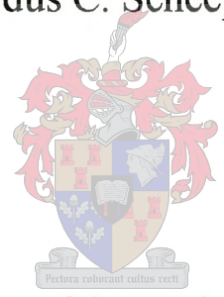
ENZYMATIC PITCH CONTROL IN THE KRAFT PULPING AND BLEACHING OF *EUCALYPTUS SPP.*

by

Gerhardus C. Scheepers



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DECLARATION

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

G.C. Scheepers

SUMMARY

The extractive materials in wood often cause pitch problems in pulp mills. During pulping and bleaching extractives are released from the wood and pulp and later stick to ceramic and metal parts, forming pitch deposits. Pitch deposits impair both product quality and production rates. It decreases the efficiency of pulp washing, screening, centrifugal cleaning, and refining, and can disrupt many paper machine operations. The deposits also break loose from equipment and cause spots in the final product. There are a few triggering mechanisms that induce pitch deposition. Hydrodynamic or mechanical shear can destabilise the colloidal pitch emulsion, causing pitch to agglomerate and deposits to form. Similarly, sudden temperature drops and/or pH shocks and/or the introduction of water hardness ions from fresh water inlets or showers can also cause pitch deposits by destabilising the colloidal pitch emulsion. Inorganic salts, such as calcium carbonate, can catalyse pitch deposition by acting as the building blocks for the sticky pitch. Calcium ions in the white water can react with fatty acids, forming insoluble, sticky calcium soaps. Triglycerides have also been shown to be a major contributor to pitch deposition in kraft pulping and bleaching mills. It forms a sticky deposit to which less sticky particles attach.

To attain an improved understanding of pitch problems associated with the kraft pulping and bleaching of *Eucalyptus spp.*, various analyses were done on wood- and pulp extractives and pitch from a South African kraft pulp mill. High molecular weight compounds (involatile) constituted a large portion of the extracts and pitch. Approximately 40% of volatile *Eucalyptus grandis* extract was β-sitosterol, with fatty acids (22.8%) and triglycerides (15.5%) also making a substantial contribution. Fatty acid amides were a prominent fraction of pulp extracts from the latter stages of bleaching. The amides constituted 38.3% and triglycerides 10.1% to total volatile pitch deposits.

Lipases hydrolyse triglycerides and could therefore help to reduce pitch problems. Consequently 381 filamentous fungi isolated from indigenous and commercial forests in South Africa were screened for lipase activity on tributyrin and Tween 80. Eight strains were selected and the tributyrin and Tween 80 assays were repeated by monitoring lipase activity over a seven-day

period. The selected strains were also assayed for their activity toward *p*-nitrophenyl palmitate. *Ophiostoma piliferum* Cartapip 58™ and *Phanerochaete chrysosporium* BKM-F-1767, two strains known for respectively their biodepitching and biopulping ability, were used as controls. A few of the strains compared well and even outperformed the control strains, indicating their potential for use in pitch control.

The effect of pretreatment with the eight selected fungal strains on *E. grandis* wood- and pulp extractives was determined. Cartapip 58™ and *P. chrysosporium* BKM-F-1767 were used as control strains. Several of the strains compared well to the control strains in their ability to reduce the triglyceride content of wood extract. The South African isolate, white-rot fungus *Phanerochaete psuedomagnoliae* nom. prov., reduced triglyceride content significantly. Consequently it can act as an agent for both biopulping and biodepitching. The treated wood samples had a lower triglyceride content than the sterile controls. Consequently more triglycerides would be released into process waters by the sterile controls than the treated samples. The effect of commercial lipases on deposited brown stock pulp extract was also evaluated. The lipases did not reduce the triglyceride content of the deposited extract. The addition of lipases in pulping and bleaching processes would therefore not affect already deposited pitch.

OPSOMMING

Die ekstrakstowwe van hout veroorsaak dikwels 'n neerslag tydens verpulping. Gedurende verpulping en bleiking kom ekstrakstowwe van die hout en pulp vry en kleef aan keramiek- en metaalonderdele. Gevolglik benadeel dié neerslag produkkwaliteit en produksietempo. Dit verlaag die effektiwiteit van pulpwas, sifting, sentrifugale skoonmaakprosesse en suiwering, en kan die werkverrigting van papiermasjiene ontwrig. Die neerslag kan ook later los breek en kolletjies op die finale produk veroorsaak. Verskeie meganismes kan die neerslag veroorsaak. Hidrodinamiese of meganiese wrywing kan die kolloïdale ekstrakstofemulsie destabiliseer en sodoende die ekstrakstof laat konglomereer en neerslaan. Op soortgelyke wyse veroorsaak skielike temperatuurverlaging en/of pH-skokke en/of die toevoeging van ione in varswater om waterhardheid te beheer ook die neerslag deur die kolloïdale ekstrakstofemulsie te destabiliseer. Anorganiese sout soos kalsiumkarbonaat kan neerslagvorming kataliseer omdat dit optree as bousteen vir die klewerige, sementagtige ekstrakstowwe. Kalsiumione in die proseswater kan ook reageer met vetsure om onoplosbare, klewerige kalsiumsepe te vorm. Dit is bewys dat trigliseriede een van die hoofoorsake is in die vorming van die neerslag tydens kraft verpulping- en bleikingprosesse.

Om die neerslagreaksie wat met die kraft verpulping en bleiking van *Eucalyptus spp.* geassosieer word, beter te verstaan, is verskeie analises op hout- en pulpekstrakte asook die neerslag van 'n Suid-Afrikaanse kraft verpulpingsaanleg uitgevoer. Hoë molekulêre massa (nie-vlugtige) stowwe het 'n groot gedeelte van die ekstrakte en neerslag uitgemaak. Ongeveer 40% van die vlugtige *Eucalyptus grandis* ekstrak bestaan uit β-sitosterol met vetsure (22.8%) en trigliseriede (15.5%) wat ook aansienlike bydraes lewer. Vetsuuramiede verteenwoordig 'n beduidende komponent van pulpekstrak by die laaste stadiums van bleiking. Die amiede het 38.3% en trigliseriede 10.1% tot die vlugtige fraksie van die neerslag bygedra.

Lipases hidroliseer trigliseriede en kan dus help om neerslagprobleme te voorkom. Gevolglik is 381 filamentagtige fungi geïsoleer uit inheemse en kommersiële woude van Suid-Afrika en hul lipase-aktiwiteit op tributyrin en Tween 80 geëvalueer. Agt rasse is geselekteer en die tributyrin en Tween 80 toetse is herhaal deur lipase-aktiwiteit oor 'n sewe-dag periode te monitor. Die geselekteerde rasse is ook getoets vir lipase-aktiwiteit met *p*-nitrofenielpalmitaat. *Ophiostoma*

piliferum Cartapip 58™ en *Phanerochaete chrysosporium* BKM-F-1767, twee rasse wat daarvoor bekend staan vir onderskeidelik hul vermoë om houtekstrakstowwe te verminder en te bioverpulp, is as kontroles gebruik. 'n Paar van die geselekteerde rasse het goed vergelyk en selfs beter presteer as die kontrolerasse; 'n aanduiding van hul potensiaal om neerslagreaksies te beheer.

Die effek van voorafbehandeling met die agt geselekteerde fungi rasse op *E. grandis* hout- en pulpekstrak is vasgestel. Cartapip 58™ en *P. chrysosporium* BKM-F-1767 is gebruik as kontrolerasse. Verskeie rasse het goed vergelyk met die kontrolerasse in hul vermoë om die trigliseriedinhoud van die houtekstrak te verlaag. Die Suid-Afrikaanse isolaat, witverrottingswam *Phanerochaete pseudomagnoliae* nom. prov., het ook die trigliseried inhoud beduidend verminder. Gevolglik sou dit as 'n middel kon dien vir beide neerslagvoorkoming en bioverpulping. Die trigliseriedinhoud van die behandelde monsters was laer as dié van steriele kontroles. Gevolglik sal meer trigliseriede in proseswater vrygestel word deur die steriele kontroles as die behandelde monsters. Die effek van kommersiële lipases op ongebleikte kraft pulpekstrakneerslag is ook geëvalueer. Omdat lipases nie die trigliseriedinhoud van die neerslag kon verlaag nie sal die gebruik van lipases dus nie die ekstrakstofneerslag in verpulpings- en bleikingsprosesse beïnvloed nie.

This dissertation is dedicated to the Lord Almighty, for I can do anything through Him who gives me strength.

Hierdie proefskrif is opgedra aan God die Almagtige, want ek is tot alles in staat deur Hom wat my krag gee.

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PREFACE

This thesis is presented as a compilation of manuscripts. Chapters 1, 2 and 6 were formatted according to the style of the scientific journal *Biotechnology Letters*. Each of Chapters 3 to 5 was formatted according to the style of the journal to which it will be submitted.

TABLE OF CONTENTS

Chapter 1: Introduction

1.1 Introduction

The vast majority of paper products are made from cellulose fibres, the aggregate of fibres being known as pulps. There are three main pulp categories: mechanical, semi-chemical and chemical pulp. World pulp production statistics are summarised in Table 1. The major products are mechanical and chemical pulps, with more than 80% of the latter being manufactured by the kraft process (Sjöström, 1993).

Table 1 World pulp production by grade in 1998 (James *et al.*, 1999)

Type of pulp	*1000 tons*
Chemical[a]	123 680
Mechanical	34 836
Other pulp[b]	17 020
TOTAL: all pulps	175 532

[a] Includes semi-chemical pulp
[b] For countries where breakdown by grade is not possible (i.e. not specified or not estimated) total ouput has been listed under the "Other pulp" category

The extractive materials in wood often deposit in pulp and bleach mills to form pitch. Not all of the extractives are troublesome, most problems occurring in pulping and papermaking when there are shifts in pH and/or temperature. Triglycerides are considered to be the cause of pitch deposition (Farrell *et al.*, 1997). During the pulping process extractives are released from wood and later stick to ceramic and metal parts as well as the wires of papermaking machines, forming pitch. The pitch also stains the felts and canvas, and eventually reaches the dryer section. Pitch accumulation can cause paper spotting and web breaks on the papermachine, causing downtime and reduced product quality. In effect pitch deposition gives rise to a production cost increase while product value decreases. The severity of pitch problems vary with wood species. Pitch from several softwoods is known to cause severe problems. Hardwood pitch, particularly from tropical hardwood species and *Eucalyptus*, can also be detrimental (Farrell *et al.*, 1997).

Traditional methods to control pitch problems include seasoning of wood before pulping and/or adsorption and dispersion of pitch particles in a pulp suspension with chemicals like talc, dispersants and cationic polymers. Seasoning requires raw wood logs to be left outdoors for several months or chips to be piled and left for weeks. Seasoning can

potentially cause losses such as decreased pulp brightness and pulp yield due to biological deterioration. It also increases working capital costs due to high wood inventory and land use. Thus, this method is often unacceptable, especially in areas where space is limited.

Recently two new and different methods of combating pitch, both of which are biotechnological in basis, have been developed independently and are now used industrially. One is the development of a pitch control method using the enzyme lipase, which catalyses the hydrolysis of triglycerides (Fujita *et al.*, 1992). Another is a pitch control method using a fungus developed in a laboratory from the ascomycete sap-staining organisms that cause natural ageing. This fungus is colourless and avoids the staining and decrease in brightness normally associated with aged wood (Farrell *et al.*, 1994).

The objective of this investigation was to relieve the pitch problems of Mondi Kraft Ltd. using enzymatic means. The bleached kraft pulp mill utilises *Eucalyptus* species and is situated in Richards Bay, South Africa.

1.2 References

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Chapter 2: Literature review

2.1 Wood stucture and characteristics with special reference to *Eucalyptus grandis*

2.1.1 Wood structure

Softwoods are members of the Gymnospermae and hardwoods are members of the Angiospermae. Although the terms softwood and hardwood were originally intended to indicate the relative hardness of the timbers, it is not an appropriate distinction. A wood anatomical difference is more descriptive. Softwoods and hardwoods have different cellular structures when viewed with a hand lens or microscope. To provide support and conducting pathways the softwoods have radial and longitudinal tracheids and the hardwoods have respectively longitudinal fibres and longitudinal vessels (Siau, 1995). In both softwoods and hardwoods parenchyma serve a storage function and can occur both longitudinally or radially. Groups of radial parenchyma cells form rays.

(A) (B)

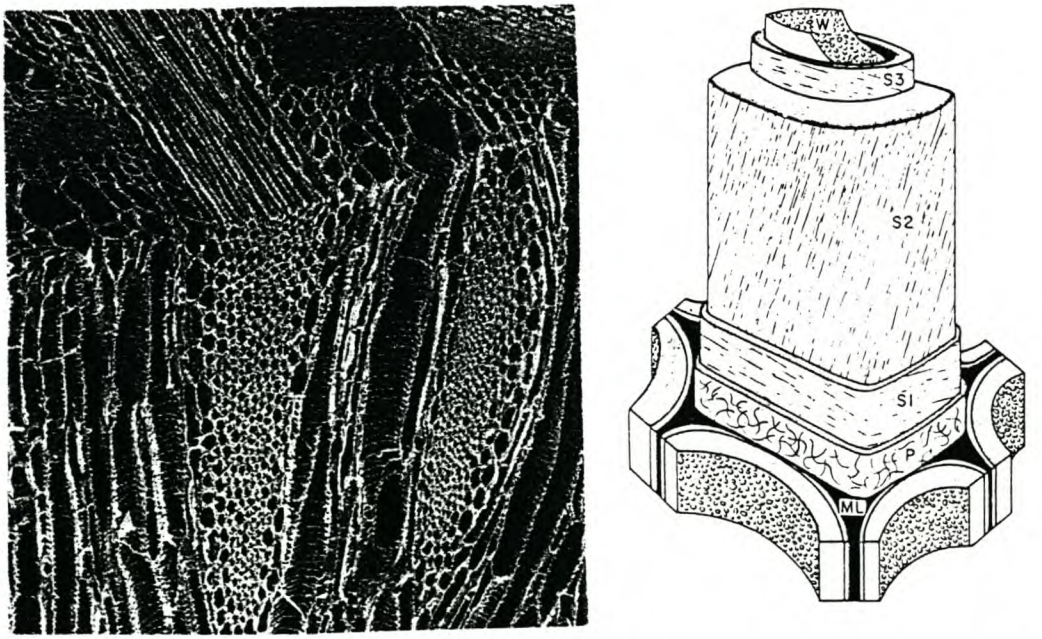


Figure 1 (A), Transverse and tangential longitudinal faces of a hardwood species. The wood comprises axial vessels surrounded by longitudinal parenchyma and fibres and radial parenchyma in broad rays. (B), Schematic structure of a woody cell wall, showing the middle lamella (ML), the primary wall (P), the outer (S_1), middle (S_2), and inner (S_3) layers of the secondary wall, and the warty layer (W) (Côté, 1967).

Figure 1a shows an electron microscope image of a hardwood where all the cells can be seen. When vessels are grouped predominantly in the earlywood, the pattern is described as ring porous. When they are distributed throughout the growth ring, it is described as diffuse porous.

Cellulose fibrils form a skeleton that is surrounded by other substances functioning as matrix (hemicelluloses) and encrusting (lignin) materials. The length of a native cellulose molecule is at least 5000 nm corresponding to a chain with about 10000 glucose units. The smallest building element of the cellulose skeleton is considered to be an elementary fibril. This is a bundle of 36 parallel cellulose molecules which are held together by hydrogen bonds. The bonding between cellulose molecules varies intermittently between crystalline structures and amorphous regions along the length of the fibril (Tsoumis, 1991). The length of the crystallites can be 100-250 nm and the cross section, probably rectangular, is on an average 3 nm × 10 nm. Elementary fibrils are arranged into microfibrils that combine to larger fibrils and lamellae. Disordered cellulose molecules as well as hemicelluloses and lignin are located in the spaces between the microfibrils. The hemicelluloses are considered to be amorphous although they apparently are oriented in the same direction as the cellulose microfibrils. Lignin is both amorphous and isotropic (Sjöström, 1993).

Figure 1b shows the cell wall structure. The cell wall is built up by several layers, namely middle lamella (M), primary wall (P), outer layer of the secondary wall (S_1), middle layer of the secondary wall (S_2), inner layer of the secondary wall (S_3) and warty layer (W). These layers differ from one another with respect to their structure as well as their chemical composition. The middle lamella is located between the cells and serves the function of binding the cells together (Tsoumis, 1991).

The primary wall is a thin layer, 0.1-0.2 μm thick, consisting of cellulose, hemicelluloses, pectin and protein, embedded in lignin. The microfibrils form an irregular network in the outer portion of the primary wall; in the interior they are oriented nearly perpendicularly to the cell axis. The middle lamella together with the primary walls on both sides, is often referred to as the compound middle lamella. Its relative lignin concentration is

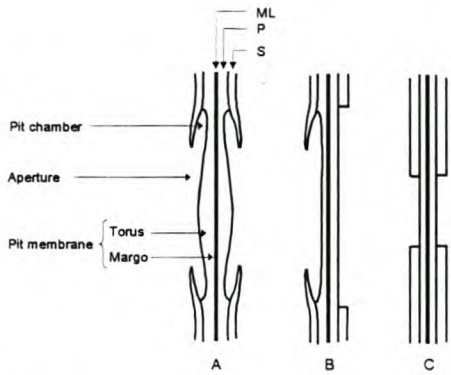


Figure 2 Types of pit pairs. A, bordered; B, half bordered; C, simple. ML, middle lamella; P, primary wall; S, secondary wall (Sjöström, 1993).

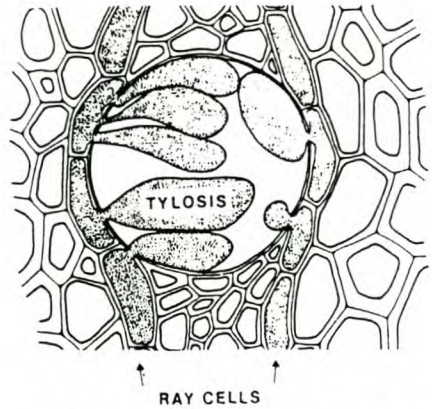


Figure 3 Development of tyloses from ray cells in oak (Sjöström, 1993).

high, but because the layer is thin, only 20-25% of the total lignin in wood is located in this layer (Sjöström, 1993).

The secondary wall consists of three layers, thin outer (S_1) and inner (S_3) layers and a thick middle (S_2) layer. These layers are built up by lamellae formed by almost parallel microfibrils between which lignin and hemicelluloses are located (Butterfield and Meylan, 1980). The outer layer is 0.2-0.3 μm thick and contains 3-4 lamellae where the microfibrils form a helix. The angle of orientation of the microfibril network varies between 50 and 70° with respect to the fibre axis. The middle layer forms the main portion of the cell wall. The inner layer is a thin layer of about 0.1 μm consisting of several lamellae, which contain microfibrils in helices with a 50-90° angle (Sjöström, 1993). The warty layer (W) is a thin amorphous membrane located in the inner surface of the cell wall in all conifers and in some hardwoods, containing warty deposits of an unknown composition (Butterfield and Meylan, 1980).

Water conduction in a tree is made possible by pits, which are recesses in the secondary wall between adjacent cells. Two complementary pits normally occur in neighbouring cells thus forming a pit pair (Wilson and White, 1986). Figure 2 shows the different types of pit pairs. Radially oriented microfibril bundles form a netlike membrane permeable to liquids (margo) in the pit. The central thickened portion of the pit membrane (torus) is rich in pectic material and in pine and spruce also contains cellulose. Bordered pit pairs are typical of softwood tracheids and hardwood fibres and vessels. In softwood heartwood the pits can become aspirated when the torus becomes pressed

against either side of the border (Siau, 1995). In some hardwoods the vessels in the heartwood can become closed by tyloses (Figure 3) when a special tylose forming membrane from adjacent parenchyma ray cells enters the vessel through the pits and forms bubbles in its inside, covering both the vessel wall and the other pits (Walker, 1993).

2.1.2 General information about *Eucalyptus grandis*

The *Eucalyptus* species from the family Myrtaceae are hardwood species indigenous to the eastern part of Australia. Because of widespread planting in South Africa, *Eucalyptus* species have become of economic importance to both the South African sawmilling and pulp and paper industries. They are planted on a fairly large scale in humid regions like Mpumalanga and Kwazulu/Natal for sawmilling, pulping and secondary wood processing. Commonly it is known as Rose gum or Saligna gum. It is a valuable wood on account of its multiple uses. It is very popular for use in constructing mine props and the treated wood is suitable for electricity poles, telephone poles and fencing. It is also suitable for frames, panels, furniture, joinery and many other purposes. The colour of the wood varies from almost white in young trees to dark red in the heartwood of old trees (Dyer, 1989). The approximate density of 12-15 year old trees is 570 kg/m^3 (Dyer, 1989). The density of 5-year-old trees is about 420 kg/m^3 (Bamber, 1985).

2.1.2.1 Macroscopic structure

E. grandis is a diffuse-porous wood. The vessels are solitary, numerous, moderately large and visible to the naked eye. A distinct diagonal arrangement forms vessel lines. Growth rings can be distinguished clearly because bands of fibre tissue containing vessels are alternated by bands without vessels. Numerous rays are visible with a hand lens, as light lines on a dark background. Parenchyma is visible with a hand lens as sheaths around the pores (Dyer, 1989).

2.1.2.2 Extractive content of various *Eucalyptus* species

The extractives of the *Eucalyptus* genera have a detrimental effect in both pulping and sawmilling (Chafe, 1987; Hillis and Carle, 1959; Nelson *et al.*, 1970; Yazaki *et al.*,

1993). Extractives of *Eucalyptus* increase chemical consumption and impair the colour and brightness of unbleached pulp (Nelson *et al.*, 1970). Table 1 gives the alcohol/benzene/hot water extractive content of twelve species of the *Eucalyptus* genus. The extractive content of these species is high with the exception of *E. diversicolor*, *E. delegatensis* and *E. marginata*. *E. globulus* has a low dichloromethane extractives content of 0.3 % (Wallis and Wearne, 1999).

It is generally assumed that lignin is responsible for the brown colour of raw pulp. In *Eucalyptus* the polyphenols produce much more colour than does lignin during mild alkaline pulping conditions (Hillis, 1971).

Table 1 Alcohol/benzene/hot water extractive content for twelve species of the *Eucalyptus* genus (Hillis, 1962)

Species	*Extractive content (%)*
E. diversicolor	7.6
E. delegatensis	8.3
E. longifolia	22.8
E. marginata	6.4
E. microcorys	17.5
E. obliqua	29.4
E. paniculata	19.4
E. polyanthemos	25.2
E. regnans	16.9
E. robusta	18.0
E. sideroxylon	34.1
E. sieberiana	19.3

2.1.2.3 Sapwood and Heartwood distribution

Extractives are usually present in greater amounts in the heartwood than in the sapwood and the change in extractive content can be very abrupt at the heartwood periphery (Breuil *et al.*, 1994; Gao *et al.*, 1995; Hillis, 1971; Höll and Poschenrieder, 1975; Sitholé *et al.*, 1992; Yanchuk *et al.*, 1988). The distribution of extractives in the stem is consequently mostly dependent on the ratio between sapwood and heartwood.

Studies done in Australia on 9½ year old *Eucalyptus grandis* show that the heartwood constitute 45% of the volume of the lower half of the stem. This percentage decreases from bottom to top (Wilkins, 1991). It has also been shown that the stems of 5 and 50-year-old *Eucalyptus grandis* have respectively 25% and 80% heartwood (Bamber, 1985). There is an indication that the number of sapwood rings in a species is a heritable feature.

For example, in the *Eucalyptus* grown in Australia there are about 5-6 sapwood rings for the greatest portion of the genus (Hillis, 1971). Bamber (1985) also reported that heartwood formation begins fairly early in *Eucalyptus*, probably in the fourth or fifth year as measured from the cambium. While in softwoods there is a striking decrease in the moisture content of the wood during heartwood transition, there is little change in the *Eucalyptus* genera (Bamber, 1985). *Eucalyptus* heartwood tends to be more acidic (± pH 4) than the sapwood (± pH 5) (Bamber, 1985).

The extractive content of heartwood from young fast grown *Eucalyptus* is, however, low when compared to the heartwood of older slow grown trees (Hillis, 1971). Fast grown trembling aspen (*Populus tremuloides*) also has a lower extractive content (Yanchuk *et al.*, 1988). Consequently the extractive content difference between the heartwood and sapwood of young fast-grown *Eucalyptus* may be less pronounced. The lipid composition of extractives may however differ from sapwood to heartwood. In Lodgepole pine (*Pinus contorta* Dougl.), triglyceride content decrease from sapwood to heartwood while fatty acid content increases (Gao *et al.*, 1995). Similar results were reported for aspen heartwood and sapwood (Breuil *et al.*, 1994). It has been suggested that the reason for the differences is the hydrolysis of triglycerides during the formation of heartwood. This reaction effects an increase in fatty acids and mono-, and diglycerides (Saranpää and Nyberg, 1987).

2.2 The extractives of wood

A large number of wood components, although usually representing a minor mass fraction, are soluble in neutral organic solvents or water. They are called extractives. The composition of wood is illustrated in Figure 4. Extractives can be regarded as non-structural wood constituents, almost exclusively composed of low molecular mass, organic compounds (Jane, 1970). Extractives comprise an extraordinarily large number of individual compounds of both lipophilic and hydrophilic types. Extractives occupy certain morphological sites in the wood structure. Lipids are concentrated in ray parenchyma cells while phenolic extractives are present mainly in heartwood and in bark.

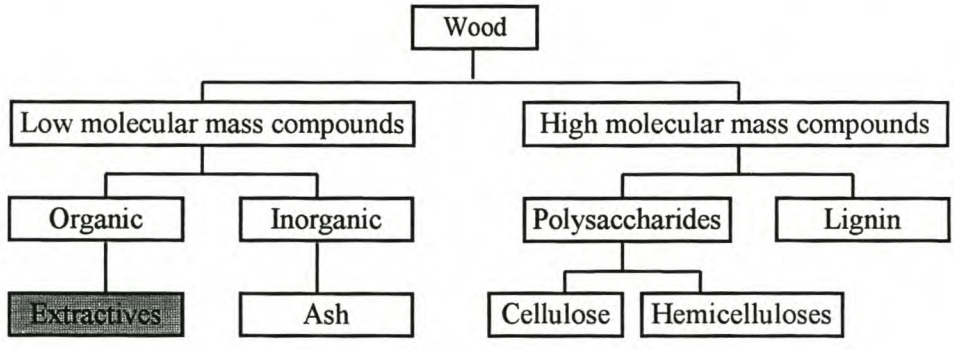


Figure 4 The composition of wood (Fengel and Wegener, 1989).

Different types of extractives are necessary to maintain the diversified biological functions of the tree. Fats constitute the energy source of the wood cells, whereas lower terpenoids, resin acids, and phenolic substances protect the wood against microbiological damage or insect attacks. The term "resin" is used as a collective name for the lipophilic extractives (with the exception of phenolic substances) soluble in non-polar organic solvents but insoluble in water (Sjöström, 1993). Dichloromethane is more selective for extracting lipids than methanol or acetone (Wallis and Wearne, 1999).

Table 2 gives the typical composition of softwoods and hardwoods. Extractives make out, on average, only 2-8% of the oven dry mass of hardwoods and 1-5% of softwoods. Extractives constitute a valuable raw material for making organic chemicals and they play an important role in the pulping and paper making processes (Sjöström, 1993).

Table 2 Typical composition of softwoods and hardwoods (Walker, 1993)

Constituent	*Softwood (%)*	*Hardwood (%)*
Cellulose	42 ± 2	45 ± 2
Hemicelluloses	27 ± 2	30 ± 5
Lignin	28 ± 3	20 ± 4
Extractives	3 ± 2	5 ± 3

2.2.1 Terpenes and terpenoides

The terpene and terpenoide fraction of extractives is built up by a building block called an isoprene unit. Their number of isoprene units present is used to classify the terpenes. Figure 5 shows the basic structures of some terpenes. To date no mention has been made of the presence of resin acids, which are diterpenes, in hardwoods. It is, however, an abundant substance in softwoods (Biermann, 1993).

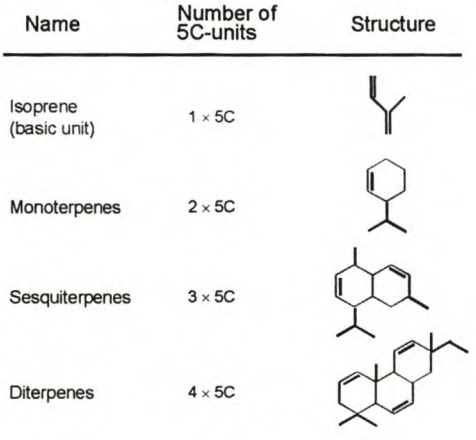


Figure 5 Basic structures of several terpenes (Fengel and Wegener, 1989).

2.2.2 Fats, waxes and their components.

A fat is an ester of glycerol and one or more fatty acids. A wax is an ester of a long chain fatty alcohol and fatty acids, thus its molecular mass is much higher than that of a fat. Examples of fats, waxes and their components are given in Figure 6. The fats are glycerol esters of fatty acids and occur in wood predominantly as triglycerides. More than thirty different fatty acids, both saturated and unsaturated, have been identified in softwoods

and hardwoods (Fengel and Wegener, 1989). Examples of the most common fatty acids are shown in Table 3.

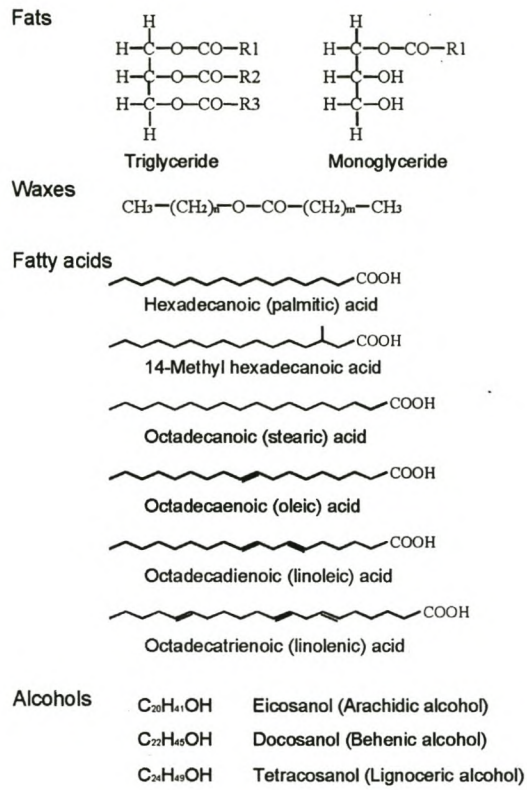


Figure 6 Fats, waxes and their components (Fengel and Wegener, 1989).

Table 3 Abundant fatty acid components of fats and waxes (Sjöström, 1993)

Trivial name	*Systematic name*	*Chain length*
Saturated		
Palmitic	Hexadecanoic	C_{16}
Stearic	Octadecanoic	C_{18}
Arachidic	Eicosanoic	C_{20}
Behenic	Docosanoic	C_{22}
Lignoceric	Tetracosanoic	C_{24}
Unsaturated		
Oleic	*cis*-9-Octadecanoic	C_{18}
Linoleic	*cis*,*cis*-9,12-Octadecadienoic	C_{18}
Linolenic	*cis*,*cis*,*cis*-9,12,15-Octadesatrienoic	C_{18}
Pinolenic	*cis*,*cis*,*cis*-5,9,12-Octadesatrienoic	C_{18}
Eicosatrienoic	*cis*,*cis*,*cis*-5,11,14-Eicosatrienoic	C_{20}

Fats and waxes (esters) are hydrolysed during kraft pulping. The fatty acids that are liberated can be recovered together with resin acids as soap skimmings from the black liquor (Biermann, 1993).

2.2.3 Phenolic substances

Extractives contain a large number of phenolic substances of simple and more complex structure. Those of more complex structure are the lignans and quinones. Some examples of the simple structure and complex phenols are displayed in Figure 7, Figure 8 and Figure 9. Phenolics give wood its resistance against microbial decay (Walker, 1993).

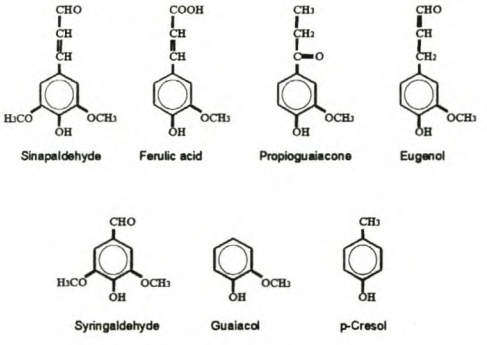


Figure 7 Some simple phenols (Fengel and Wegener, 1989).

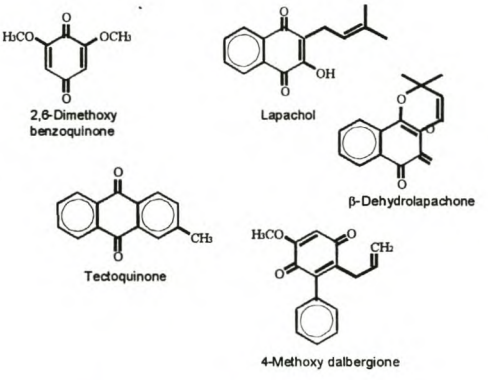


Figure 8 Kinones of hardwoods (Fengel and Wegener, 1989).

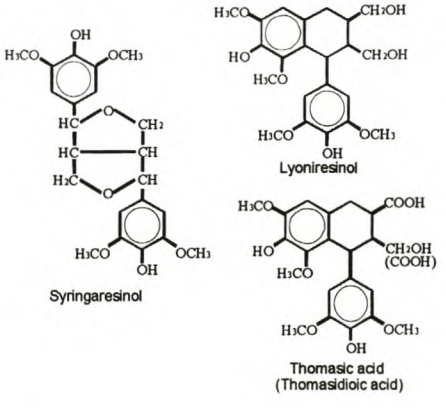


Figure 9 Well-known lignans of hardwoods (Fengel and Wegener, 1989).

2.2.4 Tannins

Two types, hydrolisable and condensed tannins, exist. Among the condensed tannins are flavonoids, a substance of widespread occurrence in the plant kingdom. Hydrolisable tannins can be viewed as polyesters of gallic acid and its dimers (Figure 10). Examples of the hydrolisable tannins and flavonoids are displayed in Figure 11 and Figure 12 (Fengel and Wegener, 1989).

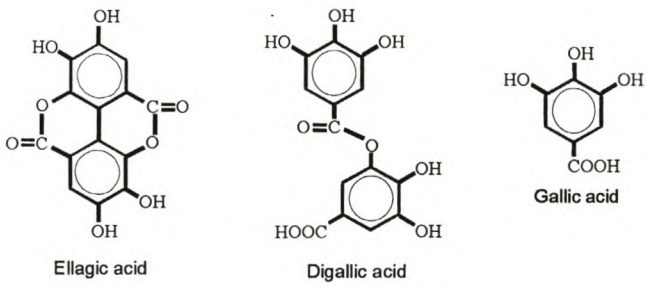


Figure 10 Galluacid and its dimers (Fengel and Wegener, 1989).

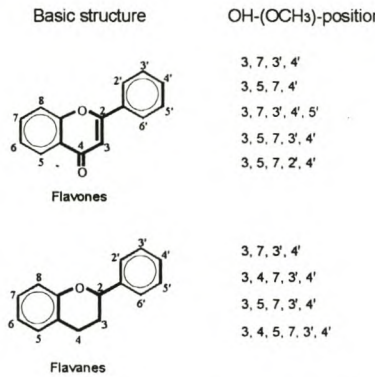


Figure 11 Some flavonoids (Fengel and Wegener, 1989).

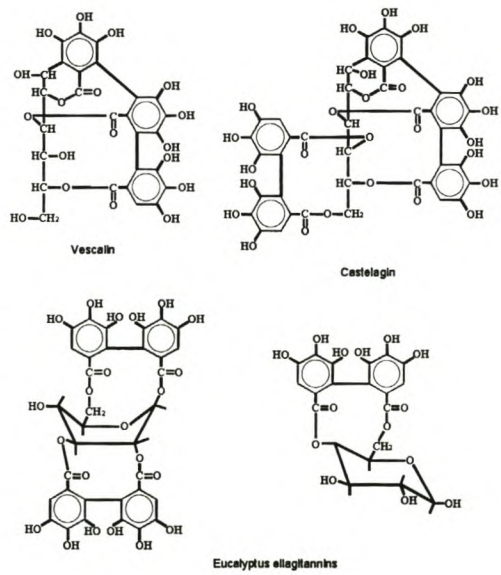


Figure 12 Some hydrolisable tannins (Fengel and Wegener, 1989).

2.2.5 Other substances present in extractives

Saturated hydrocarbons (Fengel and Wegener, 1989) and free or bonded amino acids (Hägglund, 1951) are also found in wood. The saturated hydrocarbons occur in a homologous range from C11 to C33 and in addition to the amino acids, there are also other nitrogen containing substances.

2.3 The kraft pulping process

Pulp is the basic product of wood, predominantly used for papermaking, but it is also processed to various cellulose derivatives, such as rayon silk and cellophane. The main purpose of wood pulping is to liberate the fibres, which can be accomplished either chemically or mechanically or by combining these two types of treatments. The common commercial pulps can be grouped into chemical, semichemical, chemimechanical, and mechanical types (Table 4). Chemical pulping is a process in which lignin is removed so completely that the wood fibres are easily liberated on discharge from the digester or at most after a mild mechanical treatment. Practically all of the production of chemical pulps in the world today is still based on the sulphite and sulphate (kraft) processes, of which the latter dominates. Pressurised alkaline cooking systems at high temperatures were introduced in the 1850's. In 1870 A.K. Eaton patented the use of sodium sulphate instead of sodium carbonate.

Table 4 Commercial pulp types (Sjöström, 1993)

Pulp type	*Yield (% of wood)*
A. Chemical	35-65
Acid sulphite	
Bisulphite	
Multistage sulphite	
Kraft	
Polysulphide-kraft	
Prehydrolysis-kraft	
Soda	
B. Semichemical	70-85
NSSC	
Green liquor	
Soda	
C. Chemimechanical	85-95
Chemithermomechanical (CTMP)	
Chemigroundwood (CGW)	
D. Mechanical	93-97
Stone groundwood (SGW)	
Pressure groundwood (PGW)	
Refiner mechanical (RMP)	
Thermomechanical (TMP)	

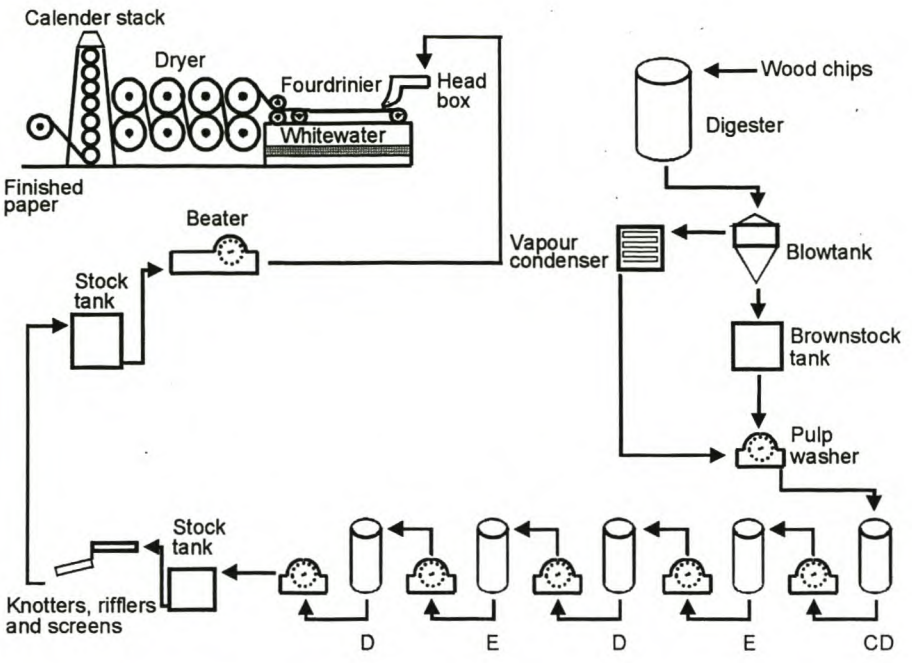


Figure 13 A schematic illustration of the kraft pulping process. Adapted from Macdonald (1969). A CDEDED bleaching process is illustrated.

The kraft process has almost completely replaced the old soda process because of its superior delignification selectivity resulting also in a higher pulp quality. More than 80% of the chemical pulp produced in the world is kraft pulp (Sjöström, 1993). A schematic illustration of the kraft pulping process is given in Figure 13.

2.3.1 Terminology

2.3.1.1 White liquor

White liquor is the solution added to the chips in the digester and consists of sodium hydroxide, sodium sulphide, water and sodium hydrosulphide (Macdonald, 1969).

2.3.1.2 Black liquor

The volatile wood extractives, consisting mainly of lower terpenes, are recovered during kraft pulping. The resin acids and fatty acids are recovered as tall soap. After acidification with sulphuric acid the resulting tall oil is finally purified and fractionated by distillation. The remaining kraft spent liquor (black liquor) contains organic constituents in the form of lignin and carbohydrate degradation products (Sjöström,

1981). Weak black liquor from the brown stock washers normally contains 12-18% solids. It contains some residual alkali not consumed during the cooking and has a pH of at least 12. Much of the sodium in the black liquor is in the form of sodium salts of organic acids. These acids are formed when lignin and carbohydrates dissolve during digesting. Inorganic components in the liquor are sodium carbonate, sodium sulphate, sodium thiosulphate, and sodium hydrosulphide. The sodium salts of resin and fatty acids are an important part of the black liquor. They are soaps that can be recovered and sold as the by-product tall oil (Mimms, 1989). Black liquor is used as a diluent of white liquor in amounts varying from 10 to 60 per cent of the total volume of the liquor charge. White liquor is added to the chips in the digester at the start of the process (Stephenson, 1950). The composition of softwood black liquor is given in Table 5.

Table 5 The composition of softwood black liquor (Sjöström, 1981)

Component		*Content (% of dry solids)*	*Composition (% of hydroxy acids)*
Lignin		47	
Hydroxy acids:		28	
	Lactic		15
	2-Hydroxybutanoic		5
	2,5-Dihydroxypentanoic		4
	Xyloisosaccharinic		5
	α-Glucoisosaccharinic		15
	β-Glucoisosaccharinic		36
	Others		20
Formic acid		7	
Acetic acid		4	
Extractives		5	
Other compounds		9	

2.3.1.3 Kappa number

The kappa number gives an indication of the lignin content of the pulp and is used for comparing different samples of pulp coming from the same wood species or wood species mix (Biermann, 1993).

2.3.2 The digestion process

There are two basic digester designs: batch digesters and continuous digesters. The principal operations in batch digesting include chip packing and steaming, liquor filling,

slow temperature rise to assure complete penetration of the chips by liquor, relief of gases, cooking at maximum temperatures, relief of pressure, and blowing the digester (Mimms, 1989).

The digestion process is essentially the treatment of wood, in the form of chips, in a pressure vessel called a digester under controlled conditions of temperature, pressure, and time, with a liquor composed mainly of an aqueous solution of sodium hydroxide or of a mixture of sodium hydroxide and sodium sulphide. The sodium hydroxide is responsible for delignification (Stephenson, 1950). The sodium hydroxide and sodium sulphide are considered to be the only active chemicals. The cooking is done at a temperature of 170-180°C for one to two hours in both batch and continuous digestors (Mimms, 1989). The sulphide reacts with water to give sodium hydroxide (NaOH) and sodium hydrosulphide (NaSH) (Equation 1).

Equation 1 The reaction of sodium sulphide with water (Mimms, 1989)

$$Na_2S + H_2O \rightleftharpoons NaOH + NaSH$$

The reaction is reversible; thus the concentrations of the different substances will reach a certain equilibrium state. As the cook proceeds, the sodium hydroxide would be consumed and the concentrations would shift, thus tending to increase hydrolysis of sodium sulphide to regain equilibrium (Stephenson, 1950). The sulphidity of the liquor in sulphate mills is usually in the range of 20 to 30 per cent (Stephenson, 1950). Constituents present in the cooking liquor are given in Table 6. Small amounts of sodium chloride, potassium salts, silicate, and calcium are also present.

All constituents of the wood are simultaneously attacked in differing degrees in the digestion process. Most of the extractives, fats, resins, etc., are saponified and dissolved in the cooking liquor. Saponification is the chemical process by which esters (e.g. mono- di- and triglycerides) are broken down into alcohols and fatty acids. Triglycerides are saponified by sodium hydroxide either partially or totally (Equation 2).

Some substances belonging to the extractives group are very difficult to dissolve and remain in the pulp. They are referred to as non-saponifiables (Stephenson, 1950). An example of a non-saponifiable is wax.

Equation 2 The reaction of triglyceride with sodium hydroxide (Stephenson, 1950)

$$\text{triglyceride} + NaOH \rightleftharpoons \text{glycerol} + 3 \times \text{fatty acid}$$

Table 6 The constituents of cooking liquor (Stephenson, 1950)

Name	*Formula*	*Concentration range (g/l as Na_2O)*
Sodium hydroxide	NaOH	81-120
Sodium sulphide	Na_2S	30-40
Sodium carbonate	Na_2CO_3	11-44
Sodium sulphate	Na_2SO_4	4.4-18
Sodium sulphite	Na_2SO_3	unknown (small amount)
Sodium thiosulphate	$Na_2S_2O_3$	4.0-8.9

2.3.3 The blow tank

The digester contents are blown tangentially into the top of the blow tank, the stock dropping into the tank and the steam and gases escaping from the top vent to a steam condenser (Stephenson, 1950). It is not until the blowing process that the chips are fully separated into fibres, if the kappa number has been lowered sufficiently. During blowing the temperature and pressure are lowered quickly. The result is that the liquor inside the chips starts to boil and the resulting pressure is enough to separate the fibres in the chips (Mimms, 1989).

2.3.4 Brown stock washing

The brown stock pulp coming from the digester has to be washed to recover process chemicals. If chemical pulps are not properly washed, foaming will be a problem, additional make-up chemicals will be needed, more bleaching chemicals will be required, and additional pollutants will result (Biermann, 1993).

2.3.5 Bleaching

The goal of bleaching is to produce whiter pulp. Some of the bleaching agents used are listed in Table 7. Between bleaching steps there are alkali extractions to wash out lignin fragments. The following sequence is an example of a bleaching process: bleach with chlorine, extraction with sodium hydroxide, bleach with chlorine dioxide, extraction with sodium hydroxide and bleach chlorine dioxide (CEDED) (Haylock, 1974).

Table 7 Nomenclature of bleaching (Walker, 1993)

Stage	*Description*
O	Delignification with oxygen
Z	Delignification with ozone
C	Delignification with chlorine, Cl_2
H	Delignification with hypochlorite, OCl^-
D	Delignification with chlorine dioxide, ClO_2
E	Extraction of lignin fragments with NaOH

2.3.6 Knotters, rifflers and screens

Knots are the uncooked wood particles, which are still not separated. The screens, rifflers and knotters serve to isolate and break down the knots into smaller particles (Mimms, 1989).

2.3.7 Refining

Refiners mechanically enlarge the specific area of the fibres by causing fibrillation and cell wall collapse. The purpose of this is to better cohesion between fibres and thus produce stronger paper. Different types of paper can be produced by controlling the degree of refining (Haylock, 1974).

2.4 Pitch

2.4.1 Defining pitch

Pitch problems arise at screens, filters, refining equipment, pulp washers and the paper machine (Macdonald, 1969). It causes clogging of equipment and results in higher production costs of paper. Allen (1988a) uses the definition of pitch as "the material in wood or wood pulps which is insoluble in water, but soluble in neutral organic solvents". This definition is also used in the book edited by Hillis (1962).

2.4.2 The composition of pitch

There are many variables (species used, specie mix, period of wood storage, stage of process, etc.) determining the composition of pitch. Thus the composition of one pitch sample would never match the other. However, an analysis of pitch does give an idea of what is to be expected. Some substances could be recognisable in all samples in larger or smaller quantities. Ohtani and Shigemoto (1991) analysed five pitch samples from four different kraft pulp mills in Japan. Suckling and Ede (1990) performed a ^{13}C nuclear magnetic resonance analysis on pitch from a pulp and paper mill that produces mechanical and kraft pulps.

Ohtani and Shigemoto (1991) analysed high molar mass components of machine and kraft pitch. They defined kraft pitch as pitch that deposits after pulping and before paper machine operations while machine pitch refers to pitch that deposits in paper machine operations. The gel permeation chromatogram (GPC) of one of the kraft pitches newly collected by these authors is displayed in Figure 14.

The machine pitch deposited in the Uhle box of a kraft paper machine was also examined by GPC. The fraction with molar mass over 1000 accounted for 85% of the total $CHCl_3$-solubles, similar to the situation in kraft pitches. The pitch was divided into four fractions according to molar mass with the same methods as used in the case of kraft pitches (Figure 14). Fraction IV (Figure 15) amounted to 15.5% of the $CHCl_3$-solubles. Fraction III (Figure 16) accounted for 22.3% of the $CHCl_3$-solubles. Fraction II

accounted for 14.5% and fraction I 47.5% of $CHCl_3$-solubles. Fractions I and II were identified as polymerised fatty and resin acids.

High amounts of aluminium were found in machine pitch due to the use of alum . Based on chemical composition, the mechanism of pitch deposition seemed to differ for these two pitches. The high molar mass fraction of machine pitches was found to be a complex consisting of aluminium and acidic wood extractives. Irrespective of difference in mill locations and pulping stages, the high molar mass fractions of most of the kraft pitch deposits gave similar carbon-13 NMR spectra and were characterised as polymerised aliphatic hydrocarbons (Ohtani and Shigemoto, 1991).

In their investigation Suckling and Ede (1990) dissolved the pitch deposit in chloroform. Seventy nine percent of the dry mass of the deposit was soluble in chloroform, the remainder consisted largely of paper fines. A NMR analysis showed that the pitch was largely composed of organic compounds present in wood extractives. The pitch sample

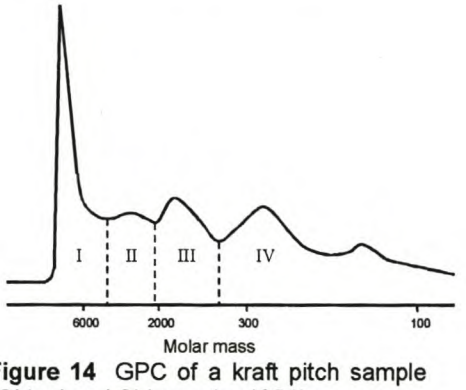


Figure 14 GPC of a kraft pitch sample (Ohtani and Shigemoto, 1991).

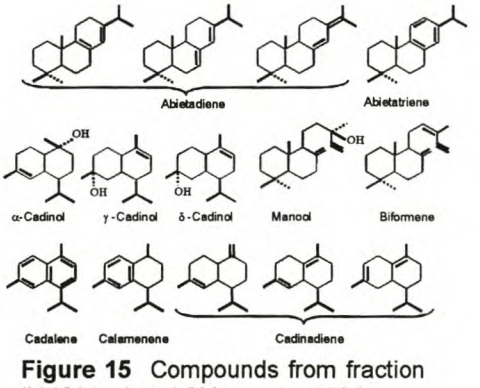


Figure 15 Compounds from fraction IV (Ohtani and Shigemoto, 1991).

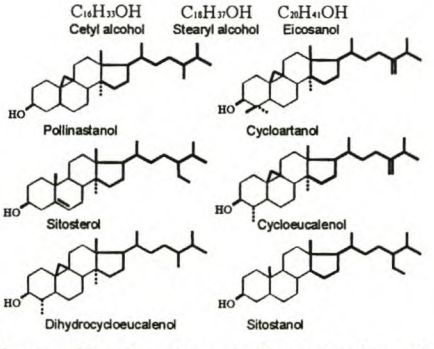


Figure 16 Compounds from fraction III (Ohtani and Shigemoto, 1991).

contained 34% triglycerides by mass, 3% fatty acids and 29% resin acids. No significant amounts of fatty acid esters other than triglycerides were present. The acids existed in salt form in the pitch. The balance of the chloroform soluble material presumably includes unsaponifiable extractives components, polymerised and oxidised resin, other added organic components such as defoamers or release aids and inorganic salts.

2.4.3 Mechanisms of deposition

2.4.3.1 Deposition due to polymerisation

Ohtani *et al.* (1986) investigated the chemical aspects of pitch deposits in five Japanese kraft pulp mills and found that most of the pitch deposits contained high molecular mass components in rather high proportions. Polymerised alkyl hydrocarbons found in pitch deposits and wood chips were assumed to be derived from fatty acids and alcohols, which exist in the wood. In a simulation to understand the chemical reactions of fatty acids and alcohols in the interior of wood some experiments were conducted where these compounds were heated in an oven at 105°C. The results are shown in Figure 17. Stearyl alcohol and stearic acid underwent very little change. Oleyl alcohol, oleic acid, and linoleic acid, all of which have one or two double bonds, gradually changed into higher molecular mass substances, and they easily underwent condensation reactions. The more double bonds they had, the faster the condensation reaction proceeded. Fatty acids are polymerised faster than fatty alcohols. It was found that polymeric materials in the pitch from four mills were condensed substances of unsaturated fatty acids or alcohols. The condensation reaction was assumed to proceed during outside chip storage. The *Eucalyptus* woods (mainly from Tasmania) pulped in Japanese mills contained large amounts of polyphenols with lesser amounts of fatty acids or fatty alcohols. According to Ohtani *et al.* (1986) most of the extractives from *Eucalyptus spp.* are dissolved in the black liquor and are easily removed from pulp by washing. The polymerised alkyl hydrocarbons derived from fatty acids and fatty alcohols are assumed not to be eliminated to any extent by cooking or bleaching and therefore induce depositions of pitch. Long-term storage of woods that contain large amounts of unsaturated fatty acids and fatty alcohols may be unfavourable for pulping because of the formation of polymeric substances, which can cause pitch deposits (Ohtani *et al.*, 1986).

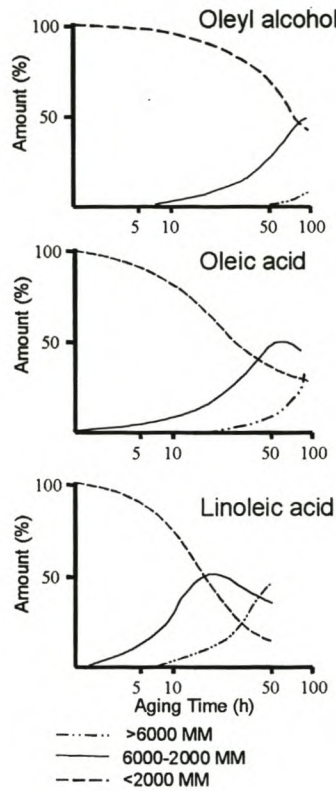


Figure 17 Changes in unsaturated fatty acids and alcohols during ageing (Ohtani *et al.*, 1986). Key: MM = molecular mass.

2.4.3.2 Metal soap deposition

The saponifiable triglycerides and other lipids break down into fatty and resin acids and alcohols in the extreme conditions of the digester. Under alkali conditions a resin or fatty acid tends to lose a hydrogen atom and becomes a soap anion. When binding with a metal ion it will form a soap (Equation 3).

Equation 3 The reaction of a metal ion with a soap anion (Allen, 1980)

$$M^{n+} + n\text{Soap}^{-} \rightarrow M\ \text{Soap}n\downarrow$$

(Metal Ion) (Soap Anion) (Metal Soap)

The resulting metal soap, which is sticky and insoluble, is sometimes found in relatively large concentrations in pitch deposits. The metal-to-soap bond appears to be strong, so that the reaction depicted in Equation 3 is effectively irreversible, even when moderately acidic conditions (e.g., pH 4) are created. Concentrations of metal ions and of fatty and

resin acids in mill white waters are usually high compared to other components. The metal ion that is most frequently involved in soap deposition in the kraft pulp mill, is calcium (Allen, 1988 b). A cationic polymer spray is available on the market to combat pitch deposition. It forms a complex with the anions and thus it hinders pitch deposition where it is applied (Aston *et al.*, 1992). The pitch particles are then fixated onto the fibres, thus reducing the concentration of pitch particles in the process water (Hassler, 1988).

The extent of the reaction described in Equation 3 is controlled by the presence or absence of soap anions in the white water and this, in turn, is controlled by pH. A pH of 6.5 and higher would result in a high concentration of anions. Allen (1988b) stated that, in all likelihood, the high temperature in the digester is responsible for a somewhat lower pH at which dissolution begins than would be found at lower temperatures. Metal soap deposition can constitute 30% of the deposited material in a part of a kraft mill where the pH is over 10 (Allen, 1988b). A mechanism for the formation of calcium soaps has been advanced where the soap anions are adsorbed onto calcium carbonate, which is also present in unbleached kraft pulps, and are then converted into calcium soaps. This mechanism is assumed to be more acceptable because the small amounts of dissolved calcium ion in unbleached kraft pulp appear to be unavailable for reaction because they are tied up with lignin (Douek and Allen, 1983).

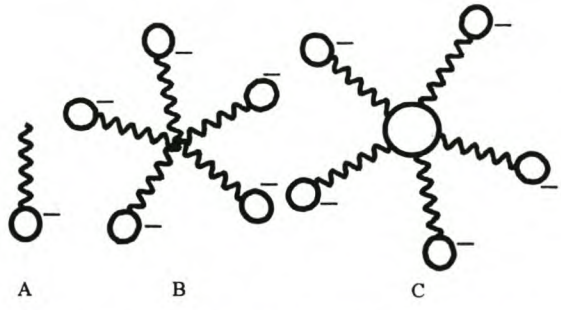


Figure 18 Schematic diagrams of: (a) a soap anion, (b) a micelle, (c) a micelle solubilising unsaponifiable material in its interior (Allen, 1988a).

If wood resin does not contain much unsaponifiable material the extent of pitch deposition is less. The saponifiable material becomes soaps which aid in removing the remainder of the resin, the unsaponifiable or non-soap portion, from the pulp. Figure 18a is a schematic representation of a soap molecule, in solution, with its hydrocarbon tail and carboxylate anion head group. Above a certain concentration, called the critical micelle concentration, these soap anions cluster together with their hydrocarbon tails on the inside and the head groups on the outside to form micelles (Figure 18b). The importance of this phenomenon, which always occurs in kraft digesters, is that these micelles are capable of solubilising some of the unsaponifiable material. The unsaponifiable material dissolves in the centres of the micelles (Figure 18c), where the hydrocarbon tails are concentrated. This phenomenon is the basis of detergency and is an important aspect of resin removal during kraft cooking. It is therefore appropriate to consider the ratio of saponifiables to unsaponifiables in the extractives of the wood. This is a good way to compare the deresination qualities of various different kinds of wood. It is estimated that a ratio of less than three will lead to poor deresination (Allen, 1988a). The waxes in hardwood resin are very difficult to saponify during alkaline pulping, but it can be enzymatically hydrolysed during storage of the wood. Thus, some of the unsaponifiables which are the most difficult to remove are hydrolysed during storage (Allen, 1988a).

2.4.3.3 Resin transfer from fibres

Resin transfer from fibres to equipment occurs when fibres in a flowing pulp suspension scrape or brush against the surfaces of equipment. In areas of intense hydrodynamic shear, such as wires and corners, resin is transferred from fibre surfaces. The deposited resin then flows along the surface to areas of less shear (Allen, 1980).

2.4.3.4 Fines and fibre deposition

When pressure is applied to a partially dried pulp such as at the press roll, it is possible for fines and fibres to adhere to the surfaces of the rollers because of the sticky resin on the surfaces involved (Allen, 1980).

2.4.3.5 Transfer of resin to the felts in press nips

In the press section, the white water is squeezed from the paper into the felt (Figure 19) and carries with it minor amounts of fibres, parenchyma cells, and dispersed resin. Deposition can occur during the felt dewatering process on the Uhle box lips (Allen, 1980).

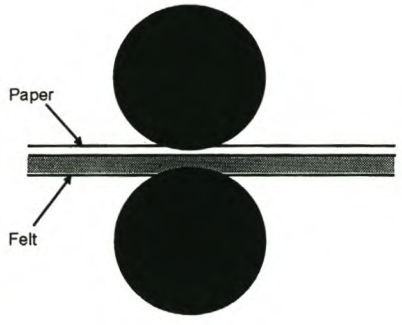


Figure 19 Transfer of resin from paper to felt in a press nip (Allen, 1980).

2.4.3.6 Deposition due to evaporation

Resin would be left behind when white water containing resin and water is evaporated. The resin will then form a deposit on the surface on which it was left behind. An example of this is the evaporation of water off the dryer rolls (Allen, 1980).

2.4.3.7 Deposition of resin due to film formation

The dispersed resin in a storage tank where the process liquid is not moving would emulsify and then rise to the surface of the tank. There it forms a ring on the tanks, which frequently breaks off and disintegrates to form deposits downstream in the equipment or on the finished product. Resin particles also attach themselves to foam or air bubbles rising from the bottom of the tank. Thus the resin is taken to the surface, also forming a ring, and the result is the same as previously mentioned (Allen, 1980).

2.4.3.8 Resin deposition in refiners and beaters

During the refining process (beater, refiner, etc.) the stock is subjected to a treatment severe enough to crush many parenchyma cells and emulsify the resin liberated both from the cells and from the resin pockets and canals. The resin then deposits inside this equipment and may break off later to cause problems downstream (Allen, 1980).

2.4.3.9 Temperature and pH fluctuations

Sudden temperature drops can decrease the solubility of pitch and cause it to precipitate. Changes in pH can also cause pitch to become less soluble and cause it to deposit in the machine system (Laubach and Greer, 1991).

2.4.3.10 Co-deposition of extraneous substances

Any material suspended in the pulp will adhere to a pitch deposit if it comes in contact with it. For example, ray cells, fibres, sand, bark, and ink particles from recycled newspaper all have a tendency to collect in pitch deposits, and these materials can on occasion affect the volume, stickiness, viscosity, and appearance of the deposit. Certain defoamers can cause deposition even in the absence of resin (Allen, 1980).

2.5 Triglyceride breakdown with lipase

Triacylglycerol acylhydrolase (EC 3.1.1.3) is also known as triacylglycerol lipase or only as lipase. It is an enzyme produced by humans and animals in the digestion canals as well as by microorganisms for the breakdown of mainly triglycerides, but also di- and monoglycerides. It can also cause ester synthesis in the presence of microamounts of water. Figure 20 illustrates all the reactions lipase can catalyse. Lipase enzymes are of common occurrence in fungi and are of two types: those for specific fatty acids chain length and those exhibiting positional specificity. The latter enzymes are further of two kinds. Type "a" hydrolyses a specific position in the acylglycerol molecule, whereas type "b" is non-specific and hydrolises all fatty acyl ester bonds (Chopra and Khuller, 1984). Lipase production is inducible in fungi by growing the organisms in a triglyceride-containing medium (Akhtar *et al.*, 1977, 1980). The enzyme may be membrane bound, as in *Saccharomyces cerevisiae* (Schousboe, 1976), or extracellular, as in *Penicillium roqueforti* (Lobyreva and Marchenkova, 1980) and *Rhizopus japonicus* (Aisaka and Terada, 1981).

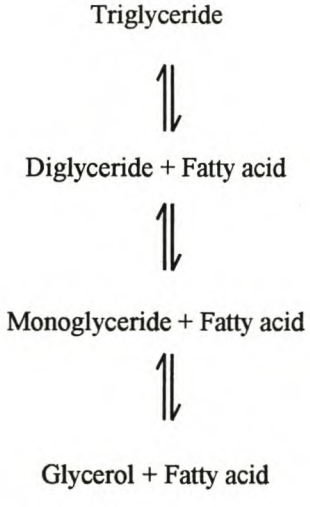


Figure 20 Reactions catalysed by lipase (Ghosh *et al.*, 1996).

Lipase is an important enzyme in industry serving as a biocatalyst for fat and oil processing in medicine, food additives, diagnostic reagents and detergents. The lipases used are usually of fungal or bacterial origin. Triglycerides have been shown to be major

contributors to pitch deposition (Allen, 1977 Fujita *et al.*, 1992; Suckling and Ede, 1990). Some success has been achieved in reducing the triglyceride content of wood, and therefore a reduction in pitch formation, by inoculating wood chips with white-rot fungi or by adding lipase to pulp (Blanchette *et al.*, 1992; Brush *et al.*, 1994; Fischer and Messner, 1992).

2.5.1 Lipase structure

Lipase, like any other enzyme, is a protein which consists of a single or a few polypeptide or amino acid chains, forming a complex folded structure. Twenty different amino acids exist and these form the polypeptide chains. The folds of the chain or chains are stabilised by disulphide bonds. The part of the enzyme that is responsible for its biocatalytic potential is called the catalytic site. Only a substrate molecule, like triglyceride in the case of lipase, which could fit into the enzyme conformation to get close enough to the catalytic site, can be attacked. The chemical mechanism of an enzyme is induced by only a few (e.g. three in the case of lipase) amino acids.

2.5.2 Hydrolysis reaction mechanism

The catalytic site of lipases usually consists of a Ser-His-Asp (serine, histidine, aspartic acid) amino acid triad (Brady *et al.*, 1990; Derewenda, 1994; Isobe and Nokihara, 1993; Jaeger *et al.*, 1993; Yamaguchi *et al.*, 1991). However, a Ser-His-Glu triad has also been identified for the lipase of the fungus *Geotrichum candidum* (Derewenda, 1994; Schrag and Cygler, 1993). The order in which Ser-His-Glu is written does not indicate any structural or chemical sequence.

Figure 21 shows the molecular structure of serine, histidine and aspartic acid. The chemically functional groups can catalyse the hydrolysis of ester bonds when the amino acids are positioned correctly, as in the case of lipase enzymes.

The following description of the mechanism of the catalytic triad is simplified, as it has to serve as a model for all lipases. The details about the angles and orientations of the different amino acids, substrate molecules, etc. differ for each type of lipase molecule.

The catalytic triad of serine proteases is chemically analogous to that of lipase and hydrolises its substrate by a mechanism very similar to that used by lipases (Brady *et al.*, 1990; Winkler *et al.*, 1990). Chymotrypsin is a serine protease. Figure 22 shows the mechanism of the chymotrypsin catalytic triad and the following discussion describes the mechanism with the help of this figure.

Some interactions between the catalytic triad are necessary to create the potential to hydrolise ester bonds. One of these is the hydrogen bond that is formed between the nucleophilic serine and histidine (Derewenda, 1994; Dressler and Potter, 1991) (Figure 22a). Another one of the vital interactions in any triad is that of the histidine imidazole (the ring structure on the side chain of histidine) and a carboxylic acid. Generally a hydrogen bonded Asp-His pair is found in the catalytic centers of a variety of enzymes (Figure 22a). The two bonded side chains assist in a nucleophilic attack during hydrolysis either directly by activating a serine hydroxyl (as in Figure 22a), a cysteine thiol, or a water molecule, or indirectly through a metal ion that polarizes the carbonyl attacked by a nucleophile (Derewenda, 1994). These two interactions appear to be necessities for the creation of a nucleophilic active site.

The net effect of these two interactions is that hydrogen is transferred to the histidine, so creating a nucleophilic serine. The nucleophilic serine then attacks the ester bond and forms a tetrahedral intermediate (Figure 22b). The tetrahedral intermediate then collapses (Figure 22c), the ester bond breaks up and an acyl-enzyme intermediate is formed (Figure 22d). Histidine then donates a hydrogen atom to the released part of the

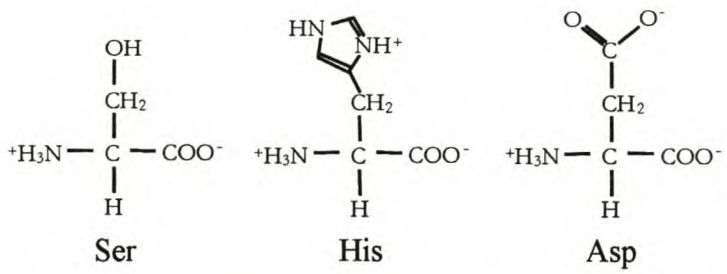


Figure 21 Serine, histidine and aspartic acid (Mathews and Holde, 1990).

substrate and returns to its original base state. Now the acyl-enzyme intermediate is broken down through the Asp-His couple which draws a hydrogen atom away from a nearby water molecule. A hydroxyl group is left over and it attacks the C=O group, a carboxyl group is formed and the remaining portion of the original substrate is released from the serine (Dressler and Potter, 1991; Scott and Sigler, 1994). It is not a necessity that an acyl-enzyme intermediate has to be formed, for in phospholipase A_2 a conserved water molecule hydrogen bonded to the catalytic histidine serves as the source of the nucleophile (Scott and Sigler, 1994).

A unique property of lipases is their action at oil-water interfaces, the presence of which greatly enhances their enzyme activity. This is called interfacial activation (Derewenda, 1994; Sarda and Desnuelle1958). However, a lipase from *Pseudomonas aeruginosa* has been characterized which does not need interfacial activation (Jaeger *et al.*, 1993). There is a definite structural difference between this lipase and the other lipases that do possess interfacial activation. Those that do have interfacial activation have a lid-like helical loop structure that covers the active site (Brady et al, 1990; Schrag and Cygler, 1993; Winkler et al, 1990). This loop is not present in *Pseudomonas aeruginosa* lipase (Jaeger *et al.*, 1993). In the right conditions of an oil-water interface, the loop would open up and thus expose the active site (Schrag and Cygler, 1993).

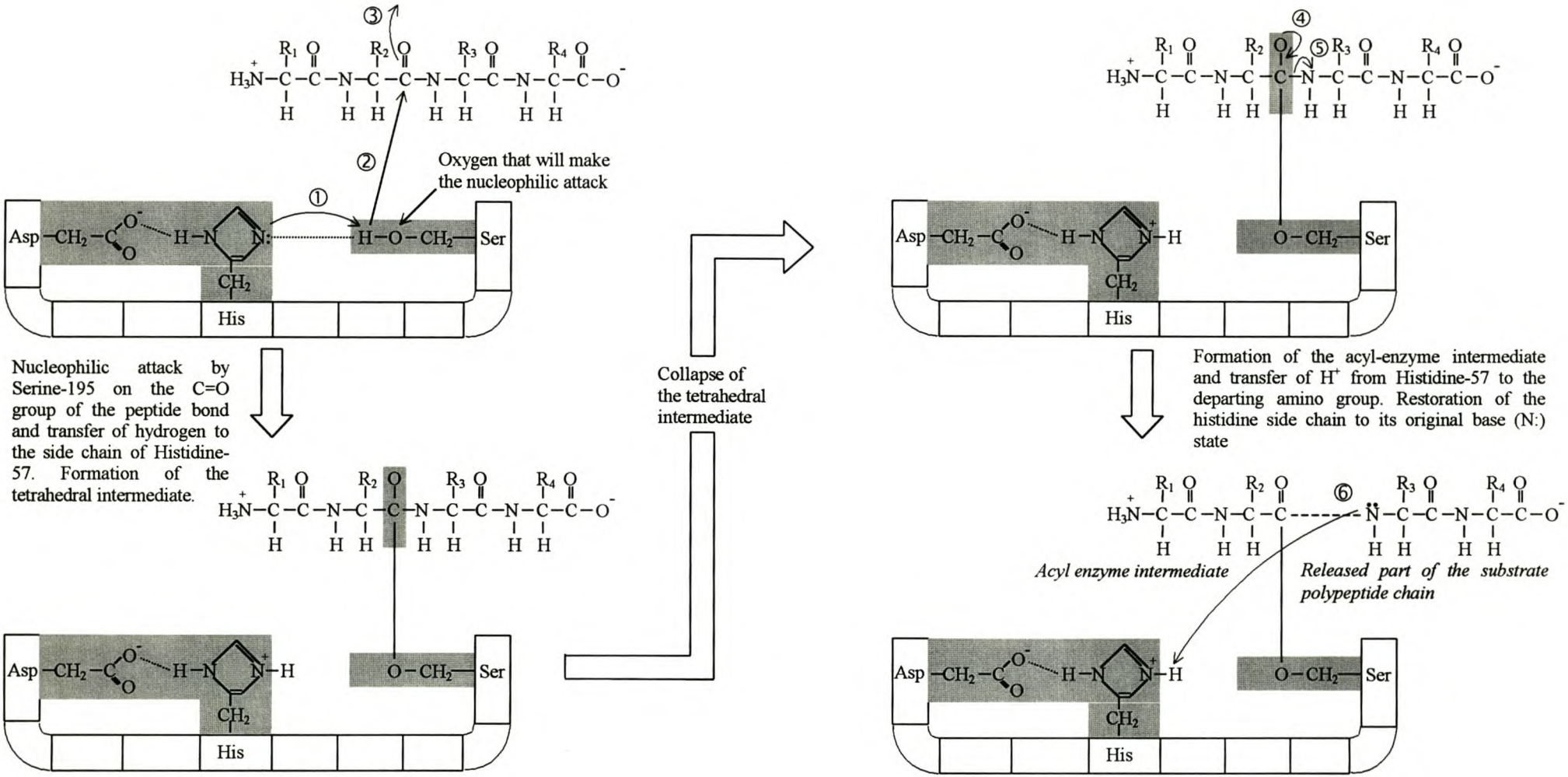


Figure 22 The role of the catalytic triad in the hydrolysis of a peptide bond by chymotrypsin. The diagram shows the involvement of acid-base catalysis in the first stage of the cleavage reaction, in which the acyl-enzyme intermediate is formed. The sequential numbering of the arrows is intended to make it easier to trace the logic of the reaction, although, in reality, the electron rearrangements may occur in a concerted way so that the various steps in the reaction occur simultaneously (Dressler and Potter 1991).

2.5.3 Homology of lipases from different species

The realisation of homology (similarity) in the gene and amino acid sequences of different species is vital for recombinant DNA technology. Tables 10 to 12 show the homology in the amino acid sequences around the active Ser residue, the active Asp residue, and the oxyanion hole. The oxyanion hole is a structurally and functionally important region around the catalytic Serine residue, which is necessary for enzymatic activity. It increases the affinity of the enzyme for lipid substrates and helps stabilise the transition state intermediate during catalysis (Winkler *et al.*, 1990). It is a strictly conserved structural element in lipases, which is formed by a region that contains a central Glycine residue (Jaeger *et al.*, 1993). Table 8 gives the names and one- and three-letter symbols of all the amino acids in order to simplify Table 9, Table 10 and Table 11. The use of shading indicates the degree of homology.

Table 8 The amino acids and their symbols (Sambrook *et al.*, 1989)

Amino acid	*One-letter symbol*	*Three-letter symbol*
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Thr
Tyrosine	Y	Tyr
Valine	V	Val

Table 9 A comparison of amino acid sequences of lipases from different origin. Homology is shown for regions around the catalytic Ser residue (Jaeger *et al.*, 1993)

SOURCE	*Sequence homologies of lipases around the active site Ser (S = Ser)*																		
Pseudomonas aeruginosa	L	S	G	Q	P	K	V	N	L	I	G	H	S	H	G	G	P	T	I
P. alcaligenes	I	S	G	K	G	K	V	N	L	V	G	H	S	H	G	G	L	T	S
P. glumae	A	T	G	A	T	K	V	N	L	V	G	H	S	Q	G	G	L	S	S
P. cepacia	A	T	G	A	T	K	V	N	L	V	G	H	S	Q	G	G	L	S	S
P. fragi	Q	V	G	A	Q	R	V	N	L	I	G	H	S	Q	G	A	L	T	A
P. fluorescens	G	L	S	G	K	D	V	L	V	S	G	H	S	L	G	G	L	A	V
Bacillus subtilis	E	T	G	A	K	K	V	D	I	V	A	H	S	M	G	G	A	N	T
B. pumilus	K	T	G	A	K	K	V	D	I	V	A	H	S	M	G	G	A	N	T
Staphylococcus aureus	W	E	P	G	K	K	V	H	L	V	G	H	S	M	G	G	Q	T	I
S. hyicus	W	K	P	G	H	P	V	H	F	I	G	H	S	M	G	G	Q	T	I
Rhizomucor miehei	Q	Y	P	S	Y	K	V	A	V	T	G	H	S	L	G	G	A	T	A
Rhizopus delemar	A	H	P	T	Y	K	V	I	V	T	G	H	S	L	G	G	A	Q	A
R. niveus	A	H	P	T	Y	K	V	I	V	T	G	H	S	L	G	G	A	Q	A
Penicillium camenbertii	Q	N	P	N	Y	E	L	V	V	V	G	H	S	L	G	A	A	V	A
Geotrichum candidum	G	G	D	P	D	K	V	M	I	F	G	E	S	A	G	A	M	S	V
Candida rugosa	G	G	D	P	T	K	V	T	I	F	G	E	S	A	G	S	M	S	V

Table 10 A comparison of amino acid sequences of lipases from different origin. Homology is shown for regions around the oxyanion hole (Jaeger *et al.*, 1993)

SOURCE	*Sequence homologies of lipases around the oxyanion hole (G = Glycine)*														
Pseudomonas aeruginosa	Y	P	I	V	L	A	H	G	M	L	G	F	D	N
P. nov. sp.	Y	P	I	V	L	A	H	G	M	L	G	F	D	N
P. alcaligenes	Y	P	I	V	L	T	H	G	M	L	G	F	D	S
P. glumae	Y	P	V	I	L	V	H	G	L	A	G	T	D	K
P. cepacia	Y	P	I	I	L	V	H	G	L	S	G	T	D	K
P. fragi	Y	P	I	L	L	V	H	G	L	F	G	F	D	R
P. fluorescens	E	I	G	I	G	F	R	G	T	S	G	P	R	E
Bacillus subtilis	N	P	V	V	M	V	H	G	I	G	G	A	S	F
B. pumilus	N	P	V	V	M	V	H	G	I	G	G	A	S	Y
Staphylococcus aureus	Y	P	V	V	F	V	H	G	F	L	G	L	V	G
S. hyicus	D	P	F	V	F	V	H	G	F	T	G	F	V	G
Rhizomucor miehei	T	I	Y	I	V	F	R	G	S	S	S	I	R	N
Rhizopus delemar	T	I	Y	L	V	F	R	G	T	N	S	F	R	S
R. niveus	T	I	Y	L	V	F	R	G	T	N	S	F	R	S
Penicillium camenbertii	A	V	V	L	A	F	R	G	S	Y	S	V	R	N
Geotrichum candidum	P	V	M	V	W	I	Y	G	G	A	F	V	Y	G
Candida rugosa	P	V	M	L	W	I	F	G	G	G	F	E	V	G

Table 11 A comparison of amino acid sequences of lipases from different origin. Homology is shown for regions around the catalytic Asp residue (Jaeger *et al.*, 1993)

SOURCE	*Sequence homologies of lipases around the active site Asp (D = Asp)*												
Pseudomonas aeruginosa	T	A	N	D	G	L	V	G	T	C	S	S	H
P. nov. sp.	T	A	N	D	G	L	V	G	T	C	S	S	H
P. alcaligenes	E	P	N	D	G	L	V	G	R	C	S	S	H
P. glumae	G	Q	N	D	G	L	V	S	K	C	S	A	L
P. cepacia	G	Q	N	D	G	L	V	S	K	C	S	A	L
P. fragi	R	E	N	D	G	M	V	G	R	F	S	S	H
Bacillus subtilis	S	S	A	D	M	I	V	M	N	Y	L	S	R
B. pumilus	S	S	A	D	M	I	V	M	N	Y	L	S	R
Staphylococcus aureus	R	K	N	D	G	V	V	P	V	I	S	S	L
S. hyicus	R	P	N	D	G	L	V	S	E	I	S	S	Q
Rhizomucor miehei	N	E	R	D	I	V	P	H	L	P	P	A	A
Rhizopus delemar	H	K	R	D	I	V	P	H	V	P	P	Q	S
R. niveus	H	K	R	D	I	V	P	H	V	P	P	Q	S
Penicillium camenbertii	H	T	N	D	P	V	P	K	L	P	L	L	S
Geotrichum candidum	Q	E	D	E	G	T	A	F	A	P	V	A	L
Candida rugosa	Q	N	D	E	G	T	F	F	G	T	S	S	L

These sequences show some homology around the catalytic site. Especially around the active nucleophile, the Serine residue, homology is clear with the G-X-S-X-G sequence present in all the sequences. The oxyanion hole also has a Glycine residue, which is present in all the sequences listed. The sequences around the Aspartic acid residue also show homology, especially with the Aspartic acid itself, which is replaced by Glutamic acid in *Geotrichum candidum* and *Candida rugosa*. The active group of Glutamic acid is precisely the same as that of Aspartic acid; the only difference is in the chain length. Thus one can expect the Glutamic acid to fulfil the same role.

The full amino acid sequences of the lipases of 14 organisms were compared by multiple alignment to produce a dendrogram (Figure 23). The dendrogram indicates how closely related the lipases from the different organisms are. It is evident from this dendrogram that the lipases are not closely related in respect to their whole amino acid sequences. The number of amino acid residues of the lipases from these organisms varies from 173 to 679. *Thermomyces lanuginoses* is a thermophilic fungus that has also been isolated in South Africa and is mentioned in chapters 4 and 5 of this thesis. It is a thermophilic fungus and from the dendrogram it is clear that its lipase is not closely related to the lipase of any of the other organisms.

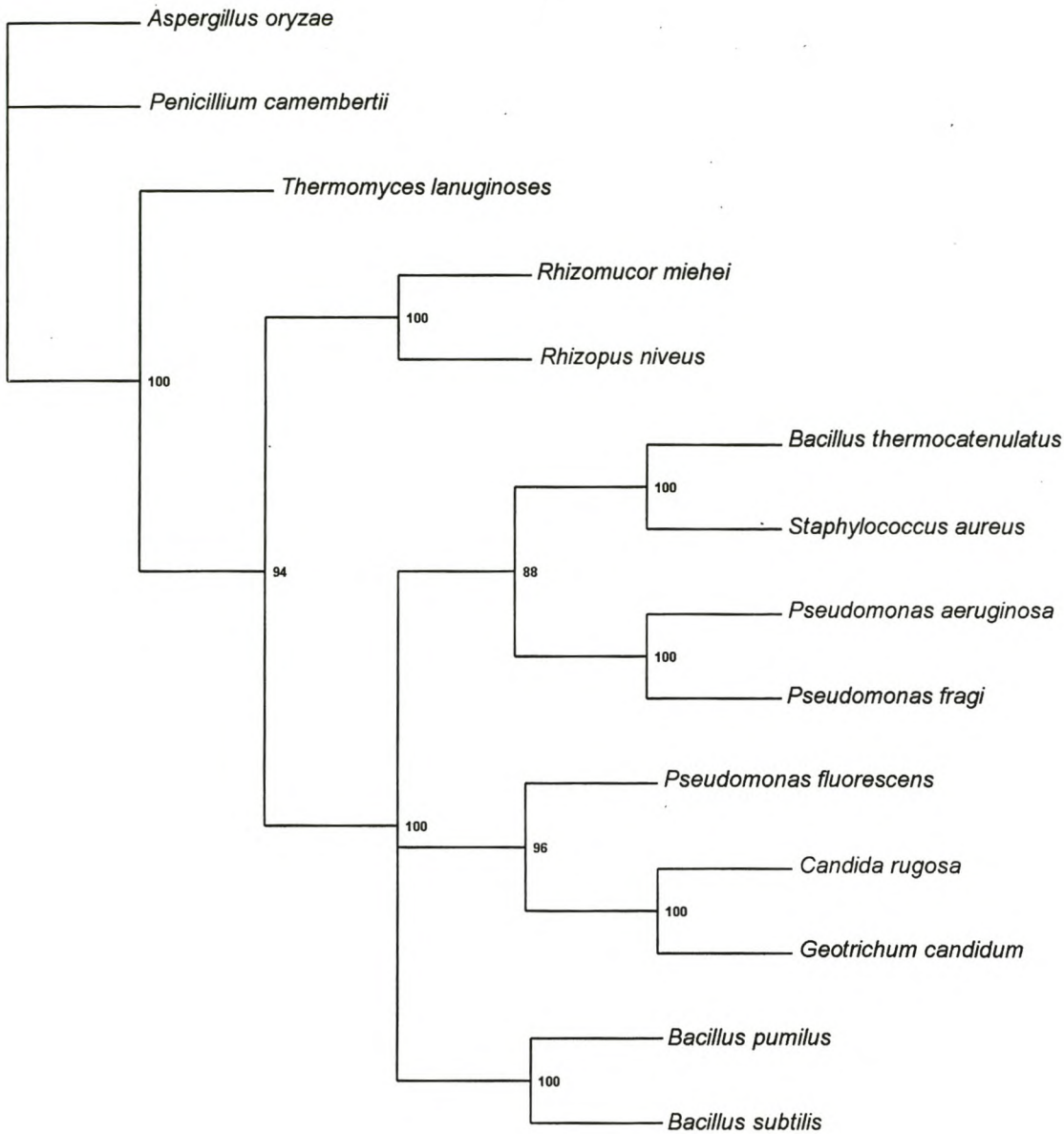


Figure 23 A dendrogram constructed from the multiple alignment of sequences of lipases from 14 different organisms. The figure at each node indicates the distance to the previous node (root node of that node). The sequences were obtained from the internet websites of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the National Biomedical Research Foundation (http://www-nbrf.georgetown.edu/).

2.5.4 Conditions for optimal lipase activity

Most lipases possess interfacial activation and have an affinity for hydrophobic surfaces since their substrates are encountered mostly in partially or fully emulsified state. The best stimulant of lipase activity is the main substrate, triglycerides. Table 12 presents a

comparison of optimal conditions for lipase activity and stability of different fungal species.

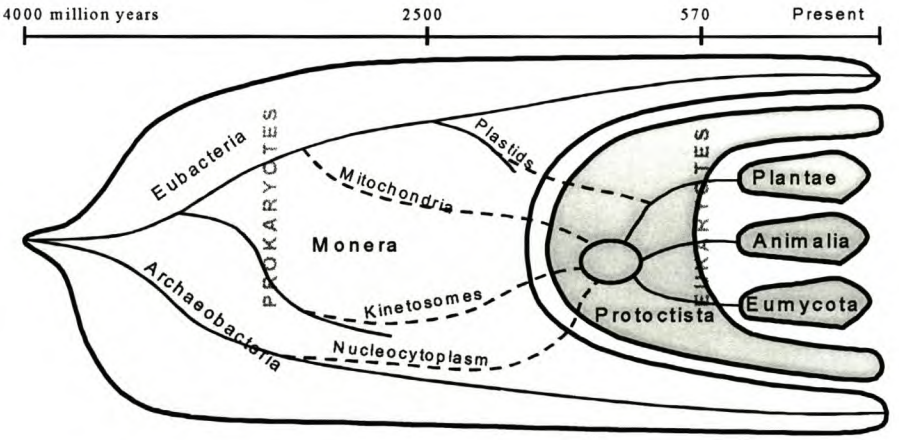
Table 12 A comparision of optimal conditions for lipase activity and stability of different fungal species

Species	*pH*	*Temp. (°C)*	*Stability*		*Lipid substrate*	*Reference*
			pH	*Temp. (°C)*		
Candida antarctica		70				Coulon *et al.*, 1997
Trichosporon asteroides	5	60	3-10	<70		Dharmsthiti et al, 1997
Candida rugosa	6.7	40-50			TG with butyric acid	Marangoni, 1994
Emericella rugulosa	8	45				Venkateshwarlu and Reddy, 1993
Humicola sp.	8	45				Venkateshwarlu and Reddy, 1993
Thermomyces lanuginoses	8	45				Venkateshwarlu and Reddy, 1993
Penicillium purpurogenum	8	45				Venkateshwarlu and Reddy, 1993
Chrysosporium sulfureum	8	45				Venkateshwarlu and Reddy, 1993
Penicillium citrinum	8	34-38			1% Olive oil	Pimentel *et al.*, 1994
Ophiostoma piceae	5.5	37			Olive oil	Gao and Breuil, 1995
Penicillium caseicolum	9	35			TG with short chain FA	Alhir *et al.*, 1990
Calvatia gigantea	7	30				Christakopoulos *et al.*, 1992
Byssochlamys fulva	8.5	25			Maize oil	Ku and Hang, 1994
Alternaria brassicicola	9	25			TG with long chain FA	Berto *et al.*, 1997
Microdochium nivale		20				Hoshino *et al.*, 1996
Aspergillus niger	7.5					Pabai *et al.*, 1995
Rhizopus oryzae	7.5					Pabai *et al.*, 1995

2.6 Wood-decay fungi: classification and characteristics

The development of the electron microscope in the 1950s expanded the dimensions of the microscopic realm a thousandfold and led to the discovery of two very different cell types, prokaryotic and eukaryotic cells. Prokaryotic cells exhibit no mitosis or cytoplasmic streaming and have no membrane-bound organelles or organised nuclei. Eucaryotic cells are larger and are present in the higher life forms. These cells divide by mitosis, exhibit cytoplasmic streaming, and have organised nuclei with double membranes, mitochondria, and plastids (Zabel and Morrell, 1992). Figure 24a shows the historical development of the living kingdoms.

(A)



(B)

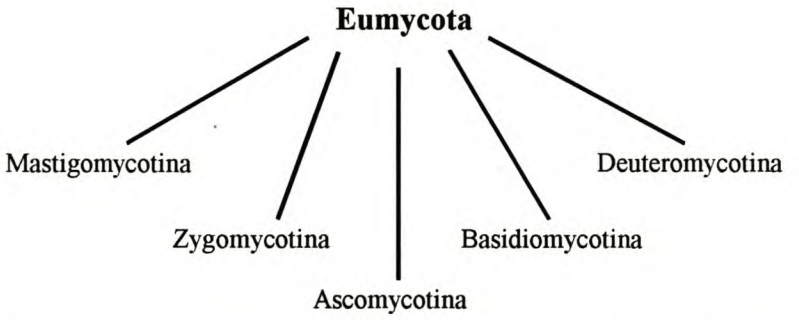


Figure 24 (a), The history of the living kingdoms (Kendrick, 1992) and (b), the classification of Eumycota (Zabel and Morrell, 1992).

Additional evidence clearly established the distinctiveness of fungi from plants based on differences in cell-wall composition, heterotrophy and the external mode of digestion

(Lindenmayer, 1965). Eumycota include primarily the filamentous fungi which incorporates wood-decay fungi. Fungal classification is based primarily on differences in the types of the reproductive structures. Currently there are five subdivisions of Eumycota, which are given in Figure 24b (Zabel and Morrell, 1992). The classification of wood-decay groups is done firstly on the sequence of cell-wall constituents utilised or altered. Secondary emphasis is placed on the mode of hyphal penetration of fibres and tracheids and the types of tissue damaged (Zabel and Morrell, 1992). Table 13 lists the major types of wood inhabiting micro-organisms as well as their anatomical and chemical features.

Table 13 A summary of the anatomical and chemical features of the major types of wood inhabiting micro-organisms (Zabel and Morrell, 1992)

Wood-inhabiting micro-organisms	*Cell-wall constituents used*	*Anatomical features*	*Causal agents*
		Decayers	
Simultaneous white-rots	All	Cell walls attacked progressively from lumen surface	Basidiomycotina Some Ascomycotina
Sequential white-rots	All, but hemicelluloses and lignin used selectively initially	Cell walls attacked progressively from lumen surface	Basidiomycotina Some Ascomycotina
Brown-rots	Carbohydrates, but lignin modified	Entire wall zone attacked rapidly	Basidiomycotina
Type 1 soft-rots	Carbohydrates	Longitudinal bore holes develop in secondary wall	Ascomycotina Deuteromycotina Some bacteria
Type 2 soft-rots	Carbohydrates	Secondary wall erosion from lumen surface	Ascomycotina Deuteromycotina Some bacteria
		Nondecayers	
Sapstainers	Wood extractives	Primarily invade parenchyma cells in sapwood	Ascomycotina Deuteromycotina
Molds	Wood extractives	Surface growth on wet wood	Ascomycotina Deuteromycotina Zycomycotina
Scavengers	Wood extractives and decay residues	Penetrate wood cells primarily through pits	Bacteria Ascomycotina Deuteromycotina Zycomycotina Basidiomycotina

The dominant available carbon sources in wood are cellulose, lignin and hemicelluloses, which are also the major structural components of wood. The fungi that decay the cell walls are divided into three classes; white-rot, brown-rot and soft-rot fungi (Cooke and Rayner, 1984).

2.6.1 Molds

Molds are capable of degrading wood extractives and grow best on wood that is very wet or that has been exposed to very high humidity for a long time. On softwoods molds mainly grow on wood surfaces. On hardwoods molds can enter the wood at exposed parenchyma, vessels, and ruptured cells and can move throughout the wood by rupturing pit membranes (Messner *et al.*, 1992). Table 14 shows the effect of various molds on the dichloromethane extractive content of non-sterile southern yellow pine when incubated at room temperature for two weeks (Farrell *et al.*, 1997). *Phlebia roqueforti* reduced the extractive content substantially more than the other fungal species.

Table 14 Dichloromethane extractive content of non-sterile southern yellow pine treated with various molds (Farrell *et al.*, 1997)

Fungal species	*Extractives (%) Control*	*Extractives (%) Treatment*	*Reduction (%)*
Phlebia roqueforti	3.34	2.16	35
Leptographium terrebrantis	2.27	1.92	15
Verticicladella truncata	2.27	1.96	14
Diplodia pinea	2.27	2.02	11
Codinaea sp.	2.27	2.07	9
Aureobasidium pullulans	3.34	3.26	2

2.6.2 Sap-stain fungi

This group is characterised by pigmentaion of the hyphal walls, which causes discolouration of infected sapwood (Levy and Dickinson, 1981). The discolouration may cause considerable reduction of the market value of wood (Tsoumis, 1991). Sap-stain fungi rapidly colonise the sapwood of logs and wood chips. These fungi grow mainly in ray parenchyma cells and are capable of deeply penetrating logs and wood chips. They can penetrate simple and bordered pits. Sap-stain fungi are not capable of degrading the major components of the wood cell wall, cellulose and lignin. Hemicelluloses are degraded to a very slight degree. Extractives and simple sugars found in the parenchyma

cells are the major nutrient source for these fungi. Sap-stain fungi cause a characteristic staining of sapwood, resulting in a blue, black, grey, or brown discoloration of the wood (Farrell *et al.*, 1997).

A variety of sap-stain fungi were screened for their ability to degrade wood extractives (Farrell *et al.*, 1997). Sterile southern yellow pine was inoculated with the sap-stain fungi listed in Table 15 and incubated for two weeks. *Ceratocystis adiposa* and *Ophiostoma piliferum* reduced the dichloromethane extractives significantly.

Table 15 Dichloromethane extractive degradation by sap-stain fungi on sterile southern yellow pine (Farrell *et al.*, 1997)

Fungal species	*Extractives (%) Control*	*Extractives (%) Treatment*	*Reduction (%)*
Ceratocystis adiposa	2.13	1.26	41
Ophiostoma piliferum	3.34	2.27	32
Ceratocystis adjuncti	1.98	1.44	27
Ceratocystis minor	2.13	1.57	26
Ophiostoma piceae	2.13	1.57	26
Ophiostoma populina	2.13	1.62	24
Ophiostoma abiocarpa	2.13	1.61	24
Ceratocystis tremuloaura	2.13	1.65	20
Ophiostoma fraxinopennsylvanica	2.13	1.71	20
Ophiostoma plurianulatum	2.13	1.73	19
Europhium aereum	1.98	1.61	19
Ceratocystis ponderosa	2.19	1.86	18
Ceratocystis penicillata	1.98	1.58	15
Ophiostoma olivaceum	2.27	1.93	15
Europhium clavigerum	2.13	1.84	14
Ceratocystis hunti	2.13	1.89	11
Ceratocystis ambrosia	2.13	1.92	10
Ophiostoma distortum	2.27	2.07	9
Europhium robustum	2.27	2.06	9
Cladobotryum virescens	2.27	2.11	7
Ophiostoma galeiformis	2.13	1.98	7
Ceratocystis coerulescens	2.13	2.13	0
Ophiostoma dryocetidis	2.24	2.27	0
Ophiostoma stenoceras	2.13	2.21	0
Xylaria. conudamae	2.44	3.16	0
Xylaria hypoxylon	2.44	2.88	0

2.6.3 White-rot fungi

White-rot fungi are able to attack and metabolise all major wood constituents. They are unique among most microorganisms in their capacity to depolymerise and metabolise lignin. The major components are used in varying orders and rates by different white-rot fungi, suggesting that these fungi probably represent a heterogeneous group with widely varying enzymatic capabilities (Zabel and Morrell, 1992). These differences were used to

group white-rot fungi into the simultaneous rotters, which utilised the components uniformly, and white-rotters, which utilised lignin initially more rapidly than cellulose (Liese, 1970). Table 16 shows that white-rot fungi like *Phanerochaete chrysosporium* not only attacks lignin, but also reduces the dichloromethane extractives content of southern yellow pine.

All cell-wall components are presumably ultimately consumed, with the exception of the minor minerals. There is considerable variation in the sequence and rate of component utilisation by both species and fungal strains within a species. Weight losses may approach 95-97% of the original wood material when prolonged exposures under optimal decay conditions prevail. At all stages of decay, the residual wood has a low solubility in 1% sodium hydroxide, suggesting that the breakdown products of decay are utilised by the fungi as rapidly as they are released. The cellulose, hemicelluloses and lignin remaining in the undecayed portions of the wood appear to be essentially unaltered, suggesting that white-rot fungi concentrate their attack on exposed cell-wall surfaces. Thus the enzymes slowly erode their way into the cell walls from lumina surfaces (Zabel and Morrell, 1992).

Table 16 Dichloromethane extractive content of sterile southern yellow pine treated with various Basidiomycetes (Farrell *et al.*, 1997)

Fungal species	*Control extractives (%)*	*Treatment extractives (%)*	*Reduction (%)*
Phanerochaete chrysosporium	2.19	1.30	41
Phanerochaete subacida	3.34	2.01	40
Phanerochaete gigantea	3.34	2.03	39
Phanerochaete tremellosa	1.98	1.21	39
Hyphodontia setulosa	1.98	1.20	39
Coriolus versicolor	1.98	1.28	36
Inonotus rheades	3.34	2.18	34
Trichaptum abietinium	4.70	3.13	33
Ceriporiopsis subvermispora	3.34	2.18	29
Trichaptum biforme	4.70	3.13	24
Schizophylum commune	2.50	2.03	17
Sistotrerma brinkmanii	2.44	2.17	11
Pleurotus ostreatus	2.44	2.30	6
Alurodiscus sp.	2.44	3.70	0
Ganoderma collosum	2.29	1.75	0
Phellinus igniarius	2.44	2.48	0

2.6.4 Brown-rot fungi

Brown rot fungi primarily decompose the cell-wall carbohydrates, leaving behind a modified, demethoxylated lignin residu. The hemicelluloses are removed more rapidly than cellulose by brown-rot fungi in the early decay states (Zabel and Morrell, 1992). Brown rots differ from white rots in the extensive depolymerisation of the carbohydrates in the secondary wall early in the decay process (Kirk and Highley, 1973). Whereas lysis zones are closely associated with white-rot hyphae, substantial cell-wall damage at distances up to several cell widths has been noted for some brown rots (Eriksson *et al.*, 1990). Figure 25 shows the mode of cell-wall attack for brown-rots, as well as white-rots and two types of soft-rot.

All carbohydrates are ultimately consumed, leaving a residuum of modified lignin in the cell wall. Large increases in water solubility and solubility in 1% NaOH occur at early decay stages, owing to the rapid carbohydrate depolymerisation. Brown-rot fungi appear

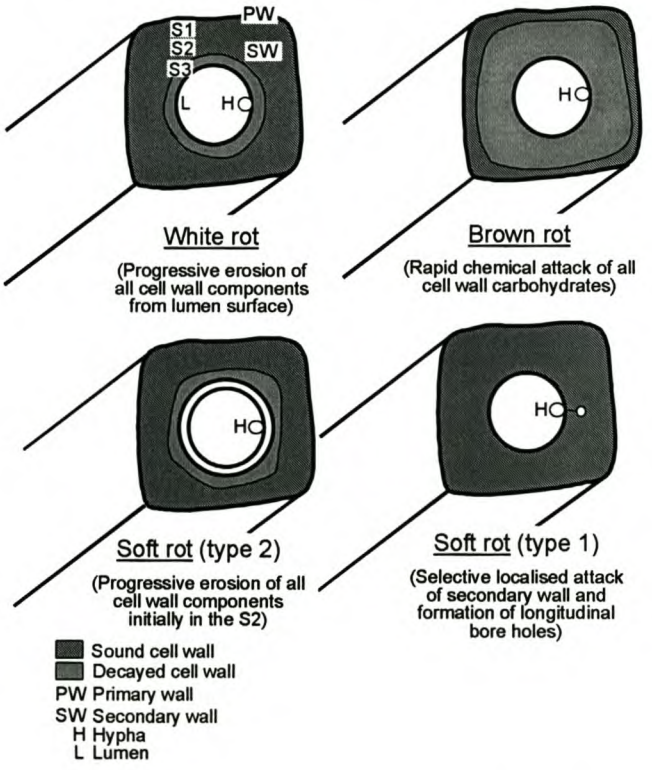


Figure 25 Diagrams showing the various modes of cell-wall destruction for white-rots, brown-rots, and the two types of soft-rots (Zabel and Morrell, 1992).

to depolymerise wood in the early decay stages much more rapidly than the decay products can be metabolised. The decay process rapidly involves the S1 and S2 layers of the cell walls, but develops irregularly. There appears to be much less variation in the sequential attack of cell-wall constituents by brown-rot fungi as compared to the white-rotters (Zabel and Morrell, 1992).

2.6.5 Soft-rot fungi

Soft-rot fungi display considerable variation in their effects on cell-wall constituents during decay development. For many species, the principle targets are the carbohydrates, whereas lignin attack is limited to minor demethoxylation (Zabel and Morrell, 1992). Some soft-rotters, however, selectively remove more lignin than carbohydrates from coniferous wood, in a manner similar to that of some white-rotters (Eslyn et al, 1975).

Wood decayed by soft-rot fungi resemble that of white-rot-degraded wood in having low alkali solubility, indicating that the degradation products are used at the same rate as they are released (Zabel and Morrell, 1992). Initially, soft rots were defined by the formation of unique longitudinal bore holes in the secondary cell wall, however, it was determined that some non-Basidiomycotina eroded the secondary wall in a manner similar to that of some white-rotters. Type 1 soft-rot fungi are able to degrade crystalline cellulose as reflected by the formation of characteristic cavities in the S2 zone of the secondary wall. The cell-wall eroders (hyphae lying on the S3 layer) are the Type 2 soft-rot fungi (Corbett, 1965). In conifers, the S3 zone of the secondary wall is resistant to soft-rot attack, but delignification substantially increases decay susceptibility and may shift the fungus from the cavity formation (Type 1) to the wall erosion (Type 2) mode (Morrell and Zabel, 1987).

2.6.6 Hyphal growth and cell wall composition

Fungi, like plants, possess firm cell walls that provide the rigidity needed for the penetration of plant cell walls. Structurally, fungal cell walls consist of an inner network of microfibrils embedded in an amorphous matrix that also forms the outer layers or often lamellae of the wall (Burnett, 1979). Chemically the walls consist of 80 to 90% polysaccharides, with the remainder composed of proteins and lipids (Zabel and Morrell,

1992). Chitin is the principal skeletal framework material (Burnett, 1979). Chitin, cellulose, and in a few cases chitosan form the microfibrils that serve as the skeletal framework of the walls (Zabel and Morrell, 1992). Table 17 shows the lipid composition of various fungi, indicating the total lipid content as well as the contribution of different lipid classes. According to this data the lipid composition of the biomass could be as little as 3% and as much as 75% of the total dry weight.

Table 17 Lipid composition of selected fungi (Lösel, 1988)

Fungi	*Total lipid (% of Wd)*	*Percentage of total lipids*								
		NL	*PL*	*PH*	*GL*	*FFA*	*TG*	*DG*	*ST*	*STE*
Ascomycotina										
Penicillium chrysogenum										
carbon source: D-glucose	12.7									
D-fructose	18.7									
sucrose	19.0									
Aspergillus niger	4.0	31.3	68.7	40.9	27.2		4.7	1.9	19.1	3.7
Humicola lanuginosa 37°C	75.1	84.4	15.6			41.0	45.6	5.5	3.0	4.9
52°C	8.5	84.9	15.1			5.8	45.0	31.3	7.7	10.2
45°C	17.0									
Sclerotium rolfsii mycelium	10.0			40.0		40.0	20.0			
Basidiomycotina										
Agaricus bisporus mycelium	5.1	68.1	20.7		2.0	10.4	13.3		3.3	1.7
Armillariella mellea	11.7					46.5				
Boletus edulis	13.4					26.3				
Cantherellus cibarius		48.6	51.4	49.6	1.7					
Lactarus trivialis	12.2					23.3				
Lepista nuda mycelium										
3 days	15.8	80.0	20.0	18.0						
4 days	20.0									
6 days	30.6									
12 days	47.7									
Polyporus ovinus	10.3					34.0				
Rhizoctonia solani mycelium	2.9	16.3		32.7						
Pisolithus tinctorius mycelium										
Tilletia controversa mycelium	5.8					48.0				
Ustilago zeae mycelium										
14 days, 5% sucrose	27.8									
22% sucrose	7.0									

NL, neutral lipids; PL, polar lipids; PH, phospholipids; GL, glycolipids; FFA, free fatty acids; TG, triacylglycerols; DG, diacylglycerols; ST, sterols; STE, sterol esters.

Hyphal growth occurs primarily by apical extension at the tips (Burnett, 1979). Vacuoles usually develop a few cells behind the hyphal tip, and the related turgor pressure within the cell protoplasts is the presumed driving force extending the plastic tip. Hence, some stain fungi are able to penetrate thin silver or aluminum foils. Hyphal branching is common and usually begins early behind the developing hyphal tips. The cytoplasmic

streaming appears to move material steadily toward the tips, leaving empty or vacuolated hyphae behind (Zabel and Morrell, 1992). Hyphae grow longitudinally along the lumina surface and pass from cell to cell through the pits (Levy and Dickinson, 1981). Growth rates longitudinally through the wood, under ideal conditions such as wood blocks in decay chambers, approach growth rates on malt-agar medium. No correlation is evident between the abundance of hyphae in cells and decay severity. The penetration modes of wood-inhabiting fungi are probably primarily enzymatic for the decay fungi that often form bore holes in the size range of hyphal diameters and primarily mechanical for the Type 2 soft-rotters (Zabel and Morrell, 1992).

2.7 Fungal growth in wood

There are several accounts on the experimental biotechnological trials of fungi on wood (Brush *et al.*, 1994, Chen *et al.*, 1994, Farrell *et al.*, 1994, Fischer *et al.*, 1995, Wang *et al.*, 1995). The degree of and method of colonisation of wood, especially wood in the form of chips or chip piles, is relevant when considering biotechnological applications. The factors influencing the colonisation of wood by fungi are the physical and chemical properties of the wood as well as the microclimate (Rayner and Boddy, 1988).

2.7.1 Nutrient sources in wood for fungi

Soluble sugars, lipids and peptides, together with the major storage compound starch, provide the main supply of readily assimilable carbon sources for fungi growing in wood. They enable development of certain microfungi such as those causing blue stain in conifers, which can not degrade cell-wall components, but are rapidly depleted during fungal colonisation. The dominant available carbon sources for fungi growing in wood are structural cell-wall components (Cooke and Rayner, 1984). The major types are cellulose, hemicelluloses and lignin. The different enzymatic affinities of fungi for these three major structural components of woody cell walls at least partially explain the three characteristically different decay types known as white-rot, brown-rot and soft-rot. Nitrogen, phosphorous and potassium are important minerals for fungal growth (Rayner and Boddy, 1988).

The presence of extractives can affect the performance of fungi in three ways: they can serve as carbon sources, they can be inhibitory or they may serve as growth stimulants. Variations between fungi in relation to these effects may be a highly significant determinant of selectivity towards different timber types (Rayner and Boddy, 1988). As the heartwood of some trees (e.g. *Eucalyptus*) ages, there is a progressive breakdown of toxic extractives to a less toxic form, with concentrations of inhibitory compounds being least in inner heartwood (Rudman, 1964, 1965). Extractives are found largely in the lumen of parenchyma cells but can also be found in the lumen of vessels, fibres and sometimes in specialised cells. They can also form coatings on the cell wall, in pits and in capillaries in the cell wall (Rayner and Boddy, 1988).

2.7.2 Factors influencing colonisation of wood

2.7.2.1 Physical and chemical properties of wood

To gain access to the nutrient sources that are held in the woody cell walls in a tree or branch the fungus first has to gain entry to the wood itself and secondly use natural passages in the wood. When colonising chips the fungus does not have to find ways to gain entry to the wood itself for the wood is already unobstructed. The fungus can achieve rapid axial access. The nature, distribution and size of the wood passages, and the interconnections (pits) that occur between them are crucial in determining patterns of colonisation. Fungal hyphae are capable of directly penetrating woody cells by their capacity for exertion of forward mechanical pressure and extracellular enzyme action, however the natural tendency is to move along avenues of least resistance. The natural pathways are provided by the axial and radial cells that are connected by pits (Rayner and Boddy, 1988).

In hardwoods the larger vessels provide the most effective channels, so that their size, number and distribution are important in determining invasion patterns (Cooke and Rayner, 1984). In diffuse-porous woods, like *Eucalyptus spp.*, penetration is limited during the early stages of colonisation when compared to ring-porous woods (Rayner and Boddy, 1988). Since the wood is already easily accessible in the chip form this feature of a specific wood specie would once again not play a significant role. The size, structure and distribution of pits are significant factors affecting the accessibility of woody tissues to fungal hyphae. When the pits are aspirated it reduces permeability (Siau, 1995), so that the breakdown of pit-closing membranes represent an early priority during fungal colonisation (Cooke and Rayner, 1984). Axial and ray wood parenchyma, with their relatively unthickened walls and large lumina, together with the presence of assimilable nutrients derived from their protoplasts, can be regarded as facilitating colonisation. Fibres with their thick, less pitted walls and narrow lumina are ineffectual as access routes, and when occurring together, form tissues which can be regarded as barriers to both axial and transverse colonisation. Little precise information is available concerning the role of parenchyma and fibres with regard to fungal colonisation (Rayner and Boddy, 1988).

It has been reported that fatty acids can inhibit fungal growth (Garg and Muller, 1993). In some wood species like aspen (*Populus tremuloides*) and lodgepole pine (*Pinus contorta*) fungal growth is inhibited in the heartwood of the tree. The heartwood of aspen has a much higher fatty acid concentration than the sapwood (Table 18). According to literature it is, however, doubtful that the fatty acid content would be high enough to inhibit fungal growth. Tests in liquid medium showed that fungi can easily grow in fatty acid concentrations of 1% and the fatty acid concentrations of the aspen heartwood which was inoculated was 0,83%. In this case fungal growth may have been inhibited because the moisture content of the heartwood (33%) was lower than that of the sapwood (43%) (Breuil *et al.*, 1994). The natural durability of wood appears to be dependent on the presence of certain phenolic extractives (Rudman, 1963), rather than on fatty acid content. The decay resistance of aspen is increased after it has been submersed in the methanol extracts of the heartwood of black locust (*Robinia pseudoacacia*), osage orange (*Maclura pomifera*), redwood (*Sequoia sempervirens*) and *Intsia bijuga* (Kamdem, 1994).

Table 18 Contents of different lipid classes (% of oven dry weight) in the sap- and heartwood of untreated aspen (Breuil *et al.*1994)

Lipid class	*Aspen sapwood*	*Aspen heartwood*
Triglycerides	1.45 ± 0.03	0.36 ± .01
Fatty acids	0.03 ± 0.01	0.83 ± 0.03
Waxes and steryl esters	0.62 ± 0.01	0.52 ± 0.02
Others	0.99 ± 0.08	0.73 ± 0.08

The pH optima for growth of most wood decay fungi is 4 to 6, which corresponds to the pH found in most woods. White-rot fungi show greater tolerance of high pH than brown-rot fungi (Cooke and Rayner, 1984). Table 19 shows the pH values of a range of hardwood tree species. The Myrtaceae family, which incorporates the *Eucalyptus* species, is also included. There can be considerable differences between sapwood and heartwood within a tree (Hartley *et al.*, 1961). Generally, the sapwood would have a lower pH than heartwood. However, the actual microenvironmental conditions experienced at the hyphal level and metabolic activity itself is likely to have both marked and highly localised effects on the pH. Nonetheless, extremes of pH have sometimes been implicated in the selectivity of fungi for particular timber types, examples being elm (*Ulmus spp.*)

with a pH above 7 (Gray, 1958) and in oak (*Quercus spp.*), which may have a pH as low as 3 and acid enough to corrode metals (Packman, 1960).

Table 19 The pH values for a range of hardwood tree species (Rayner and Boddy, 1988)

Family	*Species*	*pH range*
Aceraceae	*Acer pseudoplatanus*	4.05-6.0
Apocynaceae	*Dyera costulata*	4.65
Aquifolaceae	*Ilex aquifolium*	5.5
Araliaceae	*Acanthopanax ricinofolius*	6.7
Betulaceae	*Alnus glutinosa*, *Betula spp.*, *Carpinus betulus*	4.5-5.95
Bombaceae	*Ochroma lagopus*	6.6
Burseraceae	*Aucoumea klaineana*	4.2-4.45
Buxaceae	*Buxus sempervirens*	5.05-6.0
Combretaceae	*Terminalia spp.*	3.05-5.65
Compositae	*Brachylaena hutchinsii*	4.35-4.8
Dipterocarpaceae	*Anisoptera sp.*, *Dipterocarpus spp.*, *Shorea spp.*, *Parashorea sp.*	3.2-5.55
Ebenaceae	*Diospyros mespiliformis*	5.7
Fagaceae	*Castanea sativa*, *Fagus sylvatica*, *Quercus spp.*, *Nothofagus spp.*	3.35-6.05
Flacourtiaceae	*Scottelia coriacea*	5.35-6.8
Gonystylaceae	*Gonystylus sp.*	5.25-5.35
Guttiferae	*Calophyllum brasilience*	4.9
Juglandaceae	*Carya* sp., *Juglans regia*	4.4-5.8
Lauraceae	*Eusideroxylon sp.*, *Ocotea spp.*, *Persea sp.*	3.65-4.9
Lecythidaceae	*Cariniana sp.*	3.3
Leguminosae	18 *spp.* tested	3.65-6.15
Melastomaceae	*Dactylocladus stenostalchys*	4.55
Meliaceae	12 *spp.* tested	2.75-5.85
Moraceae	*Chlorophora exelsa*, *Piratinera guianensis*	5.4-7.25
Myrtaceae	*Eucalyptus spp.*, *Tristania conferta*	3.2-4.95
Ochnaceae	*Lophira alata*	4.2-4.45
Olaceae	*Fraxinus spp.*, *Olea sp.*	3.55-6.25
Platanaceae	*Platanus acerifolia*	4.6-6.0
Proteaceae	*Grevillea robusta*	4.95
Rhamnaceae	*Maesopsis eminii*	4.35
Rosaceae	*Prunus acium*, *Sorbus aucuparia*	4.5-4.8
Rubiaceae	*Calycophyllum sp.*, *Mitragyna sp.*	3.85-5.75
Salicaceae	*Populus spp.*, *Salix fragilis*	4.2-4.8
Sapotaceae	*Minusops heckelii*	4.75-5.1
Steruliaceae	*Sterculia oblonga*, *Tarrieta spp.*	4.9-5.5
Tiliaceae	*Cistanthera papaverifera*, *Tilia vulgaris*	3.75-5.55
Triplotichonaceae	*Mansonia altissima*, *Triplochiton scleroxylon*	4.05-6.75
Trochodendraceae	*Ceridophullum japonicum*	5.05-5.55
Ulmaceae	*Ulmus spp.*	6.45-7.15
Verbaceae	*Tectona grandis*	4.75-4.9
Vochysiaceae	*Vochysia sp.*	3.65
Zygophyllaceae	*Guaacum spp.*	3.6-5.45

Different patterns of attack of the S1, S2 and S3 layers occur. In white rots hyphae typically penetrate into the cell lumen, where they lie on the inner surface of the wood cell wall (Nasroun, 1971; Bravery, 1971, 1972, 1975, 1976; Levy and Dickinson, 1981). Erosion of the cell wall may be generalised or localised to the immediate vicinity of the hyphae, forming a groove or trough with a central ridge on which the hypha lies. This

implies that the extracellular enzymes are bound to the hyphae by an external layer of mucilage (Cooke and Rayner, 1984). Branching of the hyphae eventually results in progressive cell-wall erosion from the lumen through the S3 and S2 layers (Rayner and Boddy, 1988).

Soft rot fungi produce two distinct types of attack termed Type 1 and Type 2 (Corbett, 1963, 1965; Crossley, 1980; Nilsson, 1974, 1975, 1976, 1977; Zainal, 1976). Type 1 attack is characterised by the formation of chains of cavities with pointed ends in the S2 layer, which follow the orientation of the microfibrils (Savory, 1954). Type 2 attack is similar to localised white-rot attack, in that it works outward from the lumen and results in an eroded groove with the hypha lying on a central ridge. With brown rot the hyphae lie on the surface of the S3 layer in the lumen, but not within it. The hyphae, S3 layer and compound middle lamella alter very little but the S2 and S1 layers become extensively hollowed out owing to removal of cellulose and hemicelluloses (Bravery, 1971; Crossley, 1980; Nasroun, 1971; Wilcox, 1968).

2.7.2.2 Microclimate

Wood, which is not in contact with the ground, absorbs water by precipitation through rain, mist, hail or snow and also by absorption of water vapour from the air. The relative humidity of the surrounding air is an important factor. Air has a certain maximum potential saturation with water vapour and when the real water vapour content is expressed as a percentage of the maximum potential water vapour content it is called relative humidity. Wood will absorb water from the air or transfer water to the air until it reaches an equilibrium moisture content that is governed by the relative humidity of the air. The approximate equilibrium moisture contents of wood at varying relative humidity values are given in Figure 26. At about 30% moisture content wood reaches the fibre saturation point where the cell walls are saturated with water and the voids are empty. Above 30% moisture content, free water exists in the lumen of woody cells. Below 30% all moisture is bound to cell walls (Siau, 1995). Excessive water content can prevent fungal colonisation (Cooke and Rayner, 1984).

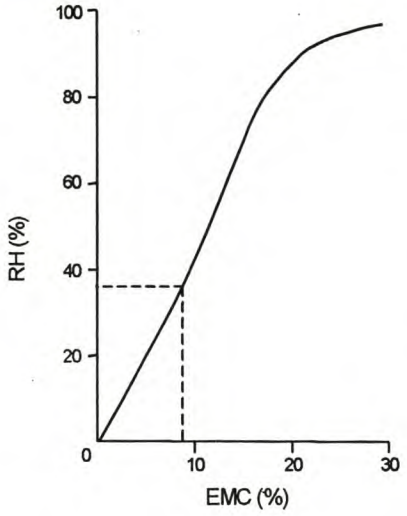


Figure 26 The equilibrium moisture content (EMC) of wood with varying relative humidity (RH) (Skaar, 1972).

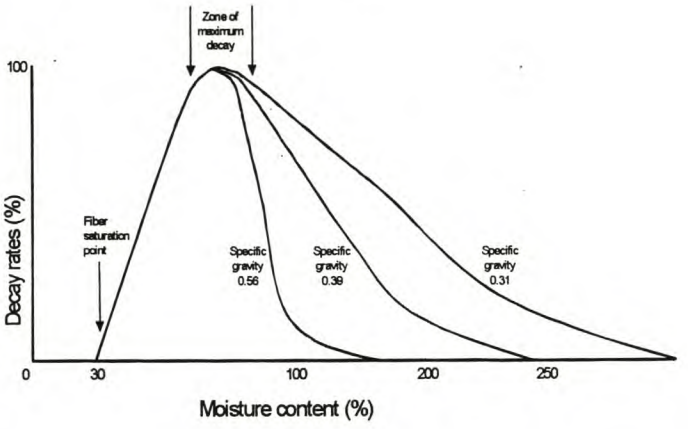


Figure 27 An approximation of the cardinal moisture contents for the rates of decay development of several common mesophilic basidiomycete decay fungi as judged from reports in the literature and experience (Zabel Morrell, 1992). Maximal moisture contents are calculated based on data from Higgins (1957) and Skaar (1972).

Moisture content crucially affects the growth of wood-decaying fungi in either of two ways. At low moisture contents limitations are imposed by restricted accessibility of water supplies to growing hyphae. At high moisture contents the presence of water-filled void space may impose a physical barrier to growth by influencing the exchange and availability of air. Under normal temperature conditions the solubility of carbon dioxide is 30-50 times greater than that of oxygen (Brock, 1966). Reduced supply of oxygen and an elevated concentration of carbon dioxide have consistently been shown to reduce growth (Jensen, 1967; Scánel, 1976). Water fill of void space would hinder oxygen supply due to its relative insolubility in water. Above 30% equilibrium moisture content the woody cells become filled with water. The lack of oxygen supply would become limiting for fungal growth with an increase in moisture content above fiber saturation point. Several species grow at lower extremes of moisture levels including *Schizopora paradoxa* which survives at a relative humidity of 35% (Theden, 1961), which would correspond to a wood moisture content of ± 8% (Figure 26). Table 20 shows the minimal moisture levels for decay development. Optimal wood-moisture levels for most decay fungi lie between 40 and 80% (Scheffer, 1973). Since the void volume of wood varies

inversely with specific gravity, the upper moisture levels limiting fungal growth will be much lower in the high-density woods (Figure 27).

Table 20 Minimal moisture levels for decay development (Zabel and Morrell, 1992)

Fungi	*Moisture level*	*Reference*
Wood-decay fungi	25-32%	Snell *et al.*, 1925
Xylobolus frustulatus	16-17%	Bavendamm and Reichelt, 1938
Schizophyllum commune	16-17%	Bavendamm and Reichelt, 1938
Antrodia sinuosa	26%	Freyfeld, 1939
Common wood-decay fungi	22-24%	Cartwright and Findlay, 1958
Ophiostoma piliferum	23%	Lindgren, 1942
Wood-inhabiting fungi	Slightly above fibre saturation point	Etheridge, 1957

Generally, wood-decay fungi have been found to be mesophilic, with a growth range within 0-45°C and an optimum between 20 and 30°C. *Phanerochaete chrysosporium*, however, has an optimum of about 40°C and a maximum of 50°C (Bergman and Nilsson, 1971).

2.7.3 Conditions in wood chip piles

Chip storage of pulpwood, because of its handling advantages and relatively low losses, has now become the predominant method of wood storage. It decreases handling cost, and requires smaller storage areas (Zabel and Morrell, 1992). In relation to the wood in a tree a chip pile has less free surface for evaporation and dissipation of metabolic heat and commonly gives rise to a self-heating system, in which the centre may be considerably hotter and moister than the outside. Temperatures as high as 48,9°C may be reached after seven days (Springer and Hajny, 1970). Further temperature rises depend, in part, on pile features. Tall piles with excessive compaction, or accumulated layers of fines reduce air circulation, and temperatures may rise to 60-71°C. At these temperatures, slow heat decomposition of wood begins, and acetic acid is released (Kubler, 1982). The exothermic reaction further elevates the temperature, and the acetic acid increases wood acidity, which may reach a pH of 3. Chips exposed at these acidity and temperature levels turn brown and become soft. Losses from chemically degraded chips and occasional fires from spontaneous combustion can occur in piles under these high temperature conditions. The principle heat sources in chip piles and the factors affecting various temperature levels are illustrated in Figure 28 (Zabel and Morrell, 1992). After

the initial rise, temperatures decline in properly constructed and managed piles, but remain higher than external ambient temperatures (Feist *et al.*, 1973). Management practices generally strive to maintain pile temperatures below 60°C.

Fungal colonisation by both stain-causing fungi and true decay species occurs mostly in the outermost layers (Lindgren and Eslyn, 1961). While the growth of most basidiomycetes is restricted, *Phanerochaete chrysosporium* has been repeatedly isolated (Bergmann and Nilsson, 1971; Tansey, 1971; Ofosu-Asiedu and Smith, 1973). A range of thermophilic fungi grows on chip piles (Tansey, 1971). Thermophilic or thermotolerant fungi predominate in the interior zone of a chip pile (Zabel and Morrell, 1992).

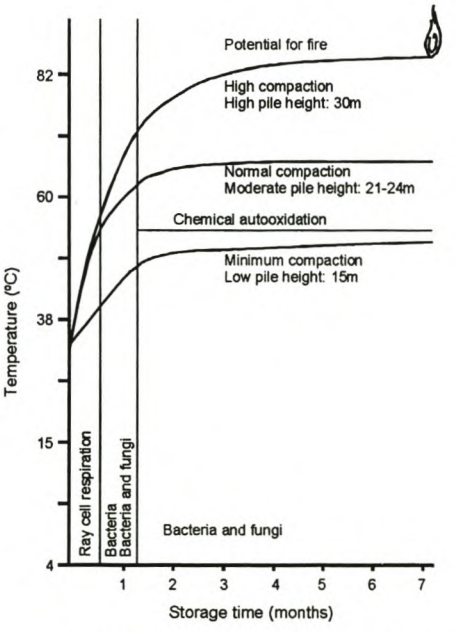


Figure 28 The principle heat sources in large piles of pulpwood chips and the various factors affecting the temperature levels and chip damage (Zabel and Morrell, 1992).

2.8 Conclusions

The temperature, pH fluctuations and chemicals used in the kraft pulping process coupled with a bleaching regime induce the formation of pitch deposits from wood extractives. Pitch deposits lead to the formation of a layer on tile and metal parts. This layer would break up into flakes and form spots on the pulp sheets and paper produced and reduce its value. Current chemical technologies of pitch reduction (e.g. surfactants and dispersants) are costly and the use of biotechnology may decrease the use of chemicals while improving product quality.

Triglyceride is the key to pitch formation in the pulping process. The products of triglyceride breakdown, fatty acids and glycerol, are more easily removable from the pulping process. This prompts the use of lipase in combating pitch. In a pulping context the use of a lipase secreting white-rot fungus is the ideal because of the capability of white-rots to remove lignin. With genetic engineering in mind, the identification of any lipase secreting fungi is of significance. *Ophiostoma piliferum* (Cartapip®) is a sapstainer and Ascomycete used industrially specifically for its ability to hydrolise triglycerides. Although it has been reported that *Ophiostoma piliferum* improves chemical pulping efficiency (due to the increased permeability of the wood) and mechanical pulp properties (Kohler *et al.*, 1995; Wall *et al.*, 1994; Wall *et al.*, 1995), a white-rot fungus which hydrolyses triglycerides and lignin and increases wood permeability would still be the ideal. The application of lipase biotechnology in the pulping process is already practised in industry with success (Fujita *et al.*, 1992).

Although *Eucalyptus grandis* is not a ring-porous but a diffuse porous hardwood, it is not a factor in the chip piles of a pulp mill when considering the inclination of the wood specie to be colonised by fungi. The presence of numerous rays in *Eucalyptus grandis* makes the wood more inclined to fungal colonisation. The wood is already well exposed in the chip form and the colonisation by fungi will be unconstrained. In addition to pitch reduction it is probable that the permeability of the wood will be enhanced by reduction of tyloses and opening up of pits (Siau, 1995), consequently ensuring that the wood is

more evenly pulped. The necessity of holding large amounts of wood inventory in the form of chip piles may be a limiting factor.

The optimal moisture content of wood for fungal growth lies between 40 and 80%. Addition of water would have to be considered to maintain this moisture content throughout a chip pile. The temperature optima for the fungus used, climate and self-heating characteristic of a chip pile are factors to be considered when contemplating temperature control of a chip pile. Aeration of chip piles may be necessary for temperature control, depending on fungal strain used, as well as oxygen supply.

The development of a fungal or enzymatic biotechnological approach to combat pitch problems in South African pulp mills that utilise *Eucalyptus grandis* could decrease chemical consumption and improve product quality. The use of biotechnology can also improve the marketability of pulp and paper products.

2.9 References

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Chapter 3: Manuscript submitted for publication in Holzforschung

3.1 An Investigation into Pitch Deposition in a *Eucalyptus spp.* Kraft Pulping and Bleaching Mill

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3.1.1 Summary

Wood and pulp extracts as well as pitch deposits were collected and their composition was analysed. Large portions of the samples were involatile and had a molecular weight above 900 Da. Approximately 40% of volatile *E. grandis* extract was identified as β-sitosterol, with fatty acids (22.8%) and triglycerides (15.5%) also making a substantial contribution. Amide derivatives of fatty acids 16:0, 18:2, 18:1 and 18:0 were a prominent fraction of the pulp extracts from the later stages of bleaching. The amide derivatives constituted 38.3% and triglycerides 10.1% of total volatile pitch deposits.

Keywords: *Eucalyptus grandis*, wood extractives, kraft pulp extractives, pitch, gas chromatography, mass spectrometry, gel permeation chromatography

3.1.2 Introduction

The extractive materials in wood often cause pitch problems in pulp mills. During pulping and bleaching, extractives are released from the wood and pulp and later stick to ceramic and metal parts. These pitch deposits impair both product quality and production rates. They decrease the efficiency of pulp washing, screening, centrifugal cleaning, and refining, and can disrupt many paper machine operations (Dreisbach and Michalopoulos 1989). There are a few triggering mechanisms that induce pitch deposition. Hydrodynamic or mechanical shear can destabilise the colloidal pitch emulsion causing pitch to agglomerate and deposits to form. Similarly, sudden temperature drops, pH shocks, or the introduction of water hardness ions from fresh water inlets or showers can

also cause pitch deposition by destabilising the colloidal pitch emulsion. Inorganic salts, such as calcium carbonate, can catalyse pitch deposition by acting as the building blocks for the sticky pitch. Furthermore, calcium ions in the white water can react with fatty acids, forming insoluble, sticky calcium soaps (Laubach and Greer 1991). Triglycerides have been identified as the major contributors to pitch deposition in kraft pulping and bleaching mills (Allen 1977; Fischer and Messner 1992; Fujita *et al.* 1992; Suckling and Ede 1990).

The extractives of the *Eucalyptus* genera have also been reported to have a detrimental effect in both pulping and sawmilling operations (Chafe 1987; Hillis and Carle 1959; Nelson *et al.* 1970; Yazaki *et al.* 1993). To attain an improved understanding of pitch problems associated with the kraft pulping and bleaching of *Eucalyptus spp.*, various analyses were done on pulp extractives and pitch from a South African kraft pulp mill in this investigation. The aim of the study was to determine (a) the composition of the wood and pulp extracts, (b) the change in pulp extract composition through the pulping and bleaching process and (c) the composition of the small amounts of pitch still depositing despite the use of pitch reducing additives.

3.1.3 Experimental

3.1.3.1 Wood, pulp and pitch sampling

Six year old *Eucalyptus grandis* logs were obtained from the logyard of a kraft pulp mill in the KwaZulu/Natal region of South Africa. The logs were left in the plantations for six weeks after harvesting prior to delivery to the pulp mill logyard. The logs were hand debarked and chipped. The pulp mill feedstock comprises several *Eucalyptus* species and hybrids with *E. grandis* comprising more than 50% of the total feedstock. The mill has a C/DEoDED bleaching regime. Pulp samples were taken from the brown stock decker mat, CD mat, D1 inlet, E2 mat and bleach stock decker mat. Pitch was sampled from the bleach stock decker outlet. All samples were stored at -20°C until analysed.

3.1.3.2 Dichloromethane extractions

Dichloromethane was selected as extraction solvent due to its ability to extract lipids (Wallis and Wearne, 1999). The moisture and extractive contents of wood and pulp samples were determined according to Tappi method T 204 om-88. The wood chips were ground in a Wiley mill with a 0.40 mm (40 mesh) sieve. Two thimbles per wood sample, each containing approximately two grams of air dry ground wood (2 g heartwood or 2 g sapwood or 1 g heartwood and 1 g sapwood), were extracted with dichloromethane for 5 hours. Two thimbles per pulp sample, each containing approximately 10 g of air dry pulp each, were extracted with dichloromethane for 5 hours. The extracts were air dried in a fume hood, weighed to determine the total amount of extractives present and analysed by gel permeation chromatography, gas chromatography and gas chromatography - mass spectrometry.

3.1.3.3 Gel permeation chromatography (GPC)

Samples were dissolved in tetrahydrofuran. Analyses were done using a Spectra Physics 8875 autosampler, HP1100 isocratic pump and Spectra Physics GPC software. The molecular mass distribution of wood and pulp extracts were determined by passing extracts through four in series 300 × 7.8 micron Phenogel 10 columns from Phenomenex with packing pore sizes of 100 Å, 500 Å, 1000 Å and 10 000 Å at a flow rate of 1.25 $ml.min^{-1}$. Molecular masses were determined using polystyrene standards and an Erma 7510 refractive index (RI) detector.

3.1.3.4 Derivatization of extracts

Diazomethane was used to methylate all carboxylic acids present in the wood or pulp extracts (Christie 1992). Extracts were also dissolved in chloroform and methylated by the addition of trimethylsulphonium hydroxide (TMSH) as described by Butte (1983). TMSH caused transesterification and methylation of carboxylic acids.

3.1.3.5 Gas chromatography (GC) of diazomethane methylated extracts

Gas chromatography analyses of diazomethane methylated extracts was carried out on a Hewlett Packard 6890 gas chromatograph equipped with a flame ionisation detector.

Column: Chrompack SimDist Ulti Metal, 10 m × 0.53 mm i.d., 0.17 µm coating. Carriergas: Nitrogen. Injection method: Samples were dissolved in 200 µl chloroform/methanol (2:1) and 2 µl was injected with a 20:1 split ratio. Temperature program: Injector 250°C, initial column temperature 130°C, ramp rate 5°C.min^{-1}, final column temperature 390°C.

3.1.3.6 Gas chromatography of TMSH methylated extracts

Gas chromatography analyses of TMSH treated extracts were carried out on a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionisation detector. Column: Supelcowax-10, 30 m × 0.53 mm i.d., 1 µm coating. Carriergas: Nitrogen. Injection method: Samples (still dissolved in the methylation solvents) were injected with a HP 7673 autosampler at a 40:1 split ratio. Temperature program: Injector 220°C, initial column temperature 145°C held for 3 min, ramp rate 3°C.min^{-1}, final column temperature 245°C.

3.1.3.7 Gas chromatography - Mass spectrometry analyses (GC-MS)

Gas chromatograph: GC-MS analysis was carried out on a Hewlett Packard 5890 Series II gas chromatograph coupled to a HP 5972 quadropole mass spectrometer. Column: HP-5 60 m fused silica capillary, 0.28 mm i.d. 0.25 µm coating. Carriergas: He. Injection method: Samples were dissolved in 200µl chloroform/methanol (2:1) and 2 µl was injected with a 50:1 split. Temperature program: Injector 250°C, initial column temperature 130°C, ramp rate 6°C.min^{-1}, final column temperature 320°C. Mass spectrometer parameters: EMV 1447, scan 20 to 690 atomic mass units, GC-MS interphase temperature 280°C.

3.1.4 Results and discussion

3.1.4.1 The composition of E. grandis wood extract

Ohtani *et al.* (1986) and Ohtani and Shigemoto (1991) reported that the high molecular mass components of several hardwoods used in Japanese kraft pulping mills as well as pitch from these mills were mostly polymerised aliphatic hydrocarbons. The polymerised

aliphatic hydrocarbons were primarily unsaturated fatty acids and alcohols that underwent condensation reactions. They found that the more double bonds the hydrocarbons had, the faster the condensation reactions took place. Fatty acids were polymerised faster than fatty alcohols. Extensively polymerised aliphatic hydrocarbons are not soluble in organic solvents (Ohtani *et al.* 1986). Consequently, it can be expected that some of the higher molecular weight polymerised compounds would not be removed from wood or pulp by dichloromethane extractions.

Triglycerides, which have molecular weights in the range of 800-900 Da, were the compounds with the highest molecular weight that could be detected by GC analysis. The <900 Da range comprised 71.4% of the total wood extract (Fig. 1). The >900 Da range accounted for 28.6% of total wood extract. The GPC peaks in the <200 Da range represent terpenes and phenols and the one in the 200-900 Da range represents fatty acids, sterols, mono-, di- and triglycerides. Gas chromatography showed that the major contributors to the volatile component of the wood extract were fatty acids, sterols and triglycerides (Table 1). One specific sterol, peak 12 in Fig. 2, comprised about 40% of the total volatile wood extract. The mass spectra of sterols are characterised by among other a combination of m/z 43, m/z 55 and m/z 81 fragments. The mass spectrum of β-sitosterol in particular is also characterised by the presence of fragments m/z 303, m/z 329 and the molecular ion at m/z 414. The mass spectrum of peak 12 is illustrated in Fig. 3 and exhibits the characteristic sterol and β-sitosterol fragments, confirming that the compound in question was β-sitosterol. β-sitosterol is reported to be a major fraction of the sterol content of many hardwoods (Fengel and Wegener 1989).

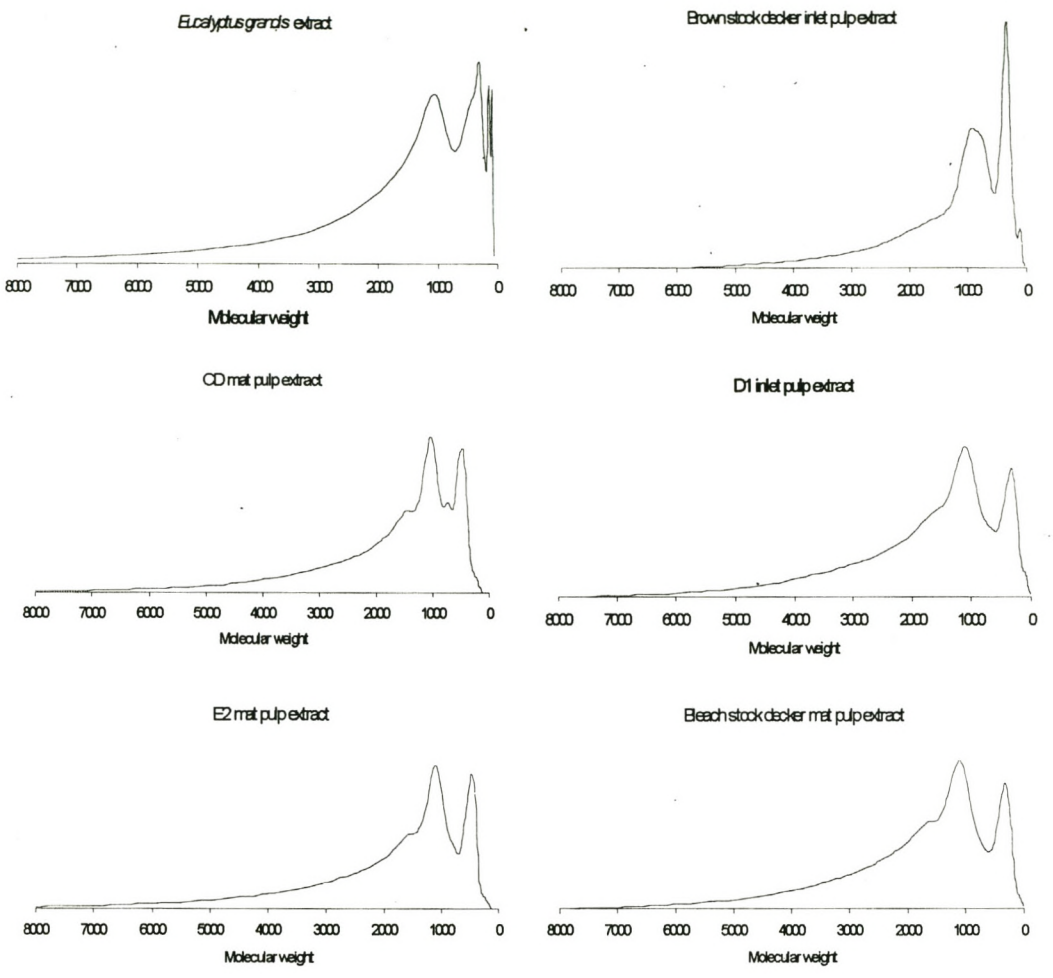

Fig. 1. Gel permeation chromatograms showing the change in extractive composition during the pulping and bleaching process.

Table 1. A comparison of the composition of *E. grandis* heartwood and sapwood extract

	Heartwood	Sapwood
Total extractives[a]	0.39	0.37
Terpenes and phenols	3.6	2.1
Free fatty acids	22.9	22.7
16:0	7.7	13.2
18:2	5.9	2.7
18:1	2.4	1.6
18:0	0.7	1.0
20:0	0.4	0.6
22:0	0.9	1.0
23:0	0.0	0.5
24:0	1.6	1.7
26:0	2.0	0.4
28:0	1.2	0.0
Sterols	47.8	47.3
β-sitosterol	39.0	45.9
Monoglycerides	3.0	2.9
Diglycerides	0.0	0.0
Triglycerides	15.9	15.1

[a]Total extractives are given as a percentage of oven dry weight. The remainder is given as a percentage of total volatile extract.

The terpene and phenol content of the heartwood extract was higher than that of the sapwood extract. Total free fatty acid content was much the same, however, there was a marked difference in the fatty acid composition of the heartwood and sapwood. Fatty acids 16:0, 18:2 and 18:1 were abundant in both, with 16:0 being much more prevalent in the sapwood than the heartwood. Upon derivatisation with TMSH the fatty acid ratio (16:0):(18:2):(18:1):(18:0) would typically change from 9:8:2:1 to 7:10:3:1, indicating an increase in fatty acids 18:2 and 18:1 relative to 16:0 and 18:0. Consequently fatty acids 18:2 and 18:1 were the predominant esterified fatty acids in the wood extract. Monoglycerides generally constituted about 3% of the total extract. Diglyceride content of the wood extract was rarely higher than 3% and frequently none could be detected. There was little difference in the triglyceride content of the sapwood and heartwood. Triglyceride content was usually within the range of 15-30% of volatile wood extract.

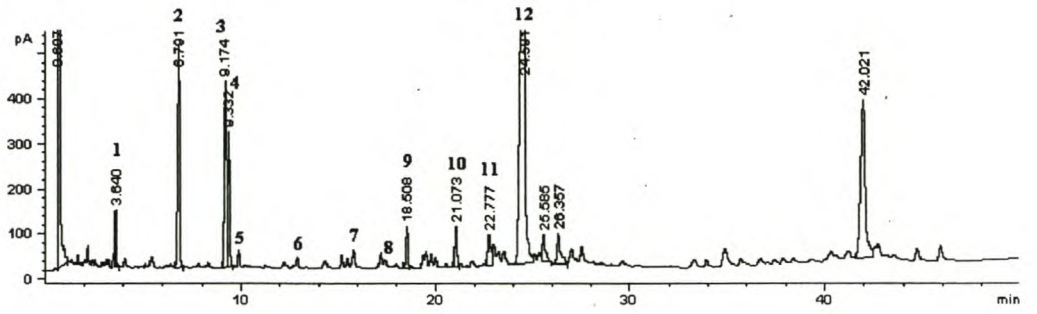

Fig. 2. Gas chromatogram of diazomethane treated *E. grandis* extract. Retention time ranges determined with standards: 1-5.6, terpenes and phenols; 19.1-20.3, monoglycerides; 22.9-30, sterols; 32-40, diglycerides; 40-46.5, triglycerides. Peaks identified by GC-MS and/or the use of standards: 1, phenol; 2, fatty acid (16:0); 3, fatty acid (18:2); 4, fatty acid (18:1); 5, fatty acid (18:0); 6, fatty acid (20:0); 7, fatty acid (22:0); 8, fatty acid (23:0); 9, fatty acid (24:0); 10, fatty acid (26:0); 11, fatty acid (28:0); 12, β-sitosterol.

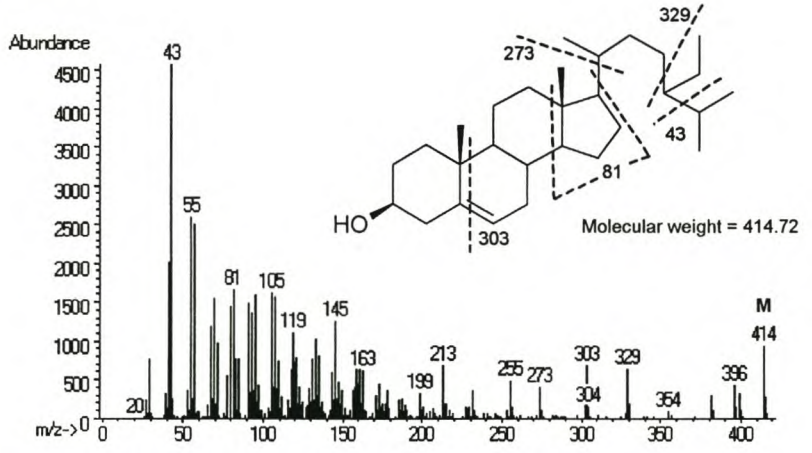

Fig. 3. The mass spectra of peak 12, exhibiting the fragmentation pattern of β-sitosterol. Key: M = molecular ion.

3.1.4.2 The composition of Eucalyptus spp. pulp extract

GC analysis of pulp and pitch revealed the formation of four compounds that did not occur in the wood extract initially (Fig. 4). The first four unknown peaks occurred at a 4.6 min retention time offset from respectively the 16:0, 18:2, 18:1 and 18:0 fatty acid peaks, indicating that the compounds are derivatives of these fatty acids. The mass spectrum of fatty acid 16:0 (palmitic acid) is compared with its corresponding peak at a 4.6 min offset in Fig. 5. The base peak of palmitic acid was formed due to the McLafferty rearrangement where a hydrogen atom is donated, resulting in the formation of fragment m/z 74 (Budzikiewicz *et al.* 1967). The mass spectrum of the peak

corresponding to palmitic acid is an identical match to the spectrum of N, N,-dimethylpalmitamide. The mass spectra of the other three peaks showed that they were also amide derivatives of respectively fatty acids 18:2, 18:1 and 18:0. Amide derivatives of fatty acids have also been detected in mechanical pulp effluents (Eriksson 1999). The origin of these compounds may be the additives utilised by the mill.

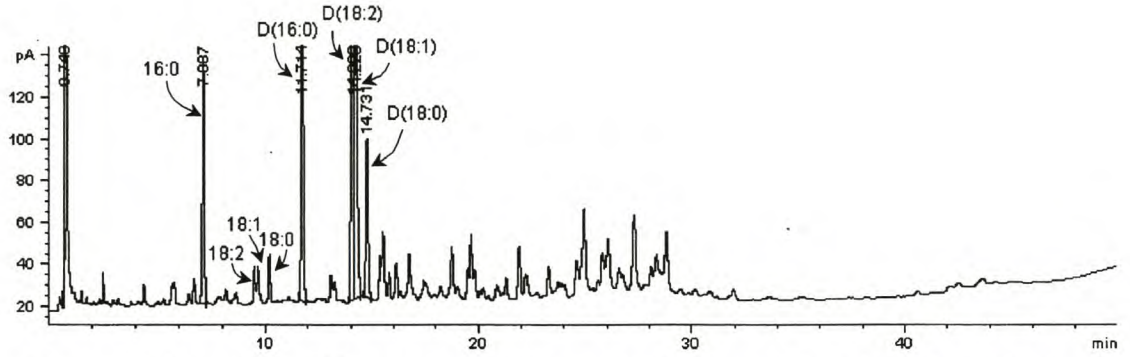

Fig. 4. Gas chromatogram of E2 mat pulp extract showing the four fatty acid derivative peaks which occurred at a 4.6 min offset from the corresponding fatty acid peaks. Key: D = derivative.

The presence of amide derivatives of fatty acids can be ascribed to the use of non-ionic organic additives provided by Buckman Laboratories (Pty) Limited. BUBREAK® 4051 is a brown stock drainage aid that also possesses the properties of a washing aid. It is added at the brown stock washing stage (1.1 kg/ton dry pulp) to improve drainage of the cooking liqour. It is added again after screening of the washed brown stock. BUBREAK® 4051 is specifically engineered to stick to the pulp. BUSPERSE® 47 is a pitch dispersant added before the screening decker (150 g/ton dry pulp), after the screening decker (250 g/ton dry pulp), before the E1 bleaching stage (400 g/ton dry pulp) and before the E2 bleaching stage (400 g/ton dry pulp). Talc is added after the E2 filter (4.2 kg/ton dry pulp). BUSPERSE® 2219, a dispersant, is added after the D2 filter (1.9 kg/ton dry pulp). The fact that the additives enter process waters at an early stage was reflected in the analysis results. The washed brown stock pulp already contained fatty acid amides (Table 2).

Table 2. A comparison of the composition of *Eucalyptus spp.* pulp extracts from different stages of the pulping and bleaching process.

	Brown stock	CD mat	D1 inlet	E2 mat	Bleach stock decker mat
Total extractive content[a]	0.15	0.26	0.19	0.16	0.22
Terpenes and phenols	1.4	5.8	1.6	1.2	1.3
Fatty acids	23.7	25.0	24.0	13.9	24.0
16:0	11.6	12.5	13.1	6.7	13.7
18:2	0.5	0.4	0.8	1.0	0.0
18:1	6.4	0.5	1.0	1.1	1.1
18:0	1.6	2.1	2.7	1.3	2.7
20:0	0.6	1.2	1.4	0.7	1.3
22:0	0.8	2.6	3.4	1.0	1.3
23:0	0.6	0.5	0.7	0.0	1.2
24:0	1.1	3.6	4.6	1.6	1.9
26:0	0.6	1.6	2.4	0.6	0.8
28:0	0.0	0.0	0.0	0.0	0.0
Sterols	51.9	28.8	30.1	21.6	20.9
β-sitosterol	30.5	5.8	6.2	4.2	3.2
Monoglycerides	1.5	3.2	8.4	3.6	8.4
Diglycerides	2.3	1.0	0.0	0.0	0.0
Triglycerides	0.0	0.0	0.0	0.0	0.0
Amide derivatives	7.9	6.1	8.7	47.9	29.0
16:0	2.2	3.1	5.1	10.5	15.0
18:2	1.3	1.3	0.0	19.2	2.7
18:1	3.3	0.3	0.6	13.5	4.6
18:0	1.0	1.5	3.0	4.7	6.6

[a]Total extractives are given as a percentage of oven dry weight. The remainder is given as a percentage of total volatile extract.

Non-ionic agents are extremely stable in acid and alkaline solutions. There are three principle types: alkyl phenol-ethylene oxide, aliphatic polyhydric alcohol esters, and fatty acid amides (Shreve 1956). The type used in this case is probably fatty acid amides, which accounts for the fatty acid amides found in the pulp extracts and pitch. Fatty acid amides used as detergents could be of polyhydroxylic constitution (Webb 1964), in which case there had to be some molecular modification of the additives, for the fatty acid amides found had no hydroxyl groups. Molecular modification is, however, improbable due to the stability of non-ionic additives. The only source for amide groups up to the point of high density storage of washed brown stock was the amide derivatives added after brown stock washing. There were not any extreme pH fluctuations after the addition of the additives before the brown stock decker and the sampling of brown stock

decker mat pulp. Taking this fact into account as well as the stability of the additives, it follows that the fatty acid amides found were constituents of the additives.

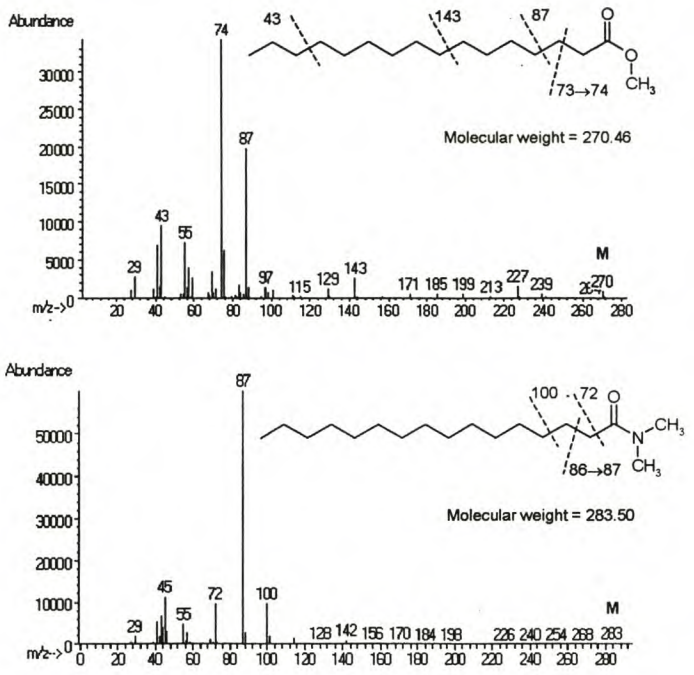

Fig. 5. The mass spectrum of methyl palmitate, methyl ester of fatty acid 16:0, and its amide derivative, N, N-dimethylpalmitamide. The McLafferty rearrangement (Budzikiewicz *et al.* 1967) caused the formation of fragment m/z 74 in the case of methyl palmitate and fragment m/z 87 in the case of N, N-dimethylpalmitamide. Key: M = molecular ion.

Affleck and Ryan (1969) reported that the ethanol/benzene (1:2) extractive content of the pulp from a kraft mill with a CEDED bleach plant decreased significantly due to washing, bleaching and caustic extraction. The total DCM extractive content of the bleached pulp is higher than that of the brown stock pulp. The first bleaching stage, the CD stage, had a significant effect on total extractive content and pulp extract composition (Fig. 1). A large portion of the sterols was washed out (Table 2) and the reduction is reflected in Fig. 1. The fatty acid amides constituted a large proportion of the pulp extracts from the latter stages of bleaching. The fatty acid ratio (16:0):(18:2):(18:1):(18:0) for the E2 mat pulp extract differs sharply with the ratios of the other stages of bleaching. Fatty acid 16:0 content is lower relative to the other fatty acids. Talc was added to the bleaching process after the E2 filter as it is known that talc

reduces the stickiness of pitch particles by adsorbing onto the particles. The mechanism does not involve attachment of particles to fibres (Hassler 1988). There was a significant reduction in the proportion of amide derivatives and increase in the proportion of fatty acids from E2 mat pulp extract to bleach stock decker mat pulp extract (Table 2).

Figure 1 shows the gel permeation chromatograms of *E. grandis* and pulp extracts from different stages in the pulping and bleaching process. The same compounds as discussed under *E. grandis* wood extract were present in the pulp extracts. However, in the latter stages of bleaching a significant proportion of the 250-350 range was represented by amide derivatives of fatty acids 16:0, 18:2, 18:1 and 18:0 (Table 2). The first graph in Fig. 1 shows the extractive composition of *E. grandis* and the second the composition of brown stock decker inlet pulp extract. Due to the digestion process the contribution of the >900 Da range changed from 28.6% to 30.1%. The >900 range comprised 51.6% of bleach stock decker mat pulp extract, indicating that the high molecular weight compounds were not easily removed.

3.1.4.3 Pitch composition

A lot of pulp fibres were attached to the sticky pitch deposits. Free fatty acids and fatty acid amides constituted a large portion of the volatile fraction of pitch (Table 3). Most of the amide derivatives were N, N-dimethylpalmitamide. It should be noted that the additives did not intensify pitch deposition. Its presence in the small amounts of pitch still depositing is only an artefact of its presence in process waters. The pulp mill experienced minimal pitch deposition since the use of additives was instigated.

Triglycerides constituted 10.1% of the volatile pitch deposits. Pulp extracts contained no triglycerides. This indicates either that triglycerides were in suspension in the white water or that very small amounts carried by the pulp deposited over an extended period of time. It is also possible that the triglycerides were deposited in periods when feedstock with a high triglyceride content was pulped.

Table 3. The composition of pitch. All values are given as a percentage of total volatile extract.

Component		Content
Terpenes and phenols		0.8
Free fatty acids		21.7
	16:0	6.7
	18:2	0.0
	18:1	0.0
	18:0	1.7
	20:0	0.6
	22:0	2.0
	23:0	2.1
	24:0	4.6
	26:0	3.6
	28:0	0.4
Sterols		11.5
	β-sitosterol	8.5
Monoglycerides		3.6
Diglycerides		3.8
Triglycerides		10.1
Amide derivatives		38.3
	16:0	20.0
	18:2	1.2
	18:1	9.8
	18:0	7.2

3.1.5 Conclusions

The dichloromethane extractable levels remained much the same despite washing and bleaching stages. High molecular weight compounds constituted a large portion of wood and pulp extracts. Approximately 40% of volatile *E. grandis* extract was identified as β-sitosterol. Triglycerides constituted 10% and amide derivatives of fatty acids 16:0, 18:2, 18:1 and 18:0 from additives made up 38.3% of the volatile fraction of pitch deposits. Wallis and Wearne (1999) found a substantial amount of steryl esters in the dichloromethane extracts of *E. globulus*. In our investigation on *E. grandis* extracts, no significant amounts of steryl esters were found, indicating that there could be significant differences in the extract composition of different *Eucalyptus* species.

The composition of the E2 mat pulp extract differed considerably from pulp extracts from other stages. The increase in fatty acid amides and decrease in fatty acids may be ascribed to extreme conditions in the second caustic extraction stage. However, the first

extraction stage does not have such a marked effect on the pulp extract. The decrease in fatty acid amides in the bleach stock decker mat pulp extract as well as the normalisation of fatty acid levels may be attributed to the addition of talc and the change in process conditions.

Kraft cooking eliminated triglycerides from the pulp extract. Wallis and Wearne (1999) also found that kraft cooking eliminated triglycerides from *E. globulus* pulp extract. The fact that triglycerides were found in the pitch deposits indicate either that triglycerides were in suspension in the white water or that minute amounts carried by the pulp deposited over an extended period of time. It is also possible that the triglycerides were deposited in periods when feedstock with a high triglyceride content was pulped. As reported by Farrell *et al.* (1997), triglycerides may also be the key to pitch deposition in this case. Due to their sticky nature only small amounts of triglycerides are needed to induce pitch deposition.

3.1.6 Acknowledgements

The authors are grateful to Mondi Kraft Ltd., The Foundation for Research and Development as well as The Technology and Human Resources for Industry Programme for supporting this project.

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Chapter 4: Manuscript submitted for publication in Biotechnology Letters

4.1 Lipase production and activity of fungi isolated from South African forests

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Filamentous fungi isolated from indigenous and commercial forests in South Africa, were screened for lipase activity on tributyrin and Tween 80. Eight strains were selected and the tributyrin and Tween 80 assays were repeated by monitoring lipase activity over a seven-day period. The selected strains were also assayed for their activity toward *p*-nitrophenyl palmitate. *Ophiostoma piliferum* Cartapip 58™ and *Phanerochaete chrysosporium* BKM-F-1767, two strains used commercially in the pulp and paper industry, were used as controls. A few of the strains compared well and even outperformed the control strains, indicating that there is potential for the use of these strains in the pulp and paper industry for pitch control.

Keywords: fungi, lipase activity, triglycerides, Tween 80, tributyrin, *p*-nitrophenyl palmitate, pitch control

4.1.1 Introduction

Lipases (acylglycerol acylhydrolases, EC 3.1.1.3) hydrolyse the ester bonds in tri-, di-, and monoacylglycerols. Some will also degrade a fairly broad range of other compounds containing an ester linkage. Lipases are important enzymes in industry, serving as biocatalysts for fat and oil processing in medicine, food additives, diagnostic reagents and detergents (Alhir *et al.*, 1990; Derewenda, 1994; Jaeger *et al.*, 1994; Soberón-Chávez and Palmeros, 1994). Animals, plants and micro-organisms produce lipases. To date, the

majority of lipases used for biotechnological purposes have been isolated from filamentous fungi since they produce extracellular enzymes (Comménil *et al.*, 1995).

The extractive materials in wood often cause pitch problems in pulp mills. During the pulping and bleaching process the extractives are released from the wood and pulp and the organic deposits formed, called pitch, later stick to ceramic and metal parts as well as the wires of the papermaking machines. Not all of the extractives are troublesome. Wood triglycerides have been shown to be a major contributor to pitch deposition (Allen, 1977; Fujita *et al.*, 1992; Suckling and Ede; 1990). Recently two new methods of decreasing triglycerides in pulping and bleaching processes, and therefore combating pitch, have been developed independently and are now used industrially. One is a method using a fungus obtained through selective breeding from the sap-staining organisms that are associated with weathering of wood. This Ascomycetous fungus is non-pigmented and avoids the staining and decrease in brightness normally associated with the ageing of wood (Farrell, 1994). It is grown on wood chip piles prior to pulping. The other method is the addition of lipase to the stock after the pulping process (Fujita, 1992). There are several reports describing the effectiveness of fungal biotechnology and lipase use in decreasing the triglyceride content of wood extractives (Blanchette *et al.*, 1992; Brush *et al.*, 1994; Farrell *et al.*, 1997; Fischer and Messner, 1992; Fischer *et al.*, 1994; Fischer *et al.*, 1995; Rocheleau *et al.*, 1998). Consequently the investigation and identification of fungi, especially white-rot fungi that produce lipase, can be of benefit to the pulping industry. In this study, 381 fungal strains were screened for lipase production and activity.

4.1.2 Materials and methods

4.1.2.1 Materials

Peptone, yeast extract, malt extract and potato dextrose agar were obtained from Merck. Lipase from *Rhizopus arrhizus*, tributyrin, Tween 80 and *p*-nitrophenyl palmitate (pNPP) were purchased from Sigma Chemical Co. All other reagents were analytical grade from commercial sources.

4.1.2.2 Fungal strains

Three hundred and eighty one fungal strains isolated from indigenous forests as well as commercial *Eucalyptus spp.* and *Pinus spp.* plantations in South Africa (De Koker *et al.*, 1998) were screened for their lipase production and activity using the described Tween 80 and tributyrin methods. The eight best strains were re-evaluated over a seven-day period with the same methods and were also assayed for their lipase production and activity with pNPP as substrate. Cartapip 58™ (*Ophiostoma piliferum)*, a commercial fungal strain known for its depitching potential, was used as a control in the Tween 80, tributyrin and pNPP assays. *Phanerochaete chrysosporium* BKM-F-1767, a fungal strain known for its biopulping ability, was also used as control in the pNPP assay.

4.1.2.3 Tween 80 agar plate method

A basal medium consisting of 10 g peptone, 15 g potato dextrose agar, 5 g NaCl, 25 mg bromocresol purple and 0.1 g $CaCl_2.2H_2O$ made up to 1 litre with distilled water was prepared. The pH was adjusted to 5.4 with hydrochloric acid. A 10% (v/v) aqueous Tween 80 solution was prepared by slowly adding 10 ml Tween 80 to 90 ml warmed (60-70°C) distilled water. The agar and Tween 80 solutions were sterilised separately by autoclaving for 15 min at 121°C. After the media cooled down to 65-70°C, one part Tween 80 solution was added to nine parts basal medium. The medium was inoculated with an agar plug and incubated for 7 days at 23°C except for the thermophilic fungi, which were incubated at 50°C. A positive reaction was recorded as the development of a purple zone. Every 24 hours the diameters of the purple zones were measured. Due to the size of the plates, the growth and lipase active zones could only reach a maximum diameter of 35 mm.

4.1.2.4 Tributyrin deep agar diffusion method

The medium, consisting of 0.5% peptone, 0.3% yeast extract, 1.5% potato dextrose agar, and 0.1% tributyrin in distilled water, was sterilised by autoclaving for 15 min at 121°C. The sterilised medium was dispensed into sterile test tubes (7 ml per test tube) and allowed to cool down. The medium was inoculated with agar plugs of growing cultures and incubated for 7 days at 23°C except for the thermophilic fungi, which were incubated

at 50°C. A positive reaction was recorded as a clearing of the opaque medium. Every 24 hours the depth of the clearing zones was measured.

4.1.2.5 Assay of enzyme activity with pNPP as substrate

A pH 8 phosphate buffer containing 1 part 0.05 M KH_2PO_4 and 19 parts 0.05 M Na_2HPO_4 was prepared. Thirty milligrams of pNPP was dissolved in 10 ml propan-2-ol. A solution containing 0.207 g sodiumdeoxycholate (Sigma Chemical Co.) and 0.1 g gum arabic (Sigma Chemical Co.) was dissolved in 90 ml of the prepared phosphate buffer. The pNPP solution was added in small quantities to the sodiumdeoxycholate/gum arabic solution while stirring continuously until all was dissolved. This solution, which served as substrate, was dispensed into test tubes in 2.5 ml quantities. The pNPP substrate was prewarmed at 37°C. Lipase containing culture supernatant (100 µl) from the liquid medium was added to the pNPP substrate and incubated at 37°C for 15 minutes. The reaction was stopped by boiling the substrate for 3 minutes and then placed on ice. Absorbance was measured at 410 nm against a blank consisting of water. The commercial lipase from *Rhizopus arrhizus* was used to create calibration curves for lipase activity. Fresh solutions were made up daily for the assay and to generate calibration curves. According to Sigma Chemical Co. one unit of lipase will hydrolyse 1.0 microequivalent of fatty acid from a triglyceride in 1 hr at pH 7.7 at 37°C.

The selected strains were grown in 100 ml liquid medium containing 1% olive oil and 1.5% malt extract medium prior to inoculation in triplicate into the same medium for the assay. The inoculum (10 ml) consisted of 10 day old fungal biomass which was fragmented in a Waring blender. The medium was incubated for 7 days at 23°C except for the thermophilic fungi, which were incubated at 50°C. Lipase activity in the culture supernatant was measured throughout the incubation period.

4.1.2.6 Results and discussion

The preliminary Tween 80 agar plate trial results of the 381 screened fungal strains are shown in Table 1. Most of the strains did not cause discolouration while 42 strains gave a high degree of discoloration. In the following Tween 80 trial, the eight selected fungal strains, except GTB 69, all gave a larger lipase active zone than the control Cartapip 58™

(*O. piliferum* CAR 58) and all of them had a faster growth rate (Figs. 1 and 2). All the strains, except GTB 69, MTZ 95 and *P. pseudomagnoliae* nom. prov. did not clear the medium as well as *O. piliferum* CAR 58 (Table 1). From these results it is clear that most of the strains tested, are better than the control strain *O. piliferum* CAR 58.

Table 1 Results of the Tween 80 agar plate screening of the 381 fungal strains.

Discoloration	*Number of strains*
-	203
+	91
++	45
+++	42

- no effect, + weak effect, ++ moderate effect, +++ striking effect

The results of the eight selected strains and the control, *O. piliferum* CAR 58, when grown on tributyrin are shown in Figure 3 and listed in Table 2. The two thermophilic *Thermomyces lanuginoses* strains, MED 2D and MED 4B1, performed better than Cartapip 58™ (*O. piliferum* CAR 58) in terms of lipase production (size of lipase active zone) and was equal in lipase activity (clarity of lipase active zone). Strain MTZ 95 was comparable in terms of the size of the lipase active zone, but did not match the lipase activity of the control and the two thermophilic strains.

Table 2 Lipase activity of the eight selected strains and the control, *O. piliferum* CAR 58, on tributyrin and Tween 80

Fungal strain	Degree of clarity *(Tributyrin)*	*Degree of discoloration (Tween 80)*
BLK 10A	+++	+++
O. piliferum CAR 58	+++	++
GTB 69	++	+
KWA 16	++	+++
T. lanuginoses MED 2D	+++	+++
T. lanuginoses MED 4B1	+++	+++
MTZ 95	++	+
MTZ 97	+++	+++
P. pseudomagnoliae nom. prov.	++	+

+ weak effect; ++ moderate effect; +++ striking effect

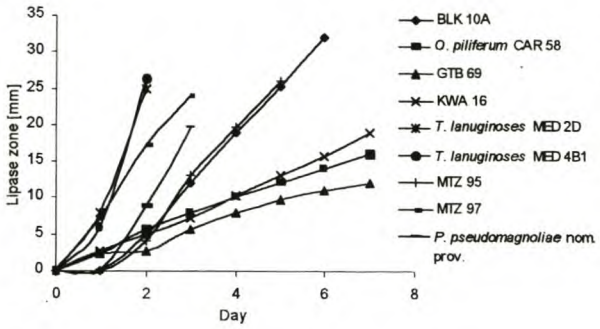

Figure 1 The size of the zones of the eight selected strains and the control, CAR 58, on Tween 80.

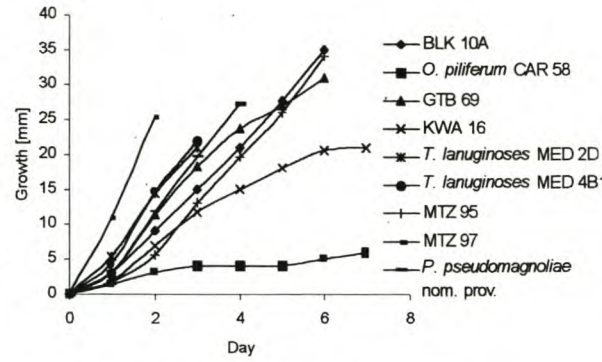

Figure 2 Growth rate of the eight selected strains and the control, CAR 58, on Tween 80.

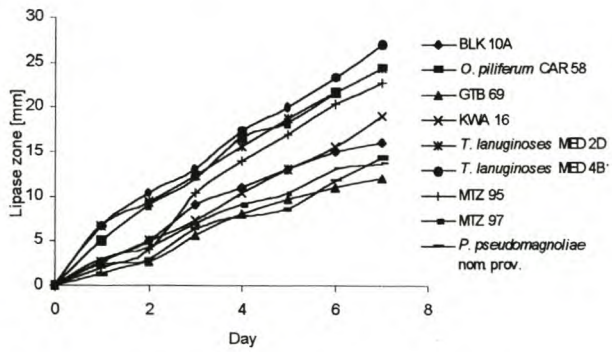

Figure 3 The size of the zones of the eight selected strains and the control, CAR 58, on tributyrin.

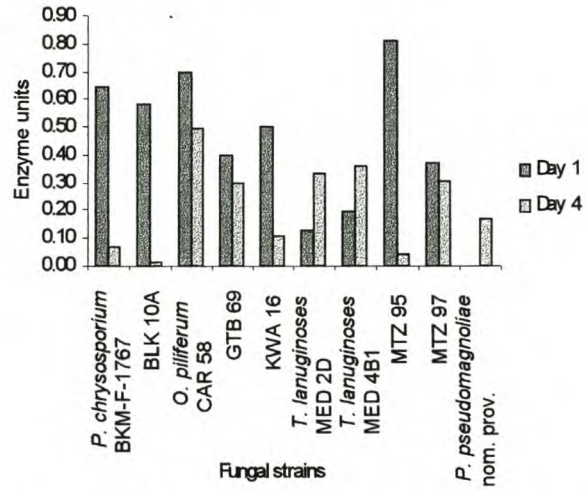

Figure 4 Cell-associated (Day 1) and free (Day 4) lipase activity of eight selected strains and the controls, CAR 58 and BKM-F-1767, on pNPP.

Rapp and Backhaus (1992) reported that the rate of hydrolysis of pNPP by microbial lipases was in the range of 0.1-6% of the activity toward triolein, indicating that pNPP is a poor substrate for many microbial lipases. In our study, low absorbance values were also detected. White-rot erosion of the wood cell wall may be widespread or localised to the immediate vicinity of the hyphae, forming a groove or trough with a central ridge on which the hypha lies. If erosion is localised, the extracellular enzymes are associated with the hyphae by an external layer of mucilage (Rayner and Boddy, 1988). Figure 4 shows the lipase activity in culture supernatant inoculated with fragmented biomass on day 1 as well as the activity in the culture supernatant on day 4. Due to the fragmentation

of the biomass prior to inoculation, cell-associated lipases (intracellular and/or mucilage-bound lipases) were released into the culture supernatant giving cell-associated lipase activity on day 1. The lipase activity found in the culture supernatant on day 4 is attributed solely to lipases released into the supernatant without any agitation of the biomass. These lipases are defined as free lipases. Three of the strains; *Thermomyces lanuginoses* MED 2D, *T. lanuginoses* MED 4B1 and *P. pseudomagnoliae* nom. prov., showed a higher lipase activity on day 4 than day 1, indicating higher free lipase activity. The control strain, *O. piliferum* CAR 58, had both high cell-associated and free lipase activities. The other control strain, *Phanerochaete chrysosporium* BKM-F-1767, which is also known to reduce the amount of total dichloromethane wood extractives (Farrell *et al.*, 1997), has a high cell-associated lipase activity and a low free lipase activity. Since both control strains reduce total dichloromethane wood extractives, the inability to produce free lipases does not seem to have a negative effect on the ability of a fungus to hydrolyse wood extractives while growing on wood.

4.1.3 Conclusions

The strains used in this study showed different levels of lipase activity toward the different substrates, indicating that the lipases are substrate specific. A few of the strains compared favourably and even outperformed the control strains, indicating the potential for the use of these strains to break down the triglycerides in wood extractives. Consequently these strains have the potential for application in the pulp and paper industry for pitch control. The lipases from the *Thermomyces lanuginoses* strains, MED 2D and MED 4B1, have even more potential due to their ability to hydrolyse triglycerides at the higher temperatures commonly found in industrial processes. The eight strains isolated in South Africa will also be evaluated in our laboratory for their biopulping and depitching potential.

4.1.4 Acknowledgments

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Chapter 5: Manuscript submitted for publication in Biotechnology Letters

5.1 Enzymatic Pitch Control in the Kraft Pulping of Eucalyptus grandis

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The effect of pretreatment with eight fungal strains isolated in South Africa on *Eucalyptus grandis* wood- and pulp extractives was determined. *Ophiostoma piliferum* Cartapip 58™ and *Phanerochaete chrysosporium* BKM-F-1767 were used as control strains. Several of the strains compared well to the control strains in their ability to reduce the triglyceride content of wood extract. The white-rot *Phanerochaete psuedomagnoliae* nom. prov. gave better results than both the control strains. Consequently it can act as an agent for both biopulping and biodepitching. Treated samples did not show a significant difference in pulp triglyceride content or pulp characteristics compared to the controls. The effect of commercial lipases on deposited brown stock pulp extract was also evaluated. The lipases did not reduce the triglyceride content of the deposited extract.

Keywords: Fungi, biotechnology, pulp, pitch, extractives, triglycerides, lipase

5.1.1 Introduction

Because of extensive planting in the humid regions of South Africa, *Eucalyptus* species have become of economic importance to the South African sawmilling and pulp and paper industries. The extractives of the *Eucalyptus* genera have a detrimental effect during both pulping and sawmilling operations (Chafe, 1987; Hillis and Carle, 1959; Nelson *et al.*, 1970; Yazaki *et al.*, 1993). The extractives increase consumption of pulping chemicals and impair the colour and brightness of unbleached pulp (Nelson *et al.*, 1970). Pitch is a sticky organic deposit found on pulping equipment that is caused by wood extractives. During the pulping and bleaching process the extractives are released from the wood and pulp and later stick to ceramic and metal parts as well as the wires of

the papermaking machines. Wood triglycerides are major contributors to pitch deposition (Allen, 1977; Fischer and Messner, 1992; Fujita *et al.*, 1992; Suckling and Ede, 1990). Recently two new methods of decreasing triglycerides in pulping and bleaching processes have been developed independently and are now used industrially. Both methods are aimed at hydrolysing the triglycerides. One is the use of fungal biotechnology to decrease triglyceride content of wood (Blanchette *et al.*, 1992; Brush *et al.*, 1994; Farrell *et al.*, 1997; Fischer *et al.*, 1994; Fischer *et al.*, 1995; Rocheleau *et al.*, 1998). The other method is the addition of lipase to pulp in a watery suspension (Fujita *et al.*, 1992; Fischer and Messner, 1992).

The screening of fungi that hydrolyse wood triglycerides, especially white-rot fungi, is of importance to the pulping industry. It would be ideal to use one fungal strain for both biopulping and biodepitching. In this study the ability of fungal strains isolated in South Africa to decrease wood and pulp triglyceride content was evaluated. Commercial lipases decrease triglyceride content of pulp in suspension (Fujita *et al.*, 1992; Fischer and Messner, 1992). In this study the effect of lipase addition on deposited brown stock pulp extract was determined as well.

5.1.2 Experimental

5.1.2.1 Wood and fungal strains

Freshly delivered 6 year old *Eucalyptus grandis* logs were obtained from the KwaZulu/Natal region in South Africa. The logs are left in the plantations for a few weeks before delivery. The logs were hand debarked and chipped. Eight fungal strains isolated in South Africa were selected on the basis of their lipase activity (Scheepers *et al.*, 2000). *Ophiostoma piliferum* Cartapip 58™, a commercial fungal strain known for its depitching potential, was used as a control strain in the evaluation of the effect of the fungi on wood extractives. *Phanerochaete chrysosporium* BKM-F-1767, a fungal strain known for its biopulping ability, was used as control strain in the pulping trial.

5.1.2.2 Dichloromethane extractions

The moisture and extractive contents of wood and pulp samples were determined according to Tappi method T 204 om-88. The wood to be extracted was ground in a

Wiley mill with a 0.40 mm (40 mesh) sieve. Two thimbles per wood sample, each containing approximately two grams of air dry ground wood (1 g heartwood and 1 g sapwood), were extracted with dichloromethane for 5 hours. Two thimbles per pulp sample, each containing approximately 10 g of air dry pulp, were extracted with dichloromethane for 5 hours. The extracts were dried in a fume hood, weighed to determine the total amount of extractives present and analysed by gas chromatography.

5.1.2.3 Wood chip treatment trial

The strains used in this trial are listed in Table 1. The strains were incubated in triplicate in liquid medium containing 1.5% malt extract, 0.5% yeast extract and 1% olive oil until a mycelial mat has formed. The mycelia were fragmented in a Waring blender prior to inoculation in triplicate on 100 g (oven dry equivalent) of autoclaved (121°C for 15 min) *Eucalyptus grandis* wood chips at 70% moisture content based on dry weight. The samples were incubated for four weeks in a cotton wool stoppered container at 30°C except for *Thermomyces lanuginoses* MED 2D and *Thermomyces lanuginoses* MED 4B1, which were incubated at 50°C. After incubation the total dichloromethane extractives of each sample were determined. The dried extract was stored at -20°C for GC analysis.

Table 1 The strains used for the two fungal biotechnology trials

Strains used for wood chip treatment trial	*Strains used for pulping trial*
BLK 10A	*P. chrysosporium* BKM-F-1767
O. piliferum CAR 58	BLK 10A
GTB 69	*Thermomyces lanuginoses* MED 4B1
KWA 16	MTZ 95
T. lanuginoses MED 2D	*P. pseudomagnoliae* nom. prov.
T. lanuginoses MED 4B1	
MTZ 95	
MTZ 97	
P. psuedomagnoliae nom. prov	

5.1.2.4 Pulping trial

The strains used in this trial are listed in Table 1. Inoculum was prepared in the same way as with the wood chip treatment trial. 500 g (oven dry equivalent) of autoclaved (121°C for 15 min) *Eucalyptus grandis* wood chips at 70% moisture content based on dry weight was inoculated. The samples were incubated for four weeks in cotton wool stoppered containers at 30°C except for *Thermomyces lanuginoses* MED 4B1, which was

incubated at 50°C. Two hundred grams of the wood chips were removed after incubation for dichloromethane extractions while the rest was kraft pulped. Triplicates were pooled and pulped together to produce only one pulp sample per fungal strain. Pulp characteristics were evaluated. The pulp was also extracted with dichloromethane. Samples were stored at –20°C for GC analysis.

5.1.2.5 Lipase treatment of deposited brown stock pulp extractives

Three brown stock pulp extract samples were incubated with purified lipase from *Mucor javanicus* and another three with purified lipase from *Rhizopus arrhizus* (Sigma Chemical Co.). One negative control with only buffer and two positive controls with autoclaved enzyme solutions from each of the lipases were used. Thirty milligrams of brown stock pulp dichloromethane extractives was dried down in glass vials, forming a deposit on the glass. The deposited extract was incubated with 0.5 mg of the lipases in 10 ml pH 7.7 phosphate buffer at 37°C for 4 hours. 0.5 mg yields 200 U of lipase from *Mucor javanicus* and 195 U *Rhizopus arrhizus* lipase. One unit of lipase will hydrolyse 1.0 microequivalent of fatty acid from a triglyceride in 1 hr at pH 7.7 at 37°C. At these conditions only 68 U would be needed to hydrolyse 30 mg of triglycerides in 1 hour. Because of this and since triglycerides constitute a small fraction of the total brown stock pulp extract, the amount of lipase used was more than adequate to hydrolyse all the triglycerides present in 4 hours. After incubation all the samples were freeze dried, the triplicate samples were combined and stored at -20°C.

5.1.2.6 Gas chromatography of wood and pulp extracts

All carboxylic acids present in the wood or pulp extracts were methylated by using diazomethane. The methylation with and preparation of diazomethane was done according to the methods of Christie (1992). GC analyses of diazomethane methylated extracts were carried out on a Hewlett Packard 6890 gas chromatograph equipped with a flame ionisation detector. Column: Chrompack SimDist Ulti Metal, 10 m × 0.53 mm i.d., 0.17 µm coating. Carriergas: Nitrogen. Injection method: 10 mg of each sample was dissolved in 200 µl chloroform/methanol (2:1) and 2 µl was injected with a 20:1 split

ratio. Temperature program: Injector 250°C, initial column temperature 130°C, ramp rate 5°C min^{-1}, final column temperature 390°C.

5.1.3 Results and discussion

5.1.3.1 Wood chip treatment trial

The extractive composition of the sterile controls varied considerably and made it difficult to compare strains in their ability to reduce triglyceride content. The major components of the extract of the *E. grandis* used in this study have been identified (Scheepers *et al.*, 2000). The wood extract consists of approximately 30% high molecular weight compounds and 70% low molecular weight (volatile) compounds. GC analyses only describe the composition of the low molecular weight compounds. Table 2 presents the effect of the different fungal strains on the composition of the volatile wood extract. All eight strains were evaluated with *Ophiostoma piliferum* CAR 58 as control strain. BLK 10A, GTB 69, MTZ 95 and *P. pseudomagnoliae* nom. prov. all whitened the wood during growth. None of the nine fungal strains decreased the total extractive content although all, except *T. lanuginoses* MED 4B1, decreased the triglyceride content. The increase in extractive content could be attributed to the presence of fungal mycelia.

KWA 16 was the only fungal strain that decreased free fatty acid content, while BLK 10A was the only fungus that decreased diglyceride content compared to the sterile controls. Fatty acid 18:1 increased substantially in most cases. Analyses done on *E. grandis* wood extracts have shown that most of the esterified fatty acids are 18:1 and 18:2 fatty acids (Scheepers *et al.*, 2000). Hydrolysis of triglycerides would therefore result in an increase in fattty acid 18:1 and 18:2 content. *O. piliferum* CAR 58 gave results comparable to the white-rots. All the white-rots except GTB 69 decreased triglyceride content to a lower final level than CAR 58. The final triglyceride content of MTZ 97 treated samples was substantially lower than that of the other strains. The initial triglyceride content of the treated samples may have been very low, giving a false impression of the ability of the fungus to reduce the triglyceride content. *T. lanuginoses* strains, MED 2D and MED 4B1, had little effect on total extractive content. However, the terpene and phenol as well as sterol contents were considerably reduced by the *T. lanuginoses* strains. Both increased the triglyceride, diglyceride and fatty acid contents

with fatty acid 18:1 content changing most. It is important to note that the most abundant fatty acids in fungi are 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 (Kock and Botha, 1998). A dramatic increase in fatty acid 18:1, under conditions where the wood esters were not hydrolysed, could therefore be linked to the fatty acid contribution of the fungus. KWA 16 decreased terpene and phenol, fatty acid and triglyceride content while increasing sterol content. MTZ 97 increased total extractive content fourfold due to a dramatic increase in fatty acid content as well as diglyceride content. Terpene and phenol, sterol and triglyceride content did not decrease much when the increase in total extract is considered.

Table 2 Average extractive content and composition of sterile *E. grandis* treated with nine different fungal strains

		BLK 10A		*O. piliferum CAR 58*		*GTB 69*		*KWA 16*		*T. lanuginoses MED 2D*	
		Sterile controls	*Treated samples*	*Sterile controls*	*Treated samples*	*Sterile controls*	*Treated samples*	*Sterile controls*	*Treated samples*	*Sterile controls*	*Treated samples*
Total extractives [a]		0.33	0.44	0.31	0.32	0.32	0.34	0.31	0.31	0.34	0.36
Terpenes and phenols		5.0	6.9	5.7	4.6	5.7	4.0	7.1	3.6	9.2	3.1
Free fatty acids total		23.4	30.6	23.9	25.4	25.0	34.4	28.7	19.1	25.9	33.9
	16:0	8.8	10.9	6.3	8.4	8.4	7.8	10.9	3.3	8.1	6.4
	18:2	7.4	7.6	8.9	6.6	8.1	7.0	9.0	5.5	7.6	5.3
	18:1	1.6	5.8	2.0	4.0	1.9	12.8	2.2	4.4	2.3	14.1
	18:0	0.5	0.8	0.6	0.5	0.6	1.2	0.8	0.5	0.9	1.4
	20:0	0.3	0.4	0.4	0.3	0.4	0.5	0.4	0.4	0.5	0.6
	22:0	0.6	0.6	0.7	0.7	0.7	0.7	0.7	0.7	0.8	0.7
	23:0	0.6	0.5	0.4	0.4	0.4	0.4	0.6	0.5	0.6	0.8
	24:0	1.4	1.3	1.5	1.5	1.6	1.4	1.4	1.2	1.8	1.6
	26:0	1.3	1.2	1.7	1.5	1.7	1.4	1.2	1.3	2.3	1.9
	28:0	1.0	1.5	1.4	1.4	1.2	1.2	1.6	1.3	0.8	1.0
Sterols total		33.5	44.6	42.1	43.6	39.3	32.0	40.2	55.9	43.4	34.4
	β-sitosterol	29.2	32.2	33.7	34.7	32.7	23.6	32.7	30.7	33.0	26.0
Monoglycerides		2.4	1.9	2.6	1.9	2.7	1.8	3.0	1.8	3.0	2.2
Diglycerides		7.2	2.7	1.5	3.7	1.2	3.4	0.5	3.2	0.9	4.7
Triglycerides		26.6	11.0	20.9	17.2	22.9	20.6	15.2	12.0	12.3	18.0

Table 2 continued		*Thermomyces lanuginoses MED 4B1*		*MTZ 95*		*MTZ 97*		*P. pseudomagnoliae* nom. prov.	
		Sterile controls	*Treated samples*	*Sterile controls*	*Treated samples*	*Sterile controls*	*Treated samples*	*Sterile controls*	*Treated samples*
Total extractives		0.33	0.33	0.31	0.39	0.32	1.28	0.33	0.36
Terpenes and phenols		8.9	3.4	6.3	9.9	6.9	2.6	4.2	4.5
Free fatty acids total		27.4	37.5	28.2	31.8	26.1	73.9	21.7	33.5
	16:0	8.2	8.7	10.4	6.0	7.2	10.5	6.9	7.8
	18:2	8.6	5.6	9.0	6.3	9.3	55.7	7.2	7.5
	18:1	2.5	15.6	2.0	10.8	2.4	3.2	1.6	10.9
	18:0	0.9	1.4	0.6	1.4	0.7	0.2	0.5	1.1
	20:0	0.6	0.6	0.4	0.8	0.5	0.6	0.3	0.5
	22:0	0.9	0.8	0.7	1.0	0.8	0.3	0.6	0.7
	23:0	0.5	0.4	0.4	0.5	0.3	2.1	0.5	0.7
	24:0	2.0	1.6	1.6	1.7	1.8	0.4	1.3	1.4
	26:0	2.4	1.9	1.7	1.5	2.1	0.5	1.3	1.4
	28:0	0.9	1.2	1.3	1.8	1.1	0.5	1.4	1.5
Sterols total		44.7	35.5	41.1	35.2	43.2	9.9	33.9	29.2
	β-sitosterol	34.3	26.3	34.0	19.9	33.8	8.1	27.3	20.5
Monoglycerides		3.1	1.6	2.5	2.3	2.7	1.2	2.3	1.7
Diglycerides		0.6	3.3	0.6	2.7	1.1	7.1	6.3	10.2
Triglycerides		10.0	15.0	17.6	12.4	15.5	2.9	29.4	16.5

[a] Total extractive content is given as a percentage of oven dry wood weight. All other values are given as a percentage of total volatile extract.

5.1.3.2 Pulping trial

Four fungal strains were selected for the pulping trial with *P. chrysosporium* BKM-F-1767 as control strain. Table 3 displays the effect of the strains on the wood extract content and composition. The extractive content of the *E. grandis* used in this trial was higher. Nevertheless, the effect of the selected fungal strains on the wood extract was the same except for *Thermomyces lanuginoses* MED 4B1, which decreased the triglyceride content. Pulping halved the total extractive content with sterols being most difficult to remove. Only *P. chrysosporium* BKM-F-1767 treated samples showed a significant decrease in pulp triglyceride content compared to the sterile controls (Table 4). Consequently, more triglycerides would be released into process waters by the sterile controls than the treated samples. There were no significant differences in pulp properties of treated and sterile control samples except for the high percentage of rejects and low percentage of screened yield in the *P. chrysosporium* BKM-F-1767 treated pulp sample (Table 5). The abnormal amount of rejects and low screened yield could be ascribed to a process problem rather than an effect from pretreatment.

Table 3 Extractive content and composition of sterile *E. grandis* treated with five different fungal strains

		P. chrysosporium BKM-F-1767		*BLK 10A*		*Thermomyces lanuginoses* MED 4B1		*MTZ 95*		*P. pseudomagnoliae* nom. prov.	
		Sterile controls	*Treated samples*	*Sterile controls*	*Treated samples*	*Sterile controls*	*Treated samples*	*Sterile controls*	*Treated samples*	*Sterile controls*	*Treated samples*
Total extractives [a]		0.41	0.48	0.42	0.40	0.44	0.36	0.42	0.46	0.45	0.45
Terpenes and phenols		8.7	9.7	7.9	6.8	7.5	6.5	7.6	9.2	6.6	9.0
Free fatty acids total		21.7	27.0	19.9	19.8	22.6	20.1	19.2	20.1	22.0	22.3
	16:0	7.4	6.2	7.2	6.6	7.1	6.1	6.6	4.8	7.3	6.2
	18:2	5.4	4.3	5.2	4.4	5.7	3.6	4.9	3.8	5.9	5.1
	18:1	2.0	8.8	1.8	2.3	2.8	2.9	1.8	4.2	1.9	3.4
	18:0	1.0	1.6	1.0	0.9	1.2	1.0	1.0	1.1	1.3	1.2
	20:0	0.7	0.9	0.5	0.5	0.8	0.6	0.5	0.6	0.4	0.6
	22:0	1.0	1.1	0.6	0.9	1.0	1.1	0.8	1.3	0.8	1.2
	23:0	0.5	0.7	0.4	0.5	0.3	0.4	0.4	0.5	0.5	0.5
	24:0	1.8	1.7	1.6	1.6	1.7	2.0	1.6	1.9	1.8	2.1
	26:0	1.3	1.1	1.1	1.1	1.5	1.6	1.2	1.4	1.3	1.4
	28:0	0.6	0.6	0.5	0.9	0.6	0.9	0.6	0.6	0.8	0.7
Sterols total		43.0	37.1	39.0	44.1	43.2	47.6	39.1	44.2	45.1	42.2
	β-sitosterol	36.5	27.7	36.0	35.2	38.0	37.6	34.4	30.1	40.5	31.0
Monoglycerides		3.5	2.5	2.6	2.3	2.7	2.9	2.9	2.8	3.3	3.2
Diglycerides		0.0	2.7	2.4	1.2	0.0	1.8	2.4	2.6	0.0	1.5
Triglycerides		14.8	11.7	21.4	16.6	15.8	12.1	22.0	12.0	16.7	11.0

[a] Total extractive content is given as a percentage of oven dry wood weight. All other values given as a percentage of total volatile extract.

5.1.3.3 Lipase treatment of brown stock pulp extract

Mucor javanicus and *Rhizopus arrhizus* lipase had no effect on the triglyceride content of the deposited brown stock pulp extract (Table 6). The high molecular weight compounds

of the extract as well as the other volatile compounds made the triglycerides inaccessible for hydrolysis. Pitch deposits in a pulp mill would therefore be unaffected by the addition of lipase.

Table 4 Extractive content and composition of the kraft pulp from the treated wood

	P. chrysosporium BKM-F-1767		BLK 10A		*Thermomyces lanuginoses* MED 4B1		MTZ 95		*P. pseudomagnolia* e nom. prov.	
	Sterile controls	*Treated samples*	*Sterile controls*	*Treated samples*	*Sterile controls*	*Treated samples*	*Sterile controls*	*Treated samples*	*Sterile controls*	*Treated samples*
Total extractives [a]	0.26	0.26	0.21	0.24	0.23	0.21	0.21	0.21	0.26	0.27
Terpenes and phenols	2.5	3.8	0.9	1.8	1.4	1.3	0.9	1.0	2.5	2.2
Free fatty acids total	9.6	12.8	8.4	5.5	9.4	5.1	8.4	6.7	9.6	10.3
16:0	4.8	4.7	3.9	1.9	4.7	2.0	3.9	2.1	4.8	2.9
18:2	0.4	3.6	0.6	0.3	0.4	0.4	0.6	0.3	0.4	1.3
18:1	1.1	3.6	1.2	0.9	1.2	0.7	1.2	1.9	1.1	1.3
18:0	1.5	1.6	0.8	0.8	1.1	0.7	0.8	0.8	1.5	0.9
20:0	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.4	0.3	0.4
22:0	0.5	0.5	0.4	0.4	0.5	0.3	0.4	0.3	0.5	0.5
23:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24:0	0.8	1.1	0.9	0.6	0.8	0.5	0.9	0.6	0.8	1.2
26:0	0.3	0.5	0.2	0.3	0.4	0.2	0.2	0.2	0.3	0.5
28:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1
Sterols total	71.9	70.4	79.5	77.6	79.8	81.0	79.5	80.9	71.9	72.6
β-sitosterol	61.0	60.3	63.5	68.1	67.6	72.4	63.5	68.6	61.0	63.8
Monoglycerides	1.4	2.1	1.1	1.4	1.6	1.3	1.1	0.9	1.4	1.8
Diglycerides	0.9	0.0	1.3	0.2	0.7	0.6	1.3	1.3	0.9	0.4
Triglycerides	8.6	5.6	4.9	9.7	2.7	7.2	4.9	4.8	8.6	9.1

[a] Total extractive content is given as a percentage of oven dry wood weight. All other values given as a percentage of total volatile extract.

Table 5 Properties of pulp from treated and control samples

Sample	*Residual Black Liqour (g/l)*	*Screened Yield (%)*	*Rejects (%)*	*Kappa No.*	*Weighted Fibre Length (mm)*	*Fibre Coarseness (mg/100m)*	*Fibres Per Gram (millions)*
P. chrysosporium BKM-F-1767	5.68	41.3	13.94	36.3	0.82	8.40	18.9
BLK 10A	6.33	48.2	1.45	33.0	0.79	8.00	20.2
Thermomyces lanuginoses MED 4B1	6.18	49.3	0.66	29.0	0.79	7.90	20.4
MTZ 95	7.50	48.1	0.65	27.2	0.79	6.60	24.0
P. pseudomagnoliae nom. prov.	6.89	55.1	0.50	28.2	0.78	6.50	24.8
30°C sterile controls	6.99	50.5	0.61	30.6	0.80	7.10	22.4
30°C sterile controls	5.91	43.9	3.37	37.0	0.86	8.80	17.2
50°C sterile controls	6.90	55.4	0.42	28.2	0.79	6.50	24.0

Table 6 The effect of commercial lipases on deposited brown stock pulp extract

		Rhizopus arrhizus lipase		*Mucor javanicus lipase*	
	Negative control	*Positive control*	*Treated sample*	*Positive control*	*Treated sample*
Terpenes and phenols	0.6	0.3	0.5	0.4	0.4
Free fatty acids	20.1	19.6	18.4	17.7	18.8
16:0	6.8	7.2	6.4	5.8	6.2
18:2	4.6	4.5	3.9	4.2	4.3
18:1	4.6	4.5	3.9	4.2	4.3
18:0	1.1	1.1	1.2	1.0	1.2
20:0	0.5	0.5	0.6	0.5	0.6
22:0	0.8	0.7	0.7	0.7	0.6
23:0	0.0	0.0	0.1	0.0	0.1
24:0	1.1	0.8	0.9	0.9	0.9
26:0	0.6	0.4	0.7	0.5	0.6
28:0	0.0	0.0	0.0	0.0	0.0
Average sterols	61.4	59.9	59.1	61.2	57.2
β-sitosterol	44.0	47.1	46.0	49.1	45.3
Monoglycerides	1.4	1.8	2.2	2.0	2.6
Diglycerides	0.6	0.0	0.9	0.0	0.3
Triglycerides	5.2	7.6	7.8	7.5	8.8

5.1.4 Conclusions

Although none of the strains reduced the total extractive content of the wood chips, the triglyceride content was decreased by seven of the nine fungal strains. Wallis and Wearne (1999) obtained similar results when they seasoned *Eucalyptus globulus* wood chips. The total extractive content they determined for *E. globulus* is similar to that of the *E. grandis* of this study. This result indicates that the reduction of the total extractive content can not be used as a measure of the ability of a fungal strain to combat pitch. The effect on the triglyceride content should rather be employed as measure. More triglycerides would be released into process waters by the sterile controls than the treated samples due to the higher triglyceride content of sterile wood controls.

The apparent natural variation in extractive composition of the sterile controls made it difficult to compare the ability of different fungal strains to reduce extractive and triglyceride content. All the white-rot fungi compared well to *O. piliferum* CAR 58 and *P. chrysosporium* BKM-F-1767 in reducing the triglyceride content of *E. grandis*. The South African isolate white-rot *P. pseudomagnoliae* nom. prov. gave good results and can therefore act as an agent for both biopulping (Cerff, 1999) and biodepitching.

Pulping results were variable. Treated pulp samples did not show a significant difference in triglyceride content or pulp characteristics compared to the sterile controls. Triglycerides were not eliminated from the pulp extract as reported in a previous study on the effect of industrial pulping on pulp extracts (Scheepers *et al.*, 2000). Laboratory scale pulping gave variable results and did not correlate well with industrial scale pulping results. Consequently, no outright conclusions could be made on the basis of laboratory scale pulping results before industrial scale trials are done.

The addition of commercial lipases did not affect the triglyceride content of deposited pulp extract. The addition of lipases in pulping and bleaching processes would therefore not affect already deposited pitch.

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Chapter 6: General discussion and conclusions

6.1 General discussion and conclusions

In recent times the pulp and paper industry has looked to fungal and enzymatic biotechnology to reduce chemical consumption and cut production costs. Some pulp mills have already employed these technologies successfully. Pulping is no longer confined to the cooking process in digesters. Since the advent of fungal biopulping, pulping can commence on the chip piles, so reducing chemical requirements in the caustic digestion process. Fungal growth on wood chip piles and lipase addition to pulp in suspension can also decrease the use of chemical pitch control agents. The identification of a fungal strain that performs the dual task of biopulping and depitching would thus be beneficial to the pulping industry.

Chapter 2 introduces all the relevant factors to combating pitch with either fungal or enzymatic means. Triglycerides are thought to be the key to pitch troubles. They tend to adhere to hydrophobic surfaces and build up to become pitch deposits. Commercial lipases are already employed cost effectively in some pulp mills to hydrolyse triglycerides in a pulp suspension. The result is improved product quality and less downtime to clean forming wires and other equipment. Wood structure is such that wood is easily penetrable by fungal hyphae. Consequently, resin deposits incorporating triglycerides are accessible to fungi. Some fungal strains can decrease the triglyceride content of wood while growing on wood chip piles. In this way less triglycerides enter the papermaking process and thus the propensity for pitch deposition is reduced. Pulp mills in South Africa pulping *Eucalyptus spp.* could benefit from fungal pitch control, especially those in the warm, humid climate areas of South Africa where conditions favour accelerated fungal growth.

The results of analyses of wood and pulp extracts as well as pitch presented in Chapter 3 indicate either that triglycerides were in suspension in the white water or that very small amounts carried by the pulp deposited over an extended period of time. As reported by Farrell *et al.* (1997), triglycerides may also be the key to pitch deposition in this mill situation. The presence of amide derivatives of fatty acids can be ascribed to the use of non-ionic organic additives provided by Buckman Laboratories (Pty) Limited. The

composition of the E2 mat pulp extract differed considerably from pulp extracts from other stages. The increase in fatty acid amides and decrease in fatty acids may be ascribed to extreme conditions in the second caustic extraction stage. However, the first extraction stage did not have such a marked effect on the pulp extract. The decrease in fatty acid amides in the bleach stock decker mat pulp extract as well as the normalisation of fatty acid levels could be attributed to the addition of talc and the change in process conditions.

The lipase production capability of several strains isolated in South Africa was compared to that of commercial control strains (*Phanerochaete chrysosporium* BKM-F-1767 and *Ophiostoma piliferum* CAR 58) in Chapter 4. *P. chrysosporium* BKM-F-1767 is a biopulping and *O. piliferum* CAR 58 a biodepitching strain. Results indicated that a few of the strains compared favourably and even outperformed the control strains, indicating the potential for the use of these strains to break down wood triglycerides. Some of the isolated strains were white-rot fungi, which could also serve as biopulping agents.

Chapter 5 illustrates the effect of the isolated strains compared to the control strains on wood extract composition. All the white-rot fungi compared well to *O. piliferum* CAR 58 and *P. chrysosporium* BKM-F-1767 in reducing the triglyceride content of *Eucalyptus grandis*. The South African white-rot isolate *Phanerochaete pseudomagnoliae* nom. prov., known for its biopulping potential, reduced the triglyceride content substantially. Results indicate that the reduction of total extractive content can not be used as a measure of the ability of a fungal strain to combat pitch. The effect on the triglyceride content should rather be employed as measure. The addition of commercial lipases did not affect the triglyceride content of deposited pulp extract. The addition of lipases in pulping and bleaching processes would therefore not affect already deposited pitch.

The South African isolate *P. pseudomagnoliae* nom. prov. proved to be the best candidate for combined biopulping and pitch control in one step. Its use could therefore reduce the need for pulping and pitch control chemicals.