp.KN1 invaded							
KN1-KN3	-0.017	0.03	0.15	2.22552E-05	0.000222552	0.022255193	**0.02**
KN1-KN3	-0.017	0.024	0.12	1.78042E-05	0.000178042	0.017804154	**0.02**
KN1-KN3	-0.017	0.023	0.115	1.70623E-05	0.000170623	0.017062315	**0.02**
KN1-GL	-0.019	0.014	0.07	1.03858E-05	0.000103858	0.010385757	**0.01**
KN1-GL	-0.019	0.013	0.065	9.64392E-06	9.64392E-05	0.009643917	**0.01**

KN1-GL	-0.019	0.013	0.065	9.64392E-06	9.64392E-05	0.009643917	**0.01**
When *Rhizopus microsporus* KN1 invaded							
KN2-GL	0.011	0.02	0.1	1.48368E-05	0.000148368	0.014836795	**0.01**
KN2-GL	0.011	0.02	0.1	1.48368E-05	0.000148368	0.014836795	**0.01**
KN2-GL	0.011	0.02	0.1	1.48368E-05	0.000148368	0.014836795	**0.01**
KN2-KN9	0.018	0.019	0.095	1.4095E-05	0.00014095	0.014094955	**0.01**
KN2-KN9	0.018	0.019	0.095	1.4095E-05	0.00014095	0.014094955	**0.01**
KN2-KN9	0.018	0.019	0.095	1.4095E-05	0.00014095	0.014094955	**0.01**
KN2-KN4	-0.006	0.015	0.075	1.11276E-05	0.000111276	0.011127596	**0.01**
KN2-KN4	-0.006	0.015	0.075	1.11276E-05	0.000111276	0.011127596	**0.01**
KN2-KN4	-0.006	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN2-KN3	-0.018	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN2-KN3	-0.018	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN2-KN3	-0.018	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
KN2-KN6	-0.01	0.016	0.08	1.18694E-05	0.000118694	0.011869436	**0.01**
KN2-KN6	-0.01	0.016	0.08	1.18694E-05	0.000118694	0.011869436	**0.01**
KN2-KN6	-0.01	0.016	0.08	1.18694E-05	0.000118694	0.011869436	**0.01**
KN1-KN2	-0.022	0.01	0.05	7.4184E-06	7.4184E-05	0.007418398	**0.01**
KN1-KN2	-0.022	0.01	0.05	7.4184E-06	7.4184E-05	0.007418398	**0.01**
KN1-KN2	-0.022	0.009	0.045	6.67656E-06	6.67656E-05	0.006676558	**0.01**
When *Coriolopsis polyzona*KN6 invaded							
COMBINATION NOT DONE DUE TO NON-VIABILITY OF KN12 AND KN5 ON SBA							
KN5-KN6	0	0	0	0	0	0	**0.00**
COMBINATION NOT DONE DUE TO NON-VIABILITY OF KN12 AND KN5 ON SBA							
KN5-KN10	0	0	0	0	0	0	**0.00**
KN2-KN5	0	0	0	0	0	0	**0.00**
KN1-KN5	0	0	0	0	0	0	**0.00**
KN1-KN12	0	0	0	0	0	0	**0.00**

KN2-KN12	0	0	0	0	0	0	**0.00**
KN10-KN12	0	0	0	0	0	0	**0.00**

Monocultures Enzyme Assay Results on WSA

LIGNIN PEROXIDASE ASSAY ON WSA (Wheat Straw Agar)							
Ligninolytic Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
KN3	0.325	0.468	2.34	0.002516129	0.02516129	2.516129032	**2.52**
KN3	0.325	0.468	2.34	0.002516129	0.02516129	2.516129032	**2.52**
KN3	0.325	0.468	2.34	0.002516129	0.02516129	2.516129032	**2.52**
KN6	0.378	0.451	2.255	0.002424731	0.024247312	2.424731183	**2.42**
KN6	0.378	0.451	2.255	0.002424731	0.024247312	2.424731183	**2.42**
KN6	0.378	0.451	2.255	0.002424731	0.024247312	2.424731183	**2.42**
KN4	0.269	0.438	2.19	0.002354839	0.023548387	2.35483871	**2.35**
KN4	0.269	0.438	2.19	0.002354839	0.023548387	2.35483871	**2.35**
KN4	0.269	0.435	2.175	0.00233871	0.023387097	2.338709677	**2.34**
KN10	0.846	0.439	2.195	0.002360215	0.023602151	2.360215054	**2.36**
KN10	0.846	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
KN10	0.846	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
KN1	0.382	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
KN1	0.382	0.433	2.165	0.002327957	0.02327957	2.327956989	**2.33**
KN1	0.382	0.433	2.165	0.002327957	0.02327957	2.327956989	**2.33**
KN5	0.237	0.426	2.13	0.002290323	0.022903226	2.290322581	**2.29**
KN5	0.237	0.424	2.12	0.00227957	0.022795699	2.279569892	**2.28**
KN5	0.237	0.424	2.12	0.00227957	0.022795699	2.279569892	**2.28**
GL	0.839	0.417	2.085	0.002241935	0.022419355	2.241935484	**2.24**
GL	0.839	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
GL	0.839	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN9	0.555	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN9	0.555	0.414	2.07	0.002225806	0.022258065	2.225806452	**2.23**
KN9	0.555	0.414	2.07	0.002225806	0.022258065	2.225806452	**2.23**
KN2	0.311	0.386	1.93	0.002075269	0.020752688	2.075268817	**2.08**

KN2	0.311	0.381	1.905	0.002048387	0.020483871	2.048387097	**2.05**
KN2	0.311	0.381	1.905	0.002048387	0.020483871	2.048387097	**2.05**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**

MANGANESE PEROXIDASE ASSAY ON WSA(Wheat Straw Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X3451 X0.5**	**FX10D.F**	**GX100**	**Enzyme Activity**
KN9	0.284	0.889	4.445	0.002576065	0.025760649	2.576064909	**2.58**
KN9	0.284	0.889	4.445	0.002576065	0.025760649	2.576064909	**2.58**
KN9	0.284	0.889	4.445	0.002576065	0.025760649	2.576064909	**2.58**
GL	0.11	0.829	4.145	0.002402202	0.024022023	2.40220226	**2.40**
GL	0.11	0.828	4.14	0.002399305	0.023993045	2.399304549	**2.40**
GL	0.11	0.817	4.085	0.00236743	0.023674297	2.367429731	**2.37**
KN6	0.105	0.747	3.735	0.00216459	0.0216459	2.164589974	**2.16**
KN6	0.105	0.723	3.615	0.002095045	0.020950449	2.095044915	**2.10**
KN6	0.105	0.723	3.615	0.002095045	0.020950449	2.095044915	**2.10**
KN3	0.155	0.648	3.24	0.001877717	0.018777166	1.877716604	**1.88**
KN3	0.155	0.648	3.24	0.001877717	0.018777166	1.877716604	**1.88**
KN3	0.155	0.645	3.225	0.001869023	0.018690235	1.869023471	**1.87**
KN4	0.198	0.651	3.255	0.00188641	0.018864097	1.886409736	**1.89**
KN4	0.198	0.629	3.145	0.00182266	0.018226601	1.822660099	**1.82**
KN4	0.198	0.629	3.145	0.00182266	0.018226601	1.822660099	**1.82**
KN10	0.408	0.625	3.125	0.001811069	0.018110693	1.811069255	**1.81**
KN10	0.408	0.625	3.125	0.001811069	0.018110693	1.811069255	**1.81**
KN10	0.408	0.625	3.125	0.001811069	0.018110693	1.811069255	**1.81**
KN1	0.053	0.61	3.05	0.001767604	0.017676036	1.767603593	**1.77**
KN1	0.053	0.588	2.94	0.001703854	0.01703854	1.703853955	**1.70**
KN1	0.053	0.588	2.94	0.001703854	0.01703854	1.703853955	**1.70**
KN2	0.143	0.557	2.785	0.001614025	0.016140249	1.61402492	**1.61**
KN2	0.143	0.554	2.77	0.001605332	0.016053318	1.605331788	**1.61**
KN2	0.143	0.554	2.77	0.001605332	0.016053318	1.605331788	**1.61**
KN5	0.061	0.437	2.185	0.0012663	0.012662996	1.266299623	**1.27**
KN5	0.061	0.437	2.185	0.0012663	0.012662996	1.266299623	**1.27**

KN5	0.061	0.434	2.17	0.001257606	0.012576065	1.257606491	**1.26**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**

LACCASE ASSAY ON WSA (Wheat Straw Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**A X 5**	**E / 2 X 6740X1**	**FX10D.F**	**GX100**	**Enzyme Activity (X10)**
KN9	0.034	0.792	3.96	0.000293769	0.002937685	0.293768546	**0.29**
KN9	0.034	0.785	3.925	0.000291172	0.002911721	0.291172107	**0.29**
KN9	0.034	0.785	3.925	0.000291172	0.002911721	0.291172107	**0.29**
GL	-0.013	0.748	3.74	0.000277448	0.002774481	0.277448071	**0.28**
GL	-0.013	0.746	3.73	0.000276706	0.002767062	0.276706231	**0.28**
GL	-0.013	0.746	3.73	0.000276706	0.002767062	0.276706231	**0.28**
KN10	0.225	0.704	3.52	0.000261128	0.002611276	0.261127596	**0.26**
KN10	0.225	0.689	3.445	0.000255564	0.002555638	0.255563798	**0.26**
KN10	0.225	0.689	3.445	0.000255564	0.002555638	0.255563798	**0.26**
KN3	-0.004	0.518	2.59	0.000192136	0.001921365	0.192136499	**0.19**
KN3	-0.004	0.518	2.59	0.000192136	0.001921365	0.192136499	**0.19**
KN3	-0.004	0.5	2.5	0.00018546	0.001854599	0.185459941	**0.19**
KN2	-0.009	0.367	1.835	0.000136128	0.001361276	0.136127596	**0.14**
KN2	-0.009	0.367	1.835	0.000136128	0.001361276	0.136127596	**0.14**
KN2	-0.009	0.362	1.81	0.000134273	0.00134273	0.134272997	**0.13**
KN6	0.011	0.359	1.795	0.00013316	0.001331602	0.133160237	**0.13**
KN6	0.011	0.359	1.795	0.00013316	0.001331602	0.133160237	**0.13**
KN6	0.011	0.359	1.795	0.00013316	0.001331602	0.133160237	**0.13**
KN4	0.015	0.357	1.785	0.000132418	0.001324184	0.132418398	**0.13**
KN4	0.015	0.357	1.785	0.000132418	0.001324184	0.132418398	**0.13**
KN4	0.015	0.351	1.755	0.000130193	0.001301929	0.130192878	**0.13**
KN5	0.016	0.35	1.75	0.000129822	0.00129822	0.129821958	**0.13**
KN5	0.016	0.35	1.75	0.000129822	0.00129822	0.129821958	**0.13**
KN5	0.016	0.347	1.735	0.000128709	0.001287092	0.128709199	**0.13**
KN1	-0.012	0.082	0.41	3.04154E-05	0.000304154	0.03041543	**0.03**
KN1	-0.012	0.082	0.41	3.04154E-05	0.000304154	0.03041543	**0.03**

KN1	-0.012	0.082	0.41	3.04154E-05	0.000304154	0.03041543	**0.03**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**
KN12	0	0	0	0	0	0	**0.00**

Dual Cultures Enzyme Assays on WSA with Antagonistic Invasion Interactions Results

DUAL CULTURES LIGNIN PEROXIDASE ASSAY ON WSA (Wheat Straw Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X9300 X0.1**	**FX10D.F**	**GX100**	**Enzyme Activity**
When *Trichoderma* sp.KN10 invaded							
KN10-KN9	0.826	0.439	2.195	0.002360215	0.023602151	2.360215054	**2.36**
KN10-KN9	0.826	0.439	2.195	0.002360215	0.023602151	2.360215054	**2.36**
KN10-KN9	0.826	0.434	2.17	0.002333333	0.023333333	2.333333333	**2.33**
KN3-KN10	0.678	0.439	2.195	0.002360215	0.023602151	2.360215054	**2.36**
KN3-KN10	0.678	0.439	2.195	0.002360215	0.023602151	2.360215054	**2.36**
KN3-KN10	0.678	0.437	2.185	0.002349462	0.023494624	2.349462366	**2.35**
KN1-KN10	0.707	0.418	2.09	0.002247312	0.022473118	2.247311828	**2.25**
KN1-KN10	0.707	0.418	2.09	0.002247312	0.022473118	2.247311828	**2.25**
KN1-KN10	0.707	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN5-KN10	0.643	0.392	1.96	0.002107527	0.021075269	2.107526882	**2.11**
KN5-KN10	0.643	0.39	1.95	0.002096774	0.020967742	2.096774194	**2.10**
KN5-KN10	0.643	0.39	1.95	0.002096774	0.020967742	2.096774194	**2.10**
KN4-KN10	0.66	0.377	1.885	0.002026882	0.020268817	2.02688172	**2.03**
KN4-KN10	0.66	0.372	1.86	0.002	0.02	2	**2.00**
KN4-KN10	0.66	0.372	1.86	0.002	0.02	2	**2.00**
KN6-KN10	0.726	0.425	2.125	0.002284946	0.022849462	2.284946237	**2.28**
KN6-KN10	0.726	0.425	2.125	0.002284946	0.022849462	2.284946237	**2.28**
KN6-KN10	0.726	0.423	2.115	0.002274194	0.022741935	2.274193548	**2.27**
KN10-GL	0.659	0.392	1.96	0.002107527	0.021075269	2.107526882	**2.11**
KN10-GL	0.659	0.39	1.95	0.002096774	0.020967742	2.096774194	**2.10**
KN10-GL	0.659	0.39	1.95	0.002096774	0.020967742	2.096774194	**2.10**
When *Fomitopsis* sp. KN1 invaded							
KN1-KN5	0.309	0.425	2.125	0.002284946	0.022849462	2.284946237	**2.28**

KN1-KN5	0.309	0.424	2.12	0.00227957	0.022795699	2.279569892	**2.28**
KN1-KN5	0.309	0.424	2.12	0.00227957	0.022795699	2.279569892	**2.28**
KN1-KN3	0.402	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN1-KN3	0.402	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN1-KN3	0.402	0.416	2.08	0.002236559	0.022365591	2.23655914	**2.24**
KN1-GL	0.323	0.418	2.09	0.002247312	0.022473118	2.247311828	**2.25**
KN1-GL	0.323	0.415	2.075	0.002231183	0.022311828	2.231182796	**2.23**
KN1-GL	0.323	0.415	2.075	0.002231183	0.022311828	2.231182796	**2.23**
When *Coriolopsis* sp. KN6 invaded							
KN5-KN6	0.36	0.41	2.05	0.002204301	0.022043011	2.204301075	**2.20**
KN5-KN6	0.36	0.41	2.05	0.002204301	0.022043011	2.204301075	**2.20**
KN5-KN6	0.36	0.41	2.05	0.002204301	0.022043011	2.204301075	**2.20**
KN2-KN6	0.34	0.377	1.885	0.002026882	0.020268817	2.02688172	**2.03**
KN2-KN6	0.34	0.377	1.885	0.002026882	0.020268817	2.02688172	**2.03**
KN2-KN6	0.34	0.373	1.865	0.002005376	0.020053763	2.005376344	**2.01**
When *Rhizopus microsporus* KN1 invaded							
KN2-KN5	0.513	0.439	2.195	0.002360215	0.023602151	2.360215054	**2.36**
KN2-KN5	0.513	0.433	2.165	0.002327957	0.02327957	2.327956989	**2.33**
KN2-KN5	0.513	0.433	2.165	0.002327957	0.02327957	2.327956989	**2.33**
KN2-KN9	0.533	0.42	2.1	0.002258065	0.022580645	2.258064516	**2.26**
KN2-KN9	0.533	0.42	2.1	0.002258065	0.022580645	2.258064516	**2.26**
KN2-KN9	0.533	0.42	2.1	0.002258065	0.022580645	2.258064516	**2.26**
KN2-KN3	0.853	0.378	1.89	0.002032258	0.020322581	2.032258065	**2.03**
KN2-KN3	0.853	0.378	1.89	0.002032258	0.020322581	2.032258065	**2.03**
KN2-KN3	0.853	0.378	1.89	0.002032258	0.020322581	2.032258065	**2.03**
KN2-GL	0.436	0.368	1.84	0.001978495	0.019784946	1.978494624	**1.98**
KN2-GL	0.436	0.364	1.82	0.001956989	0.019569892	1.956989247	**1.96**
KN2-GL	0.436	0.364	1.82	0.001956989	0.019569892	1.956989247	**1.96**

KN2-KN4	0.441	0.424	2.12	0.00227957	0.022795699	2.279569892	**2.28**
KN2-KN4	0.441	0.424	2.12	0.00227957	0.022795699	2.279569892	**2.28**
KN2-KN4	0.441	0.421	2.105	0.002263441	0.022634409	2.26344086	**2.26**
KN1-KN2	0.399	0.429	2.145	0.002306452	0.023064516	2.306451613	**2.31**
KN1-KN2	0.399	0.429	2.145	0.002306452	0.023064516	2.306451613	**2.31**
KN1-KN2	0.399	0.427	2.135	0.002295699	0.022956989	2.295698925	**2.30**
COMBINATION NOT DONE DUE TO KN10E NON-VIABILITY OF KN12 ON WSA							
KN1-KN12	0	0	0	0	0	0	**0.00**
KN2-KN12	0	0	0	0	0	0	**0.00**
KN10-KN12	0	0	0	0	0	0	**0.00**

DUAL CULTURES MANGANESE PEROXIDASE ASSAY ON WSA (Wheat Straw Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**E= Absorbance X5**	**E/1X3451 X0.5**	**FX10D.F**	**GX100**	**Enzyme Activity**
When *Trichoderma* sp.KN10 invaded							
KN3-KN10	0.292	2.706	13.53	0.007841205	0.078412054	7.841205448	**7.84**
KN3-KN10	0.292	2.705	13.525	0.007838308	0.078383077	7.838307737	**7.84**
KN3-KN10	0.292	2.652	13.26	0.007684729	0.076847291	7.684729064	**7.68**
KN5-KN10	0.337	2.629	13.145	0.007618082	0.076180817	7.618081715	**7.62**
KN5-KN10	0.337	2.628	13.14	0.007615184	0.07615184	7.615184005	**7.62**
KN5-KN10	0.337	2.592	12.96	0.007510866	0.075108664	7.510866416	**7.51**
KN1-KN10	0.406	2.594	12.97	0.007516662	0.075166618	7.516661837	**7.52**
KN1-KN10	0.406	2.594	12.97	0.007516662	0.075166618	7.516661837	**7.52**
KN1-KN10	0.406	2.594	12.97	0.007516662	0.075166618	7.516661837	**7.52**
KN10-KN9	0.515	2.604	13.02	0.007545639	0.075456389	7.545638945	**7.55**
KN10-KN9	0.515	2.594	12.97	0.007516662	0.075166618	7.516661837	**7.52**
KN10-KN9	0.515	2.594	12.97	0.007516662	0.075166618	7.516661837	**7.52**
KN4-KN10	0.326	2.498	12.49	0.007238482	0.072384816	7.2384816	**7.24**
KN4-KN10	0.326	2.498	12.49	0.007238482	0.072384816	7.2384816	**7.24**
KN4-KN10	0.326	2.494	12.47	0.007226891	0.072268908	7.226890756	**7.23**
KN6-KN10	0.636	2.591	12.955	0.007507969	0.075079687	7.507968705	**7.51**
KN6-KN10	0.636	2.586	12.93	0.00749348	0.074934802	7.493480151	**7.49**
KN6-KN10	0.636	2.586	12.93	0.00749348	0.074934802	7.493480151	**7.49**
KN10-GL	0.442	2.603	13.015	0.007542741	0.075427412	7.542741234	**7.54**
KN10-GL	0.442	2.577	12.885	0.007467401	0.074674008	7.467400753	**7.47**
KN10-GL	0.442	2.577	12.885	0.007467401	0.074674008	7.467400753	**7.47**
When *Fomitopsis* sp. KN1 invaded							
KN1-GL	0.146	0.893	4.465	0.002587656	0.025876558	2.587655752	**2.60**
KN1-GL	0.146	0.896	4.48	0.002596349	0.025963489	2.596348884	**2.59**
KN1-GL	0.146	0.891	4.455	0.00258186	0.025818603	2.58186033	**2.58**

KN1-KN3	0.088	0.886	4.43	0.002567372	0.025673718	2.567371776	**2.57**
KN1-KN3	0.088	0.877	4.385	0.002541292	0.025412924	2.541292379	**2.54**
KN1-KN3	0.088	0.877	4.385	0.002541292	0.025412924	2.541292379	**2.54**
KN1-KN5	0.134	0.853	4.265	0.002471747	0.024717473	2.47174732	**2.47**
KN1-KN5	0.134	0.853	4.265	0.002471747	0.024717473	2.47174732	**2.47**
KN1-KN5	0.134	0.839	4.195	0.002431179	0.024311794	2.431179368	**2.43**
When *Coriolopsis* sp. KN6 invaded							
KN2-KN6	0.149	0.876	4.38	0.002538395	0.025383947	2.538394668	**2.54**
KN2-KN6	0.149	0.876	4.38	0.002538395	0.025383947	2.538394668	**2.54**
KN2-KN6	0.149	0.871	4.355	0.002523906	0.025239061	2.523906114	**2.52**
KN5-KN6	0.145	0.856	4.28	0.00248044	0.024804405	2.480440452	**2.48**
KN5-KN6	0.145	0.855	4.275	0.002477543	0.024775427	2.477542741	**2.48**
KN5-KN6	0.145	0.853	4.265	0.002471747	0.024717473	2.47174732	**2.47**
When *Rhizopus microsporus* KN2 invaded							
KN2-KN9	0.235	0.797	3.985	0.002309476	0.023094755	2.309475514	**2.31**
KN2-KN9	0.235	0.797	3.985	0.002309476	0.023094755	2.309475514	**2.31**
KN2-KN9	0.235	0.793	3.965	0.002297885	0.022978847	2.297884671	**2.30**
KN2-KN5	0.192	0.769	3.845	0.00222834	0.022283396	2.228339612	**2.23**
KN2-KN5	0.192	0.768	3.84	0.002225442	0.022254419	2.225441901	**2.23**
KN2-KN5	0.192	0.751	3.755	0.002176181	0.021761808	2.176180817	**2.18**
KN2-KN3	0.157	0.648	3.24	0.001877717	0.018777166	1.877716604	**1.88**
KN2-KN3	0.157	0.648	3.24	0.001877717	0.018777166	1.877716604	**1.88**
KN2-KN3	0.157	0.646	3.23	0.001871921	0.018719212	1.871921182	**1.87**
KN2-GL	0.177	0.401	2.005	0.001161982	0.01161982	1.161982034	**1.16**
KN2-GL	0.177	0.382	1.91	0.001106926	0.011069255	1.106925529	**1.11**
KN2-GL	0.177	0.382	1.91	0.001106926	0.011069255	1.106925529	**1.11**
KN2-KN4	0.151	0.424	2.12	0.001228629	0.012286294	1.228629383	**1.23**
KN2-KN4	0.151	0.423	2.115	0.001225732	0.012257317	1.225731672	**1.23**

KN2-KN4	0.151	0.423	2.115	0.001225732	0.012257317	1.225731672	**1.23**
KN1-KN2	0.196	0.789	3.945	0.002286294	0.022862938	2.286293828	**2.29**
KN1-KN2	0.196	0.789	3.945	0.002286294	0.022862938	2.286293828	**2.29**
KN1-KN2	0.196	0.788	3.94	0.002283396	0.022833961	2.283396117	**2.28**
COMBINATION NOT DONE DUE TO KN10E NON-VIABILITY OF KN12 ON WSA							
KN1-KN12	0	0	0	0	0	0	**0.00**
KN2-KN12	0	0	0	0	0	0	**0.00**
KN10-KN12	0	0	0	0	0	0	**0.00**

DUAL CULTURES LACCASE ASSAY ON WSA (Wheat Straw Agar)							
Fungi	**Enzyme Extract Absorbance**	**Reaction Absorbance**	**A X 5**	**E / 2 X 6740X1**	**FX10D.F**	**GX100**	**Enzyme Activity**
When *Trichoderma* sp.KN10 invaded							
KN4-KN10	0.119	0.678	3.39	0.000502967	0.005029674	0.502967359	**0.50**
KN4-KN10	0.119	0.678	3.39	0.000502967	0.005029674	0.502967359	**0.50**
KN4-KN10	0.119	0.678	3.39	0.000502967	0.005029674	0.502967359	**0.50**
KN3-KN10	0.118	0.633	3.165	0.000469585	0.004695846	0.46958457	**0.47**
KN3-KN10	0.118	0.633	3.165	0.000469585	0.004695846	0.46958457	**0.47**
KN3-KN10	0.118	0.631	3.155	0.000468101	0.004681009	0.46810089	**0.47**
KN1-KN10	0.123	0.24	1.2	0.000178042	0.001780415	0.178041543	**0.18**
KN1-KN10	0.123	0.239	1.195	0.0001773	0.001772997	0.177299703	**0.18**
KN1-KN10	0.123	0.239	1.195	0.0001773	0.001772997	0.177299703	**0.18**
KN5-KN10	0.088	0.626	3.13	0.000464392	0.004643917	0.464391691	**0.46**
KN5-KN10	0.088	0.624	3.12	0.000462908	0.00462908	0.462908012	**0.46**
KN5-KN10	0.088	0.62	3.1	0.000459941	0.004599407	0.459940653	**0.46**
KN10-KN9	0.105	0.15	0.75	0.000111276	0.00111276	0.111275964	**0.11**
KN10-KN9	0.105	0.147	0.735	0.00010905	0.001090504	0.109050445	**0.11**
KN10-KN9	0.105	0.145	0.725	0.000107567	0.001075668	0.107566766	**0.11**
KN6-KN10	0.275	0.287	1.435	0.000212908	0.00212908	0.212908012	**0.21**
KN6-KN10	0.275	0.287	1.435	0.000212908	0.00212908	0.212908012	**0.21**
KN6-KN10	0.275	0.287	1.435	0.000212908	0.00212908	0.212908012	**0.21**
KN10-GL	0.151	1.011	5.055	0.00075	0.0075	0.75	**0.75**
KN10-GL	0.151	0.877	4.385	0.000650593	0.006505935	0.650593472	**0.65**
KN10-GL	0.151	0.875	4.375	0.00064911	0.006491098	0.649109792	**0.65**
When *Fomitopsis* sp.KN1 invaded							
KN1-KN5	-0.016	0.696	3.48	0.00051632	0.005163205	0.516320475	**0.52**
KN1-KN5	-0.016	0.696	3.48	0.00051632	0.005163205	0.516320475	**0.52**
KN1-KN5	-0.016	0.694	3.47	0.000514837	0.005148368	0.514836795	**0.51**

KN1-GL	-0.021	0.65	3.25	0.000482196	0.004821958	0.482195846	**0.48**
KN1-GL	-0.021	0.648	3.24	0.000480712	0.004807122	0.480712166	**0.48**
KN1-GL	-0.021	0.648	3.24	0.000480712	0.004807122	0.480712166	**0.48**
KN1-KN3	-0.024	0.214	1.07	0.000158754	0.001587537	0.158753709	**0.16**
KN1-KN3	-0.024	0.214	1.07	0.000158754	0.001587537	0.158753709	**0.16**
KN1-KN3	-0.024	0.214	1.07	0.000158754	0.001587537	0.158753709	**0.16**
When *Coriolopsis* sp.KN6 invaded							
KN5-KN6	0.007	0.193	0.965	0.000143175	0.001431751	0.143175074	**0.14**
KN5-KN6	0.007	0.185	0.925	0.00013724	0.001372404	0.137240356	**0.14**
KN5-KN6	0.007	0.185	0.925	0.00013724	0.001372404	0.137240356	**0.14**
KN2-KN6	-0.021	0.577	2.885	0.000428042	0.004280415	0.428041543	**0.43**
KN2-KN6	-0.021	0.575	2.875	0.000426558	0.004265579	0.426557864	**0.43**
KN2-KN6	-0.021	0.569	2.845	0.000422107	0.004221068	0.422106825	**0.42**
When *Rhizopus microsporus* KN1 invaded							
KN2-KN3	-0.002	0.73	3.65	0.000541543	0.00541543	0.541543027	**0.54**
KN2-KN3	-0.002	0.73	3.65	0.000541543	0.00541543	0.541543027	**0.54**
KN2-KN3	-0.002	0.67	3.35	0.000497033	0.004970326	0.497032641	**0.50**
KN2-GL	0.016	0.628	3.14	0.000465875	0.004658754	0.465875371	**0.47**
KN2-GL	0.016	0.628	3.14	0.000465875	0.004658754	0.465875371	**0.47**
KN2-GL	0.016	0.624	3.12	0.000462908	0.00462908	0.462908012	**0.46**
KN2-KN9	0.043	0.353	1.765	0.000261869	0.002618694	0.261869436	**0.26**
KN2-KN9	0.043	0.353	1.765	0.000261869	0.002618694	0.261869436	**0.26**
KN2-KN9	0.043	0.351	1.755	0.000260386	0.002603858	0.260385757	**0.26**
KN2-KN5	-0.012	0.556	2.78	0.000412463	0.004124629	0.412462908	**0.41**
KN2-KN5	-0.012	0.556	2.78	0.000412463	0.004124629	0.412462908	**0.41**
KN2-KN5	-0.012	0.556	2.78	0.000412463	0.004124629	0.412462908	**0.41**
KN2-KN4	-0.011	0.192	0.96	0.000142433	0.001424332	0.142433234	**0.14**
KN2-KN4	-0.011	0.192	0.96	0.000142433	0.001424332	0.142433234	**0.14**

KN2-KN4	-0.011	0.192	0.96	0.000142433	0.001424332	0.142433234	**0.14**
KN1-KN2	-0.018	0.083	0.415	6.15727E-05	0.000615727	0.0615727	**0.06**
KN1-KN2	-0.018	0.083	0.415	6.15727E-05	0.000615727	0.0615727	**0.06**
KN1-KN2	-0.018	0.083	0.415	6.15727E-05	0.000615727	0.0615727	**0.06**
COMBINATION NOT DONE DUE TO KN10E NON-VIABILITY OF KN12 ON WSA							
KN1-KN12	0	0	0	0	0	0	**0.00**
KN2-KN12	0	0	0	0	0	0	**0.00**
KN10-KN12	0	0	0	0	0	0	**0.00**

APPENDIX E

Statistical Analysis

Monoculture Analysis of Variance of the Enzyme Activities

Factorial AOV Table for LiP

Source	DF	SS	MS	F	P
Rep	2	0.0006	0.00031		
Fungi	9	34.4544	3.82827	17315.45	0.0000
Substrate	3	5.0794	1.69315	7658.20	0.0000
Fungi*Substrate	27	21.3971	0.79248	3584.44	0.0000
Error	78	0.0172	0.00022		
Total	119	60.9488			

Grand Mean 2.1203
CV 0.70

Factorial AOV Table for MnP

Source	DF	SS	MS	F	P
Rep	2	0.0028	0.0014		
Fungi	9	4.2070	0.4674	4026.58	0.0000
Substrate	3	54.6752	18.2251	156991.09	0.0000
Fungi*Substrate	27	9.8714	0.3656	3149.34	0.0000
Error	78	0.0091	0.0001		
Total	119	68.7654			

Grand Mean 0.5537
CV 1.95

Factorial AOV Table for Lacc

Source	DF	SS	MS	F	P
Rep	2	0.00003	1.583E-05		
Fungi	9	0.13976	0.01553	4015.13	0.0000
Substrate	3	0.47123	0.15708	40614.23	0.0000
Fungi*Substrate	27	0.24788	9.181E-03	2373.80	0.0000
Error	78	0.00030	3.868E-06		
Total	119	0.85920			

Grand Mean 0.0509
CV 3.86

Enzyme Activities on Substrates Correlations in Monocultures

Correlations (Pearson)

	LiP	MnP	Lacc
LiP	1.0000		
p-value	0.0000		
MnP	0.2084	1.0000	
	0.0224	0.0000	
Lacc	0.1822	0.8328	1.0000
	0.0464	0.0000	0.0000

Cronbach's alpha 0.3678
Standardized Cronbach's alpha 0.6738

Fleiss' Kappa -0.297

Cases Included 120 Missing Cases 0

Tukey HSD All-Pairwise Comparisons of Enzyme Activities and Substrates in Monocultures

Tukey HSD All-Pairwise Comparisons Test of LiP for Fungi

Fungi	Mean	Homogeneous Groups
KN10	2.4225	A
KN6	2.3992	B
KN9	2.3525	C
KN1	2.3475	C
KN4	2.3458	C
KN3	2.3442	C
GL	2.3200	D
KN2	2.3175	D
KN5	1.7417	E
KN12	0.6125	F

Alpha 0.05 Standard Error for Comparison 6.070E-03
Critical Q Value 4.604 Critical Value for Comparison 0.0198
There are 6 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of LiP for Substrate

Substrate	Mean	Homogeneous Groups
PDA	2.4290	A
CCA	2.1233	B
WSA	2.0773	C
SBA	1.8517	D

Alpha 0.05 Standard Error for Comparison 3.839E-03
Critical Q Value 3.713 Critical Value for Comparison 0.0101
All 4 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of LiP for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN2	PDA	2.5367	A
KN10	PDA	2.5300	A
KN3	WSA	2.5200	AB
KN1	PDA	2.4733	BC
KN4	PDA	2.4533	CD
KN12	PDA	2.4500	CDE
KN9	PDA	2.4300	CDEF
KN10	CCA	2.4233	DEFG
GL	PDA	2.4200	DEFG
KN6	WSA	2.4200	DEFG
KN6	CCA	2.4033	EFGH
KN6	PDA	2.4033	EFGH
KN10	SBA	2.3967	FGHI
KN3	CCA	2.3867	FGHIJ
KN9	CCA	2.3767	GHIJK
KN6	SBA	2.3700	HIJK
KN9	SBA	2.3700	HIJK
KN5	PDA	2.3533	IJKL
KN4	WSA	2.3467	JKL
KN10	WSA	2.3400	JKL
KN4	CCA	2.3400	JKL
KN2	CCA	2.3367	KL
KN2	SBA	2.3367	KL

KN1	CCA	2.3300	KLM
KN1	WSA	2.3300	KLM
KN5	CCA	2.3300	KLM
GL	SBA	2.3133	LM
GL	CCA	2.3067	LM
KN5	WSA	2.2833	MN
KN1	SBA	2.2567	NO
KN4	SBA	2.2433	NO
GL	WSA	2.2400	NO
KN3	PDA	2.2400	NO
KN9	WSA	2.2333	O
KN3	SBA	2.2300	O
KN2	WSA	2.0600	P
KN12	CCA	0.0000	Q
KN12	SBA	0.0000	Q
KN12	WSA	0.0000	Q
KN5	SBA	0.0000	Q

Alpha 0.05 Standard Error for Comparison 0.0121
Critical Q Value 5.716 Critical Value for Comparison 0.0491
There are 17 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Fungi

Fungi	Mean	Homogeneous Groups
KN9	0.7758	A
GL	0.7325	B
KN6	0.6800	C
KN3	0.6100	D
KN4	0.5875	E
KN10	0.5842	E
KN1	0.5467	F
KN2	0.5258	G
KN5	0.4192	H
KN12	0.0750	I

Alpha 0.05 Standard Error for Comparison 4.399E-03
Critical Q Value 4.604 Critical Value for Comparison 0.0143
There are 9 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Substrate

Substrate	Mean	Homogeneous Groups
WSA	1.7220	A
PDA	0.2037	B
CCA	0.1537	C
SBA	0.1353	D

Alpha 0.05 Standard Error for Comparison 2.782E-03
Critical Q Value 3.713 Critical Value for Comparison 7.304E-03
All 4 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN9	WSA	2.5800	A
GL	WSA	2.3900	B
KN6	WSA	2.1200	C
KN3	WSA	1.8767	D

KN4	WSA	1.8433	DE
KN10	WSA	1.8100	E
KN1	WSA	1.7233	F
KN2	WSA	1.6100	G
KN5	WSA	1.2667	H
KN12	PDA	0.3000	I
KN5	PDA	0.2500	J
KN10	CCA	0.2400	JK
KN3	PDA	0.2333	JKL
KN6	PDA	0.2300	JKL
KN10	SBA	0.2267	JKL
GL	PDA	0.2133	KLM
KN9	PDA	0.2033	LMN
KN2	PDA	0.1867	MNO
KN6	CCA	0.1867	MNO
KN4	CCA	0.1833	MNO
KN4	PDA	0.1833	MNO
KN6	SBA	0.1833	MNO
KN1	PDA	0.1767	NO
GL	CCA	0.1733	NOP
KN2	SBA	0.1733	NOP
KN3	CCA	0.1667	OPQ
KN3	SBA	0.1633	OPQ
KN9	CCA	0.1600	OPQ
KN5	CCA	0.1600	OPQ
KN9	SBA	0.1600	OPQ
GL	SBA	0.1533	OPQ
KN1	SBA	0.1533	OPQ
KN4	SBA	0.1400	PQ
KN1	CCA	0.1333	Q
KN2	CCA	0.1333	Q
KN10	PDA	0.0600	R
KN12	CCA	0.0000	S
KN12	SBA	0.0000	S
KN12	WSA	0.0000	S
KN5	SBA	0.0000	S

Alpha	0.05	Standard Error for Comparison	8.797E-03
Critical Q Value	5.716	Critical Value for Comparison	0.0356

There are 19 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Fungi

Fungi	Mean	Homogeneous Groups
KN10	0.1275	A
KN9	0.0825	B
GL	0.0758	C
KN3	0.0575	D
KN2	0.0383	E
KN6	0.0375	EF
KN5	0.0350	FG
KN4	0.0333	G
KN12	0.0125	H
KN1	0.0092	I

Alpha	0.05	Standard Error for Comparison	8.029E-04
Critical Q Value	4.604	Critical Value for Comparison	2.614E-03

There are 9 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Substrate

Substrate	Mean	Homogeneous Groups
WSA	0.1577	A
PDA	0.0337	B
SBA	0.0083	C
CCA	0.0040	D

Alpha 0.05 Standard Error for Comparison 5.078E-04
Critical Q Value 3.713 Critical Value for Comparison 1.333E-03
All 4 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN9	WSA	0.2900	A
GL	WSA	0.2800	B
KN10	WSA	0.2600	C
KN10	PDA	0.2200	D
KN3	WSA	0.1900	E
KN2	WSA	0.1367	F
KN4	WSA	0.1300	G
KN5	WSA	0.1300	G
KN6	WSA	0.1300	G
KN12	PDA	0.0500	H
KN1	WSA	0.0300	I
KN10	SBA	0.0200	J
KN3	PDA	0.0200	J
KN9	PDA	0.0200	J
KN9	SBA	0.0200	J
GL	CCA	1.00E-02	K
GL	SBA	1.00E-02	K
KN10	CCA	1.00E-02	K
KN2	PDA	1.00E-02	K
KN3	CCA	1.00E-02	K
KN3	SBA	1.00E-02	K
KN5	PDA	1.00E-02	K
KN6	CCA	1.00E-02	K
KN6	SBA	1.00E-02	K
KN1	SBA	6.67E-03	KL
KN2	SBA	6.67E-03	KL
GL	PDA	3.33E-03	LM
KN4	PDA	3.33E-03	LM
KN1	CCA	0.0000	M
KN1	PDA	0.0000	M
KN12	CCA	0.0000	M
KN12	SBA	0.0000	M
KN12	WSA	0.0000	M
KN2	CCA	0.0000	M
KN4	CCA	0.0000	M
KN4	SBA	0.0000	M
KN5	CCA	0.0000	M
KN5	SBA	0.0000	M
KN6	PDA	0.0000	M
KN9	CCA	0.0000	M

Alpha 0.05 Standard Error for Comparison 1.606E-03
Critical Q Value 5.716 Critical Value for Comparison 6.489E-03
There are 13 groups (A, B, etc.) in which the means
are not significantly different from one another.

Dual Cultures Analysis of Variance of the Enzyme Activities involving invasion/replacement by *Trichoderma* sp. KN10

Factorial AOV Table for LiP

Source	DF	SS	MS	F	P
Rep	2	2.392E-04	0.00012		
Fungi	16	90.9522	5.68451	20281.95	0.0000
Substrate	2	3.20483	1.60241	5717.30	0.0000
Fungi*Substrate	32	19.0788	0.59621	2127.24	0.0000
Error	100	0.02803	0.00028		
Total	152	113.264			

Grand Mean 1.9798
CV 0.85

Factorial AOV Table for MnP

Source	DF	SS	MS	F	P
Rep	2	0.007	3.459E-03		
Fungi	16	168.391	10.5245	29386.14	0.0000
Substrate	2	500.381	250.191	698576.25	0.0000
Fungi*Substrate	32	293.710	9.17845	25627.84	0.0000
Error	100	0.036	3.581E-04		
Total	152	962.526			

Grand Mean 1.4580
CV 1.30

Factorial AOV Table for Lacc

Source	DF	SS	MS	F	P
Rep	2	0.00019	0.00010		
Fungi	16	0.83193	0.05200	779.02	0.0000
Substrate	2	1.52726	0.76363	11441.01	0.0000
Fungi*Substrate	32	1.03969	0.03249	486.78	0.0000
Error	100	0.00667	0.00007		
Total	152	3.40575			

Grand Mean 0.0976
CV 8.37

Tukey HSD All-Pairwise Comparisons of Enzyme Activities and Substrates involving invasion/replacement by *Trichoderma* sp. KN10

Tukey HSD All-Pairwise Comparisons Test of LiP for Fungi

Fungi	Mean	Homogeneous Groups
KN9-KN10	2.4189	A
KN1-KN10	2.4178	A
KN6	2.3978	AB
KN6-KN10	2.3933	AB
KN10	2.3867	B
KN3-KN10	2.3856	B
KN3	2.3789	B
KN9	2.3267	C
KN10-GL	2.3156	C
KN4	2.3100	CD
KN1	2.3056	CD
GL	2.2867	D
KN4-KN10	2.2822	D
KN5	1.5378	E
KN5-KN10	1.5133	E
KN10-KN12	0.0000	F
KN12	0.0000	F

Alpha 0.05 Standard Error for Comparison 7.892E-03
Critical Q Value 5.015 Critical Value for Comparison 0.0280
There are 6 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of LiP for Substrate

Substrate	Mean	Homogeneous Groups
CCA	2.1404	A
WSA	2.0094	B
SBA	1.7896	C

Alpha 0.05 Standard Error for Comparison 3.315E-03
Critical Q Value 3.365 Critical Value for Comparison 7.889E-03
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of LiP for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN1-KN10	CCA	2.6000	A
KN3	WSA	2.5200	B
KN10-GL	CCA	2.5133	B
KN6-KN10	CCA	2.5100	B
KN4-KN10	CCA	2.4900	BC
KN3-KN10	CCA	2.4733	BCD
KN9-KN10	CCA	2.4667	BCD
KN9-KN10	SBA	2.4400	CDE
KN5-KN10	CCA	2.4367	CDE
KN10	CCA	2.4233	DEF
KN6	WSA	2.4200	DEF
KN1-KN10	SBA	2.4067	EFG
KN6	CCA	2.4033	EFGH
KN10	SBA	2.3967	EFGHI
KN6-KN10	SBA	2.3933	EFGHIJ
KN3	CCA	2.3867	EFGHIJ
KN9	CCA	2.3767	FGHIJK

KN6	SBA	2.3700	FGHIJK
KN9	SBA	2.3700	FGHIJK
KN3-KN10	WSA	2.3567	GHIJKL
KN9-KN10	WSA	2.3500	HIJKL
KN4	WSA	2.3467	IJKL
KN4-KN10	SBA	2.3467	IJKL
KN10	WSA	2.3400	JKL
KN4	CCA	2.3400	JKL
KN1	CCA	2.3300	KLM
KN1	WSA	2.3300	KLM
KN10-GL	SBA	2.3300	KLM
KN5	CCA	2.3300	KLM
KN3-KN10	SBA	2.3267	KLM
GL	SBA	2.3133	LM
GL	CCA	2.3067	LMN
KN5	WSA	2.2833	MNO
KN6-KN10	WSA	2.2767	MNO
KN1	SBA	2.2567	NO
KN1-KN10	WSA	2.2467	O
KN4	SBA	2.2433	O
GL	WSA	2.2400	O
KN9	WSA	2.2333	O
KN3	SBA	2.2300	O
KN10-GL	WSA	2.1033	P
KN5-KN10	WSA	2.1033	P
KN4-KN10	WSA	2.0100	Q
KN10-KN12	CCA	0.0000	R
KN10-KN12	SBA	0.0000	R
KN10-KN12	WSA	0.0000	R
KN12	CCA	0.0000	R
KN12	SBA	0.0000	R
KN12	WSA	0.0000	R
KN5	SBA	0.0000	R
KN5-KN10	SBA	0.0000	R

Alpha	0.05	Standard Error for Comparison	0.0137
Critical Q Value	5.840	Critical Value for Comparison	0.0564

There are 18 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Fungi

Fungi	**Mean**	**Homogeneous Groups**
KN3-KN10	2.7444	A
KN1-KN10	2.7356	A
KN10-GL	2.6922	B
KN6-KN10	2.6778	B
KN9-KN10	2.6689	B
KN5-KN10	2.6289	C
KN4-KN10	2.5733	D
KN9	0.9667	E
GL	0.9056	F
KN6	0.8300	G
KN10	0.7589	H
KN3	0.7356	HI
KN4	0.7222	I
KN1	0.6700	J
KN5	0.4756	K
KN10-KN12	0.0000	L
KN12	0.0000	L

Alpha 0.05 Standard Error for Comparison 8.921E-03
Critical Q Value 5.015 Critical Value for Comparison 0.0316
There are 12 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Substrate

Substrate	Mean	Homogeneous Groups
WSA	4.0151	A
CCA	0.2186	B
SBA	0.1402	C

Alpha 0.05 Standard Error for Comparison 3.748E-03
Critical Q Value 3.365 Critical Value for Comparison 8.917E-03
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN3-KN10	WSA	7.7867	A
KN5-KN10	WSA	7.5833	B
KN9-KN10	WSA	7.5300	BC
KN1-KN10	WSA	7.5200	BC
KN6-KN10	WSA	7.4967	C
KN10-GL	WSA	7.4933	C
KN4-KN10	WSA	7.2367	D
KN9	WSA	2.5800	E
GL	WSA	2.3900	F
KN6	WSA	2.1200	G
KN3	WSA	1.8767	H
KN4	WSA	1.8433	HI
KN10	WSA	1.8100	I
KN1	WSA	1.7233	J
KN5	WSA	1.2667	K
KN1-KN10	CCA	0.4700	L
KN10-GL	CCA	0.3833	M
KN6-KN10	CCA	0.3167	N
KN5-KN10	CCA	0.3033	NO
KN4-KN10	CCA	0.2933	NO
KN9-KN10	CCA	0.2800	NOP
KN3-KN10	CCA	0.2667	NOP
KN10	CCA	0.2400	OPQ
KN10	SBA	0.2267	PQR
KN6-KN10	SBA	0.2200	PQRS
KN1-KN10	SBA	0.2167	PQRST
KN10-GL	SBA	0.2000	QRSTU
KN9-KN10	SBA	0.1967	QRSTUV
KN4-KN10	SBA	0.1900	QRSTUV
KN6	CCA	0.1867	QRSTUV
KN4	CCA	0.1833	QRSTUV
KN6	SBA	0.1833	QRSTUV
KN3-KN10	SBA	0.1800	QRSTUV
GL	CCA	0.1733	RSTUV
KN3	CCA	0.1667	RSTUV
KN3	SBA	0.1633	RSTUV
KN9	CCA	0.1600	STUV
KN5	CCA	0.1600	STUV
KN9	SBA	0.1600	STUV
GL	SBA	0.1533	TUV
KN1	SBA	0.1533	TUV
KN4	SBA	0.1400	UV

KN1	CCA	0.1333	V
KN10-KN12	CCA	0.0000	W
KN10-KN12	SBA	0.0000	W
KN10-KN12	WSA	0.0000	W
KN12	CCA	0.0000	W
KN12	SBA	0.0000	W
KN12	WSA	0.0000	W
KN5	SBA	0.0000	W
KN5-KN10	SBA	0.0000	W

Alpha 0.05 Standard Error for Comparison 0.0155
Critical Q Value 5.840 Critical Value for Comparison 0.0638
There are 23 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Fungi

Fungi	Mean	Homogeneous Groups
KN10-GL	0.2678	A
KN4-KN10	0.2044	B
KN3-KN10	0.1867	C
KN5-KN10	0.1833	C
KN6-KN10	0.1200	D
KN1-KN10	0.1100	DE
KN9	0.1033	E
GL	0.1000	E
KN10	0.0967	E
KN3	0.0700	F
KN9-KN10	0.0689	F
KN6	0.0500	G
KN4	0.0433	G
KN5	0.0433	G
KN1	0.0122	H
KN10-KN12	0.0000	H
KN12	0.0000	H

Alpha 0.05 Standard Error for Comparison 3.851E-03
Critical Q Value 5.015 Critical Value for Comparison 0.0137
There are 8 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Substrate

Substrate	Mean	Homogeneous Groups
WSA	0.2384	A
CCA	0.0376	B
SBA	0.0169	C

Alpha 0.05 Standard Error for Comparison 1.618E-03
Critical Q Value 3.365 Critical Value for Comparison 3.850E-03
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN10-GL	WSA	0.6833	A
KN4-KN10	WSA	0.5000	B
KN3-KN10	WSA	0.4700	C
KN5-KN10	WSA	0.4600	C
KN9	WSA	0.2900	D
GL	WSA	0.2800	DE

KN10	WSA	0.2600	E
KN6-KN10	WSA	0.2100	F
KN3	WSA	0.1900	FG
KN1-KN10	WSA	0.1800	G
KN4	WSA	0.1300	H
KN5	WSA	0.1300	H
KN6	WSA	0.1300	H
KN1-KN10	CCA	0.1200	HI
KN9-KN10	WSA	0.1100	HIJ
KN6-KN10	CCA	0.1000	IJK
KN5-KN10	CCA	0.0900	JKL
KN4-KN10	CCA	0.0833	JKLM
KN10-GL	CCA	0.0800	KLM
KN3-KN10	CCA	0.0700	LMN
KN9-KN10	CCA	0.0567	MNO
KN6-KN10	SBA	0.0500	NO
KN10-GL	SBA	0.0400	OP
KN9-KN10	SBA	0.0400	OP
KN1	WSA	0.0300	OPQ
KN1-KN10	SBA	0.0300	OPQ
KN4-KN10	SBA	0.0300	OPQ
KN10	SBA	0.0200	PQR
KN3-KN10	SBA	0.0200	PQR
KN9	SBA	0.0200	PQR
GL	CCA	1.00E-02	QR
GL	SBA	1.00E-02	QR
KN10	CCA	1.00E-02	QR
KN3	CCA	1.00E-02	QR
KN3	SBA	1.00E-02	QR
KN6	CCA	1.00E-02	QR
KN6	SBA	1.00E-02	QR
KN1	SBA	6.67E-03	QR
KN1	CCA	0.0000	R
KN10-KN12	CCA	0.0000	R
KN10-KN12	SBA	0.0000	R
KN10-KN12	WSA	0.0000	R
KN12	CCA	0.0000	R
KN12	SBA	0.0000	R
KN12	WSA	0.0000	R
KN4	CCA	0.0000	R
KN4	SBA	0.0000	R
KN5	CCA	0.0000	R
KN5	SBA	0.0000	R
KN5-KN10	SBA	0.0000	R
KN9	CCA	0.0000	R

Alpha	0.05	Standard Error for Comparison	6.671E-03
Critical Q Value	5.840	Critical Value for Comparison	0.0275

There are 18 groups (A, B, etc.) in which the means
are not significantly different from one another.

Dual Cultures Analysis of Variance of the Enzyme Activities involving invasion/replacement by *Rhizopus microsporus* KN2

Factorial AOV Table for LiP

Source	DF	SS	MS	F	P
Replicate	2	0.001	0.00050		
Fungi	16	85.577	5.34856	22668.41	0.0000
Substrate	2	2.399	1.19963	5084.31	0.0000
Fungi*Substrate	32	20.372	0.63664	2698.22	0.0000
Error	100	0.024	0.00024		
Total	152	108.373			

Grand Mean 1.9380
CV 0.79

Factorial AOV Table for MnP

Source	DF	SS	MS	F	P
Replicate	2	0.004	0.0020		
Fungi	16	12.192	0.7620	4842.04	0.0000
Substrate	2	83.567	41.7837	265508.35	0.0000
Fungi*Substrate	32	17.365	0.5427	3448.22	0.0000
Error	100	0.016	0.0002		
Total	152	113.144			

Grand Mean 0.6598
CV 1.90

Factorial AOV Table for Lacc

Source	DF	SS	MS	F	P
Replicate	2	0.00015	0.00007		
Fungi	16	0.49093	0.03068	2210.24	0.0000
Substrate	2	1.40464	0.70232	50590.82	0.0000
Fungi*Substrate	32	0.83569	0.02612	1881.20	0.0000
Error	100	0.00139	0.00001		
Total	152	2.73280			

Grand Mean 0.0767
CV 4.86

Tukey HSD All-Pairwise Comparisons of Enzyme Activities and Substrates involving invasion/replacement by *Rhizopus microsporus* KN2

Tukey HSD All-Pairwise Comparisons Test of LiP for Fungi

Fungi	Mean	Homogeneous Groups
KN6	2.3978	A
KN3	2.3789	A
KN1-KN2	2.3411	B
KN9	2.3267	BC
KN2-KN9	2.3256	BC
KN2-KN4	2.3233	BC
KN4	2.3100	CD
KN1	2.3056	CD
GL	2.2867	D
KN2	2.2444	E
KN2-KN3	2.2089	F
KN2-KN6	2.2033	F
KN2-GL	2.1767	G
KN2-KN5	1.5789	H
KN5	1.5378	I
KN12	0.0000	J
KN2-KN12	0.0000	J

Alpha 0.05 Standard Error for Comparison 7.241E-03
Critical Q Value 5.015 Critical Value for Comparison 0.0257
There are 10 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of LiP for Substrate

Substrate	Mean	Homogeneous Groups
CCA	2.0671	A
WSA	1.9784	B
SBA	1.7684	C

Alpha 0.05 Standard Error for Comparison 3.042E-03
Critical Q Value 3.365 Critical Value for Comparison 7.238E-03
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of LiP for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN3	WSA	2.5200	A
KN6	WSA	2.4200	B
KN6	CCA	2.4033	BC
KN2-KN5	CCA	2.3967	BCD
KN3	CCA	2.3867	BCDE
KN2-KN9	CCA	2.3767	BCDEF
KN9	CCA	2.3767	BCDEF
KN6	SBA	2.3700	BCDEFG
KN9	SBA	2.3700	BCDEFG
KN1-KN2	CCA	2.3667	CDEFG
KN1-KN2	SBA	2.3500	DEFGH
KN2-KN4	SBA	2.3500	DEFGH
KN4	WSA	2.3467	DEFGH
KN2-KN4	CCA	2.3467	DEFGH
KN2-KN5	WSA	2.3400	EFGH
KN2-KN9	SBA	2.3400	EFGH
KN4	CCA	2.3400	EFGH

KN2	CCA	2.3367	EFGH
KN2	SBA	2.3367	EFGH
KN1	CCA	2.3300	FGHI
KN1	WSA	2.3300	FGHI
KN5	CCA	2.3300	FGHI
KN2-KN3	CCA	2.3233	GHIJ
KN2-GL	SBA	2.3200	GHIJ
GL	SBA	2.3133	HIJ
KN2-KN6	SBA	2.3100	HIJK
KN1-KN2	WSA	2.3067	HIJKL
GL	CCA	2.3067	HIJKL
KN5	WSA	2.2833	IJKLM
KN2-KN6	CCA	2.2767	JKLMN
KN2-KN3	SBA	2.2733	JKLMN
KN2-KN4	WSA	2.2733	JKLMN
KN2-KN9	WSA	2.2600	KLMN
KN1	SBA	2.2567	LMN
KN2-GL	CCA	2.2433	MN
KN4	SBA	2.2433	MN
GL	WSA	2.2400	MN
KN9	WSA	2.2333	MN
KN3	SBA	2.2300	N
KN2	WSA	2.0600	O
KN2-KN3	WSA	2.0300	O
KN2-KN6	WSA	2.0233	O
KN2-GL	WSA	1.9667	P
KN12	CCA	0.0000	Q
KN12	SBA	0.0000	Q
KN12	WSA	0.0000	Q
KN2-KN12	CCA	0.0000	Q
KN2-KN12	SBA	0.0000	Q
KN2-KN12	WSA	0.0000	Q
KN2-KN5	SBA	0.0000	Q
KN5	SBA	0.0000	Q

Alpha	0.05	Standard Error for Comparison	0.0125
Critical Q Value	5.840	Critical Value for Comparison	0.0518

There are 17 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Fungi

Fungi	**Mean**	**Homogeneous Groups**
KN9	0.9667	A
KN2-KN6	0.9611	A
KN2-KN9	0.9089	B
GL	0.9056	B
KN1-KN2	0.8578	C
KN6	0.8300	D
KN2-KN5	0.7856	E
KN3	0.7356	F
KN2-KN3	0.7322	F
KN4	0.7222	F
KN1	0.6700	G
KN2	0.6389	H
KN2-KN4	0.5267	I
KN2-GL	0.5000	J
KN5	0.4756	K
KN12	0.0000	L
KN2-KN12	0.0000	L

Alpha 0.05 Standard Error for Comparison 5.914E-03
Critical Q Value 5.015 Critical Value for Comparison 0.0210
There are 12 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Substrate

Substrate	Mean	Homogeneous Groups
WSA	1.7049	A
CCA	0.1480	B
SBA	0.1265	C

Alpha 0.05 Standard Error for Comparison 2.484E-03
Critical Q Value 3.365 Critical Value for Comparison 5.911E-03
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN9	WSA	2.5800	A
KN2-KN6	WSA	2.5333	B
GL	WSA	2.3900	C
KN2-KN9	WSA	2.3067	D
KN1-KN2	WSA	2.2867	D
KN2-KN5	WSA	2.2133	E
KN6	WSA	2.1200	F
KN2-KN3	WSA	1.8767	G
KN3	WSA	1.8767	G
KN4	WSA	1.8433	G
KN1	WSA	1.7233	H
KN2	WSA	1.6100	I
KN5	WSA	1.2667	J
KN2-KN4	WSA	1.2300	J
KN2-GL	WSA	1.1267	K
KN2-KN9	CCA	0.2300	L
KN2-GL	SBA	0.1933	LM
KN2-KN9	SBA	0.1900	LM
KN6	CCA	0.1867	M
KN4	CCA	0.1833	MN
KN6	SBA	0.1833	MN
KN2-GL	CCA	0.1800	MNO
KN2-KN3	CCA	0.1800	MNO
KN2-KN4	CCA	0.1800	MNO
KN2-KN6	CCA	0.1800	MNO
GL	CCA	0.1733	MNOP
KN2	SBA	0.1733	MNOP
KN2-KN4	SBA	0.1700	MNOP
KN2-KN6	SBA	0.1700	MNOP
KN3	CCA	0.1667	MNOPQ
KN3	SBA	0.1633	MNOPQ
KN9	CCA	0.1600	MNOPQ
KN1-KN2	SBA	0.1600	MNOPQ
KN5	CCA	0.1600	MNOPQ
KN9	SBA	0.1600	MNOPQ
GL	SBA	0.1533	MNOPQ
KN1	SBA	0.1533	MNOPQ
KN2-KN5	CCA	0.1433	NOPQ
KN2-KN3	SBA	0.1400	OPQ
KN4	SBA	0.1400	OPQ
KN1	CCA	0.1333	PQ
KN2	CCA	0.1333	PQ

KN1-KN2	CCA	0.1267	Q
KN12	CCA	0.0000	R
KN12	SBA	0.0000	R
KN12	WSA	0.0000	R
KN2-KN12	CCA	0.0000	R
KN2-KN12	SBA	0.0000	R
KN2-KN12	WSA	0.0000	R
KN2-KN5	SBA	0.0000	R
KN5	SBA	0.0000	R

Alpha 0.05 Standard Error for Comparison 0.0102
Critical Q Value 5.840 Critical Value for Comparison 0.0423
There are 18 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Fungi

Fungi	Mean	Homogeneous Groups
KN2-KN3	0.1856	A
KN2-GL	0.1656	B
KN2-KN6	0.1544	C
KN2-KN5	0.1433	D
KN9	0.1033	E
KN2-KN9	0.1011	E
GL	0.1000	E
KN3	0.0700	F
KN2-KN4	0.0533	G
KN6	0.0500	G
KN2	0.0478	GH
KN4	0.0433	H
KN5	0.0433	H
KN1-KN2	0.0300	I
KN1	0.0122	J
KN12	0.0000	K
KN2-KN12	0.0000	K

Alpha 0.05 Standard Error for Comparison 1.756E-03
Critical Q Value 5.015 Critical Value for Comparison 6.228E-03
There are 11 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Substrate

Substrate	Mean	Homogeneous Groups
WSA	0.2122	A
CCA	0.0106	B
SBA	0.0073	C

Alpha 0.05 Standard Error for Comparison 7.378E-04
Critical Q Value 3.365 Critical Value for Comparison 1.756E-03
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN2-KN3	WSA	0.5267	A
KN2-GL	WSA	0.4667	B
KN2-KN6	WSA	0.4267	C
KN2-KN5	WSA	0.4100	D
KN9	WSA	0.2900	E
GL	WSA	0.2800	E

KN2-KN9	WSA	0.2600	F
KN3	WSA	0.1900	G
KN2-KN4	WSA	0.1400	H
KN2	WSA	0.1367	H
KN4	WSA	0.1300	H
KN5	WSA	0.1300	H
KN6	WSA	0.1300	H
KN1-KN2	WSA	0.0600	I
KN2-KN9	CCA	0.0333	J
KN1	WSA	0.0300	JK
KN2-KN6	CCA	0.0267	JK
KN1-KN2	CCA	0.0200	KL
KN2-GL	CCA	0.0200	KL
KN2-KN3	CCA	0.0200	KL
KN2-KN5	CCA	0.0200	KL
KN9	SBA	0.0200	KL
GL	CCA	1.00E-02	LM
GL	SBA	1.00E-02	LM
KN1-KN2	SBA	1.00E-02	LM
KN2-GL	SBA	1.00E-02	LM
KN2-KN3	SBA	1.00E-02	LM
KN2-KN4	CCA	1.00E-02	LM
KN2-KN4	SBA	1.00E-02	LM
KN2-KN6	SBA	1.00E-02	LM
KN2-KN9	SBA	1.00E-02	LM
KN3	CCA	1.00E-02	LM
KN3	SBA	1.00E-02	LM
KN6	CCA	1.00E-02	LM
KN6	SBA	1.00E-02	LM
KN1	SBA	6.67E-03	M
KN2	SBA	6.67E-03	M
KN1	CCA	0.0000	M
KN12	CCA	0.0000	M
KN12	SBA	0.0000	M
KN12	WSA	0.0000	M
KN2	CCA	0.0000	M
KN2-KN12	CCA	0.0000	M
KN2-KN12	SBA	0.0000	M
KN2-KN12	WSA	0.0000	M
KN2-KN5	SBA	0.0000	M
KN4	CCA	0.0000	M
KN4	SBA	0.0000	M
KN5	CCA	0.0000	M
KN5	SBA	0.0000	M
KN9	CCA	0.0000	M

Alpha	0.05	Standard Error for Comparison	3.042E-03
Critical Q Value	5.840	Critical Value for Comparison	0.0126

There are 13 groups (A, B, etc.) in which the means
are not significantly different from one another.

Dual Cultures Analysis of Variance of the Enzyme Activities involving invasion/replacement by *Fomitopsis* sp. KN1

Factorial AOV Table for LiP

Source	DF	SS	MS	F	P
Replicate	2	0.0001	0.00003		
Fungi	6	8.3862	1.39771	7602.69	0.0000
Substrate	2	6.9616	3.48080	18933.50	0.0000
Fungi*Substrate	12	15.5348	1.29457	7041.68	0.0000
Error	49	0.0090	0.00018		
Total	71				

Note: SS are marginal (type III) sums of squares

Grand Mean 2.0959
CV 0.65

Factorial AOV Table for MnP

Source	DF	SS	MS	F	P
Replicate	2	0.0036	0.0018		
Fungi	6	1.8507	0.3085	2106.50	0.0000
Substrate	2	59.7225	29.8613	203930.60	0.0000
Fungi*Substrate	12	3.2944	0.2745	1874.87	0.0000
Error	49	0.0072	0.0001		
Total	71				

Note: SS are marginal (type III) sums of squares

Grand Mean 0.7952
CV 1.52

Factorial AOV Table for Lacc

Source	DF	SS	MS	F	P
Replicate	2	0.00004	2.222E-05		
Fungi	6	0.26517	0.04420	9745.08	0.0000
Substrate	2	0.90520	0.45260	99798.30	0.0000
Fungi*Substrate	12	0.46884	0.03907	8614.86	0.0000
Error	49	0.00022	4.535E-06		
Total	71				

Note: SS are marginal (type III) sums of squares

Grand Mean 0.0919
CV 2.32

Tukey HSD All-Pairwise Comparisons of Enzyme Activities and Substrates involving invasion/replacement by *Fomitopsis* sp. KN1

Tukey HSD All-Pairwise Comparisons Test of LiP for Fungi

Fungi	Mean	Homogeneous Groups
KN3	2.3789	A
KN1-GL	2.3356	B
KN1	2.3056	C
KN1-KN3	2.2989	CD
GL	2.2867	D
KN5	1.5378	E
KN1-KN5	1.5278	E

Alpha 0.05 Standard Error for Comparison 5.535E-03 TO 6.392E-03
Critical Q Value 4.346 Critical Value for Comparison 0.0170 TO 0.0196
There are 5 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of LiP for Substrate

Substrate	Mean	Homogeneous Groups
CCA	2.3400	A
WSA	2.3043	B
SBA	1.6433	C

Alpha 0.05 Standard Error for Comparison 4.032E-03
Critical Q Value 3.419 Critical Value for Comparison 9.747E-03
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of LiP for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN3	WSA	2.5200	A
KN1-GL	CCA	2.3900	B
KN3	CCA	2.3867	B
KN1-GL	SBA	2.3800	B
KN1-KN3	CCA	2.3333	C
KN1	WSA	2.3300	C
KN5	CCA	2.3300	C
KN1	CCA	2.3300	C
KN1-KN3	SBA	2.3233	CD
GL	SBA	2.3133	CDE
GL	CCA	2.3067	CDE
KN1-KN5	CCA	2.3033	CDE
KN5	WSA	2.2833	DEF
KN1-KN5	WSA	2.2800	EFG
KN1	SBA	2.2567	FGH
KN1-KN3	WSA	2.2400	GH
GL	WSA	2.2400	GH
KN1-GL	WSA	2.2367	H
KN3	SBA	2.2300	H
KN5	SBA	2.22E-15	I
KN1-KN5	SBA	1.55E-15	I

Alpha 0.05 Standard Error for Comparison 7.828E-03 TO 0.0111
Critical Q Value 5.330 Critical Value for Comparison 0.0295 TO

0.0417
There are 9 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Fungi

Fungi	Mean	Homogeneous Groups
KN1-GL	0.9700	A
KN1-KN3	0.9478	B
GL	0.9056	C
KN1-KN5	0.8622	D
KN3	0.7356	E
KN1	0.6700	F
KN5	0.4756	G

Alpha 0.05 Standard Error for Comparison 4.940E-03 TO 5.704E-03
Critical Q Value 4.346 Critical Value for Comparison 0.0152 TO 0.0175
All 7 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Substrate

Substrate	Mean	Homogeneous Groups
WSA	2.1219	A
CCA	0.1510	B
SBA	0.1129	C

Alpha 0.05 Standard Error for Comparison 3.599E-03
Critical Q Value 3.419 Critical Value for Comparison 8.699E-03
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Fungi*Substrate

Fungi	Substrate	Mean	GL,CCA	GL,SBA	GL,WSA	KN1,CCA
GL	CCA	0.1733				
GL	SBA	0.1533	0.0200			
GL	WSA	2.3900	2.2167*	2.2367*		
KN1	CCA	0.1333	0.0400*	0.0200	2.2567*	
KN1	SBA	0.1533	0.0200	1.67E-16	2.2367*	0.0200
KN1	WSA	1.7233	1.5500*	1.5700*	0.6667*	1.5900*
KN1-GL	CCA	0.1500	0.0233	3.33E-03	2.2400*	0.0167
KN1-GL	SBA	0.1700	3.33E-03	0.0167	2.2200*	0.0367*
KN1-GL	WSA	2.5900	2.4167*	2.4367*	0.2000*	2.4567*
KN1-KN3	CCA	0.1433	0.0300	0.0100	2.2467*	1.00E-02
KN1-KN3	SBA	0.1500	0.0233	3.33E-03	2.2400*	0.0167
KN1-KN3	WSA	2.5500	2.3767*	2.3967*	0.1600*	2.4167*
KN1-KN5	CCA	0.1300	0.0433*	0.0233	2.2600*	3.33E-03
KN1-KN5	SBA	8.33E-17	0.1733*	0.1533*	2.3900*	0.1333*
KN1-KN5	WSA	2.4567	2.2833*	2.3033*	0.0667*	2.3233*
KN3	CCA	0.1667	6.67E-03	0.0133	2.2233*	0.0333*
KN3	SBA	0.1633	1.00E-02	0.0100	2.2267*	0.0300
KN3	WSA	1.8767	1.7033*	1.7233*	0.5133*	1.7433*
KN5	CCA	0.1600	0.0133	6.67E-03	2.2300*	0.0267
KN5	SBA	2.64E-16	0.1733*	0.1533*	2.3900*	0.1333*
KN5	WSA	1.2667	1.0933*	1.1133*	1.1233*	1.1333*

Fungi	Substrate	Mean	KN1,SBA	KN1,WSA	CCA	SBA
KN1	WSA	1.7233	1.5700*			
KN1-GL	CCA	0.1500	3.33E-03	1.5733*		
KN1-GL	SBA	0.1700	0.0167	1.5533*	0.0200	

KN1-GL	WSA	2.5900	2.4367*	0.8667*	2.4400*	2.4200*
KN1-KN3	CCA	0.1433	1.00E-02	1.5800*	6.67E-03	0.0267
KN1-KN3	SBA	0.1500	3.33E-03	1.5733*	2.50E-16	0.0200
KN1-KN3	WSA	2.5500	2.3967*	0.8267*	2.4000*	2.3800*
KN1-KN5	CCA	0.1300	0.0233	1.5933*	0.0200	0.0400*
KN1-KN5	SBA	8.33E-17	0.1533*	1.7233*	0.1500*	0.1700*
KN1-KN5	WSA	2.4567	2.3033*	0.7333*	2.3067*	2.2867*
KN3	CCA	0.1667	0.0133	1.5567*	0.0167	3.33E-03
KN3	SBA	0.1633	0.0100	1.5600*	0.0133	6.67E-03
KN3	WSA	1.8767	1.7233*	0.1533*	1.7267*	1.7067*
KN5	CCA	0.1600	6.67E-03	1.5633*	0.0100	1.00E-02
KN5	SBA	2.64E-16	0.1533*	1.7233*	0.1500*	0.1700*
KN5	WSA	1.2667	1.1133*	0.4567*	1.1167*	1.0967*

Fungi	**Substrate**	**Mean**	**WSA**	**CCA**	**SBA**	**WSA**
KN1-KN3	CCA	0.1433	2.4467*			
KN1-KN3	SBA	0.1500	2.4400*	6.67E-03		
KN1-KN3	WSA	2.5500	0.0400*	2.4067*	2.4000*	
KN1-KN5	CCA	0.1300	2.4600*	0.0133	0.0200	2.4200*
KN1-KN5	SBA	8.33E-17	2.5900*	0.1433*	0.1500*	2.5500*
KN1-KN5	WSA	2.4567	0.1333*	2.3133*	2.3067*	0.0933*
KN3	CCA	0.1667	2.4233*	0.0233	0.0167	2.3833*
KN3	SBA	0.1633	2.4267*	0.0200	0.0133	2.3867*
KN3	WSA	1.8767	0.7133*	1.7333*	1.7267*	0.6733*
KN5	CCA	0.1600	2.4300*	0.0167	0.0100	2.3900*
KN5	SBA	2.64E-16	2.5900*	0.1433*	0.1500*	2.5500*
KN5	WSA	1.2667	1.3233*	1.1233*	1.1167*	1.2833*

Fungi	**Substrate**	**Mean**	**CCA**	**SBA**	**WSA**	**KN3,CCA**
KN1-KN5	SBA	8.33E-17	0.1300*			
KN1-KN5	WSA	2.4567	2.3267*	2.4567*		
KN3	CCA	0.1667	0.0367	0.1667*	2.2900*	
KN3	SBA	0.1633	0.0333	0.1633*	2.2933*	3.33E-03
KN3	WSA	1.8767	1.7467*	1.8767*	0.5800*	1.7100*
KN5	CCA	0.1600	0.0300	0.1600*	2.2967*	6.67E-03
KN5	SBA	2.64E-16	0.1300*	1.80E-16	2.4567*	0.1667*
KN5	WSA	1.2667	1.1367*	1.2667*	1.1900*	1.1000*

Fungi	**Substrate**	**Mean**	**KN3,SBA**	**KN3,WSA**	**KN5,CCA**	**KN5,SBA**
KN3	WSA	1.8767	1.7133*			
KN5	CCA	0.1600	3.33E-03	1.7167*		
KN5	SBA	2.64E-16	0.1633*	1.8767*	0.1600*	
KN5	WSA	1.2667	1.1033*	0.6100*	1.1067*	1.2667*

Alpha 0.05 Standard Error for Comparison 6.986E-03 TO 9.880E-03
Critical Q Value 5.330 Critical Value for Comparison 0.0263 TO 0.0372
The homogeneous group format can't be used
because of the pattern of significant differences.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Fungi

Fungi	**Mean**	**Homogeneous Groups**
KN1-KN5	0.1789	A
KN1-GL	0.1722	B
GL	0.1000	C
KN3	0.0700	D
KN1-KN3	0.0667	E
KN5	0.0433	F
KN1	0.0122	G

Alpha 0.05 Standard Error for Comparison 8.694E-04 TO 1.004E-03
Critical Q Value 4.346 Critical Value for Comparison 2.672E-03 TO 3.085E-03
All 7 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Substrate

Substrate	Mean	Homogeneous Groups
WSA	0.2552	A
CCA	0.0124	B
SBA	0.0081	C

Alpha 0.05 Standard Error for Comparison 6.333E-04
Critical Q Value 3.419 Critical Value for Comparison 1.531E-03
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Lacc for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN1-KN5	WSA	0.5167	A
KN1-GL	WSA	0.4800	B
GL	WSA	0.2800	C
KN3	WSA	0.1900	D
KN1-KN3	WSA	0.1600	E
KN5	WSA	0.1300	F
KN1	WSA	0.0300	G
KN1-GL	CCA	0.0267	G
KN1-KN3	SBA	0.0200	H
KN1-KN3	CCA	0.0200	H
KN1-KN5	CCA	0.0200	H
KN3	CCA	0.0100	I
KN3	SBA	0.0100	I
GL	CCA	0.0100	I
GL	SBA	0.0100	I
KN1-GL	SBA	0.0100	I
KN1	SBA	6.67E-03	I
KN1	CCA	2.50E-16	J
KN5	CCA	1.67E-16	J
KN5	SBA	8.67E-17	J
KN1-KN5	SBA	1.39E-17	J

Alpha 0.05 Standard Error for Comparison 1.230E-03 TO 1.739E-03
Critical Q Value 5.330 Critical Value for Comparison 4.634E-03 TO 6.553E-03
There are 10 groups (A, B, etc.) in which the means
are not significantly different from one another.

Dual Cultures Analysis of Variance of the Enzyme Activities involving invasion/replacement by *Coriolopsis sp.* KN6

Factorial AOV Table for LiP

Source	DF	SS	MS	F	P
Replicate	2	0.0004	0.00021		
Fungi	2	4.5978	2.29890	3412.80	0.0000
Substrate	2	14.1798	7.08991	10525.23	0.0000
Fungi*Substrate	4	6.7218	1.68046	2494.71	0.0000
Error	16	0.0108	0.00067		
Total	26	25.5107			

Grand Mean 1.8144
CV 1.43

Factorial AOV Table for MnP

Source	DF	SS	MS	F	P
Replicate	2	0.0006	0.0003		
Fungi	2	0.8782	0.4391	3039.77	0.0000
Substrate	2	20.3313	10.1656	70377.46	0.0000
Fungi*Substrate	4	1.5097	0.3774	2612.96	0.0000
Error	16	0.0023	0.0001		
Total	26	22.7221			

Grand Mean 0.7289
CV 1.65

Factorial AOV Table for Lacc

Source	DF	SS	MS	F	P
Replicate	2	9.041E-34	4.521E-34		
Fungi	2	4.667E-04	2.333E-04	2.6197830043625744E30	0.0000
Substrate	2	0.09647	0.04823	5.41546572473235392E32	0.0000
Fungi*Substrate	4	5.333E-04	1.333E-04	1.49701885963575296E30	0.0000
Error	16	1.425E-33	8.907E-35		
Total	26	0.09747			

Grand Mean 0.0489

WARNING: The model error mean square is too small to continue.
The model may fit the data exactly.

Tukey HSD All-Pairwise Comparisons of Enzyme Activities and Substrates involving invasion/replacement by *Coriolopsis* sp. KN6

Tukey HSD All-Pairwise Comparisons Test of LiP for Fungi

Fungi	Mean	Homogeneous Groups
KN6	2.3978	A
KN5	1.5378	B
KN5-KN6	1.5078	B

Alpha 0.05 Standard Error for Comparison 0.0122

Critical Q Value 3.651 Critical Value for Comparison 0.0316
There are 2 groups (A and B) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of LiP for Substrate

Substrate	Mean	Homogeneous Groups
CCA	2.3522	A
WSA	2.3011	B
SBA	0.7900	C

Alpha 0.05 Standard Error for Comparison 0.0122
Critical Q Value 3.651 Critical Value for Comparison 0.0316
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of LiP for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN6	WSA	2.4200	A
KN6	CCA	2.4033	AB
KN6	SBA	2.3700	ABC
KN5	CCA	2.3300	BCD
KN5-KN6	CCA	2.3233	CD
KN5	WSA	2.2833	D
KN5-KN6	WSA	2.2000	E
KN5	SBA	0.0000	F
KN5-KN6	SBA	0.0000	F

Alpha 0.05 Standard Error for Comparison 0.0212
Critical Q Value 5.035 Critical Value for Comparison 0.0754
There are 6 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Fungi

Fungi	Mean	Homogeneous Groups
KN5-KN6	0.8811	A
KN6	0.8300	B
KN5	0.4756	C

Alpha 0.05 Standard Error for Comparison 5.666E-03
Critical Q Value 3.651 Critical Value for Comparison 0.0146
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Substrate

Substrate	Mean	Homogeneous Groups
WSA	1.9544	A
CCA	0.1711	B
SBA	0.0611	C

Alpha 0.05 Standard Error for Comparison 5.666E-03
Critical Q Value 3.651 Critical Value for Comparison 0.0146
All 3 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of MnP for Fungi*Substrate

Fungi	Substrate	Mean	Homogeneous Groups
KN5-KN6	WSA	2.4767	A
KN6	WSA	2.1200	B
KN5	WSA	1.2667	C

KN6	CCA	0.1867	D
KN6	SBA	0.1833	D
KN5-KN6	CCA	0.1667	D
KN5	CCA	0.1600	D
KN5	SBA	0.0000	E
KN5-KN6	SBA	0.0000	E

Alpha	0.05	Standard Error for Comparison	9.813E-03
Critical Q Value	5.035	Critical Value for Comparison	0.0349

There are 5 groups (A, B, etc.) in which the means
are not significantly different from one another