ASSESSMENT OF WOOD DEGRADATION BY *PYCNOPORUS SANGUINEUS* WHEN CO-CULTURED WITH SELECTED FUNGI.

By

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DECLARATION

I, the undersigned declare that the work contained in this thesis is my own original work and has not in its entirety or part been submitted at any university for a degree.

SIGNITURE: ..... DATE: .....

A. VAN HEERDEN

Summary

It is commonly known that a diversity of fungi, including yeasts, may occur on plant surfaces. Similarly, on fallen trees an ecological succession of different fungal species is known to occur during wood degradation. Some of these fungi may be pioneer fungi contributing to the initial degradation process, while others may be yeasts associated with the fruiting bodies of macro-fungi which in turn are able to utilize the more recalcitrant polymers in wood. Previously, it was revealed that an increase occurs in the wood degradation rate of certain white-rot fungi when co-cultured with selected yeast species.

A well known inhabitant of decomposing trees is the white rot fungus *Pycnoporus sanguineus.* It was found by some that this fungus is capable of selective delignification while growing on the wood of poplar trees, while other authors found a simultaneous delignification pattern on *Eucalyptus grandis* trees. In the latter case cellulose and lignin are degraded simultaneously.

We were interested in how yeasts occurring on the surface of *P. sanguineus* fruiting bodies, and the pioneer fungus *Aspergillus flavipes,* impact on wood degradation by this white-rot fungus. Restriction Fragment Length Polymorphisms (RFLP) analyses were used to obtain an indication of the species composition of the culturable yeast community associated with fruiting bodies of *P. sanguineus*. The impact of the most dominant of these yeasts species, i.e. *Pichia guilliermondii* and *Rhodotorula glutinis*, as well as *A. flavipes,* on wood degradation by *P. sanguineus* was then determined by analyzing the major wood components after growth of co-cultures on hot water washed *E. grandis* wood chips. Co-cultures of *P. sanguineus* with the other fungi were prepared by inoculating the wood chips, contained in solid state bioreactors and supplemented with molasses and urea, with the an appropriate volume of fungal inoculum, resulting in an initial moisture content of 60%. After two weeks of incubation at 30°C with constant aeration, the chips were harvested. Standard

protocol (TAPPI Standard Methods), commonly used by the paper and pulp industry, were then employed to determine the percentage cellulose, Klason Lignin, as well as polar and solvent-borne extractives in the chips. The resulting data were analyzed using box plots, as well as biplots. No degradation of Klason lignin was observed, while the percentage cellulose did decrease during fungal degradation. Taking into account the inherent shortcomings of the Klason Lignin determination, the results supported the findings of others that *P. sanguineus* shows a simultaneous delignification pattern while growing on *E. grandis* wood. In addition, it was found that the yeasts played no significant role in the degradation ability of *P. sanguineus*, while *A. flavipes* showed an antagonistic effect on *P. sanguineus* with respect to cellulose degradation. However, it was clear that the analytical methods used in this study were inadequate to accurately determine fungal degradation of wood. In addition, it was obvious that the methods used did not distinguish between fungal biomass and wood components. Nevertheless, the methods provided us with a fingerprint of each culture growing on *E. grandis* wood, allowing us to compare the chemical composition of the different cultures and the un-inoculated hot water washed wood chips. The question, therefore, arose whether the effect of a particular co-culture, on the chemical composition of wood, differs between tree species. Consequently, chemical alterations in different tree species, induced by a *P. sanguineus* / *A. flavipes* co-culture, were investigated in the next part of the study. Wood chips originating from four tree species, i.e. *Acacia mearnsii, Eucalyptus dunnii, E. grandis,* and *Eucalyptus macarthurii*, were inoculated with this co-culture. The culture conditions and subsequent analyses of the wood components were the same as in the first part of the study. From the box- and biplots constructed from the resulting data, it was clear that the chemical composition of each tree species were altered in a different manner by the co-culture. Lignin content showed an apparent increase in *A. mearnsii*, while *E. dunnii* showed a decrease in cellulose content. The results indicate that wood of different tree species are degraded in a different manner and this phenomenon should be taken into account in selecting fungi for biopulping.

Samevatting

Dit is algemeen bekend dat 'n verskeidenheid fungi, insluitend giste, op plantoppervlaktes mag voorkom. Dit is ook bekend dat 'n ekologiese opeenvolging van verskillende fungusspesies tydens hout-afbraak op omgevalle bome voorkom. Van hierdie fungi mag pionierfungi wees wat bydra tot die aanvanklike afbraakproses, terwyl ander giste mag wees wat geassosieer word met die vrugliggame van makro-fungi, wat op hul beurt weer in staat is om die meer weerstandbiedende polimere in hout te benut. Dit is voorheen bekendgemaak dat daar 'n toename plaasvind in die tempo van houtafbraak deur sekere witvrot-fungi wanneer dit in ko-kulture met geselekteerde gisspesies voorkom.

'n Bekende bewoner van verrottende bome is die wit-vrotfungus *Pycnoporus sanguineus.* Dit is gevind dat hierdie fungus tot selektiewe delignifikasie in staat is terwyl dit op die hout van populierbome groei, terwyl ander outeurs 'n gelyktydige patroon van delignifisering op *Eucalyptus grandis* bome gevind het. In laasgenoemde geval is sellulose en lignien gelyktydig afgebreek.

Ons was geïnteresseerd in die effek van giste op die oppervlak van vrugliggame van *P. sanguineus*, en die pionierfungus *Aspergillus flavipes,* op die houtafbraak deur hierdie wit-vrotfungus. Restriction Fragment Length Polymorphisms (RFLP) analises is gevolglik gebruik om 'n aanduiding te kry van die spesiesamestelling van die kweekbare gisgemeenskap wat met die vrugliggame van *P. sanguineus* geassosieer word. Die impak van die mees dominante van hierdie gisspesies, naamlik *Pichia guilliermondii* en *Rhodotorula glutinis*, asook *A. flavipes,* op houtafbraak deur *P. sanguineus* is voorts bepaal deur die analise van die belangrikste houtkomponente na die kweek van ko-kulture op *E. grandis* houtskyfies wat met warm water gewas is. Ko-kulture van *P. sanguineus* met die ander fungi is voorberei deur die houtskyfies in vaste fase bioreaktore, aangevul met melasse en ureum, te inokuleer met 'n toepaslike volume van die fungus-

inokulum om 'n aanvanklike voginhoud van 60% te verkry. Na twee weke se inkubasie by 30°C met konstante belugting is die skyfies ge-oes. Standaard protokol (TAPPI Standard Methods), algemeen deur die papier en pulpindustrie gebruik, is ingespan om die persentasie sellulose, Klason Lignien, asook polêre en oplosmiddel-gedraagde ekstrakte in die skyfies te bepaal. Die gevolglike data is geanaliseer deur gebruik te maak van box plots en biplots. Daar is geen afbraak van Klason Lignien bespeur nie, terwyl die persentasie sellulose wel toegeneem het tydens fungus degradasie. Met die inherente tekortkominge van die Klason Lignien bepaling inaggenome, het die resultate die bevindings ondersteun van andere wat getoon het dat *P. sanguineus* 'n gelyktydige delignifikasiepatroon openbaar terwyl dit op *E. grandis* hout groei. Daarby is dit gevind dat die giste geen beduidende rol in die afbraakvermoeë van *P. sanguineus* gespeel het nie, terwyl *A. flavipes* 'n antagonisiese effek ten opsigte van die sellulose degradering van *P. sanguineus* getoon het. Dit was egter duidelik dat die analitiese metodes wat in hierdie studie gebruik is, onvoldoende was om die degradering van hout akkuraat te bepaal. Daarby was dit duidelik dat die metodes nie tussen fungus biomassa en houtkomponente kon onderskei nie. Nogtans het die metodes 'n vingerafdruk verskaf van elke kultuur wat op *E. grandis* hout groei, wat ons toegelaat het om die chemiese samestelling van die verskillende kulture en die ongeïnokuleerde, met warm water gewasde houtskyfies te vergelyk. Die vraag het gevolglik ontstaan of die effek van 'n bepaalde ko-kultuur op die chemiese samestelling van hout van boomspesie tot boomspesie verskil. Gevolglik is die chemiese wisselinge in verskillende boomspesies, geïnduseer deur 'n *P. sanguineus* / *A. flavipes* ko-kultuur, in die volgende gedeelte van die studie ondersoek. Houtskyfies van vier boomspesies, naamlik *Acacia mearnsii, Eucalyptus dunnii, E. grandis,* en *Eucalyptus macarthurii*, is met hierdie ko-kultuur geïnokuleer. Die kultuurkondisies en daaropvolgende analises van die houtkomponente was dieselfde as in die eerste deel van die studie. Van die box- en biplots wat van die resultate getrek is, is dit duidelik dat die chemiese samestelling van elke boomspesie op 'n verskillende manier deur die ko-kulture verander is. Lignien-inhoud het ’n waarskynlike

toename getoon in *A. mearnsii*, terwyl *E. dunnii* 'n afname in sellulose-inhoud getoon het. Die resultate toon dat hout van verskillende boomspesies op verskillende maniere afgebreek word en dat hierdie fenomeen in aanmerking geneem moet word wanneer fungi vir bioverpulping geselekteer word.

This thesis is dedicated to my parents, Tjokkie and Nobie.
Thank you for all your love and support. You are the best parents anyone could ask for.

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CONTENTS

Chapter 1

Introduction

1.1 Motivation

Basidiomycetous white-rot fungi play a pivotal role in forest ecosystems (Otjen & Blanchette, 1986; Myneni *et al*., 2001). They are the only fungal group capable of degrading all three chemical constituents of wood, namely cellulose, hemicellulose and lignin. White-rot fungi secrete hydrolases that target cellulose and hemicellulose, while lignin degradation requires more complex enzymes such as lignin peroxidase, manganese peroxidase, and laccase (Zabel & Morrell, 1992; Leonowicz *et al*., 1999). Two wood degradation patterns are known to occur in this group of fungi. The fungus can either degrade all three chemical components simultaneously, or select for the degradation of lignin (Schwarze *et al*., 2000). The latter is important for the paper and pulp industry, as the residual cellulose fibers are the main component for paper. Not surprisingly, many researchers have embarked upon studying the so-called biopulping process (Akhtar *et* al., 1993; Luna *et al*., 2004). The main drive behind this research was to reduce costs of chemicals needed and the resulting pollution during the pulping process (Guitiérrez *et al*., 1999).

The majority of these investigations however, focused on the degradation of wood from a single tree species by pure cultures of white-rot fungi (Luna *et al*., 2004) and very few studied the effect of co-cultures on wood degradation. The latter scenario would be closer to the situation in nature, where consortia of microbes are known to degrade lignocellulosic material (Watanabe *et al*., 2003). Studies conducted by Blanchette and co-workers in 1978, showed an increase in the degradation rate of certain white-rot fungi when co-cultured with selected bacterial and yeast species. When a co-culture of a known pioneer fungus of wood, *Aspergillus flavipes*, and a common white-rot fungus, *Pycnoporus sanguineus*, were evaluated in a biopulping process, it was found that the pulping properties of *E. grandis* were enhanced (Domisse, 1998).

Since it is known that fungal fruiting bodies may harbor yeasts (Kurtzman & Fell, 1998) we were interested in the identity of yeast populations occurring on the fruiting bodies of *P. sanguineus* and how these yeasts, as well *A. flavipes,* impact on the degradation of *E. grandis* wood by this white-rot fungus. Since contradicting results regarding the wood degradation pattern of *P. sanguineus* on different wood species exists in literature (Ferraz *et al*. 1998; Luna *et al*. 2004), this phenomenon needs to be further investigated.

With the above as background, the aim of this study was: 1) To characterize the natural yeast population on the fruiting bodies of a common white-rot fungus *Pycnoporus sanguineus* and to study the impact of yeasts originating from these fruiting bodies on the degradation of *Eucalyptus grandis* wood chips by this white-rot fungus. 2) To study the influence of wood species on the degradation pattern of *Pycnoporus sanguineus* when co-cultured with a known wood pioneer fungus.

1.2. Basidiomycetes and wood degradation

Basidiomycetes are regarded as the most important fungi that inhabit the forest floor (Otjen & Blanchette, 1986; Myneni *et al*., 2001). It is thought that their principle role within the forest ecosystem is to degrade woody material, since they are the only known fungi capable of degrading all the major cell wall components of wood (cellulose, hemicellulose, and lignin). These fungi however, may differ in the extent to which the different wood components are degraded and much research has been conducted to understand these wood degrading processes. Many of these studies were conducted on fungi that selectively degrade lignin resulting in residual cellulose components in the wood. The latter fungi thus found potential application in the pulping industry. To appreciate the role of these fungi in wood degradation, a better understanding of the general structure of wood is necessary.

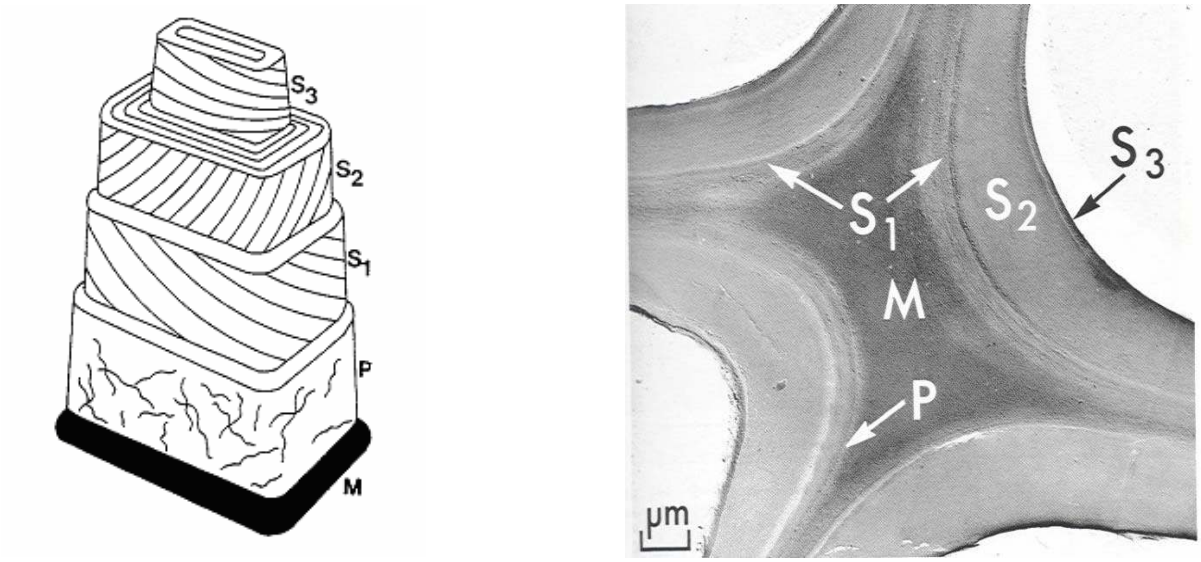
1.3. Wood tissue elements

Cells that compose wood differ in type and arrangement (Sjöström, 1993; Wiedenhoeft & Miller, 2005). These differences are used to classify wood as either soft-, or hardwood. Softwood has a simple basic structure, while hardwood is much more complex regarding cell morphology and functionality (Wiedenhoeft & Miller, 2005). Only two cell types occur in softwood. The first, called tracheids, is the major component of this type of wood serving a conductive and mechanical role. The second type is the parenchyma cells that may either be ray parenchyma or axial parenchyma. Parenchyma cells play an important role in the synthesis, storage and lateral transport of biochemicals.

Hardwoods have characteristic conducting cells called vessel elements (Wiedenhoeft & Miller, 2005). These cells are stacked on top op each other and connected with pores to form vessels. Other cell types like fibers only play a role in support and the amount of strength depends on the thickness of the fiber cell wall. Axial parenchyma cells in hardwoods also contain storage material and are either associated with the vessels (Paratracheal) or not (Apotracheal). Rays in hardwoods are more diverse than that found in softwoods and generally span more than one cell in width. Despite different tissue and cellular morphology, the cell walls of all the cell types mentioned above, contain a number of characteristic layers.

1.4. Cell wall structure.

A typical lignified cell wall consists of five cell-wall layers (fig. 1); the middle lamella (M) on the outer side, the primary wall (P), and a three–layer secondary wall consisting of the outer (S_1), middle (S_2) and inner (S_3) secondary cell wall layers on the inner side (Schwarze *et al.*, 2000). These layers differ in their fine structure, orientation of the microfibrils, and chemical composition.



(**a**) (**b**)

Figure 1. (a) A cell wall model showing the five different cell wall layers (Schwarze *et al*., 2000). (b) A transmission electron micrograph of earlywood tracheids showing the different layers of the cell wall. Scalebar = 1 µm. (Sjöström, 1993).

1.4.1. Middle lamella

The middle lamella (fig. 1) connects neighboring cells to allow for movement of biochemicals and water (Wiedenhoeft & Miller, 2005). This layer consists mainly of amorphous substances like pectin and lignin (Schwarze *et al*., 2000). Pectin acts as a cement-like substance for cell elements in non-woody organs, while lignin provides rigidness in the wood cell.

1.4.2. Primary wall

In general, the primary wall (fig. 1) in wood is thin and indistinguishable from the middle lamella (Schwarze *et al*., 2000; Wiedenhoeft & Miller, 2005). These two layers are, therefore, called the compound middle lamella. The primary wall consists of randomly orientated cellulose microfibrils providing strength to this layer.

1.4.3. Secondary wall

This three layer cell wall (fig. 1a), comprising of 94% cellulose, represents the largest part of the cell wall (Schwarze *et al*., 2000; Wiedenhoeft & Miller, 2005). Its primary function is to provide strength to the cell. The outer secondary wall (S_1) is a thin layer (0.2 µm thick in Birch fibers) next to the primary wall (fig. 1b). The cellulose fibers of this layer show a weak parallel arrangement to the longitudinal axis of the cell (Wiedenhoeft & Miller, 2005).

The middle secondary wall (S_2) forms the largest part of the secondary wall (1 to 5 µm thick in Spruce tracheids) and the most important in establishing the properties of the cell. The fibrils are arranged parallel to each other in a spiral in the direction of the cell's longitudinal axis. This layer has a low lignin and high cellulose content and it was found to be the preferred substrate for brown and soft rot fungi as these two groups can only degrade cellulose (Schwarze *et al*., 2000; Wiedenhoeft & Miller, 2005).

The inner secondary wall (S_3) is a relatively thin layer (0.1 to 0.15 µm in spruce tracheids) and separates the cell wall from the lumen (Schwarze *et al*., 2000). The arrangement of the microfibrils in this layer resembles those of the primary cell wall. The inner secondary cell wall has the lowest percentage of lignin compared to the other layers of the secondary wall (Wiedenhoeft & Miller, 2005). The reason for this low lignin content may be found in the basic physiology of a tree. Water needs significant adhesion to the cell walls to move upwards via transpiration and since lignin is a hydrophobic polymer, low concentrations in the S_3 layer will allow transpiration to occur.

1.5. Cell wall chemistry

It is obvious from the preceding paragraphs that cellulose, comprising ca. 45% (w/w) of wood, plays a pivotal role in the morphology of the wood cell (Rowell *et*

al., 2005). Individual cellulose molecules are arranged in bundles known as microfibrils which in turn are arranged in lamellae in the cell wall plane. Within these microfibrils, the cellulose has a crystalline appearance due to its highly ordered orientation. Despite the crystalline structure of some cellulose components, this carbohydrate homopolymer and hemicellulose, a carbohydrate heteropolymer, are readily utilized by many microorganisms. In contrast, lignin is an aromatic heteropolymer that consists of phenylpropane monomers and is only utilized by a few specialized fungal groups or bacteria. Hemicellulose and lignin are covalently bonded to one another and form a coating around the cellulose microfibrils. This coating protects the easily degradable cellulose from microbial attacks (Rayner & Boddy, 1988; Zabel & Morrell, 1992). Since most of the plant cell wall consists of cellulose, hemicellulose, and lignin, these polymers represent the majority of organic compounds in the biosphere and are the most important carbon sink in terrestrial ecosystems.

1.5.1. Cellulose

Cellulose is a long, linear homopolymer (fig. 2) consisting of β-D-glucose residues with (1→4) glucosidic linkages (Zabel & Morrell, 1992; Rowell *et al*., 2005). The anhydroglucose monomers on the surface of the cellulose molecules each contain three hydroxyl groups. These groups determine the physical and chemical properties of the wood, as well as the structural properties in the cell wall.

Cellulose molecules tend to form intra- and intermolecular hydrogen bonds. Crystalline regions (fig. 3) are then formed as the packing densities of cellulose increases. As much as 65 % of wood derived cellulose may be crystalline. Apart from being crystalline or non crystalline, cellulose may also be classified as accessible or non-accessible. This refers to the accessibility of the cellulose to water and microorganisms.

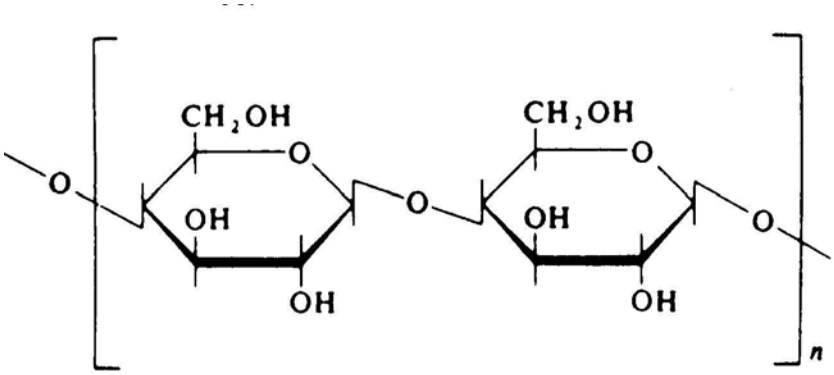


Figure 2. The structural formula of cellulose (Rayner & Boddy, 1988)

Crystalline cellulose is accessible on the surface but not inside the crystal. Non-crystalline cellulose is mostly accessible, but some, as already mentioned, are covered with hemicellulose and lignin rendering the molecule non-accessible.

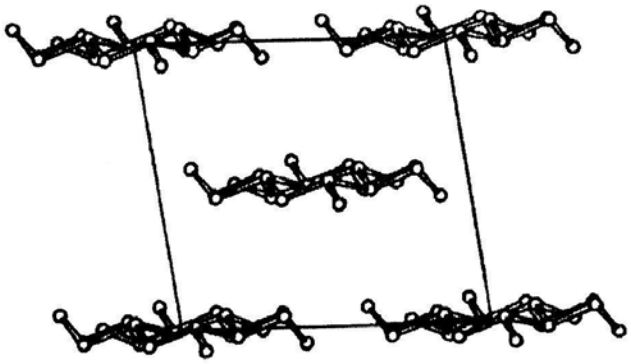


Figure 3. Crystalline structure of cellulose showing the planar orientation of the glucose monomers in relation to each other (Rowell *et al*., 2005).

1.5.2. Hemicellulose

Hemicellulose differs from cellulose as it consists of a shorter carbohydrate backbone containing other sugar monomers than just glucose, and side chains that can be branched (Rayner & Boddy, 1988; Rowell *et al*., 2005). The polymer backbone of hemicellulose consists mainly of D-xylopyranose, D-glucopyranose, D-galactopyranose, L-arabinofuranose, D-mannopyranose, D-

glucopyranosyluronic acid, and D-galactopyranosyluronic acid (fig. 4). More hemicellulose is present in hardwoods than in softwoods. Hemicelluloses in hardwoods are referred to as glucuronoxylan and are characterized by a backbone of D-xylopyranose monomers that are β-(1→4) linked to acetyl groups (Rowell *et al.*, 2005). In the backbone, side chains of 4-*O*-methylglucoronic acid monomers are linked to the xylan and substitute the xylan with intervals (fig. 5a).

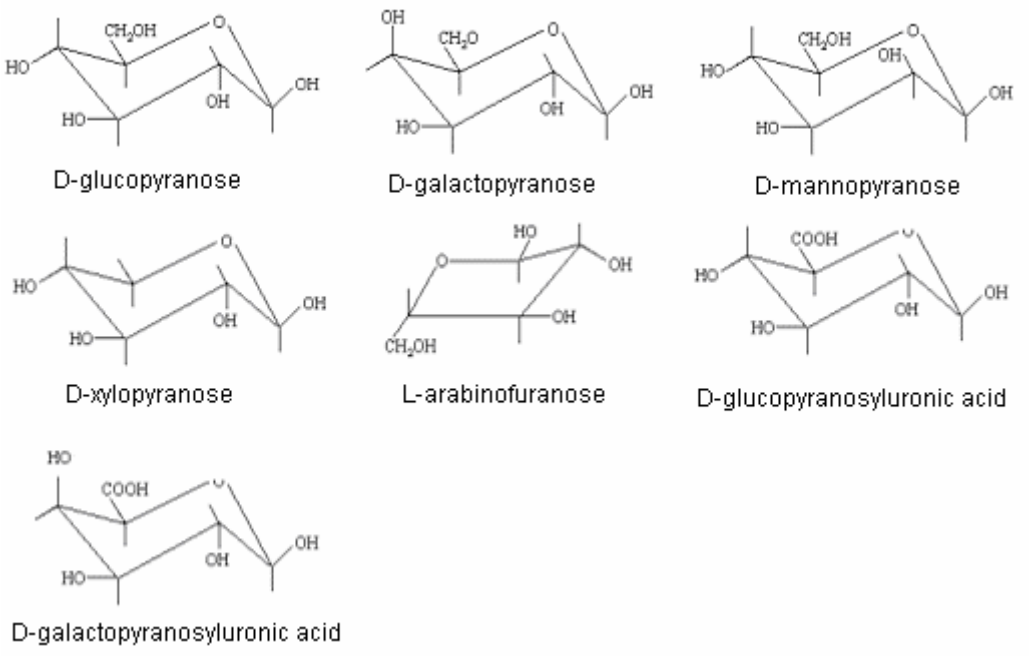


Figure 4. Structural monomers of hemicellulose (adapted from Resende, 2005).

In softwood, the hemicellulose consists of glucomannans and has a slightly branched chain with β-(1→4) linkages (fig. 5b). Another hemicellulose polymer in softwoods is an arabinoglucoronoxylan consisting of a backbone of β-(1→4) xylopyranose units and branches containing *D*-glucopyranosyluronic acid and *L*-arabinofuranose (Rowell *et al.*, 2005). The fact that hemicellulose has short chain lengths and is situated on the outer surface of the microfibrils may explain why these cell wall components are attacked first by decay fungi. Since this polymer coats the cellulose microfibrils, it possibly serves a structural role in cell walls.

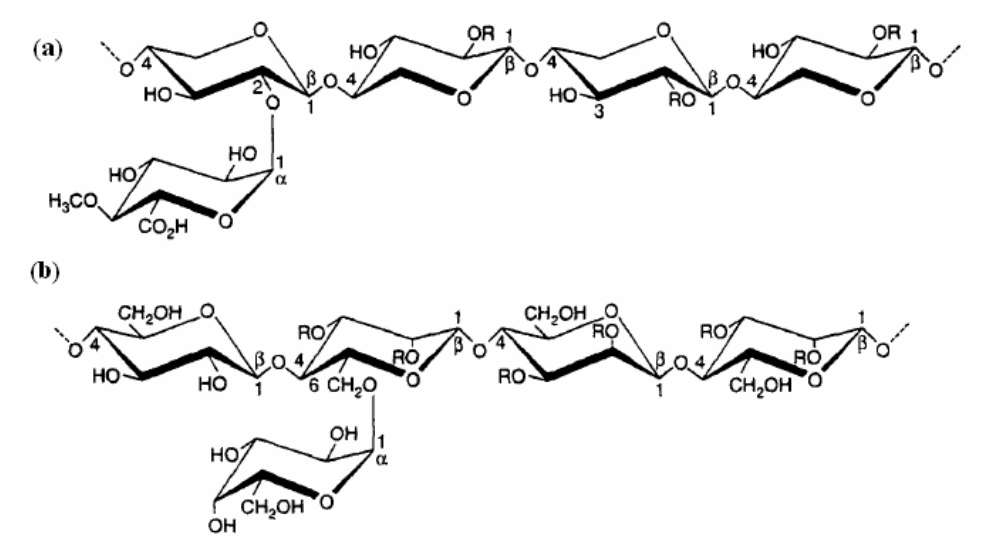


Figure 5. Partial chemical structures of the predominant hemicellulose polymers found in wood. (a) Structure of O-acetyl-4-O-methylglucuronoxylan, the major hemicellulose of hardwoods. (b) Structure of O-acetylgalactoglucomannan, the major hemicellulose of softwoods (Kirk & Cullen, 1998).

1.5.3. Lignin

Lignin occurs in all vascular plants and comprises 20 to 30 % of the wood cell wall (Zabel & Morrell, 1992). It protects the stem tissue and strengthens the plant. Lignin is a polyphenolic polymer consisting of phenylpropane units, (fig.6) and is the most complex of the plant cell wall constituents. Its monomers are held together by C-O-C and C-C linkages.

Different types of lignin are classified according to their structural elements, while lignin in general consists mainly of dimethoxylated (syringyl), monomethoxylated (guaiacyl), and non-methoxylated (*p*-hydroxyphenyl) phenylpropanoid monomers (Zabel & Morrell, 1992; Rowell *et al*, 2005). The precursors of lignin biosynthesis are *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (fig. 7).

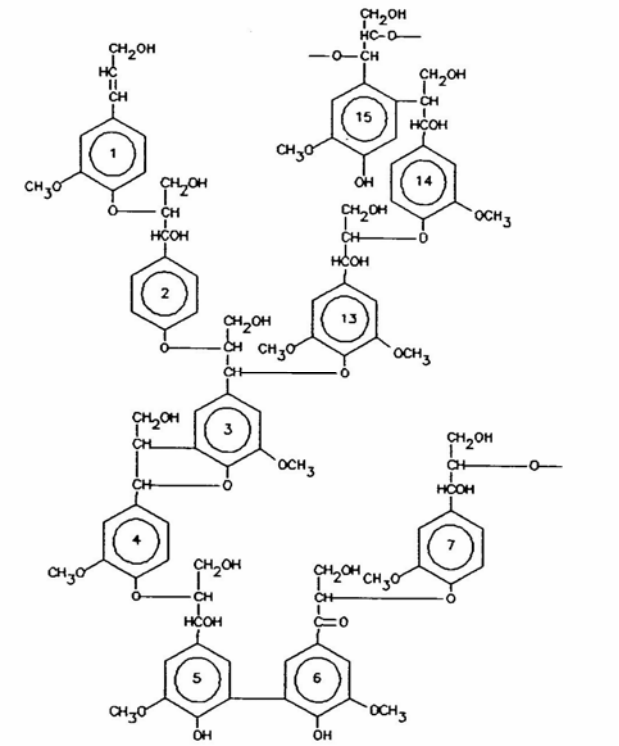


Figure 6. A structural model of lignin found in spruce (Zabel & Morrell, 1992).

These units are compressed by free radical polymerization to form the heterogeneous aromatic biopolymer (Rayner & Boddy, 1988). *P*-coumaryl is a minor precursor of soft- and hardwood lignins, coniferyl is the major precursor of softwood lignin, while coniferyl and sinapyl are both precursors of hardwood lignin.

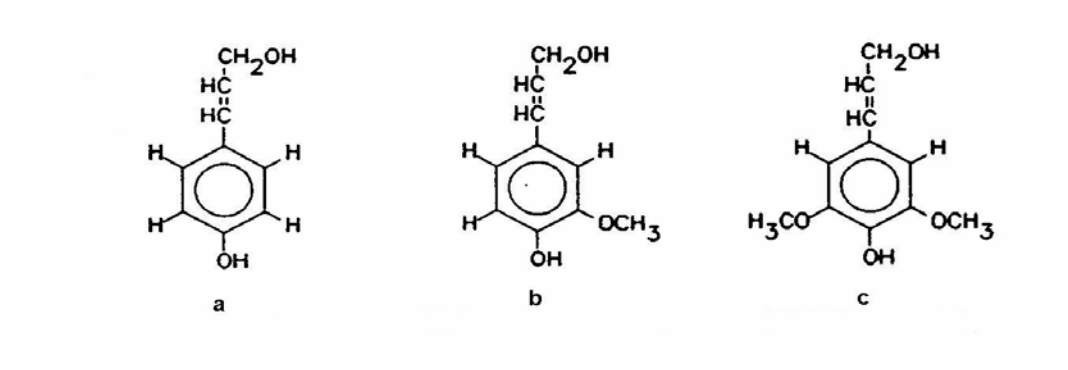


Figure 7. Chemical structures of lignin precursors. (a) *p*-coumaryl alcohol, (b) coniferyl alcohol, (c) sinapyl alcohol (Zabel & Morrell, 1992).

The highest concentration of lignin is found in the middle lamellae, but almost 70% of total lignin in wood is located in the secondary cell wall due to the difference in volume of middle lamella to secondary wall.

With the above as background, it is possible to construct a schematic illustration of the wood cell (fig. 8) showing the relative position of the main chemical components of the cell. Such an illustration is essential to explain the mechanics of fungal degradation of these cells.

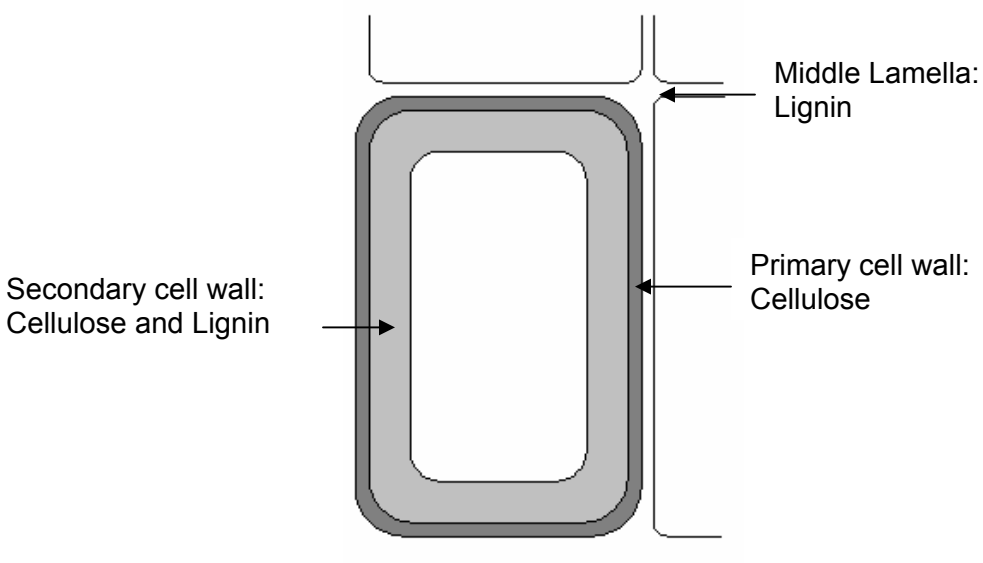


Figure 8. A schematic illustration of the wood cell showing the cell wall structures and their main chemical components.

1.6. Features of fungal wood decay.

Fungal decay of wood results in major economical losses and can be grouped into *brown, white,* and *soft* rots (Martínez *et al*., 2005). This classification is based on the properties and colors of the residual wood. In the case of brown-rot, the brownish colored lignin remains after decay. White rot is characterized by the white-colored cellulose that remains after decay. Soft-rot decay is characterized by surface softness of the wood. Both brown and white-rot fungi are

basidiomycetes that are able to overcome low nitrogen conditions, toxins and antibiotics present in wood. Soft-rot fungi are ascomycetes that are able to degrade wood under extreme environmental conditions such as high or low water potential.

1.6.1 Brown-rot

Brown-rot fungi grow mainly on softwoods and represent only 6% of the known wood-rotting Basidiomycetes (Schwarze *et al*., 2000). Common brown-rot fungi are listed in table 1. These fungi degrade carbohydrates in the cell wall at a distance from the hyphae by a diffusion mechanism leaving a modified, demethoxylated lignin residue behind. This diffusion mechanism is based on the ability of fungi to secrete hydrolases that use cellulose and hemicellulose as substrate (Zabel & Morrell, 1992).

Table 1. Fungal species that result in different decay patterns (Martínez *et* al., 2005).

Brown-rot	***Soft-rot***	***White-rot***
Gloeophyllum trabeum	*Ustulina deusta*	*Trametes versicolor*
Laetiporus sulphureus	*Alternaria alternata*	*Heterobasidium annosum*
Piptoporus betulinus	*Thielavia terrestris*	*Phlebia tremellosa*
Postia placenta		*Pycnoporus sanguineus*

The different stages of brown-rot, brought about by the synergistic action of a number of fungal enzymes, are illustrated in figure 9. Hydrogen peroxide, formed as the result of glucose oxidase, glyoxal oxidase, and aryl alcohol oxidase (Evans & Hedger, 2001), penetrates the cell wall and depolymerizes the lignocellulose matrix (Schwarze *et al*., 2000; Rayner & Boddy, 1988). This results in cellulose and hemicellulose being more accessible to fungal hydrolases.

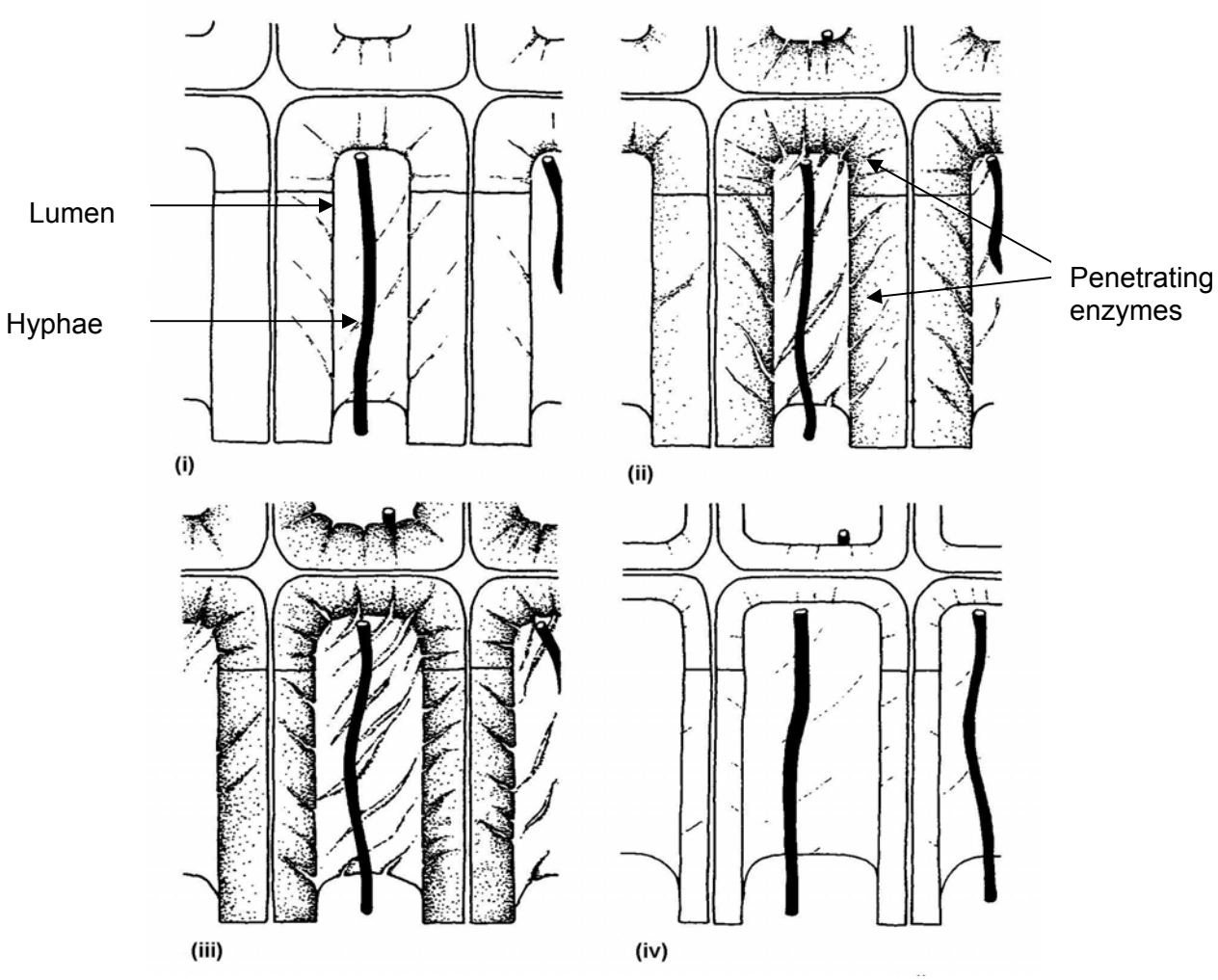


Figure 9. A schematic illustration of the stages of brown-rot. (i) The enzymes start to penetrate the cell wall from the lumen. (ii) The degree of degradation starts to increase as enzymes penetrated the secondary wall. (iii) Cracks appear in the cell wall and the volume of the latter starts to decrease. (iv) Only modified lignin remains at this stage of the degradation (Adapted from Schwarze *et al.*, 2000).

After separation of the cellulose chains, endo-1,4-β-glucanases cleave the cellulose molecule and 1,4-β-glucosidases transform the cellobiose to glucose. Due to the rapid depolymerization of carbohydrates, the water solubility of the lignocellulose may also increase during this stage of the degradation process. This type of wood depolymerization occurs more rapidly than the metabolization of the resulting degradation products. Consequently, the partially degraded lignocellulosic material and smaller degradation products become available to

scavenger fungi and bacteria on the wood. The final product of the decayed wood is brown, dry, brittle and powdery. This residue predominantly composes of modified lignin (Rayner & Boddy, 1988).

1.6.2. Soft-rot

Soft-rot is caused by a small group of fungi (Table 1) that mainly attacks hardwoods rendering it soft and crumbly (Rayner & Boddy, 1988; Schwarze *et al.*, 2000). Fungal species associated with this kind of wood degradation may vary in their effects on the cell wall, while sharing features of both white and brown-rot fungi.

Similar to brown-rot fungi, soft-rot fungi target the carbohydrates in the cell wall, but as with white-rot fungi they also contain oxidative enzyme systems (Rayner & Boddy, 1988). Soft-rot fungi however, are characterized by their preferred growth within the cellulose rich secondary cell wall where they form a series of successive cavities with conically shaped ends that follows the direction of the microfibrils in the S_2 layer (fig. 10).

Some soft-rot fungi are able to degrade cellulose using exo-1,4-β-glucanases, endo-1,4-β-glucanases, and 1,4-β-glucanases (Schwarze *et al.*, 2000). Other species do not utilize exo-1,4-β-glucanases and only degrade the amorphous cellulose zones in the microfibrils.

1.6.3. White-rot

The group of fungi resulting in this type of rot is able to degrade lignin, hemicellulose, and cellulose (Rayner & Boddy, 1988; Schwarze *et al.*, 2000). Ligninolytic fungi use hydrolases to produce monosaccharides from polysaccharide components in wood (Leonowicz *et al.*, 1999). However, when

these components are in a complex with lignin, hydrolytic breakdown does not occur. Thus, lignin appears to inhibit hydrolytic activity (Martínez *et al.*, 2005).

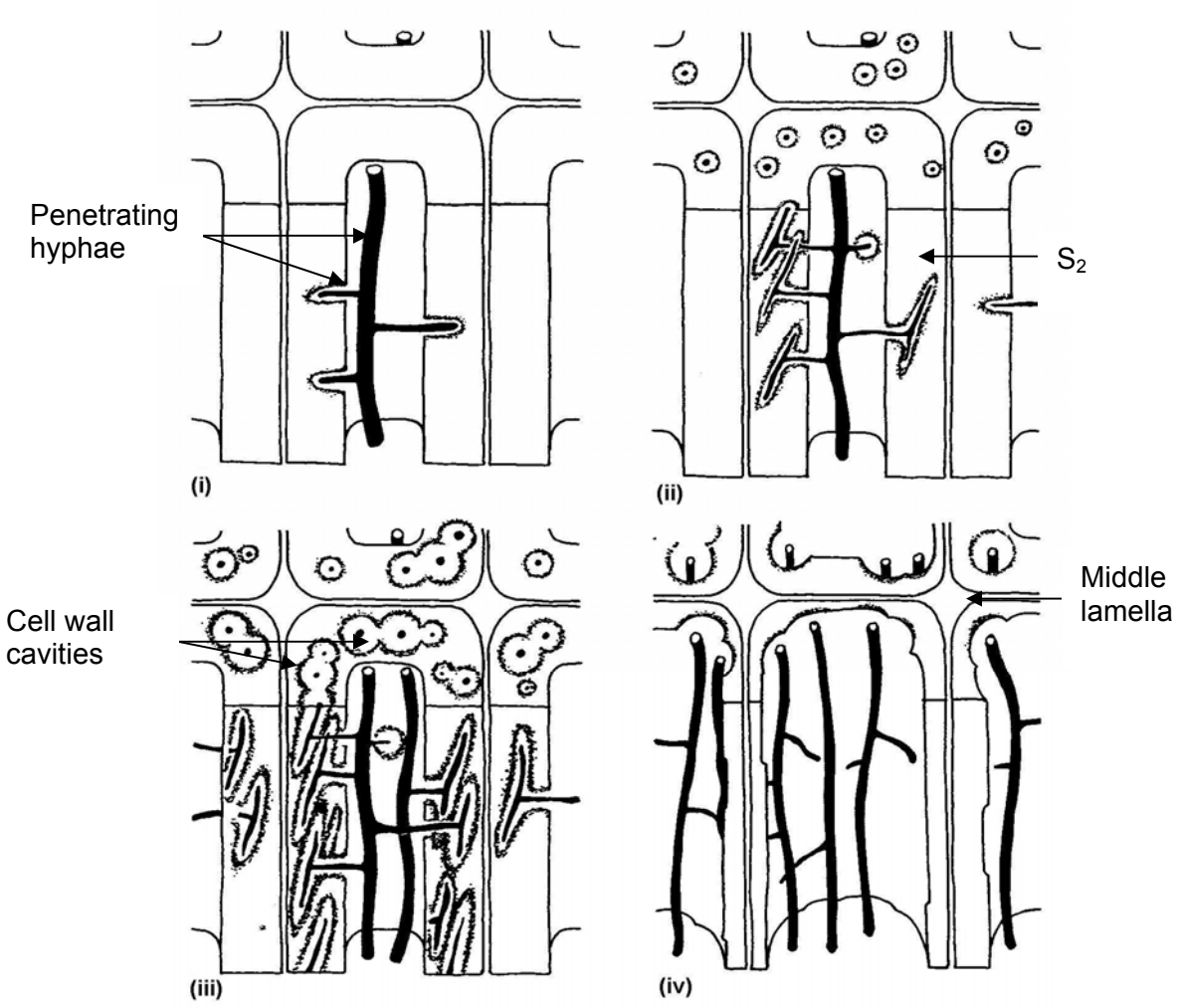


Figure 10. A schematic illustration of the stages of-soft rot. (i) The hyphae penetrate the lignified cell wall. (ii) Hyphae form branches parallel to the direction of the cellulose microfibrils in the S_2 layer. (iii) Cavities form in the cell wall due to degradation. (iv) Here the secondary wall is almost completely degraded, while the compound middle lamella stays intact (Adapted from Schwarze *et al.*, 2000).

Two patterns of lignin degradation have been identified. The first, selective delignification, occurs when lignin is degraded before hemicellulose and cellulose. This leads to dissolution of the middle lamella and defibrillation. The other type of lignin degradation is simultaneous delignification, where lignin and structural

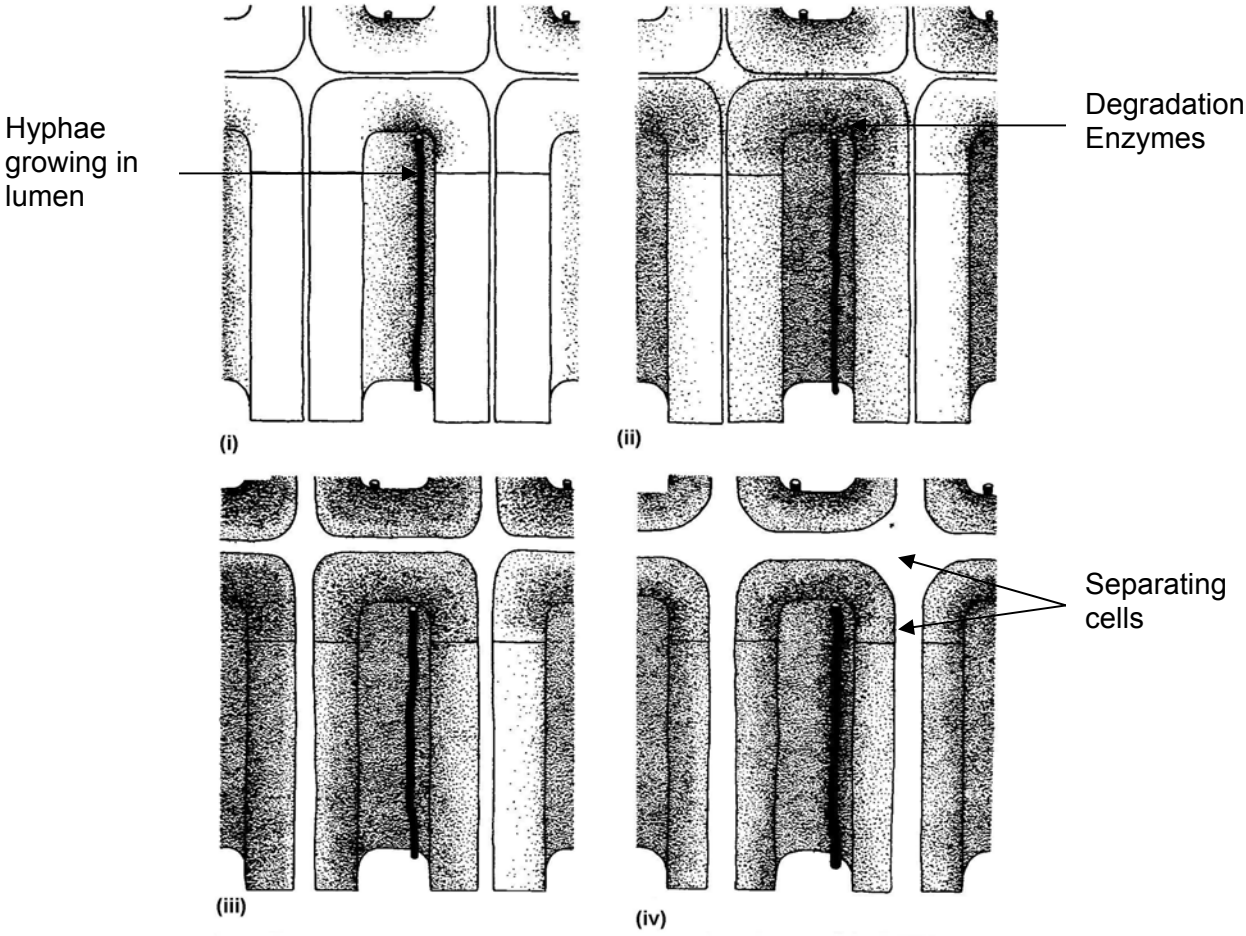
carbohydrates are removed at the same rate (Pandey & Pitman, 2003). This pattern occurs mainly on hardwoods while selective rot may occur on both hard and softwoods.

1.6.3.1. Selective delignification

During selective delignification lignin is the first wood component to be degraded. A typical example of this is the wood rot brought about by the fungus *Phellinus pini* (Schwarze *et al*., 2000). Firstly, the middle lamella is degraded together with the secondary wall (fig. 11). Later, individual cells will become separated from their matrix. This results in fibrous and stringy wood that has lost it stiffness and compression strength (fig. 13a). Cellulose is degraded at a slower rate than in brown or soft rot, and the reduction in wood strength is not as severe as in the latter two cases.

1.6.3.2. Simultaneous delignification

This type of white-rot occurs mainly on broad-leaved trees when the fungal enzymes are able to degrade all the main components of the lignified cell wall simultaneously (Schwarze *et al*., 2000). Degradation takes place in the immediate vicinity of the hyphae that grows in the lumen and leads to the formation of erosion channels (fig. 12). The degradation of the cell wall is enhanced by a biofilm coating around the hyphae that result in closer contact between the hyphae and the cell wall components (Lynd *et al*., 2002). The cell wall gradually becomes thinner from the inside out as degradation continues. In contrast to selective delignification, the wood in this case becomes brittle because of the degradation of the cellulose-rich secondary wall (fig. 13b). Regardless the pattern of lignin degradation in wood, the process of delignification is brought about by the action of three enzymes.



Figuur 11. A schematic illustration of the stages of selective delignification. (i) Hyphae grow in the lumen and the degradation enzymes diffuse into the secondary wall where lignin is degraded. (ii) Degradation of secondary wall lignin spreads to the middle lamella. (iii, iv) Later during lignin degradation, the individual cells separate from one another (Adapted from Schwarze *et al*., 2000).

1.6.4. Lignin degrading enzymes.

The pivotal enzymes in lignin degradation are, lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Zabel & Morrell, 1992). LiP and MnP were first discovered in *Phanerochaete chrysosporium* in the mid-1980s. These enzymes were described as true ligninases because of their high redox potential (Gold *et al*., 2000; Martínez, 2002).

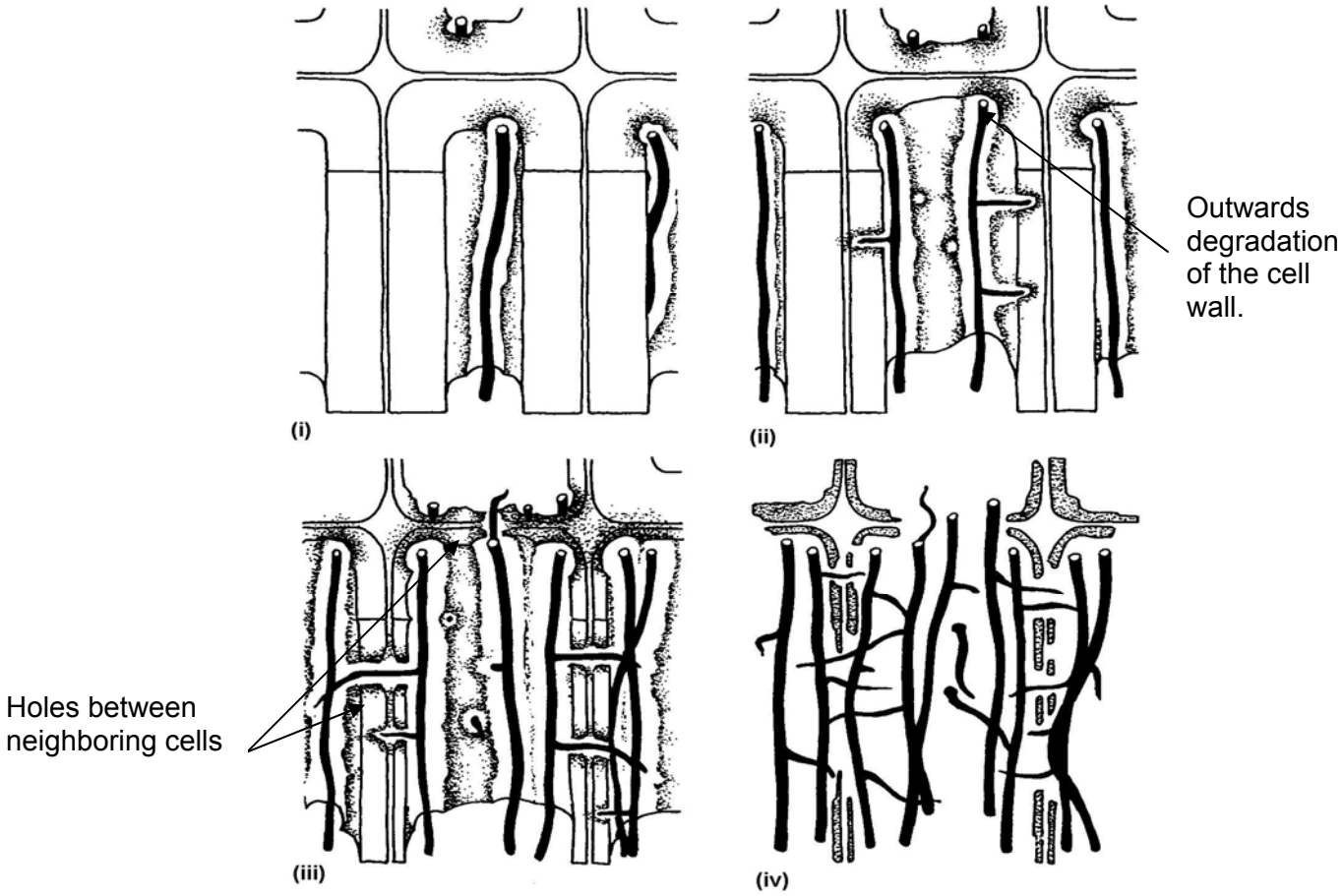


Figure 12. A schematic illustration of the stages of simultaneous delignification. (i) The degradation enzymes from the hyphae start to attack the cell wall in their immediate vicinity. (ii) The cell wall is degraded from the lumen outwards. (iii) The cell wall becomes thinner and holes appear between neighboring cells. (iv) At the final stage of degradation, the middle lamella and cell corners are degraded (Adapted from Schwarze *et al.*, 2000).

Other enzymes that are involved in lignin degradation are H_2O_2 generating oxidases, and mycelium associated dehydrogenases that reduce compounds derived from lignin (Gutiérrez *et al.*, 1994; Guillén *et al.*, 1997). LiP is able to degrade non-phenolic lignin units to aryl cation radicals which then use non enzymatic reactions to cleave C-C and C-O bonds. MnP on the other hand oxidize Mn^{2+} to Mn^{3+} (Jansen *et al.*, 1996). The latter then acts on phenolic or non-phenolic lignin units as a diffusible oxidizer via lipid peroxidation reactions.

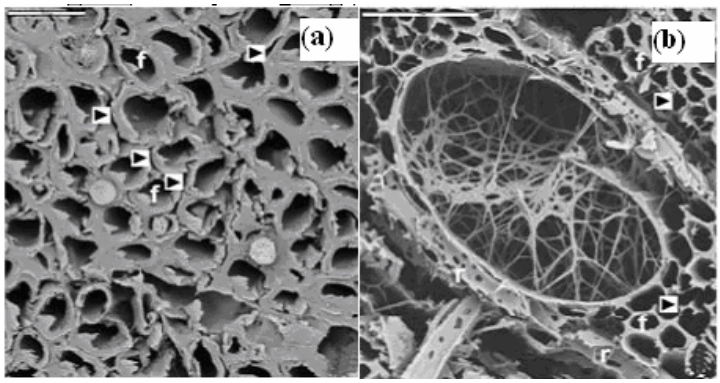


Figure 13. Scanning-electron microscopy images showing the wood anatomy after fungal degradation. (a) Selective delignification of wood. Black arrows indicate the degradation of lignin in the fiber cell walls. (b) Simultaneous delignification of wood. All the cell wall components are degraded and the fungal hyphae can be seen in the center. Black arrows indicate the separation of the fibers. Bars: (a) 20 μm, (b) 50 μm (Martínez *et al.*, 2005).

Laccases are known to commonly occur in plants, insects, and fungi where they play a role in detoxification, fruiting body morphogenesis, or pigment synthesis (Mayer & Staples, 2002). Because laccases have low redox potentials, they only allow for the direct oxidation of phenolic lignin units. Laccase cause oxidation of the alpha carbon, demethoxylation cleavages in phenyl groups, and Cα – Cβ cleavage in syringyl structures (Fig.14). As the result of lignin decomposition, laccases also provide the quinones and phenoxyradicals that are important in the decomposition of cellobiose through the action of cellobiose dehydrogenase (Zabel & Morrell, 1992). Figure 14 illustrates the degradation of lignin via enzymatic reactions.

Laccase, LiP, and MnP oxidize the lignin polymer and generate aromatic radicals (a). These radicals may be involved in a number of non-enzymatic reactions including C4-ether breakdown (b), the cleavage of the aromatic ring (c), cleavage of the Cα – Cβ bond (d), and demethoxylation (e). The cleavage of the Cα – Cβ bond in lignin releases aromatic aldehydes that are the substrate for H_2O_2 generation by aryl-alcohol dehydrogenases and aryl alcohol oxidase in cyclic redox reactions. If phenoxy radicals from C4-ether breakdown (b) are not

reduced by oxidases to phenolic compounds (i), they can repolymerize on the lignin polymer (h). Laccase or peroxidases can reoxidize the phenolic compounds formed (j). Phenoxy radicals may also undergo Cα – Cβ breakdown (k), resulting in the formation of *p*-quinones. These quinines indicated by (g) and (k) in figure 14 play a role in oxygen activation in redox cycling reactions (l, m). The ferric iron present in wood is reduced (n) and reoxidized while H_2O_2 is reduced to a hydroxyl free radical (OH^-) (o). The latter is a strong oxidizer and plays an important role in the initial stages of wood degradation, as it attacks the lignin (p) when the pore sizes are still too small for penetration by other ligninolytic enzymes.

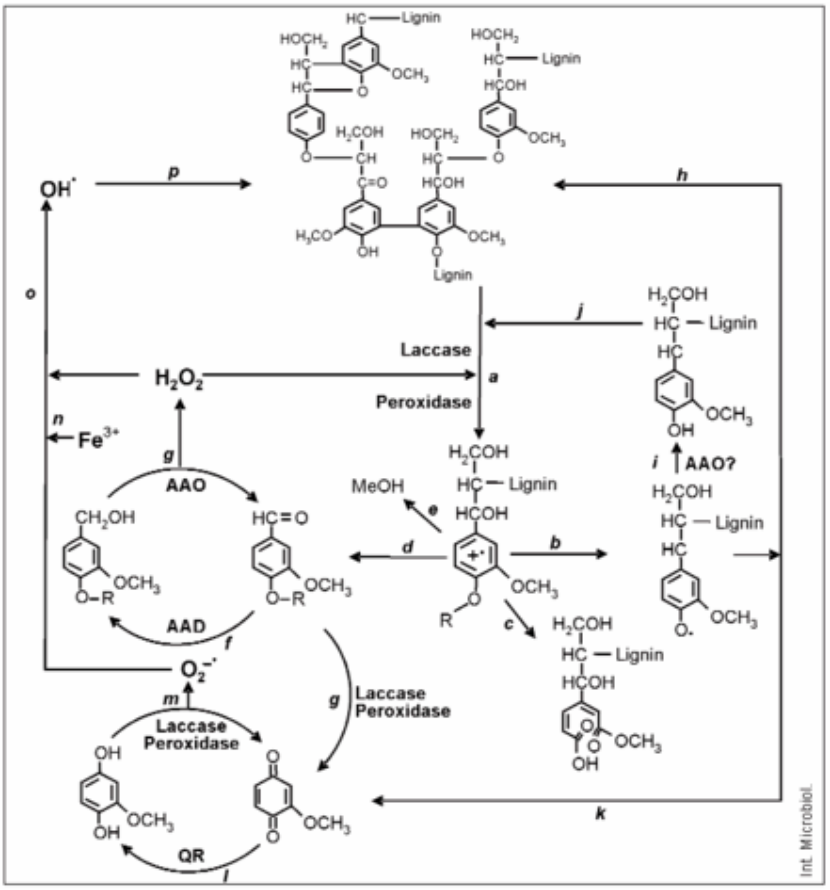


Figure14. An illustration of the chemical and enzymatic degradation of lignin (Martínez *et al.*, 2005).

From the above paragraphs, it is clear that basidiomycetous white-rot fungi use hydrolytic enzymes to degrade cellulose and hemicellulose, and oxidative

enzymes for the degradation of lignin. The carbohydrate monomers and organic acids formed by these degradation activities are re-absorbed by the fungal hyphae (fig. 15) and converted to new fungal biomass, water and carbon dioxide (Kirk & Cullen, 1998). This fungal growth and the concomitant production of degradation enzymes however, are subjected to environmental conditions.

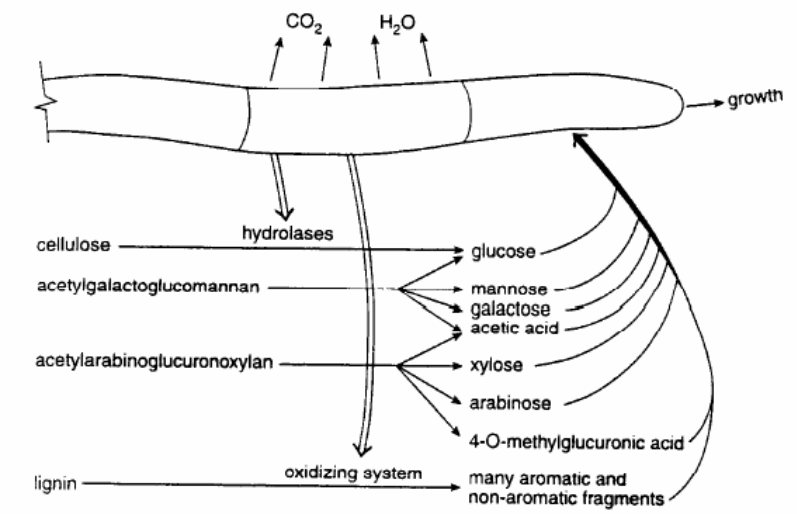


Figure 15. Schematic illustration of the degradation of wood polymers by the extracellular enzymes of white rot fungi (Kirk & Cullen, 1998).

1.7. Factors influencing wood degradation.

Physical conditions that play a role in the ability of fungi to degrade wood include temperature, concentration of oxygen in the substrate, and moisture content (Schwarze *et al*., 2000). Fungi grow optimally at temperatures between 20 and 30°C. In the dormant state however, many fungi are able to tolerate extreme temperatures (-5 to +55°C). Fungi appear to be selective regarding the colonization of certain wood. This may be related to temperature optima. For example, some fungal species show selectivity for wooden slats of cooling towers where temperatures are higher. Others are associated with utility poles below ground zones where temperatures are low (Rayner & Boddy, 1988).

It was stated that the optimum oxygen concentration for fungal degradation is ca. 10% (Schwarze *et al*., 2000). This concentration allows for degradation of wood while an oxygen concentration of ca. 1% will only ensure the survival of the fungus. However, the most important factor impacting on fungal wood degradation is the moisture content of the wood (Rayner & Boddy, 1988). Very few fungus species are able to degrade wood below a moisture content of 25%, whereas the optimum moisture content for wood degradation ranges from 40 to 70% (Schwarze *et al*., 2000). Various authors, however, demonstrated that fungal degradation of wood may even occur at a moisture content of 200% and higher. Interestingly, the activities of wood degradation fungi are known to impact on the moisture content of wood during degradation. The degradation of lignin and hemicellulose by white-rot fungi results in an increase in the moisture absorption capacity as a result of a relative increase in cellulose content. Brown-rot fungi on the other hand result in a decrease in moisture absorption as the hydrophilic cellulose and hemicellulose are degraded first.

Another factor that plays a role in wood degradation is the chemical composition of wood, such as the relative proportions of cellulose, hemicellulose, lignin monomers and anti-fungal compounds, as well as the nitrogen content of the wood (Schwarze *et al*., 2000). Phenolic substances are known to protect the wood and inhibit the activity of degradation fungi. In contrast, it was found that an increased nitrogen concentration resulted in an increased rate of wood degradation by the fungus *Heterobasidium annosum* (Schwarze *et al*., 2000). As in many natural habitats, a consortium of microbes may occur on the wood and interactions between the individuals are inevitable. These interactions may take the form of different symbiotic relations among the fungi occurring on the wood.

1.8. Interspecific fungal interactions in wood degradation

The different types of fungal symbioses are classified into competitive, neutralistic, and mutualistic interactions (Rayner & Boddy, 1988). With

competitive interaction, the outcome is detrimental to either or both of the species involved. In the case of neutralistic and mutualistic interactions, no detrimental effects to either species are involved, and benefits may be absent, unilateral, or bilateral. Such benefits may result for various reasons: one organism may provide waste products or exudates as a resource for the other; the vegetative or reproductive development of one organism may be stimulated by products from the other; or a complementary enzyme action may be achieved for both organisms.

Wood inhabiting fungi rarely show a truly non-antagonistic interaction with each other. Rather, it is common to find deadlock where neither mycelium can enter the other's domain or where one is replaced by the other. True symbiotic interaction between fungi was studied by Maijala (2005) when he co-cultured different white rot fungi on wood. This co-culturing lead to enhanced lignin degradation. The degree of enhancement varied between different co-culture combinations. Elevated levels of laccase and manganese peroxidase activity were observed through experimental work, and *Pleurotus ostreatus* was identified as a promising partner fungus for species such as *Ceriporiopsis subvermispora*, *Physisporinus rivulosus* and *Phanerochaete chrysosporium* (Maijala, 2005). Recently, co-cultures of a known pioneer fungus of wood, *Aspergillus flavipes*, and a common white-rot fungus, *Pycnoporus sanguineus*, were evaluated in a biopulping process (Domisse, 1998). It was found that this co-culture enhanced the pulping properties of *E. grandis* wood.

Previously, associations between bacteria (*Enterobacter* spp.) and white rot fungi were studied and indications of mutualistic interactions were found (Blanchette & Shaw, 1978). It was stated that within these interactions, the bacteria supply vitamins and growth stimulating substances to the fungi, while they utilize nutrients originating from the wood cell wall that is being degraded by the fungal enzymes. Some bacteria have the ability to fix atmospheric nitrogen (Blanchette & Shaw, 1978). This may also have enhanced mycelial growth and promote the

rate of wood degradation. The role of yeasts in the colonization of wood has also been studied (Blanchette & Shaw, 1978; González *et al*., 1989). Since yeasts lack the ability to penetrate wood, they need to form an association with mycelial fungi. Studies by Blanchette showed an increase in wood degradation rate by white rot fungi in the presence of yeasts such as *Saccharomyces bailii* var. *bailii* (syn. *Zygossaccharomyces bailii*) and *Pichia pinus* (syn. *Pichia pini*). These yeasts species are normally associated with spoiled food and decaying trees (Kurtzman & Fell, 1998). Since it is known that fungal fruiting bodies may harbor yeast populations, the question arose whether yeasts naturally occurring on *P. sanguineus*, a common white-rot fungus, may impact on the degradation of wood by this macro fungus. Furthermore, studies by Dommisse (1998) showed that a co-culture of *P. sanguineus* and *A. flavipes* improved the pulping properties of *E. grandis* wood chips. Consequently, we were interested to determine whether the degradation pattern of this co-culture will vary when grown on different tree species.

1.9. Purpose of study

With the above as background, the first goal of this study became to characterize the natural yeast populations on the fruiting bodies of *Pycnoporus sanguineus* (Chapter 2). The impact of some of these yeasts, as well as the pioneer fungus *A. flavipes,* on wood degradation by *P. sanguineus* was then determined by analyzing the major wood components after growth of co-cultures on *E. grandis* wood chips. Standard protocols, commonly used by the paper and pulp industry, were employed to measure parameters of the wood and boxplots, a Principal Component Analysis (PCA) biplot, a Canonical Variate Analysis (CVA) biplot, as well as an analysis-of-distance (AOD) biplot were subsequently used to analyse the data. Biplots were used since it is known that these graphs provide a means for displaying in a single graph all samples that were measured together with information of all parameters measured for all the analysed samples (Gabriel, 1971). In Chapter 3 the same protocols were used to investigate chemical

alterations in different tree species, induced by a *P. sanguineus* / *A. flavipes* co-culture.

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Chapter 2

Symbiosis between *Pycnoporus sanguineus* and other fungi associated with the woody phylloplane

2.1. Introduction

It is commonly known that a diversity of yeasts may occur on plant surfaces (Fonseca and Inácio, 2006) and that the composition of the phylloplane yeast community is influenced by a number of factors. The latter may include the chemical and physical composition of the plant and its leachates, relative humidity, heat and sunlight. Basidiomycetous yeasts containing photoprotective carotenoids, such as *Rhodotorula* and *Sporobolomyces,* are frequently encountered on the phylloplane (Bai *et al*., 2002). However, ascomycetous yeast species were also observed on plant surfaces. A number of yeasts were found as endophytes within plants (Camatti-sartori *et al*., 2005; Nassar *et al*., 2005). The latter include representatives of the genera *Sporobolomyces*, *Rhodotorula*, *Debaryomyces*, *Cryptococcus*, and *Williopsis*. It is thus inevitable that filamentous fungi growing on wood as substrate (Van der Westhuizen & Eicker, 1994), such as the cosmopolitan white rot *Pycnoporus sanguineus,* will encounter yeasts during the course of its life cycle. This ligninolitic fungus, that also produces extracellular cellulases for the utilization of carbohydrates (de Almeida *et al*., 1997), forms large conspicuous dimidiate fruiting bodies on a variety of fallen tree species. *Pycnoporus sanguineus* degrades the lignin in wood by using oxidative enzymes systems. However, contradicting results were obtained with regards to its pattern of delignification. Luna *et al*. (2004) indicated selective delignification of poplar trees, while Ferraz *et al*. (1998) found a simultaneous delignification pattern on *Eucalyptus grandis* trees.

The colonization of fallen trees by *P. sanguineus* is part of an ecological succession of different fungal species (Schwarze *et al*., 2000). Prior to the degradation by this white-rot, pioneer fungi such as *Aspergillus flavipes* will colonize the wood. These fungi utilize readily available sugars and do not cause extensive structural changes in the wood. Strains representing *A. flavipes* have been isolated from decaying vegetation and were previously used in co-culturing studies on wood chips (Domisse, 1998).

Studies by Blanchette and co-workers (1978) showed elevated levels of wood degradation when white rot fungi such as *Coriolus versicolor* were co-cultured with *Saccharomyces bailii* (syn. *Zygosaccharomyces bailii)* and *Pichia pinus* (syn. *Pichia pini*). It is known that macro-fungal fruiting bodies may harbor yeast populations i.e. *Cryptococcus humicola* on *Amanita muscaria* and *Dipodascus armillariae* on decaying *Armillaria* fungi (Kurtzman & Fell, 1998).

Restriction Fragment Length Polymorphisms (RFLP) analysis were recently applied to estimate the diversity of large populations of microbes such as mycotoxin-producing *Fusarium* isolates from different hosts (Llorens *et al*., 2006) and genotypes of *Mycobacterium tuberculosis* isolates from patients with tuberculosis (Chan-Yeung *et al*., 2006). Previously RFLP analyses were applied to estimate the diversity of yeast communities associated with wine and food and it was concluded that the method is reproducible, easy and useful to rapidly identify different species (Guillamón *et al*., 1998; Esteve-Zarzoso *et al*., 1999).

We were interested in the composition of the yeast community associated with the fruiting body of *P. sanguineus,* and how these yeasts and the pioneer fungus *A. flavipes* impact on wood degradation by this white-rot fungus. With the above as background, the aim of this study was to utilize RFLP analyses to obtain an indication of the species composition of the culturable yeast community associated with fruiting bodies of *P. sanguineus*. The impact of some of these yeasts, as well as *A. flavipes,* on wood degradation by *P. sanguineus* was then determined by analyzing the major wood components after growth of co-cultures on *E. grandis* wood chips. Standard protocols, commonly used by the paper and pulp industry, were employed to analyze the wood chips.

2.2. Materials and methods

2.2.1. Enumeration and isolation of yeasts

Yeasts on the surface of three fruiting bodies of *Pycnoporus sanguineus* were enumerated and randomly isolated. During June and July 2004 swabs were applied to take yeast samples from 2 cm^2 surface areas on fruiting bodies growing on weathered tree stumps near Stellenbosch, South Africa. Two samples were taken from the upper surface of each fruiting body, two from the lower surface in the pore area, and two from the woody phylloplane next to the fruiting body. The swabs were vortexed (Vortex Genie-2 at setting eight, from Scientific Industries) for ten seconds in 10 ml physiological salt solution (PSS) to wash the microbes from each swab. Dilutions of suspended organisms were transferred to plates with malt extract-agar (MEA) containing 50 $mg.l^{-1}$ streptomycin. After three days of incubation at 22°C, yeast colonies larger than one millimeter in diameter were counted. To estimate the yeast species composition on the surface of the fruiting bodies, yeasts were randomly isolated from the plates using a modification of the Harrison's disc method as described by Harrigan and McCance (1967). Successive inoculation and incubation on MEA at 22°C were used to purify the isolates.

To verify the identity of the white rot, a section of the fruiting body was first used to inoculate plates containing benomyl–dichloran–streptomycin medium (BDS-medium, Appendix A, Table A, Worrall, 1991). After two weeks of incubation at 22°C the culture was purified by successive inoculation and incubation on BDS-medium, before identification using sequence analyses of selected ribosomal genes.

2.2.2. Classification of yeast isolates using RFLP analysis

Yeast isolates were incubated for three days in 10 ml yeast-peptone-dextrose (YPD) broth [2% glucose (Saarchem), 2% peptone (Biolab), 1% yeast-extract (Biolab)]. Genomic DNA was then extracted according to the method of Hoffman and Winston (1987). The rRNA gene region was amplified in a Perkin-Elmer thermal cycler. Primer pairs used to amplify the ITS region were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The parameters for thermal cycling were an initial denaturation at 95°C for 3 min, followed by 36 cycles of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 4 min. The PCR products were then digested with the restriction endonucleases *Hin*fI, *Hin*6I, and *Mbo*II according to their specific instructions (Fermentas Life Sciences). The restriction fragments were then electrophoresed on a 5% polyacrylamide gel stained with ethidium bromide and photographed. A 50 bp DNA ladder marker (Hyperladder v, Bioline) was used as the size standard.

2.2.3. Identification of yeast and white rot fungal isolates

Yeast isolates, representative of the different yeast RFLP profiles originating from the white rot fruiting bodies, were incubated for three days in 10 ml YPD broth. Genomic DNA was then extracted according to the method of Hoffman and Winston (1987). The D1/D2 600-650 bp region of the large subunit of ribosomal DNA (rDNA) was subsequently amplified using the polymerase chain reaction (PCR). The DNA was amplified with the forward primer F63 (5'-GCA TATA CAA TAA GCG GAG GAA AAG-3') and the reverse primer LR3 (5'- GGT CCG TGT TTC AAG ACG G-3') in a Perkin-Elmer thermal cycler (Fell *et al.,* 2000). The PCR products were purified with Nucleospin® (Separations) chromotography columns. Sequences representing the D1/D2 of the rDNA from the strains were then obtained using an ABI PRISM model 3100 genetic sequencer. The forward and reverse sequences were aligned with DNAMAN Version 4.13 for WINDOWS

(Lynnon Biosoft). The yeast strains were then identified by comparing the sequencing results with known sequencing results using the BLAST program (www.ncbi.nlm.nih.gov/blast).

The identity of the white rot isolates was confirmed using sequence analyses of the two internal transcribed spacers (ITS 1 and ITS 2) of the ribosomal gene cluster. Genomic DNA was extracted using a method based on the protocol of Raeder & Broda (1985). Using acid-washed sand, frozen mycelia were ground to a fine powder in a mortar and pestle. The powdered mycelia were transferred to 4 ml ice cold extraction buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The resulting suspension was extracted on ice, using 3 ml phenol and 1.2 ml chloroform:isoamylalcohol (24:1). The supernatant was removed, re-extracted with phenol and centrifuged (6000 g, 50 min at 4°C). The aqueous phase was subsequently treated with RNase, then extracted twice using chloroform. Afterwards the nucleic acids were precipitated with 0.54 vol. of isopropanol. After washing, the DNA pellet was dissolved overnight in TE (10 mM Tris-HCl pH 8.0, 1mM EDTA) at 4°C.

Using the polymerase chain reaction (PCR), the DNA was amplified with Expand™ High Fidelity DNA Polymerase from Boehringer Mannheim (South Africa) in a Perkin-Elmer 2400 thermal cycler. Boehringer Mannheim, Germany, synthesized primers used for the PCR experiments. Primers ITS 5 (5' -GGA AGT AAA AGT CGT AAC AAG G - 3') and ITS 4 (5' - TCC TCC GCT TAT TGA TAT GC - 3') were used to amplify the ITS region according to the method of White *et al.* 1990. The PCR products were purified by column chromatography (Sephadex G-50, Sigma) and sequenced using an ABI PRISM model 3100 genetic sequencer. The forward and reverse sequences were aligned with DNAMAN for WINDOWS Version 4.13. The fungal strains were identified by comparing the sequencing results with known sequences using the BLAST program (www.ncbi.nlm.nih.gov./blast).

2.2.4. Assessing the degradation of wood components by yeast/white rot fungal co-cultures

A fungal strain representing *P. sanguineus* was obtained from the fungal culture collection of the ARC-Plant Protection Research Institute (PPRI), Pretoria, South Africa. This white rot fungus (*P. sanguineus* PPRI 6762), as well as *A. flavipes* J11904 and selected yeast isolates (Table 1) are being maintained at 22°C on MEA in the fungal culture collection of the Department of Microbiology, University of Stellenbosch, South Africa.

Twelve year old *E. grandis* trees were obtained from plantations on the Eastern Highveld of South-Africa. The trees were chipped and only the fraction greater than 6 mm and less than 9 mm in thickness was retained for experimentation. To enhance weathering, the wood chips were pre-treated in a pressure vessel of 15 dm^3 capacity with a hot water wash at 150°C for two hours. To ensure that the water mixes well with the wood chips and fibres, the vessel oscillated through 45° to either side. When the temperature reached 150°C, the vessel degassed automatically and the pressure dropped in about 12 minutes from 800 kPa to 0 kPa. Thereafter, the pressure was increased until it reached the maximum 800kPa where it was maintained for 25 minutes.

In order to obtain a final moisture content of 60% (Wolfaardt *et al.,* 2004), a nutrient supplement [5% (w/v) molasses and 0.28% (w/v) urea], as well as an appropriate volume of fungal inoculum, were added to the chips. Inocula of *A. flavipes* J11904 and *P. sanguineus* PPRI 6762 were prepared by growing these strains at 30°C in 5% (w/v) molasses broth. After one week of incubation, the fungal biomass of each culture was homogenized using a blender (Pineware) for 30 s. This homogenized fungal biomass was subsequently used to inoculate the woodchips, resulting in a final concentration of 1.8×10^{-4} g and 1.7×10^{-4} g dry biomass per gram of oven dried wood for *A. flavipes*, and *P. sanguineus* respectively. A monoculture of *P. sanguineus* was prepared by inoculating 1.7 x

10^{-4} g dry fungal biomass per gram of oven dried wood. To prepare *P. sanguineus* / yeast co-cultures each of the selected yeast isolates (Table 1) was inoculated onto wood containing *P. sanguineus* (1.7 x 10^{-4} g dry fungal biomass per gram of oven dried wood). In each case 9 ml (A_{600} = 0.21) of a liquid culture in stationary phase, suspended in YPD broth, was transferred to a solid state bio-reactor.

These bio-reactors consisted of closed cylindrical plastic vessels (17 cm high and 23 cm in diameter), each containing 1 kg of wood chips resting on a grid 5 cm from the bottom to allow for aeration. After inoculation, each bio-reactor was incubated at 30°C, while being aerated from below the grid with 10 $L.min^{-1}$ sterile moist air blown through a water trap using an electro-magnetic air compressor (Style King, Model ACQ-009A). After 14 days of incubation the cultures were harvested and the chemical properties of the residual wood were analyzed using standard TAPPI methodology.

Table 1. Combinations of filamentous fungal strains and yeast isolates used to inoculate hot water washed *E. grandis* wood chips.

Culture name	**Culture combination**
Mono culture	*Pycnoporus sanguineus* PPRI 6762
Co-culture 1	*P. sanguineus* PPRI 6762 + *Pichia guilliermondii* ABA006
Co-culture 2	*P. sanguineus* PPRI 6762 + *P. guilliermondii* ABA006 + *Rhodotorula glutinis* ABA003
Co-culture 3*	*P. sanguineus* PPRI 6762 + autoclaved *P. guilliermondii* ABA006 + autoclaved *R.glutinis* ABA003
Co-culture 4	*P. sanguineus* PPRI 6762 + *Aspergillus flavipes* J11904
Un-inoculated	Un-inoculated hot water washed wood chips
Untreated	Un-inoculated unwashed wood chips

*Yeasts were autoclaved at 121°C for 20 minutes.

2.2.5. Chemical analyses of wood chips.

To provide an indication of the chemical changes that occurred during treatment of the wood chips, the residual wood chips obtained after fungal cultivation, the un-inoculated hot water washed chips, as well as un-inoculated untreated wood chips were analysed. Alcohol-benzene and water soluble extractive contents as well as lignin and cellulose content were determined during this process.

Extractions were conducted by boiling 3 g of the residual wood chips in either 200 ml ethanol-benzene or water for six to eight hours (TAPPI Standard Methods T 264 om-88). After ethanol-benzene extraction, the wood was washed with 95% ethanol to remove the benzene. This was followed by washing the chips with distilled water to remove ethanol. The chips were subsequently boiled in 500 ml distilled water for one hour, whereafter it was washed with 500 ml boiling water and air dried. Subsequently, the moisture content was quantified to calculate the percentage extractives in the wood.

Klason Lignin is defined as the wood components that are insoluble in sulphuric acid (72%) [TAPPI Standard Methods T 222 om-88]. For the determination of lignin content, 15 ml sulphuric acid (72%) was added to 5 g oven dried extractive free wood. This reaction mixture was subsequently incubated at 20°C for two hours. After the incubation period, the material was suspended in water until a 3% concentration of the sulfuric acid was reached. The resulting suspension was boiled for four hours at a constant volume. After boiling, the insoluble material (lignin) was allowed to settle. The supernatant was subsequently discarded and the precipitate washed with water, dried and weighed.

The Seifert method was used to gravimetrically determine cellulose in the residual wood chips (Browning, 1967). Using a boiling water bath, 1 g of extractive free wood meal was refluxed in a solution containing 6 ml acetylacetone, 2 ml dioxane and 1.5 ml hydrochloric acid. After 30 min the

mixture was then washed successively with methanol, dioxane, hot water, dioxane, methanol and ether. The residue was subsequently weighed after drying at 105°C.

2.3. Results and Discussion

2.3.1. Yeast numbers and community composition

The quantity of yeasts on the fruiting bodies of *P. sanguineus* were log 4.1 ± 1.6 per cm^2. The majority of the yeasts on these structures belonged to the ascomycetous species, *Pichia guilliermondii* while the rest were representatives of the basidiomycetous species *Rhodotorula glutinis* (Table 2). Both species could be differentiated by characteristic RFLP banding patterns formed in polyacrylamide gels (Figure 1).

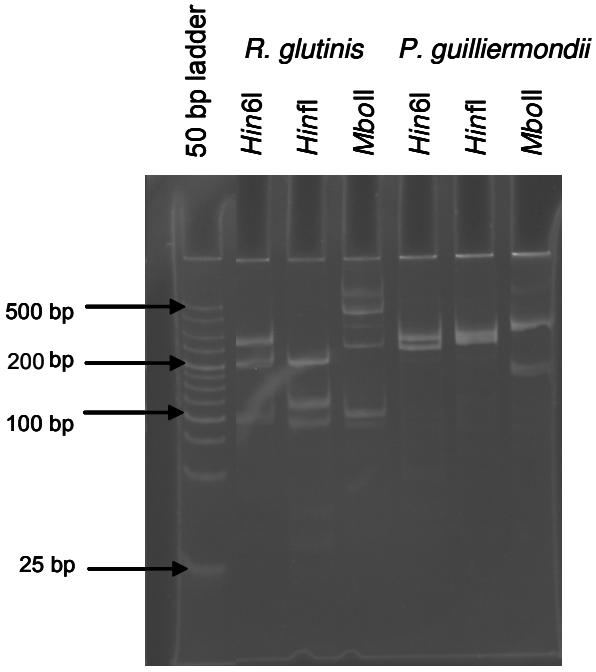


Figure 1. Polyacrylamide gel containing digestion products that originate from the ITS region within the rRNA gene cluster of *P. guiliermondii* and *R. glutinis.* The restriction enzymes *Hin*6I, *Hin*fI, and *Mbo*II, were used in the experimentation.

Rhodotorula glutinis is known to occur on plant surfaces (Fonseca and Inácio, 2006), while *P. guilliermondii* and related species were found to be associated with basidiocarps and xylophagous beetles that may be found on these structures (Zacchi and Vaughan-Martini, 2002; Suh and Blackwell, 2004; Ganter, 2006). The yeast community on the woody phylloplane seemed to be smaller (log 2.3 ± 1.1) but more diverse than the yeast community on the fruiting bodies since four basidiomycetous species, belonging to the genera *Fellomyces* and *Rhodotorula*, as well as the ascomycete *Pichia guilliermondii* were recovered from this habitat (Table 2).

Table 2. Yeast community composition associated with the fruiting bodies of *P. sanguineus* and the adjacent decaying wood surface. The identity of the yeasts was determined by RFLP analyses, followed by sequence analyses of ribosomal gene sequences in yeasts representing different RFLP profiles.

Habitat	***Yeast species***	***Percentage of species associated with habitat***
Fruiting bodies of *P. sanguineus*		
	Pichia guilliermondii	74 %
	Rhodotorula glutinis	26 %
Woody phylloplane next to fruiting bodies		
	Fellomyces penicillatus	7 %
	Pichia guilliermondii	65 %
	Rhodotorula ingeniosa	7 %
	Rhodotorula glutinis	15 %
	Rhodotorula mucilaginosa	7 %

Each of these species was characterized by a unique RFLP profile (Table 3). The fact that notably more yeasts occurred on the fruiting bodies of the white rot than on the adjacent wood, indicated that the yeasts may have been deposited on the fruiting bodies by foraging insects known to be associated with *Pichia guilliermondii* (Zacchi and Vaughan-Martini, 2002). Another possibility is that a symbiosis exists between the yeasts and this basidiomycetous macro-fungus. It was, therefore, decided to test co-cultures of *P. sanguineus* and representative isolates of the two dominant yeast species for the synergistic degradation of wood.

Table 3. Size in bp of restriction fragments obtained from yeasts species isolated from the fungal fruiting bodies and adjacent wood

Species	*Hin*6I	*Hin*fI	*Mbo*II
Pichia guilliermondii	274+255+49	293+287	337+187+64
Rhodotorula glutinis	283+182+80	180+94+70	422+358+216+81
Fellomyces penicillatus	195+147+63	206+105+68	262+233+162+93
Rhodotorula ingeniosa	250+218	245+176+147	393+273+235+96
Rhodotorula mucilaginosa	295+172+96	287+210+58	232+150+104+77

2.3.2. Motivation for and results of statistical analyses

Three replicate culture treatments were performed for each of the culture combinations: Mono culture, Co-culture 1, Co-culture 2 and Co-culture 3 (Table 1). Three further chemical analysis replicates were obtained for each of the above samples. One sample was obtained for each of the culture combinations: Co-culture 4 and Un-inoculated. Three chemical analysis replicates were obtained for each of these samples. The Untreated data consisted of three chemical analysis replicates of chip samples originating from two trees randomly selected from five locations representing the area of largest biological variation. The complete data set is given in Table 4.

Table 4. Measurements (%) obtained during chemical analyses described in section 2.2.5. "Class" depicts the culture name as listed in Table 1, A = Mono culture; B = Co-culture 1; C = Co-culture 2; D = Co-culture 3; E = Co-culture 4; F = Un-inoculated; Untreat = Untreated. Abbreviations: Cel = % Cellulose; Lig = % Lignin; EBE = % Solvent-borne extractives; EH2 = % Polar extractives.

Class	Cel	Lig	EBE	EH2
A	54.41	25.97	3.40	1.87
A	54.04	30.08	3.33	1.46
A	50.51	27.71	2.97	2.50
A	58.24	26.55	2.70	4.41
A	57.70	28.35	3.58	1.89
A	55.13	26.38	3.71	4.62
A	54.90	32.95	2.87	2.92
A	57.12	30.72	2.77	1.67
A	55.35	28.83	2.75	2.50
B	54.56	32.83	3.33	3.37
B	53.46	29.84	2.31	1.89
B	53.38	29.54	2.82	1.89
B	56.45	30.45	3.02	1.87
B	55.92	30.84	7.91	1.87
B	55.17	30.35	2.13	1.67
B	57.28	30.95	1.94	2.50
B	56.25	33.34	2.48	1.87
B	55.66	31.04	1.54	2.50
C	57.49	32.85	2.67	1.89
C	55.13	29.25	2.03	1.89
C	58.04	32.28	1.73	2.10
C	56.09	31.35	2.20	1.69
C	59.63	29.46	1.98	1.90
C	58.60	30.36	2.76	1.90
C	55.57	30.53	1.97	2.30
C	54.36	30.44	2.24	1.25
C	55.57	30.85	2.20	1.46
D	53.94	28.13	2.85	1.47
D	68.24	28.38	2.71	2.30
D	64.29	26.12	2.64	1.68
D	54.79	30.64	2.19	1.46
D	58.43	31.00	1.83	2.08
D	55.50	30.96	1.91	1.67
D	55.45	28.31	2.92	1.89
D	57.77	30.77	3.26	2.10
D	56.97	30.88	2.70	1.47

Class	Cel	Lig	EBE	EH2
E	61.28	26.68	8.51	2.98
E	61.13	28.49	8.51	2.13
E	60.01	28.58	9.35	2.55
F	75.83	26.30	7.58	2.53
F	62.95	26.35	7.79	2.74
F	74.04	24.18	8.21	2.95
Untreat	46.39	26.09	3.20	2.00
Untreat	45.55	22.15	3.00	3.60
Untreat	45.29	26.03	3.00	4.00
Untreat	47.14	18.07	2.00	2.00
Untreat	46.39	25.93	1.80	2.00
Untreat	48.34	26.16	1.80	2.20
Untreat	52.85	26.02	3.00	1.80
Untreat	53.46	22.03	2.60	2.00
Untreat	56.14	20.02	2.40	1.60
Untreat	46.31	22.08	3.00	3.00
Untreat	46.48	20.02	3.40	3.60
Untreat	49.10	21.93	3.20	2.40
Untreat	48.00	17.96	2.40	4.20
Untreat	48.05	23.93	2.20	4.20
Untreat	48.02	20.15	2.60	1.60
Untreat	52.80	23.83	3.40	2.00
Untreat	54.38	20.07	3.60	1.60
Untreat	52.90	20.06	3.40	1.40
Untreat	51.02	20.08	2.20	4.60
Untreat	49.92	18.00	2.60	5.20
Untreat	48.10	15.90	2.20	4.20
Untreat	47.21	20.03	3.80	3.80
Untreat	47.21	18.03	3.20	3.60
Untreat	46.24	24.20	3.20	3.60
Untreat	57.19	18.00	2.20	2.20
Untreat	58.40	18.04	2.40	2.00
Untreat	56.51	22.06	2.80	2.40
Untreat	49.15	20.06	1.20	4.40
Untreat	49.27	18.12	1.00	4.60
Untreat	49.01	16.02	1.60	3.40

Since in data “there are many patterns and relationships that are easier to discern in graphical displays than by any other data analysis method” (Everitt, 1994), box plots and biplots were extensively used for analysing the data presented in Table 4. Notched box plots not only provide for a visual appraisal of data, but also make available approximate hypothesis testing in providing an approximate confidence interval for the median: if two notches do not overlap (i.e. if the approximate 95% confidence intervals for the corresponding medians do not overlap) this can be considered as rejecting the hypothesis of equal medians at an approximate 5% significance level (McGill, Tukey & Larsen, 1978). However, the box plots depicted in Figure 3 are univariate displays of the data. Since four chemical properties were considered, a graphical display taking into account the multivariate character of the data contained in Table 4 was a necessity. Consequently, biplot representations of the data were considered.

Although users of statistics are often bewildered by all the statistical procedures available for analysing their data, it is not inconceivable for them to agree with Chambers, Cleveland, Kleiner and Tukey (1983) that “there is no statistical tool that is as powerful as a well-chosen graph”. Indeed, scatterplots and box plots are simple graphical devices almost universally understood and used in data analysis. Unfortunately, only two characteristics can be visually displayed in an ordinary scatterplot. However, in this study we investigated four chemical properties of wood chips. The biplot, introduced by Gabriel (1971) provides a means for displaying in a single graph all samples that were measured together with information on all characteristics measured for all of these samples.

The fact that distances in the classical Gabriel biplot are to be interpreted in terms of inner products between vectors complicates interpretation of these distances for the untrained eye. However, Gower and Hand (1996) show how biplots can be constructed so that it can be interpreted analogous to ordinary scatterplots. The basic idea is to construct a two dimensional (displaying a biplot in three dimensions is also possible) scatterplot of all the sample points together

with p > 2 axes – an axis for each of the properties measured. These axes are calibrated in the original units of measurement just as in an ordinary scatterplot. Although the biplot axes do not intersect at right angles they are used exactly as in an ordinary scatterplot: a line is drawn perpendicular from any point on the graph to any biplot axis and the value for that variable or property is read from the scale on the particular axis. Distances among the various points (samples) on the biplot are interpreted exactly as distances measured by an ordinary ruler. However care must be taken if a new point is to be added to the graph. In fact since p > 2 properties are to be taken into account statistical theory tells us that we should construct two different sets of biplot axes: one to read-off values as described above and another set for adding new points to the graph. This of course is different from ordinary scatterplots where the two rectangular axes are used for both these processes. All biplots presented here are equipped with calibrated axes allowing the reading-off of values as described above (Figures 4, 5 and 6). If a new point is to be added to this biplot, it can easily be done algebraically by utilizing the computer program used for constructing the biplot. Since p > 2 variables are represented in a two-dimensional graph, there is some loss of information. The *quality* of display provides a numerical indication (expressed as a percentage) of this loss. Similarly the *adequacy* associated with an axis provides a measurement of how successful that particular axis is represented in the two-dimensional biplot display.

Finally, it can be stated that several different types of biplots can be constructed. Here only the following are mentioned: Principal Component Analysis (PCA), Canonical Variate Analysis (CVA) and analysis-of-distance (AOD) biplots (Figures 4, 5 and 6). A PCA biplot show distances among the various samples and their *variation* with respect to the various properties measured (Gower & Hand, 1996) However, when the researcher is interested in displaying differences and overlap among different classes a CVA or perhaps an AOD biplot should be considered (Gower & Krzanowski, 1999). In a CVA biplot the aim is to maximise the between class variation with respect to the within classes variation.

When the different classes have covariance matrices that differ significantly, then AOD biplots are more appropriate for investigating differences among the class means.

A one-way multivariate analysis of variance (MANOVA) was performed on the data contained in Table 4. The null hypothesis of no significant differences with respect to the mean vectors associated with the four chemical properties measured for the seven treatment classes was rejected with a p-value approaching zero. This indicates that some differences between the classes are present. Accordingly, the MANOVA was followed by performing one-way univariate analysis of variance (ANOVA) procedures on the seven treatment classes treating in turn each of % Cellulose, % Lignin, % Solvent-borne extractives and % Polar extractives as the response variable. Table 5 contains the means and standard deviations for the data used in the ANOVA procedures while Table 6 contains the associated ANOVA tables.

Table 5. Means and, in brackets, standard deviations of the four chemical properties measured for each of seven treatment classes.

Treatment class	% Cellulose	% Lignin	% Solvent-borne extractives	% Polar extractives
Mono culture	55.27 (2.32)	28.62 (2.31)	3.12 (0.39)	2.65 (1.15)
Co-culture 1	55.35 (1.33)	31.02 (1.28)	3.05 (1.90)	2.16 (0.54)
Co-culture 2	56.72 (1.78)	30.82 (1.19)	2.20 (0.33)	1.82 (0.31)
Co-culture 3	58.38 (4.80)	29.46 (1.77)	2.56 (0.48)	1.79 (0.32)
Co-culture 4	60.80 (0.70)	27.92 (1.07)	8.79 (0.49)	2.55 (0.42)
Un-inoculated	70.94 (6.98)	25.61 (1.24)	7.86 (0.32)	2.74 (0.21)
Untreated	49.89 (3.77)	21.04 (3.10)	2.61 (0.70)	2.97 (1.13)

Table 6. ANOVA tables associated with one-way ANOVAs performed on four chemical properties measured for seven different treatment classes

	Df	*Sum of Sq*	*Mean Sq*	*F Value*	*Pr(F)*
Response: % Cellulose					
Class	6	1757.752	292.9586	24.5	0.000
Residuals	65	777.35	11.9592		
Response: % Lignin					
Class	6	1356.02	226.0033	38.99	0.000
Residuals	65	376.745	5.7961		
Response: % Solvent-borne extrs					
Class	6	181.995	30.3325	41.04	0.000
Residuals	65	48.0402	0.73908		
Response: % Polar extractives					
Class	6	16.85328	2.808881	3.49	0.005
Residuals	65	52.3104	0.804775		

It follows from Table 6 that each of the four null hypotheses, stating that the seven treatment classes have similar mean % Cellulose values; similar mean % Lignin values; similar mean % Solvent-borne extractive values and similar mean % Polar extractive values, is to be rejected with a p-value ≤ 0.005.

Consequently, in order to investigate the differences among the seven treatment classes, 99% simultaneous confidence intervals for all pair-wise differences between the means of the treatment classes were calculated according to Tukey's method (Scheffé, 1959). This was done for each of the four chemical properties. These confidence intervals are listed in Table 7. An interval excluding zero suggests the rejection at a 1% significance level of the null hypothesis that the corresponding means are equal. Such intervals are marked in grey in Table 7.

Perusal of Table 7 leads to the following conclusion in the case of % Cellulose: The Untreated mean value is statistically significantly lower than the mean value of the other treatment classes while the Un-inoculated mean value is significantly higher than the mean value of all other treatment classes. These findings are supported when comparing the notches in Figure 3 A. However, Figure 3 A also suggests the mean value of Co-culture 4 to be significantly higher than those of the other Co-cultures as well as the Mono culture.

Table 7. 99% Tukey simultaneous confidence intervals for pair-wise differences among treatment class mean values. Intervals excluding zero appear in grey. ("Treatment classes" are the culture names as listed in Table 1, A = Mono culture; B = Co-culture 1; C = Co-culture 2; D = Co-culture 3; E = Co-culture 4; F=Un-inoculated; Untreat = Untreated).

% Cellulose				
	estimate	**stderr**	**lower**	**upper**
A–B	–0.08	1.63	–5.97	5.81
A–C	–1.45	1.63	–7.35	4.44
A–D	–3.11	1.63	–9.00	2.79
A–E	–5.54	2.31	–13.87	2.80
A–F	–15.67	2.31	–24.01	–7.34
A–Untreat	5.37	1.31	0.62	10.12
B–C	–1.37	1.63	–7.27	4.52
B–D	–3.03	1.63	–8.92	2.87
B–E	–5.46	2.31	–13.79	2.88
B–F	–15.59	2.31	–23.93	–7.26
B–Untreat	5.45	1.31	0.70	10.2
C–D	–1.66	1.63	–7.55	4.24
C–E	–4.09	2.31	–12.42	4.25
C–F	–14.22	2.31	–22.56	–5.89
C–Untreat	6.82	1.31	2.07	11.58
D–E	–2.43	2.31	–10.76	5.91
D–F	–12.56	2.31	–20.9	–4.23
D–Untreat	8.48	1.31	3.73	13.23
E–F	–10.14	2.82	–20.34	0.07
E–Untreat	10.91	2.09	3.34	18.48
F–Untreat	21.05	2.09	13.48	28.62

% Solvent-borne extractives				
	estimate	**stderr**	**lower**	**upper**
A–B	0.07	0.41	–1.40	1.53
A–C	0.92	0.41	–0.54	2.39
A–D	0.56	0.41	–0.90	2.03
A–E	–5.67	0.57	–7.74	–3.60
A–F	–4.74	0.57	–6.81	–2.67
A–Untreat	0.51	0.33	–0.68	1.69
B–C	0.86	0.41	–0.61	2.32
B–D	0.50	0.41	–0.97	1.96
B–E	–5.74	0.57	–7.81	–3.67
B–F	–4.80	0.57	–6.88	–2.73
B–Untreat	0.44	0.33	–0.74	1.62
C–D	–0.36	0.41	–1.82	1.11
C–E	–6.59	0.57	–8.66	–4.52
C–F	–5.66	0.57	–7.73	–3.59
C–Untreat	–0.42	0.33	–1.60	0.77
D–E	–6.23	0.57	–8.31	–4.16
D–F	–5.30	0.57	–7.37	–3.23
D–Untreat	–0.06	0.33	–1.24	1.12
E–F	0.93	0.70	–1.60	3.47
E–Untreat	6.18	0.52	4.30	8.06
F–Untreat	5.24	0.52	3.36	7.13

% Lignin				
	estimate	**stderr**	**lower**	**upper**
A–B	–2.40	1.13	–6.51	1.70
A–C	–2.20	1.13	–6.31	1.90
A–D	–0.85	1.13	–4.95	3.26
A–E	0.70	1.61	–5.10	6.50
A–F	3.01	1.61	–2.80	8.81
A–Untreat	7.58	0.91	4.27	10.89
B–C	0.20	1.13	–3.90	4.30
B–D	1.56	1.13	–2.55	5.66
B–E	3.10	1.61	–2.70	8.90
B–F	5.41	1.61	–0.39	11.21
B–Untreat	9.98	0.91	6.68	13.29
C–D	1.35	1.13	–2.75	5.46
C–E	2.90	1.61	–2.90	8.70
C–F	5.21	1.61	–0.59	11.01
C–Untreat	9.78	0.91	6.47	13.09
D–E	1.55	1.61	–4.26	7.35
D–F	3.85	1.61	–1.95	9.66
D–Untreat	8.43	0.91	5.12	11.74
E–F	2.31	1.97	–4.80	9.42
E–Untreat	6.88	1.46	1.61	12.15
F–Untreat	4.57	1.46	–0.70	9.84

% Polar extractives				
	estimate	**stderr**	**lower**	**Upper**
A–B	0.49	0.42	–1.04	2.02
A–C	0.83	0.42	–0.70	2.36
A–D	0.86	0.42	–0.67	2.39
A–E	0.09	0.6	–2.07	2.26
A–F	–0.09	0.6	–2.25	2.07
A–Untreat	–0.33	0.34	–1.56	0.91
B–C	0.34	0.42	–1.19	1.87
B–D	0.37	0.42	–1.16	1.90
B–E	–0.39	0.6	–2.56	1.77
B–F	–0.58	0.6	–2.74	1.59
B–Untreat	–0.81	0.34	–2.05	0.42
C–D	0.03	0.42	–1.50	1.56
C–E	–0.73	0.6	–2.90	1.43
C–F	–0.92	0.6	–3.08	1.24
C–Untreat	–1.16	0.34	–2.39	0.08
D–E	–0.76	0.6	–2.92	1.40
D–F	–0.95	0.6	–3.11	1.22
D–Untreat	–1.18	0.34	–2.42	0.05
E–F	–0.18	0.73	–2.83	2.46
E–Untreat	–0.42	0.54	–2.39	1.54
F–Untreat	–0.24	0.54	–2.20	1.73

This finding is not supported by the simultaneous confidence intervals in Table 7. However, the number of observations available for Co-culture 4 is rather small. Therefore, the differences between the Mono-culture and the four Co-culture treatment classes were also investigated by means of permutation tests (see Good, 2000). Figure 2 contains the results of several permutation tests, each based upon 5000 permutation repetitions, performed to investigate these differences.

In Figure 2 the 5000 permutation repetitions for each test is indicated by a density estimate. The observed value (B) is indicated by a vertical line. Having an observed value significantly different from the permutation repetitions (under the null hypothesis), i.e. very far to the right, will provide evidence that the null hypothesis is not true for the observed data. To assess the evidence against the null hypothesis the area under the density estimate to the right of B (p-value) is calculated.

Inspection of Panel A in Figure 2 reveals that the null-hypothesis, stating that the mean % Cellulose obtained with Mono culture, Co-culture 1 and Co-culture 2 are similar, can not be rejected at a 20% significance level; inspection of Panel B reveals that, the null-hypothesis stating that the mean % Cellulose obtained with Mono culture, Co-culture 1, Co-culture 2 and Co-culture 3 are similar, should be rejected at a 7.7% significance level, while inspection of Panel C reveals that, the null-hypothesis stating that five culture classes do not differ with respect to their mean % Cellulose, should be rejected at a 1.8% level of significance. Taking into account the effect of the two outliers obtained with Co-culture 3 (see Figure 3 A), it can thus be concluded that the results with the permutation tests support the suggestion of the notched box plots in Figure 3 A that the Co-culture 4 treatment class has a higher mean % Cellulose than the other culture classes.

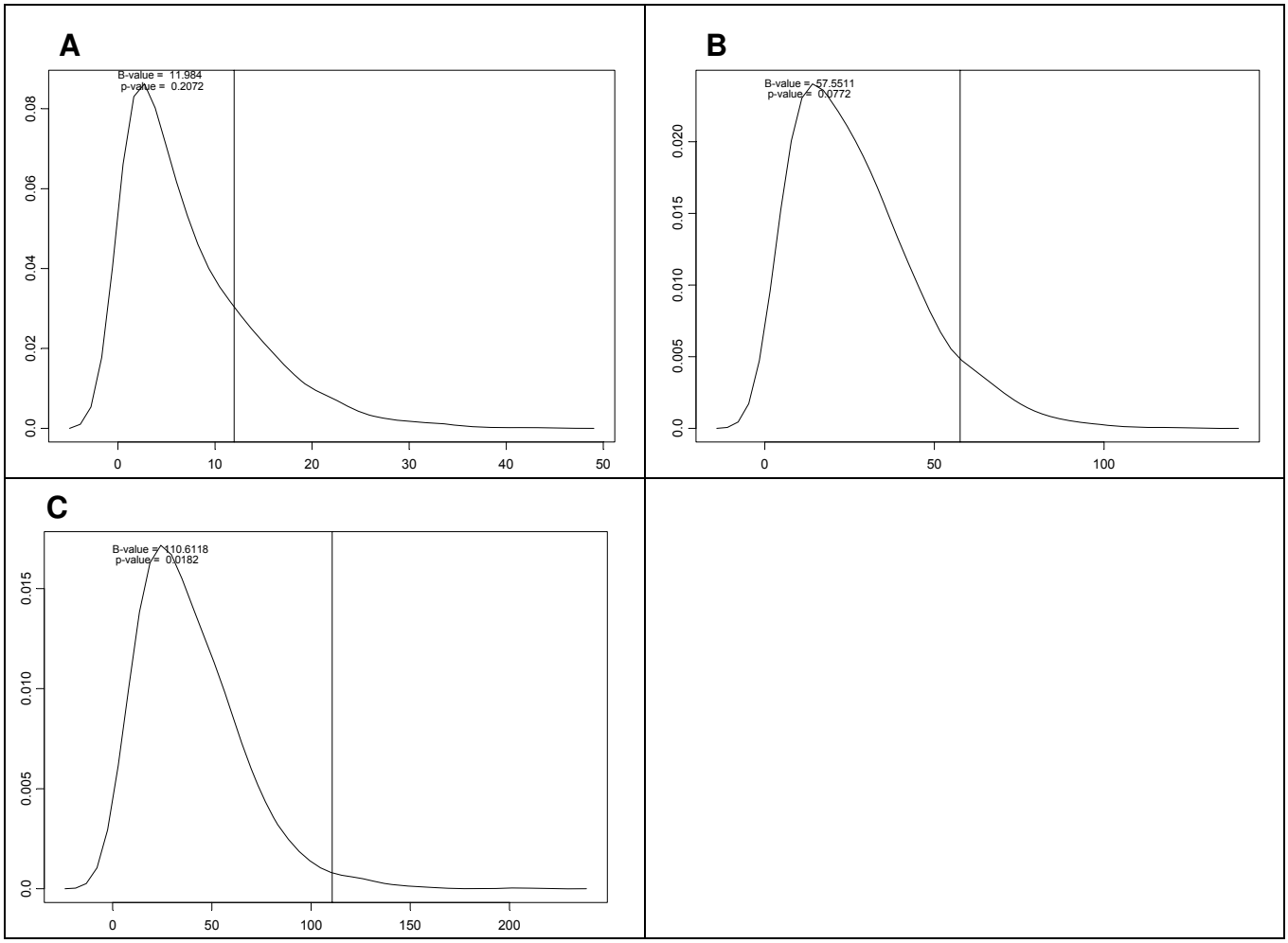


Figure 2. Results of permutation test investigation into the mean % Cellulose difference among treatment classes Mono culture, Co-Cultures 1, 2, 3 and 4.

Returning to Table 7, it follows that the simultaneous confidence intervals are in agreement with the deductions suggested by the notched box plots in Figure 3 B: The five culture treatment classes do not differ with respect to their mean % Lignin. These five classes have mean % Lignin values statistically significantly higher than that of the Un-inoculated class that in turn has a statistically significantly higher mean % Lignin value than the Untreated class.

The simultaneous pair-wise confidence intervals for mean % Solvent-borne extractives (Table 7) support what can be deduced from the notched box plots in

Figure 3 C: The Co-culture 4 class and the Un-inoculated treatment class have similar mean values statistically significantly larger than those of all other treatment classes. Note the large outlier in the case of Co-culture 1. The size of this outlier leaves some doubt about the correctness of that measurement.

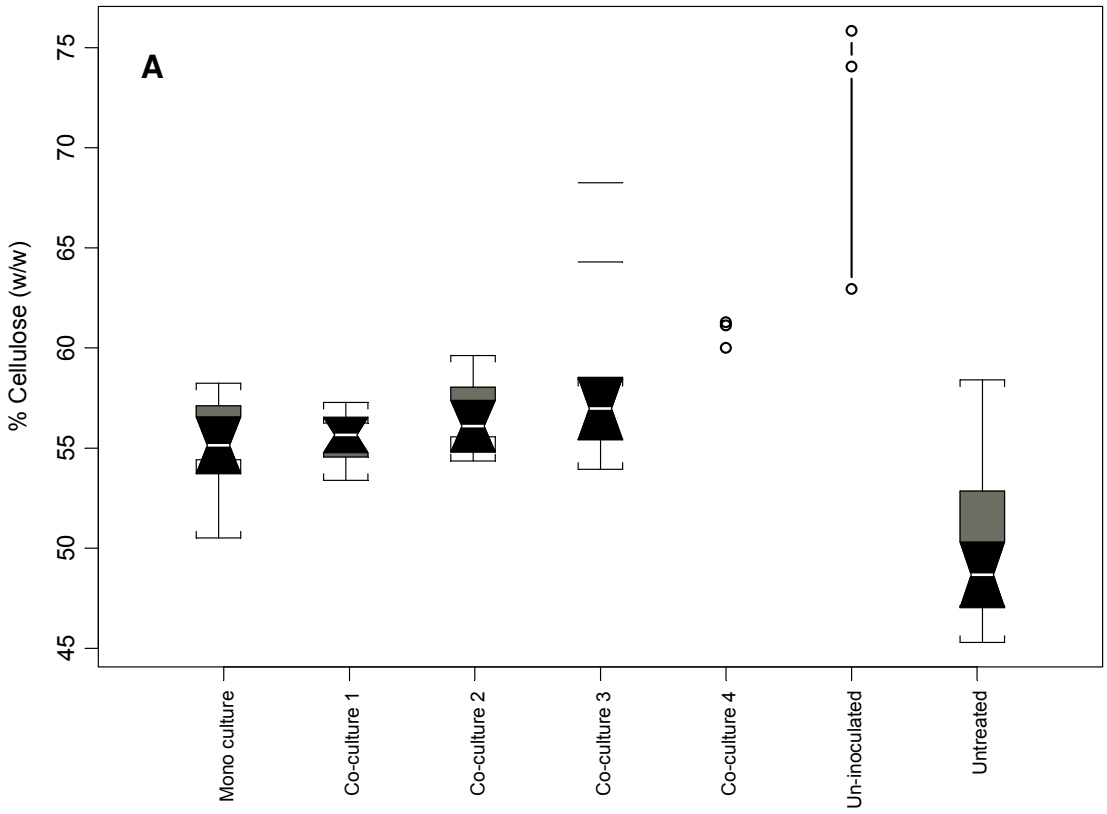
The simultaneous confidence intervals for the pair-wise differences in mean % Polar extractives (Table 7) support what can also be deduced from Figure 3 D: No statistically significantly differences between any two treatment classes are obtained. Note however, the large variation obtained in some of the treatment classes; especially with the Untreated and the Mono culture treatment classes.

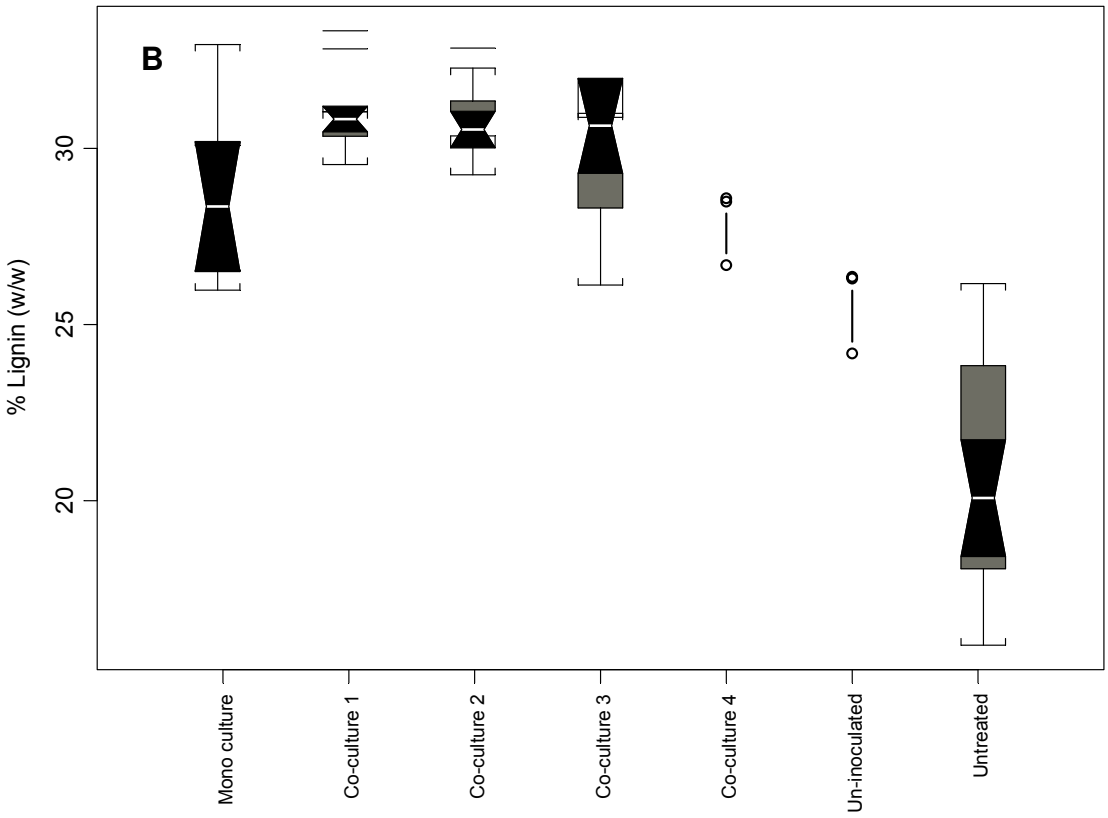
2.3.3. Analyses of residual wood components following growth of *P. sanguineus* on *E. grandis* woodchips

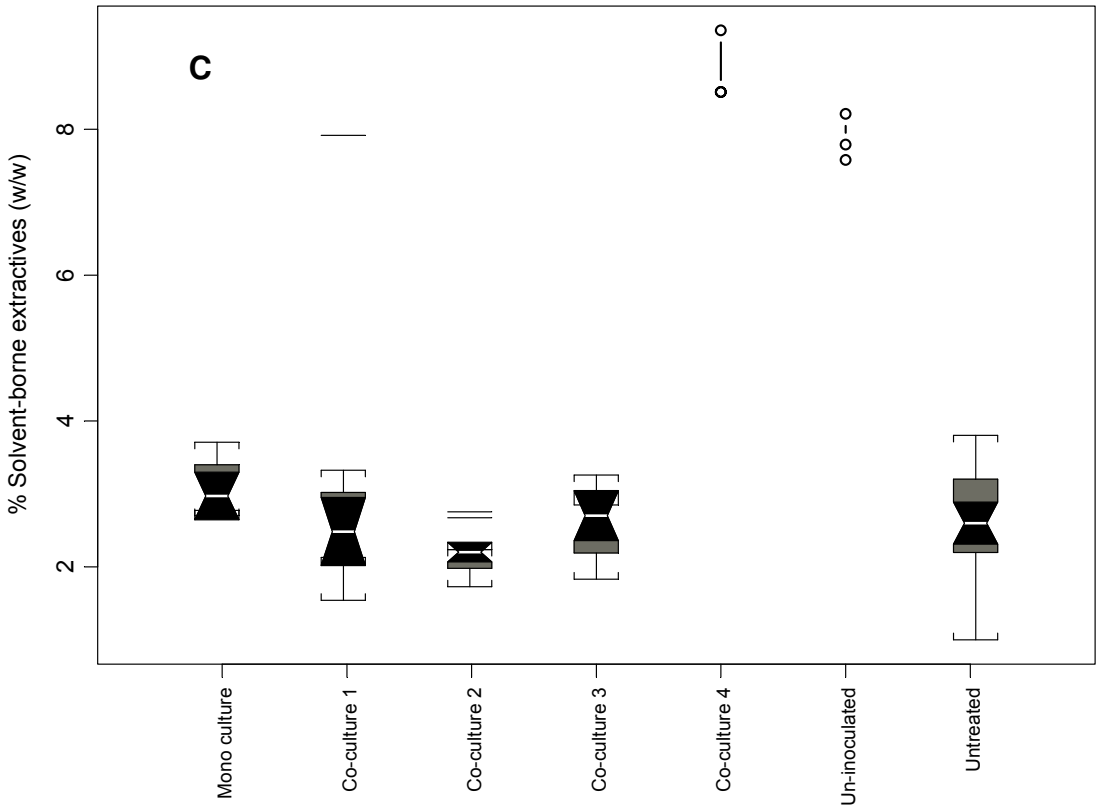
The results of Seifert analyses conducted on the residual wood chips, following a two week incubation period of *P. sanguineus* culture combinations (Table 1), are depicted in Figure 3A. When the performance of the *P. sanguineus* monoculture was compared to the different co-culture combinations, it is obvious that the yeasts had no significant impact on the degradation of cellulose by the white-rot fungus. In contrast, it seemed that the presence of *A. flavipes* resulted in inhibition of the degradation of this polymer. This may point to a potential antagonistic interaction between *P. sanguineus* and the hyphomycetous pioneer wood colonizing fungus.

Our results on cellulose degradation by *P. sanguineus* are in contrast to the findings of Luna *et al*. (2004) who observed that *P. sanguineus* selectively delignified poplar wood however, it corroborates the results of Ferraz *et al.* (1998). The latter authors found that *P. sanguineus* exerts simultaneous delignification of *E. grandis* wood, resulting in the concurrent degradation of cellulose and lignin.

Interestingly, the cellulose content of the untreated wood chips, as determined using the Seifert method, seemed to increase with ca. 30% after the hot water wash (Figure 3A). This may be explained by removal of the hemicellulose component during the latter process with the concomitant increase in the relative cellulose content. It is known that hemicellulose is hydrolyzed by hot water and steam, leading to leaching of the resultant monosaccharides from the wood (Rowell *et al*., 2002; Williams, 2005). In addition, some of the hemicelluloses could also have been removed by the formation of volatile furan-type breakdown products (Rowell *et al*., 2002).







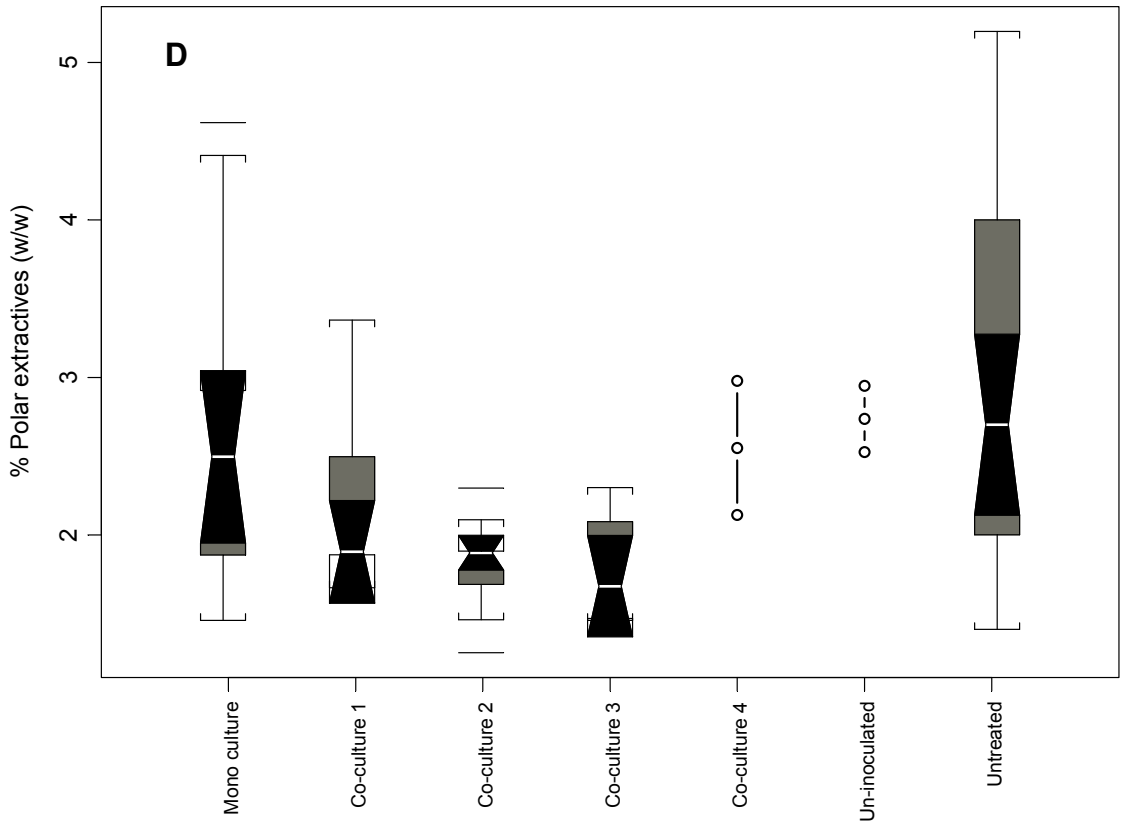


Figure 3. Notched Box plot presentation of the chemical analysis on wood chips after biotreatment. The three observations made respectively for classes Co-culture 4 and Un-inoculated are shown as line plots. (A) Percentage cellulose in wood after treatments. (B) Percentage lignin in wood after treatments. (C) Percentage solvent-borne extractives in wood after treatments. (D) Percentage polar extractives in wood after treatments. Monoculture = *Pycnoporus sanguineus* PPRI 6762; Co-culture 1 = *P. sanguineus* PPRI 6762 + *Pichia guilliermondii* ABA006; Co-culture 2 = *P. sanguineus* PPRI 6762 + *P. guilliermondii* ABA006 + *Rhodotorula glutinis* ABA003; Co-culture 3 = *P. sanguineus* PPRI 6762 + autoclaved *P. guilliermondii* ABA006 + autoclaved *R.glutinis* ABA003; Co-culture 4 = *P. sanguineus* PPRI 6762 + *Aspergillus flavipes* J11904; Un-inoculated = Un-inoculated hot water washed wood chips ; Untreated = Un-inoculated unwashed wood chips.

The results of Klason Lignin analyses conducted on the residual wood, following growth of *P. sanguineus* culture combinations on *E grandis* wood chips, are

depicted in Figure 3B. Strikingly, the lignin content of the wood appeared to increase during growth of all the cultures. Although no significant difference in lignin content was observed between co-cultures, the lignin content of the *P. sanguineus* / *Pichia guilliermondii* co-culture seemed slightly more than that of the *P. sanguineus* monoculture. The apparent increase in lignin content may be explained by the removal of celluloses and other bio-degradable wood components during fungal growth, resulting in a relative increase in the polyphenolic compound. The latter phenomenon may be especially evident where readily available cellulose is utilized to the expense of lignin degradation (Evans & Hedger, 2001).

In addition, as argued by Weiland and Guyonnet (2003), the heat from the warm water wash may have modified the lignin polymer to such an extent that the degradative enzymatic systems of the fungi were no longer effective. Also, it is known that protein contamination as a result of fungal growth may result in inflated Klason lignin values (Hatfield & Fukushima, 2005).

Similar to the apparent increase in cellulose content during hot water wash, the lignin content also appeared to increase during this process (Figure 3B). This may also be explained by removal of the hemicellulose component during the latter process with the concomitant increase in the relative lignin content (Nuopponen *et al.*, 2004; Garcia *et al.*, 2006).

Analyses of the solvent-borne extractives obtained from the residual wood, following growth of *P. sanguineus* culture combinations on *E. grandis* wood chips, revealed significant differences between the cultures (Figure 3C and Table 7). These extractives that typically consist of mixtures of aliphatic ketones, alkanes, fatty acids, sitosterol esters, triglycerides and waxes (Gutiérrez *et al.*, 1999) were significantly more in residual wood following growth of the *P.*

sanguineus / *A. flavipes* co-culture compared to residual wood of the other cultures. The lipid compounds in the residual wood may have originated both from degradation of wood components by the fungi such as hydrolyses of esters, as well as from fungal anabolism (Gutiérrez *et al*., 2002). Since *Aspergillus* is not a known oleogenous genus such as *Mucor* (Ratledge & Wilkinson 1988; Knutzon *et al*. 1998), and the monoculture of *P. sanguineus* contained significantly less lipids than the *P. sanguineus* / *A. flavipes* co-cultures, the relatively high lipid content of the latter may been the result of interactions between these two fungi within the co-culture.

The significant increase in solvent-borne extractives after the hot water wash of the untreated woodchips, but before inoculation (Figure 3C and Table 7), may also be explained by removal of the hemicellulose component during the latter process with the concomitant relative increase in lipophilic compounds. In addition, the hot water wash may have increased the availability of these lipophilic compounds to the extraction process, which involves boiling of the chips in ethanol-benzene (TAPPI Tests Methods, T264 om-88).

Polar extractives from wood mainly consist of amino acids, phenols, simple sugars and starches (Martin & Aber, 1996). Analyses of these polar compounds after fungal growth on the wood chips, revealed no significant difference between cultures (Figure 3D and Table 7). Although the co-cultures containing the mixed yeast species (Co-culture 2) and the autoclaved yeasts (Co-culture 3) contained significantly less extractives than the untreated wood chips, no difference was observed between the untreated wood chips and the wood treated with the hot water wash. It may be assumed that these extractives include enzymatic degradation products of wood components, as well as water extractable components of fungal biomass.

2.4. CONCLUSIONS

The Seifert analysis is known to be accurate for the determination of the cellulose content of wood (Browning, 1967). In contrast, no test method currently exists for the accurate determination of the total lignin content of wood (Hatfield & Fukushima, 2005). The Klason Lignin analysis however, is known to be repeatable, showing a relatively small standard deviation between repetitions (Hatfield & Fukushima, 2005; TAPPI Tests Methods, T222 om-88). It was, therefore, suggested that the most important consideration during wood analyses should be consistency in the methods used despite inherent shortcomings. From the above it is clear that the analytical methods used in this study are inadequate to accurately determine fungal degradation of wood. In addition, it is obvious that the methods used did not distinguish between fungal biomass and wood components. Nevertheless, the methods provided us with a fingerprint of each culture growing on the wood allowing us to compare the chemical composition of the different cultures and the un-inoculated hot water washed wood chips (Figures 4, 5 and 6).

A PCA biplot of the data showed that growth of all the cultures impacted on the chemical composition of the washed wood, because the latter formed a separate group from the cultures on the resulting biplot (Figure 4). From the graph, it is also clear that significant variation was present among the repeats of specific yeast co-culture combinations, as well as among the repeats of the mono culture. Despite these variations, the yeast co-culture combinations and the mono culture grouped separately from the *P. sanguineus* / *A. flavipes* co-culture on the PCA biplot (Figure 4). To compare these differences between the culture combinations used, a CVA biplot was constructed (Figure 5).

The construction of a CVA biplot (Figure 5) makes use of equality of the within classes covariance matrices. However, inspection of the box plots in Figure 3 as

well as the PCA biplot in Figure 4 cast a shadow of doubt on the truth of this assumption. Consequently, an AOD biplot that does not require this assumption was constructed (Figure 6). Strikingly, similar results were obtained for the two biplots (Figures 5 and 6) that were interpreted in the same manner.

The chemical composition of the hot water washed wood chips (Treatment class "un-inoculated" in Figures 5 and 6) was significantly different from the fungal cultures grown on these chips, indicating that the fungi impacted on the wood components. However, the chemical composition of the monoculture of *P. sanguineus* was similar to that of the *P. sanguineus* / yeast co-cultures, indicating that the yeasts had no effect on the growth of *P. sanguineus* and its degradation of *E. grandis* woodchips (Figures 5 and 6). These figures clearly show that the nature of the differences in the chemical composition described above consisted of the Un-inoculated and *P. sanguineus* / *A. flavipes* classes to have a larger cellulose and solvent-borne extractives content and a lower lignin content than the *P. sanguineus* / yeast co-cultures together.

The greater numbers of these yeasts, especially that of *Pichia guilliermondii*, on the fruiting bodies than on the adjacent woody phyloplane, may be as a result of the deposition of yeasts by basidiocarp-feeding insects. The yeast *P. guilliermondii* is associated with a wide diversity of insects (Zacchi & Vauchan-Martini, 2002), while closely related species were isolated from the gut of basidiocarp-feeding beetles (Suh & Blackwell, 2004). The greater numbers of yeasts on the fruiting bodies may also have been as a result of a commensalistic relationship between the white-rot fungus and the yeasts, the latter being the commensals. This potential relationship should be investigated in future.

In contrast to the results obtained with the *P. sanguineus* / yeast co-cultures, the presence of *A. flavipes* impacted on the chemical composition of *P. sanguineus*

cultures (Figures 5 and 6). It seems that the pioneering hyphomycetous fungus (Schwarze *et al*., 2000) exerts an antagonistic effect on the white-rot fungus known to occur later in the succession of fungi growing on decaying wood. This supports the contention that the fungal succession on wood is not only as a result of the availability of nutrients, but is also as a result the inhibitory effects of fungal competitors.

The protocol used in the analyses of the cultures discerned between different fungal co-cultures growing on a particular wood species. The question, therefore, arose whether the effect of a particular co-culture, on the chemical composition of wood, differs between tree species. Consequently chemical alterations in different tree species, induced by a *P. sanguineus* / *A. flavipes* co-culture, were investigated in the following chapter.

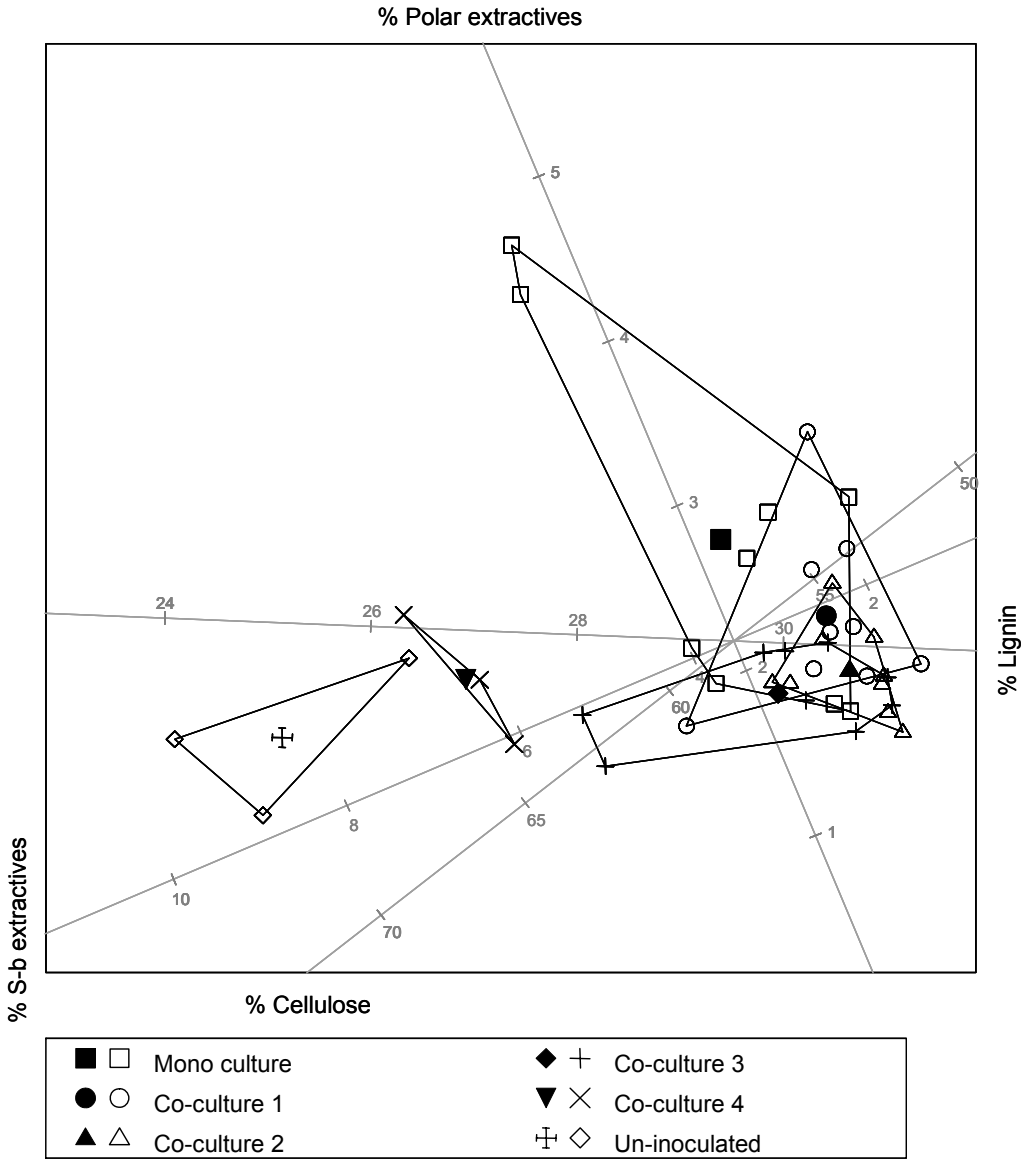


Figure 4. A PCA biplot showing distances among the various samples and their variation with respect to the chemical properties measured. The means of the parameters measured for each culture are indicated as a solid symbol. (S-b extractives = solvent borne extractives). Monoculture = *Pycnoporus sanguineus* PPRI 6762; Co-culture 1 = *P. sanguineus* PPRI 6762 + *Pichia guilliermondii* ABA006; Co-culture 2 = *P. sanguineus* PPRI 6762 + *P. guilliermondii* ABA006 + *Rhodotorula glutinis* ABA003; Co-culture 3 = *P. sanguineus* PPRI 6762 + autoclaved *P. guilliermondii* ABA006 + autoclaved *R.glutinis* ABA003; Co-culture 4 = *P. sanguineus* PPRI 6762 + *Aspergillus flavipes* J11904; Un-inoculated = Un-inoculated hot water washed wood chips.

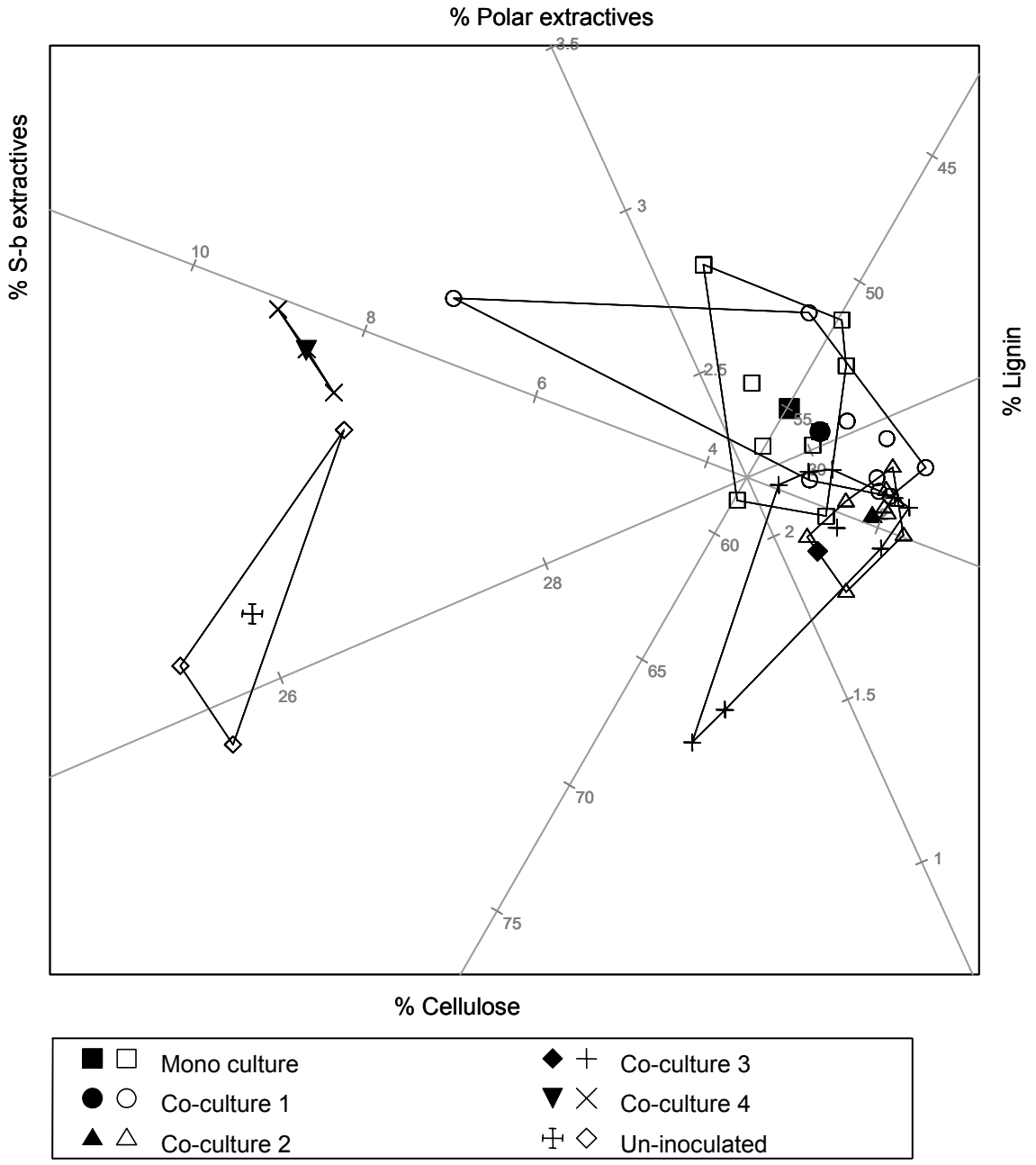


Figure 5. A CVA biplot displaying the differences and overlap between the culture combinations. (S-b extractives = solvent borne extractives). Monoculture = *Pycnoporus sanguineus* PPRI 6762; Co-culture 1 = *P. sanguineus* PPRI 6762 + *Pichia guilliermondii* ABA006; Co-culture 2 = *P. sanguineus* PPRI 6762 + *P. guilliermondii* ABA006 + *Rhodotorula glutinis* ABA003; Co-culture 3 = *P. sanguineus* PPRI 6762 + autoclaved *P. guilliermondii* ABA006 + autoclaved *R.glutinis* ABA003; Co-culture 4 = *P. sanguineus* PPRI 6762 + *Aspergillus flavipes* J11904; Un-inoculated = Un-inoculated hot water washed wood chips.

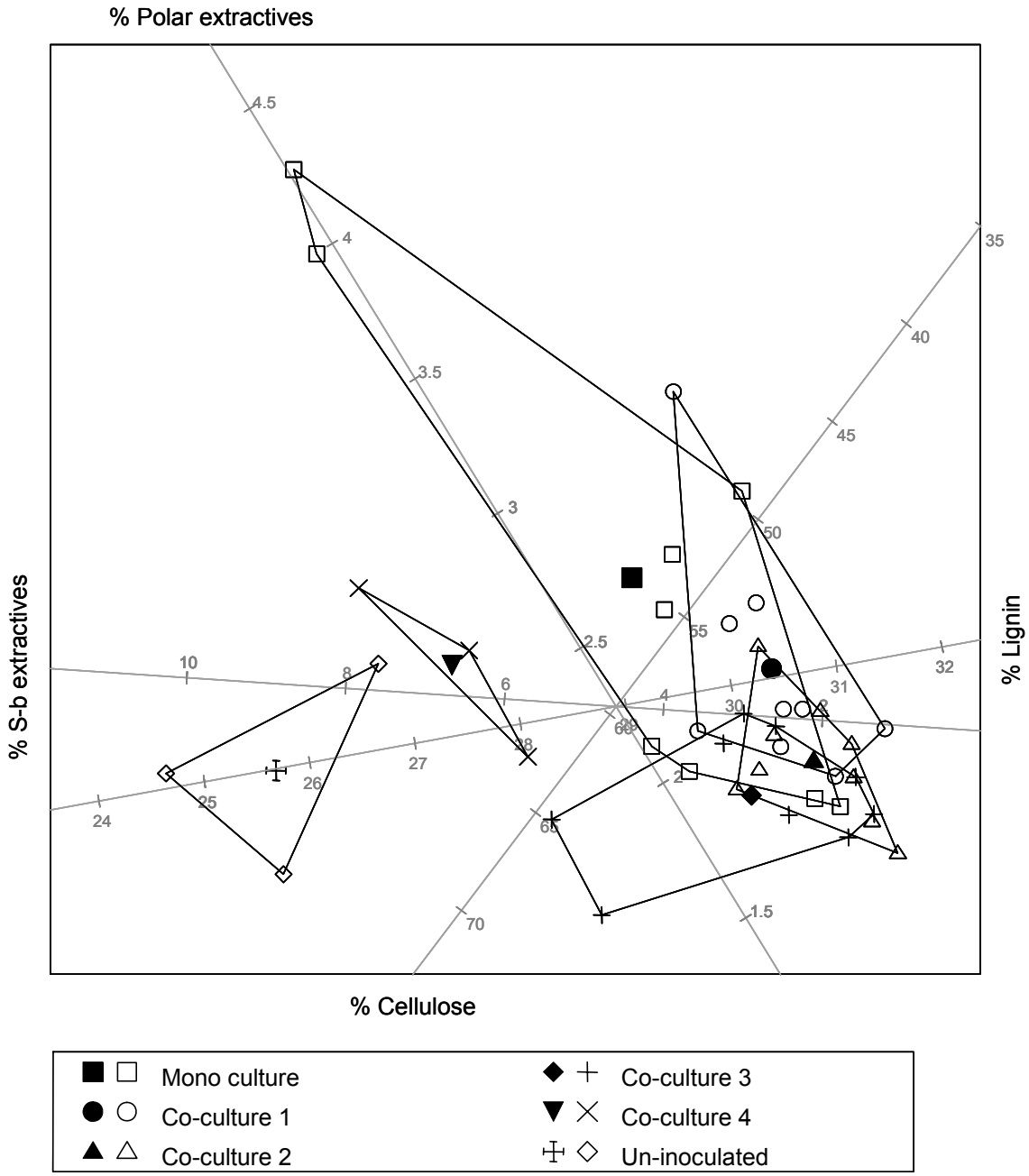


Figure 6. An AOD biplot displaying the differences and overlap between the culture combinations. (S-b extractives = solvent borne extractives). Monoculture = *Pycnoporus sanguineus* PPRI 6762; Co-culture 1 = *P. sanguineus* PPRI 6762 + *Pichia guilliermondii* ABA006; Co-culture 2 = *P. sanguineus* PPRI 6762 + *P. guilliermondii* ABA006 + *Rhodotorula glutinis* ABA003; Co-culture 3 = *P. sanguineus* PPRI 6762 + autoclaved *P. guilliermondii* ABA006 + autoclaved *R.glutinis* ABA003; Co-culture 4 = *P. sanguineus* PPRI 6762 + *Aspergillus flavipes* J11904; Un-inoculated = Un-inoculated hot water washed wood chips.

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Chapter 3

Chemical alterations of wood induced by *Pycnoporus sanguineus* / *Aspergillus flavipes* co-cultures while growing on different tree species

3.1. Introduction

Among the microorganisms responsible for wood degradation, fungi are the dominant group in terrestrial ecosystems (Schwarze *et al*., 2000). They use various processes to degrade woody tissues resulting in three forms of decay: Brown-rot, soft-rot and white-rot. The latter form of decay is caused by a group of fungi that are able to degrade lignin in addition to cellulose and hemicellulose (Otjen & Blanchette, 1986; Myneni *et al*., 2001). White-rot fungi, for example *Fomes fomentarius*, simultaneously degrade cellulose, hemicellulose and lignin, while *Ganoderma pheifferi* selectively degrades lignin and hemicelluloses first (Schwarze *et al*., 2000). *Pycnoporus sanguineus*, a known white-rot fungus, showed a selective delignification pattern on poplar trees, while exerting a simultaneous delignification pattern when cultured on *Eucalyptus grandis* wood (Ferraz *et al*., 1998; Luna *et al*., 2004). This indicates that this white-rot fungus may shift between delignification patterns depending on the wood it grows on.

The majority of studies on delignification of wood focused on pure cultures of white-rot fungi (Luna *et al*., 2004). The effect of co-cultures on wood is, however, closer to the situation in nature, as consortia of microbes are known to degrade lignocellulosic material (Watanabe *et al*., 2003). Studies by Dommisse in 1998 indicated that a co-culture of *Pycnoporus sanguineus* and *Aspergillus flavipes* enhanced the pulping properties of *E. grandis*. Our own results (Chapter 2) indicated that the *P. sanguineus* / *A. flavipes* co-culture caused less cellulose degradation compared to the mono cultures of this white-rot fungus.

Since literature indicates that *P. sanguineus* may have the ability to shift between degradation patterns, the question arose whether the effect of a *P. sanguineus* / *A. flavipes* co-culture on the chemical composition of wood, will differ between tree species. Subsequently, the aim of this study was to investigate chemical alterations in different tree species, induced by a *P. sanguineus* / *A. flavipes* co-culture.

3.2. Materials and Methods

3.2.1. Assessing the degradation of wood components by *P.sanguineus/A. flavipes* co-cultures

A strain representing *P. sanguineus* was obtained from the fungal culture collection of the ARC-Plant Protection Research Institute (PPRI), Pretoria, South Africa. This white rot fungus (*P. sanguineus* PPRI 6762), as well as *A. flavipes* J11904 are being maintained at 22°C on MEA in the fungal culture collection of the Department of Microbiology, University of Stellenbosch, South Africa.

Twelve year old *Acacia mearnsii, Eucalyptus dunnii, Eucalyptus grandis,* and *Eucalyptus macarthurii* trees were obtained from plantations on the Eastern Highveld of South-Africa. The trees were chipped and only the fraction greater than 6 mm and less than 9 mm in thickness was retained for experimentation. To enhance weathering, the wood chips were pre-treated in a pressure vessel of 15 dm^3 capacity with a hot water wash at 150°C for two hours. To ensure that the water mixes well with the wood chips and fibres, the vessel oscillated through 45° to either side. When the temperature reached 150°C, the vessel degassed automatically and the pressure dropped in about 12 minutes from 800 kPa to 0 kPa. Thereafter the pressure was increased until it reached the maximum 800kPa where it was maintained for 25 minutes.

To determine the moisture content of the wood a sub-sample of these chips (± 20g) was oven dried for 14 h at 100°C and weighed. In order to obtain a final moisture content of 60% (Wolfaardt *et al.,* 2004) a nutrient supplement [5% (w/v) molasses and 0.28% (w/v) urea], as well as an appropriate volume of fungal inoculum, were added to the chips.

Inoculums of *A. flavipes* J11904 and *P. sanguineus* PPRI 6762 were prepared by growing these strains at 30°C in 5% (w/v) molasses broth. After one week of

incubation, the fungal biomass of each culture was homogenized using a blender (Pineware) for 30 s. This homogenized fungal biomass was subsequently used to inoculate woodchips from different tree species (Table 1). The latter were contained in bio-reactors, respectively each receiving an inoculum of 1.8 x 10^{-4} g and 1.7 x 10^{-4} g dry biomass per gram oven dried wood for *A. flavipes*, and *P. sanguineus.*

Table 1. Different treatments of wood chips originating from the different tree species including inoculation with *P. sanguineus* PPRI 6762 / *A. flavipes* J11904 co-cultures.

Acronym for treatment class	***Tree species and treatment of wood chips***
AmUN	*Acacia mearnsii*; no hot water wash; no inoculum
AmWO	*Acacia mearnsii*; hot water wash; no inoculum
AmWB	*Acacia mearnsii*; hot water wash; co-culture inoculum
EdUN	*Eucalyptus dunnii*; no hot water wash; no inoculum
EdWO	*Eucalyptus dunnii*; hot water wash; no inoculum
EdWB	*Eucalyptus dunnii*; hot water wash; co-culture inoculum
EgUN	*Eucalyptus grandis*; no hot water wash; no inoculum
EgWO	*Eucalyptus grandis*; hot water wash; no inoculum
EgWB	*Eucalyptus grandis*; hot water wash; co-culture inoculum
EmUN	*Eucalyptus macarthurii*; no hot water wash; no inoculum
EmWO	*Eucalyptus macarthurii*; hot water wash; no inoculum
EmWB	*Eucalyptus macarthurii*; hot water wash; co-culture inoculum

These bio-reactors consisted of closed cylindrical plastic vessels (17 cm high and 23 cm in diameter), each containing 1 kg of wood chip woods resting on a grid 5 cm from the bottom to allow for aeration. After inoculation each bio-reactor was incubated at 30°C, while being aerated from below the grid with 10 L/ min sterile

moist air blown through a water trap using an electro-magnetic air compressor (Style King, Model ACQ-009A). After 14 days of incubation the cultures were harvested and the chemical properties of the residual wood were analyzed using standard TAPPI methodology.

3.2.2. Chemical analyses of wood chips.

To provide an indication of the chemical alterations that occurred in the wood chips during treatment of the wood chips, the residual wood chips obtained after fungal cultivation, the un-inoculated hot water washed chips, as well as un-inoculated untreated wood chips (Table 1) were analysed. Alcohol-benzene and water soluble extractive contents as well as lignin and cellulose content were determined during this process.

Extractions were done by boiling 3 g of the residual wood chips in either 200 ml ethanol-benzene or water for six to eight hours (TAPPI Standard Methods T 264 om-88). After ethanol-benzene extraction, the wood was washed with 95% ethanol to remove the benzene. This was followed by washing the chips with distilled water to remove ethanol. The chips were subsequently boiled in 500 ml distilled water for one hour, whereafter it was washed with 500 ml boiling water and air dried. Subsequently, the moisture content was determined to calculate the percentage extractives in the wood.

Klason Lignin is defined as the wood components that are insoluble in 72% sulphuric acid (TAPPI Standard Methods T 222 om-88). For the determination of lignin content, 15 ml 72% sulphuric acid was added to 5 g oven dried extractive free wood. This reaction mixture was subsequently incubated at 20°C for two hours. After the incubation period, the material was added to a beaker of water until a 3% concentration of the sulfuric acid was reached. The resulting suspension was boiled for four hours at a constant volume. After boiling, the insoluble material (lignin) was allowed to settle. The supernatant was

subsequently discarded and the precipitate washed with water, dried and weighed.

The Seifert method was used to gravimetrically determine cellulose in the residual wood chips (Browning, 1967). Using a boiling water bath, 1 g of extractive free wood meal was refluxed in a solution containing 6 ml acetylacetone, 2 ml dioxane and 1.5 ml hydrochloric acid. After 30 min the mixture was then washed successively with methanol, dioxane, hot water, dioxane, methanol and ether. The residue was subsequently weighed after drying at 105°C.

3.3. Results and Discussion

3.3.1. Motivation for and results of statistical analyses

Similar to the data described in section 2.3.2, three replicate chemical analyses were performed on a single co-culture treated sample of each species, as well as three replicate chemical analyses on an un-inoculated sample obtained for each tree species after the hot water wash. As is described in section 2.3.2 the Untreated data for each tree species consisted of a total of 30 observations: Three chemical analysis replicates of chip samples originating from two trees randomly selected from five locations representing the area of largest biological variation. Unfortunately, the results for three chemical analyses of *A. mearnsii* were lost during the chemical analysis process. The complete data set is provided in Table 2.

Table 2. Measurements (%) obtained during chemical analyses as described in section 3.3.1.

Treatment	Species	Class	Cel	Lig	EBE	EH2
HWE	Amea	AmWO	59.18	17.36	5.65	2.72
HWE	Amea	AmWO	65.44	19.05	4.81	3.14
HWE	Amea	AmWO	66.15	21.40	5.02	3.56
HWE	Edun	EdWO	67.14	22.38	10.10	4.54
HWE	Edun	EdWO	67.23	24.00	10.68	2.26
HWE	Edun	EdWO	70.19	21.91	10.25	2.61
HWE	Egran	EgWO	75.83	26.30	7.58	2.53
HWE	Egran	EgWO	62.95	26.35	7.79	2.74
HWE	Egran	EgWO	74.04	24.18	8.21	2.95
HWE	Emac	EmWO	57.03	25.96	7.07	3.33
HWE	Emac	EmWO	56.43	26.14	6.66	4.58
HWE	Emac	EmWO	55.51	21.42	7.07	2.08
HWEBP	Amea	AmWB	71.56	22.11	6.60	1.92
HWEBP	Amea	AmWB	65.78	22.21	7.88	1.49
HWEBP	Amea	AmWB	69.35	24.54	7.67	1.28
HWEBP	Edun	EdWB	68.23	24.26	9.92	1.90
HWEBP	Edun	EdWB	67.33	23.96	9.50	2.53
HWEBP	Edun	EdWB	65.58	24.09	9.92	2.53
HWEBP	Egran	EgWB	61.28	26.68	8.51	2.98
HWEBP	Egran	EgWB	61.13	28.49	8.51	2.13
HWEBP	Egran	EgWB	60.01	28.58	9.35	2.55
HWEBP	Emac	EmWB	56.79	25.86	6.56	1.90
HWEBP	Emac	EmWB	55.08	25.37	8.04	1.48
HWEBP	Emac	EmWB	56.31	28.12	7.19	1.69
UNTREAT	Amea	AmUN	45.36	20.09	2.60	2.00
UNTREAT	Amea	AmUN	45.61	21.83	2.60	2.00
UNTREAT	Amea	AmUN	50.84	17.95	3.20	2.20
UNTREAT	Amea	AmUN	49.20	19.85	3.20	1.40
UNTREAT	Amea	AmUN	48.94	20.14	2.80	1.60
UNTREAT	Amea	AmUN	55.66	24.17	1.80	2.20
UNTREAT	Amea	AmUN	55.67	20.04	1.20	1.00
UNTREAT	Amea	AmUN	57.18	19.97	1.60	1.20
UNTREAT	Amea	AmUN	54.39	21.97	1.40	0.80
UNTREAT	Amea	AmUN	46.36	19.83	2.20	2.40
UNTREAT	Amea	AmUN	46.47	21.83	2.20	2.40
UNTREAT	Amea	AmUN	48.08	22.13	2.20	2.20
UNTREAT	Amea	AmUN	48.15	23.80	2.60	2.60
UNTREAT	Amea	AmUN	46.31	20.01	2.40	2.20
UNTREAT	Amea	AmUN	48.06	22.12	2.60	2.60
UNTREAT	Amea	AmUN	45.45	19.83	3.40	1.60
UNTREAT	Amea	AmUN	46.36	22.00	3.60	2.00
UNTREAT	Amea	AmUN	46.53	20.04	3.00	2.00
UNTREAT	Amea	AmUN	51.39	24.04	2.60	1.80
UNTREAT	Amea	AmUN	50.49	21.82	2.60	2.00
UNTREAT	Amea	AmUN	48.08	21.90	3.00	1.80
UNTREAT	Amea	AmUN	47.44	25.84	2.80	1.80
UNTREAT	Amea	AmUN	49.14	19.85	3.20	1.60
UNTREAT	Amea	AmUN	49.98	19.83	2.00	1.20
UNTREAT	Amea	AmUN	48.10	20.13	1.60	2.60
UNTREAT	Amea	AmUN	54.71	24.03	1.60	1.80
UNTREAT	Amea	AmUN	54.56	21.97	1.20	1.20
UNTREAT	Edun	EdUN	49.08	21.89	3.20	2.60
UNTREAT	Edun	EdUN	49.21	22.06	3.60	2.40
UNTREAT	Edun	EdUN	34.68	17.92	2.60	2.20
UNTREAT	Edun	EdUN	48.09	19.96	2.60	2.40
UNTREAT	Edun	EdUN	55.60	22.15	2.80	2.40
UNTREAT	Edun	EdUN	49.86	24.15	2.40	2.20
UNTREAT	Edun	EdUN	46.48	15.99	2.80	2.40
UNTREAT	Edun	EdUN	45.33	15.98	2.20	2.40
UNTREAT	Edun	EdUN	47.13	13.98	2.60	2.00
UNTREAT	Edun	EdUN	46.19	16.02	2.20	2.20
UNTREAT	Edun	EdUN	43.67	23.94	2.00	2.00

Treatment	Species	Class	Cel	Lig	EBE	EH2
UNTREAT	Edun	EdUN	46.31	18.01	2.60	1.80
UNTREAT	Edun	EdUN	47.38	19.98	1.80	2.20
UNTREAT	Edun	EdUN	47.37	23.94	1.60	2.60
UNTREAT	Edun	EdUN	48.19	19.88	1.60	2.60
UNTREAT	Edun	EdUN	58.40	18.09	1.40	1.40
UNTREAT	Edun	EdUN	45.32	19.88	1.80	1.20
UNTREAT	Edun	EdUN	42.64	15.87	1.60	1.80
UNTREAT	Edun	EdUN	43.57	22.11	2.40	2.40
UNTREAT	Edun	EdUN	41.89	19.84	2.20	3.40
UNTREAT	Edun	EdUN	43.79	22.04	2.80	4.00
UNTREAT	Egran	EgUN	46.39	26.09	3.20	2.00
UNTREAT	Egran	EgUN	45.55	22.15	3.00	3.60
UNTREAT	Egran	EgUN	45.29	26.03	3.00	4.00
UNTREAT	Egran	EgUN	47.14	18.07	2.00	2.00
UNTREAT	Egran	EgUN	46.39	25.93	1.80	2.00
UNTREAT	Egran	EgUN	48.34	26.16	1.80	2.20
UNTREAT	Egran	EgUN	52.85	26.02	3.00	1.80
UNTREAT	Egran	EgUN	53.46	22.03	2.60	2.00
UNTREAT	Egran	EgUN	56.14	20.02	2.40	1.60
UNTREAT	Egran	EgUN	46.31	22.08	3.00	3.00
UNTREAT	Egran	EgUN	46.48	20.02	3.40	3.60
UNTREAT	Egran	EgUN	49.10	21.93	3.20	2.40
UNTREAT	Egran	EgUN	48.00	17.96	2.40	4.20
UNTREAT	Egran	EgUN	48.05	23.93	2.20	4.20
UNTREAT	Egran	EgUN	48.02	20.15	2.60	1.60
UNTREAT	Egran	EgUN	52.80	23.83	3.40	2.00
UNTREAT	Egran	EgUN	54.38	20.07	3.60	1.60
UNTREAT	Egran	EgUN	52.90	20.06	3.40	1.40
UNTREAT	Egran	EgUN	51.02	20.08	2.20	4.60
UNTREAT	Egran	EgUN	49.92	18.00	2.60	5.20
UNTREAT	Egran	EgUN	48.10	15.90	2.20	4.20
UNTREAT	Egran	EgUN	47.21	20.03	3.80	3.80
UNTREAT	Egran	EgUN	47.21	18.03	3.20	3.60
UNTREAT	Egran	EgUN	46.24	24.20	3.20	3.60
UNTREAT	Egran	EgUN	57.19	18.00	2.20	2.20
UNTREAT	Egran	EgUN	58.40	18.04	2.40	2.00
UNTREAT	Egran	EgUN	56.51	22.06	2.80	2.40
UNTREAT	Egran	EgUN	49.15	20.06	1.20	4.40
UNTREAT	Egran	EgUN	49.27	18.12	1.00	4.60
UNTREAT	Egran	EgUN	49.01	16.02	1.60	3.40
UNTREAT	Emac	EmUN	43.78	21.83	1.40	1.80
UNTREAT	Emac	EmUN	50.91	22.00	1.80	2.40
UNTREAT	Emac	EmUN	43.58	22.07	2.20	2.20
UNTREAT	Emac	EmUN	44.60	26.20	1.80	2.20
UNTREAT	Emac	EmUN	46.47	26.18	2.20	1.80
UNTREAT	Emac	EmUN	45.36	25.90	2.20	2.00
UNTREAT	Emac	EmUN	41.02	26.03	2.20	2.60
UNTREAT	Emac	EmUN	39.91	25.94	2.40	2.60
UNTREAT	Emac	EmUN	41.02	25.82	2.60	2.00
UNTREAT	Emac	EmUN	41.77	30.22	2.20	3.00
UNTREAT	Emac	EmUN	41.66	19.93	2.40	3.60
UNTREAT	Emac	EmUN	40.88	27.75	2.40	2.80
UNTREAT	Emac	EmUN	46.51	24.15	3.20	3.00
UNTREAT	Emac	EmUN	42.63	24.05	3.00	3.40
UNTREAT	Emac	EmUN	43.56	21.90	3.60	2.80
UNTREAT	Emac	EmUN	44.62	18.09	1.80	2.20
UNTREAT	Emac	EmUN	45.61	22.18	1.60	3.00
UNTREAT	Emac	EmUN	46.34	21.88	2.00	2.00
UNTREAT	Emac	EmUN	42.72	28.18	2.40	1.20
UNTREAT	Emac	EmUN	47.21	29.92	2.20	2.60
UNTREAT	Emac	EmUN	47.41	28.13	2.60	2.80
UNTREAT	Emac	EmUN	46.27	23.99	2.40	3.00

Table 2. (Continued)

Treatment	Species	Class	Cel	Lig	EBE	EH2
UNTREAT	Edun	EdUN	48.09	22.16	2.00	2.60
UNTREAT	Edun	EdUN	40.14	23.87	2.00	2.80
UNTREAT	Edun	EdUN	42.68	25.82	2.20	2.60
UNTREAT	Edun	EdUN	43.54	21.92	2.40	2.40
UNTREAT	Edun	EdUN	42.89	21.92	2.40	3.60
UNTREAT	Edun	EdUN	42.67	23.89	2.20	2.60
UNTREAT	Edun	EdUN	41.79	24.10	2.20	2.80
UNTREAT	Edun	EdUN	40.89	16.04	2.40	1.60
UNTREAT	Edun	EdUN	48.08	20.02	2.20	1.80

Treatment	Species	Class	Cel	Lig	EBE	EH2
UNTREAT	Emac	EmUN	48.00	23.95	2.20	2.80
UNTREAT	Emac	EmUN	47.14	24.09	2.20	2.20
UNTREAT	Emac	EmUN	40.11	25.87	3.00	2.20
UNTREAT	Emac	EmUN	40.85	23.82	2.80	2.20
UNTREAT	Emac	EmUN	44.61	25.80	3.20	2.00
UNTREAT	Emac	EmUN	40.14	17.86	2.60	2.60
UNTREAT	Emac	EmUN	39.88	25.92	2.60	2.40
UNTREAT	Emac	EmUN	40.15	26.10	2.80	3.00

Explanations for acronyms listed in the "treatment column" are; HWE = Hot water extracted wood chips; HWEBP = Hot water extracted wood chips, followed by inoculation of fungal co-culture; UNTREAT = untreated wood chips. The acronyms in the "species column" designate different tree/wood species; Amea = *Acacia mearnsii*; Edun = *Eucalyptus dunnii*; Egran = *Eucalyptus grandis*; Emac = *Eucalyptus macarthurii.* Explanations for acronyms listed in the "class column" designate different treatment classes and are provided in Table 1. Abbreviations for measured variables: Cel = % Cellulose; Lig = % Lignin; EBE = % Solvent-borne extractives; EH2 = % Polar extractives.

The above data are visually displayed in the form of notched box plots in Figure 1. Before discussing this figure however, the following had to be taken into account. The statistical analysis of the data in Table 2 (Chapter 3) differed in an important aspect from the statistical analysis of the data presented in Table 4 of Chapter 2: The data in Table 2 (Chapter 3) constitute two-way data viz. tree species x treatment. Therefore the first question that had to be addressed was the significance of the interaction between these two factors. This was done by performing a two-way MANOVA on the data. Since each cell in the two-way table associated with the data in Table 2 (Chapter 3) did not contain the same number of replicates, the order of terms entering the MANOVA model had to be taken into account. Table 3 contains the results of these MANOVAs.

Table 3. Results of MANOVA procedures performed on the two-way data in Table 2.

	Df	Pillai Trace	Approx. F	Num. df	Denom. df	P-value
Species	3	0.8844	13.3781	12	384	0
Treatment	2	1.1165	40.1221	8	254	0
Species:Treatment	6	0.8353	5.6744	24	516	0
Residuals	129					

Table 3. (Continued)

	Df	Pillai Trace	Approx. F	Num. df	Denom. df	P-value
Treatment	2	1.1164	40.1191	8	254	0
Species	3	0.9	13.7141	12	384	0
Species:Treatment	6	0.8353	5.6744	24	516	0
Residuals	129					

It is clear from Table 3 that the species x treatment interaction was statistically highly significant. Accordingly, the MANOVA was followed by performing two-way univariate analysis of variance (ANOVA) procedures on the data treating in turn each of % Cellulose, % Lignin, % Solvent-borne extractives and % Polar extractives as the response variable. Table 4 contains the mean values associated with the ANOVA procedures

Table 4. Means associated with the two-way ANOVA procedures

		Chemical property			
		% Cellulose	**% Lignin**	**% Solvent-borne extractives**	**% Polar extractives**
Species x Treatment combination	**AmUN**	49.57	21.37	2.41	1.86
	AmWB	68.90	22.95	7.38	1.56
	AmWO	63.59	19.27	5.16	3.14
	EdUN	45.70	20.45	2.29	2.38
	EdWB	67.04	24.10	9.78	2.32
	EdWO	68.18	22.76	10.34	3.14
	EgUN	49.89	21.04	2.61	2.97
	EgWB	60.80	27.92	8.79	2.55
	EgWO	70.94	25.61	7.86	2.74
	EmUN	43.82	24.52	2.40	2.48
	EmWB	56.06	26.45	7.26	1.69
	EmWO	56.32	24.51	6.93	3.33

The two-way ANOVA tables for % Cellulose, % Lignin, % Solvent-borne extractives and % Polar extractives are provided in Tables 5, 7, 9 and 11, respectively.

Table 5. ANOVA table for two-way ANOVA performed with % Cellulose as response variable.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Species	3	1083.507	361.169	26.740	0.0000
Treatment	2	5589.150	2794.575	206.902	0.0000
Species:Treatment	6	416.266	69.378	5.137	0.0000
Residuals	129	1742.371	13.507		

It follows from Table 5 that the species x treatment interaction for % Cellulose is statistically highly significant. Therefore simultaneous Tukey 99% confidence intervals (Scheffé,1959) were calculated for all pair-wise comparisons between the species x treatment combinations. These intervals are displayed in Table 6. The interpretation of the results of table 6 is deferred to section 3.3.

Table 6. Simultaneous Tukey 99% pair-wise confidence intervals between all species x treatment combinations in the case of mean % Cellulose values. An interval excluding zero (coloured in grey) suggests the rejection at a 1% significance level of the null hypothesis that the corresponding means are equal.

Contrast	Estimate	Stderr	Lower	Upper
AmUN–AmWB	–19.32	2.24	–27.91	–10.73
AmUN–AmWO	–14.02	2.24	–22.61	–5.43
AmUN–EdUN	3.88	0.97	0.13	7.62
AmUN–EdWB	–17.47	2.24	–26.06	–8.88
AmUN–EdWO	–18.61	2.24	–27.20	–10.02
AmUN–EgUN	–0.32	0.97	–4.06	3.43
AmUN–EgWB	–11.23	2.24	–19.82	–2.64
AmUN–EgWO	–21.37	2.24	–29.96	–12.77
AmUN–EmUN	5.75	0.97	2.01	9.49
AmUN–EmWB	–6.49	2.24	–15.08	2.10
AmUN–EmWO	–6.75	2.24	–15.34	1.84
AmWB–AmWO	5.31	3.00	–6.22	16.83
AmWB–EdUN	23.2	2.23	14.65	31.75
AmWB–EdWB	1.85	3.00	–9.67	13.38
AmWB–EdWO	0.71	3.00	–10.81	12.24
AmWB–EgUN	19.00	2.23	10.45	27.55
AmWB–EgWB	8.09	3.00	–3.43	19.62
AmWB–EgWO	–2.04	3.00	–13.57	9.48
AmWB–EmUN	25.07	2.23	16.52	33.62
AmWB–EmWB	12.83	3.00	1.31	24.36
AmWB–EmWO	12.57	3.00	1.05	24.10
AmWO–EdUN	17.89	2.23	9.34	26.44
AmWO–EdWB	–3.45	3.00	–14.98	8.07
AmWO–EdWO	–4.59	3.00	–16.12	6.93
AmWO–EgUN	13.70	2.23	5.15	22.25
AmWO–EgWB	2.79	3.00	–8.74	14.31
AmWO–EgWO	–7.35	3.00	–18.88	4.18
AmWO–EmUN	19.77	2.23	11.22	28.31
AmWO–EmWB	7.53	3.00	–4.00	19.05
AmWO–EmWO	7.27	3.00	–4.26	18.79
EdUN–EdWB	–21.35	2.23	–29.89	–12.8
EdUN–EdWO	–22.49	2.23	–31.03	–13.94

Table 6. (Continued)

Contrast	Estimate	Stderr	Lower	Upper
EdUN–EgUN	–4.20	0.95	–7.84	–0.55
EdUN–EgWB	–15.11	2.23	–23.65	–6.56
EdUN–EgWO	–25.24	2.23	–33.79	–16.69
EdUN–EmUN	1.87	0.95	–1.77	5.52
EdUN–EmWB	–10.36	2.23	–18.91	–1.82
EdUN–EmWO	–10.62	2.23	–19.17	–2.08
EdWB–EdWO	–1.14	3.00	–12.67	10.39
EdWB–EgUN	17.15	2.23	8.60	25.70
EdWB–EgWB	6.24	3.00	–5.29	17.77
EdWB–EgWO	–3.90	3.00	–15.42	7.63
EdWB–EmUN	23.22	2.23	14.67	31.77
EdWB–EmWB	10.98	3.00	–0.54	22.51
EdWB–EmWO	10.72	3.00	–0.80	22.25
EdWO–EgUN	18.29	2.23	9.74	26.84
EdWO–EgWB	7.38	3.00	–4.15	18.91
EdWO–EgWO	–2.76	3.00	–14.28	8.77
EdWO–EmUN	24.36	2.23	15.81	32.91
EdWO–EmWB	12.12	3.00	0.60	23.65
EdWO–EmWO	11.86	3.00	0.34	23.39
EgUN–EgWB	–10.91	2.23	–19.46	–2.36
EgUN–EgWO	–21.05	2.23	–29.59	–12.50
EgUN–EmUN	6.07	0.95	2.42	9.71
EgUN–EmWB	–6.17	2.23	–14.72	2.38
EgUN–EmWO	–6.43	2.23	–14.98	2.12
EgWB–EgWO	–10.14	3.00	–21.66	1.39
EgWB–EmUN	16.98	2.23	8.43	25.53
EgWB–EmWB	4.74	3.00	–6.78	16.27
EgWB–EmWO	4.48	3.00	–7.04	16.01
EgWO–EmUN	27.12	2.23	18.57	35.66
EgWO–EmWB	14.88	3.00	3.35	26.40
EgWO–EmWO	14.62	3.00	3.09	26.14
EmUN–EmWB	–12.24	2.23	–20.79	–3.69
EmUN–EmWO	–12.50	2.23	–21.05	–3.95
EmWB–EmWO	–0.26	3.00	–11.79	11.27

Table 7. ANOVA table for two-way ANOVA performed with % Lignin as response variable.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Species	3	305.1844	101.7281	13.716	0.0000
Treatment	2	143.5718	71.7859	9.679	0.0001
Species:Treatment	6	106.6522	17.7754	2.397	0.0315
Residuals	129	956.7328	7.4165		

It follows from Table 7 that the species x treatment interaction for % Lignin is statistically significant at an approximate 3% level of significance. Therefore simultaneous Tukey 95% confidence intervals (Scheffé, 1955) were calculated for all pair-wise comparisons between the % Lignin means for all species x treatment combinations. These intervals are displayed in Table 8.

Table 8. Simultaneous Tukey 95% pair-wise confidence intervals between all species x treatment combinations in the case of mean % Lignin values. An interval excluding zero (coloured in grey) suggests the rejection at a 1% significance level of the null hypothesis that the corresponding means are equal.

Contrast	Estimate	Stderr	Lower	Upper
AmUN–AmWB	–1.58	1.66	–7.10	3.93
AmUN–AmWO	2.10	1.66	–3.42	7.62
AmUN–EdUN	0.92	0.72	–1.48	3.33
AmUN–EdWB	–2.73	1.66	–8.25	2.78
AmUN–EdWO	–1.39	1.66	–6.91	4.13
AmUN–EgUN	0.34	0.72	–2.07	2.74
AmUN–EgWB	–6.55	1.66	–12.06	–1.03
AmUN–EgWO	–4.24	1.66	–9.76	1.28
AmUN–EmUN	–3.15	0.72	–5.56	–0.75
AmUN–EmWB	–5.08	1.66	–10.60	0.44
AmUN–EmWO	–3.13	1.66	–8.65	2.38
AmWB–AmWO	3.68	2.22	–3.72	11.08
AmWB–EdUN	2.51	1.65	–2.98	8.00
AmWB–EdWB	–1.15	2.22	–8.55	6.25
AmWB–EdWO	0.19	2.22	–7.21	7.59
AmWB–EgUN	1.92	1.65	–3.57	7.41
AmWB–EgWB	–4.96	2.22	–12.37	2.44
AmWB–EgWO	–2.66	2.22	–10.06	4.75
AmWB–EmUN	–1.57	1.65	–7.06	3.92
AmWB–EmWB	–3.50	2.22	–10.90	3.91
AmWB–EmWO	–1.55	2.22	–8.95	5.85
AmWO–EdUN	–1.18	1.65	–6.66	4.31
AmWO–EdWB	–4.83	2.22	–12.23	2.57
AmWO–EdWO	–3.49	2.22	–10.89	3.91
AmWO–EgUN	–1.76	1.65	–7.25	3.73
AmWO–EgWB	–8.65	2.22	–16.05	–1.24
AmWO–EgWO	–6.34	2.22	–13.74	1.06
AmWO–EmUN	–5.25	1.65	–10.74	0.24
AmWO–EmWB	–7.18	2.22	–14.58	0.22
AmWO–EmWO	–5.23	2.22	–12.64	2.17
EdUN–EdWB	–3.66	1.65	–9.15	1.83
EdUN–EdWO	–2.32	1.65	–7.81	3.17
EdUN–EgUN	–0.59	0.70	–2.93	1.75
EdUN–EgWB	–7.47	1.65	–12.96	–1.98
EdUN–EgWO	–5.16	1.65	–10.65	0.33
EdUN–EmUN	–4.08	0.70	–6.42	–1.74
EdUN–EmWB	–6.00	1.65	–11.49	–0.51
EdUN–EmWO	–4.06	1.65	–9.55	1.43
EdWB–EdWO	1.34	2.22	–6.06	8.74
EdWB–EgUN	3.07	1.65	–2.42	8.56
EdWB–EgWB	–3.81	2.22	–11.22	3.59
EdWB–EgWO	–1.51	2.22	–8.91	5.90
EdWB–EmUN	–0.42	1.65	–5.91	5.07
EdWB–EmWB	–2.35	2.22	–9.75	5.06
EdWB–EmWO	–0.40	2.22	–7.80	7.00
EdWO–EgUN	1.73	1.65	–3.76	7.22
EdWO–EgWB	–5.16	2.22	–12.56	2.25
EdWO–EgWO	–2.85	2.22	–10.25	4.55
EdWO–EmUN	–1.76	1.65	–7.25	3.73
EdWO–EmWB	–3.69	2.22	–11.09	3.71
EdWO–EmWO	–1.74	2.22	–9.15	5.66
EgUN–EgWB	–6.88	1.65	–12.37	–1.39
EgUN–EgWO	–4.57	1.65	–10.06	0.92
EgUN–EmUN	–3.49	0.70	–5.83	–1.15

Table 8. (Continued)

Contrast	Estimate	Stderr	Lower	Upper
EgUN–EmWB	–5.41	1.65	–10.90	0.08
EgUN–EmWO	–3.47	1.65	–8.96	2.02
EgWB–EgWO	2.31	2.22	–5.09	9.71
EgWB–EmUN	3.39	1.65	–2.10	8.88
EgWB–EmWB	1.47	2.22	–5.93	8.87
EgWB–EmWO	3.41	2.22	–3.99	10.81
EgWO–EmUN	1.09	1.65	–4.40	6.58
EgWO–EmWB	–0.84	2.22	–8.24	6.56
EgWO–EmWO	1.10	2.22	–6.30	8.51
EmUN–EmWB	–1.93	1.65	–7.42	3.56
EmUN–EmWO	0.02	1.65	–5.47	5.51
EmWB–EmWO	1.94	2.22	–5.46	9.35

Table 9. ANOVA table for two-way ANOVA performed with % solvent-borne extractives as response variable.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Species	3	6.4727	2.1576	6.338	0.0005
Treatment	2	609.1101	304.5550	894.610	0.0000
Species:Treatment	6	48.5598	8.0933	23.774	0.0000
Residuals	129	43.9159	0.3404		

It follows from Table 9 that the species x treatment interaction for % solvent-borne extractives is statistically highly significant. Therefore simultaneous Tukey 99% confidence intervals (Scheffé, 1955) were calculated for all pair-wise comparisons between the species x treatment combinations in the case of % Solvent-borne extractives. These intervals are displayed in Table 10.

Table 10. Simultaneous Tukey 99% pair-wise confidence intervals between all species x treatment combinations in the case of mean % Solvent-borne extractives values. An interval excluding zero (coloured in grey) suggests the rejection at a 1% significance level of the null hypothesis that the corresponding means are equal.

Contrast	Estimate	Stderr	Lower	Upper
AmUN–AmWB	–4.97	0.36	–6.33	–3.60
AmUN–AmWO	–2.75	0.36	–4.11	–1.39
AmUN–EdUN	0.12	0.15	–0.47	0.72
AmUN–EdWB	–7.37	0.36	–8.73	–6.00
AmUN–EdWO	–7.93	0.36	–9.29	–6.57
AmUN–EgUN	–0.20	0.15	–0.79	0.40
AmUN–EgWB	–6.38	0.36	–7.74	–5.01
AmUN–EgWO	–5.44	0.36	–6.81	–4.08
AmUN–EmUN	0.01	0.15	–0.58	0.61
AmUN–EmWB	–4.85	0.36	–6.21	–3.48
AmUN–EmWO	–4.52	0.36	–5.88	–3.15
AmWB–AmWO	2.22	0.48	0.39	4.05
AmWB–EdUN	5.09	0.35	3.73	6.45
AmWB–EdWB	–2.40	0.48	–4.23	–0.57

Table 10 . (Continued)

Contrast	Estimate	Stderr	Lower	Upper
AmWB–EdWO	–2.96	0.48	–4.79	–1.13
AmWB–EgUN	4.77	0.35	3.41	6.13
AmWB–EgWB	–1.41	0.48	–3.24	0.42
AmWB–EgWO	–0.48	0.48	–2.31	1.35
AmWB–EmUN	4.98	0.35	3.62	6.34
AmWB–EmWB	0.12	0.48	–1.71	1.95
AmWB–EmWO	0.45	0.48	–1.38	2.28
AmWO–EdUN	2.87	0.35	1.51	4.23
AmWO–EdWB	–4.62	0.48	–6.45	–2.79
AmWO–EdWO	–5.18	0.48	–7.01	–3.35
AmWO–EgUN	2.55	0.35	1.19	3.91
AmWO–EgWB	–3.63	0.48	–5.46	–1.80
AmWO–EgWO	–2.69	0.48	–4.52	–0.86
AmWO–EmUN	2.76	0.35	1.41	4.12
AmWO–EmWB	–2.10	0.48	–3.93	–0.27
AmWO–EmWO	–1.77	0.48	–3.60	0.06
EdUN–EdWB	–7.49	0.35	–8.84	–6.13
EdUN–EdWO	–8.05	0.35	–9.41	–6.69
EdUN–EgUN	–0.32	0.15	–0.90	0.26
EdUN–EgWB	–6.50	0.35	–7.86	–5.14
EdUN–EgWO	–5.56	0.35	–6.92	–4.21
EdUN–EmUN	–0.11	0.15	–0.69	0.47
EdUN–EmWB	–4.97	0.35	–6.33	–3.61
EdUN–EmWO	–4.64	0.35	–6.00	–3.28
EdWB–EdWO	–0.56	0.48	–2.39	1.27
EdWB–EgUN	7.17	0.35	5.81	8.52
EdWB–EgWB	0.99	0.48	–0.84	2.82
EdWB–EgWO	1.92	0.48	0.09	3.75
EdWB–EmUN	7.38	0.35	6.02	8.74
EdWB–EmWB	2.52	0.48	0.69	4.35
EdWB–EmWO	2.85	0.48	1.02	4.68
EdWO–EgUN	7.73	0.35	6.37	9.09
EdWO–EgWB	1.55	0.48	–0.28	3.38
EdWO–EgWO	2.49	0.48	0.66	4.32
EdWO–EmUN	7.94	0.35	6.59	9.30
EdWO–EmWB	3.08	0.48	1.25	4.91
EdWO–EmWO	3.41	0.48	1.58	5.24
EgUN–EgWB	–6.18	0.35	–7.53	–4.82
EgUN–EgWO	–5.24	0.35	–6.60	–3.89
EgUN–EmUN	0.21	0.15	–0.37	0.79
EgUN–EmWB	–4.65	0.35	–6.01	–3.29
EgUN–EmWO	–4.32	0.35	–5.68	–2.96
EgWB–EgWO	0.93	0.48	–0.90	2.76
EgWB–EmUN	6.39	0.35	5.03	7.75
EgWB–EmWB	1.53	0.48	–0.30	3.36
EgWB–EmWO	1.86	0.48	0.03	3.69
EgWO–EmUN	5.46	0.35	4.10	6.81
EgWO–EmWB	0.59	0.48	–1.24	2.42
EgWO–EmWO	0.92	0.48	–0.91	2.75
EmUN–EmWB	–4.86	0.35	–6.22	–3.51
EmUN–EmWO	–4.53	0.35	–5.89	–3.18
EmWB–EmWO	0.33	0.48	–1.50	2.16

Table 11. ANOVA table for two-way ANOVA performed with % Polar extractives as response variable.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Species	3	16.2522	5.4174	10.0923	0.0000
Treatment	2	7.0090	3.5045	6.5287	0.0020
Species:Treatment	6	4.1298	0.6883	1.2823	0.2699
Residuals	129	69.2453	0.5368		

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Treatment	2	6.9441	3.4720	6.4682	0.0021
Species	3	16.3172	5.4391	10.1327	0.0000
Species:Treatment	6	4.1298	0.6883	1.2823	0.2699
Residuals	129	69.2453	0.5368		

Table 11 consists of two sections: The upper one shows the ANOVA table where Species was fitted before Treatment and in the lower one Treatment is fitted before Species. Perusal of Table 11 leads to the following conclusions: When only % Polar extractives are considered, the interaction between species and treatment is negligible. Therefore, the species and treatment main effects can be considered separately. It is also clear that both these effects are statistically highly significant. The means associated with these two main effects are given in Table 12 and the associated pair wise 95% simultaneous confidence intervals in Tables 13 and 14, respectively.

Table 12. Mean % Polar extractives associated with the species and treatment main effects.

HWE	3.09
HWEBP	2.03
UNTREAT	2.44
Amea	1.95
Edun	2.44
Egran	2.92
Emac	2.49

Table 13. Simultaneous pair-wise 95% confidence intervals associated with the species main effect for % Polar extractives.

Contrast	Estimate	Stderr	Lower	Upper
Amea-Edun	-0.492	0.178	–0.95	–0.03
Amea-Egran	-0.972	0.178	–1.43	–0.51
Amea-Emac	-0.539	0.178	–1.00	–0.08
Edun-Egran	-0.481	0.174	–0.93	–0.03
Edun-Emac	-0.047	0.174	–0.50	0.41
Egran-Emac	0.434	0.174	–0.02	0.89

Table 14. Simultaneous pair-wise 95% confidence intervals associated with the treatment main effect for % Polar extractives.

Contrast	*Estimate*	*Stderr*	*Lower*	*Upper*
HWE–HWEBP	1.05	0.30	0.34	1.77
HWE–UNTREAT	0.66	0.22	0.13	1.19
HWEBP–UNTREAT	–0.39	0.22	–0.92	0.14

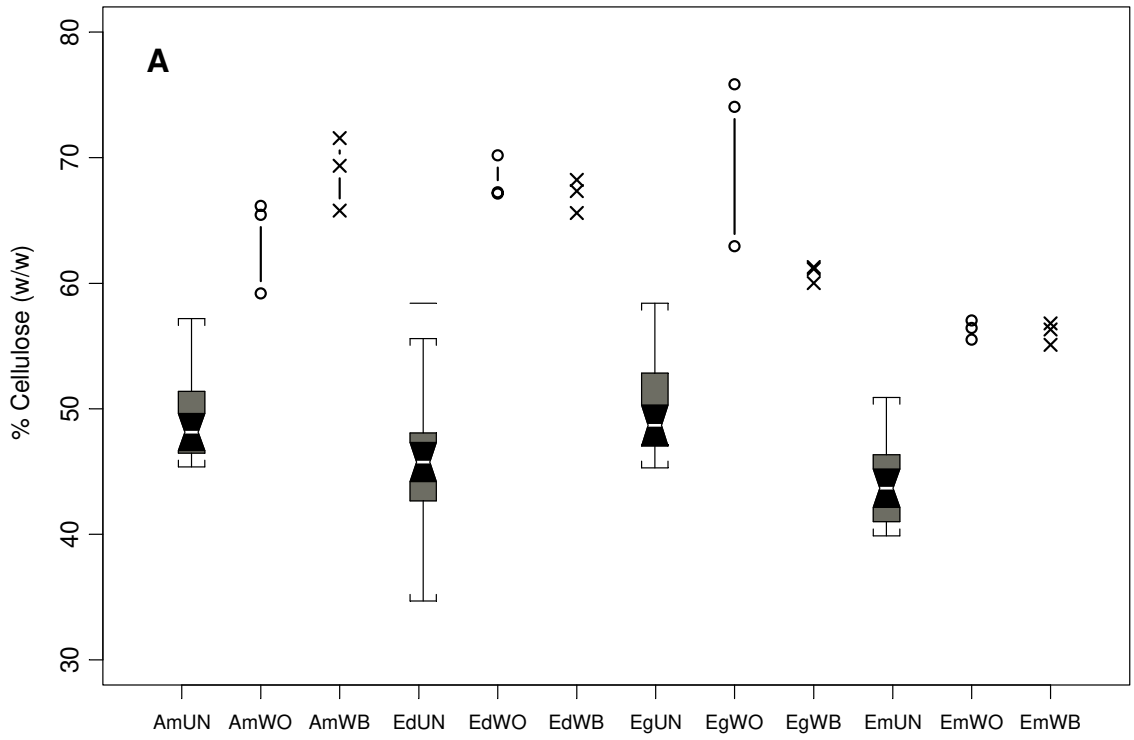
3.3.2. Degradation of wood components by *P.sanguineus/A. flavipes* co-cultures

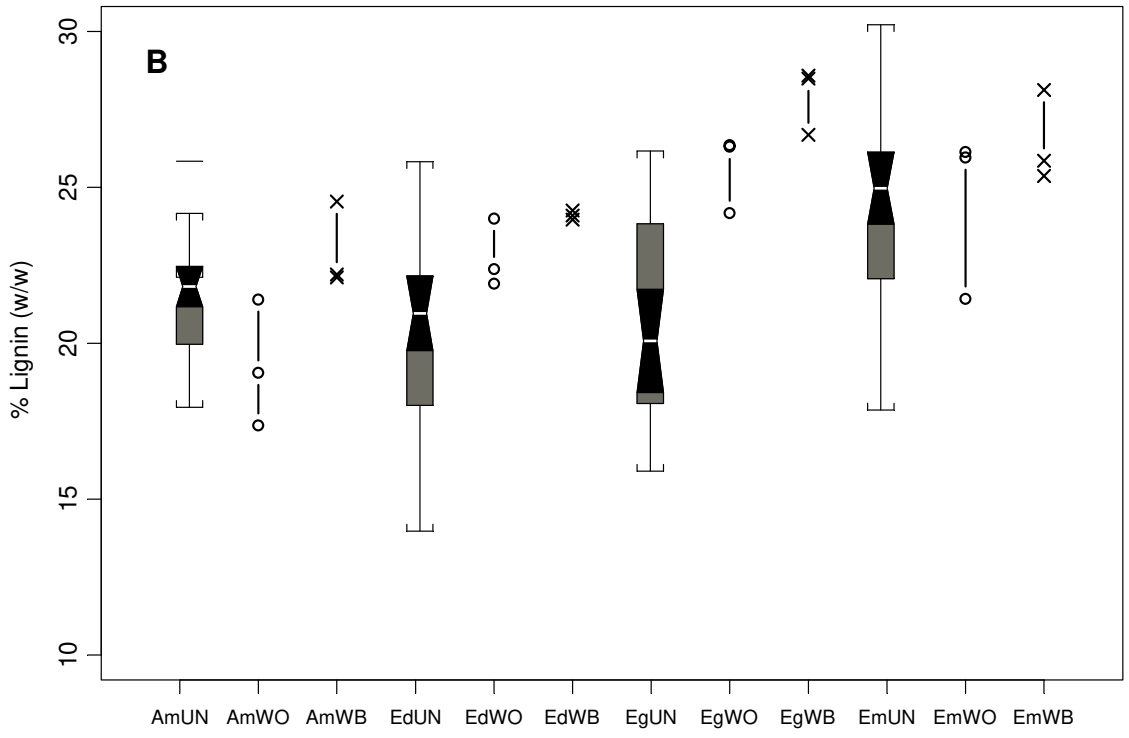
The results of Seifert analyses conducted on untreated wood chips, on hot water washed wood chips, and on residual wood chips after a two week incubation period of a *P. sanguineus / A. flavipes* co-culture, are depicted in figure 1A. Interestingly, the cellulose content of all four wood species, which were all similar in the untreated state, increased after the two hour hot water wash at 150°C. These findings were supported by Tukey's simultaneous confidence intervals (Table 6). As pointed out in Chapter 2 this increase may be as a result of the removal of the hemicellulose component during the latter process with the concomitant increase in the relative cellulose content. It is known that hemicellulose is hydrolyzed by hot water and steam, leading to leaching of the resultant monosaccharides from the wood (Rowell *et al*., 2002; Williams, 2005).

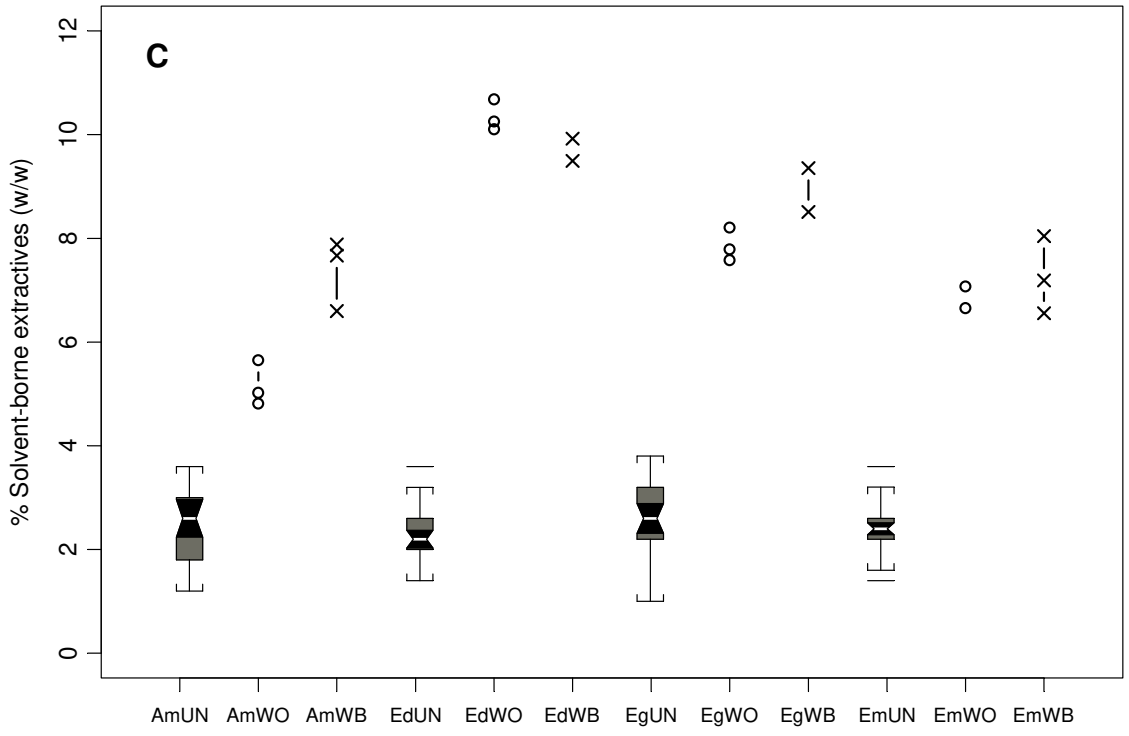
Fungal treatment of *A. mearnsii*, *E. dunnii* and *E. macarthurii* hot water washed wood chips, resulted in no significant change in the cellulose content of the wood (figure 1A, Table 6). Interestingly, although the results depicted in Figure 1A

suggested that the fungal co-culture decreased the cellulose content of *E. grandis* hot water washed wood chips, these findings were not supported by Tukey's simultaneous confidence intervals (Table 6).

The results of Klason Lignin analyses conducted on untreated wood chips, on hot water washed wood chips, and on residual wood chips after a two week incubation period of a *P. sanguineus* / *A. flavipes* co-culture, are depicted in figure 1B. Although a tendency was noted in figure 1B for the hot water wash to increase the lignin content of untreated *E. dunnii* and *E. grandis* woodchips, which may be ascribed to the removal of the hemicellulose component during the hot water wash with the concomitant increase in the relative lignin content (Nuopponen et al., 2004; Garcia et al., 2005), these findings were not supported by Tukey's simultaneous confidence intervals (Table 6).







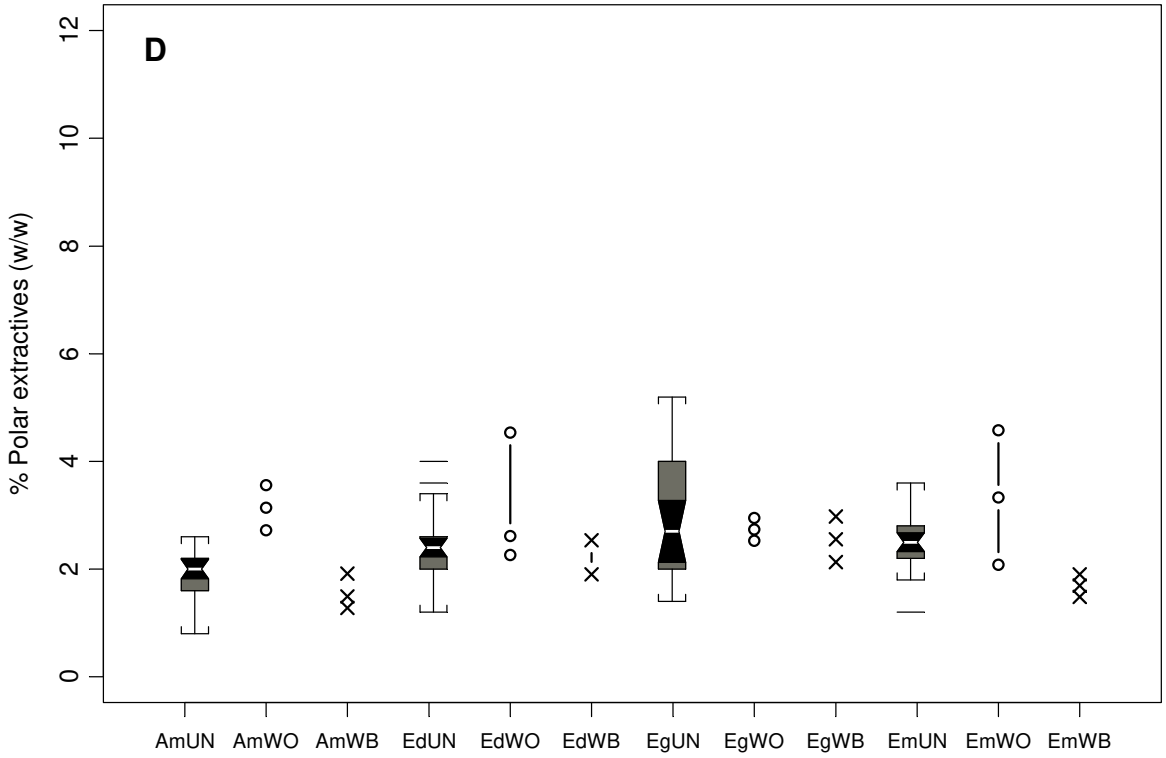


Figure 1. Notched box plot representation of differences between tree species. (A) Percentage cellulose in wood after treatments. (B) Percentage lignin in wood after treatments. (C) Percentage solvent-borne extractives in wood after treatments. (D) Percentage polar extractives in wood after treatments. Explanations of the acronyms on the x-axes are listed in Table 1.

Similar to the findings reported in Chapter 2, though also not supported by Tukey's simultaneous confidence intervals (Table 6), a tendency was noted for fungal growth to increase the lignin content of the wood (figure 1B). This may be ascribed to modification of the lignin polymer during the hot water wash as a result of excessive heat, rendering it recalcitrant against fungal degradation (Weiland & Guyonnet, 2003), thus increasing the relative content of this polymer during degradation of other wood components. The perceived increased lignin content during fungal growth may also have been as a result of protein contamination (Hatfield & Fukushima, 2005).

Analyses of the solvent-borne extractives obtained from the residual wood revealed significant increases in all the wood after the hot water wash (Figure 1C, Table 6). The hot water wash may have removed some of the hemicellulose components, thereby, increasing the relative quantities of solvent-borne extractives, and/or may have increased the availability of the lipophilic compounds during analysis (Chapter 2; TAPPI Tests Methods, T264 om-88).

Fungal treatment of *A. mearnsii* wood chips resulted in an increase in the solvent-borne extractives present in hot water washed wood (Figure 1C, Table 6). Though not supported by Tukey's simultaneous confidence intervals (Table 6), a tendency was noted for these extractives to increase during fungal treatment of hot water washed *E. grandis* wood chips (Figure 1C). As pointed out in Chapter 2 these increases may be the result of fungal anabolism or the degradation of wood components by the fungi. Fungal treatment of *E. macarthurii* had no effect on the solvent-borne extractives (Figure 1C) while this treatment tended to decrease the solvent-borne extractives in *E. dunnii* wood chips. The latter was not supported by Tukey's simultaneous confidence intervals (Table 6). Nevertheless, these results on solvent-borne extractives indicate differences in the lipid metabolism and interactions within the fungal co-culture when growing on different wood species.

The data obtained after analyses of the polar extractives in the wood were displayed in the form of notched box plots in Figure 1D. No obvious difference between the treatment classes was revealed after the hot water wash, except for an increase in the quantities of these polar compounds after the hot water wash of *A. mearnsii* wood chips. However, a Two-way ANOVA performed with "% Polar extractives" as response variable, revealed negligible interaction between "species" and "treatment" (Table 11), as well as that both these effects were highly significant and could be considered separately (see Figure 2). Consequently, perusal of Table 12 that lists the mean % Polar extractives associated with the tree species and Table 13 that depicts simultaneous pair-

wise 95% confidence intervals associated with the species main effect, revealed that differences do exist between some tree species regarding the polar extractive content. Thus, as shown in Figure 2A, the concentrations of these polar compounds that include amino acids, phenols, simple sugars and starches (Martin & Aber, 1996) were significantly lower in *A. mearnsii* than in wood chips from all the other species. The polar compounds concentration in *E. dunnii* was significantly lower that of *E. macarthurii* (Tables 12, 13) although not reflected in median differences in Figure 2A.

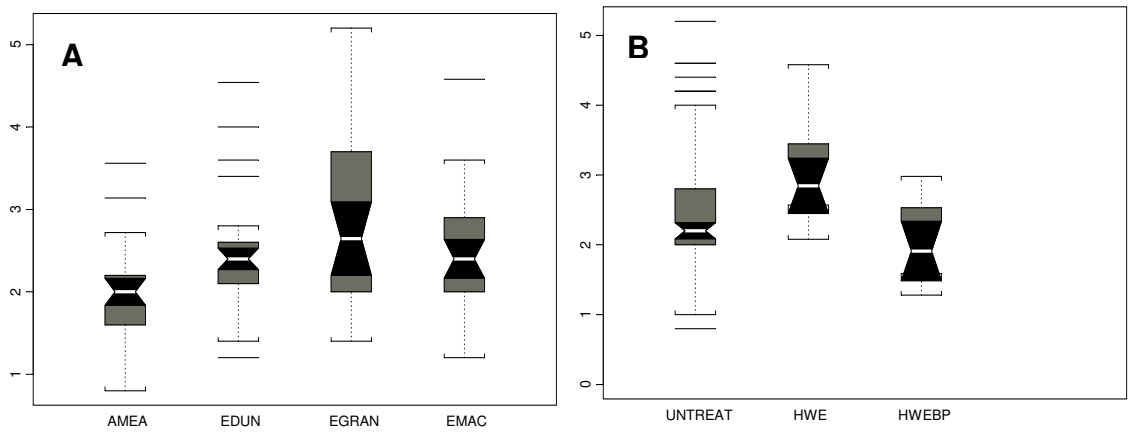


Figure 2. Notched box plots representation of differences in polar extractives. (A) Main effect of tree species. (B) Main effect of treatment.

Furthermore, when untreated wood chips were hot water washed, a significant increase in the polar extractives generally occurred in all the wood species (Tables 12, 14 and Figure 2B). The polar extractives, which after fungal cultivation may include degradation products of wood components and the water extractable fraction of fungal biomass, generally decreased statistically significantly upon fungal treatment (Tables 12, 14 and Figure 2B).

3.4. Conclusions

To investigate chemical alterations in the different tree species induced by the fungi while growing on the hot water washed wood chips, a series of biplots were

constructed (Figures 3, 4 and 5). A PCA biplot of the data revealed that despite the relative large variation observed within some treatments (*A. mearnsii* inoculated with the co-culture and hot water washed *E. dunnii* and *E. grandis* wood chips) the fungal co-culture altered the chemical composition of all the tree species (Figure 3). Notice that the biplot provides a detailed display of the differences in chemical composition. To investigate whether the *P. sanguineus / A. flavipes* co-culture impacted differently on the chemical composition of wood from different tree species a CVA biplot was constructed (Figure 4). The construction of a CVA biplot however, makes use of equality of the within classes covariance matrices. Inspection of the box plots in Figure 1 as well as the PCA biplot in Figure 3 may cast a shadow of doubt on the truth of this assumption. Consequently, an AOD biplot that does not require this assumption was constructed (Figure 5). From the resulting biplot it was obvious that the fungal co-culture impacted differently on the chemical components measured in wood chips from the various tree species. For example, despite the relative large variation observed within some treatments, such as *A. mearnsii* inoculated with the co-culture and hot water washed *E. dunnii* wood chips, it was clear that the co-culture notably increased the perceived Klason lignin content of the *A. mearnsii* wood chips, while such an obvious fungal induced increase was not observed for the *E. dunnii* chips. In the latter case the most prominent alteration was a decrease in the cellulose content of the wood chips during fungal growth. In contrast, growth of the fungal co-culture increased the relative cellulose content of the *A. mearnsii* chips.

Our results indicated that the *P. sanguineus / A. flavipes* co-culture altered the chemical composition of each tree species in a different manner. This phenomenon may have implications in the ecology of lignocellulosic degradation in the natural environment. Since lignocellulosic degradation is important in the biopulping industry, our results also indicate that optimization of the biopulping process may depend both on the fungi used and the tree species involved.

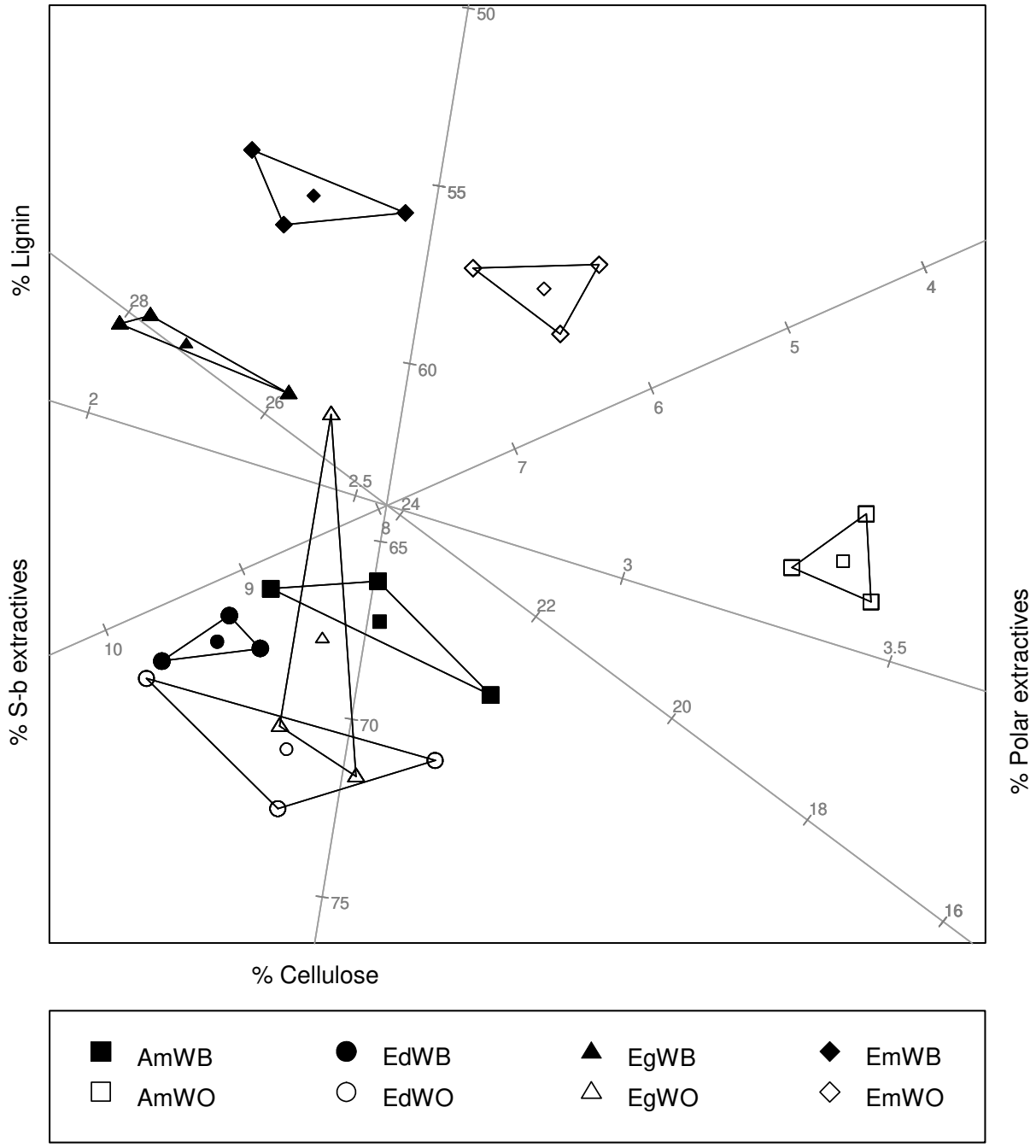


Figure 3. A PCA biplot showing the differences among the class means in the center of each group. Solid symbols = Bio treated wood chips; Open symbols = Untreated wood chips.

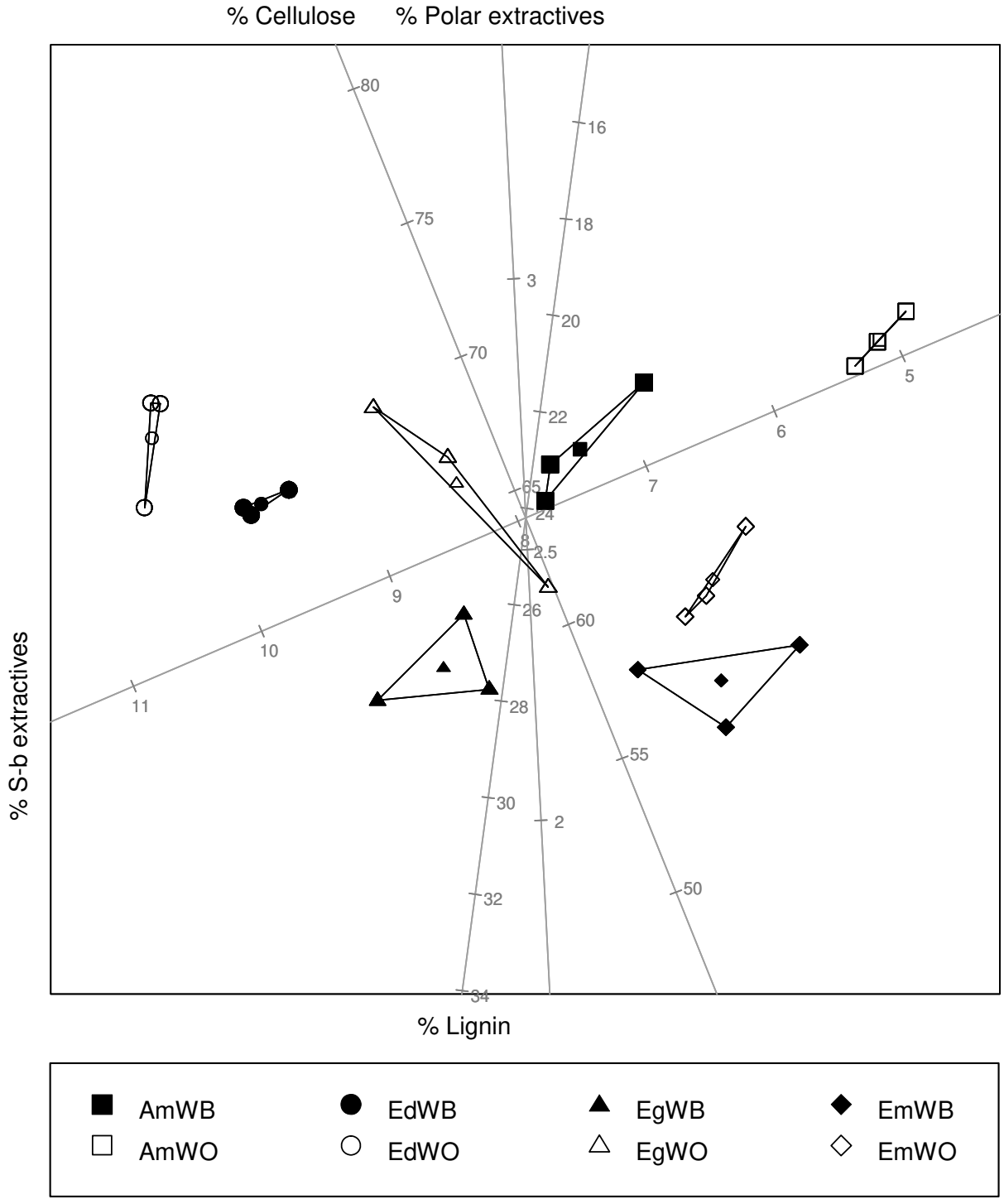


Figure 4. A CVA biplot displaying the differences between the culture combinations. Solid symbols = Bio treated wood chips; Open symbols = Untreated wood chips.

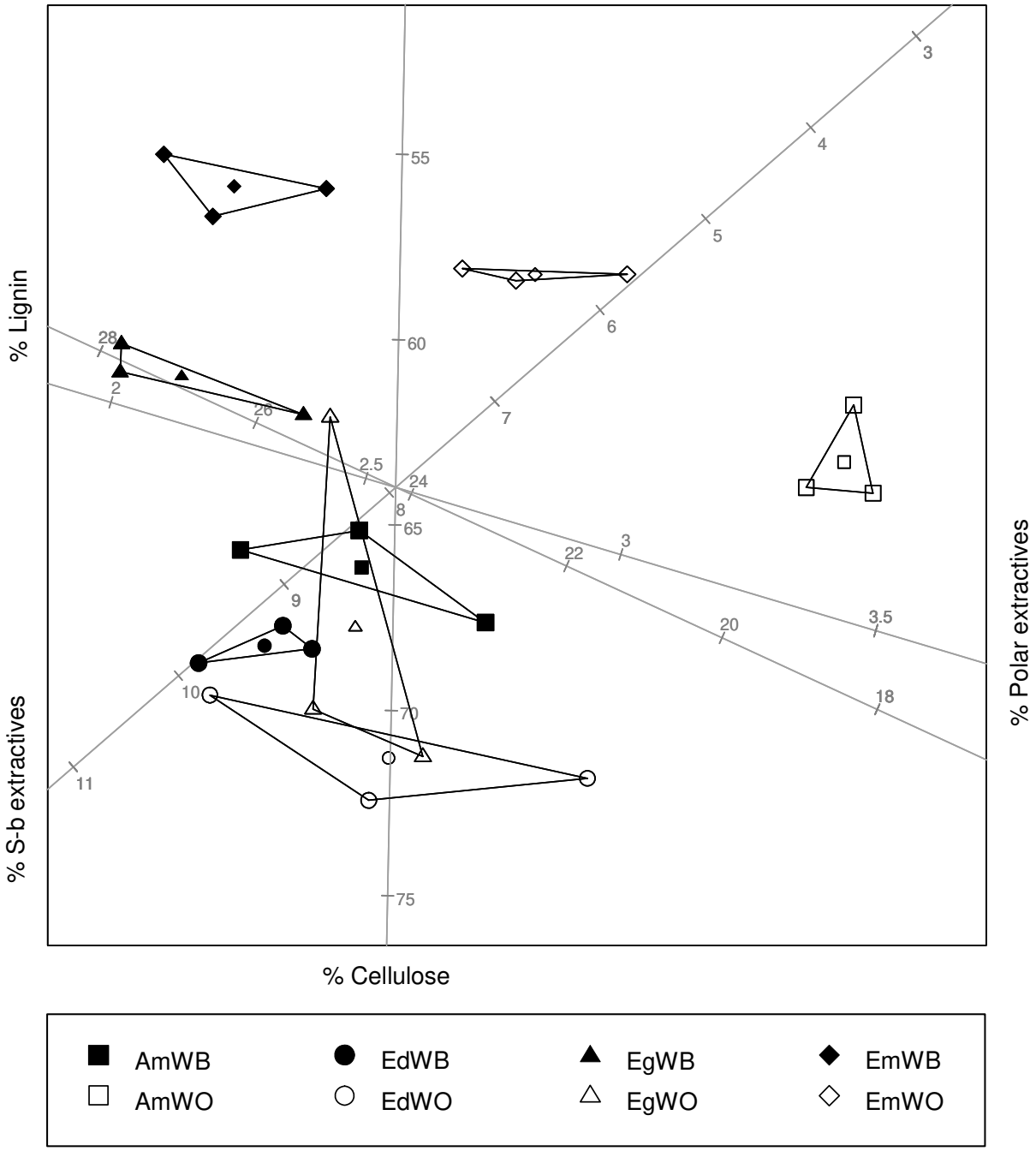


Figure 5. An AOD biplot displaying the differences between the culture combinations. Solid symbols = Bio treated wood chips; Open symbols = Untreated wood chips.

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Appendix A

Table A Composition of benomyl–dichloran–streptomycin medium (BDS-medium) used for the enumeration of hymenomycetous fungi present in the soil (Adapted from Worrall, 1991).

Components per liter of distilled water	
Carbon source	
Malt extract (g)	15.00
Anti-fungal agents	
Benomyl (mg)	2.00
[1]Dichloran (mg)	2.00
Phenol (ml)	0.05
Anti bacterial agent	
Streptomycin sulfate (mg)	100.00
Solidifying agent	
Agar (g)	15.00