KRAFT BIOPULPING OF *Eucalyptus grandis.*

by

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DECLARATION

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

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SUMMARY

Biotechnology has been earmarked as the field that will shape the future of the world as we know it and its advance has not excluded the pulp and paper industry. The importance of biotechnology in the paper industry is emphasized by the large amount of research that has been performed in the various fields of its application ranging from biopulping, biobleaching to deinking technology. However, biopulping research has focused on biomechanical pulping, while little attention has been placed on chemical pulping methods and the advantages derived from fungal pre-treatment. Therefore, the effect of the pretreatment of *Eucalyptus grandis* wood chips, with white-rot fungi, on the Kraft pulping process was investigated. *Eucalyptus grandis* is the predominant hardwood species used in the pulp and paper industry of South Africa. Initially a screening program was performed in order to evaluate the biopulping potential of 100 different fungal cultures, isolated in South Africa. Wood chips were inoculated with selected fungal cultures and incubated for a two-week period, at the relevant optimum growth temperatures, under aseptic conditions. Bio-degraded wood chips were pulped by means of the Kraft pulping process and the resultant pulp and black liquor were analysed for yield, shives, kappa number and residual active alkali. Twelve cultures, which showed the most potential for biopulping, were selected from the screening program for further evaluation and statistical validation. Results indicate that certain selected cultures affect significant improvements in the residual active alkali of just under 21%, while the kappa number was reduced by just less than 9%. Under the prevailing conditions, the chemical consumption decreased significantly by ca. 2%. No significant differences, between the test cultures and the control, were observed for yield and shive content. These results prove conclusively that Kraft biopulping of *Eucalyptus grandis* wood chips has a significantly positive effect on the pulp produced by this process. The implication of savings in both pulping and bleaching chemicals are enormous. It is strongly suggested that a mill-scale trial be performed, upon which a full investigation into the financial implications of Biokraft pulping should be determined.

OPSOMMING

Biotegnologie word as 'n veld beskou wat die toekoms van die wêreld kan verander. Diè vooruitgang het nie die pulp en papier bedryf onaangeraak gelaat nie. Die belang van biotegnologie vir die papierbedryf word beklemtoon deur die groot hoeveelheid navorsing wat in die verskillende aanwendingsgebiede, soos bioverpulping, biobleiking en ontinkingtegnologie, plaasgevind het. Bioverpulpingsnavorsing het egter gekonsentreer op biomeganiese verpulping, terwyl min aandag aan chemiese verpulpingsmetodes en die voordele verbonde aan fungale voorafbehandeling gegee is. Daarom is die effek wat voorafbehandeling van *Eucalyptus grandis* houtspaanders, met witverottingsfungi, op die Kraft verpulpingsproses het, ondersoek. *Eucalyptus grandis* is die vernaamste loofhoutspesies in die pulp en papierbedryf van Suid-Afrika. Eerstens is 'n siftingsprogram uitgevoer om die bioverpulpingspotensiaal van 100 verskillende funguskulture, wat in Suid-Afrika geïsoleer is, te evalueer. Houtspanders is toe met geselekteerde funguskulture geinokuleer en vir 'n tweeweeklange periode, by optimale groeitemperature en onder aseptiese toestande, geïnkubeer. Die houtspaanders wat sooende deur die fungus afgebreek is, is verpulp deur gebruik te maak van die Kraft-proses. Die pulp en swartloog is getoets vir opbrengs, veselbondels, kappa nommer en oorblywende aktiewe alkali. Die twaalf kulture wat die beste potensiaal vir bioverpulping getoon het, is gekies vir verdere evaluering en statistiese ontleding. Resultate toon aan dat sekere kulture 'n beduidende verbetering in oorblywende aktiewe alkali van ongeveer 21% bewerkstell het, terwyl die kappa nommer met net 9% verlaag is. Onder hierdie omstandighede is chemikalieë verbruik met ongeveer 2% verlaag. Geen beduidende verskille is tussen die kulture en kontrole gevind in terme van opbrengs en veselbondels nie. Hierdie resultate bewys onomstootlik dat Kraft-bioverpulping van *Eucalyptus grandis* houtspaanders 'n beduidend positiewe invloed op pulpproduksie toon. Reuse besparings op beide verpulpings- en moontlikke bleikingschemikalieë word deur die resultate geimpliseer. Dit word dus ten sterkste aanbeveel dat 'n ondersoek op industrieële skaal uitgevoer word, sodat die ekonomiese haalbaarheid bepaal kan word.

BIOGRAPHICAL SKETCH

Craig Bradley Cerff was born in Cape Town, South Africa, on 16 September 1972. He matriculated at Fairmont High School, Durbanville, Cape Town in 1990. In 1991 he enrolled at the University of Stellenbosch and obtained his B.Sc. (Wood Science) degree in 1994. He spent most of 1995 obtaining practical experience while working at Gruber and Weber, a German Carton-board manufacturer. He enrolled as an M.Sc. (Wood Science) student in the Department of Wood Science at the University of Stellenbosch. He is currently employed as an Engineer in Training at Mondi Kraft Ltd., an integrated pulp and paper mill. He has also enrolled at Heriot-Watt University, Edinburgh, Scotland for his Masters in Business Administration (MBA).

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CHAPTER 1

BACKGROUND AND PROJECT AIMS

1.1. BACKGROUND

Pulp and paper-making technologies undergo constant improvements due to market demands and developments in research. The need for sustainable technologies has also brought biotechnology into the realm of pulp and paper-making. Enzymatic as well as fungal treatments are being developed to increase pulp brightness, to reduce troublesome pitch, to improve the quality of waste paper and to purify bleach plant effluent. Biotechnological attempts to improve primary pulp producing processes by using isolated ligninolytic enzymes, have so far been inhibited by the complex chemistry of the ligninolytic enzyme system, low yields in enzyme production, the ultrastructure of the wood itself (the close association of lignin, hemicelluloses and cellulose in the cell wall may hamper the specific action of enzymes) and rather long reaction times (two or more weeks). White-rot fungi, however, have a great potential for biotechnological applications. They not only produce a whole set of enzymes necessary for lignin degradation, but can also act as a transport system for these enzymes by bringing them into the interior of the wood chips (by means of hyphae) and creating the physiological conditions for the enzymatic reactions. The use of white-rot fungi for the treatment of wood chips prior to mechanical pulping or chemical pulping is defined as 'biopulping'. In mechanical pulping, the aim is to reduce the energy demand and to increase paper strength properties. For chemical pulping, biopulping objectives include: a reduction in the amount of pulping chemicals required, a possible yield increase, an increase in the cooking capacity (throughput), or to enable extended cooking, resulting in lower consumption of bleaching chemicals. When applied prior to mechanical pulping as well as to chemical pulping, biopulping will also help to decrease the negative environmental impact associated with pulp and paper production (1).

Although solid state delignification of wood chips is relatively new to the pulp and paper industry, a number of biotechnological processes have already successfully been applied. The well known Sandoz Cartapip process for decreasing the pitch content of wood chips is a similar process to biopulping.

Other biotechnological advances in the paper industry include enzymatic bleaching, enzymatic deinking, effluent treatment and secondary fibre treatment. Ordinary composting, used commercially in many locations around the world, is a good example of the viability of commercial biopulping (2).

As an industrial process, biopulping would entail large-scale 'solid-state fermentation' of wood chips before they are pulped. Chips would be steamed briefly to reduce the natural microflora, then inoculated with a fungal suspension. The chips would then be incubated for 2 weeks or less, probably with forced aeration in chip piles. The 'softened' chips would be pulped via various methods i.e. chemically, mechanically or thermomechanically. One of the fundamental problems involved in any biological treatment processes, is the time involved for the reactions to occur. Time is the major factor frustrating the implementation of biopulping in the paper industry today. However, the lower limit, in days, necessary for successful biopulping has not yet been determined and is influenced by a large number of factors. Substantial research is needed to establish the minimum amount of time needed to obtain the maximum benefit of biopulping without disturbing the pulping process significantly. It is most likely possible that the reaction time of lignin degrading fungi may be reduced (to less than ten days), through various means, in order to satisfy the constraints set by the pulp and paper industry.

A second challenge is contamination by external competing microflora. These are often the brown-rot (cellulose degrading) fungi, which display extensive antagonism towards the cultures used during biopulping. Solutions, as will be seen later, have already been discovered for the thermomechanical process but these may prove to be impractical for the large volumes of chips consumed during chemical pulping procedures. Only once these two primary challenges have been solved, can biopulping make positive advancements in the pulp and paper industry!

1.2. PROJECT AIMS

In the study, by means of a preliminary screening process, a variety of 100 unidentified South African fungal strains were tested for their biopulping potential (ligninolytic activity).

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Fungi displaying the best biopulping potential were subsequently evaluated on *Eucalyptus grandis* wood chips using the Kraft pulping process and their activity tested against pulp yield, kappa number and residual active alkali. Finally, the most promising fungal strains were to be used in an experiment to statistically verify the results.

1.3.　LITERATURE CITED

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CHAPTER 2

BIOPULPING: AN OVERVIEW

2.1. INTRODUCTION

Wood decay and degradation has been known to man since before he started using wood as a building material.   However, the optimum use of micro-organisms to achieve set objectives, necessitates a better understanding of the substrate (wood and all it is elements) as well as the biochemical action of fungi involved during the biodegradation of wood.

The first part of this chapter attempts to briefly describe the nature of wood, the various fungi involved in its biodegradation and the action of their various enzymes on specific wood components.   The second part describes the practical aspects involved in the application of biopulping and summarises the results achieved to date.

Biopulping is defined as the treatment of wood chips with lignin-degrading fungi prior to pulping.   The use of white-rot fungi for the biological delignification of wood was first studied at the West Virginia Pulp and Paper Company (now Westvaco) in the 1950s (62).   The researchers published a survey of the literature (covering 72 lignin-degrading fungi), which pointed to the dearth of knowledge about the fungal degradation of lignin.   In the 1970s, Eriksson and co-workers at the Swedish Forest Products Laboratory (STFI) in Stockholm demonstrated that fungal treatment could result in significant energy savings for mechanical pulping.   That research resulted in a U.S. patent, which described a "method for producing cellulose pulp." Considerable efforts at STFI were directed towards developing cellulase-less mutants of selected white-rot fungi to improve the selectivity of lignin degradation and thus the specificity of biopulping. However, the researchers encountered some difficulties in attempting to scale-up the process. Further research is needed to solve the above problems (62).

Most early work concentrated on the use of white-rot fungi as a pre-treatment step for mechanical pulp production (1).

2.2. THE STRUCTURE AND COMPOSITION OF WOOD

Wood is cellular wall material produced by the cambium in a living tree and contains cellulose, hemicellulose and lignin as major components. It is a porous material consisting of a matrix of plant cell walls and air spaces. The air spaces exist in the form of cavities (lumens) and to a much lesser extent as voids within the cell walls.

Wood can be grouped into two major groups: softwoods, i.e., gymnosperms (pine) and hardwoods, i.e., angiosperms (*Eucalyptus*, wattle, etc.) both of which differ considerably in cell type. Softwoods have mainly two types of cells, long (2-5mm) trachieds, which provide strength to wood and are responsible for vertical water transport, and smaller ray cells, which transport water in the horizontal direction. In addition, there are both vertical and horizontal resin canals.

Hardwoods possess more diverse types of cells. In general, wood composed of an array of vessels, fibres and ray parenchyma cells in various arrangements. These cells provide for water conduction, strength, as well as transport and storage for nutrients (3).

Figure 2.1 shows a schematic microscopic composition of a wood fibre illustrating its layered structure. It consists of the primary wall (P), secondary wall, and the middle lamella (ML). The secondary wall is further subdivided into three layers (S_1, S_2 and S_3) each composed of cellulose microfibrils in distinct orientations with reference to the main vertical fibre axis. The S_2 is about 1-10 micron thick and contains 80-95% of the cell wall material. The primary wall contains a network of cellulose microfibrils and significantly higher lignin concentrations than the secondary wall, while the middle lamella is composed predominantly of lignin. However, due to its large comparative volume, the secondary wall contains approximately 70% of the lignin (4).

2.2.1. CELLULOSE

Cellulose is considered to be a linear homopolymer of anhydroglucose units linked with β-1,4 glucosidic bonds. However, the true repeating

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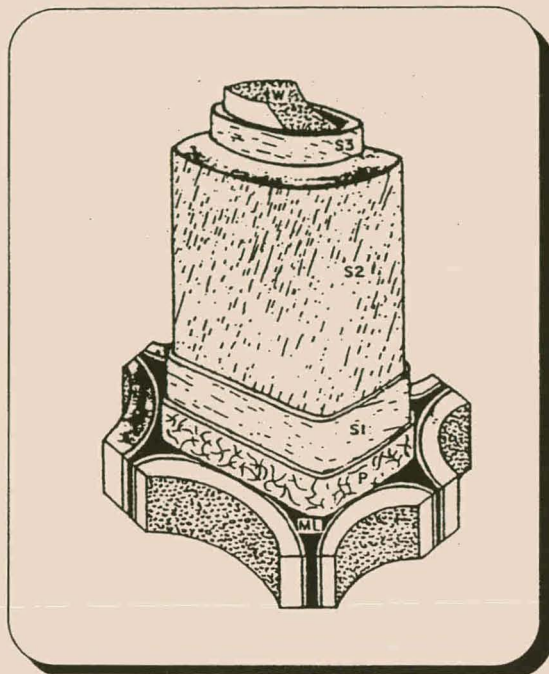
Figure 2.1. Diagrammatic representation of a softwood trachied[3].

stereochemical unit of cellulose is cellobiose, (β-1,4-D-glucosyl-D-glucose), (**Figure 2.2**). Some natural materials, such as cotton, are almost pure cellulose. Cotton is mostly α–cellulose, which is insoluble in 17.5% NaOH.
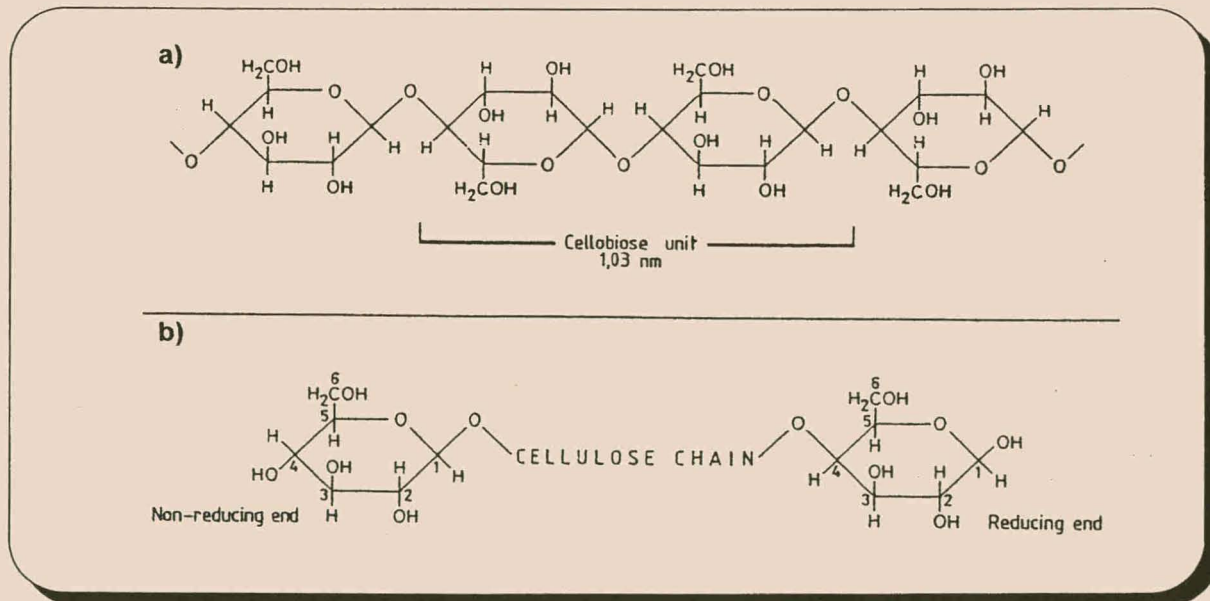
Figure 2.2. Formula of cellulose[4].

a) Central part of the molecular chain.

b) Reducing and non-reducing end group of the molecule.

The cellulose molecule is a polymer with a high degree of polymerisation (DP) of up to about 15000. The cellulose molecules are thread-like and form bundles known as fibrils which are laterally stabilised by hydrogen bonds that are formed between hydroxyl groups of adjacent molecules. The cellulose chains are packed in groups of about 30 to form microfibrils which are in turn packed in units of a hundred to form fibrils. Fibrils are further packed to form the cellulose fibre. The microfibril has been considered to be stronger than steel of corresponding size and thus significantly contributes to the strength of wood (3).

2.2.2. HEMICELLULOSES

Hemicelluloses are short chains of branched hetero-polysaccharides composed of both hexoses and pentoses. D-xylose and L-arabinose are the major constituents of pentosans (xylans), while D-glucose, D-galactose and D-mannose are the constituents of the hexosans (mannans). The major hemicelluloses component of softwood is mannan-based whilst the hemicelluloses in hardwood are xylan-based. They comprise 20-25% of hardwoods and 7-12% of softwoods. The close association of hemicelluloses with cellulose and lignin in the fibre cell walls contributes both to rigidity and flexibility. Hemicelluloses are composed both of neutral sugars, all present as their respective anhydrides, e.g., xylan, arabinan, glucan and mannan (substituted with acetyl groups), and of uronic acids. Hemicelluloses, in total, constitutes approximately 26% of hardwood and 22% of softwood and have degrees of polymerisation (DP's) of 100 to 200. Apart from galactose-based hemicelluloses, which are characterised by β-1,3-linkages, most of the hemicelluloses are built up by β-1,4-linkages between their backbone sugars. The type and amount of hemicelluloses vary widely, depending on plant materials, type of tissues, growth stage, growth conditions, storage and method of extraction. The mannans, galactoglucomannans and glucomannans in softwoods and hardwoods, are branched heteropolysaccharides.

Xylans appear to be a major interface between lignin and other carbohydrate components in many isolated phenolic-carbohydrate complexes, where they are probably covalently linked to phenolic residues via arabynosil and/or glucuronosyl residues. Xylans tend to adsorb onto cellulose and to aggregate with other hemicellulose components, probably as a result of hydrogen-bonding. Xylan may play a major role in cell wall cohesion, since its selective removal from delignified wood fibre results in substantial increases in fibre porosity(3).

2.2.3. LIGNIN

Second only to cellulose, lignin is the most abundant biopolymer on earth. It is estimated that the planet currently contains 3×10^{11} metric tons of lignin with an annual biosynthetic rate of approximately 2×10^{10} tons. Lignin constitutes approximately 30% of the dry weight of softwoods and about 20% of the weight of hardwoods. It is found in the highest concentration in the middle lamella, but is most abundant in the secondary wall due to the comparative volume of this part of the cell wall compared to the rest of the cell components. Lignification is associated with the development of vascular systems in plants, resulting in resistance to biodegradation and environmental stresses such as changes in the water balance and humidity. The presence of lignin within the cellulosic fibre wall, mixed with hemicelluloses, creates a naturally occurring composite material that imparts strength and rigidity to trees and plants. It acts as a permanent bonding agent between cells as well as a UV light stabiliser, antioxidant and water-proofing agent. The water permeation-reducing property of lignin plays an important role in the internal transport of water, nutrients and metabolites in the plant. Lignin is not a uniform compound, but is a collective form of substances that have similar chemical properties but very different molecular masses. The molecular mass of lignins may be 100 kDa or greater. Lignins are highly branched molecules consisting of phenyl-propane-based monomeric units (**Figure 2.3**) linked by different types of bonds, such as alkyl-aryl, alkyl-alkyl, aryl-aryl ether and carbon-carbon bonds. The relative proportions of three cinnamyl

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alcohol precursors incorporated into lignin, i.e., *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, vary not only with the plant species but also with the plant tissues and the location of lignins within the plant cell wall. Ecological factors such as the age of the wood, climate, plant fertilisation and the amount of sunlight also affect the chemical structure of lignins. Physically, lignins are amorphous polymers that have no crystallinity. Native lignins are insoluble in water and difficult for micro-organisms to penetrate and degrade. They are generally acid stable but can be solubilized under the alkaline conditions as used during the Kraft pulping processes (**3, 4**).
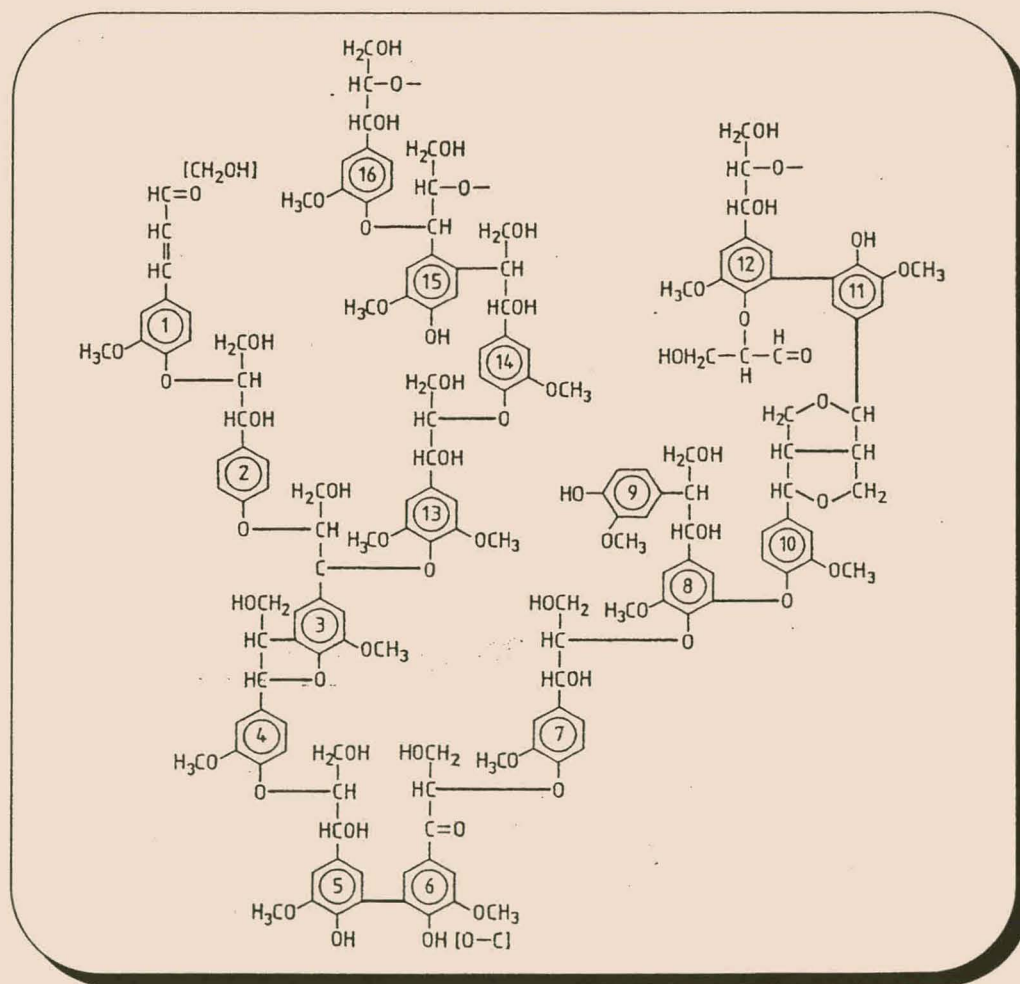
Figure 2.3. Structural scheme of spruce lignin[4].

2.2.4. OTHER CELL WALL COMPONENTS

The plant cell wall also contains structural proteins called extensions. Three kinds of cell wall proteins, which differ in amino acid composition, have been identified in plants. The existence of lignin-protein complexes suggests a role for proteases in the degradation of woody plant materials, particularly in the delignification processes, e.g., pulp bleaching, and may prove beneficial for pulp and paper manufacturing processes. Plant cell walls also contain extraneous materials, including extractives and non-extractives. Depending upon species and growth conditions, wood contains from 0.4 to 8.3% extractives on a dry weight basis. The extractives can be broadly divided into three groups, e.g., terpenes, resins and phenols. Non-extractives make up about 0.2-0.8% of the dry weight and include inorganic components such as silica, carbonates, oxalates (organic salts) and non-cell wall substances such as starch, pectin and protein (**3, 4**).

2.3.   FUNGAL DEGRADATION OF WOOD

2.3.1. WHITE-ROT

The white-rot fungi belong to the *Basiodimycetes* family and are thought to be able to decompose all the structural components of wood (cellulose, hemicelluloses and lignin). White-rot fungi appear to preferably attack and degrade hardwood species. Selective white-rot, in contrast to simultaneous white-rot, is characterised by a preferential removal of lignin throughout the wood cell wall (**5-10**). It is in part the removal of the lignin that gives 'white-rotted' wood its whitened appearance, although oxidative bleaching reactions also cause whitening. Once the delignification process has started in wood cells, the secondary cell walls are delignified quite rapidly (**7**). Middle lamellae and especially cell corners are more resistant but are finally degraded as well. Vessel elements seem to be most resistant to degradation (**8**). In the late stages of decay, lignin and probably also hemicelluloses are completely removed, while cellulose remains relatively intact (**7, 9**). The most

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remarkable example of selective delignification is 'palo podrido', which are whole logs that have been extensively delignified by *Ganoderma* species (**11**), and which are used as a forage feed for cattle in southern Chile. This type of wood decay seems to occur only under special environmental conditions and impressively demonstrates the delignification potential of certain white-rot fungi. Microscopical examination of degradation patterns of selective white-rot fungi under laboratory conditions has shown that delignification does not occur uniformly throughout the wood (**7**). Advanced degradation stages can be found adjacent to apparently unaffected areas. Selective white-rot fungi usually also cause simultaneous rot (removal of all cell wall components), and both types of degradation can be found at the same time in localised areas of a wood sample. These alternating and morphologically distinct areas give decayed wood a typical macroscopical appearance which has been referred to as white-pocket rot and white-mottled rot (**5**).

There are several important facts that have to be kept in mind when discussing the mechanism of biopulping. The greatest biopulping effect can be obtained at only a few percentage weight losses, which may be attributed to the degradation of the nutrient-rich ray cells. At this stage of degradation, the vast majority of the wood fibres are morphologically no different from those in sound wood. This means that all major structural components, including lignin, are still present in the wood cell walls after biopulping. It is also evident from biopulping studies that considerable chemical changes inside the wood cell walls take place during fungal treatment because much less energy is needed to separate wood cells in the subsequent refining process (**1**).

Based on laboratory cultures, several white-rot fungi have been selected as being potentially important for biopulping operations. These fungi exhibit selective white-rot attack. However, the distinction is not widely made between simultaneous and preferential white-rot because the two types of decay may occur together and culture conditions (presence of nitrogen sources, etc.) may influence the type of decay produced (**12**).

2.3.2. BROWN-ROT

These fungi also belong to the *Basidiomycetes* family and essentially degrade the polysaccharides in wood. A small amount of lignin modification also takes place. A brown residue (rich in tannin and extractives) is left behind on the wood and the wood appears brittle. Most brown-rot fungi tend to preferentially attack softwood species.

Two subgroups of brown-rot have been identified:

a) Dry-rot

Dry-rot is well known and causes severe damage to wooden structures. The dry-rot fungus, *Serpula lacrymans*, is found in buildings in temperate regions and sometimes in wooden structures used in mine shafts. In North America the species, *Poria incrassata*, is responsible for a similar type of damage and is able to withstand dry periods. Although, a *Poria incrassata* fungus cannot grow on dry wood, it differs from all the other fungi in that it has special hyphae (sometimes > 2 mm od) that can transport water over obstacles for a number of meters.

b) Wet-rot

This type of brown-rot is much more prevalent and is commonly referred to as the cellar fungi (*Coniophora puteana*). This is the most important wet-rot fungus and grows best on woods having a moisture content higher than 45%. It is often found in roofs, cellars and other places in buildings in the presence of leaking water or high rates of condensation. Degradation often occurs internally with no surface evidence of rotting. However, wet-rotted wood appears very dark brown; nearly black.

2.3.3. SIMULTANEOUS OR SOFT-ROT

This group of fungi belongs to the *Ascomycetes* and *Fungi Imperfecti* families and degrade lignin, as well as polysaccharides, in wood under very wet

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conditions, such as cooling towers or in the ground. The rate of degradation for the various wood components varies by species. Simultaneous-rot attacks both soft and hardwood species but preferentially degrades hardwoods. The wood loses its strength and becomes discoloured, soft and has an aged appearance.

2.4. FUNGAL DEGRADATION OF LIGNIN

Biodegradation of lignin has been studied for more than 80 years with only incremental progress over much of the time. Early assays employed the disappearance of acid-insoluble Klason lignin. One of the major bottlenecks in lignin degradation research, until about 15-20 years ago, was the lack of simple, sensitive, and accurate assays for measuring lignin degradation and decomposition. The development of synthetic ^{14}C-labelled lignins enabled rapid radiorespirometric determination of lignin degradation under controlled conditions, and a series of experiments using this assay led to improved understanding of the culture and nutritional conditions under which biodegradation occurs. However, there are important limitations to these methods. Firstly, they do not take into account the degradation of lignin into water-soluble intermediates. Secondly, the radiolabeled synthetic lignins are still relatively expensive and not readily available. Furthermore, care should be taken to ensure that the synthetic lignin prepared is polymeric, which is a potential problem with DHPs (Dehydrogenative Polymerizates). Polymeric dyes, such as poly R, poly B, remazol blue, and others, have been used as indicators of lignin degradation, but these are qualitative at best and any results obtained must be verified by studying the degradation of [^{14}C]DHPs, milled wood lignin, or other substrates (13).

Numerous studies involving lignin model compounds have been published. These model compounds have proven invaluable for the elucidation of the key steps involved in the degradation of the lignin polymer and for studying the mechanism of action of enzymes implicated in lignin degradation. The complexity of lignin prevents meaningful biochemical studies on the polymer itself; so concepts about the biodegradative mechanisms are derived from

the studies with the biodegradation or modification of model compounds and direct chemical characterisation of products from wood decayed under ligninolytic conditions. It is not completely clear whether the reactions observed in oligomeric substrates are completely related to what occurs in the native lignin polymer. One of the principal problems in employing model compounds in whole cultures is to carry out the cleavage of interest while preventing subsequent reactions that lead to degradation or polymerisation. The problem of sequential reactions is particularly acute because the initial reaction of the interest is often rate-limiting. A breakthrough in the design of model lignin substrates came with the use of the non-phenolic model compound 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol (**Figure 2.4**). Biodegradation in ligninolytic cultures shows that primary cleavage occurs between C_{α}- C_{β} with incorporation of molecular oxygen (**14**).
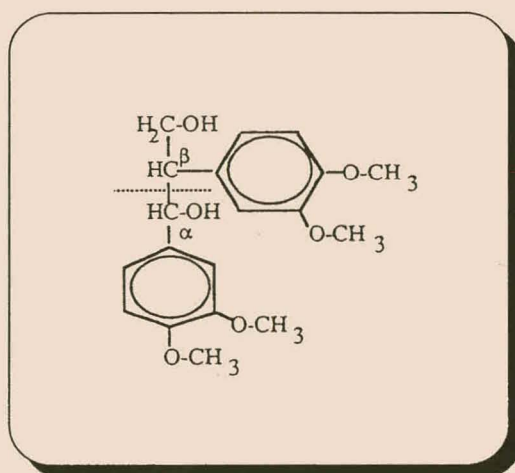
Figure 2.4. A non-phenolic model compound. 1,2-bis(3,4-dimethoxyphenyl) propane-1,3-diol[14].

2.4.1. CHEMISTRY OF LIGNIN DEGRADATION BY WHITE-ROT FUNGI

Lignin biodegradation has been studied through two complementary approaches: (i) Degradation of the polymeric lignin (**15**) such as synthetic lignin (DHP), milled wood lignin (MWL), and (ii) degradation of protolignin in the xylem cell walls by lignin-degrading *Basidiomycetes* (**16**).

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Lignin degraded by white-rot fungi contains less carbon, hydrogen and methoxyl groups but more oxygen, carbonyl and carboxyl groups. The increase in the carbonyl groups is largely due to the conjugation of the aromatic rings although about one third of the carboxyl groups attaches this way. The remainder of the new carboxyls are conjugated to α,β-unsaturated aliphatic structures. Demethoxylation of the lignin can be up to 25%. These modifications therefore involve oxidative reactions of both the aromatic nuclei and the propyl side chains. The degradative system involved shows little substrate specificity and will oxidise a variety of different lignin compositions. During lignin degradation, a variety of low molecular weight compounds are produced (**17, 18**). The most common of these in spruce wood is vanillic acid (20% of the acid fraction). Other compounds are 2,6-dimethoxy-*p*-benzoquinone, isovanillic acid, veratric acid, meta-hemipinic acid, isohemipinic acid, 2-(2-hydroxy-3-methoxy-5-carboxyphenyl)-4-carboxy-β-butenolide and 5-(2-oxo-ethyl)vanillic acid. Each formed between 3 to 10 percent of this fraction. For different wood species, different fractions are obtained with varying complexity. The essential reactions of degradation involve cleavage of the side chains (C_α-C_β, C_β-C_γ) and oxidative cleavage of the aromatic nucleus. To cleave the aromatic ring, micro-organisms require two hydroxyl groups adjacent to each other or alternatively, three hydroxyl groups attached to the ring (**12**).

2.4.2. PHYSIOLOGY OF LIGNIN DEGRADATION BY WHITE-ROT FUNGI

Under laboratory liquid culture conditions, lignin degradation by *Phanerochaete chrysosporium* is favoured at an optimum pH of 4.5, with no agitation, high partial pressures of oxygen and the limitation of certain essential nutrients, principally nitrogen, although sulphur and carbon limitation are also effective (**12, 19**). The increase in the ligninolytic activity may be related to the very low levels of nitrogen found in 'solid' wood because wood is poor in nitrogen. However, in some studies, it has been shown that a high nitrogen concentration in the growth medium does not decrease ligninolytic activity. Thus the link between nitrogen depletion and ligninolysis is not

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universal amongst white-rot fungi (**23, 24**). It has further been noted that lignin degradation, by *P. chrysosporium,* is improved when grown as mycelial mats rather than submerged pellets in agitated cultures (**12**). The correct choice of buffer and the correct concentrations of certain minerals and trace elements have also been found to play an important role in lignin degradation by *P. chrysosporium* (**20**). In certain fungi a decrease in Zn^{2+}, Fe^{2+} and Mo^{2+} has shown some stimulatory effect (**19**). In addition, Ca^{2+} concentration is important with 1 mM being favourable. Ligninase production was increased by increasing either Cu^{2+} or Mn^{2+} (**21, 22**).

Increasing the oxygen level has a multiple effect on lignin degradation. It leads to an increase in the titre of the ligninolytic system, including ligninase and the peroxide-producing system, and it also increases the activity of the existing lignin-degrading system evidently by increasing the supply of O_2 for degradative reactions and for peroxide production (**25, 26**).

In *P. chrysosporium* a co-metabolizable carbon source, e.g., glucose, is essential for lignin biodegradation and it occurs in response to nitrogen starvation. Ligninase production appears to form part of secondary metabolism in this organism. Carbohydrate starvation leads to a rapid but transient onset of ligninolytic activity. Carbon balance in wood is probably maintained and possibly limited by the rate of carbohydrate wood component breakdown in the cell wall during degradation. Nevertheless, the balance between energy producing and energy-yielding reactions in lignin mineralization is not known (**20**).

Ligninolysis is initiated when the growth of the fungus slows down and enters the stationary phase (**Figure 2.5**). Carbohydrate uptake and utilisation by the fungus are active during growth and are essential to maintain the primary metabolism within the hyphae. Carbohydrate assimilation still continues after ligninolysis has begun. However, when growth conditions become limiting for the hyphae, i.e. nitrogen becomes depleted, the mycelium enters a secondary metabolic phase and the lignin-degrading system is released by the fungus. This stage is characterised by the production of a marker metabolite, namely veratryl alcohol. Veratryl alcohol (**Figure 2.6**) is similar to lignin monomeric compounds and is derived from phenylalanine by the fungus (**27**).

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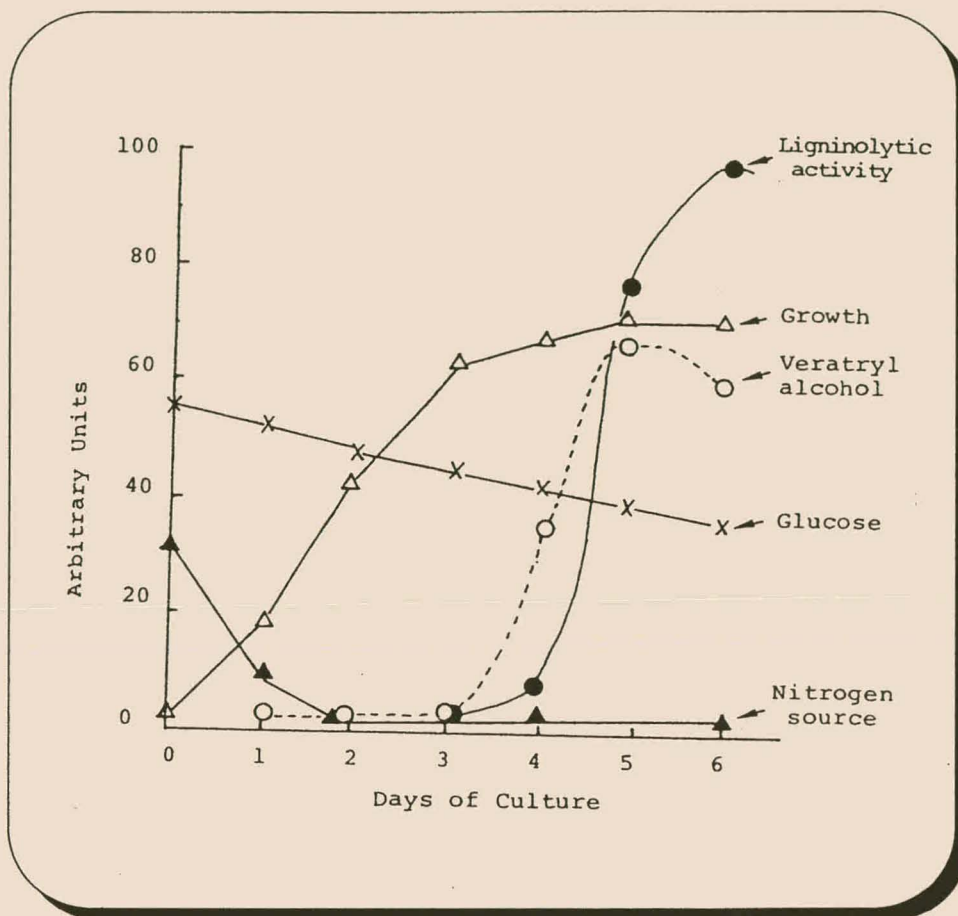
Figure 2.5. The relationship between growth, glucose depletion, nitrogen availability, veratryl alcohol release and the onset of ligninolytic activity in *Phanerochaete chrysosporium*[12].

The transition from primary to secondary metabolism is also associated with a transient increase in intracellular glutamate. Addition of exogenous glutamate or other nitrogen sources sharply suppresses secondary metabolism, including activity of enzymes involved in lignin degradation (**27, 28**).
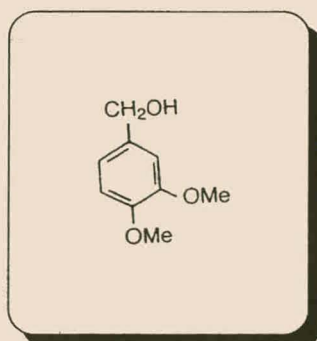
Figure 2.6. Veratryl alcohol[27].

2.4.3. ENZYMOLOGY

Enzymes are biocatalysts which accelerate and control biochemical reactions. Many of them show a high specificity, i.e. their reaction is restricted to certain molecules or molecular structures. Enzymes are often named according to the substrate on which they act or according to their action, whereby the suffix -*ase* is used, e.g. the cellulose splitting enzyme is named cellulase and enzymes effecting hydrolytic cleavage are called hydrolases. Apart from systematic names many enzymes bear trivial names unrelated to their function. Enzymes are produced intracellularly or extracellularly, and they may be secreted from the cell without losing their activity. Thus enzymatic reactions can be carried out in the absence of the parent cell from which the enzymes have been secreted. The biocatalysts are macromolecular proteins with a defined supramolecular structure containing an active centre, which is generally situated within a three-dimensional structure (**Figure 2.7**). In many cases the active centre consists of a complex organic molecule or a metal-ion (cofactor), and may or may not be chemically bound to the protein. The complex of protein (apoenzyme) and cofactor is called a holo-enzyme (**29**).

During the 1990's, in addition to the detailed studies on catalytic and enzymatic properties of lignin-modifying peroxidases and their molecular biology, major lines of research have involved applications of enzymes in biopulping and pulp bleaching, and a search for the enzymes responsible for lignin degradation in more selective lignin degraders, i.e. fungi which degrade larger amounts of lignin relative to carbohydrates. This led to a reassessment of the biotechnical potential of white-rot fungi other than *P. chrysosporium* and investigation of their ligninolytic enzyme systems. Since the discovery of two important peroxidases in the 1980's, namely lignin peroxidase (LiP) and mangenese dependant peroxidase (MnP), an array of ligninolytic enzymes has been isolated. According to their typical production patterns of extracellular ligninolytic enzymes, white-rot fungi may be divided into three main groups: (i) LiP-MnP group, (ii) MnP-laccase group, and (iii) LiP-laccase group, although overlaps and exceptions certainly occur. A fourth group may be the Laccase-AAO group, AAO denoting aryl alcohol oxidase (**30**).

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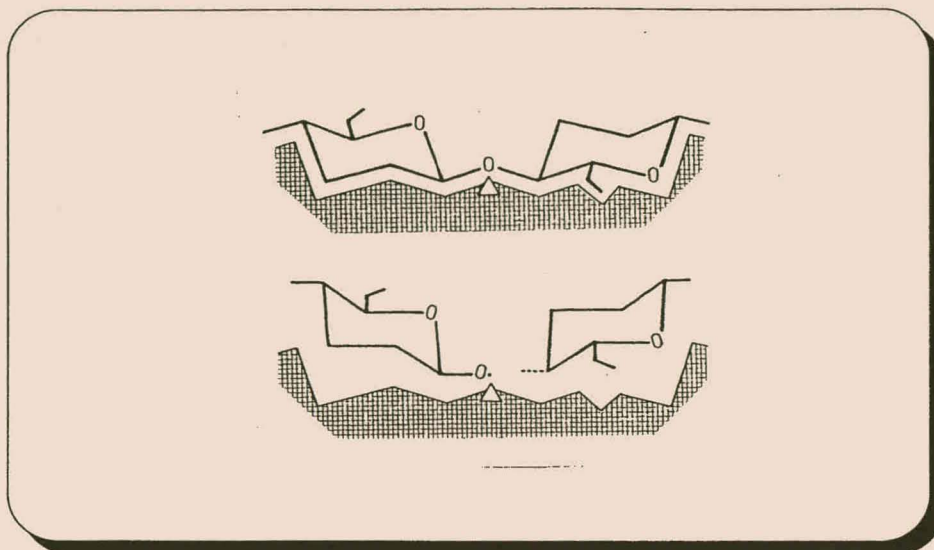
Figure 2.7. Splitting of a β-1,4-glycosidic linkage at the active centre of an enzyme. Triangle = cofactor, checked area = apo-enzyme[29].

LiP and MnP are heme-containing glycoproteins that require hydrogen peroxide as an oxidant. Fungi secrete several isoenzymes into their cultivation medium (**Table 2.1**), although the enzymes may also be cell-wall bound. LiP oxidises non-phenolic lignin substructures by abstracting one electron and generating cation radicals that are then decomposed chemically. It has been demonstrated that with synthetic lignin models, LiP is capable of C_α-C_β bond cleavage, ring opening and other reactions. However, its exact role in lignin biodegradation and in other related processes such as pulp bleaching, have been questioned (**31**).

MnP oxidises Mn(II) to Mn(III) which then oxidises phenol rings to phenoxy radicals that results in the decomposition of compounds. Laccase (benzenediol : oxygen oxidoreductase) is a copper-containing oxidase which utilises molecular copper as an oxidant and also oxidises phenolic rings to phenoxy radicals. It also has the capacity to oxidise non-phenolic compounds under certain conditions (**30**). Extracellular laccases are typically produced by white-rot fungi. Another extracellular enzyme involved indirectly in lignin degradation is the hydrogen peroxide-producing enzyme, glyoxal oxidase (**30**). Other enzymes important in lignin breakdown include: demethoxylases and the lignin monomer degrading enzymes, cellobiose dehydrogenase, vanillic acid hydroxilase and trihydroxybenzene dioxygenase (**12**).

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Table 2.1. Molecular mass (M) and isoelectric points of ligninolytic enzymes from selected white-rot fungi[30]

Fungus	Isoenzyme	M(kDa)	pI	Fungus	Isoenzyme	M(kDa)	pI
Phanerochaete	H1	38	4.7	*Phlebia brevispora*	MnP1	ND	ND
chrysosporium	H2	38	4.4		MnP2	ND	ND
BKM-F-1767	H6	40, 42	3.7		Laccase1	ND	5.2
(ATCC 24725)	H7	42	ND		Laccase2	ND	ND
	H8	42	3.5				
	H10	43	3.3	*Panus tigrinus*	MnP	43	3.2
	H3	ND	ND		MnP		2.95
	H4	ND	ND		Laccase1	64	3
	H5	ND	ND		Laccase2		2.9
	H9	ND	ND				
				Rigidoporus	MnP Form1	42	3.5
				lignosus			
Phlebia radiata	LiP1	42	4.1		MnP Form2	42	3.7
	LiP2	45	3.9		Laccase L1	53	3.8
	LiP3	44	3.2				
	MnP	47-48	3.8	*Ceriperiopsis*	MnP1	ND	ND
				subvermispora	MnP2	ND	ND
	MnPx	47	Ca. 9		Laccase	ND	ND
	Laccase	64	3.5				
				Junghuhnia	Laccase1	62	3.6
				separabilima	Laccase2	60	3.4
Phlebia radiata	PrS-L1	39	4.2		Laccase3	58	3.5
L 12-41	PrS-L2	39-40	3.1		LiPH2	47	3.5
	PrS-Mn1	44-45	3.7		LiPH3	44	3.4
	PrS-Mn2	47-48	3.75		LiPH4	43	3.4
	Laccase	ND	ND		Hemeprotein	44	3.4
Phlebia tremellosa	Mt-L1	35-36	3.1	*Phlebia*	LiP1	40	ND
				ochraceofulva	LiP2	44	ND
2845 (ATCC 48745)	Mt-L2	38-39	3.5		LiP3	38	ND
	Mt-L3	40	4		LiP4	43	ND
	MnP	ND	ND		LiP5	44, 46	ND

ND, not reported or determined.

Table 2.1. Continued

Fungus	Isoenzyme	M(kDa)	pl	Fungus	Isoenzyme	M(kDa) pl
Lentinula edodus	MnP1	44.6				
	Peroxidase2					
	Peroxidase3					
	Laccase1					
	Laccase2					

2.4.3.1. LIGNIN PEROXIDASE

Phanerochaete chrysosporium, when cultivated under ligninolytic conditions produces two types of extracellular glycosylated heme proteins: lignin peroxidases and manganese-dependent peroxidases. LiP performs many reactions with lignin and lignin model compounds and it has been heralded as the enzyme responsible for lignin degradation (**12**).

The lignin peroxidases family contains multiple iso-enzymes, ranging from as low as two to as high as fifteen (**32**). The iso-enzymes in, *P. chrysosporium* BKM-F-1767, were arbitrarily designated as H1 to H10. These reported differences in the number of iso-enzymes in different fungi reflect differences in strains, culture conditions, and purification/fractionation techniques employed. H8 has been most extensively characterised. Results show that by appropriate changes in the strains or culture conditions employed, the levels of various extracellular peroxidases can be selectively manipulated (**33**).

The essential substrate oxidation mechanism of LiP enzymes appears to be peroxidative. Lignin peroxidase (H8) catalyses stoichiometric oxidation of 1 mol of veratryl alcohol to 1 mol of veratralaldehyde per mol of H_2O_2 consumed. Steady-state kinetic results indicated a "ping-pong" mechanism in which the LiP enzyme is first oxidised by H_2O_2, and the oxidised enzyme then reacts with veratryl alcohol to form veratraldehyde. The latter can be converted to native enzyme in the presence of excess veratryl alcohol. Since veratryl alcohol is a normal

secondary metabolite produced by the lignin-degrading culture, it has been suggested that LiP catalyses the oxidation of veratryl alcohol to a cation radical and the latter acts as a diffusible one-electron oxidant to interact with lignin and lignin model compounds. However, the results of others do not appear to be consistent with this hypothesis (**20, 34**). Lignin peroxidase (H8) catalyses the H_2O_2 -dependent oxidation of a variety of nonphenolic lignin model compounds (**35**). These reactions are consistent with a mechanism involving the initial one-electron oxidation of a variety of aromatic substrates by the oxidised LiP enzyme intermediates to form aryl cation radicals followed by a series of non-enzymatic reactions to yield a multiplicity of products. A unified general scheme has been proposed for the reactions catalysed by LiPs (**Figure 2.8**). These enzymes, like other peroxidases, first react with H_2O_2 to form a two-electron oxidised intermediate (compound I). Compound I oxidises the one-electron oxidation of lignin substrate or a
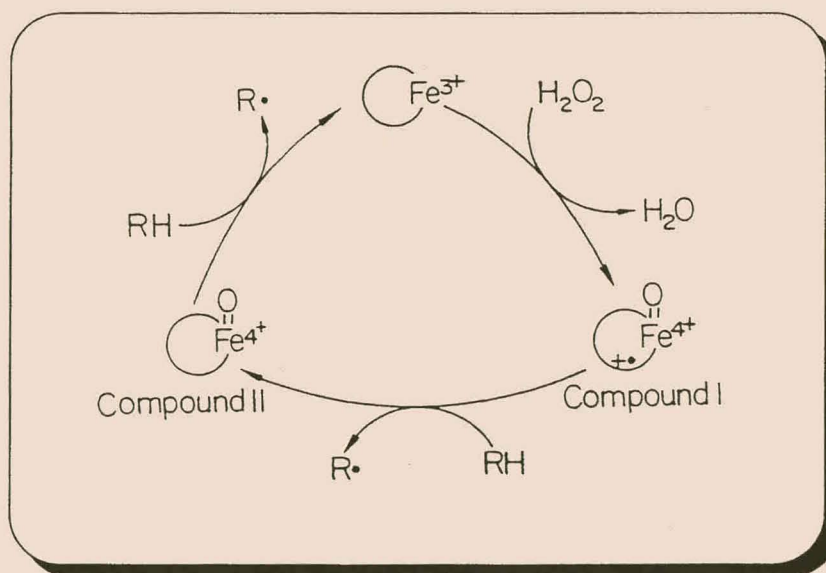
Figure 2.8. Catalytic cycle of lignin peroxidase indicating various reduction states of the heme. Resting enzyme reacts with H_2O_2 to produce the two-electron oxidised intermediate, compound I, which in turn oxidises lignin substrate (RH) to yield the one-electron oxidised intermediate, compound II, and a substrate radical. Compound II returns to resting enzyme by oxidising a second substrate molecule. The free radical (R) can undergo a variety of reactions[39].

variety of model lignin compounds (RH) to yield the one-electron oxidised intermediate compound II, and a lignin substrate cation radical. The substrate subsequently undergoes a variety of reactions. Compound II returns to resting enzyme by oxidising a second substrate. LiPs can oxidise a spectrum of substrates, some of which and their formation products are shown in **Figure 2.9**. The reactions can be classified into five categories such as benzylic alcohol oxidation (A); carbon-carbon bond cleavage (B); hydroxylation (C); phenol dimerization of polymerisation (D); and demethylation (E). These reactions have further been classified as follows : (a) cleavage of β-0-4
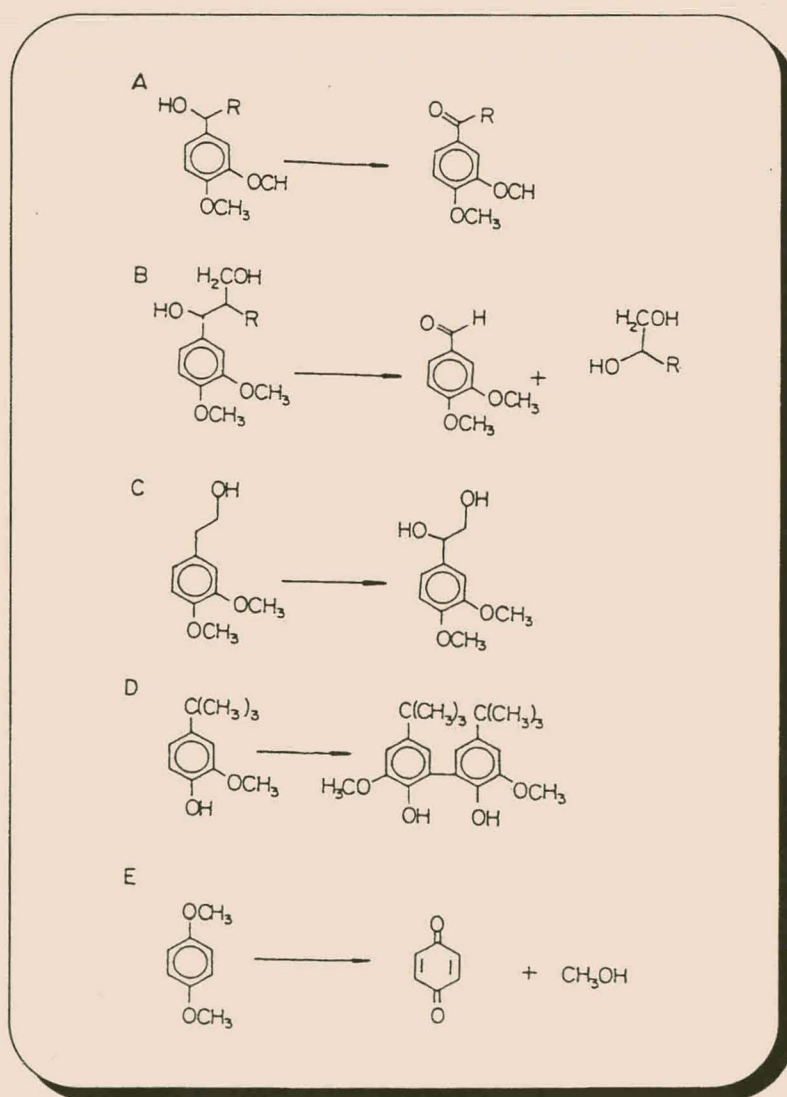
Figure 2.9. The reactions catalysed by lignin peroxidases of *P. chrysosporium*. Most of these reactions can be accounted for by cation radical formations[36].

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linkages, (b) cleavage of C_α-C_β bonds, (c) cleavage of C_β-C_γ bonds, (d) oxidative cleavage of aromatic rings, (e) oxidative cleavage of C_γ-C_1 bonds, (f) oxidative decarboxylation of 5-aroxyvanillic acid (**12**).

Researchers have reported the presence of an aromatic cleavage system and they proposed a scheme for the aromatic ring cleavage of β-0-4 lignin substructure model compounds (**Figure 2.10**) (**36**). The mechanism involves one-electron oxidation of the aromatic ring to the corresponding cation radical [substrate to (a)] followed by attack of nucleophile [H_2O (a) to (b) or the hydroxyl groups of C_α and C_γ positions of propyl side chain] [(a) to (g)] and coupling with oxygen [(b) to (c), (e) to (f), (g) to (h)], with intramolecular addition of peroxy radicals to double bonds to form cyclic peroxide [(c) to (e)] and subsequent coupling with oxygen. The reactions are summarised as follows :

Unstable cationic radicals are produced by the single electron oxidation of susceptible aromatic nuclei; these unstable aryl cation radicals may act as mediators and undergo a variety of reactions which include (a) nucleophilic attack by water of an internal hydroxyl group, (b) loss of the acidic proton at $C\alpha$, $C\alpha$-$C\beta$ cleavage, (c) addition of molecular oxygen to carbon-centred radicals, (d) single electron oxidation or reduction and, (e) in the absence of oxygen radical-radical coupling. Radicals may undergo further reactions. The aromatic ring cleavage reactions catalysed by LiPs appear to be different from that of ring cleavage of aromatic compounds catalysed by dioxygenases.

The catalytic cycle of lignin peroxidase explains its ability to catalyse one-electron oxidations (**Figure 2.11**) (**37**). The native enzyme contains a protoporphyrin prosthetic group with a high-spin ferric iron, Fe(III). Oxidation by hydrogen peroxide removes two electrons and converts the prosthetic group of the enzyme to an oxo-iron (IV) porphyrin radical cation, an enzyme form designated compound I. A one-electron reduction by abstraction of an electron from a donor molecule (such as an aromatic nucleus) produces an enzyme with an oxo-iron (IV) porphyrin, a form designated compound II. A second one-electron reduction completes the catalytic cycle by regenerating
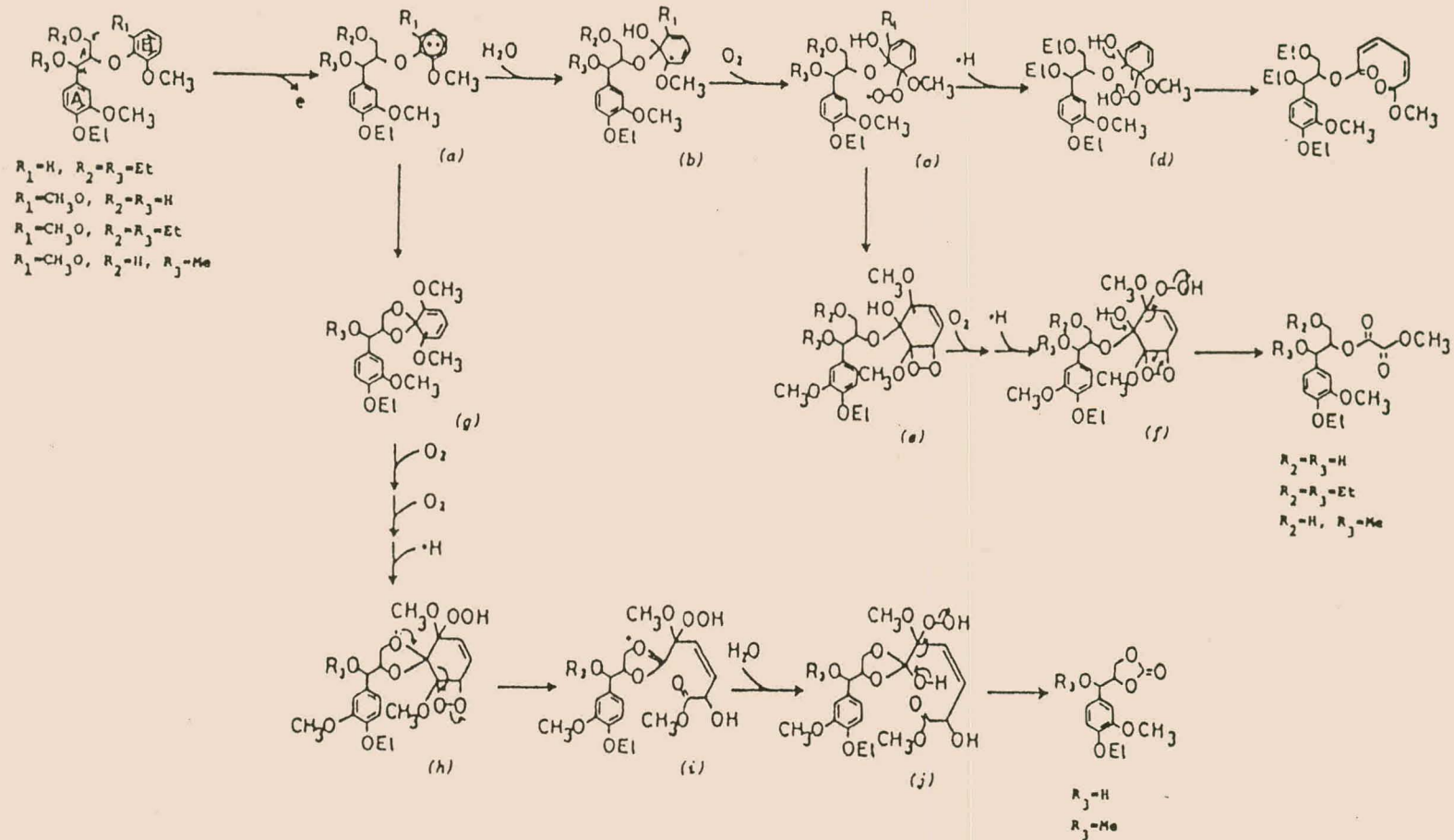
Figure 2.10. Mechanism for the formation of aromatic ring cleavage products from β-O-4 lignin model compounds.   Formation of the α,β-cyclic carbonate can be explained in the same way as for β,γ-cyclic carbonate exept for nucleophilic attack of CαOH of (a) instead of CγOH.[39]
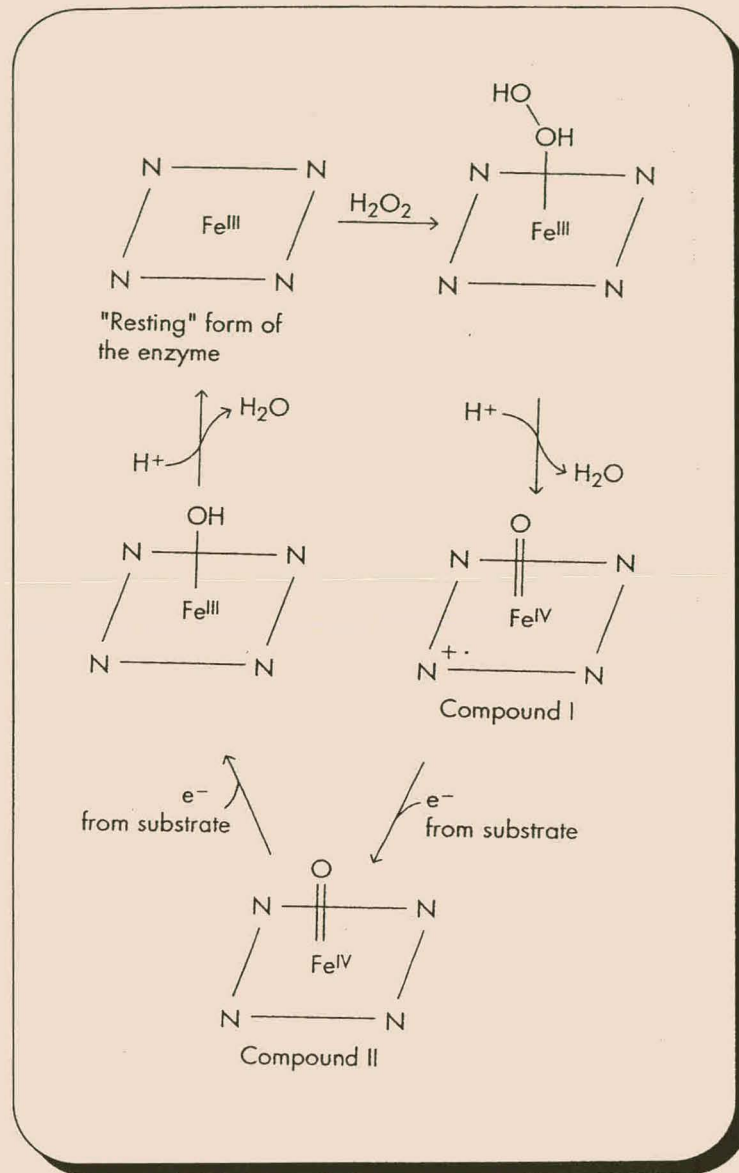
Figure 2.11. The catalytic cycle of lignin peroxidase. Compound I is an iron(IV) porphyrin π cation radical, where one of the electrons has been removed from the iron and one from the porphyrin ligand[38].

the native ferric enzyme (**38**). A major unsolved question in lignin degradation is whether each LiP has a specific role in lignin degradation and what that role is (**39**).

2.4.3.2.   MANGANESE PEROXIDASE

The successful degradation of lignin requires attacks on both the non-phenolic and the phenolic lignin components. The extracellular Mn(II)-dependent peroxidases oxidise phenolic compounds of lignin (**Figure 2.12**), but they cannot oxidise the non-phenolic substrates of lignin peroxidase such as veratryl alcohol or the non-phenolic model compounds of the lignin substructure (**38**).

Syringylglycerol-β-guaiacyl ether
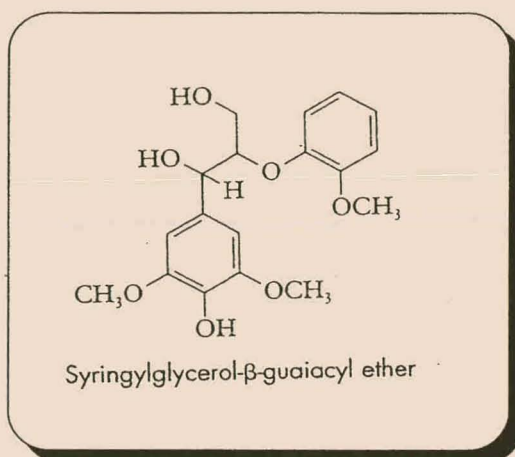
Figure 2.12. A model compound for study of the degradation of the phenolic β-0-4 components of lignin[38].

Phanerochaete chrysosporium produces a family of four to six or more manganese-dependant peroxidases. MnPs are similar to LiPs and are glycoproteins that contain 1 mol of iron protoporhydrin IX per mol of enzyme, and have a molecular mass of approximately 46 kDa (**40, 41, 42**). MnPs oxidise Mn(II) to Mn(III) and can be activated by lactate which probably acts by chelating Mn(II) to form stable complexes with a high redox potential (**41, 42, 43**). The Mn(III) in turn oxidises phenols to phenoxy radicals.

MnPs have essentially the same catalytic cycle as LiPs except that Mn(II) is obligatorily required for oxidation of phenols and other substrates. As shown in **Figure 2.13**, the native enzymes first reacts with H_2O_2 and is oxidised to compound I, which is rapidly converted to compound II by reacting with one equivalent of Mn(II) resulting in the formation of Mn(III). A second unit of Mn(II) reduces compound II to
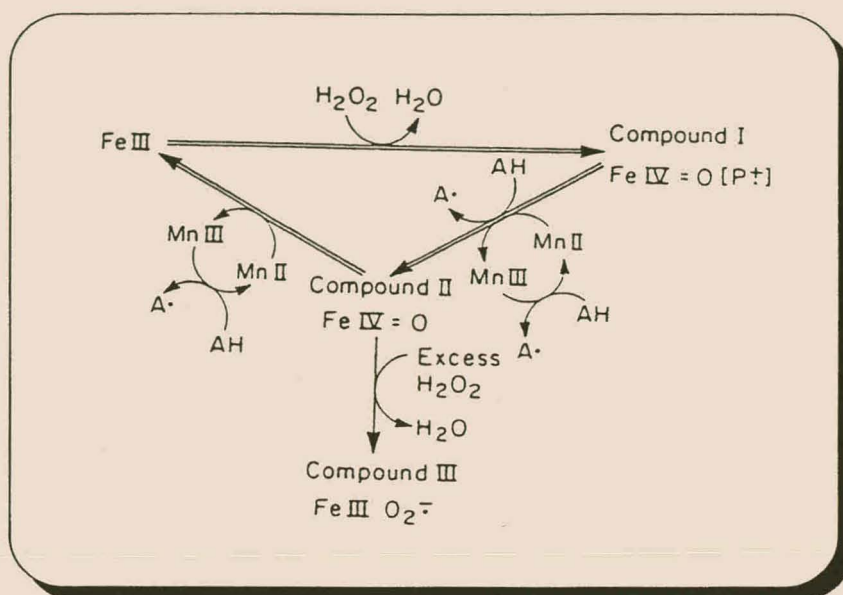
Figure 2.13. Catalytic cycle of manganese-dependent peroxidase of *P. chrysosporium*[39].

the resulting native ferric enzyme. The Mn(III) formed is involved in the oxidation of various phenolic substrates (**44**). These enzymes could not efficiently complete the catalytic cycle in the absence of Mn(II), suggesting the absolute requirement of Mn(II) (**42**). The MnPs apparently utilise freely diffusible Mn(II)/Mn(III) as an obligatory redox couple to oxidise terminal phenolic substrates such as lignin. About 10-15% of the phenolic groups in lignin are free and it is likely that the Mn(III) can diffuse into the polymeric matrix and oxidise the polymeric phenolic groups. Boominathan *et al* (**45**), have demonstrated with the aid of a LiP-negative mutant that strains producing only MnPs can depolymerize and degrade lignin albeit to a limited extent (**45**). A correlation between LiP production and ^{14}C-ring-labelled synthetic lignin degradation was also observed (**46**).

2.4.3.3. LACCASE

Laccase is a blue copper oxidase that catalyses the one-electron oxidation of phenols to phenoxy radicals, eventually transferring four

electrons to 0_2 (**47**). The effect on the substrate phenols is the same as that of horseradish peroxidase, despite fundamental differences in enzyme mechanism.

Laccase apparently has a role in sexual fruiting and in lignin degradation. Work with various phenolic model compounds and isolated laccase or horseradish peroxidase (HRP) shows that certain degradative reactions occur, particularly with syringyl models (**48, 49**). Among the consequences of the one-electron oxidation in lignin related phenols are C_α-oxidation, lignin degradation, limited demethoxylation, and aryl-C_α cleavage (**49**).

Phenol-oxidizing enzymes account for many of the degradative reactions in phenolic models seen in intact cultures of lignin-degrading fungi (**48, 50**). Coupling/polymerization is a major consequence of one-electron oxidation of lignin-related phenols and isolated lignins (**51**).

Phanerochaete chrysosporium is widely quoted as an example of a white-rot fungus that does not produce laccase (**52**). It is not known whether the inability to demonstrate the presence of laccase in *P. chrysosporium* cultures was due to the use of culture conditions that are not favourable for laccase production by this organism, or whether the organism lacks the genetic machinery for producing laccase. It has been shown that this fungus (*P. chrysosporium* BKM-F-1767) produces low but consistent levels of laccase. Laccase is present in *Phanerochaete chrysosporium* grown in low-nitrogen (2.4 mM) or high-nitrogen (24mM) defined media containing cellulose as the carbon source (**53**).

The laccase activity in extracellular culture fluids of *P. chrysosporium* grown in cellulose medium at 25 and 37°C is shown in **Table 2.2**. Cultures grown in high nitro-cellulose medium showed relatively more laccase activity than those grown in low N-cellulose medium and this was better for high N-cellulose cultures incubated at 25°C.

Table 2.2. Laccase activity of *P. chrysosporium* BKM-F-1767 as affected by temperature and nitrogen concentration in the medium[53]

Medium[a]	Days post-inoculation	Laccase activity (nkat/ml)[b] at:	
		25°C	37°C
Low-N-cellulose	5	6.8	3.1
	7	3.4	1.1
High-N-cellulose	5	4.6	2.8
	7	27.4	5.7

[a] Cultures were grown in low-N-cellulose medium (2.4 mM) or high-N-cellulose medium (24 mM) and incubated at 25 or 37°C. The cultures were oxygenated at the time of inoculation and then on days 2,3 and 4 after inoculation.

[b] Laccase activity in the 40 x concentrated extracellular culture fluid was assayed as previously described with ABTS as the subculture.

The time course of laccase activity in high N-cellulose medium is shown in **Figure 2.14**. Laccase activity first appeared on day 4, reached its peak on day 6, and began to decline from day 7 onwards. Oxygen appears to have a marked influence on the laccase levels. Un-oxygenated cultures, that were grown in high N-cellulose medium seldom showed detectable levels of laccase activity. Laccase production in *P. chrysosporium* appears to be relatively low (1.7 nkat/ml of concentrated extracellular fluid). The low level of laccase production, in *P. chrysosporium,* plus the lack of detectable laccase activity in low N-or high N-medium with glucose as substrate, might be the reason why previously laccase was not detected in this organism (**53**).
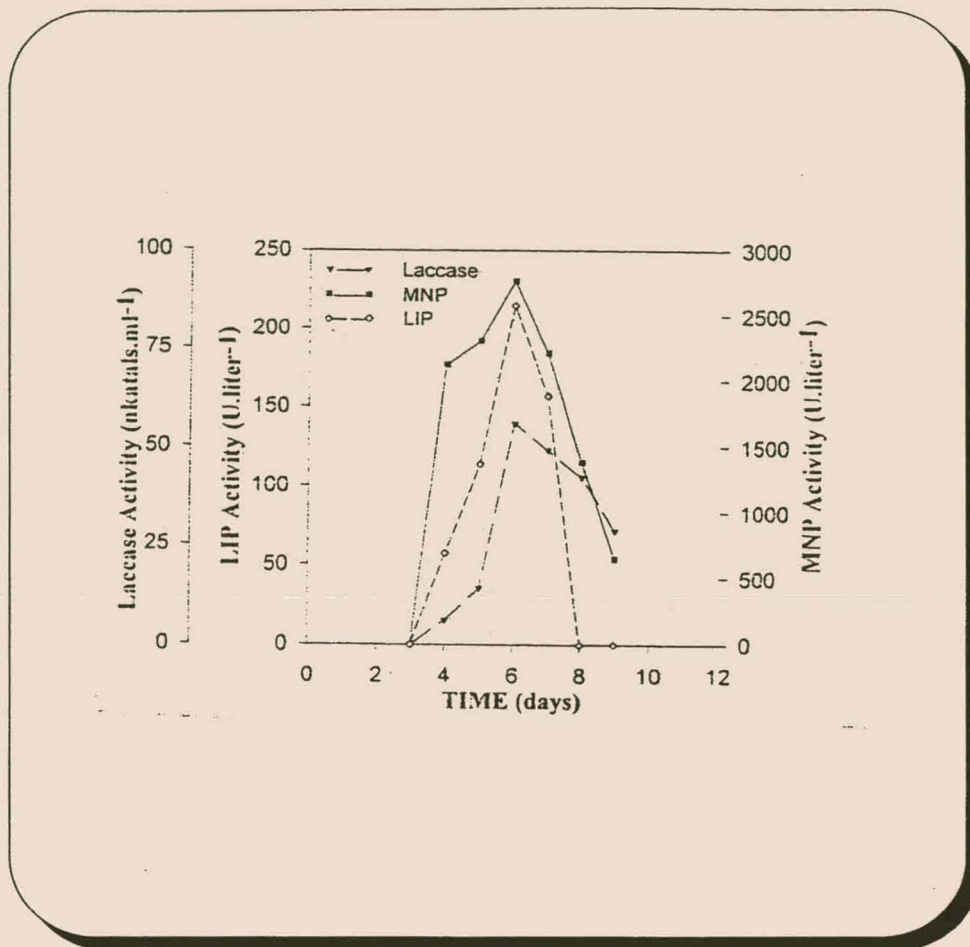
Figure 2.14. Time course of laccase, LiP, and MnP production by *P. chrysosporium* BKM-F-1767. Cultures were grown in high-N-cellulose medium (10 ml contained in a 125-ml Erlenmeyer flask) under static conditions at 25°C. All cultures were flushed with oxygen for the first six days of incubation. Three replicate flasks were used each day from days 3 through 9, and the extracellular culture fluids were harvested and concentrated (40 x) and were used for assaying laccase[53].

2.4.3.4. H_2O_2 - PRODUCING ENZYMES

Hydrogen peroxide production was shown to be temporarily correlated with lignin degradation (**54**). The production of H_2O_2, similar to the lignin-degrading activity, was shown to be triggered in response to nitrogen or carbohydrate starvation (**55**). Unique H_2O_2 producing, periplasmically located, microbody-type structures are observed only in lignin-degrading cultures grown in nitrogen-limited medium but not in non-lignin-degrading cultures grown in relatively high nitrogen media

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(56). The strongest evidence for the importance of H_2O_2 in lignin degradation is the discovery of H_2O_2-dependent LIPs and MNPs that are believed to be important in lignin mineralization (57). Results indicate that glucose oxidase is an important source of H_2O_2 in lignin-degrading cultures of *P. chrysosporium* (58). A large number of other H_2O_2-producing enzymes have also been isolated and characterised in ligninolytic cultures of *P. chrysosporium*. It is evident that *P. chrysosporium* has several enzymes that are capable of generating hydrogen peroxide. The relative contribution of each enzyme to the overall lignin degradation is still being investigated (39). In summary, it appears that the H_2O_2 required for ligninase activity may be supplied by several different oxidases; supply by intracellular enzymes, however, has not been shown directly (20).

2.4.3.5. OXIDOREDUCTASES

Other enzymes important in lignin degradation are NAD(P)H: quinone oxidoreductase, aryl alcohol dehydrogenase, and cellobiose dehydrogenase CDH(cellobiose: quinone oxireductase CBQ). *Phanerchaete chrysosporium* produces at least two intracellular NAD(P)H: quinone oxidoreductase. These enzymes reduce methoxyquinone using either NADH or NAD(P)H as electron donors. Laccase can interact with the enzyme to increase decarboxylation of lignin model compounds. In addition, glucose-1-oxidase from *Ceriperiopsis versicolor* could function as glucose: quinone oxidoreductase and use quinone as co-substrate in place of O_2 at low O_2 tension. CDH or CBQ (the proteolytic breakdown product of CDH) have probably not beyond doubt been shown to play an important role in lignin degradation but, when first discovered, CBQ was suggested to be linked to cellulose and lignin degradation. Both enzymes reduce a number of electron acceptors including quinones, phenoxy- and cation-radicals, complexed ferric ions, compound II of LiP, MnP and molecular oxygen.

Quinone reduction is more rapid than oxygen reduction, oxygen being slowly reduced to superoxide and/or H_2O_2 (3).

2.5. *Phanerochaete chrysosporium* - A MODEL FOR LIGNIN DEGRADATION

Lignin biodegradation studies have been carried out mostly using *P. chrysosporium* Burdsall. The popularity of *P. cysorsporium* in experimental work is well justified. *P. chrysosporium* is a very efficient degrader of lignin, and some *P. chrysosporium* strains also possess industrially useful properties such as selective lignin degradation to promote biopulping. Originally described as *Chrysosporium lignorium* due to it's *Chrysosporium* imperfect state, it has also been described as *Sporotrichum pulverulentum.*

Phanerochaete chrysosporium has a classical tree-stage life cycle (homokaryotic haploid, multikaryotic haploid, and homozygotic diploid), and it is homothallic or self-fertile. Prolific conidiation (abundant formation of conidiospores), which is unusual among white-rot fungi, gives *P. crysosporium* distinct advantages over other species for mutant production (20).

It occurs in both hardwood and softwood pulpwood chip storage piles and in chipboards used in warm places, e.g. ceiling panels. It is widespread in UK, USA, Europe, the former USSR and has recently been isolated in South Africa. Optimum growth occurs at a temperature of 39°C and it can withstand a maximum temperature of 50°C. The extension rate at 25°C is 15-20 mm/day and at 40°C is 35-42 mm/day on malt extract agar. Due to its high temperature tolerance and its ability to produce large numbers of spores, this fungus should be contained with care in the laboratory.

As mentioned previously, the normal way for white-rot fungi to degrade wood is to degrade the cellulose and lignin simultaneously. It seems that the need for energy in lignin degradation demands that a further, easily metabolised energy source be available. The polysaccharides of the wood, therefore, function as co-substrates.

Even if an absolutely specific attack on the lignin cannot be achieved, it has been shown that enough lignin can be degraded to cause a decreased need

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for energy in mechanical pulping if the wood chips are pre-treated with cellulase-less mutants of white-rot fungi (59).

Table 2.3 lists the major described genetic mutants of *P. chrysosporium*. Eriksson and co-workers (60) developed various cellulase negative (Cel⁻) as well as phenol oxidase positive strains (POx⁺) in attempts to improve selective lignin degradation. Crossbreeding of homokaryotic Cel⁻ strains has improved lignin-degrading ability (61). Unfortunately, highly lignin-degrading strains derived from crossbreeding may not be inter-crossable, since 75% of the strains did not fruit (20).

Table 2.3. Various mutants of *Phanerochaete chrysosporium*[20]

Organism (ATCC number)	Strain or mutation	Phenotype[a]
32629	44	C⁻: X⁻: POx⁻
32629	44-2	C⁻: X⁻: POx⁺
32629	63-2	C⁻: X⁻: POx⁺
32629	31	C⁻: L⁺
32629	3113	C⁻: L⁻
32629	85118	C⁻: L⁻
24725	SC26	L-enhanced
34571	*leu3.1.2*	Leucine auxotroph
34571	*ade1.2*	Adenine auxotroph
34571	*rib1*	Riboflavin auxotroph
34571	LMT 320	Riboflavin auxotroph
34571	*nic1*	Nicotinamide auxotroph
34571	*arg1.3.4*	Arginine auxotroph
34571	*his1.2*	Histidine auxotroph
34571	LMT 31	Histidine auxotroph
34571	*met1*	Methionine auxotroph
34571	*leu1*	Leucine auxotroph
34571	LMT 30	Leucine auxotroph
34571	LMT 26	POx⁻: L-enhanced

[a] C = cellulase: X = xylan degradation: POx = phenol oxidase activity: L = lignin degradation:

+ = positive.

- = Negative

2.6. OBJECTIVES IN BIOPULPING

The objectives of biopulping vary widely and depend on the pulping process, product, type of organism used and what one actually wishes to achieve. The two main pulping processes are mechanical and chemical, and as stated before, most research has concentrated on biomechanical pulping. During thermomechanical biopulping, the objective is energy savings with improvement in paper properties being more a consequence than an objective. In chemical pulping objectives may include such things as; lower chemical consumption, higher throughput, improved paper properties, possible yield increases, more effective pulping and use of digesters and decreased kappa number which implies less bleaching. Other areas in the pulp industry where white-rot fungi may be used are; deinking, effluent treatment and pulp bleaching.

2.7. METHODS IN BIOPULPING

All biopulping processes thus far, by their nature, operate on a similar basis and vary only by specific pulping conditions. However, there are two laboratory scale methods and a pilot plant process that have been proposed.

2.7.1. LABORATORY METHODS

2.7.1.1. ROTATING DRUM REACTOR

Myers *et al.,* (63) designed a so-called rotating drum bioreactor to enable constant mixing during incubation (**Figure 2.15**). The bioreactor consists of two major parts: (a) an outer case of specified dimensions, with a large access door and ports for aeration and nutrient addition and (b) an inner drum for holding the wood chips. The rotation of the drum can be varied from 1 rev/min to 1 rev/day. The drum shell is made from a perforated plate having 3-mm-diameter holes occupying 40% of the surface area. This allows aeration of the

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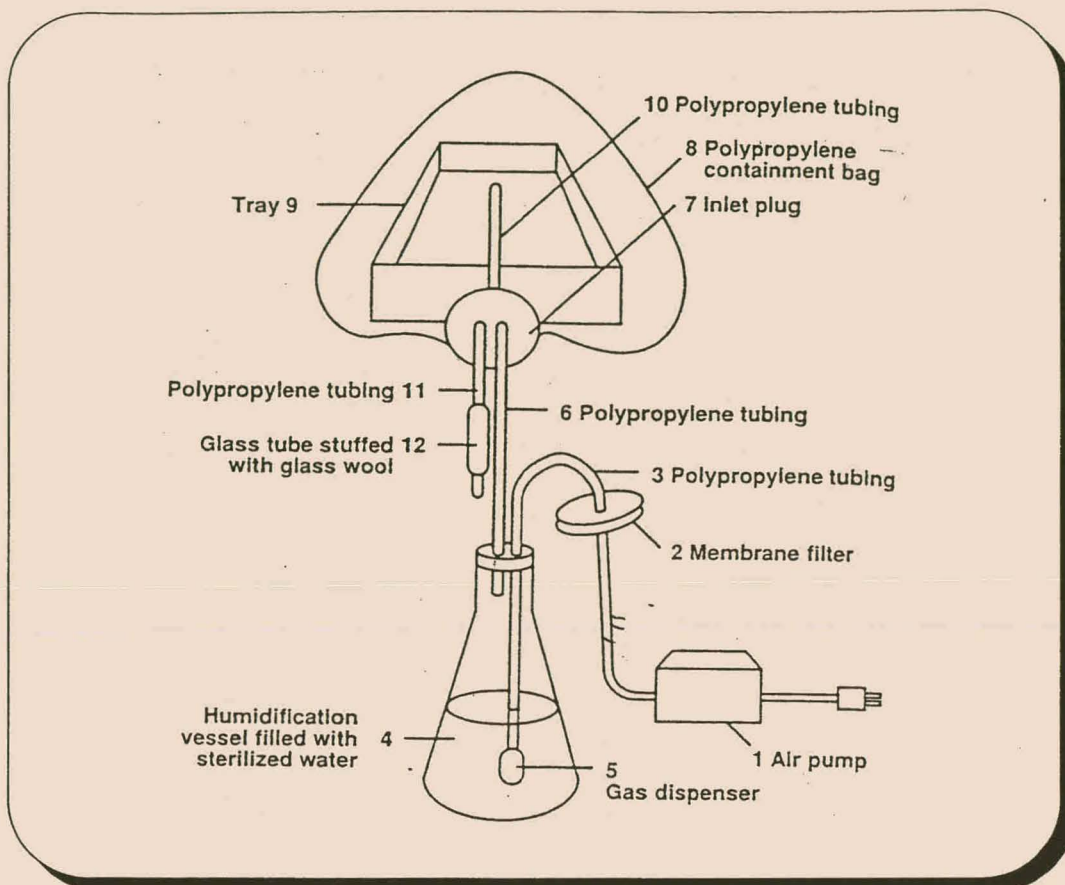
Figure 2.15. A pilot-scale, solid-substrate bioreactor used to treat wood chips[63].

wood chips and drainage of metabolically produced drainage water. The reactor fits into an autoclave for sterilisation. During operating the reactor resides within a temperature controlled incubator (**63**).

2.7.1.2.   STATIC BED REACTORS

These can further be subdivided into two groups: (a) without forced air/oxygen flow, (b) with forced air/oxygen flow.

The first method encompasses standard microbiological techniques whereby a cotton wool stoppered flask, containing the inoculated chips, is incubated at the required temperature. The role of the cotton wool stopper is to allow air to diffuse into the flask while maintaining asepsis. The second method is similar, baring the fact that moist, filtered, sterile air, is past through the container at fixed flow rates. This typically consists of a container into which inoculated chips are placed and

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incubated at a desired temperature as indicated in **Figure 2.16**. The bioreactor (L), may be fabricated from any autoclavable material to enable sterilisation prior to inoculation. The top of the vessel is sealed with a lid (M) which is vented to the atmosphere through an exit tube (N). Suspended above the bottom of the reactor (L) is a perforated plate (J), supported by a stand (K). Regulated air for the bioreactor passes through tubing (A) to a fitted glass gas dispenser (B) in a humidification flask (C) containing sterile water. Humidified air passes through tubing (D) to a rotameter (E) and through tubing (F) to a manifold (G). Humidified air from manifold (G) is passed through a filter (H), before passing through tubing (I) the reactor (L) base (**64**).
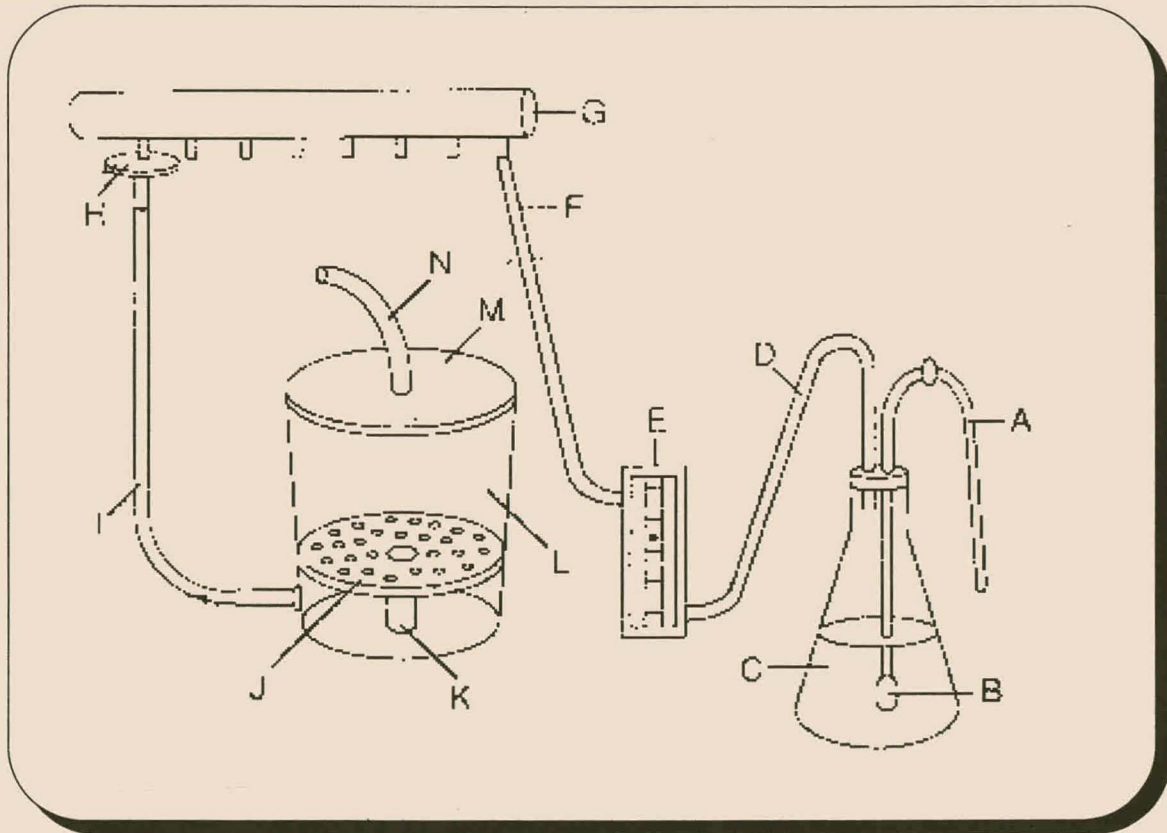
Figure 2.16. Diagram of a static-bed bioreactor configuration[64].

Figure 2.17 represents a similar apparatus but instead of a rigid container a polypropylene containment bag is used (**65**).
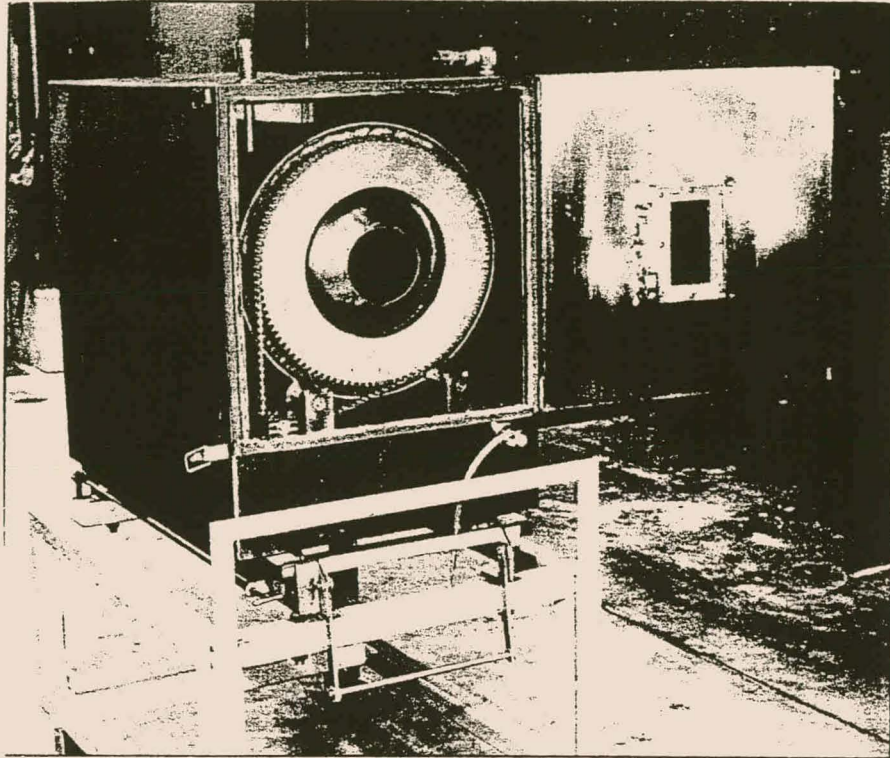
Figure 2.17. Stationary tray bioreactor used for fungal treatments[65].

2.7.2. PILOT PLANT/INDUSTRIAL SCALE BIOPULPING

Figure 2.18 represents a process flow sheet for a biopulping system on a larger scale. Trees are harvested, debarked and chipped. Chips are pre-steamed on a conveyor to effect a surface sterilisation of the chips. This is followed by inoculation with the medium and piling up of chips. Moist air is forced up from the bottom of the pile. The pile may be 'stirred' or turned, with

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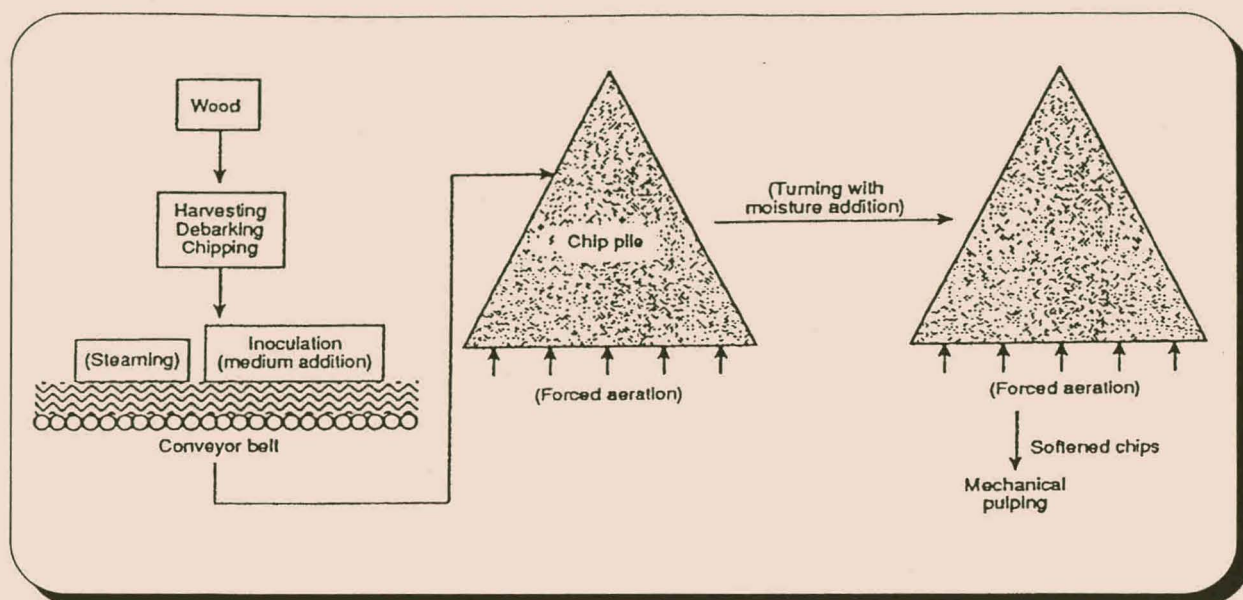
Figure 2.18. Process flowsheet for chip pile-based system. Processes in parenthesis are optional[66].

moisture being added, to ensure good contact with fungal spores and mycelium as well as improving aeration (**66**).

2.8.   BIOPULPING VARIABLES

2.8.1. AIR FLOW

As stated previously, cotton wool stoppered type reaction vessels only allow air to diffuse slowly through the porous cotton wool. However, the rate of air flow through the chips in bioreactors was found to be important (**65**). A flow rate of 0.001 l/l min (volume of air per volume of chips a minute) was found to be too low, whereas 0.022 and 0.100 l/l min were equally effective (**66**). Researchers studied the influence of three different aeration modes on the decomposition of lignin prior to TMP pulping: 1) flushing at three-day intervals with air (21% Oxygen); 2) flushing at three day interval with oxygen and 3) free air exchange. Results (**Table 2.4**) indicated that cultures flushed with oxygen gave the highest average rate of lignin and carbohydrate metabolism over the two-week incubation (**67**).

Table 2.4. Effect of Different Modes of Aeration on Lignin Decomposition in Red Alder TMP by *P. chrysosporium* 6251 in two weeks[67]

Aeration mode	Percent decomposition[a]		
	Lignin	Carbohydrates	Total Weight
Air (21% O_2), flushed every 3 days	16.5	14.3	13.6
100% O_2, flushed every 3 days	44.2	31.5	34.1
Free air exchange (cotton plugs)	29.8	21.0	22.8

2.8.2. MOISTURE CONTENT

Most experimentation has been carried out at around 55-60% chip moisture content based on wet mass **(66)**. This value has also been increased to between 70 and 80% moisture content **(68)**. Lignin in pulp samples was degraded most rapidly when the pulps were completely saturated and free water was present. Increasing the amount of water had little influence until aeration apparently became impaired **(67)**. With too much excess water, anaerobic conditions may occur in the wood cell which inhibits lignin degradation.

2.8.3. TEMPERATURE

Temperature is one of the main factors affecting growth of the fungi. According to various researchers, 39°C is the optimum temperature of *P. chrysosporium* while the requirements of other species differ: i.e. the optimum temperature for *Ceriporiopsis subvermispora* is 27 ± 1°C **(66, 69)**.

2.8.4. NUTRIENTS

The addition of nutrient nitrogen in various forms may stimulate fungal activity and lead to refiner energy savings, but the nitrogen may also promote unacceptable losses in wood substance and damage to cellulose (66). Degradation of lignin appears to increase with increasing levels of nitrogen. Too little nitrogen appears to hamper growth of the fungus and metabolism of the lignin, whereas too much might improve growth of the fungi but inhibits the secondary ligninolytic activity. The optimum appears to be around 0.5-2% based on the dry mass of the wood chips (16). It has been postulated that the only nitrogen in wood is contained in the lignin and the white-rot fungi will only attack the lignin as a secondary metabolism (low nitrogen induced ligninolysis) (70). Thus a limited amount of nitrogen will enable the primary growth of the fungi. However, as the nitrogen becomes depleted, secondary degradation of the lignin occurs. Glucose is added to wood chips to increase fungal biomass and suppress cellulose degradation. Initial amounts of glucose added were around 4% (69) but this is hoped to be decreased. Other nutritional glucose/nitrogen additions include malt extract, corn steep liquor and yeast extract from breweries etc. Corn steep liquor is preferred by such researchers as Akhtar *et al* (69), as good results have been achieved with additions of this nutrient and it is a cheap by-product of the corn producers in Northern America. It was found that 3 kg of fungal inoculum would be needed per o.d. (oven dry) ton of chips to obtain optimum growth of fungal biomass and savings in energy. However, to achieve similar or even better results this amount could be reduced to just 5 g or less with the addition of corn steep liquor (71).

Other elements such as sulphur, phosphorus and trace elements such as Ca^{2+} and Mg^{2+} also have an influence in ligninolytic activity, with the limiting of sulphur triggering such activity. The balance of these trace metals also seem to be of importance in lignin degradation (72).

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2.8.5. CHIP AGITATION

Chip movement in rotating drums affected both chip degradation and the resulting handsheet strength properties. It can be seen from **Table 2.5** that movement decreased the chip mass loss, or inhibited fungal degradation, for all the white-rot fungi tested. Movement with *P. chrysosporium* BKM-F-1767 (but not strain SC-26) increased the burst strength by 26% over the value for the untreated control; movement with *Dichomitus squalens* decreased the burst strength by 21% over the value of the untreated control (**Figure 2.19**). The effect of chip movement appears to be a function of the fungal species used with some doing well in the rotating drum and others doing well in stationary bed reactors (**73**).

Table 2.5. Effect of different fungal treatments on chip weight and composition compared to untreated control chips[73]

Bioreactor type and fungal species	Chip weight, %	Lignin content, %							Hydrolyzate sugar content, %		
		Klason	AcidSol	T_{Acid}	Syringyl	Guaicyl	S/G	T_{NBO}	Glucose	Xylose	Mannose
				Stationary wire tray							
P. tremellosa	-10.2	-33.9	4.4	-28.1	-41.4	-43.6	0.95	-42.6	-12.5	-9.7	-34.4
P. subserialis	-2.5	-32.5	7.2	-26.4	-40.2	-34.1	1.18	-37.0	-0.5	-17.3	-43.8
D. squalens	-2.6	-18.8	-4.8	-16.7	-22.0	-20.5	1.07	-21.2	-5.3	-12.7	-28.8
P. brevispora	-6.6	-39.9	10.4	-32.2	-42.1	-49.2	0.86	-45.8	-8.5	-7.6	-31.7
P. chrysosporium (SC)	-6.3	-17.7	-2.7	-15.6	-18.9	-29.2	0.65	-24.3	-7.7	1.3	-20.7
P. mutabilis	-2.2	-18.9	-6.3	-16.8	-15.7	-29.4	0.53	-22.8	-4.4	-3.8	-32.3
P. chrysosporium (BKM)	-13.0	-18.2	0.0	-18.2	-21.8	-30.8	0.71	-26.3	-21.5	-8.1	-32.1
P. medulla-panis	-11.5	-24.5	-6.9	-21.8	-25.4	-45.1	0.56	-35.7	-17.9	-8.6	-28.5
T. versicolor	-5.1	-15.7	-0.9	-13.8	-14.8	-41.7	0.36	-28.8	-6.9	-7.2	-23.4
				Rotating perforated drum							
P. chrysosporium (BKM)	-2.4	-0.5	-5.1	-1.2	-9.6	-14.3	0.67	-12.1	-20.0	-2.9	-17.4
P. tremellosa	-4.5	-0.3	-12.8	-2.1	-12.9	-18.6	0.69	-15.9	-17.4	1.2	-33.9
P. medulla-panis	-0.7	-3.1	-4.0	-3.2	-16.8	-10.9	1.54	-13.7	-14.3	-6.9	-4.5
P. chrysosporium (SC)	-2.0	-0.1	-8.8	-1.2	-10.6	-18.7	0.57	-14.8	-13.8	2.8	-9.5
D. squalens	-2.1	-12.2	-17.1	-12.9	-6.4	-3.9	1.67	-5.1	-4.8	-3.8	-11.5
T. versicolor	-4.6	-10.6	-0.9	-9.4	-9.7	-29.6	0.33	-20.1	-9.0	0.1	-11.9

[1]Fungal treatments are ranked in decreasing order based on improved burst strength index of handsheets from chips in trays and drums (Fig. 1).

[2]Values for chip weight and composition are given as percentage of change from control. Values for control (untreated aspen chips) were determined by (a) acid hydrolysis for Klason lignin (Klason; 20.5), acid soluble lignin (AcidSol; 3.62), total lignin (T_{Acid} = Klason + AcidSol; 24.1), and sugar content in acid hydrolyzates including glucose (54.2), xylose (18.4), and mannose (2.6) and (b) alkaline nitrobenzene oxidations for syringyl propane lignin residues (Syringyl; 11.5), guaicyl propane lignin residues (Guaicyl; 12.6), ratio for syringyl compared to guaicyl propane unit loss (S/G; 0.91), and total lignin content (T_{NBO}; 24.1).
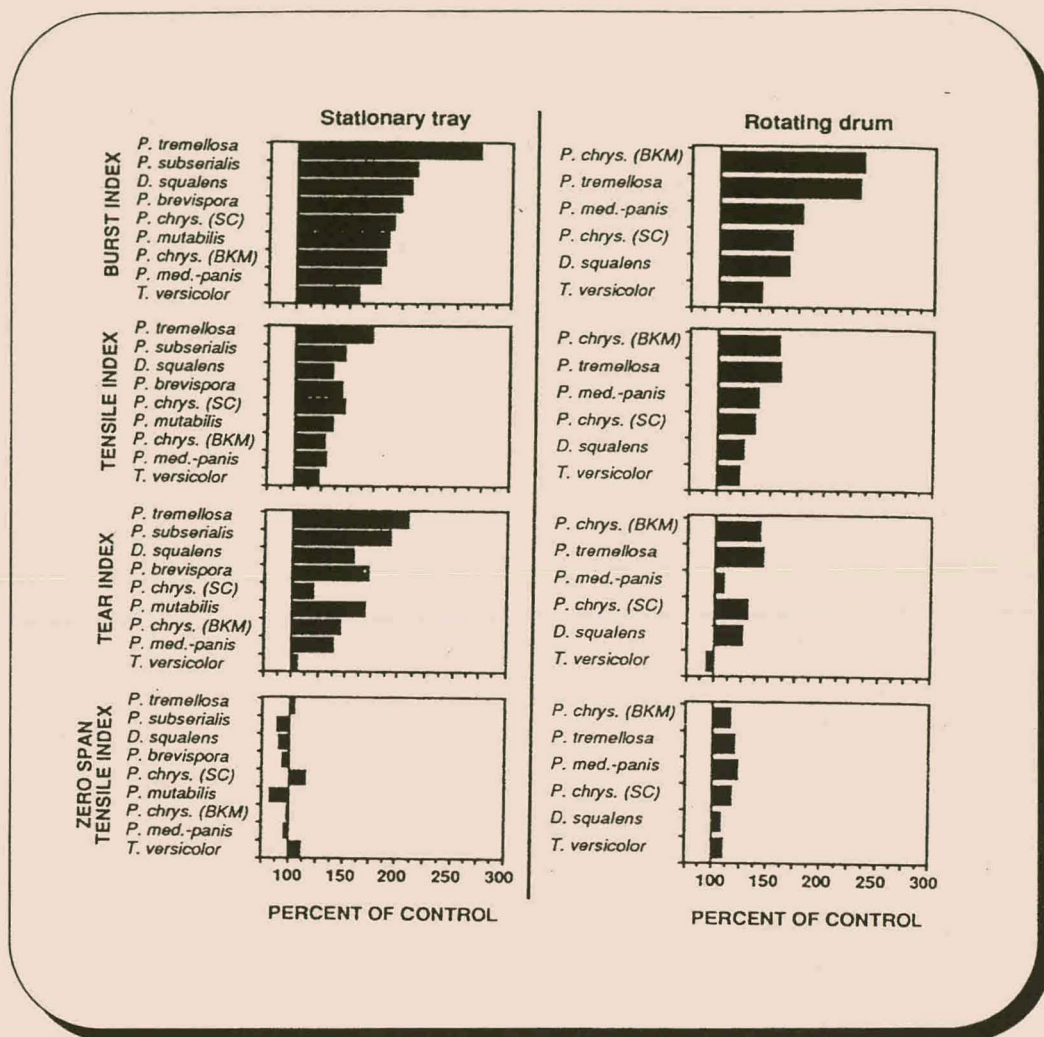
Figure 2.19. Strength properties for 60 g/m^2 handsheets made from 100 mL CSF refiner mechanical pulps produced from untreated and fungal-treated chips. Aspen chips were treated with different white-rot fungi for four weeks in either stationary wire trays or perforated drums. Handsheets made from untreated chips had the following strength properties: burst, 0.82 kPa*m^2/g; tensile, 31.3 N*m/g; tear, 2.81 mN*^{2}/g; and zero span tensile, 93.5 N*m/g. Values for treatments are ranked in descending order based on improved burst strength index[73].

2.8.6. TREATMENT DURATION

Throughout the literature, times used to treat the chips have varied substantially depending on the end objective. Biopulping incubation periods have lasted as long as three months and as short as ten days (**2**). Further research found that maximal treatment efficiency for *P. chrysosporium* on Aspen wood chips to be around four weeks (**Figure 2.20**). On the other hand, the pros and cons of the treatment times (capital investment in

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incubating chip piles) and amount of improvement/savings (energy, chemicals, improved paper properties) must be weighed against each other (**74**). Researchers have found this to be around two weeks and hopefully with fine tuning of the process similar results could possibly be achieved in ten days (**71**).
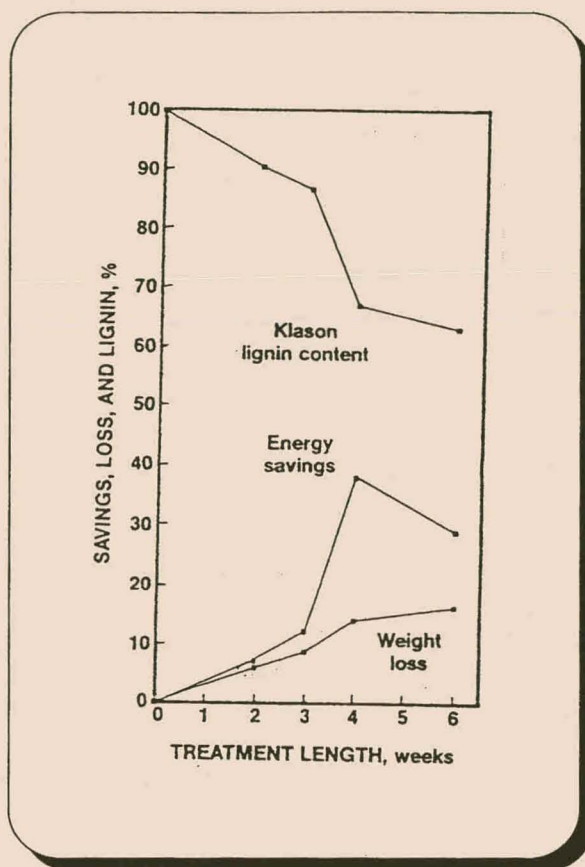
Figure 2.20. The effect of treatment duration on Aspen chip weight loss, Klason lignin content, and energy savings[73].

2.8.7. INOCULUM

The amount of inoculum was found to be critical up to a certain level, above which increases were without influence (**2**). This parameter, as stated previously, is also dependent on the type and amount of nutrient addition (**71**). Akhtar *et al.,* (**62**) found that 0.3% inoculum (dry weight basis) saved

19% energy and improved paper strength properties, such as tear index, compared to controls. This amount of fungal inoculum is quite high. However, it was discovered that the amount of inoculum can be lowered to 0.0005% (dry weight basis) or less by adding corn steep liquor.

2.8.8. CHIP SIZE

In a study on the effect of chip size using Aspen wood chips and *P. chrysosporium*, it was concluded that more biomass was found on the surface and interior of a 19-mm chip than a 6-mm chip due to its larger surface area. No major differences were observed between the two chip sizes, and the degree of attack and degradation appeared similar (**75**). It has been concluded that chip size had an important influence on reaction time (**68**). The minichips (Bruk chipper), which are smaller than commercial size chips, with their greater surface are per unit of weight, reacted with the fungal mycelium more quickly than the commercial-size chips. Studies have demonstrated that weight loss caused by white-rot fungi is inversely proportional to the thickness of the wood chips (**76**).

2.8.9. ASEPSIS

A major problem that needs to be solved is the degree of asepsis required for the process. There exists two extreme options: 1) an advanced chip pile with little process control, 2) a highly controlled enclosed type reactor. The first type of biopulping process will require either a robust fungus, competitive against autochonous fungal and bacterial strains or a decontaminating step prior to inoculation. A fungus like *P. chrysosporium* meets these demands, while *C. subvermispora* needs a higher degree of asepsis. Unfortunately, the benefits of *P. chrysosporium* on softwood seem to be minimal.

C. subvermispora is much more effective, but taking into account the enormous amounts of wood chips to be pre-treated by the white-rot fungi, the highly controlled reactor necessary for asepsis does not seem to be economically feasible (**1**). However, Aspen wood chips, have successfully

been treated with *C. subvermispora* by pre-steaming the chips for about 30 seconds (**71**). This surface sterilisation of the wood chips is said to give the inoculated fungi a foothold and an advantage over the local 'contaminating' fungi and bacteria. In this way, promising results have been achieved in large scale trials.

2.8.10. ADDITIONAL FACTORS

Many other factors are involved in biopulping, with the most obvious being wood species, fungal species and strains and age of chips. Experiments involving fresh, frozen and dried chips that have been rewetted, showed no significant difference in results.

Not much success has been obtained using *Eucalyptus* chips as a substrate (**68**), while many pine species and birch have already given good results (**68, 77**).

2.9. CHARACTERISTICS OF BIOPULP/BIOPAPER AS COMPARED TO 'STANDARD' PULP/PAPER

The conformability of BMP (Bio-mechanical Pulp) in handsheets is similar to that of chemically prepared pulps; BMP shows good compressibility and fibre-to-fibre conformability. In contrast, handsheets prepared from conventional mechanical and chemi-mechanical pulping methods produce stiff fibres that do not compress readily and that result in poor conformability, which reduces the bonding strength between fibres.

The BMP resembles Kraft pulp because of the uniform length and the collapsed appearance of fibres, plus excellent bonding of fibres, which in part may have resulted from the release of fibril ribbons by the enzymes of *Phanerochaete chrysosporium*. BMP fibres show clear qualitative differences in length, width, compressibility and conformability (**Tables 2.6** and **2.7**).

The BMP fibres (**Figure 2.21**) are woolly and loose; these fibres are of more uniform length and show more abundant fibrillation than the RMP fibres (**Figure 2.22**). The RMP fibres are substantially thinner and shorter than the

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Table 2.6. Comparison of Aspen biomechanical pulp with pulps produced by conventional methods[78]

Pulping process	Apparent fiber length	Fiber shape	Debris	Fibrillation	Compressibility and conformability	Collapsed fibers
SGW	Varied	Different width	Yes	Reduced	Poor	No
RMP	Shorter than SGW	Thinner than SGW	Yes	Moderate	Poor	No
TMP	Longer than RMP	Thinner than SGW, twisted fibers	Yes	Reduced	Poor	No
CTMP	Longer than RMP	Thinner than SGW, twisted fibers	Yes	Reduced	Poor	No
NSSC	Longer than CTMP	Various widths, not twisted	Moderate	Reduced	Moderate	Yes
Kraft	Longest fibers, fairly uniform	Not twisted	Reduced	Abundant	Good	Yes
BMP	Moderate length, fairly uniform	Not twisted	Reduced	Abundant	Good	Yes

Table 2.7. Comparison of yield and handsheet data for different pulping processes[78]

| | | | Handsheet properties* | | | | | | Bauer-McNett screen analysis for pulp | | | | |
| | | | | | | | | | Retained, | | | | Passed, | |
Pulping process	Yield, %	Freeness, mL	Burst index, kPa·m²/g	Tear index, mN·m²/g	Tensile index, N·m/g	Scattering coef., N·m/g	Opacity, %	Density, kg/m³	0.595-mm screen	0.297-mm screen	0.149-mm screen	0.075-mm screen	0.075-mm screen	Fiber length index
SGW	95	115	0.91	1.9	28	77	97	421	0.70	7.15	2.15	48.05	41.95	0.062
RMP	95	110	0.92	2.0	30	57	92	389	6.45	27.90	24.00	17.65	24.00	0.101
TMP	95	111	1.0	3.8	28	59	92	417	8.40	25.10	25.60	8.60	32.20	0.088
CTMP	94	120	2.1	6.7	47	41	84	499	22.15	28.00	20.00	8.85	21.00	0.120
NSSC	76	230	5.5	4.8	106	14	62	717	9.20	34.50	28.10	8.10	19.80	0.120
Kraft	56	315	7.1	6.4	116	18	82	773	9.20	45.90	24.90	6.75	13.25	0.154
BMP	95	100	2.0	4.6	52	40	95	402	5.30	60.00	24.00	7.25	3.45	0.243

*Properties for 60-g/m² handsheets.
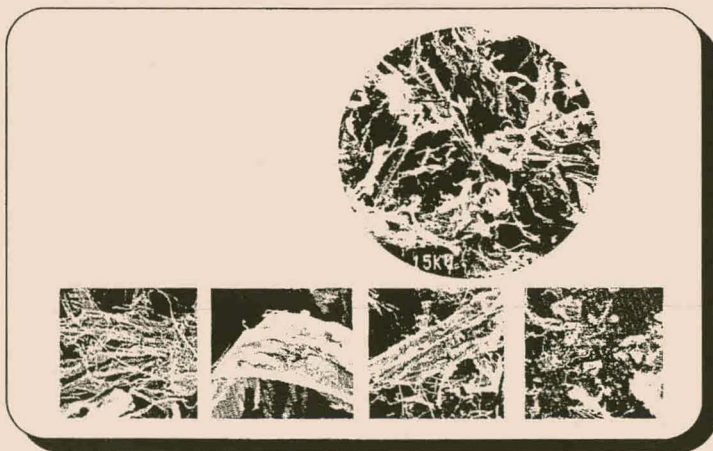
Figure 2.21. Biomechanical pulp (BMP) fibres are fairly uniform in length (x 170), with abundant fibrillation (a, x 100 and x 2200), and good compressibility and conformability, i.e. flat fibres (x 1200). Debris (x 110)[78].
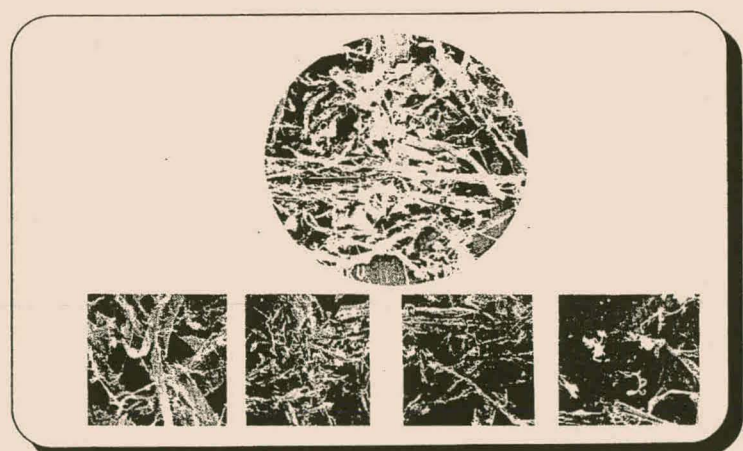
Figure 2.22. Refiner mechanical pulp (RMP) fibres are thin and short with little fibrillation (a, x 100), they are stiff (x 90), and they have various lengths and withds (x 90). Debris (x 160)[78].

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BMP fibres and had more moderate fibrillation. Both the TMP (**Figure 2.23**) and SGW pulp (**Figure 2.24**) contained stiff fibres and debris; these pulps had less fibrillation than the BMP.
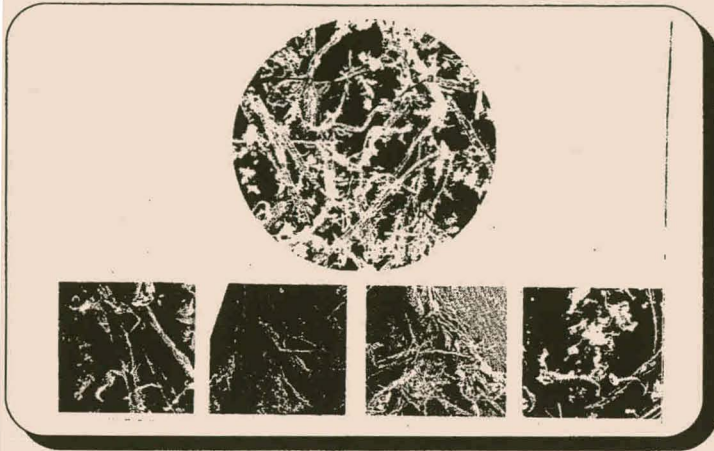
Figure 2.23. Thermomechanical pulp (TMP) fibres of various lengths and widths (a), and have less fibrillation (b) than fibres of BMP and RMP (x 100 and x 430). Fibres are stiff (x 22) and of various lengths and widths (x 22). Debris (x 150)[78].
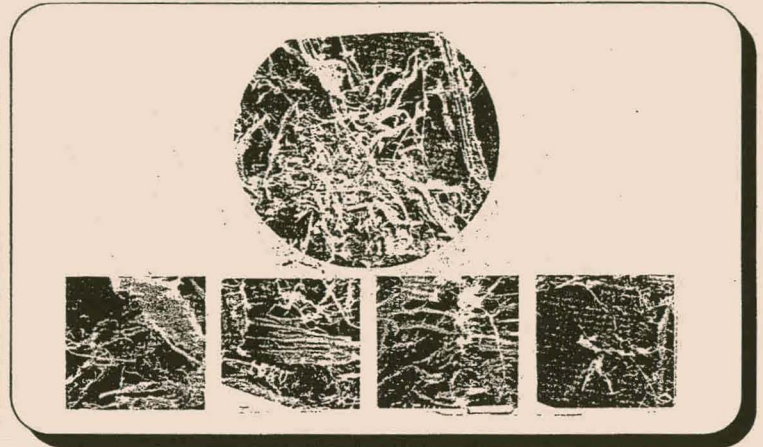
Figure 2.24. Stone groundwood pulp (SGW) fibres (a) are of various lengths and widths (c), and have less fibrillation (b) than fibres of BMP and RMP (x 100 and x 850).   Fibres are stiff (x 170) and are of various lengths and widths (x 400).   Debris (x 170)[78].

The BMP fibres are less twisted than the TMP and CTMP fibres (**Figure 2.23** and **Figure 2.25**).   Generally, chemical pre-treatment of chips before thermomechanical and neutral sulphite semichemical pulp (NSSC) (**Figure 2.26**) pulping results in stiffer fibres of various lengths and widths with reduced fibrillation and much wood cell wall debris as compared to Kraft pulp and BMP.   Kraft pulp shows the most uniformly separated and collapsed fibres and the highest degree of conformability (**Figure 2.27**).

Results (**Table 2.7**) show that BMP produces a stronger pulp than SGW, RMP, and TMP pulps. Handsheets from BMP were most similar in strength properties to handsheets produced from CTMP. Handsheets produced by Kraft pulp were far superior in strength to the rest (**78**).
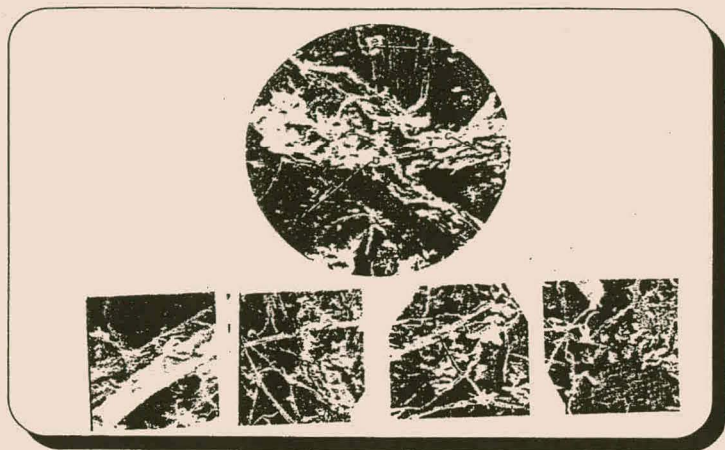
Figure 2.25. Chemithermomechanical pulp (CTMP) fibres (a) are of various lengths and widths (c), and show some twisting (e) and much cell wall debris (b) (x 100). Reduced fibrillation (x 850). Various lengths and widths and stiffness of fibres are also shown at lower magnification (x 55). Debris (x 55)[78].
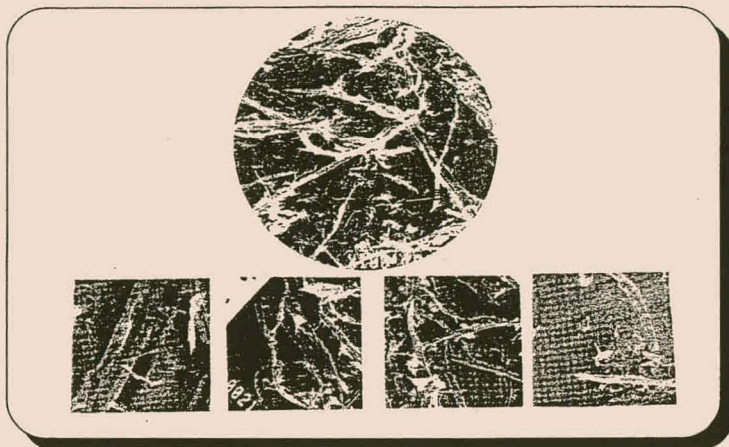
Figure 2.26. Neutral sulphite semichemical pulp (NSSC) fibres are stiff and of various lengths and widths. Reduced fibrillation (a) and cell wall debris (x 100). Reduced fibrillatin, stiffness, and various lengths and widths are also shown at higher magnification (x 750, x 110). Debris (x 170)[78].

Figure 2.27. Kraft fibres (a) are quite uniform and are usually separated; many are collapsed (x 100). Abundant fibrillation (x 600). Collapsed and conforming fibres (x 180). Uniform fibre length (x 180)[78].

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2.10. BIOPULPING RESULTS TO DATE

2.10.1. BIOMECHANICAL PULPING

Work on the application of *P. chrysosporium* for pre-treating wood prior to mechanical pulping resulted in a patent, where it was claimed that the addition of glucose and the use of mutants, with reduced ability to produce cellulose-degrading enzymes, led to an increase in selective lignin degradation **(79)**. Studies have shown that a two week incubation, of spruce and pine, resulted in up to 23% energy savings, and an increase in tensile index, but a decrease in optical properties **(59)**. Trials on a 2-6 kg scale have been carried out using various reactor formats. The best results of these were obtained using *C. subvermispora* on Aspen and Loblolly pine **(64)** **(Table 2.8.)**.

Table 2.8. Weight loss, energy saving, and physical properties of handsheets[1]

Parameters	Aspen		Loblolly pine	
	Control	Fungus-treated	Control	Fungus-treated
Weight loss (%)	–	6	–	5
Energy savings (%)	–	47	–	37
Burst index (g⁻¹)	1.01	1.23	0.66	0.93
Tear index (mN n² g⁻¹)	1.65	3.62	2.18	3.36
Density (kg m⁻³)	440	378	404	382
Brightness (%)	61.4	49.9	45.5	36.1
Opacity (%)	96.3	94.6	95.6	94.7
Scattering coefficient (m² kg⁻¹)	65.3	42.6	44.1	32.1

On aspen, energy savings of 47% were accompanied by an increase in burst and tear indices of 22% and 119%, respectively. The effects on loblolly pine amounted to a 37% energy savings and 41% and 54% increase in burst and tear indices, respectively. The optical properties decreased in handsheets

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made from pulp of both types of wood, as had been noted earlier by other authors.

New results also show that an additional benefit of fungal pre-treatment is a 30% decrease in DCM extractable resin (80), including a 60% reduction of triglycerides, said to be responsible for sticky deposits on the paper machine (81). In yet another study, strength properties of Aspen and Spruce also increased but no increase was found with *Eucalyptus*. Large scale experiments have recently been conducted at the USDA Forest Products Laboratory, Madison, Wisconsin and have given results comparable to laboratory scale bioreactors. These trials included a 50 ton and a 500 ton trial, in which the larger trial gave better results, with regards to energy savings, than the smaller one (71).

2.10.2. BIOCHEMICAL PULPING

2.10.2.1. KRAFT PULP

The effect of fungal pre-treatment on chemical pulp production has been investigated to a much lesser extent. However, it has been noted that pre-treatment of chips with white-rot fungi *Phellinus pini* and *Stereum hirsutum* resulted in a decrease in refining energy needed for Kraft pulping (82). Glucose-supplemented aspen chips, pre-treated with *P. chrysosporium* BKM-F-1767, led to Kappa reductions of 3% and 9% after 20 and 30 days, respectively (83). A marked decrease in beating time was only observed after an incubation period of 30 days, while in the same period, the water retention value increased from 102% to 137%, and the fines also increased. Brightness of handsheets, made from unbleached pulp, was reduced drastically by 35%, 54% and 62% after 10, 20 and 30 days, respectively, of fungal treatment. Tensile strength increased by 21% after 30 days, while the tear index decreased. Pulp yield increased regardless of Kraft cooking time (Figures 2.28 - 2.31) (83).

A reduction in Kappa and improved paper quality was also reported when pine wood was pre-treated with South African white-rot fungi (**77, 83, 84**).
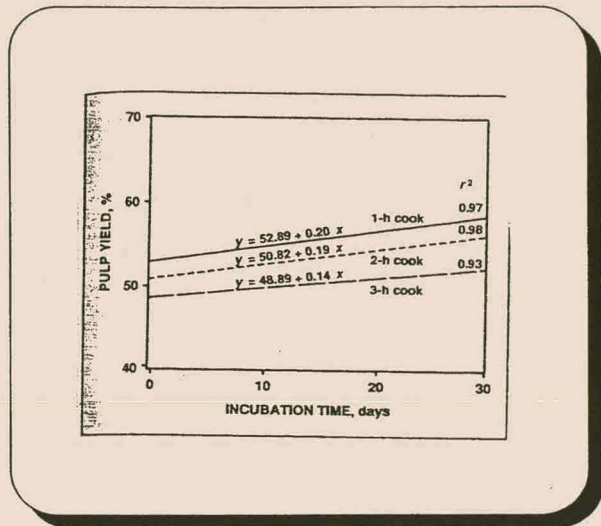
Figure 2.28. Pulp yield as a function of fungal incubation time for kraft cooks of 1, 2 and 3 hour duration[83].
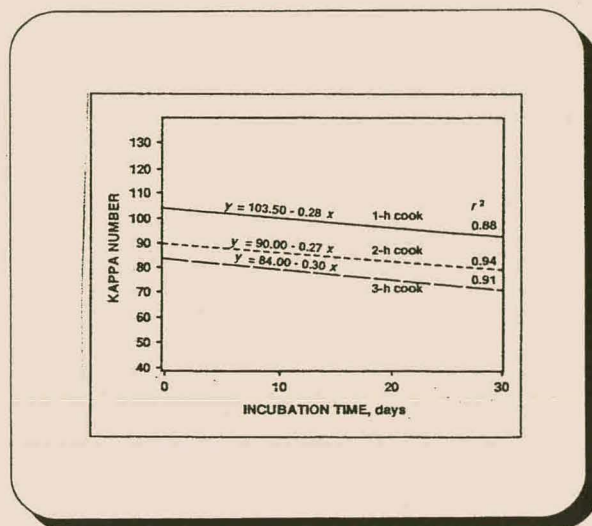
Figure 2.29. Kappa number as a function of fungal incubation time for kraft cooks of 1, 2 and 3 hour duration[83].
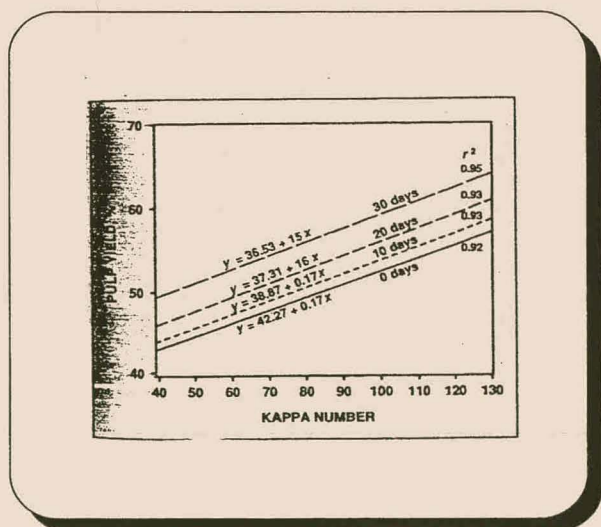
Figure 2.30. Pulp yields as a function of kappa number for aspen chips subjected to fungal incubation of 0, 10, 20 and 30 days[83].
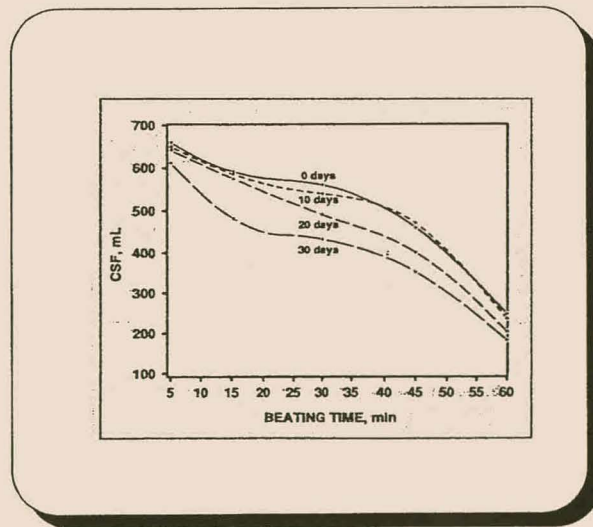
Figure 2.31. Canadian Strandard Freeness as a function of beating time for aspen chips subjected to fungal incubation of 0, 10, 20 and 30 days[83].

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2.10.2.2.   SULPHITE PULPING

In trials where five fungi (**Table 2.9**) were applied, prior to sulphite pulping, *C. subvermispora* achieved the highest Kappa reductions on both birch and spruce (**85**).

Figure 2.32 shows that on both wood types, approximately the same Kappa reduction takes place in the same period of time when wood
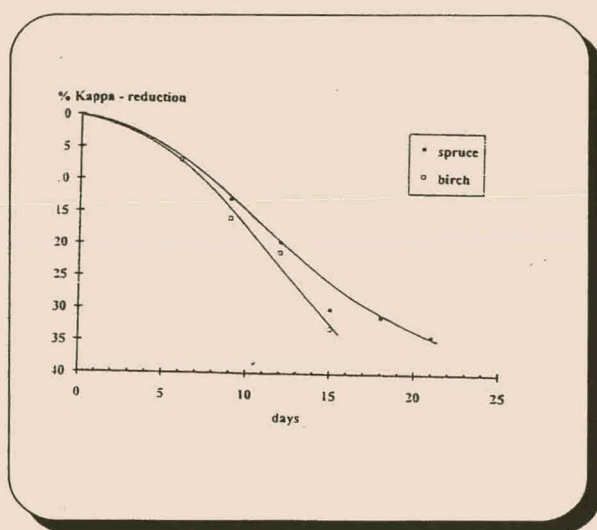
Figure 2.32. Kappa reduction caused by *Ceriporiopsis subvermispora* on birch and spruce wood chips with course of time after pulping[1].

Table 2.9. Biosulphate pulping: percent decrease in Kappa number[1]

	Birch		Spruce
	2 weeks	4 weeks	2 weeks
Ceriporiopsis subvermispora TUB 51	33	48	30
Phlebia tremellosa TUB 52		40	
Phlebia brevispora TUB 53		35	
Dichomitus squalens TUB 54		33	
Phanerochaete chrysosporium TUM B4	12	20	0

chips are incubated with *C. subvermispora*. Although no visible attack on the wood cell walls and no delignification was observed microscopically after two weeks, the Kappa number was decreased dramatically after sulphite cooking. This indicates a modification of lignin must take place during the initial phase of delignification, making lignin more susceptible to the cooking chemicals, which suggests that fungal delignification per se is not the reaction to be aimed for. This matches well with the cooking process, as the lignin fraction obtained is used as thermal energy source. The reduction in Kappa number, can be translated into a shorter cooking time, an increase in pulp produced, or eventually in a reduction in the amount of bleaching chemicals. The

use of *P. chrysosporium* did not prove to be advantageous for sulphite cooking, at least not on spruce (85). When birch chips were incubated with *C. subvermispora* for four weeks, an overall 10% decrease in strength of handsheets occurred (**Table 2.10**). Brightness of handsheets made of unbleached birch pulp was increased by 4% ISO after pre-treatment with *C. subvermispora* and sulphite pulping (**Table 2.11**)(1).

Table 2.10. Biosulphate pulping: effect on paper strength (birch, 4 weeks)[1]

	Tensile (%)		Tear (%)	
	10 [a]	20	10	20
C. subvermispora TUB 51	−8	−12	−7	+6
Ph. tremellosa TUB 52	−6	−11	−3	−14
Ph. brevispora TUB 53	−10	−12	−7	−31
D. squalens TUB 54	−8	−9	−2	−12

[a] beating time (min).

Table 2.11. Biosulphite pulping: brightness effect (birch, 4 weeks)[1]

	% ISO
C. subvermispora TUB 51	+4.4
P. tremellosa TUB 52	−0.3
P. brevispora TUB 53	+0.7
D. squalens TUB 54	+2.7
P. chrysosporium TUM B4	−1.5

2.10.2.3.   ORGANOSOLV PULPING

Eucalyptus grandis wood chips were degraded by two white-rot fungi: *P. chrysosporium* BKM-F-1767 or *Tametes versicolor*, prior to organosolv pulping (87). Weight and component losses for wood chips degraded for 1, 2 and 4 months are shown in **Figure 2.33**. During the experiments it was observed that *P. chrysosporium* did not grow abundantly on the wood chips of *E. grandis*, suggesting an inhibition effect of this wood species on *P. chrysosporium* growth. Therefore low values for mass and lignin losses were obtained. Bettucial *et al.*, (**86**) also reported a limited capacity of *P. chrysosporium* to degrade *Eucalyptus* species. On the other hand, *T. versicolor* grew abundantly on *E. grandis* wood, covering all chips after 15 days of culturing. Pulping results indicated that energy savings could be expected from organosolv pulping of samples previously delignified. (**87**)
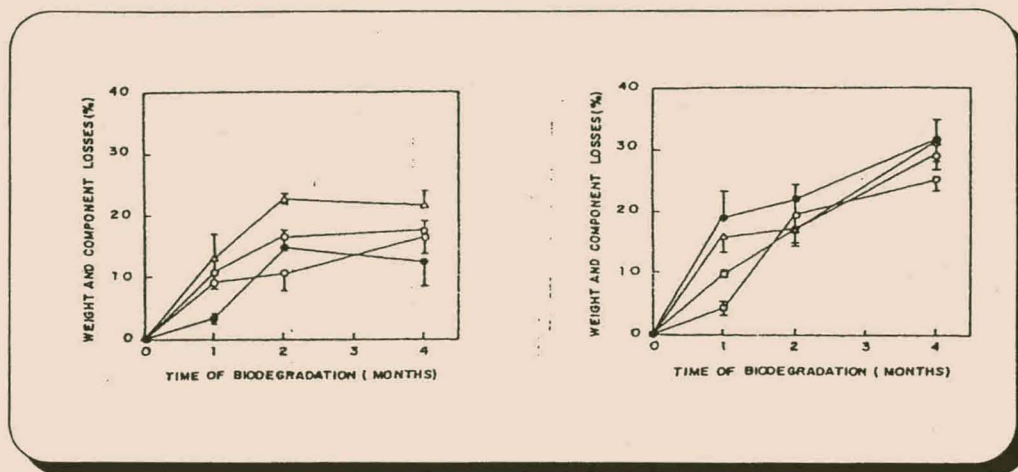
Figure 2.33. Weight and component losses of *Eucalyptus grandis* wood decayed by: A) *P. chrysosporium* and B) *T. versicolor.* (_) weight, (•) lignin (_) glucan and (_) xylan loss, respectively. Bars are standard deviation of three replicate experiments[87].

2.11.   ECONOMIC EVALUATION OF BIOPULPING

In 1987 a Biopulping Consortium (Biopulping Consortium I) was established involving the Forest Products Laboratory of the USDA Forest Services, the University of Wisconsin and the University of Minnesota, and nine pulp and paper associated companies. The engineering and economic studies related to this consortium are summarised in the Ph. D. thesis of Wall (**88**).

Early in the biopulping research, Harpole *et al.,* (**89**) conducted an economic evaluation based on a thermomechanical process model. Results indicated that a 25-percent reduction in pulping energy by fungal pre-treatment would save $21 (U.S. dollars) per air-dry ton (adt) of pulp ($33 with 40-percent energy savings based on 1989 figures). The capitalised value of the energy savings was estimated to be about $250 000, in 1989, for each percentage of energy saved, at an electricity cost of $0.035/kW-h. Thus, a sizeable capital expenditure for the biotreatment could be accommodated. Later, an economic model based on mass and energy balances was made for a controlled aerated static bed process and a chip pile-based system (**90**). The controlled static bed process does not appear to be economically

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attractive (return on investment is 15-percent); the chip pile-based system promises greater return. The chip pile-based system is illustrated in **Figure 2.18**. Calculations include duct work to provide aeration, pipes for steam to pre-steam the chips on the conveyor, and a sprayer to apply inoculum to the chips on the conveyor. Calculations were based on 25 percent energy savings, 300 ton/day mill, 2-week treatment time, 95 percent yield, and 0.59 VVM aeration. Operating costs included steam, inoculum, and electricity for aeration. Capital costs included cost for fans, duct work and steam pipe system, inoculum tanks, and humidification. The total capital investment was conservatively estimated at $500 000. **Table 2.12** shows the results of calculations for three cases based on three different costs of the inoculum, which is a major cost element. The calculated pretax return on investment (ROI) is 106 to 217 percent, making the system economically attractive **(2, 88)**.

Table 2.12. Economic feasibility of chip pile based system[88]

Item	U. S. dollars		
	Case 1	Case 2	Case 3
Installed equipment costs	500,000	500,000	500,000
Working capital investment	206,750	206,750	206,750
Total capital investment	706,750	706,750	706,750
Utility costs	2.46	2.46	2.46
Inoculum costs	3.00	5.00	10.00
Labor	0.76	0.76	0.76
Yield losses	2.46	2.46	2.46
Depreciation	0.76	0.76	0.76
Total operating costs	9.35	11.35	16.35
Pretreatment value	23.49	23.49	23.49
Gross profit	14.14	12.14	7.14
Pretax ROI	217%	180%	106%

[a]*P. chrysosporium* on aspen; 300 metric ton/day; 14-day treatment; 95-percent yield; 0.59 VVM air.
[b]Operating costs are expressed in dollars per air-dry ton.

2.12. CONCLUSION

It is apparent, from the number of recent publications, that research into the biopulping field has intensified over the last few years. Although research into biopulping started more than 40 years ago, it is only recently that there have been plans to commission the first pulp and paper mill, in the USA, using biopulping technology (**92**). As previously mentioned, this work has primarily focused on biopulping in the mechanical and thermo-mechanical pulping processes. It appears that the application of biopulping in these pulping processes has been proved to be economically and otherwise beneficial. There are still obvious opportunities for further improvement in fungal strains and mutants through bio-engineering. Many possibilities also exist for the refinement and optimisation of the process itself, by the incorporation of various additives to improve growth rates and gene expression of the fungi. The technology for bio-mechanical pulping has thus been established and it is time to shift our attention to biochemical pulping procedures. Literature concerning this field is very limited and the scope for future research is immense and somewhat daunting but the benefits achieved through biochemical pulping could far outweigh any of those already achieved through the biomechanical pulping process.

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CHAPTER 3

KRAFT BIOPULPING OF *Eucalyptus grandis*

KRAFT BIOPULPING OF *Eucalyptus grandis*

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ABSTRACT

Eucalyptus grandis wood chips were inoculated with selected white-rot fungi and incubated for two weeks. Degraded wood chips were subsequently pulped according to the Kraft pulping process. The resultant pulp was analysed for kappa number, yield and shive content, while the black liquor was analysed for residual active alkali and compared to an untreated control sample. One hundred cultures were screened in this manner. Cultures showing biopulping potential were further evaluated. Statistically significant improvements in the residual active alkali of just under 21% were observed, resulting in a reduction in chemical consumption of over 1.7%. When compared to the control, a significant reduction in kappa number of almost 9% was determined, while no significant difference in yield and shive content could be identified.

3.1. INTRODUCTION

Enzymes play an important role in all living organisms: in their development, their growth and their death. In trees the biosynthesis of wood components and the formation of cell walls are controlled by enzymes. The natural degradation of wood also occurs due to the action of enzymes produced by wood-destroying organisms. These enzymes degrade the insoluble components of wood into soluble products, and finally simple chemical compounds which, are then used by the organisms (1).

White-rot fungi are able to decompose all of the main wood components (cellulose, hemicelluloses and lignin). It is in part the removal of lignin that gives 'white-rotted' wood its appearance, although, oxidative bleaching reactions also cause whitening. Use of the term 'preferential white-rot' clearly identifies the preferred early attack of lignin by these fungi (2). However, a definite distinction between simultaneous (degradation of two or more of the wood components simultaneously) and preferential white-rot cannot be made, because the two types of decay may occur simultaneously and culture conditions (e.g. the presence of a nitrogen source) may influence the type of decay produced. *Phanerochaete chrysosporium*, has extensively been used as a model organism to characterise preferential white-rot attack (2, 3).

Biopulping is defined as 'the concept of deliberately harnessing white-rot fungi as a pre-treatment to pulping' (3). The use of white-rot fungi for the biological delignification of wood was first studied at the West Virginia Pulp and Paper Company (now Westvaco) in the 1950's. In the 1970's, Eriksson and co-workers at the Swedish Products Laboratory (STFI) in Stockholm demonstrated that fungal treatment could result in significant energy savings for mechanical pulping (4). It appears, from the literature available, that the major proportion of biopulping research has focused on biomechanical pulping (5-13). Research pertaining to biochemical pulping appears vague and incomplete with little reference to kraft biopulping of *Eucalyptus spp.* Sulphite, organosolv, soda and kraft pulping are all included in the limited number of publications concerning this field of research (14-20).

Positive results in biomechanical pulping research have led to the application and granting of a number of patents, some as early as 1976 (21, 22). A 500-ton (dry wood basis) outdoor chip pile experiment conducted at the USDA Forest Products Laboratory, Madison, Wisconsin, USA, yielded results comparable to those obtained using laboratory scale bioreactors. Based on energy savings alone, calculations indicate the economics of biopulping as being very favourable (23). Hence, it appears that the biomechanical pulping process has developed enough as to be implemented in practice. Conversely, research into biochemical pulping is still in its infancy, yet the potential improvements, both financially and environmentally, in chemical pulping

procedures, due to the considerably greater volume being processed during chemical pulping, may far exceed those already obtained in biomechanical pulping experiments. Numerous potential savings could be attained: possible yield increases, reduction in the use of pulping chemicals, increased production during pulping, savings in bleaching chemicals as a result a lower kappa number of the pulp exiting the digester, possible savings in refining energy and lastly reduction in effluent treatment costs.

In this study, 100 different white-rot fungi, previously isolated by the Department of Microbiology, Stellenbosch, South Africa, were studied to determine their effect on the Kraft pulping process. After initial laboratory screening, selected strains were subjected to further analysis to determine their effect on *Eucalyptus* wood chips. Results from this study indicate that many different white-rot strains can have a positive effect on *Eucalyptus* Kraft pulping.

3.2. EXPERIMENTAL

3.2.1. WOOD CHIP PREPARATION

Eucalyptus grandis pulp-wood logs were obtained from Mondi Kraft Richards Bay, South Africa. The trees were felled and sawn into lengths. The ends of the logs were sealed with a wood sealant to maintain 'freshness' and moisture content. Logs were transported to the Forestry Faculty, University of Stellenbosch, South Africa, where they were sawn into more manageable size boards.

Boards were chipped in a Wigger pilot plant chipper. The resultant chips were well mixed and a representative sample was screened, using a Wennberg chip screen, to a thickness of between 2 and 8 mm. Any chips falling outside of these limits were rejected. Screened chips were placed in plastic bags and stored at 0°C.

The moisture content of the chips were determined by means of the oven-dry method. Moist wood chips (100 g O.D.) were placed in 1 litre wide neck Erlenmeyer flasks. The desired moisture content (60% wet basis) was

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adjusted by adding sufficient volume of water. The flasks were stoppered with cotton wool and autoclaved (121°C) for 30 minutes.

3.2.2. FUNGAL INOCULUM

Malt extract agar petri dishes were prepared with 1,5% malt extract and 1,5% agar. Cultures used in this study are maintained in the culture collection at the Department of Microbiology, University of Stellenbosch, South Africa. Malt extract (ME) plates were inoculated with the respective fungi and incubated at 30 or 37°C, depending on the particular culture, for approximately 7 days. In the case of sporulating strains, three millilitres of sterile water was pipetted onto the plates, the spores removed, and a spore suspension was generated. A spore count was performed to calculate the numbers used for inoculation. For non-sporulating cultures, plugs from the mother cultures were inoculated into 50 ml ME medium and incubated statically at 30°C, until substantial pellicle growth was obtained. The medium was then decanted and the pellicle was ground in a Warring blender, with 40 ml of sterile water, for 20 seconds. ,

The spore or fungal suspension was poured over the wood chips and mixed well, under aseptic conditions. The flasks of inoculated chips were incubated for two weeks at the required temperatures (*Phanerochaete spp.* at 37°C and the remaining cultures at 30°C). The control chips were treated in exactly the same fashion as the inoculated wood chips i.e. autoclaved, incubated etc., without the addition of any inoculum.

3.2.3. KRAFT PULPING

After completion of incubation, the degraded chips were weighed and the moisture/solid content was determined. An equivalent mass of moist wood chips (75 g O.D.) was placed in sealed pressure cylinders with a maximum capacity of 500 cm^3. The moisture content of the various fungally degraded wood chips, as well as the control chips, was equalised by the addition of water. The appropriate amount of chemicals were mixed with a measured

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amount of water and added to the wood chips. The cylinders, three containing bio-degraded wood chips and one containing un-degraded control chips, were sealed and placed in a 15 dm³ batch-type laboratory digester equipped with pressure and temperature gauges. The digester was filled with warm water and sealed. During the cooking cycle, the temperature was automatically controlled by a programmable logic controller while the digester oscillated through 45°, to either side, to ensure proper chip liquid contact during the cook. Pulping conditions are listed in **Table 3.1**.

Table 3.1. Pulping parameters used during investigation

Active alkali	18% as Na_2O
Sulphidity	22% as Na_2O
Liquid/solid ratio	4,5:1
Maximum temperature	170°C
Digesting time at 170°C	25 minutes
Total pulping cycle	142 minutes

After completion, the digester was opened and the cylinders removed and placed in cool water to enable handling and to reduce the internal pressure of the bomb. The cooled bombs were opened and the black liquor decanted and placed in glass bottles for further analysis. The pulped chips were washed out into a 5 litre container. The container was filled with water to cool and rinse the pulp. The pulp was subsequently washed through a 10-mesh screen with a high pressure hose to separate fibres from rejects. Rejects were dried at 105°C and weighed. The washed pulp was collected on a 150-mesh screen and was re-screened with the aid of a 'Packer' screen equipped with a pulsating diaphragm and 0.15 mm slot size. The pulp was again collected on a 150-mesh screen and the shives were washed out of the Packer screen and collected in a plastic container. The shive suspension was filtered through a Büchner funnel, dried at 105°C and weighed.

The final screened pulp was transferred to a plastic container. The excess water was removed with the aid of a Büchner funnel and the pulp dried at 105°C for 24 hours.

The percentage rejects (mass of particles > 2 mm thickness), percentage shives (mass of particle between 0.15 and 2 mm in thickness) and percentage pulp yield (mass of fibres with thickness less than 0.15 mm) were calculated, based on the total O.D. mass of chips (75 g) placed in the bombs.

3.2.4. BLACK LIQUOR ANALYSIS

The residual active alkali concentrations in the black liquor of the various pulp batches were calculated by using the volume of 0,1 M hydrochloric acid solution required to titrate a prepared formaldehyde treated sample of black liquor to pH 8.3. Potentiometric determination of residual active alkali (RAA) in Kraft black liquor was determined using tha **SAPPI Method D3110/77**.

3.2.5. KAPPA NUMBER ANALYSIS

A known mass (O.D.) of pulp was disintegrated in distilled water and allowed to react with a specified amount of potassium permanganate solution for a given time. The amount of pulp was chosen so that about 50% of the permanganate was left unconsumed at the end of the reaction time. The reaction was terminated by the addition of a potassium iodide solution, and the free iodine was titrated against sodium thiosulphate solution. The value obtained was corrected to exactly 50 percent consumption of the permanganate. The Kappa number was used as an indirect approximation of the lignin content of the pulp. **DIN 54357** was the standard method used in the determination of the Kappa number .

3.2.6. STATISTICAL VERIFICATION OF SELECTED FUNGI

Twelve of the one hundred cultures, initially investigated during the screening process, were selected to statistically verify the results (**Table 3.2**). A control

(untreated) sample was also used for comparison purposes and made up a thirteenth sample.

Based on an Analysis of Variance performed on the digester, a fully randomised incomplete block design was used to account for the variation that occurred between the various digester batches. A type IV randomised incomplete block design was used: t = 13, k = 4, b = 13, M = 1, E = 0.81 (24)

Table 3.2. Cultures selected for statistical evaluation
Control is represented by CONT*

BG 3A	KKP 16	RRY 25
BG 78	MTZ 32	STB 15
BKM-F-1767	PC 38	CONT*
HB 4	PP25	
H 15A	RRY 23	

3.3. RESULTS AND DISCUSSION

The results of the screening trials are summarised in **Figure 3.1.** **Figure 3.1** is divided into two parts, the first part being plotted based on the Kappa number, in yellow, starting with the 'worst' value (an increase in Kappa number, expressed as a percentage, over control value) and ending with a zero percent difference in Kappa number over the control. The second part of the graph starts at a Kappa number improvement of just over zero percent and continues to the best value (the highest percentage improvement in Kappa number as a result of fungal degradation). The associated difference in percentage yield and residual active alkali are plotted with the Kappa numbers. Anything above zero percent on the x-axis signifies a positive difference (higher yield, higher residual active alkali and/or lower Kappa number) and anything below signifies a negative difference. Cultures for further research were chosen on their ability to decrease the Kappa number of the pulp and increase the residual active alkali of the black liquor without decreasing the yield significantly. In some cases the yield appeared to increase. Some cultures indicated a marked improvement in the residual active alkali, while others tended to increase in yield. However, only those

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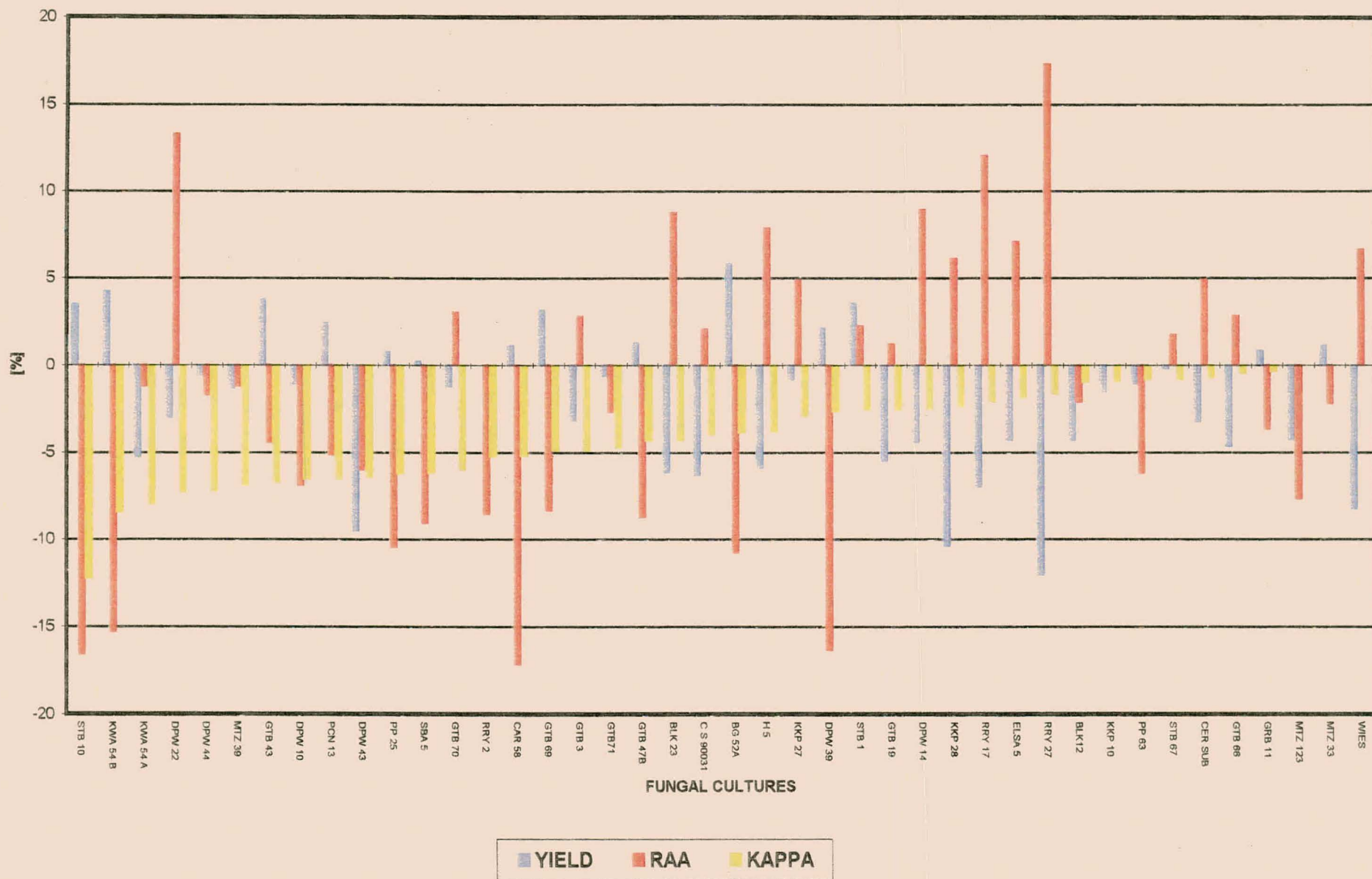
Figure 3.1. The percentage differences for the relevant criteria: Yield, Residual Active Alkali (RAA) and Kappa number, are plotted together for the various cultures. A negative result i.e. an increase in Kappa number or a decrease in Yield and/or RAA, compared to the control, is represented by a negative percentage. The graph is arranged with the worst Kappa results, and its corresponding Yield and RAA results, on the far left to a zero difference in the Kappa on the far right.
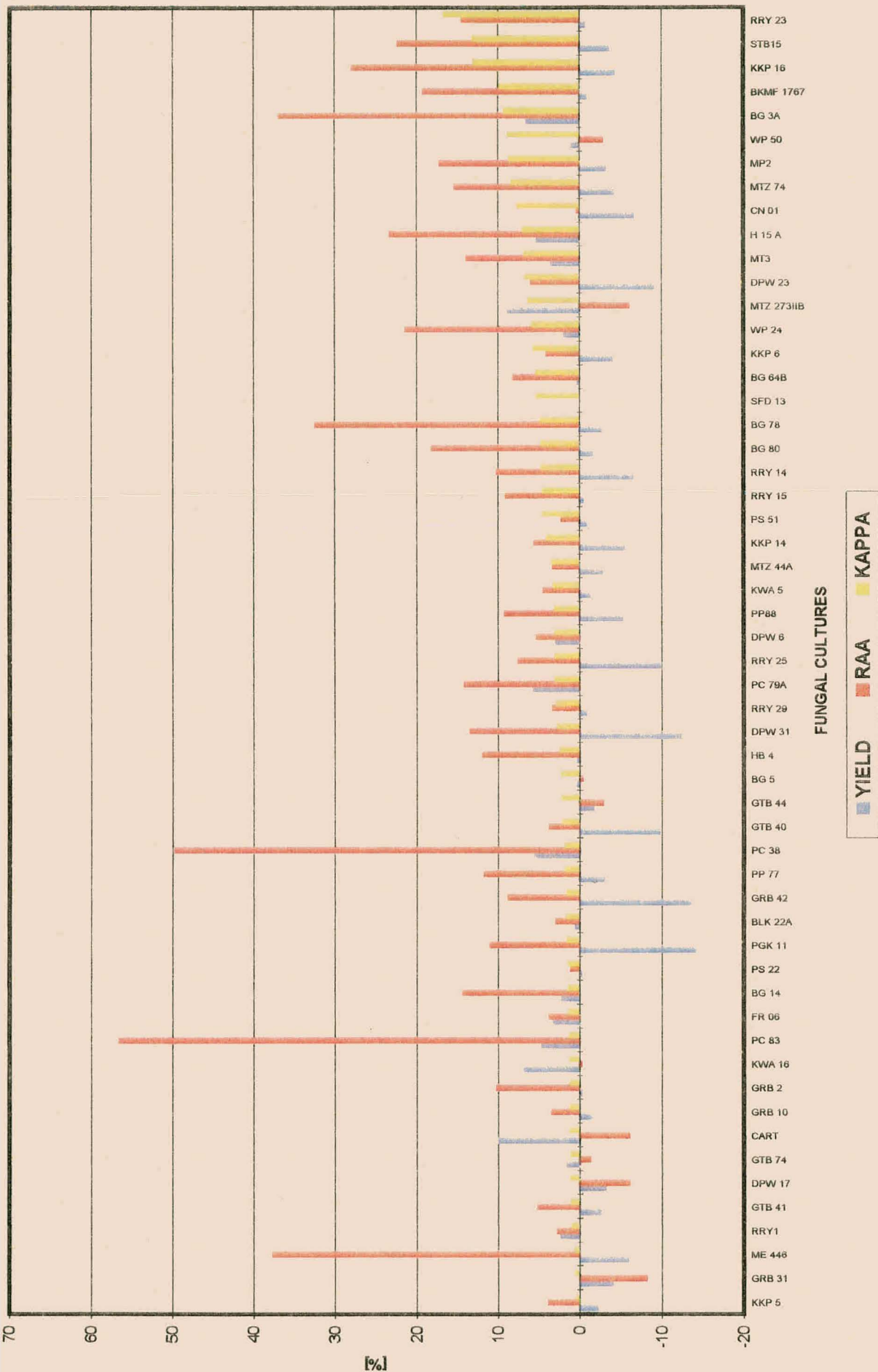
Figure 3.1. (continued)

cultures that showed improvements in both the Kappa number and the RAA were selected. The average f and p values of the Analysis of Variance, for the twelve cultures selected in **Table 3.2**, are shown in **Table 3.3**. There were no significant differences (p=0.05) in the means for the various cultures tested with regards to total yield, screened yield and shives, with p-values of 0.51, 0.56 and 0.29, respectively. In general, the means for the yields and shives tend to be lower than that of the control, although there can be no certainty of this effect.

Table 3.3. Resultant f and p values for trials

Annova Results.

	Yield	Screen. Yield	Shives	Kappa	RAA	Chem. Cons.
f Value	0.96	0.9	1.28	1.53	2.49	2.49
p > f	0.5111	0.5623	0.2856	0.1727	0.0238	0.0239

According to the generated p-value, there is no significant difference between the various means of the Kappa values. However, p=0.1727 tends to have some significance which is highlighted later in the pair-wise comparison of the cultures. The p-values for residual active alkali and chemical consumption indicate significant differences in the means of these two parameters. The p-value of 0.023, is the same for both variables as they are directly related, the chemical consumption being determined by the following equation:

(TAA - RAA)\TAA X 100%, where **TAA** is the Total Active Alkali applied expressed as Na_2O, and **RAA** is the Residual Active Alkali after pulping expressed as Na_2O.

The mean results of the statistical analysis of the twelve cultures and the un-innoculated control are shown in **Table 3.4**.

The means of the Kappa numbers, residual active alkali and the chemical consumption have been plotted in **Figures 3.2 a-c**, with their error bars at p=0.05 and degrees of freedom equal to 27. All the chosen cultures show a positive improvement in the above three categories. Isolate **PC 38** had the best reduction in the Kappa number over the control (2.5 points), while **RRY 23** resulted in the best improvement in residual active alkali and chemical consumption.

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Table 3.4. Statistical means and standard errors of the pertinent variables for selected cultures. Each culture is represented by its code, i.e. PP 25, while the control is represented by CONT and is number four on the list

No.	Fungi/Strain	Yield	Screen. Yield	Shives	Kappa	RAA	Chem Cons
1	PP 25	42.92	40.86	2.06	28	4.72	90.84
2	RRY 23	41.68	39.82	1.86	26.94	5.15	89.97
3	BG 3A	42.76	40.69	2.08	27.53	4.69	90.88
4	CONT	43.2	41.05	2.15	28.68	4.26	91.72
5	BG 78	43.45	41.41	2.04	27.87	4.5	91.25
6	H 15A	42.86	41.21	1.66	26.6	4.78	90.71
7	PC 38	41.34	40	1.33	26.2	5.1	90.09
8	RRY 25	41.14	39.52	1.62	26.57	5.12	90.04
9	MTZ 32	42.21	40.16	2.05	26.68	5.07	90.15
10	BKMF	43.41	41.44	1.97	27.31	4.62	91.03
11	HB 4	43.27	41.52	1.76	27.26	4.87	90.53
12	STB 15	41.15	39.32	1.83	27.57	4.93	90.43
13	KKP16	43.29	41.5	1.79	27.26	4.6	91.07
Std Error		0.9084	0.8335	0.2043	0.5459	0.1716	0.3332
Std Deviation		1.547	1.4194	0.3479	0.9297	0.2922	0.5674

Figure 3.3 shows the percentage improvements, compared to the control, in the Kappa number, residual active alkali and chemical consumption; included are the corresponding error bars in percent (p= 0.05) and is plotted with increasing RAA percentages. It is evident that all cultures have a positive influence on the results of the Kraft process. **RRY 23** indicates the best performance in RAA at a 20.9% increase over the control or 1.75% reduction in chemical consumption. It also ranks number five with a 6.07% reduction in Kappa number compared to the control. **PC 38,** which is the highest ranking according to Kappa number (8.65%), is third with RA (19.72%) and chemical consumption (1.63%); possibly making it the better isolate. Ultimately, the best culture will depend upon which factor is more important: a reduction in Kappa number or a reduction in chemical consumption.

As mentioned earlier, a pair-wise comparison was also performed and these p-values are contained in **Table 3.5**. Where the mean of the culture is better than that of the control, at a 95% confidence level, its p-values are marked

MEAN KAPPA VALUES

Figure 3.2a.

MEAN RESIDUAL ACTIVE ALKALI

Figure 3.2b.
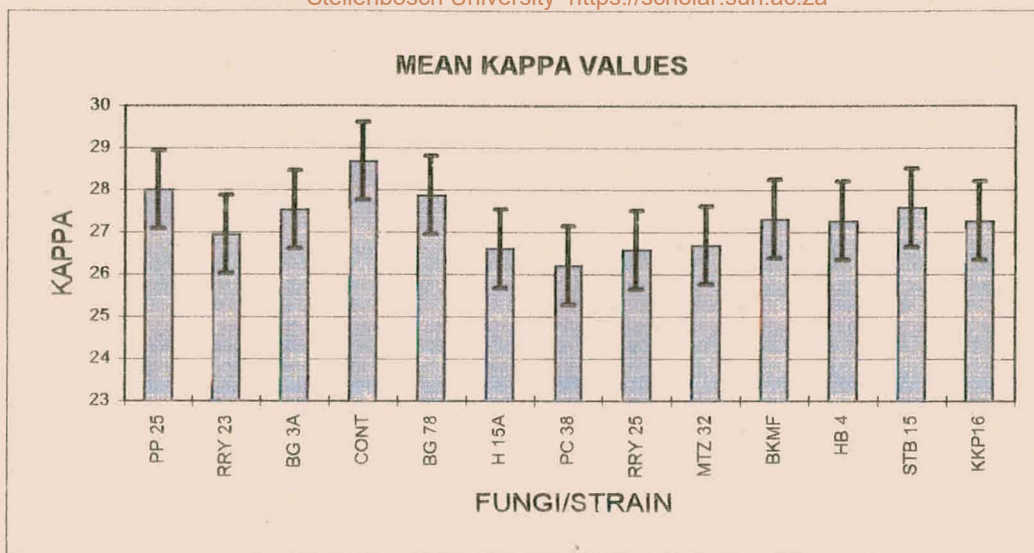
MEAN PERCENTAGE CHEMICAL CONSUMPTION
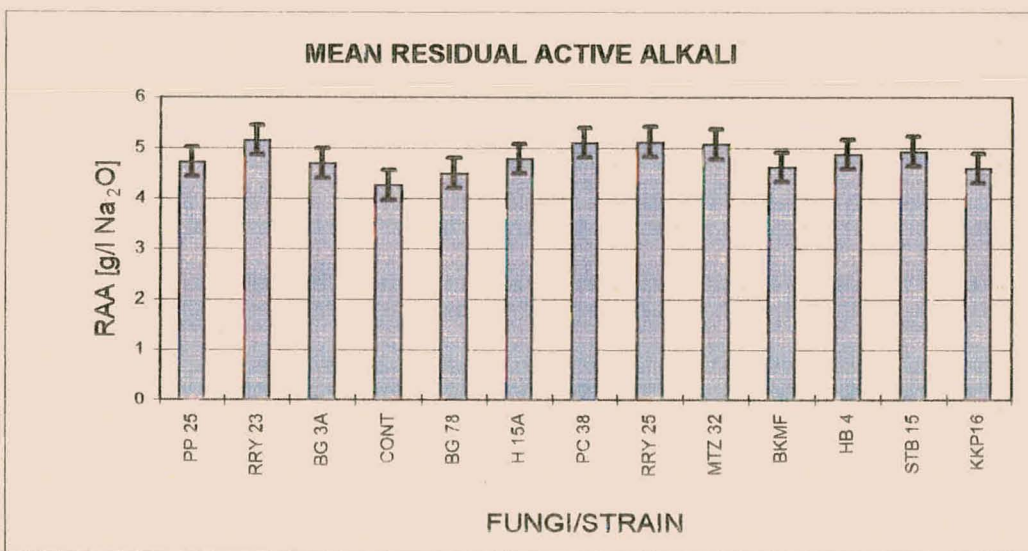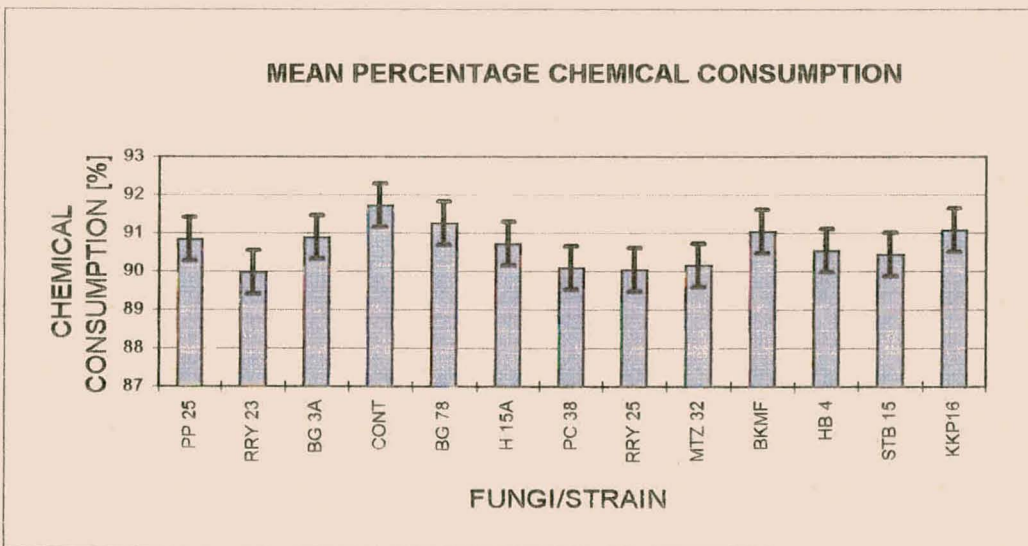
Figure 3.2c.

Figures 3.2 (a-c). The plotted means of the Kappa numbers, residual active alkali and chemical consumption with corresponding error bars at p = 0.05.

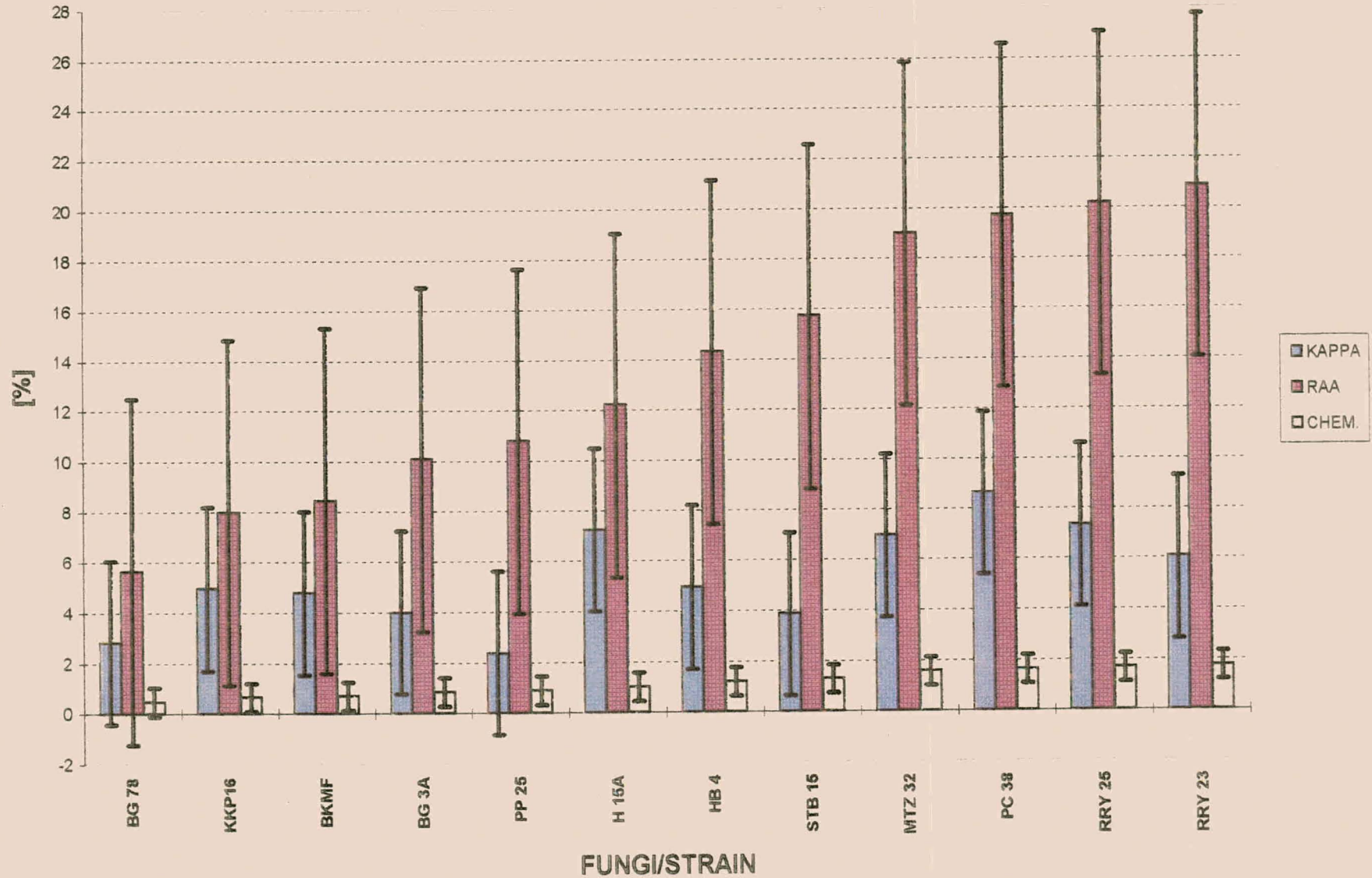**Figure 3.3.** Graph of the percentage improvement of Kappa number, Residual Active Alkali and Chemical consumption, compared to the control.   It is plotted with increasing RAA percentages with the corresponding Kappa and Chemical consumption improvements.   Error bars, in percent, have also been included at P - 0.05

Table 3.5. The p-values for a pair-wise comparison of the means or the Kappa number, RAA and chemical consumption compared to the control

Fungi/Strain	Kappa No.	RAA	Chemical Cons.
PP 25	0.3864	0.0733	0.0743
RRY 23	0.0331*	**0.0011**	**0.0011**
BG 3A	0.1495	0.0898	0.0887
BG 78	0.3037	0.3378	0.3318
H 15A	0.0124*	0.0418*	0.0423*
PC 38	**0.0036**	**0.0019**	**0.0019**
RRY 25	0.0115*	**0.0015**	**0.0014**
MTZ 32	0.0159*	**0.0026**	**0.0026**
BKMF	0.0896	0.1557	0.1579
HB 4	0.0783	0.0187*	0.0186*
STB 15	0.163	0.011*	0.0111*
KKP16	0.0783	0.1793	0.1793

with an asterix and those that are at a 99% confidence level, are in bold type. Five of the cultures resulted in a significant Kappa improvement at a p-value of 0.05 and included in these are isolate **PC 38** at a confidence level of above 99%. The results of the residual active alkali and chemical consumption indicate that the means of seven of the cultures show an improvement at a 95% confidence level, while four of these are at 99%. It appears that most of those cultures that are positive with regards to Kappa number, are also positive with regards to RAA and chemical consumption, although two extra cultures resulted in significant improvements with respect to RAA and chemical consumption only.

It is not possible, at this stage, to determine which is the best isolate out of the top three performers to the right of **Figure 3.3** namely, **RRY 23, RRY 25** and **PC 38**, without further experimentation. If Kappa improvement is of the importance then cultures **H 15 A** and **MTZ 32** also come into contention.

3.4. CONCLUSIONS

- The savings of up to 1.75% in chemical consumption and 8.7% in Kappa are remarkable and imply extensive savings and improvements in the Kraft pulping process.

- Not all cultures selected from the pre-screening trial showed significant improvements and only those showing any promise should be used on scale-up trials.

- Optimisation of the process is needed; the two week incubation period and the high concentration of inoculate could be reduced by the addition of nutrient adjuncts (by-products such as malt and yeast extracts).

- The question of asepsis when treating large wood piles will have to be addressed.

- A quicker more efficient screening method is needed to screen the many hundreds of other fungi and their various strains, for their biopulping potential of the numerous commercial wood species.

- An economic feasability of biochemical pulping should also be carried out to asses its financial viability.

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CHAPTER 4

GENERAL DISCUSSION

4.1. DISCUSSION:

This thesis emphasises the importance of biotechnology in the modern era. It also stresses the need for greater association between various research departments if any of these projects are to mature into industrial scale application. Chapter 1 describes the reasons why biopulping is so important and why specifically *Eucalyptus grandis* is of such economic importance to the South African industry. The first part of Chapter 2 provides a brief background on the fundamental aspects involved in biopulping. The second part of this chapter summarises the techniques, fungal species and pulping methods that have been focused on and results achieved, to date. Chapter 3 provides the proof of biopulping's positive influence on the Kraft pulping of *Eucalyptus grandis.*

Up until this point, there has been no explanation or investigation of the mechanisms involved in the biopulping effect during Kraft pulping of *Eucalyptus grandis.* The principal view has focused on either lignin degradation or lignin modification. However, an alternative proposal is that the enzymes secreted by the hyphae are essentially ' drilling minuscule holes or capillaries' into the wood chips. It may be argued that it is these capillaries that allow faster and more even penetration of pulping liquors, thus improving the pulping process. This would also reduce the chance of the surface of chips being over-pulped in order to allow the centre of the chip to be digested sufficiently. In support of such an argument, was the fact that no biopulping effect was observed when wood chips were oven-dried and then re-soaked, prior to pulping, after fungal pre-treatment. It could thus be hypothesised that if there were such passages or holes created by the hyphae, these would collapse during drying and not re-open during re-soaking, negating the biopulping effect. Indeed, such effects are noted during wood drying. This is commonly known as 'collapse' and is a result of the capillary forces present when water is driven out of the numerous capillaries found in wood, during drying.

One of the major challenges involved in a project of this nature, is the time constraints due to the shear number of fungal cultures and their strains, as well as the incubation time needed in order to effectively determine biopulping potential. To this effect, screening was carried out on a one-off basis. The best cultures, that were identified from this study, were then used to statistically validate their biopulping potential of *Eucalyptus grandis* in the Kraft pulping process (raw data is contained in the addendum at the end of the thesis). It is therefore quite possible that some beneficial cultures may have been overlooked during this screening process due to the inherent variation. It should also be noted that only 100 cultures were tested out of the more than 600 that exist in the culture collection kept at the Department of Microbiology, Stellenbosch, South Africa. Consequently, pre-screening should be carried out on a statistical basis right from the beginning, to ensure that no repetition of the same work is necessary. Unfortunately, analysing such a large number of cultures on a statistical basis would take an extremely long time. It would be most beneficial if a much faster method were found to identify and screen cultures with biopulping potential.

The cultures used in the study described in Chapter 3, certainly exhibit biopulping potential. However, there are a few challenges which will have to be surmounted. The degree to which asepsis must be maintained is important. Contaminating local cultures tend to compete with and defeat the culture used during biopulping, especially on the *Eucalyptus grandis* wood chips. Other important challenges are the quantity of inoculant and the time period needed for incubation. These two problems have already been investigated by Akthar *et al.*, (**1**) and possible improvements using nutrient adjuncts, such as corn steep liquor, have been demonstrated.

This study focused only on the reduction in Kappa number, chemical consumption and yield. A more integrated study is needed where the effects of biopulping on bleaching, paper properties and effluent are also examined. The effect of enzymes on pitch reduction has attracted much attention, however, these studies are normally performed independently of one another. Only recently has the influence of biopulping in its entirety been investigated by various research groups (**2**). Once the fundamentals of Kraft Biopulping

have been fully established, an economic feasibility study of the process is needed and should form part of a fully integrated approach.

In conclusion, it must be said that the results obtained are impressive and could have a large impact on the pulping industry in the near future. These results will form the basis of the next phase that would involve a pilot plant scale trial. Typically, a large chip pile (approximately one ton) would be inoculated with the best identified culture and the resultant chips pulped by means of the Kraft process in a laboratory digestor and further statistical results generated and compared to control samples. Pending the success of such a project, mill-scale trials should be carried out and the results critically evaluated.

4.2. LITERATURE CITED

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ADDENDUM

RESULTS

Table of raw results obtained during statistical varification trial

Block	Treat.	Yield	Syield	RAA	Kappa	Shives	Chem Cons
1	1	42.39	40.97	4.84	25.50	1.41	90.59
1	2	42.97	41.63	5.02	25.00	1.35	90.24
1	4	44.60	43.16	4.17	25.70	1.44	91.89
1	10	43.12	41.52	4.34	26.20	1.60	91.57
2	2	41.59	40.36	4.86	24.30	1.23	90.56
2	3	44.00	42.16	4.28	25.30	1.84	91.68
2	5	42.57	40.79	4.45	24.40	1.79	91.36
2	11	44.96	43.17	4.50	25.80	1.79	91.25
3	3	39.93	38.71	4.91	26.30	1.23	90.46
3	4	44.64	42.47	4.12	28.30	2.17	91.99
3	6	42.33	40.76	5.21	25.80	1.57	89.87
3	12	39.87	38.09	5.05	27.20	1.77	90.19
4	4	37.69	34.73	5.24	35.60	2.96	89.82
4	5	45.36	41.84	5.02	34.90	3.52	90.24
4	7	39.85	37.73	5.72	32.20	2.12	88.89
4	13	41.40	38.21	5.80	33.40	3.19	88.73
5	5	41.73	40.56	4.81	23.40	1.17	90.64
5	6	44.87	43.48	4.26	24.20	1.39	91.73
5	8	41.56	40.05	4.99	24.50	1.51	90.30
5	1	44.44	42.56	4.86	24.50	1.88	90.56
6	6	43.41	41.29	4.72	27.50	2.12	90.83
6	7	42.99	41.25	5.24	28.60	1.73	89.82
6	9	42.67	40.49	5.18	28.90	2.17	89.93
6	2	41.84	39.53	5.10	29.20	2.31	90.08
7	7	39.27	37.88	5.87	25.30	1.39	88.60
7	8	42.40	40.69	5.21	26.30	1.71	89.87
7	10	42.87	40.40	5.21	26.70	2.47	89.87
7	3	44.65	42.08	4.72	29.10	2.57	90.83
8	8	41.64	40.40	4.99	25.10	1.24	90.30
8	9	43.16	41.15	4.61	26.10	2.01	91.04
8	11	43.55	41.92	4.47	25.80	1.63	91.30
8	4	44.56	42.32	4.12	27.70	2.24	91.99
9	9	42.39	40.25	4.83	24.70	2.13	90.61
9	10	43.44	42.11	4.75	24.90	1.33	90.77
9	12	42.25	40.72	4.86	25.50	1.53	90.56
9	5	43.88	41.93	4.06	28.20	1.95	92.10
10	10	44.57	42.24	4.42	29.50	2.33	91.41
10	11	42.16	40.13	5.10	28.40	2.03	90.08
10	13	42.71	40.13	4.66	28.80	2.57	90.93
10	6	40.60	38.93	5.21	28.80	1.67	89.87
11	11	42.44	41.00	4.94	26.80	1.44	90.40
11	12	40.21	38.84	5.10	26.30	1.37	90.08
11	1	42.08	40.08	4.64	27.50	2.00	90.99
11	7	41.79	40.05	4.77	26.70	1.73	90.72
12	12	41.31	39.03	4.61	33.60	2.28	91.04
12	13	43.44	42.08	4.45	31.40	1.36	91.36
12	2	41.91	39.59	5.24	31.00	2.32	89.82
12	8	40.38	38.31	5.29	30.50	2.07	89.71
13	13	43.38	42.11	3.82	25.00	1.27	92.58
13	1	43.53	42.08	3.77	25.70	1.45	92.67
13	3	42.57	40.60	4.53	23.10	1.97	91.19
13	9	42.82	41.39	4.69	23.20	1.43	90.88