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**APPLICATION OF FUNGI IN BIOTECHNOLOGICAL
PROCESSES FOR THE PULP AND PAPER INDUSTRY**

by

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“If one sentence can’t achieve its purpose then a thousand words would also be in vane.”

Unknown

Dedicated in loving memory to my Grandfather

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PREFACE

The pulp and paper industry is a very important industry in South Africa that utilises wood, bagasse and wheat straw as sources of fibre (Fernández *et al.*, 1989; Barrasa *et al.*, 1995; Rockey, 1998). Lignin must, however be removed from these lignocellulosic materials to utilise the cellulose and hemicellulose in this industry. Biotechnology could benefit this process and increase the production rate (Lascaris *et al.*, 1997), product quality (Buchert *et al.*, 1994; Popius-Levlin *et al.*, 1997; Viikari *et al.*, 1993) and reduce environmental impact (Eriksson, 1991). Biodegradation of lignin has come under consideration as an alternative to the conventional methods used in industries such as the pulp and paper industry (Reddy, 1978). Biotechnology has a smaller impact on the environment, because it is natural reactions catalysed by microorganisms (Eriksson, 1990). Biodegradation has been applied to many fields in the forest products industry, with notable applications in biopulping, biobleaching and wastewater treatment (Eriksson, 1991). Fungi, especially white-rot Basidiomycetes, can degrade most components of cell walls including lignin, which is the most important component that needs to be degraded (Reid, 1995; Akhtar *et al.*, 1997; Tanesaka *et al.*, 1993). When suitable strains of fungi are selected, many other useful products can be obtained from the lignin breakdown process (Crawford & Crawford, 1980). Industrial biodegradation of lignocellulosic materials can also be a low-energy process that can lead to cost savings in the pulp industry (Reddy, 1978).

The primary goals of this thesis were to evaluate two biopulping processes that could lead to an increased production rate or reduced cost of pulp production in South Africa. These studies focussed on wood chips treated with Cartapip 97[®] and bagasse treated with different white-rot fungi (*Lenzites betulina* and *Pycnoporus sanguineus*). Wood chips were chosen, because the chips are more economical to handle than logs

(Zabel & Morrell, 1992) and bagasse was chosen because it is a waste product from the sugar extraction process and vast amounts of bagasse are available in South Africa (Venter, 1978). Before evaluation of Cartapip 97[®] was allowed, the Department of Agriculture had to certify that the fungus (*Ophiostoma piliferum*) contained in the product, was safe for release in South Africa. The certification and evaluation of the Cartapip fungus are discussed in Chapter 2.

Ophiostoma piliferum is unable to degrade cellulose or lignin (Farrell *et al.*, 1994), but is the only fungus that is applied commercially in pulping (Schmitt *et al.*, 1998). The fungus is a primary coloniser, therefore it has the ability to outcompete the growth of other staining fungi and decay fungi that have a negative influence on the quality of wood (Farrell *et al.*, 1993). By outcompeting the staining fungi, bleaching requirement is reduced and pulp yield improved (Blanchette *et al.*, 1992). The fungus also utilises pitch in wood and this improves the chemical pulping process and paper quality (Farrell *et al.*, 1993). Some of the benefits obtained by pre-treatment of wood chips with Cartapip before pulping, include stronger paper with better optical properties (Farrell *et al.*, 1993), an increase of pulp yield and viscosity and also a reduction in chemical consumption (Wall *et al.*, 1994).

According to the quarantine regulations in South Africa, confirmation on the identity of the Cartapip 97[®] fungus as a strain of *Ophiostoma piliferum* was required and, therefore, the morphology of the fungus had to be studied. The anamorph characteristics of the fungus were examined microscopically and compared with characteristics described for *Ophiostoma piliferum* (Upadhyay, 1981). The pathogenicity of the fungus to pine species had to be investigated under South African conditions, because the non-pathogenicity of the Cartapip 97[®] fungus could lead to the release of the fungus for pilot trials in biopulping. The pathogenicity of the fungus was compared with that of other fungi (*Ophiostoma ips* and *Sphaeropsis sapinea*) that cause sap stain, by inoculating different branches on pine trees at three locations during autumn and spring. The effect of the treatment of wood chips with *O. piliferum* before pulping was investigated on softwood and hardwood species

grown in South Africa. Biokraft pulping was done on softwood (*Pinus patula* and *P. elliottii*) chips and kraft, sulphite and Soda-AQ biopulping on treated hardwood (*Acacia mearnsii* and *Eucalyptus grandis*) chips.

Lenzites betulina and *P. sanguineus* were chosen after screening trials done by Grimbeek *et al.* (1997), on account of the high yield and low lignin content that were obtained after treatment of bagasse. Bagasse has a limited cutting season and has to be stored for long periods to be able to supply it for pulping throughout the year (Venter, 1978). During the storage, special measures such as wet bulk storage are used to reduce the decay of the fibres. The quality of the bagasse can potentially be improved with selected fungi before pulping (Wolfaardt & Grimbeek, 1997).

The studies on pulping of fungal treated bagasse to optimise pulping parameters are discussed in Chapter 3. This chapter covers the determination of the most effective inoculum concentration for bagasse, the evaluation of different methods for inoculum production and also biopulping processes on bagasse. Biopulping was done by pretreatment of bagasse with two selected strains of white-rot fungi (*L. betulina* and *P. sanguineus*). The influence of the incubation time on pulping parameters was also investigated. Ultrastructural studies (TEM, SEM and light microscopy) of treated bagasse were undertaken to investigate the colonisation strategy of the fungi and the mechanism of lignin degradation.

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CHAPTER 1

FUNGAL DEGRADATION OF LIGNIN AND ITS INDUSTRIAL IMPLICATIONS



Gymnopilus penetrans on a pine log

ABSTRACT

Lignin, hemicellulose and cellulose are natural polymers that occur together in wood, bagasse and wheat straw, which are used as fibre in the pulp and paper industry. Lignocellulose degradation is a very complex process, because of the recalcitrance of lignin. The understanding of lignin degradation could lead to the optimisation of biotechnological processes for industry. White-rot fungi can degrade most components of cell walls while brown-rot fungi modify lignin slightly, but can break down cellulose and hemicellulose. Four dominant groups of decay have been identified including white rot, soft rot, brown rot and bacterial degradation. Manganese peroxidase, lignin peroxidase and laccase are extracellular enzymes that are produced by fungi to degrade lignin. Other less important enzymes that are also involved in lignin degradation include cellobiose:quinone oxidoreductase, aryl-alcohol oxidase, aryl-alcohol dehydrogenase and NADH:quinone oxidoreductase. Organic material provides an opportunity for decay fungi to establish and lignocellulosic material is degraded during the colonisation process. It is, however, important to distinguish between primary and secondary colonisers during degradation, because of succession that takes place. Primary colonisers start the succession of fungi on wood and slowly give way to competitive secondary colonisers. Biodegradation could be applied in industries such as the pulp and paper industry to save cost and reduce environmental impact. Biodegradation has been applied to many fields in the pulp and paper industry, with the most notable effects seen in biopulping, biobleaching and wastewater treatment.

INTRODUCTION

Lignin, hemicellulose and cellulose are the most abundant polymers in nature (Boominathan & Reddy, 1992) and occur together in lignocellulosic materials (Sarikaya & Ladisch, 1997). Lignocellulosic materials such as wood, bagasse and wheat straw are used as sources of fibre by the pulp and paper industry (Fernández *et al.*, 1989; Barrasa *et al.*, 1995; Orlando *et al.*, 2002).

The process of lignocellulose degradation is complex and very slow and still not completely understood (Crowder *et al.*, 1978; Breen & Singleton, 1999; Donaldson, 2001), because it is difficult to obtain pure forms of lignin, hemicellulose and cellulose without breaking the covalent bonds in these polymers (Odier & Artaud, 1992). A number of industries such as the pulp and paper industry and the food and feed industry utilise cellulose and hemicellulose. These molecules must, however, be purified from lignin that is bound to it. The understanding of the lignin degradation process could lead to the optimisation of biotechnological processes (Breen & Singleton, 1999) to improve production rate (Lascaris *et al.*, 1997), product quality (Buchert *et al.*, 1994; Popius-Levlin *et al.*, 1997; Viikari *et al.*, 1993) and reduce environmental impact (Eriksson, 1991) of pulp and paper production.

The removal of lignin can be applied in the pulping of paper, biobleaching and treatment of mill effluents (Blanchette *et al.*, 1988a; Lawson & Still, 1957). The biomass of lignocellulosic residues can be applied as a source of food for animals and people, but the amount of lignin present in the biomass presents a problem (Crowder *et al.*, 1978; Lawson & Still, 1957). However, if the lignin present in, for example, bagasse could be removed biologically it would present great opportunities in the food and feed industry (Blanchette *et al.*, 1988a; Draude *et al.*, 2001).

Certain fungi, especially white-rot fungi, can degrade most components of cell walls of which lignin is the most important component that needs to be degraded (Reid, 1995; Tanesaka *et al.*, 1993; Nüske *et al.*, 2001). Brown-rot fungi also play a role in lignocellulose degradation, but they can only break down cellulose and

hemicellulose (Tanesaka *et al.*, 1993). Brown-rot fungi can, however, modify lignin by demethoxylation reactions (Ritschkoff *et al.*, 1992). The biodegradation of lignin can be utilised in biotechnological processes (Boominathan & Reddy, 1992) and, therefore, degradation of lignin needs to be understood for optimal application (Eggert *et al.*, 1995). The aim of this paper is to review the role of fungi in the degradation of lignin in lignocellulosic materials and also the potential role of lignin degrading fungi in biotechnological processes.

OCCURENCE AND DISTRIBUTION OF LIGNIN

Lignin is a natural product arising from a polymerisation of three precursors namely trans- ρ -coumaryl alcohol, trans-coniferyl alcohol and trans-sinapyl alcohol (Figure 1) (Sarikaya & Ladisch, 1997). Lignin units are connected through non-hydrolysable bonds such as carbon-carbon bonds and ether bonds. Carbon-carbon bonds are rarely formed, but can link aromatic nuclei and propyl side-chains. Ether bonds are the most abundant and bind propyl side-chains (C α -C β) to aromatic nuclei (Odier & Artaud, 1992).

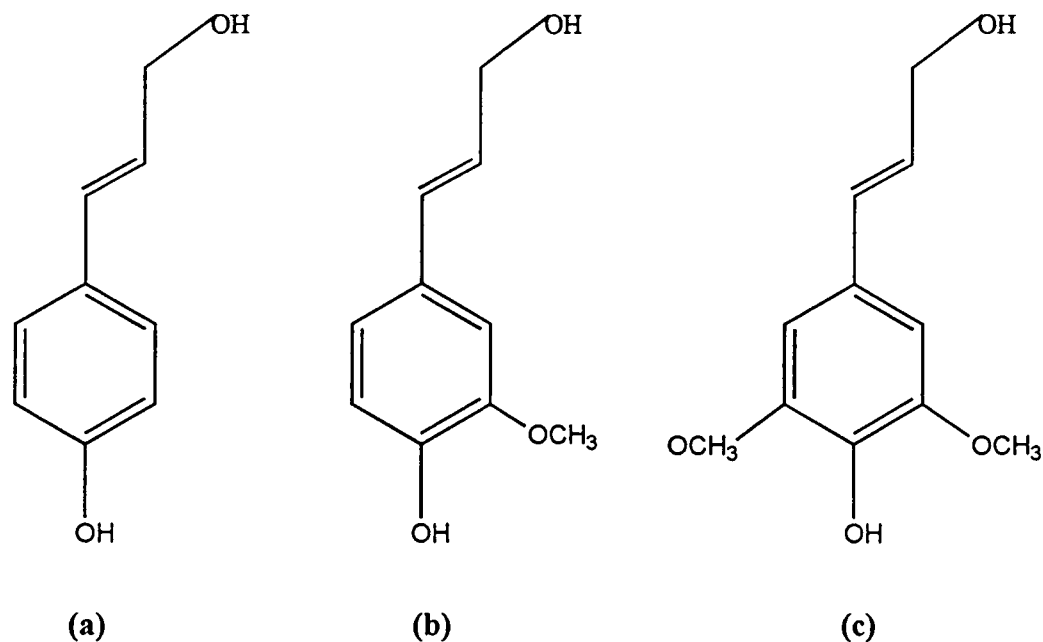


Figure 1. The chemical structures of the precursors of lignin: (a) trans-p-coumaryl alcohol (b) trans-coniferyl alcohol (c) trans-sinapyl alcohol.

Lignin units do not exist in isolation but is also bound to hemicellulose through covalent bonds (Sarikaya & Ladisch, 1997). Hemicellulose consists of xylans, mannans, galactans and glucans (Robards, 1970) and has a more complex structure than cellulose (Sarikaya & Ladisch, 1997). Hemicellulose, therefore, requires more complex enzyme systems for degradation (Rogalski *et al.*, 1993; Robards, 1970).

Cellulose consists of glucose units with 1,4- β -D-glycosidic linkages and it is embedded in a matrix that is formed through covalent bonds between lignin and hemicellulose. Cellulose is hydrolysed to glucose when the lignin and hemicellulose matrix is removed (Sarikaya & Ladisch, 1997). Cellulose fibrils are usually found in the primary and secondary walls of plants and also in the middle lamellae where they act as a connection between cells (Sarikaya & Ladisch, 1997).

The lignin content in softwood was determined to be between 26 and 32 % by applying the Klason method (Sjöström, 1993) and contains mostly guaiacyl units (Reid, 1995). In hardwoods the lignin content varies between 20 and 25 % (Sjöström, 1993) and contains guaiacyl and syringyl units (Reid, 1995). In gramineous plants the lignin content is calculated to be between 10 and 15 % (Odier & Artaud, 1992).

Ultrastructural studies by Jurasek (1995), based on computer-generated, three-dimensional models of lignin, showed that lignin appears as round particles in the middle lamella. The particles later form a strong compact structure or a continuous space-filling structure (Jurasek, 1995). The middle lamella contains a high concentration of lignin (60 to 90 %), it amounts to 10 to 30 % of the total lignin content in wood fibres (Reddy, 1978; Boomnathan & Reddy, 1992) and is between 0.2 and 1.0 μm thick (Sjöström, 1993). The secondary wall contains lignin between the cellulosic lamellae, to which it is linked by hemicellulose (Jurasek, 1995), and is approximately 5.4 μm thick (Sjöström, 1993). The primary (0.1 to 0.2 μm thick) and secondary cell walls of wood fibres contain low concentrations of lignin but it amounts to 70 to 90 % of the total lignin content in wood fibres, because of the large volume of these layers (Reddy, 1978; Sjöström, 1993; Boomnathan & Reddy, 1992). The concentration of lignin in the middle lamellae is the highest (Reddy, 1978), which makes this layer the most difficult to degrade.

The thickness of the walls is not the only factor contributing to the recalcitrance of lignin. According to Sarikaya & Ladisch (1997), the recalcitrance of lignin is caused by the ether and ester bonds between the hydroxyl groups of hemicellulose and α -carbonyl of phenyl propane subunits found in lignin. The continuous cross-linking and the three dimensional orientation of the lignin polymer, that give it a unique structure, make the degradation of lignin even more complex (Crowder *et al.*, 1978; Odier & Artaud, 1992).

EXTERNAL FACTORS INFLUENCING DEGRADATION

Lignin degradation is an oxidative process, requiring free oxygen and, therefore, lignin will not be degraded under anaerobic conditions (Breen & Singleton, 1999; Lawson & Still, 1957). When free oxygen is available, lignin degradation is increased, but also the degradation of polysaccharides (Rios & Eyzaguirre, 1992). The non-enzymatic pathway of lignin degradation includes reactions with molecular oxygen and water that decompose unstable lignin radical cations (Breen & Singleton, 1999; Lawson & Still, 1957). Too much water presents a problem, because it limits the transfer of oxygen.

Very low moisture contents, on the other hand, result in less decay (Sierota, 1997; Breen & Singleton, 1999), because fungal metabolic activity is reduced (Eriksson *et al.*, 1980). Eriksson *et al.* (1980) ascribed reduced decay of birch chips treated with a cellulaseless mutant of *Phanerochaete chrysosporium* to too much water that led to a decrease in oxygen consumption by the fungus. Excess of water could, therefore, lead to less decay because transfer of oxygen is limited.

Nitrogen also has an influence in the degradation of lignin, because the ligninolytic enzyme system will only be activated in low nitrogen environments. High amounts of nitrogen will, therefore, inhibit lignin decomposition (Keyser *et al.*, 1978). Lignin degradation, thus, starts during the secondary metabolism when a shortage of sugars or nitrogen occurs (Boominathan & Reddy, 1992; Nowak, 2001).

TYPES OF DEGRADATION

There are many chemical and morphological differences in the degradation processes caused by different groups of decay fungi. Four dominant groups of decay have been identified and these include three types of decay caused by fungi (white rot, soft rot, brown rot) and bacterial degradation (Blanchette, 1995). Bacterial

degradation will not be discussed in this paper, because this study will focus on fungal decay.

White rot

Two types of white-rot fungi can be distinguished according to Ander & Eriksson (1978). The first type is a simultaneous rot where the three most important components of wood, namely lignin, cellulose and hemicellulose, are degraded simultaneously. White-stringy rot is also a type of white rot, but very unusual. White-stringy rot causes a barrier in the final phases of decay, and is caused by *Armillaria mellea* (Vahl : Fr.) Quèl and *Bondarzewia berkeleyi* (Fr.) Sing (Blanchette *et al.*, 1988b).

The second type is a selective white rot, where lignin is degraded faster than cellulose or hemicellulose (Ander & Eriksson, 1978; Watanabe *et al.*, 2001). However, the order in which the cell wall components are attacked and the level of decay have to be studied thoroughly to make a distinction possible (Ander & Eriksson, 1978). White-pocket rot is also a selective rot, but differs in the decay pattern that forms in the wood (Blanchette *et al.*, 1988b). During white-pocket rot hyphae of white-rot fungi colonise the lumen of the cell and utilise the cell wall leading to an intense decay process. The selective delignification leads to white zones in the wood and it is, therefore, known as white-pocket rot (Breen & Singleton, 1999; Blanchette, 1995).

Wood degradation patterns formed by different white-rot fungi varied significantly according to Blanchette *et al.* (1988a). *Coriolus versicolor* (Linnaeus: Fries) Quélet, *Phellinus pini* (Brotero: Fries) A. Ames, *Phlebia tremellosus* (Schrad.) Burds. & Nakas, *Poria medullapanis* (Jacq. Ex Fr.) Donk and *Scytinostroma galactinum* (Fries) Donk sensu Donk were used to treat wood and it was observed, through transmission electron microscopy, that *C. versicolor* degraded lignin in the cell walls adjacent to hyphae and caused non-selective degradation of cell wall components. *Phellinus pini*, *P. tremellosus*, *P. medulla-panis* and *S. galactinum*

caused selective lignin degradation and the removal of middle lamellae (Blanchette *et al.*, 1988a).

It was further noticed in the studies of Blanchette (1980) that in the early stages of white-rot decay of wood, troughs were formed in the presence of hyphae and degradation occurred in the zones surrounding the fungal hyphae. Destruction of all cell wall components occurred leaving holes in the tracheid cells. During later stages of decay, masses of fungal hyphae could be observed. The last cells to be attacked were the ray parenchyma cells (Blanchette, 1980). The ray parenchyma cells were then degraded completely and the primary and secondary walls were left without lignin. The middle lamellae in the tracheid cells were also degraded.

Akhtar *et al.* (1997) used the application of histological stains and electron-dense compounds such as KMnO_4 that react with lignin, to investigate the delignification of wood by white-rot fungi. Lignin was first removed from the secondary wall and then from the middle lamellae. After the breakdown of the middle lamellae the cells without lignin broke away from other cells (Akhtar *et al.*, 1997). When fungi started to attack the lignin in the secondary wall the electron dense zone in the secondary wall became transparent and as the lignin was removed, the transparent zone extended into the secondary wall. The middle lamellae between the cells and cell corners were then removed and the electron dense zone in the middle lamellae also became less dense (Akhtar *et al.*, 1997). In the same study Akhtar *et al.* (1997) used a light-based microscopic method to indicate the difference between less dense and non-decayed cells. The experiment was based on gold labelling and the results showed that delignified wood still had crystalline and amorphous cellulose, but lesser amounts of xylan. The hemicellulose in delignified wood was also depleted as degradation increased.

Fernández *et al.* (1989) used electron microscopy to examine the mode of degradation of the different layers of bagasse cells. A wild type strain of the white-rot fungus *Phanerochaete chrysosporium Novabronova* and a cellulase-deficient mutant of the same strain were used to inoculate the bagasse. Two main patterns of

degradation were observed in this study. The pattern for the wild type strain, after a seven-day incubation period, showed that the middle lamella, primary layer as well as the secondary layers were degraded. This mode of action suggested that lignin and polysaccharides were randomly attacked. Decay caused by the mutant strain was documented after seven and 21 days. After seven days selective delignification was seen when layers and sub-layers began to appear. Hemicellulose degradation was also indicated after the seven days. After 21 days the S2-layer became softer, swollen and less rigid and the outer parts of the cell wall did not seem to be affected. The swollen S2-layer indicated that the lignin and hemicellulose were degraded (Fernández *et al.*, 1989).

Johnsrud *et al.* (1987) did TEM studies on bagasse and also found that lignin was selectively removed when treated with a cellulase-restricted strain of the white-rot fungus, *Phanerochaete chrysosporium* Burdsall. It was also observed that the bagasse cells separated, as the middle lamella was decayed (Johnsrud *et al.*, 1987). These results were compared with chemically enhanced cell wall degradation where the lignin and hemicellulose were extracted selectively. The chemical extraction resulted in layering of the cell wall and swelling of the S2-layer similar to the fungal degradation (Fernández *et al.*, 1989).

Ander & Eriksson (1978) studied the mode of action of white-rot fungi in the decay process. White-rot fungi began to cause degradation at the cell lumen and then moved on to the middle lamella. In this process the secondary wall of the cell became thinner. Some white-rot fungi loosened cells when degradation began at the S3-layer and then attacked the middle lamella.

Soft rot

Fungi that belong to the Ascomycota and Deuteromycota can cause soft rot. Soft-rot fungi differ in their decaying abilities making it difficult to differentiate and classify decayed lignocellulosic materials without ultrastructural or chemical analysis (Blanchette, 1995). Soft rot usually occurs in environments too extreme for white and brown-rot fungi e.g. in very wet habitats or where it is extremely dry (Blanchette,

1995). Wood edges turn brown when soft rot is present and the edges of the wood will crack when the wood dries out. Soft rot can be subdivided into Type-I and Type-II rot that form cylindrical cavities in the secondary walls and a total degradation of the secondary wall respectively (Blanchette, 1995). Type-I soft rot is usually associated with coniferous wood and Type-II soft rot with angiosperms (Blanchette, 1995). The middle lamellae stay intact even at late stages of soft rot. Lignin degradation is very slow indicating that soft-rot resembles the actions of brown-rot fungi (Eriksson & Kirk, 1986; Tanaka *et al.*, 1992) in the sense that brown-rot fungi modify lignin and do not completely degrade it (Blanchette, 1995).

Brown rot

Some fungi that belong to the Basidiomycota cause brown rot (Blanchette, 1995). Brown-rot fungi attack the cellulose and other polysaccharides very early in the decay process that leads to a decrease in the strength of the wood (Blanchette, 1995). The lignin is left undegraded, but with slight chemical modifications that leave the wood with a brown colour (Blanchette, 1995). Scanning electron microscopy demonstrated that cellulose degradation was dominant in brown rot and that the lignin was left intact (Blanchette, 1980). Brown rot breaks down wood into fragments making it easy to identify. Brown rot also has benefits e.g. in forest ecosystems where wood decayed by brown-rot fungi can retain moisture and nutrients in dry periods that benefits ectomycorrhizal fungi and tree feeder roots (Blanchette, 1995).

Poria placenta (Fries) Cooke and *Gloeophyllum trabeum* (Pers.: Fr.) Murr. are two brown-rot fungi that were the subjects of a study to characterize the steps of early degradation in softwoods (Wilcox, 1993). The morphological changes occurring during degradation were studied by means of light and scanning electron microscopy and it was observed that degradation of the wood occurred first in the earlywood. Degradation of earlywood led to the utilisation of cellulose in small patches (birefringence) that fulfils an important role in the diagnosing of early stages of wood decay (Wilcox, 1993).

The action of brown-rot fungi in the decay process starts by removal of cell-wall substances in the S2-layer of the secondary wall and later in the S1-layer (Ander & Eriksson, 1978). However, the high concentrations of lignin that the primary wall and middle lamella contain make it resistant to attack by brown-rot fungi. When most of the wood polysaccharides were consumed the cell wall crumbled (Ander & Eriksson, 1978).

ENZYMES INVOLVED IN DEGRADATION

Wood-decaying fungi produce different enzymes that attack the cell during lignocellulosic degradation. The complexity of lignin makes it very difficult for single enzymes to degrade the lignin (Eriksson & Kirk, 1986; Reid, 1995). Therefore, enzymes are produced that have a low specificity and that could also start oxidation of lignin (Reid, 1995). Oxidation was one of the earliest mechanisms proposed for wood degradation (Kirk *et al.*, 1978). Oxidation implies that organic nutrients are oxidised to carbon dioxide and water under aerobic conditions (Schlegel, 1993). Two major types of oxidation were identified. The first type involves ring cleavage while the ring is still attached to the polymer while the other oxidation step occurs when the propyl side chains, at the α -position, are oxidised resulting in the formation of carbonyl groups. Some oxidative action also occurs when terminal side chains are shortened, which leads to the formation of aromatic acid residues. It was further proposed by Kirk *et al.* (1978) that phenols in different regions are oxidized to catechol by demethylation or aromatic hydroxylation. The dihydroxy units are cleaved through oxidation with the production of aliphatic carboxyl-rich residues and these aliphatic residues enter hyphae for further metabolism. New phenolic groups are released for further attack by demethylation, aromatic hydroxylation and also through direct oxidative cleavage of β -ether linkages. When β -ether linkages are cleaved, aromatics are released in the cell to be degraded. The demethylation of methoxyl groups is, therefore, important in initiating the ligninolytic process (Kirk *et al.*, 1978).

During enzymatic oxidation the sub-units in a lignin polymer, joined through carbon-carbon and ether bonds are cleaved (Breen & Singleton, 1999). Different enzymes can be combined to produce different strategies of lignin biodegradation (Reid, 1995). Different enzyme classes participate in the wood degradation process (Tuor *et al.*, 1995) with different systems that are essential in converting substrates biologically for utilisation by fungi (Crowder *et al.*, 1978). The enzymes attack wood cell walls, breaking down most of the components of the cell wall enabling fungi to utilise substrates in the cell wall (Tanesaka *et al.*, 1993).

Manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Lac) are extracellular enzymes (Breen & Singleton, 1999; Li *et al.*, 2001) that are produced by white-rot fungi (Hatakka, 1994). These enzymes, collectively known as phenol oxidases (Tuor *et al.*, 1995; Li *et al.*, 2001), are the most important enzymes in the degradation, but not the only ones actively involved (Ander & Eriksson, 1978). Other enzymes involved in lignin degradation include cellobiose:quinone oxidoreductase (CBQase) (Ander & Eriksson, 1978), aryl-alcohol oxidase (AOO), aryl-alcohol dehydrogenase (AAD) and NADH: quinone oxidoreductase (Fiechter, 1993). The presence of these enzymes in degradation, especially the phenol oxidases (LiP, MnP and Lac) is required very early in wood decay, because they play an important role in lignin degradation which activate the cleavage of bonds between an aromatic ring and propane side chain (Ander & Eriksson, 1978).

Lignin Peroxidase

Lignin peroxidase is a glycoprotein with a heme group (Boominathan & Reddy, 1992) and is produced by a variety of white-rot fungi, such as *Phanerochaete chrysosporium*, *Trametes versicolor* (Linnaeus: Fries) Pilát, *Pleurotus ostreatus* (Jacquin: Fries) Kummer, *Bjerkandera adusta* (Willd.: Fr.) Karst., *Lentinus edodes* (Berkeley) Singer and *Merulius tremellosus* Shrad.: Fries (Reid, 1995; Boominathan & Reddy, 1992; Martínez, 2001). This enzyme is produced where either nitrogen or carbon limitations are experienced during secondary metabolism (Evans, 1991).

Lignin peroxidase is protected against inactivation by veratryl alcohol (VA) (Reid, 1995) and the enzyme can also use veratryl alcohol as a substrate. The enzyme is an efficient oxidizer of phenols, aromatic amines, aromatic ethers and polycyclic aromatic hydrocarbons. It reacts with lignin compounds by initially attacking the lignin molecule at the non-phenolic β -O-4 or β -1 bonds. The attack on these bonds leads to the oxidization of methoxylated aromatic rings with the formation of cation radicals (Breen and Singleton, 1999; Evans, 1991; Reid, 1995). Lignin peroxidase is, therefore, an important enzyme during the primary degradation steps of lignin (Fiechter, 1993).

Lignin peroxidase oxidizes non-phenolic compounds in lignin by reacting with H_2O_2 and forming a two-electron oxidized intermediate (Boominathan & Reddy, 1992; Gunnar, 2001). A one-electron oxidized intermediate is then produced by the oxidation of the lignin substrate and a cation radical is formed. The cation radical could play a role in various reactions, e.g. in the cleaving of carbon-carbon bonds, hydroxylation and demethylation (Boominathan & Reddy, 1992).

Lignin peroxidase has been found in close association with the plasma membrane of cells and in the extracellular wall layers and mucilage layer around fungal hyphae (Evans, 1991) and, therefore, lignin degradation will occur relatively close to the hyphae. High concentrations of lignin peroxidase were found in degraded cell walls and at sites where the middle lamellae showed signs of degradation (which is also seen with Manganese peroxidase) (Akhtar *et al.*, 1997).

Further studies by Akhtar *et al.* (1997) showed, with more accuracy, where enzymes penetrate the cell wall of decayed wood. Akhtar *et al.* (1997) combined immunological cytochemistry with electron microscopy in determining the location of different enzymes associated with lignin degradation in wood. Polyclonal and monoclonal antibodies bound to lignin peroxidase and manganese peroxidase indicated the presence of the enzymes in cell walls of decaying wood (Akhtar *et al.*, 1997). Lignin peroxidase and manganese peroxidase were found in the secondary wall near electron dense regions or where the middle lamella was degraded. These

enzymes were always located where alterations of the cell wall were evident (Akhtar *et al.*, 1997). After treatment of wood with lignin peroxidase, manganese peroxidase or an extracellular extract from white-rot fungi, gold-labelled antibodies were applied to determine if there was any penetration into the cells. Cells that were not degraded were also studied and showed no penetration of the enzyme into the secondary wall. However, enzymes were found in the lumen on the surface of the cell wall. The treated wood showed that penetration could be achieved into the peripheral zones in the secondary wall. Penetration could also be seen in the middle lamellae at less electron dense zones (Akhtar *et al.*, 1997).

Manganese Peroxidase

Manganese peroxidase is a glycosylated enzyme that contains heme and requires small amounts of H₂O₂ to function (Breen & Singleton, 1999; Evans, 1991). Many white-rot fungi, for example *P. chrysosporium* and *Ceriporiopsis subvermispora* (Pilát) Gilbertson & Ryvardeen, can produce MnP (Boominathan & Reddy, 1992; Breen & Singleton, 1999; Reid, 1995; Vicuña *et al.*, 2001).

The enzyme produces oxidizing agents that can diffuse away from the enzyme (Breen & Singleton, 1999). Manganese peroxidase attacks phenolic β-1-bonds and oxidizes phenolic substrates to phenoxy radicals indicating a similar action to laccase (Reid, 1995). The enzyme has an affinity for Mn(II) that acts as a reducing substrate. Mn(II), therefore, gets oxidized to produce Mn(III) that binds with organic acids such as malonate to oxidize phenolic residues in lignin (Reid, 1995; Breen & Singleton, 1999; Hofrichter *et al.*, 2001). High concentrations of manganese peroxidase were found in degraded cell walls and at sites where the middle lamellae showed signs of degradation. (Akhtar *et al.*, 1997).

Laccase

Laccase is known as a multicopper-containing enzyme (Thurston, 1994). Many kinds of white-rot fungi, such as *Coriolus versicolor* (Wulf.: Fr.) Quél., *Pycnoporus cinnabarinus* and *Pleurotus ostreatus* produce laccase to degrade lignin

(Reid, 1995; Eggert *et al.*, 1996; Eggert *et al.*, 1995; Bonnen *et al.*, 1994; Li *et al.*, 2001).

Laccase interacts directly with the phenolic compounds of lignin and reduces molecular oxygen to water that leads to a one-electron oxidation of an aromatic substrate (Breen & Singleton, 1999; Sannia *et al.*, 2001). The action of laccase can also be initiated via a number of mediators that allow laccase to oxidise a larger range of substrates (Breen & Singleton, 1999). The best-known mediator is 2,2'-azinobis-(3)-ethylbenzothiazoline-6-sulphonate (ABTS) that can oxidize lignin by diffusing through the intact cell wall through which laccase cannot penetrate (Breen & Singleton, 1999).

Cellobiose:quinone oxidoreductase

Cellobiose:quinone oxidoreductase is a heme-flavin enzyme (Reid, 1995) that has been identified in *C. versicolor*, but brown-rot fungi cannot produce the enzyme (Ander & Eriksson, 1978). Cellulose:quinone oxidoreductase is an important enzyme in the degradation of lignin and cellulose, because it is involved in ring cleavage by oxidizing cellobiose from cellulose to cellobiono- δ -lactone (Ander & Eriksson, 1978; Schlegel, 1993). Phenoxy radicals and quinines from lignin act as electron acceptors during this process (Kirk *et al.*, 1978). Cellobiose:quinone oxidoreductase acts as a reducer of phenoxy radicals and quinones to reduce the toxicity of quinones and also to prevent unwanted polymerization in lignin (Ander & Eriksson, 1978; Kirk *et al.*, 1978).

Auxiliary enzymes

Aryl-alcohol oxidase (AAO), aryl-alcohol dehydrogenase (AAD) and NADH:quinone oxidoreductases are three relatively unknown enzymes that also play a role in lignin degradation (Fiechter, 1993). These enzymes are less important compared to the oxidases and will, therefore, not be discussed in detail.

Aryl-alcohol oxidase produces H_2O_2 , through the transfer of electrons to O_2 , which is used by peroxidases. The transfer of electrons occurs when the aryl-alcohol

oxidase-enzyme catalyses the conversion of benzyl alcohol to an aldehyde (Fiechter, 1993). The formation of the aldehyde includes the transfer of an O_2 to form H_2O_2 (Breen & Singleton, 1999).

Phanerochaete chrysosporium produces aryl-alcohol dehydrogenase, an enzyme that is active in the last reduction step of veratryl alcohol synthesis (Fiechter, 1993). It plays a role in stabilizing lignin peroxidase (LiP) and it also acts as a mediator between LiP and lignin. Aryl-alcohol dehydrogenase is localised intracellularly (Fiechter, 1993).

NADH:quinone oxidoreductase is produced by white-rot fungi such as *P. chrysosporium* (Fiechter, 1993). This enzyme attacks the phenoxy radicals and toxic quinones produced through the action of phenol oxidases on lignin (Machuca & Duran, 1993) and is found intracellularly (Fiechter, 1993). Glyoxal oxidase is produced by *P. chrysosporium* (Fiechter, 1993). Glycol oxidase is an extracellular enzyme that also produces H_2O_2 (Breen & Singleton, 1999).

COLONISATION BY FUNGI

Organic material provides an opportunity for fungi to establish in the decaying lignocellulosic material during the colonisation process (Breen & Singleton, 1999). Fungi establish in decaying material by colonisation of the sapwood through penetration or by infecting the decaying material through wounds (Pearce, 1996). It is, however, important to distinguish between primary and secondary colonisers of decaying material, because a process of succession takes place.

Primary colonisers

Primary colonisers start the succession of fungi on wood (Niemelä *et al.*, 1995). These pioneer fungi can live on dead trees for many years and then slowly give way to competitive secondary colonisers or saprotrophs until they are eventually replaced. Brown and white-rot fungi can be primary colonisers and

colonise vast areas of wood (Niemelä *et al.*, 1995; Hawksworth *et al.*, 1995). *Ophiostoma piliferum* (Fries) H. and P. Sydow and *Ceratocystis* spp. are examples of primary colonisers (Haller & Kile, 1992; Blanchette *et al.*, 1992) amongst the ascomycetes. *Ophiostoma piliferum*, as a primary coloniser, can outcompete other fungi for nutrients and so minimize the colonisation by other staining fungi (Haller & Kile, 1992). The ability of primary colonisers to outcompete other fungi could be ascribed to the extracellular metabolites they release that inhibit the development and colonisation of other fungi (Niemelä *et al.*, 1995).

Secondary colonisers

Secondary saprotrophs are the successors to primary colonisers and usually occur more abundantly in natural forests where dead trees are not removed (Niemelä *et al.*, 1995). Successors occupy small volumes of wood and the basidiocarps of primary colonisers can act as a substrate for the fruiting bodies of the successor. Successors can be divided into two groups of fungi. The first group specializes in utilising tree trunks at the last stages of decay and usually causes white rot. This group quickly appears on wood and includes fungi from the Corticiaceae, eg. *Serpula himantoides* (Fr.: Fr.) P. Karsten as well as some polypores, eg. *Tyromyces canadensis* Overh. These fungi occupy small pieces of wood and form basidiocarps in shaded spaces under the tree trunk (Niemelä *et al.*, 1995). The second group of secondary saprotrophs can only survive in conditions that stay stable for long periods, because this group is usually very slow to establish (Niemelä *et al.*, 1995). The second group also includes a few corticiaceous species, eg. *Tylospora fibrillosa* (Burt) Donk (Niemelä *et al.*, 1995).

Interaction between fungi during colonisation

When mycelia from different individuals meet, antagonistic interactions occur. Interactions between fungi lead to "deadlock" or "replacement" and these interactions are important in the patterns of community development and the rate of wood degradation. "Deadlock" occurs when two species of fungi are present and neither can dominate the other, leading to a zone that is not colonised (Owens *et al.*, 1994). "Replacement" occurs when one fungus can dominate another by colonising its decay

column completely. Owens *et al.* (1994) paired different species of brown-rot fungi and found that interspecific mycelial interactions usually lead to deadlock or to the replacement of one fungal strain by another. No mutualistic interactions were observed when brown-rot fungi were paired. Owens *et al.* (1994) also paired brown-rot fungi with white-rot fungi showing that some brown-rot fungi might be capable of successfully colonising zones already colonised by white-rot fungi during wood decay. When *P. chrysosporium* was paired with other strains of white-rot fungi, it was found that *P. chrysosporium* replaced or deadlocked all the other white-rot fungi involved. It was, therefore, concluded that *P. chrysosporium* is more combative than the other fungal species (Owens *et al.*, 1994).

INDUSTRIAL APPLICATIONS

Biodegradation is a low-energy process that could be applied in industries such as the pulp and paper industry to save money (Reddy, 1978; Kang *et al.*, 2001). Lignin can be converted to low-molecular-weight chemicals that can replace the use of petroleum (Crawford & Crawford, 1980). When the suitable organisms are selected, many other useful products can be obtained from the lignin breakdown process (Crawford & Crawford, 1980). Industrial application of biodegradation of lignocellulosic materials can, therefore, play an important role in the world economy.

The chemical removal of lignin on an industrial scale is done by using acids or alkali while the physical separation is done using fine milling and ultrasonic excitation (Reddy, 1978). These methods are expensive, non-specific and lead to tremendous amounts of hazardous chemical wastes. Biodegradation of lignin has, therefore, come into consideration by the pulp and paper industry (Reddy, 1978). Biodegradation has been applied to many fields in the forest products industry, with the most notable applications in biopulping, biobleaching and wastewater treatment (Eriksson, 1991).

Biopulping is defined as the pretreatment of wood by lignin degrading fungi before applying mechanical or chemical pulping processes (Messner *et al.*, 1992). Biopulping, therefore, improves the pulping process, but the treatment time of the biological treatment must be minimized (Crowder *et al.*, 1978, Eriksson *et al.*, 1980).

Biomechanical pulping

Separation of fibres by physical processes is known as mechanical pulping (Reid, 1991). This pulping method has high yields and is not very expensive. Some disadvantages of this method of pulping are, a) paper with reduced optical properties; b) low paper strength; c) yellowing of the paper when exposed to light and d) high electrical energy inputs (Reid, 1991). When wood for mechanical pulping was treated with fungi it led to energy savings, strength increases in the pulp and improved brightness of the paper (Crowder *et al.*, 1978; Behrendt *et al.*, 2000). Reid (1991) applied *T. versicolor*, *P. chrysosporium* and *Pleurotus ostreatus* in the treatment of wood which led to a better paper strength and also to reduced energy required in pulping. However, biological treatment with *P. chrysosporium* before kraft pulping led to improved burst and tensile strength of the pulp but influenced the tear strength, brightness and yield negatively (Reid, 1991). These fungi secrete LiP, MnP and laccase (Reid, 1995; Boominathan & Reddy, 1992; Breen & Singleton, 1999) that directly influence lignin degradation and, therefore, pulp quality (Pilon *et al.*, 1982). It was shown in an economic evaluation that savings of up to US\$33 per ton of pulp could be achieved using biomechanical pulping due to the saving in refining energy (Reid, 1991). The lower energy input that is needed during biomechanical pulping and the elimination of waste streams from pulp manufacturing, that is difficult to treat, make the process more environmental friendly (Reid, 1991).

Biochemical pulping

Chemical pulping is used to remove lignin to separate wood fibres and in comparison with mechanical pulping, it has a lower yield and is, therefore, much more expensive (Reid, 1991). The most common chemical pulping processes are based on the sulfite process and kraft processes where kraft pulp yields 80 % of the chemical pulp produced in the world (Sjöström, 1993). Biological treatment of wood

with *P. chrysosporium* before kraft pulping led to improved burst and tensile strength of the pulp but influenced the tear strength, brightness and yield negatively (Reid, 1991).

Biobleaching

In traditional pulp bleaching the aim is to remove or decrease the residual lignin content in kraft pulp using oxidising chemicals such as chlorine. Chlorine can, however, react with organic molecules to produce potentially hazardous compounds. Biobleaching is a biotechnological process where microorganisms can initialise natural reactions to degrade lignin with less environmental impact (Eriksson, 1990; Eriksson, 1991).

Xylanases are utilised commercially for the biobleaching of pulp (Dunlop-Jones & Grönberg, 1994; Bermek *et al.*, 2000) and reduce the amounts of chlorine used in bleaching processes (Madlala *et al.*, 2001). It is, therefore, a more environmental friendly approach towards bleaching (Dunlop-Jones & Grönberg, 1994). Xylanase does not cause lignin degradation, but could break the bonds between lignin and xylan that lead to the release of lignin (Dunlop-Jones & Grönberg, 1994).

Extracellular ligninolytic enzyme systems including LiP, MnP and laccase have been studied in bleaching processes (Li *et al.*, 2001). Poppius-Levlin *et al.* (1997) studied the effect of HBT-mediated and ABTS-mediated laccase systems on three different chemical pulps in biobleaching. Pulp bleaching with a laccase-mediated system was very successful (Poppius-Levlin *et al.*, 1997). Camarero *et al.* (2001) used high-quality pulps from non-woody fibres to determine the effect of fungal laccases together with ABTS or HBT mediators in the development of a totally chlorine-free (TCF) process to bleach different pulps. This study demonstrated that laccases from *Pycnoporus cinnabarinus* (Jacquin: Fries) Karsten and *Trametes versicolor* were the most effective with HBT as a mediator. The results showed an increase of 10 to 15 % in ISO brightness as well as a decrease in kappa number (Camarero *et al.*, 2001). Surma-Ślusarska and Leks-Śtepień (2001) used laccase

together with HBT on hardwood and softwood pulp and showed that it was possible to improve the brightness of both pulps. This trial also indicated that less chemicals could be used during TCF bleaching (Surma-Ślusarska & Leks-Stepień, 2001).

Bleaching of kraft pulp with MnP had a notable effect on the brightness and kappa number of the pulp compared to untreated samples (Kondo *et al.*, 1994; Bermek *et al.*, 2000). According to Kondo *et al.* (1994) the brightness of the pulp increased with approximately 10 points and the kappa number showed a decrease of six points. Archibald (1992) examined the effect of LiP secreted by *T. versicolor* on the bleaching of pulp and found that LiP did not play a very dominant role in the process. This could be due to the fact that LiP proteins will only appear when low manganese concentrations are present, when no or gentle shaking is applied and when a surfactant, eg. Tween 20, is applied in the production of the supernatant. These special conditions have to be maintained for optimal production of LiP, but are not present in the biobleaching systems (Archibald, 1992).

Biobleaching of pulp could lead to lower costs in the industry and the application of MnP and Lac seems to be the most effective lignin degrading enzymes for biological treatment in pulp bleaching. When Lac, together with a mediator system, is applied for bleaching it seems to be more effective than MnP. Lignin peroxidase does not seem to have such a notable effect in bleaching and, therefore, bleaching processes utilising Lac in association with a mediator system should be optimised.

CONCLUSIONS

Lignocellulosic materials such as wood and bagasse are utilised as sources of fibre in pulping (Fernández *et al.*, 1989). The process of lignin degradation is not yet fully understood (Crowder *et al.*, 1978; Breen & Singleton, 1999) and, therefore, the degradation of these fibre sources must still be optimised. Lignin is a very complex molecule, because of different bonds that form between the precursors of lignin. The

bonds between the monomers make the lignin units very recalcitrant, which makes it difficult to obtain pure forms of lignin, hemicellulose or cellulose without breaking the bonds (Odier & Artaud, 1992). Lignin concentrations are very low in the primary and secondary walls of fibres. The middle lamella, however, contains the highest concentration of lignin (Reddy, 1978), making this layer the most difficult to degrade.

To be able to apply biodegradation effectively, certain conditions have to be maintained. Aerobic conditions, low nitrogen environments and suitable water availability during degradation are very important for optimal results (Breen & Singleton, 1999; Lawson & Still, 1957; Eriksson *et al.*, 1980). Selective or simultaneous degradation can occur during the decay process. Simultaneous degradation, however, presents a problem in that lignin, cellulose and hemicellulose are degraded. Selective rot, on the other hand, degrades lignin faster than cellulose or hemicellulose (Breen & Singleton, 1999; Ander & Eriksson, 1978) and this type of decay could be the most significant for application in the pulp and paper industry.

Lignin peroxidase, MnP and laccase are the most important enzymes involved during lignin degradation (Ander & Eriksson, 1978). These phenol oxidases are secreted by different groups of white-rot fungi and are required early in the decay process (Hatakka, 1994; Ander & Eriksson, 1978). Lignin peroxidase and MnP have been found in the middle lamella (Akhtar *et al.*, 1997), indicating that these enzymes can penetrate the layer that is most difficult to degrade. Laccase can also oxidise a wider range of substrates with the help of a mediator (Breen & Singleton, 1999).

Biodegradation has many applications in industrial processes (Reddy, 1978; Eriksson, 1991; Eriksson, 1990) especially in the pulp and paper industry. These biotechnological applications can lead to lower energy consumption and better product quality in comparison with conventional processes (Lascaris *et al.*, 1997; Buchert *et al.*, 1994; Viikari *et al.*, 1993).

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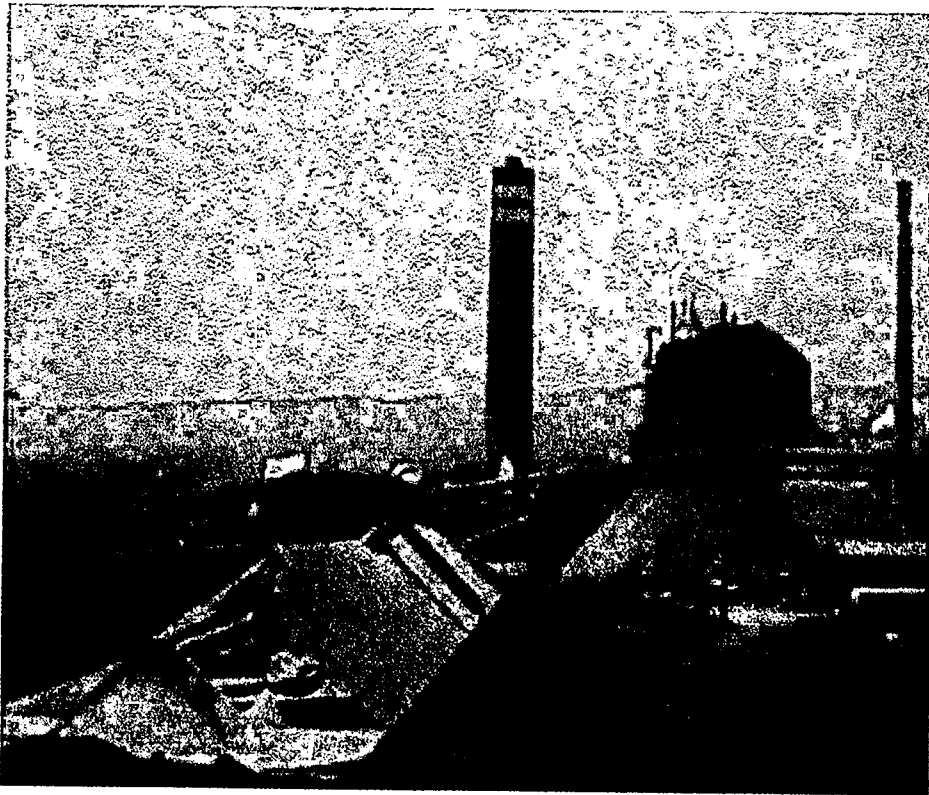
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CHAPTER 2

CERTIFICATION AND EVALUATION OF

CARTAPIP 97[®]



**Softwood (foreground) and hardwood (background) chip piles at the
Sappi Ngodwana kraft mill**

ABSTRACT

Cartapip 97[®] was developed for treatment of wood chips during storage periods and is produced from a melanin deficient strain of *Ophiostoma piliferum*. This fungus was not available in South Africa because of a possible threat to South African forest species. However, South African forestry companies wished to test Cartapip 97[®] in industrial processes and the fungus had to be certified as safe before importation for industrial trials. The pathogenicity of the Cartapip fungus had to be verified and it had to be confirmed that the fungus is a strain of *O. piliferum* before certification. The cultural characteristics and the morphology of the teleomorph and the anamorph were, therefore, studied. The cultural and morphological characteristics of the anamorph of the Cartapip fungus were similar to those of *O. piliferum* in every aspect. Cartapip 97[®] was consequently released for field testing. The pathogenicity of the fungus was compared with common causes of sapstain on *Pinus* spp. in South Africa (*Ophiostoma ips* and *Sphaeropsis sapinea*), to demonstrate that *O. piliferum* does not pose a threat to forestry. Different pine species were inoculated during autumn and spring at three different locations. The results obtained led us to conclude that *O. piliferum* should not be regarded as a pathogen and that it is safe to use Cartapip 97[®] in South Africa. Cartapip 97[®] was subsequently evaluated for its effect on wood chips before pulping. Softwood chips (*Pinus patula* and *Pinus elliottii*) were pulped according to the kraft pulping process and hardwood chips (*Acacia mearnsii* and *Eucalyptus grandis*) were pulped according to the sulphite, kraft and Soda-AQ pulping methods. The benefit of the chip treatment with *O. piliferum* was not obvious, but it is possible that the extractives content of the chips was reduced. The application of *O. piliferum* in pulping did, however, show a slight increase in strength of kraft pulp obtained from softwood and hardwood and also of Soda-AQ pulp obtained from *A. mearnsii*.

INTRODUCTION

The aim of this study was to obtain evidence to allow the release of Cartapip 97[®] in South Africa and to evaluate the potential benefit of the product on different chemical pulping processes. Cartapip 97[®] (AGRASOL Inc, Raleigh, NC) is a fungal product that was developed for application to wood chips during chip storage for the prevention of blue stain and the utilization of pitch (Zimmerman *et al.*, 1995). It is produced from a strain of *Ophiostoma piliferum* (Fries) H. and P. Sydow (Zimmerman *et al.*, 1995). This product is, however, not available in South Africa, because of the possible threat to South African forest species. In recent years, South African forestry companies have wished to test Cartapip 97[®] in various industrial processes. The product could, however, not be imported into South Africa until it had been certified as safe for general release by the Department of Agriculture. At that time, *O. piliferum* was not known to occur in South Africa and no information was available on the effect of Cartapip 97[®] on the *Pinus* spp. commonly grown in South Africa.

Ophiostoma piliferum occurs commonly on softwoods throughout the United States of America and the taxonomy, biology and economic importance of the fungus have been well documented (Upadhyay, 1981; Wingfield *et al.*, 1993). The EPA and USDA view *O. piliferum* as a fungus that is non-pathogenic or toxic to plants and animals (Wall *et al.*, 1994). It has, furthermore, never been associated with bark beetle vectors and is known as an exclusively saprobic fungus (Dowding, 1969; Farrell *et al.*, 1993). As a saprophyte, it colonises dead organic material and does not infect living trees. *Ophiostoma piliferum* has been tested and approved for release in countries including Australia, Brazil and New Zealand (Kay, 1997) that also have stringent quarantine requirements. Since these releases there have been no negative effects associated with this fungus (Blanchette & Farrell, 1997).

In order to meet quarantine regulations in South Africa, the identity of the Cartapip 97[®] fungus as a strain of *O. piliferum* had to be verified. The cultural

characteristics, morphology and mating behaviour of the fungus have to be studied to verify the identity of Cartapip 97[®] as a strain of *O. piliferum*. The Cartapip 97[®] strain is unable to produce the teleomorph when grown in pure culture, because the fungus is heterothallic and also unable to produce melanin that is required in the fruiting bodies (Zimmerman *et al.*, 1995). The production of the sexual state can be obtained by crossing strains from complimentary mating types and adding the melanin precursor, Scytalone (Zimmerman *et al.*, 1995). *Ophiostoma piliferum* is similar to other *Ophiostoma* spp. in that it has two mating types, A and B (Zimmerman *et al.*, 1995). When sexually compatible individuals belong to the same biological species it should be able to produce progeny that is also sexually fertile (Li & Graur, 1991). Mating type studies were, therefore, done with different strains of *O. piliferum*. After the identity of the Cartapip fungus was confirmed, its pathogenicity to pine species was investigated under South African conditions. During this study the pathogenicity of the fungus was compared with that of other fungi (*O. ips* and *Sphaeropsis sapinea*) that cause sap stain.

Biopulping can be defined as the application of fungi to raw material for improvement of pulping characteristics (Wall *et al.*, 1996). Biopulping can lead to savings in energy and reduce the amount of chemicals used in the pulping process (Wall *et al.*, 1996). One of the first fungi to be applied for biopulping was *O. piliferum* (Cartapip 97[®]) that assists in the reduction of the pitch in wood chips for mechanical pulping (Farrell *et al.*, 1993). The utilisation of pitch improved the chemical pulping process and resulted in stronger paper with better optical properties (Farrell *et al.*, 1993). Cartapip 97[®] has also been applied in chemical pulping of wood resulting in an increase of pulp yield and viscosity and also a reduction in chemical consumption (Wall *et al.*, 1994). The influence of Cartapip on chemical pulping of soft and hardwood was, therefore, evaluated. Biokraft pulping was done on softwood (*P. patula* and *P. elliottii*) chips and biokraft, sulphite and Soda-AQ pulping on hardwood (*A. mearnsii* and *E. grandis*) chips.

2.1. MORPHOLOGY OF THE CARTAPIP FUNGUS

MATERIALS AND METHODS

Purification and cultivation of the fungus

The Cartapip fungus (*O. piliferum*) was purified from the inoculum of Cartapip 97[®] (Clariant South Africa, Durban). A spatula tip of the commercial Cartapip inoculum was stirred into 10 ml of sterile distilled water. The suspension was transferred to Potato Dextrose Agar (PDA) (BIOLAB Merck, Midrand) plates (800 µl per plate) and cultivated at 24 °C for eight days. Pure cultures of the fungus were then transferred to Malt Extract Agar (2 % Malt extract, 2 % Agar) (BIOLAB Merck) slants.

Morphology of the anamorph

The fungus isolated from Cartapip was grown on Malt Extract Agar (MEA) for 18 days at 22 °C. Fungal material was mounted in lactophenol with cotton blue and examined microscopically. Phase contrast and differential interference contrast (DIC) microscopy were used to study the anamorph characteristics of the fungus. This examination included the measurements of 50 conidia. These characteristics of the Cartapip fungus were compared with characteristics described for *O. piliferum* (Upadhyay, 1981).

Cultural characteristics

Six strains of *O. piliferum* (Table 1) and the strain from Cartapip, were cultivated at different temperatures to compare their growth rates and cultural characteristics. The inoculum was produced by cultivating the strains on MEA and PDA plates for five days at 23 °C. A 4-mm cork borer was used to remove plugs of colonised agar that were transferred to MEA and PDA plates for growth studies. The cultures were incubated at 10, 15, 20, 25 and 30 °C. Growth rates of different strains at different temperatures were, therefore, compared in a factorial experiment with three treatments (strains, temperature and media). Each treatment was replicated

three times. Colony diameters were monitored daily and recorded on the day before the fungal growth reached the edge of the Petri dishes. Data were subjected to analysis of variance and significance of differences in mean growth rate tested with Tukey's test (Winer, 1971) at $p \leq 0.05$.

Table 1. Culture number and source of strains used in studies of cultural characteristics.

Strain number	Source
SCC 308	Cartapip
CMW 1758	<i>P. ponderosa</i> , U.S.A.
CMW 1024	<i>P. sylvestris</i> , Netherlands.
CMW 2481	Spruce lumber, Canada
CMW 2623	Unknown
CMW 2626	Unknown
CMW 2627	Unknown

Studies of the teleomorph

A purified culture from the commercial Cartapip 97[®] preparation was crossed with four single-ascospore strains of each of the A and B mating type strains. These single-ascospore strains were produced by Z. W. de Beer (Forestry and Agricultural Biotechnology Institute, University of Pretoria) from strains CMW 2481 and CMW 2482. All the crosses, including crosses between the A and B mating type strains, were made in duplicate.

The pairings were completed on water agar (1 % Agar) and sterile pine twigs were placed between the two test strains. Cultures were incubated for 50 days at room temperature until proto-perithecia formed. The proto-perithecia were flushed with scytalone (0.01 %) according to the method of Zimmerman *et al.* (1995). The presence of fertile perithecia on the twigs and media was noted after a further 30 days and the morphological characteristics were studied. The diameter of the ascocarp base, the length of the perithecium neck and the width of the perithecium neck at the apex were measured and the shape of the ascospores recorded (Upadhyay, 1981).

Twenty measurements were made for each character and the data were subjected to analysis of variance.

Production of single-ascospore cultures

The ascospores produced during successful crosses were transferred to 1 % water agar and flushed with water to disperse the spores for single-ascospore colonies to develop. Four single-ascospore cultures resulting from crosses between the Cartapip fungus with *O. piliferum* were transferred to 1 % MEA to determine if the single-ascospore progeny were also fertile. These strains were crossed with both the A and the B mating type strains of *O. piliferum*.

RESULTS AND DISCUSSION

Morphology of the anamorph

The cultural and morphological characteristics of the Cartapip fungus were similar to those of *O. piliferum* (*sensu* Upadhyay, 1981) in almost every aspect (Table 2). The simple, septate, hyaline conidiophores (Figure 1 & 2) with terminal or intercalary denticles (Figure 3 & 4) were unmistakable. Conidia were holoblastic, hyaline, one-celled, and cylindrical to ellipsoidal, often pointed at the base (Figure 5). Conidiogenesis occurred, either sympodially (Figure 1 & 2) or in acropetal chains with ramoconidia (Figure 6 & 7).

Table 2. Comparison of characteristics of *Ophiostoma piliferum* with the Cartapip fungus (SCC 308).

Characteristics	Upadhyay (1981)	Cartapip fungus (SCC 308)
Colony morphology	Effuse, cottony	Effuse
Colony colour	White, might become brownish black	White
Superficial hyphae	Hyaline to subhyaline	Hyaline
Submerged hyphae	Hyaline to subhyaline or brown	Hyaline
Conidiophores	Mononematous, simple, septate, hyaline	Simple, septate, hyaline
Conidiogenous cells	Terminal or intercalary, denticulate, cicatrized	Terminal or intercalary, denticulate, cicatrised
Conidiogenesis	Sympodially like <i>Sporothrix</i> , subsequently in acropetal chains typical of <i>Hyalodendron</i>	Sympodially like <i>Sporothrix</i> , or in acropetal chains typical of <i>Hyalodendron</i>
Conidia	Holoblastic, hyaline, one-celled, cylindrical to ellipsoidal, often pointed at the base	Holoblastic, hyaline, one-celled, cylindrical to ellipsoidal, often pointed at the base
Conidium length	3-9 μ m	4-12 (-25) μ m *
Conidium width	1-3 μ m	1-3 μ m *

* 50 conidia were measured

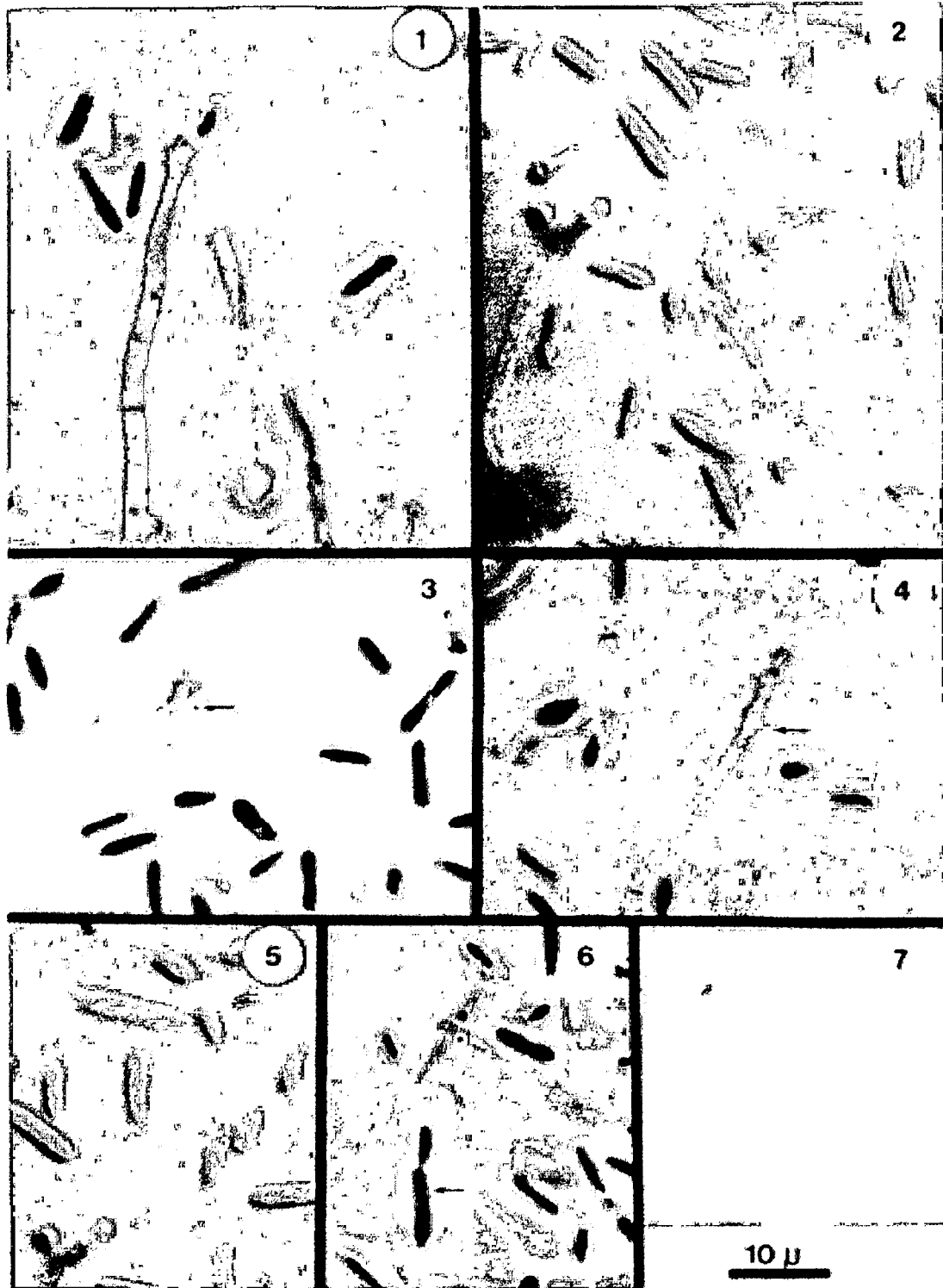


Figure 1-7. The Cartapip fungus. 1-2. Simple, septate, hyaline conidiophores. 3-4. Terminal, denticulate conidiogenous cells, showing sympodial proliferation. 5. Holoblastic, hyaline, one-celled, cylindrical to ellipsoidal conidia. 6-7. Budding conidia showing ramoconidia.

Cultural characteristics

Mean colony diameter of cultures was recorded after four days on MEA and after six days on PDA. All interactions between temperatures, colony diameter of different strains and media were significant. Growth rates for the different strains were, therefore, compared at each temperature and on different media. The colony diameter of the Cartapip fungus agreed with that of other cultures of *O. piliferum*. However, some exceptions were observed. The fastest (CMW 1024) and the slowest growing cultures (CMW 1758 and CMW 2623) differed significantly from the Cartapip fungus on MEA at 30 °C (Table 3). The slowest growing culture (CMW 1024) also differed significantly from the Cartapip fungus on PDA at 10, 15 and 20 °C (Table 4). However, this strain appears to be thermotolerant and grew significantly faster than all other strains at 30 °C. Some of the other strains (CMW 1758, CMW 2623 and CMW 2481) grew significantly slower than the Cartapip fungus at this temperature. The most variable growth rates were, therefore, observed at 30 °C.

Table 3. Mean colony diameter (mm) of *Ophiostoma piliferum* cultures after four days growth on malt extract agar.

Strain number	Temperature (°C)				
	10	15	20	25	30
SCC 308	4.50a	7.10a	5.50a	28.20ab	18.70b
CMW 1758	4.40a	6.40a	7.30a	25.80b	4.00c
CMW 1024	4.00a	4.00a	4.00a	23.80b	48.10a
CMW 2623	9.10a	10.70a	10.90a	33.00ab	4.00c
CMW 2626	5.50a	8.60a	6.40a	27.90b	17.60bc
CMW 2627	4.18a	5.80a	4.00a	25.60b	15.30bc
CMW 2481	4.40a	6.50a	4.40a	42.20a	4.40bc

abc: Mean colony diameters, in columns, followed by the same letter do not differ significantly ($p \leq 0.05$; Tukey's test).

Table 4. Mean colony diameter (mm) of *Ophiostoma piliferum* cultures after six days growth on potato dextrose agar.

Strain number	Temperature (°C)				
	10	15	20	25	30
SCC 308	12.5ab	18.1ab	14.5ab	44.9a	28.0b
CMW 1758	8.7b	16.1b	12.7bc	40.8ab	4.0c
CMW 1024	4.1c	7.6c	8.7c	41.2ab	67.3a
CMW 2623	15.2a	20.5a	16.6ab	48.8a	4.0c
CMW 2626	11.2ab	17.4ab	12.8bc	42.6ab	29.4b
CMW 2627	10.2b	14.9b	14.5ab	44.2ab	29.2b
CMW 2481	9.8b	14.4b	17.4a	39.8b	5.1c

abc: Mean colony diameters, in columns, followed by the same letter do not differ significantly ($p \leq 0.05$; Tukey's test).

Studies of the teleomorph

Successful crosses were made between the Cartapip strain and the B mating types as well as between the A and B mating types (Table 5). This indicated that the Cartapip fungus represented the A mating type.

Table 5. Results of crosses between the Cartapip strain and strains of mating type A and B.

	Cartapip (SCC 308)	A1	A2	A11	A21	B3	B4	B13	B24
B24	+	+				-	-	-	?
B13	+	+				-	-	-	
B4	+	-				-	?		
B3	+	?	+	+	+	-			
A21	?	-	-	-	-				
A11	-	-	-	?					
A2	?	-	-						
A1	-	-							
Cartapip (SCC308)	-								

+: Fertile cross, perithecia with ascospores formed.

-: Unfertile cross

?: Result of cross uncertain, protoperithecia formed but no ascospores observed

The morphology of the teleomorph produced from crosses that included Cartapip did not differ significantly ($p \leq 0.01$) from crosses between *O. piliferum*

strains (Table 6). The neck lengths of the perithecia, however, differed at $p \leq 0.05$. The measurements of the fruiting bodies and the ascospores produced by the Cartapip fungus and the B mating type fell within the ranges as described by Upadhyay (1981) (Table 6). The measurements from the crosses between the *O. piliferum* strains also fell in ranges as described in Upadhyay (1981) except for the ascospores that were longer (Table 6).

Table 6. Characteristics of the sexual states of the Cartapip fungus and *O. piliferum* in comparison with descriptions in literature.

Characteristics	Cartapip x B type	A type x B type	Significance	Upadhyay (1981)
Perithecium base diameter (μ)	166.50	197.30	n.s.	75-250
Perithecium neck length (μ)	577.00	872.30	$p \leq 0,05$	300-3000
Perithecium neck width (μ)	12.00	11.00	n.s.	≤ 20
Ascospore length (μ)	4.70	5.25	n.s.	3-5
Ascospore width (μ)	1.55	1.55	n.s.	1.50-2.00
Ascospore shape	Orange section	Orange section		Orange section

Production of single-ascospore cultures

Four single-ascospore cultures were obtained from the crosses between the Cartapip fungus and *O. piliferum* (Table 5). When these cultures were paired with the tester strains, fertile perithecia formed (Table 7). The fertile progeny proved that the Cartapip fungus is of the same biological species as *O. piliferum* (Li & Graur, 1991).

Table 7. Results of crosses of the Cartapip progeny, two tester strains and a back cross with the Cartapip strains.

Parents	Progeny (Strain #)	Result of cross with tester strains		
		A1	B4	Cartapip
Cartapip x B3	1-9	-	+	-
Cartapip x B4	6-1	-	+	-
Cartapip x B4	6-9	-	+	-
Cartapip x B13	8-9	+	-	+

+ : Indicates fertile perithecia.

2.2. PATHOGENICITY OF *Ophiostoma piliferum*

MATERIALS AND METHODS

Tree species and location

Three major pine-growing regions of South Africa (Table 8) were selected for the pathogenicity tests with Cartapip 97[®]. Trials were conducted during the autumn and spring of 1997 on *Pinus elliotii* Engelm. var *elliotii*, *P. patula* Schl. & Cham, *P. greggii* Engelm. and *P. radiata* D. Don. Softwood species were targeted, because the strain of *O. piliferum* used for Cartapip 97[®] was isolated from *Pinus taeda* (Farrell *et al.*, 1997) and applications in South Africa would also be on softwoods. The number of trees of each species inoculated, differed from site to site depending on availability. At Longmore *P. radiata* (13 trees) and *P. elliotii* (20 trees) were inoculated. At Ugie *P. patula* (15 trees) and *P. greggii* (14 trees) were used and at Jessievale *P. patula* (16 trees) and *P. elliotii* (12 trees) were available (Table 8). In fulfilment of quarantine requirements, the trial sites were isolated from human activity and surrounded by an isolation area of four meters that was free from all vegetation.

Table 8. Location and description of different trial sites used for pathogenicity trials.

Location and province	Latitude	Longitude	Altitude (m)	Mean annual rainfall (mm/a)	Tree Species
Longmore (Southern Cape)	33° 49' S	25° 08' E	530	702	<i>P. radiata</i> <i>P. elliotii</i>
Ugie (North E Cape)	31° 06' S	28° 14' E	1320	875	<i>P. patula</i> <i>P. greggii</i>
Jessievale (Mpumalanga)	26° 24' S	30° 11' E	1700	878	<i>P. patula</i> <i>P. elliotii</i>

Inoculum

Inoculum of *O. piliferum* was produced by cultivating Cartapip 97[®] on PDA plates. For comparative purposes, trees were also inoculated with strains of *Ophiostoma ips* (Rumb.) Nannf. (CMW 0386) and *Sphaeropsis sapinea* (Fr.:Fr.)

Dyko & Sutton in Sutton (CMW 1184). *Ophiostoma ips* was chosen because it is a sap-stain fungus with a similar biology to *O. piliferum* (Upadhyay, 1981) that occurs commonly in South Africa (Wingfield & Marasas, 1980). *Sphaeropsis sapinea* is one of the most important pathogens of *Pinus* spp. in South Africa (Swart & Wingfield, 1991; Swart *et al.*, 1985). Strains of the latter fungi had been selected for pathogenicity in previous trials (unpublished results) and all strains used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Inoculum of each strain was grown on PDA at 24 °C for eight days. The control treatment consisted of sterile PDA disks.

Inoculation of the pine trees

All treatments were replicated on 12 to 20 trees, depending on the availability of suitable trees in selected areas. Four branches of each tree were selected and inoculated at random with *O. piliferum*, *O. ips*, *S. sapinea* and the control. Trees were inoculated by removing the outer bark from the branches with a 9-mm diameter cork borer and agar discs overgrown with the test fungi or the control were inserted, mycelium facing downwards, into the wounds. Inoculation wounds were covered with masking tape to restrict contamination and desiccation of the inoculum.

Re-isolations of the inoculum

Lesions that developed as a result of the inoculations were examined and measured after six weeks. Re-isolations of the test fungi were also made from the inoculated branches by cutting out small pieces of wood, away from the point of inoculation, but within the confines of the discoloured tissue. The pieces of wood were then placed on different media and incubated at 24 °C. Malt Extract Agar (MEA) supplemented with streptomycin (5 ppm) was used for the re-isolation from the *S. sapinea* and control treatments. Malt extract agar supplemented with cycloheximide (5 ppm) was used for the re-isolation of *O. ips* and *O. piliferum*. The resulting colonies were transferred to new MEA plates to purify cultures. Tissue infected with *O. piliferum* was removed from the trial sites and autoclaved in compliance with quarantine specifications.

Trial design and statistical analysis

A factorial experiment with two factors was used to test the influence of inoculum and season. A randomised block experimental design was applied at each locality and for each tree species. Two-way analysis of variance was done to compensate for differences between the individual trees that were used as replications. Means of different treatments were compared using Tukey's test (Winer, 1971) at a 99 % confidence level. The trial design had limitations in that the same pine species were not available at all test sites, due to the different climates in the various forestry regions.

The influence of the inoculum on different tree species at different locations was tested by combining data for tree species, location and season for each of the test fungi and the control. The smallest number of trees available at the different locations was 12 at Jessievale (*P. elliotii*). The data for 12 trees were, therefore, selected at random for each species, location and season. A completely randomised trial design was used and the data subjected to one-way analysis of variance. The means of different treatments were tested for significant differences using Tukey's test at the 95 % level of confidence.

RESULTS AND DISCUSSION

Inoculation of the pine trees

After six weeks, discolouration of the cambium could be seen for all the inoculations. Interactions between season and inoculation were significant ($p \leq 0.01$) in all trials except at Ugie where season did not influence the development of lesions on *P. greggii*. In this trial, *S. sapinea* resulted in the longest lesions followed by *O. ips*, *O. piliferum* and the control (Figure 8). At other locations, the pathogenicity of the test organisms during autumn and spring had to be examined individually, due to the significant interaction between season and inoculated fungi.

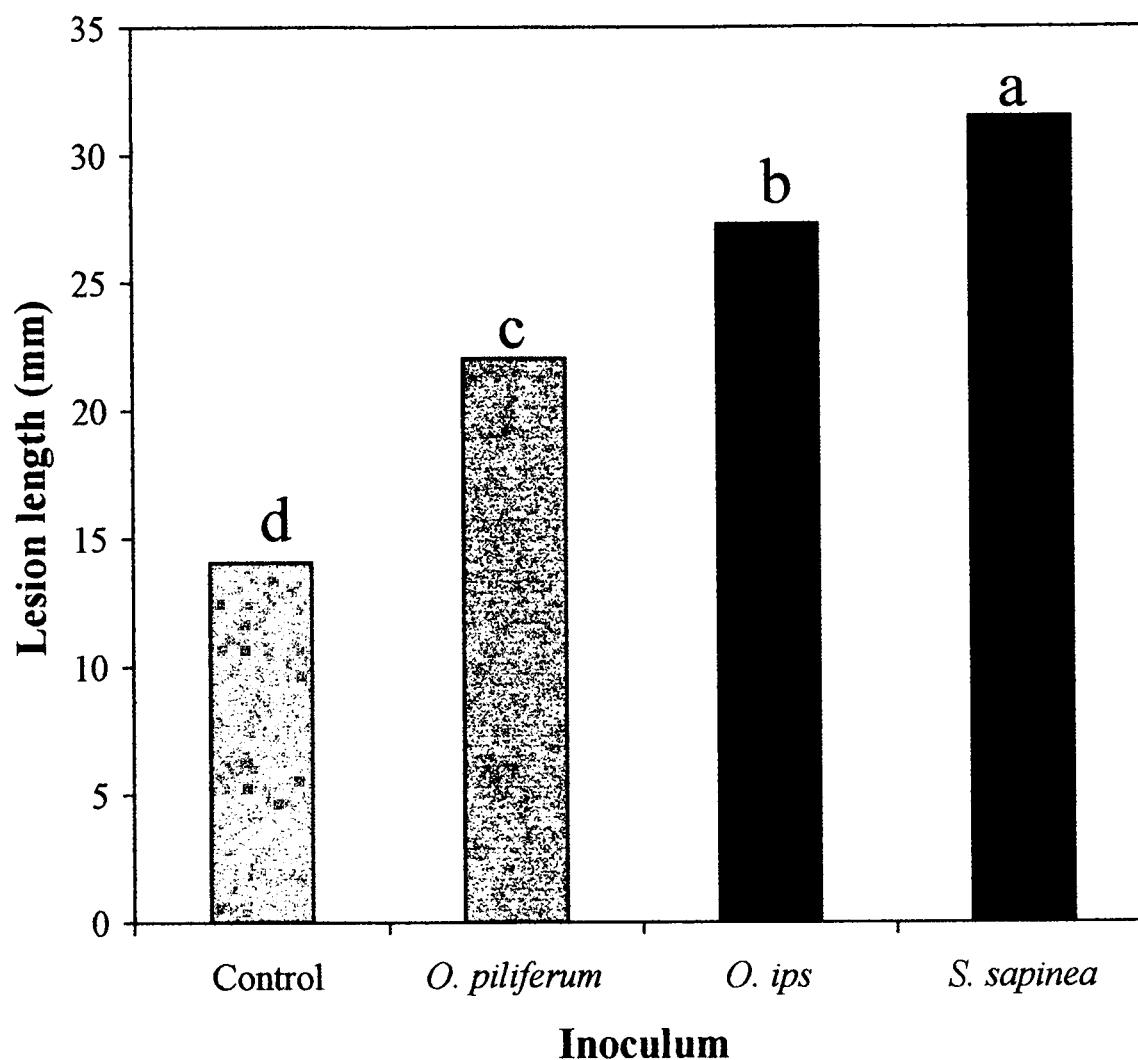


Figure 8. Mean lesion length observed during two seasons on *Pinus greggii* at Ugie. Different letters indicate that bars differ significantly ($p \leq 0.01$; Tukey's test).

Ophiostoma piliferum caused significantly ($p \leq 0.01$) longer lesions than the control on *P. greggii* during both seasons (Figure 8). During the autumn, *O. piliferum* caused longer lesions than the control on *P. patula* and *P. radiata*, but not on *P. elliotii* (Table 9). During the spring, *O. piliferum* gave rise to longer lesions than the control only on *P. patula* at Jessievale (Table 10). However, *O. piliferum* caused significantly shorter lesions ($p \leq 0.01$) or lesions not significantly different from those associated with *O. ips* in all trials (Figure 8, Table 9 and Table 10). These results

support previous reports that *O. piliferum* is not pathogenic; since *O. ips* is not considered to be an important pathogen. The EPA and the USDA have also treated *O. piliferum* as non-pathogenic and non-toxic to plants and animals (Wall *et al.*, 1994).

With the exception of spring inoculations on *P. elliotii* at Jessievale, *S. sapinea* caused significantly ($p \leq 0.01$) longer lesions than other fungi in all trials (Figure 8, Table 9 and Table 10). On average, these lesions were 123 % and 87 % longer than those associated with *O. piliferum* and *O. ips* respectively. *Sphaeropsis sapinea* is a well-known and important pathogen in South Africa (Swart & Wingfield, 1991; Smith *et al.*, 1996; Swart *et al.*, 1985) and its high level of pathogenicity was not surprising.

Table 9. Mean length of lesions after inoculation of *O. piliferum*, *O. ips* and *S. sapinea* on different *Pinus* species at different sites during autumn.

Trial site	Treatment	Mean lesion length (mm)		
		<i>P. patula</i>	<i>P. radiata</i>	<i>P. elliotii</i>
Ugie	<i>S. sapinea</i>	41.5a	-	-
	<i>O. ips</i>	32.1b	-	-
	<i>O. piliferum</i>	25.3b	-	-
	Control	13.8c	-	-
Longmore	<i>S. sapinea</i>	-	35.6a	153.3a
	<i>O. ips</i>	-	23.9b	29.0b
	<i>O. piliferum</i>	-	23.4b	35.8b
	Control	-	16.1c	17.0b
Jessievale	<i>S. sapinea</i>	35.6a	-	56.8a
	<i>O. ips</i>	25.8b	-	21.4b
	<i>O. piliferum</i>	24.1b	-	19.5b
	Control	15.1c	-	13.0b

a,b,c : Mean lesion lengths for each pine species at a specific site, followed by the same letter do not differ significantly ($p \leq 0.01$; Tukey's test). Each value represents the mean of 14 to 20 replications.

- : Pine species not available for inoculation.

Table 10. Mean length of lesions after inoculation of *O. piliferum*, *O. ips* and *S. sapinea* on different *Pinus* species at different sites during spring.

Trial site	Treatment	Mean lesion length (mm)		
		<i>P. patula</i>	<i>P. radiata</i>	<i>P. elliottii</i>
Ugie	<i>S. sapinea</i>	41.1a	-	-
	<i>O. ips</i>	31.6b	-	-
	<i>O. piliferum</i>	18.4c	-	-
	Control	15.1c	-	-
Longmore	<i>S. sapinea</i>	-	77.2a	110.6a
	<i>O. ips</i>	-	38.4b	44.7b
	<i>O. piliferum</i>	-	27.2b	29.5bc
	Control	-	35.9b	18.6c
Jessievale	<i>S. sapinea</i>	35.6a	-	21.9a
	<i>O. ips</i>	28.9b	-	21.6a
	<i>O. piliferum</i>	18.7c	-	16.8ab
	Control	11.6d	-	11.2b

a,b,c,d: Mean lesion lengths for each pine species at a specific site, followed by the same letter do not differ significantly ($p \leq 0.01$; Tukey's test). Each value represents the mean of 12 to 20 replications.

- : Pine species not available for inoculation.

The relative susceptibility of pine species was determined by comparing lesion development on pairs of species that were inoculated at the same location (Table 11). None of the control inoculations resulted in significantly different lesions on the various pine species. During the autumn, inoculations at Longmore with *O. piliferum* caused longer lesions on *P. elliottii* than on *P. radiata*. During the spring, inoculations at Ugie with *O. piliferum* resulted in the longest lesions on *P. patula* when compared to *P. greggii*. *Ophiostoma piliferum* also caused longer lesions on *P. patula* than on *P. elliottii* at Jessievale during the spring. Significant differences between tree species were observed when *O. ips* was inoculated during the autumn, but not during the spring (Table 11). During autumn at Longmore, *O. ips* caused significantly longer lesions on *P. elliottii* than on *P. radiata*. At Ugie, this fungus was more pathogenic on *P. patula* than on *P. greggii* and at Jessievale it was more pathogenic on *P. patula* than on *P. greggii*.

Table 11. Mean lengths of lesions on different pine species at Longmore, Ugie and Jessievale during the autumn and spring.

	Location	Tree species	Mean lesion length (mm)			
			Control	<i>O. piliferum</i>	<i>O. ips</i>	<i>S. sapinea</i>
Autumn	Longmore	<i>P. elliotii</i>	17.8a	29.7a	36.9a	157.2a
		<i>P. radiata</i>	16.3a	23.3b	24.0b	39.1b
	Ugie	<i>P. patula</i>	13.7a	26.8a	34.2a	38.9a
		<i>P. greggii</i>	13.7a	25.5a	25.1b	39.1a
	Jessievale	<i>P. elliotii</i>	13.1a	22.0a	19.3b	54.2a
		<i>P. patula</i>	15.0a	24.2a	30.0a	35.3a
Spring	Longmore	<i>P. elliotii</i>	18.8a	27.3a	42.8a	99.5a
		<i>P. radiata</i>	37.1a	25.2a	42.8a	79.5a
	Ugie	<i>P. patula</i>	21.2a	28.7a	39.1a	56.8a
		<i>P. greggii</i>	14.8a	17.8b	28.3a	31.8b
	Jessievale	<i>P. elliotii</i>	11.2a	14.0b	21.9a	21.7a
		<i>P. patula</i>	11.4a	20.2a	26.9a	35.1a

a,b : Comparison of lesion lengths on different pine species, caused by the same fungal strains and at the same location. Pairs of mean values followed by the same letter do not differ significantly ($p \leq 0.05$; Tukey's test). Each value represents the mean of 12 replications.

Sphaeropsis sapinea caused significantly longer lesions on *P. patula* than on *P. greggii* during spring inoculations at Ugie (Table 11). The pathogen also resulted in longer lesions on *P. elliotii* than on *P. radiata* at Longmore in the same season. *Pinus elliotii* was more susceptible than other pine species at Longmore only. According to observations made during previous field trials, *P. radiata* and *P. patula* were more susceptible to *S. sapinea* than *P. elliotii* (Swart & Wingfield, 1991). Our results do not contradict these observations, but demonstrate the influence of a location-species interaction on general susceptibility.

The inoculation of *P. elliotii* and *P. patula* at two locations each, demonstrated the significant influence ($p \leq 0.05$) of location on lesion development (Table 12). Longer lesions developed on *P. elliotii* at Longmore than at Jessievale, with all fungi and the control during autumn. These results were repeated in the spring, but the control inoculations did not differ significantly from each other. Location did not play an equally important role in lesion development on *P. patula* at

Ugie and Jessievale. Only *O. piliferum* and *O. ips* caused significantly longer lesions at Ugie than at Jessievale during the spring (Table 12).

Table 12. Mean lesion lengths on *Pinus elliottii* and *P. patula* at different locations during the autumn and spring.

	Tree species	Location	Mean lesion length (mm)			
			Control	<i>O. piliferum</i>	<i>O. ips</i>	<i>S. sapinea</i>
Autumn	<i>P. elliottii</i>	Longmore	17.80a	29.70a	36.90a	157.20a
		Jessievale	13.10b	22.00b	19.30b	54.20b
	<i>P. patula</i>	Ugie	13.70a	26.80a	34.20a	38.90a
		Jessievale	15.00a	24.20a	30.00a	35.30a
Spring	<i>P. elliottii</i>	Longmore	18.80a	27.30a	42.80a	99.50a
		Jessievale	11.20a	14.00b	21.90b	21.70b
	<i>P. patula</i>	Ugie	21.20a	28.70a	39.10a	56.80a
		Jessievale	11.40a	20.20b	26.90b	35.10a

A,b : Comparison of lesion lengths at different locations, caused by the same fungal strains on the same tree species. Pairs of mean values followed by the same letter do not differ significantly ($p \leq 0.05$; Tukey's test). Each value represents the mean of 12 replications.

Re-isolations of the inoculum

Re-isolations confirmed the presence of the inoculated fungi in the lesions. *Sphaeropsis sapinea*, *O. ips* and *O. piliferum* were re-isolated from the infected branches at Longmore, Jessievale and Ugie, during both seasons. Isolations made from the control inoculations yielded *S. sapinea* and *Trichoderma* spp. Re-isolations of *S. sapinea* from control treatments were probably due to its endophytic occurrence in *Pinus* spp. in South Africa (Smith *et al.*, 1996; Smith *et al.*, 2000). The re-isolation of *S. sapinea* was not consistent on the controls, but occurred in approximately 10 % of the re-isolations.

2.3. BIOPULPING WITH CARTAPIP 97[®]

MATERIALS AND METHODS

Viability of Cartapip inoculum

A dilution series was made of commercial Cartapip inoculum to determine the viability for biopulping before each of the trials. Three replications of the product (1.0 g) were suspended in 100 ml of sterile distilled water and a dilution series made to 1×10^{-10} . Each of the dilutions (1 ml) was plated in duplicate onto MEA. The plates were incubated at 24 °C and the number of Colony Forming Units (CFUs) counted after nine days. Three batches of the Cartapip fungus were tested respectively before treatments of each pulping trial. To determine if the different batches were contaminated, a sample (5 g) of the fungus was transferred to 10 ml sterile distilled water. Inoculum (800 µl) was pipetted onto PDA plates and incubated at 24 °C for 11 days.

Inoculum preparation

Samples of the freeze-dried product were weighed into sterile containers for transportation to the trial sites. The freeze-dried inoculum was added to sterile distilled water before inoculation and shaken well. The amounts of inoculum and water used for these suspensions varied according to the activity of the product and the size of the trial.

Microbial contamination

Freshly cut wood chips were collected at each trial before inoculation with Cartapip. Twenty-five pieces (1.5 x 15 mm) of wood were used for each species and were plated onto 2 % MEA to detect the presence of contaminating microorganisms on the freshly cut wood. Plates were incubated for seven days at 23 °C and the microorganisms enumerated. The same process was repeated to determine the presence of contaminating microorganisms after treatment of the wood chips.

Biopulping of softwood chips

Pinus patula and *P. elliotti* logs were chipped (12 mm long) at the Ngodwana mill within two weeks of felling. Approximately 2.8 kg (dry weight) of *P. patula* chips (64 % moisture, wet basis) were placed in one set of six 25 L bioreactors and approximately 3.3 kg (dry weight) of *P. elliottii* chips (54 % moisture, wet basis) in another six bioreactors. Chips of each species in five bioreactors were sprayed with Cartapip 97[®] and the sixth untreated one used as control. The inoculum for each bioreactor consisted of Cartapip (12.5 g) suspended in 500 ml sterile distilled water. The activity of the inoculum was 1.95×10^9 CFUs/g. The treatments were incubated for two weeks at room temperature and the chips bulked and dried to 3 % moisture (wet basis) before pulping.

Kraft pulping of 1.0 kg samples was done in a 10 L rotating digester for 70 minutes at 170 °C. Duplicate samples of each treatment were pulped using 22 % active alkali at a liquor to wood ratio of 5.4 to 1. Pulp yield was determined on the basis of the weight of the chips before inoculation. Kappa number, Canadian Standard Freeness (CSF) (at 500 revolutions), tear and burst indices (for 425 ml CSF) as well as breaking length (for 425 ml CSF) were determined according to Tappi Test Methods T236, T227, T414, T403 and T220 respectively. A strength index was calculated from Tear index + 2x Burst index, because it is the preferred index used at some Sappi mills.

This experiment was later repeated, but on a smaller scale. Approximately 2.7 kg (dry weight) of *P. patula* chips (53.5 % moisture, wet basis) were placed in one set of two 25 L bioreactors and approximately 2.6 kg (dry weight) of *P. elliottii* chips (56.4 % moisture, wet basis) in another two bioreactors. Chips of each species in one bioreactor were sprayed with Cartapip 97[®] and the second untreated one used as control. The activity of the inoculum was 1.31×10^{12} CFUs/g. Kraft pulping was done for 80 minutes at temperature. Only the yields and kappa numbers of pulp were determined.

Biopulping of hardwood chips

Eucalyptus grandis and *Acacia mearnsii* logs were chipped (12 mm long) at Sappi Forest Research (Tweedie) within two weeks of felling. Approximately 2.3 kg (dry weight) of *E. grandis* chips (48.9 % moisture, wet basis) were placed in one set of three 25 L bioreactors and approximately 2.7 kg (dry weight) of *A. mearnsii* chips (36.4 % moisture, wet basis) in another three bioreactors. Chips of each species in two bioreactors were sprayed with Cartapip 97[®] and the third used as control. The inoculum for each bioreactor consisted of Cartapip (12.5 g) suspended in 500 ml sterile distilled water. The activity of the inoculum was 1.95×10^9 CFUs/g. After the two-week incubation period the chips were not dried, but pulped immediately.

Sulphite pulping of 1.0 kg hardwood was done in a 21 L digester for 450 minutes (*E. grandis*) and 405 minutes (*A. mearnsii*) at 140 °C. Pulping was done with 8.52 % SO₂ and 1.05 % CaO at a liquor to wood ratio of 4.7 to 1. Pulp yield was determined on the basis of the weight of the chips before inoculation. Kappa number, pulp brightness as well as pulp viscosity were determined according to Tappi Test Methods T236, T452 and T230 respectively. One sample of each treatment was pulped.

Kraft pulping of hardwood was done on a micro scale (100 g) in 800 ml canisters for 40 minutes at 170 °C. The cooking liquor consisted of 16 % active alkali at a liquor to wood ratio of 5 to 1. Pulp yield was determined on the basis of the weight of the chips before inoculation. Kappa number, Canadian Standard Freeness (CSF) (at 500 resolutions), tear and burst indices (for 425 ml CSF) as well as breaking length (for 425 ml CSF) and black liquor quality were determined according to Tappi Test Methods T236, T227, T414, T403, T220 and T625 respectively. The results of tear and burst indices are given as Tear + 2x Burst index because it is the standard method used at the mills. One sample of each treatment was pulped.

Soda-AQ pulping of hardwood was done on a micro scale (100 g) in 800 ml canisters for 40 minutes at 170 °C. The cooking liquor consisted of 16 % active alkali and 0.05 % Anthraquinone at a liquor to wood ratio of 5:1. Pulp yield was

determined on the basis of the weight of the chips before inoculation. Kappa number, Canadian Standard Freeness (CSF) (at 500 resolutions), tear and burst indices (for 425 ml CSF) as well as breaking length (for 425 ml CSF) and black liquor quality were determined according to Tappi Test Methods T236, T227, T414, T403, T220 and T625 respectively. One sample of each treatment was pulped.

RESULTS AND DISCUSSION

Viability of Cartapip inoculum

Wall *et al.* (1994) recommended an inoculum activity larger than 1.1×10^5 CFUs/g in the product for it to be viable. The activity in three batches of Cartapip tested, varied between 1.95×10^9 and 1.31×10^{12} CFUs/g. All the batches were, therefore, viable and suspensions could be prepared for biopulping trials. No contamination of the product was observed in any of the cultures.

Microbial contamination

All the wood chips used in this study were contaminated by at least one organism (Table 13). Softwood chips had a high percentage of bacterial and yeast contamination, but the hardwood chips showed no contamination with yeasts or bacteria. Yeast and bacterial contamination could possibly be ascribed to higher moisture content of softwood chips. *Trichoderma* sp. was the major cause of contamination on softwood, while *Penicillium* sp. and *Rhizopus* sp. caused most of the contamination on *E. grandis* and *A. mearnsii* respectively.

Table 13. Comparison of the isolations taken from the controls for each inoculation.

Tree species	Contaminant	Contaminated chips (%)
<i>Pinus patula</i>	<i>Trichoderma</i> sp.	68
	Bacteria and yeasts	96
<i>Pinus elliottii</i>	<i>Trichoderma</i> sp.	68
	Unidentified fungi	20
	Bacteria and yeasts	76
<i>Eucalyptus grandis</i>	<i>Penicillium</i> sp.	96
	Unidentified fungi	4
<i>Acacia mearnsii</i>	<i>Rhizopus</i> sp.	60
	Unidentified fungi	40

After treatment, the most abundant fungus present on the control sample of *P. patula* and *P. elliottii* was *Trichoderma* spp. A strong terpene smell from the untreated *P. elliottii* chips could be detected but not from the chips that were treated with Cartapip. It, therefore, appears that the Cartapip treatment was able to reduce colonisation by *Trichoderma* spp. and also reduce the content of some volatile components. Treated chips were apparently colonised by Cartapip only. No microbial growth was observed on the treated or untreated *E. grandis* and *A. mearnsii* chips after two weeks of incubation. The apparent lack of growth can possibly be ascribed to the very low moisture content of hardwoods.

Biopulping of softwood chips

Treatment of *P. patula* and *P. elliottii* chips with *O. piliferum* did not cause notable changes in the kappa number, pulp yield or strength indices of either wood species (Table 14). *Ophiostoma piliferum* reduced the freeness of pulp from both wood species. It also reduced the breaking length on *P. patula*, but increased breaking length on *P. elliottii* pulp. Cartapip had a similar influence on the pulp yield and kappa number of pulp when the trial was repeated. These results were also similar to those obtained by Farrell & Allison (1997), who pre-treated radiata pine with *O. piliferum* before kraft pulping.

Table 14. Characteristics of kraft pulp from untreated softwood chips and chips treated with Cartapip.

Tree species	Tested parameter	Treatment		Change (%)
		Control	Cartapip	
<i>P. patula</i>	Kappa number	32.24	33.70	+1.46
	Yield (%)	45.12	45.15	+0.07
	CSF (ml)	440	378	-14.09
	Strength index	20.60	21.20	+2.91
	Breaking length (km)	18.10	9.80	-45.86
<i>P. elliotii</i>	Kappa number	29.14	27.88	-1.26
	Yield (%)	43.27	43.27	0.00
	CSF (ml)	363	350	-3.58
	Strength index	20.90	21.20	+1.44
	Breaking length (km)	8.40	9.00	+7.14

Biopulping of hardwood chips

Sulphite pulping of hardwood chips after treatment with Cartapip did not cause notable changes in the pulp quality (Table 15 and Table 16). An increase in the yield of *E. grandis* and *A. mearnsii* pulp was observed, but there is no plausible reason for the improvement (Table 15 and 16). I, therefore, ascribe the change in yield to experimental variability. Kraft pulping of hardwood chips after treatment with Cartapip also did not cause notable improvement in the pulp quality. The kappa number of *E. grandis* pulp even increased (Table 15 and 16). The tear and burst index for the *E. grandis* chips improved in comparison with the tear and burst index of *A. mearnsii* chips. Soda-AQ pulping of hardwood chips after treatment with Cartapip showed no improvement in pulp quality (Table 15 and 16).

Values for black liquor quality remained similar for treated and control samples (Table 15 and 16). However, a consequential reduction of residual alkali was observed when the Soda-AQ process was used (Table 15 and 16). The tear and burst strengths, however, showed an improvement when hardwood chips were pulped (Table 15 and Table 16). The residual alkali in kraft black liquor and Soda-AQ black liquor decreased when pulping was done on *E. grandis* chips treated with Cartapip.

Soda-AQ black liquor also showed a decrease when pulping was done on *A. mearnsii* chips but kraft black liquor showed an increase (Table 15 and Table 16).

Table 15. Characteristics of different pulps from untreated *Eucalyptus grandis* chips and chips treated with Cartapip.

Tree species	Pulping process	Tested parameter	Treatment		Change (%)
			Control	Cartapip	
<i>E. grandis</i>	Sulphite	Kappa number	3.12	3.12	0.00
		Yield (%)	42.48	44.35	+4.40
		Brightness (%)	51.40	49.00	-4.40
		Viscosity (mPa.s)	81.60	81.10	-0.60
<i>E. grandis</i>	Kraft	Kappa number	20.68	21.63	+4.60
		Yield (%)	43.34	43.24	-0.20
		CSF (ml)	450	420	-6.70
		Strength index	19.30	20.20	+4.70
		Breaking length (km)	8.00	8.00	0.00
		Black liquor residual alkali	14.56	14.41	-1.00
		Black liquor pH	13.41	13.36	-0.40
		Black liquor solids (%)	17.28	17.34	+0.30
<i>E. grandis</i>	Soda-AQ	Kappa number	24.46	24.93	+1.90
		Yield (%)	45.66	47.97	+5.10
		CSF (ml)	425	425	0.00
		Strength index	20.30	20.20	-0.50
		Breaking length (km)	8.10	7.80	-3.70
		Black liquor residual alkali	7.05	5.79	-17.90
		Black liquor pH	13.39	13.27	-0.10
		Black liquor solids (%)	15.30	15.14	-1.00

Table 16. Characteristics of different pulps from untreated *Acacia mearnsii* chips and chips treated with Cartapip.

Tree species	Pulping process	Tested parameter	Treatment		Change (%)
			Control	Cartapip	
<i>A. mearnsii</i>	Sulphite	Kappa number	3.94	3.91	-0.80
		Yield (%)	51.32	51.98	+1.30
		Brightness (%)	33.60	35.60	+6.00
		Viscosity (mPa.s)	102.20	103.20	+1.00
<i>A. mearnsii</i>	Kraft	Kappa number	11.89	11.93	+0.30
		Yield (%)	50.64	50.01	-1.20
		CSF (ml)	480	480	0.00
		Strength index	15.80	16.10	+1.90
		Breaking length (km)	6.50	6.30	-3.10
		Black liquor residual alkali	17.38	16.91	+2.70
		Black liquor pH	13.49	13.51	+0.10
		Black liquor solids (%)	16.36	16.65	+1.80
<i>A. mearnsii</i>	Soda-AQ	Kappa number	14.89	15.07	+1.20
		Yield (%)	52.42	51.96	-0.90
		CSF (ml)	530	510	-3.80
		Strength index	16.50	16.80	+1.80
		Breaking length (km)	6.70	6.50	-3.00
		Black liquor residual alkali	10.80	9.71	-36.50
		Black liquor pH	13.50	13.50	0.00
		Black liquor solids (%)	14.32	14.49	+1.20

CONCLUSIONS

The cultural and morphological characteristics of the Cartapip fungus were similar to those of *O. piliferum* in almost every aspect (Upadhyay, 1981) and we could, therefore, conclude that Cartapip 97[®] was a strain of *O. piliferum*. The crosses of the Cartapip fungus with B mating type strains proved that the Cartapip fungus represented an A mating type. Single-ascospore cultures paired with tester strains formed fertile perithecia that proved that the Cartapip fungus and *O. piliferum* are of the same biological species.

This study demonstrated that lesions associated with *O. piliferum* were marginally longer or did not differ significantly from those of the control inoculations.

These lesions were also smaller than those caused by the weak pathogen and biologically similar *O. ips*. It was concluded that the melanin free strain of *O. piliferum* used in the production of Cartapip 97[®] is not pathogenic and that releasing Cartapip 97[®] would not pose a threat to the South African forestry industry. The non-pathogenicity of the fungus demonstrated in field trials and the lack of an associated insect vector reduce the opportunity for transformation and spread of the fungus (Farrell *et al.*, 1993; Zimmerman *et al.*, 1995). In the absence of sexual reproduction, the potential for genetic recombination is also limited. As a result of these trials, Cartapip 97[®] has been certified by the South African Department of Agriculture as safe for general release.

The benefit of chip treatment with Cartapip to pulp properties is not obvious. There is, however, a slight increase in the strength properties of kraft pulp obtained from *P. patula*, *P. elliottii*, *E. grandis* and *A. mearnsii* and also of Soda-AQ pulp obtained from *A. mearnsii* chips. It is also possible that the extractives content of the control chips was reduced during treatment, which could reduce contamination of paper machines by resins. Cartapip is apparently not able to colonise hardwood chips. These results are not promising, but were expected, because other researchers also obtained unfavourable results when doing trials with hardwood (De Beer & Wingfield, Personal Communication).

Characteristics of Kraft black liquor did not show a notable change, but the Soda-AQ black liquor values are lower and must, therefore, be monitored where recovery processes are implemented. The ideal is to concentrate the solids in black liquor as much as possible to make optimal energy recovery possible (Biermann, 1996).

Cartapip 97[®] can play an important role in the South African pulp and paper industry, but a pulping process involving Cartapip 97[®] must first be optimised before application. Such a process can contribute to the decrease of chemical consumption during pulping because of pitch reduction and can, therefore, have a significant effect on the economy in the pulp and paper industry. The release of the Cartapip fungus in

South Africa makes it possible to evaluate the effect of the fungus on other pulping processes.

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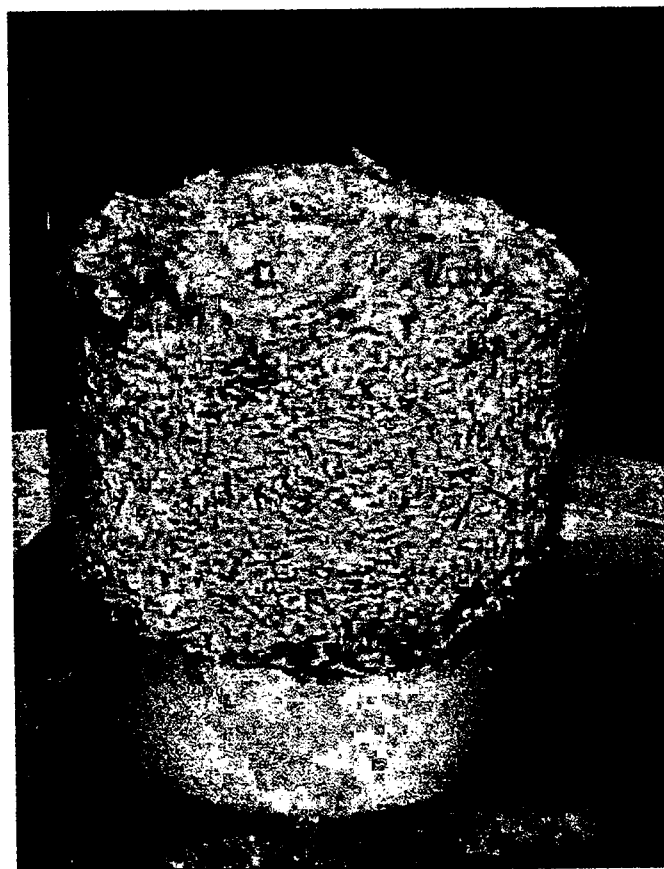
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CHAPTER 3

OPTIMISATION AND EVALUATION OF A BIOPULPING PROCESS FOR BAGASSE



Bagasse colonised by *Pycnoporus sanguineus* after incubation of three
weeks

ABSTRACT

Bagasse is a source of cellulose containing fibres that can be used in the production of paper. Bagasse has to be stored for long periods to maintain sufficient supplies for pulping throughout the year. The long storage periods usually lead to degradation of the fibres and, therefore, provide an opportunity for a biopulping treatment to improve the properties of the fibre. Two white-rot fungi, *Lenzites betulina* and *Pycnoporus sanguineus*, were selected as the most suitable strains for biopulping of bagasse. The inoculum size, different processes for inoculum production, different pulping processes (Soda, Soda-AQ and SASAQ) and different incubation periods were evaluated to optimise a pulping process for bagasse. Pulp yield, kappa number, black liquor solids, residual alkali, acid-insoluble lignin content and 1-% NaOH solubility were determined for the optimisation of pulping with bagasse. Ultrastructural studies were also done to determine the effect of fungal treatment on the bagasse. These studies included light microscopy, scanning and transmission electron microscopy. Pulping results obtained with bagasse treated with *L. betulina* were variable, but pulping of bagasse treated with *P. sanguineus* showed no improvement. Ultrastructural studies indicated that the test fungi caused non-selective delignification. This study demonstrated that economically viable biopulping processes could only be developed if the mechanism of colonisation and degradation is fully understood.

INTRODUCTION

Bagasse is a waste product that remains after sugar milling and it is also rich in cellulose containing fibres that can be utilised in the production of paper (Venter, 1978). The main components of bagasse are the rind, vascular fibre bundles and parenchyma or pith cells (Venter, 1978). The pith cells have a negative effect on the pulping process of bagasse, because where pith is present the pulp quality is lower and more chemicals are absorbed during pulping. High pith content also leads to clogging of paper machines during the paper-making process (Venter, 1978). Bagasse has a limited cutting season and, therefore, has to be stored for long periods of up to eight months, to ensure a sufficient supply of bagasse for pulping (Venter, 1978). However, the long storage periods for bagasse can lead to the degradation of the fibres and consequently provide the opportunity for biopulping of the bagasse (Wolfaardt & Grimbeek, 1997).

Johnsrud *et al.* (1987) used a chemimechanical process to determine the properties of bagasse pulp after treatment with *Phanerochaete chrysosporium* for 10 to 20 days. The fungal-treated bagasse was pulped according to conditions used for the Cuba-9 process and resulted in a good quality pulp. Lower energy consumption was also observed during the pulping process. Bustamante *et al.* (1999) studied biomechanical pulps from bagasse treated with *Ceriporiopsis subvermispora* and *Pleurotus ostreatus*. The fungal pre-treated bagasse was incubated for 15 days and the results obtained for both fungal treatments showed a decrease in energy consumption as well as in lignin content (Bustamante *et al.*, 1999). Pre-treatment of bagasse with white-rot fungi could also reduce the discolouration of the bagasse and improve the cellulose preservation that could lead to a higher yield and to the degradation of lignin (Wolfaardt & Grimbeek, 1997). The pith content in the bagasse could potentially be reduced with fungal pre-treatment (Wolfaardt & Grimbeek, 1997). Biopulping of bagasse, treated with different white-rot fungi, has already been studied by Grimbeek *et al.* (1997). In this study, screening experiments were completed to determine which white-rot fungi had the most desirable influence on the pulp properties (Grimbeek *et al.*, 1997). The selected fungi

were *Lenzites betulina* (L.: Fr.) Fr. (SCC 274) and *Pycnoporus sanguineus* (L.: Fr.) Murr. (SCC 87).

The aim of the present study was to evaluate and optimise a biopulping process for bagasse. This study included the optimisation of inoculum production, because it is the largest contributing factor to variable cost in a biopulping process (Wolfaardt *et al.*, 1998). The effect of different pulping processes and incubation periods on treated bagasse was also evaluated. Different pulping processes were used including Soda, Soda-AQ and SASAQ pulping. Ultrastructural studies were also conducted on treated bagasse to determine the effect of fungal colonisation and degradation on the bagasse. Light microscopy, scanning electron microscopy and transmission electron microscopy were used in these studies.

3.1. OPTIMISATION OF SOLID SUBSTRATE FERMENTATION

MATERIALS AND METHODS

Evaluation of inoculum size

Lenzites betulina (SCC 274) and *Pycnoporus sanguineus* (SCC 87) were selected after screening experiments done by Grimbeek *et al.* (1997). These strains were grown on potato dextrose agar (PDA) (2 % Potato dextrose, 2 % Agar) (BIOLAB Merck) for four to six days. Nine pieces of agar (~ 4 mm²) overgrown with the two respective fungi were transferred to 100 ml of diluted molasses (1 %). The cultures were incubated at 25 °C for seven days. The cultures were homogenised after seven days and a pre-inoculum of 10 ml transferred to 200 ml diluted molasses (1 %). These cultures were incubated on a shaker at 25 °C for seven days at 100 rpm, homogenised and used for inoculation of the bagasse. Samples (50 ml) were removed from each culture before inoculation of the bagasse. The samples were filtered through a crucible (30 ml; porosity 2), dried and weighed to determine the biomass.

Fresh bagasse was collected at the Sappi Stanger mill after moist depithing. The bagasse was air-dried to approximately 3 % moisture content for storage. Before inoculation, the bagasse was soaked in water for approximately seven hours and the excess water drained. Bagasse samples (~60 g dry weight) were placed in glass bioreactors (2 L), covered with cotton wool to allow aeration and autoclaved on each of two consecutive days.

The bagasse in the bioreactors was treated with 2.0, 4.0, 10.0, 20.0 and 40.0 ml of inoculum and incubated at room temperature for three weeks. After inoculation bagasse had a moisture content between 70 and 74 %. After harvesting, the treated bagasse was dried and milled.

Small samples (6.0 g) of bagasse were removed from each treatment for analysis. Two grams of the bagasse were tested for its 1-% NaOH solubility and 4.0 g were used in

determining the acid-insoluble lignin content. Previous studies indicated that 1-% caustic solubility and acid-insoluble lignin of bagasse were correlated with the pulp yield and kappa number of pulp from treated bagasse (Grimbeek *et al.*, 1997). The 1-% NaOH solubility of treated bagasse was determined by Tappi test method T212 for pulp. The acid-insoluble lignin content was determined according to Tappi test method T222 with the following modifications: Sodium hydroxide (200 ml; 5 %) was added to bagasse samples in a tall form beaker. The beaker with its contents was covered with a watch glass and incubated in a water bath at boiling point for 60 minutes to remove partly degraded lignin. The sulphuric acid hydrolysis that followed was done in an autoclave for one hour and not in a water bath as described in Tappi test method T222. The ratio of 1-% NaOH solubility to acid-insoluble lignin was also calculated as an indication of the fibre quality (fibre quality ratio). The ratio of 1-% NaOH solubility to the product of the acid-insoluble lignin and inoculum dry mass was calculated as an indication of the efficiency of the inoculum (inoculum efficiency ratio).

All treatments were replicated three times. A completely randomised trial design was used and the data subjected to a two-way analysis of variance.

Methods of inoculum production

Two methods of inoculum production from the two white-rot fungi, *L. betulina* and *P. sanguineus*, were evaluated. The first method involved cultivation for 14 days and the method of inoculum production was similar to the one described previously. The second method of inoculum production involved cultivation for 12 days with two pre-inoculum steps instead of one. The cultures were homogenised every four days and transferred to 200 ml of diluted molasses (1 %). Biomass was determined at each step. Each treatment was replicated three times and a completely randomised trial design was used. The data were subjected to a one-way analysis of variance.

RESULTS AND DISCUSSION

Evaluation of inoculum size

The average biomass in the three replicated cultures was 0.2214 g/50 ml. The different volumes of inoculum did not cause any significant changes in the 1-% NaOH solubility and the acid-insoluble lignin (Table 1). An inoculum volume of 2.0 ml/60 g bagasse was selected as the best inoculum size because of the highest inoculum efficiency ratio (Table 1).

Table 1. Mean 1-% NaOH solubility and mean acid-insoluble lignin content of bagasse treated with *Lenzites betulina* (SCC 274).

Inoculum size (ml/60 g bagasse)	Dry weight inoculum (g/100 g bagasse)	1-% NaOH solubility	Acid-insoluble lignin (%)	Fibre quality ratio	Inoculum efficiency ratio
2.0	0.0148	30.59	9.83	3.11	210.24
4.0	0.0295	32.80	9.33	3.52	119.19
10.0	0.0738	28.03	10.21	2.75	37.20
20.0	0.1493	32.09	9.01	3.56	23.86
40.0	0.2952	34.72	9.60	3.62	12.25

Methods of inoculum production

The different methods of inoculum production from *L. betulina* and *P. sanguineus* did not result in increased biomass (Table 2). Method one was, therefore, selected for the production of inoculum, because the more frequent homogenisation and transfer in the second method could increase the risk of contamination.

Table 2. Fungal biomass produced using two production methods for *L. betulina* and *P. sanguineus*.

Inoculum	Treatment	Biomass (g/210 ml)
<i>Lenzites betulina</i>	Method 1 (14 days)	0.1587
	Method 2 (12 days)	0.1503
<i>Pycnoporus sanguineus</i>	Method 1 (14 days)	0.1428
	Method 2 (12 days)	0.1194

3.2. OPTIMISATION OF A PULPING PROCESS FOR BAGASSE

MATERIALS AND METHODS

Treatment with *L. betulina* for two weeks

Pre-inoculum and inoculum from *L. betulina* (SCC 274) were produced as described previously with a total incubation period of 14 days. Dry bagasse samples (500 g dry weight) were soaked in water for seven hours and drained of excess water. The bagasse was transferred to bioreactors (25 L) with sieves in the bottom to allow drainage of excess water and to improve aeration. The bioreactors with the bagasse were covered with paper lids to allow passive aeration and then autoclaved on each of two consecutive days.

The bagasse samples (500 g) were inoculated with 210 ml homogenised mycelium of *L. betulina* and untreated bagasse was included as a control. An overdose of inoculum was used to assure sufficient colonisation of the larger volume of bagasse. Eight bioreactors were used for each treatment. The treatments were incubated at 22 °C for two weeks, harvested, air-dried and bulked for pulping. Bagasse samples were pulped according to the Soda pulping (16.0 and 14.0 % active alkali), Soda-AQ pulping (14.0 % active alkali) and SASAQ pulping (15.0 and 12.5 % active alkali) processes. Pulp yield, kappa number, black liquor solids and residual alkali were determined for each process. Pulp yield was determined on the basis of the weight of the bagasse before pulping. Kappa number and black liquor properties were determined according to Tappi Test Methods T236 and T625 respectively. Duplicate samples were pulped for each process.

Treatment with *L. betulina* for three weeks

Biopulping treatment with *L. betulina* was repeated as above except that an incubation period of three instead of two weeks was used. Four bioreactors were incubated with each treatment (*L. betulina* and a control). Bagasse samples were pulped using SASAQ conditions (with 15.0 and 12.5 % active alkali). Duplicate samples of each treatment were pulped.

Treatment with *L. betulina* for different incubation periods

Inoculum from *L. betulina* was produced and bagasse treated as described previously. Procedures for solid substrate fermentation were also repeated as described previously except for different incubation periods of 11, 13, 15, 17, 19 and 21 days. The bagasse was harvested, bulked and dried after treatment. Untreated bagasse was included as a control treatment. Each treatment was replicated three times.

The bagasse was pulped with the SASAQ process. The cooking conditions used in the pulping process were approximately 53 g of bagasse; 13.5 % active alkali and ten minutes at temperature (170°C). Pulp yield and kappa number as well as the pulp yield to kappa number ratio (pulp quality ratio) were determined. Pulp yield was determined on the basis of the weight of the bagasse before pulping and the kappa number was determined according to Tappi test method T236. The 1-% NaOH solubility as well as the acid insoluble lignin content were determined as described previously.

Treatment with *P. sanguineus* for three weeks

Inoculum from *P. sanguineus* was produced as described previously for *L. betulina*. The bagasse was also treated as described previously and incubated for three weeks. Bagasse samples were pulped in a 10 L rotating digester with a liquor to bagasse ratio of 6 to 1. The SASAQ conditions (with 15.0 and 12.5 % active alkali), which had given positive results with *L. betulina*, were used again. One sample of bagasse was pulped. Two bioreactors were used for each treatment.

Treatment with *P. sanguineus* for different incubation periods

Inoculum from *P. sanguineus* was produced and bagasse treated with *L. betulina* as described previously for different incubation periods. Procedures for solid substrate fermentation were also repeated as described previously with different incubation periods of 11, 13, 15, 17, 19 and 21 days. After days 11, 13, 15, 17, 19 and 21 the bagasse was harvested, bulked and dried. Untreated bagasse was included as a control treatment. Each treatment was replicated three times.

Two of the replicates of each treatment were pulped using a Soda-AQ process (14.0 % active alkali) and one replicate pulped using the SASAQ process (13.5 % active alkali). Kappa numbers, total yields and screened yields as well as the pulp yield to kappa number ratio were determined for each pulp sample. High values for the pulp yield to kappa number ratio were preferred, as it was regarded as an indication of efficient lignin degradation and little cellulose loss. Pulp yield was determined on the basis of the weight of the bagasse before pulping and the kappa number was determined according to Tappi test method T236. Chemical analyses were done according to the same methods as described previously. The 1-% NaOH solubility to acid-insoluble lignin content was also determined.

RESULTS AND DISCUSSION

Treatment with *L. betulina* for two weeks

Studies done by Johnsrud *et al.* (1987) on pulp properties of bagasse after an incubation period of 10 to 20 days, showed lower energy consumption during the chemimechanical pulping process. Bustamante *et al.* (1999) studied biomechanical pulps from bagasse treated for 15 days and the results also showed a decrease in energy consumption as well as in lignin content. It is difficult to compare the results of the studies done by Johnsrud *et al.* (1987) and Bustamante *et al.* (1999) to the results obtained in this study, because their focus was on mechanical pulping processes.

Good fungal growth was obtained in most cases and the colonised bagasse had a very light colour. However, colonisation by *L. betulina*, was not homogenous. This can possibly be ascribed to the poor distribution of the inoculum as well as to differences in the distribution of moisture.

Results of the Soda pulping method were not very promising (Table 3). The pulp yield decreased with both the active alkali charges (16.0 and 14.0 %). Kappa number

showed an increase with the 14.0 % active alkali charge, but a very slight decrease was observed with the 16.0 % active alkali charge. Black liquor solids increased with the use of 16.0 % active alkali and decreased when 14.0 % active alkali was used. The residual alkali decreased with both alkali charges.

Table 3. Pulp and black liquor characteristics after Soda pulping of bagasse treated with *L. betulina*.

Alkali charge (%)	Pulp yield (g/100g)		Kappa number		Black liquor solids (g/100ml)		Residual alkali (g/l)	
	16.0	14.0	16.0	14.0	16.0	14.0	16.0	14.0
Control	52.26	52.58	14.02	13.36	9.86	10.76	8.83	10.17
<i>L. betulina</i>	50.72	51.32	13.40	15.27	10.34	9.29	8.08	6.29
Change (%)	-2.9	-2.4	-4.4	+14.3	+4.87	-13.66	-8.49	-38.15

All values are means of duplicate pulping

Treatment of bagasse with *L. betulina* did not improve pulping with the Soda-AQ process (Table 4). Kappa number, black liquor solids and pulp yield showed an increase, however, not significantly. The residual alkali showed a drastic decrease (Table 4).

Table 4. Pulp and black liquor characteristics after Soda-AQ pulping of bagasse treated with *L. betulina*.

	Pulp yield (g/100g)	Kappa number	Black liquor solids (g/100ml)	Residual alkali (g/l)
Control	53.90	10.47	9.50	7.11
<i>L. betulina</i>	53.93	11.32	9.75	4.34
Change (%)	+0.1	+8.1	+2.6	-39.0

All values are means of duplicate pulping

The results obtained with bio-SASAQ pulping were very promising (Table 5) and showed an increase in pulp yield and a decrease in kappa number. The best results were obtained when 12.5 % active alkali was used. The chemical consumption decreased by 31 %, and with the pulp yield increase and possible savings in bleaching chemicals, this process should be economically viable. The improvement could possibly be ascribed to protection of the hemicelluloses by anthraquinone and degradation of pith by *L. betulina*.

Table 5. Pulp and black liquor characteristics after SASAQ pulping of bagasse treated with *L. betulina*.

	Pulp yield (g/100g)		Kappa number		Black liquor solids (g/100ml)		Residual alkali (g/l)	
	15.0	12.5	15.0	12.5	15.0	12.5	15.0	12.5
Control	56.50	52.44	12.97	16.59	10.42*	9.99	19.1*	14.4
<i>L. betulina</i>	57.73	58.01	11.12	11.54	10.96	10.87	18.3	18.8
Change (%)	+2.2	+10.6	-14.3	-30.4	+5.2	+8.8	-4.2	+30.6

All values except those indicated with an asterisk are means of duplicate pulping

Treatment with *L. betulina* for three weeks

Good fungal growth was observed with this trial and the bagasse also had a very light colour. The pulping results obtained were contradictory to results obtained with incubation of bagasse treated with *L. betulina* for two weeks. Pulp yields were lower and kappa number higher than that of the control (Table 6 & 7).

Table 6. Comparison of pulp properties of bagasse treated for two and three weeks with *L. betulina* after SASAQ pulping with 15.0 % active alkali.

Incubation time	Pulp yield (g/100g)		Kappa number	
	Two weeks	Three weeks	Two weeks	Three weeks
Control	56.5	58.7	12.97	11.84
<i>L. betulina</i>	57.7	54.7	11.11	13.14
Change (%)	+2.1	-6.8	-14.3	+11.0

All values are the means of duplicate pulping

Table 7. Comparison of pulp properties of bagasse treated for three weeks with *L. betulina* after SASAQ pulping with 12.5 % active alkali.

Incubation time	Pulp yield (g/100g)		Kappa number	
	Two weeks	Three weeks	Two weeks	Three weeks
Control	52.4	60.0	16.59	16.17
<i>L. betulina</i>	58.0	53.3	11.54	17.86
Change (%)	+10.7	-11.2	-30.4	+10.5

All values are the means of duplicate pulping

It is difficult to explain the contradictory results obtained in the two trials. One possible explanation is that incubation for two weeks benefited the pulping process by removing pith. Incubation for three weeks did not benefit pulping, because fungal growth was so vigorous that pith as well as fibre were degraded.

Treatment with *L. betulina* for different incubation periods

The kappa number of biopulped bagasse varied between 11.4 and 12.8 and seems to decrease with longer incubation time. Pulp yields between 50 and 85 g/100 g were obtained for the different incubation periods and appeared to increase over time. The increased incubation time did not influence kappa number or pulp yield significantly.

The highest pulp quality ratio was seen after an incubation period of 21 days and showed an increase from day 13 (Figure 1). However, a more realistic trend was observed for the fibre quality ratio that showed an increase from day 11 to 15 (Figure 1). At day 15 the ratio was the highest and from there it decreased drastically. The best biopulping results were, therefore, obtained after an incubation period of 15 days.

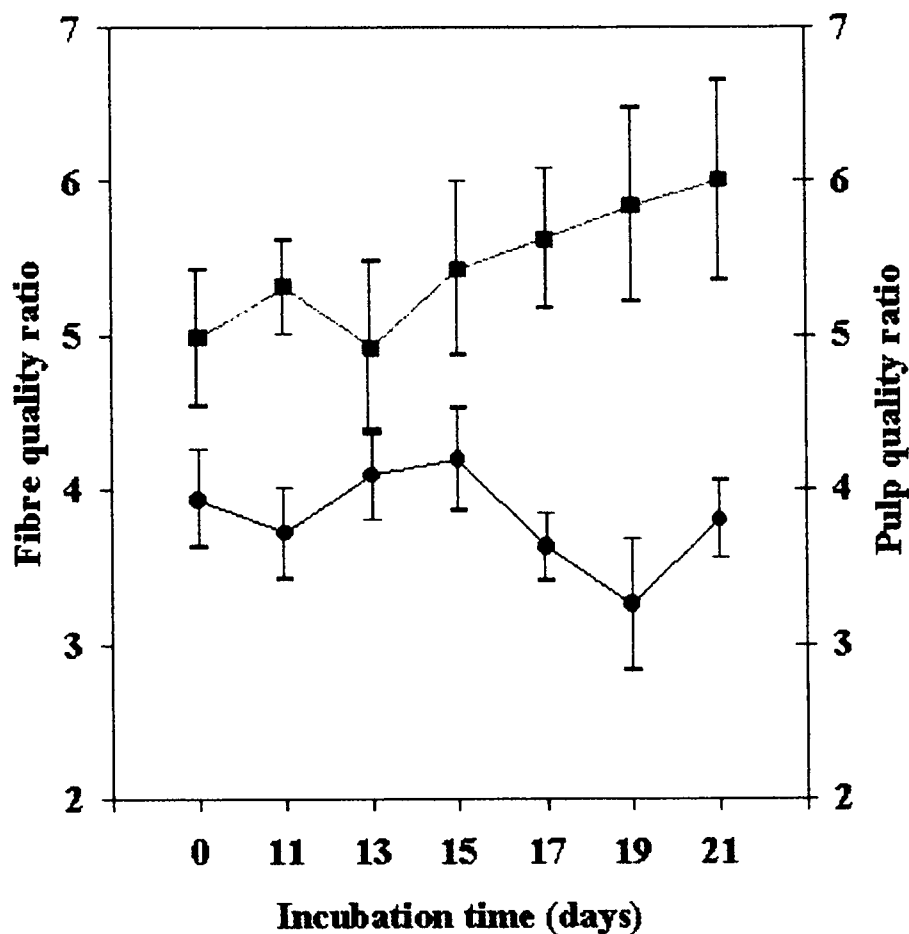


Figure 1. The fibre quality ratio and pulp quality ratio (SASAQ; 13.5 % active alkali) obtained from bagasse treated with *Lenzites betulina*. (Bars indicate standard error for each treatment).

Treatment with *P. sanguineus* for three weeks

Excellent fungal colonisation of bagasse was also observed during harvesting. The pulping results from the *P. sanguineus* treatment were similar to results obtained in the incubation time of three weeks with *L. betulina* that was well colonised. Pulp yields were lower and kappa number higher than that of the control (Table 8).

Table 8. Pulp properties of bagasse treated with *P. sanguineus* for three weeks after SASAQ pulping with different concentrations active alkali.

	Active alkali (%)			
	15.0		12.5	
	Pulp yield (g/100g)	Kappa number	Pulp yield (g/100g)	Kappa number
Control	59.7	11.6	58.9	18.7
<i>P. sanguineus</i>	52.6	18.5	50.1	22.2
Charge (%)	-11.9	+59.5	-14.9	+18.7

Treatment with *P. sanguineus* for different incubation periods

The kappa numbers of biopulped bagasse treated with *P. sanguineus* varied between 9.05 and 10.45 and seemed to increase with longer incubation time. Pulp yields between 51.25 and 53.7 g/100 g were obtained for the different incubation periods and seemed to decrease over time. Both the pulp quality and fibre quality ratios showed a decrease over time (Figure 2). Biopulping with *P. sanguineus* should not be considered further.

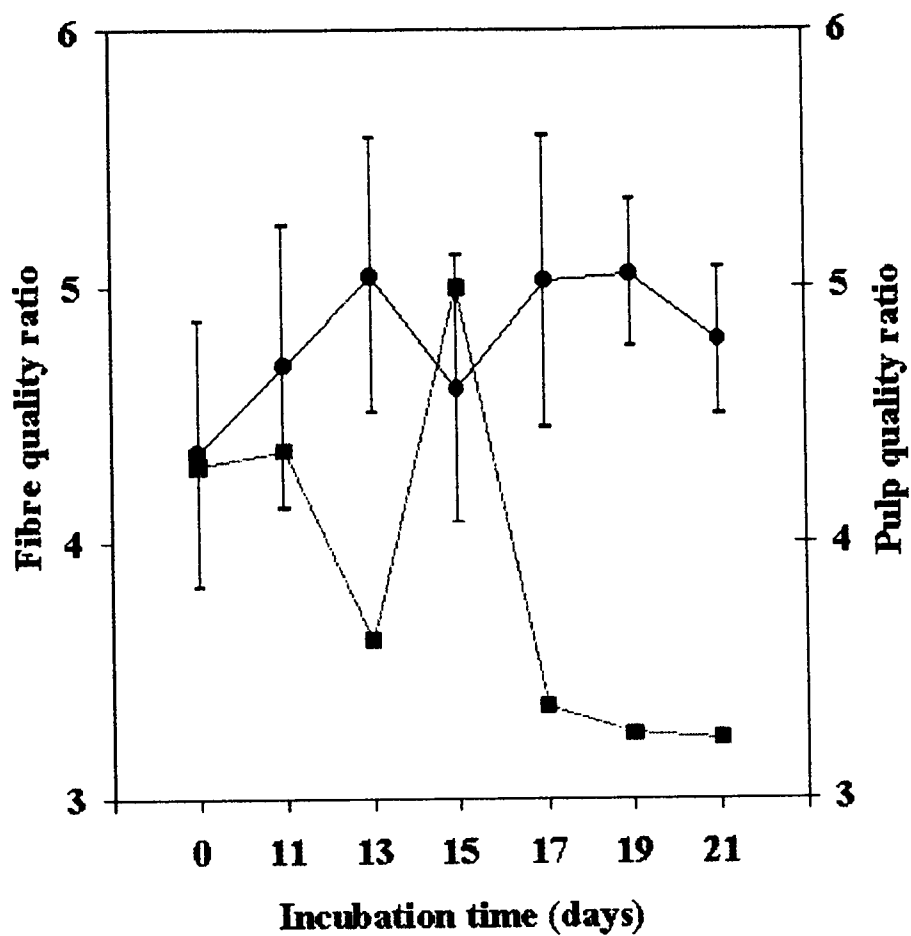


Figure 2. The fibre quality ratio and pulp quality ratio (SASAQ; 13.5 % active alkali) obtained from bagasse treated with *Pycnoporus sanguineus*. (Bars indicate standard error for each treatment).

3.3. ULTRASTRUCTURAL STUDIES OF TREATED BAGASSE

MATERIALS AND METHODS

Sample preparation

Inoculum from *L. betulina* and *P. sanguineus* was prepared, as described previously. Samples for light microscopy as well as scanning and electron microscopy were treated for different incubation periods as described previously. The samples were bulked and dried after incubation. An untreated sample was kept as a control for these studies.

Light microscopy

Pieces of bagasse treated with *L. betulina* and *P. sanguineus* were placed in 3.0 % Glutardialdehyde (Merck, Midrand) for three days. The samples were then dehydrated in a *tert*-Butanol GR series and imbedded in paraffin wax. The samples were sectioned with a rotary microtome and the sections (12 μm) were transferred to slides.

Staining of the samples for light microscopy studies was done in 14 steps. The first step was to put the slides in pure xylene (10 minutes) followed by 10 minutes in a xylene/alcohol mixture. The slides were put in absolute ethanol for five minutes and in 50 % ethanol for another five minutes. The slides were then stained with safranin (1 %) for 10 minutes, washed with 100 % ethanol, 50 % ethanol and acetic acid (3 %) respectively. The samples were next stained with Alcian Blue 8 GX (Aldrich, Steinheim, Germany) for ten minutes and washed twice with 1 x distilled water, 50 % ethanol and again with 100 % ethanol. The cycle was completed by washing the slides with the xylene/ethanol mixture and finally with the pure xylene. The specimens were sealed with Entellan (Merck) and a coverslip to make it permanent. The slides were examined microscopically using a Zeiss Axioskop at 200x with phase contrast and 1000x oil immersion with brightfield.

Scanning and transmission electron microscopy

Samples were fixed in a buffered (pH 7.0, 0.1 M sodium phosphate) solution of glutardialdehyde (3.0 %) for five days, post-fixed in similarly buffered osmium tetroxide (0.5 %) for one hour and dehydrated in an acetone series. These samples were dried using a critical point dryer, sputter-coated with gold and examined with a JEOL Winsem scanning electron microscope (SEM).

Duplicate samples for transmission electron microscopy (TEM) were imbedded in Spurr-resin after the dehydration in an acetone series. Ultrathin sections (60 nm) were cut with an LKB III Ultratome microtome and were stained with uranyl acetate and lead citrate solutions. The ultrathin sections for TEM were examined in a Philips EM 300 transmission electron microscope.

RESULTS AND DISCUSSION

Light Microscopy

Light microscopy has been previously used by Messner & Srebotnik (1994) to examine delignification in wood. Selective staining methods were used and astra blue stained fibres blue when highly delignified. Safranin on the other hand, stained lignin red in the presence or absence of cellulose. Distinction was, therefore, possible between fibres with a high degree of delignification and intact cells.

The untreated bagasse showed a single layer of epidermis cells followed by bundle sheaths imbedded in layers of sclerenchymatic tissue (Figure 3). Phloem, protoxylem and metaxylem could be distinguished in the bundle sheaths. Smaller bundle sheaths were observed nearest to the epidermis layer and bigger ones towards the centre, imbedded in layers of parenchymatic ground tissue (Figure 3). The parenchyma cells are also known as pith cells.

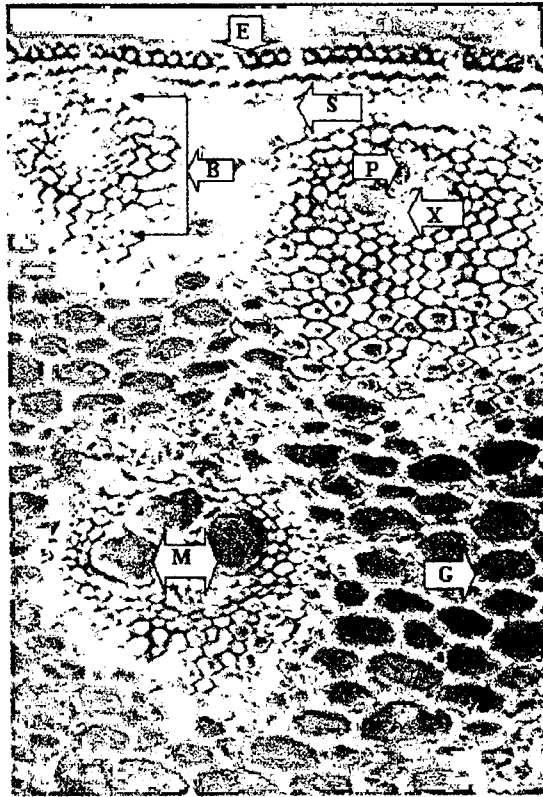


Figure 3. Light micrograph (200x; phase contrast) of a cross section through untreated bagasse showing the epidermis layer (E), the schlerenchymatic tissue (S), the phloem (P), the protoxylem (X), the parenchymatic ground tissue (G), and the metaxylem (M).

Examination of treatments with *L. betulina* showed that the epidermis layer has been attacked very aggressively and that the cells were broken down completely after 19 days. The vascular bundle disintegrated and the pith cells were also vigorously attacked (Figure 4A). Higher magnification (1000x) showed that non-selective degradation occurred (Figure 4B) since safranin staining appears to have the same intense colour as the untreated sample. The middle lamellae also showed no signs of degradation (Figure 4B). However, the secondary walls appeared lighter in colour, indicating a slight degree of decay (Figure 4B and Figure 5B).

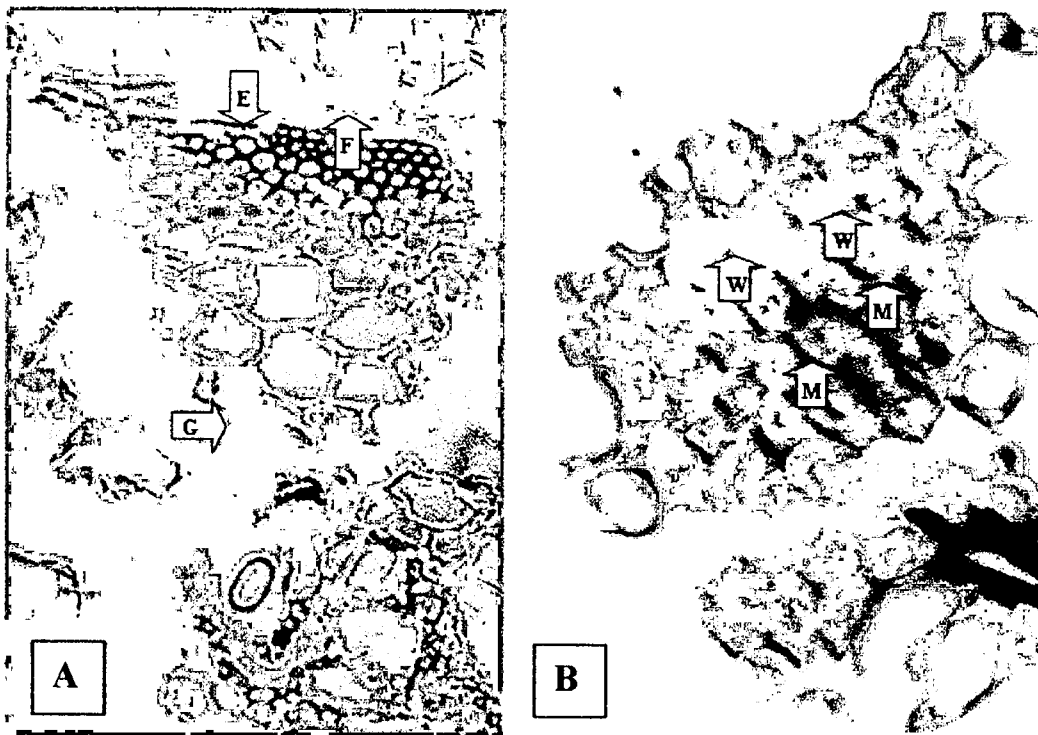


Figure 4A. Light micrograph (200x; phase contrast) of bagasse treated with *L. betulina* for 19 days showing the epidermis layer (E), the parenchymatic ground tissue (G), and the fungi attacking the epidermis (F).

Figure 4B. Light micrograph (1000x; brightfield) of bagasse treated with *L. betulina* for 19 days showing the middle lamellae (M), and the lighter coloured secondary walls (W).

The micrograph of bagasse treated with *P. sanguineus* showed that the cells has been degraded and were breaking up. The epidermis was also completely degraded. No signs of selective degradation were observed and it seemed that the parenchyma cells has been degraded first (Figure 5A).

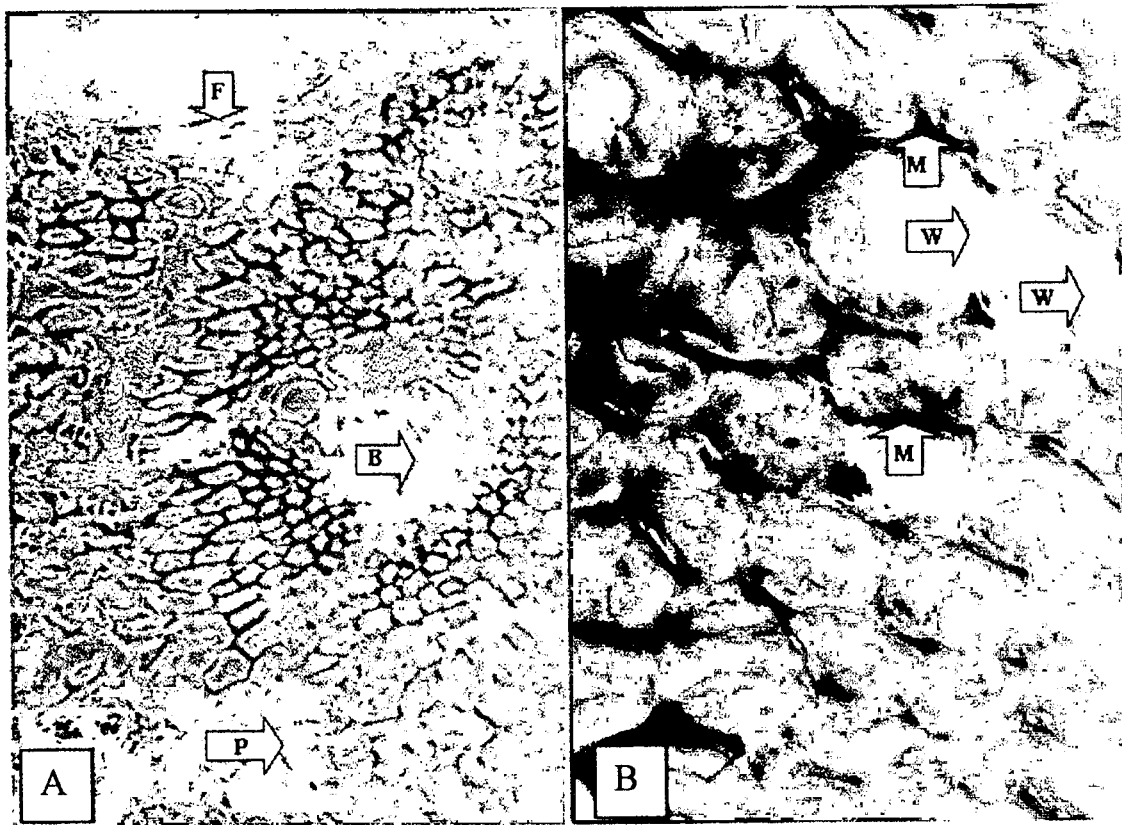


Figure 5A. Light micrograph (200x; phase contrast) of bagasse treated with *P. sanguineus* for 21 days showing the fungi attacking the epidermis (F), the bundle sheath that is degrading (B), and the parenchyma cells that are degrading (P).

Figure 5B. Light micrograph (1000x; brightfield) of bagasse treated with *P. sanguineus* for 19 days showing the middle lamellae (M), and the lighter coloured secondary walls (W).

Scanning and transmission electron microscopy

The SEM studies showed that both fungi preferentially colonised the vascular bundles (Figure 6a and Figure 6b) and that the pith was not colonised during the initial stages of decay. The pith is apparently very compact, making it difficult for the fungi to penetrate.

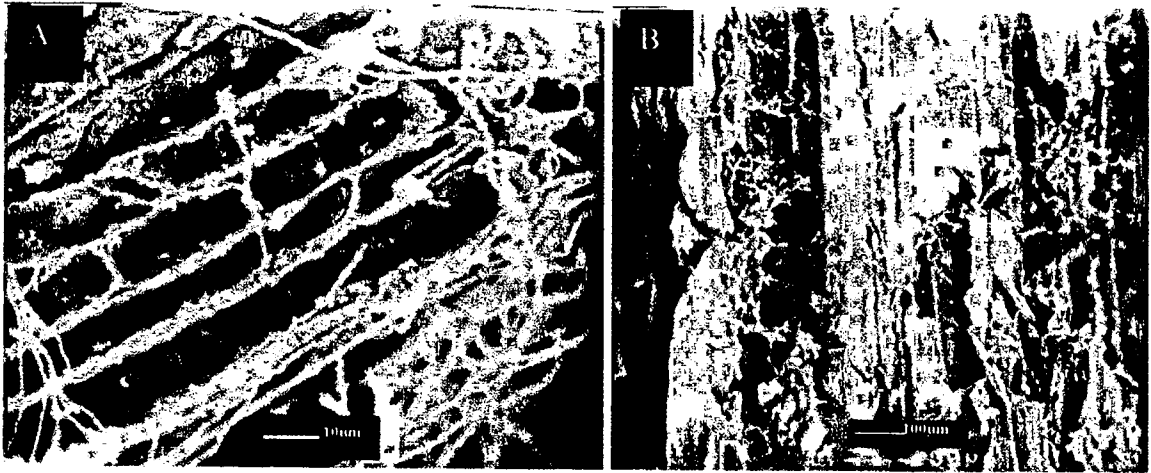


Figure 6. Scanning electron micrographs of vascular bundles of bagasse colonised by *Pycnoporus sanguineus* (A) and *Lenzites betulina* (B).

Transmission electron microscopy showed that both fungi grew within the cell lumen. Some sections also showed attachment of the fungi to the cell wall (Figure 7A) and degradation of the secondary cell wall (Figure 7B). Degradation of the middle lamella was not detected. The tested fungi did not selectively degrade lignin in any specific layer of the cell walls.

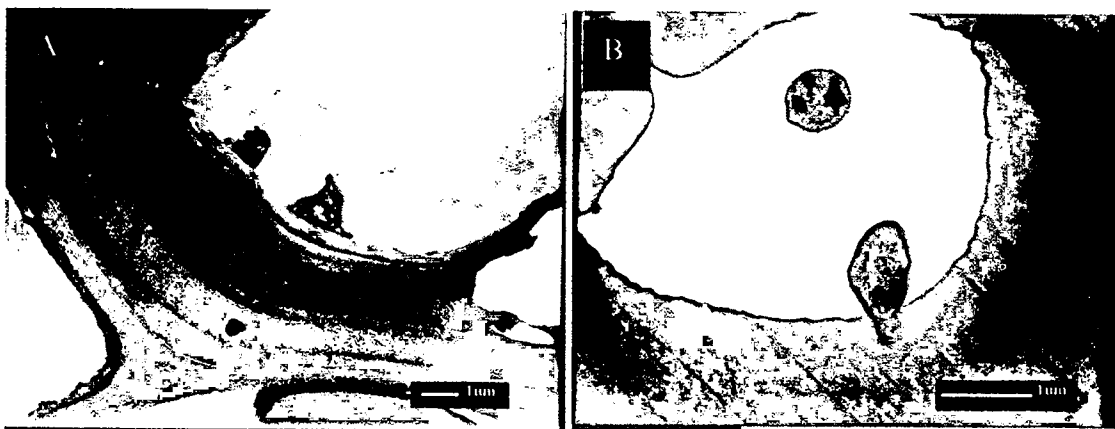


Figure 7. Transmission electron micrographs of bagasse cell lumens colonised by *Pycnoporus sanguineus* (A) and *Lenzites betulina* (B).

CONCLUSIONS

Pulping of fungal treated bagasse did not always result in improved pulp. Variable results have been obtained with pulping of fungal treated bagasse. The most promising results were obtained with bagasse treated with *L. betulina* (SCC 274) for two weeks and pulped according to the SASAQ method. The kappa number and pulp yield were improved and this method also resulted in reduced chemical consumption. This process could, therefore, be more economical and environmentally friendly than conventional processes. The variability of the results is, however, a concern.

Good fungal growth was observed in all the trials and the bagasse had a very light colour and did not darken as experienced with conventional storage methods. Increased incubation time appeared to result in fibre losses. The incubation time should, therefore, be closely monitored and could restrict commercial application of biopulping. Treatment of non-sterile bagasse should also be studied to compare results with studies on sterile bagasse, because it is important for industrial scale optimisation.

Ultrastructural studies showed that the tested fungi caused non-selective decay, which reflects the results obtained with biopulping. Microscopy could, therefore, be useful in the evaluation of biopulping processes for bagasse to determine the colonisation and degradation. It is important that more white-rot fungi should be studied to determine their colonisation and degradation patterns to be able to implement biotechnology.

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SUMMARY

Keywords: Bagasse, biodegradation, biopulping, Cartapip, enzymes, lignocellulose *Ophiostoma piliferum*, white-rot fungi, wood chips

Lignin, hemicellulose and cellulose occur together in wood and agricultural wastes that are used in industries such as the pulp and paper industry. Biodegradation could be applied by these industries to save cost and reduce environmental impact. Lignocellulose degradation is very complex and must be understood for the optimisation of biotechnological processes. White-rot fungi degrade lignin, cellulose and hemicellulose while brown-rot fungi modify lignin slightly, but also break down cellulose and hemicellulose. The most important enzymes in lignin degradation are manganese peroxidase, lignin peroxidase and laccase, which these fungi produce extracellularly. Decay fungi establish on organic material, which leads to the degradation of lignocellulose during the colonization process. A distinction must be made between primary and secondary colonisers during degradation, because of succession that takes place.

Ophiostoma piliferum is an example of a primary coloniser, which is used to produce Cartapip 97[®]. This product was developed to treat wood chips during storage to reduce fibre degradation. The fungus was previously not available in South Africa because of a possible threat to local forest species. South African forestry companies wanted to test Cartapip 97[®] in industrial processes and it was, therefore, necessary to demonstrate that the fungus is not pathogenic. It also had to be confirmed that the fungus is a strain of *O. piliferum* before certification and importation into South Africa would be allowed. Cultural and morphological characteristics of the anamorph of the Cartapip

fungus were found to be similar to those of *O. piliferum* and it was released for field trials. The pathogenicity of the fungus was compared with *Ophiostoma ips* and *Sphaeropsis sapinea*, causes of sapstain on *Pinus* spp. in South Africa, to demonstrate that *O. piliferum* does not pose a threat to forestry. Different pine species were inoculated and the results indicated that *O. piliferum* is not a pathogen and it is, therefore, safe to use Cartapip 97[®] in South Africa. Hardwood and softwood chips were subsequently treated with Cartapip 97[®] and pulped using different pulping methods, but the benefits were not obvious. It is, however, possible that the extractives content of the chips was reduced. A slight increase in strength of kraft pulp from softwood and hardwood and also of Soda-AQ pulp from *A. mearnsii*, was observed after pre-treatment with Cartapip 97[®].

Bagasse contains fibres that can be used for the production of paper. However, bagasse has to be stored for long periods during which time decay occurs. Stored bagasse could be pre-treated with fungi to preserve and possibly improve the quality of the bagasse. In this study, *Lenzites betulina* and *Pycnoporus sanguineus* were used to treat bagasse before pulping. Inoculum production, pulping processes and different incubation periods were evaluated to optimise biopulping. Ultrastructural studies of treated bagasse were used to determine the effect that fungal treatment has on bagasse. Pulping results obtained from bagasse treated with *L. betulina* were variable and *P. sanguineus* did not improve the pulping. These results indicated that colonisation and degradation strategies of biopulping fungi must be fully understood before an attempt is made to optimize pulping processes.

OPSOMMING

Sleutelwoorde: Bagasse, bio-afbraak, bioverpulping, Cartapip, ensieme, houtskerfies, lignosellulose, *Ophiostoma piliferum*, witvrot fungi

Lignien, hemisellulose en sellulose kom saam voor in hout- en landboureste wat in nywerhede soos die pulp- en papiernywerheid gebruik word. Die toepassing van bio-afbraak kan koste bespaar en die omgewingsimpak van die bedryf verminder. Bio-afbraak word gevolglik op baie terreine in die pulp- en papierindustrie toegepas. Lignosellulose-afbraak is ingewikkeld en moet volkome verstaan word vir die optimalisering van biotegnologiese prosesse. Witvrot fungi breek lignien, sellulose en hemisellulose af terwyl bruinvrot fungi lignien tot 'n mate kan modifiseer, maar ook sellulose en hemisellulose afbreek. Die belangrikste ensieme in lignienafbraak is mangaan-peroksidase, lignienperoksidase en lakkase wat buitesellulêr deur die fungi vervaardig word. Verrottingsfungi vestig hulself op organiese materiaal wat lei tot die afbraak van lignosellulose tydens die koloniseringsproses. Onderskeid moet getref word tussen primêre en sekondêre koloniseerders tydens afbraak, want 'n opeenvolging vind plaas.

Ophiostoma piliferum is 'n voorbeeld van 'n primêre koloniseerder en word gebruik om Cartapip 97[®] te vervaardig. Hierdie produk is ontwikkel om houtskerfies tydens berging te behandel om veselafbraak te verminder. Die fungus was voorheen nie in Suid-Afrika beskikbaar nie, want dit kon moontlik gevaar inhou vir spesies in plaaslike plantasies. Die Suid-Afrikaanse bosbou nywerheid het belanggestel om Cartapip 97[®] in industriële prosesse te toets en gevolglik was dit nodig om te demonstreer dat die fungus nie patogeen is nie. Dit moes ook bevestig word dat die fungus wel 'n isolaat van *Ophiostoma piliferum* is voordat dit as veilig gesertifiseer kon word vir invoer in Suid-Afrika. Kultuur- en morfologiese eienskappe van die anamorf van die Cartapip-fungus

het getoon dat dit soortgelyk was aan die eienskappe van *O. piliferum* en dit is gevolglik vrygestel vir veldproewe. Die patogenisiteit van die fungus is vergelyk met *Ophiostoma ips* en *Sphaeropsis sapinea* wat houtverkleuring van *Pinus* spp. in Suid-Afrika veroorsaak. Die vergelyking is getref om te demonstreer dat *O. piliferum* nie 'n gevaar inhou vir bosbou nie. Verskillende dennespesies is geïnokuleer en die resultate het getoon dat *O. piliferum* nie 'n patogeen is nie en dat dit ook veilig is om Cartapip 97[®] in Suid-Afrika te gebruik. Hardehout- en sagtehoutskerfies is gevolglik behandel met Cartapip 97[®] en verpulp volgens verskillende metodes, maar geen duidelike voordele is waargeneem nie. Dit is wel moontlik dat die harsinhoud van die hout verminder is. 'n Toename in die sterkte-eienskappe van kraftpulp van sagte- en hardehout en ook van Soda-AQ pulp van *A. mearnsii*, is waargeneem na die voorafbehandeling met Cartapip 97[®].

Bagasse bevat vesels wat gebruik kan word in papiervevaardiging. Bagasse word egter vir lang tydperke gestoor wat lei tot die afbraak van die vesels. Fungi kan moontlik gebruik word om bagasse te preserveer en moontlik die kwaliteit te verbeter. *Lenzites betulina* en *Pycnoporus sanguineus* is in hierdie studie gebruik om bagasse voor verpulping te behandel. Inokulumvervaardiging, verpulpingsprosesse en verskillende inkubasie-tydperke is geëvalueer om bioverpulping te optimiseer. Studies van die ultrastruktuur van behandelde bagasse is gebruik om die uitwerking van voorafbehandeling op bagasse te bestudeer. Verpulpingsresultate wat verkry is van bagasse wat met *L. betulina* behandel is, het baie gewissel en *P. sanguineus* het verpulping nie verbeter nie. Hierdie resultate het getoon dat die kolonisasie- en afbraakstrategieë van fungi vir bioverpulping ten volle verstaan moet word voordat dit aangewend kan word om verpulpingsprosesse te optimiseer.